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TITLE:

Simultaneous Quantification of Anti-Vector and Anti-Transgene-Specific CD8$^+$ T cells via MHC I Tetramer Staining after Vaccination with a Viral Vector

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KEYWORDS:

Mouse, blood, antigen-specific CTLs, MHC I, FACS, tetramer, phenotyping, quantification, immunization

SUMMARY:

Here, we present a protocol for the *ex vivo* qualitative detection of antigen-specific CD8$^+$ T cells. Analysis is possible with single cell suspensions from organs or from small amounts of blood. A broad range of studies require the analysis of cytotoxic T cell responses (vaccination and cancer immunotherapy studies).

ABSTRACT:

Upon viral infection, antigen-specific CD8$^+$ cytotoxic T cells (CTLs) arise and contribute to the elimination of infected cells to prevent the spread of pathogens. Therefore, the frequency of antigen-specific CTLs is indicative of the strength of the T cell response against a specific antigen. Such analysis is important in basic immunology, vaccine development, cancer immunobiology and the adaptive immunology. In the vaccine field, the CTL response directed against components of a viral vector co-determines how effective the generation of antigen-specific cells against the antigen of interest (*i.e.,* transgene) is. Antigen-specific CTLs can either be detected by stimulation with specific peptides followed by intracellular cytokine staining or by the direct staining of antigen-specific T cell receptors (TCRs) and analysis by flow cytometry. The first method is rather time-consuming since it requires sacrificing of animals to isolate cells from organs. Also, it requires isolation of blood from small animals which is difficult to perform. The latter method is rather fast, can be easily done with small amounts of blood and is not dependent

on specific effector functions, such as cytolytic activity. MHC tetramers are an ideal tool to detect antigen-specific TCRs.

Here, we describe a protocol to simultaneously detect antigen-specific CTLs for the immunodominant peptides of the viral vector VSV-GP (LCMV-GP, VSV-NP) and transgenes (OVA, HPV 16 E7, eGFP) by MHC I tetramer staining and flow cytometry. Staining is possible either directly from blood or from single cell suspensions of organs, such as spleen. Blood or single cell suspensions of organs are incubated with tetramers. After staining with antibodies against CD3 and CD8, antigen-specific CTLs are quantified by flow cytometry. Optionally, antibodies against CD43, CD44, CD62L or others can be included to determine the activation status of antigen-specific $CD8^+$ T cells and to discriminate between naïve and effector cells.

INTRODUCTION:

The aim of this method is to assess the frequency of cytotoxic T lymphocyte (CTL) responses to (multiple) antigens in the mouse by flow cytometric analysis without the need for time-consuming peptide stimulation. This method analyses the phenotype and antigen specificity of CTL subsets, in a single staining. We optimized the Major Histocompatibility Complex I (MHC I) tetramer staining protocol to analyze efficacy of new vaccine approaches, such as VSV-GP, a new variant of the vesicular stomatitis virus (VSV), where the glycoprotein G of VSV has been replaced by the glycoprotein GP of the lymphocytic choriomeningitis virus (LCMV)[1,2]. In addition to the humoral response, an important read-out of the effectivity of a vaccine is the induction of a CTL response against one or several antigens. As the consistency and durability of the cellular response are important in this context, it is favorable to monitor kinetics of CTL responses from the same animal. This will also lead to a reduction of animal numbers, an important aspect regarding the principles of the "3Rs"[3]. Hence, analysis from as little as 20 µL of blood is optimal for this purpose.

Tetramers were developed in the late 90s[4] and became an important tool in the field of T cell immunology. Tetramers are fluorescently-labeled complexes of four MHC I/peptide molecules, which bind to TCRs, specific for a single peptide. Nowadays, they can be either bought ready-made[5], custom-ordered free of charge at the NIH Tetramer Core Facility at Emory University[6] or produced in the lab[7]. MHC I and II tetramers are available*, i.e.* for $CD8^+$ and $CD4^+$ T cells, respectively. The potency of tetramer staining lies in time-savings, rather simple and easy to standardize[8] protocols, and sensitivity[9]. Also, if working with blood, animals do not need to be sacrificed and minimal amounts of sample are required. One measurement is not limited to a single antigen, but several antigens can be analyzed in one staining when combining tetramers conjugated with different fluorophores. Newly discovered antigens, *e.g.* from peptide screens, can easily be incorporated in tetramers and used for quantification of the T cell subset.

Tetramer staining will not give information about CTL functionality (*i.e.*, cytokine production, effector functions), but only specificity. To gain information about T cell functionality, intracellular cytokine staining (ICS) or Enzyme Linked Immuno Spot (ELISpot) Assay needs to be performed[8,10]. Tetramer staining and ICS/ELISpot, however, are not redundant but rather complement each other. *In vitro* stimulation to induce cytokine production for ICS/ELISpot will

alter the original T cell phenotype. Tetramer staining, in contrast, leaves the T cell untouched; the original phenotype is preserved and can be analyzed. Also, another big advantage of tetramers is that staining can be combined with magnetic sorting and enrichment of antigen-specific cells[11]. This allows for the analysis of rare populations, as well as culturing of sorted cells with defined antigen-specificities – a feature that is not be possible with other methods.

Using the protocol described here, tetramer staining, as well as ICS/ELISpot can be performed from one organ, because only very little material (blood: 20 µL; spleen: 1 x 10^6 cells) is required for tetramer staining. However, depending on the frequency of the antigen-specific cells of interest, the strength of the respective TCR and the experimental context, the amount of cells required might need to be scaled up or magnetic enrichment might need to be applied.

Tetramers are widely used, for example to assess effectivity of (antitumor) vaccines[12-15] or immunotherapy[16,17], phenotypic analysis and spatial localization of antigen-specific T cell subsets[18-23]. The method described here is suited for studies, which aim to include the quantification and phenotypic analysis of murine antigen-specific $CD8^+$ T cells in their analysis in a fast and convenient way.

PROTOCOL:

All methods described were performed in compliance with the Austrian National Animal Experimentation Law ("Tierversuchsgesetz"), and animal trial permission was granted by Austrian National Authorities.

1. Buffer Preparation and Sample Collection

Note: The mouse strain used depends on the epitope analyzed. Choose an appropriate tetramer that binds to an MHC type expressed in the mice, *e.g.,* H-2Kb for C57BL/6 mice. The gender and age of the animals will depend on the scientific question. For most of the experiments described here, use female mice at 6-8 weeks of age at the start of the experiment, *i.e.,* first immunization.

1.1 Prepare FACS Buffer (Phosphate-buffered saline (PBS) + 1% fetal calf serum (FCS) + 0.1% sodium azide + 2 mM Ethylenediaminetetraacetic acid (EDTA) and FACS fixing buffer (1.5% (v/v) formaldehyde in PBS).

Note: It is recommended that both buffers are prepared in advance. Stored at 4 °C until use.

1.2 Blood: Collect 20 µL of blood per mouse from the tail vein of the mouse in EDTA-coated tubes, as described previously[24].

Note: Blood may also be collected by other routes, *e.g.,* vena facialis or retro-orbital sinus. However, the method of blood collection has to be in compliance with the national animal experimentation law and animal trial applications. Collection of blood from the tail vein is ideal

for studies where repeatedly small amounts of blood are needed. Additional material is required for compensation controls and the non-stained control.

1.3 Spleen: Isolate the organ and, with the help of the plunger of a syringe, press through a 70 nm and 40 µm cell strainer. Perform lysis of erythrocytes, as described in step 6 and count. Adjust the concentration to 1 × 10^7 cells/mL in PBS. Per sample, 1 x 10^6 cells are required.

Note: Always include some mock immunized or control vector immunized animals as negative control. For ovalbumin (OVA)-tetramer, a sample from OT-1 mice might be used as positive control. Do not forget unstained and compensation controls in the calculation. If necessary, samples from different animals in the experiment might be pooled for this.

1.4 After sample collection, directly proceed with the staining.

2. Staining Set-Up

2.1 Prepare one FACS tube for each sample. Label tubes properly and transfer 100 µL of organ suspension (1 × 10^6 cells) or 20 µL of blood into each tube.

2.2 Spleen: Centrifuge for 5 min at 600 × g at 4-8 °C and discard the supernatant. Vortex to resuspend the cell pellet.

Note: This will result in a remaining volume of around 20 µL, similar as the volume for the blood samples.

2.3 For each channel to be used also prepare one FACS tube for a compensation sample. Prepare one additional sample as an unstained control.

3. Tetramer Staining

3.1 For each sample, use 50 µL of tetramer dilution. For suggested tetramers and optimized dilutions, refer to **Table 1**.

[Place Table 1 here]

Note: When working with tetramers or antibodies, turn off the light of the safety cabinet and protect samples from light.

3.1.1 Prepare a tube with FACS buffer (volume = 50 µL × number of samples plus additional 10% of the total volume to compensate for pipetting errors).

3.1.2 Add tetramer(s) at optimal dilution, as listed in **Table 1**. Vortex the solution.

Note: When using the whole antibody panel (CD3, CD8, CD43, CD44, CD62L) with listed

fluorophores, two tetramers (in PE and APC) can be included. Both tetramers can be combined in a single staining, *i.e.,* staining of cells with both tetramers can be performed simultaneously.

3.2 Add 50 µL of tetramer dilution to each sample and vortex gently. Add FACS buffer only (without tetramer) to compensation controls and to the unstained sample.

3.3 Incubate samples for 20 min at 37 °C, protected from light. To ensure a seamless transition from tetramer to antibody staining, prepare the antibody mix as described in step 4 during the incubation time.

Note: For each individual tetramer, optimal conditions (dilution, incubation time and temperature) need to be adjusted.

4. Preparation of Antibodies

4.1 For each sample, prepare 50 µL of antibody mix.

4.1.1 Prepare a tube with FACS buffer (volume = 50 µL × number of samples plus additional 10% of the total volume to compensate pipetting errors).

4.1.2 Add antibodies in the dilutions as listed in **Table 2**.

[Place Table 2 here]

Note: Depending on the scientific question, other marker combinations apart from the one described here might be used. Make sure to always include antibodies against CD3 and CD8 in the panel.

4.1.3 Vortex the solution.

4.2 Prepare antibodies for compensation controls. For each compensation control, use an antibody against CD8 in the respective color.

4.2.1 For each channel, prepare a tube with 200 µL of FACS buffer and add 1 µL of a 1:200 dilution antibody against CD8 in the respective color.

4.2.2 Vortex the tubes.

4.3 Immediately proceed with staining.

5. Staining of Samples

5.1 Wash samples once by adding ~1 mL of FACS buffer and centrifuge for 5 min at 600 × g at 4-8 °C. After centrifugation, discard the supernatant and drain off remaining liquid on a stack of

paper towels.

Note: When working with blood, be cautious when draining off remaining liquid. Prior to the lysis of erythrocytes, blood will not stick to the bottom of the FACS tube. Alternatively, aspirate the supernatant.

5.2 Add 50 µL of the antibody mix to each cell pellet and vortex gently.

5.3 Add 50 µL of each compensation mix to the cell pellet of the corresponding compensation control and vortex gently.

5.4 Add 50 µL of FACS buffer to the cell pellet of the unstained control and vortex gently.

5.5 Incubate all samples for 30 min at 4 °C, protected from light.

5.6 When working with organs: skip step 6. Wash once by adding ~ 1-2 mL of FACS buffer and centrifuge for 5 min at 600 × g at 4-8 °C. After centrifugation, discard the supernatant and drain off remaining liquid on a stack of paper towels.

5.7 When working with blood: proceed to step 6 (lysis of erythrocytes).

6. Lysis of Erythrocytes

6.1 Add 500 µL of ACK (Ammonium-Chloride-Potassium) buffer[25] to each sample and gently vortex.

Note: ACK buffer will lead to osmotic swelling and lysis, specifically of erythrocytes.

6.2 Incubate for 5 min at room temperature in the dark.

6.3 Add 1 mL of FACS buffer and centrifuge for 5 min at 600 × g at 4-8 °C. After centrifugation, discard the supernatant and drain off the remaining liquid on a stack of paper towels.

Note: When the pellet is rather red, repeat the lysis of erythrocytes.

6.4 Wash once by adding ~ 1-2 mL FACS buffer and centrifuge for 5 min at 600 × g at 4-8 °C. After centrifugation, discard the supernatant and drain off remaining liquid on a stack of paper towels.

7. Flow Cytometric Measurement and Analysis

7.1 Add 150 – 300 µL of FACS fixing buffer to each tube and mix by vortexing. For 20 µL of blood, 150 µL of buffer is sufficient.

Note: Before fixation, make sure that cells are well re-suspended in order to prevent formation

of clumps. Proceed with flow cytometric measurement as quickly as possible.

7.2 Measure the compensation controls and correct any spectral overlaps.

7.3 Set up sequential gates, as depicted in **Figure 1** to select for $CD3^+/CD8^+$ cells.

7.3.1 Gate on lymphocytes using forward and sideward scatter (Area) (non-logarithmic scale).

7.3.2 Within the lymphocyte population, gate on single cells using forward scatter width vs area (non-logarithmic scale).

7.3.3 Plot single cell lymphocytes using CD3 and CD8 channels (logarithmic scale). Identify $CD8^+$ T cells by gating on $CD3^+/CD8^+$ cells.

7.3.4 Plot $CD8^+$ vs $Tetramer^+$ cells and gate on $CD8^+$ $Tetramer^+$ cells, as depicted in **Figure 2**.

7.4. If possible, record 20,000 cells (at least 5,000 cells from blood) in the $CD3^+/CD8^+$ gate for each sample and save as a FCS file.

Note: The amount of cells to record might need to be adjusted according to the frequency of the antigen-specific cells of interest.

7.5 Analyze FCS files with appropriate analysis software. Use the gating strategy, as described above (section 7.3) and quantify $CD8^+$ $Tetramer^+$ cells.

REPRESENTATIVE RESULTS:

Figure 1 demonstrates how to gate correctly on the target cells of this protocol, namely $CD3^+/CD8^+$ cells. It is to note that activated cells often downregulate the T cell receptor[26,27] and, therefore, $CD3^{low}$ cells should also be included in the gating. After gating the $CD3^+/CD8^+$ cells, tetramer positive cells can be identified (**Figure 2**). Representative blots for a negative control (naïve) mouse, as well as animals either vaccinated with either OVA-secreting Adenovirus 5 (Adeno-OVA) or OVA-expressing VSV-GP (VSV-GP-OVA) are shown. As seen in the lower blots two different tetramers can be combined in the same tube for staining. This allows simultaneous quantification of two different CTL specificities: virus-specific (VSV N) and transgene-specific (OVA) CTLs. We confirmed that single and double tetramer stainings give similar percentages of positive cells for each tetramer. Using this protocol, other virus-specific (*e.g.*, LCMV GP, HPV 16 E7) or transgene-specific (*e.g.,* GFP) T cell populations can be analyzed (**Supplemental Figure 1**). In **Supplemental Table 1**, results for five animals after immunization with VSV-GP-OVA are shown – indicating robustness of tetramer staining.

[Place Figure 1 here].

[Place Figure 2 here].

[Place Supplemental Figure 1 here].

One big advantage of the protocol described here is that T cell responses from the same mouse can be followed over time as only small amounts of blood are needed for each measurement. **Figure 3** shows exemplary results for T cell responses over time. In addition to quantities of antigen-specific CTLs, their phenotype can also be analyzed using this protocol **(Figure 4**).

[Place Figure 3 here].

[Place Figure 4 here].

FIGURE AND TABLE LEGENDS:

Table 1: Suggested tetramers and optimal dilutions. Recommended tetramers for some immunodominant peptides of model antigens (Ovalbumin (OVA) and enhanced green fluorescent protein (eGFP)) or pathogen components (Vesicular stomatitis virus (VSV) nucleoprotein (NP), Lymphocytic Choriomeningitis Virus (LCMV) Glycoprotein (GP) and human papillomavirus (HPV) E7 oncoprotein (E7)). For each, the peptide sequence and corresponding allele, as well as recommended fluorophore and optimized dilution is listed.

Table 2: Antibodies used in this protocol and optimal dilutions. Recommended surface markers (CD3, CD8, CD43, CD44 and CD62L) are listed in the first column. For each, the recommended fluorophore, optimized dilution and amount of antibody/sample are listed. In the last column, the cell type identified with each marker is specified.

Supplemental Table 1: Percentages of activated and antigen-specific $CD3^+/CD8^+$ cells after vaccination. Mice were either naïve or immunized with ovalbumin (OVA)-expressing VSV-GP (VSV-GP-OVA) (n = 5). Blood was collected from tail vein at day 7 after immunization and stained with tetramers (VSV-N and OVA). Activated ($CD43^+$) and antigen-specific ($tetramer^+$) $CD3^+/CD8^+$ cells were quantified by flow cytometry.

Figure 1: Representative gating strategy to analyze $CD8^+$ T cells in blood. Schematic representation of the gating strategy used for flow cytometric analysis. After tetramer staining and flow cytometric measurement, data was analyzed. Lymphocytes were identified with forward and sideward scatter (Area) (non-logarithmic scale). From those, single cells were identified by applying forward scatter width vs area (non-logarithmic scale). $CD8^+$ T cells were identified by gating on $CD3^+/CD8^+$ cells (logarithmic scale).

Figure 2: Representative gating strategy to quantify OVA- and N-specific $CD8^+$ T cells in blood. Schematic representation of the gating strategy used for flow cytometric quantification of $tetramer^+$ cells. $CD3^+/CD8^+$ cells were used for tetramer analysis. Upper and middle panel: the CD8 marker was plotted against respective tetramer (logarithmic scale). Lower panel: both tetramers were plotted against each other (logarithmic scale). **Left:** control (naïve) mouse; **middle:** mouse was immunized with OVA-secreting Adenovirus 5 (Adeno-OVA); **right:** mouse was immunized with ovalbumin (OVA)-expressing VSV-GP (VSV-GP-OVA). Blood was collected from

tail vein at day 7 after immunization.

Figure 3: Representative result of CD8$^+$ T cell kinetic in blood after vaccination. Schematic representation of the gating strategy used for flow cytometric quantification of tetramer$^+$ cells. CD3$^+$/CD8$^+$ cells were used for tetramer analysis and both tetramers were plotted against each other (logarithmic scale). Upper panels: mouse was immunized with OVA-secreting Adenovirus 5 (Adeno-OVA); lower panels: mouse was immunized with ovalbumin (OVA)-expressing VSV-GP (VSV-GP-OVA). Blood was collected from tail vein at day 3, 7, 10 and 14 after immunization.

Figure 4: Representative result of CD8$^+$ T cell activation and differentiation into naïve and effector cells after vaccination. Schematic representation of the gating strategy used for flow cytometric quantification of activated (CD43$^+$), naïve (CD44$^-$/CD62L$^+$) and effector (CD44$^+$/CD62L$^-$) CD3$^+$/CD8$^+$ cells (logarithmic scale). **Left:** control (naïve) mouse; **middle:** mouse was immunized with OVA-secreting Adenovirus 5 (Adeno-OVA); **right:** mouse was immunized with ovalbumin (OVA)-expressing VSV-GP (VSV-GP-OVA). Blood was collected from tail vein at day 7 after immunization.

Supplemental Figure 1: Representative result of CD3$^+$/CD8$^+$ tetramer$^+$ cells after vaccination. Schematic representation of flow cytometric quantification of tetramer$^+$ cells. CD3$^+$/CD8$^+$ cells were used for tetramer analysis and the CD8 marker was plotted against respective tetramer (logarithmic scale). **Left:** mice were naïve, **right:** mice were immunized with VSV-GP (upper panel), enhanced Green Fluorescent Protein (eGFP)-expressing VSV-GP (middle panel) or VSV-GP expressing human papillomavirus (HPV) E7 oncoprotein (E7) (lower panel). Blood was collected from tail vein at day 7 after immunization and stained with tetramers (LCMV-GP, eGFP and HPV E7).

DISCUSSION:

Tetramer staining is a rather straightforward and uncomplicated protocol to analyze phenotype and peptide specificity of a T lymphocyte. The usage of blood for analysis, as described here, is minimally invasive and allows continuous monitoring, for example in vaccination studies. In the field of vaccination, the quantification of vector- and antigen-specific responses is of interest, as vector-specific responses might hinder an effective immune response against the vaccine antigen[28]. Of note is that with the protocol described here, both populations can be quantified simultaneously in a single tetramer staining, thereby reducing staining variability and sample amounts. However, a few steps need to be done carefully to ensure proper measurement and reliable data. If using blood from the tail vein for analysis, one should make sure to pre-warm the animals to induce vasodilation[24]. Thereby, sufficient blood can be collected in a short time, stress on the animals is reduced and the analysis is much better, as compared to if the blood is collected slowly. Also, after sample collection (either blood or organs), direct staining is recommended to avoid false negative results due to TCR downregulation. The same applies for all subsequent steps: the procedure should not be interrupted and all washing steps reduced to the minimal number (as stated in the protocol). To ensure proper staining, care should be taken to vortex all solutions and samples prior to and after incubation. This is especially important before fixing the samples to avoid clumping of cells.

In terms of modifying the protocol, other surface markers and tetramers can be used, depending on the aim of the analysis. However, all reagents then need to be titrated, optimally in combination with the whole staining panel. For some of the tetramers specified here, optimization revealed that we can increase the dilution recommended by the manufacturer (1:10 recommended, optimized 1:25) (**Table 1**). To compensate spectral overlap, compensation beads can be used instead of stained cells. Regarding the choice of the tetramer-coupled fluorochrome, one should envisage to use bright fluorochromes, as this facilitates detection – especially when the signal is low. As Dolton and colleagues[29], we prefer to use PE- or APC-coupled tetramers, which can be perfectly combined in a single staining and $CD8^+$ T cells with a single antigen specificity can be nicely detected (**Figure 2**). Regarding temperature and incubation times, a variety of tetramer staining conditions exist. In our optimization process, we addressed this issue and performed tetramer staining at different conditions (*e.g.* 4 °C, room temperature or 37 °C). From the results obtained, we recommend to stain for 20 min at 37 °C, which is in concordance with literature[30,31]. Prolonged incubation should be avoided, as this can lead to internalization of the tetramer[30] and false negative results.

The choice of the right antibody for detection of $CD8^+$ cells is another important issue, which has to carefully considered (and potentially adapted). This arises from the fact that certain anti-CD8 antibody clones block binding of tetramers to the TCR, in human[32] as well as mouse[33] samples. For our tetramer staining protocol, we selected clone 53-6.7 to stain for murine $CD8^+$ cells – a clone which does not block, but rather enhances tetramer staining.

Tetramer staining is rather uncomplicated when analyzing prominent immune responses at the peak of the T cell response, for example. However, there might be populations which are a bit more 'problematic'. Such examples include cells specific for low affinity antigens (tumor, self), recently activated cells which subsequently down-regulated their receptors or rare cell subsets (*e.g.* naïve precursor or memory cell populations). In these cases, the classical tetramer staining protocol might need to be improved or combined with other methods. For example, the protein kinase inhibitor (PKI) dasatinib inhibits TCR internalization and might be included prior to tetramer staining. Tetramers can also be stabilized by including anti-fluorochrome unconjugated primary Abs after tetramer staining. Additionally, fluorescence intensity can be increased by addition of a second anti-Ab fluorochrome-conjugated Ab[29,34-36]. We optimized the conditions selectively for the tetramers specified in this protocol and did not include PKI or additional Abs. However, for any other tetramer, the optimal conditions have to be adjusted individually. With regard to rare populations, tetramer staining might need to be combined with magnetic enrichment[11].

To facilitate and validate FACS analysis of tetramer staining, negative and positive controls should be included. As a negative control, we always stain cells of a naïve mouse of the same strain with our tetramer of interest. Alternatively, samples can be stained with tetramers with irrelevant peptides, but with the same fluorochrome as the tetramer of interest. Such controls are essential to exclude false positive signals, *e.g.,* originating from dying cells. In addition to this, it is recommended to include a live/dead stain, such as propidium iodide (PI). This is of special

importance if cells are not stained directly after isolation. Another strategy to remove autofluorescence background might be to include several non-T cell markers in one channel. By excluding cells positive in this channel, non-T cell populations can be excluded. As a positive control, a sample from an OT-1 mouse can be used for OVA tetramer, for example. For other tetramers, this has to be chosen individually (*e.g.,* sample from a mouse, which was immunized several times). Alike others[37], we also observe a down-regulation of the CD8 receptor during CTL activation at day 7 of the effector T cell response. Therefore, to avoid loss of activated tetramer$^+$ effector T cells, we recommend to include the CD8^{low} cells in the analysis (at least if measuring in the effector phase).

The quality and amount of information one can retrieve from this protocol is dependent on the knowledge about the antigen to be studied, the availability and specificity of tetramer and the quality of the FACS machine (number of lasers and available detectors). If working with animal samples, variation in the immune response is natural and inevitable. Therefore, to gain meaningful results from tetramer staining, at least 3-5 animals should be analyzed. If done so, the protocol described here will give reliable and reproducible results (exemplary result from one experiment can be found in **Supplemental Table** 1). As mentioned before, this method is perfectly suited to quantify the phenotype and antigen-specificity of CD8$^+$ T cells (**Figure 3, 4; Supplemental Figure 1**), not only in the mouse but also in humans. However, to analyze CD8$^+$ T cell effector functions, such as granzyme-induced cell death, ICS and/or ELISpot need to be performed. However, one should keep in mind that T cell functions, as measured by *in vitro* stimulation might not represent the actual situation *in vivo*. *In vivo*, a suppressive environment might prevent T cell functions which are measured *in vitro*.

On its own, tetramer staining does not provide all information, but it evolved to become an essential method to characterize T cell responses and quantify T cell subsets *in vitro* in a very sensitive manner[38]. Tetramers cannot only be used to quantify certain subsets, but also to isolate those[39], localize them by in situ hybridization[19,20] and study low-affinity antigens, such as tumor-associated[40,41]. Since the discovery of tetramer technology[4], tetramer staining has become an essential tool in T-cell analysis and the range of applications.

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DISCLOSURES:

Dorothee von Laer is an inventor of VSV-GP and holds minority shares in the biotech company ViraTherapeutics GmbH, which holds the intellectual property rights for VSV-GP. For the other authors, no competing financial interests exist.

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Figure 1

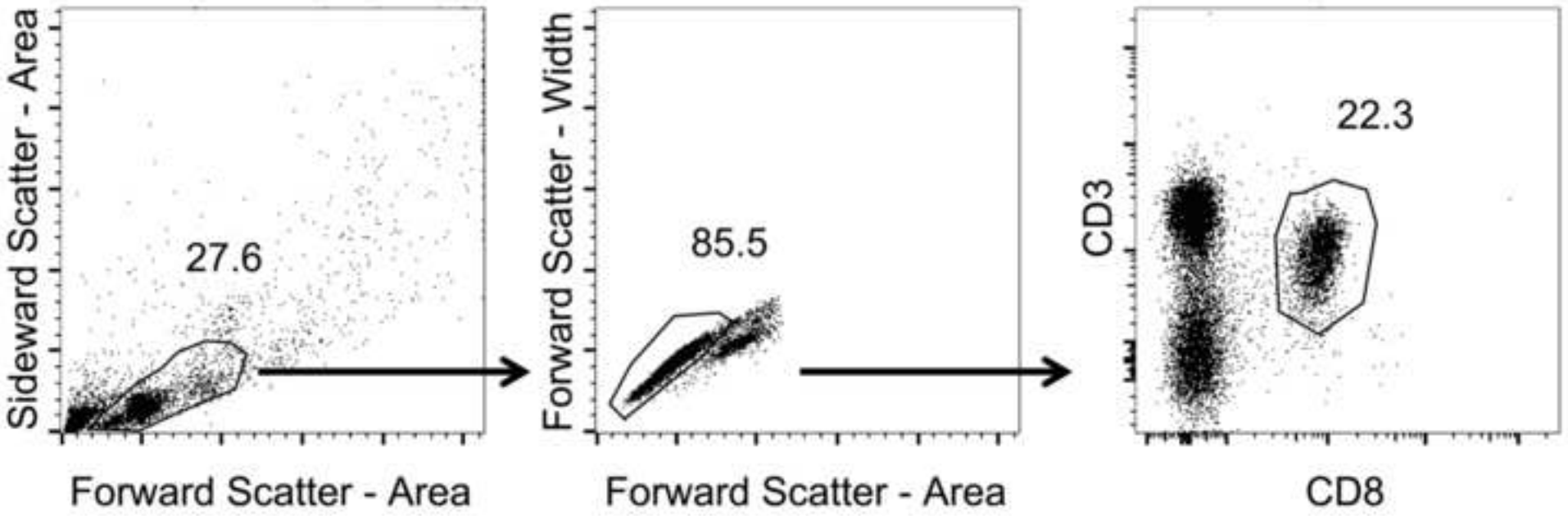

Figure 2

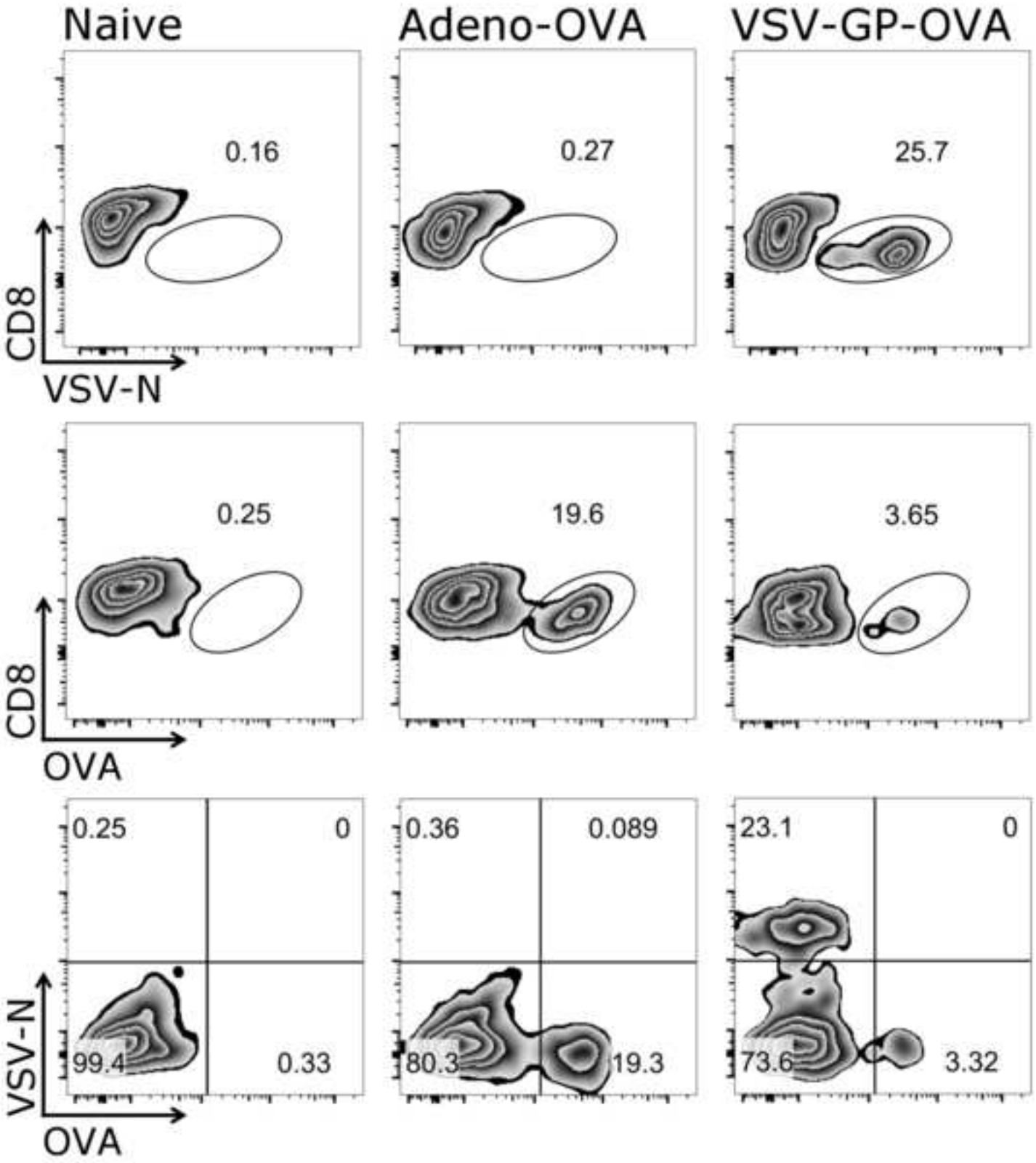

Figure 3

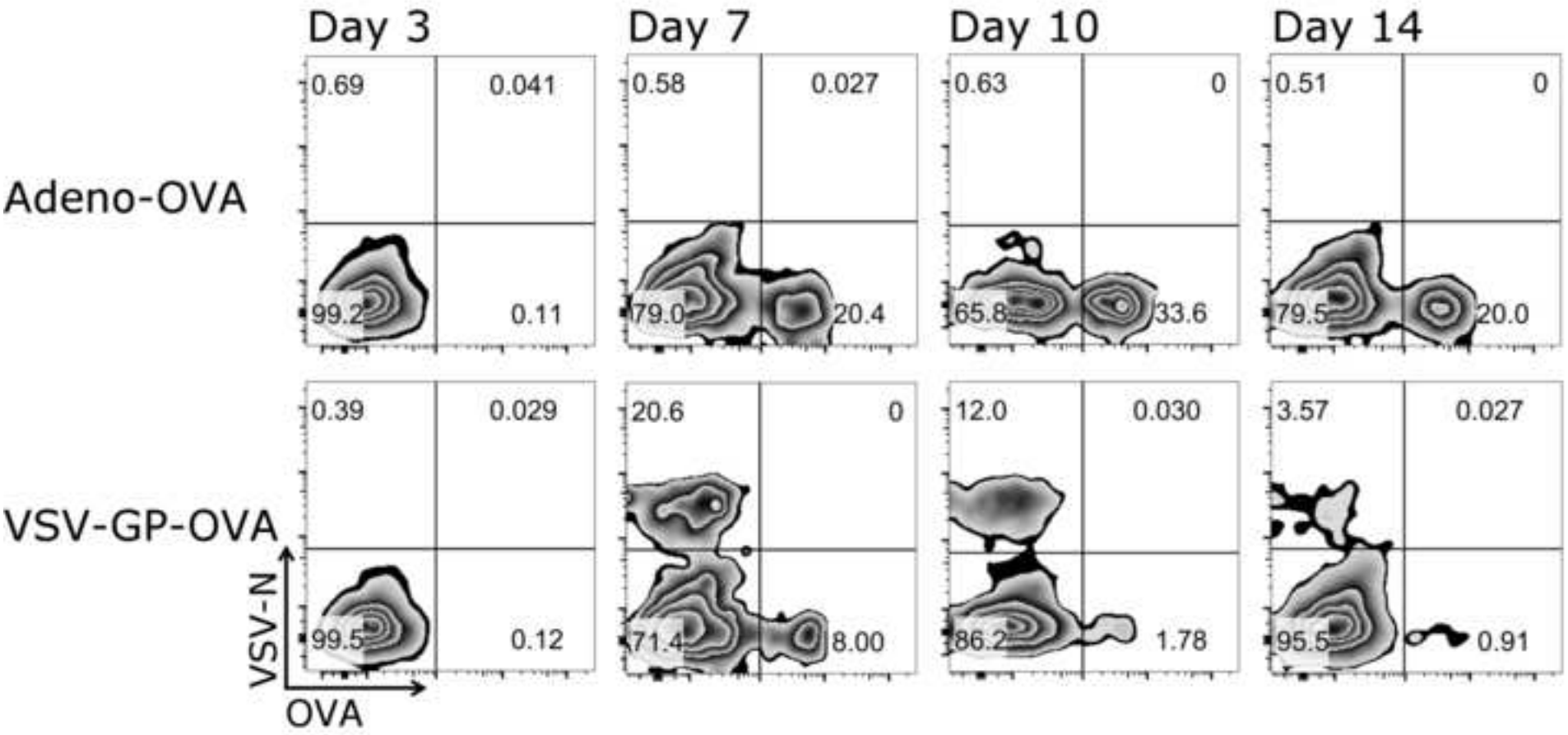

Figure 4

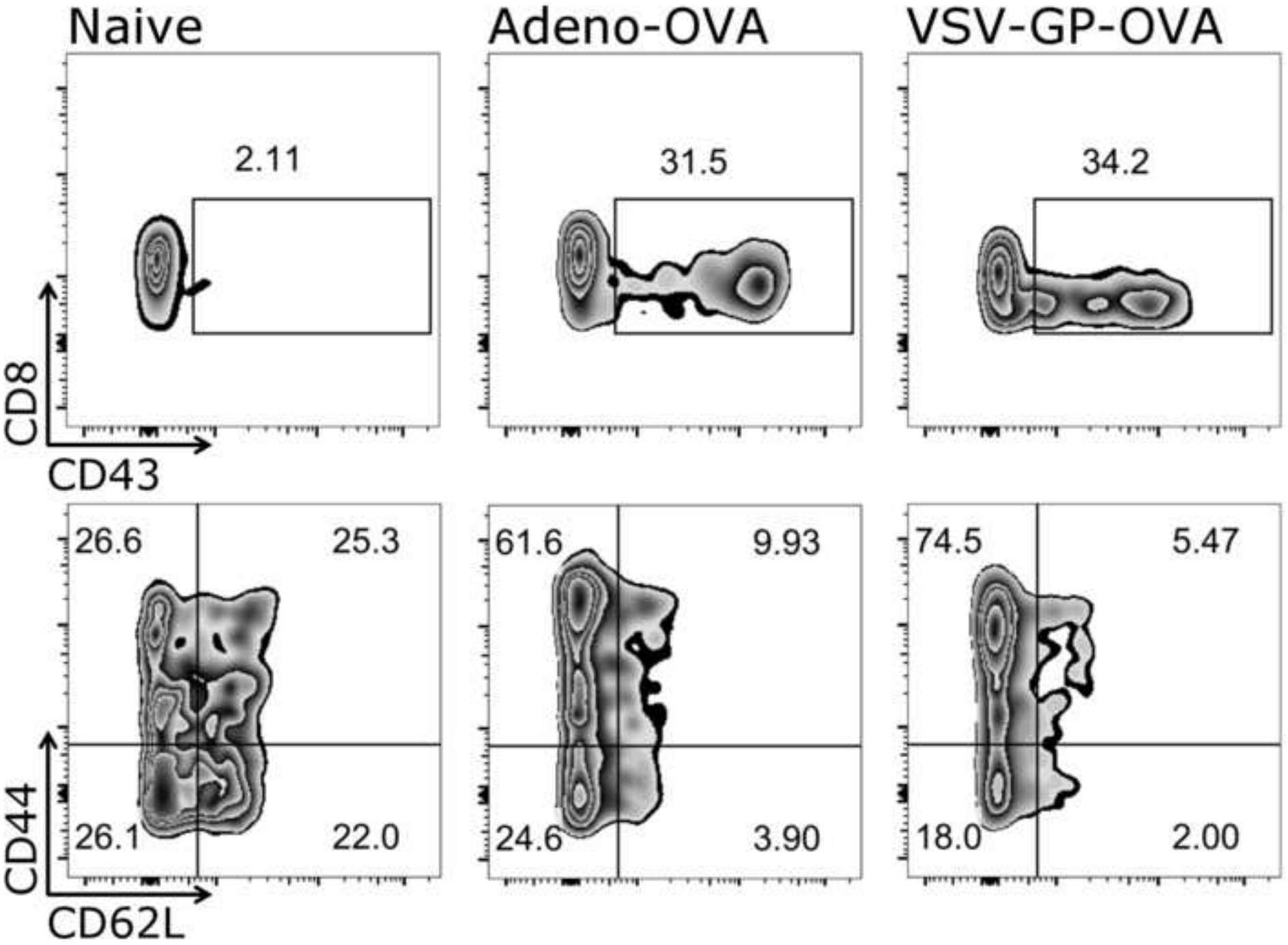

Table 1

Tetramer	Peptide sequence	Allele	Fluorophore	Dilution
MHC I/OVA	SIINFEKL	H-2Kb	APC	1:25
MHC I/VSV-NP	RGYVYQGL	H-2Kb	PE	1:25
MHC I/EGFP	HYLSTQSAL	H-2Kd	PE	1:25
MHC I/LCMV-GP	KAVYNFATC	H-2Db	APC	1:25
MHC I/HPV 16 E7	RAHYNIVTF	H-2Db	APC	1:10

Table 2

Antibody	Fluorophore	Dilution	µg/sample	Marker
CD3	PE-Cy7	1:200	0.05	CTLs ($CD3^+CD8^+$)
CD8	Pacific Blue	1:750	0.013	
	V450	1:100	0.1	
CD43	FITC	1:100	0.25	Activation ($CD43^+$)
CD44	PE-Cy5	1:250	0.04	Naive ($CD44^-CD62L^+$) & effector ($CD44^+CD62L^-$)
CD62L	APC-Cy7	1:500	0.02	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Safety cabinet class 2	VWR	LBCP302411030	
Flow cytometer (e.g. FACSCanto II)	BD	338962	
Analysis platform for flow cytometry analysis (e.g. FlowJo)	Fisher Scientific Co. L.L.C.	NC0887833	
Binocular microscopes, VisiScope 100	VWR	630-1553	
Vortex mixer	Phoenix Instrument	RS-VA 10	
Centrifuge suitable for FACS tubes (e.g. Rotanta 460R)	Hettich	5660	
Sterile Scalpel Blades Nr. 10	Braun	BB510	
Cell strainer 40 µm	Sigma	CLS431750	
Cell strainer 70 µm	Sigma	CLS431751	
Neubauer counting chamber	VWR	630-1506	
Pipettes (20 µL, 200 µL and 1000 µL)	Eppendorf	4924000037, 4924000061, 4924000088	
Pipette tips, sterile (20 µL, 200 µL, 1000 µL)	Biozym	770050, 770200, 770400	
Pipet Boy	Integra	155 000	
Sterile pipettes (5 mL, 10 mL, 25 mL)	Sarstedt	86.1253.001, 86.1254.001, 86.1685.001	
Multistep Pipette, HandyStep S	BRAND	705110	
12.5 ml Combitips for Multistep Pipette	BrandTech Scientific	702378	
Microvette CB 300 K2E	Sarstedt	16.444	
Sterile reaction tubes (1.5 mL, 50 mL)	Sarstedt	72.692.005, 62.547.254	
FACS tubes (non-sterile)	Szabo Scandic	BDL352008	

PBS	Lonza	LONBE17-516F	
Heat-inactivated FCS	ThermoFisher Scientific	10500064	
Formaldehyde	Roth	4979.1	
Sodium azide	Roth	K305.1	
PE-Cy7 Rat Anti-Mouse CD3 Molecular Complex	BD	560591	Clone 17A2; Lot # 7235504
Pacific Blu Rat Anti-Mouse CD8a	BD	558106	Clone 53-6.7; Lot # 5058904
V450 Rat anti-Mouse CD8a	BD	560469	Clone 53-6.7; Lot # 5205945
FITC anti-mouse CD43	BioLegend	121206	Clone 1B11; Lot # B233778
PE-Cy5 Rat Anti-Mouse CD44	BD	553135	Clone IM7; Lot # 85660
APC-Cy7 Rat Anti-Mouse CD62L	BD	560514	Clone MEL-14; Lot # 7215801
OVA-tetramer/APC	MBL	TB-5001-2	SIINFEKL, H-2Kb; Lot # T1702008
VSV NP-tetramer/PE	MBL	TS-M529-1	RGYVYQGL, H-2Kb; Lot # 007
EGFP-tetramer/PE	MBL	TS-M525-1	HYLSTQSAL, H-2Kd; Lot # 004
LCMV-GP-tetramer/APC	MBL	TB-5002-2	KAVYNFATC, H-2Db; Lot # T1412006
HPV 16 E7-tetramer/APC	MBL	TB-5008-2	RAHYNIVTF, H-2Db; Lot # T1804003

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Author(s): Sarah Wilmschen, Zoltan Banki, Dorothee von Laer, Janine Kimpel

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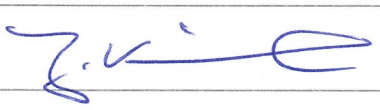
Signature:	
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Resubmission of manuscript JoVE58680 to JoVE

Dear Dr. Bajaj,

We would like to thank you and the reviewers for your evaluation of our manuscript 'Simultaneous quantification of anti-vector- and anti-transgene-specific $CD8^+$ T cells via tetramer staining'. We found the comments very helpful and have revised our manuscript accordingly. Please find below a point-by-point response.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

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We have done this.

4. 1.2: Please specify the gender, age and strain of mouse.

The details were added.

5. 1.3: What is isolated here? More details are needed here.

Isolation of spleen was specified.

6. 7.5: Please add more details here.

More details regarding analysis were added.

7. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included.

An introducing paragraph was added.

8. References: Please do not abbreviate journal titles.

We have selected the JoVE endnote style.

9. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. Please use SI abbreviations for all units: L, mL, µL, etc.

We have done this.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a protocol to identify antigen-specific CD8+ T cells from blood or lymphoid tissue samples by staining with peptide-MHCI tetramer reagents. This is a very simple procedure that has been in common use for two decades, and has already been published in JoVE in greater detail, albeit with T cells from macaques instead of mice (Gonzalez-Nieto, Martins, et. al., 2016). Aside from the different species of animal T cells being studied, there is very little value added by this protocol. Novelty notwithstanding, the protocol could be greatly improved with attention given to the following issues:

Major Concerns:

1. The title is a bit confusing because it is unclear what "anti-vector" and "anti-transgene" refer to until one reads through the whole protocol. The word "vaccine" and "MHCI" should be included. The abstract should also explain the benefit of simultaneously tracking CD8+ T cell responses to both the vaccine epitope and epitopes within the vector, if this is indeed a selling point.

As recommended, we modified the title to “Simultaneous quantification of anti-vector- and anti-transgene-specific CD8+ T cells via MHC I tetramer staining after vaccination with a viral vector”. The abstract has also been updated.

2. The authors should discuss how CD8 binds to MHCI and contributes to tetramer staining. A major issue with tetramer staining is the use of proper CD8 antibody clones to prevent blocking of this interaction.

This aspect has been added to the discussion (see lines 387-391).

3. The concentration of tetramer used should be explicitly stated as a molar or mass/volume quantity, not just a dilution factor of whatever stock they are using. The same holds true for antibodies. If each tetramer needs to be titrated, the authors should state this.

As the manufacturer does not give information about the molarity and recommends to use the tetramers in certain dilutions, these dilutions are stated. The information, that each tetramer needs to be titrated was further emphasized.

4. The tracking of cell, tetramer, and antibody volumes through the steps is confusing, especially because the authors describe two different starting volumes, 100 ul for cells from lymphoid tissues, and 20 ul for blood samples. Are these sample types supposed to be stained at different tetramer concentrations? In addition, they don't mention what volume the cells are in when they add the antibody cocktail.

We have clarified this point in the protocol (see 2.2). As the splenocytes are pelleted prior to addition of the tetramer and resuspended in the volume that remains in the tube after discarding the supernatant, the volume prior to adding the tetramer will be ~20 µL (same as for the blood). Therefore, both types of samples are stained at the same tetramer concentration.

The antibody cocktail is added to the cell pellet after washing the samples. We amended this information in the protocol.

5. A dump channel to remove non T cell populations (e.g. B cell, CD4+ T cell, and macrophage markers) would help reduce autofluorescent background.
The reviewer is correct that a dump channel is a good option to reduce autofluorescence. We included a comment to this in the discussion (see lines 414-416).
6. The CD3+CD8+ population in Figure 1 appears to be largely CD3-. Is this a compensation issue?
I has been reported that T cell activation correlates with T cell receptor downregulation (PMID: 10714682; 8187769). We indeed see this also in our hands after immunization of mice with viral vectors and therefore do not believe that this is a compensation issue. We included a comment to this in the results section (see lines 270-271).
7. If the authors are going to include the phenotypic markers CD43, CD44, and CD62L in their protocol, they should show some representative data in which they use these to identify naïve and effector T cell subsets.
Representative data were added (Figure 4).
8. There are many different incubation times and temperatures used for tetramer staining throughout the literature. The authors should discuss this variable and how they arrived at their conditions.
We emphasized this aspect in the discussion (see lines 381 ff.).
9. The authors should discuss the issue of low antigen-specific T cell frequencies in naïve and memory populations and whether enrichment strategies are needed for the analysis of these.
This issue has been added to the discussion (see lines 393 ff.).

Minor Concerns:
1. Why are compensation samples stained in 1.5 ml tubes when the other samples are stained in FACS tubes (5 ml)?
All samples are stained in FACS tubes. We clarified this in the protocol.
2. Why is the ACK lysis performed after tetramer and antibody staining?
As we also outline in the discussion (lines 385-386), loss of signal due to TCR internalization over time is a problem for tetramer staining. We therefore perform the tetramer staining in the first step and do the lysis of erythrocytes afterwards. It might also be possible to do the lysis in the first step. However, we did not try this.

Reviewer #2:

Manuscript Summary:
The Manuscript describes a protocol for monitoring antigen-specific CD8+ T cell responses by direct staining of specific T cell receptor with tetramers. In particular the authors describe a method allowing the simultaneous detection of CD8 T cells specific against two different antigens from blood or organs. Furthermore, the protocol includes information to determine the activation status of analyzed T cells by CD44, CD62L staining.
A detailed overview of all required buffers, tetramer and antibody mix staining solutions (including dilution) is given. Organ preparation, staining procedure for tetramer and antibody including controls as well as flow cytometric setting is depicted clearly. In addition, the all material needed is listed.
In general the protocol is written in good English, well stuctured and generally intelligible. All procedures are described in detail and are easy to follow.

Major Concerns:
None

Minor Concerns:

Line 81: Please give the minimal cell number needed rather the information "very little cells".

We have added this information in line 90.

Line 94: Can buffer be stored or should it be prepared fresh? Please give a defined temperature rather the information "cold".

We added the comment that buffers can be prepared in advance and stored until use at 4 °C.

Line 130: The authors mentioned that two tetramers (in PE and APC) can be included in the panel. It is not clearly written whether it is necessary to stain first with one tetramer and afterwards with the second or can the staining be done with two tetramers at the same time.

The information that both tetramers can be combined in a single staining, *i.e.* staining of cells with both tetramers can be performed simultaneously, was added.

Line 146: Might be helpful to mention that antibody mix should be prepared in advance (or during the 20 min incubation time "step 3.3") to directly proceed with Ab staining after tetramer staining.

We added a comment to this.

Line 152: Table 1 is pasted twice in the outline (line 127 + line 152). Alternatively, one might split the table 1 into table 1 "tetramers" and table 2 "antibodies".

As suggested, table was split into table 1 (tetramers) and table 2 (antibodies).

Line 178: Include the information that samples should be protected from light.

The information, that samples should be protected from light was included.

Figure 2: It's pivotal to provide the information about the frequencies (%) of tetramer positive cells (especially in B) + C)). Does simultaneous use of two tetramers results in the same frequencies of tetramer positive cells as staining with single tetramers of the same sample?

Frequencies were added to all figures. Yes, we have confirmed that the simultaneous use of two tetramers results in the same frequency of tetramer$^+$ cells as if using a single tetramer and have added a comment to this in lines 277/278.

Figure 2: The Scale in B) and C) is different and should be aligned.

All scales were aligned.

Figure 2C: CD8+ population is very close to zero and it gives the impression that specific proportion of VSV-N and OVA tetramer positive cells are in cutt-off.

This impression was due to the different scale in figure 2C. We double checked that no tetramer positive cells were cut off.

Reviewer #3:

Manuscript Summary:

Wilmschen and colleagues describe the procedure to stain T cells against two different antigens (vector- and transgene-specific) using tetramer technology in addition to staining of CD8+ T cell markers. In this respect, the authors show staining of CD8+ T cells with VSV-N and OVA tetramer as cross-sectional (Fig. 2) and longitudinal analysis (Fig. 3).

It is appreciated that the authors included important hints, e.g. lines 142-144 ("prior to lysis of erythrocytes, blood will not stick to the bottom of the FACS tube."), lines 272-273 ("pre-warm the animals to induce vasodilation"), lines 284-286 ("for some of the tetramers specified here, … we can increase the dilution recommended by the manufacturer"), lines 290-291 ("we recommend to stain for 20 min at 37 °C … prolonged incubation should be avoided"), and lines 305-307 ("… observe a down-regulation of the CD8 receptor during CTL activation … recommend to include the CD8(low) cells in the analysis").

Major Concerns:

- Fig. 2: include percentages of gated cells with tetramer staining. How often was this experiment repeated? Please include data about the variability. It remains unclear whether the percentage of OVA tetramer+ cells in OVA-immunized mice is larger or similar to OVA- and N-immunized mice. Have the authors checked whether staining with just one of the tetramers reveals similar percentages of positive cells compared to staining with both tetramers?

Frequencies were added to all figures. Also, a supplemental table (Supplemental Table 1) was added with data from several mice from one experiment to indicate variability. We have performed several independent experiments and always measure similar numbers of Tetramer$^+$ cells after vaccination. Yes, we have confirmed that the simultaneous use of two tetramers results in the same frequency of tetramer$^+$ cells as if using a single tetramer and have added a comment to this in lines 277/278.

- The authors do not show data for all immunodominant peptides mentioned in the abstract. These data should be added as supplementary figures.

We have added a supplementary figure 1 showing exemplary results for HPV 16 E7, GFP and LCMV GP tetramers.

Minor Concerns:

- Introduction, lines 53-54: "… pseudotyped with the glycoprotein of the lymphocytic choriomeningitis virus (VSV-GP)". Either LCMV or VSV-GP is wrong in this sentence.

VSV-GP is a chimeric VSV variant where the glycoprotein G of VSV has been replaced by the glycoprotein GP of LCMV. We clarified this in the introduction.

- Protocol, lines 93-94: what is meant by "optionally"? Do the authors use EDTA or not? This is important because EDTA helps to maintain single-cell suspensions.

EDTA is used and sentence was changed accordingly.

- Protocol, line 96: the authors describe to collect blood from the tail vein. However, they emphasize that a small amount of 20 µl is sufficient to perform the analyses, which should also be obtained from the eye. Correct?

This is correct. A note was added that blood can also be collected by other means.
However, regulations for animal experiments are rather strict in Austria/ the EU recommending retroorbital blood sampling only as terminal method. We recommend to collect blood from the tail vein as we believe that this is a good method to repeatedly collect small amounts of blood from the same animal. However, it is correct that blood samping always needs to be adapted to national regulations and animal trial applications.

- Protocol, line 119: the reference to Table 1 should already be included here.

Reference to Table 1 was included at the recommended location.

- Protocol, line 187: please explain ACK buffer.

An explanation for ACK buffer was added.

- Protocol, line 223: the number of events to record depends on the frequency of tetramer-positive cells.

A note was added that the amount of cells to record might need to be adjusted according to the frequency of the antigen-specific cells of interest.

- In the discussion, the main point of the manuscript, namely concomitant detection of vector- and transgene-specific CD8+ T cells should be emphasized.

The point was emphasized in the discussion.

Reviewer #4:

Manuscript Summary:

This study provides a protocol for staining antigen-specific murine T-cells in direct ex vivo samples. The staining results and methodology are generally good but there are some important omissions that

ought to be included as detailed below.

Major Concerns:

1. Just as for isotype controls during antibody staining, it is vitally important to demonstrate antigen specificity by including control staining with an 'irrelevant' tetramer labelled with the same fluorochrome and bearing a different peptide. Without such a control, the results could represent non-specific staining so it is crucial that this methodology study makes the importance of staining with a control tetramer crystal clear. The VSV-N tetramer serves as a 'sort of' control in the Adeno-OVA samples but this is not ideal as it appears to be conjugated to a different fluorochrome than the OVA tetramer.

In our case, naïve ('mock') mice serve as control, as they are stained with the same tetramer (peptide and fluorochrome). We only see low background signal for the naïve mice for all tetramers (see Figure 2 and Supplementary Figure 1). We addressed this issue in the discussion (see lines 409 ff.).

2. Why is no live/dead stain included (or mentioned)? Admittedly, such stains are often less relevant directly ex vivo (providing staining is performed immediately). Nonetheless the authors should mention that it is highly desirable to include a live/dead stain in all experiments. Dead and dying cells take up tetramers non-specifically. Performing tetramer staining in the absence of a control tetramer and live/dead staining could be a recipe for disaster! At the very least, please make this point clear but, better still, include a control stain as mentioned above.

We included a comment on live/dead staining in the discussion (see lines 412ff.). We always include naïve mice in our experiments, and stain these cells with the same tetramer (in that case than an irrelevant tetramer for the naïve mice). However, we also included staining with another irrelevant tetramer as potential additional control (see lines 410/411).

3. The figures are said to be "representative". It would be helpful to show summary graphs/tables of tetramer percentage. How does the data look over several mice? Did the approach work on all mice? Everybody knows that "representative data" = "the best example" so it would be helpful for the reader to get a feel for just how "representative" these data are.

As suggested, a summary table for percentages of tetramer positive cells for several animals from one experiment was included as supplement (Supplement Table 1).

4. Where are the phenotyping data? Phenotyping is included in the methods so I think it is important that this document includes some examples. CD43, CD44 and CD62L are included in Table 1 but no data are shown.

Representative data were added (Figure 4).

5. The use of PKI is mentioned on line 286 but it is not included in any experiments. It would be helpful to include a +/- comparison with this reagent as it has been reported to make the mean fluorescence of staining brighter in addition to lowering the TCR affinity threshold required for tetramer staining. It would be further helpful if the use of cross-linking antibody were also mentioned (and preferably included) as this simple addition has also been reported to offer considerable improvements in some cases (PubMed ID: 25452566). A recent study shows how even fully functional anti-viral T-cells can express TCRs that are below the threshold of detection for peptide-MHC tetramers (PMID: 29483360). While this might not represent the norm, it ought to be included for the sake of completeness. Another recent study reviews the recent literature demonstrating how T-cells with very weak affinity TCRs can still contribute to immune responses (PMID: 30008714). Such cells do not stain with standard tetramer staining protocols as used here. Consequently, there is ample room to "beef up" the section on potential protocol modifications (Lines 282-298) and why these improvements might be important. Indeed, it would be preferable to suggest that users try several modifications to the technique to optimize experimentation for their own individual experimental system(s).

Above mentioned references were included and possible modifications were discussed in more detail. Also, it is emphasized that all conditions and modifications have to be adjusted individually for each tetramer.

Point 5 is relevant to lines 76-77 "results from MHC tetramer staining with those from ELISpot or ICS correlate well in terms of magnitude of the response" as clearly this is not always the case. The work of the Evavold laboratory (PMIDs: 27976744 and 21220453) and recently backed up by the Davis lab (PMID: 26979955) and others demonstrates how the results of tetramer staining and functional assay can be very different (mainly for MHC II but also for MHC I). Some tempering of the message in this respect would produce a more balanced and honest document.

We agree. This statement was rather related to MHC I, but might be misleading. To avoid misunderstanding, we have deleted the respective paragraph.

6. Several tetramer specificities are mentioned in the materials and methods and throughout but only OVA and VSV-N tetramer are shown. Table 1 mentions EGFP, LCMV-GP and HPV 16 E7 tetramers but I see no data from these. It would also be helpful if the authors included the precise restricting element and peptide sequence for these tetramers in the table as saying that they are simply "MHC I" is insufficient detail and requires that the reader be a specialist in this area (unlikely the target audience for this protocol).

Above mentioned details were included in the table.

Minor Concerns:

Abstract: "Antigen-specific CTLs can either be detected by stimulation with specific peptides followed by intracellular cytokine staining or by direct staining of antigen-specific T cell receptors (TCRs)."

There are also many other ways to detect antigen-specific T-cells. The abstract should specify "by flow cytometry" (or similar) as this is what the authors mean. Even when limited to flow cytometric methods of detection then cells can also be detected by upregulation of CD107a (PMID: 14580882) and/or TNF cell surface staining (PMID: 21501617) and other activation markers such as CD69. A key advantage of physical detection with peptide-MHC multimers is that cells are not required to exhibit a specific effector function.

Respective specification was added to the abstract, as well as mentioning the effector functions.

Lines 77/78: "In vitro stimulation to induce cytokine production for ICS/ELISpot will alter the original T cell phenotype. Tetramer staining, in contrast, leaves the T cell 'untouched' and the original phenotype is preserved and can be analysed"

True, but surely the biggest advantage of tetramers is that they allow the sorting of live, intact cells that can be subsequently cultured or clonotyped. This is not possible following ICS or ELISpot so it is important to mention this advantage of tetramers too.

This is completely true and was included.

It would be helpful to add % to Figure 2 as has been done in Figure 3.

As proposed, % were added to the figure.

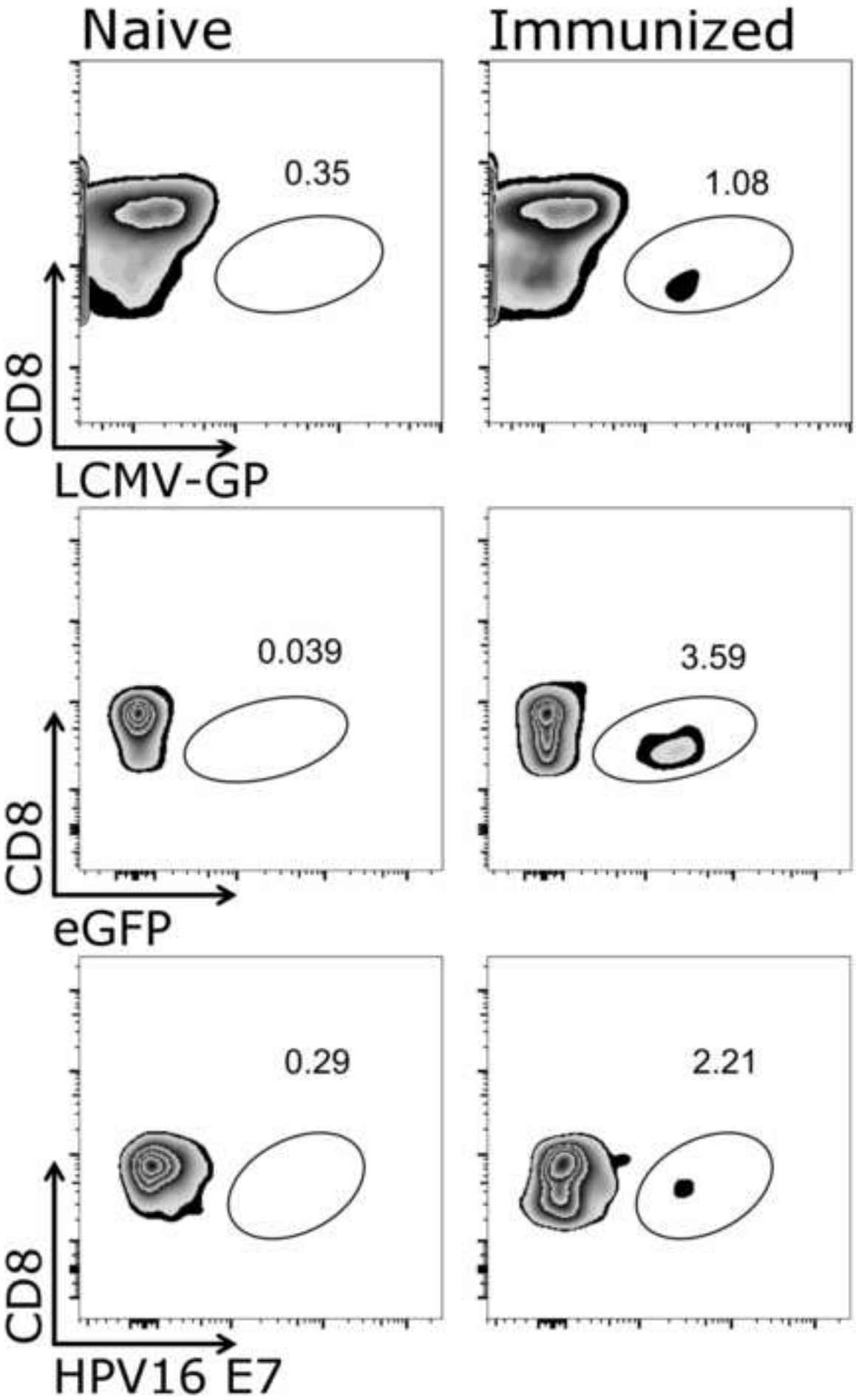
A version of the manuscript with changes indicated in yellow highlighting is attached. We hope the revised manuscript is now suitable for publication in JoVe and thank you and the reviewers again for evaluation of our manuscript.

Yours sincerely,

Janine Kimpel

Supplementary Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 1.tif



Supplemental Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Table1.xls

	Mouse #	% CD43^{+}	% VSV-N Tetramer^{+}	% OVA Tetramer^{+}
mock	1	1.7	0.45	0.41
	2	0.77	0.69	0.15
	3	1.34	0.35	0.31
	4	1.68	0.83	0.26
	5	0.84	0.47	0.23
VSV-GP-OVA	1	23	13.2	3.69
	2	32.6	15.9	3.54
	3	22.1	11.7	2.43
	4	15.1	8.89	1.79
	5	29.1	14.7	5.64