Direct isolation, phenotyping and cloning of low-frequency antigen-specific cytotoxic T lymphocytes from peripheral blood

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Cytotoxic T lymphocytes (CTLs) play an important role in controlling viral infections and certain tumours, but characterising specific CTL responses has always been technically limited. Fluorogenic 'tetramers' of major histocompatibility complex (MHC) class I complexes have been exploited recently to quantify the massive expansion of specific CTLs in human immunodeficiency virus (HIV) infection [1]. Here, we use MHC class I complex tetramers to isolate low-frequency antigen-specific CTLs directly from human peripheral blood, allowing the simultaneous phenotypic and functional characterisation and cloning of these CTLs. We synthesised a tetramer that specifically stained human leukocyte antigen (HLA)-A2.1-restricted CTL clones recognising the influenza matrix protein peptide 58–66, matrix 58–66 [2]. This tetramer stained between 1 in 1,500 and 1 in 58,000 peripheral blood mononuclear cells (PBMCs) from HLA-A2.1$^+$ individuals. The surface phenotype of these cells could be analysed by fluorescence-activated cell sorting (FACS), and the cells could be directly sorted into enzyme-linked immunospot (ELISpot) plates, where they released interferon-γ (IFN-γ) within 1 day of antigen exposure. The same population was cloned by FACS, and the specificity of several expanded clones was confirmed. Cloning was greatly simplified and accelerated compared with standard protocols, and was highly efficient. We also used tetramer-based sorting to enrich melanoma-specific CTLs derived from a tumour-infiltrated lymph node. Direct cloning of specific CTLs from peripheral blood can provide important information about immunological memory, CTL responses against tumour antigens and CTL proliferation and function, and opens up new possibilities for generating CTLs for adoptive immunotherapy.

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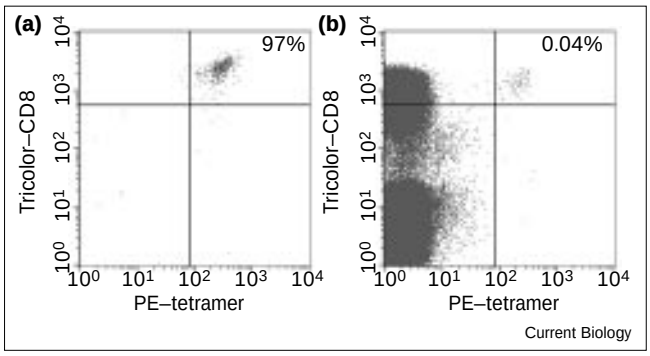
Results and discussion

HLA-A2.1 molecules (A2) were refolded *in vitro* [1] with β2 microglobulin and matrix 58–66 (peptide GILGFVFTL, in single-letter amino-acid code). Complexes were biotinylated and bound to phycoerythrin (PE)-labelled streptavidin at a 4:1 ratio, to form an A2–matrix tetramer [1]. This reagent stained CTL clones specific for matrix 58–66 (Figure 1a) but did not stain other A2-restricted clones that had different specificities (data not shown). The A2–matrix tetramer was used to study matrix-specific CTLs in the PBMCs of individuals not suffering from known influenza infection to assess whether tetramers could be used to analyse low-frequency CTLs, such as memory CTLs, directly *ex vivo*.

A distinct population of CD8$^+$ cells was repeatably stained by the A2–matrix tetramer in all PBMC samples from A2$^+$ individuals (Figure 1b), whereas no cells in up to 400,000 PBMCs from A2-mismatched donors were double-stained with equivalent intensity. Staining patterns were unaffected by raising tetramer concentration beyond that required for maximal clone staining. Frequencies of A2–matrix tetramer$^+$ CD8$^+$ cells in A2$^+$ individuals (Table 1) were considerably higher than those previously estimated by limiting dilution analysis (LDA), but are close to those obtained by ELISpot analysis for peptide-specific cytokine release [3]. This result suggests that CD8$^+$ cells stained by the A2–matrix

Figure 1



FACS profiles of A2–matrix tetramer-stained cells. (a) A CTL clone recognising matrix 58–66 and (b) PBMCs from a healthy HLA-A2.1$^+$ individual were double-stained with PE-labelled A2–matrix tetramer and Tricolor–anti-CD8 (Caltag) for 15 min each, then washed extensively in FACS buffer (1% foetal calf serum in PBS) before analysis on a FACSort (Becton Dickinson). Cells shown are small lymphocytes gated by forward and side scatter profile.

Table 1

Frequencies of A2–matrix tetramer$^+$ CD8$^+$ cells per total PBMCs in HLA-A2.1$^+$ individuals H1–H11.

Individual	Frequency
H1	1 in 1,500
H2	1 in 3,100
H3	1 in 3,700
H4	1 in 10,900
H5	1 in 12,900
H6	1 in 19,100
H7	1 in 20,100
H8	1 in 22,000
H9	1 in 34,000
H10	1 in 40,300
H11	1 in 58,000

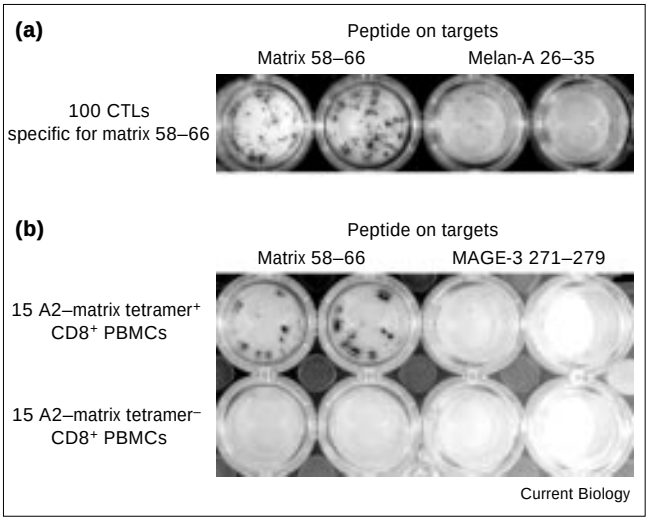
tetramer include a proportion of cells that would fail to register in LDA because of their limited proliferative potential [3].

To determine the phenotype of the A2–matrix tetramer$^+$ CD8$^+$ cells, we used triple-staining to characterise surface markers, and developed novel FACS-based assays to test the effector function and proliferative potential of these cells. Such coordinated assessment of the phenotype and function of low-frequency CTLs from peripheral blood has not previously been possible with other techniques.

A large number of PBMCs were obtained from one A2$^+$ individual (termed H3). The frequency of A2–matrix tetramer$^+$ CD8$^+$ cells in this sample was consistently about 1 in 3,700 PBMCs and 1 in 2,500 small lymphocytes (Figure 1b). These cells were CD45RO$^+$ and CD45RA$^-$, consistent with them having previously been exposed to antigen. At least 74% of the A2–matrix tetramer$^+$ CD8$^+$ cells in the PBMCs of H3 expressed the Vβ17 T-cell receptor (TCR) chain, compared with 4% of the A2–matrix tetramer$^-$ CD8$^+$ PBMCs (data not shown). These data confirm the bias towards the use of the Vβ17 chain that had been observed previously in cloned CTLs recognising matrix 58–66 [4]. Only 36% of A2–matrix tetramer$^+$ CD8$^+$ cells in the PBMCs of H3 expressed CD28, compared with 75% of A2–matrix tetramer$^-$ CD8$^+$ cells (data not shown). This finding is consistent with the majority of A2–matrix tetramer$^+$ CD8$^+$ cells having a reduced proliferative potential due to a longer replicative history, as has been reported previously for other CD28$^-$ CD8$^+$ cells [5].

The specificity and activation of A2–matrix tetramer$^+$ CD8$^+$ PBMCs was studied by the ELISpot assay, which produces 'spots' of colour wherever individual CTLs release IFN-γ. In control experiments, a CTL clone specific for matrix 58–66 was stained with the A2–matrix tetramer and anti-CD8; 100 A2–matrix tetramer$^+$ CD8$^+$ cells were then sorted directly into duplicate wells of an ELISpot plate containing HLA-A2.1$^+$ target cells (T2) that had been pre-pulsed with either matrix 58–66 or the negative-control peptide melan-A 26–35 (EAAGIGILTV) [6]. No IFN-γ was released from the A2–matrix tetramer$^+$ CD8$^+$ clones exposed to control peptide (Figure 2a), indicating that tetramer staining does not by itself activate clones to produce cytokine. IFN-γ was released from clones exposed to matrix 58–66, however, with approximately one IFN-γ spot produced per CTL within 1 day, indicating that all the cells had been activated by recognition of their cognate peptide. We then stained PBMCs from H3, and sets of 15 cells were sorted into duplicate ELISpot wells and incubated with peptide-pulsed targets for either one or two days. IFN-γ release was observed only when A2–matrix tetramer$^+$ CD8$^+$ PBMCs were exposed to targets pulsed with matrix 58–66 (Figure 2b); these cells did not recognise targets pulsed with the negative-control peptide MAGE-3 271–279 (FLWGPRALV) [7]. A2–matrix tetramer$^-$ CD8$^+$ PBMCs did not produce IFN-γ in response to either peptide. Results were essentially the same after either 1 or 2 days incubation, although the IFN-γ spots were less intense in colour at the earlier time point. The

Figure 2



ELISpot analysis of A2–matrix tetramer-stained cells. (a) A CTL clone recognising matrix 58–66 and (b) PBMCs from a healthy HLA-A2.1$^+$ individual were double-stained and sorted directly into a 96-well ELISpot plate (MAIP S45; Millipore) using a FACS Vantage (Becton Dickinson) running CellQuest and CloneCyt software. Each well contained 100 μl of RPMI 1640 medium with 5% human serum and 5,000 HLA-A2.1$^+$ target cells (T2) that had been pre-incubated overnight in serum-free medium with 10 μM of either matrix 58–66 or the negative-control peptides melan-A 26–35 and MAGE-3 271–279, then washed before plating. Sorted A2–matrix tetramer$^+$ CD8$^+$ or A2–matrix tetramer$^-$ CD8$^+$ cells were cultured with targets for 1 or 2 days before developing the ELISpot for IFN-γ as described by the manufacturer (Mabtech). (b) Results after 2 days incubation.

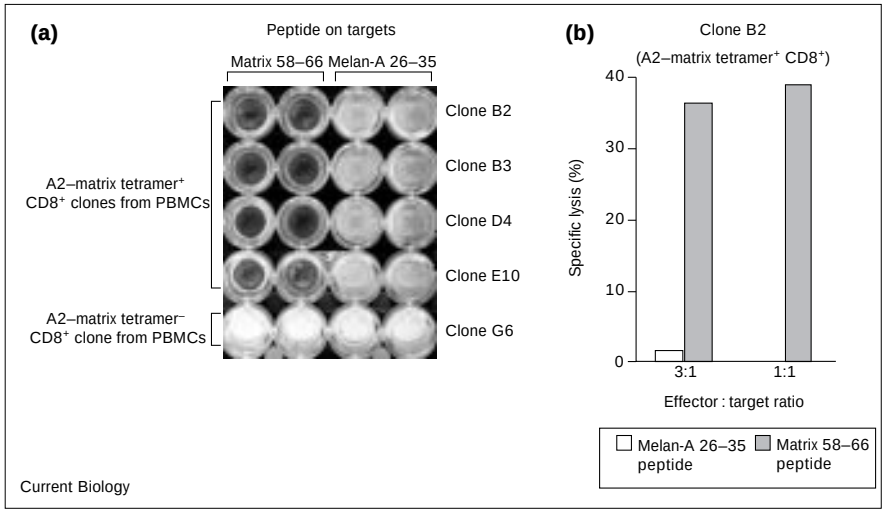
number of spots formed was similar to the number of PBMCs placed in the wells, suggesting that the majority of A2–matrix tetramer$^+$ CD8$^+$ PBMCs were capable of responding specifically to antigen within 1 day. These data support the concept that most circulating influenza-specific memory CD8$^+$ cells are capable of rapid effector function when triggered by exposure to cognate peptide in the absence of cytokine [3], even when the peptide is presented by cells that are not professional antigen-presenting cells.

To further confirm the specificity of A2–matrix tetramer$^+$ CD8$^+$ PBMCs, and to study their proliferative potential, single cells from the PBMCs of H3 were sorted directly into cloning wells. Out of 60 wells seeded with A2–matrix tetramer$^+$ CD8$^+$ cells, 15 contained proliferating blasts after 2 weeks, and the four most proliferative clones were tested in duplicate ELISpot assay for antigen-specificity, along with one proliferating A2–matrix tetramer$^-$ CD8$^+$ clone. As shown in Figure 3a, all four A2–matrix tetramer$^+$ CD8$^+$ clones specifically synthesised IFN-γ when exposed to targets pulsed with matrix 58–66, but not when exposed to targets pulsed with control peptide, whereas the A2–matrix tetramer$^-$ CD8$^+$ clone failed to respond to the same target cells. One expanded A2–matrix tetramer$^+$ CD8$^+$ clone was also tested in a chromium-release assay, where it specifically lysed targets pulsed with matrix 58–66 (Figure 3b).

Finally, to demonstrate the potential of tetramer-based FACS protocols for the analysis of tumour-specific CTL responses, we synthesised an A2 tetramer with the melan-A 26–35 peptide, which derives from a common melanoma antigen [6]. This tetramer was used to stain and sort a polyclonal CTL line, MM7, which had been generated from an A2$^+$ melanoma-infiltrated lymph node. MM7 contained only a small proportion of cells (6%) that could be double-stained with A2–melan-A tetramer and anti-CD8. MM7 specifically killed A2-matched targets pulsed with melan-A 26–35, but it also poorly killed two A2-matched melanoma lines expressing melan-A, and demonstrated small background killing of target cells pulsed with a negative-control peptide (Figure 4a). An aliquot of MM7 was then enriched for A2–melan-A tetramer$^+$ CD8$^+$ cells by FACS, to form the line MM7-E, which contained 42% A2–melan-A tetramer$^+$ CD8$^+$ cells. MM7-E killed the melanoma lines and targets pulsed with melan-A 26–35 much more efficiently than MM7, with no background killing (Figure 4b). Hence, specific cytotoxicity correlates entirely with the percentage of tetramer staining, indicating that tetramer staining followed by FACS can be used to generate highly effective tumouricidal CTL lines.

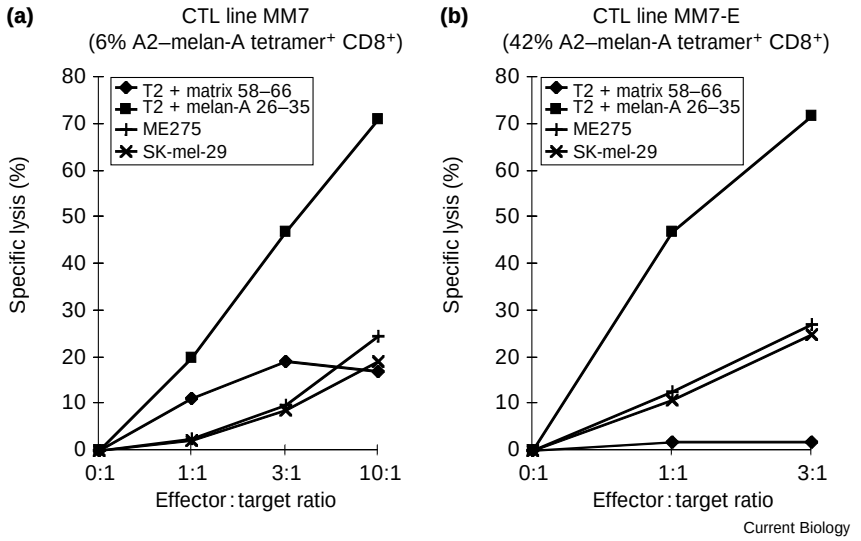
In conclusion, we have demonstrated that tetrameric MHC class I complexes can be used to identify and characterise low-frequency CTLs of defined specificity directly *ex vivo*. In peripheral blood, CTLs specific for the influenza matrix 58–66 epitope were more frequent than recognised previously and were rapidly reactive to antigen, although only a minority had significant proliferative potential. Heterogeneity of CD28 expression in this CTL population suggests that functional differences, such as the ability to generate large numbers of progeny by clonal expansion, may co-segregate with surface phenotype.

Figure 3



Cloning of specific CTLs directly from PBMCs using A2–matrix tetramer. PBMCs were stained with A2–matrix tetramer and anti-CD8, and sorted as for Figure 2 as single cells directly into cloning wells containing 100,000 irradiated mixed PBMCs from three donors, in 100 μl Iscove's medium, supplemented with 5% human serum and 5 μg/ml phytohaemagglutinin. Clones were fed with IL-2 to 15 U/ml and Lymphocult T (Biotest) to 10% on day 4, and with IL-2 to 50 U/ml on day 10 and every third day subsequently. (a) After 14 days culture, ELISpot analysis was performed as for Figure 2, using 10% of each clone per well. (b) One expanded clone was subsequently tested in a standard 4 h chromium-release assay for its ability to lyse HLA-A2.1$^+$ target cells (the B-cell lymphoblastoid line .45) pulsed with either matrix 58–66 or the negative-control peptide melan-A 26–35. Percentage specific lysis was calculated by the formula: 100× (specific release – spontaneous release)/(maximal release – spontaneous release).

Figure 4



Sorting of melanoma-specific CTLs using A2–melan-A tetramer. Polyclonal CTL line MM7 was generated from an HLA-A2.1+ melanoma-infiltrated lymph node by repeated rounds of stimulation with melan-A 26–35 peptide and tissue culture in the presence of IL-2. MM7 was stained with A2–melan-A tetramer and anti-CD8, as described for Figure 1, and shown to be 6% double-positive. Double-positive cells were enriched in an aliquot of MM7 by FACSorting to form the polyclonal CTL line MM7-E, which was 42% double-positive. Both CTL lines were simultaneously tested in a standard 4 h chromium-release assay for their ability to lyse HLA-A2.1+ target cells. Targets were T2 cells, pulsed with either melan-A 26–35 or matrix 58–66 as the negative control, and the HLA-A2.1+ melanoma cell lines SK-mel-29 and ME275, both of which express melan-A. Percentage specific lysis was calculated as for Figure 3b.

We have also demonstrated that antigen-specific CTLs can be cloned directly from PBMCs using tetramers. In marked contrast to traditional methods, only a small number of clones need to be seeded and screening of the clones requires minimal effort. This technique will accelerate advances in our understanding of CTLs and might also offer new opportunities in adoptive immunotherapy, particularly in rapidly progressive conditions, such as metastatic malignant melanoma, where the time available for intervention is limited.

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