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Infection of human dendritic cells with recombinant vaccinia virus MVA reveals general persistence of viral early transcription but distinct maturation-dependent cytopathogenicity

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Abstract

Vector-infected dendritic cells (DC) are evaluated for antigen delivery in experimental therapy of cancer and infectious diseases. Here, we investigated infections of immature or mature, monocyte-derived human DC with recombinant vaccinia virus MVA producing human Her-2/neu, a candidate tumor-associated antigen. Assessment of the molecular virus life cycle in infected DC revealed a general arrest at the level of viral early gene expression. When monitoring the phenotype of MVA-infected DC, including expression of cell surface markers, we found immature cells readily undergoing apoptosis. Nevertheless, we detected significant populations of viable DC being characterized by high level Her-2/neu expression and unimpaired display of costimulatory molecules. While infected viable immature DC failed to undergo maturation despite cytokine treatment, both DC populations efficiently presented MVA-produced target antigen. These findings allow to better define the requirements for MVA-mediated antigen delivery to DC and help to derive optimized vectors for this advanced therapy option.

Keywords: Poxvirus; Vector; Gene expression; Antigen presentation; Tumor antigen; Immunotherapy

Introduction

Several poxviral vector systems are under clinical evaluation for vaccine development against infectious diseases and cancer. One of these vectors is based on the highly attenuated modified vaccinia virus Ankara (MVA) that was originally generated in chicken embryo fibroblast cultures and became growth-restricted in many mammalian cells including those of human origin (Carroll et al., 1997; Drexler et al., 1998; Meyer et al., 1991). Importantly, upon infection of non-permissive human cell lines (Ludwig et al., 2005; Sutter and Moss, 1992), MVA can still initiate the full cascade of vaccinia viral gene expression allowing for recombinant genes to be placed under transcriptional control of vaccinia virus-specific late promoters and for abundant synthesis of heterologous antigens. Indeed, recombinant MVA vaccines have been shown to stimulate immune responses against a large variety of different antigens with protective efficacy against viral infections, bacterial as well as parasitic diseases and tumors (Carroll et al., 1997; Drexler et al., 2004; McConkey et al., 2003; Sutter et al.,

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1994), and several prototype MVA vector vaccines have already been tested in phase I or phase I/II clinical trials (Cebere et al., 2006; Corona Gutierrez et al., 2004; Cosma et al., 2003; Di Nicola et al., 2003; Di Nicola et al., 2004; Harrer et al., 2005; Liu et al., 2004; McConkey et al., 2003; McShane et al., 2004; Moorthy et al., 2004; Moorthy et al., 2003; Mwau et al., 2004; Rochlitz et al., 2003; Smith et al., 2005). One interesting approach is based on the combined use of dendritic cells (DC) and recombinant MVA for delivery of vaccine antigens. The idea behind joint application of viral vector vaccines and professional antigen-presenting cells is to induce more potent target antigen-specific immune responses in the context of DC-mediated immune signaling. This strategy could be advantageous to enhance the immunogenicity of tumor-associated antigens (TAA) being affected by immunological tolerance. In a recent clinical phase I trial protocol, autologous CD34+ cell-derived DC were ex vivo infected with an MVA vector virus expressing the human melanoma-associated antigen tyrosinase and transferred to melanoma patients (Di Nicola et al., 2003; Drexler et al., 1999). This therapeutic approach resulted in a significant activation of tyrosinase-specific CD8+T cells, suggesting efficient antigen presentation upon MVA infection of DC (Di Nicola et al., 2004). In contrast, more conventional vaccination of melanoma patients with the same MVA vector virus failed to induce detectable T-cell responses directed against the human self antigen tyrosinase (Meyer et al., 2005). To better understand the potential advantage of TAA delivery by MVA-infected DC, we analyzed the effect of MVA infection in human immature (iDC) or mature (mDC) antigen-presenting cells. To assess transcriptional, translational and post-translational levels of target gene expression, we used recombinant MVA delivering human Her-2/neu, a 185-kDa cell surface protein, to be easily monitored by antibody surface staining and FACS analysis. Her-2/neu is also a potentially useful target antigen for tumor immunotherapy because it was found to be overexpressed in human malignant cells and to induce specific cellular and humoral responses in breast cancer patients (Disis et al., 1994; Disis et al., 1997; Park et al., 2005). Upon infection of human DC, we found persisting MVA early gene expression to result in substantial production of Her-2/neu antigen. However, based on the expression of relevant cell surface markers, infected iDC were unable to undergo maturation even in the presence of maturation inducing cytokines. In contrast, we found fully preserved cell surface expression of costimulatory markers on infected, but importantly, non-apoptotic mDC. Both infected iDC and mDC were able to translate, process and present recombinant antigen in an MHC-restricted manner providing a rational basis to explain the in vivo immunogenecity of DC-delivered MVA vaccines. Finally, our work with MVA suggested a surprising difference to previous infection experiments with standard replication competent vaccinia virus resulting in downregulation of costimulatory markers as well as MHC class I and class II molecules on human DC.

Results

Her-2/neu is produced by recombinant MVA and transported to the cell surface

The recombinant virus MVA-Her-2/neu allowing expression of the human *her-2/neu* gene sequence under transcriptional control of the VV early/late promoter P7.5 was generated by using the host range gene *K1L* as a transient selectable marker (Fig. 1A). To confirm the synthesis of recombinant Her-2/neu protein upon infection, we analyzed total cell extracts from

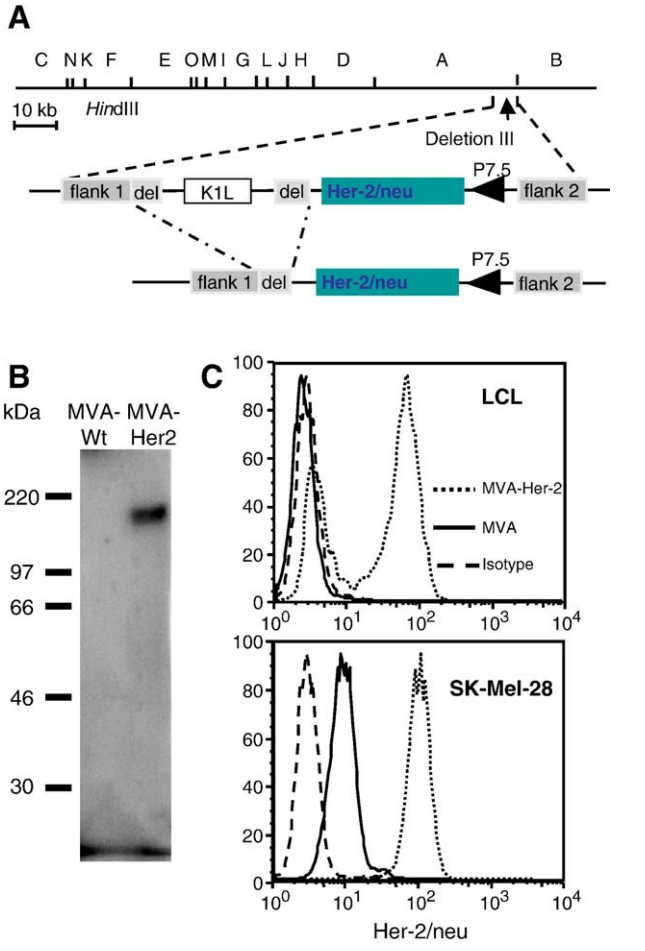

Fig. 1. Construction of recombinant virus MVA-Her-2/neu and expression of Her-2/neu target protein. (A) A schematic map of the MVA genome for the restriction endonuclease *Hin*dIII is shown on top. The coding gene sequence of *her-2/neu* was placed under the transcriptional control of VV early/late promoter P7.5 and inserted by homologous recombination at the site of deletion III within the MVA genome. Flank 1 and flank 2 refer to MVA-DNA sequences that are adjacent to the site of deletion III and serve to target the recombination into the MVA genome. Del indicates the positions of 283-bp repetitive MVA-DNA sequences corresponding to the right end of flank 1 and allowing the removal of the K1L selectable marker from the genome of final recombinant viruses by homologous recombination. (A) Representation of the genomic structure of recombinant MVA-Her-2/neu is shown at the bottom. (B) Western blot analysis of HeLa cell lysates prepared at 12 h post-infection with MVA or MVA-Her-2/neu. The numbers to the left refer to the molecular weights of marker proteins (in kilodalton) used during electrophoresis. (C) FACS analysis of Her-2/neu cell surface expression in human LCL and a Her-2/neu-positive human tumor cell line (SK-MEL-28) after MVA-Her-2/neu infection.

human HeLa cells by Western blot (Fig. 1B). Among the proteins harvested at 12 h after infection, the anti-Her-2/neu antibody Ab-2 revealed a single protein band migrating at the molecular weight of 185 kDa and precisely corresponding to the expected size of human Her-2/neu. Since Her-2/neu is a transmembrane protein usually located on the cell surface, we expected it to be detectable on MVA-Her-2/neu-infected cells by FACS analysis using antibody staining. Indeed, as shown in Fig. 1C, after infection of human LCL with MVA-Her-2/neu, we found more than 50% of the cell population carrying the target molecule while cells infected with non-recombinant MVA as control remained negative upon staining. Additionally, infection of human melanoma cells SK-Mel-28 which naturally produce Her-2/neu resulted in obvious enhancement of Her-2/neu cell surface expression (Fig. 1C). Therefore, MVA infection by itself seemed not to impair the surface presentation of endogenous Her-2/neu, allowing for efficient production and transport of additional target protein.

MVA infection of human DC is restricted to the production of viral early proteins

While recent data (Di Nicola et al., 2004) suggest that MVA-infected human DC can be successfully used for recombinant antigen delivery, there is still limited information on the MVA molecular life cycle in these antigen-presenting cells. Moreover, immune evasion rather than immune stimulation could be expected from the results obtained with conventional VV infection in human DC (Engelmayer et al., 1999). To monitor for viral proteins being produced in infected DC, we performed an [S^{35}]methionine metabolic labeling of polypeptides in iDC and mDC at various times after infection with MVA (Figs. 2A, B) or MVA-Her-2/neu (Fig. 2C). To allow for comparison with a well-characterized pattern of viral protein synthesis, infections of human HeLa cells served as positive control (Figs. 2A, B). Compared to the labeling of cellular proteins in mock-infected cells (U), MVA infection resulted in the typical shut-off of host cell protein synthesis in both DC and HeLa cells (Fig. 2A). At 2 h of infection, the pulse labeling revealed the characteristic pattern of early viral polypeptides being made in iDC and mDC which also resembled the pattern found for typical early viral proteins made in HeLa cells (marked by white arrow heads in Fig. 2A). At later time points of infection, the onset of abundant late viral protein synthesis became very obvious in MVA-infected HeLa cells illustrated by multiple new polypeptides of varying molecular weight (typical late viral proteins are indicated by black arrow heads in Fig. 2A). In contrast, we failed to detect these late viral proteins in MVA-infected DC, suggesting a failure of viral late gene expression in these professional antigen-presenting cells. This notion was corroborated by our finding of close to identical viral protein patterns in MVA-infected DC in the presence of cytosine arabinoside (AraC) serving as potent inhibitor of vaccinia virus DNA replication and, in consequence, as inhibitor of viral intermediate and late gene expression (Fig. 2B). In addition, the synthesis of recombinant Her-2/neu in MVA-Her-2/neu-infected DC also suggested a restriction to viral early gene

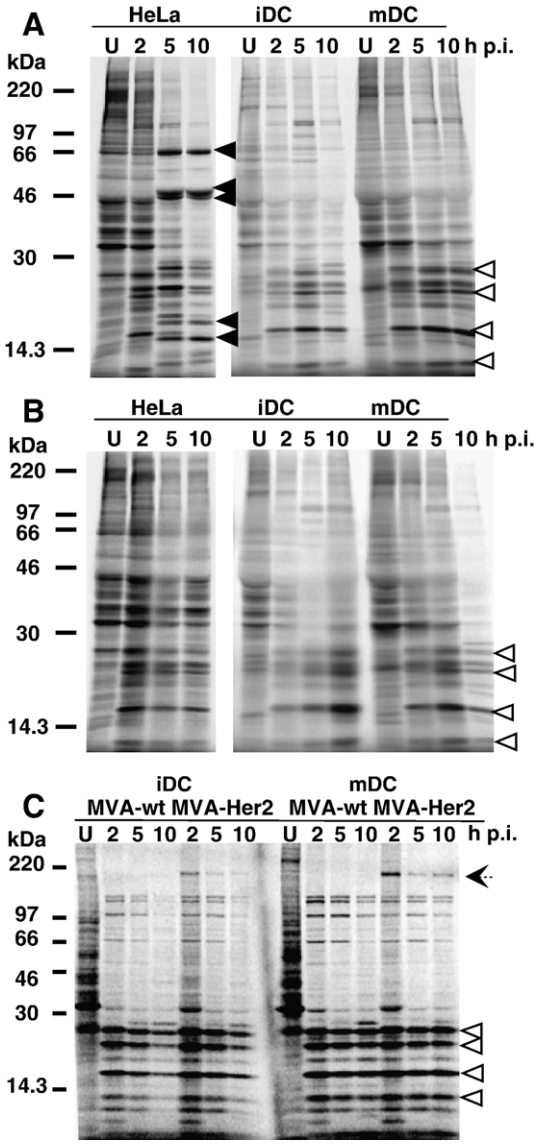

Fig. 2. Polypeptide synthesis in HeLa cells and DC infected with MVA or MVA-Her-2/neu. HeLa cells and DC were infected at MOI 20 in the absence (A) or presence (B) of AraC, polypeptides were metabolically labeled with [S^{35}] methionine and cell extracts were prepared at indicated times post-infection (h p.i.). Proteins were separated by SDS-PAGE and analyzed by autoradiography. U refers to lanes with extracts from uninfected cells. (A) MVA produces typical late viral proteins (indicated by black arrowheads) in HeLa cells, but not in DC. (B) In the presence of AraC, no late viral proteins are produced (white arrowheads indicate typical early proteins). (C) Viral early proteins are made in mature and immature DC infected with MVA or MVA-Her-2/neu, respectively. An arrow indicates the expected molecular weight of recombinant Her-2/neu protein.

expression (Fig. 2C). Already at 2 h post-infection, we detected a protein being exclusively made in MVA-Her-2/neu, but not in MVA-infected DC, signifying it as the recombinant Her-2/neu (marked by an arrow in Fig. 2C). The labeling of this polypeptide was clearly most intense at early times of infection without indication of increased production late in infection despite the recombinant gene being transcriptionally controlled by the robust vaccinia virus early/late promoter P7.5. Thus, we speculated that upon infection of both DC populations the MVA

life cycle was arrested at the early phase with some level of viral early gene expression being possibly maintained through later times of infection.

Efficient Her-2/neu surface expression in DC after infection with MVA-Her-2/neu

Unlike DC, the human cell lines SK-Mel-28 and LCL allow for viral late gene expression (unpublished observations), and we had confirmed high level synthesis of recombinant Her-2/neu in these cells upon MVA vector infection (Fig. 1). In order to obtain more detailed information on the efficacy of MVA-driven target antigen production in DC, we performed a series of infection experiments applying various doses of vector virus MVA-Her-2/neu (ranging from MOI 1 to 100) to iDC or mDC. Subsequently, Her-2/neu surface expression was monitored by FACS (Fig. 3). At 12 h after infection, the percentage of Her-2/neu-positive iDC increased from about 50% using MOI 1 up to >90% using MOI 10. After infection of mDC, the number of Her-2/neu-positive cells continuously augmented from about 20% at MOI 1 up to 99% at the highest MOI. To obtain similar levels of Her-2/neu surface expression, MVA infection of mDC appeared to require about two-fold more virus particles as compared to infection of iDC. On the other hand, as judged by the uniformity of the Her-2/neu surface expression, it looked as if iDC were more fragile than mDC when exposed to unusual high infectious doses of MVA (MOI 100). Remarkably, the overall amount of Her-2/neu being detectable by FACS on infected DC seemed comparable to levels measured on human LCL which can promote late viral gene expression.

Human-monocyte-derived DC do not facilitate intermediate and late viral gene expression upon MVA infection

Next, we determined whether the lack of MVA late protein synthesis in monocyte-derived DC is associated with a failure of viral late mRNA synthesis. For this purpose, we used a recently established VV-specific RNase protection assay (RPA) as a highly sensitive method to simultaneously monitor transcription of all three classes of VV transcripts (Ludwig et al., 2005). The assay includes three different RNA probes targeting viral early (ORF 005R), intermediate (ORF 078R) and late (ORF 047R) MVA transcripts and one probe to assess cellular transcription (GAPDH). Despite being unable to productively replicate in HeLa cells, MVA can amplify its genomic DNA and accomplish synthesis of early, intermediate and late viral gene products (Drexler et al., 1998; Ludwig et al., 2005; Sutter and Moss, 1992). Thus, the analysis of RNA isolated from MVA-infected HeLa cells revealed the typical cascade-like pattern of vaccinia virus transcription (Fig. 4A). First, early gene transcription was detected at about 1 h post-infection followed by intermediate gene transcription with highest level at 4 h after infection. Finally, abundant late gene transcription was prominent at later times of infection when expression levels of viral early and intermediate genes had ceased again, coinciding with the shut-down of cellular transcription. After MVA infection of both iDC and mDC, however, we found unusual patterns of viral transcripts (Fig. 4B). To our surprise, high level of viral early gene expression appeared to continue until at least 12 h post-infection together with intermediate and late gene transcripts being detectable from 5 h and 10 h post-infection on,

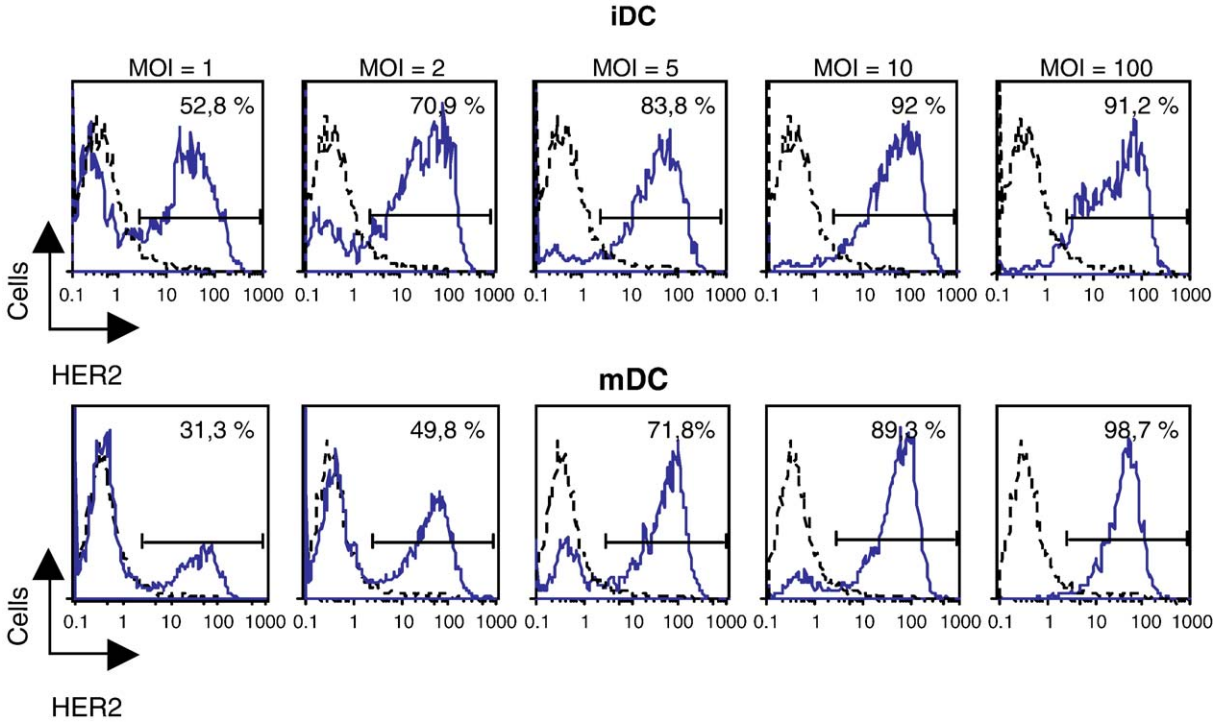

Fig. 3. Cell surface expression of Her-2/neu in human DC. iDC (upper panel) or mDC (lower panel) were infected at different MOI with MVA-Her-2/neu for 12 h. Cells were then recovered and analyzed by flow cytometry. The histograms display the number of cells counted with various intensities of fluorescence. The broken line represents the isotype control.

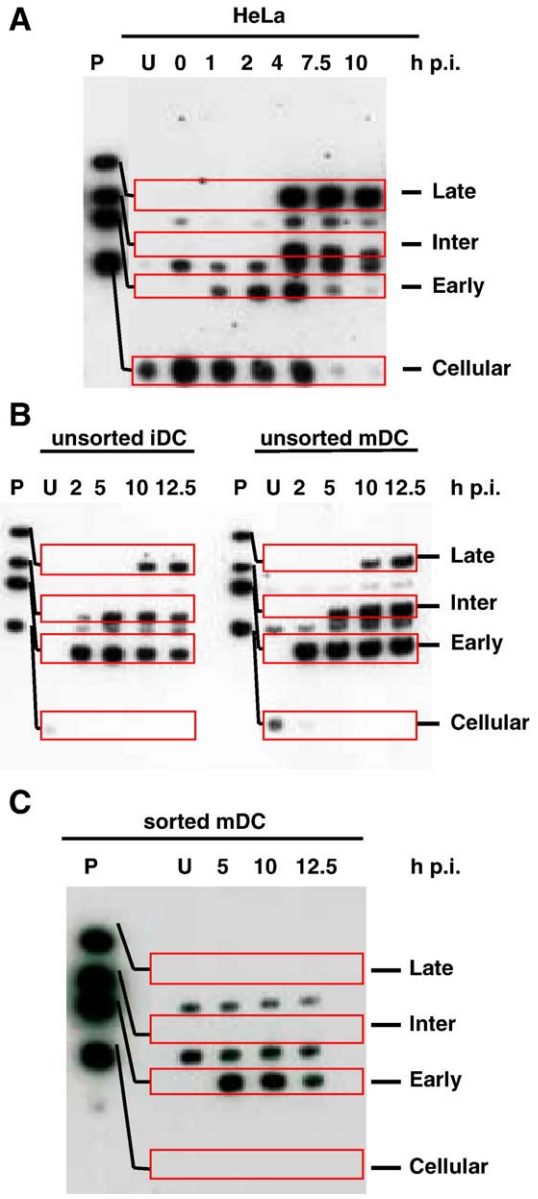

Fig. 4. Analysis of viral transcription in human DC. RNase protection assays were performed on total RNA isolated from MVA-Her-2/neu-infected DC (MOI 20) at indicated times post-infection (h p.i.) using biotinylated probes (P) designed to monitor typical viral early (Early), intermediate (Inter) and late (Late) mRNAs as well as cellular GAPDH message (Cellular). Bands that shift to the expected size (framed bands) are representative for the corresponding transcripts. Left-over bands are due to incomplete RNase digest of GAPDH and ORF 005R probe. P indicates full-length probes, U samples from uninfected controls. (A) In MVA-infected HeLa cells, early, intermediate and late viral transcripts are synthesized in a cascade-like pattern. Cessation of cellular transcription is indicated by the disappearance of protected cellular probes at 7.5 h post-infection. (B) Ongoing viral early transcription in iDC and mDC with delayed detection of intermediate and late viral RNA. The weak signal of the uninfected control in the cellular box was confirmed by RT-PCR (not shown). (C) Analysis of RNA from sorted and infected mDC (CD83+, CD19−) reveals early viral gene expression only.

respectively. In general, a rapid shut-down of cellular transcription occurred in both types of infected DC. As active transcription of viral early and late genes is unlikely to simultaneously take place in an infected cell, we considered heterogeneous cell populations in DC preparations being responsible for the detection of apparently mixed transcription patterns. We suspected a possible contamination of DC preparations with, e.g. B cells, because this cell type is also contained in human PBMC and is already known to allow for MVA late gene expression (Drexler et al., 1998). Indeed, FACS analysis showed about 5–10% B cells (gated on CD19) being present in all DC preparations used in this study (data not shown). Indeed, FACS analysis showed about 5–10% B cells being present in all DC preparations used in this study (data not shown). To clearly reveal the transcriptional phenotype of MVA infection in DC, we depleted B cells by sorting mDC for CD83-positive and CD19-negative cells using a MoFlo cytometer. As shown in Fig. 4C, the infection of sorted DC populations (>95% CD83-positive cells) resulted in high level synthesis of early viral RNA whereas intermediate or late viral gene products were not detected. Thus, our data clearly demonstrate that MVA gene expression in DC is exclusively restricted to transcription of viral early genes.

Viability of DC following MVA-Her-2/neu infection

Since cell viability may be a critical factor, e.g. for the efficacy of immunotherapeutic approaches based on adoptive transfer of virus-infected cells, we wished to analyze the onset of cell death upon MVA infection of iDC or mDC. We monitored for signs of apoptosis or necrosis in cells that had been differentially stained with PI (necrotic cells) and with Annexin V (apoptotic cells) at 12, 24, 36, 48 or 60 h post-infection with MVA-Her-2/neu (Koopman et al., 1994; Vermes et al., 1995). These cells were analyzed by FACS using different gating strategies based on cell morphology determined by forward (FS) and sideward scatter (SS) intensity (Figs. 5A–C). In general, we found two major distinct populations within infected DC preparations, either (i) living cells being negative for PI and Annexin V or (ii) apoptotic/necrotic dead cells positively stained for PI and Annexin (Fig. 5A). Interestingly, these two populations strictly correlated to morphological parameters based on cellular size and granularity. Analysis of large iDC or mDC only detected cells remaining negative upon staining with PI and Annexin V and therefore identified the living cell population (Fig. 5B). The gating on morphologically smaller cells determined the population of necrotic or apoptotic cells which positively stained with PI and Annexin V (Fig. 5C). Fig. 5 shows the analysis of MVA and mock-infected iDC and mDC 36 h post-infection and is representative for the kinetic analysis, summarized in Table 1. Briefly, at 36 h after infection with MVA-Her-2/neu MOI 10, we found more than 30% of iDC and about 75% of the mDC population alive. However, 60 h post-infection, we detected only 8% viable iDC, while living mDC still accounted for more than 50% of the total cell population. These data suggest that mDC compared to iDC could tolerate MVA infection for a longer time period. Nonetheless, more than half of both DC populations (iDC 60%, mDC 83%) remained alive for at least 24 h using a dose of recombinant MVA infecting more than 90% of all cells.

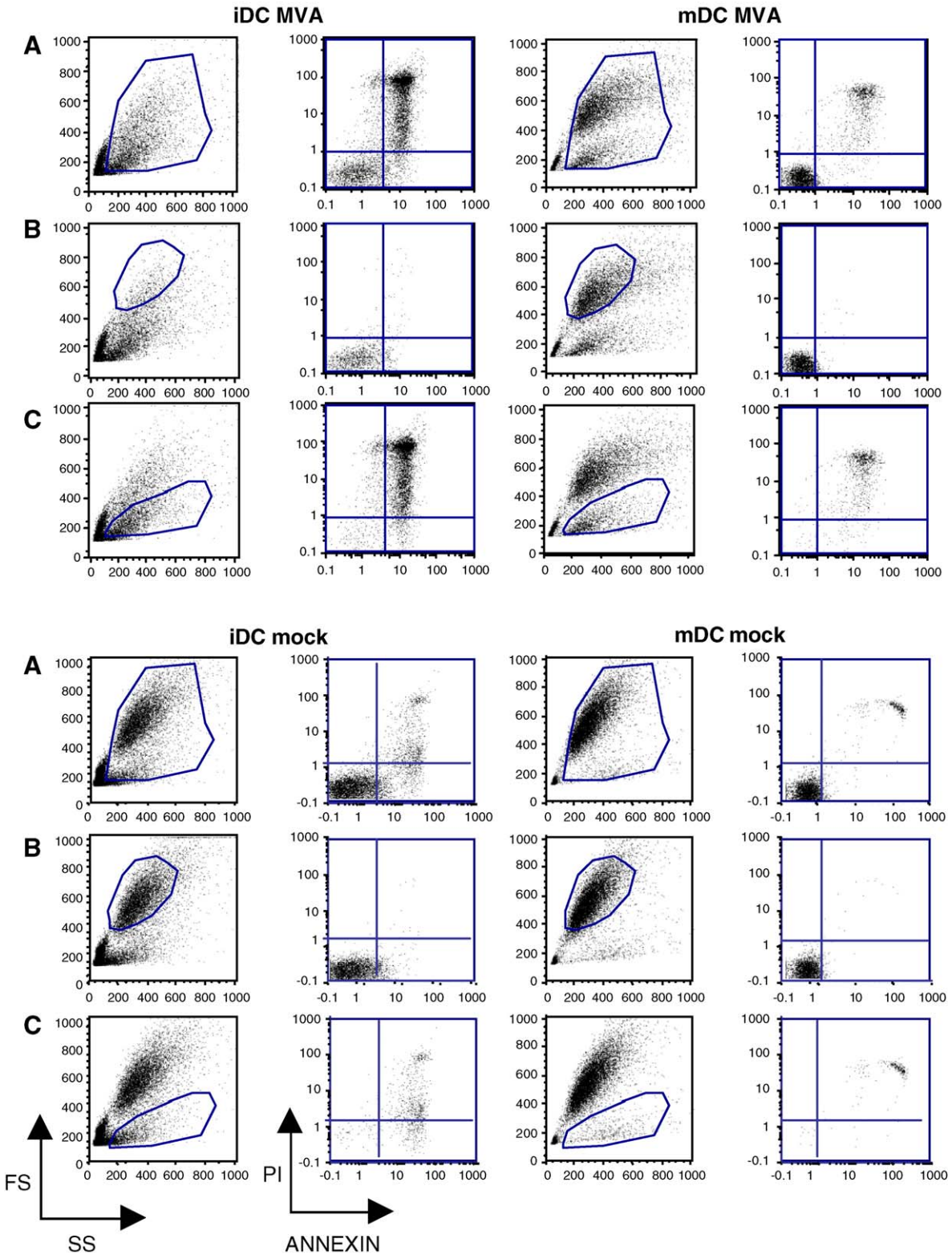

Fig. 5. MVA-Her-2/neu-infected iDC are more susceptible to apoptosis than mDC. Panels A–C show a representative FACS analysis of infected DC using three different gating strategies based on cell morphology determined by forward (FS) and sideward scatter (SS) intensity at 36 h after infection. To obtain comparable levels of infection, MVA-Her-2/neu was added to mDC at an MOI of 10 and to iDC at an MOI of 5. Viability was monitored by staining dead cells using PI and Annexin V. (A) The gating comprises of all cells of various sizes contained in the DC preparations including viable and apoptotic DC. (B) Viable DC. (C) Double positive, apoptotic/necrotic DC.

Table 1
Apoptosis in MVA-Her-2/neu-infected DC MOI 10 (% poptotic cells, Annexin V positive)

h p.i.	12 h	24 h	36 h	48 h	60 h
mDC mock	15	12	4	5	10
mDC MVA	11	17	25	39	44
iDC mock	10	10	12	10	11
iDC MVA	19	40	68	85	92

This should be a sufficient time to allow for ample production and presentation of heterologous antigens.

MVA-Her-2/neu infection of iDC leads to an inhibition of maturation

The induction of apoptosis in DC by MVA infection in combination with the profound shut-off of host mRNA (Fig. 4) and protein synthesis (Fig. 3) leads to the question if an infected iDC is still able to mature. To answer that question, we compared the maturation capacity of infected iDC (MOI 5) with uninfected iDC. Fig. 6 shows that infected, viable iDC are still able to upregulate CD80, CD83 and CD86 24 h after treatment with maturation inducing cytokines compared to uninfected non-cytokine-treated iDC. However, the upregulation of these molecules is markedly reduced compared to uninfected and cytokine-treated iDC. CD71, the transferrin receptor, was used as a non-immunological control marker. Since the functionality of DC as professional antigen-presenting cells is believed to be highly dependent on expression of maturation markers such as costimulatory molecules CD80 and CD86, and MHC class II molecules, the impaired maturation of infected iDC could be interpreted as a DC-specific immune evasion mechanism. However, it seems more likely to reflect a consequence of the general host protein shut-off typically found in VV-infected cells.

MVA-Her-2/neu infection of mDC allows for unimpaired surface expression of costimulatory molecules and other maturation markers

Next, we wished to investigate the influence of MVA infection on the maturation status of mDC by determining the expression of these molecules on the surface of infected cells. Mature DC can be distinguished from iDC by high expression levels of MHC class II, costimulatory molecules and the DC-restricted marker CD83. These surface proteins are essential for mature DC to develop their full capacity as professional antigen-presenting cells. To investigate the expression level of maturation markers in infected DC, we performed a double staining for Her-2/neu and the respective marker molecules to ensure the analysis of exclusively infected cells. To scrutinize changes in kinetics and levels of marker expression after MVA-Her-2/neu infection, mDC were infected at MOI 5, 10 and 100 and then analyzed by FACS at 12, 36 or 60 h after infection. Fig. 7 shows a representative analysis at MOI 10. We simultaneously used the two different gating strategies for discrimination of alive or dead MVA-Her-2/neu-infected cells based on i) morphological discrimination in the FS/SS diagram (see also Fig. 5) as well as ii) analysis of Her-2/neu in the corresponding histogram (Fig. 7A). DC populations were compared for expression of the activation markers CD80, CD86, CD83, HLA-DR and as control marker the transferrin receptor CD71 (Fig. 7B). Importantly, in living cells, we detected a slight reduction of CD86 on MVA-infected DC as compared to mock-infected DC early in infection, while at later times CD86 was found to be expressed at comparable levels on both populations. In contrast, however we observed a gradual reduction of CD86 in apoptotic Her-2/neu-positive DC that became particularly prominent at 60 h after infection. CD83- and HLA-DR expression in MVA-infected and mock-infected DC remained comparable at all times analyzed. In contrast, CD80 was expressed at slightly lower levels on MVA-infected cells compared to mock-infected mDC. CD71 expression seemed even slightly upregulated in infected DC which might be due to a cellular activation mediated by MVA infection.

Generally, apart from CD83, the surface expression of all maturation markers tested was reduced in the apoptotic/necrotic DC population, with some variation depending on the time of analysis. Importantly, however, monitoring exclusively living and MVA-Her-2/neu-infected mDC, we detected no down-regulation of costimulatory molecules or other maturation markers.

MVA-vector-infected iDC and mDC are specifically lysed by a human CTL line

Next, we tested MVA-vector-infected iDC or mDC for their capacity to endogenously process and present peptides from recombinant antigen in an MHC-class-I-restricted manner. After

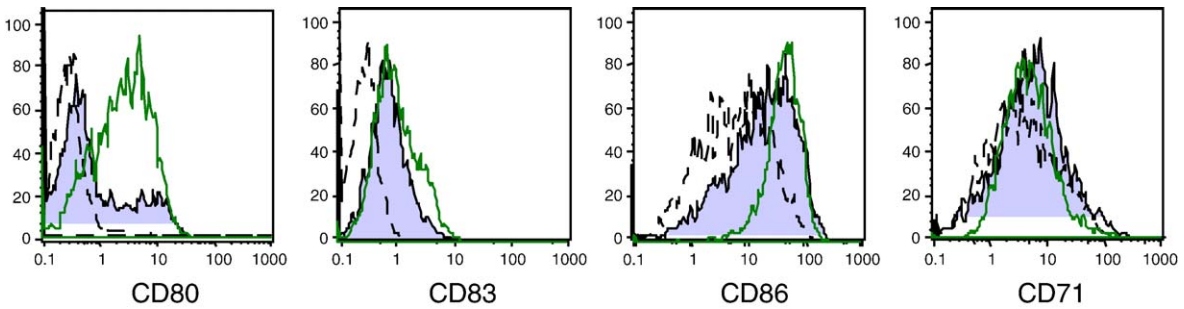

Fig. 6. MVA-Her-2/neu infection inhibits the maturation of immature DC. iDC have been mock-infected and left untreated (dotted line), mock-infected and treated with maturation inducing cytokines for 24 h (bold line), or MVA-Her-2/neu-infected (MOI 5) and treated with cytokines for 24 h (shaded).

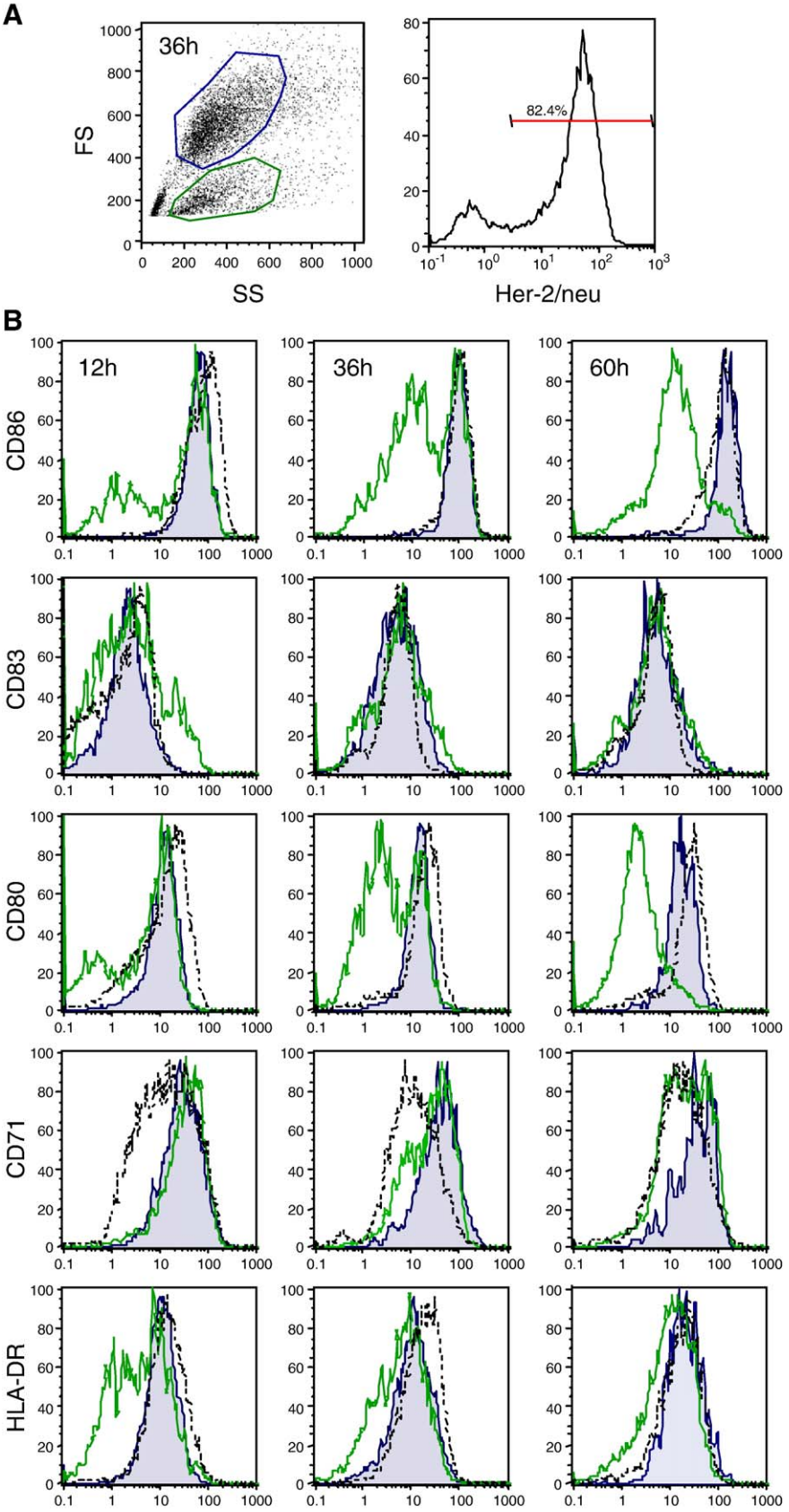

Fig. 7. MVA-Her-2/neu-infected mDC express Her-2/neu and maturation markers at high levels. (A) Depiction of the gating strategy to analyze live or dead DC expressing Her-2/neu after infection. (B) Staining of cell surface markers on MVA-Her-2/neu-infected mDC at 12, 36 or 60 h post-infection (with MOI 10). Viable cells from the upper gate are represented by filled histograms. Apoptotic cells from the lower gate correspond to light lines. Mock-infected DC are shown by dotted lines.

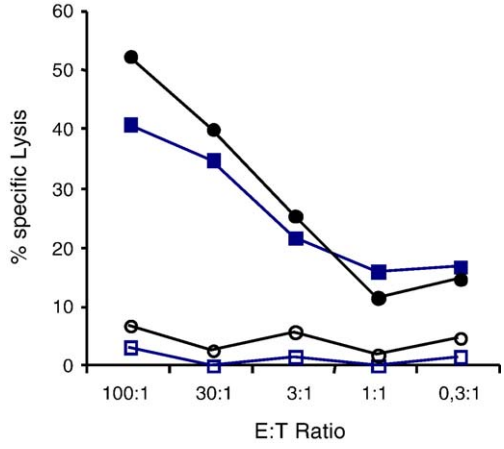

Fig. 8. Recognition of MVA-hTyr-infected DC by the tyrosinase 369–377 peptide-specific human CD8+ CTL line BST5. Specific ^{51}Cr release is shown at the indicated E:T ratios. iDC (circles) or mDC (squares) were either infected with MVA-hTyr (closed symbols) or mock-infected (open symbols).

infection with recombinant MVA expressing the human TAA tyrosinase (MVA-hTyr) (Drexler et al., 1999), iDC and mDC were specifically lysed by the human CD8+ T-cell line BST5 recognizing the human tyrosinase peptide 369–377 presented by HLA-A*0201 (Fleischer et al., 2004) (Fig. 8). Comparing the lytic activities at various effector to target ratios, we found very similar CTL-mediated recognition of MVA-hTyr-infected iDC and mDC. These data clearly suggested that MVA-vector-infected mature and immature human DC can effectively translate, process and present MVA produced proteins to activate antigen-specific CTL.

Discussion

Previous work characterizing infection of human DC with replication-competent conventional VV had suggested viral interference with DC function (Drillien et al., 2000; Engelmayer et al., 1999; Jenne et al., 2000). Suspecting a particular benefit of antigen delivery by replication-deficient vaccinia virus MVA, we extended these investigations and analyzed monocyte-derived immature and mature DC after MVA infection. Recent clinical evaluations suggest the transfer of antigen-loaded autologous monocyte or CD34+ progenitor-cell-derived DC as a promising approach to elicit a specific immune response against HIV or human melanoma (Di Nicola et al., 2004; Lu et al., 2004). In particular, the success of a protocol using CD34+ cell-derived DC infected with recombinant MVA expressing human tyrosinase as a tumor antigen had spurred our interest to study the interactions of DC and MVA vectors in more detail. In this study, we determined the effect of MVA infection on the viability, maturation and antigen presentation capacity of human DC. We clearly demonstrate that MVA gene expression in both mature and immature monocyte-derived DC was restricted to viral early gene transcription. This finding is supported by other work reporting the relevance of early gene expression when using replication-competent VV or avipox-virus vectors for infection of human macrophages and human or murine DC (Broder et al., 1994; Bronte et al., 1997; Brown et al., 1999; Subklewe et al., 1999). In fact, this restriction has a major consequence in our understanding of the induction of an immune response against MVA or the delivered recombinant antigen since direct presentation of late gene products seems impossible in infected DC. Indeed, using a highly sensitive detection system based on RNase protection, we also revealed minor amounts of viral late transcripts in MVA-infected DC populations. Yet, our experiments suggested that this late gene expression is due to infected B cells usually being present at low numbers in human DC preparations (Drexler et al., 1998). Nevertheless, we cannot formally rule out the possibility that subset(s) of human DC due to a different development stage or distinct origin are permissive for viral late gene expression as reported for MVA infection of unpurified CD34+ progenitor produced DC (Di Nicola et al., 1998). In general, MVA infection of DC was more efficient with iDC preparations requiring less MOI for maximum infection as compared to mDC. This finding might be attributed to the high phagocytic activity typically found in iDC (Banchereau and Steinman, 1998; Caux et al., 1997) which possibly supports MVA entry as suggested for infections with conventional VV (Drillien et al., 2000). Despite the restriction to early viral gene expression, infection of both mDC and iDC resulted in production of surprisingly high amounts of recombinant Her-2/neu. In this respect, the lower viral early promoter activity might be compensated by a prolonged maintenance of early transcription of the target gene in MVA-infected DC. In fact, when focusing our analysis on living cells, we detected cells strongly expressing Her-2/neu even at 60 h after MVA-Her-2/neu infection. Infected mDC maintained the costimulatory molecules CD80, CD86 as well as the maturation marker CD83 at high levels. We did not observe a significant down- or upregulation of cell surface markers in infected living cells. However, we found a slight reduction of CD80 expression which is in line with recent findings characterizing conventional VV infections in DC (Drillien et al., 2000; Nagorsen et al., 2003; Prabakaran et al., 2002). Data from other experiments demonstrating severe changes of surface marker expression in VV-infected DC and differing from our results may be explained by the use of the more cytopathogenic VV strain Western Reserve, possibly resulting in high levels of cell death among infected cells (Engelmayer et al., 1999; Jenne et al., 2000; Nagorsen et al., 2003). This might be of particular relevance since in these studies apoptotic cells have not been unambiguously excluded from analysis. Interestingly, Drillien et al. reported a moderate maturation of MVA-infected human DC without further maturation stimulus and independently of viral gene expression as shown by UV inactivation (Drillien et al., 2004). We extended that finding by additionally treating MVA-infected DC with maturating cytokines. Our data revealed that MVA-infected iDC do not fully mature compared to mock-infected iDC, despite initial upregulation of CD80, CD83 and CD86. A comparable, impaired maturation of iDC upon infection with replication-competent VV has been interpreted as a specific mechanism of immune evasion (Engelmayer et al., 1999; Jenne et al., 2000; Nagorsen et al., 2003). However, in the light of the severe shut-down of cellular protein biosynthesis

(Figs. 2 and 3), which is a general consequence of VV infection in various cell types (Moss and Salzman, 1968; Esteban and Metz, 1973), we interpret this finding to apply to all newly synthesized proteins, regardless of their immunological relevance.

We have shown that human iDC and mDC infected with recombinant MVA can serve as functional antigen-presenting cells to stimulate target antigen-specific and MHC-class I-restricted T-cell responses in vitro (Fig. 8) (Di Nicola et al., 1998; Drexler et al., 1999). For replication-competent VV, a number of other studies confirm similar functional activity of infected DC in various in vitro and in vivo models (Bonini et al., 2001; Norbury et al., 2002; Prabakaran et al., 2002; Yang et al., 2000; Yee et al., 1996). However, we still have very limited knowledge whether vector virus-infected DC act directly as the key inducers of a target antigen-specific cellular immune response after in vivo DC transfer to patients. At least with regard to antigens driven by late viral promoters, direct presentation by dendritic cells is very unlikely to occur (Fig. 4C). In vivo, T cells can also be primed by means of antigen cross-presentation (Cresswell, 2004; Norbury et al., 2004; Shen et al., 2002), and DC are known for their particular capacity to cross-present viral antigens and apoptotic cells (Ackerman et al., 2003; Houde et al., 2003; Larsson et al., 2004; Sigal et al., 1999). Therefore, it is likely that both direct priming and cross-priming contribute to the induction of antigen-specific CD8+ CTL in vivo. MVA-infected DC can be specifically recognized by T cells (Fig. 8) in vitro. Whether this interaction leads to direct priming in vivo still remains unclear. Detailed in vivo studies will be required to finally understand the specific advantage of transferred MVA-infected DC compared to MVA alone. One interesting aspect will be the evaluation and comparison of vector and target antigen-specific immune responses under both conditions. In that respect, MVA-infected DC might have a specific phenotype of anti-vector immunity due to restricted viral gene expression which could possibly lead to reduced vector-specific immune responses. Thereby, infected DC might have a specific benefit and advantage in prime boost regimen. Yet, our in vitro data might help to explain the successful use of adoptively transferred MVA-infected human DC in several aspects. First, MVA-infected iDC and mDC produce substantial amount of recombinant protein despite restricted viral gene expression. Second, induction of apoptosis upon MVA infection is varying between iDC and mDC, with the latter being more resistant. Third, viable MVA-infected mDC do not downregulate costimulatory molecules in contrast to reports on replication-competent VV. Finally, although MVA-infected iDC do not fully mature under established maturation stimuli, they can process and present peptides derived from the recombinant antigen to a comparable level as mDC. In this study, we show that viability and maturation status are differentially influenced in MVA-infected human iDC and mDC. We also demonstrate previously unknown aspects of MVA and DC interaction with regard to viral transcription and in comparison reveal new differences concerning phenotypic changes reported on replication competent VV.

Materials and methods

Viruses

Vaccinia virus (VV) strain MVA was originally obtained from Anton Mayr (University of Munich, Germany). MVA from the 582nd (cloned isolate F6) passage on CEF were used for this study. Recombinant MVA expressing the human *her-2/neu* gene and the human tyrosinase gene, respectively, under control of the VV-specific natural early/late promoter P7.5 (MVA-Her-2/neu) were constructed as described previously (Staib et al., 2000; Staib et al., 2004). DNA genomes of recombinant viruses were analyzed by Southern blot hybridization and PCR. All viruses were propagated and titrated following standard methodology.

Protein analysis

Protein lysates from infected human HeLa cells were resolved by electrophoresis on a SDS–6% polyacrylamide gel and electroblotted onto nitrocellulose at 15 V for 1 h in a buffer containing 25 mM Tris, 192 mM glycine, 0.05% SDS and 20% methanol. Blots were blocked overnight at 4°C in a phosphate-buffered saline blocking buffer containing 1% bovine serum albumin (BB) and incubated for 2 h at RT with Her-2/neu-specific c-neu-antibody (9G6) (Oncogene) diluted in BB at a final concentration of 2.5 μg/ml. After washing and incubation for 1 h at RT with 125I-labeled sheep anti-mouse IgG (Amersham), these are diluted 500-fold in BB, washed again and exposed to a phosphor imaging plate (Type BAS-IIIs, Fuji) for evaluation with a phosphor imaging analyzer (Personal Molecular Imager FX, Bio-Rad). For analysis of [S^{35}] methionine-labeled polypeptides, cells were infected and labeled as previously described (Sutter and Moss, 1992). Cytoplasmatic extracts were prepared, and samples were analyzed by SDS-PAGE.

Cell lines

SK-MEL-28 (ATCC HTB 72) is a human melanoma cell line. EBV-transformed B-LCL were established by culturing peripheral blood mononuclear cells (PBMC) with supernatant from B95.8 cells in the presence of 1 μg/ml of cyclosporin A. Cells were cultured in RPMI 1640 or DMEM supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin.

CTL line

The human HLA-A*0201-restricted CD8+ CTL line BST5 specific for the human tyrosinase peptide 369–377 was maintained as described (Fleischer et al., 2004). Briefly, responding T cells were restimulated weekly with peptide-pulsed DC in the presence of rIL-2 and rIL-7.

Chromium release assay

The cytolytic activity was analyzed as described previously (Drexler et al., 1999). Briefly, DC were infected with MVA at an

MOI of 10, washed once, labeled for 1 h at 37 °C with 100 μCi Na^{51} CrO_4 and then washed four times. Labeled target cells were then cocultivated with effector cells at different E:T ratios for 4 h. Thereafter, supernatants were collected and specific ^{51}Cr release was determined.

DC isolation, culture and infection

Immature DC (iDC) from different healthy donors were generated by culturing adherent monocytes with GM-CSF and IL-4 for 7 days (purity 70–90%, analyzed by FACS) and were matured into CD83+ mDC in the presence of cytokines (TNFα, IL-1β, IL-6 and PGE_2) as previously described (Fleischer et al., 2004; Jonuleit et al., 1997). Cells to be infected were counted and washed in RPMI, as described (Drexler et al., 1999; Staib et al., 2000). Briefly, for infection, purified virus was added to the cells at an MOI (multiplicity of infection) ranging from 1 to 100 for 2 h. After infection, cells were washed three times and further cultivated.

Ribonuclease protection assay (RPA)

HeLa cells and DC were mock-infected or infected with MVA at MOI 20 and incubated at 4 °C for 30 min to allow virus adsorption and synchronization of infection. Subsequently, cells were washed and further incubated at 37 °C. Total RNA was isolated at varying periods post-infection with TRIzol reagent (Invitrogen) following the manufacturer's instructions. For RPA, 5 μg total RNA per reaction was applied as described previously (Ludwig et al., 2005). Briefly, biotinylated anti-sense riboprobes specific for MVA ORF 005R, 078R and 047R transcripts and human GAPDH were included. Electrophoretic separation of RPA samples occurred in pre-cast 6% polyacrylamide urea gels, and RNA was transferred onto positively charged nylon membrane via semi-dry blotting. For detection, North2South Chemiluminescent Hybridization and Detection Kit (PIERCE) was used.

Flow cytometric analysis and antibodies

Cells were harvested, washed and resuspended in PBS containing 0.5% BSA. After Fcγ receptor blocking (Milteny-Biotec) for 30 min at 4°C, phenotypic analyses were performed by flow cytometric using saturating concentrations of the following PE-conjugated antibodies: CD71 (M-A712) and CD83 (HB15A) (Immunotech), CD80 (BB1), CD86 (IT2.2) and HLA-DR (TÜ 36); HLA-DR (G46-6) FITC-conjugated (all PharMingen). Conjugated isotype-matched mAbs were used as controls. Her-2/neu-specific staining was performed using mAb c-neu (Ab-2) (9G6) and FITC-conjugated $F(ab')_2$ goat anti-mouse Ig (Zymed). Apoptosis and necrosis were assessed by staining with FITC-Annexin and propidium iodide (PI) using Annexin V Kit (Immunotech), according to the manufacturer's instructions. Fluorescence analyses were performed with EPICS Elite ESP flow cytometer (Coulter Electronics).

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