The Thyroid Tumor Microenvironment: Potential Targets for Therapeutic Intervention and Prognostication

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Abstract

Thyroid cancer is the most common endocrine malignancy and incidences are rising rapidly, in both pediatric and adult populations. Many thyroid tumors are successfully treated which results in low mortality rates, but there is often a significant morbidity associated with thyroid cancer treatments. For patients with tumors that are not successfully treated with surgical resection or radioactive iodine treatment, prognosis is dramatically reduced. Patients diagnosed with anaplastic thyroid cancer face a very grim prognosis with a median survival of 6 months post-diagnosis. There is a critical need to identify patients who are at greatest risk of developing persistent disease and progressing to poorly differentiated or anaplastic disease. Furthermore, development of treatments associated with less morbidity would represent a significant improvement for thyroid cancer survivors. It is well established the stromal cells and components of the tumor microenvironment can drive tumor progression and resistance to therapy. Here we review the current state of what is known regarding the thyroid tumor microenvironment and how these factors may contribute to thyroid tumor pathogenesis. Study of the tumor microenvironment within thyroid cancer is a relatively new field, and more studies are needed to dissect the complex and dynamic crosstalk between thyroid tumor cells and its tumor niche.

Keywords Thyroid cancer · Tumor microenvironment · Extracellular matrix · Immune suppression

Introduction

Thyroid cancer is one of the fastest growing malignancies worldwide and is predicted to be the 4th most commonly diagnosed cancer in the USA by the year 2030 [1]. The factors driving the dramatic rise in thyroid cancer incidence are incompletely understood. Some hypothesize that the increased incidence is driven by overdiagnosis of indolent disease [2–5], while others hypothesize that increases maybe driven by increased exposure to environmental chemical exposure [6]. Increased thyroid tumor incidence in the pediatric population is associated with a concomitant increase in larger tumors with more advanced disease, indicating increases are not only associated with indolent tumors [7]. Thyroid cancer is a slow-growing malignancy, and therefore, time is needed to fully understand whether the rising incidence will also be associat-

ed with increased mortality and aggressive disease. Although many thyroid cancers are successfully treated with aggressive surgical resection and radioactive iodine treatment, there are a subset that progress to therapeutically refractive disease. Furthermore, the aggressive treatment strategy of surgical resection and radioactive iodine therapy leads to lifelong thyroid hormone replacement for patients and places a large financial burden on the healthcare system. With rapidly rising rates of incidence and diagnosis, it is critical to identify and stratify patients who need aggressive therapy versus those who would benefit from a more conservative approach. Identification of tumor biomarkers that might predict disease progression is also critically needed.

Over the past decade, it has become clear that our understanding of the composition of cancer cannot be limited only to tumor cells. Rather, cancer is comprised of a complex ecosystem of stromal cells, immune cells, and a tumor microenvironment that plays a critical role in disease progression. Hanahan and Weinberg expanded their seminal review on the Hallmarks of Cancer [8] to include many of these microenvironmental and stromal changes that drive tumor initiation and progression [9]. Here, we will review stromal cells and components of the tumor microenvironment in thyroid cancer

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that contribute to thyroid cancer progression including pericytes, extracellular matrix components, cancer-associated fibroblasts, and immune cells. We focus primarily on how these components drive thyroid cancer progression. Research on the thyroid tumor microenvironment is constantly evolving and expanding with the hope that novel targets for prognosis and clinical intervention will be identified.

Subtypes of Thyroid Cancer and Molecular Drivers

Cancers of thyroid follicular cell origin are subcategorized into well differentiated, poorly differentiated, and anaplastic thyroid cancer. Well-differentiated thyroid cancers are further divided into follicular thyroid cancer and papillary thyroid cancer. Many thyroid tumors harbor mutations that lead to the constitutive activation of the mitogen-activated protein kinase (MAPK) pathway [10–13], and activating mutations of MAPK pathway effectors are seen throughout the spectrum of disease from well-differentiated tumors through anaplastic thyroid cancers. Poorly differentiated and anaplastic tumors carry the worst prognosis for patients and grow quickly. They often invade local soft tissue and metastasize to distant sites. Anaplastic thyroid cancer is the most severe form of the disease and the median survival for these patients is less than 1 year [14]. Most follicular thyroid cancer and papillary thyroid cancer patients have an excellent prognosis; however, recurrent disease or metastatic spread outside of the thyroid bed occurs in 10–30% of patients [15–17]. Papillary thyroid cancers are characterized by unique histopathologic features including papillae formation, nuclear clearing, and nuclear inclusion bodies. The tumors often present in both lobes, and metastatic spread is most common to local thyroid draining lymph nodes. The most common genetic mutation observed in papillary thyroid tumors is the BRAF^{V600E} mutation, with a prevalence of 36–69% [18–22]. By contrast, follicular thyroid cancers most commonly present as focal lesions present in only one thyroid lobe have dense cellular growth, and metastasis is most common to distant sites including the lungs, bone, and brain. Follicular thyroid cancers commonly harbor RAS mutations, predominantly in the genes encoding NRAS and HRAS [23–25]. It is possible, and likely, that the different driver mutations associated with different subtypes of thyroid cancer contribute to different microenvironments and differential recruitment of stromal cells to thyroid tumors. Unfortunately, many studies only stratify or identify the subtype of thyroid cancer and do not further distinguish between mutational drivers of tumorigenesis. It will be critical in future studies to investigate and identify both the subtype of tumor investigated as well as the genomic mutations associated with that tumor.

Stromal Elements Driving Thyroid Tumorigenesis

Thyroid Cancer Tumors Recruit Pericytes and Drive Progression Through Thrombospondin-1

Stromal elements of the tumor microenvironment including angiogenic factors, extracellular matrix components, and stromal cells have well-documented roles in the development and progression of cancer. To begin to understand how angiogenesis plays a role in thyroid cancer progression, Nucera and others used gene set enrichment analysis on microarray data to identify families of genes associated with BRAF^{V600E} mutations and angiogenesis [26]. Their study identified a strong role for thrombospondin-1 in the progression of papillary thyroid cancer [26, 27]. Thrombospondins are a family of anti-angiogenic proteins secreted by early-stage tumor cells and stromal cells such as fibroblasts and pericytes, which can induce protective effects through the upregulation of survival signals in target cells [28]. Their results demonstrated that *thrombospondin-1* (TSP-1) expression correlated strongly with and was dependent on the presence of BRAF^{V600E} mutations, as shRNA silencing of BRAF^{V600E} in anaplastic thyroid cancer cells reduced TSP-1 expression [26]. Moreover, silencing of BRAF^{V600E} or TSP-1 expression in anaplastic thyroid cancer cells diminished proliferation, adherence to collagen, migration, and invasion, suggesting that TSP-1 signaling is linked to enhanced tumorigenic behavior [26]. Finally, knockdown of either BRAF^{V600E} or TSP-1 decreased tumor size in vivo, highlighting the importance of TSP-1 in cancer progression [26].

Recently, it was suggested that pericytes could be a potential source of TSP-1 in the tumor microenvironment of papillary thyroid cancers driven by BRAF^{V600E} mutations [29]. Pericytes are heterogeneous stromal cell populations that aid in the stabilization of blood vessels through secretion of a number of angiogenic stimulating factors including PDGFRB, VEGF, and others [30]. In other cancers, pericytes have been documented to provide survival signals for endothelial cells through secretion of proangiogenic factors that promote endothelial cell survival, which may limit the effectiveness of anti-angiogenic chemotherapies [31]. However, in models of papillary thyroid cancer driven by BRAF^{V600E} mutations, recent studies suggest that pericytes are present in the tumor microenvironment and can induce resistance to chemotherapies in papillary thyroid cancer tumor cells through a thrombospondin-1-dependent mechanism [29]. Conditioned media harvested from human pericytes increased thyroid cancer cell survival in the presence of two FDA-approved multi-kinase inhibitors, vemurafenib, and sorafenib, when used in either isolation or as combination therapy. Protective effects induced in papillary thyroid cancer cells are likely due to increased survival signaling through increased AKT, SMAD3, and ERK phosphorylation, as survival signaling was depleted when pericyte secretion of TSP-1 was negated

through shRNA knockdown or chemical antagonism of secreted TSP-1 in vitro [29]. Additionally, thyroid cancer cells lacking a BRAF^{V600E} mutation displayed decreased pericyte-mediated protection, implying that this protective effect is BRAF^{V600E} dependent [29]. Pericyte-conditioned media also increased expression and secretion of TSP-1 and TGFβ1 in thyroid cancer cells, likely leading to a positive feedback mechanism for sustained protective effects [29]. Additionally, papillary thyroid cancer cells containing a BRAF^{V600E} mutation secreted more TSP-1 than thyroid cancer cells with wild-type BRAF, further implicating the BRAF^{V600E} mutation in thyroid cancer aggressiveness [29]. Together, these data suggest that in papillary thyroid cancers driven by a BRAF^{V600E} mutation, pericytes present in the tumor microenvironment increase resistance to chemotherapeutic interventions through secretion of TSP-1, which upregulates survival signals to tumor cells.

In addition to upregulating survival signals in tumor cells during treatment with chemotherapeutics, TSP-1 likely also plays important roles in regulating extracellular matrix (ECM) crosstalk. In other cancers, TSP-1 binds integrins, matrix proteins, and proteases, highlighting TSP-1's role in governing signaling crosstalk of ECM-related signaling pathways [28, 32–34]. In anaplastic thyroid cancer, it has been shown that the silencing of TSP-1 decreased integrin expression and focal adhesion kinase (FAK) activation in anaplastic thyroid cancer cell lines derived from a BRAF^{V600E} mutation. Additionally, cells were less responsive to collagen signaling and displayed altered adhesive characteristics, suggesting that TSP-1 is required for appropriate collagen signaling. Together, these data suggest an important role for TSP-1 in the progression of thyroid cancer.

Collagen Deposition and Fibroblast Presence in the Tumor Microenvironment of Papillary Thyroid Cancer Correlates with Poor Prognosis and Survival Outcomes

Collagen is the most abundant protein in the body and serves as a scaffold upon which cells grow and divide [35]. Regulation of the collagen matrix drives tumor progression in a number of cancers diverse in mutational profile, tissue type, and progression status. For example, collagen IV increases proliferation, resistance to apoptosis, and cell migration in pancreatic cancer cell lines, and type I collagen has been shown to play important roles in the progression of pancreatic cancer, breast cancer, and ovarian cancer [36–38].

Recently, it was shown that fibroblasts and collagen are present in the tumor microenvironment in mouse models of poorly differentiated thyroid cancer that develop from papillary thyroid cancer driven by BRAF^{V600E} mutations and loss of *Pten* [39]. Collagen I was present in the tumor microenvironment, likely deposited by cancer-associated fibroblasts, and its presence correlated with decreased patient survival. Collagen fibers were

heavily cross-linked throughout the tumor and especially on the periphery, which supports studies done by Tokarz and others demonstrating that the ultrastructure of collagen is significantly different between normal thyroid tissue and papillary thyroid cancer tumor tissue as indicated by polarization second harmonic generation microscopy [40]. While the specific mechanism of cross-linking is yet to be elucidated, lysyl oxidase expression was significantly upregulated in tumors harboring BRAF^{V600E} mutations, which likely plays a significant role in the establishment of mature and cross-linked collagen fibers [39]. Moreover, inhibition of lysyl oxidase reduced extrathyroidal invasion, metastasis, and cell proliferation in anaplastic thyroid cancer [41]. Overexpression of lysyl oxidase in patient samples also correlated with decreased survival [39, 42]. Together, these studies highlight a correlation between collagen cross-linking with poor patient prognosis. Less is known about why the presence of cross-linked collagen leads to poor patient survival, but increased motility was observed in papillary thyroid cancer cell lines with a BRAF^{V600E} mutation grown on collagen, suggesting that collagen increases the metastatic potential of papillary thyroid cancer cells in this context. Increased migration likely occurs through an integrin-dependent signaling mechanism. Integrins are the major arbiters of signaling responses to the extracellular matrix and are the primary receptor of extracellular matrix constituent proteins including collagen. Activated integrins activate the focal adhesion kinase (FAK) signaling cascade which can induce cell migration in an integrin-linked kinase-dependent manner [43].

In the Braf/Pten model described by Jolly and others, significant fibroblast infiltration was observed throughout the tumor and along the periphery [39]. The areas enriched with fibroblasts colocalized with areas of increased collagen density as determined via picrosirius red staining. In patient thyroid tissue, α-smooth muscle actin-positive cancer-associated fibroblasts were significantly higher in thyroid tumors compared to non-neoplastic tissue and were highest at the invasive front of thyroid tumors. Additionally, α-smooth muscle actin-positive areas were greatest in tumors harboring BRAF^{V600E} mutations. Similar to observations in the Braf^{V600E}/Pten murine tumors, Minna and others showed that α-smooth muscle actin, collagen, and lysyl oxidase colocalized in patient thyroid tumors, supporting the hypothesis that cancer-associated fibroblasts play a role in thyroid cancer ECM remodeling, likely driving progression [44]. Cancer-associated fibroblasts were closely associated with senescent thyroid tumor cells, particularly in tumors with BRAF-like signaling, which they postulate demonstrates stromal-tumor crosstalk at the tumor invasive front [44]. Conditioned media from Braf-driven murine tumor cells lead to both proliferation and migration of cancer-associated fibroblasts in vitro, which was not observed in the presence of conditioned media from Hras-driven thyroid cancer cells, suggesting that fibroblast recruitment and maturation is specific to Braf-driven thyroid cancer, suggesting that fibroblast recruitment and maturation is specific to Braf-

driven thyroid cancer [39]. Combined, these data suggest that the interaction between fibroblasts and tumor cells is dynamic and bi-directional.

While the connection between collagen and patient prognosis continues to be elucidated, less is known about how other components of the tumor microenvironment might impact progression. A major focus of early studies addressing the tumor microenvironment composition focused on identifying the presence of matrix metalloproteinases (MMPs). MMPs are a family of proteins whose expression correlates with cancer malignancy and increased invasion and metastasis [45, 46]. An early study by Nakamura and others revealed a correlation between expression and activation of MMP-2 and advanced papillary thyroid cancer [47]. Additionally, MMP-9 expression is upregulated in papillary thyroid cancer samples [48–51] and maybe a prognostic indicator for advanced-stage disease. MMP-1 and membrane-type-1 matrix metalloproteinase have also been implicated in the progression of papillary thyroid cancer, but it is yet unclear which components of the tumor microenvironment are responsible for its secretion [52–55]. Clinical studies have focused on identifying MMPs as prognostic indicators but to date, there have been no studies that have determined a definitive mechanism for how MMPs might play a role in thyroid cancer progression, highlighting a significant need for further research. Additionally, studies are needed to identify additional acellular components of the tumor microenvironment. The only other acellular component described in thyroid cancers is fibronectin, which was associated with papillary thyroid cancer tumors described as “intermediate risk” or harboring a BRAF^{V600E} mutation. When fibronectin was silenced in human papillary thyroid cancer cell lines, migration, adhesion, and proliferation were reduced, suggesting that fibronectin may be important for disease progression [56]. However, the specific role of fibronectin in thyroid cancer or how it arises in the tumor microenvironment is yet unknown.

The Extracellular Matrix Induces Tumor Cell Intracellular Signaling Cascades

Most studies that have focused on characterizing the composition of the extracellular matrix in the tumor microenvironment of thyroid cancer have been done in poorly differentiated and anaplastic thyroid cancer models that arise from papillary thyroid cancer, highlighting a need for further research on other subtypes and stages. Additionally, few studies have addressed potential signaling mechanisms and the molecular scaffolds that drive tumor cell behavior and crosstalk with other components of the tumor microenvironment. Identifying components of the tumor microenvironment that drives cancer as potential targets for therapeutic intervention has become a major area of research in all cancers, but elucidating mechanisms for tumor cell responses to components of the tumor microenvironment in the context of thyroid cancer has been more limited. Here again, most efforts

have focused on signaling mechanisms driven by the BRAF^{V600E} mutation.

As mentioned previously, integrins are a primary receptor for extracellular matrix proteins found in the tumor microenvironment of papillary thyroid cancer. Once activated, integrins often activate focal adhesion kinase signaling which increases cell proliferation, migration, and resistance to apoptosis [43]. Notably, components of this signaling pathway are overexpressed and implicated in the progression of papillary thyroid cancer. Integrin receptors for collagen including $\alpha2\beta1$ are expressed in papillary thyroid cancer cell lines, as is integrin-linked kinase (ILK), which is upregulated in papillary thyroid cancer and anaplastic thyroid cancer cell lines [57, 58]. Moreover, inhibition of ILK decreased AKT, mTOR, and MLC activation in papillary and anaplastic thyroid cancer cell lines, suggesting an important role for ILK in tumor cell survival signaling that occurs in response to extracellular matrix components [58]. FAK is overexpressed in papillary and anaplastic thyroid cancer patients, and Schweppe demonstrated that inhibition of FAK activity using small molecule inhibitors resulted in a decrease in AKT phosphorylation, suggesting FAK may contribute to tumor cell survival in an AKT-dependent manner [59]. Each of these studies supports a model in which signaling from collagen in the extracellular matrix occurs through integrins that activate a variety of pro-survival mechanisms. However, the exact mechanism is yet to be elucidated. Future studies should further dissect the molecular mechanisms underpinning the interplay between thyroid cancer tumor cells, fibroblasts, and the extracellular matrix if this interface is to be seriously considered for therapeutic intervention. We summarize what is known about stromal cells and the extracellular matrix in papillary thyroid cancer in Fig. 1.

The role of mechanical forces impacting both physiological and pathological processes has gained interest, and studies in other cancers have shown that tissue density can impact tumor aggressiveness. Increased collagen and extracellular matrix stiffness has been associated with tumor progression [36, 60, 61], increased metastasis [62], and can enhance tumor cell invasiveness [63]. Tumor cells respond to mechanical signals, resulting in altered cell signaling, alterations in intracellular tensions and cytoskeletal rearrangement, and even gene expression signatures [64]. Stiffness of the extracellular matrix increased glycolysis in both cancer cells and cancer-associated fibroblasts [65]. Similarly, it has been shown that breast cancer cells and fibrosarcoma reduce glucose and glutamine uptake when grown in dense or crowded cellular matrices, and gene set enrichment analysis demonstrated an associated decreased expression of genes involved in the TCA cycle and pyruvate metabolism [66]. Morris and others demonstrated that a metabolic shift occurred in breast cancer cells grown in 3D gels with increasing collagen density, whereby oxygen consumption and glucose utilization by the TCA cycle decreased in high-density collagen gels [67]. Recently, biophysical forces and alignment of fibers

Fig. 1 Tumor cells recruit stromal cells, leading to upregulation of pro-survival signaling. BRAF^{V600E}-positive papillary thyroid cancer cells recruit pericytes and fibroblasts to the tumor microenvironment. Pericytes secrete thrombospondins, which leads to upregulation of integrins and

other survival pathways. Cancer-associated fibroblasts secrete extracellular matrix proteins, which tumor cells respond to through integrins, likely amplifying tumor cell survival responses

have been shown to impact Hras-driven follicular thyroid cancer cells [68]. Fiber spacing significantly impacted thyroid tumor cell migration persistence, cell shape, nuclear shape, and expression of metabolic genes [68]. In contrast to what was hypothesized, cells grown on densely spaced fibers (3–6 μm) migrate persistently at high speeds with 3-dimensional morphologies, whereas those on less densely spaced fibers (18–36 μm) attain more 2-dimensional morphologies with longer focal adhesion cluster lengths and migrate randomly. These data are consistent with increased aggressiveness and migration associated with dense extracellular matrices. Growth on differentially aligned fibers significantly affected transcription, resulting in a generalized decrease in transcriptional output compared to growth on tissue culture plastic [68]. However, gene set enrichment analysis demonstrated the upregulation of families of genes associated with protein synthesis and translation as well as a set of genes regulating metabolic processes. These studies suggest that structural changes and mechanical properties within the tumor microenvironment may induce transcriptional, translational, and metabolic alterations in tumor cells, all contributing to enhancing the transformational capacity of tumor cells [68].

The Yin-yang of the Immune System in Cancer

The immune system in cancer is a double-edged sword. It is vital for preventing and clearing cancers but can also play a

role in initiation and progression. Immune cells that normally perform an immune surveillance/anti-tumor function can be co-opted by the tumor and made pro-tumorigenic. The tumor microenvironment can often be immunosuppressive, either directly or indirectly dampening immune signaling and preventing tumor clearance or promoting its development. A classic example of this yin-yang effect is inflammation. Acute inflammation is characterized by the recruitment of immune cells to clear an infection or resolve acute tissue injury. This initiates a healing process and is often anti-tumorigenic. By contrast, chronic inflammation is a prolonged abnormal immune response that does not resolve through normal feedback signals. Chronic inflammation is thought to be tumor promoting by creating a tumor-supportive microenvironment fueled by increased levels of cytokines and chemokines. Cancer is often referred to as a wound that never heals. Here, we will discuss the current knowledge of immune components in thyroid cancer and what is known about their role (Fig. 2).

Tumor-Associated Macrophages Are Associated with Poor Prognosis

Macrophages are essential innate immune cells with roles in fighting infection and facilitating tissue repair. Macrophages display transcriptomic plasticity and have a complex polarization spectrum with a variety of distinct characteristics and behaviors that are activated depending on the environmental

Fig. 2 Immune infiltration to thyroid tumors. Immune cells have the ability to both support and inhibit tumor growth. It is critical to assess both surface markers as well as functionality of immune cells specific to the tumor and its niche. Pan-CAF markers may contribute to disparate reports that have indicated TAM presence in the tumor may be associated

with improved outcomes; however, most studies indicate TAM presence is associated with tumor progression. Significant diversity is observed in the role of T cells and even dependent on the stage of the tumor where they are present

context [69]. The macrophage spectrum is often simplified to 3 main types of macrophages: M0, M1, and M2. M0 macrophages are considered at rest and unsolicited. M1 macrophages are classically activated, mediating pro-inflammatory responses and are typically involved in acute conditions such as viral or bacterial infections. Conversely, M2 macrophages, considered alternatively activated, mediate multiple functions including immunoregulation and wound healing by producing anti-inflammatory cytokines, ECM components, and angiogenic factors. They are typically active in tissue repair processes, chronic infections, parasitic infections, or conditions such as fibrosis [69]. However, M2 macrophages represent a wide spectrum of cells with various functions and can be further stratified into M2a, M2b, M2c, and M2d [70, 71]. Unfortunately, many cancer studies do not address the vast spectrum of macrophage and plasticity of these immune cells. It is critical that future studies more specifically define the various subtypes of macrophages and functionality within tumors as lack of specificity may account for limited consensus regarding their role in thyroid tumorigenesis.

Tumor-associated macrophages (TAMs) are typically characterized as M2 [72, 73] {Biswas, 2006 #459}, but their function and polarization in thyroid cancer progression are unknown. Most of our knowledge of macrophage polarization characteristics is derived from their roles in infection studies. However, applying these findings directly to tumor biology is likely naïve and must be done with caution. Studies must independently validate the function of these macrophages between infection and cancer and not assume surface markers and function are always comparable between models. Despite sharing markers, TAMs likely perform very different roles and may have very different characteristics than their counterparts involved in infection. Furthermore, the complex polarization spectrum, numerous markers of polarization, and plasticity of macrophages have made establishing macrophage

polarization states across the fields challenging. Patient-derived TAMs are most commonly identified using the pan-macrophage marker CD68 in addition to a specific M2 macrophage marker CD163 or CD206 [74, 75]. It is difficult to ascertain and ensure that all groups referring to TAMs are indeed identifying the same population of macrophages. Consequently, it is difficult to conclude whether the diverse role of TAMs in the tumor microenvironment is due to heterogeneity of function or alternatively a diverse subset of cells that have been grouped under the umbrella of TAMs. Uniform identification of macrophage polarization states and the definition of TAMs throughout the cancer field is needed.

In papillary thyroid cancer patients with lymph node metastasis, the density of TAMs in tumors varied from comprising 5 to 70% of the total cell population [76]. Patients with higher TAM density (25% or greater) had significantly larger tumors than those with low TAM density (less than 25%) [76]. TAM densities play a role in predicting prognosis, as Jung and others previously observed that higher densities of TAMs were associated with poor survival rates in thyroid cancers. Surveying clinical data from the Korean population, they noticed an inverse correlation between the 5-year survival rate and the density of CD163+ TAMs [77]. This decrease in survival rates could be related to findings by Qing. In their analysis, the TAM density correlated with lymph node metastasis in papillary thyroid cancers. The TAMs identified appear to be of an M2-like phenotype and displayed an upregulation of the M2 characteristic genes IL-10 and mannose receptor when compared to peripheral monocytes [78]. Unfortunately, while TAM presence correlates with poor prognosis in clinical data, the actual role of TAMs in thyroid tumor progression has yet to be fully understood. Compared to patients with high recruitment of CD68+ cells, low recruitment was associated with shorter survival. Additionally, it was observed that *TGFBI* expression correlated with increased recruitment of CD68+

cells, but that it also inhibited their anti-tumor functions [79], thus rendering them with a pro-tumorigenic phenotype. These data suggest that merely the presence or absence of TAMs is not enough to be prognostic, but that their functionality and signaling must also be assessed [79].

Studies to date have primarily investigated the role of TAMs in papillary thyroid cancer, but less is known regarding the role of TAMs in follicular thyroid cancer. Huang and others reported increased recruitment of TAMs in follicular thyroid cancer patient tumors when compared to patients with benign follicular adenomas. They also observed the upregulation of the chemokine CCL15 and demonstrated that conditioned media from follicular thyroid cancer cell lines recruited macrophages, which could be inhibited by treatment with a CCL15 blocking antibody [80]. CCL15 is a chemokine that recruits both leukocytes and endothelial cells [81, 82] both of which could be contributing to enhanced malignancy. TAM recruitment was also observed in the follicular thyroid cancer mouse model *Hras*^{G12V}/*Pten*^{-/-}/*TPO-cre*, as was increased expression of Ccl9, the mouse homolog of CCL15 [83]. Together, these data suggest that CCL15 may be an important mediator of TAM recruitment and tumor progression in follicular thyroid cancer. More murine studies are needed to determine if Ccl9 is essential for TAM recruitment and contributes to the polarization of macrophages within the tumor microenvironment. Future studies, in both patients and murine models, should also address the functional role of TAMs in FTC progression and more precisely define the polarization of this subset of macrophages within the thyroid tumor microenvironment.

As thyroid cancer progresses from well differentiated to poorly differentiated and anaplastic disease, TAM density increases. Ryder and others showed that more than 50% of all nucleated cells in anaplastic thyroid cancer were TAMs [84]. Additionally, decreased survival rates are associated with advanced thyroid cancers that have increased TAM density. Together, these data suggest that TAMs likely promote tumor progression [84, 85]. Kim and others showed additionally that CXCR4 expression correlated with TAM density [85]. Upon binding its ligand, CXCL12, CXCR4 activates G protein-mediated signaling pathways including AKT, JAK/STAT, and MAPK [86]. These pathways are known to be activated in various forms of thyroid cancer and their downstream effects promote proliferation, cell survival, and chemotaxis. Additionally, there is evidence CXCR4 can interact with other ligands like macrophage migration inhibitory factor (MIF). When MIF binds CXCR4, the MAPK, AKT, and integrin signaling pathways can be stimulated leading to changes in processes such as migration, adhesion, proliferation, and gene expression. Signaling can also result in the recruitment of various cells including T cells, B cells, eosinophils, endothelial progenitor cells, and mesenchymal stromal cells [87–91], all of which can contribute to the

development of tumor permissive niche facilitating tumor growth and progression.

To add to the complexity, Cailluo and colleagues identified a specific subset of anaplastic thyroid cancer-associated TAMs referred to as “ramified TAMs” (RTAMs), which are classified by their cytoplasmic extensions [92]. RTAMs were unique to anaplastic thyroid cancers and were not observed in any well-differentiated tumors. Furthermore, these RTAMs appear to form a dense support network throughout the tumor, potentially mimicking a pseudo-vascular network to supply nutrients to the tumor and remove reactive oxygen species [92]. While the functions of RTAMs have yet to be determined, this study suggests they may represent a unique form of TAMs that can supplement the tumor vasculature system and drive/support anaplastic thyroid cancer progression [92]. These data indicate TAMs are present early on in thyroid cancer development and that they are essential for progression and the aggressive phenotype of more advanced thyroid cancers. These studies also highlight the need for consistent markers to identify TAMs across all tumor subtypes, and for studies to identify the functional significance of the presence of these cells within the tumor microenvironment.

The Prognostic Value of T Lymphocytes (T cells) Is Unclear

T cells are lymphocytes that are essential in adaptive immune responses. T cells can be characterized into three categories based on their function and the expression of cell surface factors: CD8+ cytotoxic T cells, CD4+ helper T cells, and Foxp3+ regulatory T cells. Cytotoxic T cells are stimulated to produce an antigen-specific immune response by binding major histocompatibility complex-1 molecules on antigen-presenting cells. Conversely, helper T cells do not have direct cytotoxic effects but rather assist in cytotoxic responses by signaling to other cells, such as B cells. Finally, regulatory T cells are essential in tolerance and preventing autoimmunity. However, in cancer, they can also suppress anti-tumor immune responses, aiding in immune escape and tumor progression. A balance of these 3 cell types is essential for recognition, elimination, and resolution of potential tumors. When this balance is offset, the immune system can fail to control tumors.

The impact of helper T cell recruitment on papillary thyroid cancer prognosis is still unclear [93, 94]. In a retrospective study of 100 papillary thyroid cancer tumors, the majority of tumor-associated T cells were identified as CD4+. Additionally, they observed that CD4+ FoxP3+ regulatory T cells were consistently present in the center of lymphocytic aggregates. There was also a strong correlation between the presence of regulatory T cells, disease severity, and lymph node metastasis. This study suggests that elevated numbers

of regulatory T cells could be used to predict disease outcomes in papillary thyroid carcinoma patients [95].

French and others further evaluated the function of CD4+ T cells in lymph nodes of patients with metastatic papillary thyroid cancer [96]. Lymph nodes involved in metastasis displayed enrichment of regulatory T cells. This study revealed that a significant portion of CD4+ and CD8+ T cells within tumor-involved lymph nodes were positive for programmed cell death-1 (PD-1), which is an inhibitory molecule and a marker of exhausted T cells [96]. PD-1 is found on the surface of T cells. When bound to its ligand on neighboring cells, it limits immune recognition and cytotoxic killing. Immune therapy using checkpoint inhibitors block the association between PD-1 and PD-L1, thus activating the immune system and potentially allowing T cells to recognize and destroy tumor cells. Exhausted T cells fail to activate properly, despite the presence of a stimulus. T cell exhaustion results in reduced proliferation, lack of production of T cell-specific cytokines, and T cells unable to produce an anti-tumorigenic immune response.

The French laboratory additionally characterized PD-1+ T cells isolated from tumor-involved lymph nodes and discovered that while their previous study indicated decreased proliferation and impaired activation [96], there was no functional evidence of T cell exhaustion despite displaying molecular markers for T cell exhaustion [97]. Unfortunately, these studies did not stratify tumors based on the mutational driver. Mutational drivers in these tumors could account for heterogeneity observed in T cell response, and it will be critical to not only identify a subtype of tumors but also mutational drivers in future studies. These findings suggest that the T cells in tumor-involved lymph nodes minimize tumor growth and further metastasis despite failing to eliminate the tumor. This work also highlights the need for concurrent functional studies, with molecular markers.

An analysis of retrospective studies resulted in differing opinions on whether increased T cell recruitment results in positive patient outcomes [94, 98, 99]. Retrospective studies rely on classically defined immune markers that are unable to assess the functionality of these cells due to their retrospective design. The limitations of relying solely on molecular markers to infer functionality are well documented, and such studies often misrepresent the functional consequences of the identified cells [96, 97]. Future studies are needed to characterize the functionality of the recruited immune populations to truly dissect their role in thyroid cancer.

The role of T cells in follicular and anaplastic thyroid cancer is largely unexplored. In the follicular thyroid cancer mouse model, *Hras*^{G12V}/*Pten*^{-/-}/*TPO-cre*, Jolly and colleagues identified significant immune cell recruitment. Approximately 65% of the total cell population was CD 45+ [83]. The majority of T cells recruited to the tumor microenvironment were Foxp3+ regulatory T cells, and only 1% of the

entire T cell population was CD8+ [83]. These data indicate that there is substantial T cell recruitment to follicular thyroid tumors that likely suppress anti-tumor immune responses. Batsman and others showed that poorly differentiated and anaplastic thyroid cancers are enriched with both CD8+ T cells and PD-1+ Tregs [100]. They also observed PD-L1 expression throughout anaplastic thyroid tumors. Additionally, PD-L1 expression was higher in patients with metastasis [100]. These data are further evidence that targeting the PD-1 pathway may be beneficial for the treatment of advanced thyroid cancers. Future studies characterizing the functionality and the development of the T cell populations in follicular and anaplastic thyroid tumors are needed.

Thyroid Toxicity and Side Effects Associated with Immune Checkpoint Inhibitor Therapy

The clinical application of immune checkpoint inhibitor antibodies has revolutionized cancer therapy for many advanced-stage cancer patients. The most common targets of these therapies are CTLA-4 (cytotoxic T lymphocyte-associate protein 4) and PD-1/PD-L1. Blocking of these receptors through antibody therapy releases the breaks of the immune system and the cytotoxic effects of T cells are enhanced. However, despite the profound success of this therapeutic strategy in some patients, only a subset of patients respond positively to this type of therapy, and many patients develop significant side effects. Adverse events can occur in up to 90% of patients receiving anti-CTLA-4 and approximately 70% of patients treated with PD-1/PD-L1 antibody [101]. The different adverse events occurring between the different checkpoint inhibitors are not completely understood but may be related to the different functions of CTLA-4 and PD-1/PD-L1 in mediating T cell activation [101]. One side effect, or adverse event related to immune checkpoint inhibitor therapy, is thyroid dysfunction resulting in hypo- or hyperthyroidism or generalized thyroiditis [102, 103]. Approximately 1–4% of patients treated with anti-CTLA-4 monoclonal antibodies develop thyroid toxicity [104]. These adverse events vary in severity and are often successfully managed with thyroid hormone replacement therapy. However, the duration of the adverse event varies by the patient with some events transient while others persist. Together, these studies suggest that there may be an endogenous immune suppressive microenvironment within the thyroid, even in the absence of thyroid malignancy. A more thorough and comprehensive understanding of the physiological immune landscape within the thyroid gland, even in the absence of malignancy may lend insight to the rational design of immune-modulating therapeutic strategies for the treatment of thyroid tumors. Evaluation and understanding of the native immune populations within the thyroid, especially through all stages of development, will be critical as we attempt to translate immune-modulation into clinical care, particularly

as related to treating pediatric versus adult patients, and those with refractive or recurrent thyroid cancer.

Future Directions and Questions that Remain

Current research is beginning to highlight the importance of non-immune components of the tumor microenvironment in thyroid cancer progression. Of note, important roles for pericytes, cancer-associated fibroblasts, and collagen have been identified and correlated with poor prognosis. However, most of the work has been focused on models of poorly differentiated and anaplastic thyroid cancers driven by *BRAF*^{V600E} mutations. Further studies are needed to address the relative paucity of information known regarding the composition of the tumor microenvironment in other subtypes of thyroid cancer. Moreover, to date, there are no studies that address changes and remodeling of the tumor microenvironment as thyroid cancer progresses. While it is clear that pericytes, CAFs, and collagen are important drivers of progression, there are likely to be other mediators. Additionally, while both CAFs and pericytes are recruited to the tumor microenvironment of papillary and anaplastic thyroid cancers, their origins and recruitment are poorly understood. Further work is needed to elucidate the molecular mechanisms through which the tumor microenvironment influences tumor cell behavior and cancer progression.

The studies of other cancers provide some insights into how components of the non-immune tumor microenvironment may play a role in thyroid cancer progression. In breast cancer, TSP-1 signaling is mediated through the $\alpha 3 \beta 1$ /insulin-like growth factor axis [33]. Similarly, the $\beta 1$ portion of the integrin heterodimer activates FAK and controls cell proliferation and metastasis in lung cancer models [105]. Therefore, the role of integrin signaling in thyroid cancer progression is a potential avenue for future targeting, as integrins have been highlighted as effective targets for chemotherapy in preclinical models of other cancers [106–108]. Additionally, while collagen has been identified as a major constituent of the ECM component that drives papillary thyroid cancer, it is possible that cancer-associated fibroblasts secrete other ECM proteins that have known roles in cancer progression. In melanomas harboring a *BRAF*^{V600E} mutation, fibronectin drives cancer metastasis and invasion, and in models of prostate cancer, cancer-associated fibroblasts generate fibronectin highways that guide cancer cells to migrate in specific directions through $\alpha 5 \beta 1$ integrin binding [109, 110].

The tumor microenvironment of thyroid cancer is a rich landscape for future study and the possibility for the development of chemotherapeutic efficacy. To date, a number of RAS, RAF, and MEK inhibitors have been shown to be effective in vitro, but clinical outcomes remain are not nearly as robust for patients with poorly differentiated and anaplastic disease [111]. Lack of clinical efficacy can likely be attributed

to many variables, but the tumor microenvironment undoubtedly plays a central role. The tumor microenvironment can be thought of as a multipronged armory of defense that impacts whether therapies are effective, most of which we know very little about in the context of thyroid cancer. To be effective, a therapeutic must first gain access to the tumor, but many physical and chemical barriers can impede this process. The tumor microenvironment can often be an acidic and hypoxic place, which alters the chemical properties of chemotherapies. Moreover, vasculature properties within a tumor may prevent the delivery of a chemotherapy drug through high static pressure. Additionally, elements of the tumor microenvironment may engage tumor cells in crosstalk that leads to the upregulation of drug exporters or other survival signaling pathways sufficient to overcome therapy effectiveness. It is unknown how current therapeutics might impact the cellular composition of the tumor microenvironment in thyroid cancer. Remodeling of the tumor microenvironment that occurs through chemotherapeutic intervention may increase tumor cell aggressiveness. However, no studies have addressed these elements of the tumor microenvironment, and it is clear that improving our understanding of the thyroid cancer tumor microenvironment is imperative for improving chemotherapeutic efficacy and patient prognosis.

Prognostic indicators are not simply the presence or absence of a single factor in a tumor. There are dynamic interactions between multiple cell types in the tumor microenvironment and particularly between different subsets of immune cells. It is not sufficient to just identify these cells but to also determine their function. The multitude of identified immune cells, as well as their diverse functions in different contexts, make it difficult to determine whether their presence confers a better or worse prognosis. Future studies must not only identify these cells but determine the functional role of these stromal cells, particularly immune cells. Furthermore, it will be critical to determine whether the function of these cells evolves or changes through the progression of the disease. The tumor microenvironment represents a new frontier for the development of cancer therapeutics, and adjuvant therapeutic strategies to improve current standard care of which could lead to new, less aggressive treatments that will enhance the quality of life for well-differentiated patients and more effective treatments to improve clinical outcomes for patients with advanced disease.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflicts of interest.

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