Supplemental information for:

Temporal Response of the Human Virome to Immunosuppression and Antiviral Therapy

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Extended experimental procedures:

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This analysis represents a substudy of a prospective cohort study funded by the National Institutes of Health (RC4 AI092673) to study the clinical utility of a donor-derived cell-free DNA assay for the diagnosis of acute and chronic rejection and allograft failure after thoracic organ transplantation. Patients were enrolled if they received a heart or lung transplant at Stanford University Hospital (SUH) or Lucile Packard Children's Hospital (LPCH), and were excluded if they were recipients of multi-organ transplants or if they were followed at centers other than SUH or LPCH post-transplant. This study was approved by the Stanford University Institutional Review Board (protocol # 17666) and enrollment commenced in March 2010.

Details of the post transplant therapeutic protocol:

Adult heart transplant recipients

Post-transplant immunosuppression consisted of methylprednisolone 500 mg administered immediately post-operatively followed by 125 mg every 8 hours for three doses. Anti-thymocyte globulin (rATG) 1 mg/kg was administered on post-operative days 1, 2, and 3. Maintenance immunosuppression consisted of prednisone 20 mg twice daily starting on post-operative day 1 and tapered to <0.1 mg/kg/day by the 6^{th} post-operative month and tapered further if endomyocardial biopsies showed no evidence of cellular rejection. Tacrolimus was started on post-operative day 1 and dosing was further adjusted to maintain a level of 10-15 ng/ml during months 0-6, 7-10 ng/ml during months 6-12, and 5-10 ng/ml thereafter. Mycophenolatemofetil was started at 1,000 mg twice daily on post-operative day 1 and dose adjustments were made, if required, in response to leukopenia. All patients received standard CMV (anti-viral) prophylaxis consisting of ganciclovir 5 mg/kg IV, adjusted for renal function, every 12 hours starting on post-operative day 1 unless both donor and recipient were CMV negative. When able to tolerate oral medications, recipients were started on valganciclovir 900 mg twice daily for 2 weeks, then 900 mg daily until 6 months post-transplant, followed by 450 mg daily until 12 months post-transplant, at which point anti-viral prophylaxis was discontinued. Valganciclovir dose reductions were made in the setting of leukopenia. CMV-recipients of a CMV+ allograft also received CMV hyperimmune globulin, 150 mg/kg IV, within 72 hours of transplant, 100 mg/kg at post-transplant weeks 2, 4, 6, and 8, and 50 mg/kg at weeks 12 and 16 post-transplant.

CMV- recipients of CMV- allografts were not treated with anti-viral prophylaxis until May 2012; subsequently, these recipients were treated with acyclovir 400 mg twice daily for one year. Anti-fungal prophylaxis consisted of itraconazole 300 mg daily for the first 3 months post-transplant, and prophylaxis against pneumocystis jiroveci infection consisted of trimethoprim/sulfamethoxazole, 80 mg TMP component daily. Prophylaxis against pneumnocystis infection was continued indefinitely, and patients intolerant of TMP-SMX were treated with atovaquone, dapsone, or inhaled pentamidine.

All heart transplant recipients were monitored for acute cellular rejection by surveillance endomyocardial biopsies performed at scheduled intervals after transplant: weekly during the first month, biweekly until the 3rd month, monthly until the 6th month, and then at months 9, 12, 16, 20, and 24. Biopsies were graded according to the ISHLT 2004 revised grading scale (0, 1R, 2R, 3R) (29). Blood samples were collected from heart transplant recipients at the following time points post-transplant: weeks 2, 4, and 6; months 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 20, and 24. A subset of heart transplant recipients also had blood samples collected on post-transplant day 1. If blood sampling and endomyocardial biopsies were performed on the same day, care was taken to ensure that blood was collected prior to the biopsy procedure.

Pediatric heart transplant recipients

Induction immunosuppression initially consisted of daclizumab 1 mg/kg IV every 2 weeks for a total of 5 doses, and was switched to basiliximab 10-20 mg IV on post-operative days 0 and 4 beginning in August 2011. Recipients were also treated immediately with pulse methylprednisolone 10 mg/kg IV every 8 hours for 3 doses, followed by prednisone 0.5 mg/kg twice daily for the first 14 days post-transplant; corticosteroids were subsequently tapered off during the first post-transplant year, in the absence of acute rejection. Calcineurin inhibition consisted primarily of cyclosporine, with goal levels of 300-350 ng/ml for months 0-3, 275-325 ng/ml for months 4-6, 250-300 ng/ml months 7-12, and 200-250 after month 12 post-transplant. Patients intolerant of cyclosporine were treated with tacrolimus. Protocols for prophylaxis against opportunistic infections and surveillance endomyocardial biopsies were similar to adult heart transplant recipients.

Lung transplant recipients

Post-transplant immunosuppression consisted of methylprednisolone 500-1000 mg administered immediately post-operatively followed by 0.5 mg/kg IV twice daily. Basiliximab, 20 mg IV on days 0 and 4, was given for induction immunosuppression. Maintenance immunosuppression consisted of methylprednisolone 0.5 mg/kg IV twice daily on post-operative days 0-3, followed by prednisone 0.5 mg/kg daily until day 30, and subsequently tapered every 2-3 months to 0.1 mg/kg daily during months 6-12 post-transplant. Tacrolimus was started on post-operative day 0 and dosing was adjusted to maintain a level of 12-15 ng/ml during months 0-6, 10-15 ng/ml during months 6-12, and 5-10 ng/ml thereafter. Mycophenolatemofetil was initiated at 500 mg twice daily on post-operative day 0 and dose adjustments were made, if required, in response to leukopenia. Antiviral, antifungal, and PCP prophylaxis were similar to the adult heart transplant cohort.

All lung transplant recipients were monitored for acute cellular rejection by protocol transbronchial biopsies performed at months 1.5, 3, 6, 12, 18, and 24 post-transplant. Biopsies were also performed if indicated for clinical reasons, based on symptoms or pulmonary function test results. Blood samples were collected from lung transplant recipients for study purposes at the following intervals: thrice on day 1, twice on day 2, and once on day 3 post-transplant, followed by weeks 1 and 2, and months 1.5, 2, 3, 4.5, 6, 9, 12, 18, and 24. Blood samples were drawn prior to performance of per-protocol and clinically-indicated biopsies.

Workflow for the identification of pathogen-derived sequences

Exact duplicates were removed using the C-based utility fastq.cpp. Low quality reads were removed using the quality filter that is part of the fastx package (fastq_quality_filter -Q33 -q21 - p50). The remaining reads were subsequently aligned using BWA to the human reference genome build hg19 (bwaaln - q25). Unmapped reads were collected using samtools (samtools view -f4) and low complexity reads were removed using Seqclean (seqclean -l 40 -c 1, see https://sourceforge.net/projects/seqclean/). Reads were subsequently aligned to a selection of viral, baterial and fungal reference genomes (ncbi_viruses/all.fna.tar.gz, ncbi_bacteria/all.fna.tar.gz and all references in ncbi_fungi downloaded from ftp://ftp.ncbi.nih.gov/genomes on June 14, 2012). Fig. S1A shows the distribution of the genome sizes. The following parameters were used for the BLAST alignment: reward = 1, penalty = -3, word_size = 12, gapopen = 5, gapextend = 2, e-value = 10^{-4} ,perc_identity = 90, culling_limit = 2. Blast hits with alignment length shorter than 45 were removed. For a subset of samples longer reads were available (2x100bp, n =

55). To test the robustness of the genomic abundance estimates, the length dependence of the composition measurement was examined. Here, reads were trimmed to 40, 50, 65, 80 and 100 bp lengths (fastx_trimmer) and analyzed using the above-described workflow. Here the blast hits with alignment lengths shorter than 37, 45, 59, 72, and 80 bp were removed for the 40, 50, 65, 80 and 100 bp reads respectively.

Genome abundance estimation

Relative genome abundance estimation was calculated with GRAMMy(*18*). This tool utilizes the BLAST-derived nucleic acid sequence-similarity data to perform a maximum likelihood estimation of the relative abundance of species in the sample. GRAMMy filters hits by BLAST alignment metrics (E-score, alignment length and identity rate) and accounts for the target genome size and the ambiguity of read assignments in assessing the relative abundance of the candidate reference genomes. Grammy was called using following parameters: python grammy_rdt.py; python grammy_pre.py -q "40,40,1" input set; python grammy_em.py -b 5 -t .0001 -n 100 input.mtx; grammy_post.py input.est setinput.btp. Custom scripts (available at https://sourceforge.net/projects/viromedynamics) were used to combine the strain level abundance estimates to obtain the abundance at higher taxonomic level abundances. Here, a minimal taxonomy for the reference database was built using Taxtastic (available at http://github.com/fhcrc/taxtastic).

Quantification absolute viral load

To quantify the load of infectious agents in the samples the blast hit results were collected and the best hits selected for each read using a custom script (Bioperl). Figure S1B shows the distribution of the number of unique viral, bacterial and fungal blast hits per million unique molecules sequenced. Figure S1C shows the number of viral, bacterial and fungal genome copies relative to the number of human genome copies present in the sample. The coverage of the genome of the infectious agent was normalized with respect to the human genome coverage.

qPCR validation of sequencing results for selected viral targets

Standard qPCR kits for the quantification of Human Herpes Virus 4, 5 and 6 and parvovirus (PrimerDesign™, genesig) were used to validate the sequencing results for a subset of cell free DNA samples. qPCR

assays were run on cfDNA extracted from ~1 ml of plasma and eluted in a 100 µl Tris buffer (50 mM pH 8.1-8.2). The plasma extraction and PCR experiments were performed in different facilities. No-template controls were ran to verify that the PCR reagents were included in every experiment. Figure S1D compares the relative number of blast hits per million reads acquired to the concentration of viral genome copies as determined using qPCR.

No-template control

A no-template control experiment was performed. A sequencing library was generated from nuclease-free water (S01001, Nugen). The library was prepared together with 7 additional sample libraries (cell-free human DNA) to test for possible sample-to-sample crosstalk during library preparation. To ensure formation of clusters with sufficient density on the Illumina flow cell, the sample was sequenced together with a sample unrelated to the study. Whereas the sample unrelated to the study recruited 16 million reads, the no-template control library generated just 15 reads that mapped to two species in the reference database, the methanocalcodoccus janaschii (9 hits) and Bacillus subtillis (5 hits) genomes. No evidence was found for human related sequences, indicating that sample-to-sample contamination was low.

Figure S1 related to Figure 1.Genome sizes and hit statistics, qPCR assay and influence of read length on measured relative abundance of species at different levels of taxonomic classification A. Distribution of genome sizes in the reference database with, 1401 viral genomes, 32 fungal genomes and 1980 bacterial genomes. **B.**Distribution of unique blast hits per million unique molecules sequenced (average number of hits specified in the x-axis label). **C.**Distribution of genome equivalents (infectious agents/diploid human) for viruses, bacteria and fungi (average number of genome equivalents specified in the x-axis label). **D.**Comparison of sequencing hits found per million of total reads sequenced to the number of viral copies detected using qPCR.For the qPCR assays, DNA was purified from ~ 1ml of plasma and eluted in a 100 µl volume.**E.**Measurements of CMV and parvovirus burdens for selected cases. The highest loads of CMV virus (genome equivalents, viral/human diploid, G.E.) measured for all samples corresponded to two cases of clinically diagnosed disseminated CMV infection (panels a and b, shaded area denotes time-window of clinical diagnosis, * denotes time of death). Panel c shows a time trace of a pediatric patient that suffered from CMV viremia.. Parvovirus was detected in one pediatric heart transplant patient

immediately post-transplant (panel d). **F**. Influence of read length on measured relative abundance of species at different levels of taxonomic classification (n =52). Spearman sample-to-sample correlation, r, and p value, p, (two-sample Mann-Whitney U test) for the abundance of the most abundant node extracted from the 50 and 100 bp datasets: r = 0.80, p = 0.8 (panel a), r = 0.86, p= 0.4 (panel b), r = 0.92, p = 0.6 (panel c), r = 0.84, p = 0.5 (panel d), r = 0.7, p =0.28 (panel e), r = 0.99, p = 1 (panel f).

Figure S2 related to Figure 2. Average drug doses and measured levels for adult heart and lung transplant patients after transplantation and influence of drug dosage on viromecomposition.A-C. Average dose of valganciclovir and prednisone (A and C) administered and measured level of tacrolimus in blood (B) for the adult heart and lung transplant patients part of this study. **D.** Compared to the viral component, the composition of the bacterial component of the microbiome is relatively insensitive to antivirals and immunosuppressants. **E.**Virome composition as function of dose of anti-CMV drug (valganciclovir) and immunosuppressant (Prednisone).

Figure S3 related to Figure 3. Temporal dynamics of the bacterial component of the microbiome post-transplant. A. Relative abundance of bacterial phyla as function of time. **B.** Relative abundance of bacterial genera as function of time.

Figure S4 related to Figure 4. Virome composition and total viral burden for different patient classes. Viral load and composition for CMV positive adult heart (**A**), adult lung (**B**), and pediatric heart (**B**) transplant recipients, treated with bothimmunosuppressants and antivirals.