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TGF- β biology in cancer progression and tumor immunotherapy

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TGF- β signaling plays key roles in cancer progression. Most carcinoma cells have inactivated their epithelial antiproliferative response but benefit from increased TGF- β expression and autocrine TGF- β signaling through effects on gene expression, release of immunosuppressive cytokines and epithelial plasticity, which enables invasion and dissemination, increased stem cell properties and cancer drug resistance. TGF- β released by cancer cells, stromal fibroblasts and other cells in the tumor micro-environment further promotes cancer progression by shaping the architecture of the tumor and suppressing anti-tumor activities of immune cells, thus generating an immunosuppressive environment that prevents or attenuates the efficacies of cancer immunotherapies. Repression of TGF- β signaling is therefore seen as a prerequisite and major avenue to enhance the efficacies of current and forthcoming immuno-oncological therapies, also when the tumor cells themselves are not TGF- β responsive. We discuss approaches to TGF- β signaling blockade, their use in cancer immunotherapies, and possible adverse effects.

Since its discovery as a secreted factor that induces reversible phenotypic transformation of select fibroblast cell lines^{1–3}, the roles of TGF- β have been studied in tumorigenesis, particularly carcinomas. These studies were encouraged by three sets of observations: (1) TGF- β expression in tumor cell lines and tissues is often increased when compared to normal cells or healthy tissue^{4,5}, (2) the growth inhibitory response to TGF- β seen in normal epithelial cells is often impaired in carcinomas^{6,7}, and (3) the TGF- β response of epithelial and carcinoma cells confers attenuation of epithelial characteristics and increased cell migration and invasion, which contribute to cancer progression^{8,9}. Yet, tumorigenesis depends on the tumor cells' ability to generate and condition a tumor micro-environment (TME) containing diverse stromal cell types that enables cancer cells to thrive, and promote cancer progression^{10–20}. Most if not all cells of the TME have the potential to respond to

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Rik Derynck is a co-Founder of Pliant Therapeutics. Shannon Turley is an employee of Genentech Inc., and Rosemary Akhurst has sponsored research agreements with Pfizer Corp. and BMS and is a co-inventor of a patent on the use of anti-TGF- β antibodies that is co-owned by UCSF and Xoma.

TGF- β , although their responses depend on the cell type, are highly contextual, and show considerable signaling heterogeneity. Consequently, understanding the roles of TGF- β in cancer progression requires a full appreciation of the complex interplay of TGF- β responses in cancer cells and non-cancer cells of the tumor. That such insights might enable new therapeutic approaches has become apparent with the success of anti-cancer therapies based on immune checkpoint blockade, and observations that increased TGF- β signaling in the TME correlates with poor overall survival and resistance to immunotherapeutic blockade of PD-L1 or its receptor, PD-1²¹⁻²⁴. Complementing previous reviews²⁵⁻²⁷, this critical review aims to convey insight into the roles of TGF- β signaling in carcinomas and their TME, including immune cells, and addresses expected outcomes of targeting TGF- β signaling in cancer progression to enhance and benefit immunotherapy.

Carcinomas and their tumor micro-environment

As epithelial cells proliferate, initiate tumor formation, and transform into carcinoma cells, their ability to establish tumors and the behavior of those tumors depend on conditioning a micro-environment conducive for cancer progression. The tumor and TME evolve coordinately with reciprocal signaling between epithelial and TME cells^{10,11}. An early tumor-promoting microenvironment may develop in response to insults, or chemical or radiation-induced damage^{11,28-30}. Paracrine signals from tumor cells with activated oncogenes or lost tumor suppressor genes are likely to continue to condition the TME and help instruct the behavior of its cell populations. Conversely, signals from distinct TME cell populations instruct the behavior of tumor cells while also defining the behavior and functions of each other through a web of instructive and inhibitory autocrine and paracrine interactions. Many interactions occur locally through cell-cell interactions, or between cells and extracellular matrices, including contacts between T and NK cell receptors and peptide-MHC complexes³¹⁻³³. Most cytokines, chemokines and differentiation factors, including TGF- β , also act primarily locally.

Dependent on tumor type, grade and stage, carcinoma cells might represent a minority of tumor cells, with the bulk of the tumor consisting of stromal cells including fibroblasts, myeloid and lymphoid cells, as well as pericytes and endothelial cells (Figure 1). In most human carcinomas, the majority of tumor cells retain epithelial characteristics, including epithelial cell junctions and gene expression signatures, whereas some cells, mostly at invasive fronts, attenuate these characteristics through a transdifferentiation process, named epithelial-mesenchymal transition (EMT)³⁴⁻³⁷.

Stromal fibroblasts and fibroblast-like cells, referred to as carcinoma-associated fibroblasts (CAFs), play key roles in providing a beneficial tumor stroma, are interspersed between populations of tumor cells, and thus are well positioned to communicate with carcinoma cells (Figure 1). CAFs are thought to originate from fibroblasts that were activated in response to inflammatory and tumor-derived signals, and further expand through cell proliferation³⁸⁻⁴⁰. However, CAFs may additionally originate from epithelial and endothelial cells through EMT, thought to occur in response to cytokines, most prominently but not exclusively TGF- β ^{35,41,42}. CAFs support tumor growth and progression through secreted or membrane-associated signals that benefit the carcinoma cells, and deposition of

extracellular matrix (ECM) proteins, which confer instructive signals, and retain and thus store secreted chemokines and cytokines, including TGF- β ^{40,43}. The ECM network of the TME helps define tumor architecture. Tumor growth depends on extensive vascularization to transport oxygen and nutrient into the tumor mass, thus allowing both the tumor cells and non-malignant stromal cells to thrive^{18,44–47}. The vascular capillary system, elaborated by endothelial cells and pericytes that organize into vessels, supply blood, oxygen and nutrients to the TME, facilitates entry of immune and other cell types into the tumor^{13–17}, whereas the lymphatic system enables exit of immune cells, antigens, interstitial fluid, live and dying tumor cells, and particles from the tumor into tumor-draining lymph nodes^{18,19}.

The TME contains diverse types of innate and adaptive immune cells (Figure 1); these include monocytes and macrophages, myeloid-derived suppressor cells (MDSCs), neutrophils and other granulocytes, dendritic cells (DCs), conventional and non-conventional T cells, B cells and NK cells, all of which are thought to contribute to or control cancer progression. Among these, T cells and NK cells exert cytotoxic anti-tumor effects^{48,49}, DCs present tumor antigens to T cells^{50–54}, and macrophages and neutrophils remove cells and cell debris by phagocytosis⁵⁵. Local cytokines in the TME, including TGF- β , direct the behavior of these cell types, often resulting in functional changes that convert them to support cancer progression rather than direct tumor destruction. IgA-producing plasmablasts derived from B lymphocytes can contribute to tumor progression, and also their activities are controlled by TGF- β ⁵⁶. Among the T cell populations that, together with NK cells and B cells, control cancer progression, are CD8⁺ T cells whose differentiation and anti-tumor killer cell activity can be repressed by several cell types, including MDSCs⁵⁷ and regulatory T (Treg) cells⁵⁸. TGF- β promotes Treg cell differentiation^{59,60} and enables Treg cells to suppress adaptive and innate immune responses^{61–64}.

Fundamentals of TGF- β biology with relevance to cancer

To understand the roles of TGF- β in cancer, we introduce some fundamentals of TGF- β biology that are more extensively summarized elsewhere⁶⁵. The mammalian genome encodes three TGF- β s, TGF- β 1, - β 2 and - β 3, which act as disulfide-linked dimers. Each gene encodes a precursor protein with a short amino-terminal signal peptide required for secretion and a carboxy-terminal 112 amino acid, mature TGF- β polypeptide. These two domains are linked by a long pro-segment. During secretion, the pro-segments are cleaved from the mature polypeptides, yet remain associated with mature TGF- β to act as chaperones. Consequently, the mature TGF- β dimer is secreted in “latent” complex with two copies of the non-covalently associated pro-segment, often called “latency-associated polypeptide” (LAP), that prevent TGF- β binding to its cell surface receptors (Figure 2A).

Since TGF- β 1, purified from platelets, was biochemically seen to primarily exist as homodimer⁶⁶, it is assumed that TGF- β 1, TGF- β 2 and TGF- β 3 are always expressed and act as homodimers. This notion is reinforced by the commercial availability of TGF- β homodimers that are used to evaluate effects of TGF- β s; however, the isolation of natural TGF- β 1: β 2 heterodimers⁶⁷ raises the possibility that TGF- β heterodimers, e.g. TGF- β 1: β 3, may also be naturally expressed in tumors. Carcinoma cells express TGF- β 1, although its expression is heterogeneous and dynamic, and may occur with or without TGF- β 2 or

- $\beta 3^{21,41,68}$. MDSCs, type II macrophages and stromal CAFs are also predominant sources of TGF- $\beta 1$, and CAFs and poorly differentiated spindle cell carcinomas additionally express TGF- $\beta 3^{41,69,70}$. Hematopoietic cells, including immune cells, predominantly express TGF- $\beta 1$, thus making TGF- $\beta 1$ the predominant but not the only TGF- β actor in the TME⁷¹.

Latent complexes of the TGF- $\beta 1$ homodimer, often have one of the dimeric pro-segments covalently linked to a fibrillin-like latent TGF- β binding protein 1 (LTBP1) that enables their deposition in the ECM⁴³ (Figure 2B). Secretion of such complexes does not result in release of soluble active TGF- β , but in localized deposition of inactive complexes in proximity to TGF- β expressing cells. As a consequence, subsequent activation is required to release mature TGF- β that can bind and activate TGF- β receptors on cells, in close proximity⁴³. Alternatively, the pro-segments in latent TGF- $\beta 1$ complexes also associate with transmembrane proteins that belong to a subset of LRRC proteins and allow for cell-associated retention of latent TGF- $\beta 1$. Indeed, latent TGF- $\beta 1$ associates with LRRC32, also known as GARP (“glycoprotein-A repetitions predominant”), at the surface of Treg cells, activated B cells, mesenchymal stromal cells and platelets, thus enabling GARP to control retention of latent TGF- β complexes^{72,73} (Figure 2B). GARP is also expressed by endothelial cells, fibroblasts and megakaryocytes⁷⁴, raising the possibility for an even broader role for GARP in TGF- $\beta 1$ latency or distinct functions with no direct link to TGF- β activation. The GARP-related LRRC33 may play a similar role for other cell types, including myeloid cells, since it also associates with latent TGF- $\beta 1$, and consequently also controls TGF- $\beta 1$ latency and activation^{75,76}. Other closely related LRRC proteins, such as LRRC15, which is expressed by stromal fibroblasts in advanced stage cancers^{77,78}, may play similar roles in cell-associated TGF- β retention.

Since many, if not all, cell types of the tumor deposit or retain latent TGF- β complexes locally, and have variable degrees of responsiveness to TGF- β , the TGF- β activation mechanisms and tumor architecture of cancers are key determinants of TGF- β actions in the TME. How latent TGF- $\beta 1$ complexes, with or without LTBP1, are activated has been extensively studied⁴³; however, no studies elucidate the activation of TGF- $\beta 2$ or - $\beta 3$ homodimers or TGF- β heterodimers. Together, many findings highlight (1) key roles of structural interactions between the TGF- $\beta 1$ prosegment and selected integrins in maintaining TGF- $\beta 1$ latency and TGF- $\beta 1$ activation, (2) contributions of proteases, often metalloproteases, in degrading the prosegments and associated proteins, thus leading to release of active TGF- $\beta 1$, and (3) cell type- and context-dependent diversity of the activation mechanisms as determinants of the spatiotemporal control of TGF- β actions in the TME⁴³. While structural deformation of the integrin-prosegment interaction can release TGF- $\beta 1$ from latency⁷⁹, physiological TGF- $\beta 1$ activation scenarios are likely to combine molecular deformation of this interface with metalloprotease activities⁴³ (Figure 2C). Which integrin is involved in TGF- $\beta 1$ latency and activation depends on cell type and context, and often involves heterotypic cell interactions. Thus $\alpha v\beta 6$ and $\alpha v\beta 1$ mediate TGF- $\beta 1$ activation at the surface of epithelial cells and fibroblasts^{80,81}, respectively, whereas $\alpha v\beta 8$ is required for activation of GARP-bound TGF- $\beta 1$ at the surface of Treg cells^{81,82}, and endothelial cells during retinal and neurovascular development^{83,84}. TGF- $\beta 1$ and $\alpha v\beta 8$ also associate with LRRC33 at the surface of microglial cells⁷⁵, enabling LRRC33 to control TGF- β signaling. The expression of $\alpha v\beta 8$ by carcinoma cells suggests that this integrin controls

TGF- β 1 activation on or adjacent to malignant cells⁸⁵. Thus, targeted interference with the integrin-prosegment interface represents an approach to selectively inhibit or attenuate TGF- β 1 activation in some cell populations. The diversity of local TGF- β 1 activation mechanisms may help explain differences in the roles of TGF- β in the TME, and in susceptibility to TGF- β inhibition, depending on the architectural organization of the TME.

Following activation, TGF- β binds a tetrameric combination of two types of transmembrane kinases, type I and type II receptors, that are able to phosphorylate serine, threonine and tyrosine⁶⁵ (Figure 2D). TGF- β binding to these receptor complexes activates the receptors, and, consequently, Smad2 and Smad3 through C-terminal serine phosphorylation by the type I receptor kinases. These effector Smads, in combination with Smad4, then translocate into the nuclei, where they combine with DNA sequence-specific transcription factors and coregulators at regulatory gene sequences, and thus activate or repress target gene transcription in response to TGF- β ^{65,86,87} (Figure 2D). Detection of C-terminally phosphorylated Smad2 and/or Smad3 is indicative of TGF- β /Smad signaling, yet may also result from their activation in response to other TGF- β family members, such as activins, myostatin and GDFs^{65,88}. While Smad signaling uniquely defines “canonical” signaling for TGF- β family proteins, TGF- β also activates non-Smad signaling pathways, including the PI3K-Akt-mTOR pathway, Erk MAPK and p38 MAPK signaling, and Rho GTPases^{65,89} (Figure 2D). These pathways, however, are not diagnostic of TGF- β signaling, since they are strongly activated by receptor tyrosine kinases⁹⁰.

The number of TGF- β receptors at the cell surface is low, especially when compared to receptor tyrosine kinases⁶⁵. Unlike receptor tyrosine kinases, the bulk of TGF- β receptors are retained intracellularly, not available for TGF- β binding but ready for transport to the cell surface⁹¹. Cells have a remarkable ability to rapidly upregulate TGF- β responsiveness by inducing receptor transport to the cell surface, thus enhancing the availability of receptors for TGF- β binding^{91,92}. Additionally, ubiquitylases and de-ubiquitylases extensively control the stability and degradation of TGF- β receptors^{88,93}, whereas activation of the metalloprotease TACE, e.g. in response to inflammatory stimuli or growth factors, induces ectodomain cleavage of one of the two TGF- β receptors, thus attenuating TGF- β responsiveness. Consequently, metalloprotease inhibition can prevent TACE activity, thus enhancing TGF- β responsiveness and signaling⁹⁴.

TGF- β signaling defines the phenotype and behavior of carcinoma cells.

While resting epithelial cells show very low, if any, TGF- β expression in vivo, hyperplasia and neoplasia confer increased expression of TGF- β 1^{5,29} that can suppress the growth of benign or low-grade cancers, or stimulate cancer progression of malignant and metastatic tumors^{41,95} (Figure 3). The *TGFBI* gene is transcriptionally activated by AP-1 or E2F transcription factors in response to activities of oncogenic proteins such as mutant Ras, growth factors and/or tumor promoters^{96,97}. TGF- β receptor expression is transcriptionally enhanced concomitantly with hyperplasia⁹⁸, and Akt activation promotes transport of intracellular TGF- β receptors to the cell surface, thus enhancing TGF- β responsiveness^{65,91}. Increased glucose uptake, a hallmark of carcinoma cells, and hyperglycemia induce Akt activation and thus promote an increase in cell surface TGF- β receptors and TGF-

β responsiveness⁹². Enhanced Akt activation also results from mutations, e.g. *PTEN* inactivation, or increased signaling of IGF-1 receptors or other receptor tyrosine kinases, which is common in carcinomas and promotes cell proliferation or survival^{99,100}. We therefore surmise that most carcinoma cells have increased sensitivity to autocrine or localized TGF- β signaling, when compared to normal epithelial cells.

In addition to a plethora of changes in gene expression, TGF- β induces or represses expression of microRNAs^{101,102} and enables Smad-mediated control of the maturation of microRNAs^{65,103}, with each microRNA regulating translation of many target gene transcripts. TGF- β /Smad signaling also directs extensive changes in mRNA splicing, thus generating different protein isoforms^{104,105}. Many autocrine TGF- β -induced responses control differentiation and behavior of carcinoma cells, while others affect cell metabolism and additional activities of direct relevance to the carcinoma cells and/or their TME. Among these, TGF- β induces ECM protein expression, and induces or represses the expression of proteases and protease inhibitors^{106,107}. These responses to autocrine TGF- β signaling allow carcinoma cells to contribute to the composition of the ECM, which serves as a reservoir of latent TGF- β and controls its activation (Figure 3). The ECM in turn helps direct the differentiation and behavior of different cell populations, and contributes to mechanical properties of tumors, with an ensuing increase in intratumoral interstitial fluid pressure that contributes to poor accessibility of cancer drugs in the tumor¹⁰⁸. Overall, though, how TGF- β responsiveness of carcinoma cells affects the TME has been minimally characterized.

TGF- β inhibits cell proliferation in normal epithelial cells by inhibiting cell cycle progression through G1¹⁰⁹⁻¹¹¹, which is seen as a tumor suppressor pathway that is incompatible with malignant cell transformation and cancer progression^{111,112}. However, carcinoma cells develop strategies to inactivate this growth inhibitory response^{110,111}. Colorectal and other carcinomas with microsatellite instability (MSI) commonly have inactivating mutations in *TGFBR2*, which encodes the TGF- β type II receptor, T β RII, thus rendering cells unresponsive to TGF- β . Such microsatellite-unstable colon carcinomas have a lower capacity for dissemination than their more common MSI-negative counterparts¹¹³. Genetic loss of *TGFBR2* also occurs at very low frequency in other tumor types, such as head and neck squamous cell carcinoma (SCC)¹¹⁴, and genetic inactivation of the *TGFBR1* gene, encoding the TGF- β type I receptor T β RI, also known as ALK5, is also seen in gastric and several other carcinomas^{115,116}. While *TGFBR2* mutations should affect all TGF- β responses, it is unclear whether this holds for *TGFBR1* mutations.

Mutations in the gene encoding Smad3, the major effector of TGF- β signaling, are infrequent. However, deletions in the gene encoding Smad4, the co-Smad for all effector Smads, are frequent in pancreatic ductal adenocarcinomas (PDAC) and other gastrointestinal tract cancers¹¹⁷, but do not necessarily inactivate TGF- β /Smad signaling^{118,119}. In PDAC, *SMAD4* inactivation is a late event in cancer progression that may selectively inhibit TGF- β -induced growth inhibition, and, by extrapolation to loss of *Tgfbr2*¹²⁰, might change the profile of cytokines and chemokines released from tumor cells. By some accounts, loss-of-function TGF- β receptor or Smad mutations occur in the majority of head and neck SCCs¹²¹, and are frequent in other carcinomas¹¹⁶. *TGFBR1* mutations in SCCs are unlikely to drive cancer progression, since they are subclonal, and loss of heterozygosity is rare¹²¹.

However, this may illustrate the role of tumor heterogeneity in cancer progression, whereby tumor cells with TGF- β receptor or Smad mutations interact with those that do not, to cooperatively promote tumor outgrowth.

Also, non-mutational mechanisms impair the growth inhibitory response to TGF- β . In advanced tumors, *TGFBR2* or *SMAD2* genes are frequently silenced by histone and DNA methylation^{122,123}. Additionally, increased inhibitory Smad6 or Smad7 expression in various carcinomas, including PDAC, attenuates Smad signaling^{124,125}, whereas increased expression of the Smad corepressors c-Ski or SnoN, observed in some carcinomas, selectively impairs Smad-mediated target gene transcription^{87,125}. Highly active PI3K-Akt signaling prevents FoxO nuclear import, and thus impairs Smad-mediated induction of p21^{Cip1} expression¹¹¹, and increased expression of the c-Myc-binding protein Miz represses TGF- β -induced expression of cdk inhibitors¹¹¹. Such scenarios enable carcinoma cells to overcome the tumor suppressive activity of TGF- β signaling, while maintaining TGF- β responses that benefit cancer progression.

TGF- β promotes differentiation plasticity, stem cell properties and cancer drug resistance.

An increasingly appreciated response to TGF- β signaling in normal and transformed epithelial cells is the epithelial plasticity response, which represses epithelial characteristics and promotes transdifferentiation toward a mesenchymal phenotype (Figure 3). This EMT process results in deconstruction of epithelial cell-cell junctions and reorientation of apical-basal polarity toward a front-rear polarity, elongation of cells, and increased motility that enables directed migration and invasion through ECM. Accompanying these phenotypic and behavioral changes are extensive changes in gene reprogramming and mRNA splicing^{34–36,126,127}. The ability of TGF- β to promote epithelial plasticity and EMT results primarily from activation of T β RI, enabling Smad3/4-mediated activation of genes that encode master EMT transcription factors, such as Snail1 and Snail2/Slug, and ZEB1 and ZEB2. These in turn cooperate with Smad3/4 complexes to repress epithelial and activate mesenchymal genes¹²⁸. Additionally, TGF- β signaling through another type I receptor, ACVR1/ALK2, which activates Smad1 and Smad5, is also required for TGF- β -induced EMT¹²⁹. Added to extensive gene expression changes are abundant changes in miRNA expression and mRNA splicing that alter the diversity of protein isoform expression^{37,102,128,130}. Some changes in gene expression are seen as diagnostic for decreased epithelial character and the EMT process^{127,128,131}. Since Smads control transcription through cooperation with high-affinity DNA-binding transcription factors⁶⁵, TGF- β -induced EMT requires cooperation with other pathways, most prominently Wnt and MAPK signaling^{128,132}, and mTOR signaling downstream from Akt is required for progression and completion of EMT^{128,132}. In human carcinomas, extensive changes that include an elongated phenotype and mesenchymal gene expression suggestive of full EMT are rarely seen^{41,42}. Most commonly, human carcinoma cells acquire an intermediate, partial EMT, i.e. a hybrid epithelial/mesenchymal state that allows for coexistence of epithelial and mesenchymal characteristics^{37,127,133–135}. Furthermore, gradations in EMT are often apparent, with leading cells at the tumor periphery having more pronounced EMT than those

that follow them^{37,127,133,134}, reminiscent of collective cell migration, when groups of cells move together directionally^{135,136}. Decreased adhesion and increased motility and invasion enable carcinoma cell dissemination and thus provide carcinoma cells their metastatic potential^{37,135}.

In a variety of carcinomas, EMT increases the number of carcinoma cells with stem cell properties, i.e. cancer stem cells (CSCs)^{34,37,127,137}. Their “stemness” is defined by an ability to initiate tumor formation in vivo, but is more conveniently scored by expression of cell surface markers, exclusion of certain dyes and drugs, and generation of multicellular spheres from single cells^{34,37,127,137,138}. Under the influence of TME factors, particularly TGF- β ^{9,37,139}, epithelial cells acquire CSC-like phenotypes, providing a dynamic balance of interconversion between a small population of CSCs with EMT properties and a much larger population of differentiated carcinoma cells^{37,140}. How, at the cellular and molecular levels, stem cell properties relate to EMT is unclear, but EMT is thought to be required for and enables stemness^{34,127,140,141}. A linear relationship may not exist between progression along the EMT spectrum and stem cell properties, a notion that is supported by the observation that in mammary carcinoma cells partial EMT or stabilized EMT correlates with more pronounced stemness characteristics than full, reversible EMT^{36,134,142}.

Of further therapeutic relevance, EMT enables carcinoma cells to exert local immunosuppression^{143,144}. EMT confers repression of major histocompatibility complex class I (MHC I) antigen presentation by tumor cells, which is required for recognition by CD8⁺ T cells, and thus indirectly suppresses cytotoxic activities of CD8⁺ T cells and cytolytic activities of NK cells^{141,143–145}. TGF- β -induced EMT also suppresses the expression of other components of the antigen processing and presentation machinery that is required for cancer cell recognition and killing by cytotoxic T lymphocytes¹⁴⁵. Local immunosuppression induced by EMT also correlates with increased expression of immunosuppressive cytokines and chemokines^{141,143,144}. Among these, increased TGF- β 1 release and activation¹⁴¹ may play a key role since TGF- β promotes Treg cell differentiation and represses tumor cell killing by CD8⁺ T cells. EMT also confers increased cancer cell expression of PD-L1, the ligand for the immune-inhibitory PD-1 receptor on CD8⁺ T lymphocytes^{141,146}. These and other observations predict that EMT allows for escape from antitumor immunity, and decreases susceptibility to immunotherapy^{37,143,146}. Accordingly, in mice, breast carcinoma cells with mesenchymal characteristics show resistance to checkpoint blockades, and those that remain largely epithelial are more susceptible^{34,141,143}. On the other hand, triple-negative breast cancer in humans, which are marked by the mesenchymal appearance of cancer cells, show susceptibility to anti-PD-L1 checkpoint inhibition^{147,148}.

EMT also correlates with increased resistance of carcinoma cells to chemotherapies^{149–152}. The underlying basis of this correlation needs to be further explored, although cancer drug resistance correlates with stem cell-like properties^{149,153–155}, and cancer drug resistance and stem cell properties are coordinately repressed by mTOR inhibition¹⁴². Additionally, EMT in carcinoma cells is accompanied by changes in genomic stability and DNA repair. Although divergent observations are reported^{156,157}, TGF- β treatment and EMT-associated CSC properties correlate with a decreased ability of carcinoma cells to repair double-

stranded DNA breaks and increased genomic instability^{158,159}. The resulting accumulation of genomic changes may contribute to heritable genotypic diversity that contributes to tumor evolution during cancer progression.

Considering the roles of TGF- β sensitivity and autocrine TGF- β signaling in defining the behavior of carcinoma cells (Figure 3), one may speculate how therapeutic inhibition of TGF- β signaling might directly affect TGF- β -responsive cancer cells. Since TGF- β signaling promotes TGF- β 1 expression, TGF- β inhibition should decrease the de novo synthesis, release and activation of TGF- β 1. Furthermore, considering its role in driving epithelial plasticity and EMT-associated changes, TGF- β inhibition should enhance the epithelial characteristics of cancer cells, and decrease their dissemination and tumor re-initiation capacity. Moreover, localized EMT-associated immunosuppression, cancer drug resistance, and increased genomic instability, might be repressed by TGF- β inhibition, thus impairing long-term cancer progression. However, TGF- β inhibition is not expected to lead to cancer cell death or inhibition of tumor cell proliferation. Ultimately, however, these effects on tumor cells need to be conceptualized in the context of anti-tumor immunity driven by TGF- β inhibition.

Diverse roles of TGF- β in innate immune cell populations in cancer

Early in tumor development, the immune system exerts surveillance, destroying genetically or epigenetically abnormal cells through actions of NK cells, macrophages and intra-epithelial T cells. Mutational activation of a single or multiple oncogenes is, however, insufficient for immune eradication of an oncogene-activated cell, reflected by the existence of oncogenic mutations at a high rate within normal tissues¹⁶⁰. Nevertheless, immunosurveillance-mediated destruction of cells with dominant neoantigens shapes the tumor's antigenic repertoire¹⁶¹. Concomitantly, increased TGF- β 1 expression and activation, seen as initial events in tumorigenesis, are induced by insults²⁹, and expose immune cells, pre-neoplastic and neoplastic tumor cells to epithelial cell-derived TGF- β from the earliest stages of tumorigenesis. At some critical point during tumor progression, cancer cells prevail over immune surveillance, which allows the tumor to progress towards full-blown malignancy¹⁶¹. Once established, the tumor comprises a mix of immune cell types including monocytes, macrophages, DCs, granulocytes, T cells, B cells, NK cells and various subsets thereof, with considerable heterogeneity within each tumor and between tumor types. Innate and adaptive immune cells interact with each other as well as the tumor and its microenvironment. Several types of innate and adaptive immune cells respond to TGF- β that can be released by cancer cells, stromal cells and immune cells themselves, resulting in an immunosuppressive TME (Figure 4).

Among the innate immune armory, NK cells exhibit tumor suppressive functions during early and late stages of anti-cancer immunity. Ligation of activating receptors, such as NKG2D, by MHC class I-related (MIC)-A or MICB proteins on target cancer cells can incite activation, cytokine production, degranulation and cytotoxic potential in NK cells¹⁶². Notably, TGF- β 1 represses NKG2D expression directly and through induction of miR-183 that targets the expression of an NKG2D adaptor protein, and suppresses MICA and MICB expression by tumor cells^{163–167}. As in other immune cell types, TGF- β /Smad signaling

represses interferon- γ (IFN- γ) expression that supports myeloid cell and CD8⁺ T cell proliferation and differentiation¹⁶⁸.

TGF- β additionally affects myeloid cell types within the tumor, including MDSCs, macrophages and neutrophils. These cells normally accumulate at sites of infection and wounding, where they contribute to elimination of infectious agents and resolution of inflammation. They accumulate early during tumor outgrowth in response to chemokines and cytokines, including TGF- β , that are produced by tumor cells and in response to tumor promotion. While macrophages and neutrophils evolved to eliminate infectious agents and damaged cells, they adopt a tumor-promoting type II phenotype during cancer progression in response to factors released by the tumor and its microenvironment¹⁶⁹, and in response to TGF- β ^{170–173}. Within the tumor, the phenotypes of MDSCs overlap with those of monocytes and immature neutrophils. MDSCs are highly plastic in their migratory behavior and phenotype, suppress NK cell activities¹⁷⁴, and contribute to metastatic effects of TGF- β ^{57,174}. TGF- β represses antigen presentation, skews cytokine production and promotes immunosuppressive activity in DCs and other myeloid cells^{175–177}. TGF- β also suppresses reactive oxygen species and NO production, which is required for the destructive properties of macrophages^{178,179}. TGF- β signaling in myeloid cells of the pre-metastatic niche is essential to breast cancer metastasis, and *Tgfbr2* inactivation within the myeloid compartment dramatically reduces lung colonization by mammary tumor cells¹⁸⁰.

DCs specialize in antigen processing and presentation, and function as liaisons between the innate and adaptive arms of the immune system. They normally patrol tissues, an example being the Langerhans DCs in the epidermis, which depend on TGF- β signaling, initiated by paracrine TGF- β activation by keratinocytes¹⁸¹. Multiple types of DCs, including the CD103⁺ subset, are detected in tumors^{54,182}. DCs internalize soluble and particulate antigens, including tumor cell fragments, and transport their antigenic cargo to draining lymph nodes through lymphatic vessels. Although DCs constitute only a small fraction of immune cells of the tumor, they are critical to an adaptive immune response^{54,182}. En route to lymph nodes, DCs process tumor antigens into peptide ligands for presentation to CD4⁺ and CD8⁺ T cells¹⁸³. TGF- β suppresses antigen presentation by DCs, thus impairing T-cell-mediated anti-tumor responses¹⁸³, and may suppress DC cell migration^{184,185}. Additionally, intestinal DCs can induce Treg cell differentiation through activation of TGF- β , possibly involving cell-intrinsic expression of integrin $\alpha v \beta 8$ ^{186,187}.

Control of intratumoral T cell-mediated cytotoxicity by TGF- β

Immune-mediated tumor eradication requires cytotoxic CD8⁺ T cells, except in tumors with lost or reduced HLA/MHC expression, in which case NK cells may serve this role¹⁸⁸. Additionally, memory CD8⁺ T cells provide long-term immunity against key tumor epitopes¹⁸⁹. Accordingly, activation of CD8⁺ T cells is a major focus of cancer immunology, and in the design of effective immunotherapies, illustrated by the engineering of adoptive CD8⁺ T cell therapies¹⁹⁰, chimeric antigen receptor-expressing CD8⁺ T cells (CAR-T cells) of different functionalities^{191–193}, and checkpoint blockade inhibitors that boost the tumoricidal activity of CD8⁺ T cells^{194–196}. The consecutive steps leading to CD8⁺ T cell-mediated tumor cell killing, including T cell activation, expansion, differentiation and

infiltration, are all regulated by immune and non-immune cell types, including myeloid cells, Treg cells, endothelial cells, fibroblasts and tumor cells. Each of these cell types are themselves TGF- β -responsive and support or control cytolytic T cell activity, and thus help define the outcome of tumor rejection. Furthermore, TGF- β impinges on key steps of the T cell response to tumor, including T cell activation, proliferation, differentiation and migration, in both the TME and tumor-draining lymph nodes. TGF- β represses CD8⁺ T cell-mediated anti-tumor immunity, through direct effects on the CD8⁺ T cells, and effects on accessory cells that govern CD8⁺ T cell function (Figure 4). Key roles of TGF- β 1 in suppressing the adaptive immune system are apparent from the lymphoproliferative autoimmune phenotype of *Tgfb1*^{-/-} mice¹⁹⁷⁻¹⁹⁹, and similar phenotypes following selective ablation of TGF- β signaling components within CD4⁺ and CD8⁺ T cells²⁰⁰.

Activation of CD8⁺ T cells involves signals provided by APCs; T cell receptor (TCR)-mediated recognition of peptide-MHCI complexes at the surface of any cell, including tumor cells, and interaction of the T cell-restricted co-activating receptor, CD28, with one of its APC-expressed ligands, CD80 (B7.1) or CD86 (B7.2) are both required. This dual APC-dependent ligation mechanism initiates TCR signaling to drive T cell activation, proliferation and differentiation into cytotoxic CD8⁺ T cells with antigen-specific tumoricidal potential²⁰¹. TGF- β signaling suppresses processes that lead to CD8⁺ T cell activation and maturation, including, in APCs, expression of components of the antigen processing and presentation machinery, such as HLA/MHC^{175,183,202}. Furthermore, TGF- β signaling in T cells activates SHP1 expression, which represses protein tyrosine kinase activities required for TCR signaling^{203,204}. In addition, TGF- β inhibits CD8⁺ T cell proliferation, as shown in mice with T cell-specific inactivation of TGF- β signaling that develop multifocal lymphoproliferative inflammation similar to *Tgfb1*^{-/-} mice²⁰⁰. CD8⁺ T cell proliferation additionally requires interleukin (IL)-2 expression and responsiveness, and TGF- β suppresses the expression of IL-2 and its receptor by effector and memory T cells²⁰⁵. Finally, TGF- β signaling represses expression of IFN- γ and genes for cytolytic machinery components or transcription mediators that orchestrate their expression in activated CD8⁺ T cells^{206,207}.

With a focus on anti-tumor immunity by cytotoxic CD8⁺ T cells, CD4⁺ T lymphocytes and their roles in tumor rejection have received less attention. However, renewed interest in CD4⁺ T cells has arisen from studies of CAR-T cells²⁰⁸⁻²¹⁰, and growing evidence that TGF- β blockade targets CD4⁺ CAR-T cells in anti-cancer therapy²¹¹. CD4⁺ T cells, activated by MHCII-presented peptides on the surface of APCs, are classically associated with their helper function for CD8⁺ T cells and other immune cells. For example, release of Th1 and Th2 cytokines by activated CD4⁺ T helper (Th) cells supports tumor cell killing by cytotoxic T cells, macrophages and granulocytes²¹². However, tumor rejection that depends on CD4⁺ Th cells and NK cells but not on CD8⁺ T cell activity has been observed²¹³⁻²¹⁵. Indeed, CD4⁺ T cells can adopt tumor-reactive cytotoxic activity that contributes to tumor eradication, as shown in mice with inactivated TGF- β signaling in CD4⁺ T cells, leading to NK- and cytotoxic T cell-like phenotypes²¹⁶. Notably, CD4⁺ CAR-T cells are more effective cytolytic effectors than CD8⁺ CAR-T cells, demonstrating CD28-dependent granzyme and perforin-mediated cytotoxicity²⁰⁸. They are also less susceptible to exhaustion following TCR activation²⁰⁹. Opposing the activities of CD4⁺ Th cells, CD4⁺ Treg cells repress the

functional differentiation and cytolytic activities of CD8⁺ T cells, and anti-tumor activities of myeloid cells²¹².

TGF- β orchestrates the development and plasticity of CD4⁺ T cells. In much the same way as cytotoxic CD8⁺ T cells are suppressed, TGF- β suppresses CD4⁺ Th cell proliferation and expression of master transcription factors that drive TNF- α and IFN- γ expression²¹⁷. Furthermore, TGF- β skews the differentiation of CD4⁺ Th1 cells along alternative pathways, dependent on the cytokine context, e.g. supporting differentiation of Th2 and Th17 cells in the presence of IL-4, and IL-6 and IL-1 β , respectively²¹⁷. Importantly, TGF- β in the presence of IL-2 promotes functional differentiation and survival of CD4⁺ Treg cells. In naïve CD4⁺ T cells, TGF- β /Smad3 signaling activates *Foxp3*, which encodes a master transcription factor that directs differentiation into activated CD4⁺Foxp3⁺CD25⁺ Treg cells⁶⁰. These then functionally repress, through various activities, including TGF- β 1 activation^{82,145}, the tumor-killing activity of CD8⁺ T cells^{58,63,82,145,218,219}, suppress differentiation of NK and DCs²²⁰, and contribute to macrophage and neutrophil polarization towards a regenerative rather than inflammatory cell state^{58,63,82,145,172,173,218,219}. As single cell analyses reveal extensive phenotype heterogeneity among Treg cells²²¹, it will be important to differentiate the effects of TGF- β and its inhibitors on distinct Treg cell sub-populations.

Taken together, TGF- β signaling exerts profound immunosuppressive activities on key cell types that orchestrate innate and adaptive immunity, thereby attenuating the intrinsic tumoricidal potential of immune cells within the TME. Inhibition of TGF- β signaling is therefore expected to enhance the anti-tumor responses of both myeloid and T cells.

TGF- β signaling and stromal fibroblasts

In addition to immune cells, the TME comprises a large CAF population with subsets of fibroblasts of diverse phenotypes^{77,222–226}. In primary and metastatic tumors, cancer cells reside adjacent to these stromal cells, and tumor cells play key roles in their recruitment, differentiation and behavior. In turn, CAFs define and support the properties, compartmentalization and behavior of cancer cells. Additionally, they can help define the immunosuppressive environment, through effects on immune cell activation and impeding infiltration of immune cells into tumors^{227–231}. The interplay between cancer cells, stromal fibroblasts and immune cells drives and defines cancer progression.

Fibroblasts play central roles in connective tissue by maintaining tissue homeostasis and enabling wound healing. In healthy adult tissues, they show a “resting” phenotype with low levels of proliferation and metabolism. Upon tissue injury or in response to inflammation, they are “activated”, with enhanced proliferation and protein synthesis, and are more metabolically active. They can further differentiate into myofibroblasts that express α -smooth muscle actin²³². CAFs show characteristics of activated fibroblasts and/or myofibroblasts, consistent with the chronic inflammation and mechanical tension in tumors^{12,233,234}. Depending on the tumor type, fibroblasts can represent a sizable component of primary tumors and metastatic lesions that proportionally expands with increased tumor size.

Stromal fibroblasts are key determinants of the architectural and functional organization of the tumor, in part through abundant secretion of ECM proteins and protease-mediated remodeling of the ECM. Indeed, activated fibroblasts and myofibroblasts abundantly secrete various ECM proteins, and matrix metalloproteases, cathepsins and other ECM remodeling enzymes^{77,224,235,236}. They also express lysyl oxidases such as LOXL2, which crosslinks collagen fibers yet also promotes TGF- β signaling in CAFs^{237,238}. Additionally, the markedly adhesive and contractile myofibroblasts sense and incite mechanical tension and tissue rigidity²³⁹. The ECM deposition and remodeling by stromal fibroblasts and contractility of myofibroblasts define the mechanical properties and stiffness of the tumor, and position stromal fibroblasts as determinants of therapeutic efficacy. Finally, CAFs secrete a plethora of soluble and ECM-associated factors, including cytokines, chemokines and growth factors that promote tumor growth and cancer cell dissemination^{40,240}. Some of these act directly on tumor cells, while others act on immune and endothelial cells, thus influencing trafficking and functions of myeloid and T cells, or act through autocrine and paracrine mechanisms on the stromal cell populations²⁴¹. Conversely, in a PDAC mouse model, depletion of proliferative α -smooth muscle actin expressing cells, which may comprise a mixture of CAFs and other stromal cells, increased the ratio of Treg and effector T cells in late stage tumors, and extended survival following treatment with a CTLA-4 blocking antibody²⁴².

Since TGF- β is activated locally and unlikely to diffuse, CAFs respond locally to TGF- β provided by cancer cells, immune cells or other cell types in close proximity. The localized exposure of stromal fibroblasts to TGF- β produced by cancer cells may involve filopodial extensions over several cell diameters^{243,244}. The response of stromal fibroblasts to TGF- β released by hyperplastic epithelial cells might be particularly relevant early in tumor formation when pre-neoplastic cells recruit fibroblasts to generate an ECM and TME. However, inactivation of TGF- β signaling in stromal fibroblasts potentiates neoplasia in adjacent epithelia²⁴⁵. The communication between CAFs and carcinoma cells through TGF- β is likely to contribute to tumor evolution throughout cancer progression.

Stromal fibroblasts are a major source of TGF- β in the TME^{40,227}. Since TGF- β induces *TGFBI* expression²⁴⁶, TGF- β 1 released by cancer cells may activate TGF- β 1 expression in CAFs. Consequently, CAFs show amplified autocrine TGF- β expression and activation, conceptually similar to autocrine TGF- β signaling in the cancer cell population. Whether stromal fibroblasts express primarily TGF- β 1, similarly to carcinoma cells, and how latent TGF- β released by CAFs is activated are questions that remain to be addressed. However, TGF- β 3 is clearly expressed by stromal CAFs and mesenchymal tumor cells^{41,70,247}, and its role in relation to TGF- β 1 remains to be better defined.

TGF- β signaling in stromal fibroblasts induces changes in their physiology and behavior that help define their roles in cancer progression. At very low levels, TGF- β act as chemo-attractant for fibroblasts^{248,249}. Thus, TGF- β released and activated by CAFs may promote recruitment of fibroblasts, similarly to TGF- β secreted by cancer cells. Additionally, TGF- β promotes fibroblast proliferation¹¹⁰, and in this way may contribute to the expansion of the fibroblast population²⁵⁰. TGF- β signaling in fibroblasts also protects against cell death and

promotes survival²⁵⁰. Conversely, however, pharmacological inhibition of the T β RI kinase enhances stromal fibroblast proliferation in a mouse melanoma model³⁸.

TGF- β /Smad signaling, in cooperation with Erk MAPK and Akt-mTOR signaling, promotes activation and myofibroblast differentiation of CAFs, similar to fibroblast differentiation upon wounding and inflammation^{228,251,252}. Persistent TGF- β signaling in the TME may drive fibroblast activation and myofibroblast differentiation in the tumor. It may also explain the expression and functions of EMT transcription factors in a fraction of stromal fibroblasts^{253,254}. EMT transcription factor expression in response to TGF- β may be intrinsic to fibroblasts, but may also reflect an epithelial or endothelial origin of these CAFs. Indeed, epithelial and endothelial cells contribute, through EMT or endothelial-mesenchymal transition (EndMT), to the CAF population^{255,256}, although the extent of their participation is a matter of debate. Concomitant with fibroblast activation, TGF- β induces ECM protein expression and ECM remodeling, thus making increased ECM deposition and remodeling a direct consequence of increased TGF- β signaling in CAFs. Additionally, with CAFs as a major source of TGF- β expression in the TME, CAF-mediated deposition of TGF- β in the ECM generates a reservoir of latent TGF- β for subsequent activation. Consequently, the architectural organization of TGF- β expression and signaling in stromal fibroblasts controls intratumoral immune cell functions and cytotoxic activities of NK and T cell populations (Figure 5), and thus cancer progression. Finally, TGF- β influences CAF expression of cytokines and chemokines that act on cancer, endothelial and/or immune cells, such as HGF²⁴⁵, VEGF, IL-6, IL-8, MCP1, PGE2 and CXCL12^{229,257–262}. Release of such factors by CAFs influences the TME and may thus contribute to a pro-tumorigenic and immunotolerant state with e.g. inhibition of T cell trafficking and exclusion of T cells. TGF- β signaling in CAFs is increasingly seen as a determinant of resistance to immunotherapy^{21,23,77}.

Control of endothelial cell function and angiogenesis by TGF- β

The ability of tumor cells to induce new blood vessel formation is essential for tumor growth and metastatic cancer dissemination^{44,263}. Various observations highlight activities of TGF- β on endothelial cells that may promote or repress angiogenesis, depending on the experimental setting^{264–266}. Pro-angiogenic activities of TGF- β on endothelial cells are mediated by the ALK1 type I receptor and endoglin, which promote endothelial cell proliferation and migration²⁶⁵, whereas its anti-angiogenic activity signals through the canonical T β RI/ALK5 receptor²⁶⁶. TGF- β 's pro-angiogenic activities include promotion of capillary tube formation²⁶⁷, and expression of angiogenic factors, including VEGF-A^{262,268}. However, TGF- β can also induce mesenchymal stem cells to differentiate into endothelial cells²⁶⁹. Overall, TGF- β has been convincingly shown *ex vivo* and *in vivo* to induce a pro-angiogenic environment and stimulate tumor angiogenesis^{270–272}. Additionally, TGF- β secreted by endothelial cells promotes recruitment of perivascular pericytes and myofibroblasts that support functional vascular integrity²⁷³, and interaction between endothelial cells and pericytes confers localized and constitutive TGF- β activation, which is required for microvascular integrity^{274,275}. It should be noted that increased TGF- β expression has been linked to increased microvessel density in certain tumor types^{271,272}. TGF- β also indirectly acts on endothelial cells, through Treg cells, to

establish an immune-protected endothelium with suppressed endothelial cell activation²⁷⁶. Conversely, endothelial cell-specific inactivation or repression of TGF- β signaling can suppress atherosclerosis-associated vascular inflammation by enhancing vascular integrity through reversal of EndMT²⁷⁷. These and other observations link TGF- β to tumor-induced angiogenesis and functional integrity of the tumor vasculature.

Lymphangiogenesis is essential for trafficking of immune cells and cellular debris, as well as drainage of interstitial fluid. Lymphatic vessels can also carry tumor cells and tumor-derived factors to draining lymph nodes, and thus contribute in multiple ways to tumor physiology, immunosuppression and cancer progression^{278,279}. However, the roles of TGF- β in tumor-associated lymphangiogenesis and lymphatic function have been less studied²⁸⁰.

Therapeutic approaches to inhibit TGF- β signaling

As is apparent from the roles of TGF- β signaling in cancer cells and different cell types of the TME, increased TGF- β signaling promotes progression of carcinomas. Hence, inhibition of TGF- β signaling is expected to attenuate cancer progression through direct effects on cancer, immune and stromal cells. The notion of TGF- β signaling inhibition as a therapeutic anti-cancer approach was met with considerable hesitation for about two decades, and took many years to mature and be evaluated. A primary reason for this delay was that TGF- β was initially found to potently inhibit proliferation of epithelial cells, and therefore seen as a tumor suppressor of carcinomas^{110,111,281–284}. The elucidation of underlying mechanisms of cell cycle inhibition^{281,285–287} provided high visibility to this concept, and justifiably raised concerns that TGF- β inhibition might give rise to tumors from pre-neoplastic epithelial cells²⁸⁷. Furthermore, the notions that TGF- β promotes EMT, that EMT contributes to cancer dissemination, and that EMT associates with an increase in cancer stem cell properties took a long time to become accepted¹²⁷. Finally, although it was realized early on that immunosuppressive actions of TGF- β contribute to cancer progression²⁸⁸, the clinical successes with immune checkpoint blockade stimulated renewed interest in harnessing TGF- β signaling as an approach to enhance cancer therapies. Recent results in fact highlight the role of increased TGF- β signaling in intrinsic resistance to checkpoint blockade therapy^{21,23,38,71,145}. The immunosuppressive role of TGF- β reminds us of earlier concerns, based primarily on knock-out mouse studies, that TGF- β 1 depletion would lead to excessive and de-repressed inflammation and auto-immune responses^{197,199}.

An array of therapeutic approaches that target individual steps of the canonical TGF- β /Smad signaling pathway and cell-directed approaches to inhibit TGF- β signaling have been developed and are under evaluation, with several of these in clinical trials. Besides inhibition of *TGFB1* transcription²⁸⁹ or targeting *TGFB1* or *TGFBR2* mRNA for degradation²⁹⁰, most approaches to inhibit TGF- β signaling fall into four classes (Table 1). One class, exemplified by galunisertib (LY2157299; developed by Eli Lilly) as a well-studied therapeutic^{291,292} consists of small molecule inhibitors of TGF- β receptor kinases. These prevent ATP binding to the ATP binding pockets of the TGF- β receptors, primarily T β RI, thus blocking Smad2 and Smad3 activation in response to TGF- β binding. However, although simple to deliver orally, these inhibitors have poor pharmacokinetics and pharmacodynamics. Additionally, they do not target TGF- β receptors specifically, since they equally effectively inhibit

related type I receptors for several other TGF- β -related proteins, such as activin, nodal and myostatin²⁹³, and may also inhibit other kinases, such as p38 MAPK^{292,294}. Furthermore, while they prevent TGF- β -induced Smad activation, it is unclear whether they prevent TGF- β -induced PI3K-Akt-mTOR activation, since Akt activation through T β RI-TRAF6-TAK1 appears unaffected by this type of inhibitor in some cells but not others^{65,295,296}. Consequently, lack of specificity and poor pharmacokinetics of current TGF- β receptor kinase inhibitors make dosing strategies difficult. Nevertheless, low production costs, ease of administration and promising results encourage the clinical evaluation of several such inhibitors, including Eli Lilly's next generation LY3200882 (NCT02937272²⁹⁷) and vactosertib from Medpacto^{298,299}(NCT03732274²⁹⁷).

Another class of therapeutics comprises monoclonal antibodies that prevent TGF- β receptors from binding ligand^{145,300}. While neutralizing TGF- β antibodies have exquisite ligand specificity, they need to effectively interfere with the very high binding affinity (10^{-11} M range) of TGF- β to cell surface receptor complexes⁶⁵. Antibodies are expensive to manufacture and may be less efficiently distributed within the tumor than small molecule inhibitors, but provide superior pharmacokinetic properties. The TGF- β antibody fresolimumab, a human IgG4k monoclonal antibody based on the 1D11 antibody that neutralizes all three TGF- β isoforms, was developed by Cambridge Antibody Technology/ Genzyme-Sanofi, and showed promising results in a phase 1 clinical trial³⁰⁰. A next generation pan-TGF- β antibody, SAR439459 (NCT03192345²⁹⁷), was developed by Sanofi, and is seen as a lead candidate in anti-cancer mono- and combination therapies. Xoma Corporation developed TGF- β blocking antibodies with distinct specificities for the three TGF- β s³⁰¹, and selected one that neutralizes TGF- β 1 and - β 2 but not TGF- β 3 for clinical testing, with the rationale that TGF- β 3 may oppose effects of TGF- β 1 and - β 2, and may be less commonly expressed in tumors³⁰². However, CAFs do express TGF- β 3, as do some mesenchymal tumors^{21,23,41,69}. TGF- β 3's role in cancer progression needs to be better defined. Novartis is pursuing a clinical phase 1/1b dose escalation study to evaluate anti-TGF- β 1/ β 2 antibody, NIS793, in combination with anti-PD-1 antibody in patients with advanced cancers (NCT02947165²⁹⁷). An antibody, developed by Eli Lilly, that blocks activated TGF- β 1 but not TGF- β 2 or TGF- β 3, completed phase 1 clinical evaluation as monotherapy³⁰³. Finally, a T β RII receptor antibody (IMC-TRI, LY3022859) under development by Eli Lilly showed efficacy in preclinical evaluation against mammary cancer³⁰⁴, but was not further pursued due to onset of uncontrolled cytokine release syndrome at higher doses before therapeutic benefit was observed³⁰⁵ (Table 1).

Other inhibitors are designed as soluble, high-affinity ligand traps to prevent TGF- β binding to its receptors^{306,307}. These comprise Fc-stabilized dimers of T β RII extracellular domains that are aimed to sequester TGF- β 1 and - β 3, but not TGF- β 2, and thus prevent their binding to transmembrane T β RII receptors³⁰⁷⁻³⁰⁹. The utility of a TGF- β ligand trap in blocking tumor dissemination was first shown in mice that express such a trap and were challenged by intravenous or orthotopic implantation of breast carcinoma cells³⁰⁶. Distinct TGF- β traps have now been developed with differences in ligand binding specificities, and incorporation of the betaglycan ectodomain was shown to enable TGF- β 2 binding³¹⁰. Further engineering led to the development of a smaller ligand trap with a 100–1000-fold increased ligand-

binding efficacy³¹¹. Based on this design, AVID200, developed by Forbius, entered the clinic for therapeutic evaluation against advanced malignancies (NCT03834662²⁹⁷). The Fc-T β RII trap was also the basis for the development of a bi-specific trap that combines TGF- β binding by the T β RII ectodomains with a human PD-L1-blocking IgG1^{308,309,312}.

Finally, some antibodies and small molecules target the TGF- β activation process, and thus provide selective cell- or tissue-type inhibition of TGF- β signaling. Latent TGF- β 1 activation involves interaction at the cell surface of the prosegments of TGF- β 1 with selected β integrins in complexes with α v integrin. Since α v β 1, α v β 6 and α v β 8 mediate TGF- β 1 activation⁸¹, targeted interference with these interactions prevents TGF- β 1 activation in a cell type-selective manner without systemic TGF- β inhibition. Antibodies against integrins β 1, β 6 and β 8 were shown to selectively impair TGF- β 1 activation^{85,313–315}, and small molecules have been developed that prevent integrin β 1- and integrin β 6-mediated latent TGF- β 1 activation^{316–318}. Such antibodies and small molecules are now under preclinical and clinical evaluation. Notably, antibodies against integrin β 8, which is expressed on tumor cells, Treg cells and DCs, have anti-tumor activity as monotherapy in some syngeneic cancer models^{82,85,319}. This activity, thought to result from interference with TGF- β activation, is the basis of a clinical evaluation of PF-06940434, which targets α v β 8 (NCT04152018²⁹⁷). Since GARP, in cooperation with integrin α v β 8, is required for TGF- β 1 activation at the surface of Treg cells^{82,320}, antibodies that block GARP-mediated TGF- β 1 activation have also been evaluated for their efficacy in enhancing the cytotoxic immune response in cancers⁶⁴. Since GARP is also expressed by endothelial cells, fibroblasts, megakaryocytes and platelets^{73,74}, the selectivity of such anti-GARP antibodies for the cytotoxic immune response remains to be further validated. Finally, an antibody was developed that binds the TGF- β 1 prosegment and prevents dissociation of mature TGF- β 1 from LAP, thus keeping TGF- β 1 latent without affecting TGF- β 2 or TGF- β 3 activation⁷¹. This antibody is expected to systemically prevent TGF- β 1 activation and is under preclinical evaluation⁷¹.

Efficacy of anti-TGF- β monotherapy

Success of classical cancer therapies, i.e. chemotherapy, radiation and molecular targeted therapies, is based on early regression of the primary tumor, using Response Evaluation Criteria In Solid Tumors, or RECIST³²¹, which commonly results from direct cytotoxic effects on cancer cells. However, while some anticipated direct antitumoral activities of TGF- β inhibition, TGF- β signaling inhibitors in general do not incite direct cytotoxic effects on malignant cells. Inhibition of cancer cell dissemination or cancer stem cell properties is expected from anti-TGF- β therapy; however, these are not primary RECIST criteria and not easily scored. Success of TGF- β inhibition in anticancer therapy is likely to arise from combined effects on cells of the TME, e.g. inhibition of immunosuppressive activities, effects on stromal fibroblasts that repress ECM production and allow for immune cell infiltration (Figure 5), and impaired angiogenesis. These effects are not easily assayed in immuno-compromised human xenograft models, but require syngeneic immune-competent mouse tumor models, and clinical outcomes should be assessed using guidelines developed for immune checkpoint blockade therapies^{322,323}. Notably, therapeutic effects of anti-TGF- β

monotherapy or combination therapies on immune or other cells in the TME do not depend on TGF- β responsiveness of the cancer cells^{23,324}.

TGF- β inhibition as monotherapy, using neutralizing antibodies, ligand traps or receptor kinase inhibitors, has seen some success in mouse models of carcinomas, primarily in reducing metastatic spread rather than reducing growth of the primary tumor³²⁵ (Table 1). Early examples showed reduction of breast cancer metastasis in response to anti-TGF- β antibodies or soluble T β RII-based ligand trap^{306,326,327}. Indeed, transgenic expression of a ligand trap suppressed metastatic outgrowth of mouse mammary tumors, with no apparent adverse effects³⁰⁶. Other studies using T β RI small molecule inhibitors confirmed attenuation of metastasis in various carcinoma types³²⁵.

In clinical trials that used conventional RECIST criteria, monotherapy with the T β RI kinase inhibitor galunisertib provided unimpressive clinical responses, although success may have been pre-empted by restricted dosing regimens imposed to avoid possible non-tolerated adverse effects^{328–330} (Table 1). The anti-pan-TGF- β antibody fresolimumab showed promising results in a phase 1 trial of 29 melanoma and renal cell carcinoma patients, without dose-limiting toxicity up to 15 mg/kg dose. One melanoma patient achieved a partial response, and six had stable disease with a median progression-free survival of 24 weeks³⁰⁰. In contrast, Eli Lilly's TGF- β 1-specific neutralizing antibody, developed for renal fibrosis, did not show clinical benefit in a small biomarker study of patients with metastatic cancer³⁰³. Additional, ongoing studies in monotherapy are designed to assess potential toxicities of novel TGF- β inhibitors. However, based on increasing usage of combination therapies, and restrictions due to compliance with standard of care practices, TGF- β inhibition as monotherapy is unlikely to be a clinical path forward.

Potential Adverse Effects of TGF- β signaling blockade

Although anti-TGF- β treatment is well documented to attenuate cancer cell dissemination and metastasis in mouse models, some pre-clinical data nevertheless raise concerns about possible adverse outcomes. Increased mammary cancer metastasis was seen in response to the T β RI kinase inhibitor, LY364947 (Eli Lilly)³³¹. Additionally, treatment with a pan-TGF- β antibody reduced metastasis in only three of twelve syngeneic mouse mammary carcinoma models, and increased cancer dissemination in another three³³². The suppression of cancer dissemination by anti-TGF- β was not seen in immune-deficient mice, whereas its prometastatic activity was immune-independent, associated with a gene expression signature similar to ER⁺ human breast cancers. This effect may result from enhanced cancer cell proliferation since ER⁺-like carcinomas retain growth inhibition by TGF- β ³³². Additionally, TGF- β 2 was shown in a mouse model to support metastatic breast cancer dormancy within bone, thus raising concerns about tumor promoting effects of TGF- β 2 inhibition^{333,334,335}. These cautionary observations illustrate the importance of identifying biomarkers that predict responses to TGF- β inhibition of distinct tumor types and distinguish effects of TGF- β blockade on cancer cell proliferation and dissemination.

Like many small molecule inhibitors, resistance to TGF- β receptor kinase inhibitors may develop. Long term LY2109761 treatment of mice harboring chemically induced SCCs

resulted in outgrowth of tumors with increased, drug-refractile Smad2 activation and phenotypes suggestive of elevated TGF- β signaling³³⁶. Treatment of colon carcinoma cell lines with galunisertib resulted in activation of AXL and p38 MAPK, suggesting an adaptive mechanism of cancer cell resistance³³⁷. Short-term treatment schedules and/or “drug holidays”, routinely used with targeted therapies, may avoid acquisition of such resistance. Consequently, most clinical trials utilizing galunisertib or next generation relatives follow a regimen of two weeks on and two weeks off drug treatment.

Clinical studies of TGF- β antibodies or TGF- β signaling inhibitors allow an evaluation of undesirable clinical adverse effects, including those that had been major causes for concern and contributed to extensive delays in clinical evaluation. Such fears were that inhibition of TGF- β signaling might induce metaplasia and tumor outgrowth, consistent with the tumor suppressor role of TGF- β signaling during carcinoma development, or that de-repressed immune responses might lead to inflammation and auto-immune manifestations. Our current knowledge, primarily derived from clinical trials with galunisertib, fresolimumab, and a bi-specific anti-PDL-1/anti-TGF- β chimeric trap, M7824, indicate that some patients on high drug doses develop sporadic keratoacanthomas^{338,339}. Since such low-grade pre-malignant cutaneous squamous lesions are common manifestations with several other oncology drugs, and easily monitored and surgically managed, their occurrence is less of a barrier than originally perceived, especially with the hope for complete and durable remission of the treated malignancy. Additionally, grade 1 or 2 skin rashes have been reported with fresolimumab and M7824 inhibition^{338,339}. No dose-limiting adverse effects on the immune system have been reported in clinical trials using small molecule T β RI kinase inhibitors or TGF- β antibodies. However, escalating doses of a T β RII antibody (LY3022859, Eli Lilly) in patients with advanced carcinomas resulted in effects indicative of a cytokine release syndrome, prior to reaching a dose that was considered efficacious for cancer treatment³⁰⁵. The anti-TGF- β 1 antibody LY2382770 was found to be safe, with fatigue, nausea and diarrhea as common side effects, in a phase 1 study in metastatic cancer patients, but without efficacy³⁰³.

Initial dose escalation studies of galunisertib or other T β RI kinase inhibitors mandated monitoring for cardiotoxicity due to heart valve dysplasia that was observed in rats and dogs receiving high doses of drug^{340,341}. However, valve malformations were seen only at doses beyond those required for anti-cancer efficacy. Cardiovascular toxicity with histopathological changes in the heart was also seen in mice and Cynomolgus monkeys treated with an antibody that potently neutralizes all three TGF- β s³⁴². However, in contrast to treatment with the T β RI kinase inhibitor LY2109761 or a pan-TGF- β antibody, antibody-mediated repression of TGF- β 1 activation did not result in cardiac valvulopathies in a rat toxicity model⁷¹. In human clinical trials using galunisertib, cardiac monitoring revealed treatment-related cardiovascular effects, but these did not reach grade 3 toxicity even at the highest doses³⁴¹.

Additionally, concerns about effects on vascular integrity leading to hemorrhagic lesions need to be considered when designing anti-TGF- β therapy regimens, especially since TGF- β 1 is required for endothelial integrity through effects on pericytes^{273,275}. Treatment of mice and monkey in toxicology models with a potent, neutralizing pan-TGF- β antibody resulted

in persistent hemorrhagic bleeding and associated pathologies³⁴². Clinical trials of M7824 reveal an association with manageable mucosal bleeding attributed to TGF- β blockade³³⁹. Overall, these findings strongly suggest that potent and systemic inhibition of all three TGF- β s may confer substantial toxicity that, however, is attenuated with less efficient inhibition or targeting TGF- β 1 only. Nevertheless, small molecule inhibitors, anti-TGF- β -antibodies and M7824, using appropriate dosage and treatment regimens, have manageable safety profiles, even though the therapeutic window is narrow. Ongoing studies will expand our knowledge on adverse effects and their impact on therapeutic modalities.

Combining TGF- β blockade with established anti-cancer therapies

Since inhibition of TGF- β signaling is expected to repress immunosuppressive activities, angiogenic responses and activation of the CAF population in the TME, TGF- β inhibition lends itself to enhance the efficacies of other therapeutic approaches. Furthermore, EMT of cancer cells, driven by TGF- β signaling, also promotes cancer drug resistance and cancer stem cell properties. Consequently, targeted inhibition of TGF- β signaling and/or EMT of cancer cells is expected to inhibit cancer progression when combined with cytotoxic or radiation therapies (Table 1).

Radiation induces both a rapid release of bioactive TGF- β 1 from latent complexes and increased TGF- β 1 expression^{343,344}. Consequently, tumor irradiation enhances TGF- β 1 activation within the TME that promotes ECM deposition and fibroblast proliferation, and contributes to fibrosis³⁴⁵. Enhanced TGF- β may also promote radiation-induced increase in drug resistance, and p53-ATM-mediated DNA mismatch repair as a protective mechanism against radiation-induced damage¹⁵⁶. Radiation also increases tumor antigenicity by increasing antigen presentation on cancer cells and release of tumor neoantigens from necrotic cells³⁴⁶. This anti-tumor immune activation in response to irradiation accounts for the abscopal effect of radiation therapy, whereby irradiation of a single metastatic lesion can induce regression of dispersed non-irradiated lesions within the same animal or patient³⁴⁶.

Several preclinical and clinical trials combine irradiation with TGF- β signaling inhibition (Table 1), with the expectation of maximizing DNA damage, reducing fibrosis, and elevating immune-mediated tumor clearance³⁴⁷. A small clinical trial with fresolimumab in combination with radiotherapy for metastatic breast cancer failed to meet the endpoint of activation of an abscopal effect following radiation therapy, but did show enhanced overall survival in patients receiving a high drug dose³⁴⁷. In this study, elevated memory CD8⁺ T cells led the authors to postulate that a combination of PD-1/PD-L1 blockade with fresolimumab and radiation would be required for durable anti-tumor responses³⁴⁷.

Combinations of T β RI kinase inhibitors with chemotherapy are also pursued³⁴⁸ (Table 1). Most completed studies used galunisertib in combination with the methylating agent lomustine for recurrent glioblastoma^{291,349}, sorafenib for unresectable hepatocellular carcinoma^{350,351}, or gemcitabine for unresectable pancreatic cancer³⁵². Eli Lilly's T β RI small molecule inhibitor, LY3200882, is also under evaluation in combination with capecitabine, a fluoropyrimidine, for advanced colorectal cancer, and other chemotherapeutic agents for a variety of solid tumor types ([NCT02937272](#)²⁹⁷). Other

T β RI kinase inhibitors, including vactosertib²⁹⁸, are being evaluated in combination with paclitaxel in metastatic gastric cancer patients (NCT03698825²⁹⁷), pomalidomide for multiple myeloma (NCT03143985²⁹⁷), and FOLFOLI for pancreatic cancer (NCT03666832²⁹⁷).

Combining TGF- β blockade with immune checkpoint blockade

In light of the current emphasis on immunotherapies for cancer treatment, and appreciation of TGF- β 's immunosuppressive activities on innate and adaptive immune cells, avenues towards combining anti-TGF- β with checkpoint inhibitors are under intense investigation. An underlying and predominant rationale focuses on the ability of TGF- β released and activated by cancer cells, stromal fibroblasts and/or immune cells to dampen anti-tumor immunity and responses to immunotherapeutics through direct and indirect mechanisms^{21,23,71,145,353,354}. Thus, blocking TGF- β signaling should represses these immunosuppressive activities and enhance the success of checkpoint blockade inhibition or other immunotherapeutic approaches in cancer patients.

Complementary to immunosuppression by TGF- β , the transmembrane PD-L1 ligand, expressed by carcinoma cells, tumor infiltrating DCs and macrophages, binds the PD-1 receptor on CD8 T lymphocytes and represses their anti-tumor functions²⁰¹. Notably, EMT of carcinoma cells in response to increased TGF- β signaling is accompanied, in some tumors, by increased PD-L1 expression^{37,355,356}. CTLA-4, another inhibitory receptor that is expressed on CD4⁺ Treg and activated CD8⁺ T cells, restrains costimulation of T cells and thus also serves as immune checkpoint^{357,358}. Blockade of the CTLA-4 receptor or PD-1/PD-L1 interaction enables treatment of cancers in which either checkpoint restricts immune rejection of the tumor. Anti-CTLA-4 therapy has been somewhat effective in melanoma and several other cancers, and blockade of the PD-1/PD-L1 axis is now first-line therapy for various tumor types, particularly melanoma and non-small cell lung carcinoma, with their combination being superior at the expense of enhanced adverse effects^{359,360}. Unfortunately, the therapeutic response to anti-CTLA-4, anti-PD-1 or anti-PD-L1 antibodies is limited, not only by tumor type, but also by other parameters, such as mutation load and immune cell penetration into the tumor parenchyma. For most tumor types, response rates to single agent therapies in clinical trials ranges between 10 and 25 %, depending on tumor type. Melanomas and urothelial carcinomas that are intrinsically resistant to anti-PD-1/PD-L1 therapy show elevated TGF- β signaling, particularly in the stromal compartment of immune excluded tumors^{21,77,228,361}. Moreover, anti-PD-1 therapy by itself promotes intratumoral TGF- β signaling in mouse models¹⁴⁵. The contributions of TGF- β expression, activation and responsiveness in the different cell populations of different tumor types and grades, and the mechanisms by which T cell infiltration into the tumor are impaired remain to be defined; they involve activated CAFs, immunosuppressive myeloid cells, Treg cells and the ECM architecture of the TME^{21,145,361}.

Considering that TGF- β , CTLA-4 and PD-L1/PD-1 signaling act as parallel immunosuppressive pathways that repress cytotoxic T cell, NK cell and macrophage activities through distinct mechanisms, treatments that combine TGF- β inhibition with immune checkpoint inhibition are likely to increase therapeutic efficacy^{21,23,71,145,346,362}.

The rationale for such combinations is strengthened by observations that TGF- β -induced EMT enhances PD-L1 expression by tumor cells³⁶³, TGF- β /Smad3 signaling enhances antigen-induced PD-1 expression on tumor infiltrating T lymphocytes³⁶⁴, and anti-PD-1 treatment increases Smad3 activation and Treg cell differentiation in carcinomas¹⁴⁵, conferring increased TGF- β -driven immunosuppression by cancer cells.

Combination therapies have been pre-clinically evaluated in murine cancer models. Depending on the model and experimental design, anti-PD-1 or anti-PD-L1 enhance the anti-tumor responses of anti-TGF- β , and vice versa, and result in potent and durable cytotoxic T cell responses that can clear even large and aggressive primary tumors, and suppress cancer metastasis^{21,23,145,346,353,362}. Galunisertib^{23,353}, neutralizing anti-TGF- β antibodies^{21,145}, an antibody that prevents TGF- β 1 activation⁷¹ and anti-integrin β 8 antibodies that prevent TGF- β activation^{85,319} were shown to enhance the anti-tumor efficacy of PD-1/PD-L1 blockade. Increased efficacy of anti-PD-1/PD-L1 with these blocking antibodies was accompanied by enhanced T cell penetration into the tumor parenchyma and activation of CD8⁺ cytotoxic T cell and CD4⁺ Th cell populations^{145,319} (Figure 5). With rapidly expanding preclinical evaluation of combinations of immune checkpoint blockade, with TGF- β signaling inhibition, clinical trials have commenced (Table 1). A Novartis trial of anti-PD-1 and anti-TGF β 1/2 (NCT02947165²⁹⁷) initiated following preclinical studies on chemically induced carcinoma and other syngeneic models. A major question in this context relates to the extent to which TGF- β 2 and/or TGF- β 3 inhibition contribute to the therapeutic efficacy of TGF- β blockade. TGF- β 2 was reported to contribute to metastatic tumor dormancy^{333–335}, and TGF- β 3 was seen, in some cases, to antagonize TGF- β 1 activity³⁰². It is also unknown to what extent anti-integrin β 8 antibody affects integrin β 8 functions beyond TGF- β 1 activation. Other questions relate to possible adverse effects of TGF- β inhibition on previously established therapeutic windows for anti-PD-1, anti-PD-L1 and anti-CTLA-4 therapies. Combined on- and off-target toxicities will determine clinical design, therapeutic regimens and efficacies.

Bifunctional fusion proteins that bind and block both TGF- β and CTLA-4 or PD-L1 have been generated, thus biochemically linking immune checkpoint inhibition with blocking TGF- β signaling^{308,309,365}. By design, these chimeras, based on the T β RII ligand trap, should sequester TGF- β 1 and - β 3 only, similarly to dimerized T β RII receptor⁶⁵. However, M7824, the chimeric fusion protein that combines dimerized T β RII ectodomains with a monoclonal antibody to PD-L1 depletes both TGF- β 1 and TGF- β 2 in patients³³⁹, likely due to altered TGF- β binding properties of the T β RII trap when fused to anti-PD-L1. This bi-specific drug may enable targeting of TGF- β blockade to sites of high PD-L1 expression, i.e. within tumor or its draining lymph nodes. Whether a single molecule can simultaneously neutralize TGF- β and either PD-L1 or CTLA-4 in vivo may depend on steric constraints related to localized TGF- β activation and PD-L1 or CTLA-4 expression in the tumor.

Preclinical evaluation of an anti-CTLA-4-T β RII chimera in comparison with anti-CTLA-4 showed increased repression of Treg cell specification and a switch from IL17-expressing CD4⁺ Th17 cells to IFN- γ expressing Th1 cells³⁰⁹. Anti-CTLA-4-T β RII had superior anti-tumor activity in a breast cancer mouse model, compared with anti-TGF- β or a combination of anti-CTLA-4 and anti-TGF- β ³⁰⁹. The anti-PD-L1-T β RII chimera, M7824,

also demonstrated increased efficacy of tumor regression in some mouse models, when compared to T β RII trap alone or anti-PD-L1 monotherapy³⁶⁶, consistent with results from combinations of anti-TGF- β or anti-PD-1/PD-L1¹⁴⁵. M7824 and an independently developed anti-PDL-1-T β RII chimera also outperformed a combination of T β RII-Fc with anti-PD-1 antibody in tumor growth inhibition, and were found to repress mesenchymal properties of carcinoma cells in culture and in vivo^{309,367}, consistent with TGF- β 's role as driver of EMT in cancer progression. Furthermore, M7824 reduced Treg-mediated repression of CD8⁺ cytotoxic T cell activation in culture, and tumor infiltration of T cells and NK cells in vivo^{312,366}. Clinical studies suggest that M7824 exerts more durable responses than historic responses for anti-PDL-1 monotherapy³³⁹. With M7824 under clinical evaluation³⁶⁵, its safety profile is seen as manageable, with a narrow therapeutic window. Keratoacanthomas, skin rashes, and impaired vascular integrity that results in mucosal bleeding are expected side effects of TGF- β inhibition, whereas other adverse effects, including immune-related changes, resemble those seen with anti-PD-1 or anti-PD-L1 treatment.

With these successes, the key role of CD4⁺ T cells in driving anti-TGF- β tumor immune responses is increasingly recognized. CD4⁺ T cells are more prevalent than CD8⁺ cytotoxic T cells and, as mentioned, CD4⁺ T cells can acquire cytotoxic activity when TGF- β responses are inactivated²¹⁶. Importantly, CD4⁺ T cells show considerable plasticity within tumors, dependent on the cytokine milieu, and particularly TGF- β levels. The balance of CD4⁺ Th1, Th2, Th17 and Treg cells can shift dramatically following therapy, with reprogramming from one CD4⁺ T cell subset towards another^{368,369} (Figure 4). In a preclinical study of six SCC lines, the CD4⁺ T cell composition distinguished responsive lines from those refractile to anti-TGF- β /anti-PD-1 combination therapy in vivo¹⁴⁵. Checkpoint blockade enhanced their Th1/ Treg ratio, and this was reversed and further diminished by TGF- β blockade, accounting for anti-tumor synergy between both drugs¹⁴⁵. In a mouse model of anti-CTLA4-resistant metastatic prostate cancer, high CD4⁺ Th17 cell levels in bone metastases provided vulnerability towards TGF- β blockade by reprogramming these cells towards an anti-tumorigenic CD4⁺ Th1 phenotype³⁵⁴. Thus, high CD4⁺ T cell content, whether Treg or Th17 cells, may be prognostic for favorable response to TGF- β blockade.

TGF- β blockade promotes therapeutic activity of cancer vaccines and CAR-T cells

Before the synergy of TGF- β signaling inhibition with checkpoint blockade was discovered, the potentiating activity of TGF- β blockade towards cancer vaccines was recognized. Indeed, the earliest clinical trials targeting the TGF- β pathway for cancer treatment were designed to potentiate anti-tumor immunity using a tumor vaccine for non-small lung carcinoma, genetically modified by including an anti-sense TGF- β 2 RNA³⁷⁰. The concept was ahead of its time; the technology and choice of *TGFB2* as target now seem outdated, and a phase 3 clinical trial did not meet its endpoint of increased overall survival³⁷¹. TGF- β blockade using a T β RI kinase inhibitor, SM16, enhanced the antitumor effects of an

immune-activating adenoviral vector encoding IFN- β , and an adenoviral HPV E7 vaccine therapy in models of mesothelioma and lung cancer³⁷².

Adoptive transfer of ex vivo-expanded tumor-homing T lymphocytes for immunotherapy was clinically introduced over thirty years ago³⁷³. In preclinical studies, TGF- β blockade using a T β RI kinase inhibitor, SM16, was shown to augment the persistence of adoptively transferred T cells in spleen, lymph nodes, and tumors of thymoma-bearing mice, showed increased activation of tumor infiltrating T cells, enhanced tumor necrosis factor- α and decreased arginase expression in the TME³⁷⁴. Clinically, adoptive T cell therapy was rapidly followed by genetic manipulations to enhance targeting of T cells to tumor cells and T cell activation using chimeric antigen receptors (CARs)³⁷⁵, and CAR-T cell therapy has been successful for hematological malignancies. Expansion and adoptive transfer of autologous cytotoxic T lymphocytes from patients, selected for targeting one or two key Epstein-Barr Virus Latent Membrane Proteins expressed on B-lymphoma cells, resulted in sustained complete responses with little toxicity³⁷⁶. Since TGF- β represses cytotoxic T lymphocyte activation, TGF- β inhibition has been pursued to enhance the efficacy of CAR-T cell therapies. The T β RI inhibitor SM16A enhanced the adoptive T cell transfer and anti-tumor efficacy in mesothelioma-bearing mice³⁷⁴. With refinements in T cell engineering, incorporation of a dominant-negative, cytoplasmically truncated T β RII receptor within T cells attenuated immunosuppression by TGF- β ³⁷⁷. In a small number of patients with relapsed and refractory EBV-positive Hodgkin's lymphoma, the engineered lymphocytes showed considerable expansion in vivo, persisting in blood, and induced complete regression without a lymphoproliferative phenotype or cytokine release syndrome²¹¹. Further modifications of CAR-T-mediated approaches resulted in engineering of T cells expressing a chimeric T cell antigen receptor with an extracellular, single chain variable fragment derived from a neutralizing TGF- β antibody. These CAR-T cells respond to TGF- β by dimerizing the TGF- β binding CAR that results in activation of T cells. Thus, rather than exerting its normal immunosuppressive role, TGF- β acts as a potent immunostimulant of these CAR-T cells^{378,379}. Such design may enable targeting of CAR-T cells to areas in the TME with high levels of TGF- β .

Summarizing these clinical developments, repression of TGF- β signaling appears to enhance the efficacy of some cancer treatments, independent of the cancer cells' TGF- β responsiveness. Preclinical studies, supported by early clinical observations, show enhanced efficacies of immunotherapies when TGF- β signaling is repressed and its immunosuppressive activities are consequently attenuated. Ongoing and future clinical trials may fully validate these findings in cancer treatment.

Concluding remarks and future directions

TGF- β signaling plays key roles in cancer progression, through direct effects on cancer cells, immune cells and other cell types in the TME. Autocrine and paracrine TGF- β signaling in cancer cells directs profound changes in gene expression that repress the epithelial phenotype and facilitate invasion and dissemination, promote stem cell properties and cancer drug resistance, and release of immunosuppressive cytokines. TGF- β released by cancer cells, stromal fibroblasts and other cell types in the TME shapes the architecture of

the TME through ECM protein expression, and suppresses cytotoxic activities of immune cells, thus making the TME an immune tolerant environment that prevents or attenuates the efficacies of cancer immunotherapies. Considering this plethora of TGF- β activities, therapeutic avenues to inhibit TGF- β signaling should target both the cancer cells and immune and stromal components of the TME to ensure durable regression.

High TGF- β signaling has been implicated in resistance to various anti-cancer treatments including chemotherapies and molecularly-targeted therapies^{149,380,381}. Increased TGF- β signaling within tumors also revealed itself as a substantial impediment of responsiveness to immunotherapies, to the extent that only a fraction of patients respond to anti-PD-1 or -PD-L1, anti-CTLA-4 and CAR-T cell therapies, and these patients show low TGF- β signaling signatures. As new immunotherapies are evaluated, TGF- β signaling in the TME will again reveal itself as impeding response and extension of survival time. These will undoubtedly benefit from the considerable effort that is directed towards therapeutically repressing TGF- β signaling to enhance anti-PD-1/-PD-L1 responsiveness. In contrast to chemotherapy or oncogene-targeted drugs, immunotherapy, including TGF- β blockade, does not routinely result in short-term endpoints that are assessed by RECIST³²¹. Rather, responses can be considerably delayed and unpredictable, in part due to the cascade of immune events that are required for a productive anti-tumor cytotoxic response³⁸². Since cytotoxic responses to anti-TGF- β monotherapies, when they occur, are immune-mediated and followed by sustained immunity to tumor challenge^{23,145,319,353}, responses to TGF- β blockade should be scored using Immune-Related Response Criteria^{322,323}. Delayed anti-tumor responses and long-term survival may take years of patient follow-up to meet statistical significance.

Considering the normal roles of TGF- β signaling in normal physiological processes, it is not surprising that systemic suppression of TGF- β signaling is accompanied by adverse effects that limit the therapeutic window and design of treatment regimens. Concerns for potential adverse effects resulting from normal physiological activities of TGF- β signaling impeded the pursuit of anti-TGF- β therapies. Together with only marginal clinical efficacy of anti-TGF- β monotherapy, these fears stalled drug development for many years, even though severe adverse effects are common when exploring novel cancer treatment modalities that interfere with normal physiology yet extend patient life for months. While therapeutic approaches and regimens clearly need further exploration, current, early clinical trials have shown adverse effects to be generally of low grade and manageable, with substantial and durable responses to TGF- β blockade, when combined with checkpoint inhibition. Going forward, selectively targeting TGF- β inhibition to the tumor should greatly decrease the severity of adverse effects and enhance therapeutic opportunities. TGF- β inhibition using bispecific drugs or through interference with TGF- β signaling components with restricted tissue expression, e.g. blocking integrin- or GARP-mediated TGF- β activation, is likely to enable novel therapies. More precise targeting may also be provided by the use of pro-bodies³⁸³. Incorporation of TGF- β inhibitors into tumor-targeted CAR-T cells, viral vectors, nanoparticles, or oncolytic viruses³⁸⁴ may provide additional opportunities. Some may require smaller TGF- β inhibitors that are packaged into viral vectors, such as TGF- β 1 mini-monomers that act as potent dominant negative inhibitors³⁸⁵, or the use of nanobodies³⁸⁶.

As we learn more about TGF- β 's activities on individual TME compartments, it becomes clear that key cellular and molecular features that define responsiveness to TGF- β blockade may differ between tumor types. For example, TGF- β blockade responses of PDAC, with its extensive stromal elaboration, frequent *SMAD4* inactivation and low tumor mutation load³⁸⁷, may differ from those of head and neck SCCs that have high tumor mutation loads and CD4⁺ T cell content^{114,388}. Distinct tumor mutation loads, oncogenic drivers, and other genetic and epigenetic differences in cancer cells dictate immune infiltration and responses to checkpoint blockade, and will help define responses to TGF- β blockade.

Additionally, germline genetic variations^{389–392} and microbiome differences between individuals^{359,393–395} are likely to influence therapeutic responses and immune-related adverse effects. GWAS studies revealed genetic associations between germline variants in genes encoding TGF- β signaling molecules and susceptibilities to cancer and autoimmune disorders^{396,397}, and preclinical studies highlight extensive influences of germline genetic modifiers of TGF- β activity on tumorigenesis, vascular integrity and allergy^{392,398–400}. Also the gut microbiome may help define responses to TGF- β blockade. For example, *Tgfb1*^{+/-} mice are healthy in a germ-free environment, but develop colon inflammation, colitis and colon adenocarcinoma when housed under less clean conditions⁴⁰¹. Furthermore, the colon microbiome can also influence systemic responses to immune checkpoint blockade^{393,394,402}. It will therefore be important to also take this variable into consideration when studying responses to TGF- β blockade.

Substantial research on cancers of different organ sites is still needed to evaluate the extent to which tumor architecture, genetic and phenotypic heterogeneity of malignant and TME cells and their TGF- β responsiveness define the efficacy of diverse immunotherapies, without or in combination with TGF- β inhibition. Only in this way can we move toward a reliable personalized prediction of responsiveness to therapy and effects of anti-TGF- β immunotherapy.

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Figure 1:

Cell type heterogeneity within the TME. This schematic diagram illustrates the irregular distribution of cancer cells within the tumor with extensive interactions with stromal fibroblasts and immune cells, and interspersed blood vessels and lymphatic vessels.

The cell types that have been discussed are shown in this cartoon format.

For Figure 1, please refer to Figure 1 of Derynck, R., Turley, S.J., and Akhurst, R.J. (2021) TGF β biology in cancer progression and immunotherapy. *Nature Reviews in Clinical Oncology*, 18, 9–34

Figure 2:

Latent TGF- β activation and initiation of TGF- β signaling through receptors and Smads. The TGF- β 1 dimer is expressed in complex with its pro-segments (a) that confer latency and associate with either LTBP1 that promotes the latent complex localization within ECM, or GARP (or related molecules) that localizes the latent complex at the cell surface (B). Activation of latent TGF- β 1 complexes involves non-covalent association of TGF- β 1's prosegment through an RGD sequence (circle) with one of several integrin β chains, and likely requires physical tension and proteases to release the active TGF- β 1 dimer from the latent complex (C). Active TGF- β 1 binds heteromeric type II-type I TGF- β receptor (RII-RI) complexes at the cell surface (D). When associated with clathrin in nascent endosomes, ligand-activated receptor complexes activate Smad2 and Smad3 that combine with Smad4 to form trimeric complexes that translocate into the nucleus. These Smad complexes cooperate with high affinity DNA binding transcription factors and coregulators to activate or repress genes. TGF- β also activates non-Smad signaling pathways from distinct receptor complexes in caveolar compartments. TGF- β -induced Erk MAPK pathway activation and Akt signaling may initiate from the same receptor complexes. However, TGF- β -induced Akt activation was also shown in different cells to require TRAF6 and not require the T β RI kinase activity, suggesting distinct receptor complexes, as shown. TGF- β -induced p38 MAPK and JNK activation initiate from similar or distinct receptor complexes in cholesterol-rich lipid raft compartments.

For Figure 2, please refer to Figure 2 of Derynck, R., Turley, S.J., and Akhurst, R.J. (2021) TGF β biology in cancer progression and immunotherapy. *Nature Reviews in Clinical Oncology*, 18, 9–34

Figure 3:

Epithelial plasticity responses of carcinoma cells to autocrine and paracrine TGF- β most commonly lead to partial EMT, in which gene expression patterns are extensively reprogrammed and cells convert from an apical-basal polarity toward front-rear polarity. These EMT-associated changes enable directed cell migration and invasion, which are prerequisites for cancer cell dissemination. Concomitant with the EMT-associated changes, cancer cells are enabled to acquire stem cell characteristics and increase their resistance to cancer drugs, and express and release immunosuppressive ligands, while also showing increased protection against senescence and decreasing DNA repair, thus enhancing genomic instability.

For Figure 3, please refer to Figure 3 of Derynck, R., Turley, S.J., and Akhurst, R.J. (2021) TGF β biology in cancer progression and immunotherapy. *Nature Reviews in Clinical Oncology*, 18, 9–34

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Figure 4:

Actions of TGF- β and TGF- β inhibition on immune cell properties. Stimulatory (black arrows) and inhibitory (red lines) activities of TGF- β on distinct cells of the immune system. Blue arrows represent activities of pharmacological and/or genetic inhibition of TGF- β signaling on specific cell types. The boxes highlight major effects of TGF- β on distinct cell types.

For Figure 4, please refer to Figure 4 of Derynck, R., Turley, S.J., and Akhurst, R.J. (2021) TGF β biology in cancer progression and immunotherapy. *Nature Reviews in Clinical Oncology*, 18, 9–34

Figure 5:

Anti-TGF- β blockade stimulates efficient penetration of T cells into the tumor core, compared to anti-PD-1 monotherapy. Mice with established CCK168 SCC subcutaneous tumors were treated with two doses of antibody therapy on day 15 and 19 after tumor cell implantation, and examined four days later by immunohistochemistry to detect CD45⁺ total immune cells and CD8⁺ T cells. Note that anti-PD-1 stimulates CD45⁺ cell levels, but fails to provide efficient infiltration into the center of the tumor, whereas anti-TGF- monotherapy results in efficient penetration of T cells into the core. Combination therapy enhances CD8⁺ cytotoxic T cell activity and CD4⁺Th1:Treg ratio, resulting in tumor shrinkage (reproduced with modifications from Dodagatta-Marri et al.¹⁴⁵).

For Figure 5, please refer to Figure 5 of Derynck, R., Turley, S.J., and Akhurst, R.J. (2021) TGF β biology in cancer progression and immunotherapy. *Nature Reviews in Clinical Oncology*, 18, 9–34

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Table 1.

Major classes of TGF- β signaling inhibitors that are pursued therapeutically

Small molecules that inhibit the TGF- β receptor kinases

- e.g. - galunisertib and next generation LY3200882 (Eli Lilly)
 - vactosertib (Medpacto)
 - PF06952229 (Pfizer)

Antibodies that prevent TGF- β binding to its receptors

- e.g. - fresolimumab and next generation SAR439459 (Genzyme-Sanofi):
 neutralizes TGF- β 1, - β 2 and - β 3
 - NIS793 (Xoma-Novartis): neutralizes TGF- β 1 and - β 2
 - IMC-TR1 (Eli Lilly): targets the T β RII receptor

TGF- β ligand traps

- e.g. - AVID200 (Forbuis): soluble, dimerized, Fc-linked T β RII ectodomains
 - M7824/Bintrafusp (EMD Serono): dimerized chimera of T β RII ectodomains and anti-PD-L1
 - dimerized chimera of T β RII ectodomains and anti-CTLA-4 (preclinical)

Selective interference with activation of latent TGF- β complexes

- e.g. - ABBV-151 (Abbvie) anti-GARP antibody that prevents latent TGF- β 1 activation
 - antibodies to integrins that selectively prevent latent TGF- β 1 activation (preclinical)
 - small molecules that selectively interfere with integrin-mediated TGF- β 1 activation (preclinical)
 - antibodies to the latent TGF- β 1 complex that prevent TGF- β 1 activation (preclinical)
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