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The ‘Danse Macabre’ – Neutrophils the Interactive Partner Affecting Oral Cancer Outcomes

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Over the past few decades, tremendous advances in the prevention, diagnosis, and treatment of cancer have taken place. However for head and neck cancers, including oral cancer, the overall survival rate is below 50% and they remain the seventh most common malignancy worldwide. These cancers are, commonly, aggressive, genetically complex, and difficult to treat and the delay, which often occurs between early recognition of symptoms and diagnosis, and the start of treatment of these cancers, is associated with poor prognosis. Cancer development and progression occurs in concert with alterations in the surrounding stroma, with the immune system being an essential element in this process. Despite neutrophils having major roles in the pathology of many diseases, they were thought to have little impact on cancer development and progression. Recent studies are now challenging this notion and placing neutrophils as central interactive players with other immune and tumor cells in affecting cancer pathology. This review focuses on how neutrophils and their sub-phenotypes, N1, N2, and myeloid-derived suppressor cells, both directly and indirectly affect the anti-tumor and pro-tumor immune responses. Emphasis is placed on what is currently known about the interaction of neutrophils with myeloid innate immune cells (such as dendritic cells and macrophages), innate lymphoid cells, natural killer cells, and fibroblasts to affect the tumor microenvironment and progression of oral cancer. A better understanding of this dialog will allow for improved therapeutics that concurrently target several components of the tumor microenvironment, increasing the possibility of constructive and positive outcomes for oral cancer patients. For this review, PubMed, Web of Science, and Google Scholar were searched for manuscripts using keywords and combinations thereof of “oral cancer, OSCC, neutrophils, TANs, MDSC, immune cells, head and neck cancer, and tumor microenvironment” with a focus on publications from 2018 to 2021.

Keywords: neutrophil, immune cells, oral cancer, tumor microenvironment, myeloid cells, interaction, innate immunity

INTRODUCTION

Head and neck cancers (HNCs) are the seventh most common cancer worldwide and have a high mortality rate, with 177,384 deaths occurring in 2018, and a poor prognosis, with a 5-year relative survival rate of 68%. This survival rate is known to be poorer in developing countries (1–4). Oral cancer is often included in head and neck cancer statistics and represents 48% of HNC cases, with oral squamous cell carcinoma (OSCC) being the most common malignant lesion (approximately 90% of these cases) (5, 6). The OSCC develops in the oral cavity (namely, the lips, gums, lining of the cheeks and lips, front two-thirds of the tongue, floor of the mouth under the tongue, and roof of the mouth) and oropharynx (7). Despite advances in diagnosis and the availability of diverse treatment modalities, the global 5-year OSCC survival rate remains below 50% (8). Generally, the data support that with an earlier diagnosis comes a higher chance of survival with treatment. As patients with early-stage oral cancer have a 75% survival rate at 5 years, this decreases sharply to only a 35% survival rate for patients with advanced stages at diagnosis (9). This makes timely diagnosis and treatment essential for a good prognosis with OSCC. Though the oral cavity can be easily examined and assessed by direct visual inspection, most OSCC cases are diagnosed at an advanced stage (10). This most likely arises from the low rates of dental visits per year by people [e.g., on average, 56% of the Australian population sees a dentist once per year (11)], and most oral cancers commence as a painless surface lesion with erythema, minor elevation, and typically mimics benign processes in the mouth (12). Once lesions become intense masses, symptoms such as altered mucosa lining sensation, persistent sore throat, or ear infection, can appear, which then prompts medical intervention (9).

The etiology of OSCC is complex and is associated with several risk factors involving the interplay of the whole immune system, and recently, neutrophils have become the focus of several investigations as a pivotal cell in cancer development, which is the focus of this review. Identification of these factors has a significant impact on the prevention and early detection of cancer development. Although there are many risk factors associated with OSCC, alcohol and tobacco consumption, namely, smoking cigarettes, cigars, pipes, and chewing tobacco, are associated with 75% of OSCC tumors. Among the different compounds of cigarette smoke, nicotine is well known for its biological effects on the brain and other organs such as the oral cavity (13). Though nicotine is commonly acknowledged as non-carcinogenic, it is always accompanied in tobacco by carcinogens such as nitrosamines [i.e., 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N²-nitrosonornicotine (NNN)] (14). It has been shown that binding of nitrosamines to the nicotinic acetylcholine receptor promotes cell proliferation and creates a microenvironment for tumor growth (15). Overall, tobacco users have a five-fold increased risk of developing oral cancer and a 10-fold increase in developing laryngeal cancer in comparison to non-users (16). Significantly, smoking increases the neutrophil to lymphocyte ratio (NLR) a known prognostic marker, in cancer patients, leading to poor prognosis (17). Alcohol is known to

decompose the lipid composition of the epithelial cell membrane of the oral mucosa, thus facilitating carcinogen penetration (18). Frequent use of alcohol alone may result in OSCC *via* three mechanisms: (i) DNA adduct formation, (ii) interference with the DNA-repair mechanism, and (iii) generation of ethanol-related reactive oxygen metabolites (19). Alcohol use is known to affect the NLR of HNC patients, leading to poor prognosis (20). The consumption of alcohol alongside tobacco is known to have a multiplicative impact on increasing the risk of oral cancer, especially when both products are used on a regular basis (21).

Chronic viral infections of human cells can induce mutagenesis, potentially commencing cellular transformation and giving rise to malignant disease (22). Human papillomavirus (HPV) infection has recently been associated with the carcinogenesis of OSCC. In particular, HPV-16 is frequently isolated from oropharynx cancers of the tonsils and base of the tongue (23). It is estimated that 15–20% of all OSCC are related to high-risk HPV infection, which is the most common sexually transmitted virus (22). Likewise, HPV-DNA can be found in up to 70% of oropharyngeal squamous cell carcinomas (OPSCCs), particularly localized to the tonsils (24). As a result of poor oral hygiene, gingival inflammation may facilitate HPV penetration through the oral epithelial superficial layers to invade the basal layer (25). The association of HPV status and neutrophil infiltration in OSCC or OPSCC tissues has yet to be fully elucidated. However, one study by Li et al. (26) found that HPV positive OSCC patients had low levels of neutrophils, in part due to HPV positive OSCC cells expression resulting in low levels of the neutrophil chemotactic factor IL-8. Although studies have found that neutrophil levels are lower in HPV positive OSCC/HNC patients, other studies of patients have found the opposite or no significant association, indicating the complexity of this association and the requirement of further investigations to understand this relationship (27–30). In addition to HPV, Epstein–Barr virus (EBV), an oncogenic double-stranded DNA virus, is known to be involved in neoplastic transformation in oral cancers such as nasopharyngeal carcinoma (31). Nearly 60% of OSCCs were EBV genome positive (32) and increased expression of EBV correlates with poor OSCC prognosis (33, 34). Compounding this is that a high EBV DNA titer has been found to correlate with a high NLR and reduce overall survival (35). Most epidemiological studies show that HNC, and specifically oral cancer, typically occurs in the fifty to seventy-year-old age group (36). Nevertheless, there are reports that show 5% of HNC patients are in younger age groups (37). This correlates with higher rates of smoking and use of other drugs in younger age groups (38), and more recently, the increased prevalence of HPV (37).

More than 700 bacterial species are reported to be part of the bacterial flora in the oral cavity. In a healthy oral cavity, bacteria interact with each other and maintain a “good” balance. However, through poor oral hygiene, diet, or infection, this balance is broken, causing dysbiosis, which favors the growth of certain oral pathogens, leading to diseases such as caries and periodontal disease (39). Recent studies have confirmed a close link between OSCC and oral bacteria, which may present a fresh view and new potential targets for diagnosis and treatment of OSCC (40, 41). A study using a 16S rRNA V3-V5 marker gene

approach to compare oral bacterial DNA isolated from oropharyngeal and oral cavity squamous cell carcinoma patients and healthy subjects demonstrated the comprehensive relationships between OSCC and specific oral bacteria (42). Also, several studies have shown that oral bacteria such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* influence the development and progression of OSCC by altering the microbiota, which contributes to cancer development by enhancing cell proliferation, inhibiting apoptosis, and improving tumor invasion and metastasis (43, 44). It has been observed that *P. gingivalis* induces an increase in the oral tumor cell proliferation rate by modifying the expression levels of oncogenic-relevant α -defensin genes (45). Furthermore, *P. gingivalis* infection in OSCC patients has been positively correlated with increased levels of tumor-associated neutrophils and poor prognosis (46).

Many other factors are associated with OSCC, such as gender, previous cancer, prolonged sun exposure, poor oral hygiene, poor diet, family history, and various genetic mutations. Generally, OSCC is 2–5-fold more common in men (47). People who have previously had oral cancer have a greater risk of developing further oral cancer, particularly if alcohol and/or tobacco use is continued. OSCC develops from pre-existing, possibly malignant disorders like oral erythroplakia, lichenoid dysplastic lesions, and leukoplakia (48). Furthermore, combinations of specific genetic mutations and polymorphisms have been associated with an increased risk and development of oral cancers (49, 50). The multitude of risk factors and mechanisms that may lead to OSCC demonstrate the complex nature of the disease and the interplay of neutrophils with these factors and other immune cells and mechanisms in the initiation and development of OSCC is an area of research that requires investigation.

In addition to all the above-mentioned predisposing conditions, there are metabolic factors that can increase the risk of oral cancer. High concentrations of reactive oxygen species (ROS) lead to oxidative stress, which plays a crucial role in the destruction of key cellular components, such as DNA, proteins, and cell membranes. Because of these destructive mechanisms, ROS may contribute to the initiation and progression of multistage carcinogenesis (51). Oxidative stress is a key factor in the pathogenesis of cancer and can arise from poor nutritional habits, mainly a diet low in vegetables and fruits, which are rich sources of antioxidants, and lifestyle choices and practices (52, 53). In one study, patients with HNC had high oxidative stress and reduced antioxidant defense (54). Furthermore, OSCC patients had significantly higher levels of ROS (55). Given that neutrophils are major producers of ROS and neutrophil infiltrate increases in OSCC tissues lead to poor prognosis, this may be a novel therapeutic target.

Following the initiation of oral cancer, its development and progression at specific sites is heavily influenced by the immune system (56). It is now understood that specific cells of the immune system can have anti- and pro-tumor effects (57). With the development of immunotherapies to complement the standard care treatments of surgery, chemotherapy, and radiotherapy, it has become increasingly important to know how specific immune cells influence tumor growth,

progression, and metastases in OSCC. It is evident that many of the risk factors for OSCC may be associated with the presence/infiltration of neutrophils in the tumor, but how this cell population interacts in the tumor environment and with other immune cells has received little attention. This review will focus on describing the interplay of neutrophils and major subpopulations of cancer-associated immune cells and other factors, focusing from tumor initiation to metastatic colonization in OSCC.

TUMOR MICROENVIRONMENT AND IMMUNE EVASION

Several studies have supported the synergistic role of the tumor microenvironment (TME) in oral cancer development (58). The TME in HNSCC comprises many different cell populations, such as tumor cells, tumor stromal cells, namely, stromal fibroblasts, endothelial, and various infiltrating immune cells (neutrophils, macrophages, regulatory T cells, myeloid-derived suppressor cells, natural killer cells, platelets, and mast cells), and heightened non-cell components of the extracellular matrix (ECM) such as collagen, fibronectin, hyaluronan, laminin, among others (57, 59–61). Within the TME, malignant cells interact with the surrounding and infiltrating cells synergistically to promote cancer progression and that both the innate and adaptive immune responses contribute to tumorigenesis (62, 63). In the initial stages of tumor development, cytotoxic immune cells such as natural killer (NK) and CD8⁺ cytotoxic T cell lymphocytes (CTLs) identify and kill the more immunogenic cancer cells (64). Cancer cells that are less immunogenic and go undetected by the immune system are therefore positively selected and the cancer grows. As the neoplastic tissue progresses to a clinically evident tumor, different subsets of inflammatory cells impact the fate of the tumor (57). Although N1 neutrophils, M1 macrophages, dendritic cells (DCs), T helper 1 (Th1), and CTLs are involved in anti-tumor immunity (65, 66), certain immune cells such as N2 neutrophils, myeloid-derived suppressor cells (MDSC), M2 macrophages, tolerogenic DCs, T helper 2 (Th2), and T regulatory cells (Tregs) play an essential role in aiding and supporting cancer cell growth (65).

Understanding the mechanisms of how cancer cells avoid the immune system is a significant and on-going challenge in oncology. There are several well described mechanisms, albeit with a T cell and DC focus, by which tumors avoid the immune system and limit effective anti-tumor immunity by the host, these being: (i) induction of Treg cells (CD4⁺ CD25⁺ CTLA4⁺ GITR⁺ FOXP3⁺) that can suppress tumor-specific CD4/CD8 T cells (67); (ii) production of immunosuppressive cytokines, e.g., interleukin (IL)-10 (IL-10) and transforming growth factor beta (TGF- β) (68); (iii) decreased MHC-I expression due to gene loss *via* structural changes or β 2-microglobulin synthesis alteration; (iv) induction of dendritic cell (DC) anergy; (v) inhibiting DC maturation *via* producing and releasing granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-10 by tumor cells; and (vi) defective MHC-I antigen

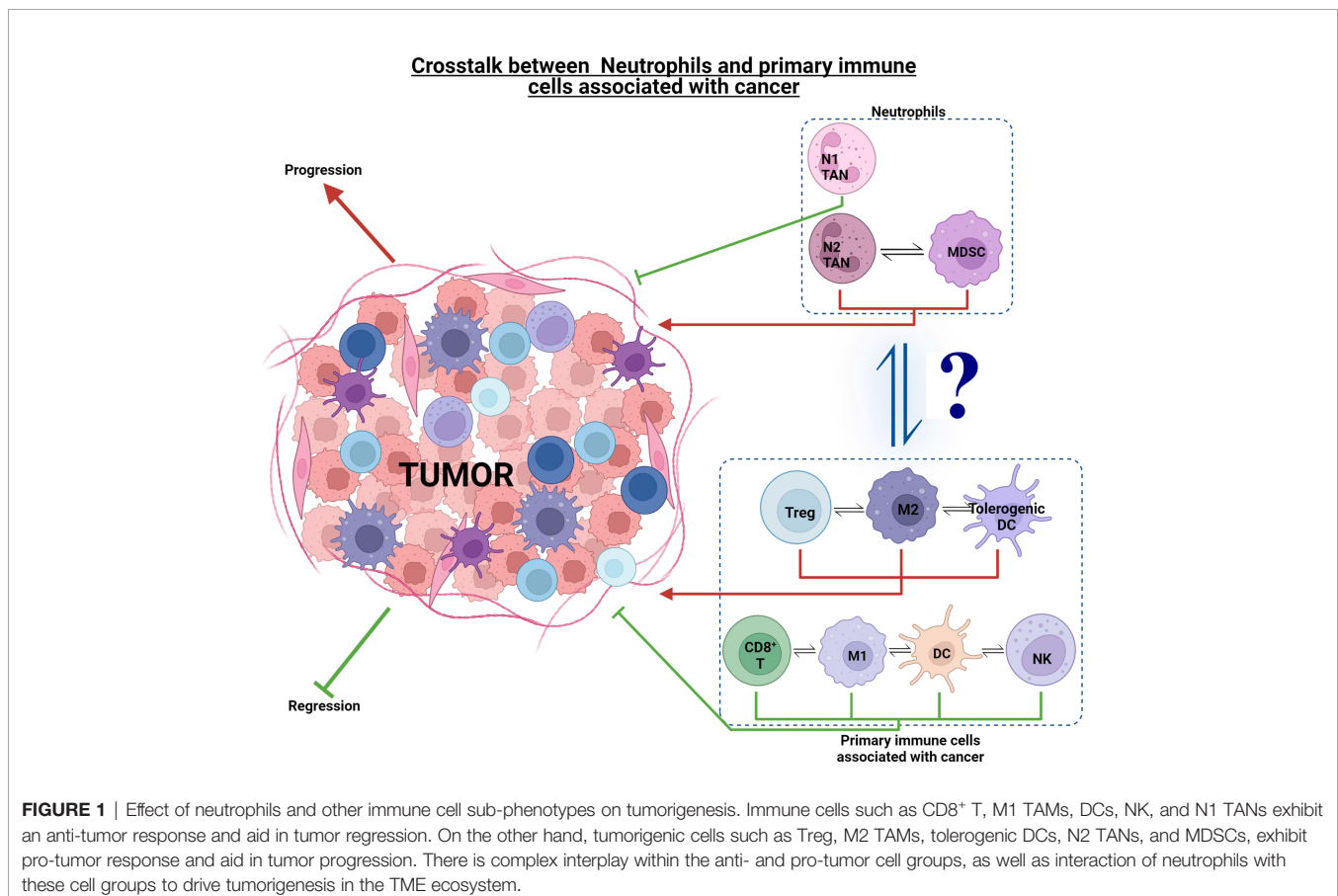
presentation *via* attenuation of the costimulatory molecule B7-1 (CD80) (69). It has been shown that CD8⁺ T cell tolerance can be induced by Gr-1⁺ immature myeloid cells (ImC) isolated from tumor-bearing mice (70). IL-6 has a suppressive action on DC maturation, which was attributed to the activation of the transcription factor STAT3 (71). IL-10 is thought to reduce costimulatory molecule expression on immature DC, resulting in tolerogenic APCs (72). Although these studies have a lymphocyte focus, these key cytokines (IL-6, IL-10, TGF- β , and GM-CSF) stimulate myeloid cells and activate the STAT3 signaling pathway in neutrophils and other myeloid cells such as macrophages (68, 71–74). All of these aid tumor growth and immune evasion, thus highlighting the complexity in cancer and the need to view the impact of one cell or chemokine/cytokine on the whole immune response and recognize that one cell ‘class,’ e.g., neutrophils, will have many faces/phenotypes in cancer (Figure 1) (73).

The type, proportion, and activation state of tumor infiltrating lymphocytes and myeloid cells are becoming increasingly important as prognostic markers for many cancers. A favorable prognosis is associated with a number of differing immune factors such as: high levels of memory CD8⁺ T cells, high expression of Th1 cytokines, i.e., interferon gamma (IFN- γ) and IL-1, the development of a tertiary lymphoid

structure (TLS) associated with the tumor, increased levels of cytotoxic mediators (granzymes, granulysin), low neutrophil-lymphocyte Ratio (NLR) and low to moderate vascularization of the tumor (75). Poor prognosis is associated with the lack of TLS, infiltration of neutrophils (particularly N2), M2 macrophages, and extensive vascularization (76). In many solid cancers, high levels of tumor-infiltrated T cells are associated with a good prognosis (77); in contrast, the influx of neutrophils and tumor-associated neutrophils (TANs), and high levels of macrophage infiltration, particularly the phenotype of tumor-associated macrophages (TAMs), are linked with a poor prognosis and a reduction in overall survival (78).

NEUTROPHILS: CELLS WITH A MULTITUDE OF ROLES

In the context of cancer, neutrophils have had less attention by comparison to other immune cells because it was thought that the lifespan of neutrophils is too short (7–10-hour circulating half-life in humans) to impact cancer development and progression (79). However, cytokines released by tumor cells, such as G-CSF, IL-1 β , IL-6, or tumor necrosis factor (TNF), have been proposed to



prolong neutrophil lifespan, indicating that neutrophils may have a significant impact on cancer (80, 81). Recently, it has been shown that uncoupled biological and chronological aging of neutrophils contributes to the progression of cancer and promotes advanced stages of malignant disease (82). Using a mouse squamous cell carcinoma cell line (SCC VII), we showed a noteworthy positive association between the RNA expression levels of formyl peptide receptor 1 (FPR1), an established marker gene of neutrophils, and of C-X-C motif chemokine receptor 4 (CXCR4), whose gene product increases during neutrophil aging on the surface of these immune cells, with higher tumor stages (83, 84). Activation of the NLRP3 inflammasome in perivascular macrophages by tumor-released Damage-associated molecular patterns (DAMPs) such as S100A8/9 induce the synthesis of inflammatory mediators that upregulate adhesion and signaling molecules on the surface of microvascular endothelial cells, in turn promoting the trafficking of aged neutrophils to the perivascular space (84). Following antibody-mediated depletion of aged neutrophils, a significant decrease in the growth of tumors was observed in experimental HNSCC (84). Aged neutrophils are related to a more pro-tumorigenic state. They support cancer cell proliferation *via* the release of neutrophil elastase (84, 85). The neutrophil-to-lymphocyte ratio (NLR) has recently been introduced as a better prognostic factor for survival in several types of solid tumors, including OSCC (86–89), as opposed to neutrophil levels alone (90), although the mechanisms involved in high NLRs (typically >3) are yet to be determined (91, 92). Several studies have found that higher NLR showed higher mortality rates compared with those with lower NLR and was associated with more advanced or aggressive cancer (93–95). In general, neutrophilia appears to be linked with a poor prognosis in cancer. However, the inverse might also be true to some extent in the context of antibody therapy, which has emerged as an important weapon in the anticancer armament (96–99). Recombinant technology presents huge opportunities to design antibodies to meet clinical requirements, including the reduction of immunogenicity (100). For example, antibodies can prevent tumor growth factors or their receptors, trigger immunologic attack on the tumor, or be used to provide payloads, for example, radioisotopes, cytotoxic drugs or toxins, and nanoparticles (101, 102).

It is known that the formation of neutrophil extracellular traps (NETs) activates platelets and stimulates thrombosis (103), and interestingly, an increased risk of cancer-associated venous thromboembolism (VTE) has been reported in numerous types of cancer, including OSCC (104). Previous studies have shown that NETs capture and operate as adhesion substrates for cancer cells, and using this process promotes metastatic dissemination (105). Park et al. (106) have shown that targeting NETs with DNase I-coated nanoparticles efficiently reduces metastasis in an *in vivo* cancer model, confirming that neutrophils and NET production are an important mechanism in cancer progression (106). A recent study investigating the myeloperoxidase (MPO) and histone expression using immunohistochemistry showed NETs in the tumor tissue of patients with OSCC (107). It has been shown, in OSCC patients, that the interaction between

cancer cells and neutrophils increases NET formation *via* the PI3K/Akt/PBK pathway (108). This co-existence of NETs and cancer demonstrates that the presence of NETs may be a marker of poor prognosis, highlighting their potential as a target for cancer therapy (109).

A novel prognostic model of HNSCC patients based on six-NET-related genes (Annexin A3 (ANXA3), lactotransferrin (LTF), colony-stimulating factor 2 (CSF2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), selectin P ligand (SELPLG), and cytochrome b-245 beta chain (CYBB)) was constructed that might be beneficial for developing personalized treatment directed at neutrophils (110). Irregular ANXA3 expression is associated with the development, occurrence, metastasis, and drug resistance of cancers (111). The LTF inhibits the development and release of NETs, which might be associated with the anticancer role of the gene (112). These studies indicate the potential clinical approaches for targeting neutrophils as a therapy in cancer treatment.

Polymorphonuclear granulocytes (PMN) from the peripheral blood of patients with late stage HNC showed a significantly lower inducible production of reactive oxygen species (ROS) and reduced spontaneous apoptosis compared with PMN from healthy donors (113). However, another clinical study showed there was an acceleration in the apoptosis of circulating PMNs of oral cavity cancer patients due to higher caspase-8 activity and elevated activity of the TRAIL-mediated mitochondrial cascade (114). Though this data may seem conflicting, it does indicate that peripheral blood PMN from HNSCC patients and healthy donors show distinct functional differences.

Several studies have shown that neutrophils have various and conflicting roles in cancer. After transmigration into tumor tissue, neutrophils [referred to as tumor-associated neutrophils (TANs)] go through dramatic changes in their activity and phenotype, depending on the cytokines and growth factors they encounter in the TME. Because of the minor size of primary tumors in HNC, data pertaining to TANs are limited (115). In a European gastric cancer cohort study, immunohistochemical staining of myeloperoxidase was used to show a correlation between TAN density and survival in women but not in men. These findings indicate a possible sex-specific prognostic effect of TANs (116, 117). In two independent clinical cohorts, the ratio of CD8⁺ T cells to TANs within the tumor was associated with anti-PD1 monotherapy failure in non-small cell lung cancer (NSCLC) patients, indicating that neutrophil antagonism may be a sustainable secondary therapeutic approach to boost ICI treatment outcomes (118). Recently, an association between the resistance of mismatch repair-deficient tumors to anti-PD-1 monotherapy and abnormal neutrophil accumulation within the tumor was reported (119).

TANs express CD11b⁺ Ly6C^{int} Ly6G^{hi} in mice and CD11b⁺ CD14⁻ CD66b⁺ CD15^{hi} in humans (120). Like the M1/M2 macrophage phenotype, TANs are suggested in cancer to exist in two polarization states, these being the anti-tumor (N1) or pro-tumor (N2) phenotypes (121). Regardless of the growing interest in TANs in recent years, our current understanding of the role of neutrophils in tumor development is primarily based

on murine models of cancer. In a human cancer population, low-density neutrophils (LDN) (N2 like) and high-density neutrophils (HDN) (N1 like) both express CD11b, CD66b, and CD15, but LDN express these at a higher level (122). Several studies in OSCC have shown neutrophils to express high levels of one or more of these markers in human cancer patients, consequently leading to poor prognosis (26, 123, 124). Furthermore, it has been shown that it is feasible to polarize blood-derived primary human neutrophils toward N1- and N2-like phenotypes *in vitro* (125). Also, human neutrophils incubated under a tumor-mimicking *in vitro* environment were found to highly express the typical N2 receptor CXCR2 on their surface and secreted elevated amounts of IL-8 (125). Thus, although human neutrophils do not have definitive N1 and N2 markers as in mice, there is, potentially, a N1 and N2 like neutrophil, i.e., HDN and LDN, respectively, which would appear to play the same role in humans. However, there is significant debate around human N1 and N2 neutrophils as a study by Brandau et al. (126) found that in peripheral blood human HNC, lung, bladder, and ureter cancer patients had a CD66+ PMN population but based on their LDN and HDN profile, the CD66+ LDN cells expressed low levels of CD11b and CXCR2. Further studies will be needed to investigate whether humans have N1 and N2 populations, as in mice the N1 and N2 phenotypes have a profound effect on cancer development, and from the few studies in humans that have described a N1-like or N2-like neutrophil phenotype they appear to also have a profound effect on tumor immunity.

At the early stages of tumor development, neutrophils mostly remain located at the edges of the tumor and have an N1 phenotype, eliminating cancerous cells and limiting metastatic seeding. As the tumor progresses, neutrophils are often found deeper within the tumor and transition toward a more aggressive N2 phenotype, enabling tumor growth to be supported (127). A humanized mouse model of hepatocellular carcinoma (HCC) showed that CCL2⁺ and CCL17⁺ chemokines, which are part of the N2 signature, promote macrophage (F4/80+) and regulatory T (Treg) cell (FoxP3+) infiltration into the TME by activating the MAPK and PI3K signaling pathways, which stimulate neovascularization, enhance growth and metastasis, and contribute to sorafenib (a kinase inhibitor drug) resistance (128). This switch of N1 to N2 with the maturation of cancer is very reminiscent of the M1 and M2 switches in TAMs, strongly suggesting that there is synergy between the immune cells and their respective functions and roles in cancer/tumor development. In the earliest stages of cancer, TANs stimulate T-cell proliferation and IFN- γ release (129), while in established tumors, TANs are immunosuppressive and linked with a more pro-tumor phenotype with tumor progression (130). In OSCC, *P. gingivalis* infection contributes to the enhanced CXCL8 and CCL2 secretion in the TME, which in turn recruits CD66b⁺ TANs to the site of neoplastic cells and the promotion of tumor development (131, 132). This strong immune induction of CXCL2, CXCL8 from neutrophils by *P. gingivalis* is well known in oral disease research and may aid in our understanding of how this bacterium is associated with a poor prognosis in OSCC patients (131, 132).

It has been shown that N2 neutrophils contribute to tumor growth by several mechanisms such as increased expression of pro-angiogenic genes (MMP9, VEGF) with absent IFN- β and is acquired by neutrophils following the TGF- β treatment/exposure (121, 133–135). MMP-9 is a protease produced predominantly by neutrophils (N2 neutrophils in mice) and located in its tertiary granules (136) and is involved in elevated cancer cell proliferation, angiogenesis induction, tumor growth, inhibition of cancer cell apoptosis, promotion of neutrophil extravasation, and migration into tissues by the degradation of the extra-cellular matrix (137, 138). An *in vitro* study using two oral squamous cell carcinoma cell lines (UT-SCC-43A and UT-SCC-43B) showed that the expression of MMP-2 and MMP-9 was downregulated in both cell lines after being incubated with human neutrophil peptide (HNP)-1 (a N1 neutrophil produced peptide), indicating a protective role of HNP-1 against the spread of metastatic cells (139). The antitumor mechanisms of another peptide, melatonin (Mel), are linked with anti-proliferation, apoptosis promotion, migration, invasion inhibition, and anti-angiogenesis (140). TANs were suppressed by Mel in a MMP-9-dependent manner in OSCC (141). These studies indicate that targeting MMP-9 expression is a possible therapeutic avenue to explore.

The serine protease neutrophil elastase (NE), located in neutrophil azurophilic granules (142), promotes the detachment of tumor cells through the degradation of the adhesion molecule E-cadherin, decreasing the stability of the tumor and increasing metastasis. Significant elastase expression has been shown to be upregulated in OSCC (143, 144). The elastase and serine protease inhibitor Secretory Leukocyte Protease Inhibitor (SLPI) was considerably reduced in OSCC compared with normal oral epithelium, and cancer cells treated *in vitro* with SLPI had reduced invasive ability, suggesting that SLPI is a therapeutic lead as it may decrease many tumor-promoting events (145). Another, neutrophil targeting therapy may be TGF- β blockade or IFN- β treatment as both promote neutrophil reversion to a cytotoxic N1 subset while expressing high levels of intercellular adhesion molecule 1 (ICAM1) and TNF- α and increasing NET formation (146, 147). Taken together, new novel cancer therapies may involve modulation of neutrophil function through alterations of the tumor microenvironment by blocking TGF- β activity or enhancing IFN- β activity instead of depleting specific neutrophil subsets such mature and immature low-density neutrophils (LDN) that accumulate continuously with cancer progression (148).

It has been shown that an increased neutrophil-to-lymphocyte ratio (NLR) is linked with poor survival in patients undertaking chemoradiotherapy or radiation for nasopharyngeal carcinoma (149). Another study revealed that in patients with nasopharyngeal carcinoma, NLR was a significant predictor of both survival and response to chemoradiotherapy (150). Several retrospective cohort studies have evaluated the prognostic significance of NLR in patients with oral squamous cell carcinoma (95, 151–153). They found that a low NLR was the only independently favorable marker of both overall survival and distant control in patients with OSCC; in contrast, a high NLR was associated with worse overall survival. These studies suggest

that preoperative NLR in the peripheral blood is an important prognostic factor for OSCC and is valuable in predicting OSCC development.

THE ANTI-METASTATIC ROLE OF NEUTROPHILS

Although numerous cancer studies support the pro-tumorigenic role of neutrophils, there is evidence that they also remove cancerous cells and limit metastatic seeding. Cytotoxic action of neutrophils towards cancer cells is mostly evident in early stages of tumor development in the form of N1 TANs, and killing has been shown to require a high level of target specificity (79). To induce tumor cell apoptosis, activated neutrophils are required to identify cancer cells as targets through Receptor for Advanced Glycation End products (RAGE)-Cathepsin G (directly) (154) or in an antibody dependent fashion (ADCC) (155). High expression of RAGE is observed in OSCC patients and is associated with depth of invasion (156, 157). It has been shown that RAGE expression is responsible for migration, invasion, and MMP-9 production in patients with OSCC, thus representing a possible therapeutic candidate in treating OSCC patients by enhancing N1 activity (158). After cancer cell identification, neutrophils then need to have physical contact with the tumor cells in order to release cytotoxic mediators such as myeloperoxidase (MPO), H_2O_2 , reactive oxygen species (ROS), and proteases (159). Neutrophil cytotoxicity is Ca^{2+} dependent and is mediated by the transient receptor potential cation channel, subfamily M, member 2 (TRPM2), a ubiquitously expressed H_2O_2 -dependent Ca^{2+} channel (160). TRPM2 expression is increased in cancerous tissues, making tumor cells more susceptible to neutrophil cytotoxicity (161). Using a breast cancer model, it has been shown that reduced expression of TRPM2 in tumor cells allowed neutrophil immune evasion but also led to tumor growth retardation, albeit accompanied by an increase in metastatic potential (162). Inhibiting the overexpression of TRPM2 in human tongue squamous samples with the small interfering RNA technique (shRNA-TRPM2) resulted in enhanced apoptosis of SCC cells and reduced the migratory abilities of SCC cells (163). Studies have shown that TRPM2 expression is elevated in circulating tumor cells (CTC) compared with the primary tumor, rendering CTC more susceptible to neutrophil cytotoxicity (162). Neutrophils as well as secreting H_2O_2 are able to suppress metastasis *via* their expression of thrombospondin 1 (TSP1) (164) and MET proto-oncogene, encoding the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF), which regulates invasive growth (165, 166). TSP1 can be induced in neutrophils by a peptide derived from prosaposin, a precursor of sphingolipid activator proteins (164). It is reported that MET, induced by tumor inflammatory stimuli such as $TNF-\alpha$, is essential for neutrophil chemoattraction and cytotoxicity in response to its ligand hepatocyte growth factor (HGF). C-MET-HGF stimulation leads to neutrophil transmigration across an activated endothelium and the production of inducible nitric oxide

synthase (iNOS). Subsequently, MET/HGF-dependent nitric oxide release by neutrophils assists in cancer cell killing, which greatly dampens tumor growth and metastasis (165). It has been shown that hypoxia activates HGF/c-Met signaling in a hypoxia-inducible factor-1 (HIF-1) dependent manner, leading to the invasive growth of cancer cells through activating the PI3K/Akt pathway (167). These studies indicate that targeting the HIF-1 α /c-Met signaling pathway using synthetic small-interfering RNA could be a useful new approach to the treatment of OSCC patients.

MYELOID-DERIVED SUPPRESSOR CELLS (MDSCs)—PATHOLOGICALLY ACTIVATED NEUTROPHILS?

Myeloid-derived suppressor cells (MDSCs) have been described in humans and mice and occur as two main sub-groups: monocytic MDSCs (Mo-MDSCs), granulocytic or polymorphonuclear MDSCs (G-MDSCs/PMN-MDSCs), and a third sub-type termed early MDSCs (eMDSCs) and their discovery has been recently reviewed (168). Of the two main sub-groups, polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and neutrophils share the same origin cell type, the differentiation pathway, and are phenotypically and morphologically alike, with both being identified in oral cancer patients (169–171). A recognized distinguishing feature of PMN-MDSCs is that they have been reported to be more immunosuppressive than immature neutrophils (172). Currently, several studies have attempted to distinguish PMN-MDSCs from activated immature or mature neutrophils as the heterogeneity of PMN-MDSCs means that they are indistinguishable from activated neutrophils as they share the same phenotypic markers: $CD14^- CD15^+ CD66b^+ CD16^+$ and $CD11b^+ CD33^+ HLA-DR^-$ (168, 173–176). Recently, for human PMN-MDSCs, the lectin-type oxidized LDL receptor 1 (LOX1) has been suggested as a distinguishing marker (177). In mice, the proportion of MDSCs has been shown to increase significantly within the tumor microenvironment (178) and represents potent suppressors of antitumor immunity (179). To regulate an immunosuppressive response, TANs and MDSCs block T-cell proliferation by releasing ARG1 and modulate PD-L1/PD-1 signaling, a potential tumor escape mechanism (Figure 2) (180, 181).

In a murine cancer model granulocytic myeloid-derived suppressor cells (G-MDSCs also known as PMN-MDSCs) and TANs induced CD8 T-cell apoptosis *via* the $TNF-\alpha$ pathway and NO production, thereby promoting a tumor-supportive environment (182). Further, using the established 4-nitroquinoline 1-oxide (4NQO)-induced oral cancer mouse model, Chu et al. (170) showed that there was a significant progressive increase in the proportion of MDSCs in the spleens and peripheral blood of 4NQO-treated mice compared to control mice, suggesting that MDSCs contribute to oral cancer progression (170). MDSCs were initially defined in HNSCC patients as immature $CD34^+$ cells presenting the ability to

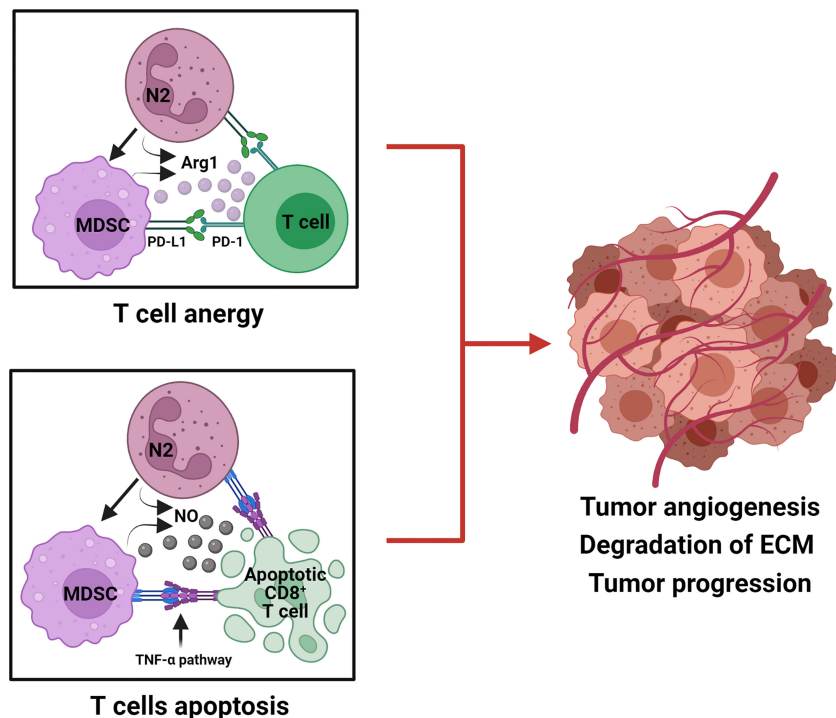


FIGURE 2 | Tumorigenic role of TANs (N2) and MDSCs in the suppression of T-cell responses. N2 TANs differentiate into MDSC, an activated and more immunosuppressive neutrophil phenotype. Both N2 and MDSC produce Arg 1 and upregulate PD-L1 to cause T-cell anergy by modulating PD-L1/PD-1 signaling. N2 and MDSC also produce nitric oxide (NO) which initiates the TNF- α pathway to induce CD8⁺ T-cell death, *via* apoptosis. N2 and MDSC hinder anti-tumor T-cell function by anergy and apoptosis and promote tumor progression.

suppress the activity of T cells (183, 184). Another study showed that CD34⁺ cells in HNSCC patients can be differentiated into cells that phenotypically and functionally resemble dendritic cells (185). Though MDSCs (pathogenically activated neutrophils) have been originally recognized for their immune-suppressive function in cancer, lately their presence has been associated with other activities within the TME, including promotion of tumor angiogenesis, degradation of extracellular matrix, and the formation of premetastatic niches (186, 187). The role of PMN-MDSCs in cancer and immunity, how they interact with other immune cells to affect their actions, is currently being defined and thus are probably a major foci of novel therapeutics, treatments and prognostic and diagnostic factors (168).

MACROPHAGES AND NEUTROPHILS: AN IMMUNOLOGICAL PARTNERSHIP AIDING CANCER GROWTH

The crosstalk between tumor cells and infiltrated neutrophils and macrophages can promote and drive tumor growth and metastasis (188). Arising from a common progenitor lineage, the multi-layered roles of TANs and TAMs are implicated in almost every step of tumor growth and metastasis. Both TAMs and TANs use multiple overlapping pathways to crosstalk with T cells, including

engagement of immune checkpoints and secretion of cytokines, resulting in tumor immune escape, as well as angiogenesis and invasion (189, 190). It is known that activated neutrophils provide signals for the activation, maturation, and recruitment of monocytes/macrophages, NK cells, and DCs by releasing IL-8, TNF- α , macrophage inflammatory protein-1 α (MIP-1 α), and MIP-1 β , indicating that neutrophils play a central role in the involvement of these three major cell phenotypes in immunity (191–194). Murine neutrophils secrete myeloperoxidase (MPO) and have a direct tissue damaging effect. They are also recognized by tissue resident macrophages expressing macrophage mannose receptors (MMRs) (195, 196). This recognition of MPO by MMR⁺ macrophages activates macrophages, which in return overproduce neutrophil survival factors, namely, IL-1, IL-6, IL-8, TNF- α , and GM-CSF, which activate neutrophils and upregulate their survival mechanisms (191, 197). Though limited direct evidence that supports TAN and TAM interaction through MPO and the MMR is available, high density MPO-positive neutrophil infiltration has been reported in colorectal cancer (198). Intriguingly, this neutrophil and macrophage interplay results in an increase in neutrophil half-life/survival, which is a feature of N2 neutrophils, TANs, and PMN-MDSCs. However this dynamic requires further investigation. The importance of murine neutrophil MPO has been shown using a novel tripeptide MPO inhibitor [N-acetyl lysyltyrosylcysteine amide (KYC)], which diminished lung tumor burden, suggesting that targeting

neutrophil MPO is a novel cancer treatment (199). A cytokine impacting the activation of macrophages and neutrophils is TGF- β , which as well as being produced by many infiltrating cells in the TME, is also highly expressed by cancer cells, including OSCC cells (200). It is thought that the interplay of TGF- β with macrophages and neutrophils generates M2-like and N2-like cells suggests a close relationship between TAMs and TANs in the same TME and the possibility that recruitment of macrophages by neutrophils may lead their N2-like polarization (201). However, studies must confirm whether the crosstalk between TANs and TAMs in the TME is comparable to the known interactions between neutrophils and macrophages in a non-tumoral chronic inflammatory environment.

Despite the significant role of macrophages in promoting host defenses, their inappropriate or extended activation can lead to immune dysregulation and the promotion of cancer. The role of macrophages in tumor progression and interaction with other infiltrating immune cells is yet to be completely clarified, partly because of the plasticity of macrophages and the conflicting roles of their different phenotypes. In response to malignant cell-derived growth factors and chemokines including colony-stimulating factor-1 (CSF-1) (202), VEGF-A (203), chemokine (C-C motif) ligand such as CCL2 (MCP1) (204), CCL18, CCL20 (MIP3a), and CXCL12 (SDF1), bone-marrow derived monocytes or tissue-resident macrophages are recruited into the tumor site and are then termed Tumor Associated Macrophages (TAMs) (205). CSF-1 in binding to its receptor on monocytes and macrophages ((macrophage colony-stimulating factor receptor (M-CSFR)) is known to have a critical role in differentiating the phenotypes of macrophage subsets in tumors (206, 207). Significantly, murine neutrophils and MDSCs have been shown to be major producers of CSF-1, resulting in macrophage polarization and an immune tolerant/suppression phenotype, further strengthening, albeit yet to be proven in cancer, the interplay of neutrophils and macrophages in the TME (177, 208).

During carcinogenesis, TAMs mainly exhibit an M1-like polarization that results in the elimination of the more immunogenic cancer cells. As the tumor progresses, the changing composition of the TME provokes an M2-like repolarization of TAMs that is pro-tumorigenic and supports primary tumor growth and metastatic spread (209, 210). The effect of TAMs on tumor progression can depend on the tumor nature, the type of TME, and the intra-tumoral location of TAMs (211). It has been suggested that TAMs can combine the properties of M1 and M2 macrophages (212). Hence, the presence of TAMs by themselves does not have prognostic value, and so an M1/M2 ratio is used. Low and high M1/M2 TAM ratios are associated with poor and good prognosis, respectively (213). The differentiation to M1 or M2 phenotype and the ratio of M1/M2 is heavily aided and/or influenced by the presence and secretion of cytokine/chemokines by neutrophils and it appears vice versa (214).

This neutrophil/macrophage interplay can be considered a crucial factor in cancer growth and prognosis as multiple studies have reported a strong association with the role of M2-like TAM

phenotypes and oral cancer aggressiveness (215–219). One of the predominant TAM markers that is correlated with a poor clinical prognosis is CD163 (220). Indeed, an increase in CD163 expression is seen in advanced OSCC compared with premalignant lesions (218, 221) and initial tumor stages (216, 220). The ratio between CD163⁺ and CD68⁺ (pan macrophage marker) increases in oral cancer with lymphogenic metastasis (222). Another TAM marker, CD206, was correlated with cancer aggressiveness and clinical prognosis (219). The significance of CD206 is evident, as a radiotracer specific to CD206 is clinically used to identify sentinel lymph nodes in oral cancer patients to aid in OSCC diagnosis and treatment decisions (223).

TAMs promote tumor progression in several different ways. TAMs not only directly provide structural support for cancer development but also contribute to tumor induction by producing signaling molecules and extracellular vesicles. These vesicles play a significant role in crosstalk between cells by transferring bioactive cargo such as microRNAs (miRNAs) to recipient cells (224). TAMs can also directly communicate with tumor stem cells to support their survival by secreting growth factors, and in return, tumor stem cells provide essential tumor-promoting signals to activate TAMs that promote tumorigenesis (225). Furthermore, TAM-secreted cytokines induce anti-apoptotic programs in cancer cells (226–228). Following activation of the STAT3 pathway due to TAM-derived IL-6, tumor suppressor miR-204-5p expression significantly decreased, increasing in the anti-apoptotic protein RAB22A and B-cell lymphoma 2 (Bcl2) expressions in cancer cells (229–231). Thus, TAMs can enhance cancer cell resistance to chemotherapy and radiotherapy.

Another crucial role of TAMs in cancer is metastasis. TAMs allow tumor cell invasion and migration *via* cathepsins, secreting matrix metalloproteinases (MMP) and serine proteases, which alter cell-cell junctions and disturb basal membranes (232). TAMs either directly or indirectly inactivate T-cell responses or facilitate immune escape within tumors (233). Direct strategies include (i) depletion of metabolites essential for T-cell proliferation such as L-arginine, which is necessary for T-cell fitness and anti-tumor activity, through the expression of arginase-1 (ARG1), (ii) production of reactive oxygen species (ROS), (iii) expression of immune checkpoint ligands such as programmed cell death ligands (PDL1 and PDL2), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (B7-1 and B7-2) and B7-H4, and (iv) producing anti-inflammatory cytokines such as IL-10 and TGF- β (209, 234). These well-known macrophage mechanisms of T-cell inactivation are present in neutrophils, which are known to express ARG1, ROS, PDL1, and IL-10 (235, 236). It must be noted here that there is debate over whether human neutrophils produce IL-10. However, Lewkowicz et al. (237) have shown that in inflammatory settings, human neutrophils do produce IL-10. Though TAMs predominantly present pro-tumorigenic roles, the plasticity of macrophages has been used in a breast cancer model to re-program pro-tumorigenic TAMs (238). It has been shown that upon treatment with the class IIa histone deacetylase inhibitor,

TMP195, TAMs become activated and reprogrammed to an extremely phagocytic phenotype, resulting in a reduction in tumor volume (238).

Clinical studies have indicated a link between the recruitment of TAMs and poor overall survival in OSCC patients and suggest this could be used as a potential prognostic marker (239, 240). Immuno-histochemistry analysis of OSCC indicated considerable TAM infiltration compared to control samples and the existence of CD68⁺CD163⁺(M2) TAMs or CD206⁺(M2-like) TAMs were linked with poor overall survival (241–243). Additionally, CD163⁺(M2) TAMs are linked with chemoresistance in esophageal cancer (244) and primary HPV-negative HNSCC (245). TAMs can adopt an extensive range of diverse activation states between M1 (classical) and M2 (non-classical), expressing both M1 and M2 markers, upregulated TNF- α (M1) (246), matrix metalloproteinase 9 (MMP-9) (M1) (246), increased levels of CCL2, CCL5, CXCL9, CXCL10, and CXCL16 chemokines (M1) (247), upregulated IL-10 (M2) (248), arginase-1 (Arg1) (M2) (249), and peroxisome proliferator-activated receptor γ (PPAR γ) (M2) (250). Though the overall number of TAMs accumulated within a tumor is not considered in the assessment of clinical prognosis, the ratio of M1/M2 is considered an important prognostic marker (251, 252). A clinical cohort study showed that high expression of receptor for activated C kinase 1 (RACK1) inhibits macrophage recruitment and decreases the M1/M2 ratio (tumor having a higher M2 proportion) *via* the NF-KB pathway, promoting the development of OSCC, indicating RACK1 and the M1/M2 ratio are predictors of a poor prognosis in (253). The increased understanding of the role of TAMs in carcinogenesis is reflected across many immune cells in the TME and, similarly, the N1/N2 ratio is currently being investigated as a prognostic marker and, along with the NLR ratio, there may come a point whereby we can use several cell-based ratios to inform more accurate treatment and prognostic outcomes.

CROSSTALK BETWEEN DENDRITIC CELLS AND NEUTROPHILS

Upon activation by numerous inflammatory stimuli, neutrophils release several inflammatory proteins (e.g., TNF- α) (254) and different alarmins such as defensins, cathelicidins (LL-37), lactoferrin, and high-mobility group box-1 (HMG-B1), with the ability to stimulate the maturation of immature DCs (255–258). Alarmins induce the maturation of immature DCs and their recruitment at the site of inflammation by acting on G α -protein-coupled-receptor (G α PCR) and activating receptors and also by stimulating the production of chemokines by leukocytes (259). It has been shown that neutrophil derived α -Defensins, human neutrophil peptide-1 and -2 (HNP-1 and -2), contribute to adaptive immunity by mobilizing DCs and T cells (260). β defensins secreted by neutrophils bind to TLR-4 receptors expressed on immature DCs, promoting their maturation and the initiation of adaptive immunity (261). HMG-B1 induces the migration and activation of immature

DCs, leading to DC stimulation of T-cell proliferation and T helper 1 polarization (262). This DC initiation of a T-cell response is reliant on the binding of neutrophil Mac-1 and CEACAM1 (carcinoembryonic antigen-related cellular adhesion molecule-1 or CD66a) to the DC-specific receptor, DC-SIGN, resulting in the delivery of activation signals and antigenic molecules to DCs and the initiation of a T-cell response (263, 264). This cellular adhesion can also regulate neutrophil proliferation and prolong the survival of neutrophil granulocytes (265, 266).

Accumulating evidence indicates that DCs play a significant role in driving immune suppression against tumor-associated antigens (**Figure 3**) (267). The migration of DCs is critical for tumor immune surveillance (268). This includes DCs migrating to tumor sites, capturing and endocytosing dead tumor cells or cellular debris, and transporting tumor-associated antigens to tumor draining lymph nodes (TDLNs), where they induce tumor-specific T-cell activation (269). DC recruitment to the TME relies on chemokines such as CCL4, CCL5, and XCL1, while CCR7 is required for migration of DCs to TDLNs (268). Neutrophils are known producers of CCL4 and CCL5, so they would contribute to DC recruitment to the TME (270). Generally, it is assumed that informative signals within the TME program DCs into a tolerogenic or immunosuppressive state rather than an inflammatory state (271, 272). The infiltration of BDCA3⁺ cDC1s in the TME has been shown to be associated with greater T-cell infiltration, improved prognosis in cancer patients, and better efficacy of cancer immunotherapies (273), emphasizing the important positive role of cDC1s in generating antitumor immune response in the TME.

Antitumor immunity has been found to be extremely dependent upon expression of the type I IFN receptor (IFNAR1) (274). Thus, administration of type I IFNs (IFN- α , IFN- β) is considered a treatment strategy in cancer (275) as they facilitate DC activation, migration, and cross-presentation, thus enhancing the DC anti-tumor immunity (276). Type I interferon treatment may also have additional benefits as type I IFN treatment will aid neutrophil antitumor activity by polarizing them to the N1 phenotype (146). In an *in vitro* study it was demonstrated that sensing of nucleic acids through the cyclic-GMP-AMP synthase (cGAS)-stimulator of the interferon genes complex (STING) pathway and interferon regulatory factor 3 (IRF3) contributes to DC activation and IFN- β production in antitumor immunity (277). DCs can also facilitate the trafficking of effector T cells into tumors by producing certain chemokines. For instance, CD8⁺ T-cell recruitment into the TME is mediated through the chemokines CXCL9 and CXCL10, which are produced by tumor-infiltrating cDC1s (278). Neutrophil migration has been shown in mice and humans to be induced *via* a CXCR3–CXCL9 and CXCR3–CXCL10 axis (279–281). Thus, cDC1s would also recruit neutrophils, and as neutrophils are also major producers of these two chemokines, there would be positive reinforcement of CD8⁺ T cells and further neutrophil infiltration into the TME (270).

The TME comprises a range of immunosuppressive factors known to inhibit DC antitumor activity and infiltration,

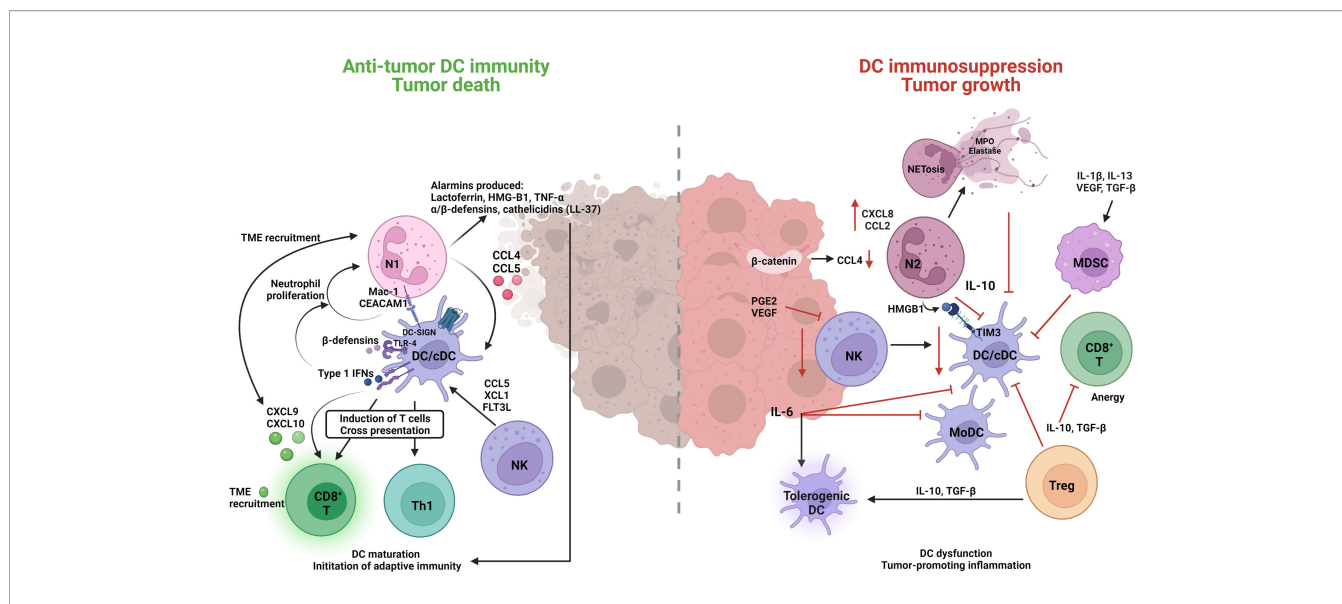


FIGURE 3 | Neutrophil engagement with dendritic cells in the TME can result in immune-suppressive or immune-promotion of cancer pathology. Classical/conventional DC1 cells (cDC1) are the predominant subtype orchestrating an anti-tumor response through the interplay with N1 TAN, CD8⁺ cytotoxic T (CD8⁺ T) and natural killer (NK) cells. N1s produce several alarmins and cytokines that induce DC maturation, TME recruitment, and the initiation of anti-tumor adaptive immunity. DCs in-turn induce N1 proliferation and survival through cytokine/chemokine release, Type I IFNs, β-defensins and direct interaction MAC-CEACAM1/DC-SIGN receptor engagement. N1-induced DCs have a greater propensity to engage with and enhance the functions of anti-tumor CD8⁺ T cells, Th1, and NK cells to induce cytotoxic killing of tumor cells. On the other hand, N2 TANs and MDSCs have a suppressive role on DC functions and promote tumorigenic tolerant DCs. Reduction in N2 produced CCL4 leads to decreased cDC tumor infiltrate, an βincrease in N2 CXCL8 and CLL2 contributes to tumor progression and invasion pathways, N2–DC cell interaction HMGB1-TIM3, and IL-10 production leads to inhibition of cDCs. The inhibition of cDCs further compromises other anti-tumor immune cells (NK, CD8⁺ T) and allows for suppressive cells (N2, MDSC, Treg, tolerogenic DCs) to promote tumor growth.

promoting immune tolerance and tumor progression (282). A high concentration of cDC1s within the TME has been correlated with good prognosis. However, tumor cell-intrinsic factors can limit cDC1 recruitment (268). It has been shown that active β-catenin in TME induces low CCL4 expression, leading to a significant reduction of cDC1 infiltrate and consequently an increase in tumor growth (283). Additionally, depending on the release of pro-inflammatory mediators, e.g., cytokines and granule contents by neutrophils-through NETosis or degranulation, neutrophils may either suppress or promote T-cell activation in the context of cancer immunity (284). For instance, the release of lactoferrin promotes the recruitment and activation of DC (285), while myeloperoxidase (MPO) and elastase, which are abundantly expressed by neutrophils, have a suppressive impact on DC migration and activation, although the role of neutrophils here is to be elucidated (286). In contrast, tumor-infiltrating NK cells have induced cDC1s recruitment by CCL5 and XCL1 production (282), and promote cDC development and proliferation, with FMS-like tyrosine kinase 3 ligand (FLT3L) (287). However, tumor cells can produce PGE2, which reduces FLT3L-producing NK cells and pro-inflammatory chemokine production. This in turn reduces cDC1 infiltration and the terminal differentiation of pre-DCs, resulting in tumor-promoting inflammation (288).

Cancer cells secrete IL-6. Although a pro-inflammatory cytokine, it reduces cDCs and MoDCs differentiation and promotes tumor DC dysfunction (289, 290). A dual function of IL-6 and M-CSF in tumor promotion is that they inhibit CD34⁺

progenitor differentiation into DCs but then induce their commitment towards CD14⁺ monocytes with an effective phagocytic capability but lacking APC functionality, thus failing to mediate allogeneic T-cell proliferation (291). Tumor-derived IL-6 is reported to be involved in the induction of tolerogenic DC phenotypes (292), but can switch the monocyte differentiation to macrophages rather than DCs (293). Several factors, such as IL-1β, IL-13, vascular endothelial growth factor (VEGF), and transforming growth factor beta (TGF-β) that are secreted by TME tumor cells, inhibit cDC maturation and survival and promote their differentiation into immunosuppressive cells, e.g., tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (294). In particular, VEGF can inhibit FLT3 ligand (FL) activity and suppress cDC differentiation (295). Treg cells are commonly found in the TME and produce IL-10 and TGFβ, which are two potent immunosuppressive cytokines resulting in DC dysfunction (296). Additionally, neutrophils have been shown in OSCC patients to express IL-10, indicating they would also contribute to DC dysfunction (297). IL-10 inhibits several aspects of DC biology, including DC maturation, IL-12 production, and antigen presentation to T cells (298). Further, it has been shown that IL-10 provokes a switch from an immunogenic DC profile toward a tolerogenic DC state and the induction of antigen-specific anergy in cytotoxic CD8⁺ T cells (299). Further, Treg produced TGF-β can inactivate DC function by inhibiting DC maturation (300).

The process of apoptotic cell death plays an important role in determining immunogenicity as it induces the activation of cDCs

and primes humoral and/or effector T cell-mediated immune responses (immunogenic cell death) (301). Immunogenic cell death depends on the alarmin high mobility group protein B1 (HMGB1) (302) as it binds nucleic acids released from dying tumor cells in the DC endosome, facilitating innate sensing of dead tumor cell nucleic acids (303). However, these processes are often inhibited in tumor-infiltrating cDCs through high expression of the inhibitory receptor T-cell immunoglobulin and mucin domain 3 (TIM3), which interacts with alarmin HMGB1, inhibiting anti-tumor responses and reducing the efficacy of cancer treatments (304, 305). In patients with resectable non-small cell lung cancer, the ratio of CD66b⁺ tumor-infiltrating neutrophils (TINs) to CD8⁺ T cells is reported as an independent prognostic factor for high tumor recurrence and poor overall survival (306). In a recent study conducted in lung adenocarcinoma, it has been shown that CD66b⁺ TIN infiltration significantly correlated with TIM3 expression (307). It has been shown that antibody crosslinking of TIM-3 results in tyrosine phosphorylation and the activation of the nonreceptor tyrosine kinases, Bruton's tyrosine kinase (Btk) and c-Src, which then suppress DC activation and maturation *via* inhibition of the NF- κ B pathway (308). These studies emulate the diversity and complexity of tumor immunity and how several immune cells are interconnected to produce a single outcome, which needs further work to be fully elucidated.

CD47, a transmembrane protein known as a "do not eat me" signal, which is highly expressed on tumor cells, interacts with signal regulatory protein α (SIRP α) expressed on dendritic cells (309). Engagement of SIRP α by CD47 promotes the phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIMs) in the cytoplasmic tail of SIRP α , which in turn recruits SHP-1 and/or SHP-2 [src homology-2 (SH2)-domain containing protein tyrosine phosphatases] to dephosphorylate motor protein myosin IIA, thus preventing phagocytosis (310). Abundant expression of CD47 has been associated with poor survival in several types of cancers (311). DCs are more dedicated in employing cytosolic DNA sensing pathways to connect innate response to adaptive response following anti-CD47-mediated phagocytosis (310, 312). It has been shown that blockade of CD47 facilitated the activation of NADPH oxidase NOX2 in DCs, which in turn prevented phagosomal acidification and decreased the degradation of tumor mitochondrial DNA (mtDNA) in DCs (312). A recent study has shown that oxidized mtDNA from irradiated cancer cells can translocate to the cytosol of dendritic cells (313), activating the STING (stimulator of interferon genes)-TBK1 (TANK-binding kinase 1)-IRF3 (transcription factor interferon regulatory factor 3)-IFN- β pathway enhancing antigen cross-presentation, CD8⁺ T-cell activation and antitumor immunity and resulting in tumor rejection (312, 313). Tumors are known to produce colony-stimulating factor-1 (CSF-1) which recruits TAMs, which in turn inhibit DC maturation (314). Additionally, it has been shown that CSF1 producing neutrophils mediate immunological tolerance by promoting the development of proliferating Ly6C^{lo} macrophages with suppressive function (208). Tumors can also induce DC dysfunction *via* altering DC

metabolism, for example, by increasing the accumulation of truncated fatty acids such as triglycerides in DCs (315). It has also been shown that a high lipid content within DCs reduces their ability to activate allogeneic T cells or present antigens, indicating cancer immune responses can be manipulated, positively or negatively, by altering the lipid levels in DCs (315).

Several signaling pathways such as β -catenin, signal transducer and activator of transcription (STAT), and mitogen-activated protein kinase (MAPK) trigger multiple immunosuppressive cascades in cancer (316). In addition to these signaling pathways, the Wnt signaling pathway is emerging as having a fundamental role in shaping the functions of DCs in the TME (317–319). Currently, nineteen Wnt proteins (lipid-modified cysteine-rich glycoproteins) typically 350–400 amino acids in length and ten cognate Frizzled (Fzd) receptors have been identified in humans (320). It has been reported that the Wnt family of ligands is highly expressed in the TME and that different tumor types have different composition profiles of Wnt proteins (320). For instance, Wnt1 is highly expressed in lung adenocarcinoma (321), while in melanoma (322), and oral carcinogenesis (323), high expression of Wnt3a and Wnt5a are found. In addition to affecting DCs in the TME, Wnt3a and Wnt5a, albeit not directly shown in cancer, affect neutrophil maturation and recruitment, with Wnt5a being shown to act as a chemoattractant and induce CXCL8 and CCL2 from neutrophils, two chemokines recently implicated in OSCC progression and invasion (324–328).

EXTRACELLULAR MATRIX (ECM) AND CANCER-ASSOCIATED FIBROBLASTS (CAFs)

In cancer, the extracellular matrix (ECM) is a non-cellular network consisting of macromolecules such as collagen, fibrous structural proteins, glycoproteins, growth factors, and proteoglycans that provide structural and biochemical support to surrounding cells (329). The formation of deregulated and disorganized ECM results in the promotion of malignant cell transformation (330). Several proteases released by neutrophils can contribute to the continuous remodeling process of the ECM and mediate immune responses (331). In the context of cancer, neutrophils release neutrophil elastase (NE) in large quantities that, through its influential protease activity, can cleave not only elastin but also other extracellular matrix proteins such as collagen, laminin, and numerous transmembrane proteins, which devastate the firm junctions between cells and provoke the exudation and migration of neutrophils (332, 333). In addition, boosted NE activity activates matrix-metalloproteinases (MMPs), which may improve the degradation of ECM and cause tissue damage (334). It is assumed that upregulation of neutrophil-derived MMP-8 and MMP-9 can degrade lung structure proteins such as collagen and elastin to produce bioactive peptides that stimulate neutrophil chemotaxis through CXCR1/2 receptor activation, supporting the occurrence of inflammatory cascades

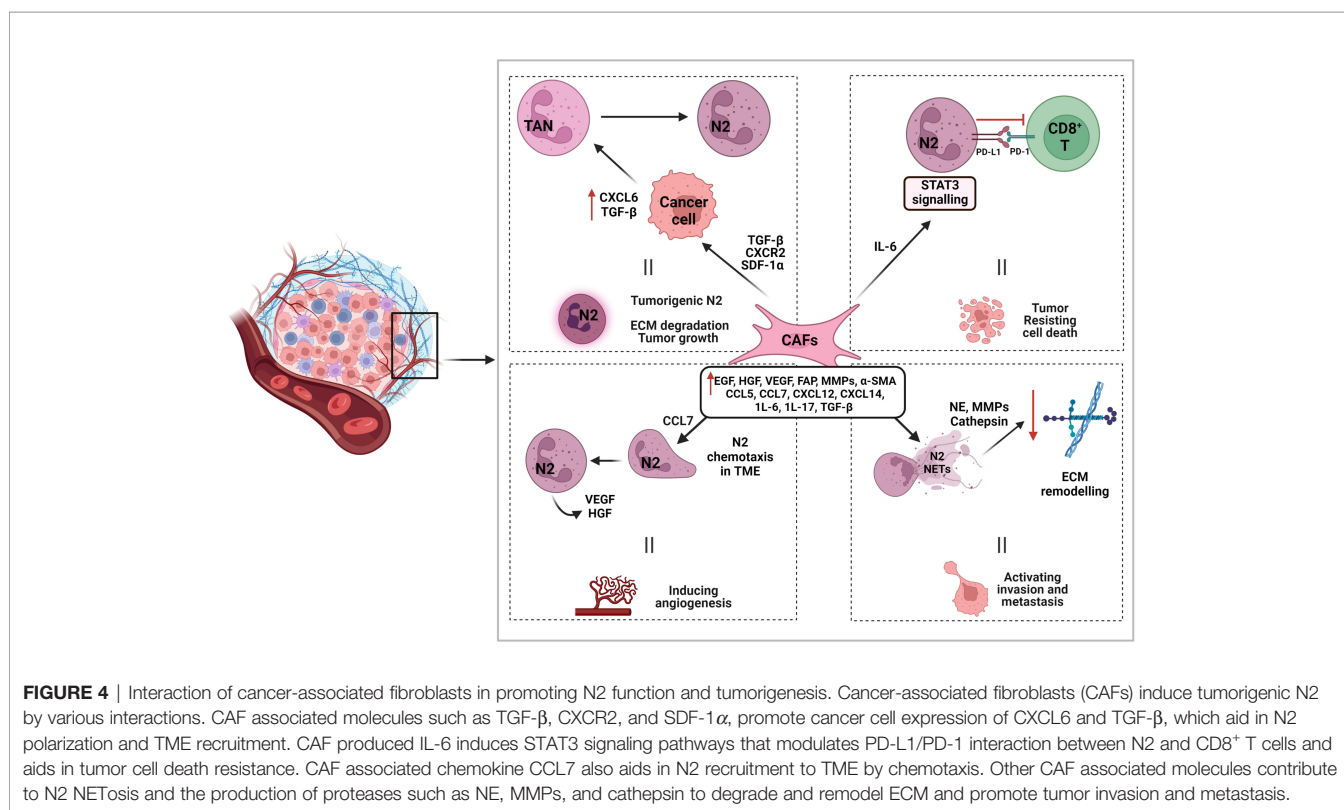
(335, 336). In an inducible colon tumor mouse model, neutrophil-secreted MMP-9 stimulates latent TGF- β in the ECM by damaging the ECM, enhancing TGF β in the TME, and resulting in suppressing the antitumor T-cell response (337). Cathepsin G, a serine protease secreted by activated neutrophils, promotes E-cadherin/catenin complex formation on fibronectin and thereby induces cell–cell adhesion of MCF-7 human breast cancer cells, suggesting that cathepsin G plays a role in tumor development and metastasis (338).

Collagen, laminin, and fibronectin are the main ECM proteins involved in HNSCC development and progression (339). Immunohistological studies of different histological grades of HNSCC indicated a direct relationship between the presence of collagen/or laminin and the degree of differentiation of oral squamous cell carcinoma (340, 341). A decreased distribution of ECM proteins was positively associated with increasing cancer stages, with the deposition of collagen or laminin decreasing with higher histopathological grades and an absence of staining associated with a poor prognosis (342). Coculturing UMSSC47 cells (OSCC cell line) and neutrophils was shown to increase UMSSC47 invasion and matrix degradation (343). In highly invasive primary OSCC tumors, the expressions of laminin, collagen type IV, and vitronectin were decreased. In contrast, the expressions of fibronectin and tenascin were increased, indicating that the composition of ECMs in OSCC is valuable in predicting tumor behavior (344).

The main function of cancer-associated fibroblasts (CAFs) main function has been shown to be in preserving the microenvironment for tumor cell growth and proliferation *via*

the secretion of a large variety of autocrine and paracrine cytokines and other tumor-promoting factors such as CCL5, CCL7, CXCL12, CXCL14, epidermal growth factor (EGF), hepatocyte growth factor (HGF), IL-6, IL-17, and VEGF (61, 345–348) (**Figure 4**). It has been shown that CCL5 is an effective inducer of neutrophil recruitment in septic lung injury through the formation of CXCL2 in alveolar macrophages (349). CCL7 generated by CAFs is the key promoter of OSCC cell migration and invasion, guides cytoskeletal transformation, and triggers membrane ruffling and cell dissemination (350). CCL7 exerts its carcinogenic properties as a chemoattractant for neutrophils involved in the formation of the tumor microenvironment (351). It has been demonstrated that neutrophils are directly angiogenic by releasing VEGF and HGF (352).

It has been shown that CAFs influence the motility of cancer cells by inducing epithelial–mesenchymal transition (EMT) *via* secreted cytokines in endometrial cancer cells (353). The best markers used to identify CAFs in the TME are (i) α -smooth muscle actin (α -SMA), a specific marker of myofibroblasts (354) and (ii) fibroblast activation protein (FAP) (355). In a clinical study, α -SMA was upregulated and correlated with poor prognosis in oral carcinoma (356). Another study found that upregulation of FAP at the mRNA level in human tongue squamous cell carcinoma was also linked to poor prognosis (357). It has been demonstrated that an abundance of myofibroblasts leads to more aggressive behavior of the squamous cell carcinomas and is associated with a worse prognosis in HNSCC patients (358). A strong association between increased CAF density and higher mortality in mobile



tongue squamous cell carcinoma has been reported (359). Numerous immunohistochemical studies have shown that HNSCC-derived CAFs express high levels of TGF β , hepatocyte growth factor, and MMPs compared with healthy fibroblasts (360–362). In the context of the TME, blockade of TGF- β results in the recruitment and activation of TANs with an antitumor phenotype, indicating a major role of TGF- β in tumor promoting N2 polarization (121). This recruitment of neutrophils upregulates the expression of MMP9 and MMP-9⁺ neutrophils play a functional and concomitant role in tumor cell angiogenesis and intravasation (363).

CAFs might be able to modulate the polarization of TANs. A recent study showed that CAF-derived cardiostrophin-like cytokine factor 1 (CLCF1) induces TAN-N2 polarization by increasing the expression of CXCL6 and TGF- β in tumor cells, thereby accelerating tumor progression (364). Another study showed that CAFs recruit neutrophils to tumors by producing stromal cell-derived factor 1 (SDF-1 α , known as CXCL12) (365). Furthermore, CAFs enhance TAN recruitment in a CXCR2-dependent manner (366). CAF-derived IL-6 induces the activation of STAT3 pathways in TANs, which are essential for the survival and function of activated neutrophils, subsequently suppressing T-cell immunity and inducing immune tolerance in a PD1/PDL1-dependent manner within the TME (364). This interaction of CAFs and neutrophils needs to be explored to understand how cancer progresses *via* this interaction and the possibilities to disrupt this mechanism through therapeutic targeting.

INTERACTION OF NATURAL KILLER (NK) CELLS AND NEUTROPHILS

Natural killer (NK) cells were first identified as a subpopulation of innate lymphoid cells (ILCs) and comprise about 5–15% of the total peripheral blood mononuclear cells (PBMCs) (367). Though NK cells and ILCs are derived from a common progenitor cell, NK cell development depends on IL-15-mediated signaling, whereas IL-7 signaling induces ILC differentiation (368). NK cells are considered the most efficient immune cells involved in immunosurveillance as they can target infected or cancer cells lacking major histocompatibility class I (MHC-I), marking them for programmed cell death (369). In fact, NK cells mainly target cells with low MHC-I expression or cells that express the cell stress markers MIC-A or MIC-B (370). In contrast, in healthy cells, the binding of MHC-I molecules to their receptors on NK cells blocks NK cell function (371). In a large OSCC cohort study, it was found that CD57⁺ NK expression is positively associated with high tertiary lymphoid structures (TLS), indicating higher overall survival rates (372).

NK cells are a heterogeneous population and in mice are recognized as CD3⁻ NKp46⁺ or more commonly as CD3⁻ NK1.1⁺ lymphocytes. In humans, NK cells have been categorized into two distinct subpopulations: immature CD3⁻ CD56^{bright} CD16⁻ cells and mature CD3⁻ CD56^{dim} CD16⁺ cells (373). Immature and mature NK cells differ in their functions and have different sensitivities to activating cytokines. After

activation by IL-2, IL-15, and IL-12, immature NK cells can activate systemic antitumor immunity indirectly by modulating the function of other innate and adaptive immune cells *via* the secretion of several cytokines such as IFN- γ , TNF- α , GM-CSF, and chemokines such as CCL1, CCL2, CCL3, CCL4, CCL5, and CXCL8 (374). It has been shown that neutrophil-derived IL-18, along with dendritic cell-produced IL-12, is critical for IFN- γ synthesis by NK cells, indicating that neutrophils are essential activators of NK cells (**Figure 5**) (375). In patients with severe congenital neutropenia, the percentage of responding NK cells is much lower in comparison with healthy control patients, indicating the significant role of neutrophils in NK cell maturation and function (376).

The majority (90%) of NK cells in PBMCs, which show less response to cytokine stimulation, are mature cells. Mature NK cells have several direct cytolytic mechanisms against tumors and pathogen-infected cells, which include (i) lysis by cytolytic granules such as granzyme and perforin, (ii) death receptor (DR) mediated apoptotic processes such as TNF-related-apoptosis-inducing-ligand (TRAIL)/TRAIL receptors or induction of apoptosis by FasL/Fas ligation, and (iii) antibody dependent cell-mediated cytotoxicity (ADCC) (377, 378). In tumor models, the cytotoxic activity of NK cells has been shown to be inhibited by the cell–cell interaction with MDSCs (pathogenically activated neutrophils), reducing NK cell activation by IL-2 and perforin production and a significant decline in the ability of NK cells to attack tumor cells (379). MDSC-derived nitric oxide impairs NK Fc receptor binding, leading to reduced ADCC and impaired signal transduction (**Figure 5**) (380). In HNSCC patients, inhibition of MDSC trafficking with SX-682, a small-molecule inhibitor of CXCR1 and CXCR2, enhances NK cell immunotherapy, indicating the important role MDSCs play in NK cell function in TME (381).

There has been improving evidence that neutrophil-derived mediators modulate NK cell effector functions in humans and mice, and in return, NK cells can modulate the survival, recruitment, and functional responses of neutrophils (**Figure 5**) (376, 382). In a murine colon cancer model (383), the association between the tumor infiltrate of the neutrophil and NK cell-mediated antitumor immunity was investigated. It was demonstrated that there was crosstalk between neutrophils and NK cells as neutrophil depletion significantly (i) decreased the frequency of IFN- γ ⁺ cells within NK cells, (ii) increased the fraction of Ki67⁺ NK cells, and (iii) increased the fraction of dead NK cells, indicating that neutrophil depletion during homeostatic proliferation induced NK cell proliferation accompanied by poor survival of NK cells (383). It has also been reported that some cytokines from neutrophils (such as IL-5 and IL-18) are involved in NK cell activation or support the survival of NK cells. IL-15, expressed in granulocytes including murine and recently human neutrophils (384), mediates a wide range of effects on mouse NK cells and is considered an important cytokine for NK cell maintenance (385) and homeostatic proliferation (386). It is interesting to note here that the Chen et al. study (384) detected IL-15 *via* mRNA expression (rather than secreted protein) from human

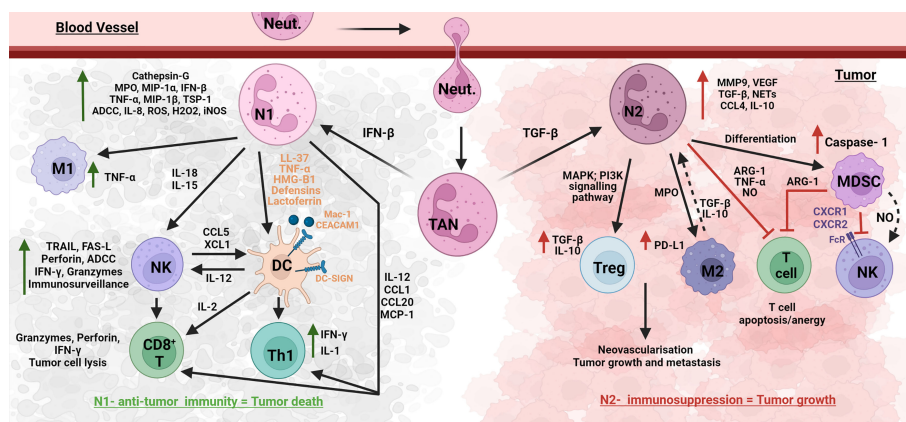


FIGURE 5 | Neutrophil networks affecting oral cancer outcomes. Neutrophils interact with anti-tumor and suppressive immune cells in the complex TME ecosystem. TME recruited neutrophils, TANs (tumor-associated neutrophils) polarize to a N1 anti-tumor phenotype in the presence of IFN- β , and to a N2 tumorigenic phenotype with TGF- β . N1 neutrophils upregulate several molecules such as cathepsin-G, MPO, MIP-1 α/β , IFN- β , TNF- α , IL-8, and TSP-1, to induce other immune cells and also execute N1 functions such as ADCC, ROS, and iNOS-induced cytotoxicity. N1 induce M1 TAM, NK (IL-18, 1L-15), T cells (IL-12, CCL1, CCL20, MCP-1) and DCs (LL-37, TNF- α , HMG-B1, defensins, lactoferrin, cell interaction Mac-1/CEACAM1-DC-SIGN). DCs further aid in N1 induced NK cell via IL-12, and NK aid in N1 induced DC via CCL5 and XCL1. N1 induced DCs further promote CD8⁺ T cells and Th1 cells. Interaction of these anti-tumor immune cells with N1 promotes tumor death. On the other hand, N2 neutrophils promote suppressive cells such as MDSC, Treg, and M2 and function via molecules such as MMP-9, VEGF, TGF- β , NETs, CCL4, and IL-10. N2 and MDSC inhibit anti-tumor T cells via Arg-1, TNF- α , and NO; and NK cells via NO and CXCR1/2. These lead to tumor growth and metastasis.

neutrophils that were under a highly inflammatory condition (sepsis), indicating that neutrophils may have an inflammatory phenotype yet to be elucidated and this may have a significant impact on cell-to-cell engagement. IL-15 and IL-18 in synergy with IL-12 produced from dendritic cells are also required for IFN- γ expression by NK cells (387–389). The proinflammatory heterodimer S100A8/A9 which is constitutively expressed by myeloid cells, including neutrophils, has been shown to directly enhance the cytotoxic activity of NK cells through binding to the receptor for advanced glycation end products (RAGE) (390).

Neutrophil-derived molecules such as azurocidin, cathepsin G, Defensins, elastase, and lactoferrin enhance NK cytotoxic activity in humans (391–393). NK cell cytolytic activity, instead of using an antigen-specific mechanism, is mediated by a broad repertoire of receptors which are engaged by ligands expressed on putative target cells (394). These receptors can be categorized either by their functions as NK cell-activating receptors and NK cell-inhibitory receptors or by their structure as Killer cell Lectin-like Receptors (KLRs) and Killer cell Immunoglobulin-like Receptors (KIRs) (395). Each NK cell typically expresses only a selection of these receptors, and thus NK-cells are quite heterogeneous and have a diverse repertoire of different MHC class I specificities (396). Stimulatory and inhibitory receptor signaling regulate NK cell activation and the balance between these two signals controls the outcome of the interaction with the target cell (397). Normal cells are shielded from killing by NK cells when signals provided by activating ligands are balanced by inhibitory signals delivered by self-MHC-I. In contrast, cells experiencing stress, such as tumor cells, downregulate their MHC-I expression, a ligand for inhibitory receptors. Simultaneously, they develop stress-associated molecules, which act as ligands for activating receptors. Consequently, the

absence of inhibitory signaling along with the induction of activating signaling shifts the balance toward NK cell activation, resulting in cytokine secretion and killing of tumor cells. This process is known as missing-self recognition (397, 398). A clinical study indicated that the rise in the expression of CD57⁺ NK cells in the tumor stroma of OSCC may serve as a good prognostic marker for the patients (399).

Monomorphic MHC-like molecule, CD1d-restricted T cells are known as NKT cells, which can be divided into two subsets based on their TCR repertoire and lipid antigenic profile specificity; type I and type II (400). Type I NKT cells play a significant role in regulating immune responses, including immune surveillance against tumors following stimulation by exogenous factors such as IL-12 or α -GalCer (401, 402). The NKT cell and neutrophil relationship has been investigated in hepatitis (403), renal ischemia–reperfusion injury (404), and pneumonia (405), and these studies showed that excluding or blocking NKT cells relieved the injury and reduced neutrophil infiltration (406). It has been shown that colitis-associated colorectal cancer was suppressed in NKT cell-deficient CD1d^{-/-} mice (406). This study suggested that NKT cells essentially act as an initiator, strongly expressing TNF- α which could stimulate epithelial chemokine secretion (CXCL1, 2, and 3), thereby mediating neutrophil recruitment indirectly. Neutrophils in turn become tissue-damaging through ROS upregulation (406), leading to colon cancer by causing DNA instability (407). It has been shown that iNKT cells can indirectly control tumor growth through targeting tumor-supportive, IL-6-producing, CD1d⁺ CD68⁺ tumor-associated macrophages (TAM) (408). Further, a deficiency in circulating iNKT cells was associated with poor clinical outcome in HNSCC patients, suggesting their critical contribution to antitumor immune responses (409, 410).

TUMOR MICROENVIRONMENT (TME) IN THE PATHOGENESIS OF HNSCC/OSCC

Though HNSCC is linked with intense immune suppression, the impact of the premalignant and TME on immune reactivity has yet to be elucidated. Significant infiltration of proinflammatory immune cells, such as CD163⁺ TAMs, CD8⁺ T cells, and NK cells, has been reported in oral leukoplakia and carcinoma (411–413). Using a mouse model of 4-nitroquinoline-1-oxide-induced oral carcinogenesis, De Costa et al. (414) investigated the shift in the immune cell phenotypes at the premalignant and malignant stages of HNSCC/OSCC (414). The development of oral premalignant lesions was shown to be associated with elevated levels of inflammatory Th1 cells, Type 1 CD8⁺ T cells (Tc1) secreting IFN- γ and Th17 cells compared with controls and HNSCC/OSCC-bearing mice, though the number of CD4⁺ regulatory T cells increased in HNSCC/OSCC-bearing mice (414). Regarding the inflammatory cytokine profile, it was shown that premalignant oral lesions are associated with an increased level of IL-17 as well as IL-23, in comparison with controls or HNSCC/OSCC, thus supporting the Th17 phenotype (415). In contrast, HNSCC tissues produce increased levels of TGF- β and skew normal spleen cells toward the Treg phenotype (415). Another study demonstrated that premalignant lesion cells released a panel of proinflammatory mediators including CCL5, G-CSF, monocyte chemoattractant protein 1 (MCP-1), and prostaglandin-E2 (PGE2) in comparison to HNSCC/OSCC cells, indicating that the premalignant microenvironment is more immune stimulatory than the microenvironment of an established HNSCC/OSCC (416). In addition, α -SMA (CAF cell marker) expression was high in premalignant lesions while it is not observed in normal epithelium (417).

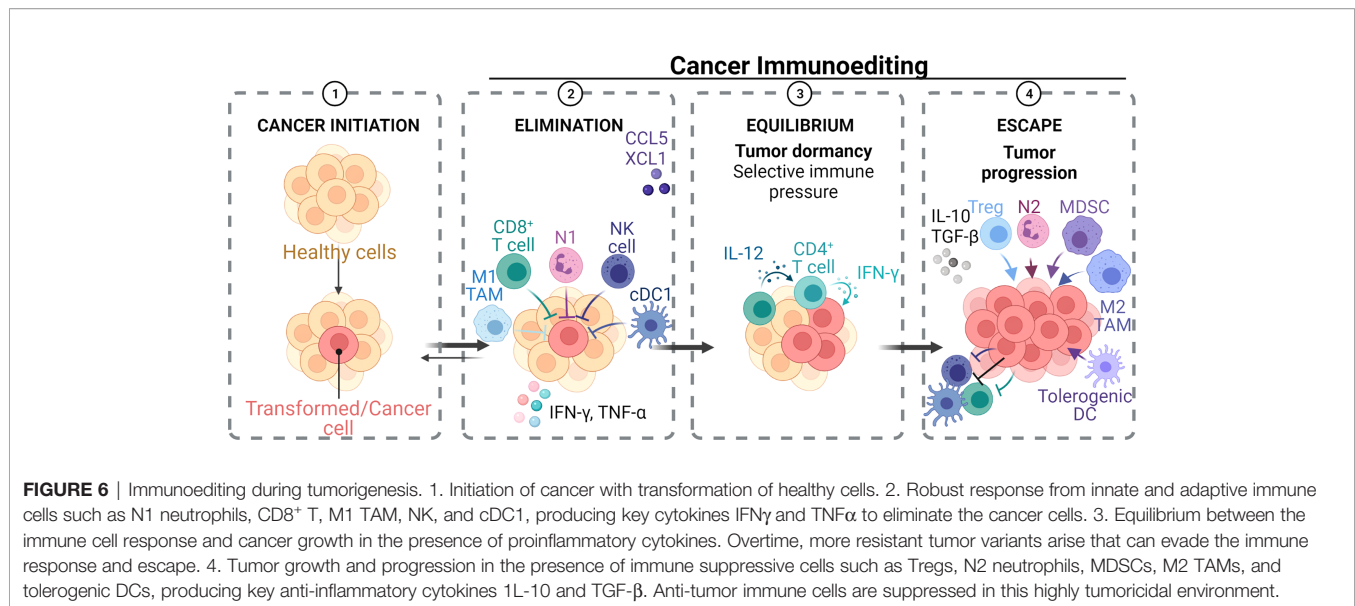
The influx, differentiation, and activation of neutrophils in the TME is indicative of a functional interaction between HNSCC/OSCC cells and neutrophils. A study by Trellakis et al. showed that high infiltration of neutrophils in OSCC is associated positively with tumor stage and negatively with overall survival times (418). HNSCC/OSCC cancer cells directly recruit neutrophils, extend their survival, and stimulate their inflammatory activity. HNSCC/OSCC cells are reported to be a crucial trigger for the recruitment of neutrophils as high serum concentrations of the inflammatory/chemotactic chemokines CCL4, CCL5, and CXCL8 in HNSCC/OSCC patients (418). The interaction of neutrophils and HNSCC/OSCC cells was found to enhance the chemotaxis of neutrophils to the TME and secretion of MMP-9 and CCL4 by neutrophils, triggering further recruitment and aiding tumor progression (418). Elevated MMP-9 has been reported to be at the invasive front of squamous cell and verrucous carcinomas in the oral cavity, indicating that MMP-9 expression is a reliable marker for invasive squamous cell carcinoma grading (419).

The abundance of circulating MDSCs is observed in HNSCC/OSCC and is associated with advanced stages of cancer (420). Though inhibiting T-cell activation is a key function of MDSCs, both *in vitro* and *in vivo* studies have demonstrated MDSC-derived

caspace-1 promotes the proliferation of HNSCC cancer in a T-cell-independent manner (421). The increase in MDSCs in HNSCC/OSCC patients has led to a few studies investigating methods to target these cells. A study by Weed et al. (422), showed that targeting MDSCs with tadalafil (10 mg/day) promotes antitumor immunity by increasing tumor-specific CD8⁺ T cells in a dose-dependent manner in patients with head and neck squamous cell carcinoma (422). Based on the observation that the expression of B7 homolog 3 protein (B7-H3) is an essential immunosuppressive mechanism in HNSCC, Mao et al. (423) conducted an HNSCC mouse model and showed that the blockade of B7-H3 decreased the levels of MDSCs and TAMs, as well as promoted IFN- γ secretion of cytotoxic T cells, resulting in enhanced antitumor immune activity (423). Another potential therapeutic target that may affect MDSCs is semaphorin 4D (Sema4D), a cytokine expressed by several epithelial malignancies and known to induce tumor angiogenesis produced by MDSC resulting in suppression of T-cell proliferation and IFN- γ production (424). Sema4D acts on immature MDSC and DC by (i) preventing their migration and (ii) inducing considerable increases in the immune-suppressive profile (425, 426). Although the MDSCs or neutrophils were not investigated, Zhou et al. (427) have shown that anti-Sema4D treatment reduced tumor growth and vascularization in an OSCC xenograft model, demonstrating a further possibility of targeting MDSCs.

A main contributor of inflammation in HNSCC is CD68⁺ TAMs, which is correlated with poor clinical outcomes in oral squamous cell carcinoma patients (428). Several studies confirmed that OSCC cells could directly suppress antitumor T-cell immunity through induction of PD-L1 expression on TAMs (429, 430). A high proportion of M2 macrophages express TGF- β and IL-10 in oral squamous cell carcinoma which was associated with a reduced patient survival time (431). TGF- β plays an important role in tumor-associated neutrophil polarization. It is shown that in the presence of TGF- β , neutrophils develop the N2 phenotype, which exhibits immunosuppressive and tumor-promoting activity, whereas blocking this molecule shifts the phenotype toward N1 (366). A recent study suggested that NET formation induced by TGF- β in oral lichen planus has substantial implications for developing oral cancer (432). The enhanced level of IL-10, which is produced by murine neutrophils (433), and inflammatory activated human neutrophils (237), leads to adverse survival in animal models and is associated with a poor prognosis in cancer patients (434). Genetic variation in IL-10, in particular IL-10 gene promote -1082 A/G (rs1800870) polymorphism, has been strongly associated with an increased risk of oral squamous cell carcinoma (435, 436) but has a non-significant association with HNC clinical stages and the association with neutrophils has not been conducted (437).

NK cells are well-known for their strong anti-tumor immunity, which is often compromised in cancer. Several studies investigating NK cells in oropharyngeal squamous cell carcinoma found that high abundance and activity of NK cells predicted improved survival, indicating that NK cells are a good prognostic marker for OSCC patients (399, 438). Tumor cells express NK activating receptor ligands *de novo*, making them susceptible to NK cell killing (439). The upregulation of the NK



cell inhibitory ligand NKG2A on tumor-associated NK cells is considered one of the biological mechanisms of immune escape in HNSCC (440). NK cells from the primary tumor present a different phenotype than NK cells from the blood of the same HNSCC patients (399). Tumor-infiltrating NK cells significantly downregulated activating receptors such as NKG2D, DNAM-1, NKP30, CD16, and 2B4, while over-expressing their inhibitory receptors (e.g., NKG2A and PD-1) compared with matched blood NK cells and thereby could not kill target cells and produce cytokines (61, 399, 441). It has been reported that tumor-infiltrating NK cells reduce cytotoxicity and produce significantly less IFN- γ (442). In colitis, NKG2A-expressing NK cells reduce inflammatory neutrophil recruitment and functions. By extension, it could be possible that the presence of NKG2A⁺ NK cells in the TME would dampen N1 neutrophil anti-tumor activity (443, 444). With this phenotype, NK cells in the primary tumor would enhance tumor progression and immune suppression.

CONCLUSION

Cancer such as HNSCC/OSCC is a major health issue globally and, with high incidence and mortality, it imposes a significant psychosocial and economic burden on individuals and society. Despite the clinical success of immunotherapies based on immune checkpoint inhibitors (i.e., antibodies against the immune regulators CTLA4 and PD-L1/PD-1), unfortunately, only a subset of patients respond to this treatment, suggesting that cancer immune evasion is a major barrier in current immunotherapy. An immune evasion characteristic that has received less attention is the crosstalk between distinctive immune cells within the TME and how this affects clinical outcomes.

Our review highlights that neutrophils do play a significant role in cancer immunology and that there are major holes in our

knowledge of how neutrophils affect tumor immune escape mechanisms, and a better understanding of this will determine more appropriate biomarkers for diagnostics and treatment of cancer. However, the TME represents a complex eco-system which alters over time, and determining how tumor cells and other constituents of the TME, such as CAFs, B cells, DC, macrophages, MDSC, NK cells, and T cells interact with each other and neutrophils will be challenging (Figure 6). It can be seen that neutrophils have a role throughout cancer initiation and progression, recruitment of DC and NK cells and consequently T cells, N1s then the suppressive N2 and the formation of MDSCs (activated neutrophils) and their effects. Although originally maligned in cancer immunology due to their short half-life, it now appears that neutrophils play a significant if not pivotal role in cancer pathology through their heterogeneity phenotypes, N2, TANs, and MDSCs interacting with other myeloid immune cells to affect disease states and overall cancer survivability and treatment success (Figure 5). Thus, there needs to be a focus on neutrophils in cancer, which in turn may yield significant knowledge gain and lead to more effective and lasting treatments. Though current evidence supports a pro-tumor role for neutrophils in OSCC, there may be an anti-tumor role for neutrophils and there needs to be more research to elucidate the complexity of the neutrophil mechanisms involved in cancer. Understanding these immune cell interactions is crucial for a better understanding of the TME and in treatment provided to HNSCC/OSCC patients.

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Conceptualization, SH and NO'B-S. Writing and original draft preparation, SH. Writing—review and editing, SH, NO'B-S, and BS. Figure drafting and editing, BS. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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