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ORIGINAL ARTICLE

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Transduction of human dendritic cells with a recombinant modified vaccinia Ankara virus encoding MUC1 and IL-2

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Abstract The epithelial mucin MUC1 is considered an opportune target antigen for cancer immunotherapy, as it is over-expressed and exhibits aberrant glycosylation in malignant cells. Because dendritic cells (DC) are powerful initiators of immune responses, efforts have focused on tumor antigen-bearing DC as potent cancer vaccines. In this study we have characterized the transduction of monocyte-derived DC with a highly attenuated vaccinia virus vector [modified vaccinia Ankara (MVA)] encoding human MUC1 and the immunostimulatory cytokine IL-2. Analysis of transduced DC cultures generated from a number of donors revealed MUC1 expression in the range of 27–54% of the cells and a co-regulated secretion of bioactive IL-2. As shown by FACS analysis with MUC1-specific antibodies, the MVA-MUC1/IL-2-transduced DC predominantly expressed the fully processed glycoform of MUC1, typical of that displayed by normal epithelia. Over a 3-day period after transduction, transgene expression declined concurrent with an increase in MVA-induced cytopathic effects. The transduced DC stimulated allogeneic lymphocyte proliferation, indicating that DC immunostimulatory function is not impaired by vector transduction. In the presence of IL-2, MVA-transduced DC were able to enhance autologous lymphocyte proliferation. Also, vector expression was analyzed in DC cultures treated with TNF-α, a known DC maturation factor. As indicated by the up-regulation of several DC maturation markers, neither virus infection nor transgene expression influenced the maturation capacity of the cells. The MVA-MUC1/IL-2 vector effectively transduced both immature and TNF-α-matured DC. Overall, our results are encouraging for the clinical application of MVA-MUC1/IL-2-transduced DC.

Keywords Dendritic cells · Modified vaccinia Ankara · MUC1 · Immunotherapy · IL-2

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Introduction

The mucin MUC1 is a large, transmembrane glycoprotein localized to the apical membrane of normal epithelial tissue. Aberrant expression of the protein is observed in a number of different carcinoma types (prostate, lung, breast, ovarian, pancreatic, renal) [for reviews, see 39, 51] and certain hematopoietic neoplasms [52]. MUC1 is considered an opportune tumor-associated antigen (TAA) for immune targeting based on several novel features observed in the malignant state: (1) over-expression; (2) aberrant glycosylation, revealing novel epitopes for targeting; (3) loss of the normal apical distribution, allowing greater exposure to the immune system. Moreover, there is evidence of occasional antibody and cytotoxic T cell (CTL) immune responses to MUC1 in cancer patients, suggesting that MUC1 immunization has the potential to stimulate stronger anti-MUC1 responses [39, 51].

For pre-clinical testing, transgenic mice that express human MUC1 in a normal tissue distribution pattern have provided an opportunity to investigate novel vaccine approaches for generating human anti-MUC1 tumor immunity [2, 13, 25, 38, 44]. Of importance, tolerance to human MUC1 can be overcome without inducing autoimmunity. In several clinical trials, cancer patients have been vaccinated by direct administration of MUC1 polypeptides [22, 26, 33]. In general, anti-MUC1 antibody responses predominate with few, if any, MUC1-specific CTL responses detected. Although neither toxicity nor autoimmunity is evident, overt tumor regression has not been observed. Recently, a vaccinia virus (VV) vector containing both a MUC1 cDNA and a cDNA encoding the immunomodulatory cytokine

interleukin 2 (IL-2) was used in a phase I/II vaccine trial of breast cancer patients [47]. Intramuscular vaccination resulted in no serious side effects, and two of nine patients generated MUC1-specific CTL.

Because strong anti-tumor CTL responses are considered vital for tumor eradication [21], dendritic cell (DC)-based tumor vaccination has emerged as a promising approach to cancer immunotherapy [for reviews, see 21, 40, 41]. DC are the most potent antigen-presenting cells for stimulating naive and memory T cell responses, including both $CD4^+$ T helper cells and $CD8^+$ CTL [21, 41]. Strategies involve loading DC with specific TAA peptide/protein or RNA; pulsing DC with whole tumor cell products, such as RNA, apoptotic bodies and peptides eluted from major histocompatibility complexes (MHCs); and directly fusing DC with tumor cells [21, 25, 37, 40]. In a recent phase I study, vaccination with DC pulsed with MUC1-derived peptides induced CTL immune responses in at least some patients with metastatic breast or ovarian cancer [10].

A further approach for loading DC with TAA is the introduction of TAA transgenes into DC [for review see 34]. Advantages include prolonged duration of TAA presentation to the immune system by the gene-expressing DC and display of multiple TAA peptide epitopes by diverse HLA alleles, obviating the requirement to define the patient's HLA haplotype when pulsing with a TAA peptide. Both recombinant viral vectors (adenovirus, poxvirus, retrovirus, herpes virus) and plasmids have been used to introduce transgenes into DC [34].

MUC1 expression in DC has been obtained by transfection with MUC1-encoding plasmid DNA or RNA [35, 45], as well as by transduction with MUC1 recombinant viruses derived from murine retrovirus [27, 28] and adenovirus [24]. Limitations for the application of plasmid DNA and RNA include relatively low transfection efficiencies and lack of durable expression, especially with RNA molecules [34]. Transduction with murine retroviral vectors requires the isolation and transduction of dividing $CD34^+$ progenitors prior to cytokine-induced DC differentiation [27, 28]. This approach is not applicable to DC differentiated from non-dividing monocytes derived from peripheral blood, a common source of cells for ex vivo DC generation [6, 21].

In this report, we have investigated the in vitro transduction of monocyte-derived DC with a MUC1/IL-2 VV-type vector. Although direct administration of this vector type demonstrated CTL responses in at least some patients [47], our rationale is that improved MUC1-specific CTL responses would be observed upon immunization with gene-modified DC. For this study, the recombinant vector was prepared using the highly attenuated VV strain, modified vaccinia virus Ankara (MVA). In contrast to conventional VV strains, MVA exhibits limited replicative capacity in mammalian cells and has lost several functional cytokine receptors that may contribute to VV immune evasion and virulence [5, 7, 50]. We demonstrate that both immature and TNF-α-matured DC are effectively transduced with MVA-MUC1/IL-2, resulting in detectable expression of both MUC1 and bioactive IL-2. Although the MVA vector can induce virus-related cytopathic effects over time, the functional capacity and maturation status of DC are not overtly altered. Our results suggest that DC modified with this MVA recombinant vector may prove clinically relevant as a vaccine for the generation of anti-MUC1 tumor responses.

Materials and methods

Dendritic cells and cell culture

DC were generated from monocyte-derived peripheral blood mononuclear cells (PBMC). Briefly, peripheral blood was obtained from healthy donors, and PBMC were purified over Ficoll-Hypaque (Pharmacia Corp., Peapack, N.J.). The cells were washed three times with sterile phosphate-buffered saline [PBS (Nexell Therapeutics, Irvine, Calif.)] and plated in 25 ml of AIM-V medium (Gibco/BRL, Gaithersberg, Md.) at a concentration of $1.5–2\times10^8$ cells per T-75 flask (Corning Inc., Corning, N.Y.). Monocytes were allowed to adhere for 2 h at 37°C in a humidified 5% CO_2 incubator. The non-adherent PBMC were removed and frozen for future use in Origen Freezing Media (IGEN International Inc., Gaithersberg, Md.) at –80 °C. The plastic adherent cells were subsequently cultured in 25 ml of AIM-V media supplemented with 1000 U/ml GM-CSF (Immunex, Seattle, Wash.), 1000 U/ml Interleukin-4 (Schering-Plough, Kenilworth, N.J.) and 5×10^{-5} M 2-ME (Gibco/BRL). The cells were then incubated for 8 days (± day). For maturation, TNF-α (R&D Systems) was added at 200 IU/ml during the last 2 days of culture. The DC yield was typically 5–10% of the input PBMC. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (Rockville, Md.) and cultured in RPMI-1640 medium (GIBCO/BRL) supplemented with 10% heat-inactivated fetal bovine serum [FBS (HyClone, Logan, Utah)] and 2 mM L-glutamine. Cell viability was evaluated by trypan blue dye exclusion.

Monoclonal antibodies and FACS analysis

The DC phenotype was assessed by immunostaining followed by FACS analysis using fluorescent-conjugated monoclonal antibodies recognizing HLA-DR (MHC class II), CD1a, CD14 (monocyte lineage marker), the co-stimulatory molecules CD86 and CD80, and the CD83 DC maturation marker (BD Pharmingen, San Diego, Calif.). The monoclonal antibodies HMFG-1, HMFG-2 and SM-3, which recognize differentially glycosylated forms of MUC-1, were kindly provided by Dr. J. Burchell (Imperial Cancer Research Fund, London, England) [11, 12, 23]. FITC-conjugated goat anti-mouse antibody (Sigma Chemical Co., St. Louis, Mo.) was used as secondary antibody. Washes were performed in PBS. Cells were subsequently fixed in 2% paraformaldehyde/PBS. Apoptosis and necrosis were assessed by staining with FITC-Annexin V/propidium iodide using the Apoptosis Detection Kit (Caltag Laboratories, Burlingame, Calif.), as described by the manufacturer. Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, Calif.) with staining pattern analysis performed using the WinMDI software (J. Trotter, Scripps Institute, La Jolla, Calif.).

Viral vectors and gene transduction of target cells

The recombinant MVA-MUC1 vector encodes the human MUC1 containing 5 copies of the tandem repeat under the control of the early/late pH5R promoter. The vector MVA-MUC1/IL-2 addi-

tionally encodes the human IL-2 cDNA under the control of the vaccinia early/late p7.5 promoter, which is placed head-to-head with the MUC1 sequence. DC were plated in 6-well dishes (Corning) at 1–2×10^{6} cells/well. Recombinant virus was added in a total of 2 ml of AIM-V media. Plates were spun at 1000 *g* for 1.5 h at room temperature. The co-centrifugation of MVA virus and DC improved transduction (data not shown), as previously reported for recombinant retrovirus and adenovirus vectors [36, 42]. Directly after the centrifugation period, the viral-containing medium was removed, and the cells were returned to AIM-V media containing 1000 IU/ml each of GM-CSF and IL-4. For DC maturation studies, TNF-α (R&D Systems, Minneapolis, Minn.) was added at 200 IU/ml to media containing GM-CSF and IL-4. With respect to the multiplicity of infection (MOI) with MVA virus, 1 plaque-forming unit (pfu) of MVA is composed of a clump of virus particles, unlike replicative VV strains [48]. Therefore, 1 pfu of MVA is likely to infect several cells.

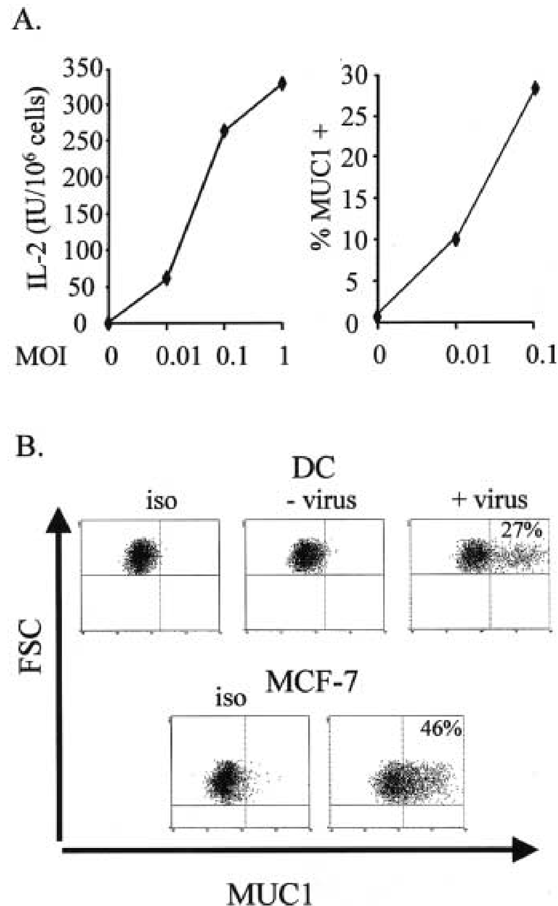

Fig. 1A, B Dose-response expression of MVA-MUC1/IL-2 vector by DC. **A** DC were transduced at the indicated MOI and analyzed for expression 24 h later. MUC1 expression was determined by immunostaining with the anti-MUC1 HMFG-1 antibody followed by FACS analysis. IL-2 levels were assessed by ELISA analysis of cell supernatants. **B** FACScan representation of MUC1 expression at 24 h post-transduction at the indicated MOI of 0.1 shown in **A**. *Panels* depict DC stained with the anti-HMFG-1 antibody with and without virus transduction and immunostaining with the control isotype (*iso*) antibody. Immunostaining of MCF-7 breast cancer cells is shown for comparison. Percent MUC1 positive cells are indicated

IL-2 cytokine analysis

Expression of IL-2 by transduced cells was determined by analyzing supernatants for levels of secreted IL-2. Cells were centrifuged at 1000 *g* for 5 min, and supernatants were removed and stored at –20 °C. Samples were thawed and analyzed for human IL-2 using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biosource International, Camarillo, Calif.). ELISA values (ng/ml) were converted to IU/ml according to the manufacturer. Standard deviations between duplicate samples were routinely less than 15%. IL-2 bioactivity was confirmed by determining whether the secreted protein supported the proliferation of activated peripheral blood lymphocytes [4]. PBMC were activated by plating cells at 1×10^{6} cells/ml in AIM-V media in 24-well dishes that were pre-coated with a solution of 2 μg/ml murine anti-CD3 monoclonal antibody (BD Pharmingen) in PBS. PBMC were maintained for 3 days, washed and plated in triplicate at 1×10^{5} cells in 96-well dishes in 100 μl of fresh AIM-V media and 100 μl of supernatant from individual DC cultures. Supernatants were derived from DC that had been transduced as described above and maintained for 3 days in AIM-V media without the addition of GM-CSF and IL-4 to avoid potential proliferative effects induced by these cytokines. Controls included supernatant from the same DC cultures that were not transduced or AIM-V media only and AIM-V media containing 100 IU/ml recombinant human IL-2 (Cetus, Emeryville, Calif.). After 5 days, PBMC proliferation was assessed by [^{3}H]-thymidine incorporation as described previously [43]. Mean counts per minute (cpm) values were calculated and standard deviations determined.

Mixed lymphocyte reaction (MLR) assays

Frozen, non-adherent PBMC that were obtained following monocyte adherence (described above) were thawed at 37 C and rested overnight in AIM-V media at 1×10^{6} cells/ml in a 24-well culture dish. PBMC (2×10^{5} cells) were added to 4×10^{4} DC plated in triplicate in 200 μl of AIM-V media in a flat-bottom, 96-well culture dish. Autologous MLR assays were performed using DC and PBMC derived from the same donor. DC and PBMC obtained from separate donors were used for the allogeneic MLR assays. To those cultures receiving exogenous IL-2, recombinant human IL-2 (Cetus, Emeryville, Calif.) was added to 100 IU/ml. After 5 days, proliferation was determined by [^{3}H]-thymidine incorporation as previously described [43].

Results

Dose-response expression of the MVA-MUC1/IL-2 vector by DC

Monocyte-derived DC obtained from culturing in GM-CSF and IL-4 generally showed characteristic expression of HLA-DR class II, CD1a, CD54, CD86 and CD80 with <15% of the cells displaying the monocyte lineage marker CD14 (data not shown). Viable staining of the cells at days 6–8 revealed the morphologic appearance of DC with veiled edges and multiple processes (data not shown). A dose-response experiment was initially performed comparing expression of the MVA-MUC1/IL-2 vector at increasing multiplicities of infection (MOIs). DC were transduced at three different MOIs and incubated for 24 h prior to determining IL-2 and MUC1 antigen expression. With respect to IL-2 secretion, increasing MOIs clearly resulted in higher levels of IL-2 (Fig. 1A). Similarly, there was a parallel increase in MUC1 expression, as determined by immunostaining

with the anti-MUC1 antibody HMFG-1, which recognizes a highly glycosylated form of MUC1 [12]. At the highest MOI tested (an MOI of 1), the DC culture contained extensive amounts of cellular debris and dead cells, based on microscopic examination, preventing accurate measurement of MUC1 expression. DC receiving lower doses of virus (MOIs of 0.01 and 0.1) were ~90% viable, as determined by trypan blue dye staining, similar to non-transduced DC. Figure 1B shows FACS analysis for MUC1 expression by DC cultures infected at an MOI of 0.1 and analyzed 24 h post-transduction. Non-transduced DC appeared negative for immunostaining with the HMGF-1 antibody. For comparison, anti-HMFG-1 staining of MUC1-expressing MCF-7 breast cancer cells is shown; the percentage of positive cells was routinely 45–55%.

MVA-MUC1/IL-2-transduced DC were further analyzed for expression of differentially glycosylated forms of MUC1. As detected by immunostaining with the HMFG-1 antibody, the cells primarily displayed highly glycosylated molecules (Table 1), which are exhibited most abundantly on normal epithelium [51]. A much lower percentage of cells was stained with the SM-3 or HMFG-2 anti-MUC1 antibodies, both of which recognize tumor-associated, under-glycosylated MUC1 molecules. Typical of the MCF-7 breast cancer cell line, a combination of all 3 glycoforms of MUC1 was expressed [11, 12]. As previously shown for MCF-7 cells, surface staining was heterogeneous with SM-3 epitopes displayed on ~50% fewer cells than HMFG-2 epitopes [31].

Expression of MVA-based MUC1 vectors by DC generated from different donors

To examine DC donor variation in the expression the MVA-MUC1/IL-2 vector, cultures of DC derived from five different blood donors were transduced at an MOI of 0.1 and analyzed for MUC1 and IL-2 expression at 24 h post-transduction. As shown in Fig. 2A, MUC1-positive cell expression varied between 27 and 54%. IL-2 secretion profiles correlated with the MUC1 levels in that higher amounts were detected in those cultures with higher numbers of MUC1-expressing DC. In addition, DC derived from three different donors were transduced with both the MVA-MUC1/IL-2 vector and an MVA-MUC1 virus, lacking the IL-2 cDNA insert. The percentages of MUC1-positive cells were comparable for the two vectors (Fig. 2 B). These results suggest that co-expression of IL-2 does not alter DC expression of the MUC1 transgene.

Table 1 DC expression of MUC1 glycoforms

Anti-MUC1 antibody	DC (% +)	MCF-7 (% +)
HMFG-1	28	57
HMFG-2	< 1	27
SM-3	< 1	14

Bioactivity of transgene-derived IL-2

The immunostimulatory activity of IL-2 was confirmed by determining whether the IL-2 secreted by DC transduced with the MVA-MUC1/IL-2 vector was capable of sustaining the proliferation of activated PBMC. Supernatants were removed from cultures 72 h after transduction with the MVA-MUC1 or MVA-MUC1/IL-2 vector (MOI 0.1) and subsequently added to previously activated PBMC cultures. As shown in Fig. 3, supernatants derived from non-transduced DC or DC transduced with MVA-MUC1 vector exhibited little, if any,

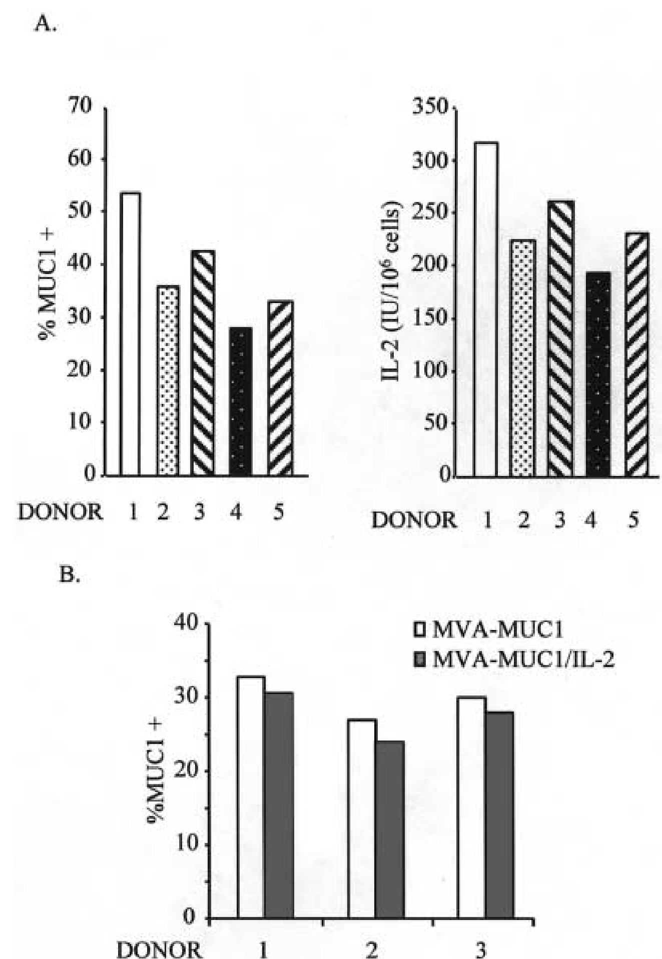

Fig. 2A, B Donor DC expression of MVA vectors encoding MUC1. DC cultures generated from separate donors were transduced at an MOI of 0.1 and analyzed 24 h later for vector expression. **A** Cells from five donors were immunostained with the anti-MUC1 HMFG-1 antibody, followed by FACS analysis. Supernatants were analyzed by ELISA for IL-2 expression. **B** Cells from three donors were transduced with either the MVA-MUC1 or MVA-MUC1/IL-2 vector. MUC1 expression was determined by HMFG-1 antibody immunostaining and FACS analysis

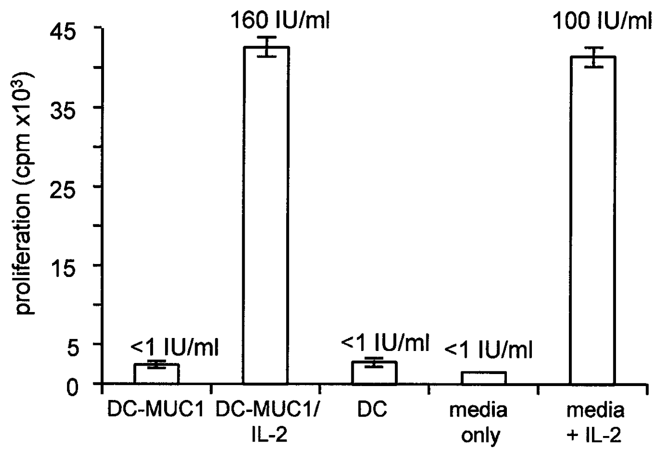

Fig. 3 Bioactivity of MVA-MUC1/IL-2 transgene-encoded IL-2. Supernatants derived from non-transduced DC or cells transduced with either MVA-MUC1 or MVA-MUC1/IL-2 were added to pre-activated PBMC, as described in the Materials and methods. ELISA values of IL-2 (IU/ml) added to each sample are indicated. Additional samples of PBMC received media only or media to which recombinant human IL-2 was added (100 IU/ml). PBMC proliferation was assessed 5 days later by measuring [^{3}H]-thymidine incorporation. Mean counts per minute (cpm) are given for triplicate samples; standard deviations are shown

IL-2, as determined ELISA analysis and failed to support high-level PBMC proliferation. In contrast, supernatant derived from DC transduced with MVA-MUC1/IL-2 and added at 160 IU/ml IL-2 maintained PBMC proliferation at levels comparable to PBMC receiving 100 IU/ml recombinant human IL-2. This data indicated that the MVA-encoded IL-2 is functionally active and capable of supporting PBMC expansion.

Time-course of MVA-MUC1/IL-2 expression and MVA-induced cytopathic effects

Expression levels of MVA-derived MUC1 and IL-2 were monitored at 24, 48 and 72 h post-transduction. As depicted in Table 2, the percentage of MUC1-positive cells declined over each consecutive 24-h time period, from 54% at 24 h post-transduction to only 10% by 72 h post-transduction. IL-2 secretion monitored over each time period was also reduced. Moreover, as determined by trypan blue dye exclusion, there was a concomitant decline in cell viability that appeared to correlate with the loss in MUC1-positive cells. Comparable results have been obtained for transduced DC generated from 2 other blood donors (data not shown).

Table 2 Time course of MUC1 expression and DC viability. DC were plated at 2×10^6 cells/well and infected at an MOI of 0.1. Medium was exchanged daily and analyzed for IL-2

Time post-transduction	% MUC1 +	IL-2 (IU/well)	% Viability
24 h	54	660	89
48 h	36	401	58
72 h	10	123	39

The cell death observed over time in the MVA-MUC1/IL-2-transduced cultures was most likely a consequence of known MVA-mediated cytopathic effects that can occur in infected human cell types, including DC [14, 16]. To confirm that DC cell death was attributable to the MVA vector and not overtly influenced by expression of MUC1 or IL-2, cells receiving either MVA-MUC1/IL-2 or a wild-type MVA virus lacking the transgenes (MVA-wt) were compared for the extent of cell death. Figure 4 shows FACS analysis of apoptotic and necrotic DC 48 h after vector transduction (MOI 0.1). Apoptosis was indicated by Annexin V staining, while necrosis was determined by propidium iodide (PI) staining. Relative to the non-transduced DC population, both the MVA-wt and MVA-MUC1/IL-2 transduced DC cultures exhibited significant numbers of cells that appeared to have experienced apoptosis, followed by necrosis based on dual staining by both Annexin V and PI (MVA-wt, 17%; MVA-MUC1/IL-2, 15%). Moreover, both populations contained higher levels of apoptotic cells at this time point, as indicated by Annexin V staining only (MVA-wt, 18%; MVA-MUC1/IL-2, 16%). With respect to viable cells that were negative for Annexin V and PI staining, the MVA-wt-transduced culture displayed only slightly fewer viable cells than the MVA-MUC1/IL-2 population (MVA-wt, 49% viable; MVA-MUC1/IL-2, 59% viable). Overall, the observed cytopathic effects appeared to be mediated by the MVA viral vector with no obvious effects of MUC1/IL-2 transgene expression on DC viability.

Functional activity of MVA-transduced DC

The ability of DC to induce proliferation of allogeneic PBMC has served as a reliable indicator of DC immunostimulatory function [21]. Recent studies have reported that transduction with a wild-type vaccinia virus vector can inhibit the ability of DC to stimulate allogeneic PBMC proliferation [19, 30]. The functional properties of non-transduced DC and DC transduced with the various MVA vectors (MVA-wt; MVA-MUC1;

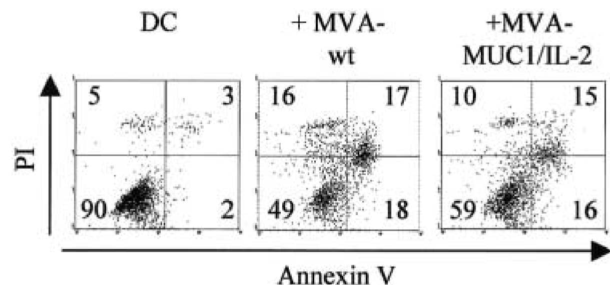

Fig. 4 MVA-mediated cell death in DC cultures. DC were transduced with either MVA-MUC1/IL-2 or MVA-wt (MOI 0.1) and analyzed 48 h later. Apoptotic and necrotic cells were determined by staining with FITC-Annexin V and propidium iodide (PI) followed by FACScan analysis

MVA-MUC1/IL-2) were compared in an allogeneic MLR assay. DC cultures were transduced at an MOI of 0.1, followed by the addition of allogeneic PBMC at a DC:PBMC ratio of 1:5. Cultures were assessed 5 days later for induced PBMC proliferation. Analysis of separate aliquots of the DC cultures at 24 h post-transduction indicated 32% and 30% MUC1-positivity in populations exposed to the MVA-MUC1 and MVA-MUC1/IL-2 vectors, respectively. Controls included non-transduced DC cultures plus PBMC co-cultivated in 100 IU/ml of IL-2, as well as PBMC alone with or without exogenous IL-2 addition. As shown in Figure 5A, comparable levels of allogeneic PBMC proliferation were observed in MVA-transduced and non-transduced cultures. Neither the IL-2 expressed by the MUC1/IL-2 transgene nor exogenous IL-2 influenced proliferative responses. Most likely, this reflects the highly stimulatory nature of the allogeneic DC; allo-PBMC proliferation would not necessarily be further enhanced by IL-2 addition. PBMC alone exhibited minimal proliferation, which was augmented by the addition of exogenous IL-2. Even though, the overall level of proliferation remained far less than cultures containing DC. These findings indicate that MVA-transduced DC cultures in general, and those expressing MUC1 and IL-2 in particular, are functionally capable of stimulating an allogeneic PBMC response.

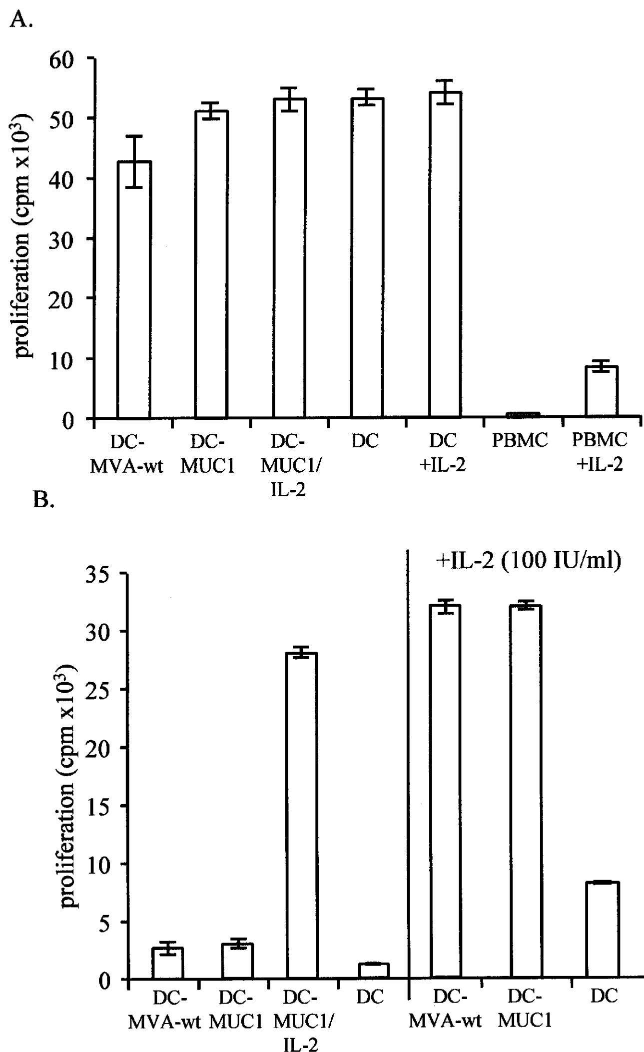

Fig. 5A, B Functional activities of MVA-transduced DC, as indicated by MLR assays. **A** DC were transduced at an MOI of 0.1 with the MVA viral vectors (MVA-wt; MVA-MUC1; MVA-MUC1/IL-2). Allogeneic PBMC ($2{\times}10^5$ cells) were cultured in the absence or presence of the DC populations ($4{\times}10^4$ cells). Recombinant human IL-2 (100 IU/ml) was added as indicated (+IL-2). [^{3}H]-thymidine uptake was subsequently measured 5 days after co-cultivation. **B** An autologous MLR assay was performed as described in **A**. Data are reported as mean counts per minute (cpm) of triplicates ±SD

The concept of including the IL-2 cDNA insert in the MVA vector was to provide further immunostimulatory support to autologous lymphocytes that would be exposed to the MVA-MUC1/IL-2-transduced DC in vivo. With this consideration, proliferation of autologous PBMC cultured in the presence of DC transduced with the MVA vectors was assessed in an autologous MLR assay. PBMC were mixed with the respective DC cultures at a ratio of 5:1 immediately following the 1.5-h transduction period. In separate cultures maintained in the absence of added PBMC, the MVA-MUC1/IL-2 DC secreted IL-2 at 228 IU/10^6 cells by 24 h post-transduction. MUC1 positivity was 31% and 28% in the MVA-MUC1 and MVA-MUC1/IL-2 DC cultures, respectively. The proliferative activity of autologous PBMC cultured in the presence of MVA-MUC1/IL-2 DC was markedly higher than that observed in samples receiving non-transduced DC or DC transduced with MVA-wt or MVA-MUC1 vector (Fig. 5B). The addition of exogenous IL-2 (100 IU/ml) to reactions containing DC transduced with MVA or MVA-MUC1 increased PBMC proliferation to levels comparable to the MVA-MUC1/IL-2 cultures (Fig. 5B). Although exogenous IL-2 addition improved proliferation of PBMC exposed to non-transduced DC, the effect was ~4-fold less than that observed in the MVA-transduced cultures. Similar results have been observed using DC and PBMC derived from a second donor (data not shown). These observations demonstrate that IL-2, either provided by transgene expression or exogenous addition, stimulates the proliferation of autologous PBMC upon exposure to MVA-transduced DC. Possibly, the donor PBMC are responding to MVA antigens.

MVA vector transduction of DC matured in the presence of TNF-α

We examined whether transduction with the MVA-MUC1/IL-2 vector would alter the maturation of DC exposed to TNF-α. This cytokine is known to induce DC maturation and, thereby, augment antigen-specific T cell responses [6, 9, 21]. Moreover, TNF-α-matured DC have been applied in cancer vaccine trials in which the DC appear to stimulate antigen-specific CTL responses [10, 37]. DC derived from two separate donors were exposed

to the MVA-MUC1/IL-2 virus (MOI 0.1) and cultured with or without TNF-α addition for 48 h. Comparable levels of MUC1 and IL-2 were observed in the presence or absence of TNF-α (Fig. 6A). As indicated by assessment of the levels of CD83 expression, a primary indicator of DC maturation [21], virus transduction did not appear to affect TNF-α-induced maturation (Fig. 6A). CD83 expression was upregulated 3–6-fold in both non-transduced and transduced cultures.

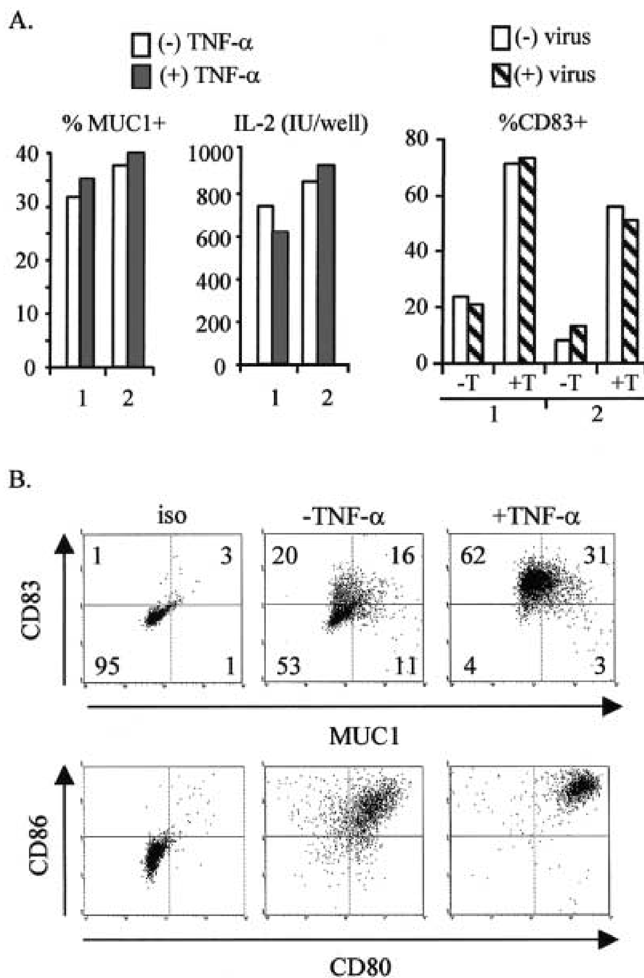

Fig. 6A, B Effect of MVA transduction on TNF-α-induced DC maturation. **A** DC populations derived from two separate donors (1 and 2) were transduced with the MVA-MUC1/IL-2 vector (MOI 0.1) and cultured for 48 h in the presence of GM-CSF and IL-4 cytokines with or without the addition of TNF-α (200 IU/ml). MUC1 expression was determined by immunostaining with the HMFG-1 antibody followed by FACS analysis. ELISA analysis of cell supernatants was used to monitor IL-2 expression and accumulation during the 48-h incubation period. Surface expression of the CD83 maturation marker was monitored by immunostaining and FACS analysis of both non-transduced and transduced cultures, with or without the addition of TNF-α (−T, +T). **B** MVA-MUC1/IL-2 DC, cultured in the presence or absence of TNF-α for 48 h, were dual immunostained and analyzed for MUC1 expression and markers of DC maturation. Analysis of staining with control, isotype antibodies is also shown (*iso*). *Upper panel*, co-staining for MUC1 and CD83. *Numbers* represent percentages of cells present in each quadrant based on FACScan analysis: *upper left*, $CD83^+$ only; *upper right*, $CD83^+$ $MUC1^+$; *lower right*, $MUC1^+$ only; *lower left*, $CD83^-$ $MUC1^-$. *Lower panel*, co-staining for CD80 and CD86 maturation markers. Note that TNF-α induced an increase in intensity of cells expressing both markers

In a separate experiment, dual flow cytometry was performed to confirm that matured DC displaying CD83 also expressed the MUC1 transgene. As shown in Fig. 6B, DC transduced and cultured in the absence of TNF-α exhibited relatively low levels of the CD83 marker (36% positive), as expected, with MUC1 expression detected in both CD83-positive (16% MUC1 positive) and -negative cells (11% MUC1 positive). Transduced DC that were subsequently matured in the presence of TNF-α displayed an overall higher level of CD83 expression with the majority of cells (93%) displaying CD83. The majority of cells exhibiting MUC1-positivity were of the $CD83^+$ mature phenotype (31%). As is well known for maturing DC populations [21], the co-stimulatory molecules CD80 and CD86 were also substantially upregulated by TNF-α treatment (Fig. 6B), along with enhanced expression of the MHC II molecule HLA-DR (data not shown). Non-transduced DC showed a similar upregulation of these markers upon TNF-α exposure (data not shown). Taken together, these results indicate that MVA-MUC1/IL-2 transduction and gene expression are not inhibitory to TNF-α-induced maturation. Conversely, TNF-α maturation does not modify expression of the MVA-encoded transgenes.

With respect to MVA-mediated cell death, Annexin-V and PI co-staining demonstrated that infection followed by TNF-α exposure results in a loss in viability, as observed for immature DC populations exposed to virus. By 48 h post-transduction with either MVA-wt or MVA-MUC1/IL-2 virus, ~50% of the cells remained viable (data not shown). TNF-α maturation of previously transduced DC did not appear to impact the cytopathic effects of the MVA virus.

If DC cultures were instead first matured in the presence of TNF-α for 48 h followed by MVA-MUC1/IL-2 exposure (MOI 0.1), expression levels of the MUC1 and IL-2 transgenes were comparable to immature, transduced DC (Fig. 7). Moreover, virus transduction and expression did not substantially alter levels of the CD83 marker induced by prior TNF-α exposure. Further FACS analysis revealed no effects of virus transduction on the upregulated expression of HLA-DR, CD80 and CD86 molecules (data not shown). Overall, TNF-α-matured DC were as susceptible to MVA virus infection and transgene expression as immature DC. Based on DC marker evaluations, the MVA-MUC1/IL-2 virus did not affect the DC maturation status.

Discussion

DC are attractive cancer vaccine agents, based on their ability to efficiently present TAAs and prime CTL antitumor responses [6, 21, 41]. Transduction of DC with

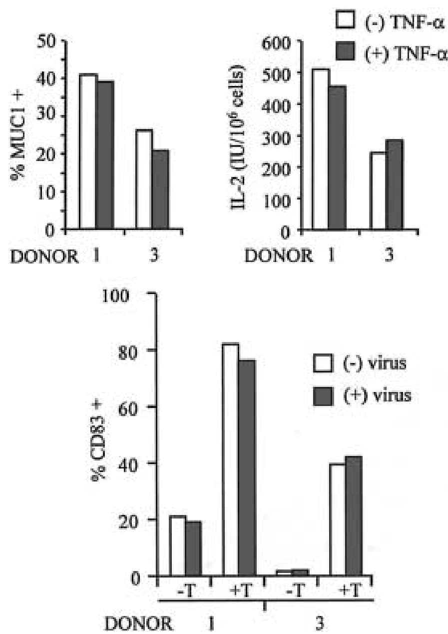

Fig. 7 MVA-MUC1/IL-2 transduction of TNF-α-matured DC. DC populations were cultured in the presence of GM-CSF and IL-4 with or without TNF-α (200 IU/ml) for 48 h prior to exposure to the MVA-MUC1/IL-2 virus (MOI 0.1). Cells were analyzed for MUC1, IL-2 and CD83 expression at 24 h post-transduction as described in Fig. 6A

recombinant virus vectors is considered an efficient means of loading DC with TAA for DC immune display of TAA antigenic peptides [34]. Herein, we have characterized the transduction of monocyte-derived DC with an MVA vector expressing genes for both the MUC1 TAA and the immunostimulatory cytokine IL-2. Moreover, we have investigated biological aspects of MVA vector-DC interactions that could influence clinical application of the MVA-MUC1/IL-2-transduced DC.

Thus far, reports of DC transduced with recombinant MVA vectors have been limited [16, 17], although there have been several studies applying recombinant VV vectors derived from conventional virus strains (Western Reserve, Copenhagen) [19, 49, 18, 30]. An MVA vector encoding the melanoma TAA tyrosinase has been shown to express in monocyte-derived DC; however, potential virus-related effects on the target DC were not addressed [17]. In a second study, MVA-mediated gene transfer into $CD34^+$ stem cells or $CD34^+$-derived DC was investigated rather than DC derived from monocytes [16].

With respect to transgene expression in MVA-MUC1/IL-2-tranduced DC cultures, an MOI of 0.1 provided significant expression of both MUC1 and IL-2, with the levels of IL-2 secretion consistently reflecting the MUC1 positivity. Following transduction with either the MVA-MUC1 or MVA-MUC1/IL-2 vector, MUC1 expression levels were virtually identical, indicating that the MUC1/IL-2 dual-expression vector is of a favorable design and that IL-2 co-expression does not alter the level of MUC1 production. A co-regulation of the MUC1 and IL-2 transgenes is anticipated, as the MUC1 and IL-2 transgenes are each driven by similar promoter elements that carry both early and late transcriptional signals (pHR5 and p7.5, respectively). In studies with other VV strains, early promoters have been shown to retain activity in monocyte-derived DC, while late promoter activity is suppressed, along with virus replication [18, 19, 30]. Consequently, known replicative strains of VV are phenotypically similar to MVA in that virus production is attenuated in DC cultures.

MVA-MUC1/IL-2-transduced DC predominantly display fully glycosylated MUC1, comparable to that found on normal epithelia. There is only low-level expression of under-glycosylated forms that are typical of tumor cells, including the MCF-7 breast cancer cells in this study [12, 13]. As a non-malignant cell type, DC would not be expected to exhibit changes in glycosyltransferase activities that are known to give rise to tumor-associated glycoforms of MUC1 [51]. Our results are compatible with one other study in which $CD34^+$ cells were transduced with a retroviral vector encoding MUC1 and subsequently differentiated into DC; under-glycosylated MUC1 molecules were only weakly detected [27]. In theory, the glycosylation status of MUC1 expressed by DC should not affect MHC class I peptide display, because class I molecules are loaded with peptide in the ER before transport and glycosylation in the Golgi apparatus [51]. Moreover, vaccination with DC transfected with human MUC1 RNA induces MUC1-specific CTL and anti-tumor immunity in mice bearing tumors expressing human MUC1 [35]. Although the MUC1 glycosylation status was not determined, this result indicates that DC expressing MUC1 are capable of processing and displaying MUC1 peptides in the context of MHC class I molecules.

The significant loss in DC expressing the MUC1/IL-2 transgenes over a 3-day period post-transduction correlates with enhanced cell death. Comparable percentages of apoptotic and necrotic cells were observed in cultures transduced with either MVA-wt or the MVA-MUC1/IL-2 vector, indicating that the loss in cell viability is MVA-related. Similarly, MVA-associated toxicity has been observed in transduced $CD34^+$-derived DC cultures [16]. The occurrence of cells staining for both Annexin-V and PI suggests that our MVA-transduced cells die via apoptosis followed by necrosis, a phenomenon previously seen in DC cultures transduced by a standard VV strain (Western Reserve) [19, 49]. MVA-induced DC death does not necessarily obviate the application of such cultures as a cancer vaccine. Previous findings have demonstrated that necrotic cell debris and apoptotic bodies derived from either DC or tumor cells can be phagocytosed by viable DC that subsequently cross-prime $CD4^+$ and $CD8^+$ antigen-specific T cells [3, 20, 29, 46].

Due to the extensive cell death observed in the MVA-transduced DC cultures, impairment of the allostimulatory capacity might be expected. However, DC cultures transduced with either the MVA-wt, MVA-

MUC1 or MVA-MUC1/IL-2 vectors displayed immunostimulatory activities equivalent to non-transduced DC. Most likely, the delayed cell death that occurs over several days provides ample time for the PBMC to mount a strong allo-proliferative response. Furthermore, neither the presence of IL-2 expressed by the MVA-MUC/IL-2 transgene nor the addition of exogenous IL-2 enhanced allo-PBMC proliferation. Because the lymphocyte response to allogeneic MHC antigens is extraordinarily robust and results in T cell secretion of immunostimulatory cytokines (including IL-2) [1], additional IL-2 would not necessarily further augment proliferation. For our MLR analyses, immature DC generated in the presence of GM-CSF and IL-4 were used. In similar studies of immature DC transduced with non-attenuated VV, both enhanced and reduced allo-MLR responses have been observed [18, 30]. These differences have been attributed to variances in individual donor lymphocyte responses [18].

In autologous MLR assays, PBMC proliferation was strongest in cultures containing MVA-MUC1/IL-2 transduced DC or upon addition of IL-2 to DC transduced with the MVA-wt or MVA-MUC1virus. These results indicate that the predominant PBMC response is against vaccinia virus antigens, as suggested by others applying VV-transduced DC [18]. Because our donors were vaccinated in childhood against smallpox, these individuals most likely possess low levels of vaccinia-specific, memory lymphocytes that are primed by the MVA-transduced DC and further expanded by IL-2 supplementation. In general, our findings portend well for the use of a recombinant MVA vector encoding IL-2 to support the outgrowth of low-frequency T cells.

Precursor lymphocytes recognizing MUC1 are rare, but can be detected in PBMC populations derived from at least some MUC1-vaccinated patients [10, 47]. Multiple in vitro stimulations with MUC1 peptide-pulsed DC have been shown to generate tumor-reactive CTL from PBMC of only a limited number of healthy donors [9]. Further experimentation will clarify presumed MVA-specific T cell responses and determine whether the MVA-MUC1/IL-2-transduced DC can prime T cell reactivity against MUC1. We do have promising preliminary results in mice immunized with DC transduced by the MVA-MUC1/IL-2 vector; vaccinated animals injected with human MUC1-expressing mouse tumor exhibit improved, tumor-free survival (manuscript in preparation).

Important issues for clinical development of MVA-MUC1/IL-2-transduced DC are whether TNF-α maturation influences recombinant MVA transgene expression and, conversely, whether MVA transduction would interfere with TNF-α induced maturation. Immature DC efficiently process antigen for MHC display, while matured DC initiate stronger antigen-specific T cell responses due to upregulation of MHC molecules, co-stimulatory molecules (such as CD80, CD86 and CD40) and certain chemokine receptors [21]. Thus, immunization with DC that have been transduced and subsequently matured would prove more effective in sensitizing anti-MUC1 T cells in vivo. Moreover, recent studies in humans have suggested that ex vivo maturation of DC is essential for the immune efficacy of DC [15, 32]. As indicated by known markers of maturation, MVA-MUC1/IL-2 transduction does not alter the potential of DC to undergo TNF-α-induced maturation or modify the maturation status. Furthermore, the levels of MUC1and IL-2 produced in immature and mature DC cultures are approximately the same.

Our results are in distinct contrast to previous findings of DC infected with non-attenuated VV strains. VV infection prior to maturation dramatically inhibits the upregulation of such molecules as HLA-DR, CD83 and the T cell co-stimulatory molecules CD80 and CD86. Maturation followed by VV transduction results in a loss of CD83, CD86 and CD80 expression [19, 30]. Consistent with the marker phenotype, the functional capacity of VV-transduced DC to stimulate PBMC responses is also reduced [19, 30]. Possibly, our results are accounted for by the type of culture media employed or the TNF-α maturation method, both of which differ from previous VV-DC studies. A more plausible explanation is that conventional VV strains, unlike MVA, express known "immune evasion genes" encoding functional receptors for TNF-α, IFN-γ and IFN-α/β [5, 7] that could well attenuate cytokine-induced DC maturation. Because MVA infection does not interfere with DC maturation, we would speculate that MVA-MUC1/IL-2 transduced DC would be more efficient in generating anti-MUC1 tumor responses than DC infected with conventional, non-attenuated VV vectors. Although further analyses are required to define the complex effects of MVA-derived vectors on target DC cells, vaccination with MVA-MUC1/IL-2-transduced DC may well elicit effective anti-tumor T cell responses and prove broadly applicable for the treatment of a wide number of known $MUC1^+$ tumor types.

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