ORIGINAL ARTICLE

Cytokine-armed vaccinia virus infects the mesothelioma tumor microenvironment to overcome immune tolerance and mediate tumor resolution

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Intratumoral (i.t.) administration of cytokine genes expressed by viral vectors represents a rational approach that induces cytokine secretion at the site they are needed, and i.t. vaccinia virus (VV) has shown promise in mesothelioma patients. However, we and others have shown that the mesothelioma tumor microenvironment includes macrophages, dendritic cells (DCs), and T cells. Therefore, we investigated which of these cell types are infected after exposure to VV or Fowlpox virus (FPV)-cytokine gene constructs. *In vitro* studies showed that mesothelioma tumor cells were resistant to FPV infection yet highly permissive to infection by VV vectors resulting in significant cytokine production and impaired proliferation. Macrophages secreted low levels of cytokine suggestive of resistance to overt infection. DCs transiently secreted virally derived cytokines, but did not mature during VV infection. VV inhibition of T cell proliferation was rescued by the interleukin (IL)-2 and IL-12 VV constructs. *In vivo* studies of murine mesotheliomas showed that i.t. injection of the parent VV could not hinder tumor progression. In contrast, the VV-cytokine constructs induced profound tumor regression. These data suggest that i.t. VV-cytokine gene constructs retard tumor growth by infecting mesothelioma cells and targeting the immune system through tumor-infiltrating DC and T cells.

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Introduction

A number of strategies have been used in an effort to boost anti-tumor immunity in cancer patients. Often these strategies involved cytokines on account of the substantial body of literature showing that cytokines, such as interleukin (IL)-2, can have powerful anti-tumor effects *in vivo*. However, intravenous delivery is associated with numerous problems including short half-life, the ability to only function at short distances, and toxic side effects. Therefore, correct application with regard to dosage, time, and mode of administration is required.

One approach that may reduce systemic toxicity and maintain high levels of cytokine at local sites is to construct a viral vector that expresses DNA coding for cytokine production and inject it directly into the tumor

site.^{1–5} The viruses introduce this genetic code into infected host cells, which then produce the cytokine. A number of cytokine-gene viral vectors have been designed and include adenoviruses, retroviruses, lentiviruses, and pox viruses.^{6–11} However, each viral vector offers specific advantages; the potential of pox viral vectors, specifically vaccinia vectors, in mesothelioma has been already shown.¹² To this effect, a phase I gene therapy trial in malignant mesothelioma patients involving weekly intratumoral (i.t.) injections of a replication-restricted vaccinia virus-IL-2 (VV-IL-2) vector was conducted¹² showing the feasibility and safety of such an approach. Despite the generation of an anti-VV antibody response, VV-IL-2 mRNA gene expression persisted in tumor biopsies for up to 3 weeks and induced a local T cell infiltrate. However, no tumor regression was observed suggesting that the optimum therapeutic regimen has not yet been identified. We earlier considered that an alternative therapeutic strategy could be one that returned *ex vivo* dendritic cell (DC) infected *in vitro* with VV-IL-2 back to patients. However, we found that this approach downregulated antigen-presenting function in mature DC and was likely to be unreliable.¹³ Thus, cytokine-armed DC may not be

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an effective alternative therapy and, as suggested by studies in mesothelioma and other cancers, regardless of the vector used, the direct i.t. approach remains a viable proposition.^{14–23}

Despite the apparent safety of the mesothelioma VV clinical trial,¹² safety issues continue to be a concern. Another member of the Poxviridae family, Fowlpox virus (FPV), undergoes abortive replication in the cytoplasm of mammalian cells allowing for transcription of inserted genes without production of functional FPV particles, thereby reducing safety concerns.²⁴ Several clinical trials using avipox recombinant vaccines have confirmed the safety and feasibility of this viral vector and, where a tumor antigen was encoded, have shown the induction of anti-tumor immunity mediated by CTL cells.^{25–28} However, VV may be particularly effective because it can infect a wide range of human and murine cells, leading to highly efficient expression of foreign genes.^{19,28,29} VV does not integrate into cellular DNA, but replicates within the cytoplasm and contains all the enzymes necessary for efficient mRNA synthesis and translation.^{30,31} The VV constructs used are attenuated and tumor selective because of the insertion of cytokine genes into the thymidine kinase (TK) region.³² Thus, in this study we compared VV and FPV viral vectors, both of which express the same marker antigen (hemagglutinin, HA).^{24–27}

As both IL-2 and IL-12 have been shown to induce anti-tumor immunity against mesothelioma,^{33–37} we compared VV with FPV vectors that encode either IL-2 or IL-12. We show here that i.t. injection of the IL-2 and/or IL-12 VV gene constructs into murine mesotheliomas provided significant survival benefits *in vivo*. We also examined the effects the VV-cytokine gene constructs have on immune cells that infiltrate the tumor micro-environment (T cells, macrophages, and DCs), as well as on tumor cells.

Materials and methods

Mice

Female C57BL/6J mice aged 6–8 weeks were obtained from the Animal Resources Centre (Perth, Western Australia) and maintained under standard animal housing conditions. All mice were used in accordance with institutional guidelines and approval of the University of Western Australia and Curtin University's Animal Ethics Committees.

Murine tumor cell lines

AE17 mesothelioma cell lines were derived from the peritoneal cavity of C57BL/6J injected with asbestos (crocidolite) fibers. Injection of AE17 into naïve mice results in mesothelioma tumors histologically similar to human mesothelioma, as described earlier.³⁵ AE17sOVA was developed by transfecting the AE17 parental cell line with cDNA coding for sOVA, as described earlier.³⁵ Both cell lines were maintained in media, which consisted of RPMI 1640 (Invitrogen, Auckland, New Zealand) supplemented with 10% turbo bovine serum (Invitrogen), 50 µg ml⁻¹

gentamicin (Pharmacia, Bentley, Australia), 60 µg ml⁻¹ benzylpenicillin (CSL, Melbourne, Australia), and 0.05 mM 2-mercaptoethanol (Merck, West Point, PA). Transfected tumor cell lines were maintained in the same medium supplemented with 400 µg l⁻¹ neomycin analog G418 (geneticin, Invitrogen). Cells were cultured at 37 °C in a 5% CO₂ humidified atmosphere; 143BTK⁻ is a human fibroblast osteosarcoma cell line lacking the TK gene (kindly donated by Jane Allen, Department of Microbiology, University of Western Australia) and was used for propagating all of the viral constructs. The 143BTK⁻ cells were maintained in minimum essential media with L-glutamine (GIBCO BRL, NY) supplemented with 10% fetal calf serum (FCS), gentamicin, and benzylpenicillin.

Cytokine constructs

FPV and VV-cytokine constructs were kindly donated by Ian Ramshaw (John Curtin School for Medical Research, Australian National University, Australia). Construction of rFPV by homologous recombination has been described earlier.³⁸ Briefly, FPV insertion plasmids were constructed containing either the HA gene or a cytokine (IL-2 or IL-12) gene and co-transfected into FPV-infected chicken embryo cells resulting in rFPV vectors containing both genes. Construction of rVV has also been described earlier³² and was similar to the construction of rFPV vectors. Briefly, plasmid transfer vectors expressing either HA or the herpes simplex virus TK gene and cytokine were inserted into the VV-TK gene. Therefore, only viral products, which go through homologous recombination will have herpes simplex virus TK and will be produced in TK⁻ cells. For TK selection, co-transfection of 143BTK⁻ cells with the two plasmids results in rVV constructs expressing HA and cytokine. A control virus expressing HA alone was similarly constructed.^{32,38}

Growth of VV constructs involved confluent 143BTK⁻ cells infected with 1×10^9 plaque-forming units (pfus) VV constructs, incubated for 2 h, and overlaid with 15 ml minimum essential media/2.5% FCS. After 3 days, cells were removed, washed in phosphate-buffered saline (PBS), and lysed by three freeze-thaw cycles. Concentrations of virus stocks were assessed using a plaque-forming assay in which confluent 143BTK⁻ cells incubated with virus constructs for 1 to 2 h at 37 °C, were overlaid with minimum essential media/2.5% FCS, and incubated for 2 days. Crystal violet (Sigma, St Louis, MO) was used to visualize plaques. The pfus were determined based on the following formula: pfus per ml = number of plaques × volume × dilution. Use of these viruses was approved of by the Genetic Manipulation Association Committee and the Office of Gene Technology Regulator.

Cell preparations

Spleen single cell suspensions of tissues harvested from mice were obtained by mashing gently with two frosted glass slides. Macrophages were obtained by flushing the peritoneal cavity of mice with cold PBS and their phenotype confirmed by FACS analysis and F4/80 expression. DCs were obtained from mouse bone marrow,

as described elsewhere.³⁹ Briefly, bone marrow cells were dispersed through a 27 g needle, washed, and resuspended at 2×10^5 cells per ml in Iscoves (GIBCO), supplemented with 10% FCS, gentamicin, benzylpenicillin, 2-mercaptoethanol, and 10% GM-CSF supernatant (GM-CSF producing cell line X-86 kindly donated by B Stockinger, NIMR, London), placed onto bacteriological grade Optilux petri dishes (Falcon, Becton Dickinson, NJ), and incubated at 37°C in 5% CO₂ for 3 days before complete media was added and regularly replenished. On day 10, non-adherent cells were gently washed and phenotyped for expression of MHC II, CD54, CD80, CD86, and CD11c by FACs analysis.

Determining the percentage of cells infected by FPV and VV constructs

Cells were incubated with a biotinylated anti-HA antibody, clone H18 (made in house), for 30 min, washed, linked to streptavidin-phycoerythrin (PE; Zymed, San Francisco, CA) for further 30 min, washed, and resuspended in PBS/2% FCS for FACs analysis.

Flow cytometry

Double staining involved 1×10^6 cells incubated with one or two primary monoclonal antibodies, washed in PBS/2% FCS, and secondary antibodies added. After the second incubation, cells were washed and resuspended in PBS/2% FCS for FACs analysis. Fluorescence intensity analysis was performed on a Becton/Dickinson FACScan or FACs Calibur using Cell Quest V3.1f Apple Software. The antibodies used were CD11c-PE directed against the integrin α -chain of CD11c on murine DCs (clone HC3), CD54, CD80 directed against murine B7.1 (clone 1G10), CD86 directed against murine B7.2 (clone GL1), plus the isotype controls rat IgG_{2a} (clone R35-95), rat IgG_{2b} (clone A95-1), and Hamster IgG (clone A19-4), all from PharMingen (San Diego, CA). N418 (anti-CD11c) and TIB-120 (anti-murine MHC class II) were gifts from Professor P Holt, Perth. F4/80-biotin (ImmunoKontakt Abingdon, UK) and Streptavidin-FITC (Amersham, UK) were also used.

T cell and tumor cell proliferation

Cells were incubated in 96-well plates (Falcon, Becton/Dickinson) in triplicate for 24, 48, or 72 h when 1 μ Ci of tritiated thymidine (³H-Thy, Amersham, UK) was added to each well and incubated for 4 h at 37°C. Cells were harvested using 96-well unfilter plates (Packard, Zurich, Switzerland) on a Filtermate Harvester (Packard) and ³H-Thy incorporation determined by scintillation (Microscint, Packard) using a β counter (Packard).

For T cell proliferation, supernatants or 200 ng ml⁻¹ recombinant murine IL-2 (rec IL-2; PharMingen) all in 100 μ l were added to the first well of 96-well plates (Falcon, Becton/Dickinson) in triplicate and then serially diluted one in two; 1×10^5 whole splenocytes were then added to each well in 100 μ l. Splenocytes were added alone or were stimulated with 2.5 μ g ml⁻¹ ConcanalavinaA (ConA) (Sigma).

IL-2 and IL-12 ELISA

The primary anti-cytokine capture antibody was added to 96-well MicroTest III flexible assay plates (Falcon, Becton/Dickinson) and incubated for 1 h at 37°C (IL-2) or overnight at 4°C (for IL-12), washed, blocked with 1% bovine serum albumin for 1 h at 37°C, washed, and samples added. Standards of rmIL-2 (PharMingen) or IL-12 (gift from Dr Wolf, Genetics Institute Massachusetts) were serially diluted. Samples and standards were incubated overnight at 4°C, washed, and the appropriate biotin anti-cytokine antibody added (anti-IL-2: PharMingen or C17.8-biotin anti-cytokine detection antibody; kindly donated by Dr Trinchieri, Philadelphia) and incubated for 30 min at 37°C. This was followed by incubation with extravidin alkaline phosphatase conjugate for 30 min at 37°C, washing, and addition of color substrate using phosphatase substrate tablets (Sigma, prepared according to manufacturer's instructions) and incubated for 30–60 min at 37°C. Cytokine levels were determined by a Spectromax 250 spectrophotometer reading absorbance at 405 nm, using Softmax Pro V2.2.1 software (both Molecular Devices).

Cytotoxic T cell line assay to determine the biological activity of IL-2

The bioactivity of recombinant murine IL-2 and of IL-2 secreted from VV and FPV-infected cells was assayed using a murine IL-2-dependent cytotoxic T cell line (CTLL), as described earlier.⁴⁰ Briefly, 1×10^4 CTLL cells per well/50 μ l were plated with 50 μ l of varying concentrations of rec IL-2 and test supernatants. CTLL proliferation was determined 24 h later by [³H]-thymidine incorporation (for 5 h) and measured by liquid scintillation counting (Hewlett Packard, Palo Alto, CA). Activity was measured as the median effective dose (ED50; U ml⁻¹) in which 1U is the amount required for 50% maximum growth from 1×10^4 CTLL cells in 0.1 ml culture volumes during a 24-h culture period.

Statistical analysis

One way analysis of variance was used to compare cytokine production from VV or FPV-infected cells, or the mean ³H-Thymidine incorporation, within each group across the different groups using GraphPad Prism (San Diego, CA).

Results

Mesothelioma cells are resistant to infection by FPV viral constructs

We first examined the ability of the FPV-HA and FPV-HA cytokine constructs to infect 143BTK⁻ cells *in vitro*. All of the vectors express HA enabling HA expression levels measured by FACS analysis to become a surrogate marker of the percent of cells infected (Figures 1a, c, and d). The 143BTK⁻ cells (1×10^6) were incubated for 24, 48, or 72 h with 5×10^6 or 1×10^6 pfus of the FPV constructs (Figure 1a). After exposure to 5×10^6 FPV-HA at a multiplicity of infection (m.o.i.) of 5:1, almost 100%

Figure 1 Mesothelioma cells are resistant to infection by FPV viral constructs. HA expression by 1×10^6 143BTK⁻ cells incubated with 1×10^6 or 5×10^6 pfus of the FPV constructs for 24 h (representative histograms of FPV-HA shown in (a)) was detected using a biotinylated anti-HA antibody linked to streptavidin-PE. Pooled data is from one experiment using 1×10^6 pfus of the FPV constructs (b) showing the percent of cells that were HA⁺. AE17 mesothelioma tumor cells were similarly incubated with the FPV constructs for 24, 48, and 72 h and stained for HA expression; representative histograms in (c) and (d), and pooled data using 1×10^7 pfus from three experiments shown in (e) as mean $\pm$ s.d.

of 143BTK⁻ cells were infected within 24 h (Figures 1a and b). In contrast, at the same time point and m.o.i., each FPV-cytokine gene construct had infected only 50% of 143BTK⁻ cells. However, by 72 h, the percent of cells infected increased; FPV-HA-IL-2 reached 80% 143BTK⁻ cells and FPV-HA-IL-12 infected 66% 143BTK⁻ cells.

AE17 mesothelioma tumor cells incubated with the same FPV constructs at the same m.o.i. showed a lower percent of infected cells (Figures 1c and d) relative to

those seen with 143BTK⁻ cells. Even at a higher m.o.i. of 10:1, the maximal percent of cells infected reached by 72 h was 60% for FPV-HA. The FPV-cytokine constructs did not infect >30% AE17 cells at any time point.

Mesothelioma cells are permissive to VV infection

As we have already shown that the VV constructs readily infect 143BTK⁻ cells,¹³ we next examined the ability of the VV-HA viral vector and its cytokine constructs to

infect 1×10^6 mesothelioma tumor cells *in vitro*. AE17 mesothelioma cells were incubated with 5×10^6 or 1×10^6 pfus of the VV constructs for 24, 48, or 72 h (Figures 2a–c). All of the VV constructs led to $>80\%$ of AE17 mesothelioma cells being infected after 72 h in culture. The infectious nature of VV is further highlighted by the lack of titratable HA expression; that is 5×10^6 pfus showed similar HA expression to 1×10^6 pfus (Figure 2).

Closer examination of the ability of the FPV versus VV vectors to infect AE17 mesothelioma cells shows that none of the FPV constructs reached the same HA expression levels as their VV counterparts (Figures 2d–h). Furthermore, FPV infection had little impact on AE17 cell viability except at the high 10^7 pfu dose (Figure 2i). In contrast, all of the VV vectors reduced AE17 viability at a log fold lower dose by around 20% (Figure 2j). However, this is less than the percent of cells that were infected ($>70\%$; Figure 2c).

The data from Figures 1 and 2 show that while FPV readily infects 143BTK[−] cells, it cannot reproduce this in AE17 mesothelioma cells, suggesting mechanistic differences with $<30\%$ of mesothelioma cells infected by FPV-cytokine gene constructs at any time point. In contrast, 143BTK[−] cells and mesothelioma cells were highly susceptible to VV infection. In the latter case, $>80\%$ of AE17 MM cells were infected after 72 h incubation with VV constructs. Thus, the following series of experiments focussed on the VV constructs.

VV-infected mesothelioma cells secrete high levels of the transfected cytokine

VV-infected AE17 mesothelioma cells and 143BTK[−] cells secreted both IL-2 and IL-12 (Figures 3a and b). Neither cell type secreted endogenous IL-2 or IL-12 after VV-HA infection (Figures 3a and b).

VV-infected mesothelioma cells are unable to proliferate

AE17 cells readily proliferate *in vitro* in complete media (shown as no VV in Figure 4). However, the presence of each VV construct prevented normal cell turnover. Log fold reductions of each VV construct restored this proliferative response. These data suggest that AE17 tumor cells are either lysed by VV or their ability to proliferate is impaired, both occurring as HA surface expression is still detectable on viable AE17 cell 3 days after VV infection. However, cells expressing HA also showed increased granularity and reduced size relative to uninfected cells indicative of cell death (data not shown).

Macrophages can be infected by VV

The next series of experiments were designed to better understand the events that may occur *in vivo* after i.t. administration of VV as per a recent clinical trial.¹² Macrophages dominate the AE17-induced tumor microenvironment and can represent up to 60% of the total tumor cellularity.^{41–43} Therefore, these cells are highly likely to encounter any viral vector that is delivered by i.t. injection. Thus, we investigated VV infection of 1×10^6 peritoneal macrophages incubated with 5×10^6 , 1×10^6 , 5×10^5 , or 1×10^3 pfus of the VV-HA cytokine constructs

for 24 h. As removal of adherent macrophages using trypsin or cell scrapers led to high levels of cell death, only IL-2 and IL-12 cytokine levels in supernatants were analyzed (Table 1). Overall levels of cytokine production were low relative to those secreted by infected mesothelioma cells. These data suggest that macrophages may be infected by VV at low levels.

VV-infected T cells require exogenous cytokines to restore proliferative responses

T cells are recognized to be critical anti-tumor effector cells, and their proliferation may be directly provoked by increased i.t. IL-2 levels,³⁵ or indirectly by increased ‘danger’ levels and/or local IL-12 concentration that impact on antigen-presenting cells.³⁶ Thus, we examined splenic cells (as indicators of T cells) exposed to each of the VV constructs. IL-2 production by VV-HA-IL-2-infected spleen cells titrated in accordance with virus concentration (Table 2). Similar to IL-2, IL-12 production by T cells infected with VV-HA-IL-12 corresponded to viral load and increased with time (Table 2).

None of the constructs, including VV-HA-IL-2, induced proliferation of resting, non-activated splenic T cells (data not shown), despite IL-2 production by VV-HA-IL-2-infected cells. A CTLL assay confirmed that this virally derived IL2 was biologically active, as the IL-2-dependent CTLL cell line proliferated in response to supernatants from 143BTK[−] cells infected with VV-HA-IL-2, but not to VV-HA or VV-HA-IL-12 (data not shown).

ConA-induced splenic T cell proliferation was significantly inhibited at a high m.o.i. (10:1) of the VV-HA constructs (Figure 5). This inhibition was ameliorated by the VV-cytokine constructs, regardless of whether the cytokine was IL-2 or IL-12.

VV constructs prevent DC maturation

Finally, DCs were examined on account of the crucial function they have in inducing immune responses and they represent approximately 12% of cells in the AE17-induced tumor microenvironment,⁴¹ thus they may be directly infected after i.t. administration of the VV constructs. In these studies, bone marrow-derived CD11c⁺ DCs (Figure 6a) incubated with varying concentrations of the VV constructs did not express HA at 24 h (Figure 6b). Similar results were seen for VV-cytokine gene constructs (data not shown). However, DCs exposed to the VV-cytokine constructs did produce cytokines (Table 3) indicative of infection. The cytokine levels titrated according to the pfus added, peaked at 24 h, and were higher than those secreted by macrophages. DC viability was compromised by high doses of VV-HA and VV-IL-12, but not by VV-IL-2 (Figure 6c). No upregulation of MHC class II expression was seen in DC infected with any of the constructs 24 or 48 h after infection with 1×10^7 pfus (Figures 6d–g), suggesting impaired maturation (Figures 6g–j). Reducing viral load by a log fold partially improved DC maturation.

Mesothelioma tumor growth is arrested after i.t. VV-IL-12
Established mesothelioma tumors were given repeated i.t. injections of the VV-HA and cytokine constructs in a

regimen that mimicked the clinical trial.¹² VV-HA did not provide a beneficial effect, as the tumors continued to grow at a similar, or faster, rate than the PBS controls (Figures 7a and b). In contrast, both VV-IL-2 and VV-IL-

12 monotherapies slowed tumor growth rate. VV-IL-2 offered cures to 20% of mice, whereas VV-IL-12 cured 80% of mice. The combination of both cytokine constructs seemed to further slow tumor growth rate,

Figure 3 VV-infected mesothelioma cells secrete high cytokine levels; 1×10^6 AE17 or 143BTK⁻ cells were incubated with 1×10^6 pfus of the VV-IL-2, VV-IL-12, or VV-HA constructs for 24 h. Supernatants were removed and assayed for IL-2 or IL-12 by ELISA. No cytokines were detected in supernatants collected from VV-HA (a, b), or in uninfected cells (data not shown). Similarly, no IL-2 was detected in VV-HA-IL-12-infected cells (a), and no IL-12 was detected in VV-HA-IL-2-infected cells (b). Values represent means of duplicates within one experiment $\pm$ s.e.m.

but this difference did not reach statistical significance compared with VV-IL-12 alone.

Discussion

Earlier studies have shown that i.t. administration of cytokine VV vectors represents a safe and rational approach that can be used in mesothelioma patients.^{5,12} Those studies were premised on the hypothesis that after

i.t. administration, tumor cells infected by the viral vector would produce the cytokine and modify the tumor microenvironment. However, most tumor microenvironments contain stromal elements that include different immune cell types. Therefore, in these studies, we aimed to identify which cell types located in the mesothelioma-induced tumor microenvironment are likely to be infected and what the consequences of this infection might be. We show, using our murine mesothelioma model, that while the parent VV vector readily infects mesothelioma tumor cells, *in vivo* tumor growth continues unimpeded

Figure 2 Mesothelioma cells are highly permissive to VV infection. FACS analyses, as for Figure 1, were used to detect HA expression by 1×10^6 AE17 mesothelioma cells incubated with 1×10^6 or 5×10^6 pfus of the VV constructs for 24, 48, and 72 h. Representative histograms of VV-HA shown in (a) and (b), and pooled data from three experiments using 1×10^6 pfus of the VV constructs showing the percent of cells that were HA⁺ is shown in (c). Representative comparisons of HA expression levels achieved by AE17 cells incubated with the VV and FPV constructs for 24 and 72 h (d–f), and pooled data from four experiments showing the mean fluorescent intensities (MFI) of HA staining at 48 h (as mean $\pm$ s.d.; (g) and (h) highlight the differences. AE17 viability was reduced after 48 h infection with the FPV (i) and VV constructs (j) (from four experiments and shown as mean $\pm$ s.d.).

Figure 4 VV-infected mesothelioma cells lose their ability to proliferate; 1×10^6 AE17 cells were incubated with 1×10^7 (m.o.i. of 10), 1×10^6 (m.o.i. of 1), 1×10^5 (m.o.i. of 0.1), or 1×10^4 (m.o.i. of 0.01) pfus for 72 h and their proliferative responses measured by ^3H -Thy incorporation and compared with uninfected (no VV) controls; * $P < 0.05$, *** $P < 0.001$. Values are shown as the mean of triplicates within two experiments $\pm$ s.e.m.

Table 1 Cytokine production (ng ml^{-1}) by VV-infected macrophages

pfus	VV-HA-IL-12	VV-HA-IL-2
5×10^6	65.6 ± 6.3	22.2 ± 2.3
1×10^6	17.1 ± 1.8	3.2 ± 0.5
5×10^5	9.9 ± 1.1	2.8 ± 0.5
1×10^5	1.4 ± 0.2	0.3 ± 0.1

Abbreviations: HA, hemagglutinin; IL, interleukin; pfus, plaque-forming units; VV, vaccinia virus.

1×10^6 macrophages were infected with differing concentrations of the VV cytokine constructs for 24 h and supernatants assayed by ELISA. Neither cytokine was detected in supernatants from VV-HA-infected nor uninfected macrophages (data not shown). Values represent means of duplicates $\pm$ s.e.m.

after i.t. injection. We conclude that this is likely because of VV interfering with the ability of infected immune cells to become activated and/or cycle during infection. However, expression of IL-2 and/or IL-12 by VV rescues these immune cells and induces profound tumor regression.

FPV vectors are considered as a safer alternative than VV vectors. Thus, we commenced our studies by comparing the two vectors for their ability to infect our murine mesothelioma tumor cells. The data show that

Table 2 Cytokine secretion (ng ml^{-1}) by VV-infected spleen cells

pfus	VV-HA-IL-2		
	24 h	48 h	72 h
1×10^6	17.1 ± 3.1	18.8 ± 4.8	18.9 ± 4.9
1×10^5	1.0 ± 0.3	1.3 ± 0.3	1.5 ± 0.2
1×10^4	0	0.1	0.1

pfus	VV-HA-IL-12		
	24 h	48 h	72 h
1×10^6	46.4 ± 0.4	42.7 ± 3.0	51.5 ± 9.8
1×10^5	3.5 ± 0.1	3.2	3.9 ± 0.5
1×10^4	0.3	0.2	0.4

Abbreviations: HA, hemagglutinin; IL, interleukin; pfus, plaque-forming units; VV, vaccinia virus.

1×10^5 spleen cells were infected with each cytokine construct at various pfus for 24–72 h. Supernatants were by ELISA. Values represent the mean $\pm$ s.e.m. No IL-2 or IL-12 was detected in supernatants collected from spleen cells without viral constructs or those infected with VV-HA (data not shown).

Figure 5 VV-infected T cells are rescued by the cytokine constructs; 1×10^5 splenocytes were incubated in triplicate with ConA or with 1×10^6 , 1×10^5 , or 1×10^4 pfus of each VV construct with ConA for 72 h. ^3H -Thy was then added and the level of ^3H -Thy incorporation determined 4 h later as CPM.

these mesothelioma tumor cells were more susceptible to infection by the VV vectors than the FVP vectors. Not only was the percent of infected cells greater, but increased staining intensities (MFI levels of HA expression) in VV-infected cells versus FVP-infected cells suggests the presence of more VV viral particles in infected cells. VV-infected mesothelioma cells produced

Figure 6 DCs do not mature after exposure to the VV constructs. After culture in GM-CSF for 10 days, bone marrow-derived DC were identified by their CD11c expression (a; filled histogram is the isotype control and open histogram is anti-CD11c); 1×10^6 DC were incubated with 1×10^7 pfus of each VV-cytokine construct for 24 or 48 h and prepared for FACs staining. No surface HA was detected for VV-HA at 24 h (b; filled histogram is the isotype control and open histogram is anti-HA). DC viability, assessed by PI staining and by gating on FCS versus SCC, was reduced after 48 h infection by high doses of VV-HA and VV-IL-12 (c shows pooled data from two experiments shown as mean $\pm$ s.d.). MHC class II expression did not change in DC exposed to each of the VV vectors (d–f) for 24 h relative to uninfected controls (filled histograms). After 48 h, MHC class II expression (black filled histograms) remained unchanged or had decreased in DC exposed to the VV vectors (g–i); (j) shows pooled data from two experiments shown as mean $\pm$ s.d.

significant levels of cytokine. Our data also show that while some mesothelioma cells die as a consequence of VV infection, the remaining viable cells are unable to proliferate. Others have shown that VV infection modulates expression of cellular regulators of the cell cycle and alters cell cycle progression resulting in more cells in S-phase,⁴⁴ which may then apoptose. Thus, VV-cytokine

gene vectors are highly likely to infect mesothelioma tumor cells after i.t. delivery, generating a cytokine-rich milieu in association with reduced tumor cell turnover and increased cell death. Future studies will show whether this contributes to increased tumor antigen presentation and tumor-specific cytotoxic T cell induction in regional lymph nodes after *in vivo* i.t. application.

Table 3 Cytokine secretion (ng ml⁻¹) by DCs

pfus	VV-HA-IL-2		VV-HA-IL-12	
	24 h	48 h	24 h	48 h
1 × 10 ⁷	77.1 ± 6.3	41.7 ± 5.2	267.7 ± 38.0	164.7 ± 36.0
1 × 10 ⁶	2.0 ± 0.5	1.5 ± 0.1	19.7 ± 4.1	16.0 ± 4.0
1 × 10 ⁵	0.1	0	2.4 ± 0.8	1.4 ± 0.7

Abbreviations: DC, dendritic cell; HA, hemagglutinin; IL, interleukin; pfus, plaque-forming units; VV, vaccinia virus.

1 × 10⁶ DCs were infected with differing concentrations of each VV cytokine construct for 24 h. Supernatants were assayed by ELISA for IL-2 or IL-12. No IL-2 or IL-12 was detected in supernatants collected from uninfected DCs or from DCs infected with VV-HA (data not shown). Values represent means of duplicates ± s.e.m.

Figure 7 Mesothelioma tumor growth is arrested by VV-cytokine constructs. Mice were subcutaneously injected with 1 × 10⁶ AE17 cells at day 0. Once palpable, tumors were injected intratumorally with 100 µl PBS or 5 × 10⁶ pfus of either VV-HA, VV-HA-IL-2, or VV-HA-IL-12 alone (all with five mice per group), or with a combination of VV-HA-IL-2 and VV-HA-IL-12 (6 mice). Weekly injections were given for 3 weeks and tumor growth (a; data shown as mean ± s.d.) and survival (b) monitored.

The differences observed between the susceptibility of mesothelioma tumor cells to the two viral constructs could be due to the differences in replicative ability. FPV undergoes abortive replication in mammalian cells, whereas VV can undergo full replication, but is attenuated and others have shown that VV is more efficient at delivering IFN γ -mediated protection than FPV.⁴⁵ It may also be that these viral constructs enter cells through different mechanisms resulting in differing intracellular pathways being used. We concluded from these data that

VV was likely to be a more useful vector for i.t. studies in our animal model.

We then turned our attention to the stromal cells that are part of the mesothelioma-induced tumor microenvironment. F4/80⁺ macrophages are the dominant cell type in this complex microenvironment and in this model represent 60% of total tumor cellularity,⁴¹ and, therefore, have a high probability of encountering an intratumorally injected cytokine vector. We used peritoneal macrophages as surrogates for tumor-infiltrating macrophages and found that they secreted low levels of cytokine regardless of the vector used suggestive of resistance to overt VV or FPV infection. These data indicate that after i.t. administration of a VV-cytokine gene construct, tumor-residing macrophages are not likely to be significantly infected.

Other important stromal cells are DCs. DCs are crucial antigen-presenting cells and low numbers of DCs are present in mesothelioma tumors.⁴¹ In these studies, bone marrow-derived CD11c⁺ DCs were examined. No HA was observed after 24 or 48 h incubation with each VV construct, suggesting that the DCs were not infected with VV. However, their cytokine production was greater than that observed with macrophages and splenocytes. These data imply that DCs were infected leading to transient cytokine production. Thus, proteosomal degradation may have prevented HA expression and resolved DC infection. However, in agreement with the earlier reports, DC maturation did not occur after infection by the VV constructs^{46–49} and indicating a potentially detrimental bystander response after i.t. VV. Furthermore, we have earlier shown that these VV vectors prevent DC maturation,¹³ whereas others have shown that DC viability is compromised after phagocytosing-killed neoplastic cells in the presence of MVA viral particles.⁵⁰ Similarly, our data show that high doses of the VV-HA and VV-IL-12 constructs produce increased DC death. In contrast, the presence of IL-2 protected DC viability, but not their maturation.

T cells can also be found in mesothelioma tumors,^{41,43} thus, they may also be infected after i.t. administration of a viral vector. T cells have an important function in anti-tumor immunity, and splenic cells were used as an indicator of T cell responsiveness to the viral constructs. VV-infected spleen cells produced low levels of cytokine. These data suggest that T cells, or other splenic components, are resistant to infection by VV. Nonetheless, biologically active IL-2 was produced by VV-HA-IL-2-infected cells. The most striking observation was that the VV-HA vector interfered with T cell proliferative responses to ConA. Similarly, others have reported a transient decrease in human T cell proliferative responses after vaccinia immunization.⁵¹ However, T cell proliferation was restored by the IL-2 and IL-12 cytokine constructs. These data imply that use of a VV vector without the rescue effects of an appropriate cytokine may impair T cell immunity.

Taken together, these data show that after i.t. injection, VV vectors infect large numbers of mesothelioma tumor cells such that the tumor cells cease to cycle and undergo

cell death. However, VV vectors also sabotage the immune system by preventing DC maturation and T cell proliferation. Indeed, i.t. injection of a VV vector without cytokine arming enables tumor progression. In contrast, VV-cytokine gene constructs infect large numbers of mesothelioma tumors cells, which are likely to generate a cytokine-rich microenvironment. Furthermore, tumor-infiltrating DCs and T cells may also be infected and secrete the same cytokines, albeit to a much lesser extent. Importantly, as a result of VV infection mesothelioma, tumor cells cease to cycle and those remaining viable may then be destroyed by T cells rescued by virally derived IL-2 and/or IL-12. Indeed, our *in vivo* studies show that both VV-cytokine gene constructs significantly restrained tumor growth. Their combination with a tumor antigen vaccination strategy may further improve this outcome. Thus, these proof-of-principle studies support the concept of using VV vectors to deliver cytokines into the mesothelioma tumor site.

Conflict of interest

The authors declare no conflict of interest.

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References

- Peplinski GR, Tsung K, Whitman ED, Meko JB, Norton JA. Construction and expression in tumor cells of a recombinant vaccinia virus encoding human interleukin-1 beta. *Ann Surg Oncol* 1995; **2**: 151–159.
- Slavin-Chiorini DC, Catalfamo M, Kudo-Saito C, Hodge JW, Schlom J, Sabzevari H. Amplification of the lytic potential of effector/memory CD8+ cells by vector-based enhancement of ICAM-1 (CD54) in target cells: implications for intratumoral vaccine therapy. *Cancer Gene Ther* 2004; **11**: 665–680.
- Greiner S, Humrich JY, Thuman P, Sauter B, Schuler G, Jenne L. The highly attenuated vaccinia virus strain modified virus Ankara induces apoptosis in melanoma cells and allows bystander dendritic cells to generate a potent anti-tumoral immunity. *Clin Exp Immunol* 2006; **146**: 344–353.
- Kudo-Saito C, Garnett CT, Wansley EK, Schlom J, Hodge JW. Intratumoral delivery of vector mediated IL-2 in combination with vaccine results in enhanced T cell avidity and anti-tumor activity. *Cancer Immunol Immunother* 2007; **56**: 1897–1910.
- Robinson BW, Mukherjee SA, Davidson A, Morey S, Musk AW, Ramshaw I *et al.* Cytokine gene therapy or infusion as treatment for solid human cancer. *J Immunother* 1998; **21**: 211–217.
- Qian C, Liu XY, Prieto J. Therapy of cancer by cytokines mediated by gene therapy approach. *Cell Res* 2006; **16**: 182–188.
- Liu M, Acres B, Balloul JM, Bizouarne N, Paul S, Slos P *et al.* Gene-based vaccines and immunotherapeutics. *Proc Natl Acad Sci USA* 2004; **101**(Suppl 2): 14567–14571.
- Wu Q, Moyana T, Xiang J. Cancer gene therapy by adenovirus-mediated gene transfer. *Curr Gene Ther* 2001; **1**: 101–122.
- Schambach A, Baum C. Clinical application of lentiviral vectors—concepts and practice. *Curr Gene Ther* 2008; **8**: 474–482.
- Bouard D, Alazard-Dany D, Cosset FL. Viral vectors: from virology to transgene expression. *Br J Pharmacol* 2009; **157**: 153–165.
- Collins SA, Guinn BA, Harrison PT, Scallan MF, O'Sullivan GC, Tangney M. Viral vectors in cancer immunotherapy: which vector for which strategy? *Curr Gene Ther* 2008; **8**: 66–78.
- Mukherjee S, Haenel T, Himbeck R, Scott B, Ramshaw I, Lake RA *et al.* Replication-restricted vaccinia as a cytokine gene therapy vector in cancer: persistent transgene expression despite antibody generation. *Cancer Gene Ther* 2000; **7**: 663–670.
- Mukherjee S, Upham JW, Ramshaw I, Bundell C, van Bruggen I, Robinson BW *et al.* Dendritic cells infected with a vaccinia virus interleukin-2 vector secrete high levels of IL-2 and can become efficient antigen presenting cells that secrete high levels of the immunostimulatory cytokine IL-12. *Cancer Gene Ther* 2003; **10**: 591–602.
- Foloppe J, Kintz J, Futin N, Findeli A, Cordier P, Schlesinger Y *et al.* Targeted delivery of a suicide gene to human colorectal tumors by a conditionally replicating vaccinia virus. *Gene Ther* 2008; **15**: 1361–1371.
- Mastrangelo MJ, Maguire Jr HC, Eisenlohr LC, Laughlin CE, Monken CE, McCue PA *et al.* Intratumoral recombinant GM-CSF-encoding virus as gene therapy in patients with cutaneous melanoma. *Cancer Gene Ther* 1999; **6**: 409–422.
- Peplinski GR, Tsung K, Meko JB, Norton JA. *In vivo* gene therapy of a murine pancreas tumor with recombinant vaccinia virus encoding human interleukin-1 beta. *Surgery* 1995; **118**: 185–190; discussion 190–191.
- Flanagan K, Glover RT, Horig H, Yang W, Kaufman HL. Local delivery of recombinant vaccinia virus expressing secondary lymphoid chemokine (SLC) results in a CD4 T-cell dependent antitumor response. *Vaccine* 2004; **22**: 2894–2903.
- Yang AS, Monken CE, Lattime EC. Intratumoral vaccination with vaccinia-expressed tumor antigen and granulocyte macrophage colony-stimulating factor overcomes immunological ignorance to tumor antigen. *Cancer Res* 2003; **63**: 6956–6961.
- Lattime EC, Lee SS, Eisenlohr LC, Mastrangelo MJ. *In situ* cytokine gene transfection using vaccinia virus vectors. *Semin Oncol* 1996; **23**: 88–100.
- Mahvi DM, Henry MB, Albertini MR, Weber S, Meredith K, Schalch H *et al.* Intratumoral injection of IL-12 plasmid DNA—results of a phase I/IB clinical trial. *Cancer Gene Ther* 2007; **14**: 717–723.
- Liu Y, Huang H, Saxena A, Xiang J. Intratumoral coinjection of two adenoviral vectors expressing functional interleukin-18 and inducible protein-10, respectively, synergizes to facilitate regression of established tumors. *Cancer Gene Ther* 2002; **9**: 533–542.

- 22 Rochlitz C, Dreno B, Jantschkeff P, Cavalli F, Squiban P, Acres B *et al.* Immunotherapy of metastatic melanoma by intratumoral injections of Vero cells producing human IL-2: phase II randomized study comparing two dose levels. *Cancer Gene Ther* 2002; **9**: 289–295.
- 23 Gambotto A, Tuting T, McVey DL, Kovessi I, Tahara H, Lotze MT *et al.* Induction of antitumor immunity by direct intratumoral injection of a recombinant adenovirus vector expressing interleukin-12. *Cancer Gene Ther* 1999; **6**: 45–53.
- 24 Leong KH, Ramsay AJ, Boyle DB, Ramshaw IA. Selective induction of immune responses by cytokines coexpressed in recombinant fowlpox virus. *J Virol* 1994; **68**: 8125–8130.
- 25 Zhu MZ, Marshall J, Cole D, Schlom J, Tsang KY. Specific cytolytic T-cell responses to human CEA from patients immunized with recombinant avipox-CEA vaccine. *Clin Cancer Res* 2000; **6**: 24–33.
- 26 Morse MA, Clay TM, Hobeika AC, Osada T, Khan S, Chui S *et al.* Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. *Clin Cancer Res* 2005; **11**: 3017–3024.
- 27 Arlen PM, Skarupa L, Pazdur M, Seetharam M, Tsang KY, Grosenbach DW *et al.* Clinical safety of a viral vector based prostate cancer vaccine strategy. *J Urol* 2007; **178**(4 Pt 1): 1515–1520.
- 28 Jolly D. Viral vector systems for gene therapy. *Cancer Gene Ther* 1994; **1**: 51–64.
- 29 Meko JB, Tsung K, Norton JA. Cytokine production and antitumor effect of a nonreplicating, noncytopathic recombinant vaccinia virus expressing interleukin-12. *Surgery* 1996; **120**: 274–282; discussion 282–283.
- 30 Ramshaw I, Ruby J, Ramsay A, Ada G, Karupiah G. Expression of cytokines by recombinant vaccinia viruses: a model for studying cytokines in virus infections *in vivo*. *Immunol Rev* 1992; **127**: 157–182.
- 31 Mackett M, Smith GL, Moss B. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. *J Virol* 1984; **49**: 857–864.
- 32 Ramshaw IA, Andrew ME, Phillips SM, Boyle DB, Coupar BE. Recovery of immunodeficient mice from a vaccinia virus/IL-2 recombinant infection. *Nature* 1987; **329**: 545–546.
- 33 Astoul P, Picat-Joossen D, Viallat JR, Boutin C. Intrapleural administration of interleukin-2 for the treatment of patients with malignant pleural mesothelioma: a Phase II study. *Cancer* 1998; **83**: 2099–2104.
- 34 Boutin C, Schlessner M, Frenay C, Astoul P. Malignant pleural mesothelioma. *Eur Respir J* 1998; **12**: 972–981.
- 35 Jackaman C, Bundell CS, Kinnear BF, Smith AM, Filion P, van Hagen D *et al.* IL-2 intratumoral immunotherapy enhances CD8+ T cells that mediate destruction of tumor cells and tumor-associated vasculature: a novel mechanism for IL-2. *J Immunol* 2003; **171**: 5051–5063.
- 36 Caminschi I, Venetsanakos E, Leong CC, Garlepp MJ, Robinson BW, Scott B. Cytokine gene therapy of mesothelioma. Immune and antitumor effects of transfected interleukin-12. *Am J Respir Cell Mol Biol* 1999; **21**: 347–356.
- 37 Caminschi I, Venetsanakos E, Leong CC, Garlepp MJ, Scott B, Robinson BW. Interleukin-12 induces an effective antitumor response in malignant mesothelioma. *Am J Respir Cell Mol Biol* 1998; **19**: 738–746.
- 38 Boyle DB, Coupar BE. Construction of recombinant fowlpox viruses as vectors for poultry vaccines. *Virus Res* 1988; **10**: 343–356.
- 39 Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N *et al.* An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 1999; **223**: 77–92.
- 40 Weston L, Geczy A, Farrell C. A convenient and reliable IL-2 bioassay using frozen CTLL-2 to improve the detection of helper T lymphocyte precursors. *Immunol Cell Biol* 1998; **76**: 190–192.
- 41 Jackaman C, Cornwall S, Lew AM, Zhan Y, Robinson BW, Nelson DJ. Local effector failure in mesothelioma is not mediated by CD4+ CD25+ T-regulator cells. *Eur Respir J* 2009; **34**: 162–175.
- 42 Jassar AS, Suzuki E, Kapoor V, Sun J, Silverberg MB, Cheung L *et al.* Activation of tumor-associated macrophages by the vascular disrupting agent 5,6-dimethylxanthone-4-acetic acid induces an effective CD8+ T-cell-mediated antitumor immune response in murine models of lung cancer and mesothelioma. *Cancer Res* 2005; **65**: 11752–11761.
- 43 Hegmans JP, Hemmes A, Hammad H, Boon L, Hoogsteden HC, Lambrecht BN. Mesothelioma environment comprises cytokines and T-regulatory cells that suppress immune responses. *Eur Respir J* 2006; **27**: 1086–1095.
- 44 Wali A, Strayer DS. Infection with vaccinia virus alters regulation of cell cycle progression. *DNA Cell Biol* 1999; **18**: 837–843.
- 45 Cheers C, Janas M, Ramsay A, Ramshaw I. Use of recombinant viruses to deliver cytokines influencing the course of experimental bacterial infection. *Immunol Cell Biol* 1999; **77**: 324–330.
- 46 Engelmayer J, Larsson M, Subklewe M, Chahroudi A, Cox WI, Steinman RM *et al.* Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol* 1999; **163**: 6762–6768.
- 47 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.
- 48 Deng L, Dai P, Ding W, Granstein RD, Shuman S. Vaccinia virus infection attenuates innate immune responses and antigen presentation by epidermal dendritic cells. *J Virol* 2006; **80**: 9977–9987.
- 49 Yao Y, Li P, Singh P, Thiele AT, Wilkes DS, Renukaradhya GJ *et al.* Vaccinia virus infection induces dendritic cell maturation but inhibits antigen presentation by MHC class II. *Cell Immunol* 2007; **246**: 92–102.
- 50 Di Nicola M, Napoli S, Anichini A, Mortarini R, Romagnoli L, Magni M *et al.* Dendritic cell viability is decreased after phagocytosis of apoptotic tumor cells induced by staurosporine or vaccinia virus infection. *Haematologica* 2003; **88**: 1396–1404.
- 51 Mathew A, Ennis FA, Rothman AL. Transient decreases in human T cell proliferative responses following vaccinia immunization. *Clin Immunol* 2000; **96**: 100–107.