Immune Cytokine Responses to Hepatitis C Virus (HCV) Proteins: A Comparison between Canadian First Nations and Caucasians

By

Koko Akoachoh Bate Agborsangaya

Department of Immunology
University of Manitoba

Winnipeg

Copyright © 2007 by Koko A. Bate Agborsangaya
Immune Cytokine Responses to Hepatitis C Virus (HCV) Proteins: A Comparison between Canadian First Nations and Caucasians

BY

Koko Akoachoh Bate Agborsangaya

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

Koko Akoachoh Bate Agborsangaya © 2007

Permission has been granted to the University of Manitoba Libraries to lend a copy of this thesis/practicum, to Library and Archives Canada (LAC) to lend a copy of this thesis/practicum, and to LAC's agent (UMI/ProQuest) to microfilm, sell copies and to publish an abstract of this thesis/practicum.

This reproduction or copy of this thesis has been made available by authority of the copyright owner solely for the purpose of private study and research, and may only be reproduced and copied as permitted by copyright laws or with express written authorization from the copyright owner.
# Table of Contents

Table of Contents......................................................................................................................... i
Dedication........................................................................................................................................ iv
Acknowledgements.......................................................................................................................... v
List of Figures.................................................................................................................................... vi
List of Tables..................................................................................................................................... viii
List of Abbreviations......................................................................................................................... ix
List of copyright materials................................................................................................................ xi

ABSTRACT.......................................................................................................................................... xii

## 1. INTRODUCTION

1. Introduction
   1.1 Viruses........................................................................................................................................ 1
   1.2 Cells of the immune system.......................................................................................................... 2
   1.3 Host defense against viral infections.......................................................................................... 4
      1.3.1 Cytokine networks in viral infections..................................................................................... 4
      1.3.2 Innate immune responses to viral infection........................................................................... 6
      1.3.3 Adaptive immune responses to viral infections ................................................................. 7

2. Hepatitis C virus (HCV)
   2.1 HCV epidemiology..................................................................................................................... 9
   2.2 HCV viral genome...................................................................................................................... 11
   2.3 Natural course of HCV infection............................................................................................... 13
      2.3.1 Acute HCV............................................................................................................................ 13
      2.3.2 Chronic HCV........................................................................................................................ 13
   2.4 Diagnosis, treatment and vaccine options................................................................................ 14
   2.5 HCV proteins and mechanisms of viral propagation.............................................................. 16
   2.6 HCV and host factors................................................................................................................. 17
   2.7 Role of cytokines in HCV........................................................................................................... 19
   2.8 Host responses to HCV infection.............................................................................................. 20

3. Canadian Aboriginals
   3.1 Geographic distribution.............................................................................................................. 21
   3.2 HCV prevalence among Aboriginals.......................................................................................... 22

4. Genetics
   4.1 Cytokine gene polymorphisms and in vitro cytokine production............................................. 24
   4.2 Link between cytokine SNP and HCV disease progression.................................................... 26
   4.3 Ethnic differences in cytokine SNP frequencies....................................................................... 26
      4.3.1 Differential cytokine frequencies between Canadian Caucasian and Aboriginals................. 27
5. Experimental models
   5.1 In vitro models.......................................................... 28
   5.2 In vivo models.......................................................... 29
   5.3 Limitations.................................................................. 30
6. Project summary............................................................. 31

7. MATERIALS AND METHODS
   7.1 Participants.................................................................. 33
   7.2 Peripheral blood mononuclear cell (PBMC) isolation.......... 34
   7.3 PBMC culture.............................................................. 35
   7.4 Cytokine enzyme linked immunosorbent assays (ELISA)..... 37
   7.5 Cytokine single nucleotide polymorphisms (SNP)............ 39
   7.6 Statistical analysis...................................................... 40

8. RESULTS
   8.1 Introduction.................................................................. 41
     8.1.2 Allele frequencies of IL-10, IL-6 and IFN-γ polymorphisms
differ significantly between FN and CA.............................. 43
   8.2 Comparison of bacterial ligand induced cytokine secretion between
   FN and CA PBMC............................................................. 47
     8.2.1 Impact of bacterial toxins on FN and CA cytokine responses... 53
   8.3 Viral induced cytokine secretion between FN and CA......... 56
   8.4 Differential cytokine responses to HCV specific antigens.... 67
     8.4.1 In vitro effects of HCV Core, NS3 and NS4 on spontaneous
           IFN-α and IL-6 production by PBMC............................. 67
     8.4.2 Effect of HCV Proteins on IFN-α responsiveness .......... 71
   8.5 Relationship between cytokine gene polymorphisms and in vitro
cytokine production......................................................... 73
     8.5.1 Relationship between IL-10 promoter genotype
           polymorphisms and functional in vitro synthesis........... 75
     8.5.2 Relationship between HCV Core induced IL-10 synthesis and
           predefined IL-10 polymorphisms................................. 79
     8.5.3 Effect of viral agent induced IL-10 synthesis on predefined
           IL-10 polymorphisms................................................. 82
     8.5.4 Correlation between LPS induced IL-6 production and
           IL-6 (-174) polymorphism........................................... 85
     8.5.5 HCV Core has a significantly higher impact on IL-6 (-174)
           High phenotype within the FN compared to CA ............. 88
     8.5.6 In vitro production of IFN-γ correlates with polymorphisms
           in IFN-γ (+874) gene................................................. 90
   8.6 Effect of high altitude on cytokine responses in healthy CA PBMC... 94

9. DISCUSSION
   9.1 Introduction.................................................................. 96
   9.2 Differences in cytokine production between populations...... 98
   9.3 Impact of HCV Core on population cytokine responses........ 102
Dedicated to
Mmammy Ebot
and to the
Loving memories of
Daddy Bate-Enow

“....wherever I walk, your counsel can lead me. When I sleep, they will protect me. When I wake up in the morning, they will advise me” (Proverbs 6:22)
Acknowledgements

To my supervisor Dr. Julia Rempel, who gave me the opportunity to work on this unique project. Working with you has been an experience that will shape all areas of my life, character and person for decades to come.

I am grateful for the dedication, commitment and patience of my committee members; Dr. Peter Nickerson for collaborating on this project, providing me with polymorphism data and for discussions on ethnographic immunological responses and Dr. Lawrence Elliott for helpful suggestions on data analysis and insights on population epidemiology. Special thanks go to the members of the Department of Immunology, at the University of Manitoba, particularly Dr. Jude Uzonna for all his encouragement and mentorship throughout my studies, and beyond.

Kudos to the research nurses; Kim Hawkins and Elaine McDougall for coordinating the patient samples. I am also thankful to the individuals who were gracious enough to participate in the study. Thanks to Iga Dembinski for the cytokine genetic polymorphisms data and Dr. Suresh Khatkar and Eng Piew Kok (Lewis) for their contributions to ELISA.

I sincerely appreciate all the members of the Section of Hepatology at the University of Manitoba. Special thanks to Dr. Gerald Minuk for his profound wisdom, constructive criticism and wit, and for creating a supportive integrated learning atmosphere. I am also thankful for Drs. Eberhard Renner and Yuewen Gong for helpful discussions on experimental design, and encouraging all the trainees during research rounds. I can't say enough about my lab colleagues who have always had my back. James and Suresh for “tea-time”, Martin for his calming presence and unending insight on troubleshooting. Carla for all the conversations, silently understanding, and mostly for tolerating my melodramatic moments. Lewis, for all his hard work and humor and Hermann for fitting right in. And finally to Charlene (and Garth) for making sure it all ran smoothly.

Last, but far from the least, I am grateful to my family and friends, whose love, support and understanding saw me through it all. Special thanks to Mmammy whose optimism helped me believe; to Sister Ozong, Onege & Etem for the conference calls and 4 way emails which kept me in the loop; to Ekeb, Oben & Offama for financial and moral support and finally to the rest of the clan (Abaleabooh et al.) for silently cheering me on. Grand Merci a Sous le Baobab, who have been a loving extended family. And finally, much love to all my own personal mendem (Iffy, Helen, Asu, Richard, Nathan, Waks, Daisy, Ngum, Tiayo, Fraud, Abuh, Bekono, Immuno peeps, etc.) for helping me keep my sanity through it all.

Mankub w'en kotiiee!!
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>HCV genome</td>
<td>11</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Host signal transduction pathways to HCV infection</td>
<td>20</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Healthy FN individuals have a lower frequency of IL-10 and IFN-γ High producer phenotypes</td>
<td>46</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Bacterial stimulants enhance PBMC IL-10 synthesis</td>
<td>49</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>FN and CA PBMC secrete similar levels of IFN-γ in response to bacterial ligands</td>
<td>51</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>No significant differences in IL-6 production between populations, in response to bacterial antigens</td>
<td>52</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>FN cells exhibit significantly lower levels of DT induced IFN-γ or IL-10 synthesis, compared to CA</td>
<td>55</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Similar IFN-α expression from FN and CA PBMC</td>
<td>57</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Viral reagents induce CA cells to secrete significantly enhanced levels of IL-10</td>
<td>60</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Poly:IC stimulated FN cells produce significantly lower levels of IFN-γ</td>
<td>61</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>No significant differences in IL-6 production between cohorts, at both days 1 and 6</td>
<td>62</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>FN PBMC exhibit significantly lower levels of IFN-α induced IL-10 and IFN-γ synthesis</td>
<td>65</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Gender disruption may result in artificially lower IL-10 levels in CA cohort</td>
<td>66</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>HCV Core has the highest impact on PBMC IL-10 and IFN-γ synthesis</td>
<td>69</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Ethnicity has a limited influence over the impact of HCV proteins IFN-α and IL-6 synthesis</td>
<td>70</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>HCV Core has a lower impact on IFN-α induced IL-10 levels in FN</td>
<td>72</td>
</tr>
</tbody>
</table>
Figure 17. Allelic distribution of IL-10 -592/819 and -1082 gene polymorphisms among FN and CA Canadians

Figure 18. Relationship between IL-10 promoter genotypes and in vitro synthesis following PBMC ConA stimulation

Figure 19. HCV Core induced IL-10 synthesis correlates inversely with predefined IL-10 -819 polymorphisms within a FN cohort

Figure 20. Effect of viral reagent induced IL-10 synthesis on pre-defined IL-10 polymorphisms

Figure 21. Frequency of IL-6 promoter genotype between FN and CA populations, and its association with in vitro synthesis

Figure 22. HCV Core has a significantly higher impact on the IL-6 -174 High (GG) phenotype within FN, compared to CA

Figure 23. Relationships between ConA induced IFN-γ production and polymorphisms among CA individuals

Figure 24. Core induced IFN-γ does not correlated with IFN-γ polymorphisms

Figure 25. High altitude may increase PBMC IL-10 production
### List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>IL-10, IL-6 and IFN-γ genotypes and phenotypes</td>
<td>25</td>
</tr>
<tr>
<td>Table 2</td>
<td>Subject Characteristics</td>
<td>33</td>
</tr>
<tr>
<td>Table 3</td>
<td>List of Reagents Used In Primary PBMC Culture</td>
<td>36</td>
</tr>
<tr>
<td>Table 4</td>
<td>List of Cytokines Analyzed by ELISA</td>
<td>38</td>
</tr>
<tr>
<td>Table 5</td>
<td>Percentage genotypes of IL-10, IL-6 and IFN-γ polymorphisms in a healthy Canadian FN and CA cohort: Genetic analysis</td>
<td>45</td>
</tr>
<tr>
<td>Table 6</td>
<td>Percentage genotypes of IL-10, IL-6 and IFN-γ polymorphisms in a healthy Canadian FN and CA cohort: Functional analysis</td>
<td>74</td>
</tr>
</tbody>
</table>
# Lists of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine aminotransferases</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody dependent cell mediated cytotoxicity</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BVDV</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>CHCV</td>
<td>chronic HCV</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytidine phosphate guanosine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>ESLD</td>
<td>End stage liver disease</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
</tr>
<tr>
<td>ETR</td>
<td>End treatment responses</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>FN</td>
<td>First Nations</td>
</tr>
<tr>
<td>γ</td>
<td>Gamma</td>
</tr>
<tr>
<td>GBVB</td>
<td>GB virus B</td>
</tr>
<tr>
<td>E1/2</td>
<td>HCV envelope protein -1 or 2</td>
</tr>
<tr>
<td>NS</td>
<td>HCV nonstructural protein</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes Simplex Virus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISDR</td>
<td>Interferon sensitivity determining region</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon stimulated response elements</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>I</td>
<td>Intermediate</td>
</tr>
<tr>
<td>IRSE</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>IDU</td>
<td>Intravenous drug users</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer cell immunoglobulin - like receptors</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>L</td>
<td>Low</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility complex</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>National health and nutrition examination survey</td>
<td>NHANES</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>NK</td>
</tr>
<tr>
<td>Natural killer T cells</td>
<td>NKT cells</td>
</tr>
<tr>
<td>Non-A non-B</td>
<td>NANB</td>
</tr>
<tr>
<td>Nuclear factor kappa B</td>
<td>NF-κB</td>
</tr>
<tr>
<td>Pattern associated molecular patterns</td>
<td>PAMP</td>
</tr>
<tr>
<td>Pattern recognition receptors</td>
<td>PRR</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>PGN</td>
</tr>
<tr>
<td>Plasmacytoid dendritic cells</td>
<td>pDC</td>
</tr>
<tr>
<td>Polyethylene glycol</td>
<td>PEG</td>
</tr>
<tr>
<td>Polymerase chain reactions</td>
<td>PCR</td>
</tr>
<tr>
<td>Protein kinase receptor</td>
<td>PKR</td>
</tr>
<tr>
<td>Retinoic acid-inducible gene</td>
<td>RIG</td>
</tr>
<tr>
<td>Ribonucleic acid</td>
<td>RNA</td>
</tr>
<tr>
<td>Signal transducers and activators of transcription</td>
<td>STAT</td>
</tr>
<tr>
<td>Single nucleotide polymorphism</td>
<td>SNP</td>
</tr>
<tr>
<td>Single strand RNA</td>
<td>ssRNA</td>
</tr>
<tr>
<td>South East Asian</td>
<td>SEA</td>
</tr>
<tr>
<td>Sustained virologic response</td>
<td>SVR</td>
</tr>
<tr>
<td>T helper (1 or 2)</td>
<td>Th</td>
</tr>
<tr>
<td>Toll-like receptor</td>
<td>TLR</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>TNF</td>
</tr>
<tr>
<td>Untranslated region</td>
<td>UTR</td>
</tr>
<tr>
<td>Virus-like particles</td>
<td>VLP</td>
</tr>
</tbody>
</table>
List of Copyrighted material

Figure 1. HCV genome

Figure 2. Host signal transduction pathways to HCV

Table 1. IL-10, IL-6 and IFN-γ genotypes and phenotypes
Abstract

Hepatitis C virus (HCV) infects about 200 million people worldwide with a chronicity rate of approximately 75%. IL-10 appears important in HCV disease persistence. First Nations people (FN) have a higher incidence of HCV infection compared to Caucasian (CA); but may have better disease outcomes, defined by a higher rate of viral clearance. Larcombe et al, previously found significant differences between FN and CA cytokine polymorphisms. Thus, we hypothesized that these groups will exhibit different early cytokine responses to HCV proteins. PBMC were obtained from healthy CA (n=40) and FN (n=25) and stimulated with medium or interferon (IFN)-α, and simultaneously with β-gal, or β-gal linked HCV Core, NS3 or NS4. ELISAs for IL-10, IL-6 and IFN-γ were conducted. HCV Core and NS3 enhanced IL-10 and IFN-γ production in both cohorts. However, FN cells appeared more resistant to the capacity of HCV Core augmentation of IL-10 synthesis. In a blinded manner, single nucleotide polymorphisms (SNP) for these cytokine genes were determined by Dr. Nickerson’s laboratory. Functional cytokine levels were analyzed against cytokine genotypes. Similar to previously published results, there was a linear relationship between ConA stimulated Caucasian cells and IL-10 polymorphisms. Core induced an inverse relationship between IL-10 SNP and functional IL-10 synthesis among First Nations. First Nations capacity to resist HCV Core induction of IL-10 may contribute to a higher rate of HCV clearance among these peoples. These results may provide a basis for understanding the population differences in infectious disease prevalence.
1. INTRODUCTION

1.1 Viruses

Viruses are obligate intracellular parasites containing small packets of protein and nucleic acid that only reproduce inside host cells (1). They were initially termed filterable agents due to their inability to be retained by an unglazed filter (2). The initial investigation into human viruses was prompted in the 18th Century when US soldiers serving in Cuba were plagued with yellow fever (2).

Wendell Stanley was the first to describe viral structure, which consists of structural proteins arranged into capsids (2). Viruses are classified based on; nature of nucleic acid, symmetry of capsid, presence of an envelope and dimensions of virion and capsid (1). Ribonucleic acid (RNA) and deoxy ribonucleic acid (DNA) are composed of either double or single stranded genome units. Viral nucleic strands can either be positive (similar sequence to mRNA and can be directly translated to proteins) or negative stranded (2).

There are several mechanisms through which viruses can cause immunopathology. These include but are not limited to; causing host cell polymerases to be redirected to viral templates, disrupting cellular DNA during viral replication, or disrupting production of host cell proteins important for maintaining cell homeostasis (1). There are a significant number of viruses known to man, many of which cause a significant burden on our health system.
1.2 Cells of the immune system

The immune response to infection consists of an innate (nonspecific) and adaptive (specific) defense system (3).

The innate immune system is the body's first response to an infection. It relies on germline encoded receptors to recognize nonself targets (3). Innate immune defenses are critical in viral infections because they are quickly activated within hours of infection (4). On the contrary, the adaptive system requires clonal expansion of antigen specific lymphocyte receptors and takes a few weeks to develop (3).

The immune defense system is made up of cells that produce cytokines and serum proteins or directly cause cell lysis (1). Innate immunity is largely dependent on granulocytes, macrophages, dendritic cells (DC) and natural killer (NK) cells (5).

Neutrophils are granulocytes found in peripheral blood that protect against bacteria and fungi infections (6). Mast cells and basophills make up < 1% of blood leukocytes, and contribute towards pathogenesis during allergy, and host resistance to some parasites. Their role in viral immunology is yet to be defined, but recent research demonstrates that they can be activated by viral peptides. Macrophages are accessory cells that recognize and destroy pathogens such as protozoa, fungi, helminthes or virus infected cells (7).
NK cells are critical for early immune response to viral infection by secreting antiviral cytokines in response to viral antigens (10). Other cells of the innate immune system included natural killer T cells (NKT) which recognize lipid antigens and activate T cells (3).

DC are accessory cells which arise from a lymphoid progenitor and are highly potent antigen presenting cells (8). They play a key role in linking innate and adaptive immunity (9).

Adaptive immune system requires the action of T and B lymphocytes (5). There are two kinds of T cells. CD8+ cytotoxic T lymphocytes (CTL) detect and destroy cells infected with pathogens. Activated T helper (Th) 1 cells secrete cytokines that favor maturation and clonal expansion of CTL while Th2 lymphocytes secrete cytokines that favor a B cell humoral response. Initial research suggested that CD4+ Th cells are biased either towards T helper TH1 or Th2 based on the type of pathogen encountered by the immune system (3). Over the past decade, there has been evidence demonstrating that CD4+ cells are not necessarily committed to a single Th lineage, but their cytokine regulation is based upon transcription factors, as well as the effect of other cells. As a result, CD4 can secrete both Th1 and Th2 cytokines. Th cells, such as suppressor T cells are responsible for dampening B and T cell function (5, 11, and 15).
1.3 Host defense against viral infections

Host defense to infection depends on interplay between virus and immune system (3). The immune system includes such components as secreted proteins, receptor-mediated signaling and intimate cell-cell contact (3).

1.3.1 Cytokine networks in viral infections

Cytokines are a group of low molecular weight soluble proteins that modulate immune function, and are induced by stimulation with bacterial and viral antigens. Most cell types produce cytokines, which are responsible for controlling the magnitude of the immune response. There are 3 main functional groups of cytokines; pro-inflammatory (promote leukocyte activation), anti-inflammatory (cause immunosuppression and prevent action of pro-inflammatory) and chemokines (recruit immune cells in early stages of the immune response) (5). The first response to a viral infection is the production of cytokines such as interferons (IFN), tumor necrosis factor (TNF) – α, interleukin (IL) – 6 and interleukin -12 (2).

IFNs are key regulators of immune cell activation, cell growth and apoptosis (12). Due to these properties, IFNs are used clinically as treatment, for certain cancers and viral diseases (14). There are 2 main classes of IFNs; Type I IFNs (IFN-α/β) are produced mainly by plasmacytoid dendritic cells (pDC) and fibroblast respectively, while Type II IFNS (IFN-γ) are activated by T and NK cells (13, 14). They are released when conserved pathogen-associated molecular patterns
(PAMPs) from viral particles are recognized by Toll like receptors. Both induce an antiviral state by impairing viral replication and signal via distinct pathways which activate differing aspects of the Jak-Stat pathway. IFN-γ receptor are regulated by γ-activated sequences, while type I are regulated by IFN-stimulated response elements. They also play different roles in viral infection, whereby Type I IFNs are responsible for inhibiting cell proliferation, stimulating growth and function of NK cells, increasing MHC I expression while decreasing MHC II expression. IFN-γ increases expression of MHC II on antigen presenting cells (APC), as well as activates macrophages and promotes adhesion of Th cells to the endothelium (2, 12). Unlike their counterparts, type II interferons play a limited role in anti-viral immunity; however are key modulators of inflammatory response by directing leukocytes to infection sites (7).

TNF-α is also crucial for potentiating lysis of viral infected cells via apoptosis, and its production is mediated by T cells and macrophages. IL-6 is produced by macrophages, T cells as well as other vascular endothelial cells. It is made in response to IL-1 and TNF-α, and stimulates hepatocytes to create acute phase proteins (15).

IL-12 is produced by macrophages or monocytes and is a key regulator of NK cell growth and its cytolytic activity. IL-12 regulates pro-inflammatory cytokines by shifting differentiation of T cells towards a Th1 profile. IL-12 production is inhibited by IL-10 which blocks macrophage function (4).
IL-10 is a pleiotropic cytokine, produced by human CD4+ T cells, monocytes, macrophages, keratinocytes and B cells. It promotes anti-inflammatory pathways by interfering with the production of inflammatory cytokines from cells (16). IL-10 also plays a key role in regulating adaptive immune pathways through its actions on antigen presenting cells or T cells.

Clearance of viral infections, such as Herpes Simplex Virus (HSV), require a strong Th1 to induce T lymphocytes maturation (help in priming secondary responses) and facilitates viral clearance (17, 18).

1.3.2 Innate immune responses to viral infections

Viral associated molecules such as genomic RNA, DNA or double stranded RNA (dsRNA) from infected cells can be recognized by host cell pattern-recognition receptor (PRR) expressed by innate immune cells. After recognition, appropriate antiviral mechanisms are initiated.

Type I IFN are key host cytokine regulators of viral invasion, and are regulated via transcription factors nuclear factor kappa B (NF-κB), interferon regulatory factor (IRF) 3 or IRF7 (19). Toll like receptors (TLR) function as PRR that recognize pathogen associated molecular patterns (PAMP) from a wide variety of pathogens, including bacteria, viruses and fungi. These receptors respond to the presence of viral components by inducing type I interferons (20). For instance
TLR7 recognizes RNA homologs such as imiquimod, synthetic guanosine or uridine derived single stranded RNA (ssRNA). Recent studies in TLR7<sup>−/−</sup> mice showed that their pDC were incapable of producing IFN-α in response to viruses (21, 22). TLR9 recognizes unmethylated 2′-deoxyribo cytidine-phosphate-guanosine (CpG) DNA motifs present in viral DNA. TLR3 is localized in endosome-like vesicles and recognizes dsRNA. Signalling via TLR3 induces DC activation and prepares the adaptive immune response by promoting cross priming of T cells (23, 24). TLR2 and 4 are very important for recognizing components of gram (+) bacterial products such as peptidoglycan (PGN) and gram (-) bacteria such as lipopolysaccharide (LPS) respectively (144).

More recently, a new PRR called Retinoic acid-inducible gene I (RIG-I) was found to recognize cytoplasmic dsRNA (25, 26). Killer cell immunoglobulin-like receptors (KIR) are expressed by NK cells and are also important in the anti-viral response. When activated, they trigger NK cell lyses of virally infected cells (27).

1.3.3 Adaptive immune responses to viral infections
The adaptive immune system employs antiviral strategies, which are either humoral or cell mediated.

Cell mediated responses remove viral related cells without causing damage to the host (2). CTL recognize viral peptides presented by major histocompatibility complex (MHC) class I and causes destruction via two main apoptosis pathways
Firstly, they can induce the secretion of pore forming proteins (perforin) at the interface between CTL and infected cell, and then secrete a protease granzyme which is responsible for cleaving peptides within the infected cells. The second mechanism requires interaction between membrane bound Fas ligand (FasL) to Fas, found on the surface of target cells (28). Alternatively, CTL may secrete cytokines to interrupt viral replication, eliminating infection without damaging the cell (11, 29). CD4+ T cells also seem to play an important role, by promoting and maintaining the effect of CD8+ T cell memory. They are dubbed "helper T cells" due to their ability to sustain memory T cells, and also for their role in isotype switching and clonal selection of immunoglobulins (3, 4, and 11).

Humoral mediated antiviral responses can occur via two pathways. Entry of viruses into host cells can be prevented by binding of antibody to viral ligands. Antibodies can also neutralize viral peptides by interfering with viral outer envelope and the cell membrane of the host. Antibodies can also recognize viral antigens on cell surfaces and lyse the cells either by activating complement, or antibody dependent cell mediated cytotoxicity (ADCC) through Fc receptors on NK cells (11, 29, 30).

Although host cells are capable of a myriad of mechanisms to both prevent and deter viral infections, viruses have also evolved over the years to evade host mechanisms and persist within the host.
2. Hepatitis C virus (HCV)

2.1 HCV epidemiology

The existence of non-A non-B (NANB) hepatitis was discovered in 1974, after new cases of viral hepatitis were observed in patients who were negative for Hepatitis A virus (HAV) or HBV (31). A major breakthrough in HCV research came over a decade later when researchers from Chiron Corporation were able to isolate complimentary DNA (cDNA) from monkeys infected with serum from humans with NANB hepatitis (32).

HCV is a small positive single strand RNA virus, with a size of < 80 nm (33). It belongs to the genus *Hepacivirus* within the *Flaviviridae* family (34). Other viruses in this family include; GB virus B (GBV-B) or Bovine viral diarrhea virus (BVDV) from the *Pestivirus* genera (35). HCV primarily targets hepatocytes, and can cause fibrosis, cirrhosis and eventually hepatocellular carcinoma if untreated. It remains the primary indication for liver transplants in Canada, USA and Europe (36).

It is estimated that about 3% of the world's population has been infected with HCV. This number may be an underestimate, given that the acute phase of disease is asymptomatic, and the virus can survive for long within its host in a quasi dormant state (36). Prevalence varies geographically from < 1% in Canada and up to 10% in Egypt (37). The high rate in Egypt largely attributed to cross-contamination during parenteral antischistosomal therapy (38, 39). The advent
of a diagnostic test for HCV has decreased the incidence of HCV in developed countries, where the principal method of transmission prior to the 1990s was blood transfusion. Presently, individuals at highest risk for contracting HCV in Canada are intravenous drug users (IDU) (40). However, introduction of safe needle programs has helped reduce infection transmission through this route (157).

Other methods of HCV contraction include: nosocomial transmission such as patient to patient (hemodialysis, organ donation, and colonoscopy) or patient to health care worker (needle-stick injury or surgery); perinatal transmission, sexual transmission; and other means such as non-sterile technique during tattoos (41).

There are 6 major genotypes of HCV, which contain genetically related but distinct subtypes (42). Molecular epidemiology has been paramount in revealing genotype distributions based on population and geographic distribution. Genotypes 1a and b are predominantly found in Northern Europe and the US, while 2 and 4 are predominant in West and Central Africa respectively (39, 43).
2.2 HCV viral genome

HCV genome is 9.6 kilobases in length, and contains a 5' and 3' untranslated region (UTR), a long open reading frame encoding ~ 3000 amino acids. The 5' UTR is composed of an internal ribosome entry site (IRES) which function as a viral protein post-translational initiative (31). The open reading frame encodes a polyprotein which is processed by cellular and viral proteases to generate structural proteins (Core, envelope glycoprotein 1 (E1), E2 and p7) in the N-terminal region, and non-structural (NS) proteins (NS2, NS3, NS4A/B and NS5A/B) located at the C terminus (44, 45).

Figure 1: HCV genome\(^1\) (46)

HCV Core is a highly conserved protein of about 21 kDa thought to interact with genomic HCV RNA inducing nucleocaspid formation (31, 33, 35). Core plays a

---

\(^1\) Reprinted from Current Opinions in Microbiology, Volume 9, Issue 4, Bartenschlager R. Hepatitis C virus molecular clones: from cDNA to infectious virus particles in cell culture, Pages 416- 422., 2006, with permission from Elsevier (November 28th, 2006)
major role in modulating host immune response to infection by affecting various aspects of gene expression, such as Nf-κb activation or inducing cellular apoptosis (31, 47). Moreover, serum HCV core antigen has been recently used to detect extent of viremia, since it corresponds with HCV RNA level (31). Further discussions on the immunomodulatory role of HCV Core will be had in the chapter 2.5.

E1 and E2 have molecular weights of about 31 and 70 kDa respectively (31, 33), and are both involved in receptor binding and cell fusion (35). The amino end of E2 exhibits a high degree of variability (hypervariable region 1 and 2), which are targets for neutralizing antibodies. Mutations in this region play a role in immune evasion (31). The role of p7 is currently undefined. NS proteins provide enzymatic activity for viral replication (31). NS2 is a short 23 kDa transmembrane protein active in the cleavage of NS3, and NS5A phosphorylation. NS3 encodes a 67 kDa protein that acts both as a serine protease (cleaves NS4A) and helicase (unravels dsRNA) (31, 33). NS3 has also been found to regulate signal transduction of protein kinase (PKA) and influence host survival. NS4 is cleaved into NS4A and NS4B, which weigh 6 and 27 kDa respectively. NS4A is mostly a cofactor for NS3 protease activity (48). NS5 is cleaved into NS5A and NS5B weighing 56 and 58 kDa respectively. NS5A contains an IFN sensitivity-determining region (ISDR) which suppresses protein kinase receptor (PKR), an intracellular enzyme mediating IFN action activity (31, 33, and 49).
2.3 Natural course of HCV Infection

The biggest challenge in assessing HCV natural history is due to the asymptomatic nature of the disease implying that the disease goes undetected. Moreover, viral replication is extremely robust leading to diverse quasispecies which may lead to false positive tests (50, 51).

2.3.1 Acute HCV

The onset of HCV is typically asymptomatic. Though symptoms of acute disease, are rarely seen, they include jaundice, malaise and nausea (52). It takes about 6 - 8 weeks to see disease symptoms after virus exposure (34). Acute infection is also characterized by elevation of liver enzyme alanine aminotransferases (ALT) (31). It lasts for 3-12 weeks and results in a self-limiting infection in approximately 15-25% of the population who are able to combat the virus (50).

2.3.2 Chronic HCV

Approximately 70% of patients infected with HCV are unable to control viral replication and ensuing symptoms, leading to chronicity (53). Chronic HCV (cHCV) leads to end stage liver disease (ESLD) in 20% of people, and fibrosis induced hepatocellular carcinoma (HCC) occur in 10-15% of these patients (54). Alcohol intake, male sex and co-infection with Hepatitis B virus (HBV) or HIV-1 are known to accelerate the course of chronic disease (50).
2.4 Diagnosis, treatment and vaccine options

Serological assays are used to detect HCV antibodies and viral load (31). Enzyme immunoassays (EIA) detect the presence of anti HCV antibodies (50). False negative test can occur in immunocompromised individuals, requiring the use of confirmatory test, such as recombinant immunoblots. A positive assay implies the recognition of more than 1 viral protein. Qualitative RNA tests are the most direct methods of determining the presence of an active infection. HCV RNA found in serum can be identified by PCR amplification reactions. Amplicor assay (Roche) has a detection limit of < 100 viral copies/µl (31, 53). There are three commercially available quantitative assays for detecting the level of viral RNA (viremia or viral load); a branched DNA (bDNA) assay (Bayer), and two RT PCR assays (Roche). Although both systems are reliable, they're not comparable, thus only 1 type is used throughout treatment duration (50).

Initial HCV treatment was with standard IFN-α for a 24 week period (145). Ribavirin and pegylated IFN-α are currently used as therapy for HCV. IFN-α is fused with polyethylene glycol (PEG) thus extending IFN half-life. Ribavirin is a guanosine analogue, thought to boost host adaptive antiviral immune response by enhancing Th1 cytokine response (55). Unfortunately, less than half of HCV patients respond to treatment (55). Non responsiveness has been partly linked to HCV genotypes or viral loads. There are six major HCV genotypes, and HCV patients with genotype 2 and 3 have been shown to have higher response rates to IFN therapy (39). Other patients relapse after treatment stops, or cease
treatment due to adverse side effects. IFN related side effects are numerous; the most severe are psychosocial side effects, such as depression or anxiety (31, 50). Due to unfavorable events related with current IFN treatment, an important research focus will be to pre-determine patients that are least likely to respond to therapy, in order to prevent treatment associated side effects. Investigational treatments include therapies such as amantadine, which have been successful in patients who failed IFN-α therapy (56).

There are several factors hindering the design of an HCV vaccine include: (1) the lack of an efficient cell culture system, (2) the quasispecies nature of the virus, (3) genotypes and (4) no small animal model (57). As addressed in a review by Torresi et. al, a potential solution to address the antigenic variability of the virus, may be a vaccine designed with multiple epitopes representing immunodominant regions from existing serotypes (57). In addition, including those epitopes conserved between genotypes that are broadly cross reactive with other quasispecies may help address this problem. An effective HCV vaccine should be capable of incorporating the use of helper T cells, as well as CTL responses and neutralizing antibodies. Such a broad vaccine strategy has been seen in studies where HCV structural proteins were incorporated into viral like particles and successfully used to protect transgenic mice when challenged with virus-like particles (VLP) (146). In addition, chimpanzee studies showed protection against HCV infection when immunized with recombinant E1 and E2 proteins (58). E1 vaccine trials in humans led to phase II clinical trials which only improved liver
histology, but showed no decrease in HCV RNA (147). The challenge remains for the formation of an inexpensive and effective vaccine.

2.5 HCV proteins and mechanisms of viral propagation

Initial research into HCV kinetics suggested that viral clearance was due to robust CD4 and CD8 specific T cell responses to viral antigen (59). However, the low rate of spontaneous clearance, as well as poor response to antiviral therapy suggests that certain viral components interfere with the induction of innate immune pathways in the host.

Deregulation of NK cell activity as well as interruption of IFN-α signaling pathways, are key properties of HCV which contribute to its capacity to evade early immune responses, establishing a chronic course of infection (60). HCV Core and NS3 were shown to stimulate proinflammatory cytokines (TNF, IL-6 and IL-8) via TLR2 in monocytes (61) and decrease DC differentiation (62). HCV Core is also responsible for interrupting IFN-α signaling by activating ISRE and GAS-containing promoters and preventing nuclear translocation of signal transducers and activators of transcription (STAT)-1 (63,64).

Other structural proteins also affect early innate immune responses. E2 has been shown to down regulate the activity of NK cells by directly binding to a receptor CD81 (66). Interestingly, E2 has the exact opposite effect on T cells, acting instead as a costimulatory signal. NK cell IFN-γ mediated antiviral activity is also
thwarted by this negative feedback mechanism. E2 also interferes with IFN-α by inhibiting PKR activity (67). NS3/4A protease was recently shown to inhibit IRF-3 function by blocking RIG-1 or TLR3 activation (68). HCV NS4 induces anti-inflammatory cytokines, while suppressing Th1 cytokine IL-12 (69).

HCV NS5A targets PKR resulting in inhibition of IFN-α induced antiviral activity (49, 70). NS5A has also been shown to up regulate TLR4 and increase B cell responses to microbial agents (71).

2.6 HCV and host factors

Genetic and biological factors can modify host response to diseases as well as determine treatment success. These host factors include, but are not limited to age, gender, body mass index (BMI) and ethnicity. In addition, other exogenous factors such as alcohol consumption and injection drug usage influence the rate or extent of chronicity, response to therapy and also risk of recurrence (72).

According to the third National Health and Nutrition Examination Survey (NHANES III), approximately 2.7 million Americans are chronically infected with HCV, with African and Hispanic Americans having a higher prevalence than White Americans (73). This analysis was done by evaluating anti-HCV antibodies, a method which is unable to differentiate between acute, chronic or resolved infections (73). African Americans with chronic HCV have been shown to have lower serum levels of liver enzymes, less advanced hepatic fibrosis and slower
rate of progression of liver disease compared with Caucasians (74-76). A recent multicenter study which eliminated genotype, age and other factors also showed that African Americans were 10 times less likely to have end treatment responses (ETR) to IFN monotherapy, compared to Caucasians (74). While the differences in natural history and treatment responses have been well documented between African American and Caucasians, studies comparing other minority populations are few and far in between. A study by Hepburn et al, observed that Asians have a higher response rate than any other ethnic group with or without similar genotype, followed by Caucasian, Hispanic and African Americans (77). A similar study by Dev et al, showed that South East Asians with genotype 1b were more likely to respond to antiviral therapy compared to Caucasian patients (78). It is currently not known why race affects likelihood to clear disease upon treatment, or disease progression rates. It will thus be important to further characterize viral-host factors which may cause these differences, by including more minorities on future clinical trials, so as to customize population specific treatments.

Better treatment responses have also been shown in patients who are young and female. It is thought that older individuals may have advanced liver disease, thus exhibit poor responses to IFN-α. In addition estrogen may increase IFN-α effectiveness. Other factors which may impact IFN-α therapy are a high BMI, though fatty deposits that induce steatosis and accelerate fibrosis (72).
Differential responses to treatment and virus have important ramifications for disease management.

2.7 **Role of Cytokines in HCV**

During liver injury, pro inflammatory cytokines such as IL-12 and TNF-α are secreted (148). There's evidence linking certain cytokines to HCV clearance or better disease outcomes. Generally Th1 cytokines have been linked with viral clearance. In contrast, anti-inflammatory cytokines such as IL-10 and IL-4 may lead to enhanced HCV chronicity (72).

IL-10 is a pleiotropic cytokine which has a significant role in HCV disease pathogenesis. It has been reported that patients with high serum and PBMC levels of HCV respond poorly to treatment, and are more susceptible to recurring infection after a transplant (79, 80).

Some studies have reported a role for HCV specific IFN-γ producing T cells in viral clearance (81, 149). However IFN-γ's role during the course of HCV or treatment still remains contradictory (150). Other pro inflammatory cytokines, such as TNF-α and IL-1β have been found in high concentrations in chronically infected patients, and are also associated with poor treatment responses (151, 152). IL-6 is an acute phase protein whose role in HCV has not been clearly defined.
2.8 Host response to HCV infection

Upon viral infection, viral PRR bind to TLR3 or RIGI on cells to induce phosphorylation of IRF-3. IRF-3 activation induced the production of IFN-β from infected cells. IFN-β in turn binds IFN-α/β receptor which leads to activation of the Jak-STAT pathway. STAT activation in turn results in localization of IFN-stimulated gene factor -3 (ISGF3) to the nucleus leading to IFN stimulated genes (ISG) and IFN-α transcription (as reviewed by 168, Figure 2).

Figure 2: Host signal transduction pathways to HCV² (168)

3. Canadian Aboriginals

3.1 Geographic distribution

Canadian Aboriginal people are the descendants of the original inhabitants of North America. They are divided up into three distinct groups; North American Indians (First Nations), Inuit and Métis, based on their place of origin, language, culture and customs as defined by Canada’s 1982 constitution (83).

First Nations (FN) are collectively indigenous people of Canada, who are neither Inuit nor Métis. There are 3 different categories of North American Indians: Status Indians (registered under the Indian act), Non-status Indians (not registered), or Treaty Indians (registered under the Indian Act and a member of a band) (84). The Inuit are the Aboriginal people of the Arctic, and they include those primarily from Nunavut, Northwest Territories, Labrador and Northern Quebec. Métis represents people with mixed FN and European ancestry (83, 85).

According to the Statistics Canada 2001 national survey (86), Aboriginals make up 3.3% of Canada’s total population. They represent one of Canada’s fastest growing populations, increasing by 22.2% from 1996 to 2001, while the non-Aboriginal population increased only by 3.4%. Manitoba, a province with the highest concentration of Aboriginals (13.6%) has seen the Aboriginal population grow by 16.6%, while the non-Aboriginal population has decreased by 1.8% over a 5 year period. This increase in Aboriginal population size has been attributed to birth rates that are almost twice that of the non-Aboriginal population (84, 86).
3.2 HCV Prevalence among Aboriginals

The prevalence of several infectious diseases, such as acute HBV and HCV is significantly higher among Aboriginal compared to Non-Aboriginal Canadians (87, 88, 89). This was determined based on a study of seroprevalence of antibody. These differences in health status have been attributed to relatively poorer socioeconomic conditions (93) and increased occurrence of high risk activities promoting infectious disease such as injection drug use (40, 89, and 90).

Despite all the above factors, there is evidence that people of indigenous heritage have better HCV disease outcomes. For instance, a higher rate of spontaneous clearance has been reported among Alaskan Natives, compared to other races (91, 92). A literature review by Minuk et. al, summarized the prevalence of viral hepatitis among First Nations and Inuit (93). Interestingly although acute HCV was higher among Aboriginals, they were 15 times more likely to be HCV RNA negative compared to Caucasians (93,153). These findings are in line with a recent report (2006) by the public health laboratory in Winnipeg, which demonstrates a significantly higher rate of self limited infection among First Nations in Manitoba, compared to non-First Nations (155). Furthermore a study of liver organ transplantation in British Columbia, reported that, the primary indication for liver transplant among First Nations is primary biliary cirrhosis (PBC, 53%) and not HCV (7%) (94). Interestingly, the percentage of both First Nations and Caucasians receiving liver transplants due to HCV is similar, indicating that
HCV has a similar impact on both populations. However PBC has more of an impact on FN than HCV does.

Prevalence of HCV is linked with socio-economic factors and behavior. Despite the socio-economic status of Aboriginals (similar to African Americans), they seem to have a lower rate of chronicity. These trends may be due to differences in host genetics or immune systems. An understanding of the molecular mechanisms behind these epidemiologic findings, may lead to better therapeutic options in all peoples.
4. Genetics

4.1 Cytokine gene polymorphisms and in vitro cytokine production

Several studies have demonstrated that the amount of cytokines produced by individuals may be influenced by gene transcriptions caused by single nucleotide polymorphisms (SNP) within their genome (95). Functional studies, have classified cytokine genotypes as high, intermediate or low producers, based on in vitro production in response to T cell stimulators (96-98, Table 4).

Several single base pair substitutions have been identified. IL-10 -1082, -819 and -592 in the IL-10 promoter region are some of the most documented which are relevant to our study (96). Biallelic polymorphism located at -174 promoter region of the IL-6 gene has also been identified (97). A polymorphism at the +874 position at intron 1 of the IFN-γ gene has also been shown to influence the amount of cytokine production (99).
Table 1: IL-10, IL-6 and IFN-γ genotypes and phenotypes

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Genotype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 592 CA</td>
<td>CC</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>Low</td>
</tr>
<tr>
<td>- 819 CT</td>
<td>CC</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>Low</td>
</tr>
<tr>
<td>- 1082 GA</td>
<td>GG</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>Low</td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 176 GC</td>
<td>GG</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>Low</td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 874 TA</td>
<td>TT</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>TA</td>
<td>Intermediate</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>Low</td>
</tr>
</tbody>
</table>

4.2 Link between cytokine SNP and HCV disease progression

It has been widely reported that polymorphisms within the promoter region of certain cytokine genes may influence various aspects of disease outcomes, such as susceptibility, degree of disease and response to treatment (95).

A number of studies have examined the role of IL-10 promoter SNP and their association with HCV susceptibility, disease severity and antiviral response. Generally, the inheritance of IL-10 -1082 High producer phenotypes, may lead to susceptibility to chronic infection (72, 100-102), Sustained virological responses (SVR) to combination therapy (103) and accelerated fibrosis (102). Other studies have linked IL-10 -819 low producer phenotype (104) and haplotype with disease clearance (105).

Only one report exists supporting a role for IL-6 -176 low producer phenotype and spontaneous viral clearance (106). There does not appear to be any relationship between IFN-γ polymorphisms and HCV disease outcomes (104, 106-108).

4.3 Ethnic differences in cytokine polymorphism frequency

Ethnic populations display heterogeneity to polymorphism inheritance. For instance, South East Asians had a significantly higher frequency of IL-10 1082A and -819T allele compared to Caucasians (109-114, 125-127). Asians were also reported to have a lower propensity of producing IFN-γ compared to Black,
Hispanic or White individuals (111). IL-10, IFN-γ and IL-2 polymorphism frequencies were similar among Caucasian populations from South East England, Manchester, Denmark and Finland (112).

4.3.1 Differential cytokine genotype frequencies between Canadian Caucasians and Aboriginals

A recent study by Dr. Rempel published with Larcombe et. al, reported significant differences between Canadian Aboriginal and Caucasian cytokine polymorphisms (113). It was reported that Aboriginals had phenotypes predisposing them to secrete significantly ($p < 0.005$, all) lower levels of IFN-γ (76% vs. 27%), IL-10 (-1082: 62% vs. 22%, -592/-819: 16% vs. 1%) and TNF-α (93% vs. 68%), and enhanced levels of IL-6 (86% vs. 35%) A similar study comparing IL-10 Polymorphisms among Norwegian Caucasians and Aboriginals had similar findings (114).

The inheritance of cytokine polymorphisms and subsequent immune profiles may influence disease outcomes. Previous studies have capitalized on correlating socio-economic factors and environment on the high prevalence of certain diseases among Canadian Aboriginals (88). A better understanding of immune cytokine networks may provide additional knowledge into present day ethnographic disease trends.
5. Experimental models

5.1 *In vitro models*

Although HCV research has been hampered by the lack of an efficient cell culture system, researchers have been able to develop in vitro techniques, which have increased our knowledge of viral life cycle and the course of infectivity.

Initial studies of HCV infectivity using primary hepatocytes from infected humans or chimpanzees yielded contradictory results (115). For this reason, and also due to difficulty in growing hepatocytes, some groups use hepatoma cell lines, such as PH5CH to assess infectivity of HCV+ sera (116). These systems are useful for evaluating drugs for antiviral activity or inhibition of HCV infection.

Several studies have shown that full length functional clones of HCV cDNA are infectious in chimpanzees, after intrahepatic inoculation (117,118). Other groups have transfected full length in vitro transcribed viral cRNA into a human hepatoma cell line (HepG2) (117). This method was also useful for conducting genetic analyses and examining the function of various viral domains.

The recent establishment of a cell culture system supporting stable replication of a subgenomic replicon is groundbreaking (158 -160). The major advantage of this system is that it will allow for studies on mechanisms of viral entry, viral clearance and also contribute to vaccine development studies.
5.2 *In vivo* models

Chimpanzees were the original source of discovery of the HCV genome and remain one of the only animal models susceptible to HCV infection (32). The availability of this model has enabled scientists to investigate the acute phase of viral infection and determinants of viral clearance. Unlike their human counterparts, they have a high degree of viral clearance, which makes them useful for memory or prophylactic vaccine studies (58). HCV can also infect hepatocytes of tree shrews (*Tupaia* spp); nonrodent animals phylogenetically close to primate (120). However the wild nature of these animals has discouraged their use as a laboratory model. Rodents are incapable of being infected with viral hepatitis, but may be useful for investigating pathogenesis of chronic infection. HCV transgenic mouse models have been used to study direct pathophysiological effects of specific viral proteins. Recent developments yielding a chimeric mouse model with humanized liver provide a lot of promise for *vivo* experiments (178).

Researchers are also investigating surrogate models of HCV. The most promising so far is use of GBV-B, shown to infect hepatocytes of tamarins and marmosets (121). Some advantages to the GBV-B tamarin model include its ability to a) induce higher viral loads than HCV, b) replicate in primary hepatocytes, and c) GBV-B clone infectivity after intrahepatic inoculation. Tamarins are also smaller than chimpanzees thus are suitable for use in a laboratory setting (120).
5.3 Limitations

The biggest challenge in cell culture models, are the low viral replication levels, as well as reproducibility problems. There are several limitations to the use of cRNA transfection models and replicon systems. cRNA is highly unstable inside a cell, and could remain detectable for a long time. The minireplicon system doesn't replicate whole HCV which hampers the process of investigating the impact of antiviral compounds on virus replication. Minireplicons are also known to have mutations, which can cause them to be non-infectious in chimpanzees (120). Finally, and this is similar for all culture systems, viral replication is inconsistent and this hinders their use to assess antiviral compounds (120).

In vivo experiments are mostly hampered by the lack of an efficient small animal model. Transgenic mice are capable of tolerating viral antigens, thus cannot be used to extrapolate studies to human's naïve to HCV, which would mount an immune response to HCV (90,120). Generations of chimeric mouse models have proven to be a laborious process, and their efficiency is yet to be verified (178). Chimpanzees, although being susceptible to viral hepatitis, are an endangered species and extremely expensive to breed and maintain. Chimpanzees also lack a chronic disease phase of infection, thus cannot be used to study chronic pathogenesis (122). In addition, Surrogate viral models, such as GBV-B or BVDV have subtle differences compared to HCV.

In conclusion humans are the most effective model for studying HCV disease pathogenesis.
6. **Project summary**

The aim of this study is to understand what differences exist between First Nation and Caucasian immunity, in order to improve our knowledge of Aboriginal immunological responses to infectious diseases and HCV.

We hypothesize that First Nation and Caucasian cells will have different cellular immune responses to HCV antigens, as defined by cytokine production levels. Additionally, we aim to determine the impact of ethnicity on the relationship between *in vitro* cytokine production and SNP expression.

A cellular model was used to evaluate the impact of HCV proteins on innate responses using freshly isolated peripheral blood mononuclear cells (PBMC) culture from healthy HCV naive individuals (117). This enabled us to characterize the immune responses of First Nation and Caucasian individuals by measuring pro-inflammatory (IFN-γ and IL-6) and anti-inflammatory (IL-10) cytokine production. We characterized the influence of ethnicity on cytokine responses to viral and bacterial stimulants, HCV proteins (Core, NS3 and NS4) alone, and in combination with other exogenous viral stimulants (Poly: IC, IFN-α). The influence of ethnicity was evaluated.

A major strength of this study is the utilization of a cellular culture model derived from healthy individuals. This gave us insight to the model of the earliest immune events upon infection. Moreover we measured actively produced cytokine
responses to HCV antigens, as opposed to mRNA cytokine levels. This study also compares in vitro cytokine function to genetic levels, to enhance understanding of ethnic immune response.
7. MATERIALS AND METHODS

7.1 Participants

Blood was collected from approximately 70 healthy individuals, who were randomly recruited in response to local advertisement. Selection was based on no evidence of substance abuse, non-diabetic, cancer or autoimmune diseases. They were divided into two groups, based on Caucasian or First Nation ethnicity. The Caucasian cohort was recruited from Winnipeg, Manitoba, and consisted of 10 men and 31 women. They had a mean age of 37 ± 10 years, ranging between 21 and 60 years and a mean BMI of 30. The Aboriginal cohort was recruited from a Northern Manitoba First Nation community and consisted of 12 men and 17 women. They had a mean age of 34 ± 10 years, ranging from 20 to 57 years, and a mean BMI of 26 (refer to Table 1). Participants gave written informed consent and this study was approved by the University Of Manitoba Faculty Of Medicine Committee on the Use of Human Subjects in Research. In addition, First Nation cohorts were established after discussion with First Nation Community Health Authority and approval from their chief of council.

Table 2: Subject characteristics

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>First Nation (FN)</th>
<th>Caucasian (CA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>29</td>
<td>41</td>
</tr>
<tr>
<td>% Males</td>
<td>41</td>
<td>24</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>34 ± 10</td>
<td>37 ± 10</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>30 ± 7</td>
<td>26 ± 6*</td>
</tr>
</tbody>
</table>

* values represent mean ± SD, * p < 0.05
7.2 Peripheral Blood Mononuclear Cells (PBMC) Isolation

Whole blood (40 ml) was collected into 10 ml EDTA tubes (BD, Franklin Lakes, New Jersey). Caucasians samples were collected locally, and stored at room temperature up to 5 hours until isolated. First Nation blood samples where collected at a Northern Manitoba community, and transported within a temperature and pressure controlled box. These blood samples were flown to Winnipeg at 16000 - 18000 ft in a PA226 Swearingen metroliner aircraft. It took approximately 5 hours, from sample collection, to arrival at our lab, where PBMC isolation was done in conjunction with Caucasian samples.

Human PBMC were obtained from whole blood, diluted with 0.85 % sodium chloride (NaCl) (Fisher Scientific, Whitby, ON) by density centrifugation on Ficoll Histopaque-1077 (Sigma, St Louis, MO) for 30 minutes at 1600 rpm (Centra GP83, Thermo IEC, Needham heights, MA). Following aspiration of plasma, cells were collected from interface, washed with saline and centrifuged twice at 1100 rpm for 10 minutes. Cells were then re-suspended in 4 ml RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY) supplemented 10% (v/v) fetal calf serum (Invitrogen), 1 % penicillin-streptomycin-glutamine (Invitrogen) and 2-ME (55 μM, Invitrogen). Cells were subsequently counted (> 99 % viability as determined by trypan blue exclusion) using a hemacytometer.
7.3 PBMC culture

To generate supernatants for measuring the level of cytokines, we used an *in vitro* primary culture system with freshly isolated PBMC from blood acquired from healthy First Nation and Caucasian individuals. PBMC were seeded at a concentration of $2.5 \times 10^6$ cells per well, in a final volume of 200 μl in 96-well round bottom plates (Corning Inc, Corning, NY). Generally, in the same plate, PBMC were cultured with all or a combination of bacterial and viral agents, IFN-α, HCV proteins with IFN-α or viral agents and toxins (refer to table 2). The rational for these reagents is explained in results section. The culture system was optimized using a range of concentrations for lipopolysaccharide (LPS, 1 ng/ml and 1 μg/ml), Toxins (Pertusis and Tetanus, 1, 10 or 20 μg/ml, Diphtheria 1, 10 or 100 μg/ml) and Reovirus serotype Type 1 Lang (T1L) and Type 3 Dearing (T3D) ($10^5 - 10^7$ PFU/ml). Cell cultures were maintained at 37°C for 1 to 6 days. Cultures were established to allow harvest of supernatant at days 1 and 6. We chose these days to look at early innate responses to the reagents. Recombinant HCV proteins Core, NS3 or NS4 used correspond to amino acids 2-192, 1450-1643 and 1658-1863 respectively of HCV polyprotein (Virogen, Watertown, MA). Proteins were fused at the N-terminus with betagalactosidase ($\beta$gal). Proteins were > 95 % pure evaluated by SDS-PAGE. Supernatants were collected, sealed and stored at -20°C until analysis by enzyme linked immunosorbent assay (ELISA).
Table 3: List of reagents used in primary PBMC culture

<table>
<thead>
<tr>
<th>Bacterial Stimulants</th>
<th>Final Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipopolysaccharide (LPS)</td>
<td>1 ng/ml and 1 µg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td><em>Staphylococcus aureus cowen</em> (SAC)</td>
<td>0.016 %</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Peptidoglycan (PGN)</td>
<td>0.01 mg/ml</td>
<td>Fluka</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T cell Activation Molecules</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concavalin A (Con A)</td>
<td>5 µg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Diphtheria Toxin (DT)</td>
<td>1 µg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Pertusis Toxin (PT)</td>
<td>1 µg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tetanus Toxin (TT)</td>
<td>1 µg/ml</td>
<td>Sigma-Aldrich</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Viral-like Stimulants</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>r IFN-α</td>
<td>1000 IU/ml</td>
<td>PBL Biomedical</td>
</tr>
<tr>
<td>Polyinosinic-polycytidylic acid (Poly IC)</td>
<td>50 µg/ml</td>
<td>ICN Pharmaceuticals</td>
</tr>
<tr>
<td>Reovirus Type 3 Dearing (T3)</td>
<td>10⁷ and 10⁵ PFU/ml</td>
<td>Dr. Kevin Coombs, University of Manitoba</td>
</tr>
<tr>
<td>&amp; Type 1 Lang (T1L)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recombinant viral proteins</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>βgal, HCV Core, NS3 or NS4</td>
<td>1 µg/ml</td>
<td>Virogen</td>
</tr>
</tbody>
</table>
7.4 Cytokine Enzyme Linked Immunosorbent Assays (ELISA)

The levels of IL-10, IL-6, IFN-γ and IFN-α cytokines in the supernatants that were collected from cell cultures were determined by sandwich ELISA technique. ELISA plates (Corning) were coated with primary antibody overnight at 4°C in a moist box with carbonate-bicarbonate buffer (0.02 % NaN₃) with a final pH of 9.6. A buffer solution (0.17 % bovine serum albumin (BSA), phosphate buffered saline (PBS), 0.02 % NaN₃, pH 7.4) was added to all wells and incubated for 2 hours at 37°C, to block the plates for binding other proteins. Plates were then washed three times (ELx405 Autoplate washer, Biotek Instruments, Inc., Winooski, VT) with wash buffer (PBS, 0.05 % Tween 20, 0.02 % NaN₃, pH 7.4). Recombinant cytokine was applied to plate and titrated 2 fold in dilution buffer (0.085 % BSA, 0.05 % Tween 20, PBS, 0.02 % NaN₃, pH 7.4) for 12 wells. Samples were appropriately diluted in dilution buffer and then titrated 2 fold for 3 wells. Dilution buffer was applied to all remaining wells. Samples were incubated in a moist box overnight at 4°C. Plates were then washed and biotinylated detection antibody diluted in dilution buffer was added to all wells. Plates were incubated for 3 hours at 37°C. After washing, streptavidin-alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) in dilution buffer was added to all wells and incubated for 45 minutes at 37°C. P-nitrophenol phosphate tablets (Sigma) dissolved in substrate buffer (MgCl₂·6H₂O, 9.7 % diethanolamine (Fisher), pH 9.8) was then added to plates. Plates were read at 405- 690 nm (ELx808, KC4, Biotek Instruments, Inc.) after the appropriate time of development. Recombinant capture and biotinylated detection antibodies for IL-10, IFN-γ and...
IL-6 were obtained from Biolegend (San Diego, CA), while IFN-α antibodies were obtained from Endogen (Rockford, IL). Recombinant proteins for IFN-γ and IL-10 assays were made by SAC-stimulated (Sigma) PBMC supernatant, calibrated against recombinant human IL-10 (Preprotech Canada Inc. Ottawa, ON) or IFN-γ (BD bioscience). IL-10 standard was prepared by Dr. Suresh Khatkar in Dr. Julia Rempel’s laboratory. IL-6 and IFN-α standard were obtained from Preprotech and PBL biomedicals respectively. See Table 4 for standard concentrations, sample dilutions and final sensitivity.

Apart from IFN-γ ELISAs, other immunoassays were conducted with the aid of Dr. Suresh Khatkar and Eng Piew Kok.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Standard Concentrations (pg/ml)</th>
<th>Sample Dilutions</th>
<th>Final Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>2000</td>
<td>1/2</td>
<td>5.72 pg/ml</td>
</tr>
<tr>
<td>IL-10</td>
<td>1000</td>
<td>1/2</td>
<td>15 pg/ml</td>
</tr>
<tr>
<td>IFN-α</td>
<td>1000</td>
<td>1/2</td>
<td>3.5 pg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>8000</td>
<td>1/4</td>
<td>23 pg/ml</td>
</tr>
</tbody>
</table>
7.5 Cytokine single nucleotide polymorphisms (SNP)

To investigate the distribution of cytokine polymorphic genotypes, Dr. Rempel and the research nurses collected blood from healthy First Nations (n = 89) and Caucasians (n = 100). Participants for genotyping were selected based on testing negative for HCV and HBV exposure. A sub cohort was evaluated for cytokine production blinded to genotype data. The blood was sent out to the Canadian Blood Services – Immunogenetics lab for genotyping performed by Iga Dembinski. Single nucleotide substitutions at the positions -592, -819 and -1082 in the IL-10 promoter, -176 in the IL-6 promoter and +874 in the IFN-γ intron 1 were determined as previously described (113). Briefly, DNA was extracted (QIAGEN protease digestion, manufacturers directions; Qiagen, Mississauga, ON) and purified (UV spectroscopy, BioRad, Mississauga, ON) from whole blood collected from healthy volunteers. Polymorphisms for IL-10, IL-6 and IFN-γ were determined using the ‘Cytokine Genotyping Tray’ (One Lambda, Inc., Canoga Park, CA) per manufacturer’s instruction. DNA bands were visualized with a UV transilluminator and photographed for further analysis. Genetic phenotypes were assigned according to previously defined allele and functional cytokine production associations (Table 1).
7.6 Statistical analysis

All samples were evaluated in at least two cytokine assays. The concentration of cytokines in each supernatant was calculated from a minimum of three points falling on the linear portion of titration curves calibrated against recombinant cytokine standards serially diluted on each plate. CM and LPS were used as internal negative and positive controls, respectively for every experiment. Data used in analysis was included if the ratio of IL-10 positive: negative control was greater than 2 fold, CM less than 500 pg/ml or LPS greater than 200 pg/ml. To test for differences in means, cytokine values were log transformed. Original rather than log transformed data was presented for clarity. The mean values for cytokine production were compared using the student's t test or one way analysis of variance (ANOVA), where appropriate.

Genotype frequencies were compared using the Fisher's exact or the \( X^2 \) test, as appropriate. Linear regression analysis examined the relationship between genotype and \textit{in vitro} cytokine production. Repeated measures ANOVA examined the differences in cytokine production between populations.

Univariate analysis was used to examine the significance of the interaction of cytokine polymorphisms and ethnicity on cytokine production. Statistical analyses were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL) statistical analysis software. GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA) was used to make figures. All \( p \) values were two tailed and a \( p \) value of 0.05 or less was considered significant.
8. RESULTS

8.1 Introduction

Cytokines are known to play a key role in HCV disease pathogenesis. Enhanced \textit{in vitro} IL-10 and expression of polymorphisms resulting in high IL-10 production are linked with viral persistence (72, 100 - 102). Other studies have reported differential cytokine genetic polymorphisms can differ between ethnic groups (109 – 114, 125 - 127). African American ethnicity has been linked with a higher rate of HCV, compared with individuals of South East Asian (SEA) or Caucasian origin, irrespective e of HCV genotype (74 - 78). Interestingly, although African Americans show a higher prevalence of HCV, they exhibit a slower progression to chronicity (76).

Our population of interest is the Canadian Aboriginals, which make up 13% of Manitoba’s population (86). Despite socioeconomic status similar to African Americans, and a higher incidence of acute HCV among Aboriginals (Aboriginal = 1 – 18%, Canadian = 0.5 – 2%), Aboriginals seem to have a higher rate of self limited infection (93, 153, 155). Our goals are thus as follows;

1) Improve general understanding of early immune responses against Infectious agents in CA and FN. There are several reports on CA immunity in terms of immune response to non-specific mitogens or specific antigens, while reports on FN are usually limited to epidemiologic data. We would specifically evaluate responses to;
a) Bacterial agents; LPS, PGN (Innate immunity), DT, PT, TT (adaptive immunity) and SAC (both),
b) Viral agents; Poly IC (viral replication intermediate), Reovirus (both) and
c) IFN-α responsiveness.

2) Evaluate FN PBMC immune cytokine responses to HCV proteins in comparison to CA controls. Our focus here will be on
   a) Effect of HCV proteins (Core, NS3 and NS4) on cytokines, and
   b) Effect of HCV proteins on IFN-α responsiveness.

3) Evaluate the interaction between cytokines, genetics and ethnicity. Our focus here will be on IL-10, IL-6 and IFN-γ cytokines.

Our overall aim is to define the immunological response of FN cells and examine how they differ from CA immune response by examining cytokine profiles. Our cytokines of interest have been shown to have an impact on HCV disease progression, thus will provide us with better understanding of ethnic response to HCV. In addition, by correlating cytokine genetic polymorphism to in vitro functional cytokine release, we aim to identify possible mechanisms responsible for the epidemiological differences in the occurrence of infectious diseases, particularly HCV in these peoples.
8.1.2 **Allele frequencies of IL-10, IL-6 and IFN-γ polymorphisms differ significantly between FN and CA populations**

A recent study by Larcombe *et al.*, investigated the distribution of cytokine polymorphic genotypes, between Canadian Aboriginals and Caucasians. The individuals in this study included end stage renal disease (ESRD) patients as well as healthy individuals. It was observed that Canadian Caucasians had a significantly lower propensity for IL-10, IFN-γ and TNF-α, and a lower propensity for IL-6 compared to Canadian Aboriginals (113). A study of IL-10 polymorphisms among Norwegian Caucasians and Aboriginals (Sami) had similar findings (114).

To investigate the distribution of cytokine polymorphic genotypes, Dr. Rempel and the research nurses collected blood from healthy FN (n=89) and CA (n=101). The blood was sent to the Canadian Blood Services for genotyping performed by Iga Dembinski. Genomic DNA was isolated from their PBMC, and SNPs for cytokines (IL-10, IFN-γ and IL-6) were determined using a commercially available PCR-SSP kit.

The distribution of cytokine polymorphic genotypes among FN and CA populations are shown in Table 5. As shown in Figure 3, FN individuals have a significantly lower frequency of IL-10 1082 High (FN = 4% vs. CA = 27%), IL-10 -819/-592 (FN = 29% vs. CA = 66) and IFN-γ +874 (FN = 4% vs. CA = 21%) producer phenotype. Interestingly, only 1 FN individual with a low IL-6 176 phenotype was detected (Fig. 3).
These differences in genetic cytokine distributions are significant and may help explain the disparity in population disease inheritance.
Table 5: Percentage genotypes of IL-10, IL-6 and IFN-γ polymorphisms in a healthy Canadian FN and CA cohort: Genetic analysis

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>First Nation (n = 89)</th>
<th>Caucasian (n = 100)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 592CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>29</td>
<td>66</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>CA</td>
<td>52</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>- 819CT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>29</td>
<td>66</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>CT</td>
<td>52</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>19</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>- 1082GA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>4</td>
<td>27</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>GA</td>
<td>31</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>64</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 176GC¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>85</td>
<td>41</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>GC</td>
<td>13</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>1</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- 874TA¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>4</td>
<td>21</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>TA</td>
<td>24</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>72</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

¹Caucasian sample numbers reduced for IL-6 (n = 99) and IFN-γ (n = 97)
Figure 3: Healthy FN individuals have a lower frequency of High IL-10 and IFN-γ producer phenotype. Single nucleotide polymorphisms (SNPs) for IL-10 -819 C/T, -592 A/C, -1082 G/A, IFN-γ +874 T/A or IL-6 -174 G/C were determined by a PCR-SSP kit, and classified into High, Intermediate or Low phenotypes. 189 virally naive individuals (FN = 89, CA = 100) were enrolled into the study. There is a significant difference ($p < 0.001$) between ethnic group and % allelic distributions. $p$ values were calculated using a $X^2$ test.
8.2 Comparison of bacterial ligand induced cytokine secretion between First Nation and Caucasian PBMC

To investigate immunity against bacterial agents, cytokine production from CA or FN cells were measured for IL-10, IFN-γ and IL-6. The purpose of these experiments is to shed more light on FN immune response, utilizing cytokine profiles as a measurement. Moreover evaluation of bacterial ligands (widely documented among CA) serves as a control to general immune response.

PBMC from healthy FN and CA (control) were cultured with medium alone, LPS (monocyte activator), SAC (activator of monocytes and lymphocytes) (123) or PGN (monocyte activator) (124). Cytokine levels were measured from supernatants harvested at day 1 and 6 by ELISA. Supernatants were harvested at day 1 to help us evaluate early innate immune response, while day 6 showcases innate, as well as the onset of adaptive immune response. In addition, day 6 analysis can address whether early innate immune response is maintained, or decreases after the initial burst.

Bacterial-ligand induced IL-10 expression was examined. Low levels of IL-10 were seen in unstimulated PBMC at day 1 (FN = 109 ± 22 and CA = 201 ± 40 pg/ml; Fig. 4). This spontaneous secretion was enhanced by the addition of LPS, with the greatest impact seen with 1μg/ml LPS (FN = 11 and CA = 7 fold increase, p < 0.001). PBMC also secreted higher levels of IL-10 in response to SAC and PGN, compared to medium control. FN cells produced two fold higher SAC induced IL-10 compared to CA (FN = 766
± 163 and CA = 368 ± 66 pg/ml, p = NS). Similar trends were observed at day 6, however overall IL-10 synthesis was lower at this time point (Fig. 4).
Figure 4: Bacterial stimulants enhance PBMC IL-10 synthesis.
FN (n = 29) and CA (n = 41) PBMC were stimulated in vitro with CM (medium), 1µg/ml or 1ng/ml LPS, 0.016% SAC or 0.01 mg/ml PGN. Supernatants were collected at 24 hours (top panel) and day 6 (bottom panel). IL-10 production was determined by ELISA. Bars represent mean values ± SEM. p values within cohorts comparing bacterial agent with medium (** p < 0.01, *** p < 0.001). Differences between FN & CA cohorts are indicated by (# p < 0.05). Statistical significance were calculated using ANOVA.
Since bacterial stimulants were found to induce IL-10 to a similar degree in both populations, we examined what effect they would have on pro-inflammatory cytokines; IFN-γ and IL-6. FN cells generally secreted lower levels of IFN-γ under all conditions (e.g. CM alone FN = 25 ± 6 and CA = 42 ± 10 pg/ml). Interestingly overall IFN-γ synthesis is significantly enhanced at day 6 compared to day 1 (Fig. 5). LPS and PGN induction of IFN-γ at day 1 is mainly due to interaction with TLR4 and TLR2 (innate) respectively, while day 6 is due to the combined action of NK (innate) as well as Th1 lymphocytes (adaptive).

Microbial agents influenced IL-6 synthesis to a similar degree in both cohorts. The greatest impact was seen with the addition of 1 μg/ml LPS, resulting in a 13 and 12 fold increases within FN and CA cells respectively, compared to CM at day 1 (FN = 1956 ± 754 and CA = 2023 ± 496 pg/ml). Similar to IFN-γ, there was an increase in overall IL-6 levels after 6 days of culture (Fig. 6).

Taken together, bacterial stimulation induced enhanced levels of both pro and anti inflammatory cytokines. The most potent responses were seen in response to LPS which is a TLR4 ligand. Decreased levels of IL-10 observed at day 6 may be due to the inhibitory effect of bacterial agent induced inflammatory cytokines (particularly IFN-γ) on IL-10 action. In addition, there were limited differences between populations in response to bacterial products.
Figure 5: FN and CA PBMC secrete similar levels of IFN-γ in response to bacterial ligands. FN (n = 23) and CA (n = 41) PBMC were stimulated in vitro with CM (medium), 1μg/ml or 1ng/ml LPS, 0.016% SAC or 0.01 mg/ml PGN. Supernatants were collected at 24 hours (top panel) and Day 6 (bottom panel). IFN-γ production was determined by ELISA. Bars represent mean ± SEM. p values within cohorts (** p < 0.01, *** p < 0.001) or between cohorts (# p < 0.05) were calculated using ANOVA.
Figure 6: No significant differences in IL-6 production between populations, in response to bacterial antigens. FN (n = 28) and CA (n = 36) PBMC were stimulated in vitro with CM (medium), 1μg/ml or 1ng/ml LPS, 0.016% SAC or 0.01 μg/ml PGN. Supernatants were collected at 24 hours (top panel) and Day 6 (bottom panel). IL-6 production was determined by ELISA. Bars represent mean ± SEM. p values within cohorts comparing bacterial agents with medium(* p < 0.05, ** p < 0.01, *** p < 0.001) were calculated using ANOVA.
8.2.1 Impact of Bacterial Toxins on FN and CA cytokine responses

In the previous section, we showed no population differences to non-specific microbial stimulants (LPS, PGN). However CA cells showed a more robust IFN-γ specific T cell response to SAC at day 6. In order to further evaluate T cell specific responses, bacterial toxins from presumably previous exposure (via vaccination or infection) were examined. Here we used toxins from diphtheria (DT), pertusis (PT) and tetanus (TT).

PBMC were cultured with medium, 1 μg/ml DT, PT or TT, and supernatants collected after 24 hours. IL-10, IFN-γ and IL-6 production were determined by ELISA.

DT did not enhance IL-10 synthesis in both cohorts. FN produce significantly lower levels of spontaneous IL-10 synthesis. The most potent IL-10 production was seen in response to PT, with approximately 4 fold increases seen in both populations (Fig. 7A).

FN cells generally did not produce IFN-γ in response to DT and TT. In contrast, PBMC isolated from CA exhibited enhanced antigenic T cell response in the presence of DT (6 fold) and TT (4 fold), compared to medium control. FN cells show a trend towards reduced antigen specific T cell IFN-γ synthesis relative to CA cells, and this relationship was significant with DT stimulation (FN = 5 and CA = 71 pg/ml; p = 0.014, Fig. 7B).

DT and TT did not enhance IL-6 production by CA cells. On the contrary, FN cells demonstrated a 5 and 2 fold increase in IL-6 synthesis (Fig. 7C).
Taken together, there is no difference in non-specific (LPS, PGN) microbial induction of cytokine production. However, CA cells seem to have more robust IL-10 and IFN-γ recall responses to microbial agents (SAC, toxins). This data suggest that CA individuals may show enhanced immune clearance upon secondary exposure of a bacterial infection. This is particularly significant, due to the higher burden of bacterial infectious diseases among FN peoples.
Figure 7: FN cells exhibit significantly lower levels of DT induced IFN-γ or IL-10 synthesis, compared to CA. FN (n = 16) and CA (n = 26) PBMC were stimulated in vitro with or without 1 µg/ml Diptheria toxin (DT), Pertussis (PT) or Tetanus (TT) toxins for 24 hours. A, IL-10, B, IFN-γ and C, IL-6 production was determined by ELISA. Data points represent mean values obtained for an individual subject, with group medians indicated by a horizontal bar. p values within cohorts for differences between toxins and medium were calculated using Wilcoxon signed rank test (* p < 0.05, ** p < 0.01, *** p < 0.001). P values between FN and CA cohorts were calculated using Mann-Whitney U test (# p < 0.05).
8.3 **Viral induced cytokine secretion between First Nation and Caucasian**

We have already shown that similar cytokine responses were observed between FN and CA to nonspecific bacterial agents, while CA showed more robust T cell specific responses. Our next step is to observe if there is a similar trend in viral stimulants.

First, we wanted to determine the ability of FN and CA PBMC to produce IFN-α in response to viral intermediates, in order to evaluate early response to viruses. Cells were cultured with 50 μg/ml Poly: IC (double stranded RNA mimic) and 10⁷ or 10⁵ PFU/ml Reovirus T3D (ubiquitous double strand RNA viruses). IFN-α levels are measured by ELISA on supernatants harvested at 24 hours. Viral agents induced similar levels of IFN-α in both populations (Fig. 8). IFN-α synthesis is enhanced by 4 and 11 fold in CA cells, while slightly higher fold increases of 19 and 14 were observed in FN cells, in response to Poly: IC and 10⁷ PFU/ml T3D respectively. At the lower T3D concentration, there was no effect on IFN-α production (Fig. 8).
Figure 8: Similar IFN-α synthesis from FN and CA PBMC. FN (n = 11) and CA (n = 10) PBMC were stimulated in vitro with medium alone, 50 μg/ml Poly: IC, 10⁷ and 10⁵ PFU/ml Reovirus T3D. Supernatants were collected at 24 hours and IFN-α production determined by ELISA. Data points represent mean values obtained for an individual subject, with group medians indicated by a horizontal bar. No statistical differences were found using a Mann-Whitney U test.
We went on to evaluate the effect of viral agents on IL-10, IFN-γ and IL-6 production. These cytokines were used because they have all been shown to play a role in general viral pathogenesis, and particularly HCV pathogenesis. Cells were cultured with medium alone, 50 µg/ml Poly: IC (double stranded RNA mimic) and 10⁷ or 10⁵ PFU/ml Reovirus T3D (ubiquitous double strand RNA viruses). Cytokine levels are measured by ELISA on supernatants harvested on day 1 and 6. The results for these experiments are shown in figures 9 – 11.

Unstimulated CA cells exhibit slightly enhanced levels of IL-10 compared to FN (FN = 120 ± 24 and CA = 200 ± 40 pg/ml; Fig. 9). CA cells secrete significantly lower IL-10 when stimulated with the lower concentration of T3D compared to CA (FN = 24 ± 4 and CA = 89 ± 32 pg/ml; p = 0.02). Overall, FN seem to have a more robust IL-10 viral antigen specific (reovirus) response compared to CA, while producing lower levels of IL-10 in the presence of non-specific viral intermediates (Fig. 9).

After 24 hours of culture, there were no significant changes in IFN-γ synthesis within the CA cells, while FN cells were significantly enhanced in reovirus. FN cells secreted significantly lower amounts of Poly: IC induced IFN-γ relative to CA (FN = 24 ± 11 and CA = 82 ± 21; p = 0.008). At day 6, we observe significantly enhanced levels of IFN-γ under all conditions (Fig.10).
IL-6 levels were significantly enhanced (3 fold) in response to Poly: IC, compared to CM (FN = 2199 ± 694 and CA = 1911 ± 423 pg/ml; p < 0.001 both). There were no differences in population responses to IL-6 (Fig.11).

Taken together, CA cells showed higher secretion of IL-10 and IFN-γ in the presence of dsRNA. There was a slight decrease in IL-10 secretion at day 6, which may be in part due to inhibitory action of inflammatory response (IFN-γ). Viral recall responses demonstrate a state of immunosuppression among FN cells, showcased by significantly higher IL-10 responses to reovirus. In addition, although CA cells had more robust innate response to nonspecific viral agents, there were no observed population differences to IFN-γ T cell specific viral response. Higher levels of IL-10 observed among CA to viral intermediates may suppress the immune system thus leading to worse outcomes or delayed clearance of an acute viral infection.
Figure 9: Viral reagents induce CA cells to secrete significantly enhanced levels of IL-10. FN (n = 26) and CA (n = 41) PBMC were stimulated in vitro with CM (medium), 50 μg/ml Poly:IC and 10^7 or 10^5 PFU/ml Reovirus T3D. Supernatants were collected at 24 hours (top panel) and Day 6 (bottom panel). IL-10 production was determined by ELISA. Bars represent mean ± SEM. *p values within cohorts comparing viral agents with medium (* p < 0.05, ** p < 0.01, *** p < 0.001) were calculated using ANOVA. P values between FN and CA cohorts were calculated using ANOVA (# p < 0.05).
Figure 10: Poly:IC stimulated FN cells produce significantly lower levels of IFN-γ. FN (n = 21) and CA (n = 41) PBMC were stimulated in vitro with CM (medium), 50 μg/ml Poly:IC and 10^7 or 10^5 PFU/ml Reovirus T3D. Supernatants were collected at 24 hours (top panel) and Day 6 (bottom panel). IFN-γ production was determined by ELISA. Bars represent mean ± SEM. p values within cohorts comparing viral agents with medium (* p < 0.05, ** p < 0.01, *** p < 0.001) were calculated using ANOVA. P values between FN and CA cohorts were calculated using ANOVA (# p < 0.05).
Figure 11: No significant differences in IL-6 production between cohorts, at both days 1 and 6. FN (n = 25) and CA (n = 36) PBMC were stimulated in vitro with CM (medium), 1000 IU/ml IFN-α, 50 μg/ml and 10^7 or 10^5 PFU/ml Reovirus T3D. Supernatants were collected at 24 hours (top panel) and Day 6 (bottom panel). IL-6 production was determined by ELISA. Bars represent mean ± SEM. p values within cohorts comparing viral agents with medium (* p < 0.05, ** p < 0.01, *** p < 0.001) were calculated using ANOVA.
Finally, we wanted to directly examine the effect that exogenous IFN-α would have on cytokine production. We did this to observe how a naïve immune system will react to anti-viral action. Cells were cultured with or without 1000 IU IFN-α (key role in anti-viral immunity) for 24hrs or 6 days. Supernatants were assayed for IL-10, IFN-γ or IL-6 levels by ELISA. At 24 hrs, FN show a trend towards lower levels of IFN-α induced IL-10 (FN = 91 ± 27 and CA = 212 ± 42 pg/ml; p = 0.04) and IFN-γ (FN = 49 ± 23 and CA = 68 ± 19; p = 0.04). The relationship between IFN-α and IFN-γ is further strengthened after 6 days of culture (p = 0.02). There are no significant differences in IL-6 production between cohorts, in response to IFN-α (Fig. 12).

Taken together FN cells produced lower levels of IFN-γ and IL-10, compared to CA. Overall, our viral results suggest TLR3 mediated differences in IFN-α responsiveness between FN and CA population.

Due to the difference in male/female ratio between populations, and we wanted to ensure that this gender imbalance doesn't alter cytokine production. We thus examined spontaneous IL-10 synthesis within cohorts based on gender. There were no gender differences in IL-10 secretion within FN. However, we observed that CA females secrete significantly lower levels of IL-10 compared to males (females = 152 ± 32 and males = 350 ± 122; p = 0.03, Fig. 13). Similar results were shown in stimulated (LPS) cells. In addition FN males seem to show significantly higher levels of spontaneous IFN-γ compared to females (data not shown). Overall, the data shown suggest that CA IL-10
levels may be artificially skewed towards lower IL-10 production due to a higher percentage of females.
Figure 12: FN PBMC exhibit significantly lower levels of IFN-α induced IL-10 and IFN-γ synthesis. FN (n = 25) and CA (n = 36) PBMC were stimulated *in vitro* with 1000 IU/ml IFN-α. Supernatants were collected at days 1 and 6. A, IL-10, B, IFN-γ and C, IL-6 production was determined by ELISA. Bars represent mean values ± SEM. *p* values within cohorts comparing bacterial toxins with medium (*p* < 0.05, **p** < 0.01, ***p*** < 0.001) were calculated using ANOVA.
Figure 13: Gender disruption may result in overall lower IL-10 levels in CA cohort. FN and CA PBMC were stimulated in vitro with CM (medium). Supernatants were collected at 24 hours, IL-10 production was determined by ELISA. Bars represent mean values ± SEM. p values within cohorts (* p < 0.05) were calculated using students t test.
8.4 Differential cytokine responses to HCV specific antigens

HCV proteins are known to promote viral persistence by modulating the host immune response (61, 62, and 69). HCV Core, NS3 and NS4 proteins have been found to activate monocytes and DC, inducing monocytes to secrete IL-10 (61, 69). Further studies have shown that Core and NS3 cause cellular abnormalities during chronic HCV infection and trigger inflammatory pathways via TLR2 (61, 62).

Our goal in this section is to investigate the impact of HCV proteins on FN PBMC. Secondly, we wanted to evaluate the effect that HCV proteins have on the induction of IFN-α and viral mediated immunity. This will be done by comparing IFN-α, IL-6, IL-10 and IFN-γ synthesis in stimulated FN and CA PBMC. We hypothesized that PBMC from healthy FN and CA would exhibit different cytokine responses to HCV Core, NS3 and NS4 proteins.

8.4.1 In vitro effects of HCV-Core, NS3 and NS4 on spontaneous IL-10, IFN-γ, IFN-α and IL-6 production by PBMC

We collected 40 mls of blood from healthy FN (n = 25) and CA (n = 40) individuals. PBMC were cultured with 1 μg/ml β-gal and β-gal fused HCV Core, NS3 or NS4 protein. ELISA were performed on supernatants collected at days 1 and 6, to determine IL-10, IFN-γ, IFN-α and IL-6 levels.

At both time points, HCV Core and NS3 induced significantly elevated IL-10 responses in both cohorts. Stimulation with HCV Core resulted in up to 3 times IL-10 production,
compared to β-gal control (FN = 157 ± 25 and CA = 401 ± 92; FN = 139 ± 49 and CA = 262 ± 46 pg/ml; days 1 and 6 respectively, p < 0.005). Similarly, at both days 1 and 6, FN PBMC released significantly less spontaneous IL-10 than CA. The same observance was made in the presence of HCV NS3 (FN = 111 ± 18 and CA = 297 ± 51 pg/ml, p = 0.03; FN = 109 ± 34 and CA = 287 ± 61 pg/ml, p = 0.005; days 1 and 6 respectively, p < 0.005) (Fig. 14A).

There is a significant boost in IFN-γ levels within FN and CA populations, when PBMC are stimulated with HCV Core, compared with β-gal at day 1 (FN = 60 ± 21 and CA = 70 ± 16 pg/ml; p < 0.05). These Core-induced IFN-γ levels are further enhanced by 9 and 6 fold, in FN and CA cells respectively, at day 6. However, in contrast to IL-10, there are no marked differences between cohorts, with respect to HCV protein induced IFN-γ production (Fig. 14B).

As shown in Figure 15A, HCV proteins did not seem to alter or induce IFN-α release in either population. In contrast, HCV proteins significantly enhanced IL-6 synthesis in both cohorts. HCV Core alone induced 4 and 2 times more IL-6 in FN and CA respectively, compared to β-gal (FN = 2661 ± 775 and CA = 4352 ± 1420 pg/ml). Similar results were seen in the presence of NS3 and NS4 (Fig.15B).

Taken together, our data suggest that HCV Core induces suppression to a greater extent in CA population.
Figure 14: HCV Core has the highest impact on PBMC IL-10 and IFN-γ synthesis. First Nation (FN, n = 25) and Caucasian (CA, n = 40) PBMC were stimulated in vitro with 1μg/ml βgal, HCV Core, NS3 or NS4. Supernatants were collected at 24 hours and Day 6. A. IL-10 and B. IFN-γ production measured by ELISA. Results indicate medians per cohort. Results indicate means ± SEM, p values within cohorts comparing HCV proteins and βgal control (∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001). Differences between FN and CA cohorts are represented (♯p < 0.05, ♦p < 0.01). Statistical test were calculated using ANOVA.
Figure 15: Ethnicity has limited influence over the impact of HCV protein IFN-α and IL-6 synthesis. First Nation (FN, n = 22) and Caucasian (CA, n = 32) PBMC were stimulated in vitro with 1μg/ml βgal, HCV Core, NS3 or NS4 simultaneously for 24hrs. IFN-α and IL-6 were measured by ELISA. Results indicate means ± SEM.
8.4.2 Effect of HCV Proteins on IFN-α responsiveness

To evaluate the effect of HCV protein IFN-α responsiveness, PBMC were co-cultured with 1000 IU/ml of IFN-α and 1 μg/ml β-gal and β-gal fused HCV Core, NS3 or NS4 protein. Supernatants were collected at days 1 and 6; for determination of IL-10 and IFN-γ production respectively.

IFN-α induced significantly lower levels of IL-10 in FN compared to CA cells at day 1 (FN = 44 ± 13 and CA = 135 ± 36 pg/ml; p = 0.03). Similar results are shown with addition of NS3, but not Core or NS4. In addition, IFN-α induced IL-10 is significantly enhanced by 4 – 3 fold in the presence of HCV Core and NS3. Similar results are seen at day 6 (Fig. 15A). Likewise, at day 1, IFN-α induced IFN-γ shows a trend towards lower production in FN cells compared to CA (FN = 30 ± 17 and FN = 72 ± 20 pg/ml; p = 0.06). However, HCV Core differentially impacts both populations' responses to IFN-α, boosting FN IFN-γ levels 5 fold, and CA levels a mere 2 fold. This trend reaches significance at day 6 (FN = 6 and CA = 2 fold difference compared to βgal, p = 0.007, Fig. 16B).

Our most significant finding in this section is the demonstration of strong early T cell responses to HCV Core among FN. Although this did not reach statistical significance, this phenomenon may provide insight on better HCV clearance rates among these peoples.
**Figure 16:** HCV Core has a lower impact on IFN-α induced IL-10 levels in FN. First Nation (FN, n = 25) and Caucasian (CA, n = 40) PBMC were stimulated *in vitro* with 1000 IU/ml IFN-α and co-stimulated with 1 μg/ml βgal, HCV Core, NS3 or NS4. Supernatants were collected at 24 hours and Day 6. A. IL-10 and B. IFN-γ production measured by ELISA. Results indicate medians per cohort. Results indicate means ± SEM. Results indicate means ± SEM. *p* values within cohorts comparing HCV proteins and IFN-α alone (*p* < 0.05, **p** < 0.01, ***p** < 0.001). Differences between FN and CA cohorts are represented (#p < 0.05, ##p < 0.01, ###p < 0.001). Statistical test were calculated using ANOVA.
8.5 Relationship between cytokine gene polymorphisms and *in vitro* production

A substantial body of research suggests that cytokine gene polymorphisms may influence *in vitro* and *in vivo* cytokine levels. For this reason, studies have been conducted to evaluate if genetic susceptibility for diseases, is related to polymorphisms of cytokine genes.

Our goals within this section are to: a) compare cytokine polymorphisms distributions within our population to previously published data, b) correlate functional cytokine production with *in vitro* cytokine levels and finally c) evaluate the impact of HCV Core and ethnicity on predefined polymorphisms.

A sub-cohort of healthy FN (n = 24) and CA (n = 35) with cytokine genes representative of bigger cohort (Table 5) were selected. The distribution of cytokine polymorphic genotypes among FN and CA populations used for functional studies are shown in Table 6. Generally, FN individuals had a significantly higher frequency of polymorphisms associated with decreased production of IL-10 and IFN-γ. This is discussed in greater detail in further figures. In contrast, there was no FN individual with a low IL-6 176 phenotype was detected (Table 6).
Table 6: Percentage genotypes of IL-10, IL-6 and IFN-γ polymorphisms in a healthy Canadian First Nation (FN) and Caucasian (CA): Functional analysis.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>First Nation (n = 24)</th>
<th>Caucasian (n = 35)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-10</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>592CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>33</td>
<td>57</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>CA</td>
<td>42</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>25</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>819CT</td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>33</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>42</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>25</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1082GA</td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>4</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>21</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>75</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>176GC²</td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>85</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>15</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td><strong>IFN-γ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>874TA²</td>
<td></td>
<td></td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>4</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>8</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>88</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

* Sample numbers reduced for IL-6 (FN, n = 20; CA, n = 27) and IFN-γ (FN, n = 22; CA, n = 34) analysis
8.5.1 Relationship between IL-10 promoter genotype polymorphisms and functional *in vitro* synthesis

IL-10 production is influenced by polymorphisms within its promoter region. There are three well described polymorphisms in the IL-10 gene promoter region, at positions -1082 (GA), -819 (CT) and -592 (CA) (96). Single base pair substitutions at this region have been associated with differential *in vitro* cytokine production. Turner et. al. were the first to find that IL-10 -1082 GG genotype was associated with significantly higher *in vitro* IL-10 production (96). CA PBMC were stimulated with 5 µg/ml Concavalin A (ConA) and ELISA conducted on supernatants collected at 48 hours.

It was observed that allele frequencies of the -819 and -592 alleles in the promoter region of the IL-10 gene were similarly inherited, suggesting a linkage disequilibrium at these loci which has been previously reported (113). Thus for the rest of the text we will refer to –592/819 together. In the entire cohort of 189 healthy individuals we observed that FN individuals had a significantly higher percentage of low phenotypes in both -592/819 (FN = 19% vs. CA = 25%) and -1082 (FN = 64% vs. CA = 25%). CA have a correspondingly higher percentage of high phenotypes among IL-10 -592/819 polymorphism (FN = 29% vs. CA = 66%) and -1082 (FN = 4% vs. CA = 25%) (Fig. 17A) (115). Among our sub-cohort of 59 previously genotyped healthy individuals, we observed a similar trend, with FN having a higher propensity for IL-10 -592/819 (FN = 25% vs. CA = 3%) and -1082 (FN = 75% vs. CA = 29%) low producer phenotype, while CA had a higher propensity for high producer phenotypes (Fig. 17B).
Figure 17: Allelic distribution of IL-10 -592/819 and -1082 gene polymorphisms among First Nation and Caucasian Canadians. A, A group of 189 viral naïve healthy volunteers were divided into two groups based on ethnicity (FN = 89, CA = 100). Single nucleotide polymorphisms (SNPs) for IL-10 -592 A/C, 819 C/T and -1082 G/A were determined by a PCR-SSP kit, and classified into High, Intermediate or Low phenotypes. B, A sub-cohort of previously genotyped individuals (n = 59) were selected based on the absence of chronic afflictions. There is a significant difference between ethnic groups in IL-10 -819/-592 and 1082 allelic expression (p = 0.02, 0.002 respectively, X² test).
Supernatants from PBMC cultured with 5 μg/ml ConA for 24 hours were measured for IL-10 levels. It should be noted that only 1 CA individual had IL-10 -819 low producer (TT) phenotype, thus PBMC culture from that individual was performed in triplicate to facilitate statistical analysis. We found a marked variation in ethnic responses to ConA induced IL-10 synthesis; whereby FN PBMC produced significantly less IL-10 when compared to CA (FN = 106 ± 16 and CA = 277 ± 51 pg/ml; p = 0.005, Fig. 18A).

Our results showed positive correlation between -592/819 (r = 0.147) and -1082 (r = 0.211) polymorphisms with ConA induced IL-10 synthesis in our entire cohort (Fig. 18B). When populations are split based on ethnicity, CA show a positive linear correlation (r = 0.116) between IL-10 -592/819 polymorphisms and Con A induced IL-10 synthesis. These findings are in line with previous publications (96). The relationship between IL-10 polymorphisms and Con A induced synthesis among the FN cohort remains very weak (-592/819; r = 0.03 & -1082 r = 0.002) (Fig. 18C.)

The lack of statistical significance in -1082/IL-10 correlation, upon differentiation based on ethnicity may be in part due to the overall number of individuals being evaluated. Our cohort shows a limited number of FN individuals with a H to I IL-10 -1082 polymorphism.
Figure 18: Relationship between IL-10 promoter genotypes and in vitro synthesis following PBMC ConA stimulation. 

A, Previously genotyped individuals (n = 56) were selected due to the absence of chronic afflictions. Blinded to their genotypes, IL-10 production following ConA (5 µg/ml) stimulation was assessed. CA produce significantly higher amounts of IL-10 compared to FN. Results represent mean ± SE. B, Correlation between IL-10 -592/-819 High (H), Intermediate (I), and Low (L) polymorphisms as defined by SNP and cytokine production as measured by ELISA to. C, Correlation between IL-10 -1082 SNP and cytokine production.
8.5.2 Relationship between HCV Core induced IL-10 synthesis and predefined IL-10 polymorphisms

Many reports have proposed an association between IL-10 genetic polymorphisms and HCV disease outcomes (100,101,105,106,169,170,172).

Studies conducted in Europe and Asia reported that individuals with polymorphisms resulting in lower IL-10 production have a significantly higher rate of self limiting infection, whereas those with propensity for higher IL-10 are linked with persistent infection (100,101,105,169,170). It has also been reported that HCV patients with SVR to IFN-α therapy posses IL-10 polymorphic genes associated with lower IL-10 synthesis, compared to non-responders (100, 101). However other studies showed no significant correlation (106, 172).

We wanted to assess how IL-10 production in response to HCV proteins correlates with IL-10 -819 and -1082 polymorphisms. Supernatants from PBMC cultured with 1 μg/ml β-gal or HCV Core for 24 hours were measured for IL-10 levels.

First, we examined the relationship between IL-10 polymorphisms and spontaneous IL-10 production. It should be noted that only 1 FN individual had IL-10 -1082 high producer (GG) phenotype. Thus we combined high and intermediate (H/I) producers and compared with low (L) when doing FN or inter cohort IL-10 -1082 analysis. There is a weak inverse correlation between producer phenotypes, and functional IL-10 production in both cohorts.
when IL-10 -592/819 (FN; \( r = -0.182 \), CA; \( r = -0.089 \)) and -1082 (FN; \( r = -0.273 \), CA; \( r = -0.089 \)) was observed.

We then proceeded to do a comparison between spontaneous and HCV Core induced cytokine synthesis within each genotypes. An intra-cohort analysis of FN IL-10 -819/592 polymorphisms distribution observed that HCV Core had a significantly higher impact (10 fold increase) on IL-10 High and Low producer phenotypes compared to spontaneous (\( \beta \text{gal}: H = 15 \pm 1, L = 31 \pm 9; \text{Core}: H = 137 \pm 47, L = 227 \pm 60 \text{pg/ml}, p = 0.03, 0.02 \) respectively, Fig. 19A). Among CA, there was a 4 fold increase among the H and I phenotype (\( \beta \text{gal}: H = 115 \pm 28, I = 163 \pm 74; \text{Core}: H = 467 \pm 150, I = 333 \pm 138 \text{pg/ml}, p = 0.02 \), Fig. 19A). Similar analysis was performed for IL-10 -1082 polymorphisms, where it was observed that HCV Core had the highest impact on the L polymorphism in both cohorts (Fig. 19B).

Finally, we wanted to examine the correlation between IL-10 polymorphisms and HCV Core induced IL-10 synthesis. Among FN, there seems to be an inverse relationship between IL-10 polymorphisms and Core induced IL-10 production: -592/819 (\( H = 137 \pm 47, I = 132 \pm 34 \) and \( L = 227 \pm 60 \text{pg/ml}; r = -0.163 \)) and -1082 (\( H/I = 79 \pm 18, L = 183 \pm 32 \text{pg/ml}; r = -0.241 \)) polymorphisms. This association however is weak (Fig.19A). Core induced IL-10 does not correlate with IL-10 polymorphisms within CA cohort (Fig. 19B).
Figure 19: HCV Core induced IL-10 synthesis correlates inversely with pre-defined IL-10 -819 polymorphisms within a FN cohort. IL-10 production following PBMC culture with 1 μg/ml βgal and HCV Core defined by ELISA. SNPs for A, IL-10 592/819 and B, IL-10 1082 were determined by PCR-SSP kit and classified into High (H), High/Intermediate (H/I), Intermediate (I), or Low (L) phenotypes based on their genotype. The mean level of production ± SEM are shown. Statistical analysis was done on log transformed data. Significant differences within cohorts indicated (Linear regression, r values indicated)
These data suggest that the involvement of HCV Core protein and IL-10 SNPs are different from its action with other T cell stimulants or other mitogens. Core interacts more strongly with IL-10 Low producer phenotypes in FN than CA. Conversely, Core interacts more strongly with High producing phenotypes in CA. Our data suggest that HCV is adapting to these populations independently, although it appears more successful in CA.

8.5.3 Effect of viral agent induced IL-10 synthesis on predefined IL-10 polymorphisms

We wanted to assess how viral agents induced IL-10 correlates with IL-10 -592/819 and -1082 polymorphisms, and also compare our results with observations made in HCV specific proteins. Supernatants from PBMC cultured with medium and 1000 IU/ml IFN-α or 50 μg/ml Poly: IC for 24 hours was measured for IL-10 levels.

There was no visible trend between IFN-α induced synthesis and IL-10 polymorphisms. We examined the effect of Poly: IC and ethnicity on functional IL-10 production with respect to pre-defined IL-10 -592/-819 polymorphisms. Both cohorts exhibit an inverse relationship between polymorphisms and functional IL-10, although this association only reaches statistical significance within the FN (H = 93 ± 40, I = 130 ± 37 and L = 450 ± 153 pg/ml; r = - 0.414, p = 0.04, Fig. 20A). A similar relationship was observed between IL-10 -1082 polymorphisms and Poly: IC induced IL-10 (FN; H/I = 100 ± 54 and L = 240 ± 67 pg/ml, r = - 0.432; CA = H = 191 ± 37 and L = 358 ± 89 pg/ml, r = - 0.386; p < 0.05 both, Fig. 20B).
Overall, our data suggest that relationships between cytokine polymorphisms and \textit{in vitro} cytokine production will differ depending on cell stimulants. Till date, the vast majority of evidence collected for the classification IL-10 polymorphisms was done with the use of ConA. In addition, Poly:IC and HCV Core stimulation correlate inversely with IL-10 polymorphisms among FN. This is extremely relevant for HCV pathogenesis where high IL-10 alleles have been linked with worse disease outcomes. Our data implies that FN individuals who have a higher genetic propensity for IL-10, secrete decreased amounts in the presence of viral intermediates. This may be a host mechanism for actively inducing an immune response by inhibiting a suppressed immune state.
Figure 20: Effect of viral reagent induced IL-10 synthesis on predefined IL-10 polymorphisms. IL-10 production following PBMC culture with 1000 IU IFN-α or 50 μg/ml Poly:IC defined by ELISA. SNPs for A, IL-10 592/819 and B, IL-10 1082 were determined by PCR-SSP kit and classified into High (H), High/Intermediate (H/I), Intermediate (I), or Low (L) phenotypes based on their genotype. The mean level of production ± SEM are shown. Statistical analysis was done on log transformed data. Significant differences within cohorts indicated (Linear regression, r values indicated, * p < 0.05, ** p < 0.01).
8.5.4 Correlation between LPS induced IL-6 production and IL-6 (-174) polymorphism.

A polymorphism located at position -174 of the promoter region of the IL-6 gene has been identified (127). Homozygotes with G allele (high producers) appear to have higher plasma IL-6 levels compared to those with a C allele (low producers) (17). Two reports which investigated the frequency of IL-6 genotypes within a large racially diverse cohort found a low frequency of IL-6 low (CC) producer phenotypes among non Caucasians (127, 161). Meenagh et al, showed a complete absence of CC genotype among Asians and Africans.

Our sub-cohort consisting of 47 healthy individuals (FN = 20, CA = 27) was genotyped for IL-6 polymorphisms by Iga Dembinski. We grouped their phenotypes (see Table 4), into High (H) and Intermediate or Low (I/L) producers due to the low occurrence of FN with a Low phenotype. There was equal distribution between phenotypes among the CA population (H = 44%, I/L = 56%, Fig. 21A). A significantly greater proportion of the High allele was represented among FN (p = 0.006, Fishers exact test, Fig. 21A).

Previous studies evaluating the association between IL-6 polymorphisms and *in vitro* production examined plasma from patients suffering from an inflammatory disorder or cells from healthy controls stimulated with LPS (97, 106). To determine if *in vitro* IL-6 production differed between ethnic populations, PBMC from healthy FN and CA individuals were cultured with 1 μg/ml LPS and supernatants collected after 24 hours. IL-6 production was determined by ELISA. Our results showed no difference in LPS induced
IL-6 synthesis between groups (FN = 28642 ± 4606 and CA = 24771 ± 4276 pg/ml; Fig. 21B).

*In vitro* IL-6 production was analyzed against genetic phenotypes. We found no correlation between IL-6 polymorphisms and LPS induced cytokine production within cohorts. The I/L phenotype had slightly less IL-6 than the H, in both cohorts. Upon further analysis, we found no differences when comparing H or I/L phenotypes between FN and CA (Fig. 21C).
Figure 21: Frequency of IL-6 promoter genotype between FN and CA populations, and its association with in vitro synthesis. A, Previously genotyped individuals (n = 47) were selected in a blinded manner for evaluation of cytokine synthesis. There is a significant difference (p = 0.0064, Fishers exact test) in IL-6 174 GC allelic expression between ethnic groups. B, IL-6 production following PBMC stimulation with 1 μg/ml LPS. Results represent mean values + SEM. C, No correlation between IL-6 production as measured by ELISA and High (H) or Intermediate/Low (I/L) IL-6 producer phenotypes.
8.5.5 **IL-6 (-174) High phenotype may have a significantly higher impact on HCV Core induced IL-6 synthesis within First Nations, compared to Caucasians**

It has been well established that the pro-inflammatory properties of IL-6 may promote HCV disease progression. However, there have been limited reports supporting a link between IL-6 genotype polymorphisms and HCV disease outcome. In an experiment comparing IL-6 genotypes between spontaneously cleared Caucasian patients versus those with a persistent infection, Barrett *et. al*, reported a significant relationship between low IL-6 genotypes and spontaneous viral clearance (106).

In order to assess the impact of HCV Core on pre-defined IL-6 - 174 polymorphisms, healthy PBMC were stimulated with 1 µg/ml HCV Core and βgal control. Supernatants were collected after 24 hour culture. IL-6 levels were determined by ELISA.

Linear regression showed a positive correlations between core induced IL-6 production and H or I/L phenotypes within the FN cohort ($H = 111583 \pm 2272$ and $I/L = 5179 \pm 2588$ pg/ml; $r = 0.338$, $p = ns$ Fig. 22B). There was no association observed within the CA cohort (Fig. 22B). Similar results were observed when cells were stimulated with 50 µg/ml Poly: IC (Fig. 22D). Moreover, there was no significant difference in IL-6 synthesis observed between cohorts, irrespective of genotype.
Figure 22: HCV Core has a significantly higher impact on the IL-6 -174 High (GG) phenotype within FN, compared to CA. IL-6 production following PBMC co-culture with 1 μg/ml βgal HCV core1000 IU IFN-α, 50 μg/ml Poly:IC and βgal control defined by ELISA, SNPs for IL-6 174 (G/C) were determined by PCR-SSP kit and classified into High (H) or Intermediate and Low (I/L) phenotypes based on their genotype. The mean level synthesis ± SEM are shown. Numbers on bar graphs indicate ratio of fold difference compared to βgal. Significant differences within cohorts are indicated (* p < 0.05, ** p < 0.01) for linear regression or paired t test in comparison to βgal where applicable.
8.5.6 *In vitro* production of IFN-γ correlates with polymorphisms in IFN-γ + 874 gene

A polymorphism at the +874 position at intron 1 of the IFN-γ gene has been shown to influence the amount of cytokine production (99). A few studies have reported that allelic inheritance may be determined by race. Hoffman *et al.*, found that Asian individuals have a higher proportion of genotypes that result in low (AA) IFN-γ production (111). Similarly, we found that FN individuals had a higher percentage of low producer (AA) phenotype (FN = 87% vs. CA = 24%, p < 0.0001; Fig. 23A)

IFN-γ is produced by T lymphocytes and NK cells. Similar to previous studies, Con A (T cell stimulant) was used to assess in vitro production (99, 179). To evaluate ethnic differences in IFN-γ production, supernatants from Con A-stimulated PBMC from 52 healthy individuals were measured for IFN-γ levels by ELISA. Supernatants were harvested after 24 hrs. Our results showed no difference in ConA induced IFN-γ synthesis between groups (FN = 272 ± 136.9 and CA = 301 ± 158.5 pg/ml; Fig. 23B).

Finally, we analyzed ConA induced IFN-γ production against polymorphisms. There is a positive correlation between *in vitro* production and cytokine polymorphisms within the CA cohort. We were unable to determine r values for the FN cohort, due to insufficient representation among the higher phenotypes (Fig. 23C).
Figure 23: Relationship between ConA induced IFN-γ production and polymorphisms among CA individuals. A, Previously genotyped individuals (n = 52) were selected. CA had a significantly greater frequency of high producer phenotypes compared to FN (p < 0.0001, X²). B, Con A induced IFN-γ synthesis. CA and FN demonstrated similar IFN-γ levels. Results represent mean values ± SEM. C, Positive correlation between IFN-γ production as measured by ELISA to cytokine phenotypes (High, Intermediate or Low) as defined by SNP in CA cohort. This correlation is not statistically significant (p = 0.405)
To assess how HCV Core impacts pre-defined IFN-γ +874 polymorphisms, supernatants from healthy CA PBMC (n = 34) were stimulated with 1 μg/ml βgal or HCV Core and collected after 6 days of culture. IFN-γ synthesis was determined by ELISA.

There is no relationship between Core induced IFN-γ production and predefined polymorphisms. Similar results were shown, in response to IFN-α or Reovirus (Figure 24).

Our data suggest that IFN-γ polymorphisms may not alter viral antigen induced IFN-γ production. This suggests that IFN-γ cytokine polymorphisms may have limited or no influence on host clearance of viral diseases.
Figure 24: Core induced IFN-γ does not correlate with IFN-γ polymorphisms. IFN-γ production following Caucasian PBMC culture with A, 1 μg/ml HCV core, B, 1000 IU IFN-α or C, 10⁷ PFU/ml Reovirus T3D. SNPs for IFN-γ - 874 (T/A) were determined by PCR-SSP kit and classified into High (H), Intermediate (I) or Low (L) phenotypes based on their genotype. The mean level synthesis ± SEM are shown. Significant differences within cohorts compared to βgal are indicated (Paired t test, ** p < 0.01)
8.6 Effect of High Altitude on cytokine response in healthy Caucasian PBMC

There is some evidence to support that high altitude can impact immune function, by increasing circulating inflammatory cytokines such as IL-6 (128,129).

A significant part of this project, involved obtaining whole blood from a Northern Manitoba community, and transporting these samples in an aircraft. We wanted to assess if this mode of transportation impacted cytokine levels. Three CA had blood drawn in Northern Manitoba, and blood samples flown to Winnipeg in an aircraft. Two to three weeks later, blood was drawn from the same individuals locally, and left at room temperature for 5 hours in the lab. At both time points, PBMC were cultured with medium, supernatants were collected after 24 hours and cytokine synthesis was determined by ELISA. In addition, the same two individuals were used as controls; that is their blood was drawn in Winnipeg at the same time as the experimental group. Cells were not stimulated, to give us a true picture of the effect of altitude.

Generally, the comparison of the experimental groups; sea level (SL) and high altitude (HA) did not demonstrate more variation than the comparison of the controls. The variation in controls severely limits the assumptions that can be made regarding the impact of HA on cytokine levels (Fig. 25). Nonetheless, if anything, HA enhances IL-10 production.

This data suggest that FN samples which were flown in through out the duration of the study may have had artificially increased levels of IL-10.
Figure 25: High altitude may increase PBMC IL-10 production
Whole blood was collected from 3 healthy individuals, and either isolated at Sea Level (SL) or transported at high altitude (HA) (16 – 18,000 ft) before isolation. Cytokine levels were determined by ELISA after a 24hour culture. Values are means ± SE.
9. DISCUSSION

9.1 Introduction

The disproportionate occurrence of infectious diseases among some ethnic populations is evidence for the key role of ethnicity in disease susceptibility. Evidence that demonstrates this more conclusively is the rate for several infectious diseases, which are twice as high among African Americans, compared to the entire US population (130). Similarly, studies have reported a higher rate of infectious diseases among Indigenous people of the Americas and Australia (87, 91–93, 131-134 and 153-155). Though over-representation of high risk behavior among these populations contributes to these findings, there is an increasing body of evidence which shows ethnic disparity in infectious disease susceptibility irrespective of environment (87 - 88).

HCV disease outcomes are partly defined by viral factors, including genotype, viral load and duration of infection. Viral genotype plays a key role and this is demonstrated by the 46% rate of clearance in genotype-1 HCV infected patients compared with 80% in genotype 2 or 3 infected patients after combination therapy (135). Host factors also contribute to disease outcomes. High serum levels of IL-10 have been linked with poor treatment outcomes and susceptibility towards recurring infection. In addition some studies have shown an association between patients with genotypes or haplotypes resulting in lower IL-10 with self limiting infection (101, 109).

There is also compelling data supporting the influence of ethnicity in HCV disease outcomes. Epidemiologic data suggest that Native Americans have higher rates of HCV
clearance, compared to other ethnic groups (91 – 93, 153 - 155). Unfortunately, there have been no studies evaluating the role of ethnically distinct immune systems in these reports.

The aim of this project was to determine whether differences exist between FN and CA immune responses, and if these contribute towards epidemiological differences in response to HCV infection. To this end we characterized FN PBMC immune cytokine responses to infectious agents, evaluated the effect of HCV proteins on cytokine production and IFN-α responsiveness. Finally, we evaluated the influence of ethnicity and genetics on cytokine production.
9.2 Differences in cytokine production between populations

Cytokines are known modulators of immune response, and their activity may be influenced by genetic polymorphisms in their genes. Recent reports by Drs. Rempel and Nickerson’s research groups demonstrate that FN individuals have differential cytokine genetic polymorphism profiles compared to CA (113, 114). Various studies have documented the effects of bacterial and viral products on *in vitro* cytokine immune responses. However, the current literature is very sparse and provides no insight into FN cytokine immune responses. Therefore our primary objective at the beginning of this study was to describe FN responses to known inducers of innate and adaptive immunity, and compare these with a CA control.

The bacterial agents used are known inducers of both innate and adaptive immune response. LPS is a component of gram negative bacterial cell wall and a known inducer of inflammatory response (144). SAC is a gram positive bacterial species while PGN is bacterial cell wall component of gram negative bacteria. LPS and PGN are used to evaluate innate immune response; SAC is used to evaluate both responses while toxins are used to evaluate adaptive immune responses. We found that PBMC stimulated with different bacterial products and heat inactivated bacteria induced enhanced levels of pro-inflammatory cytokines. Interestingly, while there was a marked increase in IFN-γ production at day 6, we observed a decrease in IL-10 production. This is expected, as there is generally a shift towards pro-inflammatory cytokines in response to bacterial stimuli. IL-12 is one of the prominent immunoregulatory cytokines involved in host response to mycobacterium, and it’s also known to suppress IL-10 synthesis and action.
Our results showed a delayed onset of bacteria induced IFN-γ synthesis with higher amounts produced at day 6 compared to day 1. IFN-γ is produced initially by NK cells; however a day 6 production is mediated by T cells. There was no difference in FN and CA bacterial ligand induced IL-6 synthesis.

Overall, the most potent bacterial induced cytokine levels were in response to LPS endotoxin, a known inducer of pro inflammatory cytokines (163). The least potent responses were in the presence of PGN, which is a component of gram negative bacteria. This is in accordance to literature where studies in whole blood or monocytes stimulated different strains of bacteria found a 100 or 1000 fold potency of IL-6 or IL-1β in gram positive compared to gram negative bacteria (163). These differences may be partly due to the induction of slightly different signaling pathways. Bacterial LPS signals via TLR4, which is a PRR expressed on a variety of immune cells (144). PGN and SAC are recognized by TLR2 which are expressed on mostly APC and epithelial cells (144). In addition, SAC is also a T cell stimulant. Although both TLR4 and TLR2 signal through MyD88 to stimulate an inflammatory response via NFκB, TLR4 also signals independent of MyD88 to induce inflammatory pathways via NFκB (144). The exacerbation of IFN-γ shown at day 6 in response to bacterial reagents is due to the action of a combination of cells (NK and T cells) to induce an inflammatory environment. Our results, which showed a significantly lower level of bacteria (SAC) and bacterial toxin (DT) induced IFN-γ synthesis among FN maybe a contributing factor towards worse outcomes in response to bacteria. Bacterial toxins were used to evaluate adaptive immunity. We observed more robust IL-10 and IFN-γ recall responses to microbial agents among CA cells. The
depression of IFN-γ exhibited by FN PBMC may contribute towards a defective host immune response to bacterial agents.

Secondly, we examined antiviral responses to viral reagents. Poly: IC is a synthetic virus replication intermediate, which mimics dsRNA and induces an antiviral pathway via TLR3 (20). FN cells produced significantly lower levels of Poly: IC induced IFN-γ, compared to CA. During an acute viral infection, pro-inflammatory cytokines induce NK cells to secrete IFN-γ (164). TLR3 are predominantly found on DC and upon detection of dsRNA, transcription factors in a signaling cascade induce IFN-α production which may result in viral clearance (168). Upon examining the effect of exogenous IFN-α on cytokine production, we observed that FN secretes lower levels of IL-10 and IFN-γ. However endogenous IFN-α is similar between cohorts. IFN-α plays a key role in viral pathogenesis, and is currently used in combination as therapy for HCV. Population differences observed in exogenous IFN-α suggest distinct early population responses to viral agents. A lower level of IL-10 among FN in response to dsRNA or IFN-α implies less immune suppression, increasing their ability to fight viral agents, which may lead to better outcomes in FN during HCV and other infections. On the contrary, higher levels of IL-10 among CA may lead to immune evasion.

On one hand, although IL-10 may decrease viral disease persistence, its immunosuppressive properties may augment susceptibility to microorganisms (177). Based on these results, we may conclude that FN and CA act in an identical manner upon encountering a bacterial antigen. In addition, reported differences between
inheritance of Tuberculosis and other bacterial diseases between populations are probably not due to differences in IL-10 immune response.

Noteworthy is the fact that our CA gender had a higher percentage of females, which may have artificially lowered overall IL-10 levels. Epidemiological studies on the natural course of HCV infection noted that males have a faster rate of HCV progression compared to females. The reason behind this finding is unknown, but scientists speculate that this is in part due to the action of sex hormones. Our data suggest that CA females inherently have lower levels of IL-10 thus are capable of mounting a suitable response against HCV infection, compared to their immunosuppressed male counterparts. This observation gives credence to our finding of overall lower levels of IL-10 among FN individuals, despite artificially lowered levels of IL-10 in a female biased CA cohort.

Overall our data suggest that CA may have strong T cell specific IFN-γ responses to bacterial agents, however they go into a state of immunosuppression or anergy upon exposure from viral agents. This may lead to better outcomes to secondary bacterial infections, and worse outcomes overall to viral agents.
9.3 Impact of HCV Core on population cytokine responses

IL-10 has also been shown to play a key role in certain viral diseases. A recent report examining the role of IL-10 in mice infected with LCMV showed that an increase in IL-10 induced T cell inactivity and viral persistence. Most importantly, T cell activity was returned to optimal functioning capacity, when mice where injected with IL-10 neutralizing antibody (138). In addition, another study which examined the effect of endogenous IL-10 on IFN-α secretion by HSV stimulated PBMC showed that blocking IL-10 increases the number of interferon producing cells and the amount of IFN-α being produced (16). These authors concluded that IL-10 has the capacity to alter early IFN transcription activities, because cells stimulated 4 hours before IL-10 blocking Ab was added had no effect on overall IFN-α production.

IL-10 is one of the key cytokines linked with HCV chronicity due to its immunosuppressive role and its ability to inhibit antigen presentation and T cell proliferation (5). Evidence for this has been demonstrated in studies showing increased serum IL-10 levels in acute patients who develop a chronic infection (165). Another study solidifying the specificity of IL-10 showed that blocking IL-10 receptor lead to enhanced CD4+ T cells from chronic HCV (166).

HCV Core, NS3 and NS4 proteins have been found to induce monocytes to secrete IL-10 and inhibiting DC maturation and allostimulatory capacity (62, 69). Similarly monocyte derived from chronic HCV patients exhibit dysfunctional stimulatory capacities in vitro (167). Recent studies suggest that HCV Core and NS3 can recognize TLR2, triggering an
inflammatory pathways (61, 62). Here, we investigated the effect of HCV proteins on PBMC. HCV Core and NS3 seem to have the highest impact on IL-10 synthesis, with FN cells producing significantly lower levels of IL-10. Similar results were seen when examining the influence of HCV proteins on IFN-α induced cytokine responses.

Our data demonstrates that FN cells secrete lower levels of IL-10 in response to viral non-specific reagents, as well as HCV specific antigens. IL-10 has been shown to affect IFN-α activity, as well as influence viral clearance (16, 138). A lower capacity of HCV Core or NS3 to induce IL-10 supports a higher rate of clearance seen among FN people. It is thus possible, that HCV Core induces signal transducers in a differential manner in both populations.

IL-10 also regulates IFN-α secretion and transcription (138 - 143). This has important ramification for IFN treatment of HCV where high IL-10 has been found in serum and PBMC of patients. It may be possible that the worse outcomes are seen among CA, due to their cells secreting higher IL-10 which in turn interferes with IFN-α signaling and prevents the initial anti-viral pathway.

The low response rates (30 – 50 %) to IFN-α combination therapy suggest that HCV may interfere with IFN-α antiviral pathways in vivo. Decreased levels of STAT1 and STAT3 protein have been seen in chronic HCV infected livers (140,141). Moreover HCV proteins have also been shown to interfere with IFN-α signaling in vitro. HCV Core proteins have been shown to inhibit the Jak-STAT pathway, probably by degrading STAT3 (62, 169). In
addition HCV NS3/4A protease has been shown to block the action of IRF-3, thus interrupting the antiviral pathway (68).

We have also shown no population differences with respect to HCV induced IFN-γ or IL-6 production. IFN-γ is important for innate anti-viral resistance by inducing NK cell activity; its role in HCV disease pathogenesis is still being debated. Previous reports have shown elevated levels of IL-6 during acute and chronic HCV infection. However apart from its role in liver regeneration, it's currently unknown how IL-6 influences HCV disease outcomes. IL-6 inflammatory role may play a role in immune protection during an acute infection; however prolonged action may lead to poor host response to an infection.

Other factors which negatively regulate STAT3 are the suppressor of cytokine signaling (SOCS) proteins. It has been shown that IL-10 can induce expression of SOCS-3 (143). IL-10 may thus play a central role in defining anti-viral responses to HCV protein, by decreasing STAT expression either directly or indirectly via SOCS.

Further studies are needed to show how the inherently higher levels of IL-10 may affect and hinder IFN-α therapy. However, there may be other factors which play attribute to the trends observed (such as interferon stimulatory genes), as cytokine networks are heavily redundant.
9.4 Relationship between HCV Core and predefined genetic polymorphisms

*In vitro* cytokine production is reported to be influenced by polymorphisms within promoter region of the gene. Thus, differential inheritance of cytokine genetic polymorphisms may explain reports on racially diverse populations having statistically different SNP profiles (111,113,114,127).

There seems to be a link between cytokine polymorphisms and HCV disease outcomes; such as HCV susceptibility, rate of disease progression or resistance to anti viral therapy.

A large study (n = 659) done on European CA showed that individuals with IL-10 -819 genotype characterized by lower functional production have a significantly higher rate of self limited infection, and those with IL-10 -1082 phenotype characterized by higher *in vitro* production are linked with persistent infection (100). Similarly, HCV patients with sustained virologic response to IFN-α therapy posses to a greater degree IL-10 -819 or -1082 genotypes resulting in reduced levels, compared to non-responders (101,102). More recently, a large multiethinic study found that SNP from IL-10 haplotypes were associated with HCV clearance among African American, and European Caucasians (105, 170). However other studies have not found a link between IL-10 polymorphisms with HCV disease natural progression (98, 104, 106, 170 – 172).

Our results showed a correlation between genetic and *in vitro* data among CA. FN have a lower genetic propensity for IL-10, and secreted significantly lower levels of ConA induced IL-10 compared to CA (Fig. 17A/B). Similar to some previous publications, we observed a linear relationship between ConA induced IL-10 levels and SNP. To our surprise, FN cells
exhibited an inverse relationship between polymorphisms and Core or Poly IC, while this trend was not seen with ConA. This suggests that viruses might interact more strongly with IL-10 Low producing phenotypes among FN. Our data clearly demonstrates that apart from cytokine polymorphisms, different stimulations also cause differential IL-10 production.

Similar to previously published findings, we observed a positive correlation between CA cytokine genes and ConA induced IFN-γ production. However in the presence of Core, there was no correlation between polymorphisms and cytokine produced. Similarly in the presence of PolyIC and Reovirus all associations were weak. This data provides evidence for the limited role of IFN-γ or its polymorphisms on HCV disease progression.

Barrett et al. reported a significant relationship between low IL-6 CC genotype and spontaneous viral clearance (106). Upon examining IL-6 allelic distributions, we found that FN had a significantly lower percentage of alleles leading to intermediate or low IL-6 production (15% vs. 55%, Fig. 18A). Despite these differences in allelic distribution, LPS stimulated cells from both populations secreted similar amounts of IL-6. We also showed that while HCV Core had a higher impact on High IL-6 phenotype among FN, it interacts identically with both phenotypes in CA. Thus, the ability of Aboriginals to preferentially clear an HCV infection may not be attributable to IL-6 polymorphisms, given that this population has a higher propensity for IL-6. Given that genetic inheritance may be influenced by environment, some may attribute the differential cytokine levels to environmental influences. However, we are assured that this is not the case, as previous
genetic studies showed no differences within Caucasian, Aboriginal or Filipino individuals irrespective of gender, geographic area, or health status (end stage renal disease patients were included (113).

However, we must be cautious when making a case for cytokine SNP in HCV disease pathogenesis. The contradictory nature of the data published, makes it difficult to compare results. The reasons for these contradictions are unknown, but could be attributed to insufficient sample size, inappropriate controls (healthy rather than healthy individuals previously exposed to HCV) or the comparison of different ethnic groups. Furthermore, the majority of studies correlating cytokine genotype with cytokine production were conducted on European Caucasians (96, 97, and 99). This could influence how SNP are being interpreted. In addition, the focus on SNP rather than haplotypes, which are more stable gene elements make it difficult to interpret results (173). Given that an organism’s genotype may not define its haplotype, thus to be cautious it is better to err on the side of haplotypes. Anthropological data suggest that Indigenous people, prior to European contact, were exposed to a limited range of communicable diseases. With the new world order and increased urbanization, it is postulated that newer infectious diseases are only now adapting to FN immunity, albeit unsuccessfully in the case of HCV (88). Based on the opposite relationship between HCV Core and predefined IL-10 polymorphisms, our data may be suggesting that HCV is adapting independently in FN populations.
9.5 Caveats and Limitations

The major caveat to these experiments is the mixture of lymphocytes and monocytes found within our PBMC population. For this reason, it is hard to pinpoint which cells are making IL-10 and clearly define which arm of the immune system is playing a role. We addressed this by collecting supernatants at 24 hours (early innate immune responses) and day 6 (innate and early adaptive immune response). On the other hand, lymphocyte separation techniques have been known to activate cells. This would introduce confounders into our results as reported. A way around this may be the use of intracellular cytokine staining techniques to analyze the percentage of cells producing each cytokine.

Another major problem with the experimental design was the geographical location of our cohorts, which led to subjection of the FN samples to different physical factors. As shown in Figure 25, blood samples transported at a higher altitude seem to have slightly higher levels of IL-10. This suggests that reported FN IL-10 levels may have been artificially enhanced due to flight times. However, despite this finding, FN still exhibited significantly lower levels of IL-10. In addition, due to an over representation of females among the Caucasian cohort, this population's represented IL-10 values may be slightly reduced. In addition data not shown here suggest a difference in IFN-γ production among FN cohorts. This is clear indication of the importance of introducing age, gender and BMI matching, to reduce confounders.
9.6 Global summary

As previously observed, ethnicity may define host outcomes in HCV. In addition, IL-10 has been shown to play a central role in viral persistence. FN cells secrete lower levels of IL-10, in response to HCV proteins and other viral intermediate reagents. Our data suggests that these populations regulate innate responses to viral proteins differently, and this may explain their overall lower rates of chronic disease. Also, the lower levels of IL-10 observed, may be a mechanism accounting for better anti-viral responses in FN, via STAT or SOCS pathways. Further studies, defining the immune cells and transcription factors responsible for these differences, are needed.

Our *in vitro* data correlates with genetic studies which show that FN have a lower propensity for IL-10 secretion. It is well established that differences in cytokine SNP frequencies exist in ethnic populations. This may be as a result of the restricted nature of initial experiments, which were conducted in a homogenous population. In addition, the inverse relationship observed between FN IL-10 SNP and core induced cytokine production; suggest that HCV may have evolved separately in these populations.

More ethnographic studies on pathogen-host immune interactions are needed, to improve our knowledge on the differences that exist at the molecular level. This may provide an explanation for epidemiological trends seen. Better understanding of ethnic responses to HCV may improve patient management for all peoples. Further studies into how cytokine SNP predict HCV disease clearance or treatment outcomes will be useful for identifying patients who are more susceptible to spontaneously clearing infection, thus avoiding adverse treatment side effects.
10. REFERENCES


(19) Bonjardim CA. Interferons (IFNs) are key cytokines in both innate and adaptive antiviral immune responses—and viruses counteract IFN action. Microbes Infect 2005 Mar; 7(3):569-78.


(27) Yokoyama WM, Scalzo AA. Natural killer cell activation receptors in innate immunity to infection. Microbes Infect. 2002 Dec; 4(15):1513-1521.


(137) Edelmann KH, Richardson-Burns S, Alexopoulou L, Tyler KL, Flavell RA, Oldstone MB. Does Toll-like receptor 3 play a biological role in virus infections? Virology 2004 May 1; 322(2):231-238.


(177) Mege JL, Meghari S, Honstetter A, Capo C, Raoult D. Lancet Infect Dis. 2006 Sep; 6(9):557-69