Human Herpesvirus 6B and Lower Respiratory Tract Disease After Hematopoietic Cell Transplantation

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PURPOSE Human herpesvirus 6B (HHV-6B) DNA is frequently detected in bronchoalveolar lavage fluid (BALF) from immunocompromised subjects with lower respiratory tract disease (LRTD). Whether HHV-6B is a pulmonary pathogen is unclear.

METHODS We tested BALF for HHV-6B DNA using polymerase chain reaction in allogeneic hematopoietic cell transplantation (HCT) recipients who underwent a BAL for evaluation of LRTD from 1992 to 2015. We used multivariable proportional hazards models to evaluate the association of HHV-6B⁺ BALF with overall mortality, death from respiratory failure, and the effect of anti-HHV-6B antivirals on these outcomes. We used branched-chain RNA in situ hybridization to detect HHV-6 messenger RNA (*U41* and *U57* transcripts) in lung tissue.

RESULTS We detected HHV-6B⁺ BALF from 147 of 553 (27%) individuals. Subjects with HHV-6B⁺ BALF, with or without copathogens, had significantly increased risk of overall mortality (adjusted hazard ratio [aHR], 2.18; 95% CI, 1.41-3.39) and death from respiratory failure (aHR, 2.50; 95% CI, 1.56-4.01) compared with subjects with HHV-6B⁻ BALF. Subjects with HHV-6B⁺ BALF who received antivirals within 3 days pre-BAL had an approximately 1 log₁₀ lower median HHV-6B BALF viral load, as well as a lower risk of overall mortality (aHR, 0.42; 95% CI, 0.16-1.10), compared with subjects with HHV-6B⁺ BALF not receiving antivirals. We detected intraparenchymal HHV-6 gene expression by RNA in situ hybridization in lung tissue in all three tested subjects with HHV-6B⁺ BALF and sufficient tissue RNA preservation.

CONCLUSION These data provide evidence that HHV-6B detection in BALF is associated with higher mortality in allogeneic hematopoietic cell transplantation recipients with LRTD. Definitive evidence of causation will require a randomized prevention or treatment trial.

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INTRODUCTION

Lower respiratory tract disease (LRTD) occurs frequently in the early period after allogeneic hematopoietic cell transplantation and is an important contributor to morbidity and mortality.¹⁻³ Despite advances in diagnostic methods, causative pathogens are often not identified, and approximately 2% to 8% of hematopoietic cell transplantation (HCT) recipients with LRTD are diagnosed with idiopathic pneumonia syndrome (IPS).⁴ Current diagnostic algorithms may miss treatable pathogens such as human herpesvirus 6B (HHV-6B),⁵ a β -herpesvirus closely related to the well-established pulmonary pathogen human cytomegalovirus (CMV).⁶

HHV-6B infects more than 95% of individuals during early childhood and establishes latency in a broad range of cells, including bronchial glands.^{7,8} HHV-6B DNA is detectable in blood in approximately 40% of allogeneic HCT recipients within 100 days and is the

most frequent infectious cause of encephalitis after HCT.⁹ HHV-6B was first demonstrated as a possible cause of post-HCT LRTD in 1991.¹⁰ Subsequent studies with small sample sizes and heterogeneous patient populations, and that used variable sample types and testing strategies frequently detected HHV-6B DNA in respiratory samples but demonstrated inconsistent associations with LRTD.¹¹⁻¹⁵ There is no consensus whether HHV-6B is a pulmonary pathogen, as reflected by a lack of testing and treatment guidelines.^{2,16} Importantly, several therapies have activity against HHV-6B.¹⁷

In this study, we addressed limitations of prior publications by using established strategies to define pathogen-disease associations in the molecular era¹⁸ and a rigorous study design to determine whether HHV-6B is a pulmonary pathogen in allogeneic HCT recipients with LRTD. Our approach included testing for HHV-6B DNA in bronchoalveolar lavage fluid (BALF) from subjects with LRTD, analyzing the dose-response

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

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FIG 1. CONSORT diagram. BALF, bronchoalveolar lavage fluid; HHV, human herpesvirus; iciHHV-6, inherited chromosomally integrated HHV-6.

association of HHV-6B⁺ BALF with mortality and death from respiratory failure, and determining the association of anti-HHV-6B antivirals with HHV-6B viral load and outcomes. We compared these findings to those for CMV DNA detection in BALF in the same cohort. Finally, we performed in situ hybridization for HHV-6 gene expression in lung tissue.

METHODS

Subjects

The study cohort consisted of patients who received allogeneic HCT between 1992 and 2015 at Fred Hutchinson Cancer Research Center (FHCRC) and who had stored BALF from clinical evaluation of LRTD within 100 days after HCT. LRTD was defined as abnormal radiographic findings with lower respiratory tract symptoms (ie, cough, shortness of breath, or decreased oxygen saturation). Leftover BALF and blood were stored at -80°C at the time of collection. Subjects were excluded if the HCT donor or recipient had inherited chromosomally integrated HHV-6 (iciHHV-6; Data Supplement).¹⁹ The study was approved by the FHCRC Institutional Review Board.

Transplantation and Supportive Care Practices

HCT regimens, supportive care, and post-HCT complications varied over time.^{3,6} Subjects in whom LRTD developed underwent a bronchoalveolar lavage (BAL) using standard procedures based on international consensus guidelines.²⁰ Subjects were not routinely tested or treated for HHV-6B in blood or BALF during the study period. Antiviral treatment with agents active against HHV-6B (namely, ganciclovir, foscarnet, or cidofovir) was primarily for CMV.⁶

Microbiologic Testing

We retrospectively tested BALF and the closest available plasma sample within 14 days before or after BAL for HHV-6B and HHV-6A DNA using real-time quantitative polymerase chain reaction (qPCR) assays, as previously described.²¹ A threshold of at least 50 copies/mL was

considered positive. We also tested BALF for CMV DNA.⁶ We performed qPCR for the human β -globin gene from BALF to confirm cellularity and the quality of DNA extraction.^{6,12} Additional microbiologic testing is detailed in the Data Supplement.

LRTD Categorization

We newly categorized the cause of LRTD according to consensus guidelines on the basis of review of clinical, microbiologic, histopathologic, and radiographic records (Data Supplement).^{2,22,23}

Outcome Measures

Primary outcome measures were overall mortality or death from respiratory failure within 100 days after the BAL. We defined death from respiratory failure as any death caused exclusively or predominantly by respiratory failure.

Lung Tissue Detection of HHV-6B DNA and RNA by Droplet Digital PCR and In Situ Hybridization

We obtained formalin-fixed paraffin-embedded lung-tissue specimens from subjects with HHV-6B DNA detection in BALF (HHV-6B⁺ BALF). We used droplet digital PCR and qPCR to determine the absolute number of HHV-6 DNA copies per cell and the HHV-6 species (Data Supplement).¹⁹ We used RNAscope branched chain RNA in situ hybridization²⁴ (RISH; ACDBio, Newark, CA) to stain tissue for early (*U41* transcript) and late (*U57* transcript) HHV-6 messenger RNA transcripts (Data Supplement).²⁵

Statistical Analysis

We abstracted clinical data from medical records and databases and were blinded to research HHV-6B test results. Descriptive statistics were used to detail the prevalence of HHV-6B⁺ BALF and the HHV-6B viral load over time; results were compared with the Wilcoxon rank-sum or Kruskal-Wallis tests as appropriate. Spearman rank correlation was used to test correlations between HHV-6B viral load and BALF cellularity.

TABLE 1. Baseline Characteristics of the Cohort and Time-Dependent Characteristics for Each BAL Event

Variable	All Patients (N = 553)	Patients With HHV-6B ⁺ BALF (n = 147)	Patients Without HHV-6B ⁺ BALF (n = 406)
Baseline variables			
Transplantation year range			
1992-1999	197 (36)	58 (39)	139 (34)
2000-2015	356 (64)	89 (61)	267 (66)
Age at transplantation, years			
≤ 20	45 (8)	12 (8)	33 (8)
21-40	167 (30)	48 (33)	119 (29)
41-60	270 (49)	73 (50)	197 (49)
> 60	71 (13)	14 (9)	57 (14)
Sex			
Female	231 (42)	51 (35)	180 (44)
Male	322 (58)	96 (65)	226 (56)
Race			
White	429 (78)	109 (74)	320 (79)
Other	113 (20)	36 (25)	77 (19)
Missing	11 (2)	2 (1)	9 (2)
CMV serostatus			
D ⁻ and R ⁻	134 (24)	39 (27)	95 (23)
D ⁺ or R ⁺	396 (72)	92 (63)	304 (75)
Missing	23 (4)	16 (11)	7 (2)
Disease risk ^a			
Standard	291 (53)	78 (53)	213 (52)
High	262 (47)	67 (47)	193 (48)
HCT comorbidity index score ^b			
Low (0)	14 (2)	3 (2)	11 (3)
Intermediate (1-2)	225 (41)	67 (46)	158 (39)
High (≥ 3)	314 (57)	77 (52)	237 (58)
HLA match			
Matched related	179 (32)	37 (25)	142 (35)
Matched unrelated	178 (32)	34 (23)	144 (35)
Mismatched related	50 (9)	15 (10)	35 (9)
Mismatched unrelated	146 (27)	61 (42)	85 (21)
Cell source			
Peripheral blood	264 (48)	55 (37)	209 (52)
Bone marrow	230 (42)	62 (42)	168 (41)
Umbilical cord blood	59 (10)	30 (21)	29 (7)
Conditioning regimen ^c			
Nonmyeloablative	218 (39)	42 (29)	176 (43)
Myeloablative	335 (61)	105 (71)	230 (57)

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TABLE 1. Baseline Characteristics of the Cohort and Time-Dependent Characteristics for Each BAL Event (continued)

Variable	All Patients (N = 553)	Patients With HHV-6B ⁺ BALF (n = 147)	Patients Without HHV-6B ⁺ BALF (n = 406)
Acute GVHD ^d			
Grade 0-2	351 (63)	77 (52)	274 (67)
Grade 3-4	202 (37)	70 (48)	132 (33)
Time-dependent variables	All BALF (N = 685)	HHV-6B ⁺ BALF (n = 165)	HHV-6B ⁻ BALF (n = 520)
HHV-6B ⁺ BALF			
Yes	165 (24)		
No	520 (76)	–	–
CMV ⁺ BALF ^e			
Yes	164 (24)	38 (24)	126 (24)
No	515 (76)	123 (76)	391 (76)
Antiviral use pre-BAL ^f			
Yes	152 (22)	32 (19)	120 (23)
No	533 (78)	133 (81)	400 (77)
Maximum oxygen use pre-BAL, L/min ^g			
≤ 2	268 (39)	83 (50)	289 (56)
> 2	372 (54)	75 (46)	193 (37)
Missing	45 (7)	7 (4)	38 (7)
Maximum steroid use pre-BAL, mg/kg/d ^h			
< 2	618 (90)	158 (96)	460 (88)
≥ 2	65 (10)	7 (4)	58 (11)
Missing	2 (< 1)	0	2 (1)
WBC count pre-BAL, cells/mm ³ⁱ			
> 1,000	440 (64)	110 (67)	332 (64)
≤ 1,000	245 (36)	55 (33)	188 (36)
ALC pre-BAL, cells/mm ^{3h}			
> 300	265 (39)	59 (36)	206 (40)
≤ 300	420 (61)	106 (64)	314 (60)
ANC pre-BAL, cells/mm ^{3h}			
> 500	436 (64)	109 (66)	327 (63)
≤ 500	249 (36)	56 (34)	193 (37)
AMC pre-BAL, cells/mm ^{3h}			
> 300	232 (34)	52 (32)	180 (35)
≤ 300	453 (66)	113 (68)	340 (65)
HHV-6B DNA in plasma pre-BAL ⁱ			
Yes	52 (8)	41 (25)	11 (2)
No	384 (56)	86 (52)	298 (57)
Missing	249 (36)	38 (23)	211 (41)

(continued on following page)

TABLE 1. Baseline Characteristics of the Cohort and Time-Dependent Characteristics for Each BAL Event (continued)

Variable	All Patients (N = 553)	Patients With HHV-6B ⁺ BALF (n = 147)	Patients Without HHV-6B ⁺ BALF (n = 406)
LRTD cause			
Bacterial	34 (5)	12 (7)	22 (4)
Viral	119 (17)	22 (13)	97 (19)
Fungal	116 (17)	24 (15)	92 (18)
Multiple pathogens	121 (18)	36 (22)	85 (16)
IPS	118 (17)	30 (18)	88 (17)
Other	177 (26)	41 (25)	136 (26)

NOTE. Data are presented as No. (%).

Abbreviations: ALC, absolute lymphocyte count; ANC, absolute neutrophil count; AMC, absolute monocyte count; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CMV, cytomegalovirus; D, donor; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; HHV, human herpesvirus; HLA, human leukocyte antigen; IPS, idiopathic pneumonia syndrome; LRTD, lower respiratory tract disease; R, recipient.

^aWe categorized underlying disease risk groups as being at standard or high risk. The high-risk group was defined as acute leukemia, chronic lymphoid leukemia, lymphoma, multiple myeloma, solid tumor not in remission, chronic myeloid leukemia in blast crisis, and all relapsed diseases after HCT. All other stages in any disease as well as myelodysplastic syndrome were categorized as standard risk.

^bBased on the HCT comorbidity index.

^cMyeloablative regimens included any regimen containing ≥ 800 cGy total body irradiation, any regimen containing carmustine/etoposide/cytarabine/melphalan, or any regimen containing busulfan/cyclophosphamide with or without antithymocyte globulin.

^dIncorporated into models as a time-dependent variable relative to the BAL date.

^eTesting for CMV DNA was not performed for six subjects, so the total number of subjects included was 679.

^fWithin 3 days before BAL.

^gWithin 24 hours before BAL.

^hWithin 14 days before BAL, based on prednisone-equivalent dosing.

ⁱClosest sample within 3 days before BAL.

^jAmong subjects not receiving anti-HHV-6B antivirals at the time of the available plasma, HHV-6B DNA detection had low sensitivity (35%; 95% CI, 26% to 45%) and high specificity (96%; 95% CI, 93% to 98%) for BALF HHV-6B DNA detection.

We calculated the cumulative incidence of overall mortality and death from respiratory failure (treating death for other reasons as a competing risk event) among subjects alive at day 60 post-HCT to graphically display associations with HHV-6B⁺ BALF in the preceding 60 days. We next performed multivariable Cox proportional hazards regression to examine the association of HHV-6B⁺ BALF, the primary predictor of interest, with outcomes of overall mortality or death from respiratory failure within 100 days after the last BAL. We modeled HHV-6B⁺ BALF as (1) positive versus negative and (2) quartiles of viral load. To evaluate the association of anti-HHV-6B antivirals with overall mortality and death from respiratory failure, we created separate Cox models incorporating a composite variable for HHV-6B⁺ BALF and antiviral therapy to illustrate the varying degrees of association depending on whether each factor was present singly or in combination. For subjects with more than one BAL, time-dependent variables were updated with each additional BAL. We used the same approach to test the association of BALF CMV DNA detection with overall mortality among subjects in whom the donor or recipient was CMV seropositive. We selected potential confounding variables a priori. Variables with $P < 0.1$ in univariable models were considered for multivariable models and excluded if their exclusion did not change the hazard ratios (HRs) for

HHV-6B-related variables by more than 10%. No adjustments were made for multiple comparisons. We used SAS, version 9.4 (TS1M3) for Windows (SAS Institute, Cary, NC) for analyses. Additional details are in the Data Supplement.

RESULTS

Subjects

During the study period, 6,126 patients received an allogeneic HCT at FHCRC and 1,391 (23%) had a BAL within 100 days after HCT. We identified 564 patients (41%) with 697 BALF samples that were available for testing (Fig 1). Eleven recipients with 12 BALF samples had icHHV-6 (species A or B) and were excluded. Characteristics of the 553 included patients are described in Table 1. The characteristics of excluded patients were largely representative of this cohort (Data Supplement).

HHV-6B Detection in BALF

We detected HHV-6B DNA in 165 of 685 BALF samples (24%) from 147 of 553 patients (27%) with LRTD. The distribution of LRTD categories between days 0 to 100 after HCT is depicted in the Data Supplement. Among those with HHV-6B⁺ BALF, the median day of the first HHV-6B⁺ BALF occurred 29 (interquartile range [IQR], 20-64) days after

FIG 2. The distribution of human herpesvirus (HHV)-6B⁺ bronchoalveolar lavage fluid (BALF) detection and viral load after allogeneic hematopoietic cell transplantation (HCT). (A) A histogram of the number of BALF samples (N = 685) and the proportion of HHV-6B⁺ BALF (n = 165) over time after allogeneic HCT. (B) A box-and-whisker plot of the HHV-6B viral load over time among patients with HHV-6B⁺ BALF. (C) A histogram of the number of BALF samples and the proportion of HHV-6B⁺ BALF samples stratified by cause of lower respiratory tract disease (LRTD). (D) A box-and-whisker plot of the HHV-6B viral load stratified by cause of LRTD among patients with HHV-6B⁺ BALF. (B, D) The boxes represent the interquartile range, the horizontal lines and diamonds within the boxes represent the median and mean, respectively, and the upper and lower whiskers extend to the third and first quartiles ± 1.5 times the interquartile range, respectively. Circles represent data points that fall outside these parameters. IPS, idiopathic pneumonia syndrome.

HCT. The median HHV-6B BALF viral load was 3.3 log₁₀ copies/mL (IQR, 2.5-4.1 copies/mL). We did not detect HHV-6A DNA in any BALF.

The majority of HHV-6B⁺ BALF and the highest HHV-6B BALF viral loads occurred between days 11 and 40 after HCT (Fig 2A and 2B). There were relatively similar proportions of HHV-6B⁺ BALF and HHV-6B BALF viral loads across strata defined by LRTD category (Fig 2C and 2D). Higher proportions of HHV-6B⁺ BALF occurred in patients with diffuse abnormalities with a mixed pattern than other radiographic patterns (Data Supplement). Median HHV-6B BALF viral loads were higher in patients with diffuse ground

glass opacities or diffuse mixed changes compared with focal changes or diffuse nodules (Data Supplement).

We performed additional analyses to test whether HHV-6B⁺ BALF was due to lower respiratory tract infection versus contamination from blood. We demonstrated that patients with diffuse alveolar hemorrhage and HHV-6B⁺ BALF had a higher median HHV-6B viral load in BALF than in plasma obtained within a median of 1 day of the BALF (IQR, 1-3 days), and the plasma was often negative (Data Supplement). Similar findings were demonstrated in patients without diffuse alveolar hemorrhage (Data Supplement). Next, we demonstrated no meaningful correlation of BALF

FIG 3. Cumulative incidence curves of overall mortality and death from respiratory failure among subjects alive at day 60 after hematopoietic cell transplantation (HCT). The graphs are stratified by whether a subject had human herpesvirus (HHV)-6B detected in a BALF sample between day 0 and day 60 after HCT. Only subjects who had a BAL for evaluation of lower respiratory tract disease after HCT were included in this study. (A) Cumulative incidence curve of overall mortality. (B) Cumulative incidence curve of deaths due to respiratory failure. Deaths due to other causes were treated as competing risk events.

total β -globin or WBC count with HHV-6B BALF viral load (Data Supplement). In comparison, there was a correlation between BALF β -globin and HHV-6 viral load in BALF from patients with iHHV-6, as expected due to latent HHV-6 DNA in every nucleated recipient cell (Data Supplement). These findings support HHV-6B replication within the lung as opposed to contamination from the blood.

HHV-6B⁺ BALF and Risk for Overall Mortality and Death From Respiratory Failure

There were 269 deaths (49%) due to any cause and 219 deaths (40%) from respiratory failure within 100 days of the last BAL per patient. Three patients died on the day of the BAL and were excluded. Among day 60 survivors, those who had a preceding HHV-6B⁺ BALF had increased overall mortality and death from respiratory failure compared with those without HHV-6B⁺ BALF (Fig 3). In adjusted Cox models incorporating the entire cohort, patients with HHV-6B⁺ BALF had a higher risk of overall mortality (adjusted hazard ratio [aHR], 2.18; 95% CI, 1.41 to 3.39; Fig 4A). Patients with HHV-6B BALF viral loads greater than the lower quartile (2.41 log₁₀ copies/mL) had a higher risk for overall mortality (Fig 4B). In univariate analysis, there was increased risk for mortality in every LRTD category when HHV-6B was detected in BALF as a copathogen (Fig 4C). Similar findings were demonstrated for death from respiratory failure (Data Supplement). These findings were recapitulated in subgroup analyses among patients receiving a HCT on or after 2006 (Data Supplement).

HHV-6B BALF Viral Load and Mortality Among Subjects Receiving Antivirals

Antivirals active against HHV-6B were administered to 152 patients within 3 days before BAL at a median of 33 days

after HCT (IQR, 23-52 days). Antivirals primarily consisted of ganciclovir or foscarnet and were indicated for CMV in 86% of patients. Patients who received antivirals within 3 days before HHV-6B⁺ BALF had lower median HHV-6B viral loads (2.6 log₁₀ copies/mL) compared with those not receiving anti-HHV-6B antivirals (3.4 log₁₀ copies/mL; Fig 5A). Among patients with HHV-6B⁺ BALF who had a subsequent BAL within 28 days, there was a greater decrease in the HHV-6B BALF viral load in patients who received anti-HHV-6B antivirals in between BALs compared with those who did not (Fig 5B; Data Supplement).

Patients with HHV-6B⁺ BALF who received antivirals within 3 days before the BAL had a lower risk for overall mortality compared with patients with HHV-6B⁺ BALF not receiving antivirals (aHR, 0.42; 95% CI, 0.16 to 1.10; Data Supplement). This approximated the HR in patients without HHV-6B⁺ BALF who did or did not receive antivirals (Data Supplement). Findings were similar for death from respiratory failure (Data Supplement).

CMV⁺ BALF and Risk for Overall Mortality

We tested BALF for CMV DNA as a comparison with this related and established pulmonary pathogen. Detection of CMV DNA in BALF was similar in HHV-6B⁺ and HHV-6B⁻ BALF (Table 1). CMV⁺ BALF was similarly associated with an increased risk of overall mortality (aHR, 1.78; 95% CI, 1.01 to 3.15; Data Supplement).

Lung Tissue Detection of HHV-6B DNA and RNA by Droplet Digital PCR and RISH

We identified six patients with HHV-6B⁺ BALF who had available formalin-fixed paraffin-embedded lung tissue

FIG 4. Forest plots of risk factors for overall mortality within 100 days after the BAL. (A) Model 1 demonstrates the results of an adjusted model in which the human herpesvirus (HHV)-6B⁺ BALF variable was incorporated as positive or negative. (B) Model 2 demonstrates the results of an adjusted model in which the HHV-6B⁺ BALF variable was incorporated in quartiles of viral load. (C) Model 3 demonstrates the results of a univariate analysis of LRTD categories stratified by HHV-6B⁺ BALF; bacterial pneumonia without HHV-6B detection in the BALF is the referent (ref) for all other categories. The variables shown in the figure were all the variables included in the final models. aHR, adjusted hazard ratio; ALC, absolute lymphocyte count; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; IPS, idiopathic pneumonia syndrome; LRTD, lower respiratory tract disease.

from within 3 weeks of the BAL. HHV-6B was identified by PCR in all samples. We performed RISH for HHV-6 messenger RNA in tissue from three patients whose samples

met prespecified quality metrics (Data Supplement). Scattered intraparenchymal cells stained with HHV-6 *U41* and/or *U57* probes in all three lung samples (Fig 6). One

FIG 5. Median HHV-6B BALF viral loads stratified by the receipt of antivirals before the BAL. (A) Among subjects with HHV-6B⁺ BALF, individuals who received anti-HHV-6B antivirals within 3 days before the BAL had significantly lower median HHV-6B BALF viral loads (2.6 $\log_{10}$ copies/mL) compared with individuals not receiving anti-HHV-6B antivirals (3.4 $\log_{10}$ copies/mL). (B) Among seven subjects and 12 subjects who did and did not receive anti-HHV-6B antivirals in between subsequent BALs, respectively, there was a greater decrease in the HHV-6B BALF viral load in the subjects receiving antivirals. Only subsequent BALF samples within 28 days of the first HHV-6B⁺ BALF were considered. The boxes represent the interquartile range, the horizontal lines within the boxes represent the median, and the upper and lower whiskers extend to the third and first quartiles ± 1.5 times the interquartile range, respectively. Circles represent data points that fall outside these parameters.

patient with clinically diagnosed IPS had the highest viral detection; virologic and clinical findings are detailed in the Data Supplement.

DISCUSSION

We found that allogeneic HCT recipients with LRTD and HHV-6B⁺ BALF had an increased risk for overall mortality and death from respiratory failure compared with those without HHV-6B detection in BALF. Anti-HHV-6B antiviral use was associated with decreased HHV-6B BALF viral load and lower mortality risk. Analogous findings were demonstrated in analyses of CMV⁺ BALF. Lung tissue from three patients with HHV-6B⁺ BALF demonstrated HHV-6 gene expression in intraparenchymal cells.

Ascribing a causal role to HHV-6B in human diseases on the basis of DNA detection is challenging in the context of observational studies because of the high prevalence of HHV-6B and its ability to establish latency in many cell types.⁷ Despite these challenges, we used multiple strategies to approach causal inference based on the Bradford Hill epidemiologic criteria²⁶ and an adaptation of these criteria that consider sequence-based identification of pathogens.¹⁸ We present data that fulfill the majority of these criteria in support of HHV-6B as a pulmonary pathogen.

The primary finding of this study is a strong association between HHV-6B⁺ BALF and increased risk for mortality. Furthermore, we demonstrated a dose-dependent response between HHV-6B BALF viral load and mortality. Low-level HHV-6B detection in BALF was not associated

with increased mortality risk and may be due to asymptomatic shedding, similar to that described for CMV.⁶ Although the increased risk for mortality did not reach statistical significance in patients in the highest viral-load quartile, the confidence intervals were wide, and interpretation is limited by fewer events in this exploratory stratified analysis. A viral load threshold may be more important, as demonstrated for CMV.⁶ We also demonstrated that patients with HHV-6B⁺ BALF who received antivirals within 3 days before the BAL, typically for CMV, had a lower risk for overall mortality and death from respiratory failure that approximated the risk in patients with HHV-6B⁻ BALF. Although the associations did not reach statistical significance in adjusted models, the power of this analysis was limited by the low number of patients with HHV-6B⁺ BALF who received antivirals before BAL. Thus, these results should be interpreted cautiously. Nonetheless, the findings are notable, given that HCT recipients receiving antiviral treatment for CMV tend to have higher mortality.²⁷ Together, the data demonstrate strong associations of HHV-6B detection with mortality and a dose-response relationship, supporting the plausibility of HHV-6B as a pulmonary pathogen.

We performed a variety of analyses to demonstrate HHV-6B replication within the lungs as opposed to detection of latently infected cells or viral DNA from blood. Findings that supported HHV-6B replication in the lungs included differences in HHV-6B BALF detection over time after HCT, lack of correlation between HHV-6B BALF viral load and

FIG 6. RNAscope branched-chain RNA in situ hybridization (RISH) for HHV-6 messenger RNA transcripts (*U41* and *U57* transcripts) in formalin-fixed paraffin-embedded lung-tissue specimens from subjects with HHV-6B⁺ BALF. (A–O) The images under the columns labeled “*U41*” and “*U57*” correspond to HHV-6 RISH targeting HHV-6 early (*U41*) and late (*U57*) messenger RNA transcripts expressed during lytic viral infection. The images under the column labeled “DAPB” represent the negative control, which is a cocktail of probes targeting bacterial genes. The images under the column labeled “PPIB” represent the positive control, which is a cocktail of normally weakly expressed human control genes. Each row represents a subject with HHV-6B⁺ BALF sample collected within 10 days of the tissue-sample collection date. Only tissue samples that met prespecified quality of RNA preservation based on PPIB staining were included (Data Supplement). (A) In case 1, the subject was diagnosed with bacterial and fungal pneumonia. The hematoxylin and eosin (H&E) stain demonstrated a thin alveolar wall, some congested capillaries and hemosiderin pigment, but no overt viral cytopathic changes. (B) RISH (with red chromogen staining) for *U41* was negative. (C) RISH for *U57* demonstrated rare positive cells. In case 2, the subject was diagnosed with bacterial and fungal pneumonia. (F) The H&E stain demonstrated diffuse alveolar damage and organizing pneumonia with significant vascular congestion but no overt viral cytopathic changes. RISH (with red chromogen staining) for (G) *U41* and (H) *U57* demonstrated rare positive cells. In case 3, the subject was diagnosed with idiopathic pneumonia syndrome and diffuse alveolar hemorrhage. (K) The H&E stain demonstrated marked destruction of normal lung parenchyma with hemorrhage and diffuse alveolar damage. There was a comparatively mild inflammatory infiltrate without overt viral cytopathic changes. RISH (with brown chromogen staining) for (L) *U41* and (M) *U57* demonstrated rare positive cells. (C, G, H, L, M) The rare positive cells highlighted by the arrowheads are intraparenchymal, often seen within alveolar walls, and, therefore, favored to represent pneumocytes or lymphocytes. (D, I, N) The negative control stains for each case demonstrated minimal to no background staining. (E, J, O) The positive control stains for each case demonstrated good staining to indicate preservation of RNA in the samples.

blood contamination, and lower HHV-6B BALF viral load among recipients of anti-HHV-6B antivirals. The similar distribution of HHV-6B BALF detection and viral loads across LRTD categories could argue in favor of detection of latent virus. However, a large study of allogeneic HCT recipients with CMV LRTD demonstrated similar findings for CMV.⁶ In addition, the time periods with the highest proportion of HHV-6B⁺ BALF samples and highest viral loads correspond with the timing of blood and CNS HHV-6B reactivation and disease.²⁸ Importantly, HHV-6B was detected in only one BALF sample (5%) at a low level from a historical cohort of 21 asymptomatic allogeneic HCT recipients who underwent a research BAL between days 35 and 45 after HCT at our center.⁵ This may reflect the incidence of pulmonary HHV-6B shedding or detection of latent virus in the absence of LRTD after HCT.

We also performed similar analyses using CMV⁺ BALF as the primary predictor of interest, given its close biologic

relationship to HHV-6B and that it is an established pulmonary pathogen.²⁹ First, we found that CMV was equally distributed among HHV-6B⁺ and HHV-6B[−] BALF samples and thus unlikely to be driving our primary findings. We demonstrated that CMV⁺ BALF was associated with an increased risk of overall mortality, similar to that of HHV-6B⁺ BALF—a finding that meets the Bradford Hill criteria of analogy.

Finally, we developed a RISH assay to detect markers of intraparenchymal HHV-6B replication in lung tissue. We identified HHV-6B RNA transcripts in lung tissue from all three tested subjects with HHV-6B⁺ BALF and well-preserved samples. Additional support for the significance of HHV-6B detection at the tissue level come from an animal model demonstrating that latent infection with murine roseolovirus, an HHV-6 homolog, can reactivate in murine lungs after HCT and induce an IPS-like phenotype.³⁰

Limitations of this study include the retrospective, single-center design with the potential for residual confounding. Lack of broad availability of the new international standard for HHV-6B PCR³¹ limits generalizability of the viral load results. This cohort was a specifically defined group of allogeneic HCT recipients with LRTD undergoing BAL, which may explain clinical features such as the high rate of GVHD grades 3 and 4, and limits generalizability. Variability in the volume of saline injected and recovered from the BAL could affect quantification of HHV-6B viral load; we used the log₁₀ scale for all viral load analyses, which may mitigate some of the differences in variable dilution. There was a possibility of misclassification of the cause of LRTD due to negative test results or lack of testing, particularly in the earlier period. However, we recapitulated our findings in analyses restricted to a more contemporary subgroup receiving a HCT on or after 2006, when comprehensive molecular testing was routinely performed. Analyses of the association of antivirals with outcomes were limited by small sample size and variable durations of antiviral

administration. Given the recent approval of letermovir for CMV prophylaxis in CMV-seropositive adult allogeneic HCT recipients,³² which is limited in antiviral effect to CMV, the epidemiology of HHV-6B and other viral infections may change with reduced use of broader-spectrum antivirals.¹⁷

In conclusion, the molecular and clinical observations from this study demonstrate that HHV-6B can reactivate in the lungs after allogeneic HCT, and detection of HHV-6B in BALF from allogeneic HCT recipients with LRTD is associated with increased mortality risk. The data presented here fulfill criteria to define pathogen-disease associations in the molecular era.¹⁸ Whether HHV-6B reactivation independently causes LRTD or is a copathogen reactivating in the context of pulmonary damage is unclear. Definitive evidence of causation will require a randomized prevention or treatment trial, although this has not been performed for most microorganisms accepted as pulmonary pathogens. Recognition of HHV-6B as a pulmonary pathogen has potential to improve outcomes after allogeneic HCT.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST AND DATA AVAILABILITY STATEMENT

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST**Human Herpesvirus 6B and Lower Respiratory Tract Disease After Hematopoietic Cell Transplantation**

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