Identification of HLA-DR1- and HLA-DR15-restricted human papillomavirus type 16 (HPV16) and HPV18 E6 epitopes recognized by CD4+ T cells from healthy young women

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Human papillomavirus (HPV) infection, particularly with types 16 and 18, is causally associated with the development of cervical cancer. Prophylactic vaccines against HPV have recently been licensed and have the primary aim of protecting children against future HPV infection and cervical cancer. However, these vaccines are unlikely to be effective in women with pre-existing HPV infection and disease. Previous studies have suggested that HPV16 E6-specific CD4+ T cells play a role in controlling viral infection; however, the epitopes recognized by such T-cells have not been defined. In this study, we analysed T-cell responses against HPV16 and 18 in ten healthy young women in an age group (21–31) with a high prevalence of HPV infection and clearance. Five individuals made HPV E6 responses, from which five candidate T-cell epitopes (three HPV16 E6 and two HPV18 E6) were identified. More detailed characterization of epitopes from HPV16 E6(127–141) and HPV18 E6(43–57) revealed HLA-DRB1*01 and HLA-DRB1*15 restriction, respectively. Furthermore, generation of a T-cell line against HPV16 E6 (127–141) demonstrated that this epitope could be presented after endogenous processing of soluble HPV16 E6 protein. Overall we demonstrate a powerful approach for defining novel CD4+ T-cell epitopes from two oncogenic HPV types. This approach could be applied to study populations in developing countries with a high incidence of cervical cancer. Such epitopes could provide a more precise way of investigating the role of natural and vaccine-induced T-cell responses against HPV in blood and at sites of disease.

INTRODUCTION

Infection with human papillomaviruses (HPV) is a common event, particularly in sexually active young women (Ho et al., 1998). Persistent infection with ‘high-risk’ HPV is causally associated with the development of high-grade cervical intraepithelial neoplasia (CIN) and cervical cancer (Walboomers et al., 1999; zur Hausen, 1996). HPV16 and HPV18 DNA is detected in over 50 and 14 % of all cervical cancer cases, respectively (Bosch et al., 1995). E6 and E7 are the major transforming proteins in oncogenic HPVs and they are constitutively expressed in high-grade cervical lesions and cervical cancer, making them attractive targets for T-cell-based immunotherapy.

Several lines of evidence suggest that the cellular immune response plays a significant role in the clearance and control of HPV infection. First, immunocompromised transplant patients and human immunodeficiency virus-positive individuals have significant increases in the prevalence of progressive HPV-induced anogenital disease (Fruchter et al., 1996; Petry et al., 1994). Second, spontaneous regression of CIN appears to correlate with delayed type hypersensitivity reaction to HPV16 antigens (Hopfl et al., 2000). Third, the cellular infiltrate of regressing HPV-induced genital warts is dominated by CD4+ T cells and macrophages (Coleman et al., 1994), suggesting a role for CD4+ T helper cells (Th) in response to HPV infection. Th play an important antiviral role during infection, in particular the activation and co-ordination of CD8+ T cell primary responses, the generation of CD8+ memory populations, and can themselves demonstrate direct effector functions (Bickham et al., 2001). This may be particularly relevant to HPV disease, as HLA (human leukocyte antigen) class II molecules are expressed in high-grade cervical neoplasia and cervical cancer (Coleman & Stanley, 1994). In contrast, levels of HLA class I expression are greatly diminished as a result of HPV infection/transformation (Ashrafi et al., 2005; Connor & Stern, 1990). It therefore stands to reason that Th epitopes identified from a memory response that correlated with viral clearance could be useful in the development of effective immunotherapeutic approaches.

Until recently, the study of oncogenic HPV T-cell responses has been restricted to women with recurrent HPV infection,
high grade anogenital neoplasia or cervical cancer (Bontkes et al., 2000; Kadish et al., 1997; Nakagawa et al., 1999). However, Welters and colleagues demonstrated that memory CD4+ T-cell responses against HPV16 E6 antigens could be frequently found in healthy subjects (Welters et al., 2003). These memory T-cell responses are likely to have been a component of an effective anti-HPV16 immune response that has limited or cleared viral infection (de Jong et al., 2003). These memory T-cell responses are likely to have been a component of an effective anti-HPV16 immune response that has limited or cleared viral infection (de Jong et al., 2003). To date, the majority of this work has focussed on evaluating the overall immune response to HPV16 viral proteins in the form of peptide pools, rather than defined peptide epitopes (de Jong et al., 2003). However, epitopes are useful in assessing dominant patterns of immune recognition and in precisely tracking immune responses following natural exposure or in response to vaccination.

The incidence of HPV infection is highest in sexually active women under 30, with peak HPV prevalence in the 25–29 age group (Jacobs et al., 2000). Therefore we studied a group of ten healthy female volunteers, aged between 21 and 31 years, for memory Th responses to HPV16 and HPV18 E6 and E7 peptides. We identified several novel candidate T-cell epitopes for HPV16 and 18 E6 and generated T-cell lines for phenotypic and functional analysis.

**METHODS**

**Volunteers and cells.** The ten healthy female volunteers in this study (aged 21–31 years) had no known history of cervical dysplasia. Since the project involved healthy volunteers with no clinical testing for evidence of HPV infection, it was deemed to raise no ethical issues. Blood samples were obtained after informed consent and with approval from the South East Wales Research Ethics Committee (panel D). Blood was taken with informed consent and peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-based density-gradient centrifugation (Sigma). PBMC were cultured directly or cryopreserved for later use (Yould et al., 2000). The HLA class I and class II haplotypes of the blood donors were typed by the Welsh Transplantation and Immunogenetics Laboratory, Pontyclun, UK. The HLA types of donors from which epitopes were mapped are summarized in Supplementary Table S1, available in JGV Online.

**Cell lines and T-cell cultures.** BLCL (Epstein–Barr virus-immortalized B-cell line) were cultured in RPMI (Invitrogen) supplemented with 10% FCS (fetal calf serum; Invitrogen), 2 mM glutamine, 100 μg streptomycin ml−1, 100 IU penicillin ml−1 and 25 mM HEPES (Sigma) (RPMI-FLCS medium). Wild-type and HLA DRβ1*01- or HLA DRβ1*15-transfected murine L cells (provided by Professor D. Altman, Imperial College, London, and Dr G. Lombardi, Kings College London, UK) were also cultured in RPMI-FCS medium. All T cells were cultured in RPMI (PAA), supplemented with 10% AB serum, 2 mM glutamine, 100 μg streptomycin ml−1, 100 IU penicillin ml−1 and 25 mM HEPES (RPMI-AB medium).

**Antigens.** HPV peptide pools consisted of 15mer peptides (overlapping by 9 aa) which span the length of the E6 and E7 proteins of HPV16 and HPV18 (Mimotopes). Peptides (purity ≤65%) dissolved in DMSO were combined to generate four peptide pools: HPV16 E6 (25 peptides), HPV16 E7 (15 peptides), HPV18 E6 (25 peptides) and HPV18 E7 (16 peptides). Each peptide was present at 4 mg ml−1. For peptide mapping, six smaller split pools from HPV16 and HPV18 E6 were created with each pool containing four or five peptides. Peptides were allocated numbers (1–81) to allow simple identification. The HPV16 E6 protein was diluted in a 1:1 mixture of DMSO and PBS at 10mg ml−1 (kind gift from Dr Marij Welters, Leiden Medical School, The Netherlands) (van der Burg et al., 2001; Welters et al., 2003). A mixture of common recall antigens (positive peptide pool, PPP) for CD4+ and CD8+ T cells was included in epitope-mapping experiments as a positive control (purity >85%, Severn Biotech) (Smith et al., 2005).

**Short-term T-cell culture and ELISpot analysis.** PBMC were plated out at 2 × 10^6 cells ml−1 in RPMI-AB medium (1 ml per well of 24-well plates). Peptides were added as follows: HPV pools or individual peptides at 5 μg ml−1 each peptide; PPP at 10 μg ml−1 each peptide. On day 2, 500 μl RPMI-AB medium containing interleukin-2 (IL-2, 20 IU ml−1; Chiron) was added to each well. On day 6, 500 μl medium was removed and replaced with 1 ml fresh RPMI-AB medium without IL-2 added. On day 7, cultures were tested for the presence of peptide-specific T cells in the ELISpot assay.

**Generation of stable T-cell lines.** PBMC isolated from donor 1 were cultured as described above in the presence of 5 μg p37 [HPV16 E6][127-141] ml−1 (>95% purity) for 7 days. T cells were harvested, washed and incubated with anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Cells were then passed over two magnetic LS columns (Miltenyi Biotec) and unlabelled cells were collected as the CD8+ enriched fraction. Purity was assessed by flow cytometry. The CD8+ enriched fraction was always >90% pure, whilst the CD4+ enriched fraction consisted of up to 20% CD8+ T cells.

For antibody blocking experiments, the anti-HLA-DR antibody L243 and the anti-HLA class I antibody W6/32 (control for non-specific blocking) were included directly to the ELISpot wells at 25 μg ml−1.

Experiments in which HPV pool responses were mapped to individual epitopes were performed using PBMC that had been cultured for 14 days with the whole peptide pool. Cells had been restimulated on day 7 with the peptide pool (5 μg ml−1) and autologous irradiated PBMC. The culture was supplemented with IL-2 (20 IU ml−1) on days 7 and 10, and half the medium was replaced (no IL-2) on day 13. Positive responses against any given peptide epitope were confirmed by culturing PBMC with the individual peptides alone before testing by ELISpot (data not shown).

For antibody blocking experiments, the anti-HLA-DR antibody L243 and the anti-HLA class I antibody W6/32 (control for non-specific blocking) were included directly to the ELISpot wells at 25 μg ml−1.

**Generation of CD8+ and CD4+ enriched fractions.** PBMC were cultured with peptide (5 μg ml−1) for 7 days. Cells were harvested, washed and incubated with anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Cells were then passed over two magnetic LS columns (Miltenyi Biotec) and unlabelled cells were collected as the CD4+ enriched fraction, whilst the column-bound cells were collected as the CD8+ enriched fraction. Purity was assessed by flow cytometry. The CD8+ enriched fraction was always >90% pure, whilst the CD4+ enriched fraction consisted of up to 20% CD8+ T cells.

**Generation of stable T-cell lines.** PBMC isolated from donor 1 were cultured as described above in the presence of 5 μg p37 [HPV16 E6][127-141] ml−1 (>95% purity) for 7 days. T cells were harvested, washed and incubated with anti-CD8 microbeads according to the manufacturer’s instructions (Miltenyi Biotec). Briefly, 10^6 cultured cells were restimulated with 5 μg p37 ml−1 plus 2 × 10^6 irradiated autologous PBMC and subjected to enrichment based on IFN-γ secretion. The enriched cells were cultured in RPMI-AB medium (20 IU IL-2 ml−1) with 5 × 10^6 irradiated allogeneic PBMC for 5 days. Supernatants were collected and tested for IFN-γ production by ELISA (data not shown).
4 weeks. The enriched p37-specific T-cell culture was used either to generate line B using an allogenic feeder expansion (Evans et al., 2001) or seeded into limiting dilution cultures. Numerous resulting lines were screened using ELISpot for recognition of p37 and line 46 was selected for expansion. The lines Belx1 (TCR Vβ16+) and Belx2 (TCR Vβ16−) were generated from line 46 through cell sorting with a MoFlo cell sorter (Dako). Cells were stained with an anti-TCR receptor (TCR) Vβ16–PE (phycoerythrin-conjugated) monoclonal antibody (AbD Serotec), and sorted into two populations based upon the presence and absence of TCR Vβ16 staining.

The p64 [HPV18 E6(1,43–57)]-specific T-cell line was generated by culturing PBMC from donor 7 for 7 days with p64 (5 μg ml−1). The cultured cells were then expanded as above.

**TCR analysis.** T-cell lines were tested using a panel of PE-conjugated anti-TCR Vβ monclonal antibodies: Vβ 2, 3, 5.1, 5.2, 5.3, 8, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 22 and 23 (AbD Serotec). Cells were co-stained with an anti-CD4 FITC-conjugated monoclonal antibody (AbD Serotec) and analysed by flow cytometry.

**IFN-γ intracellular cytokine staining (ICS).** T-cell lines were seeded at 5 × 10^5 T cells per well with BLCL at a ratio of 1:2 in 1 ml RPMI-AB medium. In some experiments, a proportion of BLCL were fixed with 2% paraformaldehyde prior to the ICS assay. Cells were incubated in the presence of either DMSO (negative control), peptide fixed with 2% paraformaldehyde prior to the ICS assay. Cells were stained with an anti-CD4 FITC-conjugated monoclonal antibody (AbD Serotec), and sorted into two populations based upon the presence and absence of TCR Vβ16 staining.

Once HPV responders were identified, PBMC cultures were tested using a panel of PE-conjugated anti-TCR Vβ monclonal antibodies: Vβ 2, 3, 5.1, 5.2, 5.3, 8, 9, 11, 12, 13.1, 13.6, 14, 16, 17, 18, 20, 22 and 23 (AbD Serotec). Cells were co-stained with an anti-CD4 FITC-conjugated monoclonal antibody (AbD Serotec) and analysed by flow cytometry.

**RESULTS**

**Detection and mapping of HPV16 and HPV18 E6-specific CD4+ T-cell responses from healthy volunteers**

To define HPV-specific T-cell epitopes, it was first necessary to identify donors that responded to HPV16 and HPV18 E6 and E7 peptide pools. Ten healthy female donors (aged 21–31 years) were recruited. Since we (Smith et al., 2005) and others (de Jong et al., 2002; Facchinetti et al., 2005; van der Burg et al., 2001; van Poelgeest et al., 2005) have demonstrated that it is difficult to detect HPV-specific T cells in healthy donors directly *ex vivo*, PBMC were cultured with HPV peptide pools for 7 days before testing for T-cell responses by IFN-γ ELISpot. We have previously shown that this protocol is a sensitive method for the detection of memory CD4+ T-cell responses in healthy donors and vaccinated patients (Smith et al., 2005).

HPV-specific T-cell responses were detected from five of the ten donors. One responded to HPV16 E6 alone, one to HPV18 E6 alone, and three responded to both HPV16 and HPV18 E6 peptides (Table 1). No donors demonstrated responses to HPV E7 peptides. This is unsurprising as several studies have shown that T-cell responses against E7 are rarely detected in healthy women (van der Burg et al., 2001; Welters et al., 2006). All donors demonstrated responses to the positive peptide pool containing common recall antigens (data not shown).

<table>
<thead>
<tr>
<th>Donor</th>
<th>HPV16 E6 peptides</th>
<th>HPV18 E6 peptides</th>
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<tr>
<td></td>
<td>Epitopes</td>
<td>Epitopes</td>
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<tr>
<td></td>
<td>Spots per 10⁶ cells</td>
<td>Spots per 10⁶ cells</td>
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<tr>
<td>1</td>
<td>Peptide 37(127–141)</td>
<td>Peptide 65(49–63)</td>
</tr>
<tr>
<td></td>
<td>293 ± 73.9</td>
<td>162.5 ± 45.2</td>
</tr>
<tr>
<td>5</td>
<td>Peptide 37(127–141)</td>
<td>18E6-positive</td>
</tr>
<tr>
<td></td>
<td>502 ± 24.0</td>
<td>No further mapping</td>
</tr>
<tr>
<td>6</td>
<td>Peptide 30(85–99)</td>
<td>Peptide 64(43–57)</td>
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<td></td>
<td>219.5 ± 25.8</td>
<td>329.8 ± 58.1</td>
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<tr>
<td>7</td>
<td>Peptide 36(121–135)</td>
<td>Peptide 64(43–57)</td>
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<td>382.8 ± 38.1</td>
<td>57.2 ± 35.2</td>
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<tr>
<td>9</td>
<td>Peptide 19(19–33)</td>
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<td>35.1 ± 13.5</td>
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Data are collated from the ELISpot-based epitope mapping experiments for all HPV-responding donors. Data represent the mean result from triplicate wells and background responses (T cells plus APC alone) have been subtracted. Background spot counts did not exceed 40 spots per 10⁵ cells.
ELISpot (Fig. 1c). In all cases the peptide response was restricted to the CD4\(^+\)-enriched fraction, with the low-level response in the CD8\(^+\) fraction likely corresponding to the 5–10% contamination with CD4\(^+\) T cells. For peptides 30 [HPV16 E6\(_{85-99}\)] and 36 [HPV16 E6\(_{121-135}\)], IFN-\(\gamma\) ICS was used as an alternative approach to the CD4/CD8-enriched populations (Fig. 1c). In both cases the peptide-specific IFN-\(\gamma\) production was restricted to the CD4\(^+\) T cell fraction with no response associated with the CD8\(^+\) T cells.

**HLA class II restriction analysis**

The HLA-DR restriction of peptides 30, 36, 37, 64, and 65 was first established using peptide-specific polyclonal CD4\(^+\) T-cell lines in ELISpot assays. The addition of the HLA-DR antibody L243 was shown in all cases to abrogate the peptide-specific T-cell response, indicating that the epitopes are HLA-DR-restricted (Fig. 2a). Furthermore, the pan-HLA class I blocking antibody W6/32 did not affect the response to the peptides, indicating that there is no...
The CD4+ T cell component in these responses. Collectively, the CD4/CD8 enrichments and ICS data provide strong evidence that the epitopes tested are recognized by CD4+ T cells. T-cell responses to p37 and p64 were selected for further characterization as they were both detectable from two donors (Table 1). Subsequently the HLA-DR allotypes involved in the presentation of p37 and p64 were investigated.

The CD4+ T-cell line B from donor 1 was generated by immunomagnetic enrichment of peptide-specific IFN-γ-secreting T-cells from PBMC cultured with p37 for 7 days. As the only HLA-DR allotype common to the two p37-responding donors (1 and 5) was HLA-DRB1*01, this allotype was the obvious candidate for a role in peptide presentation. Line B was tested in an ELISpot assay for recognition of p37 using mouse L-cells as APC that were either wild type or transfected with HLA-DRB1*01 or HLA-DRB1*15 (Fig. 2b). A strong p37 response was only detected where the DRB1*01-positive L-cells were used, clearly indicating that HLA-DRB1*01 is capable of p37 presentation. This finding has also been confirmed using panels of HLA-typed PBMC as the APC in ELISpot assays (data not shown).

The only HLA-DR allotype common to the two p64-responding donors (7 and 9) was HLA-DRB1*15, making it a strong candidate for p64 presentation. To test this hypothesis, a panel of HLA-typed PBMC was employed as APC for a p64-specific T-cell line in ELISpot assays. Of the 11 PBMC-APC tested, the six that were able to present p64 to the T-cell line were HLA-DRB1*15-positive, with no other HLA-DR allotype common to all (Fig. 2c). This suggests that DRB1*15 is capable of presenting p64. It should be noted that using DR15-transfected L-cells as APC in an ELISpot with the p64 line also resulted in a weak positive response (21.8 spots vs 0 spots using untransfected L-cell control, data not shown).

**Functional and phenotypic characterization of p37-specific T-cell lines**

The response to p37 was further investigated through the generation and analysis of oligoclonal CD4+ T-cell lines from donor 1. A proportion of the IFN-γ-secreting, magnetically enriched CD4+ T cells were seeded at varying densities into 96-well plates with allogenic feeders and cultured for several weeks before screening by ELISpot for p37 recognition. Of those screened, line 46 was examined for TCR Vβ usage as an indicator of how many T-cell populations it contained. A panel of anti-TCR Vβ antibodies (covering approximately two-thirds of the TCR Vβ chains) was used to screen line 46. Flow cytometric analysis revealed that 88% of T-cells expressed TCR Vβ16, with no other Vβ chain being detected (data not shown). The p37-specific IFN-γ production by the two T-cell populations (Vβ16+ and Vβ16−) was then investigated by IFN-γ ICS as this is a sensitive and informative flow cytometer-based assay (Fig. 3). Interestingly, the IFN-γ-positive cells consisted of both Vβ16− and Vβ16+ CD4+ T cells. Of the total Vβ16+ T-cell population, 47.5% were IFN-γ-positive (Fig. 3c), whilst 85.8% of Vβ16− cells were positive for IFN-γ (Fig. 3b). The mean fluorescent intensity for IFN-γ-staining was eightfold higher for the Vβ16− T cells, indicating that this population is superior both in the proportion of cells that were IFN-γ positive and also in the amount of cytokine produced. This suggests that the dominant T-cell population (Vβ16+) within this line is functionally inferior to the subdominant population (Vβ16−) of T cells.

In order to effectively investigate these two T-cell populations, it was necessary to purify the Vβ16+ and Vβ16− cells using a MoFlo cell sorter. The resulting populations were expanded and the lines were then assessed for their purity; Belx1 was approximately 94% positive for Vβ16 expression and Belx2 was >99% negative for Vβ16 staining (data not shown). ICS analysis demonstrated that

![Fig. 3. The HPV16 E6(127–141)-specific T-cell line 46 consists of a TCR Vβ16+ and TCR Vβ16− population. Line 46 T cells were incubated with either p37 or DMSO (negative control) in an IFN-γ ICS assay. Cells were stained with an anti-TCR Vβ16-PE antibody and anti-IFN-γ-FITC antibody. (a) The percentage of TCR Vβ16 positive and negative cells within the lymphocyte gate that were positive for IFN-γ staining. The proportion of (b) Vβ16+ and (c) Vβ16− cells that were positive for IFN-γ following incubation with p37 are depicted in histograms (black fill), with the DMSO control results shown in grey. Representative data are shown for each duplicate sample.](image-url)
the differential IFN-γ response of the Vp16+ population was maintained, with 96.4% of Belx2 cells (Vp16−) producing IFN-γ in response to p37, as compared to only 31.3% of the Belx1 line (Vp16+) (Fig. 4). Belx2 (Vp16−) was also shown to be functionally superior at lower peptide doses, with 45.5% being positive for IFN-γ at 0.05 μg ml⁻¹ compared to 0% for the Belx1 line (Vp16+) (data not shown). This differential IFN-γ response was confirmed by cytometric bead array which revealed that Belx2 (Vp16−) produces 31-fold more IFN-γ than Belx1 (Vp16+) at 6 h post-p37 activation. In the same assay, both Belx1 (Vp16+) and Belx2 (Vp16−) lines demonstrated a Th1 cytokine profile with moderate levels of IL-2, low IL-4, IL-10 and no IL-5 (Supplementary Table S2, available in JGV Online).

**Endogenous processing and presentation of the HPV16 E6(127–141) epitope**

As the Belx1 (Vp16+) and Belx2 (Vp16−) T-cell lines had been generated against a synthetic 15mer, p37, representing HPV16 E6(127–141), it was important to demonstrate that the epitope could also be presented from an endogenously processed full-length HPV16 E6 protein source. Due to the lack of HPV16− cervical carcinoma cell lines expressing HLA-DRB1*01, an alternative method was employed to investigate processing and presentation.

The initial approach involved the use of BLCL (HLA-DRB1*01+ and HLA-A*02+) infected with TA-HPV, a recombinant vaccinia virus expressing an HPV16 E6–E7 fusion protein. The recognition of E6-derived epitopes was assessed using the Belx2 T-cell line (Vp16−) and 7E7, an HPV16 E629–38-specific cytotoxic T-lymphocyte (CTL) clone (Supplementary Fig. S1, available in JGV Online).

Whilst the CTL clone 7E7 was positive for IFN-γ by ICS, demonstrating endogenous E6 expression, the Belx2 T-cells (Vp16−) did not demonstrate recognition of the cognate epitope. An alternative approach consisted of incubating full-length recombinant HPV16 E6 protein with HLA-DRB1*01-positive BLCL and the Belx2 line (Vp16+) in an ICS assay (Fig. 5). Belx2 (Vp16+) only demonstrated epitope recognition when the BLCL were pulsed with the E6 protein, with 41.1% of Belx2 cells (Vp16+) positive for IFN-γ (Fig. 5a). No IFN-γ response was generated by Belx2 (Vp16−) when fixed BLCL pulsed with the full-length E6 protein were used as APC (Fig. 5c), indicating a requirement for antigen uptake for epitope recognition by Belx2 (Vp16−). However, Belx2 (Vp16−) did demonstrate an IFN-γ response to the fixed BLCL pulsed with p37 (Fig. 5d), confirming the capacity of the fixed BLCL to present peptide. The ability of more physiologically relevant APC, such as autologous PBMC, to process and present the exogenous source of E6 protein has also been confirmed in ICS experiments (data not shown). This clearly indicates that p37 contains an epitope which can be endogenously processed and presented from the full-length E6 protein.

Belx1 (Vp16+) was also tested for E6 protein recognition, but did not demonstrate an IFN-γ response at the highest protein dose tested (40 μg ml⁻¹), further highlighting the

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**Fig. 4.** Differential IFN-γ secretion profiles of the TCRVp16+ (Belx1) and the TCR Vp16− (Belx2) T-cell lines. The T-cell lines were incubated with either p37 or DMSO (negative control), in an IFN-γ–ICS assay. Cells were stained with an anti-TCR Vp16–PE antibody (to identify the contaminating T cells in each population) and an anti-IFN-γ–FITC antibody. Histograms depict the percentage of cells that were IFN-γ-positive, with the DMSO control shown in grey and the p37 tests shown in black fill. The percentage of IFN-γ-positive cells stated refers only to the Vp16− cells in Belx1 (Vp16+) and Vp16− cells in Belx2 (Vp16−). Duplicate samples were tested and representative data are shown.

**Fig. 5.** The Belx2 (Vp16−) cognate epitope is processed and presented by BLCL from a full-length recombinant HPV16 E6 protein. Belx2 (Vp16−) T cells were incubated with either (a, b) unfixed or (c, d) fixed BLCL. Cells were tested with either DMSO, p37 [HPV16 E6(127–141)] or the HPV16 E6 protein and an ICS assay was performed. Cells were co-stained with an anti-TCR Vp16–PE antibody and an anti-IFN-γ–FITC antibody. Histograms depict the proportion of T cells positive for IFN-γ staining, with the DMSO control shown in grey and the E6 protein or p37 tests shown in black fill. The values stated refer to the percentage of IFN-γ-positive cells in the p37 or E6 protein tests. Duplicate tests were carried out, and representative data are shown.
functional inferiority of this T-cell population (data not shown).

**DISCUSSION**

In this study, ELISpot assays have been used to investigate CD4+ T-cell responses against HPV16 and 18 onco-proteins in women in the age group with the highest prevalence of HPV. CD4+ T-cell responses against HPV16 E6 have been studied previously using pooled synthetic peptides or 22mer peptides (de Jong et al., 2004; Welters et al., 2003). However, to our knowledge, this is the first report of candidate CD4+ T-cell epitopes from HPV16 E6 and derivation of HPV16 E6-specific CD4+ T-cell lines. We identified five novel 15mer peptide sequences: HPV16 E6 p19(19–33), p30(85–99), p36(121–135), p37(127–141), and HPV18 E6 p64(43–57), and one previously defined HPV18 E6 epitope [p65(49–63)]. Two were mapped to specific HLA-DR allotypes (p37 and p64) and three were HLA-DR restricted (p30, p36, and p65), making them strong candidates for further investigation of HPV16/18-specific CD4+ T-cell responses.

Initially all donors were tested for responses against peptide pools derived from HPV16 and 18 E6 and E7 oncoproteins, because these are the HPV types most often associated with cervical cancer. In our study, five of the ten healthy donors assessed responded to either HPV16 E6 or HPV18 E6 peptide pools. Interestingly, the HPV-specific responses from donors 1 and 6 had previously been detected 2 years before the initiation of this study (Smith et al., 2005). No T-cell responses were detected in the healthy donors against HPV16 or HPV18 E7 peptide pools, a finding shared by other studies (van der Burg et al., 2001; Welters et al., 2006). In contrast, T-cell responses against oncogenic HPV E7 can be readily detected in patients with cervical cancer, and several T cell epitopes have been defined (Hohn et al., 2000; van der Burg et al., 2001; Warrino et al., 2004). The current study did not investigate the role of the defined epitopes in relationship to disease. This would require larger cross-sectional and longitudinal studies of both healthy donors and patients.

Immunity to both HPV16 and HPV18 E6 was detected in three of the ten donors. Infection with multiple high-risk HPV is not uncommon in young women (Cuschieri et al., 2004) and simultaneous detection of HPV16- and HPV18-specific T-cell responses has been previously reported (Smith et al., 2005; Welters et al., 2006). This is, however, the first report in which both HPV16 and HPV18 E6 CD4+ T-cell 15mer epitopes have been defined from the same donors. It is unlikely that the peptide responses detected in this study were due to cross-reactivity between the two viruses. HPV16 and 18 are from different viral clades (A7 and A9 respectively) and although the E6 protein has an overall identity of 55%, there are no continuous stretches of identical sequence that are long enough to form CD4+ T-cell epitopes. Welters et al. (2006) recently demonstrated that T cells recognizing 52–57 aa regions of HPV16 E6 did not cross-react on the corresponding regions of HPV18 E6. We confirmed and extended this finding by mapping 15 amino acid epitopes from the majority of responses. HPV16 E6 and HPV18 E6 responses from the same donor were being directed against peptides from non-homologous regions (Table 1). Whilst it appears that the Th responses are not cross-reactive between HPV16 and HPV18 E6 peptides, the possibility that a proportion of the responses detected could represent cross-reactivity with E6 proteins from HPV's from the same viral clades cannot be excluded (Chan et al., 1995). Nevertheless, the approach described here allowed mapping of epitopes from the two HPV types most commonly associated with cervical cancer worldwide. Such an approach could be also applied to other oncogenic HPV types that have an increased prevalence in countries with a high incidence of cervical cancer (Munoz et al., 2003).

The precise allotype involved in the presentation of p37 [HPV16 E6(127–141)] was identified to be HLA-DRB1*01. The DRB1*01 allele occurs at a relatively high frequency within Caucasian (9.4%) and black (5.4%) populations, making it potentially useful for therapeutic application in these populations (Marsh et al., 2000). Although we focussed on a small cohort of young women for this study, positive T-cell responses against p37 could also be detected in older DR1+ donors. In addition to donors 1 and 5 reported here, two further female HLA-DR1+ donors (aged over 50 years, with no other HLA-DR in common), demonstrated responses against p37, after having responded to the HPV16 E6 peptide pool (K. M. E. Gallagher, unpublished observations).

The p64 epitope [HPV18 E6(43–57)] is presented by a common allotype, HLA-DRB1*15, which occurs at a frequency of 10.7% in Caucasian and 9.9% in black populations (Marsh et al., 2000). Whilst the HLA-DR allotype restriction for p65 [HPV18 E6(49–63)] was not defined in this study, a similar CD4+ T-cell epitope, HPV18 E6(52–66), was recently reported (Facchinetti et al., 2005). The E6(52–66) epitope was shown to bind promiscuously to both HLA-DRB1*03 and HLA-DRB1*16. As donor 5, from whom the p65 response was detected, is HLA-DRB1*01/14, it suggests that this epitope is more promiscuous than previously recognized. Importantly, the physiological relevance of the E6(52–66) epitope was demonstrated, as it was shown to be naturally processed (using PBMC-APC) from a full-length E6 protein (Facchinetti et al., 2005).

T-cell lines generated from donor 1 against p37 were used to examine the TCR Vβ usage of the peptide-specific CD4+ T cells. Analysis of one such line (line 46) using a panel of TCR Vβ antibodies revealed the presence of two T-cell populations, with the dominant population (80%) expressing TCR Vβ16. Interestingly, ICS analysis revealed that the subdominant population (TCR Vβ16+) was functionally superior, both in the proportion of IFN-γ-positive cells and...
in the amount of cytokine produced. Thus the dominant T-cell population generated in an in vitro response may not be the one with the best functional phenotype. This finding has implications for designing T-cell immunotherapeutic strategies based on selection of dominant TCR clonotypes responding to tumour antigens. To our knowledge, this is the first report in which the TCR Vβ usage of a CD4+ T-cell line recognizing a HPV16 E6 epitope has been described. It will be interesting to determine how frequently this TCR Vβ chain is used in the p37 response, by studying p37-specific T-cell lines from other HLA-DRB1*01+ donors. Using a PCR-based technique, the TCR Vβ chain expressed by Belx2 (Vβ16-) has subsequently been identified as TCR Vβ7 (TRBV4-2), a specificity missing from our TCR antibody panel (Dr Jonathan Boulter, School of Medicine, Cardiff University, UK, personal communication).

We demonstrated that the HPV16 E6(127–141) epitope could be processed endogenously and presented from full-length soluble E6 protein. Most peptides presented by the HLA class II pathway are derived from an exogenous source of protein antigen unless they are targeted to the lysosomes (Wu et al., 1995). Accordingly, HPV16 E6(127–141)-specific T cells were unable to recognize BLCL cells expressing HPV16 E6 endogenously after vaccinia–HPV infection (Supplementary Figure S1, available in JGV Online). This was not due to inefficient expression of HPV16 E6 because there was clear recognition of the same target cells by an HPV16 E6(29–38)-specific CTL (Supplementary Figure S1). However, there are reports of CD4+ T cells recognizing epitopes derived from endogenously expressed HPV proteins in tumour cells (Facchini et al., 2005; Hohn et al., 2000), so it will be interesting to test HPV16 E6(127–141) T cells with DRB1*01 carcinomas.

To conclude, we have developed a powerful method for epitope mapping, from which several novel candidate HPV16 and HPV18 E6 CD4+ T-cell epitopes have been identified. One of the epitopes was shown to be processed and presented from full-length HPV E6 protein. The therapeutic value of this and the other epitopes remains to be determined. Nevertheless, such epitopes could provide the basis for more precise monitoring of both naturally occurring and vaccine-induced CD4+ T-cell responses in relation to HPV disease.

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