GB virus C infection is associated with a reduced rate of reactivation of latent HIV and protection against activation-induced T-cell death

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Background: GB virus C (GBV-C; also known as hepatitis G virus) is the most closely related human virus to HCV [1]. GBV-C infection is common and viraemia prevalence is approximately 1–3% in US blood donors [1,2]. Owing to shared routes of transmission, the prevalence of GBV-C is much higher among HIV-infected individuals (20–40%) [1,2]. GBV-C infection is not clearly associated with any disease; however, several studies found a beneficial effect of GBV-C viraemia on survival or CD4+ T-cell count, HIV viral load (VL) and delayed progression to AIDS in HIV-infected individuals as compared with those without GBV-C. Moreover, a meta-analysis supported improved survival with persistent GBV-C viraemia [3–10]. Although the mechanisms for this protective effect are not entirely clear, studies of GBV-C interactions with host T-cells have suggested several potential mechanisms by which GBV-C might alter HIV infection and disease progression [1,2].

Chronic HIV infection is associated with aberrant and persistent immune activation, which is thought to contribute to the immune dysfunction observed in HIV-infected people [11–15]. Immune activation correlates with HIV VL and in some studies is a better predictor of HIV disease progression than plasma VL [16,17]. In addition, immune activation enhances HIV replication and contributes to the depletion of uninfected bystander cells by activation-induced cell death (AICD).
Treatment with combination antiretroviral therapy (cART) suppresses HIV VL and reduces mortality among HIV-infected individuals; however, viremia usually becomes detectable within a few weeks of discontinuing cART [19]. Although T-cell activation levels are reduced by cART, they do not return to levels found in healthy HIV-negative individuals.

A major barrier to eradicating HIV from infected individuals is the presence of a stable pool of latently infected resting memory CD4+ T-cells [20]. Although individuals is the presence of a stable pool of latently viremia usually becomes detectable within a few weeks of discontinuing cART [19]. Although T-cell activation levels are reduced by cART, they do not return to levels found in healthy HIV-negative individuals.

Peripheral blood mononuclear cell isolation and stimulation

PBMCs were isolated by Ficoll-Hypaque (GE Healthcare, Upplands, Sweden) density gradient centrifugation and maintained in RPMI-1640 (GIBCO, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. PBMCs (1×10^6/ml, 2 ml/well in 12-well plates; Corning Inc., Corning, NY, USA) were cultured under three different conditions and three replicates of each culture condition were performed. In the first condition studied, PBMCs were cultured without activation stimuli or healthy donor feeder cells. In the second, PBMCs were cultured with activation. These cells had supernatant removed and replaced with fresh media weekly. Activation was accomplished by adding phytohaemagglutinin (PHA M form, 1.5%; GIBCO) and IL-2 (25 U/ml; ZeptoMetrix, Buffalo, NY, USA) to the media. For the third condition, PBMCs were activated as above, and co-cultured with PHA/IL-2-stimulated healthy, GBV-C-negative donor PBMCs (1:1 ratio). Cells were fed weekly thereafter with PHA/IL-2-activated PBMCs obtained from GBV-C non-viremic healthy donors. All cultures were maintained for 5 weeks. Culture supernatants were obtained and stored at -80ºC until they were batch tested for GBV-C replication and HIV reactivation. CD4+ and CD8+ T-cells in PHA/IL-2-stimulated cultures were analysed on day 35 for viability using trypan blue exclusion or by flow cytometry. Cells were counted using the Countess automated cell counter (Invitrogen Inc., Eugene, OR, USA). Each of three wells was counted twice and the average change from baseline (day 2) was determined.

Viral quantification

GBV-C replication was assessed by measuring GBV-C RNA in serum or culture supernatant fluids by real-time RT-PCR. Briefly, RNA was extracted from 140 µl
of serum or supernatant using QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA), following the manufacturer’s instructions. Each PCR reaction contained an 11 μl aliquot of RNA (representing 25 μl of serum or supernatant), 15 μl ABI TaqMan Master Mix (2×), 0.75 μl superscript III RT platinum Taq mix, 0.18 μl each of forward and reverse primers (50 μM, Integrated DNA Technologies, Coralville, IA, USA), 0.06 μl Taqman probe-ABI (100 μM), 0.3 μl of Rnase (40 U/μl) and 1.8 μl RNase/DNase-free H₂O. Primers included the forward primer 5’-GGC GAC CGG CCA AAA-3’ (96–110), antisense primer 5’-CTT AAG ACC CAC CTA TAG TGG CTA CC-3’ (163–188) and probe 5’-FAM-TGA CCG GGA TTT AGC ACC TAC CAA CCC T-TAMRA-3’ (131–158) [34]. Quantitative one-step real-time RT-PCR was performed using an ABI 7500 system. The running conditions were 50°C for 20 min, 95°C for 2 min, 40 cycles at 95°C for 15 s and 58°C for 1 min. A GBV-C RNA quantitation standard was prepared. GBV-C genome sequences from nucelotides 1 to 850 (from GenBank AF121950) were cloned into pCR2.1 plasmid (Invitrogen) downstream of the T7 polymerase promoter. The plasmid was linearized with Kpn1 and run-off transcripts were generated (Riboprobe, Promega Inc., Madison, WI, USA). RNA was quantified by A260/280, divided into 50 μl aliquots (concentration 1×10⁹ genome copies/ml) and stored at -80ºC. For each real-time PCR experiment, a fresh aliquot was used in serial 10-fold dilutions starting stored at -80ºC. For each real-time PCR experiment, a

A total of 49 HIV-positive individuals on cART participated in this study. All subjects had non-detectable HIV viral loads for >6 months documented in their medical record prior to enrollment in the study; HIV RNA was not detected in their blood on the day blood was sampled. Subjects with known GBV-C viraemia (16) or unknown (33) agreed to participate in the study. All 16 subjects with prior GBV-C viraemia were viraemic on the day of the study and 10 of the 33 subjects with unknown GBV-C status were viraemic (30.3%). Consistent with the demographics of our HIV/AIDS clinic, approximately 20% of the subjects were women and 86% were Caucasian (Table 1). The major mode of HIV transmission was sexual and 10% of subjects had coinfection with HCV. GBV-C RNA was detected in the serum obtained from 26 of the 49 individuals. The average GBV-C VL was 8.43×10⁷ copies/ml serum in the 26 subjects with GBV-C (median 5.45×10⁷ copies/ml). There were no significant differences in baseline demographics or clinical variables in those with GBV-C viraemia and those without GBV-C, including CD4+ T-cell percentage, CD4+ T-cell nadir or the duration of non-detectable HIV RNA prior to culture (Table 1).

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CD4+ and CD8+ T-cell analysis

Following PHA/IL-2 stimulation, CD3+CD4+ and CD3+CD8+ T-cells were analysed by multicolor flow cytometry. PBMCs were pelleted at 1,500 rpm for 5 min, washed and resuspended in 100 μl of phosphate-buffered saline. Cells were stained with three antibodies from Becton Dickinson (BD, San Jose, CA, USA) per manufacturer’s recommendation: CD3 (Pacific Blue), CD4 (APC-H7) and CD8 (FITC). Live cells were gated using the LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen), as recommended by the manufacturer. Staining was performed on ice for 1 h and cells were subsequently washed three times with phosphate-buffered saline and fixed in 2% paraformaldehyde (Polysciences). Data were acquired on a BD LSR II flow cytometer using single-stained Comp-Beads for compensation. At least 100,000 total events were collected and FlowJo program (Tree Star Inc., Ashland, OR, USA) was used for data analysis.

Statistics

Statistics were performed using either SPSS Version 19 (IBM, Armonk, NY, USA) or GraphPad software V4.0 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between two groups were carried out using two-sided Student’s t-tests and more than two groups by ANOVA. The significance of differences in HIV reactivation from PBMCs of GBV-C viraemic and non-viraemic individuals was determined using the χ² test. P-values <0.05 were considered statistically significant.

Results

Study participants

A total of 49 HIV-positive individuals on cART participated in this study. All subjects had non-detectable HIV viral loads for >6 months documented in their medical record prior to enrollment in the study; HIV RNA was not detected in their blood on the day blood was sampled. Subjects with known GBV-C viraemia (16) or unknown (33) agreed to participate in the study. All 16 subjects with prior GBV-C viraemia were viraemic on the day of the study and 10 of the 33 subjects with unknown GBV-C status were viraemic (30.3%). Consistent with the demographics of our HIV/AIDS clinic, approximately 20% of the subjects were women and 86% were Caucasian (Table 1). The major mode of HIV transmission was sexual and 10% of subjects had coinfection with HCV. GBV-C RNA was detected in the serum obtained from 26 of the 49 individuals. The average GBV-C VL was 8.43×10⁷ copies/ml serum in the 26 subjects with GBV-C (median 5.45×10⁷ copies/ml). There were no significant differences in baseline demographics or clinical variables in those with GBV-C viraemia and those without GBV-C, including CD4+ T-cell percentage, CD4+ T-cell nadir or the duration of non-detectable HIV RNA prior to culture (Table 1).

PHA/IL-2 stimulation decreases GBV-C replication in PBMCs

In a clinical study of IL-2 in HIV-infected individuals, subjects who were viraemic with GBV-C demonstrated a marked reduction in CD4+ T-cell expansion following
IL-2 infusion [30, 34]. In addition, data from a single patient suggested that GBV-C replication in PBMCs might be inhibited in vitro by PHA/IL-2 activation, although this was not systematically examined [30, 34]. Although GBV-C replicates well in vivo (8.43 × 10^7 copies/ml serum in this study), in vitro replication of GBV-C is inefficient. To confirm that GBV-C replicates in PBMCs ex vivo, and to evaluate the effect of PHA/IL-2 activation on GBV-C replication, we analysed GBV-C production in PBMCs from GBV-C viraemic subjects. GBV-C replication was detected in culture supernatants of PBMCs maintained in all three culture conditions (Figure 1A). However, GBV-C production was lower when PBMCs from GBV-C RNA positive donors were co-cultured with PHA/IL-2-stimulated healthy donor PBMCs (Figure 1A). GBV-C replication was not detected in PBMCs from 2 of the 15 viraemic subjects tested. In subjects

**Table 1. Patient characteristics**

<table>
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<th>GBV-C RNA positive (n=26)</th>
<th>GBV-C RNA negative (n=23)</th>
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<tr>
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<td>Number HCV-antibody-positive</td>
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*HIV suppression = average number of months that HIV RNA was suppressed to <48 copies/ml. P-values were all >0.05. GBV-C, GB virus C; IDU, intravenous drug use.

**Figure 1. PHA/IL-2 stimulation alters GBV-C replication and PBMC proliferation**

(A) GB virus C (GBV-C) RNA released into peripheral blood mononuclear cell (PBMC) culture supernatants was significantly greater in unstimulated cells compared with cells stimulated with phytohaemagglutinin A and interleukin 2 (PHA/IL-2) or co-cultured cells. (B) PBMCs from GBV-C-infected subjects did not proliferate following PHA/IL-2 stimulation to the extent of PBMCs from subjects without GBV-C viraemia. aP < 0.001, bP < 0.01, cP < 0.05.
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PBMCs that produced GBV-C, the amount produced was greatest in the first week post-culture in all three sets of conditions, after which GBV-C production gradually declined. No GBV-C RNA was detected in cell culture supernatants from subjects without GBV-C viraemia. To ensure that GBV-C RNA detected was produced from the infected cells and did not reflect slow release of viral particles coated on antigen-presenting cells, we added high titre ($1.0 \times 10^7$ copies/ml) GBV-C RNA positive serum to healthy, GBV-C-negative PBMCs and incubated these cells overnight at $37^\circ C$. Following incubation, the cells were washed and maintained in culture with or without PHA/IL-2 in order to determine if virus that was adherent to cells was released in quantities similar to those observed in GBV-C RNA positive PBMC cultures. By day 7, no GBV-C RNA was detected in any of the three PBMC cultures studied (data not shown). In addition, when GBV-C RNA positive serum (titre $4 \times 10^7$ genome equivalents/ml) was incubated in the absence of cells, no viral RNA was detected on day 7 (data not shown). This observation further suggests that the GBV-C RNA detected in these cultures represented productive infection and not release of viral particles from antigen-presenting cells.

Effects of GBV-C on PBMC following PHA/IL-2 activation

As noted, GBV-C viraemia is associated with reduced CD4+ T-cell expansion following IL-2 therapy in HIV-positive individuals [30], suggesting an interaction between GBV-C and IL-2 signalling. To determine if GBV-C viraemia influenced proliferation of PBMCs cultured ex vivo, cells from a GBV-C viraemic patient and non-viraemic controls were activated with PHA/IL-2 and viable cells counted using trypan blue exclusion microscopy. PBMC proliferation was reduced in GBV-C viraemic subjects compared with non-viraemic controls following PHA/IL-2 activation; levels were significantly different on day 35 (Figure 1B).

GBV-C reduces HIV reactivation

Efficient HIV replication and reactivation of latent HIV in vitro requires T-cell activation [37]. To determine if GBV-C infection altered reactivation of latent HIV, we utilized PHA/IL-2-stimulated PBMC cultures as described [38]. Culture supernatants from three independent and replicate cultures were assessed for HIV p24 antigen weekly for 5 weeks. Similar to previous data obtained using this methodology, we detected reactivation of latent HIV in 16% of subjects (8 of 49) [38]. When stratified by GBV-C viraemia status, the frequency of reactivation of latent HIV was significantly greater in subjects without GBV-C viraemia (7/23) compared with those who were viraemic (1/26; $P=0.019$, $\chi^2$ test; Figure 2). HIV reactivation was not detected in any cultures maintained without PHA/IL-2 and HIV p24 antigen was only detected after three or more weeks in culture.

Effects of GBV-C on T-cell depletion following PHA/IL-2 activation

Previous clinical studies found an association between GBV-C viraemia and reduced activation and proliferation markers on T-cells studied ex vivo [29,30]. As GBV-C viraemia was associated with a reduction in PBMC proliferation in vitro compared with non-viraemic controls (Figure 1B), GBV-C might also influence activation-induced cell death (AICD) of CD4+ and CD8+ T-cells in vitro. To determine if GBV-C viraemia reduces T-cell depletion following activation, PBMCs from 17 subjects stimulated with PHA/IL-2 and maintained in culture for 35 days were analysed for viable CD4+ and CD8+ T-cells by flow cytometry. To exclude the possibility that T-cell death was induced by HIV reactivation, only subjects that did not demonstrate HIV reactivation were studied (GBV-C infected $n=6$; GBV-C uninfected $n=11$). Live and dead CD4+ and CD8+ T-cells were analysed by flow cytometry, as described in Methods (Figure 3A). Examples of cell populations stimulated with PHA/IL-2 for 35 days for one representative patient with GBV-C viraemia and
a representative patient without GBV-C viraemia are shown in Figure 3B (live cells in right lower quadrant, dead cells in right upper quadrant). At baseline (day 0) there were no differences in the number of CD4+ and CD8+ T-cells by GBV-C RNA status (Figure 4A). However, by day 35, levels of viable CD4+ and CD8+ T-cells were significantly higher in the GBV-C-positive group (GB+) compared with the GBV-C-negative group (GB-).

**Figure 3.** Determination of CD4+ and CD8+ T-cells using flow cytometry

(A) Lymphocytes were gated according to forward and side scatter and live CD3+ lymphocytes were analysed for CD4+ and CD8+ T-cell viability. (B) Flow cytometry data of peripheral blood mononuclear cells from representative GB virus C (GBV-C) viraemic and non-viraemic subjects; the cells had been maintained in culture for 35 days with phytohaemagglutinin A and interleukin 2.
Figure 4B). PBMCs maintained without PHA/IL-2 were not significantly different at day 35 (data not shown).

Discussion

Previous studies demonstrated that GBV-C replicates in T- and B-lymphocytes in vitro and one case report suggested that GBV-C replication is inhibited by PHA/IL-2 activation [34,39]. Consistent with these data, we found that GBV-C replication was significantly greater in unstimulated PBMCs compared with PBMCs that were either stimulated with PHA/IL-2 or co-cultured with PHA/IL-2-activated PBMCs (Figure 1A). GBV-C viraemia is associated with reduced T-cell activation in HIV–GBV-C coinfected individuals [29,30] and GBV-C production was significantly greater in PBMCs that were maintained without exogenous PHA/IL-2 activation [29,30]. These data suggest that T-cell activation might interfere with signalling factors required for GBV-C replication.

In a clinical study, GBV-C viraemia was found to be associated with a block in CD4+ T-cell expansion in HIV-positive individuals receiving intravenous IL-2 therapy [30]. Consistent with this observation, PBMC proliferation was reduced in GBV-C viraemic subjects following exogenous activation with PHA/IL-2 compared with non-viraemic subjects (Figure 1B). This reduction in T-cell proliferation further suggests a bidirectional interaction between GBV-C replication and T-cell activation, and raises the possibility that GBV-C replication alters activation and IL-2 signalling pathways. Studies examining the effects of GBV-C on activation and IL-2 signalling pathways are underway.

GBV-C viraemia was associated with a reduction in the frequency of reactivation of latent HIV (3.8%) when compared with reactivation in subjects without GBV-C viraemia (30.4%). Although we suspect that these data indicate that the effects of GBV-C on PHA/IL-2-mediated activation prevent reactivation of HIV transcription, an alternative explanation is that GBV-C infection alters other, undefined HIV latency and/or reactivation factors. Further studies on the effects of GBV-C on HIV reactivation appear warranted. Regardless, GBV-C coinfection is associated with an improved virological response to cART and a decreased incidence of viral blips [7,31–33]; the effect of GBV-C on HIV reactivation might contribute to the improved response to cART. Our data suggest that GBV-C viraemia status might adversely influence

Figure 4. GBV-C protects CD4+ and CD8+ T-cell depletion following PHA/IL-2 activation

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CD4+ and CD8+ T-cells (A) at baseline (day 0) and (B) following maintenance in media containing phytohaemagglutinin A and interleukin 2 (PHA/IL-2) for 35 days. Gated cells were normalized to 10^5 total events for each group analysed. Data shown represent the median value for each group with the GB virus C (GBV-C) RNA negative subjects shaded. The box plots illustrate the IQRs and the largest and smallest values. *P<0.01, **P<0.05, ns, not significant.
approaches designed to activate HIV in cellular reservoirs in subjects receiving cART.

Persistent immune activation contributes to HIV disease progression at least in part by depleting uninfected bystander cells through AICD [11,12]. CD4+ and CD8+ T-cells maintained in PHA/IL-2 ex vivo were significantly more depleted in subjects without GBV-C viraemia compared with those with GBV-C viraemia following 35 days in culture (Figure 4), suggesting that GBV-C has a protective effect against AICD. Persistent GBV-C replication in vitro appeared to be necessary for this effect, as CD4+ and CD8+ T-cells were depleted in the two GBV-C-positive subjects that did not demonstrate GBV-C replication in cell culture. The Fas/FasL pathway is suggested to be the major apoptotic pathway involved during AICD of bystander cells [11,18]; a previous study found that GBV-C infection is negatively associated with Fas expression on lymphocytes and with apoptosis [40]. Our findings further demonstrate that GBV-C is associated with a reduction in T-cell depletion following in vitro activation with PHA/IL-2. Together, these data support an inhibitory role of GBV-C in Fas-mediated T-cell apoptosis.

In summary, PHA/IL-2 stimulation significantly reduced GBV-C replication in vitro, and PBMC proliferation was significantly reduced in subjects with GBV-C viraemia following PHA/IL-2 activation compared with non-viraemic subjects. GBV-C infection was associated with a significant reduction in reactivation of latent HIV in vitro and with protection of CD4+ and CD8+ T-cells from AICD. The reduction in cell death was offset by a reduction in proliferation, suggesting that GBV-C might contribute to the regulation of T-cell homeostasis in vivo. These studies suggest potential mechanisms by which GBV-C co-infection could contribute to improved survival in HIV-infected subjects. The data support further characterization of the mechanisms by which GBV-C modulates T-cell proliferation, activation, HIV reactivation and CD4+ and CD8+ T-cell preservation following activation.

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Disclosure statement

JTS has a potential conflict of interest in that he is a co-author of patents on the use of GBV-C as potential antiviral therapy. The remaining authors declare no competing interests.

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