



## Research paper

## ELISPOT and functional T cell analyses using HLA mono-specific target cells

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## ABSTRACT

Simple T cell assays specific for any chosen HLA class I or class II/peptide combination, are of enormous value in cancer immunotherapy, clinical trials, vaccine and infectious disease research. The reliable measurement of T cell activity can be difficult due to the presence of other alleles on target cells, particularly for the non-HLA-A2 alleles, and the varying baseline characteristics of the different APCs employed. In the absence of pulsing with HLA-A2 restricted peptides, T2 cells are functionally HLA class I and II negative. By coating these cells with recombinant HLA peptide complexes, HLA mono-specific cells are produced that present only a defined single epitope, and generate minimal background immune activation. In ELISPOT, intracellular cytokine staining (ICS) and killing assays using T cells specific for HLA-A2/peptide complexes, the HLA mono-specific cells gave comparable results, to those using standard peptide pulsed HLA-A2 positive T2 cells without significant background. Successful T cell assays for non-HLA-A2 T cells were also performed, with PBMCs recognizing HLA-A24 and HLA-DR15/peptide complexes. The data, obtained with ELISPOT, ICS and FACS-based killing assays, all demonstrate high specificity of T cell activity and low levels of background activity. HLA mono-specific cells are simple to prepare, and can be used with any stable recombinant HLA allele/peptide combination; providing a useful system for improved T cell functional analyses across all HLA allotypes. This represents a significant advance in the generation of reliable functional T cell data.

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## 1. Introduction

The measurement of epitope specific T cell numbers and activity is central to the development of vaccination, and other immunotherapeutic strategies in oncology and viral

infections (Nagorsen et al., 2004). The use of HLA class I and class II tetramer technology allows the accurate enumeration of antigen specific T cells (Altman et al., 1996); however, tetramer analyses provide limited information on the functional activity of cells.

Assays that measure the activation and killing ability of antigen specific T cells require a target cell or antigen presenting cell (APC) expressing the epitope(s) of choice. In current assays, the cells employed include autologous tumour cells or peptide pulsed B cells, whilst for some HLA alleles there are HLA 'matched' standard target cells. Obtaining autologous cells and maintaining them, can be demanding, and their T cell interactions may be subject to non-specific activation from other HLA class I and II complexes expressed

*Abbreviations:* HLA, human leukocyte antigen; ICS, intracellular cytokine staining; HLA-A2, HLA-A\*0201; HLA-A24, HLA-A\*2402; HLA-DR1, HLA-DRB1\*0101; HLA-DR15, HLA-DRB1\*1501; PBMC, peripheral blood mononuclear cell.

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by the target cell, or from non-target peptides within the chosen HLA allotype. As a result the measurement of T cell activity by ELISPOT, intracellular cytokine staining (ICS) and direct lytic activity, are difficult to perform, of variable reproducibility, and subject to considerable background 'noise', particularly for the non-HLA-A2 epitopes (Keilholz et al., 2002).

For HLA-A2, an allele present in approximately 50% of the Caucasian population (Middleton et al., 2003), assays can be simplified by the use of natural or transfected APC/targets that express only this allele, such as T2, CIR-A2, or K562-A2 cells (Purbhoo et al., 2001; Britten et al., 2002; Shafer-Weaver et al., 2006). However, for the T cells that make up the non-HLA-A2 immune response, including the majority of patients worldwide who are HLA-A2 negative, performing accurate assays is problematic.

To produce APCs or target cells that present a single HLA specificity, other groups have produced single HLA class I allele transfectants with the chosen peptide introduced by peptide pulsing (Britten et al., 2002). These cells have high levels of specificity and low background when used with T cell lines. However, they have not been widely adopted due to the logistical issues of producing and keeping multiple cell lines in culture to test for the varying HLA alleles, and the potential difficulty using some of these cells directly with PBMCs populations.

We have previously described the use of an antibody delivery system to immobilize HLA class I complexes on the surface of B cells and tumour cells via binding to CD20: this targets B cells into highly effective APCs capable of producing activation and expansion of peptide specific CD8+ T cells (Savage et al., 2004; Stebbing et al., 2004). In addition, coating

B cells with HLA class I/peptide complexes, also makes them effective target cells for T cells of that specificity (Savage et al., 2002; Mous et al., 2006; Mous et al., 2008).

Herein, we have investigated the use of HLA class I and HLA class II negative T2 cells targeted with recombinant HLA class I and class II complexes (Fig. 1), in T cell functional assays, including those performed directly with fresh *ex vivo* PBMCs.

## 2. Methods

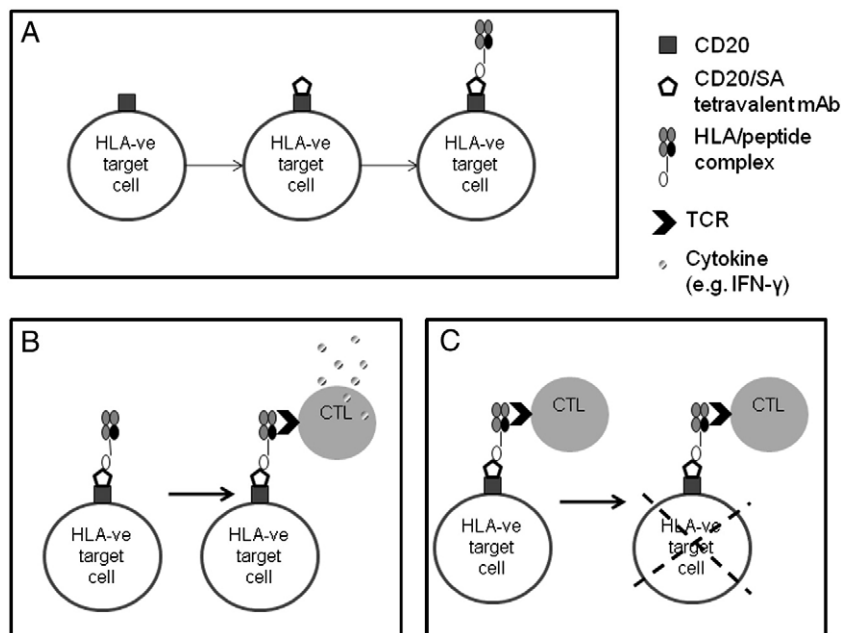
### 2.1. Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were purified from the heparinized blood of healthy volunteer donors by Ficoll–Hypaque density gradient centrifugation. HLA class I and class II genotyping was performed using PCR sequence-specific primers (Olerup SSP; Genovision, Alpha Helix). CMV status was determined by ELISA for CMV specific IgG antibodies. All samples were obtained with appropriate ethical approval in accordance with the Declaration of Helsinki.

### 2.2. Human primary cells and cell lines

#### 2.2.1. T2 cells

The T2 cell line is a CD20-positive TAP deficient B cell/T cell hybrid that expresses HLA-A2 but lacks antigenic peptides in the absence of peptide pulsing (Salter and Cresswell, 1986). T2 cells are frequently used in T cell assays for measuring HLA-A2 specific responses when pulsed with HLA-A2 binding peptides.



**Fig. 1.** Preparation of HLA mono-specific cells for use in immunological assays. A) Coating of HLA – ve cells with HLA monomers in a two-step antibody-targeted process. T2 cells are first coated with recombinant B9E9 single-chain-Fv streptavidin (ScFvSA) fusion protein, then the HLA/peptide monomer of choice is added, which binds directly to the ScFvSA. These cells can be used in a variety of assays, including those measuring cytokine release, e.g. ELISPOT and intracellular cytokine staining (B). In addition, they can be used as targets in cytolytic killing assays (C). HLA, human leukocyte antigen; TCR, T cell receptor; CTL, cytotoxic T lymphocyte.

### 2.2.2. T cell lines and clones

T cell clones recognizing HLA-A2/telomerase, from the restricted human telomerase reverse transcriptase (hTERT) epitope ILAKFLHWL (hTERT<sub>540–548</sub>), were generated as previously described (Whelan et al., 1999; Laugel et al., 2005; Lissina et al., 2009). T cell lines recognizing HLA-A2/Melan-A or HLA-A24/pp65 were generated by growing PBMCs from healthy donors in the presence of peptide (5–10 µg/ml) with restimulation with irradiated PBMCs and fresh peptide as necessary.

### 2.3. HLA monomers, tetramers and pentamers

Biotinylated class I monomers and fluorochrome conjugated HLA class I pentamers were purchased from ProImmune Ltd (Oxford, UK) or produced “in-house” as previously described (Maini et al., 1999; Wooldridge et al., 2005). Class II monomers and fluorochrome conjugated HLA class II tetramers were obtained from Beckman Coulter Ltd (High Wycombe, UK), or produced “in-house” as previously described (Cole et al., 2007).

### 2.4. Coating T2 cells with HLA monomers

T2 cells ( $1 \times 10^6$ /ml) were incubated with recombinant B9E9 single-chain-Fv streptavidin (ScFvSA) fusion protein (10 µg/ml), diluted in phosphate-buffered saline (PBS) for 1 h at 4 °C (Schultz et al., 2000). After washing in PBS, cells were incubated with biotinylated HLA class I or class II monomers (0.5 µg/ml in PBS), for 30 min at room temperature (RT). Coating with HLA monomers was verified by staining with FITC-conjugated anti-human HLA class I antibody W6/32 (Abd Serotec, Oxford, UK) or for class II with anti-human HLA-DR (Beckman Coulter, High Wycombe, UK), and analysed by flow cytometry.

### 2.5. Interferon- $\gamma$ ELISPOT

T2 cells were pulsed with HLA-A2 binding peptides in serum-free media (10 µg/ml; 37 °C; overnight), or coated with recombinant HLA/peptide monomers as described above. Cells were washed in PBS 3 times prior to using in assays.

ELISPOT plates (MAIPN45, Millipore, Billerica, MA, US) were coated with antibody recognizing human interferon-gamma (1-DIK, 15 µg/ml; 3 h RT), then washed, and cells added at various effector:target ratios. Plates were incubated overnight at 37 °C when using PBMCs or T cell lines, or for 4 h at 37 °C if using T cell clones. T2 cells were added at 50,000 cells per well, whilst PBMCs or T cell lines were added at varying cell numbers as stated for each experiment. All samples were added in triplicate.

Following incubation, cells were discarded, and the plates were washed. Biotinylated detection antibody was then added (7-B6-1, 1 µg/ml), and incubated for 2 h at RT. After washing, streptavidin-ALP was added for 1 h at RT. Finally the plate was washed and ALP colour development buffer (BioRad, Hemel Hempstead, UK) was added and the plate developed until clear spots appeared. The colour reaction was stopped with water, the plate was left to dry, and spots were then counted and verified using an AID ELISPOT reader

(version 4; AID Diagnostika GmbH, Strassberg, Germany). All antibodies for the ELISPOT system were purchased from Mabtech, Nacka Strand, Sweden.

### 2.6. Intracellular staining (ICS)

To measure intracellular cytokine production,  $0.5 \times 10^6$  PBMCs were co-cultured with  $0.25 \times 10^6$  T2 cells for 4 h, with Brefeldin A (10 µg/ml; Sigma, Poole, UK) added during the third hour of incubation. Cells were then stained with FITC-conjugated antibody specific for anti-human CD8 (OKT8) or CD4 (OKT4) at 4 °C for 20 min, cells were then permeabilized using the Fix/Perm kit from eBioscience (San Diego, CA, US), followed by intracellular staining performed with APC-conjugated antibody specific for interferon-gamma (4S.B3) according to the manufacturer's instruction. All antibodies used were obtained from eBioscience. Cells were analysed using a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ US), with a minimum of 100,000 live cells per sample collected and evaluated using FlowJo software (Tree Star Inc, Ashland, OR, US).

### 2.7. FACS-based killing assay

A FACS-based killing assay using a method modified from that published by Sheehy et al. (2001) was performed. T2 cells were pulsed with peptide, or coated with HLA/peptide monomer as above. These target cells were then stained with carboxyfluorescein succinimidyl ester (CFSE; 0.5 µM; Sigma, Poole, UK), and anti-human CD19 APC (HIB19, eBioscience, San Diego, CA, US). Following staining, T2 cells were cultured with T cell lines recognizing the specific HLA/peptide combination, at several effector:target ratios, in a 96 well U-bottom plate (Becton Dickinson, Oxford, UK) for 4 h at 37 °C. Following incubation, the contents of the U-bottom plate were transferred to FACS tubes, and washed with PBS. Supernatant was discarded, and cells were resuspended in 150 µl 1.5% paraformaldehyde, and analysed by flow cytometry within 24 h of harvesting the cells.

The total contents of each FACS tube were run on a BD FACScalibur flow cytometer and all events were collected. For analysis, dead cells were excluded by gating out small subcellular fragments via forward side scatter characteristics.

To calculate the percentage killing in the assay: target T2 cells were selected by gating on the CD19+ population, then a FL1 (CFSE) histogram for this population was created. Live target cells were gated on using their CFSE staining characteristics (live cells are CFSE<sup>high</sup>) and percentage killing was calculated by the following equations: % survival = (mean CFSE<sup>high</sup> % of test well / mean CFSE<sup>high</sup> % of spontaneous release) × 100, and, % killing = 100 – % survival. Cell lysis is demonstrated by reduction in CFSE fluorescence, whereby dead cells are detected as CFSE<sup>low</sup> or CFSE negative (Sheehy et al., 2001).

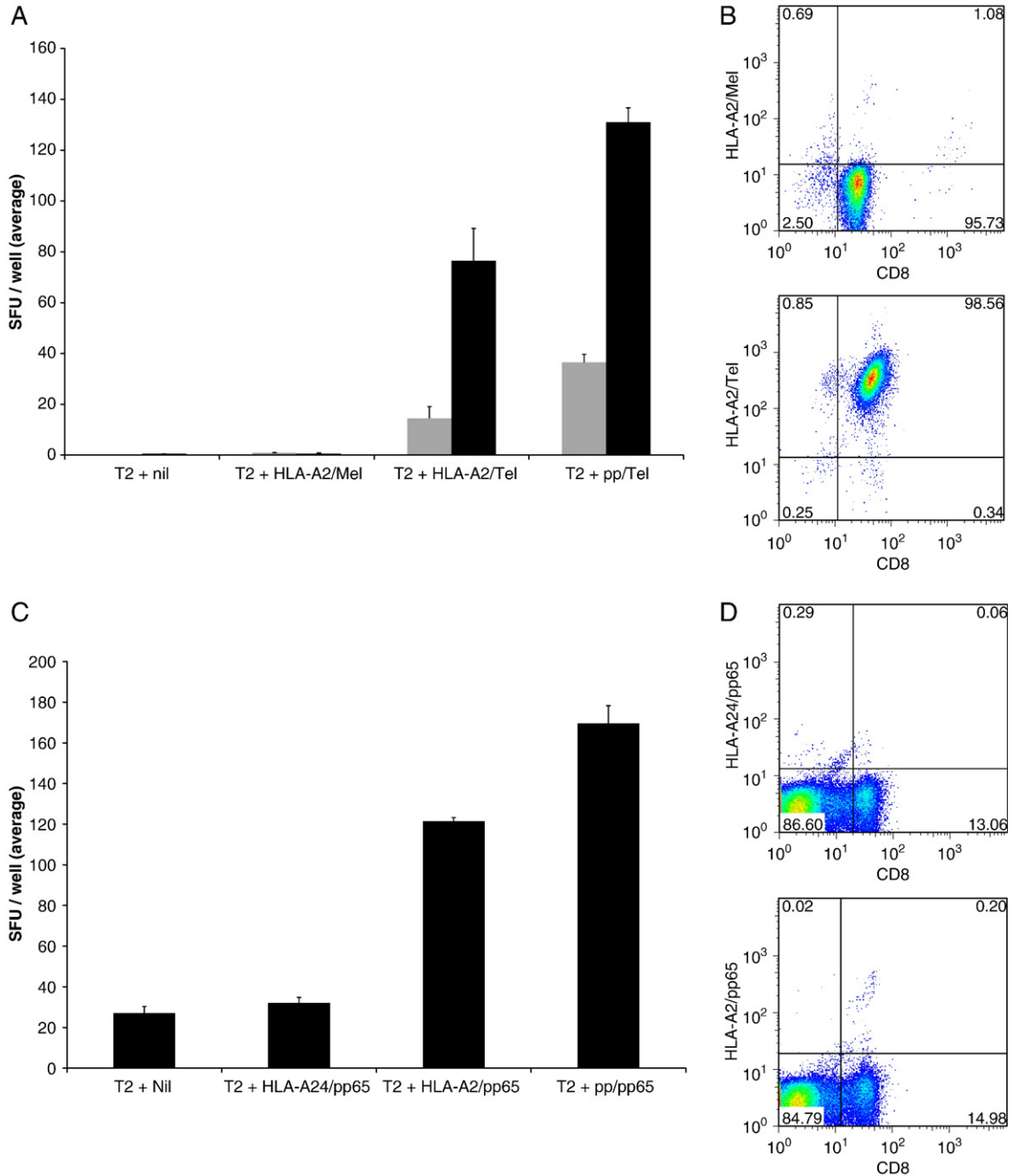
### 2.8. Tetramer and pentamer staining

To assess the antigen specificity of cells, a small sample of PBMCs or T cells used in each assay was stained with anti-

human CD4 or CD8 antibody (eBioscience, San Diego, CA, US), and with tetramers or pentamers corresponding to the HLA/peptide monomers used in the assay. HLA class I tetramers were incubated at 37 °C for 15 min, HLA class I pentamers were incubated for 5 min at 37 °C, and HLA class II tetramers were incubated at 37 °C for 2 h.

## 2.9. Flow cytometry analysis

Samples were analysed on a FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA, USA), and data were analysed using FlowJo v. 7 (Treestar Inc., Ashland, OR, USA).



**Fig. 2.** HLA-A2 ELISPOT assays using HLA mono-specific T2 cells are comparable to peptide pulsed T2s. **A)** An ELISPOT using T cell clones specific for HLA-A2/Tel. Data presented is the mean number of spot forming units (SFU) per well, with either 125 (grey), or 500 (black) T cell clones added per well; along with native T2s, or T2s coated with monomer, or, peptide pulsed (pp). The corresponding tetramer stains of the T cell clones are shown in **B**, where the top panel shows staining with a HLA-A2/Mel tetramer, and the bottom panel shows staining with a HLA-A2/Tel tetramer. The data is representative of four experiments with T cell clones. In **C**, an ELISPOT has been carried out using PBMCs from a CMV sero-positive donor. The corresponding pentamer staining of PBMCs is shown in **D**. Here the upper plot shows staining with irrelevant HLA-A24/pp65 pentamer, whilst the lower plot shows staining with HLA-A2/pp65 pentamer. Data is representative of three separate experiments with PBMCs. ELISPOTs were carried out in triplicate, and data presented is the mean of the replicates, with error bars showing  $\pm$  s.e.m.

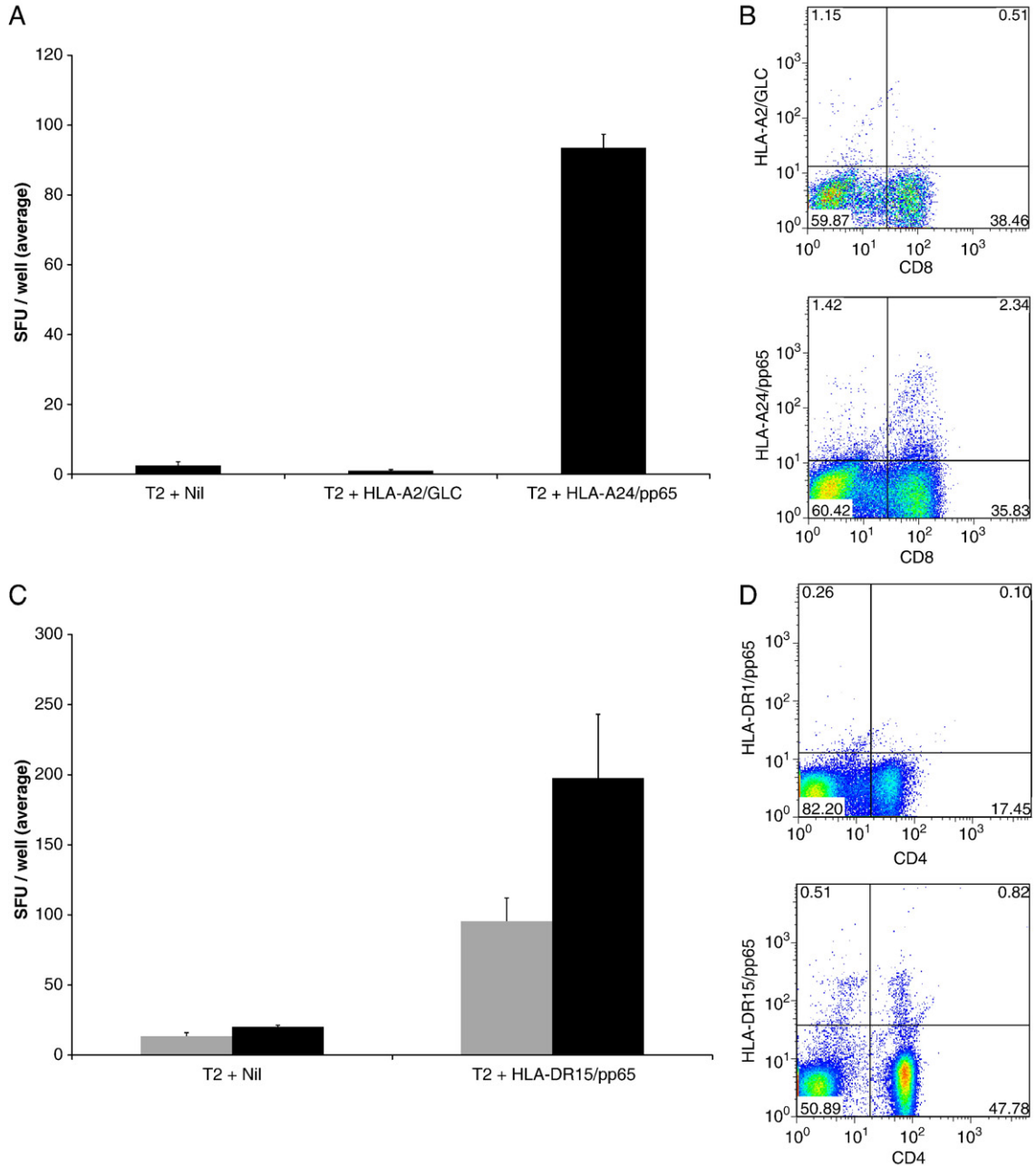
### 3. Results

#### 3.1. ELISPOT analysis

##### 3.1.1. ELISPOT assay specificity

To demonstrate the specificity of functional assays using the T2 HLA mono-specific cells, ELISPOTs were first carried

out using T cell clones specific for an HLA-A2 Telomerase epitope (HLA-A2/Tel). The ability of the T2 HLA mono-specific cells to act as APCs for ELISPOT assays is demonstrated in Fig. 2A. Here, analysis of a T cell clone specific for HLA-A2/Tel is demonstrated in an ELISPOT carried out with native T2 cells, peptide pulsed T2 cells or T2 cells coated with recombinant HLA-A2/peptide complexes.



**Fig. 3.** Non-HLA-A2 ELISPOT assays. An ELISPOT using PBMCs stimulated with HLA-A24/pp65 peptide for one week is shown in A. 40,000 PBMCs were co-cultured with native or monomer coated T2s as labeled. The corresponding flow cytometry data is shown in B. Here the upper dot plot shows staining with an HLA-A2/GLC tetramer, whilst the lower plot shows staining with an HLA-A24/pp65 pentamer. In C, ELISPOT data for an HLA-DR15 positive HLA-DR1 negative CMV sero-positive donor is shown. Here, 250,000 (grey) or 500,000 (black) PBMCs were added per well, and co-cultured with T2s as labeled. The corresponding flow cytometry data is shown in D, where the top dot plot shows PBMCs stained with an HLA-DR1/pp65 tetramer, whilst the bottom plot shows those stained with an HLA-DR15/pp65 tetramer. Data presented is representative of three experiments carried out with HLA-A24 or HLA-DR15 donors, all ELISPOTs were carried out in triplicate and data is the mean of three wells, with error bars showing  $\pm$  s.e.m.

Fig. 2B shows tetramer staining of the HLA-A2/Tel clone, with the upper dot plot showing the irrelevant staining with a HLA-A2/Melan-A (HLA-A2/Mel) tetramer (1.1% of CD8+ cells), whereas the lower dot plot shows the tetramer stain with HLA-A2/Tel tetramer (99.7% of CD8+ cells).

There were less than 2 spots per well when the T cell clone was incubated with native non-peptide pulsed T2 cells, or T2 cells bearing the inappropriate HLA-A2/Mel complex. In contrast, when the T cell clones were exposed to conventional HLA-A2 telomerase peptide pulsed T2 cells there are an average of 131 spot forming units (SFUs) per 500 T cell clones; with a slightly lower number of SFUs produced by T cell clones in the presence of T2 HLA mono-specific cells bearing the HLA-A2/Tel complex (77 per 500 T cell clones).

### 3.1.2. Comparison with peptide pulsed T2 cells for HLA-A2 specific T cells

Fig. 2C shows the ELISPOT results for unexpanded PBMCs from a CMV sero-positive HLA-A2 positive, HLA-A24 negative donor. Fig. 2D shows the corresponding pentamer staining, with the uppermost dot plot showing staining data with an irrelevant HLA-A24/pp65 pentamer (0.4% of CD8+ cells), and the bottom graph showing staining with the relevant HLA-A2/pp65 pentamer (2.1% of CD8+ cells). Here, the ELISPOT shows positive results with 120–170 SFUs in the wells containing PBMCs in combination with either T2 cells pulsed with HLA-A2/pp65 peptide, or those targeted with HLA-A2/pp65 complexes; showing comparable sensitivity of HLA mono-specific T2 cells and peptide pulsed T2 cells as APCs. The sample wells containing PBMCs co-cultured with native T2 cells, those targeted with the B9E9 antibody alone, or those targeted with inappropriate HLA-A24/pp65 complexes showed only background levels of between 27 and 32 SFUs per well.

### 3.1.3. ELISPOT for non-HLA-A2 specific T cells

The tetramer and ELISPOT analysis of PBMCs from a CMV immune HLA-A24 positive, HLA-A2 negative donor are shown in Fig. 3A + B. Here it can be seen that staining with an irrelevant HLA-A2/GLC tetramer positively stains a background level of 1.3% of CD8+ cells, whereas staining with relevant HLA-A24/pp65 pentamer revealed there to be 6.1% of CD8+ cells reacting to HLA-A24/pp65 (Fig. 3B).

In the ELISPOT assay, PBMCs co-cultured with native T2 cells produced minimal activation with only 3 SFU per well, whilst those cultured with T2 cells coated with appropriate HLA-A24/pp65 complexes produced 90 SFU from 40,000 PBMCs. Wells containing PBMCs along with T2 cells targeted with inappropriate HLA-A2/GLC complexes produced an average of 1 SFU per well.

### 3.1.4. ELISPOT for HLA class II specific T cells

Using unexpanded *ex vivo* PBMCs from an HLA-DR15 positive, HLA-DR1 negative CMV immune donor, the tetramer and ELISPOT analysis data for T cells recognizing HLA-DR15/pp65 are shown in Fig. 3C + D. PBMCs stained with irrelevant HLA-DR1/pp65 tetramer show staining of 0.07% of CD4+ cells, in comparison, 1.7% of CD4+ cells stained positively for the HLA-DR15/pp65 tetramer (Fig. 3D). ELISPOTs were carried out using either 500,000 or 250,000 PBMCs per well.

In wells containing PBMCs in the presence of native T2 cells, an average of 20 SFU per well were produced. Comparatively, in the presence of HLA mono-specific cells coated with HLA-DR15/pp65 complexes, the sample well produced an average of 198 spot forming units per 500,000 PBMCs. In addition, a dose–response was clearly shown, with an average of 96 SFU produced per 250,000 PBMCs.

## 3.2. Intracellular cytokine assays

### 3.2.1. Comparison with peptide pulsed T2 cells

The comparative abilities of peptide pulsed T2 cells, and T2 cells coated with HLA-A2/peptide complexes, to act as APCs in intracellular cytokine experiments, with a T cell line recognizing HLA-A2/Melan-A are shown in Fig. 4A + B. Fig. 4A shows the graphical representation of intracellular cytokine staining (ICS), whilst Fig. 4B shows the corresponding flow cytometry plots. Here, the peptide pulsed T2 cells give a higher signal, with 28% of CD8+ cells producing IFN- $\gamma$ , compared to 14% when the assay is carried out using the HLA mono-specific T2 cells as APCs. The controls, with either native T2s, or, irrelevant HLA-A2/WT1 coated T2s, show a background level of IFN-gamma production, with either 0.3% or 0.6% of CD8+ cells positively stained for IFN-gamma, respectively.

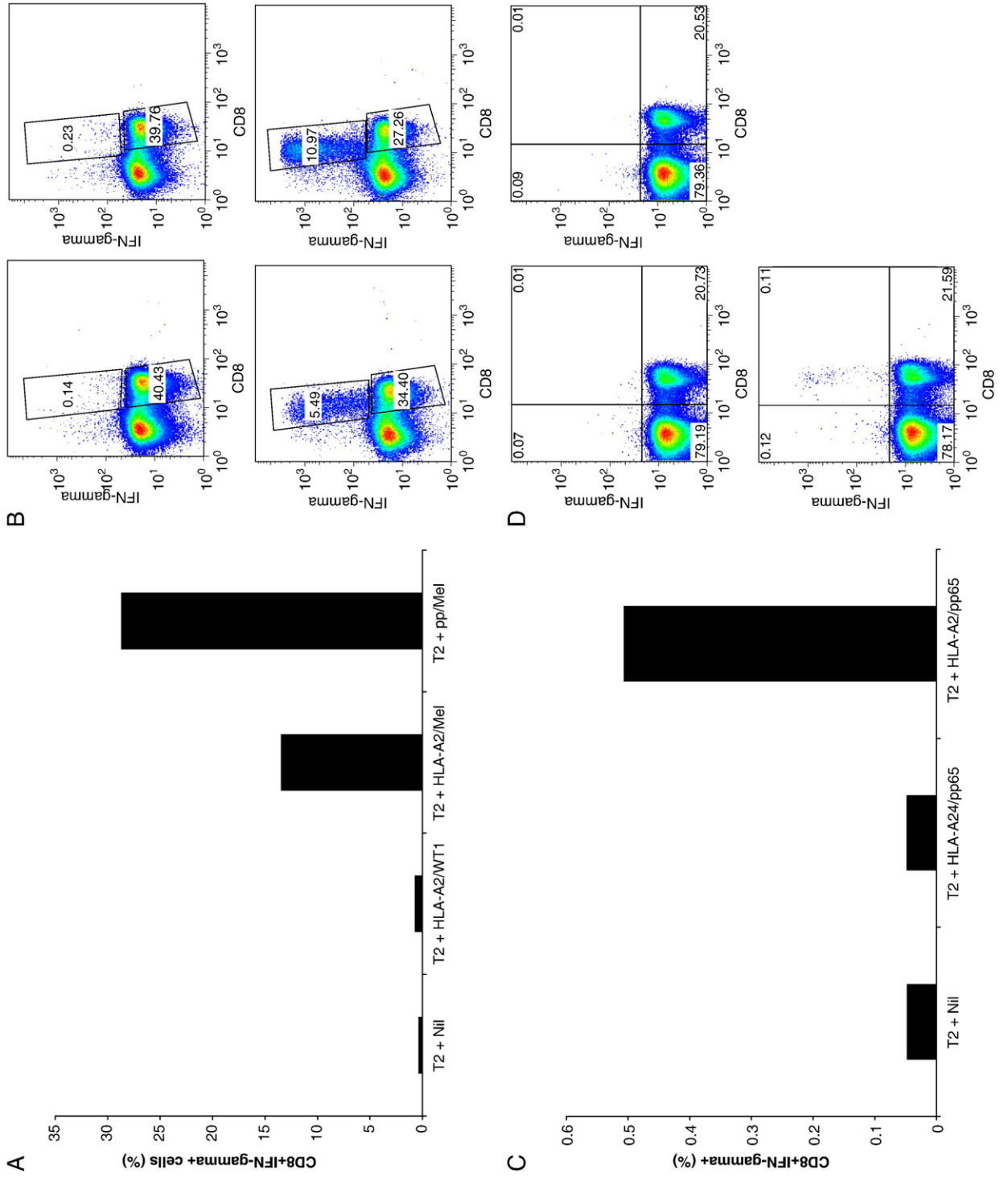
In addition, ICS using non-expanded *ex vivo* PBMCs from a CMV sero-positive HLA-A2 positive, HLA-A24 negative donor was carried out (Fig. 4C + D). Here, Fig. 4C shows the numerical data, whilst Fig. 4D shows the corresponding flow cytometry dot plots. ICS carried out on PBMCs in the presence of native T2 cells, or, T2 cells coated with an irrelevant HLA-A24/pp65 monomer, show background levels of IFN-gamma staining with both conditions revealing 0.05% of CD8+ cells staining positive. In comparison, ICS on PBMCs co-cultured with T2 cells coated with HLA-A2/pp65 monomer, show 0.51% of CD8+ cells staining positively for IFN-gamma.

### 3.2.2. ICS for non-HLA-A2 specific T cells

The results of ICS performed on PBMCs from an HLA-A24 positive, HLA-A2 negative donor are shown in Fig. 5A + B. PBMCs co-cultured with native T2 cells stimulated minimal numbers of cytokine producing cells from the PBMCs, with 0.5% of the CD8+ cells staining positive for IFN- $\gamma$ . In addition, T2 cells coated with an irrelevant HLA class I monomer (HLA-A2/GLC), produced background levels of 0.36% IFN- $\gamma$  positive CD8+ cells. Whilst, PBMCs co-cultured with the relevant HLA-A24/pp65-mono-specific T2 APCs, showed cytokine production from 2.75% of the CD8+ cells.

### 3.2.3. ICS for HLA class II specific T cells

The results of ICS performed on *ex vivo* PBMCs from an HLA-DR15 positive HLA-DR1 negative donor are shown in Fig. 5C + D. Here 0.25% of the CD4+ cells produced IFN-gamma in response to exposure to HLA mono-specific T2 cells coated with HLA-DR15/pp65 complexes compared to 0.14% or 0.12% in cells exposed to native T2 cells or HLA mono-specific cells coated with inappropriate HLA-DR1/flu complexes, respectively.



### 3.3. FACS-based killing assay

The results of a FACS-based cytotoxicity assay using an HLA-A2/Melan-A specific T cell line are shown in Fig. 6. Here target cells were stained with the fluorescent dye carboxy fluorescein succinimidyl ester (CFSE), and an antibody for the B cell antigen CD19. This enabled target cells to be easily gated on for analysis using flow cytometry. For analysis, CD19+ cells were gated, and a CFSE (FL1 fluorescence) histogram then created for each sample, as shown in Fig. 6B. Here, all live target cells can be classified as CFSE<sup>high</sup>, shown in Fig. 6B as the gated population, cell lysis is characterised by loss of CFSE fluorescence. Cell lysis is then calculated using the formula given in the methods.

At an E:T ratio of 10:1, the degree of cell lysis of the HLA-A2/Melan-A mono-specific T2 cell targets is 21%, which at an E:T of 5:1 decreases to 14%. Comparatively, using HLA-A2/pp65 peptide pulsed T2's at an E:T ratio of 10:1 there is lysis of 51% of the target cells, decreasing to 41% at 5:1. As a control, T cells in the presence of either native T2 cells, or T2 cells coated with irrelevant HLA-A24/pp65 monomer, show background lysis of less than 5%.

## 4. Discussion

The accurate measurement of T cell numbers and activity is central to the development of immunotherapy, and vaccination in the treatment of malignancy and complex infectious diseases. To date, these efforts have been hampered by the lack of reproducible assays with low background activity, particularly when testing for T cells recognizing non-HLA-A2 alleles.

We have previously demonstrated that recombinant HLA complexes targeted by an antibody delivery system to cell surfaces remain functional and that this technology could lead to developments in tumour targeting and therapeutic vaccination (Savage et al., 2002; Stebbing et al., 2004). In this current study we have developed the technology to produce an *in vitro* assay system that provides flexible T cell functional analysis, including ELISPOT, ICS or killing assays, for T cells recognizing any designated recombinant HLA class I or HLA class II/peptide complex.

In this new assay system, HLA class I or class II mono-specific APCs/target cells are produced by immobilizing HLA class I or HLA class II/peptide complexes on the surface of cells that lack any functional native HLA complexes (Fig. 1). This produces cells of a single designated immunological specificity (a single HLA/peptide complex presented on the surface), which can have the added potential to fine tune the epitope density as required, by altering the concentration of HLA/peptide complex used to coat the cell. Using this system an unlimited number of highly reproducible cells that produce low background, and have comparable characteristics between differing HLA alleles can be produced.

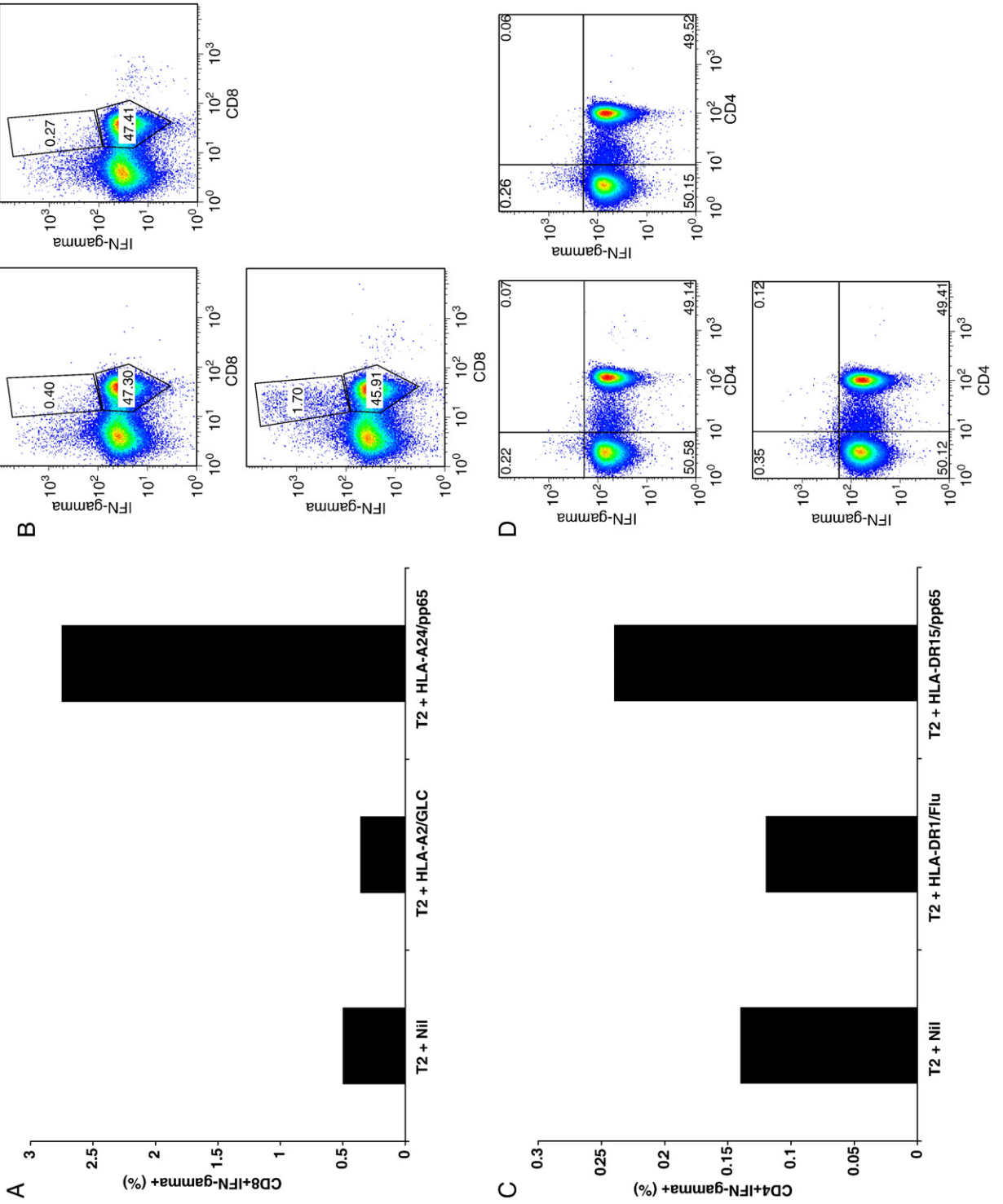
We have previously demonstrated this in combination with the Daudi cell line; a human Burkitts lymphoma cell line that is HLA class I negative but HLA class II positive, it is also EBV positive (Barber et al., 2006). This cell line worked with the described system, and was used successfully in intracellular staining assays and chromium release assays using expanded PBMCs from HLA-A2 positive donors as effector cells. However, Daudi cells could not be used successfully for HLA class II analysis, and in addition showed significantly high non-specific background in ELISPOT assays. Here, the T2 cell line has been used, this is a T cell/B cell hybrid cell line which is functionally HLA class I and II negative and expresses CD20 on the cell surface.

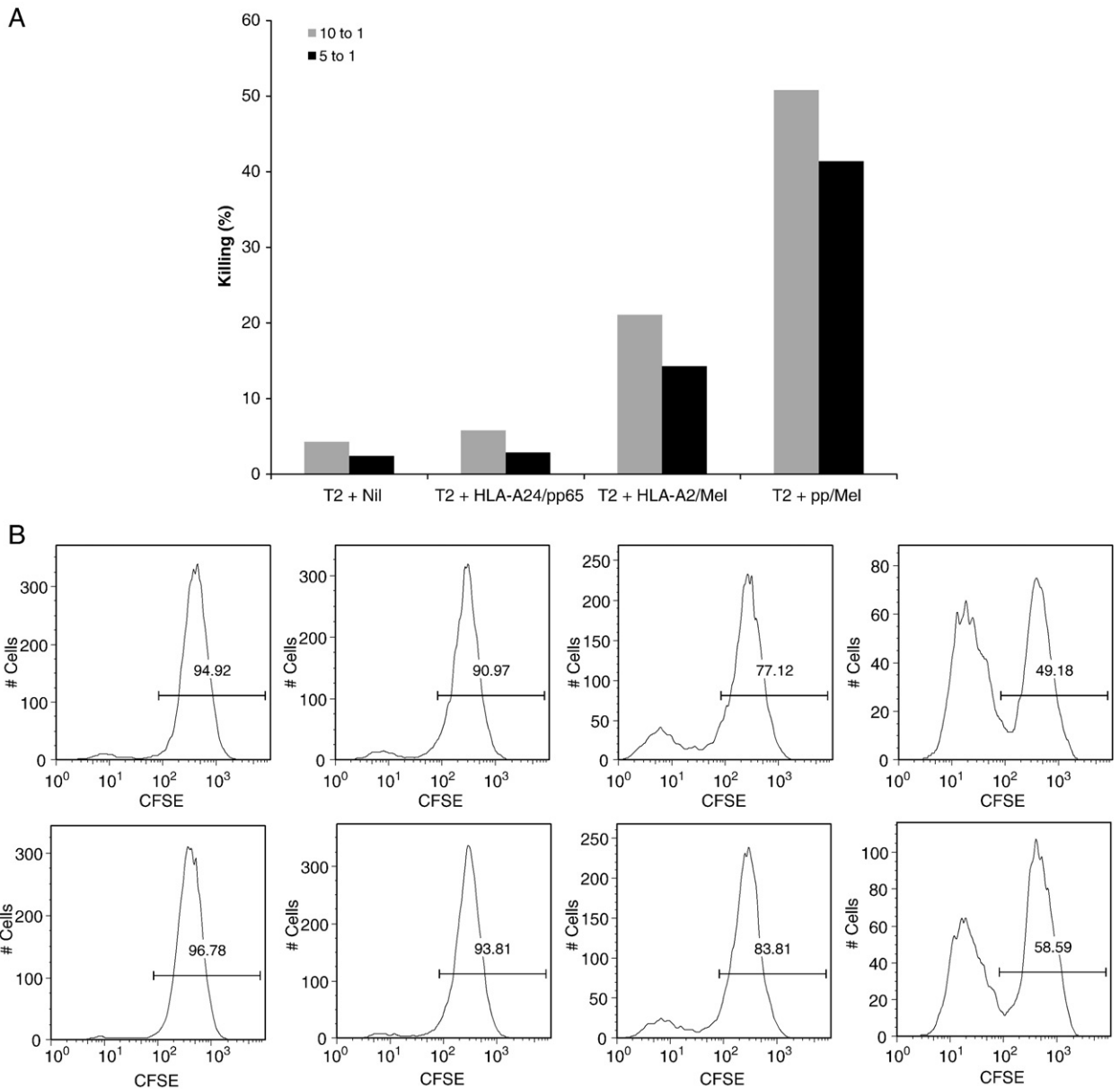
A comparison of the performance of peptide pulsed T2 cells and HLA/peptide coated T2 mono-specific cells for the ELISPOT detection of HLA-A2/Tel and HLA-A2/Melan-A specific T cells demonstrates similar high degrees of specificity, but a slightly reduced signal produced by HLA mono-specific targeted T2 cells. This tendency towards a lower signal has previously been noted in other parallel studies, and is likely to relate to the slight degree of elevation of the targeted HLA/peptide complex from the surface of the T2 cell, as compared to physiologically expressed peptide (Mous et al., 2008).

Whilst there are already high quality functional assays for HLA-A2 T cells readily available, those for non-HLA-A2 T cells are of significantly lower utility and quality. Using HLA mono-specific APCs, highly specific functional analysis for other HLA class I/peptide combinations can be simply performed. In Figs. 3A + B, and, 5A + B, the results of ELISPOT and ICS assays using PBMCs from an HLA-A24 positive, CMV sero-positive donor are shown. These assays show high levels of specificity using T2 cells coated with HLA-A24/pp65 complexes, without any significant background T cell activation using the T2 cells coated with irrelevant HLA-A2/peptide monomers.

The ability of the system to be used for functional analysis of HLA class II/peptide specific CD4+ T cells, is shown in Fig. 3C + D. Here, tetramer and ELISPOT analysis demonstrates that although the number of HLA-DR15/pp65 specific CD4+ T cells is low (1.7% of CD4 cells, Fig. 3D), the ability to detect the functional response of these cells in an interferon-gamma ELISPOT was highly specific, with 96 spot forming units per 250,000 PBMCs with the HLA mono-specific cells coated with HLA-DR15/pp65, compared to only 13 with native T2 cells. In the HLA class II ICS analysis shown in Fig. 5C the level of tetramer positivity of the HLA-DR15/pp65 CD4 T cells was even lower at 0.8% of CD4+ cells (compared with 1.7% in Fig. 3D), however the ICS assay is still able to demonstrate the functional activity of the cells within the PBMC population, with 0.25% of CD4+ cells staining positively for IFN- $\gamma$  activity when co-cultured with HLA mono-specific T2 cells, compared to the background of 0.12% with native T2 cells.

**Fig. 4.** Intracellular staining for HLA-A2+ cells. Intracellular staining (ICS) of a T cell line specific for HLA-A2/Mel was carried out (A + B). Here, T cells were co-cultured with T2s as labeled. Data is presented in a bar graph (A), with the corresponding flow cytometry data shown in B. In B, the top left hand graph shows T2 + Nil, top right hand is T2 + HLA-A2/WT1, bottom left is T2 + HLA-A2/Mel and bottom right is T2 + pp/Mel. In C + D, ICS of *ex vivo* PBMCs from an HLA-A2 positive HLA-A24 negative CMV sero-positive donor is shown. PBMCs were co-cultured with T2s as labeled. Data is representative of three separate experiments using HLA-A2 positive donors.





**Fig. 6.** Flow cytometry-based cytotoxicity assay using HLA mono-specific cells as targets. A flow cytometry (FACS)-based cytotoxicity assay was carried out with a T cell line specific for HLA-A2/Mel. Target cells were either native T2s or T2s coated with HLA/peptide monomers as labeled. In A, the percentage killing of the target cells is plotted for T cells co-cultured for 4 h in the presence of T2s. In B, the corresponding flow cytometry data is shown. Here, live target cells are characterised by their CFSE staining characteristics (CFSE<sup>high</sup> gated cells shown), whilst reduction in CFSE fluorescence represents cell lysis. Data is representative of four separate experiments.

The ability of a T cell to produce the lysis of a target cell is a key attribute, and also one of the most difficult to measure accurately. Current assays have a number of potential adverse issues, these include their relationship to potential *in vivo* lytic ability, the use of radioactivity, poor reproducibility, and for non-HLA-A2 targets, the absence of suitable surrogate target cells. The introduction of FACS-based killing assays has

helped improve some of these issues, and combined with the use of HLA mono-specific cells, may further enhance the practicality of this vital assessment of T cell function (Sheehy et al., 2001; Kim et al., 2007). In Fig. 6, the results of a FACS-based functional analysis of a T cell line specific for HLA-A2/Melan-A were demonstrated. HLA mono-specific cells were able to act as appropriate targets for these, and potentially

**Fig. 5.** Intracellular staining in non-HLA-A2 donors. A + B show ICS carried out using PBMCs from an HLA-A24 positive HLA-A2 negative CMV sero-positive donor. PBMCs were co-cultured with differentially treated T2s as labeled. In C + D, PBMCs from an HLA-DR15 positive HLA-DR1 negative CMV sero-positive donor are shown. PBMCs were co-cultured with native and monomer coated T2s as labeled. Data is representative of three experiments carried out with HLA-A24 or HLA-DR15 positive donors.

any other designated target, showing a sensitive and specific response. This assay is very simple and only requires an antibody specific to the target cells (here using CD19) in combination with CFSE. Although using the HLA monospecific cells produced a reduced level of lysis compared to conventional peptide pulsed T2 cells, there was a very low level of non-specific background lysis shown with the negative control samples, using either native, or irrelevant HLA monomer coated T2 cells.

With the combination of high specificity, reproducibility and ease of use, the HLA mono-specific cells may help provide the technology to accurately assess the immune response across the full range of the immune response in patients of all HLA class I and II allotypes. This technology employs only a single base cell across all HLA allotypes that should ease laboratory workloads, and provide a degree of comparability for testing across the width of the T cell immune response.

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