REVIEW

Tumor-Infiltrating Lymphocytes and Adoptive Cell Therapy: State of the Art in Colorectal, Breast and Lung Cancer

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Summary

Our knowledge of tumor-infiltrating lymphocytes (TILs) is dramatically expanding. These cells have proven prognostic and therapeutic value for many cancer outcomes and potential to treat also disseminated breast, colorectal, or lung cancer. However, the therapeutical outcome of TILs is negatively affected by tumor mutational burden and neoantigens. On the other hand, it can be improved in combination with checkpoint blockade therapy. This knowledge and rapid detection techniques alongside gene editing allow us to classify and modify T cells in many ways. Hence, to tailor them precisely to the patient´s needs as to program T cell receptors to recognize specific tumor-associated neoantigens and to insert them into lymphocytes or to select tumor neoantigen-specific T cells, for the development of vaccines that recognize tumor-specific antigens in tumors or metastases. Further studies and clinical trials in the field are needed for an even better-detailed understanding of TILs interactions and aiming in the fight against multiple cancers.

Key words

Tumor-infiltrating lymphocytes • Adoptive cell therapy • Solid tumors • Cancer • Immunology

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Introduction

Tumor-infiltrating lymphocytes (TILs) commonly represent the heterogeneous population of αβ T cells, comprising of both CD4+ and CD8+ subsets, resident within the tumor microenvironment (TME). However, the composition of cellular infiltrate in human cancers is complex and consists of various hematopoietic cells, stromal cells, and tumor vasculature. Usually, the immune infiltrate of most cancers is dominated by macrophages (M0/M1/M2 subtypes) and conventional αβ T cells, including CD4+ follicular helper cells, CD4+ memory cells, CD4+ regulatory cells, and CD8+ effector cells [1,2]. Smaller populations of hematopoietic cells within the tumor infiltrate consist of monocytes, dendritic cells, B cells, natural killer (NK) cells (activated and resting), mast cells, and γδ T cells subsets. Neutrophils and eosinophils subpopulations are variable [3].

TIL therapy was extensively studied in patients with metastatic melanoma and has shown remarkable results [4,5]. This immunotherapy has advanced into many other cancer diagnoses and its potential for use in solid tumor treatment is currently extensively studied. The feasibility of *ex vivo* TIL expansion has been proved in many solid malignancies, including non-small cell lung cancer (NSCLC) [6] and breast cancer [7]. However, only a few clinical trials in patients without metastatic melanoma have been published, and thus clinical efficacy and safety of adoptive cell therapy (ACT) in solid cancers is not well known yet. Generally, response rates have been lower than patients with metastatic melanoma, but durable complete responses have also been found [8-11]. Table 1 summarizes currently ongoing TIL clinical trials in breast, lung, and colorectal cancer.

Table 1. Ongoing clinical studies utilizing Tumor-Infiltrating Lymphocytes in breast, lung and colorectal cancer. Source: https://clinicaltrials.gov/ct2/home (accessed on 17th April 2023).

Clinical Trial	Malignity	Biological Treatment	Phase	Estimated Enrollment
NCT04426669	Gastrointestinal, Colorectal, Pancreatic, Gall Bladder, Colon, Esophageal & Stomach Cancer	Aldesleukin, Tumor-Infiltrating Lymphocytes	1-2	20
NCT03645928	Metastatic Melanoma, Squamous Cell Carcinoma of the Head and Neck, Non-small Cell Lung Cancer	Lifileucel, LN-145, LN-145-S1, Ipilimumab, Nivolumab	2	178
NCT04614103	Metastatic Non-Small Cell Lung Cancer	LN-145	2	95
NCT03935893	Gastric, Colorectal & Pancreatic Cancer, Sarcoma, Mesothelioma, Neuroendocrine Tumors, Squamous Cell Cancer, Merkel Cell Carcinoma, Mismatch Repair Deficiency, Microsatellite Instability	Tumor-Infiltrating Lymphocytes	2	10
NCT04643574	Solid Tumors (Adults only)	Neo Tumor-Infiltrating Lymphocytes	1	42
NCT05141474	Malignant Epithelial Tumors, Malignant Solid Tumor	Nextgen Tumor-Infiltrating Lymphocytes	Early 1	10
NCT03449108	Bone Sarcoma, Dedifferentiated Chondrosarcoma, Giant Cell Tumor of Bone, Malignant Solid Neoplasm, Ovarian Carcinosarcoma, Platinum Resistant Ovarian Carcinoma, Poorly Differentiated Thyroid Gland Carcinoma, Recurrent Osteosarcoma, Recurrent Ovarian Carcinoma, Refractory Osteosarcoma, Soft Tissue Sarcoma Thyroid Gland Anaplastic Carcinoma, Thyroid Gland Squamous Cell Carcinoma, Undifferentiated High Grade Pleomorphic Sarcoma of Bone, Triple Negative Breast Cancer	Aldesleukin, LN-145, LN-145-S1, Ipilimumab, Nivolumab	2	95
NCT01174121	Metastatic Colorectal Cancer, Metastatic Pancreatic Cancer, Metastatic Ovarian Cancer, Metastatic Breast Carcinoma, Metastatic Endocrine Tumors	Aldesleukin, Young Tumor-Infiltrating Lymphocytes	2	332
NCT03610490	Malignant Solid Neoplasm, Metastatic Colorectal Adenocarcinoma, Metastatic Ovarian Carcinoma, Metastatic Pancreatic Ductal Adenocarcinoma, Platinum-Resistant Ovarian Carcinoma, Recurrent High Grade Ovarian Serous Adenocarcinoma, Recurrent Ovarian Carcinosarcoma, Refractory Colorectal Carcinoma, Stage IV Colorectal Cancer	Interleukin-2, Autologous Tumor Infiltrating Lymphocytes MDA-TIL	2	27

Despite the promising potential of TIL therapy in solid tumors, a high percentage of patients do not respond to treatment as they develop innate or acquired resistance mechanisms, which may arise from tumor intrinsic or extrinsic factors, leading to impaired immune-mediated tumor killing and tumor progression [12]. These mechanisms are complex and still not fully understood as they likely overlap. Innate resistance to immunotherapy is mostly caused by the absence/low number of tumor-specific T cells in the infusion product or the absence of well-presented immunogenic tumor neoantigens, resulting in the inability of tumor recognition by T cells [13,14]. Due to the heterogeneity in mutational load, neoantigens and lymphocytic infiltration with various subpopulations of CD4+ and CD8+ T cells, regulatory T cells (Tregs), tumor-associated (M2) macrophages, immune checkpoint regulators expressed by myeloid-derived suppressor cells (MDSCs) and tumor cells, the production and reactivity of TIL products for solid tumors vary between patients [15]. Because of these factors, there is a need for optimization of TIL production and treatment which comprise different combinational treatments including checkpoint inhibitors and targeting patient-specific neoantigens using personalized medicine approaches.

TILs and adoptive cell therapy

TIL-based ACT typically consists of three stages – surgical resection of tumor material (at least 2-3 cm) from the patient, cultivation and *ex vivo* rapid expansion of isolated TILs, and administration of the final product to the lymphodepletion patient. The entire process usually takes 6-8 weeks. After surgical resection, the tumor sample is either mechanically cut into multiple small fragments of a few millimeters or enzymatically digested into a single-cell suspension. Such material is cultivated *in vitro* (on average around 14 days, but the range is 7 to 21 days) in the presence of interleukin-2 (IL-2). During this initial outgrowth, tumor cells "disappear" and a culture of "young" TILs (yTILs, at least 50×10^6 cells) is prepared. This cell population is then used in rapid expansion protocol (REP) [16-18]. As TIL products are heterogeneous and differ in the percentage of CD8+ versus CD4+ T cells, antigen specificity, and tumor reactivity (only a fraction of the population is tumor-reactive), most initial studies used to prepare several independently grown TIL cultures per patient which were screened for *in vitro* antitumor reactivity before REP. Cells were cultured with autologous tumor material or HLA-matched tumor cells and selection was made based on interferon-gamma (IFN-γ) production. However, this process required a long production time, and many patients were not eligible for treatment. Therefore, this strategy of TIL selection was replaced with minimally cultured non-selected yTILs that are used in REP. Such cells appeared to have comparable clinical efficacy as "selected" TILs and allow treatment of most patients in a shorter time. yTIL thus became the current standard in the field [4,19]. In REP, cells are cultivated for 14 days in the presence of irradiated feeder cells from autologous or allogenic source (peripheral blood mononuclear cells – PBMCs) in a 100-200-fold excess to the TILs, anti-CD3 antibody (OKT3 clone) and IL-2. Irradiated feeder cells release growth factors into the culture for massive TIL expansion (usually more than 1000-fold). During the last phase of REP, a bioreactor is required to allow a culture of high cell densities [16-18]. The success rate of TIL outgrowth is currently very high and varies from 75 % [4] to 97 % [20]. The CD3 antibody targets the CD3 complex of the T cell receptor (TCR), stimulates TILs, and induces extensive proliferation. IL-2 stimulates effector CD8+ T cell differentiation and synthesis of effector molecules [21,22], leading to a highly reproducible generation of sufficient numbers of TILs. However, IL-2 also leads to the generation of terminally differentiated T cells that in combination with the long production time may result in the presence of mainly exhausted T cells in the final infusion product [16,23]. Therefore, it is questionable if IL-2 is the most optimal cytokine for TIL outgrowth phase. Currently, some other cytokines are being considered as potential candidates for *ex vivo* expansion and differentiation of TILs used for therapy, amongst them IL-12, which is able to directly augment the cytolytic potential of T cells and increase antigen presentation. In murine models, activation of naive T cells mediated by IL-12 generated highly proliferative cells with increased cytolytic function and resistance to exhaustion. Furthermore, the adoptive transfer of T cells primed with IL-2 + IL-12 has led to enhanced tumor regression in several studies [24-26]. Thus, a switch to other homeostatic cytokines such as IL-7, IL-12, IL-15, and IL-21 could possibly generate a less differentiated T cell product and lead to longer engraftment and better tumor control in the recipient. Future clinical trials are needed to compare the effect of different cytokine combinations on TIL outgrowth in order to choose the superior cytokine strategy. At the end

of the expansion protocol, TILs (minimum 1×10^{10} cells; up to 2×10^{11} cells) are harvested and infused into previously lymphodepletion patients [17,18].

Lymphodepletion (non-myeloablative chemotherapy with cyclophosphamide and fludarabine, and/or total body irradiation) appeared to increase antitumor efficacy of TILs and thus the effectiveness of therapy through several mechanisms, including depletion of immunosuppressive cells (CD4+CD25+ Tregs, MDSCs), augmentation of neoantigen presentation and elimination of cellular cytokine sinks, which could weaken proliferation and persistence of effector cells [27]. Moreover, such preconditioning can induce immunogenic death of tumor cells, resulting in the release of proinflammatory cytokines and tumor neoantigens that further enhances antitumor immune response [28]. However, no randomized trials have been undertaken. The infusion of TIL product to preconditioned patients is followed by administration of high-dose IL-2 (720000 IU/kg every 8 h for a maximum of 15 doses), which supports the proliferation and activity of infused TILs [4,29-31].

In some patients, adoptively transferred TILs may induce non-antigen-specific toxicity caused by their activation – a cytokine release syndrome, rarely with the development of life-threatening complications [32]. Despite this, the most severe toxicities for TIL-based ACT are attributed to lymphodepletion and IL-2 administration. Lymphodepletive preconditioning is an essential part of TIL-based ACT and together with IL-2 therapy significantly increases ATC clinical efficacy. However, both induce transient but severe toxicities which require hospitalization and prophylactic treatment in the majority of patients and thus are one of the significant limitations that restrict widespread clinical usage of TIL-based ACT. This therapy is therefore carried out at specialized centers with experienced staff [20]. Besides lymphopenia, adverse effects of lymphodepletion include grade 3-4 severe anemia, neutrocytopenia, thrombocytopenia with a high infection susceptibility, and risk of febrile neutropenia. Restoration of peripheral hematological values usually takes around 1-2 weeks and may be supported by the administration of granulocyte colony-stimulating factor after TIL infusion. However, lymphocyte counts may remain affected also for many months after lymphodepletion [31,33,34]. Therefore, there is a need to optimize preconditioning regimens in order to minimize adverse events and thus increase the clinical utility of TIL-based ACT.

Similarly, systemic high-dose IL-2 administration is associated with severe toxicities which generally manifest in multiple organs (including vascular leak syndromes, pulmonary edema, renal failure, and neurological symptoms) and may require management at the intensive care unit [5]. Moreover, high-dose IL-2 can prolong the hematologic toxicities from conditioning therapy [10,31]. In fact, a lot of patients do not finish the entire post-transfer IL-2 procedure. To overcome this limitation, clinical trials have explored the effect of attenuated or low-dose IL-2. Indeed, the use of reduced IL-2 doses was still able to achieve objective clinical results with significantly decreased toxicity and the need for intensive care, indicating that high-dose IL-2 is not a prerequisite for clinical efficacy [20,31,33]. However, clinical trials involving low-dose IL-2 currently have a limited number of patients and do not directly compare the efficacy of high-dose versus low-dose IL-2 as adjuvants to TIL-based ACT.

TILs, checkpoint blockade therapy and tumor neoantigens

One of the major questions related to autologous TIL therapy was the antigen specificity of the cell product and the recognition of shared versus unique tumor antigens. cDNA expression cloning methods and T cell lines (derived from TILs or PBMCs) screening helped to assess recognition of autologous tumor and reveal the fact that cancer patients could develop spontaneous immunity to tumor-encoded missense mutations which lead to neoantigen production, typically expressed only in tumor cells. Many single-patient case reports utilizing this strategy were able to describe a single neoantigen per patient in melanoma, several lung carcinomas, renal cell carcinomas, and a head and neck cancer. With the onset of next-generation sequencing and robust bioinformatics pipelines, identification of tumor-encoded genomic alterations (missense mutations and indels) and characterization of all putative neoantigens encoded by high mutational burden malignancies can be conducted in a clinically relevant timeframe [35]. Checkpoint blockade cancer immunotherapy has reached some notable advances in the past few years. Tumor regressions were acknowledged in cancer patients who underwent CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), PD-1 (programmed cell death receptor 1) or PD-L1 (programmed cell death – ligand 1) blockade clinical trials [36]. Presently, the most widely accepted

hypothesis is that tumors with more mutations are likely to generate more neoepitopes, which can be recognized by TILs. Checkpoint-blocking antibodies reactivate these T cells *in vivo* and thus lead to tumor regressions. As a result, cancers with high tumor mutation burden (TMB), such as melanoma and lung cancer, are more susceptible to checkpoint blockade therapies and more likely to benefit from TIL-based ACT [37,38]. However, most solid tumors have a low TMB, resulting in a relatively small neoantigen load and thus immune evasion which represents a significant obstacle in TIL-based ACT. Such malignancies exhibit decreased response to TIL-based ACT (objective response rate 28 %) [39]. One notable exception to this hypothesis is renal cell carcinoma, which is susceptible to checkpoint blockade therapies but with a low mutation load. Recent studies have successfully explained the correlation between the number of mutations/neoantigens and clinical outcomes. In a PD-1 blockade clinical trial comparing colorectal cancer patients with or without mismatch-repair deficiency, prolonged progression-free survival was associated with more somatic mutations, found in tumors with mismatch-repair deficiency. Higher neoantigen burden also correlated with clinical benefit and progression-free survival in PD-1/CTLA-4 blockade immunotherapy in patients with non-small cell lung cancer. These studies suggested that a higher number of neoantigens positively associates with clinical benefits after immune checkpoint blockade therapies [38]. Adoptive transfer of mixed TILs could induce tumor regressions in melanoma patients. Another study reported that objective tumor regressions in three melanoma patients were associated with the adoptive transferring of TILs recognizing neoantigens. Furthermore, two TIL products were associated with complete tumor regressions observed in two melanoma patients, and each TIL could recognize one unique neoantigen [40]. Also, metastatic cholangiocarcinoma was treated with mutated ERBB2IP-reactive CD4+ T cells, with partial response ongoing for more than 2 years since treatment [41]. All these data show that neoantigen-reactive T cells likely have a dominant role in inducing tumor regressions in patients and could induce long-term tumor regressions in a variety of cancer patients.

Tumor reactive T cell identification markers

Most currently used TIL-based ACT utilizes bulk, randomly isolated TILs from the tumor tissue for subsequent *ex vivo* expansion and infusion to the patients. However, only a minor fraction of the administered T cells recognizes tumor antigens (derived either from mutation-derived neoantigens or shared tumor-associated antigens (TAAs), and subsequently eliminate the tumor. Neoantigens are expressed only in tumor cells. On the other hand, shared TAAs, like glycoprotein 100, HER2 (human epidermal growth factor receptor 2), MART-1 (melanoma-associated antigen recognized by T cells), or NY-ESO-1 (cancer-testis antigen 1) exist also in normal tissues, but typically they are significantly over-expressed in tumor cells [42]. TILs enriched to be specific to neoantigens are superior to unselected TILs at reaching complete and stable tumor regression. It has been shown in recent clinical trials that colorectal cancer and metastatic breast cancer patients obtained remission after the administration of neoantigen reactive T cells [9,43]. Moreover, the adoptive transfer of TILs enriched in neoantigen-targeted T cells seems to be a promising therapeutic strategy also for the treatment of tumors with a low TMB [44].

The frequency and presence of respective TIL subpopulations are the key determinants of their therapeutic efficacy. Tumor-reactive T cells (TRTs) are limited to a relatively small number of cells, e.g., only about 10 % of intra-tumoral CD8+ T cells can recognize autologous tumor-specific antigens in colorectal and ovarian cancers. Moreover, even no tumor-reactive T cell receptors have been found in some patients with the presence of TILs [14,45]. Thus, evaluating the proportion of TILs and their ability to recognize tumor antigens is critical for predicting the outcome of ACT. TILs can naturally recognize a wide spectrum of epitopes different from tumor antigens (e.g. virus antigens) forming so-called bystander T cells, also infiltrating the cancer tissue as effector cells. However, neoantigen-specific TILs obviously exhibit stronger anti-tumor activity and tumor specific expansion as compared to bystander TILs. To achieve optimal ACT treatments, the development of ACT protocols that specifically focus on neoantigen-specific TRTs, followed by the definition of biomarkers that can be used as surrogates of neoantigen specificity and which could be targeted in cell sorting procedures is principal. Therefore, efforts have been made to identify a specific biomarker(s) for screening of TILs in different tumor types, followed by *ex vivo* expansion to clinically relevant numbers, which should subsequently provide the best therapeutic effect. Despite the fact, there is no unique shared biomarker for TRTs in different tumor types at

present, there are several most common biomarkers originally identified in different tumor types being studied and targeted as objects of interest, including particularly the PD-1, the chronic activation marker CD39, the tissue-resident marker CD103, the costimulatory receptor CD137 and their combinations [46].

PD-1 (CD279)

PD-1 is a protein on the surface of T and B cells, highly expressed on all activated T cells, with an important role in the regulation of T cell anti-tumor immune response. It works as an immune checkpoint through the promotion of antigen-specific T cell apoptosis and reduction of Treg apoptosis, after binding its ligand PD-L1 or PD-L2, often expressed in several tumor cells [47]. Previous studies found a significantly higher proportion of PD-1+CD8+ T cells than PD-1-CD8+ T cells in melanoma-infiltrating TILs [48,49]. Sukegawa *et al.* [50] analyzed the TCR-β repertoires of PD-1- and PD-1+CD8+ TILs derived from colorectal and breast cancer. They found that PD-1+CD8+ TILs from both contained a significant number of T cells expressing the same TCR clonotype, indicating that PD-1+CD8+ TILs were clonally expanded in tumor tissues. Hence, it can be concluded that the intrinsic properties of the PD-1+ TILs determine their ability to specifically recognize tumor antigens. Furthermore, similarities in tumor antigen specificity and TCR repertoires of PD-1+CD8+ T cells both in circulating peripheral blood and tumor tissue have been described recently, suggesting that PD-1 expression could identify diverse patient-specific antitumor T cell responses also in peripheral blood, thus providing a new non-invasive approach for developing personalized therapies using neoantigen-reactive T lymphocytes [51,52]. Additionally, it has been discovered that PD-1+CD8+ T cells preserve a less differentiated "stem-like" progenitor-exhausted subpopulation of TILs with the ability of self-renewal, expansion, mediate long-term tumor control and superior anti-tumor activity [53]. To conclude, extensive studies suggested PD-1 as a stable biomarker of TRTs, suitable for identifying and sorting of CD8+ TRTs.

CD39 (Ectonucleoside triphosphate diphosphohydrolase-1 – ENTPD1)

CD39 is a cell surface enzyme participating in the degradation of adenosine triphosphate into adenosine by the CD39/CD73 pathway which leads to an anti-inflammatory environment. CD39 has been described as a marker of T cell reactivity to tumors and T cell exhaustion and is used to identify TRTs in a variety of malignancies. Its expression has been used as a marker for the identification, isolation, and expansion of TRTs in various clinical studies. Bystander CD8+ TILs have diverse phenotypes that overlap with tumor-specific cells but lack CD39 expression. Precisely, in colorectal and lung tumors, the absence of CD39 in CD8+ TILs defines populations that lack hallmarks of chronic antigen stimulation at the tumor site, supporting their classification as bystander T cells, and it was CD39+CD8+ TILs that could recognize tumor antigens specifically [54]. Presently, further studies validated the specific anti-tumor function of CD39+CD8+ TRTs in NSCLC tumor tissues [55] and similarly, defines the prognostic benefit of CD39 high tissue-resident memory CD8+ T cells in luminal-like breast cancer [56]. Even though little is known about the cell surface markers that specifically determine TRTs among CD4+ T cells, a recent study by Li *et al.* proposed that CD39 marker can guide the enrichment also for tumor-reactive CD4+ T cells and could be used to distinguish them from other CD4+ TILs such as bystander CD4+ T cells or regulatory T cells [57].

CD103 (Integrin αE – ITGAE)

CD103 is an integrin molecule involved in the migration and residency of T cells in tissues. It is widely expressed in intraepithelial T cells, TILs, and some Tregs. In combination with CD69 and CD49a markers defines a specific population of tissue-resident memory T cells (TRMs), classified as a cluster of memory T cells located in tumor tissues and not contributing to T cell recirculation [58]. Previously, CD103 expression on CD8+ TILs was emphasized as a prognostic marker for breast and ovarian cancer patient survival and positively associated with therapy outcomes in lung and bladder cancer patients [59]. Similarly, CD103+ TILs have been identified as a valuable prognostic marker associated with a good prognosis for overall and recurrence-free survival in patients with bladder urothelial cell carcinoma in the retrospective study [60]. Nowadays, studies on CD103 marker to identify TRTs are becoming more and more eminent. Several studies have identified CD103+CD8+ TRMs to be the population with the most antitumor activity in patients treated with anti-PD-1

immunotherapy by different tumor types. Single-cell analysis of breast cancer TILs also demonstrated CD103+CD8+ TRMs associated with improved prognosis in patients with triple-negative breast cancer (TNBC) and provided better prediction than CD8 expression alone. Moreover, single-cell analysis of mutation-associated neoantigens in resectable and metastatic NSCLC treated with anti-PD-1 immunotherapy revealed that roughly 90 % of mutation-associated neoantigen-specific CD8 T cells have the hallmark of transcriptional programs of TRMs, which shows the presence of TRTs within the TRM population, thus CD103 marker may well act as a marker for identification of TRTs. Overall, it seems that CD103+ cytotoxic T lymphocytes as a subpopulation of TILs are important in the antitumor immune response and favorable prognosis [61].

CD137 (tumor necrosis factor receptor superfamily member 9 – TNFRSF9)

CD137, a member of the tumor necrosis factor receptor superfamily, has been identified as the co-stimulatory marker, which is induced upon a specific interaction of T cells with their counterpart and is described as a surface marker for the identification of activated T cells. Therefore, CD137 was described as an important biomarker for the selection of TRTs [62]. Parkhurst *et al.* [63] enriched neoantigen-reactive T cells from TILs by FACS sorting for CD137+CD8+ T cells and expanded them *in vitro*. Subsequently, TCRs isolated from those cells were introduced to autologous PBMCs to induce tumor rejection *in vitro*, suggesting the potential of PBMCs to become true TRTs by introducing recognized TCRs. Furthermore, this study proves CD137 as a potential marker to isolate shared TAA as well as neoantigen-reactive TILs. Similarly, another study performed by Seliktar-Ofir *et al.* [64], demonstrated that CD137-selected TILs significantly increased antitumor reactivity and were enriched for T cells recognizing neoantigens as well as shared TAAs. They developed and validated a method for the selection of CD137-expressing T cells based on magnetic bead separation. Moreover, CD137 selection was performed with clinical grade compliant reagents, and CD137+ TILs were expanded in a large-scale manner required for the patient setting in a good manufacturing practice facility. Recently, the analysis of expression of all four most prominent biomarkers PD-1, CD39, CD103, and CD137 on TILs isolated from human ovarian tumor samples using single-cell mass cytometry, observed that PD-1+, CD103+, and CD39+ TILs all contain a CD137+ cell subset, while CD137+ TILs highly co-express all aforementioned markers. CD137+ TILs exhibit the highest expression of major histocompatibility complex-dependent INF-γ and the other effector cytokines. These findings may demonstrate that the antitumor abilities of PD-1+, CD103+, and CD39+ TILs are mainly derived from a subset of CD137-expressing TILs, implicating CD137 as a more selective biomarker for naturally occurring tumor-specific TILs [65].

All described studies indicated the diversity of TILs populations, which should be enriched as much as possible to achieve optimal therapeutic results. Despite the aforementioned study of Eiva *et al.* [65] proposing the CD137 as the most selective marker for TRTs, there are several other studies assuming, that only one single biomarker may not be adequately effective to sort specific TRTs, but the combination of two or more markers is sufficient to obtain a subpopulation of TILs that recognize tumor antigens more accurately, may represent an effective strategy for the beneficial outcome of ACT, as a personalized TRTs-based therapy. Therefore, future efforts will be needed to find the optimal strategies.

TILs in selected solid tumors

Colorectal cancer

Different subgroups of TILs could predict the prognosis of colorectal carcinoma (CRC) [1]. Further studies proved that TILs are closely related to the prognosis of CRC and tumor regression degree after neoadjuvant radiotherapy for advanced rectal cancer [2]. Cytotoxic CD8+ T cells have a crucial role in anti-tumor immunity. Several studies have demonstrated that CD8+ T cells are linked with a benign prognosis of CRC [1,2,66]. Ling *et al.* [67] found that the infiltration of CD8+ T cells in CRC tumor epithelium provided strong prognostic information. On the other hand, immune suppressive cells, such as MDSCs or M2 macrophages show their highest infiltrating expression level in CRC, but unveil poor prognosis [68,69]. Immune cells infiltrating or surrounding colorectal tumors, particularly TILs, constitute an important prognostic factor [70]. Also, TILs have been shown to have prognostic and predictive value in lung cancer [71,72], and CRC [73,74]. Different populations of TILs in primary tumors might

predict the various outcomes in different patients. Several studies have indicated that some checkpoint genes are associated with TIL load and overall survival of patients with CRC and pan-cancer [75-78]. TIL infiltration features have also been analyzed by using single-cell RNA sequencing in the Cancer Genome Atlas cohorts in a few studies. Quin *et al.* [79] showed that TIL distribution profile continued to be consistent with crosswise mutations of specific genes and somatic copy number variants. CD8+ T cells infiltration was always higher than CD4+ T cells infiltration. Furthermore, a negative correlation between patient´s age, CD8+ TILs and better response to immune checkpoint therapy in patients with CRC older than 70 years, implying that age might be an important factor in patient prognosis and immunotherapy. A higher frequency of TILs, notably CD8+ TILs, is correlated with a better prognosis in CRC [80,81]. Nevertheless, several reports described that Th2 cells and Tregs depict poor prognosis [82,83]. Therefore, determining which sub-group is the major component in TILs has crucial significance related to the prognosis of CRC. TILs subsets are relatively constant, even amongst different tumors in different patients. On the other hand, the relative amount of each TILs subgroup showed substantial heterogeneity, possibly implying that the balance between various immune cell states is a serious factor in regulating tumor-specific immune response. Thus, CD4+ and CD8+ TILs have undoubtedly application value in the prognostic prediction of CRC and can also be used as possible therapeutic indicators for new immunotherapy approaches and target population screening for suitable ACT.

Breast cancer

The ductal cellular layer in the normal breast encompasses a substantial immune cell population, namely CD8+ and CD4+ T cells, B cells, macrophages, NK cells, dendritic cells and also other immune cell subtypes [84,85]. Carcinogenesis from normal breast tissue to breast cancer is always accompanied by quantitative and qualitative changes in the nature and location of the immune cell population, including immune cell content increase in the parenchymal as well as stromal compartment. The immune cell infiltration in breast cancer consists of multiple cellular subtypes, including CD4+ and CD8+ cells [85,86]. The residence of multiple immune cell subtypes in both tumor compartments places these cells near to tumor cells and other cells in the microenvironment, thus allowing these cells to affect tumor growth in multiple ways, directly *via* CD4+ and CD8+ cell-mediated cytotoxicity, or indirectly *via* immunostimulatory or immunosuppressive effects of secreted cytokines, growth factors or other agents, which distribution and characteristics might also vary depending on cancer subtype, mutational load, formation of tertiary lymphoid structures and estrogen responsiveness. Multiple studies have shown that in HER2+ and TNBC patients, cumulative TILs increase in both, the tumor itself and its surrounding can appropriately predict improved survival and response to chemotherapy [87-89]. Bearing in mind that the size and composition of tumor immune infiltrate can affect prognosis and response to therapy for invasive breast cancer and ductal carcinoma *in situ*, the immune environment of the tumor can be used as a guide to determine what is the most appropriate therapy as well as a biomarker of an individuals' disease prognosis. In 2015, the International TILs Working Group started standardizing the evaluation of breast cancer TILs for use in clinical practice. Administering TILs to patients has resulted in durable complete regression of solid tumors as well as allowing the development of cellular therapy with gene-modified TCRs for the treatment of breast and other cancers [9].

Furthermore, the study of mastectomy samples using immunohistochemistry (IHC) and flow cytometry, observed that breast cancer tissues contained infiltrates dominated by CD4+ and CD8+ lymphocytes, with minor populations of NK cells and B lymphocytes, while in the normal breast tissue myeloid-lineage cells including macrophages, mast cells, and neutrophils were more apparent [86]. A comparable immune profile was observed in breast tissues from prophylactic mastectomies. Activated T lymphocytes also dominated in tumor tissue, with both CD4+ and CD8+ T cells displaying an increased expression of activation markers CD69 and HLA-DR (major histocompatibility complex II cell surface receptor). These findings indicated a shift within tumors toward Th2-type response in breast cancer characterized by the increased presence of B cells and CD4+ T cells, in comparison with normal breast tissue [86]. A transcriptional study compared the immune cell distribution in breast cancer with that of normal adjacent breast tissue and found a significant increase in the variety of cell conditions. This implies that the increased heterogeneity of cell conditions and marked phenotypic expansions found in the tumor were as such presumably due to more distinct local tumor microenvironments [85]. Moreover, unique subsets of enriched clonotypes in

tumor and bordering normal breast tissue, as well as identifying sequences shared among patients unlikely to be involved in specific tumor recognition have been discovered [90]. The complexity of the composition of immune cells in breast cancer demonstrates a crosstalk between components of the innate immune response as it controls the tumor microenvironment and adaptive immune response in that tumor [91]. Immunostimulatory or immunosuppressive environments affect the fate of T cells capable of guidance to the tumor [92]. In breast cancer, the distribution of immune cells might differ between the tumor parenchyma and stroma. In a study, immune cells of breast cancer mastectomy samples were outlined by IHC staining in the tumors and surrounding stroma to identify CD3 (T cells), granzyme B (a cytotoxic subset of CD3+ cells), CD20 (B cells) and CD68 (macrophages) cells. The samples covered the spectrum of breast disease: normal, benign proliferative disease, ductal carcinoma *in situ*, and invasive ductal carcinoma. A progressive increase in all cell types in both the parenchyma and stroma moving from normal mammary tissue to ductal carcinoma was seen, with the most surprising influx of CD3+ cells in the stroma. Generally, the incidence of stromal TILs varies from 15 % to 25 %, and intra-tumoral TILs from 5 % to 10 % [93]. A review of 13,914 patients, found a median of 20 % of patients with TNBC demonstrated lymphocyte-predominant breast cancer (50-60 % lymphocytic infiltrate) at the time of diagnosis compared to 16 % of HER2+ tumors and 6 % of hormone receptor-positive (HR+) cancers. A median of 60 % of TNBC samples had infiltrating CD8+ T cells in contrast to only 43 % of HR+ tumors. These findings point out that HR+ disease might be the least immunogenic of the common breast cancer subtypes [94]. Another study showed high tumor infiltration with FOXP3P+ Tregs, but not CD8+ T cells, correlating significantly with HER2 overexpression. Elevated infiltration of Tregs and cytotoxic lymphocytes was considerably more common in tumors with unfavorable histologic characteristics, such as negative estrogen and progesterone receptor status or high histologic grade [95].

Lung cancer

Lung cancer is ranked first among cancer-related death worldwide [96]. NSCLC, the most commonly diagnosed lung cancer type is a very heterogeneous disease covering multiple histologic subtypes. NSCLC is usually immunosuppressive and able of impeding the antitumor immune responses utilizing various mechanisms such as antigen processing and presentation deficiencies, immunomodulatory cytokines release and recruitment of immunosuppressive cells like MDSCs and Tregs [97,98]. An immunologically rich cluster of NSCLC patients whose tumors have ample CD8+ T cells expressing elevated levels of T cell immunoglobulin and mucin-domain containing protein 3 along with PD-1, and an immunologically feeble cluster with tumors characterized by the decreased relative abundance of CD8+ T cells and reduced expression of inhibitory markers were successfully identified. Despite the broad profile of immune cell content and function in NSCLC, the correlation between TIL phenotypes and their prognostic relevance is not conclusively proved. T cell repertoire in NSCLC shows notable overlap in TCR sequences with a high prevalence of sequences predicted to recognize viral epitopes in comparison to normal adjacent uninvolved lung tissue, implying that a major proportion of T cells in the lung might be irrelevant to tumor control. Thus, it is important to identify qualitative in lieu of quantitative differences in T cell infiltrates that could impact patient outcomes. Moreover, dual enrichment for tumor-infiltrating B cells and T cells might contribute to a favorable prognosis for NSCLC patients. A better prognosis associated with high B cell infiltration must have a parallel high level of T cells, as neither high level of B or T cells alone was significantly associated with improved regression-free survival of patients [99].

Conclusions

TILs represent a rich source of effector T cells that process the recognition and elimination of solid tumors as demonstrated by ACT approaches for high mutational burden malignancies. Our understanding of TILs is dramatically expanding. These cells have certain prognostic value for many cancer outcomes and have inter alia proven potential to treat disseminated breast cancer, CRC, or lung cancer.

This knowledge allows us to classify and modify T cells in many ways as to program TCRs to recognize specific tumor-associated neoantigens and to insert these TCRs into lymphocytes or selection of tumor neoantigen-specific T cells, for the development of vaccines that recognize tumor-specific antigens in tumors or metastases. Advances in gene editing and mass spectroscopy imaging have the potential to improve TIL products and even further enhance the efficacy of

this therapy, as it allows to insert promising artificial receptors or molecules into TILs, or precisely knock-out the ones which could reduce the effect of TILs [100]. Available data suggest that not frequencies of TILs, but their qualities such as T cell clonality, T cell subset distribution, expression of immune checkpoints, and immune microenvironments, as well as types and frequencies of suppressor cells are crucial factors for TIL treatment success and favorable prognosis of patients, thus combinatorial approaches possibly represent the key to enhance the efficacy of future TIL based medicinal products.

Conflict of Interest

There is no conflict of interest.

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