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Virus-like particles derived from major capsid protein VP1 of different polyomaviruses differ in their ability to induce maturation in human dendritic cells

Alma Gedvilaite [a,1], David C. Dorn [b,1,2], Kestutis Sasnauskas [a], Gabriele Pecher [c], Aiste Bulavaite [a], Robert Lawatscheck [b], Juozas Staniulis [d], Tina Dalianis [e], Torbjörn Ramqvist [e], Günther Schönrich [b], Martin J. Raftery [b], Rainer Ulrich [b,*]

[a] *Institute of Biotechnology, V. Graiciuno 8, LT-02241 Vilnius, Lithuania*
[b] *Institute of Virology, Charité Medical School, D-10098 Berlin, Germany*
[c] *Medical Clinic for Oncology and Hematology, Charité Medical School, D-10098 Berlin, Germany*
[d] *Institute of Botany, Zaliuju ezeru 49, LT-08406 Vilnius, Lithuania*
[e] *Department of Oncology-Pathology, Karolinska Institutet, S-171 77 Stockholm, Sweden*

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Abstract

As polyomavirus major capsid protein VP1-derived virus-like particles (VLPs) have been demonstrated to be highly immunogenic, we studied their interaction with human dendritic cells (hDCs). Exposure of hDCs to VLPs originating from murine (MPyV) or hamster polyomavirus (HaPyV) induced hDC maturation. In contrast, exposure of hDCs to VLPs derived from human polyomaviruses (BK and JC) and simian virus 40 (SV40) only marginally induced DC maturation. The hDCs stimulated by HaPyV- or MPyV-derived VLPs readily produced interleukin-12 and stimulated CD8-positive T-cell responses *in vitro*. The highest frequencies of activated T cells were again observed after pulsing with HaPyV- and MPyV-derived VLPs. Monocyte-derived hDCs both bound and internalized the various tested polyomavirus VP1-derived VLPs with different levels of efficiency, partially explaining their individual maturation potentials. In conclusion, our data suggest a high variability in uptake of polyomavirus-derived VLPs and potency to induce hDC maturation.

Keywords: Polyomavirus; Capsid; Virus-like particles; Dendritic cells; Maturation; T-cell response

Introduction

Virus-like particles (VLPs) produced by the heterologous synthesis and self-assembly of viral structural proteins can mimic viruses in their morphological and immunological properties. However, due to the absence of viral nucleic acid, they are non-infectious. The structural similarity of VLPs to the original viruses has been exploited to investigate the fine structure of viral capsids, their assembly processes and potential cell receptor–virus interactions. In addition, VLPs have been utilized for vaccine and gene transfer development as well as for serodiagnosis of virus infections (for reviews, see Ulrich et al., 1998, 2002; Pumpens and Grens, 2002; Noad and Roy, 2003; Tegerstedt et al., 2005).

Different polyomavirus-derived VLPs can be generated in an array of systems including insect cells and *E. coli* by heterologous synthesis of the major capsid protein VP1

* Corresponding author. Present address: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Novel and Emerging Infectious Diseases, Boddenblick 5a, D-17493 Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany. Fax: +49 38351 7192.
E-mail address: rainer.ulrich@fli.bund.de (R. Ulrich).
[1] These authors contributed equally to the paper.
[2] Present address: Laboratory of Developmental Hematopoiesis, Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA.

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followed by its spontaneous self-assembly (Montross et al., 1991; Forstova et al., 1993; Kosukegawa et al., 1996; Pawlita et al., 1996; Chang et al., 1997; Voronkova et al., in press). Expression of murine polyomavirus (MPyV) VP1 in *E. coli* allowed the generation of capsomer subunits that assemble to VLPs under specific ion and pH conditions (Salunke et al., 1986; Schmidt et al., 1999). A previously described yeast expression system allowed the high-level expression and self-assembly of VP1 of avian and mammalian, human and non-human, polyomaviruses, namely the avian Budgerigar fledgling disease polyomavirus (BFDPyV), hamster polyomavirus (HaPyV), MPyV, simian virus 40 (SV40), BK polyomavirus (BKPyV) and JC polyomavirus (JCPyV) (Sasnauskas et al., 1999, 2002). Although the native polyomavirus capsid contains the additional minor capsid proteins VP2 and VP3, the above cited investigations demonstrated that both proteins are dispensable for VLP assembly.

Polyomaviruses and polyomavirus-derived VLPs have become important tools to study general immunological phenomena. Differences in the susceptibility of two mouse strains to MPyV infection and tumor formation have been explained by differences in the CD8-positive T-cell response (Moser and Lukacher, 2001; Welsh et al., 2002). Moreover, CD94-NKG2A inhibitory receptors were demonstrated to induce a polyomavirus-reactive CD8-positive T-cell population to become anergic (Moser et al., 2002). Recently, quantitative differences in expression of TLR2 and TLR4 among different antigen-presenting cell (APC) subpopulations have been detected in susceptible and resistant mouse strains (Velupillai et al., 2006). Interestingly, MPyV and MPyV-VP1-derived VLPs elicited in these strains the same host-specific cytokine responses (Velupillai et al., 2006). Whereas polyomavirus infection of mice has been shown to result in the induction of an antiviral T-cell-independent antibody response, recombinant VP1 and VP1-derived VLPs failed to do so in some systems (Szomolanyi-Tsuda et al., 1998, 2001) but not in others (Vlastos et al., 2003). In addition, MPyV-derived VLPs proved to induce a protective immunity in mice against specific MPyV-induced tumors (Franzen et al., 2005).

Dendritic cells (DCs) are professional APCs which possess the capability to efficiently initiate and modulate cell-mediated immunity. Immature DCs take up and process antigens. Activation of APCs can be mediated by a wide variety of stimuli, such as inflammatory cytokines, microbial products and CD40L. This maturation is associated with upregulation of costimulatory molecules (i.e. CD80 and CD86) as well as antigen-presenting molecules (MHC classes I and II). After DCs are activated, endocytosis is down-regulated, and the mature DCs migrate toward regional lymph nodes, where they activate antigen-specific naive T cells. A wide range of pathogens trigger this kind of primary immune reaction (for reviews, see Banchereau and Steinman, 1998; Banchereau et al., 2000; Guermonprez et al., 2002). However, basic knowledge about underlying mechanisms of the immune responses induced by polyomavirus major capsid protein VP1 during viral infection or upon immunization is only very limited (Lenz et al., 2001).

In the present paper, we investigate the human DC (hDC) maturation potential of VP1-derived VLPs of various polyomaviruses. To elucidate the observed different maturation potentials, we measured the uptake level of these VLPs by hDCs.

Results

The induced level of maturation of human dendritic cells differed between VP1-derived VLPs of rodent and primate polyomaviruses

The immunophenotypic maturation of hDCs was examined after 48 h of stimulation with polyomavirus VP1-derived VLPs. More than 75% of the harvested cells visually depicted the typical morphological features of hDCs. FACS analysis showed that after differentiation to hDCs the prepared cells were CD14- and CD3-negative (data not shown).

The hDC maturation induced by polyomavirus-derived VLPs was evaluated by flow cytometry analysis of cell surface markers. Pulsing of hDCs with VLPs originating from rodent polyomaviruses HaPyV and MPyV resulted in markedly increased levels of maturation markers CD83, CD80, CD86, MHC class I (HLA *ABC*) and MHC class II (HLA *DR DQ*) (data not shown). For all surface molecules, the level of upregulation was higher for HaPyV-derived VLPs than for MPyV-derived VLPs. In contrast, incubation of hDCs with SV40- and human polyomavirus JCPyV- and BKPyV-derived VLPs resulted in only very slightly increased levels of CD86, whereas the level of all other surface molecules was not affected (CD83, CD80, MHC class I and class II). In our negative controls, untreated hDCs and hDCs treated with "mock VLPs" (for details, see Materials and methods), no increased expression of maturation markers could be detected (data not shown).

A functional test of hDC activation is the degree of reduced uptake of antigens by macropinocytosis and receptor-mediated endocytosis (Salter and Dong, 2001). The macropinocytotic capacity was measured by FACS analysis of Lucifer Yellow uptake, similarly the receptor-mediated endocytosis was studied by the FITC-conjugated dextran uptake (Fig. 1). Comparison of the uptake observed for untreated or "mock VLPs"-treated cells on the one hand and LPS-treated cells on the other demonstrated the validity of the assay. In general, the detection of the reduced uptake of FITC–dextran was more sensitive than that of Lucifer Yellow. VLPs from MPyV and HaPyV demonstrated a slight reduction of the Lucifer Yellow and a more pronounced reduction of the FITC–dextran uptake. In contrast, SV40-, BKPyV- and JCPyV-derived VLPs had no or only a very slight influence on the internalization of FITC–dextran or Lucifer Yellow.

Human dendritic cells pulsed with VLPs of rodent polyomaviruses, but not SV40, BKPyV and JCPyV showed the induction of a VP1-specific T-cell response in vitro

The secretion of interleukin-12 (IL-12) by hDCs is one of the crucial steps to induce an effective T-cell response, in particular

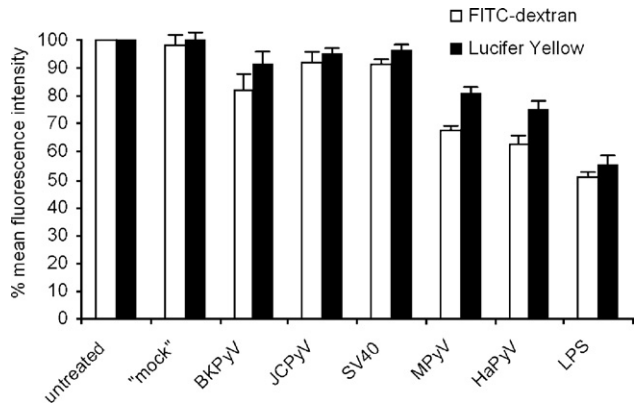

Fig. 1. Uptake levels of FITC–dextran and Lucifer Yellow by VLP-pulsed hDCs as a functional maturation marker. The hDCs were pulsed with BKPyV-, JCPyV-, SV40-, MPyV- or HaPyV-derived VLPs, "mock" VLPs and LPS or were left untreated. After 48 h of incubation, the hDCs were exposed to Lucifer Yellow or FITC–dextran for 4 h at 37 °C. As a control for unspecific binding or uptake, cells treated in parallel were kept at 4 °C. The columns represent the mean values of three independent experiments, the given error bars indicate the standard deviation.

Th1. The level of IL-12 secreted into the supernatant was measured 48 h after pulsing hDCs with VLPs (Fig. 2). The ELISA detected elevated IL-12 levels in the media of cells stimulated with LPS and rodent polyomavirus-derived VLPs. HaPyV-derived VLPs induced a higher level of IL-12 secretion than MPyV-derived VLPs. Compared to "mock VLPs"-treated cells, hDCs treated with VLPs originating from primate polyomaviruses did not show any increased level of IL-12 secretion.

DCs are the main cell type capable of inducing primary T-cell responses. The induction of T-cell responses was evaluated using autologous HLA A2-restricted T cells co-cultivated with hDCs that had been pulsed with VLPs. After two stimulation cycles of the autologous peripheral blood lymphocytes (PBL) with pulsed hDCs, the T cells were stimulated with the corresponding VLPs and stained for interferon (IFN)-gamma secreting CD8-positive T cells (Fig. 3). In the negative controls

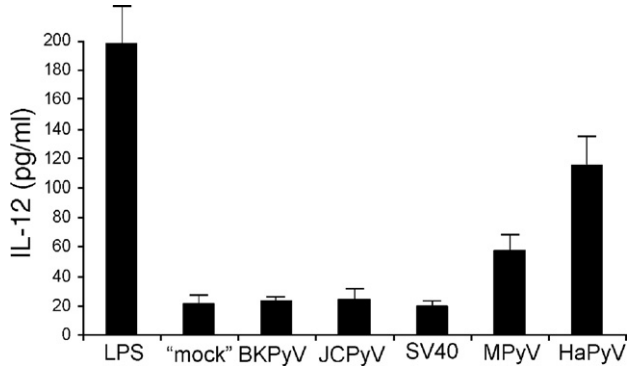

Fig. 2. IL-12 secretion of hDCs pulsed with polyomavirus-derived VLPs. The hDCs were incubated with BKPyV-, JCPyV-, SV40-, MPyV- or HaPyV-derived VLPs, "mock" VLPs and LPS. The supernatants of the hDCs were collected after 48 h of incubation and stored frozen at −80 °C until analysis by IL-12 ELISA. Shown are the mean values and standard deviations of three independent experiments.

(untreated and "mock VLPs"-treated hDCs), a very low frequency of CD8-positive, IFN-gamma secreting cells was detected. The strongest increase of the frequency of specific T cells was observed for MPyV- and in particular for HaPyV-derived VLPs.

Chimeric HaPyV-VP1-derived VLPs harboring an enhanced green fluorescent protein enabled the detection of the uptake of VLPs by human dendritic cells

To study the uptake of HaPyV-derived VLPs, a HaPyV-VP1 derivative was generated harboring the enhanced green fluorescent protein (eGFP) sequence at insertion site #4 (between amino acid residues 288 and 295) of HaPyV-VP1. The intrinsic fluorescence of the eGFP in the yeast-expressed

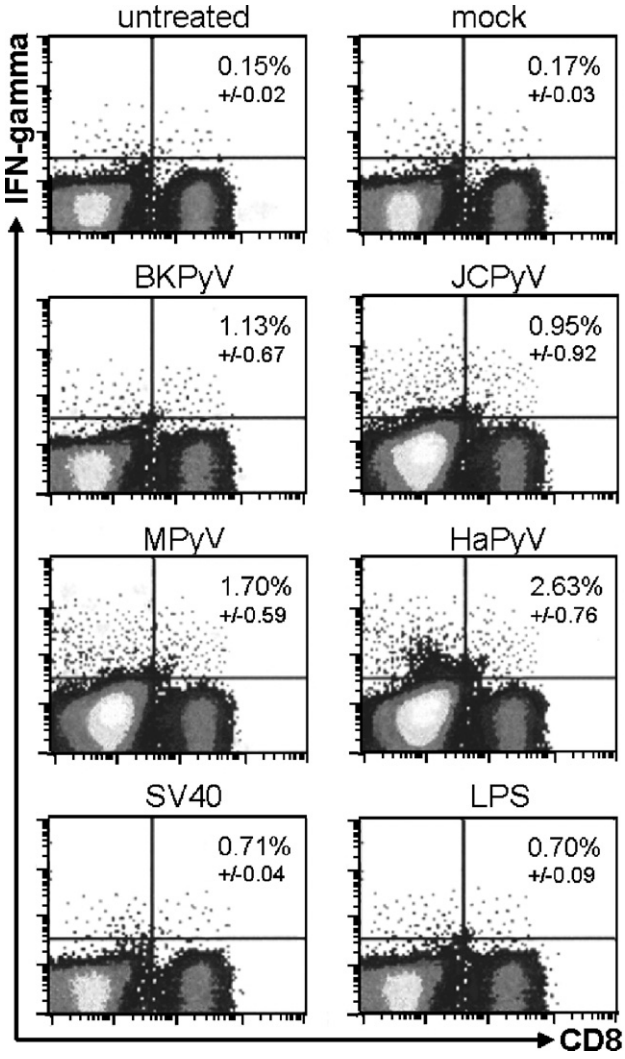

Fig. 3. Frequency of VLP-reactive CD8-positive T cells. T cells were stimulated twice with pulsed hDCs as described in Materials and methods and restimulated with VP1-VLPs 16 h before the assay. The percentage of IFN-gamma secreting cells was detected by PE-labeled IFN-specific detection antibody in a capture assay. The T cells were marked with a CD8-FITC-conjugated antibody. A representative of three independent experiments is shown. In the upper right quadrant, the mean T-cell frequencies of three experiments with standard deviation are indicated.

VP1 fusion protein allowed an easy identification of the protein band in the cesium chloride (CsCl) gradient after ultracentrifugation (data not shown). The migratory behavior of VP1/eGFP in the gradient was found to be slightly different from that of the unmodified VP1. This is reflected in the fact that the main portion of VP1/eGFP was recovered in fractions corresponding to densities of 1.27 to 1.29 g/cm^3, whereas VP1 VLPs were found in fractions with densities of 1.30 to 1.31 g/cm^3 (data not shown). A protein band of the expected molecular weight from corresponding fractions (about 66 kDa) reacted with a rabbit serum raised against the entire HaPyV-VP1 (aa 1–384) and rabbit anti-GFP serum (data not shown). The VP1/eGFP fusion protein tended to aggregate in high salt buffers and in concentrations higher than 2 mg/ml. The formation of VLPs was confirmed by negative staining electron microscopy. The electron microscopic images of VP1/eGFP VLPs (Fig. 4A) were found to be similar to those of VLPs generated by unmodified VP1 (Fig. 4B).

Purified VP1/eGFP VLPs were used to analyze the VLP uptake by monocyte-derived hDCs. Whereas after 12 and 16 h of incubation, only traces of eGFP fluorescence were observed inside or in association with human DCs, incubation for 24 h resulted in the internalization of the majority of VP1/eGFP VLPs, as determined by fluorescence microscopy. In contrast, in untreated cells (negative control), no green fluorescence was observed by fluorescence microscopy (data not shown).

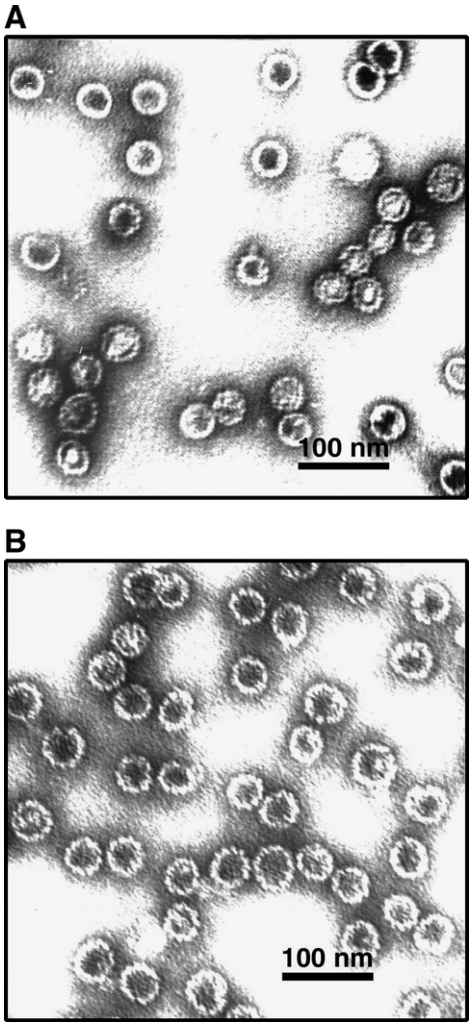

Fig. 4. Detection of VLP formation by negative staining electron microscopy for HaPyV-VP1/eGFP (A) and HaPyV-VP1 used for comparison (B).

VLPs originating from VP1 of different polyomaviruses vary in the efficiency of their uptake by human dendritic cells

To exclude a possible influence of eGFP insertion on the uptake of polyomavirus-derived VLPs, the different polyomavirus VP1-derived VLPs were labeled with CFDA before incubating with hDCs. First, a time course of the uptake of CFDA-labeled HaPyV-VLPs by hDCs was established by two alternative approaches, i.e. fluorescence microscopy (Fig. 5A) and FACS analysis of hDCs (Fig. 5B). As documented by fluorescence microscopy, the incubation of hDCs with CFDA-labeled HaPyV-VP1 VLPs resulted in an increasing number of fluorescent cells, with the majority of cells (>80%) being fluorescent after 60 min of incubation (Fig. 5A). FACS analysis of hDCs incubated with CFDA-labeled HaPyV-derived VLPs confirmed that after an incubation period of 120 min 92% of the cells had taken up CFDA-labeled VLPs (Fig. 5B), which did not increase after further incubation. Based on this protocol established for HaPyV-VP1 VLPs, the uptake of CFDA-labeled VLPs of the other polyomaviruses was measured (Fig. 5C). The observed level of uptake for HaPyV-derived VLPs at the 2 h endpoint was higher than that for VLPs originating from MPyV and from the primate polyomaviruses (BKPyV, JCPyV and SV40).

As a first approach to study receptor association, the VP1 VLPs of the different polyomaviruses (HaPyV, MPyV, SV40, JCPyV and BKPyV) were tested for their hemagglutination activity with guinea pig erythrocytes. The JCPyV-, BKPyV- and MPyV-derived VLPs all demonstrated a strong hemagglutination activity down to a concentration of 2 pg/μl. In contrast, HaPyV-derived VLPs were found to have no activity in the hemagglutination assay. As already known for SV40, the SV40-derived VLPs were also negative in this test (data not shown).

Discussion

Pulsing of hDCs with VP1-derived VLPs of rodent polyomaviruses, i.e. HaPyV and MPyV, was found to induce hDC maturation as shown by upregulation of CD80, CD83, CD86, MHC-I and MHC-II. In addition to the upregulation of maturation markers, in our approach, the VLP-induced maturation of hDCs was confirmed functionally by the reduced uptake of FITC–dextran and Lucifer Yellow. Moreover, VLP-pulsed hDCs were found to secrete IL-12 and induce a VP1-reactive CTL response *in vitro* as demonstrated by IFN-gamma secretion. In contrast, VP1-derived VLPs originating from SV40 or human polyomaviruses (JCPyV, BKPyV) demonstrated only very weak or no maturation of hDCs and correspondingly weak T-cell responses.

Potential reasons for the observed differences between rodent and primate polyomavirus-derived VLPs in the induced level of hDC maturation might be (i) intrinsic to the VLP

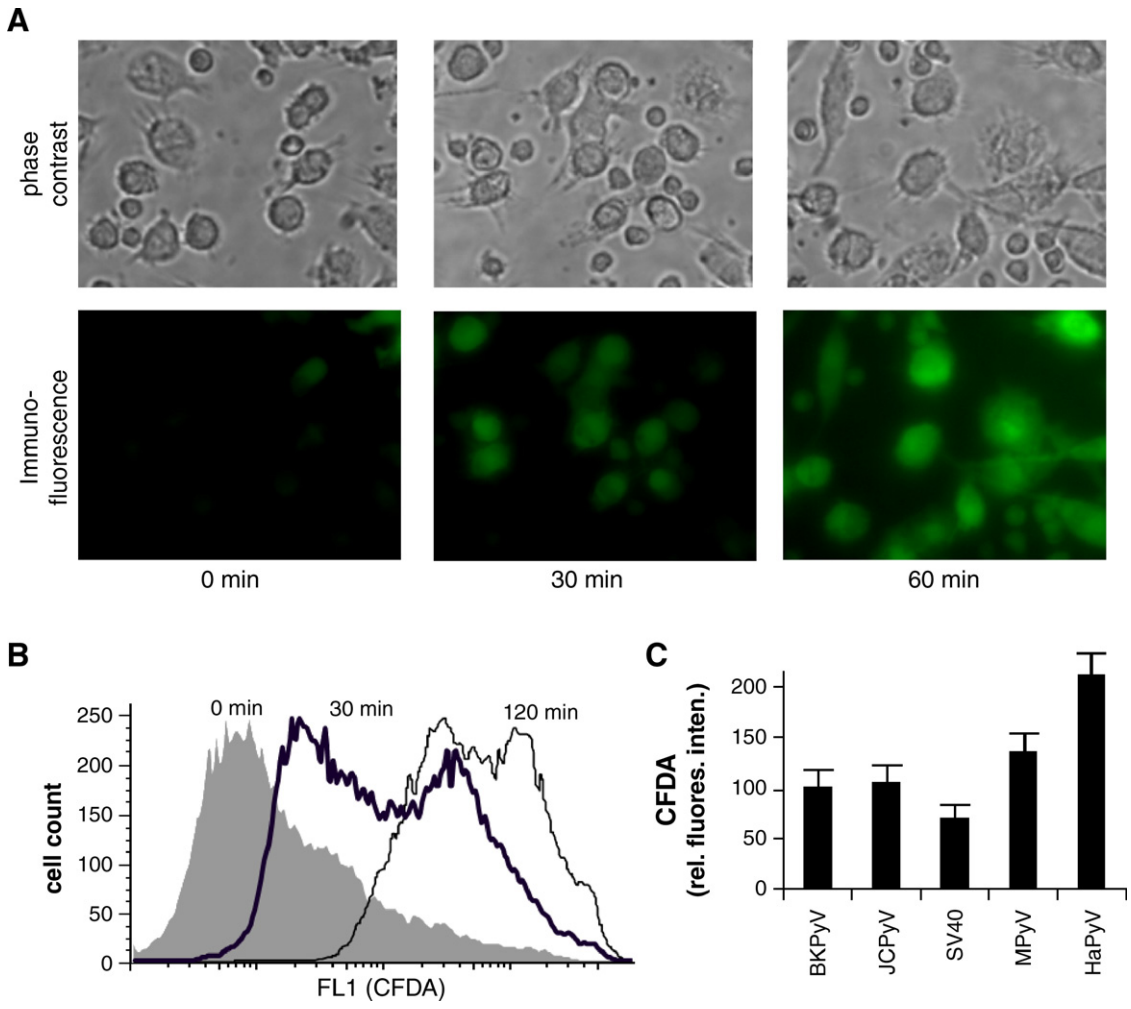

Fig. 5. Uptake of polyomavirus-derived VLPs by hDCs: time course of uptake of CFDA-labeled HaPyV-VP1 VLPs by hDCs determined by fluorescence microscopy (A; lower row; for control, the corresponding representative areas of phase contrast images are given in the upper row) and FACS analysis (B; one representative of three experiments) and comparison of the fluorescence intensity of hDCs after 2 h of incubation with CFDA-labeled VLPs of various polyomaviruses (C; the average with standard deviation of three independent experiments is given).

antigen preparation itself (quality), (ii) level of uptake and (iii) receptor association and uptake.

Recently, pentamers of carboxy-terminally truncated VP1 of MPyV were found to be unable to induce host-specific cytokines whereas on the contrary infectious virus and corresponding VP1-derived VLPs were capable to elicit a host-specific cytokine response in mice (Velupillai et al., 2006). As reported by us earlier and confirmed by a large number of independent expression and purification studies (Sasnauskas et al., 2002), VP1 proteins of mammalian polyomaviruses efficiently form VLPs. We have never seen pentamers or sub-capsid assemblies in our electron microscopic images of purified polyomavirus VLPs. If assuming that there will be a low level of non-VLP structures in our preparations (pentamers or sub-capsid assemblies) that is not detectable by electron microscopy, as we have never seen differences in the assembly capacity of these VP1 proteins, we would not expect differences in the level of these contaminations between the VLPs of the different mammalian polyomaviruses used in this study. Therefore, it seems to be very unlikely that pentamers or sub-capsid assemblies in some VLP preparations inhibit the response in our hDC studies. In contrast, the assembly capacity of VP1 of avian polyomaviruses, at least in our yeast system, was found to be less efficient (Sasnauskas et al., 2002; Zielonka et al., 2006).

To study entry pathways and uptake levels of papillomavirus-derived VLPs, VLPs were labeled with CFDA (Fausch et al., 2003) or by generating an eGFP-VLP fusion (Peng et al., 1999). As site #4 in HaPyV-VP1 has been found, in line with the corresponding site in MPyV-VP1 (Gleiter et al., 1999), to tolerate large foreign insertions, we generated a HaPyV-VP1 derivative harboring the eGFP insert at this position. The behavior of VP1/eGFP in the CsCl gradient and of the assembled VLPs in the negative stain electron microscopy confirmed the formation of VLPs, but slightly deviated from the properties of authentic VP1 VLPs. Nevertheless, these chimeric VLPs were able to demonstrate the uptake of HaPyV-derived VLPs by hDCs (this paper) and murine DCs (Zvirbliene et al., 2006) and might represent a useful tool for entry studies on HaPyV which were hampered by the lack of an efficient cell

culture system (Scherneck et al., 2001). To exclude any influence of structural peculiarities due to the large eGFP insert on the entry kinetics of HaPyV-derived VLPs, the comparative study on the uptake level of different polyomavirus VP1-derived VLPs was performed using CFDA-labeled VLPs. Based on CFDA-labeled HaPyV-derived VLPs, a routine protocol to study VLP uptake was established. This study revealed that the level of uptake corresponds to the induced maturation and functional activity of hDCs; the highest level of uptake was observed for VLPs originating from HaPyV, followed by MPyV, and the primate polyomaviruses.

The various uptake levels of the VLPs by hDCs might be the consequence of individual receptor association differences. Indeed, the tested VLPs differed in their ability to hemagglutinate guinea pig erythrocytes. In line with previous data for the corresponding infectious viruses (Bolen and Consigli, 1979; Knowles et al., 2003), JCPyV-, BKPyV- and MPyV-derived VLPs were found to agglutinate, whereas HaPyV- and SV40-derived VLPs did not. In contrast, mouse pneumotropic virus-derived VLPs were recently found to fail to hemagglutinate erythrocytes (Tegerstedt et al., 2003). Although initial data of competitive entry inhibitor studies suggest similar entry pathways for HaPyV-, SV40- and MPyV-derived VLPs, the varying levels of inhibition observed might indicate a certain degree of diversity (our unpublished data). These differences might be caused by unique receptors used for SV40 and MPyV entry (Basak et al., 1992; Breau et al., 1992; Stehle et al., 1994; Tsai et al., 2003). These variations among VP1-derived VLPs originating from different polyomaviruses in hemagglutination activity as well as entry inhibition experiments might reflect the diversity in receptor recognition among polyomaviruses.

In our study, the maturation potential of HaPyV-derived VLPs was found to be slightly stronger than that of MPyV-derived VLPs. Recently, it was described that authentic VP1 and VP1/VP3-eGFP MPyV-derived VLPs failed to induce an upregulation of hDC maturation markers but readily triggered an IL-12 secretion (Boura et al., 2005). This discrepancy to our data might be explained by the different expression systems used as well as experimental details.

The strong potential of HaPyV-derived VLPs is in line with earlier data on the strong immunogenicity of these VLPs in mice. As also observed for papillomavirus-derived VLPs, yeast-expressed chimeric HaPyV-VP1 VLPs are able to induce strong insert-specific immune responses without additional adjuvants (Gedvilaite et al., 2004). Moreover, chimeric HaPyV-VP1-derived VLPs were demonstrated to represent a useful tool for generating monoclonal antibodies with desired specificity (Zvirbliene et al., 2006). In addition, the yeast-expressed HaPyV-VP1 was found to tolerate medium-sized foreign insertions (120 aa in length) at certain insertion sites (Gedvilaite et al., 2000, 2004). This high insertion capacity of HaPyV-VP1 is here confirmed by the formation of chimeric VLPs harboring an eGFP at site #4 of HaPyV-VP1.

The observed differences between VLPs originating from rodent and primate polyomaviruses might reflect the coevolution of these viruses and their natural hosts. Another explanation for the different levels of maturation induced by VLPs from rodent polyomaviruses (HaPyV and MPyV) and from primate polyomaviruses (SV40, JCPyV, BKPyV) might be that rodent polyomavirus-derived VLPs more extensively trigger pattern recognition receptors inducing hDC maturation than primate polyomavirus-derived VLPs. As a consequence, the antiviral T-cell response can be more efficient. In line with this assumption, the frequency of JCPyV-VP1-specific CTLs directed to two HLA-A*0201-restricted epitopes was found to be low compared to CTL frequencies observed for other DNA viruses like herpesviruses EBV, CMV and HSV (Du Pasquier et al., 2003). In addition, in a previous study, baculovirus-expressed human polyomavirus JCPyV- and BKPyV-derived VP1 VLPs failed to induce maturation of murine DCs, even at a concentration 100 times higher than that sufficient for phenotypic maturation by papillomavirus-derived VLPs (Lenz et al., 2001). Similarly, MPyV was found to infect myeloid- and lymphoid-related murine DCs resulting in an efficient induction of polyomavirus-specific CD8-positive T cells (Drake et al., 2000, 2001). To allow a comparison of the maturation potential of VLPs of rodent and primate polyomaviruses in hDCs and murine DCs, future studies should explore these VLPs expressed in the same heterologous system.

In conclusion, monocyte-derived hDCs internalized the tested VP1-derived VLPs with different efficiencies. Exposure to VLPs originating from HaPyV and MPyV thereby induced maturation of hDCs resulting in activation of CD8-positive T-cells. In contrast, VLPs derived from human polyomaviruses (BK and JC) as well as SV40 were less potent. These data offer important implications for development of polyomavirus VLP-derived vaccines and gene transfer carriers and confirmed the value of the HaPyV-based VLP system for these applications.

Materials and methods

Antibodies

For the phenotypic flow cytometry analysis of hDC surface markers, the following antibodies were used: anti-CD3 (clone OKT3), anti-CD8 (clone HIT8a), anti-CD14 (clone M5E2), anti-CD80 (clone MAB104), anti-CD83 (clone HB15e), anti-CD86 (clone IT2.2), anti-HLA *ABC* (clone WG/32) and anti-HLA *DR DQ* (clone Tü39). All antibodies were obtained from BD Pharmingen (Heidelberg, Germany). The secondary antibodies, PE-labeled goat-anti-mouse-IgG and FITC-labeled anti-mouse-IgG, were purchased from Biozol Diagnostica (Eching, Germany) and BD Pharmingen, respectively.

Generation of a HaPyV-VP1/eGFP fusion construct

All DNA manipulations were performed according to standard procedures. Recombinants were screened in *E. coli* K12 DH5α cells. The coding sequence for eGFP was introduced in frame into position #4 (corresponding to aa 288–295) of the HaPyV-VP1-encoding sequence. For expression in yeast, the VP1/eGFP fusion gene was cloned into the pFX7 plasmid (Sasnauskas et al., 1999).

Expression and purification of VLPs

VLPs consisting of the major capsid protein VP1 of HaPyV, MPyV, SV40 or human polyomaviruses JCPyV and BKPyV as well as chimeric VP1/eGFP VLPs were generated in yeast *S. cerevisiae* and purified by CsCl gradient centrifugation as described previously (Sasnauskas et al., 1999, 2002). As a control ("mock VLPs"), a lysate of pFX7-transformed and induced yeast cells was treated in the same way. A fraction of the CsCl gradient corresponding to the fraction where usually VP1-VLPs can be recovered was collected. VLP-containing CsCl gradient fractions were collected and dialyzed against PBS. Subsequently, the dialyzed samples were either mixed with glycerol (50%) or lyophilized and stored at −20 °C until further use.

SDS polyacrylamide gel electrophoresis and Western blot analysis

After VLP purification by CsCl gradient ultracentrifugation and dialysis against PBS, aliquots of protein were boiled and separated by 12% SDS polyacrylamide gels. Subsequently, gels were stained by Coomassie blue or transferred onto PVDF type Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA, USA) by semidry blotting. After transfer, the membranes were blocked for 1 h with TBS (0.1 M Tris, 0.3 M NaCl, pH 7.4) with 5% dry milk/0.1% Tween 20. Thereafter, the membranes were incubated for 2 h in rabbit sera raised against *E.-coli*-expressed, entire HaPyV-VP1 (Voronkova et al., 2002) or GFP (Invitrogen, Groningen, The Netherlands) diluted 1:1000 and 1:5000, respectively, in TBS/0.1% Tween 20. As secondary antibody, the horse radish peroxidase (HRP)-labeled anti-rabbit IgG conjugate (Amersham Life Science, Hercules, CA, USA) was applied for 2 h. The HRP-mediated staining was performed by adding 4-chloro-1-naphtol and H_2O_2 (Fluka, Buchs, Switzerland).

Electron microscopy

To confirm the formation of VLPs consisting of unmodified VP1 from the different polyomaviruses and of HaPyV-VP1/eGFP, CsCl gradient fractions were placed on 400-mesh carbon coated palladium grids. Samples were stained with 2% aqueous uranyl acetate solution and examined with a JEM-100S electron microscope.

Generation of human monocyte-derived DCs

Isolation and cultivation of monocyte-derived hDCs were performed as previously described (Pecher et al., 2002). Briefly, heparinized (100 U/ml heparin, Liquemin; Hoffmann-La Roche, Grenzach-Wyhlen, Germany) blood was drawn from healthy donors and diluted with 1 volume RPMI-1640 medium (Gibco). Peripheral blood mononuclear cells (PBMC) were isolated over a Lymphoprep-Plaque (1.077 g/ml; Nycomed). The PBMC were resuspended in RPMI-1640 and thrombocytes were removed by washing four times by centrifugation (1000 rpm, 10 min, 4 °C). Then, PBMC were pelleted, resuspended in X-vivo 15 (BioWhittaker; Walkersville, Maryland) and seeded at a concentration of 10^7 PBMC in 3 ml of medium per well into 6-well plates (Falcon) to separate monocytes by adhesion to plastic. After incubating for 2 h at 37 °C, monocytes were obtained by gently washing away the contaminating non-adherent cells with pre-warmed RPMI-1640. Immature hDCs were generated from adhesion-purified monocytes by cultivating cells for 6 days in X-vivo 15 supplemented with 500 U/ml human recombinant IL-4 (AL-ImmunoTools, Friesoythe, Germany) and 1000 U/ml recombinant human granulocyte-macrophage colony-stimulating factor (hGM-CSF). Half of the medium was changed every 2 days, and cytokines added in the concentrations described above.

DC stimulation and pulsing

Immature hDCs were incubated with 10 μg/ml VLPs or controls for 48 h at a concentration of 10^6 cells/ml of medium containing 1000 U/ml hGM-CSF. As positive control, mature hDCs were generated by incubation with 1 μg/ml of LPS (Sigma, Deisenhofen, Germany). In addition to untreated cells, a parallel preparation from pFX7-transformed and induced yeast cells was used as a negative control ("mock VLPs"). Cells were harvested, washed with PBS and subsequently stained for FACS analysis or used for T-cell stimulation. Supernatants were collected for IL-12 ELISA.

Flow cytometry of human DCs

After pulsing of hDCs, cells were harvested, washed with PBS and subsequently stained for FACS analysis. To this end, hDCs (1×10^5) were resuspended in FACS buffer (PBS containing 2% FCS) and incubated with specific antibodies against the following markers: CD83, CD80, CD86, HLA ABC and HLA DR DQ. After 1 h of incubation, cells were washed and stained with a PE-labeled secondary antibody for 1 h on ice. Cells were analyzed using a FACSCalibur (Becton Dickinson, Heidelberg) with CellQuest software (BD Pharmingen).

Determination of phagocytotic capacity

Pulsed hDCs were incubated with FITC–dextran (10 μg/ml) or Lucifer Yellow (4%) for 1 h at 37 °C and 4 °C. The cells were then washed three times with PBS and analyzed by flow cytometry. The receptor-mediated uptake of FITC–dextran or macropinocytosis of Lucifer Yellow was determined by calculating the difference of the mean fluorescent intensities at 37 °C and 4 °C. FITC–dextran was purchased from SIGMA (Deisenhofen, Germany) and Lucifer Yellow from Molecular Probes (Leiden, The Netherlands).

IL-12 assay

For the IL-12 ELISA, the supernatant of 10^6 hDCs stimulated with 10 μg/ml VLPs for 48 h was collected. As negative control, "mock VLPs"-treated cells were used. As positive control, hDCs were treated with 1 μg/ml of LPS. Supernatants were collected

after spinning cells at 1000 rpm for 10 min. Subsequently, the amount of IL-12 was determined with a commercially available total IL-12 (p70 and p40) assay (Pierce Endogene, Cambridge, MA) according to the protocol of the manufacturer.

T-cell stimulation assays

The specific stimulation of CD8-positive T cells with VLPs was determined evaluating 5×10^5 autologous T cells and using a commercially available IFN-gamma secretion assay following the manufacturers' protocol (Miltenyi Biotec). T cells were placed into 24-well plates (NUNC) at 1×10^6 cells/ml and were stimulated with pulsed hDCs at a T cell/hDC ratio of 20:1 and 100 U/ml human recombinant interleukin-2 (IL-2; PeproTech, London, UK). The hDC pulsing was performed as described for the maturation analysis. As negative control, "mock VLPs"- and untreated hDCs or LPS-maturated hDCs were included. After 5 days in co-culture, T cells were supplemented with 100 U/ml IL-2 and restimulated with hDCs as before. Four days after the second stimulation, T cells were prepared for T-cell stimulation assays. The T cells were restimulated for 16 h with VLPs (10 μg/ml) at 37 °C. To prepare for staining, cells were washed twice with buffer (PBS with 1% FCS). The stimulated T cells were then incubated with the IFN-gamma catch reagent for 5 min followed by a 45 min secretion phase at 37 °C. After the secretion period, the cells were washed and incubated on ice with the PE-labeled IFN-gamma detection antibody and CD8-FITC mAb for 35 min. The number of IFN-gamma secreting CD8-positive T cells was calculated as a percentage of IFN-gamma and CD8-positive cells minus the number of IFN-gamma- and CD8-positive cells in the negative control to the total number of CD8-positive T cells.

Uptake of VLPs by human DCs

VLPs were labeled with 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFDA; SIGMA-Aldrich, Munich, Germany) according to a recently described protocol for papillomavirus-derived VLPs (Fausch et al., 2003). Briefly, VLPs were incubated with CFDA at room temperature. After 4 h of incubation, VLPs were diluted 1:15 with PBS and ultracentrifuged at 36,000 rpm at 4 °C for 1 h. The pellet was resuspended in 0.5 ml PBS and added to the hDCs at a concentration of 10 μg/ml VLP-CFDA for 12 h at 37 °C. The fluorescence intensity of the hDCs was measured by flow cytometry.

Hemagglutination (HA) assays

VLPs were tested for their ability to hemagglutinate guinea pig erythrocytes by incubating 0.1 ml of serially diluted VLPs (50 ng/μl–0.025 pg/μl) with 0.1 ml of a 0.4% guinea pig erythrocyte suspension at 4 °C for 4 h.

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