Cell-free RNA reveals host and microbial correlates of broadly neutralizing antibody development against HIV

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Abstract:

An effective broadly reactive antibody response is crucial to prevent viral infections. A small number of people living with HIV (PLWH) develop broadly neutralizing antibodies (bNAbs) targeting multiple HIV strains. Although several viral and immune factors contribute to bNAb development, the genetic and environmental factors driving this response remain largely unknown. We performed combined cell-free DNA (cfDNA) and cell-free RNA (cfRNA) sequencing in 42 plasma samples from a longitudinal cohort of 14 PLWH (7 who develop bNAbs and 7 matched controls). This approach enabled us to non-invasively monitor the host transcriptome, viral genetic variation, and microbiome composition during HIV infection, and to identify molecular correlates of bNAb development. We find that development of bNAbs is associated with a transcriptomic signature of early immune activation that involves MHC class I antigen presentation. This signature is independent of viral load or CD4 count and declines over time. In addition to host features, we recovered sufficient viral reads to reconstruct HIV consensus sequences, supporting the utility of cfRNA for viral genotyping. Finally, we also identified an enrichment of several commensal microbiota in bNAb producers

and increased coinfection with GB virus C. Our findings suggest a distinct early immune activation profile in PLWH who develop bNAbs. More broadly, we show that combined cfDNA/cfRNA sequencing can reveal relationships between a protective immunogenic response to HIV infection, the host immune system, and microbiome, highlighting its potential for biomarker discovery in future vaccine and therapeutic studies.

Introduction

HIV has infected over 36 million people worldwide, reaching epidemic proportions in Eastern and Southern Africa with an estimated adult prevalence close to 7% (1). Antiretroviral therapy (ART) has greatly improved outcomes, allowing HIV to be a manageable condition throughout life. However, global access to ART is difficult to achieve and also requires lifelong daily adherence to a pill taking regime. Moreover, despite effective treatment, transmission of HIV remains common in vulnerable populations, and people living with HIV (PLWH) are at increased risk of aging-related and chronic diseases (2).

Few PLWH develop broadly neutralizing antibodies (bNAbs), which can neutralize multiple strains of the virus (3), and when administered passively can prevent infection and suppress replication (4). There is great interest in understanding the drivers of bNAbs, as this could inform the development of an effective vaccine. Efforts to design an effective HIV vaccine have increasingly focused on strategies to induce bNAbs by guiding B-cell affinity maturation, with studies demonstrating promising immunization approaches through the rational design of immunogens (5–7). Despite these advances, which specific cellular processes give rise to bNAbs during infection remains incompletely described. So far, there is evidence that viral and host genetic factors might play a role (8–12), and that a combination of high viral titres and reduced CD4 counts early in infection is associated with the development of bNAbs in some individuals (13,14). Additionally, changes in frequency of certain immune subpopulations, such as T follicular helper cells (Tfh) might also influence affinity maturation towards bNAb production (15–19). Moreover, there is some evidence that bNAbs might arise from pre-existing memory B cells initially stimulated by bacterial antigens -likely from the gut microbiome- which later cross-react with the HIV glycan shield and develop neutralizing capabilities (20,21). Although microbial antigen cross-reactivity has also been shown to lead to non-protective immune responses in HIV vaccine trials (22), a better understanding of the interplay between the host immune response, microbiome and a protective bNAb response could aid in vaccine development.

Here we perform combined cell-free DNA (cfDNA) and cell-free RNA (cfRNA) sequencing in a longitudinal cohort of 14 PLWH from South Africa who either

developed or did not develop bNAbs (7 individuals per group). For every donor, we obtained a blood sample approximately 6 months, 1 year and 3 years after HIV acquisition and prior to administration of ART (42 samples in total) as well as measurements of their viral load and CD4+ T cell counts. Both groups had comparable CD4 counts and viral loads, with no significant differences at 6 months after HIV acquisition (first time point). The extent of bNAb development was assessed by measuring plasma neutralization breadth against a panel of 18 HIV strains representing multiple subtypes.

Sequencing of cell-free nucleic acids permits simultaneous measurement of the host genome and transcriptome and the microbiome while sampling from virtually every tissue in the body (23–26). We show that such measurements allow us to profile both molecular alterations in the immune response and microbiome of the participants during HIV infection. The signatures observed from circulating nucleic acids are combined with percentage breadth, which is a continuous measure of bNAb development. This enables us to investigate factors associated with HIV neutralization by both stratifying individuals into bNAb developers and non-developers and by assessing factors associated with the development of breadth over time. We report a set of HIV and immune associated genes that correlate with the amount of viral cell-free RNA. We also identify a distinct innate immunity signature in the transcriptome of bNAb-producing individuals that precedes the development of breadth, as well as viral and microbial taxa whose presence is associated with the production of bNAbs.

Results

HIV load in cfRNA correlates with known disease-associated genes

To investigate whether circulating nucleic acids can be used to noninvasively monitor longitudinal phenotypic changes during HIV infection, we performed cfDNA and cfRNA sequencing in a subset of samples from the CAPRISA studies (Figure 1A, Figure S1). After quality control and filtering, we obtained enough reads to sample the host transcriptome (80% of total reads for cfRNA) and microbiome (0.2% and 20% reads of cfDNA and cfRNA respectively) (Figure S2). We detected HIV in the cfRNA assay for all samples, with coverage sufficient to genotype the majority strain in 38 out of 42 samples (Figure S3A-C). For high coverage samples, the nucleotide identity of HIV clustered within individuals (Figure 1B) except for one mislabeled sample that was excluded from further analyses (Methods). We validated the accuracy of cfRNA-derived consensus sequences by comparing them to previously published single-genome amplification (SGA) sequences from the same CAPRISA participants (Table S1), observing consistent clustering and strain concordance across time points and sequencing methods (Figure S3D). By comparing differences in consensus sequences within individuals (Figure S3E), we found that most mutations occur in the *env* gene, which encodes the surface protein responsible for host cell binding. The Env protein is the sole target of bNAbs and is known to be the most rapidly evolving

part of the HIV genome (27–29). Using cfRNA we estimated that the dominant viral strain in each individual accumulates approximately 10 mutations per year (Figure S3F), in line with other estimates from virion or PBMC-derived studies (30,31). Altogether, these results show that cfRNA is a quantitative tool to perform genotype analysis of viral pathogens and to track strain-specific mutational dynamics over time.

To characterize host responses to HIV infection, we measured the correlation between the host transcriptome and the total abundance of HIV-derived reads in plasma cfRNA (cfHIV), as well as with clinical parameters such as viral load and CD4 counts. Spearman correlation of cfHIV, viral load and CD4 counts to the human transcriptome revealed several genes significantly associated with these measurements (Figure 1D, Figure S4A-B). Analysis of genes significantly correlated to cfHIV revealed 19 known HIV-associated genes whereas only 1 for each of viral load and CD4 count (Figure 1E). CD4 count levels showed a significant association to *IL4*, a cytokine inducing differentiation of naive T helper cells. Although the relation between cytokine profile and HIV progression is controversial, increased secretion of IL4 in relation to IFN-gamma is a known hallmark of switching from Th1 to Th2 lymphokine secretion in PLWH (32). Our observation of IL4 being upregulated in low CD4 samples is compatible with an increase of IL-4 levels upon disease progression and T helper cell decrease. The most correlated HIV-associated genes to cfHIV include: *CCL5*, also known as RANTES, an HIV suppression factor released by $CD8^+$ T cells, *TFRC* (CD71), a marker of T-cell activation, and *CCL2*, a chemokine that attracts immune cells to sites of infection (Figure 1F, Figure S4C). These genes do not show a differential behaviour between donors who develop bNAbs and those who did not, indicating that they represent shared responses to HIV infection. This shows that simultaneous cfRNA sequencing of the host and viral RNA is a useful tool to identify genetic covariates of HIV infection progression.

Development of bNAbs is associated with a transcriptional phenotype of immune activation

We then investigated if there are significant differences between the transcriptome of donors who develop bNAbs and those who do not. We performed differential expression analysis finding 218 genes upregulated in donors who developed bNAbs (FDR < 0.05, fold-change > 2, Figure 2A). This variation could not be attributed to environmental factors such as site of collection (urban vs rural), that showed little to no correlation to these transcriptomic signatures. Remarkably, of the 218 genes upregulated in bNAb producers, 44% (n=96) are related to the immune system. In particular, 25 of these genes are associated with production of RANTES (CCL5), a chemokine involved in homing and migration of effector and memory T cells. Although CCL5 itself is not enriched in bNAb developers in our dataset, elevated RANTES, along with other inflammatory markers, have previously been linked to the development of bNAbs in spontaneous controllers of HIV (33).

A pathway analysis confirmed these results and further revealed an enrichment of molecular functions related to an immunologic response in bNAb producers (Figure S5A). Terms such as receptor activity, IgG and peptide antigen binding, cytokine signaling and NF-kB signaling were significantly enriched in bNAb positive samples. Analyses of the genes down-regulated in the bNAb producers showed lower enrichment scores and did not point to the existence of other host immunoregulatory effects (Figure S5B). Overall, host cfRNA sequencing provides evidence for an increased immune activation in donors who developed bNAbs, despite having similar CD4 counts and viral loads to donors who did not.

To investigate this immune activation in more detail, we performed pseudotime analysis on the two groups, using the number of broadly neutralizing antibodies to define the temporal progression (Figure 2B). We found that the immunologic gene signature is elevated only early in infection for those who go on to produce bNAbs, and later converges to equivalent levels for both groups as breadth develops. This aligns with previous studies showing that factors such as CD4 levels inversely correlate with bNAb development only early in infection (34). However, in our study, both groups had similar CD4 counts at the first time point. Focusing on the genes significantly correlated with breadth in both groups (Figure S5C-D) revealed almost the entirety of the MHC class I antigen presentation pathway was anticorrelated with the development of breadth (i.e. time) and sometimes differentially expressed or correlated with the level of cfHIV measured (Figure 2C). Hence, MHC class I antigen genes are highly expressed early in infection for donors who go on to develop bNAbs and its expression decreases as breadth develops, becoming comparable to that of donors who do not develop bNAbs. MHC class I antigen presentation is present in almost all cells of the body, presents antigens to be killed by cytotoxic T cells (CD8) but is not generally associated with antigen presenting cells or the production of antibodies by B cells (34). Taken together, these results indicate that early immune activation associated with MHC class I antigen presentation, independent of viral load or CD4 count, is a relevant factor in the development of bNAbs.

Microbiome associated with bNAbs and the development of breadth

Development of bNAbs has been hypothesized to be triggered by antigen cross-reactivity between HIV and bacterial components present in the gut microbiome (20,21,35,36). To test whether a differentiated microbiota could be observed in participants who developed bNAbs, we mapped all non-human reads (both cfDNA and cfRNA) to a database of bacterial and viral genomes. After discarding reads that mapped to potential reagent contaminants (see Methods), we identified 249 taxa in cfDNA and 4366 taxa in cfRNA that could be confidently attributed to the donor microbiome. We combined data from all donors to assemble a phylogenetic tree of the human microbiome during HIV infection as measured from circulating nucleic acids (Figure S6). This accounted for ~70% of non-human reads in both cfDNA and cfRNA, and spanned several areas of the tree of life including archaea, bacteria and both DNA

and RNA viruses. Among other features, we identified the presence of several DNA and RNA viruses such as Anelloviruses, Flaviviruses, Herpesviruses indicative of concomitant viral infections for several patients. In cfRNA, we found that ribosomal reads encompass 37% of the nonhost microbiome reads, and we also identified a moderate fraction of reads (~20%) that could not be attributed to specific species and mapped to uncultured bacteria.

We then compared the microbiome of both groups (bNAbs/few bNAbs) finding 22 genera that were significantly associated with each group (Figure 3B, Figure S7). Among genera enriched in the high bNAb group, we identified several known opportunistic pathogens that colonize the gut and intestinal tract (e.g. Enterobacter, Leptotrichia, Serratia). By performing a temporal analysis we found that the detection of these pathogens does not show significant differences across timepoints and does not precede development of breadth (Figure S7). Overall, and despite the relatively small sample size of this study, our data shows the existence of differences in the microbiota compositions in patients who develop bNAbs from those who do not.

Additionally, metagenomic analysis of cell-free RNA revealed the presence of GB virus C (previously known as Hepatitis G) in several participants (Fig. 3A). GB virus C is a flavivirus that infects lymphocytes, has no known pathology, and has been associated with significantly improved survival in people with HIV infection (37,38). In our study, all of the individuals who produced high levels of bNAbs had non-zero levels of GB virus C ($p < 0.05$, one-sided Fisher exact test), frequently at very high levels (for some donors we found up to 100-fold higher counts of GB virus C than HIV itself) (Figure 3A). Differential gene expression analysis on samples with high levels of GB virus C (defined as >1/2000 nonhost reads) revealed 42 host genes that are also overexpressed in these donors. Among top candidates we identified several genes of the MHC class II antigen presentation complex (*HLA-DQA1*, *HLA-DQB1*, *HLA-DRB1*), an HIV suppressive factor involved in inflammatory signaling (*CCL3*), several proinflammatory cytokines (*IL6*, *EBI3*), and immunoglobulin receptors (*KIR3DL2*). Notably, increased expression of inhibitory KIRs, including KIR3DL2, has been associated with bNAb development, suggesting a potential link between natural killer cell maturation and the regulation of antibody production (39). These findings suggest that high levels of GB virus C might be associated with an increased activity of antigen presenting cells.

Discussion

In this work, we demonstrate simultaneous monitoring of viral infection and donor response through sequencing of circulating nucleic acids (DNA and RNA) in plasma samples of PLWH prior to antiretroviral therapy. We show that cfRNA can be used to monitor circulating HIV viral strains in these individuals. Together with viral dynamics,

the host transcriptome can also be captured through longitudinal cfRNA measurements during infection. We find that overall cfHIV levels correlate with host transcriptomic signatures of HIV infection, and also detect changes in multiple immune-related genes that we attribute to the host immune response to infection. Specifically, by comparing the response of participants who develop bNAbs to those who did not, we find that the development of bNAbs is associated with a transcriptomic signature suggestive of an enhanced immune activation early in infection. This response is independent of viral load and CD4 counts, and is enriched in genes involved in immune activation, including RANTES production and MHC class I antigen presentation.

Previous studies have highlighted the central role of lymph nodes in shaping humoral responses during HIV infection, yet their direct study remains challenging (19,40,41). Cell-free RNA may provide a non-invasive window into immune activity within these tissues, where Tfh-B cell interactions drive affinity maturation. Hence, it is plausible that the immune-related transcripts detected in plasma cfRNA that we report here, primarily originate from immune activity in lymph nodes. However, we cannot rule out the possibility that increased cfRNA levels are also partly driven by a higher number of circulating immune cells.

Finally, by measuring the microbiome of both groups, we identify a reduced set of species whose presence or absence in the microbiome is associated with the development of bNAbs. In particular, we found that donors who develop bNAbs show increased levels of GB virus C which is in turn correlated to increased expression of several proinflammatory cytokines and host adaptive immune signatures. The results presented here represent a small pilot dataset that needs to be validated in larger studies. An expanded cohort with finer time-resolution would also allow us to investigate if the development of bNAbs is influenced by the dynamics of the patient microbiota. Finally, although our work does not imply a causal relationship between the observed molecular alterations and the development of bNAbs, we provide experimental evidence of a link between an increased immune response, a particular set of microbial and viral species and the development of bNAbs.

Materials and Methods

Participants

The study utilized biobanked plasma specimen of PLWH with known neutralization status enrolled in the CAPRISA cohorts. The CAPRISA 002 Acute Infection cohort was established in 2004 and has been following women from the early/acute stage of infection (42). The CAPRISA 002 study protocol was reviewed and approved by the Biomedical Research Ethics Committees of the University of KwaZulu-Natal

(E013/04), the University of Cape Town (025/2004) and the University of the Witwatersrand (MM040202).

Isolation of cfDNA and cfRNA and library preparation

Blood samples were collected in sodium citrate tubes and plasma extracted according to the standard clinical centrifugation protocol of the HIV/AIDS Network Coordination, and stored at -80 C. Each plasma sample was thawed once and split into two aliquots for cfDNA/cfRNA extraction (0.4-1 mL per aliquot). cfDNA was extracted using the Qiagen Circulating Nucleic Acid kit. cfRNA was extracted using the Plasma/Serum Circulating RNA and Exosomal Purification kit (Norgen, cat 42800), followed by DNA digestion using Baseline-ZERO DNase (Epicentre) and then cleaned up using the RNA Clean and Concentrator-5 kit (Zymo) (26,43). cfDNA/cfRNA libraries were prepared using the automated Ovation SP Ultralow Library Systems (Mondrian ST) and the SMARTer Stranded Total RNA Pico Input Mammalian kit (Clontech) respectively (26,43). A negative control (1 mL PBS, Gibco) was processed in parallel for every batch to control for potential contaminants (23).

Sequencing methods

Paired end Illumina sequencing (2×75 bp) was performed on the Nextseq 500 for cfDNA and on the Novaseq 6000 for cfRNA. This resulted in a median of 69 million (cfDNA) and 109 million (cfRNA) reads per sample (Fig. S2).

Analysis methods

Analysis proceeded similarly for both cfRNA and cfDNA data: i) Low quality reads and adapters were trimmed by Trimmomatic (44) ii) overlapping read pairs were merged (45) (iii) sequences aligning to a database of known vectors and control sequences were removed based on UniVec database (iv) remaining reads were aligned to the human genome by Bowtie (46), and only non-human reads were retained, v) microbial taxonomic classification was performed in these reads using using Kraken (47).

For cfRNA, reads were aligned to the human genome using STAR (48) and expression of genes counted by HTSeq. HIV sequences were extracted from FASTA files and mapped to the HIV-1 group M consensus reference (CON-C.fa) using BWA-MEM. Consensus sequences were generated with BCFtools (49). Multiple sequence alignment was performed with MAFFT v7 (50), and a maximum-likelihood phylogenetic tree was constructed using IQ-TREE (51). The tree was visualized with Bio.Phylo (52), with metadata-based label coloring and shaded backgrounds highlighting predefined sample clusters. HIV reference sequences from the same CAPRISA participants were obtained from previously published studies and are available from the Los Alamos National Laboratory (LANL) HIV Sequence Database (Table S1). Sample identity was validated based on HLA typing and consistency of the HIV sequences across the three time-points for every donor (Fig. S3).

For both cfDNA and cfRNA, 6 negative controls were used to create a database of potential environmental taxa that were blacklisted in downstream analysis. This stringent quality control filtering preserved 72% of microbial reads in cfRNA and 17% in cfDNA. Background levels of sample cross-talk were estimated to be below <0.5% based on a set of non-template control barcodes (n=8) spiked into the cfRNA libraries. This threshold was used to define the detection limit for each taxon.

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Data availability

The datasets generated and analyzed in the study are available in the NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) and can be accessed upon request.

Figures

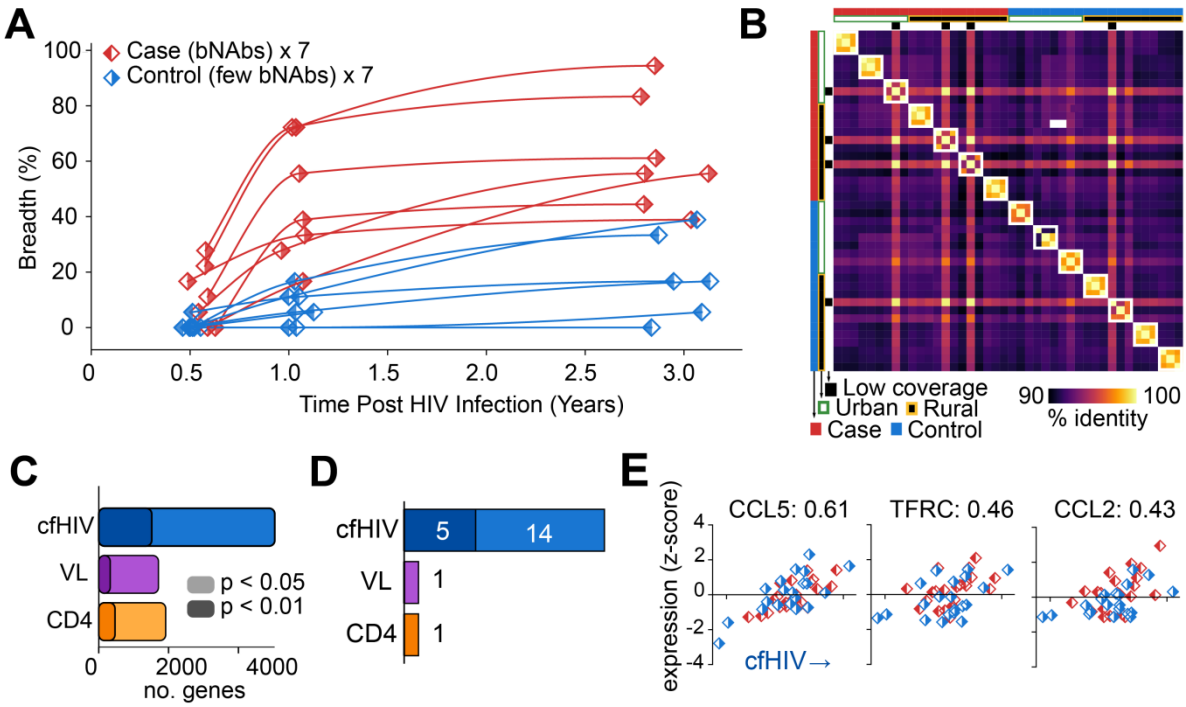

Figure 1: Production of broadly neutralizing antibodies, detection of HIV in cfRNA and correlation with viral load and host transcriptome. A) Time series comparing the neutralization breadth in participants who produce bNAbs (red, left-filled) to those who do not (blue, right-filled). Time zero corresponds to the HIV infection point, with breadth defined as the ability of plasma antibodies to neutralize a cross-clade panel of 18 env-pseudotyped viruses (Figure S1A). B) Heatmap showing the genomic similarity of consensus HIV sequences within individuals (see also Figure S3). C) Number of genes significantly correlated with cell-free HIV counts, viral load (VL) and CD4 counts (CD4). D) Number of genes known to be HIV-associated from those found in D. E) The top three known HIV-associated genes most correlated with cfHIV counts. These show no partitioning across case/control.

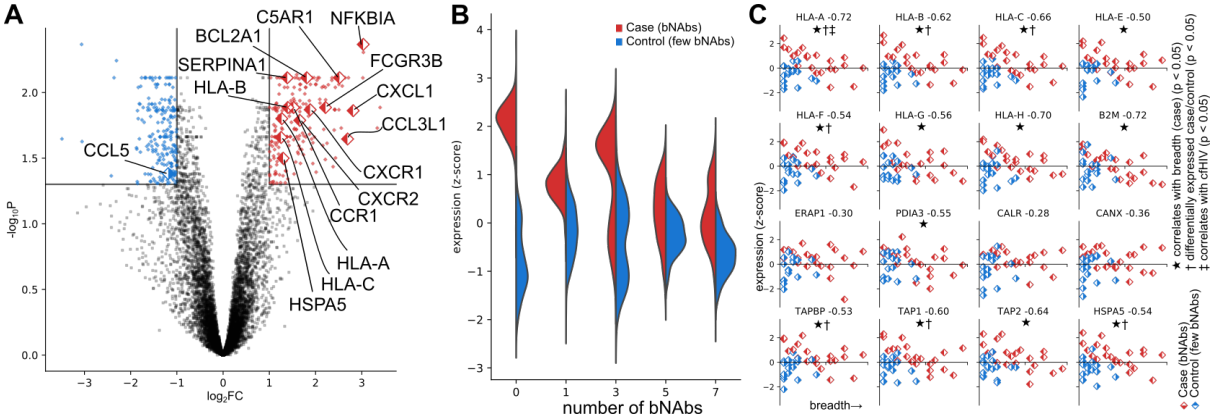

Figure 2: Gene level differences in cfRNA in participants who produce bNAbs. A) Volcano plot showing differentially expressed genes between bNAb producers and controls. Genes with a fold change 2x and greater and FDR < 0.05 are highlighted in red and blue (increased/decreased in bNAb producers respectively). Labelled points are known HIV-associated genes. B) Violin plots of the distribution of up-regulated genes in bNAbs producing patients stratified by group and number of bNAbs (i.e. breadth) at sample collection. C) Genes in the MHC class I antigen presentation pathway showing that most of them negatively correlate with breadth and that some also correlate with cfHIV levels or are differentially expressed in the two groups.

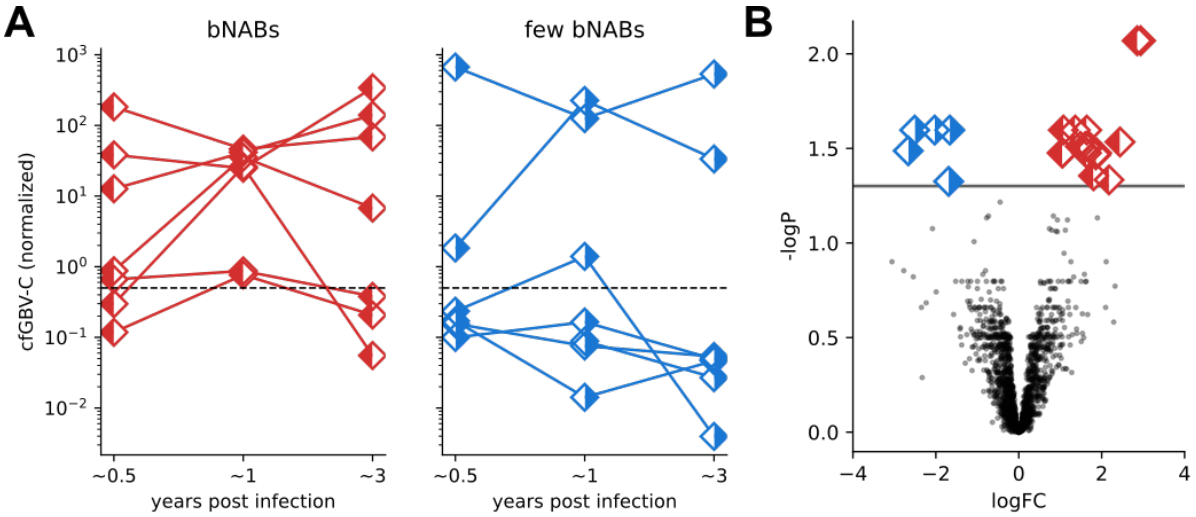

Figure 3: Microbiome differences and GBV-C enrichment in participants who produce bNAbs. A) Scatterplots showing the expression of GBV-C in samples partitioned by time and study group (left, those who produce bNAbs, right, those who produce few bNAbs). Dashed line at 1/2000 counts/1000 nonhost, indicating the division between 'high' and 'low' GBV-C levels. B) Volcano plot of the differentially abundant microbial genera (FDR < 0.05, BH method). There were 17 genera (red) that had higher abundances in those that produce bNABs, and 5 that were lower.