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The role of transforming growth factor-beta in immune suppression and chronic inflammation of squamous cell carcinomas

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Abstract

Despite a decline in incidence of SCCs over the past 20 years, their survival rate has remained nearly the same, indicating that treatment options have not improved relative to other cancer types. Immunotherapies have a high potential for a sustained effect in SCC patients, but their response rate is low. Here, we review the suppressive role of TGFβ on the anti-tumor immune response in SCC and present its potential as a therapeutic target in combination with the current range of immunotherapies available for SCC patients. We conclude that SCCs are an optimal cancer type to study the effectiveness of TGFβ inhibition due to the prevalence of dysregulated TGFβ signaling in them.

Keywords

Immunotherapy; head and neck squamous cell carcinoma; tumor microenvironment; immune checkpoint blockade

Squamous cell carcinomas (SCCs) are cancers that arise from areas of stratified epithelium and are primarily found in the skin, lung, and the epithelial lining of the oropharyngeal and nasopharyngeal cavities. Although skin SCC incidence cannot be estimated because most cancer registries do not collect data on it, deaths associated with non-melanoma skin cancer, which is primarily SCC, exceed melanoma deaths due to the high number of SCC cases[1]. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and accounts for 53,000 new cases each year in the US.[2] Although its incidence has decreased slightly, its mortality rate has slightly increased since 2012,[2] indicating that current therapeutic options for patients with HNSCC have stagnated and novel therapeutics have not yet improved the majority of patient outcomes. The current standard of care is a combination of radiotherapy, surgery, and chemotherapy; patients who do not respond to those typically have incurable recurring metastatic disease. The primary causes of HNSCC

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are either HPV or heavy tobacco and alcohol use. HPV$^-$ HNSCCs are associated with worse prognoses and a less inflammatory immune microenvironment.[3]

It is long established that mutagen-induced SCC results in elevated levels of secreted transforming growth factor-beta (TGFβ) in the tumor microenvironment.[4] The TGFβ signaling pathway has been reviewed in depth elsewhere[5,6]and is summarized here in Figure 1. Briefly, when the TGFβ receptor complex binds TGFβ ligand, it phosphorylates receptor-associated Smad2 and Smad3 proteins. Smad2 and Smad3 form a complex with Smad4, which translocate to the nucleus, bind to Smad binding elements (SBEs) on genetic loci, and regulate numerous transcriptional pathways. The TGFβ receptor complex also activates numerous non-canonical signaling pathways independently of Smad proteins. In tobacco and alcohol-associated cases, HNSCC is often preceded by dysregulation of TGFβ signaling, resulting in elevated levels of TGFβ within a tumor while tumor cells simultaneously become unresponsive to the growth-inhibitory effects of TGFβ; early mouse SCC models of dysregulated TGFβ signaling demonstrated that TGFβ no longer inhibits growth of cells that lack the TGFβ receptor.[7,8] When TGFβ expression is induced, tumor progression is exacerbated resulting in the formation and progression of premalignant lesions and subsequent metastasis.[9,10] We have shown that Smad4 is lost or downregulated in 35% of HNSCC patients[11] and TGFβRII is downregulated in 69% of HNSCC samples,[12] resulting in high levels of TGFβ secreted within the tumor microenvironment.[13] TGFβ is a daunting molecule to study because of the wide range of signaling that takes place downstream of the TGFβ receptor complex. Furthermore, it paradoxically functions as both a growth suppressor and tumor promoter, depending on the stage and TGFβ responsiveness of the tumor cells.[14] TGFβ was originally identified as a pro-inflammatory cytokine that induced the migration of lymphocytes, monocytes, neutrophils, and fibroblasts.[15] However, subsequent animal models with dysregulated TGFβ signaling (discussed below) revealed its role as a potent suppressor of inflammation. Its inhibitory effect on the anti-tumor immune response makes TGFβ an important therapeutic target as immunotherapy is further developed for use in SCC.

HNSCC is an excellent candidate for immunotherapy; it has a high mutational burden, one of the predictors of a response to immune checkpoint blockade,[16] and a high percentage of HPV-negative patients score positive for PD-L1 expression.[17] The phase II KEYNOTE 012 clinical trial of the pembroluzimab PD-1 blockade had an 18% overall response rate in recurring and metastatic HNSCC.[18,19] This led to two successful phase III trials (KEYNOTE 040 and CHECKMATE 141) with pembroluzimab and nivolumab,[20,21] resulting in FDA approval of both drugs for the treatment of metastatic HNSCC that is refractory to chemotherapy. The main success of immunotherapy has been its durability of response. However, a response rate of only 10-20% suggests that additional therapeutic targets are needed to improve the effectiveness of immunotherapy in HNSCC. Here, we review the impacts of dysregulated TGFβ signaling on immune suppression in HNSCC, and discuss how current cancer therapeutics are taking advantage of this interaction to improve the effectiveness immune checkpoint blockade.

TGFβ impacts many arms of the immune response to cancer. The effects of secreted TGFβ in the SCC tumor microenvironment on various populations of stromal and immune cells within the tumor are discussed below and summarized in Figure 2.

Reduced anti-tumor function of T cells

TGFβ plays a critical role in T cell function, as evidenced by genetically engineered mouse models with dysregulated TGFβ signaling. Mice with T cell-specific disruptions of the TGFβ signaling pathway exhibit increased T cell activation and inflammatory disease,[22-26] and mice with TGFβRII loss in mature T cells have increased levels of T cell proliferation.[27] Therefore, HNSCCs with elevated levels of TGFβ have a high potential for reduced general T cell function within them based on the inhibitory effect of TGFβ on $CD8^+$ cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs), and CD4 helper T (Th) cells.

It is generally accepted that $CD8^+$ CTLs are one of the primary effectors of anti-tumor immunity, and increased numbers of tumor-infiltrating lymphocytes correlate with improved survival in HNSCC patients.[28-30] Secreted TGFβ in the tumor microenvironment directly inhibits the expression of cytolytic genes in $CD8^+$ T cells: Fas ligand, perforin, granzyme A, granzyme B, and interferon gamma, impairing their function.[31] TGFβ signaling also contributes to CTL exhaustion; PD-1 expression on CTLs is increased by secreted TGFβ,[32] and T cells in TGFβ-rich HNSCC microenvironments are highly exhausted and incapable of mounting a cytolytic response against tumor cells.[33] Preventing TGFβ from inhibiting effector T cell function is important for the effectiveness of T-cell mediated therapies; expressing a dominant-negative TGFβRII on chimeric-antigen receptor T cells increased their proliferation, activation, and resistance to exhaustion in prostate cancer models.[34] The effects of combining TGFβ inhibition with other immunotherapies that improve T cell function within tumors are further discussed below.

Tregs are required to maintain immune homeostasis through their repression of effector T cell function. However, they are a major source of immune evasion in cancers and are often (but not always) associated with poor prognosis in solid tumors.[35] Their role in HNSCC tumor progression is particularly controversial, with studies showing that infiltrating $FOXP3^+$ Tregs are correlated both with tumor progression[36,37] and improved prognoses.[38,39] One of the reasons for this discrepancy could be the tumor-specific environment that the Tregs are found in; Tregs have distinct anti-inflammatory and pro-inflammatory phenotypes depending on the presence of cytokines like TGFβ and IL-12 within the tumor,[40] and HNSCC tumors that are high in IL-33 result in Treg-mediated immune suppression.[41] TGFβ induces the differentiation of $CD4^+$ cells into Tregs,[42] and the FOXP3 expression that drives Treg differentiation is induced by TGFβ[43,44] in a Smad2 and Smad3-dependent manner.[45] This mechanism has been shown to be relevant in tumor models; TGFβ pathway activation is correlated with increased FOXP3 expression in melanoma.[46] Additionally, Tregs themselves further increase TGFβ levels within the tumor microenvironment,[47] resulting in increased immune suppression of other infiltrating immune cells discussed here. Therapeutic targeting of surface-bound TGFβ on Tregs reduced tumor growth in mouse melanoma models,[48] showing the importance of Tregs in the tumor microenvironment.

TGFβ alters differentiation of naïve $CD4^+$ T cells into Th cells in the periphery. TGFβ prevents naïve T cells from differentiating into Th1 T cells *in vitro,*[49] and mice with a TGFβRII-knockout on T cells have increased Th1 cell activation.[23,27,50] TGFβ also silences expression of the Th1 differentiation transcription factors TBET and STAT4.[51,52] In pancreatic carcinomas, secreted TGFβ contributed to a shift in frequency from pro-inflammatory Th1 cells to tumor-permissive Th2 cells.[53] Therefore, in TGFβ-rich microenvironments, $CD4^+$ T cells can become more tolerant of tumors and inhibit the CTL response against them.

Inhibition and inactivation of natural killer (NK) cells

Even in a T cell-suppressive microenvironment, NK cells can mount an effective response against tumor cells. However, TGFβ signaling impairs NK cell effectiveness by inhibiting their secretion of IFNγ, resulting in decreased Th1 cell activation.[54,55] TGFβ signaling also silences NKG2D and NKp30 receptor expression on human NK cells *ex vivo,*[56] which results in a decreased ability of NK cells to recognize abnormal tumor cells. NK cells in a murine model of HNSCC also exhibited decreased NKG2D expression in response to TGFβ, [57] and the activating receptor NKp46 and both inhibitory and activating KIRs were decreased on NK cells in HNSCC patients exhibiting high levels of TGFβ.[58] TGFβ contributed to down-regulation of CD16 on NK cells in esophageal SCC patients that was associated with decreased NK cell function.[59] TGFβ signaling also causes NK cells to differentiate into type 1 innate lymphoid cells[60] that do not exhibit cytotoxic activity, and this process is further exacerbated by the loss of Smad4.[61] Taken together, elevated TGFβ in the tumor microenvironment of HNSCC can result in dysfunctional NK cell activity and a dramatic reduction of the innate response to cancer.

Reduced dendritic cell function in SCC

Dendritic cells are the primary antigen-presenting cells within a tumor and are often required for anti-tumor immunity to function. In HNSCC, increased infiltration of antigen-presenting cells is associated with a better prognosis.[62-64] TGFβ inhibits MHC-II expression on dendritic cells,[65,66] impairing their antigen presentation and resulting in a more tumor-permissive immune microenvironment. TGFβ signaling also induces dendritic cells to switch to an immature myeloid cell phenotype, which is associated with increased immune suppression.[67] Finally, dendritic cells are a source of TGFβ in the tumor immune microenvironment; tumor cells can induce TGFβ secretion by dendritic cells, leading to increased Treg differentiation.[68,69] This is one of the many ways that dysregulated TGFβ signaling within the tumor microenvironment can cause additional TGFβ secretion, compounding the effects of TGFβ within a tumor. Langerhans cells, dendritic cells localized in the epidermis, are often the first antigen-presenting cells to encounter SCC.[70] Akin to dendritic cells in other regions, Langerhans cells are also a major source of TGFβ secretion, [71] and TGFβ is required for their maintenance and differentiation.[72] TGFβ-rich microenvironments would, therefore, be tolerant of dendritic cell infiltration, but simultaneously inhibit their function as antigen-presenting cells.

Increased tumor-permissive macrophages in SCC

Tumor-associated macrophages (TAMs) are commonly categorized into pro-inflammatory M1 and immune-suppressive M2 polarizations. TAM accumulation is a major contributor to immune evasion and poor prognoses in HNSCCs,[73] implying that the majority of TAMs in HNSCC are M2-polarized macrophages. Indeed, HNSCC samples with high levels of TGFβ also had high levels of TAMs and their associated immune-suppressive phenotypes.[74] A potential mechanism for this is revealed by research showing that TGFβ induced a M2-like phenotype via SNAIL signaling in a human macrophage cell line *in vitro*.[75] TGFβ also inhibited toll-like receptor signaling in macrophages via inhibitory signaling of Smad6[76-78] and inhibited the pro-inflammatory tumor necrosis factor pathway by Smad7-mediated inhibition of Tak1,[79] resulting in decreased macrophage activation and reduced macrophage-induced inflammation of the tumor. Therefore, TGFβ signaling in macrophages can inhibit both the innate and adaptive responses to tumors. We found that when macrophages were ablated in SCCs, tumors had increased apoptotic tumor cells and reduced angiogenesis,[80] which could be attributed to the reduction in pro-angiogenic and anti-inflammatory cytokines released by macrophages.

Neutrophils and monocytes inhibit T cell function

Similar to macrophages, neutrophils also polarize between pro-inflammatory N1 and immune-suppressive N2 categories.[81] Increased neutrophil infiltration in HNSCCs is correlated with reduced survival,[82] suggesting that just like with macrophages, HNSCC neutrophils are predominantly N2-polarized. TGFβ signaling induces N2 polarization; in mice, treatment with a TGFβ inhibitor caused a shift from N2 to N1 tumor-associated neutrophil polarization and increased immune clearance of mesothelioma tumors.[81] Myloid-derived suppressor cells (MDSCs) encompass both granulocytic polymorphonuclear cells that are closely related (if not identical) to neutrophils, and monocytic cells that are similar to monocytes.[83,84] MDSCs strongly inhibit T cell proliferation and activation *in vitro*,[85] and are associated with reduced survival in HNSCC.[86] The expression of *TGFβ1* and MDSC marker genes is positively correlated in HNSCC, and supplemental TGFβ enhanced the ability of cultured monocytes to impair T cell proliferation and induced Treg differentiation *in vitro*.[87] Furthermore, as tumor cells lose TGFβ signaling components they enhance recruitment of MDSCs to the tumor microenvironment.[88,89] Finally, MDSC suppressor activity is not limited to CTLs; monocytic MDSCs in the tumor microenvironment secrete TGFβ that in turn inhibits NK cells.[90]

Cancer-associated fibroblasts (CAFs) and their interactions with immune cells in SCC

Late-stage HNSCCs are comprised of up to 80% CAFs[91] that secrete a variety of immunosuppressive cytokines,[92] and they are the primary source for secreted TGFβ in the tumor microenvironment.[93] The secretion of TGFβ by CAFs is increased by the loss of TGFβ signaling components in tumor cells.[94] TGFβ signaling also drives a gene expression program in HSNCC CAFs that both promotes immune evasion and predicts the failure of immune checkpoint blockade.[95] This TGFβ signaling program functions as a better

predictor of anti-PD-1 therapeutic failure than other commonly used biomarkers, including tumor mutational burden, amount of infiltrating T cells, and T cell activation markers.[95] Recent research suggests that the mechanism for this is TGFβ-mediated signaling in CAFs that results in the exclusion of $CD4^+$ and $CD8^+$ infiltrating T lymphocytes from the tumor; once TGFβ signaling was inhibited, CTLs were able to reach the tumor cells and became responsive to immune checkpoint blockade as their cytolytic signaling pathways were activated.[96]

The paradox of TGFβ as a pro-inflammatory molecule

As discussed above, TGFβ plays many anti-inflammatory roles in the tumor microenvironment. However, TGFβ also has a paradoxical pro-inflammatory effect in SCCs. We found that TGFβRII-negative HNSCCs exhibited increased $CD45^+$ and $F4/80^+$ cell infiltration and an upregulation of inflammatory cytokines when compared to wild-type mucosa,[12] and similarly Smad4-negative SCCs had increased $CD11b^+$ and Th17 cell inflammation.[13] Th17 cells are a subset of $CD4^+$ T cells that can both promote inflammation by inducing CXCL9 and CXCL10 secretion by tumor cells,[97] and suppress it via induction of Treg and Th1 cell differentiation.[98-100] Whether Th17 cells function as a tumor suppressor or promoter depends on the context and type of the tumor, but in HNSCCs they are associated with improved prognoses.[101] Th17 cells were originally identified by their propensity to secrete IL-17, a pro-inflammatory cytokine that is required for TGFβ-induced inflammation in premalignant skin lesions.[102] Th17 cells also need TGFβ to differentiate from naïve T cells,[103] and elevated levels of TGFβ in the skin are associated with increased Th17 cell infiltration in premalignant lesions.[13] However, as HNSCC tumors reach a later stage, IL-17 secretion is inhibited and the number of infiltrating Th17 cells decreases.[104] Although the significance of the inflammatory effect of TGFβ in the skin has not been fully explored in SCC treatment, it has the potential to create paradoxical effects of TGFβ inhibition in epithelial cancers as TGFβ-induced inflammation would be abrogated by TGFβ inhibitors. Indeed, when mice bearing a TGFβ1-knockout model of psoriasis were treated with a TGFβ-inhibitory drug, chronic inflammation was drastically and durably reduced.[105]

TGFβ signaling and immune checkpoints

The expression of PD-1 on tumor-infiltrating lymphocytes and the positivity of tumors for PD-L1 staining are associated with improved survival in HNSCC patients,[106] and the expression of PD-L1 is a commonly used metric to predict a response to PD-1 blockade.[107] Multiple preclinical models have demonstrated that TGFβ signaling induces expression of both PD-1 and PD-L1, for example, TGFβ induced PD-1 and PD-L1 expression on infiltrating T cells in a transplant model.[108] Furthermore, PD-1 expression on tumor-infiltrating lymphocytes was abrogated in mice treated with either a TGFβ-depleting antibody or a TGFβRII inhibitor.[32] Recent research showing that PD-L1 on human non-small cell lung cancer cell lines was induced by TGFβ *in vitro* suggests that the mechanism behind this is Smad2-mediated canonical TGFβ signaling in response to TGFβ ligand stimulation.[109] This has important implications for the combination of TGFβ inhibitors with PD-1 and PD-L1 checkpoint blockade, as TGFβ inhibition has the potential to reduce the

effectiveness of immune checkpoints in the tumor microenvironment from impairing CTLs within the tumor.

TGFβ inhibition as an immunotherapy

Because of the numerous ways TGFβ promotes immune suppression, it makes for a promising target in cancer therapeutics. However, TGFβ also functions as a growth inhibitor of tumor cells at early cancer stages until they become unresponsive to TGFβ signaling. This, combined with the cardiac toxicity reported in early-generation TGFβ inhibitors,[110] resulted in few TGFβ inhibitors being developed until the impact of immunotherapies and the importance of targeting immune evasion by cancer became apparent. More recently, TGFβ receptor inhibitors such as Galunisertib (LY2157299) have been developed with special care given to their impact on the cardiac health of patients.[111] The current range of TGFβ inhibitors and their clinical trial status has been reviewed elsewhere,[112] but they have proved to be effective both as a single agent and in combination with other therapeutics. For example, an immune-suppressive response in mice treated with an EGFR inhibitor was driven by secreted TGFβ in a preclinical model of HNSCC.[113]

Perhaps the TGFβ inhibition therapy with the most potential has been in combination with immune checkpoint blockade. As mentioned earlier, TGFβ signaling and immune checkpoint signaling are closely related. In murine SCC, anti-PD-1 treatment induces Treg expansion and elevated TGFβ signaling within the tumor that can be abrogated with the addition of a TGFβ depleting antibody.[114] Recent studies show that the mechanism behind this is TGFβ-mediated signaling in fibroblasts that causes T cell exclusion, resulting in increased T cell infiltration into tumors that were treated with a TGFβ inhibitor.[96,115,116] In addition to combining TGFβ inhibitors with PD-1 blockade, bifunctional antibodies that combine the TGFβRII ligand-binding domain with immune checkpoints blockade antibody have also been effective in both preclinical models and clinical trials. TGFβ/PD-1 and TGFβ/CTLA-4 bifunctional antibodies improved the anti-tumor response in melanoma and triple-negative breast cancer lines that are otherwise resistant to immune checkpoint blockade.[46] M7824, a combination of the TGFβRII ligand trap and anti-PD-L1, improved the response against murine breast and colon cancer models, resulting in increased T cell and NK cell activation within the tumors.[117] TGFβ inhibition also improves systemic responses to cancer. A pan-TGFβ depleting antibody improved tumor clearance in mice when used in combination with irradiation, including in non-irradiated areas.[118]

Conclusion

TGFβ has wide-ranging effects on multiple arms of the immune response to cancer. Because of the prevalence of dysregulation of TGFβ signaling in them, SCC is an optimal cancer for the application of TGFβ-inhibitory therapy. Furthermore, because the loss of TGFβ signaling components also results in increased DNA damage and tumor mutational burden, SCC is already primed for immune checkpoint blockade. However, because the tumor-infiltrating T cells in these tumors are already exhausted and incapable of mounting an anti-tumor response, immune checkpoint blockade must be combined with other therapies that release T cells from immune suppression. Therapeutically targeting TGFβ in combination

with immune checkpoint blockade has the potential to prevent immune evasion by SCC, and allows a complete anti-tumor response by reducing the immunosuppressive effects of multiple arms of the immune system on T cells within the tumor.

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Abbreviations:

TGFβ	Transforming growth factor-beta
SCC	Squamous cell carcinoma
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
SBE	Smad binding element
TGFβRI/II	TGFβ receptor I/II
PD-1	Programmed cell death protein 1
PD-L1	Programmed-death ligand 1
CTL	Cytotoxic T lymphocyte
Treg	Regulatory T cell
Th cell	Helper T cell
NK cell	Natural killer cell
TAM	Tumor-associated macrophage
MDSC	Myeloid-derived suppressor cell
CAF	Cancer-associated fibroblast

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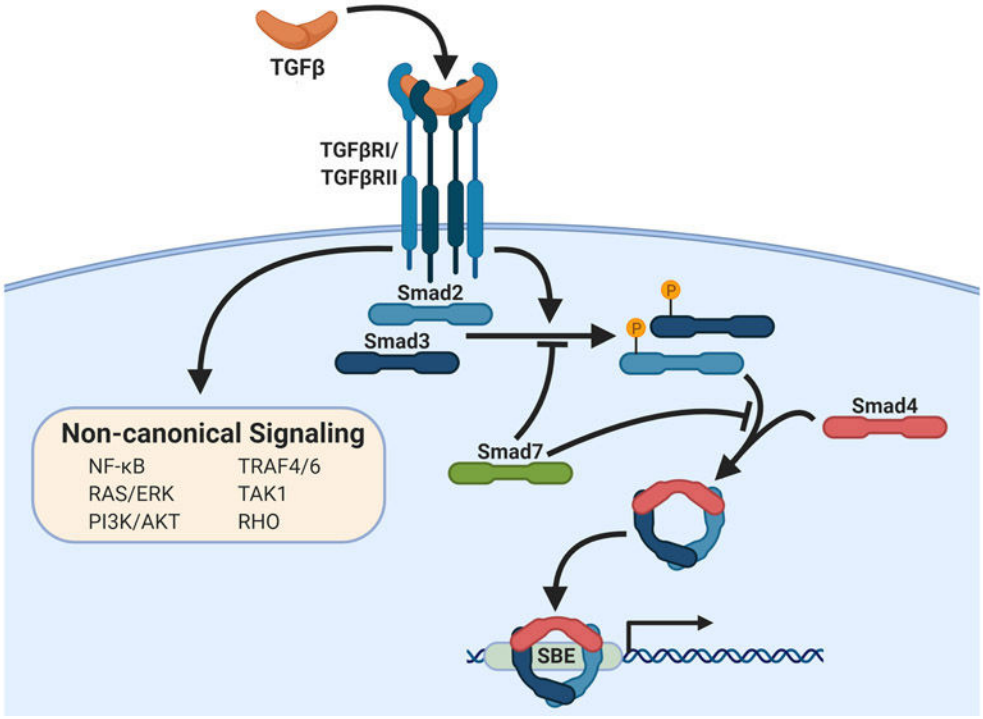

Figure 1:

A simplified schematic of the TGFβ signaling pathway. The TGFβ receptor complex is a heterotetramer of TGFβRI and TGFβRII dimers. Upon TGFβ ligand binding, the TGFβ receptor phosphorylates Smad2 and Smad3, which then form a complex with Smad4 that localizes to the nucleus, binds to SBEs at genetic loci and regulates downstream transcription. Smad7 is a genetic product of canonical TGFβ signaling that inhibits this process. Additionally, the TGFβR complex can activate non-canonical signaling pathways independently of Smad proteins.

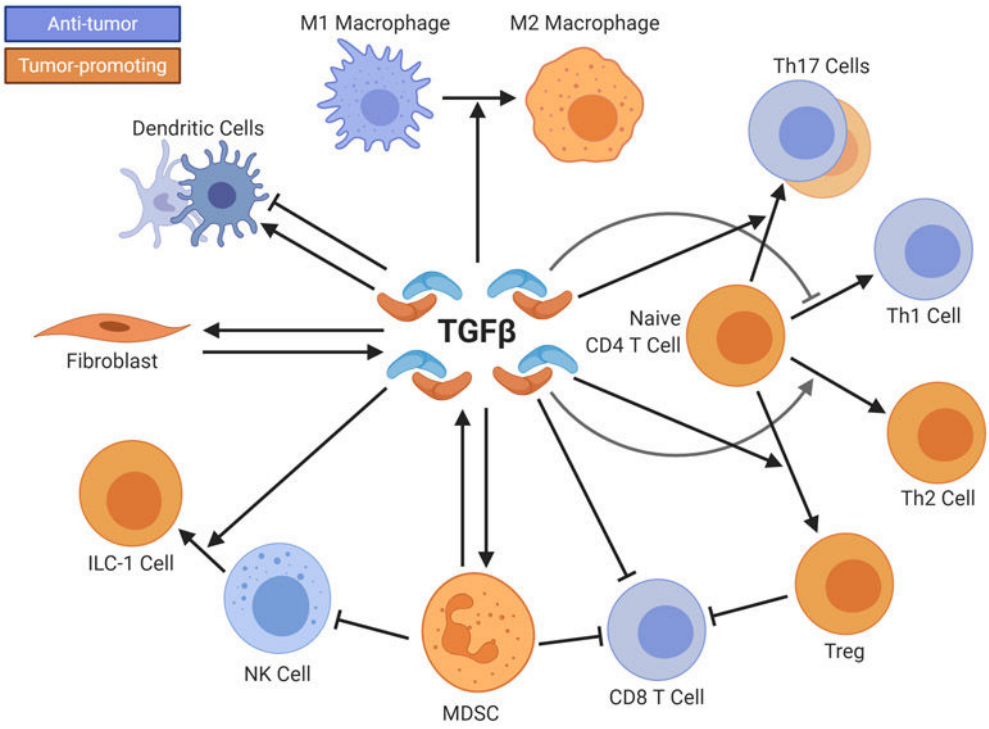

Figure 2:

The effects of TGFβ on major populations of stromal cells in SCC. TGFβ both inhibits and promotes the infiltration and function of many infiltrating immune and stromal cells in SCC tumors. These cell populations can have anti-tumor (blue) and tumor-promoting effects (orange) within the tumor microenvironment.