**TGF-β and Cancer Immunotherapy**

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The cytokine, transforming growth factor beta (TGF-β), has a history of more than 40 years. TGF-β is secreted by many tumor cells and is associated with tumor growth and cancer immunity. The canonical TGF-β signaling pathway, SMAD, controls both tumor metastasis and immune regulation, thereby regulating cancer immunity. TGF-β regulates multiple types of immune cells in tumor microenvironment, including T cells, natural killer (NK) cells, and macrophages. One of the main roles of TGF-β in the tumor microenvironment is the generation of regulatory T cells, which contribute to the suppression of anti-tumor immunity. Because cancer is one of the highest causes of death globally, the discovery of immune checkpoint inhibitors by Honjo and Allison in cancer immunotherapy earned a Nobel Prize in 2018. TGF-β also regulates the levels of immune checkpoints inhibitory receptors on immune cells. Immune checkpoints inhibitors are now being developed along with anti-TGF-β antibody and/or TGF-β inhibitors. More recently, chimeric antigen receptors (CARs) were applied to cancer immunity and tried to combine with TGF-β blockers.

**Key words** immunologic factor; immunotherapy; neoplasm; noble prize; signal transduction

INTRODUCTION

In 1978, De Larco and Todaro found many growth factors were produced by murine sarcoma virus-transformed cells including transforming growth factor beta (TGF-β). It has also reported that many types of cancer, including colorectal cancer (HT29), gastric cancer (Kato-III, OCUM-1 and HSC-39), and breast cancer (EMT-6), secrete TGF-β. In addition, tumors from a late stage of cancer patients showed high level of TGF-β expression. Therefore, concentration of TGF-β in sera from cancer patients was significantly higher than that from healthy donors. M2-like phenotype tumor associated macrophages (TAMs) is another source of TGF-β. Of note, the number of TAM and cancer stem cells was correlated in tumor microenvironment. Foxp3+ regulatory T cells (Tregs) can be generated or recruited in the tumor microenvironment and produce TGF-β, which also suppress tumor immunity. Therefore, targeting TAMs or Tregs' depletion induces strength of anti-cancer immunity. In addition, tumor infiltrate interleukin-10 (IL-10) B cells (namely regulatory B cells: Bregs) also produce TGF-β which also suppress T helper 1 (Th1) cytokine secretion and T cells proliferation. Although tumor cells help to generate Bregs, it is still unclear whether tumor-produced TGF-β contributes to the generation of Bregs. In this review, we focus on how TGF-β controls cancer immunity.

1. TGF-β CONTROLS IMMUNITY

1.1. TGF-β and Regulatory T Cells

In 2003, Chen et al. demonstrated that TGF-β can induce Foxp3+ Tregs from naïve CD4+ T cells in vitro in a dose dependent manner. The TGF-β-induced Tregs have suppressive ability against the expansion of antigen specific T cells in vitro and in vivo. T cell specific TGF-β receptor I-deficient mice (Tgbr1lox/lox Lck-cre) showed significantly less Foxp3+ Tregs population in the thymus at days 3–5 after birth, with subsequent expansion of the thymic Tregs population through IL-2 signaling. Therefore, TGF-β play crucial roles for generation of thymic Tregs (also called nTregs). Thymic Tregs maintain the suppression ability via Foxp3 stable expression. Zheng et al. identified several Foxp3-conserved non-coding sequences (CNS) and demonstrated that around 2kb downstream of transcriptional starting site of Foxp3 has conserved cytosine-phosphate-guanine (CpG) island, namely CNS2. In thymic Tregs, CNS2 region showed highly de-methylation status and binding Foxp3 itself. In addition, CNS2-deficient mice were showed easy to loss of Foxp3 expression in Tregs and also less thymic Tregs population. Therefore, CNS2 play an important role for stability of Foxp3 expression. Conversely, peripherally induced Tregs showed a less de-methylation status of CpG island in Foxp3-CNS2 and ease to loss the expression of Foxp3. TGF-β-induced Tregs are also less stable, but some components (e.g. retinoic acid, CDK8/19 inhibitor) can promote de-methylation status of CpG island in Foxp3-CNS2. It has been well demonstrated the mechanisms how TGF-β control Foxp3+ Tregs’ generation. Tone et al. demonstrated that SMAD3, a downstream of TGF-β signaling, could be enrich via the Foxp3 enhancer (called the CNS1 region) and positively regulate the generation of Tregs in cooperation with nuclear factor of activated T-cell (NFAT). These events occurred within 2 h after TGF-β stimulation. Histone/protein acetyltransferases p300 play crucial roles for TGF-β-induced Foxp3+ Tregs and cancer immunity. p300 could be enriched by the Foxp3 promoter and positively regulate Foxp3 gene expression in cooperation with several transcriptional factors (e.g. nuclear factor-kappaB (NF-κB), activator protein 1 (AP-1)), Some authors suggested that TGF-β protects nTregs from apoptosis in the thymus rather than TGF-β being required for generation of nTregs in the thymus. More recently, apoptotic cells support to produce TGF-β in thymus and it can prevent apoptotic thymic Tregs’ death. Thus, TGF-β has ability to protect the
progenitors of nTregs from apoptosis.

TGF-β suppresses interferon-γ (IFN-γ) (an inflammatory anti-cancer cytokine) expression in CD4^+ T cells.\(^{23}\) Takimoto et al. demonstrated that TGF-β-induced SMAD2/3 activation is a key pathway to Foxp3-independently suppress the expression of IFN-γ in CD4^+ T cells.\(^{24}\) Although SMAD2 or SMAD3-deficient T cells has been showed less Foxp3^+ Tregs induction in response to TGF-β1 in vitro, TGF-β1 signaling (SMAD2 and SMAD3) during Tregs’ generation steps would be complementing their function in each other (Fig. 1).

1-2. TGF-β and T Cell Immunity

In CD8^+ T cells, TGF-β suppresses activation, apoptotic cell death, and IFN-γ production.\(^{25}\) We further demonstrated that the TGF-β-SMAD-pathway induced nuclear kappa B family protein inhibitor of NF kappaB (IkB)-ζ, which negatively regulates IFN-γ gene expression in CD4^+ T cells.\(^{26}\) Therefore, IkB-ζ-deficient T cells show more IFN-γ expression even in the presence of TGF-β. Thus, the TGF-β-SMAD-IkB-ζ axis may regulate the gene expression of IFN-γ gene expression in a Foxp3-independent manner.

TGF-β also helps to generate IL-9-producing helper T cells (Th9) that enhanced anti-tumor immunity.\(^{27,28}\) Nakatsuksaka et al. demonstrated that IL-4 inhibits TGF-β-induced Id3 expression via TAK1 activation (non-SMAD pathway) and it plays a crucial role for Th9 differentiation.\(^{29}\) Therefore, anti-IL-9 antibody treatment showed tumor progression. On the other hands, IL-9-treatment in SGC-7901 (Gastric tumor cell line) xenografted nude mice showed less tumor growth.\(^{29}\) Another report has been shown that Th9 cells induce anti-tumor immunity via enhancement of IFN-γ production from CD8^+ T cells and natural killer (NK) cells.\(^{30}\) Recently, Li et al. generated 4T-Trap, anti-human CD4 antibody with TGF-βRII extra cellular domain, which is selectively blockade of TGF-β signaling in CD4^+ T cells and promotes cancer immunity-therapy.\(^{31}\) Interestingly, 4T-Trap-treated tumor bearing mice observed tumor vascular remodeling through a large amount of IL-4 from CD4^+ T cells.\(^{31}\)

Not only Tregs’ generation, TGF-β is also important for the differentiation of thymic CD8^+ T cells and IL-7 receptor α expression in T cells.\(^{32}\) In the thymus, IL-7 receptor signaling is known to be a sensor for the detection of the duration of TCR signaling and regulates the differentiation of CD8^+ T cells.\(^{33}\) Another paper demonstrated that the TGF-β-SMAD axis control the differentiation of CD8^+ T cells in thymus.\(^{24}\) In addition, the TGF-β-SMAD axis represses IFN-γ and granzyyme B expression in CD8^+ T cells.\(^{34}\) Therefore, TGF-β can suppress the tumor cytotoxicity of CD8^+ T cells,\(^{35}\) TGF-β from cancer cells also help to generate CD8^+ Foxp3^+ Tregs and it observed in peripheral blood from ovarian cancer patients.\(^{35}\) CD8^+ Foxp3^+ Tregs were also observed in tumor infiltrate lymphocytes and it can be induced and maintained by TGF-β from tumor cells.\(^{36}\)

1-3. TGF-β Regulate Innate Immunity

TGF-β from tumors could recruit CD45^+CD11c^+CD11b^+MHCII^+ macrophages but not dendritic cells.\(^{37}\) These macrophages called tumor associate macrophages (TAMs) and has been reported to produce immune suppressive cytokines (IL-10 and TGF-β) and control tumor immunity.\(^{8,38}\) Therefore, TAMs showed M2-like phenotypes. Another report showed that the TGF-β can induce M2-type macrophages through the SMAD-SNAIL pathway.\(^{39}\) Interestingly, silencing SNAIL in Bone marrow-derived macrophages showed M1-like phenotype including highly expressed Tfrα, Mcpl and IL2p40. Therefore, TGF-β may suppress M1-like macrophages and induces the polarization of M2-like macrophages in the tumor microenvironment.\(^{39}\)

NK cells express NKG2D, a transmembrane protein, which can recognize tumor surface ligands (MICA, MICB and ULBP1-6) and helps cancer immunosurveillance. TGF-β impaired NKG2D expression of NK cells, which prevent recognizing tumor surface ligands and inducing cytotoxicity.\(^{40}\) In addition, TGF-β control IFN-γ and granzyyme B from NK cells in response to CD16 and antibody dependent cellular cytotoxicity (ADCC).\(^{41}\) They demonstrated that TGF-β-SMAD3 axis play a crucial role for controlling ADCC. Another report demonstrated that NK cell specific SMAD4-deficient mice had less anti-tumor immunity, because a TGF-β-independent SMAD4 pathway promote granzyeme B production from NK cells.\(^{42}\) Gao et al. demonstrated that NK cells could be converted into type 1 ILC in the tumor microenvironment by TGF-β and lost their tumor immunosurveillance properties.\(^{43}\)

This type 1 ILC subset showed less IFN-γ production and more inhibitory immunological checkpoint receptor cytokotic T-lymphocyte antigen 4 (CTLA-4) expression. TGF-β in the tumor microenvironment also promotes IL-9 production from ILC2 and may play roles in anti-cancer immunity.\(^{27}\) TGF-β can induce Foxp3 expression in γδ (Vγ2) T cells and it shows blockade ability against proliferation of PBMC in response to TCR stimulation.\(^{44}\) Conversely, TGF-β with IL-2 stimulated γδ (Vγ2) T cells produced large amount of granzyyme B and IFN-γ and showed tumor cytotoxicity.\(^{45}\)

Thus, TGF-β controls cancer immunity through many kinds of immune cells in the tumor microenvironment (Fig. 2).

2. CANCER IMMUNOTHERAPY OF IMMUNE CHECKPOINT INHIBITORS

2-1. Immune Checkpoint Inhibitor PD-1 and TGF-β

In 1992, Ishida et al. has been performed subtraction hybridization method using cDNA libraries from PMA + Ionomycin stimulated T cells hybridoma and cultured T cell hybridoma without growth factor (meaning apoptotic cells), then identified the immunoglobulin superfamily gene Program cell Death (PD)-1.\(^{46}\) PD-1 conserved several consensus sequences in
their cytoplasmic domain, including immunoreceptor-tyrosine based inhibitory motif (ITIM). Therefore, PD-1 stimulation inhibits T cell activation (TCR-stimulation induced Syk phosphorylation) via SHP, a kinase accumulated in ITIM motif. 

Fig. 2. Roles of Regulatory T Cells, T Helper Type 9, and Tumor-Associated Macrophages in Cancer Immunity

Transforming growth factor beta (TGF-β) in the tumor microenvironment promotes interleukin-10 (IL-10) production from tumor-associated macrophages (TAMs) and regulatory T cells (Tregs). IL-10 suppresses anti-tumor immunity. Additionally, TGF-β inhibits the anti-tumor cytokine, interferon-gamma, and cytotoxic granzyme B from natural killer (NK) cells and cytotoxic (CD8) T cells. TGF-β helps to generate T helper type 9 (Th9) cells while Th9 produces IL-9/IL-21 and enhances cytotoxicity of CD8 T cells and NK cells. Thus, TGF-β plays a crucial role in anti-tumor immunity through immune regulation.

Fig. 3. Programmed Cell Death Protein 1/Programmed Death-Ligand 1 and CTLA4/CD80/86 Axes in Cancer Immunity

Programmed cell death protein 1 (PD-1) expressed on cytotoxic (CD8) T cells is recognized programmed death-ligand 1 on tumor cells, which is negatively regulated T cell activation. Transforming growth factor beta (TGF-β) from tumor induces PD-1 expression and negatively regulates tumor immunity. CTLA4 is highly expressed on regulatory T cells and predominantly binds to CD80/CD86 on tumor-associated macrophage (TAM). Therefore, CD28 molecules in CD8 T cells have little chance to bind to CD80/CD86 on TAM.

J558L melanoma growth but not B16 melanoma growth. In clinical trials, anti-PD-L1 antibody has been treated in many types of tumor patients including lung cancer, ovarian cancer and melanoma and showed pharmacological anti-tumor effects, and is now approved for cancer immunotherapy drugs: called Immune Checkpoints Inhibitors. It is also known that anti-PD-L1 antibody treatment enhances T cells infiltration in the tumor microenvironment. However, it has also been observed the tumor patients who have resistant to cancer therapy using immune checkpoint inhibitors.

To develop the effect of the immune checkpoint inhibitors, blockade of TGF-β has been tried. Anti-PD-L1 antibody with anti-TGF-β antibody treatments show strongly evoked anti-tumor immunity through the activation and penetration of T cells into the center of the tumor. A new concept of a biological drug: M7824 (MSB0011359C), a bifunctional fusion protein harboring a PD-L1 binding region and two TGF-β receptor 2 molecules (trap linker), treated tumor model showed activation of ADCC. In addition, M7824 treatment canceled suppressed T cell proliferation by Tregs. Another group also demonstrated that M7824-treated tumor bearing mice showed less tumor volume and metastasis and more CD8+ T cells and NK cells in tumor infiltrate lymphocytes than that of anti-PD-1 or TGF-β trap-treated mice. Clinical trial of M7824 showed that anti-tumor affects with a complete neutralizing effect of TGF-β in plasma and saturate PD-L1 in PBMC. Because sample size was too small, subset of immune cells in patients were no big difference even in the presence of M7824.

2-2. Regulation of PD-1 Expression and Cancer Immunity

TGF-β receptor signaling in Tregs plays crucial roles for their suppression ability. Therefore, blockade of surface bound TGF-β on the Tregs fails to suppress anti-tumor effects by CD8+ T cells. TGF-β also enhances antigen-induced PD-1 expression in CD4+ and CD8+ T cells both human and mouse. Mechanistically, TGF-β signal molecule SMAD3 directly enriched on PD-1 promoter region in response to TGF-β and positively regulated PD-1 gene expression cooperate with a transcriptional factor NFAT. Therefore, SMAD3-deficient mice (Smad3+/- CD4-cre) show less PD-1 expression in CD8 T cells in tumor microenvironment and are resistant to B16-
melanoma models. Therefore, anti-PD-1 treatment does not show anti-tumor effects in tumor bearing Smad3-deficient mice. Although the study did not demonstrate how much tumor specific Tregs were in TILs, the percentage of Foxp3+ Tregs in TILs was comparable between wild type (WT) and Smad3-deficient mice. Stephen et al. demonstrated more detail about molecular mechanisms of PD-1 expression that TCR stimulation induced histone deacetylase SATB1 enrichment on PD-1 promoter, which is negatively regulate PD-1 gene expression.59) TGF-β/SMAD axis negatively regulate SATB1 expression, therefore promote PD-1 expression in T cells through the SMAD3 binding on PD-1 promoter. Therefore, SATB1-deficient mice shows less anti-tumor immunity. Another report showed that SMAD3-deficient mice also prevented B16-melanoma progression through NK cell development.60) Conversely, SMAD3-deficient mice develop colorectal cancer with aging.61)

Previously, we demonstrated that GO-Y030 controls the TGF-β signaling and prevent Foxp3+ Tregs generation in vitro and also in tumor microenvironment.62) GO-Y030 identified a curcumin analog and has a 5-carbon space between 3,5-bis(methoxymethoxy)phenol. This phenomena is due to the strong reduction of S-phase fraction (DNA-synthesis) and apoptosis induction through the caspase-3 activation, which is more strongly than curcumin itself.63,64) In addition to colorectal carcinomas, breast and pancreatic carcinoma’s cell growth can equally be strongly inhibited by GO-Y030.65) Since anti-PD-1 treatment failed to deplete Tregs in tumor microenvironment.66) Therefore, when we try to apply GO-Y030 and cancel this negative effect of tumor immunity by anti-PD-1 treatment.67) Therefore, when we try to apply GO-Y030 and anti-PD-1 antibody in B16-F10 melanoma bearing mice, anti-tumor effects showed more strikingly than that of anti-PD-1 treatment only.

2.3. Immune Checkpoint Inhibitor CTLA4 and TGF-β

CTLA-4 is another inhibitory receptor that expresses Tregs and has a stronger affinity against CD80/CD86 compared with CD28.68) (Fig. 3). Signaling through the CTLA-4 negatively regulated T cells activation. Leach et al. showed that anti-CTLA-4 antibody treatment has potential to activate anti-tumor immunity using several types of tumor mouse model (e.g. Colon carcinoma and fibrosarcoma).69) In immunodeficient environment, Tregs showed highly expressed CTLA-4 and anti-CD-1 antibody in B16-F10 melanoma bearing mice, anti-tumor effects showed more strikingly than that of anti-CD-1 treatment only.

The deletion of TGF-β receptor II (TGFBR2) in Chimeric Antigen Receptor (CAR)-T cells by clustered regularly interspaced short palindromic repeats (CRISPR)-associated proteins 9 (CRISPR/CAS9) led to strong anti-tumor efficiency.70) These TGFBR2-edited CAR-T cells demonstrated less Tregs induction and T cell exhaustion in response to TGF-β in vitro. Another report indicated that TGF-β receptor kinase inhibitor SD-208-treated CAR-T cells induce more proliferation, more IFN-γ production and less PD-1 expression.71) In addition, the expression of dominant-negative TGF-βRII in CAR-T cells increased tumor proliferation and cytotoxicity.72) Conversely, CD28-LCK-ζ CAR-T cells are resistant to TGF-β mediated repression of tumor cytotoxicity through constitutive IL-2 secretion.73) Interestingly, CAR-T cells targeting TGF-β prevented generation of Tregs and promoted cytotoxicity of CD8+ T cells, which would be contribute to anti-cancer immunity.74) CD137 (4-1BB) is a costimulatory molecule that expressed CD4+ and CD8+ T cells.75) For anti-tumor immunity, anti-4-B11 antibody treatment has been performed and demonstrated that activation of CD8+ T cells including IFN-γ production and cytotoxicity maker-induced capability. Agonistic anti-4-1 BB humanized antibody treatment showed therapeutic effect in carcinoma (MCA5) bearing mice. However, this effect was abolished in the mice who has no CD4+ and/or CD8+ T cells.76) On the other hands, anti-4-1 BB treatment does not show anti-tumor effects in C3 tumor bearing mice (Tumorigenic HPV16-transformed embryonic cells).77) At the moment, transfected chimeric TGF-βR2-4-1BB receptor in T cells showed increase tumor clearance activity (A375 melanoma xenograft model).78) This chimeric receptor does not include the partial extra cellular domain of TGF-βR2; therefore, TGF-β consumption may occur without TGF-β signaling conduct. Thus, anti-TGF-β signaling can increase the potential of CAR-T tumor immunotherapy.

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Conflict of Interest The authors declare no conflict of interest.

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