



## REGULATORY PERSPECTIVE

**Regulatory perspective on *in vitro* potency assays for human T cells used in anti-tumor immunotherapy**CHARLOTTE DE WOLF<sup>1,2</sup>, MARJA VAN DE BOVENKAMP<sup>1</sup> & MARCEL HOEFNAGEL<sup>1</sup><sup>1</sup>Medicines Evaluation Board (CBG-MEB), Utrecht, The Netherlands, and <sup>2</sup>Department of Infectious Diseases and Immunology, Utrecht University, The Netherlands**Abstract**

The adaptive immune system is known to play an important role in anti-neoplastic responses via induction of several effector pathways, resulting in tumor cell death. Because of their ability to specifically recognize and kill tumor cells, the potential use of autologous tumor-derived and genetically engineered T cells as adoptive immunotherapy for cancer is currently being explored. Because of the variety of potential T cell-based medicinal products at the level of starting material and manufacturing process, product-specific functionality assays are needed to ensure quality for individual products. In this review, we provide an overview of *in vitro* potency assays suggested for characterization and release of different T cell-based anti-tumor products. We discuss functional assays, as presented in scientific advices and literature, highlighting specific advantages and limitations of the various assays. Because the anticipated *in vivo* mechanism of action for anti-tumor T cells involves tumor recognition and cell death, *in vitro* potency assays based on the cytotoxic potential of antigen-specific T cells are most evident. However, assays based on other T cell properties may be appropriate as surrogates for cytotoxicity. For all proposed assays, biological relevance of the tests and correlation of the read-outs with *in vivo* functionality need to be substantiated with sufficient product-specific (non-)clinical data. Moreover, further unraveling the complex interaction of immune cells with and within the tumor environment is expected to lead to further improvement of the T cell-based products. Consequently, increased knowledge will allow further optimized guidance for potency assay development.

**Key Words:** government regulation, immunotherapy, *in vitro* potency assays, neoplasms, quality control, T cells

Due to its potential to induce an anti-neoplastic response, the adaptive immune system is explored as a potential tool for cancer treatment. After being activated by e.g. dendritic cells (DCs) presenting tumor-associated antigens (TAAs), the effector role of the adaptive immune system in the anti-neoplastic response mainly consists of specific recognition and killing (cytolysis) of tumor cells [1,2]. Both CD8<sup>+</sup> and CD4<sup>+</sup> T cells are thought to play an important role in tumor control and thereby use the perforin/granzyme secretion route as well as the Fas/Fas ligand (FasL) interaction pathway, but effector cytokines (e.g., interferon gamma [IFN- $\gamma$ ] and tumor necrosis factor alpha [TNF- $\alpha$ ]) are also produced [3–8]. Nevertheless, the central principle of most cellular anti-neoplastic medicinal products is focused mostly on the expansion and survival of activated antigen-specific CD8<sup>+</sup> T cells (i.e., cytotoxic T

lymphocytes [CTLs]) that can effectively recognize and destroy target cells after infiltrating tumors, thereby resisting potential immunosuppression [9]. However, because T cell subsets appear to play a synergistic role in long-lasting immune responses, more attention is now given to anti-tumor CD4<sup>+</sup> T cells (e.g. [10–18]). Therefore, products specifically combining CD8<sup>+</sup> with CD4<sup>+</sup> T cells (or specific subsets) or only containing CD4<sup>+</sup> can be expected in phase III clinical trials in the near future. This will also require broader insight in the role of CD4<sup>+</sup> in the anti-tumor response, both directly via cytolytic mechanisms and indirectly via, for example, induction of a pro-inflammatory environment to support the activity of other immune cells and to increase recognition of the tumor.

To induce and enhance an *in vivo* anti-tumor response, tumor-antigen-specific T cells can be produced

*ex vivo* from patient-derived peripheral blood lymphocytes (PBLs; via stimulation with TAAs presented by DCs) and administered parenterally. However, other, more advanced groups of adoptive T cell therapy exist, including tumor-infiltrating lymphocytes (TILs) and T cells genetically engineered either to express a conventional T cell receptor (TCR) or a chimeric antigen receptor (CAR) [1,19,20]. We briefly introduce these three adoptive therapies and subsequently discuss the *in vitro* functionality assays proposed for these anti-neoplastic approaches.

## Adoptive T cell therapies

### TILs

To boost the patient's own immune response against a tumor, autologous T cells can be isolated from tumor fragments and cultured *ex vivo* for several weeks with high concentrations of interleukin (IL)-2 to expand antigen-specific effector cells [20,21]. Cells are subsequently harvested, formulated and stored before re-infusion into the patient [2]. A distinct advantage of this approach is the potential broad spectrum of (both defined and undefined) tumor-derived antigens that can be recognized by the expanded T cells, in contrast to the single specificity of genetically engineered T cells.

TIL-based immunotherapy has been successful in the treatment of melanoma, but the clinical application for other tumors remains doubtful, mainly due to several hurdles in TIL isolation and expansion methods [19,22]. The outgrowth of only a few T cell clones (leading to a decrease in antigen targets that can be recognized), the potential activation of immunoregulatory cells during expansion, and the presence of regulatory cells in the *in vivo* tumor environment are potential reasons for clinical failure of tumor regression [2,23]. In addition, broad use of TILs is hampered by the requirement for fresh tumor tissue containing sufficient T cells and a long, labor-intensive expansion period [2,24–26]. Many new approaches in TIL development try to solve parts of these issues, and protocols still evolve to improve the functionality and/or expansion of TILs, including a better engraftment (with longer persistence) of these T cells with the optimal anti-tumor phenotype [20,21,25]. In addition, TILs could be genetically adapted to express the anti-tumor cytokine IL-12 or high-affinity TCRs that recognize tumor-dominant epitopes, for example [25].

### T cells engineered with a transgenic TCR

An alternative approach to the use of patient-derived TAA-specific T cells could be the generation of T cells with a predetermined anti-tumor specificity via gene

therapy [27]. T cells transduced with TCRs that can recognize specific tumor antigens have been developed to target both extra- and intracellular antigens [28]. The manufacturing process of these transgenic T cells is relatively straightforward (i.e., peripheral blood mononuclear cell isolation, activation, transduction and expansion) and short (only a few weeks) compared with the expansion of TILs (usually 6–8 weeks). Also several clinical successes have been achieved, against acute myeloblastic leukemia, myeloma, metastatic sarcoma and melanoma, among others [29–31]. Nevertheless, these genetically engineered T cells have a distinct limitation: they are human leukocyte antigen (HLA)-restricted [2,20,21,24,32]. This means that only tumors that present the antigen in the matched major histocompatibility complex (MHC) class I molecule will be recognized. Downregulation of MHC molecules on tumor cells may even further limit the potential benefit of this immunotherapeutic approach [2,27,33,34].

In addition, genetically engineered T cells may have specificity issues, especially when TCR affinity is artificially augmented to increase product efficacy. Reactivity of TCR with self-peptides present in other tissues may result in life-threatening, cross-reactive toxicity, which can be divided in two main types: on-target (i.e., recognition of homologous peptides, e.g. [35,36]) or off-target (i.e., recognition of unrelated antigens, e.g. [37]) [24,32]. Recent (lethal) incidents exemplify the serious consequences of TCR-based approaches in which cross-reactivity could not be predicted with standard non-clinical screening methods [35,37]. However, identifying mechanisms behind existing toxicities and developing human-specific screening assays for potential toxicities are expected to result in rapid improvements of TCR-engineered T cell therapies [30,32,38–40].

### T cells engineered with a CAR

To circumvent at least the HLA dependency of conventional TCRs, T cells can be transduced with a CAR construct, which consists of an extracellular antibody-antigen recognition domain fused with the intracellular signaling moiety of a TCR [2,20,28,32,41,42]. On the basis of the intracellular signaling domains present, CAR constructs are grouped into four generations. The first generation contained only one intracellular domain (e.g., CD3 $\zeta$  chain), which does not lead to significant IL-2 production. Therefore, appropriate proliferation and survival signals are lacking, and the persistence of the cells *in vivo* is low. It was recognized that a costimulatory domain (e.g., CD28, CD27, OX40 or 4-1BB) is required for proper T cell stimulation and cytolytic activity [41,43]. Indeed, the second and third CAR generations (with one or two

costimulatory domains, respectively) have shown clinical superiority to the first generation [44]. To even further optimize the T cell functionality and fine-tuning of the downstream response, a cytokine-expressing construct can be added to the intracellular moiety to induce secretion of pro-inflammatory cytokines in the micro-environment of the tumor. This fourth generation of CARs is called TRUCKs (i.e., T cells redirected for universal cytokine killing) [2,27,43,45]. The extracellular domain can also be further optimized; for example, flexible spacers can be used to improve target recognition [27].

The specificity of CAR T cells is based on the targeting capability of the extracellular binding domain and, in contrast to TILs and TCR-engineered T cells, not on the TCR [27,28,46]. Therefore, this approach avoids HLA issues and can be used to target a broad range of cell surface (but usually not intracellular) antigens, including proteins, carbohydrates and glycolipids, although the expression of these antigens is generally not restricted to tumor cells [2,27,28]. Moreover, the costimulatory domain(s) present in the second, third and fourth CAR generation allows T cell expansion *in vivo*, because no additional APC stimulation is required [28]. This could facilitate the long-term persistence of the cells and thereby ongoing tumor surveillance, especially when CAR T cell infusion is preceded by a lympho-depleting treatment [46]. Nevertheless, T cells that express hyperactive CARs, which is expected to lead to significant anti-tumor activity, are more prone to apoptosis due to a higher FasL surface expression [8,47].

Although CAR T cell treatment is a promising anti-neoplastic strategy and the use of autologous cells circumvents issues with HLA-matched donor availability or potential graft-versus-host responses, many functional T cells need to be collected from (usually lymphotoxic agent-treated) patients, and it takes weeks to prepare the CAR T cells in sufficient numbers per individual patient, which may be a challenge in diseases with an acute onset or deterioration [46]. In addition, the extent of the (potential) *in vivo* expansion of CAR T cells is unpredictable [28,46]. Moreover, CAR T cell treatment still has prominent side effects, which includes cytokine release syndrome, tumor lysis syndrome, cross-reactive toxicities or other immunotherapy-related effects [11,24,25,28,32,40,46]. In addition, presence of foreign elements in the construct is thought to increase immunogenicity, resulting in a higher risk of host-directed immune responses against the adoptively transferred T cells [11,32,40]. Therefore, various possibilities to reduce these side effects are in development, for example, concomitant use of anti-IL-6 receptor antibodies, insertion of suicide gene domains in CARs or increasing CAR specificity by requiring two tumor antigens for optimal

activation, for example [24,32,41,46,48,49]. Also enrichment of specific T-cell subsets or cells with a specific differentiation status may contribute to clinical efficacy and safety, but will concurrently result in a more complex and expensive production process [2,12,25,27,50,51]. Thus, every impediment seems to be followed by promising improvements in CAR T-cell production. Nevertheless, the search for the most optimal CAR T-cell design and treatment protocols will be ongoing. Improved techniques and increased understanding of tumors and immune cells will ultimately lead to more disease-specific (or patient-specific) therapeutic approaches.

### Control of adoptive T-cell manufacture

Although the clinical application of anti-neoplastic medicinal products containing TILs and genetically engineered T cells may be promising, manufacture of these products involves several challenges. To ensure production consistency of their modified T cells, manufacturers are required to implement several in-process controls and release tests with predefined acceptance criteria. One of the most important release test parameters is functionality (potency). However, potency is a challenging parameter to test for, because it should demonstrate one or more of the cells' relevant *in vivo* biological functions related to clinical efficacy [52,53].

Several publications have dwelled on the general challenges, considerations and requirements for potency assay development and determination of their acceptance criteria [54–59]. Also the European Medicines Agency and the U.S. Food and Drug Administration have provided general guidance for potency testing of cell-based therapies, including products for the treatment of cancer. The main requirement for potency assays used for cell-based products is, in our opinion, the ability to measure product-specific biological activity and should be based on the anticipated *in vivo* mechanism of action (MoA). However, functional assays often require culture steps that last several days and a significant amount of the (autologous) cell batch. Such an approach may not be feasible for release. An acceptable strategy could be to determine relevant surrogate markers in characterization studies. Strictly speaking, all *in vitro* assays, even those measuring intended functionality (i.e., cytotoxicity for T cell-based anti-tumor therapies), could be considered surrogates. The *in vivo* clinical effect is the true potency of the product, because not all relevant details of the MoA are usually known. Therefore, a correlation between the *in vitro* potency read-out and the *in vivo* response would be ideal, but this requires considerably more clinical experience than can be expected before marketing authorization application.

In the choice and design of a potency assay for release, the ICH requirements (accuracy, precision, repeatability, specificity, linearity, robustness, etc.) for assay validation should also be taken into account [60]. The use of the ideal materials (e.g., autologous cells) and read-outs may be excluded, because their inherent variability make them unsuitable.

Despite general guidance for testing cell based product functionality, no recommendations regarding cell type-specific potency assays have yet been presented [52,61,62]. To our knowledge, only a few groups have combined a review about functionality assay development with comments on specific (surrogate) potency tests for actual cell-based medicinal products [54–57]. Nevertheless, pros and cons of these product-specific assays were not discussed in detail. In this review, we first describe several prerequisites for cell-based product functionality and subsequently provide an overview of *in vitro* potency assays most widely used and presented in literature for characterization and release purposes of T cell-based medicinal products as a quantification of their anti-tumor function. We have also taken potency assays along that have been subject of scientific advice for T cell-based medicinal products in development.

### Prerequisites for functionality of cell-based products

#### *Specificity*

The anticipated *in vivo* MoA for T cell-based anti-tumor therapies consists of tumor recognition and attack. Therefore, *in vitro* potency assays are expected to show the cytotoxic effect of the products' cells toward target cells. For an optimal clinical effect, however, T cells should also be sufficiently specific (i.e., being able to discriminate cognate antigen from bystander antigens) [32]. As already mentioned for genetically engineered T cells, potential cross-reactivity toxicity should be investigated in non-clinical studies. However, because such safety issues are not expected to differ between batches, they generally will not be part of release testing.

#### *Viability and dosing*

Other parameters (e.g., viability) that are imperative for the *in vivo* functionality of cell-based therapies are part of release testing. Specifically for genetically engineered T cell-based products, a sufficient amount of cells within the injected cell population should be transduced to be able to exert a clinical response. Therefore, the number of transduced cells belongs to dose determination and not *in vitro* potency testing.

### *Migration, proliferation and persistence*

Injected T cells need to migrate to and accumulate in the tumor tissue, where they should overcome the immunosuppressive environment and activate the endogenous immune system [32,40]. This also requires the capacity of the cells to proliferate and survive *in vivo* to combat large amounts of tumor cells (and potential relapses) [32,40]. Much work has been dedicated to unravel the mechanisms underlying these T cell capacities and to develop tools for improving these functions (e.g., reviewed in Lim and June [32]).

Mainly *in vivo* migration, expansion and persistence are related to positive clinical effects (and thus to efficacious products) and various techniques to determine or predict such T cell abilities are in advanced development [18,31,43,51]. Several studies found a relationship between reduced persistence or loss of target antigen and disease relapse and could predict maximal and/or long-term functionality via the expression of a phenotypic marker, such as programmed cell death protein 1 (PD-1) [31,50,63]. Also the use of T cells with a stem cell memory-like phenotype would contribute to an optimized *in vivo* anti-tumor response. However, studies do not yet agree on the surface markers for identifying this T cell subset [31,50,51,64]. In addition, proposed biomarkers such as PD-1 may indicate the presence of functional T cells in one study, while signal the presence of functionally impaired cells in another study [63,65,66]. Nevertheless, discovery of phenotypic markers or signaling pathways that identify CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets correlated with clinical efficacy could result in defining product-overarching (i.e., more generally applicable) biomarkers or assays. However, the need for additional product-specific markers will probably remain [50,51,63,65].

Although the capacities discussed here are prerequisites for T cells to attack the tumor, only cytotoxic ability results in reduced tumor burden. Therefore, cytotoxicity is considered in terms of actual T-cell functionality and the preferred MoA for a release assay. Other T-cell capabilities should be guaranteed via characterization studies and covered in the phenotype (biomarker expression) specifications for release. Functional release testing of such aspects will be exceptional, occurring only when the biomarkers are considered insufficient.

### *In vitro* assays to test for T-cell functionality

Although many potential *in vitro* assays are proposed for the monitoring of T cell functionality, there is a tendency toward the use of cytokine release assays for TILs and cytotoxicity assays for genetically engineered T cells [67]. However, each T cell product is unique, and so are the individual potency assays.



Therefore, these assays are assessed on a case-by-case basis. Nevertheless, in the following sections, we provide an overview of the assays proposed for the testing of the T cell frequency, activation status or cytotoxic capacity with their main advantages and limitations (summarized in Table I).

#### *Assays to test T cell frequency and activation status*

Antigen-specific T cells can be identified using flow cytometric methods based on detection of phenotypic markers, MHC class I multimeric complexes and/or intracellular cytokine production [67,70,71]. Although these methods provide a good measure of T cell frequency and activation upon antigen recognition, they only provide insight into part of the T cell functional capacities, because cytotoxic potential is not taken into account. After all, not all multimer-positive cells produce cytokines, and not all cytokine-producing cells are cytotoxic [36,67,72].

Various assays to test T cell activation status have been developed. Suni *et al.* have provided a clear overview of read-out parameters of such assays, divided in early, intermediate and late T cell signaling events [73]. Early signaling events consist of antigen-specific induction of (sub)cellular calcium flux and protein phosphorylation. Intermediate events include cytokine production and cellular degranulation, and late events involve T cell proliferation and target cell apoptosis. These factors have been proposed as indicator or surrogate markers for T cell-mediated cytotoxicity and are usually tested in a co-culture of the cells from the medicinal product (i.e., responder cell type) and antigen-loaded or antigen-expressing tumor cells (i.e., stimulator cell type). The assays proposed for intermediate and late T cell signaling events are discussed here in further detail.

#### *Effector molecule production and release*

T cell activation following antigen recognition can be determined via a cytokine release assay, in which the lymphocytes produce cytokines (e.g., IFN- $\gamma$  or IL-2) after a short co-culture period with antigen-loaded HLA-matched tumor cells [71,73]. *In vitro* cytokine production and cytolytic activity of CD8<sup>+</sup>T cells are often correlated, for example, because cytokines such as IFN- $\gamma$  enhance MHC I and Fas expression on target cells [34,72]. However, it should be noted that killing of target cells requires that the lymphocyte is capable of, among other capacities, forming a conjugate with the target cell and producing and release mediators for death induction [69]. Also, the activation of CD8<sup>+</sup>T cells can lead to generation of non-cytolytic T cells [67,74,75]. The detection of cytokine release can therefore only be regarded as a surrogate marker for cytolytic function.

Clinical relevance of a cytokine release assay used for determination of potency of a T cell product should be established before implementation but may be challenging. Seiter *et al.* showed that IFN- $\gamma$  production by melanoma antigen-specific TILs was not related to the *in vivo* clinical response [76]. Dudley *et al.* found a wide range of absolute amount of IFN- $\gamma$  produced by TILs against melanoma that were derived from different sites of the same tumor [26]. Only a few cell preparations resulted in sufficient cytokine release for potential anti-tumor therapy. The findings of the studies from Seiter [76] and Dudley [26] may be explained by the choice of antigen for *in vitro* stimulation and/or phenotypical differences (including CD8<sup>+</sup> to CD4<sup>+</sup> T cell ratio) between TIL preparations. Nevertheless, in a more recent study, Dudley *et al.* showed that IFN- $\gamma$  production by TILs from responders to treatment was significantly higher than cytokine production by TILs from non-responders [77]. Thus, in cases where a correlation between a cytokine release assay with *in vivo* functionality can be demonstrated, such an assay (even if based on a single cytokine) could be used as surrogate marker for T cell potency, provided that specifications can be set that differentiate between sub-potent and potent batches.

Assay characterization studies should address the correlation with *in vivo* functionality and killing capacity of the cytokine-producing cells, but also the cytokine release behavior of both the tumor and (sub-types of) the T cells. The most abundantly applied test to evaluate release, the enzyme-linked immunosorbent assay (ELISA), analyzes cytokine production by the total cell population present, and overestimation of this production may occur when tumor cells have also released the cytokine (e.g., IFN- $\gamma$ -producing B-cell lymphomas) [22,26,47,49,76,78,79]. Flow cytometry can be used to detect production of cytokines on intracellular protein level and provide information on expression in different cell types, but not on the actual release of cytokines that is needed to act on target cells, [71]. Instead of a flow cytometric method, an enzyme-linked immunospot (ELISpot) analyzing cytokine or cytotoxic molecule release at a single effector cell level could be used [72,80,81]. Such an assay is usually easier to perform and has sufficient reproducibility and accuracy when applying automated analysis methods [82]. ELISpot uses relatively low numbers of effector cells and measures actual molecule secretion instead of intracellular production [67]. However, compared with a flow cytometric method, no additional phenotyping can be performed, and only one or two cytokines can be detected simultaneously, although the use of fluorophores instead of enzymes would enable accurate detection of multiple cytokines (in the so-called FluoroSpot) [71,82–84]. In addition, ELISpot analysis does not directly enumerate antigen-specific

Table I. Overview of the advantages and limitations of frequently proposed *in vitro* potency assays.

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Read-out parameter	Advantages	Limitations	Key references
<b>Phenotyping assay<sup>a</sup></b>	—	Effector T cells	—	Surface expression of CD markers T cell frequency	Relatively simple and short assay Analysis at single cell level  Frequency of antigen-specific effector cells can be determined (e.g., multimer staining)	Assay measures only surrogate marker of functionality Assay requires HLA-matched multimers Assay requires known tumor-specific antigens (multimer staining)	26, 30, 31, 68, 75, 79, 85
<b>Proliferation assay</b>	Irradiated peptide-pulsed PBMCs Irradiated (peptide-pulsed) tumor cells	Effector T cells	IL-2  Nothing added	Proliferation via CFSE dilution	Antigen-specific functionality testing Analysis at single cell level Simultaneous phenotypic T cell characterization possible Correlates with other functionality assays	Long assay incubation (around 3-7 days) Assay measures only surrogate marker of functionality Assay requires autologous stimulator cells Assay requires known tumor-specific antigens	12, 17, 18, 51, 68
<b>Avidity assay</b>	MHC multimers loaded with antigens	Antigen-specific T cell clones	—	Amount/duration of TCR-MHC-peptide binding (EC <sub>50</sub> )	Short assay incubation (<1 h) Low assay variability Antigen-specific functionality testing Analysis at single cell level Simultaneous phenotypic T cell characterization possible Correlates with other functionality assays	Assay measures only surrogate marker of functionality Assay requires HLA-matched multimers Assay requires known tumor-specific antigens	68
<b>Cytokine production assay</b>	(Peptide-pulsed) tumor cells	Effector T cells	—	Expression of intracellular cytokines (e.g., IFN- $\gamma$ and IL-2)	Short assay incubation (around 5-6 h) Antigen-specific functionality testing Analysis at single cell level  Simultaneous phenotypic characterization of effector and target cells possible	Assay measures only surrogate markers of functionality Assay requires HLA-matched antigen-specific stimulator cells Intracellular production is not necessarily similar to secretion	31, 68, 78

(continued)

Table I. Continued

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Readout parameter	Advantages	Limitations	Key references
<b>Effector molecule release assay<sup>b</sup></b>	(Peptide-pulsed) tumor cells (can be patient-specific material)	PBMCs	—	Secretion of cytokines (e.g., IFN- $\gamma$ , TNF- $\alpha$ and IL-2) in supernatant or as spots	Short assay incubation (~3-8 h) for ELISpot	Relatively long assay incubation (around 18-48 h) for ELISA	10, 12–14, 17, 18, 22, 26, 39, 47, 49, 51, 75, 77–80, 84
	Peptides ( <i>ex vivo</i> restimulation)	Effector T cells		Secretion of cytotoxic factors (e.g., granzyme B) as spots	Antigen-specific functionality testing Frequency of antigen-specific effector cells can be determined (ELISpot)	Assay measures only surrogate markers of functionality Assay requires HLA-matched antigen-specific stimulator cells May cause overestimation of release in case of secretion by target cells Analysis at population level (ELISA) No simultaneous phenotypic characterization of effector or target cells possible	
<b>Degranulation assay</b>	(Peptide-pulsed) tumor cells	PBMCs Effector T cells	Anti-CD28, anti-CD49b and peptide (only in absence of tumor cells)	Surface expression of CD107a and b	Short assay incubation (~3-6 h) Antigen-specific functionality testing Analysis at single cell level Simultaneous phenotypic characterization of effector and target cells (including lysis) possible	Assay measures only surrogate markers of functionality Assay requires HLA-matched antigen-specific stimulator cells	10, 39, 68, 80, 84, 86

(continued)

Table I. Continued

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Readout parameter	Advantages	Limitations	Key references
<b>Growth inhibition assay</b>	(Irradiated) T cell clones	(Patient-specific) tumor cells	—	Inhibition of proliferation via <sup>3</sup> [H]-thymidine incorporation	Antigen-specific functionality testing Readout (indirectly) detects both cytotoxicity and suppression of proliferation	Long assay incubation (~3-5 days)  Assay requires use of radioactive compound Assay requires HLA-matched antigen-specific stimulator cells May cause overestimation of inhibition in case of non-specific cell loss Analysis at population level No simultaneous phenotypic characterization of effector or target cells possible Assay requires target cells that sufficiently proliferate <i>in vitro</i> Irradiated effector cells may behave differently than <i>in vivo</i> counterparts Difficult to create a consistent assay with patient material	<a href="#">87</a>
<b>Cytotoxicity assay (protein release)</b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Presence of intracellular proteins (e.g., LDH, β-gal or luciferase) in supernatant or as spots	Short assay incubation (~4-6 h) No radioactive compound required  Antigen-specific functionality testing Cannot cause overestimation of cytotoxicity in case of effector cell lysis (production of foreign protein such as β-gal only in transduced cells) Simultaneous cytokine- producing characterization of effector cells possible (Lysis spot)	Assay requires HLA-matched antigen-specific target cells May cause overestimation of cytotoxicity in case of effector cell lysis (readout on naturally present protein like LDH) Analysis at population level  No simultaneous phenotypic characterization of effector or target cells possible  Target cells transfected with a foreign protein may behave differently than <i>in vivo</i> counterparts	<a href="#">73, 88, 89, 90</a>

(continued)



Table I. Continued

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Readout parameter	Advantages	Limitations	Key references
<b>Cytotoxicity assay (radioactive compound determination, short term)<sup>b,c,d</sup></b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Presence of intracellular radioactive compound (e.g., <sup>51</sup> Cr) in supernatant	Short assay incubation (usually 4–6 h) for <sup>51</sup> Cr and JAM assay ( <sup>3</sup> [H]-thymidine)	Long assay incubation (~2–3 days) for <sup>125</sup> I and <sup>3</sup> [H]-thymidine (other than JAM assay)	12, 17, 18, 47, 68, 72, 74, 77, 79, 84, 91–101
	Complete TIL population			Elimination of target cells (e.g., loss of <sup>3</sup> [H]-thymidine or <sup>125</sup> I signal)	Antigen-specific functionality testing	Assay requires use of radioactive compound Assay requires HLA-matched antigen-specific target cells Analysis at population level No simultaneous phenotypic characterization of effector or target cells possible <sup>3</sup> [H]-thymidine is only suitable for dividing target cells	
<b>Cytotoxicity assay (radioactive compound determination, midterm)</b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Presence of intracellular radioactive compound (e.g., <sup>51</sup> Cr) in supernatant	Relatively short assay incubation (18 h)	Assay requires use of radioactive compound	87, 98
	Complete TIL population				Antigen-specific functionality testing Higher change on full effector T cell response detection compared with short-term assay	Assay requires HLA-matched antigen-specific target cells Analysis at population level  No simultaneous phenotypic characterization of effector or target cells possible	
<b>Cytotoxicity assay (bioluminescent compound determination)<sup>d</sup></b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Survival of target cells (e.g., remaining luciferase activity)	Short assay incubation (~6–8 hours) No radioactive compound required Antigen-specific functionality testing	Assay requires HLA-matched antigen-specific target cells Analysis at population level  No simultaneous phenotypic characterization of effector or target cells possible May cause overestimation of cytotoxicity in case of non-specific cell loss Target cells transfected with a foreign protein may behave differently than <i>in vivo</i> counterparts	47, 97, 102

(continued)

Table I. Continued

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Readout parameter	Advantages	Limitations	Key references
<b>Cytotoxicity assay (fluorescent compound determination, short term)<sup>b,c,d</sup></b>	PBMCs	(Peptide-pulsed) tumor cells	—	Elimination of target cells (e.g., loss of fluorescent signal or positive 7-AAD staining)	Short assay incubation (usually ~3-6 h)	Assay requires HLA-matched antigen-specific target cells	10, 13, 72, 74, 78-80, 84, 86, 93-95, 103-109
	Effector T cells			Survival of target cells (e.g., remaining fluorescence or negative 7-AAD staining)	No radioactive compound required	May cause overestimation of cytotoxicity in case of non-specific cell loss (when readout is based on a single fluorescent marker)	
				Induction of apoptosis in target cells (e.g., caspase staining)	Antigen-specific functionality testing Analysis at single cell level Simultaneous phenotypic characterization of effector and target cells possible		
<b>Cytotoxicity assay (fluorescent compound determination, midterm)</b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Elimination of target cells (loss of fluorescent signal)	No radioactive compound required	Relatively long assay incubation (~48-60 h)	49, 86
		Peptide-pulsed B cells		Survival of target cells (phenotypic markers)	Antigen-specific functionality testing Analysis at single cell level Simultaneous phenotypic characterization of effector and target cells possible	Assay requires HLA-matched or autologous antigen-specific target cells May cause overestimation of cytotoxicity in case of non-specific cell loss (when readout is based on a single fluorescent marker)	
<b>Cytotoxicity assay (fluorescent compound determination, long term)</b>	Effector T cells	(Peptide-pulsed) tumor cells	IL-2	Survival of target cells (e.g., crystal violet staining or remaining fluorescence)	No radioactive compound required	Long assay incubation (~3-5 days)	96, 98, 99
	HLA-matched T cell clone		Nothing added		Antigen-specific functionality testing Analysis at single cell level (only via fluorescent labeling)	Assay requires HLA-matched or autologous antigen-specific cells Analysis at population level (via crystal violet staining)	
					Simultaneous phenotypic characterization of effector and target cells possible (only via fluorescent labeling)	No simultaneous phenotypic characterization of effector or target cells possible (via crystal violet staining)	
					May give insight in tumor immune escape	Potential proliferation of target cells may interfere with analysis Clinical relevance of assay (as release) for T cell functionality is not clear	

(continued)

Table I. Continued

Proposed <i>in vitro</i> potency assay	Stimulator cells	Target or responder cells	Additional stimuli	Readout parameter	Advantages	Limitations	Key references
<b>Cytotoxicity assay (detachment detection)</b>	Effector T cells	(Peptide-pulsed) tumor cells	—	Removal of target cells by detachment (e.g., via loss of fluorescent intensity or change in electrical impedance)	<p>No radioactive compound required</p> <p>Antigen-specific functionality testing</p> <p>Analysis at single cell level (only via fluorescent labeling)</p> <p>Simultaneous phenotypic characterization of effector and target cells possible (only via fluorescent labelling)</p> <p>Low amount of cells required</p> <p>Real-time monitoring possible (xCELLigence system)</p>	<p>Assay measures only surrogate marker of functionality</p> <p>Long assay incubation (&gt;24 h)</p> <p>Assay requires HLA-matched antigen-specific target cells</p> <p>Analysis at population level (via electrical impedance testing)</p> <p>No simultaneous phenotypic characterization of effector or target cells possible (via electrical impedance testing)</p> <p>May cause overestimation of cytotoxicity in case of non-specific cell loss</p> <p>Assay is only suitable for adherent target cells</p> <p>Potential proliferation of target cells may interfere with analysis</p>	<a href="#">85</a> , <a href="#">113</a>

$\beta$ -gal,  $\beta$ -galactosidase; EC<sub>50</sub> = half maximal effective concentration; <sup>125</sup>I, radioactive iodine; LDH, lactate dehydrogenase; PBMC, peripheral blood mononuclear cell.

<sup>a</sup>References are literature examples where this assay is mainly used for identity and frequency purposes.

<sup>b</sup>References are literature examples where these similar assays are described.

<sup>c</sup>Cytotoxicity assays described by Höppner *et al.* [68] and Kim *et al.* [69] were performed with NK cells instead of T cells.

<sup>d</sup>Several cytotoxicity assays were performed with murine effector and target cells instead of human cells.

T cells, because cytokine secretion may require a certain threshold that not all these T cells may reach upon stimulation [76]. Nonetheless, ELISpot detection of components released by T cells could be used as a surrogate marker of potency.

To combine determination of antigen-specific cytokine-release with cytolytic capacity, Snyder *et al.* suggested a combination of an ELISpot with a LysisSpot [74]. The ELISpot determines whether effector cells have produced IFN- $\gamma$ , and the LysisSpot detects leakage of a foreign non-secreted protein (here:  $\beta$ -galactosidase) from transduced target cells, both at the single cell level. As is known from several other studies, the authors confirmed that most cytolytic cells also produced IFN- $\gamma$  (dual functionality), but that not all cytokine-producing cells could lyse target cells (single functionality). Nonetheless, this 3-h assay could be useful in *in vitro* or *ex vivo* determination of effector cells potentially able to induce a successful *in vivo* response, especially because the assay could be adapted to analyze, for example, granzyme release to elucidate cytotoxic mechanisms. However, also this modified assay has some limitations. First, as was already mentioned for the ELISpot assay, the phenotype of the lysed and non-lysed target cells and the single or dual functional effector cells cannot be further elucidated. Furthermore, variability in protein expression in the target cells may occur, and therefore a very sensitive assay is required. This indicates that a relatively large amount of target cells is needed so that each individual effector cell can find at least one target to display cytotoxicity. Therefore, the relevance of the corresponding effector to target ratio for the *in vivo* situation would require justification. Finally, although it is expected that many target cell types can be transfected via the proposed protocol, the significance of manipulated cells expressing a foreign protein should be clarified.

#### *T cell degranulation*

Enumeration of antigen-specific T cells that have released effector molecules can also be performed with a degranulation assay [81,86,103]. Hereby, exposure of cytolytic granule-associated membrane proteins (mainly CD107a and b) on the surface of T cells acts as a marker for cell activation and subsequent degranulation of vesicles with cytolytic molecules.

A degranulation assay is based on flow cytometry, and thus this test can be combined with cellular phenotyping and analysis of effector molecule production (both cytokines and cytolytic components), for example, during characterization. Because not all cells contain sufficient amounts of these intracellular effector molecules to directly measure them, CD107 surface detection is thought to be a superior alternative (especially for quality control release) [103]. Moreover, in contrast to intracellular cytokine detec-

tion, CD107 detection requires no fixation of the cells before staining. In addition, there appears to be a good correlation between degranulation and actual killing by cytotoxic T cells, but this may depend on the stages of T cell differentiation, because only effector T cells possess immediate cytolytic activity, whereas the memory types do not [73,86].

#### *T cell proliferation*

Apart from the capacity of T cells to produce effector molecules and degranulate upon target cell contact, their proliferation ability in response to recognition of their cognate antigen is also used as a measure for their activation status [70]. Proliferation can be detected by the incorporation of a DNA label like  $^3\text{H}$ -thymidine and expressed as a stimulation index (i.e., ratio of  $^3\text{H}$ -thymidine incorporation in response to antigen vs. negative control), for example. However, it should be taken into account that this degree of proliferation does not necessarily correlate with the antigen-specific T-cell frequency within the product, as many cells may proliferate a bit, or a few cells may proliferate a lot. The use of a fluorescent marker such as carboxyfluorescein succinimidyl ester (CFSE) can provide more insight in individual T-cell responses, but setting appropriate specifications for this assay readout may be challenging. T-cell proliferation can be used to determine a T-cell response before and after *in vivo* immunotherapeutic treatment. However, proliferation is not directly linked to tumor cytotoxicity and, therefore, to clinical outcome [71]. The value of T-cell proliferation as surrogate marker for *in vivo* potency thus requires further evaluation. Nevertheless, if no suitable alternative potency assays are available, it may be a valuable marker to ensure the consistent quality of a T-cell product.

#### *Assays to test T cell cytotoxic capability*

Cellular cytotoxicity, via secretion of cytotoxic molecules such as perforin and granzymes or engagement of cell surface death receptors such as Fas, is one of the most important effector functions of T cells required during an anti-tumor response [4–8]. The tumor-attacking function of T cells is generally determined in tests that are collectively called cytotoxicity assays, which consist of a co-culture of T cells as effectors and responding tumor target cells. These target cells can be tumor-derived or tumor antigen-presenting primary cells or cell lines, each having their advantages and limitations.

Although the aim of cytotoxicity tests is induction of target cell killing, the read-out can vary considerably between individual assays. Read-outs are based on, for example, the induction of the apoptotic pathway within the target cells, loss of target mem-

brane integrity or leakage of specific components from the target cells [73]. Several of these tests are discussed in more detail below.

#### *Induction of apoptotic pathways in target cells*

Killing of target cells is preceded by the activation of several intracellular pathways, which ultimately leads to apoptosis and cytolysis. Induction of apoptotic pathways downstream of both the cytotoxic route and the death receptor route includes activation (i.e., cleavage) of several caspases [80,104]. Because post-translational modifications can considerably influence caspase activity, the presence of cleaved caspase may nevertheless not be the best indication of induction of apoptotic pathways [105]. Instead, several groups proposed the detection of cleavage of fluorogenic caspase substrates as read-out for potency assays [104,105]. The use of synthetic substrates that become fluorescent when cleaved by activated caspases may give a good indication of induction of the apoptosis pathway.

Next to detection of apoptosis, induction via caspase activity, analysis of other stages of the cytolytic cascade is frequently used to determine T cell cytotoxic capacity. One event includes translocation of phospholipid phosphatidylserine to the outer leaflet of the plasma membrane, which can be stained with annexin V [81,91]. Liadi *et al.* proposed a high-throughput assay with a single effector cell and a single target cell per well [91]. Cytolysis (via annexin V staining) was used as read-out but can be easily adapted or combined with analysis of, for example, cytokine production. However, for use as a potency assay, it would be required to determine the clinically relevant effector to target cell ratio. The proposed combination of a single effector cell and a single target cell may lead to underestimation of cytolysis, especially when one effector cell could induce cytotoxicity in several target cells or when interaction of a target cell with several (different) effector cells would be required before irreversible cell death is induced [3].

Relatively few assays have been proposed to specifically detect and quantitate death receptor-induced apoptosis of target cells. To analyze Fas-mediated apoptosis, Hoves *et al.* adapted the JAM assay, which was earlier described by Matzinger [92,106]. The JAM assay is based on the principle that dying cells fragment their DNA before membrane integrity is lost and measures labeled DNA retained by living cells. A drawback of this assay is that DNA is labeled with  $^3\text{[H]}$ -thymidine, which requires ongoing cell division, but not all target cells will easily divide *in vitro*. Moreover, not all apoptotic pathways lead to cleavage of DNA into small fragments, so target cell killing could be underestimated with a JAM assay [110].

To justify their use in a potency assay for cytotoxicity, for all read-outs discussed here, it should be

demonstrated that they correlate with actual cell death [21,110]. In addition, it should be taken into account that not all types of cytotoxic cell death involve the mentioned apoptotic pathways [4–6,110]. Thus, before developing a potency assay for a T cell product, its main mechanism of cell killing should be determined. Moreover, it is noted that not only target cells but also effector cells may undergo apoptosis [110]. This is especially the case when tumor cells express or secrete FasL, because activated T cells are prone to Fas-induced cell death [34,111]. This T cell apoptosis may result in underestimation of *in vivo* efficacy of a product due to loss of cytotoxic cells. In terms of assay read-out, this also stresses the importance to aim for a way to distinguish between effects in target and effector cells.

#### *Loss of target cell membrane integrity and cell death*

The most conventional assay to test for cytolytic activity is the chromium ( $^{51}\text{Cr}$ ) release assay, in which target cells are intracellularly labeled with  $^{51}\text{Cr}$ , and after short incubation with effector cells, leakage of this radioactive compound due to loss of target membrane integrity is determined [112]. Advantages of this assay are that antigen-specific activity of the medicinal product can be analyzed (provided that the responder cells are HLA-matched) and that the assay can be performed in only a few hours. Nevertheless, use of radioactive material (including potential difficulties with target cell labeling), detection of lysis only at total target cell population level (although there may be heterogeneity in the apoptotic susceptibility and other characteristics of individual target cells), and the need for relative high amounts of effector and target cells makes the assay less practical for today's use [67,72,110]. Moreover, release of  $^{51}\text{Cr}$  through pores in cells that are attacked by perforin from CTLs will lead to a positive signal in the assay, although these leaky cells will not necessarily die, because this will require subsequent intracellular activation of an apoptosis pathway by, for example, granzymes [7,110]. In addition, longer incubations required for the analysis of death receptor-mediated cytolysis can lead to a high rate of spontaneous  $^{51}\text{Cr}$  release from or  $^{51}\text{Cr}$ -mediated cytotoxicity of target cells, resulting in high background values [72,88]. These drawbacks also hold true for other membrane integrity tests, including leakage of cell-specific components (e.g., enzymes such as lactate dehydrogenase) [67,107].

To overcome the limitations of the conventional cytolytic (leakage) assay, several alternative approaches have been proposed. As outlined subsequently, these methods are often based on flow cytometry and have the advantage that they are non-radioactive. Nonetheless, flow cytometric-based assays also have their restrictions, because completely destroyed cells can no



longer be detected, and some labeling dyes (used to tag a specific cell population before culture) are not stably integrating in the membrane of particular cell types, leading to leakage to unstained neighboring cells [68]. Further, labeling dyes used in the cytosol of tagged cells may leak if there is increased membrane permeability [105]. In addition, use of internal controls (i.e., non-antigen-specific cells or cells harboring another HLA type) appears to be essential to obtain an accurate estimate of target cells that lost membrane integrity due to T cell effects, thus requiring the addition of another cell population in the sample [75,93,94].

Determination of increased membrane permeability by staining with specific dyes that bind to intracellular components is commonly performed. The DNA intercalators propidium iodide and 7-amino-actinomycin D (7-AAD) are frequently used in conjunction with annexin V labeling (see previous section) to measure induction of apoptosis and subsequent loss of membrane integrity in the same sample. Simultaneous phenotyping of effector and target cell (sub)populations, determination of the cytolytic function against multiple antigens and additional immunophenotyping could further improve this type of assay for characterization purposes (e.g. [67,68,93,95]). One example is given by Kim *et al.*: they proposed a four-color flow cytometric method to label target cells, detect cell death (7-AAD staining) and measure the effector cell phenotype [69]. Although this assay was performed with natural killer (NK) cells, the principle may be applicable for T cells as well.

Sheehy *et al.* proposed the simple FATAL assay, in which they labeled the target cells with two fluorescent dyes to detect their presence via membrane labeling and their membrane integrity by loss of intracellular dye [94]. This analysis could be easily extended by the addition of other fluorescent labels to determine, for example, effector cell phenotype and cell death. In addition, the use of target cells expressing a fluorescent protein compared with temporarily labeling the cells with a specific dye could improve the reproducibility of this kind of assay [75,93].

Several flow cytometry-based cytotoxicity tests have been used to quantify target cell survival instead of killing. Kochenderfer *et al.* proposed using the ratio of surviving target cells to surviving negative control cells present in a single test sample to determine antigen-specific killing, as this generates a more suitable background value for nonspecific cytotoxicity [79]. Also in the VITAL assay developed by Hermans *et al.*, survival of fluorescent antigen-pulsed target cells by CD8<sup>+</sup> T cells (and NK T cells) was monitored relative to differently labeled non-pulsed cells in the same sample [108]. The great advantage of this assay is the potential to label target cells with different fluorescent

intensities, in that way permitting the simultaneous analysis of the T cell cytotoxic capacity against multiple target cells (e.g., loaded with a titrated dose of antigen). In addition, it was shown that the VITAL assay could be used both *in vitro* and *in vivo*. Although the study was performed with murine cells, the use of this assay principle with human cells is conceivable.

In a survival assay using human cells, antigen-specific loss of CFSE-labeled autologous tumor cells was followed over a 5-day culture period, allowing for detection of both rapid (i.e., perforin/granzyme secretion) and slow (Fas/FasL interaction, among others) cell death-inducing pathways [96]. This assay also enabled determination of which tumor cell subpopulations had survived T cell-mediated cytotoxicity. Because tumor cells could be harvested from the blood (leukemia), the potency assay setup is relatively simple and may even be suggested for release. This is, however, not expected to be feasible for non-hematologic malignancies. In addition, proliferation of surviving tumor cells during the culture period may underestimate the T cell cytotoxic capacity measured at the end of the culture, thereby interfering with quantification of cytotoxicity. Therefore, such an assay would probably be more useful in non-clinical studies to obtain knowledge with respect to pharmacodynamics and potential tumor escape.

Another way to measure cytotoxicity via analysis of the remaining viable target cells is using target cells expressing the specific antigen in combination with a luciferase reporter and where the cytotoxicity level of effector cells is based on the remaining luciferase activity in viable target cells [47,97,102]. The advantage may be to overcome or reduce problems related to peptide loading, for example. However, it should be demonstrated that the transduced cells are sufficiently representative for the *in vivo* target cells of the immunotherapy. Moreover, Künkele *et al.* showed the need to properly correlate *in vitro* and *in vivo* potency and to justify the choice of *in vitro* assay, because medicinal products (in this case, CAR T cells) showed discordance between *in vitro* potency and *in vivo* anti-tumor effects [47]. A more complex *in vitro* potency characterization assay with repeated effector cell activation showed that insufficient (*in vivo*) anti-tumor activity was the result of activation-induced cell death of highly active T cells [47].

#### *Alternative assays*

Instead of detecting specific signals of apoptosis induction or cell death (after loss of membrane integrity), cytotoxicity can also be determined in terms of detachment of target cells from a surface. A sensitive semi-automated detachment assay requiring a relatively small amount of target cells was proposed by Wang *et al.*, where adherent target cells detached from a plastic

surface upon death due to the cytotoxic function of effector CD8<sup>+</sup> T cells. Counting the remaining viable attached target cells was used as a measure of cytotoxicity [113]. Peper *et al.* proposed a detachment assay, in which the xCELLigence system was used to detect detachment via changes in electrical impedance [85]. Although this system can be used to determine target cell lysis in real time, the assay requires many more target cells than the one described by Wang *et al.* Drawbacks of this type of assays are that they take several days to execute and can only be performed with adherent target cells, which may not be sufficiently relevant for specific tumors. For use as a release potency assay, appropriate controls would need to be in place to account for potential proliferation of target cells during culture, and it would need to be demonstrated that detachment is indeed due to T cell function and correlates with cell death.

Van der Hoorn *et al.* took an entirely different approach to determine the anti-tumor capability of T cells. Instead of a “standard” cytotoxicity assay, inhibition of tumor cell proliferation (which could be partly due to cytolysis) in the context of graft-versus-leukemia was analyzed [87]. Leukemic progenitor cells proliferated in the presence of several cytokines and irradiated T cells were added to inhibit this proliferation, which was analyzed at population level via <sup>3</sup>[H]-thymidine incorporation after several days of co-culture. Although clear inhibition of target cell proliferation was detected, this read-out cannot be considered the same as cell death and therefore measures a different effector cell function. Moreover, this assay takes several days to perform and should include the determination of the most optimal cell concentration and harvest day for maximal proliferation (which requires additional culturing days and valuable patient material), because this appeared to differ between tumor material. Therefore, this assay seems to be less practical for release. Further, although this kind of read-out may be useful (especially when the radioactive compound is replaced by flow cytometric signals, for example), several adaptations would be required to use this assay for other target cells, because not all cells divide easily *in vitro* [110]. In addition, the effect of irradiation on different effector T cell functions should be further investigated.

As a final example of an alternative functional assay, Allard *et al.* determined the dissociation rate of the TCR and MHC-peptide complex and stated that this rate is a major determinant controlling the functions of CD8<sup>+</sup> T cells both *in vitro* and *in vivo* [70]. Indeed, the dissociation rate appears to be a stable and robust biomarker independent of the T cell’s activation status. Nevertheless, this marker remains a surrogate one, because more than TCR stimulation is required for effective T cell activation [9]. The authors therefore

analyzed other *in vitro* parameters usually tested to determine functionality (including cytokine production, cell proliferation and cytotoxicity). They observed a positive correlation of the *in vitro* dissociation rates with these parameters, but also with *in vivo* anti-tumor potency in mice [70]. Although their approach may be suitable for the development of a release assay properly correlated with results found in characterization studies, some limitations should be mentioned. First, the requirement of patient-matched MHC molecules presenting defined tumor antigens would make this assay less practical for release of autologous products. In addition, an assay based on the TCR-MHC-peptide dissociation rate is only possible when dominant tumor-specific antigens are known and sufficiently stable expressed *in vivo*. Moreover, the authors used clones of antigen-specific T cells, so comparability with activation of primary T cells remains to be shown. Therefore, instead of using the proposed assay for as release test, it may be more suitable for selection of cells with a slow dissociation rate to obtain a product with enhanced functionality.

### Selection and design of potency assays

Despite the frequent use of cytotoxicity assays for the determination of T cell functionality, it is still uncertain which specific assays and readouts would be most appropriate to assess potency of T cell-based medicinal products. In addition, because T cell products are unique in their manufacturing process and main functional properties, the relevance of a potency assay should be justified on a case-by-case basis. This requires sufficient characterization data of the phenotypic and functional properties of the medicinal product, both *in vitro* and *in vivo*. In addition, the following topics mentioned should be taken into account in the selection and design of an *in vitro* potency assay.

#### Assay and read-out type

The choice of the assay principle is one of the most important and first decisions to be made in the development of a potency assay (for release), because the read-out of the assay should be relevant for and related to the patient’s *in vivo* situation. Most assays proposed are based on the actual cytotoxic potential capacity of T cells instead of the T cell activation status, because T cell-based medicinal products for anti-neoplastic purposes are expected to result in target (i.e., tumor) cell death. Measurement of the T cell activation status is only regarded as surrogate for cytolytic capability. However, assay read-outs testing for cytotoxicity can also be surrogates, because, for example, induction of early apoptosis markers do not necessarily result in cell death. Nevertheless, surrogate markers or tests may be appropriate for an *in vitro*

release assay, especially because (autologous) cell material may be too limited for extensive testing.

A recent paper from Dwarshuis *et al.* noted that use of surface marker determination and simple “killing” assays will not be sufficient for proper quality control of T cell functionality, due to, for example, too limited reflection of the complex *in vivo* tumor micro-environment [21]. Functional assays may therefore be replaced or augmented by more advanced models and technologies. Indeed, the development of assays based on factors reflecting the *in vivo* MoA and complex tissue micro-environment are essential to guarantee the quality of the product and will require extensive attention in characterization studies. Nevertheless, performing advanced approaches during release testing may be needlessly time-consuming and costly. Therefore, relatively simple assays could be used for release. Characterization data are then imperative in determining the product characteristics essential for *in vivo* potency to support the choice of the read-out parameter(s) for release and to correlate this (surrogate) read-out with actual antigen-specific T cell cytotoxicity and with true target cell lysis. Characterization should also identify the specific mechanism of killing per individual medicinal product, because the type of proposed cytotoxicity assay is usually dependent on one of the main cell death-induction routes (which is partly relying on the target cell type) [7,80]. This may include the contribution of knowledge obtained from studies using inhibitors of specific cytotoxic routes or pathway initiators. Finally, to ensure a functional medicinal product, it should also be shown that secretion of effector molecules, T cell proliferation or changes in target cell intracellular pathways and membrane integrity induced by the *in vitro* stimulus is an actual reflection of the antigen-specific function of the cells *in vivo* (in the tumor environment) [71].

#### Assay optimization

In addition to the choice of an appropriate assay type and read-out, optimization of assay conditions is essential for accurate measurement of cytolytic activity. A study by Finke *et al.* using short-term (4 h), midterm (18 h) and long-term (3 days) cytotoxicity assays suggests that cytolysis by different effector cells in TIL preparations is dependent on culture duration [98]. CD56<sup>+</sup> cells in the effector population displayed most cytolytic activity in the short-term assay, while CD8<sup>+</sup> and CD4<sup>+</sup> T cells only showed significant lytic activity in the midterm and long-term cytotoxicity assays. Nevertheless, antigenic restimulation of T cells preceding the co-culture period resulted in T cell-mediated cytolysis in the short-term assay as well.

The culture duration may also influence the ability to detect production of effector molecules. For example,

in a 4 day-culture of T cell subsets and target cells, the secretion kinetics of cytokines and lytic molecules varied over time [13]. This indicates that a standard culture duration for ELISA (generally 24 or 48 h) may not be optimal, depending on the read-out.

Also the technique used to read out the assay can influence potency assay suitability. In the aforementioned study by Finke *et al.*, short-term and midterm assays were performed with chromium-labeled target cells, while the long-term assay determined survival of target cells via crystal violet staining. The latter may overestimate survival (and thereby underestimate effector cell potency) when target cells proliferate during culture [114]. In addition, these more conventional assays are not able to distinguish between both actors in cytolysis: effector cell activation (such as degranulation capacity and expression of FasL) and target cell killing (e.g., induction of apoptotic pathways and actual death). Although simple single-color assays with only target cell labeling and dual-color assays with cell-specific CD marker labeling could distinguish between effector and responder cell populations, such an assay will not be sufficient for proper potency testing, because, for example, specific and non-specific target cell loss cannot be differentiated [49,109]. Therefore, a multi-color analysis using flow cytometry would be most appropriate (for characterization studies) as distinct effects on the different cells can be evaluated simultaneously [82]. Moreover, this technique can combine frequency and different functions at a single cell level. For example, the simultaneous measurement of several CD markers and cytokines would lead to information about the direct and indirect (i.e., micro-environmental change) influence of different effector cell types (NK cells, CD8<sup>+</sup> T cells, etc.) on potential tumor rejection [67]. Nevertheless, flow cytometric-based assays may require more cells than available and a company may therefore have to choose a more material-suited potency test [71].

#### Cell types

Characterization studies are required not only to substantiate assay and read-out type and additional assay optimization, but also to determine the most relevant effector and responder cell types, ratios and interaction times. Testing several effector to responder cell ratios (that appeared physiologically relevant in non-clinical studies) will result in choosing the optimal ratio and at the same time prevent misinterpretation of data and needlessly rejecting a useful potency assay. Within the effector cell population, it may also be necessary to determine the most relevant CD4<sup>+</sup> to CD8<sup>+</sup> T cell ratio. In addition, the optimal interaction time between effector and responder cells needed for induction of cell death (both

via rapid and slow routes), and the time needed for actual cell death to occur (i.e., able to measure) should be evaluated during potency assay development.

#### *Cell types in the product*

For TIL therapy both CD4<sup>+</sup> and CD8<sup>+</sup> T cells may be used to attack the malignancy, whereas for anti-neoplastic CART cell manufacture, mostly CD8<sup>+</sup> T cells are used. However, medicinal products could still consist of a population with (potentially essential) heterogeneity in cell fate and function and other immune cells than T cells can also significantly contribute to the anticipated anti-tumor response [2,3,115,116]. For example, Finke *et al.* showed that *ex vivo* TIL expansion with IL-2 resulted in an increase in CD56<sup>+</sup> cells (next to T cells), which may display significant (non-specific) cytotoxicity [98]. Moreover, a medicinal product is expected to contain cells that have a long-term *in vivo* survival or can form a pool of memory cells. Especially CD4<sup>+</sup> T cells appear to be important for the induction of proper CD8<sup>+</sup> T cell memory functions [64]. As mentioned earlier, non-clinical and clinical studies are nowadays focusing on the specific role of CD4<sup>+</sup> T cell subsets (alone or in combination with CD8<sup>+</sup> subsets) in the anti-tumor response [10–18]. Due to inherent differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells, their MoA in the anti-tumor response is also expected to be different. It has already been shown that, for specific medicinal products, CD4<sup>+</sup> T cells have a higher capacity to proliferate or produce specific cytokines but have no or less cytolytic activity than CD8<sup>+</sup> T cells [10,12,13,18]. Still, *in vitro* and *ex vivo* functionality of these CD4<sup>+</sup> T cells before and during treatment is determined with the same assays as for products containing only CD8<sup>+</sup> T cells or the complete CD3<sup>+</sup> T cell population. The suitability of *in vitro* potency assays for products containing a significant amount of CD4<sup>+</sup> T cells will require further evaluation.

Taken together, for the design of a relevant potency assay, the contribution of the different cell (sub-)types to the *in vivo* potency should be evaluated.

#### *Tumor cells*

The type and quality of the antigen-specific tumor cells as responders (or stimulators) is essential to induce an optimal *in vitro* T cell response. For example, Baldan *et al.* showed that the digestion method of the tumor cells influenced the amount of surface MHC molecules and thereby the antigen presentation capacity [22]. Therefore, improved methods for isolation, digestion and culture of tumors are required to produce (stimulator or responder) cells that are consistently recognized by T cells. Furthermore, the targets in the potency assay are usually surrogate (including artificial antigen loading instead of endogenous antigen processing) or easy-to-kill instead of autologous tumor

cells and may not reflect the actual *in vivo* situation in which tumor cells may up- or downregulate specific surface proteins or display an altered antigen expression [71]. In some cases, non-tumor cells are proposed as *in vitro* targets, for example, autologous antigen-presenting DCs or CD19<sup>+</sup> B cells. Tumor cells, however, are expected to have a different metabolic activity and genetic background (with more aberrations) than normal cells, making the latter less representative for the *in vivo* situation. However, use of primary tumor cells also has limitations, such as difficulties with the isolation and *in vitro* culture of viable cells (without contaminating non-tumor cells) or significant heterogeneity between and within individual tumors [117]. Use of cell lines may therefore lead to more practical and robust assays. Especially when cell lines with different levels of the target antigen (such as higher CD19<sup>+</sup> and CD22<sup>+</sup> expression on Raji cells vs. Daudi cells [118]) are used. The suitability of these target cells, including their antigen-presentation capacity or lysability in the assay, and the ratio and interaction time between target and effector cells used, should then be evaluated with respect to the relevance for the *in vivo* target cells [67,107,119].

Because the T cell cytotoxic potential is related to, among other factors, target cell lysability and effector to target cell ratio, the method of quantifying this cytotoxic potential requires attention. Cytotoxicity is usually quantitated as the percentage specific lysis (or survival) of target cells. However, Finke *et al.* expressed cytotoxicity as lytic units (i.e., the number of effector cells required for 15% specific lysis) [98]. The advantage of lytic units is that the real potency of cells for a specific amount of lysis is determined. Nevertheless, the clinical relevance of the percentage lysis used as potency threshold would have to be made clear.

It may also be useful to gain more insight in the local (immunosuppressive) tumor environment and mechanisms of tumor immune escape—for example, by identifying the phenotype of surviving target cells and suppressive immune cells in a co-culture assay, but also by determining whether target cells and the culture conditions used may be sufficiently representative for the *in vivo* situation [21,34,96,116,117].

Together, this implicates that a potency assay for release or characterization should contain several control samples that at least exclude the presence of antigen-a-specific effects (e.g., by using target cells expressing an unrelated antigen), effects by other cell types than the supposed effector cells (e.g., by using a target sensitive to NK lysis) and effector cell a-specific effects (e.g., by culturing target cells without effectors).

#### *Other influencing factors*

It should be acknowledged that *in vitro* immunological assays only represent a snapshot of the dynamic



immune response taking place. *In vivo*, significant changes within the immune response systemically, as well as locally in the tumor environment, take place, due to differentiation of cells, variations in immune cells homing receptor expression and adaptations of the antigenic profile of target cells [33,82]. This demonstrates the challenge and importance of product characterization to identify markers that reflect the *in vivo* MoA and dynamics. Consistency of the product characteristics that determine *in vivo* potency should be ensured by the *in vitro* potency test and other release assays.

Also, the use of concurrent therapies (e.g., high doses of IL-2 during TIL administration or the use of cyclophosphamide to deplete regulatory cells) can influence the general *in vivo* immune response or the response of specific cells and should therefore be taken into account in the development of a relevant potency assay [27,44,77,120]. Moreover, addition of specific cytokines (e.g., IFNs) during the culture and expansion of (tumor) cells could influence the expression of certain cell surface receptors, for example, MHC molecules, and thereby affect the applicability of these cells in a relevant potency assay [119]. Furthermore, restimulation of antigen-specific T cells to obtain more cells is suggested in case insufficient patient material is available [67]. However, this approach could lead to artificial induction of antigen-specific cells and should only be considered (for characterization purposes) when the functionality of the cells is not altered.

### Immunomonitoring

T cell functionality testing is required not only for release; during the treatment period in clinical trials, it is also often monitored whether T cells retain their functionality *in vivo* (including the prerequisites for this functionality, as mentioned earlier). A comparison of the *in vitro* potency test results and the *in vivo* immunomonitoring could thereby further corroborate the relevance and representativeness of the *in vitro* potency assay.

Nevertheless, cells for immunomonitoring purposes are usually taken from blood instead of tissue, and the loss of cell-cell contacts may influence their *ex vivo* behavior. To detect the presence and determine the functionality of antigen-specific T cells, inclusion of a short pre-culture of blood-derived cells at high density would allow restoration of original reactivity via cell-cell contacts (i.e., the so-called RESTORE protocol) [121]. By increasing T cell antigen sensitivity, *ex vivo* activity was expected to more or less mimic the responses within the tumor tissue. These may differ substantially from responses in peripheral blood, where relative numbers of specific immune cells are different and local immunosuppression is absent.

In that study, however, relevance of optimizing antigen specificity for T cell functionality was only determined as enhanced IFN- $\gamma$  secretion, but was not further analyzed via actual cytotoxicity testing [121].

### Future perspectives and conclusion

We have focused on potency assays for T cell-based medicinal products as anti-neoplastic therapies, because this is the majority of products currently registered or in clinical development. However, T cells or specific subsets are also tested for their usability in the treatment of autoimmune diseases, transplantation and immune reconstitution, among other applications. For example, CAR techniques are also used to develop regulatory T cells with specificity to a disease-relevant antigen for the establishment of immune tolerance and reduction of unwanted immune responses (e.g. [122–126]). Several strategies are attempted to optimize cell isolation and transduction methods or to combine therapeutic approaches for improvement of CAR constructs and production of antigen-specific effector T cells (or T cell subsets). Using directed functions or multiple target recognition capabilities, overcoming physical barriers and immunosuppression in the tumor environment without increasing the risk of self-recognition and destruction has been attempted [1,9,12,23–25,27,32,33,40,41,45,46,48,64,99,120]. Although some applications are still in their infancy and many unanswered questions remain, promising results drive further investigation to ultimately overcome the hurdles for clinical translation of experimental cellular therapies.

Whatever their indication, each *ex vivo* expanded (transduced) T cell-based medicinal product is considered unique, because of disease impact on (autologous) starting material and differences between isolation, culturing and manipulation methods, for example. Consequently, individual T cell products require product specific characterization studies and quality control assays, including a functionality test. Such assays should be based on the (predicted) *in vivo* MoA. Nevertheless, potency assays for T cell-based medicinal products are assessed on a case-by-case basis, and no tests can be precluded in advance. Therefore, assays based on T cell properties directly related to the MoA as well as tests based on surrogate markers may be appropriate, provided that sufficient product-specific nonclinical and clinical data can justify the relevance of the proposed assay and read-out.

For tumor-specific T cells, the anticipated *in vivo* MoA consists of tumor recognition and attack. Thus, *in vitro* potency assays based on the cytotoxic potential of T cells resulting in target cell death are most evident. As discussed, several good assays for measuring



this tumor-specific immune response *in vitro* are available. However, a potent *in vitro* response may not always correlate with, for example, clinical tumor regression, prolonged time to progression or patient survival. Therefore, it is important to include multiple parameters during characterization studies. A combination of identification of antigen-specific T cells and determination of their cytotoxic (and cytokine-producing) functionality would be optimal. Homing or persistence markers could also be included given that the presence of specific receptors on the cell surface could identify cells that would be able to migrate toward and into the tumor tissue [27]. Thus, the combination of (i) T cell phenotypic markers and (ii) (surrogate) markers for T cell cytotoxic potential and/or (iii) cell death markers to detect killing of (labeled) target cells could be used as a combined determination of identity, frequency, activation status and functionality of the drug product in one multi-color flow cytometric-based assay. This extensive characterization should then identify the most optimal and robust (surrogate) potency assay for release, based on a proper correlation with clinical results [116]. Obviously, this will include the determination of appropriate acceptance criteria to distinguish potent and sub-potent batches.

Nevertheless, it is acknowledged that variability in the (patient-specific) starting material may result in considerably variable potency results, making it challenging to set appropriate specifications. Clinical results should therefore be taken into account in setting these criteria. Ideally, the individual patient clinical outcomes should be linked to the potency data of the individual batches. This would provide evidence that the proposed potency assay(s) and the range of acceptance are related to a positive patient response.

Many studies have already been performed to start unraveling the complex interaction of immune cells with and within the tumor environment. With increased *in vivo* knowledge, improved and adapted manufacturing processes for T cell-based products are expected to be established. This will inevitably have implications for quality control testing and lead to optimized guidance for potency assay development.

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