More tricks with tetramers: a practical guide to staining T cells with peptide–MHC multimers

Garry Dolton,[1,]* Katie Tungatt,[1,]* Angharad Lloyd,[1] Valentina Bianchi,[1] Sarah M. Theaker,[1] Andrew Trimby,[1] Christopher J. Holland,[1] Marco Donia,[2] Andrew J. Godkin,[1] David K. Cole,[1] Per Thor Straten,[2] Mark Peakman,[3] Inge Marie Svane[2] and Andrew K. Sewell[1]

[1]*Division of Infection and Immunity, Cardiff University School of Medicine, Cardiff, UK,* [2]*Centre for Cancer Immune Therapy, Herlev University Hospital, Herlev, Denmark and* [3]*Peter Gorer Department of Immunobiology, King's College London, Guy's Hospital, London, UK*

*These authors contributed equally.
Correspondence: Andrew K. Sewell, Division of Infection and Immunity, Henry Wellcome Building Cardiff University School of Medicine, University Hospital, Cardiff, Wales CF14 4XN, UK.
Email: sewellak@cf.ac.uk
Senior author: Andrew Sewell

Summary

Analysis of antigen-specific T-cell populations by flow cytometry with peptide–MHC (pMHC) multimers is now commonplace. These reagents allow the tracking and phenotyping of T cells during infection, autoimmunity and cancer, and can be particularly revealing when used for monitoring therapeutic interventions. In 2009, we reviewed a number of 'tricks' that could be used to improve this powerful technology. More recent advances have demonstrated the potential benefits of using higher order multimers and of 'boosting' staining by inclusion of an antibody against the pMHC multimer. These developments now allow staining of T cells where the interaction between the pMHC and the T-cell receptor is over 20-fold weaker ($K_D > 1$ mM) than could previously be achieved. Such improvements are particularly relevant when using pMHC multimers to stain anti-cancer or autoimmune T-cell populations, which tend to bear lower affinity T-cell receptors. Here, we update our previous work to include discussion of newer tricks that can produce substantially brighter staining even when using log-fold lower concentrations of pMHC multimer. We further provide a practical guide to using pMHC multimers that includes a description of several common pitfalls and how to circumvent them.

Keywords: peptide–MHC dextramer; peptide–MHC tetramer; T-cell receptor; T cell.

Introduction

The $\alpha\beta$ T-cell antigen receptor (TCR) allows T cells to inspect the proteome for anomalies by sampling peptide antigens cradled in either MHC class I or II molecules at the cell surface.[1,2] The interaction between TCR and peptide–MHC (pMHC) is weak, and typically only lasts a few seconds. Multimerization of soluble pMHC can considerably extend the half-life of this interaction due to the avidity effect,[3] and can thereby produce reagents that stably adhere to the cell surface of T cells bearing a cognate TCR. Peptide-MHC multimers in the form of avidin–biotin-based pMHC tetramers were first used to stain T cells by Altman *et al.* in 1996[4] and have gone on to transform the analysis of antigen-specific T-cell populations. Peptide-MHC multimers have been used in many thousands of studies and spawned the generation of several commercial companies that sell various forms of these reagents.[3] Moreover, pMHC multimers can be used in conjunction with a cocktail of antibodies raised against other cell surface proteins. This enables co-staining of antigen-specific T cells and segregation into various phenotypic populations without the distortion associated with function-based profiling.[5–9] Such phenotyping can be informative on antigen-experience, effector function, and also location of original antigen encounter, thereby allowing researchers to begin to deconvolute the complexities of T-cell immunity.

Peptide–MHC multimers are most commonly linked to fluorochromes, and used to detect T cells by conventional flow cytometry,[10] although next-generation technology uses pMHC multimers and antibodies that are linked to rare metal ions (typically lanthanides), which are then detected via mass spectrometry (MS).[6,7,11] This cytometry by time of flight, or 'mass cytometry', offers several advantages over conventional fluorescent cell sorting, but also comes with some major disadvantages. One advantage of MS-based detection is that heavy metal ion-based detection is not limited to the ∼ 20 parameters possible

with conventional flow cytometers; metal-ion-conjugated antibodies and pMHC multimers could, in theory, be used to separate cells in over 100 dimensions. MS detection of heavy metal ions also allows greater sensitivity, and does not suffer from the spectral overlap or spreading error that is associated with detection of emission from fluorochromes. One major drawback of mass cytometry is that it does not yet allow cell sorting on the light-based properties of forward and side scatter. Furthermore, it incinerates cells, so making it impossible to collect them. These shortcomings mean that mass cytometry is unlikely to fully supplant conventional, fluorochrome-based cytometry for the analysis of antigen-specific T-cell populations.[11]

Limitations of pMHC multimer staining

Peptide–MHC multimers have excelled for analyses of pathogen-specific CD8^{+} T-cell responses, but their use for dissection of autoimmune or anti-cancer T cells or CD4^{+} T cells is less widespread.[3] We have demonstrated that the TCR–pMHC affinity required for pMHC tetramer binding exceeds that required for T-cell activation.[12] This difference in affinity threshold means that conventional pMHC tetramer staining can fail to detect functional T cells.[12–15] Failure to stain cognate T cells that have a low affinity TCR is likely to be a more serious problem when pMHC multimers are used to stain self-specific (anti-cancer and autoimmune) T-cell populations, which tend to express lower affinity TCRs.[3,16–19] This issue is even greater when staining pMHCII-restricted T cells because the CD4 co-receptor, unlike the CD8 molecule, does not cooperate to aid TCR–pMHC binding.[3,20–26] Evavold and colleagues recently highlighted the potential level of under-estimation of antigen-specific CD4^{+} T-cell populations when staining with pMHCII tetramers *ex vivo*.[19] This study found a high prevalence of low-affinity pMHCII tetramer-negative effectors during polyclonal CD4^{+} T-cell responses, and demonstrated that myelin oligodendrocyte glycoprotein (35–55) and lymphocyte choriomeningitis virus glycoprotein (61–80) CD4^{+} T-cell populations were under-estimated by eightfold and fourfold, respectively by pMHCII tetramer staining. We have further demonstrated that the majority of the Melan A-specific CD8^{+} T cells in tumour-infiltrating lymphocyte (TIL) populations derived from malignant melanoma samples were not detected by conventional pMHC tetramer staining.[13] T-cell clones derived from these TILs that failed to stain by conventional pMHC tetramer staining were efficient killers of autologous tumour, indicating that pMHC tetramers missed fully functional cognate T cells.[13] These demonstrations highlight the pressing need to extend pMHC multimer technology to a point where it can be used to stain all T cells capable of responding to a given pMHC antigen.[13,27] Fortunately, new develop-

ments in the last 12 months considerably lower the TCR–pMHC affinity threshold required for efficient pMHC multimer staining. These, and other 'tricks' for improving staining with pMHC multimers are described below.

Materials and methods

This study aims to provide an appraisal of the tricks that can be used to produce enhanced staining of cognate T cells with pMHC multimers. We review a number of tricks below. The methodologies for these individual techniques have been published elsewhere as indicated. Here we apply these procedures to the staining of a number of different samples in order to demonstrate how they can be of benefit. We also detail the optimized staining protocol that we use in Cardiff within the results, and demonstrate what each step adds to the procedure. We therefore only document the materials used in this section.

Manufacture of pMHC tetramers and dextramers

The following streptavidin conjugates were used: streptavidin-allophycocyanin (APC) and -R-phycoerythrin (PE) (Life Technologies, Paisley, UK); streptavidin-brilliant violet (BV) 421 and -FITC. (Biolegend, London, UK). Peptide-MHC tetramer and dextramer were assembled as previously described.[13] Depending on experiment, either 0·3 µg or 0·5 µg (6 or 10 µg/ml) of tetramer or dextramer were used per stain, and equivalent amounts were used when tetramer and dextramer were being compared.

T-cell clones

The following HLA-A*0201 (HLA A2) -restricted CD8^{+} T-cell clones were used: ST8.24 and VB25D12.24, which recognize the peptide EAAGIGILTV, in addition to the heteroclitic version of the peptide, ELAGIGILTV, from Melan A (residues 26–35)[28,29] and were derived from TILs of a patient with stage IV malignant melanoma (patient MM909.24); GD.GIL influenza-specific clone which recognizes GILGFVFTL from the matrix protein (residues 58–66); 1E6, which was grown from a patient with type 1 diabetes[30] and recognizes the preproinsulin (PPI) epitope, ALWGPDPAAA (residues 15–24).[31] The CD4^{+} HLA-DRB*0101 (HLA DR1) clone, DCD10, which recognizes PKYVKQNTLKLAT (residues 307–319) from haemagglutinin of influenza A virus.

Antibody clones

Mouse anti-PE (clone PE001, BioLegend) and anti-APC (clone APC003, BioLegend) primary (1°) unconjugated monoclonal antibodies were used at a concentration of 10 µg/ml (0·5 µg/test). Goat anti-mouse conjugated secondary (2°) antibodies (multiple adsorbed PE- or

APC-conjugated immunoglobulin polyclonal; BD Biosciences, Oxford, UK) were used at 2 µg/ml (0·1 µg/test). We used the violet LIVE/DEAD Fixable Dead Cell Stain, Vivid (Life Technologies). The following monoclonal antibodies were used as indicated for individual experiments: anti-CD8-PE and anti-CD8-APC (clone BW135/80; Miltenyi Biotec, Bergisch Gladbach, Germany); anti-CD3-peridinin chlorophyll protein (PerCP) (clone BW264/56; Miltenyi Biotec); anti-CD19-Pacific blue (clone HIB19; BioLegend); and anti-CD14-Pacific blue (clone M5E2; BioLegend). Anti-rat CD2-PE (clone OX34; BioLegend) was used to stain lentivirally transduced T cells.

Dasatinib

The protein kinase inhibitor (PKI), Dasatinib (Axon Medchem, VA), was reconstituted in dimethylsulphoxide (1 mM) and stored frozen in 5 µl one-use aliquots. Each 5 µl aliquot only costs < £0·02 and makes enough reagent to treat 1000 samples (100 µl volume at 50 nM).

MEL5 TCR transduced CD8 T cells

The HLA A2-restricted Melan A-specific TCR MEL5 recognizes the natural 10-mer peptide, EAAGIGILTV. The TCR α and β chains[32] were cloned into the pELN third-generation lentivirus vector (a kind gift from James Riley, University of Pennsylvania). The pELN lentiviral vector contained a rat CD2 marker to determine the frequency of transduction, and the TCR α and β chains were separated by a 2A cleavage sequence. Integrase proficient lentivirus stocks were prepared by co-transfecting 293T/17 cells by calcium phosphate precipitation with the transfer vector and packaging plasmids – pRSV.REV (Addgene #12253), pMDLg/p.RRE (Addgene #12251)[33] and pCMV-VSV-G (Addgene #8454).[34] Supernatant was collected after 24-hr and 48-hr incubations, and the lentivirus stocks were concentrated by ultracentrifugation and used to transduce Dynabead (Life Technology) stimulated CD8+ T cells. The efficiency of lentivirus transduction was assessed by flow cytometry staining.

Results

Important tricks for improving staining efficiency

Several tricks for improving T-cell staining with pMHC multimers have been described and are reviewed elsewhere.[3] Here we detail the five most important tricks that we currently apply within our laboratory when staining antigen-specific T cells. These techniques are: (i) using a bright fluorochrome, (ii) inclusion of a PKI during staining, (iii) staining with anti-coreceptor antibody after staining with pMHC multimer, (iv) use of higher-order multimers, and (v) signal boosting with an anti-multimer antibody. The

benefits of the latter two techniques were published in 2014. We refer the reader to our previous review[3] for a full list of tricks and a detailed explanation of the benefits of PKI and correct anti-coreceptor antibody usage.

Using bright fluorochromes

It stands to reason that, when high staining intensity with pMHC multimer is required, it is better to use reagents coupled to a bright fluorochrome. This aspect is irrelevant when using MS-based detection methodology, and is less important when using pMHC dextramers that can be constructed to carry multiple fluorochrome molecules and also work well with FITC.[13] Our favourite 'flavours' of pMHC multimers are constructed with PE and APC. We have found wide variations in the quality of these reagents between different manufacturers. We currently use fluorochrome-conjugated streptavidin from Life Technologies for pMHC tetramers. However, researchers should remain mindful of the fact that we have not exhaustively tested all of the various products on the market so there may be better preparations available. Quantum dots (Q-dots) offer a good way of making very bright and robust pMHC multimers.[35] Although we have used Q-dot pMHC multimers, we do not have extensive experience with these reagents. Corry and colleagues directly compared Q-dots and tetramers for staining the same sample, and while Q-dots gave brighter staining in this comparison there was a noticeable staining of the general CD8+ cell population.[36] We have also noticed a similar phenomenon when some pMHC dextramers are used to stain some peripheral blood mononuclear cell (PBMC) populations.[13] Restriction of this non-specific staining to CD8+ T cells suggests that it is due to the avidity of pMHCI–CD8 interactions made possible with higher order multimers like Q-dots and dextramers. However, we remain unsure of why this background staining of CD8+ T-cell populations is only observed with some combinations of pMHC and PBMC. Figure 1(a) shows staining of HLA A2-restricted, influenza-specific CD8+ T-cell clone GD.GIL with pMHC tetramer manufactured with identical biotinylated HLA A2-GILGFVFTL monomer, and streptavidin linked to FITC, PE, APC and BV421 (see Materials and methods for details). These reagents gave mean fluorescence intensities (MFI) of 474, 4545, 3886 and 3684, and staining indices of 5·4, 52·8, 44·2 and 32·3, respectively. The BV421 reagent gave a higher background than the other fluorochromes when staining a T-cell clone (Fig. 1a) and PBMC (see Supplementary material, Fig. S1). Indeed, at the time of writing this review a higher degree of background on PBMC with BV421 cytomegalovirus (CMV) tetramers, compared with PE tetramers, is displayed on the manufacturer's website. We do not know the reason for the increase of non-specific staining with the BV421-containing reagents, but with optimization its use may offer an

alternative channel if those for PE and APC are being used for other cell markers.

The benefits of protein kinase inhibitors

Staining with pMHC multimers is critically dependent on the TCR density at the T-cell surface as effective capture of a pMHC multimer from solution requires that a second TCR engages further pMHC in the multimer during the duration of the first TCR–pMHC engagement, to establish an initial avidity effect.[3] TCRs are known to trigger and internalize after engaging cognate antigen,[37] and we have shown that pMHC tetramers can fail to stain anti-cancer and anti-human pancreatic β-cell T cells after these cells have been exposed to cognate antigen.[13,14] Fortunately, TCR internalization can be inhibited by inclusion of a PKI, such as dasatinib for as little as 30 seconds before pMHC multimer staining, resulting in substantially enhanced staining intensities.[15] This increase in staining can be > 50-fold when TCR affinity is extremely low.[15] Incubation of T cells in 50 nM dasatinib for 60 min increases surface concentrations of both TCR and co-receptor at the cell surface.[15] We assume that this increase is due to inhibition of normal turnover of these molecules because of inhibited down-regulation. We recommend researchers to avoid repeated freeze–thawing of dasatinib by storing it as frozen one-use aliquots for use within 1 week of being defrosted. PKI treatment also enhances staining with higher-order multimers such as pMHC dextramers,[13] and has been used in conjunction with pMHC multimer detection by MS.[6] We now include dasatinib during pMHC multimer staining as a matter of routine. Inclusion of PKI prevents cellular activation, and so it is incompatible with function-based profiling techniques such as intracellular cytokine staining. Figure 1(b) shows that a fully functional T-cell clone, VB25D12.24, isolated from the TILs of a patient with Stage IV melanoma, recognizes Melan A peptide (left panel), and responds to autologous tumour (middle panel). However, the clone fails to stain with HLA A2-Melan A tetramer in the absence of PKI treatment (right panel). Nevertheless, the clone stains well with cognate tetramer after pre-treatment with 50 nM dasatinib for 30 min. These data serve to highlight what could be missed during regular pMHC tetramer staining without added 'tricks'. Figure 1(c) demonstrates the benefits of dasatinib when staining antigen-specific T cells in PBMC, the situation where they are most commonly used. Inclusion of 50 nM dasatinib increases by fourfold the number of cells detected when staining with HLA A2-Melan A tetramer.

The importance of anti-coreceptor antibody

Peptide–MHC multimers are normally used in conjunction with an antibody for the relevant T-cell co-receptor (anti-CD4 for pMHCII multimers, and anti-CD8 for pMHCI multimers). It is well established that some antibody clones can disrupt staining of cognate T cells, while some antibodies can augment the interaction of pMHC multimers with cell surface TCR.[22,24,25,38–40] It is consequently preferable to use an anti-coreceptor antibody that aids pMHC multimer binding, or to stain with pMHC multimer before staining the T-cell co-receptor.[3] Figure 1(c) demonstrates that staining with CD8 antibody clone BW135/80, a clone we like to use in our laboratory, before staining of PBMC with HLA A2 Melan A tetramer, blocks staining in the absence of PKI and also reduces by half the number of cells that stain when PKI is included. The effects are less pronounced for an antiviral (cytomegalovirus) response, but there is still a reduction in the intensity of overall staining if anti-CD8 antibody is added first.

Higher valency pMHC multimers

We recently compared staining of antiviral, anticancer and autoimmune T cells with pMHC tetramers and pMHC dextramers.[13] Peptide-MHC dextramers are dextran-based multimers that can carry greater numbers of both pMHC and fluorochrome per molecule, due to the larger scaffold. When staining was compared, we found that dextramers stain more brightly than tetramers and outperformed them when TCR–pMHC affinity was low. Dextramers also outperformed tetramers with pMHC class II reagents where there was an absence of co-receptor stabilization. Importantly, we also found that staining with pMHC dextramers was additionally enhanced when PKI was included, demonstrating that the two techniques are compatible.[13] Figure 2 shows pMHC staining of a Melan A-specific T-cell clone, ST8.24, with a full range of conditions. Staining with dextramer + PKI was more than threefold brighter than with tetramer + PKI. Dextramers + PKI uncovered 25-fold more Melan A-specific cells when compared with regular tetramer staining of HLA A2[+] PBMC (Fig. 5).

Signal boosting with antibodies

We have also recently made use of the ILA1 T-cell clone that recognizes the pseudo HLA A2-restricted hTERT-derived epitope ILAKFLHWL,[41] and for which we have characterized a wide range of altered peptide ligands that act as agonists. These agonist peptides bind to HLA A2 equally well, but exhibit binding affinities for the ILA1 TCR that range from $K_D \sim 3$ μM to $K_D \sim 2$ mM by surface plasmon resonance.[12,42] The weakest ligand, 8E, still acts as a good agonist of ILA1 T cells when supplied exogenously at a concentration of 1 μM,[12,42] yet binds to the TCR with a $K_D \sim 2$ mM by extrapolation of response
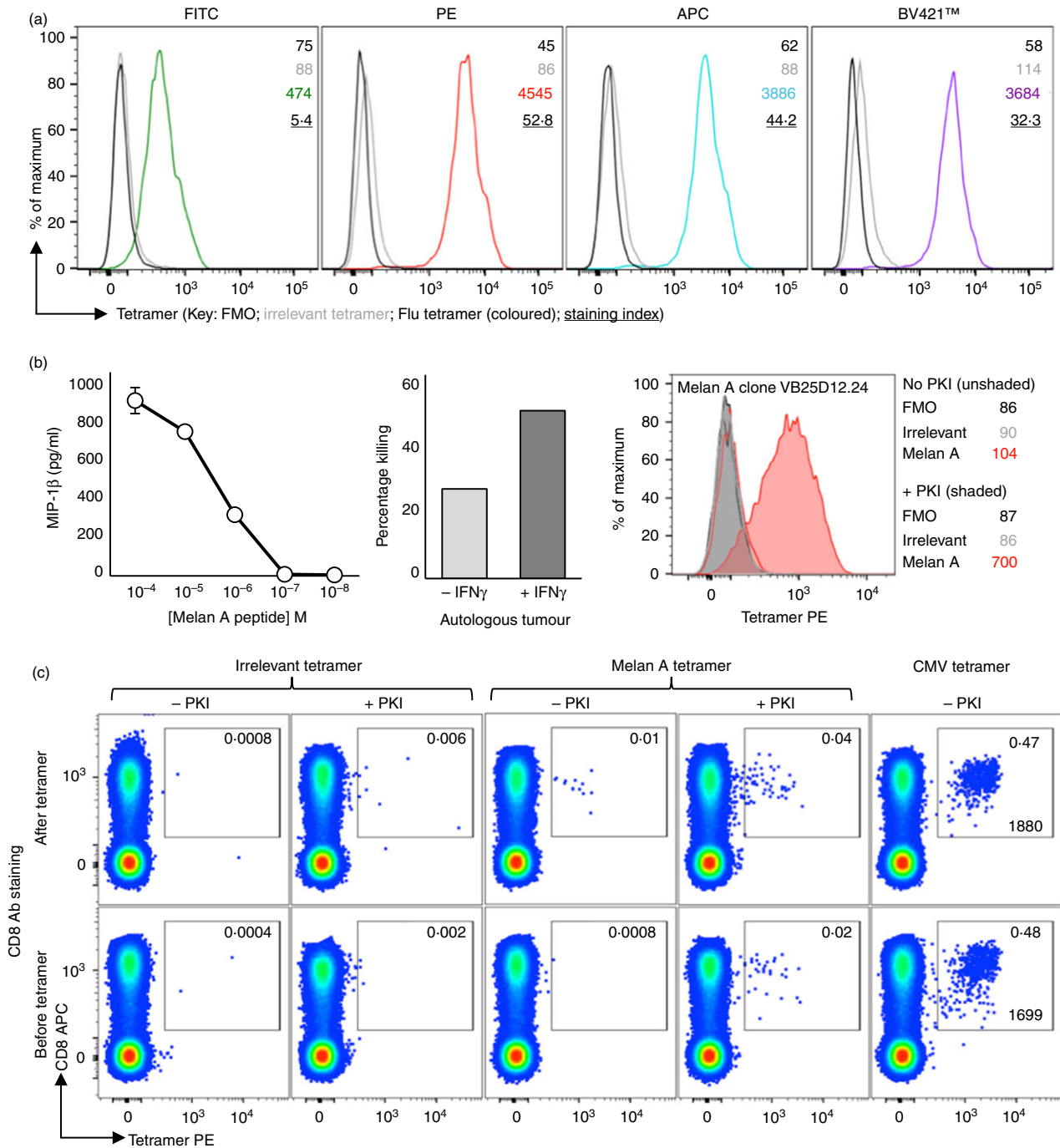
Figure 1. Tetramer staining of T cells is improved by using bright fluorochromes, protein kinase inhibitor treatment and addition of anti-CD8 antibody after tetramer staining. (a) HLA A2-restricted, influenza (flu) specific T-cell clone (GD.GIL) was stained with FITC, phycoerythrin (PE), allophycocyanin (APC) and brilliant violet (BV)421-conjugated cognate (matrix protein, GILGFVFTL) or irrelevant (preproinsulin; PPI; AL-WGPDPAAA) tetramers. The staining index [mean fluorescence intensity (MFI) of flu stain/MFI of PPI stain] is shown underlined. (b) T-cell clone (VB25D12.24) that recognizes a peptide from Melan A (left) and kills autologous tumour [± pre-treatment with interferon-γ (IFNγ)], at a T-cell to tumour ratio of 10:1 (middle), was stained with PE-conjugated Melan A (ELAGIGILTV) and PPI tetramers ± protein kinase inhibitor (PKI) pre-treatment (right). (c) HLA A2⁺ PBMC used from frozen ± PKI were stained with PE-conjugated PPI, Melan A and cytomegalovirus (CMV) (pp65, NLVPMVATV) tetramers (no PKI for the latter), with anti-CD8 APC antibody added before (lower panel) or after (upper panel) tetramer staining. The percentage of CD8⁺ cells that were also tetramer⁺ is shown inset for each gate and MFI displayed for the CMV tetramer stain.
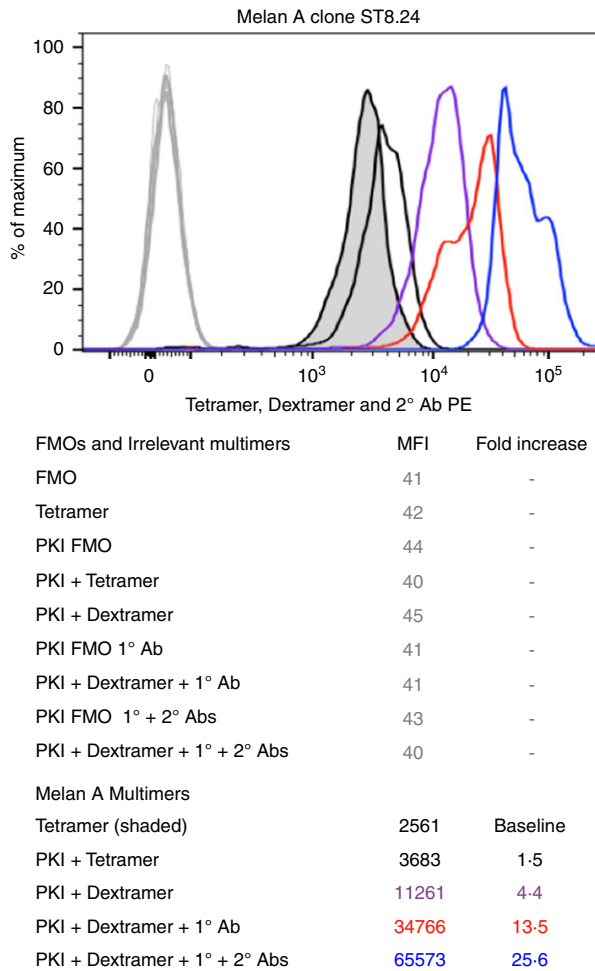
Melan A clone ST8.24

FMOs and Irrelevant multimers	MFI	Fold increase
FMO	41	-
Tetramer	42	-
PKI FMO	44	-
PKI + Tetramer	40	-
PKI + Dextramer	45	-
PKI FMO 1° Ab	41	-
PKI + Dextramer + 1° Ab	41	-
PKI FMO 1° + 2° Abs	43	-
PKI + Dextramer + 1° + 2° Abs	40	-
Melan A Multimers		
Tetramer (shaded)	2561	Baseline
PKI + Tetramer	3683	1·5
PKI + Dextramer	11261	4·4
PKI + Dextramer + 1° Ab	34766	13·5
PKI + Dextramer + 1° + 2° Abs	65573	25·6

Figure 2. Chronological improvements to staining T cells with peptide–MHC (pMHC) multimers. 'Tricks' shown by our group to improve the staining of T cells with pMHC multimers were compared with tetramer alone (baseline) when staining a Melan A-specific CD8^{+} clone (ST8.24). ST8.24 was pretreated with protein kinase inhibitor (PKI) and stained with Melan A (ELAGIGILTV) or irrelevant [preproinsulin (PPI); ALWGPDPAAA] multimers (tetramer and dextramer) followed by the addition of 1° antibody (Ab) ± phycoerythrin (PE)-conjugated 2° Ab as indicated. Mean fluorescence intensity (MFI). Fluorescence Minus One (FMO).

units from surface plasmon resonance experiments. Until recently, we had failed to stain the ILA1 T-cell well with pMHC multimers made with HLA A2-ILAKFLHEL (8E), suggesting that even a combination of the techniques described above does not allow pMHC multimer staining with the very weakest TCR ligands.

It has long been known that T cells rapidly internalize pMHC multimers when stained at room temperature or physiological temperatures.[43] Some internalization is also observed during regular staining protocols on ice when cells are not pre-chilled.[13] Treatment with a PKI, like dasatinib, prevents TCR triggering and internalization of the TCR and any pMHC multimer bound to

it.[13,15] We reasoned that this would leave pMHC multimers available at the T-cell surface for further signal boosting with anti-pMHC multimer antibody. In testing this hypothesis we discovered that just adding anti-multimer antibody during stains with pMHC dextramers or pMHC tetramers boosted the MFI of staining, and lowered the TCR–pMHC affinity that was amenable to detection with these reagents. Indeed, adding anti-PE antibody to staining with PE-conjugated tetramer in the presence of PKI boosted staining to a point where the ILA1 T-cell clone could be recovered from HLA A2[+] PBMC using tetramer made with HLA A2-ILAKFLHEL, a ligand that binds to the cognate TCR with extremely low affinity ($K_D \sim 2$ mM).[14] This unexpected enhancement was shown to be the result of a substantial reduction in the off-rate of pMHC tetramer during the process of staining and washing before flow cytometric analysis.[14] This boost in staining is so powerful that it enabled brighter staining of cognate T cells, even when log-fold lower concentrations of pMHC multimer were used; thereby allowing for a considerable reduction in costs.[14] Further enhancements were observed when a fluorochrome-conjugated antibody against the original anti-pMHC multimer antibody was also included, or when pMHC dextramers were used. However, this extra level of enhancement is unlikely to be necessary during the vast majority of pMHC multimer stains. Staining of Melan A clone, ST8.24, with PE-conjugated pMHC dextramer in the presence of a 1° antibody increased the MFI of staining from 11261 to 34766 (Fig. 2). Further inclusion of a PE-conjugated 2° antibody additionally increased staining to 65573, while the intensity of background staining with an irrelevant HLA A2 multimer remained unchanged at an MFI of ∼ 40. The benefits of signal boosting with antibody are also evident in Fig. 3(a), where HLA A2 Melan A tetramer was used to stain cells that were lentivirally transduced with Melan-specific TCR MEL5, as described in the Materials and methods. The lentivirus also expressed rat CD2, allowing identification of transduced cells with PE-conjugated anti-rat CD2 antibody (Fig. 3a). Transduced TCRs have to compete with the natural endogenous TCR for expression at the T-cell surface, such that the introduced TCR may be present at a low surface density. Low TCR density is also a problem during pMHC multimer staining of autoimmune or cancer-specific T cells that may have recently encountered their cognate antigen *in vivo*.[13,14] Seventeen per cent of the transduced cells stained with APC-conjugated HLA A2-ELAGIGILTV tetramer (Fig. 3a); this increased to 39% when PKI was included. Further addition of 1°, anti-APC antibody, or 1° anti-APC antibody + APC-conjugated 2° antibody increased this percentage to 53% and 65% of lentivirally transduced cells, respectively, and demonstrated the benefits of antibody boosting when surface

TCR density is low. Furthermore, we recently showed that signal boosting with antibody can enhance the ability of tetramers to stain, and detect both autoimmune and CD4^{+} HLA class II restricted T cells.[14] Figure 3(b) shows tetramer staining of PBMC spiked with the HLA A2 restricted CD8^{+} T-cell clone, 1E6. This clone was derived from a patient with type I diabetes,[30] and has a TCR that binds weakly to a PPI-derived peptide (ALWGPDPAAA) with a K_D of >200 μM.[31] This is a feature that often precludes the effective staining of autoimmune T cells when tetramers are used without 'tricks'. Tetramer in combination with PKI recovered 6% of 1E6 from the spiked PBMC, and addition of 1° anti-PE antibody, alone or in combination with a PE-conjugated 2° antibody, gave 100% recovery with clear 1E6 T-cell discrimination from non-specific CD8^{+} T cells (Fig. 3b). The complete recovery of 1E6 with tetramer + 1° + 2° antibodies was achievable with eightfold less tetramer compared with tetramer alone, an observation we had previously shown with even less tetramer (25-fold).[14] CD4^{+} HLA class II restricted T cells also present a challenge when staining with tetramers, due to a lack of co-receptor help from CD4[3] and possession of TCRs with weaker average affinities.[16] Figure 3c demonstrates that the addition of a 1° antibody, with or without a 2° antibody, enhances the staining of the HLA DR1 restricted influenza-specific clone, DCD10, with 1·8-fold and 2·8-fold increases in staining, respectively.
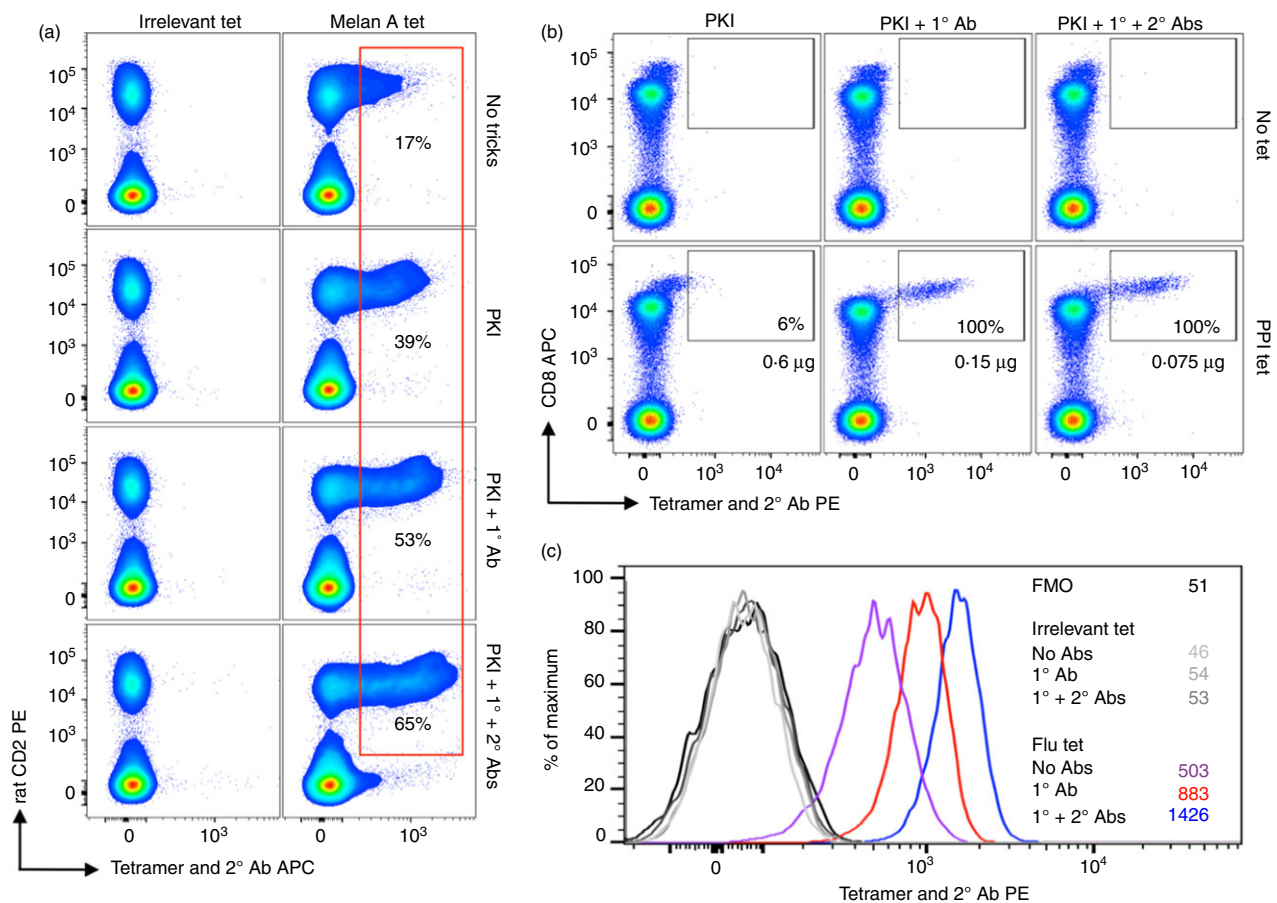

Figure 3. Improved staining of T-cell receptor (TCR)-transduced, autoimmune and MHC class II restricted T cells with tetramers by applying various tricks. (a) CD8^{+} T cells lentivirally co-transduced with a Melan A-specific TCR and rat CD2 ± protein kinase inhibitor (PKI) were stained with allophycocyanin (APC)-conjugated Melan A or irrelevant [preproinsulin (PPI); ALWGPDPAAA] tetramers. In addition to tetramer, PKI-treated cells were also labelled with unconjugated 1° antibody (Ab) ± APC-conjugated 2° Ab, as indicated. The percentage of Melan A tetramer^{+} cells of rat CD2^{+} cells is displayed. (b) The PPI-specific CD8^{+} T-cell clone, 1E6, which was grown from a patient with type I diabetes, was spiked into HLA A2^{+} peripheral blood mononuclear cells (PBMC), PKI treated and stained with PPI tetramer (amounts shown for each plot) ± 1° Ab ± phycoerythrin (PE)-conjugated 2° Ab. The 1E6 clone is CD8^{high} allowing it to be seen within the PBMC population based on CD8 staining alone. The percentage recovery of 1E6 with PPI tetramer is shown for each condition. (c) An influenza-specific HLA DR restricted CD4^{+} T-cell clone was stained with cognate (PKYVKQNTLKLAT from haemagglutinin) or irrelevant (DRFYKTLRAEQASQ from p24 Gag of HIV) PE-conjugated tetramer ± 1° Ab ± PE-conjugated 2° Ab.

We are currently exploring the advantages of using boosted MHC class II tetramers on anti-tumour CD4^{+} T-cell clones and *ex vivo* PBMC samples.

An optimized staining protocol

The techniques described above provide multiple ways to enhance staining with pMHC multimers. The most sensitive staining we have seen to date included use of: (i) bright fluorochrome, (ii) PKI, (iii) addition of anti-coreceptor antibody after pMHC multimer staining, (iv) higher order multimers (pMHC dextramers), (v) 1° anti-pMHC dextramer antibody, and (vi) fluorochrome-conjugated 2° antibody[14] as summarized in Fig. 2. However, use of all these tricks together is well beyond what is necessary for most pMHC multimer stains. Since publishing the above tricks, we have been asked many times by other researchers to provide an optimal pMHC staining protocol. We provide our own optimized protocol in Fig. 4, but in doing so we advise that researchers adjust their staining method using the above tricks so as to produce the best results in their own individual experimental systems.

The optimal pMHC multimer protocol will vary depending on the particular assay, the nature of the T cells and pMHC multimers being used. The tricks described above work well in conjunction, allowing researchers to tailor pMHC multimer staining to their own individual requirements. The most sensitive staining technique of using a bright fluorochrome using PKI and pMHC dextramers in conjunction with an anti-multimer 1° antibody and fluorochrome-conjugated 2° antibody (Fig. 2) is well beyond the requirements of most experiments. Inclusion of fluorochrome-conjugated 2° antibody adds expense, and the possibility of off-target staining due to the addition of fluorochrome that is not conjugated to pMHC; whereas pMHC dextramers are difficult to assemble in-house. Hence our own preferred standard protocol uses pMHC tetramers, PKI and anti-tetramer 1° antibody, as described in Fig. 4 (shown by the red arrows and text). This combination is sufficient for staining T cells with very-low TCR affinity ($K_D > 1$ mM), and so is more than adequate for staining the vast majority of antigen-specific T cells.[14] Figure 5 demonstrates the improvements possible by applying the various tricks above to the staining of HLA A2[+] PBMC with HLA A2 Melan A tetramer. Addition of PKI and 1° antibody to the stain increases the number of cells recovered by sixfold compared with no tricks (baseline). Use of PKI and 1° antibody with pMHC dextramer instead of pMHC tetramer recovers 25-fold more Melan A-specific cells than regular pMHC tetramer staining. Further addition of 2° antibody increases staining with tetramer and dextramer to 7·5-
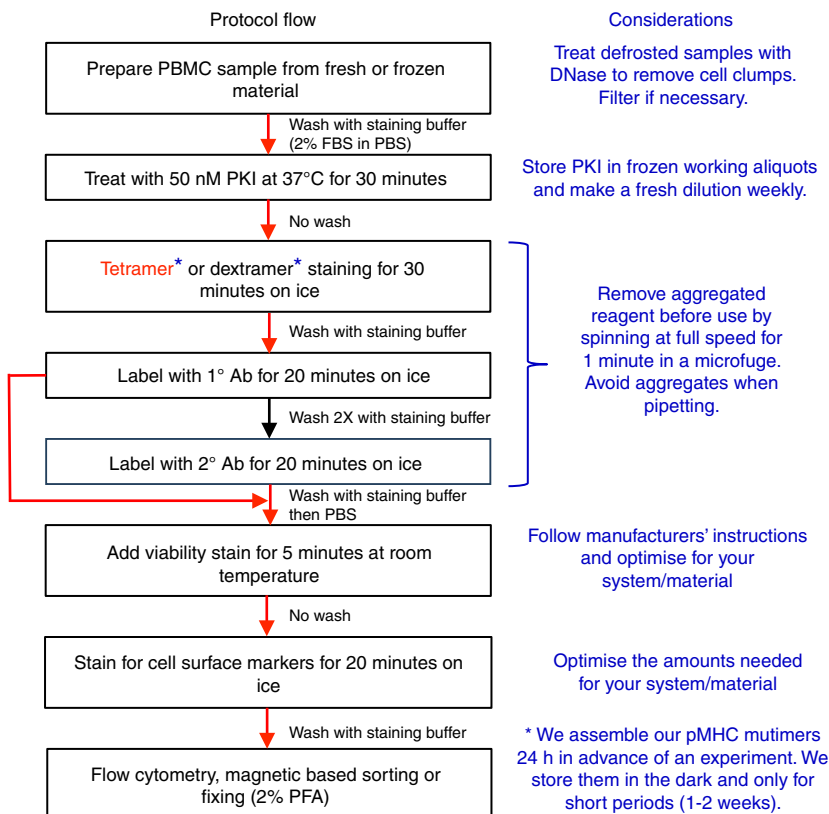

Protocol flow

Prepare PBMC sample from fresh or frozen material

↓ Wash with staining buffer (2% FBS in PBS)

Treat with 50 nM PKI at 37°C for 30 minutes

↓ No wash

Tetramer* or dextramer* staining for 30 minutes on ice

↓ Wash with staining buffer

Label with 1° Ab for 20 minutes on ice

↓ Wash 2X with staining buffer

Label with 2° Ab for 20 minutes on ice

↓ Wash with staining buffer then PBS

Add viability stain for 5 minutes at room temperature

↓ No wash

Stain for cell surface markers for 20 minutes on ice

↓ Wash with staining buffer

Flow cytometry, magnetic based sorting or fixing (2% PFA)

Considerations

Treat defrosted samples with DNase to remove cell clumps. Filter if necessary.

Store PKI in frozen working aliquots and make a fresh dilution weekly.

Remove aggregated reagent before use by spinning at full speed for 1 minute in a microfuge. Avoid aggregates when pipetting.

Follow manufacturers' instructions and optimise for your system/material

Optimise the amounts needed for your system/material

* We assemble our pMHC mutimers 24 h in advance of an experiment. We store them in the dark and only for short periods (1-2 weeks).

Figure 4. Our protocol for staining peripheral blood mononuclear cells (PBMC) with peptide–MHC (pMHC) multimers including the 'tricks' discussed in this review. Staining is performed in 5 ml 'FACS' tubes. Our default protocol flow is shown by red arrows/text and involves using tetramer, protein kinase inhibitor (PKI) and 1° antibody (Ab). PKI is sometimes used without other tricks. Dextramers and/or 2° Ab are used when T cells are particularly challenging to stain. The same protocol can be used for staining T cell clones and lines.
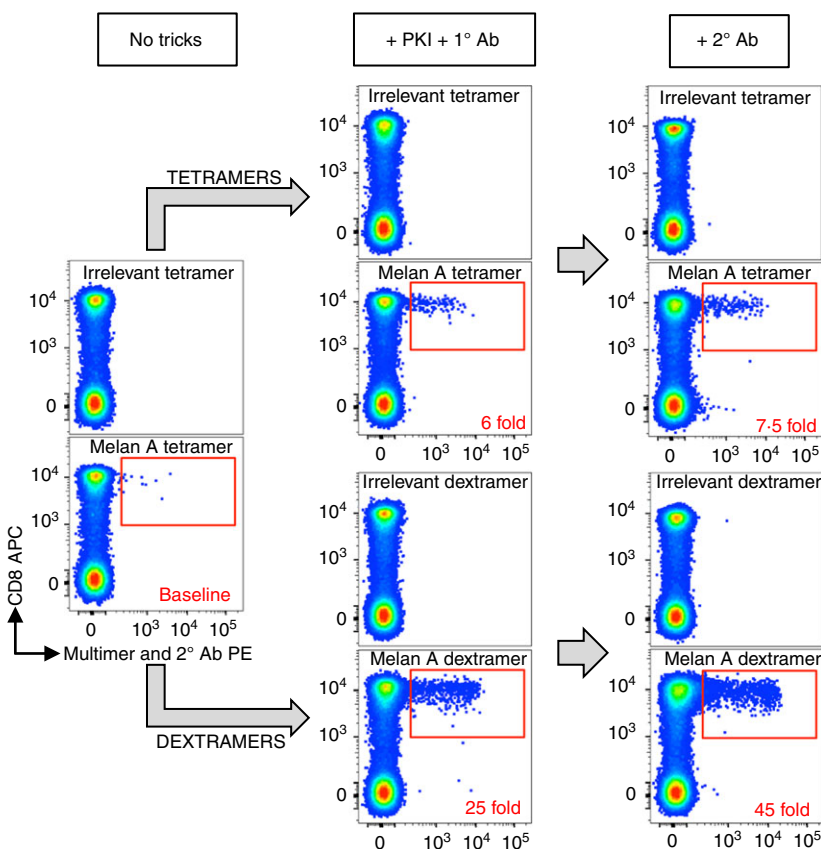
Figure 5. The combination of tricks greatly improves the recovery of T cells from peripheral blood mononuclear cells (PBMC) with pMHC multimers. HLA A2$^+$ PBMC were stained with Melan A (ELAGIGILTV) and irrelevant [preproinsulin (PPI); AL-WGPDPAAA] phycoerythrin (PE)-conjugated tetramers or dextramers ± protein kinase inhibitor (PKI) and unconjugated 1° antibody (Ab) ± PE-conjugated 2° Ab, as indicated. The recovery of Melan A tetramer or dextramer$^+$ cells (red box) is compared to the staining with tetramer alone (baseline).

fold and 45-fold above standard pMHC tetramer staining without tricks, respectively. These data demonstrate the additive nature of these tricks when applied to pMHC multimer staining of PBMC.

Troubleshooting

Various issues can arise when staining with pMHC multimers and we are occasionally asked to try and troubleshoot issues for other laboratories. One of the commonest problems seems to arise due to aggregation of the pMHC multimer preparation. This problem can be quickly eliminated if pMHC multimer is spun in a microfuge at top speed for 1 min before use to precipitate any aggregates present. Figure 6(a) shows a clean staining of HLA A2$^+$ PBMC with spun HLA A2 Melan A tetramer adjacent to the identical stain with this reagent before spinning. In the absence of centrifugation there is considerable background in the CD8-negative cell population, in addition to some high intensity staining in the CD8$^+$ population. Similar issues are also apparent, although to a lesser degree, when the same PBMC are stained with HLA A2-GILGFVFTL influenza-specific reagents. It is also important to include a 'dump channel' during pMHC multimer analyses that eliminates dead/dying cells, B cells and CD14$^+$ cells that can

take up pMHC multimers non-specifically without the need for cognate TCR expression. Figure 6(b) shows the staining of HLA A2$^+$ PBMC with HLA A2-PPI and HLA A2-CMV tetramers (peptide sequences ALWGPDPAAA and NLVPMVATV, respectively) ± a viability stain, to demonstrate how dumping of dead and dying cells, as well as those capable of non-specific pMHC multimer uptake via macropinocytosis and other mechanisms, can substantially improve staining with pMHC multimers.

Detection of non-classical T cells

Recent advances have described how T cells are able to recognize lipid antigens in the context of CD1a, CD1b, CD1c and CD1d molecules, or bacterial metabolites in the context of MHC-related protein (MR)1.[1,2] Multimeric forms of CD1-lipid and MR1-metabolite can also be used to identify and phenotype the T cells that respond to these antigens.[44–52] TCR interactions with these non-classical ligands tend to be relatively robust, such that regular tetrameric versions of these molecules appear to stain cognate cells well. Nevertheless, it is likely that the application of higher-order multimers, PKI and signal boosting with antibodies (as described for conventional pMHC multimers) will enhance the intensity of staining, with concomitant poten-
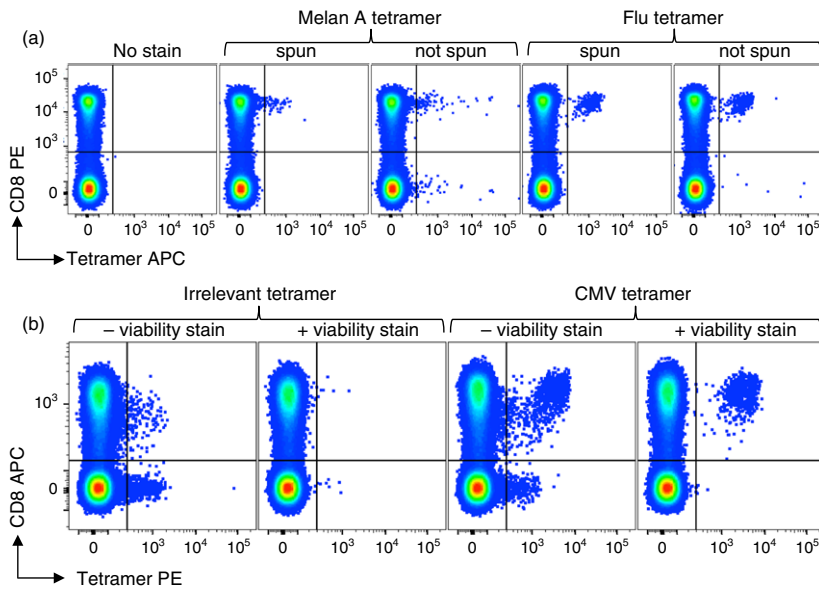
Figure 6. Removal of aggregated tetramer and including a viability stain improves the clarity of tetramer staining. (a) HLA A2$^+$ peripheral blood mononuclear cells (PBMC) from frozen were stained with allophycocyanin (APC) -conjugated Melan A (ELAGIGILTV) and influenza (flu) (matrix protein; GILGFVFTL) tetramers that were either spun (microfuge at full speed for 1 min) before use to remove aggregates or left unspun. (b) HLA A2$^+$ PBMC from frozen were stained with phycoerythrin (PE)-conjugated cytomegalocirus (pp65, NLVPMVATV) and irrelevant (preproinsulin (PPI), AL-WGPDPAAA) tetramers and co-stained for CD3, CD8, CD19 and CD14 $\pm$ a viability stain and the plots shown gated on CD3$^+$(viable when viability stain was present)/CD19$^-$/CD14$^-$ cells.

tial for also using less multimerized non-classical ligand during staining. It remains to be seen whether the application of more sensitive techniques to the staining of non-classical T cells with multimeric ligands will lead to the discovery of new subsets of T cells.

Conclusions

Fluorochrome-conjugated pMHC multimers have already revolutionized the study of antigen-specific T cells. Until recently, the major problem with these reagents has been that the TCR affinity threshold required for pMHC multimer staining exceeded that required for T-cell activation, resulting in a failure to detect all T cells capable of responding to a particular pMHC.[12–15] This problem is far more likely when staining anti-cancer T cells, autoimmune T cells, or MHC class II-restricted T cells, as such populations tend to bear TCRs with lower affinity for cognate antigen.[16,17] There is a further issue caused by low TCR surface densities when staining T cells that have been recently exposed to antigen.[13,14] Recent antigen exposure can occur naturally in vivo, or artificially during functional profiling following antigen exposure in vitro (e.g. intracellular cytokine staining).[13,14] Newer developments including staining in the presence of PKI,[15] using higher order pMHC multimers,[13] and boosting staining by including anti-multimer antibody,[14] have lowered the TCR affinity required for effective pMHC multimer staining by > 20-fold, while increasing staining intensity. These advances enhance pMHC multimer technology to a point where it can be used to stain T cells where the affinity between the TCR and antigen exceeds a K_D of 1 mM.[13] It remains to be determined whether this improvement takes pMHC multimer staining to a point where it stains all functional T cells and thereby allows these reagents to realize their full potential for immune monitoring. Recent addition of MS-based detection to pMHC multimer staining increases the number of cell surface molecules that can be studied simultaneously, and also circumvents the requirement for spectral overlap compensation during antibody phenotyping so increasing the power of this influential technology further still.[6,11] Contemporary developments using pyrosequencing of TCRs to quantify T cells or inform on their function[53] are still far from routine, so we anticipate that pMHC multimers will continue to remain prominent for T-cell detection for many years to come.

Disclosures

AKS is inventor on patent application numbers EP 09737105.8 and US 13/119,795 which has been

licensed to a company by University College Cardiff Consultants Limited. He has not benefitted personally from the exploitation of the technology at the current time.

References

1 Rossjohn J, Gras S, Miles JJ, Turner SJ, Godfrey DI, McCluskey J. T Cell antigen receptor recognition of antigen-presenting molecules. *Annu Rev Immunol* 2014; **33**:169–200.

2 Attaf M, Legut M, Cole DK, Sewell AK. The T cell antigen receptor: the Swiss Army knife of the immune system. *Clin Exp Immunol* 2015; **181**:1–18.

3 Wooldridge L, Lissina A, Cole DK, van den Berg HA, Price DA, Sewell AK. Tricks with tetramers: how to get the most from multimeric peptide-MHC. *Immunology* 2009; **126**:147–64.

4 Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996; **274**:94–6.

5 Fergusson JR, Smith KE, Fleming VM et al. CD161 defines a transcriptional and functional phenotype across distinct human T cell lineages. *Cell Rep* 2014; **9**:1075–88.

6 Newell EW, Sigal N, Bendall SC, Nolan GP, Davis MM. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity* 2012; **36**:142–52.

7 Newell EW, Sigal N, Nair N, Kidd BA, Greenberg HB, Davis MM. Combinatorial tetramer staining and mass cytometry analysis facilitate T-cell epitope mapping and characterization. *Nat Biotechnol* 2013; **31**:623–9.

8 Appay V, van Lier RA, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 2008; **73**:975–83.

9 Appay V, Dunbar PR, Callan M et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat Med* 2002; **8**:379–85.

10 Klenerman P, Cerundolo V, Dunbar PR. Tracking T cells with tetramers: new tales from new tools. *Nat Rev Immunol* 2002; **2**:263–72.

11 Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. *Trends Immunol* 2012; **33**:323–32.

12 Laugel B, van den Berg HA, Gostick E et al. Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *J Biol Chem* 2007; **282**:23799–810.

13 Dolton G, Lissina A, Skowera A et al. Comparison of peptide-major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells. *Clin Exp Immunol* 2014; **177**:47–63.

14 Tungatt K, Bianchi V, Crowther MD et al. Antibody stabilization of peptide-MHC multimers reveals functional T cells bearing extremely low-affinity TCRs. *J Immunol* 2015; **194**:463–74.

15 Lissina A, Ladell K, Skowera A et al. Protein kinase inhibitors substantially improve the physical detection of T-cells with peptide-MHC tetramers. *J Immunol Methods* 2009; **340**:11–24.

16 Cole DK, Pumphrey NJ, Boulter JM et al. Human TCR-binding affinity is governed by MHC class restriction. *J Immunol* 2007; **178**:5727–34.

17 Aleksic M, Liddy N, Molloy PE, Pumphrey N, Vuidepot A, Chang KM, Jakobsen BK. Different affinity windows for virus and cancer-specific T-cell receptors: implications for therapeutic strategies. *Eur J Immunol* 2012; **42**:3174–9.

18 Bridgeman JS, Sewell AK, Miles JJ, Price DA, Cole DK. Structural and biophysical determinants of alphabeta T-cell antigen recognition. *Immunology* 2012; **135**:9–18.

19 Sabatino JJ Jr, Huang J, Zhu C, Evavold BD. High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses. *J Exp Med* 2011; **208**:81–90.

20 Choi EM, Chen JL, Wooldridge L et al. High avidity antigen-specific CTL identified by CD8-independent tetramer staining. *J Immunol* 2003; **171**:5116–23.

21 Choi EM, Palmowski M, Chen J, Cerundolo V. The use of chimeric A2K(b) tetramers to monitor HLA A2 immune responses in HLA A2 transgenic mice. *J Immunol Methods* 2002; **268**:35–41.

22 Wooldridge L, Hutchinson SL, Choi EM et al. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *J Immunol* 2003; **171**:6650–60.

23 Wooldridge L, Laugel B, Ekeruche J, Clement M, van den Berg HA, Price DA, Sewell AK. CD8 controls T cell cross-reactivity. *J Immunol* 2010; **185**:4625–32.

24 Wooldridge L, Lissina A, Vernazza J et al. Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *Eur J Immunol* 2007; **37**:1323–33.

25 Wooldridge L, Scriba TJ, Milicic A, Laugel B, Gostick E, Price DA, Phillips RE, Sewell AK. Anti-coreceptor antibodies profoundly affect staining with peptide-MHC class I and class II tetramers. *Eur J Immunol* 2006; **36**:1847–55.

26 Wooldridge L, van den Berg HA, Glick M et al. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. *J Biol Chem* 2005; **280**:27491–501.

27 Massilamany C, Upadhyaya B, Gangaplara A, Kuszynski C, Reddy J. Detection of autoreactive CD4 T cells using major histocompatibility complex class II dextramers. *BMC Immunol* 2011; **12**:40.

28 Cole DK, Edwards ES, Wynn KK et al. Modification of MHC anchor residues generates heteroclitic peptides that alter TCR binding and T cell recognition. *J Immunol* 2010; **185**:2600–10.

29 Madura F, Rizkallah PJ, Holland CJ et al. Structural basis for ineffective T-cell responses to MHC anchor residue-improved "heteroclitic" peptides. *Eur J Immunol* 2015; **45**:584–91.

30 Skowera A, Ellis RJ, Varela-Calvino R et al. CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. *J Clin Invest* 2008; **118**:3390–402.

31 Bulek AM, Cole DK, Skowera A et al. Structural basis for the killing of human beta cells by CD8(+) T cells in type 1 diabetes. *Nat Immunol* 2012; **13**:283–9.

32 Cole DK, Yuan F, Rizkallah PJ et al. Germ line-governed recognition of a cancer epitope by an immunodominant human T-cell receptor. *J Biol Chem* 2009; **284**:27281–9.

33 Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998; **72**:8463–71.

34 Stewart SA, Dykxhoorn DM, Palliser D et al. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 2003; **9**:493–501.

35 Hadrup SR, Bakker AH, Shu CJ et al. Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* 2009; **6**:520–6.

36 Zhu J, Koelle DM, Cao J, Vazquez J, Huang ML, Hladik F, Wald A, Corey L. Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation. *J Exp Med* 2007; **204**:595–603.

37 Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 1995; **375**:148–51.

38 Clement M, Ladell K, Ekeruche-Makinde J et al. Anti-CD8 antibodies can trigger CD8+ T cell effector function in the absence of TCR engagement and improve peptide-MHCI tetramer staining. *J Immunol* 2011; **187**:654–63.

39 Daniels MA, Jameson SC. Critical role for CD8 in T cell receptor binding and activation by peptide/major histocompatibility complex multimers. *J Exp Med* 2000; **191**:335–46.

40 Holman PO, Walsh ER, Jameson SC. Characterizing the impact of CD8 antibodies on class I MHC multimer binding. *J Immunol* 2005; **174**:3986–91.

41 Purbhoo MA, Li Y, Sutton DH et al. The HLA A*0201-restricted hTERT(540-548) peptide is not detected on tumor cells by a CTL clone or a high-affinity T-cell receptor. *Mol Cancer Ther* 2007; **6**:2081–91.

42 Laugel B, Price DA, Milicic A, Sewell AK. CD8 exerts differential effects on the deployment of cytotoxic T lymphocyte effector functions. *Eur J Immunol* 2007; **37**:905–13.

43 Whelan JA, Dunbar PR, Price DA et al. Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J Immunol* 1999; **163**:4342–8.

44 de Jong A, Cheng TY, Huang S et al. CD1a-autoreactive T cells recognize natural skin oils that function as headless antigens. *Nat Immunol* 2014; **15**:177–85.

45 Kasmar AG, Van Rhijn I, Magalhaes KG et al. Cutting Edge: CD1a tetramers and dextramers identify human lipopeptide-specific T cells ex vivo. *J Immunol* 2013; **191**:4499–503.

46 Kasmar AG, van Rhijn I, Cheng TY et al. CD1b tetramers bind alphabeta T cell receptors to identify a mycobacterial glycolipid-reactive T cell repertoire in humans. *J Exp Med* 2011; **208**:1741–7.

47 Van Rhijn I, Kasmar A, de Jong A et al. A conserved human T cell population targets mycobacterial antigens presented by CD1b. *Nat Immunol* 2013; **14**:706–13.

48 Ly D, Kasmar AG, Cheng TY et al. CD1c tetramers detect ex vivo T cell responses to processed phosphomycoketide antigens. *J Exp Med* 2013; **210**:729–41.

49 Sidobre S, Kronenberg M. CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells. *J Immunol Methods* 2002; **268**:107–21.

50 Gadola SD, Karadimitris A, Zaccai NR, Salio M, Dulphy N, Shepherd D, Jones EY, Cerundolo V. Generation of CD1 tetramers as a tool to monitor glycolipid-specific T cells. *Philos Trans R Soc Lond B Biol Sci* 2003; **358**:875–7.

51 Karadimitris A, Gadola S, Altamirano M et al. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci U S A* 2001; **98**:3294–8.

52 Corbett AJ, Eckle SB, Birkinshaw RW et al. T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* 2014; **509**:361–5.

53 Attaf M, Huseby E, Sewell AK. alphabeta T cell receptors as predictors of health and disease. *Cell Mol Immunol* 2015;. doi:10.1038/cmi.2014.134.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Comparison of phycoerythrin (PE) and brilliant violet (BV)-conjugated tetramers on peripheral blood mononuclear cells (PBMC). HLA A2$^+$ PBMC + PKI were stained with Melan A (ELAGIGILTV) and influenza (flu) (matrix protein; GILGFVFTL) PE-conjugated and BV-conjugated tetramers. More background staining was evident on the CD8$^-$ cells with the BV tetramer (red arrows) as observed in Fig. 1.