

**PROJECT 1: AN INVESTIGATION OF FcRL4 POSITIVE B
CELLS IN THE RHEUMATOID SYNOVIUM**

**PROJECT 2: THE DEVELOPMENT OF A CULTURED ELISPOT
ASSAY FOR THE DETECTION OF LOW FREQUENCY T CELL
RESPONSES.**

By

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The Development of a Cultured ELISPOT Assay for the Detection of Low Frequency T cell Responses

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide, and HPV-16 is found in ~60% of cases. The oncogenic nature of HPV is primarily attributed to proteins E6 and E7 which are up regulated in infected host cells. T cells have been detected in the tumour of patients and correlate with improved prognosis. As a step towards the analysis of HPV-specific T cell responses in tumours of HNSCC patients we have worked towards optimising a cultured IFN- γ ELISPOT assay using Epstein Barr virus (EBV) peptide CLG as a model. Optimal culture conditions and blocking reagents for use in the IFN- γ ELISPOT assay were investigated. We found lowest backgrounds when cells were cultured in serum-free AIM-V medium, and ELISPOT plates are blocked with 10% albumin from bovine serum (BSA) or 10% autologous plasma. We have demonstrated that culturing PBMCs with peptide prior to performing the ELISPOT assay significantly amplifies the antigen-specific T cell response detected compared to standard ELISPOT assays performed overnight on PBMCs *ex vivo*. Future work must now focus on the translation of this optimised cultured ELISPOT assay for the detection of HPV-specific T cells in the tumours and peripheral blood of patients with HPV⁺ HNSCC.

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Introduction

Human papillomaviruses (HPV) in head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide, with an estimated 500,000 new cases each year[42]. A number of genetic influences have been linked to the development of HNSCC and major environmental risk factors include smoking and alcohol consumption [43]. HPV is the most common sexually transmitted disease and infection with this virus has been causally linked to invasive cervical carcinoma [44, 45]. More recently, infection with high risk HPV-subtypes, and in particular HPV-16 and HPV-18, have been associated with HNSCC independent of other risk factors [44, 46, 47]. HPV-16 is estimated to be found in ~60% of oropharyngeal SCCs[48, 49]. HPV-positive HNSCCs are considered to be a distinct subset of HNSCC with improved prognosis and better response to treatment.[50] Evidence that higher viral loads in tonsil cancer correlates to better disease outcomes, suggests that the host is able to mount an active immune response to HPV, however this has not been thoroughly investigated.[51]

HPV-16 preferentially invades cells in epithelial surfaces and integrates its genome into the host nucleus [48]. The oncogenic nature of high risk HPV is primarily attributed to proteins E6 and E7 which are up regulated in infected host cells[52]. E6 inhibits and degrades tumour suppressor p53 in infected cells, while E7 binds to retinoblastoma suppressor pRb and causes the cell to enter the S-phase of the cell cycle [53]. Together these oncogenic proteins induce transformation and immortalisation of infected cells[54, 55].

Since the successful development of a vaccine for HPV-induced invasive cervical carcinoma, attention has turned to the investigation of immune responses to HPV in HNSCC. It has been shown that HPV oncogenic proteins E6 and E7 are preferentially and exclusively expressed in HPV infected tumour cells and HPV specific antibodies have been described in oral rinses and sera from HNSCC patients[56] [57]. T cells have been identified infiltrating tumours, and higher titres have been associated with improved prognosis although these responses are not well examined [58, 59]. The identification of active immune responses in HNSCC patients highlights the potential for the development of novel prophylactic vaccines, and suggests that there may be scope for the creation of immunotherapies for the treatment of this prevalent cancer.

Detection of T cell responses – the ELISPOT assay

Several methods for the detection of T cell responses are commonly used each method with its advantages and pitfalls. MHC-tetramer staining is a powerful tool for the identification of antigen specific T cells in a mixed population and works by binding of the T cell receptor (TCR) to known peptide-MHC complexes[60]. This method involves the production of multimeric MHC-peptide complexes labelled with streptavidin-biotin. Using four MHC molecules coupled together increases TCR-MHC binding avidity and therefore stable binding of the tetrameric structure to the cell surface and detection by flow cytometry methods[61]. However, tetramers alone cannot indicate the functional nature of the identified T cells, but must be used in conjunction with other flow cytometry protocols. Furthermore, developing new MHC tetramers is very expensive and most importantly requires prior knowledge of the target peptide antigen. Although peptide-specific responses have been described in cervical cancer, the epitopes of E6 and E7 which may be significant in HNSCC have not been thoroughly investigated[62]. For the purpose of this investigation, peptide pools spanning the full lengths of E6 and E7 will be used to ensure novel responses are not overlooked. MHC tetramer staining is therefore not a suitable method for use in this project [63].

Multi-parameter flow cytometry is a valuable method for revealing the cytokine profile and functional characteristics of antigen specific T cells by the simultaneous staining for intracellular cytokines and membrane bound cellular markers using fluorescently labelled monoclonal antibodies [63]. Cells are stimulated with peptide prior to staining to ensure detection of antigen specific responses above the level of spontaneous cytokine production [64]. Flow cytometry is expensive, and while it can be used to quantify intracellular levels of cytokines such as IFN- γ , these levels may not correspond to the quantity of cytokine secreted by the cells. Furthermore cytokine flow cytometry is reported to be less sensitive for detecting low level responses compared to the enzyme-linked immunospot (ELISPOT) assay[65].

ELISPOT assays are relatively inexpensive and enable quantification and functionality of T cells to be assessed simultaneously directly after antigen stimulation. A number of different ELISPOT assays have been developed to assess the production of various cytokines by immune cells, but the IFN- γ ELISPOT is the most commonly used. The ELISPOT assay is a plate 'sandwich' assay during which live cells are cultured overnight with antigenic stimulation. Cytokines produced and secreted by activated cells during the assay are captured by primary cytokine-specific monoclonal antibodies which are used to coat the wells of the ELISPOT plate. A secondary, biotinylated monoclonal antibody is added and spots are developed by incubation with conjugated streptavidin and the addition of a chromogen substrate. [66] Each spot represents a single cell producing the cytokine of interest and spot size correlates to the amount of cytokine produced by that cell. In contrast to MHC tetramer staining, the ELISPOT assay is not restricted to distinct peptides as peptide pools or target cells expressing whole proteins may be used for *in vitro* stimulation of the T cells applied to the assay. ELISPOT assays are regularly used in vaccine trials, but have also been utilized for the investigation of viral immunity and for the diagnosis of bacterial infection [67] [63-65] [68, 69]. IFN- γ ELISPOT assays have been successfully employed for the detection of HPV-specific T cell responses in cervical cancer studies, suggesting they may be suitable for use during

this project [70]. Cultured ELISPOT assays, in which PBMCs are cultured for several days prior to the assay, have also been described for detection of low frequency T cell responses.[71-73] Culturing cells with the antigen of interest allows for preferential amplification of peptide specific T cells and subsequent detection of a T cell response which may have been overlooked by standard ELISPOT analysis. Amplification of T cell responses may be maximised by supplementation with IL-2, which causes T cell proliferation [74]. Since HPV-specific T cell responses in HNSCC are expected to be relatively low frequency and the functional capacity of the cells is not well investigated, cultured ELISPOT assays may be the best method for their detection[58, 63, 75].

Numerous protocols for standard and cultured ELISPOT assays have been described and the importance of optimising and harmonising the assay is emphasised in the literature [67, 76]. Small protocol changes have been reported to dramatically affect ELISPOT sensitivity. When considering the development of an assay involving clinical samples it should be noted that delays in processing blood samples may have dramatic effects on the sensitivity of the standard ELISPOT assay [77]. The culture medium used during the assay is an important variable that is often overlooked. RPMI 1640 supplemented with whole serum is frequently used for cell culture. However, some recommend serum-free medium such as AIM-V for cultured ELISPOT assays as it avoids the introduction of foreign antigens which may be present in fetal bovine serum or human serum and which may induce T cell activation[67, 78]. It is clear that when designing an ELISPOT protocol (*ex vivo* or cultured), variables at every stage of the assay should be carefully considered, most notably; culture medium, length of culture, cell numbers applied per well and plate preparation and blocking reagents [67].

Aims

1. To optimise the cultured ELISPOT assay for the detection of low frequency T cell responses to defined EBV peptide epitopes.
2. To compare the magnitude of antigen specific responses detected using *ex vivo* and cultured ELISPOT assays.
3. To investigate HPV-specific T cell responses in the tumours and peripheral blood of patients with HNSCC.

Materials and Methods

Epstein Barr virus as a model for HPV responses

Patients with HNSCC are expected to have low frequency T cell responses to HPV-peptides. For the optimisation of the ELISPOT assays healthy donors known to have weak responses to the EBV peptide CLG were selected and tested[79]. CLG and IED are HLA A2- and B40- restricted epitopes respectively from the latent membrane protein 2 (LMP2) of EBV.

Healthy donors and cone cells

In accordance with ethical approval and following informed written consent, blood samples were taken from 5 healthy donors (3 men and 2 women) known to be HLA-A2 positive, from the Cancer Sciences faculty of the University of Birmingham. These samples were used for the detection of weak T cell responses to EBV peptide CLG.

Frozen aliquots of peripheral blood mononuclear cells (PBMC) derived from leukoreduction system chambers obtained from NBS platelet donors (cone cells) from 8 donors were selected at random and used for the detection of T cell responses to HPV-16 proteins E6 and E7. Donor age and gender of these samples were unknown.

Isolation of PBMC

Heparin was added to the syringe prior to blood collection to avoid clotting. The blood sample was diluted in an equal volume of RPMI 1640. 30ml diluted blood was then carefully layered on to 15ml Lymphoprep (Axis-Shield, Norway). The tubes were then centrifuged at 800g for 30 minutes. The plasma layer was carefully removed and stored for use in subsequent experiments. PBMCs were aspirated from the layer at the interface between the blood and the Lymphoprep. The isolated PBMCs were washed 3 times RPMI 1640: at 700g for 10 minutes, at 600g for 5 minutes and at 400g for 5 minutes. Finally, the PBMCs were counted using a haemocytometer.

PBMCs not being immediately used in ELISPOT assays were stored in freezing medium (10% Dimethyl sulfoxide (DMSO) and 90% FCS) at -80°C for up to 3 days and if still unused, were transferred to -180°C until ELISPOT analyses could be carried out.

Peptides

EBV peptides CLG and IED (Alta Bioscience) stock was kept at -20°C at 14mg/ml and was diluted to 40 $\mu\text{g}/\text{ml}$ for use in the ELISPOT assays. Pepmixes contained peptide fragments of 15 amino acids which overlapped by 11 amino acids and covered the full length of the proteins HPV-16 E6 (37 peptides) and E7 (22 peptides) and human actin (92 peptides) (JPT Peptide Technologies, Germany). The pepmixes were diluted in DMSO to a final concentration for individual peptides of 0.2 $\mu\text{g}/\mu\text{l}$ and stored at -20°C in 10 μl aliquots to avoid multiple freeze thawing.

ELISPOT assays

Where possible, PBMCs from healthy donors were used on the day of isolation. However, if this was not possible the PBMCs were stored at -80 C until use. Frozen healthy donor PBMCs and cone cells were thawed, washed with RPMI 1640 and rested overnight in RPMI 1640 with 10% fetal bovine serum (FBS), 1% GPS (penicillin, streptomycin and L-glutamine) in a 37°C incubator before use. 'Ex vivo' and cultured ELISPOT assays were carried out in parallel.

PBMCs from healthy donors were tested for T cell responses to EBV peptide CLG. First, suitable culture medium and culture conditions were investigated. 3×10^6 PBMCs were cultured for 6 days (on the advice of a faculty member at the Institute of Cancer Sciences) in a 24 well tissue culture plate in 2ml of AIM-V, Opti-Mem, RPMI 1640 +10% FBS or RPMI + 10% autologous plasma. 10µg CLG was added to each culture. The cells were cultured with or without 20 IU/ml IL-2. Half of the cells had the IL-2 washed off on day 5 and were rested overnight in their respective culture medium.

Cone cell PBMCs were tested for T cell responses to HPV-16 proteins E6 and E7. 1.5×10^6 cells were cultured for 6 days in a 48 well tissue culture plate. On day 1, 0.2µg E6 and 0.2 µg E7 were added to the cells in 0.5ml AIM-v medium. On day 2 a further 0.5ml AIM-V was added and the cells were incubated at 37 C until use. On day 6 cultured cells were washed 3 times in RPMI 1640 the pellet was resuspended in the AIM-V at a concentration of 1×10^6 cells/ml for the ELISPOT assay.

ELISPOT kit for human IFN-γ (Mabtech, Sweden) was used. First, MultiScreen 96- well plates (Millipore) were prewetted with 70% ethanol and washed twice with sterile filtered PBS. 50µl of the IFN-γ capture antibody was added to the plates at 7.5µg/ml and the plates were left at room

temperature for 4 hours. The plates were then washed 4 times with RPMI 1640 and blocked for 1 hour at room temperature with 100µl of 10% bovine serum albumin (BSA) (Sigma-Aldrich), 10%FBS or 10% autologous plasma. For ELISPOT assays detecting T cell responses to EBV peptide CLG, 1×10^5 PBMCs were added to 3 replicate wells along with 4µg CLG (dissolved in DMSO), an equivalent concentration of DMSO solvent (negative control) or 8µg PHA (positive control) , to a total volume of 100µl/well. For ELISPOT assays detecting T cell responses to HPV peptides, 1×10^5 PBMCs were added to 3 replicate wells along with 0.1µg E6 + 0.1µg E7, 8µg PHA (positive control) or an equivalent dilution of DMSO (negative control) or 0.1µg human actin (negative control) to a total volume of 110µl/well.

The ELISPOTs were incubated for 18 hours at 37 C and then washed 4 times with 0.05% Tween 20 solution in PBS. 50µl of the detection antibody was then added at 1µg/ml and the plates were incubated at room temperature for 3 hours. The plates were again washed 4 times with 0.05% Tween 20 solution in PBS. 50µl Streptavidin-Alkaline Phosphatase was added at 1µg/ml and the plate was incubated at room temperature for 1.5 hours. The wells and the undersides of the plates were washed 8 times with 0.05% Tween 20 solution in PBS, and 4 times with PBS and the chromogen substrate was added at 100µl/well. After 1 hour the plates were rinsed several times under the tap and left overnight to dry. Spots were counted using an ELISPOT reader system (Autoimmun Diagnostika GMBH). All reader settings were kept consistent throughout. Brightness was set at 128, sharpness at 184, white balance at 135, gamma at 128, exposure at 2384, and gain at 1, hue at 139 and saturation at 145.

Analysis of results

Results were standardised to spot forming cells (SFCs) per 10^6 cells. The mean and standard error of the triplicate wells was calculated using GraphPad Prism and presented on graphs. In order to

assess the significance of peptide specific T cell responses detected, one tailed paired T-tests were carried out. $p < 0.05$ was considered to be statistically significant[80].

Results

IFN- γ ELISPOT assays are useful for assessing the frequency and functionality of antigen specific T cells in a sample. T cells specific for oncogenic HPV proteins E6 and E7 are expected to be at low frequency in the tumour and peripheral blood of patients, and it was therefore important to optimise an assay for the detection of these rare cells[59, 63, 75, 81]. For the purpose of optimisation, T cell responses of healthy HLA-A2 donors to EBV peptide CLG were investigated. This project focused on the optimisation of a cultured ELISPOT assay which would allow for detection of low frequency T cell responses which may not be detectable by standard ELISOPT assay.

Standard ELISPOT assays can detect T cell responses to EBV peptide IED

In order to demonstrate that a IFN- γ ELISPOT assay could be effectively utilised for the detection and quantification of T cell responses, a standard ELISPOT assay was carried out on PBMCs tested immediately ex vivo. Blood was taken from a healthy donor with known responses to the EBV peptide IED, and an ELIPSPOT assay was carried out following the manufacturer's instructions. During this investigation the plates were blocked with 10%FBS in RPMI 1640, and 1×10^5 cells were added to each well. The assay was carried out twice on different days, with cells tested in triplicate wells to determine the reproducibility of the assay.

The well containing cells stimulated with PHA (positive control) well was fully saturated with spots which indicate that the cells used during this assay were healthy and functional. These wells could not be accurately read by the ELISPOT plate reader as there are so many spots that they overlapped and covered the entire well(#).The negative control wells, in which cells were stimulated with DMSO alone, showed no IFN- γ production which was indicated by a lack of spots

in these wells (figure 1c). Via paired T-test, the antigen specific response was shown to be statistically significant compared to the negative control in both repeats 1 and repeat 2. $p=0.003$ and 0.0007 respectively (figure 1a, 1b). Spot numbers were consistent both within internal replicates and also between the two experiments carried out on the same donor on different days. The negative control wells (DMSO) appeared clean and clear spots were visible in response to the IED peptide (figure 1c).

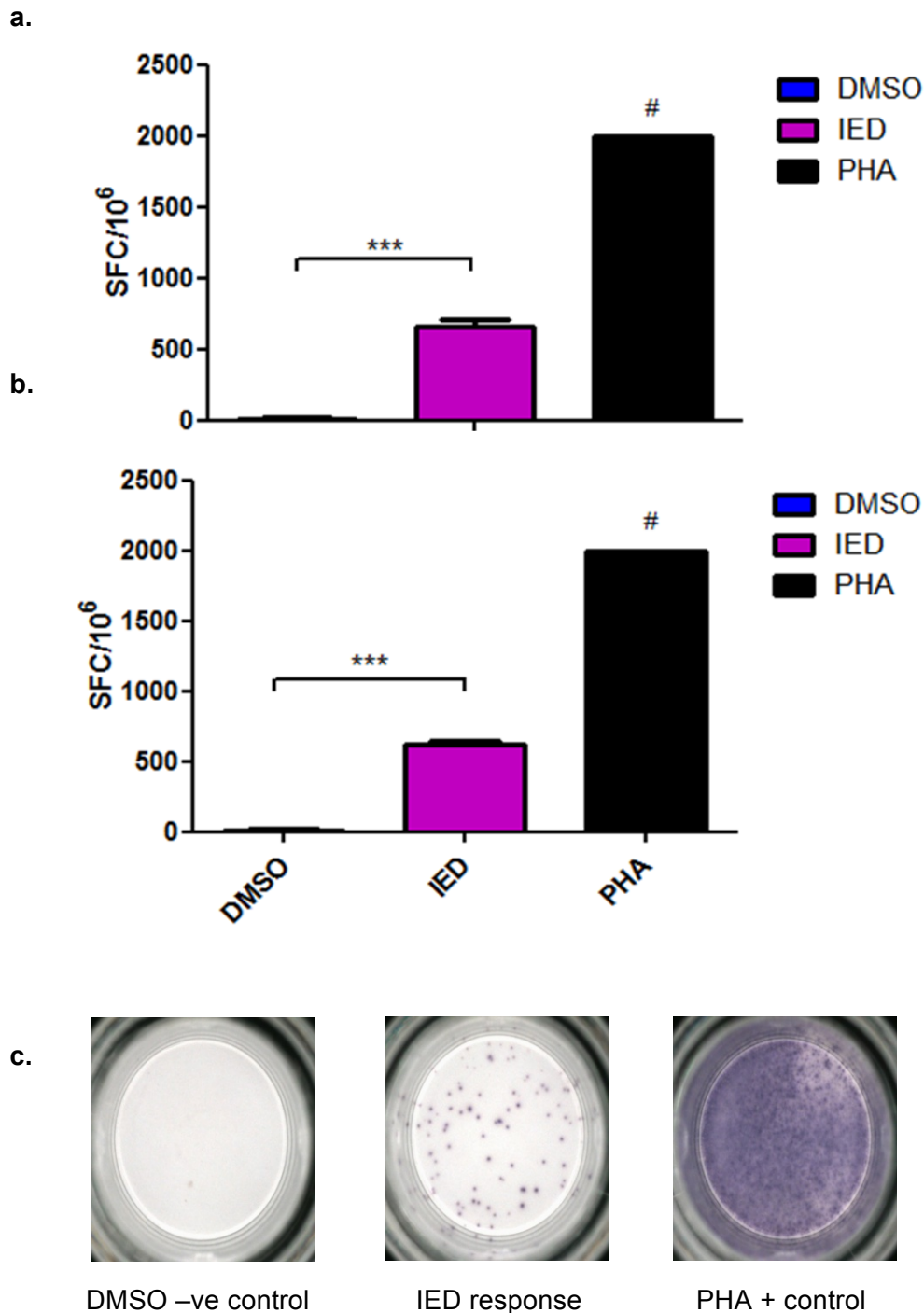


Figure 1. Standard ELISPOT assay carried out on PBMCs isolated from a healthy donor with a known response to EBV peptide IED. Plates were blocked with 10%FBS in RPMI 1640, and 1×10^5 cells were added per well. Antigen specific responses to IED are shown, as well as negative and positive controls, in which cells were tested against DMSO and PHA respectively. # indicates the PHA well was fully saturated and therefore the number of spots cannot be determined accurately. Data shown as SFC/ 10^6 PBMCs (+standard error of the mean). Paired T-test was performed *** $p < 0.005$ (a) Graph to show mean spot forming cells (SFC) per million repeat 1. (b) Graph to show mean spot forming cells (SFC) per million repeat 2 (c) Representative images of control and test wells.

Blocking with BSA results in a speckled appearance of the wells.

This project focused on the development of a cultured ELISPOT for the amplification and detection of low frequency T cell responses to known antigens. Standard 'ex vivo' and cultured ELISPOT assays were carried out in parallel on PBMCs isolated from 2 HLA-A2 donors so that the effect of culturing the cells could be seen by direct comparison. In an attempt to standardise the assay and identify a suitable media to culture the cells in, PBMCs were cultured in AIM-V, Opti-Mem, 10%FBS in RPMI 1640 or 10%autologous plasma in RPMI 1640 medium (3×10^6 cells in 2ml) for 6 days in the presence of CLG and were subsequently applied to a standard ELISPOT assay. Following the advice from a faculty member at the Institute of Cancer Sciences at the Birmingham University, 10% BSA in AIM-V was used to block the plates as this had been shown to produce consistent results in standard ex vivo ELISPOT assays in a clinical trial[82].

Background on the cultured ELISPOT plates was so high it was not possible to draw conclusions from the data obtained (figure 2a,2b). When the wells were inspected visually, a speckled appearance was observed (figure 2d). The same speckled appearance of the wells was also seen in plates from the 'ex vivo' ELISPOT assay which had been conducted in parallel using the same blood sample (figure 2c). No antigen specific responses could be detected above background in either assay blocked with BSA. Clearly it was not possible to draw firm conclusions on optimal culture conditions from this investigation, but this batch of BSA was disregarded as a potential blocking reagent for subsequent investigations.

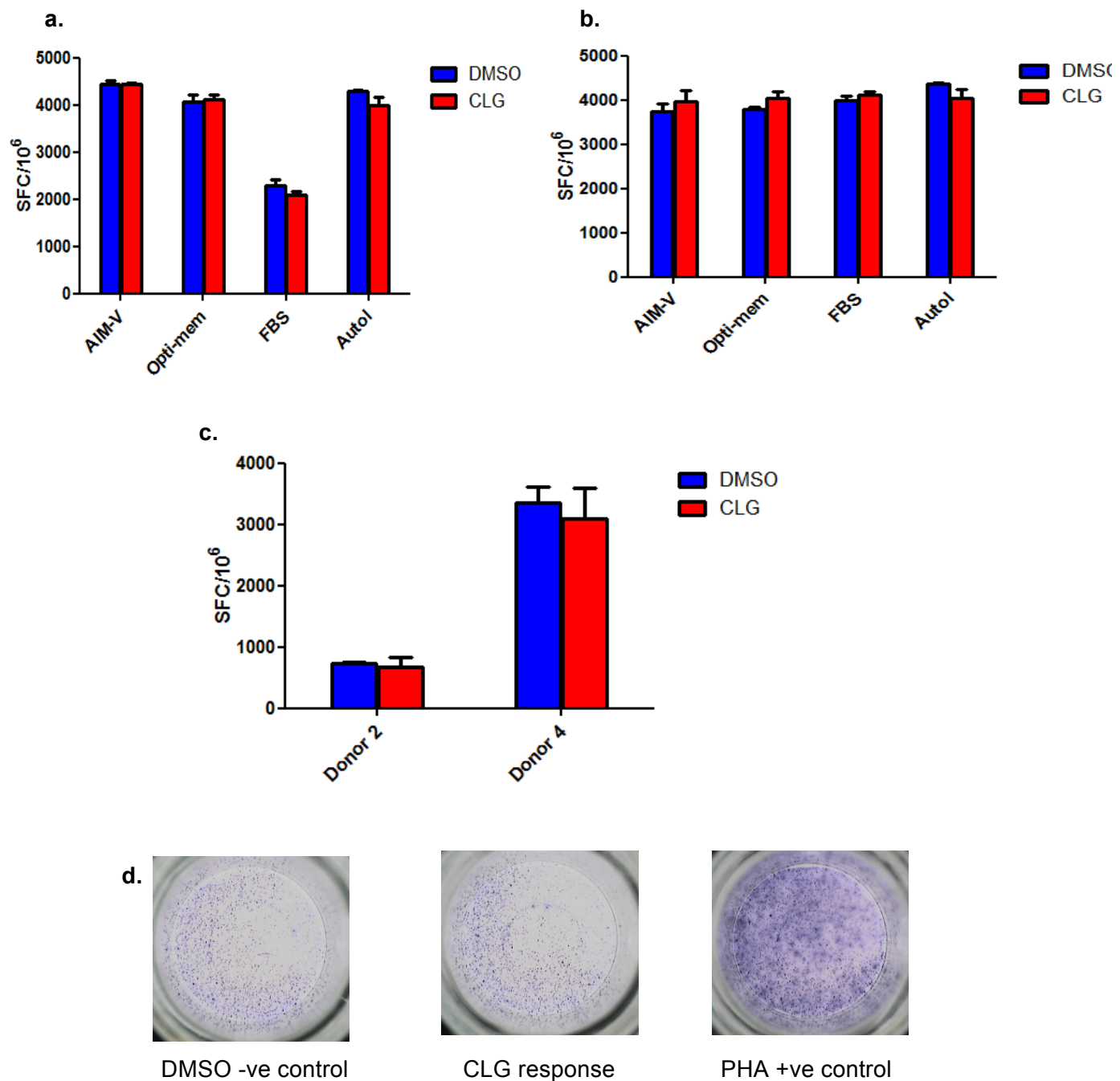


Figure 2. Cultured and 'ex vivo' ELISPOT assays carried out on PBMCs isolated from 2 HLA-A2 positive healthy donors. For cultured assays, PBMCs were cultured for 6 days with CLG peptide in AIM-V, Opti-mem, 10%FBS in RPMI 1640 (FBS) or 10% autologous plasma in RPMI 1640 (autol). Plates were blocked with 10% BSA in AIM-V medium. Antigen specific responses to CLG peptide, as well as negative controls in which cells were supplemented with DMSO alone are shown. PHA positive control wells were fully saturated with spots in all cases (data not shown) Data shown as SFC/ 10⁶ PBMCs (+standard error of the mean). Paired T-test was performed **p*<0.05 **(a)** Donor 2 cultured ELISPOT. **(b)** Donor 4 cultured ELISPOT **(c)** Donors 2 and 4 'ex vivo' ELISPOT assays **(d)** Representative images of wells from cultured ELISPOT assay.

Blocking with autologous plasma reduces background

Due to high backgrounds resulting from blocking the ELISPOT plates with BSA, the blocking reagent was changed to 10% autologous plasma in RPMI 1640. Autologous plasma may be a good alternative to FBS because it is less likely to contain foreign antigens that could lead to high backgrounds. Standard 'ex vivo' and cultured ELISPOT assays were again carried out in parallel on PBMCs isolated from 2 HLA-A2 donors so that the effect of culturing the cells could be seen by direct comparison. In an attempt to identify a suitable media to culture the cells in, PBMCs were once more cultured in AIM-V, Opti-Mem, 10%FBS in RPMI 1640 or 10%autologous plasma in RPMI 1640 medium (3×10^6 cells in 2ml) for 6 days in the presence of CLG and were subsequently applied to a standard ELISPOT assay.

The plates from both the 'ex vivo' and cultured ELISPOT assays appeared much cleaner than the plates in the previous experiment which had been blocked with BSA, and background readings were reduced (figure 3a-e). In cultured ELISPOT assays very little difference was seen between the negative control and the antigen specific response. However, a statistically significant difference was seen between antigen specific and background when PBMCs from donor 5 were cultured in 10%FBS in RPMI 1640 and the plate was blocked with 10%autologous plasma ($p=0.027$) (figure 3 a, 3b). A smaller CLG specific response was also detected when PBMCs from the same donor were cultured in RPMI supplemented with 10% autologous plasma. Background in the 'ex vivo' assays was low and antigen specific responses could be detected. Donor 5 showed a statistically significant antigen specific T cell response compared to the negative control ($p=0.027$). results suggest that culturing in RPMI supplemented with 10% FBS may be optimal based on results from 1 donor but tends to give high backgrounds in cultured ELISPOT assays, and therefore some antigen specific response may not be detected above background levels. Donor 3 is expected to respond to CLG, however a significant response is not seen ex vivo and culturing

does not amplify it. Donor 5 has a significant CLG response *ex vivo* which is increased after culture.

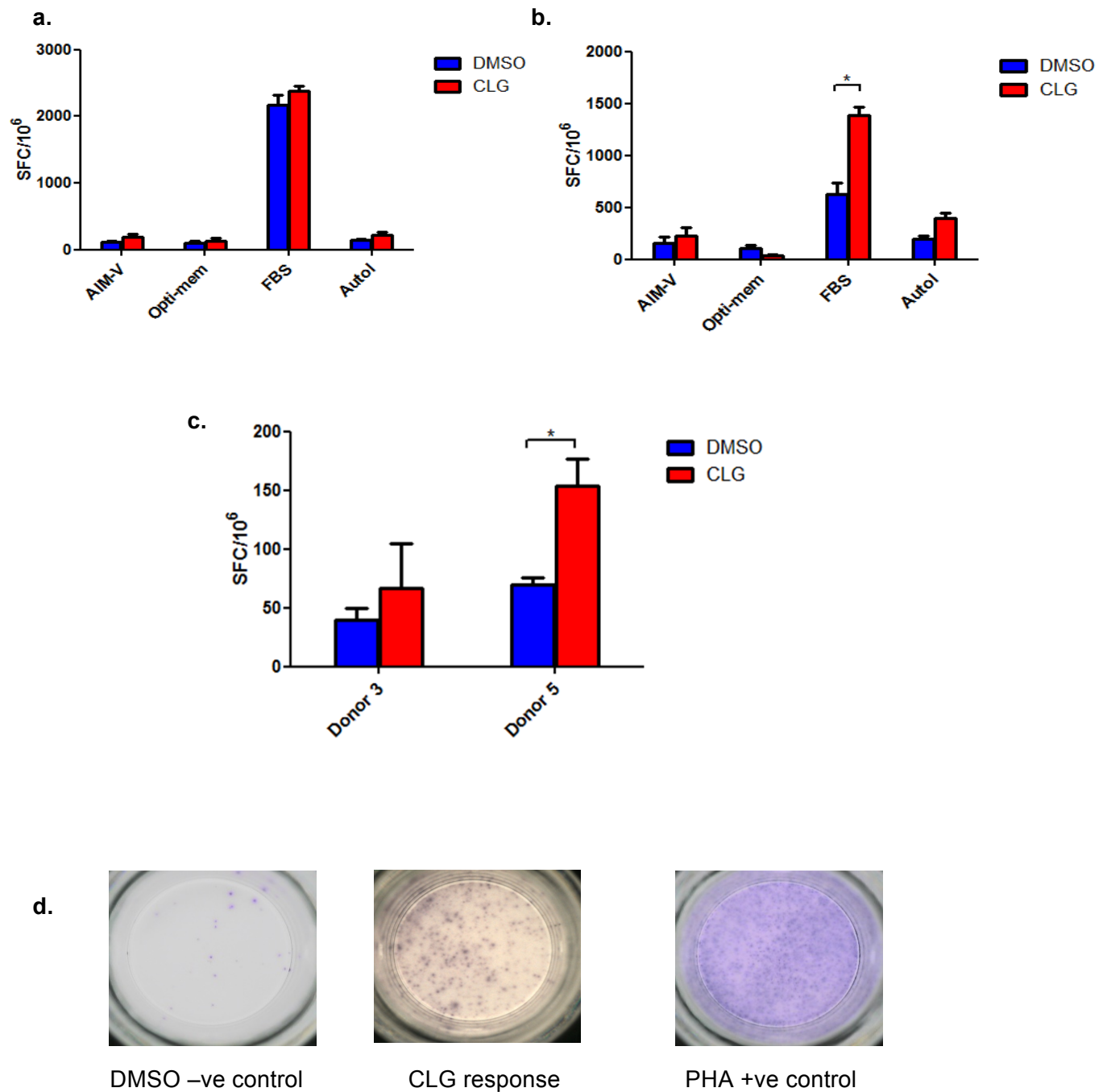


Figure 3. Cultured and 'ex vivo' ELISPOT assays carried out on PBMCs isolated from 2 HLA-A2 positive healthy donors. For cultured assays, PBMCs were cultured for 6 days with CLG peptide in AIM-V, Opti-mem, 10%FBS in RPMI 1640 (FBS) or 10% autologous plasma in RPMI 1640 (autol). Plates were blocked with 10% autologous plasma in RPMI 1640. Antigen specific responses to CLG peptide, as well as negative controls in which cells were supplemented with DMSO alone are shown. All PHA positive control wells were fully saturated with spots (not shown). Data shown as SFC/ 10⁶ PBMCs (+standard error of the mean). Paired T-test was performed *p<0.05 **(a)** Donor 3 cultured ELISPOT. **(b)** Donor 5 cultured ELISPOT **(c)** Donors 3 and 5 'ex vivo' ELISPOT assays **(d)** Representative images of wells from cultured ELISPOT assay.

A significant antigen specific response is seen when cells are cultured in AIM-V.

Due to inconclusive results from previous experiments, and upon further inspection of the literature and advice from colleagues, we decided to focus on culturing cells in serum free AIM-V medium or RPMI 1640 supplemented with 10% autologous plasma. Standard 'ex vivo' and cultured ELISPOT assays were carried out in parallel on PBMCs isolated from 1 HLA-A2 donor. PBMCs were isolated and cultured for 6 days with CLG peptide (3million cells in 2ml). In an attempt to reduce background which may occur if stimulated cells were already producing IFN- γ when they were applied to the ELISPOT assay, half of the cells were washed on day 5 to remove the peptide and left to rest overnight in their appropriate medium. On day 6 the cells were applied to a standard ELISPOT assay at 1×10^5 cells per well. Plates were blocked with 10%FBS in RPMI 1640 or 10% autologous plasma in RPMI 1640. A new batch of BSA was also retested as a blocking agent. PBMCs were also isolated from a second donor and the for the sake of time efficiency, the effects of altering the blocking reagent used were tested by standard 'ex vivo' ELISPOT assay.

When the PBMCs were cultured in autologous plasma overall spot numbers are low, but background levels are relatively high, with no significant CLG-specific response detected with any blocking reagent (figure 4a). When cells were cultured in AIM-V medium, antigen specific responses were clearly visible when using some blocking agents. A statistically significant CLG-specific response was seen in wells blocked with BSA ($p=0.04$) and autologous plasma ($p=0.007$) (figure 4b). When cells which had been cultured in AIM-V were applied to wells blocked with FBS, background was very high and the wells appeared speckled (figure 4c). Washing and resting the cells before conducting the cultured ELISPOT assays did not reduce background relative to the antigen specific response (figure 4a,4b). The new batch of BSA did not elicit the same speckled appearance of the wells and significant antigen specific responses were detected when cells were cultured in AIM-V medium. Due to concerns that autologous plasma may not always be available

and would therefore make an unsuitable blocking reagent, subsequent cultures were set up in AIM-V and plates were blocked with BSA.

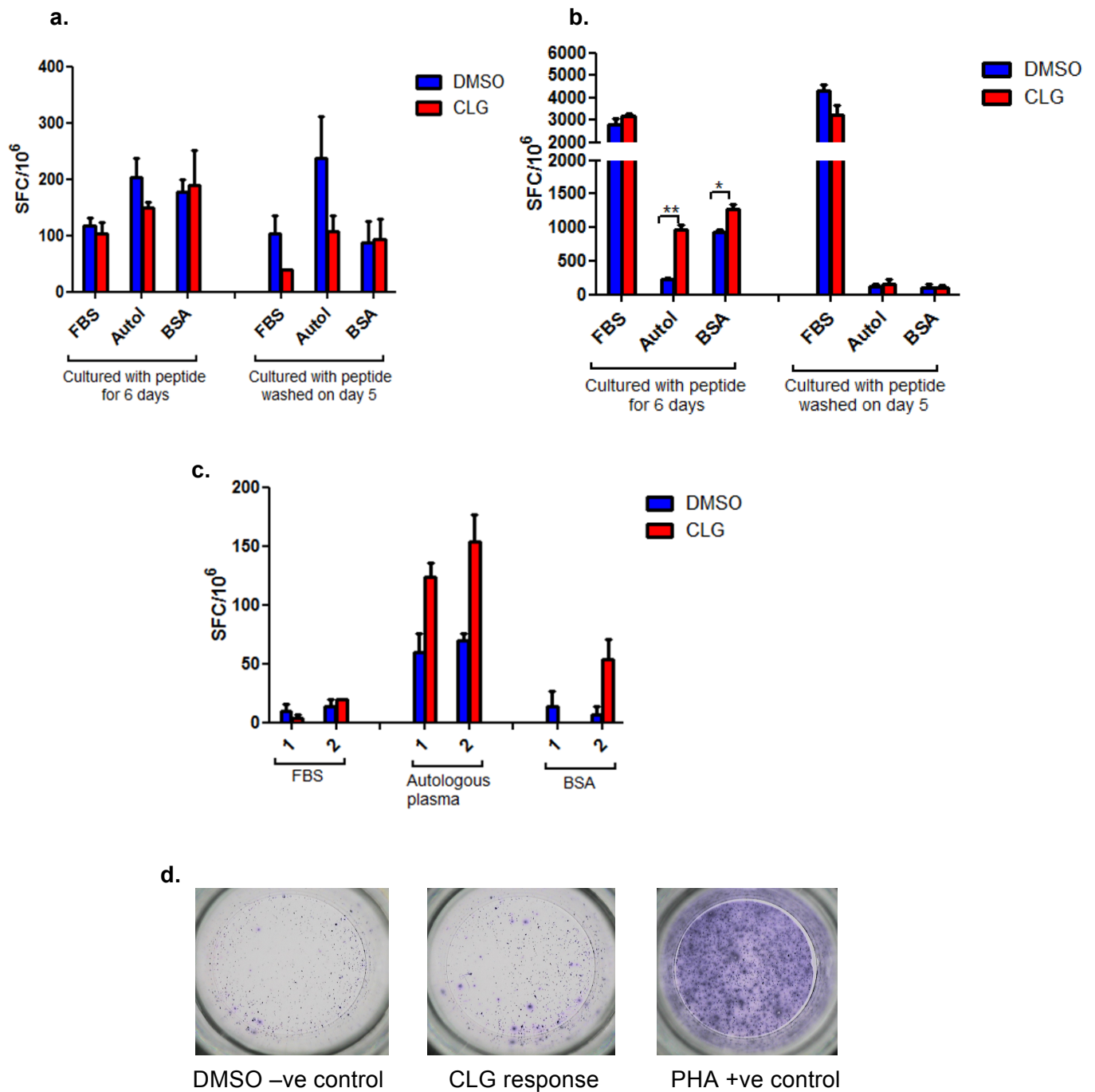


Figure 4. Cultured and 'ex vivo' ELISPOT assays carried out on PBMCs from 1 healthy donor known to have a detectable response to CLG. Plates were blocked with 10%FBS in RPMI 1640, 10% autologous plasma in RPMI 1640 (autol) or 10% BSA in AIM-V. 1×10^5 cells were added per well. Antigen specific responses to CLG peptide, as well as negative controls in which cells were tested with DMSO are shown. PHA positive control wells were fully saturated with spots (not shown) Data shown as SFC/ 10^6 PBMCs (+standard error of the mean). Paired T-test was performed * $p < 0.05$, ** $p < 0.01$ (a) PBMCs cultured in RPMI 1640 supplemented with 10% autologous plasma. (b) PBMCs cultured in AIM-V medium. (c)'ex vivo' ELISPOT assays carried out on two donors tested with each of the 3 blocking reagents (d) Representative images of wells blocked with 10%FBS in RPMI 1640 and cells cultured in AIM-V were added

Culturing PBMCs significantly amplifies the antigen specific response

In order to test whether culturing PBMCs in AIM-V and blocking plates with 10% BSA produced consistently low negative control backgrounds, and to investigate whether culturing the cells with peptide significantly amplified the antigen specific response, 'ex vivo' and cultured ELISPOT assays were carried out in parallel on 5 HLA-A2 positive healthy donors. The mean number of spots in the negative control wells on the ELISPOT assay was subtracted from the corresponding peptide exposed wells. Donors 1 and 3 were tested twice to examine the reproducibility of the data.

Throughout this investigation, the PHA positive control wells were fully saturated with spots. All donors showed an increase in spot number after culture. This increase was statistically significant in assays carried out on PBMCs from donor 3 ($p=0.04$) and donor 5 ($p=0.02$), and approached significance in the assay on PBMCs from donor 4 ($p=0.054$). Repeat assays which tested T cell responses from Donor 1 results closely matched with little variability indicating that the result was reliable. In contrast, donor 3 showed huge variability between repeats. CLG specific response was not amplified during culture for one investigation on PBMCs isolated from donor 3. (figure 5).

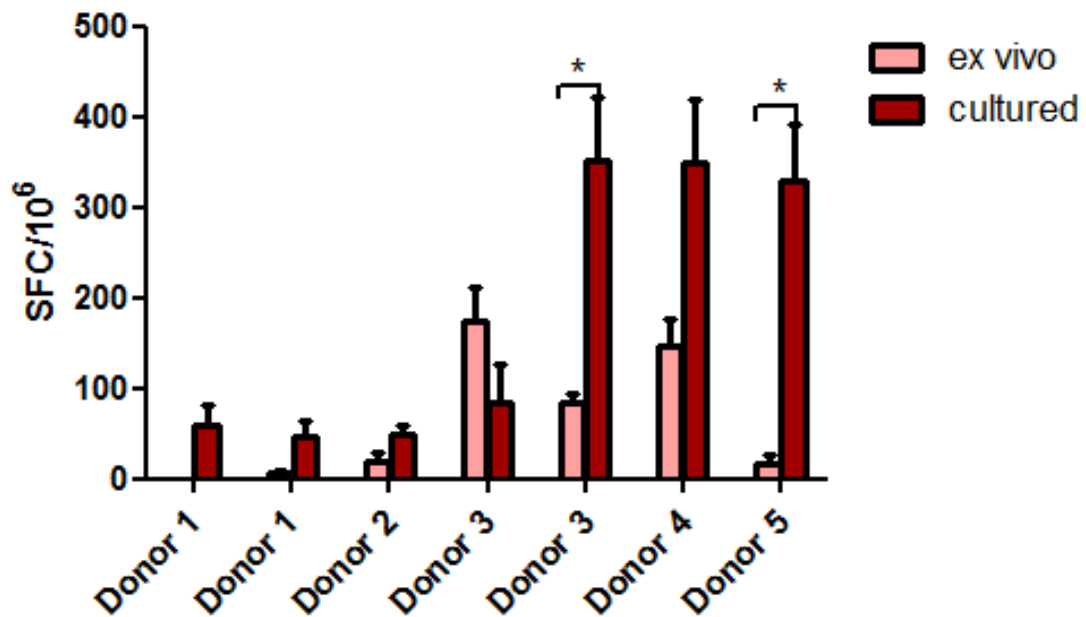


Figure 5. Comparison of standard ‘ex vivo’ and cultured ELISPOT assays carried out in parallel on PBMCs isolated from 5 HLA-A2 positive healthy donors. PBMCs were cultured in AIM-V medium supplemented with CLG. All plates were blocked with 10% BSA and 1×10^5 cells were added per well. In all cases background was low and subtracted from the CLG response results. PHA positive control wells were fully saturated with spots (not shown) Data shown as mean spot forming cells (SFC) per million (+ standard error of the mean). Paired T tests were performed * $p < 0.05$

Addition of IL-2 increases background

In order to test whether adding IL-2 to the culture mix would significantly amplify the antigen specific response, cultured ELISPOT assays were carried out on 2 HLA-A2 positive healthy donors previously seen to have good CLG-specific responses. As previously described, PBMCs were cultured for 6 days in AIM-V supplemented with CLG (3×10^6 cells in 2ml). On day 3 IL-2 was added to half of the cells in culture. Plates were blocked with 10% BSA in AIM-V.

Addition of IL-2 to the culture mix increased the number of cells secreting IFN- γ . However, this increase was not restricted to CLG-specific responses. Negative control wells had dramatically higher spot numbers, and no significant antigen specific response was detected. (Indeed an antigen-specific response detected with donor 5 was lost when IL-2 was added to the culture). It was concluded that addition of IL-2 did not improve responses and should not be used in subsequent investigations.

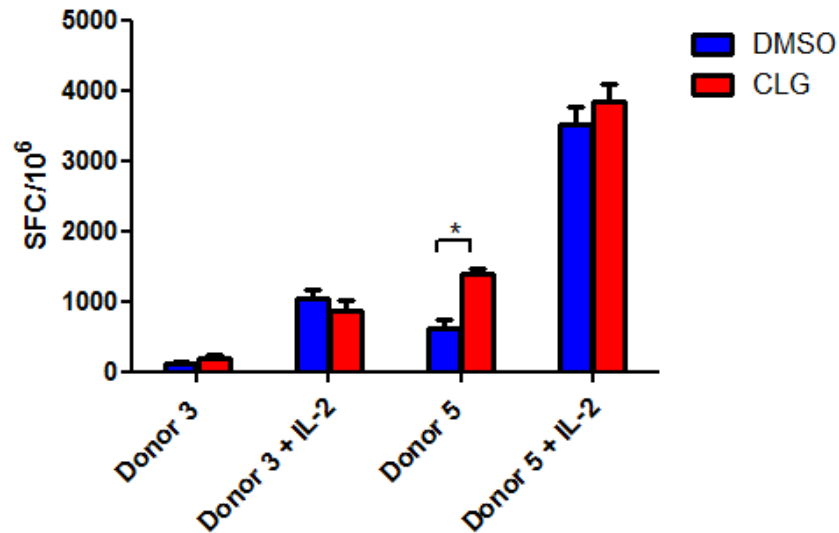


Figure 6. ELISPOT assays carried out to investigate the effect of adding IL-2 to the culture mix. PBMCs isolated from HLA-A2 positive healthy donors. PBMCs were cultured in AIM-v medium supplemented with CLG. 20IU/ml IL-2 was added to half of the cells on day 3. All plates were blocked with 10% BSA and 1×10^5 cells were added per well. PHA positive control wells were fully saturated with spots (not shown) Data shown as SFC/ 10^6 PBMCs (+standard error of the mean). Paired T-test was performed * $p < 0.05$

Culturing with HPV16 peptide pools for E6 and E7 reduced PHA response

Before committing to an ELISPOT protocol for investigation of HPV 16 E6 and E7 responses from cancer patients, it was important to ensure that the optimised protocol for detection of EBV peptide responses was transferable and could be used in combination with commercially available pepmixes. Frozen aliquots of PBMCs from 7 healthy donors were selected at random. In parallel, standard 'ex vivo' and cultured ELISPOT assays were carried out. PBMCs were thawed and rested overnight to eliminate cells which has been damaged by the freezing/ thawing process. Cells were then applied to a standard ELISPOT, or cultured for 6 days in AIM-V supplemented with HPV16 peptide pools (pepmixes) for oncogenic proteins E6 and E7 before the assay was conducted. To use less of the pepmixes and reduce costs, cells were cultured in a smaller volume of medium (1.5million cells in 1ml).

Antigen specific responses were not detected from any of the cone cells in either standard or cultured ELISPOT assays. Of course this could be a true result as it was not known whether the randomly selected donors carried HPV-specific T cells. However, it was noted that cultured cells showed dramatically reduced PHA responses. In all 'ex vivo' assays the PHA positive control wells were fully saturated with spots, whereas PHA wells from cultured cell experiments were much cleaner with relatively few spots (figure 7a,7b)

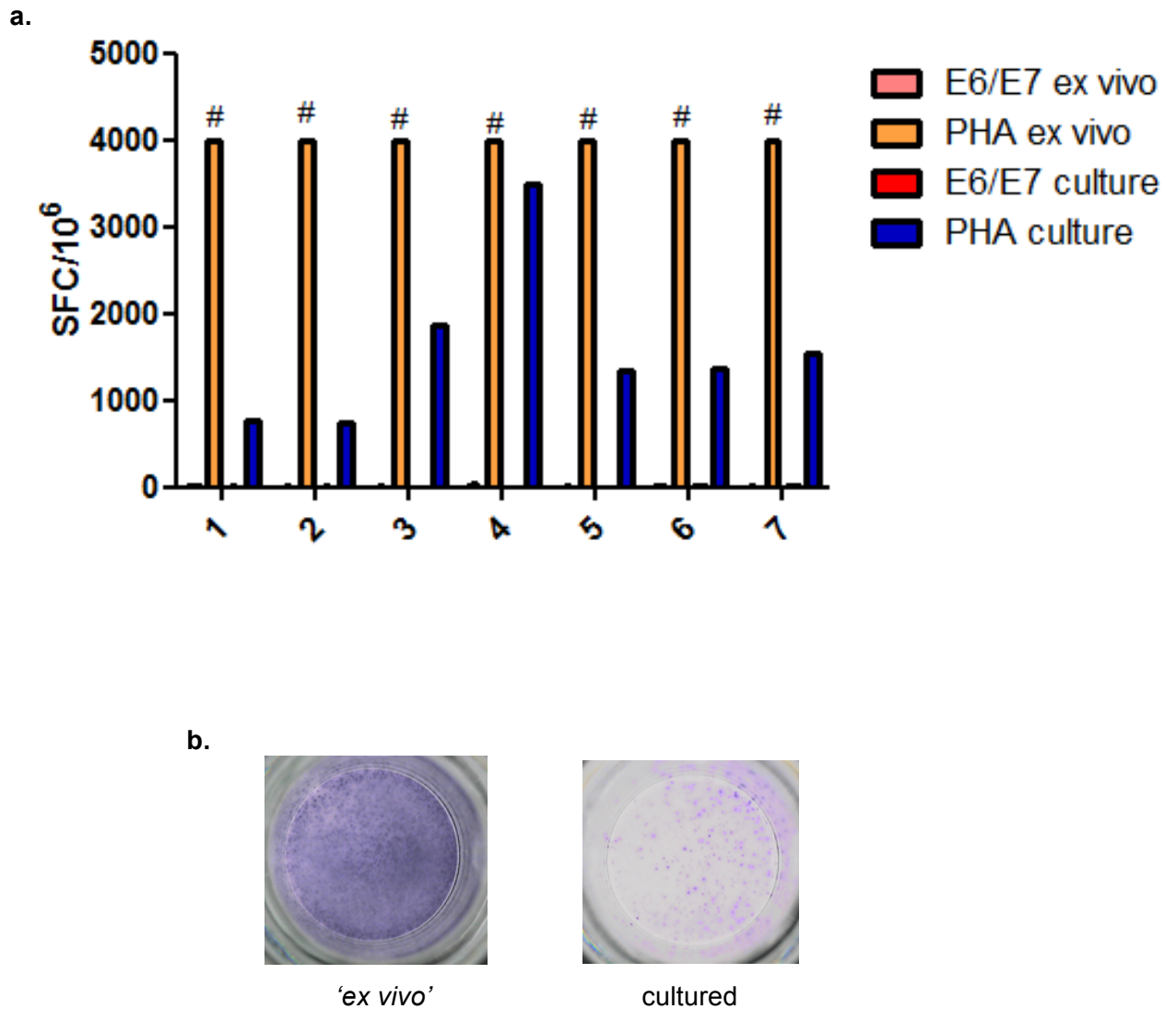


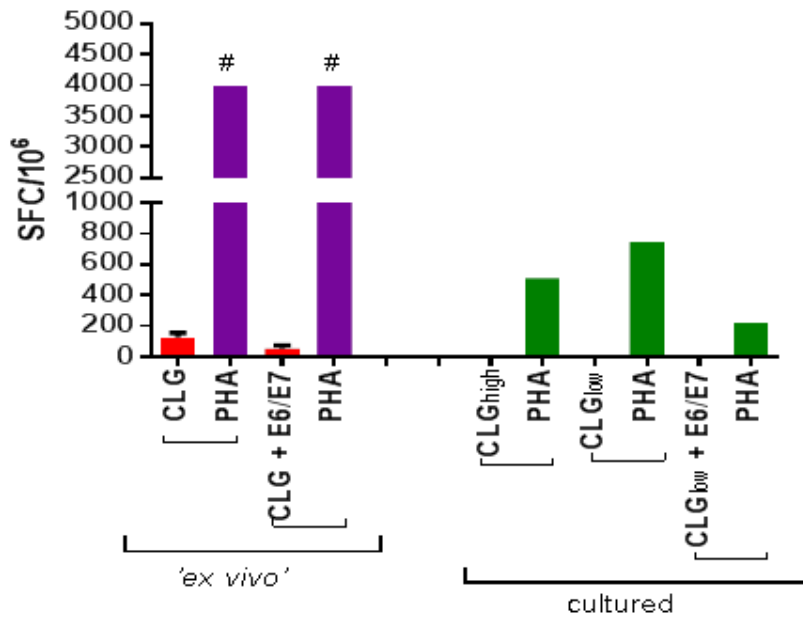
Figure 7. Standard 'ex vivo' and cultured ELISPOT assays carried out in parallel on PBMCs. PBMCs were cultured in AIM-V medium supplemented with HPV-16 E6 and E7 pepmixes. All plates were blocked with 10% BSA and 1×10^5 cells were added per well. In all cases background was low and subtracted from the CLG response results. # indicates the PHA well was fully saturated. **(a)** Data shown as SFC/ 10^6 PBMCs (+standard error of the mean). Antigen specific responses and PHA positive controls are shown. **(b)** Representative images from PHA positive control wells from standard 'ex vivo' and cultured ELISPOT assays.

Culturing cells in a reduced volume of medium may diminish their ability to produce IFN- γ

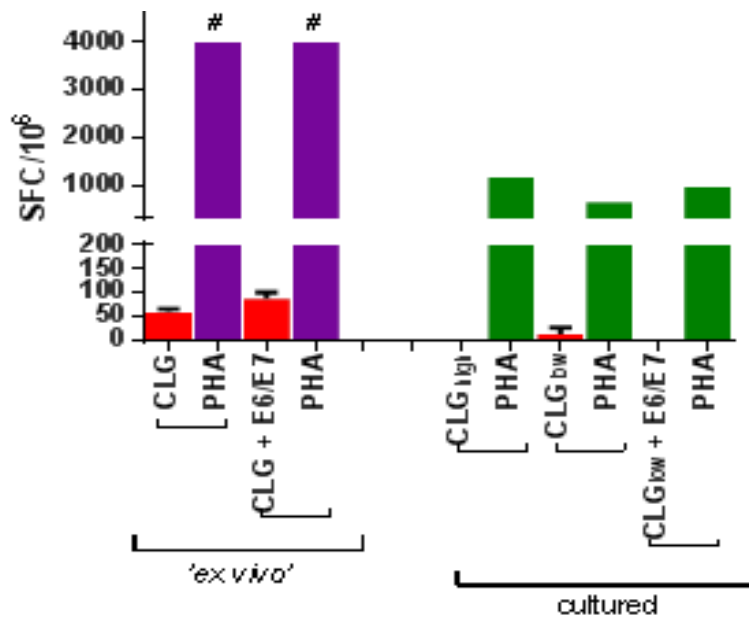
In order to investigate whether cells had become reduced in their ability to produce IFN- γ due to the smaller culture volume or inhibitory effects of supplementing the cells with E6 and E7 peptide pools, standard 'ex vivo' and cultured ELISPOT assays were set up in parallel on 2 donors previously seen to respond to EBV peptide CLG. PBMCs were isolated from donors 4 and 5. PHA positive and DMSO negative controls were run as well as test wells supplemented with either CLG alone, or CLG, E6 and E7 together in order to assess whether addition of E6 and E7 immediately ex vivo inhibited T cells production of IFN- γ . PBMCs were also set up in culture in AIM-V supplemented with CLG at the concentration previously used, CLG at a lower concentration (comparable to the concentration of HPV peptides used) and CLG at this lower concentration with E6 and E7 peptide pools. 1.5×10^6 cells were cultured for 6 days in 1 ml of medium before a standard ELISPOT assay was carried out testing for CLG responses.

Donors 4 and 5 both showed similar 'ex vivo' T cell responses to CLG as was seen in previous assays. Donor 4 and donor 5's 'ex vivo' responses were ~ 150 and ~ 25 SFC/ 10^6 cells respectively in this and prior assays. Addition of E6 and E7 premixes did not significantly impact antigen specific responses (figure 5, 8a,8b). PHA responses were high for both donors during the 'ex vivo' assays and the wells were saturated with spots (figure 8c). No antigen specific responses were seen after 6 days of culture with any of the peptide combinations (figure 8a,8b). Furthermore the PHA response was significantly reduced. PHA wells were not saturated with spots for any of the cultured ELISPOT assays suggesting the cells were not healthy or were functionally impaired (figure 8c).

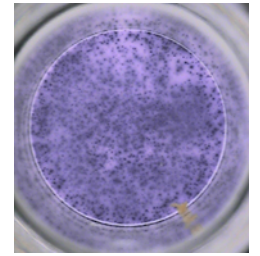
a.



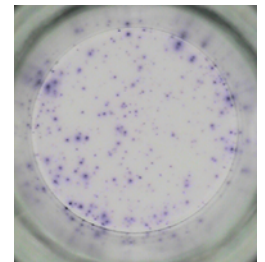
b.



c.



'ex vivo'



cultured

Figure 8. Standard 'ex vivo' and cultured ELISPOT assays carried out in parallel on healthy donors with a known response to EBV peptide CLG. PBMCs were cultured in AIM-V medium supplemented with CLG at the same concentration previous experiments (CLG_{high}), CLG at the same concentration as HPV pepmixes had been used (CLG_{low}), or CLG and HPV-16 E6 and E7 pepmixes together. All plates were blocked with 10%BSA and 1×10^5 cells were added per well. In all cases background was low and subtracted from the CLG response results. Graph shows mean spot forming cells (SFC) per million. Antigen specific responses and corresponding PHA positive controls for each assay are shown. # indicates the PHA well was fully saturated (**a**) Donor 4 standard and cultured ELISPOT (**b**) Donor 5 standard and cultured ELISPOT (**c**) Representative images from PHA positive control wells from standard 'ex vivo' and cultured ELISPOT assays.

Discussion

This project has clearly demonstrated the sensitivity of the cultured IFN- γ ELISPOT assay, and the importance of carefully considered variables such as culture conditions, blocking reagents and environmental changes which may drastically impact results. Multiple protocols have been described in the literature, and were adapted and tested during the course of this study[67, 71]. It was important to identify a protocol which would be broadly suitable for all donors and patients and to optimise a single protocol for both standard 'ex vivo' and cultured ELISPOT assays so that results of parallel experiments could be compared. In accordance with previously described data, we have shown that small variations in the protocol can make huge differences to the results obtained and thus leads to unreliable data [67, 83]. Harmonisation of the assay, which involves a process of development, optimisation and validation, was required before a chosen protocol was utilised for the investigation of patient samples[76]. This study has focused on the optimisation of a cultured ELISPOT assay which has been shown to be effective for amplification and detection of low frequency memory T cell responses[71, 73].

Preliminary standard ELISPOT assays testing for IED responses, clearly demonstrated the proficiency of the ELISPOT assay. The negative control wells appeared clean and a significant antigen specific T cell response was detected. However, there was concern that FBS may not be broadly compatible for blocking plates as it may introduce variation such as batch disparity. It is also possible that foreign antigens in FBS may cause T cell activation in some donors or patients[78]. Cross-reactivity with detection antibodies was also a concern and for these reasons FBS was side-lined as a potential blocking reagent[84].

The significance of identifying a suitable blocking reagent was highlighted during the course of this project. Blocking with BSA was highly recommended by a colleague at the Institute of Cancer

Sciences, who had used this blocking reagent during clinical trials and achieved consistently low background results. However, we found that blocking with BSA in initial cultured and 'ex vivo' assays resulted in a speckled appearance of the wells which has not been previously reported. This appearance may have been due to contaminants in the BSA which could have caused non-specific T cell activation in all wells. However, the speckles were distinctly different in size, shape and intensity from spots seen in other assays. It is possible that components of the BSA may have formed particulates which were detected in the wells. In order to confirm the reason for the speckled appearance of the wells, an assay could be carried out in which the plates were blocked with BSA, but no PBMCs were added to the reaction, thus any spots seen could be attributed to issues with the BSA and not to IFN- γ production by T cells. This batch of BSA was discarded and a new batch was subsequently tested with improved results. Autologous plasma was collected during PBMC isolation and tested for efficacy as a blocking reagent for the ELISPOT assay. It was expected that plasma isolated from the donor being tested would make a good blocking reagent because donors would be unlikely to mount T cell responses to their own plasma and thus, compared to FBS, autologous plasma may make a good alternative. The use of this blocking reagent dramatically reduced background in both *ex vivo* and cultured ELISPOT assays compared to earlier experiments blocked with BSA. Blocking with autologous plasma yielded a particularly good spontaneous background: antigen specific response ratio when cells were cultured in AIM-V supplemented with CLG before being applied to the assay (figure 4b). This result should be validated with numerous repeats with PBMCs from several donors.

The optimal culture medium was investigated as multiple culture conditions were described in the literature [67, 71, 72]. It was hypothesised that cells should be cultured in serum free medium to reduce background as supplementation with whole serum may result in undesired T cell activation during culture and subsequent responses detected may not be specific to the antigen of interest. We were concerned that the PBMCs would not survive and remain healthy after 6 days of culture in serum free medium. However, initial investigations did not indicate that changing the culture

medium resulted in significant differences in survival or functionality of the cultured cells and so only AIM-V medium (serum free) and RPMI 1640 supplemented with 10% autologous plasma were investigated further.

Interestingly upon further inspection, when cells were cultured in RPMI 1640 supplemented with autologous plasma SFC per million PBMCs were low and no significant antigen specific response was detected. In contrast, when cells from the same donor were cultured in serum-free AIM-V medium significant antigen specific responses were seen in plates blocked with autologous plasma or BSA (a new batch). Although the response detected in plates blocked with autologous plasma was greater than the response seen in wells blocked with BSA, BSA was used for all subsequent investigations as autologous plasma was not available for all samples tested during this project. For example, when screening for responses to HPV peptides we decided to use frozen stocks of PBMCs from consenting anonymised donors, as this avoided potential ethical issues that might occur with blood from laboratory colleagues. However autologous plasma was not available for these anonymised donors. Additionally, BSA was considered to be the preferred blocking reagent for use on patient samples as tumour samples may not always come with matching peripheral blood samples and so again autologous plasma may not be available. As previously mentioned absolute consistency is essential for reproducible ELISPOT data. My preliminary data suggest blocking with autologous plasma may be a good means to detect weak antigen specific responses in the ELISPOT assay and so in situations where such plasma is available it may well be worth further testing to see if this is the best approach to use. However, there is still the concern that autologous plasma will be different for every donor, and in some cancer patients may contain some immune suppressive components, which introduces another variable into the assay; in contrast, blocking with BSA offers a standardised reagent.

In accordance with previous studies, we have demonstrated that culturing the cells with peptide before the assay successfully amplifies the T cell response detected by the ELISPOT assay[71, 73, 85]. This optimised protocol developed for the detection of CLG responses in healthy donors should be retested on multiple donors to ensure reproducibility. During this investigation, we cultured PBMCs for 6 days before they were applied to the ELISPOT assay on the advice of a colleague at the Institute of Cancer Sciences at the University of Birmingham. This varies from culture periods described in the literature. Some studies have suggested that 24 hour incubation with peptide may be sufficient to activate T cells in the sample sufficiently for detection without significantly increasing background [67]. Several other studies have suggested a longer incubation of 10 days including supplementation with IL-2 (which causes proliferation of T cells) is optimal to maximise T cell expansion[71]. We have found that culturing the cells before completing the ELISPOT assay results in increased spontaneous T cell activation and higher background levels compared to *ex vivo* assays. This is particularly evident in cultures that were set up supplemented with IL-2 where non-specific T cell amplification was such that no antigen specific responses could be detected above background levels. Further culturing conditions should be investigated for the optimisation of T cell expansion for future cultured ELISPOT assays. IL-7 and IL-15 are also implicated in inducing T cell proliferation and may be useful for maximising T cell expansion during the culture phase. [74]

In the timescale of this project it was not possible to successfully translate the optimised cultured ELISPOT protocol, which had been effective in detecting of T cell responses to CLG, for the detection of T cell responses to HPV proteins E6 and E7. Initial ELISPOT assays did not detect T cell responses to E6 or E7 in PBMCs from any of the randomly selected donors. It is estimated that only ~23% of the population are infected with high risk HPV (16, 18 or 31) and only a small proportion of these will be HPV-16[86]. It is therefore possible that the results we obtained from our investigation were real and the donors did not have E6 or E7-specific T cell responses. Alternatively pepmixes were not functioning properly to activate T cells to produce IFN- γ . This may

be because the pepmixes were added at too low a concentration to elicit a response, although EBV pepmixes were successfully used at a matching concentration during a clinical trial at the Institute of Cancer Sciences[82]. The major difference between the protocol used for these assays and my previous assays was the reduced volume of medium in which the cells were cultured. In previous assays 3 million cells were cultured for 6 days in 2ml AIM-V. In assays assessing T cell responses to E6 and E7, 1.5 million cells were cultured for 6 days in 1ml AIM-V, but were first incubated overnight in just 0.5ml of medium to allow the cells to encounter a higher concentration of peptide. It was surprising that there was dramatically reduced T cell activation seen in PHA wells after culture compared to cells that were tested immediately after thawing. In both *ex vivo* and cultured ELISPOT assays 1×10^5 cells were added per well and so differences cannot be accounted for by differing cell numbers between assays. Cells were inspected and counted under a microscope and appeared healthy after culture. Cells may have been alive when they were applied to the ELISPOT but have undergone apoptosis during the assay period[66]. Over stimulation of T cells may have caused the cells to lose function, although this is unlikely because only peptide-specific T cells were stimulated during culture so only these cells would have become unresponsive[87] [88]. Other factors which may be responsible for these unexpected results include environmental changes such as problems with the thermostat of the incubator in which the cells were cultured, or potential issues with the PHA or culture medium although I have no evidence for this (e.g. the same batches of PHA and culture medium were used for both *ex vivo* and cultured ELISPOT assays).

In order to identify and rectify the issue causing unresponsiveness of the T cells in these final assays, the original, optimised, cultured ELISPOT assay should be repeated with another donor with a known response to CLG peptide as this will suggest whether the issue stems from problems with the reagents. Next, the culture process should again be scaled down so that again, 1.5 million cells are cultured in AIM-V supplemented with CLG at the concentration originally used. This will elucidate whether the problem is associated with the cells being cultured in a small volume of medium. If at this stage the assay is successful and PHA responses are strong and CLG specific

responses are amplified, then the reduced response seen here is likely to be attributed to transient environmental changes which may have occurred in the laboratory.

ELISPOT assays have been shown to be a very effective method of detection and quantification of T cell responses [65] [22]. Here we have demonstrated the sensitivity of the assay and the scope of the cultured ELISPOT assay for the detection of low frequency T cell responses. We had intended to investigate T cell responses in patients with HPV⁺ oropharyngeal head and neck cancer. However, due to delays in receiving ethical approval it was not possible to analyse these responses. Future work must now focus on the transition of the optimised cultured ELISPOT assay described in this project into use for the detection of HPV-specific T cells in the tumours and peripheral blood of patients with head and neck cancer. Uncovering these responses may prove significant for the development of novel immunotherapy and vaccine strategies to combat this life threatening cancer.

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