

Review

# Integrin-Mediated TGF $\beta$ Activation Modulates the Tumour Microenvironment

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**Abstract:** TGF $\beta$  (transforming growth factor-beta) is a pleiotropic cytokine with contrasting effects in cancer. In normal tissue and early tumours, TGF $\beta$  acts as a tumour suppressor, limiting proliferation and inducing apoptosis. However, these effects are eventually abrogated by the loss or inactivation of downstream signalling within the TGF $\beta$  pathway, and in established tumours, TGF $\beta$  then acts as a tumour promotor through multiple mechanisms including inducing epithelial-to-mesenchymal transition (EMT), promoting formation of cancer-associated fibroblasts (CAFs) and increasing angiogenesis. TGF $\beta$  is secreted as a large latent complex and is embedded in the extracellular matrix or held on the surface of cells and must be activated before mediating its multiple functions. Thus, whilst TGF $\beta$  is abundant in the tumour microenvironment (TME), its functionality is regulated by local activation. The  $\alpha$ v-integrins are major activators of latent-TGF $\beta$ . The potential benefits of manipulating the immune TME have been highlighted by the clinical success of immune-checkpoint inhibitors in a number of solid tumour types. TGF $\beta$  is a potent suppressor of T-cell-mediated immune surveillance and a key cause of resistance to checkpoint inhibitors. Therefore, as certain integrins locally activate TGF $\beta$ , they are likely to have a role in the immunosuppressive TME, although this remains to be confirmed. In this review, we discussed the role of TGF $\beta$  in cancer, the role of integrins in activating TGF $\beta$  in the TME, and the potential benefits of targeting integrins to augment immunotherapies.

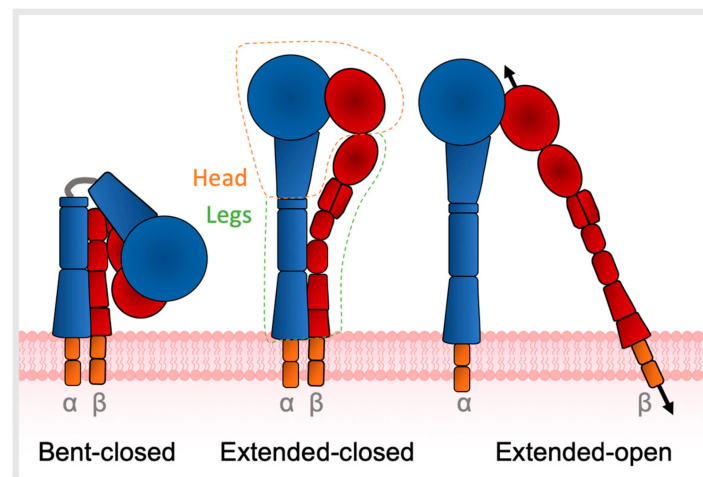
**Keywords:** integrins; TGF $\beta$ ;  $\alpha$ v $\beta$ 6; tumour microenvironment

## 1. Introduction

The extracellular matrix (ECM) serves both as a scaffold for cells and as an information-rich system that cells decipher through interacting sensory inputs in which integrins and transforming growth factor-beta (TGF $\beta$ ) are highly influential [1]. Homeostasis regulates the balance of cytokines and cell surface receptors to mediate intricate interactions between cells and the ECM. TGF $\beta$  is the most pleiotropic known cytokine and almost every cell produces TGF $\beta$  and has receptors for it [2,3]. It is an effective growth inhibitor of epithelial, haematopoietic and immune cells and is locally activated during tissue remodelling to regulate growth and repair. Dysregulation of TGF $\beta$  is implicated in a number of pathologies, most notably cancer and tissue fibrosis [3,4]. Whilst almost ubiquitously secreted, TGF $\beta$  is held within the ECM in an inactive state, requiring activation to mediate its effects. As discussed below, the  $\alpha$ v $\beta$  integrins are major activators of TGF $\beta$  in both normal tissue and cancer.

Integrins are heterodimers that mediate bidirectional signalling across cell membranes, coupling diverse extracellular ligands to the cytoskeleton [1,5,6]. Integrin ectodomains comprise an  $\alpha$ - and  $\beta$ -subunit non-covalently joined at the head, each connected to a flexible leg which traverses the cell membrane to a short cytoplasmic domain (Figure 1) [7]. The endodomains link with cytoskeletal components and thus allow integrins to act as mechanotransducers between the cell and ECM [8–10].

Regulation of ligand affinity and signalling is mediated by a series of coupled motions of the headpiece with leg domains that change the overall shape from bent to extended [11]. Integrin structure is described in three main conformations—bent-closed, extended-closed, or extended-open, with low ligand affinity when in the bent state [10,12]. A total of 24 different  $\alpha\beta$  heterodimers exist, composed of 18  $\alpha$ - and 8  $\beta$ -subunits, each with different functional and tissue specificity. The primary binding site of integrins to their target ligands is via recognition of short peptide motifs, the most common of which is arginine-glycine-aspartic acid (RGD) motif; eight integrins ( $\alpha\text{IIb}\beta_3$ ,  $\alpha_5\beta_1$ ,  $\alpha_8\beta_1$ ,  $\alpha\nu\beta_1$ ,  $\alpha\nu\beta_3$ ,  $\alpha\nu\beta_5$ ,  $\alpha\nu\beta_6$ ,  $\alpha\nu\beta_8$ ) recognize the RGD motif [13].



**Figure 1.** Integrin  $\alpha\nu\beta_6$  conformation states. Integrins comprise an  $\alpha$ - and  $\beta$ -subunit associated non-covalently at the head, each connected to a flexible leg that traverses the cell membrane to a short cytoplasmic domain. Ligand affinity is mediated by changes in conformation, with affinity highest in the extended-open state and lowest in the bent-closed state. The black arrows indicate the direction of force that integrins mediate between the extracellular matrix and actin cytoskeleton adapted from [9].

Integrin functions are myriad and include cell adhesion, migration, proliferation, differentiation, survival and invasion but the  $\alpha\nu$ -integrins, particularly  $\alpha\nu\beta_6$  and  $\alpha\nu\beta_8$ , are specialised to activate TGF $\beta$  [14]. Since integrins and their ligands are amongst the plethora of TGF $\beta$ -regulated transcriptional targets, TGF $\beta$ -integrin interactions are bilateral.

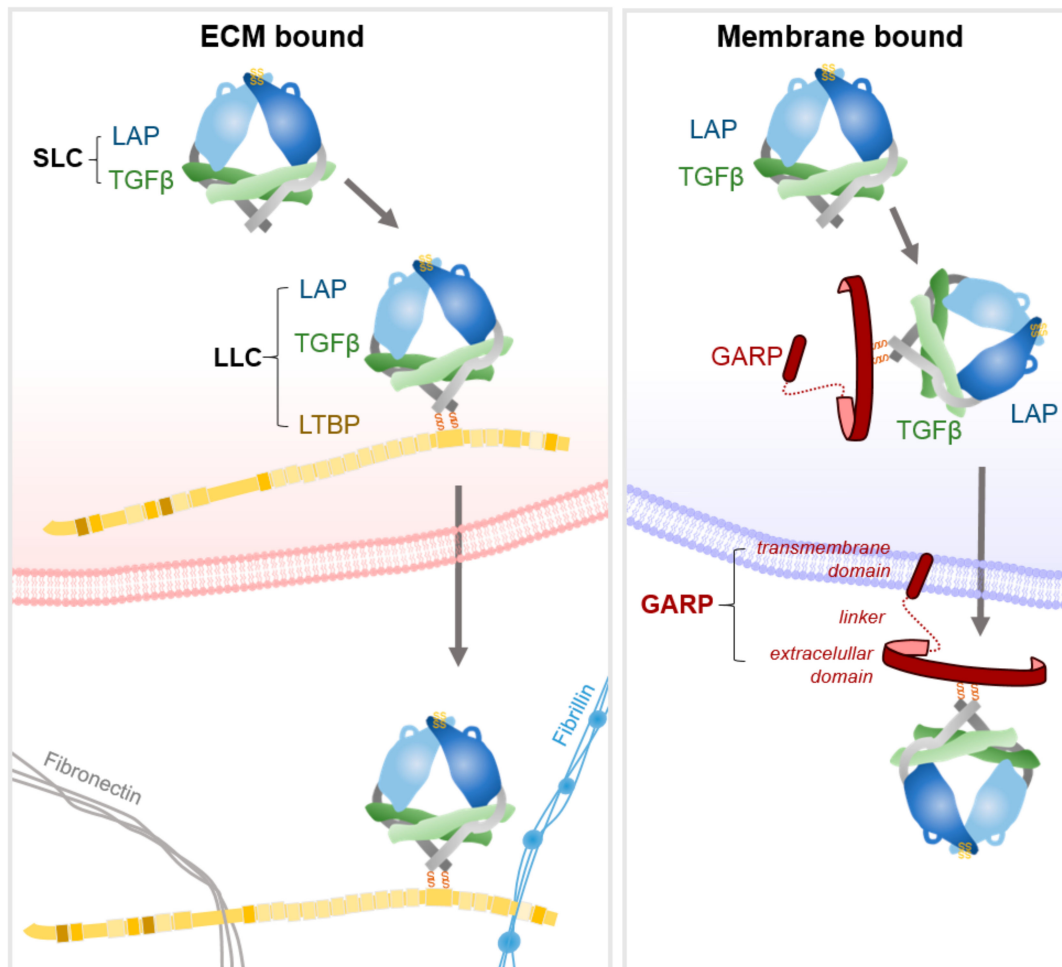
In this review, we discussed TGF $\beta$  activation by integrins and its consequences by promoting cancer through regulating the the immune and non-immune tumour microenvironment (TME).

## 2. TGF $\beta$ Structure and Secretion

The transforming growth factor  $\beta$  (TGF $\beta$ ) family is encoded by 33 genes and includes the three TGF $\beta$  isoforms (TGF $\beta$ 1, TGF $\beta$ 2, TGF $\beta$ 3) relevant to this review [15–17]. The three isoforms are 75% homologous with similar signalling activities but have variable expression in different cells and tissues, and distinct phenotypes are observed in vivo in knockout models [18]. TGF $\beta$ 1 is predominantly expressed in endothelial, hematopoietic, and connective-tissue cells, TGF $\beta$ 2 in epithelial and neuronal cells, and TGF $\beta$ 3 in mesenchymal cells [19]. TGF $\beta$ 1 is the most abundant, most widely studied, and most commonly dysregulated isoform in cancer [20,21], and thus, will be the focus of this review.

TGF $\beta$  is initially translated as a propeptide comprising the growth factor and latency-associated peptide (LAP). A disulphide-linked dimer of the propeptide is formed, which is cleaved by furin to release the mature TGF $\beta$  from LAP. However, the affinity of the propeptide LAP for TGF $\beta$  is such that it assembles into a non-covalent complex, termed the small latent complex (SLC) that comprises TGF- $\beta$  and homodimers of LAP (Figure 2) [2,16,22]. The SLC is then further processed before it exits the cell. In non-leukocyte cells, a pair of disulphide bonds link LAP to a latent-TGF $\beta$  binding protein (LTBP) [22]. LTBPs are large glycoproteins that serve as chaperones for pro-TGF $\beta$  by enhancing folding

and secretion [22]. In leukocytes, the glycoprotein-A repetitions predominant protein (GARP) bind to LAP, again via two disulphide linkages and again serving as chaperone for correct presentation of the complex [23–25]. As GARP is a transmembrane molecule, it holds the SLC at the membrane (discussed in [25]). Thus, TGF- $\beta$  is secreted as a tripartite complex of TGF- $\beta$ , LAP and either LTBP or GARP, termed the large latent complex (LLC) [9,22,26]. Once secreted, LTBPs localise latent-TGF $\beta$  to the ECM by interactions with fibronectin and fibrillin [22,27], the four LTBPs being expressed in a tissue specific fashion [2]. The majority of latent-TGF $\beta$  is sequestered within the ECM, with the remaining latent-TGF $\beta$  anchored to immune cell surfaces or stored in  $\alpha$ -granules within platelets and mast cells [28].



**Figure 2.** TGF $\beta$  (transforming growth factor-beta) structure and secretion. Dimers of TGF $\beta$  and latency-associated peptide (LAP) linked by non-covalent bonds form the small latent complex (SLC). Left: Covalent bonds link the small latent complex to latent TGF $\beta$  binding protein (LTBP) which together form the large latent complex (LLC). Once secreted, TGF $\beta$  is held in an inactive state, anchored by LTBP to fibronectin and fibrillin in the extracellular matrix. Right: In some cells, notably regulatory T-cells, the SLC binds covalently to glycoprotein-A repetitions predominant (GARP), and following secretion is bound to the cell membrane. Adapted from [15,16,29]. SS, disulphide bonds.

### 3. TGF $\beta$ Activation

TGF $\beta$  is stored within the ECM at concentrations that are several orders of magnitude higher than required to produce potent biological effects [30]. Thus, most TGF $\beta$  regulation occurs at the level of activation of latent-TGF $\beta$  sequestered within the ECM by LTBP or on cell-surface scaffold proteins by GARP [9,22,26,30]. Active TGF $\beta$  has a considerably shorter half-life than latent TGF $\beta$

and is rapidly cleared from the extracellular space if not associated with its receptor [31]. Thus, the activation of latent-TGF $\beta$  permits tight spatial and temporal regulation of TGF $\beta$  signalling [32]. Latent-TGF $\beta$  is activated in vitro by a variety of protease and non-protease-dependent mechanisms, including physiochemical conditions. However, the major activators of TGF $\beta$  in vivo are integrins, most prominently  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8.

### 3.1. Knockout Mouse Phenotypes

Early indications of integrin involvement in TGF $\beta$  activation came from similarities in knockout mice phenotypes. TGF $\beta$ 1<sup>-/-</sup> mice die shortly after birth from multi-organ inflammation and vasculogenesis defects. TGF $\beta$ 2<sup>-/-</sup> mice die around the time of birth with multi-organ developmental defects. TGF $\beta$ 3<sup>-/-</sup> mice develop cleft palate (reviewed in [32]). Integrin  $\beta$ 6<sup>-/-</sup> mice develop lung and skin inflammation [33]. Moreover, they are protected from bleomycin-induced lung fibrosis, established as TGF $\beta$ 1 dependent, and gene expression profiles of the  $\beta$ 6<sup>-/-</sup> lungs show the majority of TGF $\beta$  responsive genes are not upregulated [14,34]. In addition,  $\beta$ 6<sup>+/+</sup> mice dramatically upregulate  $\alpha$ v $\beta$ 6 in response to bleomycin, suggesting that  $\alpha$ v $\beta$ 6 expression is important for local activation of TGF $\beta$ 1 in epithelial cells [14]. These correlations between increased TGF $\beta$ 1 activity and  $\alpha$ v $\beta$ 6 expression were confirmed by showing  $\alpha$ v $\beta$ 6-specific antibodies blocked activation of TGF $\beta$ 1 [14]. The vital role of the RGD integrin-binding sequence in TGF $\beta$ 1 activation was demonstrated in vivo by generating mice whose TGF $\beta$ 1 lacked the integrin recognition motif RGD in LAP $\beta$ 1 which is replaced with the inactive motif RGE. TGF $\beta$ 1<sup>RGE/RGE</sup> mice exhibit the TGF $\beta$ 1<sup>-/-</sup> phenotype despite normal levels of latent-TGF $\beta$ 1 [35,36].

Whilst most integrin- $\beta$ 6<sup>-/-</sup>/ $\beta$ 8<sup>-/-</sup> double knockout mice die in utero, those that survive develop cleft palate [37], a phenotype replicated in the *garp* knockout mouse [38], showing that GARP regulates TGF $\beta$ 3. Interestingly, replacement of the TGF $\beta$ 3 gene with TGF $\beta$ 1 at the TGF $\beta$ 3 locus partially rescues palate closures, highlighting that TGF $\beta$ 3- and TGF $\beta$ 1-LAP share critical features but also display isoform-specific roles [39]. Integrin- $\beta$ 8<sup>-/-</sup> mice have abnormal cerebral and yolk sac vasculogenesis. Whilst the yolk sac defect is seen in TGF $\beta$ 1<sup>-/-</sup> mice, the cerebrovascular defect is not apparent in TGF $\beta$  single isoform knockouts, suggesting overlapping functions in the TGF $\beta$  isoforms [40]. A key observation was that conditional deletion of  $\alpha$ v $\beta$ 8 in dendritic cells (DCs) resulted in widespread inflammation in the intestines, attributed to failure of DCs to activate TGF $\beta$  and thus regulate Treg activity (discussed below) [41]. Furthermore, pharmacological inhibition of  $\alpha$ v $\beta$ 6 in  $\beta$ 8<sup>-/-</sup> mice causes a similar phenotype to TGF $\beta$ 1<sup>-/-</sup> mice, consistent with  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8 as dominant latent TGF $\beta$ 1 activators.

### 3.2. Ligand Affinity

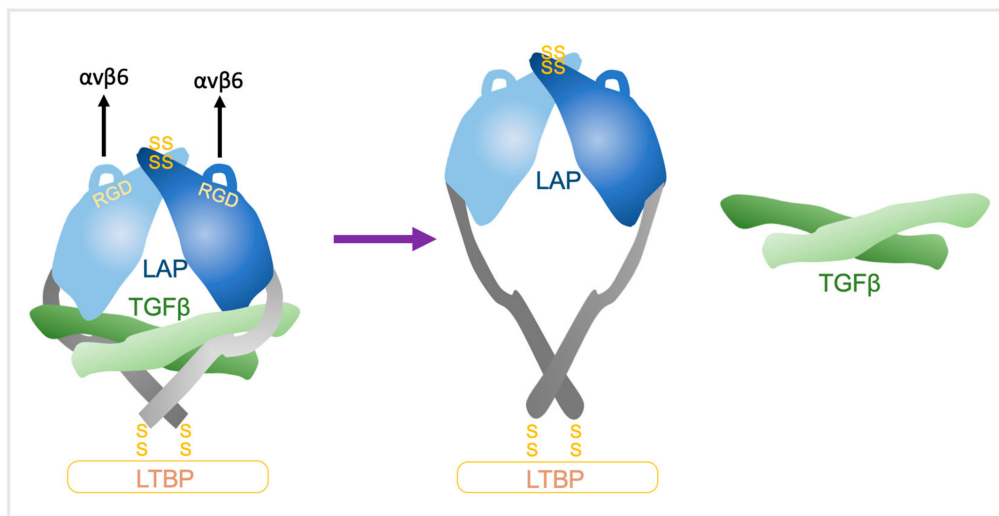
The RGD integrin-binding motif is present on the LAP propeptides of TGF $\beta$ 1 and TGF $\beta$ 3, which have been shown to bind  $\alpha$ v $\beta$ 1,  $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5,  $\alpha$ v $\beta$ 6,  $\alpha$ v $\beta$ 8, and  $\alpha$ 8 $\beta$ 1 [14,36,42–46]. The homologous latency associated peptide from pro-TGF $\beta$ 2 has SGD (serine-glycine-aspartic acid) in place of RGD and binds to  $\alpha$ v $\beta$ 6, but with a thousand-fold lower affinity than LAP $\beta$ 1 [47] and thus, is not activated by integrins [48]. LAP-TGF $\beta$ 1 binds strongly to  $\alpha$ v $\beta$ 6 (10.3 nM) and  $\alpha$ v $\beta$ 8 (13 pM) but with a significantly lower affinity for  $\alpha$ v $\beta$ 3 (8.5  $\mu$ M). This nanomolar affinity is unusual in integrins, which typically bind with lower affinity to allow the reversal of adhesion in retracting regions of migrating cells. Thus, this high affinity may reflect specialisation to support TGF $\beta$ -activation over cell migration [36,47]. The higher affinity of  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8 for LAP-TGF $\beta$ 1 is due to the ability to bind both to RGD and to a second binding motif, LXXL/I which  $\alpha$ v $\beta$ 3,  $\alpha$ IIb $\beta$ 3, and  $\alpha$ 5 $\beta$ 1 are unable to recognise [47].

### 3.3. Force-Mediated Activation of ECM Bound Latent TGF $\beta$ by $\alpha$ v $\beta$ 6

When integrins bind to the RGD motif on LAP, association with the actin cytoskeleton triggers conformational changes in the LLC that releases TGF $\beta$  [49,50].  $\alpha$ v $\beta$ 6 activates latent-TGF $\beta$  even in

the presence of a cocktail of protease inhibitors, indicating a non-protease-dependent mechanism [14]. Furthermore, binding alone of integrins to LAP does not lead to TGF $\beta$  activation [1,14,15,22,49,50]; traction forces generated through  $\alpha\text{v}\beta 6$  binding to the LAP of latent TGF $\beta$  are required. This activation is abolished by actin cytoskeleton inhibitors, truncation of the  $\beta 6$ -endodomain residues that bind to the cytoskeleton, or by deletion of the binding site for the latent TGF $\beta$  to bind to the ECM that is required to generate tensile force across the pro-domain [22]. Thus, LAP anchored to the ECM by LTBP and secured to the cell surface by integrins is distorted by traction between the matrix and cells that permits liberation of active TGF $\beta$  [49–51].

The underpinning mechanism was solved by the Springer group whereby crystal structures of latent TGF $\beta$  revealed a ring-like shape with two LAP prodomain ‘arms’ connected at the ‘elbows’ to crossed ‘forearms’ formed by two TGF $\beta$  monomers and by LAP prodomain ‘straitjacket’ elements that surrounded each TGF $\beta$  monomer (Figure 3). The arms come together at the neck, disulphide linked in a ‘bowtie’, with RGD motifs located at each ‘shoulder’. The RGD motifs are accessible for integrins and nearby exposed hydrophobic sidechains on the body of the arm increase affinity for integrins [15].  $\alpha\text{v}\beta 6$  binds in a 1:2 complex of the  $\alpha\text{v}\beta 6$  head bound to one monomer of the latent-TGF $\beta$  dimer [9]. The actin cytoskeleton generates the force necessary for TGF $\beta$  release from the  $\alpha\text{v}\beta 6$ /pro-TGF $\beta$  complex through the  $\beta 6$ -subunit cytoplasmic domain, with LTBP anchored to the ECM providing countertraction [9]. Integrin headpiece opening increases affinity for TGF $\beta$  by altering a  $\beta$ -leg domain orientation and thence, the direction of the force when traction force is applied to the  $\beta$ -subunit by the actin cytoskeleton [9]. The LAP lasso holds TGF $\beta$  within the straitjacket and covers all of the TGF $\beta$  receptors contact sites [15]. RGD motifs are located on opposite sides of the lasso, so when tensile forces are exerted across them, the lasso is elongated and TGF $\beta$  is liberated [9,52].



**Figure 3.** Force-mediated liberation of TGF $\beta$  by  $\alpha\text{v}\beta 6$ . Left: TGF $\beta$  is held in a ‘straitjacket’ by LAP with the ‘latency lasso’ preventing covering TGF $\beta$  receptor binding sites.  $\alpha\text{v}\beta 6$  activates TGF $\beta$  by binding to RGD motifs on the shoulders of LAP and transducing a force (black arrows) from the actin cytoskeleton. A resistant counter-force is generated by LTBP which are bound to LAP by disulphide bonds (SS). Right: When tensile force is exerted across the structure, the lasso is elongated, the straitjacket unfastens, and active TGF $\beta$  is liberated. Adapted from [15].

This force-dependent mechanism is supported by experiments that demonstrated  $\alpha\text{v}\beta 5$  integrin-dependent release of active TGF $\beta$  by contracting myofibroblast cytoskeletons occurs in a wholly mechanical manner in detergent-treated cells that lack cell membranes or cytosol [53]. Moreover, ferromagnetic beads coated with integrins or anti-LAP beads are capable of liberating TGF $\beta$  from a cell-free matrix [52].

TGF $\beta$  activation by  $\alpha$ v $\beta$ 6 is also dependent on the covalent attachment of LAP to LTBP, specific ECM-binding regions in LTBP-1, and the presence of fibronectin in the matrix [49,51]. LTBP supports  $\alpha$ v $\beta$ 6-mediated activation by concentrating and fixing the latent complex. Recombinant SLC that lacks LTBP fails to activate TGF $\beta$ , and LTBP1-derived TGF $\beta$  binding cassettes that cannot bind to ECM prevent  $\alpha$ v $\beta$ 6-mediated activation. However, if the complex is artificially fixed to the pericellular environment, activation can occur [49]. Fixation of LTBP to the mechano-resistant ECM creates a resistant 'holding force' to the transmembrane 'pulling force' of integrins attached to the actin cytoskeleton, and together, these generate sufficient force to unfasten the straitjacket and liberate TGF $\beta$ .  $\alpha$ v $\beta$ 6 activation is LTBP1-specific, as  $\alpha$ v $\beta$ 6 can activate TGF $\beta$  bound to LTBP1, but not LTBP3 [49].

#### 3.4. Activation of GARP-Bound Latent TGF $\beta$ on Treg Cells

One of the most powerful pro-tumourigenic functions of TGF $\beta$ 1 is to promote an immunosuppressive environment in the TME and recent data highlights the central role of  $\alpha$ v $\beta$ 8 in this process. It has been known for some time that TGF $\beta$  signalling in T cells was required to maintain immune tolerance, since the genetic abrogation of T-cell TGF $\beta$  signalling resulted in lethal autoimmunity [54,55]. The source of the TGF $\beta$  required to promote these signals is only recently becoming clearer. It had also been established that regulatory T (Treg) cells can produce activated TGF $\beta$ 1 that can suppress the activity of helper-T (Th) cells [56,57] and had latent TGF $\beta$ 1 on their surface membranes [58] in association with GARP [59]. Genetic suppression [59] or antibody [60] inhibition of GARP confirmed that GARP was required for activation of TGF $\beta$ 1 by Treg cells but was not sufficient to activate TGF $\beta$ 1. Thus, the expression of GARP in HEK293 cells resulted in the surface expression of latent TGF $\beta$ 1 but no TGF $\beta$ 1 activity. Developing upon the mechanisms identified for the activation of ECM bound latent TGF $\beta$  all five  $\alpha$ v integrins were assessed for their capacity to activate latent TGF $\beta$  on the GARP expressing HEK293 cells; only  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8 generated active TGF $\beta$ , but only if the GARP retained its transmembrane domain; "soluble" GARP-LAP-TGF $\beta$  released from cells expressing a transmembrane-deletion mutant of GARP was not activated by any  $\alpha$ v integrins [23]. These data imply (i) that  $\alpha$ v $\beta$ 8 is the principal activator of GARP-LAP-TGF $\beta$  complexes on Treg cells since  $\alpha$ v $\beta$ 6 is epithelial-specific, a prediction that is now confirmed since either genetic deletion or antibody inhibition of  $\alpha$ v $\beta$ 8 on Treg cells inhibits activation of cell surface TGF $\beta$  [61–63], and (ii) that the GARP-LAP-TGF $\beta$  must be tethered to permit activation to occur, similar to that of LTBP bound latent-TGF $\beta$ . Intriguingly, while  $\alpha$ v $\beta$ 6 forms strong association with the cytoskeleton through conserved domains [64] within its cytoplasmic tail,  $\alpha$ v $\beta$ 8 has a divergent cytoplasmic tail that lacks the conserved domains [65] that predict cytoskeletal interaction. Thus, if  $\alpha$ v $\beta$ 8 mediates the activation of GARP-LAP-TGF $\beta$  through force generation, as  $\alpha$ v $\beta$ 6 does for LTBP1-LAP-TGF $\beta$ , the mechanism for generating transmembrane cytoskeletal force remains unclear.

Another similarity between the activation of LTBP1-LAP-TGF $\beta$  and GARP-LAP-TGF $\beta$  is that the cell responding to the release/exposure of the mature TGF $\beta$  needs to be in contact with the cell where TGF $\beta$  was activated [14,65]. This suggests that either the mature 'free' TGF $\beta$ 1 cytokine is not released from the Treg surface and remains associated with the GARP-LAP complex, or that, if it is released, it does not reach the threshold concentration required to suppress Th cells; biologically the former model is more conservative and provides a much more secure spatio-temporal mechanism for regulating TGF $\beta$  activation in our tissues. The relative importance of one or both mechanisms *in vivo* remains unclear.

GARP is expressed by cells other than Tregs, including platelets, megakaryocytes, fibroblasts, hepatic stellate cells, endothelial cells, some carcinomas, as described in the excellent review by Stockis et al. [25]. Few formal studies have examined if they also exhibit integrin-dependent local activation of TGF $\beta$ . However, in the context of cancer it is perhaps worth noting the platelets. In a recent study of mice whereby the GARP protein was selectively deleted from platelets, the amount of circulating active TGF $\beta$ 1 reduced significantly and the tumour growth rate was reduced, corresponding with an increased immune infiltrate into the TME [66]. Thus, platelets require GARP to produce activated TGF $\beta$  and appear to be an important source of both systemic and tumour-associated TGF $\beta$ 1

mediating immunosuppression. What is not clear is what, if any, roles integrins play in activating platelet TGF $\beta$ 1.

### 3.5. Metalloprotease-Dependent TGF $\beta$ Activation

$\alpha$ v $\beta$ 8 binds LAP- $\beta$ 1 with high affinity (Kd 13 pM).  $\alpha$ v $\beta$ 8 mediated TGF $\beta$ -activation is illustrated by the inhibition of activation with anti- $\alpha$ v $\beta$ 8 antibodies [36]. However, the mechanism of activating ECM-bound TGF $\beta$  is distinct from that of  $\alpha$ v $\beta$ 6. As mentioned previously, the  $\beta$ 8 cytoplasmic-domain is dissimilar to any other  $\beta$ -integrins [65] and may be incapable of linking to the actin cytoskeleton [67], and thus, incapable, directly, of TGF $\beta$ -activation by mechanical transduction [36].

The current understanding of  $\alpha$ v $\beta$ 8-mediated TGF $\beta$ -activation is that LAP $\beta$ 1 and LAP $\beta$ 3 bind with high affinity to  $\alpha$ v $\beta$ 8 on cell surfaces, and  $\alpha$ v $\beta$ 8 brings the latent complexes into juxtaposition with a membrane metalloprotease (MMP) which cleaves LAP, releasing TGF $\beta$ . This is based on a number of findings from a single study. Consistent with a proteolytic event, active TGF $\beta$  is liberated into tumour cell line supernatants and into the aqueous phase of lung cancer xenografts by an  $\alpha$ v $\beta$ 8-dependent mechanism.  $\alpha$ v $\beta$ 8 and MMP-14 co-localise in LAP $\beta$ 1 substrate contacts and  $\beta$ 8-specific RGD inhibitors and MMP-14 inhibitors both block  $\alpha$ v $\beta$ 8-mediated TGF $\beta$  activation, indicating that  $\beta$ 8 and MMP-14 are both required. MMP-14 deficient H1264 lung cancer cell lines are unable to activate TGF $\beta$  via  $\alpha$ v $\beta$ 8, whilst the restitution of MMP-14 rescues  $\alpha$ v $\beta$ 8-mediated TGF $\beta$  activation. Consistent with LAP- $\beta$ 1 as the proteolytic substrate of  $\alpha$ v $\beta$ 8-MMP-14-mediated TGF $\beta$  activation,  $\beta$ 8-overexpressing/MMP-14 expressing H1264 cells cleave and inactivate LAP- $\beta$ 1, whereas  $\beta$ 8-overexpressing/MMP-14 deficient H1264 do not [36].

The mechanism was further supported by the finding that the ability of monocytes to activate TGF $\beta$  also relates to both  $\alpha$ v $\beta$ 8 and MMP-14. TGF $\beta$  activation in monocytes is almost abrogated with an anti- $\beta$ 8 antibody. Whilst all monocyte subsets express similar amounts  $\alpha$ v $\beta$ 8, only CD14+ monocytes activate TGF $\beta$ , suggesting that as with  $\alpha$ v $\beta$ 6, the mere expression of  $\alpha$ v $\beta$ 8 is insufficient to activate TGF $\beta$ . MMP-14 expression varies ten-fold between CD14+ and CD14- monocytes, and expression correlates with the ability to activate TGF $\beta$ . Antibody inhibition of MMP-14 diminished TGF $\beta$  to a similar degree to anti- $\beta$ 8 antibodies [68].

However, MMP knockout mice do not recapitulate the TGF $\beta$ <sup>-/-</sup> phenotype. Furthermore, whilst it has been shown that  $\alpha$ v $\beta$ 8 expression on Tregs is vital for TGF $\beta$  activation, the authors stated that the role of MMP-14 was unclear given that Tregs did not have increased expression of MMP-14 compared with naïve T cells [62]. Moreover, protease inhibitors do not reduce TGF $\beta$  activation [63], indicating that at least in Tregs,  $\alpha$ v $\beta$ 8-mediated TGF $\beta$  activation can occur in a non-MMP dependent manner.

### 3.6. Other Mediators of TGF $\beta$ Activation

The higher affinity for LAP $\beta$ 1 of  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8 possibly explains their role as the major activators of TGF $\beta$ . However, TGF $\beta$  activation can be activated by a number of other integrins.

$\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 3 are widely expressed and although their respective knockout mice do not display phenotypes associated with decreased TGF $\beta$  activation, they have been shown to activate TGF $\beta$  in vitro when expressed in fibroblasts [45,69]. While the mechanisms were uncertain, there are hints that MMPs may play a role for some integrins. There is a correlation between activated  $\alpha$ v $\beta$ 3 and MMP-9 expression in breast cancer cells [70] and the localisation of MMP-2 to the surface of melanoma cells upon interaction with  $\alpha$ v $\beta$ 3 [71]. Both MMP-2 and MMP-9 are capable of activating TGF $\beta$  in vitro [72]. Evidence for mechanical release of TGF $\beta$  by  $\alpha$ v $\beta$ 5,  $\alpha$ v $\beta$ 3, and an unidentified  $\beta$ 1 integrin was demonstrated in contracting myofibroblast cytoskeletons, with stretching of adherent myofibroblasts increasing their ability to liberate TGF $\beta$  [53]. Based on separate studies, it is likely that the  $\beta$ 1 integrin is  $\alpha$ v $\beta$ 1. Mice with hepatic stellate cells with integrin- $\alpha$ v<sup>-/-</sup> do not develop carbon tetrachloride-induced liver fibrosis. However, mice with global knockout of  $\beta$ 3,  $\beta$ 5 or  $\beta$ 6 or conditional knockout in hepatic stellate cells of  $\beta$ 8 do develop liver fibrosis, unless pharmacological blockade of  $\alpha$ v-integrins is administered [73].  $\alpha$ v $\beta$ 1 has also been implicated as a promotor of bleomycin-induced

lung fibrosis. Given that this pathology is also established as  $\alpha\text{v}\beta\text{6}$ -mediated, this suggests that multiple integrins can operate simultaneously to regulate TGF $\beta$ -mediated stromal responses [14,73].

$\alpha\text{v}\beta\text{3}$ ,  $\alpha\text{v}\beta\text{5}$ ,  $\alpha\text{v}\beta\text{6}$ , several  $\beta\text{1}$  integrins and MMP genes are themselves TGF $\beta$  transcriptional targets. Thus, integrin-mediated TGF $\beta$  activation can generate self-amplifying feed-forward loops. In addition, activated TGF $\beta$  induces local fibroblasts to differentiate into myofibroblasts which contracts the ECM. This places anchored LLC under tension and lowers the threshold for activating latent-TGF $\beta$ , augmenting the feed-forward loop and permitting activation by integrins with lower LAP-binding affinity [45]. Crosstalk amongst integrins also modulates TGF $\beta$  activation. Integrin- $\beta\text{1}^{-/-}$  mice or pharmacological inhibition of  $\beta\text{1}$  induces compensatory upregulation of  $\beta\text{3}$  and increases TGF $\beta$  activation in breast cancer cells. However, the overexpression of  $\beta\text{3}$  did not replicate this phenotype after  $\beta\text{1}$  loss, implying that there are other modulators of TGF $\beta$  in this setting [74].

Physiochemical conditions such as heat and low pH can also unfasten the straitjacket and either activate TGF $\beta$  or lower the activation threshold [32]. Of note is the diverse range of non-integrin proteins that have been associated with TGF $\beta$  activation and are discussed comprehensively elsewhere [22].

#### 4. TGF $\beta$ Signalling

Having liberated/exposed the mature TGF $\beta$  cytokine, it can interact with its cognate receptors and promote a variety of intracellular pathways. TGF $\beta$  signaling is described by many excellent reviews including [3,4,17,21] and the following is just a brief overview.

Once activated, TGF $\beta$  triggers signalling in cells by binding to the TGF $\beta$  serine/threonine kinase receptor complex. Upon binding to TGF $\beta$  receptor 2 (TGF $\beta\text{R2}$ ), TGF $\beta$  receptor 1 (TGF $\beta\text{R1}$ ) is recruited, transphosphorylated and activated. Serine/threonine protein kinases in the intracellular domain leads to the recruitment and phosphorylation of the intracellular downstream mediators Smad2 and Smad3. Oligomerisation of phosphorylated Smad2 and Smad3 with Smad4 permits translocation to the nucleus where they act as a transcriptional complex. Inhibitory Smads, including Smad6 and Smad7, regulate the pathway [16,17,21,32,75–79]. The oligomers of Smads acquire different stoichiometries, permitting different signalling thresholds of gene expression [21,80]. TGF $\beta\text{R3}$  ( $\beta$ -glycan) can amplify the signalling cascade by binding TGF- $\beta$  and promoting presentation to TGF $\beta\text{R2}$  [16,17,78]. TGF $\beta\text{2}$  has a lower binding affinity than TGF $\beta\text{1}$  and TGF $\beta\text{3}$  and therefore, relies on  $\beta$ -glycan for high-affinity binding to the TGF $\beta$  receptor complex. Therefore, in endothelial and haematopoietic cells that do not express  $\beta$ -glycan, TGF $\beta\text{2}$  has a limited activity [81,82].

Smad target genes interact with a range of transcription factors. Interactions between these co-activators and co-repressors define the degree of transcription. Although Smads bind to DNA with a 100-fold lower affinity than high-affinity transcription factors, they are required for transcriptional activation [21]. TGF $\beta$  receptors remain active for several hours after ligand binding, and repeated receptor activation maintains Smad complexes within the nucleus [83].

The canonical TGF $\beta$  pathway is Smad-mediated. Smad-independent TGF $\beta$ -activated signalling pathways mediate a non-canonical pathway that includes the PI3K-AKT, Ras/ERK, p38 kinase, and small GTPase (RHOA, PKN, Rock) pathways [16,21,75,84]. Both Smad- and non-Smad dependent pathways may induce TGF $\beta\text{1}$  expression, thus amplifying the TGF $\beta$  response [85]. Although the converging pathways usually results in cooperativity, pathways may counteract each other [21].

#### 5. TGF $\beta$ in Cancer

TGF $\beta$  has a dichotomous role in cancer, emerging as a positive prognostic factor in early tumours, yet as a poor prognostic marker in advanced tumours [77]. A definitive “switch” from tumour suppressor to promoter is not evident. Rather, an accumulation of genetic, epigenetic and cellular events in canonical and non-canonical pathways within cancer cells and the TME drive a phenotype of decreased TGF $\beta$  responsiveness and the increased expression or activation of TGF $\beta$  ligands [17,86,87]. The role of TGF $\beta$  in cancers can broadly be categorised into effects on cell proliferation, induction



of epithelial to mesenchymal transition (EMT), modulation of the TME, and dampening of immune surveillance [88–90]. Below we outline these actions, focusing on the role of integrins.

### 5.1. Proliferation

In normal tissues, TGF $\beta$  counteracts proliferation primarily by inhibiting cell-cycle progression with G1 arrest and stimulation of cyclin-dependent kinase inhibitors [2,20]. This is demonstrated in vivo where integrin  $\beta$ 8-expressing cancer cells are less tumorigenic and have slower growth mediated by increased TGF $\beta$  activation [36]. However in cancer, growth-inhibitory effects of TGF $\beta$  are eventually overcome by either “decapitation” of core pathway components—TGF $\beta$  receptors or Smad transcription factors, or the loss of downstream signalling targets [77]. Within the TGF $\beta$  pathway, loss of function mutations are most common, particularly in gastrointestinal cancers [91], notably in pancreatic cancers and colorectal cancers (up to 100% and 83% of patients respectively) [92,93]. Truncated, functionally inactive TGF $\beta$ R2 is associated with high microsatellite instability cancers [2,77] and Smad4 is mutated in about half of pancreatic, colon, and oesophageal cancers [77].

A number of experimental findings support the increased tumorigenicity associated with abrogated or dysfunctional TGF $\beta$  pathways. In normal keratinocytes, TGF $\beta$ 1 knockout or expression of dominant-negative TGF $\beta$ R2 causes genomic instability and malignant transformation [94]. Cancer cells that have lost sensitivity to TGF $\beta$  due to mutations in TGF $\beta$ R1 but can be re-sensitised by re-expression of TGF $\beta$ R1, also resulting in reduced tumour formation [95].

Integrins are also implicated. A large fraction of integrin- $\beta$ 6<sup>-/-</sup> mice have spontaneously occurring tumours, with the phenotype linked to deficient TGF $\beta$  activity [96].  $\alpha$ v $\beta$ 6 blockade increases tumour cell proliferation in both early and late stage disease, depending on the presence of Smad4 [97]. In addition, inappropriate  $\alpha$ 6 $\beta$ 4 expression in stratified squamous epithelia has been found to inhibit TGF- $\beta$  signalling and increase tumorigenicity by impeding TGF $\beta$ -mediated suppression of clonal expansion of initiated cells within the epidermal basal layer [98,99].

### 5.2. EMT & Metastasis

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose or reduce cell–cell and cell–substrate adhesion and transition to a more mesenchymal-like phenotype, aiding migration, invasion, and metastatic spread [100]. The loss of E-cadherin, typically located at cell–cell adhesion junctions and essential for maintaining an epithelial phenotype, is a hallmark of EMT [101]. The TGF $\beta$  pathway can mediate the entire switch from epithelial to mesenchymal phenotype by repression of epithelial gene signatures, including E-cadherin, and the elevation of mesenchymal genes such as  $\alpha$ -SMA and vimentin [42,102]. Smad-mediated pathways induce the loss of cellular adhesion and non-canonical pathways, particularly as RHO and AKT promote migration and invasion [103–105].

Integrins potentiate TGF $\beta$ -mediated E-cadherin downregulation. In renal cancer cells, cyclo-RGD peptide  $\alpha$ v $\beta$ 3 ligand and TGF $\beta$ 1 inhibit E-cadherin with a synergistic effect [106]. During EMT of mammary epithelial cells,  $\beta$ 1-integrins induce TGF $\beta$  dependent p38 MAPK activity [107]. In basal cell carcinoma organotypics,  $\alpha$ v $\beta$ 6-mediated TGF $\beta$  activation induces differentiation of fibroblast into myofibroblasts with subsequent induction of tumour cell invasion [108]. Similar effects have been shown with  $\alpha$ 5 $\beta$ 1,  $\alpha$ v $\beta$ 3, and  $\alpha$ v $\beta$ 5. These other integrins are not normally highly expressed in epithelial cells but are induced by TGF $\beta$  signalling, consistent with the feed-forward loop discussed earlier. Antagonising TGF $\beta$  in these cells blocks induction of these integrins and reduces invasion and metastasis [109], indicating that the inhibition of integrin-mediated TGF $\beta$  pathways may reduce metastasis. Indeed, TGF $\beta$  responsive stromal signalling has been demonstrated to drive metastasis. In breast cancer cells, it has been shown to promote single cell motility and intravasation and to be essential for blood-borne metastases [110]. In Her2-positive breast cancer xenografts,  $\alpha$ v $\beta$ 6-expression is associated with increased metastases, and treatment with the anti- $\alpha$ v $\beta$ 6/ $\alpha$ v $\beta$ 8 antibody causes decreased metastases and pSmad2 [111]. In breast ductal carcinoma in situ,  $\alpha$ v $\beta$ 6 expression is associated with progression to invasive cancer. In studies to elucidate the mechanism, overexpression

of  $\beta 6$  in myoepithelial cells was found to activate TGF $\beta 1$  and upregulate MMP9. The blockade of either MMP or TGF $\beta$  inhibited the ability of  $\beta 6+$  myoepithelial cells to promote invasion, suggesting that  $\alpha v\beta 6$  mediates TGF $\beta$  promotion of an invasive phenotype [112].

In colon cancer organoids and in vivo models, transcriptional activation of integrin- $\alpha v\beta 6$  enhances tumorigenicity, with autocrine TGF $\beta$  implicated as the mediator. In patients with colon cancer, tumour  $\alpha v\beta 6$ -expression is a powerful prognostic marker with a median survival of 16.5 vs 4.8 years in those with low vs high expression [113]. Similarly, in squamous cell carcinoma organotypics, collagen-7 deletion induced increased invasion,  $\alpha v\beta 6$  upregulation, and increased matrix fibronectin. These findings were suppressed by co-inhibition with TGF $\beta R1$  inhibitors. In vivo similar results were observed, along with increased pSmad2/3 signalling in the stroma of collagen-7 deficient SCC tumours, implying that collagen-7 acts as a TGF $\beta$  suppressor, and consistent with  $\alpha v\beta 6$ -dependent TGF $\beta 1$  activation inducing fibroblast to myofibroblast transition [114].

TGF $\beta$  signalling is central to the formation of bone metastases and osteolytic destruction of adjacent bone in both prostate and breast cancers, with TGF $\beta$  blockade inhibiting bone metastases development [115–117].  $\alpha v\beta 6$  has been shown to be expressed in prostate cancer bone metastases, and in vivo models of bone metastasis have shown that  $\alpha v\beta 6$  promotes osteolysis through upregulation of MMP-2 and parathyroid hormone-related protein [118]. A subsequent study revealed that  $\alpha v\beta 6$  was required for TGF $\beta 1$ -mediated MMP-2 expression in prostate cancer cells [119]. Of note is the fact that in this study,  $\alpha v\beta 6$  induced TGF $\beta$  signalling by interacting with TGF $\beta R2$ ; although a direct association between and  $\alpha v\beta 6$ -TGF $\beta R2$  was not fully confirmed [119].

### 5.3. Angiogenesis

Blood vessel proliferation permits tumour growth by providing adequate nutrient supplies, removal of waste products, and routes for metastasis [120]. Pancreatic cancer cells with activated TGF $\beta$  induce angiogenesis when implanted into mice [121] whilst TGF $\beta$ -blocking antibodies or targeted deletion of TGF $\beta$  in mice results in decreased angiogenesis [122]. Epithelial cells with activated TGF $\beta$  show increased gene expression of the pro-angiogenic vascular endothelial growth factor (VEGF) and thrombospondin [17]. Of note, the TGF $\beta$  effects appear concentration-dependent. In endothelial cells, sheets derived from mouse metatarsals low TGF $\beta$  concentrations are pro-angiogenic, but high concentrations are anti-angiogenic. Furthermore, in this setting, integrin- $\alpha 5\beta 1$  is a key mediator of TGF $\beta R1$  and VEGF promotion of angiogenesis. TGF $\beta R1$  inhibitors and VEGF synergistically upregulate  $\alpha 5$  and  $\beta 3$  integrin gene expression, with downregulation or antibody blockade of  $\alpha 5$  inhibiting this co-operative effect [123,124]. Similarly,  $\alpha v\beta 3$  and  $\alpha v\beta 5$  deletion accelerates tumour angiogenesis through increased expression of VEGF2 and sensitivity to VEGF-A [125,126].

$\alpha v\beta 8$  is also implicated in tumour angiogenesis. Integrin- $\beta 8^{-/-}$  mice develop abnormal cerebral vasculogenesis [37,127]. In glioma, orthotopically implanted  $\beta 8$ -high astrocytoma cells develop microscopic non-haemorrhagic tumours with uniform vessels, whereas  $\beta 8$ -low cells cause large haemorrhagic tumours with an abundant vasculature and a lower Smad2/3 expression in endothelial cells, implying TGF $\beta$ -mediation [128].

### 5.4. Stroma

TGF $\beta$  modulates the ECM by different mechanisms. TGF $\beta$  promotes fibroblast-to-myofibroblast transdifferentiation and stimulates cancer-associated fibroblasts (CAF) to produce ECM- and cell-adhesion proteins including integrins, collagen, and fibronectin [17,129]. Integrins are also implicated as mediating this process in CAFs—another feed-forward loop [10,130]. TGF $\beta$ -induced mesangial cell collagen expression has been shown to require integrin-mediated FAK activation [131] and  $\alpha 3\beta 1$  potentiates TGF $\beta$ -induction of MMP-9 in immortalised keratinocytes [132].

Stromal TGF $\beta R2$  expression decreases with tumour progression and is a poor prognostic marker in colorectal cancer [17,133]. The attenuation of TGF $\beta$  at either receptor or Smad level results in increased myeloid-derived suppressor cells and stromal fibroblast activation, driven by increases in

CXCL1 and CXCL5 expression, which are normally inhibited by TGF $\beta$ . This is demonstrated in vivo by increased desmoplasia and angiogenesis in mice pancreatic epithelium expressing a dominant-negative TGF $\beta$ -receptor [17].

A number of studies have implicated  $\alpha\text{v}\beta 6$  as a mediator of TGF $\beta$ -induced stromal changes.  $\alpha\text{v}\beta 6$  blockade with the antibody 6.3G9 in Detroit-562 pharyngeal tumour cells had no effect on TGF $\beta$ -mediated proliferation in vitro, but did inhibit in vivo xenograft tumour growth, suggesting that the TME has an important regulatory role.  $\alpha\text{v}\beta 6$  expression in the stroma correlated with  $\alpha$ -SMA, although treatment had no effect on stromal collagen and smooth muscle actin levels [134]. A similar study in Detroit-562 also found limited effects in vitro when treated with 264RAD and a dose-dependent reduction of tumour growth in vivo, which, in this study, was associated with reduced stromal fibronectin and  $\alpha$ -SMA [134–136]. This difference may be due to the additional blockade of  $\alpha\text{v}\beta 8$  with 264RAD, which was not explored at the time. 264RAD also reduces stromal  $\alpha$ -SMA expression in MCF7 and HER2-18 breast cancer xenografts with associated Smad2 reductions [111]. Similarly, a subset of non-small cell lung cancer (NSCLC) cell lines co-cultured with fibroblasts induced an activated CAF phenotype with  $\alpha$ -SMA expression. CAF activation was associated with  $\alpha\text{v}\beta 6$ , and activation was blocked by either 264RAD or the TGF $\beta$ R kinase inhibitor SB321542. However, after three days co-culture, only SB321542 was effective, indicating that whilst initial CAF activation was  $\alpha\text{v}\beta 6$ -dependent, once activated, alternative TGF $\beta$  activating pathways maintain the phenotype [53,136]. Treatment of  $\alpha\text{v}\beta 6$ -expressing transgenic pancreatic ductal adenocarcinoma (PDAC) with 264RAD plus gemcitabine significantly increased overall survival compared with gemcitabine alone and was associated with reductions in both pSmad3 and nuclear Smad4 levels, as well as reductions in  $\alpha$ SMA-positive fibroblasts and blood vessel density, both targets of TGF $\beta$  signalling [137].

Whilst the consensus is that TGF $\beta$ 1 activated CAF rich stroma is tumour-promoting, conflicting data exist. For example, in transgenic PDAC mice models, depletion of  $\alpha$ -SMA-expressing cells, designed to eliminate CAFs, unexpectedly induced invasive undifferentiated tumours with poorer survival, despite associated reductions in fibrosis in both advanced and precursor lesions. Fewer tumour myofibroblasts were also associated with poorer survival in patients with pancreatic cancer. In the mice, the reduced fibrosis was strongly associated with increased Tregs [138]. Separate studies have also found that sonic hedgehog pathway inhibition in mouse pancreatic cancer models reduces stromal density but causes more aggressive tumours [139]. A subsequent study showed that PDAC stroma has at least two types of CAFs, inflammatory (iCAF) and  $\alpha$ SMA-expressing myofibroblasts (myCAFs) that had distinct transcriptional and secretory profiles and were likely to affect tumour growth differently [140]. Thus, the earlier study [138] would have eliminated only the myCAFs and left the iCAFs present. In a recent study, at least four types of pancreatic stellate cell (a specialized pancreatic fibroblast) existed, suggesting even greater heterogeneity [141]. It has not been established whether each subtype responds differently to TGF $\beta$ , nor which are tumour-promoting or suppressive. Thus, caution would be prudent when therapeutically targeting CAFs in PDAC, and one assumes cancers in other tissues as well, as it is not clear which types of CAF are the tumour-promoting cells.

Recently, studies have identified exosomes as mediators of TGF $\beta$  activation between cancer cells and the TME through horizontal propagation of integrin-associated phenotypes in a paracrine manner. Integrin-expressing exosomes can directly transfer integrins to target cells [142,143], with transfer from cancer cells to benign cells inducing an  $\alpha\text{v}$  integrin-mediated aggressive migratory phenotype [144]. Exosomally transported  $\alpha\text{v}\beta 6$  from gut epithelial cells have been shown to be transferred to mucosal dendritic cells, expressed on the surface, activate local TGF $\beta$ , and confer tolerogenic properties to dendritic cells including induction of TGF $\beta$  production by Tregs [145]. Within cancer, prostate cancer cells have been shown to efficiently transfer  $\alpha\text{v}\beta 6$  via exosomes to  $\alpha\text{v}\beta 6$  negative cells and localize to the cell surface with subsequent enhanced adhesion and LAP-TGF $\beta$  migration in vitro [146], although it remains to be seen whether this occurs in stromal cells within the TME. Cancer exosomes are also implicated in increased TGF $\beta$  signaling. Exosomes purified from stromal fibroblasts from patients

with oral squamous cell cancer (SCC) have been demonstrated to contain TGF $\beta$ R2, and when these exosomes are transferred into TGF $\beta$ R2-deficient SCC keratinocytes, TGF $\beta$  signaling increases [147].

### 5.5. Immunomodulation

Tumour progression is aided by promoting regulatory immune cell subsets and dampening anti-tumour immunity. TGF $\beta$  influences these processes in both the innate and adaptive immune systems. High tumour TGF $\beta$  concentrations attracts myeloid and lymphoid cells as described previously [17,87,133,148]. Accordingly, it may be that the immunosuppressive function of TGF $\beta$  can be modulated or even abrogated by modulating the extent of TGF $\beta$  activation rather than TGF $\beta$  production [149], inferring a possible role for integrins.

Many of the effects of TGF $\beta$  on immune cells result from an ability to polarise cells towards an alternative differentiation status that is immunosuppressive and pro-tumorigenic (excellently reviewed in [150]). TGF $\beta$  induces pro-tumourigenic 'M2'-macrophages and their presence is a poor prognostic marker in many cancers including ovarian, breast, gall-bladder, oral, oesophageal, and non-small cell lung carcinoma [151,152].  $\alpha$ v $\beta$ 6 is independently associated with the induction of M2 macrophages, with demonstration in prostate cancer that  $\alpha$ v $\beta$ 6 containing exosomes are transferred to monocytes and promote M2 polarisation, whereas  $\alpha$ v $\beta$ 6 downregulation in exosomes inhibited M2 polarisation [153]. The study implicates  $\alpha$ v $\beta$ 6-mediated modulation of the STAT1/MX1/2 signaling pathway, but whether  $\alpha$ v $\beta$ 6-mediated TGF $\beta$  activation contributes is unclear. Neutrophils can also acquire a tumour permissive 'N2' phenotype in a TGF $\beta$ -dependent manner, with a subset of low-density neutrophils shown to accumulate with tumour progression [154,155]. TGF $\beta$  also inhibits NK cell maturation [156,157].

The loss of Smad4 signalling in T cells, but not in epithelial cells, is associated with spontaneous epithelial cancer formation throughout the gastrointestinal tract in mice [158]. TGF $\beta$  has been shown to directly suppress CD8+ cytotoxic T-lymphocyte (CTL) function through transcriptional repression of key proteins including perforin, granzyme, and cytotoxicins [159]. Tregs act to counter CTL activity and maintain immunological tolerance. Treg accumulation is associated with poor survival, particularly in cervical, renal, melanoma, breast, liver, and gastric cancer [160]. Thus, TGF $\beta$  promotion of Treg activity is a significant contributor to tumour progression. Similar multi-organ inflammation of TGF $\beta$ <sup>-/-</sup> mice is seen with TGF $\beta$  signalling abrogation specifically in T-cells, implying T-cell mediation of the TGF $\beta$ <sup>-/-</sup> phenotype [54,161]. Treatment of naïve T-cells with TGF $\beta$  can induce differentiation to Tregs [87,159,162], which, in liver cancer has been shown to be mediated by CCL22 promotion [163].

$\alpha$ v $\beta$ 8-mediated TGF $\beta$  activation is crucial in preventing anti-tumour immunity through enhanced Treg activity. Integrin- $\alpha$ v $\beta$ 8 is expressed in Tregs, but not naïve T-cells. Studies have indicated that Tregs require  $\alpha$ v $\beta$ 8 to liberate TGF $\beta$ 1 from the GARP complex, that  $\alpha$ v $\beta$ 8-deficient Tregs are incapable of inducing differentiation of naïve T cells, and that  $\beta$ 8 expressed by Tregs can suppress aberrant T-cell-mediated inflammation [61,62]. Accordingly, mice with conditional knockout of  $\alpha$ v $\beta$ 8 on antigen-presenting DCs develop increased T-cell activation with consequential autoimmunity [41,68,164]. It is possible that tolerance is induced by  $\alpha$ v $\beta$ 8 on DCs releasing TGF $\beta$  from Tregs via cell-cell contact, inducing a larger Treg pool [23]. As described above, Tregs have been shown to be specialised activators of TGF $\beta$  via  $\alpha$ v $\beta$ 8 expression. Tregs express 50–100-fold higher levels of integrin- $\beta$ 8 and increased ability to activate latent-TGF $\beta$  than naïve and effector memory T cells. Unlike Tregs,  $\beta$ 8-knockout Tregs are unable to suppress CD4+ T cell expansion in vivo, indicating  $\alpha$ v $\beta$ 8 is necessary to limit this effector T-cell function [62]. In addition, anti- $\beta$ 8 antibodies have been shown to block Treg-mediated immunosuppression in vivo [63].

Macrophages also activate TGF $\beta$  through an  $\alpha$ v $\beta$ 8-dependent mechanism that can counter pro-inflammatory cytokine production. This is supported by the finding that  $\alpha$ v $\beta$ 8 is upregulated on M2-macrophages and downregulated on pro-inflammatory M1-macrophages, and also by the ability to almost fully suppress TGF $\beta$  activation in monocytes treated with anti- $\alpha$ v $\beta$ 8 antibodies [68].

Crucially, TGF $\beta$  inhibition has been shown to modulate antitumour immune responses with enough potency to mediate tumour regression. Preclinically, transgenic mice with EL-4 thymoma or B16-F10 melanoma xenografts grown in mice with T cells deficient in TGF $\beta$  signalling generate tumour-specific CTLs that eradicate tumours [165]. In colorectal cancer mouse models, TGF $\beta$  is a primary mechanism of immune evasion that promotes T-cell exclusion and inhibits CTL maturation, and although PD-1 or PD-L1 immune checkpoint inhibition has limited efficacy, TGF $\beta$ R1 inhibition with galunisertib produces potent and enduring anti-tumour T-cell responses, and rendered tumours susceptible to anti-PD-L1 therapy. Galunisertib efficacy was abolished with the depletion of CD8 T-cells, indicating efficacy was through augmentation of anti-tumour immunity rather than other TGF $\beta$  mechanisms [166]. Similarly, in a melanoma model, B16 tumours-derived Tregs were able to suppress CTL-mediated killing of explanted tumour cells, with restoration by neutralising antibodies against TGF $\beta$  on the Tregs [167].

Excitingly, TGF $\beta$  has recently been identified as a key source of resistance to immune checkpoint inhibitors in patients. In a large trial of patients with metastatic urothelial cancer treated with the anti-PD-L1 antibody atezolizumab, a lack of response was associated with a TGF $\beta$  signature on fibroblasts in the TME. Tumours were found to exhibit three distinct immune phenotypes—inflamed, excluded, or desert [168,169]. The TGF $\beta$  signature was only associated with lack of response in the excluded tumours, consistent with TGF $\beta$ -mediated fibroblast activation in the TME. In associated studies in mice with EMT6 breast cancer tumours, which display an immune-excluded phenotype, treatment with either TGF $\beta$  or PD-L1 blocking therapy had a minimal effect, but combination treatment led to increased tumour infiltrating T-cells and tumour regression. Combination therapy had limited impact on T-cell or macrophage TGF $\beta$  signatures, and thus, the efficacy was attributed to reprogramming stromal fibroblasts and increased TME CTLs [170].

## 6. Therapeutic Targeting of TGF $\beta$

TGF $\beta$  inhibitors have as yet failed to deliver the anticipated clinical efficacy [86,171,172]. Recent findings have highlighted that the principal anti-tumour effect of global TGF $\beta$  inhibitors may not be against cancer cells as originally believed, but by modulating the TME, as discussed above. Thus, combination therapy with agents that target cell proliferation may elucidate a clearer role for TGF $\beta$  inhibitors. Given the role of  $\alpha$ v-integrins in liberating active TGF $\beta$ , therapeutic inhibition of surface  $\alpha$ v-integrin expression may be a more effective method of suppressing local TGF $\beta$  activation than current therapies which predominantly inhibit TGF $\beta$  receptor activation or downstream signal transduction. Furthermore, the inhibition of  $\alpha$ v-integrins may interrupt the feedforward loop of TGF $\beta$  activation described several times in this review and thus, prevent self-amplifying tumour-stroma interactions that lead to malignant progression. The most significant potential role will be in patients with tumours where  $\alpha$ v $\beta$ 6 is a poor prognostic marker, notably colon [113], cervix [173], lung [124], and breast cancers [111], and in cancers with high  $\alpha$ v $\beta$ 6 expression, notably pancreatic [174], oesophageal [134], and skin cancer [134]. Certainly, some pre-clinical data suggest that blockade of  $\alpha$ v $\beta$ 6 does suppress local TGF $\beta$  signaling [114,132,135].

Of the integrins that activate TGF $\beta$ , only anti- $\alpha$ v $\beta$ 3 antibodies and peptides have been evaluated in patients, none with clinical success [175,176]. The pan- $\alpha$ v blocking antibody abituzumab was evaluated with or without standard therapy in metastatic colorectal cancer. Whilst overall, there was no survival benefit, in patients with high  $\alpha$ v $\beta$ 6 tumour expression, the risk of death was reduced by 59% with abituzumab [177]. However, no endpoints relating to TGF $\beta$  are available. Cilengitide was evaluated in glioblastoma in two large clinical trials: CENTRIC (Phase 3) [178], and CORE (Phase 2) [179]. Tumour  $\alpha$ v $\beta$ 3 expression was associated with improved survival in patients treated with cilengitide in CORE, but not CENTRIC. However, in CORE there was no evidence that pSMAD levels changed in relation to  $\alpha$ v $\beta$ 3/ $\alpha$ v $\beta$ 5 levels, indicating that survival may not be controlled by  $\alpha$ v $\beta$ 3/ $\alpha$ v $\beta$ 5-dependent TGF $\beta$  process [180]. It is worth noting that the discovery that GARP is required for Treg activation of TGF $\beta$  and thus functionality and that antibodies to GARP can inhibit this

process has resulted in investigations of GARP as a therapeutic target for suppressing Treg-dependent pathologies (discussed in [25]).

Anti-PD-1, PD-L1, and CTLA-4 immune checkpoint inhibitors are licensed in a number of cancers, with remarkably potent and durable anti-tumour effects in a subset of patients [181]. Given that around 70–80% of patients experience side effects, yet less than a third of patients benefit from the expensive therapy, elucidating biomarkers for response or resistance is vital [182–185]. Thus, the ability to use a TGF $\beta$  signature to identify patients with urothelial cancer that are unlikely to benefit from PD-L1 inhibition is clinically very useful, and it will be interesting to see if it translates to other cancers. The finding that combination TGF $\beta$  and PD-L1 overcomes resistance to PD-L1 checkpoint-blockade is exciting and will undoubtedly be evaluated clinically to see if the benefits of checkpoint inhibitors can be extended to more patients [170]. However, over 70% of patients treated with checkpoint inhibitors develop autoimmune related toxicity [186], and given the TGF $\beta^{-/-}$  inflammatory phenotype discussed earlier, a potential concern is that combination therapy may be too toxic. Again, local targeting of TGF $\beta$  by integrin inhibition may overcome this issue, although as yet, very little has been published on the effect on the immune microenvironment when integrins are targeted in cancer. In advanced carcinomas, targeting of both  $\alpha\nu\beta 6$  and  $\alpha\nu\beta 8$  to eliminate the TGF $\beta$  producing capacity of the tumour, monocytes, dendritic cells and Tregs, would seem an appropriate future strategy.

## 7. Conclusions

TGF $\beta$  has a dichotomous role in cancer. In normal tissue and early tumours, it acts as a potent tumour suppressor, but with loss or inactivation of TGF $\beta$  downstream signalling it becomes a tumour promotor, inducing EMT, metastasis, angiogenesis, stromal changes and an immunosuppressive TME. TGF $\beta$  has been implicated as a key mediator of resistance to immune checkpoint inhibitors in patients, and combination therapy in mice with TGF $\beta$  inhibitors has been shown to overcome this resistance. However, global inhibitors of TGF $\beta$  have failed to achieve the clinical benefits expected owing to co-suppression of TGF $\beta$ -dependent homeostatic activities that resulted in off-target toxicity. Thus local tissue inhibition of TGF $\beta$  activation may offer improved therapeutic outcomes.

Integrins  $\alpha\nu\beta 6$  and  $\alpha\nu\beta 8$  are specialised to liberate TGF $\beta$  from the latent complex in which it is embedded in the ECM or on the surface of immunosuppressive Tregs and monocytes. Experimentally studies inhibiting of  $\alpha\nu\beta 6$  or  $\alpha\nu\beta 8$  have resulted in reduced TGF $\beta$  signaling, reduced cancer growth, reduced tumour immunosuppression and changes in the TME that suggest a less permissive environment. These data offer hope that similar observations might be possible in humans. Additionally, antibody inhibition of GARP may offer a more selective means to reduce tumour immunosuppression mediated by Tregs and monocytes. Integrins other than  $\alpha\nu\beta 6$  and  $\alpha\nu\beta 8$  may activate TGF $\beta$  when the threshold for activation is lowered, such as when the ECM is under tension, as it would be during a fibrotic stage of growth. This can result in cross-talk between integrins and TGF $\beta$  production and activation that can mediate a self-amplifying feed-forward loop of TGF $\beta$  activation.

As yet, it remains unclear how anti-integrin therapies affect the tumour immune microenvironment. However, it is worth investigating if such therapies may be superior to specific TGF $\beta$  or TGF $\beta$  pathway inhibitors in reducing TME immunosuppression and augmenting the anti-tumour immunity mediated by checkpoint inhibitors and other biotherapeutics such as chimeric antigen receptor-T cells.

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