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REVIEW

Adoptive transfer with high-affinity TCR to treat human solid tumors: how to improve the feasibility?

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Abstract The adoptive transfer of tumor antigen-specific T cells recently achieved clinical efficacy for a fraction of melanoma patients refractory to other therapies. Unfortunately, the application of this strategy to the remaining melanoma and most other cancer patients is hampered by the difficulty to generate high-affinity tumor-reactive T cells. Two strategies are currently developed to extend the feasibility of this therapeutic approach: clinical grade tool production for MHC-peptide multimer-driven sorting of antigen-specific T cells from the endogenous peripheral T cell repertoire and de novo engineering of the missing repertoire by genetic transfer of cloned specific T cell receptor (TCR) into T cells. The expected multiplication of adoptive transfer treatments, by these strategies, and their careful evaluation should enable the cure of a number of otherwise compromised cancer patients and to gain insight into the characteristics of transferred T cells best fitted to eradicate tumor cells, in terms of antigen specificities, phenotype, and functions. In particular, identification of tumor-rejection antigens by this approach would improve the design and efficacy of all immunotherapeutic approaches.

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Introduction

CD8 T cells play a major role in preventing the development of virus-associated human cancers. Indirect evidence, such as correlations between the amount of tumor-infiltrating T cells (TIL), and better prognosis also support their role in the control of non-virally-induced tumors. Therefore, the goal of most cancer immunotherapies has been to increase the number of tumor-reactive CD8 T cells capable to traffic to the tumor and mediate tumor cell lysis.

In the 1980s, it was shown that many tumors elicit the development of a specific CD8 T cell response [1–4]. Nonetheless, in cancer patients, this response ultimately fails to control the tumor. Many potential reasons for this failure have been identified. One is the limited anti-tumor strength of the TIL due to the condition of their priming in the absence of appropriate danger signal or of proper inflammation, and/or the lack of a high-affinity T-cell repertoire for most tumor-associated antigens. Other is the selection of tumor variants resistant to immune effectors due to both immunoediting and genomic instability of tumor cells and another, the progressive development in the tumor bed of an immunosuppressive environment of multiple origins, such as pro-oncogenic inflammation [5, 6], tumor cell-associated/derived factors, and regulatory pathways of the immune system, which are safeguards against the risk of autoimmune attacks [7].

Tumor-specific CD8 TIL recognize, through their (T cell receptor) TCR, peptides derived from intracellular tumor cell proteins attached to MHC class-I molecules and exposed on the tumor cell surface. Identification of these

tumor antigens started in 1991 [8], raising the hope that stimulation of specific T cell responses might become feasible. Unfortunately, in humans, the induction or boosting of T cell responses towards a tumor protein, by a vaccine-like approach, resulted more challenging than anticipated. Therefore, the alternative approach of transferring tumor-reactive T cells, isolated from tumor samples and expanded ex-vivo, was developed. This approach, called adoptive cell therapy (ACT), has recently been proven to be powerful, by inducing durable complete responses in metastatic melanoma patients resistant to all other treatments, which represents an important breakthrough in cancer immunotherapy [9, 10]. Compelling evidence from animal and human studies established two major conditions for the efficacy of these treatments: the transfer of highly tumor-reactive and long-lived T cells [11, 12] and previous lympho depletion [13], to favor the survival and/or expansion of the transferred T cells, their access to the tumor and/or the neutralization of immunosuppressive immune cells at work within tumors. Adequate selection and high expansion in culture of T cells and heavy patient conditioning are the main limiting factors for the development of ACT.

Here, following a review of ACT-based clinical studies (Table 1), we will present the technological advances under development to reproducibly obtain high-affinity T cells of defined tumor antigen specificities (Fig. 1), that currently make antigen-targeted ACT feasible for the treatment of various tumors, along with the many prospects for improvement and the potential future of ACT.

ACT using TIL in metastatic melanoma patients: the NIH group experience

The first ACT treatments were done using TIL and IL-2 in melanoma and renal cell carcinoma patients. Although clinical responses to IL-2 had been reported in both cancers [14, 15], the coadministration of TIL with IL-2 resulted more efficient only in melanoma patients [16]. This was attributed to the paucity of tumor-specific CD8 T cell among IL-2-expanded TIL from renal cell carcinomas [17, 18], in sharp contrast with the frequent presence of such cells among melanoma TIL [2]. Therefore, ACT using TIL were essentially pursued in melanomas.

In the first generation of ACT, $>10^8$ TIL, obtained by enzymatic digestion of cutaneous or lymph node metastases, were expanded to 10^{11} by a bulk culture with high doses of IL-2 before re-infusion [19, 20]. This method had two important drawbacks: in many cases it did not yield the TIL number expected (10^{11}) and, reaching this number often required long culture periods (up to 100 days), during which many patients dramatically progressed and/or many lymphocytes became exhausted or senescent.

Nonetheless, using this method, TIL could be obtained and injected to 86 patients in two cycles, following, or not, a cyclophosphamide injection (25 mg/kg) to induce lymphodepletion. An objective response rate (OR) of 34% and complete responses (CR) in 6% of the treated patients were reported [16, 19].

From these pioneer studies, the NIH group established the higher efficacy of TIL ACT together with IL-2 infusion versus IL-2 administration alone. Unfortunately, the difficulty of other clinical centers, with less T cell culture experience to grow TIL and, likely for this reason, the lack of clinical responses obtained by these groups generated a great deal of skepticism about the feasibility and the efficacy of this approach in the oncologist community [21, 22].

Thanks to the significant size of their first TIL ACT trial, the NIH group could retrospectively compare the characteristics of TIL infused to responder and non-responder patients, so as to identify potential cues for ACT improvement. This revealed that responder patients had been treated with TIL that were more cytotoxic and obtained through shorter culture periods than those infused to non-responder patients. In addition, a tendency towards a higher OR rate was observed in the group of patients pre-treated with cyclophosphamide [19]. Based on these data, a second generation of TIL protocol was designed [9]. This protocol differed from the previous one by three main modifications: the selection of tumor-reactive TIL before amplification [23], an efficient T cell expansion protocol using allogeneic feeder cells [24], and systematic patient lymphodepletion [25], akin of conditions shown to favor transferred T cell survival in the mouse. TIL were administered intravenously in a single injection, followed by high doses IL-2 administration. A recent update of the results obtained following the treatment of 93 patients by this general approach was just published [10]. The authors report impressive objective response rates of 49%, 52% and 72%, and complete response rates of 12%, 20%, and 40%, according to the type of lymphodepletion regimen: chemotherapy alone or in association with total body irradiation [10]. Importantly, among the 20 patients who achieved a complete tumor regression, 19 have ongoing complete regressions beyond 3 years. Compared with the 34% and 31% response rates reported previously by the same group with or without lymphodepletion respectively [19], these results suggest a strong impact of selecting tumor-reactive T cells and/or of a shorter TIL culture and/or of lymphodepletion on the clinical efficacy of TIL ACT.

The prior selection of TIL able to secrete IFN-γ in response to autologous tumor cells allows both to minimize the transfer of non-tumor-specific TIL and to enrich the infused population with T cells reactive against tumor-associated antigens. However, this selection significantly limited the fraction of enrolled patients who could benefit from the treatment, because of the threshold of detection of

Table 1 Advantages and drawbacks of the various ACT strategies

ACT strategy	Production method	Clinical responses	Advantages	Drawbacks	Improvements	Ref
Non selected melanoma TIL and high doses IL-2	Enzymatic tumor dissociation and TIL expansion with high doses IL-2	34% OR, 6% CR	Relatively simple procedure	Long culture period, infusion of non-tumor reactive T cells, high dropout of enrolled patients due to cancer progression or TIL expansion failure	Shorter expansion procedure and selection of tumor specific TIL	[16, 19]
Myelo and/or lymphodepletion; Melanoma TIL enriched in tumor reactive cells and iv high doses IL-2	Selection of tumor reactive TIL by mixed lymphocyte-tumor cell culture and expansion on feeder cells	49–72% OR, 12–40% long term CR	Enrichment in tumor specific T cells, rapid TIL expansion phase, TIL with long telomers, best clinical efficacy for treated metastatic melanoma patients	Autologous or HLA-matched tumor cells required, lack of tumor specific TIL in some cultures, high dropout of enrolled patients, adverse events related to myelo and lymphodepletion	Simpler methods to select tumor-specific T cells and less toxic patient conditionings	[10, 13, 21, 32, 33]
Non selected TIL from metastatic lymph nodes of melanoma patients and sc low doses IL-2, Adjuvant setting	Expansion of metastatic lymph node T cells on feeder cells	Prolonged RF survival for patients with a single LN metastasis; Prolonged RF survival for patients treated with tumor-reactive TIL	Rapid and reproducible TIL expansion, all the patients enrolled can be treated	Infusion of non-tumor reactive T cells for a fraction of patients	Selection of tumor or tumor antigen-specific T cells	[25, 35–37]
Melanoma patient PBL enriched in T cells specific for the autologous tumor and sc low doses IFNα	Repeated PBL simulation with autologous melanoma cells	1 CR, 1 PR (out of 10 patients)	Enrichment in CD8 and CD4 tumor-specific T cells	Autologous tumor cells required, infusion of a fraction of uncharacterized T cells	Confirm results in a phase II study and identify factors correlating with clinical responses.	[33]
Melanoma patient PBL enriched in CD8 T cells specific for a melanoma antigen peptide and sc low doses IL-2	Peptide stimulation of PBMC	1 CR, 1 PR (out of 12 patients)	Enrichment in CD8 antigen-specific T cells	Short survival of specific infused T cells, infusion of a fraction of uncharacterized T cells	Develop methods to obtain pure polyclonal populations of non exhausted specific T cells	[43, 44]
antigen-specific CD8 T cell clones derived from melanoma patient PBL and IL-2	Peptide stimulation of PBMC, cloning and expansion	Best results: 2 CR, 4 PR (out of 14) [50, 51]	Pure antigen-specific T CD8 cells, induction of repertoire spreading [50, 51]	Technical difficulty of T cell cloning, short survival of infused cells potentially due to T cell exhaustion, absence of CD4 help	Develop methods to obtain pure polyclonal populations of non exhausted specific T cells and co-infusion of antigen-specific CD4 T cells	[45, 50–52]
antigen-specific CD4 T cell clones derived from melanoma patients	PBL stimulation by autologous moDC DC pulsed with CD4 tumor-antigen peptides, cloning and expansion	1 CR, 4PR (out of 9 patients)	Pure antigen-specific CD4 T cells, induction of CD8 T cells specific for non targeted antigens	Technical difficulty of T cell cloning	Develop methods to obtain polyclonal antigen-specific CD4 T cells and co-infusion of antigen-specific CD8 T cells	[42, 53]
Poorly expanded TCR transduced PBL from melanoma patients and IL-2	Transduction of specific TCR chains into autologous PBL, and in vitro expansion	2 OR (out of 17 patients)	Autologous T cells with a desired specificity, non exhausted T cells	Mispairing of transferred TCR chains with endogeneous α and β chains, autoreactivity, low expression of specific TCR	Improved pairing and expression of transduced TCR and co-transduction of CD3	[57–61]
Non expanded HLA-peptide multimers based sorted T cells from CMV infected stem cell transplant patients	Sorting of CMV specific T cells with HLA-peptide multimers and expansion	Clearance of CMV infection in 8 out of 9 patients	Specific and polyclonal non exhausted antigen-specific T cells		Develop clinical grade HLA multimer-based sorting procedures for tumor-specific lymphocytes	[63]

TIL tumor infiltrating lymphocytes, *OR* objective response, *CR* complete response, *PR* partial response, *LN* Lymph node, *RF* Relapse-free

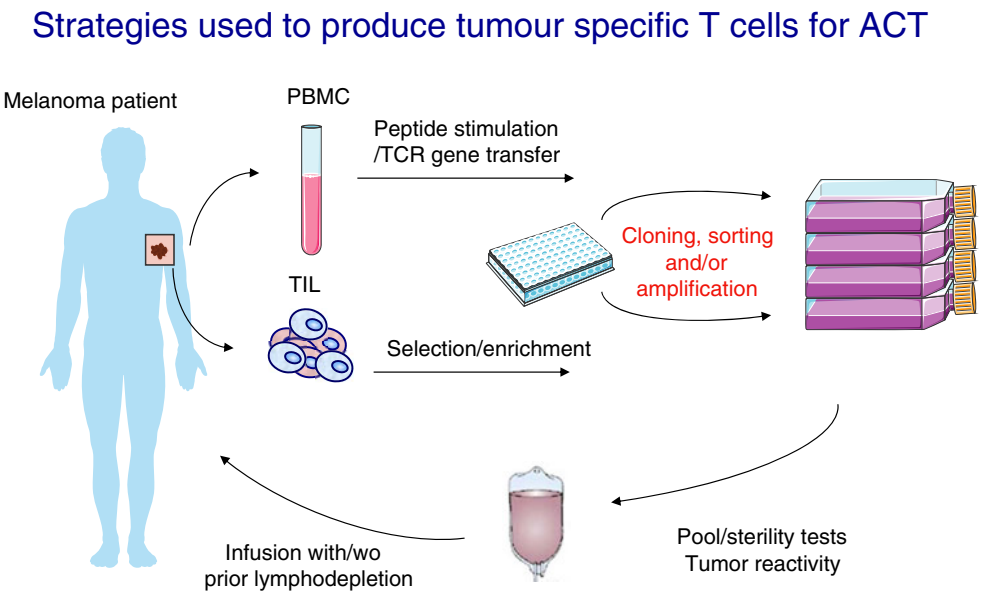


Fig. 1 The production of autologous tumor-specific T cells for a use in ACT trials can be achieved either from TIL (spontaneously enriched in tumor-specific T cells) or PBMC. PBMC are enriched in antigen-specific T cells, either by peptide stimulation or TCR gene transfer. TIL populations can be also enriched in antigen-specific T cells by additional steps of selection (based on tumor reactivity or antigen specificity). Thereafter, tumor specific T cells can be sorted or cloned and/or in vitro expanded. After the final expansion step, T cells are pooled and tested for sterility and tumor reactivity, just before their transfer to the autologous patients

specific T cells, leading to withdraw TIL cultures containing low fractions of tumor-specific T cells [26]. Therefore, alternative means to select tumor-reactive T cells should be developed. A correlation between short TIL culture duration and overall response rate (OR) was reported both in the first and second generations of TIL ACT [10, 27]. It has been attributed to T cell exhaustion or senescence and telomere shortening, induced by high numbers of T cell divisions.

In the second generation of TIL-based ACT, TIL expansion was obtained by anti CD3 antibody stimulation, in the presence of allogeneic irradiated "feeder cells". This procedure induced a more rapid division and/or reduced mortality of TIL, which strongly shortened the culture period, compared with the IL-2 driven expansion used before. Although the exact mechanisms through which irradiated feeder cells (allogeneic PBMC) boost T cell expansion are poorly known, both soluble and membrane bound signals may be involved and it has been suggested that these signals are both pro-mitotic and anti-apoptotic. The latter signals in particular might have contributed to the longer in vivo survival of TIL in the second generation of TIL-based ACT of the NIH group.

Patient lymphodepletion and myeloablation is the third factor considered to be critical for the improved efficacy of the second generation of TIL treatments. Nonetheless, the impact of these conditionings has not been formally addressed, i.e., independent of specific T cell selection and of improved culture procedure. Mouse studies suggested that this conditioning may improve ACT efficacy through eliminating suppressor cells such as CD4+ CD25+ Foxp3+ regulatory T cells [28] and/or myeloid derived suppressor cells, and by triggering homeostatic mechanisms, such as IL-7 and IL-15 bioavailability [29]. Increased levels of serum IL-15 were indeed reported in lymphodepleted patients [13]. In addition, also resulting from endogenous lymphocyte depletion, high frequencies of CD8 tumor-reactive T cells could be achieved in the blood of most treated patients, which favored the access of these cells to the tumor tissues. The recent comparison of three conditioning regimens suggests that lymphodepleting chemotherapy plus myeloablation induced by high total body irradiation (12GY) has the highest impact on the overall and complete response rate [10]. However, the 72% overall response rate reported in 25 patients treated with this conditioning remains not statistically different so far from the 49% and 52% responses rates in the groups treated with chemotherapy alone or chemotherapy and 2-Gy TBI. In addition, this very heavy/drastic conditioning led to a strong immunosuppression, which requires a rapid hematopoietic reconstitution of the patient. Reconstitution was done using autologous CD34 peripheral stem cells, obtained by GM-CSF mobilization before treatment, but a sufficient number of these cells cannot be obtained from all the patients, increasing significantly the dropout of patients initially included. In the first and second TIL ACT generations, autoimmune manifestations were observed in a low fraction of patients. These were essentially vitiligo and uveitis, and for most patients, but one, these autoimmune after-effects were mild and reversible with topical corticosteroid therapy. Since similar autoimmune symptoms were also observed following other melanoma treatments, such as antibody-mediated blockade of CTLA-4 or successful surgical tumor removal, it has been suggested that they might be inherent consequences of melanoma therapies [30].

Overall, the second generation of TIL ACT is an effective therapy for patients with metastatic melanoma. Nonetheless, these treatments remain technically and logistically difficult

and suffer from a major drawback, the very high dropout (about two third) of the enrolled patients, due to the TIL selection and growing process requirements [23, 31, 32] and to patient conditioning requiring a sustained and heavy surveillance of toxic autoimmune and immune defects. More easy and reproducible methods to obtain tumor-reactive T cells and less heavy patient conditioning are therefore desired. Two strategies currently under development should allow reaching the first of these goals, the production of genetically engineered autologous cells and the tetramer driven sorting.

ACT using TIL in an adjuvant setting

It is generally assumed that the limited success of immunotherapy results in great part from its use late after cancer discovery, when all available conventional treatments failed and when the cancer has become the most aggressive and immune escape mechanisms maximal. Nonetheless, the interest of performing ACT at earlier stages of the diseases has been poorly addressed. Because of the limited efficacy of approved treatments, such as dacarbazine, to induce complete regression at the metastatic stage, we had performed a randomized TIL ACT trial in stage III melanoma patients, following metastatic lymph node resection. Eighty-eight patients were randomly assigned to receive either TIL plus IL-2 or IL-2 alone. To avoid toxicity, low doses of IL-2 were used and injected intradermally [33]. The rapid T cell expansion protocol described before [24] was used, allowing to achieve within 2 weeks a huge expansion of T cells by a factor ranging from 1,200 to 18,000. Amounts of infused TIL ranged from 0.22 to 3.34×10^{10} and clinical follow-up was performed after the treatment, to detect an eventual relapse. After a median follow-up of 60 months [33], and of 7 years [34], we found no impact of TIL infusion on relapse-free or overall survival. Nonetheless, a retrospective study of TIL specificity and of patient's characteristics revealed that two variables, not used in intention to treat, had a significant impact on these parameters. In the subgroup of patients with only one invaded lymph node, the estimated relapse rate was significantly lower (P(adjusted) = 0.0219) and the overall survival increased (P(adjusted) = 0.0125) in the TIL + IL-2 compared with the IL-2 arm [34]. In addition, we measured the tumor reactivity of infused TIL for patients whose tumor cell line had been established. This showed that only two third of the patients had received tumor-reactive TIL. Interestingly, relapse-free survival evaluated in 2002 and in 2007 was significantly correlated with the infusion of tumor-reactive TIL [34, 35]. These results and the compatibility of this ACT treatment with normal daily activity strongly support the interest of addressing in a similar setting the efficacy of ACT using T cells selected for a strong tumor reactivity. In addition, analysis of the antigen specificity of the tumor-reactive TIL infused in this trial revealed significant correlations between the infusion of TIL specific for two melanoma-associated antigens, and the prevention of relapse of HLA-A2 patients. One of these antigens is Melan-A/MART-1 [36–38]; the other was unknown before and could be identified by our group using TIL infused to a relapse-free patient [39]. This new melanoma-associated antigen is called MELOE-1 and contains an epitope presented in the HLA-A*0201 context. Importantly, among the 21 HLA-A2 patients treated by TIL, the five who received TIL containing CD8 T cells specific for this particular epitope experienced a prolonged relapse-free survival whereas all the 21 who did not experienced relapse. These results establish the strongest correlation between antigen reactivity of infused T cells and relapse prevention compared with other antigens recognized by TIL populations, and thus strongly suggest the particular relevance of this new melanoma epitope. This is further supported by our recent results showing that a large and tumor-reactive repertoire specific for this epitope is present in all HLA-A2 melanoma patients tested [40].

Actually, all the patients who remained relapse-free, following MELOE-1 specific TIL transfer, had also received Melan-A specific TIL. It is therefore possible that T cells of both specificities are important to avoid tumor recurrence, following the ablation of all detectable metastasis. This strongly supports the development of ACT using CD8 T cells of these two specificities for the treatment of HLA-A2 melanoma patients.

ACT using the peripheral repertoire of tumor-reactive T cells

Recently, a phase I/II study was conducted to test the feasibility and safety of ACT with PBL enriched in tumor-reactive T cells and daily injections of IFN-α in metastatic melanoma patients with progressive disease [41]. Tumor-reactive T cells, CD8 and CD4, were obtained by repeated mixed lymphocyte-tumor stimulation using autologous PBL and melanoma cell line. Ten patients were treated by infusion of relatively low numbers of T cells (about 10^9 in three infusions) along with low-dose IFN-α. All the patients experienced an IFN-α induced transient toxicity including a cellular neutropenia, according to RECIST criteria. Five patients showed clinical benefit from the treatment, including one complete response (above 51 months), one partial response (above to 22 months), and three disease stabilizations (15 months and above 8 and 47 months). The other five patients progressed and died rapidly. There was no correlation between clinical outcomes and the number of tumor-reactive T cells infused or their level of tumor cell lysis, nor with the frequency

of CD4 CD25 FoxP3 regulatory T cells in infused T cells. In contrast, a high expansion rate of T cells, a preferential Th1 profile, and a high level of neutrophil reduction tended to correlate with clinical responses and survival. The main interest of these data is to suggest that infusion of relatively low doses of T cells and low dose of IFN-α, a cytokine much less toxic than high doses IL-2, could be efficient ACT treatments for metastatic melanoma patients without the need of lymphodepletion and irradiation. Nonetheless, this conclusion needs to be tested in the context of a phase II study.

ACT using the endogenous peripheral repertoire of defined antigen specificity

The search of proteins recognized on tumor cells by CD8 cytotoxic T cells from patients led to identify a number of tumor-associated antigens (TAA) and epitopes presented by several tumor types, in the context of frequent HLA alleles (www.cancerimmunity.org/peptidedatabase). A number of studies therefore addressed the capacity of T cells specific for some shared tumor antigens to induce tumor regressions. Strongly facilitating this approach and its evaluation, the use of tetramers (biotinylated MHC-peptide complexes linked to a fluorescent labeled streptavidin molecule) allows the direct visualization and the quantification of specific T cells.

Compared with TIL, an advantage of antigen-specific T cells for ACT is that these cells can be obtained from patients blood, therefore not requiring the availability of a tumor sample. However, on the other hand, the frequency of tumor antigen-reactive cells is considerably lower among PBL than among TIL. Therefore, in most cases, the isolation of tumor antigen-specific blood T cells requires previous antigen stimulation in vitro to reach detectable frequencies, followed by cloning or selection procedures.

Only a limited number of ACT studies using tumor antigen-specific PBL have been performed so far. Most of these studies used CD8 T cells specific for HLA-A2 restricted epitopes of Melan-A (26–35, 27–35 or A27L) or gp100 (152–164 or 209–217) melanocytic differentiation proteins. Either T cell lines enriched in these specific CD8 T cells or CD8 T cell clones were used. Recently, the transfer of CD4 T cells specific for the NYESO-1 antigen was initiated in metastatic melanoma patients [42]. So far, no serious toxicity was reported in these trials.

Mackensen et al used CD8 T cell lines containing a median fraction of 50% Melan-A/A2 specific CTL in a phase I study including eight patients [43]. These HLA-A2 patients were infused three times with $0.2–11\times10^8$ Melan-A specific CTL along with subcutaneous injection of low doses IL-2 (10^6 IU daily). The fate of transferred antigen-specific T cells was followed by tetramer labeling in the peripheral blood. The frequency of these cells among CD8 PBL always below 0.1% before transfer rose after transfer to up to 2%. In most cases, this increase disappeared during the second week post transfer, becoming undetectable at day 14 in most patients. The infusion to three of these patients of $Indium^{-111}$-labeled T cells demonstrated their localization to metastatic sites as early as 48 h after injection. This study was then extended to 12 metastatic melanoma patients [44]. Out of 11 clinically evaluable patients, two responses, according to the WHO classification, were reported: one complete (24 months +) and one partial (11 months). In addition, the expression of the target antigen Melan-A was lost in several metastatic lymph nodes. Therefore, despite the clear demonstration that transferred T cells could migrate to metastatic lymph nodes, the rapid disappearance of these cells and the limited clinical results were disappointing. It may be that stimulation by the modified short peptide primed short-lived CD8 specific T cells due to the epitope presentation by non-professional antigen presenting cells. In addition, this modified peptide might have induced the expansion of regulatory CD4 T cells [45], therefore co-infused with CD8 T cells. Unfortunately these two questions were not addressed.

Three clinical studies had used CD8 CTL clones in ACT trials. In a pioneer study from the Rosenberg group, gp100 specific clones were obtained by repeated PBL stimulation by an anchor-optimized analog of the gp100/A2 209–217 epitope. Very rapid disappearance of infused cells and lack of clinical response led the authors to suggest that CD8 T cells were unable to persist and be effective when infused in the absence of CD4 helper T cells [46]. This has been contradicted by recent studies in which CD8 clones or genetically modified CD8 T cells persisted for a relatively long period post-infusion and were followed by tumor regressions. In addition, the transfer of CD8 T cells has been highly successful for prophylaxis of CMV- and EBV-associated diseases in the post-transplantation setting [47, 48]. The possible explanations for the poor survival of gp100 T cell clones may be an exhaustion or senescence due to their long-term culture (in the absence of feeder cells) as suggested recently by the Rosenberg group itself for TIL and/or a limited TCR strength of the clones for the natural epitope, due to their generation against an analog epitope. Indeed, analog epitope design requires to extensively control that T cell repertoires for the natural and the modified peptides efficiently cross-react. Such a control has not been reported for the gp100 epitope. It was performed to select the Melan-A 26–35 A27L analog among a number of other analog candidates, which had revealed more limited cross-reactivities [49, 50].

Therefore, we used this analog to generate Melan-A/A2 specific T cell clones that were used to treat 14 HLA-A2 patients at the metastatic stage in a phase II study. Each patient received a T-cell clone suspension ($0.14–2.10^9$ cells), followed by subcutaneous injections of interleukin 2 and interferon alpha. CTL clone survival was followed by quantitative clonotypic PCR and the frequency of Melan-A/A2 specific T cells by tetramer labeling. Out of 14 patients treated, six (43%) experienced an objective response (CR + PR) with long-term complete remission for two patients (1 CR for 5 years and 1 CR for 28 months). All these clinical responses were significantly associated with in vivo expansion of the Melan-A-specific T-cell repertoire, independently of T cell clone persistence [51, 52]. These results suggest that infused CTL clones may have initiated an anti-tumor response that may have induced the expansion of Melan-A-specific CTL and contributed to the clinical responses observed. Thus, over the course of an adoptive cell transfer, monitoring this melanoma-specific T-cell expansion in patient blood may be crucial for predicting the clinical efficiency of such an immunological approach.

In another ACT study [53], T cell clones specific for the Melan-A 27-35 and gp100 154-162/A2 restricted epitopes were used to treat ten metastatic melanoma patients. Those patients received four infusions of $3.3\ 10^9$ cells/m^2, the first one without Il-2 and the following with sc infusion of increasing doses IL-2 twice daily for 14 days. Half patients received Melan-A specific CTL and half gp100 specific ones. The fate of infused T cells was followed by specific tetramer labeling. IL-2 infusion significantly prolonged median T cell survival in vivo from 6.68+/-0.9 to 16.92+/-1.37 days. Nonetheless, the preferential use of tetramer to measure this survival cannot exclude that infused CTL clone survival had been overestimated if some repertoire spreading had occurred. In addition, selective loss of the target antigen in residual tumors was observed in three patients suggesting a CTL effect. However, CTL clone infusions resulted essentially in disease stabilization in five patients with only minor or mixed responses in three others.

Overall, in these studies, the transfer of antigen-specific CD8 T cells was safe and, in some cases, had induced a low rate of clinical responses including CR. Several reasons may explain the lower efficacy of these ACT compared with TIL ACT: (1) the lack of patient conditioning and the use of much lower IL-2 doses, (2) the lack of CD4 helper cells potentially present among TIL, (3) the short life expectancy of some antigen-specific populations due to extended culture period in vitro, and finally, (4) the fact that antigen-specific T cell transfer studies were performed without prior patient's selection, whereas TIL ACT were performed in patients [31], selected in particular for the capacity to grow tumor-reactive TIL which might be associated with a less immunosuppressive tumor micro-environment and potentially with a better response to ACT treatments.

Importantly, ACT using antigen-specific T cell transfer would permit to address the relative immunogenicity of known tumor antigens. They could also permit to elucidate other characteristics of T cells required for an effective ACT, such as the effector/memory CD8 subsets, markers associated with optimal function proliferation and survival and the need and effector memory status of helper CD4 T cells. Importantly, this knowledge might secondarily permit to identify potential obstacles to effective antigen-specific therapeutic vaccinations and may be used to establish requirements for antigen-specific immunotherapy.

ACT using CD4 T cells

Helper CD4 T cells are crucial for both the initiation and effector phases of tumor immunity. Through interactions with DC, they help to the priming, survival, and function of tumor-specific CD8 T cells. They may also play roles in tumor cell destruction, either directly by killing MHC class-II expressing tumor targets or indirectly by activating innate effector cells such as macrophages. Nonetheless, the presence and eventual role of CD4 T cells in human ACT remains largely ignored. The difficulty to address their roles comes from the large diversity of MHC class-II molecules, from the limited number of known class-II restricted tumor antigen epitopes and also from the diversity of functional CD4 T cell subsets among which Th2 and regulatory CD4 FoxP3 T cells are detrimental to tumor immune responses.

In a few studies, the presence among infused TIL of IFN-γ-secreting tumor-reactive CD4 T cells was documented [35, 41]. However, neither their antigen specificity nor their impact on the clinical response was analyzed. To address the potential of tumor antigen-reactive CD4 T cells in ACT, Hunder et al. recently derived Th1 CD4 T cell clones specific for a NY-ESO-1 epitope and infused up to 10^{10} of these cells/m^2 together with IL-2 to nine metastatic melanoma patients [42, 54]. Four patients experienced PR or disease stabilization and one patient underwent durable CR of more than 3 years. Infused CD4 T cell frequencies as high as 3% were detected in patient blood up to 2 months after treatment. Interestingly, in some patients, including the patient having developed a CR, T cell responses to non-targeted antigens were durably observed, a phenomenon called antigen spreading previously described in the context of vaccine treatments [55–57]. This phenomenon appears crucial when targeting a single or a few antigens, not expressed by all cells in the tumor since it broadens the

induced immune response to other tumor antigens including even mutated and unknown ones, and thus may prevent tumor escape by the selection of antigen loss variants.

ACT using genetically modified lymphocytes

One of the main limitations of ACT strategy is the difficulty to produce high amounts of antigen-specific T cells for each patient, from blood or tumor samples. Retroviral TCR gene transfer is an attractive strategy by which large numbers of autologous, antigen-specific T cells can be generated. A recent clinical trial has demonstrated the feasibility of this technique in melanoma patients [58]. Retroviral gene transfer was used to transduce peripheral blood lymphocytes from melanoma patients, with the genes encoding the α and β chains of a TCR specific for the Melan-$A_{26–35}$/HLA-A*0201 epitope. Seventeen patients were lympho-depleted before the transfer of transduced autologous T cells. The engineered T cells persisted in 15 patients, but only two of these (with the highest levels of circulating anti-melanoma T cells) showed objective regression of metastatic lesions and remained in remission 18 months after treatment. Several factors could explain these rather disappointing clinical results. One could be the expression level of the introduced TCR. Indeed, the specificity of transferred alpha and beta chains can be altered due to their unwanted pairing with endogenous αβ TCR chains of the recipient cells. Any mispairing may reduce the expression density and, hence, the efficacy of the desired TCR, since the density of TCR expression on the cell surface has been shown to correlate with avidity. Furthermore, such mispairing could also lead to TCRs with unpredictable specificities [59].

In recent years, several groups have explored the means to optimize TCR gene transfer. In particular, efforts have been directed towards increasing the preferential pairing of exogenous α and β chains. A number of strategies have been employed to address this issue. TCRs have been engineered to include an additional cysteine residue in the constant regions of the α and β chains, resulting in the formation of a second disulphide bond between them. T cells transduced with cysteine-modified receptors showed increased tetramer binding and increased reactivity against specific tumor cell lines, compared with T cells expressing wild type TCR [60]. Hybrid TCRs have also been designed to incorporate murine constant regions and human variable regions. These hybrid TCRs show reduced mispairing with endogenous TCR chains when introduced into human T cells, combined with superior cell surface expression, and biological activity [61]. However, there is a possibility that a human host will mount an immune response against the murine component of such a TCR. These approaches also improve the safety of TCR gene transfer by preventing the infusion of potentially self-reactive T cells.

Furthermore, it has been recently shown that some transduced TCR turned to be weakly expressed because they poorly compete with the endogeneous TCR chains for CD3 molecules [59]. Thus, another strategy to enhance the expression of the transduced TCR is to co-transduce with TCR chains the genes coding for CD3 complex. This would increase the availability of CD3 molecules for the transduced TCR, and thus its expression at the cell surface [62].

In conclusion, although TCR gene transfer holds promise, there are still obstacles to overcome with respect to either the safety or the efficacy of this strategy. In this respect, many efforts are made to address the safety issues and to improve the expression of retrovirally introduced TCRs.

Technical strategies for the production of high-affinity T cells

In recent years, an important development of the tetramer technology has been to allow not only specific T cell quantification but also their sorting by flow cytometry or with magnetic beads. Cobbold et al. used this technology to select ex-vivo CMV-specific CD8+ T cells from sero-positive stem cell donors. These T cells expanded in vivo after their transfer and cleared CMV infection in the majority of recipients [63]. Until now, this approach has not been tested in cancer immunotherapy. We developed a sorting procedure based on the use of multimer-coated magnetic beads that will allow the development of clinical grade reproducible ACT assays with tumor antigen-specific T cells in melanoma patients. Initially, we described a sorting procedure that relied on biotinylated HLA/peptide-coated onto streptavidin-coupled magnetic beads to isolate epitope-specific T lymphocytes. Several technical improvements could then be made to enhance the sorting efficiency of this procedure and to meet clinical requirements. First, we documented that a mutation in the α3 domain of the heavy chain of HLA-A201 (A245V) reduced the non-specific binding of the HLA-A2/peptide complexes to the co-receptor CD8 and critically improved the efficiency and specificity of sorts of T lymphocytes specific for viral epitopes [64]. This demonstration was then extended to melanoma epitopes. However, many variables remained to be explored, including antigen-specific T cell sources. We compared TIL and PBMC as sources of antigen-specific T cells. As stated above, the strong advantage of PBMC is that blood sample is available for all the patients, at variance with tumor samples required to derive TIL. However, the frequency of tumor antigen-reactive cells is considerably lower among PBL than among melanoma TIL. Therefore, in most cases, the isolation of tumor

antigen-specific T cells requires previous antigen stimulation in culture to reach detectable frequencies. Using multimer-coated beads, we demonstrated that antigen-specific T cells can be sorted from TIL or enriched PBMC with the same efficiency. Moreover, specific T cells sorted under these conditions (more than 95% purity) were able to proliferate in vitro, were polyclonal and possessed longer telomers (Mean 12 kb) than T cell clones obtained by limiting dilution of bulk cultures (Mean 6 kb) ([65] and unpublished data). We also showed that a preliminary step of PBMC peptide stimulation did not significantly alter the antigen-specific T cell repertoire.

Finally, in order to produce antigen-specific T cells that can be used in a clinical setting, we modified the sorting procedure by replacing the biotin-streptavidin interaction on magnetic beads by an antibody specific for a Tag peptide located at the end of the HLA heavy chain. We documented the high efficiency of antibody-coated beads loaded with HLA-A2/peptide complexes to sort antigen-specific T cells from polyclonal PBMC [66]. Furthermore, an additional advantage of this procedure is that it induces less apoptosis of sorted T cells than streptavidin coated beads because of a different distribution of HLA-peptide multimers at the surface of magnetic beads [67]. These tools are currently under GMP production by a biotechnology company, and a clinical trial using the transfer of sorted-antigen specific T cells will be performed in 2012 on metastatic melanoma patients.

Other issues

The development of ACT for cancer patients that do not benefit already from this treatment relies on the identification of TAA presented by a significant fraction of these tumors and fulfilling all the requirements to induce high-affinity CD8 T cell responses. Cancer testis antigens most identified as T cell targets expressed by melanoma tumors are among the most shared TAA. Nonetheless, antigens of more restricted expression might be better expressed in individual tumor types in relation with specific oncogenic mechanisms. Systematic studies to identify such antigens are therefore required. It might be done by stimulating patients PBL by autologous tumor cells in vitro as initiated by the group of T Boon or by cloning tumor-reactive TIL. However, in some tumor types, despite the high infiltration of tumor tissue by T cells and strong correlations between the amount of these cells and patient prognostic, the identification of conventional CD8 tumor-reactive TIL has been unsuccessful [68]. Once tumor-associated antigens recognized by CD8 T cells have been identified, their immunogenicity in an ACT context has to be addressed. For pathogen-derived antigens, it has been shown that their immunogenicity is related to the frequency of naïve specific T cell precursors [69]. For many tumor antigens, this frequency appears highly variable but in most cases very low [70]. The recent development of clinically approved procedures allowing to create de novo a missing repertoire, by transducing foreign TCR into PBL, will permit to overcome this potential limit. Another critical factor for tumor antigen immunogenicity is the avidity of the T cell repertoire for antigen presenting cells. Genetic TCR engineering should also allow generating a high-affinity repertoire, by appropriately modifying transduced TCR. However, CD8 T cell avidity also depends on the density of MHC-peptide expression by tumor cells, which cannot be controlled.

In melanoma tumors, the antigens expressed at the highest levels are melanocytic differentiation antigens, also shared by skin melanocytes, as well as retinal pigmentary cells. Targeting these antigens by ACT has been shown to be clinically effective but may induce in a few cases important ocular and cutaneous autoimmune reactions [30]. For most other tumors, the use of differentiation antigens is excluded, as far as normal cells sharing these antigens are critical for survival. The antigen choice therefore remains a major issue for the development of ACT for non-melanoma cancers. This question has been well reviewed recently [71].

Perspectives

The important clinical results obtained recently in metastatic melanoma patients treated by TIL-based ACT [10] have finally convinced the oncologist community of the interest of this clinical approach. Importantly, several obstacles to ACT that have been limiting its development were overcome. On one hand, methods to reproducibly select or engineer T cells of desired avidity and specificities will be soon available. On another hand, the T cell expansion process might become significantly alleviated if, as suggested recently [41], the number of T cell required to achieve clinical efficacy is much lower than initially thought, and thanks to the availability of clinical grade fast expansion procedures.

In addition, several other improvements of ACT efficacy are awaited from (1) the identification of qualitative attributes of T cells best fitted to induce clinical responses, such as expression of CD27, CD28, CD62L, high levels of cytotoxic molecules, or long-life expectancy; (2) the development of procedures, sorting or culture protocols, favoring the production of such T cell subsets; (3) the in vivo development of treatments able to bring the benefits attributed to lympho- and myeloablation with less toxic effects, such as homeostatic cytokine administration to

favor the survival of transferred T cells, or neutralization of suppressive cells at work inside tumors. In this context, the replacement of r-IL-2, that favors Treg growth by other cytokines, such as IL-15, IL-7, IL-21, or IFN-α, could be in itself a major advance. Other major improvements of ACT efficiency will likely come from combining these treatments with either conventional treatments such as immunogenic chemo- or radio-therapy, or with vaccination by the target antigen of adoptively transferred T cells. In support of this, a study recently showed that mouse vaccination with a long peptide from the melanocytic antigen gp100 following the transfer of gp100-specific CD8 T cells, strongly increased the number of circulating transferred cells, suggesting that such an approach might boost ACT efficiency [72].

Genetic modifications of transferred T cells distinct from TCR transduction also represent interesting means to improve ACT efficacy, although may be within a longer delay. Genetic modifications proposed in this context are for example the constitutive expression of IL-2 by activated specific T cells and the expression of anti-apoptotic molecules.

Due to this extremely favorable context, it may be hoped that ACT will be developed for the treatment of diverse tumors.

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