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TGF- β AND TUMORS – AN ILL-FATED ALLIANCE

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SUMMARY

Mechanisms of host defense can form an unwitting alliance with tumor cells to promote tumor progression, invasion and dissemination to distant sites. By secreting TGF- β , an immunoregulatory molecule designated for both promoting inflammation and dampening immune responses, the tumor tricks the host into supporting its expansion and survival. TGF- β not only recruits leukocytes to secrete chemokines, growth factors, cytokines and proteases in support of a tumor-friendly niche, but also in a context-specific manner, incapacitates the emergent immune response. As a profound immunosuppressant, TGF- β , both directly and through the generation of regulatory T cells, blunts immune surveillance, favoring tumor escape. Collectively, the ability of the tumor to hijack these host defense pathways can tip the balance in favor of the tumor.

INTRODUCTION

Tumorigenesis, a complex, multigenic process, generally occurs as cells acquire abnormal resistance to growth inhibition, proliferate autonomously, promote angiogenesis, invade, and/or metastasize. Cancers thus arise when somatic cells become subjugated to pro-oncogenic signals, while eluding tumor suppressor mechanisms. Although host anti-tumor defense revolves around immune-mediated mechanisms of recognition and removal, paradoxically, in addition to immune surveillance, inflammation may actually promote tumor progression [1].

The link between inflammation and cancer is well recognized and chronic wound and inflammatory states can be considered risk factors for cancer development. Since the observations, many years ago, that the tumor microenvironment resembles normal wound healing, it has been proposed that tumor stroma is “normal wound healing gone awry” [2]. Recapitulation of normally reparative processes within the tumor milieu may have a powerful inductive influence on cellular transformation and underlie tumor engraftment, invasion and/or metastasis [3,4]. Normal growth and development can be deviated by factors generated during overzealous or persistent inflammation, whether in response to tumor antigens (TA), pathogens or other danger signals. Infiltrating inflammatory cells produce a myriad of chemokines, growth factors, angiogenesis factors, damaging oxidant-generating enzymes and proteases that may encourage tumor growth and invasion. Whereas inflammatory molecules such as NF κ B2/p100 overactivate the developmental pathway and dysregulated signaling networks can trigger malignant growth[5], other inflammatory

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molecules also profoundly influence tumorigenesis. One such factor with recognized involvement in inflammation, inflammatory pathogenesis and tumorigenesis is TGF- β [6].

In the context of a malignant niche, tumor cells themselves secrete TGF- β , as well as the stromal cells and infiltrating immune cells (Fig. 1), and the increasingly TGF- β -enriched environment profoundly alters the outcome of the contest between host and tumor. Evidence supports a role for genetic mutations and aberrant TGF- β signaling pathways in cancer risk [7,8], outside the scope of this review. Beyond establishment of primary tumors, most cancer-related deaths stem from metastasis, which requires transformed cells to enter the vasculature, then breach the endothelium to extravasate and establish a new metastatic outgrowth, and leukocyte infiltrates have been temporally and spatially linked to basement membrane breach and tumor cell egress [4]. Continuing efforts to pinpoint genes linked to these events revealed 4 genes that synergistically promote tumor progression, notably, epiregulin, matrix metalloproteinases(MMP)1, MMP2 and COX-2 [9], co-opted from normal vascular remodeling, inflammation and wound healing. Tellingly, several of these pivotal genes can be regulated by TGF- β . While indisputable that TGF- β plays both direct and indirect roles in tumor development and progression, which depending on context, may facilitate or impede cancer cells gaining a foothold [8], it is also a dominant force in dictating the host response to invading tumor cells [10], as discussed in this brief review.

TGF- β in Head and Neck Squamous Cell Carcinoma (HNSCC)

One of the tumors associated with high mortality rates, due to its high infiltrative potential, is HNSCC which represents about 6% of all new cancers in the US [11] and for which the survival rate has not improved significantly in decades. Although a heterogeneous disease, a major and common etiological risk factor is continuous exposure of head and neck epithelia to environmental carcinogens, with genetic and epigenetic alterations in oncogenes or tumor suppressor genes. TGF- β is reportedly overexpressed in pre-neoplastic lesions adjacent to HNSCC, relative to uninvolved mucosa [12], implicating TGF- β early during HNSCC development. In a mouse model, TGF- β 1 transgene induction in head and neck epithelia caused severe inflammation and angiogenesis, evidence that TGF- β 1 overexpression does provide a tempting tumor milieu [12]. As a model to dissect unequal relationships between host and tumor, we have examined immune cells and their products in the context of HNSCC.

TGF- β in immune dysregulation

Within the tumor site, TGF- β is sequestered in the extracellular matrix via latent TGF- β binding protein (LTPB) and secreted from resident or recruited leukocytes, stroma and tumor cells [10](Fig. 1). In this dynamic microenvironment, TGF- β is a potent recruitment factor for inflammatory cells, which may participate in immune surveillance, but also generate a sanctuary conducive to tumor escape. Despite local infiltration of the key players needed to orchestrate anti-tumor immunity (Fig. 1,2), the response is typically ineffective as the immune cells are outmaneuvered by the cancer cells. Recognition of and response to TA involves CD4⁺ T cell activation with assistance from antigen presenting cells (APC), including dendritic cells(DC, CD1a⁺) and macrophages(CD68⁺) (Fig. 1D). CD8⁺ T cells within tumor infiltrates (Fig. 1F) should represent key mediators of the adaptive anti-tumor response, exerting their effector functions through cytolytic activity and secretion of IFN γ . Nonetheless, natural killer(NK) cells and cytotoxic T-cells(CTL) appear functionally inactivated in oral cancers [11] in the context of increased expression of cell-associated and matrix-linked TGF- β and its downstream signaling molecules.

TGF- β biases CD4⁺ T helper(Th) populations (Fig. 2) toward or away from commitment to Th1, Th2, Th3, Th17 or regulatory T cell lineages in the context of other requisite regulatory

factors. As a differentiation factor for Th17 effector cells, TGF- β [13], together with IL-6 and/or IL-21 [14], activates Stat3 and induces the transcription factor, retinoic acid-related orphan receptor (ROR γ t), a controller of Th17 differentiation [15](Fig. 2). In so doing, TGF- β engages IL-17 production, important in defense against extracellular bacterial pathogens, but now linked to tumorigenesis. Functionally, over-expression of IL-17 in tumor sites correlated with recruitment of phagocytes, along with increased angiogenesis [16]. Recognized for its ability to induce cytokines, chemokines, colony stimulating factors and pro-angiogenic factors in multiple cell types, IL-17 enhanced tumor vascularity was inhibitable by CXCR2 neutralization [16]. Coordinately, this local repository of IL-17 can be incriminated in the intricate web supporting local malignant cell expansion and survival, but conceivably, systemic levels of IL-17 may also program pre-metastatic niches in distant sites. Th17 cells not only produce IL-17, but also the proinflammatory cytokines IL-21, IL-22 and TNF α , potential contributors to unresolved inflammation and autoimmune diseases [17,18] that may foment a tumor-supportive niche. In advanced tumors, both Th17 cells and suppressive Foxp3⁺ regulatory T cells(Tregs)(Fig. 1G) reach maximal levels [17], although excess Tregs could reflect either Th17 inhibition or potentiation via TGF- β . Perhaps relevant is the recognition of IL-21, one of the T cell-derived gamma-chain receptor cytokine family(IL-2, IL-7, IL-15), in the tumor microcosm (Fig. 3A). IL-21 co-operates with TGF- β to induce Th17 differentiation, while suppressing Foxp3 through Stat3 and ROR γ t signaling [14,19] and increasing T cell resistance to Treg [20]. In this regard, in mice bearing subcutaneous HNSCC, intravenous injection of a plasmid encoding IL-21, either alone or in combination with IL-15, led to increased NK and CTL activity with significant suppression of tumor growth and increased survival [21], reflecting similar promising results in other pre-clinical tumor models and in phase I clinical trials [20].

Although murine Th17 differentiation involves TGF- β , dependence on TGF- β does not appear to be a feature of human Th17 polarization, and a role for IL-1, IL-6 and IL-23 has been established [22]. In the flurry of interest now surrounding this unique population, additional communication signals that preferentially drive lineage commitment will most likely become evident, as will the mechanisms by which tumor niches become a repository for IL-17-secreting cells. Evidence of tumor-associated IL-23(Fig. 3B), recently linked to Th17 amplification and stabilization by mechanisms under close scrutiny, but which may involve activation of Stat3 [22], may orchestrate local polarization. APC-derived IL-23(p19/p40), like its relative IL-12(p35/p40), can also influence Th1 activation and may synergistically or antagonistically impact on tumor growth and progression, as suggested by conflicting data regarding IL-23 in tumors. Since IL-12 favors infiltration of CTL, but IL-23 promotes upregulation of MMP9, increased angiogenesis and reduced CD8⁺ T cell infiltration, the high tumor levels of IL-23 bode poorly for tumor suppression. And in fact, elimination of IL-23 led to increased infiltration of CTL, rendering a protective effect against chemically-induced murine carcinogenesis [23]. Paradoxically, high levels of exogenous IL-23 have also been associated with a beneficial response [24,25], a discrepancy to be resolved.

TGF- β suppression of tumor immunity

The tumor appears undaunted by the complexity and intensity of the immune cell interactions coordinated as an impediment to its existence and in fact, seems to exploit these pathways to create a beneficial tolerogenic environment. In the evolving events, tumor-derived TGF- β and/or other mediators may effectively impair the function of immune cells involved both in tumor recognition and effector anti-tumor responses. For example, APC, notably DC [26], as well as macrophages are critical elements in anti-tumor immunity and are abundant in HNSCC (Fig. 1), but apparently fail to do their job (Fig. 2). By immobilizing DCs, TGF- β interferes with migration and antigen transport to draining lymph

nodes and may also decrease DC by escalating apoptosis [27], along with inhibiting maturation and expression of MHC class II and co-stimulatory molecules [28]. While it is bad enough that inadequate APC cannot generate an appropriate T cell response, these cells also release a plethora of mediators, including TGF- β , supportive of malignancy [4]. TGF- β also stimulates production of vascular endothelial growth factors(VEGF) and other angiogenic factors, expression of which (VEGFR-3 and VEGF-C) in HNSCC is not only ominously associated with delayed neck metastasis [29], but may further obscure DC function. In patients with HNSCC, increased VEGF-A correlated with microvessel density, disease progression, reduced mature DC, but increased peripheral immature DCs with aberrant functional capacity. When co-cultured with these immature DCs, allogeneic T cells expressed markers consistent with regulatory T cells and secreted TGF- β , IL-10 and VEGF-A. Consequently, VEGF-A may not only be instructional in angiogenesis and tumor progression, but also foster immune tolerance [30].

When the immune system fails to eliminate all transformed cells, tumors with reduced immunogenicity emerge that are capable of escaping immune destruction [1], in part through hijacking of protective immunosuppressive pathways [10]. To this end, TGF- β influences most aspects of CD4⁺ and CD8⁺ effector functions including their proliferation, differentiation and acquisition of effector molecules [28]. In TGF β RII transgenic mice, loss of TGF- β signaling in T cells resulted in the generation of tumor-specific CTL responses which otherwise would be repressed [31]. TGF- β can repress CTL through blunting expression of cytolytic gene products [32], through a mechanism involving CD1d-restricted natural killer T cells(NKT) in concert with IL-13 [33], and also through induction of Treg.

Regulatory T cells and TGF- β

In addition to its direct immunoregulatory powers, TGF- β also coordinates its immunosuppressive functions through the conduit of regulatory T cells. Different subtypes of regulatory T cells, molded within the tumor milieu, contribute to a tolerogenic environment. Regulatory type 1(Tr1) lymphocytes, influenced by Cox2 and detected primarily by expression of IL-10 and TGF- β , are associated with inhibition of DC maturation and immunosuppression in HNSCC [34]. Naturally occurring CD4⁺CD25⁺Foxp3⁺ Treg whose primary function is to control immunologic tolerance to self-antigens [35] lose their focus within the tumor milieu. Among the mechanisms for improvising niches of immune tolerance is the recruitment and generation of these immunosuppressive cells that are dependent on TGF- β for lineage commitment through induction of Foxp3, a master lineage regulator gene, and for function [10,36,37]. Elevated percentages of CD4⁺CD25⁺ Treg in the T cell population within or isolated from tumor tissues in HNSCC(Fig. 1) are also seen in the circulation [38,39]. Tregs within the tumor mediate suppression through TGF- β and/or IL-10, compromising tumor-specific CD8⁺ responses [38].

Tumor cells generate chemokines that attract Treg to foster immune privilege [40] and a more hospitable tumor site. Tregs within the tumor microenvironment are likely adaptive and induced (iTreg) in the periphery by their encounter with TGF- β [36], although they may differentiate from natural thymus-derived Tregs (nTreg) or be induced locally from another precursor or through expansion. Treg expansion and tolerance show a newly appreciated dependence on IL-2, consistent with the presence of IL-2R α (CD25) on their cell surface [17], but induction is abated in the presence of IL-6. Different pathways of Treg differentiation in tumors probably lead to heterogeneous populations of accumulating Tregs and might account for conflicting reports regarding their attributes and mechanisms of suppression [40]. At least some tumor-associated Tregs are specific for TA, although once activated, they further place a stranglehold on responses through bystander mechanisms.

In the tumor setting, Tregs inhibit the function of an expanding repertoire of target cells, including NK cells [39,41] and *in vivo* depletion of Tregs leads to restoration of both NK and CD8⁺ anti-tumor functions. NK cells, innate effectors aimed at defending against foreign invaders and transformed cells, accumulate in the tumor microenvironment, yet are often incompetent in eliminating malignant cells. Identifying their targets through receptors that recognize pathogen-encoded molecules or self proteins whose expression is altered in transformed or infected cells, NK cells are susceptible to TGF- β due to their stable expression of functional TGF β RII [39,41]. Not only does TGF- β interact with NK TGFBR directly, but NK cells are susceptible to control by Treg in a cell-cell contact dependent mechanism [39,41]. The consequence of engaging TGF- β signal transduction in NK cells is to suppress lytic and secretory activities and to downregulate NKG2D tumor cell recognition receptors [42].

That Tregs play a tumor-endorsed role in suppression of immunogenicity is supported by evidence that abrogation of Tregs results in enhanced protective anti-tumor immunity [43]. Depletion of Tregs was shown to be most efficient in early stages of tumor progression, since elimination of Treg before tumor challenge resulted in 70% tumor-free survival, while depletion in animals with established tumors lacked therapeutic effect [44]. Studies have also linked the prevalence of Treg with poor patient prognosis [40], and the ratio of CD8⁺ T cells to Tregs was considered a better predictor of patient survival than Treg alone [45]. In one report, increased Treg prevalence in HNSCC was surprisingly associated with a better disease prognosis [46], requiring further elucidation. Response to treatment has been linked to an expansion of Tregs in the circulation, although this may not be indicative of what occurs within the tumor sites [38]. The balance between helper T cells and Treg has also been shown to be a critical parameter in the response to cancer vaccines [47].

Conclusions

Clearly, within the context of a tumor environment, the forces impacting on the tumor and the infiltrating components of the immune system, whose initial attempts to attack the tumor may be subverted to assist the tumor, are extremely complex. No one cell type or its product(s) can be held accountable, but TGF- β appears to pervade the milieu and profoundly influence the behavior of not only transformed cells, but also stromal cells and immune cells. TGF- β may directly support tumor growth through its conditioning of the local microenvironment with growth and angiogenic factors, modulation of extracellular matrix, and distortion of the protease/antiprotease balance, but equally disconcerting is its ability to dramatically suppress host-tumor immune responses. Using TGF- β as a coalition builder, the tumor attenuates protective anti-tumor responses through suppression of T cell activation and accumulation of Tregs to create a safe haven.

Interrupting TGF- β 's stranglehold on immune surveillance may offer an interesting strategic option and the rationale for evaluating TGF- β signaling inhibitors as cancer therapeutics is compelling [48]. Nonetheless, since TGF- β has complex and often opposing context-linked effects on its cellular targets involving TGF- β -specific pathways and crosstalk with other signal transduction pathways, will delivery of TGF- β inhibitors shift the anti-tumor response from tolerogenic to immunogenic in concert with positive effects on the tumor or will the lack of synchronous effects have disparate effects on tumorigenesis and immunogenesis [49]? More directed approaches such as eliminating Treg through Foxp3 vaccination or suppressing Foxp3 via IL-21 [20] may provide more specific and effective modes of enhancing tumor immunity [50]. From the immunologic perspective, targeting TGF- β and/or its sources may offer respite from the ill-fated alliance between the tumor and TGF- β .

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•• of outstanding interest

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Figure 1. Expression of TGF β and TGF β -related molecules in OSCC

A) In HNSCC, tumor cells and inflammatory cells in the stroma as well as in a necrotic region express high levels of TGF β as detected by immunohistochemistry. B) Within regions of inflammatory cell infiltrates, identified by CD3⁺ T lymphocyte staining, are C) CD1a⁺ dendritic cells, D) CD68⁺ macrophages, E) IL-17⁺ lymphocytes, F) CD8⁺ T cells, G) Foxp3⁺ Treg, and H) intracellular phosphoSmad2 positive cells indicative of TGF β signaling.

Figure 2. Putative immunoregulatory roles of TGFβ in tumor immunity

TGF-β secreted by tumor cells and infiltrating leukocytes and sequestered in the extracellular matrix, may represent an early communication between host cells and tumor cells. In its pro-inflammatory role, TGFβ exerts potent chemotactic effects on leukocytes (Recruitment) and participates in the differentiation of CD4⁺ T helper (Th) cell responses, including Th17 (RORγt⁺) and Treg (Foxp3⁺) (T cell differentiation) activated by antigen presenting cell/dendritic cell (DC) presented tumor antigens (TA). Such pro-inflammatory events, intended to contribute to immune surveillance and to orchestrate an anti-tumor response, may be usurped by the tumor cells to provide growth factors, angiogenic factors (VEGF, etc.) and proteases (MMP1/2, plasmin, etc.) necessary to optimize tumor growth, invasion and metastasis. On the other hand, the immune suppressive effects of TGF-β assist the tumor in evasion of immune surveillance (Suppression). Unstimulated CD56⁺ NK cells and activated CD4⁺ and CD8⁺ T responder cells express TGFβR, which makes them vulnerable to secreted TGF-β and to suppression mediated through contact with Treg that express membrane TGF-β. Taking advantage of these pathways, the tumor often outmaneuvers the host defense system. While TGF-β represents but one of multiple contributing factors, its role is not insignificant in the growth and immune escape of the heterogeneous cellular constituents of a tumor. Like tissue repair, the evolution of tumors and host failure to eradicate them are regulated by complex interactions between malignant cells, epithelial cells, stromal cells, inflammatory cells, extracellular matrix, proteases, and a bevy of soluble mediators of communication.

Figure 3. IL-21 and IL-23 expression in HNSCC

Tissue sections from squamous cell carcinoma (A, B) and normal oral epithelium (C) were stained with antibodies for the cytokines IL-21 and IL-23. A) Inflammatory cell infiltrates that characterize squamous cell carcinoma contain cells consistent with lymphocytes that stain positively for IL-21. B) IL-23 staining included macrophage-like cell populations within the stromal cell regions. C) Little or no IL-23 and IL-21 stained inflammatory cells were identified within the relatively normal oral epithelium.