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Polyomavirus EGFP-pseudocapsids: Analysis of model particles for introduction of proteins and peptides into mammalian cells

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Abstract A vector for preparation of mouse polyomavirus capsid-like particles for transfer of foreign peptides or proteins into cells was constructed. Model pseudocapsids carrying EGFP fused with the C-terminal part of the VP3 minor protein (EGFP-VLPs) have been prepared and analysed for their ability to be internalised and processed by mouse cells and to activate mouse and human dendritic cells (DC) in vitro. EGFP-VLPs entered mouse epithelial cells, fibroblasts and human and mouse DC efficiently and were processed by both, lysosomes and proteasomes. Surprisingly, they did not induce upregulation of DC co-stimulation molecules or maturation markers in vitro; however, they did induce interleukin 12 secretion.

Keywords: Mouse polyomavirus; Empty artificial virus-like particle; Dendritic cell activation; Vector for peptide transfer

1. Introduction

Viral coats were selected by evolution for efficient entry of viruses through the plasma membrane into cells and for delivery of genetic information into the cell nucleus or other cell compartments for virus gene expression. Viruses thus represent natural, highly specialised transfer vehicles.

Polyomaviruses, small non-enveloped DNA viruses, have a wide range of hosts, including human and different pathogenic responses in the infected organisms. Despite this variation, these viruses share similar virion structure. The icosahedral capsid formed by three structural proteins, VP1, VP2 and VP3, is arranged around the nucleocore consisting of 5.3 kbp circular genomic DNA, cellular histones (except H1) and VP1 protein. The crystal structure of two polyomaviruses (mouse polyomavirus (PyV) and Simian virus 40 (SV40)) has been determined. The capsid shell is composed of 72 capsomeres – pentamers of VP1 molecule. The major structural protein, VP1, of the PyV is formed by a sandwich core of β-sheets with several outfacing loops [1,2], it is able to self-assemble into capsid-like particles and is responsible for interaction with sialic acid of ganglioside receptor [2,3]. VP1 non-specific DNA-binding activity [4] suggests its role in the nucleocore assembly. Neither VP2 nor VP3, which seem to interconnect the VP1-surface lattice and the nucleohistone core [5], are required for the capsid-like structure assembly and their functions are still unclear. Nevertheless, PyV with the genome mutated in the ATG codon for either VP2 or VP3 is not infectious. Also, the absence of myristyl moiety on the N-terminal amino acid of VP2 results in a substantial decrease of polyomavirus infectivity [6,7]. On the basis of the unique properties of VP1 (ability of spontaneous self-assembly, receptor recognition, nuclear localisation signal, DNA non-specific binding activity), studies have been performed with the aim to use empty artificial murine polyomaviral virus-like particles (VLPs or VP1 pseudocapsids) for gene transfer [8–10]. VLPs derived from mouse PyV (PyV-VLPs) can cross the outer membrane of a variety of cell types – including cells of human origin [9,11]. Studies in vivo demonstrated: (i) the ability of PyV-VLPs to target many organs/tissues of mouse and (ii) the possibility to introduce them into experimental animals by different routes, including intranasally. VLPs were shown to be able to cross the blood–brain barrier since gene expression of the delivered gene in the brain was observed [12]. Several studies were performed to follow humoral and cellular immune responses to PyV VP1 pseudocapsids. They showed that such VLPs provoked a strong cellular T helper 1 (Th1) and humoral response and could protect mice against polyomavirus infection [13–15]. High immunogenicity of VLPs offers an exploitation of virus structures for development of vaccines based on VLPs as carriers for the delivery of other disease antigens.

In this study, we have focused on development of chimeric polyomavirus capsid structures for the transfer of epitopes

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Abbreviations: AcNPV, *Autographa californica* nuclear polyhedrosis virus; BMDC, bone marrow-derived dendritic cells; CFSE, carboxyfluorescein diacetate succinimidyl ester; CM, complete culture medium; DC, immature human monocyte-derived dendritic cells; EEA1, early endosomal antigen; EGFP, enhanced green fluorescent protein; FCS, foetal calf serum; FSC and SSC, forward and side scatter characteristics; EM, electron microscopy; GM-CSF, granulocyte macrophage-colony stimulating factor; LAMP2, lysosomal associated membrane protein; LPS, lipopolysaccharide; NMuMG, normal murine mammary gland cells; p.a., post adsorption; p.i., post infection; PFU, plaque forming unit; poly(I:C), inosine-cytosine dsRNA polynucleotide; PyV, mouse polyomavirus; t-VP3, truncated, C-terminus of VP3 minor capsid protein; VLPs, empty artificial virus-like particles; VP1-VLPs, pseudocapsids composed of VP1 major capsid protein; EGFP-VLPs, pseudocapsids composed of VP1 major capsid protein and EGFP-t-VP3 fusion protein

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and other therapeutic peptides into mammalian cells. As a model protein to be transferred via PyV VLPs, we have chosen the enhanced green fluorescent protein (EGFP). We examined the ability of "green" VLPs to enter cells and followed their fate in the cells. Finally, we examined the effects of "green" particles on activation of dendritic cells.

2. Materials and methods

2.1. Plasmid construction

Sequences encoding the C-terminal fragment of protein VP3 (AA 105–204, nucleotides # 4345-4659) were amplified by PCR using primers: forward: 5′-CATCAGCGAGCTCAGGGTACTC-3′, reverse: 5′-TTAGAGGATCCTTAGAGACGCCGCTT-3′, and the plasmid pMJG (containing the whole PyV genome open in the unique *Eco*RI site; kindly provided by B.E. Griffin) as a template. The PCR fragment as well as the plasmid pEGFP-C2 (Clontech, Palo Alto, CA) were cut with *Sac*I/*Bam*HI and ligated to generate the plasmid pEGFP-t-VP3, where EGFP sequences were fused in frame with truncated VP3 (t-VP3). To generate baculovirus transfer plasmid pAcDB3/VP1/EGFP-t-VP3, sequences encoding the fusion protein EGFP-t-VP3 were amplified by PCR using primers: forward: 5′-AGATAGGATCCACCATGGTGAGCAAG-3′, reverse: 5′-TTAGAGGATCCTTAGAGACGCCGCTT-3′, and the pEGFP-t-VP3 as a template. The PCR product was cut with *Bgl*II/*Bam*HI and inserted into the plasmid pAcDB3/VP1 (with the gene for the major structural protein VP1 under the control of late p10 promoter; kind gift of T. Ramqvist and T. Dalianis) into the *Bgl*II position under the control of a second p10 promoter.

2.2. Cell cultures and viruses

Spodoptera frugiperda (Sf9) cells were grown as monolayer cultures at 27 °C in standard TNF-FK medium containing 10% foetal calf serum (FCS) as described by Hink [16]. *Autographa californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculoviruses were propagated as described by O'Reilly et al. [17]. Recombinant baculovirus AcDB3/VP1/EGFP-t-VP3 was prepared by in vivo allelic exchange between AcNPV DNA and recombinant transfer plasmid pAcDB3/VP1/EGFP-t-VP3, and purified by plaque assays as previously described [17].

Normal murine mammary gland (NMuMG) epithelial cells purchased from the American Type Culture Collection (no. CRL-1636) were grown in Dulbecco's modified Eagle's medium suplemented with 10% FCS (Sigma).

2.3. Human and mouse dendritic cell generation

Complete culture medium (CM) was used for the culture of human lymphocytes and dendritic cells and consisted of RPMI (BioWhittaker, Berkshire, England) supplemented with 10% heat-inactivated FCS (BioWhittaker), 2 mM L-glutamine, and 1% penicillin/streptomycin (BioWhittaker). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. Immature human monocyte-derived dendritic cells (DC) were generated as described previously [18]. Briefly, peripheral blood mononuclear cells were obtained from buffy coats of healthy donors and monocytes were separated by 2 h adhesion in 75 cm^2 culture flasks. Adherent monocytes were cultured for 5 days in CM in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) (Leukine^R) and 15 ng/ml of IL-4 (Peprotech). When indicated, immature DC were activated by the addition of either inosine-cytosine dsRNA polynucleotide (poly(I:C)) 50 μg/ml or lipopolysaccharide (LPS) 10 μg/ml. For generation of mouse bone marrow dendritic cells (BMDC), bone marrow was flushed from femurs of C57BL/10Sn mice. Cells were plated for 5 days in 6 well plates (5×10^5 cells/ml) in 4 ml RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 10 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml) and 5% Ag hybridoma culture supernatant as a source of GM-CSF [19]. Every second day the cultures were fed by aspirating 50% of medium and adding fresh medium with GM-CSF. Mature DC were generated by incubating BMDC with GM-CSF plus 1 μg/ml LPS for the last 18 h.

2.4. Interaction of DC with polyomavirus capsid-like particles

On day 5, immature DC (human or mouse) were seeded in 24-well plates at 5×10^5 cells/ml in CM and cultured with artificial polyomavirus capsid-like particles at the DC:VLPs ratios of $1{:}10^3$, $1{:}10^4$, $1{:}10^5$ and $1{:}10^6$. Functional and phenotypic consequences of DC interaction with VLPs were investigated after 24 h.

2.5. Antibodies

Following primary antibodies were used for cell immunostaining: anti-PyVP1 A mouse monoclonal [8] or mouse or rabbit polyclonal antibody (prepared in our laboratory) against polyomavirus VP1; anti-PyVP2/3A mouse monoclonal antibody against the common region of VP2 and VP3 [8]; goat anti-EEA1 (Santa Cruz) against early endosome antigen EEA1, mixture of mouse anti-α and anti-β tubulin (Exbio) for microtubule staining; mouse monoclonal anti-ubiquitin antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-GFP antibody for EGFP staining (AbCam antibodies); rabbit anti-BiP purified antiserum (Alexis) against the BiP (GRP78) marker of endoplasmic reticulum; rat monoclonal anti-LAMP-2 antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City) against lysosomal associated membrane protein (LAMP2). Secondary antibodies: Alexa Fluor-546 (red) goat anti-mouse, anti-rabbit or anti-rat antibody (Molecular Probes), Alexa Fluor-546 (red) donkey anti-goat antibody (Molecular Probes) or the Alexa Fluor-488 (green) goat anti-mouse or anti-rabbit antibody (Molecular Probes) were used for fluorescence staining. For immunoelectron microscopy, goat anti-mouse IgG antibody conjugated with 5 nm colloidal gold was used (British Biocell Int.). DNA was stained by DAPI. For flow cytometry, FITC or PE-conjugated monoclonal antibodies (mAbs; PharMingen, San Diego, CA) against following molecules were used: CD80, CD83, CD86, CD11c, CD40, CD11c, CD40, HLA-ABC, HLA-DR for human dendritic cells and CD80, CD86, CD11c and MHC class II I-A^b for mouse cells.

2.6. Flow cytometry

DC were stained with antibodies for 30 min at 4 °C, washed twice in PBS + 0.1% bovine serum albumin (BSA) and analyzed in FACS Calibur (Becton Dickinson) using Cell Quest software. DC were gated according to their FSC and SSC properties and dead cells were excluded by TO-PRO3 (Molecular Probes) staining. Appropriate isotype controls were always included and 5×10^3 viable DC were obtained in each experiment.

2.7. FITC-dextran endocytosis

DC (2×10^5 cells) were incubated with 1 mg/ml of FITC-dextran (40 000 mw, Sigma). After 1 h, cold medium was added to stop the experiment. Cells were washed three times with ice-cold PBS and analyzed in FACS Calibur.

2.8. Cytokine detection

After the interaction with polyomavirus capsid-like particles, IL-12 p70 production by human DC and IL-12 p40 production by mouse DC was analyzed in culture supernatants by a standard ELISA kit (Immunotech).

2.9. Induction of lymphocyte proliferation

CD4+ T cells were positively selected using CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of CD4 population used for further studies always exceeded 95%. CD4+ T cells were subsequently labelled by 2 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) [20]. DC (10^4 cells) activated by Poly(I:C), LPS or DC that interacted with VP1 particles were cultured with allogeneic CFSE-labelled CD4+ T cells (10^5 cells) for 4 days and the induction of T-cell differentiation was evaluated by gradual CFSE dilution in dividing T cells by flow cytometry.

2.10. Chemotaxis

DC (5×10^3 cells) in 100 μl of CM were plated in the upper chamber of 24-well transwell plate with 5 μm porous inserts (Corning Costar). Lower chambers contained 600 μl of medium or medium with 50 ng/

ml of chemokine MIP-3β. After 2 h incubation at 37 °C, inserts were lifted and the cells migrated to the lower surface were washed into the lower chamber and counted.

2.11. Isolation of capsid-like particles from insect cells

Insect cells were infected with recombinant baculovirus (10 PFU/per cell). Cells were harvested 72 h post infection (p.i.), lysed, and VLPs were purified by CsCl and sucrose gradients as described previously [8].

2.12. Immunofluorescence of cells

VLPs were applied to cells grown on coverslips. At the indicated times post adsorption (p.a.), the cells were washed with PBS, fixed with 3% paraformaldehyde in PBS (15 min at 4 °C) and permeabilised with 0.5% Triton X-100 (Sigma) in PBS (5 min). Fixed cells were washed with PBS and blocked in 0.25% BSA (Sigma) and 0.25% porcine skin gelatine (Sigma) in PBS. Incubation with primary and secondary antibodies was carried out for 1.5 and 0.5 h, respectively, with extensive washing with PBS after each of incubations. Coverslips were mounted on droplets of glycerol with or without DAPI and samples were observed in a Leica TCS SP2 confocal microscope.

2.13. Electron and immunoelectron microscopy

Mouse 3T6 or NMuMG cells grown on coverslips were infected with pseudocapsids composed of VP1 major capsid protein and EGFP-t-VP3 fusion protein (EGFP-VLPs) (10^4–10^5 particles/cell). At appropriate times p.a., cells were processed for transmission electron microscopy. Briefly, the infected cells were washed in PBS, fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide, dehydrated through increasing ethanol series (including 30 min contrasting in 1.5% uranylacetate in 70% ethanol) and propylenoxide, infiltrated, and flat embedded in Agar 100 resin (Gröpl, Tulln, Austria). Ultrathin sections were contrasted with a saturated uranyl acetate solution and Reynold's lead citrate. Isolated VLPs (or capsomeres) were immunostained against VP1 and EGFP by the direct immunoelectron microscopy technique. VLPs were adsorbed on carbon-coated parlodion membrane on electron microscopy (EM) grids, activated with glow discharge. Grids were then incubated on drops of primary antibodies, washed intensively in PBS and exposed to colloidal gold-conjugated secondary antibodies. Grids were washed again in PBS and redistilled H_2O, contrasted on a drop of 2% phosphotungstic acid (pH 7.0) and dried. Samples were observed in a JEOL 1200EX electron microscope operating at 60 kV.

3. Results

3.1. Construction of a baculovirus for the production of mouse polyomavirus EGFP-VLPs and their characterisation

The construction has been designed to introduce EGFP as a fusion protein with the sequence of PyV minor capsid proteins responsible for their interaction with the central cavity of polyomavirus VP1 pentameric capsomeres (Fig. 1(a)). EGFP fused with the C-terminal VP3 sequence was cloned into the *Bgl*II site (under the baculovirus late promoter p10) in baculoviral transfer plasmid pAcDB3 carrying the VP1 gene (in the *Sma*I site, under the second p10 promoter). Isolated recombinant baculovirus obtained by recombination in Sf9 insect cells gave green plaques (Fig. 1(b)) and cell lysates were positive for both, VP1 and fusion protein composed of EGFP and t-VP3 (not shown). Confocal microscopy showed nuclear localisation of the EGFP-t-VP3 product (Fig. 1(c)). We have observed previously that neither VP2 nor VP3 protein have nuclear localisation in insect cells when expressed separately. VP3 remained spread in the cytoplasm and its nuclear localisation appeared only in cells co-expressing the VP1 gene. This suggests that VP3 and apparently also EGFP-t-VP3 were transported into the cell nucleus as a complex with the major structural protein, VP1 [3].

When lysate of cells containing VP1 and EGFP-t-VP3 proteins was analysed by CsCl or sucrose gradient ultracentrifugation, we observed the green coloured band to co-purify with the VP1 band as proved by gradient fractionation and immuno-dotblot (not shown). EM analysis (Fig. 2) proved that this fraction contains high concentration of capsid-like particles. Particles isolated from cell lysates containing VP1 only are presented for comparison (Fig. 2, third panel).

In agreement with our assumption that EGFP-t-VP3 should be situated inside the capsid-like particle, antibody against GFP (5 nm gold) was observed mainly in connection with single capsomeres, or with partially disassembled but, not with intact VLPs (Fig. 2, left panel), while antibody against VP1 decorated particles with a high efficiency (Fig. 2, middle panel). Often imperfectly assembled "green" VLPs were of irregular shape in comparison to pseudocapsids formed by VP1 only. Nevertheless, green particles were stable during gradient centrifugations, dialysis and during (at least) 1 month storage at 4 °C.

3.2. EGFP-VLPs enter mammalian cells efficiently

Green particles were adsorbed efficiently on the surface of mouse NMuMG epithelial cells and became internalised by tightly fitted smooth invaginations (Fig. 3). Thirty minutes p.a., fusions of vesicles carrying particles into endosomes containing more then one particle could be observed by EM (Fig. 4A). However, these membrane structures could also represent one multivesicular body of inter-connected membrane cisternae. At the same time (30 min p.a.), confocal fluorescence microscopy showed co-localisation of particles with the early

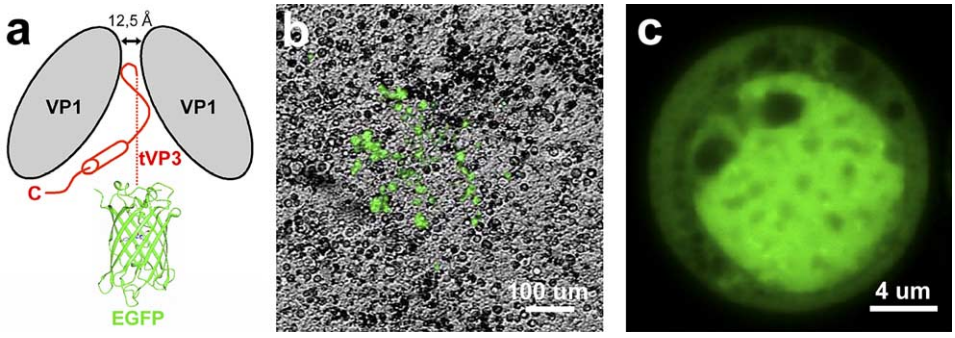

Fig. 1. Design, production and detection of EGFP-VLPs. Schematic view of constructed EGFP-VLP (a). Plaque of the recombinant baculovirus producing the EGFP-t-VP3 and VP1, selectable on the basis of its green fluorescence (b). The fusion protein EGFP localised in the nucleus of insect Sf9 cells (confocal section of a live cell) infected with recombinant baculovirus Ac-VP1-EGFP-t-VP3 60 h p.i. (c).

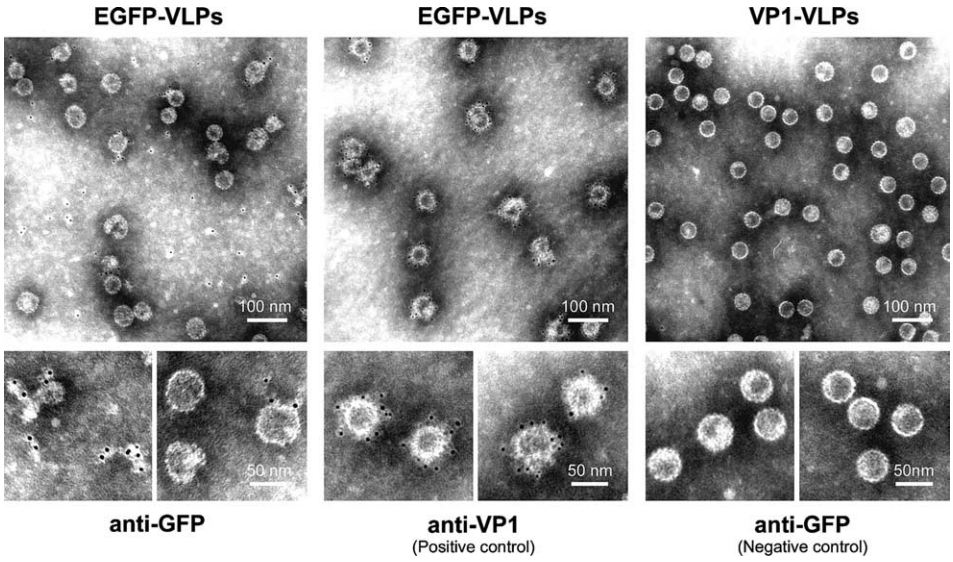

Fig. 2. Direct immunoelectron microscopy of EGFP-VLPs. Isolated "green" VLPs adsorbed on EM grids and immunostained by rabbit anti-GFP and rabbit anti-VP1 polyclonal serum, respectively, followed by goat anti-rabbit immunoglobulin antibody conjugated with 5 nm gold particles. VP1-VLPs were used as EGFP negative control. Lower panel represents detailed enlargements.

endosome antigen (EEA1) (Fig. 4B) and later (3 h p.a.), the signal of particles merged partially with BiP-marker of endoplasmic reticulum (ER) (Fig. 5). To enhance green EGFP signal of VLPs, VP1 protein of VLPs was immunostained using Alexa Fluor-488 (green) secondary antibody (with the exception of Fig. 7).

3.3. VLPs are degraded by both lysosomes and proteasomes

Before the signal of EGFP pseudocapsids began to cumulate in perinuclear space (90 min p.a.), co-localisation of VLPs and LAMP2 marker of lysosomes (Fig. 6(b) and (c)) was observed, suggesting that a subpopulation of VLPs became degraded in lysosomal compartments. EM of ultrathin cell sections also confirmed fusion of vesicles carrying VLPs with lysosomes (Fig. 6(a)).

Later, 6 h p.a., the majority of the VP1 and EGFP-t-VP3 was cumulated in perinuclear space, partly still in the co-localising pattern (Fig. 7, upper panel). At the same time, fluorescent signals of VP1 (red) and EGFP-t-VP3 (green) began to translocate from perinuclear space into the cell periphery and were separated from each other suggesting previous dissasembly of "green" VLPs (Fig. 7, lower panel). At that time, no in-

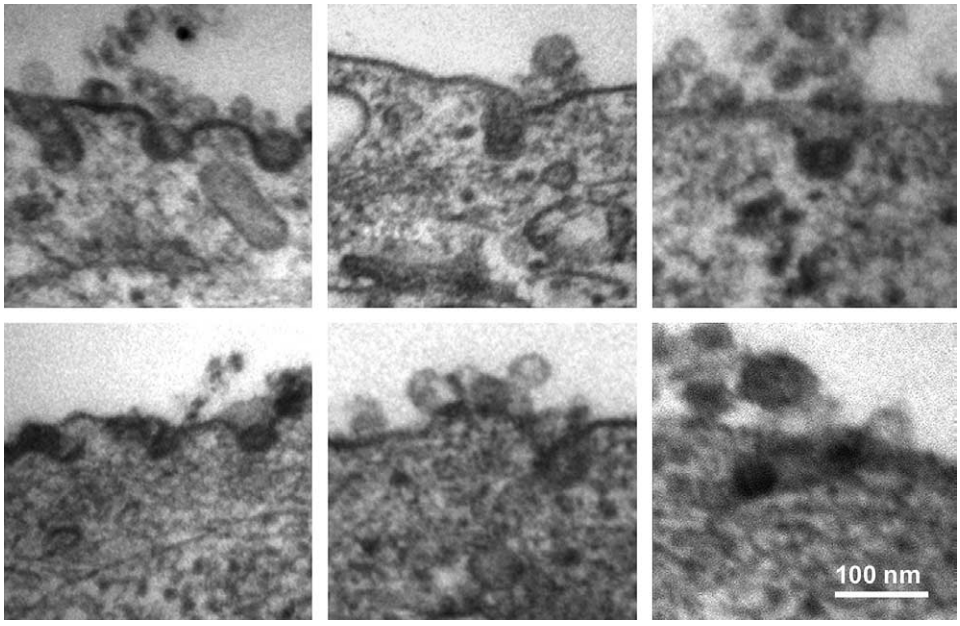

Fig. 3. EGFP-VLPs are efficiently internalised by NMuMG cells. After binding to the cell surface, EGFP-VLPs enter the cytoplasm in smooth membrane invaginations. Electron microscopy of ultrathin sections. Adsorption performed with multiplicity of 10^4 particles per cell; Cells were fixed 20 min p.a.

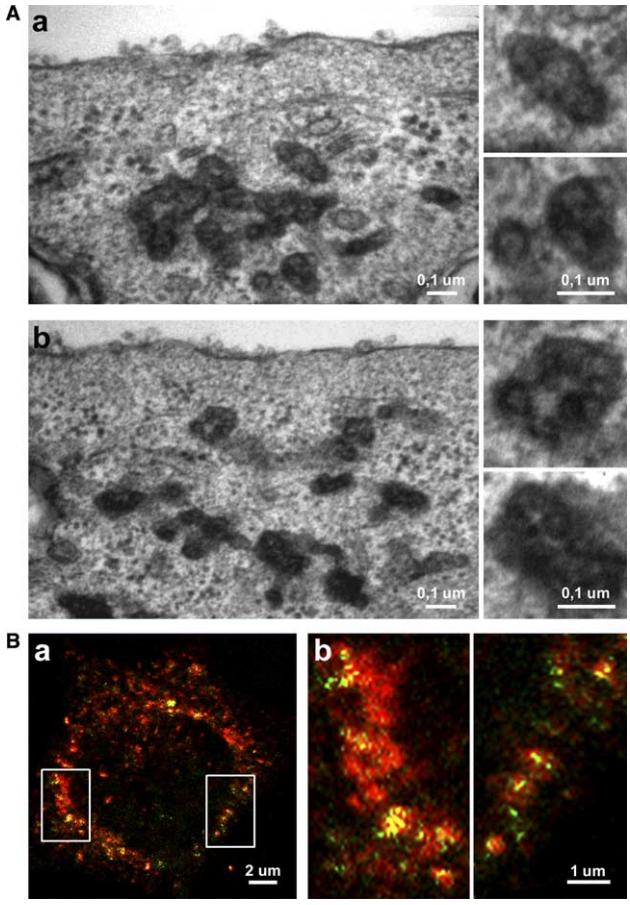

Fig. 4. Monopinocytic vesicles carrying EGFP-VLPs fuse with early endosomes. (A) Electron microscopy of 3T6 (a) and NMuMG (b) cells 30 min p.a. Adsorption performed with multiplicity of 10^4 particles per cell. Enlarged details of ultrathin sections (on the right). (B) Co-localisation of VLPs (green) with EEA1 marker of early endosomes (red). Merged image of a confocal section of the NMuMG cell fixed 30 min p.a. Adsorption performed with multiplicity of 10^2 particles per cell (a) with enlarged details (b).

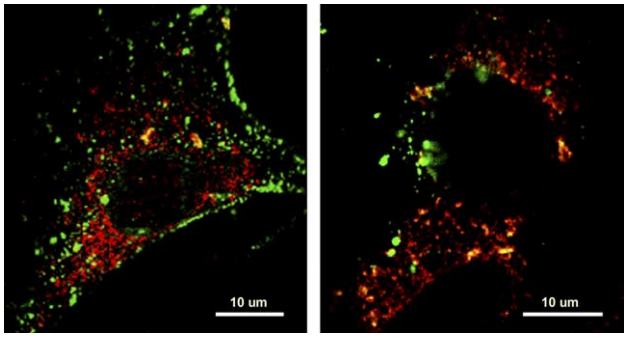

Fig. 5. Co-localisation of EGFP-VLPs with the Bip marker of ER. NMuMG cells fixed 3 h p.a. Adsorption performed with multiplicity of 10^2 particles per cell. (EGFP-t-VP3 + VP1 - green, Bip - red). Sections of different cells, merged images.

tact virus particles were detected by EM on ultrathin sections (not shown). Massive co-localisation of ubiquitin and EGFP-VLP signal was observed at 8 h p.a., when the majority of VLP signal appeared back in the cytoplasm (Fig. 8).

3.4. EGFP particles enter mouse and human dendritic cells

The important point in exploitation of artificial mouse VLPs carrying specific epitopes as vaccines is whether these particles are able to adsorb on, enter and activate professional antigen presenting cells (APCs). Therefore, we followed (in vitro) entry and trafficking of green VLPs into human and mouse dendritic cells and examined markers of their activation. Confocal and EM revealed that VLPs enter both mouse and human DC efficiently. The endocytic pathway of VLPs in DC resembled that observed in NMuMG cells. The VLP signal (EGFP signal enhanced by "green" immunostaining of VP1) moved via early endosomes into perinuclear space and later (from 6 h p.a.), the VLP signal moved back into the cytoplasm. Finally, 24 h p.a., it could be observed near to and on the surface of dendritic cells (Fig. 9). At that time, substantial co-localisation of VLP signal with ubiquitin was observed (not shown).

3.5. Mouse polyomavirus VLPs induce production of IL-12 by DC but do not activate other DC markers in vitro

Untreated human DC produced already relatively high levels of CD80 and especially CD11c, but low levels of CD83 and 86. When inoculated with pseudocapsids composed of VP1 major capsid protein (VP1-VLP) or VP1/EGFP-t-VP3 VLPs for 24 h, no substantial upregulation of CD80, CD83, CD86 and HLA-DR markers was observed (Fig. 10). Also, analysis of functional characteristics: (i) phagocytic capacity, (ii) migratory capacity of DC in response to MIP-3β and (iii) T-cell allostimulatory capacity of DC did not demonstrate convincing activation of human DC by polyomavirus VLPs (Fig. 11a, b and c). On the other hand, secretion of interleukin 12 (p70 IL-12 heterodimer) was induced by incubation of human DC with EGFP-VLPs (Fig. 11d).

Similar to human DC, no substantial upregulation of CD80, CD86 or MHCII molecules was observed in mouse DC 24 h p.a. of VLPs composed of the VP1 only or VLPs carrying EGFP-t-VP3 fusion proteins (not shown). The induction of (p40) IL-12 secretion in mouse DC was achieved by high concentrations of VP1-VLPs or EGFP-VLPs (10^6–10^7 per cell; Fig. 11e). Nevertheless, the same protein concentrations of bovine serum albumin added to DC did not induce IL-12 production (not shown). There was a different correlation between production of human and mouse DC and a total dose of VLPs used for activation of cells. Production of IL-12 by human cells increased with higher VLPs concentration up to 10^4 VLPs/cell but, decreased when higher dose was used (10^5 VLPs/cell). On the other hand, IL-12 production by mouse DC increased with higher doses of VLPs up to 10^7 VLPs/cell. This observation can be a result of different sensitivity of human and mouse DC to the presence of PyV VLPs. However, in contrast to flow cytometry, in these experiments, no correction for numbers of dead cells was performed.

4. Discussion

Virus-like particles of many viruses were found to be potent inducers of immune responses against viral capsid proteins without the need for adjuvans [21–23]. Immunisation with papillomavirus L1 VLPs can induce high titres of neutralising antibodies that confer protection against experimental papillomavirus challenge in animal papillomavirus models [24–26].

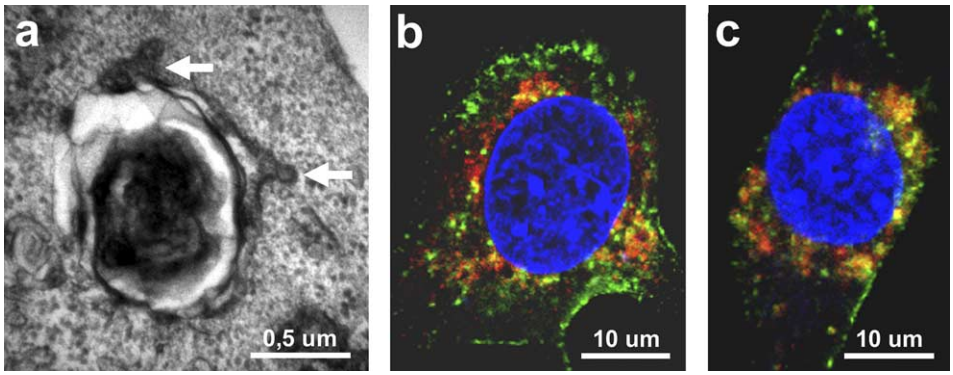

Fig. 6. Subpopulation of EGFP-VLPs is degraded in lysosomes. Electron microscopy of ultrathin section (a): white arrows show fusion of VLP-containing vesicles with the lysosome. Co-localisation of LAMP2 (red) and EGFP-t-VP3 +VP1 (green) on confocal sections of two different cells, merged images (b,c). NMuMG cells fixed 90 min p.a. Adsorption performed with multiplicity of 10^3 particles per cell.

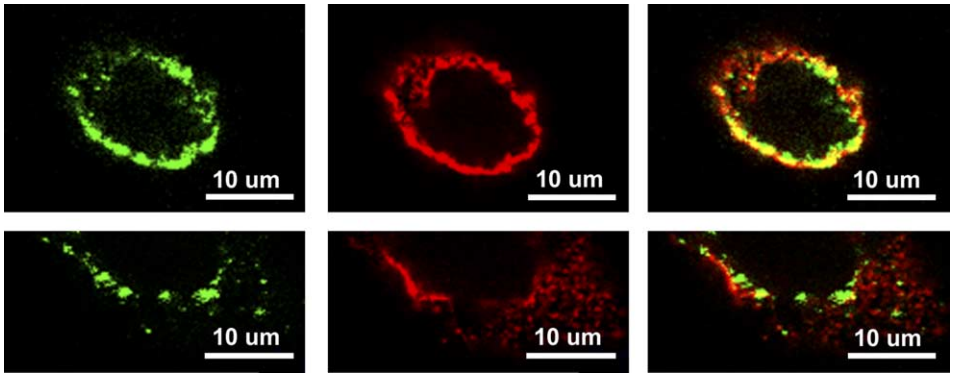

Fig. 7. Co-localisation of EGFP-t-VP3 (green) **and VP1** (red) in NMuMG cell 6 h p.a. The majority of the VLPs in the perinuclear space and their EGFP-t-VP3 and VP1 signals in partial co-localisation (upper panel). Separation of VP1 and EGFP-t-VP3 signals in the cytoplasm of the same cell, shown in different confocal section (lower panel).

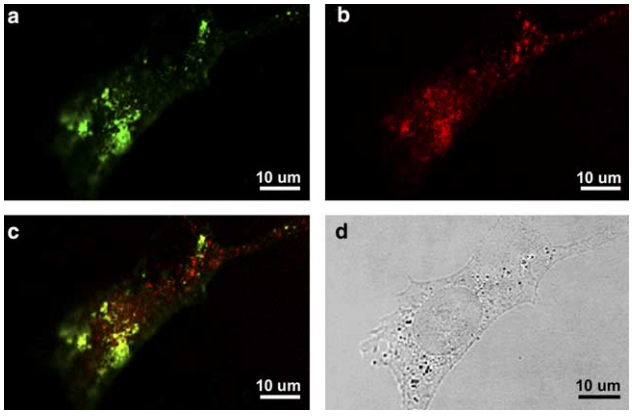

Fig. 8. Co-localisation of EGFP signal with ubiquitin. EGFP-t-VP3 + VP1 – green (a), ubiquitin – red (b), merged images of cell section (c) and Nomarski contrast (d). Cells were fixed 8 h p.a. (multiplicity 10^3 VLPs per cell).

Also in human, an efficacy trial has shown that systemic vaccination with L1 VLPs induced high serum antibody titres and can protect against persistent HPV 16 infection [27]. The VLPs can also be engineered to carry exogenous epitopes for induction of specific immune responses [28,29]. Chimeric VLPs based on murine polyomaviruses can be prepared by inserting

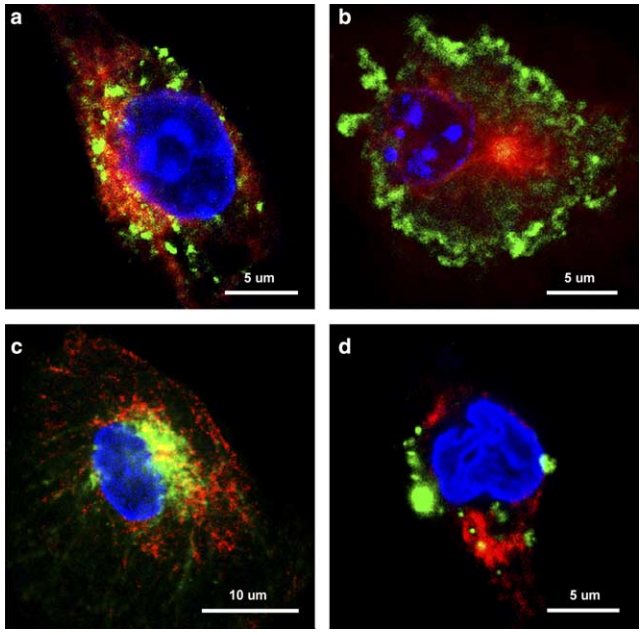

Fig. 9. EGFP-VLPs in human and mouse dendritic cells. Mouse (a,b) and human (c,d) dendritic cells 3 h (a,c) and 24 h (b,d) p.a. of EGFP-VLPs (multiplicity 10^3 VLPs/cell). Confocal sections of different cells, merged images; EGFP-t-VP3 + VP1 green, tubulin red and nucleus (DAPI) blue.

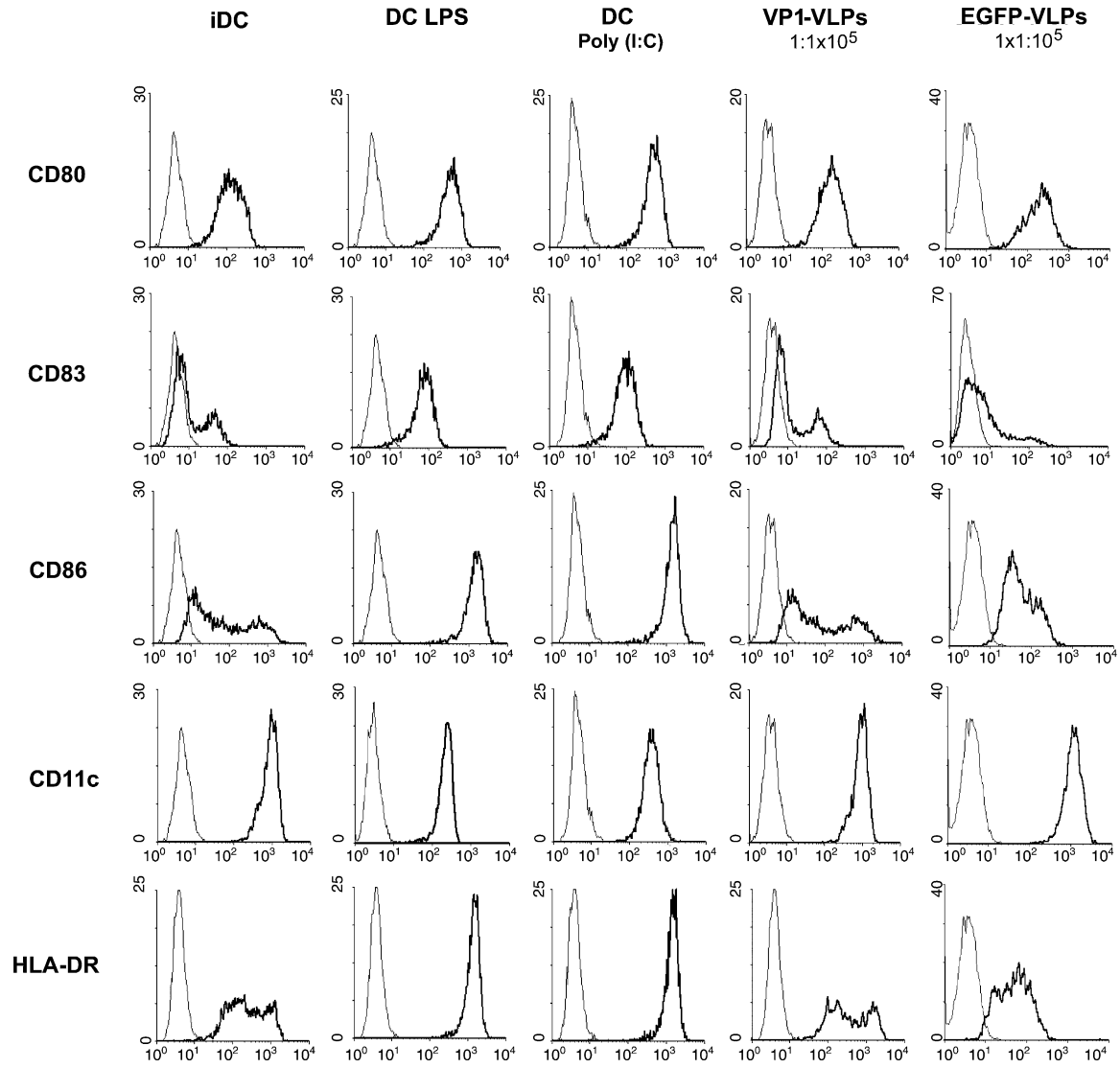

Fig. 10. Activation of human dendritic cells: Flow cytometry of immature DC (iDC) and of DC activated by the treatment with LPS, poly(I:C), VP1-VLPs and EGFP-VLPs. DC were gated according to their morphological properties and only viable cells (propidium iodide negative) were included in the final analysis. The thick lines represent the specific expression of investigated molecules, whereas the thin line represents the isotype control staining. The representative profiles of three independent experiments with DC generated from different donors are shown.

exogenous sequences into one of the surface loops of VP1 structural protein [30,31]. However, this approach has to overcome the restriction given by the requirement of VLP assembly. VLPs with insertion in the HI loop are unstable (our unpublished observation) and, moreover, they cannot be efficiently internalised by cells as they loose the ability to bind sialic acid moiety of a receptor molecule. Therefore, we exploited the C-terminal part of minor structural proteins, responsible for their interaction with VP1 pentamers, for the introduction of foreign peptides into VLPs. We examined the feasibility of introduction of an exogenous protein (EGFP) into capsid protein coding sequences as a fusion tag with a truncated peptide (99 amino acids) of the common C-terminus of VP2/VP3 minor structural proteins. The engineered construct was used to study the possibility of artificial capsid formation in the nuclei of insect cells infected by recombinant baculovirus carrying both VP1 and EGFP-truncated VP3 (each under a strong late baculovirus promoter).

Inserted protein should be exposed inside the particle, noncovalently connected with the central cavity of VP1 capsomeres. Theoretically, 72 fusion proteins could be present in one virus-like particle. Recently, such particles were assembled in vitro from VP1 pentamers and fusion protein GFP-46 C-terminal amino acids of VP2, both isolated from *Escherichia coli* [32]. They achieved an average loading capacity of 64 GFP molecules per particle. We showed that such particles can be efficiently assembled in nuclei of insect cells. The yield of VLPs accumulating in the nuclei of baculovirus-infected insect cells was particularly high and they could be easily purified by gradient centrifugations.

To further exploit chimeric VLPs carrying the desired epitopes connected with t-VP3 instead of EGFP, we examined the potential of EGFP-VLPs to induce maturation of DC in vitro. Previously, VLPs derived from the mouse polyomavirus major structural protein, VP1, were analysed for the stability and immunogenicity following intranasal administration

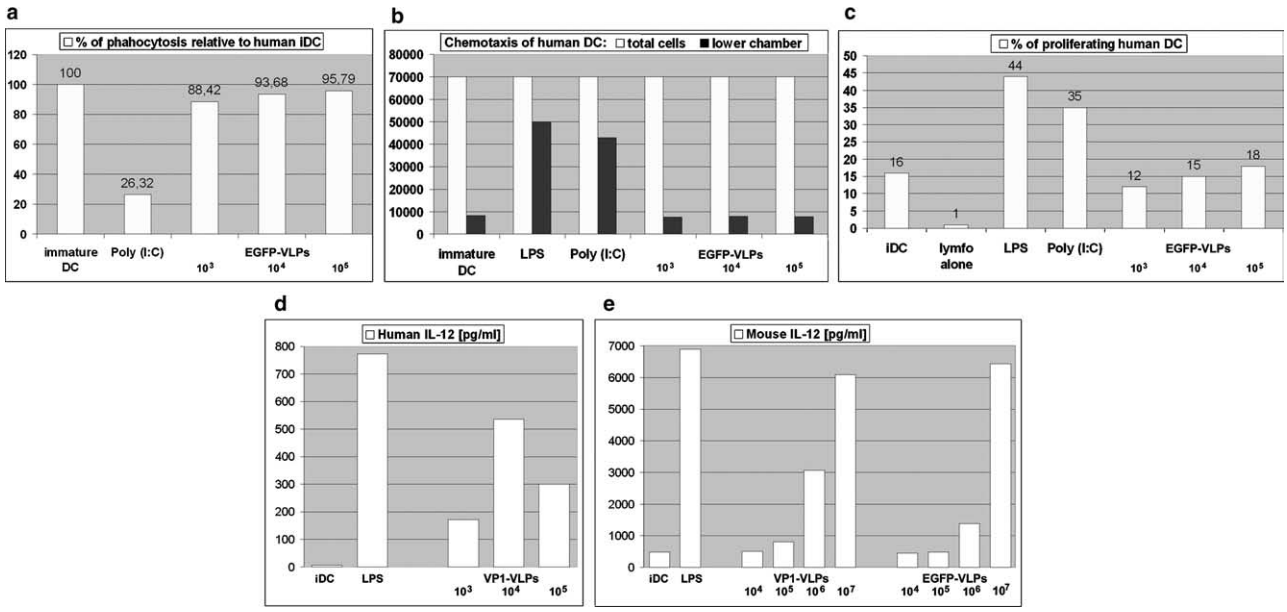

Fig. 11. Functional characteristics of DC after the interaction with EGFP-VPLs. FITC-dextran uptake (a) DC were pulsed with FITC-dextran (1 mg/ml) and analyzed by FACS. Values represent relative endocytic activity of DC compared to the immature DC. Representative result of three independent experiments with different DC preparations is shown. Migratory capacity of DC in response to MIP-3β: (b) white bars represent the number of DC seeded in the upper wells. Black bars show the number of cells that migrated into the lower wells of the transwell chamber. T-cell allostimulatory capacity of DC: (c) DC that interacted with EGFP-VLPs were used as stimulators of CFSE-labelled CD4 T lymphocytes. Activation capacity is expressed as a percentage of proliferating T cells on day 5. Secretion of IL-12 by DC interacting with EGFP-VLPs: (d,e) the quantity of biologically active p70 IL-12 heterodimer was analyzed in supernatants from cultures of human DC 24 h p.a. of EGFP-VLPs (d) and p40 IL-12 production by mouse DC was analysed from culture supernatants 24 h p.a. of VP1-VLPs or EGFP-VLPs (e). Experiments were done three times for each experiment with DC cultured for 5 days before addition of particles. Results of one representative experiment are shown.

without adjuvans into mice [14,15]. Strong humoral and cellular Th1 responses were observed after the first dose of VLPs and were efficiently boosted after a second dose [15]. Mechanism of immune system induction by PyV-VLPs remains unclear. Dendritic cells are recognised as the most potent professional APCs, which interact with naive T cells and thus initiate primary immune responses. Exogenous antigens need to be internalised and processed in the endo-lysosomal compartments of APCs to be presented to T cells in complex with MHC II molecules. Recently, increasing evidence appeared that in DC, internalised exogenous antigens, including VLPs, can also access the MHC class I presentation pathway (cross-presentation). After being captured by endocytosis, some exogenous non-cytosolic antigens (immune complexes, heat shock proteins, apoptotic cells and also VLPs) can gain access to the MHC class I pathway of antigen presenting cells by cross-presentation through several alternative processing pathways [33,34]. Two main routes of cross-presentation have been proposed: one of them involves the escape of antigens from endosomes to the cytosol following further the cytosolic pathway used by endogenous antigens [23,35] and in the second one, antigens are fully processed inside the endosomes binding to recycling MHC class I molecules [36,37]. It was shown that VLPs of hepatitis C virus [38], SHIV VLPs [39] and also porcine parvovirus VLPs [23] entered DC efficiently and the uptake was followed by DC activation, which indicates that the uptake led to antigen processing. Also non-enveloped human or bovine papillomaviruses were reported to induce maturation of dendritic cells in vitro by upregulating MHC class I and II molecules, CD80, CD86, CD40, CD54, and to induce production of IL-12 p70 [40]. In the same study, human polyomaviruses BK and JC failed to induce maturation of DC cells in vitro [40]. We showed that neither mouse polyomavirus chimeric EGFP-VLPs nor VLPs derived solely from the VP1 structural protein did upregulate CD80, CD83, CD86 of human or mouse DC. Also, in in vitro experiments, PyV-derived VLPs did not change (i) the migratory capacity of human DC in response to MIP-3β, (ii) the competence to stimulate T-cell proliferation and (iii) the ability of phagocytosis. Notwithstanding, EGFP-VLPs had adsorbed on human and mouse DC and were internalised efficiently. We did not observe differences in VLP trafficking in fibroblasts and epithelial and dendritic cells. The endocytic pathway used by mouse polyomavirus and VLPs is not completely understood. In the cell types used, PyV-VLPs were internalised via a sialysed receptor into smooth monopinocytic vesicles, which fused particularly with early endosomes and later appeared further in recycling endosomes and in ER cisternae compartments. Substantial fraction of monopinocytic vesicles was also positive for caveolin-1 immunostaining [41]. Nevertheless, the question how VLPs or other antigens enter the cross-presentation pathway is currently not fully elucidated.

In contrast, as mentioned above, in vivo experiments proved the ability of mouse polyomavirus VLPs to induce strong humoral and cellular responses in experimental animals [13–15]. Differences in behaviour between in vitro and in vivo treated cells may exist. One possible explanation for induction of cellular immunity in vivo can be concealed in

a recently described novel alternative mechanism of cross-presentation by gap-junction-mediated immunological coupling [42]. Neijssen et al. [42] showed that peptides generated by proteosomal degradation in one cell could diffuse from the cytoplasm through gap junctions to the neighbour cells. The authors also showed that monocytes can use coupling through gap junctions to load its own MHCI molecules with viral peptides derived from adjacent infected cell. We observed that a subpopulation of mouse PyV-VLPs appeared in lysosomes after internalisation by DC and other cell types. Later, the majority of VLPs entered endoplasmic reticulum and were dispatched to the cytoplasm for degradation in proteasomes. However, the signal of VP1 protein moving in the direction to the cell periphery could be still seen 24 h p.i. (Fig. 9). Further experiments are required to test whether gap-junction-mediated immunological coupling plays a role in the induction of strong immune responses to PyV-VLPs in experimental animals. Interestingly, production of IL-12, one of the major DC derived factors regulating T and B cell activation was induced by mouse PyV VLPs in human and also in mouse DC. IL-12 is a heterodimeric pro-inflammatory cytokine that induces the production of interferon-γ, favours the differentiation of Th1 cells and forms a link between innate resistance and adaptive immunity. IL-12 might feed back to regulate APC in in vivo conditions. Both macrophages and DC express functional IL-12 receptors [43].

In conclusion, we constructed VLPs derived from mouse polyomavirus that contain foreign protein (EGFP) fused to C-terminal sequences of minor antigens, responsible for their interaction with the central cavity of VP1 capsomeres. These chimeric VLPs were assembled in the nuclei of insect cells infected by recombinant baculovirus and efficiently purified by gradient centrifugations. Pseudocapsids were irregular in size and often imperfectly assembled, apparently due to steric stress caused by the presence of fusion proteins. Despite their disturbed shape, they delivered foreign protein into host cells and were efficiently internalised and processed also by human and mouse dendritic cells. Neither VLPs composed of VP1 only, nor EGFP-VLPs upregulated co-stimulation molecules and other markers of DC maturation in vitro. On the other hand, they did induced production of the IL-12. Despite extensive research, more detailed studies, both in vitro and in vivo, will be necessary for reasonable designing of protective vaccines.

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