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Herpes Simplex Virus Type-1-Induced Activation of Myeloid Dendritic Cells: The Roles of Virus Cell Interaction and Paracrine Type I IFN Secretion

Gabriele Pollara,* Meleri Jones,‡ Matthew E. Handley,* Mansi Rajpopat,* Antonia Kwan,‡ Robert S. Coffin,* Graham Foster,‡ Benjamin Chain,* and David R. Katz2*}

Adaptive cellular immunity is required to clear HSV-1 infection in the periphery. Myeloid dendritic cells (DCs) are the first professional Ag-presenting cell to encounter the virus after primary and secondary infection and thus the consequences of their infection are important in understanding the pathogenesis of the disease and the response to the virus. Following HSV-1 infection, both uninfected and infected human DCs acquire a more mature phenotype. In this study, we demonstrate that type I IFN secreted from myeloid DC mediates bystander activation of the uninfected DCs. Furthermore, we confirm that this IFN primes DCs for elevated IL-12 p40 and p70 secretion. However, secretion of IFN is not responsible for the acquisition of a mature phenotype by HSV-1-infected DC. Rather, virus binding to a receptor on the cell surface induces DC maturation directly, through activation of the NF-κB and p38 MAPK pathways. The binding of HSV glycoprotein D is critical to the acquisition of a mature phenotype and type I IFN secretion. The data therefore demonstrate that DCs can respond to HSV exposure directly through recognition of viral envelope structures. In the context of natural HSV infection, the coupling of viral entry to the activation of DC signaling pathways is likely to be counterbalanced by viral disruption of DC maturation. However, the parallel release of type I IFN may result in paracrine activation so that the DCs are nonetheless able to mount an adaptive immune response. The Journal of Immunology, 2004, 173: 4108–4119.

Herpes simplex virus type-1 infection resolves in the periphery predominantly as a result of local CD4+ and CD8+ T cell responses (1). In common with most other T cell responses, this HSV response is believed to be initiated by dendritic cells (DCs) from the injured site (2, 3). Several pieces of evidence point to a role for these cells in HSV-1 infections in vivo, including an inverse correlation between DC number and HSV-1-induced skin lesion severity (4). The Th1-skewed T cell response that is important in clearing HSV-1 from the periphery is dependent on IL-12 (5), which is known to be a cytokine secreted by DC during the early phase of Ag presentation (6).

In the local lesion sites, at the time of herpetic infection, the majority of professional APCs in the skin are epidermal Langerhans cells and dermal DC. Both of these cell types are known to be of myeloid origin (2). Plasmacytoid DCs (PDC) (7) are not present in the human skin under these conditions (8). Therefore, following cutaneous infection by HSV, the virus will initially interact with either Langerhans cells or dermal DC. Several recent studies have therefore focused on the interaction between HSV-1 and human myeloid DC (9–12). These studies, however, have predominantly focused on the negative effects of HSV on DC function, identifying disruption of several critical aspects of DC physiology, including their morphology, cytokine secretion, and T cell stimulation (9, 12). Nevertheless, HSV infection is generally accompanied by strong cellular as well as humoral immunological responses (1). Therefore, the question arises as to the nature of the “danger” signals induced by HSV, which trigger DC activation and migration and which are necessary for an adaptive immune response to develop.

A number of observations suggested that the infection of DC by HSV-1 did indeed induce some aspects of DC activation (12). Firstly, infected DCs release a soluble factor with a paradoxical effect, activating neighboring uninfected DC and priming them to secrete elevated levels of IL-12. Secondly, HSV-1-infected DC showed a more mature phenotype than uninfected DC, characterized by elevated MHC class II and CD86 expression. This up-regulation was, however, unable to render the DC potent T cell stimulators, due to the ability of the virus to disrupt the DC maturation process.

In this study we dissect two pathways activated by HSV-1 infection of DC, which when unchecked by the products of viral transcription, can both stimulate DC maturation. The first pathway is mediated via the release of a soluble factor, identified in this study as type I IFN, which can act as paracrine activator of bystander uninfected cells. In addition, HSV-1 can induce DC activation directly, via binding of glycoprotein D (gD) to the DC surface and activation of NF-κB and p38 MAPK. This pathway, however, is inhibited by expression of one or more HSV-1 gene

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3 Abbreviations used in this paper: DC, dendritic cell; PDC, plasmacytoid DC; MDDC, monocyte-derived DC; MOI, multiplicity of infection; MFI, mean fluorescence intensity; IFNAR, type I IFN-α receptor; VHS, virus host shutoff; HVEM, herpesvirus entry mediator; WT, wild type; gD, glycoprotein D.
products. We suggest that the balance between activation via these two pathways and inhibition by viral products is a key factor in determining the immunological outcomes of HSV-1 infection.

Materials and Methods

Antibodies

The following mAbs were used: CD3 (supernatant mouse mAb UCH T1, IgG1, gift of Prof. P. Beverley, Edward Jenner Institute for Vaccine Research, Newbury, U.K.); CD2 (mouse mAb MAS 593, IgG2b; Harlan Sera Lab, Loughborough, U.K.); CD19 (supernatant mouse mAb BU12, IgG1, gift of D. Hardie, Birmingham University, Birmingham, U.K.); HLA-DR (supernatant mouse mAb L243, IgG2a, gift of Prof. P. Beverley); CD14 (supernatant mouse mAb HB246, IgG2b, gift of Prof. P. Beverley); CD1a (supernatant mouse mAb NA1/54, IgG2a, gift of Prof. A. McMichael, John Radcliffe Hospital, Oxford, U.K.); CD13-FTTC (mouse mAb WM15, IgG1; DPC Biemann, Bad Nauheim, Germany); IgG1 isotype control Ab to Aspergillus niger glucose oxidase (mouse mAb DAK-G01, IgG1; DAKOCytomation, Ely, U.K.); HSV-VP16 (supernatant mouse mAb LPI1, IgG1, gift of Prof. T. Minson, Cambridge University, Cambridge, U.K.); neutralizing HSV-gD (supernatant mouse mAb LP2, IgG1, gift of Prof. T. Minson); non-neutralizing HSV-gD (supernatant mouse mAb AP7, IgG1, gift of Prof. T. Minson); human IFN-α (mouse mAb MMHA-11, IgG1; IDS, Boldon, U.K.), which is an Ab that recognizes largest number of IFN-α subtypes; IgG2a isotype control Ab anti-mouse MHC class I (TIB92, IgG2a; American Type Culture Collection, Manassas, VA); and type I IFN-α receptor (JFNAR) chain 2 (MMHAR-2, mouse IgG2a; EMD Biosciences, San Diego, CA).

Cell preparation

Monocyte-derived DC (MDDC) were prepared from 120 ml fresh whole blood from healthy volunteers. Mononuclear cells separated on lymphoprep (Nycomed Pharma, Oslo, Norway) (400 g, 30 min) were incubated in six-well tissue culture plates for 2 h at 37°C/5% CO2 in RPMI 1640 (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (H9262). The supernatant was centrifuged in a minifuge at 16,000 g, and passed through a 0.2-μm pore-size filter (Sartorius, Goettingen, Germany) to remove any viral particles and cell debris, before adding to autologous DC. Direct assay of these supernatants found no remaining live virus, both by plaque assay and by GFP expression. Supernatant-treated DCs were then incubated for 16 h and examined for surface phenotypic changes.

Flow cytometric analysis

DCs were stained for surface markers by incubation first with the relevant mAb (30 min, 4°C) followed by 1/25 diluted PE-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) (30 min, 4°C). Detection of opsonized HSV-1 particles was achieved by staining infected cells only with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). For intracellular IFN-α staining DCs were cultured in complete medium supplemented with 2 μM monensin for a maximum of 12 h, fixed in 4% formaldehyde (10 min, 4°C) at a concentration of 10^6 cells/ml. This was followed by a wash in HBSS supplemented with 2% goat serum (FACS buffer). All subsequent washes and incubations were conducted in FACS buffer supplemented with 0.5% saponin (Sigma-Aldrich). Nonspecific Ab binding was blocked with 10% goat serum solution (15 min, 4°C) before addition of the relevant primary mouse mAb (30 min, 4°C). This was followed by PE-conjugated goat anti-mouse Ig (30 min, 4°C). Staining of other intracellular Ags was conducted as previously mentioned in the absence of monensin, at the times previously detailed. The stained cells were examined by flow cytometry immediately, using a FACScan flow cytometer (BD Biosciences, Oxford, U.K.) and analyzed with WinMDI software. Results are expressed as median fluorescence intensity (MFI).

Cytokine measurement

Secreted type I IFN was measured using a bioassay as described (13). Secreted IL-12 was measured by ELISA: IL-12 p40 (R&D Systems, Abingdon, U.K.) and IL-12 p70 (eBiosciences, San Diego, CA).

Microscopy

DCs were analyzed by confocal microscopy Bio-Rad Confocal microscope (Hercules, CA). GFP fluorescence was recorded by a 488 nm excitation laser and detected in the fluorescence channel by a 522 ± 32 nm emission filter. The images were analyzed using Confocal Assistant and Adobe Photoshop software.

Cell viability

DC viability was assessed by MTT reduction assay as previously described (12).

Proliferation assays

T cell proliferation was assessed by culturing DC, either uninfected or infected for 16 h with allogeneic T cells (10^5/well) as previously described (12). Results are expressed as counts per minute.

Western blotting

DCs were incubated in complete medium containing 0.5% FCS v/v for at least 24 h before treatment. The levels of phospho-p38, total p38, and IκB-α were assayed as previously described (14), using rabbit polyclonal Abs to phospho-p38 (9211; Cell Signaling Technology, Frankfurt am Main, Germany), total p38 (9212, Cell Signaling Technology), and IκB-α incubated in equal volumes of virus (at 2 × 10^7 PFU/ml) and LP2 Ab ± 12.5 μg/ml heparin (C. P. Pharmaceuticals, Rexam, U.K.) at 37°C for 15 min and then added to DC directly. AP7 non-neutralizing anti-gD Ab was used as a control. All virus stocks tested negative for the presence of mycoplasma.

Infection of DCs

DCs were infected with HSV-1 as previously described (12) at a multiplicity of infection (MOI) of 1 (or equivalent for inactivated viruses) unless otherwise stated. For all virus infections, uninfected groups received RPMI 1640 treated in the same manner as the virus. Previous experiments had determined that mock viral preparations had equivalent effect on DC as RPMI 1640 (data not shown) (12).

Supernatant transfer studies

In some experiments, the effect of supernatant of infected cells on normal uninfected DCs was tested. Supernatants from uninfected and infected DCs were taken at 16 h incubation, centrifuged in a minifuge at 16,000 × g for 30 min, and passed through a 0.2-μm pore-size filter to remove any viral particles and cell debris. Supernatant-containing DCs were then incubated for 16 h and examined for surface phenotypic changes.

Virus preparation

HSV-1 was derived from HSV-1 strain 17+ containing a GFP cassette, as previously described (12). The virus was propagated on confluent baby hamster kidney BK cells and harvested by freeze-thaw. Cellular debris was removed by centrifugation at 3500 rpm for 45 min at 4°C. The supernatant was then immediately removed, filtered through a 0.5-μm filter followed by a 0.45-μm filter and spun at 12,000 rpm for 2 h at 4°C. The supernatant was discarded and the subsequent viral pellet was gently resuspended in 1 ml HBSS. The resuspended pellet was then sonicated three times for 10 s in a water bath sonicator and stored at −70°C. The virus stock used in this entire study had a titer of 1 × 10^7 PFU/ml as determined by plaque assay. This virus preparation was referred to as wild-type HSV (WT-HSV). The dilution of the virus during preparation makes it unlikely that another danger signal was active at the time of initial HSV exposure. For UV inactivation, the HSV-1 preparation (10^7 PFU/ml) was exposed for 20 min to UV-C light source at a distance of 6 cm delivering 20 mW/cm². This virus preparation is referred to as UV-HSV. HSV-1 was formaldehyde inactivated by incubating HSV-1 (10^9 PFU/ml) with equal volume of 2% formaldehyde (BDH, Poole, U.K.). The virus stock was then diluted to 10^5 PFU/ml with RPMI 1640 and excess formaldehyde neutralized by adding 0.175% sodium bisulphite (Sigma-Aldrich) solution (final concentration). This virus is referred to FIX-HSV. Exposure of DC to the equivalent concentrations of fixative and neutralizing agent (formaldehyde and sodium bisulphite) did not alter DC surface phenotype, excluding indirect effects on the DC by the viral treatment protocol (data not shown). HSV-1 was incubated in equal volumes of virus (at 2 × 10^7 PFU/ml) and LP2 Ab ± 12.5 μg/ml heparin (C. P. Pharmaceuticals, Rexam, U.K.) at 37°C for 15 min and then added to DC directly. AP7 non-neutralizing anti-gD Ab was used as a control. All virus stocks tested negative for the presence of mycoplasma.
(sc-371; Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry analysis of the blots was performed using GeneSnap software (Syngene, Frederick, MD). The ratios of the densities of the phospho-p38 to total-p38 were calculated to give an indication of the proportion of p38 phosphorylated in each condition.

**Statistical analysis**

Where appropriate, the means of paired groups were analyzed by a two-tailed Student’s *t* test.

**Results**

**Characterization of MDDC and HSV-1 infection**

The MDDC cultures used in this study contained predominantly myeloid DC as shown by high CD1a, low CD14 expression and >99% expression of both CD11c and CD13 (Fig. 1A). HSV-1 infection of MDDC was previously shown to inhibit their Ag-presenting capacity (9, 12). However, using an HSV-1 strain engineered to express GFP, we observed that following infection, two markers of DC maturation, CD86, and HLA-DR, were elevated in both GFP− and GFP+ DC, relative to uninfected DC (Fig. 1B). GFP may not be expressed in all infected DC, thus underestimating infection efficiency. However, almost 100% of DC expressing GFP following infection also expressed the late HSV proteins VP16 (intracellular) and gD (extracellular) at high levels. There was no significant population of VP16 or gD expressing DC that did not express GFP, confirming that this transgene is indeed expressed in all infected DC in this system and can be used as a parameter for gating on the direct effects of HSV infection (Fig. 1, C and D).

**Type I IFN secretion by MDDC**

The elevated expression of CD86 and HLA-DR in the GFP− DC population, suggested that uninfected DCs in these cultures were receiving a soluble maturation stimulus. Indeed, culturing autologous DC in the supernatants from HSV-infected DC cultures

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**FIGURE 1.** Characterization of MDDC infection by HSV-1. A. The expression of myeloid markers by the MDDC was assessed by flow cytometry. MDDC were infected with HSV-1 and incubated for 16 h. The expression of GFP transgene was assessed together with the expression of CD86 (B), HLA-DR (B), HLA-ABC (B), HSV-1 VP16 (C), and HSV-1 gD (D). Numbers in dot plot quadrants refer to MFI expression of Ab on y-axis. Representative of three independent experiments.
(supHSV-DC), but not supernatant from uninfected DC (supCON-DC), induced up-regulation of costimulatory and MHC class II molecules, albeit less than that observed with LPS stimulation (Fig. 2A). SupHSV-DC did not contain significant levels of the proinflammatory cytokines IL-1, IL-6, TNF-α, or IFN-γ (Y. McGrath, unpublished observations and data not shown). In contrast, HSV-1 infection induced the release of significant levels of biologically active type I IFN (Fig. 2B).

Despite the purity of the myeloid DC population used in this study (Fig. 1), human PDC are known to secrete large quantities of type I IFN in response to viral stimulation (7). Therefore, it was important to demonstrate that the type I IFN released following HSV-1 infection did not derive from small numbers of contaminating PDC. This was confirmed by intracellular staining for IFN-α, in which the increase in IFN staining was mediated by a shift in fluorescence of the entire DC population, not by a subset of IFN-secreting DC (Fig. 2, C and D). As the type I IFN secretion system responds to a well-described autocrine- and paracrine-positive feedback loop (15), DCs in these experiments were infected at a high MOI of 3. The resulting infection efficiency was near 90%, reducing to a minimum the number of uninfected DC, and ensuring that the stimulus for IFN secretion was coordinated and originated directly from the viral infection.

Type I IFN-mediated activation of DC

Analogous to the effects of supHSV-DC, recombinant IFN-α was a maturation stimulus for DC, up-regulating CD86 and HLA-DR molecules in a dose-dependent manner (Fig. 3A). Of note, in the concentration range of type I IFN secreted following HSV infection (~250 U/ml), the maturation was submaximal relative to that of LPS, analogous to that seen when DCs were cultured in supHSV-DC (Fig. 2A).

We have previously shown that supHSV-DC primed DC for increased IL-12 p40 secretion following LPS stimulation (12) and this priming effect is also observed for the secretion of the functional heterodimer of this cytokine, IL-12 p70 (data not shown). To assess whether type I IFN could reproduce this effect, DCs were cultured in the presence of recombinant IFN-α and stimulated with LPS. Consistent with the type I IFN activity in supHSV-DC, IFN-α itself was not a stimulus for DC secretion of IL-12 p40 but could prime DC for increased LPS-mediated IL-12 p40 secretion (Fig. 3B). This synergistic action was also observed for IL-12 p70 secretion (Fig. 3C). Furthermore, IFN-α increased the IL-12 p70: p40 ratio (the proportion of functional heterodimer secreted) (Fig. 3D), suggesting a regulatory role for IFN-α also on the IL-12 p35 subunit.

Role of type I IFN in HSV-1-mediated DC maturation

To confirm that the type I IFN in the supHSV-DC was responsible for the maturation effects observed, we attempted to neutralize its activity in the supernatants by preventing binding of IFNAR. Culturing DCs in supHSV-DC in the presence of a neutralizing mAb to one of the two IFNAR chains (IFNAR2) abrogated the ability of the supernatant to up-regulate CD86 (Fig. 4A) and HLA-DR (data not shown), confirming that type I IFN secreted after infection was the major active component in supHSV-DC.

As shown in Fig. 1B, DCs infected with HSV-1 also displayed a more mature phenotype. To investigate whether this could be attributed to the autocrine activity of type I IFN, we infected DC in the presence of anti-IFNAR2 Abs. HSV-1 induced up-regulation of CD86 (Fig. 4B) and HLA-DR (data not shown) on DC was not abrogated by this treatment. Thus, we concluded that HSV-1 infection of DC resulted in a maturation process independent of the autocrine activity of type I IFN secreted.

In summary, Figs. 2, 3, and 4 show that following HSV-1 infection, DCs of myeloid origin can secrete biologically significant amounts of type I IFN to induce partial maturation of neighboring uninfected DCs but this is not required for the maturation observed in infected DC.

UV-inactivated HSV-1 infection of MDDC abolishes inhibition of WT-HSV and induces maturation

We investigated which step of the virus infection process resulted in DC maturation. This strategy also allowed us to identify the level at which HSV-1 exerts its main inhibitory effects on DC function. To remove the effects of de novo viral gene expression, we inactivated the virus using UV light. Intracellular staining for the HSV-1 tegument protein, VP16, 2 h postinfection confirmed that UV-inactivated HSV-1 (UV-HSV) was able to infect DC with equal efficiency to WT-HSV (Fig. 5A). Inactivation of the viral genome was confirmed by the absence of GFP expression following overnight culture (Fig. 5B). In contrast to WT-HSV, DC viability was not compromised following UV-HSV infection (Fig. 5C) and the strong defect in T cell stimulation observed with WT-HSV was also lost in the absence of HSV gene expression (Fig. 5D).

Similarly, DC morphology was not altered following UV-HSV infection, and the DC showed increased dendrite formation in response to LPS, like uninfected cells. In contrast, WT-HSV-infected DC lost dendrites and became rounded up both in the presence and absence of LPS (Fig. 5E).

UV-HSV infection of DC resulted in similar changes in surface phenotype to those seen with WT-HSV, up-regulating CD86 (Fig. 6) and HLA-DR (data not shown) expression. However, in contrast to WT-HSV, LPS stimulation of UV-HSV-infected DC resulted in further up-regulation of CD86 and MHC class I (Fig. 6).

In summary, Figs. 5 and 6 show that HSV-1 gene expression is responsible for the deleterious effects of HSV-1 infection on DC, rendering DC morphology and phenotype refractory to further stimulation by LPS stimulation, and also inducing cell death and preventing efficient T cell stimulation. However, the changes in phenotype also demonstrate that viral receptor binding and/or entry into DC is a maturation stimulus.

HIV interaction with DC surface up-regulates CD86 and activates p38 MAPK and NF-κB

To identify this activation event in more detail, we modified WT-HSV particles further. Formaldehyde fixation of HSV virions (FIX-HSV) rendered them unable to fuse with the cell membrane and therefore noninfective, as determined by the absence of VP16 staining 2 h postinfection (data not shown) and lack of GFP expression after overnight culture (Fig. 7A). However, the virus’ ability to bind to the DC surface remained intact, as determined by flow cytometry by the presence of HSV-1 gD on the surface of DC 1 h after infection (Fig. 7B). DC exposed to FIX-HSV up-regulated the surface marker, CD86 (Fig. 7B) similarly to WT-HSV. Furthermore, like UV-HSV, FIX-HSV-infected DCs were not refractory to further LPS stimulation, again in contrast to the effects of WT-HSV (Fig. 7B).

HSV-1 infection has been shown to activate NF-κB and p38 MAPK previously (16, 17), but the role of early viral interaction with receptors on the cell surface in initiating this activation has not been documented. Therefore we assessed the ability of viral infection to induce the degradation of IkB-α, a cytosolic inhibitor of the NF-κB subunits. Western blotting showed that WT-HSV infection of DC induced significant IkB-α degradation within 30 min after infection (Fig. 8A). This is regarded as indicative of...
FIGURE 2. Type I IFN secreted by MDDC infected with HSV-1. A, Supernatants from uninfected and HSV-1 infected cultures in Fig. 1 (supCON-DC and supHSV-DC, respectively) were harvested and used to culture autologous DC for 16 h. Surface markers of DC maturation were assessed. The data are the mean of three independent experiments. B, Supernatants from uninfected and HSV-1 infected DC were harvested 16 h after infection and type I IFN secretion determined by an antiviral bioassay. The data are the mean of four independent experiments. Error bars represent SEM; *, p < 0.01 relative to uninfected DC. C, MDDC were infected with HSV-1 at MOI of 3 and incubated for 12 h in the presence of 2 μM monensin. Production of IFN-α was assessed by intracellular staining. Representative of three independent experiments. D, MDDC as in C, but displayed as frequency histogram. Representative of three independent experiments. Bar chart (inset) represents mean ΔMFI IFN-α staining of three independent experiments. Error bars represent SEM.
NF-κB activation, as loss of the IκB-α protein permits NF-κB transcription factor subunits to translocate to the nucleus and induce transcription of NF-κB controlled genes, such as CD86 (18). As the activation of the p38 MAPK pathway, which is another important modulator of DC maturation (19–21), is also induced in DC, the same membranes were also assayed for p38 phosphorylation. WT-HSV induced significant phosphorylation of p38 after infection (Fig. 8A). This was quantified relative to total p38 by densitometry (Fig. 8B). Interestingly, LPS mediated greater p38 activation than WT-HSV, correlating with the degree of maturation seen with LPS relative to the (submaximum) maturation observed following HSV-1 infection (Fig. 7B) (12).

**FIGURE 3.** Type I IFN is responsible for maturation of bystander DC but not for infected DC. A, MDDC were cultured for 16 h in a range of concentrations of recombinant IFN-α (left) and changes in surface phenotype assessed. Data are mean MFI of three independent experiments. Error bars represent SEM. MDDC were cultured for 16 h in 1000 IU/ml IFN-α or 100 ng/ml LPS (right) and changes in surface phenotype were assessed. Representative of three independent experiments. B, MDDC were cultured in the presence or absence of recombinant 1000 IU/ml IFN-α ± 100 ng/ml LPS for 16 h. Supernatants were assayed for IL-12 p40 or IL-12 p70 (C) by ELISA. Results are mean of three independent experiments. Error bars represent SEM; *, p < 0.01 relative to LPS only stimulated DC. D, Bar chart of IL-12 p70:IL-12 p40 ratio calculated from bar charts B and C.
Glycoprotein D neutralization of HSV-1 abolishes CD86 up-regulation and type I IFN secretion

HSV-1 entry into cells is mediated by glycoprotein B and glycoprotein C attachment to cell surface heparan sulfate, followed by gD-mediated entry via one of the entry receptors (22). Therefore, to determine the viral factors responsible for the up-regulation of CD86, we neutralized FIX-HSV and WT-HSV virions with a mAb to HSV-1 gD (LP2 clone) and observed that the stimulus required for the up-regulation of CD86 expression was abrogated (Fig. 9A). The effect on FIX-HSV confirmed that extracellular binding of gD to a cell surface receptor was a sufficient activating signal to induce partial DC maturation seen following HSV-1 infection. To exclude the possibility that the presence of Abs on the viral envelope could sterically hinder the interaction of viral ligands with the cell surface, HSV particles were also precoated with a non-neutralizing anti-gD mAb (AP7 clone) before addition to DC. As determined by GFP expression, this Ab did not prevent infection to any significant degree, in contrast to LP2 (Fig. 9B). Furthermore, staining for the presence of mouse IgG on the surface of DC 16 h after infection demonstrated that DC infected with AP7 coated HSV (i.e., the GFP− population), expressed mouse IgG on the cell surface. This is likely to have originated from non-neutralized viral envelope that fused with the DC membrane.

DC exposed to FIX-HSV coated in AP7 demonstrated an equally mature phenotype as virus treated with an isotype control Ab, in contrast to the neutralizing effect of LP2. Thus Fig. 9, B and C demonstrate that the ability of neutralizing anti-gD Abs to prevent DC maturation is likely to occur by preventing specific sites on gD from interacting with activatory surface receptors, rather than through nonspecific steric effects or possibly by interacting with inhibitory FcγR on DC.

The neutralization of gD did not totally exclude the role of other viral structures in the activation of DC. Two of these are glycoprotein B and glycoprotein C that attach HSV-1 virions to heparan
sulfate on the DC surface (22). It was possible that gD neutralization also prevented heparan sulfate binding and that this interaction was an important activation signal in DC, as proposed for CMV (23). However, binding of HSV-1 virions neutralized with LP2 mAb on DC could still be detected by flow cytometry. Furthermore, this binding was sensitive to prior incubation of the neutralized virions with heparin, which can bind glycoprotein B and glycoprotein C, preventing viral attachment to heparan sulfate (Fig. 9D). Therefore, gD neutralization of HSV-1 still permitted virion attachment to heparan sulfate on the cell surface, and attachment per se is not an activatory signal in DC.

Similar to the changes in surface phenotype, gD neutralization of WT-HSV with LP2 also abolished DC secretion of type I IFN (Fig. 9E). We also observed equivalent IFN secretion as WT-HSV in five of seven individuals’ DCs infected with UV-HSV and in two of three individuals’ DCs exposed to FIX-HSV. Therefore, HSV-DC surface interaction is important for inducing IFN secretion and viral envelope gD plays an important role in inducing this effect.

Discussion
DCs play a central role in the initiation of T cell-mediated antiviral immune responses (2). This is particularly important in the context of resolving immunity to HSV-1 in vivo (3, 4) and therefore the study of the interaction between this virus and DC has attracted considerable interest.
HSV-1 infection of human DC results in a functional impairment of the infected cells (9, 12). HSV infection, however, is generally associated with humoral and cellular immune responses, implying that DC activation, as well as DC impairment must occur. In this study, therefore, the mechanisms involved in the initial activation have been analyzed in detail. We propose a mechanism by which myeloid DC, the first APC to encounter HSV-1 in vivo, can bypass the block in viral Ag presentation by releasing type I IFN that can mature bystander DC. In parallel, however, DC interaction with HSV envelope results in NF-κB and p38 MAPK activation and direct maturation of infected DC. This dual effect has important implications both for the use of HSV-1 as a gene delivery vector and in vaccine design.

**Viral inhibitory mechanisms**

De novo synthesis of viral gene products was predominantly responsible for the inhibitory effects seen following WT-HSV infection of immature DC. DC infected with UV-HSV remained viable, could stimulate T cell proliferation efficiently, had normal morphology, and were able to respond to further maturation stimuli through changes in cytoskeletal shape and through up-regulation of CD86 and MHC class I to maximal levels, in sharp contrast to the effects seen with WT-HSV (9, 12) (Figs. 5 and 6). These findings agreed with previous studies showing that UV-HSV infection of mature DC results in no loss of function (10), but it is important to note that mature DC are generally more resistant to viral infection than immature DC (9) and therefore subtler effects of viral proteins might not have been observed.

The specific viral components responsible for the changes observed have not been defined. Recently, a key role has been suggested for the tegument protein virion host shutoff (vhs), which inhibits protein synthesis by accelerating mRNA degradation (24). However, the precise targets for vhs and other candidate viral immunomodulatory molecules remain to be elucidated.

**Bypassing the roadblock and HSV-induced production of type I IFN by myeloid DC**

Given the importance of DC in the initiation of the antiviral T cell response, mechanisms to by-pass the loss in Ag-presenting function resulting from HSV-1 infection are necessary to develop effective antiviral immunity. Previously, we observed that bystander uninfected DC had a more mature phenotype and that this effect was mediated by soluble factors (12). In this study, we have concluded that this paracrine signal is type I IFN released from infected myeloid DC and that this is responsible for the maturation of bystander DC and can prime DC for increased IL-12 secretion in the presence of LPS (Figs. 2–4).

Type I IFN is a family of cytokines made up of many subtypes that were originally identified for their antiviral properties and that can be secreted by all cell types (15). A subset of DC, PDC secrete particularly high levels of type I IFN when stimulated with certain viruses (7). However, the inhibitory effect of IL-4 on PDC survival (25) and their CD2 surface expression (26) (a marker used to deplete contaminating T and NK cells in our experimental protocol) made the presence of such DCs in our studies unlikely. The combination of myeloid markers expression in >99% of these DC (Fig. 1) and intracellular staining for IFN-α secretion confirmed that the source of type I IFN secreted into the supernatant of HSV infected cultures was undeniably MDDC (Fig. 2). This is consistent with a recent study that showed that myeloid DC can produce as much type I IFN as PDC through a protein kinase R-dependent mechanism, if the viral RNA genome is transfected directly into the cytoplasm (27), but add to it with the observation that significant functionally active type I IFN is also secreted following natural infection of DC by the DNA virus HSV-1. Type I IFN release was observed in the majority of individuals following DC infection with UV- and FIX-HSV, in a gD-dependent manner (Fig. 8), consistent with previous reports (28). The DC receptors involved in inducing this IFN secretion are currently unspecified. Important candidates include chemokine receptors (28) and the mannose receptor, which has been implicated in the secretion of type I IFN by DC in response to HSV-1 (29, 30). Nevertheless, the data presented in this study strongly suggest that the stimulus for type I IFN secretion in MDDC to this DNA virus is independent of viral replication and therefore of the production of viral RNA, and thus differs from the mechanisms proposed for some RNA viruses (27).

**DC response to type I IFN**

To confirm that the type I IFN in supHSV-DC was responsible for the paracrine effects on DC function, we used recombinant IFN-α and showed that this cytokine had the same effect on DC as supHSV-DC (Fig. 3). Although previous studies have demonstrated that IFN-α induces submaximal DC maturation (31–33), our data show that the response is dose-dependent. Sufficient IFN-α can result in a degree of maturation equal to that seen with LPS (data not shown). It is important to note that the amount of type I IFN secreted following HSV infection (~250 IU/ml) closely reproduced the submaximal up-regulation of CD86 and HLA-DR observed in DC cultured in supHSV-DC. In vivo, the localized...
concentration of IFN-α achieved may be much higher, and drive full DC maturation.

We have also shown that type I IFN secreted from HSV-1 infected DCs can overcome the HSV-1 induced block in IL-12 secretion (12) by priming bystander uninfected DCs for enhanced IL-12 secretion. Although the synergistic effects observed with IFN in this study are similar to those reported by others (33, 34), the regulation of IL-12 secretion in human DC by type I IFN remains an area of controversy, in relation to the subtypes of IFN and the precise timing of IFN exposure. In contrast to IFN-α (Fig. 3) (33), IFN-β does not have a priming effect on DC IL-12 secretion (35) and addition of type I IFN during DC differentiation generates more mature DCs with impaired ability to secrete IL-12, consistent with an “exhausted” DC phenotype (36–38).

**DC response to HSV-1**

Despite the similar effects of IFN-α and HSV-1 on DC phenotype, the maturation of DC infected with WT-HSV was not dependent on the secretion of type I IFN (Fig. 4). The autocrine loop may have been disrupted by HSV-1 interference with type I IFN induced signaling pathways (39). However, even after FIX-HSV infection, in which this disruption presumably does not occur, DC maturation also occurred independent of the autocrine activity of type I IFN (data not shown). HSV-1 is not unique in inducing type I IFN independent activation, as some RNA viruses, such as influenza and Sendai virus, also activate DCs in this way (40). However, in other instances, viral interaction with the DC surface induces autocrine cytokine feedback loops that are necessary to mature DC (41). Therefore, the critical factors in determining direct DC maturation by viral infection are likely to be the viral ligands, the cellular receptors they interact with, and the subsequent downstream signaling events induced.

HSV-1 entry into DCs requires glycoprotein B and glycoprotein C attachment to surface heparan sulfate followed by gD binding to one of two surface receptors, either herpesvirus entry mediator (HVEM) or poliovirus related receptor-1 (22). UV-HSV and FIX-HSV induced CD86 up-regulation on DC (Fig. 9). Notably, gD-neutralized HSV virions were still able to attach to heparan sulfate, but did not up-regulate CD86 (Fig. 9), excluding HSV attachment to
heparan sulfate as an activation stimulus for DC, contrary to the effects of CMV (23). Binding non-neutralizing anti-gD mAb to HSV-1 did not prevent the activation, excluding the role of inhibitory FcγR or non-specific sterol inhibition by the mAb. In addition it emphasized that gD interaction with receptors on the DC surface plays a critical role in the activation of DC. This was underlined by the activation 30 min after infection of both p38 and NF-κB (Fig. 8), two pathways that are necessary to complete the full program of DC maturation (18–21).

The ability of gD to bind to the surface of cells to exert functional effects has been previously described (28). However, the receptors that exert its function on DC have not yet been specified. One candidate is HVEM, a member of the TNFR superfamily that is expressed on DCs (9, 42) (data not shown) and has been shown to activate NF-κB upon ligation in other cell types (43). Although a nonviral HVEM ligand, LIGHT, can induce maturation of DC and enhance their function as APC (44), gD and LIGHT bind to different sites on HVEM (45) and the signaling consequences of binding these ligands may differ. HVEM has not been shown to be involved in p38 MAPK activation. Nevertheless, it is interesting that in this study there was correlation between activation of p38 involved in p38 MAPK activation. Nevertheless, it is interesting that in this study there was correlation between activation of p38 and NF-κB, and the phenotype of HSV-infected DC compared with LPS, which supports the hypothesis that these pathways are important in the maturation of DC following HSV-1 infection.

The ability of HSV-1 to activate DC directly improves the therapeutic prospects of replication incompetent vectors. Infection of DC and expression of the desired transgenes in the context of a mature DC will favor the induction of a potent T cell response (24). Furthermore, identification of the viral proteins that have adjuvant capacity on DC may also identify novel immunogenic vaccine candidates (3).

The direct activation of DC by this virus also raises important issues with regards to host-pathogen recognition. Recent studies have focused on the ability of the innate immune system, of which DCs are a central component, to recognize pathogen-associated molecular patterns and swiftly initiate anti-pathogen responses. In this respect, the ability of DC to recognize conserved envelope structures of HSV-1 is consistent with such a model of pathogen recognition. Further studies are required to elucidate whether gD is the sole stimulatory ligand in this system or whether proteins involved in envelope-membrane fusion are also critical in inducing activating signals in DC (46). Nevertheless, as the activation of both p38 MAPK and NF-κB is required for several facets of DC maturation (18–21), it is perhaps surprising that HSV-1 has conserved structures that can activate both these proinflammatory pathways that could promote antiviral responses by DC. However, HSV-1 activation of both NF-κB and p38 MAPK can enhance the efficiency of viral replication (16, 17). Therefore, the reliance on the activation of these host signaling pathways for efficient viral replication may have provided the driving force for the evolution of viral mechanisms to dampen the function of DC that are activated early in the initial infection process (9, 12). In this way, the ability of DC to mature in response to HSV-1 infection may result primarily from the dependence of the virus to activate certain signaling pathways for its own replicative advantage. Further studies are required to determine whether these events benefit to a greater extent the survival of the virus or the host.

Role of DC in resolution of HSV-1 lesions

This study also addresses important issues regarding the role of DC in a peripheral herpetic skin infection. Signals from the resident DC, in combination with those from neighboring non-APC (e.g., keratinocytes, see Ref. 47), may be required both to initiate the maturation of neighboring uninfected myeloid DC and to limit local viral replication. The important role of type I IFN in the differentiation of monocytes to DC (48) may also result in the selective local recruitment of myeloid DC. Murine models have shown that uninfected myeloid submucosal and lymph node resident DC subsets are responsible for HSV-specific T cell activation after peripheral infection (49–51). The implied reliance on DC cross presentation in these models underlies the importance of innate immune responses to HSV-1. This study proposes that early local release of type I IFN by myeloid DC drives DC maturation, enhances cross-priming by DC and skew toward Th1 responses (52). This tilts the immunological balance from HSV-1 tolerance to HSV-1 immunity (53).

References


