

# Antigen-specific activation and cytokine-facilitated expansion of naive, human CD8<sup>+</sup> T cells

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**Antigen-specific priming of human, naive T cells has been difficult to assess. Owing to the low initial frequency in the naive 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 evaluate antigen-specific priming of CD8<sup>+</sup> cells 10 d after a single specific stimulation. The assay provides reference conditions, which result in the expansion of a substantial population of antigen-specific T cells from the naive repertoire. Various conditions and modifications during the priming process (e.g., testing new cytokines, co-stimulators and so on) can now be directly compared with 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 naive 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 before their activation with cytokines or reagents regardless of the T cell receptor (TCR) trigger, but in the context of this paper we will use priming to reflect the initial activation of naive T cells after an encounter with their respective cognate peptide in the context of a major histocompatibility complex (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 by the signals provided during this initial priming step<sup>1</sup>. Thus, the priming process shapes the resulting immune response and is key to our understanding of how T cell responses evolve<sup>2,3</sup>.

## Methods for investigating antigen-specific priming

Systematic studies of antigen-specific priming have been hampered by the exceedingly low frequency of each TCR specificity within the vast diversity of the repertoire of naive T cell precursors. Animal models enable analysis of evolving immune responses to infectious model antigens, such as lymphocytic choriomeningitis virus (LCMV) in mice, which simulates effective or dysfunctional T cell responses depending on the viral variant of LCMV<sup>4</sup>. Furthermore, TCR-transgenic mice, in which virtually all T cells are specific for a defined epitope, have been extremely valuable for our understanding of the basic concepts regarding T cell and tumor immunology<sup>5–7</sup>. However, mouse immunology differs in many aspects from that of humans<sup>8</sup>, 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<sup>9</sup>. Vaccinologists and virologists have increasingly resorted to testing nonhuman 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 a few specialized laboratories<sup>10,11</sup>.

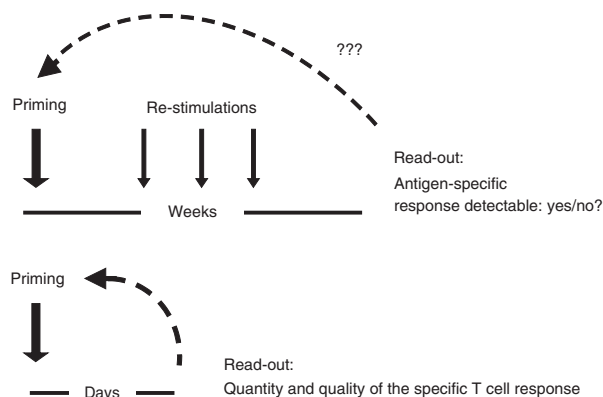
Developing principles of antigen-specific priming of human T cells *in vitro* has been hindered by the variability of T cell responses observed not only between individual donors but also, more importantly, in experiments performed from the same individual. This variability is generally attributed to 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 level of detection. However, such repetitive stimulation that requires 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 that was overexpressed in melanoma, which was recognized by a large number of tumor-infiltrating T cells isolated from patients. The gene, officially known as *MLANA*, was independently termed *Melan-A*<sup>12</sup> or *MART-1* (ref. 13) (for simplicity, we will refer to the protein encoded by this gene as Melan-A). An immunogenic peptide, restricted to the human leukocyte antigen (HLA)-A0201 allele, was identified as AAGIGILTV, corresponding to positions 27–35 of the Melan-A protein<sup>14</sup>, and was the target of the immune-dominant response in HLA-A0201<sup>+</sup> individuals. In addition to its use as a major immunogenic target in melanoma<sup>15</sup>, which we will not discuss in detail, this epitope has become the prototypical antigen for probing naive T cell responses in human T cells.

The peptide epitope Melan-A<sub>27–35</sub>, or the corresponding decamer from positions 26–35, can be used as a model antigen because the frequency of naive T cells specific for this epitope is about tenfold higher than the frequencies observed against other self- or tumor-associated antigens<sup>16,17</sup>. Precisely what is responsible for this increased frequency (~1 in 1,000 naive T cells) is not completely understood, but enhanced positive

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

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



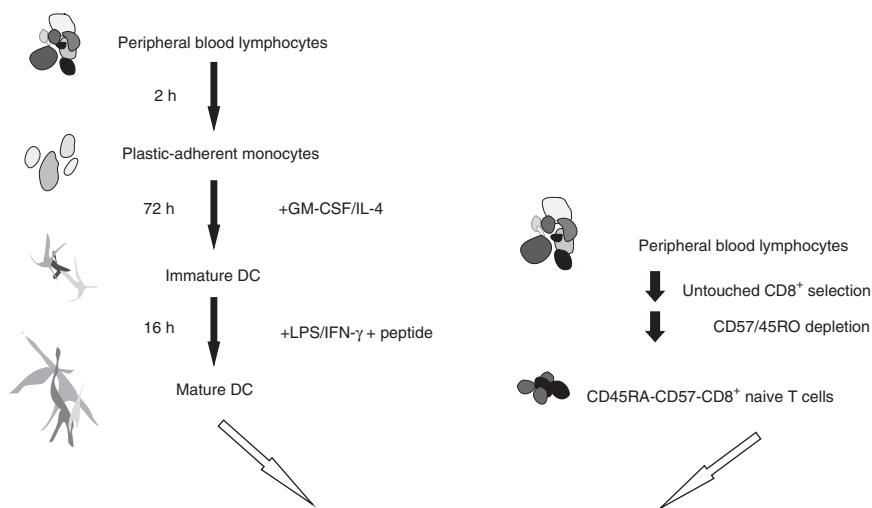
selection in the thymus<sup>18</sup> and/or cross-reactivity of T cells with various other specificities have been described<sup>19</sup>. In addition to cell phenotype, analyses of TCR excision circles and telomere length have shown that Melan-A-specific T cells in normal persons have characteristics similar to naive T cells, with none or only one potential additional round of expansion detected<sup>18</sup>. Valmori and colleagues<sup>18</sup> discovered that exchange of the alanine to leucine in position 27 greatly increased binding to HLA-A0201, thereby enhancing the immunogenicity of this epitope. Marked chemical and immunological differences between the known epitopes, the nonamer 27–35, the decamer 26–35 and the heteroclitic peptide 26–35<sub>A27L</sub>, exist. An extensive summary of the biochemical properties, immunogenicity and clinical use of this peptide can be found in Romero *et al.*<sup>15</sup>.

**Advantages and applications of the current protocol**

By using this model antigen (Melan-A 26–35<sub>A27L</sub>), which has a distinctly high frequency of reactive naive T cells, we developed

an *in vitro* priming system to reliably assess priming conditions for CD8<sup>+</sup> T cells<sup>20</sup>. This method, which we call ACE-CD8 for antigen-specific activation and cytokine-facilitated expansion of naive, human CD8<sup>+</sup> T cells, focuses on the encounter of effectively matured, peptide-loaded dendritic cells (DCs) with highly purified naive CD8<sup>+</sup> T cells (Fig. 2). ACE-CD8 defines conditions that, after a single stimulation, will lead to the rapid expansion of Melan-A-specific T cells within 10 d of culture. The protocol described here for ACE-CD8 is highly reproducible; thus, the experimental variability frequently reported when using other published protocols is seen to a much lesser extent in this protocol (Table 1 and Fig. 3). ACE-CD8 therefore allows an analysis of the effect of further variables (e.g., new cytokines, chemical compounds or drugs) at the time of priming with an antigen-specific T cell readout 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, the use of ACE-CD8, including the Melan-A peptide as a reference peptide, provides a validated, internal positive control for the priming and expansion conditions, and should clarify whether the experimental conditions of a specific experiment have been suboptimal.

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, and



- Day 0: start of coculture (+IL-21), 48-well plate
- Day 3: add medium + IL-7/15
- Day 5: transfer to 12-well plate; add medium + IL-7/15
- Day 7: transfer to six-well plate; add medium + IL-7/15

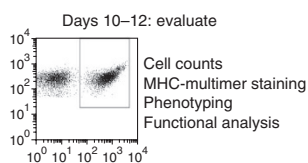


Figure 2 | Experimental outline. Start with monocyte isolation on day -4 of culture (Step 1), followed by T cell isolation on day -1 of culture (Step 1B). Initiation of coculture of peptide-pulsed DCs with T cells is termed day 0 (Steps 2–12). Expansion may be extended to more than 10 d, but the phenotype of responding cells may change gradually. Analysis earlier than day 10 is possible, but the variation is bigger and the yield is substantially lower, as increased proliferation is generally seen between day 7 and day 10.

**Figure 2** | Experimental outline. Start with monocyte isolation on day -4 of culture (Step 1), followed by T cell isolation on day -1 of culture (Step 1B). Initiation of coculture of peptide-pulsed DCs with T cells is termed day 0 (Steps 2–12). Expansion may be extended to more than 10 d, 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 substantially lower, as increased proliferation is generally seen between day 7 and day 10.



TABLE 1 | Literature search regarding studies on priming of Melan-A-specific T cells.

Reference (in order of publication date)	Starting T cell population	Antigen presentation	Maturation cocktail	No. of repetitive stimulations	Peptide epitope used	% MHC multimer <sup>+</sup> T cells
Oelke <i>et al.</i> <sup>57</sup>	Purified CD8	moDC	LPS	5	26–35, 30 µg/ml	3
Blanchet <i>et al.</i> <sup>58</sup>	Purified CD8	CD8-PBMC	None	2	26–25 A27L, 1 µM	2.8
Von Bergwelt-Baildon <i>et al.</i> <sup>26</sup>	Purified CD8	B cells	CD40 ligand	4	26–25 A27L, 10 µg/ml	21
Oelke <i>et al.</i> <sup>30</sup>	Purified CD8	Artificial APC/Dimer Ig	Not applicable	3	26–25 A27L 30 µg/ml	62
Xu <i>et al.</i> <sup>59</sup>	Purified CD8	fastDC	IFN-γ/TNF-α/CD40L	1	27–35, 10 ng/ml	7.5
Li <i>et al.</i> <sup>42</sup>	Purified CD8	moDC	IL-1β, TNF-α, PgE <sub>2</sub> , IL-6	2	27–35, 40 µg/ml	12 <sup>a</sup>
Hirano <i>et al.</i> <sup>60</sup>	Purified CD8	Artificial APC/K562	Not applicable	6	27–35, 10 µg/ml	12
Rudolf <i>et al.</i> <sup>61</sup>	Purified CD8	Artificial APC	Not applicable	4	26–25 A27L, concentration not applicable	72
Dubsky <i>et al.</i> <sup>62</sup>	Naive T cells	IL-15-moDC	LPS	1	27–35 10 µg/ml	18
Dauer <i>et al.</i> <sup>63</sup>	CD3 <sup>+</sup> T cells	fastDC	LPS/R848	3	26–35 A27L, 10 µM	25
Chauvin <i>et al.</i> <sup>64</sup>	PBL	moDC	Poly I:C/TNF-α	1	8 µM, 26–35 and 26–35 A27L	2
Hervas-Stubbs <i>et al.</i> <sup>65</sup>	Naive T cells	fastDC	Poly I:C/TNF-α	1	26–35, 10 µM	20

<sup>a</sup>10 µg/ml of a peptide with a molecular mass of 985 g/mol (corresponding to 26–25 A27L) equals 10 µM.

to analyze 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 DC and/or T cell function during the initial priming step. By using ACE-CD8 and slightly modified previous versions of this protocol, we probed the naive T cell repertoire of T cells specific for viral variants of HCV<sup>21</sup>,

assessed the effect of differentially matured DCs on priming<sup>22</sup> and identified novel peptide epitopes for the Wilms tumor antigen 1 (ref. 23), 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<sup>24</sup>. This drug, which is used clinically as a bcr/abl-kinase inhibitor, can also block TCR triggering through inhibition of the src-kinase Lck<sup>25</sup>. By using ACE-CD8, however, it was observed that dasatinib has a strong stimulatory activity on DCs, synergistically enhancing IL-12 in combination with a toll-like receptor 4 (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 an as-yet unknown mechanism of a widely used drug. Interestingly, when testing mouse and rhesus macaque DCs, we did not see the same effect, possibly owing to different binding properties of the drug<sup>24</sup>. Therefore, the results emphasize the need for a valid immunologic test system using human cells.

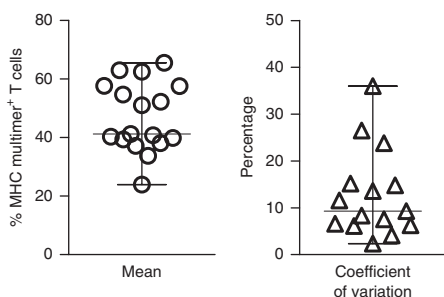


Figure 3 | Inter-assay and intra-assay variability of ACE-CD8. In all, 17 consecutive experiments performed by two technicians were evaluated. Experiments with at least three wells for standard conditions were included, and only the first three wells were counted if more than three wells had been setup. Depicted are the means of these experiments. Bars indicate median and range (left). As an indicator of intra-assay variation, the coefficient of variation was calculated (right, median and range) for each of these experiments.

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 that use the Melan-A epitope as a model antigen. Differently generated and differently matured DCs, as well as CD40L-activated B cells<sup>26</sup> and artificial APCs<sup>27</sup> 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 DCs'. 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. By 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 this has revealed how variable and versatile this APC remains<sup>22</sup>.

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 the 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<sup>27</sup>. By using this approach with mouse 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<sup>28</sup>. 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<sup>29,30</sup> to help understand the role of individual molecules for priming. The strength of such a system lies in its modular design as well as in the possibility to process large numbers of T cells, even if functional, autologous DCs are not available. Moreover, defined characteristics of such stimulatory beads are advantageous when re-stimulation 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 by using nonspecific stimuli such as phorbol 12-myristate 13-acetate (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 APCs 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 supra-physiological 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<sup>31</sup>. However, as all cells are stimulated simultaneously, results need to be interpreted with caution, as the magnitude and qualities 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. Cytomegalovirus (CMV), adenovirus (ADV) or Epstein-Barr virus (EBV) responses in healthy previously infected donors are generally robust, and specific T cells for the respective immunodominant epitopes can be tracked easily by using MHC multimers<sup>32</sup>. 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 after 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 to answer questions about the secondary response after *in vivo* priming.

### Experimental design

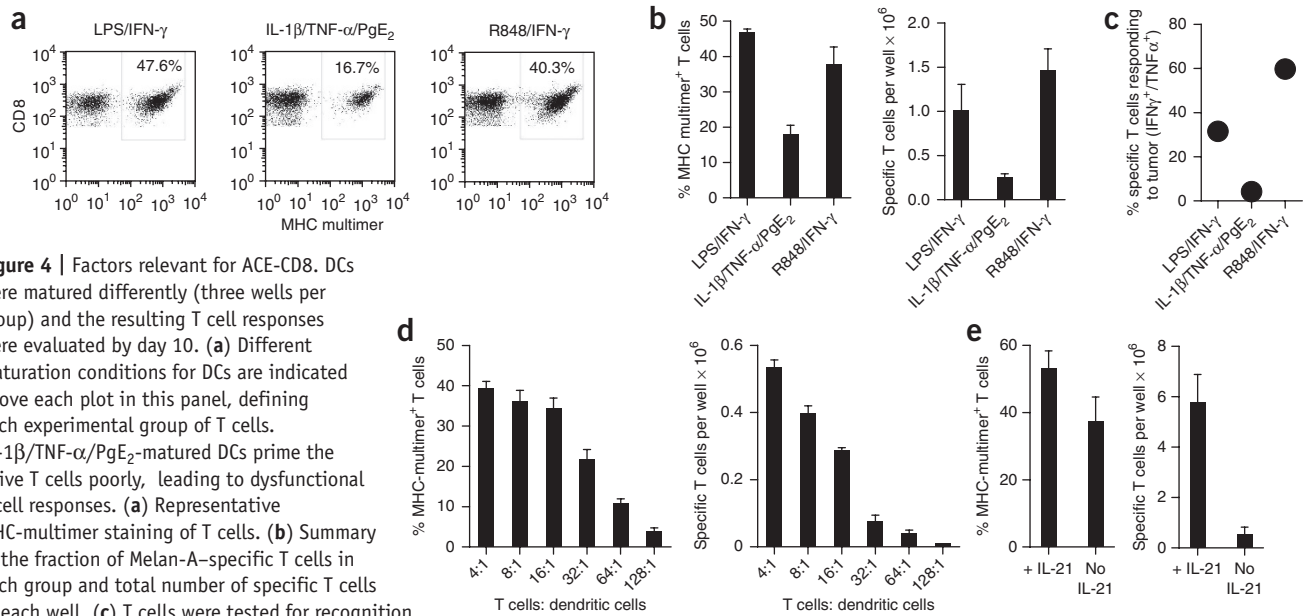
The ACE-CD8 protocol can be divided in two major parts: first, the preparation of DCs (Step 1), and then the stimulation and expansion of the T cells (Steps 2–26). The following subsections discuss key aspects of the protocol.

**DC preparation.** The preparation of DCs and the quality of the maturation trigger are crucial to achieving maximal expansion of the antigen-specific T cells. In this protocol, we use monocyte-derived DCs cultured for 72 h in granulocyte macrophage colony-stimulating factor (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 lipopolysaccharide (LPS) and interferon (IFN)- $\gamma$ . At this time, the peptide epitope is added to the culture as well; 16 h later, these cells are used for stimulating the naive 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 nonadherent fraction. Rinsing the plate with the appropriate pressure requires practice: rinsing with too little pressure will result in a large number of contaminating lymphocytes, rinsing with too much pressure will wash away some monocytes and lead 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 IFN- $\gamma$  are used as the essential reagents to induce IL-12 production. IFN- $\gamma$  primes the promoter region of IL-12p40 (ref. 33), whereas LPS activates IL-12 production via TLR4 triggering. In accordance with the kinetics of IL-12 induction, maturing DCs need to be used for priming within 16 h, as longer incubation with the maturing reagents only leads to DC exhaustion<sup>34</sup>. Unfortunately, the widely used maturation cocktail based on a mixture of pro-inflammatory cytokines (IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and PGE<sub>2</sub>)<sup>35</sup> does not lead to IL-12 production by DCs, as IL-12p35 is dose-dependently inhibited by prostaglandin E<sub>2</sub> (ref. 36), and, as a consequence, such DCs show only limited priming capacity (**Fig. 4**)<sup>24</sup>.





**Figure 4** | Factors relevant for ACE-CD8. DCs were matured differently (three wells per group) and the resulting T cell responses were evaluated by day 10. (a) Different maturation conditions for DCs are indicated above each plot in this panel, defining each experimental group of T cells. IL-1 $\beta$ /TNF- $\alpha$ /PgE<sub>2</sub>-matured DCs prime the naive T cells poorly, leading to dysfunctional T cell responses. (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<sup>+</sup>, Melan-A<sup>+</sup> melanoma cell line at a 2:1 effector:target ratio. The fraction of IFN- $\gamma$ /TNF- $\alpha$  double-positive cells/MHC multimer<sup>+</sup> 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. Experiments shown in d,e were matured under standard conditions. Experiments were performed in triplicate; error bars represent the s.d.

R848, a TLR7/8 agonist, in combination with IFN- $\gamma$  also induces IL-12 in the DCs, leading to a good specific expansion and full T cell function (Fig. 4a,b). It may therefore serve as an alternative maturation cocktail, although it has not been tested in our hands as extensively as LPS/IFN- $\gamma$ .

IL-12 production has been, in our hands, the only factor that correlated with fully functional T helper cell 1 (T<sub>H</sub>1)-primed T cell populations. Thus, quality controls on DC preparation should include assessment of IL-12 either by ELISA (for IL-12p70) or by intracellular cytokine staining after a 16-h stimulation in the presence of brefeldin A. Note that there are marked differences between the staining quality of different antibodies. We routinely use the Alexa Fluor 488-labeled IL-12p40 antibody from Affymetrix/eBiosciences (Clone: C8.6, order no. 53-7129). Surface marker expression has not been sufficiently reliable to distinguish between optimal (IL-12 producing) and suboptimal (IL-12 nonproducing) DC populations.

The matured DCs are irradiated (30 Gy) before co-incubation with T cells for priming. This prevents contamination with natural killer (NK) cells or non-naive T cells present in the DC preparation. Unless this fraction is irradiated, memory and NK cells may proliferate vigorously 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 experiment results. For DCs, effects of irradiation have been controversially discussed<sup>37,38</sup>. Treatment with mitomycin C (followed by extensive washing) may be an alternative method, but it has not been validated in our hands so far.

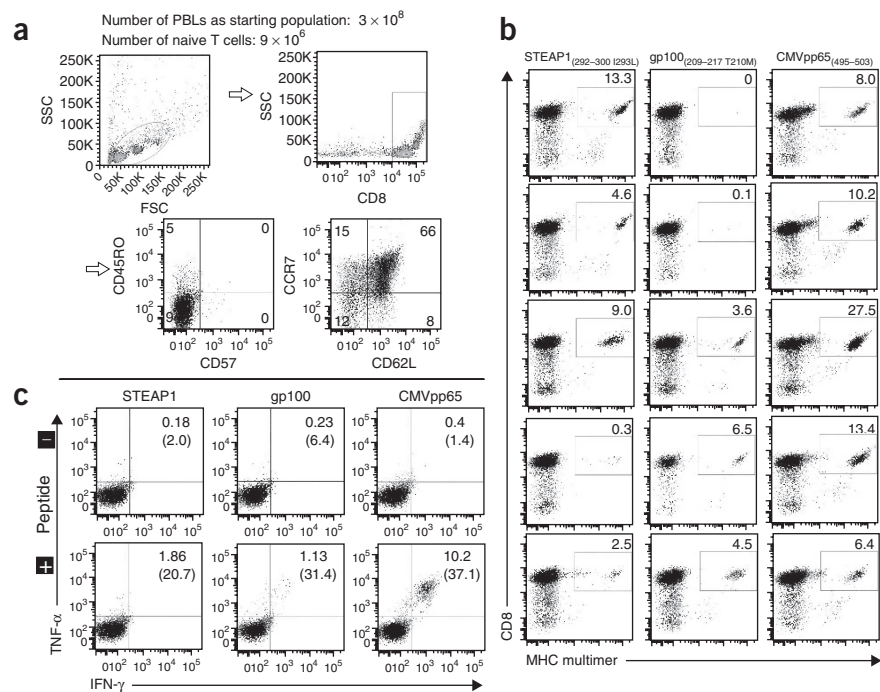
**Purity of the T cell preparation.** There are several reasons why highly purified naive T cells are a more suitable starting population than total CD8 preparations or whole peripheral blood leukocytes (PBLs). First, the fraction of naive T cells within the CD8 compartment varies from donor to donor. Therefore, unless

purified naive T cells are used, the number of naive cells in the starting well will vary from donor to donor. Moreover, the purity of naive T cells is crucial, as effector memory T cells may be cross-reactive and therefore may 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<sup>39</sup>, 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 PBLs, and the influence of such cells on the initial priming may add to the variability between individuals<sup>40</sup>. Each T cell preparation is therefore evaluated for the purity of the preparation by using CD62L and CCR7 as positive markers for naive T cells, and CD45RO and CD57 as exclusion markers. We generally demand that the CD62L<sup>-</sup>CCR7<sup>-</sup> fraction and/or the CD57<sup>+</sup> fraction and/or CD45RO<sup>+</sup> fraction be less than 10%, indicating a naive population of more than 90% (Fig. 5).

**Initial cell numbers and cell ratios.** ACE-CD8 starts with a T cell number of  $5 \times 10^5$  naive T cells per well in 0.5 ml of complete medium in a 48-well plate. Given the approximate frequency of 0.1% Melan-A-specific T cells within the naive T cell repertoire of a healthy donor, ~500 specific precursor T cells will be present in each well. The T cell:DC ratio is adjusted to 4:1. Note that ACE-CD8 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 markedly and requires side-by-side comparison (Fig. 4d). If alternative peptide epitopes are investigated, the precursor frequency is likely to be lower than that 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 a few



**Figure 5** | Expansion of T cells against antigens other than Melan-A. **(a)** Example of the evaluation of the initial population of naive 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<sub>292–300</sub>(I293L) and gp100<sub>209–217</sub>(T210M), and the CMV-related peptide antigen pp65<sub>(495–503)</sub> were evaluated by using the same naive T cell preparation. **(c)** Functional testing of the lines depicted in row 3 of **b**, using peptide-loaded autologous monocytes as APCs (bottom); top, monocytes without peptide were used as control stimulators. Numbers in brackets indicate the percentage of cytokine<sup>+</sup> T cells in relation to MHC multimer<sup>+</sup> cells in the respective well. T cells were stimulated for 5 h in the presence of brefeldin A. FSC, forward scatter; SSC, side scatter.



positive cultures at the end of the 10-d period. Thus, this microculture approach enables the detection of rare cells by limiting the number of irrelevant cells per well (the rare events follow a Poisson distribution) (Fig. 5)<sup>41</sup>.

Note that the setup of a reliable experiment requires relatively large amounts of PBLs as a starting cell population. Downscaling to very few cells, in our hands, negatively affects the overall experimental outcome, but it may become feasible when using a 96-well format at the initial priming step. Upscaling this procedure for clinical use is an ongoing project. It is possible to switch to larger flasks by using higher cell numbers, but careful re-evaluation is needed regarding the initial culture volume, cell density and so on.

**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.*<sup>42</sup>. 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 suggests 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)<sup>43</sup>. For robust and strong expansion, addition of this cytokine is essential (Fig. 4d). Adding IL-7 and IL-15 in a relatively low concentration (5 ng/ml each), 3 d after the initial priming step (and subsequently every 2 d), is based on the observation that T cell responses to cognate antigen are boosted by these cytokines<sup>39</sup>. During the first 3 d of culture in the absence of IL-7 and IL-15, a proliferative advantage is provided to cells receiving a TCR stimulus, whereas nonspecific cells may be ‘starved’ during this period<sup>44</sup>. 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, nonspecific homeostatic proliferation<sup>45</sup>.

Moreover, addition of IL-2, which is used in many protocols, is not necessary in this protocol, as it is known to drive cells toward terminal differentiation<sup>46</sup>. Note that for extended culture, beyond day 15, the 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 considerable 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 in which 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 markedly reduces inter-assay variation. One way to start the comparison of different serum batches is to include autologous serum off the clot from the healthy donor of the PBLs. This may not necessarily be the best serum batch, but it qualifies as a baseline. For the analysis of patient cells, autologous serum is generally not recommended, as interference with disease-related factors in the serum (drugs, inhibitory cytokines and so on) cannot be excluded<sup>47</sup>. Also, note that the serum concentration in the medium has a substantial effect, 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% (vol/vol) human serum (HS) rather than the standard 5% (vol/vol) HS resulted in a 66% decrease in the absolute number of Melan-A-specific T cells (Table 2)). A frequently asked question is whether medium *x*, *y* or *z* may be equally suitable for expansion. We have only tested a certain number of different culture media and found the

**TABLE 2** | Influence of different serum concentrations or an alternative medium.

Experimental group	Total cell number	Viability (%)	% MHC multimer <sup>+</sup>	Absolute no. of specific cells	x-fold expansion <sup>a</sup>	% of standard conditions
Standard conditions	19 × 10 <sup>6</sup>	95	80	15.2 × 10 <sup>6</sup>	30,000x	Not applicable
3% (vol/vol) HS well 1	8 × 10 <sup>6</sup>	96	70	5.6 × 10 <sup>6</sup>	11,000x	36.0
3% (vol/vol) HS well 2	7 × 10 <sup>6</sup>	98	69	4.8 × 10 <sup>6</sup>	9,600x	31.0
1% (vol/vol) HS well 1	3 × 10 <sup>6</sup>	98	38	1.1 × 10 <sup>6</sup>	2,200x	7.2
1% (vol/vol) HS well 2	3.5 × 10 <sup>6</sup>	98	49	1.7 × 10 <sup>6</sup>	3,400x	11.1
Medium B, 1% (vol/vol) HS, well 1	4 × 10 <sup>6</sup>	96	42	1.7 × 10 <sup>6</sup>	3,400x	11.1
Medium B, 1% (vol/vol) HS, well 2	3.5 × 10 <sup>6</sup>	95	45	1.7 × 10 <sup>6</sup>	3,400x	11.1

<sup>a</sup>Calculated as the x-fold expansion from an average well, containing 500 specific naive precursor T cells.

CellGenix DC medium to be the best in terms of expansion of specific cells and total proliferative capacity of the T cells. Side-by-side comparisons should be performed when introducing a new medium (Table 2).

**Experimental variation.** One major 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 three wells of each experiment from the standard expansion group is presented (Fig. 3a). The coefficient of variation (CV), indicating the ratio of s.d. and mean in percent, is plotted in Figure 3b to illustrate the spread of the data. Except for three outliers with higher CVs, the majority of experiments showed a CV between 3% and 20%. Given the nature of a biological assay, which extends over a 10-d period, and the small *n* (three 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<sup>+</sup> T cell lines: the fifth 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 serve as a positive control. From a few donor leukapheresis specimens, we observed a particularly high cell expansion (e.g., compare absolute numbers in Fig. 4d with those in Fig. 4e). Whether such ‘super donors’ reflect biological 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). For in-depth evaluation of certain conditions, or for testing new peptide epitopes with an unknown precursor frequency, higher numbers of parallel wells (depending on the statistical considerations) are beneficial.

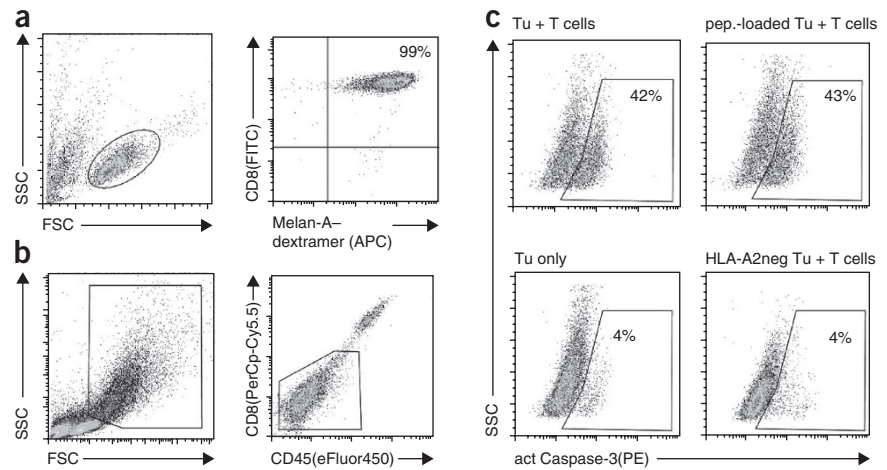
Experimenter variability is an important factor for observed inter-assay variability and robustness. Adherence to the protocol should greatly reduce such experimenter variability. The biggest variation may occur at the time of removing nonadherent cells from the monocyte layer (see above and Step 1A(v)). Washing intensity varies from person to person and with respect to training, and frequent controls based on microscopic analysis are required. In our laboratory, 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 STEAP<sub>1292–300</sub>(I293L) (ref. 48), PRAME<sub>435–443</sub> (ref. 49), gp100<sub>209–217</sub>(T210M) or MELOE1<sub>(36–44)</sub> (ref. 50). In contrast, responses to glioma-associated antigens EphA2<sub>(288–891)</sub> or IL13Rα<sub>(345–353)</sub> have been sporadic at best.

At very low precursor frequencies, the experimental setup is similar to classical limiting dilution assays in which each positive well indicates at least one specific T cell precursor<sup>51</sup>. Thus, the minimum precursor frequency may be estimated by the number of positive wells divided by the number of total input cells. For example, the frequency of gp100-specific T cells in the donor shown in Figure 6 must be at least 3/2.5 × 10<sup>6</sup> (three positive wells, three in 5 × 10<sup>5</sup> naive T cells). Distribution of such rare events follows a Poisson distribution, as has been discussed for limiting dilution assays<sup>51</sup>. Precision will be increased in tests of a larger number of wells (e.g., 20 parallel wells). Note that such calculations are estimates only; more elaborate statistical analyses are required for an exact determination of the precursor frequency<sup>52,53</sup>. For exact quantification of single precursors only, MHC multimer-guided enrichment starting with a large quantity of CD8<sup>+</sup> T cells may provide a more direct approach to quantifying very low frequency cell numbers<sup>16</sup>. In contrast, advantages of ACE-CD8 include obtaining sufficient specific cell numbers for



**Figure 6** | Representative results from a cytotoxicity assay. **(a)** Melan-A-specific T cells were expanded by using the ACE-CD8 procedure and purified on day 10 of culture with Melan-A dextramer as outlined in **Box 1**. Gating strategy and purity of the antigen-specific T cells. **(b)** Gating strategy for the caspase-3 assay: large cells according to forward scatter (FSC)/side scatter (SSC) are included and further analyzed for CD8 and CD45 staining (right). **(c)** CD8-CD45- tumor cells are included in the analysis. The upper left panel shows activated caspase 3 in the Melan-A<sup>+</sup>, HLA-A0201<sup>+</sup> melanoma cell line FM55 (gift from J. Becker, Würzburg). As a positive control for maximum caspase activation achievable within 4 h, 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 image shows spontaneous caspase-3 activation in tumor (Tu) cells without T cells. An HLA-A2 negative, Melan-A negative tumor cell line remains unaltered by the T cells (lower right).



functional characterization and having the means to dissect the priming process into different steps.

**Experimental readout**

ACE-CD8 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 d under strong T<sub>H</sub>1-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 owing to a lack of sufficient antigen-specific T cells. Detection and analysis of T cells by using MHC multimers can be done for Melan-A as early as 5 d

**Box 1 | Purification of MHC multimer<sup>+</sup> T cells**

Purification of MHC multimer<sup>+</sup> T cells is generally not necessary for assays focusing on effector T cells with a FACS-based method (intracellular cytokine staining, degranulation assays), as numbers can be correlated to the percentage of MHC multimer<sup>+</sup> T cells. 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 are highly recommended. Purification of antigen-specific T cells by using MHC multimers 2 d before the assay represents a simple and reliable approach to achieve comparable cell populations.

**Additional materials**

- APC-labeled MHC multimer of the chosen epitope (Immudex or Proimmune)
- Anti-APC beads (Miltenyi Biotec, cat. no. 130-090-855)
- MS column (Miltenyi Biotec, cat. no. 130-041-305) and corresponding magnet
- Anti-CD8 antibody for subsequent quality control

**Procedure**

1. Start with an antigen-specific T cell line of your choice (pooled cells from parallel wells).
2. Count the cells, as this protocol is suitable for up to 10<sup>7</sup> T cells.
3. Incubate the cells with 50 μl of cold buffer and resuspend.
4. Add 5 μl of APC-labeled MHC multimer and resuspend.
5. Incubate for 20 min at room temperature (20–24 °C).
6. Add 10 μl of anti-APC beads.
7. Incubate for 15 min at 4 °C. Gently shake every 5 min.
8. In the meantime, prepare MS column by placing it on the magnet and rinsing it with 2 ml of cold buffer.
9. Wash the cells in 5 ml of cold buffer.
10. Resuspend the cells in 0.5 ml of cold buffer and transfer to column.
11. Let the cell suspension enter the column, and then gradually add 1 ml of cold buffer.
12. Cells retained on the column represent the antigen-specific population.
13. After 2 ml have passed through the column, remove the column from the magnet, place it on a 15-ml tube and add 2 ml of cold buffer.
14. Gently push out the retained cells with the plunger (avoid strong pressure).
15. Count the cells and use aliquots for FACS staining (add anti-CD8 antibody—keep in mind that the cells are already stained for MHC multimer).
16. Spin the cells and transfer them into T cell medium containing IL-7 (5 ng/ml) and IL-15 (5 ng/ml).
17. After 48 h, purified cells may be used for functional assays.





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 owing 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 may be assessed after 10 d 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 that require large cell numbers, but evaluation of the individual wells by MHC-multimer staining and cell counts before 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 before the assay may increase nonspecific activation, whereas failure to refresh the medium for longer than 72 h increases the fraction of apoptotic or dysfunctional cells. T cells may be kept in culture for up to 21 d without re-stimulation. After this time point, re-stimulation is necessary to maintain sufficient cell numbers. Note that the T cells tested later than day 18 of culture may already display a different degree of differentiation. For cytotoxicity assays (Fig. 6), selection of Melan-A-specific T cells using MHC multimers and magnetic beads allows differential comparison of generated T cell 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 1). Note that to avoid interference of the multimer with the TCR at the time of the assay, this purification is recommended to be performed 2 d prior to the assay.

**MATERIALS**

**REAGENTS**

- Peripheral blood leukocytes, see Reagent Setup
- Biocoll, density 1,077 g/ml (Merck Millipore, cat. no L6113)
- CellGro dendritic cell medium (CellGenix, cat. no. 2005)
- 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. We recently tested Biochrom's AB serum (no. S 01049.2-0.1; lot no. 0672B) with good results.
- Recombinant human IL-4 (CellGenix, cat. no. 1403; alternative source: Peprotech)
- Recombinant human GM-CSF (Gentaur, cat. no. 04-RHUGM-CSF-300MCG)
- Human IFN- $\gamma$  (Peprotech, cat. no. 300-02)
- Human IL-7 (CellGenix, cat. no. 1410; alternative source: Peprotech)
- Human IL-15 (CellGenix, cat. no. 1413; alternative source: Peprotech)
- Human IL-21 (CellGenix, cat. no. 1419; alternative source: Peprotech)
- Melan-A (26-35(A27L), peptide, immunograde (ELAGIGILTV; JPT)
- CD8<sup>+</sup> T cell 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<sup>+</sup> column (Miltenyi Biotec, cat. no. 130-042-401)
- Countess cell-counting chambers (Invitrogen, cat. no. C10228)

**Antibodies and staining reagents**

- Anti-human CD8- $\alpha$  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 (eBioscience, 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

**Plasticware**

- Plate, 48 wells, sterile (GBO, cat. no. 677 180)
- Plate, 12 wells, sterile (GBO, cat. no. 655 180)
- Plate, six wells, sterile (GBO, cat. no. 657 160)

**EQUIPMENT**

- Countess cell counter (Invitrogen) or Neubauer hemocytometer
- Microscope (Leica DM IL)
- FACS instrument (two lasers minimum)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- QuadroMACS separator (Miltenyi Biotec, cat. no. 130-090-976)
- Cell incubator (temperature 37 °C, CO<sub>2</sub> level 5%)
- Temperature-controlled freezing container (Mr. Frosty)

**REAGENT SETUP**

**PBLs** We obtain these from unstimulated apheresis products from HLA-A2<sup>+</sup> healthy donors. We immediately process apheresis products (no overnight storage). PBLs are separated with a Ficoll (Biocoll) gradient and subsequently stored as aliquots in vials containing  $3 \times 10^8$  or  $1 \times 10^8$  cells. Freezing medium contains CellGro 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 they are transferred to liquid nitrogen. To thaw cells, vials are quickly placed in a water bath and transferred to cold PBS/5% (vol/vol) HS (20 ml) immediately after thawing. PBLs are spun down (5 min, 400g, 4 °C) and resuspended once more in PBS/1% (vol/vol) HS, spun again and then used for further assays. Frozen PBLs are best used within 1 year of cryoconservation, as viability will decrease with longer storage.

**! CAUTION** Apheresis products and cells are blood products. Universal safety precautions apply, such as the use of gloves and protective eyewear.

**! CAUTION** Experiments must conform to all relevant institutional and governmental ethics regulations, and appropriate informed consent must be obtained for the proposed use of human blood. **▲ CRITICAL** To be able to use the Melan-A26-35-peptide, expression of HLA-A201 is a requirement. Candidate donors can be screened by flow cytometry using an antibody against HLA-A2 (e.g., Affymetrix/eBioscience, clone BB7.2, cat. no. 17-9876-41).

**Human AB serum** We purchase the serum in frozen flasks of 500 ml and store them directly at -20 °C. Individual flasks are slowly thawed (at 4 °C overnight), and serum aliquots are prepared in 50-ml tubes and frozen again (see note regarding differences in serum quality). The serum can be stored for up to 2 years at -20 °C.

**DC medium** For DC preparation, supplement CellGro DC medium with 1% (vol/vol) penicillin-streptomycin and 1% (vol/vol) human AB serum. Store this medium at 4 °C.

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

**Peptides** Dissolve peptides in DMSO to a concentration of 5  $\mu$ g/ $\mu$ l. For the Melan-A<sub>26-35(A27L)</sub> peptide, which has a molecular weight of 985 g/mol, this stock solution thus equals 5 mM. Store the aliquots at -80 °C. Note that a peptide's stability in DMSO is limited to ~2 months, depending also on the specific peptide sequence.

**Cytokines** Many cytokine concentrations are indicated in IU/ml, which is the correct form for comparison 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



the 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 when cytokines from different vendors are being used. The biggest difference between this protocol and the values published in many other protocols is the concentration of IL-4. Originally, concentrations of 1,000 IU/ml were reported for DC differentiation<sup>35</sup>. We titrated down this concentration and found 10 ng/ml, which equals 50 IU/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 them in aliquots at -20 °C. Cytokines (prepared in stock solution as mentioned above) can be stored at 4 °C for no longer than 4 weeks. The concentrations vary, as indicated in the following table:

Cytokine	Stock concentration
IL-4	10 ng (50 IU)/μl
GM-CSF	86 ng (800 IU)/μl
IL-7	10 ng/μl
IL-15	10 ng/μl
IL-21	30 ng/μl
IFN-γ	10 ng/μl

## PROCEDURE

### Generation of monocyte-derived DCs and naive T cells

**1|** Generate monocyte-derived DCs and prepare naive T cells as described in options A and B, respectively. Both options must be performed. Option A should be started first, and option B should be started 1 d before T cell stimulation (Step 12) and on the same day that DC maturation is induced (Step 1A(x), denoted as day -1). PBLs from the same HLA-A2<sup>+</sup> donor must be used for both options A and B.

#### (A) Generation of monocyte-derived DCs

- (i) Thaw a vial of PBLs rapidly by diluting the cells in at least 20 ml of cold wash buffer. Count the cells and perform a routine spin. (A routine spin as mentioned throughout this protocol consists of 5 min of centrifugation at 400g and 4 °C.) For standard-sized assays, start with at least  $1 \times 10^8$  PBLs.
- (ii) Resuspend the cells in prewarmed Cellgro DC medium/1% HS to achieve a concentration of  $1 \times 10^7$  cells per ml.
- (iii) Pipette 2 ml of this cell suspension into each well of a six-well plate. Prepare at least 2–5 six-well plates.
- (iv) Incubate the cells for 2–3 h at 37 °C to allow adherence to plastic.
- (v) Gently resuspend nonadherent cells by using a blue 1-ml tip. Allow the medium to run over the bottom of the well to wash nonadherent cells off. Also rinse the corners of each well.
  - ▲ **CRITICAL STEP** Applying the correct pressure for 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 plates from other manufacturers are used, adherence and DC recovery may vary.
- (vi) Add 3 ml of warm Cellgro DC medium supplemented with 1% (vol/vol) HS and 10 ng/ml of IL-4 and 800 IU/ml of GM-CSF.
- (vii) Incubate the cells for 2 d at 37 °C.
- (viii) Add 1.5 ml of fresh Cellgro DC medium supplemented with 10 ng/ml of IL-4 and 1,600 IU/ml of GM-CSF.
- (ix) Incubate the cells for an additional 24 h in the cell incubator.
- (x) *Collection of immature DCs.* Collect 3 ml of the supernatant first, then vigorously resuspend the remaining medium to remove all cells. Check under the microscope and, if necessary, wash the plate again with cold wash buffer. Pool the cells from all the wells and count.

#### ? TROUBLESHOOTING

- (xi) Spin the cells and resuspend them in prewarmed Cellgro DC medium, supplemented with 1% (vol/vol) HS, GM-CSF (800 IU/ml), IL4 (10 ng/ml), LPS (10 ng/ml) and IFN-γ (100 IU/ml). Calculate the volume depending on the cell number and the number of groups needed for the assay. A minimum of  $5 \times 10^5$  cells per well (optimal is  $\sim 2 \times 10^6$  cells per well; 2 ml per well) is necessary to obtain sufficient numbers of semi-mature DCs.
- (xii) Add the peptide (2.5 μg/ml = 2.5 μM) to each well individually. (Alternatively, if a large batch of DCs is required to be loaded with the same peptide, peptide can be added to the initial Cellgro DC medium mix.)
  - ▲ **CRITICAL STEP** The assay is validated for DCs that have been pulsed with single peptides for each individual well. Peptide mixtures can be used, but interference with peptide binding between peptides with high and low binding affinities is theoretically possible.
- (xiii) Incubate the cells at 37 °C for 16 h.

#### (B) Preparation of naive T cells

▲ **CRITICAL** This option should be done on the day when DC maturation is induced (Step 1A(x)).

- (i) Thaw PBLs as described in Step 1A(i). For a normal-sized experiment,  $1-2 \times 10^8$  PBLs are required.
  - ▲ **CRITICAL STEP** For each experiment, naive T cells are isolated freshly from PBLs. We do not recommend freezing purified naive T cells.
- (ii) Resuspend the thawed cells in cold wash buffer; count and spin.
- (iii) Use the CD8 untouched isolation kit (Miltenyi) (**Box 2**, Steps 1–11). CD8-negative cells should be retained on the LS column, whereas CD8<sup>+</sup> cells should be selected in the flow-through.

## PROTOCOL

- (iv) Directly continue with CD45RO/CD57 depletion (**Box 2**, Steps 12–22).  
▲ **CRITICAL STEP** CD57 depletion is necessary, as it depletes most of the terminally differentiated cells, which may be CD45RO<sup>-</sup>, CD45RA<sup>+</sup> (T<sub>EMRA</sub>). The size of this subset greatly varies from donor to donor<sup>31</sup>.
- (v) Count the cells, remove an aliquot for FACS analysis, and spin the remaining cells.

### ? TROUBLESHOOTING

- (vi) Resuspend cells in Cellgro DC medium supplemented with 5% (vol/vol) HS and 5 ng/ml of IL-7. The final cell concentration should be  $3 \times 10^6$  per ml.  
▲ **CRITICAL STEP** Preincubation at this cell density in IL-7-containing medium allows for optimal stimulation.
- (vii) Transfer cells to six-well plates, 2 ml per well, and incubate overnight.
- (viii) 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 naive cell population should be higher than 90% (**Fig. 5a**). Check the purity for each experiment, as variations in the final results could be due to initial impurities in cell preparation.

### Start of DC/T cell coculture

2| Collect DCs (from Step 1A) by vigorously resuspending the cells. Empty the wells and add cold wash buffer. Incubate for 20 min on ice and check adherence frequently by using a microscope. Ensure that all DCs are collected by pooling the initial culture medium and the wash buffer.

▲ **CRITICAL STEP** In contrast to the classical cytokine-based maturation cocktail (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, PGE<sub>2</sub>), DCs matured with LPS/IFN- $\gamma$  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 the cells and resuspend in 10 ml of wash buffer.

4| Count the DCs.

5| Irradiate the DCs with 30 Gy.

▲ **CRITICAL STEP** Ensure that the cells are kept cool during transport to the radiation source.

## Box 2 | Purification of naive CD8<sup>+</sup> T cells

We use the CD8<sup>+</sup> T cell isolation kit human, CD45RO beads and CD-57 beads from Miltenyi to perform Step 1B(iii,vi). The procedures to for using these kits are described here so that they can be easily substituted if an alternative kit is used. There are other alternatives with different kits from different companies to isolate naive CD8<sup>+</sup> T cells. However, depleting the terminally differentiated effector T cell population (T<sub>EMRA</sub> cells) is important to get consistent results. Therefore, selection for CD45RA or depletion of CD45RO<sup>+</sup> T cells alone is insufficient.

1. Start with  $2 \times 10^8$  PBLs (minimum  $1 \times 10^8$ ).
2. Incubate the cells with 40  $\mu$ l of cold buffer per  $10^7$  cells and resuspend.
3. Add 10  $\mu$ l of biotin-antibody cocktail per  $10^7$  cells.
4. Incubate for 15 min at 4 °C. Gently shake every 5 min.
5. Add 30  $\mu$ l of cold buffer per  $10^7$  cells and 20  $\mu$ l of microbeads per  $10^7$  cells.
6. Incubate for 15 min at 4 °C. Gently shake every 5 min. In the meantime, prepare an LS<sup>+</sup> column by placing it on the magnet and rinsing it with 3 ml of cold buffer.
7. Wash cells in 5 ml of cold buffer.
8. Resuspend cells in 1 ml of cold buffer and transfer to column.
9. Let the cell suspension enter the column, and then gradually add 3 ml of cold buffer.
10. Collect the flow-through representing the CD8<sup>+</sup>, untouched cell population.

### Depletion of the memory T cell fraction

11. Spin the CD8<sup>+</sup> cell population.
12. Resuspend the cells in 40  $\mu$ l of cold buffer per  $10^7$  cells.
13. Add 10  $\mu$ l of anti-CD57 beads per  $10^7$  cells and 10  $\mu$ l of anti-CD45RO beads per  $10^7$  cells.
14. Incubate for 15 min at 4 °C. Gently shake every 5 min.
15. Prepare an LD column by rinsing it with 3 ml of cold buffer (placed on the magnet).
16. Wash the cells with 5 ml of cold buffer.
17. Resuspend the cells in 1 ml of cold buffer and transfer them to the LD column.
18. Allow the cells to enter the column.
19. Rinse the column with 3 ml of cold buffer.
20. Collect the flow-through representing the naive T cell population
21. Return to PROCEDURE at Step 1B(v).

**▲ CRITICAL STEP** Irradiation may have an additional influence on DC maturation. However, the crucial 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 the DCs in warm Cellgro DC medium/5% (vol/vol) HS at a concentration of  $5 \times 10^5$  cells per ml.
- 7| Collect the T cells (from Step 1B); count and spin.
- 8| Resuspend the T cells in CG-DC medium/5% (vol/vol) HS at a concentration of  $2 \times 10^6$  cells per ml.
- 9| Add IL-21, 60 ng/ml, to the T cell fraction. (This will result in a final concentration of 30 ng/ml after the addition of the DCs).
- 10| Mix the DCs with T cells at a 1:1 (vol/vol) ratio (resulting in a 4:1 T cell:DC ratio): calculate for at least 3–5 wells per group (condition). If alternative peptides are to be tested, always include a preparation of Melan-A–pulsed DCs, matured under standard conditions, as an internal positive control (provided that the experiment is performed for HLA-A2–restricted peptides). Always mix the cells for each group first in one larger tube. Do not add T cells and DCs separately to the individual wells, as variation owing to pipetting errors will increase. Groups consisting of a single well do not provide sufficient data on the variation within your experiment.
- 11| Transfer 500  $\mu$ l of the cell mix into individual wells of a 48-well plate.
- 12| Incubate at 37 °C for 72 h.

**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 medium to give 0.5 ml per well.
- 14| Make up warm Cellgro DC medium containing 5% (vol/vol) HS and a 5 ng/ml concentration each of IL-15 and IL-7 (referring to the final concentration in the culture medium).
- 15| Add 500  $\mu$ l of this cytokine-supplemented medium to each well.
- 16| Incubate the cells for 72 h.

**Second feeding**

- 17| Calculate the required amount of medium to give 1 ml per well.
- 18| Make up warm Cellgro DC medium containing 5% (vol/vol) HS and a 5 ng/ml concentration each of IL-15 and IL-7 (referring to the final concentration in the culture medium).
- 19| Add 1 ml of this cytokine-supplemented medium to each well of a 12-well plate.
- 20| Transfer the cells and medium from each well of the old plate (from Step 16) 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 the cells for 48 h.

**Third feeding**

- 22| Calculate the required amount of medium to give 2 ml per well.
- 23| Make up warm Cellgro DC medium containing 5% (vol/vol) HS and a 10 ng/ml concentration each of IL-15 and IL-7 (referring to the final concentration in the culture medium). Note that now the cytokine concentration is doubled.
- 24| Add 2 ml of this cytokine-supplemented, freshly prepared medium to each well of a six-well plate.





### Box 3 | Methods we use to evaluate T cells

**Cell number.** Although 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 adds a considerable amount 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 internal control, these responses can be measured by using MHC multimers. Staining of an aliquot of cells is performed in 96-well plates by using a staining volume of 50  $\mu$ l of 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 for APC-labeled MHC multimers). To reduce internalization of the multimer, the staining buffer is substituted with 100 nM dasatinib<sup>66</sup>. Cells are incubated at room temperature for 20 min. Additional antibodies, including anti-CD8, are added only after this staining period. After an additional 20-min staining period, cells are washed twice and analyzed.

**Absolute number of antigen-specific cells.** Values obtained from counting cell numbers 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 depend 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 by 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.

**25|** Transfer the cells and medium from each individual well of the 12-well plate (from Step 21) to individual wells of the new 6-well plate. Incubate for 72 h.

#### Analysis on day 10 of culture

**26|** Analyze cells. The T cells are routinely analyzed on day 10 of culture. Analysis may vary depending on the research question. We briefly describe the methods that we routinely use for evaluation in **Box 3**. If T cells are to be evaluated for cytotoxic activity, purification by MHC multimers is recommended (**Fig. 6** and **Box 1**). In **Box 4**, we present 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 were no antigen-specific T cells in the starting well). However, if the wells are pooled before analysis, the specific T cell lines will become diluted with nonspecific T cells from the negative wells.

#### ? TROUBLESHOOTING

### Box 4 | Cytotoxicity assay with activated caspase 3 as readout

Use T cells purified with MHC dextramers (**Box 2**). The test should be performed 48 h 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.

#### Additional Materials

Tumor cells as target cells (ideally, test antigen-negative and antigen-positive cells in parallel; alternatively, include a cell line lacking the respective HLA allele as a negative control).

Activated caspase-3 antibody, PE-labeled, rabbit anti-active caspase-3 (BD Pharmingen, cat. no. 51-68655X)

Fix/Perm and Perm/Wash solution (BD Biosciences)

Anti-CD8 and anti-CD45 antibodies for surface staining

#### One day before the assay

1. Collect the T cells and count them.
2. Spin the T cells, keep the supernatant and adjust the cell concentration to  $1 \times 10^6$  cells per ml by using the supernatant. (This way, one can avoid adding fresh cytokines, which would potentially result in increased nonspecific activation.)
3. Transfer the T cell suspension to a 96-well plate; 200  $\mu$ l per well ( $2 \times 10^5$  cells per well). The outline of the plate should match your assay outline (see below).
4. Collect the tumor cells and count them.
5. Resuspend the tumor cells in the appropriate fresh culture medium (e.g., RPMI/10% (vol/vol) FCS) and adjust cell numbers to  $5 \times 10^5$  cells per ml.
6. Transfer the tumor cells to another 96-well plate (round bottom), 200  $\mu$ l per well ( $= 1 \times 10^5$  cells per well). The outline should match the T cell plate. Include wells for the following controls: tumor cells only and peptide-loaded tumor cells. If enough T cells are available, duplicates of each well are recommended.

(continued)

## Box 4 | (continued)

7. Add peptide (1 µg/ml) to the control wells with tumor cells as the maximum lysis control. (Note that this maximum control substantially differs from the maximum lysis control used in <sup>51</sup>chromium-release assays, which is usually obtained by lysing all cells with a detergent.)
8. Incubate the T cells and the tumor cells in their respective plates overnight at 37 °C.

### Day of the experiment

9. Warm Cellgro DC medium/1% (vol/vol) HS to 37 °C (20 ml per plate).
10. Spin the plate containing tumor and T cells. Discard the supernatant by flicking the plate over the sink.
11. Resuspend the tumor cells in 200 µl of Cellgro DC medium per well and spin again. Discard the supernatant (this step is meant to wash off the residual culture medium from the tumor cells as well as the peptide in the peptide-control wells).
12. Resuspend T cells in 200 µl of warm Cellgro DC medium/1% (vol/vol) HS, 200 µl per well.
13. After discarding supernatant of the tumor cell plate, transfer the T cells to the tumor cells with a multichannel pipette (Note: do not transfer tumor cells to T cells, as tumor cells may be adherent).
14. Spin the plate for 30 s to allow quick sedimentation of the T cells. (Do not discard.)
15. Incubate the plate for 4 h at 37 °C.

### Staining procedure

▲ **CRITICAL** All staining/washing steps are performed with a multichannel pipette, including the transfer of cell samples to microtubes for acquisition.

16. Perform staining directly in the 96-well plate. Spin the cells and discard supernatant by flicking the plate.
17. Resuspend once in 200 µl of PBS/1% (vol/vol) HS and spin again, then discard the wash buffer.
18. Prepare staining mix with wash buffer (50 µl per well) and the appropriate amount of antibody (CD8-PerCP-Cy5.5 and CD45-eFluor450). We generally use 1:50 dilutions, but staining intensity may vary depending on the antibodies used.
19. Incubate for 10 min at RT.
20. Wash the cells once.
21. Fix and permeabilize the cells by resuspending them in 100 µl of Fix/Perm buffer.
22. Incubate for 10 min at RT.
23. Wash the cells by adding permeabilization buffer.
24. Resuspend the cells by adding 50 µl of permeabilization buffer and anti-activated caspase-3 antibody (1:25).
25. Incubate for 20 min at RT.
26. Wash the cells with permeabilization buffer and transfer them to FACS microtubes.
27. Acquire at least 5,000 tumor cells per sample with a flow cytometer by following the gating strategy described in **Figure 5**.

## ? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

**TABLE 3** | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(x)	Low yield of DCs	Extensive washing during purification of plastic adherent monocytes (Step 1A(v))	Reduce the washing steps and control frequently using a microscope
	Strong contamination of the DC population with other cell types	Insufficient separation during plastic adherence (Step 1A(v))	Increase the washing steps and control frequently using a microscope
1B(v)	Low yield of naive T cells	Low number of naive T cells in the particular donor; elution phases on the magnetic column are suboptimal/elution phases on magnetic column suboptimal	Check initial PBLs for naive cell population by FACS. Check each fraction of the separation process by FACS to track naive T cells
26	Expansion of CD8 <sup>-</sup> cell populations during culture	Insufficient naive T cell purification leading to expansion of NK cells or CD4 <sup>+</sup> T cells; insufficient irradiation of DC-containing population, thereby introducing contaminating lymphocytes	Check purification steps by FACS
	T cells do not expand properly	Possible reasons include serum quality, cytokines and cell density	Check several donors and use autologous serum

(continued)

**TABLE 3** | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	T cells do not respond to endogenously presented antigen (e.g., by tumor cells)	Initial priming may still be suboptimal	Check DCs for IL-12 production
	T cells kill tumor cells nonspecifically	May be caused by even small fractions of contaminating NK cells	Select for MHC multimer <sup>+</sup> CD8 <sup>+</sup> T cells 2 d before the functional assay

**● TIMING**

The protocol as described here stretches over a total experimental time of 14 d, up until day 10 of T cell culture. For convenience, starting with a DC preparation on a Monday (Step 1) and priming T cells on Friday (Steps 2–12) 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 readout 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 16 h.

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

Step 1A(i–vii), day –4: 3 h plus 3 h of incubation

Step 1A(viii, ix), day –3: 30 min

Step 1A(x–xiii), day –1: 1 h

Step 1B(i–viii), day –1: 3 h

Steps 2–12, day 0: 3 h

Steps 13–16, day 3: 30 min

Steps 17–21, day 5: 1 h

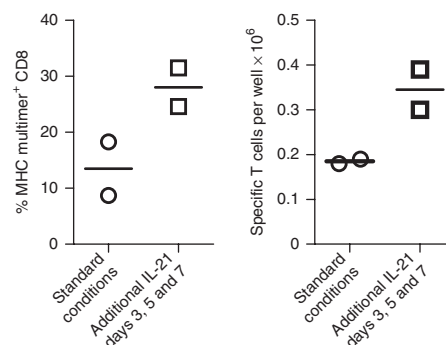
Steps 22–25, day 7: 1 h

**ANTICIPATED RESULTS**

Examples for typical results of ACE-CD8 are shown in **Figures 4–6**. **Figure 4** highlights important aspects of 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: T cells primed with IL-12 nonproducing DCs and matured with a cytokine cocktail including PGE<sub>2</sub>, only poorly recognize endogenously presented antigen (**Fig. 4c**). Other factors crucial to optimal priming are the T cell:DC ratio (**Fig. 4d**) and the presence of IL-21 at the initial priming step (**Fig. 4e**).

On day 10–12 of culture, T cells display an early effector memory cell type. They are CD45RO<sup>+</sup>, but still express CD45RA to some level. Moreover, the T cells still express CD62L and, to some degree, CCR7, which gradually declines further as the cells expand. Co-stimulatory molecules such as CD28 and CD27 are still expressed at variable degree. The phenotype after 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*<sup>54,55</sup>. 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 (ref. 56). In this case, this was observed even after a prolonged culture time due to the cloning of individual cells and their massive expansion.

**Figure 5** provides data on a representative experiment targeting other peptide antigens with lower precursor frequency. First, the quality of the naive T cell preparation was evaluated (**Fig. 5a**). The experiment was subsequently



**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 under standard conditions did not meet the predefined criteria of >24% MHC multimer<sup>+</sup> T cells. In addition, the assay had not been performed in triplicate, which would have been desirable. Repeat experiments revealed a problem with this particular leukapheresis.



set up in five parallel wells for each peptide tested, and MHC-multimer staining was performed for each individual well (Fig. 5b), 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 in 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. 5c). T cells expanded over only a short time period remain reactive against endogenously presented antigen. Figure 6 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 4. 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 otherwise<sup>42</sup>.

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