OVERCOMING TGFβ-MEDIATED IMMUNE EVASION IN CANCER

Daniele V.F. Tauriello[1†], Elena Sancho[2,3] and Eduard Batlle[2,3,4†]

1. Department of Cell Biology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, PO Box 9101, 6500 HB Nijmegen, The Netherlands.
2. Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Baldiri i Reixac 10, 08028 Barcelona, Spain.
3. Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Barcelona, Spain.
4. ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain.

[†]Email: Daniele Tauriello (Daniele.Tauriello@radboudumc.nl) and Eduard Batlle (eduard.batlle@irbbarcelona.org).

ABSTRACT

Transforming Growth Factorβ (TGFβ) signalling controls multiple cell fate decisions during development and tissue homeostasis. Hence, dysregulation of this pathway can drive several pathologies, including cancer. Here we discuss the influence that TGFβ exerts on the composition and behavior of different cell populations present in the tumour immune microenvironment, and the context-dependent functions of this cytokine in suppressing or promoting cancer. During homeostasis, TGFβ controls inflammatory responses triggered by exposure to the outside milieu in barrier tissues. Lack of TGFβ exacerbates inflammation, leading to tissue damage and cellular transformation. In contrast, as tumours progress, they leverage TGFβ to drive an unrestrained wound-healing program in cancer-associated fibroblasts, as well as to suppress the adaptive and innate immune system. In consonance with this key role in reprogramming the tumour microenvironment, emerging data demonstrate that TGFβ inhibitory therapies can restore cancer immunity. Indeed, this approach can synergize with other immunotherapies—including immune checkpoint blockade—to unleash robust anti-tumour immune responses in preclinical cancer models. Despite initial challenges in clinical translation, these findings have sparked the development of multiple therapeutic strategies that inhibit the TGFβ pathway, many of which are currently in clinical evaluation.

Introduction

The role of TGFβ signalling during cancer progression is complex, as it can have both tumour-suppressive and tumour-promoting functions[1–4]. Virtually all cell types are responsive to TGFβ, but its role has been particularly well characterized in epithelial cells. In organs such as skin, colon, breast or pancreas, TGFβ signalling regulates homeostatic growth, inhibiting cell proliferation and transformation during the early stages of tumourigenesis (Figure 1). Cancers arising in these tissues can avert the tumour-suppressive effects of TGFβ by acquiring inactivating mutations in pathway components. In other cases, tumour cells remain responsive to TGFβ during disease progression but, in crosstalk with several oncogenic alterations such as *KRAS*

mutations, rewire the signalling pathway's outcome to promote epithelial-to-mesenchymal transition (EMT), dissemination, dormancy, and metastasis (Figure 1). The context-dependent roles of TGFβ signalling in healthy and tumorigenic epithelial cells have been reviewed elsewhere[2–4].

Whereas research on TGFβ signalling in cancer has been predominantly tumour cell-centric, the pioneering works on TGFβ signalling in the 1980's and 1990's already addressed the profound effects that this cytokine exerts on the tumour microenvironment (TME)[5].These early studies showed that inoculation of mice with TGFβ accelerated wound healing by stimulating both the recruitment of immune cells and the production of multiple extracellular matrix (ECM) components by fibroblasts[6–8]. These findings were linked to a pivotal role for TGFβ in the differentiation of cancer-associated fibroblasts (CAFs), as well as to the generation of the desmoplastic reaction that characterizes many prevalent tumour types[9], fueling the notion that "tumours are wounds that do not heal"[10]. In parallel, TGFβ signalling was discovered to suppress the function of adaptive and innate immune cells[11–14], a mechanism that a decade later was associated to cancer immune evasion[1,15–17].

We now know that TGFβ controls immune homeostasis in several tissues, and genetic defects in pathway components are linked to loss of immune tolerance and autoimmunity[18,19]. Moreover, in mouse models, exacerbated inflammation associated with the loss of TGFβ signalling in several immune cell types leads to enhanced cancer formation (Figure 1). In contrast, as tumours progress the levels of TGFβ increase, concurrent with marked remodelling of the TME (Figure 1). Combined with the well-documented cancer cell-intrinsic effects of TGFβ on invasion and metastasis[2–4], the net result is a systematic disposition to tumour progression, immune evasion, and therapy resistance (Figure 1).

Here we describe how the complex cellular ecosystem of the TME responds to TGFβ throughout the evolution of the disease. We first summarize the basics of the TGFβ signal transduction pathway, emphasizing the mechanisms of TGFβ production, storage and release within the TME. We then review the current knowledge of the role of TGFβ signalling in immune homeostasis and its link to tumour initiation in pathogen-exposed organs such as the gut. Subsequently, and forming the main focus of this Review, we discuss how TGFβ signals facilitate malignant tumour growth, dissemination, and immune evasion by instructing gene programs in different TME cell types. We conclude with the current translational and clinical efforts to block the TGFβ signalling pathway, recognizing a promising role of this strategy in immuno-oncology.

Regulation of TGFβ bioavailability

The three TGFβ isoforms, TGFβ1, TGFβ2, and TGFβ3, belong to a 33-member family of structurally related cytokines known as the TGFβ superfamily[20,21]. These cytokines share many features, including structurally related receptors and downstream signalling effectors, yet they often play functionally distinct roles in physiology and disease[20,21] (Box 1). The TGFβ pathway has been extensively investigated, and several excellent reviews cover its molecular biology[2,21,22]. As a reference, we summarize below the essential components and critical regulatory steps (Figure 2). In essence, TGFβ triggers a classical membrane to nucleus signal transduction pathway whereby upon binding to type I and type II TGFβ receptors (TGFBRs) at the cell surface, intracellular SMAD effector proteins translocate into the nucleus and activate transcriptional programs. The specificity of SMAD DNA binding and transcriptional regulation is achieved through their interaction with lineage-determining transcription factors and signal-driven transcription factors[2]. As a result, TGFβ regulates specific transcriptional programs depending on the cell type and context, which explains its

diversity of roles in physiological and pathological processes[2]. This is particularly relevant in the TME, where TGFβ can instruct disparate gene programs in each of the different cell types present. It is also important to note that whereas most TGFβ responses involve SMAD-driven transcription, several alternative (non-canonical) pathways can transduce TGFBR signals[21,22] (Figure 2).

Production and storage of latent TGFβ

TGFB1, *TGFB2*, and *TGFB3* genes encode pro-hormones that include a large N-terminal domain called the latency-associated peptide (LAP) and a short C-terminal domain that corresponds to the mature, bio-active cytokine[19]. In the Golgi complex, the TGFβ pro-hormone dimerizes through the formation of disulfide bonds and is subsequently cleaved by the Furin protease. However, the bioactive and LAP portions that result from this cleavage remain non-covalently linked (Figure 2). This conformation, known as latent TGFβ (L-TGFβ), impedes signal transduction because the LAP domain obstructs binding of the active portion of TGFβ to the receptors[23]. The LAP dimer is often crosslinked to latent TGFβ binding proteins (LTBPs), which results in the formation of the large L-TGFβ complex (LLC)[19].

TGFβ can be found in the plasma of patients with cancer with poor prognosis[24–26], suggesting that it can freely diffuse. However, large L- TGFβ complexes are generally retained by the ECM through interaction of LTBPs with, or crosslinking to, several glycoproteins such as Fibrillins[27],[19] (Figure 2). These act as reservoirs from which the active cytokine can be released in a tightly regulated manner. The relevance of these interactions is exemplified by the effect of germline mutations in *FBN1* (which encodes Fibrillin-1), present in patients with Marfan syndrome. These mutations interfere with the retention of L-TGFβ in the ECM and the resulting elevated levels of TGFβ signalling cause hypermobile joints, skeletal deformities, and aortic aneurysms[28]. In particular cell types, newly synthesized L-TGFβ is not crosslinked to LTBPs but forms disulfide bonds with leucine-rich repeat-containing protein 32 (LRRC32, also known as GARP)[29,30] or with the related LRRC33[31]. After Furin cleavage, GARP- or LRRC33-bound L-TGFβ is loaded onto the cell membrane, enabling spatially controlled TGFβ1 release and signalling (Figure 2). GARP tethers L-TGFβ onto the surface of regulatory T cells (Treg cells), endothelial cells, and platelets[29], whereas LRRC33 plays an equivalent function in macrophages and microglia[31].

Release of active TGFβ in the TME

Like TGFβ production and storage, its release is conducted by a variety of tightly regulated processes. Active TGFβ can be liberated from latent ECM complexes by proteolytic cleavage mediated by various serine proteases such as plasmin or cathepsin D[19] and, particularly, by matrix metalloproteinases present in the TME[32,33]. The protease thrombin can also cleave GARP on the surface of platelets, releasing active TGFβ and contributing to tumour immune evasion[34]. However, mounting evidence suggests that the main mechanism of TGFβ release from latent deposits depends on integrin activity. In particular, the αvβ6 and αvβ8 integrins bind to an Arg-Gly-Asp (RGD) motif present in the LAP portion of L-TGFβ1 and L-TGFβ3 with very high affinity, which may reflect a specialized function of these integrin isoforms in TGFβ activation rather than cell adhesion or migration[35]. In this context, αvβ6 integrin translates tension resulting from actomyosin-mediated cell contraction on the L-TGFβ molecule, which results in the unfolding of the LAP domain and the release of the active hormone[23,36] (Figure 2). This mechanical process is mainly performed by highly contractile cells such as cancer cells, myeloid cells, and myofibroblasts, and it is facilitated by the tethering of L-TGFβ to stiff substrates through LTBPs[37–39]. In the context of cell surface-bound TGFβ, GARP operates as a chaperone that orients L-TGFβ for binding to the αvβ8 integrin[36]. Of note, the cytoplasmatic tail of the αvβ8 does not interact with the actin cytoskeleton and cannot transmit cell contraction forces onto

the L-TGFβ molecule. Instead, αvβ8 integrin enforces a change of L-TGFβ conformation that enables activation of the TGFβ receptors while the ligand is still bound to GARP[40]. The pivotal role of αvβ8 integrin in the regulation of TGFβ availability is further supported by the analyses of mice with conditional deletion of the gene encoding the β8 subunit in dendritic cells, monocytes and macrophages, all of which develop loss of TGFβ-mediated immune tolerance and inflammatory pathology in barrier tissues (reviewed in refs[19,41]).

Both GARP- and LTBP-bound TGFβ are main sources of the cytokine in the TME, and cancer cells leverage integrin activity to regulate its bioavailability. Expression of αvβ6 integrin predicts poor prognosis in colorectal cancer (CRC), and its activity mobilizes TGFβ, inducing EMT in cell line models[42]. TGFβ released by tumour cells through αvβ8 integrins also facilitates immune evasion[43,44]. GARP is upregulated in breast, colon, and lung cancers, and enforced expression of GARP in breast cancer cells increases TGFβ bioactivity and blocks anti-tumour responses through Treg cells[45]. In other cases, cells of the TME operate as TGFβ suppliers. As mentioned above, platelets carry L-TGFβ bound to GARP on the cell surface; these are a primary source of TGFβ in tumours that provoke thrombocytosis[46]. Conditional GARP deficiency in platelets decreases levels of TGFβ signalling in the TME, leading to robust immune responses in mouse models of melanoma and CRC[46]. In tissue fibrosis, contractile myofibroblasts use integrins to liberate TGFβ bound to the ECM through LTBPs, resulting in a paracrine loop that reinforces the fibrotic program[47]. A wealth of evidence also suggests that CAFs help release active TGFβ from ECM stores present in the TME (reviewed in ref[48]) although formal in vivo proof supporting the relevance of this activity in tumour models is still lacking. Moreover, the regulatory mechanisms that determine the location, timing and level of active TGFβ release in the complex TME—a critical step in the stromal TGFβ cascade—remain poorly understood. Despite these knowledge gaps, it is becoming increasingly clear that preventing TGFβ mobilization represents a viable therapeutic option. For example, blocking the release of active TGFβ from both GARP and LTBPs deposits using antibodies that target either the LAP domain,[49,50] or αvβ6 and αvβ8 integrins[43,51–54] facilitates anti-tumour immune responses.

TGFβ deficiency, inflammation, and cancer

TGFβ signalling regulates peripheral tolerance in adulthood. Deletion of *Tgfbr2* in CD4+ and CD8+ T cells after thymocyte selection has occurred does not cause overt alterations in immune homeostasis[55,56]. Yet, these conditional mutant mice show enhanced T cell receptor (TCR) activity and immune responses to weak antigens in peripheral T cells[55,56]. The key role of TGFβ in regulating the immunological balance is particularly evident in the gastrointestinal tract, which requires both tolerance for commensal bacteria and food-borne antigens, and vigilance against pathogens[57]. In the latter case, inflammatory responses can be vital, yet need to be tightly regulated to prevent tissue damage. Colonic tumours can be induced by the treatment with the carcinogen AOM and the detergent DSS, which cause mucosal damage, disruption of the barrier function, and both inflammatory and regenerative signalling; these tumours are delayed by transgenic overexpression of TGFβ1 in T cells[58]. Conversely, the expression of a truncated form of TGFBR2 that acts as a dominant-negative receptor accelerates tumourigenesis[58]. This study showed that TGFβ signalling negatively regulates the production of pro-inflammatory cytokine interleukin 6 (IL-6) by lamina propria-resident CD4+ T cells. IL-6 signals to colonic epithelial cells and promotes their survival and proliferation in an inflamed environment, eventually resulting in dysplasia[58]. Similarly, deletion of *Smad4* in mouse T cells also elevates the production of several pro-inflammatory cytokines by T cells, including IL-6 and IL-11, and predisposes mice to spontaneous epithelial neoplasia throughout the gastrointestinal tract[59,60].

In addition to its role in suppressing T cell responses, TGFβ signalling in stromal cells also contributes to limiting chronic epithelial inflammation. Mice with fibroblast-specific *Tgfbr2* knockout develop prostatic intraepithelial neoplasia and invasive squamous cell carcinomas in the forestomach[61]. It was initially proposed that *Tgfbr2* deficiency causes the production of hepatocyte growth factor (HGF) by fibroblasts, which acts as a mitogen for adjacent mucosa cells[61]. However, an alternative mechanism linking TGFβ signalling deficiency in fibroblasts and formation of epithelial neoplasia was later identified. It was found that tissues surrounding *Tgfbr2*-null fibroblasts are inflamed and show signs of DNA damage—possibly caused by reactive oxygen species and nitrogen radicals that occur during persistent inflammation. Indeed, the forestomach mucosa exhibits loss of genomic regions encoding the tumour suppressor genes *Cdkn2b* and *Cdkn2a*[62]. Moreover, this phenotype is delayed by treatment with anti-inflammatory drugs and aggravated by *Helicobacter pylori* infection[62]. Overall, these observations imply that during barrier tissue homeostasis, TGFβ signalling in both T cells and fibroblasts is necessary to control inflammatory responses triggered by exposure to harmful antigens (Figure 1). The lack of TGFβ results in exacerbated inflammation, leading to tissue damage and cellular transformation.

Innate immune evasion by TGFβ

During advanced stages of cancer, TGFβ plays a central role in the coordination of immune evasion (Figure 1). In addition to fighting infectious diseases, the innate immune system possesses mechanisms to identify transformed cells. This is partly based on molecular recognition patterns. These danger- or pathogen-associated molecular patterns (DAMPs or PAMPs, respectively) include many molecules that are released from damaged or dying cells, and activate inflammatory responses in a number of stromal cell types[63]. These processes occur in cancer and, in principle, can alert the immune system[64]. As discussed below, TGFβ signalling broadly attenuates this vigilance, generally skewing innate immunity towards tolerance or dysfunction (Figure 3).

Macrophages and monocytes

Macrophages are abundant and highly plastic, phagocytic cells that can polarize into phenotypes that range across the inflammatory–anti-inflammatory spectrum, sometimes referred to as M1–M2, respectively[65]. During early stages, M1-like macrophages can promote tumourigenesis[66], whereas factors including TGFβ tend to induce M2-like states in more mature TMEs. Furthermore, TGFβ may help attract circulating monocytes to the tumour[67], where this cytokine inhibits interferon-γ (IFNγ)-mediated expression of inducible nitric oxide synthase (iNOS)[68], and stimulates arginase activity[69]. Tumour-associated macrophages (TAMs) commonly suppress immune responses[70], involving both the expression and activation of TGFβ via αvβ8 integrin[71] (Figure 3a).

Genetic experiments in mice revealed that TGFβ signalling in myeloid cells, which includes macrophages and monocytes, can promote tumour growth[72], progression[73], and metastasis[74]. More recently, TGFβ was linked to increase programmed death-ligand 1 (PDL1) expression on lung adenocarcinoma-associated macrophages[75], which is consistent with our own finding of myeloid PDL1 involvement in a TGFβ-mediated immune evasion mechanism in CRC liver metastasis[76]. Furthermore, TGFβ1 induces the expression of microRNA (miRNA) 494 in myeloid-derived suppressor cells (MDSCs), mediating their accumulation in the TME and exacerbating their immunosuppressive functions[77]. In fact, TGFβ-induced miRNAs with effects on immunosuppression in cancer have been observed in multiple immune cell types[78].

Granulocytes

Of all types of granulocytes, neutrophils have been studied most in the context of cancer, although intratumoural eosinophils have also been identified to produce TGFβ[79]. Neutrophils are highly prevalent and upon infection are one of the first cell types to be recruited to eliminate pathogens and raise acute inflammation[80]. Given the overlap of some of the DAMPs and PAMPs involved in infection and oncogenic transformation, neutrophils can be recruited to, recognize and eliminate cancer cells[81]. However, increased numbers of infiltrating as well as circulating neutrophils have been associated with a worse prognosis for most cancer patients[81], indicating these cells commonly fail their role in immunosurveillance. Accordingly, neutrophils have been ascribed tumour-supportive functions, mediated by exposure to signals in the TME[81–83]. Indeed, tumour-associated neutrophils (TANs) can adopt a markedly pro-tumoural polarization, sometimes called N2, mediated by TGFβ signalling[84] (Figure 3b). Blockade of this pathway in mice induced the influx of proinflammatory, cytotoxic N1-like neutrophils, impinging on tumour growth[84,85]. Moreover, a recent study with a mouse model for poor prognosis serrated CRC found that liver metastasis was driven by NOTCH1 through TGFβ2-mediated recruitment of neutrophils[86].

Natural Killer cells

Natural killer cells (NK cells) play a role in immunosurveillance[87,88]. The cytotoxic powers of NK cells are not indiscriminate, and are controlled by an array of cell surface receptors and regulatory pathways, by which NK cells can adapt to their environment[89]. This intricate regulatory balance can be exploited by the TME, leading to NK cell exhaustion, desensitization or exclusion.

Stromal TGFβ can increase the expression levels of inhibitory cues on cancer cells such as non-classical major histocompatibility complex (MHC) molecules and immunological checkpoint molecules[90–93]. Furthermore, TGFβ plays multiple roles in shaping NK cell anergy: it inhibits TBET (also known as TBX21), a transcription factor that drives IFNγ expression[94,95], it regulates activating[96,97] or inhibitory[98] surface receptors, and it represses NK cell metabolism and effector function[99,100] (Figure 3b). Additionally, TGFβ constrains CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) by NK cells[101]. Apart from as a soluble ligand, membrane-bound TGFβ on MDSCs[102], Treg cells[103], or on exosomes[104,105] can also abrogate NK cell function. NK cells can be grouped among a growing family of innate lymphoid cells (ILCs), which functionally and phenotypically mirror several T cell subtypes, except for their antigen specificity[106]. Interestingly, TGFβ can convert NK cells into type 1 ILCs that, especially under the control of the immunosuppressive cytokine, fail to control local tumour progression[107–109] (Figure 3b). Furthermore, TGFβ was reported to change the phenotype of type 2 ILC cells into an IL-17-producing type 3 ILC phenotype[110], analogous to a shift in response from a T helper 2 (TH2)- type response to a TH17-type response.

TGFβ and adaptive cancer immunity

The functions of TGFβ signalling in reducing pro-tumourigenic inflammation during early stage cancer are deflected into creating a permissive TME during disease progression. Below, we describe how tumours that have co-opted a TGFβ-rich, anti-inflammatory TME evade antitumor T cell responses (Figure 3c-e).

Suppression of DC function

One of the critical roles in orchestrating immunity is antigen presentation: dendritic cells (DCs) are professional antigen presenting cells, known for their ability to mature in inflammatory conditions and phagocytose tumour cells. They can then migrate to lymphoid structures and present tumour antigens on the two types of major

histocompatibility complex (MHC), interacting with both CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ T-helper (Th) cells[111].

Active TGFβ can avert immature myeloid cells from DC differentiation, a process driven by the SMAD-regulated transcription factor ID1[112]. In the case of monocytes, this process may additionally involve an autocrine TGFβ-mediated feedback loop[113] (Figure 3c). After DC differentiation, immature DCs promote tolerance and mediate the generation of Treg cells during homeostasis[114,115]. Elevated TGFβ can impede DC maturation and lower the expression levels of MHC molecules and inflammatory cytokines, reducing the ability of DCs to activate T cells[116,117]. This regulatory function of TGFβ is critical in preventing auto-immunity[118], but can limit immunity in the TME. Furthermore, tumour-associated DCs produce TGFβ1 that primes the differentiation of Treg cells[119,120]. In addition, DCs express αvβ8 integrin, which enables the release of active TGFβ from the ECM. In mice, DCs lacking αvβ8 integrin fail to induce Treg cells and cause autoimmunity[121–123].

DC migration is another critical function in steering immunity, and TGFβ has been reported to restrict DC chemotaxis by regulating chemokine receptor expression[124,125] (Figure 3c). In *in vivo* cancer models, DC trafficking to lymph nodes was reduced by TGFβ1[126], and blockade of TGFβ signalling improved the antitumor efficacy of DC vaccines[127]. Finally, a recent study found that TGFβ can also inhibit the function of plasmacytoid DCs (pDCs), which includes secretion of type-I IFN and activating NK cells[128,129]. This corroborates findings in breast and head and neck cancer, where TGFβ played a role in suppressing pDC-derived IFNα and IFNβ[130,131].

Regulation of TCR signalling

TCRs can recognize a large variety of epitopes, including tumour neoantigens, cancer germline antigens, and viral oncoproteins, bound to MHC at the surface of antigen-presenting cells. The strength of the TCR–MHC-bound antigen interaction determines whether or not the downstream signal is sufficiently robust to activate the T cell. *In vitro* experiments showed that the earliest biochemical events detectable upon TCR triggering, such as tyrosine phosphorylation and calcium ion influx, are suppressed by TGFβ signalling[132]. Indeed, *Tgfbr1* deficient mouse T cells can be activated by weaker TCR stimuli compared to their wild-type counterparts[133]. These observations are further supported by the finding that CD4+ T cells isolated from conditional *Tgfbr2* mutant mice display accelerated calcium influx and TCR activation upon suboptimal stimulation[55,56]. Indeed, blockade of proximal TCR signalling by TGFβ has been observed in cancer[134,135] and genetic inhibition of the TGFβ pathway in CD8+ T cells potentiates antitumor adaptive immune responses by lowering the TCR activation threshold[136] (Figure 3c).

Th cell proliferation and differentiation

$CD4^+$ T cells are able to redirect their differentiation program in response to different threats and acquire distinct functions to combat specific pathogens. Extracellular signals from the environment control this process. Amongst them, TGFβ signalling exerts a powerful influence in the polarization of the four major CD4+ T cell subsets; it prevents Th1 and Th2 differentiation while promoting Th17 and Treg cell programs. This role is also evident in the TME and represents an important mechanism of immune evasion (Figure 3d).

Th1 cells

Th1 cells are mainly characterized by the production of IFNγ and tumour necrosis factor (TNF), and are primarily responsible for activating and regulating phagocytic and cytotoxic responses against pathogens and tumour cells. The abundance of CD4+ Th1

cells in the TME predicts good prognosis and productive immunotherapy responses against cancer[137]. TGFβ impedes the differentiation of naive T cells towards the Th1 subsets[138]. Mechanistically, TGFβ operates during the antigen priming phase via SMAD2 and SMAD3 signalling to inhibit the expression of TBET and STAT4, the two master transcription factors of the Th1 gene program[139–141]. TGFβ also suppresses the levels of the transcription factor MYC and upregulates the cell-cycle inhibitors CDKN1A and CDKN1B (ref[142,143]), thus promoting cytostasis and apoptosis in CD4+ T cells. Accordingly, mice deficient for TGFβ signalling in T cells exhibit exacerbated Th1 responses[55,56,144–146]. In cancer, TGFβ restrains immune responses by antagonizing Th1 differentiation, as shown by genetic or pharmacological inhibition of TGFβ signalling in multiple tumour types, including CRC[76], prostate cancer[147], and melanoma[148].

Th2 cells

Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and mediate humoral responses to pathogens. TGFβ signalling also suppresses Th2 differentiation[138]. The mechanism proposed involves blockade of the Th2 lineage transcription factor GATA3 by the TGFβ transcriptional target *SOX4*[149]. Although the role of Th2 immunity in cancer is still debated, the TME of several tumour types, including subsets of CRC, squamous lung cancer, and luminal A breast cancer, exhibit upregulation of Th2 gene signatures[137]. Early works showed that combined blockade of IL-10 and TGFβ signalling in tumour-bearing mice elicited Th2 responses[15]. Further evidence of TGFβ-mediated suppression of Th2 immunity in tumours came from analysis of the MMTV-PyMT breast cancer mouse model. In this strain, genetic or pharmacological blockade of TGFBR2 in CD4+ cells (but not in CD8+ cells) promotes a Th2 response that depends on IL-4, and that results in blood vessel reorganization in the TME leading to tumour hypoxia and death[150,151].

Th17 cells

Th17 cells constitute a proinflammatory CD4+ T cell subset that is characterized by the production of IL-17A and IL-22 cytokines. The Th17 response is adept at fighting extracellular pathogens and fungi, and has been associated with tumour-promoting inflammation and autoimmune diseases[152]. TGFβ, together with the proinflammatory cytokine IL-6, fosters Th17 cell fate by elevating the levels of orphan nuclear receptor RORγt[153], a key Th17 differentiation regulator. In addition, RORγt interacts with SMAD2 to drive the Th17 differentiation program in mice[154]. Several studies have demonstrated the Th17-polarizing nature of a TGFβ-rich TME. For example, in human melanoma cell-bearing immunodeficient mice that were immune reconstituted, pharmacological blockade of TGFβ signalling using a bifunctional TGFβ ligand trap and cytotoxic T-lymphocyte associated protein 4 (CTLA4) antibody antagonizes Th17 differentiation, and fosters the generation of IFN-γ-expressing CD4+ T cells[148]. In a mouse model of serrated CRC, treatment with a TGFBR1 inhibitor diminished the number of infiltrating Th17 cells[155]. In mice with prostate cancer bone metastasis, TGFβ is released from the bone matrix as a result of osteolysis. This TGFβ-rich TME skews newly primed CD4+ T cells towards a Th17 program instead of the Th1 lineage, preventing effective anti-tumour responses by anti-CTLA4 plus anti-programmed cell death protein 1 (PD1) treatment[147]. In this model, TGFβ blockade restores Th1 polarization in bone metastasis, potentiating the effects of immune checkpoint inhibitor (ICI) therapy[147].

Treg cells

Treg cells present in healthy tissues maintain immune homeostasis by inhibiting the function of effector T cells. TGFβ drives the expression of FOXP3[156,157], the master transcription factor of the Treg cell program. Mechanistically, SMADs, in combination with the NFAT transcription factor, bind to a distal enhancer in the *FOXP3* gene driving

its expression[141,158]. The acquisition of the Treg cell phenotype is, however, counterbalanced by Th1 and Th2 polarizing cytokines such as IFN-γ and IL-4[159]. In addition, low TGFβ concentrations synergize with IL-6 to promote Th17 instead of Treg differentiation[160]. In turn, Th17 cells can transdifferentiate into Treg cells by the action of TGFβ and the aryl hydrocarbon receptor (AHR) during the inflammation resolution phase[161].

The TGFβ-rich TME can promote CD4+ T cell polarization to a Treg cell phenotype as a mechanism to enforce tumour antigen tolerance, as seen in pancreatic cancer models[162]. Indeed, *FOXP3* gene expression correlates with *TGFB1* mRNA levels in patient cohorts of several tumour types[148]. Furthermore, pharmacological inhibition of TGFβ signalling results in decreased Treg cell numbers in the TME of tumour models[148,163]. However, the relative contribution of these effects to the outcome of anti-TGFβ therapy remains to be established. For example, conditional deletion of *Tgfbr1* in Treg cells does not influence CRC growth or radiation response in syngeneic tumour cell implantation in mice, whereas *Tgfbr1* deficiency in CD8+ T cells results in potent antitumor immune responses, implying a minor role for TGFβ-induced Treg cell polarization in this model[136].

Treg cells produce and carry GARP-bound L-TGFβ1 at the cell surface, which can be activated by αvβ8 integrins. However, the relevance of this mechanism is controversial, as TGFβ1 knockout Treg cells can still enforce tolerance[164,165]. Consistent with this finding, in a mouse model of prostate cancer, CD4+ T cell- but not Treg cell-specific ablation of *Tgfb1* enhanced immune responses against the tumour[166]. On the other hand, activated Treg cells upregulate integrin αvβ8 expression, and integrin β8-deficient Treg cells cannot suppress active T cell-mediated inflammation in an experimental model of colitis[167]. As discussed above, antibodies that target β8 integrin prevent TGFβ mobilization from latent deposits and potentiate antitumor cytotoxic T cell responses[43]. Similarly, antibodies that prevent TGFβ release by targeting GARP inhibit the immunosuppressive capacity of Treg cells in a graft *versus* host disease model[168]. Anti-GARP antibodies also promote tumour immunity and synergize with ICI therapy[45,168,169]. However, it remains to be proven that these effects occur due to inhibition of active TGFβ derived from Treg cells, as multiple other cell types—including platelets—carry GARP–L-TGFβ1 on their cell surface[45,46]. Indeed, deletion of *Garp* in Treg cells does not trigger overt immune responses against tumour cells in mice[170].

Inhibition of CTL activity

CTLs are central players in adaptive immune responses and play a critical role in anti-tumour immunity. They release cytolytic granules in response to binding specific antigen peptides presented on MHC class I by target cancer cells. Conditional deletion of *Tgfbr2* in T cells exacerbates the effector phenotype of CD8+ T cells, which encompasses increased production of granzyme B and IFNγ[55,56]. A pioneering study demonstrated that transgenic mice that express a truncated, defective TGFBR2 in CD4+ and CD8+ T cells mount potent immune responses against tumour cells, characterized by expansion of tumour-specific CD8+ cells[17]. Furthermore, CD8+ T cell-specific *Tgfbr1* knockout mice reject tumour cells efficiently, whereas *Tgfbr1* deficiency in Treg cells or macrophages does not modify the anti-tumour immune response[136]. Immunotherapy based on the adoptive transfer of autologous tumour-reactive CTLs is improved if transferred T cells are rendered insensitive to TGFβ by expressing a dominant-negative TGFBR2[171–173].

Taken together, these observations imply that in several tumour types, the immuno-suppressive function of TGFβ is exerted, to a large extent, by direct inhibition of CD8+ T cell function (Figure 3e). Besides lowering the TCR activation threshold (discussed

above), TGFβ suppresses CTL activity through several mechanisms. First, TGFβ downregulates transcription of genes encoding critical elements of the lytic machinery such as granzyme A, granzyme B, perforin, Fas ligand, and IFNγ by directly repressing their promoters[174]. Also proliferation is inhibited through TGFβ-mediated silencing of *Myc* and *Jun* gene expression[175]. SMADs drive these effects in complex with the transcription factors ATF1[174] and FOXP1[175]. Observations in melanoma mouse models and in T cell isolated from melanoma patients also indicate that the genes encoding the transcription factors TBET and EOMES, two enforcers of the CD8+ effector program[176,177], are downregulated by TGFβ[178,179]. Another mechanism involves the inhibition of CD8+ T cell migration to tumour beds by TGFβ-mediated silencing of the gene encoding C-X-C chemokine receptor 3 (CXCR3), a receptor for the chemoattractant C-X-C motif chemokine 10 (CXCL10)[136].

Promoting CD8+ T_{RM} cells

Besides suppressing the cytotoxic effector program of CTLs, TGFβ signalling can also stimulate their conversion to a tissue-resident memory T cell (T_{RM} cell) phenotype (Figure 3e). CD8+ T_{RM} cells are important mediators of adaptive immunity in peripheral tissues and provide long-lived protection against re-infection. TGFβ downregulates TBET and EOMES transcription factors during the maturation of T_{RM} cells, initiating a departure from the Th1 program[180]. In addition, TGFβ signalling promotes T_{RM} cell residence in epithelial tissues such as skin, intestine, or lungs by increasing the levels of αE (also known as CD103) and β7 integrin subunits in T_{RM} cells, which interact with the epithelial adhesion molecule E-cadherin[181–185]. In mice, the induction of lung CD8+ T_{RM} cells by TGFβ does not require SMAD4, suggesting that this subset is specified by non-canonical signalling[186]. It has been observed that TGFβ increases the abundance of CD8+CD103+ T cells in the TME[187,188]. These findings are at odds with the immunosuppressive role of TGFβ in the TME, as the presence of CD8+ T_{RM} cells in tumours is associated with anti-tumour immune responses and predicts good prognosis[187–191]. However, it has also been described that in mice, TGFβ induces a tolerogenic CD8+CD103+ cell subset that expresses immune suppressive molecules such as CTLA4 and IL-10 and helps tumours evade immunity[49].

TGFβ-activated CAFs and immune evasion

In healthy tissues, fibroblasts remain largely quiescent but become activated in the event of tissue damage to help wound healing by depositing ECM and contracting the wound. The role of TGFβ in these processes has been extensively investigated[39,192,193]. In cancer, persistent inflammation and other signals sustain continuous fibroblast activation and exacerbate TGFβ production, resulting in a permanent and pathogenic wound-healing program (Figure 3). Furthermore, CAF generation is also affected by cancer-derived exosomes, carrying nucleic acids (including mRNAs, miRNAs, or other non-coding RNAs)[194] or proteins such as surface-bound TGFβ1. The latter was shown to induce tumour-promoting CAFs *in vitro* in a distinct manner from soluble TGFβ1[195]. Solid tumours recruit fibroblasts without exception, but the microenvironment of some subtypes is particularly CAF-rich, exhibiting widespread TGFβ signalling in stromal cells and prominent ECM deposition. This phenomenon has been associated with poor prognosis and lack of immunotherapy responses in multiple studies (Box 2).

The mechanisms behind the role of TGFβ-activated CAFs in immune evasion remain, however, partially understood. TGFβ produced by CAFs, either through direct secretion or by release from latent deposits stored in the ECM, can directly suppress tumour immunity through signalling in cells of the innate and adaptive immune system. Evidence also suggests that the composition, extent of crosslinking, and stiffness of the ECM, all of which are the consequence of the fibrogenic program controlled by

TGFβ, regulate T cell infiltration in tumours[196–200]. In addition, TGFβ signalling stimulates the production of a plethora of cytokines and growth factors by fibroblasts[9,201], including IL-6[202], leukemia inhibitory factor (LIF)[203,204], CXCL12[205], and prostaglandin E2 (PGE2)[206–208], which impact the immune environment and contribute to immune evasion. Of note, these molecules are not only produced by CAFs but also by other TME cell types or even by cancer cells and, therefore, the relative contribution of CAFs to their expression varies from tumour to tumour depending on the TME composition.

TGFβ-inhibition-based immunotherapies

In light of the determining effect of TGFβ signalling in the TME on cancer progression, immune evasion and therapy resistance, a wide range of therapeutic modalities have been developed. These include TGFβ mRNA-directed agents, ligand traps, antibodies, fusion proteins, and small molecule kinase inhibitors against TGFBRs (Table 1). However, progress to bring these drugs to the clinic has faced important challenges. There are three main reasons for hesitation; first, TGFβ is a tumour suppressor for early neoplastic lesions. Indeed, a common side effect observed in patients treated with the anti-TGFβ-blocking antibody fresolimumab is the development of acanthomas, a type of benign neoplastic skin lesion that regresses after treatment cessation[209]. Second, genetic loss-of-function studies in mice caution for the possibility of serious inflammatory disease in gastrointestinal tissues caused by global TGFβ inhibition[58–61]. Third, and more critical, animal studies with small molecule TGFBR1-inhibitors such as AZ12601011 and AZ12799734[210] and pan-TGFβ antibodies have confirmed a risk for overt cardiovascular toxicity characterized by heart valve thickening, hemorrhage, inflammation, and endothelial and stromal hyperplasia[211–213] (Box 1). Mice with *Tgfbr2* deficiency in postnatal smooth muscle cells develop similar cardiovascular pathology implying that the deleterious effects triggered by TGFβ inhibitors are to a large extent due to alterations in vascular smooth muscle cells[214,215].

Selected for its relatively safe toxicology profile[212], the TGFBR1 kinase inhibitor galunisertib entered clinical investigation more than a decade ago. In phase I trials, an intermittent dosing schedule was found to be well-tolerated, demonstrating a therapeutic window[216–218]. Since then, many other clinical trials have assessed galunisertib alone or in combination with other chemotherapies with manageable safety (Reviewed in references [219–221]). However, this drug achieved only modest responses in phase II trials, including as monotherapy for patients with refractory hepatocellular carcinoma [222] (Table 2). The reasons are unclear but may partly be due to suboptimal patient stratification and insufficient inhibitory potency of this molecule at the intermittent dosing strategy used. New TGFBR1 inhibitors more potent and specific than galunisertib have been developed and are currently being tested in patients (Table 3).

Beside small molecule TGFBR1 inhibitors, other early agents were a phosphorothioate antisense oligodeoxynucleotide specific for *TGFB2* mRNA (trabedersen[223]); a vaccine derived from an irradiated and *TGFB2*-antisense transfected non-small cell lung cancer cell line (belagenpumatucel-L[224,225]); and a monoclonal antibody against all 3 TGFβ ligands (fresolimumab). Clinical development of trabedersen has slowed down, but second-generation antisense molecules targeting either *TGFB1*, *TGFB2* or *TGFB3* are still in development[226,227]. At present, of these first-generation agents only galunisertib and fresolumumab remain in active trials; however, they have not shown sufficient clinical activity and, as we discuss herein, several second generation TGFβ pathway inhibitors have already reached clinical trials.

Despite the complexity and risks of clinically targeting the TGFβ pathway, an enduring interest is demonstrated by the long list of recent agents and active trials (Tables 1, 3). A number of these strategies target one or two specific TGFβ isoforms, in an attempt to avoid toxicity issues seen with pan-inhibitory antibodies used in the past (Box 1). Pre-clinical evidence suggests that individual TGFβ ligands may be safe to target[50,228] and—in combination with ICIs—could be sufficient in some cancer types[50,229].

Other ligand sequestering approaches have been taken using TGFBR2 ectodomain fusion proteins, engineered into bi-specific drugs. Of these, the most advanced is bintrafusp alfa (also known as M7824) that has an ecto-TGFBR2-derived ligand trap fused to a human monoclonal antibody against PDL1[230]. This agent, as well as the similar molecule SHR-1701, is currently being evaluated in the clinic[231]. Similarly, a ligand trap fused to anti-CD73 (GS-1423) has entered clinical trials (Table 2), and a ligand trap fused to an antibody targeting the immune checkpoint molecule CTLA4 has shown promising results in preclinical studies[148]. Furthermore, the above-mentioned preclinical $CD4^+$ T_h-cell-specific TGFBR2 blockade strategy also involves a fusion protein, consisting of the TGFBR2 ectodomain attached to ibalizumab—a non-immunosuppressive CD4 antibody that was previously used to block HIV infection[150,232].

Most ongoing strategies to block TGFβ signalling involve combination therapies, either together with standard-of-care agents or, increasingly, with immunotherapeutic regimens such as ICIs (Table 3 and Box 2). The latter is supported by a growing number of promising results in preclinical studies, pointing to synergistic immunomodulatory actions of TGFβ blockade[76,148,155,229,230,233–237]. The prevailing rationale is that TGFβ pathway inhibition can overcome immunosuppressive signalling in the TME, facilitate T cell tumour infiltration and cytotoxicity, among a number of other relevant factors that are, unsurprisingly, implicated in failure of ICIs. Indeed, several studies have found elevated TGFβ programs in ICI-nonresponding cancers[50,76,229,236,238–244] (Box 2).

Furthermore, TGFβ is increasingly recognized as a key immunosuppressor that can diminish tumour infiltration and efficacy of adoptive immune cell transfer therapy, especially for solid cancers. In that field, chimeric antigen receptor (CAR) T cell approaches are actively investigated[245]. There have been a number of approaches to make CAR T cell products resistant to TGFβ. These include the overexpression of a dominant-negative TGFBR2[246] or of a constitutively active AKT[247], or using CRISPR/Cas9 to knock out the endogenous *TGFBR2*[248]. Alternatively, the lymphocyte-inhibitory TGFβ ligand has been rewired into a stimulatory signal via a chimeric switch receptor[249]. One approach combined the extracellular ligand-binding parts of TGFBR1 and 2 with intracellular IL-12Rβ1 and IL-12Rβ2 signalling domains, expressed on a CAR T cell[250]. A second study used a pooled CRISPR knockin screen to evaluate a panel of transgenes, among them an engineered TGFBR2–4-1BB switch receptor[251]. Furthermore, a TGFβ-CAR has been reported that switches T cells from immunosuppressed to proliferating, Th1 cytokine-producing T cells that can activate neighbouring CTLs[252,253]. In these studies, the transferred CAR T cells show both a better activity and fitness over TGFβ pathway wild-type CAR T cells. Together, these developments demonstrate a broad investment in combining immunotherapeutic strategies with targeted TGFβ inhibition.

Parallel efforts to induce tumoural T cell infiltration and subsequent immunotherapeutic efficacy led to the auspicious combination of ICIs with radiotherapy[254,255]. Interestingly, TGFβ plays a key role in limiting the effect of *in situ* vaccination, a key therapeutic benefit of radiation, advancing the rationale for a triple combination of an ICI, radiotherapy and TGFβ blockade in a preclinical breast cancer model[256]. Similarly,

such a strategy was reported for mouse models of CRC and melanoma[257]. Furthermore, a feasibility trial of the combination of fresolimumab with focal irradiation in patients with metastatic breast cancer was successful[258] (Table 2). A similar clinical trial is ongoing for early-stage non-small cell lung cancer (Table 3). Other potential TGFβ inhibition-based combinatorial immunotherapies may include oncolytic viruses[259], NK cell therapy[260], DC vaccination[127,261], vaccine-based approaches such as gemogenovatucel-T[262] or blockade of monocyte recruitment[263].

Discussion and Future perspectives

TGFβ is a powerful cytokine capable of dominating the behaviour of most cells present in the TME. Generally, TGFβ enforces immune tolerance, suppresses inflammation, and regulates wound healing during homeostasis. These mechanisms are often co-opted during tumour evolution to evade the immune system. However, as we have described herein, the effect of TGFβ signalling can differ substantially depending on the tumour type, organ affected, and disease stage. Beyond the findings that genetic or pharmacological TGFβ blockade triggers potent anti-tumour responses in several pre-clinical model systems, it is becoming increasingly clear that the type and extent of this response are largely context-dependent and the sum of disparate processes. Therefore, how TGFβ remodels different cancer ecosystems remains an important question for the coming years: which cell types are essential in each context, and how are distinct responses coordinated in space and time? It is also worth bearing in mind that our current understanding of the roles of TGFβ in cancer emerges from decades of studies of this cytokine in tissue development and organ homeostasis. Yet, chronic or elevated TGFβ signalling may affect the TME beyond the range of functions identified in homeostatic conditions. Research on all these topics is key to identifying which tumour types or subtypes can benefit from TGFβ inhibitory therapies, interpreting the results of upcoming clinical trials, and optimizing the use of TGFβ inhibitors in combination with other therapeutic modalities. These efforts should include the application of TGFβ-related predictive biomarkers, such as the fibroblast TGFβ response gene signature (F-TBRS, Box 2). In our view, progress in translational research also demands a shift from the simplistic subcutaneous tumours commonly used in immunological studies to cancer models that more faithfully reproduce key aspects of TGFβ signalling in human disease.

Despite the impressive effects of TGFβ inhibitory therapies in pre-clinical cancer models, the benefits of this therapy have not yet been translated to patients. Research in mouse models has revealed a strong synergism between TGFβ pathway inhibitors and ICIs. To date, combinatorial TGFβ blockade and ICI strategies have not yet been extensively tested in patients, in part due to the scarcity of TGFβ inhibitors in advanced clinical stages. As this situation is rapidly changing (Table 3), the field eagerly anticipates the results of these studies, keenly aware of the pending safety concerns. In this regard, a better understanding of the biological basis for the cardiovascular toxicities shown by many TGFβ inhibitors is crucial for their systematic implementation in the clinical setting. Are the TGFβ isoforms that are important in shaping the TME the same as those that regulate the cardiovascular system? What is the relative contribution of canonical versus non-canonical signalling in the cardiovascular defects triggered by TGFβ inhibition? New strategies, including TGFβ isoform-specific blocking antibodies—some of which already under clinical investigation—antibodies capable of inhibiting the TGFβ pathway in specific TME cell types, and tumour-specific delivery of TGFβ inhibitors may also help reduce side-effects. In addition, novel small molecule TGFBR1 inhibitors are advancing with apparently manageable toxicity. Finally, a growing group of agents aim at preventing TGFβ activation. Although our knowledge of this area is relatively limited, TME-specific upstream mechanisms have an unmistakable therapeutic potential.

As the number of possible combinations of (immuno) therapies grow exponentially, one inevitable challenge of near-future clinical practice concerns the choice for the best suited targets and therapies on a per-patient level. This requires a much better understanding of the most relevant tumour-specific mechanisms in the TME, and their relation to the individual immunological status[264]. For the moment, relevant questions that have already emerged for trials that involve TGFβ blockade include patient stratification or selection, treatment duration, and therapy resistance. It is thus imperative that we keep unraveling the complex biology of TGFβ signalling in the TME.

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samples reveal TGFβ- and IL-1-induced CAFs. The transcriptome of the TGFβ-activated LRRC15+CAF subset predicts poor clinical response to ICIs.

Grauel AL, Nguyen B, Ruddy D, Laszewski T, Schwartz S, Chang J, Chen J, Piquet M, Pelletier M, Yan Z, et al. 2020. TGFβ-blockade uncovers stromal plasticity in tumours by revealing the existence of a subset of interferon-licensed fibroblasts. *Nat Commun* **11**.

- **Authors investigate CAF heterogeneity upon TGFβ inhibition in mouse tumour models using single-cell RNA sequencing. The study reveals the emergence of an interferon-licensed CAF subset characterized by upregulation of antigen presentation machinery and expression of cytokines.**

GLOSSARY

danger- or pathogen-associated molecular patterns
Molecules, molecular motifs or epitopes that are upregulated or exposed in the presence of pathogens, or on damaged or dying cells. Specialized receptors on innate immune cells recognize these signals and trigger an inflammatory response.

tumour-associated macrophages
a heterogeneous population in the TME originating from tissue-resident macrophages or monocytes

Granulocytes
(also known as polymorphonuclear cells) – a group of myeloid cell comprising neutrophils, basophils, eosinophils, and mast cells

natural killer cells
innate cytotoxic immune cells that can kill tumour cells (or pathogen-infected cells) without any priming or prior sensitization

antibody-dependent cellular cytotoxicity
cell killing by virtue of target cell-specific antibodies and effector cells, such as NK cells, that express antibody receptors.

myeloid-derived suppressor cells
a heterogeneous group of myeloid immune cells that are characterized by their immunosuppressive functions. They can accumulate in cancer or infections, impinge on the function of other immune cells, and are often poorly differentiated or immature.

innate lymphoid cells
cells from the lymphoid lineage with innate immune functions, regulating other immune cells and producing signalling molecules. These lymphocytes without a T cell receptor are functionally analogous to helper T cells (Th1, Th2 and Th17) and are classified accordingly (type 1, 2 and 3, respectively).

dendritic cells
innate immune cells that respond to DAMPs and PAMPs, induce inflammation, and can stimulate NK cells in the TME.

plasmacytoid dendritic cells
a subset of DCs that are mostly found in circulation, lymph nodes, and the spleen. They have important roles in antiviral immunity, immune regulation, and are implicated in certain immune disorders.

desmoplastic reaction
the growth of fibrous tissue around the tumour

thymocyte positive selection
in the thymus, T cells whose TCRs bind strongly to MHC complexes and will likely be self-reactive, are killed in the process of negative selection

serrated CRC
a non-classical type of colorectal cancer that derives from an alternative carcinogenesis pathway and has a ‘saw-tooth’-like histological appearance.

MMTV-PyMT breast cancer mouse model
A mouse model of breast cancer generated by the mammary specific expression of polyomavirus middle T antigen driven by a mouse mammary tumour virus (MMTV) element.

chimeric switch receptor
fusion proteins that link the binding of (immuno-) inhibitory ligands to the activation of intracellular stimulatory signal elements, or vice versa.

in situ vaccination
the effect of therapeutically elevating the release of tumour-associated antigens, combined with innate immune cell activation, which results in (more) effective antigen presentation and T or B cell priming. Triggers include immunogenic cell death, radiotherapy, or oncolytic viruses.

Table of contents summary

This review discusses the context-dependent functions of transforming growth factor β (TGFβ) on the composition and behavior of different cell populations in the tumour immune microenvironment, as well as emerging data that demonstrate that TGFβ inhibition can restore cancer immunity.

Table 1. An overview of TGFβ targeting agents

Class	Strategy	Name	Target	Status	Reference
Small molecule inhibitors	Block receptor signalling	Galunisertib (LY2157299)	TGFBR1	Phase I/II	216–218
		Vactosertib (TEW7197)	TGFBR1	Phase I/II	265
		LY3200882	TGFBR1	Phase I/II	266
		PF-06952229	TGFBR1	Phase I	NA
		TD-1058	TGFBR1	Phase I	NA
		YL-13027	TGFBR1	Phase I	267
		AZ12601011, AZ12799734	TGFBR1	Preclinical	210
		GFH-018	TGFBR1	Preclinical	268
Antibodies	Trap ligand(s)	Fresolimumab (GC1008)	TGFβ1, 2 and 3	Phase II	269
		SAR439459	TGFβ1, 2 and 3	Phase I/II	270
		NIS793	TGFβ1, 2 and 3	Phase II	229,271
		TβM1 (LY2382770)	TGFβ1	Phase I	272
		XPA-42-089	TGFβ1 and 2	Preclinical	235,271
	Prevent ligand activation	SRK-181	Latent TGFβ1	Phase I	50
		ABBV-151	GARP-TGFβ1	Phase I	168
		Anti-LAP	Latent TGFβ	Preclinical	49
		264RAD	Integrin αvβ6	Preclinical	51,52
		Anti-αvβ8 (C6D4)	Integrin αvβ8	Preclinical	43
		Anti-αvβ8 (ADWA-11)	Integrin αvβ8	Preclinical	53,54
	Receptor blockade	IMC-TR1 (LY3022859)	TGFBR2	Phase I	273,274
Engineered fusion proteins	Trap ligands	AVID200	TGFβ1 and 3	Phase I	275,276
		sBetaglycan/TGFBR3	TGFβ ligands	Preclinical	277
		sTGFBR2-Fc	TGFβ ligands	Preclinical	278
	Trap ligands and checkpoint blockade	Bintrafusp alfa (M7824)	TGFβ ligands and PDL1	Phase II/III	230
		SHR-1701	TGFβ ligands and PDL1	Phase I	NA
		GS-1423	TGFβ ligands and CD73	Phase I	NA
		anti-CTLA4-TGFBRII	TGFβ ligands and CTLA4	Preclinical	148
	Trap ligands and bind T_H cells	4T-Trap	TGFβ ligands and CD4	Preclinical	150
Peptides	*Competitive ligand binding*	YH14618	TGFβ1	Phase I	279
Antisense oligonucleotides	*Prevent ligand expression*	Trabedersen (AP 12009)	*TGFB2* mRNA	Phase I/II/III	223
		ISTH0036	*TGFB2* mRNA	Phase I	280
		ISTH0047; ISTH1047	*TGFB2* mRNA	Preclinical	226,227,281
Cancer vaccines	*Boost immune response*	Belagenpumatucel-L	*TGFB2*-antisense-modified NSCLC[a] cells	Phase III	224,225
		Gemogenovatucel-T	Autologous tumour cell vaccine + GM-CSF + FURIN shRNAi (reduces TGFB1, -2 levels)	Phase II	282

NA, not available.

Table 2. Completed trials targeting TGFβ in clinical immuno-oncology

Agent	Strategy	Cancer type	Phase	Outcome	Trial ID and/or Reference
Galunisertib	With anti-PD-L1 (durvalumab)	Advanced PDAC	I	Acceptable safety/tolerability, low clinical activity (ORR 3%)	NCT02734160[283]
Fresolimumab	With radiation	Metastatic BC	II	Acceptable safety/tolerability, low clinical activity but favorable immune responses were observed	NCT01401062[258]
Bintrafusp alfa	Monotherapy	Advanced solid tumours; NSCLC; HNSCC	I	Acceptable safety/tolerability, clinical activity: ORR 16%; 18%; 13%	NCT02517398[284–286]
		BTC; ESCC	I	ORR 20%; 10%	NCT02699515[287,288]
Belagenpumatucel-L	Monotherapy	NSCLC	II	Acceptable safety/tolerability, ORR 15%	NCT01058785[224]
		NSCLC	III	No significant clinical benefit	NCT00676507[225]
Gemogenovatucel-T	Monotherapy	ES, NSCLC or HCC	I	Acceptable safety/tolerability, benefit in mOS (ES)	NCT01061840[289,290]
		Melanoma	I/II	Acceptable safety/tolerability, mOS 20 vs 7 months	NCT01453361[291]
		Stage IIIb-IV ovarian cancer	II	Well-tolerated maintenance therapy, mRFS 11.5 vs 8.4 months	NCT02346747[262]
	With chemotherapy	ES	II	NA	NCT02511132
	With anti-PD1 (pembrolizumab)	Melanoma	I	NA	NCT02574533
	With anti-PDL1 (durvalumab)	Advanced women's cancers	II	NA	NCT02725489

HCC: hepatocellular carcinoma; HNSCC: head and neck squamous cell carcinoma; BTC: biliary tract cancer; ESCC: esophageal squamous cell carcinoma; BC: breast cancer; ES: Ewing sarcoma; ORR: objective response rate; mOS: median overall survival; mRFS: median recurrence-free survival; NSCLC, non-small cell lung cancer; PDAC, pancreatic ductal adenocarcinoma. NA, not available.

1929 **Table 3. Ongoing trials targeting TGFβ in clinical immuno-oncology**

Agent	Strategy	Cancer type	Phase	Reference
Galunisertib	With radiation	Adv. HCC	I	NCT02906397
	With anti-PD1 (nivolumab)	HCC, NSCLC, Advanced solid tumours	I/II	NCT02423343
Vactosertib	With anti-PD1 (pembrolizumab)	Metastatic CRC or gastric cancer	I/II	NCT03724851
		PDL1⁺ NSCLC	II	NCT04515979
	With anti-PDL1 (durvalumab)	Advanced NSCLC	I/II	NCT03732274
		Urothelial carcinoma	II	NCT04064190
Fresolimumab	With radiation	Early-stage NSCLC	I/II	NCT02581787
SAR439459	Monotherapy and with anti-PDL1 (cemiplimab)	Advanced solid tumours	I	NCT03192345
			I	NCT04729725
	With dexamethasone and anti-CD38 (isatoxumab)	Relapsed/refractory multiple myeloma	I/II	NCT04031872
NIS793	Monotherapy and with anti-TIM3 (MBG453)	Myeloproliferative tumours	I	NCT04810611
	With anti-PD1 (spartalizumab)	Advanced solid tumours	I	NCT02947165
	With chemotherapy and anti-PD1 (spartalizumab)	Metastatic pancreatic cancer	II	NCT04390763
SRK-181	Monotherapy and with anti-PD1 or PDL1	Advanced solid tumours*tumour*	I	NCT04291079
ABVV-151	Monotherapy and with anti-PD1 (budigalimab)	Advanced solid tumours	I	NCT03821935
Bintrafusp alfa	Monotherapy	Metastatic CRC or advanced microsatellite-instable solid tumours	I/II	NCT03436563
		Stage II-III HER2⁺ breast cancer	I	NCT03620201
		Advanced NSCLC	III	NCT03631706
		Metastatic second-line BTC	II	NCT03833661
		Platinum-refractory cervical cance	II	NCT04246489
		Adv. urothelial cancer	I	NCT04349280
		Adv. nasopharyngeal carcinoma	II	NCT04396886
		Thymoma and thymic carcinoma	II	NCT04417660
		Operable and untreated head and neck squamous cell carcinoma	II	NCT04428047
		Checkpoint inhibitor-naïve and -refractory urothelial carcinoma	II	NCT04501094
	Neo-adjuvant	First-line resectable NSCLC	II	NCT04560686
		Resectable BTC	II	NCT04727541
	With chemotherapy	Relapsed small cell lung cancers	I/II	NCT03554473
		Metastatic triple-negative breast cancer	I	NCT03579472
		Advanced NSCLC	I/II	NCT03840915
		BTC	II/III	NCT04066491
		Advanced NSCLC	II	NCT04396535
		HMGA2-expressing triple-negative breast cancer	II	NCT04489940
		Advanced gastric cancer	I/II	NCT04835896
	With TKIs	Brain metastases	I/II	NCT04789668
	With chemotherapy and immunocytokine (IL-12)	Prostate cancer	I/II	NCT04633252
		Advanced solid tumours	I/II	NCT04708470

	With radiation	Hormone receptor$^+$, HER2$^-$ adv. breast cancer	I	NCT03524170
		Recurrent or second primary head and neck squamous cell carcinoma	I/II	NCT04220775
		Advanced intrahepatic cholangiocarcinoma	I	NCT04708067
	With chemo-radiation	Unresectable stage III NSCLC	II	NCT03840902
		Adv. cervical cancer	I	NCT04551950
		Esophageal SCC	II	NCT04481256 NCT04595149
	With immunocytokine (IL-12) and radiation	Metastatic non-prostate genitourinary malignancies	I	NCT04235777
		Pancreatic cancer	I/II	NCT04327986
		Hormone receptor$^+$, HER2$^-$ metastatic breast cancer	I	NCT04756505
	With vaccines HPV vaccine	Human papilloma virus-associated cancers	I/II	NCT04432597
	CXCR1/2 inhibitor CEA/MUC-1 cancer vaccine	Advanced solid tumours	I/II	NCT04574583
	Brachyury vaccine	Advanced breast cancer	I	NCT04296942
SHR-1701	Monotherapy	Advanced solid tumours	I	NCT03710265 NCT03774979 NCT04324814
		Advanced head and neck SCC	II	NCT04650633
	With chemotherapy	Stage III NSCLC	II	NCT04580498
		Pancreatic cancer	I/II	NCT04624217
		Advanced nasopharyngeal carcinoma	I	NCT04282070
	And with anti-VEGF	Metastatic CRC	II/III	NCT04856787
	With radiation	Second-line metastatic NSCLC	II	NCT04560244
	With anti-VEGF (BP102)	Advanced solid tumours	I/II	NCT04856774
	With TKIs (famitinib)	Advanced solid tumours	I/II	NCT04679038
		Advanced NSCLC	II	NCT04699968
	With histone methyl-transferase inhibitor	Adv. solid tumours and B cell lymphomas	I/II	NCT04407741
GS-1423	With chemotherapy	Adv. solid tumours	I	NCT03954704
Gemogenovatucel-T	Monotherapy	Advanced solid tumours		NCT03842865
	With chemotherapy	Ewing's sarcoma	III	NCT03495921
	With anti-PDL1 (atezolizumab)	Adv. gynecological cancers	II	NCT03073525

FIGURE LEGENDS

Figure 1. TGFβ functions in healthy tissues and during cancer progression. a. In healthy epithelial tissues and during the early stages of tumourigenesis, TGFβ signalling regulates homeostatic growth and imposes a cytostatic program to premalignant epithelial cells that suppresses tumour progression. In advanced cancers, tumour cells rewire the TGFβ pathway to avoid the cytostatic response. TGFβ then promotes epithelial-to-mesenchymal transition (EMT). Many prometastatic genes are also under the control of TGFβ in late-stage cancer. In some cancer types, tumour cells often acquire loss of function mutations in TGFβ pathway components, mainly in

SMAD4 and *TGFBR2*. These tumour cells are blind to the action of the cytokine and can expand in a TGFβ-rich TME. Furthermore, during cancer dissemination, TGFβ can impose cell cycle arrest to tumour cells, which results in a dormant state, a phenomenon associated with metastatic latency, chemotherapy resistance, and disease relapse. **b.** In the immune environment of healthy tissues, particularly in the gastrointestinal tract and the skin, TGFβ is necessary to induce tolerance and regulate responses to harmful antigens of commensal bacteria. In this context, TGFβ is a potent suppressor of inflammation, and its lack triggers an excessive inflammatory response that predisposes to tumour formation. TGFβ is also necessary to regulate wound-healing responses. As cancer progresses, tumours hijack these TGFβ functions to promote immune evasion and a continuous wound-healing response.

Figure 2. TGFβ production, release, and signalling. a. Cells deploy different mechanisms to release active TGFβ from latent deposits. When the TGFβ molecule is either covalently linked to LTBPs and tethered to the ECM, or localized to the cell surface-bound to GARP, integrin-transmitted tension generated by cell contraction releases active TGFβ. Extracellular proteases can also cleave the LAP domain. **b.** The active TGFβ dimer triggers TGFBR1 and TGFBR2 dimerization. In this ligand-induced receptor complex, TGFBR2 phosphorylates TGFBR1, which in turn recognizes and phosphorylates SMAD2 and SMAD3 proteins, the cytoplasmatic mediators of TGFβ signalling. Phosphorylated SMAD2 and SMAD3 interact with SMAD4 to form a trimeric complex that travels to the nucleus. Dimers of phosphorylated SMAD2 and SMAD3 together with SMAD4 form complexes with different signal-driven transcription factors (SDTFs) and lineage-determining transcription factors (LDTFs) to regulate transcription of target genes. Several cofactors (CoFs) are also recruited to these transcriptional complexes. The stability of the nuclear SMAD complex is negatively controlled by poly (ADP-ribose) polymerase (PARP)-mediated PARylation, which causes SMAD dissociation from DNA. **c**. The main non-canonical TGFβ pathway involves TRAF6 and TAK1 signals in combination with SMAD7, and activates downstream kinases in the JNK, p38 and NF-κB pathways independently of SMAD-driven transcription. Other non-canonical pathways that activate mTOR, RHOA or KRAS signalling downstream of TGFBR receptors are also detailed.

Figure 3. Regulation of TME cell types by TGFβ in advanced cancer. Schematic summary of the effects of TGFβ in innate immunity, where it drives pro-tumourigenic cell polarization and impinges on NK cell cytotoxicity (a, b). Adaptive anti-tumour immunity is abrogated by TGFβ by DC dysfunction and reduced antigen presentation (c), Treg- and Th17-skewed CD4+ T cell polarization (d), and dysfunctional cytotoxic CD8+ T cell responses (e). Furthermore, TGFβ is a key regulator of myofibroblast-like CAF specification and inhibits the formation of inflammatory CAFs (iCAFs) and interferon-licensed CAFs (ilCAFs) (f).

BOX 1 – TGFβ isoform-specific functions and therapies.

Despite binding the same receptors, TGFβ1, TGFβ2, and TGFβ3 isoforms exhibit distinct expression patterns and their bioavailability is differentially regulated. In particular, the TGFβ2 latency-associated peptide (LAP) domain lacks the RGD motif present in TGFβ1 and TGFβ3. As a consequence, latent TGFβ2 (L-TGFβ2) is not activated by αvβ6 or αvβ8 integrins[292], implying the existence of specific mechanisms to release this isoform. Indeed, *Tgfb1*, *Tgfb2*, and *Tgfb3* knockout mice show non-overlapping developmental defects. Global knockout of the *Tgfb1* gene results in multifocal inflammatory disease owing to an important role of this isoform in setting immune tolerance[293,294]. *Tgfb2* null mice exhibit a range of developmental abnormalities including heart, lung, craniofacial, limb, spinal column, eye, inner ear

and urogenital defects[295]. *Tgfb3* knockout mice develop cleft-palate[296,297]. The role of each isoform in the adult organism has been less well characterized as most pharmacological and genetic approaches used to investigate TGFβ functions during tissue homeostasis and disease disrupt signals from all three isoforms. Correlative analyses indicate that TGFβ1 shows higher and more widespread upregulation in the tumour microenvironment than the other two isoforms and is more robustly associated with failure of immune checkpoint inhibitor responses in cancer patients[50]. In turn, TGFβ3 plays specific roles during wound healing and fibrosis[298] and is highly upregulated in cancer-associated fibroblasts[299]. A role for TGFβ2 in breast cancer cell dormancy has been demonstrated[300]. In addition, TGFβ2 has been associated with neutrophil recruitment in models of metastatic colorectal cancer [86]. Of particular interest is the finding that adult *Tgfb2* haploinsufficient mice phenocopy patients with germline loss-of-function mutations in the *TGFB2* gene, which develop thoracic aortic aneurysm dissections and other cardiovascular abnormalities[301]. This pathology is similar to that observed in preclinical models treated with pan-TGFβ inhibitors[50,211–213], suggesting that toxicities associated with these drugs are due to TGFβ2 inhibition in the cardiovascular system. These findings inspired the development of therapeutic antibodies that target specifically the TGFβ1 isoform and that avoid the cardiotoxicity associated with pan-TGFβ approaches in experimental models[50]. TGFβ ligand traps able to preferentially block TGFβ1 and TGFβ3 have also been engineered[275,276], and integrin-targeted strategies are also selective for these two isoforms (Table 1).

BOX 2. Linking TGFβ signalling in CAFs to immunotherapy responses.

In colorectal cancer (CRC), cancer-associated fibroblast (CAF) abundance and elevated expression of fibroblast TGFβ response signature (F-TBRS), which includes primarily genes encoding for extracellular matrix (ECM) proteins and cytokines induced by TGFβ, predict risk of relapse after therapy and metastasis robustly[299,302]. It was also found that upregulation of a similar F-TBRS identified urothelial cancer patients exhibiting poor therapeutic responses to anti-PDL1 therapy in a clinical trial[236]. ECM-encoding genes induced by TGFβ also predict lack of responses to immune checkpoint inhibitors (ICIs)[238]. Subsequent studies characterized CAF heterogeneity, and its association with response to ICIs [303–306]. These studies revealed the existence of two major CAF subsets: one exhibits an ECM-producing and contractile (α-smooth muscle actin (αSMA+)) phenotype enforced by TGFβ, whereas the other upregulates proinflammatory mediators such as IL-6[304–306] (Figure 3f). The abundance of the fibrogenic TGFβ-activated CAF subset associates with a poor response to anti-PDL1 therapy in clinical trials and experimental models[76,236,304,305]. Emerging evidence also suggest an essential role for TGFβ signalling in shaping CAF heterogeneity (Figure 3f). Whereas IL-1 promotes the acquisition of a inflammatory program in CAFs (iCAF) of pancreatic cancer, TGFβ suppresses IL-1 receptor expression in this population and impedes their specification[306]. Besides, an IFN-gamma licensed CAF (iICAF) population emerges upon TGFβ blockade in mouse tumour models[304] (Figure 3f). This subset expresses MHC molecules and other factors involved in antigen processing and presentation, implying an immunomodulatory role[304].

CRCs, urothelial carcinomas, and possibly other tumour types that exhibit elevated levels of TGFβ-driven CAF gene expression program are immune-excluded and insensitive to ICI immunotherapy[76,236,238,239]. Using human-like mouse models of CRC, we showed that treatment with a TGFBR1 inhibitor enables T cell infiltration and renders metastases susceptible to anti-PDL1 therapy[76]. Another study demonstrated that treatment with a pan-TGFβ antibody prevents T cell exclusion and enhances responses to anti-PDL1 treatment in tumour models characterized by TGFβ-activity in

CAFs[236]. The synergism between TGFβ inhibition and ICIs was subsequently corroborated in multiple mouse cancer models and experimental settings[148,155,229,230,233,235,237,304]. It however remains unclear to what extent CAFs are the culprits of immune evasion and anti-PD1 or anti-PDL1 therapy failure in these models.

Figure 1

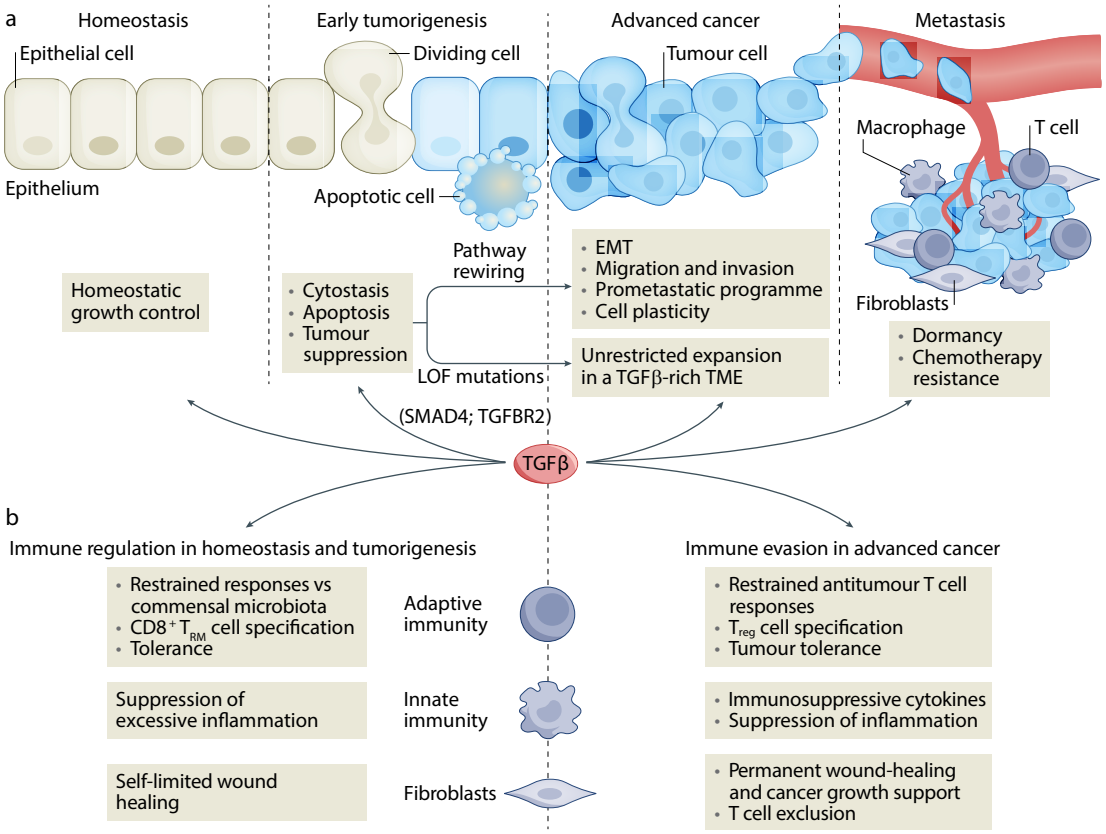

a
Homeostasis
Early tumorigenesis
Advanced cancer
Metastasis
Epithelial cell
Dividing cell
Tumour cell
Epithelium
Apoptotic cell
Macrophage
T cell
Fibroblasts
Pathway rewiring
Homeostatic growth control
Cytostasis
Apoptosis
Tumour suppression
LOF mutations
EMT
Migration and invasion
Prometastatic programme
Cell plasticity
Unrestricted expansion in a TGFβ-rich TME
Dormancy
Chemotherapy resistance
(SMAD4; TGFBR2)
TGFβ
b
Immune regulation in homeostasis and tumorigenesis
Immune evasion in advanced cancer
Restrained responses vs commensal microbiota
CD8+ TRM cell specification
Tolerance
Adaptive immunity
Restrained antitumour T cell responses
Treg cell specification
Tumour tolerance
Suppression of excessive inflammation
Innate immunity
Immunosuppressive cytokines
Suppression of inflammation
Self-limited wound healing
Fibroblasts
Permanent wound-healing and cancer growth support
T cell exclusion

Figure 2

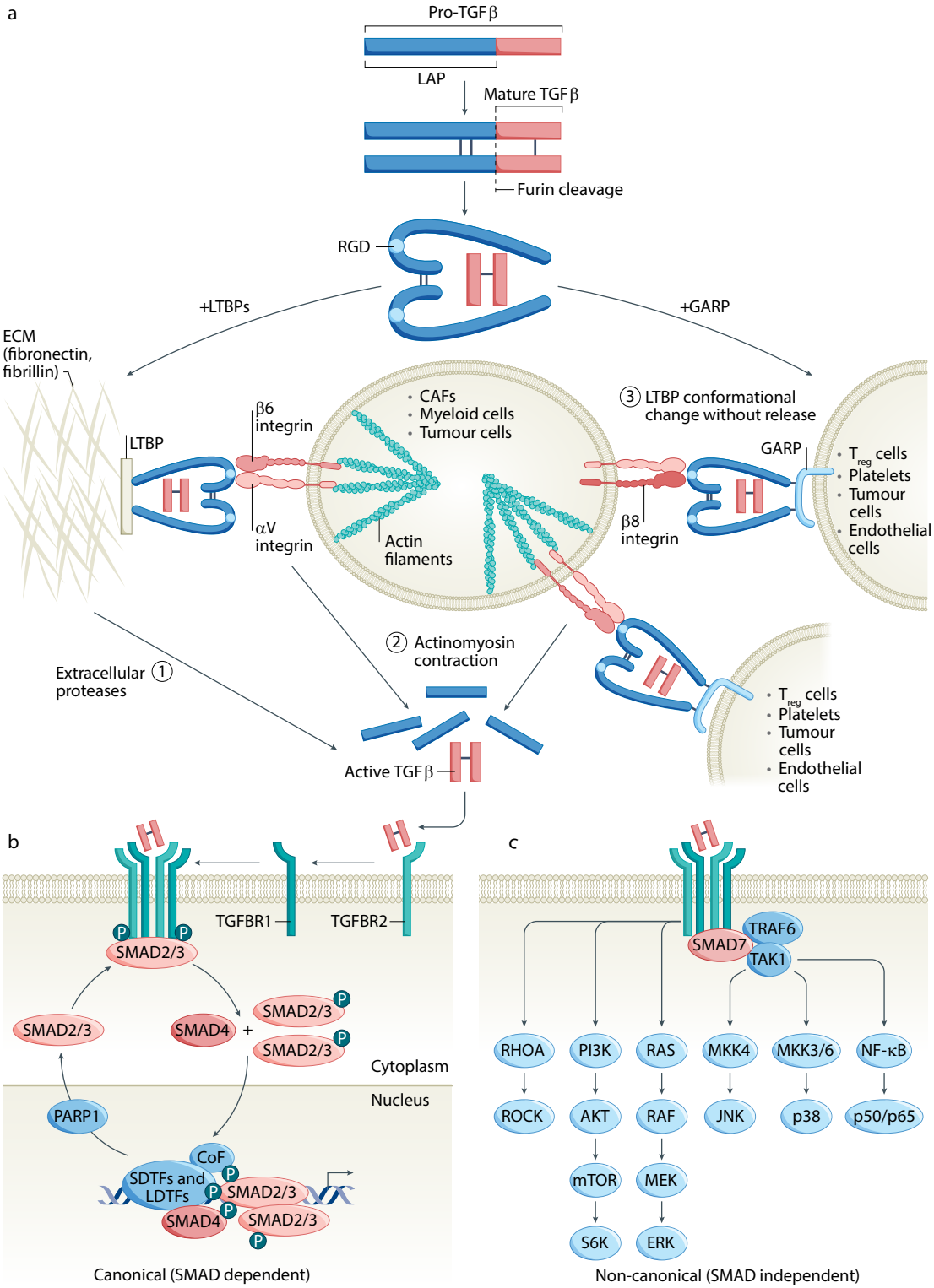
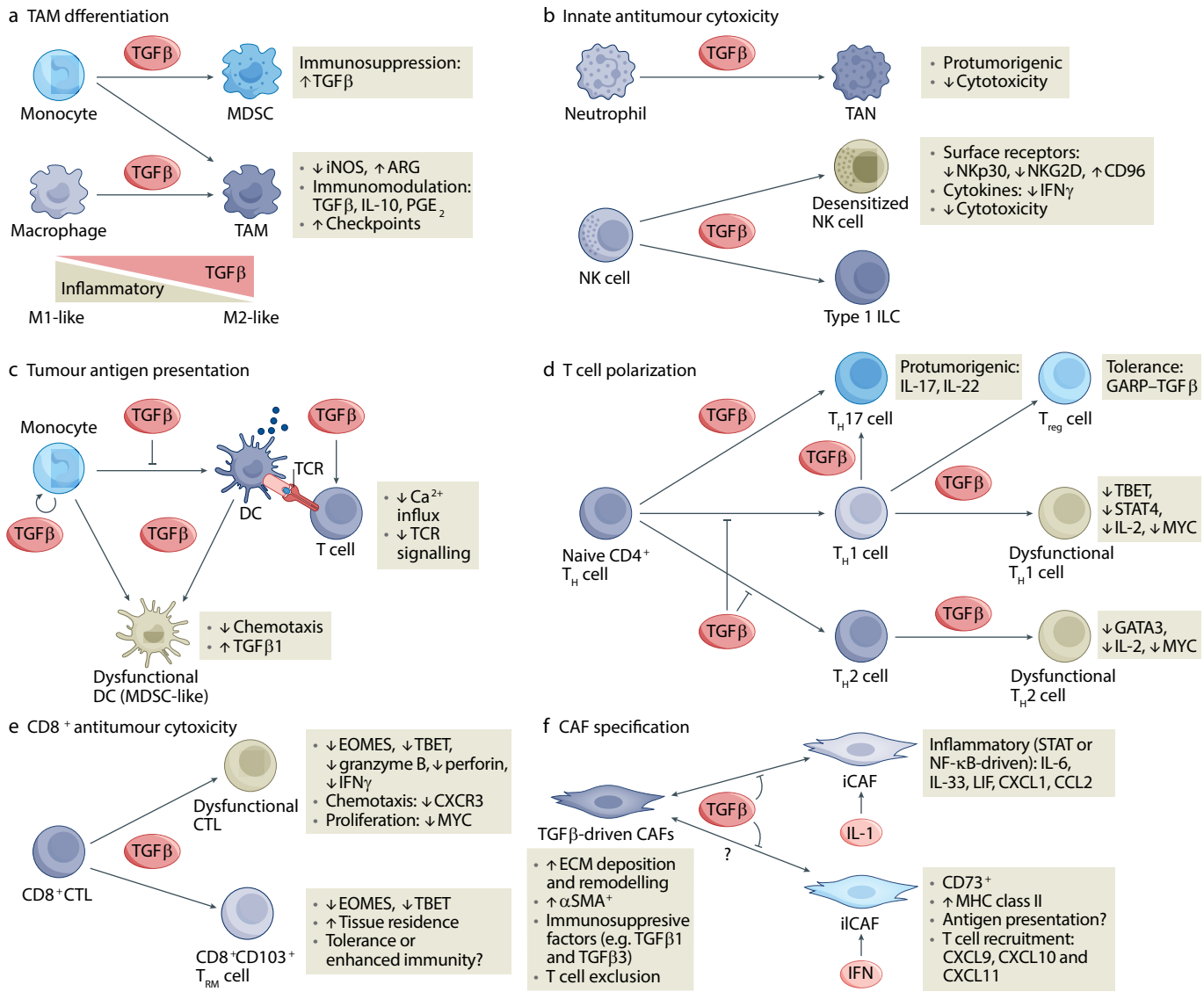

a
Pro-TGFβ
LAP
Mature TGFβ
Furin cleavage
RGD
+LTBPs
+GARP
ECM
(fibronectin,
fibrillin)
LTBP
β6
integrin
αV
integrin
• CAFs
• Myeloid cells
• Tumour cells
Actin
filaments
③ LTBP conformational
change without release
GARP
β8
integrin
• Treg cells
• Platelets
• Tumour
cells
• Endothelial
cells
② Actinomyosin
contraction
Extracellular ①
proteases
Active TGFβ
• Treg cells
• Platelets
• Tumour
cells
• Endothelial
cells
b
TGFBR1
TGFBR2
SMAD2/3
SMAD2/3
SMAD4 +
SMAD2/3
SMAD2/3
Cytoplasm
Nucleus
PARP1
CoF
SDTFs and
LDTFs
SMAD2/3
SMAD4
SMAD2/3
Canonical (SMAD dependent)
c
SMAD7
TRAF6
TAK1
RHOA
PI3K
RAS
MKK4
MKK3/6
NF-κB
ROCK
AKT
RAF
JNK
p38
p50/p65
mTOR
MEK
S6K
ERK
Non-canonical (SMAD independent)

Figure 3


a TAM dfferentiation
TGFβ
Monocyte
MDSC
Immunosuppression: ↑TGFβ
TGFβ
Macrophage
TAM
• ↓iNOS, ↑ARG
• Immunomodulation: TGFβ, IL-10, PGE2
• ↑Checkpoints
TGFβ
Inflammatory
M1-like
M2-like
b Innate antitumour cytoxicity
TGFβ
Neutrophil
TAN
• Protumorigenic
• ↓Cytotoxicity
Desensitized NK cell
• Surface receptors: ↓NKp30, ↓NKG2D, ↑CD96
• Cytokines: ↓IFNγ
• ↓Cytotoxicity
TGFβ
NK cell
Type 1 ILC
c Tumour antigen presentation
Monocyte
TGFβ
TGFβ
TCR
DC
T cell
TGFβ
TGFβ
• ↓Ca2+ influx
• ↓TCR signalling
• ↓Chemotaxis
• ↑TGFβ1
Dysfunctional DC (MDSC-like)
d T cell polarization
TGFβ
TH17 cell
Protumorigenic: IL-17, IL-22
Treg cell
Tolerance: GARP–TGFβ
TGFβ
TGFβ
Naive CD4+ TH cell
TH1 cell
↓TBET, ↓STAT4, ↓IL-2, ↓MYC
Dysfunctional TH1 cell
TGFβ
TGFβ
TH2 cell
↓GATA3, ↓IL-2, ↓MYC
Dysfunctional TH2 cell
e CD8+ antitumour cytoxicity
Dysfunctional CTL
• ↓EOMES, ↓TBET, ↓granzyme B, ↓perforin, ↓IFNγ
• Chemotaxis: ↓CXCR3
• Proliferation: ↓MYC
TGFβ
CD8+CTL
CD8+CD103+ TRM cell
• ↓EOMES, ↓TBET
• ↑Tissue residence
• Tolerance or enhanced immunity?
f CAF specification
iCAF
Inflammatory (STAT or NF-κB-driven): IL-6, IL-33, LIF, CXCL1, CCL2
TGFβ
TGFβ-driven CAFs
IL-1
?
• ↑ECM deposition and remodelling
• ↑αSMA+
• Immunosuppresive factors (e.g. TGFβ1 and TGFβ3)
• T cell exclusion
ilCAF
• CD73+
• ↑MHC class II
• Antigen presentation?
• T cell recruitment: CXCL9, CXCL10 and CXCL11
IFN