Modified vaccinia virus Ankara induces moderate activation of human dendritic cells

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Modified vaccinia virus Ankara (MVA) is a highly attenuated strain known to be an effective vaccine vector. Here it is demonstrated that MVA, unlike standard vaccinia virus (VACV) strains, activates monocyte-derived human dendritic cells (DCs) as testified by an increase in surface co-stimulatory molecules and the secretion of pro-inflammatory cytokines. Inhibition of virus gene expression by subjecting MVA to UV light or heat treatment did not alter its ability to activate DCs. On the other hand, standard VACV strains activated DCs if virus gene expression was prevented by prior UV light or heat treatment. These results suggest that MVA or standard VACV particles are responsible for DC activation but, in the case of standard VACV strains, virus gene expression prevents activation. Additional experiments showed that DCs were activated by MVA-infected HeLa cells and, under these conditions, could induce secretion of gamma interferon from T lymphocytes more efficiently than if a replication-competent VACV strain was employed. These data provide one explanation for the remarkable immune-stimulating capacity of MVA in the absence of virus multiplication.

INTRODUCTION

Modified vaccinia virus Ankara (MVA) is a vaccinia virus (VACV) strain that fails to multiply in mammalian cell lines, with the notable exceptions of the permissive hamster BHK21 cell line and the semi-permissive monkey MA104 cell line (Mayr et al., 1975; Meyer et al., 1991; Carroll & Moss, 1997; Drexler et al., 1998). Primary or established human cell lines are non-permissive for MVA, although some tumour cell lines enable very low levels of virus multiplication when high multiplicities of infection are employed (Carroll & Moss, 1997; Blanchard et al., 1998).

MVA infection in HeLa cells is arrested at a late stage of the virus cycle, after the accumulation of immature virus particles (Sutter & Moss, 1992) but before the association of these particles with nascent DNA (Sancho et al., 2002), a step required for the formation of intracellular mature virus. The defectiveness of MVA makes it an attractive candidate for use as a viral vector, as well as a smallpox vaccine if such a vaccine is again needed. Studies in animal models have demonstrated that MVA is indeed an effective vaccine vector (Moss et al., 1996; Sutter & Staib, 2003) and that it is safe even in immunocompromised animals (Stittelaar et al., 2001). Although higher MVA titres are often used to induce an immune response similar to that in a standard VACV infection (Belyakov et al., 2003), the titres employed may be lower than the final body titres of replicating VACV strains. This suggests that MVA displays some unique features that enable it to induce an immune response without propagating. One explanation may lie in the fact that MVA lacks many of the genes found in standard VACV strains that counteract the immune response (Antoine et al., 1998; Blanchard et al., 1998). Another key factor may be the ability of MVA to induce human fibroblasts and human leukocytes to secrete type I interferons known to boost T cell responses (Buttner et al., 1995; Blanchard et al., 1998). In fact, MVA has been shown to efficiently re-stimulate human cytotoxic T lymphocytes in vitro (Dorrell et al., 2000). In this study we focus on the interactions of MVA with human dendritic cells (DCs), as a considerable amount of work has demonstrated the importance of these antigen-presenting cells in the induction of the immune response (Banchereau & Steinman, 1998). Prior investigations into the interaction of VACV with DCs have mainly been conducted with replicating VACV strains. Such studies have shown that DCs allow the expression of early virus genes, but are non-permissive for viral DNA replication or late protein synthesis (Bronte et al., 1997; Engelmayr et al., 1999; Drillien et al., 2000; Jenne et al., 2000). Furthermore, VACV-infected DCs fail to respond to agents that induce their activation (Engelmayr et al., 1999; Jenne et al., 2000), a step also termed maturation, which is required for de novo stimulation of T lymphocytes but not necessarily for memory responses. In contrast, it has been reported that MVA-infected DCs can be activated by tumour necrosis factor alpha (TNF-α) (Trevor et al., 2001), but no study has examined whether MVA on its own may activate DCs.

Thus we investigated the level of cell surface co-stimulatory molecules and pro-inflammatory cytokine secretion in MVA-infected DCs. We also studied the influence of MVA-infected
HeLa cells on DCs by following the level of co-stimulatory molecules and the ability of such mixed populations to induce gamma interferon (IFN-γ) secretion from T lymphocytes. These studies highlight the unique interactions of MVA with human dendritic cells.

METHODS

Cells. Leukocytes were isolated from healthy donors in the French Blood Centre (EFS-Alsace) after obtaining written informed consent. All donors were old enough to have undergone prior smallpox vaccination, and the majority displayed proliferative memory T cell responses in vitro in the presence of VACV. Bulk cell populations were isolated from donors by continuous flow centrifugation leukopheresis, and their monocytes were separated from other cell types by counterflow elutriation as described previously (Faradji et al., 1994). Fractions containing either lymphocytes or monocytes were frozen separately in liquid nitrogen. When required, the monocytes were thawed and differentiated into DCs for 5–6 days at a density of 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 40 ng interleukin 4 (IL4) ml^{-1}, 50 ng granulocyte macrophage–colony stimulating factor (GM-CSF) ml^{-1}, 0-11 mg sodium pyruvate ml^{-1}, non-essential amino acids for MEM, and 10% FBS (Sallusto & Lanzavecchia, 1994). After the first 3 days’ culture, half of the initial volume of fresh medium was added. When DCs were used for T cell stimulation, monocytes were differentiated in the presence of 2% pooled human serum (PHS) and heparin (50 IU ml^{-1}) as described previously (Xia & Kao, 2002). All media contained 50 µg gentamicin ml^{-1}. When required, bulk lymphocyte preparations maintained in liquid nitrogen were thawed and cultured in RPMI 1640 supplemented with 0-11 mg sodium pyruvate ml^{-1}, non-essential amino acids for MEM and 5% PHS. Hamster BHK21 cells were cultured in BHK21 medium supplemented with tryptose phosphate broth and 10% FBS. Human HeLa cells were grown in MEM with 10% FBS.

Virus. VACV strains Copenhagen (VACV-Cop), Lister (VACV-Lis) and MVA (a clonal isolate designated MVA-N33 and kindly provided by Jean-Marc Balloul, Transgene, Strasbourg, France) were grown in BHK21 cells and purified by the standard sucrose gradient procedure (Joklik, 1962). The ratio of virus particles to p.f.u. was determined by counterflow elutriation as described previously (Faradji et al., 1994). Fractions containing either lymphocytes or monocytes were frozen separately in liquid nitrogen. When required, the monocytes were thawed and differentiated into DCs for 5–6 days at a density of 10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 40 ng interleukin 4 (IL4) ml^{-1}, 50 ng granulocyte macrophage–colony stimulating factor (GM-CSF) ml^{-1}, 0-11 mg sodium pyruvate ml^{-1}, non-essential amino acids for MEM, and 10% FBS (Sallusto & Lanzavecchia, 1994). After the first 3 days’ culture, half of the initial volume of fresh medium was added. When DCs were used for T cell stimulation, monocytes were differentiated in the presence of 2% pooled human serum (PHS) and heparin (50 IU ml^{-1}) as described previously (Xia & Kao, 2002). All media contained 50 µg gentamicin ml^{-1}. When required, bulk lymphocyte preparations maintained in liquid nitrogen were thawed and cultured in RPMI 1640 supplemented with 0-11 mg sodium pyruvate ml^{-1}, non-essential amino acids for MEM and 5% PHS. Hamster BHK21 cells were cultured in BHK21 medium supplemented with tryptose phosphate broth and 10% FBS. Human HeLa cells were grown in MEM with 10% FBS.

Flow cytometry. Five to 6 days after differentiation of monocytes into DCs, the cells were pelleted, counted and resuspended at 5 x 10^6–10^7 cells ml^{-1} in fresh culture medium containing 50 ng GM–CSF ml^{-1} and 40 ng IL4 ml^{-1}. The DCs were then infected with purified virus that had been subjected to 5 s ultrasonic treatment to disaggregate clumps. Lipopolysaccharide (LPS) endotoxin from Escherichia coli (serotype O128:B12) was routinely employed to check for the ability of DCs to undergo maturation. Eighteen to 24 h after infection the cells were pelleted, washed once in PBS and resuspended for 30 min in PBS containing 5% normal goat serum (NGS) and a 20-fold dilution of a specific primary mouse monclonal antibody or a control antibody of the same isotype. The cells were then pelleted, washed once in cold PBS and incubated for 30 min in PBS containing 5% NGS and F(ab’)^2 sheep antibodies directed against mouse IgG that had previously been coupled with FITC. The cells were pelleted again, washed once in PBS, resuspended in PBS and fixed with 1% paraformaldehyde. Cells were then examined by flow cytometry using a Becton Dickinson Facscan and CellQuest software. In some experiments, direct immunolabelling was performed with FITC- or phycoerythrin (PE)-coupled mouse antibodies. Alternatively, HeLa cells in monolayers were infected with MVA or VACV-Cop for 15 h, then detached from the culture flasks with 0-02% EDTA in PBS, pelleted, washed twice with PBS to remove any input virus, and added to fresh DCs for an additional 24 h (one HeLa cell per four DCs). The mixed population was then double-labelled with a mouse anti-CD1a antibody (HI149) coupled to FITC, which binds only to DCs, and anti-CD86 antibodies coupled with PE. The cells were then examined by flow cytometry in the FL1 and FL2 channels, and only CD1a^+ cells in the FL1 channel (FITC) were gated to plot histograms of fluorescence in the FL2 channel (PE).

Cytokine assays. Dendritic cells cultured at 10^6 cells ml^{-1} as described above were infected at different multiplicities for the chosen time interval, after which they were pelleted and the culture medium was recovered and stored in aliquots at −80°C for cytokine assays. TNF-α and IL6 were determined by standard sandwich ELISA tests using reagents from Beckman Coulter. For IFN-γ assays, 10^6 HeLa cells in 2 cm² tissue culture plates were infected with MVA or VACV-Cop for 1 h in PBS, then fresh RPMI containing 5% PHS was added. After 4 h infection, the culture medium was removed and the cells were washed twice with PBS. Human DCs (10^6) in RPMI containing 5% PHS were then added to each culture plate; alternatively, DCs were directly infected with MVA or VACV-Cop. Autologous T lymphocytes were then prepared by depletion of B cells, NK cells, monocytes, activated T cells and granulocytes from total lymphocytes using a combination of antibodies coupled to magnetic beads (anti-CD14, anti-CD16, anti-CD56, anti-HLA class II DR/DP from Dynal) and added 2 h later to the mixture of infected HeLa cells and DCs (10^5 T lymphocytes per 10^6 DCs and 10^5 HeLa cells) or to 10^5 infected DCs. After overnight incubation, the cells in suspension were removed from the culture dishes, pelleted once and resuspended in fresh RPMI and 5% PHS. The cells were assayed for IFN-γ production using an enzyme-linked immunospot (ELISPOT) method and reagents obtained from Diatec.
RESULTS

MVA infection activates human DCs

Dendritic cells were prepared by in vitro differentiation of human monocytes for 5 to 6 days in medium containing IL4, GM–CSF and 10% FBS. Under these conditions the majority of the cells remained in suspension, displaying dendritic veils and a high level of CD1a, a characteristic DC surface antigen (not shown). For further phenotypic analysis we assayed for the presence of CD83, a molecule found on the DC surface only after activation, and for the presence of CD80 and CD86, two co-stimulatory molecules that are expressed strongly on activated DCs and are critical for their function as antigen-presenting cells (Banchereau & Steinman, 1998). When analysed by flow cytometry, uninfected DCs displayed almost no CD83 on their surface and only moderate levels of CD80 and CD86, indicating that they were in an immature state (Fig. 1a). A striking increase in all three cell surface antigens was observed after 1 day’s culture in the presence of 10 ng ml⁻¹ LPS, which is a potent maturation factor for DCs (Fig. 1b). Infection of the DCs for 1 day with MVA (5 p.f.u. per cell) led to a moderate increase in cell surface CD80 and CD83, and a more marked increase in CD86 (Fig. 1c). The extent of increase in these surface molecules was proportional to the m.o.i. employed, being barely detectable at 0·1 p.f.u. per cell and reaching a maximum at 20 p.f.u. per cell (not shown). Infection of the DCs with VACV-Cop resulted in no change in the level of CD83 and CD86, and a decrease in the baseline level of CD80 (Fig. 1d), consistent with our previous studies (Drillien et al., 2000). Thus MVA, in contrast to VACV-Cop, is able to induce a moderate level of DC activation as judged by an increase in cell surface maturation markers. However, in no instance did MVA infection, whatever the multiplicity (0·1–20 p.f.u. per cell), lead to the striking increase in all three maturation markers that was observed in the presence of LPS.

To investigate DC activation by MVA further, we assayed for the release of several pro-inflammatory cytokines (Table 1). Infection by MVA led to a significant, albeit low, level of TNF-α and IL6 secretion that increased with m.o.i. The level of cytokine secretion after MVA infection was much lower than that induced by LPS. IL1β secretion was also detected in some experiments, but not consistently (results not shown). VACV-Cop failed to induce any TNF-α and induced IL6 secretion barely above the background level of uninfected DCs.

In further analyses of DC activation by MVA we routinely assayed for an increase in cell surface CD86, as this marker proved to be the most sensitive. It was particularly important to rule out the possibility that endotoxin contamination accounted for DC activation by MVA. For this purpose, DCs were infected in the presence of polymyxin B, a...
A potent inhibitor of a variety of endotoxins (Rifkind, 1967). Whereas polymyxin B significantly reduced the high level of DC activation observed in the presence of as much as 1 mg ml\(^{-1}\) LPS, it was unable to alter the moderate activation of DCs induced by MVA (Fig. 2).

### Table 1. Pro-inflammatory cytokines in culture medium from infected DCs

DCs were left uninfected; infected with MVA 32 (MVA) or VACV-Cop 32 (VACV-Cop) at the multiplicities indicated, or incubated in the presence of LPS. After 24 h the cell culture medium was collected, stored at \(-80^\circ\)C and thawed, and the amount of TNF-\(\alpha\) or IL6 was determined. The results are representative of two independent experiments for determination of IL6 and three for TNF-\(\alpha\) using DCs from different donors. ND, No detection above background.

<table>
<thead>
<tr>
<th>Infection</th>
<th>TNF-(\alpha) (pg ml(^{-1}))</th>
<th>IL6 (pg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
<td>58</td>
</tr>
<tr>
<td>MVA (1 p.f.u. per cell)</td>
<td>ND</td>
<td>461</td>
</tr>
<tr>
<td>MVA (2 p.f.u. per cell)</td>
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<td>612</td>
</tr>
<tr>
<td>MVA (5 p.f.u. per cell)</td>
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</tr>
<tr>
<td>MVA (20 p.f.u. per cell)</td>
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<td>970</td>
</tr>
<tr>
<td>VACV-Cop (1 p.f.u. per cell)</td>
<td>ND</td>
<td>34</td>
</tr>
<tr>
<td>VACV-Cop (2 p.f.u. per cell)</td>
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<td>29</td>
</tr>
<tr>
<td>VACV-Cop (5 p.f.u. per cell)</td>
<td>ND</td>
<td>79</td>
</tr>
<tr>
<td>VACV-Cop (20 p.f.u. per cell)</td>
<td>ND</td>
<td>56</td>
</tr>
<tr>
<td>LPS (10 ng ml(^{-1}))</td>
<td>90 000</td>
<td>37 928</td>
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</tbody>
</table>

MVA activates DCs in the absence of virus gene expression

Previous studies have shown that standard VACV strains express only early virus genes in DCs, and we confirmed these findings for MVA (not shown). To investigate whether DC activation by MVA requires this early gene expression, we infected cells with a recombinant virus encoding GFP (MVA 32). The MVA 32-infected DCs displayed the expected increase in CD86 and a distinct population of GFP-positive cells (Fig. 3, compare c and a). Interestingly, both GFP\(^-\) and GFP\(^+\) cells showed an increase in the level of CD86, indicating that virus gene expression may not be

\[\text{CD86} \quad \text{GFP}\]

**Fig. 2.** Activation of MVA-infected DCs is insensitive to polymyxin B. DCs were mock-infected, treated with LPS (1 \(\mu\)g ml\(^{-1}\)) or infected with MVA (5 p.f.u. per cell) in the absence (open bars) or continuous presence (solid bars) of polymyxin B (20 \(\mu\)g ml\(^{-1}\) added 1 h before LPS treatment or infection). After 26 h, cells were collected and labelled with mouse anti-CD86 followed by FITC-coupled sheep anti-mouse antibodies. Cells were examined by flow cytometry, and mean fluorescence intensity was calculated for each sample and plotted. The results are representative of two experiments.

**Fig. 3.** MVA activates DCs in the absence of virus gene expression. DCs were mock-infected (a), treated with 10 ng ml\(^{-1}\) LPS (b), infected with MVA 32 (c), infected with UV-inactivated MVA 32 (d), infected with VACV-Cop 21 (e) or infected with UV-inactivated VACV-Cop 21 (f). Infections were performed with 5 p.f.u. per cell or the equivalent amount of UV-inactivated virus. After 24 h cells were collected and labelled with PE-coupled mouse antibodies directed against CD86. Cells were examined by flow cytometry for GFP fluorescence in the FL1 channel and PE fluorescence in the FL2 channel after adjustment of the windows to remove overlapping signals. The intensity of the CD86 signal for each cell is plotted versus the intensity of the GFP signal. Quadrants were overlaid to visualize distinct cell populations (CD86\(^-\), CD86\(^+\), GFP\(^-\), GFP\(^+\)) and the percentage of total cells in the fluorescence-positive quadrants is indicated. The experiment is representative of three experiments performed using DCs from different donors.
an absolute requirement for DC activation. However, the absence of GFP expression did not necessarily suggest that the DCs had not been infected, as some DCs may undergo abortive infection characterized by virus binding/uptake but no gene expression. To reduce virus gene expression, DCs were infected with MVA 32 that had undergone prior inactivation by UV light in the presence of psoralen. Considering both CD86− and CD86+ DCs, only 8.8% (7.2 + 1.6) of cells infected with the UV-inactivated MVA 32 expressed GFP (Fig. 3d), whereas 21.3% (10.9 + 10.4) of the DCs infected with the control virus expressed GFP (Fig. 3c), indicating that UV inactivation of the virus led to a significant reduction in its gene expression. However, no decrease but rather an increase in the number of CD86+ cells was observed in the DCs infected with UV-inactivated MVA 32, suggesting that gene expression was not a prerequisite for DC activation. VACV-Cop 21, as reported above for VACV-Cop, failed to increase the level of CD86 on the DC surface (Fig. 3e). However, DCs infected with UV-inactivated VACV-Cop 21 displayed a reduced level of GFP expression and, most surprisingly, an increase in CD86 expression (Fig. 3f) which was nearly comparable to the level achieved after MVA 32 infection.

We next investigated whether another method of virus inactivation, i.e. heat treatment, could be employed to abolish virus gene expression completely yet retain the ability of MVA to activate DCs. Prior heat treatment of MVA at 55 °C, for 1 h, led to a total loss of GFP expression in DCs (not shown), but did not affect its enhancement of CD86 expression (Fig. 4b). Heating MVA at a higher temperature (1 h at 65 °C) completely destroyed its ability to activate DCs (Fig. 4c). As endotoxins are notoriously resistant to mild heat treatments (such as 1 h at 65 °C), these results also indicate that they are not involved in DC activation by MVA. We also examined the ability of heat-treated VACV to activate DCs and, in this case, used either VACV-Cop or VACV-Lis (results shown for Lister strain). Untreated VACV-Lis failed to activate DCs (Fig. 4a) but prior heat treatment of the virus (55 °C for 1 h) unveiled its ability to activate DCs (Fig. 4b). This feature was further confirmed by the ability of VACV-Cop (treated at 55 °C) to induce TNF-α and IL6 secretion (not shown). Heat treatment of VACV-Lis (65 °C for 1 h) destroyed its capacity to activate DCs (Fig. 4c).

**MVA-infected HeLa cells activate DCs and stimulate IFN-γ release by T lymphocytes**

Under in vivo conditions a large number of DCs would probably escape virus infection, particularly if a non-replicating virus such as MVA is employed. Therefore, it was of interest to examine whether DC activation could occur indirectly through contact with MVA-infected cells. To investigate this, HeLa cells in monolayers were infected with MVA or VACV-Cop for 15 h, then detached from the culture flasks, pelleted, washed to remove any input virus and added to fresh DCs for an additional 24 h (one HeLa cell per four DCs). The mixed population was then double-labelled for CD1a (coupled with FITC), which binds only to DCs, and CD86 (coupled with PE). CD1a+ cells were gated to plot histograms of CD86 fluorescence. This procedure eliminated from the analysis both HeLa cells and the small proportion of CD1a− cells in the DC population. Under these conditions the addition of uninfected HeLa cells to DCs had no effect on CD86 surface expression (Fig. 5, compare thin lines in a and b). Addition of MVA-infected HeLa cells to DCs induced a large increase in the level of CD86 (Fig. 5b, thick line) demonstrating that MVA-infected cells activated DCs, as reported above for direct virus infection. However, HeLa cells infected with MVA did not induce as high a level of DC activation as was reached after LPS treatment (Fig. 5a). When HeLa cells were infected with VACV-Cop instead of MVA, and then mixed with DCs, there was no increase in

![Fig. 4. DC activation with heat-treated virus. DCs were infected at 5 p.f.u. per cell with untreated MVA or VACV-Lis (a), MVA or VACV-Lis inactivated at 55 °C for 1 h (b) or MVA or VACV-Lis inactivated at 65 °C for 1 h (c). After 20 h infection cells were collected and labelled with PE-coupled mouse antibodies directed against CD86. Cells were examined by flow cytometry and histograms of fluorescence plotted. Thin lines are histograms of control DCs immunolabelled with anti-CD86; thick lines are histograms of infected DCs immunolabelled in the same manner. The mean fluorescence intensity calculated for each sample is indicated above the corresponding histogram. The experiment is representative of four performed with MVA and either VACV-Cop or VACV-Lis.](http://vir.sgmjournals.org)
the level of CD86 (Fig. 5b, dashed line). To determine whether DC activation required active virus infection, HeLa cells infected with MVA for 12 h were fixed for 30 min with 1 % paraformaldehyde (c). In all instances, HeLa cell infection was carried out for 15 h before mixing with DCs. Cells were then collected and labelled with FITC-coupled anti-CD1a and PE-coupled anti-CD86 antibodies. Cells were analysed by flow cytometry and CD1a+ cells were gated to plot histograms of DCs labelled for CD86. The results are displayed for one experiment representative of four performed with DCs from different donors.

To examine the functional consequences of MVA infection of DCs, we studied the stimulation of T lymphocytes by assaying for the number of IFN-γ-producing T cells. For these experiments DCs were differentiated from monocytes in the presence of 2 % PHS, instead of FBS, to avoid presentation of bovine antigens to T cells, and 50 units ml⁻¹ heparin to ensure they were bona fide DCs expressing the CD1a antigen (Xia & Kao, 2002). We found that DCs infected directly with MVA or VACV-Cop did not induce a significant number of IFN-γ-producing T cells above the background level (Fig. 6a). Therefore, further experiments were conducted in which HeLa cells were infected, washed to remove any input virus, then mixed with DCs, and finally autologous T cells were added. The combination of MVA-infected HeLa cells and DCs induced a much higher number of IFN-γ-producing lymphocytes than the combination of VACV-Cop-infected cells with DCs (Table 2). No IFN-γ-producing lymphocytes were noticed in the absence of DCs or in the presence of uninfected HeLa cells, and only a small number of spots were visualized with uninfected DCs alone. To extend this analysis we investigated the influence of m.o.i. on the number of IFN-γ-producing lymphocytes (Fig. 6b). The DCs combined with VACV-Cop-infected HeLa cells induced no IFN-γ-producing lymphocytes, or only a small increase above background level in some experiments (not shown). The DCs combined with MVA-infected HeLa cells
The number of IFN-γ-producing T cells was then determined in quadruplicate and the mean number of spots for 2 × 10^4 cells calculated. The results represent four experiments using DCs from different donors.

<table>
<thead>
<tr>
<th>HeLa</th>
<th>DCs</th>
<th>Spots for 2 × 10^4 T lymphocytes</th>
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</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>MVA-infected</td>
<td>+</td>
<td>138</td>
</tr>
<tr>
<td>VACV-Cop-infected</td>
<td>+</td>
<td>19</td>
</tr>
<tr>
<td>Uninfected</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>MVA-infected</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>VACV-Cop-infected</td>
<td>−</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. ELISPOT detection of IFN-γ-producing T lymphocytes

Uninfected or infected HeLa cells (10 p.f.u. per cell) were cultured in the presence or absence of DCs and T lymphocytes overnight. The number of IFN-γ-producing T cells was then determined in quadruplicate and the mean number of spots for 2 × 10^4 cells calculated. The results represent four experiments using DCs from different donors.

DISCUSSION

These studies demonstrate that MVA infection of human DCs or incubation of MVA-infected cells with DCs induces their partial activation, characterized by the upregulation of co-stimulatory molecules and secretion of pro-inflammatory cytokines. The results were obtained reproducibly in repeated experiments with DCs from different donors. In no instance, however, did MVA infection boost the level of co-stimulatory molecules or induce cytokine secretion as efficiently as LPS, a potent maturation factor from Gram-negative bacteria. Importantly, addition of polymyxin B, an inhibitor of LPS, was able to prevent activation induced by LPS, but could not prevent the weaker activation induced by MVA or MVA-infected cells, indicating that neither MVA nor the HeLa cells were contaminated with LPS. Moreover, heat treatment of MVA at 65 °C, which does not inactivate LPS, prevented activation of DCs by MVA. Our findings show that MVA behaves distinctly from standard VACV strains that are unable to activate DCs, as shown in this report for the Copenhagen and Lister strains, and in previous reports for the WR strain (Engelmayr et al., 1999; Jenne et al., 2000). The latter two studies demonstrated that infection with standard VACV strains interferes with DC activation by a number of agents. In contrast, MVA-infected DCs responded to activation by TNF-α treatment (Trevor et al., 2001), but no direct activation of DCs by MVA was reported previously, possibly because only a low m.o.i. (0.1 p.f.u. per cell) was employed.

The use of UV- or heat-treated virus (55 °C) enabled us to demonstrate that DC activation did not require virus gene expression, suggesting that virus binding or uptake is sufficient. Similar conclusions have been reached for activation of human DCs by measles virus (Bieback et al., 2002) or activation of murine DCs by mouse mammary tumor virus (Burzyn et al., 2004) or human adenovirus 5 (Hirschowitz et al., 2000). Intriguingly, we found that VACV-Cop or VACV-Lis subjected to UV light or heat treatment at 55 °C activated DCs as efficiently as MVA. Furthermore, VACV-Cop-infected cells, which failed to activate DCs, acquired this capacity if they had previously been fixed with paraformaldehyde. These data indicate that standard VACV strains are capable of inducing direct or indirect DC activation if virus gene expression is prevented, and suggest that standard VACV strains, but not MVA, express one or several genes that prevent activation. It should be noted that poxviruses belonging to the genus Avipoxvirus have been shown to activate human DCs (Ignatius et al., 2000) and murine DCs (Brown et al., 2000), a finding that may be correlated with the lack of virus functions that interfere with the activation pathway in mammalian cells. However, it is not yet known whether the avipoxviruses activate DCs in the absence of virus gene expression.

What might be the mechanism that enables MVA to activate DCs? MVA infection was found to induce NF-κB activation in human embryonic kidney cells, while a replication-competent VACV strain or cowpox virus inhibited TNF-α-induced NF-κB activation (Oie & Pickup, 2001). Relevant to these findings, it has been reported that NF-κB activation was essential for DC activation and antigen presentation, as both are prevented by overexpression of the natural NF-κB inhibitor, IκBz (Yoshimura et al., 2001). Taken together, these data suggest that MVA could activate DCs via an NF-κB-dependent mechanism. Interestingly, the VACV A52R ORF, which is deleted from the MVA genome (Antoine et al., 1998), has been shown to belong to the IL1/Toll-like receptor family and to interfere with the activation of NF-κB by a number of effectors (Bowie et al., 2000; Harte et al., 2003). However, our data show that deletion of this gene from the MVA genome cannot be the only critical factor that allows DC activation, as the recombinant VACV-Cop encoding GFP used in this work was also deleted in A52R yet was unable to activate DCs.

Although DCs infected with MVA or VACV-Cop were able to stimulate the proliferation of autologous T lymphocytes equally well, when the cells were derived from individuals vaccinated against smallpox (not shown) we could not detect a significant boost in the number of IFN-γ-secreting lymphocytes using infected DCs as stimulators. On the other hand, we report here a very effective induction of IFN-γ-secreting lymphocytes by a mixture of MVA-infected HeLa cells and DCs, which was much higher than if VACV-Cop-infected HeLa cells were employed. These results highlight the efficiency of the cross-presentation pathway for the stimulation of IFN-γ-producing T cells, and presumably cytotoxic T cells, compared with the direct
presentation pathway. Other studies have documented the ability of DCs to cross-present antigens from VACV-infected cells (Larsson et al., 2001; Ramirez & Sigal, 2002). These studies employed cells that were non-permissive for VACV replication (monocytes, macrophages, DCs), a situation that may closely mimic MVA infection of HeLa cells. Clearly the stimulation of T lymphocytes with a mixture of VACV-Cop-infected HeLa cells and DCs is very poor, and this may be explained by the continual production of virus in HeLa cells which will then inhibit DC activity. We can speculate from these findings that, under circumstances of in vivo infection with replication-competent VACV, non-permissive cells are the most important for transferring antigens to DCs, in much the same way as MVA-infected HeLa cells are able to do so in vitro. Finally, our data also help explain why a non-replicating virus such as MVA is an efficient vaccine strain provided high input titres are used.

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REFERENCES


