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CD34+ Cells in the Peripheral Blood Transport Herpes Simplex Virus DNA Fragments to the Skin of Patients with Erythema Multiforme (HAEM)

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Herpes simplex virus (HSV)-associated erythema multiforme (HAEM) is a recurrent disease characterized by the presence and expression of HSV DNA fragments in lesional skin. Our studies examined the mechanism of viral DNA transport to the skin of HAEM patients. CD34+ cells were isolated from the blood of normal subjects and HSV and HAEM patients during acute lesions and at quiescence. They were cultured with cytokines that favor their differentiation into Langerhans cells (LC) precursors (CD1a+/CD14−) and examined for HSV replication, HSV-induced cellular alterations, viral DNA fragmentation, and clearance. CD34+ cells from all study groups were non-permissive for HSV replication but infection favored their differentiation into CD1a+/CD14− LC precursors and upregulated E-cadherin expression, thereby assisting LC targeting to the skin. Only HAEM patients had CD34+ cells that retained viral DNA fragments, notably polymerase DNA, for at least 7 d of *in vitro* culture. The percentages of circulating CD34+ (and CD34+/CLA+) cells were significantly higher in HAEM patients at the time of acute lesions. A similar increase was not seen for HSV patients. The data are the first report implicating CD34+ cells in HAEM pathogenesis, likely by transporting HSV DNA fragments to lesional skin.

Key words: HSV/HAEM/CD34+ cells/langerhans cells/DNA transport
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Erythema multiforme (EM) is a polymorphic, often recurring disease caused by exposure to medication or various infections, notably with herpes simplex virus (HSV). HSV-associated EM (HAEM) lesions are virus-free (Miura *et al*, 1992; Aurelian *et al*, 1998, 2003), but they contain HSV DNA fragments, most often comprising sequences that encode the viral DNA polymerase (Pol) (Darragh *et al*, 1991; Aslanzadeh *et al*, 1992; Miura *et al*, 1992; Imafuku *et al*, 1997; Kokuba *et al*, 1998, 1999a, b; Aurelian *et al*, 2003; Ng *et al*, 2003; Sun *et al*, 2003). Lesion development is initiated by expression of HSV DNA sequences in the skin (Imafuku *et al*, 1997; Kokuba *et al*, 1998, 1999a, b) and the recruitment of a restricted population of virus-specific T-helper type 1 (Th1) cells that produce interferon-γ (IFN-γ) in response to the viral antigen in the skin (Kokuba *et al*, 1998, 1999b; Aurelian *et al*, 2003). This is followed by an autoimmune pathogenesis component resulting from the recruitment of T cells that respond to auto-antigens that are likely released by lysed/apoptotic virus-infected cells (Aurelian *et al*, 2003). Because HSV DNA is transported to peripheral skin sites of HAEM lesion development before each recurrent HAEM episode (Kokuba *et al*, 1999a), transport plays a pivotal role in HAEM pathogenesis. The identity of the transporting cells and their role in DNA fragmentation, however, are still unknown.

Dendritic cells (DC) form a complex network of cells that are distributed throughout the body and whose primary function is antigen presentation and the stimulation of antigen-specific immune responses (Banchereau and Steinman, 1998; Banchereau *et al*, 2000). They are critically involved in immune responses to viral antigens and the initiation of autoimmune diseases (Salio *et al*, 1999). Human DC are generated from CD14+ peripheral blood monocytes (Randolph *et al*, 1998) and bone marrow-derived CD34+ hematopoetic progenitor cells that exist as a trace population in normal adult blood (Reid *et al*, 1992). *In vitro*, CD34+ cells differentiate into DC by culture with tumor necrosis factor α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Differentiation generates two types of intermediate precursors (immature DC) that can be identified by the mutually exclusive expression of CD1a or CD14 antigens. The CD14+ cells mature into interstitial DC-like cells; the CD1a+ cells mature into DC bearing phenotypic and functional properties of Langerhans cells (LC) (Sallusto and Lanzavecchia, 1994; Bernhard *et al*, 1995; Caux *et al*, 1996, 1997; Strunk *et al*, 1996; Strobl *et al*, 1998; Arrighi *et al*, 2001; Strobl, 2003). Differential expression of the skin homing cutaneous leukocytes antigen (CLA) identifies the CD34+ cell subset (CD34+/CLA+) that gives rise to LC (Herbst *et al*, 1996; Strunk *et al*, 1997; Arrighi *et al*, 2001; Strobl, 2003).

Abbreviations: CLA, cutaneous leukocytes antigen; DC, dendritic cells; EM, erythema multiforme; FITC, fluorescein isothiocyante; HAEM, HSV-associated erythema multiforme; HSV, herpes simplex virus; LC, Langerhans cells; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PFU, plaque-forming units; p.i., post-infection; Pol, polymerase; TUNEL, TdT-mediated dUTP nick end labeling

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The epidermis is continually repopulated by bone marrow-derived LC progenitors (Jakob and Udey, 1998; Banchereau *et al*, 2000). E-cadherin, which is expressed on LC, but not other DC, is responsible for LC interaction with keratinocytes (KC) (reviewed in Udey, 1997). CD34+ cells and/or CD34+-derived LC precursors in the blood stream could be infected with HSV during their traffic through HSV lesional skin, and transport the viral DNA to distal skin sites. Previous studies have shown that several human viruses can infect monocyte-derived DC (Grosjean *et al*, 1997; Cella *et al*, 1999; Engelmayer *et al*, 1999; Ho *et al*, 2001; Kakimoto *et al*, 2002; Kawamura *et al*, 2003). HSV infection of monocyte-derived DC inhibits their antigen-presenting function, causing immune evasion, long-term immunosuppression, and/or virus spread (Kruse *et al*, 2000; Mikloska *et al*, 2001; Pollara *et al*, 2003). But the ability of HSV to infect CD34+ cells, the effect of infection on their differentiation, particularly into LC precursors, and the potential role of the infected cells in HAEM pathogenesis are still unknown. The studies described in this report were designed to address these questions. They indicate that CD34+ cells in the blood increase in number during acute HAEM episodes and are involved in disease pathogenesis, likely by transporting HSV DNA fragments to the skin of HAEM patients.

Results

CD34+ cells do not sustain HSV replication The hypothesis that CD34+ cells transport HSV DNA to the skin carries the implicit interpretation that they can be infected with HSV. To test this interpretation, CD34+ cells obtained with the magnetic curve sorting (MACS) kit from normal healthy individuals and from lesion-free HAEM and HSV patients were infected with 10^6 plaque-forming units (PFU) of HSV-1 or HSV-2 (MOI $\approx$ 10) and cultured in complete medium, as described in Materials and Methods. They were studied for virus growth (by plaque assay) at 4, 12, and 24 h, and at 7 d post-infection (p.i.) These time points were selected in order to encompass the virus replicative cycle in permissive cells (24 h) and the endpoint of CD34+ cell differentiation into immature DC (7 d). In all three study groups (normal, HSV, and HAEM), virus titers decreased precipitously (within 4 h) after infection. Residual input virus (10–1000 PFU per mL) was still seen at 4–24 h p.i., but it was completely cleared by day 7 p.i. Cord blood CD34+ cells, which differ from the CD34+ cells in peripheral blood in terms of differentiation-related properties and their response to cytokine stimulation (van den Oudenrijn *et al*, 2000; Schipper *et al*, 2003), were similarly non-permissive for HSV replication. Virus titers decreased by 4 h and all virus was cleared by 7 d p.i. The data indicate that HSV infects CD34+ cells, which is consistent with recent findings that they express the virus receptor nectin 1 (Belaaloui *et al*, 2003). CD34+ cells, however, do not sustain HSV replication and are not latently infected with whole virus that is capable of reactivation. This is independent of the origin (disease status) of the CD34+ cells.

HSV infection favors CD34+ cells differentiation into LC precursors CD34+ cells in peripheral blood are committed to differentiation along two independent pathways that are characterized by the mutually exclusive expression of the CD1a or CD14 antigens. CD34+/CLA+ cells are committed to become CD1a+ LC precursors that eventually reside in the skin (Caux *et al*, 1996, 1997; Herbst *et al*, 1996; Strunk *et al*, 1996, 1997; Arrighi *et al*, 2001; Strobl, 2003). Because cellular gene expression can be altered by non-productive HSV infection (Taddeo *et al*, 2002), we wanted to know whether the differentiation of CD34+ cells is affected by HSV infection, and whether this differs in HAEM as compared with HSV patients. Two series of experiments were carried out. First, CD34+ cells from two normal subjects and from quiescent HSV and HAEM patients were infected with HSV-1 or HSV-2 (10 PFU per cell) or mock-infected with phosphate-buffered saline (PBS), cultured for 7 d in complete medium, and examined for CD1a and CD14 antigen expression by two-color flow cytometry. As shown in Fig 1*A* for HSV-infected CD34+ cells from one normal subject, the percentages of CD1a+/CD14− cells (LC precursors) were, respectively, 5%–5.2% and 8.1%–10.3% in mock- and HSV-infected cultures, independent of disease status (normal, HSV, or HAEM). By contrast, the CD14+/CD1a− cells (progenitors of interstitial-type DC) were minimal in all cultures (0.1%–0.3%), suggesting that HSV infection favors differentiation along the LC pathway. In addition, the % double-positive cells that could be gated out by PI staining (dead cells) was also increased in HSV relative to mock-infected CD34+ cell cultures (19%–43% and 2%–5% dead cells, respectively).

In a second series of experiments to examine the effect of HSV infection on CD34+ cell differentiation, we asked whether infection alters expression of the homophilic cytoadhesion molecule E-cadherin that is involved in the adhesion of LC to KC (reviewed in Udey, 1997). Peripheral blood mononuclear cells (PBMC) were obtained from two normal subjects and two quiescent patients, one with HSV and one with HAEM. They were fractionated with the MACS kit and the CD34+ cells were mock infected or infected with HSV (10 PFU per cell) and cultured in complete medium. At 10 and 24 h p.i., cells were examined for E-cadherin expression by immunoblotting, a sensitive and specific method to verify gene expression. A431 cells that express E-cadherin served as positive control (Fig 1*B*, *lane 4*). The % expressing cells were determined by immunofluorescence flow cytometry using duplicates of the mock and 10 h HSV-infected cultures. E-cadherin was barely detectable in immunoblots from mock-infected CD34+ cells (Fig 1*B*, *lane 1*), but its levels were significantly higher at 10 h (Fig 1*B*, *lane 2*) and 24 h (Fig 1*B*, *lane 3*) p.i. (Densitometric integration units = 7.3, 102, and 70 for mock, 10 and 24 h p.i., respectively). Increased expression does not reflect improper gel loading or other technical difficulties, because the levels of actin were virtually identical in all lanes (Fig 1*B*). The % E-cadherin+ cells were also 3–3.8-fold higher in HSV (Fig 1*D*) than mock (Fig 1*C*)-infected cultures, supporting the conclusion that HSV favors CD34+ cell differentiation into LC precursors that can reside in the skin. This was also independent of disease status.

CD34+ cells from HAEM, but not HSV, patients retain Pol DNA Having observed that HSV infection of CD34+

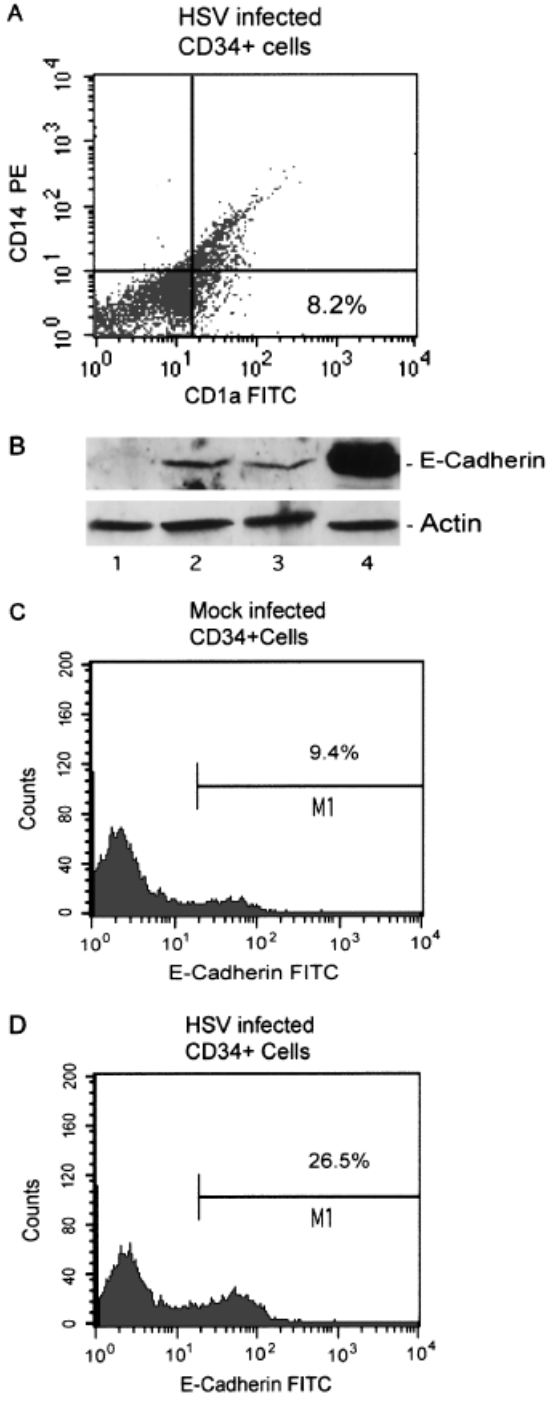

Figure 1
Herpes simplex virus (HSV) infection favors CD34+ differentiation along the Langerhans cells pathway. (*A*) CD34+ cells from a normal subject infected with HSV-1 and cultured in complete medium for 7 d were analyzed for CD1a (fluorescein isothiocyanate (FITC) fluorescence intensity) and CD14 (phycoerythrin (PE) fluorescence intensity) antigen expression by two-color flow cytometry. Mock-infected cells studied in parallel had approximately 2-fold lower levels of CD1a+/CD14− cells and double-positive cells could be gated by PI staining dead cells (data not shown). Similar results were obtained for HSV and HSV-associated erythema multiforme (HAEM) patients. (*B*) CD34+ cells obtained with the MACS kit from a quiescent HSV patient were mock infected (*lane 1*) or infected with HSV and cultured in complete medium for 10 h (*lane 2*) or 24 h (*lane 3*). A431 cells (*lane 4*) served as positive control. Cell extracts were examined for E-cadherin expression by immunoblotting with E-cadherin antibody. The blots were stripped and re-probed with antibody to actin. Similar results were obtained in CD34+ cells from HAEM patients or normal adult subjects. (*C*, *D*) Duplicates of the mock (*C*) or 10 h HSV (*D*)-infected samples in (*B*) were stained with E-cadherin antibody and examined by flow cytometry.

cells is non-permissive, we wanted to verify the state of the viral DNA in the infected cells. Duplicates of the cultures studied for infectious virus were examined for the presence of viral DNA by PCR with Pol primers at 1, 5, and 7 d p.i. The results are shown in Fig 2*A* for infected CD34+ and CD34−

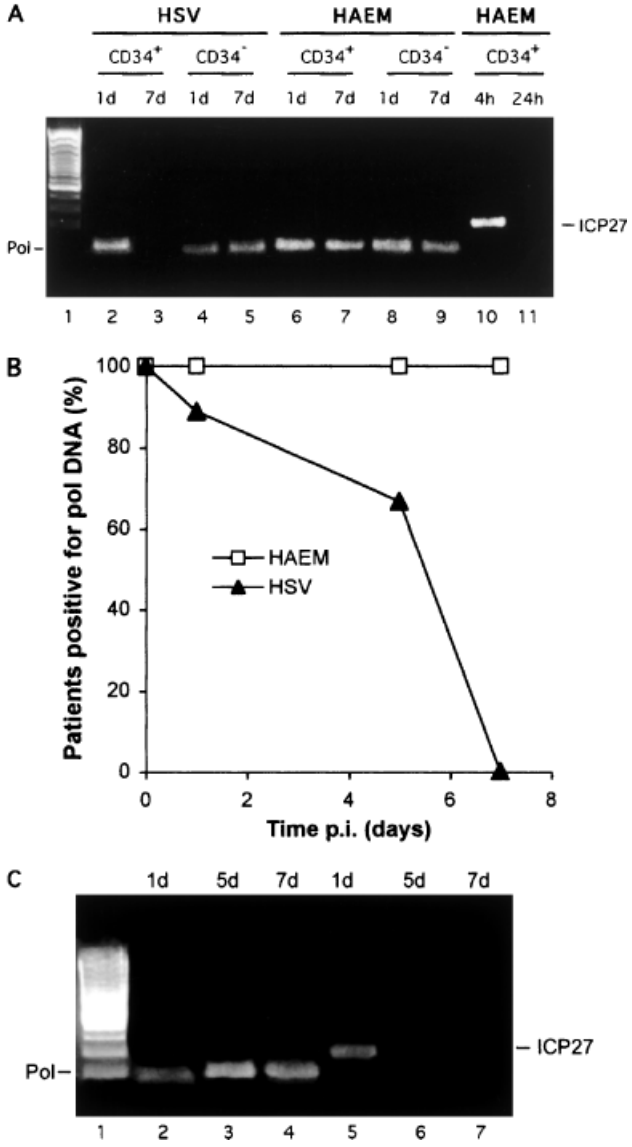

Figure 2
CD34+ cells from herpes simplex virus (HSV)-associated erythema multiforme (HAEM) patients are impaired in viral DNA clearance. (*A*) CD34+ and CD34− cells obtained with the MACS kit from quiescent HAEM and HSV patients were infected with 10^6 plaque-forming units of HSV-2 and examined for viral DNA by PCR with polymerase (Pol) and ICP27 primers. The 92 bp Pol amplificant is shown in lanes 2–9 for CD34+ and CD34− cells from HSV (*lanes 2–5*) and HAEM (*lanes 6–9*) patients at 1 and 7 d post-infection (p.i.) The 169 bp ICP27 amplificant is shown in lanes 10–11 for an HAEM patient at 4 and 24 h p.i. Similar results were obtained for cells infected with HSV-1. Lane 1 represents molecular weight markers. (*B*) Percentage of HAEM and HSV patients with CD34+ cells positive for Pol DNA at various times p.i. with HSV-1. Similar results were obtained for HSV-2. (*C*) CD34− cells from a quiescent HAEM patient (obtained with the MACS kit) were enriched in CD14+ cells by adhesion to plastic as described in Materials and Methods, and the CD14+ cells were infected with HSV-1. They were assayed for viral DNA by PCR with primers for Pol (*lanes 2–4*) or ICP27 (*lanes 5–7*) at 1–5 and 7 d of culture in complete medium. Similar results were obtained for HSV patients and in cells infected with HSV-2. Lane 1 represents molecular weight markers.

cells from two patients, and summarized in Fig 2*B* for CD34+ cells from all patients. CD34+ cells from cord blood, normal adult subjects, and HSV patients showed a time-dependent clearance of the viral DNA, with all subjects becoming DNA-free on day 7 p.i. (Fig 2*A*, *lanes 2* and *3*; Fig 2*B*). By contrast, CD34+ cells from the HAEM patients were positive for Pol DNA throughout the 7 d of *in vitro* culture (Fig 2*A*, *lanes 6* and *7*; Fig 2*B*), indicating that they are impaired in viral DNA clearance.

To examine whether the viral genome is fragmented in infected CD34+ cells, duplicates of the HSV-infected CD34+ cultures from HAEM patients were assayed by PCR with infected cell protein 27 primers at 4 and 24 h p.i. These primers were selected because: (i) ICP27 is located close to the opposite end of the viral genome relative to Pol, (ii) ICP27 and Pol primers are equally sensitive (Imafuku *et al*, 1997), and (iii) Pol, but not ICP27, DNA is present in lesional skin from most HAEM patients (Imafuku *et al*, 1997; Aurelian *et al*, 2003), a finding confirmed in our patients (data not shown). As shown in Fig 2*A*, lanes 10 and 11 for one HAEM patient, ICP27 DNA was seen in CD34+ cultures from 30% of the patients at 4 h p.i. One-third of these patients (10%) were still positive for ICP27 DNA at 24 h p.i. (data not shown). Because all the HAEM patients were positive for Pol DNA as late as 7 d p.i., the data suggest that HSV DNA is rapidly fragmented in these cultures, likely as an outcome of non-permissive infection. The mechanism responsible for the lower stability of ICP27 as compared with Pol DNA is still unknown.

As further control for these data, CD34− cell fractions obtained from the same patients as a result of the MACS fractionation were studied in parallel. Virus titration at 4, 12, and 24 h and at 7 d p.i. indicated that CD34− cells are also non-permissive for virus replication. CD34− cells from both HAEM (Fig 2*A*, *lanes 8* and *9*) and HSV (Fig 2*A*, *lanes 4* and *5*) patients, however, were positive for Pol DNA at 1–7 d p.i., indicating that the differential DNA clearance seen in CD34+ cells is specific for HAEM patients. Studies of CD34− fractions enriched in CD14+ cells (monocytes) by adhesion to plastic (2 h at 37°C) suggested that the viral DNA is in monocytes. Thus, the adherent cells (80%–85% CD14+) were infected with HSV-1 or HSV-2 (10 PFU per cell), cultured in complete medium, and examined for viral DNA by PCR with Pol and ICP27 primers at 1–7 d p.i. Pol DNA was seen in the CD14+ cells from both HAEM and HSV patients at all times in culture whereas ICP27 DNA was seen in 20% of the patients at 1 d p.i., but not later (Fig 2*C*). HSV DNA was not seen in CD19+ cells (B cells) fractionated from the CD34− population with the appropriate MACS separation kit and used as control (data not shown). Collectively, the data indicate that viral DNA clearance is only impaired in CD34+ cells from HAEM patients. Since the CD34+ fractions contain 7%–15% CD34− cells that retain Pol DNA, the failure to detect viral DNA in the CD34+ cultures from normal subjects and other HSV patients suggests that it is degraded by viral or virus-induced/activated cellular nuclease(s) that are not expressed/functional in CD34+ cells from HAEM patients. The data support the conclusion that CD34+ and CD34− cells do not retain the whole virus that is capable of reactivation.

CD34+ cells from HAEM patients evidence increased HSV-induced apoptosis Having seen that HSV infection can also cause cell death, we wanted to know whether it is because of apoptosis (as previously shown for monocyte-derived DC; Salio *et al*, 1999; Kruse *et al*, 2000; Mikloska *et al*, 2001; Pollara *et al*, 2003), and whether this differs for HAEM as compared with HSV patients. CD34+ cells from quiescent HSV and HAEM patients were infected with 10 PFU per cell of HSV-1 or HSV-2 and cultured in complete medium. At various times p.i. (0, 4, 12, and 24 h, 5 and 7 d), the cells were centrifuged onto glass slides and assayed for apoptosis by TdT-mediated dUTP nick end labeling (TUNEL). Cells in five different fields were counted (at least 350 cells) and the % TUNEL+ cells were calculated. Results are expressed as % TUNEL+ cells ± SEM. The % TUNEL+ (apoptotic) cells in mock-infected CD34+ cells were very low for both HAEM and HSV patients (1% ± 0.2%–3.9% ± 0.1%). HSV infection caused a significant

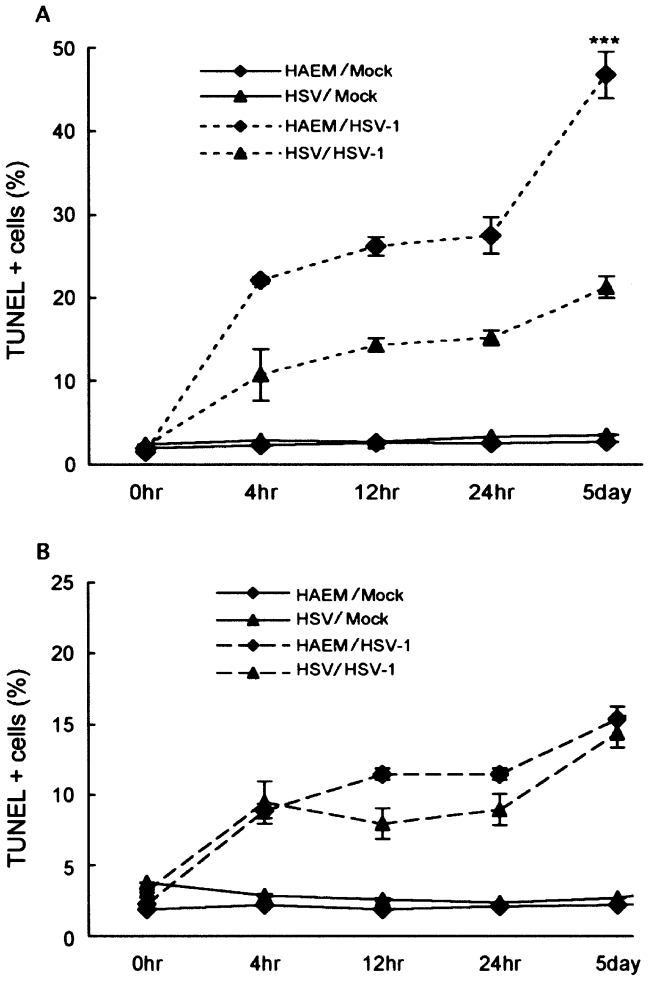

Figure 3
CD34+ cells from herpes simplex virus (HSV)-associated erythema multiforme (HAEM) patients evidence increased HSV-induced apoptosis. CD34+ (*A*) and CD34− (*B*) cells from quiescent HAEM and HSV patients were mock-infected or infected with 10 plaque-forming units per cell of HSV-1 and cultured in complete medium. At various times p.i., the cells were centrifuged onto glass slides and assayed by TdT-mediated dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit. Cells in five different fields were counted (at least 350 cells) and the % TUNEL+ (apoptotic) cells were calculated. Results are expressed as % TUNEL+ cells ± SEM (***$p < 0.001$ by ANOVA *vs* HSV patients).

($p < 0.001$) increase in the % TUNEL+ cells in the HAEM patients (45% on days 5 p.i.), but the % TUNEL+ cells in infected CD34+ cells from HSV patients were significantly ($p < 0.001$) lower (20% on day 5 p.i.) (Fig 3*A*). The % TUNEL+ cells in CD34− cell cultures (used as control) were similar in HAEM and HSV patients, and similar to those seen for CD34+ cells from HSV patients (15% on day 5 p.i.) (Fig 3*B*). The data indicate that: (i) HSV triggers apoptosis in CD34+ and CD34− cells, and (ii) CD34+ cells are significantly more susceptible to HSV-induced apoptosis in HAEM than HSV patients.

The % CD34+ cells in peripheral blood is increased during acute HAEM episodes Having seen that CD34+ cells from HAEM and HSV patients respond differently to HSV infection, we wanted to know whether patients in the two study groups differ in terms of the numbers of CD34+ cells at various stages of the disease. PBMC were obtained from HAEM and HSV patients at the time of acute disease (days 1–3 after HSV and 2–8 after HAEM lesion onset) and during quiescence (free of clinical symptoms). They were examined for CD34+ and CD34+/CLA+ cells by double immunofluorescence with phycoerythrin (PE)-labeled CD34 and fluorescein isothiocyante (FITC)-labeled CLA antibodies by flow cytometry. CD34+/CLA+ cells were examined because they give rise to LC (Herbst *et al*, 1996; Strunk *et al*, 1997; Arrighi *et al*, 2001; Strobl, 2003). PBMC from three normal subjects served as control. The results of these studies are shown in Fig 4 for two patients and one normal subject and summarized in Table I, for all patients. The per-

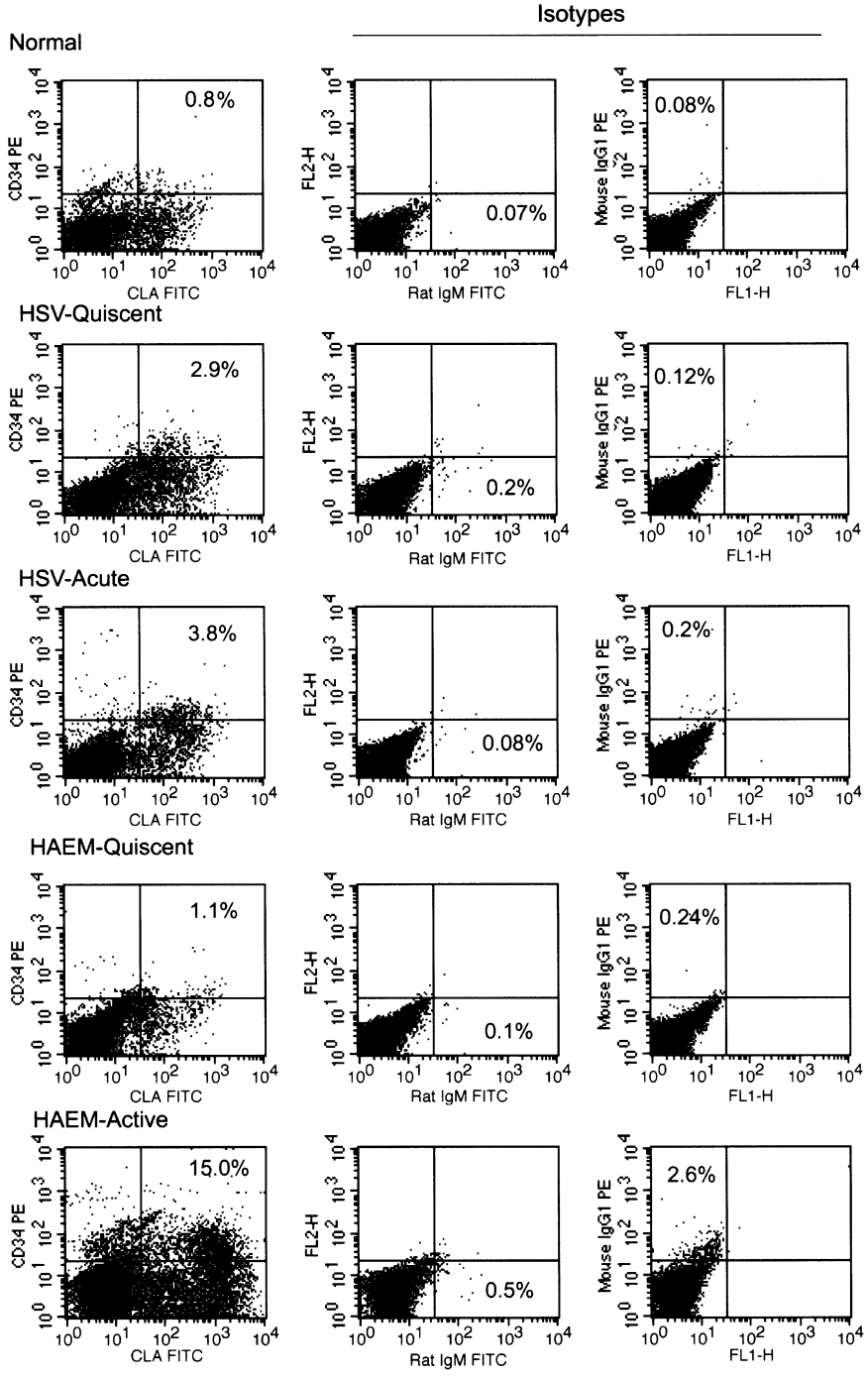

Figure 4
The percentages of CD34+ and CD34+/CLA+ (cutaneous leukocytes antigen) cells are increased during acute herpes simplex virus (HSV)-associated erythema multiforme (HAEM) episodes. Peripheral blood mononuclear cells obtained from a normal HSV-negative subject and from an HAEM and an HSV patient during quiescence and at the time of an acute episode were examined for CD34 and CLA expression by two-color flow cytometry. CLA expression was determined by measuring fluorescein isothiocyante (FITC) fluorescence intensity, and CD34 expression was determined by measuring phycoerythrin (PE) fluorescence intensity. Similar results were obtained for all patients (Table I).

Table I. CD34+ and CD34+CLA+ cell numbers are increased during acute HAEM

Diagnosis	Total CD34+ (%)	CD34+CLA+ (%)
Normal	2.0 ± 0.1 ($p < 0.001$)	0.9 ± 0.2 ($p < 0.001$)
Acute HAEM	27.0 ± 2.0	17.5 ± 2.5
Quiescent HAEM	4.6 ± 1.8 ($p < 0.001$)	2.0 ± 0.7 ($p < 0.001$)
Acute HSV	5.6 ± 1.1 ($p < 0.001$)	3.8 ± 0.8 ($p < 0.001$)
Quiescent HSV	3.9 ± 1.8 ($p < 0.001$)	2.7 ± 1.9 ($p < 0.001$)

PBMC were collected from three normal subjects and from four HAEM and eight HSV patients. They were obtained at the time of acute disease (days 1–3 after onset of HSV and 2–8 after onset of HAEM lesions) and during quiescence (3–6 wk after lesion resolution; free of clinical symptoms). Cells were analyzed by double immunofluorescent staining with PE-labeled CD34 and FITC-labeled CLA antibodies and flow cytometry. Results are expressed as the mean cell population number ± SEM.

Statistical analysis compared each cell population with the acute HAEM patient group by ANOVA.

Differences between the acute and quiescent HSV and the quiescent HAEM groups were not statistically significant ($p > 0.05$). These three groups were somewhat, but not significantly, higher than normal subjects.

CLA, cutaneous leukocytes antigen; HAEM, herpes simplex virus (HSV)-associated erythema multiforme; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; FITC, fluorescein isothiocyante.

centages of CD34+ cells in the quiescent HSV (3.9% ± 1.8%) and HAEM (4.6% ± 1.8%) patients were similar, but somewhat higher than those (2.0% ± 0.1%) in normal adult blood. Similar percentages of CD34+ cells (5.6% ± 1.1%) were seen in patients with acute HSV episodes, but the percentages of CD34+ cells in patients with acute HAEM lesions were significantly higher (27% ± 2.0%). The CD34+/CLA+ cells were 50%–65% of the total CD34+ cell populations in all study groups, being 2.0% ± 0.7% and 2.7% ± 0.9% in quiescent HAEM and HSV patients and 17.5% ± 2.5% and 3.8% ± 0.8% in acute HAEM and HSV patients, respectively. The CD34+/CLA+ cells in the normal subjects were 0.9% ± 0.2%. The increased percentage of CD34+ and CD34+/CLA+ cells during acute HAEM episodes is not because of improper patient selection, because the same patients were studied during quiescence and acute disease episodes. In this context, it may also be important to point out that the percentage of CLA+/CD34− cells (8.4%–11%) were similar in HAEM patients with quiescent or acute episodes. The data suggest that CD34+ cells are involved in HAEM pathogenesis, presumably by transporting viral DNA to distant skin sites.

HSV, but not HAEM lesional skin, contains Pol DNA+ CD34− cells To further test the hypothesis that CD34+ cells transport viral DNA to HAEM skin, we asked whether CD34+ and/or CD34− cells from HSV and HAEM lesional skin are positive for viral DNA. Infiltrating PBMC were isolated by Ficoll–Hypaque gradient centrifugation from punch biopsies (4 mm) obtained from HSV and HAEM lesions from one patient (day 2 post-HSV and day 8 post-HAEM lesion onset) and further fractionated with the MACS kit. The numbers of CD34+ cells (8×10^2 and 3×10^3 for HSV and HAEM lesions, respectively) were close to the sensitivity limit of the PCR assay (Imafuku *et al*, 1997). By contrast, the numbers of CD34− cells (2×10^4 and 5×10^4 for HSV and HAEM lesions, respectively) were sufficient for PCR analysis and they were examined in parallel with DNA extracted from the skin tissues. Pol was amplified from the HAEM and HSV skin tissues (Fig 5, *lanes 1* and *3*) and from the CD34− fractions isolated from the HSV skin lesion (Fig 5, *lane 2*). CD34− cells from the HAEM skin lesion were negative for Pol DNA (Fig 5, *lane 4*), suggesting that the DNA in the HAEM skin is not in CD34− cells.

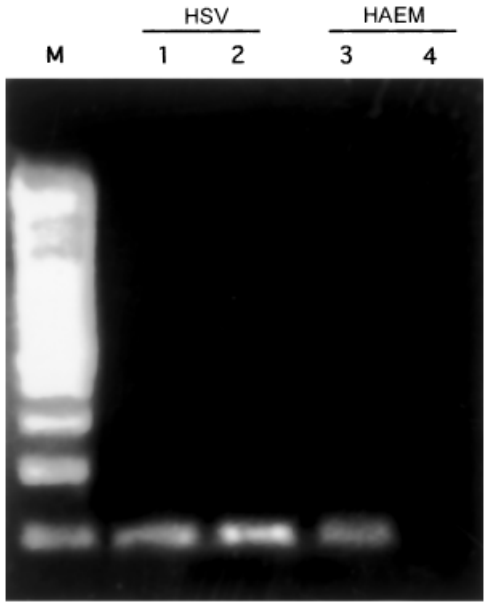

Figure 5
CD34− cells from herpes simplex virus (HSV), but not HSV-associated erythema multiforme (HAEM) lesions, are positive for polymerase (Pol) DNA. Biopsy samples were minced with forceps and scissors in complete medium, and infiltrating lymphocytes were separated by Ficoll–Hypaque density gradient centrifugation. CD34+/− fractions were obtained with the MACS kit. DNA extracted from the lesional skin and from CD34− cell fractions was assayed by PCR with Pol primers. The 92 bp Pol amplificant was seen in skin from HSV (*lane 1*) and HAEM (*lane 3*) lesions and in CD34− cells isolated from the HSV lesion (*lane 2*). CD34− cells isolated from the HAEM lesion were free of Pol DNA (*lane 4*). The numbers of isolated CD34+ cells were too small for study.

Discussion

HAEM pathogenesis is associated with the presence and expression of HSV DNA fragments that are transported to HAEM lesional skin before each recurrent episode (Imafuku *et al*, 1997; Kokuba *et al*, 1998, 1999a, b; Aurelian *et al*, 2003). The salient feature of the data presented in this report is the finding that transport appears to be mediated by increased numbers of circulating CD34+ cells. The following comments seem pertinent with respect to these findings.

These studies were stimulated by previous observations that HSV DNA is transported to the HAEM skin by the vascular route (Aurelian *et al*, 1998; Kokuba *et al*, 1999a), and its deposition is likely to involve increased expression of the adhesion protein ICAM-1 in microvascular endothelial cells (Bruynzeel *et al*, 1993; Larcher *et al*, 2001). They focused on CD34+ cells because these cells traffic from the bone marrow through the blood stream, undergoing a maturation

process that begins with immature DC. In peripheral blood, CD34+ cells represent early committed progenitors. At 5–7 d of *in vitro* culture with TNF-α and GM-CSF, they give rise to two subsets of DC precursors, one of which (CD1a+/CD14−) develops into LC that continually repopulate the epidermis (reviewed in Udey, 1997). We found that under the experimental conditions used in our studies, CD34+ cells from normal adult subjects or cord blood, as well as CD34+ cells from the blood of adult HSV and HAEM patients were non-permissive for the growth of both HSV serotypes. Virus titers decreased precipitously within the first 4 h p.i. and residual virus was completely lost by 7 d p.i., indicating that CD34+ cells do not sustain virus replication and they are not carriers of latent HSV. But infection favored cell differentiation along the LC pathway (2-fold increase in the % CD1a+/CD14− cells relative to mock-infected cultures) and it increased expression of E-cadherin, which favors LC residence in the epidermis (reviewed in Udey, 1997). This likely reflects virus-induced upregulation of cellular genes that is replication independent (Taddeo *et al*, 2002). The percentages of CD14+/CD1a− cells (progenitors of interstitial-type DC) were minimal, and similar for both HSV- and mock-infected cultures. E-cadherin upregulation early p.i. may represent a mechanism of virus spread that is limited by the non-permissive nature of the CD34+ cell infection and is independent of the origin of the CD34+ cells (normal subjects or patients with HSV or HAEM).

CD34+ cells from normal subjects, cord blood, and HSV patients evidenced a time-dependent loss of viral DNA that was no longer seen at 7 d p.i. By contrast, CD34+ cells from the HAEM patients were impaired in viral DNA clearance, thereby providing a unique opportunity for its delivery to the skin. We conclude that DNA is fragmented in CD34+ cells, because ICP27 DNA was lost by CD34+ cells from most HAEM and HSV patients (70%) during the first 4 h p.i., indicative of the different stability of the various fragments of viral DNA. Pol DNA retention by CD34+ cells from HAEM patients indicates that these cells process the viral DNA differently than CD34+ cells from other subjects, including HSV patients. Differential processing of the viral DNA was not seen in CD34− cells from the same patients, underscoring the distinct interaction of HSV with CD34+ cells from HAEM patients. In this context, it is important to point out that the CD34+ cell fractions used in these studies were contaminated by approximately 7%–15% CD34− cells that retain viral DNA at 7 d p.i. Therefore, detection of Pol DNA in CD34+ cells from HAEM patients but not other study groups suggests that viral or virus-induced/activated cellular nuclease(s) are not upregulated/functional in CD34+ cells from HAEM patients.

Despite the high MOI (10 PFU per cell) used in these studies, HSV infection caused cell death in a proportion, but not all, CD34+ cells. Death was because of apoptosis, as determined by a higher % TUNEL+ cells in HSV than mock-infected cultures. The highest % TUNEL+ cells was seen in HAEM patients (45%), however, supporting the conclusion that their CD34+ cells respond differently to HSV infection. Presumably, infection affected different cellular genes, with apoptotic cascades stimulated in 45% of the cells whereas 10% of the cells were stimulated to differentiate into CD1a+ LC precursors. Mechanisms responsible for the differential response of various infected cells are still unknown. But the higher percentage of apoptotic CD34+ cells in HAEM patients could contribute to the autoimmune component of HAEM (Aurelian *et al*, 2003) because apoptotic DC are likely to be ingested by normal uninfected DC, LC, and/or macrophages involved in antigen presentation (von Bubnoff *et al*, 2001).

The % CD34+ and CD34+/CLA+ cells detected by FACS analysis in PBMC from normal healthy subjects were somewhat higher than those previously reported after extensive cell fractionation procedures (Reid *et al*, 1992) and may reflect the use of different technology for their isolation/identification. We do not believe that our data are because of technical errors, because: (i) the FACS assay is established and internally controlled, (ii) our findings for CD4+/CD25+ cells studied in parallel for some of these patients (6.2%–10.4% of CD4+ T cells) were similar to those (5%–15% of CD4+ T cells) independently reported by others (Tang *et al*, 2003), and (iii) approximately one half of the CD34+ cells were CLA+, as also reported by others for normal adults (Strunk *et al*, 1996). The somewhat increased percentages of CD34+ and CD34+/CLA+ cells in HSV patients may represent a host attempt to overcome immune evasion resulting from the inhibitory effects of virus infection of monocyte-derived DC (Kruse *et al*, 2000; Mikloska *et al*, 2001; Pollara *et al*, 2003). The present data do not address the mechanism responsible for this increase. It is particularly relevant, however, that the percentages of circulating CD34+ (and CD34+/CLA+) cells were significantly increased during acute episodes of HAEM, when viral DNA is transported to the skin (Kokuba *et al*, 1999a). The increase was disease related, because: (i) the same patients were studied during acute episodes and at quiescence, and (ii) a similar increase was not seen during acute HSV episodes. This conclusion is also supported by the findings that: (i) CD34+ cells from HAEM patients preferentially retain Pol DNA throughout their differentiation into LC precursors, and (ii) HSV infection favors their differentiation into LC precursors, and increases the expression of E-cadherin that facilitates LC population of the epidermis. Presumably, CD34+ cells and/or LC precursors derived from them pick up the virus when they migrate through HSV lesional skin, whether through direct infection or by endocytic capture of dying infected cells. Although our data indicate that the viral DNA is fragmented in the infected CD34+ cells, we do not exclude the possibility that the migrating CD34+ cells may also pick up viral DNA fragments. Either way, however, the outcome is Pol DNA transport to distal skin sites by the LC precursors. Significantly, because LC have a relatively long life-span in the periphery under steady-state conditions (Merad *et al*, 2002), our data carry the implicit conclusion that HSV infection accelerates LC replacement in the skin.

Consistent with the conclusion that CD34+ cells transport Pol DNA to the skin of patients with HAEM, Pol DNA was found in CD34− cells from HSV, but not HAEM lesional skin, although both skin tissues were DNA+. Additional CD34+ cells, immature DC, and auto-antigen-specific Th1 cells are further mobilized to the skin by chemokines, such as MCP-1 and RANTES (Xu *et al*, 1996; Dieu *et al*, 1998), that are expressed in lesional skin (Spandau *et al*, 2002).

Indeed, media conditioned by CD34+ cells contain chemoattractants for other CD34+ cells (Majka *et al*, 2001) and antigen- or chemokine-stimulated CD34+ cells produce/secrete regulatory mediators that may contribute to inflammatory and immune processes (Umland *et al*, 2004). The unique response of CD34+ cells from HAEM patients is consistent with the genetic theory of HAEM pathogenesis (Khalil *et al*, 1991). Indeed, five of 11 (45.5%) independently studied HAEM patients had HLA DQB1 0301 allele and of these five patients, four also had a DQA10505 allele. The latter subset included our HAEM patients with the more severe and longer-lasting lesions (unpublished).

Our data do not exclude the possibility that monocytes can also transport viral DNA to the skin (Aurelian *et al*, 1998), at least under specific conditions. If various PBMC subpopulations pick up HSV from skin lesions, HAEM development after some but not other HSV episodes experienced by the same patient (Kokuba *et al*, 1999a) may be determined by the specific cell subset that is involved in DNA transport (e.g. macrophages, monocyte-derived DC, or CD34+-derived LC precursors). However, random virus uptake is unlikely to be responsible for HAEM development in some, but not other HSV patients, because IFN-γ and chemokines in HSV lesions (Kokuba *et al*, 1999b) contribute to the mobilization, homing, and recruitment of most PBMC subpopulations. This includes CD34+ cells, which express the CXCR3 receptor during proliferation and differentiation (Jinquan *et al*, 2001). Ongoing studies are designed to examine the presence of HSV DNA in circulating CD34+ cells from HAEM and HSV patients at different stages of the disease, and determine the contribution of genetic components (Khalil *et al*, 1991) to the distinct response of CD34+ cells from HAEM patients to virus infection.

Materials and Methods

Study groups Thirteen patients with a history of recurrent HAEM and 18 patients with a history of recurrent HSV-1 (12 patients) or HSV-2 (six patients) were studied. Diagnosis was confirmed by virus isolation (HSV patients) and by PCR of lesional skin (obtained by punch biopsy) with Pol primers, as previously described (Miura *et al*, 1992; Imafuku *et al*, 1997; Kokuba *et al*, 1998, 1999a, b). Blood samples (50 mL) were obtained at the time of acute lesions (days 1–3 after HSV lesion onset; days 2–8 after HAEM lesion onset) and during lesion-free intervals (at least 3 wk after lesion healing). Four adult subjects who denied a previous history of HSV infection and were seronegative for both HSV-1 and HSV-2 determined by gG ELISA (Focus Technologies, Cyprus, California) served as normal control. The medical ethical committee of the University of Maryland approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

Cells and viruses Vero (African green monkey kidney) cells were grown in Eagle's-modified minimal essential medium (EMEM) (Invitrogen, Carlsbad, California) with 10% fetal bovine serum (FBS) and 100 U per mL penicillin–streptomycin (Gibco-BRL, Gaithersburg, Maryland). HSV-1 (strain F) and HSV-2 (strain G) were as previously described (Gyotoku *et al*, 2002). They were grown in Vero cells and clarified of cell debris by centrifugation at 20,000 $\times$ *g*. The clarified supernatant was subjected to ultracentrifugation (2 h, 114,000 g) in a Beckman SW501 rotor and the pelleted virus (partially purified) was resuspended in fresh MEM and stored at -80°C until use. Virus titers were determined by plaque assay on Vero cells and expressed as PFU per mL (Aurelian, 2000). Mock infection with PBS served as control.

Antibodies Mouse monoclonal antibodies (Mab) to human CD34 (581), CD1a (HI149), CD14 (M5E2), and CD19 (HIB19) antigens and rat anti-human Mab to CLA (HECA-452) antigen were obtained from BD Biosciences PharMingen (San Diego, California). The antibodies to CD14, CD19, and CD34 were conjugated to R-PE. Those against CLA and CD1a were conjugated to FITC. R-PE-conjugated mouse IgG2a k, mouse IgG1 k, and rat IgM K and FITC-conjugated mouse IgG1 k were also obtained from BD Biosciences Pharmingen and, respectively, used as matched isotype controls for CD14 (mouse IgG2a), CD34, CD1a, and CD19 (mouse IgG1), and CLA (rat IgM). Affinity-purified rabbit anti-E-cadherin IgG (H-108) and goat anti-actin IgG (C-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, California), normal rabbit IgG from Calbiochem (San Diego, California), and HRP-conjugated anti-rabbit IgG was obtained from Cell Signaling Technology (Beverly, Massachusetts) and used according to the manufacturer's instructions.

PBMC collection and culture of CD34+, CD34−, and CD14+ cells PBMC were isolated using discontinuous Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. PBMC obtained by Ficoll–Hypaque centrifugation from 50 mL of blood collected during quiescence were fractionated with the MACS kit, according to the manufacturer's instructions. CD34+ and CD34− fractions were obtained from the PBMC using the MACS Direct CD Progenitor Cell Isolation Kit and MiniMACS separation columns (Miltenyi Biotec, Auburn, California), according to the manufacturer's instructions. CD34+ cells averaged $2.1 \pm 1.2 \times 10^5$ and $1.4 \pm 0.8 \times 10^5$ cells for HAEM and HSV patients, respectively. For all patients, the purity of the CD34+ population ranged between 85% and 93% as determined by staining with CD34-specific antibody and flow cytometry analysis. Two-color flow cytometry with PE- and FITC-labeled CD34 and CLA antibodies indicated that 54%–60% of the CD34+ cells expressed CLA (CD34+/CLA+). Cord blood CD34+ cells were purchased from Cambrex BioScience (Rockland, Maryland).

Primary cultures of CD34+ cells were established in 24-well plates (Costar, Cambridge, Massachusetts) seeded with 10^4 cells per well at 37°C in a humidified atmosphere in the presence of 5% CO_2. The medium consisted of RPMI 1640 containing 2.05 mM L-glutamine (Invitrogen) with 10% heat-inactivated FCS (Gemini Bio-Products, Woodland, California) and supplemented with optimized concentrations of GM-CSF (50 ng per mL) (Sigma, St Louis, Missouri), TNF-α (10 ng per mL) (R&D Systems, Minneapolis, Minnesota) (Caux *et al*, 1996, 1997; Strunk *et al*, 1996, 1997), and IL-4 (20 ng per mL) (Sigma) (complete medium). IL-4 was added to the medium because it blocks the CD14 maturation pathway when added to CD34+ cells and enhances the survival of LC-like DC (Romani *et al*, 1994; Canque *et al*, 1998; Rougier *et al*, 1998). Primary cultures of CD34− cells were set up in parallel and served as control. CD14+ enriched populations were obtained from the CD34− fraction by adhesion to plastic (2 h at 37°C) followed by removal of the contaminating cells by four washes with PBS. More than 60% of the attached cells expressed the CD14 marker, as determined by flow cytometry. The CD14+ enriched cell populations were cultured in RPMI-10% FBS supplemented with GM-CSF (50 ng per mL) and IL-4 (20 ng per mL) for 5 d (generate immature DC) and for 2 additional days in the same medium supplemented with TNF-α (50 ng per mL). Cells were infected with HSV-1 or HSV-2, as described (Aurelian, 2000). Adsorption was for 1 h at 37°C. Unbound virus was removed by extensive washing and the cells were re-incubated in complete medium. Cultures were collected at various times p.i. and duplicates were examined for virus titers or viral DNA by PCR.

Immunofluorescence and flow cytometry For membrane staining, 50 μL of cells (5×10^6 to 10^7 per mL) were incubated (30 min)

with 20 μL of conjugated Mab. Cells were washed and expression of the cell surface molecules was examined by flow cytometry using the FACScalibur system equipped with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, California), as previously described (Aurelian *et al*, 2001). Fluorescence from FITC-labeled cells was collected using a 530 per 30 nm band pass filter whereas PE fluorescence was measured with a 580 per 25 nm band pass filter. After gating with the respective isotype-matched immunoglobulin, percentages of staining cells and mean fluorescence calculations were determined from the histogram and dot plot displays using CellQuest software (Becton Dickinson Immunocytometry Systems).

PCR amplification DNA was extracted from HSV or mock-infected CD34 +, CD34−, or CD14 + cells and amplified by PCR with the HSV-type common primers for HSV DNA Pol (5′-CATCACCG ACCCGGAGAGGGAC-3′ and 5′-GGGCCAGGCGCTTGTTGGTG TA-3′) or ICP27 (5′-AGGACATTGCATCCTTCGTG-3′ and 5′-GTGC GTGTCTAGGATTTCGA-3′). The sensitivity and specificity of the primers were previously described (Miura *et al*, 1992; Imafuku *et al*, 1997). Thermal cycles included 1 min denaturation (94°C) followed by 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1min. PCR was completed with an extension step of 7 min at 72°C. The Pol and ICP27 genes were targeted because they are located at distant sites on the HSV genome and the primers used for their detection are equally sensitive (Imafuku *et al*, 1997). Consequently, detection of both Pol and ICP27 DNA is indicative of the presence of the entire viral genome, whereas detection of only one of these DNA sequences is evidence of fragmentation (Aurelian *et al*, 2003). Negative controls present between samples included DNA from uninfected Vero cells and amplification with β-globin primers (Miura *et al*, 1992; Imafuku *et al*, 1997).

TUNEL assay CD34 + and CD34− cells were infected with 10 PFU per cell of HSV-1 or HSV-2 and cultured with cytokines that induce DC development, as above. At various times p.i. (0, 4, 12, 24 h, 5, 7 d), the cells were centrifuged onto glass slides and assayed by TUNEL using the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, Indiana) according to the manufacturer's instructions. Cells in five different fields were counted (at least 350 cells) and the % TUNEL + cells were calculated. Results are expressed as % TUNEL + cells ± SEM, as previously described (Perkins *et al*, 2003).

Immunoblotting CD34 + cells (3.3×10^5) and CD14 + cells (1×10^7) were mock infected or infected with HSV-2 (10 PFU per cell) and incubated in complete medium for 10 or 24 h. At this time, the cells were washed with PBS and resuspended in 70 μL of RIPA buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with phosphatase and protease inhibitor cocktails (Sigma). For each time point, proteins in the entire extract of CD34 + cells or 100 μg of the CD14 + cells extract were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked for 1 h at room temperature with blocking buffer (TN-T buffer, 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20) containing 5% nonfat milk (w/v) and incubated with antibody to E-cadherin (1:200 dilution) for 1 h at room temperature. After three washes, blots were incubated with a 1:2000 dilution of HRP-conjugated anti-rabbit IgG (in blocking buffer) for 1 h at room temperature followed by four additional washes. Detection was with ECL reagents (Amersham Life Science, Arlington Heights, Illinois) followed by exposure to a high-performance chemiluminescence film (Hyperfilm ECL, Amersham Life Science). Quantitation was by densitometric scanning using the Bio-Rad GS-700 Imaging Densitometer (Smith *et al*, 2000; Perkins *et al*, 2003).

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