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REVIEW

Tumor-infiltrating lymphocyte expansion protocols for adoptive cell therapy in cancer

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Abstract

Adoptive cell therapy (ACT) with tumor infiltrating lymphocytes (TILs) has emerged as an innovative strategy in cancer immunotherapy, especially in the treatment of solid tumors. This review provides a comprehensive analysis of current TILs expansion protocols, with a focus on strategies aimed at optimizing their performance, feasibility and functionality. The classical TILs expansion protocol is based on a two-step process: a rapid pre-expansion phase (Pre-REP), followed by a rapid expansion phase (REP), in which high concentrations of interleukin-2 (IL-2) are used. In recent years, various modifications have been incorporated into the protocol with the aim of improving its efficiency, including the use of alternative combinations of cytokines such as IL-7, IL-15, and IL-21, as well as the replacement of irradiated *feeder* cells or anti-CD3 antibodies with other strategies. Furthermore, advances such as the targeting of TILs specific to neoantigens, the development of CAR-TILs, the application of artificial intelligence, and the genetic modification of TILs have contributed to expanding both the applicability and efficacy of this therapy. The clinical success of *Lifileucel* with the recent FDA approval of underscore the translational potential of TILs-based therapies. However, significant challenges remain, including time and production costs. This review examines ongoing clinical trials and combination therapeutic strategies, such as the use of immune checkpoint inhibitors. As research progresses, optimization of TILs expansion protocols will be critical to improving clinical outcomes and broadening the applicability of this personalized immunotherapy in oncology.

Keywords Tumor-infiltrating lymphocytes (TILs) · Adoptive cell therapy (ACT) · TILs expansion protocols · Immunotherapy · Neoantigen-reactive t cells

Clinical trial number

Not applicable.

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1 Introduction

Immunotherapy has emerged as a cornerstone in the therapeutic landscape for cancer patients. Its efficacy is rooted in the strategic manipulation of the immune system, including various immune cells and molecular mediators, to target and eliminate tumor cells while sparing normal tissues. The initial breakthroughs in immunotherapy were achieved with immune checkpoint inhibitors (ICIs) or monoclonal antibodies (mAbs), which have revolutionized cancer treatment by enabling the patient's immune system to affectively attack tumor cells, achieving remarkable outcomes across multiple cancer types [1, 2]. Today, ICIs have become fundamental in oncology and have replaced conventional therapy in many types of tumors, such as PD-1 inhibitors (Nivolumab, Pembrolizumab, Cemiplimab), PD-L1 inhibitors (Atezolizumab, Durvalumab, Avelumab) and CTLA-4 inhibitors (Ipilimumab, Tremelimumab), along with the LAG-3 inhibitor Relatlimab, positioning themselves as the first line of therapy both individually and in combination with other therapies [3]. Despite these successes, ICIs present certain challenges, such as resistance and limited efficacy [4] Therefore, the need to overcome their limitations has driven the development of new therapies.

ACTs have revolutionized the treatment of neoplasms, offering promising antitumor activity in several types of cancer. The main current therapies include chimeric antigen receptors (CAR) [5] and genetically modified T cells with T cell receptors (TCR-T) [6], natural killer (NK) cells [7], gamma-delta T cells (γδT) [8], dendritic cells (DC) [9], and finally TILs [10]. CAR T-cell therapy involves genetically modifying T cells to express receptors that specifically target tumor antigens and has shown significant clinical efficacy in the treatment of hematological malignancies but any solid tumors [11]. In contrast, TILs therapy involves extracting immune cells from a patient's tumour, expanding them ex vivo with high doses of interleukin-2 (IL-2) and reinfusing them to attack and eliminate cancer cells [12]. TILs therapy has demonstrated clinical efficacy, particularly in metastatic melanoma, which historically has had limited options and poor clinical outcomes, and its application is now being explored in a wider range of solid tumors [13, 14]. This approach was first developed by Rosenberg and colleagues, who demonstrated its potential to induce significant clinical responses [12].

In this article we provide a comprehensive overview of the historical context, current clinical developments, and future directions of TILs-ACT. We will delve into the advancements in understanding genetic intratumor heterogeneity, neoantigen identification, and strategies to overcome T-cell exhaustion and tumor immunosuppression, which have collectively enhanced the efficacy of TILs therapy.

Furthermore, we will discuss the characteristics of TILs products and innovative strategies, such as selective expansion of specific cell fractions and genetic modifications, aimed at improving the in vivo survival and functionality of TILs. This review aims to underscore the potential of TILs-ACT as a personalized therapeutic approach for tumors and its expanding applicability to other cancer types.

1.1 Overview of historical context and technical challenge

The first study was developed by Rosenberg et al. developed an innovative protocol to produce autologous cultures of TILs extracted directly from the patient's tumor, allowing the selection of lymphocytes with specificity towards tumor cells and the expansion in large quantities to obtain antitumor effect [12, 15].

The procedure consists of obtaining the tumor tissue by surgical resection, which will provide the source of TILs. It is essential that the sample is processed quickly and carefully to preserve cell viability. Subsequently the therapeutic approach consists in two main phases: laboratory phase and clinical phase. The laboratory phase involves the large-scale expansion of specific T cells derived from patient´s tumor tissue. This process includes the selection and proliferation of lymphocytes with specificity towards tumor cells. A commonly used protocol is the Rapid Expansion protocol (REP), which involves stimulating TILs with interleulin-2 (IL-2) [16]. In the infusion phase, lymphodepleting chemotherapy was administered to the patient before TILs infusion. This regimen creates a favorable environment for antitumor lymphocytes, followed by a high dose of IL-2 that maintains T-cell activity after infusion. [17, 18].

The efficacy of TILs therapy is highly dependent on the specificity, viability and functionality of these cells. A successful expansion process ensures that the infused lymphocytes maintain their cytotoxic capacity and specificity against tumor cells, which is crucial for improving therapeutic response and clinical outcomes in patients.

Compliance with Good Manufacturing Practice (GMP) standards in the production of TILs is essential to ensure the safety and efficacy of the treatment. This involves controlled environments to prevent contamination, the implementation of standardized protocols at each stage of the process, the qualification and validation of equipment and processes, and the application of rigorous quality control to ensure that the TILs produced meet the required criteria of specificity, viability and functionality [18–20].

2 Cell production: optimization of TIL expansion protocols

The classical scheme for the procurement, expansion and testing of TILs consists of a lengthy multi-step open or closed system process, termed the Pre-Rapid Expansion Protocol (Pre-REP) followed by a Rapid Expansion Protocol (REP). The Pre-REP phase aims to select and condition the patient's TILs before subjecting them to massive expansion in the REP phase, which lasts approximately 14 days. During the REP phase, TILs are cultured under optimal conditions designed to promote their accelerated proliferation, with the goal of generating a cell population large and functional enough to be therapeutically effective when reinfused into the patient (Fig. 1). The classic and standardized TILs expansion was developed by Rosenberg and coworkers in the early stages of this research [15, 16, 21].

Although it remains the most efficient and rapid method for TILs expansion, several variations on the original protocol have emerged. Therefore, the classical TILs expansion protocol will be describing and subsequently documented alternatives will be discussed.

2.1 Procedure for isolation of TILs from tumor tissue

Tumor samples are obtained from patients after surgery, followed by extraction of TILs from the tissue [22]. Tumor tissue is transported in a sterile container with sterile saline solution, optimized for storing fresh samples without inducing apoptosis or cell activation. Upon arrival at the laboratory, all procedures must be performed in a biosafety cabinet with laminar flow [23].

2.2 Pre-rapid expansion protocol (pre-REP) phase

There are different methods to obtain TILs such as mechanical or enzymatic disaggregation or a combination of both [24]. Disaggregation of the tumor into different fragments, which are cultured independently in 24-well plates with complete T-cell medium consisting of RPMI-1640 (Roswell Park Memorial Institute), with 1% L-glutamine (2 mM), supplemented with 10% human serum and 1% penicillin-streptomycin and high doses of interleukin-2 (IL-2). Plates are placed in an incubator at 37 °C with 5% CO2. When the medium is consumed it is replaced in all wells at the same time. Each initial well is considered a separate TILs culture and is kept always separate from the others. This process lasts 15 to 30 days and is concluded when the TILs culture reaches confluence in four wells of a 24-well plate, at which time cell counts and phenotypic characterization by flow cytometry are performed to observe the percentages of T cell populations [15, 16, 21, 25].

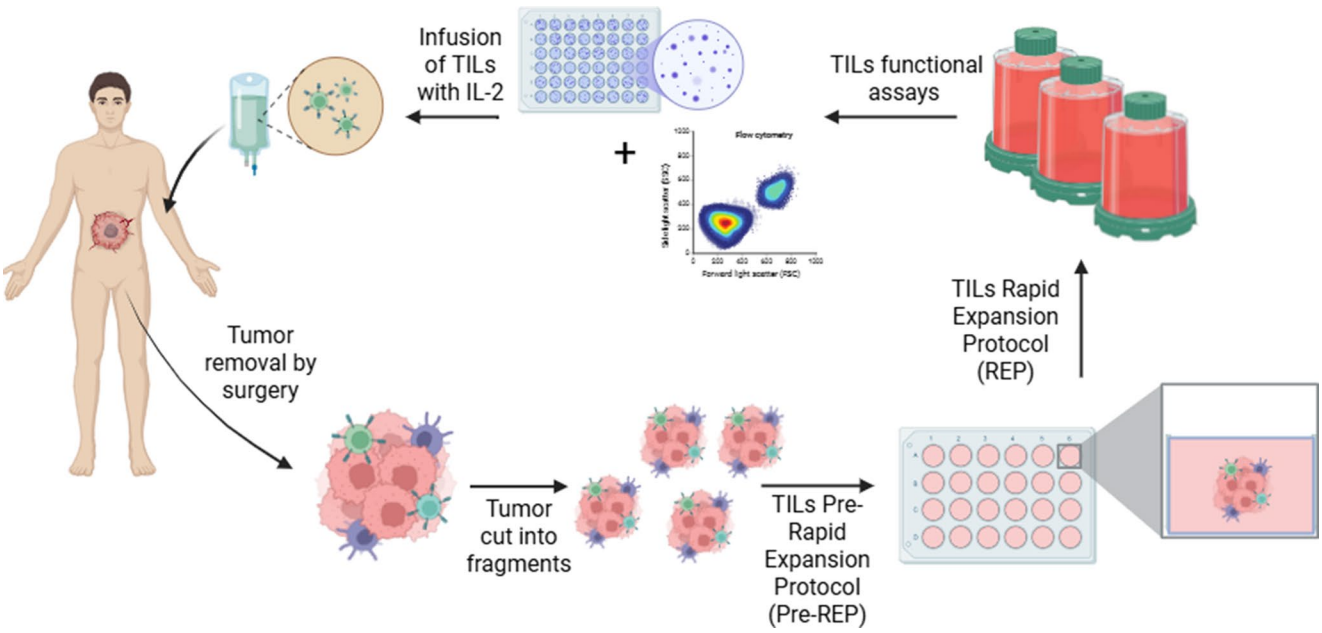

Figure 1 General process of the protocol for the production of cell therapy based on TILs. The process begins with surgical resection of the tumor and its fragmentation for the removal of tumor cells. The TILs are isolated from the tumor tissue and undergo an initial expansion phase with high doses of IL-2. Subsequently, the main expansion phase is performed using IL-2, OKT3 and feeder cells. At the end of the expansion protocol, the functionality of the TILs is assessed by specific assays. Finally, the activated TILs are infused into the patient together with IL-2 to enhance the antitumor immune response. Created in https://BioRender.com

2.3 REP phase: fundamentals, challenges and improvements in the expansion of TILs

2.3.1 Preclinical optimization

In the REP phase, CM and AIM V® media are mixed in a 50/50 ratio, supplemented with IL-2 interleukins, anti-CD3 antibody OKT3, and a 200:1 ratio of irradiated feeder cells (50 Gy) to TILs from the Pre-REP phase. The cells are incubated under controlled conditions (humidity 90–95%, CO2 5%, and temperature 37°C) to promote effective TILs expansion. After the REP phase, TILs are collected, centrifuged, washed, and split for phenotypic analysis by flow cytometry and evaluation of T-cell reactivity [15, 16, 21, 25].

GMP-compliant production

In GMP-compliant TILs production, culture vessels such as *T*-175 flasks, G-Rex culture bags, and Wave or Xuri bioreactors are used. G-Rex flasks are the most commonly used as they reduce the need for media changes and minimize contamination risks [26–29]. On the other hand, automation and system shutdown represent an important evolution in the manufacture of cellular products. First, studies have been carried out on the CliniMACS Prodigy system (Miltenyi Biotec) which has been adapted to produce TILs in a fully closed, automated and controlled environment, from the initial isolation of cells to their expansion and final formulation. This platform offers additional advantages such as automated traceability and reduced manual handling, which improves reproducibility and facilitates regulatory compliance [30].

2.4 Defining quality assays in TILs

The determination of interferon gamma (IFN-γ) concentration by ELISA, ELISPOT, and/or intracellular cytokine flow cytometry and the detection of the expression level of the surface marker 4-1BB after co-culturing TILs and tumor cells has been established as the reference method for evaluating TIL functionality in vitro due to its key role in the antitumor immune response. When TILs are exposed to specific tumor antigens, IFNγ production is a reliable indicator of their cytotoxic capacity and functional activation [15, 22, 31, 32].

Next, we focused on optimizing key steps of the TILs expansion protocol such as interleukins, feeders and mitogens to improve the efficacy of adoptive cell therapy, reduce production time and improve the viability and functionality of tumor infiltrating lymphocytes.

2.5 Role of interleukins in the expansion and functionality of TILs

Interleukins act synergistically and specifically on the activation, proliferation, and differentiation of T cells, playing key roles in both innate and adaptive immunity, as well as in cell development and specialization [33]. However, not all responses induced by these cytokines are beneficial in the context of TILs therapy. For example, increased NK cells can displace cultured T cells [33–35], and the expansion of regulatory T cells (Tregs) can compete with $CD8^+$ T cells and suppress their effector responses [36, 37].

Among the most relevant interleukins in the field of adoptive immunotherapy with TILs, those belonging to the IL-2 family stand out, such as IL-2, IL-7, IL-15, and IL-21, due to their ability to modulate the fate and functionality of different T cell subpopulations [33, 38]. These cytokines have been extensively studied for their potential to induce more effective cellular profiles in the antitumor response (Table 1).

Historically, IL-2 has been the central interleukin in standard protocols for TILs expansion, due to its ability to activate potent immune responses in the tumor microenvironment [39, 40]. However, several studies have shown that high and sustained levels of IL-2 induce a preferential expansion of more differentiated T cells, with less persistence and greater expression of markers associated with functional exhaustion [41]. This signaling, mediated by STAT5, promotes the expression of tryptophan hydroxylase 1 (TPH1) and the production of 5-HTP, which activates the AhR receptor, with the consequent dysfunction of $CD8^+$ T cells [41]. However, Beltra et al. [42] demonstrated that a targeted and temporary activation of STAT5a in exhausted T cells can antagonize the TOX factor, reprogramming these cells towards functional intermediate states and a more favorable epigenetic landscape [42].

In contrast, cytokines such as IL-7 and IL-15 offer significant advantages in terms of generating fewer exhausted profiles with greater memory capacity. IL-7 is essential for the survival, proliferation, and maintenance of naive and memory T cells, through the activation of the JAK/STAT5 pathway and the induction of anti-apoptotic genes such as Bcl-2 and Mcl-1, while inhibiting pro-apoptotic genes such as Bax and Bak [43, 44]. IL-15, on the other hand, promotes the expansion of $CD8^+$ T cells and favors the proliferation of Th1-type T cells, without significantly stimulating the expansion of Treg lymphocytes, which is highly desirable in this context [45, 46].

Finally, IL-21 has emerged as a promising cytokine due to its ability to enhance the effector function of TILs and promote the generation of central memory T cells, essential for a long-lasting immune response [47]. Unlike IL-2,

Table 1 Advantages and disadvantages of different interleukin. This table summarizes the advantages and disadvantages of the interleukins IL-2, IL-7, IL-15, and IL-21 in the context of TILs therapy. Each interleukin plays a critical role in immune cell activation, proliferation, and differentiation, impacting the effectiveness of antitumor responses. Understanding these factors is essential for optimizing TILs therapy and enhancing patient outcomes

Interleukin	Advantages	Disadvantages
IL-2	- This is the classic and most studied protocol, used in multiple clinical trials with proven efficacy. - IL-2 promotes massive proliferation of activated T lymphocytes.	- High levels can induce exhaustion in tumor-reactive $CD8^+$ T cells. - IL-2 also stimulates Treg, which can suppress the antitumor response.
IL-7	- Stimulates proliferation of naive and less differentiated T cells. - Activates anti-apoptotic genes and inhibits pro-apoptotic genes. - Not stimulate Treg or NK cells.	- Less effective in expansion performance. - Potentially limited in promoting highly differentiated T cell responses.
IL-15	- Contributes to cell maintenance in a dose-dependent manner. - Facilitates expansion of memory T cells.	- May promote expansion of NK cells, which can compete with T cells in culture. - Excessive expansion of $CD8^+$ T cells can lead to potential functional impairment.
IL-21	- Promotes lymphocyte proliferation and generation of central memory TILs. - Reduces expansion of Treg and promotes a younger, more functional phenotype of $CD8^+$ T cells.	- Less established in clinical use compared to IL-2. - Requires further investigation to fully understand long-term effects on TILs therapy and patient outcomes.

Abbreviations: TILs: Tumor-infiltrating lymphocytes; Tregs: Regulatory T cells; NK cells: Natural Killer cells; IL: Interleukin; Tcm: Central memory T cells

IL-21 reduces Treg expansion and favors a more youthful and functional phenotype in $CD8^+$ T cells [48], positioning itself as a solid candidate in optimized expansion protocols.

Taken together, these findings underscore the limitations of using IL-2 alone in current protocols and support the exploration of alternative cytokine combinations that promote the expansion of more persistent and functional TILs.

Based on a review of studies, it can be inferred that the combined and moderate administration of IL-2 and IL-7 induces a synergy that optimizes lymphocyte proliferation, particularly of the $CD4^+$ subgroup, without causing a notable increase in cell death [49] likewise, in the stimulation of TILs by artificial antigen-presenting cells (aAPCs) expressing IL-2, IL-15, or IL-21 or lacking cytokine production, conditions with IL-2-producing aAPCs exhibit significantly higher total expansion and a phenotype characterized by a high proportion of $CD3^+$ cells compared to those without cytokine or with IL-15/IL-21 [50].

2.5.1 Clinical evaluation

Several clinical trials are evaluating interleukins to optimize TIL expansion. High-dose IL-2 remains the standard option, with notable clinical results in the C-144–01 study (NCT02360579) in melanoma, which reported an objective response rate (ORR) of 31–72% and a complete remission (CR) rate of 8–20%. Alternatives such as IL-7 (NCT06204991), IL-15 in the form of modified TILs (NCT05470283), and IL-21 combined with cyclophosphamide and IL-2 (NCT01106235) are currently being investigated, although results have not yet been published. These strategies seek to improve the efficacy of TILs in immunotherapy.

2.6 Ex vivo activation of T cells by anti-CD3/CD28/4-1BB co-stimulation

Ex vivo activation of TILs by monoclonal antibodies in the absence of antigen takes advantage of conserved signal transduction pathways common to all T cells. Activation is typically initiated by stimulation of the T cell receptor (TCR) using anti-CD3 antibodies, which bind to the TCR/CD3 complex and provide the primary activation signal [51]. This is often combined with a secondary costimulatory signal, usually via CD28 and/or 4-1BB, to enhance activation and promote downstream signaling cascades [52, 53]. This strategy more closely mimics physiological T cell activation than traditional mitogens, as it reproduces the natural receptor-ligand interaction and better supports T cell proliferation and differentiation [51, 54, 55]. CD28-mediated co-stimulation amplifies TCR signaling, leading to increased IL-2 production and improved T cell survival, proliferation, and differentiation factors that are critical for the success of adoptive immunotherapies. Anti-CD3/CD28-coated microspheres are widely used for robust $CD4^+$ T cell expansion; however, studies have shown that using soluble anti-CD3 alone can offer distinct advantages for $CD8^+$ T cell expansion, particularly when the goal is to preserve a less differentiated phenotype. This includes populations such as central memory T cells or even naive T cells, which are associated with increased proliferative capacity and persistence in vivo desirable characteristics in many clinical settings. [54–56].

An alternative or complementary strategy consists of the use of anti-4-1BB agonist antibodies, which further reinforce the expansion and effector function of $CD8^+$ T cells, improving their ability to recognize and eliminate tumor cells [18, 53].

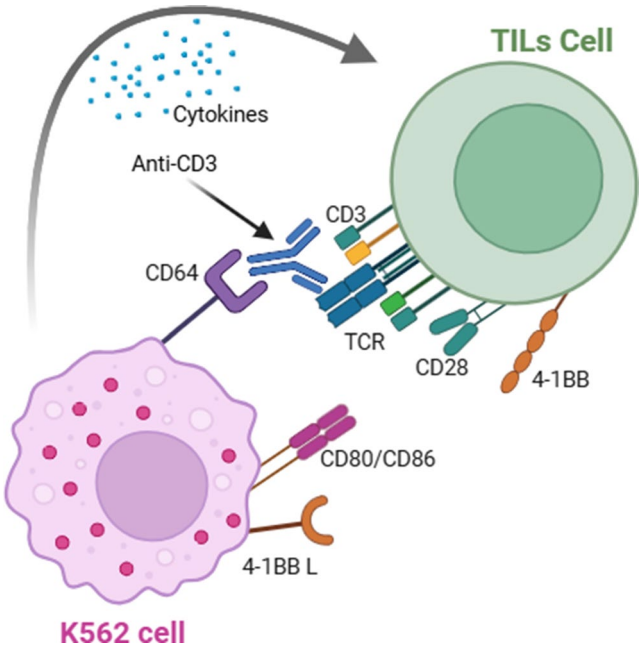

Figure 2 Interaction between K562 cells and TILs. The diagram depicts the interaction between a K562 cell and a TILs cell. This configuration is used in the REP of TILs REP. The K562 cell is modified to express key co-stimulatory ligands, such as anti-CD64, CD80/CD86 and 4-1BBL, which interact with their respective receptors on T cells: CD3, CD28 and 4-1BB. This contributes to the efficient activation and expansion of TILs. Created in https://BioRender.com

2.7 Advances in T Cell Support Systems: Comparing Classical Feeders and Innovative Approaches

Feeder cells are used in the rapid expansion of TILs to secrete growth factors and provide essential signals that support T cell survival and proliferation. Irradiated allogeneic PBMCs from at least three healthy donors are commonly used in a 1:200 ratio (TILs:feeders). Irradiation (50 Gy) inactivates the proliferative capacity of these cells while preserving their cytokine secretion function, creating a nutrient-competition-free environment [21, 57].

K562 cells are immortal hematopoietic cells of myeloid origin that carry the t(9;22) translocation, leading to the formation of the Philadelphia chromosome (Ph) and the expression of the chimeric BCR-ABL1 protein, whose constitutive tyrosine kinase activity promotes uncontrolled cell proliferation. These cells are genetically modified to stimulate T-cell responses in vitro by incorporating costimulatory molecules such as CD86, CD64, and 4-1BBL, along with the expression of cytokines such as IL-7 or IL-15. This strategy enhances T-cell activation, proliferation, and function, promoting more efficient expansion and improving their effector capacity. Their use in adoptive TILs therapy protocols represents a promising alternative to optimize the antitumor immune response and improve the efficacy of immunotherapy-based treatments [57–59]. K562 cells induce a numerical expansion of TILs that is statistically similar to a rapid expansion method established with a proportion of irradiated feeder cells [60] (Fig. 2).

Table 2 Phenotypic and functional markers are used to characterize T cell subsets and immune status. This table summarizes various types of immune markers used in T cell characterization and analysis. It includes activation markers, T cell subpopulations, T helper profiles, exhaustion markers, memory/naive markers, Treg markers, proliferation markers, as well as cytokines and chemokines that play critical roles in immune responses

Marker Type	CD Markers	Associated Subset
Activation Marker	CD25, CD69, CD137 (4-1BB), CD134 (OX40)	Activated T cells
T cell subpopulations	CD3, CD4, CD8, TCR α/β, TCR γ/δ	Cytotoxic T cells, γδ T cells
Th profile	CXCR3, CCR4, CCR6, CCR10	Th1 (CXCR3), Th2 (CCR4), Th17 (CCR6), Th22 (CCR10)
Exhaustion	PD-1, CD39, TIM-3, LAG-3, CTLA-4	Exhausted T cells
Memory/naive markers	CD45RA, CD45RO, CCR7, CD62L	Naive ($CD45RA^+$ $CCR7^+$), Central memory ($CD45RO^+$ $CCR7^+$), Effector memory ($CD45RO^+$ $CCR7^-$)
Treg	CD4, CD25, FOXP3	Regulatory T cells
Cytokines	IL-2, IL-6, IL-10, IL-12, TNF-α	Various T cell responses
Chemokines	CCL2, CCL5, CXCL8, CXCL10	Recruitment of immune cells

2.8 Immunophenotypic characterization of TILs in preclinical and clinical contexts

To evaluate TILs, it is essential to characterize surface markers to identify T-cell subpopulations with the highest therapeutic potential, based on their activation status, memory, homing, exhaustion, and responsiveness. While there is no consensus on the most appropriate markers, the following were commonly used to assess TILs status (Table 2 & Fig. 3).

Recent studies highlight the therapeutic value of intervening on both exhausted T cells and stem cell-like memory T cells, as they are essential to achieve a long-lasting and effective response in adoptive immunotherapies [61].

Traditionally, $CD8^+$ T cells have been the primary focus of cancer immunotherapy. However, recent evidence highlights the crucial role of $CD4^+$ T cells in the antitumor response. These cells not only collaborate with $CD8^+$ lymphocytes but also have direct effector functions, such as tumor cell destruction, macrophage activation, induction of tumor cellular senescence, elimination of tumor vasculature, and enhancement of other immune responses [62, 63].

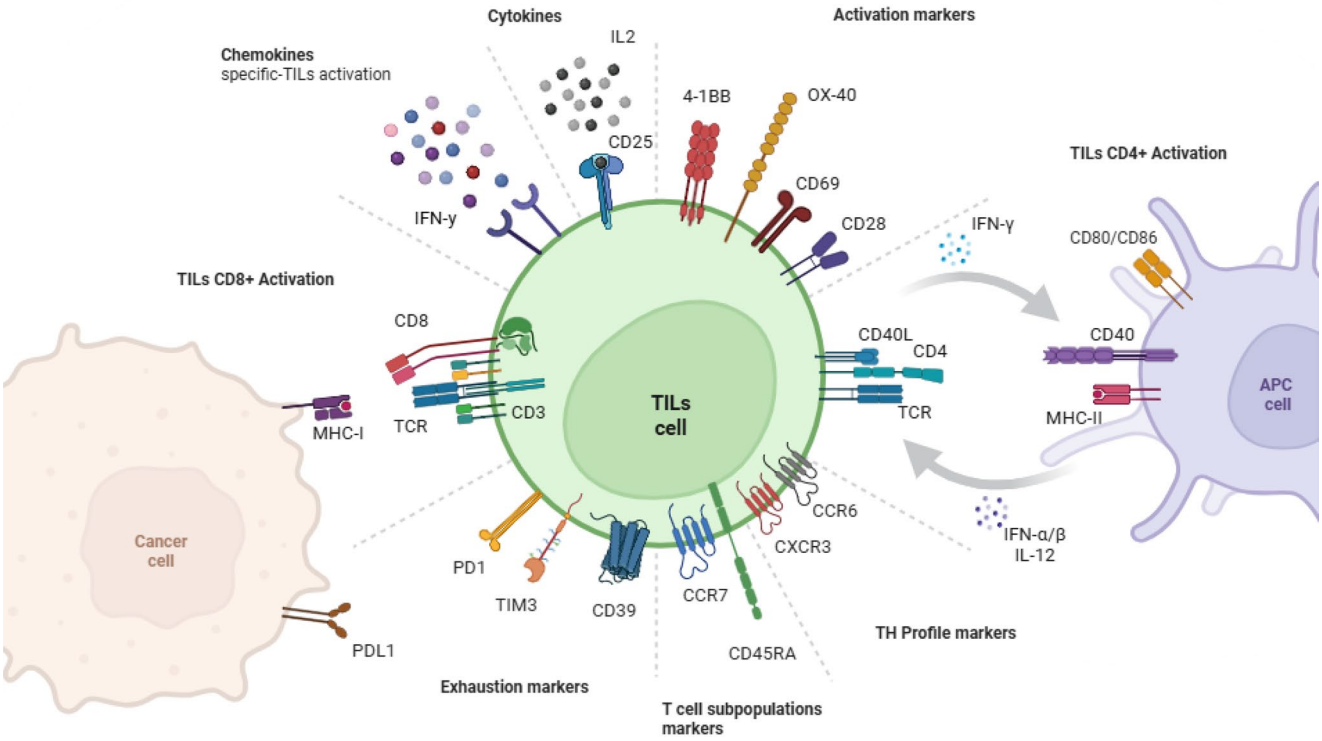

Figure 3 Schematic representation of TILs and its interaction with presenting cells (APCs) and tumor cells. The most important markers in immunophenotyping are shown including activation markers (CD28, CD69, OX-40, 4-1BB), $CD4^+$ TILs subpopulations (CCR7, CXCR3, CD45RA), cytokines and chemokines involved in their activation, as well as depletion markers (PD-1, TIM-3, CD39). Activation of $CD8^+$ TILs is illustrated by interaction with the MHC-I complex of tumor cells, whereas activation of $CD4^+$ TILs occur through MHC-II-mediated antigenic presentation on antigen-presenting cells. Created in https://BioRender.com

Furthermore, the differentiation mechanisms and signals that regulate $CD4^+$ T cell function during tumor progression are being elucidated, reinforcing their relevance in the design of immune-restorative strategies [64]. Specific subpopulations, such as PD-1^+ $ICOS^+$ $CD4^+$ T cells, have demonstrated a remarkable ability to recognize tumor-associated antigens and actively cooperate with agent-presented cells and $CD8^+$ lymphocytes, making them promising candidates for advanced adoptive therapies and personalized vaccines [65].

One key activation marker is 4-1BB/CD137, A key activation marker is 4-1BB/CD137, which indicates antigenic stimulation in the tumor microenvironment and therefore the expression of 4-1BB in TILs is associated with a higher specificity of antigenic recognition against tumor cells. CD137 expression on T cells can mediate activation, making it a useful biomarker for evaluating TILs activation in expanded protocols [18, 66, 67]. Other activation markers are also of interest, such as OX-40, which is a co-stimulatory receptor that, upon binding to its ligand OX40L, promotes T-cell activation, proliferation, survival, and memory formation, while reducing Treg suppression [68].

CD69 is a marker of early activation in leukocyte cells, and it has been shown that by interacting with the S1P1 receptor (sphingosine-1-phosphate receptor), it induces their internalization. This interaction reduces the availability of S1P1 in the membrane and favors the retention of T cells in lymphoid tissues, which may favor processes such as maturation or longer activation in these environments [69]. $CD69^+$ $CD103^+$ TRM-like $CD8^+$ TILs play a key role in tumor-reactive immunity in ICC and may serve as promising therapeutic targets. Enhancing $CD69^+$ TILs responses or blocking immune evasion pathways could inform future immunotherapy strategies [70].

CD25 and FoxP3 are markers of Tregs, and their expression plays a role in inhibiting tumor immunity through mechanisms such as immunosuppressive cytokine production, extracellular ATP depletion, and inhibitory cell contacts. However, CD25 is also essential for the optimal expansion and activity of effector T cells in peripheral tissues [71]. However, recent studies suggest that the $CD25^+$ $FoxP3^-$ subset correlates with better patient survival, despite high PD-1 levels [71]. In triple-negative breast cancer (TNBC) study analyzed the composition of $CD4^+$ $CD25^+$ TILs, finding that $Foxp3^+$ Treg were a minority subset and that the expression of immunosuppressive molecules, such as CTLA-4, LAG-3, TGF-β and IL-10, differed between

Foxp3$^+$ and Foxp3$^-$ TILs, the latter showing greater plasticity and potential to differentiate into Th17 cells under specific stimuli [72].

PD-1 (CD279) is exhaustion marker expressed on activated T cells [73]. In the tumor context. PD-1$^+$ TILs are more likely to have interacted with tumor cells, which suggest some specificity for this tumor [74]. Fernandez-Poma et al. identified that PD-1 expression on CD8$^+$ TILs allowed precise definition of tumor-reactive cells and evaluated the antitumor activity of CD8$^+$ PD-1$^+$ TILs in in vivo models, directly supporting this idea [75, 76].

TOX is a key transcription factor that acts as a master regulator in the establishment and maintenance of the exhausted state of CD8$^+$ T lymphocytes that driven by chronic T cell receptor stimulation and NFAT activation [77]. Furthermore, recent studies have shown that TOX is not only essential for initiating this dysfunctional state, but also for sustaining it over time [78]. In human TILs, inhibition of TOX by RNA interference leads to a decrease in the expression of PD-1, as well as other inhibitory receptors such as TIM-3, TIGIT, and CTLA-4 [79].

TIM-3, an important marker of immune exhaustion, negatively affects the functional status of TILs when co-expressed with PD-1. Because cells that express TIM-3 along with PD-1 exhibit dysfunctional signaling pathways, this has a significant positive impact on tumor progression [80]. High CD39 expression on TILs also indicates an exhaustion phenotype. CD8$^+$ T cells expressing CD39 have been found in metastatic tumors and lymph nodes, and this marker is associated with tumor reactivity in breast cancer and melanoma [81]. Unlike other exhaustion markers, CD39 expression correlates with clinical benefit in lung cancer patients and can be used to identify neoantigen-specific T cells [82]. Additionally, CD8$^+$ TILs co-expressing CD39 and CD103 are enriched in tumor-reactive T cells and show a distinct TCR repertoire, contributing to better outcomes in HPV-negative HNSCC [83].

Integrin CD103 binds to E-cadherin expressed on epithelial cells, contributing to the characterization of TILs as tissue-resident T cells within the tumor microenvironment, alongside the C-type lectin CD69 [84]. A higher frequency of CD103$^+$ CD39$^+$ CD8$^+$ TILs has been associated with an enhanced ability to destroy tumor cells in a major histocompatibility complex (MHC)-dependent manner [83]. Additionally, their presence correlates with a better prognosis and increased survival in patients with head and neck cancer, particularly in epithelial-origin tumors [85]. This correlation has also been observed in non-small cell lung carcinoma, where the presence of CD103$^+$ TILs is linked to improved patient survival [86]. Furthermore, the interaction between CD103 and E-cadherin has been implicated in the recognition and elimination of tumor cells by cytotoxic T lymphocytes in colorectal, lung, and pancreatic cancer [85, 87].

The phenotypic characterization of TILs is a crucial step in developing cell selection strategies. Identifying specific markers allows for the selection of subpopulations with greater therapeutic potential, such as those previously mentioned in this section. This approach facilitates the expansion of TILs that are more specific and reactive against tumor cells, thereby optimizing their efficacy in immunotherapy.

3 Clinical role of lymphodepletion and cytokines in enhancing TIL therapy response

Non-myeloablative lymphodepletion (NMA) by chemotherapy prior to infusion of TILs is essential in TILs therapy as it enhances their efficacy. Based on murine studies, lymphodepletion enhances the effect of TILs by eliminating Treg, increasing homeostatic cytokines (IL-7 and IL-15) and reducing endogenous lymphocyte competition for these cytokines [88]. It also promotes the activation of APCs, which regulate transferred T cells [9]. Studies by Rosenberg et al. and Dudley et al. demonstrated that lymphodepletion improves the objective response rate (ORR) in patients with metastatic melanoma treated with TILs [16, 89]

Cytokines administered to the host influence the persistence of adoptive T cells. IL-2 can promote the expansion of effector CD8$^+$ T cells, but it also reduces memory T cells and increases Tregs. In contrast, IL-7 and IL-15 promote the persistence of memory CD8$^+$ T cells and decrease Tregs, showing a more favorable profile for adoptive therapies [90].

4 Current research on TILs therapy and advances in TILs expansion protocols

TILs expansion protocols for adoptive cell therapy in cancer have undergone significant advances in recent years. These advances focus on improving the selection and expansion of tumor-reactive lymphocytes, as well as increasing their functionality and persistence in TME. A recent study has described a novel TILs expansion process that utilizes a different combination of cytokines during the Pre-REP and REP. This process not only increases TILs yield and viability, but also preserves cells in a less differentiated, more stem cell-like state, resulting in increased cytotoxicity and persistence [91].

Furthermore, single-cell sequencing technologies have improved our understanding of the composition and phenotype of TILs in the tumor microenvironment. These technologies allow for the selection of neoantigen-reactive

TILs through cell sorting or selective expansion strategies, improving the efficacy of the final product [92].

Neoantigen-based targeting utilizes peptides from tumor-reactive mutations, offering precise targets for immunotherapy [93]. Rosenberg et al. combined whole exome sequencing (WES) with bioinformatic analysis to identify mutations and the subsequent selection of peptides with high affinity for HLA class I and HLA class II [94, 95]. Despite the high non-synonymous mutations load detected by WES, only a few mutated peptides are immunogenic. Tumor heterogeneity, low HLA affinity and limitations of the TCR repertoire complicate their identification, requiring strategies like *in silico* prediction and Immunopeptidomics, followed by TILs functional screening. These methods have improved clinically relevant neoantigen identification [94–97]. TILs reactivity is evaluated by synthesizing mutant epitopes for APC presentation via HLA II or electroporating APCs with neoantigen-loaded mini-genes for HLA I presentation. TILs, upon antigen recognition, exhibit phenotypic changes, including cytokine secretion and activation marker upregulation [67, 93]. However, not all tumors generate neoantigens, limiting applicability. Using a tandem minigene library (TMG) can streamline the process, reducing time and cost by bypassing peptide identification and synthesis [98]. Factors like TILs count, tumor mutational load, and neoantigen-specific lymphocyte frequency correlate with therapy efficacy, even in ICI-refractory advanced melanoma patients [99–103] suggesting that enriching or selecting patients based on TILs reactivity could improve outcomes [67, 104]

Several studies have shown that neoantigen-specific TCRs can be isolated from TILs, which can enhance adoptive transfer therapy specificity [93]. Further research have mapped neoantigen-specific TCR clonotypes in metastatic tumors, showing tumor-reactive expansion, but often with exhaustion phenotypes [105]. This highlights the need for strategies to preserve or reactivate TILs function. TILs with high structural and functional avidity for neoantigens have been found to be associated with increased tumor infiltration and superior cancer cell killing efficacy [106].

MART-1 (Melan-A) and the MAGE family of antigens have been the subject of numerous studies in melanoma and other tumors. However, MART-1 carries a risk of autoimmunity and central tolerance as it is also expressed in melanocytes, while MAGE, although tumor-reactive, exhibits low immunogenicity, heterogeneous expression and tumor antigen loss. These limitations illustrate the difficulty of finding tumor targets that are specific, immunogenic and uniformly expressed, highlighting the need for new antigens and strategies to overcome tolerance and heterogeneity [107–109].

In summary, integrating neoantigen-reactive TILs identification and expansion into adoptive therapy offers a promising strategy for personalized cancer treatment that combines immune specificity with personalized treatment. As identification and selection methods improve, this approach could provide new, more effective treatments for advanced cancer patients.

Recent research in the field of adoptive cell therapy has focused on improving the efficacy of TILs by integrating new strategies. One of these explores a strategy that combines the specificity of CARs with their polyclonality, with the aim of maximizing antitumor efficacy in solid tumors. Conventional CAR-T cells, despite their high specificity for recognizing defined tumor antigens, have been found to have limitations in the context of solid tumors due to extratumoral toxicities derived from the expression of antigens shared with healthy tissues and the immunosuppressive microenvironment [110–112]. TILs, on the other hand, are a polyclonal population capable of recognizing multiple antigens, including specific neoantigens derived from mutations, as discussed above [93]

Furthermore, the insertion of CAR into TILs offers the advantage of overcoming classic tumor escape mechanisms, such as loss of HLA-I expression or defects in the antigen processing machinery, and allows independent recognition of MHC through costimulatory signals such as CD28, which is effective even in tumors with low expression of B7 costimulatory molecules [110].

Preclinical studies have shown that TILs isolated from metastatic tumors and transduced with an anti-Her2 CAR exhibit increased IFN-γ production and antitumor activity against Her2-positive cell lines, as well as tumor regression in xenograft models [113]. On the other hand, platforms have been developed for the in vivo engineering of CARs in TILs using implantable bio-instructive structures that recruit, modify, and release lymphocytes directly into the tumor, optimizing local activation and reducing systemic toxicities [114].

CRISPR/Cas9 technologies are being used to optimize CAR-T and TILs cell therapies. In CAR-T, proinflammatory genes and immune checkpoints (such as PD-1 or CTLA-4) are inactivated, and loci such as TRAC are modified to improve persistence and resistance to the tumor microenvironment. Allogeneic CAR-T cells are also being developed by removing TCR and HLA-I. In addition, CRISPR allows key regulators to be identified to improve proliferation and prevent cell exhaustion. Although preclinical studies show promising results in solid tumors, editing methods and safety controls still need to be refined before widespread clinical application [115]. In contrast, in TILs, editing trials with SOCS1 CRISPR/Cas9 technology have revealed that inactivation of this gene increases the accumulation and

persistence of TILs in preclinical models, improving their antitumor efficacy [116]

In TILs, other genetic modifications have been explored to improve their function, such as the activation of Toll-like receptors, the expression of cytokines (IL-2, IL-12, IL-15) or constitutively active receptors (caTLR4, caCD40), with positive effects on their expansion, persistence, and tumor migration. For example, Rosenberg's group demonstrated retroviral transduction of TILs with the IL-2 gene, while Forget's group described a protocol for transducing TILs with the gene encoding the chemokine receptor CXCR2 [117]. In addition to these strategies, emerging new lines of research include the incorporation of gene editing technologies such as CRISPR/Cas9 to improve the persistence, trafficking, and resistance of TILs to immunosuppressive signals within the tumor microenvironment. For example, disruption of immune checkpoint inhibitory molecules such as PD-1 or CTLA-4 [118].

The combination of TILs with oncolytic viruses could synergistically improve tumor recognition and overcome immune escape mechanisms, as viral DNA integrates into the host genome, leading to the restricted and durable expression of virus-specific antigens in the tumor [119].

Artificial intelligence (AI) is transforming medicine and immunotherapy, especially through multiomic analysis of TILs, which allows the identification of subpopulations with greater antitumor potential and improves the prediction of clinical response, facilitating more personalized and effective immunotherapy. However, its implementation poses ethical and social challenges, such as transparency, bias control, and protection of the doctor-patient relationship. Therefore, ethical and responsible AI is promoted to complement, rather than replace, human intelligence in healthcare [120, 121].

In summary, advances in TILs engineering, such as gene editing, the use of modified cytokines, oncolytic viruses, and artificial intelligence, are transforming adoptive therapy toward more effective and personalized approaches to solid tumors (Fig. 4). However, significant challenges remain in safety, toxicity control, editing efficiency, biomarker validation, and large-scale feasibility. Overcoming these challenges through well-designed clinical studies and rigorous monitoring will be key to consolidating a new generation of safer and more precise TILs therapies.

5 Therapeutic translation of TILs: ongoing clinical trials and future directions

In recent years, multiple clinical trials have evaluated the efficacy and safety of this therapy in different types of cancer, ranging from melanomas and ovarian cancer to lung and breast tumors. Supplementary Table 1 compiles a selection of ongoing Phase II clinical trials with results and ongoing Phase III clinical trials investigating the use of TILs in various malignancies.

Clinical trials with TILs have mainly focused on metastatic melanoma, but their application has been extended to other tumor types such as human papillomavirus-associated cervical cancer (NCT01585428), non-small cell lung cancer, squamous cell carcinoma of the head and neck, triple negative breast cancer and various gastrointestinal tumors. The therapeutic strategies most used in these studies include the infusion of autologous TILs after a lymphodepletion regimen, combined with IL-2 at various doses (NCT01369875, NCT01468818, NCT01814046) and, in some cases, with immune checkpoint inhibitors such as nivolumab or pembrolizumab (NCT01993719, NCT03215810, NCT02500576, NCT02818920, NCT05727904, NCT03215810, NCT06640582, NCT06538012).

Other strategies explored in these trials include targeting based on 4-1BB expression (NCT02111863), genetic modification for IL-12 secretion (NCT01236573) or combination with chemotherapy (NCT01807182) or radiotherapy.

In addition, some phase III trials evaluate different clinical applications of TILs therapy, such as its use in combination with an adjuvant after tumor resection, using low-dose subcutaneous IL-2 (NCT00200577), the results of which have not yet been published. In pretreated advanced melanoma, a randomized trial versus ipilimumab demonstrated a clear benefit in favor of TILs (NCT02278887). Finally, a study in first-line advanced melanoma investigating the early combination of TILs with PD-1 inhibitors is ongoing (NCT05727904).

In 2021, Iovance Biotherapeutics (U.S.) successfully completed Phase II trials with the TILs product Lifileucel (LN-144) in patients with ICI-resistant advanced melanoma, achieving an 80% disease control rate and 36% objective response rate [123, 124]. These results highlight TILs therapy as a potential treatment for advanced melanoma, being used mainly in trials as a second-line treatment [76]. In 2024, the FDA approved Lifileucel, the first cell therapy with TILs for metastatic melanoma, offering an option for patients resistant to other therapies. This strategy, based on ex vivo expansion of tumor T lymphocytes, boosts the immune response and represents a breakthrough in personalized oncology [125]. For its part, the EMA has not yet approved of any TILs product, although it has already received the marketing application for Lifileucel, whose evaluation is expected in 2025.

Among the most innovative therapeutic strategies are clinical trials exploring the genetic modification of TILs using CRISPR technology to inactivate the PD-1 receptor (NCT06783270), aiming to enhance their functionality

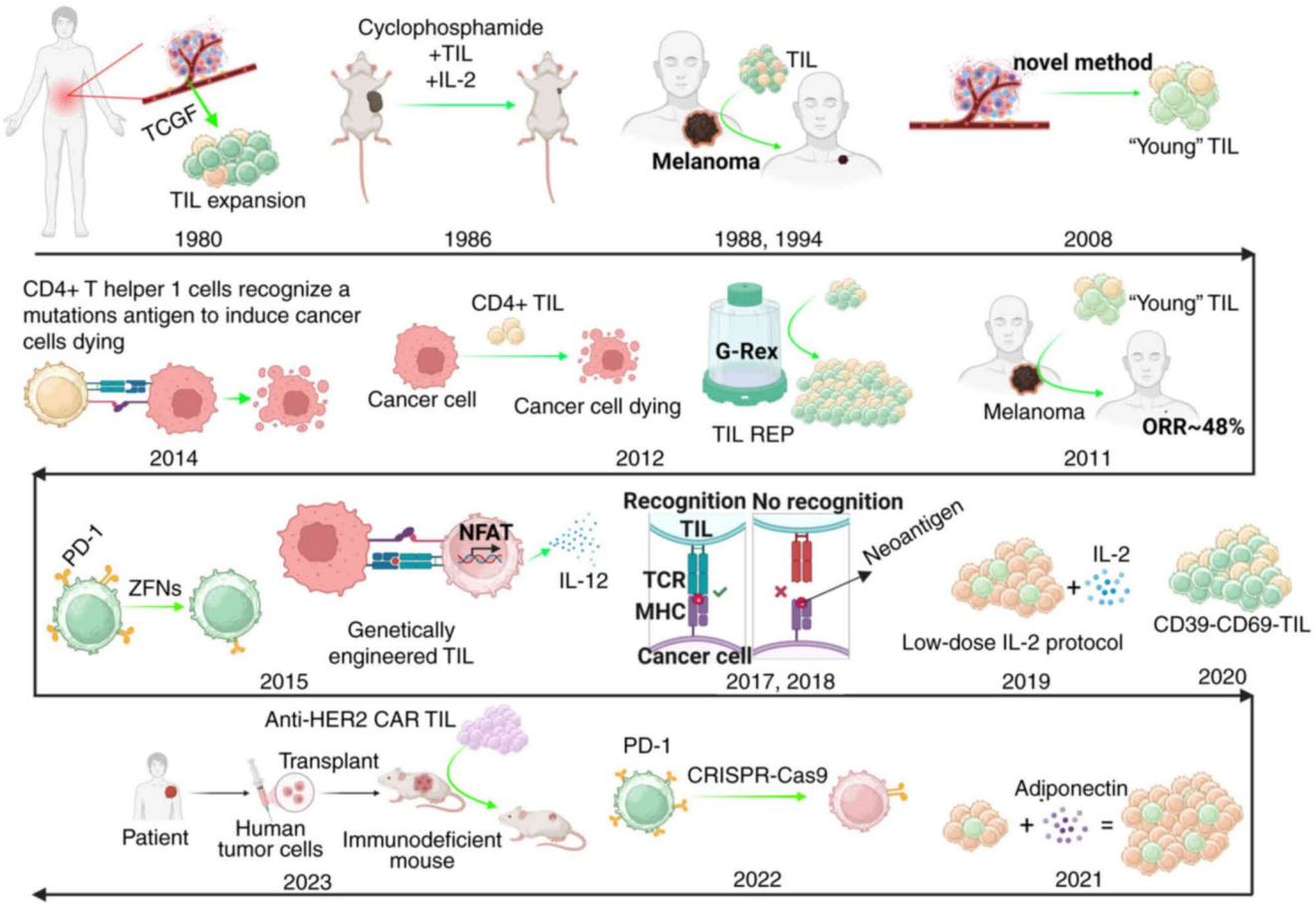

Figure 4 Historical evolution and key advances in the development of tumor TILs therapy. From the first cell expansion protocols and clinical trials in melanoma in the 1980s to current strategies in genetic engineering, gene editing and culture optimization, the figure summarizes the main scientific and clinical milestones in the evolution of this therapy. Noteworthy events include the introduction of 'young' TILs, the use of G-Rex platforms for expansion REP, the development of neoantigen-specific TILs, and new strategies such as IL-12-modified TILs, CAR-TILs, low-dose IL-2 protocols, and selection based on biomarkers such as CD39 and CD69. The timeline also includes emerging technologies such as CRISPR-Cas9 and combinations with metabolic factors such as adiponectin. This overview illustrates the dynamism and continuous progress in the field of TIL-based adoptive immunotherapy [122]

in the immunosuppressive tumor microenvironment. In this context, recent studies have shown that dendritic cells (DCs) can express high levels of PD-L1, which reduces TILs activity by interfering with their proper stimulation. Therefore, combining TILs with PD-L1 inhibitors is considered a promising strategy to counteract this suppression [126–128].

Additionally, other combination approaches aim to boost the antitumor efficacy of TILs using dendritic cell vaccines (NCT01946373) or oncolytic viruses (NCT00720031), to reverse immunosuppression within the tumor microenvironment. Synergistic effects have also been observed when combining TILs with targeted therapies such as adenoviral vectors or BRAF inhibitors (NCT04217473, NCT02354690), as well as with immunomodulators like Aldesleukin (NCT01955460), which are intended to optimize T-cell activation and expansion.

Taken together, combination therapies with TILs represent a promising strategy to overcome tumor resistance and enhance the antitumor immune response. These approaches reflect an evolution toward more effective and personalized treatments, supported by encouraging results from preclinical studies and ongoing clinical trials.

6 Conclusions

Overall, the findings presented in this review underscore the relevance of advances in TILs expansion protocols, originally developed by Rosenberg and colleagues, as a key tool in adoptive cancer immunotherapy. The implementation of innovative strategies and continuous improvement of culture processes have made it possible to generate cell products with greater viability, functionality, and therapeutic potential. However, significant challenges remain, such as

the limited availability of viable cells, immunosuppression of the tumor microenvironment, high costs, and the need for surgical procedures to obtain tumor samples.

Future prospects include the optimization of expansion protocols, CAR-TILs fusion, genetic editing with CRISPR/Cas9, cytokine gene transduction, stimulation with constitutive receptors, combination with oncolytic viruses, the use of artificial intelligence, and the utilization of neoantigen-reactive TILs.

These advances promise to drive the development of effective and accessible therapies for cancer patients. In summary, the evolution of TILs therapy consolidates its role as an essential strategy within the immunotherapeutic arsenal and paves the way for a more precise and effective approach to cancer.

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