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Protein Kinase C Overexpression Does Not Enhance Immune-Stimulatory Surface Markers of Vaccinia-Infected Dendritic Cells and DC Cell Lines

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One of the shortcomings of vaccinia virus (VACV) as immunization vector is the downregulation of HLA and costimulatory molecules in antigen presenting cells. To overcome this problem we investigated the use of protein kinase C (PKC) as immune stimulatory agent. Thus several classical and atypical PKCs were inserted into wild-type or attenuated VACV using recombination into the hemagglutinin gene and the expression driven by the VACV 7,5K-IE gene promoter. Recombinant constructs expressing PKC-alpha, -beta, -theta as well as wild-type, constitutive active or dominant negative PKC-zeta constructs were generated. Additional constructs expressing PKB/Akt1 and ICAM-1 were used for comparison. Immature and mature peripheral blood derived-dendritic cells (DC) as well as lymphoid cell lines capable of obtaining a DC-like phenotype upon mitogen stimulation were infected. Disappointingly, VACV-driven PKC overexpression did not significantly enhance expression of various activation markers or costimulatory molecules tested. Neither CD86 nor HLA-DR expression was upregulated and also no influence on the maturation of DC, as measured by DC-SIGN and CD83, was observed. However, VACV did

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not interfere with LPS induced up-regulation of CD83 and did not lead to substantial apoptosis of infected DC within the first 24 hours.

Keywords Proteinkinase C, PKC, Dendritic cell, DC, Maturation, Immune Stimulation, Vaccinia.

INTRODUCTION

Vaccinia virus (VACV) is a well-documented vaccine that has been used for eradication of smallpox virus. Due to its long track record of relatively safe use in immune competent humans it has been also applied with variable success as a vaccine vector for the expression of various antigens. VACV induces a broad range of immunological pathways but also has developed numerous escape mechanisms to evade the host immune response (for review see Bahar et al., 2011). These include the down-regulation of immune stimulatory molecules as well as a reduced expression of HLA antigens in VACV-infected cells and destruction or conversion of antigen presenting cells like dendritic cells (DC) to an immature phenotype (Engelmayer et al., 1999).

Therefore VACV is less suitable for the construction of immunization vectors for antigens with low immunogenicity, e.g., tumor antigens, etc.

An ideal VACV immunization vector should have features leading to an enhancement of the immune stimulatory phenotype of DC. The aim of our depicted constructs was to activate pathways that lead to a broad range of cell activating and stimulatory effects. Such constructs would not only allow the expression of single defined antigens, which are difficult to identify especially among highly variable (tumor) antigens, but might also be applied for enhancing the immune stimulatory phenotype of cells in general.

The protein kinase C (PKC) appeared to be one of the candidates to achieve rather broad cell stimulation, as several immune pathways have been shown to be associated with the activation of various PKC isoforms. Thus members of the classical and novel PKCs play an important role in T cell signaling, and PKC-alpha, PKC-beta, and PKC-theta isotypes determine the nature of specific lymphocyte responses (for review see Lee et al., 2008). Additionally, influences also on the humoral immune response are well established facts (Leitges et al., 1996). Most recent data indicate that classical as well as atypical PKCs (e.g., PKC-zeta) may also have profound effects on toll- like receptor signaling (Loegering and Lennartz, 2011), which plays an important role in the immune response against viral infections too. Furthermore, PKC-zeta appeared as a promising candidate as up-regulation of the potent T-cell activator IL-15 in herpes simplex-infected monocytic cells has been shown to be dependent on the activation of PKC-zeta/lambda (Ahmad et al., 2007).

Recent publications have suggested also profound effects of PKC on DC maturation and function. Thus, PKC-beta2 seems to influence DC maturation

(Cejas et al., 2005) and PKC-delta mediated phosphorylation has been suggested to be crucial for the differentation to DC from hematopoetic stem cells (Hamdorf et al., 2011). Recently, PKC-alpha has been shown to be involved in TLR/IL-1R signaling and cytokine production in mouse and human dendritic cells (Langlet et al., 2010).

Activation of PKC by cytokines or phorbol esters drives human hematopoietic CD34(+) progenitors and several myelo-blastoid cell lines to differentiate into DC like phenotype (Cejas et al., 2005). Additionally, PKC inhibitors have been shown to inhibit calcium ionophor-mediated DC maturation (Li et al., 2005). This is a strong indication that the various PKC pathways are involved in DC maturation and function. Therefore our intention was to counteract the known inhibitory effect on the DC maturation by DNA-virus infection (Jenne et al., 2001) by use of overexpression of PKC constructs.

MATERIAL AND METHODS

Cells and Viruses

The established VACV laboratory strain WR (Western Reserve) has been propagated on the rabbit kidney RK13 cell line (ATCC # CCL-37) grown in DMEM supplemented with antibiotics, glutamine and 10% bovine serum. Recombinant viruses grown under selection pressure and isolated by repeated plaque purification were used to generate virus stocks with titers of about 10^8 plaque forming units per ml as determined in standard dilution assays ($TCID_{50}$) and plaque assays with soft agar overlay and cresol red staining on RK13 cells. The attenuated MVA (modified vaccinia Ankara) strain, which is adapted to chicken cells and not replicating in most mammalian cells, has been propagated on primary chicken fibroblast (CHF) cultures as described earlier (Huemer et al., 2000a, 2000b).

Preparation of viral pools, determination of viral titers and plaque assays were performed on CHF accordingly. DC preparations were produced from peripheral blood derived mononuclear cells as described below. Additionally, lymphocytic cell lines, which have been shown to lead to a DC-like phenotype after cytokine and mitogen stimulation, were used under the published conditions. The respective properties of myelomonocytic cell line THP-1 (ATCC # TIB-202) and of MUTZ-3 cells, which were kindly donated by Dr. deGruijl, Amsterdam, Netherlands, have been described in detail elsewhere (Berges et al., 2005; Masterson et al., 2003). THP-1 cells were grown in RPMI1640 supplemented with 10% FCS, MUTZ-3 cells in alpha-MEM supplemented with antibiotics, glutamine, nonessential amino acids and 2-mercaptoethanol. Activated by GM-CSF (100 ng/ml = 1500 IU/ml), IL4 (200 ng/ml = 3000 IU/ml) and TNF-alpha (20 ng/ml = 2000 IU/ml), followed by

E. coli derived bacterial lipopolysaccharide (LPS, 100 ng/ml) or calcium ionophore Ionomycine (200 ng/ml) stimulation, those cells show the expression of typical DC markers and also immune presentation functions have been described (Santegoets et al., 2008).

Generation of Human Monocyte-Derived DC

DCs were produced according to a standard protocol of 5 days duration. In brief, peripheral mononuclear cells (PBMC) were isolated from whole blood of human donors by purification on Ficoll density gradients. Monocytes were isolated using human CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were cultivated at a density of 1×10^6 cells/ml medium in 6-well plates (Costar) in RPMI 1640/10% FCS/2 mM L-glutamine containing 1000 U/ml IL-4 and 1500 U/ml GM-CSF to generate monocyte-derived DC. New IL-4 (1000 U/ml) and GM-CSF (1500 U/ml) were added to the medium after 2 days in culture without medium change.

On day 5 those inmature dendritic cells (IDC) cells were harvested and controlled by FACS analysis for the expression of DC phenotypic markers. In the following experiments IDC were incubated/stimulated for up to 2 more days in the above cytokine/growth factor cocktail supplemented with either bacterial lipopolysaccharide (LPS, 100 ng/ml) as positive control for maturation, or the different indicated recombinant viruses tested. For co-culture/Transwell experiments, PBMC and DC from the same donor were used.

Recombinant VACV Constructs

The generation and properties of the used recombination vector pHA7.5K/gpt has been described earlier (Huemer et al., 2000a, 2000b). It allows the recombination of foreign antigens into the VACV hemagglutinin (HA) gene and the protein expression under the control of the VACV 7,5K- immediate early gene promotor. An additional guanosyl-phosphoribosyl-transferase (gpt) gene on the plasmid allows for the selection of the recombinant viruses with integrated constructs.

The production of cDNA and sub-cloning into plasmid vectors of the used PKC genes (alpha, beta, theta) and PKB/Akt1 has been described in detail earlier (Baier et al., 1994; Bauer et al., 2001; Thuille et al., 2004).

The generation of wild-type (WT), constitutive active (AE) and dominant negative (KW) mutants of PKC-zeta, leading to the expression of functional or non-functional versions of this atypical PKC isoform, have been described in detail (Kampfer et al., 1998). The coding regions of the different PKCs and PKB/Akt1 were excised by restriction enzyme digest and ligated into the multicloning site of pHA7.5K/gpt recombination vector by standard cloning techniques and

the intact reading frame of the genes verified by automated DNA sequencing on an ABI3100 automated sequencer (Applied Biosystems).

Generation of the recombinant viruses was performed according to standard procedures. In brief, plasmid DNA was transfected into VACV infected cells and then a selective pressure was applied by the addition of xanthin/hypoxanthine and mucophenolic acid. After 2 rounds of selection the remaining clones were subcloned by repeated plaque purification in 6-well culture plates with soft agar overlay and tooth-picking of isolated recombinant isolates. The insertion of the foreign genes was verified by PCR amplification and immune fluorescence with PKC isotype specific antibodies.

The PKC -alpha, -beta, -theta and PKB/Akt1cDNAs were inserted into VACV wildtype strain WR. The 3 mutants of PKC-zeta were inserted into the chicken adapted MVA strain as a safety measure due to the yet insufficient knowledge about the biological properties of this isoform. An additional VACV recombinant containing the intracellular adhesion molecule-1 (ICAM-1) has been obtained from Christine Berling, Department for Technology Transfer, INSERM, Paris, France and propagated on RK13 cells and used under the same conditions.

PKC Protein Expression

The recombinant VACV constructs were tested for PKC over-expression by western blotting as described recently. In brief, lysates of RK13 cells infected for 48 hours with the respective recombinant constructs were run on SDS gels as described earlier (Kampfer et al., 1998). Protein expression was identified with the following monoclonal antibodies specific for PKC-alpha (clone M4 from Upstate, Lake Placid, N.Y., USA) and PKC-beta (clone 36), PKC-theta (clone 27), PKB/Akt1 (clone 55) all from BD Bioscience, Life Science Research Inc. A PKC-zeta specific antibody was obtained from Santa Cruz Biotechnology Inc., USA. The Blots were developed with alkaline phosphatase-labeled secondary antibody followed by a chemiluminescence substrate reaction (Amersham ECL, from GE Life Sciences) and exposure to photographic films.

Testing for Immunostimulatory Molecules and Surface Markers, and Assessment of DC Maturation by Flow Cytometry

Immature DC (IDC) or the respective cell lines were infected with the different recombinant VACV constructs at a multiplicity of infection of >1 at a cell density of 1×10^6 cells/ml and were harvested after various time points (12, 24, 48 hours) post-infection. To analyze the maturation and activation status unstimulated, LPS-stimulated, or IDC incubated with the various compounds were stained for 1 h at 4°C with characteristic differentially labeled monoclonal antibodies. Antibodies against DC-SIGN labeled with allophycocyanin (APC),

CD86 labelled with fluorescein isothiocyanate (FITC), CD83 labeled with phycoerythrin (PE), CD11b labeled with PE and cyanine 7 (PE/Cy7), or HLA-DR labeled with APC and cyanine 7 (APC/Cy7) were used according to the conditions and wavelength described by the protocol of the manufacturer (BD Biosciences). The same markers were also used following co-culture/Transwell experiments, which were performed in 24-well plates with Corning^{R}Transwell inserts of 12 mm diameter and a pore size of 0.4 μm (from SIGMA-Aldrich).

To evaluate indirect cytokine effects, the PBMC (1×10^6 cells/ml) were infected with the different VACV constructs for 24 hours at a multiplicity >1, thereafter the cells were washed with full medium and added into the Transwell microchambers placed above the uninfected DC preparations for further 1–2 days. After harvest of the DC by washing with ice cold PBS antibody staining was performed for 1 hour on ice, the cells then washed again in PBS, and finally fixed in PBS/4% paraformaldehyde. The following FACS analyses were performed using a FACS CantoII flow cytometer and FACS Diva software (both from BD Biosciences).

Assessment of Apoptotic Cells

Testing for apoptosis of the DC harvested after the various time points was performed with single laser FACS analysis by staining the unfixed cells with 7-amino-actinomycin D (7-AAD, from Calbiochem) according to standard protocols (Schmid et al., 1994). Additionally, the LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation was applied according to the instructions of the manufacturer (Molecular Probes/ Invitrogen).

RESULTS

PKC Expression by VACV Recombinants

Several recombinant viruses were obtained after repeated rounds of selection and subcloning. They were tested by means of Western blotting for the protein expression of the different PKC isoforms. As shown in Figure 1a staining with PKC-alpha specific antibody led to an intense band of about 80kDa in cells infected with the appropriated recombinant VACV clones, which fits to the expected molecular size of PKC-alpha (82kDa) whereas no signal was detected in cells infected with the other isoforms.

Accordingly PKC-beta (80kDa) and PKC-theta (79kDa) were also detected only in cells infected with the respective VACV clones (Figures 1b, 1c). Additionally, a clear expression of PKB/Akt1 with a MW of 59kDa was also detected in one of the 2 recombinants shown (Figure 1d).

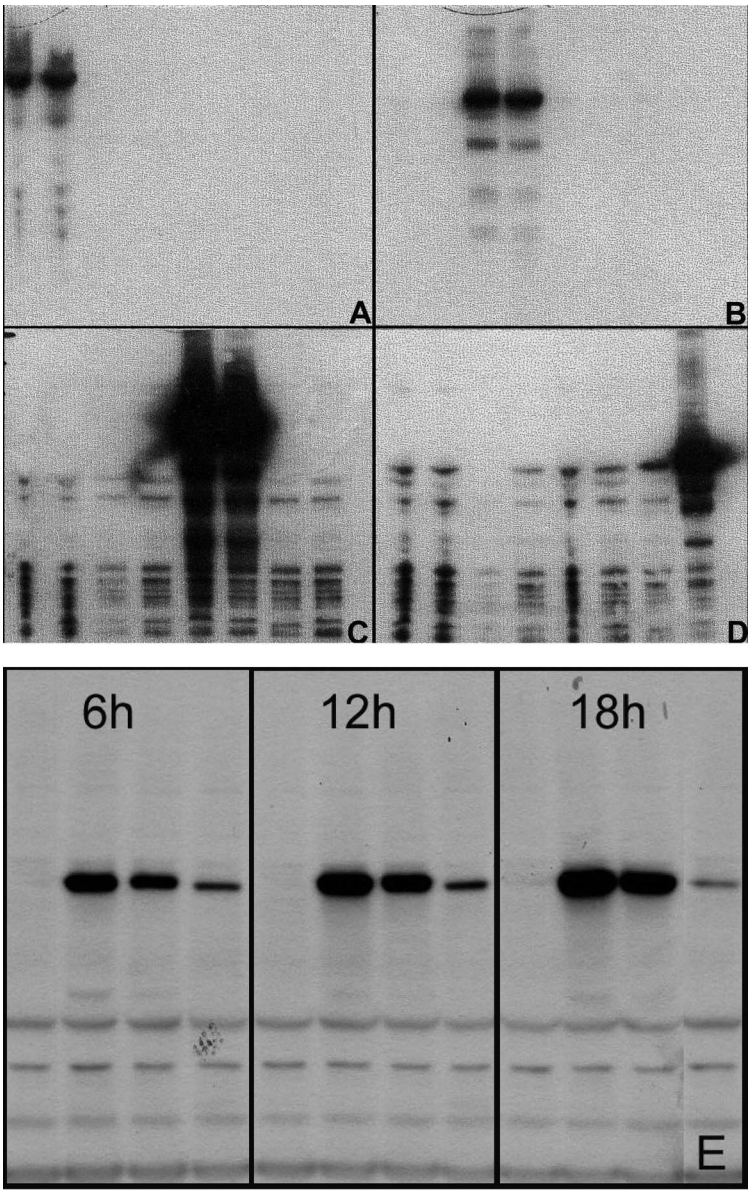

Figure 1: Expression of PKC proteins by recombinant VACV constructs. Cells infected with the different recombinant viruses were tested for PKC protein expression by western blotting. In lanes 1/2 lysates of PKC-theta construct infected cells were analyzed; PKC-alpha, PKC-beta and PKB/Akt1 construct-infected cells were analyzed on lanes 3/4, lanes 5/6 and lanes 7/8 respectively. The blots were reacted with antibodies against PKC-theta (shown in A), PKC-alpha (B), PKC-beta (C) and PKB/Akt1 (D). The PKC proteins led to clear signals at the expected size of about 80kD and 59kD for PKB/Akt1 which was specific for the respective isoforms. In Figure 1E, a time course of the expression of wild-type (lanes 2), constitutive active (lanes 3) and dominant negative (lanes 4) mutants of PKC-zeta by the recombinant viruses is shown. Lanes 1 showing the cell/vector background.

The protein expression of PKC-zeta wild-type (WT), constitutive active (AE) and dominant negative (KW) mutations was also clearly observed 6, 12 and 18 hours after infection (Figure 1e).

Expresssion of Costimulatory Molecules and Surface Markers in VACV-infected DC

As depicted in Figure 2 treatment of immature DC with PKC expressing VACV constructs did not lead to significant enhancement of costimulatory molecules like CD86 or DC maturation marker CD83 as compared to the control viruses, i.e., strain WR for the classical PKCs and strain MVA for the atypical PKC-zeta constructs. Similar negative results were observed using THP-1 or MUTZ-3 cells infected with the PKC constructs (not shown). LPS was used as a positive control and led to a clear increase of those activation markers (Figures 2 and 3).

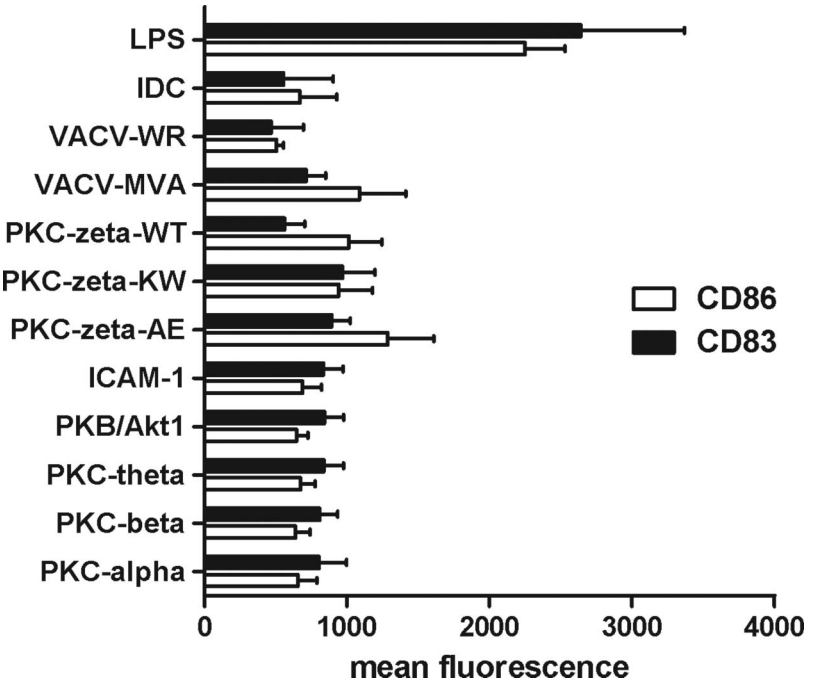

Figure 2: Expresssion of costimulatory molecule CD86 and CD83 in infected DC. Immature dendritic cells (IDC) derived from human peripheral blood were infected for 24 hours with the recombinant viruses expressing the indicated PKC isoforms (PKC-alpha, beta, theta) or mutants thereof, i.e., PKC-zeta wild-type (WT), constitutive active (AE) and dominant negative (KW). Controls consisted of the ancestor vaccinia virus strains, i.e., wild-type strain WR (VACV-WR) and attenuated strain MVA (VACV-MVA) as well as VACV constructs containing ICAM-1 and PKB/Akt1. Uninfected IDC and IDC stimulated with lipopolysaccharide (LPS) served as negative and positive control. Columns represent the mean of 5 measurements of the intensities of fluorescence from 3 independent FACS experiments with the standard error indicated as bars.

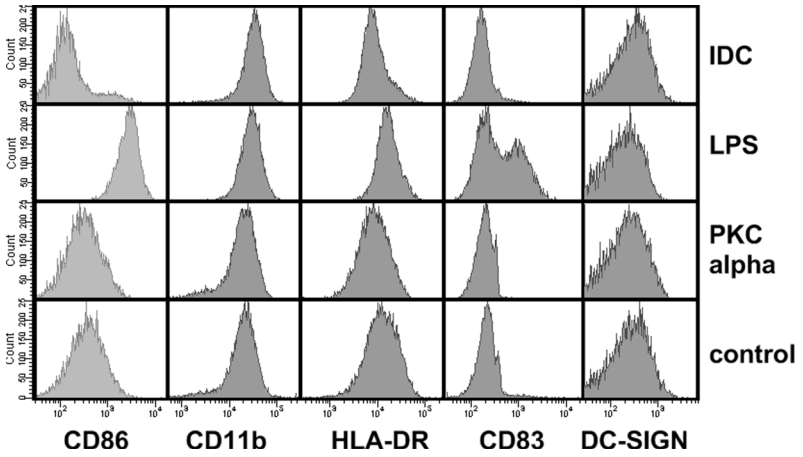

Figure 3: Expression of surface markers on unstimulated, stimulated and infected DC. The expression of CD86, CD11b, HLA-DR, CD83 and DC-SIGN is shown on human peripheral blood-derived immature dendritic cells (IDC) and IDC after treatment/infection for 2 days with lipopolysaccharide (LPS), VACV expressing PKC-alpha and the non-recombinant VACV control.

This stimulatory effect was not abolished by the VACV infection at least within the first 24 hours, leading to clearly enhanced CD83 expression upon LPS stimulation also in infected DC (Table 1).

No Enhanced DC Maturation or Apoptosis in Transwell Experiments

Co-cultivation of the DC with PBMC infected with the various recombinant constructs did not lead to enhanced expression of DC activation/maturation markers and also no enhanced rate of apoptosis of the DC was observed as compared to the respective control viruses (not shown).

Table 1: Effect of LPS stimulation on CD83 expression in VACV infected immature dendritic cells.

CD83 mean (% apoptotic)	IDC unstimulated	IDC + LPS	IDC + VACV unstimulated	IDC + VACV + LPS
12h	1251 (11.5%)	4029 (13.3%)	1527 (4.5%)	3975 (14.8%)
24h	1763 (6.2%)	7575 (10.2%)	1311 (9.9%)	6769 (10.5%)

Mean channel fluorescence of anti-CD83 and percentage of apoptosis in uninfected and VACV infected immature dendritic cells (IDC) without stimulation or stimulated with lipopolysaccharide (LPS) is shown at 12 and 24 hours after infection. Then, 48 hours after infection the CD83 expression data were not valid anymore, as the VACV infected cells showed substantial levels of apoptosis (IDC+$VACV_{48h}$: 85.9%; IDC+LPS +$VACV_{48h}$: 46.6%).

Viability of the Infected DC

No increased apoptosis was observed in the infected DC within the first 24 hours after infection. However, after 36 hours the infected cells started to detoriate with nearly 90% of dead cells at the time point of 48 hours (Table 1). Interestingly the stimulation of the infected DC with LPS seemed to delay the apoptosis to a certain extent, resulting in clearly higher viability of the stimulated cell population (IDC+VACV: 85,9%; IDC+LPS +VACV: 46,6% apoptosis after 48 hours).

DISCUSSION

Several members of the poxvirus family can infect dendritic cells (DC). Vaccinia virus (VACV), canary pox and fowlpox virus have been used to insert foreign genes into DC in an attempt to stimulate their immunogenicity, but unfortunately DC infected with poxviruses seem to fail to undergo maturation (for review see Engelmayer et al., 1999; Jenne et al., 2001).

These direct viral effects on the cells must be distinquished from indirect effects like the stimulation of pro-inflammatory cytokines IL-1 and IFN-beta, which have been shown to depend on simulation of TLR2 receptors and can be also achieved by UV-inactivated virus (Zhu et al., 2007).

Presumably the enhanced expression of CD86 observed in spleen derived DC from VACV-infected animals in the same study might be also due to similar mechanisms. That would be in agreement with our finding that VACV infection of human DC did not lead to enhanced CD86 expression. However, it would contradict recent reports of an up-regulation of CD86 observed in peripheral blood-derived CD8 positive and negative mouse DC infected with an green fluorescent protein expressing VACV strain. These effects seemed to occur rather early within 6 to 24 hours after infection via the intravenous route (Liu et al., 2008; Yammani et al., 2008). This would be an indication that VACV-infected mouse DC might indeed be capable to undergo maturation *in vivo*, which was not observed in our human *in-vitro* system.

The rational for using a VACV strain replicating in mammalian cells like WR instead of replication deficient strains like the chicken adapted MVA strain, which we have used successfully for vaccination purposes (Huemer et al., 2000a), is based on the overall higher expression rate and broader range of susceptible cells expected with the “wild-type” strains. Additionally, their virulence was also expected to be reduced by using our recombination vectors disrupting the VACV hemagglutinin (HA) gene (Huemer et al., 2000b). Thus several highly attenuated vaccines and strains proposed for therapy including Lister derivative GLV-1h68 (Yu et al., 2009), HA mutants of the NYCBH strain (Lee et al., 1992) and the established NYVAC (vP866) vaccine strain are lacking a functional HA and have been showing greatly reduced virulence in mice (Tartaglia et al., 1992). This was considered a safety measure as in addition to immunosuppressive disorders also

certain drug use might render people more vulnerable for infection/vaccination with orthopoxviruses (Huemer et al., 2010, 2011).

In the mouse model the MVA strain has been suggested to preferentially target antigen presenting cells among the subsets of hemato-lymphoid cells leading as expected to early gene expression only in DC followed by decreasing rates of viability starting after 12 hours (Liu et al., 2008). In our experiments with human DC, no significant apoptosis was observed within the first 24 hours post-infection but decreased viability due to viral effects was observed after 36–48 hours with both the wild-type and attenuated strains. In those mouse experiments also an increase in the expression of CD86 and HLA-DR was described within 12 to18 hours post-infection with MVA (Liu et al., 2008), a finding we did not observe in our human DC preparations or cell lines.

Our finding that LPS stimulation was able to increase expression of activation markers like CD83 also in VACV infected DC, could indicate that those infected cells might be still capable to act as antigen expressing targets in the human system, but perhaps not in mouse models where they are dying early. Interestingly LPS stimulation also seemed to delay apoptosis of our infected DC to a certain extent, suggesting that additional induction of unknown anti-apoptotic pathways by the LPS stimulation might be a strategy to counteract the known fact of apotosis induction by abortive VACV infection of human DC (for review see Engelmayer et al., 1999; Jenne et al., 2001).

Although the role of DC in immune stimulation is mainly the processing and presentation of antigens, also the production of cytokines may play a role in addition to cell surface activation markers (for review see Blanco et al., 2008). Even if we did not check a direct influence of the VACV constructs on cytokine production profiles by infected DC's on the cellular level, major indirect effects via secreted cytokines can be excluded as no cytokine effects were observed in coculture experiments with MVA infected PBMCs separated by Transwell^{R} culture inserts from uninfected DC.

These data suggest that the stimulation and antigen presentation of human DC after VACV infection might be also due to cross presentation of viral material or destructed cell material by non infected antigen presenting cells, which has been shown to have an important role in the immune response against poxvirally expressed antigens in the mouse system (Shen et al., 2002).

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ABBREVIATIONS

DC dendritic cells
IDC immature dendritic cells
DMEM Dulbeco modification of minimal essential medium
FACS fluorescence activated cell sorter
GM-CSF granulocyte macrophage colony-stimulating factor
ICAM intracellular adhesion molecule
IL-4 interleukin 4
LPS lipopolysaccharide
MEM minimal essential medium
MVA modified vaccinia Ankara strain
PBMC peripheral blood derived mononuclear cells
PBS phosphate-buffered saline solution
PKB proteinkinase B
PKC proteinkinase C
PKC-zeta WT wild-type PKC-zeta
PKC-zeta AE constitutive active mutant
PKC-zeta KW dominant negative mutant
RPMI1640 medium developed at Roswell Park Memorial Institute in the 60s
TCID50 tissue culture infective dose
TNF-alpha tumor necrosis factor alpha
VACV vaccinia virus
WR Western Reserve vaccinia strain

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