

Transforming Growth Factor- β Signaling in Immunity and Cancer

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Transforming growth factor (TGF)- β is a crucial enforcer of immune homeostasis and tolerance, inhibiting the expansion and function of many components of the immune system. Perturbations in TGF- β signaling underlie inflammatory diseases and promote tumor emergence. TGF- β is also central to immune suppression within the tumor microenvironment, and recent studies have revealed roles in tumor immune evasion and poor responses to cancer immunotherapy. Here, we present an overview of the complex biology of the TGF- β family and its context-dependent nature. Then, focusing on cancer, we discuss the roles of TGF- β signaling in distinct immune cell types and how this knowledge is being leveraged to unleash the immune system against the tumor.

Introduction

Correct operation of the immune system in vertebrates requires constant regulation to ensure protection against extraneous agents and tolerance of self-antigens. To achieve this critical balance, several types of regulatory components act to impose restraint on the immune system. These components include dedicated cell types such as regulatory T (Treg) cells, which limit the expansion of immune effector cells, checkpoint molecules such as CTLA-4 and PD-1, which counterbalance antigen receptor signaling, and immunosuppressive cytokines (Li and Flavell, 2008), the most prominent of which is transforming growth factor β (TGF- β).

TGF- β regulates the generation and effector functions of many immune cell types (Flavell et al., 2010; Sanjabi et al., 2017). It controls adaptive immunity by directly promoting the expansion of Treg cells and by inhibiting the generation and function of effector T cells and antigen-presenting dendritic cells (DCs). TGF- β similarly controls the innate immune system by inhibiting natural killer (NK) cells and regulating the complex behavior of macrophages and neutrophils, thus forming a network of negative immune regulatory inputs.

These effects of TGF- β in immune regulation fall within a wider role of this cytokine and other members of its family in development, homeostasis, and tissue regeneration. Malfunctions of this pathway cause congenital defects, fibrotic diseases, immune dysregulation, and cancer. Most adult mammalian cell types respond to TGF- β with effects on cell proliferation, differentiation, adhesion, movement, metabolism, communication, and death. Of particular interest here, TGF- β functions as a potent tumor suppressor by inducing growth inhibition and apoptosis in pre-malignant cells. Mutations that eliminate the TGF- β pathway or decouple it from apoptosis not only convert these cells into a full-blown malignant state, but also allow them to use TGF- β to create an immune-suppressive tumor microenvironment (TME) and produce additional stromal modifiers that foster tumor progression and metastasis.

The composition and function of the TGF- β signaling pathway and the extensive role of the TGF- β family in development, homeostasis, and diseases, including cancer, have been reviewed in detail elsewhere (David and Massagué, 2018; Mullen and Wrana, 2017; Oshimori and Fuchs, 2012). Here, we focus on the role of TGF- β in immune regulation and its relevance to cancer. We provide an overview of the TGF- β signal transduction pathway and summarize current knowledge about the production and mobilization of TGF- β from latent stores in the TME. We review how the effects of TGF- β are switched from tumor suppressive to pro-metastatic as cancers advance. We then focus on the profound effects of TGF- β on major cellular components of the adaptive and innate immune systems, and in this context, we discuss recent progress in elucidating how the immunosuppressive role of TGF- β is enlisted by cancer cells to avert immune surveillance and to thwart cancer immunotherapy. We conclude with comments on the prospect of circumventing TGF- β signaling to improve the effectiveness of cancer immunotherapy.

Sources of TGF- β and Regulation of Its Bioavailability

The 32 members of the TGF- β superfamily of ligands encoded in the human genome are grouped into the TGF- β and the bone morphogenetic protein (BMP) subfamilies based on sequence similarity and functional criteria (David and Massagué, 2018). The TGF- β subfamily comprises three TGF- β ligands (TGF- β 1, TGF- β 2, and TGF- β 3), two activins (A and B), Nodal, growth and differentiation factor 1 (GDF1), GDF3, GDF8 (also known as Myostatin), GDF9, and GDF11. The BMP subfamily includes 10 BMPs, several GDFs, and the anti-muellerian hormone. Additional members encode antagonistic ligands and a few distant outliers. The three TGF- β s, and in particular, TGF- β 1, are the most relevant members of the family from the standpoint of immune regulation (Figure 1). Although not discussed here, emerging evidence also suggests important roles for BMPs



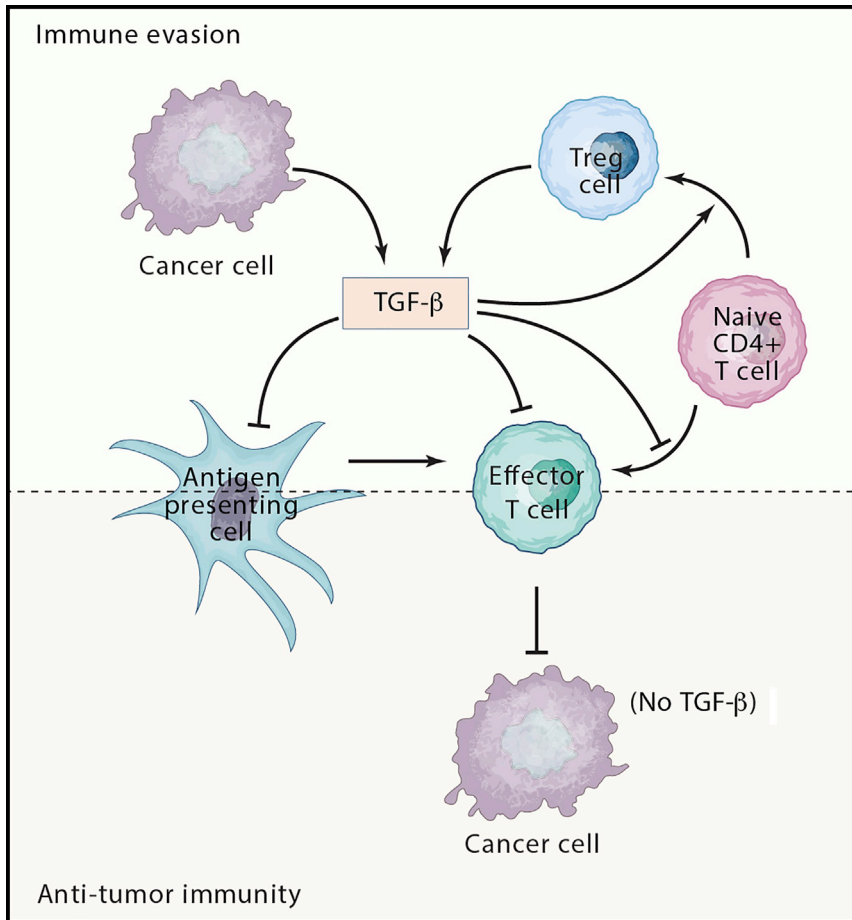


Figure 1. Key Players in TGF- β Suppression of Tumor Adaptive Immunity

Several prevalent cancer types exhibit a TGF- β -rich TME. TGF- β is produced by cancer cells and by several other cell types present in the TME, including Tregs. Fibroblasts, macrophages, and platelets are also significant TGF- β producers in tumors (not shown). Elevated TGF- β -levels block naive T cell differentiation toward a Th1 effector phenotype, promote their conversion toward the Treg subset, and dampen antigen-presenting functions of dendritic cells.

expression of GARP is limited to Treg cells, endothelium, and platelets (Tran et al., 2009), whereas LRRC33 is expressed in macrophages and microglia (Qin et al., 2018), suggesting that these proteins regulate TGF- β signaling in restricted regions and particular contexts.

The release of active TGF- β from latent complexes is a tightly regulated process achieved through enzymatic and non-enzymatic activities present in the extracellular space. The ECM protein Thrombospondin 1 binds to a specific sequence in LAP and prevents its association with the active TGF- β molecule. Supporting this mechanism, mutations in thrombospondin 1 phenocopy several alterations present in TGF- β 1 mutant mice including inflammation and epithelial hyperplasia (Crawford et al., 1998).

and other TGF- β superfamily members in the immune system (Chen and Ten Dijke, 2016).

TGF- β 1, TGF- β 2, and TGF- β 3 are synthesized as pro-hormones that include a signal sequence, a large N-terminal portion called the latency-associated peptide (LAP), and a short C-terminal segment, which corresponds to the mature active cytokine monomer (Travis and Sheppard, 2014). Upon cleavage by the protease Furin in the Golgi complex, the dimeric bioactive portion remains non-covalently associated with the disulfide-linked LAP homodimer (Figure 2A). The crystal structure of this complex revealed that LAP encircles the active TGF- β portion and hinders the relevant contact sites of the cytokine with cognate heterotetrameric receptors (Shi et al., 2011). In many cell types, the LAP homodimer is crosslinked by disulfide bonds to a family of proteins called latent TGF- β binding proteins (LTBPs) forming the so-called large latent complex (LLC) (Travis and Sheppard, 2014). After secretion, LLCs interact with Fibrillin, an extracellular matrix (ECM) protein of the elastic fibers. Mutations in the Fibrillin 1 gene cause Marfan syndrome, a human disease characterized by joint laxity, skeletal deformities, and aortic aneurysms due to excessive TGF- β signaling (Verstraeten et al., 2016). TGF- β can also be anchored to the cell surface through association with transmembrane Glycoprotein A Repeats Predominant Protein (GARP/LRRC32) (Tran et al., 2009; Wang et al., 2012) (Figure 2A) or the related Leucine-Rich Repeat-Containing Protein 33 (LRRC33) (Qin et al., 2018). The

Cleavage by extracellular serine proteases, such as plasmin and cathepsin D, and several metalloproteases, including matrix metalloproteases MMP9 and MMP14, also release active TGF- β from its latent form (Travis and Sheppard, 2014) (Figure 2A).

The best-understood mechanism of active TGF- β release from latent complexes involves either $\alpha v\beta 6$ or $\alpha v\beta 8$ integrin heterodimers, which bind to an Arg-Gly-Asp (RGD) integrin recognition motif present in the LAP domain of TGF- β 1 and - β 3 (Shi et al., 2011) (Figure 2A). Upon cell contraction, tension exerted by the cytoskeleton is converted by integrins into a physical force on the latent TGF- β complex that unfolds LAP and releases the active cytokine (Shi et al., 2011). This phenomenon depends critically on the actin/myosin-mediated contraction machinery (Giacomini et al., 2012), and it is facilitated by stiff substrates and the presence of highly contractile cells such as myofibroblasts (Wipff et al., 2007). It also requires tethering of the small latent complex to the ECM via LTBP1 (Annes et al., 2004). Supporting the validity of these findings, conditional ablation of integrins $\beta 6$ or $\beta 8$ in Treg cells, DCs, monocytes, or macrophages leads to inflammatory phenotypes and loss of TGF- β -mediated immune tolerance (Kelly et al., 2017, 2018; Travis and Sheppard, 2014). GARP also acts as a chaperone that orients latent TGF- β 1 for activation by integrins (Liénard et al., 2018) (Figure 2A). As discussed above, particular cell types, including Tregs, platelets, and macrophages, expose TGF- β bound to either GARP or LRRC33 on the cell surface, facilitating local TGF- β 1 release and paracrine

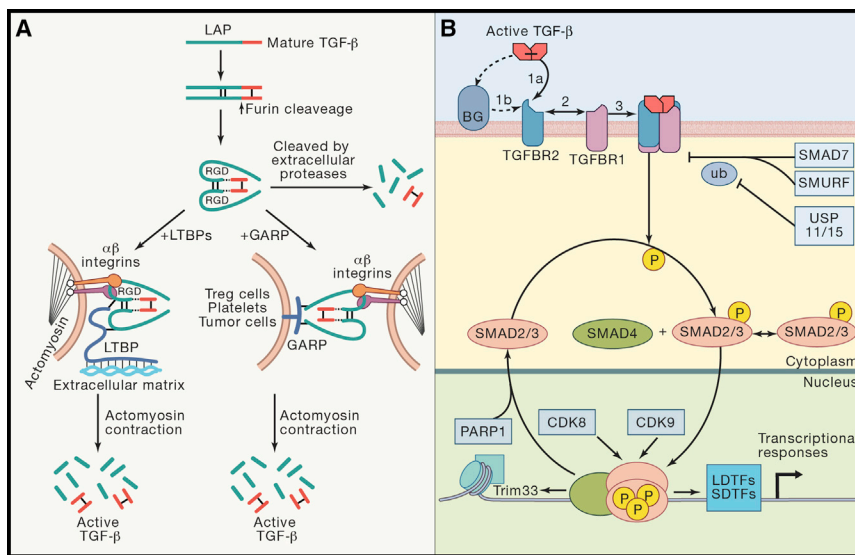


Figure 2. The TGF- β Signaling Pathway

(A) Synthesis and release of active TGF- β (adapted from Kelly et al., 2017). In the endoplasmic reticulum, each pro-TGF- β molecule is assembled into a dimer via three interchain disulfide bonds. Following cleavage by the endoprotease Furin, the C-terminal fragment remains non-covalently associated with the disulfide-linked LAP homodimer. This molecule is termed the small latent complex. Three main mechanisms of release of active TGF- β are represented: (1) extracellular protease cleavage of LAP domain, (2) tethering of small latent complex to ECM through LTBP1 and release of active TGF- β by integrin-transmitted tension upon cell contraction, and (3) tethering of small latent complex to GARP on the cell surface and release of active TGF- β by integrins.

(B) Signal transduction by TGF- β . The fundamental steps of ligand-induced formation of a paired-kinase receptor complex and receptor-mediated phosphorylation of R-SMAD proteins for the formation of a trimeric receptor complex are shown. The accessory receptor proteoglycan BG presents TGF- β to the signaling receptors. In the nucleus, the activated SMAD heterotrimeric complex binds to target *cis*-regulatory sites as determined by interactions with LDTFs and other SDTFs. CDK8 and CDK9 phosphorylate SMADs in this complex for further activation and eventual degradation. SMAD phosphatases (not shown) reverse these phosphorylations, and PARP-mediated parylation causes SMAD dissociation from DNA. The inhibitory SMAD7 recruits SMURF ubiquitin ligases to target the receptor for degradation, whereas ubiquitin-specific peptidases USP11 and USP15 counterbalance this process.

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signaling (Liéart et al., 2018; Qin et al., 2018; Stockis et al., 2017; Tran et al., 2009).

In cancer cells, the release of active TGF- β 1 mediated by GARP promotes TGF- β -driven epithelial-to-mesenchymal transition and immune evasion due to an expansion of tolerogenic Treg compartment (Metelli et al., 2016). Cancer cells can also evade the immune system by mobilizing active TGF- β 1 through α v β 8 integrins (Takasaka et al., 2018). In some contexts, cells recruited to the TME act as mediators of this process. A prime example are platelets, which carry GARP-bound TGF- β on the cell surface. Mice knockout for GARP in platelets exhibit reduced TGF- β signaling in the TME and enhanced anti-tumor immune responses (Rachidi et al., 2017).

The TGF- β Signaling Pathway

The TGF- β pathway is a classic membrane-to-nucleus signaling process involving direct receptor-mediated activation of SMAD transcription factors. Activated SMAD proteins bind to multiple loci throughout the genome as dictated by partner transcription factors whose availability in a particular cellular context determines the response of this cell to TGF- β (Figure 2B). The components and operating logic of the pathway have been recently reviewed elsewhere (David and Massagué, 2018) and are only briefly summarized here.

TGF- β family members signal through paired transmembrane serine/threonine protein kinases known as the type I and type II receptors (Figure 2B). Mammalian genomes encode seven type I receptors, five type II receptors, and eight SMAD proteins. TGFR1 (also known as T β R-I and ALK5) and TGFR2 (also known as T β R-II) function as TGF- β receptors. TGF- β 1, TGF- β 2, and TGF- β 3 are the only ligands for the TGFR1/TGFR2 combination. SMAD2 and SMAD3 are substrates for TGF- β subfamily receptors and SMAD1, SMAD5, and SMAD8 for BMP subfamily receptors; these are referred to as receptor-regulated SMADs, or R-SMADs. SMAD4 is not a receptor sub-

strate but binds to activated R-SMADs forming heterotrimeric transcriptional complexes. SMAD6 and SMAD7 are inhibitory molecules that suppress receptor and SMAD signaling functions. SMAD7-recruited SMURF1/2 ubiquitin ligases and counteracting ubiquitin specific peptidases (USP11, USP15) regulate TGF- β receptor degradation. The induction of SMAD6 and SMAD7 expression by TGF- β and BMP family members creates negative feedback loops.

SMAD proteins consist of globular N-terminal and C-terminal domains known as the MH1 and MH2 domains, respectively, connected by a linker region. The MH1 domain in R-SMADs and SMAD4 binds DNA, and the MH2 domain binds other SMADs, cooperating transcription factors, and chromatin reader and modifying factors. The components of the TGF- β -SMAD pathway are highly conserved, and the structural basis for many aspects of their interactions and function are known (Macias et al., 2015).

TGF- β released from latent complexes binds to the receptors either directly or with the assistance of the accessory co-receptor betaglycan (BG, also known as TGF- β receptor type III [TGFR3]) (Figure 2B). Betaglycan is particularly important for TGF- β 2 binding. In the TGF- β -driven receptor complex, TGFR2 phosphorylates and activates TGFR1, which then phosphorylates SMAD2 and SMAD3 at two C-terminal serine residues. Receptor-phosphorylated SMAD2 and SMAD3 then form heterotrimeric complexes with SMAD4.

In the nucleus, activated SMAD complexes bind to hundreds of regulatory regions by interacting with other transcription factors (Mullen et al., 2011; Trompouki et al., 2011; Wang et al., 2017; see below). Additional interactions with co-activators and co-repressors determine the transcriptional effect. In these complexes, R-SMADs are phosphorylated by the RNA polymerase II kinases CDK8 and CDK9, which create sites for recruitment of additional co-factors. CDK8/9-mediated phosphorylation additionally primes SMADs for phosphorylation by

glycogen synthase kinase 3 β (GSK3 β), which marks SMADs for polyubiquitination by the HECT-domain ubiquitin ligases NEDD4L and SMURF1, leading to SMAD degradation. Alternatively, R-SMADs are dephosphorylated and dissociated from DNA for new rounds of signal transduction.

SMAD Signaling Variants and Non-SMAD TGF- β Signaling

The phenotypes of SMAD knockouts demonstrate a central role of the SMAD pathway in the effects of TGF- β in different contexts, including in the regulation of the immune system. However, variant forms of SMAD signaling and TGF- β signaling mechanisms not involving SMAD proteins should be noted. SMAD4 is essential for most, but not all, TGF- β family responses. Exceptions include the development of the pancreas in mice (Bardeesy et al., 2006) and the induction of the master transcription factor SOX4 in pancreatic epithelial progenitors (David et al., 2016), which require SMAD2/3, but not SMAD4. SMAD proteins also have non-transcriptional roles, a notable example being the regulation of Drosha-mediated microRNA maturation by R-SMADs (Davis et al., 2008).

SMAD-independent forms of TGF- β signaling have been described (Heldin and Moustakas, 2016; Massagué, 2012; Moustakas and Heldin, 2005). TGFBR2 directly phosphorylates the cell polarity regulator PAR6, which regulates tight junctions and cell migration (Ozdamar et al., 2005; Yi et al., 2010). TGF- β activates members of mitogen-activated protein kinase (MAPK) cascades, including TGF- β -activated kinase 1 (TAK1) (Sorrentino et al., 2008), ERK (Lee et al., 2007), p38MAPK, JNK (Lee et al., 2007), and phosphatidylinositol 3-kinase (PI3K) in cell cultures (Heldin and Moustakas, 2016). The adaptor protein TRAF6 was shown to link TGF- β receptors to TAK1, p38MAPK, and JNK activation (Sorrentino et al., 2008; Yamashita et al., 2008), but beyond this, the structural basis for coupling of TGF- β receptors to these various pathways remains unknown. Moreover, MAPK and PI3K pathways have their own potent agonists, including mitogen receptor tyrosine kinases and cell metabolism sensors. Likewise, stress sensors regulate p38MAPK and JNK activation, whereas tumor necrosis factor (TNF), interleukin (IL)-1, and Toll-like receptors (TLRs) control TAK1 activation, making the role of TGF- β as a regulator of these pathways in physiology and disease quite difficult to ascertain at present.

Basis for Contextual Responses

Although the MH1 domain of all effector SMADs binds GC-rich, 5-bp motifs (CAGAC, GGCGC, and others), the affinity of SMADs for these motifs is neither high nor specific enough to explain the selective recognition of target loci by TGF- β and BMP pathways or the context-dependent nature of TGF- β action. Rather, activated SMAD complexes bind to hundreds of regulatory regions as dictated by context-defining transcription factors. In progenitor cells, lineage-determining transcription factors (LDTFs) play a dominant role in recruiting activated SMAD complexes (David and Massagué, 2018). This is exemplified by the interaction of the LDTF FOXH1 with Nodal-activated SMADs in mesendoderm progenitors. FOXH1 is a pioneer factor that binds to chromatin independently of TGF- β signaling and is then poised for recruitment of activated SMAD complexes (Charney et al., 2017; Chen

et al., 1996). Similarly, TGF- β -activated SMADs co-bind the genome with MYOD1 in myogenic progenitors to regulate myogenic differentiation and with PU.1 in pro-B cells to regulate B cell differentiation (Mullen et al., 2011).

Several signal-driven transcription factors (SDTFs) participate, together with LDTFs, as determinants of SMAD binding, providing integration of multiple inputs. Focusing on the immune system, TGF- β -activated SMADs cooperate with STAT5 and nuclear factor of activated T cells (NFAT) to induce FOXP3 expression in naive CD4⁺ T cells, thus promoting their differentiation to a Treg phenotype (Tone et al., 2008), and with ROR γ 2 to induce a T helper 17 (TH17) phenotype (Martinez et al., 2010; Zhou et al., 2008). SMADs, together with the transcription factor RUNX3, regulate immunoglobulin class switching in B cells (Park et al., 2005). In TGF- β -stimulated CD8⁺ T cells, SMADs collaborate with the transcription factor ATF1 to repress the expression of several cytolytic genes (Thomas and Massagué, 2005).

TGF- β and Tumor Progression

TGF- β functions as a tumor suppressor that can induce apoptosis in pre-malignant cells and inhibit proliferation in carcinoma cells. However, cancer cell clones that inactivate the TGF- β pathway or decouple it from tumor suppressive effects under this selective pressure can use TGF- β for tumor progression. In this altered context, tumor-derived TGF- β can induce tumorigenic and pro-metastatic responses in cancer cells and the stroma, including the formation of an immune-suppressive TME (Figure 3). The basis for this dual tumor-suppressive and tumor-promoting role of TGF- β has been reviewed in detail elsewhere (David and Massagué, 2018; Pickup et al., 2017) and is summarized hereunder.

Based on current human TCGA datasets, esophageal, gastric colorectal (CRC), and pancreatic (PDA) adenocarcinomas contain mutations or deletions in SMAD and TGF- β receptor genes in 25%–50% of cases. Head and neck, bladder, and endometrial adenocarcinomas and cervical and lung squamous carcinomas harbor such mutations in 10%–20% of cases. SMAD4-inactivating mutations predominate in PDA and CRC, but mutations in SMAD2, SMAD3, TGFBR1, and TGFBR2 are also frequently observed. Collectively, these loss-of-function mutations provide evidence for the tumor suppressor role of the TGF- β pathway in human cancer. Genetically engineered mouse models of PDA and CRC indicate that TGF- β exerts its tumor-suppressive functions mostly by blocking the transition of pre-malignant cells to a more malignant phenotype (Figure 3). Evidence from human cancer also suggests that TGF- β pathway mutations accumulate during the malignant conversion step.

TGF- β is an archetypical growth-inhibitory cytokine and inhibits cell proliferation through increased activity of the cyclin-dependent kinase (CDK) inhibitors p15INK4, p21CIP1, p27KIP1, and/or p57KIP2 (Hannon and Beach, 1994; Polyak et al., 1994; Reynisdóttir et al., 1995; Scandura et al., 2004; Seoane et al., 2004) and downregulation of MYC expression (Chen et al., 2002; Pietenpol et al., 1990). However, oncogenically transformed cells harbor strong CDK activating signals that thwart the effectiveness of TGF- β -induced cytostasis as a tumor-suppressive effect. Moreover, the capacity to enter a slow-cycling state is a basic feature of stem cells, one that enables these cells to evade immune surveillance and antimetabolic

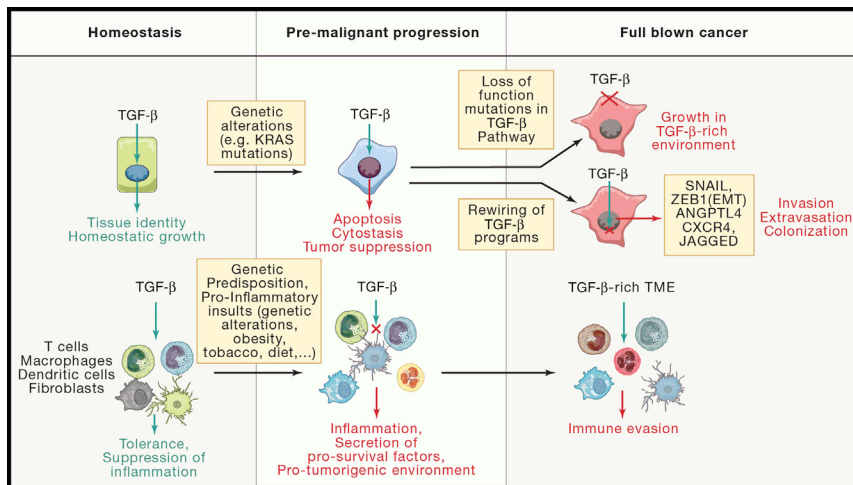


Figure 3. Tumor-Suppressor and Tumor-Promoting Functions of TGF- β Signaling

(Left) In homeostasis, TGF- β signals regulate key processes in multiple tissues, including growth, regeneration, and identity. In the immune system, TGF- β instructs tolerance and suppresses inflammation. This function is particularly relevant in the gastrointestinal tract. (Center) Genetic alterations can modify the output of TGF- β signals in tumor-initiating cells. During the initial stages of carcinogenesis, TGF- β operates as a key tumor suppressor by triggering cytosclerosis and apoptosis programs in cancer cells. A pro-inflammatory environment fosters the onset of cancer. Loss of TGF- β signals in the microenvironment contributes to exacerbated inflammation in this context. Secretion of pro-survival factors and cytokines by stromal and immune cells pushes continuous regeneration in a harsh inflammatory environment, which eventually leads to the onset of cancer. (Right) During tumor progression, selective pressure promotes loss of the tumor-suppressor function of TGF- β in cancer cells. In general, this

process occurs via two distinct mechanisms. Acquisition of loss-of-function mutations in TGF- β pathway components renders tumor cells resistant to TGF- β , thus enabling growth in a TGF- β -rich environment present in many advanced cancers. Alternatively, TGF- β signals are somehow decoupled from apoptosis in cancer cells and instead instruct tumor-promoting functions, such as the ability to migrate and colonize distant organs. In the TME of several prevalent tumor types, TGF- β operates as central mechanism of immune evasion.

chemotherapy. Therefore, not only is cytosclerosis a milder form of tumor suppression compared to apoptosis, but cytosclerosis also may ultimately enable disseminated tumor cells to survive for the eventual initiation of metastasis (Malladi et al., 2016).

TGF- β triggers apoptosis in pre-malignant cells that suffer oncogenic stress, in particular, stress from RAS oncogenes, as documented in genetically engineered mouse models of cancer and human cancer cell lines. Notably, TGF- β -SMAD signaling causes apoptosis in pancreatic progenitors harboring KRAS mutations (Bardeesy et al., 2006). Mouse skin and mucosal epithelia showed hyperproliferation offset by TGF- β -dependent apoptosis when harboring a HRAS oncogene, mild hyperplasia when harboring TGFBR2 deletion, and overt tumor formation when both alterations were combined (Guasch et al., 2007). Thus, in these and other cancers, oncogenic mutations sensitize premalignant cells to TGF- β -induced apoptosis.

Epithelial-to-mesenchymal transitions (EMTs) are processes by which epithelial progenitor cells lose polarity, downregulate cell-cell adhesions, and migrate and invade for the purpose of generating or regenerating tissues (Nieto et al., 2016). EMTs occur during development and wound healing and in pathologies including fibrosis and cancer. EMTs are driven by master EMT regulators including SNAIL, ZEB, and TWIST, which function as transcriptional repressors of epithelial genes. TGF- β is a potent inducer of EMTs, but this function critically depends on inputs from the RAS-MAPK pathway (David et al., 2016; Horiguchi et al., 2009; Janda et al., 2002). However, TGF- β -induced EMT is pro-apoptotic and tumor suppressive in pre-malignant pancreatic progenitors, owing to a conflict with a pro-epithelial program that TGF- β concomitantly induces in these cells (David et al., 2016). A pro-apoptotic role of TGF- β -induced EMT is also observed in mouse mammary epithelial cells (Gal et al., 2008).

Cancer cells with an intact TGF- β pathway can avert its pro-apoptotic effect by somehow decoupling EMT from apoptosis, which in turn allows the cancer cells to use EMT for tumorigenic advantage. Beyond the contribution of TGF- β -induced EMT to tumor invasion and metastatic dissemination, the TGF- β

pathway induces gene responses that support the ability of cancer cells to infiltrate and colonize specific organs (David and Massagué, 2018). Examples include the ability of hormone-receptor-negative breast cancer cells in primary tumors to respond to TGF- β with induction of angiopoietin-like 4, which enhances the extravasation of these cells as they move into the circulation and lodge into capillaries in the lungs (Padua et al., 2008). Breast cancer cells that instead colonize the bone marrow respond to bone-derived TGF- β with induction of CXCR4, PTHrP, IL-11, CTGF, and JAGGED1, which promote osteoclast mobilization and osteolytic metastasis (Figure 3) (Kakonen et al., 2002; Kang et al., 2003; Sethi et al., 2011; Yin et al., 1999). In addition to these effects, growing evidence points at a major role of TGF- β as a mediator of immune suppression in the TME.

TGF- β Suppression of Tumorigenic Inflammation

The master role of TGF- β signaling in controlling immune tolerance and inflammatory responses was first revealed by the analysis of mice with germline null mutations in the TGF- β 1 gene that die early after birth of multiorgan inflammation reminiscent of an autoimmune disorder (Kulkarni et al., 1993; Shull et al., 1992). Subsequently, it was shown that this phenotype can be rescued by loss-of-function mutations in either major histocompatibility complex (MHC) class II (Letterio et al., 1996) or β 2-microglobulin genes (Kobayashi et al., 1999), implying that loss of TGF- β 1 causes an unrestrained adaptive T cell response. Similarly, mice expressing a dominant-negative TGFBR2 construct under the control of the CD4 promoter (Gorelik and Flavell, 2000) or bearing T cell-specific deletion of either *Tgfb2* (Li et al., 2006; Marie et al., 2006), *Tgfb1* (Liu et al., 2008), or *Tgfb1* (Li et al., 2007) by means of a CD4-Cre driver develop enhanced T cell activation, leading to a severe inflammatory disease similar to that observed in *Tgfb1*^{-/-} mice. These phenotypes are consequence of CD4⁺ T cell activation by self-antigens present in the periphery (Robinson and Gorham, 2007). Overall, these pioneering genetic experiments demonstrated that TGF- β signaling is

required for the establishment and maintenance of T cell tolerance during thymic development.

When *Tgfb2* was ablated in T cells using the dLck-Cre driver, which is only turned on after thymocyte-positive selection, mice survived to adulthood and neither displayed overt T cell activation nor inflammatory disease in homeostasis (Zhang and Bevan, 2012). However, T cells in these mice exhibited enhanced proliferation and acquisition of an exacerbated effector phenotype in a lymphopenic environment, which was triggered by weak T cell receptor (TCR) stimuli (Zhang and Bevan, 2012). Similar observations were made upon deletion of *Tgfb2* in peripheral T cells of adult mice using inducible a CD4-creERT2 allele (Sledzińska et al., 2013). Therefore, in peripheral T cells, a key function of TGF- β signaling is to restrain T cell expansion and activity in response to exogenous stimuli.

The inflammatory disorders caused by genetic loss of TGF- β signaling are particularly severe in the gastrointestinal tract. TGF- β pathway mutant mice develop either spontaneous colitis or are more susceptible to experimental colitis induced by treatment with dextran sulfate sodium (DSS) (Ihara et al., 2017; Kulkarni et al., 1993; Shull et al., 1992). These phenotypes are also evident in mice with TGF- β pathway deficiencies in either T cells or DCs (Gorelik and Flavell, 2000; Ihara et al., 2017; Ramalingam et al., 2012; Sledzińska et al., 2013). In agreement with these findings, multiple studies have linked dysregulation of TGF- β signaling with pathogenesis of ulcerative colitis or Crohn's disease (Ihara et al., 2017). These inflammatory syndromes predispose to the development of cancer. Transgenic mice that overexpressed TGF- β 1 in T lymphocytes under control of the CD2 promoter exhibited delayed tumor development in experimental models of azoxymethane-induced colonic tumorigenesis, whereas expression of dominant-negative TGFBR2 in T cells accelerated tumor progression in this model (Becker et al., 2004). This effect was linked to the modulation of IL-6/STAT3 signaling in tumor cells, a cytokine input that promotes growth and survival of intestinal cancer cells in the context of inflammation (Becker et al., 2004). Similarly, deletion of *Smad4* in T cells led to spontaneous formation of epithelial cancers throughout the gastrointestinal tract as a result of increased levels of pro-inflammatory cytokines IL-5, IL-6, IL-11, and IL-13 (Hahn et al., 2011; Kim et al., 2006).

TGF- β signaling also instructs a non-inflammatory program in macrophages of the intestinal epithelium, which exhibit a profound loss of response to inflammatory insults while retaining a bactericidal function (Smythies et al., 2005). This anergic phenotype is partially driven by TGF- β produced by intestinal stromal cells (Smythies et al., 2005). Mice that express dominant-negative *Tgfb2* under the control of a macrophage-specific promoter display exacerbated colitis in response to the DSS treatment, an effect that correlates with increased production of pro-inflammatory cytokines, such as IL-33 (Rani et al., 2011).

Finally, fibroblast-specific *Tgfb2* knockout mice develop intraepithelial prostate neoplasias and invasive squamous cell carcinomas in the forestomach (Bhowmick et al., 2004). It was initially proposed that hepatocyte growth factor (HGF) secretion by *Tgfb2* mutant fibroblasts led to hyperproliferation of adjacent epithelial cells in the affected tissues (Bhowmick et al., 2004). However, another study revealed DNA damage in epithelial cells of the forestomach due to extensive inflammation caused by loss

of *Tgfb2* in fibroblasts (Achyut et al., 2013). This phenotype was enhanced by *Helicobacter pylori* infection and ameliorated by anti-inflammatory drugs (Achyut et al., 2013).

Altogether, these studies highlight the pivotal role of TGF- β signaling in regulating tolerance and suppressing inflammatory reactions triggered by commensal and harmful antigens. Loss of TGF- β signaling in tissues exposed to these stimuli, such as the gastrointestinal tract, promotes exacerbated T cell activity and an unrestrained inflammatory reaction. These processes eventually result in DNA damage and increased levels of cytokines and growth factors, which foster an uncontrolled regenerative response and the onset of cancer (Figure 3).

Inhibition of Th1 Helper and Cytotoxic T Cell Responses by TGF- β

The most prominent and best-characterized T cell responses against cancers are mediated by cells of the Th1 subset. Naive T cells cultured with TGF- β cannot differentiate into the Th1 phenotype (Sad and Mosmann, 1994). Conversely, mice lacking TGFBR2 specifically on T cells display enhanced Th1 responses (Gorelik and Flavell, 2000; Li et al., 2006; Marie et al., 2006; Sledzińska et al., 2013; Zhang and Bevan, 2012). TGF- β signaling impinges on the earliest phase of T cell activation by dampening the initial Ca²⁺ influx-triggered TCR stimulation (Chen et al., 2003a). Consistent with this finding, *Tgfb2* mutant T cells exhibit increased sensitivity to TCR stimulation (Sledzińska et al., 2013). In addition, TGF- β signaling exerts an inhibitory role on T cell differentiation by silencing the expression of two Th1 master transcription factors, TBET and STAT4 (Gorelik et al., 2002; Lin et al., 2005). Blockade of STAT4 activation prevents the production of interferon (IFN)- γ during the priming phase, whereas loss of TBET expression impairs IFN- γ production during re-stimulation of T cells after initial priming (Gorelik et al., 2002; Lin et al., 2005).

In addition to blocking Th1 differentiation, TGF- β inhibits T cell proliferation and effector function. Early studies revealed that during the priming phase, TGF- β silences the expression of IL-2, the cytokine that elicits subsequent CD4⁺ T cell proliferation (Brabletz et al., 1993). This effect is mediated by SMAD3 (McKarns et al., 2004) with SMAD4 in association with co-factor TOB1 (Tzachanis et al., 2001). A cytostatic and apoptotic response of T cells to TGF- β has also been linked to the control of several downstream cell-cycle regulators, including c-Myc, p21^{Cip1} and p27^{Kip1} (Genestier et al., 1999; Wolfrum et al., 2004). As we discussed in the previous section, *in vivo* experiments using genetically modified mouse models revealed that the main alteration of TGFBR2-deficient peripheral CD4⁺ and CD8⁺ T cells is enhanced TCR-dependent activation upon weak antigenic stimulation (Sledzińska et al., 2013; Zhang and Bevan, 2012). These studies also demonstrated that TGFBR2-deficient T cells exhibit enhanced effector phenotypes and functions, such as expression of the receptor KLRG1 and production of granzyme-B and IFN- γ (Sledzińska et al., 2013; Zhang and Bevan, 2012). TGF- β signaling directly inhibits the cytotoxic program of CD8⁺ T cells (Thomas and Massagué, 2005). Mechanistically, TGF- β -stimulated SMADs together with transcription factor ATF1 repress the promoters of several genes involved the lytic function of CD8⁺ T cells including granzyme B and IFN- γ (Figure 4).

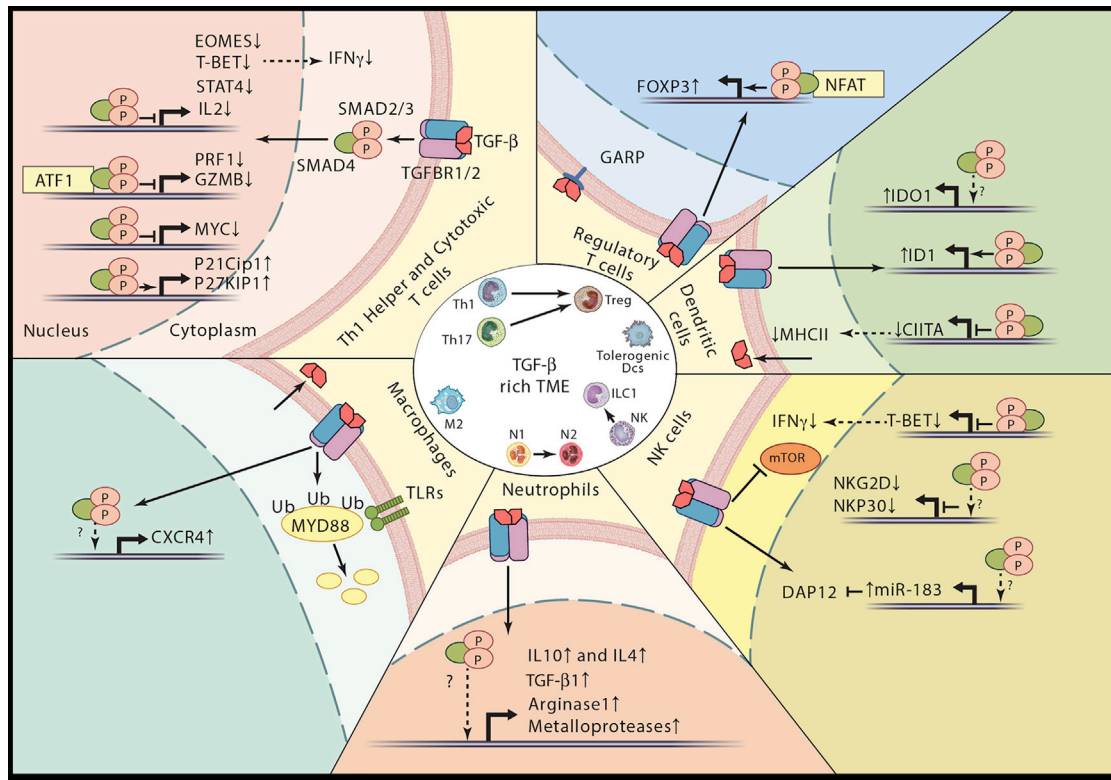


Figure 4. Immune Evasion Mediated by TGF- β Signaling in Late-Stage Cancers

The central circle depicts phenotypic transitions instructed by TGF- β in the distinct immune cell types present in the TME as described in the text. The outer part summarizes the key processes regulated by TGF- β signaling in immune cells. We depict direct transcriptional responses driven by SMADs, in some cases in cooperation with cell-specific transcription factors.

Echoing the above observations, widespread evidence supports a role for a TGF- β -rich TME in suppressing anti-tumor Th1 responses. Transgenic mice engineered to express dominant-negative TGFBR2 in CD4+ and CD8+ T cells are resistant to tumor formation upon inoculation of syngeneic cancer cell lines, and this phenomenon is associated with a large expansion of tumor-reactive CD8+ T cells (Gorelik and Flavell, 2001). In an autochthonous genetic model of prostate cancer, TGF- β 1 secreted by T cells was shown to mediate tumor evasion from adaptive immunity (Donkor et al., 2011). Authors reported that transgenic dn-TGFBR2 T cells infiltrate both the tumor beds and the tumor-draining lymph nodes and produce high amounts of IFN- γ and GZMB. These findings suggest a role for TGF- β signaling in inhibiting tumor-antigen-specific T cell priming (Donkor et al., 2011). Similarly, adoptively transferred tumor-specific CD8+ T cells modified to express a dominant-negative TGFBR2 infiltrate the tumor mass and display enhanced secretion of IFN- γ and cytotoxic products, leading to anti-tumor responses in models of renal cancer (Wang et al., 2010). TGF- β attenuates the effector function of antigen-specific CD8+ cells obtained from melanoma patients, a phenomenon that segregates with decreased expression of TBET (Ahmadzadeh and Rosenberg, 2005). Repression of eomesodermin (EOMES), a homolog of TBET that is required to establish the gene program of effector cytotoxic T cells (Cruz-Guilloty et al., 2009; Pearce et al., 2003), has been also correlated with TGF- β -driven immune evasion in mouse models of melanoma (Yoon et al., 2013).

Tissue resident CD8+ memory cells T cells are maintained, associated to tissues through expression of the α E β 7 integrin homodimer (also known CD103), which binds E-cadherin present in the epithelial cell junctions. TGF- β upregulates the expression of α E and β 7 subunits, thus facilitating the residence of CD8+ cell in tissues (El-Asady et al., 2005; Zhang and Bevan, 2013). In addition, downregulation of the transcription factors TBET and EOMES by TGF- β is necessary to specify the CD8+CD103+ phenotype (Mackay et al., 2015). The role of CD8+CD103+ in cancer is not completely understood. Several studies have shown that a TGF- β -rich TME fosters CD8+CD103+ cells and that abundance of this T cell subset confers good prognosis (Mami-Chouaib et al., 2018). In addition, checkpoint immunotherapies reinvigorate their cytotoxic function, leading to anti-tumor responses (Edwards et al., 2018; Mami-Chouaib et al., 2018). In sharp contrast, a recent study has provided evidence that TGF- β -induced CD8+CD103+ cells present in tumor beds exhibit a tolerogenic phenotype that facilitates immune evasion (Gabrieli et al., 2017).

Induction of T Regulatory Phenotype by TGF- β Signaling

Tregs suppress the function of effector T cells to maintain immune homeostasis and are present in low frequency in healthy tissues. However, their numbers increased in many cancers, where they enforce tolerance to tumor antigens and facilitate immune evasion. TGF- β is key to instruct the regulatory program on T cells. For example, suboptimally stimulated peripheral CD4+

T cells produce TGF- β , which in turn promotes their differentiation to the Treg stage (Strainic et al., 2013; Windhagen et al., 1995). TGF- β in combination with IL-2 enforces a suppressor phenotype in *ex vivo* naive CD4⁺ T cells by triggering expression of FOXP3, the master transcription factor of the Treg program (Chen et al., 2003b; Fantini et al., 2004). Consistent with these findings, genetic deletion of both Smad2 and Smad3 resulted in loss of FOXP3 upregulation by TGF- β (Takimoto et al., 2010). Downstream of TGF- β receptors, SMADs cooperate with NFAT transcription factor to bind a distal regulatory element in the FOXP3 locus (Tone et al., 2008) (Figure 4). TGF- β -driven induction of Tregs is counteracted by an environment rich in pro-inflammatory cytokines that favors differentiation of CD4⁺ T cells toward an effector phenotype (Battaglia et al., 2013; Wei et al., 2007).

FoxP3 expression correlates with TGF- β levels in transcriptomic datasets of skin cutaneous melanoma and breast cancer (Ravi et al., 2018), and extensive evidence suggests that the TGF- β -rich environment characteristic of late-stage cancers promotes differentiation of T cells to a Treg phenotype. High TGF- β levels secreted by cancer cells induce the Treg program on CD4⁺ T cells in models of pancreatic cancer (Moo-Young et al., 2009). Furthermore, TGF- β together with prostaglandin E2 trigger Th17-to-Treg transdifferentiation in tumors (Downs-Canner et al., 2017). Conversely, the inhibition of TGF- β and VEGF signaling synergistically reduces the number of Tregs and restores sensitivity to anti-PD-1 and anti-CTLA-4 treatment (Courau et al., 2016).

In addition to inducing a T regulatory program, TGF- β is utilized by Tregs to suppress anti-tumor immune responses (Chen et al., 2005; Mempel et al., 2006). As we discussed above, Tregs carry latent TGF- β 1 on their surface via disulfide linkage to transmembrane protein GARP for activation by α V integrins. In B16 melanoma explants, Tregs inhibit the cytotoxic function of CD8⁺ T cells, whereas this immunosuppressive mechanism is prevented by neutralizing antibodies against surface-bound TGF- β (Budhu et al., 2017). Indeed, expression of GARP in tumor-infiltrating Tregs characterizes an enhanced suppressive phenotype that correlates with impaired anti-tumor T cell responses (Kalathil et al., 2013).

TGF- β Subversion of Dendritic Cell Function

DCs are a central node in regulation of Th1 and Treg-mediated immune responses by TGF- β signaling. DCs are the most potent antigen-presenting cells and play key roles in tumor immunity. TGF- β inhibits antigen presentation capabilities of DCs *in vitro* by suppressing expression of MHC-II genes (Nandan and Reiner, 1997; Piskurich et al., 1998). *Tgfb2* deletion in DCs using CD11c-Cre driver causes multiorgan inflammation (Ramalingam et al., 2012). In tumor models, cancer cells instruct DCs to secrete TGF- β , which in turn promotes acquisition of a Treg phenotype by CD4⁺ T cells (Dumitriu et al., 2009; Ghiringhelli et al., 2005). Furthermore, the TME redirects DC differentiation toward an immature myeloid cell phenotype with potent immune suppressor functions (Papaspnyridonos et al., 2015). This phenotypic switch is mediated by transcriptional regulator ID1 downstream of TGF- β signaling (Papaspnyridonos et al., 2015) (Figure 4). The tolerogenic program of DCs is in part mediated by induction of immunosuppressing molecules indoleamine 2,3-dioxygenase (IDO) and arginase by TGF- β signaling (Belladonna et al.,

2008; Pallotta et al., 2011). In models of breast cancer and melanoma, TGF- β signaling mediates immune evasion by upregulating IDO in plasmacytoid DCs and the CCL22 chemokine in myeloid DCs (Hanks et al., 2013). These altered DCs facilitate Treg cell infiltration and immune suppression (Hanks et al., 2013).

TGF- β Suppression of Natural Killer Cells

NK cells respond rapidly to virus-infected cells and tumor cells through their unique capacity to recognize stressed cells in the absence of an adaptive response, allowing a rapid immune reaction. TGF- β signaling blocks NK function at multiple levels. It silences IFN- γ and TBET expression in NK cells, thus inhibiting Th1 responses (Laouar et al., 2005; Yu et al., 2006). This mechanism is counteracted by inflammatory signals, which decrease TGFBR2 levels and suppress downstream SMAD signaling in NK cells (Yu et al., 2006). The expression of NKG2D and NKp30, two surface receptors of NK cells that mediate the recognition of stressed and malignant transformed cells, is silenced by TGF- β signaling (Castriconi et al., 2003) (Figure 4). Indeed, there is an inverse correlation between TGF- β 1 and NKG2D levels in lung, CRC carcinomas, and glioblastoma (Crane et al., 2010; Lee et al., 2004). In addition, the levels of DAP12, an adaptor of several cytotoxic receptors, including NKG2D, are downregulated by TGF- β -induced miR-183 (Donatelli et al., 2014) (Figure 4).

A controversy exists regarding the role of TGF- β signaling in the ontogeny of NK cells. Early studies found increased NK cell numbers and accelerated maturation during infancy in transgenic mice that express a dominant-negative TGFBR2 in NK cells under the control of the *cd11c* promoter (Laouar et al., 2005; Marcoe et al., 2012). In contrast, ablation of *Tgfb2* in NK cells by means of the *Ncr1-cre* deleter strain did not cause alterations in NK cell distribution or development. However, these mice exhibited a reduced NK-mediated anti-tumor response to lung metastasis formation (Viel et al., 2016). This study also revealed that TGF- β -mediated suppression of NK activity is due to impairment of mTOR metabolic signaling downstream of stimulatory cytokines, such as IL-15 (Viel et al., 2016). Finally, NK cells are plastic and can switch phenotype in response to TGF- β (Cortez et al., 2016). Analysis of tumor models revealed that a TGF- β -rich TME facilitates immune evasion by enforcing transdifferentiation of NK cells to innate lymphoid cells type 1 (ILC1), which are devoid of cytotoxic function (Gao et al., 2017; Cortez et al., 2017) (Figure 4). This process apparently requires SMAD4-independent TGF- β signaling (Cortez et al., 2017).

Regulation of Macrophage Behavior by TGF- β Signaling

It is well established that the TME polarizes macrophages toward a phenotype with anti-inflammatory, immune-suppressive, and pro-angiogenic functions, referred to as the M2 phenotype. TGF- β is one of the main immunosuppressive cytokines produced by tumor-associated macrophages (TAMs), and as already mentioned, subsets of human macrophages can mobilize active TGF- β through the activity of integrin α v β 8 and MMP14 (Kelly et al., 2018).

TGF- β instructs distinct programs in cells of the monocyte/macrophage lineage depending on the differentiation state and context. Early studies provided evidence that TGF- β acts as

chemoattractant for monocytes to the sites of inflammation and upregulates adhesion molecules that enable monocyte attachment to the ECM (Allen et al., 1990; Wahl et al., 1993). A similar mechanism may operate in tumors. For example, in models of breast cancer, tumor cells secrete TGF- β , which upregulates CXCR4 in monocytes, while CXCL12 secreted by perivascular fibroblasts attracts these monocytes to tumor beds (Arwert et al., 2018). Subsequently, monocytes differentiate into perivascular macrophages and facilitate tumor cell extravasation by promoting blood vessel leakiness (Arwert et al., 2018).

TGF- β signaling in macrophages also inhibits anti-inflammatory responses mediated by transcription factor NF- κ B. The inhibitory SMADs, SMAD6 and SMAD7, have been implicated in this process. TGF- β promotes degradation of MYD88, an adaptor protein utilized by all TLRs, except TLR3, to activate NF- κ B signaling. SMAD6 induced polyubiquitination of MYD88 through recruitment of E3-ubiquitin ligases SMURF1/2 in TGF- β -stimulated peritoneal macrophages (Lee et al., 2011; Naiki et al., 2005) (Figure 4). SMAD6 also sequesters adaptor protein Pellino-1, which is required downstream of IL-1R/TLR to promote an inflammatory response (Choi et al., 2006). Furthermore, TGF- β 1 suppresses the inflammatory phenotype of macrophages by crosstalk with TNF signaling pathway through SMAD7, which blocks the activity of the TNF-induced kinase TAK1 (Hong et al., 2007). Although these mechanisms have not been formally investigated in the context of cancer, it is conceivable that a TGF- β -rich TME may contribute to immune evasion by dampening the inflammatory functions of macrophages. Indeed, the acquisition of a M2 phenotype in TAMs correlates with inhibition of NF- κ B activity (Porta et al., 2009).

TGF- β Signaling in Myeloid Cells of the Neutrophil Lineage

Neutrophils represent around 80% of all blood leukocytes and play key roles in controlling infection. Patients with various cancer types, including breast, lung, and CRC, exhibit increased numbers of circulating neutrophils, often associated with poor prognosis (Gentles et al., 2015; Templeton et al., 2014). Functional analyses in models of cancer have revealed important roles of neutrophils during tumor progression (Coffelt et al., 2016). Paralleling the phenomenon of TAM polarization, tumor-associated neutrophils (TANs) adopt either an anti-tumorigenic or pro-tumorigenic program (Fridlender et al., 2009) (Figure 4). In mice bearing mesothelioma tumors, treatment with TGF- β inhibitors enforces the anti-tumorigenic phenotype in TANs that includes cytolytic activity and elevated expression of pro-inflammatory cytokines (Fridlender et al., 2009).

A debate exists about whether TANs represent mature neutrophils or rather immature polymorphonuclear cells (Coffelt et al., 2016; Gabrilovich, 2017). It is well established that chronic infection, inflammation, or cancer induce a persistent stimulation of myelopoiesis that results in the production of cells similar to neutrophils and monocytes in morphology and phenotype yet with potent ability to suppress immune responses (Coffelt et al., 2016; Gabrilovich, 2017). These cells, termed myeloid-derived suppressor cells (MDSCs), infiltrate tumors and are important players during cancer immune evasion. 80% of MDSCs display a polymorphonuclear phenotype (PMN-MDSCs) similar to that of neutrophils and can be recognized in flow cytometry analysis

by the neutrophil surface marker combination CD11b+Ly6G+ (Coffelt et al., 2016; Gabrilovich, 2017). TGF- β signaling in MDSCs enhances tumor progression and metastasis in animal models. Depletion of MDSCs in a breast cancer model inhibited the therapeutic effects exerted by systemic administration of anti-TGF- β antibodies (Li et al., 2012). Indeed, mice with myeloid-specific deletion of *Tgfb2* were largely resistant to metastasis formation upon transplantation of syngeneic breast cancer, lung cancer, and melanoma cell lines (Pang et al., 2013). This effect was linked to downregulation of type 2 cytokines in MDSCs and increased expression of IFN- γ in CD8+ T cells, leading to enhanced anti-tumor responses (Pang et al., 2013). Interestingly, in this study, primary tumor growth was not affected by loss of *Tgfb2* in myeloid cells, which suggests a specific role for TGF- β -activated MDSCs in the metastatic niche.

In contrast to the tumor-promoting effects of TGF- β signaling in neutrophils and MDSCs, several studies have shown that genetic inhibition of TGF- β signaling in cancer cells results in recruitment of immature myeloid cells and MDSCs to the TME. A genetic mouse model of invasive CRC generated by inactivating *Apc* and *Smad4* in the intestinal epithelium exhibited prominent infiltration with CCR1+ immature myeloid cells (Kitamura et al., 2007). Loss of TGF- β signaling in *Apc* mutant colon cancer cells led to upregulation of chemoattractant CCL9, which in turn mediates recruitment of the immature myeloid cell population (Kitamura et al., 2007). Furthermore, immature myeloid cells present in the TME expressed matrix metalloproteinases that facilitate tumor cell invasion and metastasis (Kitamura et al., 2007, 2010). In a similar study, knockout of *Tgfb2* in mammary carcinoma cells increased MDSC infiltration. In this case, the effect was mediated by upregulation of CXCL5 in *Tgfb2*-deficient breast cancer cells (Yang et al., 2008). MDSCs also produced matrix metalloproteases that contributed to tumor cell invasion and metastasis in this model (Yang et al., 2008). Therefore, the anti-tumoral effects of TGF- β on cancer cells result, at least in part, from suppression of expression of myeloid cell chemoattractants.

Cancer-Associated Fibroblasts and TGF- β -Driven Immune Evasion

TME of advanced-stage cancers is often characterized by an abundance of fibroblasts. These cancer-associated fibroblasts (CAFs) are a heterogeneous group of cells dedicated to producing the main components of the ECM, including collagens, elastins, and fibronectin, but also an array of cytokines that regulate tumor properties. CAFs represent the main TGF- β producers in many tumor types. A prime example is CRC, where CAFs secrete TGF- β 1 and TGF- β 3, and their abundance correlates with TGF- β pathway activity in distinct TME cell types, including T cells and macrophages (Calon et al., 2012, 2015). In addition, TGF- β activates a gene expression program in CAFs that is tightly associated with poor prognosis in CRC patients (Calon et al., 2012, 2015; Isella et al., 2015).

These findings are further strengthened by the fact that distinct molecular classifications of CRC have consistently identified a poor prognosis subtype characterized by elevated TGF- β levels and abundant CAFs (Calon et al., 2015; Guinney et al., 2015; Isella et al., 2015). The generation of genetic mouse

models that develop human-like CRCs has offered insights into the interaction of the CAF-rich TGF- β -activated TME with the immune system (Tauriello et al., 2018). Elevated TGF- β levels produced by CAFs and other TME cell types exclude CD4+ and CD8+ T cells from the tumor center (Tauriello et al., 2018). T cell exclusion is associated with adverse outcome in CRC patients (Galon et al., 2006). Treatment of these mouse CRC models with the TGFBR1 inhibitor Galunisertib triggered T cell infiltration, and upon treatment, primary tumors and metastases became susceptible to checkpoint therapies (Tauriello et al., 2018). Dual Galunisertib/PDL1 therapy induced a potent immune response, with increased TBET and IFN- γ levels in CD4+ T cells and elevated granzyme B production in CD8+ cells, which eradicated overt metastatic disease (Tauriello et al., 2018). Similar therapeutic responses to dual Galunisertib/PDL1 treatment were observed in genetic mouse models of serrated CRC (Nakanishi et al., 2018).

A link between CAFs and immune evasion is further supported by another study that searched for determinants of response to checkpoint immunotherapies (Mariathasan et al., 2018). By interrogating a transcriptomic cohort of urothelial cancer, authors found that a subset of patients that did not respond to PD-L1 treatment bore tumors rich in TGF- β -activated CAF gene program. These tumors presented an immune-excluded phenotype (Mariathasan et al., 2018). This study further showed that treatment with an anti-Pan-TGF- β antibody reverted T cell exclusion and sensitized mouse tumor models to PD-L1 treatment (Mariathasan et al., 2018).

At present, the association between TGF- β -activated CAFs and cancer immune evasion remains largely correlative, and therefore, we can only speculate about the mechanisms utilized by CAFs to block T cell infiltration and function. CAFs secrete TGF- β , which can directly suppress several immune cell types through the mechanisms described herein. In addition, and by analogy with tissue fibrosis (Kim et al., 2018; Wipff et al., 2007), highly contractile CAFs may facilitate the release of active TGF- β from latent complexes stored in the TME. Alternatively (or additionally), particular genes activated by TGF- β signaling in CAFs may inhibit the migration and function of immune cells present in the tumor stroma. Supporting this hypothesis, a transcriptomic signature of ECM genes driven by TGF- β in CAFs predicts failure of PD-1 therapy (Chakravarthy et al., 2018).

TGF- β -Based Immunotherapies

The development of therapies based on inhibiting the TGF- β pathway has had a slow progress. Its low priority in the pharmaceutical industry pipeline is likely explained by two observations: first, the longstanding evidence supporting a tumor-suppressor role for the TGF- β pathway in several contexts, which raised concerns regarding the possibility that inhibition of TGF- β signaling could worsen rather than cure cancer, and second, the finding that first-generation TGFBR1 inhibitors triggered overt cardiac toxicity in experimental models (Anderton et al., 2011). Nevertheless, as we discussed throughout this review article, over the past years, it has become evident that genetic ablation of TGF- β signaling pathway components in distinct immune cell types, including CD4+, CD8+ T cells, NK cells, or dendritic cells, triggers robust anti-tumor responses in pre-clinical models of

cancer. These studies, together with the overwhelming evidence that late-stage tumors exploit TGF- β for invasion and metastasis, have sparked the development of multiple programs aimed at blocking TGF- β signaling in cancer. Table 1 summarizes the main strategies, some of which are currently in clinical trials. The most extensively tested compound is Galunisertib (LY2157299), a small molecule that inhibits TGFBR1 kinase activity. Galunisertib in combination with gemcitabine has shown modest but significant therapeutic activity in a phase 2 clinical trial for pancreatic cancer (Melisi et al., 2018). Another phase 2 clinical trial demonstrated therapeutic responses in a subset of hepatocellular carcinoma patients treated with Galunisertib as monotherapy (Favre et al., 2014). Of note, Galunisertib displayed a safe profile across various clinical trials without discernable cardiac toxicities (Kovacs et al., 2015).

Pre-clinical studies suggest that the immunological effects of Galunisertib are strongly potentiated by combination with checkpoint inhibitors, whereas TGF- β inhibition as monotherapy may exhibit limited efficacy (Holmgaard et al., 2018; Mariathasan et al., 2018; Nakanishi et al., 2018; Tauriello et al., 2018). Based on these findings, several clinical trials aimed at testing Galunisertib in combination with anti-PD1 antibodies have been recently launched. In addition, TGFBR1 small-molecule inhibitors that are more potent and specific than Galunisertib have been developed and are currently being tested in patients. One of them, Vactoserib (TEW-7197), in combination with chemotherapy or antibodies against immune checkpoint molecules, is in phase 1/2 clinical trials for several cancer types.

The use of antibodies that block systemic TGF- β has shown promising anti-tumor responses in multiple pre-clinical studies. For instance, treatment with the 1D11 anti-pan-TGF- β antibody enhances the priming of tumor-reactive CD8+ by dendritic cells upon irradiation of subcutaneous tumors (Vanpouille-Box et al., 2015). This combinatorial therapy inhibits tumor growth and metastases and is further enhanced by treatment with PD-1 antibodies (Vanpouille-Box et al., 2015). In a similar study, radiotherapy in combination with blocking TGF- β antibodies enhances systemic anti-tumor responses as shown by analysis of non-irradiated lesions in the same mice—i.e., abscopal effects (Rodríguez-Ruiz et al., 2019). A humanized derivative of the 1D11 antibody, Fresolimumab, is being evaluated for the treatment of renal cell carcinoma, melanoma, non-small cell lung cancer, and metastatic breast cancer. Reported results from this clinical trial show that Fresolimumab in combination with focal radiotherapy increases the overall survival of patients with metastatic breast cancer (Formenti et al., 2018). This effect correlated with a large increase in the number of CD8+ central memory cell pool, suggesting that the treatment triggered an immune response against the tumor (Formenti et al., 2018). Several other pan-TGF- β blocking antibodies are in phase 1 clinical trials (Table 1).

There are ongoing efforts to develop TGF- β isoform-specific therapies. For example, the antibody XPA-42-089 binds TGF- β 1 and TGF- β 2, but not TGF- β 3, and exhibits a robust synergism with PD-1 during the treatment of both genetic and mutagen-induced mouse squamous cell carcinomas (Dodagatta-Marri et al., 2019). AVID200 is a computationally designed receptor ectodomain-based trap that binds and neutralizes TGF- β 1 and TGF- β 3, but not TGF- β 2 (O'Connor-McCourt et al., 2017, Cancer

Table 1. Therapeutic Strategies to Block TGF- β Signaling in Cancer and Their Progress to the Clinic

Therapy	Target	Drug	Progress to Clinic
Small-molecule inhibitors	TGFBR1 kinase	Galunisertib	Multiple completed safety and efficacy phase 1 clinical trials in HCC, glioblastoma and other tumor types. Several ongoing phase 1/2 trials: metastatic breast cancer (GAL + radiation therapy), metastatic pancreatic cancer (GAL + Durvalumab), advanced hepatocellular carcinoma (GAL + stereotactic radiotherapy), metastatic androgen receptor negative triple, negative breast cancer (Galunisertib and Paclitaxel), colorectal cancer (GAL + Capecitabine), rectal cancer (GAL + chemoradiation), advanced refractory solid tumors (GAL + Nivolumab), and metastatic prostate cancer (GAL + Enzalutamide).
		Vactosertib	Safety and efficacy phase trials in advanced-stage solid tumors. Several ongoing phase 1/2 trials: metastatic gastric cancer (VAC + Paclitaxel), advanced NSCLC (VAC + Durvalumab), metastatic colorectal and gastric cancer (VAC + Pembrolizumab), advanced desmoid tumors (VAC + Imatinib)
		LY3200882	Phase 1: safety and dose escalation in solid tumors.
		PF-06952229	Phase 1: safety and dose escalation in breast cancer and prostate cancer in monotherapy or combination with several drugs (palbociclib, Letrozole, Enzalutamide).
		AZ12601011 AZ12799734	Pre-clinical development (Spender et al., 2019).
Antibodies	Blocking pan-TGF- β (TGF- β 1, TGF- β 2, TGF- β 3)	Fresolimumab	Completed safety and efficacy phase 1 trials in renal cell carcinoma, melanoma, and glioma. Ongoing phase 1/2 trials: relapsed malignant mesothelioma, advanced renal cell carcinoma and melanoma, early-stage NSCLC (FRESO + stereotactic ablative radiotherapy), and metastatic breast cancer (FRESO + radiation).
	Blocking pan-TGF- β	SAR439459	Phase 1 (safety and dose escalation) for advanced solid tumors in monotherapy or combination with anti-PD1 antibodies.
	Blocking pan-TGF- β	NIS793	Phase 1 in combination with anti-PD-1 antibodies for patients with advanced malignancies (breast, lung, HCC, CRC, pancreatic cancer, renal cancer).
	Blocking TGF- β 1 and TGF- β 2 specific	XPA-42-089	Pre-clinical cancer models (Dodagatta-Marri et al., 2019).
	Chimeric antibody-TGF- β traps	CTLA4-TGF β RII PDL1-TGF β RII (M7824)	Pre-clinical cancer models (Ravi et al., 2018). Ongoing phase 1/2 trials: NSCLC (compared to Pembrolizumab), triple-negative breast cancer (M7824 + Eribulin), prostate cancer, metastatic colorectal cancer, and cholangiocarcinoma and gallbladder.
	GARP	ABBV151	Phase 1 trials for advanced solid tumors as monotherapy or in combination with PD-1 antibodies.
	α v β 8 Integrins LAP		Pre-clinical cancer models (Takasaka et al., 2018). Pre-clinical cancer models (Gabriely et al., 2017).
Receptor-based TGF- β traps		AVID200	Phase 1 trials (safety and dose escalation) for advanced and metastatic solid tumors.
Adoptive cell transfer		Autologous CD8+ T cells expressing a dnTGFR2	Phase 1 trial in chemorefractory Epstein Barr Hodgkin lymphoma.

Res., abstract). It is currently in phase 1 trials for patients with advanced solid tumors.

Another interesting approach are therapies based of anti-CTLA4 or anti-PD-L1 antibodies engineered as fusions with the extracellular domain of TGFBR2 (Ravi et al., 2018). These bi-functional molecules potentiate the efficacy of immunotherapies in mouse models of cancer by quenching TGF- β . The anti-CTLA4-TGFBR2 molecule reduced accumulation of Treg cells in immune-reconstituted NOD scid gamma (NSG) mice bearing patient-derived melanoma and exhibited enhanced anti-tumor efficacy compared with standard anti-CTLA4 monotherapy (Ravi et al., 2018). Likewise, an anti-PDL1-TGFBR2 chimera exerted more pronounced anti-tumor responses than PD-L1 antibodies

(Ravi et al., 2018). The chimeric anti-PDL1-TGFBR2 antibody is currently being tested in phase 1 clinical trials for several indications.

Blockade of TGF- β signaling also improves the outcome of immunotherapies based on adoptive cells transfer. For instance, infused CD8+ cells reactive against an autochthonous tumor model of prostate cancer mount enhanced anti-tumor responses if TGF- β signaling is prevented by transgenic expression of a dominant-negative TGFBR2 (Bendle et al., 2013; Zhang et al., 2005). This strategy was recently tested in patients with chemorefractory Epstein Barr Hodgkin lymphoma (Bollard et al., 2018). The authors reported that autologous CD8+ cells directed against the Epstein-Barr virus-derived tumor antigens that have been

engineered to express a dominant-negative TGFBR2 triggered complete responses in four out of seven patients (Bollard et al., 2018). Although the patient number is small, the study suggests improved efficacy compared to a previous trial using TGF- β sensitive CD8+ T cells (Bollard et al., 2018). This strategy might be widely applicable to a wide range of adoptive cell transfer therapies.

Finally, the complex mechanism of active TGF- β release from latent complexes also offers opportunities for therapeutic intervention. Antibodies against GARP that blocked its binding to latent TGF- β prevented the formation of lung metastases generated by breast cancer cell lines implanted orthotopically (Metelli et al., 2016). Another study characterized the activity of antibodies that inhibit active TGF- β release from Treg cells through binding to a conformational epitope in GARP (Cuende et al., 2015). One of them, ABBV151, is being tested in phase 1 clinical trials for advanced solid tumors. Antibodies that bind the LAP domain of latent TGF- β elicit immune responses against tumors in models of melanoma, CRC, and glioblastoma (Gabriely et al., 2017). Similarly, an antibody against α v β 8 integrin that blocks the release of active TGF- β by cancer cells unleashed the immune system against tumors in pre-clinical models, and this therapeutic effect was largely potentiated by combining anti-PD1 antibodies (Takasaka et al., 2018).

Concluding Remarks

The basic elements in the TGF- β signaling cascade were elucidated more than a decade ago. During the past few years, it has become evident that the pleiotropic nature of TGF- β responses is the result of context-dependent transcriptional programs orchestrated through the interaction of SMADs with distinct tissue- and cell-specific transcription factors. Rewiring of transcriptional circuits in cancer cells—as result of genetic and epigenetic alterations—changes the output of TGF- β signaling from a tumor-suppressing function to different tumor-promoting programs that facilitate growth, invasion, and metastasis. Concomitantly, TGF- β signaling operates as a major suppressor of the adaptive and innate immune responses during tumor progression. The inhibition of the immune system by TGF- β signaling leads to opposing effects depending on the context. In tissues subjected to continuous antigenic stimuli, such as the gastrointestinal tract, TGF- β limits adaptive responses and dampens inflammation. In this setting, loss of TGF- β signaling leads to a pro-inflammatory environment that facilitates the onset of cancer. In contrast, in the advanced stages of the disease, TGF- β signaling acts as an important mechanism of immune evasion in several tumor types.

The snapshots of TGF- β effects on tumor immunity covered here illustrate how distinct immune cell types present in the TME react to TGF- β in a variety of experimental systems. In most cases, observations in tumor models echo the function of the TGF- β pathway in promoting tolerance and suppressing immune responses during homeostasis and infection. It remains unclear, however, how the individual cell responses described here are orchestrated in a given tumor. Do they occur in concert or sequentially along tumor progression? Which TGF- β -activated immune cell types are relevant and which tumor subtypes utilize them? Addressing these questions will require cancer models that faithfully reproduce human disease. Genomic analyses, including single-cell profiling, should also help under-

stand the ecology of TGF- β -mediated immune evasion. These are particular important aspects that may contribute to rationalize the use of upcoming TGF- β -based immunotherapies. In this regard, the observed synergism between TGF- β inhibition and checkpoint blockade perhaps reflects a hierarchy of immune-suppressive events whereby TGF- β dampens the initial immune response, such as the ability of T cells to infiltrate tumors and the acquisition of effector program, whereas PD1/PDL1 signaling operates at a later stage by suppressing T cell effector functions and causing exhaustion. These investigations, together with the development of more potent and specific TGF- β pathway inhibitors, hold promise for the treatment of prevalent tumor types that thrive in a TGF- β -rich environment.

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DECLARATION OF INTERESTS

J.M. is a scientific advisor and owns company stock in Scholar Rock. J.M. is an author in the following patents: U.S. Patent 5,688,665, U.S. Patent 5,719,120, U.S. Patent 5,830,847, U.S. Patent 6,015,693. E.B. research is sponsored by Merus.

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