

## HUMAN T CELL RESPONSES TO HPV 16 E2 GENERATED WITH MONOCYTE-DERIVED DENDRITIC CELLS

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**Persistent infection with human papillomavirus (HPV) type 16 has been implicated in the etiology of cervical cancer. The E2 protein is required early in viral infection and therefore may serve as a useful immune target for a vaccine aimed at prevention or therapy of premalignant lesions. Dendritic cells (DC) prepared from monocytes and pulsed with bacterially produced HPV 16 E2 C-terminus protein were used to stimulate autologous T cells over several rounds of stimulation. T cells were tested for  $\gamma$ -interferon release by ELISPOT and for cytotoxic activity by <sup>51</sup>chromium release assays. To generate E2-expressing target cells for cytotoxicity assays, we constructed a recombinant vaccinia virus encoding HPV 16 E2, which was used to infect autologous Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL). The results show that DC pulsed with E2 C-terminus protein induce  $\gamma$ -interferon-releasing T cells as demonstrated by ELISPOT. Furthermore, we demonstrate E2-specific lysis of vaccinia-E2 infected autologous LCL by CD8+ cytotoxic T lymphocytes (CTL). E2-specific CTL did not lyse untreated autologous LCL or LCL infected with wild-type vaccinia and showed low levels of cytotoxicity against natural killer cell-sensitive K562 cells. In addition, T cells stimulated with DC in the absence of E2 failed to demonstrate lysis of vaccinia-E2-labeled targets. Phenotypically, CTL populations were CD3+/CD8+. These results will facilitate the study of naturally occurring T-cell responses to HPV E2 in patients with cervical intraepithelial neoplasia and the development of immunotherapeutic strategies designed to treat this and other HPV-associated diseases.**

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**Key words:** human papillomavirus (HPV); E2 protein; dendritic cells; cytotoxic T lymphocytes (CTL);  $\gamma$ -interferon ELISPOT; primary immune responses; cervical intraepithelial neoplasia (CIN); cervical cancer; vaccines

Persistent infection with oncogenic human papillomaviruses (HPV) such as HPV 16 is necessary for the development of invasive cervical carcinoma.<sup>1–4</sup> Natural immunity plays an important role in the control of HPV infection,<sup>5–7</sup> and the identification of HPV-specific immunity in patients<sup>8–11</sup> has provided the impetus for the development of vaccines to both prevent and treat HPV-associated disease. Most therapeutic vaccines have focused on stimulating specific cell-mediated immunity to HPV 16 E6 and E7 proteins, which are expressed in all stages of HPV-related disease development and are therefore appropriate immune targets in high grade and malignant disease.<sup>12,13</sup>

Other early viral proteins could also be immune targets for vaccines aimed at treating HPV-associated disease. HPV 16 E2 is a 42-kDa protein comprising 3 functional domains: a 200-amino acid amino-terminal (N-terminal) activation domain<sup>14</sup> and a 100-amino acid carboxy-terminal (C-terminal) DNA-binding domain,<sup>15</sup> joined by a flexible hinge region.<sup>16</sup> E2 is necessary for viral replication<sup>17,18</sup> and for the transcriptional control of other viral proteins, including E6 and E7.<sup>19</sup> In cervical dysplasia, E2 expression is highest in koilocytes and decreases with increasing cervical intraepithelial neoplasia (CIN) grade.<sup>20</sup> In the cottontail rabbit papillomavirus (CRPV) model, immunization of rabbits

with DNA plasmid-encoding CRPV E2 prevented the development of skin papillomas.<sup>21,22</sup> These studies support the use of E2 as an immunologic target in vaccines for the protection or therapy of HPV infection and CIN. Thus virus-like particles composed of the viral capsid proteins (L1 and L2) for use in prophylaxis of HPV-associated disease have been modified to include E2 and E7 proteins. The idea is that generation of cellular immunity against the early genes may induce the regression of subclinical infections that result from imperfect neutralization of virus by antibodies.<sup>23</sup> However, E2 gene disruption is associated with invasive disease, mechanistically via deregulation of viral oncogene expression. This association could provide an escape from E2-specific immunity, although integration into the cellular genome is not always seen in higher grade lesions and carcinomas.<sup>24</sup>

Whether E2-specific cellular immune responses play a role in the natural history of HPV infection is not known. Two studies have demonstrated helper T-cell responses to HPV 16 E2 in patients with cervical neoplasia. Lehtinen *et al.*<sup>25</sup> showed T-cell proliferation in response to E2 peptides in 3 of 6 women with CIN 3 or cervical carcinoma. Bontkes *et al.*<sup>26</sup> investigated helper T-cell responses to the C-terminus and N-terminus of the HPV 16 E2 protein in a cohort of 74 women with CIN. Although responses to the N-terminus were nonspecific, longitudinal analysis revealed that responses to the C-terminus occurred coincident with viral clearance.<sup>26</sup> Cytotoxic T lymphocyte (CTL) responses to HPV 16 E2 have not been extensively studied. Konya *et al.*<sup>27</sup> identified a human leucocyte antigen (HLA)-A2 E2 epitope that was capable of eliciting peptide-specific CTL in normal donors. However, the peptide-based epitope approach is restricted by the necessary use of immunodominant or subdominant peptides and by the requirement for a particular HLA type.<sup>28,29</sup>

In the present study we investigated whether HPV 16 E2-specific CTL can be induced in normal volunteers using E2 C-terminus protein-pulsed autologous dendritic cells (DC). Using the intact protein provides all potential peptide epitopes if processed and presented by efficient antigen-presenting cells (APC) like DC, which are capable of activating naïve T cells.<sup>30</sup> A recombinant

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vaccinia virus encoding HPV 16 E2 was constructed and used to infect autologous Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL) as targets in cytotoxicity assays and CTL were also tested by  $\gamma$ -interferon ( $\gamma$ -IFN) ELISPOT.

#### MATERIAL AND METHODS

##### *Production of E2 C-terminus protein*

The 86-amino acid HPV 16 E2 C-terminus protein was expressed in *Escherichia coli* XL1-blue cells using the expression vector pKK-E2Ct and purified according to Webster *et al.*<sup>31</sup> The protein was shown to be a single band by Coomassie-stained SDS-PAGE and reactive with a polyclonal rabbit anti-E2 C-terminus antibody by Western blot (data not shown).

##### *Description of vaccinia-E208 construction*

The E2 open reading frame (ORF) from HPV 16 (nucleotides 2756–3851) was amplified by PCR and cloned into vaccinia virus insertion vector p1108, which placed the ORF under the control of the 7.5K early/late vaccinia virus promoter. The expression cassette was then inserted into the TK locus of vaccinia virus strain WR by homologous recombination, followed by selection for a cotransferred *gpt* marker (guanine phosphoribosyltransferase). Five clones were isolated by several rounds of plaque purification and tested for successful insertion of the E2 gene into the TK locus using PCR. Expression of the E2 protein by recombinant virus was investigated by immunofluorescence microscopy of infected HeLa cells using a rabbit polyclonal antibody against the C-terminal region of the E2 protein<sup>31</sup> at a concentration of 1:100 and a swine anti-rabbit-fluorescein isothiocyanate antibody at 1:40 (Dako). Vaccinia-E2 infected CV-1 cells were also analyzed by Western blot for E2 protein expression. Cells infected with wild-type vaccinia and cells infected with vaccinia-E2 were prepared 24 hr before analysis by SDS-PAGE to allow maximal protein expression by the cells. Cellular protein was extracted, reduced and fractionated by 10% SDS-PAGE before being transferred to nitrocellulose. Rabbit anti-E2 C-terminus polyclonal antibody was used at a concentration of 1:50 followed by development using enhanced chemiluminescence (Amersham-Pharmacia).

##### *Isolation of peripheral blood mononuclear cells (PBMC) and culture of DC*

Peripheral blood of 3 healthy donors was obtained every 7 days. Donor A was HLA A2 negative; donors B and C were HLA A2 positive. Venous blood was heparinized (6000 IU heparin/20 ml blood) and 10 ml layered onto 10 ml Lymphoprep lymphocyte separation medium (LSM, Nycomed) and centrifuged at 900g for 20 min at 20°C. PBMC were collected from the LSM interface and washed by centrifuging at 400g in PBS for 3 × 10 min. PBMC at 4 × 10<sup>6</sup> cells/ml in X-VIVO 10 medium (Biowhittaker UK Ltd) supplemented with 2 mM glutamine were transferred to tissue culture plates and incubated at 37°C for 2 hr. The nonadherent peripheral blood lymphocytes (PBL) were collected and 3 ml of X-VIVO 10 supplemented with 2 mM glutamine (Life Technologies), 100 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 50 ng/ml interleukin-4 (IL-4; both from R & D systems) was added to each well of adherent cells and the plates incubated at 37°C in 5% CO<sub>2</sub>. Fresh medium supplemented with GM-CSF and IL-4 was added to the cultures on day 4. All T-cell stimulation was with day 7 DC. As described below, the DC were matured with lipopolysaccharide (LPS) on day 6 and E2 antigen added for effective processing at this time. Peptides do not need the latter so were added on day 7.

##### *DC pulsing with E2 C-terminus protein*

N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP) liposomal transfection reagent (25  $\mu$ g) (Roche) and bacterially produced purified HPV 16 E2 C-terminus protein (10  $\mu$ g) were mixed at room temperature in polystyrene tubes for 20 min. After this time, 3 × 10<sup>5</sup> DC in 400  $\mu$ l X-VIVO 10 with 100 ng/ml GM-CSF were added to the DOTAP/protein

mixture. The DC were incubated for 4 hr at 37°C, washed twice, resuspended in 1 ml X-VIVO 10 supplemented with 100 ng/ml GM-CSF, 50 ng/ml IL-4 and 10 ng/ml LPS (Sigma) and incubated at 37°C to mature the DC. After 24 hr, DC were harvested, washed twice and resuspended in 100  $\mu$ l RPMI 1640 (Life Technologies).

##### *DC pulsing with influenza matrix peptide*

Day 6 DC were suspended in 1 ml X-VIVO 10 supplemented with 100 ng/ml GM-CSF, 50 ng/ml IL-4 and 10 ng/ml LPS, and matured at 37°C for 24 hr. After this time, 3 × 10<sup>5</sup> DC in 400  $\mu$ l X-VIVO 10 with 100 ng/ml GM-CSF was added to 10  $\mu$ g HLA A2.1 binding influenza matrix peptide 58–66, sequence GILG-FVFTL (Alta Bioscience) and incubated for 4 hr at 37°C. The DC were washed twice and suspended in 100  $\mu$ l RPMI.

##### *CD4 depletion of PBL*

PBL were suspended in 80  $\mu$ l of ice cold Minimacs buffer (0.5% BSA, 0.005 M EDTA in PBS, filter sterilized) and 20  $\mu$ l CD4+ beads (Miltenyi Biotec) per 10<sup>7</sup> cells. The cells were incubated at 4°C for 20 min, then washed with Minimacs buffer at 400g for 10 min. The Minimacs column (Miltenyi Biotec) was prepared by attaching to the magnet and washing twice with 0.5 ml Minimacs buffer. The cells were suspended in 0.5 ml buffer and added to the top of the prepared column. The CD4-depleted fraction was collected as the column flow-through. The rationale was to favor CTL responses without removing all the T-helper activity required for optimal activation of CD8 cells.

##### *Generation of E2 CTL*

Autologous CD4-depleted PBL at a concentration of 3 × 10<sup>6</sup>/ml in RPMI 1640 supplemented with 10% human AB serum (Quest Biomedical), 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin (Life Technologies) were added to E2 protein-loaded DC with 10 ng/ml IL-7 (R & D systems) and cultured for 7 days. Every 2 days, 1 ml of spent medium was replaced with 1 ml fresh medium supplemented with 10 ng/ml IL-7. After 7 days in culture, T cells were restimulated using fresh day 7 DC pulsed with protein/peptide according to the methods described above. Cultures were incubated overnight at 37°C before being supplemented with 10 ng/ml IL-7 and 20 IU/ml IL-2 (R & D systems). The cultures were then incubated for 7 days. Every 2 days, 1 ml spent medium was replaced with 1 ml fresh medium supplemented with 10 ng/ml IL-7 and 20 IU/ml IL-2. T cells were tested in cytotoxicity assays after 1 or several rounds of stimulation.

##### *ELISPOT assay*

CD4-depleted PBL as above were added to autologous DC (loaded or not with E2 protein/flu peptide) and cultured in RPMI + 10% AB serum for 4 or 7 days. The 7-day cultures were restimulated with either autologous PBMC or DC (with or without antigen). ELISPOT<sup>2</sup> was performed 4 days after the first or second stimulation. The cultures were harvested, washed and seeded in 3 replicate wells at 4 doubling dilutions from 2 × 10<sup>5</sup> cells/well of a multiscreen 96-well Immobilon-P filtration plate (Millipore) coated with anti  $\gamma$ -interferon antibody [capture mAb (1-D1K), ELISPOT assay kit, Mabtech] and processed as per kit instructions. Initial experiments incubated PBMC at 2 × 10<sup>6</sup>/ml with 10  $\mu$ g/ml of flu peptide or E2 protein in the ELISPOT plates for 48 hr. Spots were counted at each cell density using a dissecting microscope, scores normalized and the mean and standard errors calculated. The number of specific events was calculated by subtracting the normalized number of spots in control wells from those in antigen-stimulated wells.

##### *Cytotoxicity assay*

*Preparation of target cells.* First, 10<sup>7</sup> PBMC were suspended in 200  $\mu$ l of B95-8 Epstein Barr virus stock (donated by Dr. M. Mackett, Molecular Biology Group, PICR, UK). The cells were incubated at 37°C for 1 hr, washed and resuspended in RPMI + 10% FCS, 2 mM glutamine, 100  $\mu$ g/ml streptomycin and 100 IU/ml penicillin at 5 × 10<sup>5</sup>/ml. Phytohaemagglutinin (Murex

Diagnostics) was added at 0.5  $\mu\text{g}/\text{ml}$  and cells plated out at 2 ml/well. The cultures were checked weekly and 1 ml medium/well replaced with fresh RPMI + 10% FCS as appropriate.  $1 \times 10^6$  autologous LCL cells were pelleted and infected for 1 hr at 37°C with 10 pfu/cell of virus, 24 hr before the assay. Viruses used were WR (wild-type vaccinia) and vE208 (recombinant vaccinia virus encoding HPV 16 E2). After 1 hr the cells were washed 3 times and incubated overnight in RPMI + 10% FCS before labeling with  $^{51}\text{Cr}$ . All target cell lines were labeled with 100  $\mu\text{Ci}$  (20  $\mu\text{l}$ ) of  $\text{Na}_2^{51}\text{CrO}_4$  (NEN) for 1 hr on the day of the assay, washed 3 times and placed in a 96-well plate in aliquots of  $2 \times 10^3$  targets/well.

**Preparation of effector cells.** T cells were washed and resuspended in RPMI + 10% AB serum to give a maximum concentration of  $2 \times 10^5$  effectors/well. Serial dilutions were prepared, with each cell concentration represented in triplicate. Maximum  $^{51}\text{Cr}$  release was determined from supernatants of target cells that were lysed with 100  $\mu\text{l}$  of PBS/2% Tween 20 and spontaneous  $^{51}\text{Cr}$  release was determined by adding target cells only to the wells. Plates were incubated at 37°C for 4 hr and 100  $\mu\text{l}$  of the supernatant from each well transferred to Lumaplates (Packard) and left to dry overnight. The plates were read in a Top Count plate reader (Packard). Specific lysis was calculated as: (specific release - spontaneous release)  $\div$  (maximum release - spontaneous release)  $\times 100$ . Spontaneous release was less than 20% of maximum release. Samples were considered positive when the difference in specific lysis between the recombinant vaccinia virus infected LCL and the wild-type infected LCL was more than 10% over 2 or more E:T ratios.

## RESULTS

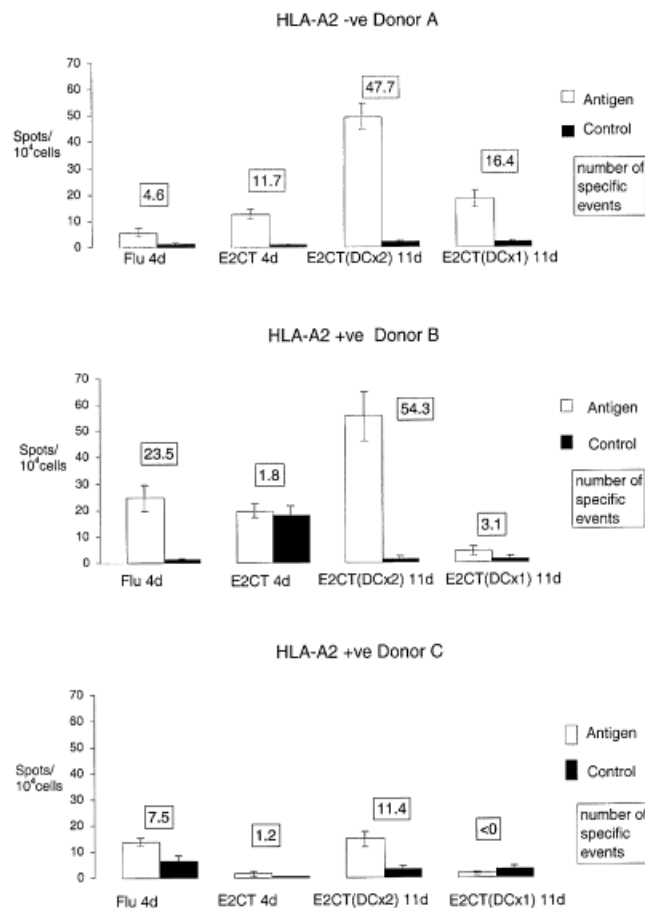
### Phenotypic analysis of DC

DC were prepared from adherent PBMC in serum-free medium with GM-CSF and IL-4. After 6 days of culture, DC were pulsed with E2 C-terminus protein and DOTAP or with DOTAP alone for 4 hr and then matured with LPS for 24 hr. Flu peptide was added on day 7 to matured DC. The purity of the resulting DC populations was determined by flow cytometry for the cell surface markers CD1a, HLA-DR, CD80 and CD86. High proportions of the monocyte-derived DC populations were positive for CD1a (66%), HLA-DR (96%), CD80 (49%) and CD86 (61%).

### E2-specific CD8+ T-cell responses: ELISPOT

PBMC were pulsed with E2 C-terminus protein and added to the ELISPOT plate at  $2 \times 10^6/\text{ml}$  for 48 hr. Untreated PBMC were included as a negative control and HLA A2 influenza peptide-pulsed PBMC as a positive control. A response for the influenza peptide was detected in the 2 HLA A2 -positive donors (12.5 spots/ $10^5$  cells for donor B; 8.5 spots/ $10^5$  cells for donor C), but not in the HLA A2-negative donor (0 spots/ $10^5$  cells for donor A). No responses were detected for PBMC pulsed with E2 C-terminus protein for any of the donors (data not shown). Experiments were repeated 3 times for each donor with consistent results. These results reflect immunologic memory of exposure to influenza by HLA A2-positive individuals. This is because primary *in vitro* antigen stimulation of naïve cells does not lead to  $\gamma$ -IFN release. The lack of response to E2 protein may indicate a low frequency of E2-specific CTL in the peripheral blood of normal volunteers or poor processing and presentation of the whole protein to CTL by nonprofessional APC.

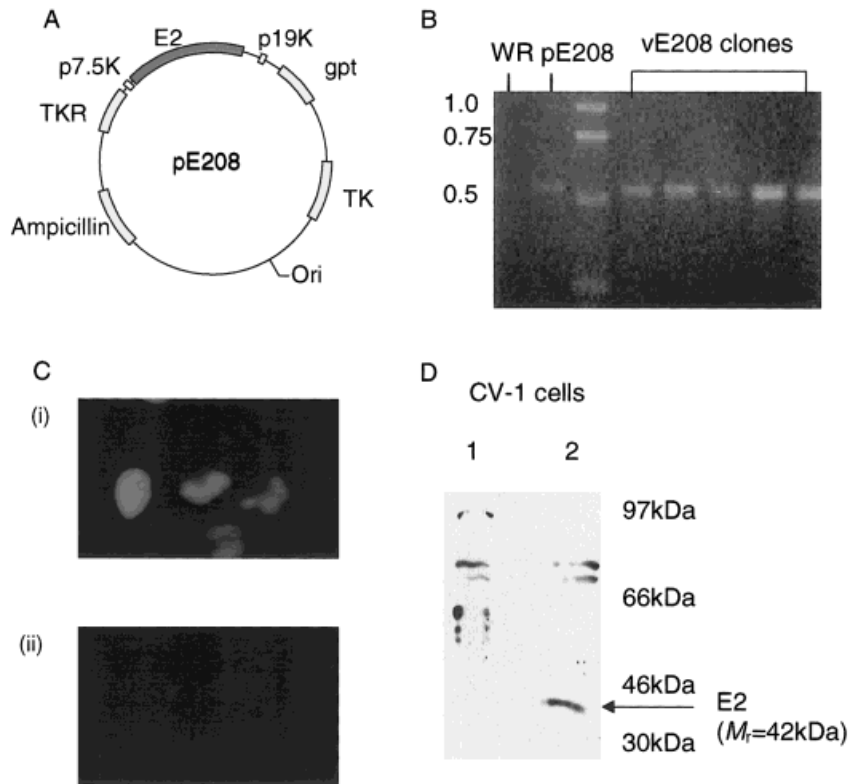
This phenomenon was studied further using functional monocyte-derived DC pulsed with E2 C-terminus protein, HLA A2 influenza peptide or no antigen, to stimulate autologous CD8+ enriched T cells. After 7 days, the T cells were restimulated with fresh E2 C-terminus-pulsed DC or DC alone, or with E2 C-terminus-pulsed PBMC or PBMC alone.  $\gamma$ -IFN ELISPOT was performed at 4 days after the first or second stimulation (Fig. 1). Experiments were repeated 3 times for each donor with consistent results.



**FIGURE 1** – ELISPOT data for 3 normal donors showing the mean number of spots and standard errors per  $10^4$  cells after 4 days stimulation with dendritic cells (DC) pulsed with flu peptide (Flu 4d) or E2 C-terminus protein (E2CT 4d) and after 11 days stimulation with DC pulsed with E2 C-terminus protein [E2CT(DCx2) 11d] or 11 days stimulation with DC pulsed with E2 C-terminus (first 7 days) followed by stimulation with PBMC pulsed with E2 C-terminus (second 4 days) [E2CT(DCx1) 11d]. The number of specific events = number of spots in control wells - number of spots in antigen-stimulated wells.

For the HLA A2-negative donor A, 11.7 specific events/ $10^4$  cells were seen after the first stimulation with DC pulsed with E2 C-terminus protein compared with 4.6 specific events/ $10^4$  cells with HLA A2-restricted flu peptide. For HLA A2-positive donors B and C, 1.8 and 1.2 specific events/ $10^4$  cells, respectively, were seen with DC pulsed with E2 C-terminus protein compared with 23.5 and 7.5 specific events/ $10^4$  cells after 4 days' stimulation with DC pulsed with flu peptide. These results show evidence of a higher frequency of memory T cells to the flu peptide in HLA A2-matched individuals than that detected after PBMC stimulation.

Four days after a second stimulation with DC + E2 C-terminus protein, the number of specific events for donors B and C was 54.3 and 11.4 per  $10^4$  cells, respectively, compared with 1.8 and 1.2 per  $10^4$  cells after the first stimulation. The same increase in specific events was not seen when the restimulation at 7 days was with PBMC + E2 C-terminus (3.1 and <0 specific events/ $10^4$  cells for donors B and C, respectively). For donor A, the 2 stimulations with DC loaded with HPV 16 E2 C-terminus caused the number of specific events to increase 4-fold from 11.7 per  $10^4$  cells to 47.7 per  $10^4$  cells. Unlike the other donors, donor A also showed an increase in the number of specific events after DC + E2 C-



**FIGURE 2** – Characterization of vE208, a recombinant vaccinia virus encoding human papillomavirus (HPV) type 16 E2 protein (HPV 16 E2). (a) Plasmid map of the vaccinia insertion vector encoding HPV 16 E2. The expression cassette of HPV 16 E2 driven by the p7.5K promoter and *gpt* (guanine phosphoribosyltransferase) driven by the p19K promoter is flanked by vaccinia *tk* sequences to allow homologous recombination. (b) Clones with the correct size of insert by PCR. WR, wild-type vaccinia; pE208, E2 vaccinia transfection plasmid (1 ng); vE208, vaccinia-E2. MBI 1Kb DNA ladder. 25 cycles PCR, annealing temperature 40°C for 1 min. (c) Immunofluorescent-staining of HeLa cells infected at 10 pfu/cell with (i) vE208 and (ii) WR, stained with polyclonal rabbit anti-E2 C-terminus antisera at dilutions of 1:100. (d) Western blot of E2 protein expression by CV-1 cells. Reduced 10% SDS-PAGE of cells infected with WR (lane 1) and cells infected with vE208 (lane 2), detected with 1:50 dilution of polyclonal rabbit anti-E2 C-terminus antibody.

terminus for 7 days followed by PBMC + E2 C-terminus for 4 days (16.4 per  $10^4$  cells).

#### Characterization of vaccinia-E2

To generate E2-expressing target cells for cytotoxicity assays, we constructed a recombinant vaccinia virus encoding HPV 16 E2, which was used to infect autologous Epstein-Barr virus-transformed LCL (Fig. 2). Five clones of vaccinia-E2 were isolated after 3 rounds of plaque purification and tested for the successful insertion of the E2 gene by PCR (Fig. 2b). Recombinant virus was used to infect HeLa cells at 10 pfu/cell and expression of the E2 protein was confirmed by immunofluorescence microscopy, using a rabbit anti-E2 C-terminus polyclonal antibody. All 5 clones proved positive for E2 protein expression (Fig. 2c). A single clone was selected and designated vE208. vE208-infected CV-1 cells were also analyzed by Western blot for E2 protein expression. The Western blot (Fig. 2d) shows a band near the predicted molecular weight of the full-length E2 protein of 42 kDa with the rabbit anti C-terminus antibody in the CV-1 cells infected with vE208, but not in the CV-1 cells infected with WR.

#### E2-specific CD8+ T-cell responses: cytotoxicity assays

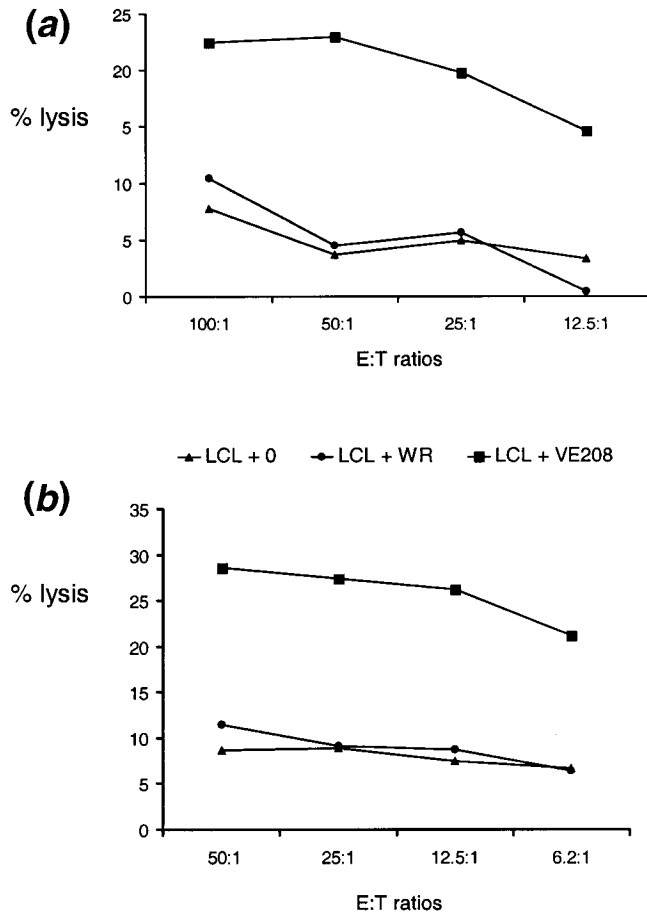
T-cell responses were tested by  $^{51}\text{Cr}$  release assays after 2, 3, 4 and 5 stimulations with autologous monocyte-derived DC pulsed with E2 C-terminus protein or with DC alone. Target cells were autologous LCL alone, autologous LCL infected with WR, autologous LCL infected with vE208 and K562 cells. Target cell lysis by T cells stimulated with DC pulsed with E2 C-terminus protein was compared with T cells stimulated with DC in the absence of E2. Assays were repeated 4 times for each donor with comparable results. Donors B and C consistently showed evidence of E2-specific lysis of vE208-infected autologous LCL (Fig. 3). E2-specific CTL did not lyse untreated autologous LCL or LCL infected with WR and showed low levels of cytotoxicity against natural killer cell-sensitive K562 cells (less than 6% lysis). Furthermore, T cells stimulated with DC in the absence of E2 failed to demonstrate lysis of vE208-labeled targets. Phenotypically,

CTL populations were CD3+/CD8+ and CD4- as assessed by flow cytometry. E2-specific lysis first became apparent after 3 or 4 stimulations of T-cell cultures, depending on the donor. Donor A did not demonstrate E2-specific lysis of vE208-infected autologous LCL.

#### DISCUSSION

In this study, we have investigated T-cell responses to the HPV 16 E2 C-terminus in normal volunteers. Populations of functional monocyte-derived DC were generated with surface markers as previously described.<sup>33–36</sup> They were pulsed with E2 C-terminus protein and used to stimulate autologous T cells over several rounds of stimulation and these were tested for specific activity by ELISPOT and  $^{51}\text{Cr}$ -release assay. ELISPOT is a rapid and sensitive assay that detects  $\gamma$ -IFN secretion by antigen-specific memory T cells in response to antigen exposure.<sup>32,37,38</sup> Usually, the ELISPOT assay uses HLA-restricted peptides as a source of antigen, but the assay was adapted in the present study to allow the potential detection of memory and primary T-cell responses to an HPV protein antigen after it has been processed and presented by autologous APC. This approach has the obvious advantage that it more closely resembles antigen presentation *in vivo* and allows CTL responses to be studied regardless of an individual's tissue type.

Primary *in vitro* stimulation of naïve T cells can lead to IL-2 production and proliferation but not  $\gamma$ -IFN secretion; this occurs following re-encounter with antigen.<sup>39</sup> Unstimulated PBMC pulsed with E2 C-terminus protein did not induce specific  $\gamma$ -IFN secretion by T cells in any of the donors. This finding is consistent with a frequency of memory CTL in the peripheral blood of normal volunteers below the threshold of detection. However, T-cell responses to HPV 16 E2 were demonstrated by ELISPOT after a single stimulation of T-cell cultures with protein-loaded DC for donor A, and after 2 stimulations for donors B and C. Thus in individual A there may be a memory response that is detectable after appropriate antigen presentation or a small expansion *in vitro*. This finding was in contrast to the  $^{51}\text{Cr}$ -release assays in which a



**FIGURE 3** – Cytotoxicity assays for donors B and C after stimulation of T-cell cultures with autologous dendritic cells (DC) pulsed with E2 C-terminus protein. (a) Donor B anti E2 T cells after 3 stimulations and (b) donor C anti E2 T cells after 4 stimulations *in vitro*. E2-specific lysis is measured by comparing lysis of  $^{51}\text{Cr}$ -labeled autologous lymphoblastoid cell lines (LCL) infected with vaccinia-E2 (LCL + vE208) with  $^{51}\text{Cr}$ -labeled LCL infected with wild-type vaccinia (LCL + WR) or unlabeled LCL (LCL + 0) at a number of different effector:target (E:T) ratios.

minimum of 3 *in vitro* stimulations were required before E2-specific lysis became apparent. Donor A was female, HLA A2 negative with no history of influenza; donor B was male, HLA A2 positive with a history of 2 recent episodes of influenza; and donor C was male, HLA A2 positive with a questionable history of influenza. After a single stimulation with either PBMC or DC pulsed with flu peptide, T cells from donors B and C showed evidence of a memory response to influenza. However, the frequency detected was 5.3- and 11.3-fold more with DC stimulation than with PBMC stimulation for donors B and C, respectively. By contrast, no significant E2 responses were seen in these donors. Donor A showed some evidence of a “memory” response to E2 compared with the irrelevant test for an HLA A2 flu peptide response after the same 4-day stimulation procedure. This E2 memory response could be enhanced or boosted by a second stimulation with protein-loaded DC ( $47.7/10^4$  cells) or PBMC ( $16.4/10^4$  cells). This result might reflect priming of naïve T cells or

an expansion of the existing memory response with antigen effectively presented by the DC. A memory response to HPV 16 E2 in a healthy volunteer may be explained by previous subclinical exposure to the virus. Genital HPV infection rates in women with normal cervical cytology varies with age and the number of lifetime sexual partners, but may be as high as 25% in women in their early 20s.<sup>40</sup> Many of these infections are transient and disappear without ever causing dysplasia.<sup>41–43</sup>

Donors B and C did not have evidence of memory T-cell responses to HPV 16 E2, but putative primary T-cell responses could be generated *in vitro* after 2 stimulations with E2 C-terminus protein-loaded DC and quantified by ELISPOT assays. These apparent primary T-cell responses required antigen processing and presentation by DC as PBMC appeared to be ineffective and insufficient for eliciting the response. This finding is in contrast to donor A in whom expansion of the existing memory response to E2 could be supported by DC or PBMC, although the magnitude of response was 3-fold lower in the latter case. This finding is consistent with the theory that PBMC are able to restimulate previously activated T cells but are unable to prime naïve T cells. Studies by van der Burg *et al.*<sup>44</sup> have shown that after primary activation of T cells,  $\gamma$ -IFN release only begins after stimulation with antigen for a second time. Using overlapping peptides to HPV 16 E7 they have mapped the E7 peptide epitope responses in normal individuals and patients with CIN or cervical cancer. The exposure to the pathogen is correlated with positive short-term ELISPOT assays in about 30% of cases.<sup>44</sup>

In the present study, all 3 donors demonstrated T-cell responses to E2 by  $\gamma$ -IFN ELISPOT, and only 2 of the 3 showed E2-specific cytotoxicity by  $^{51}\text{Cr}$ -release assays. This result required a minimum of 3 stimulations so it is not possible to know whether these are primary or memory-derived effectors. It is possible that the anti-E2 T cells generated for donor A were  $\gamma$ -IFN-releasing but not active in CTL assays. In any case, the specific release of  $\gamma$ -IFN by CTL may be sufficient to promote the eradication of HPV infection even in the absence of cytotoxicity. Although little is known about natural anti-HPV immunity, cytotoxicity and the release of type 1 cytokines such as  $\gamma$ -IFN and IL-2 are thought to be essential for effecting viral clearance.<sup>45</sup> Whether antigen-specific cytotoxicity or antigen-specific cytokine release plays the dominant role in viral clearance has yet to be established, but efficient anti-HPV immunity probably depends on a complex interaction of both these and other immune mechanisms.

In this report we have demonstrated that HPV 16 E2-specific CTL can be elicited in normal volunteers using E2 C-terminus protein-pulsed autologous DC. The resulting T-cell populations are active in the  $\gamma$ -IFN ELISPOT system after 1 or 2 stimulations *in vitro*, and by  $^{51}\text{Cr}$ -release assays after 3 or 4 stimulations for 2 of the 3 donors. The utility of this type of methodology in the study of natural immune responses to E2 in cervical neoplasia patients now needs to be investigated and compared with the overlapping peptide approaches that have been used for HPV oncogenes.<sup>44</sup>

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