Therapeutic targeting of TGF-β in cancer: hacking a master switch of immune suppression

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Review Article

Therapeutic targeting of TGF-β in cancer: hacking a master switch of immune suppression

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Cancers may escape elimination by the host immune system by rewiring the tumour microenvironment towards an immune suppressive state. Transforming growth factor-β (TGF-β) is a secreted multifunctional cytokine that strongly regulates the activity of immune cells while, in parallel, can promote malignant features such as cancer cell invasion and migration, angiogenesis, and the emergence of cancer-associated fibroblasts. TGF-β is abundantly expressed in cancers and, most often, its abundance associated with poor clinical outcomes. Immunotherapeutic strategies, particularly T cell checkpoint blockade therapies, so far, only produce clinical benefit in a minority of cancer patients. The inhibition of TGF-β activity is a promising approach to increase the efficacy of T cell checkpoint blockade therapies. In this review, we briefly outline the immunoregulatory functions of TGF-β in physiological and malignant contexts. We then deliberate on how the therapeutic targeting of TGF-β may lead to a broadened applicability and success of state-of-the-art immunotherapies.

Introduction

Transforming growth factor-β (TGF-β) comprises a large family of structurally related and multifunctional cytokines that include the TGF-β1, -β2, and -β3 isoforms; activins and bone morphogenetic proteins (BMPs) are also considered to be part of the TGF-β family but are not the focus of this review. These secreted dimeric proteins assume crucial roles in embryonic development, in tissue repair, and in homeostasis of the skeleto-muscular, cardiovascular, nervous, endocrine, and immune systems [1,2]. Genetic studies in mice and humans have revealed that perturbation of TGF-β activity can lead to a large variety of developmental disorders as well as pathologies. For example, a majority of *Tgfb1*-knockout mice die just after birth due to profound multifocal inflammatory disease [3], and mutations in the *TGFB1* gene have been linked to Camurati–Engelmann disease and inflammatory bowel disease [3–5].

TGF-β signals by inducing heteromeric complexes of selective cell surface TGF-β type I and type II receptors, i.e. TGFβRI and TGFβRII [6,7]. Upon heteromeric complex formation, the constitutively active TGFβRII kinase phosphorylates TGFβRI, leading to its activation and phosphorylation of downstream effector proteins (Figure 1A). Receptor regulated SMADs, i.e. SMAD2 and SMAD3, can be recruited to activated TGFβRI, and become phosphorylated at two serine residues, at their carboxy-termini. Activated SMAD2 and SMAD3 can form heteromeric complexes with SMAD4 that translocate to the nucleus where, together with co-activators and co-repressors, they regulate the expression of target genes. The affinity of SMAD proteins for DNA is weak, and interaction of other DNA-binding transcription factors is needed for efficient gene regulation [8,9]. These co-factors are subjected to regulation by extracellular and intracellular cues, which contributes to the highly context and tissue-dependent effects of TGF-β [10] (Figure 1A).

Each step of the pathway is stringently regulated. An important control mechanism is that TGF-β is secreted in a latent form where the amino terminal remnant of TGF-β precursor protein (also termed

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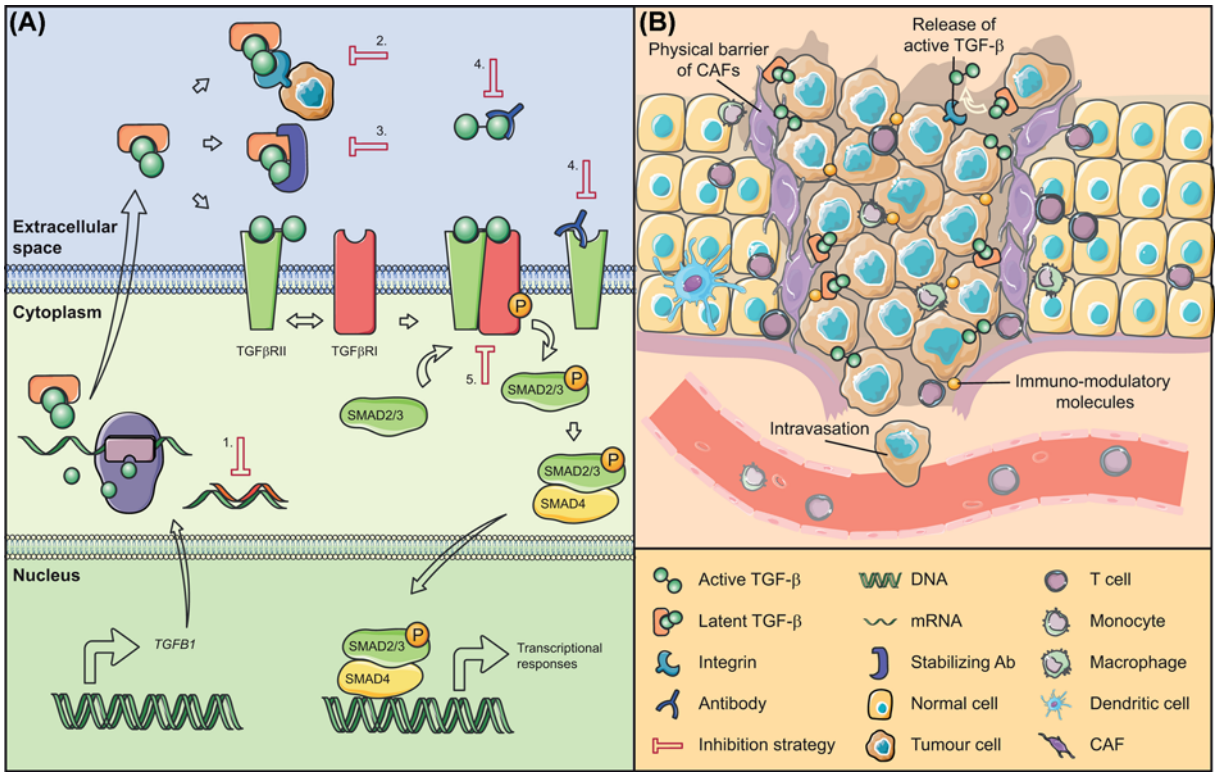

Figure 1. Targeting TGF-β, a pleotropic pathway with effects on cancer cells and tumour microenvironment

(**A**) TGF-β is secreted by cells in an inactive form in which the latency associated peptide (orange) is wrapped around the mature TGF-β (green), preventing it from binding to cell surface receptors. Latent TGF-β can be activated by integrins or metalloproteases, among other mechanisms. Once activated, TGF-β binds initially to TGFβRII and thereafter recruits TGFβRI, thereby forming a heteromeric or heterotetrametric (not drawn) complex. Upon ligand-induced complex formation, TGFβRII kinase phosphorylates TGFβRI, which propagates the signal into the cell by phosphorylating SMAD2/3 molecules. Activated SMAD2/3 partner with SMAD4, translocate into the nucleus, where this complex can interact with DNA in a sequence-specific manner and regulate transcriptional responses. The TGF-β signalling pathway can be targeted at several levels indicated by the red symbols: 1 – Transcription/translation of TGF-β genes with siRNAs or antisense oligonucleotides; 2 – Release of active TGF-β via integrins; 3 – Release of active TGF-β from LAP; 4 –TGF-β ligands and TGF-β receptor binding; 5 –TGFβRI kinase activity. (**B**) Schematic overview of the effects of TGF-β on the tumour microenvironment (TME). (I) Latent TGF-β is present in high amounts in the TME and, when locally activated by i.e. integrins or metalloproteases. TGF-β can affect cells locally. (II) TGF-β induces the activation of tumour supporting cancer-associated fibroblasts (CAFs), which create a physical barrier around the TME that hampers the influx of immune cells. Moreover, CAFs produce high amounts of TGF-β themselves. (III) Immune-modulatory molecules that further enhance the immunosuppressive milieu are being upregulated by tumour and resident immune cells (i.e. PD-L1 and IDO, respectively) and being secreted (i.e. arginase). (IV) High amounts of TGF-β increase the tumour cellular motility leading to an invasive phenotype contributing to metastasis.

latency associated protein (LAP)) is wrapped around the active carboxy domain, shielding it from receptor binding [11]. LAP cooperates with the latent TGF-β binding protein (LTBP) (or related proteins) at the extracellular matrix (ECM), or with GARP, a cell surface docking receptor that mediates cell surface display of the latent complex [11]. Activation of latent TGF-β can be achieved via proteases that cleave the LAP portion of the complex or via integrins that upon mechanical forces can dissociate LAP from functional TGF-β. An inhibitory SMAD, SMAD7, antagonizes TGF-β/SMAD signalling by competing with SMAD2/SMAD3 for TGFβRI binding and by recruiting E3 ubiquitin SMURF ligases and, thereby, targeting TGFβRI for proteasomal degradation [12,13]. SMAD7 is transcriptionally induced by TGF-β, and thereby constitutes an important negative feedback mechanism of TGF-β activity [12]. Finally, TGF-β bioavailability is controlled by axillary receptors and soluble ligand-binding proteins that can mediate the interaction of TGF-β with its receptors [14].

TGF-β has been proposed to have a biphasic role during cancer progression [15]. At early stages of oncogenesis, it acts as tumour suppressor by mediating growth arrest but, at advanced stages, it can act as tumour promotor by supporting invasion and metastasis of cancer cells while its cytostatic effects are blocked by the rewiring of its signalling during malignant transformation [16,17]. Importantly, TGF-β also exerts profound effects on other cells that compose the tumour microenvironment (TME) as it promotes angiogenesis, the emergence of cancer-associated fibroblasts, and thwarts anti-cancer immunity [18,19]. This latter aspect of TGF-β activity will be the focus of this review.

We start with a discussion on the immunoregulatory functions of TGF-β in the development of the immune system and inflammatory processes, followed by a description of its role in the TME. Finally, we describe how state-of-the-art immunotherapies can synergize with the therapeutic targeting of TGF-β signalling, albeit the challenging nature of TGF-β as a therapeutic target.

The physiological role of TGF-β in the development of the immune system

The immune system is vital in conferring protection from pathogens [20]. In vertebrates, it is comprised by a myriad of cell types and molecules that cooperate during immune defence processes. On one hand, the immune system has evolved to react robustly against external insults, on the other hand, it ensures a swift resolution of inflammatory responses and, importantly, the discrimination between self and non-self antigens [21–23]. It thereby prevents abnormal or exacerbated responses like the ones observed in autoimmunity [20]. TGF-β is essential for the development of the immune system and during inflammatory processes, but is also responsible for dampening ongoing immune responses [24]. Its relevance in immunity is best highlighted by the fact that most immune cells are capable of producing TGF-β and potently respond to its effects.

Hematopoietic stem cells reside in the bone marrow where they give rise to myeloid and lymphoid progenitors that can further differentiate into all immune cell types of their respective lineages [25]. The generation of hematopoietic stem cells requires Tgf-β signalling at embryonic stages [26] and, at later stages of development, Tgf-β can have a systemic impact on the immune system by regulating hematopoietic stem cell fate. High levels of TGF-β keep hematopoietic stem cells in a quiescent state that ensures a permanent pool of stem cells throughout life [27,28]. Furthermore, fluctuations in TGF-β levels can push differentiation processes towards particular immune cell subsets [28]: for example, high TGF-β1 levels favour megakaryopoiesis over the generation of other myeloid descendants [29]. Of note, most research thus far has been performed on the effects mediated by the TGF-β1 isoform while the complete scope of TGF-β activity on the development of the immune system remains to be fully revealed.

TGF-β in innate immunity

In most vertebrates, the immune system can be subdivided into two main compartments: the innate and the adaptive immune system. The innate immune system is composed by both myeloid cells including monocytes, macrophages, and dendritic cells (DCs), granulocytes as well as innate lymphocytes, most notoriously natural killer (NK) cells. These subsets provide the first line of defence against pathogens as they can engage threats in a non-specific manner [30]. Furthermore, subsets such as DCs play an essential role in bridging innate and adaptive immune responses. NK cell development and differentiation is strongly influenced by TGF-β as it was demonstrated that activation of the TGF-β pathway counteracts the development of mature NK cells from its progenitors and, thereby, can increase susceptibility of hosts to viral infections [31,32] (Figure 2). Moreover, the effector functions of NK cells are also impaired in presence of TGF-β as their cytolytic activity is inhibited via decreased degranulation as well as diminished production of granzyme B, perforin, and interferon (IFN)-γ [33]. In myeloid cells, TGF-β stimulates the survival of monocytes via increased expression of transcription factors RUNX1 and SOX4 and tumour necrosis factor ligand TNFSF14 that, together, inhibit apoptosis and support cell survival [34]. Alveolar macrophages and Langerhans cells require Tgf-β for their differentiation as well as for the maintenance of mature cell pools where autocrine signalling plays a major role [35,36]. Other macrophage subsets seem to be less dependent on the effects of Tgf-β activity during their developmental phase [35]. Nevertheless, TGF-β does impact their phagocytic activity, namely, through the down-regulation of CD36 and class A scavenger receptors [37,38]. DCs combine their capacity to phagocyte extracellular material with a special status as professional antigen presenting cells. They are essential for triggering adaptive immune responses through (cross-) presentation of antigens and provision of co-stimulatory signals (e.g. CD80/86) that are essential for T-cell activation [39,40]. The maturation and differentiation of DCs is impaired by TGF-β [41] as its presence can result in sub-optimal DC-mediated T-cell activation. More specifically, differentiated DCs were found to have impaired expression of major histocompatibility class (MHC)-II and co-stimulatory molecules upon

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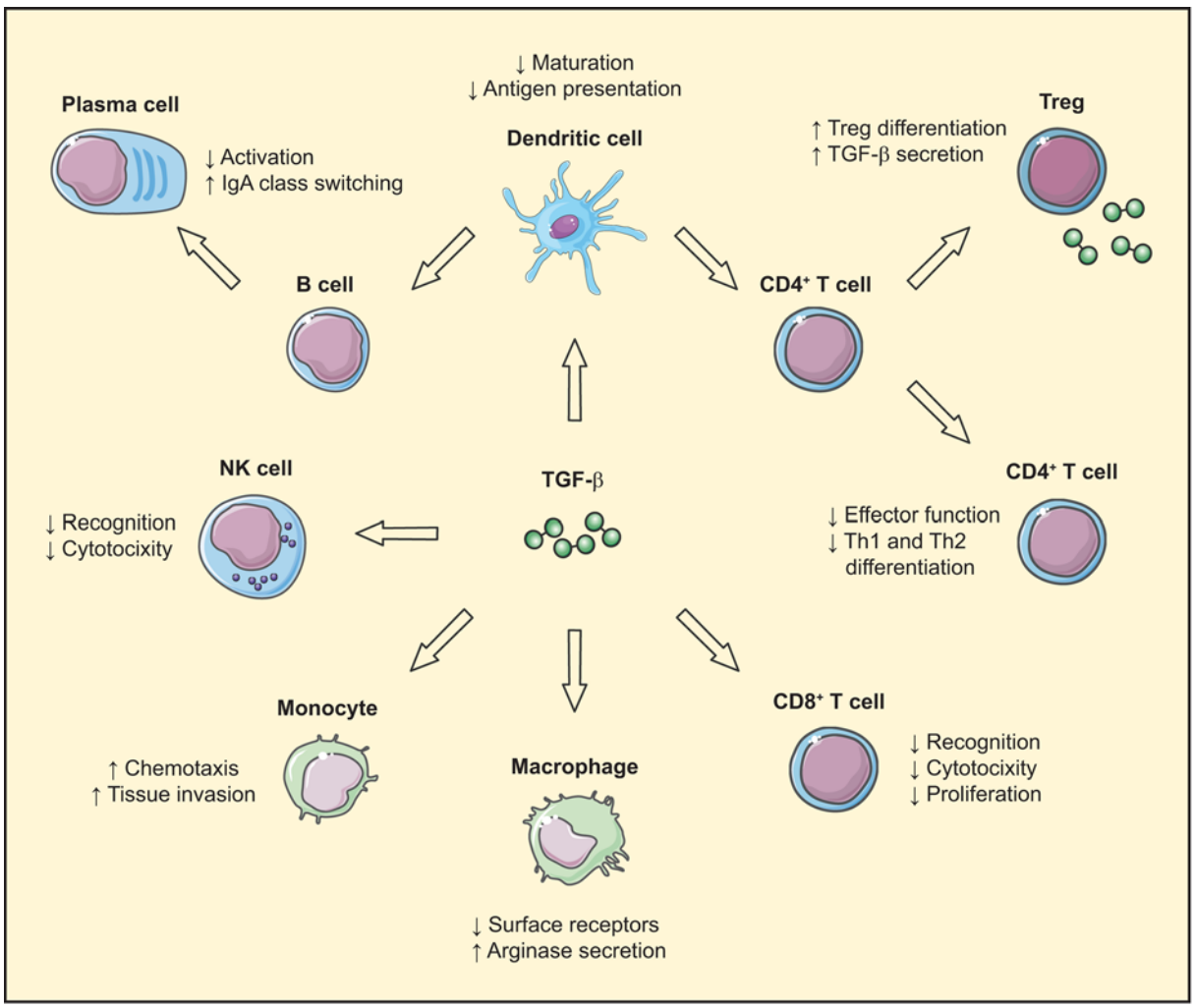

Figure 2. TGF-β effects on immune cell subsets

TGF-β regulates proliferation, activation, and differentiation of immune cells. More specifically, the recognition of target cells and cytotoxic effector functions of NK cells are inhibited by TGF-β. TGF-β mediates the recruitment of monocytes and impairs the expression of cell surface receptors in macrophages. Macrophages and neutrophils secrete immune suppressive molecules instead of inflammatory compounds (i.e. iNOS and ROS) in response to TGF-β. The maturation and antigen presentation capacity of dendritic cells is decreased upon challenge with TGF-β, which subsequently influences the stimulation of B and T cells. B-cell activation is diminished and class switching to most immunoglobulin (Ig) isotypes is hampered. $CD4^+$ T cells are driven by TGF-β to differentiate into Tregs instead of the Th1 or Th2 phenotypes. Features of $CD8^+$ T cells like target recognition, cytotoxicity, and proliferation are all thwarted by TGF-β.

stimulation with lipopolysaccharides and TGF-β, resulting in impaired antigen processing and presentation [42–44]. Furthermore, DCs with such an immature phenotype displayed immune suppressive features conferred by the production of Indoleamine-pyrrole 2,3-dioxygenase (Ido)1 and Tgf-β [45,46].

TGF-β in adaptive immunity

T cells together with B cells compose the adaptive immune system, which is responsible for the generation of antigen-specific responses and the establishment of immunological memory. T cells develop in the thymus, where positive selection of T cells that can recognize MHC–peptide complexes takes place, while cells that exhibit reactivity towards autologous antigens are deleted (negative selection) [47]. Tgf-β is constitutively expressed in the thymus and forms an essential factor during negative selection as it leads to increased expression of pro-apoptotic Bcl2-like protein Bim, specifically in autoreactive T cells, resulting in apoptosis via caspase-3 [48]. In addition, up-regulation

of chemokine receptor Cxcr3, in the absence of Tgf-β, results in aberrant localization of T cells in the thymus and escape from negative selection as they avoid interaction with medullary thymic epithelial cells [48,49]. Furthermore, the maturation of medullary thymic epithelial cells is also impaired in the absence of Tgf-β which further supports the accumulation of autoreactive T cells [48].

T cells are composed of several subsets, including $CD8^+$ cytotoxic lymphocytes and $CD4^+$ T cells that can be further divided into Th1, Th2, Th17, and regulatory T cell (Tregs) subsets [50]; TGF-β exerts distinct effects on all these populations due to the combinatorial activity of other signalling pathways that are differentially active in those subsets [51]. Naïve $CD4^+$ T cells are steered by TGF-β to differentiate into Tregs through up-regulation of the FOXP3 transcription factor and subsequent down-regulation of the TGF-β signalling inhibitor Smad7, thereby increasing the susceptibility of these cells for TGF-β pathway activation [52–54]. Also, differentiation of naïve T cells into the Th17 phenotype is induced by Tgf-β1 in the presence of IL-6 [55]. Th17 cells play a role in the defence against pathogens at mucosal barriers and their strong pro-inflammatory features can facilitate tumorigenesis [56]. In opposition, the differentiation of helper phenotypes, Th1 and Th2, which support cell-mediated and humoral immune responses is inhibited by TGF-β activity through decreased transcriptional activity of TBX21 (T-bet) and GATA3, respectively [57–59]. In line with the suppression of Th1 responses, activated $CD8^+$ T cells do not acquire cytotoxic features in presence of high levels of Tgf-β [60]; IFN-γ, granzyme A and B, perforin, and Fas ligand expression are all down-regulated by this cytokine [61–64]. The decrease in IFN-γ production by $CD4^+$ T cells was found to be mediated by inhibition of mitochondrial respiration via phosphorylation of SMAD2/3 proteins [65].

B cells develop in the bone marrow and undergo selection based on the specificity of their immunoglobulin receptor to warrant central tolerance (negative selection) and antigen affinity (positive selection) [66]. The whole process of differentiation and maturation from pre-B cell to plasma cell is under control of TGF-β [67,68]. At secondary lymph nodes, and upon antigen recognition via the B-cell receptor, naïve B cells undergo somatic hypermutation accompanied by clonal selection, and class switch recombination of the immunoglobulin locus further differentiating into memory B cells or plasma cells. Class switching replaces the heavy chain constant region of the immunoglobulin locus so that IgG, IgA, and IgE antibodies can be produced. High concentrations of TGF-β inhibit switching to most IgG isotypes, while switching to IgA isotypes and secretion of IgA by plasma cells is promoted by TGF-β in a SMAD-dependent manner [67–69]. Immunoglobulins of the IgA isotype are involved in the primary immune response against pathogens, whereas IgG plays a fundamental role in the generation of adaptive and memory immune responses. IgG isotypes can induce antibody-dependent cellular cytotoxicity (ADCC) [70], and antigen-bound IgG and IgM molecules can activate the complement system [70]. Consequently, switching the balance towards the IgA isotype generally results in less efficient cytotoxic immune responses.

TGF-β as master regulator of tumour microenvironment

In homeostasis, a delicate balance controls the pleiotropic functions of TGF-β but this balance is often disrupted in cancers. TGF-β generally functions as a tumour suppressor gene; inhibiting proliferation, regulating differentiation and, eventually, promoting apoptosis. Thereby, the presence of TGF-β in the TME can be a strong selective agent for the clonal outgrowth of cancer cells that acquire (epi) genetic changes that disrupt or rewire the TGF-β pathway. Moreover, cancer cells can become abundant sources of TGF-β and thereby, impose its inhibitory effects on other cells of the TME while benefiting themselves from malignant properties provided by this cytokine such as an increased invasive and metastatic potential. Unsurprisingly, genetic alterations affecting members of the TGF-β pathway can be observed in approximately 40% of cancers [71]. Mutations in genes encoding TGF-β signalling components, including *TGFBR2* and *SMAD4*, are particularly common in gastrointestinal malignancies, which might relate to the important role that inflammation plays in the aetiology of gastrointestinal tumours [71].

Immune cells within the tumour microenvironment

The effects of TGF-β on the development of the immune system are somewhat recapitulated in the TME, in particular its immune suppressive effects (Figure 1B). TGF-β acts as a chemo-attractant for monocytes [72] and induces the expression of integrins in these cells with affinity to collagen type IV, laminin and fibronectin thereby supporting their embedding in the ECM of tumours [73,74]. TGF-β further stimulates the secretion of matrix metalloproteinases that digest surrounding tissues thereby facilitating the entrance of immune cells like monocytes into tumours [74]. There, TGF-β drives the differentiation of monocytes into macrophages and, from that moment onwards, the role of TGF-β switches from pro-inflammatory to immune suppressive on this myeloid subset. Macrophages, as well as neutrophils, were found to adopt pro-tumorigenic phenotypes in the TME under TGF-β control [34,75]. These pro-tumorigenic

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macrophages and neutrophils secrete immune suppressive molecules like Arginase 1 that replace molecules like inducible nitric oxide synthase (iNOS) or reactive oxygen species (ROS), which are produced by their pro-inflammatory counterparts [34,75]. The increased Arginase levels result in L-arginine depletion and, consequently, decreased expression of CD3ζ. The latter thwarts T-cell activation through decreased T-cell receptor (TCR) expression, thereby supporting immune escape [76,77].

In addition to the previously discussed impact of TGF-β on lymphocyte development and activity, it has recently been described that Tgf-β inhibits anti-cancer activity of $CD4^+$ T cells in the TME of breast cancer in mice [78,79]. Conditional deletion of *TgfbrII*, specifically in $CD4^+$ T cells, stops cancer progression via increased levels of IL-4 that are secreted by $CD4^+$ Th2 cells. The IL-4 levels induce remodelling of the vasculature which subsequently results in hypoxia at avascular areas leading to cancer cell death [78]. Similar effects were observed after treatment with a bispecific antibody, 4T-Trap, targeting CD4 and TgfβrII [79]. The anti-tumour efficacy of NK cells is also hampered by TGF-β. Engagement of cancer cells by NK cells is diminished due to decreased expression of natural cytotoxicity receptor NKp30, NK glycoprotein DNAM-1 and Killer Cell Lectin Like Receptor (NKG2D) in the presence of TGF-β [80,81]. This, together with the fact that in a TGF-β-rich environment $CD8^+$ T cells may be improperly primed and have reduced cytotoxic activity, differentiation of $CD4^+$ T cells is swung towards a regulatory phenotype, and IgG production is inhibited, all favours immune escape in cancers.

Remodelling the extracellular matrix

Beyond cancer and immune cells, there are other important players to account in the TME such as fibroblasts and vessels but also the ECM where cells are embedded and which is essentially composed of collagen fibres, proteoglycans and other proteins. A pan-cancer analysis of ECM-coding genes revealed the dysregulation of 58 (30 up-regulated and 28 down-regulated) genes in cancers in comparison with healthy tissues [82]. Genes that were up-regulated, i.e. matrix metalloproteinases and collagens, positively correlated with high mutation burden and neoantigen availability and elevated expression of immune suppressive molecules (IDO1, B7-H3, PD-L2) [82]. This observation suggests that the remodelling of the ECM in cancers may also constitute a mechanism of immune evasion [83–85]. Interestingly, it was shown that this dysregulated ECM signature was largely derived from transcriptional profiles of cancer-associated fibroblasts (CAFs), and that the most prominent molecular driver of their activity was TGF-β and its associated molecules [82]. It is now known that CAFs actively remodel the ECM, thereby creating a physical barrier that impairs immune cell infiltration [86]. TGF-β induces the formation of CAFs not only from fibroblasts, but also from endothelial cells and mesenchymal stem cells [87–89], thereby creating a self-enabling response connecting TGF-β availability, malignant features, and immune suppression. Not surprisingly, an abundance of fibroblasts in cancer, and particularly CAFs, is often related to advanced disease and predicts poor patient prognosis [90,91].

Integrins as gatekeepers of TGF-β availability

Communication between ECM components and cells is largely mediated by integrins; transmembrane receptors that mediate signalling and control processes of cell survival, proliferation, and others, based on the availability of extracellular ligands. αVβ6 and αVβ8 are two integrins that are potent activators of latent TGF-β, which is abundantly present in the ECM [92,93]. By locally releasing active TGF-β they play a role in the bioavailability and, consequently, the immunosuppressive character of the TME. Both integrins are exclusively expressed on epithelial cells [93,94] and release active TGF-β from LAP in cooperation with metalloproteases. Furthermore, αVβ6 can liberate active TGF-β in a force-dependent manner through contraction of the actin cytoskeleton [94]. A similar process can be mediated by αVβ8 in order to release TGF-β from the adaptor protein GARP [95]. GARP is mainly expressed by Tregs and platelets but also aberrantly expressed in several cancers [96]. Accordingly, increased GARP surface expression on tumour cells was found to be correlated with worse overall survival for patients of several tumour types [96]. The potential of Garp as a therapeutic target was illustrated in xenogeneic NSG mice models, which lack mature T, B, and NK cells. Treatment with anti-Garp antibodies decreased the immunosuppressive function of Tregs in a graft-versus-host setting by diminishing the availability of active Tgf-β in the TME [97]. GARP forms, in our opinion, a promising treatment target to diminish the release of active TGF-β in the TME although studies on GARP are still in pre-clinical phase. Minimizing the amount of active TGF-β would break the feed-forward loop that promotes the differentiation to CAFs and the subsequent production of more integrins, collagen and fibronectin as well as other cytokines [98]. Following the same line of thought, a phase I clinical trial was initiated to diminish the release of active TGF-β making use of an αVβ8 antagonist (Table 1; NCT04152018). Finally, integrin αE(CD103)β7 is up-regulated in T cells by TGF-β and is an important molecule to mount anti-tumour immune responses as it supports T-cell effector functions

Table 1. Ongoing interventional clinical trials that combine immunotherapeutic strategies with the targeting of TGF-β

Therapy class	TGF-β/ Immunotherapy component	Additional treatment	Cancer type	Phase	Clinical Trial ID	Status
Autologous tumour vaccin	Vigil; Atezolizumab (aPD-L1)		Gynecological cancers	2	NCT03073525	Active, not recruiting
Autologous tumour vaccin	Vigil		Variety of solid cancers	1	NCT01061840	Completed
Autologous tumour vaccin	Vigil		Colorectal cancer	2	NCT01505166	Terminated
Autologous tumour vaccin	TGF-β2 Antisense-GMCSF		Variety of solid cancers	1	NCT00684294	Terminated
Autologous tumour vaccin	Lucanix		Non-small cell lung cancer	2	NCT01058785	Completed
Autologous tumour vaccin	Vigil; Durvalumab (aPD-L1)		Breast and gynecological cancers	2	NCT02725489	Active, not recruiting
Autologous tumour vaccin	Vigil		Melanoma	2	NCT01453361	Terminated
Fusion protein	aPD-L1/TGFβRII M7824		HER2+ breast cancer	1	NCT03620201	Recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Brachyury-TRICOM; Entinostat; Ado-trastuzumab emtansine	Triple negative and HER2+ breast cancer	1	NCT04296942	Not yet recruiting
Fusion protein	aPD-L1/TGFβRII M7824; M9241 (IL-12)	M9241; Radiation	Non-prostate genitourinary cancers	1	NCT04235777	Not yet recruiting
Fusion protein	aPD-L1/TGFβRII M7824; TriAd vaccine	N803	Head and neck squamous cell cancer	1/2	NCT04247282	Not yet recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Topotecan; Temozolomide	Small cell lung cancer	1/2	NCT03554473	Recruiting
Fusion protein	aPD-L1/TGFβRII M7824		Colorectal cancer and non-colorectal MSI-H cancers	1/2	NCT03436563	Recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Eribulin Mesylate	Triple negative breast cancer	1	NCT03579472	Recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Radiation	Hormone positive, HER2- breast cancer	1	NCT03524170	Recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Radiation	Head and neck squamous cell cancer	1/2	NCT04220775	Not yet recruiting
Fusion protein	aPD-L1/TGFβRII M7824	Gemcitabine; Cisplatin	Biliary tract cancer	2/3	NCT04066491	Recruiting
Cellular therapy	GPC3/TGF-β targeting CAR-T cells		GPC3+ hepatocellular cancer	1	NCT03198546	Recruiting
Cellular therapy	TGF-β resistant HER2/EBV-CTLs		HER2+ solid cancers	1	NCT00889954	Completed
Cellular therapy	HPV Specific CTLs; Nivolumab (aPD-1)	Fludarabine; Cytoxan	HPV+ cancers	1	NCT02379520	Recruiting
Cellular therapy	Aldesleukin; TGF-β.DNRII-transduced Autologous TIL	NGFR-transduced CTLs; Cyclophosphamide; Fludarabine	Melanoma	1	NCT01955460	Recruiting
Cellular therapy	DNR.NPC-specific CTLs	Cyclophosphamide; Fludarabine	EBV+ nasopharyngeal cancer	1	NCT02065362	Active, not recruiting
Cellular therapy	TGF-β resistant LMP-specific CTLs		Lymphoma	1	NCT00368082	Active, not recruiting
Kinase inhibitor	Vactosertib; Durvalumab (aPD-L1)		Urothelial cancer	2	NCT04064190	Not yet recruiting
Monoclonal antibody	NIS793; PDR001 (aPD-1)		Variety of solid cancers	1	NCT02947165	Recruiting
Monoclonal antibody	LY3200882 (TGFβRI); LY3300054 (aPD-L1)	Gemcitabine; Nab-Paclitaxel; Cisplatin; Radiation	Variety of solid cancers	1	NCT02937272	Active, not recruiting
Small molecule inhibitor	Galunisertib; Durvalumab (aPD-L1)		Pancreatic cancer	1	NCT02734160	Completed
Antagonist	PF-06940434; PF-06801591 (aPD-1)		Variety of solid cancers	1	NCT04152018	Recruiting

CTL, cytotoxic T lymphocyte; TIL, tumour infiltrating lymphocytes; GPC3, Glypican 3; HER2, human epidermal growth factor receptor 2; HPV, Human Papilloma Virus; EBV, Ebstein Barr Virus.

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and enables them with tumour residency features as it is a receptor for E-cadherin which is expressed on epithelial cancer cells [99,100].

TGF-β pathway in the age of checkpoint blockade

The field of immuno-oncology received a major boost in the last decades with the identification of immune checkpoints in T cells and their targeting with therapeutic antibodies [101,102]. In particular, CTLA-4, PD-1 and PD-L1 targeting have formed the basis for checkpoint blockade therapies that provided impressive results, particularly in cancers with a strong immunogenic profile [103–106]. Although the overall response rate to checkpoint blockade is still low, the induced responses can be long-lasting and have remarkably improved cancer patient survival, especially the ones affected by cancers that present with high mutation burden (e.g., lung cancer and melanoma). In advanced melanoma, response rates to checkpoint blockade immunotherapies can be as high as 50% depending on therapeutic regimens (single agent vs. combination therapies) and previous treatment history [107,108]. It is of utmost importance to further understand the biological basis of checkpoint blockade response to identify patients that are most likely to benefit from these interventions in order to avoid fruitless treatment cycles and unnecessary exposure to side-effects. In addition, the understanding of factors determining resistance to treatment in non-responsive patients would stimulate the development of alternative treatment strategies that could sensitize patients to immunotherapeutic interventions.

Checkpoint blocking antibodies prevent receptor–ligand binding of inhibitory molecules, thereby blocking the transmission of inhibitory signals that induce an anergic state and dysfunctionality in T cells [109,110]. Recently, the Fc receptor of checkpoint blocking antibodies was also proposed to play an important role through the induction of antibody-dependent cellular cytotoxicity (ADCC) via macrophages and NK cells, and complement activation [111–113]. This composes an additional route of efficacy for IgG1 anti-CTLA-4 and anti-PD-L1 antibodies. Whereas binding of the Fc receptor from anti-CTLA-4 and anti-PD-L1 antibodies results in enhanced anti-tumour efficacy by, respectively, depleting Tregs and destroying cancer cells [111,112,114], the opposite effect is observed with anti-Pd-1 antibodies where the elimination of Pd-1$^+$ T cells and NK cells diminishes its anti-tumour efficacy [114,115].

Inhibiting the TGF-β signalling pathway

A number of strategies to interfere with the activation of TGF-β signalling in cancer have been proposed (Figure 1A). Their successes and limitations have been reviewed elsewhere [116,117]. In short, available therapeutic agents can either target the bioavailability of TGF-β, ligand–receptor interactions, the kinase domain of TGF-β receptors or other signalling molecules. They are available in the form of neutralizing antibodies, ligand traps, small molecule inhibitors or antisense oligonucleotides. However, thus far, all these different treatment approaches did not surpass phase II clinical trials due to severe adverse events or lack of clinical benefit. Toxicities associated with TGF-β targeting agents comprise cardiac toxicity, including inflammatory, degenerative and haemorrhagic lesions in the heart valves, and skin lesions such as cutaneous squamous-cell carcinomas, basal cell carcinomas, eruptive keratoacanthomas, and hyperkeratosis [116]. The type of toxicities observed is highly dependent on the therapeutic agent employed to target TGF-β: ligand traps and antibodies are more likely to induce skin toxicity, whereas small molecule kinase inhibitors are associated with cardiovascular toxicity. Of note, and considering the role of TGF-β as tumour suppressor, it will be important to understand whether TGF-β-targeting treatments can increase the risk for secondary malignancies in addition to skin neoplasias. In the next sections, we will discuss pre-clinical combination therapies followed by the ongoing interventional clinical trials that combine immunotherapy with TGF-β targeting and are registered at clinicaltrials.gov (Table 1).

Combined targeting of TGF-β and immune checkpoint molecules

A conspicuous TGF-β-associated transcriptional signature can discriminate specific molecular subtypes in several cancers [118–120]; in colorectal cancers, transcriptomic profiling revealed four consensus molecular subtypes (CMS), where CMS4 is characterized by prominent mesenchymal features that are driven by TGF-β activation. This subtype is also associated with worst clinical prognosis [119,121,122]. Patients diagnosed with CMS4 colorectal cancers were shown to carry neoantigen-reactive T cells despite the fact that these patients do not benefit from checkpoint blockade in an advanced setting [123,124]. In addition, these tumours display some hallmarks of ongoing anti-tumour immunity and inflammatory processes, indicating a certain degree of immunogenicity that might be counterbalanced by TGF-β [123]. In mice models that recapitulate the CMS4 molecular subtype, exclusion of CD4$^+$ and CD8$^+$ T cells could be explained by the presence of high Tgf-β levels that were produced by CAFs and other cells of the

TME. Monotherapy treatment with the TgfβrI small molecule inhibitor galunisertib increased both $CD4^+$ Th1 responses and cytolytic activity of the $CD8^+$ T cells, resulting in decreased tumour volume and reduced metastasis formation [125]. Moreover, the combination of galunisertib with anti-Pd-l1 enhanced the number of therapy responders remarkably compared with either monotherapy [125]. Melanoma and breast cancer mice models also showed increased immunity and survival benefits with galunisertib monotherapy or combined with checkpoint blockade antibodies [126,127]. Tgf-β-induced immune cell exclusion was also observed in an urothelial mice model, where non-responders to anti-Pd-l1 agent atezolizumab showed high Tgf-β signalling activity in CAFs [128]. Again, addition of anti-Tgf-β enhanced T-cell infiltration into the tumour core and induced tumour regression [128].

In a squamous cell carcinoma model a synergistic effect of a pan-Tgf-β neutralizing antibody and anti-Pd-1 therapy was observed and resulted in a higher influx of cytotoxic and helper T cells, accompanied by a decrease in the proportion of Tregs, compared with anti-Pd-1 monotherapy [129]. Recently, an antibody was developed, termed SRK-181, that interferes with Tgf-β signalling by keeping Tgf-β1 in a latent form [130]. It interacts with LAP and inhibits activation of TGF-β1 in all latent LTBP- and GARP-containing complexes. Combinatorial treatment with SRK-181 and an anti-Pd-1 antibody, in mice with syngeneic tumours that are resistant to anti-Pd-1 treatment, led to decreased tumour growth and improved survival with an increase in intratumoral cytotoxic T cells and a decrease in immunosuppressive myeloid cells. Importantly, no (cardio)toxicity was observed, which could be due to the specific targeting of the TGF-β1 isoform; TGF-β2 and TGF-β3 have been shown to have important functions in the cardiovascular system in humans [131–134]. Also, an antibody that targets Garp and Tgf-β1 in a colon carcinoma mouse model was found to increase the anti-tumour efficacy of anti-Pd-1 therapy [135]. This observation was explained by immune-related effects that included increased activation of $CD8^+$ T cells and inhibition of TGF-β-mediated, Treg immune suppressive activity [135]. Also, thrombin was found to mediate the release of active Tgf-β by cleaving Garp from platelets, resulting in systemic activation of latent Tgf-β in tumour bearing mice. Consequently, the inhibition of thrombin resulted in enhanced efficacy of checkpoint blockade therapy in murine breast and colon cancer models [136].

Clinical exploitation of the synergistic potential of TGF-β inhibitors with immunotherapies

Galunisertib is a thoroughly studied small molecule, TGFβRI kinase inhibitor. Pre-clinical mouse models showed promising treatment effects but, at the same time, cardiac toxicity [137,138]. An intermittent treatment schedule, or so-called 'drug holiday', was found to minimize toxicity in mice and humans [139,140]. The importance of the treatment schedule on the clinical outcome [127] was further illustrated by the enhanced efficacy of anti-Ctla-4 antibody during concurrent galunisertib treatment in mice with melanoma, while the efficacy of anti-Pd-(l)1 antibodies was only apparent when galunisertib was initiated 3 weeks after the start of anti-Pd-1 treatment [127]. Nevertheless, the first clinical studies on galunisertib, performed with glioma patients, did not result in improved overall survival [141,142], possibly due to the combined use of chemotherapeutic agent lomustine, which frequently results in leukopenia. Subsequent studies, however, performed in advanced hepatocellular carcinoma showed overall survival benefits [143]. Currently, a phase I dose-escalation study is being conducted exploring the combined use of galunisertib and durvalumab, an anti-PD-L1 antibody (NCT02734160). This therapeutic strategy seems to stand on a good rationale as T cells in galunisertib-treated tumours were found to acquire high Pd-1 expression while tumour-associated macrophages frequently expressed Pd-l1 [125]. Another TGFβRI kinase inhibitor, vactosertib, was already found to be safe and is now being tested for its effectiveness in combination with durvalumab for urothelial cancers that previously failed to obtain a complete response upon checkpoint blockade therapy alone (NCT04064190).

Dual-targeting of molecules, in close physical proximity, can be achieved by employing fusion proteins that combine different antibody domains or ligand traps. Combining either anti-Ctla-4 or anti-Pd-l1 antibodies with a TgfβrII receptor domain that traps soluble Tgf-β resulted in decreased Tregs in the TME of melanoma and breast cancer, in mice, and reduced tumour growth in comparison with checkpoint blockade therapy alone [144]. In other studies, the fusion protein bintrafusp alfa that combines anti-Pd-l1 with a TgfβrII receptor domain slowed tumour growth and decreased metastasis formation in murine breast and colon cancer models, and led to increased anti-tumour activity of innate and adaptive immune cell compartments [145,146]. The TgfβrII module was able to efficiently trap the Tgf-β1, -2 and -3 isoforms [145]. In murine breast and lung cancer models, treatment with bintrafusp alfa was combined with small molecule inhibitors targeting Cxcr1/2; the latter blocked interleukin 8 (IL-8) activity and thereby prevented the acquisition of mesenchymal properties by cancer cells [147]. This combination improved anti-tumour efficacy of either monotherapy, leading to decreased tumour volumes and increased infiltration of T and NK cells in the TME [147]. These promising findings resulted in the advancement of bintrafusp alfa to clinical trials. Dose

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escalation studies (phase I) showed manageable safety profiles while some clinical efficacy seemed to be present at the administered dosage [148–154]. Currently, a number of phase I and phase I/II trials are ongoing that investigate combinatorial effects of bintrafusp alfa with radiotherapy and chemotherapeutics, among other treatments. Exploring the effect of bintrafusp alfa as monotherapy on the immune infiltrate is one of the main goals of NCT03620201. Phase II/III study NCT04066491 combining the chemotherapeutics cisplatin and gemcitabine with bintrafusp alfa in locally advanced or metastatic biliary tract cancer is currently the most advanced clinical trial in regard of this therapeutic drug.

Vaccination strategies

Therapeutic cancer vaccines aim at boosting immune responses against cancer antigens and, consequently, enhance immune infiltration in tumours and clearance of cancer cells. Vaccines exist in the form of DNA, RNA, protein or cells, and are generally accompanied by adjuvants that promote an inflammatory response. Intranasal vaccination with the B subunit of Shiga toxin showed that tissue-resident memory T cells, which express among others integrin CD103, were found to be positively correlated with tumour regression and enhanced treatment efficacy in an orthotopic mouse model of head and neck cancer [155]. However, the use of a Tgf-β-neutralizing antibody, following vaccination, blocked CD103 expression and T cell differentiation into a tissue-resident, memory phenotype and thereby diminished vaccination efficacy, which indicates that Tgf-β can also exert positive effects in cancer immunity [155]. Another study that combined mRNA-based vaccines targeting tumour-associated antigens demonstrated the merits of a combination scheme involving immune checkpoint blockade, IL-6 and Tgf-β inhibition in murine lung cancer and melanoma models [156]. Therefore, more studies will be needed to understand the combinatorial effect of TGF-β in different contexts. Also, the use of different treatment schedules might contribute to the differences observed between studies. The use of irradiated autologous tumour cells as vaccination strategy is an interesting approach because it combines the availability of numerous antigens with the possibility to modulate the expression of specific proteins by employing for instance shRNAs or protein-coding plasmids. Phase I and II clinical trials in Ewing sarcoma and advanced ovarian cancer patients showed the safety of Vigil, an autologous tumour vaccine with increased GM-CSF expression and dampening of TGF-β1 and -β2 signalling due to decreased furin expression by shRNA. Low levels of furin subsequently prevent cleavage of latent TGF-β to the active form [157,158]. The detection of increased IFN-γ response of peripheral blood lymphocytes to autologous tumour material, after Vigil vaccination, indicates that this approach is valid for the generation of anti-tumour immune responses [159]. Currently, a crossover study is being performed on patients with ovarian cancer where anti-PD-L1 or Vigil vaccine is given followed by the combination of both, in comparison with anti-PD-L1-alone in the control group. Resulting data will indicate whether the Vigil vaccine, in a combination of pre-treatment setting yields enhanced efficacy of checkpoint blockade therapy (NCT03073525). The applicability of Vigil as a treatment option will soon be investigated on various solid tumours (NCT03842865).

Cellular therapies

How to 'force' T cells to infiltrate tumour tissues, in particular in the context of cellular therapies, remains a challenge; poor vascularization and physical barriers provided by tumour stroma, low nutrient levels, the presence of immunosuppressive immune cells and expression of inhibitory molecules are some of the factors that are likely playing a major role [160]. Such barriers are often regulated by TGF-β and strongly compromise the efficacy of cellular therapies that make use of engineered TCR therapies or chimeric antigen receptor (CAR) T cells [161]. TCR therapies exploit 'natural' T cell receptors while CAR T cells express a chimeric receptor that is able to recognize cell-surface antigens in a MHC-independent manner. Further genetic manipulation of T cells is being explored to minimize or eliminate their sensitivity to TGF-β signalling.

Clinical trials making use of TCRs are all, at the moment, in phase I. Most studies target a tumour-associated (e.g., Her2 or lymphoma antigen) or virus-derived (e.g., EBV or HPV) antigen that is predetermined to occur in the diagnosed cancers. The autologous or allogeneic antigen-specific T cells have been manipulated to circumvent TGF-β signalling by, for instance, transduction of a dominant-negative TGFβRII domain. In a small group of eight lymphoma patients such cellular therapy was found to be safe and without severe adverse effects at 5 years post-infusion [162]. Several clinical studies with a similar setup using different tumour types and targets are in recruitment or follow-up phase (Table 1). One trial with melanoma patients uses a diverse population of tumour infiltrating lymphocytes (TIL) which were genetically manipulated to lose TGF-β sensitivity through transduction of a dominant-negative TGF-β receptor (NCT01955460). This strategy using TIL is particularly interesting for the treatment of cancers where targetable antigens are unknown or when antigen expression is demonstrated to be heterogeneous.

CAR T cell treatments have produced clinical benefit mainly in haematological diseases where CD19 is a commonly targeted antigen [163,164]. However, the successful application of CAR T cells for the treatment of solid tumours is thus far limited, partially due to the lack of cancer-specific targetable antigens and poor infiltration of CAR T cells into tissues. Recently, TgfβrII-knockout CAR T cells were found to improve tumour elimination in a murine squamous cell lung carcinoma model and a patient-derived xenograft pancreatic model [165]. The TgfβrII-knockout CAR T cells also showed an increased number of memory subsets compared with the conventional CAR T cells [165].

Oncolytic virus therapy

Oncolytic viruses, which specifically replicate in transformed cells, are an interesting approach to simultaneously provide targetable antigens and inflammatory signals that boost anti-tumour immune responses. Importantly, oncolytic viruses can be specifically designed to deliver additional therapeutic or immunostimulatory compounds at the cancer site. Preliminary studies making use of murine models support the notion that combination of oncolytic viruses with other treatment forms is a valid approach to produce clinical benefit. The combination of mesothelin-targeting CAR T cells with a Tgf-β targeting adenoviral vector significantly increased anti-tumour immune responses in a breast cancer model where mesothelin is overexpressed [166]. Groeneveldt and colleagues have discussed the applicability of oncolytic viruses in combination with TGF-β targeting more extensively in a review [167].

Precautions for combination therapies

Achieving synergy with different therapeutic strategies is the ultimate aim of many studies, but also concerns difficulties with potentially counteracting downstream effects of the therapeutic interventions and concurrent lack of clinical efficacy or undesirable side-effects. Moreover, promising treatment results in pre-clinical studies cannot easily be translated to the human setting, especially when it concerns such pleiotropic molecules as TGF-β. Side-effects of checkpoint blockade therapies mainly result from the interrupted peripheral tolerance of T cells, frequently affecting the gastrointestinal tract, skin, endocrine system, lungs, nervous system, and musculoskeletal tissue [168]. In many cases, these side-effects can be diminished by use of immunosuppressive drugs, but in severe cases treatment has to be discontinued.

In this review, we discussed several studies that make use of mice models that reported satisfactory clinical efficacy of combination therapies without severe side-effects. Clinical trials however have not surpassed phase II yet due to treatment-induced toxicities and the pleiotropic character of TGF-β that hampers predictability of (combination) treatments that target all TGF-β-induced responses. TGF-β controls a variety of cellular processes and specific aspects of its activity can be selectively targeted when we zoom in and unravel the pathway in more detail. Using bispecific antibodies to target TGF-β signalling in certain cell types (CAFs, immune cells), or targeting transcription co-factors or pathway modulators could be a way to avoid the toxic effects while achieving clinical benefit [117]. The toxicity profiles of targeted molecules are, accordingly, expected to result in less unwanted side-effects than the pan-TGF-β antibodies that were used in the past [112]. Of note, the systemic impact of TGF-β therapeutic inhibition on the immune system is still barely investigated, while such data could be helpful in designing strategies to prevent side-effects while achieving optimal anti-cancer efficacy. A study in patients with metastatic breast cancer patients, for example, revealed that upon treatment with radiotherapy and fresolimumab (an anti-TGF-β antibody) the number of peripheral blood mononuclear cells per millilitre increased and more central memory $CD8^+$ T cells were found in circulation at the expense of effector $CD8^+$ T cells [169].

Cancer-specific features, different checkpoint blockade molecules, the diversity of TGF-β isoforms and TGF-β-interfering strategies, as well as timing and therapy schedules are all factors to be taken into account when considering the combination of immunotherapy with anti-TGF-β therapies. Thus far, a consensus has not yet been achieved on the best approach to explore this potentially powerful combination. Overall, we observe that TGF-β inhibition seems to lead to an increase in anti-tumour immunity that includes activation of cytotoxic responses and, importantly, increased immune cell infiltration in tumours. This last observation makes TGF-β targeting a particularly attractive strategy for tumours that are generally perceived as immunologically 'cold' or 'immune-excluded'. Since checkpoint blockade therapy, particularly the one targeting the PD-1/PD-L1 axis, appears to heavily rely on the previous occurrence of natural anti-tumour immune responses, some cancer patients could benefit from an initial targeting of the TGF-β pathway (potentially in combination with chemo- or radiotherapy) and only subsequently the targeting of co-inhibitory pathways in T cells. On the other hand, for patients that experienced spontaneous anti-tumour immune responses but where TGF-β is acting as an axis of immune suppression the concomitant targeting of TGF-β and checkpoints might be more logical. In any case, more pre-clinical and clinical data are necessary

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to substantiate these hypotheses and to guide the optimal application of immunotherapy and TGF-β targeting in a combinatorial setting.

Conclusion and perspectives

We described the important role that TGF-β plays in the development and function of the immune system, with a particular focus on the TME. While immunotherapeutic strategies are being extensively studied for their anti-cancer potential, adopting TGF-β-targeting as a combination treatment seems a low hanging fruit, but remains rather challenging due to the pleiotropic character of this molecule. Taking into account the complex biology at play in the TME, one could propose several combinations for optimal synergistic effects. Lessons learned on intermittent drug schedules, combination therapies and patient selection should be considered in the design of combination treatments of TGF-β-targeting with immunotherapy, but also for the interpretation of 'unsuccessful' trials which can concern very potent drugs that were applied in an unfavourable context. Application of certain drug-delivery systems forms another possibility to diminish side-effects because of the improved selective cell targetability and thereby diminished systemic drug levels.

The potential of TGF-β is recognized by many, but broadly applicable treatment regimens have not been established as of to date. Encouraging results have been obtained in first clinical trials that combine TGF-β targeting with immunotherapeutic strategies, supporting the continuation of these investigations, and a number of clinical trials is currently ongoing that can potentially address questions raised here.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; CAF, cancer-associated fibroblast; CAR, chimeric antigen receptor; CMS, consensus molecular subtypes; DC, dendritic cell; ECM, extracellular matrix; IFN, interferon; IL-8, interleukin 8; iNOS, inducible nitric oxide synthase; LAP, latency associated protein; LTBP, latent TGF-β binding protein; MHC, major histocompatibility class; NK, natural killer cell; ROS, reactive oxygen species; TCR, T-cell receptor; TGF-β, transforming growth factor-β; TME, tumour microenvironment.

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