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Contribution of Direct and Cross-Presentation to CTL Immunity against Herpes Simplex Virus 1

Adan Chari Jirimo,* Claus-Henning Nagel,† Christof Bohnen,† Beate Sodeik,† and Georg M. N. Behrens²*²

Dendritic cells (DC), which can be subdivided into different phenotypic and functional subsets, play a pivotal role in the generation of cytotoxic T cell immunity against viral infections. Understanding the modes of Ag acquisition, processing and presentation by DC is essential for the design of effective antiviral vaccines. We aimed to assess the contribution of direct vs cross-presentation for the induction of HSV1-specific CD8⁺ T lymphocyte responses in mice. Using HSV1 strains expressing fluorescence proteins, we provide evidence for the ability of HSV1 to induce viral transcription. Using HSV1-wild-type as well as gB- or gH-deficient mutants to either directly inoculate DC or to infect target cells, which then were given to DC, we show that DC acquired viral Ag via phagocytosis of target cells and via direct inoculation of virus being released from target cells. Our study corroborates the function of the CD8⁺ DC specialized in Ag cross-presentation and confirms this specific feature for Ags that these DC acquire directly from HSV1. However, although infection of cross-presenting DC amplified T cell responses, it was not a requirement for presentation of viral Ags, both in vitro and in vivo. Finally, we provide evidence that direct presentation did not contribute to the Ag presentation capacity of CD8⁺ DC after phagocytosis of infected target cells. We conclude that cross-presentation is of major importance for the induction of CTL immunity in mice. The Journal of Immunology, 2009, 182: 283–292.

Dendritic cells (DC)³ are professional APCs capable of both priming an immune response and efficient stimulation of memory responses (1–3). Efficient Ag uptake, migration, and sustained interaction by DC with specific T cells is essential to drive the formation of effector CD8⁺ and CD4⁺ T cell populations (4). Induction of strong CD8⁺ T cell responses is a major goal in the development of preventive and therapeutic vaccines against persistent viruses and tumors.

Viruses are intracellular pathogens that utilize host-cell protein synthesis for replication. Given that endogenously synthesized Ags are predominantly directed in the “direct” MHC class I presentation pathway, this allows CTLs to target virus-infected cells for destruction. To generate effective CTL immunity, however, naive CD8 T cells must be primed to viral Ags presented on DC (5, 6). DC use multiple pathways for Ag acquisition such as phagocytosis, endocytosis, pinocytosis, and specific receptors to capture microbial pathogens, dead or dying cells, immune complexes, and other Ags for immune presentation. Exogenous Ags acquired from the extracellular environment are typically presented by MHC class II molecules, whereas MHC class I molecules bear endogenous Ags synthesized in the cytosol. DC are specifically endowed with the capacity to cross-present exogenous Ags on MHC class I complexes and to efficiently prime CTL immunity. Cross-priming has been hypothesized to be important for immunity to tissue-specific viruses that do not infect DC (7) and may also be crucial for priming CTL to viruses that can impair the direct class I presentation pathway in cells they infect (8).

Analysis of the DC subsets involved in priming CTL immunity to various viral infections, including HSV1, influenza virus, and vaccinia virus, has shown that a single subset, which expresses CD8α and CD205, are the main contributors to activation of naive virus-specific CTL in mice (9, 10). Given that these DC are also the major subset responsible for cross-presentation (11, 12), this provides a tantalizing although unproven link between cross-presentation and viral immunity. Finally, it has been proposed that after cutaneous HSV1 infection in mice, migratory DC transport viral Ags to the draining lymph node were they then transfer the Ag for efficient CTL priming to lymph node-resident CD8α DC. Whether CD8α DC become infected after uptake of these cellular Ags or solely rely on their cross-presentation capacity to induce CTL immunity to HSV1 remains elusive. HSV1 initially infects and replicates in keratinocytes and epithelial cells in the mucosa before it enters autonomous and sensory neurons and travels to the ganglia of the peripheral nervous system where a life-long latent infection is established (13). HSV1 virions consist of a DNA genome of 152 kbp, an icosahedral capsid covered by the tegument, and a envelope that contains ~12 different viral membrane proteins.

HSV1 enters many cell types (e.g., Vero cells or neurons) by direct fusion of its envelope with the plasma membrane, while other cells are productively infected by endocytosis or a phagocytosis-like uptake (e.g., HeLa cells or keratinocytes) (14, 15). To initiate an infection, viruses bind to the plasma membrane via a

*Clinic for Immunology and Rheumatology and †Institute of Virology, Hannover Medical School, Hannover, Germany

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2 Address correspondence and reprint requests to Dr. Georg M. N. Behrens, Clinic for Immunology and Rheumatology, Hannover Medical School, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. E-mail address: behrens.georg@mh-hannover.de

3 Abbreviations used in this paper: DC, dendritic cell; ONPG, o-nitrophenyl-β-D-galactopyranoside; BAC, bacterial artificial chromosome; wt, wild type; ORF, open reading frame; MOI, multiplicity of infection; RFP, red fluorescent protein; CAV, cell-associated virus; CCD, cytochalasin D; HVEM, herpesvirus entry mediator.

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from the bacterial lacZ gene under the control of the immediate-early HSV-1-ICP4 promoter (HSV1(KOS)-lacZ) (26); HSV1(17\(^+\))\(\text{Lox}\)P\_MCMV\_GFP expressing GFP under the control of a MCMV promoter (HSV1-GFP; K. Theusner, J. Schipke, and B. Sodeik, unpublished data); and HSV1(17\(^+\))\(\text{blueLox}\)RFPVP26-gDGFP expressing \(\beta\)-galactosidase under the control of a SV40 promoter, RFPVP26, instead of the small capsid protein VP26, and gDGFP instead of the envelope protein gD (HSV1-RFP-VP26-gDGFP) (27) were amplified in BHK cells and titrated in Vero cells.

**FIGURE 1.** Inoculation of spleen DC by HSV-1. A, Expression of HSV1 entry-mediating receptors on murine spleen-derived CD11c\(^+\) DC. HVEM (upper panel), nectin-2 (middle panel), and nectin-1 (lower panel) expression is depicted as filled gray histograms. Unstained cells and cells stained with the secondary swine anti-rabbit Ab are shown as black and red histograms, respectively.

**B**, Dose-dependent infection of CD11c\(^+\) DC by HSV1 using reporter gene LacZ. Inoculation with increasing MOI per DC led to integration, transcription, and eventual translation of \(\beta\)-galactosidase, whose activity was measured using its substrate ONPG. Crystal violet staining (depicted here only for 2 \(\times\) 10\(^4\) DC) confirmed equal cell density in all conditions.

**C**, Infection of spleen-derived CD11c\(^+\) DC using dual-color expressing virus (HSV1-RFP-GFP) revealed expression of major capsid viral protein gD in the perinuclear region and minor capsid protein VP26 in the nucleus of infected DC. **D**, GFP expression of infected DC after inoculation with HSV1-GFP. The specimens were analyzed by confocal laser-scanning microscopy.
cells. The amount of β-galactosidase expressed by HSV1(KOS)-loxZ was quantified using o-nitrophenyl-β-n-galactosidase (ONPG), and the cell density was estimated from a parallel plate stained with crystal violet (28). The amounts of ONPG (A650) and crystal violet (A590) were measured using a plate reader (Spectra Count Microplate Photometer; Packard Instruments). For all HSV1 strains and mutants, viral particles secreted into the medium of infected cells were harvested by sedimentation (medium pellet; cf Table I). For fluorescence microscopy experiments, such particles were further purified using linear Nycodenz gradients (gradient purified) (29). For all viral preparations used in Ag presentation assays, the genome: PFU ratios were within a range of 15–48, indicating a very low number of defective particles in our inocula (29).

Preparation of noninfectious, single-round HSV1 particles

Escherichia coli DY380 harboring pHSV1(17”)lox, a bacterial artificial chromosome (BAC) in which the thymidine locus of pHSV1(17”+blueLox (27) had been repaired and the β-galactosidase expression cassette had been removed (N. Mütter, C.-H. Nagel, B. Sodeik, and M. Messerele, unpublished data), were grown in the presence of 17 μg/ml chloramphenicol at 32°C. For BAC mutagenesis, the red recombination system of bacterio- phage λ, which has been inserted into the genome of E. coli DY380 (30), was induced by heat shock for 15 min at 42°C, then the bacteria were cooled on ice and made electrocompetent. To delete the complete open reading frame (ORF) of HSV1-gB (UL27), a kanamycin resistance cassette was amplified from pGP704-Kan (31) with primers ATG CAC ATG CGG TTT TAC ACC CAG GTT TTA TAT TTA CAA CAA ACC CCC CAC AGG AAC ACT TAA CCG CTT GAG and CCG CCC CAG GCT ACC TAC TGG CGG GCA CGG GCC GCC GTA GTC CCG CCA GGA CGA CGA CGA CAA GTA A. To delete aa 105–475 of HSV1-gB (ORF UL22) similar to a previously published mutant (19), the primers CTG GGC GCC ACT GGC TCG TAC GCC GAG GGG CTA TCC AGC AGC AAC GGC AAC ACT TAA CCG CTT GAG and TTT GTC TAT TGG TCA CCA CCG CAG ATT TTC CCG GGC ACC GCC AGA AGA CGA CGA CGA CAA CAA GTA A were used. The 50 bp of 5‘ overhanging sequences not annealing to the template correspond to the sequences upstream and downstream of the insertion site and are written in italics. The bacteria were transformed with the PCR products by electroporation and grown at 32°C with 17 μg/ml chloramphenicol and 30 μg/ml kanamycin. Restriction analysis of pHSV1(17”)lox-ΔgB-Kan and pHSV1(17”)lox-ΔgH-Kan in comparison to pHSV1(17”)lox with EcoRI, EcoRV, HindIII, BglII, BamHI, or NotI generated all of the predicted fragment sizes, calculated according to the published HSV1(17”) sequence (GenBank accession no. NC001806; data not shown). HSV1(17”)lox-ΔgH-Kan (HSV1-ΔH) lacking the gene coding for gH and HSV1(17”)lox-ΔgB-Kan (HSV1-ΔgB) deficient of gB were amplified and plaque-titrated using either the Vero cell line d6.1 expressing gB with less flanking sequences than the previously used cell lines (H. Brown and A. Minson, personal communication and Ref. 18) or the Vero cell line CR1 expressing gH (32). The particles released from infected cells still contained gB or gH in their membrane, but not the ORFs encoding these proteins in their genome. Thus, after inoculation into a noncomplementing cell, they perform a single replication cycle with viral protein synthesis, after which noninfectious particles lacking gB or gH and thus unable to fuse with a host membrane, were released. HSV-1(17”)lox-ΔgH-Kan and HSV-1(17”)lox-ΔgB-Kan are therefore single-round viruses.

Mice

C57BL/6 (B6), B6.C-H-2Kbm1 (bm1), gBT-I-1 (gBT-I) (33), OT-I (34), and OT-II (35) mice were maintained in specific pathogen-free conditions in the Animal Facility of Hannover Medical School in accordance with institutional guidelines. All experiments were reviewed and approved by an appropriate institutional review committee. CD8+ T lymphocytes of gBT-I transgenic mice express a V602/V8.1+ TCR (36) that recognizes the HSV-1 gB549–556 determinant complexed with H-2Kb. The OT-I and OT-II mice express MHC class I- and II-restricted TCR (V198/8.1+) specific for the H-2Kb-restricted OVA peptides OVA257–264 and OVA323–339, respectively. B6.C-H-2Hbm mice express a mutated H-2Kb molecule that prevents presentation of HSV-1 gB499–505 or OVA257–264 peptides to the respective TCR-transgenic T cells.

DC isolation

DC were isolated essentially as described previously (37–39). Briefly, spleen fragments were digested for 20 min at room temperature with collagenase (Cell Systems) and DNase (Roche Diagnostics) and then treated for 5 min with EDTA to disrupt T cell-DC complexes. Cells not of the DC lineage were depleted by incubating in predetermined optimal concentrations of purified Abs: anti-CD3 (KT3), anti-Thy-1 (T24/31.7), anti-CD19 (ID3), anti-GR-1 (RB6-8C5), and anti-erythrocyte (TER-119). Ab-binding cells were then removed with anti-rat Ig-coupled magnetic beads (Qiagen). The DC in the enriched populations (usually ~70% pure) were stained with CD11c (rat anti-mouse FITC), CD8 (rat anti-mouse allophycocyanin), and CD4 (rat anti-mouse PE), all from BD Pharmingen, and total CD11c+.
cells or specific subsets were sorted on a MoFlo (DakoCytomation) or FACSria (BD Biosciences).

**HSV1 receptors on DC**

Expression of HSV1 entry receptors on DC was analyzed using a set of polyclonal Abs targeting various receptors. Briefly, DC were first blocked with FcγRII/III receptor Abs (Mouse BD Fc Block, no. 553141; BD Pharmingen) followed by incubation with the rabbit polyclonal primary Abs R140 (against HveA), R146 (against HveB or nectin-2), or R166 (against nectin-1) at a dilution of 1/2000 at 4°C. DC were washed and incubated with swine anti-rabbit F(ab')2 secondary Ab conjugated with HveC or nectin-1) at a dilution of 1/2000 at 4°C. DC were washed and incubated with swine anti-rabbit F(ab')2 secondary Ab conjugated with FITC (no. F 0054; DakoCytomation) and analyzed by flow cytometry. For functional analysis, 1 × 10^5 DC were incubated with polyclonal anti-nectin-1 (R166) or irrelevant rat anti-mouse Abs before inoculating them with HSV1 virus with a MOI of 3. The amount of gBT-I proliferation induced by DC incubated with the entry receptor Abs and control DC was compared using flow cytometry.

**Phagocytosis assay**

Single-cell suspensions were made from bm1 splenocytes and then labeled with PKH26 dye according to the manufacturer's instructions (Sigma-Aldrich). Briefly, cells were resuspended in diluent C at a concentration of 2–8 × 10^6/mL combined with an equal volume of 10 μM PKH26 in diluent C and incubated at 25°C for 5 min with periodic gentle mixing. Staining was terminated with addition of PBS containing 1% (v/v) BSA and further incubated for 1 min before adding an equal volume of 10% (v/v) FCS medium for washing. Cells were then centrifuged at 400 × g for 10 min, transferred into a new tube, and further washed three more times before they were used in experiments. For in vivo assay, the PKH26-stained bm1 cells were injected into C57BL/6 mice via tail vein and 18 h later the mice were sacrificed and DC preparations were obtained from the spleens. Amount of PKH26 fluorescence was then determined in each DC subset by flow cytometry (FACSCalibur; BD Biosciences).

**CpG treatment**

Mice were injected i.v. in the tail vein with 20 nmol of synthetic phosphorothioated CpG1668 (TIB MOLBIO Syntheselabor), and 18 h later DC were isolated from the spleen and either cocultured with Ag-specific T cells.

**Inoculation of DC with HSV1 for fluorescence microscopy**

DC were inoculated with HSV1(17')Lox-P_mon, GFP or HSV1(17')blueLox-RFPVP26-pDGFP as previously described (22, 28). Briefly, the virus was attached by incubation at a MOI of 5 on ice for 1 h to CD8^+ DC. One × 10^4 CD8^+ DC and CD8^- DC were isolated from mice that had been treated with 20 nM CpG or PBS. DC were cocultured with 1 × 10^3 HSV1-wt-inoculated splenocytes (CAV) and proliferation of gBT-I cells was assessed after 60 h. CD8^- DC had a significantly reduced capacity to cross-present Ag from CAV to gBT-I cells but maintained their ability to directly present viral Ag after inoculation in vitro. In contrast, CpG treatment had no effect on the low-level basal Ag presentation capacity of CAV by CD8^- DC and their T cell stimulation capacity remained also unchanged upon direct inoculation with HSV1-wt. Bars depict the mean (±SEM) of three independent experiments with duplicate measurements.
scanned and images acquired were analyzed on a Axiovert 200M microscope equipped with an LSM 510 META confocal laser-scanning unit (Zeiss) using a ×60 oil immersion objective lens. Image acquisition and processing was performed by using the Zeiss LSM imaging software ImageJ 1.35 (W. Rasband; National Institutes of Health (http://rsb.info.nih.gov/ij/)).

Preparation of CFSE-labeled T cells

OT-I, gBT-I, or OT-II cells were purified from single-cell suspensions of pooled lymph nodes (inguinal, axillary, sacral, cervical, and mesenteric) of transgenic mice by depletion of non-CD8 T cells (non-CD8 T cells for purification of OT-II samples) using a CD8 or CD4 enrichment kit (nos. 558471 and 558131 respectively; BD Pharmingen). These cells were labeled with CFSE (Molecular Probes and Invitrogen) by incubating 10^7 purified cells/ml with 1 μM CFSE for 10 min at 37°C. Cells were then washed three times in HEPES-modified Eagle’s medium containing 2.5% FCS. The T cell preparations were routinely 85–95% pure as determined by flow cytometry (FACScalibur; BD Biosciences).

Analysis of T cell proliferation

A total of 5 × 10^3 enriched CFSE-labeled gBT-I, OT-I, or OT-II transgenic cells was added to 1 × 10^5 DC, if not otherwise indicated, in 200 μl of RPMI 1640 containing 10% FCS, 50 μM 2-ME, 2 mM t-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 96-well U-bottom plates (Falcon; BD Biosciences). Cultures were analyzed for proliferation after 60 h. Briefly, transgenic T cells were labeled with CD8, CD4, or anti-Vα2. The samples were analyzed by flow cytometry and the total number of live dividing lymphocytes (CD8^+ T cells) was assessed as above.

Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A).

Results

Inoculation of spleen DC by HSV1

Host plasma membrane proteins such as nectin-1 and herpesvirus entry mediator (HVEM) provide entry receptors for HSV1 infection (14, 40, 41). First, we assessed the surface expression of nectin-1 was expressed on spleen DC subsets (42). We purified CD8^+ T cells from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A). Next, we determined whether DC obtained from the spleen of incoming capsids, and viral gene expression within murine DC (Fig. 1A).

Inoculation of spleen DC by HSV1

Next, we used the dual-color strain HSV1-RFPVP26-gDGFP to track the expression of late structural proteins, namely, the small capsid protein VP26 and the envelope protein gD via their respective red fluorescent protein (RFP) or GFP tags. Such dual-color viral strains allow the detection of all cells and subcellular structures containing viral proteins, irrespective of their interaction and possible masking by host factors or other structural viral proteins (27). Moreover, they do not require specific Abs directed against viral proteins, which often cross-react with cellular structures in primary cells. At 8 h after infection, the nuclei showed a prominent nuclear RFPVP26 expression indicating efficient nuclear HSV1 capsid assembly. Moreover, the entire cytoplasm was labeled by gDGFP, indicating the synthesis of HSV1 envelope proteins in the endoplasmic reticulum (Fig. 1D), and there was a strong perinuclear gD signal that most likely represented the trans-Golgi network where HSV1 capsids acquire their final envelope (27). However, we were unable to detect any infectious virus particle in the supernatant of HSV1-inoculated DC by plaque assay using Vero cells (data not shown), suggesting that the DC synthesized structural viral proteins, but did not release infectious HSV1.

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MHC-restricted Ag presentation of HSV1 or soluble Ags by spleen DC populations

At least three conventional CD11c^+ DC subsets from murine spleen can be distinguished based on their surface expression of CD4 and CD8 or lack of both: the CD8^+ DC, CD4^+ DC, and CD8^-CD4^- DC (double-negative DC) subsets (42). We purified these DC populations and measured the MHC class I-restricted T
cell stimulation by pulsing individual DC subsets with the viral-derived immunodominant Kb-restricted HSV-gB498–505 epitope (SSIEFARL) (34) which lead to efficient proliferation of Ag-specific gBT-I cells, demonstrating sufficient MHC class I expression on all DC subsets (Fig. 2A). In addition, direct inoculation of DC with HSV1(F)-wt led to efficient Ag presentation and gBT-I cell proliferation by all DC (Fig. 2B), confirming inoculation and Ag processing. Given the expression of nectin-1 on murine DC, we next assessed whether blocking of virus-receptor interaction would influence the in vitro DC inoculation and Ag presentation. DC were enriched, preincubated with anti-nectin-1, or control Abs and then inoculated with HSV1-wt. Blocking the viral infection by interfering with the interaction of HSV1 and nectin-1 lead to ~2-fold reduction of T cell proliferation to viral Ags via MHC class I (Fig. 2C). Together, these results demonstrate that splenic DC express at least nectin-1 as a surface receptor for viral entry of HSV1. Inoculation with HSV1 was followed by early and late gene expression which contributed to efficient Ag presentation by all DC subsets.

**MHC class I presentation of cell-associated noninfectious (OVA) and infectious (HSV1) Ags by DC**

We next compared the ability of splenic DC subsets to cross-present cell-associated nonviral and viral Ags. Purified DC subpopulations were cocultured with either OVA-coated splenocytes or with HSV1-wt inoculated splenocytes (cell-associated virus, CAV). Given our interest in the unique characteristics of CD8+ DC in viral infections (43), CD4+ DC and double-negative DC were classified in the following experiments as CD8− DC. To prevent direct presentation of OVA or HSV1 by the target cells, H-2Kbm1 (bm1) splenocytes were used, which cannot present the Kb-restricted OVA257–264 or HSVgB498–505 epitopes to OT-I cells or gBT-I cells, respectively (44). These experiments confirmed that efficient cross-presentation of cell-associated OVA to OT-I cell via MHC class I was confined to the CD8+ DC population (Fig. 3A). In contrast, although the CD8+ DC subset was the dominating DC population to present viral Ag obtained from infected target cells, CD8− DC clearly stimulated significant proliferation of gBT-I cells (Fig. 3B). We speculated that under these experimental conditions, in addition to cross-presentation of acquired exogenous Ags by CD8+ DC, infective virions, produced and released by the target cells, had led to DC inoculation, which then contributed to the CD8+ DC and CD8− DC-mediated Ag presentation. In addition, we hypothesized that CD8+ DC and potentially CD8− DC could become infected upon uptake of HSV1-positive target cells, which would result in production of viral Ags and favor direct rather than cross-presentation (45).

To confirm the first hypothesis, we inhibited actin polymerization of CD8+ DC by treating them with 10 μM cytochalasin D (CCD) for 30 min to reduce their phagocytic activity. Inhibiting phagocytosis led to a 4-fold reduction in the ability of CD8+ DC to present cell-associated viral Ag and to a 2-fold reduction of T cell stimulation by CD8+ DC (Fig. 3C). To rule...
out the possibility that this resulted from a direct effect of CCD on Ag processing and presentation, we infected CCD-treated DC directly with HSV1 and observed no effect on the presentation of viral Ag to Ag-specific gBT-I T cells (data not shown). Preactivation of DC by CpG (or stimulating TLR receptors) inhibits cross-presentation while maintaining the ability for classical MHC class I Ag presentation, partially due to reduced uptake by phagocytosis (46). Therefore, we pretreated mice with CpG and 18 h later isolated their spleen CD8<sup>+</sup> and CD8<sup>−</sup> DC for Ag presentation of CAV. Indeed, CpG-activated CD8<sup>−</sup> DC demonstrated a significantly reduced Ag presentation (about 5-fold) of cell-associated HSV1 but had no influence on direct inoculation (Fig. 3D). The Ag presentation of CD8<sup>−</sup> DC was almost unaffected by CpG treatment, both when given cell-associated HSV1 or when directly infected with the virus (Fig. 3E). Taken together, these data indicate that cross-presentation of phagocytosed viral Ags by CD8<sup>−</sup> DC was the major pathway for Ag acquisition and T cell activation by this DC subpopulation, whereas CD8<sup>+</sup> DC required direct inoculation with HSV1 for effective MHC class I-mediated Ag presentation.

We adopted an alternative approach to specifically address the possibility of infection by released virions and generated a reinfection-incompetent virus lacking gp H (HSV1-ΔgH). This virus does not synthesize gH, which is essential for viral core entry (14, 19) and thus enabled us to study the contribution of viral release from the target cells to the MHC class I presentation. HSV1-ΔgH was generated using BAC mutagenesis and a complementary cell line that provided gH in trans for the virion production. The resulting single-round HSV1-ΔgH particles lacking the gH gene in their genome but containing gH proteins in their envelope were used to inoculate bm1 splenocytes, which then were cocultured with the respective DC populations and gBT-I cells. The bm1 splenocytes inoculated with HSV1-ΔgH could become inoculated, could synthesize all HSV1 proteins but gH, and were thus unable to produce infectious virions. Interestingly, under these in vitro conditions, only the CD8<sup>−</sup> DC presented the MHC class I-restricted gB epitope to gBT-I cells, indicating that indeed released virions from the infected target cells contribute to the overall T cell proliferation obtained with HSV1-wt (Fig. 4). The proliferation induced by CD8<sup>−</sup> DC and cell-associated HSV1-ΔgH was negligible. As a control, HSV1-ΔgH particles containing cell-derived gH in their envelope but lacking the gH gene were equally well presented by the various DC subsets after direct inoculation, confirming that the mutant virus could enter DC and synthesize more gB. More importantly, in vitro DC inoculation with HSV1-ΔgH particles produced in noncomplementing Vero cells and thus lacking gH both in the envelope and in the genome did not result in any T cell proliferation, validating the experimental approach (data not shown). These data also corroborate our earlier observation that HSV1 inoculation of DC was facilitated by specific receptor-mediated uptake, as HSV1-ΔgH released from the infected target cells or used for direct DC inoculation did not lead to adequate Ag presentation.

Next, we addressed the hypothesis that CD8<sup>−</sup> DC could become infected after uptake of infected target cells and that gB being produced within this DC subset contributes to MHC class I presentation. For this, we used the single-round viral mutants HSV1-ΔgH and HSV1-ΔgB to inoculate target cells and compared the resulting T cell proliferation stimulated by the DC subsets. In case of any contribution of gB synthesis upon infection due to uptake of infected target cells to the MHC class I response, only the HSV1-ΔgH mutant would be able to synthesize more gB in the CD8<sup>−</sup> DC, which then should result in a relatively increased Ag presentation and T cell proliferation as compared with HSV1-ΔgB. The T cell proliferation resulting from coculture with either HSV1-ΔgH- or HSV1-ΔgB-infected target cells was almost equal supporting the notion that CD8<sup>−</sup> DC infection was not required and the uptake of inoculated target cells containing newly synthesized gB (HSV1-ΔgH) or even incoming gB (HSV1-ΔgB) sufficed to trigger T cell proliferation (Fig. 5). As a further control, CD8<sup>−</sup> DC could even efficiently cross-present the small amount of gB Ag derived from the incoming HSV1-ΔgB particles after inoculation, which was in marked contrast to the inability of CD8<sup>−</sup> DC inoculated with HSV1-ΔgB particles to stimulate T cell proliferation.

Ag presentation after uptake of HSV1-infected cells in vivo
To test the ability of the DC subsets to acquire viral Ags from infected cells in vivo, splenocytes from bm1 mice were stained with the membrane dye PKH26, infected with HSV1-wt, and injected into B6 mice. Eighteen hours later, we isolated DC from the spleen and quantified their amount of PKH26. Flow cytometry showed that both CD8<sup>−</sup> DC and CD8<sup>−</sup> DC had taken up injected HSV1-wt (Fig. 6A). We sorted for CD8<sup>−</sup> DC and CD8<sup>−</sup> DC to stimulate CFSE-labeled gBT-I cells ex vivo. Similar to earlier descriptions using CFSE-labeled gBT-I cells ex vivo. Similar to earlier descriptions using CFSE-labeled gBT-I cells, proliferation of CFSE<sup>low</sup> gBT-I cells was quantitated by flow cytometry. Results show the mean (±SEM) of three independent experiments with three mice per group.
and generation of HSV1-specific CTL immunity in mice after systemic infection.

Discussion
Generating optimal vaccines ultimately requires a rational design based upon the detailed knowledge of how CTL against viral infections are generated. We wanted to dissect the contribution of direct vs cross-presentation to antiviral CTL immunity and took advantage of the differing constitutive cross-presentation capacities of conventional murine DC subtypes (48) as well as of genetically modified single-round HSV1 mutants. Based on our findings, we conclude that upon systemic HSV1 infection, CD8+ DC presented viral Ag by cross-presentation of virally infected cells. Moreover, viral targeting and nuclear transcription either of free virus or virus being released after phagocytosis of infected cells by CD8+ DC contributed only marginally, if at all, to antiviral immunity after systemic inoculation. This is in contrast to exposing DC to HSV1 in vitro, where all conventional murine DC subsets of the spleen were capable of acquiring free virus or virus released from infected cells, where HSV1 lead to expression of viral proteins and where viral inoculation significantly contributed to classical MHC class I presentation, but did not lead to productive infection. We speculate that in contrast to conditions of in vitro experiments, antiviral mechanisms of the innate immune system such as IFN-α, TNF-α, or NK cells can prevent direct inoculation of DC in vivo. This could result in significantly reduced amounts of infectious particles for direct inoculation of DC in the spleen after systemic exposure to the virus. In addition, these factors could prevent infected target cells from releasing infectious HSV1 for inoculation of DC.

The CD8+ DC is the dominating DC subset involved in Ag presentation via MHC class I after footpad or s.c. infection with HSV1, and cross-priming is a likely pathway for antiviral CTL immunity (6, 49). These studies suggest that CD8+ DC reside in the draining lymph node and receive Ags for CTL priming from other DC that have emigrated from the periphery. Other experimental evidence has shown that the ability of CD8+ DC to cross-presentation cell-associated Ags is not dictated by their capability for Ag capture but by their intracellular Ag-processing machinery (47). These studies, however, did not address the way of acquisition of pathogen Ags or used cell-associated OVA as model Ag. Specifically, factors like virus release from infected cells or viral infection of DC after uptake of infected cells, which could be important factors for antiviral CTL immunity, were not considered.

Several groups have shown that virus-infected cells in various forms can be cross-presented in vitro (50, 51) or can cross-prime in vivo, resulting in the generation of CTL responses to viral proteins (52–55). In addition, detection of priming in the presence of virus-encoded mechanisms that impair direct presentation implicates a component of cross-priming in viral immunity (56, 57). We observed in our study that under conditions that allow DC ample access to infected cells, all DC subsets could efficiently present viral Ags via MHC class I, independent of their constitutive cross-presentation capacity. We believe that this was most likely due to inoculation of all DC subsets with virus released from infected target cells. Two independent experimental approaches support this interpretation. First, impairing cross-presentation of cell-associated (viral) Ags after preactivation of DC with TLR ligands or inhibition of phagocytosis with CCD led to levels of CD8+ T cell stimulation by cross-presenting CD8+ DC similar to those by CD8+ DC, which showed no or little alteration in T cell stimulation after CpG or CCD pretreatment. Second, abrogation of viral inoculation of DC by using the single-round mutant HSV1-ΔH, which cannot infect DC after release from inoculated target cells, resulted in a complete loss of CD8+ T cell proliferation induced by CD8+ DC but did not reduce proliferation obtained with cross-presenting CD8+ DC. Thus, if direct inoculation or release of HSV1 from other cells after systemic infection would occur and gain access to splenic DC, all DC subsets would be able to contribute to MHC class I presentation in vivo. This, however, has not been demonstrated in the previous studies (6) and also our experiments using infected target cells in vivo demonstrate that the CD8+ T cell stimulation was restricted to the CD8+ DC that had captured target cells, although phagocytosis of HSV1+ target cells was also readily detectable in the splenic CD8+ DC compartment.

Numerous alternative approaches have been used to dissect the role of direct and cross-presentation (23). It was shown that trafficking of class I molecules to the phagosome directly from the endoplasmic reticulum requires a specific signal sequence in the cytoplasmic tail of class I (58). When this sequence is mutated, normal traffic through the Golgi apparatus is maintained, leaving direct presentation intact, but limiting the extent of cross-presentation. To assess the contribution of cross-priming, transgenic mice expressing this form of mutant class I molecule have been examined for their response to vesicular stomatitis virus and Sendai virus. In this case, CTL responses are reduced by ~90%, implying that cross-priming is important in CTL priming to these viruses. The question remains whether the residual priming is due to Ag processed by the direct class I pathway in infected DC or to a residual degree of cross-presentation not impaired by this mutation.

One of the best demonstrations that cross-priming can facilitate CTL immunity during actual virus infection was reported by Sigal et al. (24). These authors transgenically expressed the human cellular receptor for polio virus in mice, which normally lack this receptor and are therefore not infected by this virus. When CTL immunity was examined in chimeric mice that only express the polio virus receptor on non-bone marrow-derived cells, efficient priming of CTL to viral Ags was shown to occur via presentation by bone marrow-derived cells. This, Segal et al. (24) concluded, could only occur if host DC capture viral Ags from infected non-bone marrow-derived cells. Although this provides strong evidence that cross-priming can facilitate CTL immunity during a virus infection, it fails to determine the contribution of direct vs cross-priming when both pathways are unimpaired by experimental design. Moreover, Freigang et al. (25) argued compellingly that polio virus replication in some bone marrow chimeras used by Sigal et al. (24) most likely amplified the virus dose, resulting in inoculation of DC in the B6 mice and therefore leading to direct priming. Although viral RNA was found in splenic DC after infection, translation of viral genes was not demonstrated. Given that murine DC are deficient of any known polio virus entry receptor, the authors speculated that other substituting molecules on DC may facilitate viral entry, that infection intermediates could infect DC, or that DC nonspecifically acquire pathogens or translatable pathogens.

We hypothesized that DC could become infected upon uptake of infected cells, which would also circumvent the need for cross-presentation and should most likely occur in all DC subsets. When target cells inoculated with HSV1-ΔgH or HSV1-ΔgB were given to splenic DC, neither HSV1-ΔgH nor HSV1-ΔgB acquired through phagocytic uptake by DC could result in viral egress from the phagosome in nuclear targeting and in viral transcription of gB. Under these two conditions, CD8+ T cell proliferation induced by both CD8+ DC and CD8+ DC was still within the same range, suggesting that direct Ag presentation of endogenous synthesized gB after phagocytosis of infected cells did not contribute in any of
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