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Dendritic cells infected with recombinant fowlpox virus vectors are potent and long-acting stimulators of transgene-specific class I restricted T lymphocyte activity

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The identification of dendritic cells (DC) as the major antigen-presenting cell type of the immune system, combined with the development of procedures for their ex vivo culture, has opened possibilities for tumour immunotherapy based on the transfer of recombinant tumour antigens to DC. It is anticipated that the most effective type of response would be the stimulation of specific, MHC class I restricted cytotoxic T lymphocytes capable of recognising and destroying tumour cells. In order to make this approach possible, methods must be developed for the transfer of recombinant antigen to the DC in such a way that they will initiate an MHC class I restricted response. Here, we demonstrate that murine DC infected with a recombinant fowlpox virus (rFWPV) vector stimulate a powerful, MHC class I restricted response against a recombinant antigen. A rFWPV containing the OVA gene was constructed and used to infect the DC line DC2.4. The infected DC2.4 cells were found to stimulate the T-T cell hybridoma line RF33.70, which responds specifically to the MHC class I restricted OVA peptide SIINFEKL. The stimulatory ability of the rFWPV-infected DC2.4 cells lasted for at least 72 h after infection and was eventually limited by proliferation of uninfected cells. By comparison, DC2.4 cells pulsed with synthetic SIINFEKL peptide stimulated RF33.70 well initially, but the stimulatory ability had declined to zero by 24 h after pulsing. FWPV infection of DC2.4 up-regulated MHC and costimulatory molecule expression. rFWPV was also found to infect both immature and mature human DC derived from cord blood CD34$^+$ progenitors and express trangenes for up to 20 days after infection. We conclude that rFWPV shows promise as a vector for antigen gene transfer to DC in tumour immunotherapy protocols. Gene Therapy (2000) **7,** 1680–1689.

Keywords: *Dendritic cells; fowlpox virus; transgene; immunotherapy; MHC class I; hybridoma*

Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the immune system and are crucial for the initiation of T cell responses to pathogens and tumours.[1,2] It is possible to generate clinically relevant quantities of DC from a variety of sources: by expansion and differentiation of CD34$^+$ cells from bone marrow,[3] cord blood[4] and the peripheral circulation[5] or by *in vitro* differentiation and maturation of peripheral blood mononuclear cells.[6] This has encouraged the development of protocols involving the readministration to patients of *ex vivo*-modified autologous DC with the aim of initiating and directing an immune response against a particular pathogen or tumour target. With respect to cancer targets expressing tumour antigens intracellularly, it is anticipated that the most effective type of immune response would be stimulation of specific, MHC class I restricted, cytotoxic T lymphocytes (CTL). In order to make such approaches possible, methods must be developed for the *ex vivo* modification of DC in such a way that they will stimulate a CTL response against the desired target antigen.

Previous studies have shown that it is possible to transfer specific antigens to DC to induce an immune response both in animal models and in humans. DCs pulsed with MHC class I-binding peptide epitopes have been shown to induce CTL responses against model antigens in mice and provide protection against challenge from tumour cells encoding these antigens.[7–9] In humans, DC pulsed with HIV peptides were found to initiate primary *ex vivo* CTL responses from CD8$^+$ T lymphocytes isolated from healthy donors.[10] Peptide-pulsed DC from B cell lymphoma, disseminated prostatic carcinoma, and melanoma patients have been shown to stimulate specific CTL activities.[11–13]

There are, however, limitations associated with the use of MHC class I-binding peptides as a means of modifying DC to induce specific CTL responses in patients. Detailed

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knowledge is required of the binding and immunodominance of each candidate peptide. The epitopes thus identified are suitable only for patients with the appropriate HLA type and the peptides chosen may not include epitopes capable of stimulating $CD4^+$ help. Peptide binding to MHC class I molecules has been shown to be both rapid and reversible,[14] whereas prolonged exposure to antigen may be necessary for the induction of an efficient T cell response.[15] Moreover, repeated stimulations of T cells with peptide-loaded DC can lead to the emergence of non-cytolytic $CD4^+$ cells which block the activation of fresh T lymphocytes, leading to immune unresponsiveness.[16]

It has been known for some time that DNA vaccination using direct injection or gene gun can produce immune responses in experimental animals. However, this procedure is inefficient requiring tens or hundreds of micrograms of plasmid DNA for inoculation of mice and much larger amounts for primates.[17,18] The amounts of DNA required can be reduced significantly by *ex vivo* transfection of DC before readministration, nevertheless, transfer of plasmid DNA to DC is relatively inefficient.[19]

Viral vector systems have the potential advantage of high gene transfer efficiencies. Arthur *et al*[20] conducted a comparison of methods for gene transfer to human DC and found that gene transfer using recombinant adenoviruses (rAd) was far superior to any of the transfection methods tested. Additionally, because of their capacity to encode whole antigens or even several antigens, viral vectors may be able to address the epitope and HLA type restrictions imposed by peptide pulsing approaches. Moreover, intracellular synthesis of recombinant antigen by virally transduced DC may provide a longer duration of stimulation than peptide-pulsed DC, which may be crucial to the success of DC reinfusion protocols. Brossart *et al*[21] showed that murine DC infected with recombinant vaccinia virus (rVV) or rAd expressing an OVA peptide were able to stimulate CTL responses efficiently *in vitro* and *in vivo*. In humans, recombinant adenoviruses expressing *LacZ* have been shown to infect both mobilised $CD34^+$ cells and mature DC.[22] Using EBV seropositive donors, PBMC-derived DC infected with rAd expressing the EBV LMP2B antigen induced EBV-specific CTL responses.[23] Infection of human PBMC-derived DC requires high multiplicities of rAd infection for maximal expression and induces some maturation of the DCs.[24]

Recombinant poxviruses are extremely efficient gene transfer agents and have a long history of ability to generate CTL responses against recombinant antigens in both animal models and humans.[25] Human and mouse DC can be infected with rVV expressing antigen genes from early, but not late promoters.[26,27] DC infected with a rVV were able to protect mice from challenge with tumour cells expressing a model antigen.[26] In humans, rVV infection of peripheral blood preparations enriched for DC was shown to generate CTL reactive against the transgene.[28]

The use of rVV based on replication-competent strains of vaccinia virus is undesirable due to concerns over the safety of such agents.[29–31] This problem has been addressed by using host range restricted poxviruses such as Modified Vaccinia Ankara or fowlpox virus (FWPV) to infect DC.[22,27,32] Avipoxviruses such as FWPV are naturally host range restricted to avian cells. However, they can initiate an abortive infection in mammalian cells, demonstrating early and in some cases late gene expression without production of viable progeny.[27,33,34] Immunisation of mice with recombinant FWPV expressing *LacZ* has been shown to protect mice from challenge against a tumour line which had been transfected with the *LacZ* gene.[35]

In this study we used a murine model system to investigate whether recombinant FWPV could infect DC and stimulate an MHC class I restricted immune response. rFWPV were found to be capable of infecting an immortalised DC line and expressed the transgene from an early, but not a late promoter. FWPV infection up-regulated the expression of surface molecules required for antigen presentation which are associated with DC maturation. DC infected with rFWPV expressing the chick ovalbumin gene were able to process and present the MHC class I restricted peptide SIINFEKL, and stimulated a T-T hybridoma line that expresses a T cell receptor specific for the SIINFEKL peptide. Immunostimulatory activity of rFWPV infected DC far outlasted the activity of DC pulsed with synthetic SIINFEKL peptide. We further showed that rFWPV can infect human DC derived from $CD34^+$ progenitors, and that detectable expression lasted for up to 20 days after infection. These observations encourage the development of rFWPV as an additional modality for *ex vivo* antigen transfer to DC.

Results

Infection of DC2.4 dendritic cells using rFWPV

Recombinant fowlpox viruses expressing *LacZ* were examined for their abilities to infect and express the transgene in the immortalised murine DC cell line DC2.4.[36] The cells were infected at a multiplicity of 10 p.f.u. per cell with rFWPV expressing *LacZ* under the control of an early (FpEFL2) or late (FpEFL29) promoter. Expression of β-galactosidase was monitored every 24 h for 5 days and at 10 and 20 days after infection (Figure 1a). β-Galactosidase expression was observed in cells infected with FpEFL2, but not in cells infected with FpEFL29, wild-type Fowlpox virus (Fp9) or uninfected cells. This observation concurred with our previous findings, made using human DC, that rFWPV must utilise early promoters for transgene expression in DC.[27] β-Galactosidase expression was detected in the cultures up to 20 days after infection, however, the levels reached their maxima after 3–5 days. Since β-galactosidase is a very stable protein, these observations suggested that synthesis of the protein under control of the early promoter stopped after 3–5 days, while the protein remained present in the cultures for the duration of the experiment.

In order to confirm that cells with the appropriate morphology had been infected, DC2.4 cells were cultured on poly-L-lysine-coated slides then infected with FpEFL2 for 3 days, stained with X-gal and observed by phase contrast microscopy. Figure 1b shows that cells with a typical DC morphology were infected and expressed β-galactosidase.

rFWPV infection up-regulates immunostimulatory cell surface molecules

Poxviruses have a large repertoire of non-essential genes involved in the modulation of the host immune response to infection.[37,38] We investigated the effects that wild-type

FWPV infection had on the expression by DC2.4 of cell surface molecules involved with antigen presentation or capture. DC2.4 cells were infected with Fp9 for 3 days then analysed by FACS for surface expression of the peptide-presenting molecules MHC class I, class II, costimulatory molecules B7.1, B7.2, ICAM-1 adhesion molecule and the endocytosis mediator DEC-205. Infection with Fp9 led to an increase in expression of all six immunostimulatory molecules investigated (Figure 2). This suggested that DC2.4 cells infected with FWPV are likely to retain, or indeed enhance, their antigen-presenting capabilities.

Construction of rFWPV expressing chick ovalbumin

We aimed to investigate the capacities of rFWPV infected DC2.4 to stimulate an MHC class I restricted immune response, by employing a T-T cell hybridoma (RF33.70) which expresses a T cell receptor specific for the MHC class I H-2K^b restricted chick ovalbumin peptide SIINFEKL.[39] RF33.70 responds to engagement of the T cell receptor by secretion of IL-2.[39] In order to utilise this model, it was necessary to produce a rFWPV containing the chick ovalbumin (OVA) gene. An OVA cDNA was placed under the control of a synthetic early/late promoter and inserted into ORF-1 of the 6.8 kb terminal *Bam*HI fragment of Fp9 (Figure 3a.). To facilitate selection of recombinant virus, the *E. coli gpt* gene under the control of the vaccinia virus 7.5K promoter was inserted into the same site, transcribing in the opposite orientation. The resulting vector (pEFOVA) was used to insert the OVA cDNA into Fp9 using homologous recombination and *gpt* selection. Six *gpt*-resistant clones were isolated, of which five clones proved positive for OVA expression by Western blot. One clone (designated FpEFOVA) was expanded, retested for expression in QT35 cells (Figure 3b.) and used in all further experiments. Expression from FpEFOVA in DC2.4 cells was monitored over a period of 5 days using an OVA recombinant vaccinia virus (VV-OVA) as a control. As shown in Figure 3c, DC2.4 cells infected with either virus expressed OVA for at least 5 days after infection.

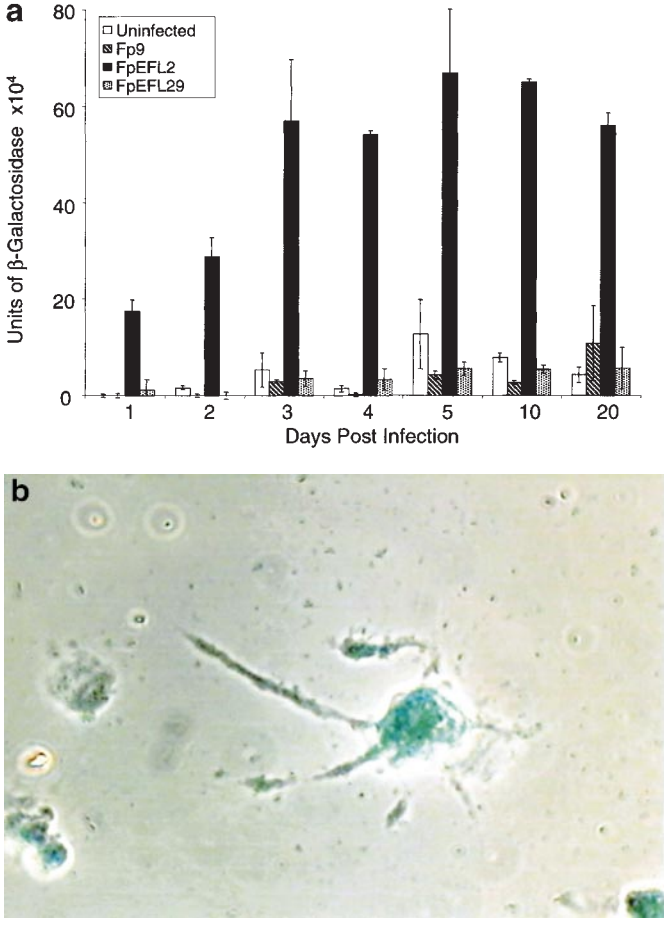

Figure 1 *rFWPV infection of and transgene expression in DC2.4 dendritic cells. (a) DC2.4 cells were infected with 10 p.f.u. per cell of rFWPV expressing LacZ from either an early viral promoter (FpEFL2) or late viral promoter (FpEFL29) or were infected with wild-type FWPV (Fp9). β-Galactosidase activity was determined by CPRG assay at various time-points after infection. (b) Photomicrograph of DC2.4 infected with 10 p.f.u. per cell of FpEFL2 then stained for β-galactosidase at 3 days after infection.*

DC2.4 cells infected with FpEFOVA stimulate an MHC class I restricted response against the SIINFEKL epitope

DC.4 cells were infected with FpEFOVA, VV-OVA or the corresponding parental viruses Fp9 or vaccinia strain WR. Controls included untreated DC2.4 cells or DC2.4 cells pulsed for 1 h with synthetic SIINFEKL peptide. At various time-points after infection or peptide pulse, the cells were used to stimulate the RF33.70 T-T hybridoma. The level of IL-2 released by activated RF33.70 cells was then determined by using a cell proliferation assay with the IL-2 growth-dependent line HT-2. As shown in Figure 4a, DC2.4 infected with FpEFOVA or VV-OVA stimulated IL-2 release by RF33.70 at 24 h after infection while Fp9 or WR infected DC2.4 were unable to do so. No stimulatory activity was detected 24 h after pulsing DC2.4 with synthetic SIINFEKL peptide. For comparison, the level of stimulation observed 2 h after pulsing DC2.4 with synthetic peptide is shown in Figure 4a.

As shown in Figure 4b, both FpEFOVA and VV-OVA infected DC2.4 retained the capability to stimulate IL-2 release from RF33.70 at 72 h after infection. At this time-point the stimulatory capacity of VV-OVA exceeded that of FpEFOVA, whereas at 24 h after infection these rankings were reversed. By 120 h (Figure 4c) only slight activity from VV-OVA was detected. No activity was detected at 10 or 20 days after infection (not shown). We concluded that DC2.4 cells infected with either FpEFOVA or VV-OVA were capable of processing and presenting SIINFEKL peptide in an MHC class I restricted manner and that the immunostimulatory ability of the infected cells was retained for at least 3 days after infection. In contrast, peptide-pulsed DC2.4 could present antigen very effectively 2 h after pulsing, but this activity had declined to zero 24 h after treatment.

DC2.4 cells infected with FWPV or vaccinia virus lose viability within 3–5 days

To investigate the apparent 72–120 h limit on the immunostimulatory capacity of infected DC2.4 we assessed the viability of the infected cultures over the 20 day period of the assays. As shown in Figure 5a, uninfected DC2.4 cells remained viable and continued to proliferate over the 20 day observation period. However, DC2.4 cells infected with 10 p.f.u. per cell of Fp9 or WR showed a rapid decline in cell viability for the first 3 days

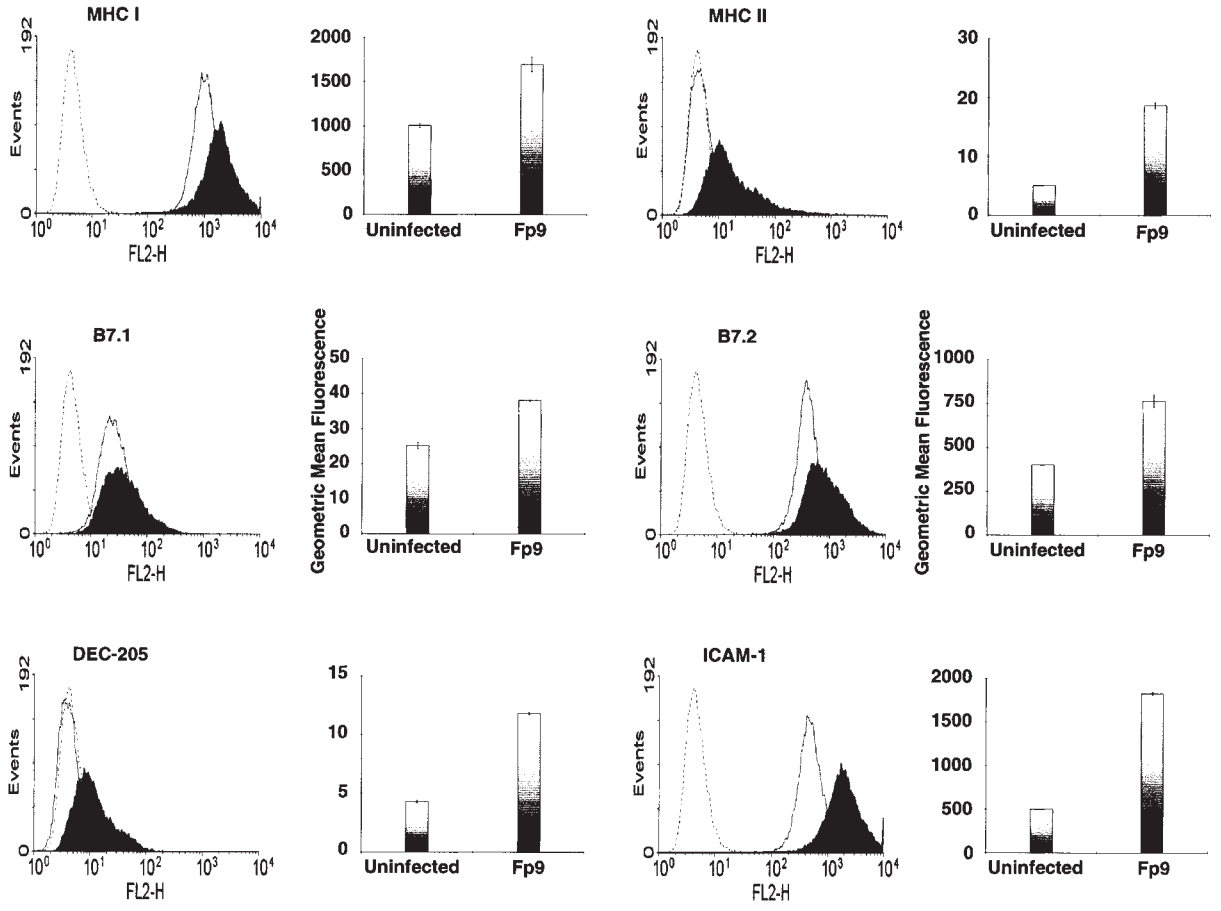

Figure 2 *rFWPV infection up-regulates immunostimulatory cell surface molecules on DC2.4 dendritic cells. DC2.4 cells were infected with 10 p.f.u. per cell of Fp9. Phenotypic analysis of cell surface marker expression (MHC I, MHC II, B7.1, B7.2, ICAM-1 and DEC205) was conducted by FACS at 3 days after infection. Histograms show controls with no primary antibody (dotted lines), uninfected cells (solid lines), Fp9 infected cells (shaded areas). Bar charts show geometric mean fluorescent intensities.*

after infection followed by a recovery in the proportion of viable cells (Figure 5a). The reduction in viability was more pronounced for the WR infected cells than for those infected with Fp9. The total number of viable cells also decreased for the first 3 days after infection, after which the numbers began to increase again (not shown). This suggested that the infected cells were losing viability and the cultures were subsequently being overgrown by cells which had escaped infection.

In order to confirm this interpretation, DC2.4 cells were infected with the *LacZ* recombinant fowlpox virus FpEFL2 or vaccinia virus v182. At various time-points after infection the proportion of cells in the culture that had been infected was determined by scoring for β-galactosidase expression. As shown in Figure 5b, the proportion of cells detectably expressing *LacZ* rose to a peak at 3 days after infection after which it began to decline. Moreover, the proportion of infected, β-galactosidase-positive cells observed at 3 days (50% for FpEFL2, 93% for v182) corresponded well to the proportion of cells that were inviable at that time-point (compare Figure 5b with a). We concluded that the infected DC2.4 cells lost viability and/or the ability to proliferate within 3–5 days and this was likely to account for the 72–120 h limit on immunostimulatory activity we had observed. This observation would also account for the observed lack of accumulation of β-galactosidase after 3 days (Figure 1a). However, the inviable cells must persist in the cultures because β-galactosidase and OVA protein remain detectable beyond this point (Figures 1a and 3c).

Antigen gene transfer to human CD34⁺-derived DC using fowlpox virus vectors

We have shown previously that rFWPV can infect and express transgenes from early promoters in human DC derived from CD14⁺ PBMC, and that expression of the transgene could be detected for at least 5 days after infection.[27] Antigen uptake is mediated by immature DC while presentation of antigen to T cells is carried out by mature DC.[2] Experiments were conducted to assess the ability of rFWPV to infect different classes of DC and to determine the levels and duration of expression achieved. Immature and mature DCs cultured from cord blood CD34⁺ progenitor cells [40] were infected with FpEFL2, expressing *LacZ* from an early promoter, or with the corresponding recombinant vaccinia virus v182. Trafficking DC, isolated using a MACS kit, and the permissive quail cell line QT35 were also infected. The levels of β-galactosidase produced were determined at 5, 10 and 20 days after infection (Figure 6). When infected with FpEFL2, the permissive line QT35 expressed high levels of β-galactosidase at day 5, which declined over the next 15 days.

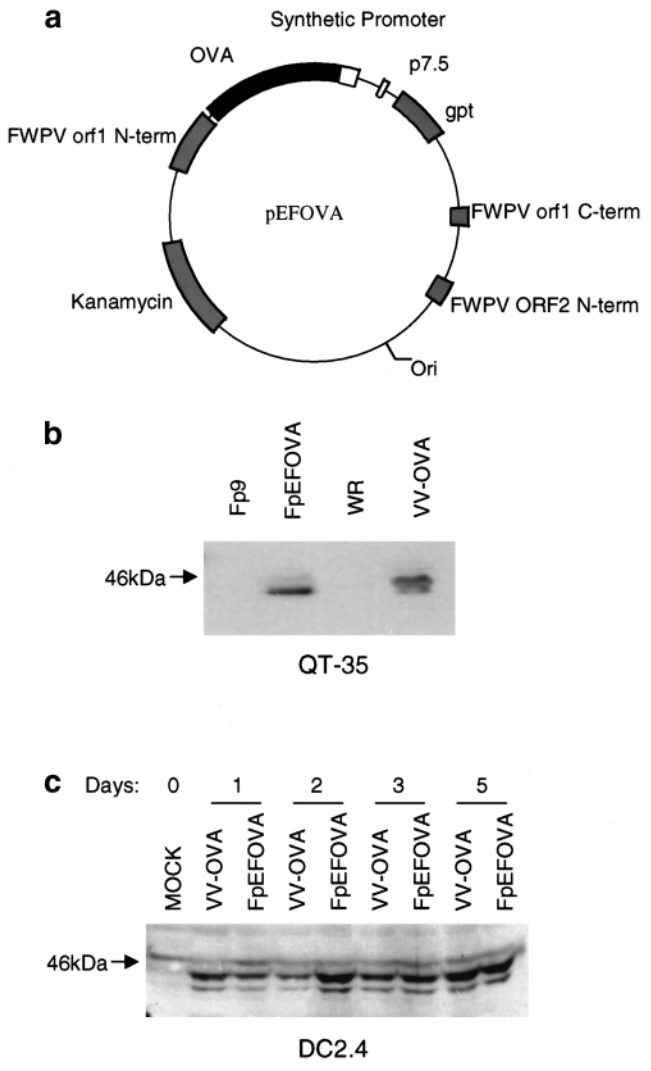

Figure 3 *Construction of rFWPV expressing OVA. (a) To produce pEFOVA, an OVA cDNA was inserted downstream of a synthetic fowlpox early/late promoter, with the E.coli gpt gene cloned under control of the vaccinia virus 7.5K promoter transcribing in the opposite direction. This unit was flanked by sequences from open reading frames (ORF) 1 and 2 derived from the non-essential BamHI terminal fragment of the FWPV genome. The OVA/gpt expression cassette was then inserted into the Fp9 genome by homologous recombination to produce FpEFOVA. (b) Western blot of OVA expression from FpEFOVA: QT35 cells were infected overnight at 10 p.f.u. per cell with either FpEFOVA, VV-OVA or the parental strains Fp9 and WR, respectively. Cell lysates were analysed for OVA expression by Western blot using anti-OVA monoclonal antibody OVA-14. The position of the 46 kDa size marker is indicated. (c) Western blot of OVA expression in DC2.4 cells. Cells were infected with FpOVA or VV-OVA then harvested at various times after infection. OVA expression was detected by Western blotting as in (b).*

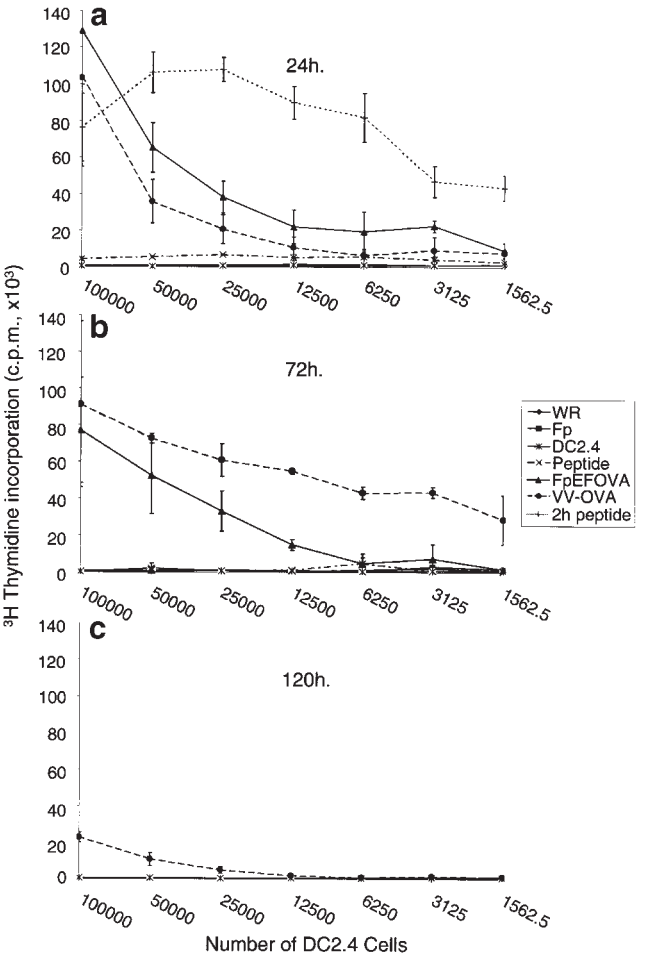

Figure 4 *FpEFOVA infected DC2.4 cells stimulate an MHC class I restricted response against the SIINFEKL epitope. DC2.4 were infected with FpEFOVA or VV-OVA or the parental strains Fp9 and WR, respectively. Other samples were pulsed with synthetic SIINFEKL peptide for 1 h at (a) 24 h , (b) 72 h and (c) 120 h after infection or peptide pulsing. DC2.4 cells were used to simulate RF33.70 responder cells at a range of stimulator:responder ratios. Release of IL-2 from RF33.70 was determined by ^{3}H thymidine uptake using the IL-2-dependent line HT-2. For comparison, the level of stimulation obtained from SIINFEKL peptide-pulsed DC2.4 assayed 2 h after pulsing was also determined and is shown as '2h peptide' in (a).*

The recombinant vaccinia virus v182 showed little expression in QT35 by 5 days because the cells had, at this point, been killed by the infection. In the immature DC infected with FpEFL2, β-galactosidase expression was detected at 5 days after infection and continued to accumulate over the next 15 days. The level of expression in the FpEFL2-infected mature DC was lower and did not accumulate significantly after 5 days. DC isolated using the MACS kit displayed a similar profile to the immature DC. Expression from the recombinant vaccinia virus v182 was lower than from FpEFL2 in all DC types.

The observation that β-galactosidase continued to accumulate in immature DC and MACS-derived DC over the 20-day observation period suggested that the FWPV infection of human DC did not result in the loss in viability of infected cells as had been observed with the murine DC2.4 line. In order to investigate this we performed viability assays on Fp9 and WR-infected human DC cultures (Figure 7). Infection of the permissive cell line QT35 with either Fp9 or WR resulted in a marked loss of viability probably due to lysis of the productively infected cells. In the infected immature DC, mature DC and MACS-derived DC, there was no corresponding decrease in cell viability. This indicated that, unlike the murine DC2.4 cell line, rFWPV infected human DC can retain viability and continue to express transgenes under an early promoter for up to 20 days after infection.

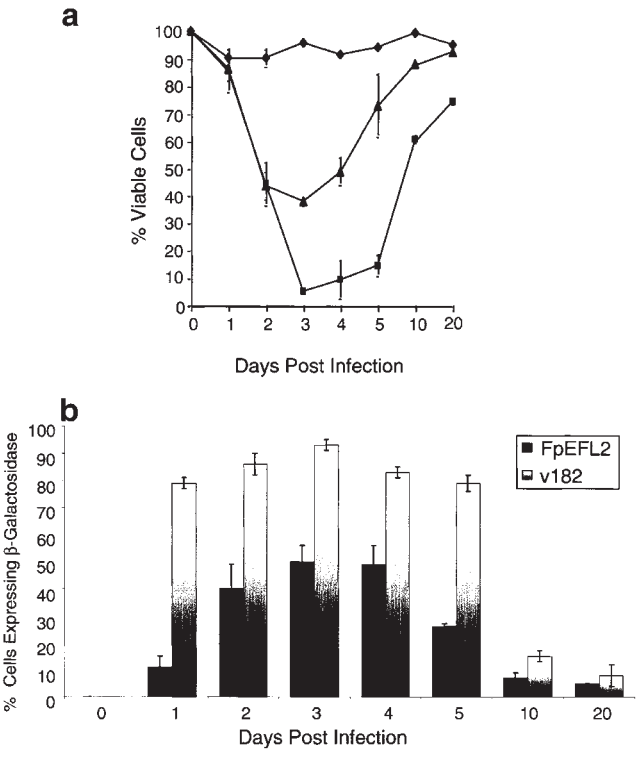

Figure 5 *DC2.4 cells infected with FWPV or vaccinia virus die within 3–5 days. DC2.4 cells were infected with 10 p.f.u. per cell of Fp9 or WR and viability was determined by trypan blue exclusion at various times after infection. (a) shows the percentage of viable cells in each culture and (b) DC2.4 cells were infected with the LacZ recombinant viruses FpEFL2 or v182 then stained with X-gal at various times after infection, followed by scoring of the fraction of cells staining blue.*

Discussion

The advent of *in vitro* culture systems to generate large numbers of DC provides the possibility of *ex vivo* modification of DC to produce a desired immune response upon reinfusion to the patient. In this study, we have examined the use of rFWPV to transfer antigens to DC with the aim of stimulating MHC class I restricted responses. Such methodologies are expected to have utility in gene therapy for cancers where target antigens are expressed intracellularly and CTL killing of the antigen-bearing cells is the desired result. Using a murine DC cell line model we showed that rFWPV can infect and express transgenes in such a way as to stimulate an MHC class I restricted response. Although peptide-pulsed DC were able to produce high levels of stimulation initially, they were ineffective by 24 h after pulsing, whereas strong stimulation by rFWPV infected DC was still evident 72 h after infection. This difference may be important *in vivo* since long-term presentation of antigen might be important for an effective CTL response.[15]

The limitation of antigen presentation by rFWPV infected DC2.4 cell line was most probably due to loss of viability of the infected cells after 3–5 days. Recombinant protein could still be detected after this time, possibly due to the persistence of the inviable cells in the culture or engulfment by neighbouring cells.[41] However, levels of the recombinant protein did not continue to accumulate after 3–5 days, suggesting that synthesis had been shut down. Using primary human material we found that both immature and mature human DC could be infected with rFWPV without significant loss of viability over a period of 20 days. In the immature human DC, β-galactosidase continued to accumulate over the 20 day period indicating not only that the cells remained viable, but also that the early promoter carried by the rFWPV

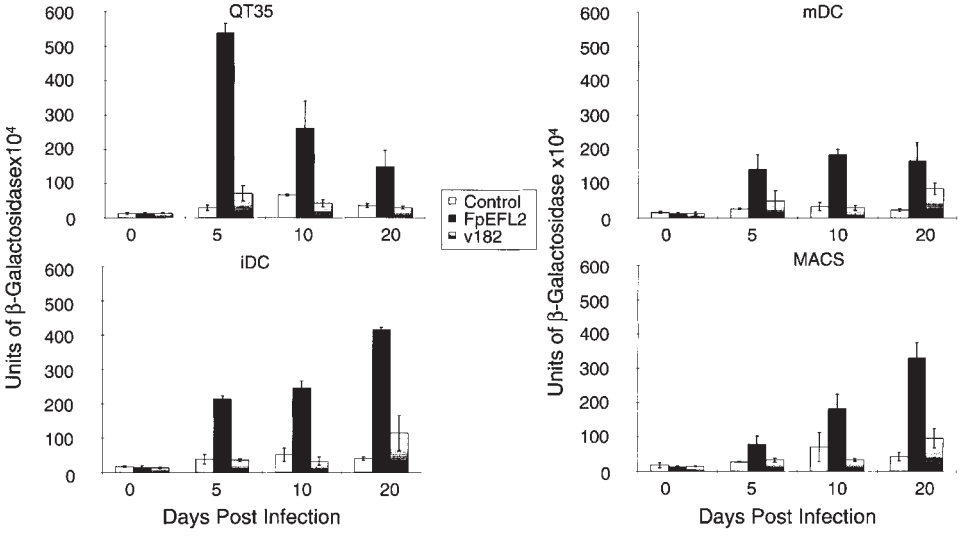

Figure 6 *Fowlpox virus vector infection and transgene expression in immature and mature human DC. Cord blood-derived CD34⁺ cells were cultured to produce immature dendritic cells (iDC) or mature dendritic cells (mDC). Trafficking DC were isolated from peripheral blood using a magnetic bead isolation kit (MACS). Cultures were infected with 10 p.f.u. per cell of LacZ recombinant viruses FpEFL2 or v182 and β-galactosidase expression was determined by CPRG assay at various times after infection. For comparison, the permissive line QT35 was infected. Control cultures were mock infected.*

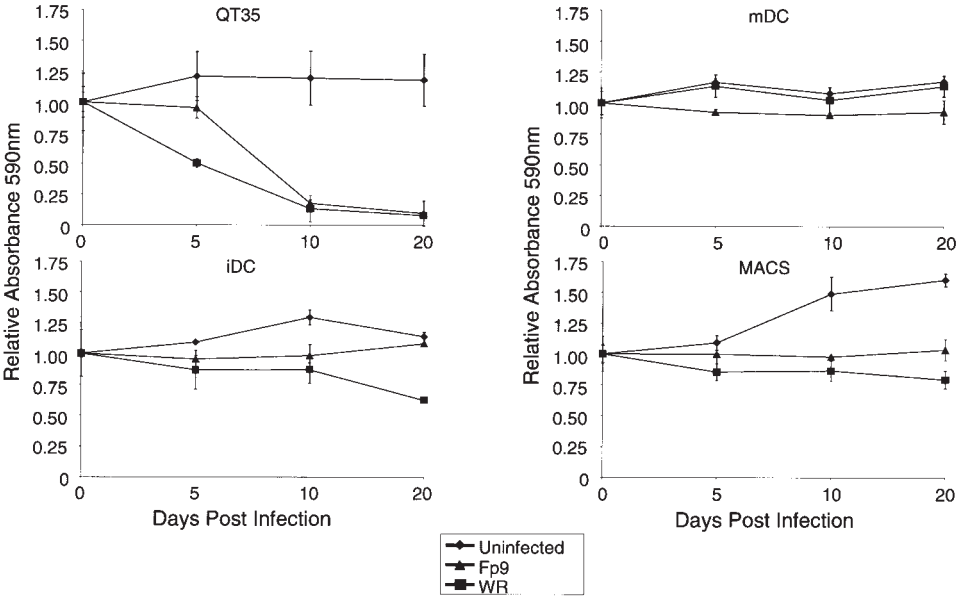

Figure 7 *Human DC infected with fowlpox virus retain viability for up to 20 days in vitro. DC isolated from PBMCs via the MACS magnetic bead isolation kit (MACS) or cultured from cord blood-derived CD34⁺ progenitors to produce immature (iDC) or mature dendritic cells (mDC) were infected with 10 p.f.u. per cell of Fp9 or WR. Viability was determined at various times after infection by MTT assay. For comparison, the permissive line QT35 was infected. Control cultures were mock infected.*

remained active during this time. It is possible that the abortive nature of the infection prevents the down-regulation of early promoter activity that would normally occur as the virus goes into its replicative phase. A similar behaviour of several early promoters has been observed in vaccinia virus infections when the early-to-late switch is inhibited by AraC.[36,42–44] The extended period of transgene expression observed in the primary human DC cultures suggests that antigenic stimulation provided by these cells may be long lasting. It will be important to define further the antigen-presenting and immunostimulatory capacities of rFWPV infected primary human DCs.

One of the primary attractions towards the use of FWPV vectors is that their natural host range restriction to avian cells, combined with their ability to set up an abortive infection in a wide range of mammalian cell types, represented a considerable safety advantage over replication-competent strains of vaccinia virus.[33,34] Although replication-competent vaccinia virus vectors have been used in cancer immunotherapy trials in humans,[45–48] their use is undesirable due to the risks of skin and central nervous system complications and potential spread to unvaccinated contacts.[29–31] Such risks are particularly acute in adjuvant cancer immunotherapy protocols, where patients may be in an immunocompromised state because of their disease or primary therapy. The use of replication-defective vaccinia viruses such as MVA[49] may go some way to addressing these problems, however, rFWPV may have particular advantages. The host range restriction may not be so complete for MVA as for FWPV.[34,50] Moreover, vaccinia viruses encode a battery of gene products which are involved in immune evasion.[37,38] While it may be anticipated that FWPV also contains similar immune evasion genes, such as the SERPIN gene homologue SPI-3,[51] their functions may be compromised in a heterologous host.

There are, in addition, strong arguments in favour of the development of a diversity of viral vector systems for immunotherapy. Although it is unlikely that large-scale smallpox vaccination will ever be required again, many cancer patients are in an age group where they will have been given smallpox vaccinations as children. The development of vaccinia virus vectors for a variety of applications means that future candidates for immunotherapy are also likely to have prior immunity to vaccinia virus. It has been shown that prior immunisation with one vaccinia virus recombinant inhibits subsequent responses to a second recombinant vaccinia virus immunisation.[52] This has led to the development of prime-boost protocols in which the vector used to boost differs from the vector used to prime. Priming with rFWPV-*LacZ* followed by boosting with rVV-*LacZ* induced a specific CTL response in mice which protected against a tumour challenge, whereas priming and boosting with the same agent failed to protect.[53] Replication-defective recombinant poxvirus vectors have worked well in heterologous vaccination schemes in mice and primates using plasmid as the primary inoculation followed by boosting with MVA or FWPV.[54–57] Little work has been carried out so far using mixed vector prime-boost protocols in human DC. Kim *et al*[32] generated human anti-MART-1 CTL *ex vivo*, using DC infected with a rVV for primary stimulation, followed by DC infected with rFWPV for secondary stimulation.

The findings in the present article demonstrate that rFWPV infected DC are able to express, process and present recombinant antigens in an MHC class I restricted context. While the ability of rFWPV infected murine DC to stimulate a MHC class I response appears to be limited by the proliferation of uninfected cells, the continued expression without loss of cell viability observed in the rFWPV infected human DC suggests that the immunostimulatory period of human primary DC may be longer. Taken together, these findings indicate that

rFWPV shows promise for use in immuotherapy protocols involving the *ex vivo* infection of DC followed by reinfusion to patients in order to generate a CTL response against the target antigen.

Materials and methods

Cell culture

The Japanese Quail Fibrosarcoma cell line QT35 was grown in 199 medium with 10% FCS. CV-1 monkey kidney cells were grown in MEM with 10% FCS. The DC2.4 murine dendritic cell line[58] was grown in RPMI 1640 with 10% FCS and supplemented with 0.05mM β-mercaptoethanol. The RF33.70 T-T cell hybridoma[39] was grown in DMEM with 10% FCS and supplemented with 0.05 mM β-mercaptoethanol. DC2.4 and RF33.70 were kindly provided by Dr K Rock (Dana Farber Cancer Institute, Boston, MA, USA). The mouse IL-2-dependent T cell line, HT-2, was grown in Iscove's DMEM with 10% FCS and supplemented with 0.02 mM β-mercaptoethanol and 200 IU/ml IL-2 (Cetus, Marburg, Germany). Before assay, the HT-2 cells were starved of IL-2 for 2 h.

Human $CD34^+$ progenitor cells were harvested from umbilical cord blood as described previously.[59] Briefly, $1–2 \times 10^8$ mononuclear cells in 300 μl PBE (PBS/0.5% BSA/5 mM EDTA) were incubated at 4–8°C for 30 min in the presence of 100 μl CD34 antibody (QBEND) directly conjugated to magnetic beads (Multisort beads, Miltenyi Biotec, Surrey, UK) and 100 μl of 8 mg/ml human IgG. Cells were washed with ice-cold PBE then loaded on to a magnetic separation column (Miltenyi Biotec). After washing with PBE, the $CD34^+$ cells were recovered from the column by removal of the magnetic field and manually flushing cells from the column. The eluted cells were then expanded and differentiated as described by Herbst *et al*.[40] Briefly, immature DC were generated from $CD34^+$ cells cultured in the presence of 100 ng/ml IL-3, IL-6 and 10 ng/ml SCF for 8 days followed by culture with 50 ng/ml IL-4 and 100 ng/ml GM-CSF for 7 days. $CD1a^+$ cells were then harvested by cell sorting on a FACS Vantage flow cytometer. Mature DC were cultured from $CD34^+$ progenitors as for immature DC except that 60 h before infection, 20 ng/ml TNFα was added to the culture to facilitate maturation. The immature and mature DC phenotypes were confirmed by monitoring expression of CD1a, CD14, and CD11b using FACS, as described by Herbst *et al*.[40] DCs were isolated from peripheral blood mononuclear cells using a MACS blood dendritic cell isolation kit (Miltenyi Biotec) as follows: PBMC were depleted of T, NK and monocytic cells by indirect magnetic labelling with haptenised CD3, CD11b and CD16 antibodies and anti-hapten magnetic microbeads, before passing the cells through a magnetic separation column. The cells in the flow through were labelled with CD4 microbeads and immobilised on a second magnetic column. After washing, the magnetic field was removed and the $CD4^+$ DC collected.

Recombinant poxviruses

rFWPV were based on the Fp9 strain. FWPV stocks were produced from QT35 (8×10^7 cells) inoculated with virus at a multiplicity of 1 p.f.u. per cell. Five days after infection, cells were scraped up into the medium and lysed by freeze–thawing. Cellular debris was removed by low speed centrifugation. The medium/cell lysate was then centrifuged at 40 000 *g* for 80 min at 4°C. The virus pellet was resuspended in 2 ml of 10 mM Tris pH 9.0, titrated and stored at −20°C. Typical titres were of the order 10^8 p.f.u./ml.

The *LacZ* FWPV recombinant with an early viral promoter, FpEFL2, contained the *LacZ* gene driven by the vaccinia virus p7.5K early/late promoter. The *LacZ* recombinant FpEFL29 utilised the FWPV p4b late promoter to drive *LacZ* expression. The ovalbumin FWPV recombinant, FpEFOVA, contained the chicken ovalbumin gene driven by a synthetic hybrid early/late promoter. Details of this promoter (designed by Lorenz Rindisbacher and Riccardo Wittek) will be published elsewhere. Expression cassettes for all rFWPV were inserted into the non essential ORF-1 within the terminal *Bam*HI fragment of Fp9. All rFWPV were propagated and titrated on QT35 cells. For infection of mammalian cells FWPV was diluted in PBS containing 0.5% BSA, 0.1% FCS and applied to cell cultures at a multiplicity of infection of 10 p.f.u. per cell. After 1 h the inoculum was removed and medium replaced.

Vaccinia virus strain WR was used as both wild-type and base for recombinant vaccinia viruses. In v182 the *LacZ* expression cassette, driven by the p7.5K early/late promoter, was inserted into the *tk* locus of WR. v182 was kindly provided by Dr M Mackett. Vaccinia virus VV-OVA contained chicken ovalbumin gene under the control of the p7.5K promoter, cloned into the *tk* locus and was kindly provided by Drs JW Yewdell and JR Bennink. Vaccinia viruses were maintained, expanded and titrated on CV-1.

Western blotting

QT35 cells infected overnight with either WR, Fp9, FpEFOVA or VV-OVA at 10 p.f.u. per cell, were then lysed in RIPA buffer before loading on a 10% SDS polyacrylamide gel. Resolved proteins were transferred to Immobilin P membranes then probed with mouse monoclonal anti-chicken egg albumin (OVA) Clone OVA-14 (Sigma, Poole, UK) followed with a HRP-conjugated goat anti-mouse secondary antibody (Dako, Cambridge, UK). Blots were developed by ECL (Amersham, Bucks, UK).

β-Galactosidase assay

Cells were infected with *LacZ* recombinant FWPV or vaccinia virus at a multiplicity of 10 p.f.u. per cell before plating on 96-well plates at 10^4 cells/100 μl per well. At various time-points the level of β-galactosidase was determined by the addition of 50 μl per well of 2.5 mM chlorophenol red β-D-galactopyranoside (CPRG; Boehringer Mannheim) in 2% SDS, 50 mM KCl, 5mM $MgSO_4$, 1.3% β-mercaptoethanol followed by incubation at 37°C for 2 h. Absorbance was then determined at 590 nm using an automated microplate reader (Molecular Devices). Units of β-galactosidase were calculated by reference to a standard curve generated using purified protein (Boehringer Mannheim, Lewes, UK). Three separate infections were used for each condition.

IL-2 release assay

DC2.4 stimulator cells were infected at a multiplicity of 10 p.f.u. per cell with either Fp9, FpEFOVA, WR, VV-OVA or were pulsed with the synthetic OVA peptide SIINFEKL at 10 μg/ml. After a 1 h incubation at 37°C,

5% CO_2 the cells were washed three times and resuspended at 5×10^5 cells/ml in RPMI 1640 (10% FCS, 0.05 mM β-mercaptoethanol) in a six-well plate. At the indicated time-points after infection/pulsing the DC2.4 stimulators were exposed to 50 Gy of γ-irradiation. RF33.70 responder cells were added to two-fold serial dilutions of DC2.4 stimulators in triplicate wells of a 96-well plate, creating responder:stimulator ratios from 1:2 to 32:1. Responders were incubated with stimulators at 37°C, 5% CO_2 for 48 h. The supernatant was then assayed for the presence of IL-2 by HT-2 cell proliferation assay: 10^4 HT-2 cells were starved of IL-2 for 2 h before the addition of 100 μl of supernatant from the stimulated RF33.70 cell culture. The cells were then incubated for 24 h at 37°C, 5% CO_2 before metabolic labelling with 1 μCi per well ^{3}H thymidine for 18 h. Cells were then harvested on to UniFilter –96, GF/C plates (Packard, Berkshire, UK) by a 96-well plate harvester (Filtermate cell harvester, Packard) and the plates analysed for ^{3}H thymidine incorporation using a Packard Topcount Microplate Scintillation counter. All infections were carried out in triplicate.

MTT assay

Dendritic cell cultures were either mock infected with PBS or infected at 10 p.f.u. per cell for 1 h with FWPV or vaccinia virus. Cells were then washed and plated out in triplicate at 1000 cells/100 μl per well in a 96-well plate. MTT assay for cell viability was conducted at 0, 5, 10 and 20 days after infection according to the manufacturer's instructions (Chemicon International, Harrow, UK). At each time-point, 0.01 ml of solution AB (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide) 50 mg in 15 ml PBS (pH 7.4), was added to the culture before incubation for 4 h at 37°C. Following incubation, 0.1 ml isopropanol, 0.04 N HCl was added, and the absorbance measured at 590 nm using an automated microplate reader (Molecular Devices, Wokingham, UK).

Light microscopy

DC2.4 cells were infected at a MOI of 10 p.f.u. per cell for 1 h with *LacZ* recombinant FWPV or vaccinia virus or were mock infected with PBS. Cells were then washed and replated on poly-L-lysine glass microscope slides at 1×10^5cells per slide in RPMI 1640 medium supplemented with 10% FCS and 0.05 mM β-mercaptoethanol. At 1, 2, 3, 4, 5, 10 and 20 days after infection, cells were stained by the addition of 330 mg/ml X-gal followed by incubation overnight. Slides were washed three times in PBS before fixing with 0.5% paraformaldehyde in PBS. β-Galactosidase expression was then assessed by light microscopy.

Flow cytometric analysis

DC2.4 infected with Fp9 (3 days after infection) were analysed by flow cytometry using antibodies either unconjugated or directly conjugated to RPE. Unconjugated rat anti-mouse H-2IA (MHC II molecules of mice), CD54, CD80, CD86, DEC205 and RPE-conjugated mouse anti-mouse MHC I H-2K^b were purchased from Serotec (Oxford, UK). A RPE F(ab)$_2$ anti-rat IgG (Serotec) was used as the secondary antibody.

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