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Novel strategies to improve efficacy of treatment with tumor-infiltrating lymphocytes (TILs) for patients with solid cancers

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Purpose of review
Treatment with tumor-infiltrating lymphocytes (TILs) has shown remarkable clinical responses in patients with advanced solid tumors. Although the TIL production process is very robust, the original protocol stems from the early nineties and lacks effective selection for tumor-reactivity and functional activity. In this review we highlight the limitations of the current production process and give an overview of improvements that can be made to increase TIL efficacy.

Recent findings
With the recent advances in single cell sequencing technologies, our understanding of the composition and phenotype of TILs in the tumor micro environment has majorly increased, which forms the basis for the development of new strategies to improve the TIL production process. Strategies involve selection for neoantigen-reactive TILs by cell sorting or selective expansion strategies. Furthermore, gene editing strategies like Clustered regularly interspaced short palindromic repeats-Cas (CRISPR-Cas9) can be used to increase TIL functionality.

Summary
Although combining all the possible improvements into a next generation TIL product might be challenging, it is highly likely that those techniques will increase the clinical value of TIL therapy in the coming years.

Keywords
adoptive cell therapy, next-generation strategies, tumor-infiltrating lymphocytes

INTRODUCTION

Since the first reports describing treatment with *in vitro* expanded tumor-infiltrating lymphocytes (TILs) in 1986, many patients have been treated with this therapy in clinical trial settings [1–3]. The majority of these trials were phase I/II trials showing the potential of TIL therapy for patients with advanced stages of cancer, mostly melanoma. However, in the current era in which immunotherapy with immune checkpoint inhibitors (ICI) dominates treatment of especially solid cancers, the role of adoptive T-cell therapies still needs to be established. Recently, our group published the results of a randomized controlled phase III trial comparing TIL therapy to ipilimumab in patients with mostly anti-programmed death-1 resistant metastatic melanoma. This study showed a statistical significant and clinically relevant progression-free survival benefit of TIL therapy over ipilimumab, and an overall response rate of 49% with 20% complete remission rate, thereby for the first time establishing a position of a cell therapy in the current melanoma treatment landscape [4▪].

An important strength of TIL therapy, that is likely responsible for its treatment success, is the polyclonal character of the TIL products. TIL therapy is a highly personalized treatment with unique properties for every individual patient [5,6▪]. However, precise definition of its content remains challenging [3]. With the recent advances in single cell sequencing technologies, our understanding of tumor resident TILs has majorly increased. In this

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KEY POINTS

- The original TIL production protocol lacks selective outgrowth of tumor-reactive cells and the end products contain T cells with different functional activities.
- TIL reactivity can be improved by increasing the number of neoantigen-specific T cells in the final TIL products, for example by selective expansion or selection strategies.
- TILs can be modified *in vitro* to become less susceptible to immunosuppressive mechanisms, for example, by inhibition of suppressor molecules like tumor growth factor-β or interference with downstream T-cell receptor signaling.

review we provide first a summary of the limitations of current TIL therapy. Subsequently, we will make suggestions on how to improve the TIL production process, eventually leading to better TIL products (summarized in Fig. 1).

CURRENT STATE OF TUMOR-INFILTRATING LYMPHOCYTES THERAPY AND ITS LIMITATIONS

The process of TIL manufacturing starts with isolating TILs from freshly obtained tumor tissue by enzymatic digestion and/or fragmentation and subsequent culturing as single cell suspensions or as small tumor fragments in culture medium with high concentrations of interleukin-2 (HD-IL-2). Following an initial outgrowth of TILs, that is accompanied by clearance of malignant cells, the TILs are expanded to very high cell numbers in a rapid expansion protocol using polyclonal T-cell stimulation with soluble anti-CD3 antibodies and irradiated allogeneic feeder cells, in the presence of HD-IL-2. This manufacturing process lasts about 4 to 6 weeks and yields between 5×10^9 to 2×10^{11} cells, mostly containing CD3+ T cells. Prior to intravenous infusion of the expanded TILs, patients undergo lymphodepleting chemotherapy, which aims to remove endogenous cytokine sinks consuming homeostatic cytokines IL-7 and IL-15 and to make physical space for the survival and expansion of the transferred TILs [3,5,7]. It is likely that the chemotherapy also impacts the myeloid compartment, but to date little is known about these consequences. Finally, following infusion of TILs, patients receive intravenous boluses of IL-2 to further support outgrowth of the infused cells [3,5].

Although this manufacturing protocol is robust, recent studies have shown that final TIL infusion products remain rather heterogeneous in terms of antigen specificities and T-cell differentiation stages [5,6▪]. Three important observations have been made: (1) Several groups, including ours, have shown that only a fraction of tumor resident TILs in human cancers can recognize autologous tumor cells and that a substantial part of TILs recognize antigens unrelated to cancer, so called 'bystander' TILs [8,9]. (2) TILs have hallmarks of tissue residency and prior antigenic stimulation, and exist in different states of T cell dysfunction, expressing elevated or high levels of inhibitory molecules, including PD-1, LAG-3 and TIGIT. Alternatively, they belong to the so-called progenitor dysfunctional T cells with self-renewing capacity [10,11]. (3) Spatial transcriptomics and proteomics have revealed that tumor-reactive TILs may cluster in tertiary lymphoid structures or are in close proximity to other immune and tumor cells. This knowledge will be important to design novel production protocols aiming for TIL products enriched for tumor-reactivity and optimal functional states, which we think are required to overcome some of the current limitations of TIL therapy.

TUMOR REACTIVITY

To improve TIL efficacy by increasing the number of tumor-reactive cells, we first need to better understand which antigens may trigger antitumor reactivity. Tumor associated antigens (TAA) can be subdivided into different subclasses. A frequently used classification divides TAA in shared and private antigens. The first group contains antigens derived from cell-lineage specific proteins, cancer/testis gene products and overexpressed self-antigens [12]. The latter category contains antigens that are classified as neoantigens. Neoantigens are mutant epitopes, resulting from tumor-specific mutations [12,13]. Unlike most tumor-associated shared antigens, neoantigens are foreign to the immune system, and therefore T cells specific for these antigens have not been subjected to negative thymic selection, thereby increasing the likelihood of finding high affinity TCRs against these antigens [12,14,15]. The potential to have high affinity T cells against these antigens and the absence of expression on normal cells makes neoantigens attractive targets for T-cell therapy. The contribution of T cells targeting shared antigens in the clinical response upon TIL therapy remains poorly understood and is perhaps underestimated. Findings that the frequency of neoantigen-specific T cells in TIL products is associated with improved response after TIL therapy may suggest that selection for neoantigen-specific T cells can be used to increase the tumor-reactive potential of TIL therapy [15,16▪]. One of the difficulties of such a

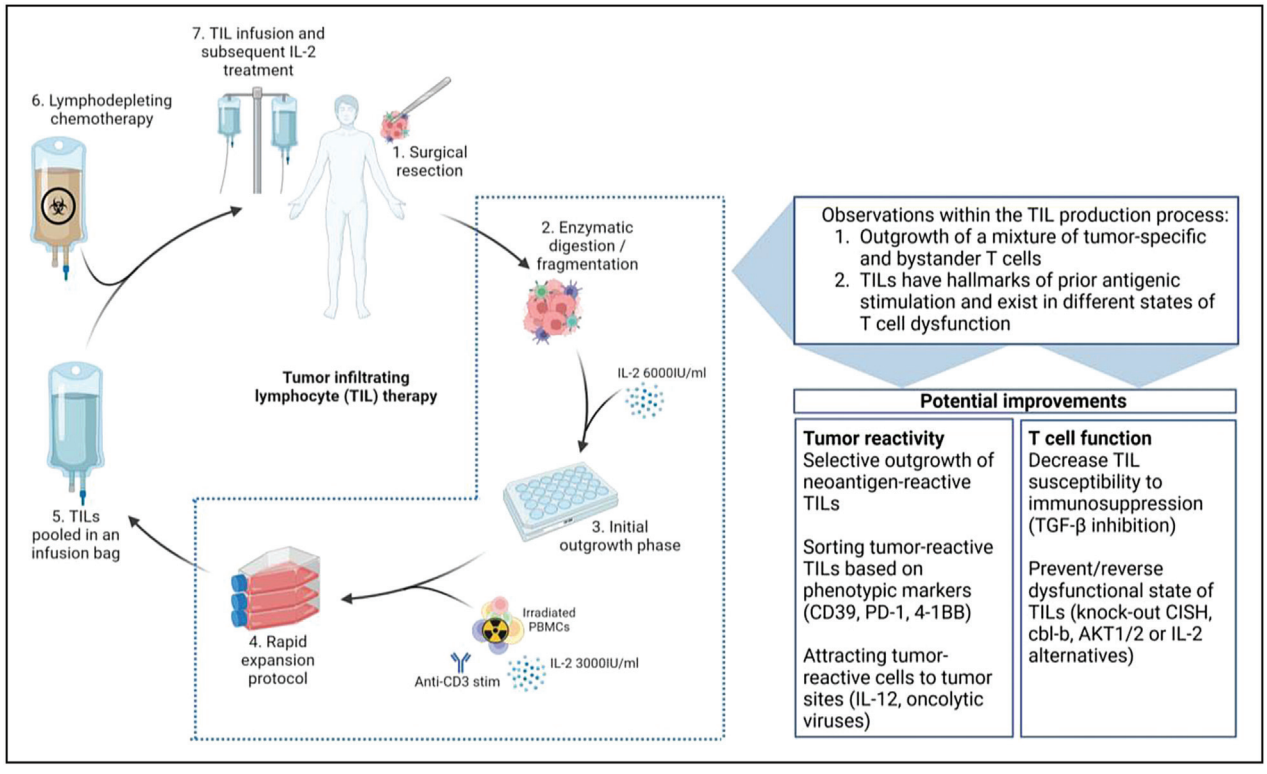

FIGURE 1. Schematic overview of the current TIL production process and potential improvements. TILs are isolated from single cell suspensions or tumor fragments generated from freshly resected tumor material and initially grown in the presence of high dose IL-2. Subsequently, the cultured TILs go through a rapid expansion phase using polyclonal T-cell stimulation with soluble anti-CD3 antibodies, irradiated allogeneic feeder cells and high dose IL-2. Prior to intravenous infusion of the expanded TILs, patients are treated with lymphodepleting chemotherapy. Finally, following infusion of the TILs, patients receive intravenous boluses of high dose IL-2. The original TIL production protocol lacks selective outgrowth of tumor-reactive cells and as a result TIL products contain T cells with different functional activities. Potential improvements are aimed at enriching the final TIL product for tumor-specificity and optimal functional states, which we think are required to overcome some of the current limitations of TIL therapy. CISH, cytokine-induced SH2 protein; IL-12, Interleukin-12; IL-2, Interleukin-2; PBMCs, peripheral blood mononuclear cells; TGF-β, tumor growth factor-β; TILs, tumor-infiltrating lymphocytes.

strategy is that the identification of suitable neoantigens based on algorithms predicting binding to HLA-molecules of the patient currently does not take factors like translation and processing into account. A recent study of Parkhurst *et al.* demonstrated that T-cell responses are elicited against less than 2% of the predicted neoantigens in tumors, which supports the need for validation of T-cell responses against these predicted neoantigens, but may also provide relevant insight in the breadth of the T-cell repertoire against these antigens [17].

More recently, new methods have been developed to identify tumor-reactive T cells directly in the tumor micro environment (TME) based on phenotypic markers. Multiple studies report that CD8+ TILs expressing PD-1, CD39 and CD103, are enriched for tumor-specific reactivity, especially targeting neoantigens [18–20,21▪]. Selection for tumor-resident T cells bearing these hallmarks may be sufficient to improve the tumor-reactive potential of a TIL product.

Selective outgrowth strategies for tumor-reactive tumor-infiltrating lymphocytes

A strategy to selectively expand neoantigen-specific TILs is currently being tested in two clinical studies with a product named ATL001 for patients with non-small cell lung cancer (NCT04032847) and melanoma (NCT03997474) [13]. In this manufacturing process (VELOS^{TM}), TILs are isolated from a patient's tumor and co-cultured with autologous dendritic cells loaded with peptides corresponding to the patient's identified neoantigens. By recognizing their cognate antigen on dendritic cells, neoantigen-reactive TILs are selectively expanded to high numbers and re-infused into the patient [13]. Alternatively, one could sort tumor-reactive TILs based on predefined phenotypic markers described earlier and expand these T cells for the manufacturing of TIL products. One such a marker is PD-1, which is highly expressed on tumor-reactive cells. Several groups have tried to improve antitumor reactivity

by enriching for CD8+PD1+ TILs utilizing flow cytometry or magnetic bead-based cell sorting [22,23]. A disadvantage of this approach is that PD-1+ TILs appeared to reside in a more dysfunctional state, thereby limiting subsequent *in vitro* outgrowth potential and possibly further *in vivo* expansion as well [22]. These studies focused on selective outgrowth of CD8+ cells. However, the importance of CD4+ T cells in the final TIL products to provide CD8 T-cell help, and to contribute to antitumor reactivity, should not be neglected [24].

Costimulatory molecule 4–1BB is another candidate for selective outgrowth as its expression on T cells is restricted to cells that are recently activated by TCR engagement and signalling [25,26]. Seliktar-Ofir *et al.* developed a successful method to generate TIL products by co-culturing TILs with autologous tumor and subsequent magnetic bead separation to select for 4–1BB-expressing T cells [26]. Finally, Duhen *et al.* sorted the CD39+CD103+ TIL fraction from tumor digest, expanded them *in vitro* and found that for six melanoma patients, the median percentage of tumor-reactive cells in the expanded TIL products was 51% [18]. Data showing which of these enrichment strategies improves current TIL therapy efficacy most efficiently and should be further developed for clinical application are currently lacking.

CREATING AN IMMUNE INFLAMMATORY ENVIRONMENT

A very different approach to improve the outcome of TIL therapy could be to reshape the immune suppressive TME [27]. By modifying TILs to secrete an immune stimulatory cytokine, such as IL-12, adoptive transfer of these T cells could induce stronger antitumor immune responses [28–31]. A clinical trial with T cells engineered to produce IL-12 elicited strong antitumor efficacy, but showed an unacceptable safety profile, because IL-12 production could not be controlled well after adoptive cell transfer and was not confined to the TME, leading to toxic levels in the circulation [32]. More recent studies tried to overcome this by engineering TILs to transiently produce IL-12 [33] or to tether the cytokine to the surface of tumor-reactive cells by making fusion proteins of cytokines and cell surface receptor-specific antibodies, resulting in a better safety profile and enhanced cytolytic activity of the TILs [34].

Another strategy to attract more T cells to the TME is by direct modification of the TME itself, for example, by combining intratumoral delivery of oncolytic viruses (OV) with TIL therapy [35,36]. In a first study, an herpes simplex virus-1 based OV encoding OX40L and IL-12 was administered shortly before TIL infusion, resulting in complete tumor regressions in patient-derived xenograft mouse models [35]. In a second study, an oncolytic poxvirus was given intratumorally, which led to attraction of tumor-specific T cells into the tumor tissue. These tumors appeared a great source for TIL harvest. TILs isolated from these tumors contained fewer Tregs, had a less exhausted phenotype and showed improved survival after adoptive transfer [36].

Lastly, more direct ways to recruit TILs to the TME, and thereby creating an immune inflammatory environment, involve the use of chemokines. Chemokines can improve migration of adoptively transferred TILs to reach the tumor sites, a strategy which is currently being tested in a clinical trial (NCT01740557) where CXC motif chemokine receptor 2 and Nerve Growth Factor Receptor are transduced into TILs for treatment of metastatic melanoma patients, after it showed increased *in vivo* homing capacity in a melanoma mouse model [37].

IMPROVING T-CELL FUNCTION

The combination of checkpoint inhibitors being readily available for cancer treatment and the finding that efficacy of anti-PD1 therapy is mediated by TILs present in the tumor micro-environment, lead to several clinical trials exploring the possibility of TIL therapy in combination with anti-PD1 therapy [38,39]. Although this strategy was found feasible and safe in those trials, a recent study found that the efficacy of TIL therapy in combination with ICI might mainly be the consequence of improved function of endogenous T cells, rather than an effect on the transfused T cells [40]. This has to be taken into consideration when deciding on the timing of the combination of checkpoint inhibitors with TIL therapy.

Other strategies to improve TIL function already focus on the start of TIL culture, by improving the dysfunctional state of TILs at the time of tumor harvest. Approaches involve counteracting immunosuppression on one hand or improving T-cell fitness on the other hand.

Overcoming immunosuppression

Besides studies combining TIL therapy with ICI, TILs can be modified to become less susceptible to immunosuppressive mechanisms. An interesting target is Tumor Growth Factor (TGF)-β, a pleiotropic molecule with key immunosuppressant features in the TME [41]. TGF-β normally binds to the TGF-β receptor present on TILs, preventing them from cytokine production and tumor cell killing [41,42]. Fix *et al.* utilized Clustered regularly interspaced short palindromic repeats-Cas (CRISPR-Cas9) to knock out (KO) the TGF-β receptor 2 (TGFBR2) in TILs from ovarian

cancer patients. Expanded TGFBR2 KO TILs demonstrated improved cytotoxicity compared to wild type TILs. Additionally, CRISPR/Cas-modification did not alter the *ex vivo* TIL expansion and TCR clonal diversity, nor did it have significant off-target effects [43].

Preventing T-cell exhaustion

Negative regulators responsible for TIL exhaustion are mostly located downstream of the TCR [44]. Interference with those mechanisms can help to maintain T-cell function of TILs upon recognition of their antigens. A recent study by Palmer *et al.* demonstrated GMP-compliant CRISPR/Cas-mediated KO of cytokine-induced SH2 protein (CISH), a member of the family of Suppressor of Cytokine Signaling molecules, and a marker associated with T-cell maturation, activation and exhaustion. The function of *in vitro* generated CISH KO TILs was tested with or without PD-1 blockade in a preclinical mouse tumor model (C57BL/6) and it was found that combination therapy resulted in impressive tumor regression [45]. Another suppressor of T-cell activation is cbl-b, a ubiquitin ligase, which is upregulated in dysfunctional TILs with high expression of PD-1 and TIM3. KO of cbl-b chimeric antigen receptor-expressing T cells prevented dysfunction, as shown by lower TIM3 expression and an increased tumor killing capability compared to cbl-b wild type T cells [46]. These T-cell modifications may lead to TIL products with a lower propensity to immune dysfunction and possibly a better cytolytic function *in vivo*.

Another key regulator of T-cell activation and proliferation following TCR triggering is the Phosphoinositide 3-kinase - protein kinase B pathway [47]. Activation of this pathway upon TCR signaling eventually leads to quiescence of T cells. Therefore, modulating this pathway with the purpose to reinvigorate activation and proliferation of T cells could be of interest [48]. Two strategies have been used so far, either by growing TILs in the presence of AKT1/2 inhibitors or by knocking out AKT1/2 using CRISPR/Cas in TILs. With both methods, TIL products with less features of dysfunction, with stem cell memory characteristics and with improved killing capacity have been generated, without compromising TIL expansion [49–53].

Interleukin-2 alternatives

Already since the development of TIL therapy, IL-2 has played a central role in the TIL production process as it is required for effector T-cell expansion, survival and function [3]. Also after TIL administration, IL-2 is essential, as demonstrated by the increased survival rates of patients that were treated with high dose IL-2 compared to low dose IL-2 [2]. Nevertheless, TIL culture with HD-IL-2 leads to significant limitations as well, for example, driving TILs towards a more differentiated phenotype and *in vivo* IL-2 administration is associated with significant toxicities [52,54,55]. Finding alternatives for recombinant IL-2 has therefore been amongst one of the strategies to improve TIL stemness and reduce treatment-related toxicity. Of particular interest is a recent study describing the use of an IL-2 variant that binds in *cis* to PD-1 [55]. This study builds on the finding that differentiation of PD-1+ T cells is mediated by IL-2 binding to the IL-2 receptor α-chain [56]. The PD-1 *cis*-targeted IL-2 variant consists of an engineered IL-2 that can initiate signaling by binding to the IL-2 β- and γ-chain of the IL-2 receptor, thereby preventing the differentiation fate of PD-1+ T cells associated with binding to the IL-2R α-chain. Using this IL-2 variant instead of recombinant IL-2 during *in vitro* TIL outgrowth can potentially restore effector functions of otherwise exhausted PD-1+ T-cells. Furthermore, administration of *cis*-targeted PD-1 IL-2 instead of recombinant IL-2 after *in vivo* TIL administration could be of great interest, as CD25 is present on endothelial cells, leading to severe side effects like capillary leak syndrome [54]. Lastly, an alternative to recombinant IL-2 that could reduce IL-2-related *in vivo* toxicity is the use of orthogonal IL-2/IL-2 receptor mutant pairs, described by Sockolosky *et al.* [57▪] Conceptually, this could be incorporated in the TIL production process by transducing TILs with the orthogonal IL-2 receptor during *in vitro* outgrowth, so that treatment with the orthogonal IL-2 after TIL administration will solely act on the transferred TILs, limiting effects of IL-2 on other cells and thus potentially decrease toxicity.

CONCLUSION

TIL therapy has shown promising results in multiple clinical trials, and now even shows to be better than standard of care in patients with advanced stage melanoma failing first-line immunotherapy [2,4▪]. In this review, we provided an overview of methods to improve the final TIL products with regards to tumor reactivity and TIL functionality. The decision on which strategy or combination of strategies should be implemented into a next generation TIL product should be partly based on the evaluation of TIL product potency. Unfortunately, to date the availability of robust potency assays for TIL products is lacking. Furthermore, it should be taken into consideration that the production process - which is already complex and warranting highly specialized production staff – should not become even more difficult and time consuming. Nevertheless, it is

highly likely that within this rapidly developing field of technologies, the clinical value of TIL therapy will increase in the coming years.

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Figure 1 was created with BioRender.com

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Conflicts of interest

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This study highlights a new technique that could be incorporated in the TIL production process, leading to reduced toxicty and improved TIL stemness.

