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VU CANTERO, Diem-Lan, et al.

Abstract

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Reference

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Human pegivirus persistence in human blood virome after allogeneic haematopoietic stem-cell transplantation


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Abstract

Objectives: Because commensal viruses are defined by the immunologic tolerance afforded to them, any immunomodulation, such as is received during haematopoietic stem-cell transplantation, may shift the demarcation between innocuous viral resident and disease-causing pathogen.

Methods: We analysed by deep-sequencing the plasma virome of 40 allogeneic haematopoietic stem-cell transplantation patients 1 month after transplantation. Because human pegivirus (HPgV) was highly prevalent, we performed a 1-year screening of 122 plasma samples by specific real-time reverse transcription PCR assay. We used the log-rank test and the Gray test to assess association with outcomes, and the Mann-Whitney test and multivariable linear regression model to assess association with T-cell reconstitution.

Results: Polyomaviruses (PyV) (20/40 patients), anelloviruses (16/40), pegiviruses (14/40) and herpesviruses (14/40) were most frequently identified, including ten cytomegalovirus; three Epstein-Barr virus; two herpes simplex virus type 1; one human herpesvirus 6b and one human herpesvirus 7; 18 Merkel cell—PyV; two BK-PyV; three PyV-6; and one JC-PyV. Papillomavirus and adenovirus were identified in 11 and two patients, respectively. The HPgV specific real-time reverse transcription PCR screening identified 51 of 122 positive samples, high virus loads and persistent infections up to 1 year after transplantation. Comparison between patients with or without HPgV infection at time of transplantation did not reveal a significant difference in infections, engraftment, survival, graft vs. host disease, relapse or immune reconstitution.

Conclusions: The blood virome after allogeneic haematopoietic stem-cell transplantation includes several DNA viruses, notably herpesviruses and PyV. Among RNA viruses, HPgV is highly prevalent and persists for several months, and it thus may deserve special attention in further research on immune reconstitution.

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Introduction

Throughout life, humans encounter countless viruses. While the majority do not cause overt disease, each leaves an indelible mark on the immune system, and some may persist as commensal residents, tolerated under tight immunologic restraint [1]. Defining pathology as the binary presence or absence of a pathogen is
Therefore a reductive analysis of a more complex interaction, where immunologic cross-talk from commensal tolerance is able to influence the immune response to pathogens, determine their pathology or have an indirect effect on host health [1]. Next-generation sequencing (NGS) can elucidate the true diversity of the human virome, providing a more holistic definition of viral pathogenicity in context of the greater microbiome. The human virome plays a diverse and important role in host biology, either by interacting with its immune system or with other components of its microbiome [2]. Because the virome is essentially composed of a collection of antigens and pathogen-associated molecular patterns, it can influence the host response to other infections [3] or enhance susceptibility to inflammatory diseases [4].

Allogeneic haematopoietic stem-cell transplant (allo-HSCT) recipients’ virome is of particular interest because of their high state of immunosuppression. The consequences of shifts in immune competence towards commensal viruses are still unknown. Investigating the HSCT virome could change the shape of routine viral screening in transplant donors and recipients; there is already evidence that complications experienced by HSCT recipients such as graft vs. host disease may be modulated by the presence of commensal viruses [5].

Using NGS, we analysed the human blood virome of HSCT patients at the peak of immunosuppression and after multiple transfusions. According to the high prevalence of human pegivirus (HPgV) identified and its potential for immune modulation [6], we then more deeply investigated its potential influence on immune reconstitution and patient outcome.

**Methods**

**Study design**

The primary cohort, composed of 40 plasma specimens of allo-HSCT recipients (Supplementary Table S1), was retrospectively analysed by NGS. Plasma specimens were collected at a median of 33 days (range, 26–40 days) after transplantation (Supplementary Fig. S1).

A specific HPgV investigation included the first cohort plus 82 additional allo-HSCT patients. We performed an HPgV specific real-time polymerase chain reaction (PCR) for establishing the presence of HPgV in the plasma of HSCT recipients.

**Data**

Data are presented as n (%) unless otherwise indicated. Statistical analyses were performed by chi-square or Fisher exact tests for categorical variables and Mann-Whitney test for continuous variables. Myeloid malignancies include acute myeloid leukaemia, MDS/MPS/MPD and chronic myeloid leukaemia. Lymphoid malignancies include acute lymphoid leukaemia, lymphoma/chronic lymphoid leukaemia and myeloma.

**ATG, antithymoglobulin; BM, bone marrow; CMV, cytomegalovirus; CNI, calcineurin inhibitor; Cy, cyclosporine A; D, donor; HPgV, human pegivirus; MAC, myeloblastic conditioning; MDS, myelodysplastic syndromes; MMF, mycophenolate mofetil; MPS, myeloproliferative syndrome; MTX, methotrexate; MPD, myelodysplastic/myeloproliferative syndrome; NA, not applicable; PBSC, peripheral blood stem cell; R, recipient; RIC, reduced intensity conditioning; TCD, T-cell depletion.**

**Table 1**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
<th>All patients</th>
<th>HPgV+</th>
<th>HPgV−</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td></td>
<td>117</td>
<td>78</td>
<td>39</td>
<td>0.2879</td>
</tr>
<tr>
<td>Follow-up (months), median</td>
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<td>22 (5–40)</td>
<td>22 (5–39)</td>
<td>26 (10–40)</td>
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<td>Age (years), median (range)</td>
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<td>51 (18–70)</td>
<td>52 (19–70)</td>
<td>50 (18–69)</td>
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<td>Sex</td>
<td></td>
<td>78 (67)</td>
<td>59 (76)</td>
<td>19 (49)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>39 (33)</td>
<td>19 (24)</td>
<td>20 (51)</td>
<td></td>
<td></td>
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<td>Primary disease</td>
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<td>Acute myeloid leukaemia</td>
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<td>26 (33)</td>
<td>23 (59)</td>
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<td>MDS/MPS/MPD</td>
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<td>25 (32)</td>
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<tr>
<td>Acute lymphoid leukaemia</td>
<td>8 (7)</td>
<td>4 (5)</td>
<td>4 (10)</td>
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<tr>
<td>Chronic myeloid leukaemia</td>
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<td>10 (13)</td>
<td>0 (0)</td>
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<tr>
<td>Multiple myeloma</td>
<td>4 (3)</td>
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<td>2 (5)</td>
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<td>Severe aplastic anaemia</td>
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<td>7 (9)</td>
<td>1 (3)</td>
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<tr>
<td>Other</td>
<td>4 (3)</td>
<td>3 (4)</td>
<td>1 (3)</td>
<td></td>
<td></td>
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<tr>
<td>Disease status at transplant</td>
<td></td>
<td>68 (58)</td>
<td>41 (53)</td>
<td>27 (69)</td>
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<tr>
<td>Partial remission/progression/relapse</td>
<td>44 (38)</td>
<td>33 (42)</td>
<td>11 (28)</td>
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</tr>
<tr>
<td>Very high</td>
<td>5 (4)</td>
<td>4 (5)</td>
<td>1 (3)</td>
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<tr>
<td>Disease risk index</td>
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<td>4 (5)</td>
<td>4 (10)</td>
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<td>Low</td>
<td>64 (55)</td>
<td>45 (58)</td>
<td>19 (49)</td>
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<tr>
<td>Intermediate</td>
<td>32 (27)</td>
<td>20 (26)</td>
<td>12 (31)</td>
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<tr>
<td>High</td>
<td>4 (3)</td>
<td>1 (1)</td>
<td>3 (8)</td>
<td></td>
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<tr>
<td>Conditioning</td>
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<td>56 (48)</td>
<td>33 (42)</td>
<td>23 (59)</td>
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<tr>
<td>MAC</td>
<td>61 (52)</td>
<td>45 (58)</td>
<td>16 (41)</td>
<td></td>
<td></td>
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<tr>
<td>RIC</td>
<td>43 (37)</td>
<td>30 (38)</td>
<td>13 (33)</td>
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<td>Donor type</td>
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<td>Matched sibling</td>
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<td>32 (41)</td>
<td>17 (44)</td>
<td></td>
<td></td>
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<tr>
<td>Mismatched relative</td>
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<td>11 (14)</td>
<td>3 (8)</td>
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<td></td>
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<tr>
<td>Mismatched unrelated donor</td>
<td>11 (9)</td>
<td>5 (6)</td>
<td>6 (15)</td>
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<tr>
<td>Cell source</td>
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<td>66 (85)</td>
<td>34 (87)</td>
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<td>PBSC</td>
<td>17 (15)</td>
<td>12 (15)</td>
<td>5 (13)</td>
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<td>BM</td>
<td>45 (38)</td>
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<td>Ex vivo TCD</td>
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<td>43 (55)</td>
<td>29 (74)</td>
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<td>ATG</td>
<td>39 (33)</td>
<td>22 (28)</td>
<td>17 (44)</td>
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<td>CMV status</td>
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<td>25 (32)</td>
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<td>0.2303</td>
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<tr>
<td>D+/R+</td>
<td>9 (8)</td>
<td>6 (8)</td>
<td>3 (8)</td>
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<tr>
<td>D+/R+</td>
<td>17 (15)</td>
<td>12 (15)</td>
<td>5 (13)</td>
<td></td>
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<tr>
<td>Immunosuppression</td>
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<td>38 (49)</td>
<td>17 (44)</td>
<td>0.300</td>
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<td>CNI, MTX</td>
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<td>28 (36)</td>
<td>19 (49)</td>
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<tr>
<td>Cy, tacrolimus, MMF</td>
<td>15 (13)</td>
<td>12 (15)</td>
<td>3 (8)</td>
<td></td>
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</table>

Data are presented as n (%) unless otherwise indicated. Statistical analyses were performed by chi-square or Fisher exact tests for categorical variables and Mann-Whitney test for continuous variables. Myeloid malignancies include acute myeloid leukaemia, MDS/MPS/MPD and chronic myeloid leukaemia. Lymphoid malignancies include acute lymphoid leukaemia, lymphoma/chronic lymphoid leukaemia and myeloma. ATG, antithymoglobulin; BM, bone marrow; CMV, cytomegalovirus; CNI, calcineurin inhibitor; Cy, cyclosporine A; D, donor; HPgV, human pegivirus; MAC, myeloblastic conditioning; MDS, myelodysplastic syndromes; MMF, mycophenolate mofetil; MPS, myeloproliferative syndrome; MTX, methotrexate; MPD, myelodysplastic/myeloproliferative syndrome; NA, not applicable; PBSC, peripheral blood stem cell; R, recipient; RIC, reduced intensity conditioning; TCD, T-cell depletion.

* Category ‘other’ includes haemoglobinopathies.
time reverse transcription PCR (rRT-PCR) assay on stored plasma at days 0 and 30 after transplantation for all 122 samples and at four additional time points (day before, then +100, +180 and +365 days after transplantation), when specimens were available. For the immune reconstitution and outcome analyses, we selected patients receiving transplants for the first time (excluding 5/122 patients) and considered the time point day 0 after transplantation to categorize patients as infected (HPgV +) or noninfected (HPgV -). The study was approved by the cantonal ethics committee.

NGS and sequence analysis

Unbiased NGS (DNA and RNA-Seq library preparation paired-end sequencing by using the 100 bp protocol with indexing on a HiSeq 2500 sequencer [Illumina, San Diego, CA, USA] was performed on plasma samples. We analysed results using the ezVIR pipeline [7].

HPgV rRT-PCR assay

A total of 190 μL of plasma was spiked with 10 μL of standardized canine distemper virus of known concentration and extracted with the NucliSENS easyMAG (bioMérieux, Marcy l’Etoile, France) nucleic acid kit in a 25 μL elution volume, according to the manufacturer’s instruction. Extracted RNA was used for HPgV-specific rRT-PCR screening analysis using a previously published assay [8]. PCR assay reaction was performed using the QuantiTect Probe RT-PCR Kit (Qiagen, Germantown, MD, USA) on a StepOne-Plus instrument (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA) under the following cycling conditions: 50 °C for 30 minutes; 95 °C for 15 minutes; 45 cycles of 15 seconds at 94 °C; and 1 minute at 55 °C. Data were analysed by StepOne 2 (Applied Biosystems; Thermo Fisher Scientific). Analytical sensitivity was assessed with a plasmid-derived transcribed RNA including the target region (kindly provided by J. T. Stapleton, Iowa City, IA, USA) and showed a 100% detection limit corresponding to five RNA copies per reaction (approximately 132 RNA copies/mL of plasma).

Flow cytometry

Anticoagulated blood (0.5–1 mL) obtained at 1, 3, 6, 9 and 12 months after transplantation was lysed with ammonium chloride and stained with fluochrome-conjugated antibodies directed against CD3, CD4, CD8, CD56, CCR7 and CD45RA. Samples were incubated for 15 minutes in the dark, and fluorescence-activated cell sorting (FACS) analysis was performed using a Navios flow cytometer (Beckman Coulter, Brea, CA, USA). FACS data were
analysed by FlowJo software (Treestar, Ashland, OR, USA). NK cells were identified as CD3<sup>+</sup>CD56<sup>+</sup> lymphocytes and further divided in CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells. CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T-cell subpopulations were further identified on the basis of CCR7 and CD45RA expression.

**Statistical analyses**

The chi-square or Fisher’s exact tests were used for categorical variables. Mann-Whitney test or Wilcoxon matched pairs signed rank test were used for continuous variables. Log-rank test was used for survival analyses. We used multivariable regression models to adjust for confounding factors (Table 1). Cumulative incidence estimates of acute graft vs. host disease, relapse and infections were calculated by the Gray test with death from other causes as a competitive event. \( p < 0.05 \) was considered statistically significant. Statistics were performed by GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA), R 3.2.0 with the EZR graphical user interface (R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org/) and Stata/IC 13.1 (StataCorp, College Station, TX, USA).

**Results**

**NGS allo-HSCT blood virome study**

Among the 40 allo-HSCT patients screened by NGS, three (7.5%) were negative for any virus sequences. Thirty-two (80%) and 19 (47.5%) had at least one detectable DNA and RNA virus sequence, respectively. Among the 37 positive patients, the number of concomitantly detected viruses ranged from one to 5 viruses in a single patient. Fig. 1A provides the virus families identified and the number of patients concerned. Among DNA virus sequences, the most frequent were polyomaviruses (PyV) (20/40 patients, 50%), followed by anelloviruses (40%), herpesviruses (35%), human papillomaviruses (HPV) (27.5%) and adenoviruses (5%). For RNA virus sequences, the most frequent were HPgV (35%), followed by hepatitis C virus (5%) and Rubella virus (2.5%).

Fig. 1B shows the specific virus sequences identified for each patient according to their respective number of reads. The anelloviruses were mostly Torque Teno (TT) virus (15/21); the other cases were TT Midi Virus and TT Mini Virus. Five patients had more than one species detected. Among herpesviruses, cytomegalovirus (CMV) was the most frequent (10/17), followed by Epstein-Barr virus (EBV), herpes simplex (HSV) 1, human herpesvirus (HHV) 6b and HHV-7. Two different types of herpesviruses were simultaneously detected in three patients. The PyV included Merkel cell (MCPyV) (18/24), BK virus, PyV-6 and JC virus. Four patients were positive for two different PyV. HPV matching to the 107 genotype was predominant among HPV (6/11), followed by genotypes 17, 4, 15 and FA75. No multiple infections were identified for HPV.

There was no differences in the blood virome composition between patients receiving non- and myeloablative conditioning (Mann-Whitney test, data not shown).

**Confirmatory testing by PCR**

Our r(RT)-PCR-specific assays for HPgV, hepatitis C virus, adenovirus, BK, JC PyV, CMV, EBV and HHV-6 confirmed all cases except two EBV, one BK PyV and one adenovirus. Oppositely, three CMV and one EBV, with relatively low virus loads (<3.2 \( \times \) 10E3 copies/mL) were detected by rPCR but missed by NGS. One BK virus was filtered by NGS as a contaminant although then confirmed by rPCR. The HSV-1 and the rubella cases were not confirmed by r(RT)-PCR. We did not screen the HHV-7, the anelloviruses nor the HPV by rPCR. Supplementary Fig. S2 provides the HPgV-positive samples phylogenetic tree.

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Given the high frequency of HPgV detection, the lack of knowledge on its infection kinetics and its potential immunomodulatory role, we performed an HPgV-specific rRT-PCR screening on 122 consecutive allo-HSCT patients with a mean follow-up of 339 days after transplantation (Fig. 2). Overall, 51 patients (41.8%) were positive for HPgV at least once during the follow-up period. 32% of patients were positive before transplantation, of whom all except one patient remained positive until the last time point (days 180–365). In seven of 51 positive patients, the infection appeared after transplantation; we screened the donor serum from 6 of these patients: one donor was positive for HPgV, whose recipient became viraemic at the time of transplantation. The median virus load of positive cases ranged between $1.7 \times 10^4$ and $5.2 \times 10^5$ copies/mL of plasma (Fig. 2).

**HPgV investigation**

We next assessed the association between HPgV infection and transplantation outcomes. Table 1 presents the patients' characteristics. HPgV$^+$ group included higher proportions of patients with myeloid malignancies (85% vs 68%; $p = 0.0072$) and lower proportions of patients with lymphoid malignancies (13% vs 27%; $p = 0.0208$). Accordingly, higher proportions of HPgV$^+$ patients received a myeloablative conditioning. No difference was observed in the time to neutrophil engraftment (Fig. 3A), in overall survival, progression-free survival or graft vs. host disease, GRFS, graft vs. host disease-free, relapse-free survival (Fig. 3B), nor in cumulative incidences of grades II–IV acute graft vs. host disease (Fig. 3A), disease relapse (Fig. 3D) or infections (Supplementary Fig. S3 and Supplementary Table S2 for microbial agents identified) between HPgV$^+$ and HPgV$^-$ patients.
HPgV association with cellular and humoral immunity

We compared cellular immune reconstitution in HPgV+ and HPgV− patients over 1 year after transplantation. At 1 and 12 months after transplantation, we detected significantly lower numbers of CD4 and CD8 and higher NK cell counts in HPgV+ patients, respectively (Fig. 4A). However, multivariable linear regression analysis, taking into account clinical variables differing between both groups at the studied time points (i.e. sex and disease status at 1 month and sex, donor type and T-cell depletion at 12 months), did not support these differences. Similar analyses on cell subsets distributions among T and NK cells identified lower proportions of CD8 TEMRA T cells in HPgV+ patients at 12 months, although this difference was not confirmed after adjustment (Fig. 4B).

Our analyses also failed to detect any significant impact of intravenous immunoglobulin administration on HPgV titres (Supplementary Fig. S4).

Discussion

We reported all RNA and DNA viruses detectable by unbiased NGS after allo-HSCT. Our results reveal that so-called commensal viruses are a major component of the allo-HSCT blood virome, where HPgV was identified in more than 30% of patients.

Overall, PyV, anellovirus and pegivirus were most frequently identified. The median number of concomitant virus sequences was two per patient, with a maximum of five distinct sequences identified in four patients. According to a previous study, the DNA blood virome of solid organ transplant recipients is composed of 70%...
Anelloviridae, 13% Herpesviridae, 5% Polyomaviridae and 0 Papillomaviridae [9]. The equivalent findings in our cohort is 28%, 22%, 32% and 14%, respectively. This divergence could be partially explained by critical differences in the protocols used for genetic material extraction and bioinformatics pipelines. If comparing with the gut virome of allo-HSCT patients [5], the same persistent viruses than ours were identified (Anelloviridae, Polyomaviridae, Herpesviridae and Papillomaviridae found in 70%, 34%, 27% and 18% of patients, respectively); differences between both studies mainly rely on the viruses’ tropism (Flaviviridae is found exclusively in blood and exogenous enteric viruses in the gut).

Anelloviruses are known to cause prolonged viraemia and multiple coinfections. Two thirds of our study population was positive for at least one anellovirus, corresponding to previously reported prevalence [10]. While it has not been linked with overt organ disease, torque teno virus (TTV) has been proposed as a proxy of immunosuppression in both solid organ transplant [11] and allo-HSCT recipients [12].

MCPyV and PyV-6 are found on the skin of healthy individuals. While MCPyV has been associated with Merkel cell carcinoma, PyV-6 is currently not associated with any disease. Peripheral blood mononuclear cells (PBMC) are a reservoir for many Polyomaviridae [13]. We studied cell-free plasma samples, which could explain the relatively low number of reads in the PyV+ samples. Similarly, HPV can persist in PBMCs. and HPV DNA is generally found in 8% to 15% of healthy blood donors [14], suggesting that HPV can circulate in blood and are not necessarily skin contaminants.

The rRT-PCR assays could not confirm the HSV-1 and the rubella results. Clinical history did not indicate any HSV infection and the Rubella virus—positive patient was serologically positive before transplantation, as for measles and mumps, suggesting previous vaccination. Although the low number of reads detected in these cases suggests nonspecific sequences, HSV viraemia can occur in up to 26% of critically ill patients [15], including immunocompromised patients without organ disease [16]. Moreover, the cumulative burden of double-stranded DNA viruses detected in plasma has been associated with increased mortality in HSCT recipients [17], although they rarely caused end-organ disease and were infrequently the direct cause of death. Similarly, in children with primary immunodeficiencies, cases of chronic skin granuloma induced by persistent rubella vaccine strains exist [18]. Thus, communal viruses may either have an indirect role in pathology or cause unexpected clinical syndrome.

HPgV viraemia is found in 1% to 5% of healthy blood donors in developed countries [6]. Virus load can reach 10^7 copies/mL, and transmission through blood transfusion is well described [19]. HPgV primarily infects haematopoietic stem cells [20] but can also be found in T, B and NK cells and in monocytes [6]. The HPgV genome can persist in serum microvesicles and be further transmitted to PBMCs in vitro, leading to active replication and production of infectious virus particles [8]. The HPgV prevalence we found is similar to that in other studies [21–26] (Supplementary Table S3), but our investigation could suggest an HPgV transmission by the donor in one patient, although a pretransplantation-undetectable but positive low virus load is not excluded, and firm evidence are lacking in the absence of sequencing. Previous studies have demonstrated that HPgV can persist for up to 9 years [26]. We provided detailed quantitative viraemia kinetics definitely proving that HPgV is a persistent infection. Although there is no known HPgV-associated organ disease, HPgV has an immunomodulatory effect in HIV patients [6]. We assessed the potential impact of HPgV on T-cell reconstitution. We identified a difference in the total CD4 and CD8 cell count at 1 month and the rate of CD8 TEMRA at 12 months between HPgV-positive and -negative cases, which could be comparable to what is seen in HIV patients [27]. The effect did not persist after multivariable analyses, and our results thus cannot identify any effect of HPgV on cell reconstitution; nevertheless, many confounders can attenuate the potential effect of HPgV, as this was the case in HPgV-infected HIV patients who experienced immune reconstitution after highly active antiretroviral therapy [28]. Similarly, our results did not uncover an association between HPgV infection and allo-HSCT outcomes, but we found a significant difference in the primary disease between HPgV+ and HPgV− patients, potentially suggesting an association between HPgV and some haematologic malignancies, similar to what we recently reported for TT virus [12].

Our virome NGS study focused on a single time point after transplantation without comparison to the virome before transplantation, and we focused our investigation on HPgV because of its immunomodulatory properties, which of interest in the context of allo-HSCT. Yet Legoff et al. [5] have recently demonstrated an increase in the proportion of the eukaryotic viruses in the gut virome after allo-HSCT; in addition, picobirnavirus infection was specifically predictive of acute graft vs. host disease, reinforcing the need to investigate the role of so-called orphan disease viruses in noninfectious complications.

In conclusion, our study highlights that most of allo-HSCT recipients have at least one virus circulating in their blood after transplantation. Our observations that HPgV is predominant and persistent in the allo-HSCT blood virome should promote further research into the potential impact that communal viruses can have on immunosuppression.

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Transparency declaration

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cmi.2018.05.004.

References

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