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The polarization of immune cells in the tumour environment by TGFβ

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Abstract

Transforming growth factor-β (TGFβ) is an immunosuppressive cytokine produced by tumour cells and immune cells that can polarize many arms of the immune system. This Review covers the effects of TGFβ on NK cells, dendritic cells, macrophages, neutrophils, $CD8^+$ and $CD4^+$ effector and regulatory cells, and NKT cells in preclinical animal tumour models and in patients with cancer. Collectively, many recent studies favour the idea that blocking TGFβ signalling in the tumour microenvironment enhances antitumour immunity and may be beneficial for cancer therapy. An overview of the current drugs and reagents for inhibiting TGFβ signalling and their phase in clinical development is also provided.

Introduction

As most tumours present self antigens, peripheral tolerance [G] has an important role in contributing to immune evasion by tumours. In addition, the overproduction of immunosuppressive cytokines, including transforming growth factor-β (TGFβ), by tumour cells and tumour-infiltrating lymphocytes also contributes to an immunosuppressive microenvironment. Many studies indicate that TGFβ can promote cancer metastasis through effects on the tumour microenvironment, by enhancing tumour cell invasion and by inhibiting the function of immune cells [1, 2]. These findings have promoted interest in targeting TGFβ and its signalling pathway in patients with cancer. However, such targeting of TGFβ could result in adverse effects in normal tissues, as this pathway is also involved in multiple homeostatic processes (Figure 1). For example, TGFβ can function as a tumour suppressor to prevent tumorogenesis; however, overproduction of TGFβ is frequently associated with tumour metastasis and poor prognosis in patients with cancer (Figure 1). Although the molecular mechanisms behind this dichotomy of TGFβ functions are not fully elucidated, progress has been made in understanding the role of TGFβ in different stages of cancer. This topic has recently been reviewed and is not discussed here [1, 3–5].

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This Review focuses on the tumour-promoting properties of TGFβ, which prevent effective antitumour immune responses once cancer has been established in the host. A successful immune response requires the proper activation and maturation of antigen-presenting cells (APCs) of the innate immune system that present antigen to adaptive immune cells. TGFβ can suppress or alter the activation, maturation and differentiation of both innate and adaptive immune cells, including natural killer (NK) cells, dendritic cells (DCs), macrophages, neutrophils, and $CD4^+$ and $CD8^+$ T cells [6]. A dampened innate immune response leads to poor adaptive immunity, resulting in persistence of the tumour. In addition, TGFβ has an important role in the differentiation and induction of natural and induced regulatory T (T_{Reg}) cells, which also contribute to the tolerizing environment. Furthermore, in the presence of IL-6, TGFβ induces the differentiation of IL-17–producing $CD4^+$ T helper 17 (T_H17) cells and $CD8^+$ cytotoxic T cells; although the role of IL-17-producing cells still remains controversial in tumour biology, given that these cells can exhibit both tumour-promoting and antitumour activities [7]. As we discuss, many recent discoveries have been made towards understanding the biological effects of TGFβ on different immune cells, although multiple areas require further investigation. Finally, there is compelling evidence to support targeting TGFβ with inhibitors to enhance antitumour immunity in patients with cancer.

Effects of TGFβ on innate immune cells

NK cells—NK cells are innate lymphoid cells that have an important role in the antitumour response by recognizing and directly killing tumours and by rapidly producing chemokines and cytokines crucial for this function. For example, interferon-γ (IFNγ) production by NK cells is important for stimulating effector $CD4^+$ T_H1 cells that are required for clearing tumours. TGFβ attenuates IFNγ production by and the lytic activity of NK cells [8, 9]. These might be direct effects of TGFβ or might result indirectly from cell-cell contact between NK cells and regulatory T cells producing this cytokine [10]. In support of a direct effect, TGFβ signalling can suppress IFNγ production through SMAD3, a transcription factor downstream from this pathway, resulting in suppression of T-bet, a transcription factor required for IFNγ production [11].

Targeted killing by NK cells requires stimulating the NK activating receptors NKG2D, NKp46, NKp44 and NKp30 [12]. It has been shown that exogenously administered TGFβ inhibits NKp30 and NKG2D expression, leading to decreased ability of NK cells to kill target cells [13]. TGFβ also decreases expression of NKG2D by NK cells and $CD8^+$ T cells from glioma patients with a high tumour burden [14]. In patients with lung and colorectal cancer, the downmodulation of NKG2D has been associated with increased serum levels of TGFβ [15]. Furthermore, recent studies of isolated NK cells from healthy donors have shown that platelet-derived TGFβ results in downmodulation of NKG2D, causing a decrease in IFNγ production and degranulation functions essential for tumour destruction by these cells [16]. Finally, surface-bound TGFβ on myeloid-derived suppressor cells [G] can inhibit NK cell cytolytic activity in an orthotopic liver cancer model [17]. These observations indicate that TGFβ has immunosuppressive effects on NK cell killing functions in patients with cancer, and therefore might be a target for enhancing NK cell- mediated antitumour immune responses.

Dendritic cells—DCs are APCs that have a major role in the initial activation and subsequent regulation of immune responses [18]. In addition to activating adaptive immunity mediated by T cells, DCs can also activate NK cells [19]. DCs can present antigen in an immunogenic or tolerogenic manner, and so they have an important role in determining the host response to tumours [18, 20]. DC activation involves the upregulation of MHC and costimulatory molecule expression, alteration in motility and the formation of dendrites to

increase the surface area for antigen presentation and interaction with lymphocytes [21]. Non-activated or immature DCs can still present antigen, but in the absence of proper costimulation this results in T cell tolerance [22, 23]. In the presence of immune-inhibitory signals such as IL-10 and/or corticosteroids, DCs can induce tolerance by T cell deletion and/or the activation and induction of T_{Reg} cells [24, 25]. Hence, DCs can induce either immunity or peripheral tolerance and are an essential component of tumour immunity. TGFβ affects DC biology in several ways. TGFβ can immobilize DCs, thereby interfering with their migration and the transport of antigen to draining lymph nodes for presentation to adaptive immune cells, and might also directly induce DC apoptosis [26–28]. Tumour-infiltrating DCs secrete TGFβ and respond to TGFβ, either in an autocrine or paracrine manner, by down-regulating expression of MHC class II molecules, the co-stimulatory molecules CD40, CD80 and CD86, and tumour necrosis factor (TNF), IFNα, IL-12 and CC-chemokine ligand 5 (CCL5) [6, 29]. These immature or tolerogenic DCs promote the formation of T_{Reg} cells that potently inhibit the function of other T cells [30, 31]. Activated DCs are also able to activate both natural and induced T_{Reg} cells [32–35]; interestingly, the capacity of DCs to induce both types of T_{Reg} cell is greatly increased by TGFβ and IL-10 [36, 37]. So, in the context of these immunosuppressive cytokines in the tumour microenvironment, DCs take up tumour cells, become tolerogenic TGFβ-secreting cells, and promote the induction of tumour-specific T_{Reg} cells in both mice and humans [38–41]. Therefore, a growing body of evidence points to the induction of T_{Reg} cells by TGFβ produced by DCs; as T_{Reg} cells are a major obstacle to tumour immunity, this indicates that the TGFβ pathway might be targeted to augment antitumour immunity in patients with cancer.

Macrophages—The mass of most solid tumours is made up of ~50% macrophages, and high levels of tumour- associated macrophages (TAMs) [G] are correlated with poor cancer prognosis [42, 43]. Macrophages are a heterogeneous population and are typically defined as being of an M1 (classically activated) [G] or M2 (alternatively activated) [G] phenotype, similar to the $CD4^+$ T_H1 versus T_H2 cell paradigm. M1 macrophages are induced by IFNγ and other proinflammatory stimuli and are efficient at presenting antigens, producing proinflammatory cytokines, activating T_H1 responses, and in general mediating anti-tumor responses. In contrast, there are a variety of M2 macrophages induced by IL-10, immune complexes, glucocorticoids, IL-4 and IL-13 with various phenotypes that are involved in remodeling and repair of damaged tissue, parasite resistance, immune regulation and/or tumour promotion [44]. Both TAMs and myeloid-derived suppressor cells (MDSCs), which are a heterogeneous population of immature DCs, macrophages, granulocytes, and other myeloid cells in early stages of their differentiation and have properties similar to those that have been described for M2 cells, are involved in cancer progression and metastasis. In skin cancer, TGFβ-mediated recruitment of macrophages into tumours has an important role in immune escape, as it converts a regressing tumour into a progressing tumour [45]. It is suggested that TGFβ-recruited TAMs are highly phagocytic and can compete with DC function, thereby markedly decreasing the ability of DCs to present tumour antigens to the adaptive immune system [45]. TAMs acquire their phenotype by expressing high levels of TGFβ, IL-10, macrophage galactose N-acetyl-galactosamine-specific lectin 1 (MGL1), Dectin-1, CXC-chemokine ligand 10 (CXCL10), CXCL9 and other IFN-responsive genes. By contrast, they produce low levels of IL-12, TNF and nitric oxide synthase 2 (NOS2). Interestingly, this cytokine phenotype is mediated by the inhibitory NF-κB subunit p50 and it is thought that the cytokine milieu of the tumour microenvironment is necessary to maintain the phenotype[46–48]. During tumour progression, TAMs can switch from an M1 to M2 phenotype, which is paralleled by a gradual inhibition of NF-κB activity [49].

Peritoneal macrophages from tumour-bearing hosts produce increased levels of TGFβ, are less differentiated and have deficiencies in inflammatory cytokine production owing to decreased expression of NF-κB and C/EBP transcription factors [50]. In this model, tumour-

derived factors TGFβ and prostaglandin E_2, individually and additively downregulate NF-κB and C/EBP. There is also evidence that tumour-infiltrating MDSCs secrete high levels of TGFβ, which upregulates CD206 (a deactivation marker characteristic of M2 cells) expression in an autocrine manner [51]. It is unclear how TGFβ production is induced in MDSCs and macrophages, but the mechanism might involve IL-13 and glucocorticoids [52, 53]; the induced TGFβ might then contribute to the alternative activation of M2 macrophages by downregulating NF-κB expression.

Neutrophils—Neutrophils are short-lived polymorphonuclear leukocytes with potent antimicrobial and inflammatory capacities. Despite their known function as professional phagocytes, the role of neutrophils in tumour progression has been controversial and has received little attention compared with that of macrophages. Initial studies characterizing the effect of TGFβ on the control of inflammatory responses showed that this factor was a potent chemotactic factor for neutrophils, promoting their migration but not degranulation or activation [6]. Subsequent studies showed that neutrophil migration could also be indirectly affected by TGFβ through regulating the expression of adhesion molecules in the endothelium [54, 55], and that TGFβ could inhibit neutrophil cytotoxicity, suggesting that TGFβ might influence human neutrophil activity *in vivo*[56]. Recently, the contradictory role of neutrophils in both tumour suppression and tumour promotion either by directly or indirectly controlling tumour growth, angiogenesis and metastasis (reviewed in [57]) was reevaluated in terms of the characterization of different types of tumour associated neutrophil (TAN) with polarized N1 or N2 phenotypes [58]. These polarized populations are similar to those that have been described for macrophages; they are influenced by the microenvironment and seem to be controlled by TGFβ in the tumour proximity. N2 cells are characterized by an expression profile that promotes tumour angiogenesis and metastasis [59–61], and inhibits the antitumour immune response by the secretion of reactive oxygen species (ROS). ROS normally act as potent microbicidal agents but in the context of the tumour microenvironment, ROS could lead to oxidative damage and inhibition of T cell function [62]. Depletion of this neutrophil subpopulation in untreated tumour-bearing mice was sufficient to inhibit tumour growth, even when $CD8^+$ T cells were absent; highlighting the immunosuppressive potential of N2 cells [58–60]. Under TGFβ-inhibiting conditions, as well as in response to certain activation signals, neutrophils acquire an antitumour N1 phenotype that promotes tumour death and inhibits tumour growth [57, 58, 63, 64]. The lack of systemic effects on neutrophil polarization during TGFβ neutralization experiments indicated that the effect is mainly intratumoral, characterized by increased numbers of N1 TANS that express activating chemokines and cytokines as well as by changes in endothelial adhesion molecules expression [58]. Interestingly N1 and N2 neutrophils were shown to control the activation status of $CD8^+$ T cells. This interplay seemed to be reciprocal as activated $CD8^+$ T cells were controlling the activation and migration of neutrophils to the tumour microenvironment as well[65]. Clearly a reevaluation of the role of neutrophils in tumour immunology as well as characterization of this polarized subpopulation by TGFβ may be required to design more effective immunotherapies.

Effects of TGFβ on effector T cells

$CD8^+$ T cells—$CD8^+$ T cells are a crucial component of antitumour immunity, as tumour antigen-specific cytotoxic T lymphocytes (CTLs) have an important role in the cytolytic killing of tumour cells. Several studies have shown a direct correlation between the frequency of CTLs in tumour infiltrating lymphocytes (TILs) and the overall survival of cohorts of patients with cancer; in particular, the high ratio of intratumoral activated cytotoxic $CD8^+$ T cells to Tregs leads to improved prognosis [66–69]. TGFβ signalling in tumour-specific CTLs dampens their function and frequency in the tumour [70], and blocking TGFβ signalling on $CD8^+$ T cells results in more rapid tumour surveillance and the presence

of many more CTLs at the tumour site[71]. Several experimental protocols have been used to render $CD8^+$ T cells unresponsive to TGFβ. In a model where a dominant-negative form of TGFβ receptor II (TGFβRII) is expressed by both $CD4^+$ and $CD8^+$ T cells, a strong antitumour immune response was associated with the proliferation and increased activity of tumour-specific CTLs [72, 73]. Similarly, when tumour-specific $CD8^+$ T cells are rendered unresponsive to TGFβ signalling by transduction with a similar TGFβRII dominant-negative construct before adoptive transfer, these cells infiltrate into tumours, secrete cytokines such as IFNγ and can successfully kill tumour cells [74, 75].

In some tumour models, systemic blockade of TGFβ using a monoclonal antibody or kinase inhibitors to block downstream signalling prevents tumour recurrence by impacting the activity of various cell types, including an increase in the cytotoxic activity of CTLs[76–79]. However, inhibition of TGFβ by using the monoclonal antibody alone is not always sufficient to promote tumour rejections in all animal tumour models. In such models, the combination of a TGFβ-specific antibody with a vaccine resulted in a synergistic improvement in the inhibition of tumour growth that is mediated by increased number and activity of $CD8^+$ T cells [80–82]. It is speculated that in these models, the source of inhibitory TGFβ is the immune system itself and not the tumour, because the antibody-mediated blockade is effective at enhancing antitumour immune responses even when antibody is administered with a prophylactic vaccine before injection of tumour cells[80]. This effect of TGFβ is consistent with the recent finding where TGFβ was shown to be responsible for the apoptosis of short-lived effector T cells that comprise more than 90% of the effector pool after immunization with *Listeria monocytogenes* [83]. TGFβ promotes the apoptosis of these effector T cells by downregulating the expression of BCL-2, which opposes the survival function of IL-15 on the short-lived effector population [83]. It is possible that blocking TGFβ signalling with the neutralizing antibody during administration of the tumour vaccine inhibits the apoptosis of tumour-specific short-lived effector $CD8^+$ T cells and therefore prevents the termination of expanding CTLs.

TGFβ-mediated inhibition of CTL function during tumour immunity might be through several mechanisms. TGFβ directly inhibits CTL function by suppressing the expression of several cytolytic genes, including the genes encoding granzyme A, granzyme B, IFNγ and Fas ligand [70]. TGFβ also attenuates the effector function of antigen-specific memory $CD8^+$ T cells by inhibiting T-bet expression resulting in inhibition of IFNγ production [84]. TGFβ might also block T cell receptor (TCR) signalling of TILs by upregulating the expression of SPRED1 (sprouty-related, EVH1 domain containing 1), which is an inhibitor of the Ras/MAPK pathway [85]. Interestingly, TGFβ can also influence $CD8^+$ T cell-mediated tumour immunity by inducing IL-17 production by $CD8^+$ T cells, although the effect of IL-17 on tumour growth versus immune surveillance remains controversial [86–89].

$CD4^+$ T cells—$CD4^+$ T cells are central orchestrators of adaptive immunity; however, their role in antitumour immune responses has largely been overlooked, mainly owing to the lack of MHC class II expression by tumour cells. TGFβ has been shown to have effects on all subsets of $CD4^+$ effector T cells controlling the expression of master transcriptional regulators in these cells. TGFβ inhibits T-bet and GATA-3 expression (which determine CTL, T_H1 and T_H2 cell differentiation) while it promotes FOXP3 and Rorγt expression (which determine T_{Reg} and T_H17 cell differentiation) (reviewed in [90]). The role of $CD4^+$ T cells in tumour biology has been classically studied in the context of T_{Reg} cells, which are covered in a separate section of this Review. This section focuses on the specific role of T_H cell subpopulations in the control of antitumour immune responses and how TGFβ within the tumour microenvironment could influence the polarization of each subset.

Early studies trying to understand the mechanism of tumor-induced immunosuppression [91] identified TGFβ as one of the major inhibitors of immune responses in the tumour microenvironment. Tumour-derived TGFβ was shown to inhibit T_H1 responses by shifting infiltrating T cells towards a T_H2 phenotype [92], and hence promoting a less efficient anti-tumor immune response. However, later studies comparing the efficacy of T_H1 and T_H2 effector cell subsets in mediating anticancer responses showed that both T_H1 and T_H2 cells increased the CTL-mediated antitumour response, although T_H1 cells secreting IFNγ seemed to be more effective by promoting APC activation [93, 94]. Studies using TCR-transgenic mice further support the requirement for $CD4^+$ T cells to activate memory CTLs *in vivo* [95], and interestingly show tumour eradication by $CD4^+$ T cells even in cases where tumours were resistant to $CD8^+$ T cell- mediated rejection [96]. These findings suggested a potential benefit of T_H cells in cancer immunotherapy and started a search for the most effective anticancer $CD4^+$ effector T cell population.

Contradictory reports regarding the role of IL-17 in cancer have made it difficult to conclude whether or not T cells expressing this cytokine would be beneficial against tumours [97, 98]. Accumulation of T_H17 cells in the tumour microenvironment has been reported in several types of cancer, as well as the expression of IL-6, IL-1β and TGFβ by tumour cells, which are key cytokines controlling T_H17 cell differentiation and proliferation [99, 100]. T_H17 cell-polarized tumour specific $CD4^+$ T cells were shown to be more efficient than T_H1 -polarized cells in tumour rejection after adoptive transfer and this efficiency was probably dependent on IFNγ rather than IL-17 production [88]. Similar observations, transferring $CD8^+IL\text{-}17^+$ cells which then become $IFN\gamma^+$ producing cells were reported; however, some discrepancies have been found regarding the role of IFNγ in these models as the use of lymphopenic hosts promotes loss of a T_H17 cell phenotype and acquisition of a T_H1 cell phenotype in the transferred cells, potentially masking the real effects of IL-17 in controlling antitumour immunity [87, 89, 101].

Recent findings indicate that the differentiation state of T cells, naïve versus effector/memory, might also be important for mounting more efficient antitumour responses as single transfers of naïve $CD4^+$ T cells were able to eradicate established tumours independent of $CD8^+$ T cells, NK cells and NKT cells [102, 103].

Growing evidence suggests that control of the cytokines expressed in the tumour microenvironment can promote tumour eradication by controlling T_{Reg} and T_H17 cell polarization in the tumour. Exogenous administration of IL-2 in tumour-bearing mice increased T_{Reg} cell and decreased T_H17 cell frequencies in the tumour [104], whereas antagonizing the effects of TGFβ by administering IL-7 has been shown to be useful in the promotion of T_H17 cells [105]. A complete understanding of the dynamic cytokine network, including the role of TGFβ in controlling T cell polarization in the tumour, as well as characterization of the molecular signals mediating T_H cell differentiation will be crucial for dissecting the beneficial use of T_H cells in future immunotherapies against cancer.

Effects of TGFβ on regulatory cells

$CD4^+$ T_{Reg} cells—T_{Reg} cells are an immunosuppressive T cell population that express the forkhead family transcription factor, FoxP3, and can suppress antitumour immune responses [106]. These cells are a heterogeneous population containing at least two distinct subsets known as natural T_{Reg} (nT_{Reg}) cells and adaptive or induced T_{Reg} (iT_{Reg}) cells [106]. nT_{Reg} cells develop in the thymus, express the IL-2 receptor α chain (CD25) and maintain self tolerance in an antigen-independent manner. iT_{Reg} cells, by contrast, develop in the periphery in response to self- or tumour- antigens and express variable levels of CD25 [106]. Although nT_{Reg} cells and iT_{Reg} cells have been identified as separate subsets of regulatory T cells, their phenotype and function have not been fully established in preclinical tumour-

bearing animal models and patients with cancer. TGFβ could be involved in generating T_{Reg} cells *in vivo* and this cytokine may assist subsets of T_{Reg} cells in suppressing effector cell function in the tumour microenvironment (reviewed in [2] and covered in more detail in the section detailing effects of TGFβ on effector cells). High levels of T_{Reg} cells in patients with cancer can be inversely correlated with survival [107, 108]. Although the precise mechanism(s) behind increased T_{Reg} cells in malignancies are unknown, TGFβ, as well as other tumour-produced chemical mediators working in concert with this cytokine, PGE_2 and H-Ferritin have been implicated in inducing T_{Reg} cells [109, 110]. In addition, the production of CCL22 by TAMs surrounding tumours might mediate T_{Reg} cell trafficking to the tumour bed through CCR4 [111]. Recently, IL-23 production in the tumour microenvironment has been implicated in promoting the proliferation of intratumoural T_{Reg} cells as these cells express the IL-23 receptor, have evidence of STAT3 signalling (downstream of IL-23 receptor) and are decreased in number in tumour-bearing animals treated with a blocking antibody specific for the IL-23 receptor [25]. This cytokine may complement the effects of TGFβ, which also seems to increase the number of intratumoural T_{Reg} cells.

Recently a new regulatory T cell subtype has been identified that can be induced and expanded in mice bearing orthotopic liver, lung and melanoma tumours [112]. Unlike the conventional T_{Reg} cells described above, this regulatory subtype lacks expression of CD25 and FOXP3. Instead, the cells express the IL-2 receptor β chain (CD122), IL-10, TGFβ1 and the early activation marker, CD69 [112]. Activation of CD69 by the agonistic Ab against CD69 (H1.2F3) results in high levels of membrane-bound TGFβ expression by these cells through the activation of ERK and this might contribute to the ability of this regulatory T cell subset to suppress $CD4^+$ T cell proliferation and promote the growth of established tumours [112]. Although these results suggest that another subset of regulatory T cells associated with TGFβ production can suppress the antitumour response, the role of these cells in patients with cancer remains to be determined. TGFβ, in combination with IL-2, is required for the conversion of naïve T cells to iT_{Reg} cells *in vitro* [113, 114]. Furthermore, the induction of T_{Reg} cells by TGFβ might be a mechanism by which tumours escape the antitumour immune response as several tumours can produce TGFβ[2]. Blockade of TGFβ with antibodies or genetic manipulation leads to decreased numbers of induced T_{Reg} cells in some models of tumour-bearing animals [39, 115]. Therefore, targeting TGFβ signalling in the tumour microenvironment could attenuate the immunosuppressive effects of iT_{Reg} cells, resulting in increased antitumour immunity.

$CD8^+$ regulatory T cells—$CD8^+$ T cells can become suppressor cells similar to $CD4^+$ T_{Reg} cells, and TGFβ can induce $CD8^+$ T cells to express FOXP3 [116, 117]. $CD8^+$ regulatory T cells are induced under immunosuppressive conditions such as the tumour microenvironment [118–121]. The role of tumour-infiltrating $CD8^+$ regulatory T cells is less well understood than that of $CD4^+$ T_{Reg} cells. It was recently shown that infiltration of $CD8^+$ T cells into the immunosuppressive microenvironment of prostate tumours can convert tumour-specific $CD8^+$ effector T cells into regulatory cells, and that this regulatory activity could be blocked by a TGFβ-specific antibody[119]. In another recent study, $CD8^+CD25^+FOXP3^+$ regulatory T cells were isolated from colorectal cancer tissue and shown to have suppressive activity *ex vivo.* In this study, TGFβ and IL-6 induced the generation of $CD8^+$ regulatory T cells in a synergistic manner [120]. However, as these $CD8^+$ regulatory T cells represent only a small number of $CD8^+$ T cells *in vivo,* more investigation is needed to fully understand how they are induced and what is their clinical relevance in patients with cancer.

NKT cells—NKT cells are a heterogenous subset of T cells that also have properties of NK cells, and thus bridge the innate and adaptive immune responses. Unlike other T cells that recognize MHC class I-presented peptides, NKT cells recognize self and foreign glycolipids

presented by the nonclassical MHC class I molecule CD1d [122]. There are two main subtypes of NKT cells that have opposing roles in the antitumour immune response: Type I NKT cells (invariant NKT or iNKT cells) and Type II NKT cells.

iNKT cells are defined by use of a semi-invariant TCR involving Vα14Jα18 in mice and Vα24Jα18 in humans, and respond to α-galactosylceramide, resulting in increased antitumour responses through IFNγ production that activates $CD8^+$ T cells and NK cells [123, 122]. Defects in iNKT cells have been identified in patients with cancer in later stages of the disease and increased numbers of circulating and intratumoural iNKT cells have been associated with improved prognosis [124]. Targeting iNKT cells with activating agents is being evaluated in clinical trials (reviewed in [123]). TGFβ has been implicated in suppressing this cell subset in patients with cancer and a recent evaluation of iNKT cells from patients with metastatic melanoma and renal cell carcinoma suggested that blocking TGFβ *in vitro* could enhance iNKT cell activation *ex vivo* [125].

Type II NKT cells have diverse repertoires of TCRs and, in contrast to iNKT cells, suppress the antitumour response through several mechanisms, including TGFβ production [122]. In a murine fibroblast tumor model this subset of NKT cells can express high levels of IL-13, leading to the production of TGFβ by MDSCs, which in turn results in attenuated antitumour responses by $CD8^+$ effector T cells [76]. However, in a different tumour model, evaluating antibody-mediated blockade of TGFβ combined with a peptide vaccine against a lung cancer tumor line (TC1) suggested that the IL-13 pathway augmented by type II NKT cells might not be the mechanism behind the enhanced antitumour activity observed in vaccinated mice [81]. These results suggest a more complex interplay, yet to be determined, between TGFβ, type II NKT cells and effector immune cells responsible for the antitumour response.

Summary

Successful cancer immunotherapy depends on overcoming the immunosuppressive milieu in the tumour microenvironment in patients with cancer. TGFβ has a crucial immunosuppressive role in both the innate and the adaptive arms of the immune response (Figures 2 and 3). In terms of the innate immune response, TGFβ inhibits IFNγ production by NK cells causing dampened $CD4^+$ T_H1 cell responses. It downregulates expression of the activating receptor NKG2D on NK cells resulting in decreased cytolytic activity and overall poor antitumour responses. TGFβ also influences tumour antigen presentation by decreasing DC migration and promoting DC apoptosis in some tumour models. In general, TGFβ inhibits DC maturation and cytokine production, thereby promoting a tolerogenic environment. In addition, TGFβ produced by tolerogenic DCs contributes to T_{Reg} cell differentiation. TGFβ can also favour the differentiation of M2 versus M1 macrophages by inhibiting NF-κB activation. TAMs are a subtype of M2 cells that are recruited to the tumour by TGFβ and also produce high levels of TGFβ. TAMs in the tumour microenvironment compete with DCs for antigen uptake but cannot properly present antigen. TGFβ also promotes the differentiation of N1 to N2 neutrophils, which similar to M2 macrophages, are less cytotoxic. So, blocking TGFβ can induce an expression profile in the tumour microenvironment that promotes better antigen uptake and presentation, resulting in more robust priming and activation of the adaptive anti-tumour immune response.

In terms of the adaptive immune response, TGFβ can also directly dampen the function of $CD8^+$ and $CD4^+$ T cells while promoting the recruitment and differentiation of regulatory T cells at the tumour bed (Figure 3). This cytokine inhibits the cytotoxic function of tumour specific CTLs and promotes apoptosis of the short-lived effector $CD8^+$ T cells. TGFβ also controls the differentiation of several key $CD4^+$ T cell subsets in tumour immunology, including T_H1, T_H17 and T_{Reg} cell subpopulations. Importantly, the effect of TGFβ on the differentiation of $CD4^+$ T cells is influenced by the cytokine milieu in the tumour

microenvironment, suggesting that modulating the relative abundance of such factors could probably promote antitumour responses *in vivo*. It is well documented that both TGFβ and regulatory T cells have key roles in suppressing the antitumour immune response; however, the precise contributions of TGFβ and different regulatory T cell subsets in suppressing effector cell function are still being evaluated (Figure 4). For example, although TGFβ can induce $CD8^+$ T cells to become regulatory cells expressing FOXP3, the precise role of $CD8^+FOXP3^+$ T cells in tumour immunity remains unclear. TGFβ is also implicated in suppressing antitumour iNKT cell function; however, the interplay between TGFβ and immunosuppressive Type II NKT cells is less clear. Given that TGFβ can actively modulate inflammation and tolerance induction in the many ways described above, TGFβ blockade might enhance antitumour immunity through effects on numerous components of the immune response.

Targeting TGFβ signalling for immunotherapy of cancer

The immunosuppressive effects of TGFβ on immune cell subsets leading to dampened antitumour immune responses as described above strongly support the development of TGFβ inhibitors to treat patients with cancer. Several inhibitors of TGFβ signalling, summarized in Table 1, are in various stages of development, targeting several steps in the TGFβ signalling cascade (Figure 5). Although most of these approaches are in preclinical studies, several clinical trials have evaluated TGFβ inhibition in patients with cancer using an antibody (GC1008), blocking oligonucleotides (AP12009), small molecule inhibitors (LY373636 and LY2157299) and a vaccine approach [126–133]. The results from these trials evaluating TGFβ blockade alone indicate that there is limited clinical benefit; however, trials evaluating the small molecule inhibitors and antibody-mediated blockade are ongoing (Table 1).

Although systemic blockade of TGFβ has been well-tolerated in preclinical studies, given the pleiotropic effects of this cytokine, one potential concern of this type of therapy is the development of significant autoimmune toxicities in humans. This could be particularly problematic if TGFβ blockade is used in combination with other immune-activating agents, such as CTLA-4- or PD1-specific antibodies (which are also being evaluated as single agents in clinical trials and have shown several autoimmune toxicities) [134]. Other potential toxicities of blocking TGFβ might result from the cytokine's homeostatic functions in other tissues outside of the immune system, including angiogenesis and the development of musculoskeletal tissues.

The manipulation of local TGFβ sources in the tumour should be considered in the future as a strategy to inhibit the dominant immunosuppressive intratumoural environment while promoting antitumour immunity. Challenges to this approach will include being able to target the tumour microenvironment with TGFβ inhibitors without affecting TGFβ function in the rest of the host to maintain systemic homeostatic processes. This might require novel delivery systems, as well as effective TGFβ-specific drugs that have minimum systemic toxicities to the recipient.

Future Directions

Given that TGFβ affects the activity and differentiation of numerous immune cell types, it is unclear which of the effects of TGFβ is most important in the tumour microenvironment. This is, in part, due to the pleiotropic nature of TGFβ and the contextual and combinatorial effects that this cytokine can have at a range of biological concentrations on different cell types at various stages of their development. Therefore, there remain several questions regarding the basic biology of this cytokine as well as regarding the best strategy to

modulate this pathway alone and in combination with other pathways to enhance antitumour immunity in patients with cancer.

Although it is thought that tumour cells are an important source of TGFβ in the tumour microenvironment, immune cells themselves might in fact be a larger source of this cytokine, which is produced by effector T cells, regulatory T cells, APCs and MDSCs. Identifying the most relevant source of TGFβ will be important, as localized immunotherapy in the tumour microenvironment might be safer than systemic therapies that could interfere with the systemic homeostatic functions of TGFβ. In addition, TGFβ is expressed and synthesized as an inactive latent form unable to bind to its receptor. TGFβ becomes activated by interacting with molecules such as plasmin, matrix metalloproteinase, reactive oxygen species, thrombospondin-1, or integrins αvβ6 or αvβ8 [6, 90]. Notably, the cells that can activate TGFβ may be different than those that produce this potent cytokine, and thus this activation step provides a means for TGFβ to integrate signals from multiple cell types [90]. We know little about how TGFβ is activated in a tumour-bearing environment and whether tolerogenic DCs, TAMs or MDSCs have a greater capacity to activate TGFβ than their immunogenic counterparts. Therefore, a precise understanding of the mechanisms by which immunosuppressive cell subsets work alone and together, and their specific involvement in producing and/or activating TGFβ, may improve cancer immunotherapies.

Further studies are also warranted to evaluate the effect of increasing innate and adaptive immune responses in a tumour-bearing host. For example, inhibition of TGFβ offers a means to manipulate T cell polarization *in vivo* that can change an immunosuppressive environment into a more antitumour environment once a tumour has established in the host, as is the case in cancer patients when these types of therapies are generally being considered. Once the exact role of IL-17 and T_H17 cells in antitumour immunity has been defined, modulation of TGFβ levels might also be used to alter the ratio between T_{Reg} cells and T_H17 cells in the tumour microenvironment. In addition, blocking TGFβ signalling in combination with tumour vaccines promotes antitumour immunity that is mediated, in part, by $CD8^+$ T cells[80–82, 135], which could lead to a long-term response with immunological memory.

Additional areas for future research related to the development of agents that efficiently block TGFβ and its activity include pharmacodynamic profiling of tissue TGFβ concentrations and the optimization of strategies to block the most appropriate TGFβ-dependent signalling pathways. For example, the blockade of SMAD-independent pathways of TGFβ-dependent signalling, including MAPKs, Rho GTPases and PI3K that are involved in tumour progression and metastasis, could lead to new strategies to enhance antitumour immunity (Figure 5). Mutations in SMAD2, SMAD3 and SMAD4 lead to cancer progression [136, 137], indicating that the tumour-suppressor properties of TGFβ involve SMAD-dependent signalling and therefore SMAD-dependent pathways may not be ideal therapeutic targets. Understanding the intricate signalling pathways controlled by TGFβ, as well as the mechanism(s) leading to its opposing effects in tumour biology, could lead to new strategies against cancer [138].

Furthermore, identifying the ideal timing of TGFβ blockade in the host, if used in combination with vaccines, cytokine therapies (IL-2 and IL-15) or other immune-activating antibodies (such as CTLA4- or PD1/PD1L-specific antibodies), would be very informative [139]. Finally, designing optimal methods to deliver the most effective TGFβ inhibitor to the tumour microenvironment and the evaluation of exposing expanded T cell subsets to these drugs *ex vivo* to enhance adoptive T-cell based immunotherapies are all areas requiring further investigation.

So far, the clinical trials evaluating blockade of TGFβ in patients with cancer do not show a clear clinical benefit. Therefore, larger studies are warranted to clarify the toxicity and efficacy of these strategies. In addition, the optimal dose and timing of TGFβ blockade, as well as the ideal combination of this approach with other immunotherapies, remain unknown. These questions are currently being addressed in ongoing preclinical studies and are likely to be the focus of future clinical trials.

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Glossary

Peripheral tolerance	The lack of responsiveness of mature lymphocytes in the periphery to specific self antigens. These mechanisms can control potentially self-reactive lymphocytes that have escaped central tolerance or prevent immune responses to specialized self proteins that were not present during establishment of central tolerance. Peripheral tolerance is associated with suppression of self-reactive antibody production by B cells and inhibition of self-reactive effector cells, such as T helper cells and cytotoxic T lymphocytes
Myeloid-derived suppressor cells (MDSCs)	A group of immature $CD11b^{+}GR1^{+}$ cells (which include precursors of macrophages, granulocytes, dendritic cells and myeloid cells) that are produced in response to various tumour-derived cytokines. These cells have been shown to induce tumour-associated antigen-specific $CD8^{+}$ T-cell tolerance
Tumour-associated macrophages (TAMs)	An important component of the tumour microenvironment. These cells differentiate from circulating blood monocytes that have infiltrated tumours. These cells can have positive or negative effects on tumorigenesis (that is, tumour promotion or immunosurveillance, respectively)
M1 (classically activated) macrophage	A macrophage that is activated by Toll-like receptor ligands (such as LPS) and interferon-gamma and that express, among others, inducible nitric-oxide synthase and nitric oxide
M2 (alternatively activated) macrophage	A macrophage that is stimulated by interleukin-4 (IL-4) or IL-13 and that expresses arginase-1, the mannose receptor CD206 and the IL-4 receptor alpha-chain

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Text box 1

Non-immune effects of TGFβ in tumours

In addition to the effects of TGFβ on various immune cell subsets to promote tumour progression, this pleiotropic cytokine enhances tumourigenesis in a number of pathways not directly involving the antitumor response. For example, TGFβ promotes autocrine mitogen production to enhance tumor cell proliferation. In addition, TGFβ augments a number of biologic process supporting metastasis formation, including: tumor cell motility, cancer cell priming for metastasis development, extravasation of tumor cells from the primary site, osteoclast mobilization which can support osseus metastasis deposits and angiogenesis to nourish primary tumor and secondary metastases. These effects are beyond the scope of this review and are discussed in detail in References 1 and 2.

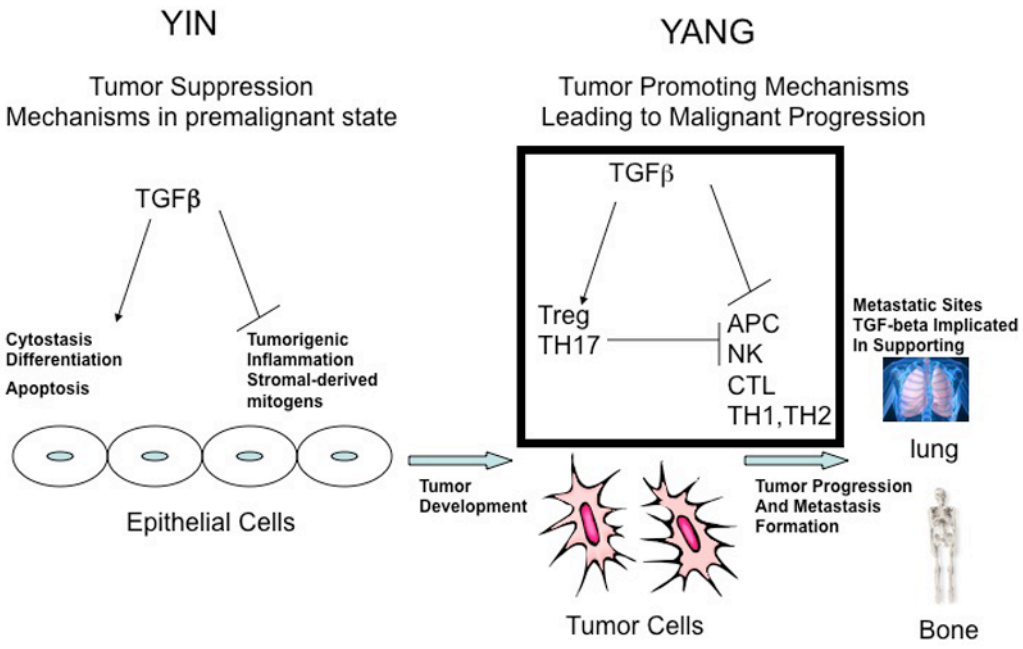

Figure 1. The yin and yang of TGFβ in tumour development maintenance, and metastasis formation

Before epithelial cells transform into a malignant tumour, transforming growth factor-β (TGFβ) functions as a tumour-suppressor, by blocking expression of stromal-derived mitogens and suppressing pro-tumourogenic inflammation. Furthermore, TGFβ supports the cytostasis, terminal differentiation and apoptosis of premalignant cells which harbor either an overexpressed oncogene or suppressed tumour suppressor gene. Once the epithelial cells become fully malignant, TGFβ has the opposite effect by blocking the antitumour immune response through support for the activity of regulatory cells and through direct inhibition of effector cell mechanisms from clearing the established tumour, as described in the main text and summarised in Figure 2. Once tumours are established, TGFβ further supports the formation of metastases to several sites, including bone and lung tissues. Additional nonimmune mechanisms outside the scope of this Review that support tumorigenesis and metastasis formation are addressed in References 1 and 2.

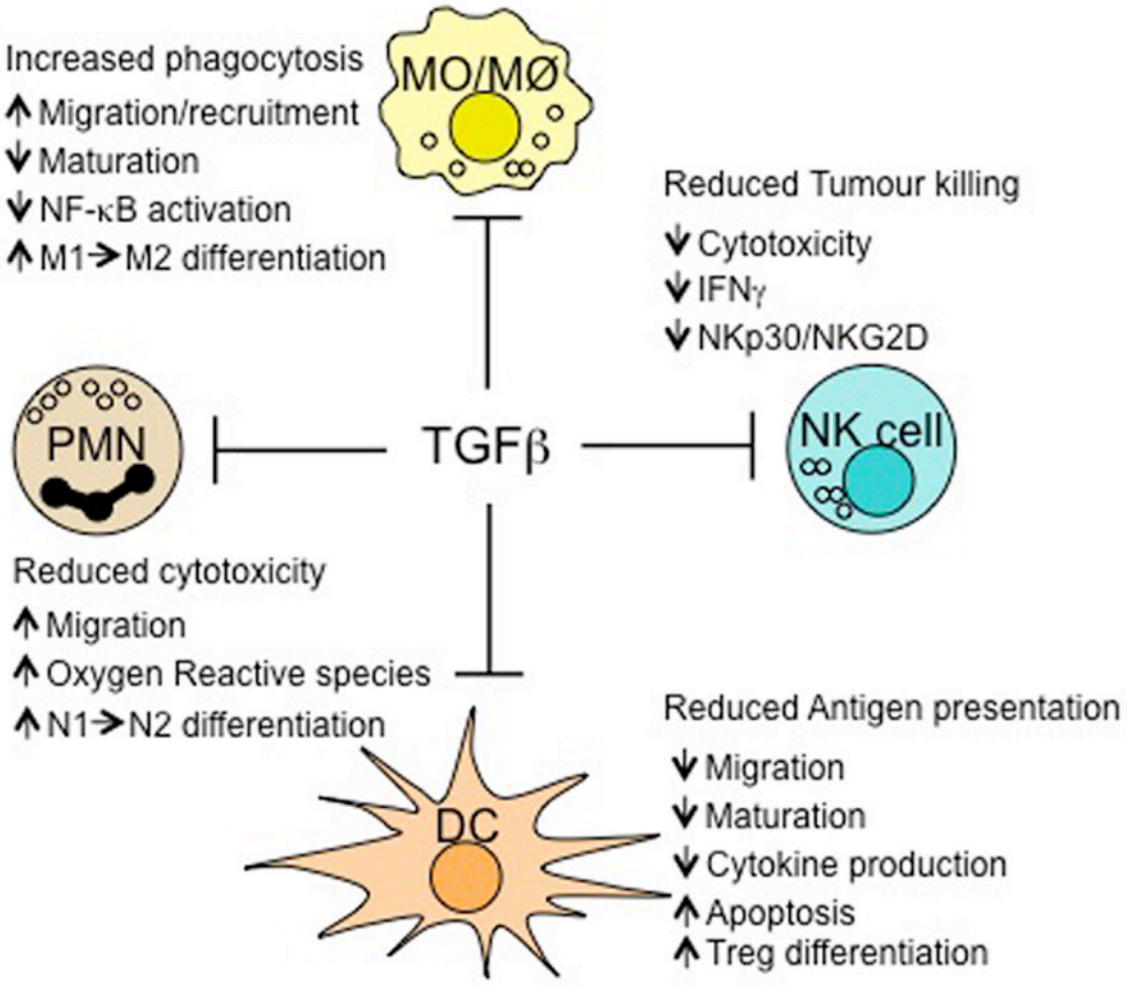

Figure 2. Effects of TGFβ on innate immune cells

Transforming growth factor-β (TGFβ) has an inhibitory effect on innate immunity in the tumour microenvironment through several pathways. It inhibits natural killer (NK) cell function by downregulating interferon-γ (IFNγ) production and expression of the activating receptors NKp30 and NKG2D, thereby decreasing NK cell killing activity. In the presence of TGFβ, dendritic cells (DCs) acquire a tolerogenic phenotype involving decreased migration, maturation and cytokine production and increased apoptosis; they gain the ability to induce regulatory T (T_{Reg}) cell differentiation. TGFβ can also convert N1 neutrophils to a N2 phenotype, which is less cytotoxic. Similarly, TGFβ can promote the recruitment of M2 over M1 macrophages and of tumour-associated macrophages (TAMs), and can decrease cytokine production by these macrophages by inhibiting nuclear factor-κB (NF-κB) activity.

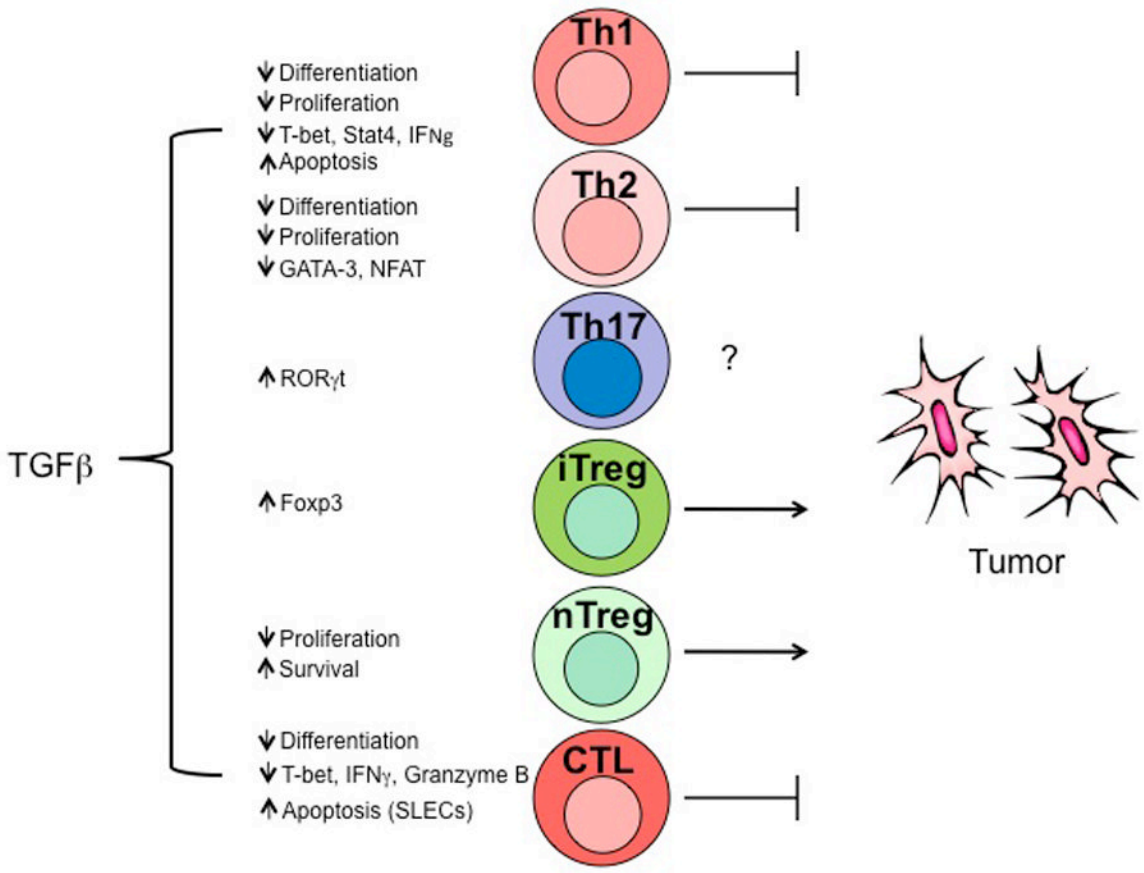

Figure 3. Effects of TGFβ on effector T cells

Transforming growth factor-β (TGFβ) differentially regulates the survival, differentiation, proliferation and apoptosis of T cell subsets. Among T_H subpopulations, both T_H1 and T_H2 can provide antitumour responses, however T_H1 seem to be more efficient. Both nTreg and iTreg populations inhibit antitumour immune responses. Within the tumour microenvironment, TGFβ can promote tumour growth by the maintenance of T_{Reg} cell and differentiation of iTreg subpopulations. TGFβ can also inhibit T_H1 cell and CTL functions by downregulating T-bet and IFNγ expression and probably promoting a shift towards T_H2 differentiation. CTLs are potent antitumour effector cells. TGFβ could also inhibit tumour immune surveillance by the induction of apoptosis in short-lived effector CTLs. The role of T_H17 cells in tumour biology is still controversial and requires further characterization.

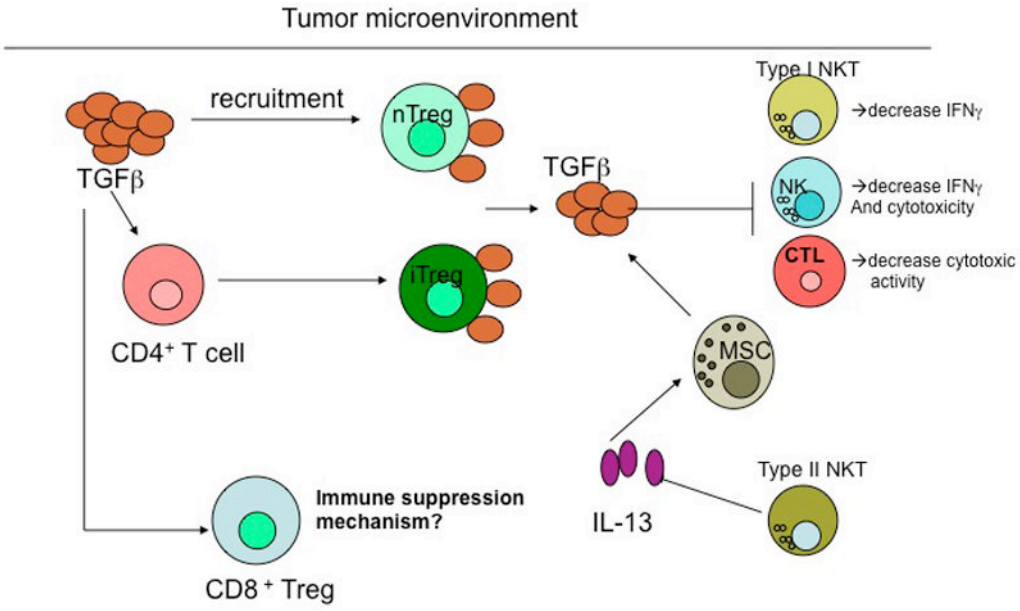

Figure 4. Effects of TGFβ on regulatory cells

Within the tumour microenvironment, transforming growth factor-β (TGFβ) has been implicated in recruiting natural regulatory T (nT_{Reg}) cells as well as converting $CD4^+$ effector T cells to induced T_{Reg} (iT_{Reg}) cells. These T_{Reg} cells can express cell surface-bound TGFβ and can inhibit effector cells, including natural killer (NK) cells and $CD8^+$ T cells, in the tumour microenvironment by cell–cell contact to dampen the antitumour response. Type I NKT cells, which are responsible for recruiting effector immune cells to the tumour through the production of large amounts of IFNγ, can be suppressed by intratumoural TGFβ, whereas Type II NKT cells support increased TGFβ production by myeloid-derived suppressor cells (MDSCs) through the generation of interleukin-13 (IL-13). $CD8^+$ regulatory T cells have been observed in lung tumours and these might result from the production of IL-10 by antigen-presenting cells, leading to increased TGFβ production in the tumour microenvironment. The precise immunosuppressive mechanisms of $CD8^+$ regulatory T cells in regulating the antitumour immune response have yet to be identified.

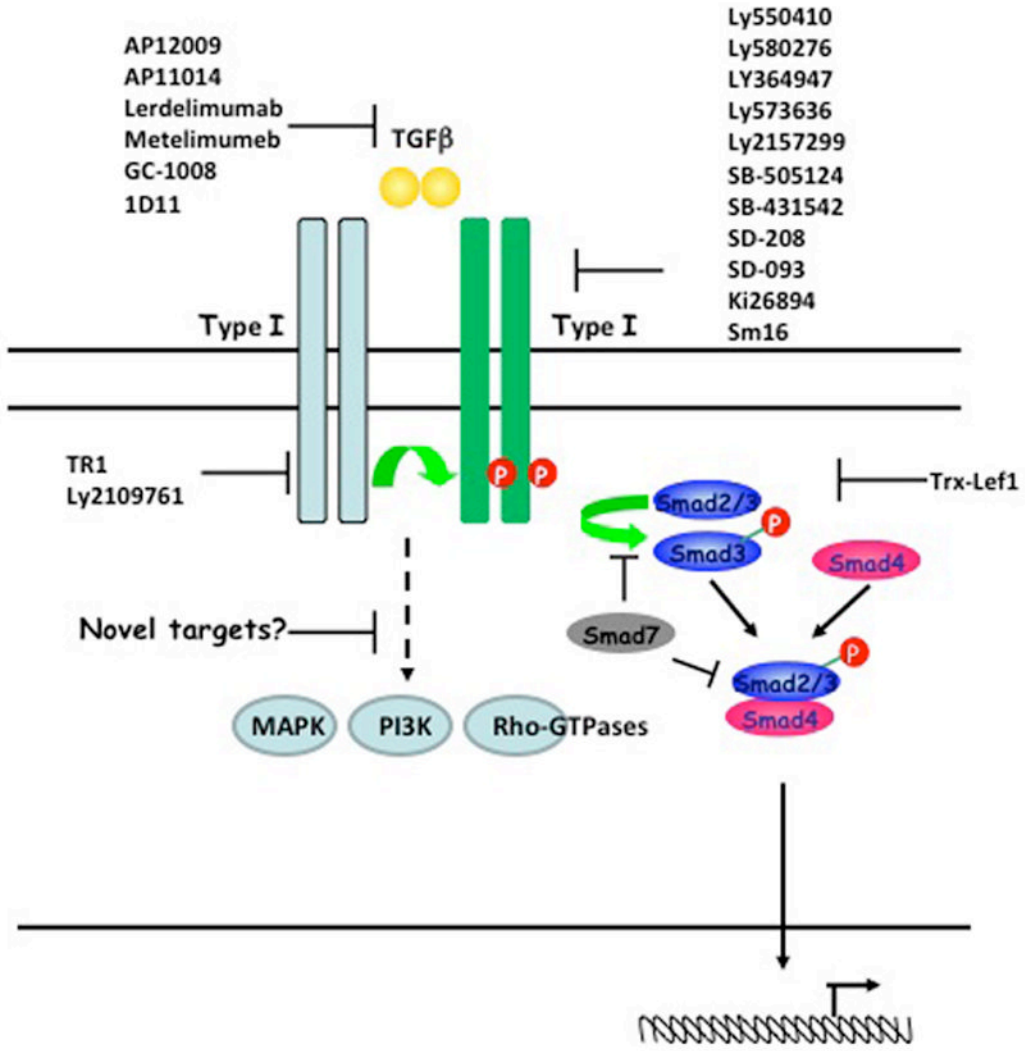

Figure 5. Targets for inhibiting TGFβ and downstream signalling events

The transforming growth factor-β (TGFβ)-dependent signalling pathway depends on Type I and Type II serine-threonine kinase receptors and transcription factors known as SMADs. The dimeric bioactive ligand binds to a Type II receptor, which in turn phosphorylates and activates a Type I receptor. Once the Type I receptor is activated, it phosphorylates the receptor SMADs (R-SMADs: SMAD2 and SMAD3), which promotes their interaction with the common mediator SMAD (SMAD4) and translocation to the nucleus. The inhibitory SMAD7 negatively regulates TGFβ signalling by competing with R-SMADs for interaction with the Type I receptor or SMAD4. Current TGFβ signalling inhibitors (listed in Table I ans shown in scheme) include ligand, receptor and SMAD antagonists. However, additional SMAD-independent pathways have been reported to be induced in response to TGFβ, including the activation of MAPK, Rho-like GTPase and phosphatidylinositol 3-Kinase (PI3K) signalling pathways; a complete understanding of these alternative pathways could potentially offer additional downstream molecules that could be targeted in future therapeutic approaches. (Figure adapted from [138])

Table 1

Summary of TGFβ signalling inhibitors being evaluated as immunotherapies.

Drug	Mechanism of Action	Stage of Development	Referenced Summary of Results
AP12009	Antisense Oligonucleotide against TGFβ2	Phase I/II and Phase III (enrolling)	AP 12009 was safe and well-tolerated in high grade glioma patients. 7/24 patients with stable disease and 2/24 patients with complete response 25 months after initiating therapy [130, 132].
AP-11014	Antisense Oligonucleotide Against TGFβ1	Preclinical	Decreases TGFb1 secretion by and subsequent proliferation of lung, colon and prostate cancer cell lines [140].
Lerdelimumab (CAT 152)	Antibody specific for TGFβ2	Phase III	Evaluated for eye surgeries to prevent scarring after primary traveculectomies. Deemed safe but ineffective at improving scarring following eye surgery when compared to placebo [141]
Metelimumab (CAT 192)	Antibody specific for TGFβ1	Phase II	May prevent excessive post operative scarring for glaucoma surgery; also when added to ACE-I drug can arrest diabetic nephropathy in rats [142]
GC-1008	Antibody specific for all isoforms of TGFβ	Phase I	Safe and well-tolerated in advanced melanoma and kidney cancer patients [143].
ID11	Antibody specific for all isoforms of TGFβ	Preclinical	One study indicates that pretreatment of mice engrafted with syngeneic breast cancer cell line with this antibody suppresses breast cancer metastases to lungs [144].
TR1 and MT1	Antibodies specific for TGFβRII	Preclinical	Enhances antitumor responses against murine breast and colon cell lines by increasing CTL and NK activity while decreasing Tregs and myeloid derived suppressor cells in mice treated with these antibodies [145].
Ly550410 Ly580276 Ly364947 Ly2109761	Small Molecule Inhibitors of TGFβRI kinase	Preclinical	While majority of these inhibitors have not been assessed in animal models, nanoparticle delivery Ly364947 has resulted in antitumor activity against human pancreatic and gastric xenografts in immunodeficient mice.[146, 147].
Ly573636	Small Molecule Inhibitor of TGFβRI kinase	Phase II	Results unavailable as Phase II trial evaluating this drug in advanced stage melanoma patients is ongoing [133].
Ly2157299	Small Molecule Inhibitor of TGFβRI kinase	Phase I	40mg and 80mg daily doses of this drug were safe and well-tolerated in colon cancer, prostate cancer, adrenal gland cancer, breast cancer and melanoma patients enrolled in this study[126].
SB-505124 SB-431542	Small Molecule Inhibitor of TGFβRI kinase	Preclinical	No in vivo data available at this time. Inhibition of TGF-beta signaling has been established in vitro[148, 149].
SD-208 SD-093	Small Molecule Inhibitor of TGFβRI kinase	Preclinical	Inhibits multiple myeloma (SD-093) and glioma (SD208) growth in vivo[150, 151].
Ki26894	Small Molecule Inhibitor of TGFβRI kinase	Preclinical	Inhibits breast cancer metastasis and enhanced survival in preclinical murine model treated with this drug [152].
Sm16	Small Molecule Inhibitor of TGFβRI kinase	Preclinical	In vivo effects include inhibiting murine mesothelioma tumor growth and recurrence following resection of this tumor in a mouse model[153,154].
Trx-xFoxH1b Trx-Lef1	Interacting peptide aptamers against smads	Preclinical	Bind to smads to inhibit TGF-beta mediated gene expression using in vitro luciferase reporter assays [155].

Drug	Mechanism of Action	Stage of Development	Referenced Summary of Results
P144, P17	14mer peptide blocking TGFβ binding to TGFβRI and TGFRβRII	Preclinical	Administration of both peptides with adjuvant molecules poly(I:C) and agonistic anti-CD40 antibody increased antitumor activity against the ova-expressing lymphoma cell line E.G7 ova in mice by enhancing NK, tumor- specific CTL, DC activity while suppressing MDSCs and inhibition of TGFb production by Tregs[156].
Belagenpumatucel-L: Antisense- transfected tumour cells	Vaccine against TGFβRII	Phase I and II	No significant toxicities observed in cancer patients enrolled in the studies. When stratified for circulating tumor cells patients with less than 2 circulating tumor cells per 7.5mL of blood demonstrated median survival of 660 days vs 150 days in patients with greater circulating tumor cells [127–129].
Soluble TBR2-FC	Stabilized soluble protein against TGFβRs	Preclinical	Lifetime exposure was tolerated in mice and decreased incidence of metastasis formation in a metastatic melanoma model and inducible transgenic breast cancer model[157].
Plasmid	Plasmid DNA encoding TGFβRII fused to human IgG heavy chain	Preclinical	Administration of this plasmid to tumor draining lymph nodes from implanted E.G7 lymphomas and B16 melanomas in mice inhibited lymphoma tumor growth and primary melanoma growth and lung metastases [158].