**United States Patent**

**Stapleton et al.**

(54) GB VIRUS C (HEPATITIS G VIRUS) E2 GLYCOPROTEIN AS AN IMMUNOMODULATORY AGENT

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None
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(57) ABSTRACT

GB virus C (GBV-C or hepatitis G virus) is a flavivirus that frequently leads to chronic viremia in humans. The invention provides compositions and methods involving GBV-C E2 polypeptides and peptides for use in modulating immune
responses, including inhibition inflammation related to pathogenic T-cell activation.

19 Claims, 27 Drawing Sheets

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FIG. 1C
A

IL-2 secretion 
(fold increase vs. unstimulated)

VC  GBV-C E2  FS  E2 (1-219)  E2 (219-331)

ns  *  ns

αCD3/CD28

B

IL-2 mRNA 
(relative expression)

VC  GBV-C E2  FS  GBV-C E2 +dox

**

αCD3/CD28

FIG. 2A-B
FIG. 3A-B
**FIG. 3C**

<table>
<thead>
<tr>
<th>Frame Shift (FS)</th>
<th>GBV-C E2</th>
<th>IL-2 (15 mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- pSTAT5 (Tyr694)
- Total STAT5
FIG. 6A-B
FIG. 7
FIG. 8
FIG. 9
FIG. 11A-B
FIG. 13A-D
A. Phospho-LAT (Y191) OD 450

B. pZAP-70/Total ZAP-70

FIG. 19A-B
A.

Unstained

GBV-C E2

FS control

% of Max

CD45

B.

FS control

GBV-C E2

[anti-CD3]

Csk

GAPDH

C.

CD45 Activity

NS

10 IgG 5 E2-Fc 10 μg protein

FIG. 20A-C
A. PQYVYGSVS  B. TGGFYEPLV  C. PNGP  D. PGTP

A  
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
GBV-C_{hum}

A
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
GBV-C_{cpz}

R  
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
--- ---
aa 83-91  aa 281-289  aa 48-51  aa 257-260

FIG. 22A-D
FIG. 23
A. No Peptide

B. TAT only

C. GBV-C E2 (Y87)

D. GBV-C E2 (Y87H)

FIG. 24A-D
GB VIRUS C (HEPATITIS G VIRUS) E2 GLYCOPROTEIN AS AN IMMUNOMODULATORY AGENT

RELATED APPLICATIONS


This invention was made with government support under Grant No. RO1 AI-58740 awarded by the National Institutes of Allergy and Infectious Disease and Merit Review Grant 101BX000207 awarded by the Department of Veterans Affairs. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The sequence listing that is contained in the file named “IOWAP0113US_ST25.txt”, which is 117 KB (as measured in Microsoft Windows®) and was created on Sep. 11, 2014, is filed herewith by electronic submission and is incorporated by reference herein.

I. Field of the Invention

The present invention relates generally to the fields of molecular biology and virology. More particularly, it concerns methods and compositions to treat inflammatory conditions, in particular those resulting from pathologic T-cell activation.

II. Description of Related Art

GB virus C (GBV-C) is a human virus of Flaviviridae family that is most closely related to hepatitis C virus (HCV) (reviewed in Stapleton et al., 2011; Mohr and Stapleton, 2009; Stapleton et al., 2003). GBV-C infection is common, and about 1% to 4% of US blood donors are viremic at the time of donation. Due to shared route of transmission, the virus is highly prevalent among HIV-infected individuals (up to 42%) (Stapleton et al., 2011; Mohr and Stapleton, 2009; Rey et al., 2000). GBV-C infection is not clearly associated with any disease; however, several studies found an association between persistent GBV-C infection and prolonged survival in HIV-positive individuals (Williams et al., 2004; Nunnari et al., 2003; Xiang et al., 2001; Tillmann et al., 2001; Yeo et al., 2000; Lefrere et al., 1999; Toyoda et al., 1998; Herlinglake et al., 1998). GBV-C is lymphotropic, and GBV-C infection modulates multiple host factors that play a critical role in HIV infection including the expression of cytokines, chemokines and their receptors (reviewed in Bhattarai and Stapleton, 2012). The alteration of host factors involved in HIV replication by GBV-C could limit HIV infection and contribute to the protective effect of GBV-C coinfection observed in HIV-positive individuals.

Chronic HIV infection is characterized by persistent immune activation which contributes to T cell depletion, altered cytokine expression and loss of T cell function (reviewed in Pett, 2009; Abrams et al., 2009; Sodora and Silvestri, 2008). Interleukin-2 (IL-2) is a critical cytokine required for T cell activation, proliferation, and function (reviewed in Pett et al., 2010; Nel, 2002). However, IL-2 also induces secretion of proinflammatory cytokines like IL-6, IL-β and tumor necrosis factor alpha (TNF-α) (Fortis et al., 2002; Sereti et al., 2001; Heaton et al., 1995), and is associated with increased levels of inflammatory markers like C-reactive protein (CRP) and D-dimer in the plasma of HIV-infected subjects independent of HIV viral load (Porter et al., 2009). In addition, in vitro activation of peripheral blood mononuclear cells (PBMCs) with IL-2 increases HIV production (Kinter et al., 1995; Morgan et al., 1976). Thus, IL-2 may also promote HIV replication and contribute to HIV associated immune activation. Immune activation not only supports HIV replication but it is suggested to be a better predictor of HIV disease progression than plasma HIV viral load (VL) (Giorgi et al., 1999; Hazenberg et al., 2003).

In studies of HIV-infected people, GBV-C viremia is associated with lower surface expression of T cell activation markers compared to GBV-C non-viremic controls independent of HIV VL (Maidana-Giret et al., 2009; Schwarze-Zander et al., 2010; Nattermann et al., 2003). Surface expression of T cell activation markers CD38 and/or CCRR were significantly lower in CD4+ and CD8+ T cells from GBV-C viremic subjects compared to non-viremic controls. Among HIV-infected subjects receiving intravenous IL-2, GBV-C viremic subjects had significantly reduced CD44+ T cell expansion compared GBV-C non-viremic controls (Stapleton et al., 2009) suggesting GBV-C infection may alter T cell activation and IL-2 signaling pathways. In addition, GBV-C produced by peripheral blood mononuclear cells (PBMCs) in vitro was significantly reduced following activation with IL-2 and phytohemagglutinin (PHA) (George et al., 2006) further suggesting an interaction between GBV-C and IL-2. Since IL-2 plays a critical role in HIV infection and disease progression, the effects of GBV-C on IL-2 may contribute to the protective effect of GBV-C coinfection in HIV infected individuals. Previous studies demonstrated that addition of the GBV-C envelope glycoprotein (E2) inhibits HIV replication when added to cells (Mohr and Stapleton, 2009; Koedel et al., 2011; Jung et al., 2007), or when expressed in a CD4+ T cell line (Xiang et al., 2006). However, the full impact of these interactions on immune signaling is not understood.

SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a method a method of inhibiting immune cell activation comprising administering to a mammalian subject in need thereof a GBV-C E2 peptide or polypeptide comprising 10 contiguous residues of the GBV-C E2 protein including Tyrosine 87. The subject may suffer from a T cell- or B-cell-mediated inflammatory disease or an IL-2-mediated inflammatory disease.

The peptide or polypeptide may comprise 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 75, 100, 150, 175, 200, 219, 250 consecutive residues of GBV-C E2 or full length GBV-C E2. The peptide or polypeptide may be about 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 175, 200, 219 or 250 residues in length. The peptide may comprise the sequence VYGSVSTCVWG (SEQ ID NO: 9), QPYVYGVS (SEQ ID NO: 10) VYGGSVS (SEQ ID NO: 11) or QYYVYGSGVS (SEQ ID NO: 12). The peptide or polypeptide may comprise a non-GBV-C E2 sequence, such as a cell permeability peptide, such as HIV TAT. The GBV-C E2 sequences may be SEQ ID NOS: 5, 6 or 8. The peptide or polypeptide may comprises all L amino acids, all D amino acids, or a mix of L and D amino acids.

The immune cell may be a T cell, a B cell, such as a helper T cell suppressor T cell, or a killer T cell or NK cell. The subject may a human. Administering comprises intravenous, intra-arterial, oral, subcutaneous, topical or intraperitoneal administration. The method may further comprising admini-
BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-C. Summary of GBV-C E2 proteins stably expressed in Jurkat CD4+ T cells. Schematic of GBV-C envelope protein E2 (at 1167-2161; 331aa), E2 deletion mutants, N terminal 219 amino acids (at 1167-1824) and C terminal 112 amino acids (at 1824-2161) and frame-shift control (FS) (FIG. 1A). Boxes indicate cell lines in which IL-2 release was not inhibited (white) or inhibited (shaded) compared to the vector control following TCR engagement. GEP expression in cell lines following TCR activation. A, GBV-C E2 protein, deletion mutants (E2 1-219; E2 219-331), frame shift (FS) control or empty vector (VC) (FIG. 1B). E2 protein expression detected in Jurkat cell lysates with tetra-his antibody directed to C-terminal his-tag fused with E2 protein (FIG. 1C).

FIGS. 2A-B. GBV-C E2 protein inhibits TCR-induced IL-2 in Jurkat CD4+ T cells. Following TCR engagement IL-2 secretion was significantly inhibited in Jurkat cells expressing GBV-C E2 protein and N terminal 219 amino acids compared to the vector control. Expression of GBV-C E2 RNA (FS) or C terminal 112 amino acids did not alter IL-2 synthesis (FIG. 2A). IL-2 transcription was significantly inhibited by GBV-C E2 protein expression and culturing E2 expressing Jurkat cells in doxycycline rescued the inhibitory effect of GBV-C E2 protein on IL-2 transcription (FIG. 2B). *P<0.05; **P<0.01.

FIGS. 3A-C. GBV-C E2 protein alters IL-2 signaling pathways. GBV-C E2 protein expression reduced CD25 expression in Jurkat cells which was rescued by culturing cells in doxycycline. Following activation with anti-CD3/CD28, CD25 expression was significantly lower in E2 expressing Jurkat cells compared to the control cells or E2 expressing cells cultured in doxycycline (FIG. 3A). CD25 transcription was significantly inhibited by GBV-C E2 protein expression compared to control cells and E2 expressing Jurkat cells cultured in doxycycline (FIG. 3B). Following stimulation with IL-2 for 15 minutes STAT5 phosphorylation was inhibited in Jurkat cells expressing GBV-C E2 protein compared to frame shift (FS) control cells. Total STAT5 expression was not altered by GBV-C E2 protein expression (FIG. 3C). *P<0.05; **P<0.01.

FIGS. 4A-B. GBV-C E2 protein expression inhibits proliferation of Jurkat cells following anti-CD3/CD28 stimulation (FIG. 4B). *P<0.05.

FIGS. 5A-C. GBV-C E2 protein inhibits IL-2 secretion from PBMCs and CD25 expression on primary CD4+ and CD8+ T cells. SDS-PAGE and immunoblot analysis of purified recombinant GBV-C E2 protein fused to human IgG Fc (FIG. 5A). Following stimulation with anti-CD3/CD28, IL-2 secretion by PBMCs (FIG. 5B) and CD25 expression on CD4+ and CD8+ T cells (FIG. 5C) were significantly reduced in PBMCs from healthy donors (n=4) incubated...
with GBV-C E2 compared to the human IgG control following stimulation with anti-CD3/CD28. **p<0.01; ***p<0.001.

FIGS. 6A-B. GBV-C E2 expression inhibits T cell activation. Jurkat (CD4+) cells expressing GBV-C E2 or the GBV-C RNA with a frameshift introduced to abolish translation (FS) were stimulated with anti-CD3/CD28. (FIG. 6A) Surface expression of the activation marker CD69 measured 24 hrs post-activation. (FIG. 6B) The reduction in activation was reversed by growing E2 expressing cells in doxycycline (Tet-Off cells). * p<0.001.

FIG. 7. GBV-C E2 expression inhibits LAT phosphorylation. Jurkat cells expressing GBV-C E2 or the frameshift control were stimulated with anti-CD3. LAT phosphorylation (Tyr191) was quantified by ELISA. * p<0.01.

FIG. 8. GBV-C E2 inhibits TCR signaling. Jurkat cells stably expressing GBV-C E2 or the FS control were stimulated with anti-CD3. Phosphorylation of LAT (Y226), ZAP-70 (Y319) and Lck (Y349 and Y505) was determined by immunoblot. Phosphorylation of LAT, ZAP-70 and Lck (Y349) was inhibited by E2 expression compared to FS control.

FIG. 9. T-cell signaling pathways. Following engagement of the T cell receptor (TCR), monophosphorylated Lck is dephosphorylated by CD45 (phosphatase), leading to a conformational change exposing tyrosine (394) that allows phosphorylation and activation. This leads ZAP-70 and LAT phosphorylation, downstream signaling and activation.

FIG. 10. Lck phosphorylation is not diminished due to altered CD45 phosphatase activity. CD45 is required for the activation of Lck. Cell surface CD45 was not different between E2-expressing cells and the FS control.

FIGS. 11A-B. GBV-C E2 protein interacts with Lck. Parental Jurkat cell lysate (JCL) was incubated with recombinant GBV-C E2-Fc fusion protein or IgG and precipitated with protein A/G beads. JCL control, JCL-E2-Fc, and JCL-IgG pull-downs are shown as indicated. Lck (FIG. 11A) and E2-Fc or IgG (FIG. 11B) were identified by immunoblot and anti-Lck or anti-Fc antibodies.

FIGS. 12A-F. Extracellular microvesicles from GBV-C infected human serum inhibit T cell receptor (TCR) signaling in human T cells. Quantification of GBV-C RNA in peripheral blood mononuclear cells (PBMCs) and purified CD4+ and CD8+ T cells obtained from nine GBV-C infected subjects (FIG. 12A). Quantification of GBV-C RNA in the serum, extracellular microvesicles (EMVs) purified from the serum and supernatant after isolating microvesicles from five individuals with GBV-C infection (FIG. 12B). Quantification of GBV-C RNA in the top and bottom fraction after GBV-C-positive serum was subjected to saline flotation gradient (FIG. 12C). IL-2 release (FIG. 12D), CD69 and CD25 cell surface expression (FIGS. 12E-F) in PBMCs from healthy donors incubated with GBV-C-positive (GB+) or -negative (GB-) serum derived EMV and stimulated with CD3 and CD28 antibodies. -Fold change was calculated by measuring CD69 and CD25 levels before and after stimulation. US=unstimulated, MFI=mean fluorescence intensity. Data represent the average of three independent cultures. *p<0.05; **p<0.01.

FIGS. 13A-D. GBV-C E2 protein expression inhibits T cell receptor (TCR) mediated activation of human T cells. Jurkat (tet-off) cells stably expressing GBV-C E2 protein or the same GBV-C sequence with a plus one frame shift to abolish translation (FS) were incubated with or without doxycycline (dox; 1 µg/ml) for 5 days. Dox treatment significantly reduced E2 protein expression (FIG. 13A). Twenty-four hours after TCR stimulation with CD3 and CD28 antibodies, CD69 surface expression was significantly reduced in Jurkat cells expressing E2 protein, and this was reversed by maintaining cells in doxycycline (FIG. 13B). Data represent the -fold increase in CD69 expression before and after TCR stimulation from three independent cultures. Following TCR stimulation with CD3 and CD28 antibodies, Jurkat cells expressing E2 had a reduction in phosphorylation of linker for activation of T cells (LAT) (FIG. 13C) and zeta-chain-associated protein kinase (ZAP)-70 (FIG. 13D) compared to the frameshift control (FS). MFI=mean fluorescence intensity. Each experiment was repeated at least three times with consistent results. *p<0.05; **p<0.01.

FIGS. 14A-D. GBV-C E2 protein interacts with and inhibits Lck activation. Lck activation (phosphorylation of Lck Y394) was reduced in Jurkat cells expressing GBV-C E2 protein compared to the frameshift (FS) control following TCR stimulation with CD3 antibody (A, B). Recombinant GBV-C E2-human Fc fusion protein but not human IgG precipitated Lck, but not Zap-70 or LAT from Jurkat cell lysates (C). Similarly, precipitation of Lck from Jurkat cells expressing GBV-C E2 protein also precipitated E2 protein (D). Each experiment was repeated at least three times with consistent results.

FIGS. 15A-E. Characterization of a peptide domain within GBV-C E2 that inhibits T cell receptor (TCR) signaling. Panel A illustrates Jurkat cells lines that stably expressed GBV-C E2 proteins (amino acid numbers shown).

*previously described cell lines. Shaded box represent cell lines that did not inhibit TCR signaling (FIG. 15A). IL-2 release was inhibited following TCR stimulation with CD3 and CD28 antibodies in all cell lines expressing E2 amino acids 86-98 (FIG. 15B). Reconstituted E2 protein incubated with recombinant Lck demonstrated E2 tyrosine phosphorylation and when CD45 was added, E2 was dephosphorylated (FIG. 15C). TCR-induced IL-2 release was not inhibited in Jurkat cells expressing the chimpanzee GBV-C E2 protein (GBV-Cepz) or the human E2 with a tyrosine to alanine substitution (Y87A) (FIG. 15D). Expression of E2 amino acids 86-98 did not inhibit activation by PMA (50 ng/ml) and Ionomycin (1 µg/ml) when compared to Jurkat cells without E2 (FIG. 15E). -Fold change in IL-2 release was calculated by measuring IL-2 at baseline (~5 µg/ml) and after anti-CD3/CD28 stimulation for 24 hours. Data represents average from three independent cultures. Each experiment was repeated at least three times with consistent results. *p<0.05; **p<0.01.

FIGS. 16A-D. Synthetic GBV-C E2 peptides inhibit TCR activation in primary human T cells. Following TCR-stimulation with CD3 and CD28 antibodies, healthy donor PBMCs incubated with GBV-C E2 86-101 peptides containing an HIV Tat protein transduction domain (TAT-Y87) had reduced IL-2 production (FIG. 16A); CD69 and CD25 expression on CD4+ (FIG. 16B) and CD8+(FIG. 16C) T cells compared to no peptide, a Tat only peptide, or the 86-101 peptide that substituted a histidine for the tyrosine (TAT-Y87). Lck mediated phosphorylation of TAT-Y87 and TAT-276-292 peptide was detected in dose-dependent manner compared to TAT-Y87 peptide synthetically phosphorylated (TAT-Y87P04) or the TAT-276-292 peptide synthesized in a scrambled order TAT-SCR (FIG. 16D). RLU=relative luminescence units. Each experiment was repeated at least three times with consistent results. *p<0.05; *p<0.01.

FIGS. 17A-G. GBV-C E2 protein inhibits T cell receptor (TCR) signaling in bystander cells. Following TCR stimulation with CD3 and CD28 antibodies, IL-2 release (FIG. 17A), surface expression of CD69 (FIG. 17B) and CD25
(FIG. 17C) was inhibited in Jurkat cells (JC; GFP-negative) co-cultured with GBV-C E2 expressing cells (GFP-positive) compared to Jurkat cells (JC; GFP-negative) co-cultured with vector control cells (VC; GFP-positive). Detection of GBV-C E2 protein and CD63 (a marker for microvesicles of endothelial origin) in extracellular microvesicles (EMV) purified from the clarified supernatant of Jurkat cells expressing GBV-C E2 protein or control cells containing the GBV-C E2 coding region with a frasment to abolish translation (FIS) (FIG. 17D). Following stimulation with anti-CD3/CD28 antibodies, IL-2 release (FIG. 17E), CD69, and CD25 cell surface expression (FIGS. 17F-G) was significantly inhibited in PBMCs from healthy donor inoculated with GBV-C E2-positive secreted microvesicles (E2 EMV) compared to E2-negative microvesicles (Em EMV). Fold change was calculated by measuring IL-2, CD69, and CD25 levels before and after stimulation. US-immunostained, MFI mean fluorescence intensity. Data represent the average of three independent cultures. *P<0.01, **P<0.001.

FIG. 18. Purification of CD4+ and CD8+ T cells from GBV-C viremic subjects. PBMCs from nine GBV-C viremic subjects were subjected to immunoaffinity selection for CD4+ T cells using magnetic beads followed by flow cytometric (FACS) purification of CD4+ and CD8+ T cells using antibodies. Purity of sorted CD4+ and CD8+ T cells were above 99%.

FIGS. 19A-B. GBV-C E2 protein expression reduces LAT and Zap70 phosphorylation. Phosphorylation of LAT (Y191) was significantly inhibited following TCR activation in Jurkat cells expressing GBV-C E2 protein compared to the frasment control (FS) as determined by ELISA (FIG. 19A). Fold change in phosphorylation of Zap70 (Y319) following TCR activation (FIG. 19B). ELISA data represent the average LAT phosphorylation from three independent cultures. Each experiment was repeated at least three times with consistent results. *P<0.05, **P<0.001.

FIGS. 20A-C. GBV-C E2 protein does not alter CD45 and Csk expression. Expression of CD45 (FIG. 20A) and Csk (FIG. 20C) was not different in GBV-C E2 expressing cells compared to the FS control. Reombinant GBV-C E2 protein did not affect CD45 enzymatic function (FIG. 20B). NS—not significant.

FIG. 21. GFP expression by Jurkat cell lines. Jurkat cell lines expressing human GBV-C E2 protein truncated mutants and E2 protein from chimpanzee GBV-C (GBV-C cch) isolate stably expressed GFP as determined by flow cytometry.

FIGS. 22A-D. Sequence alignment of E2 protein from human and chimpanzee GBV-C isolates. GBV-C E2 protein sequence from human USPV-C (GBV-C cch) and chimpanzee GBV-C (GBV-C cch) representing two predicted Lck substrate motifs (aa 83-91) (FIG. 22A) and (aa 281-289) (FIG. 22B), and two SH3 binding motifs (aa 48-51) (FIG. 22C) and (aa 257-260) (FIG. 22D).

FIG. 23. Phorbol-12-myristate-13-acetate (PMA) does not induce Lck activation. Jurkat cells were either unstimulated or stimulated with PMA (50 ng/ml) or anti-CD3 (5 μg/ml) for two minutes. Lck phosphorylation (Y394) was only detected in Jurkat cells incubated with anti-CD3 but not with PMA.

FIGS. 24A-D. Uptake of TAT-fused peptides in PBMCs. Cellular uptake of FITC labelled TAT-fused synthetic GBV-C E2 peptides representing (86-98) aa region (FIGS. 24C-D) and TAT-only control peptide (FIG. 24B) or no peptide (FIG. 24A) by PBMCs after 24 hours as determined by flow cytometry.

FIGS. 25A-C. Proposed model for inhibition of T cell receptor (TCR) signaling in infected and bystander T cells during GBV-C infection. GBV-C infection of T cells results into exposure to envelope protein E2 which competes for Lck and inhibits Lck function in the infected T cells (FIG. 25A). GBV-C infected T cells secrete GBV-C E2 proteins and RNA in microvesicles along with virus particles. Newly released virus particles can infect and inhibit TCR signaling in permissive T cells by inhibiting Lck (FIG. 26B). In addition, E2-containing extracellular microvesicles are taken up by bystander non-infected T cells where E2 can compete for Lck and inhibit Lck activation (FIG. 25C).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Persistent immune activation and immune dysfunction are characteristic features of chronic HIV infection and contribute to HIV-mediated immunodeficiency (reviewed in Pett, 2009; Sodorn and Silvestri, 2008). GBV-C viremia is associated with reduced T cell activation markers on CD4+ and CD8+ T cells in HIV-infected subjects compared to controls without GBV-C viremia, independent of HIV viral load (Maida-Giret al., 2009). HIV-positive individuals with GBV-C viremia had reduced expression of T cell activation markers compared to non-viremic controls, suggesting that GBV-C may modulate T cell activation responses (Maida-Giret al., 2009; Schwarze-Zander et al., 2010; Nattermann et al., 2003). IL-2 is a critical cytokine which regulates T cell activation and proliferation (reviewed in Pett, 2009; Pett et al., 2010), and previous studies suggested an interaction between GBV-C and IL-2 in vitro and in vivo (Sampson et al., 2009; George et al., 2003). Thus, GBV-C effects on T cell activation may be mediated in part by altering IL-2 expression.

In this study, the inventors found that the GBV-C envelope protein E2 expression significantly inhibited IL-2 production following TCR engagement (FIG. 2A) by reducing IL-2 transcription (FIG. 2B). The region within the E2 protein required to alter IL-2 expression resided in the N terminal 219 amino acids (aa), as the expression of amino acids 219 to 331 of the GBV-C E2 protein did not affect IL-2 release (FIG. 2C). In addition, since proximal TCR signaling contributes to IL-2 transcription (reviewed in Nel, 2002), studies into the effects of GBV-C infection and GBV-C E2 protein on TCR signaling appear warranted. The GBV-C effects on IL-2 observed in this study may contribute to lower immune activation in HIV-positive individuals and limit HIV mediated immunopathogenesis.

GBV-C viremic HIV-positive subjects had significantly reduced CD4+ T cell expansion following intravenous IL-2 therapy compared to GBV-C non-viremic controls (Stapleton et al., 2009) suggesting GBV-C may alter IL-2 signaling pathways. IL-2 signaling is initiated upon binding to the high affinity IL-2 receptor (IL-2R), as noted above (reviewed in Cheng et al., 2011). CD25 expression is upregulated upon activation, promoting IL-2 mediated T cell activation and proliferation. Expression of CD25 on Jurkat cell was impaired by GBV-C E2 protein expression and was significantly reduced following activation compared to the FS controls. Furthermore, this was due to E2 expression, as CD25 expression increased in the E2 expressing, Tet-off Jurkat cells maintained in doxycycline (FIG. 3A). The effect of GBV-C E2 protein on CD25 was mediated in part by reducing CD25 transcription (FIG. 3B). STAT5 phosphorylation was inhibited in GBV-C E2 expressing Jurkat cells following stimulation with IL-2 (FIG. 3C) suggesting a
significant alteration of IL-2 signaling pathway. Consistent with these data, GBV-C/HIV viremic subjects had reduced CD25 expression on CD4+ and CD8+ T cells compared to HIV mono-infected subjects (Maidana-Giret et al., 2009).

The reduction in IL-2 and CD25 transcription reduced both IL-2 signaling (FIG. 3C) and cellular proliferation (FIG. 4), providing an explanation for why GBV-C infection is associated with reduced CD34+ T cell expansion following IL-2 therapy in HIV-positive subjects (Stapleton et al., 2009). Consistent with the Jurkat cell data, incubation of PBMCs from healthy subjects with recombinant GBV-C E2 protein stimulated with anti-CD3/CD28 demonstrated a reduction in IL-2 secretion and CD25 expression on both CD4+ and CD8+ T cells compared to control PBMCs (FIGS. S8B-C).

These data support in vivo findings that GBV-C viremia modulates T cell activation, IL-2 mediated proliferation (Maidana-Giret et al., 2009; Stapleton et al., 2009) and suggest that the effect is mediated at least in part by the viral envelope glycoprotein E2. Previous studies found that GBV-C E2 protein inhibits HIV replication at the entry step, and recently this was mapped to amino acids 276-292 on the GBV-C E2 protein (Mohr and Stapleton, 2009; Jung et al., 2007; Xiang et al., 2006). The GBV-C E2 region required for downregulation of IL-2 mRNA and IL-2 release appears to be distinct from that involved in HIV inhibition as the E2 region from 1-219 did not inhibit HIV (Xiang et al., 2006) and expression of GBV-C E2 amino acids 219-331 did not alter IL-2 release, while expression of amino acids 1-219 of E2 blocked IL-2 release, transcription and CD25 upregulation in response to T cell activation. Thus, similar regions of the GBV-C E2 protein have distinct functional roles in the interaction between HIV infection and modulation of T cell activation and proliferation. The effects of GBV-C E2 protein on IL-2 signaling pathways may contribute to the reduction in HIV-associated immune activation observed in GBV-C/HIV coinfected individuals, and may also play an important role in regulating other immune cell function including regulatory T cells (Tregs), B cells, natural killer (NK) cells, macrophages, monocytes and dendritic cells, all of which are important in maintaining immune homeostasis (Morgan et al., 1976; Kim et al., 2006; Green et al., 2003).

The present invention, therefore, seeks to exploit this newly identified function of GBV-C E2 to modulate a variety of immune cells in pathologic settings. In particular, the inventors contemplate the treatment of hyperinflammatory conditions, including those linked to IL-2 release. These and other aspects of the invention are discussed in detail below.

I. GBV-C VIRUS

Like other members of the Flaviviridae, GBV-C is a positive-strand RNA virus that encodes a single long open reading frame (Leary et al., 1996). GBV-C does not cause acute or chronic hepatitis, yet it is the family member most closely related to HCV, the cause of hepatitis C. Sequences of GBV-C have been previously reported, for example in U.S. Pat. No. 5,874,563, which is specifically incorporated by reference. In particular, an infectious GBV-C clone has been described in the PCT application WO 01/77157, which is incorporated herein by reference.

The GBV-C polypeptide is predicted to be cleaved into two envelope proteins (E1 and E2), referred to collectively as GBV-C envelope protein, an RNA helicase, a trypsin-like serine protease, and an RNA-dependent RNA polymerase. A major difference between GBV-C and HCV is in the amino terminus of the polyprotein. In many isolates, this region is truncated, and no core (or nucleocapsid) protein is present (Simons et al., 1995; Xiang et al., 1999). In vitro translation experiments suggest that the AUG immediately upstream of the putative E1 protein is preferentially used to initiate translation, although there may be as many as four AUG’s in frame with the polyprotein upstream of this AUG (Simons et al., 1996).

The site of GBV-C replication has not been clearly identified, but it appears that replication in the hepatocyte, if it occurs, is not the primary source of virus in infected individuals (Laskus et al., 1998; Pessoa et al., 1998; Seipp et al., 1999). Recently, there were reports that human peripheral blood mononuclear cells (PBMC’s) and interferon-resistant Daudi cells are permissive for GBV-C replication (Fogedu et al., 1999; Shimizu, 1999). In addition, transient replication of GBV-C was described in MT-2 cells (a human T-cell line), and PHSCH (a human hepatocyte line immortalized with simian virus 40 large T antigen) (Seipp et al., 1999).

II. GBV-C E2 POLYPEPTIDES

In certain aspects, the invention is directed to the GBV-C virus E2 protein. The expression or provision of GBV-C E2 peptides and polypeptides can be used to modulate immune function. SEQ ID NO: 2 and 3 represent the translated product of SEQ ID NO:1 (GBV-C polyprotein) and 4 (GBV-C E2 protein), respectively. It is contemplated that the compositions and methods disclosed herein may be utilized to express all or part of SEQ ID NO: 3 and derivates thereof. In certain embodiments, compositions of the invention may include the peptides as set forth in SEQ ID NOs: 5, 6 or 8. Other peptides include the sequence VYYSVSSTCVWGS (SEQ ID NO: 9), FYVYGYGVS (SEQ ID NO: 10) VYGYGVS (SEQ ID NO: 11) or QYYGVS (SEQ ID NO: 12).

The method of claim 1, wherein said peptide comprises about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 75, 100, 150, 175, 200, 219, 250 consecutive residues of GBV-C E2 or full length GBV-C E2. Determination of which peptides possess activity may be achieved using functional assays measuring T-cell activation and proliferation as well as cytokine production, which are familiar to those of skill in the art.

In certain embodiments, the GBV-C E2 peptide comprises at least about 15 residues of the N-terminal 100 residues of full-length GBV-C E2 protein and 100 residues or less in length. Certain embodiments of the invention include various peptides and/or fusion proteins of GBV-C polypeptides, in particular GBV-C E2 protein. For example, all or part of a GBV-C E2 protein as set forth in SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO:8 may be used in various embodiments of the invention. In certain embodiments, a fragment of the GBV-C E2 may comprise, but is not limited to about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about
It also will be understood that amino acid sequences may include additional residues, such as additional N- or C-terminal amino acids, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological activity (e.g., immunogenicity) where protein expression is concerned. Theses non-GBV-C E2 sequences may be termed “heterologous.”

A. Variants of GBV-C Polypeptides

Embodiments of the invention include various GBV-C polypeptides, peptides, and derivatives thereof. Amino acid sequence variants of a polypeptide can be constructed by insertional, deletion or variants. Deletion variants lack one or more residues of the native protein that are not essential for immunosuppressive activity. Insertional mutants typically involve the addition of material at a non-terminal point in the polypeptide. Terminal additions, called fusion proteins, are discussed below.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide, such as stability against proteolytic cleavage, without the loss of other functions or properties. Substitutions of this kind preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamic acid to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

The term “biologically functional equivalent” is well understood in the art and is further defined in detail herein. Accordingly, sequences that have between about 70% and about 80%, or more preferably, between about 81% and about 90%, or even more preferably, between about 91% and about 99%, of amino acids that are identical or functionally equivalent to the amino acids of the GBV-C E2 polypeptide, provided the biological activity of the protein or polypeptide is maintained.

The term “functionally equivalent codon” is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine, and also refers to codons that encode biologically equivalent amino acids (see Table 1, below).

The following is a discussion based upon changes in the amino acids of a GBV-C E2 polypeptide or peptide to create an equivalent, or even an improved, second-generation molecule. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures. Since it is the interactive capacity and nature of a protein that defines that protein’s biological functional activity, certain amino acid substitutions can be made in a protein sequence, and in its underlying DNA or RNA coding sequence, and nevertheless produce a protein with like properties. It is thus conceived by the inventors that various changes may be made in the DNA or RNA sequences of genes or coding regions without appreciable loss of their biological utility or activity, as discussed herein. Table 1 shows the codons that encode particular amino acids.

<table>
<thead>
<tr>
<th>Codon Table</th>
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</thead>
<tbody>
<tr>
<td>Amino Acids</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Histidine</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Lysine</td>
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<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Asparagine</td>
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<tr>
<td>Proline</td>
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<tr>
<td>Glutamine</td>
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<tr>
<td>Arginine</td>
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<tr>
<td>Serine</td>
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<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Tryptophan</td>
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<tr>
<td>Tyrosine</td>
</tr>
</tbody>
</table>

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules.

It also is understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydropathic index of a protein, as governed by the hydropathicity of its adjacent amino acids, correlates with a biological property of the protein.

It is understood that an amino acid substituted for another having a similar hydropathic value still produces a biologically equivalent and immunologically equivalent protein.

In certain embodiments, a GBV-C E2 polypeptide may be a fusion protein. Fusion proteins may alter the characteris-
tics of a given polypeptide, such antigenicity or purification characteristics. A fusion protein is a specialized type of insertional variant. This molecule generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusions typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification.

The present invention may employ peptides that comprise modified, non-natural and/or unusual amino acids. A table of exemplary, but not limiting, modified, non-natural and/or unusual amino acids is provided herein below. Chemical synthesis may be employed to incorporate such amino acids into the peptides of interest.

## TABLE 2

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Amino Acid</th>
<th>Abbr.</th>
<th>Amino Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aad</td>
<td>2-Aminoadipic acid</td>
<td>Eta-M</td>
<td>N-D-ethylaspartagine</td>
</tr>
<tr>
<td>BAal</td>
<td>3-Aminoadipic acid</td>
<td>Hyl</td>
<td>Hydroxylglycine</td>
</tr>
<tr>
<td>BAal</td>
<td>beta-aminocarbonyl</td>
<td>Aillyl</td>
<td>ala-Hydroxyllysine</td>
</tr>
<tr>
<td>4Aa</td>
<td>2-Aminobutyric acid</td>
<td>3Hyp</td>
<td>3-Hydroxyproline</td>
</tr>
<tr>
<td>4Aa</td>
<td>4-Aminobutyric acid</td>
<td>4Hyp</td>
<td>4-Hydroxyproline</td>
</tr>
<tr>
<td>Aep</td>
<td>6-Aminoisopropionic</td>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>Ahe</td>
<td>2-Aminoadipic acid</td>
<td>Aile</td>
<td>ala-Isoleucine</td>
</tr>
<tr>
<td>Aib</td>
<td>3-Aminoisobutyric</td>
<td>Methylglycine, saccosine</td>
<td></td>
</tr>
<tr>
<td>Apn</td>
<td>2-Aminopimelic acid</td>
<td>Methylglycine, saccosine</td>
<td></td>
</tr>
<tr>
<td>Dsu</td>
<td>2,4-Dihydroxybutyric acid</td>
<td>Methylglycine, saccosine</td>
<td></td>
</tr>
<tr>
<td>Des</td>
<td>D-serine</td>
<td>Nva</td>
<td>Norvaline</td>
</tr>
<tr>
<td>Dprn</td>
<td>2,5-Dihydroxybutyric acid</td>
<td>Methylglycine, saccosine</td>
<td></td>
</tr>
<tr>
<td>Dpr</td>
<td>2,4-Dihydroxybutyric acid</td>
<td>Methylglycine, saccosine</td>
<td></td>
</tr>
<tr>
<td>Ethy</td>
<td>N-Ethylglycine</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In addition to the variants discussed above, the present inventors also contemplate that structurally similar compounds may be formulated to mimic the key portions of peptide or polypeptides of the present invention. Such compounds, which may be termed peptide mimetics, may be used in the same manner as the peptides of the invention and, hence, also are functional equivalents.

Certain mimetics that mimic elements of protein secondary and tertiary structure are described in Johnson et al. (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and/or antigen. A peptide mimic is thus designed to permit molecular interactions similar to the natural molecule.

Some successful applications of the peptide mimetic concept have focused on mimetics of beta-turns within proteins, which are known to be highly antigenic. Likely beta-turn structure within a polypeptide can be predicted by computer-based algorithms, as discussed herein. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

Other approaches have focused on the use of small, disulfide-containing proteins as attractive structural templates for producing biologically active conformations that mimic the binding sites of large proteins (Vita et al., 1998). A structural motif that appears to be evolutionarily conserved in certain toxins is small (30-40 amino acids), stable, and high permissive for mutation. This motif is composed of a beta sheet and an alpha helix bridged in the interior core by three disulfides. Betas turn have been mimicked successfully using cyclic L-peptidopeptides and those with D-amino acids (Weisshoff et al., 1999). Also, Johansson et al. (1999) report on bicyclic tripeptides with reverse turn inducing properties.

Methods for generating specific structures have been disclosed in the art. For example, alpha-helix mimetics are disclosed in U.S. Pat. Nos. 5,446,128; 5,710,245; 5,840,883; and 5,859,184. These structures, which render the peptide or protein more thermally stable, also increase resistance to proteolytic degradation. Six, seven, eleven, twelve, thirteen and fourteen membered ring structures are disclosed.

Methods for generating conformationally restricted beta turns and beta bulges are described, for example, in U.S. Pat. Nos. 5,440,013; 5,618,914; and 5,670,155. Beta-turns permit changed side substituents without having changes in corresponding backbone conformation, and have appropriate termini for incorporation into peptides by standard synthesis procedures. Other types of mimetic turns include reverse and gamma turns. Reverse turn mimetics are disclosed in U.S. Pat. Nos. 5,475,085 and 5,929,237, and gamma turn mimetics are described in U.S. Pat. Nos. 5,672,681 and 5,674,976.

The present invention may utilize an L-configuration amino acids, D-configuration amino acids, or a mixture thereof. While L-amino acids represent the vast majority of amino acids found in proteins, D-amino acids are found in some proteins produced by exotic sea-dwelling organisms, such as cone snails. They are also abundant components of the pedipalpal cell walls of bacteria. D-serine may act as a neurotransmitter in the brain. The L- and D-convention for amino acid configuration refers not to the optical activity of the amino acid itself, but rather to the optical activity of the isomer of glyceraldehyde from which that amino acid can theoretically be synthesized (D-glyceraldehyde is dextro-rotary; L-glyceraldehyde is levorotatory).

One form of an “all-D” peptide is a retro-inverse peptide. Retro-inverse modification of naturally-occurring polypeptides involves the synthetic assembly of amino acids with α-carbon stereochemistry opposite to that of the corresponding L-amino acids, i.e., D-amino acids in reverse order with respect to the native peptide sequence. A retro-inverse analogue thus has reversed termini and reversed direction of peptide bonds (NH—CO rather than CO—NH) while approximately maintaining the topology of the side chains as in the native peptide sequence. See U.S. Pat. No. 6,261,569, incorporated herein by reference.

B. In Vitro Production of GBV-C E2 Polypeptides or Peptides

Various types of expression vectors are known in the art that can be used for the production of protein products. Following transfection with a expression vector, a cell in culture, e.g., a primary mammalian cell, a recombinant product may be prepared in various ways. A host cell strain may be chosen that modulates the expression of the inserted sequences, or that modifies and processes the gene product in the manner desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen
to insure the correct modification and processing of the foreign protein expressed. In order for the cells to be kept viable while in vitro and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented (for exemplary methods see Freshney, 1992).

Animal cells can be propagated in vitro in two modes: as non-anchorage-dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (i.e., a monolayer type of cell growth).

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large-scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

In further aspects of the invention, other protein production methods known in the art may be used, including but not limited to prokaryotic, yeast, and other eukaryotic hosts such as insect cells and the like.

C. Protein Purification

It may be desirable to purify anti-GBV-C E2 polypeptides and peptides, or variants and derivatives thereof. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, hydrophobic interaction chromatography, exclusion chromatography, polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even FPLC.

Certain aspects of the present invention concern the purification and, in particular embodiments, the substantial purification, of an encoded protein or peptide. The term “purified protein or peptide” as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, “purified” will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term “substantially purified” is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a “-fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme.

D. Peptide Synthesis

GBV-C E2-related peptides may be generated synthetically for use in various embodiments of the present invention. Because of their relatively small size, the peptides of the invention can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young (1984); Tam et al. (1983); Merrifield (1986); Barany and Merrifield (1979), each incorporated herein by reference. Short peptide sequences, or libraries of overlapping peptides, usually from about 5 up to about 34 to 40 amino acids, which correspond to the selected regions described herein, can be readily synthesized and then screened in screening assays designed to identify reactive peptides. Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes a peptide of the invention is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression.

It may be desirable to purify GBV-C E2 polypeptide and peptides. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the cellular milieu to polypeptide and non-polypeptide fractions. Having separated the polypeptide from other proteins, the polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term “purified protein or peptide” as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

Generally, “purified” will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term “substantially purified” is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.
Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity, herein assessed by a “—fold purification number.” The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater “—fold” purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi et al., 1977). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified expression products may vary.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

Gel chromatography, or molecular sieve chromatography, is a special type of partition chromatography that is based on molecular size. Hence, molecules are eluted from the column in decreasing size, so long as the shape is relatively constant. Gel chromatography is unsurpassed for separating molecules of different size because separation is independent of all other factors such as pH, ionic strength, temperature, etc. There also is virtually no adsorption, less zone spreading and the elution volume is related in a simple matter to molecular weight.

Affinity Chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule that it can specifically bind to. This is a receptor—ligand type interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (alter pH, ionic strength, temperature, etc.).

The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way so as not affect its binding properties. The ligand should also provide relatively tight binding. It should be possible to elute the substance without destroying the sample or the ligand. One of the most common forms of affinity chromatography is immunoadfinity chromatography. The generation of antibodies that would be suitable for use in accord with the present invention is discussed below.

III. GBV-C E2 POLYNUCLEOTIDES

Certain embodiments of the invention include GBV-C E2-coding polynucleotides or nucleic acid molecules and fragments thereof. The polynucleotides of the invention may be isolated and purified from GBV-C virus or cells infected or transfected with GBV-C polynucleotides. The term isolated indicating they are free or substantially free from total viral or cellular genomic RNA or DNA, and proteins. It is contemplated that an isolated and purified GBV-C nucleic acid molecule may take the form of RNA or DNA. A GBV-C E2 nucleic acid molecule refers to an RNA or DNA molecule that is capable of yielding all or part of a GBV-C E2 protein from a transfected cell.

As used in this application, the term “polynucleotide” refers to a nucleic acid molecule, RNA, or DNA that has been isolated free of total genomic nucleic acid. Therefore, a “polynucleotide encoding all or part of GBV-C E2” refers to a nucleic acid segment that contains GBV-C E2 coding sequences, yet is isolated away from, or purified and free of, total viral genomic RNA and proteins; similarly, a “polynucleotide encoding full-length GBV-C E2” refers to a nucleic acid segment that contains full-length GBV-C E2 coding sequences yet is isolated away from, or purified and free of, total viral genomic RNA and protein.

The term “cDNA” is intended to refer to DNA prepared using RNA as a template. The advantage of using a cDNA, as opposed to genomic RNA or an RNA transcript is stability and the ability to manipulate the sequence using recombinant DNA technology (See Maniatis, 1989; Ausubel, 1994). There may be times when the full or partial genomic sequence is preferred. Alternatively, cDNAs may be advantageous because it represents coding regions of a polypeptide and eliminates introns and other regulatory regions.

It also is contemplated that a given GBV-C E2 may be represented by natural variants or strains that have slightly different nucleic acid sequences but, nonetheless, encode the
same viral polypeptides (see Table 1 above). Consequently, the present invention also encompasses derivatives of GBV-C E2 with minimal amino acid changes in its viral proteins, but that possesses the same activities.

The term "gene" is used for simplicity to refer to the nucleic acid giving rise to a functional protein, polypeptide, or peptide-encoding unit. As will be understood by those in the art, this functional term includes genomic sequences, cDNA sequences, and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, domains, peptides, fusion proteins, and mutants. The nucleic acid molecule encoding GBV-C E2 may contain a contiguous GBV-C E2 nucleic acid sequence of the following lengths: about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 441, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 770, 780, 790, 800, 810, or more nucleotides, nucleosides, or base pairs. Such sequences may be identical or complementary to all or part of SEQ ID NO:1, 4 or Genbank Accession numbers AY196904 or AF070476.

"Isolated substantially away from other coding sequences" means that the gene of interest forms part of the coding region of the nucleic acid segment, and that the segment does not contain large portions of naturally-occurring coding nucleic acid, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the nucleic acid segment as originally isolated, and does not exclude genes or coding regions later added to the segment by human manipulation.

In particular embodiments, the invention concerns isolated nucleic acid segments and recombinant vectors incorporating DNA sequences that encode GBV-C E2 polypeptides or peptides that include within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially corresponding to GBV-C E2 polypeptides.

The nucleic acid segments used in the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA or RNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

The nucleic acid segments used in the present invention encompass biologically functional and/or immunologically equivalent GBV-C E2 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalence that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally and immunologically equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by human may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein.

D. Vectors Encoding GBV-C E2

The present invention encompasses the use of vectors to encode for all or part of the GBV-C E2 polypeptide. The term “vector” is used to refer to a carrier nucleic acid molecule into which a nucleic acid sequence can be inserted for introduction into a cell where it can be replicated. A nucleic acid sequence can be “exogenous,” which means that it is foreign to the cell into which the vector is being introduced or that the sequence is homologous to a sequence in the cell but in a position within the host cell nucleic acid in which the sequence is ordinarily not found. Vectors include plasmids, cosmids, viruses (bacteriophage, animal viruses, and plant viruses), and artificial chromosomes (e.g., YACs). In particular embodiments, gene therapy or immunization vectors are contemplated. One of skill in the art would be well equipped to construct a vector through standard recombinant techniques, which are described in Maniatis et al., 1988 and Ausubel et al., 1994, both incorporated herein by reference.

The term “expression vector” or “expression construct” refers to a vector containing a nucleic acid sequence coding for at least part of a gene product capable of being transcribed. In some cases, RNA molecules are then translated into a protein, polypeptide, or peptide. In other cases, these sequences are not translated, for example, in the production of antisense molecules or ribozymes. Expression vectors can contain a variety of “control sequences,” which refer to nucleic acid sequences necessary for the transcription and possibly translation of an operably linked coding sequence in a particular host organism. In addition to control sequences that govern transcription and translation, vectors and expression vectors may contain nucleic acid sequences that serve other functions as well and are described infra.

1. Promoters and Enhancers

A “promoter” is a control sequence that is a region of a nucleic acid sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. The phrases “operatively positioned,” “operatively linked,” “under control,” and “under transcriptional control” means that a promoter is in a correct functional location and/or orientation in relation to a nucleic acid sequence to control transcriptional initiation and/or expression of that sequence. A promoter may or may not be used in conjunction with an “enhancer,” which refers to a cis-acting regulatory sequence involved in the transcriptional activation of a nucleic acid sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5’ non-coding sequences located upstream of the coding segment and/or exon. Such a promoter can be referred to as “endogenous.” Similarly, an enhancer may be one naturally associated with a nucleic acid sequence, located either downstream or upstream of that sequence. Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid sequence in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers may include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not “naturally occurring,” i.e., containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification
technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Pat. No. 4,683,202 and U.S. Pat. No. 5,928,906, each incorporated herein by reference). Furthermore, it is contemplated the control sequences that direct transcription and/or expression of sequences within non-nuclear organelles such as mitochondria, chloroplasts, and the like, can be employed as well.

Naturally, it will be important to employ a promoter and/or enhancer that effectively directs the expression of the nucleic acid segment in the cell type, organelle, and organism chosen for expression. Those of skill in the art of molecular biology generally know the use of promoters, enhancers, and cell type combinations for protein expression, for example, see Sambrook et al. (1989), incorporated herein by reference. The promoters employed may be constitutive, tissue-specific, inducible, and/or useful under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins and/or peptides. The promoter may be heterologous or exogenous, i.e., from a different source than GBV-C sequence. In some examples, a prokaryotic promoter is employed for use with in vitro transcription of a desired sequence. Prokaryotic promoters for use with many commercially available systems include T7, T3, and Sp6.

Table 3 lists several elements/promoters that may be employed, in the context of the present invention, to regulate the expression of a gene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof. Table 4 provides examples of inducible elements, which are regions of a nucleic acid sequence that can be activated in response to a specific stimulus.

**Table 3**

<table>
<thead>
<tr>
<th>Promoter/Enhancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin Heavy Chain</td>
<td>Banerji et al., 1983; Gilles et al., 1983; Groenscheldt et al., 1985; Aichinson et al., 1986, 1987; Inzer et al., 1987; Weinberger et al., 1984; Kiledjian et al., 1988; Porton et al.; 1990</td>
</tr>
<tr>
<td>Immunoglobulin Light Chain</td>
<td>Queen et al., 1983; Picard et al., 1994</td>
</tr>
<tr>
<td>T-Cell Receptor</td>
<td>Lucia et al., 1987; Winoto et al., 1989; Redondo et al.; 1990</td>
</tr>
<tr>
<td>HLA DQ a and/or DQB</td>
<td>Sullivan et al., 1987</td>
</tr>
<tr>
<td>β-Interferon</td>
<td>Goodbourn et al., 1986; Fujita et al., 1987; Goodbourn et al., 1988</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>Greene et al., 1989</td>
</tr>
<tr>
<td>Interleukin-2 Receptor</td>
<td>Greene et al., 1989; Lin et al., 1990</td>
</tr>
<tr>
<td>MHC Class II 5</td>
<td>Koch et al., 1989</td>
</tr>
<tr>
<td>MHC Class II HLA-DRα</td>
<td>Sherman et al., 1989</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Kawamoto et al., 1988; Ng et al.; 1989</td>
</tr>
<tr>
<td>Muscle Creatine Kinase (MCK)</td>
<td>Jaynes et al., 1988; Horlick et al., 1989; Johnson et al., 1989</td>
</tr>
<tr>
<td>Prealbumin (Transthyretin)</td>
<td>Costa et al., 1988</td>
</tr>
<tr>
<td>Elastase I</td>
<td>Omitz et al., 1987</td>
</tr>
<tr>
<td>Metallothionein (MTII)</td>
<td>Karin et al., 1987; Culeotta et al., 1989</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Pinkert et al., 1987; Angel et al., 1987</td>
</tr>
<tr>
<td>Albumin</td>
<td>Pinkert et al., 1987; Tronche et al., 1989, 1990</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
<td>Godbout et al., 1988; Campore et al., 1989</td>
</tr>
<tr>
<td>γ-Globin</td>
<td>Bodine et al., 1987; Perez-Stable et al., 1990</td>
</tr>
<tr>
<td>β-Globin</td>
<td>Trotel et al., 1987</td>
</tr>
<tr>
<td>c-fos</td>
<td>Cohen et al., 1987</td>
</tr>
<tr>
<td>c-FA-ras</td>
<td>Triesman, 1986; Deschamps et al., 1985</td>
</tr>
<tr>
<td>Insulin</td>
<td>Edlund et al., 1985</td>
</tr>
<tr>
<td>Neural Cell Adhesion Molecule (NCAM)</td>
<td>Heimb et al., 1990</td>
</tr>
<tr>
<td>α,-Anitrypsin</td>
<td>Latimer et al., 1990</td>
</tr>
<tr>
<td>H2B (TH2B) Histone</td>
<td>Hwang et al., 1990</td>
</tr>
<tr>
<td>Mouse and/or Type I Collagen</td>
<td>Rape et al., 1989</td>
</tr>
<tr>
<td>Glucose-Regulated Proteins</td>
<td>Chang et al., 1989</td>
</tr>
<tr>
<td>Rat Growth Hormone</td>
<td>Larsen et al., 1986</td>
</tr>
<tr>
<td>Human Serum Amyloid A (SAA)</td>
<td>Edbrooke et al., 1988</td>
</tr>
<tr>
<td>Tropinin I (TN I)</td>
<td>Yurzy et al., 1989</td>
</tr>
<tr>
<td>Platelet-Derived Growth Factor (PDGF)</td>
<td>Pech et al., 1989</td>
</tr>
<tr>
<td>Duchenne Muscular Dystrophy</td>
<td>Klarnet et al., 1990</td>
</tr>
<tr>
<td>SV40</td>
<td>Banerji et al., 1981; Moreau et al., 1981; Sleigh et al., 1985; Fink et al., 1986; Herr et al., 1986; Inzena et al., 1986; Kadesch et al., 1986; Wang et al., 1986; Onode et al., 1987; Kahl et al., 1987; Schallher et al., 1988</td>
</tr>
<tr>
<td>Polyoma</td>
<td>Swartendbruber et al., 1975; Vasseur et al., 1980; Kashiwa et al., 1980, 1981; Tyndell et al., 1981; Duvaldo et al., 1983; de Villien et al., 1984; Hen et al., 1986; Satako et al., 1988; Campbell and/or Villareal, 1988</td>
</tr>
<tr>
<td>Retroviruses</td>
<td>Kriegl et al., 1982, 1983; Levinson et al., 1982; Kriegl et al., 1983, 1984a, b, 1988; Bonze et al., 1986; Miksieck et al., 1986; Celerier et al., 1987; Thiesen et al., 1988; Celerier et al., 1988; Chol et al., 1988; Reisman et al., 1989</td>
</tr>
</tbody>
</table>
TABLE 3-continued

<table>
<thead>
<tr>
<th>Promoter/Enhancer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papillomas Virus</td>
<td>Campo et al., 1983; Lusky et al., 1983; Spanidios and/or Wilkie, 1983; Spallholz et al., 1985; Lusky et al., 1986; Cripe et al., 1987; Glosset al., 1987; Hirochika et al., 1987; Stuphen et al., 1987; Gluc et al., 1988</td>
</tr>
<tr>
<td>Hepatitis B Virus</td>
<td>Billa et al., 1986; Inoue et al., 1986; Shaul et al., 1987; Spandau et al., 1988; Vannice et al., 1988</td>
</tr>
<tr>
<td>Human Immunodeficiency Virus</td>
<td>Muesing et al., 1987; Hausner et al., 1988; Jakobovits et al., 1988; Fong et al., 1988; Takebe et al., 1988; Rosen et al., 1988; Berkley et al., 1989; Laapia et al., 1989; Sharp et al., 1989; Bradock et al., 1989</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>Weber et al., 1984; Bosshart et al., 1985; Foecking et al., 1986</td>
</tr>
<tr>
<td>Gibbon Ape Leukemia Virus</td>
<td>Holbrook et al., 1987; Quinn et al., 1989</td>
</tr>
</tbody>
</table>

TABLE 4

<table>
<thead>
<tr>
<th>Inducible Elements</th>
<th>Inducer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT II</td>
<td>Phorbol Ester (TFA)</td>
<td>Palmiter et al., 1982; Halsinger et al., 1985; Searle et al., 1985; Stuart et al., 1985; Imagawa et al., 1987; Karin et al., 1987; Angel et al., 1987b; McNeall et al., 1989</td>
</tr>
<tr>
<td>MMTV (mouse mammary tumor virus)</td>
<td>Glucocorticoids</td>
<td>Huang et al., 1981; Lee et al., 1981; Majors et al., 1983; Chandler et al., 1983; Lee et al., 1984; Ponta et al., 1985; Sakai et al., 1988</td>
</tr>
<tr>
<td>β-Interferon</td>
<td>poly(rI)poly(rC)</td>
<td>Tavernier et al., 1983</td>
</tr>
<tr>
<td>Adenovirus 5 E2</td>
<td>ElA</td>
<td>Imperiale et al., 1984</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Phorbol Ester (TFA)</td>
<td>Angel et al., 1987a</td>
</tr>
<tr>
<td>Stromelysin</td>
<td>Phorbol Ester (TFA)</td>
<td>Angel et al., 1987b</td>
</tr>
<tr>
<td>SV40</td>
<td>Phorbol Ester (TFA)</td>
<td>Angel et al., 1987b</td>
</tr>
<tr>
<td>Murine MX Gene</td>
<td>Interferon, Newcastle</td>
<td>Hug et al., 1988</td>
</tr>
<tr>
<td>GRP78 Gene</td>
<td>Disease Virus</td>
<td>Resendez et al., 1988</td>
</tr>
<tr>
<td>α2-Macroglobulin</td>
<td>IL-6</td>
<td>Kunz et al., 1989</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Serum</td>
<td>Ritting et al., 1989</td>
</tr>
<tr>
<td>MHC Class I Gene H-2kb</td>
<td>Interferon</td>
<td>Bianco et al., 1989</td>
</tr>
<tr>
<td>HSP70</td>
<td>ElA, SV40 Large T</td>
<td>Taylor et al., 1989, 1990a, 1990b</td>
</tr>
<tr>
<td>Proliferin</td>
<td>Phorbol Ester-TPA</td>
<td>Mordacai et al., 1989</td>
</tr>
<tr>
<td>Tumor Necrosis Factor</td>
<td>PMA</td>
<td>Hensel et al., 1989</td>
</tr>
<tr>
<td>Thyroid Stimulating Hormone α Gene</td>
<td>Thyroid Hormone</td>
<td>Chatterjee et al., 1980</td>
</tr>
</tbody>
</table>

The identity of tissue-specific promoters or elements, as well as assays to characterize their activity, is well known to those of skill in the art. Examples of such regions include the human I.MK2 gene (Nomoto et al., 1999), the somatostatin receptor 2 gene (Kraus et al., 1998), murine epidermal retinoic acid-binding gene (Larrey et al., 1999), human CD4 (Zhao-Emonet et al., 1998), mouse alpha2 (XI) collagen (Tsumaki et al., 1998), DIA dopamine receptor gene (Lee et al., 1997), insulin-like growth factor II (Wu et al., 1997), human platelet endothelial cell adhesion molecule-1 (Almendro et al., 1996).

2. Initiation Signals and Internal Ribosome Binding Sites

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon or adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be “in-frame” with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements.

In certain embodiments of the invention, the use of internal ribosome entry sites (IRES) elements are used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome-scanning model of 5′ methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, 1988). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, 1988), as well an IRES from a mammalian message (Macejak and Sarnow, 1991). IRES elements can be linked to heterologous open reading frames.
Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Pat. Nos. 5,925,565 and 5,935,819, herein incorporated by reference).

3. Multiple Cloning Sites
Vectors can include a multiple cloning site (MCS), which includes a unique nucleic acid region that contains multiple restriction enzyme sites, any of which can be used in conjunction with standard recombinant technology to digest the vector. (See Carbonelli et al., 1999, Levenson et al., 1998, and Cocea, 1997, incorporated herein by reference.) “Restriction enzyme digestion” refers to catalytic cleavage of a nucleic acid molecule with an enzyme that functions only at specific locations in a nucleic acid molecule. Many of these restriction enzymes are commercially available. Use of such enzymes is widely understood by those of skill in the art. Frequently, a vector is linearized or fragmented using a restriction enzyme that cuts within the MCS to enable exogenous sequences to be ligated to the vector. “Ligation” refers to the process of forming phosphodiester bonds between two nucleic acid fragments, which may or may not be contiguous with each other. Techniques involving restriction enzymes and ligation reactions are well known to those of skill in the art of recombinant technology.

4. Splicing Sites
Most transcribed eukaryotic RNA molecules will undergo RNA splicing to remove introns from the primary transcripts. Vectors containing genomic sequences may require donor and/or acceptor splicing sites to ensure proper processing of the transcript for protein expression. (See Chandler et al., 1997, herein incorporated by reference.)

5. Termination Signals
The vectors or constructs of the present invention generally comprise at least one termination signal. A “termination signal” or “terminator” is comprised of the DNA sequences involved in specific termination of an RNA transcript by an RNA polymerase. Thus, in certain embodiments a termination signal that ends the production of an RNA transcript is contemplated. A terminator may be necessary in vivo to achieve desirable message levels.

In eukaryotic systems, the terminator region may also comprise specific DNA sequences that permit site-specific cleavage of the new transcript to expose a polyadenylation site. This signal includes endogenous polymerase to add a stretch of about 200 A residues (polyA) at the 5’ end of the transcript. RNA molecules modified with this polyA tail appear to more stable and are translated more efficiently. Thus, in other embodiments involving eukaryotes, it is preferred that the terminator comprises a signal for the cleavage of the RNA, and it is more preferred that the terminator signal promotes polyadenylation of the message. The terminator and/or polyadenylation site elements can serve to enhance message levels and/or to minimize read through from the cassette into other sequences.

Terminators contemplated for use in the invention include any known terminator of transcription described herein or known to one of ordinary skill in the art, including but not limited to, for example, the termination sequences of genes, such as for example the bovine growth hormone terminator or viral termination sequences, such as for example the SV40 terminator. In certain embodiments, the termination signal may be a lack of transcribable or translatable sequence, such as due to a sequence truncation.

6. Polyadenylation Signals
For expression, particularly eukaryotic expression, one will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and/or any such sequence may be employed. Preferred embodiments include the SV40 polyadenylation signal and/or the bovine growth hormone polyadenylation signal, convenient and/or known to function well in various target cells. Polyadenylation may increase the stability of the transcript or may facilitate cytoplasmic transport.

7. Origins of Replication
In order to propagate a vector in a host cell, it may contain one or more origins of replication sites (often termed “ori”), which is a specific nucleic acid sequence at which replication is initiated. Alternatively, an autonomously replicating sequence (ARS) can be employed if the host cell is yeast.

8. Selectable and Screenable Markers
In certain embodiments of the invention, the cells containing a nucleic acid construct of the present invention may be identified in vitro or in vivo by including a marker in the expression vector. Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression vector. Generally, a selectable marker is one that confers a property that allows for selection. A positive selectable marker is one in which the presence of the marker allows for its selection, while a negative selectable marker is one in which its presence prevents its selection. An example of a positive selectable marker is a drug resistance marker.

Usually the inclusion of a drug selection marker aids in the cloning and identification of transformants, for example, genes that confer resistance to neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol are useful selectable markers. In addition to markers conferring a phenotype that allows for the discrimination of transformants based on the implementation of conditions, other types of markers including screenable markers such as GFP, whose basis is colormetric analysis, are also contemplated. Alternatively, screenable enzymes such as herpes simplex virus thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be utilized. One of skill in the art would also know how to employ immunologic markers, possibly in conjunction with FACS analysis. The marker used is not believed to be important, so long as it is capable of being expressed simultaneously with the nucleic acid encoding a gene product. Further examples of selectable and screenable markers are well known to one of skill in the art.

E. Host Cells
As used herein, the terms “cell,” “cell line,” and “cell culture” may be used interchangeably. All of these terms also include their progeny, which refers to any and all subsequent generations. It is understood that all progeny may not be identical due to deliberate or inadvertent mutations. In the context of expressing a heterologous nucleic acid sequence, “host cell” refers to a prokaryotic or eukaryotic cell, and it includes any transformable organisms that is capable of replicating a vector and/or expressing a heterologous gene encoded by a vector. A host cell can, and has been, used as a recipient for vectors. A host cell may be “transfected” or “transformed,” which refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A transformed cell includes the primary subject cell and its progeny. Host cells may be derived from prokaryotes or eukaryotes, depending upon whether the desired result is replica-
the vector, expression of part or all of the vector-encoded nucleic acid sequences, or production of infectious viral particles. Numerous cell lines and cultures are available for use as a host cell, and they can be obtained through the American Type Culture Collection (ATCC), which is an organization that serves as an archive for living cultures and genetic materials. An appropriate host can be determined by one of skill in the art based on the vector backbone and the desired result. A plasmid or cosmids, for example, can be introduced into a prokaryote host cell for replication of many vectors. Bacterial cells used as host cells for vector replication and/or expression include DH5α, JM109, and KC8, as well as a number of commercially available bacterial hosts such as SURE® Competent Cells and SOLOPACK™ Gold Cells (STRATEGENE®, La Jolla). Alternatively, bacterial cells such as E. coli LE392 could be used as host cells for phage viruses. Examples of eukaryotic host cells for replication and/or expression of a vector include HeLa, NIH/3T3, Jurkat, 293, Cos, CHO, Saos, and PC12. Many host cells from various cell types and organisms are available and would be known to one of skill in the art. Similarly, a viral vector may be used in conjunction with either an eukaryotic or prokaryotic host cell, particularly one that is permissive for replication or expression of the vector.

F. Expression Systems

Numerous expression systems exist that comprise at least all or part of the compositions discussed above. Prokaryotic- and/or eukaryote-based systems can be employed for use with the present invention to produce nucleic acid sequences, or their cognate polypeptides, proteins and peptides. Many such systems are commercially and widely available.

The insect cell/baculovirus system can produce a high level of protein expression of a heterologous nucleic acid segment, such as described in U.S. Pat. Nos. 5,871,986 and 4,879,256, both herein incorporated by reference, and which can be bought, for example, under the name MaxBAC® 2.0 from INVITROGEN® and BACTOPACK™ BACULOVIRUS EXPRESSION SYSTEM from CLONTECH.

Other examples of expression systems include STRATEGENE®'s COMPLETE CONTROL™ Inducible Mammalian Expression System, which involves a synthetic ecodysone-inducible receptor, or its pET Expression System, an E. coli expression system. Another example of an inducible expression system is available from INVITROGEN®, which carries the T-REX™ (tetracycline-regulated expression) System, an inducible mammalian expression system that uses the full-length CMV promoter. The Tet-On™ and Tet-Off™ systems from CLONTECH can be used to regulate expression in a mammalian host using tetracycline or its derivatives. The implementation of these systems is described in Gossen et al. (1992) and Gossen et al. (1995), and U.S. Pat. No. 5,650,298, all of which are incorporated by reference.

INVITROGEN® also provides a yeast expression system called the Pichia methanolica Expression System, which is designed for high-level production of recombinant proteins in the methylotrophic yeast Pichia methanolica. One of skill in the art would know how to express a vector, such as an expression construct, to produce a nucleic acid sequence or its cognate polypeptide, protein, or peptide.

G. Introduction of Nucleic Acids into Cells

In certain embodiments, a nucleic acid may be introduced into a cell in vitro for production of polypeptides or in vivo for immunization purposes. There are a number of ways in which nucleic acid molecules such as expression vectors may be introduced into cells. The ability of certain viruses to enter cells via receptor-mediated endocytosis, to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986).

"Viral expression vector" is meant to include those vectors containing sequences of that virus sufficient to (a) support packaging of the vector and (b) to express a poly-nucleotide that has been cloned therein. In this context, expression may require that the gene product be synthesized. A number of such viral vectors have already been thoroughly researched, including adenovirus, adeno-associated viruses, retroviruses, herpesviruses, and vaccinia viruses.

Delivery may be accomplished in vitro, as in laboratory procedures for transforming cells lines, or in vivo or ex vivo, as in the treatment of certain disease states. One mechanism for delivery is via viral infection where the expression vector is encapsidated in an infectious viral particle. Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa et al., 1986; Potter et al., 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley et al., 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer et al., 1987), gene bombardment using high velocity microprojectiles (Yang et al., 1990), liposome (Ghosh and Bachulw, 1991; Kaneda et al., 1989) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for in vivo or ex vivo use.

In certain embodiments, the nucleic acid encoding a gene or genes may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchrony with the host cell cycle. How the expression vector is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression vector employed.

Transfer of a nucleic acid molecule may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer in vitro, but it may be applied to in vivo use as well.

IV. GBV-C RELATED IMMUNOTHERAPY

A. Inflammatory Conditions

The present invention relates to the use of GBV-C E2 compositions (polypeptides, peptides, nucleic acids coding therefor, and mimetics) for the modulation of immune responses, particularly those relating to pathologic inflammation. In one embodiment, the pathologic inflammation relates to interleukin-2 (IL-2) expression. IL-2 has multiple, sometimes opposing, functions during an inflammatory response. It is a potent inducer of T cell proliferation and T-helper 1 (Th1) and Th2 effector T cell differentiation and provides T cells with a long-lasting competitive advantage
resulting in the optimal survival and function of memory cells. In a regulatory role, IL-2 is important for the development, survival, and function of regulatory T cells, it enhances Fas-mediated activation-induced cell death, and it inhibits the development of inflammatory Th17 cells. Thus, in its dual and contrasting functions, IL-2 contributes to both the induction and the termination of inflammatory immune responses.

The present invention would therefore seek to intervene in those disease whre, for example, IL-2 is activating T cells and leading to inflammatory states. Such diseases include autoimmune diseases like multiple sclerosis, psoriasis, inflammatory bowel disorders, early arthritis, juvenile arthritis, rheumatoid arthritis, enteropathic arthritis, psoriatic arthritis, ankylosing spondylitis, familial Mediterranean fever, amyotrophic lateral sclerosis, systemic lupus erythematosus, ulcerative colitis, inflammatory bowel disease, Sjögren’s syndrome, or Crohn’s disease. Other inflammatory conditions include cardiovascular disease, trauma, or pancreatitis.

B. Combinations with Anti-Inflammatories

It is common in many fields of medicine to treat a disease with multiple therapeutic modalities, often called “combination therapies.” Inflammatory disease are no exception. To treat inflammatory disorders using the methods and compositions of the present invention, one would generally contact a target cell or subject with a GBV-C E2 agent and at least one other therapy. These therapies would be provided in a combined amount effective to achieve a reduction in one or more disease parameter. This process may involve contacting the cells/subjects with the both agents/therapies at the same time, e.g., using a single composition or pharmacological formulation that includes both agents, or by contacting the cell/subject with two distinct compositions or formulations, at the same time, wherein one composition includes the GBV-C E2 agent and the other includes the other agent.

Alternatively, the GBV-C E2 agent may precede or follow the other treatment by intervals ranging from minutes to weeks. One would generally ensure that a significant period of time did not expire between the time of each delivery, such that the therapies would still be able to exert an advantageously combined effect on the cell/subject. In such instances, it is contemplated that one would contact the cell with both modalities within about 12-24 hours of each other, within about 6-12 hours of each other, or with a delay time only of about 12 hours. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either the GBV-C E2 agent or the other therapy will be desired. Various combinations may be employed, where the GBV-C E2 agent is “A,” and the other therapy is “B,” as exemplified below:

```
A/B/A/B/B
A/B/A/B/B
A/B/A/B/B
A/B/A/B/B
```

Other Combinations are Contemplated.

Agents or factors suitable for use in a combined therapy against an inflammatory disorder include steroids, glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDS; including COX-1 and COX-2 inhibitors), aspirin, ibuprofen, and naproxen. Analgesics are commonly associated with anti-inflammatory drugs but which have no anti-inflammatory effects. An example is paracetamol, called acetaminophen in the U.S. and sold under the brand name of Tylenol. As opposed to NSAIDS, which reduce pain and inflammation by inhibiting COX enzymes, paracetamol has recently been shown to block the uptake of endocannabinoids, which only reduces pain, likely explaining why it has minimal effect on inflammation.

The skilled artisan is directed to “Remington’s Pharmaceutical Sciences” 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologies standards. It also should be pointed out that any of the foregoing therapies may prove useful by themselves in treating inflammation.

V. PHARMACEUTICAL COMPOSITIONS AND ROUTES OF Administration

Where clinical applications are contemplated, it will be necessary to prepare pharmaceutical compositions in a form appropriate for the intended application. Generally, this will entail preparing compositions that are essentially free of pyrogens, as well as other impurities that could be harmful to humans or animals.

One will generally desire to employ appropriate salts and buffers to render proteins stable. Buffers also will be employed when proteins are introduced into a patient. Aqueous compositions of the present invention comprise an effective amount of the protein or polypeptide, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous media. Such compositions also are referred to as inocula. The phrase “pharmaceutically or pharmacologically acceptable” refer to molecular entities and compositions that do not produce adverse, allergic, or other untoward reactions when administered to an animal or a human. As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the vectors or cells of the present invention, its use in therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

The percentage of active compound in any pharmaceutical preparation is dependent upon both the activity of the compound. Typically, such compositions should contain at least 0.1% active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful compositions is such that a suitable dosage will be obtained.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile
powders for the extemporary preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy injection is possible. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, phenylmercuric nitrate, m-cresol, and the like. In many cases, it will be possible to use isotonic solutions, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate, and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by sterile filtration. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying techniques that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The present invention contemplates GBV-C E2 peptide and polypeptides, and nucleic acid molecules coding therefore. In some embodiments, pharmaceutical compositions are administered to a subject. Different aspects of the present invention involve administering an effective amount of an aqueous composition. In another embodiment of the present invention, therapeutic GBV-C E2 compositions are administered to a subject. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Those of skill in the art are well aware of how to apply antibodies or other binding agents, as well as gene delivery to in vivo and ex vivo situations.

The phrases “pharmaceutically acceptable” or “pharmacologically acceptable” refer to molecular entities and compositions that do not produce an adverse, allergic, or other untoward reaction when administered to an animal, or human, as appropriate. As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-cancer agents, can also be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g., tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalants and the like.

The active compounds of the present invention can be formulated for parenteral administration, e.g., formulated for injection via the intravenous, intramuscular, intrathoracic, subcutaneous, or even intraperitoneal routes. Administration by i.v. or i.m. are specifically contemplated.

The compositions may be formulated as neutral or salt forms. Pharmaceutically acceptable salts, include the acid salts and those which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylaminoethanol, histidine, proline, and the like.

In certain embodiments, it may be desirable to provide a continuous supply of compositions to the patient. For intravenous or intraarterial routes, this is accomplished by drip system. For various approaches, delayed release formulations could be used that provided limited but constant amounts of the therapeutic agent over and extended period of time. For internal application, continuous perfusion, for example with a GBV-C peptide, may be preferred. This could be accomplished by catheterization followed by continuous administration of the therapeutic agent. The time period for perfusion would be selected by the clinician for the particular patient and situation, but times could range from about 1-2 hours, to 2-6 hours, to about 6-10 hours, to about 10-24 hours, to about 1-2 days, to about 1-2 weeks or longer. Generally, the dose of the therapeutic composition via continuous perfusion will be equivalent to that given by single or multiple injections, adjusted for the period of time over which the injections are administered. It is believed that higher doses may be achieved via perfusion, however.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, Remington’s Pharmaceutical Sciences, 1990). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

An effective amount of the therapeutic composition is determined based on the intended goal. The term “unit dose” or “dosage” refers to physically discrete units suitable for use in a subject, each unit containing a predetermined quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

Peptides may be administered in a dose that can vary from 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 mg/kg of weight to 50, 60, 70, 80, 90, 100, 110, 120,
In many instances, it will be desirable to have multiple administrations of the peptides or other compositions of the invention. The compositions of the invention may be administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times. The administrations will normally be from one to twelve week intervals, more usually from one to four week intervals.

Dosages commonly used for formulations that provide passive immunity are in the range of from 0.5 ml to 10 ml per dose, preferably in the range of 2 ml to 5 ml per dose. Repeated doses to deliver the appropriate amount of active compound are common. Both the age and size by weight of the recipient must be considered when determining the appropriate dosage of active ingredient and volume to administer.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment (amelioration of symptoms versus cure) and the potency, stability, and toxicity of the particular therapeutic substance.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

As used herein, the term in vitro administration refers to manipulations performed on cells removed from an animal, including, but not limited to, cells in culture. The term ex vivo administration refers to cells that have been manipulated in vitro, and are subsequently administered to a living animal. The term in vivo administration includes all manipulations performed on cells within an animal.

VI. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1

Materials and Methods

Expression of GBV-C E2 Proteins.

The near full-length GBV-C E2 protein (nt 1167-2161, GenBank AF 121950) coding sequences, E2 deletion mutants (N-terminal 219 amino acids (nucleotides 1167-1824) and C terminal 112 amino acids (nucleotides 1824-2161)) and control sequences were ligated into a modified pTRE2-Hyg plasmid (Clontech Laboratories, Mountain View, Calif.) as described (Xiang et al., in press). This plasmid generates a bicistronic message encoding the viral sequence followed by the encephalomyocarditis virus (EMC) internal ribosomal entry site (IRES) that directs translation of GFP as previously described (Xiang et al., 2006).

Jurkat (tet-off) cell lines (Clontech, Inc.) were transfected with plasmids encoding GBV-C E2 proteins, an E2 plasmid in which frameshift mutation was inserted to abrogate protein expression (FS) or the empty vector expressing green fluorescent protein (GFP) only (vector control; VC). Insert and control sequences were confirmed by sequencing plasmid DNA (University of Iowa DNA Core Facility). All transfections were accomplished using the Nucleofector II device (Lonza Inc.). Stable cell lines were generated after selection in hygromycin and neomycin (200 μg/ml) and GFP-positive cells were bulk sorted using a BD FACSDiva (University of Iowa Flow Cytometry Facility). Expression of GBV-C E2 protein or E2 mutants were analyzed either by western blot using tetra-his antibody (Qiagen) or GFP expression by flow cytometry (BD FACScan). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (heat-inactivated), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin with hygromycin and neomycin (200 μg/ml).

Recombinant GBV-C E2 protein fused to Fc at the C terminus was constructed by inserting the human IgG Fc coding sequence at nt 2161 of GBV-C E2 in the pTRE2-Hyg vector. CHO cells were transfected and selected by hygromycin and GFP expression. Following stable transfection, cells were adapted to serum free media as described (McLinden et al., 2006). Fusion protein expressed in CHO cells was purified by protein G affinity chromatography as described (Mohr et al., 2010), and analyzed by SDS-PAGE and immunoblot analysis as described (McLinden et al., 2006).

Cell Stimulation

Jurkat cells (1x10⁶ cells/ml) were stimulated with plate-bound anti-CD3 (5 μg/ml, OKT3 clone, ebioscience) and soluble CD28 (5 μg/ml, clone CD28.2, BD Biosciences). Following obtaining written consent peripheral blood mononuclear cells (PBMCs) from four healthy subjects were isolated by Ficoll-Hypaque density gradient centrifugation. PBMCs were washed with PBS and incubated with purified GBV-C E2 protein (20 μg/ml) or purified human IgG (20 μg/ml, Sigma) for 48 hours and stimulated with anti-CD3 (500 ng/ml) and soluble CD28 (500 ng/ml). Following 24 hours of stimulation, cells were analyzed for measurement of cytokine and cellular receptor expression. To measure STS phosphorylation Jurkat cells were prepared as described (Camargo et al., 2009). Briefly, cells were stimulated with 1 μg/ml of anti-CD3 and soluble CD28 for 48 hours followed by 24 hours of serum starvation. Cells were washed and incubated with IL-2 (250 U/ml; Zephtometric) for 15 minutes. Cellular lysates were separated by polyacrylamide gel electrophoresis using 10% gels and membrane was incubated with anti-phospho Stat5 (pY694; BD Biosciences) or anti-Stat5 (BD Biosciences). Phosphorylation of Stat5 and total Stat5 expression was detected with Amer sham ECL (GE Healthcare) using a Kodak Imager.

Cytokine Quantification

IL-2 cytokine released into cell culture supernatant was quantified using human IL-2 quantikine ELISA kit (R&D Systems, Minneapolis, Minn.) according to manufacturer's instructions.

mRNA Expression

Total cellular RNA was extracted using RNeasy Mini Kit (Qiagen) following DNase treatment (RNase-Free DNase Set, Qiagen). Complementary DNA (cDNA) was generated using RT² First Strand cDNA Kit (SABiosciences) and relative expression of IL-2 and CD25 mRNA was deter-
mined using RT-qPCR primer assay for human IL2 and human CD25 (SABiosciences) and normalized to 18S rRNA using ABI 7500 Real Time PCR system.

Flow Cytometry—CD25 Surface Expression.
Cells were stained with the following antibodies from Becton Dickinson (BD) per manufacturer’s recommendations: CD3 (V450), CD4 (PE), CD8 (Alexa 700) and CD25 (APC) Staining was performed on ice for 1 hour and cells were subsequently washed 3 times with PBS. Data was acquired on BD LSR II flow cytometer using single stained CompBeads (BD Biosciences) for compensation. At least 10,000 total events were collected and FlowJo program (Tree Star Inc.) was used for data analysis.

Cell Proliferation.
Jurkat cells expressing GBV-C E2 protein or vector control (FS) were stained with cell proliferation dye (eFlour450, eBioscience) and stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble CD28 (1 μg/ml) and cell proliferation was measured by gating eFlour450-positive cells in flow cytometry.

Statistics.
Statistics were performed using GraphPad software V4.0 (GraphPad Software Inc.). Comparisons between two groups were carried out using two-sided Student's t tests. P values less than 0.05 were considered statistically significant.

Example 2

Results

GBV-C E2 Protein Expression Inhibits IL-2 Production.
CD4+ Jurkat (tet-off) T cell lines that stably expressed the GBV-C E2 protein (331 amino acids), the N terminal 219 amino acids, and the coding region for the E2 protein with a frame-shift to abolish protein translation (FS) were previously described (reference—Jinhuai). In addition, a stable cell line expressing the E2 protein region from amino acid 219 to 331 was generated (FIG. 1A). GFP expression in these cell lines was determined by flow cytometry (FIG. 1B), and the e2 protein expression was detected in Jurkat cell lysates with tetra-his antibody directed to C-terminal his-tag fused with e2 protein (FIG. 1C). Expression of GBV-C E2 RNA in Jurkats expressing E2 frameshift construct was confirmed by RT-PCR and DNA sequencing. E2 expressing Jurkat cells cultured with doxycycline (doxy, 1 μg/ml) had significantly reduced expression of E2 as determined by immunoblot (FIG. 1C) and flow cytometry (data not shown).

IL-2 production was significantly inhibited in Jurkat cells expressing GBV-C E2 compared to the VC following activation through T cell receptor (TCR) engagement with anti-CD3 and soluble CD28 antibodies (FIG. 2A). IL-2 inhibition required GBV-C E2 protein expression as Jurkat cells expressing E2 RNA but not protein (FS) did not inhibit IL-2 production (FIG. 2A). Jurkat cells expressing the N terminal 219 amino acids of GBV-C E2 protein (E2 1-219) also reduced IL-2 production compared to the VC; however, the C-terminal E2 amino acids (219 to 331) did not inhibit IL-2 production following stimulation (FIG. 2A). Stadye state IL-2 mRNA levels were significantly reduced in GBV-C E2 expressing cells compared to the VC and E2 RNA expressing cells (FS), suggesting that the reduction is due to a reduction in IL-2 mRNA transcription (FIG. 2B). Growing GBV-C E2 expressing Jurkats in doxycycline (dox) reduced E2 expression (FIG. 1C), and this restored the concentration of IL-2 mRNA suggesting GBV-C E2 protein expression alters TCR-mediated IL-2 transcription (FIG. 2B).

GBV-C E2 Protein Expression Inhibits CD25 Expression and STATS Phosphorylation.

Surface expression of CD25, the alpha chain of IL-2 receptor is upregulated following activation of T cells (reviewed in Cheng et al., 2011; Kim et al., 2006). Following upregulation, CD25 interacts with CD122 (IL-2 beta) and CD132 (IL-2-gamma) to form the high affinity IL-2 receptor (IL-2R) that binds IL-2 and initiates IL-2 signaling (reviewed in Cheng et al., 2011; Kim et al., 2006). Since GBV-C viremia is associated with altered response to IL-2 therapy in HIV-infected subjects (Stapleton et al., 2009), the inventors examined the effect of GBV-C E2 protein on CD25 expression. The percentage of CD25+ cells was significantly lower in Jurkat cells expressing GBV-C E2 compared to controls and Jurkat cells cultured in doxycycline (FIG. 3A). Furthermore, GBV-C E2 expression abrogated the upregulation of CD25 following TCR engagement, and this was prevented by growing the Jurkat cells expressing E2 in doxycycline (FIG. 3A). GBV-C E2 expression resulted in a reduction in CD25 mRNA levels, suggesting that the mechanism of CD25 reduction is mediated specifically by GBV-C E2 protein effects on CD25 transcription (FIG. 3B). Since CD25 expression is essential for IL-2 signaling we further investigated the effect of GBV-C E2 protein expression on IL-2 signaling pathways. Phosphorylation of STATS is rapidly detected after IL-2 and IL-2R interaction and is critical for IL-2 signaling (Lin and Leondard, 1997). Following stimulation with IL-2 for 15 minutes, STATS phosphorylation was detected in FS control cells but was completely inhibited in Jurkat cells expressing GBV-C E2 protein (FIG. 3C). Since total STATS protein expression did not alter by GBV-C E2 protein expression (FIG. 3C) these data indicate a significant alteration of IL-2 signaling pathway by GBV-C E2 protein.

GBV-C E2 Protein Reduces Activation Induced Proliferation in Jurkat Cells.

To examine if GBV-C E2-expression induced inhibition of IL-2 release, CD25 expression and STATS phosphorylation affected T cell proliferation, the inventors assessed Jurkat cells expressing GBV-C E2 or FS by flow cytometry. At baseline, there was no difference in the percentage of Jurkat cells positively stained for the proliferation dye eFlour450 compared to control cells (Day 0, FIG. 4A). However, following TCR engagement with anti-CD3/CD28, GBV-C E2 protein reduced proliferation in Jurkat cells compared to the FS control (Day 5, FIG. 4A). Cell proliferation was significantly reduced in Jurkats expressing E2 protein during five days of anti-CD3/CD28 stimulation (FIG. 4B).

GBV-C E2 Protein Inhibits IL-2 Production and CD25 Expression in Primary CD4+ and CD8+ T cells.

Recombinant GBV-C E2 protein fused to human IgG Fc was analyzed by SDS-PAGE and immunoblot analysis. Purified protein fractions were enriched for GBV-C E2 and reacted with anti-human IgG antibodies (FIG. 5A). Following stimulation with anti-CD3/CD28, IL-2 secretion was significantly reduced in PBMC’s from healthy subjects incubated with GBV-C E2 protein compared to human IgG (n=4; P<0.008) (FIG. 5B). Consistent with findings in Jurkat cells, following stimulation with anti-CD3/CD28, CD25 expression was significantly reduced in CD4+ and CD8+ T cells from healthy subjects incubated with GBV-C E2 protein compared to the IgG control (FIG. 5C).
Mechanism(s) for GB Virus C (GBV-C) E2 Protein Inhibition of T Cell Activation.

In humans infected with GBV-C, circulating CD4+ and CD8+ T cells have significantly lower levels of surface receptors that associated with T cell activation (Maidana-Giret et al., 2009). To date, no mechanism for how this phenotype has been described. The inventors investigated the hypothesis that GBV-C envelope glycoprotein E2 may alter T cell signaling, leading to a block in activation following activation stimuli. They expressed the GBV-C E2 protein truncated at the C-terminus to remove the transmembrane domain (res 1-331), or nucleotides 1167-2161 based on GenBank Accession No. AF121950) in a Jurkat (CD4+) T cell line. Cell lines expressing smaller portions of E2 were also generated (1-219, 219-331), and control cells including a vector control and a control cell line in which 1167-2161 were interchanged. To insert the C-terminus was inserted to abolish translation of the E2 (1-331) protein.

Following stimulation with anti-CD3 and anti-CD28 antibody, cells expressing GBV-C E2 had significantly less activation, as measured by the activation-regulated marker CD69, than did the frame shift control cells (FS: FIG. 6A). Reduction in E2 expression by growing cells in dexamethasone (these are Tet-Off cells) restored activation response (CD69 surface expression) in the cells containing E2 protein (FIG. 6B). To assess T cell receptor (TCR)-related signaling pathways, LAT phosphorylation was assessed by ELISA, and E2 protein expression was associated with significantly less phosphorylation of LAT following TCR activation (FIG. 7).

Further assessment using immune blot analysis revealed that E2 protein expression reduced activation of LAT, ZAP 70, and Lck at the activation site of amino acid 394, all critical molecules in the activation cascade initiated by engagement of the TCR (FIGS. 8-9). As shown in FIG. 9, CD45 phosphorylation of Lck at position 505 is required for activation of Lck. Lck505 is not diminished following TCR engagement (FIG. 8). This was due to altered CD45 expression on the GBV-C E2 expressing Jurkat cells (FIG. 10). To determine if GBV-C E2 interacted with Lck, Jurkat cell lysates were incubated with recombinant GBV-C E2 (fused to human Fc protein) or human IgG and precipitated with protein A/G beads. Lck was readily detected in the control lysate, E2-Fc precipitated Lck while human IgG did not (FIG. 11A). Similarly, GBV-C E2 expressing Jurkat cell lysates were incubated with anti-Lck antibody or isotype control IgG and precipitated with protein A/G beads. GBV-C E2 was precipitated by Lck (FIG. 11B). Thus, GBV-C E2 directly interacts with the Src-family kinase Lck.

In summary, expression of the GBV-C envelope glycoprotein E2 in Jurkats (CD4+ T cells) significantly inhibited activation (CD69 surface expression) upon TCR stimulation suggesting T cell activation pathways are modulated by E2 protein expression. The activation of proximal signaling downstream of the TCR, specifically LAT, ZAP-70 and Lck, was inhibited by GBV-C E2 protein expression. The Lck activation was not due to altered CD45 phosphate levels or activity (data not shown). Immuno-precipitation and pull down experiments demonstrated interactions between GBV-C E2 protein and Lck. Thus, GBV-C directly interacts with Lck, the critical T cell tyrosine kinase in the Src kinase family, reducing downstream signaling. This leads to a reduction in T cell activation, release of proinflammatory cytokines, and proliferation. These represent a novel approach to modulating T cell activation and inflammation.

Example 3

Materials and Methods

Expression of GBV-C E2 protein. Tet-off Jurkat cell lines expressing GBV-C E2 protein (at 1167-2161 based on GenBank AF121950), the vector control (expressing GFP) and E2 coding sequence with a plus one frameshift mutation inserted to abolish protein expression (FS control) were previously described. Six truncated E2 proteins were cloned into a modified pIRE2-HYG plasmid (Clontech, Inc.) as described. This plasmid generates a bicistronic messaging encoding the GBV-C E2 sequence followed by the encephalomyocarditis virus (EMC) internal ribosomal entry site (IRES) that directs translation of GFP. Jurkat (tet-off) cell lines (Clontech, Inc.) were transfected (Nucleofector II, Lonza Inc.) and cell lines were selected for resistance to hygromycin and neomycin. GFP-positive cells were bulk sorted using a BD FACS Diva (University of Iowa Flow Cytometry Facility). Protein expression was analyzed by measuring GFP by flow cytometry (BD LSR II) and by immunoblot using antibodies directed against a C-terminal histidine tag (Qiagen). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum (heat-inactivated), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin with hygromycin and neomycin (200 μg/ml). Insert sequences were confirmed by sequencing plasmid DNA (University of Iowa DNA Core Facility).

Cell Stimulation.

Jurkat cells (5×10⁶ cells/ml) were stimulated with plate-bound anti-CD3 (5 μg/ml, OKT3 clone, eBioscience) and soluble CD28 antibody (5 μg/ml, clone CD28.2, BD Biosciences) unless stated otherwise. For co-culture experiments, non-transfected GFP-negative Jurkat cells were (5×10⁵ cells/ml) were incubated with either transfected GFP-positive vector control or GFP-positive GBV-C E2-expressing cells (1×10⁶ cells/ml) for 72 hours prior to stimulation with anti-CD3/CD28. Following 24 hours of stimulation, cellular receptor expression and cytokine release were measured by flow cytometry in GFP-negative cells and by ELISA respectively.

Flow Cytometry.

Cellular receptor expression was measured using the following antibodies per manufacturer’s recommendation: CD69 (PE), CD25 (APC), CD45 (PE) (BD Biosciences). Cells were incubated on ice for 1 hour, washed 3 times with PBS and fixed in 2% paraformaldehyde (Polysciences). Data was acquired on BD LSR II flow cytometer using single stained CompBeads (BD Biosciences) for compensation. At least 10,000 total events were collected in each experiment and the FlowJo program (Tree Star Inc.) was used for data analysis. All flow cytometry experiments were repeated at least three times with consistent results.

Immunoblot Analysis.

Jurkat cells (5×10⁶) were stimulated with anti-CD3 (5 μg/ml) for indicated times prior to the addition of cell lysis buffer (Cell Signaling) for 15 minutes and briefly sonicated. Lysates were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Membranes were incubated in protein-free blocking buffer (Thermo Scientific) for 1 hour at room temperature followed by incubation with primary antibodies Immunoreactive proteins were detected with Amersham ECL (GE Healthcare) using a Kodak Imager. Protein phosphorylation was quantified using ImageJ (NIH) and normalized to total protein levels. Primary antibodies used were: pLAT (Y226; BD Biosciences); total LAT (Biolegend); CD63 antibodies
and pZAP70 (Y319); total ZAP70; pLck (Y505); pLck (Y394/SerY416); total Lck (Y394) and total Csk (all from Cell Signaling Technology). For immune precipitation studies, Jurkat cell lysates were incubated with recombinant Fe fused GBV-C E2 protein\(^5\) or alternatively GBV-C E2 expressing Jurkat cell lysates were incubated with anti-Lck antibodies overnight at 4°C, as described. Protein complexes were isolated from the cellular lysates using protein A/G agarose beads (Thermo Scientific) and precipitated proteins were detected as described above.

ELISA.

pLAT (Y191) was quantified using PathScan ELISA kit (Cell Signaling Technology) and IL-2 cytokine released into cell culture supernatant was quantified using human IL-2 quantikine ELISA kit (R&D Systems) according to manufacturer’s instructions.

Enzyme Assays.

CD45 activity was measured using CD45 tyrosine phosphatase assay kit (Enzo Life Sciences) following the manufacturer’s instructions. Purified recombinant GBV-C E2 protein expressed in CHO cells was described previously\(^3\). Enzymatic activity was evaluated with or without GBV-C E2 protein (10 μg) or human IgG control (10 μg; Sigma) at room temperature. Following 1 hour incubation, the reaction was terminated and absorbance determined by a Microplate reader (Model 680, Bio-Rad) at OD\(_\text{405nm}\). Phosphorylation of GBV-C E2 protein by Lck was measured by incubating recombinant E2 protein (40 μg) with or without human Lck (500 μg; R&D Systems) as recommended by the manufacturer. Samples were subjected to immunoblot analysis as described above. Phosphorylation was determined by immunoblot analysis with phosphorytoseine antibodies (Invitrogen) and GBV-C E2 protein was identified using an anti-E2 monoclonal antibody. Lck mediated phosphorylation of GBV-C E2 derived TAT-peptides were performed using Lck kinase enzyme system (Promega) as recommended by the manufacturer.

GBV-C E2 Synthetic Peptides.

FITC labelled synthetic peptides with an N-terminal HIV Tat protein transduction domain (TAT) alone (GGGGGRKKRRQRRR; SEQ ID NO: 13), or with the GBV-C E2 aa 86-101 (GGGGGRKKRRQRRRYPGVSSTCVWGVS; SEQ ID NO: 14; Y87), or the Y87H mutation (GGGGGRKKRRQRRRVYPLGGVSSTCVWGVS; SEQ ID NO: 15) were obtained from Ana Spec, Inc. Peptides with the TAT domain and GBV-C E2 aa 276-292 (GGAGLGGRKVEPLVRC; SEQ ID NO: 16), or the same amino acids in a scrambled order (GRCARGLVTPTGEGFY; SEQ ID NO: 17) as previously described\(^2\). Peptides were dissolved in RPMI with 10% DMSO. The TAT domain enhances cellular uptake of the peptide. Healthy donors PBMCs (1x10\(^6\) cells/ml) were incubated with 20 μg peptide at 37°C over night before stimulation with 500 ng/ml anti-CD3/CD28. IL-2 release and cellular receptor expression was analyzed 24 hrs later.

GBV-C RNA Quantification.

GBV-C viremic HIV-infected subjects receiving ART who were attending the University of Iowa HIV Clinic and healthy volunteer blood donors were invited to participate. HIV-infected subjects’ HIV viral load (VL) was below the limit of detection (<48 copies/mL) for a minimum of 6 months and at the time of blood donation. All subjects provided written informed consent and the study was approved by the University of Iowa Institutional Review Board. PBMCs were prepared as described\(^4\). For sorting experiments, CD3+ T cells were enriched using Automacs (Miltenyi Biotech), and CD3+ T cells were sorted into CD4+ and CD8+ populations by FACS (BD ARIA II) using CD3 (Y450), CD4 (FITC), CD8 (Alexa700) antibodies (all BD Biosciences). Sorted cells were counted using Countess\(^\text{TM}\) automated cell counter (Invitrogen). Total cellular RNA from specific T cell populations was isolated and GBV-C RNA was quantified by real-time RT-PCR as described\(^6\).

EMV were purified from the clarified cell-culture supernatant or from human serum using ExoQuick reagent (Systems Biosciences) according to the manufacturer’s instructions. This commercial reagent has been previously reported to yield EMV from cell culture supernatant and human serum\(^20-32\). Sodium chloride (NaCl) density flotation was performed as described\(^3\). Briefly, 1 ml of undiluted serum was mixed with 35 ml of NaCl solution (1.063 g/ml), and centrifuged in a Beckman SW28 rotor (112,000xg, 4°C). After 65 hours of spin, top and bottom fractions were collected for subsequent analysis. PBMCs from healthy donors were incubated with EMV purified from 5 ml of GBV-C-positive or GBV-C-negative serum or EMV purified from 10 ml of culture supernatant overnight and stimulated with anti-CD3/CD28 antibodies (500 ng/ml) for 24 hours before analysis.

Statistics.

Statistics were performed using GraphPad software V4.0 (GraphPad Software Inc.). Two-sided Student's t test was used to compare results between GBV-C E2 protein expressing cells and controls. P values less than 0.05 were considered statistically significant.

Example 4

Results

Extracellular microvesicles from GBV-C infected human serum inhibit TCR signaling in primary human T cells. GBV-C infection is associated with global reduction in T cell activation and reduced IL-2 signaling in the peripheral blood mononuclear cells (PBMCs)'s/Rydzew, et al., 2012; Bhattarai et al., 2012; Stapleton, 2009; Maida-Miguel, 2009 and Stapleton, 2012). Since the frequency of GBV-C infected lymphocytes in peripheral blood is unknown, we quantified GBV-C RNA copy number within CD4+ and CD8+ T cells obtained from nine GBV-C viremic subjects. Using immuno-affinity selection and fluorescent activated cell sorting (FACS) to sort CD4+ and CD8+ T cells from peripheral blood mononuclear cells (PBMCs), we recovered highly purified (>99%) populations (Fig. 18). GBV-C RNA was detected in PBMCs obtained from all nine subjects with an average of 879 genome equivalents (G.E.) per 10\(^6\) cells (Fig. 12A). GBV-C RNA was detected in both CD4+ T cells (average 146 G.E per 10\(^6\) cells) and CD8+ T cells (average 77 G.E per 10\(^6\) cells) in all but two subjects. One of these subjects had GBV-C RNA only detected in CD44+ T cells while the other had GBV-C RNA present in only the CD84+ T cell population (Fig. 12A). Thus, assuming that there is only one GBV-C RNA produced per cell, less than 10% of PBMCs are infected. However, it is likely that each cell contains multiple copies of viral RNA and that the proportion of GBV-C infected PBMCs is much lower than 10%. Given the low frequency of circulating infected cells, GBV-C infection must alter TCR activation in uninfected T cells to explain the global reduction in T cell activation as observed in previous clinical studies.

Since a closely related virus HCV transmits viral RNA and proteins to bystander cells via extracellular microvesicles (EMV) (Dreux, 2012 and Dreux, 2012), the inventors
hypothesized that GBV-C may utilize a similar mechanism to interact with bystander cells. To test this hypothesis, they examined EMV from the serum of GBV-C viremic subjects for the presence of GBV-C RNA. EMV purified using a commercial reagent (Exoquick) contained GBV-C RNA (Fig. 12B). Above 98% of GBV-C RNA in the serum was associated with EMV and only less than 2% of RNA was found in the leftover supernatant (Fig. 12B). Consistent with a previous study (Xiang, 1998), saline flotation gradient centrifugation of GBV-C-positive serum yielded two populations of RNA-containing particle with distinctly different densities (Fig. 21D). Viral RNA was concentrated in a low density fraction (Top; -1.07 g/ml), consistent with LDL-associated particles, and a heavier fraction (Bottom; -1.16 g/ml). The heavier particles had a density similar to that described for vesicles of endocytic origin (exosomes; 1.10-1.19 g/ml) (Meckes, 2011) and were precipitated by Exoquick reagent. In contrast, the lower density particles did not precipitate with Exoquick reagent suggesting microvesicles of endocytic origin are preferentially precipitated by Exoquick reagent (data not shown). Incubation of primary human CD4+ and CD8+ T cells from healthy blood donors with the EMV prepared from GBV-C viremic sera (GB+EMV) inhibited TCR signaling, as measured by the release of IL-2 and cell surface expression of CD69 and CD25 (T cell activation markers) following TCR engagement with CD3/CD28 antibodies compared to cells incubated in EMV obtained from GBV-C non-viremic controls (GB-EMV) (Figs. 12E-G).

GBV-C E2 Protein Inhibits TCR-Mediated Activation of CD4+ T Cells.

Previous study suggested that GBV-C E2 protein inhibits T cell activation and IL-2 signaling pathways in human T cells (Xiang, 1998). To determine if TCR activation was altered by E2 protein, E2 RNA or both, activation was measured in tet-off Jurkat (CD4+) T cells before and following TCR stimulation with CD3/CD28 antibodies. Tet-off Jurkat cells stably expressing the GBV-C E2 protein or the GBV-C E2 sequence in which a plus one frame shift was inserted to abolish translation (FS) were incubated with or without doxycycline (1 μg/ml) for 5 days to reduce expression of GBV-C E2 (Fig. 13A). Following TCR stimulation, surface expression of CD69 (a marker for T cell activation) was significantly inhibited in E2 expressing Jurkat cells compared to the control FS cells expressing the E2 RNA sequence, and this inhibition was reversed in cells maintained in doxycycline (Fig. 13B). Thus, GBV-C E2 protein and not the E2-coding RNA was responsible for the reduced activation following TCR stimulation.

Since GBV-C E2 protein expression inhibited surface expression of CD69 following TCR stimulation (Fig. 13B), the effects of E2 protein on proximal TCR signaling pathways were assessed. Following TCR stimulation, phosphorylation of the linker for activation of T cells (LAT) (Fig. 13C, Fig. 19A) and zeta-chain-associated protein kinase (ZAP)-70 (Fig. 13D, Fig. 19B) was reduced in GBV-C E2 expressing cells compared to the FS control. This reduction in phosphorylation was due to inhibition of TCR signaling, as the total cellular LAT and ZAP-70 protein levels were not different between the E2 expressing and FS control Jurkat cells (Fig. 13C-D).

GBV-C E2 Protein Inhibits Lck Activation.

Lymphocyte specific protein tyrosine kinase (Lck) activation is required for signaling through the TCR (Davis and van der Merwe, 2011). Inactive Lck is phosphorylated at tyrosine 505 (Y505) by the C-src tyrosine kinase (Csk). Following TCR engagement, phosphorylated Y505 is dephosphorylated by CD45 tyrosine phosphatase, leading to a change in conformation and subsequent auto-phosphorylation of Lck tyrosine 394 (Y394) in trans (Davis and van der Merwe, 2011). Phosphorylated Y394 in Lck is the active form, is required for ZAP70 phosphorylation, and drives downstream signaling through the TCR pathway.

Following TCR engagement with anti-CD3, Lck activation, as measured by phosphorylation of Y394, was reduced in Jurkat cells expressing E2 protein compared to FS controls (Figs. 14A-B). This inhibition was not due to altered Lck regulation, as CD45 and Csk expression levels were similar in both GBV-C E2 expressing cells and the FS control cells (Figs. 20A-B). Furthermore, CD45 phosphatase activity was not altered in vitro by incubation with recombinant GBV-C E2 protein (Fig. 20C). To determine if GBV-C E2 interacted directly with Lck, recombinant GBV-C E2 protein incubated with the Jurkat cell lysate was precipitated and probed for the presence of co-precipitating signaling molecules. GBV-C E2 protein specifically co-precipitated Lck but not ZAP-70 or LAT from Jurkat cell lysate (Fig. 14C). Immunoprecipitation in the reversed order confirmed the association of GBV-C E2 and Lck as immunoprecipitation of Lck co-precipitated E2 protein from Jurkat cells expressing E2 protein (Fig. 14D).

Expression of the N-terminal region 219 aa of GBV-C E2 protein is sufficient to inhibit IL-2 production following TCR stimulation (Bhattarai, 2012). To map the site(s) in the GBV-C E2 protein that mediated changes in TCR signaling, we generated a series of Jurkat cells expressing GBV-C E2 deletions (Fig. 15A). All cell lines stably expressed GFP, as demonstrated by flow cytometry (Fig. 21). Following TCR stimulation with anti-CD3/CD28, IL-2 production was blocked in all cell lines that expressed a 13 amino acid motif within GBV-C E2 (aa 86-98), but not in cell lines that expressed other regions of E2 without this motif (Fig. 15B). Utilizing kinase-specific phosphorylation substrate prediction programs, the inventors identified a tyrosine residue at position 87 (Y87) in GBV-C E2 that is predicted to be Lck (Src-kinase) target (Fig. 15A) (Xiang, 1998 and Meckes, 2011). Confirming this prediction, in vitro GBV-C E2 protein was found to be phosphorylated by Lck as demonstrated by immunoblot analysis using phospho-tyrosine specific antibodies (Fig. 15C). Similar to Lck, the GBV-C E2 protein was dephosphorylated by CD45 tyrosine phosphatase (Fig. 15C).

The predicted Lck substrate motif within GBV-C E2 protein (aa 83-91; PQYYGGSVS) is highly conserved and there is no sequence variation detected among 39 of the 42 complete human GBV-C isolates that represent the seven identified genotypes (Fig. 16A). The three isolates that differ do so at a single aa (Q84I or V90A), and neither of these changes altered predicted Lck phosphorylation site. In contrast, there were significant differences in this region of the E2 protein of the chimpanzee variant of GBV-C (GBV-Ccpz) sequences (83-91 aa; PRYVHGHIT; Fig. 22A). In addition, GBV-Ccpz E2 protein contained a histidine residue at position 87 (H87) instead of a tyrosine and expression of this protein in Jurkat cells did not inhibit IL-2 production following TCR stimulation (Fig. 15D). Furthermore, mutation of the tyrosine (Y87) in the human GBV-C E2 (aa 86-98) peptide to an alanine (Y87A) reversed the inhibition of TCR signaling as measured by IL-2 release (Fig. 15D). To further assess the specificity of GBV-C E2 protein for TCR signaling inhibition, control Jurkat cells or Jurkat cells expressing the human GBV-C E2 (86-98 aa) were stimulated with phorbol-12-myristate-13-acetate (PMA) and ionomycin. PMA-ionomycin stimulation does
not activate Lck (FIG. 23) and PMA-ionomycin induced T cell activation as measured by IL-2 release was not inhibited by the GBV-C E2 (aa 86-98) peptide (FIG. 15E). These data suggest that GBV-C E2 protein specifically inhibits TCR signaling pathways at the level of Lck activation.

To confirm that the predicted Lck substrate motif within GBV-C E2 protein was sufficient to inhibit TCR-mediated signaling in primary human CD4+ and CD8+ T cells, the inventors compared the inhibitory capacities of a series of synthetic peptides with native or mutated (Y887H) sequences in the region of interest (residues 86-101). The peptides were biotinylated to monitor cell uptake and included an N-terminal HIV Tat protein transduction domain (TAT) to promote internalization by target cells. A TAT-only synthetic peptide served as a negative control. All three biotinylated peptides were internalized by healthy human PBMCs, as demonstrated by flow cytometry (FIGS. 24A-D). Following TCR stimulation, IL-2 production by PBMCs was inhibited in cells incubated with the TAT-Y87 peptide, but not in those incubated with either the TAT-Y87H or the TAT control peptide (FIG. 16A). Consistent with this selective inhibition of IL-2 production, surface expression of T cell activation markers (CD69 and CD25) was significantly reduced in primary human CD4+ and CD8+ T cells incubated with the TAT-Y87 peptide compared to mutant or control peptide (FIGS. 16B-C). In addition, the TAT-Y87 peptide served as an Lck substrate in vitro, and was phosphorylated by Lck in a dose-dependent manner (FIG. 16D). In contrast, a synthetically phosphorylated Y87 peptide (TAT-Y87P04) did not serve as an Lck substrate (FIG. 16D), supporting the hypothesis that this region on the GBV-C E2 protein competes for phosphorylation with Lck. There is one additional predicted Lck substrate motif within the GBV-C E2 protein (aa281-289, TGGFYEPLV; FIG. 22B). A synthetic peptide containing this motif (TAT-276-292) also served as a Lck substrate in vitro compared to a control peptide (TAT-SCR) which was synthesized with the same amino acids as the TAT-276-292 in a scrambled order to disrupt predicted Lck substrate sequence (FIG. 16D). Although TAT-276-292 peptide served as a Lck substrate in vitro, expression of this region of E2 protein did not inhibit TCR-induced IL-2 production (Bhattarai, 2012).

GBV-C E2 Protein Inhibits T Cell Activation in Bystander Cells.

Since expression of GBV-C E2 protein alone inhibited TCR signaling (FIGS. 13A-D), the inventors hypothesized that E2-expressing cells may inhibit TCR signaling in bystander cells contributing to global reduction in TCR signaling observed in GBV-C infected subjects (Bhattarai et al., 2012; Stapleton, 2009; Maidana-Giret, 2009 and Stapleton, 2012). To test this hypothesis, GBV-C E2-expressing (GFP-positive) or vector control Jurkat cells (VC; also GFP-positive) were co-cultured with Jurkat cells not expressing GFP. Following TCR engagement, IL-2 secretion and surface expression of the activation markers CD69 and CD25 were significantly inhibited in the bystander Jurkat cells co-cultured with GBV-C E2 expressing cells compared to bystander cells co-cultured with the vector control cells (FIGS. 17A-C). Since, extracellular microvesicles (EMV) purified from the serum of GBV-C infected subjects inhibited TCR signaling when incubated with primary human T cells (FIGS. 12A-F), the inventors tested if E2 protein may get released in EMV from E2 expressing cells. GBV-C E2 protein was detected in EMV purified from E2-expressing Jurkat cell culture supernatant but not the VC supernatant fluid (FIG. 17D). These EMV also contained CD63 suggesting that they were of endocytic origin (Meckes, 2011) (FIG. 17D) consistent with the EMV obtained from GBV-C infected human serum (FIGS. 12B-C). To determine if GBV-C E2 protein released from Jurkat cells reduced TCR signaling in bystander T cells, primary human CD4+ and CD8+ T cells from healthy blood donors were incubated with EMV purified from E2-expressing Jurkat cells (E2 EMV) or VC control Jurkat cells (VC EMV). Following TCR engagement, IL-2 release and cell surface expression of CD69 and CD25 was significantly reduced in cells incubated with E2 EMV compared to cells incubated with VC EMV (FIGS. 12E-G).

Example 5

Discussion

GBV-C is an RNA virus that replicates only in the host cell cytoplasm and like the related HCV, it is capable of causing persistent human infection. Among HIV-infected people, persistent GBV-C co-infection is associated with reduced T cell activation and inhibition of IL-2 signaling (Rydz, et al., 2012; Bhattachar, 2012; Stapleton, 2009; Maidana-Giret, 2009 and Stapleton, 2012). The IL-2 signaling defect is due, at least in part, to effects of the envelope glycoprotein E2 (Bhattarai, 2012). The effects of GBV-C on T cell activation and IL-2 signaling may contribute to viral persistence (Bhattarai and Stapleton, 2012). In addition, there is little evidence that antibodies to GBV-C proteins develop during viremia, suggesting an impairment in B cell function which may reflect altered antigen presentation (Stapleton et al., 2011). Although clinical studies demonstrate an association between GBV-C infection and a global reduction in T cell activation (Bhattarai et al., 2012, Maidana-Giret, 2009 and Stapleton, 2012), only a small proportion of T cells contained viral genomes (FIG. 11A). Thus, the virus and viral proteins contained in extracellular microvesicles (EMV) or virus-infected cells must interact with and inhibit activation of uninfected bystander T cells.

In this study we demonstrate that extracellular microvesicles (EMV) present in the serum of GBV-C infected subjects and EMV released by E2-expressing Jurkat cells inhibit TCR signaling in primary human T cells. This is accomplished by reducing the activation of Lck, the proximal tyrosine kinase phosphorylated in the TCR signaling cascade. The data are consistent with the transfer of GBV-C E2 protein within virus particles or in EMV to bystander cells with resultant TCR-signaling inhibition. Since the average GBV-C RNA concentration in infected humans is greater than 1x10^9 (Stapleton, 2003) genome copies/ml of plasma and the virus is produced by T cells (Rydz, et al., 2012), lymphoid tissue is constantly exposed to high concentrations of GBV-C E2 protein in infected humans.

Synthetic peptides containing only one of the two predicted Lck substrate motif inhibited TCR signaling in the CD4+ T cell line and in primary human CD4+ and CD8+ T cells (Y87). Although the tyrosine at aa 285 was phosphorylated by Lck in vitro (FIG. 16D), this region of E2 did not inhibit TCR-mediated activation (Bhattarai et al., 2012). This may be due to inaccessibility of this region of E2 to Lck, as the Y285 is not likely to be exposed on the surface of the protein based on structural models of the related HCV E2 protein (Krey, 2010). This also suggests that not all predicted tyrosine kinase substrate motifs in viral structural proteins will display functional activity.

In addition to the two predicted Lck phosphorylation substrate motifs (aa 83-89 and 281-289; FIG. 22A, 5B), GBV-C E2 protein also contains two well conserved Src
homology domain 3 (SH3) binding domains (PXXP; aa 48-51 and 257-260; FIGS. 22C-D). Although, GBV-C E2 protein interacted with Lck (FIGS. 14C-D) most likely through interactions between SH3 binding domain on GBV-C E2 protein and SH3 domain present on Lck, these two SH3 binding regions were not required for TCR signaling inhibition as expression of amino acids (3-72) and (220-331) of E2 did not inhibit IL-2 release following TCR activation (FIG. 15B) (Bhattarai, et al., 2012). However, it is possible that either these SH3 binding domains may contribute to inhibition of Lck activation in the setting of natural infection.

The predicted Lck substrate motif (aa 83-91) that inhibits TCR signaling is conserved in all human GBV-C (GBV-C_{human}) E2 protein studied, but is not present in the E2 protein from chimpanzee GBV-C (GBV-C_{chimpanzee}) isolates (FIG. 22A) and the expression of the GBV-Cpze E2 protein did not inhibit TCR signaling (FIG. 15D). Based on this observation, it is tempting to speculate that since immune reactivity of lymphocytes from chimpanzee is significantly lower than humans (Sotob, et al., 2010), there is less selective pressure for GBV-Cpze to inhibit TCR signaling for replication. Furthermore, mutation of the conserved tyrosine residue reversed the inhibitory effects of human GBV-C E2 protein (FIGS. 15D, 16A) and Lck phosphorylated GBV-C E2 and the synthetic peptide (Y87) in vitro (FIGS. 15C, 16D). These data demonstrate that the viral envelope glycoprotein is a substrate for Lck and since CD45 dephosphorylated E2 protein (FIG. 15C), phosphorylation of GBV-C E2 may be regulated in the same manner as Lck phosphorylation.

Although GBV-C E2 inhibited TCR-mediated activation compared to control cells when stimulated with anti-CD3/CD28, there were no significant differences in unstimulated cells or cells stimulated with PMA and ionomycin (FIG. 15E) suggesting the inhibition of TCR signaling by GBV-C E2 is not absolute and specifically due to inhibition of Lck activation. Thus, GBV-C infection although affects global T cell activation, the virus does not create a state of severe immune suppression resulting in clinical disease (Bhattarai and Stapleton, 2012).

In summary, the GBV-C structural protein E2 inhibits TCR-mediated T cell activation by interacting with Lck and competing for Lck phosphorylation. The inhibition is mediated by either the expression of GBV-C E2 protein within cells, or by the transfer of E2 to bystander cells as part of the virus particle or within microvesicular particles. These data identify a novel mechanism by which a viral structural protein interfere with tyrosine kinase function resulting in global inhibition of T cell activation and support a proposed model for global alteration of T cell activation during GBV-C infection (FIGS. 25A-C). Recently, a non-biased study of interactions between 70 viral proteins from 30 different viruses and host cells identified specific viral protein interactions with 579 different host proteins. More than half of the host proteins are involved in signal transduction pathways (Pichlmaier, 2012). Since there are numerous predicted kinase binding and substrate sites encoded in viral structural proteins, it is tempting to speculate that the mechanism by which GBV-C inhibits Lck may also apply to other host cell signaling processes, and illustrate the potential for regulation of host cell function by virus particles. These interactions may influence viral persistence and viral pathogenesis. Identification of the interactions between viral structural proteins and host cells may facilitate the design of novel and specific antiviral therapies and vaccines.

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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360
tgcaaatag gtttatcccg cgagttgaca agggacctgt ggggccccgg gtttatgggga
420
agggcccaa acctggctct tccggtgggg cggggsagat cacgggcca cccagctccg
480
cggcgccctg cagcggcggtt agcacaagaa tctctggggtt gagggcgctg ggcatttttc
540
ttttctatac aca gca gtc ctt ctc ctc ctc ctc gtt ggt gag gcc
590
Met Ala Val Leu Leu Leu Leu Val Val Glu Ala
1 5 10

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ggg gcc att ctc gcc ccc gcc gcc acc gct tgt cga gcc aat ggg caa
30
Gly Ala Ile Leu Ala Pro Ala Thr His Ala Cys Arg Ala Asn Gly Gln
15 20 25
tat ttc ctc aca aat tgc tgt gcc cgg gaa gac ato ggg ttc tgc ctt
606
Tyr Phe Leu Thr Asn Cys Ala Pro Glu Asp Ile Gly Phe Cys Leu
30
40

gaa ggc gga tgc ctc ctg ggt gcc cgg tgg tgg acc gac gct
734
Glu Gly Gly Cys Leu Ala Val Leu Val Cys Thr Val Cys Thr Arg
45
50 55 60

tgctgg tga cca ctg tgt cag gtg cgt gtg tgt ggt ggg cct gcc aag ccc
792
Cys Thr Pro Leu Tyr Glu Ala Gly Leu Val Ala Val Gly Ser Lys
65 70 75

gcg cag ctc gtt ggg gaa ctc ggg acc ctt tgc cgg gcc cag gc
830
Ala Glu Gln Gln Glu Leu Gln Gly Ser Tyr Pro Leu Ser
80 90

gtc gct gct tac gta gcc ggg atc ctc gtt gtt gcc ggg ggt tac toc
tac Val Ser Ala Tyr Val Ala Gly Ile Leu Gly Leu Gly Val Tyr Ser
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878

ggg gtc ctc aca gtt ggt cgg ggg cgc cag gtc tac ctc atg
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926

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tgc Phe Thr Arg Thr Tyr Glu Leu Asn Ser Tyr Thr Ile Leu Glu
145 150 155

1022

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160 165 170

1070

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1118
Thr Pro Leu Thr Val Val Ala Leu Leu Leu Gly Arg
175 180 185

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tgc Ile Val Met Val Phe Leu Leu Val Thr Met Ala Gly Met Leu Gly
190 195 200

1166

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1214
Ala Pro Ala Ser Val Leu Gly Ser Arg Pro Phe Asp Tyr Gly Leu Lys
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Trp Gln Ser Cys Ser Cys Arg Ala Asn Gly Ser Arg Ile Pro Thr Gly
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Gly Asp Pro Ile Thr His Thr Ser His Gly Glu Asn Glu Trp Pro Leu
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(Continued)

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590 595 600

56

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3278

3326

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Gln Cys Val Met Gly Pro Val Ala Arg Arg Gly Arg Val
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pro Thr Ala Pro Val Ile Arg Arg Cys Gly Lys Gly Phe Leu Gly
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cat cca cgg
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Ile Ala Thr Pro Val Gly Ala Leu Lys Pro Arg Trp Trp Ser Ala Ser
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Pro Cys Thr Cys Gln Ala Gly Ser Cys Trp Val Ile Ser Arg Asp Gly
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Pro Thr Gly Ala Gly Lys Ser Thr Arg Val Pro Leu Gly Thr Gly
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Ann Met His Gly Leu Val Ile Leu Aen Pro Ser Val Ala Thr
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1125 1130 1135 3971

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1155 1160 1165 4061

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Thr Leu Ser Ile Gly
Glus Val Leu Ser

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5771

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Ser Val Val Phe Asp
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Pro Val Leu Thr

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Gln Glu Asp Thr Pro Ser Ser Asp Ser Phe Glu Val Ile Gln Glu
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<212> TYPE: PRT
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<400> SEQUENCE: 2

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Tyr Gln Ala Gly Leu Ala Val Arg Pro Gly Lys Ser Ala Ala Gln Leu
  65  70    75   80
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Val Ala Gly Ile Leu Gly Leu Gly Glu Val Tyr Ser Gly Val Leu Thr
 100 105   110
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   145   150   155   160
Val Pro Phe Glu Phe Trp Arg Gly Val Met Ser Leu Thr Pro Leu Leu
   165   170   175
Val Trp Val Ala Ala Leu Leu Leu Glu Gln Arg Ile Val Met Val
   180   185   190
Phe Leu Leu Val Thr Met Ala Gly Met Leu Gln Gly Ala Pro Ala Ser
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Val Leu Gly Ser Arg Pro Phe Asp Tyr Gly Leu Lys Trp Gln Ser Cys
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Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro Asn Gly Pro Trp
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Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser Val Ser
   290   295   300
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   305   310   315   320
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2075  2080  2085

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Glu Pro  His Ile Asp Val Ile  Met Glu Asp Cys Ser  Thr Pro Ser
2105  2110  2115

Leu Cys  Gly Ser Ser Arg Glu  Met Pro Val Trp Gly  Glu Asp Ile
2120  2125  2130

Pro Arg  Thr Pro Ser Pro Ala  Leu Ile Ser Val Thr  Glu Ser Ser
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Pro Asp  Glu Lys Thr Pro Ser  Val Ser Ser Ser Gln  Glu Asp Thr
2150  2155  2160

Pro Ser  Ser Asp Ser Phe Glu  Val Ile Gln Glu Ser  Glu Thr Ala
2165  2170  2175

Glu Gly  Glu Glu Ser Val Phe  Asn Val Ala Leu Ser  Val Leu Lys
2180  2185  2190

Ala Leu  Phe Pro Gln Ser Asp  Ala Thr Arg Lys Leu  Thr Val Lys
2195  2200  2205

Met Ser  Cys Cys Val Glu Lys  Ser Val Thr Arg Phe  Phe Ser Leu
2210  2215  2220

Gly Leu  Thr Val Ala Asp Val  Ala Ser Leu Cys Glu  Met Glu Ile
2225  2230  2235

Gln Asn  His Thr Ala Tyr Cys  Asp Lys Val Arg Thr  Pro Leu Glu
2240  2245  2250

Leu Gln  Val Gly Cys Leu Val  Gly Asn Glu Leu Thr  Phe Glu Cys
2255  2260  2265

Asp Lys  Cys Glu Ala Arg Gln  Glu Thr Leu Ala Ser  Phe Ser Tyr
2270  2275  2280

Ile Trp  Ser Gly Val Pro Leu  Thr Arg Ala Thr Pro  Ala Lys Pro
2285  2290  2295

Pro Val  Val Arg Pro Val Gly  Ser Leu Val Leu Ala  Asp Thr Thr
2300  2305  2310

Lys Val  Tyr Val Thr Asn Pro  Asp Asn Val Gly Arg  Arg Val Asp
2315  2320  2325

Lys Val  Thr Phe Trp Arg Ala  Pro Arg Val His Asp  Lys Phe Leu
2330  2335  2340

Val Asp  Ser Ile Glu Arg Ala  Lys Arg Ala Gln  Ala Cys Leu
2345  2350  2355

Ser Met  Gly Tyr Thr Tyr Glu  Glu Ala Ile Arg Thr  Val Arg Pro
2360  2365  2370

His Ala  Ala Met Gly Trp Gly  Ser Lys Val Ser Val  Lys Asp Leu
2375  2380  2385

Ala Thr  Pro Ala Gly Lys Met  Ala Val His Asp Arg  Leu Gln Glu
2390  2395  2400

Ile Leu  Glu Gly Thr Pro Val  Pro Phe Thr Leu Thr  Val Lys Lys
2405  2410  2415

Glu Val  Phe Phe Lys Asp Arg  Lys Glu Glu Lys Ala  Pro Arg Leu
2420  2425  2430

Ile Val  Phe Pro Pro Leu Asp  Phe Arg Ile Ala Glu  Lys Leu Ile
2435  2440  2445

Leu Gly  Asp Pro Gly Arg Val  Ala Lys Ala Val Leu  Gly Gly Ala
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Tyr Ala  Phe Glu Tyr Thr Pro  Asn Gln Arg Ile Arg  Glu Met Leu
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SEQ ID NO 5
LENGTH: 389
TYPE: PRT
ORGANISM: Hepatitis B Virus C

SEQUENCE: 5

| Met Ala Val Leu Leu Leu Leu Val Val Glu Ala Gly Ala Ile Leu | 1 | 5 | 10 | 15 |
| Ala Pro Ala Thr His Ala Cys Arg Ala Asn Gly Glu Tyr Phe Leu Thr | 20 | 25 | 30 |
| Asn Cys Ala Pro Glu Asp Ile Gly Phe Cys Leu Glu Gly Gly Cys | 35 | 40 | 45 |
| Leu Val Ala Leu Gly Cys Thr Val Cys Thr Asp Arg Cys Trp Pro Leu | 50 | 55 | 60 |
| Tyr Gln Ala Gly Leu Ala Val Arg Gly Lys Ser Ser Ala Ala Gln Leu | 65 | 70 | 75 | 80 |
| Val Gly Glu Leu Gly Ser Leu Tyr Gly Pro Leu Ser Val Ser Ala Tyr | 85 | 90 | 95 |
| Val Ala Gly Ile Leu Gly Leu Gly Val Tyr Ser Gly Val Leu Thr | 100 | 105 | 110 |
| Val Gly Val Ala Leu Arg Arg Arg Val Tyr Leu Met Pro Asn Leu Lys | 115 | 120 | 125 |
| Cys Ala Val Glu Cys Asp Val Lys Trp Gly Ser Glu Phe Trp Arg Trp | 130 | 135 | 140 |
| Thr Glu Glu Ala Ser Asn Tyr Thr Ile Leu Glu Gly Leu Trp Lys | 145 | 150 | 155 | 160 |
| Val Pro Phe Glu Phe Trp Arg Gly Val Met Ser Leu Thr Pro Leu Leu | 165 | 170 | 175 |
| Val Trp Val Ala Ala Leu Leu Leu Leu Glu Gin Arg Ile Val Met Val | 180 | 185 | 190 |
| Phe Leu Leu Val Thr Met Ala Gly Met Leu Gin Gly Ala Pro Ala Ser | 195 | 200 | 205 |
| Val Leu Gly Ser Arg Pro Phe Asp Tyr Gly Leu Lys Trp Gin Ser Cys | 210 | 215 | 220 |
Ser Cys Arg Ala Asn Gly Ser Arg Ile Pro Thr Gly Glu Arg Val Trp
225 230 235 240
Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro Asn Gly Pro Trp
245 250 255
Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp Gly Asp Pro Ile
260 265 270
Thr His Trp Ser His Gly Asn Gln Trp Pro Leu Ser Cys Pro Gln
275 280 285
Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser Val Ser
290 295 300
Trp Phe Ala Ser Thr Gly Gly Arg Asp Ser Lys Ile Asp Val Trp Ser
310 315 320
Leu Val Pro Val Gly Ser Ala Ser Cys Thr Ile Ala Ala Leu Gly Ser
325 330 335
Ser Asp Arg Asp Thr Val Val Glu Leu Ser Glu Gly Trp Gly Val Pro Cys
340 345 350
Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys Gly Thr Cys Val
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370 375 380
Cys Gly Thr Gly Pro
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<210> SEQ ID NO 6
<211> LENGTH: 389
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB virus C
<400> SEQUENCE: 6

Met Ala Val Leu Leu Leu Leu Val Val Val Glu Ala Gly Ala Ile Leu
1  5 10 15
Ala Pro Ala Thr His Ala Cys Arg Ala Asn Gly Gln Tyr Phe Leu Thr
20 25 30
Asn Cys Cys Ala Pro Glu Ile Gly Phe Cys Leu Glu Gly Gly Cys
35 40 45
Leu Val Ala Leu Gly Cys Thr Val Cys Thr Asp Arg Cys Trp Pro Leu
50 55 60
Tyr Gln Ala Gly Leu Ala Val Arg Pro Gly Lys Ser Ala Ala Gln Leu
65 70 75 80
Val Gly Glu Leu Gly Ser Leu Tyr Gly Pro Leu Ser Val Ser Ala Tyr
85 90 95
Val Ala Gly Ile Leu Gly Leu Gly Val Tyr Ser Gly Val Leu Thr
100 105 110
Val Gly Val Ala Leu Arg Arg Arg Val Tyr Leu Met Pro Asn Leu Lys
115 120 125
Cys Ala Val Glu Cys Asp Val Lys Trp Gly Ser Glu Phe Trp Arg Trp
130 135 140
Thr Glu Gln Leu Ala Ser Asn Tyr Trp Ile Leu Gly Tru Trp Leu Lys
145 150 155 160
Val Pro Phe Glu Phe Trp Arg Gly Val Met Ser Leu Thr Pro Leu Leu
165 170 175
Val Trp Val Ala Ala Leu Leu Leu Leu Glu Gin Arg Ile Val Met Val
180 185 190
Phe Leu Leu Val Thr Met Ala Gly Met Leu Glu Gly Ala Pro Ala Ser
Val Leu Gly Ser Arg Pro Phe Asp Tyr Gly Leu Lys Trp Gln Ser Cys
210 215 220

Ser Cys Arg Ala Arg Gly Ser Arg Ile Pro Thr Gly Glu Arg Val Trp
225 230 235 240

Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro Asn Gly Pro Trp
245 250 255

Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp Gly Asp Pro Ile
260 265 270

Thr His Trp Ser His Gly Glu Asn Gln Trp Pro Leu Ser Cys Pro Gln
275 280 285

Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser Val Ser
290 295 300

Trp Phe Ala Ser Thr Gly Gly Arg Ser Ser Lys Ile Asp Val Trp Ser
305 310 315 320

Leu Val Pro Val Gly Ser Ala Ser Cys Thr Ile Ala Ala Leu Gly Ser
325 330 335

Ser Asp Arg Asp Thr Val Val Glu Leu Ser Glu Trp Gly Val Pro Cys
340 345 350

Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys Gly Thr Cys Val
355 360 365

Arg Asp Cys Trp Pro Glu Thr Gly Ser Val Arg Phe Pro Phe His Arg
370 375 380

Cys Gly Thr Gly Pro
385

<210> SEQ ID NO: 7
<211> LENGTH: 698
<212> TYPE: DNA
<213> ORGANISM: Hepatitis B Virus C
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1) (698)

<400> SEQUENCE: 7

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1  5  10  15

tgg cag tca tgg tcc ggg ggt gct att ccc act ggg
20  25  30

Trp Gln Ser Cys Ser Cys Arg Ala Asn Gly Ser Arg Ile Pro Thr Gly
20  25  30

gag agg gtt gtg gat cga ggg aat gtc aag ctc tgg tgt gac tgc ccc
35  40  45

Glu Arg Val Trp Asp Gly Asn Val Thr Leu Cys Asp Cys Pro
35  40  45

aac ggc ccc tgg gtt gtc cgg gcc ttt tcc cag gcc gtt ggg tgg
50  55  60

Asn Gly Pro Trp Val Trp Val Trp Val Ala Phe Cys Gln Ala Val Gly Trp
50  55  60

ggc gac ccc tgg gtg gtc cgg gcc ttt tcc cag gcc gtt ggg tgg
65  70  75  80

Gly Asp Pro Ile Thr His Trp Ser His Gly Glu Asn Gln Trp Pro Leu
65  70  75  80

tca tgc ccc cca tta tcc gat ggg ttt tcc tgt tcc gta aag tgt gtt
85  90  95

Ser Cys Pro Gln Tyr Val Tyr Gly Ser Val Cys Pro Cys Thr Cys Val Trp
85  90  95

ggt tcc gtt tgg ttt gcc tcc acc ggc ggt gct gat tgg aag atc
100 105 110

Gly Ser Val Ser Trp Phe Ala Ser Thr Gly Arg Asp Ser Lys Ile
100 105 110

gat gtg tgg egt tgg gtt cgg gtt gga tct gcc aag tgc acc ata gcc
304
gct cta ggg tca tgc gat cgc gac acg gtc gtt gaa ctt gca ttc gag tgg
Ala Leu Gly Ser Ser Asp Arg Arg Thr Val Val Glu Leu Ser Glu Trp
115 120 125
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    135
    140
432

gga gtc ccc gta acg tgt att cgg gac cgg cct gct tca tgt
Gly Val Pro Cys Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys
145 150 155 160
780

ggc acc tgt gtc cgg gac tgc tgg ccc gaa acc ggg tgg gtt aga ttc
Gly Thr Cys Val Arg Asp Cys Trp Pro Glu Thr Gly Ser Val Arg Phe
165 170 175
528

cct ttc cat cgg tgc ggc acg ggg cct cgg cta cca aag gac tgt gaa
Pro Phe His Arg Cys Gly Thr Gly Pro Arg Leu Thr Lys Asp Leu Glu
180 185 190
576

gct gtt ccc ttc gtc aac agg aca act ccc ttc acc ata agg ggc ccc
Ala Val Pro Phe Val Asn Arg Thr Thr Pro Phe Thr Ile Arg Gly Pro
195 200 205
624

cgg ggg acc cag ggg gsg aac gcc acc cgg ggg ccc cgg tgt tcc
Leu Gly Asn Gin Gly Arg Gly Asn Pro Val Arg Ser Pro Leu Gly Phe
210 215 220
672

ggg tcc tac att ggc aag acc aca cg
Gly Ser Tyr Thr Met Thr Lys Ile
225 230
698

<210> SEQ ID NO 8
<211> LENGTH: 232
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB Virus C
<400> SEQUENCE: 9

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1  5 10  15
Trp Gln Ser Cys Ser Cys Arg Ala Asn Gly Ser Arg Ile Pro Thr Gly
20 25  30
Glu Arg Val Trp Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro
35 40  45
Asn Gly Pro Trp Val Trp Val Pro Ala Phe Cys Gin Ala Val Gly Trp
50 55  60
Gly Asp Pro Ile Thr His Trp Ser His Gly Gin Asn Gin Trp Pro Leu
65 70  75  80
Ser Cys Pro Gin Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp
85 90  95
Gly Ser Val Ser Trp Phe Ala Ser Thr Gly Arg Asp Ser Lys Ile
100 105 110
Asp Val Trp Ser Leu Val Pro Val Gly Ser Ala Ser Cys Thr Ile Ala
115 120 125
Ala Leu Gly Ser Ser Asp Arg Thr Val Val Glu Leu Ser Glu Trp
130 135 140
Gly Val Pro Cys Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys
145 150 155 160
Gly Thr Cys Val Arg Asp Cys Trp Pro Glu Thr Gly Ser Val Arg Phe
165 170 175
Pro Phe His Arg Cys Gly Thr Gly Pro Arg Leu Thr Lys Asp Leu Glu
180 185 190
Ala Val Pro Phe Val Asn Arg Thr Thr Pro Phe Thr Ile Arg Gly Pro
195 200 205
Leu Gly Asn Gin Gly Arg Asn Pro Val Arg Ser Pro Leu Gly Phe
210 215 220
230
Gly Ser Tyr Thr Met Thr Lys Ile
225  230

<-210> SEQ ID NO 9
<-211> LENGTH: 232
<-212> TYPE: PRT
<-213> ORGANISM: Hepatitis GB virus C

<-400> SEQUENCE: 9

Ala Pro Ala Ser Val Leu Gly Ser Arg Pro Phe Asp Tyr Gly Leu Lys
1   5   10   15
Trp Gln Ser Cys Ser Cys Arg Ala Asn Gly Ser Arg Ile Pro Thr Gly
20  25
Glu Arg Val Trp Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro
30  35  40  45
Asn Gly Pro Trp Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp
50  55  60
Gly Asp Pro Ile Thr His Thr Ser His Gly Asn Gln Trp Pro Leu
65  70  75  80
Ser Cys Pro Gln Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp
90  95
Gly Ser Val Ser Trp Phe Ala Ser Thr Gly Arg Asp Ser Lys Ile
100 105 110
Asp Val Trp Ser Leu Val Pro Val Gly Ser Ala Ser Cys Thr Ile Ala
115 120 125
Ala Leu Gly Ser Ser Asp Arg Asp Thr Val Val Glu Leu Ser Glu Trp
130 135 140
Gly Val Pro Cys Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys
145 150 155 160
Gly Thr Cys Val Arg Asp Cys Trp Pro Glu Thr Gly Ser Val Arg Phe
165 170 175
Pro Phe His Arg Cys Gly Thr Gly Pro Arg Leu Thr Lys Asp Leu Glu
180 185 190
Ala Val Pro Phe Val Asn Arg Thr Pro Phe Thr Ile Arg Gly Pro
195 200 205
Leu Gly Asn Gln Gly Arg Gly Asn Pro Val Arg Ser Pro Leu Gly Phe
210 215 220
Gly Ser Tyr Thr Met Thr Lys Ile
225  230

<-210> SEQ ID NO 10
<-211> LENGTH: 199
<-212> TYPE: DNA
<-213> ORGANISM: Hepatitis GB Virus C

<-220> FEATURE:
<-221> NAME/KEY: CDS
<-222> LOCATION: (1)..(198)

<-400> SEQUENCE: 10

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1   5   10   15
Glu Val Arg Trp Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro
20  25  30
aac ggc ccc tgt gtt gtc ccg ttc tgc cag ggc gtt ggg tgt
35  40  45
Asn Gly Pro Trp Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp
50  55  60
Gly Asp Pro Ile Thr His Thr Ser His Gly Gin Gin Trp Pro Leu
70  75  80
Gly Ser Val Ser Trp Phe Ala Ser Thr Gly Arg Asp Ser Lys Ile
90  95 100
Asp Val Trp Ser Leu Val Pro Val Gly Ser Ala Ser Cys Thr Ile Ala
110 115 120 125
Ala Leu Gly Ser Ser Asp Arg Asp Thr Val Val Glu Leu Ser Glu Trp
130 135 140
Gly Val Pro Cys Val Thr Cys Ile Leu Asp Arg Arg Pro Ala Ser Cys
150 155 160
Gly Thr Cys Val Arg Asp Cys Trp Pro Glu Thr Gly Ser Val Arg Phe
170 175
Pro Phe His Arg Cys Gly Thr Gly Pro Arg Leu Thr Lys Asp Leu Glu
180 185 190
Ala Val Pro Phe Val Asn Arg Thr Pro Phe Thr Ile Arg Gly Pro
195 200 205
Leu Gly Asn Gln Gly Arg Gly Asn Pro Val Arg Ser Pro Leu Gly Phe
210 215 220
Gly Ser Tyr Thr Met Thr Lys Ile
225  230
tca tgc ccc cca tat gtc tat ggg tct tgt tcc gta acg tgc tgt tg
Ser Cys Pro Gln Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp
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<210> SEQ ID NO 11
<211> LENGTH: 66
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB Virus C

<400> SEQUENCE: 11

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Asn Gly Pro Trp Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp
20 25 30
Gly Asp Pro Ile Thr His Trp Ser His Gly Gin Asn Gln Trp Pro Leu
35 40 45
Ser Cys Pro Gln Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp
50 55 60
Gly Ser
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<210> SEQ ID NO 12
<211> LENGTH: 66
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB virus C

<400> SEQUENCE: 12

Glu Arg Val Trp Asp Arg Gly Asn Val Thr Leu Leu Cys Asp Cys Pro
1 5 10 15
Asn Gly Pro Trp Val Trp Val Pro Ala Phe Cys Gln Ala Val Gly Trp
20 25 30
Gly Asp Pro Ile Thr His Trp Ser His Gly Gin Asn Gln Trp Pro Leu
35 40 45
Ser Cys Pro Gln Tyr Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp
50 55 60
Gly Ser
65

<210> SEQ ID NO 13
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB virus C

<400> SEQUENCE: 13

Val Tyr Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser
1 5 10

<210> SEQ ID NO 14
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Hepatitis GB virus C

<400> SEQUENCE: 14

Pro Gln Tyr Val Tyr Gly Ser Val Ser
1 5

<210> SEQ ID NO 15
Val Tyr Gly Ser Val Ser
1 5

Gln Tyr Val Tyr Gly Ser Val Ser Val Thr
1 5 10

Gly Gly Gly Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg
1 5 10

Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser
1 5 10 15

Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser
20 25

Gly Gly Gly Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Val His
1 5 10 15

Gly Ser Val Ser Val Thr Cys Val Trp Gly Ser
20 25

Gly Gly Ala Gly Leu Thr Gly Gly Arg Tyr Glu Pro Leu Val Arg Arg
1 5 10 15

Cys
What is claimed is:

1. A method of inhibiting immune cell activation comprising administering to a mammalian subject in need thereof a GBV-C E2 peptide or polypeptide, said peptide or polypeptide comprising SEQ ID NO: 8 and no more than 250 consecutive residues of GBV-C E2.

2. The method of claim 1, wherein said peptide or polypeptide comprises 232 or 250 consecutive residues of GBV-C E2.

3. The method of claim 1, wherein said peptide or polypeptide is about 232 or 250 residues in length.

4. The method of claim 1, wherein the peptide or polypeptide comprises a non-GBV-C E2 sequence.

5. The method of claim 4, wherein the non-GBV-C E2 sequence is a cell permeability peptide.

6. The method of claim 1, wherein the immune cell is a T cell or a B cell.

7. The method of claim 4, wherein the T cell is a helper T cell, a suppressor T cell, an NK cell or a killer T cell.

8. The method of claim 1, wherein said subject is a human.

9. The method of claim 1, wherein administering comprises intravenous, intra-arterial, oral, subcutaneous, topical or intraperitoneal administration.

10. The method of claim 1, further comprising administering a second anti-inflammatory agent.

11. The method of claim 1, wherein said peptide or polypeptide is administered at 0.1-500 mg/kg/d.

12. The method of claim 1, wherein said peptide or polypeptide is administered daily or weekly.

13. The method of claim 1, wherein said subject suffers from a T cell- or B-cell-mediated inflammatory disease or an IL-2-mediated inflammatory disease.

14. A method of inhibiting IL-2 release, inhibiting inflammation, and/or inhibiting STATS-mediated signaling in a mammalian subject comprising administering to said subject a peptide or polypeptide, said peptide or polypeptide comprising SEQ ID NO: 8 and no more than 250 consecutive residues of GBV-C E2.

15. A pharmaceutical formulation comprising an isolated peptide comprising (a) SEQ ID NO: 8 and no more than 250 consecutive residues of GBV-C E2 fused to a cell permeability peptide; and (b) a pharmaceutically acceptable diluent, carrier or buffer.

16. The pharmaceutical formulation of claim 15, wherein said peptide comprises 232 or 250 consecutive residues of GBV-C E2.

17. The pharmaceutical formulation of claim 15, wherein said peptide is about 232 or 250 residues in length.

18. The method of claim 5, wherein said cell permeability peptide is HIV TAT.

19. The pharmaceutical formulation of claim 15, wherein said cell permeability peptide is HIV TAT.

* * * * *