Harnessing the immunostimulatory properties of oncolytic reovirus for anticancer immunotherapy

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Blockade of TGF-β signaling to improve reovirus-based immuno-therapy

CHAPTER 6

Immunotherapeutic Potential of TGF-β Inhibition and Oncolytic Viruses

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ABSTRACT

In cancer immunotherapy, a patient's own immune system is harnessed against cancer. Immune checkpoint inhibitors release the brakes on tumor-reactive T cells and therefore are particularly effective in treating certain immune-infiltrated solid tumors. In contrast, solid tumors with immune-silent profiles show limited efficacy of checkpoint blockers due to several barriers. Recent discoveries highlight transforming growth factor-β (TGF-β)-induced immune exclusion and a lack of immunogenicity as examples of these barriers. In this review, we summarize preclinical and clinical evidence that illustrates how the inhibition of TGF-β signaling and the use of oncolytic viruses (OVs) can increase the efficacy of immunotherapy and discuss the promise and challenges of combining these approaches with immune checkpoint blockade.

HIGHLIGHTS

- Immune checkpoint blockade is not effective in immune-excluded and immune-desert tumors due to an immunosuppressive tumor microenvironment and the absence of activated T cells.
- TGF-β is a pleiotropic cytokine that contributes to immune exclusion and evasion in various cancer types.
- The therapeutic efficacy of oncolytic viruses is built on the recruitment of T cells and the induction of tumor-reactive immunity.
- Oncolytic virotherapy and inhibition of TGF-β signaling, either alone or in combination, are two emerging approaches to increase the susceptibility of immune-silent tumors to immune checkpoint therapy.

THE IMMUNE PROFILE OF SOLID TUMORS CAN DETERMINE THE EFFICACY OF IMMUNOTHERAPY

Our immune system is able to respond to invading pathogens and initiate a protective immune response. Although malignant cells are much more similar to the host than pathogens are, they still differ genetically, metabolically, and morphologically from normal cells and can therefore be recognized by the adaptive immune system, a trait called **immunogenicity** (see Glossary). In **Box 1** we provide more information about processes involved in antitumor immunity. **Immunotherapy** is being extensively studied as a new modality of cancer treatment for a wide variety of tumors. In contrast to conventional therapies that directly target the proliferation, survival, or metabolic activity of tumor cells, cancer immunotherapy is directed towards immune cells with the purpose of eliciting a durable and effective anticancer immune response.

Box 1: Priming of tumor-specific T cells

Recognition of tumors by T cells requires the expression of tumor antigens (TAs), aberrant proteins, or peptides beyond the normal repertoire that alert the adaptive immune system that the tumor cell is no longer healthy. Different classes of tumor antigens have been identified, of which **neoantigens** are the most tumor-specific. Neoantigens arise from genetic mutations in a tumor cell that give rise to a novel protein or peptide sequence. The number of mutations varies significantly per tumor type and it is believed that tumors with a high mutational burden are more immunogenic, display a higher immune infiltrate, and are more responsive to immune checkpoint inhibitors than tumors with a low mutational burden (1).

Whereas CTLs are believed to be the main T-cell subset responsible for eliminating cancer cells, $CD4^+$ T helper cells are of vital importance in shaping the tumor-specific T-cell response (2). Priming of naive $CD4^+$ and $CD8^+$ T cells towards effective tumor-specific $CD4^+$ helper and $CD8^+$ cytotoxic T cells, respectively, is a multifaceted process that requires uptake, processing, and presentation of TAs by dendritic cells (DCs) in the context of inflammation (3). The sensing of inflammatory signals, derived from **pathogen-associated molecular patterns** (PAMPs) or **damage-associated molecular patterns** (DAMPs) by a DC induces a differentiation process called maturation. DC maturation is characterized by upregulated amounts of costimulatory molecules, antigen processing, and presentation pathways, and the production of type 1 helper T cell (Th1) skewing cytokines.

The tumor microenvironment (TME) is usually not inflammatory in nature, and additionally, tumors use several strategies to actively suppress the immune system, leading to T-cell ignorance. Activating tumor-specific $CD4^+$ and $CD8^+$ T cells by applying TAs in the optimal context together with a DC maturing agent is in some cases achieved by cancer vaccine platforms, such as synthetic peptide-based vaccines or dendritic cell-based vaccines (4-6). However, tumors often have the ability to escape immune recognition and destruction, which highlights the need for effective immunotherapeutic strategies to overcome these barriers.

The tumor immune profile is an important determinant to guide immunotherapeutic strategies (7,8). Clinical responses to immune checkpoint inhibition mostly occur in patients with an **immune-infiltrated** tumor phenotype, which displays a pre-existing but often dysfunctional immune response (9). In contrast to immune-infiltrated tumors, **immune-excluded or immune-desert** (also described as immune-silent) tumors are less susceptible to checkpoint inhibition because **tumor-infiltrating T cells** (TILs) are absent (10). Strategies to convert immune-silent tumors into immune-active tumors are desperately needed to broaden the fraction of patients that might benefit from immune checkpoint therapy. In this review, we discuss two emerging approaches that

might be harnessed on their own or in combination to enhance the efficacy of immune checkpoint inhibition in immune-silent tumors (**Figure 1, Graphical Abstract**).

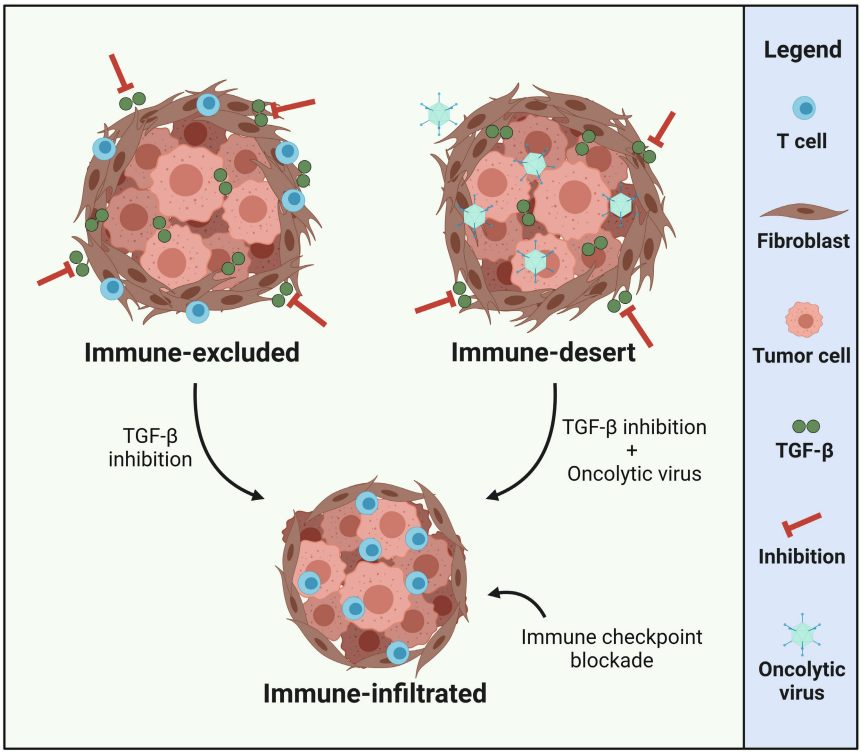

Figure 1, Graphical Abstract. Combining TGF-β inhibition with oncolytic viruses to increase efficacy of immune checkpoint blockade in solid tumors. Immune checkpoint blockade is mostly effective in immune-infiltrated tumors where T cells (blue) are present in the tumor nests (red) but may be dysfunctional. In immune excluded tumors, T cells are present but remain trapped in the stromal regions (brown) surrounding the tumor nests. TGF-β (dark green) inhibition is expected to change the phenotype of immune-excluded tumors towards an immune-infiltrated phenotype. In immune-desert tumors, a T cell response is absent. Combination strategies of oncolytic viruses with TGF-β-inhibition may also convert immune-desert tumors to immune-infiltrated tumors, facilitating effective immune checkpoint blockade for all immune phenotypes in solid tumors.

First, we discuss the inhibition of **transforming growth factor β** (**TGF-β**) signaling to enhance the efficacy of checkpoint blockade therapy given that recent evidence suggests that TGF-β may be a key factor in regulating immune exclusion and immunosuppression in solid tumors. Additionally, we highlight the potency of **oncolytic viruses** (**OVs**) to convert solid tumors from an immune-silent phenotype towards an immune-infiltrated phenotype. Lastly, we theorize how a combination of TGF-β inhibition, OVs and immune checkpoint blockade may be superior in efficacy compared to strategies that contain only two of these three aspects.

IMMUNE CHECKPOINT INHIBITION CAN REINVIGORATE DYSFUNCTIONAL ANTITUMOR RESPONSES IN IMMUNE-INFILTRATED TUMORS

The discovery of immune checkpoints boosted the development of immunotherapeutic strategies against certain cancers. **Programmed cell death protein 1** (PD-1) and **cytotoxic T lymphocyte-associated antigen 4** (CTLA-4) are well-recognized immune checkpoint receptors that can limit antitumor immunity using distinct mechanisms. CTLA-4 prevents T-cell activation by competing with the costimulatory molecule CD28 for binding to their common ligands CD80 and CD86 (11). In contrast, PD-1 induces **T-cell anergy** or **T-cell exhaustion** after binding to one of its ligands, PD-L1 or PD-L2, expressed on the surface of tumor cells and/or immune cells (10,12). The use of blocking antibodies specific for these immune checkpoint axes can prevent or overcome T lymphocyte dysfunction and reinvigorate potent $CD8^+$ T-cell-mediated antitumor immune responses, as has been demonstrated in clinical practice for hematological malignancies such as acute myeloid leukemia, as well as in solid tumors such as melanoma, lung, bladder and head and neck cancers (10,13). In addition to the CTLA4-CD80/86 and the PD-1-PD-L1/L2 axes, other coinhibitory receptor targets, such as lymphocyte activation gene 3 (LAG-3) (14), T-cell immunoglobulin and mucin-domain containing protein 3 (TIM-3) (15) and C-type lectin receptor NKG2A (16,17), are currently being investigated in either preclinical studies or clinical trials for a wide variety of both hematological and solid cancers. Although checkpoint inhibition is able to induce dramatic responses in some types of cancer, the response rate in general ranges from 10-40% and heavily depends on the cancer type and the development of resistance during disease progression (18). Factors associated with a beneficial response to checkpoint blockade therapies include a high total number of mutations in tumor cell DNA (1), the presence of an interferon gene signature, the expression of proinflammatory and T-cell-recruiting chemokines such as CXCL9 and CXCL10, the presence of $CD8^+$ T lymphocytes in close proximity to tumor cells, and high PD-L1 expression, in particular on infiltrating immune cells (10,19-22). An immune cell-infiltrated tumor without a clinical response may suggest a pre-existing but dysfunctional tumor-specific $CD8^+$ T-cell response (23).

One category of immune-silent tumors with relatively low susceptibility to checkpoint inhibition includes tumors with an immune-excluded phenotype, such as colorectal cancer, ovarian cancer, pancreatic ductal adenocarcinoma, and vulvar squamous cell carcinoma (24,25, reviewed in 26). Immune-excluded tumors are characterized by the presence of $CD8^+$ T cells in the tumor-surrounding tumor stromal regions, but these T cells fail to infiltrate into the tumor beds (**Figure 1**) (27). The presence of stroma including **cancer-associated fibroblasts** (**CAFs**), extracellular matrix components such as collagen, and cells of the myeloid lineage, such as the so-called **myeloid-derived suppressor cells** (**MDSCs**) (28) and **tumor-associated macrophages** (**TAMs**) (29), not only represents a physical barrier but also induces an

immunosuppressive **tumor microenvironment** (**TME**), which limits T-cell infiltration into tumor nests (26,30). Hence, it is necessary to overcome the physical barrier and modify the immunosuppressive TME in immune-excluded tumors to facilitate T-cell migration through the stromal region into the tumor cell nests, where these immune cells can fully exert their tumoricidal function. An additional type of immune-silent tumors that exhibits low susceptibility to checkpoint blockade is the immune-desert phenotype. Immune-desert tumors lack the presence of T cells completely and require preceding T-cell activation (31). Below, we discuss promising methods for achieving T-cell infiltration into immune-desert tumors.

OVERCOMING IMMUNOSUPPRESSION VIA TGF-β SIGNALING INHIBITION FOR IMMUNE-EXCLUDED TUMORS

TGF-β as a mediator of immunosuppression

The secreted cytokine TGF-β is one of the key factors believed to be responsible for immune exclusion and suppression in certain types of cancer, such as pancreatic cancer, non-small cell lung cancer, and colon cancer (32-34). In premalignant lesions, TGF-β signaling suppresses tumor growth by inducing apoptosis and inhibiting cell proliferation (35). However, during tumor progression, tumor cells become insensitive to TGF-β-induced cytostatic effects, and TGF-β functionally switches into acting as a tumor-promoting cytokine by promoting cancer cell migration and invasion, extracellular matrix (ECM) remodeling, **epithelial-to-mesenchymal transition** (**EMT**) and the formation of an immunosuppressive TME (36). TGF-β induces its prometastatic programs directly via cell surface TGF-β type I and type II serine/threonine kinase receptors (TGF-βRI and TGF-βRII) and intracellular SMAD-transcriptional effector proteins. Especially in human colon and pancreatic cancers, the TGF-β-induced cytostatic response is often inactivated by mutation of TGF-β receptors or SMADs (37). However, TGF-β is still produced in high amounts by cancer and stromal cells, which is associated with relapse and reduced survival (32,33,38). In **Box 2,** we provide further details regarding the TGF-β signaling pathway in cancer progression and metastasis, and how this pathway can be inhibited.

In addition to the regulation of tumor-promoting processes described above, TGF-β also inhibits the generation and function of $CD4^+$ and $CD8^+$ effector T cells and dendritic cells (DCs), while promoting the expansion of **regulatory T cells** (**Tregs**) and MDSCs (recently reviewed in 39). Early, pivotal studies showed that CD4-dnTGFβRII transgenic mice engineered to express a dominant-negative version of TGF-βRII in their $CD4^+$ and $CD8^+$ T cells rendered these mice resistant to tumor challenge with B16.F10 murine melanoma cells or EL-4 murine lymphoma cells (40). TGF-β inhibits the differentiation of $CD4^+$ T cells into effector cells by silencing the expression of master transcription factor T-bet (41), while stimulating the transition of naive $CD4^+$ cells into Tregs by inducing FoxP3 expression (42). In $CD8^+$ T cells, TGF-β represses eomesodermin (EOMES), an

important transcription factor that regulates the effector program of cytotoxic $CD8^+$ T cells (43). In a murine B16.F10 melanoma model, treatment with various small molecule kinase inhibitors specific for TGF-βRI not only directly inhibited phosphorylation of receptor-regulated SMAD proteins, but also induced ubiquitin-mediated degradation of SMAD4 mainly in $CD8^+$ **cytotoxic T lymphocytes** (**CTLs**), and thereby increased their effector function and suppressed tumor growth (43). The important role of TGF-β in T-cell suppression was further illustrated by the observation that TGF-β induced the surface expression of PD-1 on both activated human peripheral blood mononuclear cells (PBMCs) and murine B16.F10 tumor-infiltrating $CD8^+$ and $CD4^+$ T cells through SMAD3-dependent transcriptional activation, thereby reducing T-cell effector function and limiting the antitumor response (44). Additionally, T cells genetically modified to be resistant to TGF-β showed significantly enhanced tumor control in an adoptive T-cell transfer setting in a syngeneic murine B16.F10 melanoma model in comparison with T cells that could still respond to TGF-β (45).

Box 2: TGF-β signaling in cancer and metastasis

Transforming growth factor β (TGF-β) is a pleiotropic cytokine produced in a latent form by cancer cells, various immune cells, platelets, and stromal cells (39). The signaling pathways activated by TGF-β and its family members are highly conserved among species and are involved in development, homeostasis, and regeneration (46). Dysfunction of the TGF-β signaling pathway might lead to various pathologies such as fibrosis, congenital defects, dysfunction of the immune system, and cancer. In short, all three TGF-β isoforms, i.e. TGF-β1, -β2, and -β3 are cleaved into their active form by integrins or matrix metalloproteinases, resulting in a TGF-β dimer. Signaling takes place via TGF-β type I and II serine/threonine kinase receptors (TGF-βRI and TGF-βRII) that are expressed on the plasma membrane (**Figure I**). Upon TGF-β-induced heteromeric complex formation of TGF-βRI and TGF-βRII, TGF-βRII phosphorylates TGF-βRI, which subsequently leads to the phosphorylation of receptor-regulated SMADs (R-SMADs). R-SMADs form complexes with the common mediator SMAD4, which drives transcriptional regulation of various TGF-β target genes. Additionally, TGF-β family members can also signal in SMAD-independent manners by using non-canonical pathways such as the phosphatidylinositol-3 kinase (PI3K)-AKT and the p38 MAP kinase pathway (47).

TGF-β acts as a tumor suppressor in the early stages of tumor development, but this function is lost during the later phases of cancer progression. Instead, TGF-β signaling promotes epithelial-to-mesenchymal transition (EMT) by reducing the expression of epithelial markers, such as E-cadherin, while increasing the expression of mesenchymal markers, such as vimentin (48). The TGF-β-induced exploitation of EMT during cancer progression is assumed to contribute to the growth of the primary tumor as well as metastasis. Because of the various functions of TGF-β during tumor progression and metastasis, multiple strategies >>

>> have been developed to block TGF-β signaling (**Figure I**). Current TGF-β pathway inhibitors work on different levels by either (**A**) preventing TGF-β production or expression of its receptor by antisense oligonucleotides (synthetic nucleic acids with a complementary sequence that prevents mRNA translation), (**B**) preventing TGF-β activation via integrin-blocking antibodies (49), (**C**) inhibiting the interaction between TGF-β and its receptor with neutralizing antibodies to TGF-β, blocking antibodies to TGF-βRII or ligand traps (engineered soluble forms of the receptor that compete with the cell-bound receptor) or (**D**) preventing intracellular TGF-β receptor signal transduction via **small molecule kinase inhibitors** such as galunisertib (reviewed in 50).

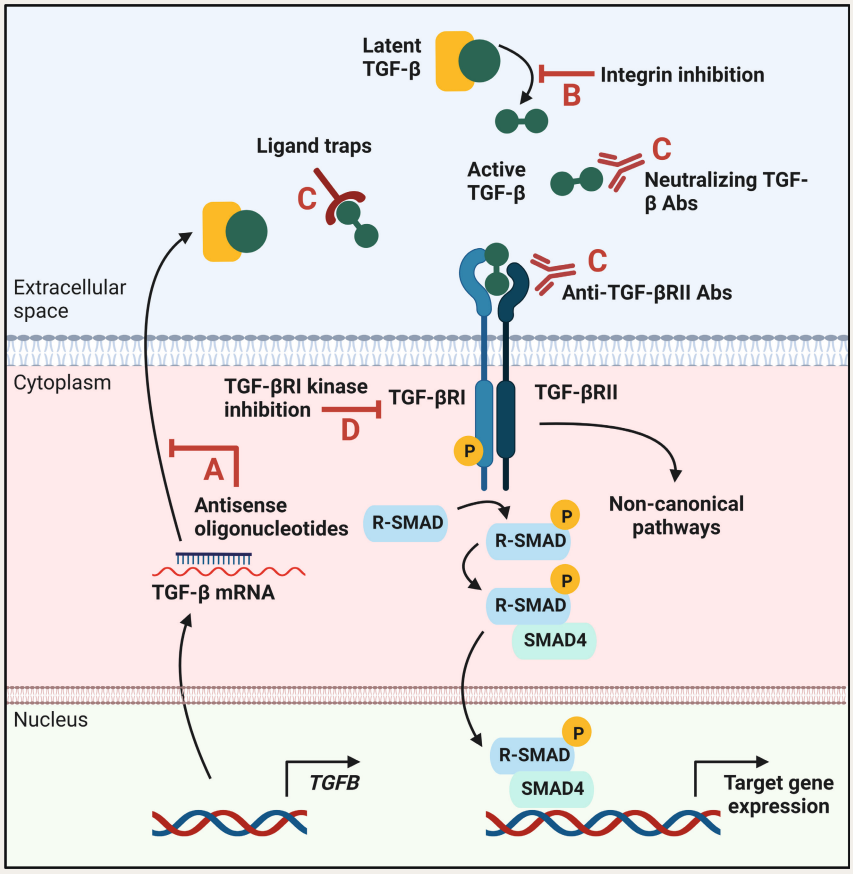

Figure I. Overview of TGF-β signaling across species. Arrows indicate processes. Latent TGF-β (dark green) is cleaved into its active form by integrins or matrix metalloproteinases, resulting in a TGF-β dimer. Active TGF-β induces heterodimerisation of TGF-βRI and TGF-βRII, ultimately leading to the phosphorylation of TGF-βRI. Subsequent phosphorylation of R-SMADs initiates the SMAD-mediated transcription of TGF-β target genes. TGF-β also signals via non-SMAD pathways, via the so-called non-canonical pathways. Interference in TGF-β signaling is possible using various strategies (indicated in dark red) by inhibiting **A:** TGF-β (receptor) production, **B:** TGF-β activation, **C:** ligand-receptor interaction or **D:** TGF-βRI receptor activation.

The results of these *in vivo* studies hint towards a potential beneficial effect of TGF-β inhibition on the induction of a potent antitumor response. Indeed, antibody-mediated inhibition of TGF-β was able to induce complete tumor regression when given as monotherapy in up to 20% of animals in the subcutaneous CCK168 model of chemically-induced cutaneous squamous cell carcinoma engrafted in FVB/NJ mice (51). Furthermore, rechallenge experiments suggested that TGF-β blockade induced immunological memory and long-term protection since both the parental cell line or similar chemically-induced cutaneous squamous cell carcinoma cell lines failed to grow in the animals that underwent complete regression (51). Similar effects were observed in a mouse model of murine 4T1-luciferase breast cancer, where complete regression was observed in 50% of animals after treatment with galunisertib (LY2157299 monohydrate), a small molecule that inhibits the kinase activity of TGF-βRI (52). Mice with durable regressions also rejected tumor rechallenge with both the 4T1-luciferase cell line and the parental, less immunogenic 4T1 cell line, thereby demonstrating established immunological memory (52). In addition, inhibition of TGF-β signaling using the same compound unleashed a potent and enduring CTL response in murine metastatic colorectal cancer models, reducing both primary tumor growth and blocking the appearance of liver metastases (53). Rechallenge experiments with the same tumor model demonstrated rejection of most tumors in the absence of any treatment, an effect that was mitigated upon antibody-mediated depletion of CTLs, again suggesting that TGF-β could limit adaptive immune responses by inhibiting CTL responses (53). Overall, TGF-β can heavily impair CTL responses and induce a generally immunosuppressed TME, thereby promoting tumor progression and metastasis.

TGF-β inhibition can increase the efficacy of immune checkpoint therapy

As described above, TGF-β inhibition induces regression of primary tumors, prevents metastasis formation, and induces protection against tumor rechallenge in various mouse tumor models when applied as a monotherapy. However, can TGF-β inhibition provide an added therapeutic effect to immune checkpoint therapy? A rationale for this strategy was demonstrated by a genomic and transcriptomic analysis that revealed enrichment in markers of EMT, cell adhesion, and ECM remodeling in PD-1 therapy-resistant melanoma patients in comparison to therapy-responding patients (54). All of these cellular processes are known to be regulated via TGF-β signaling (55). Moreover, transcriptomic analysis of human tumors from The Cancer Genome Atlas (TCGA) suggested that upregulation of ECM gene expression, such as genes encoding matrix metallopeptidases (MMPs) and collagen, was linked to the activation of TGF-β target genes in CAFs and that this pan-cancer signature predicted unresponsiveness to PD-1 blockade (56). Additionally, single-cell sequencing studies identified a population of TGF-β-driven CAFs that was associated with poor response to anti-PD-L1 therapy in human immune-excluded tumors, such as pancreatic cancer and bladder cancer (57). Finally, gene set enrichment analysis identified the genes *TGFB1* (encoding TGF-β1) and *TGFBR2* (encoding TGF-βRII) to be associated with nonresponse to anti-PD-L1 therapy and reduced overall survival in patients with urothelial cancer (58). Altogether, these

studies support the use of TGF-β signaling pathway inhibitors to sensitize immune-excluded tumors for immunotherapy. Indeed, combined treatment with anti-PD-L1 and anti-TGF-β antibodies in the immune-excluded EMT6 mouse mammary carcinoma model led to a significant decrease in the tumor burden, reprogramming of stromal fibroblasts and increased infiltration of $CD8^+$ T cells in comparison to either treatment alone (58). These effects were lost after antibody-mediated depletion of $CD8^+$ T cells, indicating that the effect of this combination therapy was based on a potent $CD8^+$ T-cell-driven antitumor immune response (58). In the 4T1 mouse model of metastatic breast cancer, TGF-β neutralization using the pan-isoform 1D11 monoclonal antibody during radiotherapy successfully decreased both primary tumor growth and the occurrence of metastasis and increased $CD4^+$ and $CD8^+$ T-cell infiltration (59). The addition of checkpoint blockade to this regimen led to complete tumor regression in 75% of mice, delayed tumor recurrence, and prolonged survival. Similar beneficial effects of combined checkpoint inhibition and TGF-β inhibition on tumor regression were observed in mouse models of 4T1 breast cancer (52), progressive metastatic liver disease (53), MC38 colorectal cancer (60), and on the metastatic spread to the lung of the colorectal tumor model CT26 (61). Additionally, *in vivo* treatment using the bifunctional fusion protein M7824, composed of an antibody targeting PD-L1 and a TGF-β ligand trap, has shown promising antitumor activity in a vast number of preclinical models, including **orthotopic** and subcutaneous mouse models of breast cancer, colon cancer, and renal adenocarcinoma, as well as in a xenograft model of human pharyngeal carcinoma (62). Last, a similar bifunctional fusion protein targeting CTLA-4 instead of PD-L1 was shown to inhibit tumor growth more efficiently than anti-CTLA-4 alone in human melanoma and triple-negative breast cancer models established in immunodeficient, humanized mice (63). Based on the promising effects observed in preclinical studies, various clinical trials are ongoing in which the combination of TGF-β inhibition and checkpoint blockade is investigated. For example, an ongoing Phase 1b/2 dose-escalation and cohort-expansion study with 75 participants (NCT02423343)[i] aims to evaluate the safety, tolerability, and efficacy of the combination of galunisertib and anti-PD-1 in advanced refractory solid tumors (Phase 1b) and in recurrent or refractory non-small cell lung cancer or hepatocellular carcinoma patients (Phase 2). This trial and others may provide more information about the ability of dual inhibition of immune checkpoint axes and the TGF-β pathway to establish tumor growth control and prevent metastasis.

RECRUITING TUMOR-SPECIFIC T CELLS IS THE FIRST PRIORITY IN IMMUNE-DESERT TUMORS

While immune-excluded tumors may benefit from combined checkpoint blockade and TGF-β inhibition, tumors with the immune-desert phenotype are less likely to benefit from this combination therapy (10,64). Immune-desert tumors are characterized by an absence of T lymphocytes in both the tumor and the surrounding stromal regions

(10). The absence of pre-existing antitumor immunity is the first barrier that needs to be overcome before checkpoint inhibitors and TGF-β blockade can be used.

Using oncolytic viruses to induce antitumor immunity

A promising immunotherapeutic strategy that may promote antitumor immunity is treatment with oncolytic viruses (OVs) (65). The use of OVs as anticancer agents is emerging and driven by the FDA approval of **talimogene laherparepvec** (**T-VEC**), a modified herpes simplex virus type 1 (HSV-1) that increased survival and demonstrated favorable tolerability in advanced-stage melanoma patients (66). OVs selectively replicate in transformed cells, either naturally or after genetic modification (**Figure 2A**). Accumulating evidence suggests that beyond their oncolytic activity, OVs have broad immunostimulatory properties. Mechanisms of action include the induction of local inflammation and priming and recruitment of tumor-reactive $CD4^+$ and $CD8^+$ T cells (**Figure 2B**) (67-70). In addition to their oncolytic and immunostimulatory properties, OVs can also be used as a delivery platform for tumor-specific expression of immunostimulatory transgenes such as cytokines, chemokines, costimulatory ligands, immune checkpoint inhibitors and TAs (**Figure 2C**) (71). More background on OVs is provided in **Box 3.**

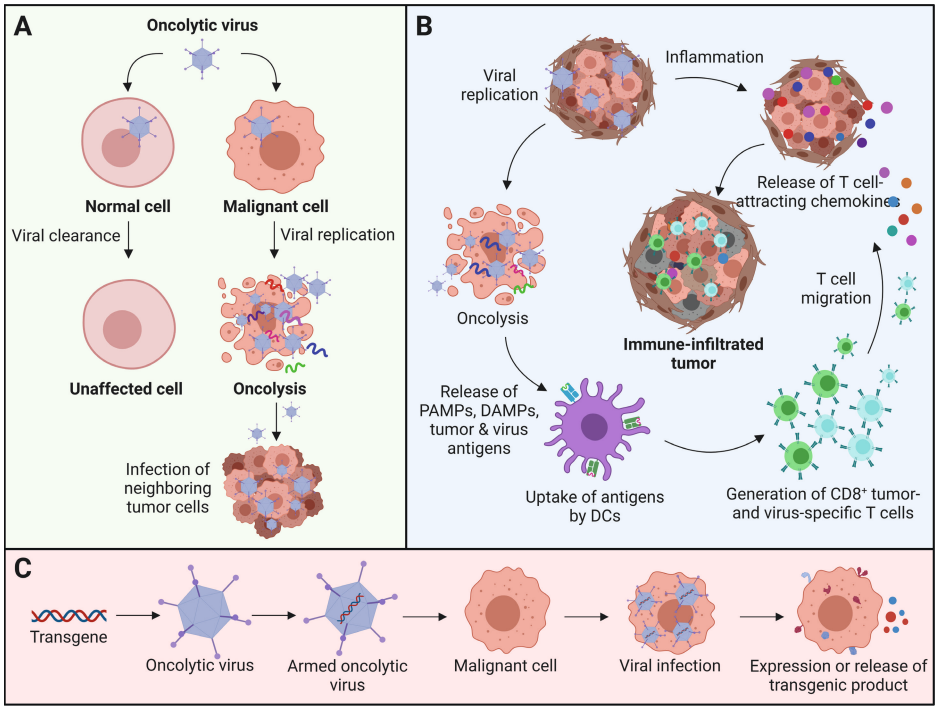

Figure 2. Properties of oncolytic viruses. (**A**) *Oncolytic properties.* Oncolytic viruses (OVs) selectively replicate in malignant cells, either naturally or after genetic modification. Normal cells remain unaffected due to viral clearance. Viral replication together with the induction of cell death pathways leads to lysis of tumor cells. Oncolysis causes the release of virus progeny, which infects new tumor cells.

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>> (**B**) *Immuno-stimulatory properties*. Virus replication causes oncolysis, which induces the release of tumor-specific and virus-specific antigens and **pathogen- and damage-associated molecular pattern molecules** (**PAMPs and DAMPs**, respectively). On the one hand, the subsequent uptake and presentation of antigens by dendritic cells leads to the induction of tumor- and virus-specific T cells. On the other hand, viral infection and replication induces an inflammatory response which causes the release of T cell-attracting chemokines. The tumor- and virus-specific T cells are attracted by these chemokines and migrate towards the tumor to exert their function. (**C**) *OVs as transgene delivery platform.* Some OVs (such as adenovirus and vaccinia virus) can be modified to encode transgenes (armed oncolytic viruses) such as cytokines or antibodies, ensuring specific delivery to the tumor microenvironment and further stimulation of an antitumor immune response.

Box 3: Oncolytic viruses as antitumor agents

Oncolytic viruses (OVs) are able to selectively infect, replicate in and lyse tumor cells that initially gained interest because of their tumor cell-lysing (oncolytic) capabilities. In contrast to normal cells, where virus infection initiates a type I interferon (IFN)-driven antiviral response program, deficiency of this pathway in many cancer cells favors cancer-specific OV replication (85,86). Tumor-specific driver mutations, such as an activated RAS pathway, and upregulation of cell-entry receptor expression can further promote selective replication in tumor cells (87,88). Furthermore, some OVs, including adenovirus and HSV, have been engineered to increase their tumor specificity (89,90), whereas a strain of oncolytic reovirus has been bioselected by growing on cells that lack expression of the entry receptor to broaden its tropism for different tumor cells (91).

Beyond their oncolytic activity, OVs are able to induce a tumor-reactive T-cell response by acting as ***in situ*** vaccines (70,79,92). The process of T-cell priming is particularly effective during virus infection because virus-derived nucleic acids optimally induce the maturation of dendritic cells (67,70). Simultaneously, dying tumor cells are a source of TAs, leading to the priming of tumor-reactive $CD4^+$ and $CD8^+$ T cells (93,94). This process is further enhanced by the OV-induced IFN response, which recruits immune cells to the tumor and promotes antigen presentation (78). Noteworthy, the OV-induced ***in situ*** vaccine strategy does not rely on prior identification of TAs or neoantigens for a given tumor or patient, which conceptually would provide a major advantage over other types of cancer vaccines (71).

OVs can also be used as platforms for the specific delivery of transgenes into the tumor bed (71). Cytokines and chemokines represent attractive transgenes because they have pleiotropic effects and are encoded by small genes (95). The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF), which promotes DC recruitment and maturation, has been the most widely studied transgene and has been encoded in many different OV platforms (96-98), including the leading clinically approved OV T-VEC (99). Other cargoes used to promote tumor-reactive

T-cell responses include ligands for costimulatory receptors such as CD40 ligand (100,101) or inducible co-stimulator (ICOS) ligand (102), checkpoint blockers such as anti-PD-1 antibodies (103), or **bispecific T-cell-engager**s (BiTEs) (104). Alternatively, OVs can also be armed with enzymes such as DNase or hyaluronidase to promote intratumoral penetration (105), or TAs such as MAGE-A3, which turn OVs into oncolytic vaccine vectors (106).

In addition to the direct elimination of primary treated tumors, OVs can induce long-term protection against secondary tumors (72-74). In an elegant rechallenge model, primary 4T1, EMT6, and E0771 murine breast cancer tumors were treated intratumorally with unarmed oncolytic Maraba virus MG1, after which the primary tumor was surgically resected (72). Thereafter, secondary tumors were implanted in the mammary fat pad and left untreated. Mice that were previously treated with Maraba virus showed significantly better tumor control of the untreated secondary tumor, and at least 20% of the animals showed complete tumor control. When T-cell deficient nude mice were used in a similar experiment, this effect was completely lost, suggesting that a functional adaptive immune system was necessary to induce T-cell memory and subsequent protection from secondary tumors. The capacity to confer immunological memory was similarly demonstrated for vesicular stomatitis virus (VSV), adenovirus, and HSV-1 in the 4T1 breast cancer model (73). Presurgical treatment with reovirus was only effective against the primary tumor in this study (73), but did induce protective memory in the EMT6 murine breast cancer model in another study (75). The OV-induced tumor-reactive immunity is believed to be not only a crucial aspect of the therapeutic efficacy of OVs (76,77) but may also be utilized to sensitize tumors for other types of immunotherapy by enhancing immunogenicity or by attracting activated $CD4^+$ and $CD8^+$ T cells to nonresponsive tumors (65,68).

Oncolytic virotherapy can synergize with immune checkpoint blockade

Several OV platforms have been demonstrated to increase the number of TILs and sensitize tumors for checkpoint therapy, both in preclinical studies and in clinical trials (68,72,75,78-81). For example, a randomized Phase 1b clinical trial (NCT02263508) [II] that investigated the combination of FDA-approved oncolytic HSV-1 T-VEC and pembrolizumab (anti-PD-1) in 21 patients with unresectable melanoma patients has shown promising results with a 61.9% objective response and a 33.3% complete response (82). Of note, responses have also occurred in patients whose tumors displayed a low $CD8^+$ T-cell density and no PD-L1 expression at baseline, which originally emerged as the first potential predictive biomarker for insensitivity to immune checkpoint blockade (83). This trial is currently continued as a Phase 3 trial to investigate the effect of combined treatment with T-VEC and pembrolizumab on progression-free survival and overall survival in comparison with pembrolizumab alone (NCT02263508)[II]. Furthermore, the combination of T-VEC and ipilimumab (anti-CTLA4 monoclonal antibody) has shown promising results in a randomized Phase 2 clinical

trial (NCT01740297)[111] with 198 patients with unresectable stage IIIB or IV melanoma that were randomly assigned half-half to combination therapy or ipilimumab alone (84). The combination therapy resulted in an objective response of 35.7% compared to 17.5% in the ipilimumab-only treated group (84). Driven by these encouraging initial studies and additional preclinical data, there are more than twenty ongoing clinical programs involving different OV platforms in combination with immune checkpoint inhibitors (65). Collectively, these studies not only highlight the potent role of OVs as anticancer agents but also illustrate their capacity to sensitize tumors for subsequent immunotherapy, although further robust testing is evidently warranted.

COMBINING OVS WITH TGF-β INHIBITION TO SENSITIZE SOLID TUMORS FOR IMMUNOTHERAPY

The lack of immunogenicity and the presence of stromal and immunosuppressive barriers are 2 major hurdles to effective immunotherapy for immune-desert tumors. Combined modulation of the stromal barrier by TGF-β inhibition and increasing immunogenicity using OVs might therefore be a potent strategy to sensitize immune-desert tumors for T-cell-based immunotherapy. Indeed, systemic treatment with a small molecule TGF-βRI inhibitor in combination with a single intratumoral injection of oncolytic HSV-1 variant MG18L resulted in complete tumor regression in 60% of treated subjects in an orthotopic model of patient-derived recurrent glioblastomas established in severe combined immunodeficient (SCID) mice lacking mature B and T cells (107). In a human MDA-MB-231 breast cancer xenograft model established in nude mice, 3 intratumoral injections of an oncolytic adenovirus armed with a soluble form of TGF-β receptor type II (sTGF-βRII) that functions as a ligand trap for TGF-β caused complete tumor regression in 7 out of 8 mice, which was better than the efficacy of the unarmed virus (3 out of 8 mice) and sTGF-βRII only (1 out of 8 mice) (108). Additionally, intravenous delivery of the same armed virus in this MDA-MB-231 breast cancer xenograft model significantly inhibited the progression of bone metastasis and prolonged survival when compared with the unarmed virus (109). A limitation of the studies performed in immunodeficient mice is that the role of T cells during the OV and TGF-β inhibition combination therapies remains underexplored. Combination treatment with intratumorally injected HSV1716, an attenuated unarmed oncolytic HSV-1, and a small molecule inhibitor of TGF-βRI was evaluated in immunocompetent models of murine rhabdomyosarcoma, resulting in tumor growth stabilization, significantly prolonged survival and even some complete responses compared to the single agents alone (89). In this study, the removal of T-cell responses via antibody-mediated depletion of $CD4^+$ and $CD8^+$ T cells or the use of athymic nude mice as recipients completely abolished the antitumor effect, indicating the importance of the T-cell response underlying efficacy of this combination treatment (89). Together, these preclinical studies suggest that the combination of TGFβ inhibition and OV therapy may be considered to putatively treat tumors with low immunogenicity and stromal or immunosuppressive barriers.

CONCLUDING REMARKS

In this review, we discussed two promising therapeutic strategies to overcome barriers to effective immunotherapy in relation to the tumor immune phenotype. For the classification of tumor immune profiles, we relied on the three main tumor immune phenotypes postulated by Chen and Mellman (9). We recognize that other classification strategies are possible, and more detailed profiles based on immunophenotyping of tumors are being investigated (26,110). Immune-infiltrated tumors have an ongoing T-cell response, but the dysfunctional state of these T cells needs to be overcome by immune checkpoint therapy. Clinical successes in various tumors with this immune phenotype have already been reported, and many efforts to identify novel targets, find biomarkers of efficacy, and understand secondary resistance mechanisms are ongoing, and more breakthroughs are anticipated. Tumors with an immune-excluded phenotype require modification of the immunosuppressive TME to allow T-cell infiltration into the tumor before checkpoint therapy can be applied. As discussed above, TGF-β inhibition has emerged as a multifunctional strategy to increase the efficacy of immunotherapy due to its capacity to modify the desmoplastic TME, increase the cytotoxic activity of $CD8^+$ (and possibly $CD4^+$) T cells, and reduce the frequency of Tregs. However, due to the pleiotropic effects on different cell types and the heterogenicity of the TGF-β superfamily, TGF-β is a challenging target in terms of pharmacology.

For immune-desert tumors, immunotherapy is a different, much harder, challenge. Treating these tumors with immune checkpoint blockade and TGF-β inhibition may be useful only when a prior treatment strategy has increased the immunogenicity of the tumor and induced tumor-reactive T-cell responses. OVs may represent potent tools to evoke potent $CD4^+$ and $CD8^+$ T-cell responses, as has been demonstrated by multiple preclinical studies mentioned above. The addition of TGF-β blockade may increase the efficacy of this combination therapy even further, but this remains to be vigorously investigated. TGF-β inhibition can not only lift the immunosuppressive and physical barriers to allow T-cell infiltration into the tumor bed, but also lift a physical barrier for penetration of OVs into tumors. Previous studies have shown that stromal components in the TME, such as TGF-β-producing CAFs and collagen, may impair viral spread in tumors, limiting the efficacy of OVs (111). Indeed, an oncolytic vaccinia virus armed with a **bispecific T-cell-engager** (**BiTE**) directed against fibroblast activation protein (FAP) and murine CD3 decreased the number of FAP-expressing CAFs, increased the viral titer and T-cell accumulation in the tumor and enhanced antitumor efficacy in comparison with the unarmed virus in the murine B16.F1 melanoma model (112). Furthermore, in a similar approach with oncolytic Adenovirus that secretes FAP-targeting BiTEs, T-cell accumulation and antitumor efficacy were enhanced in xenograft models of subcutaneous human lung carcinoma and pancreatic adenocarcinoma established in NSG mice supplemented with pre-stimulated human T cells (113). Nevertheless, a great deal of caution needs to be taken with these interpretations since TGF-β and CAFs can also promote the efficacy of OV replication. A study performed in xenografts

derived from patients with pancreatic cancer showed that tumor-derived TGF-β made the CAFs more sensitive to infection with various OVs, such as Vaccinia virus, VSV, and Maraba virus by downregulating their antiviral program (114). In turn, CAFs produced high amounts of fibroblast growth factor 2, which impeded the ability of the pancreatic cancer cells to detect and respond to virus infection.

Because of this complex interplay, the interference between TGF-β signaling and OV treatment needs to be investigated further in the context of checkpoint blockade therapy. In particular, the rational choice of targets and the timing of the combination strategy might be of key importance to effectively sensitize tumors for immunotherapy (see **Outstanding Questions**). For instance, in an inducible murine model of $BRAF^{V600E}PTEN^{-/-}$ melanoma with modest baseline responses to PD-1/PD-L1 blockade, TGF-β inhibition failed to augment the response to anti-PD-1 immunotherapy whereas anti-CTLA-4 immunotherapy did benefit from the combination, resulting in tumor growth control and increased survival (115). Mechanistic studies in mice with subcutaneously implanted $BRAF^{V600E}PTEN^{-/-}$ melanomas in C57BL/6 mice revealed that inhibition of TGF-β signaling promoted the proliferative expansion of stromal fibroblasts and increased the production of MMP9, which subsequently facilitated cleavage of PD-L1 on the surface of melanoma cells, ultimately leading to resistance to anti-PD-1 therapy (115). The authors also demonstrated that TGF-β inhibition following anti-PD-1 treatment had superior therapeutic efficacy compared to a continuous combination of TGF-β inhibition and PD-1 blockade (115).

Additionally, whether combinations of three separate strategies are achievable in terms of cost and the accumulating burden of adverse events in patients remains undetermined. Although side effects may be limited for all monotherapies (116-118), the question arises as to whether adding up these therapies still has manageable adverse effects. Encoding checkpoint blockers and TGF-β blocking agents in a single OV for intratumoral delivery may limit the therapeutic burden and systemic adverse effects (71), however, it remains to be assessed whether the antitumor efficacy of this strategy reaches its full potential when all agents are delivered to the tumor simultaneously. Additionally, not all OVs have sufficient space in their genome to allow the encoding of complicated and large molecules (119). Extensive preclinical studies need to be performed to elucidate the putative therapeutic effect of combined TGF-β inhibition and OV therapy to sensitize immune-desert tumors for immune checkpoint blockade or other immunotherapeutic strategies and to determine for which specific cancers these combinations can be helpful.

Although multiple challenges and questions remain to be addressed, combining immune checkpoint inhibition with strategies to overcome immune evasion and exclusion is expected to result in the induction of strong antitumor immune responses in a variety of cancers. It will be exciting to follow future progress in this area.

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Resources.

[I] This trial is listed in https://clinicaltrials.gov/ct2/show/NCT02423343
[II] This trial is listed in https://clinicaltrials.gov/ct2/show/NCT02263508
[III] This trial is listed in https://clinicaltrials.gov/ct2/show/NCT01740297

OUTSTANDING QUESTIONS

- Can we develop approaches to selectively inhibit TGF-β signaling in immune cells or in a specific CAF subset to restore immune surveillance and overcome immune evasion in solid tumors?
- Is replication of oncolytic virus required for its expected synergistic effect with TGF-β signaling inhibition and immune checkpoint therapy?
- Which biomarkers can predict susceptibility to the combination therapy of oncolytic viruses, TGF-β inhibition, and immune checkpoint inhibition?
- Which criteria should be used to select the appropriate oncolytic virus and immune checkpoint inhibitor for application in combination therapy? Does this differ between tumor types or even between patients?
- What would be the optimal timing for a combination approach of oncolytic virus therapy, TGF-β signaling inhibition, and checkpoint blockade?
- Would it be technically feasible and therapeutically effective to genetically engineer a single oncolytic virus expressing TGF-β signaling antagonists and immune checkpoint inhibitors to limit the therapy burden for patients?

GLOSSARY

Bispecific T-cell-engagers (**BiTEs**)**:** fusion proteins consisting of two different single-chain variable fragments of monoclonal antibodies for simultaneous tumor cell binding and T-cell activation.

Cancer-associated fibroblasts (**CAFs**)**:** cell type within the tumor stroma that can promote tumor progression by extracellular matrix remodeling and secretion of cytokines.

Cytotoxic T lymphocytes (**CTL**)**:** $CD8^+$ effector T cells, important for the elimination of intracellular pathogens and malignant cells.
Cytotoxic T lymphocyte-associated antigen-4 (**CTLA-4**)**:** immune checkpoint receptor that downregulates T-cell responses.
Damage-associated molecular patterns (**DAMPs**)**:** endogenous molecules that are released from damaged cells, initiating a noninfectious inflammatory response.
Dendritic cells (**DCs**)**:** antigen-presenting cells that are specialized in priming of naive T cells.
Epithelial-to-mesenchymal transition (**EMT**)**:** a process by which epithelial cells de-differentiate towards migratory and invasive mesenchymal stem cells.
Extracellular matrix (**ECM**): a network of extracellular macromolecules such as collagen.
Immune-desert: tumor phenotype without an evident immune response.
Immune-excluded: tumor phenotype where tumor-reactive T cells are unable to infiltrate into the tumor beds due to a physical or immunosuppressive barrier.
Immune-infiltrated: tumor phenotype where inflammation is present and T lymphocytes have infiltrated the tumor.
Immunogenicity: the ability to evoke an adaptive immune response.
Immunotherapy: treatment focused on mobilizing the host immune system to combat disease.
Myeloid-derived suppressor cells (**MDSCs**)**:** cells of the myeloid lineage with strong immunosuppressive properties that are associated with tumor progression.
Neoantigens: antigens that result from tumor-specific mutations and are absent from the normal genome.
Oncolytic viruses (**OVs**)**:** viruses that preferentially replicate in and kill cancer cells.
Orthotopic tumor model: an experimental model where a transplanted tumor is placed in the organ of the original tumor.
Pathogen-associated molecular patterns (**PAMPs**)**:** molecules derived from bacteria or viruses that evoke an inflammatory reaction.
Priming: process in which naive T cells encounter an antigen in the context of an activated dendritic cell and start clonal expansion.
Programmed cell death protein-1 (**PD-1**)**:** immune checkpoint receptor expressed on the cell surface of T cells, which negatively regulates T-cell responses.
Regulatory T cells: FoxP3-expressing $CD4^+$ T lymphocytes that functionally suppress effector T cells.
Small molecule kinase inhibitors: low molecular weight compounds that block the action of one or more enzymes called protein kinases.
Talimogene laherparepvec (**T-VEC**)**:** genetically modified oncolytic herpes simplex virus type 1, designed to produce GM-CSF. FDA-approved for the treatment of melanoma.
T-cell anergy: functionally inactivated state of T cells after antigen encounter.
T-cell exhaustion: progressive loss of effector function in T cells due to prolonged antigen stimulation

Transforming growth factor β: multifunctional secreted protein with three isoforms, involved in regulating and mediating many cellular processes.
Tumor antigens: proteins or substances produced in tumor cells that can be recognized by the adaptive immune system.
Tumor-associated macrophages (**TAMs**)**:** macrophages found in tumors that exhibit immunosuppressive properties.
Tumor-infiltrating lymphocytes (**TILs**)**:** white blood cells that have migrated into the tumor.
Tumor microenvironment (**TME**)**:** the molecules, cells, and vessels that surround and interact with the tumor cells.

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