

Review

ACT Up TIL Now: The Evolution of Tumor-Infiltrating Lymphocytes in Adoptive Cell Therapy for the Treatment of Solid Tumors

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Abstract: The past decades of cancer immunotherapy research have provided profound evidence that the immune system is capable of inducing durable tumor regression. Although many commercialized anti-cancer immunotherapies are available to patients, these treatment options only scrape the surface of the potential immune-related treatment possibilities for cancer. Additionally, many individuals are ineligible for established immunotherapies due to their cancer type. The adoptive cell transfer of autologous tumor-infiltrating lymphocytes has been used in humans for over 30 years to treat metastatic melanoma, and continued modifications are making it increasingly more effective against other types of cancer. This comprehensive review outlines this therapy from its infancy through to the present day, bringing to light modifications and optimizations to the traditional workflow, as well as highlighting the influence of new methods and technologies.

Keywords: adoptive cell therapy; tumor-infiltrating lymphocytes; cancer immunotherapy; immunotherapy; CRISPR; checkpoint inhibition; T cells; cancer; combination therapy



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1. Introduction

In 2011, the United States Food and Drug Administration (FDA) approved the checkpoint inhibitor (CPI), ipilimumab, as the first immunotherapeutic for the treatment of metastatic melanoma [1]. Following this approval, CPI therapy has been an increasingly inclusive treatment option, and continues to be the most widely used class of immunotherapeutic treatment for cancer [2]. CPI effectiveness against cancer is achieved by indiscriminately untethering the immune system, including pre-existing antitumor immunity [3–5]. Adoptive cell therapy (ACT) makes up another class of immunotherapy in which an individual's own immune cells are removed, manipulated, and administered back to them with the goal of providing a tumor-specific, cell mediated response against cancer [6]. This broad method includes chimeric antigen receptor T cells (CAR-T), engineered T cell receptor (TCR-T), and tumor infiltrating lymphocytes (TILs). CAR-T and TCR-T therapies involve extensive genetic modifications to arm the cells with unique, cancer specific receptors [6–8]. TIL-based ACT is a distinctive cell therapy, in which tumor-derived immune cells are expanded in a multi-step process and infused back into the individual (see Section 2.4). An abundance of intratumoral lymphocytes is typically a positive prognostic indicator [9–11], suggesting that tumor-infiltrating immune cells play an important role in tumor eradication. Therefore, the expansion and re-infusion of these cells is a promising approach. Significant responses can be achieved with the therapeutic use of these cells with no genetic manipulation, predominantly in patients with metastatic melanoma. With this extensive process comes numerous obstacles, including obtaining and expanding tumor-reactive TILs, managing adverse events, and increasing the effectiveness against other types of cancer. While genetic modification is not a defining component of the therapy, its integration

may be crucial in the advancement of TIL-based ACT. Here, we aim to examine and discuss the history, obstacles, and modifications of this therapy.

2. History

2.1. Early Experiments

Adoptive cell therapy using TILs as it is known today is the product of decades of landmark findings and experiments. The earliest documented appreciation of lymphocyte infiltration to tumor tissue dates back to 1921. Investigating the variable post-operative survival rates experienced by his patients, Dr. William C. MacCarty evaluated the histology of resected tumors. He found that patients who lived longer after surgery had lymphocytes that were, “intimately associated with cancer cells” [12]. The importance of infiltration of tumor tissue by lymphocytes became increasingly evident [13,14], but the therapeutic use did not come until much later.

The Surgery Branch at the National Cancer Institute (NCI) was the pioneering force behind adoptive cell therapies and conducted extensive research using lymphokine-activated killer (LAK) cells which set the stage for TIL in both the laboratory and clinic. Preparation of LAK cells involved isolating lymphocytes from peripheral whole blood of individuals with cancer, which could be then activated and expanded in culture for the adoptive cell treatment of the individual [15]. The discovery of interleukin-2 (IL-2) as an imperative agent in the function and response of immune cells allowed for the long-term culture and maintenance of T cells [16–18]. The development of recombinant IL-2 at the NCI in 1983 allowed for large-scale production of the growth factor which was instrumental in propelling the clinical use of the therapies [19]. Successful in vitro and animal models [18,20,21] gave way to a clinical trial reported in 1985 [22]. Twenty-five patients across six types of metastatic cancer were treated with the autologous adoptive cell transfer of LAK demonstrating the safety and feasibility of the therapy, with one patient experiencing complete regression of tumor [22].

The NCI subsequently demonstrated that TIL had obvious advantages over LAK. In addition to exemplifying reactive potential against LAK-resistant tumors, TIL produced better fold expansions, more successful synergy with exogenous IL-2 and cyclophosphamide, and produced more durable and powerful anti-tumor responses [23]. The NCI developed a method to obtain TILs involving the fragmentation and enzymatic digestion of resected tumor followed by culture in IL-2-containing medium for roughly 60 days [24]. After successfully using the protocol in murine experiments [23,25], a landmark study was reported in 1987 where TILs from melanoma patients were not only expanded to a clinical scale, but also showed functional reactivity against autologous tumor [26]. Pilot studies at the NCI showed that autologous adoptive transfer of these cells was safe, feasible, and improved with dose escalation of cyclophosphamide, IL-2, and TIL quantity [27,28]. In 1988, the group published a special report of preliminary clinical results in the *New England Journal of Medicine* showing that combination of cyclophosphamide, IL-2, and autologous TIL adoptive transfer had 40–60% objective response rate against metastatic melanoma [29]. This study defined TIL-based ACT as a promising treatment option and opened the door for institutions around the world.

2.2. Optimizing Methods: The Rapid Expansion Protocol

The clinical reports of the late 1980s set the standard for TIL development and administration protocol. The 1988 publication also brought insight into cell types that comprised TILs. Seventy-four to one hundred percent of the cells in the infusion product were CD3⁺ T cells, elucidating why IL-2 (formerly known as T cell growth factor) was so crucial for the feasibility of adoptive cell therapies. While essential for production, solitary use of IL-2 for cell expansion resulted in TILs lacking longevity due to dependency on the growth factor [30]. This posed a clinical barrier given the intolerability of IL-2 therapy [31].

Discoveries in T cell biology and advanced culture methods allowed for rational alteration of TIL production methods and resulted in the development of the rapid expansion

protocol (REP), now a paramount aspect of TIL-based ACT. After isolation and initial expansion of TILs from tumors, cells would undergo this process, involving the 14-day culturing of cells in medium containing IL-2, anti-CD3 monoclonal antibodies (OKT3), and allogenic feeder cells [32,33].

By the 1990s, the main components of the CD3 subcomplex (γ , δ , ϵ) were mapped and understood to play a vital role in antigen-dependent TCR signaling [34]. The addition of OKT3 to the REP was found to improve the expansion and long-term culture of T cells with antigen-independence by stimulating the TCR/CD3 complex [35–37]. The OKT3 approach also dampened the IL-2-dependency in vivo, and when applied to a murine adoptive T cell transfer model demonstrated that the autologous cells retained tumor immunogenicity and were capable of eliminating cancer in mice [38]. This 1991 study also demonstrated that in vitro expansion with anti-CD3 monoclonal antibodies resulted in improved fold expansion and produced cells with increased longevity after transfer, likely due to the upregulation of IL-2 receptors resulting from CD3-mediated activation, a mechanism not observed with IL-2 stimulation alone.

The last key component of the REP is the allogenic feeder cells. It was shown in the 1980s that accessory cells in the T cell culture environment could profoundly accentuate lymphocyte growth [39,40]. While early experiments used feeder cells derived from various anatomical sites of animals, Riddle et al. described a method to use peripheral blood-derived mononuclear cells as feeder cells to produce virus-specific T cells for murine adoptive cell transfer experiments [41]. The irradiation of these cells allowed retention of their native costimulatory abilities but rendered them incompetent for replication [42]. While the use of feeder cells transcends T cell expansion, the mechanisms by which these cells work in the context of TILs is still largely unknown. By the 2000s, the NCI reported the adoption of REP into a number of clinical trials.

2.3. Discovering Correlates of Response and Optimizations to Therapy

Since the early NCI clinical trials in the 1980s, clear correlates of response continued to be identified both in the ex vivo workflow and in the patient. An early correlate identified was the profound effect that ex vivo culture time had on the efficacy of treatment. In 1991, Aebersold et al. identified a 45-day threshold. TIL functionality was detrimentally impeded when culture times prior to infusion exceeded this threshold, and TILs cultured less than 45 days demonstrated greatly improved in vitro tumor lysis and patient outcomes [43]. Three years later, the team reported a more in-depth analysis of TIL age, supporting the need to avoid lengthy culture times before infusion [44]. Given the need for shortening ex vivo time, another method was developed.

From the compelling findings of the significant effects of culture time came the development of minimally cultured TILs, also referred to as young TILs. Until the late 2000s, only individual TIL cultures that demonstrated reactive potential would proceed through the REP process and be infused to the patient, known as the standard method. In contrast, the young TIL method involved pooling all TIL cultures from one patient into the REP [45–47], which almost halved the total ex vivo time [45,46]. An important association with shorter culture time and the young TIL method was found to be telomere length [45,48]. Telomeres are repetitions of nucleotide sequences flanking the ends of chromosomes and associate with specific trans-acting factors [49]. Every cycle of cell division results in the shortening of these regions, having downstream influences on cellular function [49,50]. The resulting consequences are detrimental in the context of TIL therapy, and include cellular senescence, loss of function, and reduced expression of CD27/CD28 costimulatory proteins important for T cell activation [48,50]. Young TIL trials have repeatedly shown that this method results in longer persistence of TIL in the patient and better clinical outcomes than the aforementioned standard method, and these benefits have been attributed to curtailing telomere shortening [45–47,51–53]. The overwhelming evidence of the inverse relation of culture time and TIL efficacy has become influential in the clinical use of the therapy.

The phenotypic variation observed in the infusion product was also linked to patient responses, although much less black and white than the effect of culture time. As previously stated, it has been known since early TIL trials that infusion products are ostensibly T lymphocytes [29]. While phenotypes of CD4-positive (CD4⁺, helper-cell functions) and CD8-positive (CD8⁺, killer-cell functions) cells were quantitated in the special report, no trend in patient outcome could be attributed to phenotype. The appreciation of increased CD8⁺ T cell populations in responding patients in a 1994 NCI publication brought to light a possible correlation between phenotype and clinical outcome [44], which could be expected due to the direct cytolytic abilities of CD8⁺ T cells. These TILs were much more functional in vitro against autologous tumor when compared to that of the non-responders, but the benefit was much less exaggerated in the clinical response, although apparent. As TIL-based ACT became more feasible, other notable groups reported the importance of CD8-dense TIL products [54–56]. Importantly, effective ACT requires phenotypic diversity. This was highlighted by clinical trials involving TIL- and PBMC-derived antigen-specific clonal CD8⁺ T cells. Unfortunately these studies showed to be largely ineffective with no subjects experiencing an overall response [57–59]. While much of the inefficacy was likely due to the narrow antigen specificity of the TILs, it is now well understood that CD4⁺ T cells are crucial for accentuating the response of CD8⁺ cytotoxic TILs [60,61]. Investigations to better understand phenotypic contributions of TILs are continually underway. In 2020 the NCI reported that metastatic melanoma responders in a TIL-based ACT trial had a subpopulation of CD8⁺ stem-like T cells that exhibited high in vivo replicative potential, longevity, and antitumor immunity after administration and correlated with positive outcome [62]. These cells were restricted to a CD39[−] and CD69[−] phenotype. Infusion products that contained only the double positive, terminally differentiated counterpart significantly hindered a successful outcome for the patient. This study further exemplifies the need for more research on ideal phenotypes and ways to identify and promote the production to increase the effectiveness of TIL-based ACT.

Lymphodepletion is a crucial component of ACT for the function and persistence of the infused cells [63,64]. While many trials investigated the ideal doses and frequencies of the non-myeloablative drugs, cyclophosphamide and fludarabine, the benefit of the more drastic depletion method of total-body irradiation (TBI) was also investigated as well as the combination of the two [63–66]. While TBI was arguably a slightly better preconditioning method, it did not yield results that outweighed the toxicities experienced by the patients [67]. Today, non-myeloablative lymphodepletion prior to ACT of low culture time TIL infusion product is the international norm. Although the general workflow of TIL-based ACT has changed very little over the decades, the multistep protocol leaves much room for optimization and alteration to improve patient outcome.

2.4. Current Standard of Care and Therapeutic Process

The cyclic workflow of TIL adoptive transfer, as it pertains to our institution, is illustrated in Figure 1 below. Following surgical removal, the tumor is manually fragmented with a scalpel. The initial expansion phase begins when the fragments are left to culture individually in a 24-well plate with IL-2 supplemented medium to produce young TILs. The lymphocytes residing in the tumor will egress the tissue and overflow into the medium, as well as exhibit cytotoxicity against the autologous tumor, typically eliminating it entirely from the culture [45]. After sufficient growth, the remaining young TILs are advanced to the REP containing irradiated allogenic feeder cells, OKT3, and IL-2. While these components are tantamount to the REP, some aspects vary between institutions, such as the use of bioreactors or specialized gas-permeable cultureware [56,68,69]. To maximize TIL proliferation, the clearance of waste and access to oxygen must keep up with the expanding cell culture. Bioreactors, such as the commonly used WAVE, oxygenate the culture through rocking and agitation while continuously renewing media [68,70]. Alternatively, gas-permeable vessels, such as G-Rex, remain static as oxygen and CO₂ are exchanged through a silicon membrane base [71]. Both methods are capable of producing high yield REPs and

fulfill good manufacturing practice requirements for clinical use [68,69]. The infusion of TILs is preceded by nonmyeloablative lymphodepletion, typically cyclophosphamide and fludarabine, then followed with IL-2 administration. Aliquots of TILs and tumor can be saved throughout the process for ex vivo experiments, and even subsequent expansion and treatment.

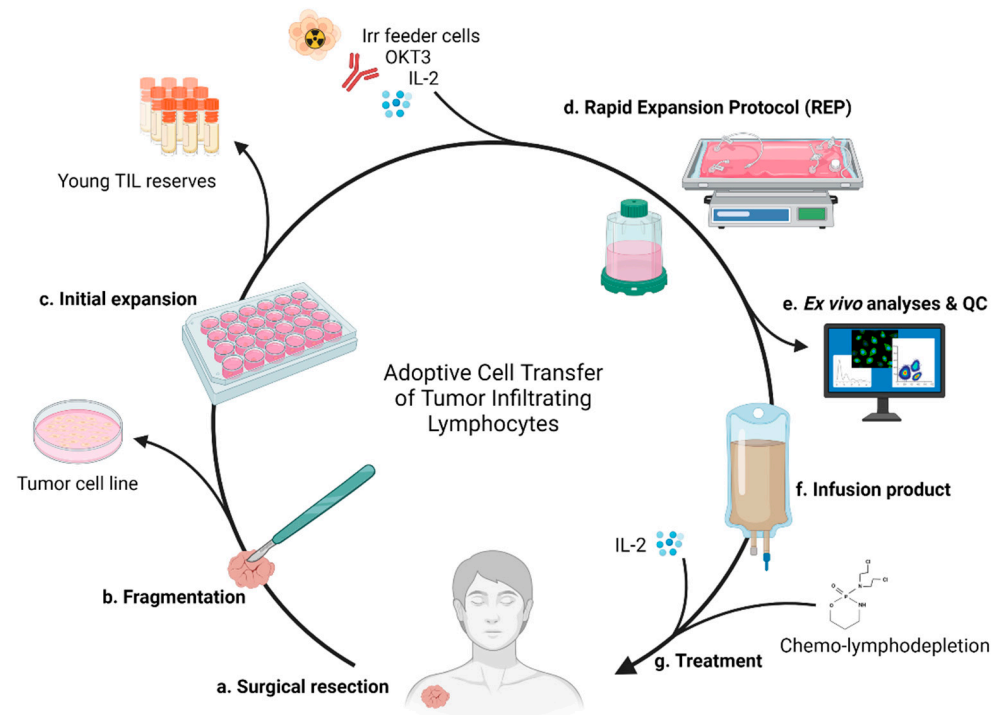


Figure 1. TIL production and administration. (a) Full or partial surgical resection of tumor. (b) The tumor is minced via scalpel. Tumor material can be saved to establish cell lines for future assays. (c) Fragments are placed into individual wells of a 24-well plate with culture medium. Harvested young TILs can be saved for future autologous therapy or experiments, while others (d) proceed to REP with irradiated allogeneic feeder cells, OKT3, and IL-2 for massive expansion. Use of bioreactor or gas-permeable flasks. (e) Quality of the cells are analyzed prior creation of (f) the infusion product. (g) Treatment of the patient includes the preparative lymphodepletion, administration of the autologous TIL product, and subsequent IL-2 administration.

3. Treatment Complications and Resolutions

3.1. Surgery

Currently, there is no clinically approved TIL therapy for the treatment of cancer. As the treatment is available only through clinical trial, there are strict eligibility requirements for admittance. One initial limiting factor for eligibility is surgical candidacy, for which a patient can be excluded for a number of reasons. Two of the most common reasons are surgical inaccessibility to the tumor and clinical deterioration of the patient. As described above, TIL therapy begins with full or partial surgical resection of the tumor which can be performed either through open surgery, or minimally invasive endoscopic surgery. Accessibility is not frequently a hurdle in case of metastatic melanoma as these tumors often metastasize to lymph nodes, cutaneous, and subcutaneous sites. Tumors from other cancer types, however, can be difficult to access surgically, or arise in sites that pose difficulty to sterily obtain. Gastrointestinal cancers are specifically difficult to resect without contamination. Though it has had little exploration in the context of TIL therapy, ultrasound-guided biopsy may address not only these issues, but also further reduce the invasiveness [72]. As surgical and imaging technologies improve, TIL adoptive therapy will become increasingly more available to patients with these types of cancer.

3.2. Expansion Complications

The ex vivo expansion phases required to produce large quantities of phenotypically effective TILs presents the next barrier to treatment. As previously discussed, a high CD8:CD4 ratio is a good indicator for effective TIL therapy alongside short ex vivo time (see Section 2.3). The outcomes of these ex vivo expansion steps can be greatly impacted by small changes in the culture environment. The development of the rapid expansion protocol demonstrated the importance of integrating discoveries in T cell biology, and the same approach should be taken to further improve the production process. Recent attention to costimulatory elements and their manipulation currently demonstrates potential for improving the speed and quality of TIL expansion for ACT.

3.2.1. Expansion Resolutions Targeting 4-1BB

In recent years, the potential of 4-1BB (CD137) as a costimulatory target for TIL-based ACT has been investigated. The theory for propagating this pathway to the benefit of TIL target is multifocal. As a “second signal” costimulatory molecule, 4-1BB ligation triggers downstream NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, driving T cell survival [73], and is highly expressed on activated CD8⁺ cells [74]. Addition of this agonistic antibody toward the costimulatory protein could drive the prolific stimulation of desired cytotoxic T cell populations within the tumor fragments. Additionally, anti-4-1BB could benefit TIL expansion by acting on intratumoral dendritic cells in tumor fragments that also express the target protein. This interaction has been shown to drive dendritic cell maturation and upregulation of MHC-II and costimulatory proteins that could augment TIL proliferation [75].

Researchers at MD Anderson Cancer Center (MDACC) experimented with the propagation of this pathway by introducing anti-4-1BB agonistic antibodies to initial expansion cultures of triple negative breast cancer with IL-2 [76]. They found that this addition not only increased CD8⁺ T cell populations, but also aided in boosting the functionality of these cells in a flow cytometry-based redirected killing assay. In a subsequent publication, the group further explored the potential of 4-1BB activation in both cutaneous and uveal melanoma [77]. Contrary to the previously described TIL-based ACT workflow above (see Section 2.4), the authors did not begin the pre-REP phase in 24-well plates but began the culture by adding tumor fragments to a specialized flask with medium containing “3-signal medium” (OKT3, anti-4-1BB, and IL-2). After 2 weeks of minor culture maintenance, cell quantities not only mirrored, but exceeded those of typical pre-REP quantities. Consistent with their previous finding, CD8⁺ phenotype was favored, and functionality was retained. The authors reason that the REP phase could altogether be bypassed if more tumor fragments were used in the initial expansion phase.

Our institution explored 4-1BB agonism in a sarcoma model [78]. The expansion and functionality of CD8⁺ TILs in this report were consistent with the previously discussed MDACC findings. In depth analysis of the TILs, however, showed that 4-1BB and CD3 combined stimulation resulted in not only favorable Th1-associated phenotype such as interferon gamma (IFN γ), but also disadvantageous Th2-associated cytokines (IL-4, IL-5, IL-13), which is supported by previous findings [79]. Additionally, while CD8⁺ T cells preferentially benefit from this costimulation, regulatory T cell populations can also counter-intuitively be expanded [80]. The extent to which this could have TIL-suppressive or antitumor effects is not clear but should be taken into consideration during future investigation.

3.2.2. Expansion Resolutions Targeting CTLA-4

While agonism of the costimulatory protein 4-1BB has a profound effect on the TILs, inhibiting negative-regulators of activation may also be a promising approach to making the therapeutic TIL product more feasible. The immunological checkpoint, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), mediates immunosuppression when agonized by its ligand, CD80/CD86, expressed by target cells [81,82]. Our group demonstrated that the addition of CTLA-4-blocking antibodies to tumor fragments from ovarian cancer metas-

tases during the pre-REP initial expansion phase improved the outcome of the expansion in a number of ways [83]. Not only did anti-CTLA-4 addition result in the expansion of TIL from more patients, but also resulted in more TILs per fragment, and better CD8:CD4 ratio. To our knowledge, this currently is the only report investigating the introduction of a checkpoint inhibitor directly to the TIL culture to assist with in vitro expansion.

Modification of the cellular environment has proven to help overcome the barriers brought forth in the ex vivo phases. 4-1BB and CTLA-4 manipulation have shown potential in broadening the availability of clinical grade TILs. While both of these approaches have been tested in diverse cancer types, it is important to recognize that expansion complications still remain a barrier to care for patients with metastatic melanoma. Other costimulatory targets and their combination have yet to be explored, but these changes could not only make treatment effective in other types of cancer, but also further improve outcome in patients with melanoma.

3.3. Severe Adverse Events

Altering the immunological landscape to boost anticancer immunity inherently has the potential to elicit immune-mediated toxicities in the patient. TIL-based ACT is a unique immunotherapy, as the risk for autoimmune side effects is less than others, such as CPI [3–5] and CAR-T [7]. This is likely because the cells administered are mostly anti-tumor with little to no off-target reactivity and not exacerbated by cutting the breaks of immunoregulatory pathways, as seen in CPI [3–5]. Some autoimmune reactions such as vitiligo, uveitis, and vasculitis are sometimes experienced [63,84,85]. Toxicities during treatment are typically due to preparatory lymphodepletion from cyclophosphamide and fludarabine, and systemic IL-2 administration [85]. Fever, chills, hypotension, hypoxia, and dyspnea are among the most common side effects experienced; vomiting, diarrhea, renal failure, and atrial fibrillation are less common [63,84,85] and are typically short-lived.

3.4. The Effect of CPI-Resistance on TIL Therapy

Cancer immunotherapies should not be thought of as one single brand. The diversity of the disease renders many cancer patients unresponsive to current commercial therapies such as ipilimumab and nivolumab. However, this should not imply that cellular immunotherapies will be unproductive in such cases, as concluded by Besser et al. in a 2013 clinical trial report showing that Ipilimumab resistance had virtually no effect on TIL therapy in melanoma [86]. In 2018, our group showed that CPI-resistant melanoma can still produce functional, tumor-reactive TILs that can mediate regression [87]. In a recent data analysis of clinical trials at our own institution, we have further concluded that disease progression after CPI therapy does not negate the ability for response to adoptive TIL therapy [88]. Even more recently the TIL-based ACT product, Lifileucel produced by Iovance Biotherapeutics, was shown to produce a durable response against metastatic melanoma in large cohorts of anti-PD-1 and PL-L1 refractory patients [89]. While CPI-resistance does not definitively nullify the success of TIL-based ACT, methods for optimizing treatment of this patient population should continue to be explored.

4. TIL-Based ACT in Non-Melanoma Solid Tumors

TIL-based ACT is arguably the best treatment option for individuals with refractory metastatic melanoma; however, few individuals with non-melanoma cancers have reaped the rewards of this therapy. Metastases from melanoma are susceptible to TIL-based ACT in all anatomic locations, including the brain [65,90] hinting at the potential of this therapy in other cancer types. While non-epithelial cancers such as non-small cell lung cancer, gliomas, hepatocellular carcinoma, and pancreatic tumors are much more resistant to the therapy, infusion-scale TIL have been produced, demonstrating the feasibility for treatment of these cancers with TIL [91–95]. Brain tumors have unique hurdles for cellular therapies, specifically the blood–brain barrier, an immunosuppressive environment [96], and invasive surgery in the case of TIL. The dearth of research in this area is likely not limited to these

factors; however, Liu et al. demonstrated that glioma-derived TILs could be efficiently expanded in 16 out of 16 cases using IL-2, -15, and -21 [95]. The improved phenotype and quantity of TILs obtained with this method could ameliorate the responses observed in a 1999 report in which one out of six individuals with malignant gliomas had a complete response after TIL-based ACT [97]. A phase I clinical trial in patients with hepatocellular carcinoma showed that 80% of the cohort had no evidence of disease after 14 months [93], but a phase II trial has yet to follow. Beyond these reports, Iovance Biotherapeutics is filling many gaps in clinical TIL-based ACT studies by including multiple solid tumor cancer types into clinical trials, some of which combine other therapies (NCT03610490, NCT03449108, NCT03215810). The success of CPIs across multiple cancer types has drawn recent attention toward other immunotherapies, namely TIL-based ACT for broader cancer types. Attempts at treating these patients with TILs has helped elucidate the factors contributing to less effective responses. Recent studies have repurposed modern therapies and technologies to make adoptive transfer of TIL work better for other groups of patients, notably combination with other immunotherapies and specific targeting.

4.1. Checkpoint Inhibition Combination

Our institution has shown that ovarian cancer is among the cancers for which ACT is safe and feasible, but it does not result in responses comparable to melanoma [56,98]. It is becoming increasingly clear that there are multiple CPI-based targets that can help augment the therapy both at the in vivo expansion phases (see Section 3.2) and as a combination therapy. While both ovarian cancer and metastatic melanoma show high T cell infiltration of the tumor, REP fold expansion, and post-REP T cell effector memory phenotype, the infusion products derived from ovarian tumors appear to have debilitating phenotypic differences such as high expression of exhaustion markers and unfavorable CD8:CD4 ratios [98,99]. This ratio is not only post-REP, but also reflected in the tumor itself, illustrating the need for a priming therapy prior to tumor resection for TIL.

To combat the evidence of exhaustion and unfavorable CD8:CD4 ratios observed in the previous studies, our group strategically combined marketed CPI therapies with TIL therapy for the treatment of six individuals with ovarian cancer [100]. The CTLA-4 inhibitor, Ipilimumab, was given in the priming phase before tumor resection, while the PD-1 inhibitor, Nivolumab, was administered after TIL infusion during the effector phase. The goal was to exacerbate the anti-tumor response, and although it is hard to compare with earlier ovarian cancer studies, CPI combination seemingly led to more lucrative expansions, more favorable CD8:CD4 ratio, and better tumor reactivity in vitro. Clinical results showed one partial response, one long-term stable disease for 12 months. The study demonstrated that CPI combination can augment the response and help skew to the desired CD8⁺ phenotype.

While we have shown that such combinations could play a role to overcome the specific barriers in ovarian cancer, there is currently very little literature on the topic. However, the plethora of ongoing clinical trials as seen in Table 1, suggest that the potential of CPI combination with TIL-based ACT will soon be better understood.

Table 1. Current TIL-based ACT clinical trials involving CPI combination.

Trial ID	Phase	Cancer Type(s)	CPI(s) Used	Estimated Completion Date	Sponsor
NCT03296137	I/II	All metastatic cancers	Ipilimumab Nivolumab	September 2020	Herlev Hospital CCIT-DK
NCT02027935	II	Metastatic melanoma Cutaneous melanoma	Ipilimumab	January 2023	MDACC
NCT03638375	I/II	Metastatic melanoma	Nivolumab	May 2023	Leiden University Medical Center
NCT03645928	II	Metastatic melanoma, Squamous cell carcinoma of the head and neck, Non-small cell lung cancer	Ipilimumab Nivolumab Or Pembrolizumab	December 2024	Iovance Biotherapeutics, Inc
NCT01993719	II	Metastatic melanoma	Pembrolizumab	September 2029	NCI
NCT01174121	II	Metastatic colorectal cancer, Progressive glioblastoma, Metastatic pancreatic cancer, metastatic ovarian cancer, Metastatic breast carcinoma	Pembrolizumab	December 2024	NCI
NCT03215810	I	Non-small cell lung cancer, Metastatic non-small cell lung cancer, Squamous cell carcinoma, Advanced non-small cell lung cancer, Adenosquamous carcinoma, Adenocarcinomas	Nivolumab	December 2023	H. Lee Moffitt Cancer Center and Research Institute
NCT03108495	II	Cervical carcinoma	Pembrolizumab	December 2026	Iovance Biotherapeutics, Inc

4.2. Specific Targeting

All anti-cancer immunotherapies are dependent on the ability of the immune system to discern healthy tissue from cancer. Metastatic melanoma is the most immunogenic cancer [101], which likely facilitates the neoantigenicity that plays a key role in its susceptibility to the administered TILs [64]. Our institution with others have investigated neoantigen-specificity in TILs with the use of barcoded neopeptide:MHC multimers [102]. Our study, currently in submission, revealed that neoantigen specificity abundance in TIL infusion product correlated with survival, while lacking such specificity resulted in progressive disease. In responders, these neoantigen-reactive TILs were engrafted into the host, detectable in peripheral blood, and active in the tumor microenvironment, acting as the driving force behind tumor regression. These findings also underline the importance of tumor neoantigenicity for successful outcome. While there are presumably many other factors, this could also help explain the inefficacy of TIL therapy in moderate to low mutational cancers such as ovarian and breast cancer [76,100,101].

In addition to combination of other therapies, TIL-based ACT could be improved by ensuring that cells infused to the patient react specifically and strongly to their own tumor cells. Broad approaches involving the adoptive transfer of clonal TILs have fallen short of traditional TIL-based ACT (see Section 2.3) and some non-melanoma cancer mutations have little overlap and require in extensive, broad screening for a highly personalized process [103,104]. Such an approach would involve the identification of cancer-restricted target(s), evidence of reactive TIL to the target(s), and isolation and expansion of these TILs.

Individual accounts of this process have been reported. In 2018, the NCI reported a case of complete durable regression of metastatic breast cancer after treatment with ACT of TIL directed towards four unique, cancer-specific, somatic mutations [105]. These mutations were identified via whole-exome sequencing and RNA sequencing. TILs expanded from the tumor recognizing peptide-MHC complexes presenting epitopes of four of these unique mutations were isolated, expanded, and shown to be functional in the ex vivo killing of autologous tumor as well as mediating tumor regression in the patient. While

this approach could be replicated, the methods used are far from practical for the treatment of many patients.

Methods of target identification and T cell reactivity are typically expensive and laborious. Whole genome, whole exome, and next generation sequencing are beyond the in-house capabilities of many institutions and cancer treatment facilities. Similarly, conventional tests to identify T cell reactivity to peptides requires special technical skill and usually industrial collaboration. Recently developed technologies are helping to mainstream these personalized approaches [106]. Some new methods depend on epitope libraries and wet lab analysis such as signaling and antigen-presenting bifunctional receptors (SABRs) [107] and peptide-MHC-TCR hybrid molecules (MCRs) [108], while others rely more heavily on epitope predictive informatic analyses such as the recently reported, ProTECT-prediction pipeline [109]. The use of DNA-barcoded multimers as described above could also be applied to other cancer types to identify and expand neoantigen-reactive TILs. Although these advances offer clear potential for broadening the treatment abilities of TIL-based ACT for individuals with non-melanoma cancers, further investigation is needed for clinical translation.

5. Genetic Modification of TILs

Genetic modification has revolutionized cell-based therapies in cancer. Though research in this area has grown in recent years, genetic modification is not new to TIL therapy. Retroviral transduction has been used at the NCI since the early 1990s in the earliest attempts to better equip these cells to eradicate cancer [110,111]. While this technique is still widely used and revised in TIL studies [112], different technologies are continuously adopted to TIL therapy as they are developed.

5.1. IL-2

While countless genetic alterations are hypothesized and tested in animal models, few have reached the clinic. In 2008, researchers at the NCI transduced TILs with a gene coding for recombinant IL-2 [113]. As stated above, IL-2 is essential for T cell survival. This factor is included in in vitro TIL cultures and expansions as well as administered to the patient during the course of TIL therapy, frequently leading to painful toxicities. The goal of this study was to compensate for the low IL-2 production capacity of the TILs by equipping them with means of autocrine IL-2 production. Success could result in increased in vivo longevity of the TILs and in turn improved patient outcome. Additionally, systemic IL-2 treatment during therapy could be decreased or discontinued. The in vitro results showed promise, as six out of the eight transduced patient samples produced IL-2 upon autologous tumor stimulation and persisted longer than non-transduced TILs. Unfortunately, the clinical trial was inconsistent with the in vitro findings. The patient outcome across the study was reduced compared to previous trials with non-genetically modified TILs. Only one patient infused with transduced product had sustained TILs after 1 month of treatment without exogenous IL-2 and experienced a partial response. The authors suggest that the extended culture time of the TILs due to lengthy transduction process was a contributing factor for the poor in vivo responses. As genetic manipulation tools improve, IL-2 may be a further investigated target of manipulation in the future to improve TIL therapy.

5.2. IL-12

IL-12 is an important cytokine in perpetuating anti-tumor Th1 responses [114,115]. However, like IL-2 and TNF α , systemic use results in painful clinical toxicity [116]. In 2015, NCI researchers transduced human TILs with an inducible gene coding for IL-12 [117]. To add a layer of regulation, an NFAT (nuclear factor of activated T cells) promoter preceded the IL-12 coding region, resulting in an NFAT-IL-12 protein. The goal of this study was to equip TILs with the ability to generate IL-12 upon activation in the tumor microenvironment after being reinfused into the patient.

The study was designed as a cell dose escalation, with a low dose cohort receiving 0.001 to 0.1×10^9 transduced TILs, and a high-dose cohort receiving 0.3 to 3×10^9 cells. Only one out of 17 patients in the low-dose cohort showed an objective response, while 10 out of 16 patients benefited in the high dose cohort. While higher cell dose correlated with tumor regression, these patients still experienced severe toxicities. Additionally, responses tended to be short lived, and after one month post infusion, only 8 of the 33 total patients showed in vivo sustenance of the transduced TILs. While the NFAT addition to the single-chain IL-12 gene was intended to confine the response to the tumor, high serum IL-12 and IFN γ levels indicated that the TILs were being activated outside of the tumor microenvironment, which was likely a contributing factor to the toxicities. This indicates that either the promotor has a basal level activity that results in constitutional expression of the transduced gene, or that non-tumor elements are causing the activation of the TILs and in turn, the expression of NFAT.IL-12. A way to overcome the latter could be to ensure that the infused TILs are tumor specific. The authors consider that a different promotor may result in a lower basal level expression of the gene.

5.3. CXCR2

While enhancing the in vivo longevity and cytotoxic capabilities of TILs could result in improved patient outcome, it is also important to ensure that infused cells localize to tumor sites. In melanoma models, CXCL1 and CXCL8 have been shown to be produced by cells in the TME [118–120], leading to the exploration of their natural receptor, CXCR2, for potential improvement of adoptive cell therapies. To overcome the lack of receptor expression in T cells, two groups, led by Patrick Hwu, have demonstrated the ability for CXCR2 retrovirally transduced T cells to chemotactically home to tumor sites both in vitro [120] and in vivo mouse model [121] with CXCR2-dependency. In these pre-clinical studies, the T cells elicit functional activity and increase the survival of animals. In 2016 our group reported a CXCR2 TIL transfection method via mRNA electroporation [122], but this method has not proceeded further. However, the adoptive cell transfer of CXCR2-retrovirally transduced TILs [123] is currently undergoing clinical trial at MDACC (NCT01740557).

5.4. Gene Knockouts

Rather than arming cells with the ability to produce immune-perpetuating and inflammatory signals, many groups have attempted to genetically eliminate immunosuppressive and exhaustive elements from them. Given the success of CPI therapy, PD-1 is seen as an attractive target. While combination studies using PD-1-targeting drugs and TIL-based ACT are being conducted (see Section 4.1), it may also be advantageous to delete PD-1 from infused TILs altogether. Anti-PD-1 monoclonal antibodies act systemically and are associated with multiple toxicities, as previously discussed. To avoid cutting the brakes from the entire immune system, deletion of PD-1 from TIL infusion products could possibly result in a more cancer-directed, exhaustion-averse immune response with less toxicity. Effective PD-1 deletion in TILs at a clinical-scale has been reported using techniques such as ZFN [124] and TALEN [125], and more recently our institute has utilized CRISPR-mediated methods to improve this efficiency even further [126]. While these studies demonstrate feasibility in clinical-scale TIL products and suggest improved performance of the product by way of increased IFN γ secretion, this combination is yet to enter clinical trials. Contrarily, two recent, unrelated clinical studies have reported successful CRISPR-mediated knockout of the PD1 gene for adoptive transfer of autologous T cells for cancer therapy [127,128], however, neither of these studies involved TILs and reported low knockout efficiency.

An internal checkpoint, cytokine-induced SH2 protein (CISH), has been found to impede anti-tumor CD8⁺ T cell responses, primarily through suppressing TCR avidity [129]. A recently reported study from the NCI and University of Minnesota used CRISPR to eliminate the CISH gene from TILs [130]. Favorable functional outcomes, such as increased tumor lysis and neoantigen reactivity, resulted from the inactivation of this internal checkpoint. Phenotypic changes include increased TCR avidity and PD-1 expression. While

the latter effect is counterintuitive to treatment, anti-PD-1 combination promoted tumor regression and survival in a murine model. A phase I/II clinical trial exploring the use of *CISH* knockout TIL-based ACT (NCT04426669) is currently underway. As chaperone technologies are developed to detect off target hits and knockout efficacy [131–133], the CRISPR/Cas9 method continues to become increasingly accessible and may soon play an integral role in TIL-based ACT. Figure 2 illustrates these interventions in the standard workflow as well as those previously discussed for the improvement of this therapy.

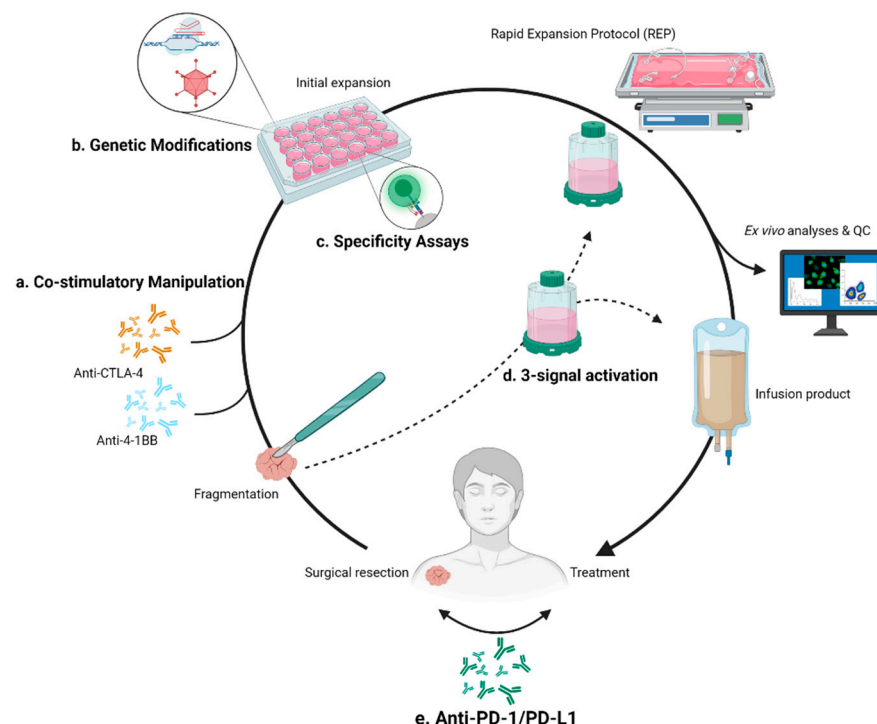


Figure 2. Modifications of TIL-based ACT workflow. (a) Addition of monoclonal antibodies targeting CTLA-4 and 4-1BB to improve the initial expansion phase of the workflow. (b) Genetic modification of TIL via CRISPR/Cas9 or viral transduction to strategically knockout or knock-in desired elements. (c) Identification of tumor-specific TILs to improve cancer-directed response. (d) A 3-signal activation method described by MDACC. Replaces initial expansion phase, and possibly could be used to bypass REP. (e) Priming and/or combination checkpoint inhibition therapies.

6. Conclusions

The recent revived attention to adoptive TIL therapy suggests that the future of the therapy will likely be similar to its past. The innovative application of the advances and discoveries in other fields continues to shape and improve this evolving therapy. Figure 2 illustrates the interventions discussed in this work that are currently being investigated. While methods capitalizing on CPI, CRISPR/Cas9 and other biotechnologies are in the process of clinical translation, few patients have felt the benefits of these laboratory accomplishments. Although further pre-clinical and clinical studies are needed for many modifications to the therapy, the future of TIL-based ACT will likely be highly personalized, interdisciplinary, and an effective treatment option in a wide variety of cancers.

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