Gene Therapy (2000) 7, 1575–1583

www.nature.com/gt

VIRAL TRANSFER TECHNOLOGY ***RESEARCH ARTICLE***

Poxvirus as a vector to transduce human dendritic cells for immunotherapy: abortive infection but reduced APC function

L Jenne[1,2], C Hauser[2,3], J-F Arrighi[2,3], J-H Saurat[2] and AW Hügin[2]

[1]*Department of Dermatology, University of Erlangen-Nürnberg, Germany;* [2]*Department of Dermatology DHURDV, and* [3]*Division of Immunology and Allergy, University Hospital, Geneva, Switzerland*

Dendritic cells (DC) are potent antigen-presenting cells (APC). Ongoing preclinical and clinical studies exploit this capacity for the immunotherapy of tumors. We tested vaccinia virus (VV) as a vector to transduce human DC. Immature and mature DC were prepared from blood monocytes and infected with (1) recombinant VV expressing GFP to analyze infection rates, virus replication in DC and the effect of infection on DC phenotype and (2) recombinant VV expressing beta-galactosidase (βGAL) under the control of viral early, intermediate and late promoters to analyze the poxvirus-driven gene expression. While the infection rate in DC was comparable to a permissive fibroblast cell line, viral βGAL gene expression was limited to early promoters. Genes under the control of virus late promoters were not expressed by VV in DC, indicating an abortive infection. VV infection selectively reduced the surface expression of the costimulatory molecule CD80 and the DC maturation marker CD83 on mature DC while other surface molecules including CD86 and MHC remained unchanged. In line with this finding, there was a pronounced reduction in the capacity of VV-infected DC to stimulate allogeneic or autologous T cells in mixed lymphocyte reactions. Furthermore, VV infection inhibited the maturation of immature DC after exposure to proinflammatory cytokines. These results indicate that VV-derived vectors may have complex effects on their target cells. In the case of DC used for immunotherapy, this may be detrimental to their function as potent APC and particularly their capacity to activate T helper cells. Gene Therapy (2000) **7,** 1575–1583.

Keywords: *dendritic cells; poxvirus; vaccinia virus; viral vector; gene therapy; immunotherapy*

Introduction

Dendritic cells (DC) play a pivotal role in the initiation of T cell-dependent immune responses.[1] Recent advances in the *ex vivo* generation of DC[2,3] have sparked interest in using DC for tumor immunotherapy. Clinical trials with DC as immune stimulators focus on loading tumor-associated antigens (TAA) on to the surface of DC by using either synthetic peptides suitable for MHC class I binding or on pulsing DC with whole tumor cell preparations.[4–6] Disadvantages of using DC pulsed with synthetic immunodominant peptides from TAA are the uncertainty regarding the longevity of antigen presentation which might be of short duration,[7] the need to determine the patient's haplotype and the profile of the individual TAAs, and the lack of $CD4^+$ helper cell-related epitopes. In addition, the CTL resulting from such protocols have a good *in vitro* capacity for killing peptide-pulsed target cells but only a modest capacity for killing tumor cells.[8] Alternatively, DC can be transduced to express full-length TAA genes. Physical methods of transfection such as DNA/liposome complexes or electroporation show a relatively low efficiency, reaching transduction levels of $<2\%$.[9–11] Some recombinant viral vectors have a high transduction efficiency and are more likely to generate a strong and sustained $CD4^+$ helper cell and a cytotoxic response against tumors. So far, adenovirus and avipoxvirus vectors have been described as efficiently transducing DC.[12–14]

Vaccinia virus, a member of the orthopox virus family, has been developed to a versatile vector system. It is able to carry at least 25 kb heterologous DNA, allowing the simultaneous expression of different heterologous genes.[15,16] Either highly attenuated virus strains,[17,18] or strains deficient for late gene expression[19] have been developed. Such vector systems are suitable for both *in vivo* vaccination and *in vitro* transduction. Numerous VV-based recombinants expressing TAAs have been described to elicit *in vivo* antibody and cellular immune responses.[20,21] We evaluated VV as vector for the transduction of human DC which could be used to generate an antitumoral or anti-infection immunity. For this purpose we infected *in vitro* generated monocyte-derived DC with green fluorescence protein (GFP)- and βGAL-expressing recombinant VV (rVV) and analyzed virological and immunological phenotypic and functional parameters of the DC.

Correspondence: A Hügin, Department of Dermatology 4-783, University Hospital, 24, rue Micheli-du-Crest, 1211 Geneva 14, Switzerland
Received 18 February 2000; accepted 22 June 2000

Results

Vaccinia virus does not replicate in DC

As a first virological parameter, VV replication was analyzed in immature and mature DC and in the permissive fibroblast cell line Mc57G. Initial virus titers at 1.5 h after infection were comparable between DC and Mc57G, indicating that virus associates equally with the two cell types (Figure 1). Nevertheless, while vaccinia virus replicates efficiently in Mc57G cells resulting in a three-log increase of the virus titer, titers dropped in both immature and mature DC over the period analyzed. In all individuals tested ($n = 6$), the drop in titers observed in DC was similar. Virus titers in DC cultures dropped even after 2 days of infection, excluding a different kinetic of virus replication in DC. This indicates that human monocyte-derived DC are not productively infected by vaccinia virus.

Vaccinia virus late gene expression is aborted in DC

Since no virus replication occurred in DC, we next analyzed if VV can penetrate DC at all. Using a series of recombinant viruses expressing βGAL under the control of specific viral early, intermediate and late promoters, viral gene expression at the different stages of viral infection can be investigated and the stage at which the virus replication cycle is arrested can be determined. Three and 6 h after infection only early and intermediate gene expression takes place in DC as indicated by the βGAL expression of vMJ343, WT-G8R and VSC56, which has a combined early/late promoter (Figure 2). βGAL expression was comparable between DC and Mc57G. This indicates that virus successfully infects both immature and mature DC. Nine and 48 h after infection, βGAL expression under the control of the p11 late promoter was extremely strong in vTFCLZ-1 infected Mc57G, but not detectable in either immature or mature DC (Figure 2). This indicates the complete lack of late gene expression in DC. To analyze further the early expression of βGAL without the interference of intermediate and late promoter components, βGAL activity was analyzed in DC and Mc57G after infection in the presence of Ara-C. This DNA synthesis inhibitor prevents protein expression that is dependent on viral DNA template amplification (virus replication), resulting in the complete suppression of gene expression under the control of virus late promoters. The βGAL expression at different times after infection remained comparable between DC and Mc57G (Figure 2). This further supports the conclusion that the early gene expression is comparable between a permissive cell line and the nonpermissive DC. Nevertheless, the shift from early/intermediate to late gene expression in DC is prevented, resulting in an abortive infection.

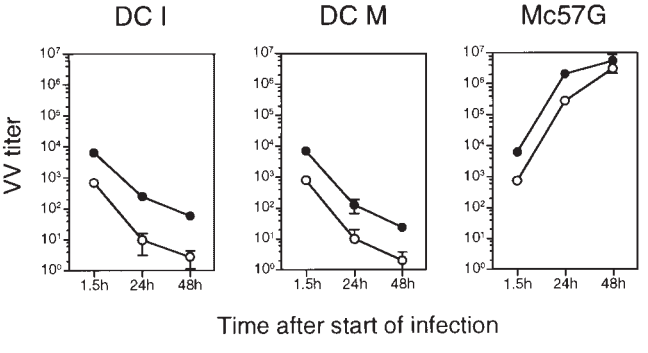

Figure 1 *Vaccinia virus replication in DC. Immature DC, mature DC and the permissive fibroblast cell line Mc57G were infected with VV at an MOI of 0.5 (closed symbols) and 0.05 (open symbols). Virus titers were determined for each culture at the times indicated after infection. Indicated are the mean ± s.d. of triplicate cultures.*

To investigate the efficiency of VV infection, we infected DC with the GFP-expressing recombinant VV strain. This allows quantification of the percentage of GFP-positive and thus infected DC. At a MOI of 2.5 (the MOI chosen for the subsequent phenotypic studies of infected DC), the percentage of VV infected (GFP^+) DC 6 h after infection was 30.9 ± 14.5 ($n = 7$) for mature and 14.1 ± 6.2 ($n = 5$) for immature DC. This compares with an infection rate of 92.0 ± 0.5 ($n = 7$) for Mc57G cells and 74.1 ± 4.4 ($n = 4$) for BS-C-1 cells, analyzed at 6 h post infection (p.i.). This time-point was chosen to exclude secondary infections. Infection rates for DC increased up to 24 h, when peak levels of GFP^+ cells were reached. The rates observed varied between individuals from 16 to 46.7% (mean: 33.7%; s.e.m.: 6.1) in immature DC and from 26 to 67.9% (mean: 57.8%; s.e.m.: 8.2) in mature DC. In all experiments performed at an MOI of 2.5, the infection rate of mature DC infected was slightly but significantly higher than in immature DC (Wilcoxon test, $P \leqslant 0.01$). At this MOI, the cell DC viability was greater than 95% at the time of analysis. Infection of DC at a MOI of up to 10 resulted in an infection rate of 86% (one experiment), suggesting that the lower infection rates of DC were relative rather than absolute. Nevertheless, trypan blue exclusion and cytofluorometric analysis of DC infected at higher MOI revealed a virus dose-dependent increase in dead cells at later times after infection (>24 h).

Vaccinia virus infection does not induce maturation of immature DC

Viruses interact with their target cells in various ways. Influenza virus induces DC maturation after infecting immature DC cells.[22] Other viruses, such as HSV, interfere with induced DC maturation.[23] Thus, we next investigated how VV affects DC maturation. Infection with GFP expressing VV at an MOI of 2.5 resulted in the transduction of 25% to 60% of DC. Thus, this infection rate allows the direct comparison of surface marker expression on infected and uninfected DC within the same culture. First, we investigated whether the VV infection induces phenotypic or functional changes in immature DC. Figure 3 shows the flow cytometric analysis of MHC molecules, costimulatory molecules CD80 and CD86, and the maturation marker CD83 expressed on immature DC 24 h after infection as compared with uninfected immature DC. There was an apparent slight increase in the surface expression of MHC-I and MHC-II in VV infected (GFP^+) DC and in the uninfected (GFP^-) population present in the same well, while the CD80, CD86 and CD83 surface expression remained unchanged. Similar results were obtained after 6 and 48 h. The slight apparent up-regulation of MHC molecules had no effect on the functional incapacity of VV infected immature DC to stimulate allogeneic or autologous PBMC in mixed leukocyte reactions. Taken together, these results indicate that the VV infection does not induce a phenotypic or functional maturation of the immature DC.

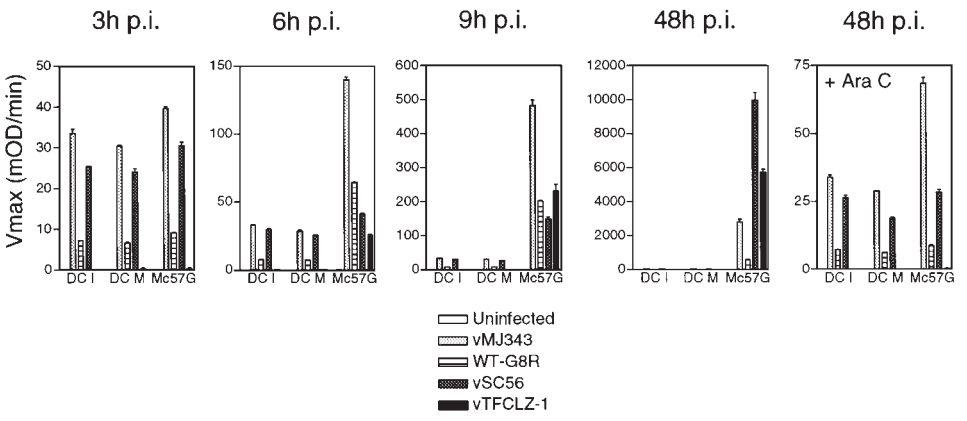

Figure 2 *βGAL expression by VV infected DC. Immature, mature DC and the permissive fibroblast cell line Mc57G were infected at an MOI of 2.5 with rVV expressing βGAL under the control of the following viral promoters: synthetic strong early promoter (vMJ343); natural intermediate promoter (WT-G8R); synthetic strong early/late promoter (vSC56); strong late promoter p11 (vTFCLZ-1). In one experimental setting (indicated by + Ara C), cytosine-1-β-arabinofuranoside was added at a concentration of 40 μg/ml to prevent virus late gene expression. At the times indicated after infection, βGAL activity was determined for each culture in a kinetic assay as described in materials and methods. Values represent the mean ± s.d. of triplicate cultures.*

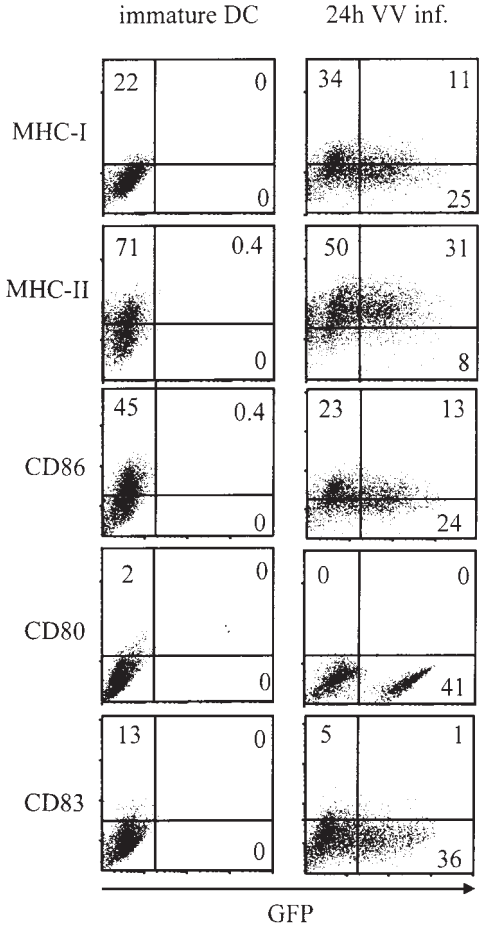

Figure 3 *VV infection does not induce maturation in immature DC. Immature DC were infected with rVV-GFP at an MOI of 2.5 and the surface marker profile was analyzed by flow cytometry 24 h later. Percent positivity of viable cells (a life gate was set using 7-AAD) is given in the dot blot analysis. Data are shown for one of five experiments; all gave similar results.*

Vaccinia virus inhibits the maturation of DC

Poxviruses have developed multiple strategies to interfere with the immune response and thus the development of an antiviral protection. Interference with DC maturation after infection would be an efficient way to subvert the immune system. Thus, we investigated if VV infected immature DC could still be matured in the presence of a maturation-inducing cocktail which includes proinflammatory cytokines. Maturation of DC induces the surface expression of the maturation marker CD83 and the up-regulation of costimulatory and MHC molecules. In addition, matured DC have an increased capacity to induce allogeneic and autologous T cell proliferation. Forty-eight hours after infection with VV and the simultaneous addition of maturation cocktail, we analyzed the DC phenotype of infected (MOI of 2.5) and uninfected DC cell cultures by flow cytometric analysis (Figure 4). First of all, VV infected DC could not be matured, as no up-regulation in the expression of CD83 and CD80 was detectable, as shown for in the histogram for all cells of the DC culture. Although the infection rate in the experiment shown was only 45%, the phenotype of uninfected and VV infected DC populations within the infected DC culture seems to be similarly affected. In addition, the expression of CD86, which is increased during maturation of immature DC but remains high and unaltered after VV infection of mature DC, remained unchanged after VV infection of immature DC in the presence of the maturation cocktail. Poly(I:C) induces DC maturation by activating NF-KB by a different pathway than the differentiation cocktail.[24–26] Addition of Poly(I:C) to infected cultures did not overcome the maturation block caused by VV infection (data not shown). Furthermore, infected DC exposed to the maturation mix were incapable to induce proliferation in MLR (data not shown). Thus, VV infection prevents the phenotypic and functional maturation of immature DC. Interestingly, this inhibition affects both VV infected (GFP^+) and uninfected (GFP^-) DC.

Vaccinia virus infection alters surface marker phenotype and function of mature DC

Mature DC are the most potent APC. This is in part due to the expression of costimulatory molecules, which are

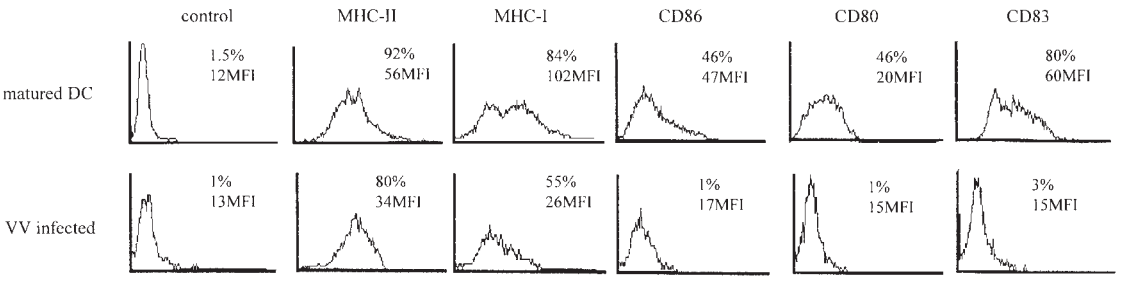

Figure 4 *VV infection inhibits DC maturation. Immature DC were either infected with rVV-GFP at an MOI of 2.5 (lower row) or left uninfected (upper row) and incubated in the presence of a maturation cocktail. Twenty-four hours after infection, the phenotype of control and infected DC was analyzed by flow cytometry for the expression of the indicated surface markers. The infection rate in the experiment shown was 45%. Surface marker expression was calculated in percent positivity and mean fluorescence intensity (MFI). One of five experiments with comparable results is shown.*

required for the induction of a strong and long-lasting immune response. To investigate whether VV infection affects the phenotype or function of mature DC, we analyzed their surface marker expression profile after infection. Figure 5a shows that the MHC-I and MHC-II expression is not altered significantly 24 h after virus infection. The same findings were confirmed at two additional time-points (6 and 48 h p.i.). In sharp contrast, the expression of the maturation marker CD83 and the costimulatory molecule CD80 was dramatically down-regulated in virus infected cells, while the CD86 surface expression remained unchanged. Interestingly, CD83 expression decreased in a time-dependent fashion in both uninfected (GFP^-) and infected (GFP^+) DC present in the same culture (Figure 5b), while CD80 expression was immediately down-regulated in both cell fractions (Figure 5c).

To test if VV infection impacts on the functional properties of DC, we performed allogeneic MLR. While mature DC induced a strong and dose-dependent proliferation, DC from VV infected cultures had no (Figure 6a) or at least a strongly reduced (in two of five experiments) capacity to induce proliferation. This happened in spite of the fact that at most, 60% of the DC were infected. Similarly, autologous MLR was completely abolished when infected DC cultures were used (data not shown). Since a reduced viability of DC might preclude a proliferative response by allogeneic T cells, the viability and number of virus infected DC was also verified microscopically by trypan blue exclusion and by flow cytometric analysis using the death cell marker 7-AAD. The viability remained greater than 90% after 48 h and dropped to 35% 72 h after infection. This excludes a strong drop of viable APC as the cause of a reduced MLR response. Mature DC are not only the best allogeneic stimulators, they are also the best APC for superantigens.[27] To test superantigen presentation by VV infected DC, we analyzed the capacity of SEA pulsed DC to induce proliferation in autologous proliferation assays (Figure 6b). Again, VV infected mature DC cultures are unable to induce superantigen-induced proliferation.

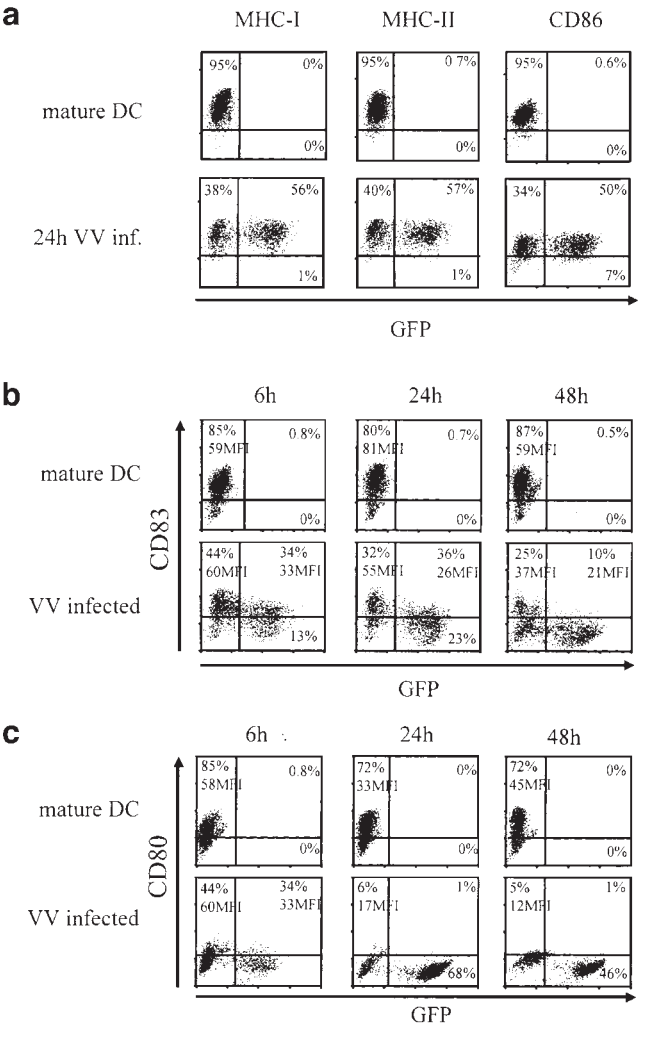

Figure 5 *VV infection induces phenotypic changes on mature DC. Mature DC were infected with rVV-GFP at an MOI of 2.5. Twenty-four hours after infection, the phenotype of control uninfected and infected DC was analyzed by flow cytometry for the expression of maturation and differentiation markers. Time-course of the loss of CD83 (b) and CD80 (c) expression in DC cultures after infection with rVV-GFP. The figures show one of five experiments, all of which showed similar alterations.*

Discussion

Dendritic cells are the most potent APC to induce both class I and class II restricted T cell responses. When using viral vectors to transduce DC, optimal stimulatory

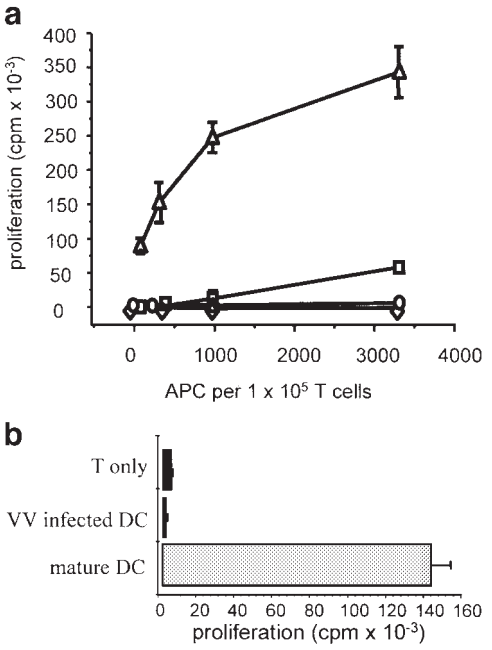

Figure 6 *VV infection reduces APC function of DC. (a) Mature DC were infected with rVV-GFP at a MOI of 2.5 resulting in 40–60% infection of DC. Six hours after infection mature DC (△), immature DC (□), mature VV-infected DC (○), and immature VV infected DC (◇) were used at different concentrations as stimulator cells in an allogeneic MLR. The proliferation of responder T cells was generally below 400 c.p.m. The experiment shown represents one of five with similar results. Data are given in mean of triplicates ± s.d. (b) VV infected DC and uninfected DC were loaded with 10 ng/ml SEA and used in a 1:30 ratio to stimulate autologous T cells. Thymidine uptake was measured 48 h after cocultivation. The experiment shown is representative for three experiments with similar results. Data are given in mean of triplicates ± s.d.*

capacity is only achieved if: (1) the virus infects the target cell efficiently, (2) expresses the specific antigens in sufficient quantities, and (3) if the fully competent phenotype of a potent APC is induced and/or maintained. At an MOI of 2.5, VV transduce up to 60% of DC, with higher infection rates at higher MOI. This places VV among the most efficient vectors. While we consistently observed a slightly better infection rate in mature DC, others[28,29] found about two-fold higher infection rates in immature DC. Results from our βGAL experiments are in line with our infection rate experiments. Beside using different methods to analyze infection rates (VV-driven GFP expression *versus* the use of antibodies to detect viral antigens), Subklewe *et al*[28] and Engelmayer *et al*[29] used monocyte-conditioned medium to induce DC maturation, while we used a well-defined cocktail. Thus, the presence of yet undefined factors may influence VV infection of DC. Nevertheless, in all instances, the infection of both immature and mature DC was always abortive. This is comparable with the resistance of mature DC to influenza virus infections,[30] but is in contrast to measles virus, which replicates in immature and even more efficiently in mature DC.[31,32] The cytokines IL-4 and GM-CSF added to immature and mature DC are not known to have antiviral effects. Possibly, the DC derived from blood monocytes as used here retained their non-permissive phenotype that has been described for VV infected blood monocytes/macrophages.[33] Interestingly, although no VV replication could be detected, the viability of DC in the infected cultures dropped after 48 h of infection. Apparently, even an abortive VV infection with expression of a restricted set of viral genes results in reduced survival of DC, which has been attributed to late apoptosis by infected DC.[28,29]

Since the VV infection of DC is abortive, a detailed analysis of gene expression will help to design vectors that still express high levels of heterologous proteins for immunization. The analysis of βGAL expression in DC showed successful virus penetration and a virus-driven gene expression that was limited to early and intermediate genes. These results are in line with results obtained with fowlpoxvirus which, as a member of the avipoxviruses, is host-range restricted and does not replicate in mammalian cells.[12] No late gene expression has been observed with other VV[28,29,34] and the modified vaccinia virus Ankara (MVA) after infection of human DC derived from $CD34^+$ hematopoietic progenitor cells.[35] Comparison of the βGAL expression in DC and a permissive cell line in the presence of Ara-C indicates that the early stages of VV infection in DC are not affected by the cellular virus restricting elements. The absence of late gene expression in DC may be due to a block in viral DNA replication, since viral DNA amplification is required for the expression of intermediate, and especially, late genes. Indeed, the absence of viral DNA replication in VV infected DC has recently been observed.[34] As a consequence, the use of poxvirus vectors would require their optimization to express specific proteins under the control of strong viral early and intermediate promoters.

Infection of DC with viruses can induce various virus-specific effects. While influenza virus infection initiates maturation of immature DC,[36] VV infection did not. On the contrary, VV even prevented the induced maturation of immature DC. Our results confirm and extend recent similar findings obtained with VV Copenhagen[34] or with another vaccinia virus.[28] Engelmeyer *et al*,[29] using monocyte-conditioned medium to mature DC, analyzed maturation of DC with an anti-DC-LAMP antibody. They observed that VV infection blocks maturation only of VV infected DC cells while uninfected cells within the same culture matured normally. This contrasts with our finding that within an infected DC culture, the surface expression of differentiation markers is attenuated in both infected and uninfected DC after VV infection. Possibly, virus infection differentially affects DC maturation markers. Alternatively, different maturation methods may account for the observed phenotypic differences after VV infection.

Since the addition of Poly(I:C) to the infected, maturation cocktail-containing cultures did not overcome this maturation block in our experiments, virus infection is likely to interfere with a key step in the induction of maturation. VV shares the inhibitory activity on DC maturation with Herpes simplex virus type 1 (HSV-1), another large DNA virus.[23] Furthermore, HSV-1 has a comparable capacity to induce CD83 down-regulation in mature DC without affecting the surface expression of CD80.[37] Most notably in our experiments, VV infection of mature DC resulted in the down-regulation of both CD80 and CD83 (Figure 5) and in reduced APC function.

The use of DC for immunization has focused on the generation of CTL responses to tumor or viral antigens.[38–40]rVV infected DC have been used to induce virus-specific CTL.[28,40] Less attention has been attributed to the induc-

tion of T helper cells by VV infected DC. Thus we analyzed the stimulatory capacity of VV infected DC in MLR and superantigen presentation, two settings in which proliferation is associated with MHC-II restricted T lymphocytes. In both tests, proliferation was strongly reduced when DC were infected with VV. Although the MHC-II expression levels on DC are unchanged after VV infection, absence of a CD80 signal and possibly the reduced expression of CD83 by mature antigen-presenting DC may interfere with the generation of a $CD4^+$ helper cell proliferative response. Thus, the functional properties of VV infected DC were consistent with the phenotype we observed after infection. Our results contrast in part with observations made previously.[29,34] Engelmeyer *et al* found strongly reduced MLR when DC were infected and matured, and subsequently used as stimulators, in spite of the presence of apparently matured DC in VV-infected DC cultures. Similarly, we observed that DC matured in the presence of virus was unable to induce a MLR. Nevertheless, MLR responses were only slightly reduced when Engelmeyer *et al* used matured DC infected with VV as stimulators. Similarly, Drillien *et al*, who used LPS matured DC, observed either enhanced or reduced MLR responses depending on the donor. It has to be established if the method used to induce matured DC may also account for these functional differences.

The generation of an anti-EBV CTL response with recombinant VV-transduced DC, using an identical VV strain, as reported by others,[28] might be explained by cross-priming mechanisms described for influenza virus.[41] Alternatively, the requirements for CTL induction may be different from the requirements for $CD4^+$ helper cells. Interference with mature DC functions has also been described for measles virus.[31,32] However, in contrast to the measles virus, VV infection of DC is abortive. This excludes secondary VV infection of T cells as the cause of a reduced proliferative response in MLR.

Since VV generally induces a good CTL response *in vivo* and *in vitro*, what are the consequences of a potentially reduced T helper cell response? While $CD4^+$ helper cells may not be so important for the induction phase of a CTL response, they may amplify a weak CTL response. In addition, the induction and persistence of $CD4^+$ helper cells may be of crucial importance to sustain a CTL response and for the long-term maintenance of a CTL memory. For humans, there is ample evidence in viral infections for a critical role of $CD4^+$ helper cell and CTL collaboration in sustaining an active cytotoxic response during a longer period and the maintenance of a strong memory CTL population.[42–46] In murine models, the importance of a concurrent $CD4^+$ helper cell response for the maintenance of a CTL memory and for the optimal protection in both, virus and tumor models, has been firmly established.[47–51] Vaccines that only induce a CTL but not a $CD4^+$ helper cell response may thus confer a transient but not prolonged protection. As a consequence, CTL vaccination protocols should also aim to induce a concurrent $CD4^+$ helper cell response.

Since VV infection of DC may preclude a $CD4^+$ helper cell response under certain circumstances, our *in vitro* observation could provide an explanation, why successful protective vaccination with a prime/boost approach requires a DNA prime/MVA boost combination, while the inverse sequence of vaccination was not or less protective.[52–54] Indeed, MVA, the host restricted modified vaccinia virus Ankara, induced in dendritic cells effects comparable with our VV strain (L Jenne and AW Hügin, unpublished results).

How could the downmodulation of CD80 and the loss of CD83 be compatible with the induction of a CTL response generally observed with rVV *in vivo* and *in vitro*? First, experiments in animal models have shown that $CD4^+$ helper cells are not required for an anti-VV CTL response.[55] Furthermore, CTL responses to VV and other viruses have been observed in CTLA-4 transgenic mice, suggesting that CD80 expression by APC is not an absolute requirement for the induction of a CTL response.[56,57] In addition, it has been shown that the costimulatory molecule CD86 (which was not affected by VV, Figure 5) can drive a CTL response in a MHC restricted manner.[58] Furthermore, the kinetic requirements of CTL cells for CD80 expression by APC may be different from $CD4^+$ helper cells. It has also been shown that human DC are able to induce efficiently a CTL response in the absence of $CD4^+$ helper cells.[59,60] In summary, these observations indicate that the specific requirements of antigen presentation may be different for the $CD4^+$ helper cell and the CTL response.

The various effects that we have seen after VV infection of DC reflect the complex interactions of large viruses with their host's immune system. This poses the question, whether the downmodulation is a consequence of the altered cellular metabolism that occurs after infection with a large DNA virus or if a specific virus factor is responsible for the effects seen. Host shut-off, the reduced synthesis of host cell proteins, is a consequence of VV infection.[61] Inhibition of the MLR occurred in the presence of a large portion of uninfected DC. In addition, MHC-I synthesis, which is sustained after DC maturation, is not diminished after VV infection of mature DC. All this indicates that host shut-off after infection cannot account for all the effects observed. Rather, this suggests a more active role for VV or virus-derived factors, respectively. Indeed, poxviruses have evolved to encode a number of proteins, generally expressed early in infection, that interfere with and subvert the host immune response.[62]

In conclusion, we have demonstrated that although VV vectors can transduce DC *in vitro*, VV infection impairs the allostimulatory capacity and thus the generation of a T helper cell proliferative response *in vitro*. Identification and elimination of the viral gene(s) that modulate APC functions from the viral genome could result in a VV vector that facilitates a concurrent helper cell response. This will result in a stronger and more persistent CTL response. In more general terms, each virus has its own pattern of effects on DC. These effects have to be taken into account when using viruses as vectors for *in vivo* vaccination or *in vitro* transduction of DC. A deeper understanding of these interactions and the identification of the factors involved will help in the design of better vector systems and vaccines.

Materials and methods

Cells

BS-C-1 (ATCC CCL-26; Rockville, MD, USA), CV-1 (ATCC CCL-70) and Mc57G cells were grown in DMEM

(Life Technologies, Basel, Switzerland) supplemented with antibiotics, L-glutamine and 10% heat-inactivated fetal calf serum (FCS) (Life Technologies).

DC generation from buffy coats

Buffy coats of healthy donors ($n = 6$) were obtained according to institutional guidelines. Peripheral blood mononuclear cells (PBMC) were prepared by density centrifugation using Ficoll–Paque (Pharmacia, Uppsala, Sweden). PBMC were resuspended (15×10^6 cells per well) in six-well plates (Nunc, Roskilde, Denmark) and incubated for 1 h at 37°C. Nonadherent cells were removed and the remaining cells were fed with 3 ml of X-VIVO 15 medium (Bio-Whittaker, Walkersville, MD, USA) containing 1% of heat-inactivated autologous plasma, 10^3 IU GM-CSF/ml (Leukomax) (Novartis, Basel, Switzerland, kindly provided by Dr PY Dietrich, University Hospital, Geneva, Switzerland) and 10^3 IU IL-4/ml (Stratagen, Hannover, Germany). Cells were refed with 0.5 ml of fresh medium containing 10^3 U GM-CSF and 10^3 U IL-4 per ml on days 2, 4 and 6. On day 7, the nonadherent cells were transferred to a new well with fresh medium. DC maturation was induced with a cocktail of cytokines and prostaglandin E2 (PGE2) as recently published.[63] The following cytokines were added: IL-4, 10^3 U/ml; IL-1β, 2×10^3/ml; IL-6, 10^3 U/ml (all from Stratagen), GM-CSF, 10^3 U/ml, PGE2, 1 μg/ml (Prostin; Pharmacia & Upjohn, Dübendorf, Switzerland); TNF-α, 10^2 U/ml (kindly provided by Prof J-M Dayer, University Hospital, Geneva, Switzerland). Cells were harvested after 2 days and used for VV infection, flow cytometric analysis and mixed leukocyte reaction.

Virus

The following recombinant viruses expressing β-galactosidase (βGAL) under the control of the indicated promoters were used: vMJ343 (synthetic strong early promoter),[64] WR-G8R (natural intermediate),[65] vSC56 (synthetic strong early/late),[66] vTFCLZ-1 (p11 late promoter) and VV-GFPS65T,[67] which expresses GFP under the control of a synthetic strong early/late promoter. Virus stocks were prepared on CV-1 cells titered on CV-1 or BS-C-1 monolayers according to standard procedures.[68] The multiplicity of infection (MOI) indicated is based on these titers.

To analyze virus replication, DC or Mc57G cells were infected in suspension in RPMI supplemented with 2% FCS. VV-GFPS65T virus was added to 10^6 cells at an MOI of 0.5 and 0.05 and the cells incubated at 37°C. After 1.5 h, the cells were washed once, resuspended in DMEM or DC growth medium and plated in triplicates of 10^5 cells per well in 24-well plates. One set of triplicate cultures was frozen immediately (first time-point). The other cultures were incubated for a total of 24 or 48 h. Virus titer was determined for each individual culture.

Analysis of βGAL expression

DC or Mc57G cells (10^6 each) were infected in suspension at an MOI of 2.5, using medium with 2% FCS and a volume of 0.5 ml. After an incubation of 1.5 h at 37°C, the cells were diluted and distributed in triplicates of 200 μl in a 96-well plate. Three, 6, 9, 24 and 48 h after infection, the plates were frozen at –80°C. For the analysis of βGAL expression, the plates were thawed and 50 μl Triton X-100 were added per well. The plates were incubated for 20 min at room temperature before being transferred on to ice. The wells were harvested individually and transferred to a 0.5 ml tube. The tubes were vortexed for 10 s and subsequently spun for 5 min at maximal speed in a microfuge. In a 96-well plate, 25 μl of the supernatant was added to 25 μl of βGAL buffer (80 mM sodium phosphate buffer pH 7.3, 100 mM 2-mercaptoethanol and 9 mM $MgCl_2$). Then, 100 μl of substrate buffer (8 mM Chlorophenolred β-D-galactopyranoside (Roche Molecular Systems, Mannheim, Germany) in βGAL buffer was added and the plates read for 20 min in kinetic mode in a vmax reader (Molecular Devices, Menlo Park, CA, USA) at 550 nm. As a standard for all assays a fixed concentration of bacterial βGAL (Sigma, St Louis, MO, USA) was used. To inhibit vaccinia virus DNA replication and thus intermediate and late gene expression, cytosine-1-β-arabinofranoside (Ara-C) (Fluka, Buchs, Switzerland) was added at a concentration of 40 μg/ml from the beginning of infection.

Antibodies and flow cytometric analysis

The following monoclonal antibodies (mAb) were used. PE-labeled murine HLA-ABC (G46–2.6), HLA-DR (G46–6), CD80 (BB1), CD40 (5C3) and CD86 (IT2.2) mAb were purchased from Pharmingen (Hamburg, Germany), PE-conjugated CD83 (Hb15a) mAb from Immunotech (Marseilles, France). Purified control IgG1-PE was purchased from DAKO (Hamburg, Germany), IgG2b-PE, IgG1-FITC and IgG2b-FITC mAb from Pharmingen. Staining and analysis of cells for three-color immunofluorescence was done according to standard procedures, using forward and side scatter to eliminate cell debris and 7-amino-actinomycin D (7-AAD) to set a life gate. The results were processed with the Cellquest software (Becton Dickinson, Mountain View, CA, USA). For the analysis of the infection rate, DC, BS-C-1 and Mc57G were infected in suspension at an MOI of 2.5 with VV-GFPS65T. Six hours later, the percentage of GFP-positive cells, indicating a successful infection, was analyzed. DC infection rate was also analyzed 24 h and 48 h after infection. This was not done for BSC-1 and Mc57G cells, as secondary infections would interfere with the correct interpretation of the initial infection rate.

Mixed leukocyte reaction and Staphylococcus enterotoxin A proliferation assay

Allogeneic T cells were obtained from buffy coats of healthy adults after Ficoll–Paque gradient separation, adherence to plastic for 1 h at 37°C, and passage over a nylon wool column (Biotest, Dreieich, Germany). Six hours after viral infection, the DC were irradiated (3000 rads, ^{137}Cs source) and graded numbers of cells were plated in round-bottomed 96-well plates in 200 μl RPMI 1640 medium containing 5% heat-inactivated human AB^+ serum (Blood Transfusion Center, Annemasse, France). A constant number of allogeneic T cells (1×10^5) was added to each well and the cultures incubated for 5 days. As additional functional test we analyzed the capacity of VV infected DC to present the superantigen *S. aureus* enterotoxin A (SEA) (a kind gift of N Bhardwaj, Rockefeller University, New York, USA) to purified T cells. SEA was added at a concentration of 10 ng/ml to autologous proliferation assays performed as described above. T cell proliferation was assessed by adding 1 μCi per well of ^3H-TdR during the last 8 to 12 h of culture.

Acknowledgements

We wish to thank Rafa Blasco and Bernard Moss for kindly providing recombinant viruses. AWH was supported by grants from the Swiss National Science Foundation and the Schweizerische Krebsliga.

References

1 Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; **392**: 245–252.
2 Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* 1995; **182**: 389–400.
3 Arrighi JF *et al*. Long-term culture of human CD34+ progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34–CD14– and a CD34–CD14+ dendritic cell precursor. *Blood* 1999; **93**: 2244–2252.
4 Hsu FJ *et al*. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nature Med* 1996; **2**: 52–58.
5 Thurner B *et al*. Vaccination with Mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999; **190**: 1669–1678.
6 Nestle FO *et al*. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nature Med* 1998; **4**: 328–332.
7 Amoscato AA, Prenovitz DA, Lotze MT. Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. *J Immunol* 1998; **161**: 4023–4032.
8 van der Bruggen P *et al*. A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur J Immunol* 1994; **24**: 3038–3043.
9 Zhong L, Granelli-Piperno A, Choi Y, Steinman RM. Recombinant adenovirus is an efficient and non-perturbing genetic vector for human dendritic cells. *Eur J Immunol* 1999; **29**: 964–972.
10 Van Tendeloo VF *et al*. Nonviral transfection of distinct types of human dendritic cells: high-efficiency gene transfer by electroporation into hematopoietic progenitor- but not monocyte-derived dendritic cells. *Gene Therapy* 1998; **5**: 700–707.
11 Arthur JF *et al*. A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther* 1997; **4**: 17–25.
12 Brown M *et al*. Antigen gene transfer to cultured human dendritic cells using recombinant avipoxvirus vectors. *Cancer Gene Ther* 1999; **6**: 238–245.
13 Dietz AB, Vuk-Pavlovic S. High efficiency adenovirus-mediated gene transfer to human dendritic cells. *Blood* 1998; **91**: 392–398.
14 Kaplan M *et al*. Induction of antitumor immunity with dendritic cells transduced with adenovirus vector-encoding endogenous tumor-associated antigens. *J Immunol* 1999; **163**: 699–707.
15 Paoletti E. Applications of pox virus vectors to vaccination: an update. *Proc Natl Acad Sci USA* 1996; **93**: 11349–11353.
16 Carroll MW, Moss B. Poxviruses as expression vectors. *Curr Opin Biotechnol* 1997; **8**: 573–577.
17 Sutter G, Moss B. Nonreplicating vaccinia vector efficiently expresses recombinant genes. *Proc Natl Acad Sci USA* 1992; **89**: 10847–10851.
18 Tartaglia J *et al*. NYVAC: a highly attenuated strain of vaccinia virus. *Virology* 1992; **188**: 217–232.
19 Holzer G *et al*. Highly efficient induction of protective immunity by a vaccinia virus vector defective in late gene expression. *J Virol* 1999; **73**: 4536–4542.
20 Perkus ME, Tartaglia J, Paoletti E. Poxvirus-based vaccine candidates for cancer, AIDS, and other infectious diseases. *J Leukoc Biol* 1995; **58**: 1–13.
21 Kantor J *et al*. Immunogenicity and safety of a recombinant vaccinia virus vaccine expressing the carcinoembryonic antigen gene in a nonhuman primate. *Cancer Res* 1992; **52**: 6917–6925.
22 Cella M *et al*. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 1999; **189**: 821–829.
23 Salio M, Cella M, Suter M, Lanzavecchia A. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur J Immunol* 1999; **29**: 3245–3253.
24 Verdijk RM *et al*. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J Immunol* 1999; **163**: 57–61.
25 Faruqi TR, DiCorleto PE. IFN-gamma inhibits double-stranded RNA-induced E-selectin expression in human endothelial cells. *J Immunol* 1997; **159**: 3989–3994.
26 Heitmeier MR, Scarim AL, Corbett JA. Double-stranded RNA-induced inducible nitric-oxide synthase expression and interleukin-1 release by murine macrophages requires NF-kappaB activation. *J Biol Chem* 1998; **273**: 15301–15307.
27 Bhardwaj N, Friedman SM, Cole BC, Nisanian AJ. Dendritic cells are potent antigen-presenting cells for microbial superantigens. *J Exp Med* 1992; **175**: 267–273.
28 Subklewe M *et al*. Induction of Epstein–Barr virus-specific cytotoxic T-lymphocyte responses using dendritic cells pulsed with EBNA-3A peptides or UV-inactivated, recombinant EBNA-3A vaccinia virus. *Blood* 1999; **94**: 1372–1381.
29 Engelmayer J *et al*. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol* 1999; **163**: 6762–6768.
30 Fonteneau JF*et al*. Heterogeneity of biologic responses of melanoma-specific CTL. *J Immunol* 1997; **159**: 2831–2839.
31 Grosjean I *et al*. Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. *J Exp Med* 1997; **186**: 801–812.
32 Fugier-Vivier I *et al*. Measles virus suppresses cell-mediated immunity by interfering with the survival and functions of dendritic and T cells. *J Exp Med* 1997; **186**: 813–823.
33 Broder CC, Kennedy PE, Michaels F, Berger EA. Expression of foreign genes in cultured human primary macrophages using recombinant vaccinia virus vectors. *Gene* 1994; **142**: 167–174.
34 Drillien R, Spehner D, Bohbot A, Hanau D. Vaccinia virus-related events and phenotypic changes after infection of dendritic cells derived from human monocytes. *Virology* 2000; **268**: 471–481.
35 Di Nicola M *et al*. Gene transfer into human dendritic antigen-presenting cells by vaccinia virus and adenovirus vectors. *Cancer Gene Ther* 1998; **5**: 350–356.
36 Cella M *et al*. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 1999; **189**: 821–829.
37 Kruse M *et al*. Mature dendritic cells infected with Herpes simplex virus type I are inhibited in their T-cell stimulatory capacity. *J Virol* 2000; **74**: 7127–7136.
38 Schuler G, Steinman RM. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med* 1997; **186**: 1183–1187.
39 Young JW, Inaba K. Dendritic cells as adjuvants for class I major histocompatibility complex-restricted antitumor immunity. *J Exp Med* 1996; **183**: 7–11.
40 Fan Z *et al*. Cultured blood dendritic cells retain HIV-1 antigen-presenting capacity for memory CTL during progressive HIV-1 infection. *J Immunol* 1997; **159**: 4973–4982.
41 Albert ML, Sauter B, Bhardwaj N. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 1998; **392**: 86–89.
42 Matloubian M, Concepcion RJ, Ahmed R. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 1994; **68**: 8056–8063.
43 Gerlach JT *et al*. Recurrence of hepatitis C virus after loss of virus-specific CD4+ T-cell response in acute hepatitis C. *Gastroenterology* 1999; **117**: 933–941.
44 Zajac AJ *et al*. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 1998; **188**: 2205–2213.

45 von Herrath MG *et al*. CD4-deficient mice have reduced levels of memory cytotoxic T lymphocytes after immunization and show diminished resistance to subsequent virus challenge. *J Virol* 1996; **70**: 1072–1079.
46 Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *J Exp Med* 1998; **188**: 2199–2204.
47 Mizuochi T *et al*. Role of lymphokine-secreting CD8+ T cells in cytotoxic T lymphocyte responses against vaccinia virus. *J Immunol* 1989; **142**: 270–273.
48 Ossendorp F *et al*. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* 1998; **187**: 693–702.
49 Hung K *et al*. The central role of CD4+ T cells in the antitumor immune response. *J Exp Med* 1998; **188**: 2357–2368.
50 Cardin RD, Brooks JW, Sarawar SR, Doherty PC. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J Exp Med* 1996; **184**: 863–871.
51 Toes RE, Ossendorp F, Offringa R, Melief CJ. CD4 T cells and their role in antitumor immune responses (comment). *J Exp Med* 1999; **189**: 753–756.
52 Plebanski M *et al*. Protection from Plasmodium berghei infection by priming and boosting T cells to a single class I-restricted epitope with recombinant carriers suitable for human use. *Eur J Immunol* 1998; **28**: 4345–4355.
53 Schneider J *et al*. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nature Med* 1998; **4**: 397–402.
54 Hanke T *et al*. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* 1998; **16**: 439–445.
55 Buller RM *et al*. Induction of cytotoxic T-cell responses *in vivo* in the absence of CD4 helper cells. *Nature* 1987; **328**: 77–79.
56 Zimmermann C, Seiler P, Lane P, Zinkernagel RM. Antiviral immune responses in CTLA4 transgenic mice. *J Virol* 1997; **71**: 1802–1807.
57 Bachmann MF, Zinkernagel RM, Oxenius A. Immune responses in the absence of costimulation: viruses know the trick. *J Immunol* 1998; **161**: 5791–5794.
58 Agadjanyan MG *et al*. CD86 (B7–2) can function to drive MHC-restricted antigen-specific CTL responses *in vivo*. *J Immunol* 1999; **162**: 3417–3427.
59 Young JW, Steinman RM. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. *J Exp Med* 1990; **171**: 1315–1332.
60 Bhardwaj N *et al*. Influenza virus-infected dendritic cells stimulate strong proliferative and cytolytic responses from human CD8+ T cells. *J Clin Invest* 1994; **94**: 797–807.
61 Moss B. Poxviridae: the virus and their replication. In: Fields PE, Knop J, Hsu FJ (eds). *Fields Virology*. Lipincott-Raven: Philadelphia, 1996, pp 2637–2671.
62 Smith GL *et al*. Vaccinia virus immune evasion. *Immunol Rev* 1997; **159**: 137–154.
63 Jonuleit H *et al*. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur J Immunol* 1997; **27**: 3135–3142.
64 Davison AJ, Moss B. Structure of vaccinia virus early promoters. *J Mol Biol* 1989; **210**: 749–769.
65 Baldick CJ, Moss B. Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes. *J Virol* 1993; **67**: 3515–3527.
66 Chakrabarti S, Sisler JR, Moss B. Compact, synthetic, vaccinia virus early/late promoter for protein expression. *Biotechniques* 1997; **23**: 1094–1097.
67 Dominguez J, Lorenzo MM, Blasco R. Green fluorescent protein expressed by a recombinant vaccinia virus permits early detection of infected cells by flow cytometry. *J Immunol Meth* 1998; **220**: 115–121.
68 Moss B, Earl PL. Expression of proteins in mammalian cells using vaccinia virus vectors. In: Renos V, Shandor B (eds). *Current Protocols of Molecular Biology*. John Wiley: New York, 1998, pp 16.15.1–16.15.5.