Interleukin-12 for Solid Tumor Immunotherapy

By

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Abstract

Immunotherapy describes a class of therapies in which the immune system is manipulated for therapeutic benefit. These treatments include immune checkpoint inhibitors, adoptive cell therapy, and vaccines. For many hematological malignancies, immunotherapy has emerged as an essential treatment component. However, this success has yet to be replicated for solid tumors, which develop advanced physical and molecular mechanisms for suppressing and evading immune destruction. Nevertheless, cytokine immunotherapy presents a potential remedy to these barriers by delivering a proinflammatory immune signal to the tumor and thereby transforming it from immunologically 'cold' to 'hot'. Interleukin-12 (IL-12), one of the most potent proinflammatory cytokines, was initially investigated for this purpose. However, initial murine and human studies in which IL-12 was administered systemically resulted in dangerous immunotoxicity associated with off-target immune activation. As a result, recent studies have employed advanced cell and molecular engineering approaches to reduce IL-12 toxicity while increasing or maintaining its efficacy such that its effective doses can be tolerated in humans. This review highlights such developments and identifies promising future directions.

Introduction

The role of the immune system in cancer has been extensively studied in recent years. This has unveiled both an antagonistic and cooperative relationship between tumors and different aspects of the immune system which shapes tumor development. Of particular interest are solid tumors, whose immobility permits the development of complex physical and molecular mechanisms for exploiting and evading the immune system. Immune antagonism towards tumors is achieved in that tumor cells are often recognized and destroyed by the immune system in a similar manner to extrinsic pathogens through presentation of abnormal antigens known as 'tumor associated antigens' (TAAs) (Coulie et al., 2014). However, the ability of T cells to recognize TAAs can be suppressed by tumors directly through upregulation of inhibitory immune checkpoint molecules such as Programmed Cell Death Ligand 1 (PD-L1), which in normal cells suppress autoimmune responses (Han et al., 2020). Furthermore, solid tumors recruit immunosuppressive cells such as regulatory T cells (Tregs) and M2 Macrophages which suppress the cytotoxic functions of CD8+ T cells that would otherwise initiate an antitumor response (Anderson & Simon, 2020). Recruitment of these immunosuppressive cells is achieved by harboring an environment favorable to their proliferation, namely through secretion of chemokines by Tregs and by inducing hypoxia and producing colony stimulating factor 1 for M2 macrophages (J.-H. Kim et al., 2020; Xu et al., 2022). Lastly, the compression of tortuous, aberrantly grown blood vessels by rapidly proliferating tumor tissue coupled with inadequate lymphatic drainage results in a high interstitial fluid pressure (IFP) (Ferretti et al., 2009). Increased IFP consequently acts as an additional physical barrier to cytotoxic lymphocyte entry into the tumor space and correlates with poor prognoses. This resulting collection of immunosuppressive features, termed the tumor microenvironment (TME), facilitates a broad escape from antitumor immune responses.

Several major classes of immunotherapy have emerged to address these immunosuppressive characteristics. Immune checkpoint inhibitors (ICI) are monoclonal antibodies that block the action of molecules such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), which are expressed on the surface of T cells and are targeted by tumors to negatively regulate antitumor immune responses (Shiravand et al., 2022). ICIs such as ipilimumab (anti-CTLA-4) and pembrolizumab (anti-PD-1) have both demonstrated success in advanced metastatic melanoma and non-small-cell lung cancer, respectively (Mansh, 2011; Reck Martin et al., 2016). Another important class of immunotherapies, Adoptive Cell Therapy (ACT), promises to target tumor cells for destruction by directly engineering patients' lymphocytes to have affinity for TAAs. Prominent examples of ACT include chimeric antigen receptor (CAR) T cell therapy and tumor infiltrating lymphocyte (TIL) therapy.

One major limitation of current immunotherapy approaches in solid tumors is that they necessarily rely on immune cell invasion into the tumor space. ICI therapy enhances T-cell antitumor activity; however, this effect is neutralized in solid tumors without sufficient populations of intratumoral T cells. ACT therapy shares this limitation, as the treatment is derived from a patient's own lymphocytes. It is not surprising, then, that immunotherapies targeting solid tumors have had limited success compared to hematological malignancies due to the TME. Indeed, ICI therapy in solid tumors is characterized by high variation in responses with a large proportion of patients exhibiting no response (Sun et al., 2023). ACT has faced similar challenges, with a general failure to translate its unprecedented successes in treating hematologic malignancies to poorly immunogenic solid tumors (Olson & Odunsi, 2023).

The solution to this broad limitation to solid tumor immunotherapy may lie in treatments that reshape the TME from immunologically 'cold' to 'hot'. Cytokine immunotherapy achieves this goal by employing an endogenous immunostimulant which improves immune cell recruitment to the tumor space, decreases immune

cell exhaustion phenotypes, promotes tumor antigen expression, and primes immune cells for increased activation leading to an enhanced antitumor response (Song, 2024).

Interleukin-12 (IL-12) is one of the most potent proinflammatory cytokines. Specifically, it activates both cytotoxic CD8 T cells and NK cells, signaling for their expansion and maturation (Gubler et al., 1991; Stern et al., 1990). IL-12 acts by binding with its receptor subunits IL-12Rβ1 and IL-12Rβ2 and downstream activation of STAT3 and STAT4, which promote expression of the highly proinflammatory interferon gamma (IFNγ) (Weaver et al., 2007). IL-12 was consequently an early target for solid tumor immunotherapy. The initial mouse trials with intravenous murine IL-12 demonstrated an ability to remodel the TME and facilitate a CD8+ T-cell-dependent antitumor immune response (Brunda et al., 1993). However, murine models also revealed extensive off-target immunotoxicity associated with IFNγ generation and IL-12 binding outside the tumor space (Gately et al., 1994). This limitation was significantly exacerbated in subsequent human studies with the first phase I and II trials resulting in severe toxicity, multiple hospital admissions, and three treatment-related patient deaths (Atkins et al., 1997a; Leonard et al., 1997). These disappointing initial findings resulted in a reduction in IL-12 cytokine related immunotherapy research. However, recent developments have been made in immunotherapy vectors which rely on immune cell invasion into the tumor space and are therefore suppressed in solid tumors by the immunosuppressive effects of TME, as well as in cellular and protein engineering. Together, these advancements have reengaged academic and industry interest in IL-12 immunotherapy. Namely, new studies attempt to employ IL-12 for solid tumor immunotherapy as either a combination or in a modified form to reduce its toxicity. This review highlights recent developments in IL-12 immunotherapy while suggesting promising future directions.

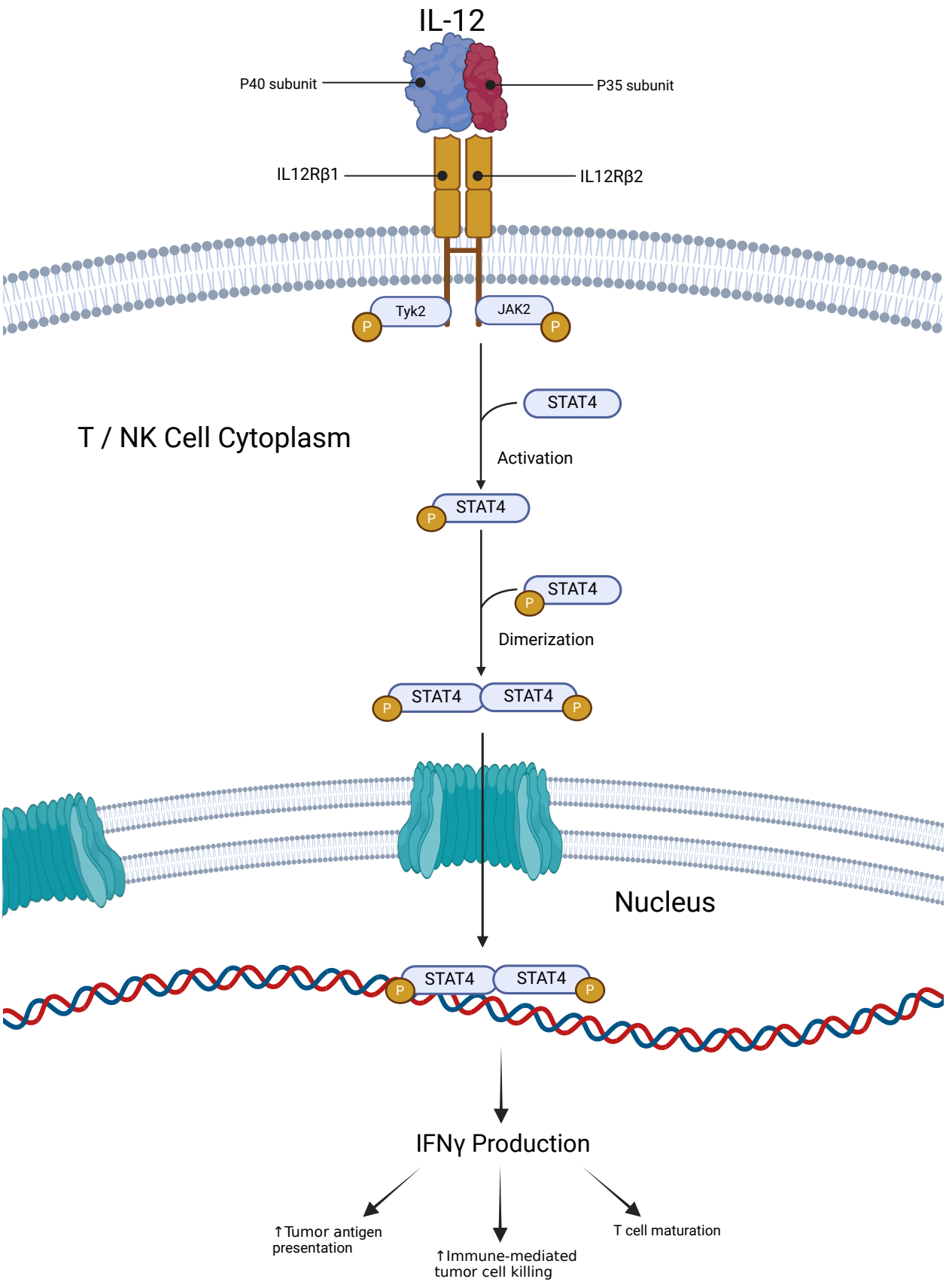

IL-12
P40 subunit
P35 subunit
IL12Rβ1
IL12Rβ2
Tyk2
JAK2
P
P
T / NK Cell Cytoplasm
STAT4
Activation
STAT4
P
STAT4
P
Dimerization
STAT4
STAT4
P
P
Nucleus
STAT4
STAT4
P
P
IFNγ Production
↑Tumor antigen presentation
↑Immune-mediated tumor cell killing
T cell maturation

Figure 1: Interleukin-12 (IL-12) Mechanism of Action (Weaver et al., 2007).
IL-12 is composed of p40 and p53 subunits which interact with the β1 and β2 subunits of the IL-12 receptor, respectively. Binding to the receptor complex triggers phosphorylation of Janus Kinase (JAK) 2 and subsequent activation of STAT4. Dimerization of phospho-STAT4 and translocation into the nucleus results in transcriptional changes, of which the primary contributor to IL-12 potency is interferon gamma (IFNγ). IFNγ acts on multiple systems to increase tumor associated antigen (TAA) presentation, promote immune-mediated tumor cell killing, and induce T cell maturation.

Protein Engineering

Systemic administration of natural cytokines as antitumor agents has long been known to cause unfavorable toxicity due to their complex pleiotropic effects, which become difficult to control or predict when administered systemically in large amounts (Santollani & Wittrup, 2023). Thus, protein engineering methods are aimed at generating artificial cytokine constructs with reduced toxicity and greater specificity for the TME. Fusion of cytokines to antibodies targeting antigens expressed in the TME, known broadly as Immunocytokines, is a promising emerging strategy (Pabani & Gainor, 2023). These fusion proteins aim to target tumor cells and the TME with greater specificity than their pro-inflammatory cytokine payloads alone, which include IL-2, tumor necrosis factor (TNF), and IL-12.

The most well-studied IL-12 immunocytokine is comprised of NHS76 fused to two IL-12 heterodimers, termed NHS-IL12. NHS76 is an engineered human monoclonal immunoglobulin G 1 antibody that targets necrotic tumor tissues by binding intracellular nucleic acids (Sharifi et al., 2001). Because solid tumors are often characterized by a necrotic core due to poor vascularization and nutrient deficiency (Z. Liu & Jiao, 2019), NHS76 was identified as a useful tool in directing IL-12 to the tumor center. Additionally, because the large size of NHS-IL12 prevents it from penetrating cells with normal cell membrane integrity, which is deficient in standard tumor and especially necrotic tumor tissue (Maeda et al., 2000), the combination was expected to exhibit reduced toxicity compared to naked IL-12. In mouse and *cynomolgus* monkey tumor model experiments, NHS-IL12 exhibited promising antitumor effects when administered intravenously (Fallon et al., 2014). However, results from human trials were disappointing. In the first phase Ib human trial of NHS-IL12, administered subcutaneously and combined with the anti-programmed death ligand 1 (PD-L1) monoclonal antibody avelumab, only 2 of 32 patients with advanced solid tumors achieved objective responses (ORs) at data cut-off (Strauss et al., 2023). In the dose expansion portion, evaluated on 16 patients with advanced urothelial carcinoma (UC), no tumor achieved ORs and, having fewer than three ORs, the treatment failed to meet the criterion for progression to stage 2. Furthermore, the results from dose-expansion were inferior to prior studies employing avelumab monotherapy in patients with advanced UC, refuting the hypothesis of a synergistic relationship between NHS-IL12 and anti PD-L1 immunotherapy (Apolo et al., 2020). It is not clear, with the small cohort of the dose expansion part, to what extent the severity of UC was replicated between the NHS-IL12-avelumab and avelumab monotherapy studies, and whether direct comparison is appropriate. Nevertheless, this underperformance may be explained in that NHS-IL12 administration was followed by an increase in expression of PD-L1 inside and outside the tumor space, acting as a pharmacological sink to avelumab. This mechanism suggests that the ability of IL-12 constructs to augment ICI therapy may be restricted when both are administered systemically. Additionally, the ability of IL-12 to exacerbate immune exhaustion by facilitating production of IFNγ, which is known to increase PD-L1 expression (Qian et al., 2018), should be considered as a major limitation to IL-12 immunotherapy.

Constructed from a human monoclonal antibody targeting fibronectin (huBC1), the development of huBC1-IL12 suffered a similar fate to NHS-IL12. This antibody targets the cryptic portion of the oncofetal B-FN isoform of fibronectin, which is a major construct in the extracellular matrices of fetal and tumor tissue but

not in healthy adult tissue (Mariani et al., 1997). In preclinical human tumor models and in xenogeneic human tumor murine models, huBC1-IL12 demonstrated promising antitumor effects (Lo et al., 2007). In a phase I human trial, evaluating intravenous huBC1-IL12 in 13 patients with malignant melanoma or renal cell carcinoma, only one partial response was observed (Rudman et al., 2011). While the treatment was generally well-tolerated and met the efficacy criteria for future trials, no such trials have been conducted.

A promising alternative target for immunocytokine therapy is fibroblast activation protein (FAP). FAP is highly expressed by cancer-associated fibroblasts (CAFs) and is used as a universal marker for these cells (Micke & tman, 2004). FAP is known to play a role in angiogenesis, extracellular matrix remodeling, and cell signaling and is primarily associated with wound healing in normal tissues (Garin-Chesa et al., 1990). The role of FAP in cancer is controversial, with different studies demonstrating both positive and negative prognostic correlations (Fitzgerald & Weiner, 2020). Nevertheless, the overexpression of FAP and thus the presence of large quantities of CAFs (20-40% of total tumor mass) in 28 common tumors including breast, colorectal, pancreatic, and lung suggests that treatments targeting FAP may have broad applications for solid tumors (Xin et al., 2021). Consequently, antibodies against FAP have emerged as an important component of antitumor therapies. Using the 7NP2 antibody to human FAP (hFAP), researchers generated two variants of a novel IL12-7NP2 fusion protein, containing either human or murine IL-12 (Nadal et al., 2022). In mouse models bearing either SKRC52 renal cell carcinoma or CT26 colon carcinoma expressing hFAP, weekly intravenous IL12-7NP2 injections achieved complete responses in 3 out of 6 and 2 out of 5 mice, respectively. In CT26 models, subsequent combination with anti PD-1 antibody (αPD1) achieved complete responses in all 3 animals compared to 0 in αPD1 monotherapy. The fully human IL12-7NP2 demonstrated induction of IFNγ release in NK-92 and human peripheral blood mononuclear cells (hPBMCs), and was tolerated in *Cynomolgus* monkeys up to 0.2 mg/kg, indicating IL12-7NP2 should be evaluated in human trials.

In addition to immunocytokines, other cytokine fusion proteins are being explored, in part with the goal to increase the therapeutic window of IL-12 by altering its half-life, specifically in the TME (Santollani & Wittrup, 2023). With their short duration of exposure, conventional cytokine therapies require repeated administration, often daily, to achieve sufficient tumor uptake. The result of frequent IL-12 dosing is a marked desensitization, with treatment cycles showing significant attenuation in IFNγ induction after two or three administrations (Atkins et al., 1997b). One of the most common modifications made to address this challenge is the fusion of a crystallizable fragment (Fc) region of the immunoglobulin class G (IgG) antibody, which improves half-life by increasing the molecular weight of the resulting peptide and through various receptor interactions (Lobner et al., 2016). Introduction of a D265A mutation prevents FcγR binding and complement activation, and thus the immunologic effects of the fusion protein are similar to the cytokine alone (Baudino et al., 2008). The Fc/IL-2 fusion protein exhibits a serum half-life roughly three times that of wild-type IL-12 (Zhu et al., 2015), but this did little to augment its efficacy against B16F10 melanoma mouse models. More recently, a similar fusion protein was generated with IL-12 as its payload (mDF6006). The IL-12-Fc substantially outperformed recombinant mouse IL-12 (rmIL-12) when administered intravenously to CT26 colon carcinoma mouse models, achieving complete tumor regression in all 10 animals compared to only 1 for rmIL-12 (Gutierrez et al., 2023). By prolonging its serum half-life from 6 to 30 hours, dosing was reduced to weekly and still facilitated stable and prolonged accumulation of IFNγ. Furthermore, the fusion protein was effective against large (~815mm^3) CT26 tumor-bearing mice, also yielding 100% complete responses without significant differences in toxicity compared to rmIL-12. These data are surprising and promising, considering large tumors are known to harbor microenvironments with greater populations of immunosuppressive Tregs, M2

macrophages, and myeloid derived suppressor cells, and thus generally respond poorly to immunotherapies compared to smaller tumors (S. I. Kim et al., 2021).

To increase the specificity of IL-12 for the TME, IL-12 was fused to a tumor-protease-cleavable peptide linker at the receptor binding site (Mansurov et al., 2022). The resulting construct M-L_6-IL-12 consisted of IL-12 fused to an IL-12 receptor β1 (IL-12Rβ1) via a substrate of matrix malloproteinases (MMPs), which are known to be overexpressed in the TME as a mediator of extracellular matrix digestion and subsequent tumor expansion (Gialeli et al., 2011). Because IL-12 has far greater affinity for the full receptor complex with both β1 and β2 subunits rather than just β2, the fused IL-12Rβ1acts as a mask which inhibits cytotoxic activity until its cleavage by proteases in the TME, thus conferring on the complex preferential activity towards tumors (Presky et al., 1998). In B16F10 and EMT6 melanoma mouse models, intravenous M-L_6-IL-12 and IL-12 exhibited similar TME remodeling and antitumor effects, though with the former having no systemic immune-related adverse events. Furthermore, M-L_6-IL-12 potentiated PD-1 checkpoint blockade by increasing CD8+ T cell infiltration into the tumor space. These data suggest that augmentation of IL-12 with peptide masks that are cleaved by proteases overexpressed in the TME may be a promising means of reducing systemic IL-12 toxicity. The choice of linker could potentially by customized to the features of each patient's TME, thus giving the cytokine greater tumor specificity.

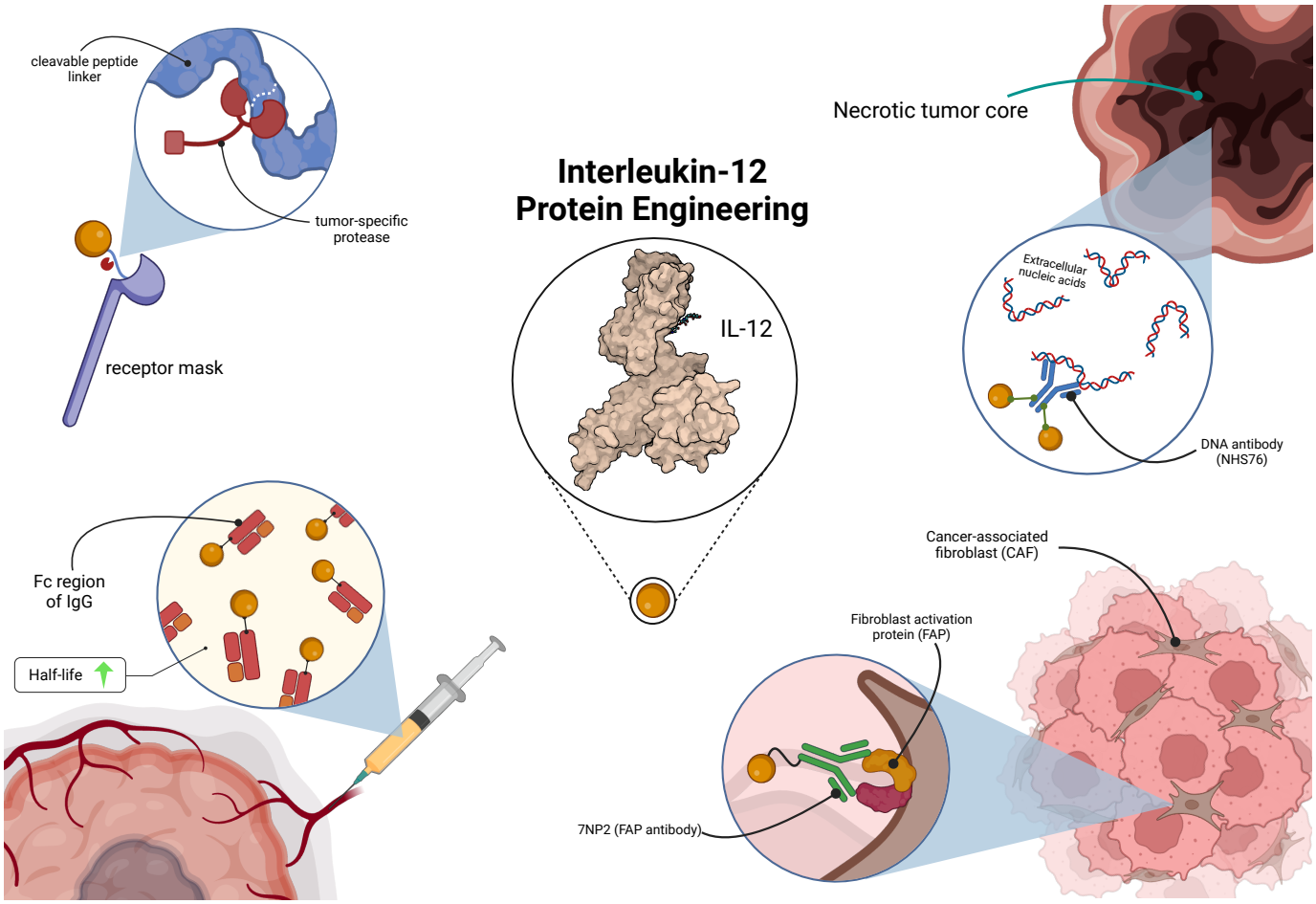


Figure 2: IL-12 Protein Engineering

Based on findings from existing protein engineering studies employing IL-12, certain approaches have the potential to augment IL-12 such that its specificity for the TME is improved and its toxicity is reduced. However, a major limitation to systemically administered fusion proteins suggested by Kin-Ming Lo et al. is that the high interstitial pressure of solid tumors may make the penetration of large peptides into the tumor space prohibitive. Future studies could attempt to address this limitation by exploring new fusion moieties of varying sizes and targets.

Adoptive Cell Therapy

In normal tissue, T cells distinguish self from non-self-antigens, which include peptides presented by cells expressing abnormal protein motifs. As a result, T cells are crucial to the identification of cancerous tissue, yet their activity is diminished due to the immunosuppressive TME of solid tumors and that cancer cells may avoid detection as 'self' cells (Fabbri et al., 2003). Adoptive cell therapy (ACT) describes a class of therapies in which immune cells are extracted from a patient, modified ex-vivo, and reintroduced, with the goal of targeting and destroying tumor tissue (Rohaan et al., 2019). Three major types of adoptive cell therapy have emerged. Tumor-infiltrating lymphocyte (TIL) therapy, the first ACT to demonstrate clinical benefit (Rosenberg et al., 1994), involves the extraction of immune cells from the tumor space, amplification in stimulatory medium, and re-administration (Zhao et al., 2022). Because TILs come directly from the TME and do not require genetic modification, they possess unique advantages compared to other ACTs in that they have a naturally greater tolerance for the immunosuppressive TME. This approach is restricted, however, to resectable tumors with sufficient populations of intratumoral T cells.

Thus, ACTs utilizing T cells isolated from peripheral blood have been developed to engineer T cells to express TCRs specific to tumor antigens (TCR-T), targeting them for destruction (Yee et al., 2002). Although this approach has achieved some success in several liquid and solid tumors (Rapoport et al., 2015; Robbins et al., 2011), it possesses an inherent limitation in that TCRs can only bind antigens presented on the major histocompatibility complex (MHC) (Szeto et al., 2021). The lifespan of many tumors is therefore characterized by a 'Darwinian' selection process of tumor cells by cytotoxic T cells towards an MHC-I-negative tumor phenotype, leading to immune evasion and subsequent resistance to TCR-T cell therapies (Aptsiauri et al., 2018). Chimeric antigen receptor (CAR) T cell therapy subverts this limitation by employing a synthetic non-MHC-restricted receptor as its targeting mechanism (Feins et al., 2019). When directed towards CD19 and CD20, CAR-T cell therapies have achieved unprecedented success against hematological malignancies including Acute Lymphoid Leukemia (ALL) and non-Hodgkin lymphoma (Grupp et al., 2013; Till et al., 2008).

However, ACTs have struggled to achieve the same clinical results for solid tumors as they have for hematologic malignancies. TIL therapy generated promising results in patients with more immunogenic solid tumor types, primarily melanoma (Rosenberg et al., 1988), but has had limited success with other solid tumors. Additionally, in contrast to blood cancers which retain their cluster of differentiation (CD) molecule following the transition to a malignant phenotype, the selection of target antigen for TCR-T and CAR-T cell therapies is made difficult by the lack of a uniformly expressed TAA in solid tumors (Martinez & Moon, 2019). These limitations are compounded by the immunosuppressive TME, which prevents their invasion into the tumor space in a similar manner to their non-engineered counterparts. While IL-12 cannot resolve the challenge associated with selecting a target antigen, its ability to reprogram the TME may act as a tool to improve ACT perfusion into solid tumors (Henry et al., 2008). Furthermore, the ability of IL-12 to prolong T cell survival and

signal for clonal expansion suggest that ACTs expressing or bound to IL-12 may be able to reflexively signal for activation upon binding to their target antigen (Starbeck-Miller et al., 2013; Valenzuela et al., 2002).

There are several ways in which IL-12 can be integrated into ACT. The first attempt to combine IL-12 with ACT therapy via genome integration was done by genetically modifying TCR-T cells to express IL-12. This approach showed promising antitumor effects in lymphodepleted B16F10 melanoma mouse models when administered intravenously by tail vein, with only 10,000 modified T cells achieving similar antitumor effects to 1 million unmodified cells lacking IL-12 expression at 25 days post-infusion (Kerkar et al., 2010). Concerns were raised over immunotoxicity as the transduced IL-12 could be expressed continuously by the modified T cells, potentially triggering severe autoimmune responses outside the tumor space. As a result, CAR-T cells with IL-12 expression controlled by a nuclear factor of activated T-cell (NFAT) promoter have been developed (Chinnasamy et al., 2012). Because NFAT-Calcineurin signaling is an essential part of T cell activation (Pan et al., 2013), these inducible IL-12 CAR-T cells express IL-12 preferentially in the tumor space upon recognition of their target antigen. With these cells exhibiting lower off-target toxicity, future trials have continued to employ NFAT to regulate IL-12 production. Employing both NFAT-IL12 and by fine-tuning CAR specificity to further for solid tumors has shown promising improvements (Yang et al., 2023). The use of single residue alanine scanning to fine-tune CARs targeted to epithelial cell adhesion molecule (EpCAM) effectively reduced CAR binding to EpCAM and therefore restricted their activity to tumor cells with high EpCAM expression. This loss of TAA affinity was compensated for by NFAT-inducible IL-12, which restored CAR activity to a level comparable to its non-affinity-mutated parent. In thyroid carcinoma and gastric cancer mouse models, subcutaneous administration of inducible IL-12 and affinity-tuned CAR-T (Y6V-iIL12 CAR-T) cells was significantly more effective compared to placebo or CAR-T cells without IL-12 in reducing tumor volume at 2-3 weeks post infusion. Furthermore, Y6V-iIL12 CAR-T exhibited no systemic toxicity or body weight loss. These results suggest that combining both affinity-tuning methods and inducible IL-12 may allow for greater specificity without compromising antitumor activity for CAR-T cells.

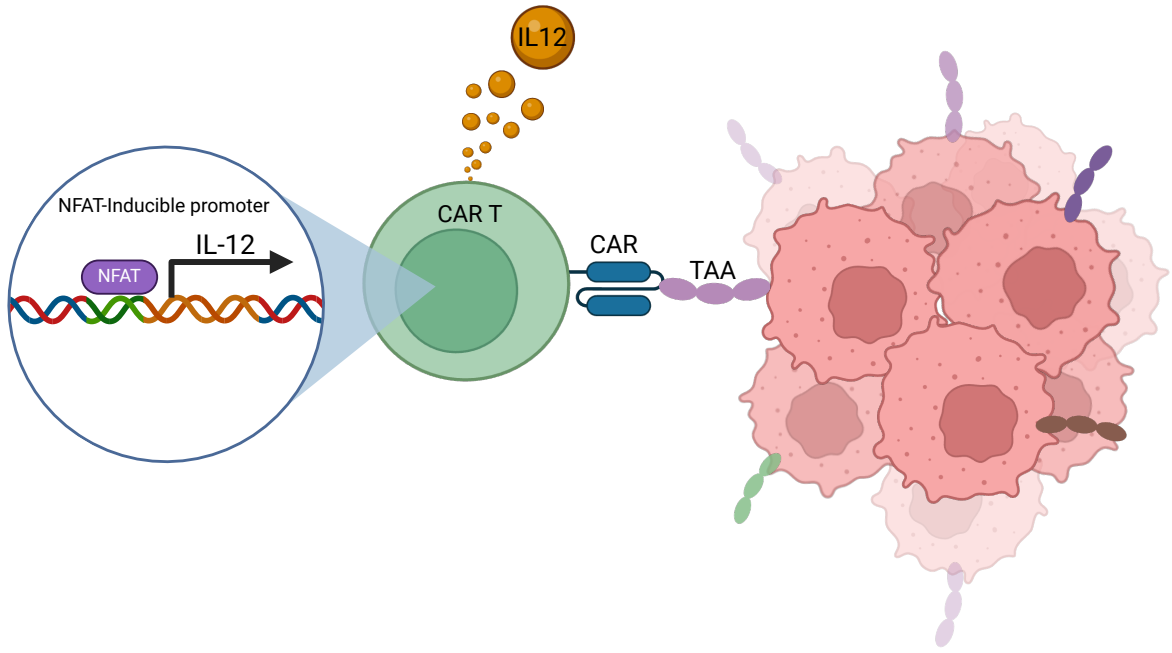


Figure 3: IL-12 Integrated into CAR T Cell Genome with NFAT-Inducible Promoter

IL12-CAR-T combinations using mRNA for IL-12 expression has also been explored. This approach is advantageous compared to direct genomic integration due to its reduced manufacturing complexity; cells can simply be stimulated to uptake mRNA through electroporation. However, it is limited in that genomic integration allows for sustained and controlled IL-12 release, while in contrast each IL-12 mRNA can be translated once before being degraded. These properties imply that mRNA-based IL12-CAR-T therapies may be useful in cases where sustained cytokine release is unfavorable. One such tumor is glioblastoma, for which the development of cytokine-CAR-T-cell therapies has been restricted, in part, due to concerns regarding neurotoxicity and cerebral oedema-related deaths observed in prior CAR-T cell trials (Turtle et al., 2016). Using a multitargeting CAR-T cell, researchers used electroporation to insert both IL-12 and IFNα2, another proinflammatory cytokine (Meister et al., 2022). Against glioma mouse models, CAR-T cells expressing IL-12 and IFNα2 achieved superior overall survival at 60 days post-infusion compared to cytokine monotherapy or standard CAR-T cells when administered both locally and systemically. While it is promising that no signs of toxicity were observed, it should be expected, as with all IL-12 therapies, that human trials will demonstrate significantly greater toxicity. Nevertheless, the use of transiently expressed IL-12 proves to achieve greater tolerability than when its expression is sustained in the case of direct genome integration.

The use of IL-12 mRNA has also been investigated in TCR-T cells. In B16F10 melanoma mouse models, intracavitary injection of ovalbumin-targeting TCR-T cells electroporated with IL-12 mRNA achieved nearly 100% survival compared to less than 25% for all other treatments including intravenous injection, TCR-T cells with irrelevant (non-IL12) mRNAs, and control (Di Trani et al., 2023). This result is significant because intracavitary administration of cytokine-engineered ACTs to treat peritoneal malignancies has been restricted by concerns over toxicity despite the well-documented superiority of locoregional immunotherapy administration for solid tumors. By exploiting the transient nature of mRNA, Di Trani et al. demonstrates how this limitation might be addressed.

ACT-IL12 combinations have also been achieved by anchoring IL-12 constructs to the membrane of CAR-T cells. By adding an antigen-dependent membrane bound IL-12 (mbIL12) to the surface of CAR-T cells, researchers demonstrated a reflexive signaling mechanism that improved antitumor efficacy in vitro and in vivo (Lee et al., 2023). When administered locoregionally to ovarian cancer mouse models, CAR/mbIL12 T cells targeting the TAG72 antigen, reduction in tumor volume was superior to those treated with TAG72-CAR-T cells lacking IL12. Similarly to ACT treatments which require IL-12 expression from genetic material, this combination demonstrated an ability to improve tumor penetration, T cell activation, and subsequent tumor killing. However, this approach whereby IL-12 is directly bound to T cells rather than expressed post-infusion is a potential remedy to the still-present challenge in adequately controlling IL-12 expression through promoters and reducing toxicity. This is because membrane bound IL-12 is unable to diffuse to other tissues as is the case when it is produced by the cell.

Researchers have also achieved membrane-binding implementations by employing nanovesicles anchored to the cell surface. One such study conjugated a nanochaperone (INS) containing IL12 by chemical attachment with dibenzocyclooctyl (DBCO) groups (Luo et al., 2022). By increasing redox activity at the cell surface upon antigen binding and T cell activation, experimental data showed that the IL12-containing nanochaperone could be disseminated through disruption of its DBCO attachment. In Luci-Raji tumor mouse models, intravenous treatment with INS-CAR-T cells resulted in little to no tumor growth at day 31 compared to significant increases in tumor volume for CAR-T cells alone or with free IL-12. The use of nanovesicles as a means of anchoring antigen-dependent IL-12 to CAR-T cells may help to further attenuate toxicity.

Certain challenges faced in applying ACT to solid tumors are yet to be resolved, including selection of tumor antigens from a vast library of heterogeneously expressed surface markers, rapid clearance from the body, and a time-consuming and laborious manufacturing process. While cytokine immunotherapy, including IL-12, cannot address these limitations, they nonetheless have the potential to improve ACT penetration into solid tumors and enhance activation and tumor killing. However, the risk of off-target toxicity already associated with many ACTs makes for the integration with further immunostimulatory particles challenging and often unfavorable. Therefore, advanced cell and protein engineering techniques are required to fully exploit the benefit of ACT for solid tumors as has been observed in hematologic cancers. As with all IL-12 immunotherapies, the ability of results from mouse models to generalize to human trials is poor, especially in the case of immunotoxicity where human trials have frequently been terminated due to patient deaths. As a result, it may be beneficial to explore tumor models that better replicate the general physiology and size of humans, including Cynomolgus monkey models, to more accurately inform translations of cytokine-ACT combinations to human trials.

Viral Vectors

Tumor cells may be targeted for destruction by gene therapy, whereby exogenous, antitumor genes are delivered in the form of DNA or RNA and subsequently expressed to achieve an antitumor effect. Viral vectors constitute approximately 70% of current gene therapy trials due to their high gene transduction efficiency compared to non-viral vectors(Arabi et al., 2022) (Arabi et al., 2022; Xie et al., 2023). Viral vector engineering can reduce pathogenicity, increase proliferation in tumors while decreasing in normal tissue, and evade the host immune response (Xie et al., 2023). Vectors which have been engineered to be replication-competent in tumor cells, or oncolytic vectors (OVs), have emerged as effective dual-action treatments by inducing lysis and cell death through both proliferation and antitumor gene expression (Tang et al., 2022).

Viral vectors encoding IL-12 offer the ability to integrate and induce expression of the cytokine directly in the TME, thus simultaneously improving tumor penetration and stimulating an antitumor response by recruiting additional immune cells. Additionally, destruction of tumor cells by OVs results in the release of a debris field consisting of pathogen-associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) to be taken up and presented by antigen presenting cells (APCs), contributing to an additional downstream recruitment of cytotoxic lymphocytes (Cirella et al., 2022). Together, these properties suggest a possible synergistic relationship between OVs and IL-12.

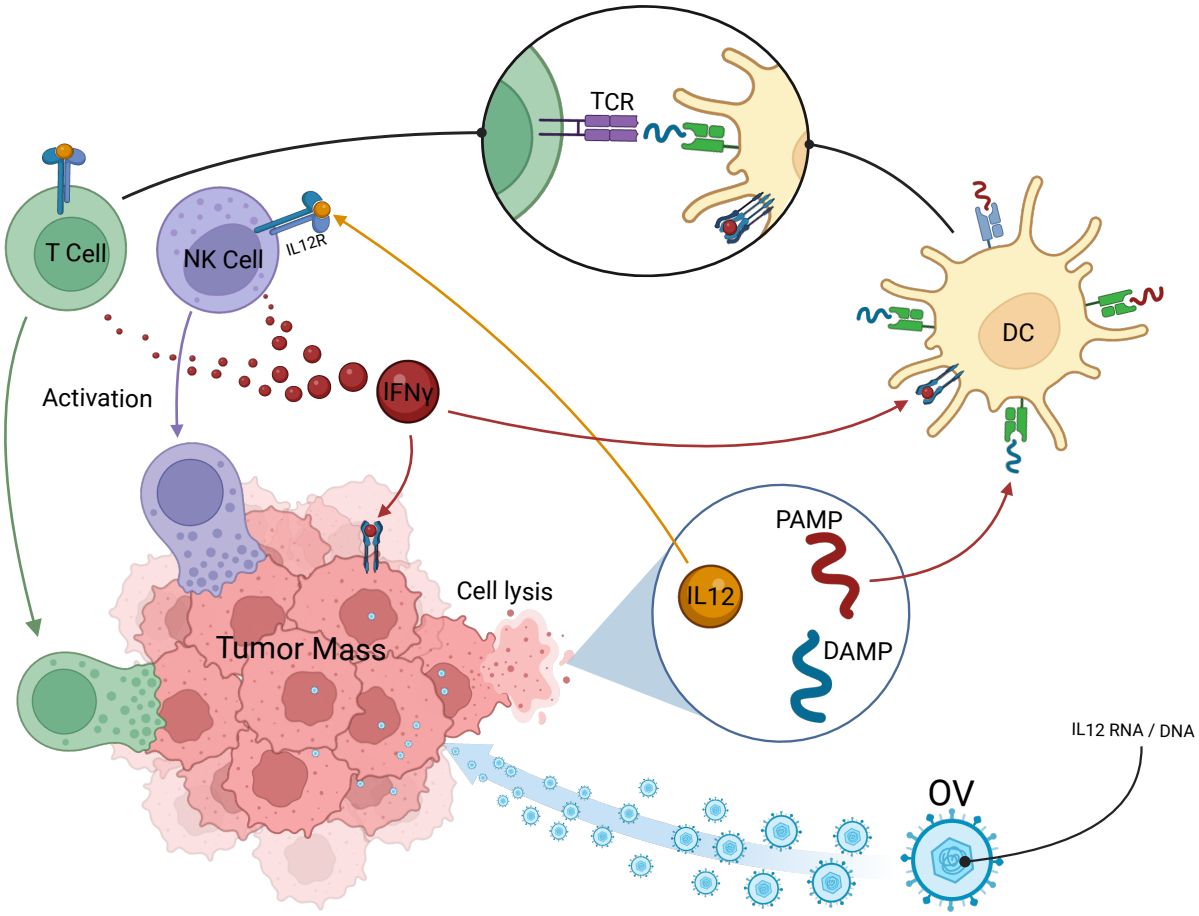


Figure 4. Interleukin-12-Expressing Oncolytic Viruses (OVs) Act on Multiple Systems to Exert Antitumor Activity (Alkayyal et al., 2016)
OVs exert antitumor activity by lysing tumor cells, thus releasing their associated antigens. These antigens can then be taken up by antigen presenting cells such as Dendritic Cells (DCs) and then presented to the adaptive immune system to activate it against tumor cells. The cell types involved in this response primarily include cytotoxic T cells and NK cells. Furthermore, OVs can integrate IL-12 directly into tumor genomes, resulting in IL-12 expression by solid tumors and subsequent activation of T and NK cells. Downstream release of IFNγ results in expression of the chemokine IP-10 by DCs, which interferes with tumor angiogenesis. Lastly, tumor necrosis factor alpha (TNF-α) released by either activated DCs or cytotoxic lymphocytes induces tumor cell death.

Initial approaches integrated IL-12 into retroviral or adenovirus platforms. However, murine models demonstrated high rates of adverse events associated with a post-treatment spike in IFNγ coupled with a risk of off-target integration into the host genome, became significant limitations (K. G. Nguyen et al., 2020). Nevertheless, an adenovirus encoding IL-12 was tested on advanced digestive tumors in human subjects by intratumoral injection (Sangro et al., 2004). Subjects exhibited mild antitumor effects with 38% having tumor progression, 48% with stable disease, and one patient achieving partial remission. These results suggest a need for novel IL-12 vectors with superior antitumor effects at tolerated doses.

Alphaviruses such as Semliki Forest virus, Sindbis virus, and Venezuelan equine encephalitis virus previously demonstrated success as antitumor agents when engineered to induce overexpression of TAAs such as human papilloma virus type 16 E7 or antitumor genes such as endostatin (Velders et al., 2001; Yamanaka et al., 2001). By delivering RNA which undergoes self-replication, alphaviruses bypass the need to transfer genetic material directly into cell nuclei and integrate into the host genome as with DNA-based vectors such as adenoviruses (Lundstrom, 2022). Furthermore, expression of corresponding peptides is superior to mRNA

delivery, for which self-replication is impossible. Alphaviruses have previously been applied as vectors for immunostimulatory agents, with the most successful thus expressing IL-12 (Lundstrom, 2022). One of the earliest attempts used a Semliki Forest virus as a mediator of IL-12 gene therapy, administered intratumorally via cannula, to a RG2 rat glioma model (Roche et al., 2010). Results from this study were promising, with low and high-dose achieving 70 and 87% reduction in tumor volume, respectively, though with a risk of lethality at high doses.

This limitation necessitated the development of novel alphavirus vectors with superior safety profiles. Sindbis virus (SV), was recently shown to be effective against Mouse Ovarian Surface Epithelial Cell Line models when encoding IL-12 and an OX40 antibody and administered systemically (Opp et al., 2022). Activation of T-cell-expressed OX40 by anti-OX40 in the TME promotes cytotoxic function and suppresses the action of Tregs, thus enhancing the anti-tumor immune response. Results showed that SV expressing IL-12 and anti-OX40 resulted in greater survival and suppression of tumor growth than for an empty vector or one encoding IL-12 or anti-OX40 alone. Due to its long half-life in the blood and resistance to neutralization by serum antibodies, SV can be administered systemically rather than intratumorally with little impact on its efficacy. This feature of SV could prove useful for patients where intratumoral administration is prohibitive, such as those where multiple lesions are present or for neurological malignancies where direct injection risks damage to surrounding tissue. Human trials are still needed to evaluate the safety and efficacy of alphavirus-IL-12 combinations.

Herpes Simplex Virus 1 (HSV-1) has been evaluated using a similar, but slightly modified strategy: using expression of IL-12 and OX40L to improve Tumor infiltrating lymphocyte (TIL). TIL has previously been limited by insufficient activation and persistence in solid tumors, this vector was seen as a potential remedy. With this strategy, transformed tumor cells express and present OX40L to function as artificial antigen presenting cells (aAPCs) to trigger T cell activation with IL-12 to transformation the TME and improve the efficacy of combination tumor infiltrating lymphocyte (TIL) therapy (Ye et al., 2022). In an *in vitro* tumor model and two oral cancer patient-derived xenograft (PDX) mouse models, the intratumor OV-mOX40L/IL12 and intravenous TIL combination was shown to be more effective in initiating tumor shrinkage than control or any of the components alone. OV was not detected in peripheral tissues, suggesting a favorable safety profile. These results suggest the potential for viral vectors incorporating IL-12 to address the limitations of combination ACT.

Viral vector approaches incorporating HSV or similar vectors may be uniquely effective in treating brain tumors due to their ability to penetrate the blood brain barrier (BBB), a major limitation observed in many treatments for neurologic pathologies. Moreover, their limited ability to replicate and spread through the TME may be augmented by combination with IL-12 (H.-M. Nguyen & Saha, 2021). Indeed, neuroblastoma murine models showed that HSV-1 engineered to express IL-12 (HSV M002) was more effective than its non-engineered counterpart when administered intratumorally (HSV R3659) in improving post-tumor-induction survival (Parker et al., 2000). In a similar study, 3D bioprinting was used to replicate the physical properties of human tumors in order test M002 against three human neuroblastoma PDXs (Quinn et al., 2022). This approach may more reliably predict the outcome of future human trials than mouse models. To this point, one phase I trial has been conducted testing a second-generation HSV-1 engineered to express IL-12 (HSV-1 M032) (Estevez-Ordonez et al., 2023). In patients with recurrent or progressive malignant glioma, an acceptable safety profile was observed for M032 administered via intratumoral catheter, with a median OS of 9.38 months. Though not placebo-controlled, this result is superior to previous recurrent glioma trials having a median OS of ~7 months (Wong et al., 1999). With glioma being one of the deadliest solid tumors, and especially because it is

characterized by one of the most intensely immunosuppressive TMEs, combination of oncolytic viruses with IL-12 proves to be a promising approach.

Engineered rhabdoviruses may also be useful as antitumor agents, with previous studies demonstrating their success as cancer vaccines due to their high immunogenicity and the precision with which their small genome can be manipulated (Tzelepis et al., 2020; Zemp et al., 2018). Of the rhabdovirus family, the Vesicular Stomatitis Virus (VSV) is the most promising. The inability of VSV to invade and infect healthy cells with normal type-I IFN expression confers a specificity for the TME, where the type-I IFN signaling axis is frequently deficient (Zhang & Nagalo, 2022). This suggests a potential to bypass off-target IL-12 toxicity. One study investigated this potential by constructing a recombinant VSVΔ51 expressing human IL-12 (Abdulal et al., 2023). Intratumoral treatment of a B16F10 melanoma mouse model showed prolonged survival for the recombinant vector compared to control and VSV alone. Furthermore, by deleting its M protein, which is responsible for VSV cytotoxicity towards healthy cells, VSVΔ51 was shown to have diminished cytotoxic effects against normal GM-38 cells with or without IL-12. Taken together, these findings suggest promising means by which VSV might be augmented to improve its safety and efficacy through incorporation with IL-12.

Systemic IL-12 toxicity remains a concern for these treatments, and mechanism to address this are being considered. Selective control of IL-12 expression after integration by a viral vector may be achieved through use of The RheoSwitch Therapeutic System® (RTS), which facilitates viral integration of a veledimex (VDX) – activated transgene. This system was shown to be effective in regulating IL-12 expression when injected intratumorally (Barrett et al., 2018). The recombinant adenovirus with RTS-mediated IL-12 expression (Ad-RTS-IL-12) allowed for precise control of intratumoral IL-12 expression with oral VDX in monkey and mouse glioma models, with higher doses resulting in significantly greater percent survival compared to the current standards of care (bevacizumab and temozolamide) and control. Importantly, the treatment resulted in no severe adverse events and 95% of animals in the Ad-RTS-IL-12 treatment groups were tumor-free at study termination. In one of the few human trials of its kind, intratumoral Ad-RTS-IL-12 expression with oral VDX was tested as a treatment for recurrent high-grade glioma (Chiocca et al., 2019). Results confirmed that hIL-12 expression was tightly controlled by RTS and that VDX successfully crosses the BBB. Adverse events were mild to moderate, with promising evidence of TME remodeling and survival probability compared to historical controls. These findings suggest that the toxicity of IL-12, which previously limited its feasible dosage and thus efficacy in human trials, could be reduced by systems which allow for ligand-controlled expression such as RTS.

By integrating IL-12 DNA or RNA directly into tumor tissue, viral vectors may improve selectivity of IL-12 treatments and thus increase the amount of IL-12 that can safely be administered to a patient without severe off-target immunotoxicity. Furthermore, the destruction of tumor cells by those vectors with oncolytic potential, thus releasing a debris field of TAAs to be recognized by cytotoxic lymphocytes recruited to the TME by by IL-12 may act as a synergistic mechanism. However, the risk of random integration into the host genome and frequent neutralization by innate and adaptive immune responses continue to pose limitations for many of these therapies.

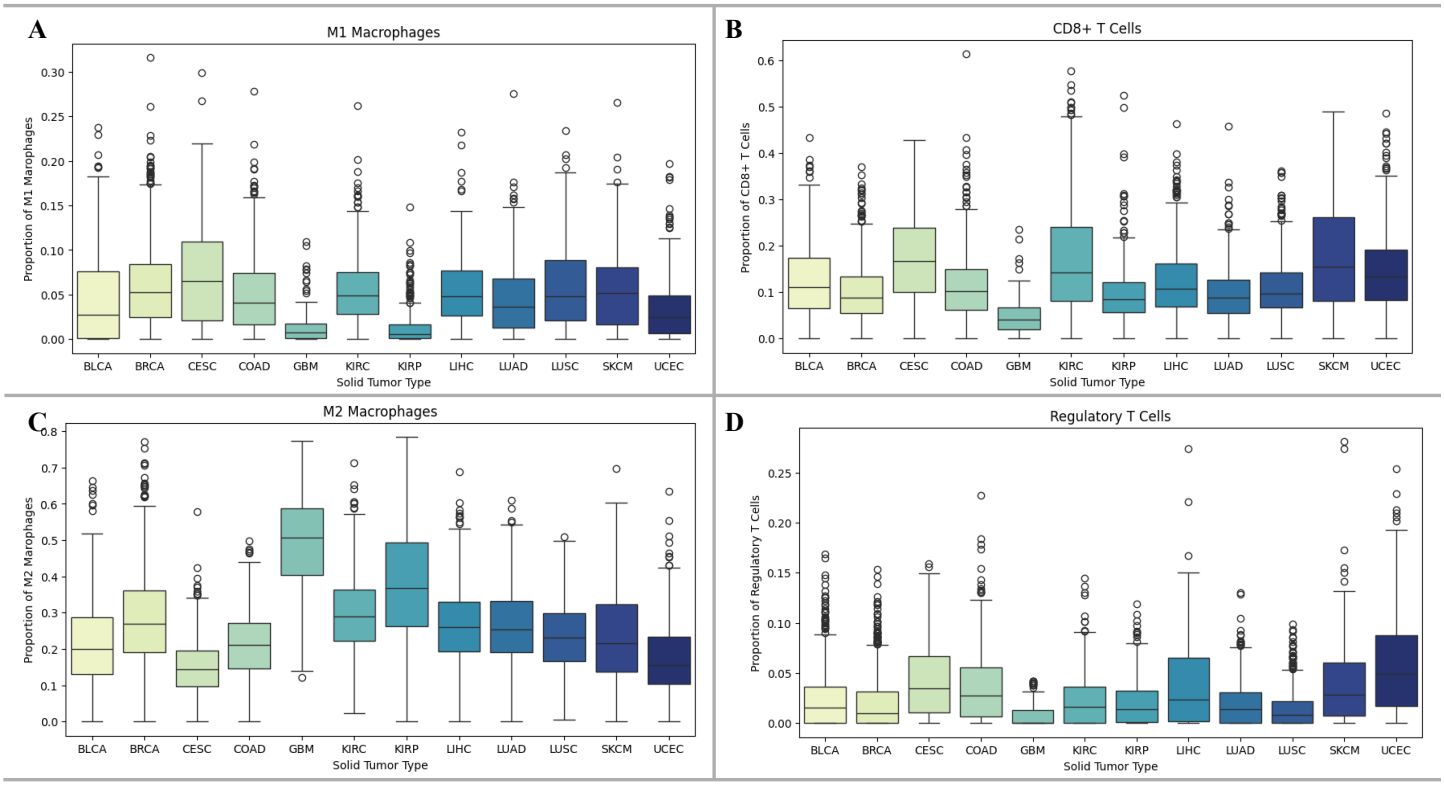
The Tumor Microenvironment

The use of IL-12 in solid tumor immunotherapy is predicated on two of its properties: the ability to remodel the immunosuppressive TME and to activate immune cells towards an antitumor response. By characterizing the TME for different solid tumor types, the precise utility of IL-12 might be elucidated. As IL-12 promotes macrophage polarization to an antitumor M1 phenotype and activation of CD8+ T cells,

proportions of immune cell populations in common solid tumor types may suggest specific cancer types that might benefit from this therapy (Figure 5). As a result, these profiles suggest that kidney renal papillary cell carcinoma (KIRP) and uterine corpus cell carcinoma (UCEC) may be a possible target for treatment. Although glioblastoma multiforme (GBM), with low populations of M1 macrophages (figure 5A), might benefit from IL-12 exposure, the low populations of CD8+ T cells may exhibit a diminished antitumor response with IL-12 treatment. Tumors with greater populations of CD8+ T cells such as skin cutaneous melanoma (SKCM) and kidney renal clear cell carcinoma may exhibit greater antitumor activity in response to IL-12. Indeed, many of the best clinical and preclinical outcomes for IL-12 immunotherapy have been observed in either advanced human melanoma or B16F10 melanoma mouse models.

The TME of GBM and KIRP also show comparatively large populations of immunosuppressive M2 macrophages (Figure 5C). As IL-12 converts M2 macrophages to their antitumor M1 phenotype, it should be expected that this feature of solid tumors can be addressed with the cytokine (Yu et al., 2016).

Lastly, high proportions of Tregs can be observed in uterine corpus endometrial carcinoma and cervical squamous cell carcinoma. The ability of IL-12 to reduce IL-2 expression, which is essential for Treg survival and expansion, and to stimulate IFNγ-dependent Treg cell cycle arrest suggests a potential corrective mechanism for tumors which exploit the immunosuppressive effects of Tregs (Mirlekar & Pylayeva-Gupta, 2021).



BLCA – Bladder urothelial carcinoma
BRCA – Breast Invasive carcinoma
CESC – Cervical Squamous cell carcinoma and endocervical adenocarcinoma
COAD – Colon adenocarcinoma
GBM – Glioblastoma multiforme
KIRC – Kidney renal clear cell carcinoma
KIRP – Kidney renal papillary cell carcinoma
LIHC – Liver hepatocellular carcinoma
LUAD – Lung adenocarcinoma
LUSC – Lung squamous cell carcinoma
SKCM – Skin cutaneous melanoma
UCEC – Uterine corpus endometrial carcinoma

Figure 5: Immune Cell Proportions in the Tumor Microenvironment of Common Solid Tumors
Proportions of immune cells in 10 most common solid tumor types based on >10,000 tumor samples. Immune fractions are calculated using CIBERSORT, which estimates cell type abundance using single-cell RNA-sequencing data (Newman et al., 2019). (A) M1 Macrophages and (B) CD8+ T cells promote antitumor immunity, while (C) M2 macrophages and (D) Regulatory T cells suppress it.

Discussion

Recent advancements in cell and protein engineering have revitalized scientific interest in cytokine immunotherapy for solid tumors, with IL-12 being a chief benefactor. The initial limitations faced by IL-12 have been addressed by integrating them into the genome of T cells as part of Adoptive Cell Therapy, transduced using oncolytic viral vectors, and engineered to better target solid tumors and persist in the TME. Nevertheless, the severe immunotoxicity observed in early IL-12 preclinical and clinical trials continues to limit safe dosages to ones with insufficient product to achieve acceptable antitumor efficacy. This is especially true in human trials, where results from murine models fail to predict the relationship between efficacy and toxicity. There are a number of possible hypotheses for why this might be the case. First, murine models with implanted tumors may not accurately represent the physical barriers posed by naturally occurring tumors, which can exist in multiple parts or integrate themselves deeply into healthy tissue. Another possible explanation is that human tumors are much smaller, as a percentage of total body weight and serum volume, than tumors used in mouse models. As an example, a 0.2 cm^3 oral squamous cell carcinoma in a mouse model with a body weight of 20g used in one IL-12 study would be the equivalent of a 12000 cm^3 tumor with a diameter of ~28.4 cm for a 60kg man (Hong et al., 2020). This size is significantly greater than those reported for the vast majority of human solid tumors, which typically average around 2-5cm in diameter (Kornprat et al., 2011; Markou et al., 2011). Lastly, differences in responses to IL-12 may be explained by differences in receptor expression, immune cell populations, and the quantity and behavior of downstream signaling components such as STAT4 and IFNγ. In order to advance IL-12 immunotherapy, it is necessary to identify tumor models that better replicate the features of human tumors.

Additionally, the method of administration of IL-12 may play a role in its efficacy. Administration either via cannula or by injection directly into the tumor space is preferable, with systemic IL-12 necessarily incurring a far greater risk of off-target tumor toxicity. However, this is prohibited in tumors for which their location prevents intratumoral administration or where multiple tumors are present. As a result, it is necessary to develop methods of administration which address these limitations. One potential approach would be the use of multiple cannulas simultaneously for fragmented tumors. For tumors such as glioblastoma, novel surgical methods may be necessary to safely administer IL-12 without damaging surrounding tissue.

IL-12 represents an important potential tool to address a major limitation in current immunotherapies: the tumor microenvironment. Its powerful proinflammatory features enable it to reshape the TME to the benefit of the patient, though with a risk of toxicity. Future IL-12 studies should continue to explore novel methods to reduce the toxicity of IL-12 therapies such that its safe dosage rises to the level of its effective dosage, and should innovate on preclinical testing and administrative methods.

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