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Antigen-specific activation and cytokine-facilitated expansion of naive, human CD8+ T cells

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Abstract

Antigen-specific priming of human, naïve T-cells has been difficult to assess. Due to the low initial frequency in the naïve cell pool of specific T-cell precursors, such an analysis has been obscured by the requirements for repeated stimulations and prolonged culture time. In this protocol, we describe how to rapidly evaluate antigen-specific priming of $CD8^+$ -cells following a single stimulation. The assay provides reference conditions, which result in the expansion of a significant population of antigen-specific T-cells from the naïve repertoire. Various conditions and modifications during the priming process (e.g. testing new cytokines, costimulators, etc.) can now be directly compared to the reference conditions. Factors relevant to achieving effective priming include the dendritic cell preparation, the T-cell preparation, the cell ratio at the time of priming, the serum source used for the experiment, and the timing of addition and concentration of the cytokines used for expansion. This protocol is relevant for human immunology, vaccine biology and drug development.

Introduction

The initial antigen encounter of a naïve T-cell with its cognate antigen is generally referred to as *priming*. It should be noted that the term *priming* has sometimes been used ambiguously to reflect incubation of cells prior to activation with cytokines/reagents regardless of the TCR-trigger, but in the context of this paper we will use priming to reflect the initial activation of naïve T-cells following encounter with their respective cognate peptide in the context of an MHC molecule.

A successful first encounter, resulting in the generation and expansion of functional T-cells, requires a sequence of signals, carefully orchestrated *in vivo* by professional antigen-presenting cells (APCs). Upon stimulation, T-cells proliferate and differentiate into effector and memory T-cells. The magnitude of this T-cell response, as well as the degree and functional characteristics acquired during differentiation are – at least in part – programmed

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by the signals provided during this initial priming step[1]. Thus the priming process shapes the resulting immune response and is key to our understanding how T-cell responses evolve [2, 3].

Methods to investigate antigen-specific priming

However, systematic studies on antigen-specific priming have been hampered by the exceedingly low frequency for each TCR-specificity within the vast diversity of the repertoire of naïve T-cell precursors. Animal models enable analysis of evolving immune responses to infectious model antigens, such as LCMV in mice, which simulates effective or dysfunctional T-cell responses depending on the viral variant of LCMV[4]. Furthermore TCR-transgenic mice, in which virtually all of their T-cells are specific for a defined epitope, have been extremely valuable to our understanding of basic concepts regarding T-cell- and tumor-immunology[5-7]. However mouse immunology differs in many aspects from the human immune system[8], and strategies to validate results from small animal models for translation to human immunobiology are needed to advance current approaches in immunotherapy and vaccine development[9]. Vaccinologists and virologists have increasingly resorted to testing non-human primates, but these studies are rightfully restricted to only very key questions. Thus, for ethical, regulatory and financial reasons, studies in monkeys are limited to few specialized laboratories [10, 11].

Developing principles of antigen-specific priming of human T-cells *in vitro* has been hindered by the variability of T-cell responses observed not only between individual donors but more importantly in- experiments performed from the same individual. This variability is generally attributed to the low and varying T-cell precursor frequency. In fact, repetitive stimulation of T-cell lines is frequently used as the method required to reach the level of detection. However, such repetitive stimulation requiring a prolonged time period has made it almost impossible to draw plausible conclusions about the initial priming process (Fig. 1).

In 1994 two groups identified an antigen overexpressed in melanoma, which was recognized by a large number of tumor-infiltrating T-cells isolated from patients. The gene was independently termed Melan-A[12] or MART-1[13] (for simplification, we will refer to this protein as *Melan-A*). An immunogenic peptide, restricted to the HLA-A0201-allele, was identified as AAGIGILTV corresponding to position 27-35 of the Melan-A protein[14], and was the target of the immune-dominant response in HLA-A0201^{+} individuals. In addition to becoming used as a major immunogenic target in melanoma[15], which we will not discuss in detail, this epitope has become the prototypical antigen for probing naïve T-cell responses in human T-cells.

The reason why this peptide epitope Melan-A$_{27-35}$, or the corresponding decamer from positions 26-35, can be used as a model antigen reflects the fact that the frequency of naïve T-cells specific for this epitope is about 10 fold higher than frequencies observed against other self- or tumor-associated antigens[16, 17]. Precisely what is responsible for this increased frequency (about 1 in 1000 naïve T-cells) is not completely understood, but enhanced positive selection in the thymus[18] and/or crossreactivity of T-cells with various other specificities have been described[19]. In addition to cell phenotype, analysis of TCR excision circles and telomere length, have shown that Melan-A-specific T-cells in normal persons display characteristics similar to naïve T-cells, with none or only one potential additional

round of expansion detected[18]. Valmori et al. discovered that exchange of the alanine to leucine in position 27 greatly increased binding to HLA-A0201, thereby enhancing the immunogenicity of this epitope. Significant chemical and immunological differences between the known epitopes, the nonamer 27-35, the decamer 26-35, and the heteroclitic peptide 26-35_{A27L} exist. An extensive summary about the biochemical properties, immunogenicity and clinical use of this peptide was provided by Romero et al[15].

Advantages and applications of the current protocol

Using this model antigen (xxx) that has a distinctly high frequency of reactive naïve T cells, we developed an *in vitro* priming system to reliably assess priming conditions for $CD8^+$ T-cells. This method, which we call ACE-CD8 for **A**ntigen-**S**pecific **A**ctivation and **P**riming of human **T**-cells, focuses on the encounter of effectively matured, peptide-loaded dendritic cells with highly purified naïve $CD8^+$ T-cells (Fig.2). ACE-CD8 defines conditions, which, following a single stimulation, will lead to the rapid expansion of Melan-A-specific T-cells within a short culture time. The protocol described here for ACE-CD8 is highly reproducible, thus the experimental variability frequently reported following the use of other published protocols is seen to a much lesser extent compared to that seen using this protocol (Table 1, Fig.3). ACE-CD8 therefore allows analysis of the impact of further variables (e.g. new cytokines, chemical compounds or drugs) at the time of priming with a T-cell read-out providing functional and quantitative data. Even if Melan-A or this specific priming regimen is not the prime focus of an intended experiment, e.g. when assessing the immunogenicity of alternative peptide antigens or proteins, employing ASAP-T8, including the Melan-A peptide as a reference peptide, provides a validated, internal control for the priming and expansion conditions, and should clarify if the experimental conditions of a specific experiment have been sub-optimal.

There are various potential applications of this protocol, depending on the specific research question: ACE-CD8 may be used, to test the immunogenicity of defined peptides and altered peptide ligands, allows analysis of the antigen-specific T-cell repertoire as well as the enumeration of T-cell precursor frequencies. Moreover, it is especially suited to screen factors that may affect dendritic cell and/or T-cell function during the initial priming step. Using ASAP-T8, and slightly modified prior versions of this protocol, we were able to probe the naïve T-cell repertoire of T-cells specific for viral variants of HCV[20], assessed the impact of differentially matured DCs on priming[21], and identified novel peptide epitopes for the Wilms Tumor antigen 1[22], a protein overexpressed in leukemia and many other malignancies. Recently, we used ACE-CD8 to analyze the impact of a small molecule drug, dasatinib, on the priming of human T-cells[23]. This drug, which is used clinically as a brc/abl-kinase inhibitor, also can block TCR-triggering through inhibition of the src-kinase Lck[24]. Using ASAP-T8, however, it was observed that dasatinib has a strong stimulatory activity on DCs, synergistically enhancing IL12 in combination with a TLR4-agonist. Thus, by dissecting the priming process into different steps – in this case incubating only the maturing DCs with the drug – it became possible to identify a so far unknown mechanism of a widely used drug. Interestingly, when testing mouse and rhesus macaque DCs, we did not see the same effect, possibly due to different binding properties of the drug, but the results emphasize the need for valid immunologic test systems using human cells.

Comparison with other methods for antigen-specific priming of human T-cells in vitro

When searching the literature, it quickly becomes clear that most protocols differ from each other in many seemingly small but in the end crucial details. In addition to the use of different epitope variants of the Melan-A-epitope (the nonamer, the decamer and the heteroclitic variant), many different forms of antigen-presentation are being used: Table 1 lists representative studies using the Melan-A-epitope as a model antigen. Differently generated and differently matured dendritic cells, as well as CD40 L-activated B-cells[25] and artificial APCs[26] have been used to present the antigen. Even when focusing on the use of only DCs as presenting cells, it has become clear that there are a multitude of ways to induce a functional state called “mature”. This mature state, however, is ill-defined, as upregulation of co-stimulatory factors, cytokine production, migration, endocytosis and priming of T-cells are rarely quantitatively assessed and compared within one set of experiments. In fact a systematic analysis of differentially matured DCs with regard to their priming capacity of T-cells is far from complete. Using ACE-CD8 as a model system, we have begun to decipher the many details in the DC maturation process that impact the resulting T-cell response, and has revealed how variable and versatile this APC remains[21].

In the studies described in table 1, additional factors confound the comparability of the individual analyses. Results from 1-6 rounds of stimulation, different use of cytokines for T-cell expansion, and variation in the purity of the starting cell population have been reported. All of these factors explain the largely variable results observed across the different studies. In fact even within the same priming system, results from repeated experiments are rarely reported. Thus, the resulting T-cell responses strongly vary from paper to paper, and presumably from experiment to experiment, making direct comparison of different variables almost impossible.

Artificial APCs have been used as one alternative for analyzing *in vitro* priming. Immortalized cell lines (such as K562 or fibroblasts) can be transfected with the desired HLA-allele along with a set of co-stimulatory molecules[26]. Using this approach with murine cells, it was shown that a single brief encounter of the T-cell with its cognate antigen is sufficient to set off the expansion and differentiation of the T-cell[27]. For human cells, in the context of stimulation with professional APCs such as DCs providing additional signals (e.g. IL-12), less information is available.

Instead of genetically modified cells, the alternative approach is to use beads and coat the necessary molecules on their surface. This approach may serve as an off-the-shelf, lego-type-system [28, 29] to help understand the role of individual molecules for priming. The strength of such a system lies in its modular design as well as the less limited supply for processing large numbers of T-cells, even if functional, autologous DCs are not available. Moreover defined characteristics of such stimulatory beads are advantageous when restimulation conditions after successful priming are in question. Whether these artificial systems really are a match for an optimally activated DC, naturally providing the full set of signals, including cytokine production in cis, is unproven.

Priming of T-cells using non-specific stimuli such as PMA, ionomycin, or anti-CD3-antibodies has often been reported as a means to analyze bulk T-cells within a short period

of time. Alternatively, fully HLA-mismatched allogeneic APC may serve as initial stimulators (a classical mixed leukocyte reaction), especially when assessing CD4 responses, as this increases the percentage of reactive naive T cells from the repertoire. All of these stimuli deliver a supraphysiological signal, and some bypass the need for co-stimulation or additional cytokines. This type of stimulation has been used to answer basic questions about proliferation and polarization characteristics of certain T-cell populations[30]. However, as all cells are stimulated simultaneously, results need to be interpreted with caution, as the magnitude and qualities if of the cells concurrently responding most likely have effects on each other, e.g. by producing cytokines and expressing ligands for stimulatory and inhibitory molecules. Therefore such results differ from a priming event, in which few individual cells are initially activated.

There have also been many reports on the activation of memory T-cells by various APCs. CMV-, ADV- or EBV-responses in healthy previously infected donors are generally robust, and specific T-cells for the respective immunodominant epitopes can be tracked easily using MHC-multimers[31]. Functional assays, such as antigen-specific proliferation assays, degranulation and cytotoxicity assays, or cytokine production in response to the respective APC are relatively easy to perform. However memory T-cells have different prerequisites for full activation as well as different expansion dynamics following a TCR trigger. Many cell types, not just professional APCs, may trigger a secondary T-cell response. Thus, memory responses cannot be used as an alternative method to study T-cell priming, but rather answer questions about the secondary response following *in vivo* priming.

Experimental design

The ASAP-T8-protocol can be divided in two major parts: first the preparation of DCs (step 1), and then the stimulation and expansion of the T-cells (steps 2-26). The following subsections discuss critical aspects of the protocol.

DC-preparation—The preparation of DCs and the quality of the maturation trigger is critical to achieve maximal expansion of the antigen-specific T-cells. In this protocol, we use monocyte-derived DCs cultured for 72h in GM-CSF and IL-4 containing medium. The cells are then lifted from the plate, resuspended in fresh medium containing GM-CSF, IL-4 and the maturation reagents LPS and IFNγ. At this time, the peptide epitope is added to the culture as well. 16 hours later, these cells are used for stimulating the naïve T-cell preparation.

Plastic-adherence is used for enriching monocytes as the initial selection step. This step includes a rinsing step, to get rid of the non-adherent fraction. Rinsing the plate with the appropriate pressure, requires practice: rinsing with too little pressure will result in a high number of contaminating lymphocytes, rinsing with too much pressure will wash away some monocytes leading to a low yield. In trained hands, this method gives rise to consistent DC populations with full stimulatory capacity.

For maturation of the DCs, LPS and IFNγ are used as the essential reagents to induce IL-12-production. IFNγ primes the promotor region of IL-12p40[32], whereas LPS activates IL-12 production via TLR4-triggering. In accordance with the kinetics of IL-12 induction,

maturing DC need to be used for priming within 16h, as longer incubation with the maturing reagents only leads to DC exhaustion[33]. Unfortunately, the widely used maturation cocktail based on a mixture of pro-inflammatory cytokines (IL1ß, TNF-α, IL-6 and PGE_2)[34] does not lead to IL-12 production by DC, as IL-12p35 is dose-dependently inhibited by prostaglandin E_2 [35], and, as a consequence, such DCs show only limited priming capacity (Fig. 4a-c)[23].

R848, a TLR7/8 agonist, in combination with IFNγ also induces IL-12 in the DCs, leading to a good specific expansion and full T-cell function. It may therefore serve as an alternative maturation cocktail, although it has not been tested in our hands as extensively as LPS/IFNγ.

IL-12 production has been, in our hands, the only factor that correlated with fully functional Th1-primed T-cell populations. Thus quality controls on DC preparation should include assessment of IL-12 either by ELISA (for IL12p70) or by intracellular cytokine staining after a 16h stimulation in the presence of brefeldin A. Note that there are significant differences between the staining quality of different antibodies. We routinely use the Alexa-Fluor488-labeled IL12p40 antibody from Affymetrix/ebiosciences (Clone: C8.6, order no. 53-7129). Surface marker expression was not sufficiently reliable to distinguish between optimal (IL-12 producing) and sub-optimal (IL-12 non-producers) DC populations.

The matured DCs are irradiated (30Gy) prior to coincubation with T-cells for priming. This prevents contamination with NK-cells or non-naïve T-cells present in the DC preparation. Unless this fraction is irradiated, memory and NK-cells may proliferate vigourously to IL-15, thus competing with the primed T-cells. In fact outgrowth of non-CD8 cells (usually NK-cells) is still sometimes observed compromising the experiments results. For DCs, effects of irradiation have been controversely discussed[36, 37]. Treatment with mitomycin C (followed by extensive washing), may be an alternative method, but has not been validated in our hands so far.

Purity of the T-cell preparation—There are several reasons why highly purified naïve T-cells are a more suitable starting population than total CD8 preparations or whole PBL: first, the fraction of naïve T-cells within the CD8 compartment varies from donor to donor. Therefore, unless purified naïve T-cells are used, the number of naïve cells in the starting well will vary from donor to donor. Moreover, the purity of naïve T-cells is critical, as effector memory T-cells may be cross-reactive and therefore interfere with T-cell priming. Furthermore, memory T-cells have a lower activation threshold and may be triggered solely by the cytokines added to the culture[38], and therefore could compete for cytokines as well as alter the relative ratio (percentage) of antigen-specific T-cells. The same argument holds true for contaminating NK-cells, which proliferate quickly in response to IL-15. Regulatory T-cells would also be present if using PBL, and the influence of such cells on the initial priming may add to variability between individuals[39]. Each T-cell preparation is therefore evaluated for the purity of the preparation using CD62L and CCR7 as positive markers for naïve T-cells and CD45RO and CD57 as exclusion markers. We generally demand the $CD62L^-CCR7^-$ fraction and/or $CD57^+$ and/or $CD45RO^+$ fraction to be less than 10%, indicating a naïve population of more than 90% (Fig. 6).

Initial cell numbers and cell ratios—ACE-CD8 starts with a T-cell number of 5×10^5 naïve T-cells per well in 0.5ml of complete medium in a 48-well plate. Given the approximate frequency of 0.1% Melan-A-specific T-cells within the naïve T-cell repertoire of a healthy donor, approximately 500 specific precursor T-cells will be in each well. The T-cell:DC ratio is adjusted to 4:1. Note that ASAP-T is validated for these conditions: changing the number of input cells, the size and volume of each well or interfering with the T cell:DC ratio may change the expansion rate significantly and requires side-by-side comparison (Fig. 4d). If alternative peptide epitopes are investigated, the precursor frequency is likely to be lower than for Melan-A. Nevertheless, we recommend using the same input-numbers, but increasing the number of parallel wells and evaluating them separately, as with lower precursor numbers there will likely be some negative wells and few positive cultures at the end of the 10 day period. Thus, this microculture-approach enables detection or rare cells by limiting the number of irrelevant cells per well (the rare events follow a Poisson distribution)(Fig. 5)[40].

Note that the set-up of a reliable experiments requires relatively large amounts of PBMC as a starting cell population. Down-scaling to very few cells in our hands negatively affects the overall experimental outcome, but may become feasible when using a 96 well format at the initial priming step.

Upscaling this procedure for clinical use is an ongoing project in the lab. It is possible to switch to larger flasks using primary cell numbers, but careful re-evaluation is needed regarding the initial culture volume, cell density, etc.

Cytokines: timing and concentration—For the initial stage of priming, IL-21 is the only exogenous cytokine added to the culture. The advantage of using IL-21 at this initial step was first shown by Li et al.[41]. All other cytokines required for sufficient priming are produced by the DCs or by the T-cells themselves. Formation of small proliferative clusters by day 3 of culture suggest that, within these clusters, secreted cytokines (e.g. IL-12, IL-2 and/or IL-15) or cell contact may provide a local advantage for the responding antigen-specific cells. In fact, when gently washing away un-clustered cells, enrichment of antigen-specific T-cells can already be observed in the clustered fraction (personal observation). IL-21 maintains the proliferative capacity of the T-cells and, to some extent, maintains the expression of a favorable phenotype (e.g. expression of CD28)[42]. For robust and strong expansion, addition of this cytokine is essential (Fig. 4d). Adding IL-7 and IL-15 in a relatively low concentration (5ng/ml each) 3 days after the initial priming step (and subsequently every 2-3 days) is based on the observation that T-cell responses to cognate antigen are boosted by these cytokines[38]. During the first 3 days of culture in the absence of IL7 and IL-15, a proliferative advantage is provided to cells receiving a TCR-stimulus, whereas non-specific cells may be “starved” during this period[43]. Indeed total cell numbers per well decrease by day 3 and dying/fading cells may be observed. Note that cytokine concentrations are kept intentionally low to limit cytokine-driven, non-specific homeostatic proliferation[44].

Moreover, addition of IL-2, which is used in many protocols, is not necessary in this protocol, as it is known to drive cells towards terminal differentiation[45]. Note that for extended culture beyond day 15, addition of IL-2 is beneficial.

Serum and culture medium—The quality of the AB serum used for culture is critical for the robustness and the strength of the proliferative response. We have noted significant serum batch-to-batch variations with regard to total proliferation of the cells. This has great implications, as sometimes several batches need to be tested: ideally a priming experiment is set up using the new serum batch is used in parallel to the old, validated serum batch. Once a suitable batch has been identified, purchase of a large amount of serum can be costly and requires room for safe storage. However having the same serum batch available throughout a whole set of experiments reduces inter-assay-variation significantly. One way to start the comparison of different serum batches is to include autologous serum off-the clot from the healthy donor of the PBMCs. This may not necessarily be the best serum batch, but qualifies as a baseline. If analyzing patient cells, autologous serum is generally not recommended, as interference with disease-related factors in the serum (drugs, inhibitory cytokines, etc.) cannot be excluded [46].

Also note, that the serum concentration in the medium has a significant impact, which may not necessarily be reflected in the percentage of antigen-specific T-cells, but rather in the total T-cell proliferation (e.g. using 3% human serum rather than the standard 5% human serum resulted in a 66% decrease in the absolute number of Melan-A-specific T-cells (Table 2)).

A frequently asked question is whether media x, y, or z may not be equally suitable for expansion. We have only tested a certain number of different culture media and found the Cellgenix DC medium to be best in terms of expansion of specific cells and total proliferative capacity of the T-cells. Side-by-side comparisons should be performed if introducing a new medium (Table 2).

Experimental variation—One critical issue is reproducibility including intra-assay and inter-assay variation. As a measure of inter-assay variation we plotted representative data from 17 independent experiments, performed by two technicians within the last 1.5 years (Fig. 3). The mean of the first 3 wells of each experiment from the standard expansion group is shown (Fig. 3a). The coefficient of variation (*CV*), indicating the ratio of standard deviation and mean in percent is plotted in Fig. 3b to illustrate the spread of the data. Except for three outliers with higher *CV*s, the majority of experiments showed a *CV* between 3-20%. Given the nature of a biological assay, which extended over a 10 day period, and the small n (3 wells per group), we consider this *CV* as an indicator for acceptably low inter-assay-variability. These data may also be used to define a lower cutoff for the percentage of Melan-A-multimer^{+} cell lines: the 5th percentile of this experimental series is 24%. Thus in any given experiment, we expect at least 24% Melan-A-specific T-cells within the experimental standard group (mean results out of three wells), which serves as a positive control (see also Fig. 6). From a few donor leukapheresis specimens, we observed a particularly high cell expansion (e.g. compare absolute numbers in Fig. 4d vs. 4e). Whether such “super-donors” reflect biologic characteristics (e.g. HLA-A02 homozygosity,

polymorphism, etc.) or depend on certain technical issues at the time of collection (apheresis, freezing) has not been studied in detail.

If different factors are to be analyzed using ACE-CD8 (e.g. novel cytokines or drugs), evaluation for each individual experimental group should be based on several separate wells per group. For screening assays, e.g. testing new compounds, the number of wells per experimental group may be reduced (for pilot experiments we often reduce the standard group to two parallel wells (Fig. 6). For in-depth evaluation of certain conditions, or testing new peptide epitopes with an unknown precursor frequency, higher numbers of parallel wells (depending on the statistical considerations) are beneficial.

Experimenter variability is an important factor for observed inter-assay variability and robustness. Adherence to the protocol should greatly reduce such experimenter variability. The biggest variation may occur at the time of removing non-adherent cells from the monocyte-layer (see above and step 5). Washing intensity varies from person-to-person and training as well as frequent controls based on analysis with a microscope are required. In our lab, students with basic cell culture experience easily pick up the protocol details, allowing them to get valid results from the very first experiment.

When assessing T-cell responses against different epitopes, experimental variation stems mostly from the sporadic events representing the clonal expansion of a single cell. Peptides differ in their response rates, and systematic evaluation of the T-cell repertoire of a specific donor is possible, revealing differences in the immunogenicity of different peptide antigens. For example peptide epitopes reliably leading to a T-cell response well above the detection limit are STEAP1(292-300 I293L)[47], PRAME(435-443)[48], gp100(209-217 T210M) or MELOE1[49]. On the other hand, reponses to glioma associated antigens $EphA2_{(288-891)}$ or $IL13R\alpha_{(345-353)}$ have been sporadic at best.

At very low precursor frequencies, the experimental set-up is similar to classical limiting dilution assays where each positive well indicates at least one specific T-cell precursor [50] . Thus the minimum precursor frequency may be estimated by the number of positive wells/ number of total input cells. For example, the frequency of gp100-specific T-cells in the donor shown in Fig. 6 must be at least $3/2.5 \times 10^6$ (3 positive wells, 3 in $5\times5\times10^5$ naïve T-cells). Distribution of such rare events follows a Poisson distribution, as has been discussed for limiting dilution assays[50]. Precision will be increased, when testing a larger number of wells (eg. 20 parallel wells). Note that such calculations are estimates only; more elaborate statistical analyses are required for an exact determination of the precursor frequency.[51, 52]

Experimental read-out

ASAP-T is designed to provide sufficient numbers of antigen-specific T-cells for quantitative, phenotypic and functional characterization.

The expanded T-cells have an expansion history of 10 days under strong Th1-driven culture conditions. This has to be kept in mind, when interpreting the data. The T-cell status immediately after priming remains difficult to assess due to a lack of sufficient T-cells. Detection and analysis of T-cells using MHC-multimers can be done for Melan-A as early

as 5 days after priming, but only few cells will be available at this early time-point. Questions regarding e.g. TCR triggering and subsequent signaling during the priming event cannot be answered properly due to the low number of specific T-cells at the time of priming. Ultimately the outcome of the priming process is of interest here, e.g. when introducing a new variable at the time of priming, and this may be assessed after 10 days of culture.

Each experimental group is started in multiple wells (3-6, depending on the analyses required) and analysis of each of the resulting cell lines separately adds to the validity of the data. Pooling cells from different wells within the experimental groups may be necessary for assays requiring large cell numbers, but evaluation of the individual wells by MHC-multimer-staining and cell counts prior to the pooling step is highly recommended. It should be noted that the timing of the assay with respect to the last medium/cytokine exchange may critically influence the functionality of the T-cells: the presence of high cytokine concentrations on the day prior to the assay may increase non-specific activation whereas failure to refresh the medium for longer than 72h increases the fraction of apoptotic/dysfunctional cells. T-cells may be kept in culture for up to 21 days without restimulation. After this time-point, restimulation is necessary to maintain sufficient cell numbers. Note that T-cells tested later than day 18 of culture may already display a different degree of differentiation. For cytotoxicity assays, selection of Melan-A-specific T-cells using MHC-multimers and magnetic beads allows differential comparison of generated T-cells lines even if the fraction of Melan-A-specific T-cells from a certain condition greatly differs from the lines generated under standard conditions (Box 2). Note that this purification is recommended 2 days prior to the assay, to avoid interference of the multimer with the TCR at the time of the assay.

Materials

Reagents

- CellGro Dendritic Cell Medium (CellGenix, cat no. 2005), store at 4°C
- Penicillin/Streptomycin (PAA, cat. no. P11-010)
- Dulbecco's PBS (PAA, cat. no. H15-002)
- Human Serum Type AB CRITICAL We have used PAA, cat. no. C15-021, however this serum is no longer available commercially. We recently tested Biochrom's AB serum with positive results.
- Recombinant Human IL4 (CellGenix, cat. no. 1403) (alternative source: Peprotech)
- Recombinant Human GM-CSF (Gentaur, cat. no. 04-RHUGM-CSF-300MCG)
- Human IFN-γ (Peprotech, cat. no. 300-02)
- Human IL7 (CellGenix, cat. no. 1410) (alternative source: Peprotech)
- Human IL15 (Cellgenix, cat. no.. 1413) (alternative source: Peprotech)
- Human IL21 (Cellgenix, cat. no. 1419) (alternative source: Peprotech)

- Melan-A (ELAGIGILTV, jpt)
- $CD8^+$ T-Zell-Isolation Kit human (Miltenyi Biotec, cat. no. 130-094-156)
- CD45RO-Beads (Miltenyi Biotec, cat. no. 130-046-001)
- CD57-Beads (Miltenyi Biotec, cat. no. 130-092-073)
- LD-Column (Miltenyi Biotec, cat. no. 130-042-901)
- LS^+ positive Column (Miltenyi Biotec, 130-042-401)
- Countess Cell Counting Chambers (Invitrogen, cat. no. C10228)

Antibodies and staining reagents

- anti-human CD8α PerCp-Cy5.5 (Biolegend, Cat. No. 300928)
- anti-human CD57-FITC (Biolegend, cat. no. 322306)
- anti-human CD45RO-PE (Biolegend, cat. no. 304206)
- anti-human CD62L-FITC (eBiosience, cat. no. 11-0629-42)
- anti-human CCR7-PE (R&D Systems, Cat. No. FAB197P)
- MelanA-Dextramer (Immudex, cat. no. WB2162-APC)

  Additional antibodies for final analysis, depending on the desired panel.

Plastic Ware

- 48-Well-Plate, sterile (GBO, cat. no. 677 180)
- 12-Well-Plate, sterile (GBO, cat. no. 655 180)
- 6-Well-Plate, sterile (GBO, cat. no. 657160)

Equipment

- Countess Cell Counter (Invitrogen) or Neubauer hemocytometer
- Microscop (Leika DMIL)
- Fluorescence-activated cell sorting (FACS) instrument (2 lasers minimum)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- QuadroMACS Separator (Miltenyi Biotec, cat. no. 130-090-976)
- Cell incubator

Reagent Setup

Peripheral Blood Leukocytes (PBL): We obtain these from unstimulated apheresis products. We immediately process apheresis products (no overnight storage). Cells are ficolled and subsequently aliquoted in vials containing 3×10^8 or 1×10^8 cells respectively. Freezing medium contains CG medium (50%), autologous plasma (40%) and DMSO (10%). Cells are then placed in a temperature controlled freezing container (Mr. Frosty) and stored

at -80°C overnight before transfer to liquid nitrogen. To thaw cells, vials are quickly placed in a water-bath and transferred to cold PBS/5%HS (20ml) immediately after thawing. PBMC are immediately spun down and resuspended once more in PBS/1%HS, spun again and then used for further assays.

Human AB serum: We purchase in frozen flasks of 500ml and store directly at -20°C. Individual flasks are slowly thawed (at 4°C overnight), aliquoted in 50ml tubes and frozen again (see note regarding differences in serum quality).

Dendritic cell medium: For dendritic cell preparation, supplement CellGro DC medium with penicillin/streptomycin (v/v) and 1% human AB serum (v/v).

T-cell medium: For T cell expansion, supplement CellGro DC medium with penicillin/ streptomycin (1% v/v) and 5% human AB serum (v/v).

Cytokines: Many cytokine concentrations are indicated in IU/ml, which is the correct form to compare on the basis of functional activity. However for some cytokines, depending on the vendor, this information is not available. Values can differ greatly from company to company, suggesting that measurement of activity may have its challenges. We therefore provide information in ng/µl referring to specific cytokines of specific companies. Adjustments need to be made, if cytokines from different vendors are being used. The biggest difference from this protocol to the values published in many protocols is the concentration of IL-4. Originally concentrations of 1000IU/ml were reported for DC-differentiation[34]. We titrated down this concentration and found 10ng/ml, which equals 50IU/ml (with our cytokine batch) to be sufficient to support DC differentiation. We dilute all the following cytokines in PBS/0.1% HS and store in aliquots at -20°C. The concentrations vary, as indicated in the following table:

Cytokine	Concentration
IL-4 10ng/µl	
GM-CSF 86ng/µl (which equals 800IU/µl) **IL-7** 10ng/µl **IL-1**5 10ng/µl **IL-21** 30ng/µl	
IFN 10ng/µl	

Peptides: Dissolve peptides in DMSO to a concentration of 5µg/µl. For the Melan-A peptide, which has a molecular weight of 982, this stock solution thus equals 5mM. Store aliquots at -80°C. Note that stability in DMSO is limited to approx. 2 months.

Procedure

Generation of monocyte-derived dendritic cells and naïve T-cells

1) Generate monocyte-derived dendritic cells and prepare naïve T cells as described in options A and B respectively. Option A should be started first and then option B should be started one day prior to T-cell stimulation (step X) and on the same day that DC maturation is induced (step X, denoted as day -1). PBL from the same donor must be used for both option A and B.

A) Generation of monocyte-derived dendritic cells

i. Thaw a vial of PBL rapidly by diluting the cells in at least 20ml of cold wash buffer. Count cells and perform a routine spin. (A routine spin as mentioned throughout this protocol consists of 5min of centrifugation at 500g.) For standard sized assays, start with at least 1×10^8 PBL.

ii. Resuspend cells in pre-warmed Cellgenix DC (CG-DC) medium/1% human serum to achieve a concentration of 1×10^7/ml.

iii. Pipette 2ml of this cell suspension in each well of a 6-well plate. Prepare at least 2-5 6-well plates.

iv. Incubate cells for 2-3h to allow adherence to plastic.

v. Gently resuspend non-adherent cells by using a blue 1ml-tip, allowing medium to run over the bottom of the well to wash non-adherent cells off. Also rinse the corners of each well.

Critical step: Applying the correct pressure in washing the cells off takes practice. Check results frequently with a microscope. Ideally, a monolayer of larger cells can be seen, with little round, small lymphocytes remaining as contaminants. Plastic adherence of monocytes differs depending on the plate manufacturer. If other manufacturers are used, adherence and DC recovery may vary.

vi. Add 3ml of warm CG-DC-medium supplemented with 1% human serum and 10ng/ml IL-4 and 800IU/ml GM-CSF.

vii. Incubate cells for 2 days at 37°C.

viii. Add 1.5 ml of fresh CG-DC-medium supplemented with 10ng/ml IL-4 and 1600 IU/ml GM-CSF.

ix. Incubate cells for an additional 24 hours.

x. *Harvesting of immature DC.* Collect 3ml of the supernatant first, then vigorously resuspend the remaining medium to remove all cells. Check under the microscope and wash plate again using cold wash buffer, if necessary. Pool cells from all the wells and count.

xi. Spin cells and resuspend in pre-warmed CG-DC-medium, supplemented with 1% HS, GM-CSF (800IU/ml), IL4 (10ng/ml), LPS (10ng/ml), IFNγ (100IU/ml). Calculate volume depending on the cell number and number of groups needed for the assay. A minimum of 5×10^5 cells/well (optimal is $\sim2\times10^6$ cells/well) (2ml/well) is necessary to obtain sufficient numbers of semi-mature DC.

xii. Add peptide (2.5ug/ml = 2.5uM) to each well individually (Alternatively, if a large batch of DC is required to be loaded with the same peptide, peptide can be added to the initial CG-DC-medium mix).

Critical step: the assay is validated for DC that have been pulsed with single peptides for each individual well. Peptide mixtures can be used but interference

with peptide binding between peptides with a high versus low binding affinity is theoretically possible.

xiii. Incubate cells at 37°C for 16h.

B) Preparation of naïve T-cells

i. Thaw PBL as described in option A step i. For a normal-sized experiment, 1-2×10^8 PBL are required.

CRITICAL STEP For each experiment, naïve T-cells are isolated freshly from PBMC. We do not recommend freezing purified naïve T-cells.

ii. Resuspend thawed cells in cold wash buffer, count and spin.

iii. Use the CD8 untouched isolation kit (Miltenyi) following the instructions of the manufacturer (Box 1, **steps 1-11).** CD8 negative cells should be retained on the LS column, whereas $CD8^+$ cells should be selected in the flow through.

iv. Count cells and spin.

v. Use CD45RO- and CD57-beads to delete memory cells (Box 1, **option B).**

Critical step: CD57 depletion is necessary, as it depletes most of terminally differentiated cells, which may be $CD45RO^-$, $CD45RA^+$ (T_{EMRA}). The size of this subset greatly varies from donor to donor[30].

vi. Count cells, remove aliquot for FACS-analysis, and spin remaining cells.

vii. Resuspend cells in CG-DC-medium supplemented with 5% human serum and 5ng/ml IL7. The final cell concentration should be 3×10^6/ml. CRITICAL STEP Preincubation at this cell density in IL7-containing medium allows for optimal stimulation.

viii. Transfer cells to 6-well plates, 2ml/well and incubate over-night.

ix. Perform FACS staining. Stain T-cells for CD62L, CD45RA, CD45RO, CD57 and CD8. If desired, stain for contaminating NK-cells (CD56). The purity of the naïve cell population should be higher than 95%(Fig. 5a). Check purity for each experiment as variation in final results could be due to initial impurities in cell preparation.

Start of DC/T-cell co-culture

2. Harvest DCs (from step 1 option A) by vigorously resuspending the cells. Empty wells and add cold wash buffer. Incubate for 20min on ice and check adherence frequently using a microscope. Ensure all DCs are harvested by pooling the initial culture medium and the wash buffer. CRITICAL STEP: in contrast to the classical cytokine-based maturation cocktail (IL1ß, TNFα, IL6, PGE2), DC matured with LPS/ IFNγ may be much more adherent to the plastic, displaying a very different morphology (mostly stretched out). Flushing cells after incubation on ice usually leads to good recovery of viable DCs.

3. Spin cells and resuspend in wash buffer.

4. Count DCs.

5. Irradiate DCs with 30Gy. CRITICAL STEP Ensure cells are kept cool during transport to the radiation source. CRITICAL STEP Irradiation may have an additional influence on DC maturation. However, the critical issue is to block potential proliferation of contaminating cells during the prolonged culture. NK-cells may respond strongly to IL-15 which is introduced later in the protocol and may therefore compete with the proliferating antigen-specific T-cells. Alternative approaches to block proliferation (such as the use of mitomycin C) have not been validated for this assay.

6. Spin and resuspend DCs in warm CG-DC medium/5% HS at a concentration of 5×10^5/ml.

7. Harvest T-cells (from step 1 option B), count and spin.

8. Resuspend T-cells in CG-DC medium 5%HS at a concentration of 2×10^6/ml.

9. Add IL-21, 60ng/ml, to the T-cell-fraction. (This will result in a final concentration of 30ng/ml after addition of the DCs).

10. Mix DCs with T-cells at a 1:1 v/v ratio (resulting in a 4:1 TC:DC ratio): calculate for at least 3 to 5 wells per group (condition) and always include a preparation of Melan-A-pulsed DC matured under standard conditions as an internal control. Always mix the cells for each group first in one larger tube. Do not add T-cells and DC separately to the individual wells, as variation due to pipetting errors will increase. Groups consisting of a single well do not provide sufficient data on the variation within your experiment.

11. Transfer 500μl of the cell mix to individual wells of a 48-well plate.

12. Incubate at 37°C for 72h.

First Feeding

13. Check cells under the microscope. When using Melan-A as a model antigen, it should be expected that a few proliferative clusters of cells will be evident. Calculate the required amount of mediumto give 0.5ml/well.

14. Make up warm CG-DC medium containing 5%HS and 5ng/ml IL-15 and IL-7 (referring to the final concentration in the culture medium). Cytokines (prepared in stock solution as mentioned above) can be kept at 4°C no longer than 4 weeks.

15. Add 500μl of the fresh medium to each well.

16. Incubate for 72h.

Second Feeding

17. Calculate the required amount of medium to give 1ml/well.

18. Make up warm CG-DC medium containing 5%HS and 5ng/ml IL-15 and IL-7 (referring to the final concentration in the culture medium).

19. Add 1ml fresh medium to each well of a 12-well plate.

20. Transfer cells and medium from each well of the old plate to the new 12 well plate. CRITICAL STEP Transfer of cells allows more room for expansion, but also should reduce the number of residual (plastic-adherent) myeloid cells from the DC preparation, which are no longer required.

21. Incubate cells for 48h.

Third Feeding

22. Calculate the required amount of medium to give 2ml/well.

23. Make up warm CG-DC medium containing 5%HS and 10ng/ml IL-15 and IL-7 (referring to the final concentration in the culture medium). Note that now the cytokine concentration is doubled.

24. Add 2ml of the freshly prepared medium to each well of a 6-well plate.

25. Transfer cells and medium from each individual well of the 12 well plate to individual wells of the new 6 well plate. Incubate for 72h.

Analysis On

26. The T-cells are analysed on day 10 of culture. Analysis varies from lab to lab, thus we briefly described methods that we routinely use for evaluation in Box 4. In Box 3 we give a protocol for one of these methods, a FACS-based cytotoxicity assay. We routinely evaluate each well individually. Especially when low frequency responses are expected, wells without antigen-specific responses may be present (because there was no antigen-specific T-cell in the starting well). However, if the wells are pooled prior to analysis, the specific T-cell lines will become diluted with non-specific T-cells from the negative wells.)

Timing

The protocol as described here stretches over a total experimental time of 14 days up until day 10 of culture. For convenience, starting with a DC preparation on a Monday and priming T-cells on Friday makes it possible to leave cultures unattended over the weekends. Day 10 of culture then again is a Monday and all necessary experiments for a functional-read-out can then be planned in this week.

Note that IL-12 production is highest in the hours after the TLR-trigger, and therefore the maturation step should not be extended for more than 16h.

The following breakdown describes how much time is required on specific days of the procedure:

Day 1: Option A, i-iv, XXX, X hours plus 3-3h incubation

Day 2: Option A, v-vii, XXX, X hours followed by 2 days incubation.

Trouble Shooting—See Table 3 for troubleshooting guidance.

Anticipated Results

Examples for typical results using ASAP-T are shown in Figure 4-6. Figure 4 highlights important aspects relevant to this protocol. The choice of DC-maturation stimulus may not only influence the percentage of specific cells, but also the total expansion of these cells. More importantly, functionality is dependent on the initial priming conditions (Fig. 4c). Other factors critical to optimal priming is the T-cell:DC ratio (Fig. 4d) and the presence of IL-21 at the initial priming step (Fig. 4e.).

T-cells at day 10 to 12 of culture display an early effector memory cell type. They are $CD45RO^{+}$ but still express CD45RA to some level. Furthermore the T-cells still express CD62L and to some degree CCR7, which gradually declines the more the cells expand. Costimulatory molecules such as CD28 and CD27 are still expressed at variable degree. The phenotype following a single stimulation and expansion in IL-7/IL-15 resembles “young” effector memory cells. For adoptive T-cell-therapy, such T-cells have been shown to be favorable in terms of efficacy and longevity after transfer in vivo [53, 54]. In fact a recent clinical trial in leukemia patients showed persistence of WT1-specific T-cells only, if cells had been cultured in the presence of IL-21 [55]. In this case, this was observed even after a prolonged culture time due to cloning of individual cells and massive expansion.

T-cells expanded over only a short time period remain reactive against endogenously presented antigen. Figure 5 shows data on a representative cytotoxicity assay using purified Melan-A-specific T-cells at a 2:1 ratio. Details of the assay are explained in box 3. Figure 6 provides data on a representative experiment targeting other peptide antigens with lower precursor frequency. First, the quality of the naïve T-cell preparation was evaluated (Fig 6a). The experiment was subsequently set up in 5 parallel wells for each peptide tested, and MHC-multimer staining was performed for each individual well (Fig. 6b), with the percentage of antigen-specific T-cells by day 10 of culture shown. Note that some wells for the gp100-epitope were negative, which emphasizes the importance with low frequency responses of using small numbers of cells in each starting well and testing multiple wells in parallel. In addition selected wells were analyzed functionally (Fig. 6c). Finally Figure 7 illustrates an experiment with suboptimal results: although differences between the experimental groups were observed, cells using standard conditions did not expand properly, thus the experimental results are not validated. Repeat experiments indicated that, in this particular case, the leukapheresis obtained was suboptimal. Whether this is due to donor-specific characteristics or to difficulties in the preparation of the leukapheresis product has yet to be determined. Additional experiments are required to determine, whether IL-21 throughout the culture may increase the T-cell yield, although previous reports suggest otherweise[41].

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Box 1

Purification of naïve CD8+ T-cells

We use the CD8+ T-Zell-Isolation Kit human, CD45RO-Beads and CD-57 beads from Miltenyi to perform option B steps iii and vi. The procedures to do this using these kits are described here so they can be easily substituted if an alternative kit is used or the manufacturers' instructions change. There are other alternatives with different kits and from different companies to isolate naïve CD8+ T-cells. However depleting the terminally differentiated effector T-cell population (TEMRA) is important to get consistent results. Therefore selection for CD45RA or depletion of CD45RO+ T-cells alone is insufficient.

Purification of untouched $CD8^+$ T-cells

$CD8^+$ T-Zell-Isolation Kit human (Miltenyi Biotec, cat. no. 130-094-156)

CD45RO-Beads (Miltenyi Biotec, cat. no. 130-046-001)

CD57-Beads (Miltenyi Biotec, cat. no. 130-092-073)

Cold PBS with 1%HSA is used as wash buffer.

For magnetic separation, LS and LD columns are routinely used on a MACS-Quattro-magnet.

1. Start with 2×10^8 PBL (minimum 1×10^8)
2. Incubate cells with 40µl cold buffer per 10^7 cells, resuspend.
3. Add 10µl biotin-antibody cocktail per 10^7 cells.
4. Incubate for 15min at 4°C. Gently shake every 5 minutes.
5. Add 30µl cold buffer per 10^7 cells and 20µl of microbeads per 10^7 cells.
6. Incubate for 15min at 4°C. Gently shake every 5 minutes. In the meantime, prepare an LS column by placing it on the magnet and rinsing it with 3ml of cold buffer.
7. Wash cells in 5ml of cold buffer.
8. Resuspend cells in 1ml cold buffer and transfer to column.
9. Let cell suspension enter the column and then gradually add 3ml of cold buffer.
10. Collect the flow-through representing the $CD8^+$, untouched cell population.
11. Resume main procedure, option B step iv.

Depletion of the memory T-cell fraction

12) Spin $CD8^+$ population.

13) Resuspend in 40µl cold buffer per 10^7 cells.

14) Add 10µl of anti-CD57-beads per 10^7 cells and 10µl anti-CD45RO-beads per 10^7 cells.

15) Incubate for 15min at 4°C. Gently shake every 5 minutes.

16) Prepare an LD column by rinsing it with 3ml of cold buffer (placed on the magnet).

17) Wash cells using 5ml of cold buffer.

18) Resuspend cells in 1ml of cold buffer and transfer to LD column.

19) Allow cells to enter the column.

20) Rinse column with 3ml of cold buffer.

21) Collect flow-through representing the naïve T-cell population

22) Return to main procedure at option B step iv.

Box 2

Purification of MHC-multimer$^+$ T-cells

Purification of MHC-multimer+ T-cells is generally not necessary for assays focusing on effector T-cells with a FACS-based method (intracellular cytokine staining, degranulation assays). For cytotoxicity assays evaluating the effect on target cells, a homogenous population of effector T-cells in all experimental groups and the absence of possibly contaminating NK-cells is highly desired. Purification of antigen-specific T-cells using MHC-multimers 2 days prior to the assay represents a simple and reliable approach to achieve comparable cell populations. We utilise xxxx and recommend you also refer to the manufacturers' instructions.

Materials required

APC-labeled MHC-multimer of the chosen epitope

Anti-APC-beads (Miltenyi Biotec, cat. no. 130-090-855)

MS column (Miltenyi Biotec, 130-041-305) and corresponding magnet

Anti-CD8-antibody for subsequent quality control

i. Start with antigen-specific T-cell line of your choice (pooled cells from parallel wells)

ii. Count the cells, as this protocol is suitable for up to 10^7 T-cells.

iii. Incubate cells with 50µl cold buffer, resuspend.

iv. Add 5 µl of APC-labeled MHC-multimer, resuspend.

v. Incubate for 20min at RT.

vi. Add 10 µl of anti-APC-Beads.

vii. Incubate for 15min at 4°C. Gently shake every 5 minutes.

viii. In the meantime, prepare MS column by placing it on the magnet and rinsing it with 2ml of cold buffer.

ix. Wash cells in 5ml of cold buffer.

x. Resuspend cells in 0.5ml cold buffer and transfer to column.

xi. Let cell suspension enter the column and then gradually add 1ml of cold buffer.

xii. Cells retained on the column represent the antigen-specific population.

xiii. After 2ml have passed, remove the column from the magnet, place it on a 15ml tube and add 2ml of cold buffer.

xiv. Gently push out retained cells using the plunger (avoid strong pressure).

xv. Count cells and use aliquot for FACS-staining (add anti-CD8-antibody; - cells are already stained for MHC-multimer).

xvi. Spin cells and transfer into T-cell medium containing IL-7 (5ng/ml) and IL-15 (5ng/ml).

xvii. After 48h, purified cells may be used for functional assays.

Box 3

Cytotoxicity assay using activated caspase 3 as read-out

Use T-cells purified with MHC-Dextramers (see box 2). The test should be performed 48h after purification and last addition of fresh cytokines. The test is designed for an Effector:Target ratio of 2:1. Adjust cell numbers according to your experimental design. Preparations on the day prior to the assay should be done under sterile conditions, whereas all steps performed on the day of the assay do not need to be done under sterile conditions.

One day prior to the assay

- **i.** Harvest T-cells and count.
- **ii.** Spin T-cells, keep the supernatant, and adjust cell concentration to 1×10^6/ml.
- **iii.** Transfer T-cells to a 96-well-plate. 200µl/well (2×10^5/well). The outline of the plate should match your assay outline (see below).
- **iv.** Harvest tumor cells and count: include negative controls such as HLA-A2 negative tumor cells or HLA-A2 positive, antigen negative tumor cells.
- **v.** Resuspend tumor cells in the appropriate fresh culture medium (e.g. RPMI/ 10%FCS) and adjust cell numbers to 5×10^5/ml.
- **vi.** Transfer tumor cells to another 96-well plate (round bottom), 200ul/well ($=1\times10^5$ cells/well). The outline should match the T-cell plate. Include well for the following controls: tumor cells only, peptide-loaded tumor cells. If enough T-cells are available, duplicates of each well are recommended.
- **vii.** Add peptide (1µg/ml) to the control wells with tumor cells as the maximum lysis control.
- **viii.** Incubate T-cells and tumor cells in their respective plates overnight at 37°C.

Day of the experiment

- **i.** Warm CG DC medium/1%HS to 37°C (20ml per plate).
- **ii.** Spin tumor- and T-cell plate. Discard supernatant by flicking the plate over the sink.
- **iii.** Resuspend tumor cells in 200µl CG medium/well and spin again. Discard supernatant (This step is meant to wash off residual culture medium from the tumor cells as well as peptide in the peptide-control wells.
- **iv.** Resuspend T-cells in 200µl of warm CG medium/1%HS, 200µl/well.
- **v.** After discarding supernatant of the tumor cell plate, transfer the T-cells to the tumor cells using a multi-channel pipette (Note: do not transfer tumor cells to T-cells, as tumor cells may be adherent).
- **vi.** Spin plate for 30s to allow quick sedimentation of the T-cells. (Do not discard!)

vii. Incubate plate for 4h at 37°.

Staining prodcedure

viii. Perform staining is in the 96 well plate using standard procedures. We first stain the T-cells using CD8-PerCP-Cy5.5 and CD45-eFluor450. After that, we fix and permeabilize cells using standard buffers for intracellular staining. We then stain the cells using anti-activated caspase-3 antibody (PE-labeled, rabbit-anti-active-caspase-3, BD Pharmingen, no. 51-68655X). Acquisition is done on a FACS Canto II flow cytometer, acquiring at least 5000 tumor cells/sample. The gating strategy is described in Figure 5. All staining/washing steps are done using a multi-channal pipette, including the transfer of cells samples to microtubes for acquisition. Alternatively a plate-reader may be used.

Box 4

Methods we use to evaluate T cells

We routinely evaluate T cells using the following methods

Cell number

While the percentage of antigen-specific T-cells between two groups may be similar, there can be big differences in the absolute expansion of the T-cells. Determination of the absolute number of cells in each well individually adds significant information about the priming condition being assessed. Use of an automated cell counter (e.g. Countess) and trypan blue exclusion if available will increase reproducibility.

MHC-multimer-staining

As responses to the Melan-A-peptide should be included as an internal control, these responses can be measured using MHC-multimers. Staining of an aliquot of cells is performed in 96-well plates using a staining volume of 50µl buffer. MHC-multimer is added to the initial staining master mix at the appropriate concentration (as tested beforehand; in general a 1:50 to a 1:100 dilution is a good starting dilution if using APC-labeled MHC multimers). To reduce internalization of the multimer, the staining buffer is substituted with 100nM dasatinib[56]. Cells are incubated at room temperature for 20min. Additional antibodies, including anti-CD8, are added only after this staining period. After an additional 20min staining period, cells are washed twice and analyzed.

Absolute number of antigen-specific cells

Values obtained from counting cell number and MHC-multimer staining. permit determination of the absolute number of antigen-specific T-cells in each well, which is the best quantitative measurement for priming.

Phenotypic analysis

Choice and design of the antibody panel used for characterization depends on the experimental question. Proposed antigens defining the phenotype of the cell population include CCR7, CD62L, CD27, CD28, CD45RO, CD45RA and PD1.

Functional assays

The quality of the resulting T-cell responses is routinely assessed using intracellular cytokine staining, cytotoxicity assays, degranulation and TCR spectratyping. A short protocol for a FACS-based cytotoxicity assay is outlined in box 4. Description of each of these assays in detail, however, is beyond the scope of this protocol.

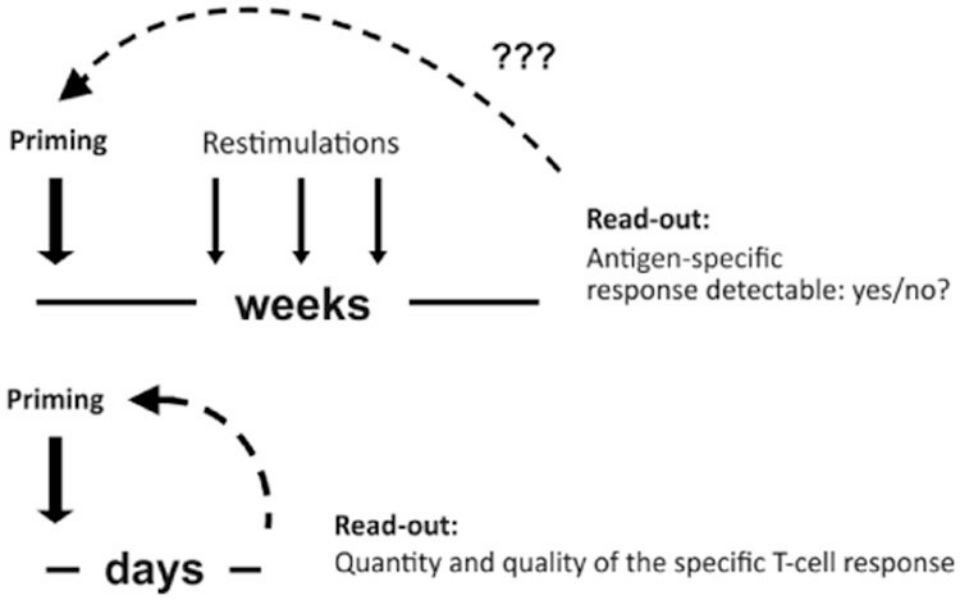

Figure 1. Advantage of a short-term T-cell expansion protocol

Upper panel: The low frequency of antigen-specific precursor T-cells requires repeated stimulations over a prolonged culture time, when sub-optimal stimulation conditions are chosen. Factors that can interfere with the results, and are independent of the initial priming, include the mode of restimulation (APC, peptide concentration), the chosen cytokines for expansion, cell density and serum quality. The need for repeated stimulations makes conclusions about the initial priming step difficult. Lower panel: short term expansion after a single peptide stimulation reduces variation within the expansion period, thus allowing conclusions about the initial priming conditions.

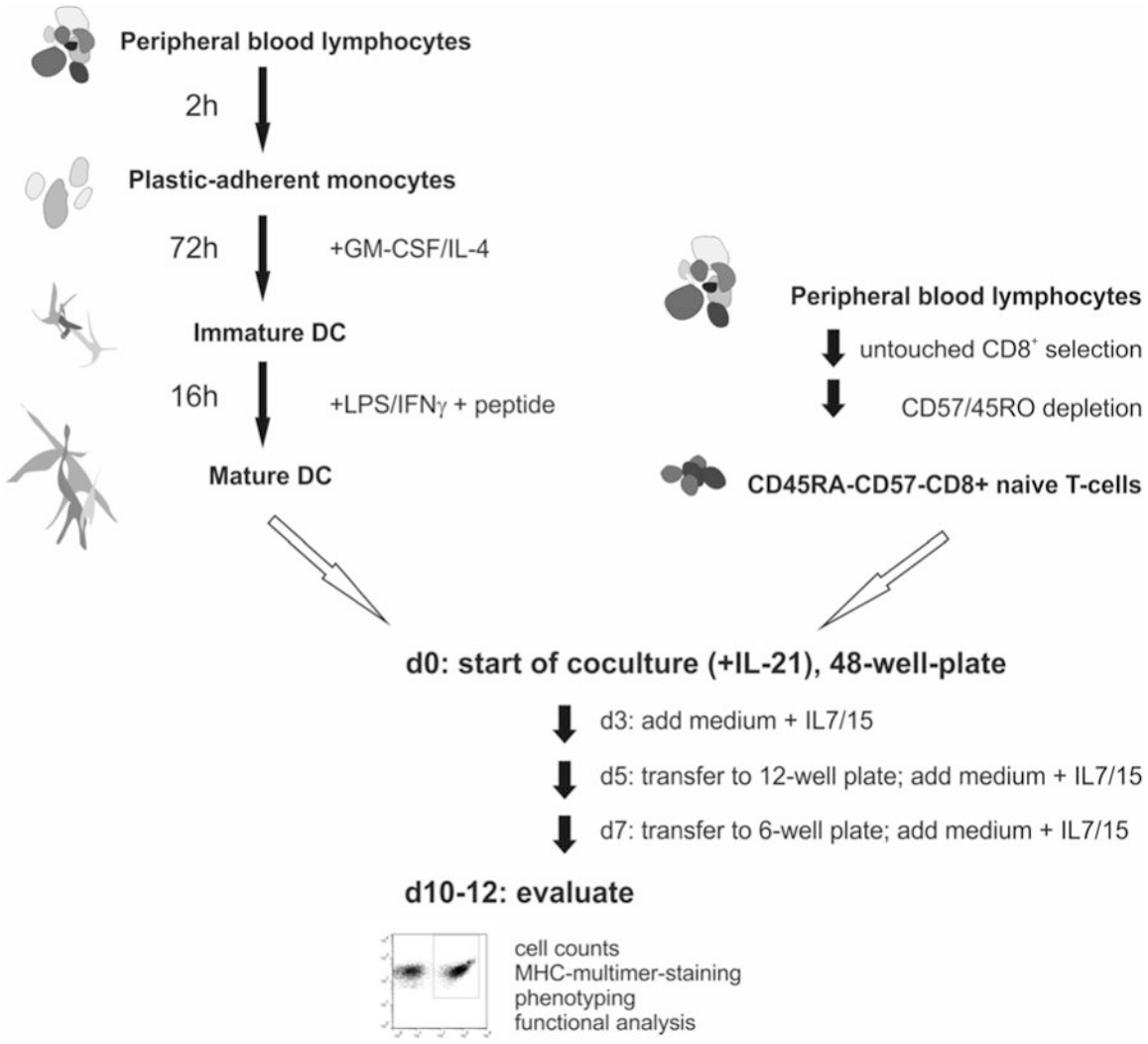

Figure 2. Experimental outline

Start with monocyte isolation on day -4 of culture, followed by T-cell isolation at day -1 of culture. Initiation of co-culture of peptide-pulsed DCs with T-cells is termed day 0. Expansion may be extended to more than 10 days, but the phenotype of responding cells may change gradually. Analysis earlier than day 10 is possible, but the variation is bigger and the yield is significantly lower, as increased proliferation is generally seen between day 7 and day 10.

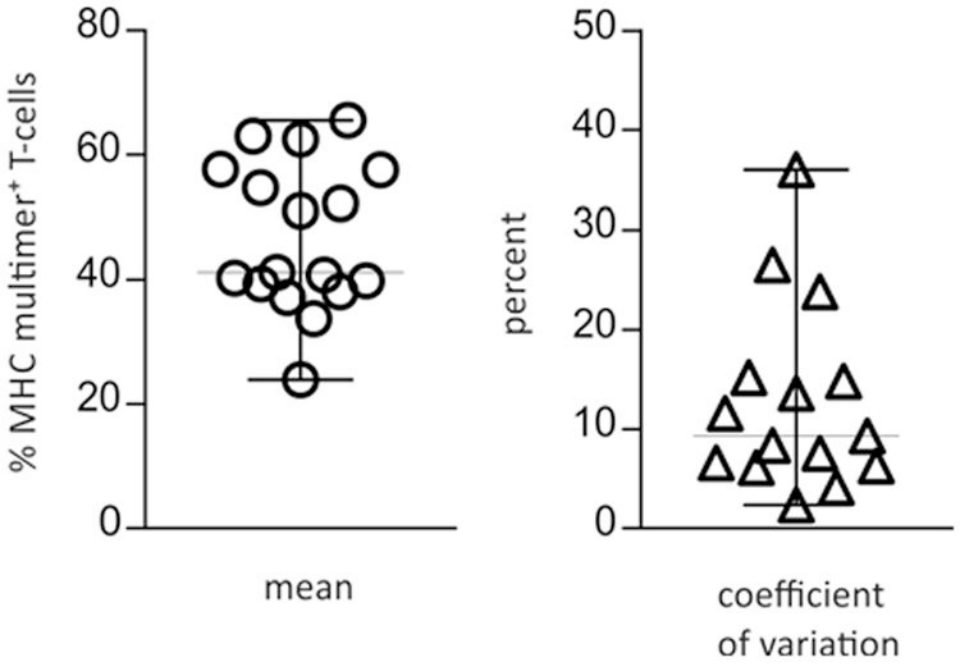

Figure 3. Inter-assay and intra-assay variability of ASAP-T

17 consecutive experiments performed by two technicians were evaluated. Experiments with at least 3 wells for standard conditions were included and only the first 3 wells were counted if more than three wells had been set up. Depicted are the means of these experiments. Bars indicate median and range (left panel). As an indicator of intra-assay variation, the coefficient of variation was calculated (right panel, median and range) for each of these experiments.

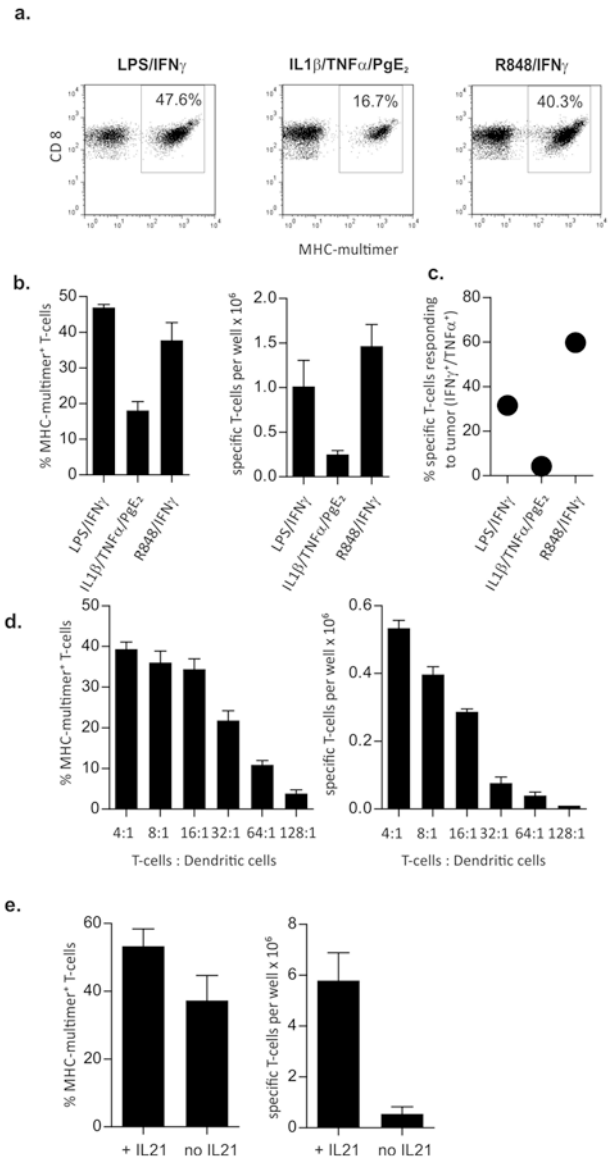

Figure 4. Factors relevant for ASAP-T

DC were matured differently (3 wells per group) and the resulting T cell responses evaluated by day 10. IL1β/TNFα/PgE_2-matured DCs prime naïve T-cells poorly leading to comparatively dysfunctional T-cell responses. Panels include: a.) representative MHC-multimer-staining of T cells; b.) summary of the fraction of Melan-A-specific T-cells in each group and total number of specific T-cells in each well. c.) T-cells were tested for recognition of a HLA-A0201$^+$, Melan-A$^+$ melanoma cell line at a 2:1 effector:target ratio. The fraction of IFNγ/TNFα double positive cells/MHC-multimer$^+$ T-cells is indicated; d.) the ratio of T-cells and DCs critically influences total expansion of Melan-A-specific cells.; e.) IL-21 greatly increases total proliferation of antigen-specific T-cells.

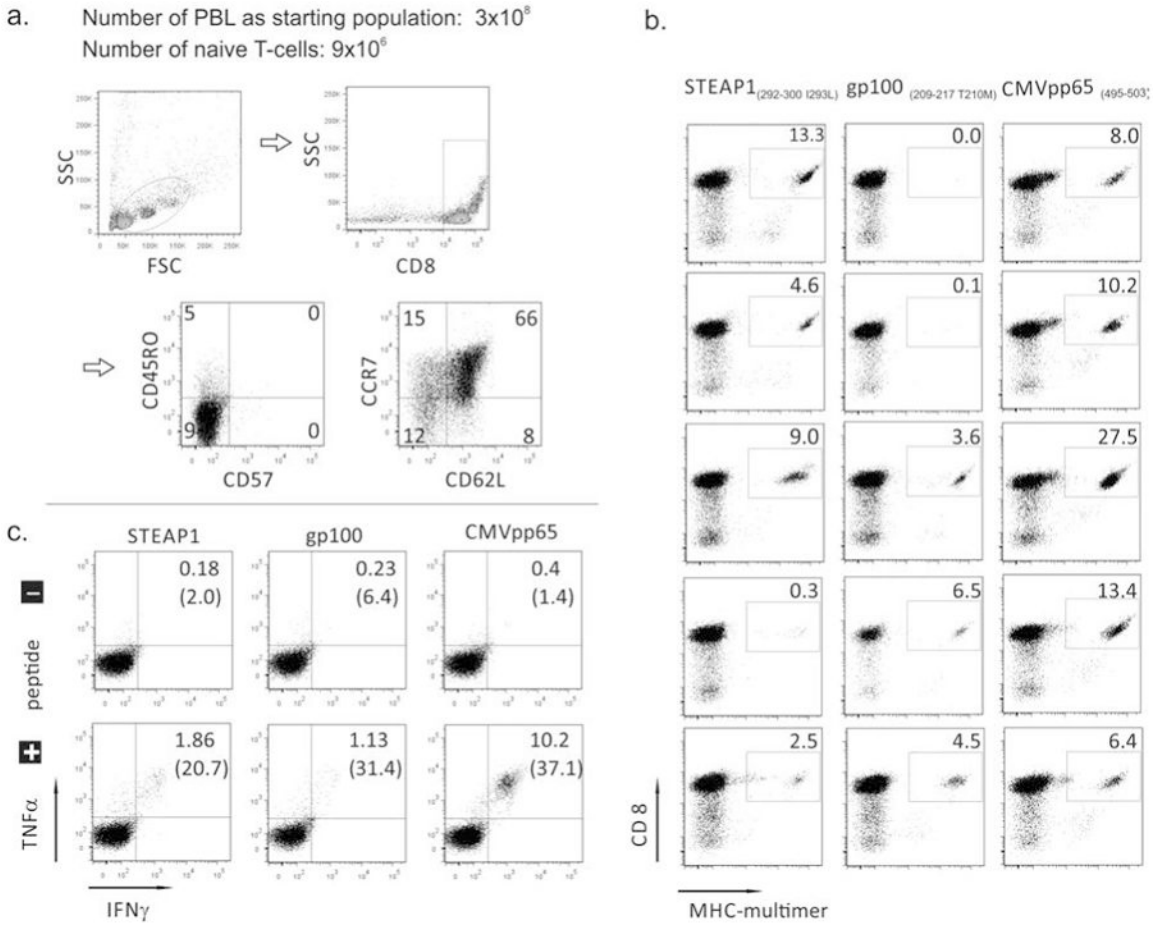

Figure 5. Representative results from a cytotoxicity assay

(a) Melan-A-specific T-cells were expanded using the ACE-CD8 procedure and purified on day 10 of culture with Melan-A-Dextramer as outlined in box 2. a) Gating strategy and purity of the antigen-specific T-cells. B) Gating strategy for the Caspase-3-assay: large cells according to FSC/SSC are included and further analyzed for CD8 and CD45 staining (right). CD8-CD45- tumor cells are then included in the analysis shown in c): the upper left panel shows activated caspase 3 in the Melan-A+, HLA-A0201+ melanoma cell line FM55 (gift from J. Becker, Würzburg). As a positive control for maximum caspase activation achievable within 4h, peptide-loaded melanoma cells are routinely used (right). Note that this maximum control differs from the “maximum lysis control” in classical chromium release assays (which usually shows lysis of targets induced by a lysing reagent). The lower left panel shows spontaneous caspase-3 activation in tumor cells without T-cells. An HLA-A2 negative, Melan-A negative tumor cell line remains unaltered by the T-cells (lower right).

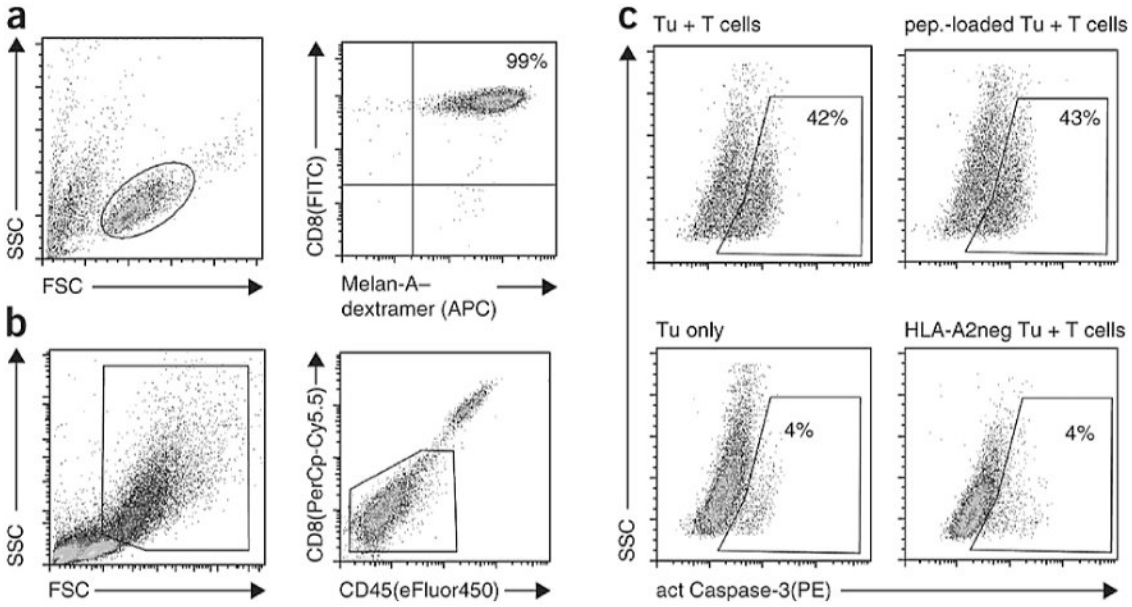

Figure 6. Expansion of T-cells against antigens other than Melan-A

a.) Example of the evaluation of the initial population of naïve T-cells (gating strategy and panel). b.) Representative results of an experiment set up in five parallel wells per tested peptide. The tumor associated peptide antigens $STEAP1_{(292\text{-}300\ I293L)}$ and $gp100_{(209\text{-}217\ T210M)}$ and the CMV-related peptide antigen $pp65_{(495\text{-}503)}$ were evaluated using the same naïve T-cell preparation; c.) functional testing of the lines depicted in row 3 of panel b, using peptide loaded autologous monocytes as APCs (lower panel); upper panel: monocytes without peptide were used as control stimulators. Numbers in brackets indicate the percentage of cytokine$^+$ T-cells in relation to MHC-multimer$^+$ cells in the respective well. T-cells were stimulated for 5h in the presence of brefeldin A.

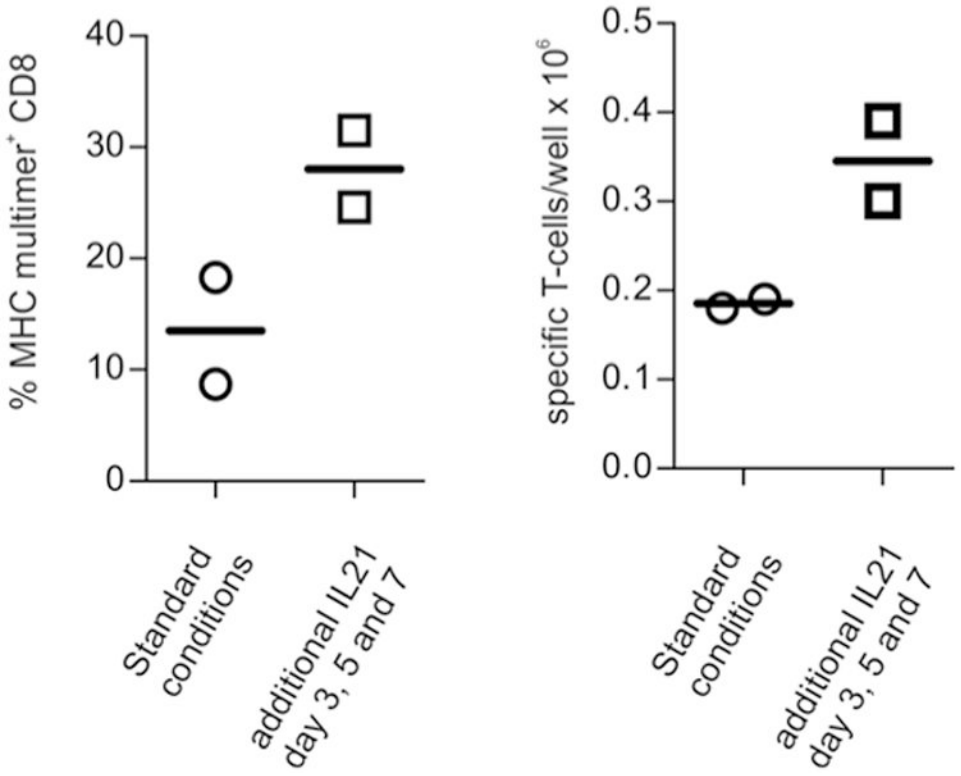

Figure 7. Example of an experiment that did not meet the validation criteria

Pilot data from an experiment assessing the effect of additional supplementation with IL-21 on days 3, 5 and 7. Although a positive effect can be observed in the experimental group with additional IL-21, the experiment does not meet the validation criteria, as the mean percentage of Melan-A-specific T-cells using standard conditions did not meet the pre-defined criteria of >24% MHC-multimer^{+} T-cells. Additionally, the assay was not been performed in triplicate, which would have been desirable. Repeat experiments revealed a problem with this particular leukapheresis.

Table 1

Literature search regarding studies on priming of Melan-A-specific T-cells

Reference (in order of pub. date)	Starting T-cell population	DC-preparation	Maturation cocktail	No. of stim.	Peptide epitope used	% MHC-multimer$^+$ T-cells
Oelke M, 2000, Clin Canc Res[57]	Purified CD8	moDC	LPS	5	26-35, 30μg/ml	3%
Blanchet JS, 2001, JI[58]	Purified CD8	CD8- PBMC	none	2	26-25 A27L, 1μM	2.8%
Von Bergwelt-Baildon MS, 2002, Blood[25]	Purified CD8	B-cells	CD40-Ligand	4	26-25 A27L, 10μg/ml	21%
Oelke M, 2003, Nat Medicine[29]	Purified CD8	artificial APC/Dimer Ig	Not applicable	3	26-25 A27L 30μg/ml	62%
Xu S, et al. 2003, JI[59]	Purified CD8	fastDC	IFNγ/TNFα/CD40L	1	27-35, 10ng/ml	7.5%
Li Y et al., 2005, JI[41]	Purified CD8	moDC	IL1ß,TNFα,PgE_2, IL6	2	27-35, 40μg/ml	12%*
Hirano N, 2006, Clin Cancer Res[60]	Purified CD8	artificial APC/K562	Not applicable	6	27-35, 10μg/ml	12%
Rudolf D, 2007, CII[61]	Purified CD8	artificial APC	Not applicable	4	26-25 A27L, conc. not applicable	72%
Dubsky et al, 2007, EJI[62]	Naive T-cells	IL-15-moDC	LPS	1	27-35 10μg/ml	18%
Dauer M, 2008, JIM[63]	CD3+	fastDC	LPS/R848	3	26-35 A27L, 10μM	25%
Chauvin JM et al, 2012 JI[64]	PBL	moDC	Poly I:C/TNFα	1	8uM, 26-35 and 26-35 A27L	2%
Hervas-Stubbs et al. 2012, JI[65]	Naive T-cells	fastDC	Poly I:C/TNFα	1	26-35, 10μM	20%

*10μg/ml of a peptide with a molecular mass of 985 (corresponding to 26-25 A27L) equals 10 μM

Table 2

Experimental data comparing different serum concentrations when using ASAP-T as well as testing an alternative medium for cell expansion

Experimental group	Total cell number	Viability	% MHC-multimer^{+}	Abs. no. of specific cells	x-fold expansion*	% of standard conditions
Standard cond.	19×10^6	95%	80%	15.2×10^6	30,000x	Not applicable
3% HS well 1	8×10^6	96%	70%	5.6×10^6	11,000x	36%
3% HS well 2	7×10^6	98%	69%	4.8×10^6	9600x	31%
1% HS well 1	3×10^6	98%	38%	1.1×10^6	2200x	7.2%
1% HS well 2	3.5×10^6	98%	49%	1.7×10^6	3400x	11.1%
Medium B, 1% HS, well 1	4×10^6	96%	42%	1.7×10^6	3400x	11.1%
Medium B, 1% HS, well 2	3.5×10^6	95%	45%	1.7×10^6	3400x	11.1%

*Calculated as the x-fold expansion from an average well, containing 500 specific naïve precursor T-cells

Table 3

Troubleshooting

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
10	Low yield of DCs	Extensive washing during purification of plastic adherent monocytes (Step 5)	Reduce wash steps and control frequently using a microscope
10	Strong contamination of the DC population with other cells types	Insufficient separation during plastic adherence (Step 5)	Increase wash steps and control frequently using a microscope
20	Low yield of naïve T-cells	Donor-related/elution phases on magnetic column suboptimal	Check initial PBL for naïve cell population by FACS. Check each fraction of the separation process by FACS to track naïve T-cells
49	Expansion of $CD8^-$ cell populations during culture	Insufficient naïve T-cell purification leading to expansion of NK-cells or $CD4^+$ T-cells; insufficient irradiation of DC-containing population, thereby introducing contaminating lymphocytes	Check purification steps by FACS
49	T-cells do not expand properly	Possible reasons include serum quality, cytokines, and cell density	Check several donors and use autologous serum
49	T-cells do not respond to endogenously presented antigen (e.g. by tumor cells)	Initial priming may still be suboptimal	Check DCs for IL12-production
49	T-cells kill tumor cells non-specifically	May be caused by even small fractions of contaminating NK-cells	Select for MHC-multimer$^+$ $CD8^+$ T-cells two days prior to the functional assay.