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Artificial antigen-presenting cells engineered by recombinant vaccinia viruses expressing antigen, MHC class II, and costimulatory molecules elicit proliferation of $CD4^+$ lymphocytes *in vitro*

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SUMMARY

The current study was designed to test the ability of recombinant Vaccinia virus (rVV) encoding essential components of an artificial antigen-presenting cell to activate antigen-specific T cells *in vitro*. We have constructed a set of rVV encoding separately or in combination a $CD4^+$ T cell-specific epitope (the 133–145 peptide of chicken conalbumin), the MHC class II molecule I-A^k, and costimulatory molecules (mB7-1 and mB7-2). Cultured cells infected with rVV encoding both the antigen and the presenting MHC, but not either one alone, could activate cloned $CD4^+$ T cells specific for the virus-encoded epitope. Additional co-expression of mB7-1 and mB7-2 resulted in further enhancement of T cell response. Thus, our rVV vector expressing four different foreign gene products elicited the highest proliferation rates of antigen-specific cloned T cells.

Keywords antigen-presenting cell engineering vaccinia virus

INTRODUCTION

Induction of optimal activation of $CD4^+$ T cells requires occupancy of the antigen-specific T cell receptor and the engagement of counter-receptors for costimulatory molecules on the antigen-presenting cell (APC) [1,2]. The second signal is provided by B7-1 (CD80) [3] and/or B7-2 (CD86) [4,5] on the surface of the APC. They both bind to the CD28 and CTLA-4 receptors on the responding $CD4^+$ T cell [6–8]. These requirements for an APC are usually reserved to highly specialized cell types, including mononuclear phagocytes, activated B lymphocytes or dendritic cells [9,10]. In light of the potential clinical relevance we investigated vector-based strategies which permit cells devoid of APC capacity to stimulate efficiently $CD4^+$ lymphocytes. Although plasmid-mediated transfection approaches have been used successfully, they are limited in the number of foreign genes which can be inserted into a single vector [11]. Therefore, we chose the vaccinia virus (VV) as a vector system due to its large capacity to carry multiple genes for simultaneous expression in infected cells. In this study, we engineered the pathway of MHC class II-restricted antigen presentation using the endogenous model antigen conalbumin (chicken ovotransferrin) together with T cell costimulatory molecules. Recently, we have shown that treatment of VV with psoralen and irradiation with long-wave UV light (PLWUV) renders the virus non-replicating and abrogates its cytopathic effect [12]. Despite complete inactivation of the viral replication and absence of cytopathic effect of PLWUV-treated recombinant VV (rVV), sufficient gene expression can be driven by early promoters. The possibility of engineering cells to provide immunologically functioning APC was successfully tested *in vitro*.

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MATERIALS AND METHODS

Cells and cell lines

D10.G4.1 cells (American Type Culture Collection (ATCC, Rockville, MD) 224-TIB) were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), L-glutamine, 2-mercaptoethanol (2-ME), sodium pyruvate, non-essential amino acids, penicillin, streptomycin, and gentamicin, at 37°C with 5% CO_2 and maintained according to the guidelines of the manufacturer. BSC40 cells, fast-growing derivates of the monkey kidney cell line BSC-1, B16.F10 melanoma cell line (H-2^b, ATCC CRL6475), and K1735 melanoma cell line (H-2^k, kindly provided by Dr J. P. Allison, University of California, Berkely, CA) were cultured in Dulbecco's minimum essential medium (DMEM; GIBCO BRL) supplemented with 10% newborn calf serum, streptomycin, gentamycin and penicillin.

(a)

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Afl II                                                          Pst I
TTAAGCCACC'ATG'AGG'TAC'ATG'ATT'TTA'GGC'TTG'CTC'GCC'CTT'GCG'GCA'GTC'TGT'AGC'GCT'GCA
    CGGTGG'TAC'TCC'ATG'TAC'TAA'AAT'CCG'AAC'GAG'CGG'GAA'CGC'CGT'CAG'ACA'TCG'CG
           M   R   Y   M   I   L   G   L   L   A   L   A   A   V   C   S   A
```

(b)

```
Pst I                                                           Apa I
      CAC'CGG'GGG'GCC'ATT'GAG'TGG'GAA'GGC'ATA'GAA'TCG'GGC'TAG'GGCC
A'CGT'GTG'GCC'CCC'CGG'TAA'CTC'ACC'CTT'CCG'TAT'CTT'AGC'CCG'ATC
   A   H   R   G   A   I   E   W   E   G   I   E   S   G  Ter
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Fig. 1. Oligonucleotide sequence and deduced amino acid sequence of the minigene encoding the signal peptide of the adenovirus glycoprotein E3/19k (a) and the 133–145 epitope of chicken conalbumin (b). The gene is flanked by the restriction sites that allow direct insertion into the expression plasmid targeting the Haemagglutinin (A56R) locus of the vaccinia virus genome.

Recombinant vaccinia virus preparation

All vectors constructed for these experiments contain multiple expression/insertion cassettes with early VV promoters [13] which allow insertion of multiple genes into several loci of the VV genome. The expression/insertion cassettes are flanked by sequences that are identical to different viral loci for homologous recombination and production of rVV. The gene for *Escherichia coli* guanine phosphoribosyltransferase (*gpt*) is co-expressed outside of the homologous sequences, which allows transient dominant selection of the rVV [14]. Complementary DNA sequences encoding the α and the β subunit of the murine MHC class II heterodimer I-A^k (Genbank accession numbers M11357 and M13538, courtesy of Dr L. Glimcher, Boston, MA) were inserted into a plasmid pKT1410 targeting the viral ribonucleotide reductase locus (I4L) [15] for integration. We then cloned the cDNA encoding the conalbumin 133–145 epitope (HRGAIEWEGIESG) together with an upstream signal peptide sequence from the adenovirus E3/19k glycoprotein (MRYMILGLLALAAVCSAA) into a VV Haemagglutinin (A56R) [15] locus-based expression plasmid pKT1330 (Fig. 1). After homologous recombination, this locus is split into a A56L and A56R fragment (Fig. 2). The genes encoding costimulatory molecules mB7-1 and mB7-2 (GenBank accession numbers X60958 and L25606, generous gifts from Dr G. Freeman, Boston, MA and Dr J. Schlom, Bethesta, MD, respectively) were cloned either as single constructs or in combination into a plasmid pKT1630 targeting the 3β-hydroxy-5-ene-steroid dehydrogenase (A44L) [15] locus of the VV genome. Since the

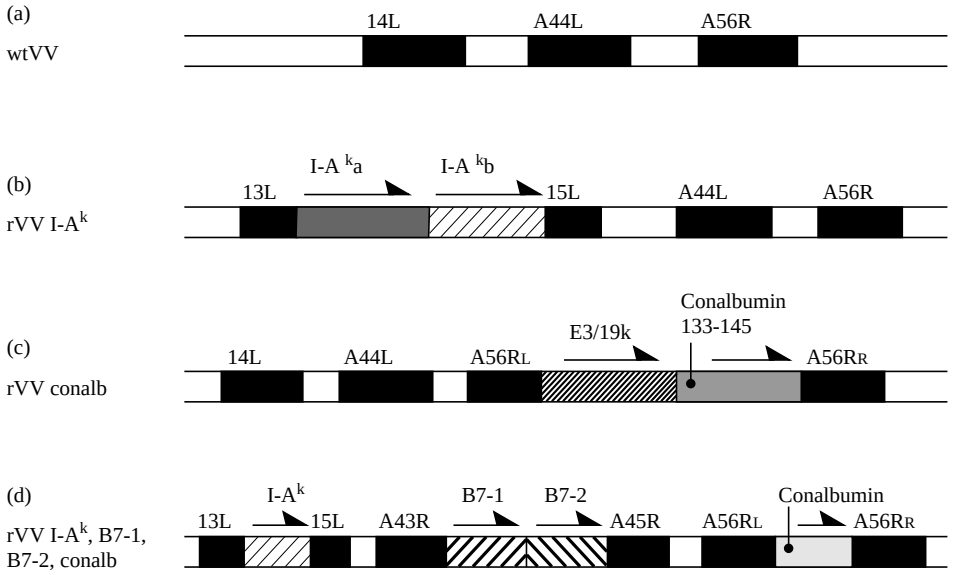

Fig. 2. Schematic representation of the genomes of wild type vaccinia virus (VV), of the single gene and multi-gene expressing rVV, respectively. (a) genome of wild type VV with three selected non-essential loci: I4L (large subunit of Ribonucleotide reductase), A44L (3β-hydroxy-5-ene-steroid dehydrogenase), and A56R (Haemagglutinin). (b) rVV encoding the α and β subunit of the murine MHC class II molecule I-A^k, replacing the I4L locus. (c) rVV encoding I-A^k and the fused minigene of conalbumin epitope and E3/19k signal sequence. (d) rVV encoding the essential genes for an artificial antigen-presenting cell (APC) (I-A^k, conalbumin epitope, mB7-1 and mB7-2). We also constructed the single B7 expressing rVV encoding I-A^k/minigene/mB7-1 and I-A^k/minigene/mB7-2, respectively, as well as control rVV deleted from the respective locus.

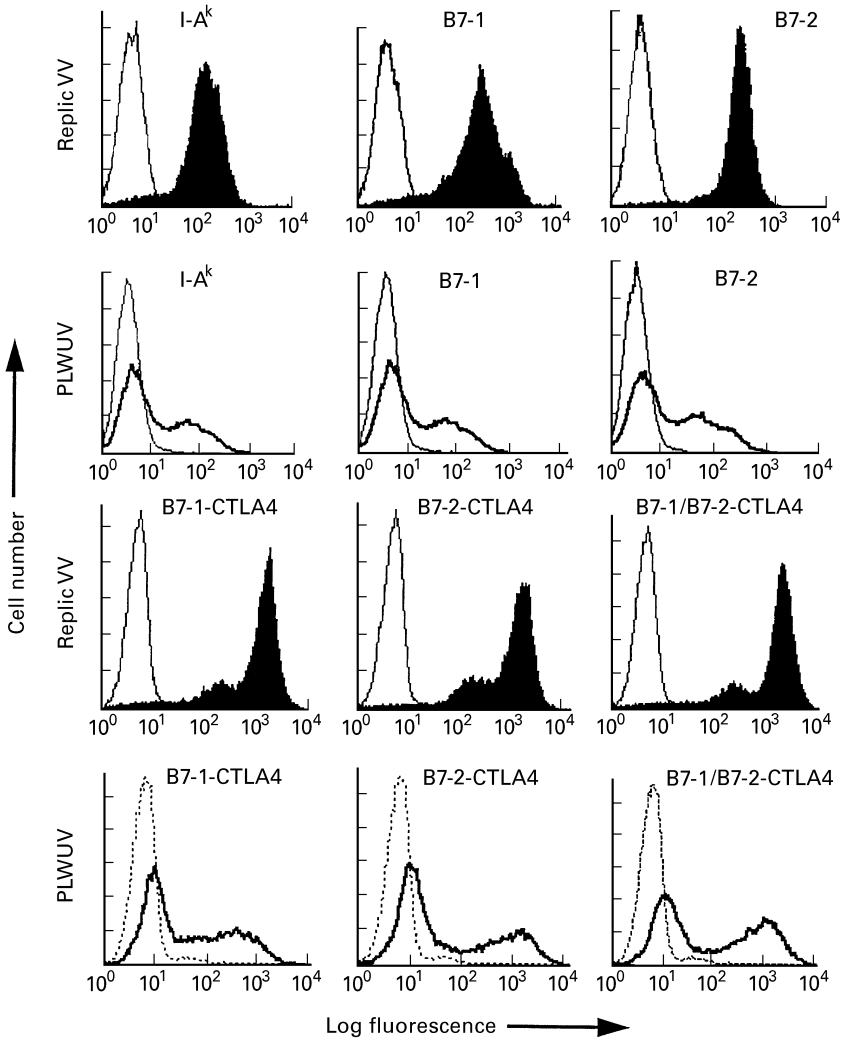

Fig. 3. Flow cytometric analysis of I-A^k, mB7-1 and mB7-2 expression in K1735 cells. Cells were infected with the respective replicating recombinant vaccinia virus (rVV) (first row) or with replication-incompetent rVV (second row) at 10 m.o.i. and cultured overnight. Cells were stained with biotinylated MoAbs against I-A^k, mB7-1, and mB7-2, followed by streptavidin–FITC conjugate. The two bottom rows represent the fluorescence analysis of CTLA-4 IgG fusion protein bound to mB7-1 and mB7-2, respectively. K1735 cells were infected with the respective replicating rVV (third row) or with replication-incompetent rVV (bottom row) at 10 m.o.i. Cells were incubated with soluble CTLA-4 IgG fusion protein and subsequently with FITC-conjugated goat anti-human (Fc-specific) IgG antibodies. As a negative control, cells were infected with the rVV deleted from I4L and A44L locus and stained with the respective antibodies. PLWUV, Treated with psoralen and irradiation with long-wave UV light.

cloned mB7 genes are inserted between the A43R and A45R locus of the viral genome, the A44L locus of the resulting rVV is deleted. For homologous recombination, CV-1 African green monkey kidney cells (ATCC CCL70) were infected with the CR-19 derivate of the Western Reserve VV strain (ATCC VR-119) and transfected with the respective insertion/expression plasmids. Recombinant viral clones were selected according to their transient expression of the *E. coli gpt* marker and candidate clones were amplified and checked for the presence of the desired inserts by polymerase chain reaction (PCR). After large scale amplification, rVV were purified and viral titre was determined as plaque-forming units (PFU) per ml.

Psoralen and long-wave UV inactivation of VV

The viral lysate was incubated with psoralen (4′ aminomethyl-trioxalen; Calbiochem, La Jolla, CA) 1·0 μg/ml and irradiated during 10 min with long-wave UV light (365 nm) in a Stratalinker 1800 UV cross-linking unit (Stratagene, La Jolla CA).

In vitro *gene transfer*

Cells were infected with rVV at a multiplicity of infection (m.o.i.) of 10, i.e. 10 PFU per cell in Hanks' balanced salt solution/bovine serum albumin (HBSS/BSA; GIBCO BRL) on a rocking device at 37°C for 60 min, washed with DMEM (GIBCO BRL) in order to remove the unabsorbed viral particles, and cultured overnight.

Fluorescence-activated cytometry

Cells were stained by indirect immunofluorescence using biotinylated MoAbs against either I-A^k, mB7-1, or mB7-2, respectively (all from Pharmingen, San Diego, CA). The samples were then incubated with streptavidin–FITC conjugate (GIBCO BRL). For

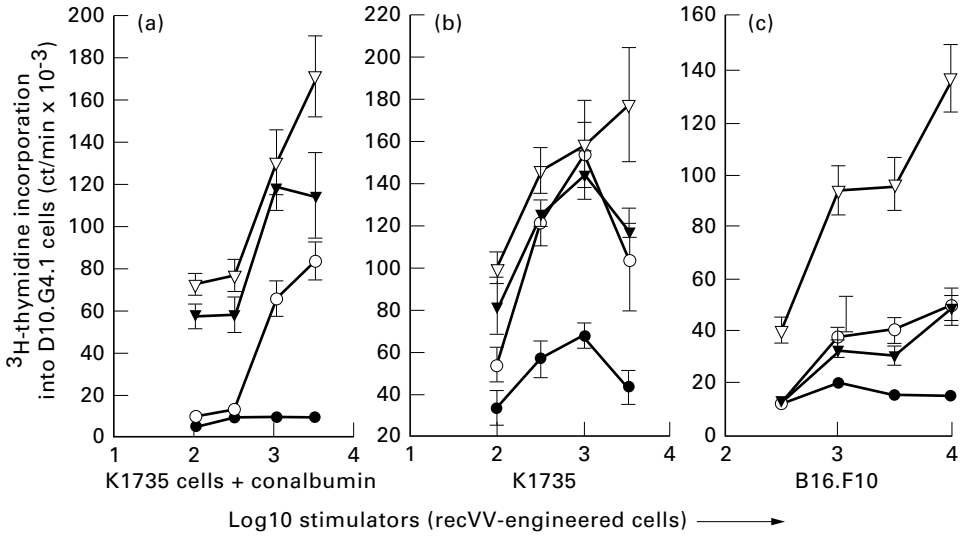

Fig. 4. Biological function of cells engineered with replication-incompetent recombinant vaccinia virus (rVV) *in vitro*. Resting D10.G4.1 were used as effectors at a concentration of 5×10^4 cells in 0·2 ml medium per well. Stimulator cells were irradiated with 50 Gy and plated in different concentrations into the wells. (a) K1735 cells were infected (10 m.o.i.) with control virus (●), rVV expressing I-A^k (○), rVV expressing I-A^k and mB7-1 (▼), and rVV expressing I-A^k, mB7-1 and mB7-2 (▽), respectively. Soluble conalbumin 133–145 peptide HRGAIEWEGIESG was added at a concentration of 1·0 mg/ml. No cytokine like IL-1 or IL-2 was added to the culture medium. (b, c) K1735 cells (b) and B16.F10 cells (c) were infected (10 m.o.i.) with rVV encoding conalbumin epitope alone (●), rVV expressing conalbumin + I-A^k (○), rVV expressing I-A^k + conalbumin + mB7-1 (▼), and rVV expressing I-A^k + conalbumin + mB7-1 + mB7-2 (▽). No soluble antigen or cytokine was added to the culture medium. Counts are given as the mean ct/min, error bars indicate s.d. of triplicate measurements. Background counts from VV-infected cells were negligible (< 4000 ct/min), indicating that the stimulator cells were not dividing and that there was no replicating intracellular VV.

CTLA-4 IgG fusion protein staining, rVV-infected cells were incubated with soluble CTLA-4 IgG [16] (kindly provided by Dr P. Linsley, Seattle, WA), followed by FITC-conjugated goat anti-human IgG (Fc-specific) antibodies (Sigma, St Louis, MO). Relative fluorescence was measured with a FACScan (Becton Dickinson, Mountain View, CA).

Proliferation assays

Effector D10.G4.1 cells were cultured in triplicate in 96-well plates together with various doses of irradiated (50 Gy) and rVV-infected stimulator cells. Cells were cocultured for 72 h and 1 μCi (37 kBq) methyl-^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) was added per well for the last 16 h of the incubation period for analysis of incorporated radioactivity. The cultures were harvested onto filter paper and incorporated radioactivity was determined with the 1450 Microbeta Plus liquid scintillation counter (Wallac Inc. Gaithersburg, MD).

RESULTS

Construction of rVV encoding the chicken conalbumin 133–145 epitope, the murine MHC class II molecule I-A^k, and costimulatory molecules mB7-1 and mB7-2

Using a series of new insertion/expression vectors, we constructed a set of single and multiple genes expressing rVV. The insertions into the genome of the different rVV are summarized in Fig. 2. As shown by FACS analysis, their encoded molecules I-A^k, mB7-1 and mB7-2 were expressed on the surface of K1735 cells (Fig. 3). Cells engineered with replication-incompetent rVV reveal a less abundant MoAb staining profile than those infected with replicating virus. This is probably due to the partial inhibition of transcription of large genes by genome cross-linking with psoralen and UV light [12]. Binding of mB7-1 and mB7-2 molecules to soluble CTLA-4 fusion protein was also clearly demonstrated. In separate experiments, we have proved the biological function of mB7-1, and mB7-2, or both together using proliferation assays with isolated naive CD4$^+$ from murine splenocytes (data not shown).

Presentation of exogenous antigen and stimulation of T cell proliferation by virus-encoded MHC class II and costimulatory molecules

The biological functions of the virus-encoded I-A^k and mB7s were assessed by their ability to present antigen and to stimulate T cell proliferation *in vitro*. The chicken conalbumin peptide 132–145 is known to be an I-A^k-restricted epitope recognized by the cloned T helper cell line D10.G4.1 [17]. To see whether this peptide can be presented by the I-A^k molecule encoded by the rVV, we infected K1735 cells with replication-incompetent rVV encoding I-A^k with or without the two B7s and cocultured the infected cells with resting D10.G4.1 cells in the presence of exogenous chicken conalbumin 132–145 epitope. As shown in Fig. 4a, a stimulation of D10.G4.1 cells was observed by cells expressing the I-A^k molecule alone in the presence of peptide antigens. However, with the expression of either one or both costimulatory molecules, further enhancement of proliferative response was observed, indicating that both the MHC class II and the costimulatory molecules encoded by the single rVV were expressed and functional.

Presentation of endogenous antigen encoded by rVV

We then tested whether the antigen can be encoded by the rVV and presented by co-expressed MHC class II molecules that were

encoded by the same virus. To do this, we infected cells with replication-incompetent viruses encoding the chicken conalbumin 132–145 peptide fused to the signal peptide of the adenovirus glycoprotein E3/19k without exogenous antigen added to the culture. As shown in Fig. 4b, c, cells infected by rVV encoding the antigenic peptide alone were unable to present the antigen to D10.G4.1 cells as tested in both K1735 and B16.F10 cells. In contrast, when both antigen and its presenting MHC class II molecule (I-A^k) were co-expressed by the infected cells, stimulation of D10.G4.1 proliferation could be induced. Again, the inclusion of recombinant costimulatory molecules seemed to enhance further the proliferative response (Fig. 4c). In all experiments control virus-infected cells or cells infected with rVV encoding I-A^k and/or mB7 molecules without conalbumin peptide were unable to stimulate D10.G4.1 cell proliferation (data not shown).

DISCUSSION

In the present study, we engineered non-immunogenic tumour cells into artificial APC by infection with a multiple gene expressing non-replicating rVV. The genes essential for mimicking professional APC were cloned into three different non-essential loci of the genome. Thus, the model antigen conalbumin, the MHC class II heterodimer I-A^k and costimulatory molecules were encoded by the same vector and therefore expressed by the same cell upon infection.

Using the Th2 clone D10.G4.1 for testing our APC constructs, we proved the bioactivity of conalbumin and that of the I-A^k heterodimer *in vitro*. Cells were then engineered to express both conalbumin peptide—linked to a signal sequence—and I-A^k together and were shown to induce a strong proliferative response in the D10.G4.1 effectors. This indicates that VV-infected non-immunogenic cells were able to produce the antigen endogenously, process and associate it with the MHC class II complex at their surfaces. By addition of costimulatory molecules to the engineered APC, proliferation of the effectors was further enhanced. These results suggest that D10.G4.1 cells responded to the stimulation by cells co-expressing the endogenously produced antigenic peptide and I-A^k similarly to cells expressing I-A^k alone in the presence of exogenously added antigen peptide. In contrast, no response to cells expressing the antigenic peptide, I-A^k, or costimulatory molecules alone, could be observed.

Unlike naive T cells needing at least one costimulatory signal during T cell receptor (TCR) activation, conalbumin-specific D10.G4.1 cells seemed to require a costimulatory stimulus for maximal activation only if TCR engagement with conalbumin and I-A^k did not occur under optimal conditions. After gene transfer, these cells may present their tumour-associated antigen in the context of the engineered molecules, thus triggering naive $CD4^+$ T cells for primary immune responses.

Despite the clear specialization of MHC class I and class II molecules for the acquisition of antigenic peptides in distinct intracellular compartments, there are several examples of class I presentation of exogenous antigen and of class II presentation of endogenously synthesized peptides without the obvious route to endosomes [10]. Some endogenously synthesized antigens with access to the secretory pathway can be presented by class II molecules, whereas its confinement to the cytoplasm is ineffective in generating the complex between peptide and MHC molecule [18]. In other situations, however, cytosolic antigen may be processed by class II molecules [19,20].

With the advent of genetic engineering of cells, it has been shown that exogenous and endogenous hen egg lysozyme (HEL) antigen can be processed and presented *in vitro* in association with the class II MHC molecule I-A^k by gene transfer [21,22]. Using rVV as a vector system, Jaraquemada *et al*. have shown evidence for an endogenous processing pathway for the presentation of cytosolic antigen. Class II^+ human B cells infected with rVV expressing the influenza A virus M1 matrix protein were lysed by HLA-DR1-restricted cytotoxic T cells [23]. *In vivo* immunization experiments in mice have demonstrated that rVV expressing a chimeric gene encoding the lysosomal-associated membrane protein LAMP-1 and the human papilloma virus E7-protein were able to generate greater E7-specific lymphoproliferative activity than the rVV encoding the E7-protein alone [24]. Thus, specific targeting of an antigen to the endosomal and lysosomal compartments enhanced class II-restricted antigen presentation and vaccine potency.

Also, studies with animal tumour models indicated that introduction of MHC class II genes into tumour cells increased their immunogenicity and protected against subsequent challenge with wild type MHC II^- sarcoma cells [25]. Tumour cells engineered to APC by transfection with genes encoding murine MHC class II molecules and mB7-1 induced protective immunity against rechallenge with wild type tumour and could rescue mice carrying established tumours in a certain percentage of cases [11]. Transfection with costimulatory molecules alone, however, could elicit antitumoural immunity only in immunogenic tumours expressing rejection antigen, but not in so-called non-immunogenic tumour types like K1735 or B16.F10 melanoma [26]. Since most tumours do not express MHC class II, transfer with genes encoding class II as well as costimulatory factors may enhance immunogenicity of tumour cells [11]. However, there are important limitations for transfection experiments in number and size of foreign genes that can be transferred into the cell by a single plasmid vector. In contrast, many dispensable viral gene loci are known in VV, that allow the multiple insertion of a broad spectrum of recombinant genes for expression.

Our data suggest that cells can be engineered with a single recombinant vector to express a model antigen epitope in the context of class II molecules and co-receptor determinants *in vitro* in a syngeneic system. These findings hold implications for the future design of artificial APC in the generation of cellular immune responses.

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