GB Virus Type C Infection Polarizes T-Cell Cytokine Gene Expression Toward a Th1 Cytokine Profile via NS5A Protein Expression

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Human immunodeficiency virus (HIV) disease progression is associated with a helper T cell 1 (Th1) to helper T cell 2 (Th2) cytokine profile switch. Persistent GB virus type C (GBV-C) infection is associated with survival and a serum Th1 cytokine profile in HIV-infected individuals. We found that GBV-C infection increased gene expression of Th1 cytokines and decreased Th2 cytokine expression in peripheral blood mononuclear cells. Furthermore, expression of GBV-C NS5A protein in a CD4+ cell line resulted in upregulation of Th1 cytokines (tumor necrosis factor α) and downregulation of Th2 cytokines (interleukin 4, interleukin 5, interleukin 10, interleukin 13). GBV-C–induced modulation in T-cell cytokines may contribute to the beneficial effect of GBV-C in HIV-infected individuals.

GB virus type C (GBV-C; also known as hepatitis G virus), identified in 1995, is a positive-stranded RNA virus classified as a member of the Flaviviridae family (reviewed in [1]). Although phylogenetically closely related to hepatitis C virus, prospective epidemiological studies of GBV-C failed to find an association between GBV-C and human hepatitis. The majority of immunocompetent individuals clear GBV-C viremia within 2 years; however, the virus may persist in some individuals for decades (reviewed in [1]). GBV-C replication in vitro is inefficient and often abortive; however, the viral RNA is found in, and produced by, B and T lymphocytes in vitro (reviewed in [2]), suggesting that the virus infects T and B lymphocytes. Only 5%–10% of peripheral blood mononuclear cells (PBMCs) express viral antigens in vitro, indicating that not all PBMCs support replication [3].

As with other lymphotropic viruses, GBV-C is transmitted vertically, sexually, and by blood exposure (reviewed in [2]). Because of shared modes of transmission, GBV-C coinfection is common in individuals infected with human immunodeficiency virus (HIV) [1]. Persistent GBV-C viremia is associated with prolonged survival in HIV-infected people in most, though not all, epidemiological studies, and a meta-analysis of studies including 1294 HIV-infected persons found a relative risk of mortality of 0.41 (95% confidence interval, .23–.69) among those with persistent infection compared to those without [4]. Lymphocyte coinfection with GBV-C and HIV demonstrates inhibition of HIV replication in vitro, and there is an inverse relationship between HIV and GBV-C load in vivo [5, 6].

Several lines of evidence suggest that the mechanisms by which GBV-C influences HIV disease relate to effects of GBV-C replication on T-cell function. Specifically, GBV-C replication induces the HIV inhibitory chemokines (RANTES, MIP-1α, MIP-1β, and SDF-1) and downregulates the two main HIV entry co-receptors (CCR5 and CXCR4) in vitro [7, 8]. In addition, the GBV-C nonstructural protein 5A (NS5A) inhibits HIV replication in vitro by inducing chemokines, decreasing CXCR4 expression, and modulating CD4 expression [8, 9]. Consistent with these in vitro findings, CCR5 and CXCR4 expression on T cells is reduced in GBV-C and HIV–coinfected people compared to HIV-monoinfected individuals [10, 11], and GBV-C coinfection is associated with reduced T-cell activation markers [11].

HIV disease progression is modulated by the cellular cytokine milieu, and the presence of helper T cell 1 (Th1) cytokines in plasma is associated with less advanced HIV disease [12]. In a Sicilian cohort of HIV-infected people, GBV-C coinfection was associated with a Th1 cytokine profile. Specifically, serum Th1 cytokines (interleukin 2 [IL-2] and interleukin 12 [IL-12]) remained stable over time and helper T cell 2 (Th2) cytokines (interleukin 4 [IL-4] and interleukin 10 [IL-10]) remained low in HIV-infected persons with GBV-C coinfection compared to those without [13]. In contrast, subjects without GBV-C viremia had significant decreases in IL-2 and IL-12 over time and increases in IL-4 and IL-10 serum levels [13].
To determine if GBV-C alters T-helper cytokine profiles in vitro, we examined cytokine gene expression and release in GBV-C-infected PBMCs and CD4⁺ T-cell lines expressing the GBV-C NS5A protein.

MATERIALS AND METHODS

Cell Preparation and Virus Infection
PBMCs from healthy (GBV-C negative, HIV-negative) donors were prepared by Ficoll-Hypaque gradient centrifugation and cultured as previously described [3]. CD4⁺ Jurkat T cells stably expressing GBV-C NS5A protein or Jurkat cells expressing the empty vector control reporter protein (VC) were utilized in these studies as described elsewhere [8, 13]. In brief, Jurkat cells were stably transfected with a tetracycline-regulated plasmid expressing the full-length GBV-C NS5A protein (nt 6149 to nt 7391 based on sequence of GenBank isolate AF121950) or VC [3, 8]. Both the NS5A and VC plasmids express green fluorescent protein (GFP), and NS5A protein expression was confirmed by Western blot analysis [8]. PBMCs (not stimulated with phytohemagglutinin or IL-2) were infected in triplicate with either a clinical GBV-C isolate or a mock control virus preparation as previously described [7]. GBV-C RNA was purified from culture supernatant fluids and detected by reverse-transcription polymerase chain reaction (PCR) or real-time PCR as described previously [14]. Experiments were performed in triplicate and repeated at least twice with consistent results. All subjects who donated PBMCs or serum samples for these studies provided written informed consent, and this project was approved by the University of Iowa Institutional Review Board.

Characterization of Th1 and Th2 Cytokine Expression
Th1 and Th2 cytokine messenger RNA (mRNA) levels in PBMCs or Jurkat cell lines were assessed by preparing total cellular RNA followed by measurement of specific cytokine mRNAs using hybridization as described [7], or by real-time PCR as recommended by the manufacturer (SA Biosciences, a Qiagen Corporation). Three separate and independent cellular RNA preparations were prepared from each PBMC culture or Jurkat cell line for testing gene expression, and the data were analyzed using the ΔΔCt method with normalization of the raw data to 5 housekeeping genes (β-actin, ribosomal protein L13a, hypoxanthine phosphoribosyl transferase 1, glyceraldehyde-3-phosphate dehydrogenase, 18S ribosomal RNA) using SA Biosciences software. Alternatively, Th1 and Th2 cytokine proteins released into culture supernatants (IL-2, IL-4, IL-10, IL-12, IL-13, interferon [IFN]-γ, and tumor necrosis factor [TNF]-α) were determined by enzyme-linked immunosorbent assay (ELISA) as recommended by the manufacturer (R&D Systems) [7]. Recombinant cytokines were obtained from PeproTech [7].

RESULTS
To determine the effect of GBV-C in vitro infection of PBMCs on cytokine gene expression, PBMCs were infected with GBV-C or mock-infected, and mRNA levels for selected cytokine genes were examined. Seven days postinfection, cytokine gene expression was polarized toward a Th1 profile, with downregulation of IL-13 and upregulation of IL-2 and IL-12A (P < .01) observed in infected PBMCs compared with mock-infected cells (Figure 1A). Of the additional cytokine genes examined in these experiments, 7 cytokines were significantly downregulated in GBV-C–infected PBMCs compared with mock-infected PBMCs (interleukin 1A, interleukin 3, interleukin 6, interleukin 7, interleukin 9, interleukin 16, interleukin 18) whereas 3 were upregulated (interleukin 1B, interleukin 8, interleukin 15; P < .05 for all, data not shown). Genes tested that were not significantly altered included IL-4, interleukin 5 [IL-5], IL-10, interleukin 11, IL-12B, interleukin 14, interleukin 17, IFN-γ, TNF-α, and TNF-β).

Unfortunately, GBV-C infection is inefficient in vitro, and no more than 5% of PBMCs support viral replication [3]; thus, in vitro infection experiments may not accurately reflect cytokine mRNA expression in individually infected cells. To overcome this limitation and to determine if a GBV-C protein is responsible for the altered cytokine expression, we assessed cytokine gene expression profiles in Jurkat cells expressing GBV-C NS5A protein. GBV-C NS5A was selected because it is closely related to the HCV NS5A protein, which has previously been shown to alter the transcription of a large number of cellular genes and signaling pathways (reviewed in [15]). Similar to the in vitro infection, GBV-C NS5A protein expression polarized gene expression in Jurkat cells toward a Th1 profile. Specifically, NS5A expression significantly downregulated IL-4, IL-5, IL-10, and IL-13 gene expression while significantly increasing TNF-α compared to the VC Jurkat cells (Figure 1B). Like HCV NS5A, GBV-C NS5A protein expression was found to regulate the transcription of many cytokine genes.

All genes included in the cytokine and Th1/Th2/Th3 pathway–focused expression arrays that were significantly upregulated or downregulated by GBV-C NS5A protein compared to the VC cells are shown in Supplementary Tables 1 and 2. Overall, GBV-C NS5A protein expression predominantly downregulated Th2-associated gene expression and had little or no effect on Th1–associated gene expression (Figure 1 and Supplementary Table 1A). The data confirmed earlier
results showing that GBV-C NS5A significantly downregulates CD4 expression (Supplementary Table 1B) [9].

Consistent with cellular feedback mechanisms, an inverse relationship was observed between mRNA expression levels of several cytokines and their corresponding receptors. For example, IL-4 and IL-13 were downregulated and their cognate receptors were upregulated (IL-4R by 15.3-fold and IL-13RA1 by 4.2-fold). Some relevant genes could not be evaluated, as they were either expressed at very low levels (eg IL-12B, IFN-γ) or not at all (IL-2, IFN-α) in either the NS5A or VC Jurkat cells (Supplementary Table 1C and 1D). Consequently, any effect of GBV-C NS5A protein on these cytokines could not be determined in this system.

To determine if cytokine protein expression of the regulated Th1 and Th2 cytokine genes was altered by NS5A expression, IL-2, IL-4, IL-10, IL-12, IL-13, and IFN-γ release into Jurkat culture supernatants in NS5A- and VC-expressing Jurkat cells was measured following 4 days in culture. The average of triplicate wells found that the concentration of IL-10 in VC-GFP day 4 supernatant was 21.4 pg/mL, whereas no IL-10 was detected in NS5A-expressing Jurkat cell supernatant fluids (P < .05), consistent with the mRNA expression levels. This experiment was repeated with similar results. The concentrations of IL-2, IL-4, IL-12, IL-13, and IFN-γ were all below the limit of detection of the ELISA.

**DISCUSSION**

Nunnari et al found an association between GBV-C infection and maintenance of a Th1 cytokine profile in a Sicilian HIV-infected cohort [13]; however, the in vitro effect of GBV-C on cytokine profiles has not been previously reported. In this study we found that GBV-C infection of PMBCs and the expression of GBV-C NS5A phosphoprotein in a CD4+ T-cell line polarized cytokine gene expression toward a Th1 profile. This outcome appears to be driven primarily by a more robust downregulation of Th2 cytokines, rather than by upregulation of Th1 cytokines. Because IL-4, IL-10, and IL-13 suppress Th1 responses, it is possible that the overall expression is driven by downregulation of these Th2 cytokines.

These results provide biological support for the mechanism proposed by Nunnari et al that suggested that GBV-C infection influences HIV disease progression by modulating Th1/Th2 cytokine profiles [13]. Because a dominant Th2 cytokine profile is associated with progression to AIDS in HIV-infected individuals [12], the effects of GBV-C infection and NS5A protein expression on Th cytokines may contribute to the beneficial clinical effects of GBV-C on HIV survival (reviewed in [1, 2]). Furthermore, loss of GBV-C might result in a Th1 to Th2 switch, leading to more rapid mortality, which has also been observed in clinical studies (reviewed in [2]).

These data illustrate that GBV-C infection, and the NS5A protein in particular, modulates T-cell cytokine gene expression. More work is needed to determine whether the effect of GBV-C T-cell cytokine polarization is only beneficial to persons coinfected with HIV, or if GBV-C influences other diseases whose pathogenesis is mediated by Th1 and Th2 cytokine balance. In theory, GBV-C modulation of cytokine expression could provide novel therapeutic approaches for diseases other than HIV.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents
of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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References