Murine gammaherpesvirus-68 productively infects immature dendritic cells and blocks maturation

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Many viruses have evolved mechanisms to evade host immunity by subverting the function of dendritic cells (DCs). This study determined whether murine gammaherpesvirus-68 (γHV-68) could infect immature or mature bone-marrow-derived DCs and what effect infection had on DC maturation. It was found that γHV-68 productively infected immature DCs, as evidenced by increased viral titres over time. If DCs were induced to mature by exposure to LPS and then infected with γHV-68, only a small percentage of cells was productively infected. However, limiting-dilution assays to measure viral reactivation demonstrated that the mature DCs were latently infected with γHV-68. Electron microscopy revealed the presence of capsids in the nucleus of immature DCs but not in mature DCs. Interestingly, infection of immature DCs by γHV-68 did not result in upregulation of the co-stimulatory molecules CD80 and CD86 or MHC class I and II, or induce cell migration, suggesting that the virus infection did not induce DC maturation. Furthermore, γHV-68 infection of immature DCs did not result in elevated interleukin-12, an important cytokine in the induction of T-cell responses. Finally, lipopolysaccharide and poly(I : C) stimulation of γHV-68-infected immature DCs did not induce increases in the expression of co-stimulatory molecules and MHC class I or II compared with mock-treated cells, suggesting that γHV-68 infection blocked maturation. Taken together, these data demonstrate that γHV-68 infection of DCs differs depending on the maturation state of the DC. Moreover, the block in DC maturation suggests a possible immunoevasion strategy by γHV-68.

INTRODUCTION

Dendritic cells (DCs) play a key role in the initiation of a primary T-cell response (Banchereau et al., 2000; Hart, 1997) and also function in the maintenance of adaptive immune responses. Several features of myeloid DCs contribute to their function. For example, DCs are very efficient at antigen uptake and processing and are able to present antigen for both MHC class I and class II pathways (Banchereau et al., 2000; Banchereau & Steinman, 1998). Of equal importance is their capacity to migrate through tissue as part of the peripheral immune surveillance (Caux et al., 2000). Induction of T-cell immunity against microbial pathogens is thought to occur following the recruitment of immature DCs from the periphery to lymphoid tissues and their subsequent differentiation into mature DCs (Kelsall et al., 2002). Numerous phenotypic and functional features distinguish immature from mature DCs. Immature DCs phagocytose pathogens; this ability is lost following maturation. Maturation of DCs is associated with increases in expression of the co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2), upregulation of MHC class I and II (Ardavin, 2003) and expression of cytokines such as interleukin (IL)-12 (Trinchieri et al., 2003). Maturation also results in the upregulation of the chemokine receptor CCR7 directing the DCs to the lymph nodes where they prime a T-cell response (Forster et al., 1999; Martin-Fontera et al., 2003). Because of the primary role of DCs in the initiation of adaptive immunity, viruses have evolved numerous strategies to subvert DC function (reviewed by Moll, 2003). One strategy observed in several viral infections is the inhibition of DC maturation (Engelmayer et al., 1999; Moutaftsi et al., 2002; Pollara et al., 2003; Salio et al., 1999; Smith et al., 2005).

Two important human pathogens, the gammaherpesviruses Epstein–Barr virus (EBV) and Kaposi’s sarcoma herpesvirus (KSHV) infect DCs (Li et al., 2002; Rappocciolo et al., 2006). EBV can infect precursor myeloid cells and interferes with DC development, thus preventing the differentiation of sufficient numbers of DCs (Li et al., 2002). Patients with Kaposi’s sarcoma have been reported to have functionally impaired DCs (Stebbing et al., 2003), but whether this is due to infection with KSHV is
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unknown. More recently, KSHV was shown to bind to DC-SIGN on myeloid DCs ex vivo, and infection of DCs inhibits endocytosis and antigen presentation (Rappocciolo et al., 2006). Although these studies suggest that infection of DCs could be an integral part of the gammaherpesvirus pathogenesis, there are limitations in the study of EBV and KSHV pathogenesis.

An amenable model system to study gammaherpesvirus pathogenesis utilizes murine gammaherpesvirus-68 (γHV-68). γHV-68-infected DCs can be isolated from lymphoid compartments (e.g. spleen and mediastinal lymph node) and from lung (Flano et al., 2000, 2003, 2005; Marques et al., 2003). More recently, Flano et al. (2005) demonstrated that bone-marrow-derived DCs could be infected with γHV-68. In their study, both productive and latent infection of DCs was detected following ex vivo infection, but they did not distinguish immature from mature DCs in their analysis (Flano et al., 2005). An important question that remains is whether γHV-68 preferentially infects immature DCs or mature DCs, or whether γHV-68 is capable of infecting both.

In this study, we tested whether γHV-68 infection of bone-marrow-derived DCs was dependent on the maturation state of the DCs and whether infection resulted in phenotypic changes. We found that γHV-68 productively infected immature DCs and blocked maturation. In contrast, bone-marrow-derived DCs driven to maturation following lipopolysaccharide (LPS) stimulation were latently infected with γHV-68. In addition, we found that γHV-68 infection of bone-marrow precursor cells drastically reduced the development of DCs. These data suggest that, like the other members of the herpesvirus family, gammaherpesviruses target DCs as one potential strategy to subvert host immunity.

METHODS

Cell lines and viruses. Owl monkey kidney (OMK) cells (ATCC CRL-1556) and NIH 3T3 cells (ATCC CRL-1658) were maintained in culture as described previously (Rochford et al., 2001). MEF-1 cells (ATCC CRL-2214) were maintained in DMEM containing 4.5 g glucose l−1, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), 4 mM glutamine, 100 IU penicillin ml−1 and 100 μg streptomycin ml−1. γHV-68 expressing enhanced green fluorescent protein (γHV-eGFP) was a generous gift of Dr Ren Sun (UCLA, Los Angeles, CA, USA) and the generation of this recombinant virus has been described previously (Cardin et al., 1994). Cells were fed on NIH 3T3 cells. To UV-inactivate virus, 1 ml of virus stock was placed in a 24-well plate and exposed to UV light for three 5 min exposures in a Stratalinker (Strategene). Plaque assays on virus stocks following UV exposure indicated that the virus had been inactivated (data not shown). For mock infection, uninfected OMK cells were processed exactly as if they had been virus infected (Rochford et al., 2001). The resulting supernatant was used for all mock infections.

Culture of DCs. Bone-marrow-derived myeloid DCs were generated by a method adapted from Lutz et al. (1999). Briefly, bone-marrow cells were flushed out of the tibia and femur of C57Bl/6 mice. After ammonium chloride lysis of red blood cells, 2 × 106 bone-marrow cells were plated on non-cell-culture-treated 100 mm Petri dishes in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 50 μM 2-mercaptoethanol, 100 IU penicillin ml−1, 100 μg streptomycin ml−1 and 10 ng granulocyte-macrophage colony-stimulating factor (GM-CSF) ml−1. GM-CSF was prepared from the culture supernatant of X63.AG-8.2C5SF cells (a gift of S. Dewhurst, University of Rochester, NY, USA) (Zal et al., 1994). Cells were fed on days 3 and 6 by adding 50% fresh medium containing GM-CSF. By day 9, cells had a typical purity of 80–90% CD11c−CD11b+ myeloid DCs (data not shown). For infections, DCs were infected with γHV-eGFP at an m.o.i. of 5 for 1 h. Unbound virus was removed by washing and cells were replated for 24–48 h. After transfer to new plates, the immature phenotype of the adherent population was verified by their low expression of CD80, CD86 and MHC II, as well as by high uptake of FITC-dextran (data not shown). In maturation experiments, non-adherent cells (mature) were removed prior to the addition of the toll-like receptor (TLR) ligands LPS 026 : B6 (0.1–1 μg ml−1) or poly(1 : C) (20 μg ml−1) (Sigma) onto the adherent, immature DCs. In some experiments, immature DCs harvested at day 8 of culture were matured with 0.1–1 μg LPS 026 : B6 ml−1 or poly(I : C), 24 h prior to infection with γHV-eGFP at an m.o.i. of 5 for 1 h.

Ex vivo limiting-dilution reactivation assays and plaque assays of infected DCs. Measurement of the frequency of viable cells that could reactivate from latency was done by a limiting-dilution reactivation assay essentially as described previously (van Dyk et al., 2000; Weck et al., 1996), with the exception that serial threefold dilutions of cells were carried out. To detect pre-formed virus, a duplicate aliquot of cells was disrupted by three rounds of freeze-thawing. To determine viral titres in infected DCs, cells were infected for 1 h, washed and plated in 12-well plates. Supernatant and cells were harvested at various times post-infection (p.i.), cells were disrupted by three rounds of freeze-thawing, and plaque assays were carried out as described previously (Cardin et al., 1996). The yield of virus for each time point was calculated as number of p.f.u. per input number of DCs in each well.

Electron microscopy. Immature and mature DCs were infected with γHV-68 for 1 h at an m.o.i. of 5. After 48 h, DCs were harvested and centrifuged. The resulting pellets were fixed overnight in 2.5% phosphate-buffered glutaraldehyde, post-fixed in 1% OsO4, dehydrated in a graded ethanol series followed by propylene oxide and then embedded in Araldite 502 epoxy resin. Ultrathin sections were hydrated in a graded ethanol series followed by propylene oxide and then embedded in Araldite 502 epoxy resin. Ultrathin sections were stained with uranyl acetate and Reynolds’s lead citrate before examination using a Tecnai BioTWIN 12 transmission electron microscope (FEI Co.). At least 30 cells were scanned in each preparation.

Immunofluorescence staining and flow cytometric analyses. All methods, media formulations, reagents and strategies for single-colour and multi-colour immunofluorescence staining and cell sorting have been detailed elsewhere (Hobbs et al., 1993), with the exception that FC-Block (BD-PharMingen) was added. Multi-colour immunofluorescent-stained cells were analysed with an LSRII flow cytometer (Becton Dickinson). Live cells were collected by gating on forward–side scatter characteristics. Data acquisition was performed with FACSDIVA software (BD Biosciences) and data were further analysed with FLOWJO (Tree Star Inc.) and WINMDI 2.8 software (The Scripps Research Institute). Anti-CD11c, anti-CD80, anti-CD86, anti-MHC class I (H-2K), anti-MHC class II (I-A/I-E) and anti-CD11b monoclonal antibodies (mAbs) were obtained from BD PharMingen.

Cytokine ELISA. Supernatants of DC cultures were harvested at 24 h.p.i. and stored at −80 °C until analysis. The p40 subunit of IL-12 (IL-12p40) was quantified by sandwich ELISA using the BD
OptEIA system (BD Pharmingen) according to the manufacturer’s instructions. IL-10 was detected by a sandwich ELISA technique as described previously (Wen et al., 2006).

Transwell migration assay. DC migration was determined by measuring cells migrating through a polycarbonate filter (5 μm pore size) in 24-well Transwell chambers (Costar Corning). γHV-eGFP-infected DCs were harvested at 24 h.p.i. and tested for migration towards CCL19 chemokine (Peprotech), a CCR7 ligand. The lower chambers of the Transwell plates were filled with 600 μl DMEM supplemented with 1% BSA and 10 mM HEPES with CCL19 (100 ng ml⁻¹). DCs (5 × 10⁵) were added in 100 μl DMEM/BSA/HEPES into the upper chamber and cells were incubated at 37 °C for 3 h. Input cells as well as migrated cells were stained with phycoerythrin (PE)-conjugated anti-CD86 and analysed by flow cytometry.

RESULTS

The maturation state of DCs affects their susceptibility to productive γHV-68 infection

To investigate the susceptibility of DCs of different maturation states to γHV-68 infection, we first isolated and cultured DCs from C57BL/6 bone marrow. After 8 days of culture, the ex vivo-expanded DCs were left untreated (immature) or treated with LPS to induce maturation. After 24 h, cells were infected with a recombinant γHV-68 that expressed eGFP under control of the human cytomegalovirus (HCMV) promoter (γHV-eGFP). Cells were harvested 24 h later and stained with allophycocyanin (APC)-conjugated anti-CD11c mAb (a DC cell-surface marker) and PE-conjugated anti-CD86 mAb (a maturation marker). In a representative set of over ten experiments, greater than 70% of cells were immature CD11c⁺ CD86⁻ DCs. Of these immature cells, 70% were infected, as indicated by the percentage of CD11c⁺ cells that were GFP positive (Fig. 1a). In contrast, DCs driven to maturation by treatment with LPS and then infected with γHV-eGFP appeared to be refractory to γHV-eGFP infection, as indicated by the very low number of GFP-expressing cells (Fig. 1a).

To determine whether the immature DCs were productively infected with γHV-68, we performed plaque assays on both cell-associated virus and supernatant harvested from immature DCs and mature DCs at various times p.i. Wells were infected in duplicate and the mean titre in p.f.u. per 10⁶ input DCs from a representative experiment is shown (Fig. 1b). We observed that the supernatant virus titre in the infected immature DCs increased between 8 and 24 h.p.i., but reached a plateau by 48 h.p.i. The cell-associated virus titre reached its peak at 24 h.p.i. and then declined. In contrast, following infection of mature DCs, virus titres did not increase significantly from the input levels, suggesting that they were not productively infected. Cell viability of both mature and immature DCs was slightly lower than mock-infected cells at 24 h.p.i. and began to decline by 48 h.p.i. (Fig. 1c). However, even at 48 h.p.i., there was still greater than 70% cell viability of infected DCs.

Although less than 8% of cells were GFP positive following infection of mature DCs, it remained possible that GFP was not expressed if the cells were latently infected. To test this possibility, we performed a limiting-dilution reactivation assay to determine whether there were latently infected mature DCs. In this assay, both viable cells and cells that had been mechanically disrupted to release pre-formed virus were plated onto susceptible MEFs and the percentage of cells showing cytopathic effect (CPE) was calculated to determine the frequency of infected cells. As shown in Fig. 1(d), for immature DCs, there was no difference in the percentage of wells showing CPE when live cells were plated compared with cell lysates, indicating that the infected immature DCs were productively infected and there were no latently infected cells. In contrast, we observed a difference in the frequency of wells showing CPE between wells that received live cells (1 : 3.5) versus disrupted cells (1 : 9). This meant that the majority of mature DCs were latently infected with γHV-68.

To compare γHV-68 infection of immature and mature DCs further, electron microscopy studies were performed. Immature and mature DCs were infected for 48 h, and cells were washed and then fixed before being processed for electron microscopy. As shown in Fig. 2(a, b), nucleated capsids were clearly seen in the nucleus of infected immature DCs. Interestingly, we also observed infected DCs being phagocytosed by other DCs in the culture (Fig. 2c). Higher magnification of the phagocytosed infected DCs revealed that the capsids appeared to be empty in contrast to the capsids from infected but non-phagocytosed DCs (compare Fig. 2b and d). No capsids were observed in the nucleus of mature DCs (data not shown). Taken together, the data presented demonstrate that the maturation state of the DCs affects their ability to sustain a productive infection (immature DCs) or a latent infection (mature DCs).

γHV-68 infection of immature DCs blocks maturation

Immature DCs are characterized by their ability to phagocytose and have relatively low-level expression of co-stimulatory molecules (CD80 and CD86), as well as MHC class I and II. In contrast, mature DCs lack the ability to phagocytose but have high-level expression of MHC class I and II as well as co-stimulatory molecules. To determine whether γHV-68 infection of immature DCs induced their maturation, DCs infected with γHV-eGFP or mock infected for 24 h were harvested and stained with APC-conjugated anti-CD11c and PE-conjugated antibodies specific for MHC class I and II, co-stimulatory molecules (CD80 and CD86) and CD11b (highly expressed on myeloid DCs). Cells were gated on CD11c⁺ cells, and GFP expression and histograms for individual surface markers are shown in Fig. 3(a). As a control, immature DCs were treated with LPS to verify that all of the cells were susceptible to a known DC maturation signal. After 24 h of
LPS treatment, we observed a significant increase in DC maturation markers. In contrast, MHC class II and CD80 and CD86 levels remained significantly lower in the infected GFP+ cells, suggesting that viral infection did not induce differentiation. No changes in MHC class I expression were observed following infection. To test that binding of virus particles induced DC maturation, DCs were infected with γHV-68 and harvested 24 h later. Cells were stained with APC-conjugated CD11c and PE-conjugated CD86 mAbs. Cells were gated on CD11c+ (top panel) and analysed for expression of CD86 (maturation marker) and GFP (virus infection) as shown in the bottom panel. Immature DCs or DCs matured for 24 h with 100 ng LPS ml⁻¹ were infected with γHV-eGFP. At 1.5, 4, 8, 24, 48 and 72 h p.i., supernatants (filled bars) and cell lysates [i.e. cell-associated virus; empty bars] were harvested and virus titres were determined by plaque assays. The number of p.f.u. is reported based on the input number of DCs per well. Immature DCs or DCs matured for 24 h with 100 ng LPS ml⁻¹ were infected or not with γHV-eGFP (filled bars, mock infection; shaded bars, γHV-68; empty bars, UV-inactivated γHV-68). After 24, 48 or 72 h p.i., cell viability was determined by a trypan blue exclusion assay. Limiting-dilution reactivation assay. Immature (upper panel) and LPS-matured (lower panel) DCs were infected for 24 h at an m.o.i. of 5. Cells were harvested and plated on MEFs in limiting dilution. In parallel, a separate aliquot of cells was disrupted by freeze-thawing and plated on MEFs. The results are shown as the percentage of wells positive for CPE relative to the value scored positive for viral CPE 3 weeks after plating. Twenty-four wells were plated per cell dilution in each experiment. The results shown are representative experiments from a minimum of three different experiments. ◆, Total; ■, lysate.

Fig. 1. γHV-68 infection of immature and mature DCs. (a) Immature DCs or DCs matured for 24 h with 100 ng LPS ml⁻¹ were infected with γHV-eGFP and harvested 24 h later. Cells were stained with APC-conjugated CD11c and PE-conjugated CD86 mAbs. Cells were stained on CD11c+ (top panel) and analysed for expression of CD86 (maturation marker) and GFP (virus infection) as shown in the bottom panel. Immature DCs or DCs matured for 24 h with 100 ng LPS ml⁻¹ were infected with γHV-eGFP. At 1.5, 4, 8, 24, 48 and 72 h p.i., supernatants (filled bars) and cell lysates [i.e. cell-associated virus; empty bars] were harvested and virus titres were determined by plaque assays. The number of p.f.u. is reported based on the input number of DCs per well. Immature DCs or DCs matured for 24 h with 100 ng LPS ml⁻¹ were infected or not with γHV-eGFP (filled bars, mock infection; shaded bars, γHV-68; empty bars, UV-inactivated γHV-68). After 24, 48 or 72 h p.i., cell viability was determined by a trypan blue exclusion assay. Limiting-dilution reactivation assay. Immature (upper panel) and LPS-matured (lower panel) DCs were infected for 24 h at an m.o.i. of 5. Cells were harvested and plated on MEFs in limiting dilution. In parallel, a separate aliquot of cells was disrupted by freeze-thawing and plated on MEFs. The results are shown as the percentage of wells positive for CPE relative to the value scored positive for viral CPE 3 weeks after plating. Twenty-four wells were plated per cell dilution in each experiment. The results shown are representative experiments from a minimum of three different experiments. ◆, Total; ■, lysate.

γHV-68 infection of DCs inhibits chemotaxis

In addition to upregulation of co-stimulatory molecules, maturation of DCs is also characterized by changes in the expression of the chemokine receptors (Sallusto et al., 1998; Vecchi et al., 1999). Maturing DCs downregulate CCR1, CCR5 and CXCR1 and upregulate CXCR4, CCR4 from six to eight experiments is shown. These data showed that infection of immature DCs did not upregulate CD80, CD86 or MHC class II compared with LPS-matured DCs or DCs infected with UV-inactivated virus.
and CCR7. In particular, CCR7, which responds to the chemokines CCL19 and CCL21, is important for directing DCs to the lymphoid organs (Martin-Fontecha et al., 2003; Sallusto et al., 1998). To test whether infected DCs were responsive to CCL19, we performed a Transwell migration assay to measure the capacity of infected DCs to migrate. DCs were infected with γHV-eGFP for 24 h and the adherent population was harvested and placed in the upper chamber of a Transwell. Cells were routinely greater than 80 % viable at the time of transfer. The lower chamber contained the CCR7 ligand CCL19 (100 ng ml⁻¹). Only a small percentage of infected DCs was able to migrate towards CCL19 (Fig. 3c). Even stimulation with LPS for 24 h after infection did not increase the number of migrating γHV-68-infected cells (data not shown). This suggested that γHV-68 infection of DCs not only inhibited the upregulation of co-stimulatory molecules, but also prevented the induction of DC migration in infected cells.

**γHV-68 infection of DCs inhibits IL-12 expression but not IL-10 expression**

Activation and maturation of DCs causes secretion of IL-12, a potent stimulator of gamma interferon (IFN-γ) and inducer of Th1 responses (Lamont & Adorini, 1996). To assess the effect of γHV-68 on the expression of IL-12, we infected DCs with γHV-eGFP or treated the cells with UV-inactivated virus or mock lysate. After 24 h, IL-12p40 and IL-10 were measured in the cell-culture supernatants by ELISA (Fig. 3d). Significantly lower levels of IL-12 were observed in the γHV-eGFP-infected wells compared with cells treated with UV-inactivated virus or mock infected. In contrast, both mock- and γHV-eGFP-infected DCs produced IL-10, although the level of IL-10 in the infected cells was not significantly higher than in the mock-infected cells. No IL-10 was observed in the DCs infected with UV-inactivated virus.

**γHV-68 infection of DCs blocks LPS and poly(I : C)-induced maturation**

DCs can be induced to mature via stimulation with signaling through TLRs. To determine whether γHV-68 infection impaired DC maturation induced by LPS (a TLR4 ligand) or poly(I : C) (a TLR3 ligand), immature DCs were infected with γHV-eGFP or mock infected. After 24 h, non-adherent cells were removed and discarded and the ability of the immature, adherent cells to mature was tested by adding 1 μg LPS ml⁻¹, 20 μg poly(I : C) ml⁻¹ or medium alone for an additional 24 h (Fig. 4). After a total of 48 h of infection, cells were stained with appropriate antibodies and flow cytometry was carried out to assess changes in DC phenotype. As expected, we observed increased levels of CD80, CD86 and MHC class II in response to LPS and poly(I : C) in mock-infected cells, demonstrating the susceptibility of *ex vivo*-generated, bone-marrow-derived DCs to maturation signals. However, the majority of infected DCs (GFP⁺ DCs) showed limited responsiveness to LPS or to poly(I : C) stimulation as measured by the low-level expression of CD80, CD86 and MHC class II. Together, these data suggested that γHV-68 infection not only failed to induce DC maturation, but also blocked maturation induced by the TLR ligands LPS and poly(I : C).

**Supernatant from mature DCs blocks productive infection of MEFs**

To test whether mature DCs express a factor that blocks lytic infection, we treated immature DCs with LPS to induce maturation or left them untreated. After 24 h, the supernatant was harvested from untreated and LPS-matured DCs. MEFs, which are very sensitive to productive γHV-68 infection, were then incubated with the supernatant for 1 or 24 h, infected for 1 h, and incubated for 6 days to determine the viral titre. We found that there was a significantly lower viral titre following infection of MEFs that had been pre-treated with the supernatant from mature DCs compared with MEFs that were treated with supernatant from immature DCs or were incubated with medium alone (Fig. 5). This suggested that the mature DCs secreted a soluble protein that actively inhibited lytic replication.
γHV-68 infection of precursor DCs blocks development

The immature DCs used in the previous studies were derived following isolation of bone-marrow cells and 9 days growth ex vivo. To determine whether γHV-68 infection blocked DC development, freshly isolated bone-marrow cells were infected with γHV-68 or UV-inactivated virus, or were mock infected or treated with LPS. Twenty-four hours later, adherent cells were harvested and stained with APC-conjugated anti-CD11c mAb and PE-conjugated anti-CD80, -CD86, -MHC class I or -MHC class II. Shown is a representative FACS analysis, gated on live cells positive for CD11c and GFP for γHV-68-infected cells. (b) Mean fluorescence intensity (MFI) of UV-inactivated virus-treated and GFP+ (infected) DCs from multiple experiments were determined at 24 h p.i. and normalized to the MFI of mock-infected cells. The graph shows the mean ± SEM of six to eight independent experiments. GFP+ DCs expressed significantly lower levels of CD80, CD86 and MHC class II compared with UV-inactivated virus-treated DCs. *P<0.05 (paired Student’s t-test). (c) DCs that had been infected for 24 h with γHV-eGFP were seeded in the upper chamber of a Transwell and allowed to migrate towards the lower chamber, which contained the CCR7 ligand CCL19 (100 ng ml–1). After 3 h, input cells and migrated cells were stained with PE-conjugated anti-CD86 mAb and analysed by flow cytometry. Circled cells represent immature DCs. (d) Immature DCs were infected with γHV-eGFP or UV-inactivated virus, or were mock infected. Supernatants were harvested at 24 h p.i., and IL-12 and IL-10 levels were determined by ELISA. Results are shown as the mean ± SEM of at least four experiments per treatment. γHV-68-infected cells produced significantly lower amounts of IL-12p40 compared with mock-infected and UV-inactivated virus-treated DCs. *P<0.05 (paired Student’s t-test).
DISCUSSION

Herpesviruses have evolved a variety of mechanisms to escape the immune response in order to establish life-long latency in the host. Because of their central role in initiating antiviral immune responses, DCs have been targeted by viruses from several different viral families (reviewed by Tortorella et al., 2000). In this study, we have reported that cHV-68 can infect immature DCs and block their maturation, suggesting that this is another strategy used to subvert host immunity.

Previous studies have found that bone-marrow-derived DCs can be infected with cHV-68 and that both latently and lytically infected cells are detected in the cultures (Flano et al., 2005). Our studies confirmed these results and extended them by demonstrating that lytic infection was predominantly restricted to the immature DCs, whilst LPS-matured DCs were latently infected. The different patterns of viral infection in immature DCs versus mature DCs is concordant with observations from in vivo infection of mice, where lung DCs, which are predominantly immature, harbour lytic infections, whereas DCs isolated from lymph nodes, which are predominantly mature, harbour latent infections (Flano et al., 2005). We initially utilized a recombinant virus, cHV-eGFP, where GFP was under the control of the HCMV promoter, to distinguish infected from uninfected DCs in our culture. However, limiting-dilution reactivation assays revealed that the mature DCs, whilst being GFP negative, were indeed latently infected with the virus. Electron microscopy also confirmed the absence of capsids in mature DCs. Whether this was due to the cellular environment or to the insertion site in the viral genome remains to be determined. Nonetheless, the demonstration that a latent infection is established following infection of LPS-matured DCs opens up a new tool to examine cHV-68 latency in an ex vivo model. Currently, the only model system for studying cHV-68 latency ex vivo is the S11 B-cell line derived from tumour cells isolated from a cHV-68-infected mouse (Usherwood et al., 1996).

We observed that if immature DCs were infected with cHV-68 for 24 h and then treated with LPS, maturation was blocked. This block was not seen following infection of
immature DCs with replication-deficient, UV-inactivated virus. This observation differs from previous studies by Flano et al. (2005) who did not observe this block in maturation following LPS stimulation of infected cells. However, in their study, they treated DCs with LPS concurrent with γHV-68 infection. Together, these results support the idea that a viral protein(s) is necessary to block maturation, possibly through blocking maturation signals. One possibility is that γHV-68 infection results in loss of TLR-4, which is essential for LPS responsiveness (Poltorak et al., 1998). We have observed that TLR4 expression is maintained in DCs following γHV-68 infection (C. Ptaschinski, R. Hochreiter & R. Rochford, unpublished observations), suggesting that the block must occur further downstream in the LPS signalling pathway. Furthermore, a similar block in DC maturation was observed after stimulation with poly(I : C), a TLR3 ligand. In contrast to TLR4 signalling, which can be MyD88-dependent and -independent, TLR3 signalling occurs only via an MyD88-independent pathway (Takeda & Akira, 2004). This again argues for a γHV-68-mediated inhibitory effect of DC maturation on a broader range, rather than interference with single TLR ligand expression or inhibition early in the signalling cascade. A general inhibitory effect on protein synthesis after γHV-68 infection was not observed up to 48 h p.i., as MHC class I, CD11b and CD11c expression were not affected. Infection of immature DCs and subsequent inhibition of maturation has also been reported for herpes simplex virus type 1 (HSV-1) (Pollara et al., 2003; Salio et al., 1999), HCMV (Moutaftsi et al., 2002), murine cytomegalovirus (Andrews et al., 2001), human herpes virus 6 (HHV-6) (Smith et al., 2005) and vaccinia virus (Engelmayer et al., 1999), suggesting that this is a common viral strategy for immune evasion. HSV-1 and HCMV have been shown to interfere with the IFN signalling JAK/STAT pathway (Miller et al., 1998; Yokota et al., 2004).

As DCs are exquisitely sensitive to maturation signals, ex vivo culture of bone-marrow-derived DCs routinely results in a mixed population of cells that contain both phenotype-immature and mature DCs. By using the γHV-68 recombinant virus expressing eGFP, we were able to determine that γHV-68 preferentially productively infects immature DCs, characterized by low-level expression of co-stimulatory molecules, low-level expression of both MHC class I and II, and an inability to migrate towards CCL19. In the infected immature DCs, we did observe a small fraction of GFP-positive cells expressing higher levels of CD86 (albeit not as high as in the mature DCs). We hypothesize that these were cells that had already received the signal for maturation before infection and thus productive infection was not blocked. This would fit in with the data of Flano et al. (2005), who found that LPS treatment simultaneous with infection did not block maturation. Our data also suggest that maturation of DCs decreases the susceptibility to productive γHV-68 infection. One possibility for decreased susceptibility is the production of IFNs that may induce an antiviral state in LPS-matured DCs, reducing virus replication. This model is supported by our observation that treatment of MEFs with the supernatant from LPS-matured DCs but not from immature DCs prior to γHV-68 infection significantly reduced the viral yield.

In order to activate T cells, DCs need to provide three signals: presentation of antigenic peptide in the context of MHC molecules, high-level expression of co-stimulatory molecules and secretion of cytokines (Lipscomb & Masten, 2002). Together, these signals not only induce T-cell proliferation but also drive CD4 T cells down the Th1 or Th2 differentiation pathways, as well as inducing T regulatory cells (Maldonado-Lopez & Moser, 2001; Maldonado-Lopez et al., 1999). Importantly, secretion of IL-12 by DCs is critical for the induction of Th1 differentiation, as well as for proliferation and enhanced cytotoxic activity of natural killer cells (Trinchieri, 2003). We observed that DCs infected with γHV-68 produced only very low levels of IL-12 compared with LPS-stimulated DCs or DCs infected with UV-inactivated virus. This suggests that γHV-68-infected DCs would have limited function in inducing a Th1 response. An inability to secrete CD40 ligation is also observed in measles virus-infected DCs as well as in HHV-6-infected DCs after LPS and IFN-γ stimulation (Smith et al., 2005), suggesting that this might be a common immunoevasion strategy (Fugier-Vivier et al., 1997). Flano et al. (2005) observed that, after an additional activation signal (LPS stimulation), γHV-68-infected DCs produced elevated levels of IL-10, which was found to be responsible for the limited proliferation of allogeneic T cells. In contrast, in the absence of LPS stimulation, Flano et al. (2005) failed to detect IL-10 with or without γHV-68 infection in DC cultures after 96 h p.i. In our study, we were unable to detect significant differences in IL-10 production between mock-treated and γHV-68-infected DCs at 24 h p.i. It is possible that differences in IL-10 levels would be more apparent by 96 h p.i. We also observed that no IL-10 was detected in immature DCs infected with UV-inactivated virus. It is possible that the higher IL-12 levels induced could counteract IL-10 production in these cells.

In addition to the inhibitory effects on DC maturation and activation, γHV-68 infection also had a dramatic effect on the generation of DCs from bone-marrow precursor cells. Inhibition of DC development from bone-marrow cells has been observed after lymphocytic choriomeningitis virus clone 13 and measles virus infection (Hahm et al., 2005; Sevilla et al., 2004). Also, the more closely related human gammaherpesvirus EBV has been found to inhibit DC development by inducing apoptosis in monocyte precursors (Guerreiro-Cacais et al., 2004; Li et al., 2002). However, Li et al. (2002) demonstrated that the apoptosis-promoting effect of EBV is restricted to the early stages of DC differentiation and is independent of viral gene expression (Li et al., 2002). This finding contradicts our results where we observed that UV-inactivated virus had no inhibitory effect on DC differentiation.
In summary, we found that γHV-68 productively infects immature DCs, whilst LPS-matured DCs are susceptible to latent infection. Importantly, γHV-68 failed to induce DC maturation following infection and infected cells could not be induced to mature fully following LPS or poly(I : C) treatment. Interference of DC maturation may be mediated by a viral protein(s) that inhibits signalling pathways involved in DC maturation. Certain viruses such as EBV, human T cell leukemia virus I, herpesvirus saimiri and KSHV can alter STAT activity to increase the virus persistence, replication and oncogenic potential (Chen et al., 2001; Lund et al., 1997; Migone et al., 1995; Weber-Nordt et al., 1996). Studies are under way to determine whether this is the critical point in DC maturation blocked by γHV-68 infection.

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