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ORIGINAL ARTICLE

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Modulation of growth factor binding properties of α2-macroglobulin by enzyme therapy

Abstract *Purpose*: To investigate the binding of transforming growth factor-beta (TGF-β) to human α2-macroglobulin upon oral treatment of patients with proteases. *Methods*: Volunteers were given a cocktail of active proteinases (Phlogenzym) composed of trypsin, bromelain and the additive rutoside orally over a period of 7 days at low dose followed by a bolus application. Before and after medication plasma was immediately withdrawn and binding of ^{125}I-TGF-β to the proteinase inhibitor α2-macroglobulin was determined by electrophoresis and γ-counting. Cell culture experiments were performed to study the effect of transformed α2-macroglobulin on TGF-β-stimulated proliferation of skin fibroblasts. *Results*: Ingestion of proteinases was found to trigger the formation of intermediate forms of α2-macroglobulin displaying high affinity to TGF-β. Maximum binding of TGF-β was observed 1–2 h after bolus ingestion, and steadily levelled off with time. In vitro experiments demonstrated that complex formation of diverse proteinases (trypsin, α-chymotrypsin, bromelain and plasmin) with α2-macroglobulin conferred binding of ^{125}I-TGF-β. α2-Macroglobulin transformed by methylamine or proteinases was found to abolish the TGF-β effect on fibroblasts in cell culture. *Conclusions*: Intestinal absorption of proteinases triggers the formation of TGF-β binding species of α2-macroglobulin in blood. Mediated by this process high concentrations of TGF-β might be reduced via enhanced clearance of α2-macroglobulin-TGF-β complexes. Thus, proteinase therapy may have beneficial effects in treatment of fibrosis and certain cancers accompanied by excessively high TGF-β concentrations.

Key words Proteinase therapy · TGF-β · α2-macroglobulin · Cancer · Fibrosis

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Introduction

Human α2-macroglobulin (α2-M) is an important proteinase inhibitor in blood. It may neutralise a wide spectrum of different proteinases due to a unique mechanism of inhibition [27]. Cleavage of a specific peptide bond in the bait region of the inhibitor induces caging of the enzyme by a conformational change. Most entrapped enzymes are still active to small substrates or polypeptides but are not able to cleave larger proteins. In contrast to the native inhibitor, α2-M-proteinase complexes have acquired new biological properties. They can bind with different affinities growth factors and cytokines [18]. Thus, they are involved in regulation of cell growth, and affect the bio-availability of regulatory polypeptides in blood and tissue [3]. In vitro, a cytokine-binding form of α2-M can be obtained by treatment with reactive primary amines, however, the reaction with proteinases is the main route of transformation of the inhibitor in vivo. Regulatory polypeptides with high affinity to α2-M are transforming growth factor-beta (TGF-β) [32], TNF-α [33] and amyloid-β [25]. Binding to α2-M may provide protection of the cytokine or degradation by the complexed proteinase, however, in most cases, it may initiate fast clearance from circulation and tissue by a specific endocytosis receptor [18]. The clearance receptor of α2-M which is identical to the low-density lipoprotein receptor-related protein (LRP) is ubiquitously distributed in the tissue [29]. High receptor expression has been found on fibroblasts [4] and hepatocytes [26]. This receptor rapidly internalises ternary complexes of α2-M with proteinases and associated polypeptides with a half-life of about 5 min [18].

The aim of the study was to investigate the effect of orally applied proteinases such as trypsin and bromelain on the growth factor binding properties of α2-M in plasma. Proteinases have already been applied in the management of glomerulonephritis [34], autoimmune diseases [31] and animal allograft arteriosclerosis [10]. However, the precise mechanism of proteinase action is still unknown. Our study indicated that oral application of proteinases triggers the conformation of endogenous α2-M leading to increased binding of TGF-β. Furthermore, cell culture experiments indicated the growth inhibition of TGF-β-stimulated fibroblasts by transformed α2-M. Modulating the activity of TGF-β may be a therapeutic strategy for fighting human diseases linked to altered TGF-β metabolism.

Materials and methods

Materials

Native α2-M, methylamine-treated α2-M (α2-M-MA), and ELISA test kits MacroTrans and MacroNat for determination of the concentrations of total and transformed α2-M were obtained from BioMac GmbH (Leipzig, Germany). Trypsin, bromelain, and α-chymotrypsin were obtained from Mucos Pharma GmbH (Geretsried, Germany). ^{125}I-TGF-β (30 TBq/mmol) was obtained from Amersham-Pharmacia Biotech (Freiburg, Germany).

Gel electrophoresis

Native (non-denaturing) gel electrophoresis was performed in 4–20% polyacrylamide gradient gels. Rate-electrophoresis was applied to separate native and transformed α2-M [2].

Preparation of α2-macroglobulin-proteinase complexes

Native α2-M was reacted with 2 *M* excess of trypsin, α-chymotrypsin, bromelain, and plasmin at 25 °C for 2 min (trypsin, α-chymotrypsin, bromelain), or 15 min (plasmin), respectively. After reaction the samples were immediately subjected to HPLC using a Bio-Sil SEC 125 column (7.8 × 300 mm) equilibrated with 50 m*M* sodium phosphate, 1 *M* NaCl, pH 7.4. The purity of the complexes was analysed by electrophoresis and western blotting. No free proteinases were detectable in the samples of the complexes. The success of transformation of α2-M by proteinases was assessed by ELISA.

Binding analysis

The binding of radiolabelled TGF-β to α2-M was studied by electrophoresis. Samples (α2-M, α2-M-proteinase complexes or plasma) were incubated with ^{125}I-TGF-β at 37 °C, and then subjected to polyacrylamide gel electrophoresis. After the run the gels were stained with Coomassie Blue R 250. After gel drying autoradiographic development was achieved using Hyperfilm MP (Amersham-Pharmacia Biotech). For quantitative analysis respective zones corresponding to exposed areas on the X-ray films were cut off the gel and γ-counted.

Methylamine treatment of plasma

To generate transformed α2-M in plasma, 1 ml fresh human plasma was treated with 200 m*M* methylamine (MA) for 2 h at 25 °C. Excess of MA was removed by intensive dialysis against PBS to obtain methylamine-treated plasma (MA-plasma).

Cell proliferation assay

Normal skin fibroblasts were isolated from the foreskin obtained by circumcision of 3 to 10-year old boys, and cultivated as recently described [4]. The effect of TGF-β and α2-M on fibroblast proliferation was measured using the WST-1 cell proliferation assay (Boehringer Mannheim GmbH).

Enzyme treatment

Two volunteers were given daily 3 × 3 tablets of Phlogenzym (48 mg trypsin, 90 mg bromelain, 100 mg rutoside each) for 7 days followed by a bolus ingestion of 30 tablets. Blood was withdrawn from the subjects before medication was started (−7 days), 1 h before bolus ingestion (−1 h) and 1 h (+1 h), 2 h (+2 h), 5 h (+5 h) and 24 h (+24 h) after bolus application. EDTA-plasma was obtained by centrifugation and the samples were immediately stored at −20 °C.

Results

Volunteers were given Phlogenzym according the schedule above, and plasma was analysed for growth factor binding to α2-M. Respective plasma samples were incubated with radiolabelled TGF-β, and separated by rate-electrophoresis. This method allows separation of native (slow-migrating form) and transformed (fast-migrating form) α2-M. Binding of ^{125}I-TGF-β to α2-M was found to be stable under conditions of non-denaturing electrophoresis. Figure 1 exemplifies binding of ^{125}I-TGF-β to α2-M of the plasma of volunteer 1 collected at different times before and after bolus administration. As expected, ^{125}I-TGF-β strongly binds to methylamine-treated α2-M present in MA-plasma. ^{125}I-TGF-β also reacts with the slow-migrating form of α2-M with a maximum binding in plasma taken 1h after bolus

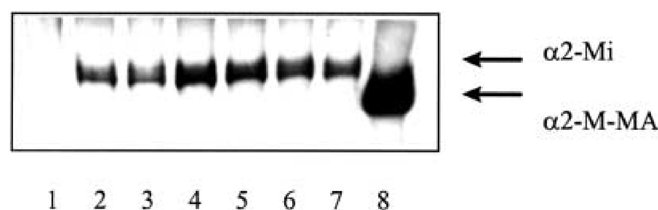

Fig. 1 Effect of proteinase ingestion on binding of ^{125}I-TGF-β to α2-macroglobulin measured by rate-electrophoresis. Plasma samples (12 µl) were incubated with 3.5 µl ^{125}I-TGF-β (250,000 cpm) in a total volume of 60 µl PBS for 4 h at 37 °C. Aliquots (20 µl) were loaded on to polyacrylamide slab gels and separated by rate-electrophoresis. After staining with Coommassie Blue R 250 the gel slabs were dried and exposed to X-ray films. *Lane 1*: ^{125}I-TGF-β without plasma; *lane 2*: ^{125}I-TGF-β + plasma (−7 days); *lane 3*: ^{125}I-TGF-β + plasma (−1 h); *lane 4*: ^{125}I-TGF-β + plasma (+1 h); *lane 5*: ^{125}I-TGF-β + plasma (+2 h); *lane 6*: ^{125}I-TGF-β + plasma (+5 h); *lane 7*: ^{125}I-TGF-β + plasma (+24 h); *lane 8*: ^{125}I-TGF-β + methylamine-treated plasma (*MA-plasma*). α2-M-MA methylamine-treated α2-macroglobulin, α2-Mi intermediate form of α2-macroglobulin

application. However, the observed autoradiographic bands on the X-ray film could not be superimposed completely on to the α2-M protein band in the polyacrylamide gel. Additional experiments revealed the association of radioactivity with the leading edge of the slow-migrating α2-M. It is known that partially transformed, intermediate forms of α2-M (α2-Mi) move at that position between native and totally transformed α2-M-MA.

Quantitative analysis of TGF-β-α2-M interaction revealed significantly increased binding of ^{125}I-TGF-β to α2-M of the plasma taken 1 h and 2 h after bolus application, compared with the blank (−7 day value), ($P < 0.05$), (Fig. 2). With further time after bolus application, binding of ^{125}I-TGF-β to α2M levels off. The results clearly indicate the creation of an α2-M species with high affinity to TGF-β by enzyme treatment.

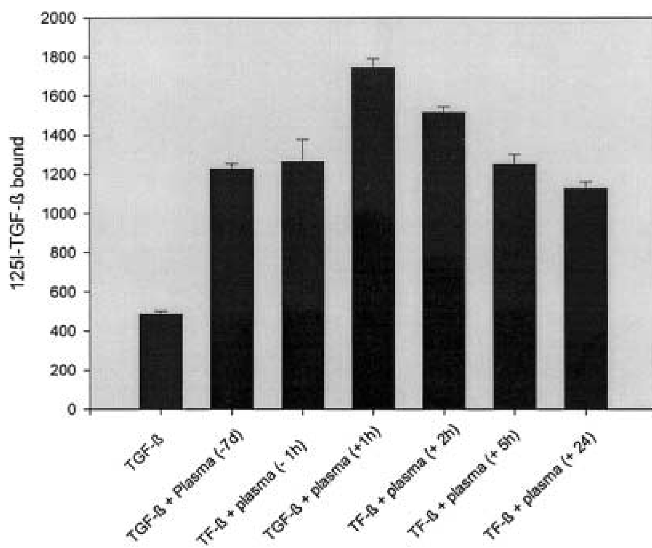

Fig. 2 Quantitative analysis of binding of ^{125}I-TGF-β to α2-macroglobulin of plasma after proteinase ingestion. Plasma samples were treated as described in Fig. 1. After electrophoretic separation the gel slabs were dried and the protein bands corresponding to the position of α2-macroglobulin (α2-M) were cut off the gel and subjected to γ-counting. The x-axis shows the time of sampling. *Column 1*: ^{125}I-TGF-β without plasma; *column 2*: ^{125}I-TGF-β + plasma (−7d); *column 3*: ^{125}I-TGF-β + plasma (−1 h); *column 4*: ^{125}I-TGF-β + plasma (+1 h); *column 5*: ^{125}I-TGF-β + plasma (+2 h); *column 6*: ^{125}I-TGF-β + plasma (+5 h); *column 7*: ^{125}I-TGF-β + plasma (+24 h). The experiments were performed in triplicates. The data were analysed by *t*-test (−7 d vs +1 h, $P < 0.05$), (−7 d vs +2 h, $P < 0.05$); (−7 d vs +24 h, $P < 0.05$). The other values when matched against the blank were not significant different

When we compared the TGF-β-α2-M interaction in two different volunteers we obtained different responsiveness to enzyme treatment (Table 1). This is probably related to a variation in absorption of the ingested enzymes in different individuals. Nevertheless, increased binding of TGF-β to α2-M was observed in either case.

From our results we can conclude that ingested proteinases may react with α2-M in plasma, and thus can induce α2-M intermediates with high affinity to TGF-β. To confirm that proteolytically transformed α2-M has TGF-β binding properties, we have analysed the binding of ^{125}I-TGF-β to α2-M complexed with trypsin and bromelain as well as with plasmin and α-chymotrypsin (Fig. 3). ^{125}I-TGF-β was found to bind to all complexes. The apparently reduced binding to α2-M-trypsin and α2-M-chymotrypsin complexes of TGF-β compared with other complexes is attributed to an increased degradation of bound TGF-β by the caged proteinases.

From our results we can expect that the high affinity of TGF-β to transformed α2-M may affect the biological activity of that growth factor. To prove this, we have investigated the effect of transformed α2-M on proliferation of TGF-β-stimulated fibroblasts in cell culture experiments (Fig. 4). In the presence of α2-M-MA, the TGF-β-stimulated proliferation of fibroblasts is significantly diminished. Furthermore, α2-M-MA was found to suppress fibroblast proliferation below the level observed without any added TGF-β. This additional effect on fibroblasts may be due to the inhibition of endogenous TGF-β, as it is known that TGF-β is synthesised by fibroblasts and acts as an autocrine growth factor. These opposing effects of α2-M-MA indicate that the mechanism of α2M-MA is neutralisation of TGF-β, most probably via an increased clearance.

Discussion

Alpha2-M exerts an important biological function because it connects metabolism of proteinases with regulation of cytokine action. A number of biological processes are triggered or initiated by proteinases, which on the other hand are under control of growth factors

Table 1 Effect of proteinase ingestion on binding of ^{125}I-TGF-β to α2-macroglobulin of plasma from two different volunteers. Two volunteers were treated orally with proteinases as described in Materials and methods. Plasma was obtained before and at different times after bolus application, and binding of ^{125}I-TGF-β to α2-macroglobulin (α2-M) was analysed as essentially described in Fig. 2. The data are means of three measurements. *MA-plasma* methylamine-treated plasma

	Time of plasma sampling					MA-plasma
	−1 h	+1 h	+2 h	+5 h	+24 h	
Volunteer 1	100%	138%*	119%	99%	89%	714%
Volunteer 2	100%	105%	140%*	131%*	114%	562%

* Significant differences from the blank value arbitrarily set at 100%

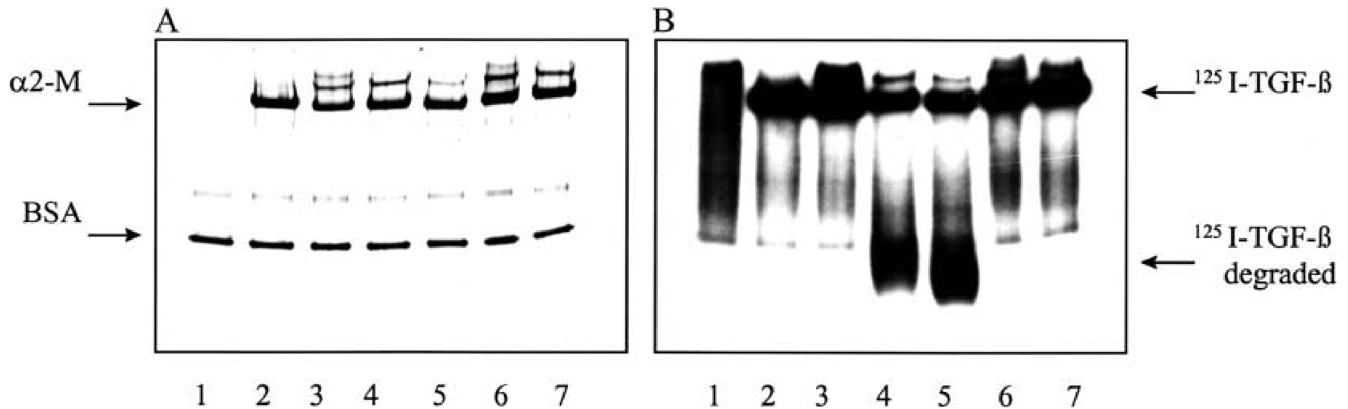

Fig. 3A, B Binding of ^{125}I-TGF-β to different α2-macroglobulin-proteinase complexes. ^{125}I-TGF-β (250,000 cpm) was incubated without or with 10 μg α2-macroglobulin-proteinase (α2-M-proteinase) complexes in a volume of 50 μl PBS-Tween for 4 h at 37 °C. Aliquots were subjected to pore gradient polyacrylamide gel electrophoresis (4–20%) under non-denaturing conditions. The gel slabs were **A** stained with Coommassie Blue R 250, dried, and **B** exposed to X-ray films. *Lane 1*: ^{125}I-TGF-β; *lane 2*: ^{125}I-TGF-β + α2-macroglobulin (α2-M) (47% transformed; showing high degree of intermediate forms); *lane 3*: ^{125}I-TGF-β + methylamine-treated α2-M (α2-M-MA); *lane 4*: ^{125}I-TGF-β + α2-M-chymotrypsin; *lane 5*: ^{125}I-TGF-β + α2-M-trypsin; *lane 6*: ^{125}I-TGF-β + α2-M-bromelain; *lane 7*: ^{125}I-TGF-β + α2-M-plasmin. The *arrows* indicate the position of **A** α2-M/α2-M complexes and bovine serum albumin and **B** the position of ^{125}I-TGF-β bound to α2-M as well as degraded TGF-β (*lanes 4* and *5*) respectively

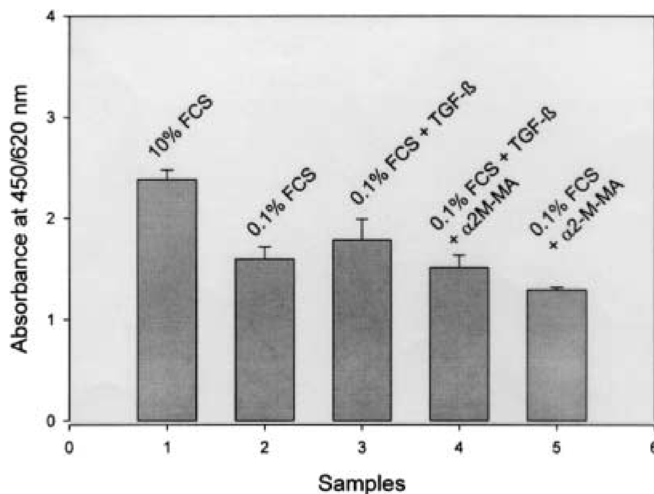

Fig. 4 Effect of transformed α2-macroglobulin on proliferation of fibroblasts. Human skin fibroblasts cultured in DMEM-10% fetal calf serum were seeded on micro-titre plates (10,000 cells/cavity) in 100 μl DMEM-0.1% fetal calf serum and incubated without or with TGF-β (5 ng/ml) in the absence or presence of methylamine-treated α2-macroglobulin (α2-M-MA) (50 μg/ml) for 24 h. After that time WST-1 reagent (10 μl) was added to the cavities, and the absorbence was read according to the instructions of the manufacturer. Statistical comparisons: column 2 vs column 3 ($P < 0.05$), column 2 vs column 4 ($P < 0.05$), column 2 vs column 5 ($P < 0.05$), column 3 vs column 5 ($P < 0.05$)

and cytokines. The inhibitor is known to bind various cytokines e.g. TGF-β, TNF-α, amyloidβ, PDGF, VEGF, IL-1, IL-6 and others [7, 17, 18]. Even different hormones are bound to, and transported by, α2-M, such as activin/inhibin [24], leptin [3], and STH [16]. Two forms of the inhibitor exist in human plasma. Under normal conditions the fraction of transformed α2-M (proteinase-complexed form) in plasma is approximately 1% of the total inhibitor concentration, amounting to 254 mg/dl [2]. Elevated concentrations of transformed α2-M up to 10% of total α2-M are found in plasma of patients suffering from acute pancreatitis [15]. In tissues and in diverse biological fluids, much higher concentrations (up to 50%) of transformed inhibitor can be measured [5]. This is due to the high proteolytic charge in those compartments. We could clearly demonstrate that ingestion of a proteinase cocktail causes formation of α2-M intermediates in blood, with high affinity to TGF-β. The appearance of these intermediates coincides with the time of absorption of proteinases through the intestine, reaching maximum values between 1 and 2 h after bolus application.

A number of publications has evidenced that proteinases can cross the intestinal epithelium in an active state [6, 9, 36]. Due to the high reactivity of trypsin and bromelain, these proteinases will preferably complex with α2-M [9, 23]. Transformed α2-M or α2-M-proteinase complexes may bind and clear TGF-β from plasma and tissue due to endocytosis via the α2-M receptor. That receptor, which is identical to the low-density lipoprotein receptor-related protein (α2-M-R/LRP), also binds Apo E-containing lipoproteins, u-PA-PAI-1 complexes and diverse other molecules. The high clearance rate of α2-M-proteinase complexes makes that elimination system extremely effective in neutralising elevated concentrations of cytokines and growth factors.

It is known that TGF-β regulates growth but also mediates far-ranging biological processes including inflammation, host defence, tissue repair and tumorigenesis. Although TGF-β facilitates resolution of inflammation, and promotes tissue repair, an excess of TGF-β leads to unresolved inflammation and fibrotic events. It may also predispose the host to serious and

recurrent infections [22]. These different effects are dependent on a critical balance of the growth factor activity and the mechanisms of its regulation.

In liver fibrosis and fibrosis of pancreas and kidney, high concentrations of TGF-β are suggested to induce and sustain the process of fibrogenesis [1]. This factor may stimulate fibroblasts and myofibroblasts to secrete collagen and matrix proteins while reducing the secretion of proteolytic enzymes. It may cause increased deposition of extracellular matrix components leading finally to thickness of extracellular space. Animal models of experimental liver and renal fibrosis support the in vivo relevance of TGF-β for fibrosis development, since increased concentrations were found very early in the course of fibrogenesis [14, 20]. Although there are conflicting data about the function of TGF-β in autoimmune and inflammatory disorders, the beneficial role of TGF-β in the maintenance of immunological homeostasis is commonly accepted.

The relationship between TGF-β expression and tumour growth has gained special interest, both for the better understanding of the process of carcinogenesis and for the development of therapeutic strategies. A number of tumours secrete substantial quantities of biologically active TGF-β, which seems to be essential for their progression [8, 11, 21]. Furthermore, TGF-β has been found to mediate immune surveillance of cancer by its T-cell suppressive activity towards cytotoxic T-lymphocytes and natural killer cells [30]. A positive correlation between invasiveness, progression, decreased TGF-β sensitivity of tumours and increased TGF-β production has been established, and may contribute to locally impaired immune response to tumour cells.

Approaches based on inhibition of TGF-β production by antisense strategy [19] or neutralisation of TGF-β by antibodies [35] have been successful in enhancing immune response in animal models, however, long-term efficacy and application in humans are still questionable. Proteoglycans which neutralise TGF-β have been exploited therapeutically, in experimental glomerulonephritis, and to enhance anti-glioma immune response in vivo [13, 28].

Recently, it could be demonstrated that in vitro conditioned cell culture supernatant of breast carcinoma cells suppress T-cell cytotoxicity due to the presence of TGF-β. It is suggested that TGF-β improves metastasis by stimulation of angiogenesis and suppression of immune defence mechanism [12]. Interestingly, the suppressive effect of TGF-β could be reversed by addition of transformed α2-M. The data clearly show that control of the action of TGF-β may be central to the management of fibrosis and malignancy in humans.

In the present study we could show that ingestion of proteinases triggers increased binding of TGF-β to α2-M. Irrespective of the type of proteinases bound to the inhibitor, the emerging complexes readily react with TGF-β and are rapidly cleared by the α2-M receptor. It is suggested that absorbed trypsin and bromelain may directly bind to α2-M, however, the induction of plasmin-α2-M complexes through activation of plasminogen by the ingested enzymes should also be considered as a possible mechanism. Nevertheless, the therapeutic potential of strategies which modulate the TGF-β action and target immune cell responsiveness to TGF-β will become an important tool for fighting human diseases including cancers.

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