2013

GB virus C interactions with HIV: effects on immunoactivation and mechanisms of immunomodulation

Nirjal Bhattarai

University of Iowa

Copyright 2013 Nirjal Bhattarai

This dissertation is available at Iowa Research Online: http://ir.uiowa.edu/etd/2437

Recommended Citation


Follow this and additional works at: http://ir.uiowa.edu/etd

Part of the Cell Biology Commons
GB VIRUS C INTERACTIONS WITH HIV: EFFECTS ON
IMMUNOACTIVATION AND MECHANISMS OF IMMUNOMODULATION

by
Nirjal Bhattarai

An Abstract
Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular and Cellular Biology
in the Graduate College of
The University of Iowa

May 2013

Thesis Supervisor: Professor Jack T. Stapleton
ABSTRACT

GB virus C (GBV-C) is a lymphotropic human virus which was recently assigned to a new genus Pegivirus within the Flaviviridae family. GBV-C infection is found worldwide, and viremia prevalence is about 1% to 4% in healthy blood donors and up to 42% in HIV-infected individuals. In clinical studies, GBV-C coinfection is associated with prolonged survival of HIV-infected individuals. GBV-C infection modestly alters T cell homeostasis in vivo through various mechanisms, including modulation of chemokine and cytokine release and receptor expression, and by diminution of T cell activation, proliferation and apoptosis, all of which may contribute to improved HIV clinical outcomes. This thesis explores the interrelationship between GBV-C infection and immunoactivation and identifies potential mechanisms by which GBV-C reduces immunoactivation.

Chronic HIV infection is associated with persistent immunoactivation which contributes to the immune dysfunction. In particular, T cell activation supports HIV replication and correlates with HIV viral load (VL). Persistent immunoactivation also contributes to the depletion of uninfected bystander cells by mechanisms of activation induced cell death (AICD). Although treatment with combination antiretroviral therapy (cART) reduces HIV VL, T cell activation does not return to levels found in HIV-uninfected individuals. Sustained immunoactivation is also associated with lower virological response to cART suggesting therapies to reduce immunoactivation in combination with cART may benefit HIV-infected individuals. Since GBV-C infection is associated with reduced immunoactivation, understanding mechanisms by which GBV-C modulates these signaling pathways may provide insights into novel approaches to treat HIV infection and chronic immunoactivation.

The effect of GBV-C infection on T cell activation and IL-2 signaling pathways were studied in a cohort of HIV-positive individuals. GBV-C viremic HIV positive
individuals on cART have reduced T cell activation which was significantly associated with higher percentage of immunomodulatory CD3⁺CD4⁻CD8⁻ T cells. *Ex vivo* GBV-C infection was associated with reduced lymphocyte proliferation in response to IL-2, lower frequency of reactivation of latent HIV and protection against AICD. *In vitro* expression of GBV-C envelope glycoprotein E2 in CD4+ T cell lines inhibited T cell receptor (TCR) induced IL-2 secretion and inhibited IL-2 signaling pathways. This effect was mediated at least in part by reducing activation of lymphocyte specific tyrosine kinase (Lck).

Through deletion mutagenesis, the inhibitory motif within the viral protein was mapped to a region that contains a predicted Lck substrate, a highly conserved tyrosine at position 87 (Y87). Lck phosphorylated GBV-C E2 protein *in vitro* and mutation of Y87 residue abolished the inhibitory effects of E2 protein. Synthetic peptides containing this inhibitory motif competed for Lck phosphorylation and inhibited TCR signaling in primary human T cells. The number of GBV-C infected T cells was found to be low *in vivo*, yet GBV-C infection reduced global TCR signaling. GBV-C RNA and E2 protein were detected in extracellular microvesicles purified from GBV-C infected human serum or the culture supernatant of E2 expressing cells, and these microvesicles inhibited TCR signaling in uninfected bystander T cells. Together, these data identify a novel mechanism by which GBV-C infection leads to global reduction in T cell activation and IL-2 signaling in the infected host, and provide a working model in which the viral envelope glycoprotein serves as a substrate for Lck and competes for Lck phosphorylation in the infected T cells and in uninfected bystander T cells.

Abstract Approved:

<table>
<thead>
<tr>
<th>Thesis Supervisor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
</tbody>
</table>

Title and Department

Date
GB VIRUS C INTERACTIONS WITH HIV: EFFECTS ON IMMUNOACTIVATION AND MECHANISMS OF IMMUNOMODULATION

by

Nirjal Bhattarai

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Molecular and Cellular Biology in the Graduate College of The University of Iowa

May 2013

Thesis Supervisor: Professor Jack T. Stapleton
CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Nirjal Bhattarai

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular and Cellular Biology at the May 2013 graduation.

Thesis Committee: 

Jack T. Stapleton, Thesis Supervisor

Wendy Maury

Jon Houtman

Chioma Okeoma

Fayyaz Sutterwala
To my family
Live as if you were to die tomorrow. Learn as if you were to live forever.

Mohandas Karamchand Gandhi
ACKNOWLEDGMENTS

First, I would like to acknowledge my parents and family for their continuous support, advice and lots of sacrifices that provided me with opportunities to get early education in Nepal, followed by undergraduate and graduate degrees in USA.

Second, I would like to thank Dr. Jack Stapleton, my Ph.D. thesis supervisor. Dr. Stapleton is a superb thesis supervisor who helped me to stay focused and excited in my research all these years. I would not have achieved this degree without him putting tireless efforts in teaching me, listening to me and making sure I was on the right path. In addition, he is the nicest person to work with and a great mentor whose life advice has helped me a lot in graduate school, which can be very tiring and frustrating at times.

I would also like to thank my thesis committee members for their advice and support, especially Dr. Jon Houtman for providing his expertise on T cell signaling.

Finally, a big thank you to Dr. Alan Landay (Rush University) for assistance with immunoactivation studies in HIV-positive cohorts, Drs. Jim McLinden and Jinhua Xiang for technical assistance, helpful discussions and entire Stapleton Lab for their assistance.
ABSTRACT

GB virus C (GBV-C) is a lymphotropic human virus which was recently assigned to a new genus Pegivirus within the Flaviviridae family. GBV-C infection is found worldwide, and viremia prevalence is about 1% to 4% in healthy blood donors and up to 42% in HIV-infected individuals. In clinical studies, GBV-C coinfection is associated with prolonged survival of HIV-infected individuals. GBV-C infection modestly alters T cell homeostasis in vivo through various mechanisms, including modulation of chemokine and cytokine release and receptor expression, and by diminution of T cell activation, proliferation and apoptosis, all of which may contribute to improved HIV clinical outcomes. This thesis explores the interrelationship between GBV-C infection and immunoactivation and identifies potential mechanisms by which GBV-C reduces immunoactivation.

Chronic HIV infection is associated with persistent immunoactivation which contributes to the immune dysfunction. In particular, T cell activation supports HIV replication and correlates with HIV viral load (VL). Persistent immunoactivation also contributes to the depletion of uninfected bystander cells by mechanisms of activation induced cell death (AICD). Although treatment with combination antiretroviral therapy (cART) reduces HIV VL, T cell activation does not return to levels found in HIV-uninfected individuals. Sustained immunoactivation is also associated with lower virological response to cART suggesting therapies to reduce immunoactivation in combination with cART may benefit HIV-infected individuals. Since GBV-C infection is associated with reduced immunoactivation, understanding mechanisms by which GBV-C modulates these signaling pathways may provide insights into novel approaches to treat HIV infection and chronic immunoactivation.

The effect of GBV-C infection on T cell activation and IL-2 signaling pathways were studied in a cohort of HIV-positive individuals. GBV-C viremic HIV positive
individuals on cART have reduced T cell activation which was significantly associated with higher percentage of immunomodulatory CD3^+CD4^-CD8^- T cells. Ex vivo GBV-C infection was associated with reduced lymphocyte proliferation in response to IL-2, lower frequency of reactivation of latent HIV and protection against AICD. In vitro expression of GBV-C envelope glycoprotein E2 in CD4+ T cell lines inhibited T cell receptor (TCR) induced IL-2 secretion and inhibited IL-2 signaling pathways. This effect was mediated at least in part by reducing activation of lymphocyte specific tyrosine kinase (Lck).

Through deletion mutagenesis, the inhibitory motif within the viral protein was mapped to a region that contains a predicted Lck substrate, a highly conserved tyrosine at position 87 (Y87). Lck phosphorylated GBV-C E2 protein in vitro and mutation of Y87 residue abolished the inhibitory effects of E2 protein. Synthetic peptides containing this inhibitory motif competed for Lck phosphorylation and inhibited TCR signaling in primary human T cells. The number of GBV-C infected T cells was found to be low in vivo, yet GBV-C infection reduced global TCR signaling. GBV-C RNA and E2 protein were detected in extracellular microvesicles purified from GBV-C infected human serum or the culture supernatant of E2 expressing cells, and these microvesicles inhibited TCR signaling in uninfected bystander T cells. Together, these data identify a novel mechanism by which GBV-C infection leads to global reduction in T cell activation and IL-2 signaling in the infected host, and provide a working model in which the viral envelope glycoprotein serves as a substrate for Lck and competes for Lck phosphorylation in the infected T cells and in uninfected bystander T cells.
TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................. xi
LIST OF FIGURES .............................................................................................................. xii
LIST OF ABBREVIATIONS ................................................................................................. xiii

CHAPTER

I. INTRODUCTION ............................................................................................................... 1

GB virus C Discovery ....................................................................................................... 1
Classification and Genome Organization ...................................................................... 1
GBV-C Structural and Nonstructural Proteins .............................................................. 2
Prevalence and Transmission ......................................................................................... 4
GBV-C Genotypes and Tropism ..................................................................................... 5
GBV-C Interactions with HIV ......................................................................................... 6
Clinical Studies ............................................................................................................... 6
Mechanisms of HIV inhibition ....................................................................................... 7
Effects on HIV entry receptors ..................................................................................... 7
Inhibition of HIV replication by GBV-C proteins ......................................................... 8
Effects on Innate Immune Response ............................................................................ 9
Th1/Th2 cytokines .......................................................................................................... 9
Modulation of Apoptosis ............................................................................................. 10
Effects on Lymphocyte activation ................................................................................. 11
Modulation of IL-2 signaling and CD4 expansion ....................................................... 13
T cell receptor (TCR) and IL-2 Signaling Pathways .................................................... 14
Exosomes ....................................................................................................................... 16
Overview of Thesis Chapters ....................................................................................... 18
Chapter II: Effects of GBV-C infection on T cell activation in HIV-positive individuals on antiretroviral therapy ............................................................. 18
Chapter III: Ex vivo activation effects on GBV-C replication, proliferation and survival of GBV-C infected primary T cells ................................................................. 19
Chapter IV: GB virus C envelope glycoprotein E2 inhibits T cell receptor induced IL-2 production and alters IL-2 signaling pathways .................................................................................................................. 19
Chapter V: GB virus C envelope glycoprotein E2 inhibits T cell receptor signaling in infected and uninfected bystander T cells via inhibiting Lck .................................................. 20
Chapter VI: General Discussion .................................................................................. 20

II. EFFECTS OF GBV-C INFECTION ON T CELL ACTIVATION IN HIV POSITIVE INDIVIDUALS ON ANTIRETROVIRAL THERAPY .......... 30

Chapter Summary .......................................................................................................... 30
Introduction ..................................................................................................................... 30
Materials and Methods ................................................................................................. 32
Study Subjects .............................................................................................................. 32
PBMC isolation and flow cytometry .......................................................................... 32
Statistics ....................................................................................................................... 32
Results ......................................................................................................................... 33
Subjects description .................................................................33
Relationship between GBV-C viremia and double negative T cells ......33
Relationship between GBV-C viremia and T cell activation .................33
Discussion .................................................................................34

III. EFFECTS OF IMMUNOACTIVATION ON GBV-C REPLICATION,
PROLIFERATION AND SURVIVAL OF GBV-C INFECTED T
CELLS .........................................................................................46

Chapter Summary ..........................................................................46
Introduction....................................................................................47
Materials and Methods ....................................................................49
Study Subjects ................................................................................49
PBMC Isolation and Stimulation ....................................................50
GBV-C Quantification ....................................................................50
HIV Reactivation .............................................................................51
CD4+ and CD8+ T cell Analysis .......................................................51
Statistics .........................................................................................51
Results ..............................................................................................52
Subjects description .......................................................................52
PHA/IL2 stimulation decreases GBV-C replication in PBMCs............52
GBV-C effects on PBMC proliferation following PHA/IL2
activation .......................................................................................54
GBV-C reduces HIV reactivation .....................................................54
GBV-C effects on T cell depletion following PHA/IL2 activation ......54
Discussion .......................................................................................55

IV. GB VIRUS C ENVELOPE GLYCOPROTEIN E2 INHIBITS T CELL
RECEPTOR INDUCED IL-2 PRODUCTION AND ALTERS IL-2
SIGNALING PATHWAYS ..................................................................66

Chapter Summary ..........................................................................66
Introduction....................................................................................67
Materials and Methods ....................................................................68
Expression of GBV-C E2 proteins ...................................................68
Cell Stimulation ..............................................................................69
Cytokine Quantification ..................................................................70
mRNA expression ............................................................................70
Flowcytometry ................................................................................70
Statistics ..........................................................................................71
Results ..............................................................................................71
GB-C E2 protein expression inhibits IL-2 production .........................71
GB-C E2 protein effects on IL-2Rα and STAT5 phosphorylation ..........72
GB-C E2 protein reduces activation induced proliferation .................73
GB-C E2 protein reduces IL-2 and CD25 expression in primary
human T cells ..................................................................................73
Discussion .......................................................................................74

V. GB VIRUS C ENVELOPE GLYCOPROTEIN E2 INHIBITS T CELL
RECEPTOR SIGNALING IN INFECTED AND UNINFECTED
Bystander T cells via inhibiting LCK ..............................................93

Chapter Summary ..........................................................................93
Introduction....................................................................................93

viii
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>95</td>
</tr>
<tr>
<td>Expression of GBV-C E2 proteins</td>
<td>95</td>
</tr>
<tr>
<td>Cell Stimulation</td>
<td>95</td>
</tr>
<tr>
<td>Flowcytometry</td>
<td>96</td>
</tr>
<tr>
<td>Immunoblot Analysis</td>
<td>96</td>
</tr>
<tr>
<td>ELISA</td>
<td>97</td>
</tr>
<tr>
<td>Enzyme Assays</td>
<td>97</td>
</tr>
<tr>
<td>GBV-C E2 synthetic peptides</td>
<td>97</td>
</tr>
<tr>
<td>GBV-C RNA Quantification</td>
<td>98</td>
</tr>
<tr>
<td>Extracellular Microvesicles (EMV) Isolation</td>
<td>98</td>
</tr>
<tr>
<td>Statistics</td>
<td>99</td>
</tr>
<tr>
<td>Results</td>
<td>99</td>
</tr>
<tr>
<td>Extracellular microvesicles from GBV-C infected human serum inhibit</td>
<td></td>
</tr>
<tr>
<td>TCR signaling in primary human T cells</td>
<td>99</td>
</tr>
<tr>
<td>GBV-C E2 protein inhibits TCR-mediated activation of CD4+ T cells</td>
<td>101</td>
</tr>
<tr>
<td>GBV-C E2 protein inhibits Lck activation</td>
<td>101</td>
</tr>
<tr>
<td>GBV-C E2 protein does not alter CD45 and Csk expression</td>
<td>102</td>
</tr>
<tr>
<td>GBV-C E2 protein interacts with Lck</td>
<td>102</td>
</tr>
<tr>
<td>A 13mer peptide motif within GBV-C E2 is sufficient to inhibit</td>
<td></td>
</tr>
<tr>
<td>TCR signaling</td>
<td>102</td>
</tr>
<tr>
<td>GBV-C E2 protein inhibits TCR signaling pathways at the level of</td>
<td></td>
</tr>
<tr>
<td>Lck activation</td>
<td>103</td>
</tr>
<tr>
<td>Synthetic GBV-C E2 peptides inhibit TCR activation in primary</td>
<td></td>
</tr>
<tr>
<td>human T cells</td>
<td>104</td>
</tr>
<tr>
<td>GBV-C E2 protein inhibits T cell activation in bystander cells</td>
<td>105</td>
</tr>
<tr>
<td>Discussion</td>
<td>106</td>
</tr>
<tr>
<td>VI. GENERAL DISCUSSION</td>
<td>143</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>151</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table

1.1 Known functions of GBV-C structural and nonstructural proteins and its effect on HIV replication

1.2 Summary of the effects of GBV-C infection in HIV-positive individuals

2.1 Subject Demographics

3.1 Subjects Demographics

21

22

37

58
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>GB virus C genome organization and polyprotein processing ..................................23</td>
</tr>
<tr>
<td>1.2</td>
<td>Prevalence and genotypic distribution of GBV-C viremia in global population ..........24</td>
</tr>
<tr>
<td>1.3</td>
<td>In vitro effects of GBV-C proteins E2 and NS5A on CD4+ T cells ..........................25</td>
</tr>
<tr>
<td>1.4</td>
<td>In vivo effects of GBV-C infection that can potentially interfere with HIV replication. ........................................................................................................26</td>
</tr>
<tr>
<td>1.5</td>
<td>Schematic of proximal T cell receptor (TCR) signaling pathways...............................27</td>
</tr>
<tr>
<td>1.6</td>
<td>Schematic of Lck domain organization and activation...............................................27</td>
</tr>
<tr>
<td>2.1</td>
<td>GBV-C viremia is inversely associated with double negative T cells .........................38</td>
</tr>
<tr>
<td>2.2</td>
<td>GBV-C viremia is associated with reduced T cell activation in subjects with undetectable HIV viral load on antiretroviral therapy ........................................41</td>
</tr>
<tr>
<td>2.3</td>
<td>Association between GBV-C viral load, DNTECs, and CD38 expression .....................43</td>
</tr>
<tr>
<td>3.1</td>
<td>PHA/IL2 stimulation alters GBV-C replication and PBMC proliferation .......................59</td>
</tr>
<tr>
<td>3.2</td>
<td>GBV-C reduces reactivation of latent HIV from cellular reservoirs ............................61</td>
</tr>
<tr>
<td>3.3</td>
<td>Flow cytometry determination of CD4+ and CD8+ T cells ........................................62</td>
</tr>
<tr>
<td>3.4</td>
<td>GBV-C protects CD4+ and CD8+ T cell depletion following PHA/IL2 activation .............64</td>
</tr>
<tr>
<td>4.1</td>
<td>Schematic of T cell activation and IL-2 signaling pathways .....................................78</td>
</tr>
<tr>
<td>4.2</td>
<td>Schematic of GBV-C envelope protein and mutants ................................................79</td>
</tr>
<tr>
<td>4.3</td>
<td>Analysis of protein expression ................................................................................80</td>
</tr>
<tr>
<td>4.4</td>
<td>Regulation of GBV-C E2 protein expression .............................................................82</td>
</tr>
<tr>
<td>4.5</td>
<td>GBV-C E2 protein inhibits T cell receptor (TCR) induced IL-2 ....................................83</td>
</tr>
<tr>
<td>4.6</td>
<td>GBV-C E2 protein reduces IL-2Rα expression and STAT5 phosphorylation ..................85</td>
</tr>
<tr>
<td>4.7</td>
<td>GBV-C E2 protein expression inhibits T cell proliferation .........................................88</td>
</tr>
<tr>
<td>4.8</td>
<td>GBV-C E2 protein inhibits IL-2 secretion and IL-2Rα (CD25) expression in primary human T cells ...........................................................................................................90</td>
</tr>
<tr>
<td>5.1</td>
<td>Extracellular microvesicles (EMV) from GBV-C infected human serum inhibit T cell receptor (TCR) signaling in primary human T cells ........................................109</td>
</tr>
</tbody>
</table>
5.2 GBV-C E2 protein expression inhibits T cell receptor (TCR) mediated activation of human T cells.................................................................114

5.3 GBV-C E2 protein inhibits Lck activation ...............................................119

5.4 GBV-C E2 protein does not alter CD45 and Csk expression.......................121

5.5 GBV-C E2 protein interacts with Lck. ......................................................124

5.6 Characterization of a peptide domain within GBV-C E2 that inhibits T cell receptor (TCR) signaling .................................................................126

5.7 GBV-C E2 protein inhibits TCR signaling pathways at the level of Lck activation.................................................................................................131

5.8 Synthetic GBV-C E2 peptides inhibit TCR activation in primary human T cells. .................................................................................................133

5.9 GBV-C E2 protein inhibits T cell receptor (TCR) signaling in bystander cells....138

6.1 Proposed model for inhibition of T cell receptor (TCR) signaling in infected and bystander T cells during GBV-C infection.......................................150
LIST OF ABBREVIATIONS

GBV-C: GB VIRUS C
HIV: HUMAN IMMUNODEFICIENCY VIRUS
HCV: HEPATITIS C VIRUS
GBV C\textsubscript{HUM}: GB VIRUS C HUMAN ISOLATE
GBV C\textsubscript{CPZ}: GB VIRUS C CHIMPANZEE ISOLATE
GBV-A: GB VIRUS A
GBV-B: GB VIRUS B
ICTV: INTERNATIONAL COMMITTEE ON THE TAXONOMY OF VIRUSES
HGV: HEPATITIS G VIRUS
HPGV: HUMAN PERSISTENT G VIRUS
IV: INTRAVENOUS
TCA: T CELL ACTIVATION
ART: ANTIRETROVIRAL THERAPY
NTR: NONTRANSLATED REGION
LTR: LONG TERMINAL REPEAT
ORF: OPEN READING FRAME
AIDS: ACQUIRED IMMUNODEFICIENCY SYNDROME
BSA: BOVINE SERUM ALBUMIN
CHO: CHINESE HAMSTER OVARY CELLS
DNTEC: DOUBLE NEGATIVE T CELLS
VL: VIRAL LOAD
FACS: FLUORESCENCE ACTIVATED CELL SORTING
RT-PCR: REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION
TCR: T CELL RECEPTOR
IL: INTERLEUKIN
IFN: INTERFERON
TGFβ: TRANSFORMING GROWTH FACTOR BETA
pDC: PLASMACYTOID DENDRITIC CELLS
PKR: PROTEIN KINASE R
MxA: MYXOVIRUS RESISTANCE 1
OAS: OLIGOADENYLATE SYNTHETASE
PHA: PHYTOHAEMAGGLUTININ
PMA: PHORBOL 12-MYRISTATE 13-ACETATE
AICD: ACTIVATION INDUCED CELL DEATH
PBMC: PERIPHERAL BLOOD MONONUCLEAR CELLS
IDU: INJECTION DRUG USERS
ELISA: ENZYME-LINKED IMMUNOSORBENT ASSAY
US: UNSTIMULATED
NTPASE: NUCLEOSIDE TRIPHOSPHATASES
NS: NOT SIGNIFICANT
STAT: SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION
LAT: LINKER OF ACTIVATED T CELLS
ZAP70: ZETA CHAIN ASSOCIATED PROTEIN 70
LCK: LYMPHOCYTE SPECIFIC PROTEIN KINASE
CSK: C-TERMINAL SRC KINASE
CD: CLUSTER OF DIFFERENTIATION
EMV: EXTRACELLULAR MICROVESICLES
DNA: DEOXYRIBONUCLEIC ACID
RNA: RIBONUCLEIC ACID
mRNA: MESSENGER RIBONUCLEIC ACID
ER: ENDOPLASMIC RETICULUM
GP: GLYCO PROTEINS
NS: NONSTRUCTURAL PROTEINS
MVB: MULTIVESICULAR BODIES
IRES: INTERNAL RIBOSOMAL ENTRY SITE
IP: IMMUNE PRECIPITATION
LDL: LOW DENSITY LIPO PROTEINS
CCR5: C-C CHEMOKINE RECEPTOR TYPE 5
CXCR4: C-X-C CHEMOKINE RECEPTOR TYPE 4
MFI: MEAN FLUORESCENCE INTENSITY
RLU: RELATIVE LIGHT UNIT
X4: CXCR4 TROPIC HIV-1 ISOLATES
R5: CCR5 TROPIC HIV-1 ISOLATES
GFP: GREEN FLUORESCENT PROTEIN
E2: ENVELOPE PROTEIN 2
NS5A: NONSTRUCTURAL PROTEIN 5A
GE: GENOME EQUIVALENTS
NK: NATURAL KILLER
MAB: MONOCLONAL ANTIBODY
IG: IMMUNOGLOBULIN
TH: T HELPER CELLS
FASL: FAS LIGAND
PBS: PHOSPHATE BUFFERED SALINE
MIP: MACROPHAGE INFLAMMATORY PROTEIN
RANTES: REGULATED AND NORMAL T-CELL EXPRESSED AND SECRETED
SDF-1: STROMAL-DERIVED-FACTOR-1
ML: MILLILITER
FITC: FLUORESCEIN ISOTHIOCYANATE
PE: PHYCOERYTHRIN
APC: ALLOPHYCOCYANIN
USA: UNITES STATES OF AMERICA
FDA: FOOD AND DRUG ADMINISTRATION
GT: GENOTYPES
CHAPTER I
INTRODUCTION

**GB virus C Discovery**

In 1995 Abbott Laboratories reported identification of a novel human virus in the serum of a West African patient with non-(A-E) hepatitis and named it GB virus C (GBV-C) based on nucleotide sequence similarities with two primate viruses called GB virus A and B (GBV-A and GBV-B) (67, 116). GBV-A and GBV-B were discovered in tamarins that developed hepatitis following inoculation with the serum of a surgeon whose initials were G. B. (110). Concurrently, Genelabs Technologies reported discovery of a novel virus in the plasma of a patient with chronic hepatitis, which they named hepatitis G virus (HGV) (76, 116). Subsequent genome sequence analysis of GBV-C and HGV revealed 96% homology, indicating that they were two isolates of the same virus (67, 76, 116). Numerous studies investigated diverse populations at risk for liver disease, and when controlled for transmission exposure risks, no conclusive association between GBV-C/HGV and hepatitis was identified (123). Since GBV-C does not cause hepatitis, the name ‘hepatitis G virus’ is misleading and in this thesis it will be referred as GBV-C.

**Classification and Genome Organization**

GBV-C is classified as a member of the family *Flaviviridae* based on its nucleotide sequence and genome organization, and it is the most closely related human virus to hepatitis C virus (HCV) (87). Recently, the executive committee of the International Committee on the Taxonomy of Viruses (ICTV) classified GBV-C, along with GBV-A and a bat GB virus (GBV-D), under a new genus ‘Pegivirus’ within *Flaviviridae* (82, 127). Although GBV-C is phlogenetically related to HCV, GBV-C is
lymphotropic, and virus is produced by T and B lymphocytes removed from GBV-C infected individuals (43).

The GBV-C genome is a positive sense, single stranded RNA (9.4 kb) that contains a long open reading frame (ORF) (67, 76, 87). The genome organization is similar to HCV with a nontranslated region (NTR) at the 5' end containing an internal ribosomal entry site (IRES) directing translation of a polyprotein of approximately 3000 amino acids (Fig. 1.1). The polyprotein is followed by a 3' NTR that is involved in RNA replication. The polyprotein is post-translationally processed into structural and nonstructural (NS) proteins by cellular signal peptidases and viral proteases (87). The structural proteins encoded by GBV-C are the envelope glycoproteins E1 and E2 and the nonstructural proteins (NS) are NS2, NS3, NS4A, NS4B, NS5A and NS5B (Fig 1.1).

**GBV-C Structural and Nonstructural Proteins**

Based on similarities between GBV-C and HCV genome organization, it is thought that similar to HCV, GBV-C also contains a core protein coding sequence upstream to coding region of E1 (87). However, the core protein of GBV-C has not been experimentally defined yet. Similar to HCV, GBV-C envelope proteins E1 and E2 contain transmembrane domain (TM) which functions as a membrane anchor and contain endoplasmic reticulum (ER) retention signals. The envelope proteins are processed by cellular signal peptidases, and are thought to be targeted to the endoplasmic reticulum (ER) where the transmembrane domain is inserted into the ER membrane to become a type I transmembrane proteins. The structural modeling of GBV-C envelope proteins suggest that the C-terminal domain of E1 adopts an amphipathic alpha-helix and beta-hairpin and E2 adopts a beta-hairpin (87). The exact mechanism of GBV-C envelope protein biogenesis is not known; however, expression of recombinant E2 protein with a signal sequence but lacking a TM results in E2 secretion. However, similar to HCV, some of the envelope proteins escape the ER retention system and may interact with host
proteins (11, 34, 40). The envelope proteins E1 and E2 are predicted to hetero-dimerize and insert into the viral membrane. The E2 protein contains a predicted receptor binding site and is thought to bind to the cellular receptor and mediate entry into the target cells (87). One study found that GBV-C envelope protein E2 may interact with CD81 (93), however; subsequent study failed to find any specific interaction between GBV-C E2 protein and CD81 concluding that CD81 does not interact with GBV-C E2 protein (61). To date, GBV-C’s cellular receptor remains unknown.

GBV-C envelope proteins have been implicated in modulating HIV infection (87). Synthetic peptides derived from GBV-C E1 and E2 protein inhibit HIV replication (64, 112). Incubation of human T cells with recombinant E2 protein leads to inhibition of HIV replication; which is at least in part due to E2-mediated block in HIV entry (87, 150). There may be additional mechanisms by which GBV-C E2 protein may modulate HIV infection. One study found that expression of E2 protein inhibited HIV-gag protein trafficking to the plasma membrane resulting into impaired virus assembly (134). Further studies are necessary to understand the molecular mechanism of HIV inhibition by GBV-C envelope proteins.

The nonstructural (NS) proteins of GBV-C are multifunctional. The NS2 protein contains serine protease function and mediates cleavage at the NS2/NS3 junction (Figure 1.1). The NS3 protease mediates NS3/NS4 and NS5A/NS5B cleavage and, together with NS4A, mediates cleavage of the NS4B/NS5A junction (87). Similar to HCV NS3, GBV-C NS3 is also predicted to contain helicase and RNA nucleoside triphosphatases (NTPase) functions. In addition to its intrinsic function to process viral polyprotein, GBV-C NS3 expression blocks HIV replication in human T cells (44). The serine protease function of NS3 was required for HIV inhibition as mutation of catalytic serine to alanine rescued the inhibitory effects of NS3 on HIV replication.

The functions of other GBV-C nonstructural proteins are not clear. NS4A is required for NS3 protease function and NS5B is an RNA-dependent RNA polymerase
The function of NS4B and NS5A are not well understood but GBV-C NS5A protein is suggested to play a role in immune evasion and expression of NS5A in human T cells also inhibit HIV replication (147, 149). The function of GBV-C structural and nonstructural proteins and their role in HIV replication is summarized in table 1.1.

**Prevalence and Transmission**

The presence of GBV-C RNA in serum indicates active GBV-C infection, while detection of antibodies to the GBV-C envelope glycoprotein E2 is associated with prior infection. Unlike HCV, simultaneous detection of viral RNA and antibodies to E2 or other viral proteins is not common (130, 132, 142). In one large multicenter HIV-infected cohort, only 1.8% of patients with GBV-C viremia had detectable E2 antibodies whereas 75% of patients who were negative for GBV-C RNA had E2 antibodies (142). GBV-C E2 antibodies are also consistently detected more frequently in individuals with GBV-C transmission risk without GBV-C viremia compared to those with GBV-C RNA (132). Finally, E2 antibodies are found more frequently than viremia among healthy blood donors (28, 130, 132), suggesting prior exposure and viral clearance. On occasion, E2 antibodies are detected in GBV-C viremic subjects; however, in longitudinal studies E2 antibodies are temporally related to subsequent viral clearance (28, 130, 132). Although E2 antibodies are a marker of prior infection, detection may be lost over time (12), and thus the presence of E2 antibodies presumably underestimates the prevalence of prior infection.

GBV-C infection is common and is distributed worldwide (Fig. 1.2). Like HCV it can establish persistent infection but without any clinical symptoms or disease in either immunocompromised or healthy individuals (87, 123). In developed countries, 1% to 4% of healthy blood donors are viremic at the time of blood donation. The prevalence is higher in developing countries, with up to 20% of blood donors viremic in some studies (87, 127). The USA FDA does not recommend blood screening for GBV-C since it is not
associated with any disease state. Based on the prevalence of GBV-C and the number of blood products transfused, approximately 1,000 people receive GBV-C viremic blood products daily in the USA (124). Similar to other lymphotropic viruses, GBV-C is transmitted sexually, vertically and by exposure to contaminated blood. Consequently, it is highly prevalent in populations with other sexually transmitted or blood-borne infections. For example, GBV-C viremia prevalence is approximately 20% among individuals with chronic HCV infection and 20% to 40% among HIV-positive individuals (87).

**GBV-C Genotypes and Tropism**

Studies of GBV-C isolates from around the world and extensive phylogenetic analysis of these isolates have identified seven GBV-C genotypes (38, 87, 92). The distribution of GBV-C genotypes throughout the world is shown in Figure 1.2. GBV-C is suggested to co-exist with humans from early ages and geographic distribution of different GBV-C genotypes is consistent with migration pattern of ancient humans out of Africa (100). However, nucleotide sequence analysis of GBV-C genotypes reveals quite remarkable lack of genetic variability (over 86% conserved sequences among genotypes) unlike closely related HCV which has less than 30% sequence similarities between different genotypes (100). In 1998, a GBV-C variant was isolated from chimpanzee (GBV-C\textsubscript{cpz}) which is quite diverse from human isolates (GBV-C\textsubscript{hum}), about 84% amino acid similarity between GBV-C\textsubscript{cpz} and GBV-C\textsubscript{hum} compared to about 95% similarity between different human GBV-C isolates (17, 87).

As noted, GBV-C is produced by T and B-lymphocytes removed from infected subjects (43, 108) and \textit{ex vivo} GBV-C RNA is detected in other immune cells including NK cells, and monocytes (E Chivero, unpublished). To date, cellular receptor(s) for GBV-C has not been identified. One study found that GBV-C envelope protein E2 may interact with CD81 (93), however; subsequent study failed to find any specific interaction
between GBV-C E2 protein and CD81 concluding that CD81 does not interact with GBV-C E2 protein (61).

The primary site of GBV-C replication is not completely characterized. Early studies found GBV-C RNA in liver samples, consistent with it being a hepatitis virus (HGV). However, subsequent studies found that the relative amount of GBV-C RNA in blood compared to liver was high, while HCV levels were higher in the liver than in blood, suggesting that the liver may not be the site of replication (87). In addition, negative strand GBV-C RNA, indicative of viral transcription within cells, was found in bone marrow and spleen samples suggesting a hematopoietic site of replication. Negative strand RNA was not found in peripheral blood lymphocytes in some studies, even though peripheral blood mononuclear cells (PBMCs) produce virus ex vivo (87, 108). These findings raise the possibility that lymphocyte progenitor cells may represent the primary permissive cell for GBV-C replication. Further studies are required to gain insights into cellular receptors and primary replication site of GBV-C in human host.

**GBV-C interactions with HIV**

*Clinical Studies*

Following the discovery of GBV-C, several studies found an association between GBV-C infection and prolonged survival of HIV-positive individuals, although a few studies failed to demonstrate this beneficial effect (54, 71, 96, 123, 133, 136, 142, 152, 157). The studies that did not show an effect were cross-sectional, conducted during early HIV disease, or were conducted after widespread use of highly active and effective HIV therapy. Two large longitudinal studies found that persistence of GBV-C viremia was significantly associated with prolonged survival compared to those who were persistently negative for GBV-C RNA or who lost viremia (138, 142). In addition, a meta-analysis found that in studies conducted ‘late’ (> 5 years) into HIV infection (n=1,294 subjects), GBV-C viremia was associated with about a 2.5-fold reduction in
mortality (Relative Hazard for mortality 0.41; 95% confidence intervals 0.23; 0.69) (158). A recent study found in a cohort of HIV-infected subjects receiving blood transfusion, subjects exposed to GBV-C through blood transfusion and became viremic had significant reduction in mortality compared to subjects who were GBV-C non-viremic (137). GBV-C viremia is also associated with improved surrogate markers of HIV disease including higher CD4+ T cell counts, lower HIV viral load, and delayed progression to AIDS in many studies (54, 123, 133, 157), however, the strongest effect has been in the most definitive clinical endpoint of mortality. GBV-C viremia is associated with improved response to antiretroviral therapy (ART) in HIV-infected individuals as measured by a greater reduction of HIV viral load, improved CD4+ T cell count and less frequent changes of ART compared with those without GBV-C viremia (106, 121, 133). GBV-C viremia is also associated with reduced mother-to-child transmission of HIV in pregnant women, particularly when the infant is infected with GBV-C during parturition (50, 129).

Although a protective effect of GBV-C infection in HIV-positive individuals has been observed in numerous studies, the mechanism by which GBV-C modulates HIV infection and AIDS progression is not fully understood. Several in vivo and in vitro studies suggest that GBV-C infection may both interfere directly with HIV replication, and affect host cell factors that support the HIV life cycle. The effects of GBV-C infection in HIV-positive individuals that may limit HIV infection are summarized in Table 1.2.

**Mechanisms of HIV inhibition**

*Effects on HIV entry receptors*

The low surface expression of the two major HIV entry co-receptors (CCR5 and CXCR4) and high plasma level of the ligands for these receptors (MIP-1α [CCL3], MIP-1β [CCL4], RANTES [CCL5], and SDF-1 [CXCL12]) have been associated with slower
HIV disease progression (68, 90). In HIV-positive individuals, GBV-C viremia is associated with low surface expression of both CCR5 and CXCR4 (93, 113). Confirming this clinical observation, in vitro GBV-C infection decreases CCR5 surface expression, induces CCR5 and CXCR4 ligands and inhibits replication of both CXCR4 (X4) and CCR5 (R5) tropic HIV isolates in PBMCs (144).

**Inhibition of HIV replication by GBV-C proteins**

Expression of the GBV-C E2 glycoprotein and the NS5A phosphoprotein in CD4+ T cells inhibit HIV replication in vitro (20, 64, 148-150). Based on single cycle replication studies, both viral proteins inhibit HIV at least in part at the HIV entry step. The GBV-C E2 protein inhibits HIV pseudovirus entry, and peptides derived from the E2 protein interfere with HIV cellular binding and fusion, independent of the viral effect on CD4 cell homeostasis (59, 64). Synthetic peptides derived from GBV-C E1 protein also inhibit HIV entry, and appear to interact with HIV fusion peptides (112). In contrast, NS5A protein expression downregulates CXCR4 surface expression and induces the release of the CXCR4 ligand (SDF-1) in CD4+ T cells (20, 149). GBV-C NS5A protein also decreases CD4 surface expression via reduction in the steady state CD4 mRNA levels (151). Thus, down regulation of HIV entry receptors and secretion of ligands for chemokine receptors by GBV-C proteins contributes to HIV inhibition. The effects of GBV-C NS5A and E2 proteins on T cells that could contribute to limit HIV replication are summarized in Figure 1.3.

As noted, GBV-C antibodies are usually not detected during viremia but antibodies against GBV-C E2 appear following viral clearance (87, 123). Although GBV-C viremia is associated with the best survival in HIV-positive individuals, GBV-C E2 antibodies are also associated with improved survival in HIV-positive individuals without GBV-C viremia or E2 antibodies (133, 142). GBV-C E2 antibodies were shown to immunoprecipitate HIV-1 particles and neutralize diverse X4- and R5-tropic HIV isolates (88), suggesting that the E2 protein may contain an immunogenic structural motif
that cross-reacts with either a cellular or an HIV antigen present on HIV particles. The E2 antibodies bind to HIV-1 particles and interfere with HIV entry. A similar finding with HIV-1 envelope glycoprotein gp41 is well characterized. HIV-1 gp41 is involved in fusion of viral membrane with the cellular membrane, and a peptide motif containing HIV gp41 can inhibit HIV replication at the fusion step. Human monoclonal antibodies that react with this peptide motif (e.g. 2F5) neutralize diverse HIV isolates in vitro (51).

**Effects on Innate Immune Response**

Innate immune responses including type I interferon (IFN) play an important role in both controlling HIV infection and in activating other components of the immune system (74). Loss of IFN-producing cells and low levels of IFN-alpha are associated with a high HIV viral load, and are thought to play an important role in HIV disease progression (120). Plasmacytoid dendritic cells (pDCs) are major producers of IFN-alpha during viral infection and suppress HIV replication (84). pDCs are frequently depleted during HIV infection and aberrant IFN-alpha production is thought to contribute to HIV pathogenesis (31, 37, 60). The frequency of activated pDCs and both IFN-gamma and RNA-dependent protein kinase R (PKR) mRNA levels were higher in GBV-C viremic individuals compared to non-viremic individuals, suggesting that GBV-C alters innate immunity (66). GBV-C viral load correlated with the frequency of activated pDCs and IFN-gamma expression, and GBV-C induced IFN-gamma expression and activated pDC in vitro (66). PBMCs from GBV-C viremic individuals also express higher levels of mRNA from IFN-related genes [2-5- oligoadenylate synthetase (OAS), MxA, IFN AR-1 and PKR] compared to PBMCs from HIV-mono-infected individuals (19). Although the mechanism by which GBV-C enhances innate immune responses is unexplored, the increased frequency of activated pDCs and IFN expression during GBV-C infection may enhance the antiviral immune response to HIV infection.

**Th1/Th2 cytokines**

Immune responses mediated by Th1 cells are involved in HIV disease, and serum
Th1 cytokine levels are typically decreased in individuals with chronic and progressive HIV infection while Th2 cytokines are increased (10, 22, 58). The cytokine disruption during HIV infection contributes to HIV and opportunistic pathogenesis (122). Nunnari et al. found that GBV-C viremia was associated with stable serum Th1 cytokine levels (IL-2 and IL-12) compared to decreased IL-2 and IL-12 levels in HIV-mono-infected individuals in a longitudinal study of HIV-infected Sicilians (96). Conversely, serum levels of Th2 cytokines (IL-4 and IL-10) increased in HIV-mono-infected people while IL-4 and IL-10 levels were low and did not increase in GBV-C coinfected individuals (96). The high level of Th1 cytokines and low level of Th2 cytokines found with GBV-C coinfection suggest that GBV-C polarizes T cells toward a Th1 cytokine profile, which may in turn be beneficial to HIV-positive individuals.

Consistent with these clinical findings, Rydze et al. recently found that in vitro infection of PBMCs, and the expression of the GBV-C NS5A protein in a CD4+ T cell line resulted in increased IL-2, IL-12b, and IFN-gamma mRNA expression, with decreased IL-4 and IL-13 mRNA expression, and with decreased secretion of IL-10 (109). These studies further suggest that GBV-C may promote Th1 differentiation and induce a Th1-specific cytokine milieu that is protective against HIV infection and that the NS5A protein contributes to this effect. Since many parasitic and allergic diseases are also influenced by Th1-Th2 cytokines, further studies on a potential role for GBV-C in the natural history of other diseases appears warranted.

**Modulation of Apoptosis**

Fas-mediated apoptosis contributes to CD4+ T cell depletion during HIV infection (29). HIV-infected individuals have an increased frequency of Fas expressing lymphocytes compared to uninfected individuals (86). However, the frequency of Fas expressing lymphocytes is significantly lower in people with GBV-C and HIV-coinfection compared to those with HIV-mono-infection (86). Fas expression on T cells
directly correlated with Fas-mediated apoptosis of T cells, thus suggesting that GBV-C may protect against CD4 T cell depletion during HIV infection.

HIV-induced CD4+ T cell depletion may result from direct lysis or lysis by HIV-specific cytotoxic T cells. However, since most CD4+ T cells do not contain HIV, CD4 depletion requires killing of bystander (uninfected) CD4+ T cells (35, 39). These bystanders are thought to be depleted through either pro-apoptotic HIV proteins released from the infected cells, or by activation-induced cell death (AICD) induced by persistent immunoactivation observed during HIV infection (3). The Fas/FasL pathway has been suggested to play a major role during AICD of bystander cells (8, 30), and GBV-C may influence CD4 cell depletion by its effects on Fas expression and Fas-mediated apoptosis.

**Effects on Lymphocyte activation**

Immune dysfunction and chronic immune activation are the characteristic features of HIV infection and AIDS progression. The aberrant activation of T cells during HIV infection is associated with an increased expression of cellular activation markers, lower CD4+ T cell gain with antiretroviral therapy, and lower CD4+ T cells counts (48, 57). Immunoactivation involves bystander T and B cells in addition to the HIV-infected CD4+ cells, and contribute greatly to HIV disease progression by enhancing HIV replication, increasing AICD of bystander cells, and perturbing immune cell function (48, 89).

Recent studies provide insight into the effects of GBV-C on reducing immunoactivation, which may also contribute to the beneficial association between GBV-C and survival of HIV-positive subjects. GBV-C viremic HIV-infected subjects have lower surface expression of T cell activation markers (CD38, CD69, CD25, CCR5) on CD4+ and CD8+ T cells than GBV-C negative subjects, independent of HIV viral load (78, 93, 113). CD38 expression on CD8+ T cells was inversely related to GBV-C viral load (78), further suggesting that GBV-C coinfection dampens T cell activation.
Since GBV-C replicates in T (CD4+ and CD8+) and B-lymphocytes, GBV-C infection may directly lower the activation state in these cells leading to clinical benefit in HIV-infected individuals. However, based on activation marker expression on bulk CD4+ and CD8+ T cells, the effect of GBV-C on activation is not limited to actively infected cells. Although GBV-C replication is inefficient in primary PBMCs (45, 153), the lower expression of T cell activation markers in bulk CD4+, and CD8+ T cells from GBV-C viremic subjects suggest that the effect of GBV-C on immune activation must result from paracrine effects of GBV-C on bystander cells. Importantly, the effect of GBV-C on immune activation is incomplete, since a more potent reduction in immune activation would result in immune deficiency.

Although understanding the cause of reduced immune activation during GBV-C co-infection requires further characterization, data identifying potential mechanisms by which GBV-C modulates T cell activation are emerging. T cell activation in response to foreign antigens is mediated by T cell receptor (TCR) signaling and IL-2 production (94). IL-2 is a key cytokine which promotes T cell activation, proliferation and supports HIV replication (99). GBV-C viremic subjects had significantly reduced response to IL-2 therapy compared to GBV-C non-viremic subjects (126) suggesting GBV-C infection may alter IL-2 signaling pathways and affect T cell activation. Consistent with this, Berzsenyi et al. found that intra-hepatic T cell signaling is impaired in GBV-C co-infected (HCV- and HIV-) positive individuals (13). Liver samples from GBV-C coinfected HIV- and HCV- positive individuals had significantly lower expression of lymphocyte-specific protein tyrosine kinase (Lck) compared to GBV-C negative HIV- and HCV- positive individuals, however Lck expression in PBMCs did not differ by GBV-C status (13). Lck plays a crucial role in proximal TCR signaling, thus down regulation of Lck expression during GBV-C co-infection could alter TCR signaling pathways. Together, these studies suggest that GBV-C might affect immunoactivation pathways resulting in reduced activation in coinfected HIV-positive individuals.
Modulation of IL-2 signaling and CD4 expansion

As noted, IL-2 (originally called ‘T cell growth factor’) is a key cytokine involved in growth, differentiation, and survival of T cells (94). Administration of recombinant IL-2 as adjunctive immunotherapy is efficacious in a subset of people with renal cell carcinoma and melanoma (7), and since IL-2 therapy leads to CD4+ T cell proliferation and expansion, IL-2 immunotherapy was extensively studied in HIV disease (99, 103). Although IL-2 infusions significantly increased CD4+ T cell counts in HIV-infected individuals, a large prospective, randomized trial conducted during the era of highly active combination antiretroviral therapy found no clinical benefit (1). In vitro GBV-C replication was reduced when PBMCs were cultured in IL-2 and PHA (43, 108). To examine the potential interaction between GBV-C and IL-2, study subjects participating in a prospective, randomized trial of IL-2 were evaluated for GBV-C (126). Subjects randomized to receive intravenous (IV) IL-2 had significantly increased CD4+ T cell counts compared to those who received either subcutaneous IL-2 or no IL-2 (126). However, when stratified by GBV-C viremia status, subjects without GBV-C who received IV IL-2 had a significantly greater increase in CD4 counts compared to GBV-C viremic subjects (859 vs. 180 cell increase at week 60). CD4+ T cells were no greater in GBV-C viremic subjects who received IV IL-2 compared to those who did not receive IV IL-2, indicating a significant interaction between GBV-C and the IL-2 cytokine. Thus, these studies suggest that GBV-C may interfere with both proliferation and IL-2 signaling pathways contributing to HIV inhibition. Figure 1.4 summarizes GBV-C infection effects on T cells which can potentially limit HIV infection.

Although GBV-C infection is common and may persist for decades, most healthy individuals clear viremia within 2 years of infection (4, 131). Although the mechanisms that control GBV-C persistence and clearance are not defined, it is unlikely due to differences in viral sequences, as full-length GBV-C genomes from different genotypes share more than 85% nucleotide and 94% amino acid sequence identity. Since no
geographic differences in clearance rates are reported (87), it is likely that, similar to HCV infection, host genetic factors are responsible for differences in viral persistence (156). Since persistent GBV-C infection is important for the beneficial effect, understanding the mechanisms by which GBV-C persists in its host should provide insights into the protective effect of GBV-C.

Like all viruses, GBV-C depends upon its human host for replication. To date, GBV-C has not been convincingly associated with any disease. However, it appears to be protective against HIV infection and may influence other diseases as well, suggesting a mutually beneficial symbiotic relationship. Clinical studies of GBV-C and its interaction with host cells provide new insights into the observed associations between GBV-C infection and improved survival in HIV-positive individuals. GBV-C infection leads to diminished immunoactivation and T cell proliferation that may limit HIV replication and slows disease progression. In this thesis, we discuss the interrelationship between GBV-C infection and immunoactivation and potential mechanisms by which GBV-C infection leads to reduction in immunoactivation and IL-2 signaling pathways.

**T cell receptor (TCR) and IL-2 Signaling Pathways**

Engagement between T cell receptor (TCR) present on the surface of T cells with peptide bound MHC complex present on the surface of the antigen presenting cells (APC) initiates rapid and complex signaling events that regulate activation and differentiation of T cells (27, 94) (Figure 1.5). The components of T cell signaling pathways assemble to form functional complexes that regulate the signal transduction leading to activation of T cells. One of the important components of this signaling pathway is protein tyrosine kinases (PTKs). Non-receptor tyrosine kinases like lymphocyte-specific tyrosine kinase (Lck) and Zeta-chain associated protein (ZAP-70) play a critical role in TCR signaling (94). Lck is one of the most proximal tyrosine kinases that is activated following TCR engagement (Figure 1.6). In resting T cells, Lck
is inactive and remains in a closed conformation with tyrosine phosphorylation at residue 505 by the activity of C-terminal Src kinase (Csk). Following TCR engagement, CD45 tyrosine phosphatase dephosphorylates tyrosine residue 505 leading to a conformation change in the protein and trans-auto-phosphorylation of Lck at tyrosine residue 394. This is an active form of Lck protein, which then phosphorylates tyrosine residues within the immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic tail of the TCR complex. The phosphorylated ITAM motifs serve as a docking site for Src homology domain 2 (SH2)-containing proteins like ZAP-70. Upon phosphorylation by Lck, the activated ZAP-70 further phosphorylates downstream effector molecules like linker for activated T cells (LAT) adaptor protein leading to functional activation of T cells. Lck activation is an important early and important step in this signaling cascade and inactivation of Lck results into defect in TCR signaling. Lck is expressed in all stages of T cell maturation and required T cell development (5). In addition to its role in TCR signaling pathways, Lck is also associated with IL-2 receptor beta chain and plays an important role in IL-2 signaling pathways (55, 85). Thus, Lck appears to play an important role in T cell activation, T cell development, and IL-2 signaling.

The Lck protein localizes in the cytoplasm where it interacts with inner leaflets of the plasma membrane via its hydrophobic N-terminus. The N-terminal region of Lck is divided into three distinct regions that can mediate interactions with other proteins (Figure 1.6). The unique domain that associates with CD4 or CD8, Src homology domain 3 (SH3) which can bind to proline-rich motifs and SH2 domain which can interact with tyrosine phosphorylated proteins. These domains play an important role during Lck function, and binding to Lck substrates via these domains may enhance or reduce Lck function.

Upon TCR engagement, T cells undergo a dramatic change in their transcriptional profile which can be measured within few hours of activation. One of the major read out during this process is expression of cytokines and receptors.
Interleukin 2 (IL-2) is a pleiotropic cytokine that is required for T cell activation, survival, and T cell effector function. Human IL-2 is a 133-amino acid protein with a molecular mass of 15-18 kDa. Upon activation, T cells upregulate IL-2 production and CD25 expression, one of the components of IL-2 receptor (IL-2R) complex. IL-2R complex consists of IL-2Rα (CD25), IL-2Rβ (CD122), and IL-2γ (CD132). In absence of IL-2Rα, signaling through IL-2R is minimal. Following T cell activation and upregulation of CD25, it interacts with IL-2Rβ (CD122) and IL-2γ (CD132) receptors to form the high affinity IL-2 receptor that binds IL-2 and induces IL-2 signaling. Phosphorylation of the cytoplasmic domain of the gamma-subunit of the IL-2R provides docking site for protein tyrosine kinase Janus kinase 3 (JAK3). JAK3 that is associated with the IL-2Rγ subunit is activated by autophosphorylation after binding of IL-2 to its receptor. One of the early events during IL-2 signaling is activation of signal transducer and activation of transcription (STAT5) by JAK3. STAT5 is a transcription factor that is found in the cytoplasm during resting condition. In response to IL-2R signaling, STAT5 is tyrosine phosphorylated; homo-dimerized and is translocated into the nucleus where it activates transcription of IL-2 responsive genes. These transcription events further support T cell activation and T cell effector function.

Thus, TCR and IL-2 signaling pathways are tightly coupled where tyrosine kinase like Lck plays a critical role in regulating signal transduction. Inactivation of Lck in T cells results into defect in TCR and IL-2 signaling which ultimately results into reduced T cell activation.

Exosomes

Intercellular communication is an important aspect of tissue function and is required for tissue homeostasis. Cells communicate with each other via various mechanisms; however, the role of extracellular microvesicles (EMVs) in intercellular communication is not completely understood and is being increasingly recognized.
EMVs are membrane-enclosed organelles which are secreted by various cells and contain proteins and RNAs. EMVs are of various types and primarily differentiated based on their origin and biophysical properties (80). The major types of EMVs include: exosomes, 40-100 nm diameter vesicles of endocytic origin; ectosomes, large membranous vesicles (50-1000 nm diameter) that are shed directly from the plasma membrane (PM) and apoptotic blebs (50-5000 nm diameter) secreted by dying cells. Of all EMVs, exosomes are the only known secreted vesicles that originate from internal membranes. Many cell types including T cells, B cells, dendritic cells, mast cells, and epithelial cells release exosomes. Exosomes are also found in body fluids including plasma, saliva, urine, ascites, and breast milk. These exosomes can transfer their cargo from secreted cells to the bystander cells and influence bystander cell function. The mechanism that determines the specific fate of exosomes are not well understood, but endosomal sorting complex required for transport (ESCRT) machinery appears to be important for exosomal maturation and are enriched with ESCRT components (e.g. Tsg101). In addition, exosomes are commonly associated with tetraspanins like CD63 and CD81.

Viruses have known to exploit cellular pathways for viral replication, escape from host immune responses, and establish persistent infections etc. Viruses that interact with or require the ESCRT pathway for release include rhabdoviruses, filoviruses, arenaviruses, paramyxoviruses, herpesviruses, HBV, and hepatitis C virus (HCV). The role of exosomes during these viral infections is not clear, but data are emerging to suggest that viruses may utilize exosomes to enhance infection and modulate host cell signaling. Exosomes released from HIV infected cells have been shown to contain co-receptors that can enhance virus entry into cells (77). Transfer of HIV Nef protein to bystander T and B cells have been implicated to increase T cell death and impair humoral immune response during HIV infection (73, 154). In case of hepatitis C virus, viral RNA and envelope protein E2 are found in exosomes (33, 79) and transfer of HCV RNA via
exosomes to bystander cells contributes to cellular activation. Similarly, exosomes containing viral factors have been reported for other viruses including hepatitis B, herpes simplex, and Epstein-barr virus (83).

Utilizing cellular microvesicles may be an effective viral strategy to modulate cellular communication and influence global cellular signaling pathways. Since viruses appear to utilize exosomal pathways, studying virally modified microvesicles may provide insights into viral infection. In addition, study of the effects of exosomal transfer of viral and cellular factors to the uninfected bystander cells, particularly in the case of persistent RNA virus infections like HCV may provide insights into mechanisms of viral persistence.

Overview of Thesis Chapters

Chapter II: Effects of GBV-C infection on T cell activation in HIV-positive individuals on antiretroviral therapy

GBV-C co-infection is associated with reduced T cell activation in HIV viremic subjects (13, 78, 113). Although HIV-positive subjects on antiretroviral therapy have lower T cell activation compared to HIV viremic subjects, activation does not return to levels observed in uninfected healthy subjects (56, 139). Furthermore, effects of GBV-C infection on T cell activation in HIV-positive subjects on antiretroviral therapy have not been studied. This chapter describes the effects of GBV-C infection on T cell activation in cohort of HIV-positive subjects on combination antiretroviral therapy (cART) with undetectable HIV viral load. The potential role of immunomodulatory double negative T cells in reducing chronic immunoactivation and relationship between GBV-C viremia and double negative T cells are discussed.
Chapter III: Ex vivo activation effects on GBV-C replication, proliferation and survival of GBV-C infected primary T cells

Clinical studies demonstrate that GBV-C infection reduces immunoactivation and alters IL-2 signaling pathways in HIV-infected subjects (78, 126); however, the interrelationship between GBV-C infection and these signaling pathways has not been well characterized. Ex vivo studies of primary T cells from GBV-C infected subjects may provide insights into mechanisms by which GBV-C alters these signaling pathways. Chapter III describes the interrelationship between immunoactivation and GBV-C infection. Utilizing primary T cells from GBV-C infected subjects, effects of immunoactivation on GBV-C replication, effects of GBV-C on IL-2 mediated lymphocytes proliferation, reactivation of latent HIV from cellular reservoirs and activation induced T cell death are described.

Chapter IV: GB virus C envelope glycoprotein E2 inhibits T cell receptor induced IL-2 production and alters IL-2 signaling pathways

GBV-C viremia is associated with blunted response to IL-2 therapy in HIV-positive subjects (126). The mechanism by which GBV-C alters IL-2 signaling pathways or viral factors that contribute to this effect are not known. Previous studies have identified anti-HIV replication effects of GBV-C envelope glycoprotein E2 (59, 150). Expression of GBV-C envelope glycoprotein E2 in a CD4+ T cell line inhibits HIV replication by interfering with HIV entry and the functional domain of E2 protein required to inhibit HIV was mapped to a C-terminal peptide motif (150). Although GBV-C E2 is a multifunctional protein, its effect on cellular activation and IL-2 signaling is not known. This chapter describes the effects of GBV-C envelope glycoprotein E2 on IL-2 signaling pathways by measuring T cell receptor induced IL-2 secretion, IL-2 receptor expression, STAT5 phosphorylation, and cellular proliferation. Purified
recombinant GBV-C E2 protein is used to study the effects on IL-2 signaling in primary human T cells. Furthermore, identification of a functional region within the viral protein E2 that alters IL-2 signaling which is distinct from HIV inhibitory motif is described.

**Chapter V: GB virus C envelope glycoprotein E2 inhibits T cell receptor signaling in infected and uninfected bystander T cells via inhibiting Lck**

Chapter V describes the potential mechanism by which GBV-C infection and envelope glycoprotein E2 alters T cell activation and IL-2 signaling pathways. Utilizing Jurkat CD4+ T cells stably expressing viral protein E2, the effects of GBV-C E2 protein on activation of proximal T cell receptor signaling pathways are described. Deletion mutagenesis studies identified a peptide motif within the viral envelope protein E2 that is required to inhibit T cell activation pathways. Utilizing kinase-substrate prediction programs and *in vitro* enzyme assays, the mechanisms by which GBV-C E2 protein inactivates a key tyrosine kinase of the TCR and IL-2 signaling pathways are described. Furthermore, the mechanisms by which GBV-C infection reduces activation of uninfected bystander T cells leading to a global reduction in immunoactivation are discussed.

**Chapter VI: General Discussion**

Chapter VI summarizes the current existing data on interactions between GBV-C and HIV. The effects of immunoactivation on HIV disease progression and GBV-C effects on reducing immunoactivation are discussed. Finally, future research required to better understand mechanisms by which GBV-C alters HIV disease progression and reduces immunoactivation are highlighted.
Table 1.1. Known functions of GBV-C structural and nonstructural proteins and its effect on HIV replication

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>Envelope protein 1, synthetic E1 peptides inhibit HIV replication.</td>
</tr>
<tr>
<td>E2</td>
<td>Envelope protein 2, E2 contains predicted receptor binding site and E2 is a major antigenic protein. E2 protein and synthetic peptides derived from E2 protein inhibit HIV replication and modulate T cell activation.</td>
</tr>
<tr>
<td>NS2</td>
<td>Nonstructural protein 2, contains serine protease function and mediates cleavage at the NS2/NS3 junction.</td>
</tr>
<tr>
<td>NS3</td>
<td>Nonstructural protein 3, contains protease function and mediates NS3/NS4 and NS5A/NS5B cleavage and, together with NS4A, mediates cleavage of the NS4B/NS5A junction. NS3 is also predicted to contain helicase and NTPase functions. NS3 expression in human T cells inhibits HIV replication.</td>
</tr>
<tr>
<td>NS4A</td>
<td>Nonstructural protein 4, co-factor for NS3 protease</td>
</tr>
<tr>
<td>NS4B</td>
<td>Nonstructural protein 4B, function not known</td>
</tr>
<tr>
<td>NS5A</td>
<td>Nonstructural protein 5A, modulates host immune response and expression of NS5A inhibits HIV replication in human T cells</td>
</tr>
<tr>
<td>NS5B</td>
<td>Nonstructural protein 5B, RNA-dependent RNA polymerase</td>
</tr>
</tbody>
</table>
Table 1.2. Summary of the effects of GBV-C infection in HIV-positive individuals

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>GBV-C infection downregulates HIV entry co-receptors CCR5 and CXCR4, and increases secretion of their ligands, RANTES, MIP-1α, MIP-1β and SDF-1.</td>
</tr>
<tr>
<td>2.</td>
<td>In vitro GBV-C NS5A and E2 proteins inhibit X4- and R5-tropic HIV replication and NS5A protein downregulates CD4 and CXCR4 gene expression.</td>
</tr>
<tr>
<td>3.</td>
<td>HIV-infected individuals positive for GBV-C E2 antibodies have survival benefit over HIV-infected individuals with neither GBV-C viremia nor E2 antibodies and in vitro GBV-C E2 antibodies immunoprecipitate HIV particles, and inhibit X4- and R5-tropic HIV replication.</td>
</tr>
<tr>
<td>4.</td>
<td>GBV-C induces activation of interferon-related genes and plasmacytoid dendritic cells (pDCs).</td>
</tr>
<tr>
<td>5.</td>
<td>GBV-C promotes Th1 polarization and the NS5A protein contributes to this effect.</td>
</tr>
<tr>
<td>6.</td>
<td>GBV-C infection reduces surface expression of activation markers on T lymphocytes, suggesting its role in T cell activation signaling pathways.</td>
</tr>
</tbody>
</table>
GBV-C genome organization

GBV-C polyprotein processing

Figure 1.1. GB virus C genome organization and polyprotein processing. GBV-C genome contains a 5’ non translated region containing internal ribosomal entry sites directing translation of poly proteins. The poly proteins are post translationally processed into structural proteins [envelope glycoproteins (E1 and E2)] by cellular signal peptidases. The nonstructural proteins NS2 and NS3 are processed by viral protease NS2 and the rest of the nonstructural proteins are processed by NS3/4A protease.
Figure 1.2. Prevalence and genotypic distribution of GBV-C viremia in global population. Based on prevalence studies of GBV-C in healthy blood donors reviewed by Mohr et al. 2009. GBV-C infection as determined by presence of viral RNA in the serum is about 1% to 4% in developed countries; and up to 19% in developing countries.
NS5A induces secretion of CXCR4 ligand, SDF-1

E2 interferes with HIV binding

NS5A downregulates HIV entry receptors

E2 and NS5A inhibit HIV replication

Figure 1.3. *In vitro* effects of GBV-C proteins E2 and NS5A on CD4+ T cells. E2 protein interferes with HIV cellular binding and/or fusion (purple box), while the NS5A protein downregulates CD4 and CXCR4 expression and induces SDF-1, soluble ligand for CXCR4. NS5A protein also induces Th1 cytokines and blocks expression of Th2 cytokines (blue boxes). GBV-C E2 and NS5A proteins inhibit HIV replication (green box).
Figure 1.4. *In vivo* effects of GBV-C infection that can potentially interfere with HIV replication. GBV-C infection reduces CCR5 and CXCR4 surface expression and induces soluble ligands for CCR5 (RANTES, MIP-1α, and MIP-1β) and CXCR4 (SDF-1). GBV-C infection reduces activation, proliferation and apoptosis in T cells. GBV-C also enhances expression of interferons, activates plasmacytoid dendritic cells and promotes Th1 cytokines leading to enhanced innate immune response. These effects can potentially limit HIV replication and slow disease progression.
Figure 1.5. Schematic of proximal T cell receptor (TCR) signaling pathways.

Engagement of TCR with peptide bound MHC complex present on the surface of antigen presenting cells (APC) initiates TCR signaling cascade. In resting T cells, the proximal non-receptor tyrosine kinase Lck is inactive and phosphorylated at inhibitory tyrosine residue Y505 by C-terminal Src kinase (Csk). Following TCR engagement, CD45 tyrosine phosphatase dephosphorylates tyrosine residue 505 leading to a conformation change in Lck and trans-auto-phosphorylation of Lck at tyrosine residue 394. This is an active form of Lck protein, which then phosphorylates tyrosine residues within the immunoreceptor tyrosine based activation motifs (ITAMs) in the cytoplasmic tail of the TCRζ chain. The phosphorylated ITAM motifs serve as a docking site for Src homology domain 2 (SH2)-containing proteins like ZAP-70. Upon phosphorylation by Lck, the activated ZAP-70 further phosphorylates downstream effector molecules like linker for activated T cells (LAT) adaptor protein leading to functional activation of T cells marked by expressing of activation receptors like CD38, CD69 and secretion of pro-inflammatory cytokines like IL-2.
Figure 1.6. Schematic of Lck domain organization and activation. The N-terminal region of Lck is divided into three distinct regions, the unique domain that associates with CD4 or CD8, Src homology domain 3 (SH3) which can bind to proline-rich motifs and SH2 domain which can interact with tyrosine phosphorylated proteins. The C-terminal region contains kinase domain via which it exerts catalytic function. Lck function is regulated by tyrosine phosphorylation of two key tyrosine residues, Y505 and Y394 (A). In resting T cells, Lck is in closed conformation and inactive due to phosphorylation of Y505 by C-terminal Src kinase (Csk). Following activation, CD45 tyrosine phosphatase dephosphorylates tyrosine residue 505 leading to a conformation change in Lck and trans-auto-phosphorylation of Lck at tyrosine residue 394. This is an active form of Lck protein (B).
Chapter II
EFFECTS OF GBV-C INFECTION ON T CELL ACTIVATION IN HIV-POSITIVE INDIVIDUALS ON ANTIRETROVIRAL THERAPY

Chapter Summary

HIV infection is characterized by chronic immunoactivation and double negative T cells (CD3\(^+\)/CD4\(^-\)/CD8\(^-\) T cells) play a role in limiting chronic immunoactivation. In clinical studies, GBV-C infection is associated with reduced T cell activation in HIV-viremic individuals. This chapter describes the effect of GBV-C infection on T cell activation in HIV-positive subjects with undetectable HIV viral load (VL). T cell activation and double negative T cells were measured in a cohort of HIV-positive subjects with undetectable HIV viral load with or without GBV-C infection. GBV-C viremic subjects had reduced CD4\(^+\) and CD8\(^+\) T cell activation (p = 0.003 and 0.034 respectively,) and higher levels of double negative T cells in the peripheral blood compared to non viremic subjects (p = 0.038). GBV-C VL significantly correlated with double negative T cell percentage (p = 0.004). In conclusion, we found that GBV-C infection was associated with an increase in double negative T cells and lower T cell activation in HIV-positive subjects on antiretroviral therapy with undetectable HIV VL. This increase in double negative T cells during GBV-C infection may contribute to reduce immunoactivation during HIV infection.

Introduction

HIV infection is associated with chronic immunoactivation, which contributes to HIV replication and AIDS progression (48, 118). Immunoactivation correlates with HIV VL and HIV disease progression (52). Effective combination antiretroviral therapy (cART) reduces T cell activation, but not to levels observed in HIV-uninfected people.
GB virus C (GBV-C) is a common human flavivirus that, due to shared modes of transmission, is highly prevalent in HIV-infected individuals (up to 42%) (87). Although GBV-C infection is not convincingly associated with any human disease, several studies and a meta-analysis including 1,294 subjects found an association between persistent GBV-C infection and prolonged survival in HIV-infected individuals (158). Consistent with these findings, GBV-C viral load (VL) is inversely related to HIV VL in co-infected individuals (133). Additionally, both GBV-C infection and expression of two GBV-C proteins inhibit HIV replication in human CD4+ T cells (16). GBV-C is lymphotropic and replicates in T and B cells (43), thus, GBV-C infection may alter lymphocyte functions which could potentially interfere with HIV infection (16).

In clinical studies, GBV-C and HIV co-infection is associated with significantly lower levels of T cell activation, and with reduced CD4 expansion in response to therapeutic interleukin 2 (IL2) infusion (15, 108, 125). The mechanisms by which GBV-C infection might lower immunoactivation and proliferation are not understood; however, these effects may limit HIV infection and potentially slow disease progression.

Double negative (DN) T cells (CD3+, CD4–, CD8–) play an important role in reducing immunoactivation and in maintaining immune homeostasis during infection, inflammation, and autoimmunity (25). Recently, the level of DN T cells was shown to inversely correlate with T cell activation and proliferation in subjects with acute HIV infection (101). In addition, DN T cells were associated with the secretion of immunosuppressive cytokines IL-10 and TGF-beta, which may contribute to the reduced activation.

Since GBV-C co-infection is associated with lower T cell activation and proliferation in HIV viremic subjects, we examined HIV-infected subjects with undetectable HIV plasma RNA levels on combination antiretroviral therapy (cART) to determine if there is an association between GBV-C viremia, DN T cells and T cell activation.
**Materials and Methods**

**Study Subjects**

Asymptomatic, HIV-positive individuals attending the University of Iowa HIV Clinic who were receiving ART and had HIV VL levels below the limit of detection (<48 copies/ml) for more than 6 months were invited to participate. The average duration of HIV VL suppression was 60 months in subjects with GBV-C viremia and 72 months in those without GBV-C viremia. Subjects were selected who had previously been tested for GBV-C viremia to provide comparable numbers of people with HIV and GBV-C coinfection and HIV monoinfection. This study was approved by the University of Iowa Institutional Review Board and all subjects provided written consent.

**PBMC isolation and flow cytometry**

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation within four hours of blood collection and evaluated by flow cytometry using the following antibodies as directed by the manufacturer (Becton Dickinson Biosciences; BD): (CD3 [Pacific Blue], CD4 [APC-H7], CD8 [FITC], and CD38 [PerpCp-Cy5.5]. LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen) was used to determine cell viability. Cells were incubated with antibodies on ice for 1 hour, washed 3 times with PBS, and fixed in 2% paraformaldehyde (Polysciences). Data were acquired using a BD LSRII flow cytometer and CompBeads (BD) were used for compensation. At least 20,000 live CD3+ events were collected. Data analysis utilized FlowJo software (Tree Star Inc.).

**Statistics**

Statistics were performed using GraphPad software V4.0 (GraphPad Software Inc.). Comparisons between two groups were carried out using Mann-Whitney nonparametric tests. Correlation between GBV-C viral load and DN T cells were analyzed in linear regression analysis and Spearman nonparametric test. *P* values less than 0.05 were considered statistically significant.
Results

Subjects descriptions

Forty two HIV-infected subjects who were effectively treated with cART were evaluated. Half (21) of the 42 subjects had GBV-C viremia with an average GBV-C VL of $1.6 \times 10^8$ genome copies/ml. There were no significant differences in age (GBV-C positive 46.5 years vs. GBV-C negative 47.7), gender (1/21 female in GBV-C positive vs. 3/21 in GBV-C negative), race (2 black, 19 white GBV-C positive vs. 3 black, 18 white in GBV-C negative), CD4 nadir (236 cells/mm3 in those GBV-C positive vs. 192 cells/mm3 in GBV-C negative), percent CD4 (29.3% in GBV-C positive vs. 32% in GBV-C negative), length of time of HIV suppression (64 months GBV-C positive vs. 76 months in GBV-C negative), or mode of HIV transmission between the GBV-C viremic and non-viremic subjects ($p>0.05$ for all, Table 2.1).

Relationship between GBV-C viremia and double negative T cells

Since GBV-C viremia is associated with lower T cell activation and DN T cells play an important role in suppressing immune activation, we compared the percentage of total CD3+ cells that lacked both CD4 and CD8 surface expression (DN T cells) in subjects with and without GBV-C viremia. Subjects with GBV-C viremia had a significantly higher percentage of DN T cells compared to GBV-C non-viremic controls (median 4.47%; interquartile range (IQ) 2.83 - 8.89 versus median 2.77%; IQ 2.10 - 4.24; $P = 0.038$; Fig. 2.1A). The GBV-C VL correlated with DN T cell levels (Fig. 2.1B and 2.1C).

Relationship between GBV-C viremia and T cell activation

Expression of CD38 on the surface of CD4+ and CD8+ T cells has proven useful in quantifying the level T-cell activation in subjects with HIV infection (56, 78, 101). Among the 42 HIV-infected individuals on cART studied, T cell activation as measured
by CD38 surface expression on CD4+ and CD8+ T cells was significantly lower in subjects with GBV-C viremia compared to those without [CD4+ T cells: median 63.6%; IQ , 44.9 - 81.5 versus median 49.35%; IQ 30.9 - 80.6 respectively; \( P = 0.003 \); Fig. 2.2A] and [CD8+ T cells: median 51.10%; IQ , 40.40 - 83.10 versus median 45.65%; IQ , 18.50 - 67.80 respectively; \( P = 0.034 \); Fig. 2.2B].

To assess relationship between levels of DN T cells and GBV-C VL with activation, we examined correlation between CD38 expression and levels of DN T cells or GBV-C VL. Although there were significant differences in levels of DN T cells and levels of activation in GBV-C viremic subjects compared to non viremic controls (Fig. 2.1; Fig. 2.2); we did not observe a significant correlation between levels of CD38 expression with either levels of DN T cells (Spearman \( r = -0.02 \); \( P = \text{ns} \)) or GBV-C VL (Spearman \( r = -0.22 \), \( P = \text{ns} \) for [CD4+CD38+]; Spearman \( r = 0.18 \); \( P = \text{ns} \) for [CD8+CD38+]) (Figure 2.3).

**Discussion**

Chronic immune activation during HIV infection is characterized by increased HIV VL, lower CD4 T cell gain after antiretroviral therapy, loss of CD4+ T cells by activation induced cell death and immune dysfunction (48, 56). Several, though not all, studies observed a beneficial association between GBV-C infection and survival in HIV-infected individuals (16). Although the mechanisms for this protective effect are not fully understood, recent studies indicate that GBV-C is associated with reduced T cell activation *in vivo* and *in vitro* (15, 108, 125). In this study, we found that GBV-C viremia is associated with a reduction of T cell activation among HIV-infected subjects with suppressed HIV VL compared to those without GBV-C viremia (Fig. 2.2). Since cART also reduces T cell activation, the effect of GBV-C viremia on T cell activation in these subjects appears to be modest compared to those with GBV-C and HIV viremia.
previously reported (78). Nevertheless, GBV-C viremia was associated with reduced T cell activation in subjects with suppressed HIV RNA on cART.

A role for DN T cells in the regulation of immune responses to viral infections is increasingly being recognized (25), and human DN T cells are important modulators of cytokine production and T cell proliferation (140). Higher levels of DN T cells are associated with reduced T cell activation and proliferation during acute HIV-1 infection (101). The effect on T cell activation appears to be related to secretion of immunosuppressive cytokines IL-10 and TGF-beta. Among HIV-positive individuals successfully treated with ART for more than 6 months we found that GBV-C viremic subjects had a significantly higher percentage of DN T cells as compared to those without GBV-C viremia (Fig. 2.1A). The percentage of DN T cells in the peripheral blood correlated with GBV-C VL, suggesting a causal relationship between GBV-C viremia and the increased percentage of DN T cells (Fig. 2.1B). Although the mechanism for higher levels of DN T cells during GBV-C infection has not been studied, the immunosuppressive functions of DN T cells may contribute to lower immune activation in these individuals. Although the levels of DN T cells were higher and CD38 expression lower in GBV-C viremic subjects, we did not observe a significant relation between levels of DN T cells and expression levels of CD38 in GBV-C viremic subjects. Thus, although the higher levels of DN T cells may contribute to the reduction in immune activation in GBV-C viremic subjects, other mechanisms of inhibition of activation by GBV-C may be involved.

In conclusion, we found that GBV-C viremia is associated with reduced T cell activation and an increase in the percentage of DN T cells in HIV-positive subjects on cART. Since chronic immune activation contributes to HIV disease progression, GBV-C infection mediated diminution of immune activation and proliferation may protect against HIV disease, and the observed increase in DN T cells among those with GBV-C infection may contribute to this reduction in immune activation during HIV-infection. Further
understanding of the mechanisms by which GBV-C alters immune activation, proliferation and regulates DN T cells may lead to novel approaches to HIV therapy.
### Table 2.1. Subject Demographics

<table>
<thead>
<tr>
<th>Variable</th>
<th>GBV-C RNA Positive (n=21)</th>
<th>GBV-C RNA Negative (n=21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46.5</td>
<td>47.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Caucasian</td>
<td>19</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HIV suppression(^1) (months)</td>
<td>64</td>
<td>76</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CD4 Nadir/mm(^3)</td>
<td>236</td>
<td>192</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CD4 Percent</td>
<td>29.3</td>
<td>32</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

\(^1\)HIV Suppression = average number of months that HIV RNA was suppressed to <48 copies/mL
Figure 2.1. **GBV-C viremia is inversely associated with double negative T cells.** The percentage of CD3+ T cells that lacked surface expression of both CD4+ and CD8+ was measured in HIV-suppressed individuals by flow cytometry (N=37). CD3+ CD4-CD28- T cells were significantly higher among the HIV-infected subjects with GBV-C viremia compared to those without GBV-C viremia (A). Among GBV-C viremic subjects, double negative T cell percentage (B) and absolute count (C) correlated with the GBV-C VL (N=21; Spearman test used to calculate r and p values and best fit linear regression analysis shown). Gated cells were normalized to $10^5$ total events.
A. GBV-C viral load (log copies/ml) and % CD3+ CD4- CD8- T cells. 

$P = 0.004$

B. GBV-C viral load (log copies/ml) and % CD3+ CD4- CD8- T cells. 

$P = 0.004$

$r = 0.589$
Figure 2.1. Continued

C.

CD3⁺CD4⁻CD8⁻ T cells (Normalized Count) vs. GBV-C viral load (log copies/ml)
Figure 2.2. GBV-C viremia is associated with reduced T cell activation in subjects with undetectable HIV viral load on antiretroviral therapy. T cell activation, as measured by the percentage of CD38 expressing CD4+ (A) and CD8+ (B) T cells, was significantly lower in cART-treated subjects with GBV-C viremia compared to those without GBV-C. N= 38; $P<0.05$. 
A. % CD38+ CD4+ T cells

GBV-C -ve

GBV-C +ve

$P = 0.003$

B. % CD38+ CD8+ T cells

GBV-C -ve

GBV-C +ve

$P = 0.034$
Figure 2.3. Association between GBV-C viral load, DN TCs, and CD38 expression. Although GBV-C infection was associated with significant increase in DN TC, we did not find any significant correlation between DN TC levels and CD38 expression (N=38; A). Similarly there was no significant correlatio between GBV-C viral load and CD38 expression on CD4+ (B) and CD8+ (C) T cells (N=18). Spearman test used to calculate r and p values and best fit linear regression analysis shown).
A. 

\[ r = -0.02 \]

\[ P > 0.05 \]

\% CD38+ CD4+ T cells vs. \% CD3+ CD4-CD8- T cells

B. 

\[ r = -0.22 \]

\[ P > 0.05 \]

\% CD38+ CD4+ T cells vs. GBV-C viral load (log copies/ml)
$r = 0.18$

$P > 0.05$
CHAPTER III
EFFECTS OF IMMUNOACTIVATION ON
GBV-C REPLICATION, PROLIFERATION AND
SURVIVAL OF GBV-C INFECTED T CELLS

Chapter Summary

In HIV-infected individuals GBV-C infection is associated with reduced immunoactivation, a block in T cell proliferation following IL2 therapy and reduced T cell death suggesting an antagonist effect of GBV-C on these signaling pathways. In this chapter, we examined peripheral blood mononuclear cells (PBMCs) from HIV-infected subjects with or without GBV-C infection to determine the effects of immunoactivation on GBV-C replication and if GBV-C infection correlated with reduction in T cell proliferation, activation induced T cell death and reactivation of latent HIV following activation with phytohemagglutinin A and IL2 (PHA/IL2).

HIV-infected subjects whose HIV viral load was suppressed on combination antiretroviral therapy (cART) for > 6 months were studied. PBMCs were cultured with and without PHA/IL2 and monitored for HIV reactivation, proliferation, and survival. GBV-C viremia and in vitro replication was detected by RT-PCR. HIV reactivation was determined by measuring HIV p24 antigen in culture supernatants. Proliferation was measured by counting viable cells and survival measured by flow cytometry.

Twenty six of forty nine HIV-infected subjects had GBV-C viremia. Significantly less HIV reactivation and PBMC proliferation following PHA/IL2 was observed in samples from GBV-C viremic subjects compared to non-viremic controls. Following 5 weeks in culture, GBV-C replication was associated with preservation of CD4+ and CD8+ T cells compared to non-viremic controls.

GBV-C replication appears to inhibit immunoactivation and IL2 signaling pathways, which may contribute to a reduction in reactivation of latent HIV from cellular
reservoirs. In addition, GBV-C viremia was associated with a reduction in activation induced T cell death. GBV-C associated T cell effects may contribute to the observed protective effect of GBV-C co-infection in HIV-infected individuals.

**Introduction**

GB virus C (GBV-C) infection is not clearly associated with any disease; however, several studies found a beneficial effect of GBV-C co-infection on survival, CD4+ T cell count, HIV viral load (VL) and delayed progression to AIDS in HIV-infected individuals compared those without GBV-C, and a meta-analysis supported improved survival with persistent GBV-C viremia (54, 69, 96, 133, 136, 142, 152, 157). Although the mechanisms for this protective effect are not entirely clear, studies of GBV-C interactions with host cells have suggested several potentials mechanisms by which GBV-C may alter HIV infection and disease progression (87, 123).

Chronic HIV infection is associated with aberrant and persistent immune activation which is thought to contribute to the immune dysfunction observed in HIV-infected people (3, 8, 48, 57, 89). Immune activation correlates with HIV VL, and in some studies is a better predictor of HIV disease progression than plasma VL (46, 52). In addition, immune activation enhances HIV replication and contributes to the depletion of uninfected bystander cells by activation induced cell death (AICD) (3, 8, 30). Treatment with combination antiretroviral therapy (cART) suppresses HIV VL and reduces mortality among HIV-infected individuals; however, viremia usually becomes detectable within a few weeks of discontinuing cART (42). Although T cell activation levels are reduced by cART, they do not return to levels found in healthy, HIV-negative individuals (139).

A major barrier in eradicating HIV from infected individuals is the presence of a stable pool of latently infected resting memory CD4+ T cells (115). Although cART decreases HIV VL to undetectable levels by current quantification methods (less than 50
copies/ml of HIV RNA in plasma), low levels of viremia can be detected in these individuals with highly sensitive assays (32, 98). Occasionally patients receiving cART have ‘viral blips’; brief periods of detectable VL in plasma (107). Although the cause of low levels of persistent viremia and occasional viral blips is not always clear, it is thought that reactivation of latent HIV from cellular reservoirs may contribute to the persistent low levels of viremia and viral blips (49, 107, 135). In the absence of therapy, HIV reactivation from cellular reservoirs is thought to be mediated by persistent, HIV and other infection-induced immune activation (24, 128, 143).

GBV-C infection is associated with reduced T cell activation (78, 126). In addition, GBV-C is associated with a reduction in CD4 T cell expansion following exogenous IL-2 therapy (78, 126). GBV-C viremia is also associated with improved response to antiretroviral therapy in HIV-infected individuals as measured by a greater reduction in HIV VL, increased CD4+ T cell count, and a reduction in the need to change ART regimens (106, 121, 133). One study found that GBV-C viremic individuals had significantly fewer viral blips during cART treatment compared to non-viremic controls (6). Taken together, these data suggest that GBV-C effects on T cell activation and proliferation may contribute to improved virological response to cART in GBV-C viremic HIV-infected individuals.

In this chapter, we tested the hypothesis that GBV-C infection downregulates T cell activation leading to reduced HIV reactivation from latent cellular reservoirs and limit HIV replication, thus protecting T cells from AICD. To test this hypothesis, we compared peripheral blood mononuclear cells (PBMCs) from subjects with and without GBV-C viremia to determine if GBV-C infection correlated with a reduction in the rate of latent HIV reactivation from cellular reservoirs in vitro, and to ascertain if GBV-C is associated with a reduction in PBMC proliferation, particularly in the setting of T cell activation in vitro.
Materials and Methods

Study Subjects

HIV-positive individuals attending the University of Iowa HIV Clinic who were receiving cART and had recorded HIV VL levels below the limit of detection (48 copies/mL) for more than 6 months were invited to participate in these studies. Following written informed consent, blood was obtained to test for GBV-C viremia and to prepare PBMCs. HIV VL and CD4 percent were tested on the day of blood sampling and the results were obtained from the medical record. HIV VL was determined using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test (Roche). CD4 T cell percent was determined by flow cytometry by the University of Iowa Hospitals and Clinics clinical laboratories. Laboratory personnel who conducted GBV-C serum RNA testing and in vitro culture studies were not aware of the clinical data. This study was approved by the University of Iowa Institutional Review Board.

PBMC Isolation and Stimulation

Peripheral Blood Mononuclear Cell (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, and maintained in RPMI-1640 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. PBMCs (1x10^6/ml, 2ml/well in 12 well plates; Costar, Inc.) were cultured under three different conditions and three replicates of each culture condition were performed. The three conditions studied were: 1) PBMCs were cultured without activation stimuli or healthy donor feeder cells; 2) PBMCs were culture with activation and healthy donor feeder cells were added to the patient samples weekly. Activation was accomplished by adding phytohemagglutinin (PHA M form, 1.5%; GIBCO) and interleukin 2 (IL2, 25U/ml; ZeptoMetrix) to the media; and 3) PBMCs were cultured with activation and were fed 7 days into culture and weekly thereafter with PHA/IL-2 activated PBMCs obtained from GBV-C non-viremic healthy donors. All cultures were maintained for 5 weeks. Culture supernatants were
obtained, stored at -80°C until they were batch tested for GBV-C replication and HIV reactivation. CD4+ and CD8+ T cells in PHA/IL2 stimulated cultures were analyzed on day 35 for viability using trypan blue exclusion or by flow cytometry (see below). Cells were counted using the Countess™ automated cell counter (Invitrogen). Each of three wells were counted twice and the average change from baseline (day 2) was determined.

**GBV-C Quantification**

GBV-C replication was assessed by measuring GBV-C RNA in serum or culture supernatant fluids by real-time RT-PCR. Briefly, RNA was extracted from 140 μL of serum or supernatant using QIamp Viral RNA Mini Kit (Qiagen), following the manufacturer’s instructions. Each PCR reaction contained an 11 μL aliquot of RNA (representing 25 μL of serum or supernatant), 15 μL ABI TaqMan Master Mix (2X), 0.75 μL superscript III RT platinum Taq mix, 0.18 μL each of forward and reverse primers (50 μM, Integrated DNA Technologies), 0.06 μL Taqman probe-ABI (100μM), 0.3 μL of Rnasin (40U/μL), and 1.8 μL RNase/DNase free H2O. Primers included the forward primer: 5’ GGC GAC CGG CCA AAA 3’ (96-110), antisense primer: 5’ CTT AAG ACC CAC CTA TAG TGG CTA CC (163-188), and probe: 5’ FAM-TGA CCG GGA TTT ACC CAC TTA CCA CCC T-TAMRA (131-158) (45). Quantitative one step RT-PCR Real Time PCR was performed using an ABI 7500 system. The running conditions were 50°C for 20 min, 95°C for 2 min, 40 cycles at 95°C for 15s and 58°C for 1 min. A GBV-C RNA quantitation standard used was prepared. GBV-C genome sequences from nt 1 to 850 (from GenBank AF121950) were cloned into pCR2.1 plasmid (Invitrogen, Inc.) downstream of the T7 polymerase promoter. The plasmid was linearized with Kpn1 and run off transcripts were generated (Riboprobe, Promega). RNA was quantified by A260/280, divided into 50 μl aliquots (concentration of 1 x 10^10 genome copies/mL) and stored at -80°C. For each real-time PCR experiment, a fresh aliquot was used in serial 10-fold dilutions, starting at a 1:100 dilution in RNAse free water. Linear regression analyses of standard curves are routinely excellent (r^2> 0.98), and variation between cycle
threshold (CT) values between experiments were within 2.5 CT. Using the GBV-C RNA transcript control, a high titer, GBV-C RNA positive serum sample was quantified. The result was within 10-fold that found by terminal dilution of RNA using nested RT-PCR (5’ non-translated region primers) previously described (data not shown) (145). Real-time PCR was between 5- to 10-fold less sensitive than nested RT-PCR for the control serum sample.

**HIV Reactivation**

HIV reactivation was determined by determining the HIV p24 antigen in culture supernatants by ELISA as previously described (144, 152). ELISA plates were read on a Beckman DU 640 spectrophotometer. Samples with HIV p24 antigen level greater than 25 pg/ml were considered positive for reactivation.

**CD4+ and CD8+ T cell Analysis**

Following PHA/IL2 stimulation, CD4+ and CD8+ T cells (CD3+) were analyzed by multicolor flow cytometry. PBMCs were pelleted at 1500 rpm for 5 minutes, washed and resuspended in 100 μL of PBS. Cells were stained with the following antibodies from Becton Dickinson (BD) per manufacturer’s recommendation; CD3 (Pacific Blue), CD4 (APC-H7) and CD8 (FITC). Live cells were gated using LIVE/DEAD fixable aqua dead cell stain kit (Invitrogen) as recommended by the manufacturer. Staining was performed on ice for 1 hour and cells were subsequently 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 100,000 total events were collected and FlowJo program (Tree Star Inc.) was used for data analysis.

**Statistics**

Statistics were performed using either SPSS Version 19 (IBM) or GraphPad software V4.0 (GraphPad Software Inc.). Comparisons between two groups were carried out using two-sided Student’s t tests and more than two groups by ANOVA. The
significance of differences in HIV reactivation from PBMCs of GBV-C viremic and non-viremic individuals was determined using chi-square test. \( P \) values less than 0.05 were considered statistically significant.

**Results**

**Subjects’ description**

Forty nine HIV-positive individuals on cART participated in this study. All subjects had non-detectable HIV viral loads for a more than 6 months documented in their medical record prior to enrollment into the study, and HIV RNA was not detected in their blood on the day blood was sampled. Subjects with known GBV-C viremia (16) or unknown (33) agreed to participate in the study. All 16 subjects with prior GBV-C viremia were viremic on the day of the study, and 10 of the 33 subjects with unknown GBV-C status were viremic (30.3%).

Consistent with the demographics of our HIV/AIDS clinic, approximately 20% of the subjects were women and 86% were Caucasian (Table 3.1). The major mode of HIV transmission was sexual and 10% of subjects had co-infection with HCV. GBV-C RNA was detected in the serum obtained from 26 of the 49 individuals. The average GBV-C VL in serum was \( 8.43 \times 10^7 \) copies/mL in the 26 subjects with GBV-C (median \( 5.45 \times 10^7 \) copies/mL). There were no significant differences in baseline demographics or clinical variables in those with GBV-C viremia and those without GBV-C, including CD4 percent, CD4 nadir, or the duration of non-detectable HIV RNA prior to culture (Table 3.1).

**PHA/IL2 stimulation decreases**

**GBV-C replication in PBMCs**

In a clinical study of IL-2 in HIV-infected people, subjects who were viremic with GBV-C demonstrated a marked reduction in CD4 cell expansion following IL2 infusion (45, 126). In addition, data from limited studies suggested that GBV-C replication in
PBMCs may be inhibited in vitro by PHA/IL2 activation, although this was not systematically examined (45, 126). Although GBV-C replicates well in vivo (8.43 x 10^7 copies/mL serum in this study), in vitro replication of GBV-C is inefficient. To confirm that GBV-C replicates in PBMCs ex vivo, and to evaluate the effect of PHA/IL2 activation on GBV-C replication, we analyzed GBV-C production in PBMCs from GBV-C viremic subjects. GBV-C replication was detected in culture supernatants of PBMCs maintained in all three culture conditions (Figure 3.1A). However, GBV-C production was higher from cells that were incubated without PHA/IL2 or healthy donor feeder cells at all-time points tested. GBV-C production was lower when PBMCs from GBV-C RNA positive donors were co-cultured with PHA/IL2 stimulated healthy donor PBMCs (Figure 3.1A). GBV-C replication was not detected in PBMCs from 2 of the 15 viremic subjects tested. In subjects PBMCs that produced GBV-C, the amount produced was greatest in the first week post culture in all three conditions, after which GBV-C production gradually declined. No GBV-C RNA was detected in cell culture supernatants from subjects without GBV-C viremia. To ensure that GBV-C RNA detected was produced from the infected cells and did not reflect slow release of viral particles coated on antigen presenting cells, we added high titer (1.0 x 10^7 copies per ml) GBV-C RNA positive serum to healthy, GBV-C negative PBMCs and incubated these cells overnight at 37°C. Following incubation the cells were washed and maintained in culture with or without PHA/IL2 in order to determine if virus that was adherent to cells was released in quantities similar to that observed in GBV-C RNA positive PBMC cultures. By day 7, no GBV-C RNA was detected in any of the three PBMC cultures studied (data not shown). In addition, when GBV-C RNA positive serum (titer 4 x 10^7 genome equivalents/mL) was incubated in the absence of cells, no viral RNA was detected on day 7 (data not shown), further suggesting that the GBV-C RNA detected in these cultures represented productive infection, and not release of viral particles from antigen presenting cells.
**GBV-C effects on PBMC proliferation following PHA/IL2 activation**

As noted, GBV-C infection was associated with reduced CD4+ T cell expansion following IL2 therapy in HIV-positive individuals (126), suggesting an interaction between GBV-C and IL-2 signaling. To determine if GBV-C replication influenced proliferation of PBMCs cultured *ex vivo*, cells from GBV-C viremic subjects and non-viremic controls were activated with PHA/IL2 and viable cells counted using trypan blue exclusion microscopy. PBMC proliferation was reduced in GBV-C viremic subjects compared to non-viremic controls following PHA/IL2 activation which was significantly different on day 35 (Figure 3.1B).

**GBV-C reduces HIV reactivation**

Efficient HIV replication and reactivation of latent HIV *in vitro* requires T cell activation (23). To determine if GBV-C infection altered reactivation of latent HIV, we utilized PHA/IL-2 stimulated PBMC cultures as described (141). Culture supernatants from three independent replicates were assessed for HIV p24 antigen weekly for 5 weeks. Similar to previous data using this methodology (141), we detected reactivation of latent HIV in 16% of patients (8 of 49). When stratified by GBV-C viremia status, the frequency of reactivation of latent HIV was significantly greater in subjects without GBV-C viremia (7/23) compared to those who were viremic (1/26; p=0.019, Chi-square test) (Figure 3.2). HIV reactivation was not detected in any of cultures maintained without PHA/IL2, and HIV p24 antigen was only detected after 3 or more weeks in culture with PHA/IL2.

**GBV-C effects on T cell depletion following PHA/IL2 activation**

Previous clinical studies found an association between GBV-C viremia and reduced activation and proliferation markers on T cells studied *ex vivo* (78, 126). Since GBV-C viremia was associated with a reduction in PBMC proliferation *in vitro* compared
to non-viremic controls (Figure 3.1B), GBV-C may also influence activation induced cell death (AICD) of CD4 and CD8 T cells in vitro. To determine if GBV-C viremia reduces T cell depletion following activation, PBMCs from 17 subjects stimulated with PHA/IL2 and maintained in culture for 35 days were analyzed for viable CD4+ and CD8+ T cells by flow cytometry. To exclude the possibility that T cell death was induced by HIV reactivation, only subjects that did not demonstrate HIV reactivation were studied (GBV-C infected n=6; GBV-C uninfected n=11). Live and dead CD4+ and CD8+ T cells were analyzed by flow cytometry as described in the Methods section (Figure 3.3A). Examples of cell populations stimulated with PHA/IL2 for 35 days for one representative subject with GBV-C viremia and a representative subject without GBV-C viremia are shown in Figure 3.3B (live cells in right lower quadrant, dead cells in right upper quadrant). At baseline (day 0) there were no differences in the number of CD4+ and CD8+ T cells by GBV-C RNA status (Figure 3.4A). However, by day 35, viable CD4+ and CD8+ T cells were significantly higher in GBV-C positive group (GB+) compared to GBV-C negative group (GB-) (Figure 3.4B). PBMCs maintained without PHA/IL2 were not significantly different at day 35 (data not shown).

Discussion

Previous studies demonstrated that GBV-C replicates in T and B lymphocytes in vitro, and one case report suggested that GBV-C replication is inhibited by PHA/IL2 activation (43, 45). Consistent with these data, we found that GBV-C replication was significantly greater in unstimulated PBMCs compared to PBMCs which were either stimulated with PHA/IL2 or co-cultured with PHA/IL2 activated PBMCs (Figure 3.1A). GBV-C viremia is associated with reduced T cell activation in HIV/GBV-C co-infected individuals (78, 126) and GBV-C production was significantly greater in PBMCs that were maintained without exogenous PHA/IL2 activation (78, 126). These data suggest that T cell activation may interfere with factors required for GBV-C replication.
In a clinical study, GBV-C viremia was found to be associated with a block in CD4+ T cell expansion in HIV-positive individuals receiving intravenous IL2 therapy (126). Consistent with this observation, PBMC proliferation was reduced in GBV-C viremic subjects following exogenous activation with PHA/IL2 compared to non-viremic subjects (Figure 3.1B). This reduction in T cell proliferation further suggests a bidirectional interaction between GBV-C replication and T cell activation, and raises the possibility that GBV-C replication alters activation and IL2 signaling pathways.

GBV-C viremia was associated with a reduction in the frequency of reactivation of latent HIV (3.8%) when compared to reactivation in subjects without GBV-C viremia (30.4%). Although we suspect that these data indicate that the effects of GBV-C on PHA/IL2 mediated activation prevent reactivation of HIV transcription, an alternative explanation is that GBV-C infection alters other, undefined HIV latency and/or reactivation factors. Further studies on the effects of GBV-C on HIV reactivation appear warranted. Regardless, GBV-C co-infection is associated with an improved virological response to cART and a decreased incidence of viral blips (6, 106, 121, 133), and the effect of GBV-C on HIV reactivation may contribute to the improved response to cART. Our data suggest that GBV-C viremia status may adversely influence approaches designed to activate HIV in cellular reservoirs in people receiving cART.

Persistent immune activation contributes to HIV disease progression at least in part by depleting uninfected bystander cells through activation induced cell death (AICD) (3, 8). CD4+ and CD8+ T cells maintained in PHA/IL2 ex vivo were significantly more depleted in subjects without GBV-C viremia compared to those with GBV-C viremia following 35 days in culture (Figure 3.4), suggesting that GBV-C has a protective effect against AICD and/or other mechanisms of cell death. Persistent GBV-C replication in vitro appeared to be necessary for this effect, as CD4+ and CD8+ T cells were depleted in the two GBV-C positive subjects that did not demonstrate GBV-C replication in cell culture. The Fas/FasL pathway is suggested to be the major apoptotic pathway involved
during AICD of bystander cells (8, 30), and a previous study found that GBV-C infection is negatively associated with Fas expression on lymphocytes and with apoptosis (86). Our findings further demonstrate that GBV-C is associated with a reduction in T cell depletion following in vitro activation with PHA/IL2. Together, these data support an inhibitory role of GBV-C in Fas-mediated T cell apoptosis.

In summary, PHA/IL2 stimulation significantly reduced GBV-C replication in vitro, and PBMC proliferation was significantly reduced in subjects with GBV-C viremia following PHA/IL2 activation compared to non-viremic subjects. GBV-C infection was associated with a significant reduction in reactivation of latent HIV in vitro, and with protection of CD4+ and CD8+ T cells from activation induced cell death. The reduction in cell death was offset by a reduction in proliferation, suggesting that GBV-C may contribute to the regulation of T cell homeostasis in vivo. These studies suggest potential mechanisms by which GBV-C co-infection may contribute to improved survival in HIV-infected people. The data support further characterization of the mechanisms by which GBV-C modulates T cell proliferation, activation, HIV reactivation, and CD4+ and CD8+ T cell preservation following activation.
Table 3.1. Subject Demographics

<table>
<thead>
<tr>
<th>Variable</th>
<th>GBV-C RNA Positive (n=26)</th>
<th>GBV-C RNA Negative (n=26)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46.5</td>
<td>47.8</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Caucasian</td>
<td>22</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>African American</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transmission</td>
<td></td>
<td></td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Sexual</td>
<td>24</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>IDU</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>HIV suppression(^1) (months)</td>
<td>60</td>
<td>72</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>CD4 Nadir</td>
<td>247</td>
<td>184</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>CD4 Percent</td>
<td>29</td>
<td>30</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>HCV Antibody</td>
<td>1</td>
<td>4</td>
<td>P&gt;0.05</td>
</tr>
</tbody>
</table>

\(^1\)HIV Suppression = average number of months that HIV RNA was suppressed to <48 copies/mL
Figure 3.1. PHA/IL2 stimulation alters GBV-C replication and PBMC proliferation. GBV-C RNA released into PBMC culture supernatants was significantly greater in unstimulated cells compared to PHA/IL2 stimulated or co-cultured cells (A). In addition, PBMCs from GBV-C infected subjects did not proliferate following PHA/IL2 stimulation to the extent of PBMCs from subjects without GBV-C viremia (B). N=52; *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
A. 

![Graph showing GBV-C RNA levels over time across different conditions.]

- Unstimulated
- PHA/IL2 Stimulated
- Co-culture

B. 

![Graph showing PBMC proliferation over time for GBV-C +ve and GBV-C -ve conditions.]

- GBV-C +ve
- GBV-C -ve
Figure 3.2. GBV-C reduces reactivation of latent HIV from cellular reservoirs. Following PHA/IL2 stimulation, reactivation of latent HIV from PBMCs was more frequent in cells obtained from subjects without GBV-C viremia compared to subjects with GBV-C viremia. N= 49; *p=0.019. p24 antigen was only detected after 3 weeks and p24 levels above 25pg/ml was considered as positive for reactivation.
Figure 3.3. Flow cytometry determination of CD4+ and CD8+ T cells. Lymphocytes were gated according to forward and side scatter and live CD3+ lymphocytes were analyzed for CD4+ and CD8+ T cell viability (A). Flow cytometry data of PBMCs from representative GBV-C viremic and non-viremic subjects that had been maintained in culture for 35 days with PHA/IL2 (B).
Figure 3.4. GBV-C protects CD4+ and CD8+ T cell depletion following PHA/IL2 activation. Live CD4+ and CD8+ T cells at base line (A; day 0) and following maintenance in media containing PHA/IL2 for 35 days (B). Gated cells were normalized to $10^5$ total events for each group analyzed. Data shown represent the median value for each group, interquartile ranges (shaded area), and the largest and smallest values. PBMCs in which HIV reactivation was detected were not included in viable analysis as HIV replication can also induce cell death. N=17, ns= not significant, *p<0.05, **p<0.01.
CHAPTER IV

GB VIRUS C ENVELOPE GLYCOPROTEIN E2
INHIBITS T CELL RECEPTOR INDUCED IL-2
PRODUCTION AND ALTERS IL-2 SIGNALING
PATHWAYS

Chapter Summary

GB virus type C (GBV-C) viremia is associated with reduced CD4+ T cell expansion following Interleukin 2 (IL-2) therapy and with a reduction in T cell activation in HIV-infected individuals. Mechanism(s) by which GBV-C might alter T-cell activation or IL-2 signaling have not been studied. In this chapter, we describe the effects of GBV-C envelope glycoprotein E2 on IL-2 release, IL-2 receptor (IL-2R) expression, IL-2 signaling, and cell proliferation following activation through the T cell receptor (TCR). TCR activation was induced by incubation in anti-CD3/CD28 antibodies. IL-2 release was measured by ELISA, STAT5 phosphorylation was assessed by immunoblot, and IL-2Rα (CD25) expression and cell proliferation were determined by flow cytometry. IL-2 and IL-2Rα steady-state mRNA levels were measured by real-time PCR. GBV-C E2 expression significantly inhibited IL-2 release, CD25 expression, STAT5 phosphorylation and cellular proliferation in Jurkat cells following activation through the TCR compared to control cell lines. Reducing E2 expression by doxycycline reversed the inhibitory effects observed in the E2-expressing cells. The N-terminal 219 a.a of E2 was sufficient to inhibit IL-2 signaling. Addition of purified recombinant GBV-C E2 protein to primary human CD4+ and CD8+ T cells inhibited TCR activation-induced IL-2 release and upregulation of IL-2Rα expression. These data provide evidence that the GBV-C E2 protein may contribute to the block in CD4+ T cell expansion following IL-2 therapy in HIV-infected individuals. Furthermore, the effects
of GBV-C on IL-2 and IL-2 signaling pathways may contribute to the reduction in chronic immune activation observed in GBV-C/HIV co-infected individuals.

**Introduction**

GBV-C is a lymphotropic virus, and GBV-C infection modulates several host factors involved in HIV infection including expression of cytokines, chemokines and cellular receptors (16). These alterations in host lymphocyte factors may limit HIV infection and contribute to a protective effect of GBV-C coinfection observed in HIV-positive individuals. Interleukin 2 (IL-2) is a critical cytokine required for T cell activation, proliferation, and function (94, 103). However, IL-2 also induces secretion of pro-inflammatory cytokines like IL-6, IL-1β and tumor necrosis factor alpha (TNF-α) (41, 53, 114), 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 (105). In addition, *in vitro* activation of peripheral blood mononuclear cells (PBMCs) with IL-2 increases HIV production (63, 91). Since chronic HIV infection is characterized by persistent immune activation which contributes to T cell depletion, altered cytokine expression and loss of T cell function (1, 102, 118), IL-2 may promote HIV replication and contribute to HIV associated immune activation *in vivo*.

In studies of HIV-infected people, GBV-C infection is associated with lower cell surface expression of T cell activation markers as compared to GBV-C non-viremic controls, independent of HIV VL (78, 93, 113). GBV-C viremia is also associated with a significant reduced CD4+ T cell expansion in HIV-infected subjects receiving intravenous IL-2 therapy compared to GBV-C non-viremic controls (126). Together, these findings suggest that GBV-C infection may alter T cell activation and IL-2 signaling pathways. In addition, GBV-C replication in peripheral blood mononuclear cells (PBMCs) is significantly reduced following *in vitro* activation with IL-2 and phytohemagglutinin (PHA) (43, 108), suggesting a potential bidirectional interaction
between GBV-C and IL-2. Since IL-2 plays an important role in HIV infection and disease progression, the effects of GBV-C on IL-2 signaling pathways may contribute to the protective effect of GBV-C coinfection in HIV infected individuals. Previous studies demonstrated that GBV-C envelope glycoprotein (E2) inhibits HIV replication when added to cells (59, 64, 87), or when expressed in a CD4+ Jurkat T cell line (150). In this study we examined the role of the GBV-C E2 protein in the modulation of IL-2 production and IL-2 signaling pathways.

Materials and Methods

Expression of GBV-C E2 proteins

The GBV-C E2 protein coding sequence without the C terminal transmembrane region (nt 1167-2161 based on GenBank AF 121950), E2 deletion mutants (N terminal 219 aa [nt 1167-1824], and C terminal 112 aa [nt 1824-2161]), and control sequences were ligated into a modified pTRE2-Hyg plasmid (Clontech Laboratories, Mountain View, CA) as previously described (150). This plasmid generates a bicistronic message encoding the GBV-C E2 sequence followed by the encephalomyocarditis virus (EMC) internal ribosomal entry site (IRES) that directs translation of GFP (149). Transcription of the bicistronic message is regulated by doxycycline as described (150). All the constructs contained a poly-histidine tag at the C-terminus of the protein.

Jurkat (tet-off) cell lines (Clontech, Inc) were transfected (Nucleofector II, Lonza Inc.) with plasmids encoding GBV-C E2 proteins. Control cell lines were generated by transfecting Jurkat (tet-off) cells with an E2 plasmid containing a frameshift mutation to abrogate protein expression (FS) or the empty vector expressing green fluorescent protein (GFP) (vector control; VC). 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 by immunoblot and GFP expression by flow
cytometry (BD FACScan). All cell lines were maintained in RPMI 1640 supplemented
with 10% fetal calf serum (heat-inactivated), 2mM L-glutamine, 100 IU/ml penicillin,
and 100 µg/ml streptomycin with hygromycin and neomycin (200 µg/ml). Insert and
control sequences were confirmed by sequencing plasmid DNA (University of Iowa
DNA Core Facility).

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 pSec vector
(Invitrogen). CHO cells were transfected and selected on zeocin (400 µg/ml) and GFP
expression. Following stable transfection, cells were adapted to serum free media as
described (81). Fusion protein expressed in CHO cells was purified by protein G affinity
chromatography and analyzed by SDS-PAGE and immunoblot analysis as described (81,
88). Purified human IgG protein was used as a negative control for the E2 – human Fc
fusion protein.

**Cell Stimulation**

Jurkat cells (1×10⁶ cells/ml) were stimulated with plate-bound anti-CD3 (5
µg/ml, OKT3 clone, eBioscience) and soluble CD28 (5µg/ml, clone CD28.2, BD
Biosciences). Following obtaining written informed consent, peripheral blood
mononuclear cells (PBMCs) were isolated from blood obtained from four healthy
subjects using Ficoll-Hypaque density gradient centrifugation. This protocol was
approved by the University of Iowa Institutional Review Board. PBMCs were washed
with PBS and incubated with purified GBV-C E2 protein (20µg/ml) or purified human
IgG (20µg/ml, Sigma) for 48 hours and stimulated with anti-CD3 (500ng/ml) and soluble
CD28 (500ng/ml). Following 24 hours of stimulation, cells were analyzed for
measurement of cytokine and cellular receptor expression. To measure STAT5
phosphorylation Jurkat cells were prepared as described (18). Briefly, cells were
stimulated with 1µg/ml of anti-CD3 and soluble CD28 for 48 hours followed by 24 hours
of serum starvation with or without doxycycline (1µg/ml). Cells were washed and
incubated with IL-2 (250U/ml; Zeptometrix) for 15 minutes. Cell 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 Amersham 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, MN) 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 determined using RT² qPCR primer assay for human IL2 and human CD25 (SABiosciences) and normalized to 18SrRNA using ABI 7500 Real Time PCR system.

**Flow cytometry**

Jurkat cells or PBMCs were incubated with the following antibodies from Becton Dickinson (BD) per manufacturer’s recommendation; CD3 (V450), CD4 (PE), CD8 (Alexa700) and CD25 (APC). Incubation 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. 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 (1µg/ml) and soluble CD28 (1µg/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.

Results

GBV-C E2 protein expression inhibits IL-2 production

Three previously described CD4+ Jurkat (tet-off) T cell lines expressing GBV-C E2 protein (331 aa), the N terminal 219 aa, and the coding region for the E2 protein with a frame-shift to abolish protein translation (FS) were studied (150). In addition, a stable cell line expressing the E2 protein region from aa 220 to 331 was generated (Fig. 4.2). All cell lines expressed GFP as determined by flow cytometry (Fig. 4.3A) and cellular lysates reacted with anti-his Ab (Qiagen) directed against C-terminal histidine (6xHis) tag on E2 protein in immunoblot analysis (Fig. 4.3B). Expression of GBV-C E2 RNA in Jurkat cells expressing the E2 frameshift construct was confirmed by RT-PCR and DNA sequencing as described (149). E2 expressing Jurkat cells maintained in doxycycline (dox, 1µg/ml) had reduced expression of E2 protein (Fig 4.4).

Following T cell receptor (TCR) activation with anti-CD3/CD28, IL-2 release into culture supernatants was significantly lower in Jurkat cells expressing GBV-C E2 protein compared to either the vector control (VC) or the frame-shift (FS) control (Fig 4.5A). The FS control confirms that IL-2 inhibition is due to GBV-C E2 protein expression and not through effects of GBV-C E2 RNA (Fig 4.5A). Examination of deletion mutants demonstrated that the N-terminal 219 a.a of E2 were required to inhibit IL-2 release following TCR stimulation, whereas the C- terminal region of E2 (aa 220 to 331) did not (Fig. 4.5A). Jurkat cells expressing GBV-C E2 protein had significantly
reduced steady state levels of IL-2 mRNA following activation compared to FS and VC control cells (Fig 4.5B). Reduction of GBV-C E2 protein expression by maintaining cells in doxycycline (dox, 1µg/mL) reversed the block in steady-state IL-2 mRNA levels following TCR activation (Fig 4.5B). Of note, no differences in 18S mRNA levels were observed between any of the Jurkat cell lines studied.

**GBV-C E2 protein effects on**

**IL-2Rα and STAT5 phosphorylation**

Surface expression of the alpha chain of IL-2 receptor (IL-2Rα; CD25) is increased by activation of T cells (21, 62). Following upregulation, CD25 interacts with IL-2Rβ (CD122) and IL-2γ (CD132) receptors to form the high affinity IL-2 receptor (IL-2R) that binds IL-2 and initiates IL-2 signaling (21, 62) (Fig. 4.1). In studies of HIV-infected people, GBV-C viremia is associated with lower CD25 expression on T cells and a reduced response to IL-2 therapy (78, 126). Consequently, we examined the effect of GBV-C E2 protein expression on Jurkat cell CD25 expression. CD25 expression was significantly reduced in cells expressing GBV-C E2 compared to the FS control following TCR activation (average fold increase in CD25 expression, 16.4 versus 2.16; p<0.001), and this was also partially reversed by growing the E2 expressing cells in doxycycline (3.7 fold; p<0.01) (Fig 4.6A). CD25 mRNA steady state levels increase following TCR activation, and this upregulation was also blocked in cells expressing the GBV-C E2 protein (Fig. 4.6B). This interference in the upregulation of CD25 was also partially reversed by maintaining the cells in doxycycline to reduce GBV-C E2 expression (Fig. 4.6B). Maintaining Jurkat cells in doxycycline did not affect cell viability as previously described (150).

Since CD25 expression is essential for IL-2 signaling, we further investigated the effect of GBV-C E2 protein expression on downstream IL-2 signaling pathways. Phosphorylation of STAT5 is rapidly detected after IL-2 interacts with the IL-2R, and is critical for IL-2 signaling (75). Following stimulation with IL-2, STAT5 phosphorylation
was inhibited in GBV-C E2 expressing cells but not in FS control cells or GBV-C E2 expressing cells grown in doxycycline (Fig 4.6C). In the absence of IL-2, addition of doxycycline to E2 expressing Jurkat cells did not induce STAT5 phosphorylation indicating that doxycycline alone does not induce activation or IL-2 signaling. Total STAT5 protein expression was not altered by GBV-C E2 protein expression (Fig 4.6C). Together, these data indicate that GBV-C E2 protein expression inhibits CD25 expression and IL-2 induced phosphorylation of STAT5.

**GBV-C E2 protein reduces activation induced proliferation**

To determine whether the effect of GBV-C E2 expression on IL-2 release, CD25 expression and STAT5 phosphorylation affected T cell proliferation, Jurkat cells expressing GBV-C E2 or FS were assessed by flow cytometry. Jurkat cells expressing GBV-C E2 or the FS control were labelled with the proliferation dye eFluor450 (Day 0, Fig. 4.7A). Following TCR stimulation with anti-CD3/CD28, the Jurkat cells expressing GBV-C E2 protein demonstrated less proliferation than the FS control (Day 5, Fig 4.7A). This difference in proliferation between E2-expressing Jurkat cells and the FS control Jurkat cells was not seen when the cells were cultured without TCR activation (data not shown). The proliferation of these cell lines over five days following anti-CD3/CD28 stimulation is shown in figure 4.7B.

**GBV-C E2 protein reduces IL-2 and CD25 expression in primary human T cells**

To determine if the addition of E2 to cells altered activation following TCR engagement, IL-2 release was measured in peripheral blood mononuclear cells (PBMCs) incubated with highly purified recombinant GBV-C E2-Fc fusion protein (Fig. 4.8A) or human IgG control protein. IL-2 production was significantly lower in PBMCs from healthy donor PBMCs (n=4) incubated with GBV-C E2 protein (20 µg/mL) compared to cells incubated with human IgG (20 µg/mL; \( P = 0.008 \); Fig. 4.8B). Similarly, and
consistent with the findings observed in the Jurkat cells expressing E2 protein, the addition of E2-Fc to primary CD4+ and CD8+ T cells obtained from the four healthy donors significantly blocked CD25 upregulation following anti-CD3/CD28 stimulation compared to cells incubated with IgG (20 µg/mL; \( P < 0.001 \); Fig. 4.8C).

Discussion

Persistent immune activation and immune dysfunction are characteristic features of chronic HIV infection that contribute to HIV-associated immunodeficiency (102, 118). Previous studies suggested a potential interaction between GBV-C and IL-2 in vitro and in vivo (43, 108, 126). IL-2 is a pleiotropic cytokine essential for normal T cell function; however, IL-2 also promotes HIV replication and activation induced cell death (AICD) of T cells (26, 63, 72). In chapter III, we found an association between GBV-C viremia and reduced T cell AICD, further suggesting that GBV-C viremia may reduce activation and alter IL-2 response (108). In this chapter, we found that GBV-C E2 protein expression significantly inhibited IL-2 production and significantly blocked the upregulation of steady-state IL-2 mRNA levels following TCR engagement (Fig. 4.5). The region within the E2 protein required to alter IL-2 expression resided in the N terminal 219 a.a, as the expression of aa 220 to 331 of the GBV-C E2 protein did not affect IL-2 (Fig 4.5A).

Clinical studies have suggested interactions between GBV-C and T cell activation and proliferation. Specifically, HIV-positive subjects with GBV-C coinfection had significantly reduced CD4+ T cell expansion following intravenous IL-2 therapy compared to subjects without GBV-C infection (126). Subjects without GBV-C infection had a significant increase in CD4+ T cell count after IL-2 therapy whereas those with GBV-C did not have a significant increase in CD4+ T cell counts following IL-2 therapy. In addition, PBMC proliferation ex vivo was reduced in GBV-C viremic subjects following activation with IL-2 compared to PBMCs from subjects without GBV-C infection (108). Furthermore, GBV-C and HIV coinfected subjects had reduced CD25
expression on CD4+ and CD8+ T cells compared to HIV mono-infected subjects (78). In this study, either the expression of the GBV-C E2 protein in Jurkat cells or the addition of recombinant E2 protein to primary CD4+ and CD8+ T cells reduced the expression of CD25 following TCR engagement compared to control cells (Fig. 4.6A and Fig. 4.8C). E2 protein expression was required for these effects, as cells expressing the GBV-C RNA region with a frame-shift to prevent E2 expression and reducing E2 expression by maintaining cells in doxycycline reversed the inhibition of CD25 expression (Fig. 4.6A).

Because GBV-C E2 expression is likely to be higher in the stably transfected Jurkat cells than that produced during natural infection, the effect of E2 on IL-2 release and IL-2 signaling may be less potent in vivo. However, as noted, GBV-C coinfection with HIV was associated with a significant block in CD4 cell proliferation following administration of recombinant IL-2 (33). The in vitro data demonstrating that E2 expression inhibits IL-2 release, CD25 (IL-2Rα) expression, IL-2 signaling (measured by STAT5 phosphorylation) and cellular proliferation following TCR activation provides evidence supporting a causal role of GBV-C for the apparent interaction observed in a clinical study (33). Reducing GBV-C E2 expression by maintaining the cells in doxycycline reduced the extent of IL-2 signaling reduction. The fact that GBV-C E2 expressing cells still inhibited IL-2 after incubation in doxycycline suggests that low levels of E2 are sufficient to have a measurable effect on TCR-mediated signaling.

GBV-C E2 protein also inhibited the upregulation of IL-2 and CD25 steady-state mRNA levels following TCR activation, contributing to the observed reduction in cellular proliferation (Fig. 4.7). Although the block in proliferation will also lead to reduced IL-2 and IL2R expression, the effect of E2 protein specifically blocked TCR signaling, as both IL-2 production and CD25 expression were blocked prior to significant cellular proliferation (within 24 hrs). Finally, exposure of primary human CD4+ and CD8+ T cells to recombinant GBV-C E2 protein recapitulated the IL-2 and CD25
findings observed in Jurkat cells expressing E2 protein (Fig. 4.8), suggesting that GBV-C particles containing E2 protein may influence TCR signaling in bystander cells.

GBV-C infection is characterized by high levels of replication in HIV-infected people, and on average, there are \(5.45 \times 10^7\) genome equivalents of GBV-C found per mL serum (35). If there are 180 copies of GBV-C envelope glycoproteins per virion as there are for other flaviviruses (2), approximately \(1 \times 10^{10}\) copies of E2 are present in each mL of plasma. Furthermore, virus is produced by B and T lymphocytes (34), thus E2 production is predominantly in lymphoid tissue. Thus, even modest effects of GBV-C E2 protein on IL-2 homeostasis and T cell activation may result in global alteration of T cell function. Since GBV-C is not associated with any known human disease, the association between GBV-C and reduced T cell activation and response to therapeutic IL-2 administration, although measurable, are not potent enough to lead to immunodeficiency. For immune-mediated disease however, these effects may be beneficial, and potentially this relates to the improved survival observed in many, though not all studies of GBV-C infection in HIV-infected cohorts (16).

In conclusion, these data provide \textit{in vitro} evidence to support a causal role for the effect of GBV-C viremia on T cell activation and IL-2 mediated proliferation observed in epidemiological studies (78, 126). The effects on T cell activation and proliferation are mediated at least in part by the N-terminal 219 aa of the GBV-C envelope glycoprotein E2 \textit{in vitro}. A previous study found that aa 276-292 of GBV-C E2 protein are sufficient to inhibit HIV replication at the entry step (150). Since GBV-C E2 protein containing this region did not interfere with activation or IL-2 signaling, it is clear that different E2 regions are involved in the interaction between HIV inhibition and the modulation of T cell activation and proliferation. Furthermore, 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. Given the pleiotropic effects of IL-2 on
immune system, future studies on the effects of GBV-C infection on other immune cell functions appear warranted.
Figure 4.1. Schematic of T cell activation and IL-2 signaling pathways. Activation of T cells through T cell receptor (TCR) induces IL-2 secretion and expression of IL-2 receptor (α chain) (blue). IL-2Rα forms a high affinity IL-2 receptor along with IL-2 receptor β (orange) and IL-2 receptor γ (green). Interactions between IL-2 and IL-2 receptor induce IL-2 signaling which results into phosphorylation of STAT5 and nuclear translocation. These events activate number of genes involved in cellular activation and proliferation.
**Figure 4.2. Schematic of GBV-C envelope protein and mutants.** GBV-C E2 (nt 1167-2161; 331aa), N-terminal deletion mutant (aa 1-219; nt 1167-1824) and C terminal deletion mutant (aa 220-331; nt 1824-2161) and the 1-331 sequence with a frame shift inserted to abolish E2 expression (FS control). All of the recombinant proteins contained signal peptide sequence (murine Igκ) on N terminus and a C-terminal poly-histidine tag. Recombinant GBV-C E2 protein that contained a C-terminal Fc region from human IgG was used for purification in Protein G column. GBV-C E2 protein represented in shaded constructs inhibited IL-2 release following T cell receptor activation, while non-shaded constructs did not.
Figure 4.3. Analysis of protein expression. GFP expression was measured by flow cytometry in the various cell lines expressing GBV-C E2 protein, truncated mutants, and the controls (A). All cell lines were bulk sorted for GFP and were 100% GFP positive. Immunoblot analysis using anti-His antibodies demonstrated recombinant GBV-C E2 protein expression in cell lines and deletion mutants (E2 1-219; E2 220-331) (B). Frame shift (FS) did not react with his antibodies. Since GBV-C E2 mutant (220-331) was expected to be small in size (~12kDa) compared to full length E2 (~42kDa), dot blot was used to confirm the expression of 220-331 mutant.
A. 

![Graph showing GFP log mean fluorescence intensity]

B. 

![Western blot analysis showing anti-His and GAPDH]
Figure 4.4. Regulation of GBV-C E2 protein expression. Jurkat (tet-off) cells expressing GBV-C E2 protein (1-331) were cultured in doxycycline (dox, 1 µg/mL) for 5 days. GBV-C E2 protein expression was reduced more than 80% by maintaining Jurkat cells in doxycycline.
Figure 4.5. GBV-C E2 protein inhibits T cell receptor (TCR) induced IL-2.
Following TCR activation with anti-CD3/CD28, IL-2 secretion was measured in Jurkat cells expressing GBV-C E2 protein (1-331), the C-terminal (1-219) and N-terminal (220-331) E2 deletions, the E2 coding region with a frame-shift control cell line (FS), or the vector control (VC). IL-2 release was significantly reduced in GBV-C E2 and E2 (1-219) expressing Jurkat cells (A). Jurkat cell IL-2 mRNA levels were measured in the various cell lines following TCR activation (B). Fold increase in IL-2 production was calculated by measuring IL-2 in the culture supernatant before and after activation. IL-2 mRNA levels were measured after activation and normalized to 18S rRNA levels. *P <0.05; **P <0.01; ns = not significant, p>0.05. Each experiment was performed in triplicate using three independent cultures and repeated at least three times on different days with consistent results.
A.

IL-2 secretion (fold change after activation)

- VC
- GBV-C E2
- FS control
- E2 (1-219)
- E2 (220-331)

B.

IL-2 mRNA (fold change after activation)

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

ns

**

*
Figure 4.6. GBV-C E2 protein reduces IL-2Rα expression and STAT5 phosphorylation. Cell surface expression of the IL-2α receptor (CD25) on Jurkat cells expressing GBV-C E2 (1-331) or the frameshift control (FS) before (unstimulated) or following T cell receptor activation (anti-CD3/CD28) as measured by flow cytometry (A). CD25 mRNA levels in Jurkat cell lines following TCR activation (B). GBV-C E2 expressing cells were maintained with or without doxycycline for 5 days (dox; 1 µg/mL). Total and phosphorylated STAT5 expression following incubation with IL-2 (see methods for details) in FS and E2 expressing Jurkat cells maintained with and without dox for 5 days (C). *P <0.01; **P <0.001
Figure 4.6. Continued

C.

<table>
<thead>
<tr>
<th>FS control</th>
<th>GBV-C E2</th>
<th>GBV-C E2+dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IL-2 (15 mins)

- pSTAT5 (Y694)
- Total STAT5
Figure 4.7. GBV-C E2 protein expression inhibits T cell proliferation. Proliferation of Jurkat T cells expressing GBV-C E2 (1-331) or the frame shift control (FS) was measured at baseline (day 0) and following five days activation with anti-CD3/CD28 by flow cytometry (eFlour450). Propidium iodide (PI) staining was used to exclude dead cells (A). Cell proliferation was quantified on days 3 to 5 by measuring decrease in percentage of cells expressing eFlour450 dye from day 0 (B). *P < 0.05. The data represent the average of three independent cultures.
A.

Day 0
Unstimulated

Day 5
αCD3/CD28

Propidium Iodide (PI)

Cell Proliferation

FS control

GBV-C E2

B.

% Cell Proliferation

Day 3  Day 4  Day 5

- FS control
- GBV-C E2

*
Figure 4.8. GBV-C E2 protein inhibits IL-2 secretion and IL-2Rα (CD25) expression in primary human T cells. SDS-PAGE and immunoblot analysis of purified recombinant GBV-C E2 protein fused to human IgG Fc (E2-Fc; panel A). Bands in the gel represents monomeric (60 kDa), dimeric (120 kDa) and trimeric (180 kDa) forms of recombinant E2-Fc protein. IL-2 release (B) from primary human peripheral blood mononuclear cells and CD25 expression (C) on CD4+ and CD8+ T cells following activation with anti-CD3/CD28 antibodies in media containing recombinant E2 (20 µg/mL) or human IgG control (20 µg/mL). Data represent average results obtained using PBMCs from four healthy subjects. IL-2 secretion by PBMCs incubated with GBV-C E2-Fc recombinant protein was normalized to IL-2 produced by control PBMCs incubated with the same concentration of human IgG.
A. kDa  MW  E2  E2

GBV-C E2 (1-331)-Fc

B. IL-2 secretion (normalized)

<table>
<thead>
<tr>
<th></th>
<th>Human IgG</th>
<th>GBV-C E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>1.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

P = 0.008
C. 

```
<table>
<thead>
<tr>
<th></th>
<th>CD4+</th>
<th>CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human IgG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GBV-C E2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

% CD25+ T cells

αCD3/CD28

P < 0.001

αCD3/CD28

P < 0.001
```
CHAPTER V

GB VIRUS C ENVELOPE GLYCOPROTEIN E2 INHIBITS T CELL RECEPTOR SIGNALING IN INFECTED AND UNINFECTED BYSTANDER T CELLS VIA INHIBITING LCK

Chapter Summary

In this chapter, we describe the identification of a novel mechanism of T cell modulation by GB virus C (GBV-C). Viruses have evolved to evade host immune responses by various mechanisms; however, the role of viral proteins within extracellular microvesicles (EMV) in modulating host immune response is not clear. EMV purified from GBV-C infected human sera contained GBV-C particles and blocked T cell receptor (TCR) signaling in primary human T cells. GBV-C E2 protein expression inhibited activation of lymphocyte-specific protein tyrosine kinase (Lck). GBV-C E2 protein directly interacted with Lck, and a conserved peptide motif within GBV-C E2 protein that represents an Lck substrate (tyrosine at position 87; Y87) was sufficient to inhibit TCR signaling. GBV-C E2 protein was found in EMV purified from the supernatant of E2-expressing cells and inhibited TCR signaling in uninfected bystander T cells. Together, these data identify a novel mechanism of T cell modulation by the envelope protein of a persistent human virus. Understanding mechanisms by which viral proteins in secreted microvesicles alter host cell signaling pathways may provide novel insights into viral persistence and allow the design of novel antiviral therapy.

Introduction

Similar to a phylogenetically related hepatitis C virus (HCV), GB virus C (GBV-C) can establish persistent infection in humans (127). In most healthy individuals, GBV-C viremia persists for one to two years following infection before clearance but
approximately 20% of those infected remain viremic for years thereafter (70, 123).

Among individuals who clear GBV-C viremia, GBV-C specific antibodies do not develop until the time of viral clearance (130). Like HCV, GBV-C has a positive strand RNA genome and the virus replicates strictly in the cytoplasm of host cells. Although, the mechanisms by which GBV-C evades host immune responses to persist are incompletely characterized, several studies suggest that GBV-C infection alters host immune signaling pathways including immunoactivation and proliferation, thereby contributing to viral persistence (14-16, 78, 93, 108, 109, 113, 125, 126). In chapter IV, we described the effects of GBV-C envelope glycoprotein E2 in reducing T cell receptor (TCR) induced IL-2 signaling pathways. In this chapter, we further characterize the effect of E2 protein in TCR signaling pathways and describe a potential mechanism by which GBV-C reduces immunoactivation in infected T cells and bystander uninfected T cells.

Engagement of the TCR initiates a cascade of intracellular signaling events required to generate a T cell response, establish immune homeostasis and clear infections (27, 94). Clinical studies previously found an association between GBV-C infection and reduced T cell activation and IL-2 signaling in HIV-infected individuals (15, 78, 108, 125, 126) suggesting that GBV-C infection alters TCR signaling pathways. This finding may explain the epidemiological studies that demonstrate an association between GBV-C infection and prolonged survival in HIV-infected people (158). Furthermore, GBV-C replication is reduced by T cell activation in vitro (108) suggesting the virus might have evolved to down modulate immune activation. In chapter IV, we described that the effects of GBV-C infection on T cell activation and IL-2 signaling pathways can be mediated at least in part by the envelope glycoprotein E2. The expression of GBV-C E2 protein in CD4+ T cell line inhibited TCR induced IL-2 production and altered IL-2 signaling pathways. In this chapter, we further characterize the role of E2 protein in reducing immunoactivation.
Materials and Methods

Expression of GBV-C E2 protein

Tet-off Jurkat cell lines expressing GBV-C E2 protein (nt 1167-2161 based on GenBank AF 121950), 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(14). Six truncated E2 proteins were cloned into a modified pTRE2-HGY plasmid (Clontech, Inc.) as described (150). This plasmid generates a bicistronic message 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), 2mM 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 (5×10^6 cells/ml) were stimulated with plate-bound anti-CD3 (5µg/ml, OKT3 clone, eBioscience) and soluble CD28 antibody (5µg/ml, clone CD28.2, BD Biosciences) unless stated otherwise. For co-culture experiments, non-transfected GFP negative Jurkat cells were (5×10^5 cells/ml) were incubated with either transfected GFP positive vector control or GFP positive GBV-C E2 expressing cells (1×10^6 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 ($5 \times 10^6$) 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 (BIORAD). 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 (Systems Biosciences); and pZAP70 (Y319); total ZAP70; pLck (Y505); pLck (Y394/ pSrcY416); total Lck(Y394) and total Csk (all from Cell Signaling Technology). For immune precipitation studies, Jurkat cell lysates were incubated with recombinant Fc fused GBV-C E2 protein(14) 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 (14). Enzymatic activity was evaluated with or without GBV-C E2 protein (10µg) or human IgG control (10µg; 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$_{620nm}$. Phosphorylation of GBV-C E2 protein by Lck was measured by incubating recombinant E2 protein (40µg) with or without human Lck (500ng; R&D Systems) as recommended by the manufacturer. Samples were subjected to immunoblot analysis as described above. Phosphorylation was determined by immunoblot analysis with phosphotyrosine 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), or with the GBV-C E2 aa 86-101 (GGGGGRKKRRQRRVYGSVSVTCVWG, Y87), or the Y87H mutation (GGGGGRKKRRQRRVHGSVSVTCVWG) were obtained from Ana Spec, Inc. Peptides with the TAT domain and GBV-C E2 aa 276-292 (GGAGLTGGRYEPLVRRC), or the same amino acids in a scrambled order
(GCRCARGVLLTPGEGYF) as previously described(150). Peptides were dissolved in RPMI with 10% DMSO. The TAT domain enhances cellular uptake of the peptide. Healthy donors PBMCs (1×10^6 cells/ml) were incubated with 20µg peptide at 37°C overnight before stimulation with 500ng/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 cART 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(108). 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 (V450), CD4 (FITC), CD8 (Alexa700) antibodies (all BD Biosciences). Sorted cells were counted using Countess™ 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(108). I would like to acknowledge Ernest Chivero for working along with me to prepare PBMCs for cell sorting and RNA quantification.

**Extracellular Microvesicles (EMV) Isolation**

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 (9, 36, 117, 159). Sodium chloride (NaCl) density flotation was performed as described (146). Briefly, 1ml of undiluted serum was mixed with 35 ml of NaCl solution (1.063 g/ml), and centrifuged in a Beckman SW28
rotor (112,000 × g, 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.

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) (15, 78, 108, 125, 126). 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. 5.1A). 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. 5.1B). GBV-C RNA was detected in both CD4+ T cells (average 146 GE per $10^4$ cells) and CD8+ T cells (average 77 GE per $10^4$ cells) in all but two subjects. One of these subjects had GBV-C RNA only detected in CD4+ T cells while the other had GBV-C RNA present in only the CD8+ T
cell population (Fig. 5.1B). 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) (33, 79), we hypothesized that GBV-C may utilize a similar mechanism to interact with bystander cells. To test this hypothesis, we 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. 5.1C). 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. 5.1C). Consistent with a previous study (146), saline flotation gradient centrifugation of GBV-C-positive serum yielded two populations of RNA-containing particle with distinctly different densities (Fig. 5.1D). 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) (83) 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) (Fig. 5.1E-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 (14). 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 (1µg/ml) for 5 days to reduce expression of GBV-C E2 (Fig 5.2A). 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. 5.2B). 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. 5.2B), 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. 5.2C-D) and zeta-chain-associated protein kinase (ZAP)-70 (Fig. 5.2E-F) 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. 5.2C, 2E).

**GBV-C E2 protein inhibits Lck activation**

Lymphocyte specific protein tyrosine kinase (Lck) activation is required for signaling through the TCR(27). 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\(^{(27)}\). 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 (Fig. 5.3A-B).

**GBV-C E2 protein does not alter**

**CD45 and Csk expression**

Inactivation of Lck 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 (Fig. 5.4A-B). Furthermore, CD45 phosphatase activity was not altered *in vitro* by incubation with recombinant GBV-C E2 protein (Fig. 5.4C).

**GBV-C E2 protein interacts with Lck**

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. 5.5A). 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. 5.5B).

**A 13mer peptide motif within GBV-C E2 is sufficient to inhibit TCR signaling**

Expression of the N-terminal region 219 aa of GBV-C E2 protein is sufficient to inhibit IL-2 production following TCR stimulation \(^{(14)}\). 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. 5.6A). All cell lines stably expressed GFP, as demonstrated by flow cytometry (Fig. 5.6B). 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. 5.6C). Utilizing kinase-specific phosphorylation substrate prediction programs (97, 155), we identified a tyrosine residue at position 87 (Y87) in GBV-C E2 that is predicted to be Lck (Src-kinase) target (Fig. 5.6A). 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. 5.6D). Similar to Lck, the GBV-C E2 protein was dephosphorylated by CD45 tyrosine phosphatase (Fig. 5.6D).

The predicted Lck substrate motif within GBV-C E2 protein (aa 83-91; PQYVYGSVS) 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. 5.6E). The three isolates that differ do so at a single aa (Q84L 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; PRYVHGHT; Fig. 5.6E). 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. 5.6I). 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. 5.6I).

**GBV-C E2 protein inhibits TCR signaling pathways at the level of Lck activation.**

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. 5.7A) 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. 5.7B). These data suggest that GBV-C E2 protein specifically inhibits TCR signaling pathways at the level of Lck activation.

**Synthetic GBV-C E2 peptides inhibit TCR activation in primary human T cells.**

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, we compared the inhibitory capacities of a series of synthetic peptides with native or mutated (Y87H) 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 (Fig. 5.8A). 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 peptides (Fig. 5.8B). 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 (Fig. 5.8C-D). In addition, the TAT-Y87 peptide served as an Lck substrate in vitro, and was phosphorylated by Lck in a dose-dependent manner (Fig. 5.8E). In contrast, a synthetically phosphorylated Y87 peptide (TAT-Y87PO4) did not serve as an Lck substrate (Fig. 5.8E), 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; Supplementary Fig. 5.6F). 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. 5.8E). 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 (14).

**GBV-C E2 protein inhibits T cell activation in bystander cells**

Since expression of GBV-C E2 protein alone inhibited TCR signaling (Fig. 5.2), we hypothesized E2 expressing cells may inhibit TCR signaling in bystander cells contributing to global reduction in TCR signaling observed in GBV-C infected subjects(15, 78, 125, 126). 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 (Fig. 5.9A-C). Since, extracellular microvesicles (EMV) purified from the serum of GBV-C infected subjects inhibited TCR signaling when incubated with primary human T cells (Fig. 5.1), we 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 FS supernatant fluid (Fig. 5.9D). These EMV also contained CD63 suggesting that they were of endocytic origin (83) (Fig. 5.9D) consistent with the EMV obtained from GBV-C infected human serum. 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 FS control Jurkat cells (FS 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 FS EMV (Fig.5.9E-G).
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. The IL-2 signaling defect is due, at least in part, to effects of the envelope glycoprotein E2 (14). The effects of GBV-C on T cell activation and IL-2 signaling may contribute to viral persistence (16). 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 (127). Although clinical studies demonstrate an association between GBV-C infection and a global reduction in T cell activation (15, 78, 125), only a small proportion of T cells contained viral genomes. 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 $1 \times 10^7$ genome copies/mL of plasma and the virus is produced by T cells (108), 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. 5D), this region of E2 did not inhibit TCR-mediated activation (14). 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 (65). This also suggests that not all predicted tyrosine kinase substrate motifs on viral structural proteins will display functional activity.

In addition to the two predicted Lck phosphorylation substrate motifs (aa 83-91 and 281-289; Fig. 5.6 E-F), GBV-C E2 protein also contains two well conserved Src homology domain 3 (SH3) binding domains (PXXP; aa 48-51 and 257-260; Fig. 5.6G-H) (2, 111). Although, GBV-C E2 protein interacted with Lck 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. 5.6C) (14). However, it is possible that either these SH3 binding domains will 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_hum) E2 protein studied, but is not present in the E2 protein from chimpanzee GBV-C (GBV-C_cpz) isolates and the expression of the GBV-Cepz E2 protein did not inhibit TCR signaling. Based on this observation, it is tempting to speculate that since immune reactivity of lymphocytes from chimpanzee is significantly lower than humans (119), there is less selective pressure for GBV-Cepz to inhibit TCR signaling for replication. Furthermore, mutation of the conserved tyrosine residue reversed the inhibitory effects of human GBV-C E2 protein and Lck phosphorylated GBV-C E2 and the synthetic peptide (Y87) in vitro. These data demonstrate that the viral envelope glycoprotein is a substrate for Lck and since CD45 dephosphorylated E2 protein, 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 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 (16).

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 either by 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 micro vesicular particles. These data identify a novel mechanism by which a viral structural protein interferes with tyrosine kinase function resulting in global inhibition of T cell activation and support a model for global alteration of T cell activation during GBV-C infection. 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 (104). 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.
Figure 5.1. Extracellular microvesicles (EMV) from GBV-C infected human serum inhibit T cell receptor (TCR) signaling in primary human T cells. PBMCs from nine GBV-C viremic subjects were subjected to immuno-affinity selection for CD3+ 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% (A). Quantification of GBV-C RNA in peripheral blood mononuclear cells (PBMC), and purified CD4+ and CD8+ T cells obtained from nine GBV-C infected subjects (B). Quantification of GBV-C RNA in the serum, extracellular microvesicles (EMV) purified from the serum and supernatant after isolating microvesicles from five individuals with GBV-C infection (C). Quantification of GBV-C RNA in the top and bottom fraction after GBV-C positive serum was subjected to saline flotation gradient (D). IL-2 release (E), CD69 and CD25 cell surface expression (F, G) 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.

I would like to acknowledge Ernest Chivero for working along with me to prepare PBMCs for cell sorting and RNA quantification in this experiment.
Figure 5.1. Continued

C.

D.
Figure 5.1. Continued

E.

![Graph showing IL-2 (pg/ml) levels for US, GB- EMV, and GB+ EMV](image)

F.

![Bar chart showing CD69 MFI (fold change) for CD4+ and CD8+ T cells](image)
Figure 5.1. Continued

G.

![Bar chart showing comparison of CD4+ T cells and CD8+ T cells with GB- EMV, GB+ EMV, and GB- EMV conditions.](image)

CD25 MFI (fold change)
Figure 5.2. 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; 1µg/ml) for 5 days. Dox treatment significantly reduced E2 protein expression (A). 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 (B). 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; C, D, E) and zeta-chain-associated protein kinase (ZAP)-70 (F, G) compared to the frame shift control (FS). MFI= mean fluorescence intensity. Each experiment was repeated at least three times with consistent results. *P< 0.05; **P< 0.001
Figure 5.2. Continued

C.

<table>
<thead>
<tr>
<th>Time (minutes) [anti-CD3]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>FS control</td>
</tr>
</tbody>
</table>

- pLAT (Y226)
- Total LAT

D.

Graph showing pLAT/Total LAT over anti-CD3 stimulation time (0-15 minutes).
Figure 5.2. Continued

E.

F.
Figure 5.2. Continued

G.
Figure 5.3. GBV-C E2 protein 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).
A.

<table>
<thead>
<tr>
<th></th>
<th>FS control</th>
<th>GBV-C E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

[Image: Western blot of plck (Y394) and plck (Y505) over time for FS control and GBV-C E2 groups.

B.

[Graph: Lck activation (pY394/pY505/Total Lck) over anti-CD3 stimulation time (0-15 minutes).]

Lines represent:
- FS Control
- GBV-C E2
Figure 5.4. GBV-C E2 protein does not alter CD45 and Csk expression. Expression of CD45 (A) and Csk (C) was not different in GBV-C E2 expressing cells compared to the FS control. Recombinant GBV-C E2 protein did not affect CD45 enzymatic function (B). NS = not significant.
A.

Unstained

GBV-C E2

FS control

% of Max

CD45

B.

<table>
<thead>
<tr>
<th>FS control</th>
<th>GBV-C E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

[anti-CD3]

Csk

GAPDH
Figure 5.4. Continued

C.
Figure 5.5. GBV-C E2 protein interacts with Lck. Recombinant GBV-C E2-human Fc fusion protein but not human IgG precipitated Lck, but not Zap-70 or LAT from Jurkat cell lysates (A). Similarly, precipitation of Lck from Jurkat cells expressing GBV-C E2 protein also precipitated E2 protein (B). Each experiment was repeated at least three times with consistent results.
Figure 5.6. 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). Shaded box represent cell lines that did not inhibit TCR signaling (A). Jurkat cell lines expressing human GBV-C E2 protein truncated mutants and E2 protein from chimpanzee GBV-C (GBV-C<sub>cpz</sub>) isolate stably expressed GFP as determined by flow cytometry (B). IL-2 release was inhibited following TCR stimulation with CD3 and CD28 antibodies in all cell lines expressing E2 amino acids 86-98 (C). Recombinant E2 protein incubated with recombinant Lck demonstrated E2 tyrosine phosphorylation and when CD45 was added, E2 was dephosphorylated (D). GBV-C E2 protein sequence from human GBV-C (GBV-C<sub>hum</sub>) and chimpanzee GBV-C (GBV-C<sub>cpz</sub>) representing two predicted Lck substrate motifs (aa 83-91) (E) and (aa 281-289) (F), and two SH3 binding motifs (aa 48-51) (G) and (aa 257-260) (H). TCR-induced IL-2 release was not inhibited in Jurkat cells expressing the chimpanzee GBV-C E2 protein (GBV-C<sub>cpz</sub>) or the human E2 with a tyrosine to alanine substitution (Y87A) (I). Fold change in IL-2 release was calculated by measuring IL-2 at baseline (~5 pg/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
A. GBV-C_hum E2 (1-331) 

<table>
<thead>
<tr>
<th>1 - 219</th>
<th>220-331</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-331</td>
<td>3 - 103</td>
</tr>
<tr>
<td>104-219</td>
<td>3-72</td>
</tr>
</tbody>
</table>

Amino Acid Sequence

VYGSVSVTCVWGS
YAGSVSVTCVWGS (Y87A)

GBV-Ccpz E2

B. 

![Bar graph showing GFP Log10 MFI](image)
Figure 5.6. Continued

C.

D.
Figure 5.6. Continued

E. PQYVYGVS F. TGGFYEPLV G. PNGP H. PGTP

GBV-C<sub>hum</sub>

GBV-C<sub>cpz</sub>

aa 83-91 aa 281-289 aa 48-51 aa 257-260
Figure 5.6. Continued

I.
Figure 5.7. GBV-C E2 protein inhibits TCR signaling pathways at the level of Lck activation. Phorbol-12-myristate-13-acetate (PMA) does not induce Lck activation. Jurkat cells were either unstimulated or stimulated with PMA (50ng/ml) or anti-CD3 (5µg/ml) for two minutes. Lck phosphorylation (Y394) was only detected in Jurkat cells incubated with anti-CD3 but not with PMA (A). Expression of E2 amino acids 86-98 did not inhibit activation by PMA (50ng/ml) and Ionomycin (1µg/ml) when compared to Jurkat cells without E2 (JC) (B). Fold change in IL-2 release was calculated by measuring IL-2 at baseline and after 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
Figure 5.8. Synthetic GBV-C E2 peptides inhibit TCR activation in primary human T cells. Cellular uptake of FITC labeled TAT-fused synthetic GBV-C E2 peptides representing (86-98) aa region and TAT-only control peptides by PBMCs after 24 hours as determined by flow cytometry (A). 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 (B); CD69 and CD25 expression on CD4+ (C) and CD8+ (D) T cells compared to no peptide, a TAT only peptide, or the 86-101 peptide that substituted a histidine for the tyrosine (TAT-Y87H). 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-Y87PO4) or the TAT-276-292 peptide synthesized in a scrambled order TAT-SCR (E). RLU= relative luminescence units. Each experiment was repeated at least three times with consistent results. *P<0.05, **P<0.01.
A.

No Peptide  
TAT only

GBV-C E2 (Y87)  
GBV-C E2 (Y87H)

FITC  
Forward Scatter

88.7%  
86.3%
Figure 5.8. Continued

B.

[Graph showing IL-2 (pg/ml) levels for different conditions: No Peptide, TAT only, TAT-Y87, and TAT-Y87H]
Figure 5.8. Continued

C.

No Peptide  TAT only  TAT-Y87  TAT-Y87H

% CD4+ T cells

CD69  CD25

D.

% CD8+ T cells

CD69  CD25
Figure 5.8. Continued

E.

![Graph showing phosphorylation (RLU x 10^6) for TAT-Y87, TAT-Y87PO4, TAT-276-292, and TAT-SCR at 1 and 5 μg/mL.](Image)
Figure 5.9. GBV-C E2 protein inhibits T cell receptor (TCR) signaling in bystander cells. Following TCR stimulation with CD3 and CD28 antibodies, IL-2 release (A), surface expression of CD69 (B) and CD25 (C) 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 endocytic 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 frameshift to abolish translation (FS) (D). Following stimulation with anti-CD3/CD28 antibodies, IL-2 release (E), CD69 and CD25 cell surface expression (F, G) was significantly inhibited in PBMCs from healthy donor incubated with GBV-C E2 positive secreted microvesicles (E2 EMV) compared to E2-negative microvesicles (FS EMV). Fold change was calculated by measuring IL-2, CD69 and CD25 levels before and after stimulation. US= unstimulated, MFI= mean fluorescence intensity. Data represent the average of three independent cultures. *P<0.01, **P<0.01.
Figure 5.9. Continued

C.

![Graph showing %GFP-CD25 cells for JC + VC, JC + E2, US, and αCD3/CD28 conditions.]

D.

![Western blot images showing GBV-C E2 and CD63 proteins for FS control and GBV-C E2 conditions.]
Figure 5.9. Continued

E.

![Graph showing IL-2 levels with bars for US, FS EMV, and E2 EMV with error bars and a significance marker ** for E2 EMV.]

F.

![Graph showing CD4+ T cell expression with bars for CD69 and CD25 with error bars and significance markers ** for E2 EMV for both CD69 and CD25.]
Figure 5.9. Continued

G.
CHAPTER VI

GENERAL DISCUSSION

This chapter summarizes existing data on interactions between GBV-C and HIV and discusses the mechanisms by which GBV-C infection may reduce immunoactivation and alter HIV disease progression.

GB virus C (GBV-C) can establish persistent human infection without any apparent clinical outcomes (127). Although GBV-C is a common infection and is highly prevalent among HIV infected subjects, it does not appear to cause any human disease (88, 127). Interestingly, acquisition of GBV and persistent GBV-C viremia appears to have a beneficial effect in HIV infected individuals. Several clinical studies including a meta-analysis of HIV-positive individuals found an association between persistent GBV-C infection and prolonged survival of HIV-infected individuals; however, some studies failed to observe this beneficial effect (69, 96, 133, 136, 138, 142, 152, 157, 158). GBV-C infection is also associated with reduced mother to child HIV transmission (50, 129) and improved response to antiretroviral therapy in HIV-positive subjects (106, 121). Studies into the possible interactions between GBV-C and HIV have demonstrated that GBV-C inhibits HIV replication in a coinfection model and GBV-C proteins contain anti-HIV replication effect (44, 59, 64, 112, 144, 149, 150). Several studies have suggested that GBV-C infection alters several host factors involved in HIV replication which may contribute to the protective effect of GBV-C coinfection observed in HIV-infected subjects (16).

Recent studies provide insights into the effects of GBV-C on immune signaling pathways that are known to contribute in HIV disease progression. Several clinical studies found that among HIV-infected subjects GBV-C infection is associated with reduced immunoactivation as measured by T cell activation and response to recombinant
IL-2 therapy (15, 78, 125, 126). Consistently, *ex vivo* GBV-C infection was associated with reduced T cell activation and protection against activation induced T cell death (108). HIV infection is associated with chronic immunoactivation, which is considered as one of the important factors in HIV disease progression (47, 95). Immunoactivation supports HIV replication, is associated with immune dysfunction, and some studies suggest that immunoactivation is a better predictor of HIV disease progression than plasma viral load (VL) (46, 52). In addition, persistent immunoactivation is associated lower virological response to antiretroviral therapy (57) suggesting modalities to treat persistent immune activation in addition to antiretroviral therapy may benefit these individuals. Thus, GBV-C effects on reducing chronic immunoactivation may benefit GBV-C infected HIV-positive subjects.

In this thesis, we describe the effects of GBV-C infection on T cell activation and IL-2 signaling pathways and the mechanism by which GBV-C infection alters these signaling pathways contributing to the observed beneficial effects in HIV-infected subjects. Although GBV-C infection is associated with reduced T cell activation in HIV-viremic subjects (78, 113, 126), the effects of GBV-C infection on T cell activation in HIV-positive subjects on cART with suppressed HIV VL has not been studied. This is particularly important as antiretroviral therapy has been suggested to lower the threshold of immunoactivation but HIV-positive subjects on cART have higher levels of immunoactivation when compared to HIV-uninfected subjects (56). We found that among cART treated HIV-positive subjects; GBV-C infected subjects have significantly lower levels of immunoactivation compared to GBV-C uninfected controls. A potential role of an immunomodulatory T cells called double negative T cells (DNTC) in regulating immunoactivation during GBV-C viremia was identified. DNTC express the CD3 receptor but lack surface expression of CD4 and CD8. DNTC also inhibits immune activation (101, 140). Subjects with GBV-C viremia had significantly increased levels of DNTC and GBV-C viral load significantly correlated levels of DNTC suggesting a causal
relationship between GBV-C infection and double negative T cells. Although the significant increase in DNTC levels in subjects with GBV-C viremia may contribute to lower immunoactivation, the mechanism for this increase proportion of DNTC during GBV-C infection requires further investigations. This study demonstrates that GBV-C infection reduces immunoactivation in cART treated HIV-positive subjects. Understanding mechanisms by which GBV-C reduces immunoactivation may provide insights into novel ways to treat immunoactivation in HIV-positive subjects effectively treated with cART.

To gain insights into the mechanisms by which GBV-C infection alters immunoactivation, lymphocytes obtained from GBV-C viremic subjects were studied *ex vivo*. GBV-C replication was significantly reduced in lymphocytes following activation with IL-2/PHA, compared to unstimulated lymphocytes. This suggests that lymphocyte activation inhibits GBV-C replication and perhaps it is of evolutionary advantage for the virus to inhibit immunoactivation. Since GBV-C coinfection is associated with blunted response to recombinant IL-2 therapy in HIV-positive subjects, effects of GBV-C infection on cellular proliferation following IL-2 treatment were studied. Consistent with the clinical studies, GBV-C replication significantly reduced IL-2 mediated cellular proliferation suggesting that GBV-C interferes with IL-2 signaling pathways. The block in immunoactivation resulted into significant reduction in reactivation of latent HIV from cellular reservoirs and protection of T lymphocytes from activation induced cell death (AICD). Given the high prevalence of GBV-C in HIV infected subjects, this observation is particularly important in designing strategies to reactivate latent HIV from cellular reservoirs. Together, these studies suggest GBV-C infection inhibits T cell activation and IL-2 signaling pathways, and immunoactivation reduces GBV-C replication providing insights into interrelationship between GBV-C, immunoactivation and HIV disease.

GBV-C envelope glycoprotein E2 was identified as a viral factor that contributes to reduce immunoactivation during GBV-C infection. Previous studies have found that
GBV-C E2 protein inhibits HIV replication (59, 64, 150) but its role in immunoactivation was not studied. Expression of GBV-C E2 protein inhibited T cell receptor (TCR) induced IL-2 production, expression of IL-2 receptor (alpha chain), and phosphorylation of STAT5 suggesting an antagonist effect of GBV-C E2 protein on IL-2 signaling pathways. These effects resulted into reduced IL-2 induced T cell proliferation as observed in clinical and ex vivo studies. Through deletion mutagenesis studies, the immunomodulatory region was mapped to the first 219 amino acid of E2 protein as expression of C terminal region (aa 220-331) did not affect TCR/ IL-2 signaling pathways.

To understand the mechanism by which GBV-C E2 protein alters TCR signaling, effects of E2 protein on proximal TCR signaling events were studied. Utilizing human CD4+ T cells stably expressing GBV-C E2 protein we found that proximal TCR signaling pathways were altered by E2 protein. Following TCR engagement with CD3 and CD28 antibodies, expression of early activation receptor CD69 was significantly inhibited in Jurkat cells expressing E2 protein, which was reversed, by growing cells in doxycycline to reduce E2 expression. GBV-C E2 protein expression inhibited TCR induced phosphorylation of linker for activation of T cells (LAT), zeta-chain associated protein kinase (ZAP-70) and lymphocyte specific protein tyrosine kinase (Lck). Lck is the most proximal tyrosine kinase in the TCR signaling pathway and activation of Lck is regulated by tyrosine phosphatase CD45 and tyrosine kinase Csk. GBV-C E2 protein expression neither altered CD45 and Csk expression levels nor CD45 function. Since the immunomodulatory region of E2 protein was mapped to the first 219 amino acids, further deletion mutagenesis studies were carried out. The region that inhibits TCR signaling was mapped to a 13 amino acid peptide motif localized in the N-terminal region of E2 protein (amino acid 86-98). This region contained a tyrosine residue at position 87 (Y87) which is a predicted Lck substrate. Confirming this prediction, we found in vitro Lck phosphorylates GBV-C E2 protein and E2 was dephosphorylated by CD45. After
analysis of E2 sequences from 42 human GBV-C isolates (GBV-C<sub>hum</sub>) from seven genotypes, we found tyrosine (Y87) is highly conserved; however, within E2 sequences of GBV-C variant isolated from chimpanzees (GBV-C<sub>cpz</sub>) we did not find Y87 residue. To study the requirement of Y87 within the E2 protein to modulate TCR signaling, we expressed E2 protein from GBV-C<sub>cpz</sub> or GBV-C<sub>hum</sub> with tyrosine (Y87) mutated to alanine (Y87A). E2 protein from chimpanzee or the human E2 protein with Y87A mutation did not alter TCR signaling. Consistent with these data, synthetic peptides representing amino acids 86-98 of human GBV-C E2 protein inhibit TCR signaling in primary human T cells. Previous studies demonstrated that similar to E2 protein from GBV-C<sub>hum</sub>, expression of E2 protein from GBV-C<sub>cpz</sub> also inhibits HIV replication in CD4+ T cells (82, 150). Thus, it appears that although HIV inhibitory effects are conserved between human and chimpanzee GBV-C E2 proteins, only human GBV-C E2 protein inhibits TCR signaling. Given the high prevalence of GBV-C in HIV-infected humans, high levels of immune activation observed in HIV-infected individuals, reduced GBV-C replication following immunoactivation and lower threshold of activation in T cells from chimpanzee compared to humans (119); it is tempting to speculate that higher immunoactivation has exerted selective pressure for human GBV-C to interfere with TCR signaling.

*In vivo* GBV-C infection is associated with global reduction in T cell activation (15, 78, 125) and since GBV-C E2 protein expression reduced T cell activation, we measured the frequency of GBV-C infected T cells in infected subjects. Interestingly, the frequency of GBV-C RNA positive cells was less than ten percent of peripheral blood mononuclear cells. This suggested that GBV-C infection alters TCR signaling in bystander uninfected T cells. We found that GBV-C E2 expression reduced TCR signaling in bystander cells. This reduction in T cell activation was only observed when E2 expressing cells were co-cultured with bystander cells. Furthermore, we identified the mechanism for inhibition of bystander cell activation by GBV-C E2 protein. Extracellular
microvesicles purified from the supernatant of E2 expressing cells contained GBV-C E2 protein and these microvesicles inhibited TCR signaling in bystander uninfected T cells. These microvesicles have biophysical properties of vesicles of endosomal origin and contained CD63, a marker commonly associated with exosomes. Consistently, extracellular microvesicles purified from the serum of GBV-C infected subjects contained GBV-C RNA and inhibited TCR signaling in bystander uninfected T cells obtained from the healthy blood donors. Together, these data support a model for GBV-C mediated global reduction in TCR signaling (Fig. 6.1). Based on this model, GBV-C E2 protein inhibits Lck activation in the infected cells by competing as a substrate for Lck. Lck phosphorylation was reduced in E2 expressing T cells, yet GBV-C E2 was phosphorylated by Lck in vitro. This suggests that E2 could compete for Lck phosphorylation due to which Lck auto-trans-phosphorylation, a critical event post TCR engagement, is inhibited resulting into defect in downstream signaling. Since activation of Lck is required for both TCR and IL-2 signaling, inactivation of Lck by GBV-C E2 protein results into inhibition of TCR and IL-2 signaling pathways. Some GBV-C RNA and/or E2 protein are released from the infected cells in microvesicles and these microvesicles transport GBV-C RNA/ E2 protein to bystander uninfected T cells which results in to inhibition of TCR signaling in these cells. Although these data cannot exclude the possibility that E2 protein and RNA is not incorporated within the microvesicles but is only associated during vesicles biogenesis, a previous study found that GBV-C RNA found in low density particles (similar to exosomes) were protected from RNase (146). This suggests that RNA and/or E2 protein gets incorporated within these microvesicles, further work is required to understand the molecular basis of GBV-C RNA and E2 protein incorporation within microvesicles.

Unlike HCV, GBV-C does not appear to encode a core protein, the cellular receptor for GBV-C is not known and in vivo multiple cell types are infected with GBV-C. In addition, GBV-C viral load in the infected human is high, and microvesicles
obtained from the serum of infected people contain abundant GBV-C RNA. These data raise possibility that GBV-C RNA within the microvesicles are infectious and are the primary mediator of viral infection. This would be quite advantageous for a positive strand RNA virus without a core protein, and these particles may form infectious units that can infect and persist within its host.

In conclusion, this thesis describes the interrelationship between GBV-C infection and immunoactivation and provides insights into potential mechanisms by which GBV-C reduces immunoactivation. The identification of immunomodulatory motif within the E2 protein and inhibition of primary human T cells by synthetic peptides representing this motif provides a promising peptide-based therapeutic intervention method to treat HIV infection and any other immunoactivation-associated diseases. Further studies are needed to examine the therapeutic potential of these peptides. Although the effects of GBV-C on immunoactivation are measurable and there is a reduction in global immunoactivation during GBV-C infection, these effects are not complete. Furthermore, GBV-C is not associated with any known human disease suggesting the association between GBV-C and reduced T cell activation and response to therapeutic IL-2 administration, although measurable, are not potent enough to lead to immunodeficiency. However, these effects may contribute to GBV-C persistence and provide a beneficial effect against immunoactivation mediated diseases. Future studies are required to understand the effects of GBV-C on other immune signaling pathways, which may lead to develop specific therapy to treat HIV infection and other immunoactivation related diseases.
Figure 6.1. 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 E2 protein expression. GBV-C E2 protein inhibits Lck activation in the infected cells by competing as a substrate for Lck (A). Lck phosphorylation was reduced in E2 expressing T cells, yet GBV-C E2 was phosphorylated by Lck in vitro. This suggest that E2 could compete for Lck phosphorylation due to which Lck auto-trans-phosphorylation, a critical event post TCR engagement, is inhibited resulting into defect in downstream signaling. Since activation of Lck is required for both TCR and IL-2 signaling, inactivation of Lck by GBV-C E2 protein results into inhibition of TCR and IL-2 signaling pathways. Along with virions, some GBV-C RNA and/or E2 protein are released from the infected cells in microvesicles and these microvesicles transport GBV-C RNA/ E2 protein to GBV-C permissive T cells (B) or bystander uninfected T cells (C) which results in to inhibition of TCR signaling in these cells. From the data presented in this thesis, we cannot conclude that GBV-C RNA within microvesicles are infectious or it cannot exclude the possibility that E2 protein and RNA is not incorporated within the microvesicles but is only associated with these vesicles during biogenesis. However, consistent with this model, a previous study found that GBV-C RNA found in high density particles (similar to exosomes) were protected from RNase (146). This suggests that RNA and/or E2 protein is incorporated within microvesicles, but further work is required to understand the molecular basis of GBV-C RNA and E2 protein incorporation within microvesicles.
REFERENCES


