Frequent Detection of Herpes Simplex Virus Antigen in Skin and Peripheral Blood CD34+ Mononuclear Cells from Patients with Graft-versus-Host Disease

Görgün Akpek ¹, Ranjit Joseph ², Canan Günay ², Irving I. Kessler ³, Marianna Shvartsbeyn ⁴, Bhavana Bhatnagar ¹, Laure Aurelian ¹, ², ³, ⁴

¹ Marlene and Stewart Greenbaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland
² Department of Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland
³ Department of Epidemiology, University of Maryland School of Medicine, Baltimore, Maryland
⁴ Department of Dermatology, University of Maryland School of Medicine, Baltimore, Maryland

ABSTRACT
Viruses are implicated in the initiation or flare of graft-versus-host disease (GVHD) by virtue of their ability to activate antigen-presenting dendritic cells (DC). Herpes simplex virus (HSV) infects circulating CD34+ stem cell progenitors, favoring their differentiation into skin homing DC (CD1a+ Langerhans cells) that contribute to the development of an inflammatory skin rash known as HSV-associated erythema multiforme (HAEM). Following these findings, we conducted a prospective study to examine whether HSV is also associated with GVHD. Skin biopsies and peripheral blood mononuclear cells (PBMC) were collected from 37 consecutive patients on admission before and after allogeneic hematopoietic stem cell transplantation (HSCT) and examined for HSV antigen (Pol) expression and the presence of Pol+CD34+ and Pol+CD1a+ cells. Sixteen patients developed a skin rash that was histopathologically consistent with GVHD (group I), 3 patients had a rash that was not GVHD (group II, EM-like) and 18 patients did not develop any rash after HSCT (group III). Skin biopsies from the group I patients were Pol negative pre-HSCT (baseline) but became Pol+ after the diagnosis of GVHD. The GVHD biopsies also contained Pol+CD34+ and Pol+CD1a+ cells, and these patients had a significant percentage of circulating Pol+CD34+ and Pol+CD1a+ PBMC. By contrast, the group II patients had Pol+ skin cells and Pol+CD34+ circulating PBMC at baseline that decreased post-HSCT. The group III patients had Pol negative skin and very few circulating Pol+CD34+ and Pol+CD1a+ PBMC at baseline that were not significantly changed post-HSCT. The data associate skin GVHD with HSV reactivation during conditioning and its propensity for nonreplicative infection of CD34+ PBMC that induces DC activation. Further studies are needed to better elucidate this association.

INTRODUCTION
Activation of host dendritic cells (DC) by tumor necrosis factor-alpha, interleukin-1, and lipopolysaccharide released from tissues damaged during high-intensity conditioning regimens and the resulting enhanced presentation of histocompatibility antigens to donor T cells is a first step in the development of acute graft-versus-host disease (GVHD) [1]. Viruses reactivated during the immunosuppressive period before and after allogeneic transplantation increase the risk of GVHD [2]. The ubiquitous herpes viruses, cytomegalovirus (CMV), Epstein-Barr virus, and human herpes virus 6 or 7 (HHV-6, -7) might participate in the induction of GVHD [3]. CMV infections that occur after but not before day 120 after cord blood transplantation, along with Epstein-Barr viremia and adenoviral enteritis, can develop in the context of GVHD [4], and DC activation via CMV-stimulated Toll-like receptor 9 (TLR9) is an established mechanism for this virus’ contribution to the GVHD reaction [5].

Our studies follow on previous findings that herpes simplex virus (HSV) infects circulating CD34+ cells, favoring their differentiation into skin-homing Langerhans cells (CD1a+) that contribute to the development of HSV-associated erythema multiforme (HAEM) through T cell stimulation. Indeed, HAEM develops after a preceding primary or recurrent HSV episode, whether clinical or subclinical, but it is independent of virus replication. HAEM lesions are free of infectious virus, but they contain viral DNA fragments as determined by polymerase chain reaction (PCR) [6-14]. Direct comparison of similarly sensitive primers for 9 genes located along the entire length of the HSV genome revealed that most of the viral DNA was lost, but 70% to 75% of the patients retained viral DNA sequences that included the HSV gene UL30, which codes for viral DNA polymerase (Pol) [14-16]. Lesion development was associated with viral DNA expression (including Pol RNA and Pol protein), which was not seen in healed lesions, even if they were still positive for viral DNA [8,14,17-20]. Skin-delivered viral antigen was shown to cause HAEM [21]. Consistent with the limited spectrum of viral antigens expressed in the HAEM lesions, the HSV-specific T cell repertoire is restricted, but the dominant CD4+/Vβ2 cells infiltrate the skin early after lesion onset and produce the inflammatory cytokine interferon-gamma (IFN-γ) [15,18,22]. This is followed by the recruitment of autoreactive T cells [15], apparently in response to cellular genes driven by the transcription factor SP-1, which is induced by Pol through molecular mimicry with a negative SP-1 regulator [23].
Viruses (including HSV) are associated with autoimmunity [24-26] and increased expression of cytokines and/or chemokines was reported in both virus and drug-induced erythema multiforme (EM) skin lesions [22,27]. In HAEM, viral DNA is fragmented in infected blood CD34+ cells and they are encouraged to differentiate into Langerhans cells (LC) that transport the viral DNA fragments to the skin [13,28]. Because (1) HSV DNA stimulates TLR9 [29], which is associated with the contribution of CMV to GVHD [5], and (2) GVHD develops after the infusion of peripheral blood CD34+ cells, we considered the possibility that HSV could contribute to GVHD development through a mechanism similar to that implicated in HAEM. This hypothesis is supported by a retrospective study, which showed that Pol was expressed in biopsies from a high proportion (79%) of patients with acute skin GVHD [30]. Here we report the results of a prospective study, which demonstrates that acute skin GVHD lesions are positive for Pol and contain Pol+CD34+ and Pol+CD1a+ cells that are not seen in the pre-transplantation skin samples obtained from the same patients. This likely involves nonreplicative (incomplete) infection of donor CD34+ cells by virus reactivated during immunosuppressive conditioning.

STUDY SUBJECTS
This prospective study enrolled adult patients with various hematologic malignancies who underwent allogeneic hematopoietic stem cell transplantation (HSCT) from related or unrelated donors. After obtaining informed consent, baseline blood samples (30 mL) and 4 mm skin punch biopsies were obtained from all the study subjects on the same day during the early phase of the conditioning regimen and before stem cell infusion. Second skin biopsies were obtained when the patients developed skin rash before or within 48-72 hours of steroid treatment. GVHD diagnosis was made by appropriate clinical presentation and confirmed histologically. The same biopsy samples were evaluated for the GVHD diagnosis, Pol antigen expression, and the infiltration by Pol+ mononuclear cells in 2 separate laboratories. A third biopsy was collected from 6 patients with a rash diagnosed as GVHD that showed increased clinical severity to evaluate the potential relationship between the levels of Pol expression and clinical severity. Second blood samples were collected from all patients after HSCT on the same day that the second skin biopsies were obtained. For uniform assessment, second blood samples were collected from the nonrash patients at the time of myeloid recovery post-HSCT (between days +14 to +28) when most acute GVHD occurs.

MATERIALS AND METHODS
Antibodies
The generation and HSV specificity of the Pol antibody were previously described. The antibody specifically identifies the Pol protein in HSV-infected cells, but it does not recognize cellular epitopes in uninfected cultured cells or patient tissues by immunohistochemistry or immunoblotting [8,14–18,22,30]. Antibody to the major HSV capsid protein VP5 was obtained from Virusys Corp (Sykesville, MD) and used to detect virion formation as a result of virus replication. Polyclonal rabbit antibody to human E-cadherin, unconjugated and fluorescein (FITC)-conjugated monoclonal antibodies (mAbs) to human CD14, and unconjugated mAb to human CD14 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated mAbs to human CD1a and CD3 and as FITC-conjugated and unconjugated mAb to human CD34 were obtained from BD Biosciences (San Diego, CA). Alexa-Fluor 594–conjugated goat anti-mouse IgG (used to detect anti-CD34, -CD14, and -CD1a in the skin) and Alexa-Fluor 488–conjugated goat anti-rabbit IgG (used to detect anti-Pol in the skin) were obtained from Invitrogen (Molecular Probes, Eugene, OR). Biotin anti-mouse/human CD11b antibody was obtained from Leinco Technologies, Inc. (St. Louis, MO), and Texas Red Streptavidin (Vector Laboratories, Burlingame, CA) was used to detect it. APC-conjugated goat anti-rabbit IgG antibody (Fab′2-specific) was purchased from Imgenex (San Diego, CA) and used in FACS analysis.

Protein Extraction and Immunoblotting
Biopsies were transferred to test tubes containing approximately 10 sample volumes of RIPA buffer (20 mM Tris-HCl [pH 7.4], 15 mM NaCl, 1% Nonidet P-40 [Sigma, St. Louis, MO], 1% sodium deoxycholate [SDS, BioRad, Hercules, CA], 5% sodium deoxycholate supplemented with protease, and phosphatase inhibitor cocktails [Sigma]) and incubated for 30 minutes on ice in an attempt grossly the tissue specimens with the help of a plastic mortar. Samples were thereafter sonicated for 60 seconds at 25% output power using the Sonicator/Ultrasonic Processor (Misonix Inc., Farmingdale, NY). Total protein was determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins were resolved by SDS-PAGE and transferred to Immobilon-P (Millipore, Billerica, MA, USA) membranes. Non specific binding was blocked by incubation (1 hour, 25° C) in TNT buffer (0.1 M Tris-HCl [pH 7.4], 15 mM NaCl, 0.5% Tween 20) containing 5% bovine serum albumin, and the blots were exposed (overnight; 4° C) to primary antibodies followed by secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technologies, Beverly, MA). Detection was with ECL reagents (Amersham Life Science, Arlington Heights, Illinois) followed by exposure to a high-performance chemiluminescence film (Hyperflirt ECL, Amersham Life Science), as described [28].

Immunofluorescence Staining of Skin Tissues
Indirect immunofluorescent staining was with Pol or VPS antibodies and differentially labeled secondary antibodies. Normal IgG served as control. Duplicate sections were stained by double immunofluorescence with antibodies to Pol and to the respective cell markers CD34+, CD1a, or CD11b. Briefly, sections (5 μm) of paraffin-embedded tissues were deparaffinized in xylene and decreasing ethanol gradients, washed in phosphate-buffered saline (PBS), and subjected to antigen retrieval using Retrievalen A (pH 6.0) (BD Biosciences) in a 95° C water bath for 10 minutes. Slides were allowed to cool in Retrievalen solution (20 minutes at 25° C), washed with PBS and blocked (1 hour at 25° C) in blocking buffer (.5% bovine serum albumin [BSA] and 5% normal goat serum [NGS] in PBS). Tissue sections were incubated with primary antibodies (18 hours at 4° C) in blocking buffer, washed with 1% Tween-20 in PBS, and exposed to secondary antibody (1 hour at 25° C). The sections were washed, mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen), and visualized with a Nikon (Nikon Inc., Melville, NY) E4100 fluorescent microscope utilizing FITC (330 to 380 nM) and UV (DAPI) (465 to 495 nM) cubes. Cells were counted in 5 randomly selected 3mm² fields (250 cells each), and the percent staining cells was calculated relative to total cell numbers determined by DAPI staining. The mean percentage of positive cells in each comparison group was calculated by dividing the total number of positive cells by the total number of patients in the group.

PCR Amplification
DNA was extracted from formalin-fixed paraffin-embedded biopsies and PCR amplified with primers for Pol and control human β-globin, as described [7,17,18].

Flow Cytometry Analysis (FACS)
FACS analysis was done as previously described [18,28]. Peripheral blood mononuclear cells (PBMC) were collected by Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare, Piscataway, NJ) according to the manufacturer’s instructions, and 2-5 × 10⁶ cells in a total volume of 0.2 mL of 2% FACS buffer (2% fetal bovine serum [FBS] in PBS) were stained in double immunofluorescence with antibodies to Pol or E-cadherin and cellular markers. The cells were washed in PBS, fixed in 1% paraformaldehyde in PBS, and stored at 4° C in the dark until analysis. Controls consisted of unstained cells, cells stained with FITC-conjugated isotype-matched antimouse IgG1 alone or with APC-conjugated goat antirabbit IgG, and cells stained only with secondary antibodies or CD3, CD34, or Pol antibodies. Two-color FACS analysis was performed using the FACSscan and LSRII analytical flow cytometers (both from Becton Dickinson Immunocytometry Systems, San Jose, CA). FITC-labeled cells were excited with a 488 nm blue laser and measured using a 530/30 nm band pass filter. Alexa 633 fluorescence was excited with a 633 nm red laser and measured with a 660/20 nm band pass filter. After gating with the respective isotype matched immunoglobulin, percentages of positive stained cells and mean fluorescence calculations were determined from the histogram and plot dot
displays using a reference point that was set so that no more than 1% of the cells were to the right of that marker. The numerical value (%) from the quadrant that reflects the double-positive cells in the control (APC-conjugated IgG and FITC-conjugated isotype-matched IgG1) was subtracted from the experimental values of the same quadrant for all patients and at all time points. The percent of cells in the specific antibody-stained samples was calculated using the FlowJo (Tree Star Inc., Ashland, OR, USA).

Statistical Analyses
Data were analyzed by between-group analysis of variance (ANOVA) and mixed repeated-measures ANOVAs followed by the Tukey’s post hoc tests. Analyses were performed with SigmaPlot 11.2 for Windows (Systat Software, Point Richmond, CA). Data were also analyzed by exact nonparametric rank test. Two group comparisons were performed using the Wilcoxon signed rank (paired samples, eg, rash with GVHD and rash without GVHD) test.

RESULTS
Patients and HSCT
The study was conducted between 2008 and 2010 at the University of Maryland Marlene and Stewart Greenebaum Cancer Center. Patients were enrolled in the study after hospitalization for an allogeneic HSCT using donor peripheral blood CD34+ cells collected by apheresis after G-CSF mobilization. Patient characteristics are summarized in Table 1. All the patients and donors were HSV seropositive (HSV-1 and/or HSV-2) as determined by ELISA. Patients had to be in hematologic remission after chemotherapy before receiving immunosuppressive conditioning regimen with or without total body irradiation. Patients received myeloablative or nonmyeloablative HSCT. Some patients received total body irradiation and others received chemo-only conditioning regimen. Tacrolimus-based GVHD prophylaxis was started on day 3, before infusion of donor stem cells. Antimicrobial prophylaxis, including acyclovir for HSV at 800 mg twice a day by mouth, was started on day 3 post-transplantation and continued throughout the follow-up. Histologic evidence provided by the pathology service was used for diagnosis of GVHD in the appropriate clinical presentation.

A total of 37 allogeneic HSCT recipients were enrolled in the study. Nineteen patients (51%) developed skin rash during the post-HSCT period. Sixteen of these 37 patients (43%) were diagnosed with GVHD within 8 to 244 days (median, 26 days) post-HSCT (group I, rash with GVHD). The rash seen in the other 3 patients (8%) was pathologically inconsistent with GVHD and it developed within 16 to 24 days (median, 16 days) post transplantation (group II; rash

Table 1. All the patients and donors were HSV seropositive (HSV-1 and/or HSV-2) as determined by ELISA. Patients had to be in hematologic remission after chemotherapy before receiving immunosuppressive conditioning regimen with or without total body irradiation. Patients received myeloablative or nonmyeloablative HSCT. Some patients received total body irradiation and others received chemo-only conditioning regimen. Tacrolimus-based GVHD prophylaxis was started on day 3, before infusion of donor stem cells. Antimicrobial prophylaxis, including acyclovir for HSV at 800 mg twice a day by mouth, was started on day 3 post-transplantation and continued throughout the follow-up. Histologic evidence provided by the pathology service was used for diagnosis of GVHD in the appropriate clinical presentation.

A total of 37 allogeneic HSCT recipients were enrolled in the study. Nineteen patients (51%) developed skin rash during the post-HSCT period. Sixteen of these 37 patients (43%) were diagnosed with GVHD within 8 to 244 days (median, 26 days) post-HSCT (group I, rash with GVHD). The rash seen in the other 3 patients (8%) was pathologically inconsistent with GVHD and it developed within 16 to 24 days (median, 16 days) post transplantation (group II; rash

Table 1. All the patients and donors were HSV seropositive (HSV-1 and/or HSV-2) as determined by ELISA. Patients had to be in hematologic remission after chemotherapy before receiving immunosuppressive conditioning regimen with or without total body irradiation. Patients received myeloablative or nonmyeloablative HSCT. Some patients received total body irradiation and others received chemo-only conditioning regimen. Tacrolimus-based GVHD prophylaxis was started on day 3, before infusion of donor stem cells. Antimicrobial prophylaxis, including acyclovir for HSV at 800 mg twice a day by mouth, was started on day 3 post-transplantation and continued throughout the follow-up. Histologic evidence provided by the pathology service was used for diagnosis of GVHD in the appropriate clinical presentation.

A total of 37 allogeneic HSCT recipients were enrolled in the study. Nineteen patients (51%) developed skin rash during the post-HSCT period. Sixteen of these 37 patients (43%) were diagnosed with GVHD within 8 to 244 days (median, 26 days) post-HSCT (group I, rash with GVHD). The rash seen in the other 3 patients (8%) was pathologically inconsistent with GVHD and it developed within 16 to 24 days (median, 16 days) post transplantation (group II; rash
without GVHD, EM-like). Two patients (1 in group I and 1 in group II) developed late GVHD (>130 days post-HSCT) and were treated with the CMV drug Cytovene(R), ganciclovir sodium IV (Roche Laboratories, Nutley, NJ) before the second biopsy was obtained. Eighteen patients (49%) never developed rash (group III, no rash). The median ages were 52, 50, and 50 years for the patients in groups I, II, and III, respectively. Ten patients (55%) in the group III received HLA-match related donor allogeneic HSCT. Eleven patients (69%) in the GVHD group received unrelated donor transplant. HLA-mismatch (7/8) was present in 2 patients in group I, 2 patients in group II, and 5 patients in group III. The majority of the patients with GVHD had 100% donor chimera.

All patients were lesion-free when the pre-HSCT blood sample and skin biopsy were taken, in a median 4 days before transplantation, ranging from 2 to 6 days. Post-transplantation skin biopsies were taken when the patients developed skin rash, before or within 48-72 hours of steroid treatment. They were histopathologically diagnosed as GVHD grade I (6 patients), GVHD grade II (5 patients), and GVHD grade III (5 patients). In 6 patients diagnosed as GVHD grades I or II, an additional post-HSCT skin biopsy was obtained at the time of increased rash severity, defined as diffuse erythema involving more than 50% of body surface area, blistering maculopapular rash, skin breakdown or sloughing, and a histopathological classification of GVHD grade III in 4 of them. Post-HSCT blood samples were collected on average twice (ranging from 2 to 4 times) during the hospitalization.

Control Subjects
The following patients served as controls: (1) Two non-transplant and 1 study cohort (MB) patient with culture-positive HSV lesions, including 1 with buttock lesions (HSV-1), 1 with vulvar lesions (HSV-2), and 1 (MB) with acyclovir-resistant HSV-2 genital ulcers erupted on day 5 post-HSCT before she developed skin GVHD at other body sites; (2) 3 patients with skin GVHD who received acyclovir prophylaxis initiated on day 3 post-HSCT according to protocol, and 1 nontransplantation patient with HAEM from whom biopsies were obtained from the respective skin lesions as well as the adjacent uninvolved skin; and (3) 2 HSV seronegative subjects who provided peripheral blood mononuclear cells.

The virus positive tissues from all 3 HSV patients were Pol positive by immunoblotting and immunofluorescence, as shown for 1 of them in Figure 1A and B, respectively. These tissues also stained with antibody to the major capsid protein VP5, which is indicative of virus replication, but not with normal IgG (Figure 1B). Intracellular localization was as described for patients with culture-confirmed HSV lesions, including those that occur post-HSCT [30]. Pol and VP5 staining was seen in the epidermis and the dermal surface of the lesion base, and it primarily localized in the cytoplasmic and intranuclear (arrow). DAPI staining to identify cell nuclei is blue. (C) Representative PCR of DNA extracted from GVHD and HAEM lesions and normal skin from patients JP and SA.
Group I Patients (with GVHD Skin Rash)

Skin findings

Skin data are available for 15 of 16 patients with GVHD and representative staining patterns of the skin biopsies from these patients are shown in Figure 2. Pol staining was not seen in the epidermis or dermis from 13 patients (87%) pre-HSCT. The other 2 patients had low numbers of Pol+ cells in the epidermis (8.5% and 15.6%), presumably reflecting virus reactivation from latency that occurred during the conditioning treatment. By contrast, the post-HSCT GVHD biopsies from all 15 patients had high numbers of Pol+ cells, with one-half of the epidermal cells (mean, 49.7% ± 6.8%) and one-third of the dermal cells (mean, 24.6% ± 3.5%) being Pol+ (Table 2, Figure 3). The percentage of Pol+ cells was also significantly increased in the 2 patients who had positive epidermal staining at baseline (78.8% and 32.6%, respectively). The difference in the percent of Pol+ cells pre- and post-HSCT was highly significant ($P < .001$ by ANOVA).

Further supporting the relationship between HSV gene expression (Pol) and disease, the percentage of Pol+ cells was strongly correlated with lesion severity, as determined in 6 patients for whom biopsies were obtained both early after lesion development and at a later time when the severity of the skin rash was significantly increased. In these patients the percentage of Pol+ cells in the more severe GVHD biopsies was 3- to 68-fold higher than in the early GVHD biopsies for the epidermis and 2- to 52-fold higher for the dermis. The GVHD biopsies did not stain with VP5 antibody, and the Pol staining was strictly cytoplasmic (Figure 2). This was also reported for HAEM and is indicative of nonreplicative (incomplete) infection associated with viral DNA fragmentation [6-18,23,30]. It is in contrast to the

![Figure 2. Representative images of immunohistochemical staining of tissues collected pre- and post-HSCT. (A) Representative skin biopsies collected at baseline from 2 patients (#1 and #2) and stained with antibodies to Pol or Pol and CD34 and secondary antibodies that are labeled with FITC (Pol; green) or Alexa-Fluor 488 (CD34; red) are signal negative. Representative biopsy of post-HSCT GI (colon) GVHD lesion from patient #1 stained with antibodies to Pol and CD34 and secondary antibodies that are labeled with FITC (Pol; green) or Alexa-Fluor 488 (CD34; red) is signal negative. (B) Representative biopsies of post-HSCT skin GVHD lesions from patients #1 and #2 stained with antibodies to Pol, VP5, or Pol and cellular markers CD34 or CD1a, and secondary antibodies that are labeled with FITC (Pol, VP5; green) or Alexa-Fluor 488 (CD34, CD1a; red). Antigen co-localization is yellow. DAPI staining to identify cell nuclei is blue.](image-url)
HSV-lesional skin, which also stained with VPS antibody and had both cytoplasmic and intranuclear Pol localization associated with viral DNA replication (Figure 1).

To test the hypothesis that the potential contribution of HSV to skin GVHD is through pathogenesis strategies similar to those involved in HAEM, the skin tissues were examined for CD34+ and CD1a+ cells that express Pol (Pol+CD34+ and Pol+CD1a+) by double immunofluorescent staining. The pre-HSCT skin tissues from all 15 patients were free of HSV antigen. The Pol+CD34+ cells in epidermis 1.6+1.2 n/a .001 49.7+6.8 0 n/a — 47.5+23 4.1+1.1 .1
Pol+ cells in dermis .1+1 24.6+3.5 <.001 0 n/a — 8.1+6.9 .3+3 .3
CD34+CD1a+ 0 5.9+2.8 .04 0 n/a — 0 3.0+2.6 .3
CD14+CD11b+ 0 3.9+2.2 .08 0 n/a — 0 0 —
CD11b+Pol+ 0 7.8+6.2 .2 0 n/a — 0 0 —
CD1a+Pol+ 0 15.5+6.8 .03 0 n/a — 0 0 —

Table 2

<table>
<thead>
<tr>
<th>Marker(s) for HSV and Cell Types</th>
<th>Rash with GVHD</th>
<th>No Rash after HSCT</th>
<th>EM-Like Rash without GVHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Pre-HSCT</td>
<td>Post-HSCT</td>
<td>Pre-HSCT</td>
</tr>
<tr>
<td>Pol+ cells in epidermis</td>
<td>n = 15</td>
<td>n = 16</td>
<td>n = 3</td>
</tr>
<tr>
<td>Pol+ cells in dermis</td>
<td>.1+1</td>
<td>24.6+3.5</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>CD34+Pol+</td>
<td>0</td>
<td>5.9+2.8</td>
<td>.04</td>
</tr>
<tr>
<td>CD14+Pol+</td>
<td>0</td>
<td>3.9+2.2</td>
<td>.08</td>
</tr>
<tr>
<td>CD11b+Pol+</td>
<td>0</td>
<td>7.8+6.2</td>
<td>.2</td>
</tr>
<tr>
<td>CD1a+Pol+</td>
<td>0</td>
<td>15.5+6.8</td>
<td>.03</td>
</tr>
<tr>
<td>Blood</td>
<td>n = 13</td>
<td>n = 16</td>
<td>n = 3</td>
</tr>
<tr>
<td>CD34+Pol+</td>
<td>.8+3</td>
<td>3.7+1.1</td>
<td>.02</td>
</tr>
<tr>
<td>CD34+E-cad+</td>
<td>.8+3</td>
<td>3.3+2.3</td>
<td>.05</td>
</tr>
<tr>
<td>CD14+Pol+</td>
<td>9.1+3.5</td>
<td>32.2+5.9</td>
<td>.002</td>
</tr>
<tr>
<td>CD11b+Pol+</td>
<td>8.8+4</td>
<td>7.9+3.0</td>
<td>.02</td>
</tr>
</tbody>
</table>

Mean percentage ± SEM of Positive Cells by Immunofluorescent Staining in Pre- and Post transplant Samples

HSV indicates herpes simplex virus; GVHD, graft-versus-host-disease; EM, erythema multiforme; HSCT, hematopoietic stem cell transplantation.

Figure 2A, suggesting that the association of HSV with GVHD is restricted to the skin.

Figure 3. Bar graphs in the top 2 panels show the percentage of Pol+ cells in the epidermal and dermal parts of the skin biopsy samples from patients with GVHD (group I) or EM-like rash with no GVHD (group II) pre- (blue bar) and post- (red bar) HSCT. Bar graphs in the bottom 2 panels show the percentage of cells with double immunostaining for Pol and cellular markers. Pol+CD14+, Pol+CD11b+, Pol+CD1a+, and Pol+CD34+ in the skin biopsies obtained pre- (blue bar) and post- (red bar) HSCT from patients with GVHD are shown. *, **, and *** represent P values calculated by ANOVA; ns indicates not significant.

Blood findings

The ability of PBMC to transport HSV antigens is established [13,27] but the contribution of CD34+ cells is still minimally documented. Data regarding the presence of Pol in circulating CD34+ PBMC are available for 13 patients in this group (Table 2, Figure 4). The percentage of Pol+CD34+ cells was minimal at baseline, essentially reflecting background staining (mean, 79% ± 35%), but it was significantly

fragmentation [28]. Three patients had increased numbers of Pol+ monocytes (CD14+ and CD11b+) in the post-HSCT biopsies compared with pre-HSCT biopsies, but for most of the other patients, the percentage of Pol+ monocytes in the pre-HSCT biopsies relative to post-HSCT biopsies was similar (Table 2, Figure 3). Significantly, 10 patients also developed extracutaneous GVHD, 8 of them in the gastrointestinal system and 2 in the liver (Table 1). However, biopsies collected from the colon, stomach, and esophagus GVHD lesions from these patients did not stain with Pol antibody and did not contain Pol+ CD34+ or Pol+CD1a+ cells (Figure 2A), suggesting that the association of HSV with GVHD is restricted to the skin.
increased ($P = .019$ by ANOVA) at the time of GVHD. This was also true for Pol$^+$ CD1a$^+$ cells that were also detected at minimal background levels in the baseline samples from 6 of 13 patients (mean, $76 \% \pm 4 \%$), but were significantly increased (mean $7.9 \% \pm 3 \%$) in 10 of 13 patients at the time that they developed GVHD ($P = .023$ by ANOVA). Because HSV infection favors the differentiation of CD34$^+$ cells into LC through increased expression of E-cadherin [28], thereby facilitating skin repopulation [31], the pre- and post-HSCT blood samples were also examined for CD34$^+$ E-cadherin$^+$ cells. Minimal background staining was seen in the pre-HSCT samples ($6 \% \pm 3 \%$) from 6 of 13 patients, but this was increased to $5.3 \% \pm 2.5 \%$ CD34$^+$ E-cadherin$^+$ cells at the time when they developed GVHD ($P = .05$ by ANOVA).

Although the percentage of Pol$^+$ monocytes was similar in the pre- and post-HSCT biopsies, 11 of 13 patients had a relatively high percentage of circulating Pol$^+$ monocytes (CD14$^+$) pre-HSCT (9.1\% $\pm 3$\%) that significantly increased in 13 of 13 patients post-HSCT (32.2\% $\pm 5.9$\%; $P = .002$ by ANOVA). This suggests that HSV infects both host and donor CD14$^+$ monocytes (pre- and post-HSCT, respectively), but they do not directly contribute to skin lesion development. By contrast, the presence of: (1) increased numbers of circulating Pol$^+$-CD34$^+$, Pol$^+$-CD1a$^+$ and CD34$^+$-E-cadherin$^+$ PBMC post-HSCT, and (2) both Pol$^+$-CD34$^+$ and Pol$^+$-CD1a$^+$ cells in the GVHD skin, associates GVHD development with LC generated from HSV-infected, likely donor, CD34$^+$ PBMC.

**Blood findings**

Relatively low numbers of circulating Pol$^+$-CD34$^+$, CD34$^+$-E-cadherin$^+$, and Pol$^+$-CD1a$^+$ PBMC (1\% to 3.4\%) and somewhat higher numbers of Pol$^+$-CD14$^+$ PBMC were seen in the blood samples from these patients pre- and post-HSCT (Table 2, Figure 4). The percentage of Pol$^+$-CD14$^+$ cells was higher in the pre-HSCT (26.6\% $\pm 9.1$\%) than post-HSCT (4.3\% $\pm 3.5$\%) samples, underscoring the potential relevance of this cell population with respect to clinical presentation, but the difference was not statistically significant (Table 2, Figure 4).

Collectively, the skin and blood data document the difference between these patients and those in group I in terms of their response to reactivated HSV. This conclusion is supported by the significant differences seen when comparing the 2 groups for Pol$^+$-CD34$^+$ and Pol$^+$-CD14$^+$ ($P > .002$) as well as CD34$^+$-Ecadherin$^+$ PBMC ($P > .005$) by the exact nonparametric rank test. It is also supported by the finding of significant differences when using the nonparametric rank test to compare the 2 groups with respect to Pol expression in the post-HSCT skin ($P = .005$ and $P = .0018$ for epidermis and dermis, respectively), but not when the 2 groups were compared for the pre-HSCT skin and blood.

**Group III (No Rash)**

**Skin and Blood findings**

Data are available for 16 of the 18 patients in this group. Their skin samples (obtained at baseline) were negative for Pol and Pol$^+$ PBMC (<.1\%) and their blood samples evidenced a low percentage of circulating Pol$^+$-CD34$^+$ and Pol$^+$-CD1a$^+$ and a higher percentage of Pol$^+$-CD14$^+$ cells pre-HSCT that were not significantly changed post-HSCT (Table 2; Figures 3 and 4).

**DISCUSSION**

The salient feature of the data presented in this report is the finding of frequent HSV antigen in acute skin GVHD lesions occurring post-HSCT and its association with increased numbers of circulating antigen positive CD34$^+$ and CD1a$^+$ cells that also infiltrate the lesions. The following comments seem pertinent with respect to these findings.

The timing of GVHD development in our patients (range, 8 to 244 days; median, 26 days) is consistent with the occurrence of acute GVHD around the time of myeloid recovery and it may be associated with engraftment syndrome, which shares a similar cytokine surge as that seen in acute GVHD. Although the total number of studied subjects is relatively small, our data establish a highly significant difference between the patients in the 3 groups in terms of HSV antigen expression in the skin and PBMC (Table 2), which is unlikely to change by the study of a larger cohort. Given the limitations imposed by the restricted availability of patient tissues and/or PBMC, the bulk of our studies relied on immunohistochemistry, which is widely used for virologic diagnosis of epithelial HSV lesions in the clinical setting [35]. The high specificity of the Pol antibody is established [8,14-18,22,23,30]. Our immunoblotting and staining experiments confirmed the presence of the Pol protein in culture positive lesions and both Pol protein and Pol DNA were seen in GVHD and HAEM lesions, but not in normal skin. The ability of the Pol antibody to stain GVHD tissue is not an artifact caused by the nonspecific recognition of human antigens, because staining was not seen in the group III patients. It is not due to nonspecific reactivity with epitopes unique to some patients (notably those in group I), because the skin tissues collected from the very same
patients before transplantation (baseline) were negative. Staining is also not due to nonspecific recognition of a GVHD-specific antigen because it was not seen in the extracutaneous GVHD lesions experienced by the very same patients. We cannot exclude cross-reactivity with host antigens newly expressed only in skin cells and PBMC, but there is no known relationship between these 2 cell types that could explain such a phenomenon. The staining data reflect expression of the viral protein because post-HSCT biopsies were positive for Pol DNA (determined by PCR) and Pol protein (determined by immunoblotting), although these were not seen in normal skin from the same patients. The significant increase in the percentage of Pol+ cells in the more severe GVHD lesions from the same patients supports the correlation between HSV and GVHD lesion development. A limitation of our study is the lack of PCR-based confirmation of HSV DNA in the plasma, in order to determine virus reactivation during conditioning. However, HSV DNAemia is limited to primary severe infections and it cannot be detected in latency reactivation [36].

The first step in the development of acute GVHD is the activation of host antigen presenting cells, resulting in enhanced presentation of major and minor alloantigens to donor T cells [1]. Virus-induced DC differentiation and its role in T cell stimulation are well established. Infection of blood monocytes with lethal influenza A, vesicular stomatitis, or vaccinia viruses induces DC differentiation within 18 hours postinfection and these CD16-CD83+ mature DCs have enhanced capacity to activate T cells [32]. Reactivation of CMV, Epstein-Barr virus, and HHV-6, -7 during the immunosuppressive period pre- and post-allogeneic HSCT, increases the risk of GVHD development [5], but the contribution of reactivated HSV to GVHD development is still unclear. We raised the possibility that HSV could contribute to post-HSCT skin GVHD, because: (1) intracellular HSV DNA activates DC through TLR9 binding [29]; and (2) CD34+ cells can differentiate into LC when infected with HSV and they contribute to HAEM development independent of virus replication [28]. Confirming our previous conclusions [30], the post-HSCT GVHD biopsies from 8 of 15 and 6 of 13 patients in this cohort, respectively contained Pol+CD34+ and Pol+CD1a+ cells that were not seen in the pre-HSCT baseline skin. The CD34+ and CD1a+ cells did not stain with V5 antibody, consistent with their role in viral DNA fragmentation [28]. The Pol+CD34+ and Pol+CD1a+ cells in the GVHD biopsies are likely to be of donor origin and reach the skin from the circulation, because the percentages of these circulating cells were significantly elevated in the blood samples collected at the time of the GVHD presentation relative to the pre-HSCT blood samples. Although it would have been desirable to stain the donor peripheral blood CD34+ cells to confirm that they do not express Pol, PBMC from normal subjects are known to be Pol negative [18], as also confirmed in our control subjects. Interestingly, 11 of 13 patients had a relatively high percentage of circulating Pol+ monocytes (CD14+) pre-HSCT and it was significantly increased post-HSCT, suggesting that HSV activates both host and donor monocytes. The increased numbers of circulating Pol+CD14+ cells seen post-HSCT might reflect amplification of the host monocytes that survived through transplantation, unrelated to the infection of the donor cells. However, there was no significant increase in the percentage Pol+CD14+ (and Pol+CD11b+) cells in the GVHD biopsies as compared with normal pre-HSCT skin.

In group II patients, we observed relatively high levels of Pol expression and Pol+CD34+ cells in the pre-HSCT baseline samples that decreased with time, potentially reflecting the infection of host cells, which declined in quantity after the conditioning regimen. Given their distinct presentation, these patients may actually have HAEM. This interpretation is supported by the shorter time to lesion development relative to the GVHD lesions (median 16 versus 26 days, respectively). However, additional studies are needed to verify whether the lesions express the HAEM-associated markers IFN-γ, SP1, TGF-β, p21(waf1), and Hsp27 [22,23], particularly since drug eruptions (which are positive for tumor necrosis factor-alpha, but not IFN-γ [22]) are not uncommon in transplant recipients.

Our study was initially designed to obtain second biopsies from all patients regardless of their rash status. However, the protocol was later amended because of ethical concerns after negative-HSV findings in 3 patients who did not develop any rash post transplantation (group III). Although the absence of post-HSCT biopsies in these patients limits our ability to verify that they did not have Pol+ cells (including CD34+ and CD1a+) post-HSCT, this is unlikely, because normal skin was free of Pol+ cells in all the studied subjects. The absence of increased Pol expression in blood CD34+ cells post-HSCT provides indirect evidence of negative Pol expression in the skin at that time.

Collectively, our data suggest that skin GVHD resembles HAEM in its association with a nonreplicative (incomplete) HSV infection that is associated with viral DNA fragmentation within infected CD34+ cells and the transportation of the DNA fragments to the skin [8,14-20]. Because similar histopathological criteria are used to identify GVHD and reactive dermatoses that include HAEM, we cannot exclude the possibility that the skin lesions experienced by the group I patients and diagnosed as GVHD are actually virus-associated erythematous lesions in the same family of reactive dermatoses as HAEM. Further studies are needed to better elucidate the role of HSV reactivation in acute GVHD induction and verify the contribution of circulating Pol+CD34+ cells to this process. Additional information about the expression of other HSV antigens and better definition of the patients who do not develop GVHD post-HSCT are particularly desirable. Still, the clinical implications of our data are noteworthy. Based on the finding of a very high proportion of patients with skin GVHD who express HSV antigens resulting from nonreplicative infection, we raise the question of whether or not current HSV prophylaxis inhibits the HSV contribution. Because antiviral therapy targets virus replication, which is not required for antigen expression by viral DNA fragments, oral acyclovir therapy initiated 3 days post-HSCT, when the virus has already had the opportunity to infect CD34+ cells and stimulate reactive T cells, is unlikely to fully suppress the effects of HSV reactivation. However, it is possible that a higher dose of acyclovir administered early and perhaps by intravenous injection may inhibit virus reactivation [33,34], thereby reducing the risk of infecting circulating CD34+ cells and the associated incidence and severity of skin GVHD.

**ACKNOWLEDGMENTS**

We thank Dr. Russell Hudson for advice with data analysis by the exact nonparametric rank test.

**Financial disclosure:** The study was supported by a grant from the Maryland Stem Cell Research Foundation to LA. The views expressed in this article do not reflect the
official policy or position of Maryland Stem Cell Research Foundation.

Conflict of Interest Statement: The authors declare no conflict of interest.

Authorship Statement: G.A., L.A., and I.I.K. designed the research; G.A., R.J., C.G., M.S., and B.B. conducted the study and performed laboratory tests; I.I.K., R.J., and L.A. analyzed the data; G.A. and L.A. interpreted the results and wrote the manuscript; L.A. contributed reagents and critically reviewed and revised the manuscript. All authors approved the final manuscript version.

REFERENCES