Targeted Deletion of \textit{Fgl2} Enhances Anti-Viral T Cell Responses and Mediates Viral Clearance in a Murine Model of Chronic Viral Infection

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Chronic viral infection is a significant burden on healthcare systems worldwide. Robust antiviral immune responses are essential for viral clearance. Persistent viruses use a variety of mechanisms to evade immune surveillance including the upregulation of host immunosuppressive factors. Secreted fibrinogen-like protein 2 (FGL2) has been identified as an inhibitory effector molecule in suppressing immune responses in patients with chronic hepatitis C virus (HCV) and hepatitis B virus (HBV) disease. In a murine model of chronic infection caused by Lymphocytic choriomeningitis virus (LCMV) clone 13, we demonstrate that mice deficient in *Fgl2* have increased numbers of mature antigen-presenting cells (APC), improved virus-specific cytotoxic T cell immunity and enhanced viral clearance when compared to wild-type mice. These results highlight the importance of the FGL2 inhibitory pathway in immune evasion and provide a rationale to investigate the effects of blocking FGL2 as a novel immune therapeutic in patients suffering from persistent infections.
Acknowledgments

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# Table of Contents

Acknowledgments ........................................................................................................ iii

List of Tables ................................................................................................................ vii

List of Figures ................................................................................................................ viii

Contribution to Science ............................................................................................... x

List of Abbreviations ................................................................................................... xi

## 1 Introduction ............................................................................................................ 1

### 1.1 Immunity in Chronic Viral Infection ............................................................... 1

#### 1.1.1 HBV, HCV and HIV Pathogenesis ............................................................. 1

- 1.1.1.1 HBV ........................................................................................................ 1

- 1.1.1.2 HCV ........................................................................................................ 2

- 1.1.1.3 HIV ......................................................................................................... 3

#### 1.1.4 Chronic Viral Infections – An Unresolved Disease .................................... 3

#### 1.1.2 Role of Innate Immune Responses in Chronic Viral Infections ................. 4

- 1.1.2.1 DCs ......................................................................................................... 4

- 1.1.2.2 Macrophages ......................................................................................... 5

- 1.1.2.3 NK Cells ............................................................................................... 7

#### 1.1.3 Role of the Adaptive Immune System in Viral Infections .......................... 9

#### 1.1.3.1 Humoral Responses are Impaired in Chronic Viral Infection ............... 10

#### 1.1.3.2 Role of Cell-Mediated Immune Responses in Chronic Viral Infection... 11

- 1.1.3.2.1 Robust CD4+ Th1 Cell Response Assists in Viral Clearance ............... 11

- 1.1.3.2.2 Cytotoxic T Cells Eliminate Virus-Infected Cells ............................... 12

- 1.1.3.2.3 Memory T Cells Protect against Re-Infection ....................................... 12

- 1.1.3.2.4 T Cell Exhaustion .............................................................................. 13

#### 1.1.3.3 Regulatory T Cells Suppress Anti-Viral Immune Responses ................ 15

### 1.2 Fibrinogen-Like Protein 2 .............................................................................. 16

#### 1.2.1 Structure of FGL2 .................................................................................... 16

#### 1.2.2 Prothrombinase Activity of Membrane-Associated FGL2 ....................... 18

#### 1.2.3 Immunosuppressive Activity of Secreted FGL2 ........................................ 19

#### 1.2.4 FGL2 Receptors - FcγRIIB/RIII ................................................................ 19

#### 1.2.5 Role of FGL2 in Viral Pathogenesis .......................................................... 20

### 1.3 Lymphocytic Choriomeningitis Virus (LCMV) ............................................... 21

#### 1.3.1 LCMV - a Model for Viral Pathogenesis ................................................... 21
1.4 Hypothesis and Aims ........................................................................................................... 26

2 Materials and Methods ....................................................................................................... 28
  2.1 Cell Culture Reagents ..................................................................................................... 28
  2.2 Mice and Infections ........................................................................................................ 28
  2.3 Blood and Tissue Collection ......................................................................................... 29
  2.4 Preparation of LCMV .................................................................................................... 29
  2.5 LCMV cl 13 Purification by CsCl Gradient Centrifugation ............................................. 29
  2.6 Preparation of α-VL4 .................................................................................................... 30
  2.7 Focus Forming Assay .................................................................................................... 31
  2.8 Measurement of Plasma FGL2 ..................................................................................... 32
  2.9 Measurement of Plasma Alanine Transaminase (ALT) .................................................. 32
  2.10 Detection of α-LCMV Antibodies by ELISA ................................................................. 32
  2.11 Neutralizing Antibody Detection ................................................................................. 33
  2.12 Histology ..................................................................................................................... 33
  2.13 Isolation of Splenic Mononuclear Cells (SMNC) .......................................................... 33
  2.14 Isolation of DCs and Macrophages from Spleen ............................................................ 34
  2.15 Analysis of T Cell Response to LCMV cl 13 ............................................................... 34
  2.16 Biotinylation of Recombinant His-FGL2 ..................................................................... 35
  2.17 Flow Cytometry ......................................................................................................... 35
    2.17.1 Antibodies and Reagents ....................................................................................... 35
    2.17.2 Cell Staining .......................................................................................................... 36
    2.17.3 Data Analysis ......................................................................................................... 36
  2.18 Statistics ...................................................................................................................... 36

3 Results .................................................................................................................................. 37
  3.1 Targeted Deletion of Fgl2 Enhances Anti-Viral Memory CD8+ T Cell Responses ........ 37
  3.2 Targeted Deletion of Fgl2 Improves Viral Clearance without Further Increasing
  Immunopathology in a Murine Model of Chronic Infection .................................................. 39
    3.2.1 FGL2 is Upregulated Following LCMV cl 13 Infection ............................................ 39
    3.2.2 Deletion of Fgl2 Leads to Reduction of Viral Load ...................................................... 41
    3.2.3 Liver Immunopathology is Similar in Fgl2−/− and Fgl2+/− Mice during Chronic Viral
      Infection ........................................................................................................................... 43
3.3 Effects of Fgl2 Deletion on Innate Immune System during Chronic Viral Infection... 46

3.3.1 Total Numbers of Mature DCs and Macrophages are Increased in Fgl2−/− Mice following LCMV cl 13 Infection ................................................................................................................................. 46

3.3.2 FGL2 Binds to NK Cells ................................................................................................................................. 48

3.3.3 NK Cell Activity during Chronic Viral Infection ............................................................................................... 50

3.4 Effects of Fgl2 Deletion on Adaptive Immune Responses during Chronic Viral Infection ................................................................. 52

3.4.1 Deletion of Fgl2 Results in Increased Titres of Neutralizing Antibodies ......................................................... 52

3.4.2 Deletion of Fgl2 Enhances Anti-Viral T Cell Responses during Chronic Infection ................................. 54

3.4.3 Fgl2 Deficient Mice Have Reduced Percentage of PD-1 Expressing T Cells during Chronic Viral Infection ........................................................................................................................................... 60

4 Discussion ............................................................................................................................................................. 62

5 Conclusion .............................................................................................................................................................. 75

6 Future Directions ....................................................................................................................................................... 76

References .................................................................................................................................................................. 77
List of Tables

Table 1-1: The comparison between membrane-associated and secreted FGL2.......................... 18

Table 1-2: Main immunological effects causing persistent LCMV cl 13 infection....................... 25
List of Figures

Figure 1-1: NK cell functions. ................................................................. 9

Figure 1-2: Markers for T cell exhaustion. ................................................... 14

Figure 1-3: Structure of FGL2. ................................................................. 17

Figure 1-4: Structure of an Arenavirus. ....................................................... 21

Figure 1-5: CTL responses to acute and persistent LCMV infection over time. ............... 22

Figure 1-6: Proposed model of FGL2 inducing chronic viral infection. .......................... 26

Figure 3-1: Anti-viral CD8\(^+\) T cell responses are enhanced in acute and re-infection studies in \(Fgl2^{+/+}\) mice. ................................................................. 38

Figure 3-2: LCMV cl 13 induces FGL2 secretion \textit{in vivo} and \textit{in vitro}. ....................... 40

Figure 3-3: Viral titre analysis in LCMV cl 13 infected \(Fgl2^{+/+}\) and \(Fgl2^{-/-}\) mice. ................. 42

Figure 3-4: ALT levels in \(Fgl2^{+/+}\) and \(Fgl2^{-/-}\) mice following LCMV cl 13 infection. ............ 43

Figure 3-5: Affects of \(Fgl2\) deletion on immunopathology in liver following LCMV cl 13 infection. ........................................................................ 45

Figure 3-6: Total numbers of mature DCs and macrophages in \(Fgl2\) deficient and wild-type (\(Fgl2^{+/+}\)) mice by day 2 LCMV cl 13 pi. ................................................................. 47

Figure 3-7: FGL2 binds to Fc\(\gamma\)RIII expressing NK cells. ........................................ 49

Figure 3-8: NK cell activity in LCMV cl 13 infected \(Fgl2^{+/+}\) and \(Fgl2^{-/-}\) mice. ...................... 51

Figure 3-9: Humoral response analysis in LCMV cl 13 infected \(Fgl2^{+/+}\) and \(Fgl2^{-/-}\) mice. ............ 53

Figure 3-10: Total numbers of CD3\(^+\), CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) T cells in spleen of LCMV cl 13 infected \(Fgl2^{+/+}\) and \(Fgl2^{-/-}\) mice. ................................................................. 55
Figure 3-11: Deletion of Fgl2 does not restore LCMV cl 13 depleted NP396+ CD8+ T cell population........................................................................................................................................ 56

Figure 3-12: Deletion of Fgl2 augments anti-viral GP33 tetramer+ CD8+ T cell responses in late phase of chronic infection. ........................................................................................................................................ 58

Figure 3-13: Analysis of Fgl2 deletion on IFNγ production by CD4+ T cell following LCMV cl 13 infection........................................................................................................................................ 59

Figure 3-14: PD-1 expression analysis on CD4+ and CD8+ T cell in Fgl2 deleted mice following LCMV cl 13 infection. ........................................................................................................................................ 61

Figure 4-1: Proposed model of Fgl2 deletion in enhancing anti-viral immune responses and viral clearance during chronic viral infection........................................................................................................................................ 74
Contribution to Science

Publications:

Abstracts:


List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Area</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<td>APC*</td>
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<td>B cell receptor</td>
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<td>Cluster of differentiation</td>
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</tr>
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<td>Cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
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<td>Cyanine</td>
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<tr>
<td>D</td>
<td>Day</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cell(s)</td>
</tr>
<tr>
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<td>Double-distilled water</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
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</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Fc</td>
<td>Constant region fragment</td>
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<tr>
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<td>Fc receptor</td>
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<tr>
<td>FGL2</td>
<td>Fibrinogen-like protein 2</td>
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<td>Figure</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Foxp3</td>
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</tr>
<tr>
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<td>Fibrinogen-related domain</td>
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<td>g</td>
<td>Gram (includes nano [n], micro [µ], and milli [m])</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GranB</td>
<td>Granzyme B</td>
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<td>H</td>
<td>Height</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HBV</td>
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<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibition morif</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>kDA</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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</tr>
<tr>
<td>L</td>
<td>Liter (includes micro [$\mu$] and milli [m])</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte activating gene-3</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic Choriomeningitis Virus</td>
</tr>
<tr>
<td>LCMV cl 13</td>
<td>Lymphocytic Choriomeningitis Virus clone 13</td>
</tr>
<tr>
<td>m</td>
<td>Meter (includes milli [m], micro [$\mu$], and centi [c]), membrane</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>M</td>
<td>Molar (includes nano [n], micro [$\mu$], and milli [m])</td>
</tr>
<tr>
<td>ME</td>
<td>Mercaptoethanol</td>
</tr>
<tr>
<td>MEM</td>
<td>Modified Eagles Media</td>
</tr>
<tr>
<td>MFI</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MHCI/II</td>
<td>Major histocompatibility complex class I/II</td>
</tr>
<tr>
<td>MHV-3</td>
<td>Murine hepatitis virus-3</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NKT cells</td>
<td>Natural killer T cells</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>p</td>
<td>Propability</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed cell death 1 receptor</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed cell death 1 ligand 1</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>Pen</td>
<td>Penicillin</td>
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<td>PerCP</td>
<td>Peridinin-chlorophyll proteins – cyanine dye</td>
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<tr>
<td>pi</td>
<td>Post-infection</td>
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<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<td>Standard error mean</td>
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<td>Splenic mononuclear cells</td>
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<td>Side scatter</td>
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<td>Streptomycin</td>
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<td>T cell receptor</td>
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<td>T follicular helper cells</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cells</td>
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1 Introduction

1.1 Immunity in Chronic Viral Infection

1.1.1 HBV, HCV and HIV Pathogenesis

Human chronic viral infection represents a serious health problem worldwide affecting over 500 million individuals and is associated with significant financial burdens and high mortality. The major etiological agents of persistent infection in humans consist of an array of viruses including hepatitis B virus (HBV), hepatitis C virus (HCV) and human deficiency virus (HIV) [1-3]. Those viruses are capable of evading immune surveillance, actively suppressing immunity and exhibit viral latency. Chronic viral infections may also induce persistent low-grade immune activation that can result in significant immunopathology and lead to the progressive loss of virus specific immune cells [4].

While significant advances have been made in treating chronic disease, a large number of individuals still remain chronically infected. Furthermore, development of drug resistance and poor tolerability to treatment regimens complicate the treatment of chronic infection [2]. Consequently, novel strategies to promote viral control need to be investigated.

1.1.1.1 HBV

HBV is a non-cytolytic hepatotropic deoxyribonucleic acid (DNA) virus and belongs to the Hepadnaviridae family [5]. HBV affects approximately 350 million people worldwide [2, 6]. Dependent on host-pathogen immune interactions, infections with HBV can result in acute or chronic liver disease (hepatitis) [5]. The development of chronic viral hepatitis (CVH) by HBV is influenced by a number of host and viral factors such as genetic background and the patient’s age at acquisition of the virus [2, 5]. The leading cause of chronic HBV infection is the vertical transmission. 90 % of perinatal infections with HBV lead to lifelong infection [2], which over
time can progress to liver cirrhosis and hepatocellular carcinoma (HCC). HCC is the largest indication for liver transplantation [7]. HBV infections in adults are generally transient and only 10 % of adults infected with HBV develop CVH [6].

Clearance of HBV infection is largely determined by the magnitude of the anti-viral immune responses [2]. A preventive vaccine against HBV exists, but the treatment success for patients with chronic HBV is limited [6]. For patients infected with HBV, lifelong anti-viral therapy is required to halt disease progression [2, 8].

1.1.1.2 HCV

HCV is a non-cytolytic hepatotropic ribonucleic acid (RNA) virus and belongs to the Flaviviridae family [2]. HCV affects 170 million people worldwide [9]. The mutation and replication rate of HCV is very high, $10^{-3}$ per nucleotide per generation and $\sim 10^{12}$ virions per day respectively [2]. HCV replicates rapidly due to highly dynamic initial RNA replication [10]. The lack of proofreading capacity of the polymerase leads to viral quasi-species [11]. This results in a rapid expansion of the virus that affects viral control by the adaptive immune responses and promotes viral persistence [2].

70-80 % of adults infected with HCV develop chronic liver disease. Acute infections usually remain undiagnosed due to mild or no clinical symptoms [12]. Treatment of chronic HCV with pegylated interferon (IFN) and Ribavirin is standard. IFN$\alpha$ treatment results in the degradation of viral RNA while Ribavirin’s anti-viral mechanism lies in the lethal mutagenesis of the RNA virus genome [13, 14]. However, 40-50% of patients, who develop CVH, remain nonresponsive or intolerant to the standard treatment and will go on to develop cirrhosis, liver failure or HCC [9, 15-17]. Currently, there is no protective vaccine against HCV infection available and anti-viral treatment can be expensive or have side-effects [9].
1.1.1.3 HIV

HIV is a RNA virus and belongs to the Retroviridae family. 34 million people worldwide are currently infected with HIV [18]. This virus infects humans and primates, and replicates preferentially in CD4\(^+\) T helper (Th) cells, which are crucial in activating adaptive anti-viral immune responses. Additionally, HIV can infect the cells of the innate immune system including dendritic cells (DCs) and macrophages, which play an important role in immune surveillance [19]. Due to great genetic diversity and antigenic variation, HIV escapes immune surveillance and complicates treatment regimens and the development of a vaccine [20].

Following HIV infection, Th cells, DCs and macrophages are depleted and individuals become prone to opportunistic infections leading to Acquired Immunodeficiency Syndrome (AIDS) [21]. In order to prevent AIDS, vaccines against HIV have been designed, but are limited due the high mutation rate of HIV. Also, HIV infects activated Th cells and vaccines that focus to enhance adaptive T cell responses have been ineffective. An opposite approach of suppressing T cell responses failed as well, leading to reduced effector T cell activity and increased viremia [22]. Therefore, high expectations are set on new strategies to treat chronic viral infection.

1.1.1.4 Chronic Viral Infections – An Unresolved Disease

The viruses HBV, HCV and HIV have evolved a repertoire of sophisticated mechanisms to suppress and escape host immunity. However, those viruses differ in their host-virus interactions. In general, the establishment of chronic viral infection is dependent on different factors such as induction of, and sensitivity to innate immune responses as well as escape from adaptive immune surveillance [2]. Understanding the virus-host interplay and the mechanisms that lead to viral persistence is essential in designing new immunotherapeutic approaches [4].
1.1.2 Role of Innate Immune Responses in Chronic Viral Infections

The innate immune system represents an evolutionarily ancient form of immune recognition, where foreign material is recognized through the expression of conserved receptors called pattern recognition receptors (PRR) that recognize conserved microbial motifs called pathogen associated molecular patterns (PAMPS). Innate immunity triggers adaptive immune responses leading to resolution of infection [23].

Many investigators have demonstrated that innate immune responses are actively suppressed in chronic infection, inhibiting virus recognition and initiation of anti-viral programmes required to prevent viral spread. Consequently, subsequent adaptive immune responses that may ensue following the failure of innate immune mechanisms to control viral infection are blunted and cannot be maintained [12, 24-30].

1.1.2.1 DCs

DCs originate from bone marrow and exist in blood as immature precursor cells. DCs are the most efficient antigen presenting cells (APCs) followed by macrophages and B cells. They migrate to peripheral tissues where they can encounter foreign antigens. Because of the wide variety of selective receptors (PRRs), DCs are capable to recognize molecular patterns on pathogens, infected or apoptotic cells [27]. After recognizing foreign antigens, DCs can undergo different maturation programs dependent on the various pathogens. DCs phagocytose foreign particles and partially digested them. The exogenous antigens are bound to major histocompatibility complex (MHC) class II (MHCII) and to MHCI (cross-presentation) [31]. Afterwards, the DCs migrate to secondary lymphoid organs such as lymph nodes where they prime immunologically resting T lymphocytes including cluster of differentiation 4 (CD4+) and CD8+ T cells [32].
In order to effectively prime a virus-specific T cell response, DCs must present antigen to virus specific T cells by expressing high levels of MHC class II and co-stimulatory molecules such as CD80 (B7-1) or CD86 (B7-2) [31]. Moreover, the secretion of cytokines including tumor necrosis factor α (TNFα), interleukin 6 (IL-6) and IFNγ further promotes differentiation into mature T effector cells [33]. T cells primed in the absence of co-stimulation progressively lose their ability to perform effector functions, become anergic and are deleted from the repertoire [34].

Two functionally distinct populations of DCs exist, the plasmacytoid (pDC) and the myeloid (mDC). High expression levels of MHCII as well as high levels of TNFα and IL-6 make mDCs capable of presenting antigen and promoting a strong anti-viral immune response. PDCs are known for their anti-viral activity by producing high levels of anti-viral IFNα [27].

Patients infected with HBV, HCV and HIV have decreased numbers of pDCs and mDCs [35-37]. Additionally, mDCs from viral infected patients have decreased expression of co-stimulatory ligands leading to a decreased ability to prime T cells [37-39]. DCs from infected patients also express immunosuppressive cytokines such as IL-10, which causes a T helper 2 (Th2) response contributing to viral persistence [27, 40]. Also, increased expression of the negative immune regulator programmed cell death-ligand 1 (PD-L1) on DCs has been reported during chronic viral infection [41].

1.1.2.2 Macrophages

Macrophages are also major participants of the innate immunity. Macrophages can mature from circulating monocytes precursors, which originate from the bone marrow [42]. The primary functions of macrophages include the removal of dead or dying cells, the containment of viral and bacterial spread by directly phagocytizing pathogens or infected cells, and the presentation
of antigens [32, 42]. Cells of the monocyte/macrophage lineage are known to be characterized by considerable diversity and plasticity [43]. Macrophages reside in different tissues and are affected by tissue-specific immune-modulating signals [28]. Based on the type and the level of stimuli, macrophages can be activated by different mechanisms. The classical activation is mediated through the inflammatory cytokine IFNγ, which is produced by Th1 cells or natural killer (NK) cells. That leads to the migration of macrophages to the site of infection or pathogen entry and induces the release of pro-inflammatory cytokines, which in return supports T cells activation [33]. In addition, cytolytic activity of macrophages can be induced through humoral activation of Fcγ-receptors (FcγR) that recognize antibody-coated pathogens. After engulfment, cell debris or pathogens are lysed within macrophages through lysosomal activity [44]. Macrophages are also capable of presenting exogenous antigens on MHCI or II to naive T lymphocytes leading to the activation of anti-viral T cell responses. However, this process is inefficient compared to DCs [32].

The classical activation of macrophages is also referred to as M1 activation and supports the Th1 response. The M2 or alternative activation is stimulated by IL-4 and IL-13 and is proposed to contribute to Th2 response [42-44]. Viral clearance is dependent on polarization towards an M1 response with robust production of IL-12 and type 1 IFNs. M2 macrophages play a critical role in the resolution of inflammation and wound-healing by producing anti-inflammatory cytokines including IL-10 and IL-4 [29].

Induction of an M2 response is a strategy adopted by many viruses including HBV, HCV and HIV to evade the immune system leading to chronic viral persistence [28]. Another mechanism of suppressing adaptive immune responses exploited in chronic viral infection is the upregulation of PD-L1 on macrophages. Due to higher expression of PD-L1 on APCs, the inhibitory receptor
programmed cell death 1 (PD-1) on T cells gets activated in turn leading to inhibition of activating signals on T cells [45].

1.1.2.3  NK Cells

NK cells as part of the innate immune system serve as a first line of defense in viral infection. NK cells are granular lymphocytic cells and originate from bone marrow. NK cells exist in different tissues, lymphoid and non-lymphoid [46, 47]. The main function of NK cells is to eliminate pathogens and virally or transformed cells through direct cytotoxic effects. The NK cell response is immediate and helps control viral spread early in infection [47]. NK cells sense pathogenic or stress signals from target cells with a variety of activating and inhibitory receptors. In order to prevent tissue damage through the cytolytic activity of NK cells upon activation, those innate cells are tightly controlled through a balanced signal input from both receptors types [48]. The differential binding of activating and inhibitory receptors influences cytokine secretion of NK cells [49]. To the group of activating receptors belong NKp46, NKG2D and CD16 (FcγRIII). Inhibitory stimuli are received from NKG2A and 2B4 (CD244) [50]. It was reported that co-ligation of both receptors leads to a dominant inhibitory effect due to the higher affinity of the ligand to inhibitory receptor [48, 51]. Blocking experiments with inhibitory receptors indicate a protective function of those receptors from the natural cytotoxicity of NK cells. The immunoglobulin G (IgG) receptor, CD16, can bind to antibody-coated pathogens and mediates antibody-dependent cellular cytotoxicity (ADCC) [51]. In general, when NK cells are stimulated by Fc portion of antibodies on pathogens through CD16, they produce the pore-forming protein perforin and other apoptosis inducing proteins to kill target cells. In addition, NK cells release IFNγ upon stimulation of CD16 [52]. For triggering NK cell cytotoxicity, antibodies must be
aggregated on cells, while monomeric antibodies have been reported to inhibit NK cell activity [53].

The immediate response of NK cells involves the release of the cytolytic mediators perforin and granzymes [46]. When NK cells sense target cells, they release granules with perforin, which lyses the target cells. Granzyme B and other pro-apoptotic mediators translocate into target cells and induce apoptosis [47]. NK cells can also trigger death receptors on target cells [54, 55]. In addition to cytolytic activity, NK cells recruit cells of the adaptive immune response by producing cytokines such as IFNγ and TNFα [47]. NK cells can also be activated by or in response to pro-inflammatory cytokines predominantly produced by DC and to a lesser extent by macrophages [56]. Notably, type I IFN triggers NK cells activation during viral infection [57].

A novel role for NK cells and other cells of the innate immune systems emerged over the past decade. Innate cells have important immunomodulatory functions. It has been reported, that NK cells regulate DC, macrophages, T and B cells. NK cells kill immature DCs to improve DC homeostasis and activation of anti-viral adaptive immune responses by mature DCs [47]. NK cells kill target cells so that DCs are exposed to greater reservoir of apoptotic cells/debris resulting in faster antigen-presentation. In addition, NK cells can kill overactive macrophages and therefore diminishing immunopathology. NK cells also directly regulate adaptive immune responses by priming Th1 cells with IFNγ or kill activated effector T cells to prevent immunopathology. Autoreactive B cells have been shown to be suppressed by NK cells (Fig 1-1) [47].

Several recent studies have dissected the role of NK cells during both acute and chronic HCV infection. Shoukry et al. has shown that during acute HCV infection in vitro, NK cells are activated irrespective of subsequent virologic outcome toward spontaneous resolution or chronic infection. The peak of NK cell activity either preceded or coincided with a peak in T cell
response. They further demonstrated that degranulation of NK cells correlates with the magnitude of HCV-specific T cell responses [58]. During chronic HCV infection, NK cells remain active and display normal or increased cytotoxicity. NK cells isolated from patients with chronic HCV, express an altered cytokine profile in vitro with higher production of immunosuppressive IL-10 and transforming growth factor (TGF) β that attenuated the adaptive immune response [59].

![Figure 1-1: NK cell functions.](image)

NK cells have regulatory activities. Upon priming by various factors (IL-15, IL-12, IL-18, type I IFN) NK cells produce IFNγ or TNFα to activate macrophages, DCs and T cells (red arrows). Conversely, NK cells can also kill (blue arrows) immature DCs, activated T cells and hyperactive macrophages. A balance of inhibitory and activating receptor stimuli controls NK cell functions. Adapted from Vivier et al. [47].

1.1.3 Role of the Adaptive Immune System in Viral Infections

To recognize a diverse array of antigens, adaptive immune cells undergo a process known as V(D)J recombination that creates a unique immune receptor capable of recognizing a single antigen. Furthermore, cells expressing an antigen receptor that is specific to self-antigens are
deleted. This process creates a diverse repertoire of immune cells capable of recognizing the universe of potential foreign pathogens [60]. The two major components of adaptive immunity are humoral and cell-mediated immunity [33].

1.1.3.1 Humoral Responses are Impaired in Chronic Viral Infection

The major mediators of the humoral immune response are B cells and antibodies. Antigens within the blood or lymphatics can bind to the B cell receptor (BCR) of a naive B cells specific to an antigen. Antigen captured by the BCR causes the aggregation of receptors on the membrane of a naive B cells [61], promoting its migration to a secondary lymphoid organ [62]. Within secondary lymphoid organs, B cells will internalize the antigen and express peptides derived from the antigen in the context of MHCII [62, 63]. A subset of CD4+ T cells called T follicular helper cells (Tfh), can recognize peptides expressed by B cells and help promoting the activation and differentiation of B cells into antibody secreting plasma cells [64]. Secreted antibodies can neutralize viral particles by interfering with the interactions of a virus with its entry receptors. Antibodies also can opsonize pathogens to promote phagocytosis by macrophages. Furthermore, antibodies can also activate the complement pathway leading to a targeted lysis of the infectious pathogen. Memory B cells provide protection from re-challenge with a previously encountered pathogen [65].

Neutralizing antibody responses are impaired during chronic viral infection as titers are low in HBV and HCV patients [66, 67]. Studies in HIV patients have shown that B and plasma cells circulate at lower percentage compared to healthy controls [68]. Antibodies in the sera of HIV infected patients had weak neutralizing activity towards HIV [69].
1.1.3.2 Role of Cell-Mediated Immune Responses in Chronic Viral Infection

The major mediators of cell-mediated immune responses are T lymphocytes. T cells share the common expression of the T cell receptor (TCR). The TCR is a multimeric protein complex that is capable of recognizing antigenic peptides expressed in the context of the major histocompatibility complex [70].

1.1.3.2.1 Robust CD4⁺ Th1 Cell Response Assists in Viral Clearance

Th cells express the co-receptor CD4, which binds to MHCII. There are different subsets of T helper cells including Th1, Th2 or Tfh cells. Th1 cells produce inflammatory cytokines IL-2, IFNγ and TNFα, while Th2 cells produce the anti-inflammatory IL-4, IL-5, IL-6 and IL-10 [71]. Th1 cells help macrophages to phagocytose pathogens and have the capacity to prime CTLs contributing to anti-viral responses [72]. Th2 cells promote IgE and eosinophilic responses in atopy and can inhibit Th1 responses [73]. Tfh cells are inefficient cytokine producing cells but have an active role in B cell helper function [74]. The magnitude of the T cell response is dependent on positive and negative regulatory signals received during differentiation [72].

Depletion or inhibition of virus-specific CD4⁺ Th cells is associated with viral evasion and persistence of viral infection [2, 58, 75]. Recent evidence suggests that viral persistence leads to a Tfh polarization [76]. Additionally, the expression of death receptors, such as PD-1 has been shown to be upregulated on virus-specific CD4⁺ Th cells [77]. In a process known as T cell exhaustion, Brooks et al. demonstrated that CD4⁺ T cells progressively lose the ability to produce inflammatory cytokines such as IFNγ and TNFα [78]. Robust helper T cell response and anti-viral cytotoxic T cell responses are not sustained leading to viral persistence [78, 79].
1.1.3.2.2 Cytotoxic T Cells Eliminate Virus-Infected Cells

Cytotoxic T lymphocytes (CTL) express the TCR and the co-receptor CD8. CD8 recognizes the constant region on MHCI molecules [70]. CTL exhibit antiviral activity through their ability to recognize and lyse virally infected cells [80]. Naive CTL are primed within the thymus to recognize a specific peptide/antigen epitope with their TCR [81]. Upon recognition of their cognate cross-presented antigens in the context of MHCI on mature DCs, CTL will proliferate within secondary lymphoid organs [82]. When the TCR is activated by peptide in the context of MHCI, necessary co-stimulatory signals are provided by CD28 (on T cells) engagement with CD80 (B7-1)/CD86 (B7-2) (on mature DC). Once activated, CTL release anti-viral cytokines such as IFN$\gamma$ and TNF$\alpha$ as well as perforin and granzyme to kill infected cells. Moreover, binding of the FasL protein on activated CTL to the Fas receptor on infected cells has the capacity to induce apoptosis in infected cells [80, 83].

During CTL activation, CTL binds to a target cells leading to the formation of an immunological synapse. For optimal CTL activation, TCRs aggregate on the surface for efficient antigen binding. In this process, inhibitory receptors join the TCR accumulation in order to diminish incoming signals and prevent tissue damage. CTL antigen 4 (CTLA-4) and PD-1 are receptors, which inhibit downstream signaling of the TCR [84]. Subsequently, TCR stimulation leads to upregulation of those inhibitory receptors. In chronic viral infection, PD-1 and other inhibitory receptors are highly expressed on CTL leading to an exhausted state of CD8$^+$ T cells [85].

1.1.3.2.3 Memory T Cells Protect against Re-Infection

Memory T cells are a dynamic population of diverse subpopulations [86]. Following T cell activation, CD4$^+$ and CD8$^+$ T cells undergo proliferation and differentiation. Once the infection
is cleared, T cells enter a contraction or death phase. A small group of T cell clones differentiate into memory CD4\(^+\) and CD8\(^+\) T cells for future protection against the same pathogen [82].

A robust memory T cell response is dependent on the duration of antigen exposure, the level and duration of inflammation, the frequency of virus-specific effector T cells during primary infection, time after resolution of primary infection and the clonal expansion of T cells during re-infection [87-90].

1.1.3.2.4 T Cell Exhaustion

High antigenic load leads to persistent upregulation of the expression of death receptors and ligands on activated T cells and can impair virus specific adaptive immune responses by reducing the pool of anti-viral T cell subsets or generating dysfunctional T cells [91-93]. The impaired state is collectively termed T cell exhaustion and was first described in chronic murine LCMV clone 13 (cl 13) infection, but is known to occur in persistent human infections including HBV, HCV and HIV [94-97]. In chronic viral infection, T cells lose the ability to produce IL-2 followed by TNF\(\alpha\) and IFN\(\gamma\) (Fig 1-2) [98]. Anti-viral CTL also lose their cytolytic ability [99]. As T cells progressively lose the capacity to exert their effector functions and proliferation, T cells specific to the virus can become physically deleted from the repertoire [92].

T cell exhaustion is associated with a persistent expression of PD-1 on CD4\(^+\) and CD8\(^+\) T cells [95]. During persistent viral infection PD-1 can also be upregulated on B cell, NK cells, monocytes and DCs. PD-1 has two ligands, PD-L1 and PD-L2. PD-L1 can be expressed on hematopoietic and non-hematopoietic cells. PD-L2 is exclusively expressed on APCs. During immunological synapse formation, PD-1 is translocated to TCR clusters and induces the dephosphorylation of TCR, diminishing the signal downstream the receptor [84]. However, persistent antigen exposure prolongs demethylation of the PD-1 gene locus leading to persistent
expression [85]. The PD-1/PD-L1 interactions were a landmark finding in T cell exhaustion [26, 92]. PD-L1 expression on hematopoietic cells was shown to induce CD8\(^+\) T cell exhaustion [26]. Blockade of this pathway restored T cell activity. More recently, it has been shown that in vitro blockade of PD-1 restored HIV, HCV and HBV specific T cell responses [100-103]. Petrovas et al. demonstrated that PD-1 expressing CTLs are more sensitive to apoptosis than non-PD-1 expressing cells [104]. The authors further showed that the sensitivity is dependent on the expression level of PD-1 indicating an important survival defect in chronic viral infection [94, 104]. Other markers of T cell exhaustion that have been reported during chronic infection include T-cell immunoglobulin domain and mucin domain 3 (Tim-3) and lymphocyte activation gene 3 (LAG-3) [105-107].

Previous studies revealed that the effects of T cell exhaustion are reversible and more importantly responsive to treatment. Strategies to promote virus-specific T cell responses have been shown to enhance viral clearance in experimental and human studies and it appears to be a promising approach to improve the treatment of patients with chronic viral infection [108, 109].

![Figure 1-2: Markers for T cell exhaustion.](image)

During chronic viral infection, T cells undergo phenotypic and functional changes. Characteristic for T cell exhaustion are upregulated expression of inhibitory PD-1, Tim-3 and LAG-3 (grey) and loss of anti-viral mediators over time such as IL-2, TNF\(\alpha\), IFN\(\gamma\) and Granzyme B (blue). Adapted from Yi et al. [110].
1.1.3.3 Regulatory T Cells Suppress Anti-Viral Immune Responses

Tregs are a subset of T cells that are recruited to sites of inflammation to reduce the magnitude of the immune response and to prevent immunopathological injury [111]. Tregs express the CD3 marker. Another identification marker for a special subtype of CD4⁺CD25⁺ Tregs is the transcription factor forkhead box P3 (Foxp3) [112]. A number of different Tregs subsets have been reported, including regulatory T cell type 1, CD8⁺ regulatory T cells, double negative (DN) T cells, γδ T cells, NKT cells and CD4⁺CD25⁺Foxp3⁺ regulatory T cells [113]. Tregs control the activity of T cells by producing inhibitory cytokines (IL-10, TGFβ and IL-35) or by metabolic disruption. They can inhibit DC maturation through binding of CTLA-4 to CD80/CD86 complex subsequently leading to inhibition of T cell activity [114]. CTLA-4 expression in Tregs was also shown to control CD4⁺ Th cell expansion [115]. Furthermore, Tregs secrete cytolytic granzyme A or granzyme B to induce cellular lysis [114]. Along with the markers IL-10 and CTLA-4, Fontenot et al. found fibrinogen-like protein 2 (FGL2) as another potential immune-suppressor gene to be highly transcribed in CD4⁺CD25⁺Foxp3⁺ Tregs [116].

Previous studies revealed that depletion or reduction of Treg numbers leads to enhanced immune responses against various infectious pathogens. Increased numbers of Tregs were present in patients with chronic HBV, HCV and HIV infection when compared with successfully treated and/or healthy controls [21, 103, 117].
1.2 Fibrinogen-Like Protein 2

1.2.1 Structure of FGL2

FGL2 was firstly identified from a murine cytotoxic T cell library by Koyama et al. 1987. FGL2 is a member of the fibrinogen superfamily due to its homology (36%) with fibrinogen β and γ chains (musfiblp) [118]. Ruegg and Marazzi et al. identified the human homologue of the FGL2 by screening a human small intestine complementary deoxyribonucleic acid (cDNA) library [119]. They referred to the human protein as fibroleukin/FGL2. The Fgl2 gene, which has been localized to chromosome 7 in humans and 5 in mice, is composed of two exons that are separated by one intron. The Fgl2 gene encodes a protein of 432 amino acids in mice and 439 amino acids in humans. Based on sequence and structural analysis, it is predicted that the encoded protein is composed of two major domains, the N-terminal domain and the carboxyl-terminus, existing as a tetrameric complex (Fig 1-3). The monomeric form of FGL2 has the size of approximately 64 kDA. The N-terminal domain is proposed to have a rigid or tubular conformation made of hydrophobic α-helicals and several conserved cysteine residues, which can promote a coiled-coil formation [120]. The 229-amino-acid-long carboxyl-terminus consists of a highly conserved globular domain, known as the fibrinogen-related domain (FRED) that is characteristic of the fibrinogen-related protein superfamily. The overall identity between the mouse and human FGL2 is 78%, but within the FRED domain the two proteins are identical up to 90% of the amino acid sequence. Yuwaraj et al. showed that the FGL2 mRNA exist in two different mRNA transcripts in the cytosol, 1.5 k and 5.0 kb long. Both FGL2 transcripts are broadly expressed to varying degrees in almost all organs [121]. The FGL2 protein contains a signal peptide which is necessary for the transport through the cell membrane [118].
Figure 1-3: Structure of FGL2.

A) shows the monomeric and B) the oligomeric structure of FGL2. The FGL2 protein contains a globular FRED domain at the carboxyl terminal region and a hydrophobic α-helical region at the amino terminus. The hydrophobic region (3-23) is a potential type II transmembrane domain for cell membrane attachment. Several conserved cysteine residues and five N-linked glycosylation sites are found throughout the molecule. Disulfide bonds can lead to oligomeric structures of FGL2. Adapted from Liu et al. [122].

According to Marazzi et al. the FGL2 protein is constitutively secreted by both CD4⁺ and CD8⁺ T cells in vitro. FGL2 mRNA transcripts were found in T cells, with higher expression in CD45R0⁺ memory T cells. When stimulated in vitro with IFNγ, IL-2 or TNFα, FGL2 mRNA transcription was induced and protein secretion was prolonged in cells including T cells and in perinatal macrophages in a dose-dependent manner. Furthermore, co-immunoprecipitation studies demonstrated that cells secrete FGL2 [123].
1.2.2 Prothrombinase Activity of Membrane-Associated FGL2

FGL2 can be expressed as a membrane-associated protein with prothrombinase activity capable of cleaving prothrombin to thrombin on cells including macrophages, vascular endothelial cells, fetal trophoblast and a subset of decidual stromal cells (Table 1-1) [120, 124-126]. This activity is only found in the amino-terminus of the protein. Site-directed mutagenesis was performed to demonstrate that serine 89 residue is essential for the coagulation activity of FGL2. A recent report has suggested that downregulation of FGL2 prothrombinase delays HCCLM6 xenograft tumor growth and decreases tumor angiogenesis indicating a role for FGL2 in liver tumor progression [127]. In addition, FGL2 was found to play a role in spontaneous abortion [124] and the pathogenesis in murine hepatitis virus-3 (MHV-3) induced fulminant hepatitis. Post MHV-3 infection, mRNA transcripts and protein levels of FGL2 were elevated in susceptible BALB/C and C57BL/6 mice, which was associated with fibrin deposition leading to ischemic hepatocellular necrosis [120].

Table 1-1: The comparison between membrane-associated and secreted FGL2.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Membrane associated</th>
<th>Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Macrophages</td>
<td>Tregs</td>
</tr>
<tr>
<td></td>
<td>Endothelial cells</td>
<td>T cells</td>
</tr>
<tr>
<td></td>
<td>Fetal trophoblast</td>
<td></td>
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<tr>
<td></td>
<td>Decidual stromal cells</td>
<td></td>
</tr>
<tr>
<td>Function</td>
<td>Prothrombinase activity</td>
<td>Inhibits maturation of DCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces B cell apoptosis</td>
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<tr>
<td></td>
<td></td>
<td>Inhibits T cell proliferation</td>
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</tbody>
</table>
1.2.3 Immunosuppressive Activity of Secreted FGL2

Secreted FGL2 has immunosuppressive properties (Table 1-1). FGL2 inhibits maturation of marrow-derived DCs suppressing T cell proliferation [128]. FGL2 also induces B cell apoptosis indicating an inhibitory effect on humoral responses [127].

Our laboratory demonstrated that FGL2 is an effector molecule of CD4⁺CD25⁺ Tregs, as the Fgl2 mRNA level is 6-fold higher in CD4⁺CD25⁺ Tregs compared with CD4⁺CD25⁻ cells. Targeted deletion of Fgl2 leads to impaired Treg activity, associated with enhanced reactivity of DC, T and B cells and manifestation of autoimmune glomerulonephritis [129]. FGL2 has an important role in the subpopulation of CD4⁺CD25⁺Foxp3⁺ Tregs. Tregs lose their suppressive function after Foxp3 deletion. Following Foxp3 deletion, Tregs also downregulated several other suppressive effector genes including Fgl2. The C-terminal globular portion of secreted FGL2 accounts for the suppressive activity of FGL2 [128].

1.2.4 FGL2 Receptors - FcγRIIB/RIII

Our laboratory found that FGL2 binds to the inhibitory receptor FcγRIIB (CD32) and the activating receptor FcγRIII (CD16) [127]. FcγR bind to the Fc portion of antibodies produced by plasma cells, and have profound effects on innate immunity and adaptive immunity. When FcγR become activated, it can induce the engulfment of antibody-coated pathogens (by DCs and macrophages) or killing of pathogens (by NK cells). Amongst the FcγR, the receptors FcγRIIB/RIII are low affinity receptors to antibodies. FcγRIIB has an immunoreceptor tyrosine-based inhibition motif (ITIM) that inhibits signals.

Macrophages and DCs express both FGL2 receptors. Previously, our laboratory reported that when the FcγRIIB is deleted in mice, FGL2 does not affect macrophages from FcγRIIB⁻/⁻ mice
suggesting that FcγRIIB is the dominant receptor for FGL2 on macrophages. FcγRIIB is a strong inhibitory receptor on DCs and macrophages. B cells express exclusively the inhibitory FcγRIIB [127]. NK cells express only the activating FcγRIII. Engagement of FcγRIII by antibody complexes activates cytokine release and cytotoxicity of NK cells [130]. Binding of FGL2 to NK cells has not yet been demonstrated. Based on the information about FcγRIII, FGL2 has the potential to activate NK cells through FcγRIII.

1.2.5 Role of FGL2 in Viral Pathogenesis

The regulatory activity of FGL2 has been implicated in inhibition of allograft rejection, in cancer and autoimmunity, and the pathogenesis of experimental and human viral infections including in patients with HIV, severe acute respiratory syndrome (SARS) and chronic hepatitis [129, 131, 132].

Our laboratory showed that patients with chronic HBV and HCV infection have increased plasma levels of secreted FGL2 as well as increased frequencies of Tregs expressing FGL2 [133-135]. Elevated expression of FGL2 predicted susceptibility and severity of disease in human hepatitis. The prothrombinase activity of FGL2 has been implicated to support liver cell necrosis in a murine model of fulminant hepatitis using MHV-3. However, using the MHV-3 model adaptive immune responses were not studied because susceptible mice died before adaptive immunity was generated [125]. A different virus model is necessary to study effects of FGL2 on adaptive immune responses.
1.3 Lymphocytic Choriomeningitis Virus (LCMV)

1.3.1 LCMV - a Model for Viral Pathogenesis

Lymphocytic Choriomeningitis Virus (LCMV) is widely used to understand the pathogenesis and biology of viral infection. Historically, the LCMV model has been used to discover insights into negative regulation of the immune response and T cell exhaustion in chronic infection [136]. Zinkernagel et al. used LCMV to define MHC restriction in transplantation [137].

LCMV is a natural pathogen of rodents such as, mice and hamster [136]. LCMV belongs to the family of Arenaviridae. LCMV is an enveloped RNA virus, whose genomic material consists of two RNA segments, a large (L) segment (7 kb) and a small (S) segment (3.4 kb) (Fig 1-4) [138]. The L-segment encodes the RNA polymerase, while the S-segment contains the information for the nucleocapsid protein (NP) and glycoproteins (GP-1 and GP-2). LCMV binds at a high affinity to the ubiquitously expressed glycoprotein alpha-dystroglycan (α-DG), whose ligand is alpha-2-laminin [139, 140].

![Figure 1-4: Structure of an Arenavirus.](image)

LCMV is an enveloped RNA virus in which the genomic material consists of two RNA polymerase segments, a large (L) segment (7 kb) and a small (S) segment (3.4 kb). On the L-segment the RNA-polymerase is encoded whereas the S-segment contains the information for the nucleocapsid protein (NP) and glycoproteins (G1 and G2). Adapted from Barthold and Smith et al. [141].
LCMV can cause an acute, chronic or protracted disease depending on inoculum viral load, route of infection, age, immunocompetence and genetic background of the host and the strain of the virus. In mice infected in utero or shortly after birth, the LCMV persists due to infection of the thymus and mice develop tolerance towards the virus. Re-infection studies revealed that mice that clear LCMV are protected from re-infection by this virus [142].

The LCMV Armstrong strain was the first LCMV strain isolated from the cerebral spinal fluid of a female patient exhibiting signs of viral encephalitis [143, 144]. Immunocompetent adult mice can clear LCMV Armstrong within 8 days. Another widely used strain of LCMV that causes acute infection is LCMV WE [145]. This clone was isolated from a patient who suffered from virus meningitis and was further propagated in laboratory mice [146]. To study chronic viral infection, LCMV cl 13 is widely used. LCMV cl 13 was isolated from the spleen of mice infected with LCMV Armstrong at birth, and persists in mice up to 120 days (Fig 1-5) [97].

**Figure 1-5: CTL responses to acute and persistent LCMV infection over time.**

In response to acute infection (LCMV Armstrong, LCMV WE-blue line), CD8+ T cells rapidly proliferate until the infection is cleared. Afterwards, CD8+ T cell numbers decline and some cells become memory cells. During chronic infection (LCMV cl 13-red line) initially a normal CD8+ T cell responses is induced, however those cells (dotted red line) lose quickly effector functions and undergo apoptosis. Only a small subset of CD8+ T cells remains, which is unable to resolve the infection (red solid line). Adapted from Pritzl et al. [4]
1.3.2 LCMV WE Induces Acute Infection

In immunocompetent mice LCMV WE is cleared after 12 day post-infection (pi) and used as a model to study the pathogenesis of acute viral hepatitis due to considerable liver injury that occurs by day 8 pi (Fig 1-5) [145].

Our laboratory has studied the immunosuppressive and prothrombinase effects of FGL2 during acute viral infection caused by LCMV WE [147]. We analyzed innate, cell-mediated and humoral anti-viral immune responses. It was observed that plasma FGL2 levels were elevated in wild-type C57BL/6 mice and that FGL2 levels remained elevated until day 42 pi. Following day 1 and day 2 pi, deletion of Fgl2 resulted in enhanced activation of splenic DCs, as indicated by enhanced expression of maturation markers CD80 and MHCII. Elevated levels of plasma IFNα were observed in Fgl2−/− when compared to Fgl2+/+ mice [147]. Furthermore, Fgl2−/− mice produced greater numbers of CD138+CD19lowCD45Rlow plasma cells by day 8 pi with increased total and neutralizing antibody titers specific to LCMV WE at all time-points measured [147]. Analysis of cell-mediated responses showed significantly greater frequencies of CD4+ and CD8+ T cells by day 8 pi. Coincidently, we found using viral titre kinetic analysis that virus elimination was increased in livers of Fgl2−/− mice when compared to Fgl2+/+ mice suggesting improved virus clearance upon deletion of Fgl2 [147].

1.3.3 LCMV cl 13 Induces Chronic Infection

LCMV cl 13 infected mice develop a chronic viral infection, which has served as a model to study the pathogenesis of chronic viral infections including HBV, HCV and HIV. LCMV cl 13 is a variant of LCMV Armstrong, therefore the RNA genome is similar but biological properties are different [139]. The LCMV clone 13 strain can be distinguished from LCMV Armstrong by two different mutations. A change of coding occurs in the RNA polymerase (K1079Q) and in
GP-1 (F260L) [148], which was identified as the α-DG receptor binding protein. The mutation in the polymerase induces higher levels of viral replication in their primary hosts, the DCs and macrophages, and is responsible for the early viremia and increased antigenic stimulation in the host [40]. In addition, due to the high expression level of α-DG on DCs and macrophages [91, 149], LCMV cl 13 inhibits the ability of the cells to provide co-stimulatory signals to activate anti-viral adaptive responses. Infected DCs are also target of CTL activity leading to a further loss of DCs leading to persistent infection [139].

LCMV cl 13 infects all organs to different degrees. Mice clear virus fastest in liver, plasma and spleen fastest (4-6 weeks) followed by lungs, brain and heart (~8-10 weeks). LCMV cl 13 persists the longest in the kidney (~120 days) [98].

LCMV cl 13 uses different mechanisms to maintain viral persistence (listed in Table 1-2). LCMV cl 13 affects innate immune responses by inhibiting DC development and maturation [25]. LCMV also disrupts secondary lymphoid follicles in infected mice. Lymphoid architecture is important for induction and maintenance of immune responses [148]. By day 8-12 pi, CD4+ and CD8+ virus-specific T cells begin to progressively lose effector function [150]. Using the LCMV cl 13 model, Brooks et al. showed that the inhibitory cytokine IL-10 produced by DCs also contributes to T cell exhaustion [151]. Furthermore, the polarization of T cells is skewed from a protective antiviral Th1 response to a non-protective Th2 response during LCMV cl 13 infection [76].

Studying LCMV cl 13 infection as a model for chronic viral infection in humans has advantages compared to humanized mouse models. LCMV is not cytolytic in vivo, which allows for a distinct separation of pathological effects caused by the host’s immune system and the virus. Furthermore, immunocompetent adult mice infected with LCMV generate a profound immune response that eliminates the virus [152, 153]. Due to the long extensive research on
LCMV, the characterization of the frequencies and functionality of virus-specific T cells through the use of tetramers (MHCI-peptide complexes) and peptides analysis are well defined.

Table 1-2: Main immunological effects causing persistent LCMV cl 13 infection.
Adapted from Pritzl et al. [4].

<table>
<thead>
<tr>
<th>System Targeted</th>
<th>Disruption of Cellular Function</th>
<th>Phenotype/Mechanism</th>
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<tbody>
<tr>
<td>Dendritic cells</td>
<td>Inhibition of DC development</td>
<td>Decrease in the frequency of CD11c⁺ cells</td>
</tr>
<tr>
<td></td>
<td>Inhibition of DC maturation</td>
<td>MHCI/II and B7-1/B7-2 upregulation impaired</td>
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<tr>
<td></td>
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<td>Increased IL-10 synthesis</td>
</tr>
<tr>
<td>T cells</td>
<td>Suppression of T cells function</td>
<td>PD-1↑, LAG-3↑, Tim-3↑</td>
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<tr>
<td></td>
<td>(exhaustion)</td>
<td>IFNγ↓, TNFα↓, IL-2↓</td>
</tr>
<tr>
<td></td>
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<td>Proliferation ↓</td>
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<td>Cytotoxic activity ↓</td>
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1.4 Hypothesis and Aims

Our laboratory has previously reported the immunesuppressive effects of FGL2. FGL2 has been shown to induce B cells apoptosis, inhibit DC maturation and T cell proliferation [127, 128] (Fig 1-6). Targeted deletion of FGL2 resulted in improved survival of susceptible mice in a model of fulminant hepatitis primarily through its effects on innate immunity [133]. In order to determine whether targeting FGL2 improves adaptive and innate anti-viral immune responses in a non-lethal model, the LCMV model was utilized in this study.

Figure 1-6: Proposed model of FGL2 inducing chronic viral infection.

FGL2 is an immunosuppressive effector molecule and binds to FcγRIIB receptor on B cells and DCs. Binding of the molecule to the receptor can inhibit DC maturation and induce B cell apoptosis. Engagement of FGL2 to FcγRIII on NK cells will lead to cytotoxic activation of NK cells, which in turn kill activated T cells. Due to inhibition of innate and adaptive immune responses, the virus LCMV cl 13 will persist in mice.
Hypothesis:

I proposed that LCMV utilizes the FGL2-FcγRIIB/RIII pathways to suppress anti-viral immune responses (Fig 1-6). I hypothesized that deletion of Fgl2 will restore effective virus-specific immunity and improve viral clearance in mice. Using the acute infection model LCMV WE, I analyzed generation of memory CD8$^+$ T cell responses in Fgl2 deficient mice during re-infection. LCMV cl 13 was utilized to study effects of FGL2 on innate and adaptive immunity, and viral clearance in chronic viral infection.

My aims were to:

1) Analyze whether targeted deletion of Fgl2 enhanced protective T cell responses during re-infection in a murine acute LCMV WE infection model.

2) Analyze whether targeted deletion of Fgl2 improved viral clearance in mice infected with LCMV cl 13, a murine model of chronic viral infection.

3) Characterize innate and adaptive immune responses in wild-type (Fgl2$^{+/+}$) and Fgl2$^{-/-}$ mice following LCMV cl 13 infection.
2 Materials and Methods

2.1 Cell Culture Reagents

Dulbecco's Modified Eagle Medium (DMEM), α-Minimum Essential Media (α-MEM), Roswell Park Memorial Institute (RPMI) media, Phosphate Buffered Saline (PBS), Hanks Balanced Salt Solution (HBSS) and Trypsin-Ethylenediaminetetraacetic Acid (EDTA) were purchased from Multicell, USA. Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (Pen/Strep) were obtained from Thermo Fisher Scientific, USA, and L-Glutamine and 2-mercaptoethanol were purchased from Gibco Life Technologies, USA.

2.2 Mice and Infections

C57BL/6 mice were purchased from Jackson Laboratory (USA). Fgl2−/− mice were generated as previously described [154]. Briefly, homologous recombination was applied to inactivate Fgl2 in 129Sv/J embryonic stem cells. Appropriately targeted ES cells clones were injected into C57BL/6 blastocysts and implanted into pseudo pregnant females. Chimeric mice were mated with C57BL/6 mice and progeny was subsequently maintained on a C57BL/6 background.

Mice were housed and maintained in micro-insulator cages and housed under specific pathogen free (SPF) conditions in the animal facility at the Ontario Cancer Institute, Toronto. Standard lab chow and water was provided ad libitum.

For infections, eight- to ten-weeks-old weight-matched littermate female mice received 2x10^6 PFU of LCMV cl 13 or 2x10^6 PFU of LCMV WE intravenously. Animal protocols were approved by the University Health Network in accordance with guidelines set by the Canadian Council on Animal Care.
2.3 Blood and Tissue Collection

Blood samples were obtained by heart puncture or saphenous venipuncture and collected into heparinized Microvettes (Sarstedt, Germany), which contain heparin and lithium to prevent blood coagulation. Blood was centrifuged and plasma was collected. Organ samples were snap frozen in liquid nitrogen, and weighed. Tissue samples were homogenized in 5 %/α-MEM complete media using the TissueLyser LT system (Qiagen, Netherlands). Homogenized organ samples were centrifuged to remove cellular debris and supernatants were collected.

2.4 Preparation of LCMV

LCMV cl 13 was obtained as a gift from laboratory of Dr. M. Oldstone (The Scripps Research Institute, USA) and LCMV WE and L929 cells were provided by Dr. Pamela Ohashi (Ontario Cancer Institute, Canada). LCMV cl 13 was propagated in BHK-21 cells (ATCC, USA catalogue # CCL-10), while LCMV WE was propagated in L929 cells. Briefly, cells were grown to 80 % confluence in 10 % FBS/α-MEM supplemented with 1xL-glutamine, 100 U/mL Pen and 100 µg/mL Strep and infected at a multiplicity of infection (MOI) of 0.01 with LCMV cl 13 or LCMV WE. Cells were incubated with the inoculum (1/5 of flask) for 1 h at 37 °C. Fresh media was added and infected cells were incubated at 37 °C. Supernatants were collected at 48 h pi, centrifuged to remove cellular debris and stored at -80 °C. Adapted from Current Protocols in Immunology “Preparation of LCMV stocks” [155].

2.5 LCMV cl 13 Purification by CsCl Gradient Centrifugation

100 mL of thawed LCMV cl 13 supernatant was mixed with 7 g polyethylene glycol (PEG, Sigma-Aldrich, Germany) and 2.32 g NaCl (Bio-Shop Canada Inc., Canada) and stirred until
both dissolved. The supernatant was kept at 4 ºC for 2 h to precipitate total protein content. Precipitated proteins were centrifuged for 30 min at 10000 rpm and 4 ºC. The protein pellet (of 100 mL supernatant) was resuspended in 2 mL of Tris buffer (pH 8.1). Ethidium bromide was added to a final concentration of 4 µg/mL to visualize viral particles. Two different CsCl (Sigma-Aldrich) gradient solutions were prepared, 1.45g/mL (heavy gradient) and 1.2 g/mL (light gradient) in double-distilled water (ddH₂O). A CsCl gradient was prepared by slowly adding first the heavy gradient in a tube and then the light gradient followed by the protein sample. Proteins were separated according to their density by ultracentrifuge (Beckman Coulter, USA) using the following settings: speed 27000 rpm, time 3h, temperature 4 ºC. The virus band was made visible by UV-light and was collected using a syringe. Virus sample was diluted 1:2 with the Tris buffer (Fisher Scientific). A second tube with CsCl gradient was prepared as previously described and centrifuged at 26000 rpm for 16 h at 4 ºC. UV light was used to visualize the virus band and was collected using a syringe. LCMV cl 13 was inactivated by UV-light exposure for 1 h. Purified LCMV cl 13 was stored at -80 ºC. This protocol was adapted from Current Protocols in Immunology “Preparation of LCMV stocks“ [155, 156].

2.6 Preparation of α-VL4

The rat hybridoma cell line VL-4 was a gift from Dr. Pamela Ohashi (Ontario Cancer Institute, Canada). This cell line was cultured in 10 % FBS/RPMI supplemented with 1xL-Glutamin, 100 U/mL Pen and 100 µg/mL Strep. The monoclonal α-VL4 (IgG2a) was collected from culture supernatant (3 days culture) and passed through a 0.2 micron filter (Pall Life Sciences, USA) [157].
2.7 Focus Forming Assay

The LCMV focus forming assay was performed as previously described [157].

*Preparation of MC57 cells.* MC57 cells (mouse fibrosarcoma, # CRL-2295) were cultured in 5 % FBS/α-MEM supplemented with 1xL-Glutamine and 100 U/mL Pen and 100 µg/mL Strep (complete media). MC57 cell were prepared by washing with PBS and trypsinized with Trypsin-EDTA solution for 5 min at 37 °C. Cells were resuspended in 5 %/α-MEM complete media to 10^6 cells/mL.

*Preparation of LCMV cl 13 containing samples.* A 10-fold dilution series was created with LCMV cl 13 containing samples. 200 µl of diluted sample was used to infect 200,000 MC57 cells in 24 well plates. Infected cells were incubated for 4 h at 37 °C. Subsequently an overlay containing 1 % methylcellulose (Sigma-Aldrich, Germany)/5% FBS/DMEM complete media was used to prevent viral spread. Plates were incubated for 48 h at 37 °C to facilitate viral growth and the formation of distinct plaques.

*Detection of plaques.* Supernatant was removed and LCMV cl 13 infected cells were fixed with 4 % Formalin (Thermo Fisher Scientific) /PBS for 30 min. Plates were washed 2x in PBS and 1 % Triton X-100 (Sigma-Aldrich)/HANKS solution was added for 20 min to permeabilize cells. Plates were washed again 2x with PBS and 10 % FBS/PBS was used as blocking solution for 1 h. LCMV nucleoprotein was detected by staining cells for 1 hour using a rat α-LCMV nucleoprotein (α-VL4). Plates were washed 2x with PBS and plaques were detected by staining cells with a goat α-rat IgG/horseradish peroxidase (HRP) (Jackson Immuno Research, USA) diluted at 1:400 for 1 h in the dark. Plates were washed twice with PBS and plaques were developed using the detection substrate (25 mL H₂O, 12.5 mL 0.1 M citric acid, 12.5 mL Na₂HPO₄, 20 mg o-phenylenediamine (Sigma-Aldrich) and 30 µL of 30 % H₂O₂ (Sigma-Aldrich)), resulting in the formation of distinct plaques. The reaction was terminated by washing the plates 5x with ddH₂O.
2.8 Measurement of Plasma FGL2

FGL2 was measured in plasma using LEGEND MAX™ Mouse FGL2 ELISA Kit with Pre-coated Plates (Biolegend, USA) according to manufacturer’s manual.

2.9 Measurement of Plasma Alanine Transaminase (ALT)

Plasma ALT was measured by transferring 10 µL of plasma onto Vitros II ALT slides. Slides were analyzed using the Vitros DT60 II Chemistry System (Ortho Clinical Diagnostics, Germany).

2.10 Detection of α-LCMV Antibodies by ELISA

Certified high-binding 96 well microplates (Corning Inc., USA) were coated with 10 ng of CsCl purified LCMV cl 13 overnight at 4 °C. Plates were washed 3x with Tris buffered saline (TBS)/0.05 % Tween-20 (TBST) (BIO-RAD, USA). Subsequently, SuperBlock (Thermo Fisher Scientific) prepared in TBST was added to the coated wells for 1 h at 37 ºC. Plates were washed 3 times with TBST. Plasma samples were diluted (1:50, 1:100, 1:500, 1:1000, 1:2000) in Superblock solution. Plasma samples were added to the antigen-coated plates for 1 h at 37 ºC. Plates were washed 4x with TBST and a 1:2000 diluted goat α-mouse IgG/HRP (Gibco Life Technologies, USA) was used to detect LCMV specific antibodies in plasma. After 1 h incubation of secondary antibody at 37 ºC in dark, plate was washed 5x with TBST. 3,3’,5,5’-tetramethylbenzidine (TMB, Sigma-Aldrich) was used as a detection substrate, resulting in a colorimetric reaction whose reaction kinetics correlate with the amount of captured total LCMV specific antibody within plasma samples. The colorimetric reaction was halted after 5 min with 2 M H₂SO₄. Absorbance was measured at 456 nm using microplate reader. This protocol was
adapted from Current Protocols in Immunology “Detection of anti-LCMV antibodies by ELISA” [155, 158].

2.11 Neutralizing Antibody Detection

LCMV neutralizing antibody titres were quantified in plasma samples from LCMV cl 13 infected mice using the plaque reduction assay as previously described [159]. In brief, plasma was diluted 1:10 in complete 2 % FBS/α-MEM media. To examine the ability for plasma samples to neutralize virus, plasma was serially diluted and mixed with 100 PFU of LCMV cl 13 for 1 h at 37 °C. Following neutralization, the samples were added to MC57 cells and plaques were visualized using a standard focus forming assay [157].

2.12 Histology

LCMV cl 13 infected tissues (liver, kidney, lung) were immersed in 10 % formalin for 48 h. Formalin fixed tissues were submitted to the Pathology Core facility at Toronto General Hospital, Canada. Tissue was embedded in paraffin, cut into 5 μm thick sections and stained with hematoxylin & eosin (H&E). Two independent liver pathologists (Dr. M. James Phillips (Sickkids, Toronto, Canada) and Dr. Oyedele Adeyi (Toronto General Hospital, Canada)) evaluated liver histology. Liver histology was scored according to Histological Activity Index (HAI) [160] and representative liver histology pictures are presented (200x magnification, Leica DFC320, Leica Mircosystems, Germany).

2.13 Isolation of Splenic Mononuclear Cells (SMNC)

Spleens were removed from anesthetized mice and filtered through a 40 micron nylon mesh (BD, USA). SMNC were washed and red cells were removed with a red cell lysis buffer (0.15 M
NH₄Cl, 0.01 M KHCO₃, 0.125 mM EDTA, Sigma-Aldrich). Red cell lysis was stopped by adding complete 10 % FBS/RPMI media (supplemented with 1xL-Glutamin, 100 U/mL Pen/100 µg/mL Strep and 1:25000 2-mercaptoethanol). SMNC were washed two times with PBS and resuspended in complete 10 % FBS/RPMI media. This protocol is adapted from Current Protocols in Immunology “Isolation of Mouse Mononuclear Cells” [161].

2.14 Isolation of DCs and Macrophages from Spleen

Isolated spleens were incubated in HBSS on ice. 1 mL of 1 mg/mL collagenase D (Roche Applied Sciences, Germany) in HBSS +Ca²⁺Mg²⁺ was injected into each spleen using a syringe. Spleens were minced and washed with 3 mL of 1 mg/mL collagenase D/HBSS+Ca²⁺Mg²⁺. Media containing cells was collected and incubated at 4 ºC. Splenic debris was resuspended in 2.5 mg/mL collagenase D/HBSS+Ca²⁺Mg²⁺ and incubated at 37 ºC for 30 min. This protocol was adapted from Current Protocols in Immunology “Isolation of Dendritic Cells” [162].

2.15 Analysis of T Cell Response to LCMV cl 13

Splenic mononuclear cells were isolated and stimulated with 5 µg/mL of the MHC class I peptides GP₃₃-₄₁ and NP₃₉₆-₄₀₄ and 2 µg/mL of the MHC class II peptide GP₆₁-₈₀ for 6 h. The LCMV peptides GP₃₃-₄₁ H-2Dᵇ (KAVYNFATC), NP₃₉₆-₄₀₄ H-2Dᵇ (FQPQNGQFI), GP₆₁-₈₀ I-Aᵇ (GLNGPDIYKGVYQFKSVEFD) were synthesized by Anaspec Inc. (USA). The protein transport inhibitor Brefeldin A (BFA, Sigma-Aldrich) was added to cultures after 1 h of peptide stimulation for 5 h at the final concentration of 10 µg/mL. Samples were further processed by flow cytometry. This protocol was adapted from Current Protocols in Immunology “Measurement of T cell responses” [155].
2.16 Biotinylation of Recombinant His-FGL2

Recombinant His (6xHis) tagged FGL2 (1-23 truncated) was produced in Chinese hamster ovary (CHO) cells as previously described [122]. Mouse serum albumin (Sigma-Aldrich) and FGL2 were biotinylated with 40-fold molar excess of biotin (EZ-Link NHS-Biotin, Thermo Fisher Scientific) according to manufacturer’s manual. Excess biotin was removed by dialysis using the Slide-A-Lyser 10 K MWCO dialysis cassettes (Thermo Fisher Scientific).

2.17 Flow Cytometry

2.17.1 Antibodies and Reagents

Detection mouse antibodies (name and dilution): fluorescein isothiocyanate (FITC)-CD4 (1:400), FITC-CD45R (1:300), FITC-CD86 (1:300), FITC-CD244 (2B4) (1:300), FITC-IFNγ (1:100), phycoerythrin (PE)-CD19 (1:300), PE-NK1.1 (1:300), PE-IL-10 (1:100), PE-MHC class II (1:300), peridinin-chlorophyll proteins – cyanine dye 5.5 (PerCP-Cy5.5)-CD3 (1:300), PerCP-Cy5.5-CD11b (1:300), PerCP-Cy5.5-TNFα (1:100), allophycocyanin (APC)-CD4 (1:400), APC-CD8α (1:400), APC-CD80 (1:300), APC-CD138 (1:300), APC-F4/80 (1:300), APC-NKG2D (1:300), APC-PD-L1 (1:300), APC-Perforin (1:100), PE-Cy7-CD3 (1:200), PE-Cy7-CD11c (1:300), PE-Cy7-NK1.1 (1:300) and PE-Cy7-PD-1 (1:300). Fixable viability dye eFluor 450 (eBioscience, USA) was used 1:1000 diluted as the viability dye. All antibodies and reagents were purchased from Biolegend (USA) except where indicated.

Biotinylated MHC class I monomers (GP33, NP396) were provided by the NIH Tetramer Core Facility, Emory University (Atlanta, USA) using the synthesized GP_{33-41} and NP_{396-404} peptides. MHC class I monomers were tetramerized with streptavidin-PE according to NIH Tetramer Core Facility instructions.
2.17.2 Cell Staining

Splenic mononuclear cells were isolated and blocked for 20 min with 5% mouse serum (Cedarlane Laboratories, Canada)/PBS at 4 ºC. Cells were surface stained with mouse antibody master mixtures containing both antibodies and MHC class I tetramer for 30 min at 4 ºC in the dark. Dead cells were stained with the fixable viability dye eFluor 450 for 30 min at 4 ºC in dark. Cells were subsequently fixed with 2% paraformaldehyde/PBS solution (Santa Cruz Biotechnology, USA) for 20 min in dark. Intracellular staining was accomplished by using 1% saponin (USB Corporation, USA)/FACS buffer (PBS supplemented 1% FBS and 5 mM EDTA, Ambion Life Technologies, USA) solution to permeabilize cells. Intracellular antibodies were diluted in the saponin solution and added to cells for 30 min at 4 ºC in dark. Cells were subsequently washed twice with FACS buffer and analyzed by flow cytometry [163].

2.17.3 Data Analysis

BD LSRII flow cytometer (BD bioscience, USA) was used for analysis. Data were analyzed using FlowJo software version 8.8.6 (Tree Star Inc, USA). The singlet gate was first set up using the side scatter height (SSC-H) and side scatter width (SSC-W). Leukocytes were gated using the forwards scatter area (FSC-A) and SSC-A. Viable cells were gated based on the eFluor 450 negative populations when appropriate. Different gating strategies using fluorescence minus one (FMO) controls were applied to select specific cell populations.

2.18 Statistics

Unless otherwise specified, statistical significance of studies was assessed using the Analysis of Variance (ANOVA) test. Statistical analysis was performed using Prism 5 software (Graphpad Software Inc., USA). Differences with $P \leq 0.05$ were considered statistically significant.
3 Results

3.1 Targeted Deletion of Fgl2 Enhances Anti-Viral Memory CD8^+ T Cell Responses

To investigate the role of targeted deletion of Fgl2 on the induction of a robust and protective anti-viral immune response, C57BL/6 wild-type (Fgl2^+/+) and Fgl2^-/- mice were infected with 2x10^6 PFU of LCMV WE, a strain of LCMV that causes a self-limited acute viral infection. On day 8 pi that Fgl2 deficient mice had greater frequencies of CD3^+CD4^+ and CD3^+CD8^+ T cells producing the inflammatory cytokine IFNγ upon \textit{ex vivo} LCMV peptide re-stimulation (GP33 or NP396) compared with wild-type mice (Fig 3-1A) [147].

We next examined whether FGL2 affects the production of memory CD8^+ T cells during a re-infection. C57BL/6 mice were infected with 2x10^6 PFU of LCMV WE and on day 45, at a time when there was no evidence of virus, mice were re-challenged with 2x10^6 PFU of LCMV WE (Fig 3-1B). On day 2 post re-challenge, our data did not indicate significant differences in percentage of IFNγ^+ cells of CD8^+ T cells following \textit{ex vivo} peptide re-stimulation (GP33 or NP396) between both mice. On day 5 post re-challenge, mice deficient in Fgl2 (Fgl2^-/-) had increased frequencies of CD3^+CD8^+ T cells producing IFNγ when compared to Fgl2^+/+ mice.
Figure 3-1: Anti-viral CD8+ T cell responses are enhanced in acute and re-infection studies in Fgl2−/− mice.

A) C57BL/6 wild-type (Fgl2+/+) and Fgl2−/− mice were infected with 2x10^6 PFU LCMV WE and sacrificed on day 8 pi. Isolated SMNC were stimulated with LCMV specific peptides GP33 and NP396. Cells were stained for the surface markers CD3, CD4 and CD8. CD8+ T cell were stained for IFNγ. Data adapted from Khattar et al. [147].

B) Mice that were previously infected with LCMV WE were re-infected with 2x10^6 PFU LCMV WE on day 45. IFNγ production was studied as described above on day 2 and 5 following re-infection.

A-B) Flow plots are representative of 4-5 mice per group. Graphs show the mean ± SEM of 5 mice per group and are representative of 2 independent experiments. *P<0.05
3.2 Targeted Deletion of Fgl2 Improves Viral Clearance without Further Increasing Immunopathology in a Murine Model of Chronic Infection

3.2.1 FGL2 is Upregulated Following LCMV cl 13 Infection

To examine the effects of FGL2 on chronic disease, we conducted a series of experiments using the murine model of chronic viral infection caused by LCMV cl 13 [97]. C57BL/6 wild-type (Fgl2+/+) mice were infected with 2x10^6 PFU of LCMV cl 13 and plasma levels of FGL2 were determined by ELISA (Fig 3-2A). Plasma levels of FGL2 increased as early as day 1 pi and peaked on day 7 pi with a 3-fold increase over uninfected mice. Plasma FGL2 levels remained significantly elevated until day 42 but normalized by day 56 pi.

To verify that LCMV cl 13 infection directly led to increased levels of FGL2, splenic mononuclear cells (SMNC) were isolated from Fgl2+/+ mice and infected in vitro with LCMV cl 13. Supernatants from infected SMNC were analyzed for the presence of FGL2 using an ELISA on day 3 pi (Fig 3-2B). An increase in levels of FGL2 in response to LCMV cl 13 infection was observed by day 3 pi. In addition, with increasing virus concentrations, SMNC produced greater amounts of FGL2.
Figure 3-2: LCMV cl 13 induces FGL2 secretion in vivo and in vitro.

A) C57BL/6 Fgl2+/+ were infected with 2x10^6 PFU LCMV cl 13 and bled weekly. FGL2 protein concentrations were measured in plasma. Dotted line represents naive FGL2 levels. B) SMNCs were isolated from spleen of Fgl2+/+ mice and infected in vitro with 10-fold serially diluted LCMV cl 13. Supernatant was collected at different time points and tested for FGL2 protein. Graphs represent the mean ± SEM of A) 4-5 mice per group and B) 3 samples in each group from two independent experiments. *P<0.05.
3.2.2 Deletion of Fgl2 Leads to Reduction of Viral Load

To examine whether deletion of Fgl2 had an effect on viral clearance, Fgl2\textsuperscript{+/+} and Fgl2\textsuperscript{-/-} mice were infected with 2x10\textsuperscript{6} PFU of LCMV cl 13 and viral titres were measured in brain, heart, kidney, liver, lung and plasma using the focus forming assay (Fig 3-3). Fgl2\textsuperscript{-/-} mice cleared LCMV cl 13 more rapidly than Fgl2\textsuperscript{+/+} mice. LCMV cl 13 was not detectable in livers in two out of eight (25 \%) Fgl2\textsuperscript{-/-} mice by day 28, while all Fgl2\textsuperscript{+/+} mice had high viral titers at this time point. By day 56 both Fgl2\textsuperscript{+/+} and Fgl2\textsuperscript{-/-} mice cleared LCMV cl 13 from the liver. By day 28 and 56, viral titres in the heart, kidney and lung were significantly lower in Fgl2\textsuperscript{-/-} mice. By day 56, Fgl2\textsuperscript{-/-} mice had significantly lower brain viral titres compared with Fgl2\textsuperscript{+/+} mice. In the lung, LCMV cl 13 was not detectable in all Fgl2\textsuperscript{-/-} mice while 5 of 8 Fgl2\textsuperscript{+/+} mice (62.5 \%) had detectable virus by day 56. Within plasma, Fgl2\textsuperscript{-/-} mice had significantly lower titres of LCMV cl 13 by day 28 and all had cleared the virus from the plasma by day 56. Only two out of eight (25 \%) Fgl2\textsuperscript{+/+} mice cleared the virus from plasma while the other Fgl2\textsuperscript{+/+} mice (75 \%) had detectable levels of replicating virus by day 56 pi.
Figure 3-3: Viral titre analysis in LCMV cl 13 infected \textit{FgL2}\textsuperscript{+/-} and \textit{FgL2}\textsuperscript{-/-} mice.

Graphs show the viral titres in organs including brain, heart, kidney, liver, lung and plasma of 2x10\textsuperscript{6} PFU LCMV cl 13 infected C57BL/6 wild-type (\textit{FgL2}\textsuperscript{+/-}) and \textit{FgL2}\textsuperscript{-/-} mice until day 56. N.D. stands for not detectable. Graphs show the mean ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05, **P<0.005.
3.2.3 Liver Immunopathology is Similar in Fgl2\(^{+/+}\) and Fgl2\(^{-/-}\) Mice during Chronic Viral Infection

Other groups have demonstrated that deletion of immunosuppressive molecules including \(Pd-1\) and \(Il-10\) can lead to improved viral clearance or reduced viral load of LCMV cl 13 [92, 147, 164]. Loss of PD-1 in particular resulted in decreased survival of mice, which died due to severe internal injuries mediated by the own immune system [92, 164]. We therefore, examined whether loss of FGL2 would lead to increased immunopathology compared to wild-type (Fgl2\(^{+/+}\)) mice. In addition to examination of liver histology, we also measured levels of alanine transaminase (ALT), a biochemical marker of liver cell death (Fig 3-4, 3-5). Plasma levels of ALT increased by day 7 pi and reached maximum levels on day 14 pi in both Fgl2\(^{+/+}\) and Fgl2\(^{-/-}\) mice and returned to normal by day 56. Levels of ALT did not reach statistical significance between Fgl2\(^{-/-}\) and Fgl2\(^{+/+}\) mice (Fig 3-4).

![Figure 3-4: ALT levels in Fgl2\(^{+/+}\) and Fgl2\(^{-/-}\) mice following LCMV cl 13 infection.](image)

C57BL/6 Fgl2\(^{+/+}\) and Fgl2\(^{-/-}\) mice were infected with 2x10\(^6\) PFU LCMV cl 13 and bled weekly. Plasma was tested for the liver injury marker ALT. Graph represents the mean ± SEM of 4 mice in each group from two independent experiments (D7-56) and 4-5 mice for the D0 time point. *P<0.05.
Examination of liver histology (Figure 3-5A) was in agreement with levels of ALT. Maximum liver injury was seen on day 14 pi and the most frequent finding in the liver was the presence of lymphocytic infiltration of portal tracts especially on day 14 pi (black arrow). Focal lobular lytic hepatic cell necrosis with focal inflammatory infiltrates was also prominent (black circles) on day 14 and day 28 pi. The presence of inflammatory infiltrates and hepatic cell necrosis were pathological findings that are also seen in patients with human viral hepatitis [165]. Additionally, marked portal and periportal lymphocyte cell infiltrates were seen within LCMV cl 13 infected livers, which extended into the surrounding lobules (day 7-day 14 pi, red arrows). These findings are similar to what is found in patients with chronic hepatitis [166]. By day 56, livers of both Fgl2+/+ and Fgl2−/− mice were near normal.

Histological evaluation of liver specimens was conducted according to the histological activity index (HAI, periportal or periseptal interface hepatitis (0-4); confluent necrosis (0-6); focal (spotty) lytic necrosis, apoptosis and focal inflammation (0-4); portal inflammation (0-4)) [160]. Histological evaluation of liver tissues showed no significant differences between LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice (Fig 3-5B).

ALT levels, histology and the HAI evaluation, showed no further increase in liver immunopathology of Fgl2 deficient mice when compared to wild-type mice following LCMV cl 13 infection.
Figure 3-5: Affects of Fgl2 deletion on immunopathology in liver following LCMV cl 13 infection.

C57BL/6 Fgl2\(^{+/+}\) and Fgl2\(^{-/-}\) mice were infected with 2x10\(^6\) PFU LCMV cl 13. Liver tissues were isolated to the indicated time points. A) H&E slides were evaluated according to B) the HAI with a maximal reachable score of 18 (periportal or periseptal interface hepatitis (0-4); confluent necrosis (0-6); focal (spotty) lytic necrosis, apoptosis and focal inflammation (0-4); portal inflammation (0-4)) [160]. Pictures (200x magnification) are representative of more than 5 analyzed histological specimens per group. Black arrows show portal inflammation, red arrows lobular infiltration and circles indicate hepatic cell necrosis. Scale bars represent 50 µm. Data represents means ± SEM of 4-5 mice in each group. *P<0.05.
3.3 Effects of *Fgl2* Deletion on Innate Immune System during Chronic Viral Infection

3.3.1 Total Numbers of Mature DCs and Macrophages are Increased in *Fgl2*<sup>−/−</sup> Mice following LCMV cl 13 Infection

LCMV cl 13 infects DCs and macrophages preferentially due to a high expression of the LCMV binding receptor α-DG on those cells and due to a high affinity of LCMV cl 13 to α-DG [24, 148]. Loss of APC (DCs and macrophages) populations has been reported in patients chronically infected with pathogenic viruses [35-37].

In order to study effects of FGL2 on APC maturation in chronic viral infection, at day 2 pi DCs and macrophages were isolated from the spleen of LCMV cl 13 infected *Fgl2*<sup>+/+</sup> and *Fgl2*<sup>−/−</sup> mice. Total numbers of mature DCs and macrophages expressing MHCII, CD80 and CD86 were determined (Figure 3-6). Total numbers of mature macrophages (CD80<sup>+</sup>, CD86<sup>+</sup> and MHCII<sup>+</sup>) were significantly higher in *Fgl2*<sup>−/−</sup> compared with *Fgl2*<sup>+/+</sup> mice on day 2 pi. Total numbers of mature DCs showed the same trend, as determined by increased numbers of CD80 and CD86 expressing DCs in spleen of *Fgl2* deficient mice. However, numbers of MHCII<sup>+</sup> DCs were not significantly different between *Fgl2*<sup>+/+</sup> and *Fgl2*<sup>−/−</sup> mice at day 2 pi. Median Fluorescence Intensity (MFI) levels of CD80, CD86 and MHCII on both DCs and macrophages did not show significant differences between *Fgl2*<sup>+/+</sup> and *Fgl2*<sup>−/−</sup> mice at day 2 pi (data not shown).

PD-L1, which binds to PD-1, is an immunosuppressive ligand and an important regulator of T cell activation [26]. We investigated, whether deletion of *Fgl2* affects PD-L1 expression on DCs and macrophages using MFI analysis during LCMV cl 13 infection. PD-L1 expression was increased on both DCs and macrophages but did not show significant differences between *Fgl2*<sup>+/+</sup> and *Fgl2*<sup>−/−</sup> mice (data not shown).
C57BL/6 Fgl2+/+ and Fgl2−/− mice were infected with 2x10^6 PFU LCMV cl 13 and sacrificed on day 2. Spleens were treated with collagenase D to isolate DCs and macrophages. Cells were stained with surface antibodies for CD11c, CD11b, MHCII, CD80 and CD86. Samples were analyzed by flow cytometry. DCs (CD11c+) and macrophages (CD11b+) were analyzed for expression of MHCII, CD80 and CD86. Left panel shows representative histogram plots of flow cytometry analysis. Graphs represent the mean ± SEM of 4-5 mice per group from one experiment. *P<0.05.

Figure 3-6: Total numbers of mature DCs and macrophages in Fgl2 deficient and wild-type (Fgl2+/+) mice by day 2 LCMV cl 13 pi.
3.3.2 FGL2 Binds to NK Cells

Previously, we demonstrated that FGL2 binds to FcγRIIB and FcγRIII, and we have postulated that FGL2 preferentially binds to FcγRIIB [127]. FGL2 binding to NK cells, which express exclusively the FcγRIII, has not been demonstrated to date.

Biotinylated FGL2 protein was incubated with isolated SMNC from C57BL/6 wild-type mice at different concentrations and detected with streptavidin (Fig 3-7). Biotinylated mouse serum albumin served as negative control. B cells (CD3^-CD45R^+), which express only FcγRIIB, were used to show positive FGL2 binding (Fig 3-7A). At a concentration of 0.15 µM FGL2 (10 µg/mL), 3.24 % of B cells showed binding of FGL2 and 3.95 % of NK cells (CD3^-NK1.1^+) were positive for FGL2. From 0.15 µM to 0.3 µM FGL2 concentration, FGL2 binding increased 4-fold on B cells. However, binding of FGL2 to B cells did not increase much further from 0.3 µM to 0.6 µM FGL2 concentration indicating saturation of FGL2 on B cells. In contrast, at a concentration of 0.3 µM of FGL2, binding to NK cells increased 4-fold over 0.15 µM FGL2 (Fig 3-7B). Our data shows a 6-fold increase of FGL2-NK cells binding from 0.15 µM to 0.6 µM FGL2 concentration.
Figure 3-7: FGL2 binds to FcγRIII expressing NK cells.

Recombinant His-tagged FGL2 was biotinylated and incubated with SMNCs from C57BL/6 mice as above described concentrations. Attached FGL2 was detected with streptavidin. Biotinylated mouse serum albumin served as a negative control. A) B cells were gated based on CD3+CD45R+ cells and represent the positive control. B) NK cells are gated based on CD3−NK1.1+ cells. Density plots are representatives from three independent experiments.
3.3.3 NK Cell Activity during Chronic Viral Infection

After confirming that FGL2 binds to FcγRIII (CD16) expressing NK cells (Fig 3-7B), the effects of binding of FGL2 to NK cells were investigated in vivo. In order to study, whether FGL2 activates or inhibits NK cells, analysis of total NK cell numbers, cytokine production and receptor expression was performed in uninfected and LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice. SMNC were isolated and NK cells (CD3− NK1.1+) were stained for the inhibitory receptor 2B4 and the activating receptor NKG2D as well as the pro-inflammatory IFNγ, the anti-inflammatory IL-10 and the cytolytic perforin. Figure 3-8A displays the percentage and total numbers of NK cells in spleen in naive and LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice. Following LCMV cl 13 infections, total numbers of splenic NK cells decreased. By day 2 pi, total NK cell numbers were reduced in LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice to half of those in naive mice. By day 7, Fgl2+/+ mice had significantly higher total NK cell numbers in spleen compared with Fgl2−/− mice (almost 2-fold reduction).

We next measured IFNγ, IL-10 and perforin production of NK cells prior and post LCMV cl 13 infection (first week pi). By day 1 pi, approximately 35 % of NK cells from both Fgl2+/+ and Fgl2−/− mice produced the inflammatory cytokine IFNγ (Fig 3-8B). By day 2, only 10 % of NK cells produced IFNγ. Interestingly, by day 7 pi Fgl2+/+ mice showed a significantly higher percentage of IFNγ+ NK cells compared with Fgl2 deficient mice. Perforin and IL-10 analysis in LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice did not show significant differences (data not shown). Further, we determined the state of NK cells by looking at expression levels of the activating (NKG2D) and inhibitory receptors (2B4). We found that these two receptors were balanced in their expression on NK cells; whenever one of those receptors was significantly increased, the other receptor showed the same trend suggesting compensatory mechanisms. No
significant differences were found in receptor expression between \textit{Fgl}2\textsuperscript{+/+} and \textit{Fgl}2\textsuperscript{-/-} mice following LCMV cl 13 infection (data not shown).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3-8.png}
\caption{NK cell activity in LCMV cl 13 infected \textit{Fgl}2\textsuperscript{+/+} and \textit{Fgl}2\textsuperscript{-/-} mice.}
\end{figure}

C57BL/6 \textit{Fgl}2\textsuperscript{+/+} and \textit{Fgl}2\textsuperscript{-/-} mice were infected with 2x10\textsuperscript{6} PFU LCMV cl 13. Isolated SMNC were stained for CD3, NK1.1 and IFN\textsubscript{\gamma}. A) CD3\textsuperscript{-}NK1.1\textsuperscript{+} cells (NK cells) were further analyzed for B) production of IFN\textsubscript{\gamma}. Samples were evaluated by flow cytometry. A-B) Left panel shows representative density and dot plots of flow cytometry analysis. A-B) On the right panel, graphs represent the mean \pm SEM of 4-5 mice per group from one experiment. *\textit{P}<0.05.
3.4 Effects of *Fgl2* Deletion on Adaptive Immune Responses during Chronic Viral Infection

3.4.1 Deletion of *Fgl2* Results in Increased Titres of Neutralizing Antibodies

We next studied humoral responses in *Fgl2* deficient mice during primary (acute) infection. We previously found enhanced humoral activity towards LCMV WE in *Fgl2*−/− mice during and after the virus was cleared [147]. In this study, we investigated effects of FGL2 on the generation of humoral responses during chronic viral infection. The percentages of B cells (Fig 3-9A) and plasma cells (Fig 3-9B) were studied in spleens of LCMV cl 13 infected *Fgl2*+/+ and *Fgl2*−/− mice. Percentages of B cells (CD19+CD45R+) in both mice were not significantly different at all time points. Figure 3-9B indicates that the percentage of plasma cells (CD19lowCD45RlowCD138+) in spleen augmented notably 10-fold by day 7 and 16-fold in both LCMV cl 13 infected mice by day 14 pi respectively, compared to uninfected mice. By day 56, the percentage of plasma returned to levels of naive mice. However, deletion of *Fgl2* had no effect on percentage of plasma cells during chronic viral infection.

In addition, we analyzed the production of non- and neutralizing antibody titres in *Fgl2*+/+ and *Fgl2*−/− mice following LCMV cl 13 infection. LCMV antibody titres were measured in plasma of infected mice (Fig 3-9C). Non-neutralizing antibody titres peaked at day 14 pi and decreased over time until day 56 pi. No significant difference in LCMV specific antibody titres between *Fgl2*+/+ and *Fgl2*−/− mice during chronic infection. Plasma isolated from LCMV cl 13 infected mice from day 28 until day 100 pi were tested using a neutralization assay. Prior to day 56 pi, titers of neutralizing antibodies were similar in *Fgl2*+/+ and *Fgl2*−/− mice but on day 56 *Fgl2*−/− mice demonstrated significantly higher neutralizing antibody titres when compared with wild-type mice.
Figure 3-9: Humoral response analysis in LCMV cl 13 infected Fgl2+/+ and Fgl2−/− mice.

C57BL/6 Fgl2+/+ and Fgl2−/− mice were infected with 2x10⁶ PFU LCMV cl 13. Isolated SMNC were stained for CD19, CD45R and CD138. Samples were evaluated by flow cytometry. A) Percentages of CD45⁺CD19⁺ B cells were determined in mice. B) Percentages of CD138⁺CD45lowCD19low plasma cells were determined in mice. C) LCMV specific antibody and neutralizing antibodies titres were measured in plasma. A-B) Left panels show representative dot plots and histograms of flow cytometry analysis. Right panel (A-B) and C) represent graphs showing the mean ± SEM of 4-5 mice per group from one experiment. *P<0.05.
3.4.2 Deletion of Fgl2 Enhances Anti-Viral T Cell Responses during Chronic Infection

Chronic viral disease is characterized by viral persistence and impaired anti-viral immune responses [93]. In chronic viral infection, it was found that T cells become exhausted, a state in which cells lose effector functions and upregulate inhibitory receptors such as PD-1. Those T cells become eventually physically deleted leading to decreased T cell numbers during chronic viral infection [92, 98].

In order to examine whether deletion of Fgl2 leads to recovery of T cell numbers, we determined total T cell (CD3$^+$ cells) numbers in spleen of LCMV cl 13 infected $Fgl2^{+/+}$ and $Fgl2^{-/-}$ mice (Fig 3-10). In addition, we performed a more detailed analysis of the T cell subsets Th cells (CD3$^+$CD4$^+$ cells) and CTL (CD3$^+$CD8$^+$ cells). All three studies did not indicate significant differences between $Fgl2^{+/+}$ and $Fgl2^{-/-}$ mice for 28 days. Total T cells from $Fgl2^{-/-}$ mice were significantly higher compared to $Fgl2^{+/+}$ mice on day 56 indicating faster recovery of the T cell population in $Fgl2^{-/-}$ mice. Significant differences in total numbers of Th cells and CTL were found on day 56, in which mice deficient in Fgl2 had increased total numbers of Th cells and CTL in spleen compared with wild-type mice.
Figure 3-10: Total numbers of CD3⁺, CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in spleen of LCMV cl 13 infected *Fgl2*⁺⁺ and *Fgl2*⁻⁻ mice.

C57BL/6 *Fgl2*⁺⁺ and *Fgl2*⁻⁻ mice were infected with 2x10⁶ PFU LCMV cl 13. SMNC were isolated and stained for CD3, CD4 and CD8. Total numbers of T cells (CD3⁺), CD8⁺ T cells (CD3⁺CD8⁺) and CD4⁺ T cells (CD3⁺CD4⁺) were determined by flow cytometry. Graphs show the mean ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05.
Our previous studies of LCMV WE showed that deletion of Fgl2 leads to augmented anti-viral T cell responses [147]. We hypothesized that the observed faster viral clearance of LCMV cl 13 in Fgl2<sup>-/-</sup> mice was due to increased anti-viral T cell responses; primarily LCMV specific CD8<sup>+</sup> T cells. To study this, T cells were isolated from spleens of LCMV cl 13 infected Fgl2<sup>+/+</sup> and Fgl2<sup>-/-</sup> mice to different time points. MHCI tetramers (GP33 and NP396) were used to determine numbers of LCMV specific CD8<sup>+</sup> T cells.

Various CD8 T cell subsets have different life spans and dynamics during viral infection such as the NP396 and the GP33 subsets. The NP396<sup>+</sup> CD8<sup>+</sup> population, which recognizes the nucleoprotein of LCMV, has been shown to be deleted rapidly in wild-type mice following LCMV cl 13 infection [98]. Percentages of NP396<sup>+</sup> CD8<sup>+</sup> T cell were measurable with higher rates only in the beginning of infection (day 7). Fig 3-11 indicates that Fgl2<sup>-/-</sup> mice had increased percentage of NP396<sup>+</sup> specific CD8<sup>+</sup> T cells compared with Fgl2<sup>+/+</sup> mice by day 7 pi. Although this subset of cells was higher in Fgl2<sup>-/-</sup> mice compared with Fgl2<sup>+/+</sup> mice at day 14-56 pi, the differences were not statistically different.

**Figure 3-11: Deletion of Fgl2 does not restore LCMV cl 13 depleted NP396<sup>+</sup> CD8<sup>+</sup> T cell population.**

C57BL/6 Fgl2<sup>+/+</sup> and Fgl2<sup>-/-</sup> mice were infected with 2x10<sup>6</sup> PFU LCMV cl 13. Isolated SMNC were stained with α-CD3, α-CD8 and NP396 tetramer. Left plots are representative dot plots of flow cytometry analysis. Right graph represent the mean ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05.
Another subset of T cells, that detects the surface glycoprotein of LCMV (GP33+ CD8 T cells), was studied and results are shown in Fig 3-12. This subset could be detected much longer in LCMV cl 13 infected mice. Effector functions of LCMV specific CD8+ T cells were also studied. Isolated SMNC were stimulated with LCMV specific peptide (GP33-41 peptide, MHC1) \textit{ex vivo} and stained for the pro-inflammatory cytokines IFN\(\gamma\) and TNF\(\alpha\). GP33 tetramer analysis showed significantly elevated percentage of LCMV specific CD8+ T cells in \(Fgl2\) deficient mice on day 28 and 56 pi. The percentage of this subset of cells remained constant in \(Fgl2^{-/-}\) mice at all time points whereas it was reduced in \(Fgl2^{+/+}\) mice.

T cell exhaustion is characterized by the loss of effector cell expression of cytokines including TNF\(\alpha\) and IFN\(\gamma\) [98]. We tested whether deletion of \(Fgl2\) improves CD8+ T cell effector cytokine production on day 28 and 56 pi (Fig 3-12). We showed that deletion of \(Fgl2\) resulted in increased total numbers of IFN\(\gamma\)\(+\)GP33 tetramer+ CD8+ T cells in spleen on day 56 pi. Furthermore, we also showed that \(Fgl2\) deletion resulted in marked increase in TNF\(\alpha\) production. Significant increase in total numbers of TNF\(\alpha\)+GP33 tetramer+ CD8+ T cells was shown by day 28 (8-fold) and 56 pi (15-fold) in \(Fgl2^{-/-}\) mice compared with \(Fgl2^{+/+}\) mice. In addition, results showed a significant rise in total numbers of IFN\(\gamma\) and TNF\(\alpha\) double positive GP33 tetramer+ CD8+ T cells in \(Fgl2^{-/-}\) mice by day 28 and 56 pi.
Figure 3-12: Deletion of *Fgl2* augments anti-viral GP33 tetramer$^+$ CD8$^+$ T cell responses in late phase of chronic infection.

C57BL/6 *Fgl2*+/+ and *Fgl2*−/− mice were infected with 2x10$^6$ PFU LCMV cl 13. Isolated SMNC were stimulated with LCMV specific peptide GP33-41 (GP33) and stained with α-CD3, α-CD8 and GP33 tetramer. Cytokine producing CD3$^+$ CD8$^+$ T cells were stained for IFN$\gamma$ and TNF$\alpha$. Left panel are representative dot plots of flow cytometry analysis. Right panel represent graphs showing the mean ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05. **P<0.005.
We further examined helper anti-viral T cell responses throughout LCMV cl 13 infection (Fig 3-13). Previously, we reported that in CD4$^+$ T cell IFN$\gamma$ production is increased during acute infection (LCMV WE) in Fgl2$^{+/+}$ mice suggesting a more robust Th1 response [147]. In order to test whether deletion of Fgl2 would lead to increased inflammatory cytokine IFN$\gamma$ in CD4$^+$ T cell post LCMV cl 13 infection, SMNC were isolated from LCMV cl 13 infected Fgl2$^{+/+}$ and Fgl2$^{-/-}$ mice and stimulated with the MHCII peptide GP$_{61-90}$ ex vivo. Stimulated CD4$^+$ T cells were stained for intracellular IFN$\gamma$. Flow analysis indicate deletion of Fgl2 had no effect on CD4$^+$ T cell effector cytokine production during chronic viral infection.

Figure 3-13: Analysis of Fgl2 deletion on IFN$\gamma$ production by CD4$^+$ T cell following LCMV cl 13 infection.

C57BL/6 Fgl2$^{+/+}$ and Fgl2$^{-/-}$ mice were infected with 2x106 PFU LCMV cl 13. Isolated SMNC were stimulated with LCMV specific peptide GP$_{61-90}$ (GP61) and stained with $\alpha$-CD3 and $\alpha$-CD4. Samples were evaluated by flow cytometry. Th cells (CD3$^+$CD4$^+$) were analyzed for IFN$\gamma$ production. Left panel are representative dot plots of flow cytometry analysis. Right panel represent graph showing the mean ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05.
3.4.3 *Fgl2* Deficient Mice Have Reduced Percentage of PD-1 Expressing T Cells during Chronic Viral Infection

PD-1 a marker of T cell exhaustion has been found to be upregulated on dysfunctional CD4 and CD8 T cells and accounts for T cell exhaustion [92]. Due to improved anti-viral T cell responses and faster viral clearance in *Fgl2* deficient mice, it was investigated whether deletion of *Fgl2* resulted in reduction of PD-1 expression on CD4⁺ and CD8⁺ T cells.

CD4⁺ and CD8⁺ T cells recovered from uninfected *Fgl2⁺/+* and *Fgl2⁻/⁻* mice expressed low basal levels of PD-1. Approximately 7 % of CD4⁺ and 4 % of CD8⁺ T cells showed PD-1 expression (Fig 3-14). Following LCMV cl 13 infection, PD-1 expression increased on CD4⁺ T cells and continued to rise to day 28 pi, where it peaked 7-fold compared to naive cells (Fig 3-14A). Afterwards, percentage of PD-1 expressing CD4⁺ T cells decreased gradually as well. Interestingly, percentages of PD-1⁺ CD4⁺ T cells were significantly higher in *Fgl2⁺/+* mice compared to *Fgl2⁻/⁻* mice at all measured time points except day 28 pi. However, the expression levels of PD-1 on CD4⁺ T cells, which were analyzed using MFI (data not shown), did not show significant differences between both groups of mice.

PD-1 expression levels on CD8⁺ T cells developed differently compared to CD4⁺ T cells. Approximately 44 % of CD8⁺ T cells in both *Fgl2⁺/+* and *Fgl2⁻/⁻* mice expressed PD-1 by day 7 pi resulting in a 11-fold increase of PD-1⁺ CD8⁺ T cells compared with uninfected mice (Fig 3-14B). Percentage of PD-1 expressing CD8⁺ T cells remained elevated throughout LCMV cl 13 infection. However, by day 56 and 100 the percentage of PD-1⁺ CD8⁺ T cells was significantly reduced in *Fgl2⁻/⁻* mice. As with CD4⁺ T cells, the MFI levels of PD-1 on CD8⁺ T cells did not show significant differences in *Fgl2⁺/+* and *Fgl2⁻/⁻* mice (data not shown).
**Figure 3-14: PD-1 expression analysis on CD4⁺ and CD8⁺ T cell in Fgl2 deleted mice following LCMV cl 13 infection.**

C57BL/6 Fgl2⁺/+ and Fgl2⁻/⁻ mice were infected with 2x10⁶ PFU LCMV cl 13. Isolated SMNC were stained for CD3, CD4, CD8 and PD-1. Samples were evaluated by flow cytometry. A) shows PD-1 expressing CD4⁺ T cells. B) shows PD-1 expressing CD8⁺ T cells. A-B) Upper panels show representative dot plots of flow cytometry analysis. Lower panel represent graphs showing the means ± SEM of 4 mice per group from one (D7 & 14) and two (D28 & 56) independent experiments. *P<0.05.
4 Discussion

Chronic viral infection in humans presents a major health burden worldwide [1-3]. It is now appreciated that multiple immunosuppressive pathways are invoked following both HBV, HCV and HIV infection that inhibit anti-viral innate and adaptive immune responses leading to the development of chronic viral persistence [2, 35, 37, 38, 69, 96, 102, 167]. Proliferation of Tregs has been proposed to account for viral persistence through inhibition of innate and adaptive immunity [103, 117]. Recently, we and other investigators have identified Fgl2 as a putative effector gene of CD4^{+}CD25^{+}Foxp3^{+} Tregs and shown its relevance to regulation of immune responses [116, 129, 133, 168, 169]. The secreted form of FGL2 binds to the FcγRIIB/RIII and has immunosuppressive properties such as inhibiting maturation of bone marrow-derived DC, suppressing T cell proliferation, and inducing B cell apoptosis [127, 128]. Our laboratory has utilized acute infection models including an MHV-3 murine model of fulminant viral hepatitis [133] and an LCMV WE murine model of acute viral hepatitis [145] to evaluate the contribution of FGL2 in regulating immune responses towards viruses, before studying the effects of FGL2 in persistent disease. Using MHV-3, we have previously demonstrated that anti-FGL2 (1F4.2) monoclonal antibody therapy reduced hepatic necrosis, limited viral replication and increased survival of MHV-3 susceptible BALB/cJ mice in a dose-dependent manner [133]. The MHV-3 model was insightful in examining innate immune responses, however, adaptive immune responses could not be analyzed because susceptible mice died at day 4 pi, prior to the development of adaptive immunity. To examine adaptive immunity, we used a self-limiting murine model of acute viral hepatitis caused by LCMV WE to examine the role of FGL2 on the generation of anti-viral immune responses. Overall we found that innate and adaptive anti-viral immune responses to LCMV WE were increased in Fgl2^{-/-} mice during acute infection [147]. In order to investigate the implication of FGL2 in restoring LCMV specific memory CD8^{+} T cell
during re-infection, I continued to use the LCMV WE model. Here I examined the immune-suppressive effects of the FGL2-FcγRIIB/RIII pathway on the generation of memory CD8⁺ T cell responses in re-infection by looking at percentage of IFNγ⁺CD8⁺ T cells after LCMV peptide *ex vivo* simulation. C57BL/6 wild-type (*Fgl2*⁺/⁺) and *Fgl2*⁻/⁻ mice were infected with 2×10⁶ PFU of LCMV WE and were re-infected with an identical dose after 45 days pi. In primary LCMV WE infection, deletion of *Fgl2* results in a greater frequency of anti-viral IFNγ⁺CD8⁺ T cells in the spleen (Fig 3-1A). Re-infection of mice following 45 days of initial infection also led to greater proliferation of memory IFNγ⁺CD8⁺ T cells. The data from the primary LCMV WE indicate that stronger clonal expansion of effector CD8⁺ T cells in *Fgl2*⁻/⁻ mice might have induced a greater percentage of memory CD8⁺ T cells [170]. However, our data showed that significant increase in percentage of anti-viral CD8⁺ T cells were achieved in *Fgl2*⁻/⁻ mice on day 5 and not day 2 following LCMV WE infection. At day 2 post re-challenge the frequencies of IFNγ⁺CD8⁺ T cells specific to GP33 and NP396 were equivalent in *Fgl2*⁺/⁺ and *Fgl2*⁻/⁻ mice. Preliminary data showed that the numbers of CD8⁺ T cells specific to GP33 and NP396 by day 120 pi are equivalent in LCMV WE infected *Fgl2*⁻/⁻ and wild-type mice (data not published). However, at day 5 post-re-infection there is an enhancement of IFNγ⁺CD8⁺ T cell responses towards GP33 and NP396 peptides in *Fgl2*⁻/⁻ mice (Fig 3-1) indicating that either inflammation, viral replication or CD8⁺ T cell proliferation may be necessary for FGL2 to play a role in the memory CD8⁺ T cell response. The potential to generate memory CD8⁺ T cells with high a proliferation is associated with robust protective immunity [170, 171]. Considering, that DCs play a critical role in re-activating memory CD8⁺ T cells [172] and FGL2 inhibits the maturation of DCs, FGL2 might have inhibited memory CD8⁺ T cell proliferation indirectly through the involvement of DCs [172, 173]. Alternately, it is known that memory and activated effector CD8⁺ T cells express the inhibitory FGL2 binding receptor FcγRIIB [150]. Therefore,
FGL2 may have a direct inhibitory role on activated and effector CD8$^{+}$ T cells. It is conceivable that FGL2 may have direct and indirect effects in ultimately inhibiting effector T cells and that deletion of Fgl2 enhanced the proliferative capacity of CD8$^{+}$ T cells during primary infection and re-infection.

Following primary LCMV WE infection, viral titre analysis revealed that both Fgl2$^{+/+}$ and Fgl2$^{-/-}$ mice cleared virus after 12 days. However, coincident with the induction of adaptive immunity at day 8 pi, there was a marked increase in the rate of clearance of LCMV WE in livers of Fgl2$^{-/-}$ mice. In order to investigate whether deletion of Fgl2 resulted in reduction of viral titres during the re-infection, plasma and liver viral titres were tested. Following day 2 post re-infection, LCMV WE was not detectable in plasma and livers in both mice suggesting that the immune response in both mice was sufficient to ameliorate disease course. Moreover, a significant rise in neutralizing antibody titres was seen in Fgl2$^{-/-}$ mice post primary LCMV WE infection [147] and the majority of virus might have been bound to neutralizing antibodies [174].

The LCMV WE model did not allow us to study the implication of FGL2 on viral clearance and the generation of innate and adaptive immune responses during chronic viral infection. Therefore, I chose to utilize LCMV cl 13 to study the effects of FGL2 on the pathogenesis of chronic viral disease. LCMV cl 13 is a viral strain of LCMV that others have utilized to study the pathogenesis of chronic viral infection. LCMV cl 13 induced viral persistence is known to induce a state of immune suppression [26, 91, 92, 159, 174]. I assessed whether targeting FGL2 would lead to a restoration of innate, cellular and humoral anti-viral immunity, and enhance viral clearance during chronic viral infection. Here, I provide evidence that LCMV cl 13 induces the upregulation of FGL2 in vivo (Fig 3-2A). In our murine study, I demonstrated that plasma FGL2 levels in wild-type mice were significantly increased as early as 2 days post-LCMV cl 13 infection. To have such an effect, it is postulated that in addition to T cells, cells of the innate
immune system may contribute to the production of FGL2 early in infection or that they secrete cytokines such as IFNγ that induces FGL2 secretion [123]. NK cells are known to produce high amounts of IFNγ during the early stages of viral infection [46]. Furthermore, levels of FGL2 remained elevated in wild-type mice as long as LCMV cl 13 was detectable in plasma (Fig 3-3) indicating that the presence of virus or more specifically inflammation was necessary to induce FGL2. In agreement with that, a study examining the plasma FGL2 levels in patients with chronic HCV and HBV demonstrated that elevated FGL2 levels correlates with chronic disease progression [134, 175]. Patients exhibiting a sustained virological response (SVR) on Ribavirin and IFN based therapy had significantly lower levels of plasma FGL2 [134].

In addition, I showed that FGL2 is secreted by SMNC in vitro following LCMV cl 13 infection and verified the correlation between LCMV infection and FGL2 production (Fig 3-2B). It was found that FGL2 production by SMNC was dependent on the initial viral load used to infect the SMNC. LCMV clone 13 utilizes a ubiquitously expressed receptor α-DG to gain entry into the cell and many splenic cell-types have the potential to become infected early in LCMV infection [140]. One possible source of FGL2 could be infected cells. Han et al. suggested that there are at least two pathways that lead to FGL2 protein upregulation, viral proteins and cytokines. Hepatitis B viral proteins such as HBc and HBx have been reported to induce FGL2 gene transcription [176]. Alternatively LCMV cl 13 infected cells can produce IFNγ in response to viral stimuli and induce the expression of FGL2 in uninfected cells [123, 177]. Marrazzi et al. demonstrated that IFNγ prolongs FGL2 expression in T cells [123]. IFNγ and TNFα can also induce FGL2 gene transcription and protein translation in macrophages and endothelial cells [120, 177]. Furthermore, our laboratory has demonstrated that FGL2 is a major effector molecule of Tregs (CD4⁺CD25⁺Foxp3⁺ Tregs) [178], which also might have contributed to FGL2 secretion in vivo and in vitro. Taken together, I confirm that persistent viruses can induce an
increased production of FGL2 in plasma [134, 135] and provide evidence that it is dose-dependent. Mueller et al. emphasized that high antigen level drives directly T cell exhaustion and viral persistence [91]. Another report suggested that high viral load affects T cell proliferation subsequently leading to viral persistence [100]. In regards to this, high antigen levels of LCMV cl 13 might have induced high FGL2 levels in mice leading to suppression of DC maturation, inhibition of T cell proliferation and viral persistence.

In order to analyze whether LCMV cl 13 utilizes the immunosuppressive FGL2-FcγRIIB/RIII pathway to escape immune surveillance and elimination, I measured viral kinetics in the brain, heart, kidney, liver, lung and plasma (Fig 3-3). Targeted deletion of Fgl2 resulted in a marked reduction in the viral load in almost all organs. By day 28, viral titres in liver, heart, kidney, lung and plasma were significantly reduced in Fgl2−/− mice. Over time, the reduction in viral load was even more pronounced in Fgl2−/− mice in all organs indicating an accelerated clearance of virus compared with wild-type control group. By day 56, there was no detectable virus in plasma in Fgl2−/− mice, while all Fgl2+/+ mice had measurable virus. Other investigators have utilized knockout systems or antibodies to achieve viral clearance by blocking immunosuppressive molecules including TGFβ, PD-1/PD-L1, CTLA-4 and IL-10. These investigators have shown enhanced reactivity towards LCMV upon deletion or treatment [92, 151, 179]. Using a murine model in which the dominant-negative form of TGF-β receptor is expressed, investigators showed serological clearance of LCMV cl 13 by day 14 following infection [179]. Barber et al. achieved faster viral clearance in plasma of LCMV cl 13 infected mice by treating with α–PD-L1 after disease onset. After 14 days of α–PD-L1 treatment, treated mice cleared LCMV cl 13 within the plasma compared to non-treated control mice [92]. Deleting Il-10 in mice improved LCMV cl 13 clearance in serum by day 9, while wild-type mice had detectable levels of virus [151]. Blockade of inhibitory receptors, such as Tim-3 and LAG, have also been shown to
significantly reduce viral titres of LCMV cl 13 infected mice [99, 180]. Taken together, I can conclude that the approach of blocking the immunosuppressive FGL2 molecule has potential in treating patients with chronic viral infection. According to Oldstone (2007) “cocktails” of antibodies or small-molecule inhibitors to key host-molecules may be a possible alternative therapy for various persistent infections in humans [153].

Observing the side effects of immunomodulatory treatment strategies is important. Here, I monitored the liver pathogenesis in \( Fgl2^{+/-} \) and \( Fgl2^{-/-} \) mice following LCMV cl 13 infection. I used biochemical liver injury marker ALT and histological evaluation. Although the first indication for effects of \( Fgl2 \) deletion was found to be on reduced viral load on day 28 pi, liver immunopathology was similar between \( Fgl2^{+/-} \) and \( Fgl2^{-/-} \) mice at all time points. Plasma ALT levels and liver histology were similar to uninfected mice in both LCMV cl 13 infected mice by day 28 and day 56 pi (Fig 3-4 and 3-5). When an immunosuppressive pathway is blocked such PD-L1/PD-1, the potential for immunopathological injury or autoimmunity is elevated [26, 92, 164]. Barber \( et \ al. \) and Chen \( et \ al. \) showed \( Pd-1^{-/-} \) mice infected with either LCMV cl 13 or MHV-3 died due to lethal immunopathology [92, 164]. In contrast, Brooks \( et \ al. \) reported that \( Il-10^{-/-} \) mice had reduced viral load and immunopathology by day 9 following LCMV cl 13 infection. The authors argued that early control of viral infection and spread is necessary to prevent further injury during LCMV cl 13 infection [151]. From studies with the LCMV WE model, which causes an acute infection, it was found that deletion of \( Fgl2 \) indeed resulted in increased immunopathology in liver [147]. However, in this study, deletion of \( Fgl2 \) did not lead to any more damage in livers during chronic viral infection indicating a safe use of \( \alpha\)-FGL2 treatment strategies.

To investigate the cause of viral clearance in \( Fgl2^{-/-} \) mice, I studied different aspects of innate and adaptive immune responses in mice. Innate immunity is the first line of host defense. DCs
and macrophages are important APCs of the innate immune response essential in initiating anti-viral adaptive immune responses [32, 181]. To assess the effect of targeted deletion of Fgl2 on APCs, I determined the total numbers of mature DCs (CD11c⁺MHC II⁺, CD11c⁺CD80⁺ and CD11c⁺CD86⁺) and macrophages (CD11b⁺MHC II⁺, CD11b⁺CD80⁺ and CD11b⁺CD86⁺) within the spleen (Fig 3-6). By day 2 pi, total numbers of mature DCs and macrophages were increased in Fgl2 deficient mice when compared to wild-type controls. Shalev et al. showed that naive Fgl2⁻/⁻ mice have increased total numbers of CD11c⁺MHCII⁺ DCs and proposed that the increased numbers of DCs in Fgl2 deficient mice are due to increased proliferation of precursor cells or an increase in DCs life span [178]. Dysfunctional and reduced numbers of DCs were observed in patients with chronic viral infections induced by persistent viruses such as HBV, HCV and HIV [35, 36, 182-184]. Virus-infected DCs are depleted after exposure to CTL activity [184] or directly by virus [24]. Lack of antigen presentation on APCs induced by IL-10 has also been reported during chronic viral infection [185]. IL-10 reduces co-stimulation and MHCII expression on APCs [186]. By blocking the IL-10 pathway Ejrnaes et al. restored CD11c⁺ and CD11b⁺ cell numbers, which in return resulted in lower viral titres and disease amelioration [185]. Taken together, here I show greater total numbers of mature DCs and macrophages in Fgl2 deficient mice indicating increased availability of functional APCs for initiating adaptive immune responses.

NK cells function as part of the innate immune system to limit viral spread by killing virus-infected cells and producing of inflammatory cytokines such as IFNγ [47]. NK cells express the activating FcγRIII [130], a receptor of FGL2 [127]. To confirm FGL2 binding to NK cells, I performed binding assays with biotinylated FGL2 (Fig 3-7). Streptavidin was utilized to detect bound biotinylated FGL2. As expected biotinylated FGL2 bound to NK cells. Although NK cells constitutively express CD16, only ~24 % of NK cells were positive for FGL2 binding at the
highest FGL2 concentration (0.6 µM). Thus, cellular receptor availability and affinity may influence the capacity for FGL2 to act on different cells of the immune response. Further studies of the FGL2-FcγRIIB/RIII pathway may provide insights into the mechanisms by which FGL2 exerts its effector function.

As FGL2 is known to bind to NK cells, I investigated whether deletion of Fgl2 had an activating effect on those cells during the early phase of chronic viral infection. I analyzed total numbers of NK cells in spleen, cytokine production (IL-10, IFNγ, Fig 3-8) and performed receptor expression analysis (NKG2D, 2B4, data not shown). Total numbers of splenic NK cell decreased over time, which could be due to migration to sites of infection (Fig 3-8A). Significantly increased numbers of splenic NK cells were found in Fgl2+/+ mice on day 7 pi, which coincides with the peak FGL2 expression in plasma. Whether FGL2 enhanced NK cell proliferation or prevented migration to other organs was not investigated within this study. However, increased frequencies of functionally skewed CD16+ NK cells were reported in patients with persistent HCV infection compared with patients, which cleared the virus [187]. Resultantly, our data support the finding that increased numbers of NK cells are disadvantageous towards viral clearance during persistent infection. Furthermore, I tested NK cells for intracellular IFNγ. I found increased frequencies of NK cells producing IFNγ in Fgl2+/+ mice on day 7 pi, which coincides with higher NK cell numbers in spleen and highest FGL2 concentration in plasma in Fgl2+/+ mice. In regards to that, the FGL2 binding assay (Fig 3-7) supports that proper FGL2 binding to FcγRIII is dose-dependent and occurs at a very high concentration of FGL2 (Fig 3-7). Here, I show that FGL2 ligation to the FcγRIII on NK cells maintains IFNγ production in NK cells making them more active in Fgl2+/+ mice compared to Fgl2−/− mice early in LCMV cl 13 infection. Recently, it has been postulated that NK cells have an immunoregulatory function and are chronically active during persistent infection when
compared to acute infection [187-189]. Furthermore, it has been shown that NK cells have an enhanced cytotoxicity during chronic viral infection [30]. Lang et al. proposed that NK cells limit CD8⁺ T cell numbers [190] while Waggoner et al. suggested that they kill CD4⁺ T cells [191], which in return leads to viral persistence. Nevertheless, further cytotoxic assays need to be performed in order to investigate whether FGL2 increases the cytotoxic abilities of NK cells during chronic LCMV cl 13 infection. In this study, I show that FGL2 binds to NK cells and that is has an activating effect on NK cells. These results give us insights into the NK cell biology and can help design appropriate strategies to treat patients with chronic viral infection.

In addition to innate immune responses, adaptive immune responses towards LCMV cl 13 were studied. In viral infection, humoral immune responses contribute to the control of virus spread. Antibody produced in response to viral infection functions can neutralize viral particles, preventing them from entering cells, and serve to protect the host from re-infection [192, 193]. Here, I show no differences in B cell or plasma cell numbers between $F_{gl2}^{+/+}$ and $F_{gl2}^{-/-}$ mice throughout LCMV cl 13 infection. While there was no significant difference observed in total LCMV antibody titres in plasma between both mice, neutralizing antibody titres were significantly higher in $F_{gl2}^{-/-}$ mice when compared to $F_{gl2}^{+/+}$ mice at day 56 pi indicating an earlier rise of neutralizing antibodies in $F_{gl2}^{-/-}$ mice. The increased levels of neutralizing antibodies coincided with serological viral clearance in $F_{gl2}^{-/-}$ mice. One possible explanation for the resurgence of antibody titres following clearance is that neutralizing antibodies were previously undetectable because they formed antibody-virus complexes [174]. In contrast, in a murine model of acute viral hepatitis caused by LCMV WE, Khattar et al. demonstrated enhanced humoral immunity in $F_{gl2}$ deficient mice with increased numbers of $CD138^{+}CD19^{low}CD45R^{low}$ plasma cells at day 8 pi, and increased total and neutralizing antibody titres [147]. In comparison to acute infection, the production of neutralizing antibody is impaired
in chronic infection [159, 192]. The neutralizing ability is low in LCMV cl 13 infected mice and occurs very late during chronic viral infection [159]. Therefore, it is not surprising that there is no difference in neutralizing antibody titres between $Fgl2^{+/+}$ and $Fgl2^{-/-}$ mice before day 56 pi. However, here I show enhanced neutralization of LCMV cl 13 in plasma, which supports the improved serological clearance in $Fgl2$ deficient mice.

Multiple reports emphasized that robust cell-mediated immune responses are important to clear viral infection [79, 92, 194, 195]. During chronic viral infection, T cell become dysfunctional, a term known as T cell exhaustion, and are deleted [93, 98, 110]. Therefore, I studied cell-mediated immune responses by analyzing T cells numbers, LCMV specific CD8+ T cells and effector cytokine production by CD8+ and CD4+ T cells in spleen of LCMV cl 13 infected $Fgl2^{+/+}$ and $Fgl2^{-/-}$ mice. Total CD4+ and CD8+ T cell numbers were increased in $Fgl2^{-/-}$ mice compared with wild-type mice at day 56 pi (Fig 3-10). It seems that deletion of $Fgl2$ helped to maintain robust T cell numbers during LCMV cl 13 infection. In addition, the IFNγ production in CD4+ T cells to LCMV specific peptide GP61 (GP$_{61-80}$) was investigated. Deleting $Fgl2$ did not improve IFNγ production in CD4+ T cells (Fig 3-13). Khattar et al. showed that deletion of $Fgl2$ enhanced IFNγ production in CD4+ T cells upon LCMV peptide stimulation during acute infection with LCMV WE [147]. IFNγ+ CD4+ T cells belong to the Th1 response, which is important in priming CTL response during viral infection [196]. Here, the Th response data in $Fgl2^{-/-}$ mice might explain the late induction of CTL responses and late viral clearance in those mice. An enhanced Th1 response might have induced a faster viral clearance in $Fgl2^{-/-}$ mice, however the immunopathology might have looked worse as well [197]. Therefore, our data are in agreement with the non-elevated immunopathology seen in both mice. Consistent with that, Khattar et al. showed that LCMV WE infected $Fgl2^{-/-}$ mice had increased anti-viral Th1 cell responses but experienced higher immunopathology when compared with wild-type mice [147].
Taken together, I show that Th1 response was not upregulated in Fgl2−/− mice, as well as liver immunopathology during viral persistence.

In order to investigate whether CD8+ T cell responses were responsible for viral load reduction in Fgl2−/− mice, the effects of FGL2 on the generation of functional LCMV specific CTLs during chronic viral infection were analyzed. It has been reported, that anti-viral CD8+ T cells become “exhausted” during persistent infection making them unable to clear the virus [96, 167, 198]. T cells progressively lose their effector functions resulting in physical deletion of T cells. The LCMV NP396+ (NP396-404) specific CD8+ T cells are deleted early in infection due to higher antigen presentation in vivo [98]. Here, I found that targeting FGL2 improved the percentage of NP396+ specific CD8+ T cells in contrast to expected deletion of the population (Fig 3-11). CD8+ T cells recognizing the GP33 epitope (GP33-41) were used to study T cell exhaustion because this subset circulates longer in mice than NP396+ CD8+ T cells [98]. Using GP33+ CD8+ T cells, I studied the effects of FGL2 on percentage of GP33+ specific CD8+ T cells and their effector functions such as the IFNγ and TNFα production (Fig 3-12). Deletion of Fgl2 improved percentage of GP33+ LCMV specific CD8+ T cells at day 28 and 56 pi. Total numbers of IFNγ+GP33+CD8+ T cells were elevated in in Fgl2−/− mice at day 28 pi while total numbers of TNFα+GP33+CD8+ T cells and IFNγ+TNFα+GP33+CD8+ T cells were elevated in Fgl2−/− mice at day 28 and 56 pi when compared to control group indicating higher numbers of functional CD8+ T cells and reduced T cell exhaustion. Here, I show that FGL2 potentially inhibits the production of TNFα and IFNγ by CD8+ T cells, or limits the proliferation of T cells secreting this cytokines.

In humans, it was shown that TNFα can reduce the half-life of viral mRNAs and can induce the degradation of viral DNA [6]. Furthermore, TNFα and IFNγ promote CD8+ T cell survival and differentiation [86] resulting in increased CTL responses. Therefore, higher numbers of TNFα+ and IFNγ+ CD8+ T cells might have contributed to viral clearance seen in Fgl2−/− mice (Fig 3-3).
During chronic viral infection, T cell exhaustion is further characterized by upregulated expression of PD-1 on T cells [92]. In this study, I aimed to analyze whether FGL2 contributes to exhaustion by examining PD-1 expression or the number of PD-1 expressing CD4⁺ T cells and CD8⁺ T cells (Fig 3-14). The percentage of PD-1⁺CD4⁺ and PD-1⁺CD8⁺ T cells was significantly lower in Fgl2⁻/⁻ mice compared with control group especially around day 56 pi. Thus, in concordance with the viral clearance and increased frequencies of IFNγ and TNFα producing LCMV specific T cells in Fgl2⁻/⁻ mice, it was found that T cells from Fgl2 deficient mice were in a less exhausted state when compared to T cells from wild-type mice. In our study, the viral load was almost the same in both mice for 28 days. By day 28, Fgl2⁻/⁻ mice showed significantly reduced viral titres when compared to wild-type mice and an even bigger difference in viral loads was found by day 56 pi between both mice. Considering that T cell exhaustion is directly driven by antigen load [91] and PD-1 is upregulated when the T cells are stimulated by antigens via TCR [84], I propose the following mechanism (Fig 4-1). Due to absence of FGL2, higher numbers of APCs (DCs and macrophages) were present leading to a higher degree of T cell activation and proliferation, which resulted in reduction of viral load. As antigen levels declined, effector T cells in Fgl2⁻/⁻ mice became less exhausted due to lower TCR stimulation. It has been shown that strong activation of the TCR increases demethylation of the PD-1 gene locus leading to a higher PD-1 expression, which in return results in a progressively dysfunctional and exhaustive state of T cells [85]. Due to the lower expression of PD-1 and greater frequencies of functional T cells, LCMV cl 13 was eliminated more efficiently leading again to lower antigen levels. Resultantly, the difference in LCMV cl 13 viral titres became more prominent over time and Fgl2⁻/⁻ mice serologically cleared LCMV cl 13 faster than Fgl2⁺/⁺ mice. Other reports showed similar findings. Brooks et al. performed a T cell exhaustion study using Il-10⁻/⁻ mice and showed that after deleting this immunosuppressor cytokine, splenic Th and CTL activity
were increased. The authors further studied the levels of PD-1 expression on CD4⁺ and CD8⁺ T cells and found that deletion of IL-10 reduced T cell exhaustion in CD8⁺ T cells, but not in CD4⁺ T cells [151]. Barber et al. analyzed the effects of PD-L1 blockade on T cell exhaustion and found that anti-viral CD8⁺ T cell responses were enhanced quantitatively and qualitatively upon treatment [92]. Taken together, different reports have identified several targets that promote and maintain the state of T cell exhaustion in chronic viral infection [24, 79, 92, 151, 199, 200]. In this study, I show that targeted deletion of Fgl2 prevents T cell exhaustion by maintaining functional T cell numbers in mice following LCMV cl 13 infection.

Figure 4-1: Proposed model of Fgl2 deletion in enhancing anti-viral immune responses and viral clearance during chronic viral infection.

Following LCMV cl 13 infection, deletion of Fgl2 resulted in enhanced numbers of mature APCs such as DCs. Due to improved antigen presentation more effector T cells were activated and could proliferate in mice. Higher numbers of functional T cells were able to reduce viral load, which in return reduced T cell exhaustion and helped further to clear the virus faster.
5 Conclusion

In conclusion, I demonstrated that targeting FGL2 is a potential approach to treat chronic viral infections. I studied the effects of FGL2 on different immune cell populations, viral load and immunopathology during chronic viral infection caused by LCMV cl 13. Cells of the innate immune system such as DCs, macrophages and NK cells as well as adaptive immune cell components (CD4, CD8, B and plasma cells) were analyzed in detail. I showed evidence that LCMV cl 13 induced increased FGL2 secretion. Following LCMV cl 13 infection, targeting FGL2 reduced the deletion of DCs and macrophages. I identified NK cells as FGL2 binding cells and showed that FGL2 had an activating effect on NK cells. Humoral responses were enhanced in Fgl2 deficient mice as shown by the increased neutralizing viral titres during chronic viral infection. Furthermore, I demonstrated that FGL2 inhibited anti-viral T responses to LCMV cl 13 and deletion of Fgl2 reduced T cell exhaustion. Using the LCMV cl 13 model, I demonstrated that by targeting the immunesuppressive molecule FGL2, anti-viral immunity can be restored and viral clearance was increased during viral persistence.
6 Future Directions

This study suggests that targeting FGL2 could be a promising therapeutic treatment for chronic viral disease. Our group has published successfully that blocking FGL2 results in increased survival of susceptible mice in the lethal MHV-3 model [125]. Using the LCMV WE model, our laboratory has shown that deleting Fgl2 enhanced anti-viral innate and adaptive immune responses [147].

Further studies will be needed to define the role of the FGL2-FcγRIIB/RIII inhibitory pathway in the pathogenesis of experimental and human chronic disease. Due to compensatory mechanisms often observed in knockout models, blocking the FGL2-FcγRIIB/RIII pathways with a humanized antibody to FGL2 alone or in combination with anti-viral therapy or siRNA might provide a novel approach to improve the treatment of patients chronically infected with HBV, HCV and HIV infections.
References


