AN ANTHROPOLOGICAL APPROACH TO IMMUNOGENETIC VARIATION IN MANITOBA FIRST NATION POPULATIONS: IMPLICATIONS FOR TUBERCULOSIS

By

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ABSTRACT

This research investigated immunogenetic variability and explored how genetics and the unique histories of First Nations may contribute to differential resistance and/or susceptibility to tuberculosis. With the support of First Nations communities, DNA samples were collected from Dene, Saulteaux, Cree and Caucasian cohorts within Manitoba. Single nucleotide polymorphisms (SNPs) in the cytokines promoter region of IL-12 (rs3212227) and in genes encoding the TLR2 (rs5743708) and TLR4 (rs4986790&4986791) were typed using PCR-RFLP analysis. Compared with the Caucasian and Saulteaux populations, the Dene and Cree were found to have a significantly higher frequency of SNPs associated with IL-12 low expression, while variation within TLRs was not statistically significant. The lower production of the IL-12 has been associated with a down-regulated Th-1 immune response, which is essential for the containment of *Mycobacterium tuberculosis*. First Nations have unique cultural, political and historic identities and the contemporary immunogenetic profiles are likely a reflection of these histories.

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CHAPTER 1 INTRODUCTION

Immunogenetics is a medically focused study that considers the interrelatedness of the immune system and the genes that control it. Differential patterns of susceptibility and/or resistance to infectious diseases can be associated with underlying variations in host genetics. However, the immune system is a complex combination of coordinated efforts by many genes. Research into these mutually dependent relationships has thus far largely focused on the cellular and molecular mechanism aspects, sometimes ignoring the evolutionary selective pressures that have produced them. Anthropology offers immunogenetic analysis a natural fit for collaborative research through the evaluation of macro and micro scales of biological and social change. In the case of disease research, anthropology shares similar aspects with immunology related to disease transmission and pathogen evolution; however with the additional scope of contributing cultural factors. Similarly, while many anthropology studies involve populations where there are increased rates of infectious disease, genetic research generally uses laboratory models which do not necessarily capture a wide spectrum of human diversity. By incorporating these different approaches in an integrative manner, a more holistic picture of genetics, disease and ultimately health can be produced.

Genetic variability between individuals can be the result of a large number of copying errors or mutations during DNA replication. While the majority of these changes are of no biological consequence, some of these variations may give individuals an enhanced survivability to particular aspects of nature, allowing their genetics to be passed on to a larger pool of descendants. However, genetic variations can also hinder survivability if they affect particular functions related to the immune response.

Functional single-nucleotide polymorphisms (SNPs) are the most abundant form of variation in the human genome (Ziegler and Konig 2010) and have been associated with altered gene function potentially resulting in differential disease susceptibility (Bellamy 2003). Any modification related to immune function will have cascade effect further impairing or benefiting the overall response. In the case of SNPs variations that result in an altered functionality, the downstream effect could be sub-optimal macrophage recruitment and resulting in repercussions for successful immunity.

The identification of various SNPs in the promoter regions of chemokine and cytokine receptors genes have been associated with increased susceptibility to tuberculosis in different ethnic populations (Selvaraj 2004). Differences in rates of tuberculosis observed in contemporary populations could be due, in part, to an underlying genetic susceptibility. In Canada, Aboriginal groups experience the highest rates of tuberculosis, being almost 6 times greater than the Canadian rate (PHAC 2010). While these incidence rates are dependent on a large number of factors including many social aspects, there is more at play. Manitoba reports the highest rates of tuberculosis (12.8/100,000) when compared to the rest of the provinces of Canada; however this appears insignificant when it's compared to specific First Nation communities in Manitoba (PHAC 2010). First Nation reserves reporting in the category of high incidence saw a mean annual incidence of tuberculosis reaching 457.4/100,000 in God's Lake Narrows and 336.7/100,000 in Pauingassi (Olson 1999). These rates are alarming, but perhaps maybe more so are the incidence rates categorized as low in Manitoba First Nation reserves, 46.2/100,000 in Cross Lake to 80.4/100,000 in Shamattawa (Olson 1999). The low incidence of tuberculosis on reserve is 3 to 6 times that of the overall

Manitoba rates. While comparatively small population numbers and periods of tuberculosis epidemics are partially responsible for these dramatically high rates on First Nation reserves (Olson 1999), genetics may be able to provide some insight into contributing immunity factors.

Specifically, within these Manitoban First Nation populations, differences in the frequencies of SNPs in certain promoter and regulatory genes involved in the immune response against tuberculosis have been observed (Larcombe et al 2008). These clusters of SNPs could be leading the First Nation populations of Manitoba to have a "diminished effective macrophage immunity towards Mycobacterium tuberculosis (MTB) and may contribute to the observed prevalence of tuberculosis within these communities" (Larcombe et al 2008:1179). This research focused on elucidating the frequency of SNPs found in three genes involved in the immune response and explored their implication in the host's ability to mount a successful immune response against MTB.

The hypothesis for this research was that the Manitoba First Nation populations would maintain a higher frequency of SNPs that may affect macrophage function in response to MTB when compared to the Caucasian population. The genes chosen for this research included Interleukin-12 (IL-12), an important Th1 immune regulator and Toll-like receptors 2 and 4 (TLR2, TLR4), as they would complement the genes previous analyzed by Larcombe and colleagues (2008). The inclusion of these three genes into the emerging immune profile of Manitoba First Nations provided additional insight into the immunogenetics of this group and the further investigation into the balance between the Th1 and Th2 immune pathways. As such, the primary objectives of this study were:

- To detect and document the occurrence of SNPs in the promoter regions of three genes: IL-12 (Genbank number rs3212227), a pro-inflammatory cytokine, and TLR2 (rs5743708) and TLR4 (rs4986790 & rs4986791), pattern recognition receptors in three Manitoba First Nation populations and a Caucasian cohort.
- To investigate how the SNPs may affect the overall immune response to MTB based on information from similar studies (Larcombe et al 2008, Larcombe 2005).
- To relate how the immunogenetic profiles may reflect the unique biocultural and environmental interactions of historical and contemporary Manitoba First Nations.

Human populations adapt biologically to their environments and to the pathogens found within. Ancestral elements of the First Nation populations of Canada can be traced to the Asian continent and have been found in the earliest populations in the New World (Wallace and Torroni 2009). In the New World (the American continent) these populations would have adapted to this local environment in order to survive. Chapter two of this thesis discusses the health of First Nations in the New World from before the arrival of European traders and explorers, throughout contact and the implications of this both from a cultural and biological standpoint. Evidence of First Nation health before the arrival of Europeans has been observed from archaeologically recovered skeletal and mummified remains. Skeletal remains affected by pathogens provide a record of individual health by having particular bone changes resulting from the pathogen environment during life. While early accounts by European explorers report First Nation health as robust and "more healthy than we" (Thwaites 1896-1901:257), the skeletal data reports a differing view. Infectious diseases, nutritional deficiencies, parasite and fungus

infections were only a few of the illnesses that affected the pre contact First Nation populations of the New World (Finch and Waddell 1996, Pfeiffer 1984, Bathurst 2005, Keenleyside 1998).

However, the First Nations populations had become adapted both culturally and biologically to the environment in the New World and it wasn't until the devastating changes resulting from European contact that the First Nation populations began to decline. The demographic collapse of First Nation populations was attributed to the introduction of new diseases carried over with Europeans as well as the immunological naive of First Nations. This immunological naivety has been described as the virgin soil hypothesis, which states that a population is at risk when it was had no previous contact with diseases that they are now infected by, and thus maintain only a minimal immunological defense or memory against them (Crosby 1976). More recently though, it has become apparent that rather than a single factor acting upon First Nation health at the time of European contact, it was a combination of changes to culture, biology and environment that resulted in the high rates of morbidity and mortality.

Contemporary First Nation health can be seen as a reflection of these complex historical influences. In Canada, the Aboriginal population, comprised of First Nations, Metis and Inuit is one of the fastest growing demographics, however as this population continues to expand, the disparity between Aboriginal and non-Aboriginal health status indicators increases (Statistics Canada 2011). New strains of tuberculosis, which were part of the disease mosaic introduced by Europeans, continues to plague the First Nation population of Canada with rates that greatly exceed the national Canadian average (Health Canada 2012). Additionally, First Nations health is also greatly affected by

chronic diseases like diabetes, ensuring that any solution to improving First Nation health will require efforts in eliminating pathogen spread as well as increasing the status of First Nation social determinants of health. These determinants are based on economic and social conditions that can play an important role in shaping overall health (Raphael 2009). Thus to better understand First Nation health, biological factors must be considered along with the social and historical influences.

Common to every living being, the physiological function of the immune system is to prevent and eradicate infections. Chapter 3 examines the manner in which the immune response is mounted against invading pathogens as well as how deviations like genetic variations in particular genes involved in the promotion or regulation of the immune response can affect its success. Generally, the immune response is characterized by the innate and adaptive immune response. Innate immunity provides the initial protection against infection, whereas adaptive immunity develops over an extended time, and therefore, can mediate a more effective and specific defense against infections. The innate immune defense is always present in healthy individuals regardless of the host's prior record of exposure to the pathogen. In contrast, adaptive immunity is a type of host defense that is stimulated by pathogens and retains a certain degree of memory of specific infections. Due to evolutionary pressures, many pathogens have adapted to resist the innate immune response, and genetic changes that influence the immune system can result in increased susceptibility to infections (Bellamy 2003, Hill 2006, Trinchieri 2003). Variations in cytokine expression during the innate and adaptive response to infection can be particularly debilitating in the generation of an optimal immune response. Cytokines are soluble proteins that mediate immune and inflammatory reactions and are responsible

for communications between leukocytes and other cells (Abbas and Lichtman 2010). Communication between cells is of paramount importance in order for the host to mount an optimal immune reaction. Any disturbances in the message relayed between cells will create a cascade effect, which can result in a down regulated or compromised immune response.

The functionality of particular genes resulting in a compromised immunogenetic profiles have been previously associated with differential susceptibly and/or resistances to infectious diseases in a variety of global populations (Flynn and Chan 2001). These population differences can inform researchers about shared or differing historical pathogen relationships. Certain SNPs may occur in regular intervals within a population. Schroder and Schumann (2005) report that certain frequencies of variations in TLR2 and TLR4 occur at 5-10% of all Caucasian populations. However, these variants are absent in Chinese (Ma et al 2010), Japanese (Noguchi et al 2004) and Taiwanese populations (Cheng et al 2007). Anthropologists can use this immunogenetic information to study the migration patterns of different populations as well as use it to measure the degree of separation or familial relationship between two populations that used to be one. Immunogenetics has also recently come to the attention of anthropologists as it can be used to research associations between ethnicity and certain diseases through the analysis of specific genetic markers. Globally, rates of diabetes are continuing to increase, reaching epidemic proportions in certain populations. Several genetic markers including SNPs located in the promoter of IL-12 have been recently associated with a diagnosis of type 1 diabetes (Davoodi-Semiromi 2002). Increasing rate of diabetes have recently become a pressing issue within First Nation communities and health care agencies,

therefore a better understanding of genetic markers and resulting immune response can provide insights into not only infectious disease susceptibility or/and resistance but also into the dynamics of syndemic diseases.

The fields of molecular biology and genetic analysis have made exponential strides over the last few decades. Chapter 4 of this thesis presents the methods used in the amplification, detection and visualization of the SNPs in the three genes analyzed. In the context of genetic epidemiological studies, genotyping is important for the identification of genes that are associated with disease or quantitative traits (Ziegler and Konig 2010). In order to determine genotypes, two different methods, whole blood and buccal swabs, were used in the collection of DNA samples from First Nation cohorts as well as a Caucasian cohort. The First Nation cohorts were from three different reserves in Manitoba, representing the Cree, Dene and Saulteaux people. The DNA results from these cohorts are presented in Chapter 5, where variations in genotypes were observed and the SNP frequencies across all cohorts were calculated. Observed in the SNPs frequency profiles were differences between First Nation cohorts and the Caucasian cohort but also among the First Nation cohorts. The differences in the genetic profiles are potentially a result of the unique evolutionary forces acting upon the different cohorts.

Chapter 6 examines the evolutionary pressures specific to First Nations populations but also the health implications of their emerging immunogenic profile. The unique genetic variations observed in each First Nation population will have an overall effect on the manner and ultimate success of immune response generated. The observed frequencies of SNPs in TLR2, TLR4 and IL-12 will be put into a larger context by examining their impact on the immune response as a whole. These genes are discussed

with results from previous studies (Larcombe et al 2008), where similar SNPs were analyzed in promoter genes and regulators of immunity in First Nation populations. By examining clusters of amplifier and regulator genes, a broader understanding of how and why alterations in immunity can occur. A variety of evolutionary forces have aided in the spread of polymorphisms in genes involved in immunity through positive selection from the interaction between host, pathogen and environment. The differences in gene frequency between different global populations are a reflection of the variations in contemporary and historical evolutionary forces which act or acted upon these populations. Global gene studies can inform on regional responses to specific infectious disease environments or larger scale global health patterns. The immunogenic profiles of Manitoba First Nations are compared to a variety of global gene studies in order to speculate on selective pressures that have helped shape their unique immunogenetic profiles. These selective pressures are part of the distinctive social and environmental niches that are a part of First Nation history in Canada.

This multi-disciplinary approach to illuminating the immunogenetic profiles of Manitoba First Nations explores the relationships between cultural and biological adaptations and complements the existing immunological literature from Indigenous populations. Underlying immunological variations resulting in impaired immune function are one of the factors associated with high rates of tuberculosis in different populations. However identifying and understanding the genetic variations that are responsible for a diminished immune response may ultimately contribute to an avenue for treatment therapy. The study of vacinomics or vaccinations specifically designed to be targeted towards a certain population “takes into account the unique combination of genomes of

the host and the prevalent pathogen" (Moller and Hoal 2010:78). With an increased understanding of which particular genes and SNPs affect the immune response these specific immune pathways can be artificially or chemically strengthened in order to achieve optimal levels of cell mediated responses. While genetic variations can arise randomly, they more often come about due to environmental and evolutionary pressures. By identifying these variations in contemporary populations, it can not only inform us about the health of our ancestors, but also can lead to enhanced treatments to improve the lives of our descendants.

CHAPTER 2 FIRST NATION HEALTH: PRE-CONTACT TO CONTEMPORARY

2.1 INTRODUCTION

The general understanding of being in "good health" is usually describes a state where an individual is free from disease or illness. There are, however, many ways to define health. Understanding health is a complex issue. Being "disease free" does not always adequately define health. The "state of well being" has become included in the board definition of health (WHO 1948). Nearest to this universally accepted definition is the standard guideline reported by the World Health Organization, which first appeared in the preamble to its charter in 1948 (WHO 1948). This definition emphasizes that health draws upon a larger understanding of the social, political, economic, spiritual aspects that, along with biological, all factor into making up "good health".

In Canada, the First Nation population has surpassed the one million mark, making up roughly 4% of the overall population (Tait 2008). This percentage differs between the provinces and territories, with Manitoba and Saskatchewan having the highest proportion of First Nation people amongst all the provinces of Canada (Statistic Canada 2001). Within the province of Manitoba the proportion of First Nation people comprises the majority in the north at 63%, compared to 10.3% in the south and 8.5% in the capital city of Winnipeg (Hallett et al 2006). As this population grows, health status is important to consider as any existing disparities will surely grow as the population grows. The indicators used by Health Canada to measure overall health use a variety of rates (life expectancy, rates of infectious and chronic disease, premature mortality) to assess the health of a population. In Manitoba, as well as for the nation as a whole, the health of

First Nations is disproportionally low compared to non-Aboriginal Canadians. First Nations measured low on several of the indicators used to assess health, likely leading to dying younger, living less years and experiencing higher rates of infectious and chronic disease than their non-Aboriginal counterparts.

The causes of the disproportional burden of disease are multi factorial and include social, biological, environmental, political and economic factors. Understanding the historical context of health can provide some insights into diseases of the past as well as elucidate the roots of some of the present, persistent health problems plaguing First Nations today. The earliest information on First Nation health and culture comes from oral traditions, archaeology and skeletal evidence from the pre-contact period. The analysis of skeletal remains from pre-contact archaeological sites in North America may provide the best insight into past health but some limitations exist. Differential preservation of remains can create sampling biases, but by using archaeology and oral traditions, a more accurate and robust picture of the past health can be reconstructed. Information on the cultural and epidemiological changes that occurred during the fur trade and subsequent contact with Europeans is somewhat more varied, with archaeology, physical anthropology and oral traditions still playing a large role, but with the addition of written reports from traders, missionaries and government officials. The conditions during the pre and post contact period have helped to shape First Nation health today.

2.2 PRE CONTACT HEALTH

The origins of the Indigenous populations in the New World are a matter of some controversy and speculation. Until relatively recently, the dominant explanation for the peopling of the Americas was the Clovis First model. This model states that human

populations travelled across the Beringia landmass around 12,900 years after the last glacial maximum (Schurr 2004). Once through the Bering Strait, these populations followed an ice-free corridor, which extended along the eastern slope of the Rocky Mountains and opened into the interior of the continent (Fagan 2000). Several scholars (Stewart 1973, Black 1975) suggest that the harsh arctic environment of Beringia was unfavourable for the spread of disease and that the earliest migrants passed through a "germ filter" essentially leaving behind the Old World diseases, like tuberculosis, once they crossed.

The number of migrations and subsequent expansions into the New World, from a variety of different entry points, is also a matter of debate. The number of migrations ranges from one to eight or more, based on mitochondrial DNA haplogroups (Mulligan et al 2004, Schurr 2004, Stone and Stoneking 1998). However, there is general agreement that the Eskimo-Aleuts and Na-Dene represent the last significant population expansion (Schurr 2004). Malhi and colleagues (2002) note that early settlement patterns likely led to biological and cultural continuity between modern and archaic groups due to the lessened effect of genetic drift and increased gene flow within local groups. This relatively localized lifeway can be inferred from archaeological excavations as well, through the specialization of specific ecological niches and in the intensification of local resources. All of these changes likely contributed to the formation of regional gene pools (Malhi et al 2002).

2.2.1 Disease in Pre-Contact Populations

Populations in the New World were exposed to a host of disease causing pathogens that were not present in the Old World. Osteological and mummified remains

are two reliable sources of information from which researchers can reconstruct the pre-contact epidemiological profiles of First Nations. Bone is a dynamic tissue that may be formed through initiation by osteoblasts or destroyed by osteoclasts throughout the life of an individual. This ongoing process of bone formation and destruction creates a record of the health that an individual experienced during life. Certain variations in the bone remodeling process can be used to infer information about sex, age, ethnicity, occupation, trauma and disease. However with osteological remains, pathogens only effect changes in the bones after an extended period of time has elapsed since initial infection. In other words, skeletal remains showing bone changes due to pathogens are, in fact, individuals who have survived with the disease for an extended time. Pathogens that cause mortality immediately or within a short period of time will not cause boney changes, which will make these individuals appear as if they have not been affected by the pathogen. This is called the osteological paradox in bioarchaeology and physical anthropology. Wood and colleagues (1992) outline three key issues that can complicate inferring health status from human remains: demographic non-stationarity, selective mortality and hidden heterogeneity in risk. Demographic non-stationarity refers to the age distributions of skeletons in cemetery populations infer more about the fertility levels of the population rather than mortality patterns (Wright and Yoder 2003). In the case of a large age distribution, when more individuals are born and given that a certain number of individuals within a population die each year -a larger age distribution would mean that there were more individuals to begin with, suggesting a higher fertility rate.

The second osteological paradox concerns selective mortality, where individuals experience different levels of health and their history with disease will affect their age at

death (Wood et al 1992). This raises the question: does a skeleton without evident lesions represent a healthy person or a weak person that died quickly after initial infection with a pathogen? A prime example of this issue can be described by the deaths resulting from *Yersinia pestis*. This bacterium was responsible for the Black Death or bubonic plague which killed one quarter to one third of the European population but because this pathogen killed so quickly it did not leave patheopathological evidence. Similarly, of concern are diseases that do not involve the bone. Smallpox and influenza leave no skeletal lesions but are historically responsible for high mortality. The use of molecular analysis in detecting pathogen DNA is one way that this problem has been circumvented.

The last issue articulated the concept of frailty within a population. The factors that create increased susceptibility to illness in a population, such as malnutrition, socio-economic status, and genetics, are not always identifiable. However, evidence of non-specific infectious diseases can provide clues for inferring individual frailty. These non-specific changes might compromise the health of the individual leading to a reduced life-expectancy, for example the skeletal lesions identified as cribra orbitalia, porotic hyperostosis and enamel hypolasia can suggest that the individual suffer from malnutrition and/or parasitic infection. The difficulties in the interpretation of past health from observations made from bone changes, while problematic, can still provide a wealth of information on the health of First Nations before contact. Besides the direct interpretation of the disease load of a population, paleopathological conditions can provide some degree of information on less tangible aspects of social and environmental factors that would have affected an individual during life.

The classification of bone changes in reaction to pathogens allows anthropologists to infer information about health and social activities at an individual, and potentially, population level. For example, environmental living conditions that allow close contact with animals and their waste products can predispose individuals to a variety of zoonotic and parasitic infections. The unique way of life of the pre contact Alaskan Eskimos and Aleuts from Point Hope and Point Barrow on the Northern Alaskan coast demonstrate that close contact with dogs was common in these cultures, largely due to their role in transportation (Keenleyside 1998). This, coupled with aspect of housing and diet, predisposed these peoples to a variety of zoonotic and parasitic infections (White and Folkens 2005, Yesner 1977). Specifically, crowded and poorly ventilated housing combined with the practice of eating raw or undercooked food places individuals at increased risk for developing parasitic infections. Kliks (1990) uses evidence from ancient human coprolites, intestinal contents and organs of preserved bodies to demonstrate that *Enterobius vermicularis* (pinworms), *Trichuris trichiura* (roundworms), diphyllobothrium species (tapeworms) and probably *Trichinella spiralis* (nematode) infected humans in the pre contact New World.

Pre contact Alaskan mummies also provide evidence of a variety of parasitic infections including: *Cypotcotyle lingua*, a fish borne parasite (Zimmerman and Smith 1975); *Trichinella spiralis*, a mammal borne parasite (Zimmerman and Aufderheide 1984) and the tapeworm genus *Echinococuss* (Ortner and Putschar 1981). In skeletal remains, parasitic infections are hard to trace, as the organism and the soft tissue have since decayed, leaving no signs of infection. Skeletal changes and coprolites are used to infer parasitic involvement during the individual's life. Evidence of roundworms,

pinworms and hookworms have all been found within coprolites from pre contact populations in the Americans (Fry 1977, Rheinhard et al 1987, Rheinhard 1992). Associated skeletal changes can come in the form of chronic anemia, a severe form of iron deficiency which often is noted as a negative side effect of parasitic infection in humans and can be identified through certain characteristic bone changes (Aufderheide and Rodriguez-Martin 1998).

Chronic anemia as well as a wide variety of other nutritional irregularities, are all classified as metabolic conditions within the study of paleopathology. Metabolic conditions are particular diseases that may loosely be considered as the abnormalities of deficiency or excess of dietary constituents (Roberts and Manchester 2007:221). Metabolic diseases resulting from nutritional deficiencies can also be used as indicators of stress. Stressors can stem from a variety of sources, however, several commonly associated conditions are: periods of food shortages and weakened immune response due to prolonged assaults and hostile environments. Other stressors resulting from infectious disease in the New World prior to contact could have included a variety of bacteria resulting in tuberculosis and other respiratory infections, treponematosis, gastrointestinal diseases, staphylococcal, streptococcal, and fungal infections (Merbs 1992).

Metabolic bone changes arise from the body's need to redistribute nutrients to areas of high significance. Anemia is somewhat unique from other metabolic conditions because it's often a co-morbidity that arises as a result of other infectious and parasitic agents. The metabolism of iron in the blood stream is a highly adaptable system which adjusts when the host has a high pathogen load. The additional stress on the immune system can cause the body to redistribute iron to areas that are pressed to clear the

infection. Conversely, in body systems that are deemed secondary, iron will be leeched away which can result in bone becoming porotic (Stuart-Macadam 1992). The appearance of the bone can become porotic or spongy which denotes areas of bone depletion where nutrients have been leeched away. When this occurs in the eye orbits, this bone depletion has been categorized as cribra orbialia, however when it occurs on the cranium it is categorized porotic hyperostosis. In the Arctic, cribra orbitalia and porotic hyperostosis have been observed in Eskimo and Aleut populations (Keenleyside 1998). While on the Plains, Finch and Waddell (1996) found a number of individuals from southern Manitoba and Red River region that displayed cribra orbitalia. Similarly on the Northwest Coast, Bathurst (2005) notes anemia indicators in several populations. This demonstrates that there was no geographic limitation to populations experiencing periods of stress in the pre contact era. Keenleyside (2003) suggests that a likely explanation for increased rates of cribra orbitalia could be increased parasitic, bacterial and viral infections associated with changes in cultural practices due to European contact.

Non- specific infections bring on changes in bone due to reactions from bacteria that are indistinguishable from one strain of bacteria to another. However, bacteria that are commonly involved in these types of bone infections bone are staphylococci, streptococci, and pneumococci (Roberts and Manchester 2007). Depending on what part of the bone becomes infected with bacteria, there are several descriptive terms that are used. Periostitis is an infection to the periosteum or bone surface compared to osteomyelitis where the infection is located in the medullary cavity. In osteomyelitis bone is destroyed and pus is formed, due to this process the bone frequently becomes enlarged in part or whole and becomes deformed (Roberts and Manchester 2007). Archaeological

recovery of skeletal remains from pre contact sites has shown a low incidence of periostitis, osteitis and osteomylitis among the Iroquois from the Uxbridge Ossuary (Pfeiffer 1984). Similar results have been reported in pre contact skeletal remains recovered from Manitoba (Finch and Waddell 1996). *Blastomyces dermatitidis,* a fungal found in cool moist areas in southern Manitoba and south western Ontario as well as in the St Lawrence River region of Quebec also can cause non specific bone modifications. While this fungal infection is rare, the lesions on the skeleton can look similar to those found on individuals infected with tuberculosis (Waldram et al 2006) and can create difficulties in diagnoses of ancient disease. Several other bacteria, staphylococcus, streptococcus, moraxella, and actinomyces species, were ubiquitous and endemic in pre contact North America (Williamson and Pfeiffer 2003).

Specific bone changes can also result if an individual is missing a specific constituent, which provides evidence of dietary deficiencies possibly relating to subsistence strategies. Anemia is an iron deficiency in the blood resulting in porotic bone, whereas rickets is a vitamin D deficiency which affects the lower limb bones, and scurvy, a vitamin C deficiency, can create bone modifications to the mandible and maxilla. Diet is especially important to consider from an evolutionary perspective as individuals with a low immune response related to malnutrition may be less able to withstand disease onslaught and will die before passing on their genes. When one or more of these metabolic changes appear in skeletal remains, physical anthropologists can infer information about the individual's diet and possibly also social status, economic position, and religion.

The classification of specific infections are those resulting from infectious diseases that have a boney reaction that is characteristic of the causative micro-organism. The three bacteria that typify this category of infections are *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Treponema pallidum*. Within pre contact North America populations, only evidence supporting *Mycobacterium tuberculosis* and *Treponema pallidum* infections have been found, although *Mycobacterium leprae* was imported into Canada post contact. *Mycobacterium leprae* is the causative bacteria of leprosy which require a warmer climatic environment than those found in North America and therefore it was not part of the disease mosaic affecting pre contact First Nations. Specific infections like tuberculosis and treponematosis cause distinctive changes to the skeleton, typically affecting the spine, ribs and pelvis and causing lytic lesions

Tuberculosis is a complex infectious disease that is commonly understood as a respiratory infection, however it can also affect internal organs and bone. This infectious disease is easily transmittable between individuals in small or crowded living conditions, particular under conditions of poor air ventilation. Factors relating to the intensity of tuberculosis propagation in different populations are often associated with social inequalities and, as Roberts and Buikstra (2003) note, tuberculosis rates typically climb as social conditions erode. The nomadic nature of pre contact First Nation populations in North America did not fulfill the crowded living conditions required for this disease to have a persistent nature. However, shifts in cultural practices from a nomadic lifestyle towards the adoption of horticultural practices could have increased sedentism and population density. These changes would have aided in creating an environment more ideal for the transmission of tuberculosis and incidence rates would have increased.

The clinical manifestations of tuberculosis are often found in the lungs and surrounding pleura, structures which do not preserve in the archaeological record. If the severity and duration of tuberculosis is increased, there may be some changes in bone structure due to the proximity to the infected lungs and pleura. In bone, changes related to tuberculosis infection appear as a destruction of tissue in a pattern of reabsorptive lesions with little evidence of proliferative, reactive changes (Aufderheide and Rodrigues-Martin 1998:134). Ribs are often the most common bone associated with lesions and/or irregular bone build up in the form of plaques on the periostium. Tuberculosis bacteria also affect the vertebrae resulting in a spine deformity called Pott's disease. In Pott's disease, the invading bacilli erode the bone in the vertebral bodies, which subsequently collapse under the strain of supporting the torso's weight (Aufderheide and Rodrigues-Martin 1998). While bone involvement during the pre contact period may have been higher due to prolonged infection and severity, bone involvement is quite rare in contemporary clinical practice. Today, skeletal lesions are observed in roughly 1% of all patients (Zimmerman and Kelley 1982), compared to 5-7% of all patients before the advent of drug therapy (Aufderheide and Rodrigues-Martin 1998).

The first appearance of tuberculosis infection in the pre contact populations of the New World was once a matter of much debate. Before Lichtor and Lichtor (1957) and Morse (1961), tuberculosis was not thought to have existed during the pre contact period. However, the first confirmed case of tuberculosis in the New World was observed in mummified remains from Peru dating to 700 AD (Allison et al 1981). The identification of tuberculosis in mummified remains is based upon the presences of lesions in the preserved soft tissue of the lungs (Allison et al 1981). Further investigation in Peru and

Chile has uncovered several more individuals displaying bone changes typified by tuberculosis (Buikstra and Williams 1991, Allison et al 1981). Advances in ancient DNA techniques have aided researchers in diagnosing tuberculosis in skeletal remains that do not display any distinctive bone modifications, clarifying the picture of health in the New World.

This ancient DNA research has successfully amplified the *Mycobacterium tuberculosis* complex in pre contact New World populations (Eisenach et al 1990 and Salo et al 1994), lending support to the mounting paleopathological evidence. The specific strain of *Mycobacterium tuberculosis* infecting New World populations however still remains uncertain. Ramenofsky (1996 and 2003) speculates that the arrival of Europeans exposed the First Nation populations to a new strain or sub strain of *Mycobacterium tuberculosis* which had a higher virulence those already endemic to the New World. The introduction of a new strain of tuberculosis would in part, account for the rates of morbidity and mortality observed within the Indigenous New World populations post contact.

While the first cases were found in South America, evidence of tuberculosis infection is not limited by geography and has been observed in skeletal populations throughout the continent. In the Arctic, among the Alaskan Eskimo and Caribou Inuit, rib lesions similar to those produced by tuberculosis have been observed (Keenleyside 1998, Buikstra 1976). Tuberculosis studies in pre contact populations of the Arctic are relatively rare, large excavations in Ontario have yielded much more information on health in the pre contact period. Lesions found on vertebrae and ribs have been observed in several ossuary sites in southern Ontario (Pfeiffer 1984, Hartney 1981, Williams and

Snortland-Coles 1986). The incidence of tuberculosis in individuals recovered from these sites suggests that tuberculosis epidemics may have occurred in these regions. These populations were particularly sensitive to tuberculosis outbreaks as they had shifted towards a horticultural subsistence, favouring maize. Maize dependency has been associated with an increase in nutritional deficiencies in different populations as it lacks many essential nutrients needed by the body (Armelagos et al 1991).The emergence of horticulture has also "been equated with higher pathogen loads, largely because settled village life is associated with accumulation of wastes, increased contact with domesticated animals, and higher populations densities, all of which enhance the opportunities for infectious disease transmission" (Waldram et al 2006:35).

Treponemal diseases are specific infections that are responsible for the non-venereal syphilis outbreaks observed in the pathogen mosaic of the New World. The bacterium, *Treponema pallidum*, has four subspecies that are responsible for the disease manifestations of syphilis, pinta, yaws, bejel or endemic syphilis (Roberts and Manchester 2007). The distribution of these treponemal diseases is often related to climatic and social conditions. Yaws is found in humid, tropical climates, whereas bejel is found in arid zones (Hackett 1983). The transmission of these diseases is through skin to skin contact and while temperate zones might prevent skin to skin contact through clothing, although sexual transmission can also play a large role. *Treponema pallidum pallidum*, resulting in syphilis, has the unique feature of being able to pass through the placental barrier between mother and fetus during pregnancy or at birth, resulting in congenital syphilis. There are several stages of treponemal infection, primary, secondary, latent and tertiary, some of which produce distinctive bone changes. Tissue damage and

localized inflammation is associated with all of the stages of treponemal infection (Harper et al. 2011). The distinctive skeletal lesions observed in paleopathology occur in the tertiary stage and can affect a wide range of skeletal elements. Long bone shafts, ribs, the pectoral girdle, the cranium, as well as the hands and feet, can all be involved in periosteal reactions. These reactions can also cause excessive periosteal deposition resulting in tibial bowing or saber shins, a distinctive diagnostic feature of syphilis (Harper et al 2011). During the later stages of syphilis infection, bone also takes on a pitted appearance, where necrosis, pitting, excessive and bone remodelling can produce a grossly thickened cranial vault covered in pits and scars (Hackett 1976).

Osteological evidence of treponemal disease in Canada is limited to a few reports, compared to studies from the United States documenting forms of treponema which are more numerous. In Manitoba, possible treponema infection was assessed by Finch and Waddell (1996) in several individuals recovered from the Whaley Cairn to which they concluded that a differential diagnosis would include probable yaws infection. In southern Ontario, several sites have yielded individuals with possible treponematosis (Lennox and Molto 1995, Saunders 1988). The northwest coast of British Columbia has also had possible tremponal infection identified in skeletal remains (Cybulski per comm., in Geise 1988). In the United States, Powell (1988) identifies bones changes in the tibia from the pre contact population at Moundville where the lesion morphology, prevalence and age distribution of modern treponemal disease are more closely related than any other disease (Powell 1988:175). Similar periosteal modifications were observed at the Tatham mound, a late pre contact site from the central Gulf Coast of Florida (Hutchinson and Norr 2006).

Degenerative diseases represent another classification of resulting bone reactions in paleopathology; these are some of the oldest aliments that have affected humans and are associated with advanced aging and continual use. While degeneration can affect soft tissue and organs, these conditions are not recognizable in past population unless they affect the skeleton. In order for these degenerative diseases to be recognizable in skeletal remains, there needs to be bone formation and destruction. Today the clinical diagnosis of these degenerative bone conditions results from the classification of bony changes which generate osteoarthritis, rheumatoid arthritis, or ankylosing spondylitis. Osteoarthritis is the most commonly occurring joint disease in the archaeological record and in clinical practice (Jurmain and Kilgore 1995).

Osteoarthritis is a non-inflammatory disease that affects the synovial joints due to repeat stress. It is due to this repeated stress that osteoarthritis involvement in particular joints can imply occupation or activities in pre contact populations (Merbs 1983). Angel (1966) suggests that the arthritis in the radiohumeral joint observed in several skeletons from California indicates excessive atlatl use and coined the term "atlatl elbow". Similarly, a Manitoba analysis found moderate osteoarthritis of the knee joint with slight involvement in the wrist, especially in females, also suggesting repeat stress due to subsistence activities (Finch and Waddell 1996). This population was also observed to have "Porter's Neck" which is osteoarthritis of the spinal column, specifically the cervical vertebra, associated with individuals carrying heavy loads on their heads or with use of head straps (Finch and Waddell 1996). Other pre contact occupation related osteoarthritic degenerations are inferred from the skeletal remains excavated from the Whiteshell area of Manitoba. In this population osteoarthritis was commonly observed in

shoulders, potentially related to the activity of canoeing (Finch and Waddell 1996). While osteoarthritis is commonly observed in the archaeological record, another form of arthritis, rheumatoid arthritis, is not. Rheumatoid arthritis is an autoimmune chronic inflammation of the connective tissue and synovial joints (Roberts and Manchester 2007). This form of arthritis is more commonly found in the hands and feet of individuals and is unrelated to an occupational stress. The cause of rheumatoid arthritis is unknown at this time although researchers suspect that a number of factors including genetics, allergies, diet and infections are connected.

In the paleopathological record in pre contact United States, Rothschild and colleagues (1992) suggest that rheumatoid arthritis was observed in skeleton remains from Archaic, Mississippian and Woodland sites in central North America. Diagnosing rheumatoid arthritis in skeletal remains can be difficult as many other arthritic conditions present with similar bone appearances; that being said, rheumatoid arthritis usually affects bones symmetrically, which is a unique feature. A compounding difficulty in the recognition of rheumatoid arthritis in skeletal remains stems from the similar appearances of bone lesions resulting from Lyme disease. *Borrelia burgdorferi* is the causative agent of Lyme disease which is transmitted through deer tick vectors primarily found along the east coast of Canada and the United States. The clinical manifestation of Lyme disease results in an arthritic-like response as it presents with chronic inflammation in certain joints such as the knee (Lewis 1998). There are overlapping similarities in the appearance of bone alterations indicative of rheumatoid arthritis and Lyme disease, therefore diagnosis requires a unique differential diagnosis and analysis of disease distribution within the population.

A similar autoimmune degenerative joint disease is ankylosing spondylitis. This disease is a progressive heritable inflammatory disease that commonly affects the axial skeleton. The sacroiliac joints are usually the first to become affected although this will progress, moving up in the vertebral column, which can lead to boney fusion or ankylosis of various joints (Bridges 1992). The cause of ankylosing spondylitis has been associated with genetic variants in the HLA-B27 antigen (Ortner 2003). Contemporarily, this condition is common in Caucasian and First Nation populations, but rarely reported in Japanese or African groups (Resnick and Niwayama 1988).

The pre contact populations of the New World were host to a wide variety of pathogens. Skeletal remains and archaeological excavations provide researchers with information that can be used to infer the health and cultural practices of these people. The New World was not a disease free nation, with a large variety of parasitic, fungal and bacterial infections being identified from the osteological record. However, disease distributions are quite susceptible to changes in environment and cultural/ social conditions. With the arrival of European settlers, traders and missionaries in the New World, the pathogen environment, health patterns and cultural practices of First Nation peoples would become forever altered.

2.2.2 Pre Contact Cultural Adaptations

Some proto-contact ethnographic accounts of the First Nation peoples painted a picture of a robust and healthy population. For example, Brebeuf describes the Huron as being "more healthy than we" (Thwaites 1896-1901:257). Similarly, Captain Bartholomew Gosnold, who explored the new England coast in 1602 found "people of a perfect constitution of body, active, strong healthful, and very witty...For ourselves, we

found ourselves rather increased in health and strength than otherwise; for all our toyle, bad dyet and lodging; yet no one of us was touched with any sickness" (Ashburn 1947:16). Combined with this idea of a "disease free people" there is a pervasive mainstream perception that before contact, the Americas were a sparsely populated wilderness, essentially an untouched paradise. However, this notion does not match the evidence supplied by research into First Nation history or health. What is known about pre contact First Nation life ways has been pieced together through a combination of oral tradition, paleo-environmental reconstruction, archaeological recovery, proto-contact ethnographic records, and skeletal analysis. These reports demonstrate that the landscape had been altered by humans, in some places, and that population density varied. Traces of agriculture, large earthworks including burial mounds, and settlements can be found across the Americas. Osteological evidence shows that severe and chronic diseases were not uncommon in the New World. A similar misconception which hinges on the belief that there was small and scattered First Nation populations is also largely unfounded. Across the Americas, there have been burials uncovered containing many individuals suggesting that the First Nation population was quite large. Further support for dispelling the myth of a pristine New World that was "untouched" by people and disease comes from the archaeological record which shows large assemblages of cultural materials. Lithic tools, pottery and faunal remains help to infer the activities of these past peoples and also help to provide clues on more intangible aspects of pre contact life. By understanding the different features of the life ways of pre contact First Nation, the osteological interpretations become contextualized. First Nation history before contact with Europeans is quite expansive with hundreds of different ethnic groups spanning

thousands of years. These different ethnic groups are all culturally, politically and economically distinct and therefore have unique responses to the pre contact environment but also unique reactions to the biological and cultural changes resulting from European contact.

2.2.3 Cultural Practices of First Nation Prior to Contact

2.2.3.1 The Saulteaux

One of the southernmost First Nation groups in Manitoba are the Saulteaux. The Saulteaux are a composite of Ojibwa peoples who came together at Sault Sainte-Marie in the seventeenth century to trap and trade (Ward 1995). The Saulteaux speak a central Algonquian language closely related to the Cree, with whom they were traditionally allied. Most of the estimated 50,000 speakers are found in the area ranging from south-western Quebec through Ontario, Michigan, northern Wisconsin and Minnesota, southern and central Manitoba, and southern Saskatchewan (Garro 2004). The expansion from the Great Lakes system in Ontario into Manitoba occurred after the Saulteaux people had made first contact with European traders. The Saulteaux encountered French traders while they were passing through the Great Lakes of Ontario and followed them out into the plains (Ward 1995).

During the pre contact period, the ancestral Saulteaux were mobile hunter gatherers who followed a seasonal rotation for harvesting resources. Historically Ojibwa society was divided into clans, each identified by clan symbols that were linked by intermarriages and common traditions (Ward 1995). These clans dispersed into family hunting units to follow seasonal cycles of hunting and gathering, assembling in great numbers for festivals and large hunts. The relationships built during these gatherings

must have continued throughout the year as long distance trade seems to be not uncommon. Archaeologically, the pre contact period in Saulteaux history is characterized by many complexes and phases defined by changes in lithic technology, style, pottery innovations and subsistence adaptations. The woodland complexes are used when classifying the different time periods and cultural changes to the First Nation groups in northwestern Ontario, including the Saulteaux. The woodland complexes are divided into three time periods: initial/early (800 or 900 BCE to 0 CE), middle (200-300 BCE to 700-900 CE) and terminal/late (estimated 500 CE) (Fagan 2000). The different time periods have been categorized by three pervasive innovations: pottery manufacture, deliberate cultivation of native plants, and interment under funerary mounds (Bense 1994, Boyd et al 2008). Archaeological sites have been excavated from east central Saskatchewan through the lakes country of central Manitoba to Lake Superior, possibly extending into north-eastern Ontario (McMillan 1995) and denote this highly mobile peoples' pre contact territories.

Towards the end of the pre contact period, the cultural practices of the Saulteaux had grown to be largely defined by the emergence of village life and increased reliance on domesticated plants, such as corn, beans and squash. The adaption of horticulture during this period did not occur uniformly across Ontario and Southern Manitoba, as colder climates in some of these regions prevented its full-scale application. Many ceramic styles have been found within the archaeological sites, suggesting that women, who were thought to be the potters in many groups, joined their husbands' band on marriage (Fagan 2000). Interband relations offered numerous opportunities for social interaction. Besides coming together for subsistence reasons, there were also annual

celebrations such a boy's first kill, naming of a child and marriages (Garro 2004). Alliances between groups were essential for creating marriages as well as organizing war parties.

2.2.3.2 The Cree

The nation of the Cree extends across Canada from Labrador to the Rocky Mountains. Within Manitoba the Cree territory encompasses the plains to the subarctic, overlapping with the Dene to the north and the Saulteaux and Dakota to the south. Scholars have divided the nation of Cree in several groups, Plains, Woodland Swampy and Moose Cree (Abel 2005). The plains environment is flat semi arid grassland, which historically, allowed this population to be highly nomadic, ranging over great distances to hunt, trade and participant in warfare. The early prehistoric period (ca 9500 to 5500 BCE) was characterized by the many fluted points discovered from sites across the plains, including early Clovis and Folsom points, and later, Plainview, Agate Basin, Hell Gap and Eden-Scottsbluff (McMillan 1995). During this period the Cree were primarily hunters, especially in the northern reaches of their geographic area. Fishing, while not as highly valued, was also important because it allowed for larger social groups to assemble (McMillian 1995). In Manitoba during the later phases of the pre contact period, the Cree people started to shift subsistence strategies. The Cree followed a seasonal round of a hunter-gather subsistence strategy. Evidence from archaeological excavations has indicated that some horticulture was practiced (Nicholson 1988, Syms 1977). In the south western region of Manitoba, some later archaeological excavations like the Lovstrom and Lockport sites have evidence to suggest that horticulture was part of the life way of this pre contact people (Nicholson 1990). This shift in subsistence strategies has important

implications for health as pathological conditions can result direct or indirectly from inadequate nutritional support.

The pre contact social dynamics of the Cree showed that generally they lived in small social groups which were constantly on the move for subsistence purposes, but did gather in larger groups for special activities like fishing. The social organization among the Cree existed on several levels: the nuclear family, the hunting group (local band) and the community (regional band) (Ward 1995). A handful of nuclear families might come together to make up a hunting group, traveling together throughout much of the year. The nuclear family was generally made up of a marriage between cross cousins, but a good hunter might take several wives, who were often sisters (Ward 1995). The community or regional band came together for important activities, generally assembling in one location during the summer months for subsistence purposes. The Cree were able to make first contact with European traders much earlier than some other First Nation groups due to their seasonal round locations close to the Hudson's Bay coastline. It was in part due to this earlier contact that the Cree started to act as middle men for the European fur trade, a position that they virtually controlled for the better part of two centuries (Ward 1995). This position allowed some of the Cree to live around the fur trading posts and become the "home guard" for the posts. Many Cree leaders encouraged marriages with British and French traders, as they assured that trade goods would be continually available (Bial 2006).

2.2.3.3 The Dene

Today, the Dene people are located across the northern parts of Manitoba, Saskatchewan and Alberta. This subarctic region is home to the Athapaskan speakers,

collectively known as the Dene, meaning "people", and is made up of chiefly but not exclusively of the Chipewyan, the Beaver, the Slavey, and the Sekani (Ward 1995). The Dene nation recognizes its members as the Gwich'in, Bearlake (or SahtuDene), Hare, Dogrib, Salvey, Chipewyan and Mountain people, and sometimes included those Cree who live in the vicinity of Fort Smith and Hay River (Abel 2005). This large subarctic region is one of the least known archaeological areas in Canada and presents major challenges in interpretation. The people who populated these regions were often made up of small groups of hunters who migrated across a vast landscape, leaving very few traces to be recovered archaeologically. When these sites are found they tend to be small compared to their contemporaneous counterparts further south and located relatively close to the surface. Due to the lack of cultural material, how and when the Athapaskans came into the western sub-arctic is still a matter of debate. Most of what is known is based on lithics; the arrival of the microblade technology typifies this population after the paleo-Arctic but predated the arrival of ancestral Eskimos populations (Snow 2010). From 2500 BCE to 1 CE, microblade technology was gradually replaced by local assemblages of side-notched bifaces (Clark 2001).

These local assemblages provide information on the subsistence pattern and way of life of these people. A seasonal migration following the animal migration patterns had a large role in Dene society. Winters were spent in the forests and as caribou moved into the barrenland during the spring thaw, the Dene people would follow (Ward 1995). It was during the spring that large gatherings would take place for communal hunting and fishing. Closely following animal migrations dictated that the Dene society had to be highly mobile. In order to achieve this, the Dene utilized conical tipis covered with hides

in the summer and banked with snow in the winter as easily transportable housing (Ward 1995). The highly mobile nature of the Athapaskans allowed them to travel into a south-eastward expansion starting in approximately 400 BCE and continued well into historic era (Ward 1995). However, the very nature of following animal migration patterns also put the Athapaskans into conflicts with neighbouring First Nation groups, like the Cree. These meetings could have been responsible for some of the traumatic injuries that "were a major cause of pain, disability and death in pre contact times" and continued into the historic period (Fortuine 1989:45) as observed from the paleopathological analysis of skeletal remains.

2.3 FIRST NATION HEALTH IN REACTION TO EUROPEAN CONTACT

The effects of European contact on the First Nation population of the New World have been varied and wide ranging. Overall, these effects have had a direct impact on the mortality rates and population sizes of these peoples. The pathogen environment prior to European contact was one that First Nation people had adapted to, though they still experienced disease. It cannot be assumed that First Nations experienced a homogenous response to the infectious disease at the time of contact with Europeans or that population decline was uniform from region to region (Joraleman 1982). The regional variations in reaction to the introduction of European settlers, traders and missionaries would have been one of cultural and biological modification. Contact with European populations would have created changes to First Nation culture in reaction to the new relationships that were built, however rapid large-scale change came when residential schools and the

reserve system were implemented. These two events radically compounded the disease epidemiology of the time and have had lasting repercussion on First Nation health.

The extent of changes in the pathogen environments and cultural practices on First Nation health is observed through comparisons of population size estimates before and after contact. While several post contact informational sources exist in the form of ethnographic and historical accounts, archaeological excavations, and mathematical modeling, pre contact population estimations have to be inferred from osteological and mathematical model data. This has lead to several difficulties in estimating the pre contact Fist Nation population as a base line to measure the effects of contact. Additional difficulties arise as post contact information sources have inherent biases due to European preconceived notions of First Nation culture. These two factors make reconstructing population profiles extremely complicated. While the exact estimation of the numbers of First Nations people before contact remains a matter of debate, one thing is certain, European contact had a devastating/significant impact on the health and culture of the First Nation people of the New World.

2.3.1 Culture Change at time of European Contact in New World

European contact in the New World had a direct biological impact on the health of First Nation peoples; however, indirectly, this contact also had cultural, political and economic ramifications. Disease patterns can be altered or even aggravated in relation to changes in subsistence and/or settlement patterns. Prior to contact, the First Nations people located on the prairies were highly mobile and participated in an intricate cycle of harvesting seasonally available resources. Subsistence strategies provided opportunities for First Nation communities to gather in large numbers for communal hunts and then

disperse into smaller family groups during the winter months to collect more scattered resources (Ward 1995). This culture practice of breaking off into smaller groups meant that when diseases were present, they did not spread into the larger population as transmission factors such as crowding were kept to a minimum.

While devastating, the alterations in First Nation culture did not occur in a homogeneous fashion. Contact with Europeans took place during different time periods depending on the geographical location of the First Nation population. There was, however, a cascade effect where First Nation practices changed as a result of nearby bands that had been impacted by Europeans even if they themselves had not. The Cree of Northern Ontario first had contact with Europeans, specifically with the La Verendrye expedition, in the early eighteenth century (Ward 1995). The commercial opportunities that the fur trade offered as well as the addition of new technology, like the firearm, resulted in the spread of some First Nation groups into new territories. In a similar manner to the Cree, the Saulteaux were pushed further west by the fur trade and eventually took up residence on the Plains (Ward 1995). The relocation of the different First Nation populations sometimes resulted in tension between neighbours. For instance, within Manitoba skirmishes between the Dene and the Cree were intensified during the fur trade period (Ward 1995). The movement and settlement patterns of First Nation populations was influenced to a large extent by the location of the fur trade posts which had become central to their subsistence strategy.

These fur trade posts became centers for disease outbreaks and transmission allowing infectious diseases to be spread far into the surrounding regions (Dobyns 1993, Waldram et al 2006). The trading centers represented a unique convergence point for

Europeans and regional First Nation groups as well as long distance First Nations and European traders (Trimble 1985). Local and non local traders, both First Nation and European alike, continually brought new pathogens to the posts, which were then disseminated out into the local areas. These pathogens could also travel long distances in human carriers allowing them to make it back to their original infection origins, most likely large urban centers. These pathogens could also sometimes arrive in a mutated form which would reinfect the population and then disseminate out again. The merging of all these cultures in one small geographic location also created higher population density in and around these posts. It was during this time that First Nations were also undergoing a transition from "small band" to "large herd" epidemiology, which had devastating results for health (Young 1994). These semi stable dense populations created an improved manner for transmission of pathogens between people as the increased likelihood of pathogens being introduced, transferred, and propagated within the population rose as the population size did. Higher population densities around static posts also resulted in more individuals relying on the same resources for subsistence. The increased pressure on bison as a primary food source for First Nation and European alike created episodes of malnutrition and chronic stresses associated with environmental depletion and food insecurities (Herring and Sattenspiel 2007). The effects of European contact on First Nations resulted in many adaptations and changes in cultural practices. Subsequently, this created an environment where the newly introduced pathogens could proliferate. The combination of trading posts acting as nodes of disease outbreak and transmission and the depletion of a main food source hastened the population decline of First Nations after European contact.

2.3.2 Disease and Population change from European contact in New World

The ethnographic accounts of disease and death during the fur trade and subsequent time periods paint a vivid picture of First Nation health. Changes in cultural practices, as well as the introduction of new pathogens, resulted in a significant population decrease in the New World. The extent of the effects of European contact on mortality rates within Indigenous populations is assumed to be directly related to the introduction of infectious diseases and the population estimations pre and post contact. These estimations are based on a variety of sources including archaeological excavations, ethnohistorical accounts, computer/mathematical modeling and epidemiological theory. While the numerous methods of estimating population sizes have all contributed to a better understanding the effects of European contact, the exact demographic profile of First Nations is still debated.

One of the earliest explanations for the significant decrease in the Indigenous population resulting from newly introduced diseases comes from the field of epidemiology. The rapid spread of disease with high mortality rates initially suggests a pattern consistent with a virgin soil epidemic (Crosby 1976). These epidemics were generally characterized by high mortality across all age groups due to the entire population lacking any degree of immunity to a new pathogen. Possible scenarios that would culminate in a population experiencing high mortality due to its inability to mount an immune response could be due to a novel pathogen or lack of herd immunity. A novel pathogen will be one that a population has never encountered and therefore never had the opportunities to acquire immunity towards. Herd immunity, on the other hand, occurs when the majority of a population possesses a degree of resistances to an invading

pathogen thus acting as a form of protection to the susceptible minority. The transmission patterns of the invading pathogen can become disrupted if herd immunity is high as susceptible individuals become less likely to encounter the pathogen. A lack of herd immunity removes this protection and the pathogen can be transmitted quicker between higher numbers of individuals.

The application of the virgin soil theory as a means of understanding the devastation of the First Nation population of North America has been criticized as being overly simplistic. Estimations of regional population densities prior and during contact in North America have lent support to this criticism. If the population decline resulting for the introduction of European infectious diseases had been solely a virgin soil response then all regions would have been affected equally. Thornton (1997) described how in certain regions, European infectious diseases were present and enlisting high mortality, but other regions had a complete absence of disease and mortality. If the virgin soil epidemic hypothesis were to hold firm, all regions would be equally affected as the entire First Nation of North America would have been immunologically naive. Region to region variation existed (Joralemon 1982, Ramenofsky 1987, Ubelaker 1976 and 1988), but variation between different populations of First Nations also occurred. While some First Nation bands were heavily affected by European disease, other bands remained unaffected or recovered after the initial introduction to disease (Dobyns 1966). The non-homogenous reaction of the First Nation population of the New World diseases demonstrates that there were many more factors influencing mortality during European contact besides the introduction of infectious disease strains.

Diffusion of settlement patterns, trade and exchange networks, and adaption of differential subsistence patterns are some of the factors offered to explain the regional variations in mortality patterns of First Nations. The extent of the depopulation following contact has been assessed on a regional and continental scale. The overall estimations of the New World Indigenous population provide a base for regional disease responses to be compared against. There have been several scholars that have estimated the pre and post contact First Nation population in the New World; however, these estimates can differ by several million individuals. Early on, Dobyns (1966) applied Malthusian theory and historical population estimates to calculate the pre contact population to be 9.8 million, which was later doubled to 18 million in a subsequent publication (Dobyns and Swagerty 1983). Other population estimations range from 2 million (Ubelaker 1988), 4 million (Denevan 1992) to roughly 7 million (Thornton 1987). Any regional adaptations in successful survivability could be inferred from demographic changes between populations. Ubelaker (1992) used ethnographic, historic and archaeological data to estimate regional population sizes from 1500 to 1970.

The coastal regions of North America appeared to have a high relative population density when compared to the subarctic, great basin and plains regions during the initial contact period (Ubelaker 1992). The subsistence strategies that were commonly practiced along the coast generally supported a larger population than those inland. The harsher environments of the subarctic, great basin and plains would have also offered a wide seasonal temperature distribution and migrating food sources. Diseases, however, can spread quite rapidly and could have started to affect these populations before Europeans had arrived in these areas. The regional variation in the population decline can also been

gleaned from the estimation of regional populations at nadir. Nadir is the lowest point that a population size can get to before it will start to recover. The reductions in population estimations were lowest in the arctic and subarctic regions, with all regions reaching nadir in the twentieth century (Ubelaker 1992). Regardless of the regional variation observed in the population estimations, due to European contact with the indigenous population of the New World there was an overall and overwhelming depopulation trend.

2.3.3 Reserve System, Residential Schools and First Nation Health

Infectious diseases imported by Europeans were in part responsible for the population decline. However, as time progressed the Canadian government introduced certain politically inspired agendas to create institutional assimilation of the First Nation population. These changes would go beyond mere enculturation of First Nation peoples into dominant European society but also had a far reaching and direct impact on contemporary First Nation health.

The reserves system in Canada was developed in 1857 prior to the Canadian confederation in 1867. Land was transferred from the Hudson Bay company ownership, made up of Rupert's Land and the North-Western territory, to the possession of the government of Canada. These lands were added to the eastern provinces and for reasons of sovereignty and jurisdiction, were claimed for the settlement of European immigrants. In order for the newly formed government to establish a somewhat peaceful coexistence between the First Nation people and the incoming European settlers, land was set aside for the sole use of the First Nation populations. In total 11 land treaties were signed

stretching across the entirety of Canada and the north between 1871 and 1921. The overall intent of the reserve system was to control and regulate the lives of Indigenous people by limiting their movement as to allow for colonial expansion (Neu and Graham 2006). The establishment of reserves was one of the first steps to convert First Nations from a semi nomadic hunting/gathering life way to that of a sedentary agricultural one.

This move towards sedentary life had several impacts on the health of the First Nation people. At the most basic level, a highly mobile population could pack up and move away from areas and infected people where there was any outbreak of disease. Sedentary life precluded that; in fact, these places acted as disease epicenters in a similar fashion as trading posts (Dobyns 1993). Highlighting this are the differences in the outcomes of the 1837-1838 smallpox epidemic in the Northern Plains. Horticultural subsistence practitioners like the Mandan, Hidatsa and Arikara, who did not have flexibility of movement due to their vested interest in particular areas of land for subsistence, suffered higher mortality rates when compared to mobile hunter gathers such as the Sioux, Iowa and Otoe (Trimble 1994). In fact, historical records suggest that horticultural subsistence populations had an estimated 50-75 percent mortality rate during certain epidemics (Dobyns 1993). The First Nation groups that could avoid infection by having fewer contacts with potential pathogen vectors and the ability to disperse were able to become some of the more powerful tribes on the Plains (Trimble 1994). The federal government limited the nomadic hunting subsistence strategy that had served the First Nation population; allowances of hunting were only for reserve and crown land which limited the amount of animals that could be hunted. Compounding this new limitation on geography was the decline of the bison herds. Large scale bison hunts had

been organized for the production of pemmican and hides for the fur markets, however with this increased demand on the bison, herds numbers slipped below regeneration sustainable levels (McQuillan 1980).The economic partnership between First Nations and the Hudson's Bay Company could not be sustained due to the depletion of the bison and other game animals. This meant that First Nation hunters could not trade for European foodstuffs but also could not find wild game to hunt. Malnutrition has previously been described as a major contributor to increased susceptibility to many infectious disease as it decreases the host's immune response to fight off invading pathogens. Famines, exhaustion of resources and epidemics reduced the First Nation population and ultimately led them into a state of dependence on government relief supplies and aid (McQuillan 1980).

The impact of reserves is still apparent in First Nation health today. There is a difference of almost ten years between the life expectancy of a First Nation living on reserve and a non First Nation individual (Hallett et al 2006). Overcrowding, inadequate housing and poor sanitation present on reserves are all contributing factors in overall health of First Nations (Clarke et al 2002; Larcombe et al 2011). Health and social conditions can vary significantly between populations living on reserve versus off and between rural and urban areas. In Canada, a recent national survey showed that the highest proportion of individuals with diabetes was First Nations living on reserve with an overwhelming majority of these individuals reporting one or more adverse health consequence associated with diabetes (PHAC 2011). First Nation individuals living on reserve are also more likely to become infected with tuberculosis. While regional tuberculosis incidence rates vary, overall the age standardized active tuberculosis rates

for First Nations living on reserve have been reported to fluctuate between 32 to 59 times higher relative to the Canadian born non Aboriginal population, and between 2 to 3 times higher than the foreign born Canadian population (PHAC 2010). First Nations have had a long history with tuberculosis, however Ferguson (1955) states that tuberculosis only became a serious problem when reserves were created.

Residential schools were also a large source of disease transmission in First Nation populations. At the time when residential schools were in operation many children attending died before they were discharged from the schools. Others became sick with different infectious diseases that propagated in the inadequate living conditions, and were sent back to their home communities. These children could have spread and infected their home communities with different pathogens that they attained while at residential schools. Dr. P Bryce, the chief medical officer of the Department of Indian Affairs reported that disease had been left unchecked with "fifty per cent of the children who passed through the schools not living to see the benefits from the education which they had received therein" (Miller 1989:213). The lasting effects of residential schools on First Nation people are the intergeneration traumas that affects survivors and their descendants. The Aboriginal Healing Foundation has defined this intergenerational trauma as "the effects of sexual and physical abuse that were passed on to the children, grandchildren and great-grandchildren of aboriginal people who attend the residential school system" (AHF 2006:ii).

The development and establishment of the residential school system in Canada occurred during the mid to late 19th century as a means to educate and assimilate First Nation children into the dominant European culture found in Canada at the time. The

process of assimilation into Canadian society for First Nation children attending residential school consisted of learning new customs, language and religion. To do this residential schools promoted, through strict rules and harsh punishment, disconnection with family and community, devaluing First Nation culture, and banning First Nation language and spirituality. Children who attended residential schools suffered physically and emotionally throughout their entire childhoods as they were removed from their families "at the earliest possible age and kept until their characters have been sufficiently formed as to ensure as much as possible against their returning to the uncivilized mode of life" (Milloy 1999:40). While there are cases of children arriving at a younger age and being sent home earlier, the federal government deemed the time needed to form "sufficiently formed characters" to be a span of six to sixteen years (Woods 2009). However, the negative impact of residential schools did not end with students being discharged after this ten year span, it affected students into their adult lives and, subsequently, their descendants.

During this ten year span, children attending residential schools were exposed to a variety of diseases and subjected to poor living conditions. Reports from many inspectors of these schools, sent to the Department of Indian affairs, pointed out the connection between poor living conditions and poor health (Milloy 1999). Besides the poor construction of buildings, overcrowding was also a major factor in creating an environment for disease transmission. A report on the Qu'Appelle school stated that the boys' dormitory was too small to meet the needs of the children living there and that "beds were packed in as closely as they can be and the ceiling only being about eight feet high, and from the deficient ventilation the boys have consequently to breathe and

rebreathe the same air during the night" (Miloy 1999:106). Tuberculosis was not the only infectious disease to circulate within residential schools, scabies, lice, chickenpox, scarlet fever, measles and influenza were all reported at high rates.

While children entering residential schools were required to provide a health certificate signed by a doctor attesting to their healthy status, in many cases children were enrolled or residing at the school prior to providing this certificate or undergoing physical examination (Milloy 1999). In other cases, sick children may have been sent to residential schools but they cleared health checks because they were asymptomatic. This is important to note as children sent to residential schools acquired different infectious diseases and then were sent back to their home communities when they were deemed too sick to stay at school. This allowed for transmission of infectious diseases into these communities and spread among the residents.

The trauma experienced at residential schools has also predisposed survivors to a variety of health complications, with higher rates of illness compared to those who did not attend (FNC 2005). Along with higher rates of health complications, recent research in the field of mental health has found that "individual trauma is so severe that clinicians have begun to identify a distinct cluster of problems and behaviours, calling it residential school syndrome" (Corrado et al 2003:23). This sub class of post traumatic stress disorder has resulted from the persistent abuse that First Nation children experienced at residential schools and has had an impact on the mental and physical health of survivors and their families.

2.4 CONTEMPORARY FIRST NATION HEALTH

The social and biological interactions between that took place during the pre and post contact periods in Canada have greatly influenced First Nation health today. The large disparity in health status indicators between Aboriginal and non Aboriginal populations are, in part, a reflection of these unique historical backgrounds. This disparity will only increase if health concerns are not met, as the Aboriginal population increases at a rate of nearly six times greater than non Aboriginal populations. In order to get a snapshot of the overall health of a population, demographic indicators are traditionally used due to their high quality and availability as well as the ease at which they are able to be compared across different populations (Health Canada 2011). These demographic indicators can be made up of simple population measures (e.g., age and sex distributions) and vital statistics (e.g., birth and death records). In order to gain a deeper understanding of the health status of a population, premature mortality rates (PMR), life expectancies and potential years of life lost (PYLL) measures are also included. Manitoba First Nations have double the premature morality rate when compared to all other Manitobans, meaning that twice as many First Nations are dying a "premature" death (e.g., before the age of 75) (MCHP 2002). Populations with a high PMR are likely to report a poorer level of overall health with an increase in illnesses and greater morbidity resulting. Interestingly enough, the Northern Regional Health Authorities report lower PMRs than those in the south (MCHP 2002). The life expectancy for First Nations in Manitoba is also lower than non First Nation individuals within the province, at about eight years less for both males and females (MCHP 2002). These statistics support similar findings for PYLL rates in Manitoba First Nations. PYLL is a measure similar to PMR but gives a

greater weight to the death of younger individuals; therefore the PYLL rates will be larger if there is a high proportion of the population that is dying young or smaller if the population is living longer and deaths occur later in life (MCHP 2002). Manitoba First Nations PYLL rate is substantially greater (2.5 to 3 times) when compared to all other Manitobans, indicating that combined with the doubled PMR there is excessive mortality in this population but proportionally it is younger First Nation individuals who are dying (MCHP 2002). There are a variety of factors that could be contributing to these rates and the general poorer overall health reported in Manitoba First Nations. These factors are social and biological in nature and require a holistic examination in order to be fully understood.

2.4.1 Non Infectious Disease

Non infectious or non communicable diseases are health conditions or diseases that are persistent or otherwise long lasting symptoms. These diseases are generally associated with lifestyle choices that will eventually increase the risk of developing these non communicable diseases. Due to their association with lifestyle choices, chronic disease are also highly influenced by social determinants of health with risk factors including poverty, housing and access to health services. In the epidemiological literature, these categories of disease are listed as “degenerative” or “manmade”, because they require such a long period to develop, treat and are likely a result of personal life style choices. Chronic diseases are occurring in high frequencies and are the number one cause of death in the Canadian population. While there have been some improvements in chronic disease care and management in western society, there are certain at risk populations, like the Aboriginal population, where chronic diseases are a growing cause

of mortality and morbidity. In Canada, almost one-third of Aboriginals over the age of 15 have been told by a health care practitioner that they have a chronic condition (MacMillan et al 1996). Diabetes, cardiovascular diseases and chronic respiratory diseases are all core disease groups that are prevalent in the Canadian Aboriginal population.

Diabetes is a chronic disease resulting from the body's inability to either use or produce insulin. The hormone insulin enables the body to absorb sugar and utilize it as an energy source. As a result, diabetic patients have a consistently high blood sugar level, called hyperglycemia. Damage to blood vessels, nerves and organs including the kidneys, eyes and heart all result from prolonged periods of hyperglycemia. The common types of diabetes occur in three forms, type 1, type 2, and gestational. Type 1 diabetes is an autoimmune disorder where the individual's own body reacts to insulin producing cells and destroys them, as it would a foreign invading pathogen (PHAC 2011). This type of diabetes is also referred to as insulin-dependent diabetes mellitus and it is not yet known the cause of this condition nor ways to prevent it (Alberti et al 1998). Type 2, on the other hand, is a metabolic disorder where either insulin is not produced in large enough quantity or the body cannot properly use the insulin it creates (PHAC 2011). Adult onset or type 2 diabetes comprises 90% of the estimated 346 million people worldwide who are diabetic, a condition which is, in part, an outcome of unhealthy behavior resulting in overweight or obese individuals (PHAC 2011). In Canada, diabetes affects close to 2.4 million people and while only being reported in Aboriginal populations in the last fifty years, type 2 diabetes has reached epidemic proportions (Young et al 2000). The prevalence of diabetes has risen significantly to almost 30% in some Aboriginal

populations; of which First Nation individuals living on reserve make up the highest portion at 17.2%, followed by First Nations living off reserve at 10.3% all after age standardized adjustments (PHAC 2011). In First Nation communities, type 2 diabetes is frequently reported in children and youth, which provides ample opportunity for the severity of the disease to increase and poses an increased risk for complications. Complications arising from diabetes commonly manifest in increased risk of heart disease and stroke, reduced blood flow and resulting neuropathy which could lead to foot ulcers and limb amputations, as well as blindness and kidney failure (PHAC 2011, Alberti et al 1998). Of diabetic First Nation adults, 89% of them reported one or more adverse health consequences of diabetes, while 25% reported four or more consequences (PHAC 2011). These pose not only an increased adverse health projection for diabetic First Nations but also put enormous strain on health care services in terms of both health care professionals and economics.

Cardiovascular diseases are another chronic disease and are the number one cause of death in the world as well as being noted as having the largest economic burden on Canada's health care system (PHAC 2010, WHO 2011). These diseases are attributed to lifestyle behaviours like high saturated fat diet, physical inactivity, and the excessive use of alcohol or use of tobacco. Cardiovascular diseases include a wide variety of disorders of the heart and circulatory system; most familiar are heart attacks and strokes (WHO 2011). Cardiovascular disease has historically observed a low prevalence rate in First Nations, however with the recent trends towards high calorie and fat diets and increasingly sedentary lifestyle, cardiovascular disease is on the rise. That being said, when First Nations living on reserve are compared to the non Aboriginal Canadian

population there is only a small difference in age standardized prevalence rates for heart disease (Health Canada 2009). While First Nation rates of heart disease are comparatively low, hypertension or high blood pressure could be on the rise. Hypertension is a co-morbidity between diabetes and cardiovascular disease, where there is excessive pressure on the walls of blood vessels and arteries. Hypertension is also precursor to heart attacks and stroke and with the dramatic increase in diabetes rates among First Nations, cardiovascular disease should soon follow.

Chronic respiratory diseases affect the airways and other structures of the lung and include two more commonly known chronic respiratory diseases: asthma and chronic obstructive pulmonary disease. These diseases interfere with the quality of breathing due to persistent blockages of airflow and have an adverse effect on quality of life. Chronic obstructive pulmonary disease is characterized by "shortness of breath, cough and sputum production", which is often irreversible and results in a slow decline of overall health (Reading 2009). Asthma however, is characterized by recurrent periods of breathlessness and wheezing which can become exacerbated by a variety of different stimulus including physical activity, allergens and temperature change. During an asthma attack the lining of the bronchial tubes swells, decreasing the amount of space avoid for airflow in and out of the lungs (Sin et al 2002). While chronic obstructive pulmonary disease is responsible for millions of deaths globally, asthma has a relatively low mortality rate although it can have a severe impact on morbidity. Risk factors for these chronic respiratory diseases encompass indoor and outdoor environmental pollutions as well as occupational allergens like moulds and dust as well as tobacco smoke (Richardson et al 2005). Cigarette smoking in Aboriginal communities is estimated to be on average two to three times

higher than in non Aboriginal communities, although variation between communities exists (Wardman et al 2007). Tobacco smoke combined with possible allergens in the home may be partially responsible for the increasing rates of asthma as well as other chronic diseases. While rates of asthma in Aboriginal children (12%) and adults (11.2%) are still within the range of the Canadian non Aboriginal (13.4% and 8.4% respectfully), they are part of an upward trend (Crighton et al 2010). Of the Aboriginal population to have been diagnosed, the severity of respiratory conditions is escalating. Aboriginal asthma patients are 2.1 times more likely to have an emergency or physician office visit, and those with chronic pulmonary disease are 1.6 times more likely when compared to non Aboriginal individuals (Sin et al 2002). Sin and colleagues (2002) suggest that that there are disproportionately higher rates of these conditions in Aboriginal communities. The discrepancy between the high rates of hospital visits and lower prevalence rates could be due to poor detection of these conditions. The location of First Nation communities, in rural and northern areas, provides challenges in accessing adequate health care services and as such could be deflating the First Nations incidence rate of chronic respiratory diseases.

2.4.2 Infectious Disease

Communicable diseases are those that are characterized by either direct or indirect transmission by which microorganisms like bacteria, viruses, parasites and fungi invade a host's immune system. The transmission of these pathogens can be through a variety of means including the air, bodily fluids, inanimate objects called fomites and vectors. The severity and prevalence of infections can vary depending on the interaction of social

factors. The two more prominent infectious diseases affecting Canadian Aboriginal populations today are human immunodeficiency virus (HIV) and tuberculosis.

First Nations in Canada have a long history with *Mycobacterium tuberculosis* and while it was once thought that tuberculosis rates were on the decline, contemporary First Nations experience rates over and above the national average. In age standardized reporting on active tuberculosis, the incidence rates for First Nations living on reserves is between 32 to 59 times higher than non Aboriginal Canadian population and between 2 to 3 times higher than foreign born Canadians (Health Canada 2012). While all Aboriginal populations share a dramatic overburden of tuberculosis infection given the elevated incidence rates, not all reserves are affected the same. In Manitoba, on reserve tuberculosis incidence rates were recorded over a four year period, this data showed a significant difference between reserves.

Out of the fifteen reserves included in the study, the highest incidence of tuberculosis was reported in God's Lake Narrows (457.4/100,000), Pauingassi (336.7/100,000) and Northlands (194.5/100,000) although some of the lowest incidence of tuberculosis were reported from Shamattawa (80.4/100,000), Little Grand Rapids (136.5/100,000) and Sayisi (76.9/100,000) (Olson 1999). The differences between high incidence reserves and close neighbouring low incidence reserves suggests that there are diverse factors unique to each reserve that are offering some degree of resistance or susceptibility to tuberculosis. The differences observed in the increased likelihood of acquiring tuberculosis stem from variations in reserve environments, location, and access to health care. Tuberculosis has been characterized as a disease of poverty because it has a strong association with adverse social conditions. An erosion of social conditions will

place individuals at an increased risk for poorer health, which is why strategies for tuberculosis control and prevention include assessing different social determinants of health.

Social factors also play a large role in the acquisition and progression of HIV. HIV is a blood born virus that attacks the immune system resulting in the progressive stage identified as the acquired immunodeficient syndrome (AIDS). It is in this stage that macrophage function is comprised making the infected individual more susceptible to opportunistic infections and cancers (NAHO 2009). In Canada, the Aboriginal population is overrepresented in reported HIV/AIDS cases. The Public Health Agency of Canada (2010) notes that HIV infection in aboriginal individuals has increased by 24%, resulting in the aboriginal population being 3.6 times more likely to acquire HIV infection when compared to the overall Canadian population. Another growing concern in the fight against HIV infection is the increased risk of co-infection with other pathogens. Tuberculosis is particularly dangerous for individuals already infected with HIV. The World Health Organization (2012) states that HIV positive individuals are 21 to 34 times more likely to have latent tuberculosis progress to its active form than individuals without HIV. At present there are numerous difficulties in assessing the rates of latent tuberculosis in Canada, however co-infection of HIV and active tuberculosis in First Nations living on reserve fluctuated between 14.3% to 38.5% over the course of an eight year span (Health Canada 2012). At the moment, in Manitoba, HIV rates are quite low in all populations; however this will not always be the case. Manitoba HIV rates will continue to rise as the national average does, particularly in the Aboriginal populations where HIV infection is being overrepresented. Similarly, as HIV rates increase so will

tuberculosis rates as well as other disease rates such as diabetes. All of these diseases can act in a synergistic manner creating various co morbidities and, furthermore, all of these diseases are overrepresented in Aboriginal populations. Unless action is taken now to prevent and treat these diseases, the future of First Nation health in Canada is troubled.

2.4.3 Social Determinants of Health

Health is now defined in a broader sense, not just characterized by the absence of disease but including aspects of mental, physical and social well-being. Social determinants of health are the economic and social conditions that aid in shaping the overall health of an individual (Raphael 2009). These factors can dictate why certain individuals are more likely to become sick and have shorter life expectancy but also if an individual has the necessary resources to obtain a good sense of well being. The quality and quantity of these factors are the underpinning of health and, while not directly related to biomedical entities such as pathogens, they play a large role in directing health. These resources can include a variety of different social and economic categories for instance-childhood conditions, income, education, employment, food insecurity, housing, and health services (Raphael 2009:7). While healthier lifestyle choices like reducing stress, eliminating tobacco use, and dietary changes are prevalent in our society, living conditions have a much larger sphere of influence on our health. Raphael (2009) puts forward several categories of social determinants of health that are especially significant for Canadians and include the previously mentioned categories with the addition of Aboriginal status.

The disparities between the Aboriginal population and the non Aboriginal Canadian populations are significant. Chronic and infectious disease show a large gap

between these two groups but there are also noted inequalities in every key category examined within social determinants of health. Income is one of the most important social determinants to good health. The level of income can affect other determinants like food security, housing and can influence behaviour by changing quality of diet, extent of physical exercise, tobacco and excessive alcohol use (MacKinnon 2010). The varying levels of income are also influenced by social determinants of health like educational attainment, social exclusion and employment. The interconnected nature of these social determinants of health demonstrates how important these factors are in overall health. Lightman and colleagues (2008) reviewed the health of the richest 20% of Canadians and the poorest one fifth. Observed in the poorer section of this population, there was double the rates of diabetes, heart disease and arthritis; a 60% increased risk of acquiring two or more chronic diseases; 3 times the rate of bronchitis when compared to the upper 20% (Lightman et al 2008). It should come as no surprise that given the increased rates of disease in the Aboriginal population that the average income is 58% and 72% less than their non Aboriginal male and female counterparts respectfully (Mikkonen and Raphael 2010).

Employment and income bracket is often correlated with the attainment of higher levels of education. Individuals with higher education tend to be healthier and wealthier than those with less education. The effects of higher education can also aid in individuals understanding and promoting their own health by adopting different lifestyle choices. Educational increases generally help to invoke a sense of agency or ability to effect change in one's own life, and without agency feelings of hopelessness and powerlessness can become prevalent. Several researchers have highlighted the relationship between

health and unemployment/poor economic conditions and have revealed that these negative feelings and behaviours can become entrenched within a community resulting in low cultural esteem, lack of cultural identity, depression and in extreme conditions self injury and suicide (Elias 1996, Warry 1998, Farmer 1996). The structural violence instilled by these conditions could be contributing, at least in part, to rates of suicide three times higher in First Nation communities when compared to the non Aboriginal population (Health Canada 2011).

Without the necessary level of education needed to attain adequate employment, options are limited and this can result in food insecurity and poor housing conditions. Both of these factors have direct links to health as malnutrition can predispose an individual to pathogen attacks by weakening the immune system while housing increases the likelihood that communicable diseases like tuberculosis are transmitted. Aboriginals living off reserve are four times more likely to experience food insecurity than non Aboriginal Canadian with this population also reporting 33% of food insecurities to be rated moderate or severe in 2004 as compared to 8.8% of non Aboriginal households (Mikkonen and Raphael 2010). Similarly the Aboriginal populations are also experiencing increased rates of crowding and substandard housing, most notably on reserves. In Manitoba, 20-30% of all on reserve housing requires major repairs or replacement (Hallett et al 2006). Aboriginal households on reserve were 60% more likely to live in a house that does not meet one or more of the adequacy or suitability standards put forth by the Canadian Mortgage and Housing Corporation (CMHC 2010). Manitoba reports similar if not higher likelihood at 65% of on reserve housing failing to meet housing standards (CMHC 2010). Compounding the severity of poor housing conditions

in Manitoba, this population also reported the highest incidence of crowding at 32% (CMHC 2010). A direct association between crowding in First Nation housing and incidence of disease has been noted for several pathogens including tuberculosis (Clark et al 2002), diarrheal illnesses (Hayward et al 2010), and bacterial dysentery (Rosenberg et al 1997). The complex interrelated nature of the different social measure of health demonstrates how there are many social and economic factors that can predispose an individual to disease. When one of these measures is subpar it can have a cascade effect creating disparities in many of the other areas. In order to improve health for Canadian Aboriginal populations there needs to be reforms to social policy.

2.5 SUMMARY

First Nation health today is a product of the complex interactions that have taken place over hundreds of years. These complex interactions are not only biological in nature but also comprise any modifications to cultural practices, social or economic conditions that could have shifted disease epidemiology. In the pre contact period, First Nation health was adapted to the parasitic, fungal and bacterial infections that predominated. Evidence of these infections comes from skeletal remains that have been excavated and analyzed using paleopathology. Certain pathogens over a prolonged period of time can invoke bone reactions. This bone remodeling can be used to infer cultural practices, nutritional status as well as denote particular infections. The First Nation populations that resided in the New World followed a nomadic hunter/gather subsistence strategy, one that was beneficial for keeping certain pathogens like tuberculosis at bay. However, changes to settlement patterns, subsistence strategy and wide-ranging cultural practices resulting from contact with European traders, settlers and missionaries, created

new diseases frequencies. From the onset of contact with Europeans, the First Nations population began to decline, due to high mortality and morbidity from new pathogens. While the responses to these new pathogens were not uniform in all First Nations populations, the overall trend was a massive decrease in population numbers. Further compounding this was the implementation of the reserve system and residential schools by the federal government to assimilate First Nations in European culture. The impact of European contact resulted in a shift in the immunological response to pathogens, as well as formulating new modes of transmission, the dispersal of infectious diseases and enduring disease pools.

Canada's health status is one of the highest rated in the world, reaching well within the top ten for developed nations. However, within Canada, Aboriginal health and living conditions are regularly described and compared to those of developing countries. The disparity that exists in First Nation health is observed across all manners of disease, measures of well being and social determinants of health. The overall trend in First Nation health is that this gap is continuing to increase, creating adverse health outcomes for First Nations and escalating demands for health services.

CHAPTER 3 THE IMMUNE RESPONSE, IMMUNOGENETICS AND HEALTH

3.1 INTRODUCTION

Immunity is defined as resistance to disease, specifically infectious disease. The role of the collection of cells, tissues and molecules that mediate resistance to infection is to identify invading micro-organisms that could harm the host and eliminate them. The coordination of the immune reactions of these cells and molecules is the immune response. The immune system response to infection is important to consider for understanding how the evolutionary forces have shaped this response can inform environmental and human histories. The record of pathogen exposure is important to consider because the longer a population has been in contact with a pathogen the better adapted the immune response will be. Perhaps the adaption of the immune system can be best described by a palimpsest metaphor, where the reuse of writing materials over time would have an imprint of what was recorded previously (Jobling et al 2004). In this sense, the genetics can be conceptualized as a palimpsest, due to an accumulation of past pathogen interactions, social interactions and environmental factors shaping contemporary genetic profiles.

This chapter will examine the immune response in the human host to infectious pathogens from the cellular level to the environmental factors that can shape it. One of the key survival factors in the immune response is how it can adapt in response to the pathogens. These adaptive capabilities in the human immune system are readily recognizable from the vast amount of variation that exists between individual immune expressions. These variations can be related to the structure of genes that function to

destroy invading pathogens. Modern day molecular technology allows researchers to detect single nucleotide polymorphisms (SNPs) in targeted genes that play an important role in mounting a successful immune response. SNPs that affect the expression of genes can impact the level of functionality of the immune response and can sway resistance and/or susceptibly to certain pathogens. While identification of these SNPs is key for understanding some contemporary health concerns, these SNPs are also being used by anthropologists to answer historical health and population relationship questions. Analyzing immunogenetic profiles offers a unique avenue for research, allowing for both a better understanding into historical pathogens environments that have aided in shaping contemporary genetic variations as well as insights into the modern health implications of these variations.

3.2 THE HUMAN IMMUNE RESPONSE

3.2.1 Introduction

The most fundamental role of the immune response is to enable the body to distinguish between "self" and "non-self", and once that division has been made, to eliminate the "non-self" material from the body. Either innate or adaptive immune pathways can characterize the process by which "self" and "non-self" are recognized within the body. Innate immunity mediates the initial protection against infections whereas adaptive immunity develops over time. Innate immunity, which is also called natural immunity, infers that this type of defense is always present in healthy individuals and reacts to every type (non-specific) of invading pathogen. Conversely, adaptive immunity, which is also called acquired immunity, is a host defense that is stimulated by particular microbes that invade the body (Abbas and Lichtman 2010). The innate immune

response occurs rapidly upon receiving a trigger by pathogen exposure and can overlap with the adaptive immune response, but whereas adaptive immunity takes days to develop, innate takes hours (Murphy et al 2008). This being said, the adaptive immune system is capable of eliminating infection more efficiently. Due to evolutionary pressures, many pathogens have adapted to resist the innate immune response, which can cause a delay in the initial immune response and result in increased disease severity.

The defense against infection comes from an immune cascade effect involving both innate and adaptive immunity. The cascade effect in the immunology literature deals with several triggers that can amplify an immune response, the strong expression of one gene will in turn help to create a strong expression in the subsequent gene. However, the cascade effect may have a negative effect on the expression of the immune response if the initial trigger of the cascade has a sub optimal expression level. This sub optimal expression level will be promoted throughout all immune pathways and can result in an overall sub optimal immune response. In order for the immune response to be successful, the immune system must fulfill four main tasks: immunological recognitions, immune effector functions, immune regulation and immunological memory (Murphy et al 2008). A sub optimal response in any of these four tasks can result in prolonged exposure to a pathogen with delayed containment and elimination, ultimately producing a poorer health outcome for the host. The initial recognition of invading pathogens is carried out by the white blood cells of the innate immune system, which provides an immediate reaction by the lymphocytes of the adaptive immune system. The immune effector function tasks are to contain the infection and to eliminate it. This task is done by a complement system of blood proteins, antibodies, and the destructive capacities of lymphocytes and the other

white blood cells (Murphy et al 2008:3). Once the immune reaction is started, it is the task of immune regulation to keep the immune response under control so that it does not damage the host while eliminating the invading pathogen. The last task of the immune system is to protect the host against recurrent infection. The adaptive immune response generates an immunological memory, so that the host will have an amplified response against subsequent infection.

3.2.2 Innate Immune Response

The first line of defense in innate immunity is provided by epithelial barriers and by specialized cells and natural antibodies present in the epithelia whose function is to block the entry of microbes into the body. Skin oils are acidic and produce chemical substances including lysozyme and phospholipase A which are antibacterial enzymes found in tears, that make it harder for bacteria to thrive on the epithelial surface (Murphy et al 2008). The mucous membranes coat microorganisms in mucus which can prevent particular bacteria and other pathogens from adhering to the internal epithelium (Murphy et al 2008). Within the stomach, natural acids and peristalsis create an inhospitable environment for pathogens and keep infectious agents moving throughout the body. Lastly, to prevent invading pathogens from colonizing the body uses coughing and sneezing reflexes.

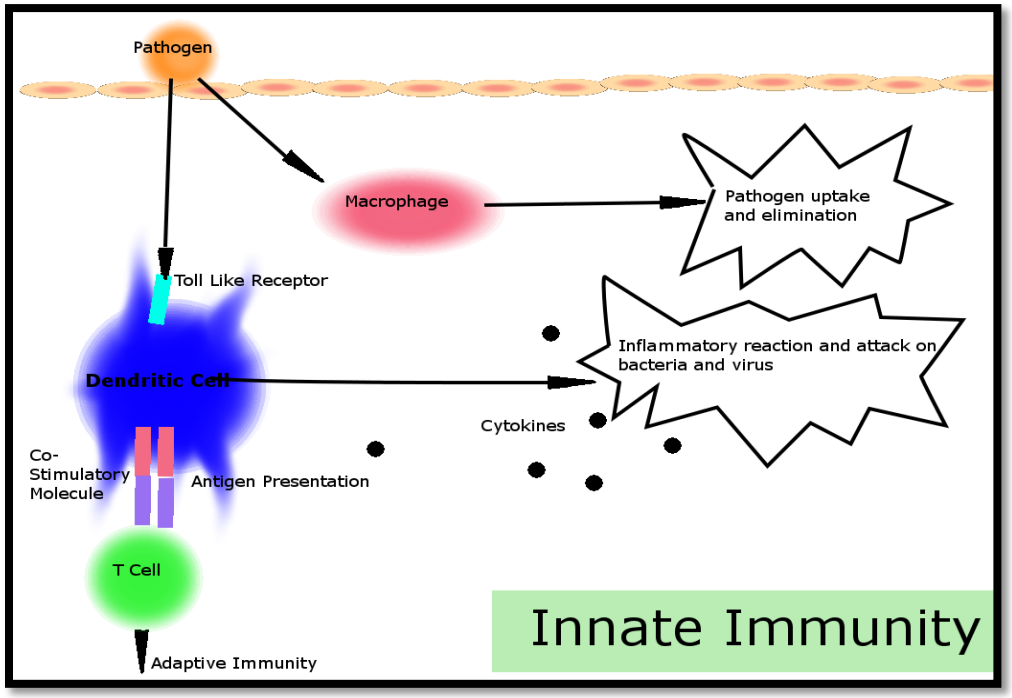
If the microbes do end up crossing the epithelial barrier and enter the host's circulation, they are attacked by phagocytes, made up of neutrophils, macrophages and dendritic cells, specialized lymphocytes called natural killer cells (NK), as well as several plasma proteins. All of these agents of innate immunity specialize in recognition and reaction against all microbes. The initial reaction against microbes is an inflammatory

reaction, characterized by the migration of cell types with defensive functions, alterations in vascular permeability and the secretion of soluble mediators (Abbas and Lichtman 2010). Inflammation is essential in eliminating invading pathogens as it delivers additional support by way of effector cells to the site of infection, induces local blood clotting which creates a physical barrier to entry into the circulatory system and lastly, promotes tissue repair (Murphy et al 2008). Inflammation recruits two main types of circulating phagocytes: neutrophils and monocytes, which mature into macrophages at the site of infection. At this site of infection during the inflammatory response, the role of the phagocytes is to recognize the microbes through specific receptors and ingest the microbes through phagocytosis. Neutrophils and monocytes identify and engulf pathogens, which become phagozomes and are eliminated through digestion by lysosomes. Monocytes give rise to dendritic cells depending on the precise signals that they receive from their environment (Murphy et al 2008), which allows the important function of phagocytosis to continue. Host cells that have become infected with microbes are then identified and killed by NK cells which produces cytokines and induces macrophages action (figure 1). Cytokines similarly stimulate the production of neutrophils which are produced in the bone marrow of the host. Cytokines are soluble proteins that affect the behaviour of other cells, mediate the immune and inflammatory reactions and are responsible for the communication between leukocytes and between leukocytes and other cells (Abbas and Lichtman 2010). Cytokines play an important role in the success of the immune response, as poor communication may ultimately lead to a down regulated immune response. The residue peptide chains inside the phagozomes are then bonded to major histocompatibility complex II (MHC II). This complex is then

presented on the surface of the phagocyte, which is also called an antigen presenting cell. Different antigens that become attached to the surface of cells through phagocytosis, will elicit particular immune amplification genes.

The cytokines produced by the macrophages induce another arm of the innate immune response. This arm involves the dendritic cells which recognize structures that are shared by various classes of microbes but are not present on host cells. These structures that are present on microbes are called pathogen-associated molecular patterns (PAMPs). Different microbes will have different PAMPs which are recognized by pattern recognitions receptors (PRR), under which Toll-like receptors (TLRs) are classified, within the immune response. The PRRs respond rapidly to infection by producing cytokines and anti-microbial agents, and TLR activation mediates the adaptive immune response (Levinson 2006).

FIGURE 1*: Innate Immune Response (Adapted from Abbas and Lichtman 2010)*



There are 10 expressed TLR in humans that act as signaling receptors distinguishing different types of pathogens and aiding in directing the immune response. Although, as there are only ten TLR genes, compared to the large diversity of pathogens, this means that the receptors have limited specificity compared to the antigen receptors of the adaptive immune system (Murphy et al 2008). There are two types of TLRs classifications based on location of antigen detection, ones that act as cell surface receptors and others that act intracellularly. TLRs are links to signal transduction pathways, which activate genes that promote inflammation (Abbas and Lichtman 2010). The signal is created with adapter proteins such as Myeloid differentiation primary response gene 88 (MyD88), Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) and TIR-domain-containing adapter-inducing interferon-β (TRIF) to initiate signaling. However, generally TLR signal transduction is through the recruitment of MyD88. MyD88 activates a cascade that results in the activation of two important transcription factors, nuclear factor-kB (NF-kB) which promotes cytokine expression and interferon response factor-3 (IRF-3) which stimulates the expression of pro-inflammatory cytokines (Turvey and Hawn 2006). These cytokines stimulate the migration of cells towards the invading microbes as well as the production of interferon-γ (IFN-γ), which enhances the ability of the phagocytes to kill invading pathogens. As already mentioned, different TLRs are specific for different components of microbes. An important TLR in the identification of tuberculosis bacterium is TLR4 which responds to *Mycobacterium tuberculosis* as well as other common bacterial infections. TLR4 is located on macrophages and responds to the presence of gram-negative bacterial lipopolysaccharides (Misch and Hawn 2008). A similarly important TLR in the detection

of tuberculosis bacterium is TLR2 which acts as a heterodimer with TLR1 and TLR6 and signals the presence of lipoteichoic acid found in gram positive bacteria and lipoproteins of gram negative bacteria, as well as lipoarabinaomannan found in mycobacteria and zymosan found in yeast (Murphy et al 2008). Other examples of TLRs that are particular to certain pathogen types are TLR5 which signals in the presence of flagellin (Misch and Hawn 2008) and TLR4 which signals in the presence of cpGDNA and has recently been identified as playing a larger role in the detection of tuberculosis bacterium as well (Bochud et al 2009).

Once recognition has taken place, the TLR signal initiates maturation of the dendritic cells which changes its behaviour from phagocytosis to activation of the adaptive immune response. The mature dendritic cells then migrate to regional lymph nodes and activate the adaptive immune response. The activation of the adaptive immune response triggers when naive T and B cells in the lymph nodes encounter the mature dendritic cells and recognize the microbial peptide chains presented. The migration of mature dendritic cells is stimulated by specific cytokines, such as tumor necrosis factor α (TNF- α) although many others cytokines also contribute (Murphy et al 2008). This response bridges innate and adaptive immunities and triggers a cascade effect, activating certain pro-inflammatory genes.

Although innate immunity can effectively combat infections, many microbes have evolved to resist innate immunity. Innate immunity is a nonspecific response to invading pathogens and as such does not have the same effectiveness in the successful elimination of pathogens when compared to the adaptive immune response. Once a pathogen resists, overcomes or circumvents the innate immune response, the task of defending against

these pathogens is left up to adaptive immunity. The adaptive immune response is stimulated by the innate immune response when antigens are produced that function to signal the activation of T and B lymphocytes. The signal is the combination of the antigens produced by invading microbes as well as the innate immune response to these microbes. Innate resistance and adaptive immunity are not simply sequential; they are in fact complementary mechanisms that regulate each other, through cellular contact and the secretion of cytokine mediators (Trinchieri 2003). Due to their complementary nature, if there is a change in the balance and levels of the cells involved in the innate immune response, the level of expression in the adaptive immune response will also be affected. These changes in levels or functionality of cells can result in a weaker immune response and can result in increased susceptibility to infections.

3.2.3 Adaptive Immune Response

An invading pathogen may not be completely eliminated by the innate immune response as there are many antigens that the innate immune response cannot recognize. Adaptive immunity is an additional level of protection in the immune response that allows for adaptations to subsequent reinfections by pathogens. Adaptive immune responses are triggered only if microbes or their antigens pass through the epithelial barrier, and are recognized by macrophages that are delivered to lymph nodes, where they can be recognized by lymphocytes (figure 2). Therefore the adaptive immunity occurs only after an initial exposure to, or immunization with a given foreign substance (Janeway et al 2001). The adaptive immune response is geared towards specialized combat specific to certain types of infections and can confer protection from new pathogens through the creation of memory cells. Adaptive immunity is characterized by

the two different pathways: humoral and cell mediated, which are mediated by different cells and molecules and are designed to provide defense against extracellular microbes and intracellular microbes, respectively (Abbas and Lichtman 2010). Cell mediated immunity functions to combat infections by intracellular microbes and it is mediated by lymphocytes; the humoral immune response is mediated by antibodies that function to neutralize and eliminate extracellular microbes. Within the two pathways classified under adaptive immunity, the two major categories or subsets of lymphocytes involved are: B- and T-cells. To oversimplify, B-cells respond to extracellular micro-organisms such as parasites and viruses by producing antibodies, whereas T-cells coordinate an immune reaction through the production of cytokines (Abbas and Lichtman 2010).

In cell mediated immunity, the responses of T lymphocytes to cells associated microbial antigens consists of a series of sequential steps that results in an increase in the number of antigen-specific T cells and the conversion of naive T cells to effector cells to memory cells. Naive T cells are antigen receptors that recognize but are incapable of performing the effector functions required for eliminating microbes. T cells originate in the bone marrow and are developed in the thymus. Before the T cells leave the thymus, they have the capacity to develop into CD4 and CD8 T cells and it is through positive selection by binding with MHC I or MHC II receptors that T cells can mature into naive T cells.

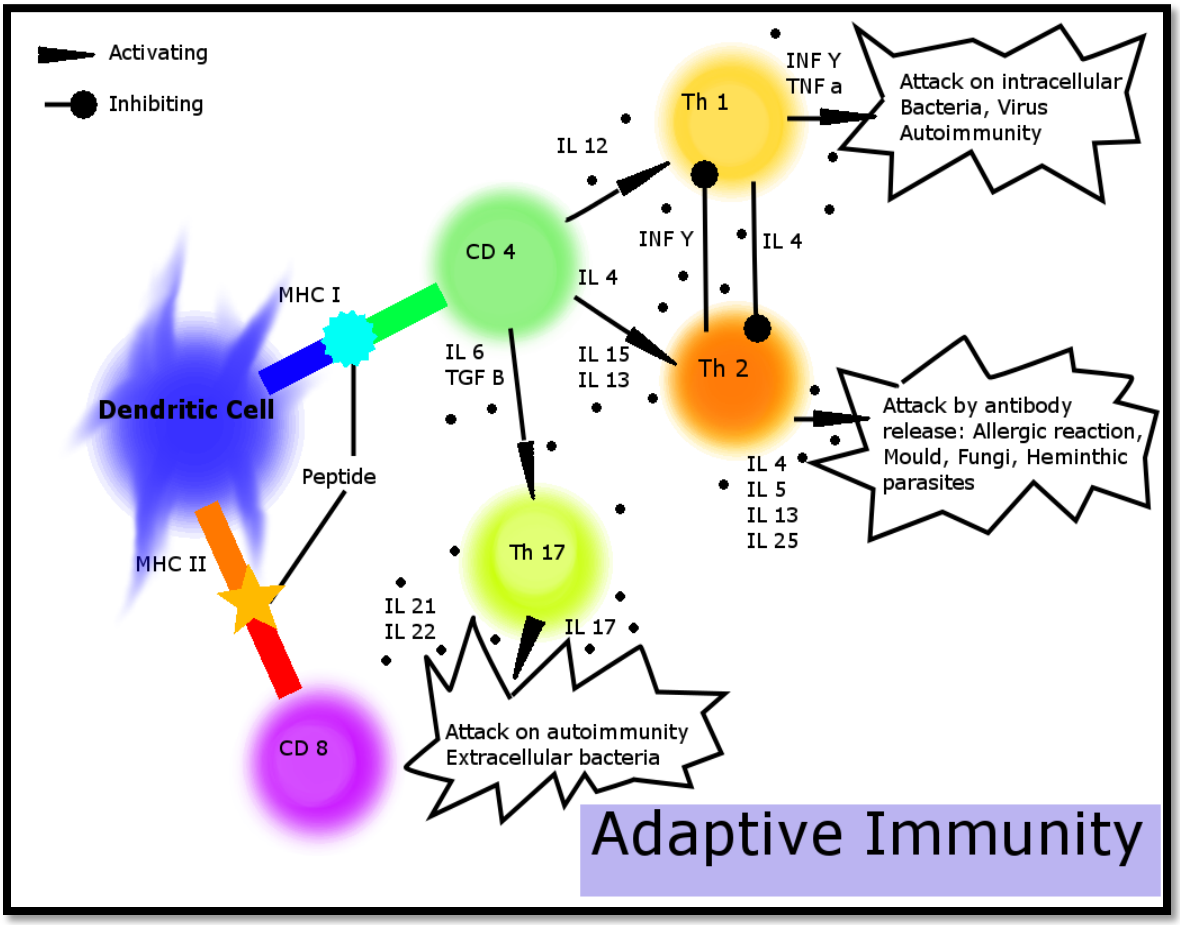
Naive T cells have yet to encounter an antigen on the MHC and migrate towards lymph nodes, once they have matured. If the naive T cells find an antigen MHC complex that fits then a primary immune response occurs in which a signal is transmitted into the interior of T cells and activates T cell responses. This response changes the role of the T

cells from microbial recognition to microbial elimination. Depending on what type of pathogen is invading, they can be targeted by either MHC I or II. Cytosolic pathogens, extracellular pathogens, toxins, tumor cells and transplanted tissues fall into MHC class I which presents to CD8 T cells (Abbas and Lichtman 2010). MHC class II target bacterial infection, viral infection, protein antigens and extracellular parasites and present to CD4 T cells. The collections of proteins that make up the MHC in humans are called human leukocyte antigens (HLAs). The MHC locus contains two sets of hugely polymorphic genes which encode class I and class II MHC molecules that display peptides to T cells (Abbas and Lichtman 2010). The genetic diversity shown in MHC relates to how pathogens are presented and process antigen in different ways, which is important for population survival in epidemics.

Once naive T cells have encountered antigens on the MHC, they differentiate into effector cells. Upon activation by antigens, the T cells begin to secrete cytokines. These cytokines help to stimulate the proliferation of antigen specific T cells, resulting in a rapid increase in the number of antigen-specific lymphocytes. Both the CD4 and CD8 cells are involved in the process of clonal expansion in adaptive immunity and specialize in specific functions. CD8 T cell recognize pathogen peptides presented by MHC class 1 molecule, and naive CD8 T cells differentiate into cytotoxic effector T cells that recognize and kill infected cells. CD4 activate phagocytes to destroy microbes residing in the vesicle of these phagocytes and they help B lymphocytes to produce antibodies (Abbas and Lichtman 2010). As well CD4 T cells differentiate down several distinct pathways that generate effector subsets with different immunological functions (Murphy et al 2008). The main CD4 effector subset currently distinguished are the Th1, Th2, and

Th17 immune response. These different pathways activate their target cells, and several regulatory T cells subsets that have inhibitory activity that limits the extent of immune activation (Murphy et al 2008).

***FIGURE 2**: Adaptive Immune Response (Adapted from Abbas and Lichtman 2010)*



It is during proliferation of effector T cells that important cytokines are released that will eventually direct which immune pathway will be amplified. Interleukins are cytokines that have the ability to communicate between leukocytes as well as between leukocytes and other cells (Abbas and Lichtman 2010). It is these cytokines, produced by CD4 T cells that also function to stimulate a Th1 or Th2 immune response. The most

important cytokine that is produced is interferon gamma (IFN-γ). IFN-γ binds to its receptor function to trigger a biochemical signal pathway that leads to the production of several transcription factors. The result is that IFN-γ mediates the stronger activation of macrophage activity. Macrophages that have encountered microbes produce IL-12. IL-12 stimulates the differentiation of naive CD4 T cell to the Th1 subset, which produces IFN-γ on encountering macrophage associate microbial antigens. IL-12 also increases the amount of IFN-γ produced by these T cells. IFN-γ then activates the phagocytes to kill and ingest microbes. IFN-γ also stimulates more IL-12 production, thus amplifying the response. In contrast, Th2 cells recognize antigens on B cells and are stimulated by a different subset of cytokines. The recognition of antigens on B cells results in the proliferation of B cells and antibody-producing plasma cells (Murphy et al 2008). The Th2 subset of CD4 T lymphocytes stimulates eosinophil-rich inflammation and also functions to limit the injurious consequences of macrophage activation.

Some of the effector T cells can then develop into memory T cells, which are long lived and functionally inactive, although ready to respond to repeat exposures to the infectious microbe. As the effector T cells eliminate the microbe, the stimuli for this process decreases and eventually the T cell expansion is eliminated.

3.2.4 Th1 verses Th2 Immune Response

The balance between activation of Th1 and Th2 cells determines the outcomes of many infections. As previously mentioned, while the CD8 cells proliferate into cytotoxic T cell, naive CD4 cells differentiate into two major pathways for effector T cells: Th1 and Th2 cells. Both of these cell types are involved in combating infectious bacteria, but the

cells involved in these pathways are quite different. Th1 cell mediated immune responses are optimized to be a more effective defense against intracellular microbes like tuberculosis. Th1 cells have a dual function in that they first control certain intracellular bacterial infections which cannot be destroyed by macrophages due to the nature of being intracellular. Th1 cells eliminate the infected macrophages by inducing the fusion of their intracellular matrix and macrophage antibacterial mechanism (Murphy et al 2008). Th1 cells also stimulate the production of antibodies by producing co-stimulatory signals through B lymphocytes. Conversely the Th2 pathway is entirely devoted to the activation of naive B cells to produce antibodies which mediates the response of the synthesis of extracellular matrix proteins, like those involved in tissue repair (Abbas and Lichtman 2010). This immune pathway is also more effective in the host defense against helminths and other parasites.

Th1 and Th2 cells can be differentiated not only in function but also in the cytokines that they produce. Th1 development is composed of two main cytokines: IFN-γ and IL-12, which pushes the CD4 cells into the Th1 differentiation the early stages of T cell activation. IL-4 and IL-5 perform a similar function for the Th2 response. The consequences of inducing the development of these CD4 subsets are that the selective production of Th1 cells leads to a cell-mediated immunity, whereas Th2 cells provide a humoral immunity. The outcome of a Th1 or Th2 immune response has implications for certain infections, as one response will be more successful in eliminating certain invading pathogens, parasitic and foreign bodies. *Mycobacterium tuberculosis* grows in the macrophage vesicles of the host, and therefore, an effective defense would require substantial macrophage activation by Th1 cells. If a Th2 immune response is produced

instead the main response is humoral and the antibodies produced cannot reach the intracellular bacteria and the bacteria continue to proliferate and the disease progresses.

3.2.5 Immunological Response to Tuberculosis

It has been known for many years that the immune system responds very differently to different microbes. Tuberculosis is a unique pathogen as it has a latent and active form, which means that the bacteria are not always eliminated. When the mycobacterium that causes tuberculosis is not eliminated from the host, it is contained within the host and has a chance of re-emergence if the host suffers a relapse in immune effectiveness. Approximately 2 million people die each year from *Mycobacterium tuberculosis* (WHO 2012) and one-third of the world's population remains infected with latent disease (van Crevel et al 2002).

Tuberculosis is caused by *Mycobacterium tuberculosis*, slow growing aerobic bacillus. They are neither gram-positive nor gram-negative, in that they stain poorly using both gram dyes (Levinson 2006). *Mycobacterium tuberculosis* is transmitted from person to person by respiratory aerosol through either repeated or prolonged exposure to the coughing of an actively infected person, although 20% of people are infected by aerosols produced by the coughing of "smear negative" people (Levinson 2006). The initial site of infection is the lungs (Pulmonary TB) but there are forms of tuberculosis where the bones of the spine are involved, called Pott 's disease (Klaus et al 2010) and miliary tuberculosis where the mycobacteria has disseminated through the entire body via the bloodstream (Burke 2011). As mentioned, there are two forms or phases of tuberculosis infection: active and latent. In the active phase, the bacteria has overcome the defense of the immune response of the host and have begun to multiply (Ernst 2012). Individuals

with active tuberculosis are considered to be infectious and can spread the bacteria to others. In the latent form of tuberculosis, the individual is not infectious and has no symptoms of the disease. This is because the host's immune system has halted the multiplication of the bacteria. However, if there are any lapses in the host's ability to put forth an effective immune response, the latent stage of tuberculosis can progress into the active form.

Once the pathogen has entered the body, phagocytosis of the bacteria begins by alveolar macrophages after recognition by TLRs. TLR2 and TLR4 have both demonstrated the ability to mediate *Mycobacterium tuberculosis*-induced intracellular signaling in vitro (Branger et al 2004). When the mycobacterium enters the body it migrates towards phagosomes. Within innate immunity the bacteria is normally ingested by alveolar macrophages and eliminated by lysosomes. *Mycobacterium tuberculosis* on the other hand, prevents the lysosomes from degrading them within the macrophages (Pieters 2008). Specifically with tuberculosis, the acquired cellular immune response is slow to be induced and to be expressed within the lung (Cooper 2009). The slowness of the infection progression allows the infection to become well established. Within 2 to 6 weeks of infection, cell-mediated immunity develops, and there is an influx of lymphoctyes and activated macrophages into the lesion resulting in granuloma formation (Raja 2004). Granulomas are epithelioid macrophages, T cells, B cells and fibroblasts that encase the bacteria and essentially starve them for nutrients (Salgame 2005). Lymphocytes are capable of mediating protection upon transfer by day 5 post primary infection (Cooper 2009) but at this time, granulomas are not formed. Around day 25, cells have started to differentiate into effector T cells (Cooper 2009). Once cells are

activated they must migrate to the primary site of infection, and this migration takes place 15-18 days post infection (Cooper 2009).

The granuloma will sequester the infection off from the rest of lung. The bacilli may remain forever within the granuloma or may get reactivated later. These granulomas can wither, heal or become fibrotic and persist for a lifetime leading to latent infection (Gerold et al 2007). There are several cytokines that are important to the pro-inflammatory response to tuberculosis. IL-6 is produced early in mycobacterium infections but also has the potential to be harmful in these infections by inhibiting the production of IFN-γ (Nagabhushanam et al 2003). IL-10 is also a pro-inflammatory cytokine that can cause a harmful effect to the host immune response by inhibiting CD4 T cell responses and the function of antigen presenting cells (Rojas et al 1999). The elimination of mycobacteria infected granulomas is mediated through the activation of killing mechanisms, particularly CD4 (Ulrichs and Kaufmann 2006). IL-12 activated CD4 T cells recognize the invading pathogen antigens in contact with MHC II molecules and secrete IFN-γ, which leads to development of a Th1 response. These cytokines combined with tumour necrosis factor (TNF-α), a secondary stimulus, stimulates the anti-microbial activity of infected macrophages through the recognition by TLRs (Gerold et al 2007). The importance of TLR signaling in host resistance to *Mycobacterium tuberculosis* is shown in MyD88-deficient mice that are more susceptible to aerosol infection that also show impaired IFN-γ, IL-12 and TNF-α production. (Fremond et al 2004).

3.3 MOLECULAR ANTHROPOLOGY

The study of Anthropology strives to understand the past holistically by drawing upon research from its four fields: archaeology, social anthropology, linguistics and biological anthropology. Archaeology seeks to understand past societies through the excavation of their material cultures whereas linguistics traces the ancestry of spoken languages. Social anthropology informs on cultural behaviours and practices of global populations. Biological anthropology on the other hand studies human development through research into different aspects of health, evolution, non-human primate biology as well as the examination of human osteological remains. Anthropological genetics or molecular anthropology is a sub-discipline of biological anthropology that applies specialized DNA analysis methodology as well as pulling literature from genetics, epidemiology, evolutionary and ecological studies to questions posed by anthropologists (Jobling et al 2004). These anthropological questions can be related to the processes of human evolution, the human diaspora out of Africa, the resulting patterns of human variation, and bio-cultural involvement in complex diseases, just to name a few. Host genetics play a major role in determining differential susceptibility to major infectious disease but genetics themselves are influenced by a variety of factors. Virtually all human traits are etiologically influenced by multiple environmental, cultural, and genetic factors (Weiss and Terwilliger 2000) so understanding that these traits cannot be studied in isolation is essential.

Molecular anthropology uses molecular markers, which are markers that are present in some populations but absent or infrequent in others (Rubicz et al 2007) to uncover and detect variations in human DNA. These markers can include portions on the

sex chromosomes such as the Y chromosome found in males as well as mitochondrial (MtDNA) located in the mitochondria of cells. These two types of genetic samples can be used in population studies to determine the familial relationship between groups. The Y chromosome is composed of large amounts of chromatin and is passed exclusively between father and son. Approximately 95% of the Y chromosome defined as the male-specific region of the Y chromosome doesn't recombine; it is because of this that the Y chromosome is useful for phylogentic reconstruction.

MtDNA on the other hand, is maternally inherited, and therefore passed from a mother to all of her children, although only her daughters will be able to pass it on to their children. MtDNA is a double-stranded, circular molecule that is located outside of the nucleus and is useful in population studies (Gray 1989). A classic example of the application of mtDNA markers to population studies is the origins of the Aleut (Rubicz 2001, Rubicz et al 2003). The Aleut are located in the Alaskan tundra and are thought to represent one of the final migrations of humans into the New World. Similar to other Aboriginal groups in North America, it has been suggested that the origins for the Aleut is in Asia where they would have crossed over the Bering land bridge into Alaska during the last Ice Age. Aleut mtDNA has been characterized by two (A and D) of the four (A, B, C, and D) haplotypes common among Aboriginal groups in North America (Rubiz et al 2003). Haplotypes represent the major branch points on the mitochondrial phylogenetic tree and are defined by differences in the hypervariable regions in the mtDNA (Hummel 2003). The Aleut have a high frequency of haplotype D which sets them apart from Eskimo, Athapaskans and other Northern Aboriginal groups, but they are genetically similar to the Chukchi Siberian Eskimo populations located in northeastern extremity of

Asia in the northern part of Russia (Rubiza 2001, Rubicz et al 2003). These findings support an early Asian origin for the Aleut people of the Alaskan tundra.

Nuclear DNA in coding and non coding regions is also used as molecular markers in anthropological genetic research. These types of markers located in non coding autosomal regions can be broken down into types including: SNPs, polymorphic sequences in short tandem repeats (STRs) which are similar to SNPs but evolve at a faster rate and lastly, minisatellites in DNA sequences called variable number of tandem repeats (VNTRs). VNTRs have been used in research exploring the Siberian origins of Aboriginals in North America (McComb et al 1995, 1996) but because of their high diversity between individuals, they are frequencly used in a contemporary forensic application in a method called DNA fingerprinting.

These molecular markers are also used in population genetic studies to measure diversity. Mutations, gene flow and other random genetic drift effects like founder's effect are all ways of classifying different models of the forces that influence allelic frequencies. Central to these models is the concept of population. Defining the population needs to be determined at the onset in order to determine the specific allele frequencies to be observed. Genetic populations can be made up of individuals who are all part of an interbreeding group or gene pool. Within a population, allele frequencies from one generation can be used to calculate the genotype proportions in the next generation if mating is random, without gene flow, mutation, natural selection and if the population is infinitely large (Jobling et al 2004). If these conditions are met, there will be no change in gene frequencies from one generation to the next. This mathematical model is called the Hardy-Weinberg Equilibrium. If the genotype proportions are not in equilibrium then it is

reasonable to conclude that there are evolutionary forces (mutation, gene flow, genetic drift, or founder's effect) acting on the population. Mutation is the only way to produce new variation by generating new alleles. Mutations can occur at the chromosomal level or a single base substitution and can potentially be passed down to offspring. Somatic mutations such as cancers are not passed on to offspring but are mutations nonetheless. Gene flow, on the other hand is a change in genetic frequencies simply by having population movement. Population movement such as "marrying out" of cultural groups creates new contributions to the gene pool. Physical barriers as well as cultural barriers can limit gene flow and reduce the amount of genetic variation in a population gene pool. However, sometimes random chance creates the phenomenon known as genetic drift which accounts for change in the allelic frequency due to random sampling or chance events (Dobzhansky 1951). Within the concept of genetic drift are two important population processes that can affect allelic frequencies. Population bottleneck or survivorship effect, and founder effect, both of these processes have similar effects as they both result in a smaller gene pool in the populations. Population bottleneck is a reduction in a population gene pool due to some random event, generally environmental, where a large section of the population is eliminated. This decrease in the gene pool will change the genetic frequency of the population by limiting the amount of choice available. Bottlenecks can cause the surviving population to have greater vulnerability to section pressures, such as disease, due to less genetic diversity.

Founder's effect is similar as this model also has a reduction in genetic diversity; however this reduction comes from the founding population's small numbers, and, therefore, small gene pool. Founder's effect can have a negative effect on the

survivorship within a population, and if the effect is strong enough, it can affect the population's genetic frequencies far into the future. For example, the Afrikaner population of South Africa was founded by one shipload of immigrants from Holland in 1652 although it was subsequently augmented in the 1700's by a small number of additional immigrants (Tipping et al 2001). Among the founding immigrants there was one carrier of the gene for Huntingtons' chorea and two carriers of lipoid protenosis and later carriers of porphyria variegate and familiar colonic polyposis arrived a few years afterward (Dean 1972, Botha and Beighton 1983). These individuals helped propel these genetic diseases to higher frequencies with more than 30,000 Afrikaner descendants carrying the genes of these founding individuals. A similar example is seen in the British colonists founding settlement on Tristan da Cunha. One of the colonists carried a recessive allele for retinitis pigementosa, a progressive from of blindness. Today the frequency of the allele is ten times higher on Tristan da Cunha (Diamond and Rotter 2002).

Many aspects of health, from increased resistance/susceptibility to diagnosing and treatment outcomes, can be influenced by host genetics. Infectious diseases have been major causes of mortality for much of human evolution and over time changes in the environment, human demography and host disease interactions have significantly altered the disease spectrum. There are two divisions of genetics that are used to describe the associations with disease variation. The first is simple genetic disease, where individuals carrying a polymorphic allele usually manifest the disease phenotype. Complex genetic diseases on the other hand, are due to individuals carrying a polymorphic allele. They are carriers of the disease phenotype and do not necessarily develop the disease. Polymorphic

sites on genes associated with disease appear to be disadvantageous but the process of selecting for that particular polymorphism may have allowed individuals carrying these genes a higher degree of fitness. Molecular anthropology can be used to piece together the disease histories that have led up to the selection for these polymorphisms. An example of complex genetic disease is the classic anthropological case study of the adaptation to malaria (Livingstone 1983, 1984).

In 2008, there were 247 million cases of malaria diagnosis and nearly one million deaths-mostly in Africa (WHO 2009). Most of these deaths are children in impoverished areas. Malaria is caused by Plasmodium parasites which are spread by the bite of an infected Anopheles mosquito. There are four types of human malaria: *Plasmodium falciparum*, the most lethal and common type, *Plasmodium vivax*, the second most common type, *Plasomodium malariae* and *Plasmodium ovale* (WHO 2003). Human immunity is an important factor in areas where malaria is prevalent, especially historically. Selective pressures from the high mortality caused by malaria have created a hemoglobin abnormality that can provide protection from this infectious disease. The abnormal haemoglobin disorder is called sickle cell anemia, in which the shape of the red blood cell is stretched into a sickle like shape instead of the normal round shape. The sickling is caused by changes to fibers in the red blood cells after oxygen that they have taken up is released. Individuals with a heterozygous genotype for the abnormal haemoglobin have some degree of immunity to malaria, compared to individuals with homozygous normal haemoglobin. Individuals with homozygous abnormal haemoglobin, haemoglobin S, develop sickle cell disease. When there is a greater demand for oxygen in the blood, for example during exercise, the deoxygenation process would cause the

abnormal haemoglobin cells to sickle and clog smaller blood vessels. To remove this blockage the immune system would have to destroy the sickled red blood cells, this would cause a reduction in the number of red blood cells and anemia and associated complications would result (Molnar 2006). The abnormal haemoglobin still appears in populations today, within descendants of those who lived in areas of endemic malaria.

Similarly, cystic fibrosis, is a genetic disorder found in high frequencies in European populations due to an autosomal recessive disease. Affected individuals carry two copies of a polymorphic gene, which causes progressive disability due to a clogging of the airways from mucus build up. Evidence for the selection of the polymorphic gene suggests that heterozygotes may be more resistant to disease like cholera, typhoid fever or bronchial asthma which would have been present in endemic forms in Europe at certain points in history (Jobling et al 2004). The polymorphic gene associated with cystic fibrosis has been dated to be much older than to when cholera was prevalent in Europe therefore it is unlikely to be the selective agent (Slatkin and Bertorelle 2001), although typhoid fever and other diarrhea causing pathogens could still be the causative agents (Wiuf 2001).

Thus, molecular markers can be used in a wide variety of applications in genetic research related to anthropological questions including interpreting fluctuations in genetic frequencies as well as identifying underlying genetic markers such as haplotypes. Population genetic models like the founder's effect and bottleneck can be applied to explain variations in allelic frequencies in different populations. In the absence of infection, genetic variations that may offer an increase in host resistance may actually carry a cost to fitness. This may become apparent in the reduced survivorship of certain

genotypes that were once adaptive but have since become maladaptive (Yan et al 1997), reduced fertility (Webster and Woolhouse 1999), reduced competitive ability (Kraaijeveld and Godfray 1997) or increased susceptibly to non communicable disease (Searle and Blackwell 1999). However, the natural protection that heterozygous genotypes provide allows certain individuals to gain a higher degree of fitness as seen in the examples of malaria and cystic fibrosis. As time progresses, a gene that may offer a positive outcome in a particular time and place may offer in a negative one if the social or biological environment changes (Woolhouse et al 2002). Using an anthropological framework to conceptualize allelic change provides a more holistic way to interpret the past, by reviewing biology without forgetting the cultural aspects that help to shape it.

3.4 PATHOGENS AND EVOLUTION

One of the major selective forces on pathogens is the host's immune system. If the host's immune response overwhelms the pathogen and the pathogen is destroyed it fails to replicate which is not evolutionary successful. However, if a pathogen overwhelms and kills the host, replication is again unsuccessful because the host is dead. The immune response has to be adaptive to the constant struggle between host and pathogen in order to survive. A successful bacterial pathogen must be able to avoid and adapt to evolving host defenses and be able to infect other hosts as well. In order for a pathogen to be truly successful, pathogens often co-evolve with the host immune response. Co-evolution is the process of reciprocal, adaptive genetic change in two or more species (Woolhouse et al 2002). This means that the variations in gene frequencies observed in populations can be a result of selective process of one population acting on the other. These strong selective forces can include: pathogen infectivity and host

resistance, host seeking and pathogen avoidance behaviors, and the ability of the host to clear an infection versus the ability of the pathogen to evade or suppress host defenses (Woolhouse et al 2002:569). However, pathogens also frequently evolve to exploit their niche. This exploitation involves pathogens selecting to exhibit environmentally responsive and adaptive molecular traits which allow adherence, entrance, and replication with the host (Brunham et al 1993).

Studies on co-evolution have been explored for the last 40 years (Mode 1958, Hamilton et al 1990) and include gene for gene, matching allele and genotype models (Morand et a 1996, Lively and Apanius 1995, Anderson and May 1991). The key feature in all of these studies has been the discussion on the interaction based on the combination of host and pathogen genotypes. Functional polymorphisms are often found in pathogen genes coding for antigens, that is, molecules that interact with the immune system, but there are also examples involving other kinds of interaction with the host, such as with host cell receptors (Woolhouse et al 2002). The human leukocyte antigen (HLA) complex is a prime example of the continual adaption of the immune response to pathogens as it is highly polymorphic. The HLA complex role in the immune system is to encode cytokines to enable them to fight the invading pathogen through the activation of T-cells. The polymorphisms in the HLA complex are a result of historical selective pressures related to past encounters with pathogens (Prugnolle et al 2005). Disease outcomes as well as resistance and/or susceptibility can be impacted by the HLA complex. Research to date has established that there is a correlation between different diseases like rheumatoid arthritis, leprosy, systemic lupus erythematosus and tuberculosis and HLA variations (Khomenko et al 1990, Zhang et al 2009, Van Gaalen et al 2004).

Evolutionary forces can affect genotypes as well as genotype frequencies in a population through selective sweeps and dynamic polymorphisms. Selective sweeps occur when new alleles appear by mutation and eventually become fixed in the population although this process is slow (Woolhouse et al 2002). The polymorphisms that arise from this type of evolutionary force are more transient because they take longer to become fixed in the population and therefore must be continually present within the gene pool in order to eventually become fixed. This type of co-evolutionary force of host immunity and pathogens can be conceptualized as a weapons arms race, where accumulated improvements in both populations (host and pathogen) are continually matched by the opposing populations (Woolhouse et al 2002). Dynamic polymorphisms conversely involve fluctuations in allele frequencies caused by selection and are inherently persistent within a gene pool (Woolhouse et al 2002). The polymorphisms that arise are represented by the Red Queen Effect, where allelic frequencies cycle as a continual adaptation in order for both populations to maintain a certain degree of fitness within the environment that they co-evolved with, essentially "running as fast as you can to stay in the same place" (van Valen 1973). Evidence for co-evolution also can be shown with temporal patterns and spatial patterns. Temporal patterns show that when new pathogens are introduced, phenotypic change occur in reaction to lessen the effect of the pathogen. A classic example of temporal co-evolutionary changes can be distinguished from the introduction of the myxoma virus into the naive rabbit populations of Europe. These changes were new genetic variations arising in both the virus phenotype as well as within the rabbit's immune reaction over several generations (Fenner and Fantini 1999). Demonstrating temporal patterns within the human/pathogen model is

more difficult due to the longer time scales involved in a human's lifetime when compared to that of any flora or fauna. Despite these difficulties, Shimizu et al. (1994) studied a patient with chronic hepatitis C virus infection (HCV) and took samples of the virus at different time points over 14 years following onset of his hepatitis infection. Plasma collected from this patient in 1990, 13 years after onset of hepatitis, contained HCV that was genetically divergent from the initial 1977 strain. This sample did not contain any antibodies capable of neutralizing either the 1977 strain of HCV or the 1990 strain. This indicates that the strain had adapted to the host's immune system some time ago and the body had defeated the original 1977 strain long enough ago that the original antibodies were no longer relevant. However the immune system had not yet been able to adapt to the 1990 strain of HCV as no antibodies were found in the plasma sample. A year later, plasma was again collected and it contained neutralizing antibodies to the 1990 strain now although still contained none to the 1977 strain (Shimizu et al 1994).

Spatial patterns of co-evolution can be demonstrated through a snapshot approach using temporal co-evolutionary model patterns. Spatial co-evolutionary patterns can be observed within a gene pool when two or more subpopulations within this main gene pool are at different stages of adaptive progress, so that spatial variations can be inferred using temporal variations (Gandon and Michalakis 2002). In this model the concept of local adaptation is of key importance with sub-populations differing in their allelic frequencies in reaction to pathogens involved within their micro-environments (Gandon et al 1996, Kawecki and Ebert 2004). Transmission pools are also important to consider within spatial patterns of co-evolution. These pools are composed of individual hosts who have had prior exposure to one or more strains of pathogens that are circulating in their

environment as well as individuals with no pervious exposure, which could be rated to being at a different stage of the temporal co-evolutionary process. Pathogens can continually be transmitted within a pool if there is not a high volume of individuals with immunity. This immunity can sometimes be classified as herd immunity. If a large enough subpopulation of the entire population is at a co-evolutionary stage where the immune system has successfully adapted to the pathogen then the rest of the individuals, who might not have immunity, are unlikely to become infected. However, because of the cyclical nature of "one-upmanship" of the pathogen/host relationship, individuals who have a rarer variant than those with herd immunity are at a greater long term selective advantage since pathogens will not have adapted to their host response yet (Bruham et al 1993).

Co-evolution can occur between any interacting populations, because of the intimate nature of the association and the strong selective pressures that each can exert on the other. By understanding how the push-pull effect that pathogens and immune system will have on each other, one can start to elucidate the underlying processes related to the changes in allelic frequencies and contemporary immunogenetic profiles.

3.5 SINGLE NUCLEOTIDE POLYMORPHISMS

3.5.1 Introduction

While certain SNPs can be quiescent and others functional, the importance of SNP diversity is highlighted by the spectrum of differing disease severities that can occur from one individual to another. In the late 1920's there was an accidental administration of a virulent dosage of *Mycobacterium tuberculosis* meant to immunize 251 children in Lubeck, Germany. Of these children, 47 did not develop clinical disease, 127 developed

radiological signs of tuberculosis, and 77 died (Moller and Hoal 2010). Why did some children develop disease and die, while others remained unaffected? A similar question can be posed when reviewing tuberculosis rates among the Qu'Appelle Aboriginals located in Saskatchewan, Canada. Initially this population had a 10% tuberculosis morality rate when it was first introduced in the 1890's, however while the first annual mortality rates were high, in the following years deaths they decreased to 0.02% (Lux 1998). Did the morality rate drop because of intervention or did the remaining individuals possess some genetic variation that allowed their immune response to be more successful?

The goal of human genome research is in a large part to answer these questions by exploring the role of common genetic variations of susceptibility to common disease. The most common type of human genetic variation is the SNP because they are the most abundant form of variation in the human genome (Ziegler and Konig 2010). SNPs can serve as genetic markers for identifying disease genes by linkage studies in families, linkage disequibilibrium in isolated populations, association analysis of patients and controls and loss of heterozygosity related to disease occurrences (Johnson et al 2001, Risch and Merikangas 1996, Janssen et al 2012). Although the majority of the nucleotide base pair changes in SNPs will have no biological consequences, there are small portions which have some functional significance. These polymorphisms can be found in both the coding (exon) and non-coding (intron) regions of the human genome. Polymorphisms arise as a result of mutation and different types of polymorphisms are typically referred to by the type of mutation that created them. The simplest types of mutations are those that are a substitution of one nucleotide for another. The variation is a single base pair

substitution and therefore, in theory, there are four possible variations. Ziegler and Konig (2010) state that two-thirds of these variations are cytosine and thymine (C and T) or guanine and adenine (G and A) alleles. Research in these areas of disease susceptibility associations has exploded since the late 1990s, with Zeigler and Konig (2010) noting that between 1998 and 2004 identified SNPs had increased by nearly 8.8 million.

3.5.2 SNPs associations with Disease

SNPs are also important tools for genetic research and as mentioned, have been used to determine underlying genetic factors in disease susceptibility. A change at the base pair level of a gene can affect the functionality of that particular gene, and in the case of genes involved in the immune response, this change can have an impact on the generation of immunity. SNPs in the immune response are very numerous, with 268 genes that contain polymorphisms reported to date (Hirschhorn et al 2002); to fully describe them and their effect on disease would require a larger platform. Therefore, for the purposes of this review, only the genes analyzed in this study, IL-12, TLR2 and TLR4, will be focused on.

SNPs occurring in the TLRs can cause decreased levels of recognition and a consequential suboptimal response from downstream elements like macrophages. TLR2 was the first human TLR involved in host immunity to be described (Yang et al 1998) and subsequent studies defined TLR4 as a receptor for lipopolysaccharides (Lien et al 2000). Studies focused on TLR4 and TLR2 gene polymorphisms demonstrate an increased susceptibility to sepsis and gram negative bacterial infections (Lorenz et al 2002, Agnese et al 2002). Lorenz and colleagues (2000) also demonstrated with function studies that the Arg753Gln polymorphism in TLR2 has significantly less responsiveness

to bacterial peptides derived from *B. burgolorferi* and *T. pallidum*. TLR2 gene may be associated with an increased risk for Mycobacterial disease, in particular *Mycobacterium leprae* in the patients with the mutations (Kang and Chae 2001, Kang et al 2002). These findings suggest that a mutation in the TLR2 gene may predispose individuals to life threatening bacterial infections. While not infectious diseases, the TLR4 (Asp299Gly and Thr399Ile) variants are also important for inflammatory function as they have been shown to be associated with decreased susceptibility to rheumatoid arthritis (Radstake et al 2004) and atherosclerosis (Schroder and Schumann 2005). Several studies have shown that Asp299Gly and Thr399Ile SNPs can affect the functionality of TLR4 and thus increase susceptibility to Legionnaires and meningococcal disease (Hawn et al 2005, Faber et al 2006). However, results in TLR4 meningococcal studies have not been consistently significant (Read et al 2001). Crohn's disease however, has been significantly correlated with the frequencies of TLR4 SNPs (Torok et al 2004). Studies focusing on the over expression of lipopolysaccharides indicated that the Asp299Gly genotypes might have a greater functional impact when compared to the Thr399Ile genotypes (Arbour et al 2000). Weiss and colleagues (2004) report that in response to Salmonella, TLR4 null mice have a suboptimal immune response allowing bacteria to harbour between 10 to 100 fold more than controls, and TLR2 and TLR4 knockout mice have between 100-1000 fold more bacteria when compared to their functioning gene counterparts. Allowing time for bacteria to amass is a concern when evaluating the immunological response since a suboptimal response will take longer to effectively destroy all bacteria, allowing the infection to increase in severity. Similarly, there were high bacteria loads of *Staphylococcus aureus* in TLR2 deficient mice with a moderate

increase in disease severity (Gonzalez-Zorn et al 2005). Disease outcomes are important to consider, as patients with type 2 diabetes had a substantially lower incidence of diabetic neuropathy if they carried the TLR4 Asp299Gly/Thr399Ile allele variant (Rudofsky et al 2004). TLR4 and TLR2 polymorphisms can provide increased resistance to certain disease conditions like arthritis and atherosclerosis and can impact, although results have varied, differential susceptibility to infectious pathogens.

Certain IL-12 studies have focused on determining the frequency of SNPs occurring in sick individuals and how SNPs may affect the functionality of the IL-12 role in the immune response. Ottenhoff and colleagues (2002) found that low production of IFN-γ was attributed to genetic defects in the production of the IL-12p40 and IL-12p70 subunits. The low production of these genes was associated with individuals who suffered from infections with salmonella (Ottenhoff et al 2002). Yin and colleagues' (2004) study on the IL-12p40 subunit appeared to have some influence on the outcome of hepatitis C virus. The frequency of A/A genotype was decreased in self-limited HCV infection, due to the SNPs' functional effect on the Th1/Th2 immune pathway balance. Davoodi-Semiromo and colleagues (2002) show that the decreased functional expression level of the IL-12p40 subunit is likely a risk factor for type 1 diabetes. IL-12 polymorphisms have been discussed at length in relation to *Mycobacterium tuberculosis* (Cooper et al 2007) and it would stand to reason that they should also have some functional significance with *Mycobacterium leprae* due to the similarities in the mycobacterium genus. A study conducted on lepromatous leprosy patients in western Mexico demonstrated that the IL-12p40 polymorphism was associated with greater

susceptibility to leprosy as the role of IL-12 in the immunological response is similar between the two mycobacterial infections (Alvarado-Navarro et al 2008).

Population genetics also have a large role to play in determining susceptibility to disease. For the most part, SNPs are common variants within a population that are found in a frequency over 1% (Schroder and Schumann 2005). The distribution of the SNPs found in IL-12 gene has been investigated frequently and disease associations have been reported. The frequency of the IL-12 polymorphism was reported in Europeans (80-83%) and higher than in Japanese (50.5%) and African (66%) and Cameroon (62.5%) populations (Huang et al 2000, Hall et al 2000, Tsunemi et al 2002, Ma et al 2003). Certain SNPs may also occur in regular intervals within a population; Schroder and Schumann (2005) report that TLR2 Arg677Try, Arg753Gln and TLR4 Asp299Gly, Thr399Ile variants occur at 5-10% in all Caucasian populations. However, these variants are absent in Chinese (Zhu et al 2008), Japanese (Noguchi et al 2004) and Taiwanese populations (Cheng et al 2007). The differences in SNP frequencies between these populations can be related to their distinctive historical relationships with certain pathogens, random evolutionary pressures and differential co-evolutionary host/pathogen interactions.

3.5.3 SNPS and Tuberculosis

It is estimated that one third of the world's population is infected with *Mycobacterium tuberculosis*, although only 10% will ever develop clinical disease (Selvaraj 2004). Even though only 10% of those infected will develop the disease, tuberculosis still causes significant morbidity and mortality annually, accounting for 1.7 million deaths and 9.4 million new cases worldwide in 2009 (WHO 2012). Tuberculosis

is transmitted through inhaled micro droplets and, therefore, for optimal transmissions individuals need to be in close living quarters. While crowded living conditions hasten the transmission, this disease has also been associated with poverty with the vast majority of deaths being located in the developing world, predominantly in African and South East Asia countries (WHO 2010). As tuberculosis is a disease associated with poverty, environmental and socio-economic factors should also been taken into consideration concerning differential susceptibility. Beyond these factors, biological variations likes SNPs may be able to account for discrepancies in disease susceptibility between individuals within the same population. A study conducted in an American nursing home found that African ancestry patients were twice as likely to be infected with *Mycobacterium tuberculosis* as individuals with European ancestry, even though they shared the same environment and socio-economic situation (Stead et al 1990). As early as 1949, it was being proposed that the maintenance of several gene variants could be creating increased susceptibilities to pathogens (Haldane 1949). Research in this area has exploded with advancements in technology and investigation into SNPs in the immune response has propelled the discipline forward. However, some aspects of research conducted into increased genetic susceptibility to pathogens has remained fundamentally unchanged: that any disease susceptibilities are likely related to immunity genes acting together in clusters to either diminish or amplify a response as genes rarely act in isolation.

Cytokines are important mediators of the immune response to tuberculosis and although they exhibit a low degree of variation, there have been an increasing number of associations between SNPs reported in the promoter regions of cytokines and

tuberculosis (Yim and Selvaraj 2010). Mutations in these genes can result in altered transcription factor recognition sites, affecting transcription activities such as altering levels of cytokine production (Pravica et al 1999). Several studies on IL-12 research have focused on determining the frequency of SNPs occurring in sick individuals and how SNPs may affect the functionality of the IL-12 role in the immune response. De Jong and colleagues (1998) found that individuals suffering from recurrent nontuberculous mycobacterial infection had higher frequencies in gene encoding IL-12p40 and IL-12R and that these patients displayed a reduced capacity to produce IFN-γ, another important inflammatory cytokine required in the successful elimination of mycobacterium. A rare genetic defect resulting in Mendelian susceptibility to mycobacterial disease has recently been linked to individuals having mutations in genes of the IL-12/IL-23/IFN-γ axis, and even having an increased susceptibility to even non-pathogenic mycobacteria (Filipe-Santos et al 2006, Casanova and Abel 2007). Similar associations between SNPs in IL-12 and increased susceptibility to tuberculosis have been found within different populations (Altare et al 1998, Flynn and Chan 2001, Morahan et al 2007, Freidin et al 2006, Tso et al 2004). Although there is not a consistent pattern of SNPs in IL-12 creating increased susceptibility to tuberculosis, Selvaraj and colleagues (2008) found no association between 3'UTR+1188 (A/C) and tuberculosis, which is similarly reported in Ma and colleagues (2003) research, although in different populations.

Closely linked to IL-12 in the immune response is INF-γ and TNF-α, as they are all involved in a positive feedback loop that promotes the differentiation of naive T cells into a Th1 immune response, a response that is essential for the containment and elimination of tuberculosis. The functional +A874T SNP which is associated with the

secretory capacity of IFN-γ, has been linked to increased tuberculosis susceptibly among Sicilians (Lio et al 2002), South Africans (Rossouw et al 2003), Hong Kong Chinese (Tso et al 2004) and Spanish (Lopez-Maderuelo et al 2003). Although again this SNP is not associated with tuberculosis in all populations, negative linkages were reported in populations from Houston, Texas (Moran et al 2007), West Africa (Cooke et al 2006) and South India (Selvaraj et al 2008). TNF- α mice models have well documented the protective role of this cytokine. Death, higher bacterial loads and inadequate granuloma formation have been observed in mice deficient in TNF- α (Flynn et al 1995, Bean et al 1999). Within human populations however, the evidence is less conclusive. TNF- α polymorphisms (-G308A, -308,-23G) were found to protect against tuberculosis in two different populations (Scola et al 2003, Correa et al 2005). Chinese, Cambodian and Indian tuberculosis patients TNF- α polymorphisms (-238, -376) were found to have no association with resistance or susceptibility to this disease (Delgado et al 2002, Selvaraj et al 2001).

One of the first lines of defense is the recognition of invading pathogens by pattern recognition receptors (PRR), one of these PPRs are TLRs, which play critical role in both the adaptive and innate immune response. Within mice models, TLR2 deficient mice demonstrate high susceptibility to tuberculosis (Reiling et al 2002, Drennan et al 2004) suggesting that TLR2 specifically, plays an important role in the detection of this pathogen. Additionally, TLR2, research on tuberculosis focused on the Arg753Gln SNP has shown an association with tuberculosis (Lorenz et al 2000, Orgus et al 2004, Bouchud et al 2003) and furthermore TLR2 deficiencies have been associated with high

bacterial loads and a moderate increase in disease (Gonzalez-Zorn et al 2005, Takeuchi et al 1999). This TLR2 Arg753Gln polymorphism has been associated with tuberculosis in several different global populations including: Turkish, Tunisian and Korean (Ogus et al 2004, Bean et al 1999, Yim et al 2006). The complexity that TLRs communicate with each other also offers an avenue for SNPs to affect tuberculosis susceptibility, as poor communication may result in a delayed detection. Bafica and colleagues (2006) report that in collaboration with TLR9, adequate TLR2 expression is required to control tuberculosis severity. The relationship between TLR2 and TLR4 may not be as direct. Shi and colleagues (2005) have observed that double knockout mice for SNPs in TRL2 and TLR4 are not particularly susceptible to *Mycobacterium tuberculosis.* However, these findings have been debated as Reiling and colleagues (2002) showed that a high dose of *Mycobacterium tuberculosis* in TLR2 and TLR4 defective mice revealed that TLR2 mice to be more susceptible than TLR4 defective mice. TLR4 mutant mice showed a reduced capacity to eliminate *Mycobacterium tuberculosis* with a lower production of TNF- α, IL-12p40 and MCP-1 suggesting a role for TLR4 in the specific defense against tuberculosis (Abel et al 2002). Lorenz and colleagues (2002) report that certain SNPs in TLR4 have been associated with gram negative bacterial infections. However, results again vary supporting an association between disease and certain SNPs, as TLR4 mutant mice show normal resistance to tuberculosis infection (Reiling et al 2002, Kamath et al 2003), directly contrasting two other studies that show increased susceptibility to this disease (Abel et al 2000, Branger et al 2004).

Historically, vitamin D supplementation had been used in the treatment of tuberculosis before pharmacological advancement had been made. Since these early

treatment days, there has been a suspicion that vitamin D plays a role in susceptibly to tuberculosis. Modern research into vitamin D deficiencies has confirmed these early suspicions and several SNPs have been found in the vitamin D receptor gene (VDR) that affects the functionality of these genes. Studies in a variety of population, including those in Gambia (Bellamy et al 1999), South India (Selvaraj et al 2008), West Africa (Bornman et al 2004), South America (Wilbur et al 2007), and South Africa (Lombard et al 2006), have associated polymorphisms in VDRs with increased susceptibility to tuberculosis.

Increased susceptibility due genetic polymorphisms is an important consideration in the on-going global eradication of tuberculosis. The underlying genetic components in many immunological pathways can be affected by SNPs and any alteration in functionality may result negative disease outcomes. Host genetics help to explain, in part, why certain individuals are more successful in eliminating this pathogen and this understanding could aid in shaping new tuberculosis control and treatment avenues.

3.6 SUMMARY

The human immune system enables the body to distinguish between self and non-self. As simplistic as that statement seems, the adaptive forces that help the immune response mount an effective reaction are complex. The manner in which the immune response is created depends on many cellular pathways and compounding interactions of PRRs, cytokines and macrophages. Heterogeneity in immunity-related genes increases the overall fitness of the host's immune response as it can increase the diversity allowing the response to cover a larger spectrum of disease strains as well as decrease the chance of acquiring inheritable genetic disorders. Human populations adapt biologically to their environment and to the pathogens found within. Gene-environment interactions are likely

to introduce another layer of complexity in understanding contemporary genetic frequencies. The genes involved in defense against infectious pathogens evolve more rapidly than others and excessive polymorphism in the human genome may result from selection pressures exerted by infectious diseases. These selective forces, mutation, gene flow, genetic drift as well as random genetic drift factors like founder's effect, are all fundamental for understanding changes in allelic frequencies. With rapidly advancing technology, information on these historical selective forces can be reverse engineered from present genetic variations, informing on historical human relationships with disease. It is therefore of the utmost importance that genetic variations related to differential disease susceptibility and/or resistance not be regarded in isolation but understood within their adapted historical and evolutionary frameworks.

CHAPTER 4 DNA METHODS AND STUDY POPULATIONS

4.1 INTRODUCTION

With the advancements that have been achieved over the past few decades, molecular research has been able to discover the genetic diversity that allows the human immune response to be such a powerful mechanism. This genetic diversity however, also can contribute to an altered immune response which while once was beneficial, with changes to the pathogen environment can become detrimental. Molecular technologies allow researchers to detect these variations and help perceive the impact that particular variations will have on health. This chapter describes the techniques involved in the amplification and detection of SNPs in IL-12p40 (rs3212227), TLR2 at site Arg753Gln (rs5743708), and TLR4 at sites Asp299Gly and Thr399lle (rs4986790 & rs4986791) in a Caucasian and three Manitoban First Nation cohorts as well as the statistical methods used to compared the relationships between ethnicity and SNP frequency.

The designation of the three First Nation cohorts as Dene, Cree and Saulteaux was purposeful, as these are the terms that the individual communities used to self identify. The classification of the Caucasian cohort as such was decided upon to conform to terminology cited in pervious literature on this particular cohort (Larcombe et al 2008) as well as to fall in line with terminology used genetic based health literature. In anthropological literature however, this racial term has become antiquated due to its contentious legacy. In this thesis, in order to stay in line with both existing anthropological and immunogenetic literature, when the term Caucasian is applied, what is meant is that this cohort is of European descent.

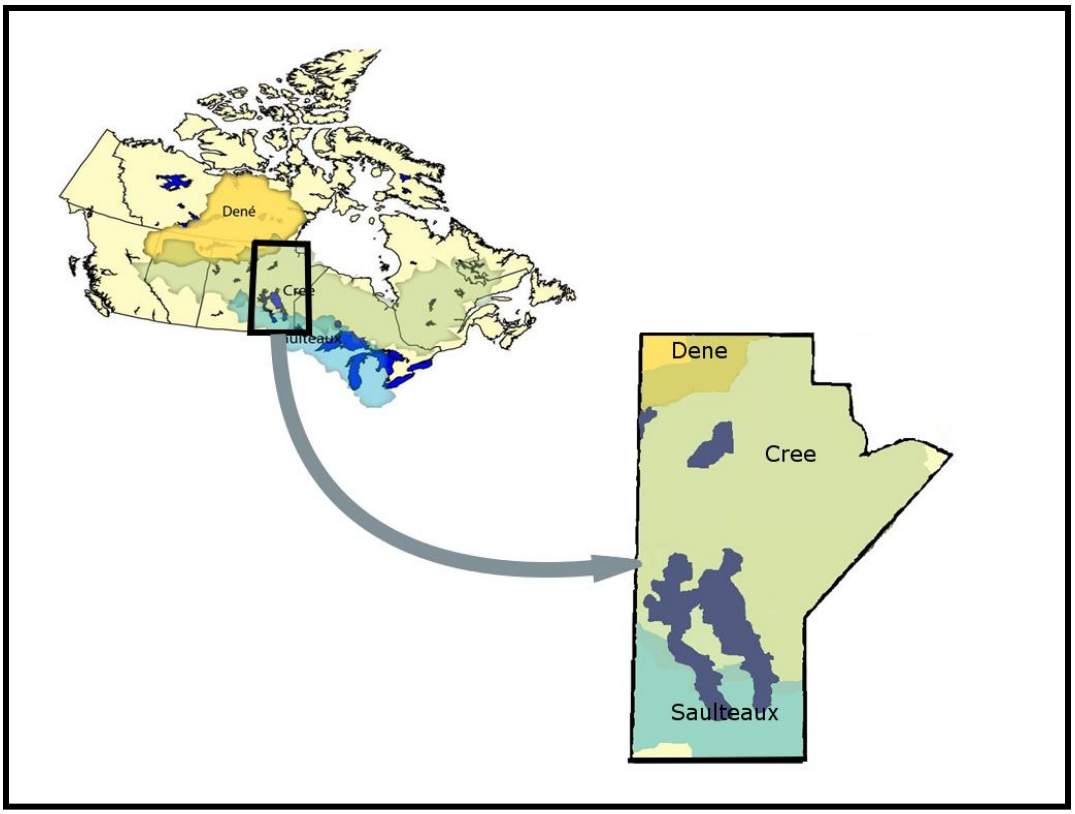
4.2 STUDY POPULATIONS

4.2.1 Study Cohorts

To explore immunogenetic variability the DNA samples used in this study were collected from three different reserve communities in the province of Manitoba, representing the Cree, Dene and Saulteaux people (figure 3). The participants from these three reserve communities self identified themselves as representing the Dene, Cree or Saulteaux people as well as the reserve communities being classified as being Dene, Cree and Saulteaux. The Caucasian sample was collected from healthy individuals within the city of Winnipeg, Manitoba. Whole blood samples and buccal swabs were collected from individuals by a registered nurse at the individual communities, or by the individuals themselves in the case of buccal swabs in view of the researcher. The four populations that made up the total study sample size consisted of: a Dene cohort (n=61), 29 males and 32 females with a mean age of 41 years, a Cree cohort (n=42), 19 males and 23 females with a mean age of 41 years, a Saulteaux cohort (n=120), 31 males and 89 females with a mean age of 44 years, and a control sample consisting of a Caucasian cohort (n=91), 28 males and 63 females with a mean age of 52 years. The combined totals from each of these individual cohorts resulted in a total study sample size of 314 individuals. The Caucasian, Cree and Saulteaux cohort did not report any known cases of tuberculosis infection, whereas 40% of the Dene cohort self-reported having had either active or latent tuberculosis. All participants gave informed consent, and approval was received from the individual Band Councils and the University of Manitoba Ethics Review Board (see appendix A). Participants from within individual communities who were related as first or second degree relatives were excluded from this study, in order to prevent biases in

genetic frequencies.

FIGURE3: *Map of Canada, Denoting the Traditional Borders of the Dene, Cree and Saulteaux Populations, with Insert of Traditional Borders reflected within the Province of Manitoba.*



4.3 DNA AMPLIFICATION, DETECTION AND ANALYSIS TECHNIQUES

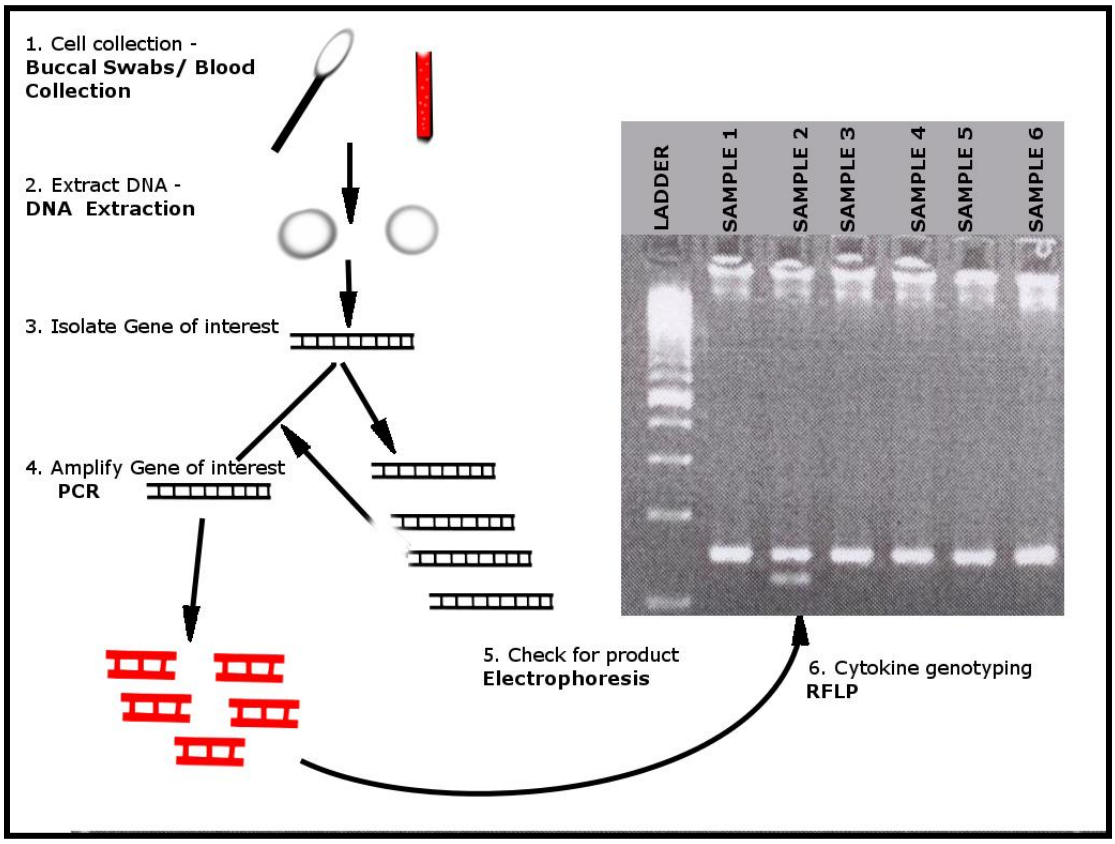
4.3.1 Cytokine Genotyping

The initial collection of whole blood and buccal swab samples occurred in each of the three First Nation communities with the aid of registered nursing staff or the participants themselves, in the case of buccal swab sample collection. Caucasian whole blood samples were collected at the University of Manitoba. Whole blood samples were collected in BD® vacutainer tubes lined with EDTA from the Dene, Cree and Caucasian participants, whereas the Saulteaux participants used Puritan cap-shure swabs™ from

VWR for buccal swab DNA collection. The protocols to assist in ensuring adequate DNA concentrations are collected with buccal swabs dictates that each of the swabs scrapes the inside surface of one check roughly a dozen times. Two swabs were collected from each individual (one per cheek) to maximize the DNA yield. The swab was allowed to dry at room temperature and then sealed to avoid contamination. The buccal swabs used in this study have been optimized for DNA collection and extraction through special design as an aerated tip type of plastic cap that slides over the stick portion of the swab, protecting the sample from contamination without allowing the sample to mold. All samples were subsequently transported to the laboratory for genetic analysis.

Gene analysis (figure 4) was conducted at the Immunogenetics Laboratory, University of Manitoba. Once at the Immunogenetics Laboratory, the whole blood samples were processed in order to achieve a useable DNA sample. In order to extract the DNA from the whole blood samples, the blood was spun at high speeds to separate the blood sample into its three component layers. The buffy coat, containing the white blood cells and platelets, was collected and the top plasma layer and bottom layer made up of erythrocytes was discarded into a solution of 10% bleach to inactivate the sample. The buffy coat samples then went through a purification process using the QIAamp DNA Blood Mini Kit (QIAGEN). Similarly, DNA extracted from the buccal swabs underwent the QIAamp DNA Blood Mini Kit (QIAGEN), although the head of the swab is snipped and the DNA is essentially washed off as part of the extraction protocols before the purification takes place.

***FIGURE 4**: Genetic Analysis (Adapted from QIAGEN Protocol Flowchart 2010)*



The genetic analysis methods used in this study were optimized for a DNA concentration of at least 100 µg/ml. Collecting whole blood samples provided a higher concentration of DNA than buccal swabs although taking blood is more invasive and can be painful which can act as a deterrent for participation. Similarly, buccal swabs are easier to transport than whole blood samples. Buccal swabs typically yielded less DNA than whole blood at a concentration which tended to be between 1-6 µg/ml far less than the optimal 100 µg/ml, however buccal swabs are less invasive and can be collected by researchers and participants rather than medical professionals.

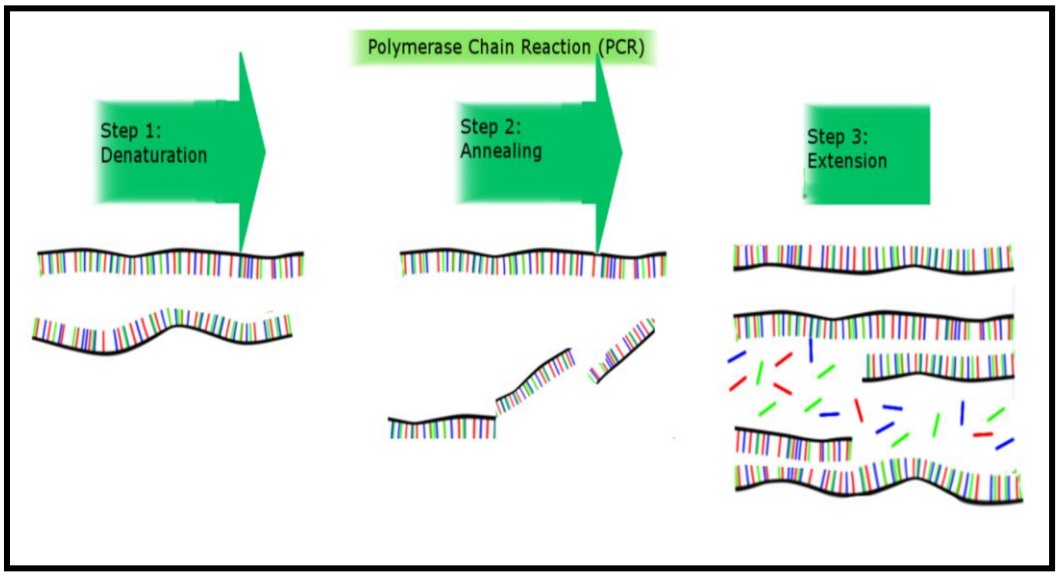
In order to use DNA collected using buccal swabs in genetic assay, the concentrations were amplified using a whole genome replication process. The REPLI-g

Mini and Midi Kits™ from QIAGEN are a whole genome amplification method which provides a rapid and reliable method for generating unlimited DNA from a few cells through multiple displacement amplification (Dean et al 2002). Amplification of the DNA concentration collected using buccal swabs was done through a QIAGEN REPLI-g mini kit, which can take DNA per 50µl reactions up to 10µg in the Mini Kit and 40µg in the Midi Kit (Qiagen 2010). In order for REPLI-g amplification to be useful, there must be a low degree of bias between the organic DNA and the amplified DNA. Tzvetkov and colleagues (2005) have investigated this bias and for that there was excellent concordance (99.95%) between SNPs in the organic and amplified DNA. All amplified DNA, from either initial whole blood or buccal swab, was quantified by spectroscopy to ensure the necessary concentration of DNA for successful genotyping.

Genotyping refers to the process of determining the genotype of an individual by the use of any biological assay. In the context of genetic epidemiological studies, genotyping is important for the identification of genes that are associated with disease or quantitative traits (Ziegler and Konig 2010). Genetic analysis involving SNP detection can use the polymerase chain reaction (PCR) method because of the short length of the DNA sequences being analyzed (figure 5). The PCR techniques used in this study were done to selectively amplify particular regions of interest within the DNA samples. The underlying principals in PCR technique is that a double stranded DNA can be melted or denatured into a single strand of that original DNA. At this point specific primers that are designed to amplify certain regions bind to original sequence so that the DNA polymerase can extend or increase the number of copies of that particular segment of the DNA. The extension of the DNA region of interest is done with the free nucleotides that

are supplied in the master mix as dNTPs. The PCR steps are repeated through a pre determined number of cycles so that the concentration of the targeted segments of DNA is high compared to that of the overall total DNA. In order for the PCR to be successful, DNA is added to a specific master mix solution and then placed onto the thermal cycler for precise cycles of denaturing, annealing and extensions.

FIGURE 5: *DNA Amplification Steps with Polymerase Chain Reaction (PCR) Method*



The PCR master mix set up involved one 25µl reaction per sample with each reaction containing 21.5µl of a master mix made up specifically for the number of reactions or number of DNA samples to be processed. Master mixes consisted of: 2.5µl of 1XPCR buffer, 1.0µl of 2.0 mM magnesium sulfate ($MgSO_4$), 0.2µl of 0.2mM dNTP mix, 0.5 µl of 0.2mM forward primer, 0.5µl of 0.2mM reverse primer, 16.7µl of sterile water and lastly, 0.1µl of 1.9 unit of Taq polymerase. The Master Mix was then aliquoted into new eppendorf tubes and 3.5µl of DNA unique to each sample was added. The forward and reverse primers that were utilized in this study were three unique sets of site

specific primers. For IL-12, the pro-inflammatory cytokine, the focus of the primer design was on the p40 region at position 1188 where there can be a shift from an A to C nucleotide (rs3212227). The primers used for this cytokine were adapted from primers described in Huang and colleagues (2000). The two pathogen recognition receptors, TLR4 and TLR2 were focused on the regions surrounding the point mutations. For TLR4 where there is an A to G nucleotide polymorphism at the Asp2999Gly site (rs4986790), C to T nucleotide polymorphism at the Thr399Ile site (rs4986791), and primers used for both TLR4 sites were described by Lorenz and colleagues (2001). The G to A nucleotide polymorphisms located at the Arg753Gln (rs5743708) site region was the focus of TLR2 analysis, with primers designed from the published set by Sanchez and colleagues (2004). All forward and backward primers were ordered from Invitrogen Life Technologies and presented in table 1.

TABLE 1: *Primer Sequences*

Gene Site	Primer	Source
TLR4 Asp299Gly	5'-GATTAGCATACTTAGACTACTACCTC**C**ATG-3' 5'-GATCAACTTCTGAAAAAGCATTCCCAC-3'	Lorenz et al (2001)
Thr399Ile	5'-GGTTGCTGTTCTCAAAGTGATTTTGGGA**G**AA-3' 5'-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3'	Lorenz et al (2001)
IL-12	5'-TTGTATAGTT**A**GATGCTAAATGCT-3' 5'-TTGTATAGTTCGATGCTAAATGCT-3'	Huang et al (2000)
TLR2 Arg753Gln	5'-AGTGAGCGGGATGCCTACT-3' 5'-CAAAATCCTTCCCGCTGAG-3'	Sanchez et al (2004)

Every sample set that underwent PCR amplification included a positive and a negative control in order to test for contamination acquired during processing. The PCR samples were then placed into the thermal cycler in order to be denatured, annealed with the primers and then an extension of the regions of interest within the DNA. The thermal cycle parameters for the specific gene sites can be found in table 2 and were carried out on the Bio-Rad MyCycler.

TABLE 2: *Thermal Cycler Parameters*

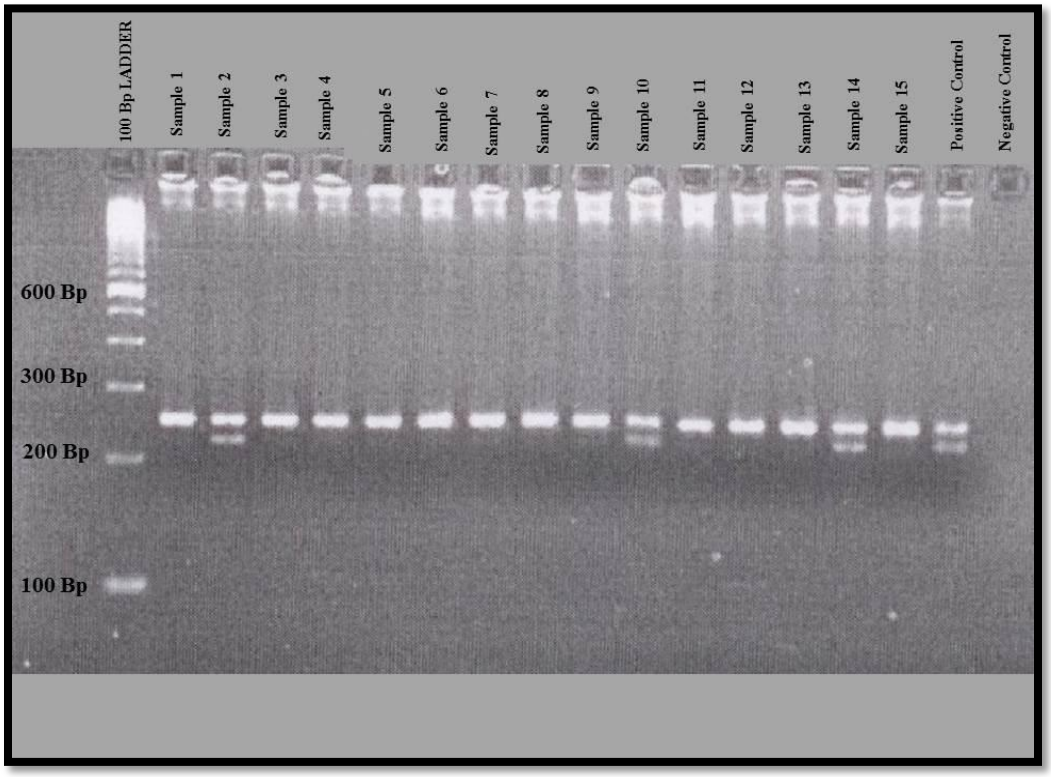
Gene	***Process***						***Total Approx. Time***
	Initial Start	**Denature**	**Anneal**	**Extend**	**Final Extension**	**Final Hold**	
IL-12	Temp 95C Length 2min	Temp 95C Length 20sec For 35 cycles	Temp 55C Length 20sec	Temp 72C Length 50 sec	Temp 72C Length 2 min	Temp 4C Length Forever	**2.5 hours**
TLR2	Temp 95C Length 4min	Temp 95C Length 1 min For 35 cycles	Temp 52.5C Length 20sec	Temp 72C Length 1 min	Temp 72C Length 7 min	Temp 4C Length Forever	**4 hours**
TLR4	Temp 95C Length 4 min	Temp 95 Length 30 sec For 30 cycles	Temp 55 Length 30 sec	Temp 72 Length 30 sec	Temp 72 Length 5 min	Temp 4C Length Forever	**1.5 hours**

Electrophoresis was used on completion of the PCR cycles to verify a successful amplification of DNA through the detection of PCR product. Electrophoresis can be described as the transportation of charged molecules through a solvent by electric fields. This is a fast and simple method for separating proteins and nucleic acids. There are two

different types of gels used in electrophoresis, agarose and polyacrylamide. The agarose gels were chosen for ease of construction and economical purposes. The 2.5% agarose gels were made up of 50ml TBE (Tris/Borate/EDTA) buffer solution, 1.25g of pure agarose and 2.5µl of ethidium bromide. Ethidium bromide is a fluorescent tag that when exposed to ultraviolet light will become visible and highlight PCR product and DNA bands. The agarose gel was then allowed to set for 20-40 minutes until firm and then loaded with a combination of DNA product and dye solution. In order to test the PCR produce, 3µl of the PCR product was mixed with 1.5µl of loading dye and was run on a horizontal 2.5% agarose gel for 45 minutes at 100V and 400 amps. The Bio-Rad gel and power supply®, Gel Logic 200 Imaging System® and Kodak 1D software® were used to run and visualize the DNA. If the visualization of the DNA was successful then a restriction digest was then conducted.

Restriction Fragment Length Polymorphism (RFLP) analysis was developed in the early 1980s and relies on the principle that specific restriction enzymes are able to cut or digest DNA at specific loci depending on the DNA sequence (Ziegler and Konig 2010). The restriction digest was used in this study to cut the DNA to determine if there were any substituted alleles at the desired locus. In order to determine if there was a substituted allele present in the DNA sample, the restriction digest was run through gel electrophoreses which separated the DNA fragments according to their lengths-number of base pairs present. Each allele that makes up the genotype combination will have a previously known base pair number and depending on the location that the individual bands run to in comparison to the 100 base pair marker ladder, the DNA sample genotype can be distinguished (figure 6).

***FIGURE 6**: Restriction Fragment Length Polymorphism (RFLP) Digestion of TLR4 Asp299Gly*



As mentioned, the amplified DNA product surrounding the desired locus can be cut with an enzyme specific to that region. For this study the enzymes that were utilized to cut the DNA samples at the desired loci were Taq-α1 for IL-12, Nco1 for TLR4 at the Asp299Gly site, whereas Thr399Ile used Hinf1. TLR2 used both Acil and Pst1 enzymes in the digestion of the Arg753Gln region. All of the enzymes were ordered though the New England Biolabs and came with a buffer solution to optimize the reaction. A master mix was also made up for the number of sample sets plus one to ensure that were was adequate master mix for all samples if any aliquoting errors occurred. The master mix required for the restriction digest contained per sample: 2.5µl sterile water, 1.0µl buffer of which buffer 3 was used for TLR2, TLR4 at the Asp299Gly site whereas buffer 4 was

used in IL-12 and TLR4 Thr399Ile site samples. For IL-12 and TLR2, there was an addition of 1.0µl of 1xBSA to the master mix which was an addition to optimize these two gene sites. In genetic analysis, when there was no required 1XBSA for the restriction digest, an additional 1.0 µl of sterile water was added. Lastly 0.5µl of the aforementioned gene/site specific enzymes was added to the master mix creating a total volume of 5µl per sample.

Once the master mix was completed, 5µl of the mix was aliquoted into new eppendorf tubes followed by5µl of DNA. The digests were then incubated in heated mineral oil for an hour at 37°C before being centrifuged and electrophoresed. The restriction digest was electrophoresed on a higher concentration of gel, 3% agarose, to create a higher degree of resolution. This resolution was needed to distinguish between the small size differences between base pair bands. The 3% agarose gel was composed of 2.25g of pure agarose, 75ml of TBE buffer solution and 3.0 µl ethidium bromide. The gel was run for 1 to 1.5 hours, depending on the gene protocol and visualized using the previously mentioned equipment.

4.3.2 Statistical Analysis

After the completion of the DNA analysis for all the cohorts and genes, the results were compared using statistical methods. The statistical analysis was done using SAS software® (SAS Institute) at the University of Manitoba. Pearsons's chi-square test was used to calculate if there was a significant association between the allelic frequencies and the different populations. The First Nation populations were tested against each other as well as against the Caucasian cohort to determine the statistical associations between SNP frequencies and ethnicity. Similarly all First Nation population SNPs frequencies

were compiled and then tested against the Caucasian cohort to determine if there was significant statistical association between Aboriginal and non-Aboriginal status in regards to SNP frequencies.

4.4 SUMMARY

The techniques, equipment and protocols used in this study are part of the contemporary suite of options available for health research concerning immunogenetic profiles. The detection of certain allele polymorphisms through DNA amplification and restriction digestion offers an avenue for better understanding global genetic variation patterns. These patterns can be used to infer pathogen/host co-evolutionary histories as well as the impact that varying immunogenetic profiles may be having on modern disease relationships. With the rapid advancements made in molecular science, their application can only help to propel health research forward.

CHAPTER 5 RESULTS: SNP FREQUENCIES IN CYTOKINE AND TOLL-LIKE RECEPTORS IN FIRST NATIONS AND CAUCASIAN COHORTS

5.1 INTRODUCTION

A great degree of genetic diversity within the immune response is of paramount importance for eliminating pathogens. Genetic diversity in a population ensures that individuals host's immune reaction will potentially be more adaptive to a larger spectrum of invading pathogens. Even with greater plasticity, the immune system is as only as strong as its weakest player and polymorphisms that affect gene functionality can cause increased disease susceptibility. Some of the key participants within the immune response are pro-inflammatory and anti-inflammatory cytokines as well as pattern recognition receptors like TLRs. Cytokines are involved in regulation, development and behavioural aspects of the immune response and therefore are needed to amplify and direct particular immunological pathways. As mentioned, TLRs are part of the pattern recognition receptor series of genes, whose role is the detection of invading pathogens. SNPs in the promoter regions of particular cytokines and TLRs have been shown to be associated with differential resistance/susceptibility to diseases, increased or decreased severity of disease outcomes due to altered gene expression functionality and their downstream immune consequences (Trinchieri 2003, Gerold et al 2007, Ogus et al 2004, Hall et al 2000, and Ben-Ali et al 2004). This chapter describes the frequency of SNP polymorphisms detected in IL-12 (p40), TLR2 (Arg753Gln) and TLR4 (Asp299Gly and Thr399lle) in a Caucasian and three Canadian First Nation cohorts as well as the genotype frequencies for each of these genes and their related phenotypic expression. Lastly this chapter will outline the statistical results related to any relationship between

ethnicity and SNP frequencies.

5.2 RESULTS

The genetic variations observed within the three First Nation cohorts were compared among one another as well as to a Caucasian cohort. This comparison was done through an examination of the allelic and genotypic frequencies. The purpose of this was to determine if the hypothesis for this research would be supported by the differential SNP frequencies among the four cohorts and within the three genes. Based on previous research (Larcombe et al 2008), the First Nation cohorts were hypothesized to maintain a higher frequency of SNPs that would affect the immune response to *Mycobacterium tuberculosis* when compared to the Caucasian population. The comparisons of immunogenetic profiles would also be statistically significant when the Caucasian cohort would be tested against the First Nation cohort. These results would provide further clarification on the mechanisms and functionality of the genes involved in mounting an immune response against *Mycobacterium tuberculosis* within First Nations as well as explore how the distinct histories of these populations have helped shape their contemporary immunogenetic profiles.

5.2.1 Allelic Frequencies

The allele counts represent the two nucleotides that were detected at the site of the particular polymorphism for each of the observed genes. The allele count for each of the cohorts is a combined total of the specific number of nucleotides observed. For the analysis of IL-12, the frequency of A and C alleles was determined with the C allele being associated as the SNP. For the two TLR genes, TLR2 had the frequency of G and

A alleles determined, with the A allele being the reported SNP. Whereas TLR4 at the Asp299Gly site had the G allele as the SNP with the frequency of A and G alleles being determined and lastly, TLR4 at the Thr399Ile site had the frequency of C and T alleles determined with the T allele being associated with the SNP (table 3).

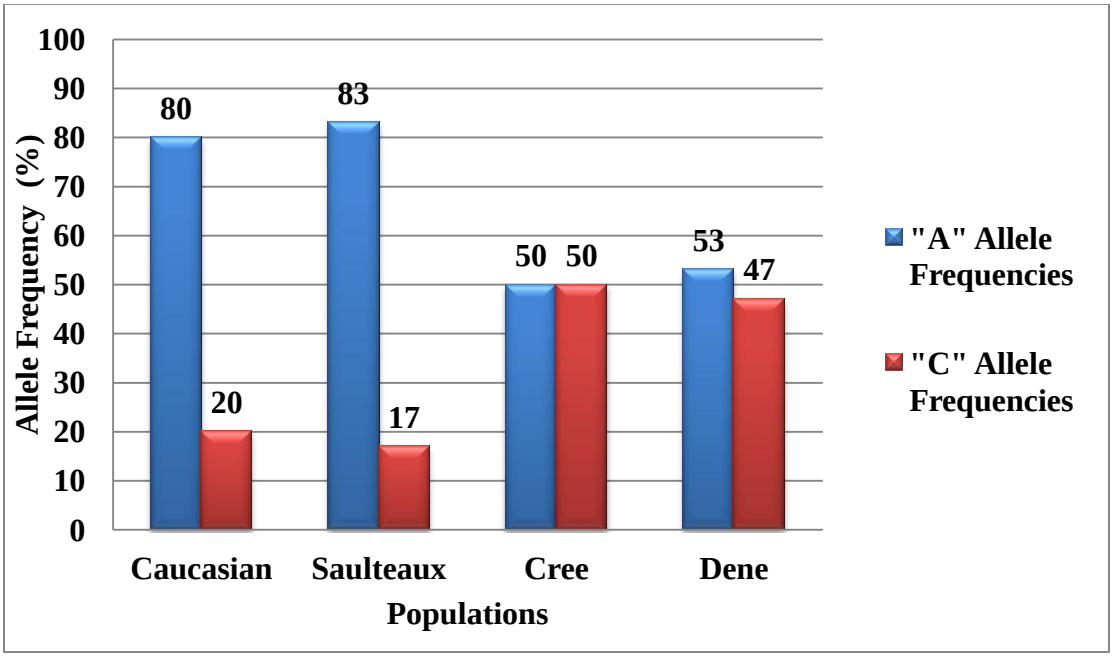
***TABLE 3**: Total Allele Counts for First Nation and Caucasian*

Cohorts	***Allele Counts** N (%)*	
	High	**Low**
IL-12	**A**	**C**
Caucasian	144 (80%)	36 (20%)
Saulteaux	192 (83.4%)	38 (16.5%)
Cree	41 (50%)	41 (50%)
Dene	65 (53.2%)	57 (46.7%)
TLR4 (Asp299Gly)	**A**	**G**
Caucasian	94 (94%)	6 (6%)
Saulteaux	224 (96.5%)	8 (3.4%)
Cree	83 (98.8%)	1 (1.1%)
Dene	125 (99.2%)	1 (0.7%)
TLR4 (Thr399Ile)	**C**	**T**
Caucasian	94 (94%)	6 (6%)
Saulteaux	226 (96.5%)	8 (3.4%)
Cree	83 (98.8%)	1 (1.1%)
Dene	125 (99.2%)	1 (0.7%)
TLR2 (Arg753Gln)	**G**	**A**
Caucasian	70 (89.7%)	8 (10.2%)
Saulteaux	230 (98.2%)	4 (1.4%)
Cree	83 (98.8%)	1 (1.1%)
Dene	124 (100%)	0 (0%)

The C allele in IL-12 SNP has been associated with a low expression of this pro-inflammatory cytokine and the overall decreased functionality of the gene, potentially resulting in down regulated cascade effect creating an overall suboptimal immune response (Alvarado-Navarro et al 2008, Zhao et al 2009). The frequencies of A and C alleles observed in IL-12 show that the Caucasian and Saulteaux cohorts maintain a

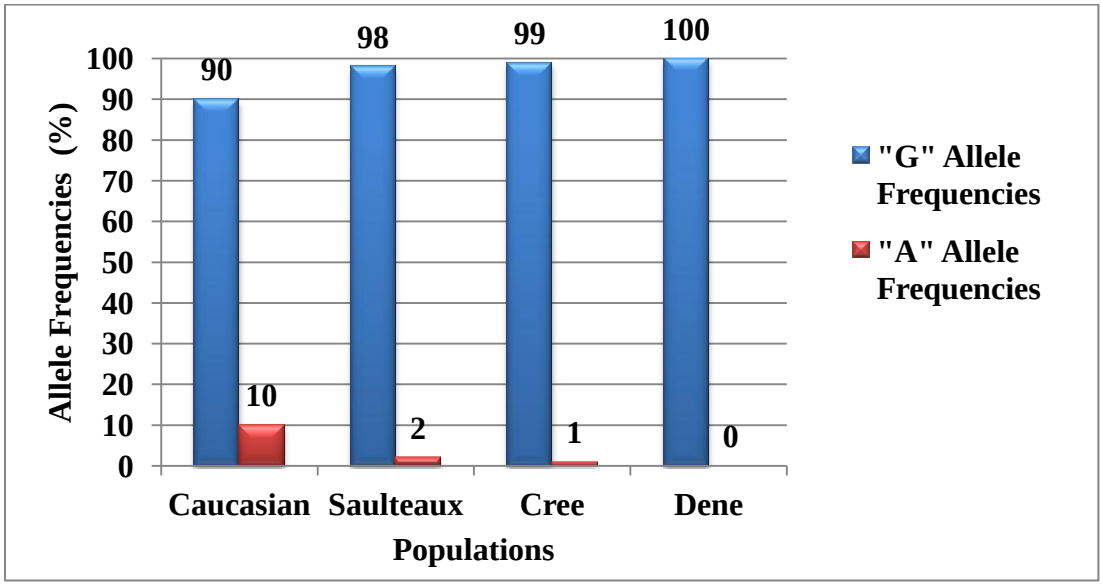
higher portion of A alleles at 80% and 83% respectfully, when compared to the Cree and Dene cohorts who reported at 50% and 53% (figure 7). The Cree have a balanced ratio of A and C alleles, although still retain a relatively high frequency of C alleles when compared to the Caucasian and Saulteaux cohorts who report at 20% and 17%. The Dene have the highest frequencies of C alleles when compared to all other cohorts at 47%, and maintain a distinct contrast in allelic frequencies when compared to the Caucasian and Saulteaux cohorts. The Caucasian and Saulteaux cohorts however, are more similar in allelic frequencies, as are the Dene and Cree cohorts. This trend is strengthened when the location of the communities is taken into account, as there is a clear north to south divide with a shift in allelic frequencies between the southern communities of the Caucasian and Saulteaux and the northern communities of the Dene and Cree.

FIGURE 7: *IL-12 Allelic Frequencies for First Nation and Caucasian Cohorts*



The allele frequencies observed in the TLR2 Arg753Gln SNP showed that all cohorts were relatively similar in their G and A allele frequencies, clearly favoring the G allele type (figure 8). The Caucasian cohort had the highest frequencies of A allele SNPs when compared to the First Nation cohorts, although all cohorts displayed a relatively low frequency of A alleles. The G allele was completely dominant in the Dene cohort with all individuals displaying the G allele. The frequency of G alleles decreased slightly through the Cree and Saulteaux, being at the lowest frequency in the Caucasian population. The A allele is the SNP that has been associated with altering the functionality of the gene, with all cohorts displaying a high frequency of the G allele, the assumed functionality of TLR2 is highly expressive.

***FIGURE 8**: TLR2 (Arg753Gln) Allelic Frequencies for First Nation and Caucasian Cohorts*



The observed SNP variants in TLR4 at sites Asp299Gly and Thr399Ile showed similar results as TLR2. Similarities were further echoed in the allelic frequencies observed at both TLR4 sites, in that they favored the non SNP alleles. All cohorts demonstrated high frequencies of A alleles compared to G alleles at the Asp299Gly site and C alleles over T alleles at the Thr399Ile site (figures 4 and 5). The SNPs variants in TLR4, the G allele at the Asp299Gly site and the T allele at Thr399Ile site have been associated with the low expression of this gene. Concerning the Asp299Gly site, all cohorts have a strong association with the A allele, with the Dene reporting the highest frequency of A alleles and the Caucasian cohort reporting the lowest, although, comparatively, a high frequency overall. This trend is continued in the Thr399Ile site by observing the strong association of C alleles in the Dene and Cree, with slightly decreasing frequencies through the Saulteaux and Caucasian cohorts, although again, a high frequency of the non SNP allele overall.

***FIGURE 9**: TLR4 (Asp299Gly) Allelic Frequencies for First Nation and Caucasian Cohorts*

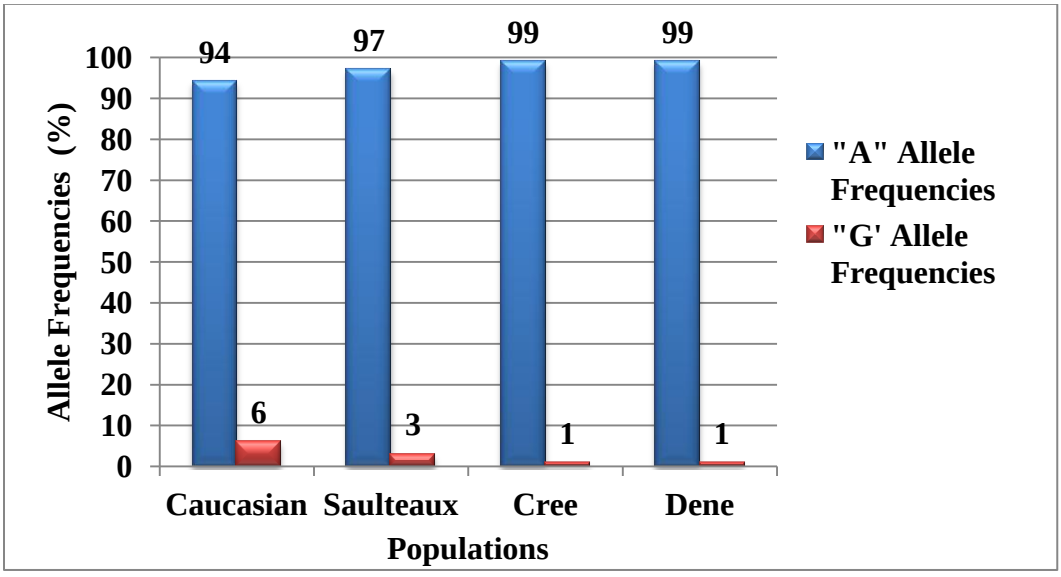
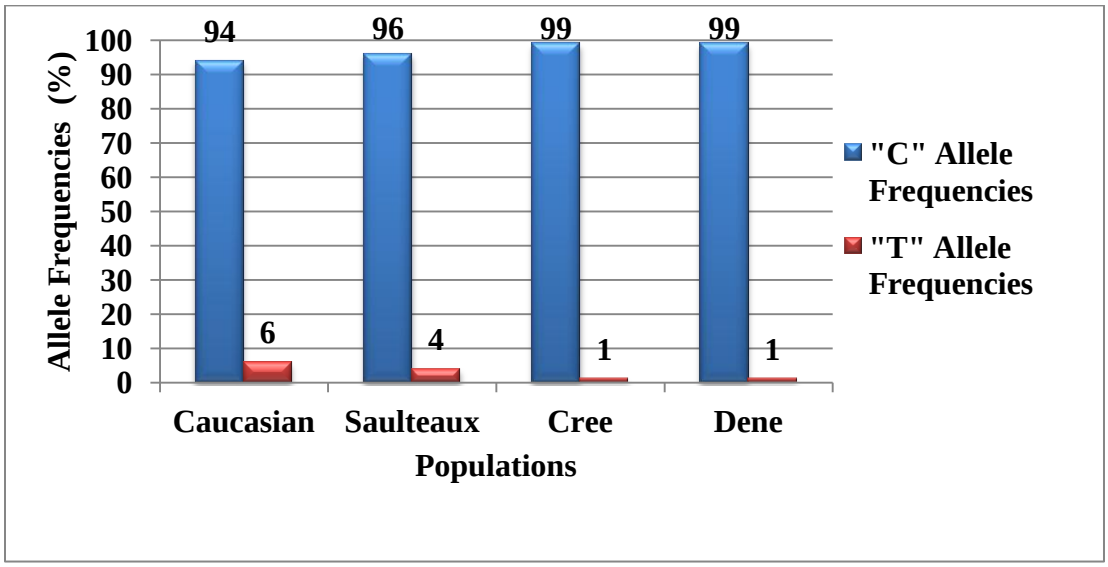


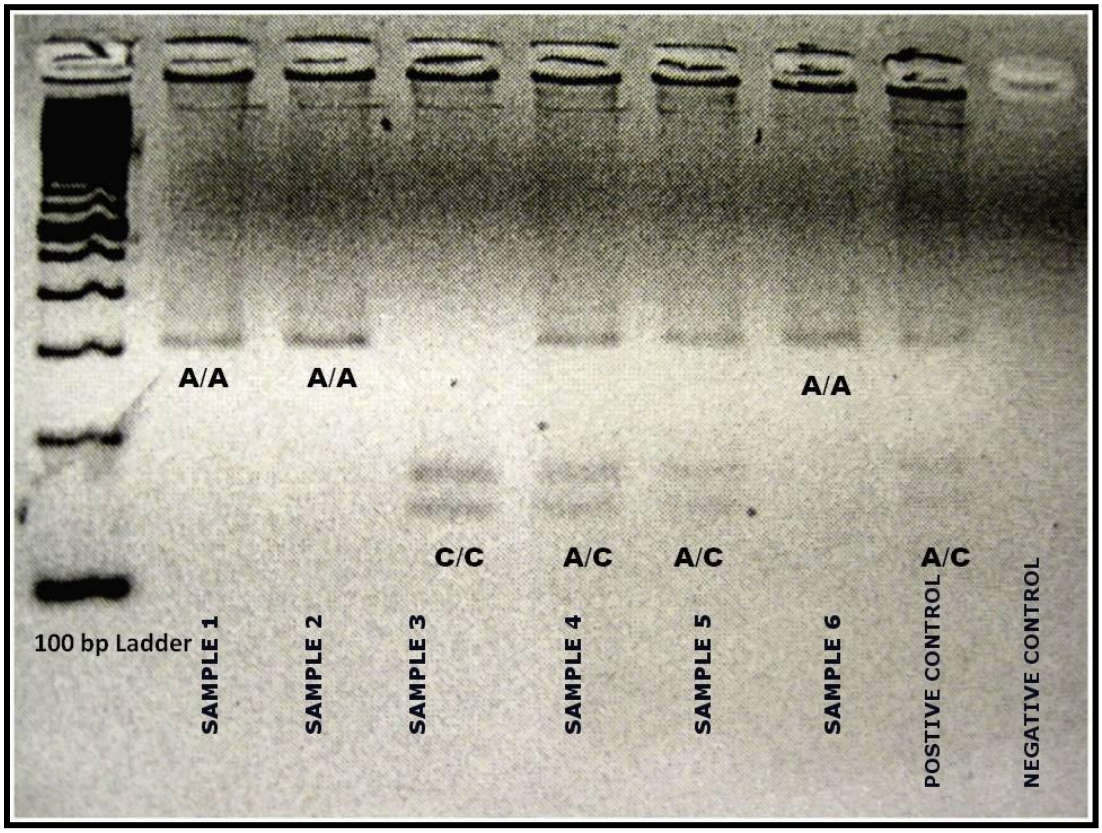
***FIGURE 10**: TLR4 (Thr399Ile) Allelic Frequencies for First Nation and Caucasian Cohorts*



5.2.2 Genotype Frequencies

The genotype of any individual gene is a combination of the two alleles or nucleotides that are present. These alleles can either make up a homozygous (same allele) or heterozygous (differing alleles) pair. The homozygous nature of the genotype pairing is further classified into either homozygous dominant or homozygous recessive informing the reader whether the two alleles present being either the expressive/functional allele type or the inactive type. In the analysis of IL-12, the A/A, A/C and C/C genotypes were documented (figure 11), whereas TLR2 had G/G, G/A and A/A genotypes documented. For TLR4, at site Asp299Gly the A/A, A/G and G/G genotypes were observed and for Thr399Ile site the C/C, C/T, T/T genotypes. The order of reporting the genotypes has been standardized in the homozygous dominant, heterozygous and homozygous recessive for all abovementioned genes.

***FIGURE 11**: RFLP Results on 3% Electrophoresis Gel with IL-12 A and C Alleles*



Upon completion of the RFLP analysis and gel electrophoresis, the alleles observed for each of the individual DNA samples were recorded according to their genotypes. These genotypes are reported by their phenotype as high, intermediate or low depending on if the SNP was present which corresponds to a level of expression previously reported (see chapter 5, section 5.2.1 and table 4).

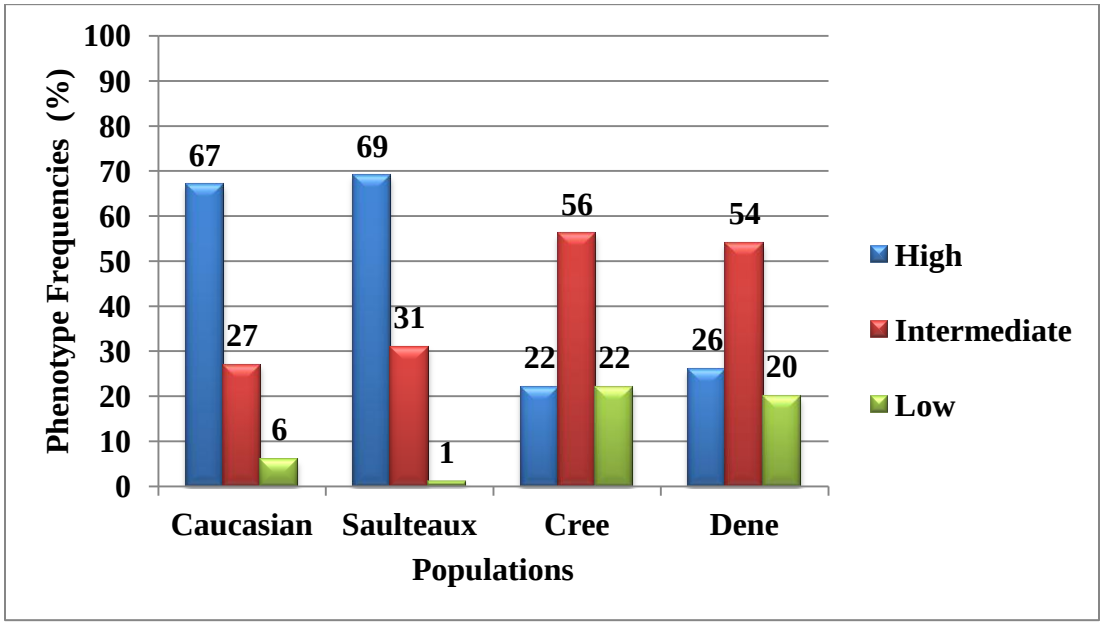
***TABLE 4**: TLR and Cytokine Genotypes and Phenotypes Expression Levels*

Gene	**Phenotypes/Genotypes**		
	High Expression	Intermediate Expression	Low Expression
IL-12	A/A	A/C	C/C
TLR2 (Arg753Gln)	G/G	G/A	A/A
TLR4 (Asp299Gly)	A/A	A/G	G/G
TLR4 (Thr399Ile)	C/C	C/T	T/T

The genotype frequency observed for IL-12 among the four cohorts showed a difference in the maintained high expression genotype frequencies between the Caucasian and Saulteaux when compared to the Cree and Dene (figure 12). The Caucasian cohorts have a high frequency of the high expression genotype at 67% with a decreasing trend in the intermediate (27%) to low expression (6%). This trend is continued in the Saulteaux cohort who has the highest rate of high expression genotypes (69%) and the lowest frequency of the low expression genotype at 1%. The Cree cohort differs from the Caucasian and Saulteaux as they have similar lower frequencies in the high and low genotype expression (both at 22%) with most of the individuals falling into the intermediate expression genotype (56%). The Dene cohort have a slightly higher frequency of individuals in the high expression genotype when compared to the Cree cohort (26% versus 22%), however, overall, the majority of the individuals were determined to have the intermediate expression genotype (54%). The discernible trend among these cohorts and the distribution of genotypes is marked in the shift towards an intermediate and low expression type among the Cree and Dene cohorts in comparison to the high frequencies of high expression genotypes among the Caucasian and Saulteaux.

This trend was previously observed in the IL-12 allelic frequencies (figure 7), as a south to north deviation where there were increasing numbers of intermediate and low phenotypes.

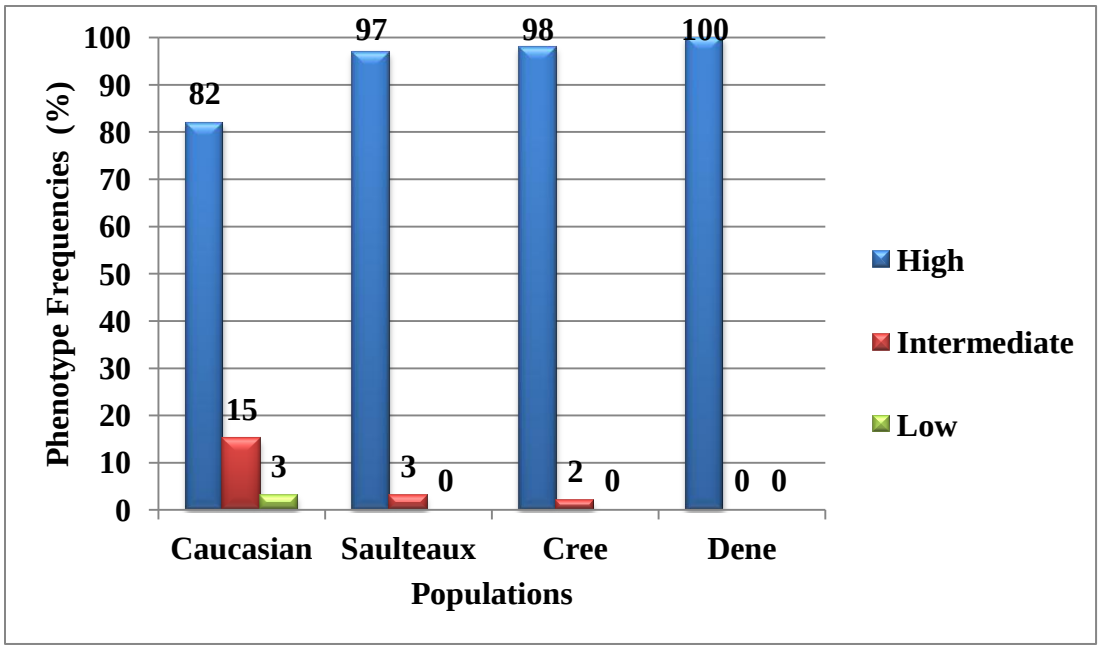
***FIGURE 12**: IL-12 Phenotype Frequencies for First Nation and Caucasian Cohorts*



The genotype frequencies for TLR2 Arg753Gln show an overall prevalence towards the high expression genotype observed within all cohorts (figure 13). All cohorts similarly reported the highest frequencies of genotypes within this category. The Dene cohort had all individuals fall in the high expression genotype for TLR2. The Cree had no individuals within the low expression genotype and only marginal amount (2%) in the intermediate genotype with the majority in the high expression genotype (98%). The Saulteaux cohorts reported similar results with the majority of individuals in the high expression genotype (97%) and a marginal amount in the intermediate genotype (3%). The Caucasian cohort had the largest distribution of genotypes relative to the First Nation

cohorts analyzed as well as the highest percentage of intermediate and low expression genotypes. The majority of individuals from the Caucasian cohort were determined to be from the high expression genotype (82%) with a decreasing frequency in the intermediate (15%) and low genotypes (3%).

***FIGURE 13**: TLR2 (Arg753Gln) Phenotype Frequencies for First Nation and Caucasian Cohorts*



The genotype frequencies observed within TLR4 were again separated into the Asp299Gly and Thr399Ile sites, although both sites reported mirror genotype frequencies. Most of the variations in genotype frequencies appeared among the Caucasian cohort when compared to the First Nation cohorts for both sites (figure 14 and 15). For the Asp299Gly site, the Caucasian cohort had the majority of its individuals falling into the high expression genotype (90%) with decreasing frequencies of individuals reporting the intermediate (8%) and low expression (2%) of this gene. This

pattern is continued in the Thr399Ile site where the Caucasian cohort reported identical results. In regards to the First Nation cohorts, the Saulteaux cohort results were comparable to the Caucasian cohort, with a high frequency of the high expression genotypes (93%) although no low expression genotypes were observed among this cohort. The Saulteaux reported the second highest frequency of intermediate genotypes at 7%, after the 8% observed in the Caucasian cohort. Again genotype results were identical for all cohorts at these two sites. The Cree and Dene cohorts were observed to have the greatest frequencies of the high expression genotypes (98%) and lowest frequencies of the intermediate expression genotypes (2%), with no low expression genotypes being present in any of the individuals in these two cohorts.

FIGURE 14: *TLR4 (Asp299Gly) Phenotype Frequencies for First Nation and Caucasian Cohorts*

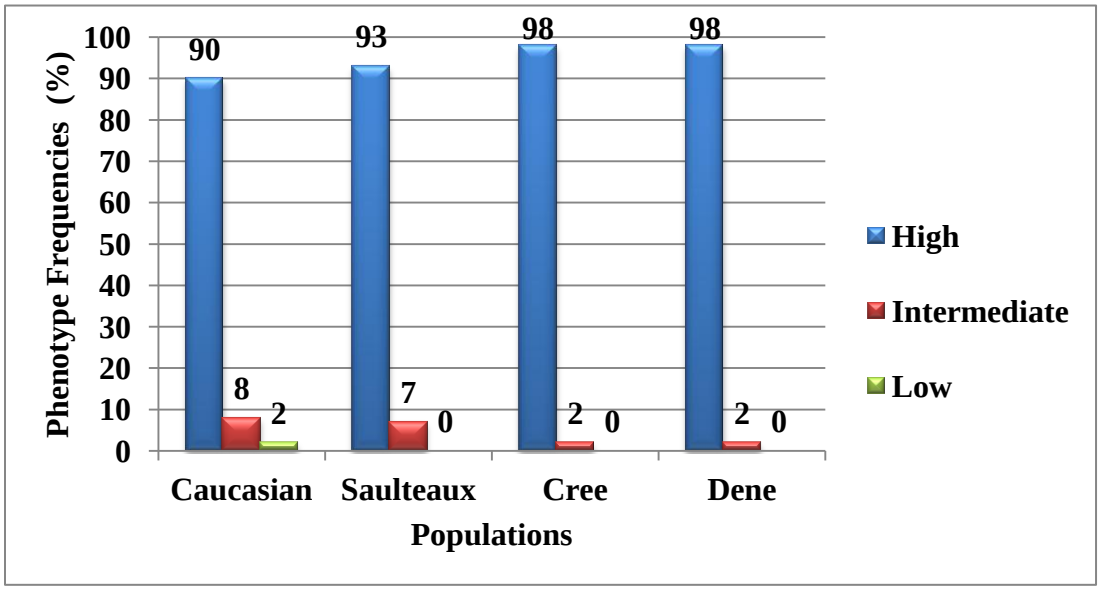
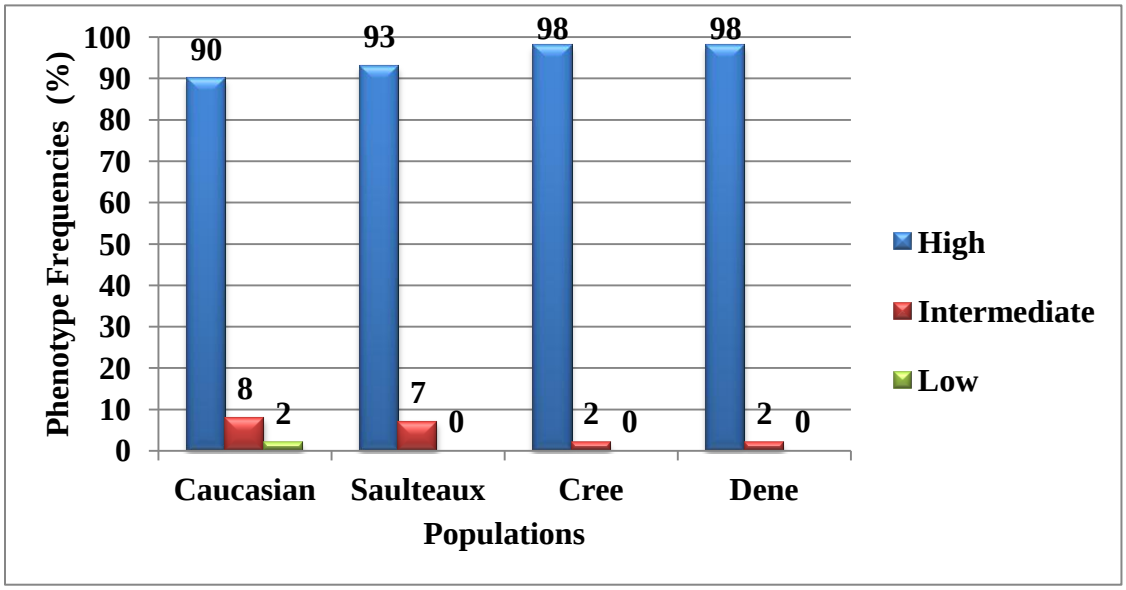


FIGURE 15: *TLR4 (Thr399Ile) Phenotype Frequencies for First Nation and Caucasian Cohorts*



5.2.3 Statistical Analysis

Statistical analyses were conducted to determine if any of the observed SNP frequencies were significantly associated with different populations. The four different cohorts were tested against each other using Pearson's chi-square test to examine any relationship between ethnicity and allelic frequency. The null hypothesis for this study stated that the cohorts were independent and homogenous. The null hypothesis was rejected if the Pearson chi-square p-value was less than 0.05. In order to assess this null hypothesis, all cohorts were compared against each other and a First Nation cohort was created with the combine allelic frequency of the Dene, Cree and Saulteaux cohorts. This First Nation cohort was tested against the Caucasian cohort. The statistical tests were chosen to examine the differences in allelic frequencies among the First Nation cohorts as well as between First Nation and Caucasian populations.

The statistical analysis for IL-12 demonstrated that when the different cohorts were compared against each other only the Dene versus Cree and Saulteaux versus Caucasian tests did not have a significant p-value, while all other comparative tests had significant differences in allelic frequencies (table 5). Non-significant allelic frequencies between the Dene: Cree and the Saulteaux: Caucasian could possibly be attributed to the close geographic locations of particular communities, the Saulteaux and Caucasian communities being the in south and the Cree and Dene communities in the remote north. Location can influence the frequency of genetic variations within a population; the remoteness and isolation of the northern communities compared to the flexibility in movement between the Saulteaux and Caucasian communities can contribute to either decreasing or increasing genetic diversity. When the allelic frequencies for the Dene, Cree and Saulteaux were combined for IL-12, there was a significant p-value reported for the test between First Nation: Caucasian cohorts. Therefore the statistical trials reported significant allele frequency differences in IL-12 amongst the First Nation cohorts as well as between the First Nation and Caucasian cohorts.

For TLR2 there is a significant difference in the allelic frequencies between the Dene: Caucasian, the Cree: Caucasian, Saulteaux: Caucasian as well as the First Nation: Caucasian. There were not a significant differences in allelic frequencies among any of the First Nation cohorts when they were tested against each other. This shows that among the First Nation cohorts there is no significant difference in allelic frequencies for TLR2 at site Arg753Gln, however, there is a difference between the First Nation (both when compared as individual cohorts as well as a whole) when compared to the Caucasian cohort.

The statistical analysis for TLR4 again separated the two different sites into Asp299Gly and Thr399Ile tests; however both sites again reported similar results and therefore will be discussed solely as TLR4. There was a significant difference between the Dene: Caucasian cohorts and the First Nation: Caucasian cohorts in allelic frequencies. The significant difference between the Dene: Caucasian (figure 9, 10) could possibly correspond to the large geographic distance between these two communities. The First Nation: Caucasian test reported that there was a significant difference between the allelic frequencies between First Nations and Caucasians, although not amongst individual First Nation cohorts. For all statistical analyses, the First Nation: Caucasian and the Dene: Caucasian trials continually reported a significant difference in allelic frequencies. For the most part, IL-12 reported the largest degree of significant difference in allelic frequencies both amongst the First Nation cohorts as well as between the First Nation: Caucasian cohorts.

***TABLE 5**: Pearson Chi Square Test and P-Values for IL-12, TLR2 and TLR4*

Cohorts	*Gene*	*Pearson Chi Square*	*P-value*
	IL-12		
First Nation: Caucasian*		8.108	0.0044
Cree: Saulteaux*		35.829	<.0001
Dene: Saulteaux*		36.896	<.0001
Dene: Cree		0.221	0.6458
Saulteaux: Caucasian		0.826	0.3635
Cree: Caucasian*		24.432	<.0001
Dene: Caucasian*		24.363	<.0001
	TLR2(Arg753Gln)		
First Nation: Caucasian*		22.649	<.0001
Cree: Saulteaux		0.108	0.7429
Dene: Saulteaux		2.144	0.1432
Dene: Cree		1.483	0.2233
Saulteaux: Caucasian*		11.556	0.0007
Cree: Caucasian*		6.335	0.0118
Dene: Caucasian*		13.242	0.0003
	TLR4(Asp299Gly)		
First Nation: Caucasian*		3.976	0.0461
Cree: Saulteaux		1.136	0.2865
Dene: Saulteaux		2.348	0.1255
Dene: Cree		0.084	0.7718
Saulteaux: Caucasian		1.127	0.2885
Cree: Caucasian		2.886	0.0894
Dene: Caucasian*		5.035	0.0248
	TLR4(Thr399Ile)		
First Nation: Caucasian*		4.016	0.0451
Cree: Saulteaux		1.116	0.2908
Dene: Saulteaux		2.316	0.1281
Dene: Cree		0.084	0.7718
Saulteaux: Caucasian		1.162	0.2810
Cree: Caucasian		2.886	0.0894
Dene: Caucasian*		5.035	0.0248

**=Significant p-value*

5.3 SUMMARY

This study examined the frequency of SNPs within three distinct First Nation communities in the province of Manitoba as well as a Caucasian cohort from Winnipeg, Manitoba. The SNPs analyzed were IL-12, a cytokine involved in the regulation and development of a Th1 immune response, and in the promoter region of two Toll-like receptors TLR2 and TLR4, that are involved the in the initial recognition of invading pathogens by the host. The genetic analysis was performed by PCR and RFLP and the results were visualized using electrophoresis.

Upon completion of the genetic analysis, the First Nation cohorts: Dene, Cree and Saulteaux showed significantly different allelic frequencies. The Dene cohort maintained a higher frequency of the SNP in IL-12, followed by the Cree cohort and lastly the Saulteaux and Caucasian. The SNP in IL-12 decreased the level of expression of the gene and can decrease the overall level of functionality of IL-12. IL-12 is required in the differentiation of naïve CD4 T cells into generating the Th1 immune response through the stimulation of IFN-γ production from T cells (Yilmaz et al 2005). The decrease in functionality caused by the polymorphism will have an impact on the cascade effect of the rest of the immune system, by limiting the number of T cells responding to the invading infection. The frequency of SNPs in IL-12 decreased in the study cohorts from northern to southern communities. The Dene in the far north had one of the highest frequencies of SNPs; the Cree had more intermediate frequencies of SNPs as well as being located intermediately between the Saulteaux and Dene. The differences in allelic frequencies between the Dene: Saulteaux and Dene: Caucasian showed a statistically significant difference which further supports the north to south SNP frequency gradient.

The genetic analysis of TLR2 and TLR4 showed that there was little difference between the First Nation cohorts although there was a statistically significant allelic frequency differences between the combined First Nation cohorts and the Caucasian cohort. The SNP frequencies found in TLR2 and TLR4 favored the higher expression of these two genes. TLR functionality is important to maintain as a decreased level of TLR signaling will not initiate adequate numbers of macrophages to mount as effective an immune response.

In all tests there was a statistically significant difference in the allelic variation between the First Nation cohort and the Caucasian cohort. However, what is more important to stress is that there were also statistically significant differences between the distinct First Nation cohorts. These genetic variations are undoubtedly a reflection of the unique histories of these different groups and may suggest that First Nation cohorts within Manitoba are mounting, to varying degrees, a suboptimal immune response in reaction to invading bacteria and viruses.

CHAPTER 6 DISCUSSION AND CONCLUSION

6.1 INTRODUCTION

The complex historical relationship between humans and disease causing pathogens can be attested to in the diversity and intricate nature of the immune system. The dynamic nature of the immune response ensures that adaptations to specific pathogen environments are possible. Without the functional and flexible nature of the immune response, the human host would not be as successful in the elimination of pathogens. In order for the immune system to adapt to a variety of pathogens, selection can occur for certain genetic mutations within a population. While some genetic mutations are quiescent, genetic variations that affect functionality can alter susceptibility or resistance to disease, thus increasing or decreasing the survivability of any individuals possessing the variation. Therefore, variants that increase the fitness of an individual may eventually become more frequent in a population. A single gene mutation is unlikely to alter the immune response completely, but when a cluster of mutations in immune promoter and regulatory genes are sustained, the cascade manner of immunity may be affected. These clusters are part of the overall immunogenetic profile of an individual and have important implications for health in contemporary and historical populations.

Manitoba First Nation populations have had various polymorphisms identified in several key genes involved in immunity (Larcombe et al 2005, 2008). The results from this study provide additional information on the unique manner of promotion and maintenance of the immune response in these populations. Specific genetic variations have been associated with increased susceptibility to tuberculosis in different ethnic populations (Flynn and Chan 2001) and these variants can be used to infer related

functionality in the immunogenetic profiles of Manitoba First Nations. These differential immunogenetic profiles of Manitoban First Nations could be a contributing factor in the high tuberculosis disease rates observed in some of these populations (Olson et al 1999, PHAC 2010). In addition to the unique immunogenetic profiles, the immunological lifecycle of tuberculosis can also provide distinctive parameters so that the effects of particular genetic variations can be inferred, as well as to highlight particular areas where SNPs may be altering optimal immunity within First Nations populations.

Genetic variations often arise in environments that are particularly demanding on optimal immunity. In areas where there is high mortality and morbidity, individuals possessing any variations that contribute to increasing their overall fitness will likely increase their biological success. This can cause the propagation of certain gene variants within the gene pool, which will then be passed on to their descendants. The frequency of variants in gene pools may increase within particular populations as time passes, creating observable differences between various ethnic populations. The differences in SNP frequencies between global populations are, in part, due to their unique historical relationships with particular pathogens, environments and cultural conditions. These forces have acted as strong selective pressures that have aided in creating differential patterns of SNP frequencies across global populations. A comparison of the immunogenetic profile of Manitoba First Nations with other global populations allows for a cross cultural evaluation of similar historical relationships with particular pathogens and environments, in an attempt to highlight particularly strong agents of evolutionary change. While cross cultural comparison can provide broader insights into large scale evolutionary pressures, the contemporary First Nation profiles are a reflection of the

diverse and unique historical interactions, both biologically and culturally, that took place in Canadian history.

The drastic changes to culture and biology resulting from the arrival of Europeans and the introduction of new diseases into the New World rapidly altered the demands of First Nation immunity. The adaptations to North American pre-contact environments and the pathogens found within are the product of years of selective pressures allowing First Nation immune responses to become optimized to these immune challenges. The immunogenetic profiles of Manitoba First Nations are no exception, with genetic variants being observed that reflect a variety of unique evolutionary pressures that may have been selective for during the pre-contact period. The historical pathogen environment of Canadian First Nations was one that brought populations into contact with higher loads of parasitic infections, which were in part related to highly mobile subsistence strategies, crowded housing and dietary food cooking methods and consumption. The immunogenetic profiles maintained today are likely successfully adapted to these historical environmental elements. However, the changes to culture and biology during the post contact period and those present today may have altered the benefits of these adaptations. Depending on the pathogen, the immune system offers different optimized pathways to efficiently contain and eliminate the invading microbes. Particular gene variations that once improved the manner in which specific immune pathways were promoted and amplified, may inhibit immunity if a different pathway is required than those which were previous selected and adapted for. During the period of European contact, the pathogen environment of the New World was transformed and First Nation immunity was required to respond in an unfamiliar manner. By identifying SNPs in key

promoters and regulators of the immune response in contemporary First Nations, these historical pathogen environments and their genetic adaptations can be deduced. Similarly, the frequency and type of gene variants allows researchers an avenue to explore historical disease epidemiology and ecological immunology, but also can provide insight into pressing disease concerns in First Nation health today.

The health of First Nations is multidimensional, relying heavily on both social determinations of health as well as biological components. The social aspects of health status can contribute to an environment, either on a small scale within the body (diet), or on a larger scale outside the body (housing), where underlying immunogenetic variations can become exacerbated. This can lead to increased susceptibility to certain infections and chronic diseases and result in poorer health outcomes. Of particular importance for Manitoban First Nations health are the emerging immunogenetic profiles and their effect on the fashion in which immunity is mounted to combat tuberculosis infection. Detecting genetic variations in immunity related to sub-optimal immune functionality offers one potential mechanism for explaining the high rates of tuberculosis disease in Manitoba First Nations. The manner in which the immune response is mounted may also have implications for other chronic and infectious diseases affecting First Nation populations like diabetes and autoimmune disorders. While solutions to improving First Nation health cannot be solely focused on biological factors, understanding the role that genetic variations play in differential disease susceptibility and the nature of host/pathogen co-evolution can provide promising leads into anthropological and health research.

6.2 MANITOBA FIRST NATION IMMUNOGENETIC PROFILES AND IMPLICATIONS FOR TUBERCULOSIS

Immunogenetic variation is an important, although not a particularly well incorporated aspect in the control of global tuberculosis infection. Of exposed individuals who have a detectable adaptive immune response, which assumedly results in the latent tuberculosis phase, only roughly 5-10% of those individuals will progress into the active tuberculosis phase (Dheda et al 2010). There are many contributing factors which aid in the progression into active tuberculosis from the latent phase, with the tuberculosis strain type and virulence featuring prominently. However, barring infection with HIV and malnutrition, most cases progress in individuals with no obvious signs of immunodeficiency (Schluger and Rom 1998). Deciphering the underlying genetic variations in key areas of the host's immune response, and how they can impact on the different phases of tuberculosis pathogenesis, can provide insight into why certain individuals remain in the latent stage of tuberculosis, while others progress.

The emerging immunogenetic profile of First Nations in Manitoba is starting to establish potentially different key immunological pathways amongst First Nation populations and Caucasian populations living within the province of Manitoba. While the SNP divergent frequencies which influence these pathways may not be surprising given the evolutionary histories of these two separate ethnic populations, differences among unique cultural groups of Manitoban First Nations have also been identified. The differences in immunogenetic profiles result from the frequencies of SNPs observed in genes involved in the promotion and maintenances of the adaptive immune response. This immune response is of paramount importance in the success of the host's ability to

clear infectious agents. Any variations resulting in altered gene expression may effect the overall adaptive immune functionality, and due to its cascade nature could result in a sub optimal response to an infectious pathogen, in this case, *Mycobacterium tuberculosis*. However, the complexity of immunity requires that many genes contribute to promoting and maintaining a response, and therefore, it would require a cluster of SNP variations to modify significant functionality of the overall immune response in order to result in sub optimal immunity.

Previous studies (Larcombe et al 2005, 2008) regarding immunogenetic profiles in Manitoba First Nation populations targeted genes that are significant promoters and/or regulators of immune pathways. The different types of immune response pathways that are triggered by invading pathogens generally fall into either the Th1 or Th2 arm of immunity. The success of the immune pathway in eliminating the pathogens rests on mounting an optimal response determined by the different levels of gene expression as well as directing the immune response towards the more effective pathway. The Th1 and Th2 pathways have different promoters and regulatory genes, and therefore, variations in SNP profiles can be associated with which pathway will be promoted. Each pathway has evolved over time to be specifically targeted for different infectious organisms, and therefore, is optimized to unique pathogen environments. Th1 immune response is promoted through a cascade effect involving IL-12 and IFN-γ and through the combined regulatory properties of IFN-γ, IL-12 and TNF-α. A promoted Th1 response is of paramount importance to the effective containment and elimination of tuberculosis and other bacterial infections. Th2 immunity however, is promoted through a similar cascade effect involving several cytokines that stimulate mucus secretions to provide a "barrier"

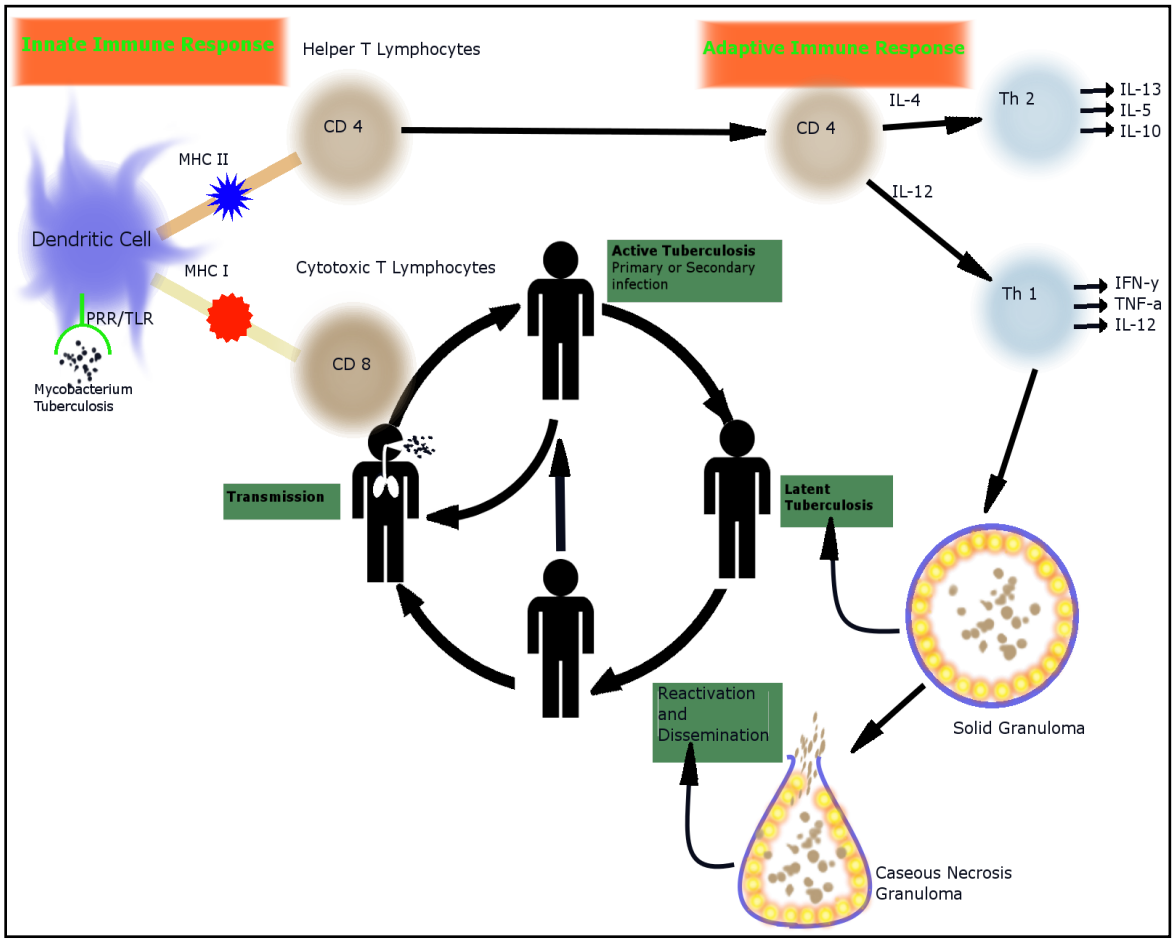
like host defense. Initially in a Th2 immune response, IL-4 stimulates the production of IgE antibodies while IL-5 activates eosinophils (Abbas and Lichtman 2010). IgE antibodies and eosinophils mediate reactions when helminthic parasites invade the host, which ultimately lead to an IL-4 and IL-13 promotion to expel any parasite from internal mucosal organs (McSorley and Maizels 2012). The induction of one arm of immunity over another has significant consequences for health outcomes and can result in an increase in the host's risk of becoming successfully infected and potentially the severity of the disease.

6.2.1 Toll-Like Receptors SNPs Profiles and Immune Response to Tuberculosis

A successful immune response to tuberculosis relies on many immunity promoters and regulators being adequately expressed after the initial infection with *Mycobacterium tuberculosis* and during this pathogen's life cycle (figure 16). Once the droplet nuclei containing *Mycobacterium tuberculosis* enter the lungs, the bacteria can either be completely killed off or can begin to multiply and progress into disease (Schluger and Rom 1998). *Mycobacterium tuberculosis* is a unique bacterium as once it has infected a host it can either become dormant/latent or present as an active infectious disease. The latent form manifests only as positive skin test, whereas the active form of tuberculosis presents with clinical symptoms (Schluger and Rom 1998). Once *Mycobacterium tuberculosis* enters the lungs it is phagocytosed by macrophages and dendritic cells (Dheda et al 2010). This initial defense against *Mycobacterium tuberculosis* infection begins with the innate immune response which is characterized by the recognition of this pathogen by pattern recognition receptors (PRRs) through conserved structures of the pathogenic microorganisms known as pathogen associated

molecular patterns (PAMPs) (Netea et al 2012). Toll-like receptors (TLRs) are among the different classes of PPRs that activate an acute inflammatory reaction after engaging with PAMPs and afterwards modulates the adaptive and humoral immune response (Netea et al 2012). As TLRs act as a detector for invading pathogens, it is logical to assume that deficiencies in the expression of these genes will have negative consequences for the immune signaling cascade.

***FIGURE 16**: Stages of Tuberculosis Infection and Associated Immune (Adapted from Dheda et al. 2010, Ernst 2012)*



Genetic variability studies have demonstrated just that, with SNPs being associated with enhanced susceptibility to and severity of infections (Schroder and Schumann 2005). Several SNPs located in TLR2, TLR4 and TLR9 appear to have a significant impact on the manner in which immunity is promoted in reaction to *Mycobacterium tuberculosis*. In this study SNPs located in TLR4 and TLR2 were of particular focus, specifically Arg753Gln (rs 5743708) in TLR2 and Asp299Gly (rs 4986796) and The399Ile (rs 4986791) in TLR4. SNPs located at Arg753Gln (rs 5743708) and Arg677Trp (rs 121917864) in TLR2 have been observed in higher frequencies among populations diagnosed with tuberculosis (Ogus et al 2004) and in Tunisian populations, Arg677Trp has been associated with increased susceptibility to tuberculosis (Ben-Ali et al 2004). Similarly, SNPs in TLR4 at position Asp299Gly (rs 4986790) and The399Ile (rs 4986791) in several in vivo studies have demonstrated a protective role against tuberculosis infection (Abel et al 2002, Fremond et al 2003, Branger et al 2004, Pulido et al 2010, Zaki et al 2012), however these sites often present with conflicting results (Shim et al 2003, Holscher et al 2008, Newport et al 2004, Xue et al 2010, Ferwerda et al 2007, Selvaraj et al 2010). The discrepancies in the protective role that TLR SNPs play in the immune response to tuberculosis may be a product of different populations that have been analyzed where evolutionary histories with particular pathogens and differential cultural and biological backgrounds may be impacted by immune cascades and bacterium strains (Caws et al 2008, Netea et al 2012).

The analysis of the First Nation and Caucasian cohorts in this study demonstrated that the prevalent allelic frequencies were the high expression genotypes, maintained in both TLR2 and TLR4 SNPs. The Dene, Cree and Saulteaux cohorts had a slightly north

to south variation in genotype prevalence, with more SNP variation being observed in the more southern cohorts, Caucasian and Saulteaux populations, when compared to the more northern located Cree and Dene cohorts. All individuals within the Dene cohort were observed to have the wild type allele genotype. The wild type genotype is the non-mutant allele pair which usually presents in the dominant form of a homozygous pairing. The Cree cohort showed the majority of individuals with a wild type genotype although marginal amounts of the Asp299Gly, Thr399Ile and Arg573Gln genotypes in heterozygous allelic pairings, a trend which intensified in the Saulteaux cohort as well. The Caucasian genotype frequencies, however, had the largest distribution of differential genotypes and the highest percentage of heterozygous and homozygous recessive genotypes, when compared to the First Nation cohorts.

As TLRs are used in the immune response to detect foreign/invading pathogens, any alteration in the genotypes that affect expression of these PRRs would be expected to modify overall immunity. Arbour and colleagues (2000) report that individuals possessing either the Asp299Gly and/or the Thr399Ile polymorphisms display a blunted pro-inflammatory immune response with decreased NF-kB activity when assaulted with inhaled lipopolysaccharides (LPS). Similarly, TLR2 SNPs have been shown to lead to decrease cellular activation (Lorenz et al 2000, Bochud et al 2003, Schroder et al 2005). TLR mediated activation up regulates the production of pro-inflammatory cytokines IL-1β, IL-12, TNF-α and IL-6, which are all an essential requirement in the immune response to control Mycobacterium infection (van Crevel et al 2002, Mortaz et al 2012). A similar TLR2 function is suspected as well, as studies involving murine models identify slight increased Mycobacterium growth in low dose aerosol infection and greatly

increased susceptibility to high dose aerosol in TLR2 knockout mice (Drennan et al 2004). The importance of adequate TLR expression is also underlined in the manner and intensity by which dendritic cells and macrophage activation occurs in response to *Mycobacterium tuberculosis*. In the absence of adequate TLR expression, there could be a down regulated pro-inflammatory response due to the diminished production of Th1 cytokines which would likely lead to severely compromised granuloma formation and a greatly reduced immune protection from the infecting Mycobacterium (van Crevel et al 2002, Stenger and Modlin 2002).

However, there exists some debate on the role of Asp299Gly polymorphism and its effect on gram negative bacterial infections. Due to the co-segregated nature of the Asp299Gly and Thr399Ile polymorphisms, elucidating the single functional consequence of one polymorphism is more difficult. Ferwerda and colleagues (2007) observed that when the TLR4 Asp299Gly and wild type heterozygous genotypes is combined with a homozygous wild type Thr399Ile genotype, a pro-inflammatory TNF-α response is increased rather than blunted when compared to a double 299/399 homozygous wild type genotype. The First Nation cohorts and the Caucasian cohort all were identified to have a predominantly wild type genotype for both TLR4 SNPs as well as the TLR2 SNP. These wild type genotypes would most likely be associated with adequate expression levels of the TLRs in the immune response, resulting in sufficient detection and identification of *Mycobacterium tuberculosis*. Nevertheless, it is too simplistic to predict the success of an immune response through the expression level of one component in isolation. The cytokine activation networks and pathways that are stimulated by the TLR detection of *Mycobacterium tuberculosis* play a crucial role in the outcome of the infection.

The signal that is transmitted by TLRs culminates in the synthesis of different cytokines and chemokines. These different cytokines promote the direction of naive CD4 T helper cells into distinct activation pathways, with specific cytokines associated with the distinctive pathways. The manner in which the adaptive immune response is directed can factor into the ability of macrophages to inhibit the growth of *Mycobacterium tuberculosis* as vigorous macrophage activation depends on the state and type of effector T cells promoted. Naive CD4 T helper cells' differentiation into either Th1, Th2, Th17, or Treg cell pathways triggered by specific cytokines that become expressed in response to a particular invading pathogen. The Th1 subset is promoted through the expression of IL-12, IL-18 and IFN-γ, while the Th2 subset is induced by IL-4, IL-5 and IL-13 (Dheda et al 2010). Once either a Th1 or Th2 pathway is selected, specific cytokines are again stimulated to amplify the immune response. Th1 cytokines are those that prompt the maturation and activation of macrophages as well as granulocytes, which are most notably IFN-γ, TNF-α, and IL-2 (Dheda et al 2010). A successful immunologic response to *Mycobacterium tuberculosis* is based on Th1 amplification due to its role in activation and maturation of macrophages and granulocytes as well as nitric oxide radical synthesis, used in efficient mycobacterium growth restriction (Dorhoi et al 2011). In contrast, Th2 cytokines have been associated with enhanced humoral immune response induced by parasitic infections and also provide a strong protective inflammatory response against a variety of allergens (Pulendran and Artis 2012).

An in vivo study of patients with positive tuberculin skin tests supported the important protective role that a Th1 response offers against increasing tuberculosis severity. Zhang and colleagues (1995) concluded that patients that had progressed to

pulmonary tuberculosis from a positive skin test had less Th1 cytokine promotion and more Th2 type response, whereas those patients who had mounted a Th1 promoted pathway were more likely to halt progression to the active form of tuberculosis. That being said, Th1 and Th2 cytokine production and promotion is not as linear as it may seem, due to a myriad of complex interactions between different cytokines which have important and often immunity altering effects. Therefore, any altering cytokine expression genotypes must be analyzed within the overall profile of immune pathways in order to understand their downstream impact on immunity.

6.2.2 Interleukin-12 SNP Profiles and Immune Response to Tuberculosis

The immunogenetic genotypes observed in Manitoban First Nation cytokine profiles, particularly those that promote and regulate the Th1 and Th2 immune pathways have revealed several important variations which could have serious implications for tuberculosis infection, severity and control. Once Mycobacterium has infected macrophages and dendritic cells, particular antigens are presented by either MHC class 1 producing CD8+ cells or MHC class II producing CD4+ cells began to differentiate (Abbas and Lichtman 2010, Dheda et al 2010). The progression of CD4+ cells into either a Th1 or Th2 immune pathway relies on the amplification of specific cytokines. The development of the Th1 immune pathway begins with the pro-inflammatory cytokine IL-12 and is continually promoted by IFN-γ, TNF-α.

The immunogenetic variation observed within IL-12p40 (rs 3212227) among the cohorts analyzed in this study show a significant trend towards the C allele SNP within more northerly located First Nation cohorts when compared to the Caucasian cohort. The Dene reported the highest frequency of the C allele SNP followed by the Cree; the

Saulteaux however, was more comparable in allelic frequencies to the Caucasian cohort than the Cree and Dene. The C allele has been associated with the lower functionality of this gene resulting in a lower production of the cytokine as well as downstream cytokines (Zhao et al 2009). A down-regulated immune response due to altered gene functionality may consequently create a sub optimal immune response to *Mycobacterium tuberculosis*, as well as to other pathogens. In contrast, the Caucasian and Saulteaux cohorts maintained a high frequency of A alleles while the Cree cohort maintained a balanced ratio of A and C alleles, albeit the C allele still remained in a relatively high frequency. The genotypes also contributed to the phenotype and general expression functionality of the gene. The wild type homozygous genotype expresses a high functionality phenotype, while a heterozygous genotype expresses an intermediate gene function and lastly, a SNP homozygous genotype has been related to low gene functionality. The high gene expression genotype was observed within the majority of Saulteaux and Caucasian cohorts, with decreasing frequencies of individuals maintaining intermediate and low expression genotypes. The Cree and Dene cohorts however, were observed to have the majority of individuals possessing the intermediate expression genotype with dramatic increase in individuals possessing the low functionality genotype when compared to the Caucasian and Saulteaux cohorts. Again, as with the TLR2 and TLR4 SNP profiles, there was a north to south trend in immunogenetic profile. However, in contrast to the TLRs, IL-12 has a significant distribution of northern cohorts (i.e., Dene and Cree) with a high frequency of the low gene expression genotype.

The tendency of an increasingly down regulated IL-12 immune response in the Dene and Cree when compared to the Saulteaux and Caucasian cohorts has important

implications for the overall amplification of the Th1 immune response in these populations. IL-12 is a pro-inflammatory cytokine that is a principle player in the cascade response involved in cytokine promotional feedback cycles, T-cell differentiation and enhanced natural killer cell production (Abbas and Lichtman 2010). Any limitation in these respective imperative focuses of immune function can result in a degraded response, allowing for increased likelihood of successful infection and re-infection, poorer disease outcomes and a greater susceptibility to environmental conditions. IL-12 specifically, increases the production of IFN-γ and TNF-α induced by T-cells and within a positive feedback loop, is in turn stimulated by IFN-γ, increasing the production of IL-12, resulting in an amplified cell mediated response where phagocytes ingest microbes. As an immunoregulatory cytokine, IL-12 bridges the innate resistance and adaptive immune response; however without adequate IL-12 expression, the cytotoxicity required for successful pathogen elimination can be lacking, allowing pathogens to proliferate. Similarly, if the balance between Th1 and Th2 T-cell differentiation is not regulated by sufficient IL-12 and IL-4 expression respectfully, then a sub optimal immune response to tuberculosis infection can be generated (Trinchieri 2003). The importance of IL-12 against *Mycobacterium tuberculosis* infection is exemplified through this function as a strong promoter of the Th1immune response. Mycobacterium is, in fact, such a strong inducer of IL-12 production that it can skew secondary antigens towards a Th1 phenotype (Sano et al 1999). In both mouse models (Cooper et al 1997, Flynn et al 1995, Holscher et al 2001) and human genetic studies (Ottenhoff et al 1998, Altare et al 1998), IL-12 is undoubtedly a critical component of mounting an adequate Th1 immune response against mycobacterium infection. Research into IL-12 SNP altered or absent expression has also

reported the inability of lung dendritic cells to prime naive T cells and decreased overall chemokine responsiveness (Khader et al 2006). The importance of this cytokine is underlined in the IL-12 induction research within the design of a tuberculosis vaccine (Lowrie et al 1999).

6.2.3 Manitoba First Nation Immunogenetic Variations and Immunity to Tuberculosis

Once IL-12 has been expressed and naive T cells being to differentiate into a Th1 subset, then these T cells produce IFN-γ to amplify the response. IFN-γ in conjunction with TNF-α, activates macrophages through nitrogen and reactive oxygen intermediates to eliminate intracellular bacteria (Dheda et al 2010). The effects of IFN-γ on macrophages extend beyond the stimulation of oxidative productions, as this cytokine also induces autophagy-a process of cell breakdown. IFN-γ induced autophagy has been demonstrated in both human and mouse models to be an essential component in antimycobacterial mechanisms (Herbst et al 2011). In contrast, the Th2 immune pathway produces cytokines that inhibit autophagy allowing for proliferation of intracellular *Mycobacterium tuberculosis* (Harris et al 2007). As such, IFN- γ is an influential activator of the antimicrobial functions of phagocytes, and plays an essential role in the resistance to many pathogenic bacteria infections (Tinchieri 2003, Raja 2004). An analysis of Manitoba First Nation populations found that the Dene and Cree cohorts maintained a high frequency of the A allele at SNP site IFN-γ (+874) when compared to a Caucasian cohort (Larcombe et al 2008). The high frequency of the A allele in IFN-γ (+874) has been associated with the low production of this cytokine (Hoffmann et al 2002, Kaur et al 2007). In regards to the control of *Mycobacterium tuberculosis* infection,

INF-γ is required for protective immunity. The cessation of bacterial growth correlates with the arrival of IFN-γ producing CD4 T cell, and that an altered or loss of these cells increases the likelihood of succumbing to tuberculosis (Havlir and Barnes 1999) as demonstrated by mice models (Ottenhoff et al 1998, Copper et al 1993, Flynn et al 1993) and within different human populations (Jouanguy et al 1996, Ting et al 1999).

Of similar importance in the control of *Mycobacterium tuberculosis* infection is the cytokine TNF-α, which is suspected to play multiple roles in the immune response against tuberculosis. TNF-α is produced by macrophages and monocytes in response to the recognition of infection by certain TLRs like TLR2 and 4 (Selvaraj 2004). Along with working in conjunction with IFN- γ in the stimulation of oxidative products and macrophage activation, TNF-α is required for granuloma formation in tuberculosis. Without adequate expression of this cytokine, the containment of intracellular bacteria may be in jeopardy. Previous analysis has demonstrated that Manitoba Dene and Cree cohorts show a significantly higher frequency of G alleles in the promoter region of TNF-α at site (-308) (Larcombe et al 2008). This SNP has again been associated with the low production of this gene. Altered functionality of TNF-α has been shown to contribute to a sub-optimal immune response, failing a primary challenge with *Mycobacterium tuberculosis* (Flynn et al 1995, Bean et al 1999). Reinfection and reactivation of tuberculosis are also highlighted threats with altered expression of this gene due to its role in maintaining the latent stage of tuberculosis in mice (Botha and Ryffel 2003) and humans (Wallis 2007, Keane 2004). The creation of granulomas is one of the hallmarks of tuberculosis infection, with TNF-α playing a key role. Granulomas are a build up of macrophages with surrounding lymphocytes that essentially trap bacilli preventing their

multiplication and dissemination (Stenger 2005). TNF-α triggers the maturation of dendritic cells and promotes their migration to the site of tuberculoid infection inducing among other immune effects, granuloma formation. In the absence of TNF-α, the granulomatous response is deficient resulting in sparse disorganized granuloma in the few that form (Flynn et al 1995). Similarly, in TNF-α deficient mice, *Mycobacterium tuberculosis* infection results in rapid death, with substantially higher bacterial burdens when compared to control mice (Garcia et al 1997) likely due to the inability of the immune response to contain and control the infection.

The immunogenetic variations observed within the Manitoban First Nation cohorts' Th1 directed cytokine profiles suggest that there are clusters of SNPs that are all associated with the low expression of regulatory and amplifier Th1 genes. These clusters can therefore be assumed to result in a down-regulated Th1 immune response against tuberculosis infection within these populations. Significant cytokines that contribute to analogous functions in the Th2 arm of immunity have also been previously analyzed and paint a different picture of the development of T-cell differentiation. The balance between these two immune pathways is modulated by specific cytokine expression, once activated by the recognition of antigens from invading microbes. If the balance becomes shifted from the pathogen optimized arm of immunity through over expression or under expression of particular cytokines, then overall immunity can be altered. A serious concern of sub-optimized immunity stems from the importance of a rapid immune response to an invading pathogen. If the response is too delayed, bacteria will proliferate and reach a point where the bacterial burden is so great that although an immune

response is being expressed, the environment that it is expressed within renders it ineffective (Cooper 2009).

The Th2 immune response is responsible for the control and elimination of helminths as well as the acute and chronic inflammatory response associated with allergic disorders. This response is characterized by the differentiation of naive T cells into the Th2 subset, stimulated by the production of IL-4, IL-5 and IL-13 cytokines (Pulendran and Atis 2012). Several other cytokines can contribute to the Th2 immune response, although they are not generally associated with its amplification. Due to the antagonistic nature of the Th1/Th2 balance, any cytokine or gene that acts to block the action of its T-helper cell pathway counterpart will modify the immune response towards or away from its original cascade (Lafaille 1998). For example, in a high IFN-γ expression environment, the proliferation of Th2 cells can become blocked, similarly, high concentrations of IL-4 impair the generation of Th1 cells (Kidd 2003). The immunogenetic profiles of Manitoba First Nations suggest that SNPs particular to cytokines that act to further diminish the Th1 immune response, favor a Th2 pathway. Within the cytokine and chemokine SNP profiles of the Dene and Cree cohorts of the previously analyzed study conducted by Larcombe and colleagues (2008), transforming growth factor beta (TGF- β (codon25)), interleukin-6 (IL-6 (-174)) and monocyte chemoattractant protein-1 (MCP-1 (-2518)) were all observed to maintain the allele and genotypes associated with high expression of the gene. The increased promotion of these cytokines and chemokines all act to skew the T-helper cell response through the inhibition of Th1 promoter cells. TGF- β is secreted by T lymphocytes and has been implicated in the suppression of the T cell response in tuberculosis patients (Selvaraj

2004, Hirsch et al 1994), through the deactivation of macrophage resulting from the inhibited IFN-γ induced oxidative products (Ding et al 1990). In the immune response to *Mycobacterium tuberculosis*, TGF- β is found within the granulomatous lesions acting as a down regulator of cell mediated immunity which blocks excessive inflammation preventing tissue damage to the lungs surrounding granuloma lesions (Toossi et al 1995, Hermandez-Pando et al 2009). Similarly, high expression of IL-6 and MCP-1 both act to limit Th1 immunity, through blocked signaling and down regulated cytokine production mediated by IFN-γ and IL-12 respectively (Nagahushanam et al 2003, Flores-Villanueva et al 2005).

All of these cascade responses act to selectively down regulate the Th1 immune pathway shifting the T cell differentiation towards a Th2 pathway. This shift has serious implications for the progression and severity of tuberculosis infection because optimal macrophage recruitment is required at the onset and early pathogenesis of this disease. However, a shift towards a Th2 immune response becomes beneficial in tuberculosis control in the later stages of this disease, when the ongoing high expression of the pro-inflammatory cytokines used in the containment of the bacteria actually start to cause tissue damage to the lungs. The suppression of these inflammatory cytokines can interrupt the process of tissue damage and aid in preventing the dissemination of Mycobacterium into the rest of the host's body. This being said, a vigorous Th1 immune response is essential for the elimination of *Mycobacterium tuberculosis* from the host.

Not all cytokine SNP profiles that have been analyzed within Manitoban First Nations support a clear shift away from a Th1 immune pathway in favor of Th2 cell differentiation. The Dene and Cree cohorts maintained a low frequency of the genotypes

associated the high and intermediate expression of IL-10 at SNP site -1082 (Larcombe et al 2008). IL-10 is an anti-inflammatory cytokine that participates in the deactivation of macrophage and down regulation of the IL-12 and IFN-γ positive feedback loop (Flynn and Chan 2001, Khader et al 2006). The high expression of this cytokine has been associated with the risk of developing tuberculosis due to its suppression of CD4+ T cells (Awomoyi et al 2002) resulting in increased bacterial growth. The First Nation cohorts of Manitoba maintain a genotype for low expression of this cytokine, and therefore, it is unlikely that the IL-10 genotypes act to skew T cell differentiation towards Th2, a sub optimal immune response to tuberculosis infection.

The pathogenesis of tuberculosis is a combination of the complex interactions of bacterial virulence, host resistance and environment. These interactions can dictate why an individual will progress into the active phase of this disease while others will remain in latency or not acquire the infection at all. The host's ability to contain and eliminate pathogens stems from the immune responses' ability to express the optimal cytokines and other immunoregulatory genes. The functionality of a gene can be altered as a result of certain SNPs being present and as a result of the cascade nature of immunity, this alteration in functionality can have "down stream" immune complications. Manitoba First Nation cohorts SNP profiles have been analyzed to elucidate any SNPs in important immune promoters and amplifier genes. The SNPs observed in these genes have suggested that SNP frequencies differ between First Nation and Caucasian cohorts as well as among the First Nation cohorts. Within the First Nation cohorts there is a trend that more northerly location populations (Dene and Cree versus Saulteaux) maintain SNPs that skew the T cell differentiation towards a Th2 pathway over Th1 immunity. The

balance between Th1 and Th2 cytokines seem to be a key factor in the immunopathogenesis of tuberculosis (Hermandez-Pando et al 2009). These SNPs, however, likely did not arise by chance but are rather the product of generations of selective pressures and co-evolutionary forces affecting the immune system and the pathogens that try to invade it.

6.3 EVOLUIONARY CONSIDERATIONS IN MANITOBA FIRST NATION IMMUNOGENETIC PROFILES

The hallmark of the immune system lies in its plastic and adaptable nature, enabling it to eliminate and/or contain a whole variety of invading pathogens, microbes and macro-parasites. This ability is one of the fundamental elements of successful immunity, as it can affect the manner in which immunity is induced and developed. Genetic variations that alter the immune response are a product of generations of modifications, resulting from both cultural and biological changes. Of these changes, pathogen/host interactions have had a significant influence on genetic variations and these interactions can be inferred from contemporary immunogenetic profiles. The reciprocal tendency in the co-evolutionary "arms race" between pathogens and the immune response dictates that any variation in one will force the other to adapt to this variation and can guide which variations are successful and inherited. Co-evolution theory also captures an essential characteristic of this adaptation trade-off experienced by hosts and pathogens, in that genetic variations that provide the highest level of fitness in one environment does not have the highest fitness in another environment (Wade 2007). Therefore population adaptations need to allow for a broad flexibility in immune response mechanisms but at the same time continually select for optimal levels of

immunity for each new particular environmental condition (Ardia et al 2011). The immunogenetic profiles that are observed today are a product of the adaptations to transitioning environments created by pathogen interactions, cultural changes and human migrations, and as such can be used to infer these co-evolutionary relationships.

6.3.1 Toll-Like Receptor Global SNP Frequencies and Evolutionary Pressures

As a member of the superfamily that comprises pattern recognition receptors it stands to reason that pathogen/host interactions supply a significant pressure on the genetic variations observed within TLR genes. Specific pathogen environments select for particular gene variants to arise within TLRs, resulting in adaptations to these unique environments. Differences in global patterns of SNP distribution within the TLR genes are a reflection of the co-evolution that occurred within various populations depending on the regional infectious pressures.

Within the TLR SNPs that have been analyzed within the Manitoba First Nation cohorts, the wild type or non-mutant allele type was found in the majority of individuals. The SNPs observed in TLR4 cause an A/G transition at the 299 Asp/Gly polymorphism and a C/T transition at the 399 Thr/Ile polymorphism and are often follow cosegregated inheritance (Ferwerda et al 2007). While the Caucasian cohort was observed to have the largest distribution of the mutant allele types at both the 299 and 399 sites, the wild type allele was still dominant within this population. In European populations however, this cosegregated allelic pairing has been found in substantial heterozygotic frequencies (Arbour et al 2000, Schroder and Schumann 2005). The relative absence of the 299 and 399 mutant allele in the First Nation cohort is similar to the populations from Asia and America, specifically the Han Chinese, Indonesian, Papuan and Trio Indians in Surinam

respectfully (Ferwerda et al 2007). While the frequency of the 299 and 399 mutant alleles being observed together within European populations suggests a cosegregrated manner of inheritance, this is not always the case in other populations. Besides the Asian and American tendency for maintaining the wild allele types at these TLR4 SNP sites, a high prevalence of the single 299 mutant allele has been observed in African populations (Ferwerda et al 2007). The differential global patterns of populations maintaining these polymorphisms are a product of the regional variations in evolutionary pressures, namely pathogen and environmental relationships. By tracing back the distribution of particular SNPs and taking into account local environmental conditions, an explanation as to TLR4 SNP frequencies within Manitoba First Nations can be generated.

It stands to reason that the global distribution of the 299 and 399 mutant alleles have followed historic human migrations into and away from particular geographic areas and that SNP patterns observed within contemporary populations are echoes of these migration patterns. Given the dispersion of the 299 and 399 SNPs, Ferwerda and colleagues (2007) suggest that an "out of Africa" model, similar to that of human migration patterns, is responsible for the global frequencies of these SNPs. The higher frequencies of the single 299 SNP in areas of Africa also supports that this mutant is older, arising in response to historical pathogen environments approximately 60,000 years ago (Ferwerda et al 2007). The variations in historical pathogen environments have also led to regional adaptations, where the functionality of the 299 and 399 sites aid in balancing immune success. The presence of the single 299 mutant allele has been associated with increased mortality to septic shock, however this association is not observed in the cosegregrated inherited pair of 299/399 alleles (Lorenz et al 2002). While

the beneficial properties of the 299 site seem to be dubious, this single mutant still appears within certain populations, namely African cohorts. This strong proinflammatory Asp299Gly phenotype enhances TNF-α production, and while it has been found to increase susceptibility to malaria, it lowers overall mortality to this disease, possibility accounting for the survival of this SNP appearing without Thr399Ile (Mockenhaupt et al 2006). In environments where malaria is not present and historically was not endemic, the success of the 299 mutant allele has become evolutionarily disadvantaged instead predisposing individuals to septic shock (Ferwerda et al 2007). The neutralizing effect of the Thr399Ile SNP is thought to have evolved to down regulate the strong proinflammatory response of Asp299Gly as humans migrated out of Africa into areas where malaria was not part of the pathogen environment mosaic (Ferwerda et al 2007). The high frequencies of European populations inheriting the cosegregrated pairing likely reflects the duration of time since these populations have migrated out of Africa and this is possibly a reason as to why these SNPs are not found in New World indigenous populations to date (Netea et al 2012, Ferwerda et al 2007). In the Manitoba First Nation cohorts, neither of these SNPs has been observed at any significant frequency, similar to the Trio Indians of the Amazon Jungle. The migration to the New World through colder climates could have removed these SNPs since they had not been a beneficial adaptation to the environment over many generations (Netea et al 2012).

The G/A nucleotide substitution polymorphism found at site Arg753Gln in TLR2 has a similar co-evolutionary history with defining relationships between particular populations and pathogens. As with the TLR4 SNPs, the frequency of the TLR2 SNP within the First Nation cohorts was relatively low to absent and while the Caucasian

cohort displayed a higher frequency of the SNP when compared to the First Nation cohorts, this SNP was not prevalent within the population. The global distribution of this SNP suggests that only European populations or those populations descended from European populations, for example New Zealand Caucasians, maintain this SNP at any significant frequency (Ioana et al 2012, Hong et al 2007). The low to absent prevalence of this SNP has been reported in several Asian populations including Taiwan (Cheng et al 2007), South Korean (Ryu et al 2006), Chinese Han (Ma et al 2010), East Indian (Biswas et al 2009); similarly in several African (Bochud et al 2009, Ioana et al 2012), South American (Zafra et al 2008, Ioana et al 2012) and now Manitoban First Nations cohorts. Therefore, the largest degree of polymorphic variation within the TLR2 gene has been observed with Indo-European populations with insignificant SNP prevalence reported in all other populations. This suggests, according to Ioana and colleagues (2012) the relatively late appearance of the TLR2 mutant allele occurring after the split of migrating populations in the Middle East who had subsequently diverged from Africa.

The regional variations within different subsets of European populations can also provide some insight into the historical host/pathogen interactions that have ultimately led to the maintenance of this SNP and its association with particular diseases. Within several European population studies, there have been associations between TLR2 polymorphisms and late stage lyme disease (Schroder et al 2005), acute rheumatic fever (Berdeli et al 2005) severe bacterial infections (Lorenz et al 2000) and of particular note, tuberculosis (Ogus et al 2004, Caws et al 2008, Kleinnijenhuis et al 2011). More interesting still are the differential reactions and resulting susceptibilities to particular strains of tuberculosis due to TLR2 polymorphisms. The Beijing genotype of *M.*

tuberculosis, the predominant form of tuberculosis affecting East Asia, has been correlated with particular SNPs in the TLR2 gene although this gene has not been observed to form any other associations between tuberculosis strains caused by the Indo-Oceanic and Euro-American isolates (Caws et al 2008). Several researchers (Kleinnigenhuis et al 2011, Caws et al 2008) cite these affinities as support for specific localized co evolutionary adaptations between global phylogeographic lineages of pathogens, in this instance tuberculosis, and certain human populations. While lineage 4 or the Euro-American lineage is the most commonplace tuberculosis strain in the Americans, it was the tuberculosis lineage ($DS6^{Quebec}$) that affected First Nation populations and was transmitted through social networks by the French Canadian fur traders into the interior of Canada (Pepperell et al 2010, Gagneux and Small 2007). Over the course of *Mycobacterium tuberculosis* evolution, this bacterium has developed a characteristic latency phase that allowed it to survive during the hunter/gatherer period when population densities were low (Gagneux 2012). Blaser and Kirschner (2007) similarly note that the reactivation and latency stages of tuberculosis infectious allow this bacterium to access new birth cohorts, in order to avoid a burn out situation where all susceptible host have been eliminated by overly virulent pathogen strains.

6.3.2 Interleukin-12 Global SNP Frequencies and Evolutionary Pressures

The major function of IL-12, a proinflammatory cytokine, is in the developmental regulation of naive CD4+ T cells and directing these naive T-cells into the Th1 immune pathway. This direction push is continually reinforced through a positive feedback loop with IFN-γ, another proinflammatory cytokine, to amplify the overall Th1 immune response (Watford et al 2003). Due to its important role in the promotion of the Th1

immune pathway, IL-12 SNP evolution is mostly likely tied to historical pathogen environments where infectious disease bore a heavy burden on the population. The C allele has been associated with the low production of IL-12 expression and the overall decreased functionality of this gene (Alvarado-Navarro et al 2008, Zhao et al 2009). In populations where there is a decreased frequency of the C allele, it stands to reason that these populations historically required an optimal Th1 immune expression to combat the pathogens that occurred at some frequency in order to survive. The Th1 immune pathway has become specialized for the elimination and containment of bacterial and viral microbes, and therefore, immunogenetic profiles that support a high expression Th1 promoter cytokine would have essentially co-evolved to withstand constant contact with these categories of pathogens.

Lending support this co-evolutionary relationship are the differences in genotypic frequencies observed within particular populations that have had a longstanding historical interaction with a large burden of infectious diseases. The SNP frequency indentified within this study's Caucasian cohort falls within a similar distribution of genotypic variation with other European/Caucasian decent populations (see table 6). The majority of individuals within these European ancestry and/or admixture populations (United States, United Kingdom, Spain, Australia, Greece, Canada, Croatia, Turkey, and Brazil) are observed to maintain the AA genotype, ranging from 53.5% to 73.6% with decreasing maintenance of the intermediate expression genotype AC(24.2%-38%) and low expression genotype (0%-8.8%) (Cargill et al 2007, Ma et al 2003, Garcia-Ganzalex et al 2005, Hall et al 2000, Windsor et al 2004, Kaarvatn et al 2012, D'Abronzo et al 2012, Yilmaz et al 2005). Unsurprising, the European ancestry and/or admixture populations

also support a high frequency of the A allele ranging from (72.4% to 85.7%). In contrast, the Asian and Indo-Asian ancestry populations as well as the Dene and Cree cohorts from this study all support a high frequency of the altered functionality C allele ranging from 39.4% to 54.5% and similarly maintain the highest frequencies of the CC genotype ranging from 14.9% to 27% (Chen et al 2011, Selvaraj et al 2008, Zhao et al 2009, Han et al 2008, Yang et al 2006). The similarities in genotype and allelic frequencies observed between the First Nation and Asian populations could possibly be related to the historic migrations over the Beringia landmass during the peopling of the New World and somewhat comparable historical pathogen environments. Supplementary evidence on mtDNA haplotypes and Y chromosome analysis has demonstrated that genetic lineages present in the Americas are also present sporadically in northern Asian populations, suggesting that these populations are comparatively closely related (Long and Bortolini 2011, Torroni et al 1992, Kolman et al 1996).

TABLE 6: *Interleukin-12 Global SNP Frequencies*

POPULATIONS	REFERENCE	AA	AC	CC	A	C
Chinese (Cancer)	Zhao et al 2009	18.1	54.8	27.1	45.5	54.5
Korea Case	Han et al 2008	21.3	58	20.7	46.4	53.6
Cree	This Study	22	56	22	53	47
Japanese	Yang et al 2006	23	53	24	49	51
Dene	This Study	26	54	20	53	47
Korea Control	Han et al 2008	29.1	49.2	21.8	46.4	53.6
Tunisia (Cancer)	Ben Chaaben et al 2011	30	50	20	55	45
Chinese Control	Zhao et al 2009	31.8	48.2	20	55.9	44.1
India (PTB)	Selvaraj et al 2008	35.6	43.8	20.6	50	50
West China	Chen et al 2011	36	49.1	14.9	60.6	39.4
Cameroon	Hall et al 2000	36.8	51.5	11.8	62.5	37.5
India (Control)	Selvaraj et al 2008	38.3	43.2	18.5	47.3	52.7
Mexico (Leprosy)	Alvarado-Navarro et al 2008	41	36	23	60	40
African American (TB)	Ma et al 2003	41.4	42.5	16.1	NA	NA
Sinaloa Mexico	Salvador et al 2012	42	44	14	64	36
African American Control	Ma et al 2003	44.9	44.3	10.8	NA	NA
Egypt	Youssef et al 2013	46	39	15	NA	NA
Mexico Control	Alvarado-Navarro et al 2008	47	49	4	73	29
Tunisia Control	Ben Chaaben et al 2011	48	41	11	68	32
Turkey	Yilmaz et al 2005	53.5	38	8.4	72.6	27.4
Croatia Control	Kaarvatn et al 2012	53.6	37.6	8.8	72.4	27.6
Brazilian Control	D'Abronzo et al 2012	60	40	0	NA	NA
Brazilian (Autoimmune anaemia)	D'Abronzo et al 2012	64.7	35.3	0	NA	NA
Croatia (Cancer)	Kaarvatn et al 2012	66	30.9	3.1	81.4	18.6
Greek	Hall et al 2000	66	25.8	8.2	78.9	21.1
Canadian Caucasian	This Study	67	27	6	80	20
Saulteaux	This Study	69	31	1	83	17
Western Australian	Windsor et al 2004	69	27	4	83	17
United Kingdom	Hall et al 2000	69.8	27.5	2.7	83.5	16.5
Spain	Garcia-Gonzalez et al 2005	70.1	25.2	4.7	82.7	17.3
United States (Texas)	Ma et al 2003	72.6	24.8	2.6	NA	NA
United States (Utah/Idaho)	Cargill et al 2007	73.6	24.2	2.1	85.7	14.3

Co-evolutionary selective pressures that could have been comparable between Asian and First Nation populations could be inferred from the genotypic frequencies that are maintained in these populations. These populations would have evolved to develop an immune response that was successful against the different microbes that made up the pathogen environment. Therefore, an assumption based on the allelic frequencies of populations, where there is an increase of low functionality IL-12 expression, is that the pathogen environment required a stronger Th2 immune response over a Th1. The polarization of the Th1/Th2 pathways impose certain constraints on individual immune responsiveness as high expression of Th1 effector cytokines actively suppresses the differentiation of Th2 cells, and vice versa (Ardia et al 2011). A host cannot simultaneously mount strong Th1 and Th2 responses, and therefore, SNPs that affect the functionality of cytokines that promote either pathway, in this case IL-12 and Th1, can be used to infer the historical pathogen environments. Asian populations and the First Nation cohorts shift towards a Th2 immune response, assumed from the genetic variations that down regulate IL-12 expression, suggesting that the parasitic burden in these populations required a significant immune investment.

6.3.3 Evolutionary Perspectives in First Nation Th1/Th2 Immune Pathways

The unique histories of Canadian First Nation people have undoubtedly influenced the conservation of particular beneficial genetic variants that have now been observed in contemporary immunogenetic profiles. These genetic variations are reflections of the adaptations that First Nation populations have undergone in response to cultural and biological evolutionary pressures. The pre-contact pathogen environment

was one that brought First Nation populations into contact with a high burden of parasitic infections and the reported differences in SNP frequencies additionally suggests that First Nation populations had successfully co-evolved to combat these infections with a strong Th2 immune response. It was not until the drastic changes to culture and the introduction of new pathogens following the arrival of Europeans that these genetic variations became disadvantageous. However, by reverse engineering the immunogenetic profiles of contemporary First Nations, the cultural and biological selective pressures that once factored into the creation of the optimized Th2 response can be deduced.

6.3.3.1 Impact of Historical Pathogen Environments on First Nation Immunogenetic Profiles

The pathogen environment that affected pre contact First Nation populations was radically different after the arrival of Europeans in the New World. While tuberculosis infection decimated the Indigenous populations of North and South America, before contact with Europeans, parasitic infection was more prevalent, and therefore, it was immunologically adapted to. The shift towards a Th2 immune response observed within the Dene and Cree displays many parallels to immune responses in other Indigenous populations of the New World, who similarly maintain a strong Th2 immune response (Longhi et al 2013). Decreased production of Th1 cytokines (IL-12, TNF-α and IFN-γ) was reported among the Warao of Venezuela and concentrations of immunoglogbulins such as IgM, IgG exhibited higher production among the the Yanomami of Brazil, the Totonaca of Mexico, the Warao of Venezuela (Sousa et al 1997, Sanchez-Rodriguez et al 2002, Araujo et al 2008, Giampietro et al 2010). This trend is also seen within a longitudinal study of the Ache of Paraguay, where a Th2 response is predominant and

helminthic infection is endemic and ubiquitous among this population (Hurtado et al 1997, Hurtado et al 2003). The warranted and successful biological adaption to the parasitic rich environments that Indigenous New World populations often found themselves in predisposed these individuals for an increased susceptibility to tuberculosis.

The nature of the Th1/Th2 balance is of particular importance for populations exposed to high levels of parasitic/helminthic/fungal infection and varied infectious diseases. Resistance to one side of T-cell differentiation results in a commitment to that arm of the immune system, the trade off between these two arms implies that co infected hosts must essentially chose which pathogen to respond to; mounting too strong a response towards one microbe type can leave the host vulnerable to infection by another (Bradley and Jackson 2008, Fenton et al 2008). In fact, chronic worm infection reduces immunogenicity or the ability of foreign microbes to provoke an immune response from the host (O'Garra et al 2013), which has been translated into the reduction of Th1 responses in active (Resende et al 2007) and latent (Babu et al 2009) tuberculosis when the patient is co-infected with helminthes. Jackson and colleagues (2008) suggest that because helminth infection has been so widespread and commonplace throughout history, the chronic exposure to a Th2 inducing pathogen would force the adaptation of the immune response towards the selection of immunogenetic variations that would be beneficial within a worm infection context. Indeed, the selective force of helminthes on interleukins and their receptors over the course of human evolution has been demonstrated to be stronger than those applied from viral and microbial agents (Fumagalli et al 2009). Essentially, from a functional co-evolutionary framework, the

strong Th2 immune pathway observed within First Nation and other Indigenous populations is one that has been successfully adapted fthrough centuries of host/pathogen interactions. The Th2 immune response is fundamentally the immunologically original or default response for the mammalian immune system due to the strong selective pressures and long historical relationship acting towards it. It was not until the arrival of new tuberculosis strains during the post contact period that this immune response and the genetic variations that had been selected to optimize it became disadvantageous.

6.3.3.2 Impact of Historical Cultural Environments on First Nation Immunogenetic Profiles

Besides pathogen/host interactions shaping the manner in which immunity is mounted, there are several other evolutionary force models that can describe how genetic variations are distributed and maintained within different populations. These evolutionary forces are used to explain the population genetic changes over time through processes like natural selection, genetic drift, and gene flow. Selection for the Th2 immunological original/default response has been a gradual process whereby certain genetic polymorphisms have been selected for through strong helminthic pressures. These pressures are not unique to any one population as the Th2 immune response is argued to be the immunological "default" of the mammalian immune system and while pertinent to the discussion of First Nation immunogenetic profiles are not unique shapers. The unique cultural histories of First Nations in Canada had distinctive events that ultimately lead to particular genetic variations being maintained in contemporary populations. These events can be characterized as gene flow and genetic drift factors like founder effects and bottlenecks within the context of First Nation history and can potentially be used to

explain the differences observed in SNP frequencies between First Nation and Caucasian cohorts.

Several lines of evidence exist that New World First Nation genetic diversity is a product of founder's effect. The founder effect is a reduction in genetic diversity stemming from a small founding number of individuals within a population. This small number of individual will result in a smaller gene pool and have certain genetic frequencies, sometimes those that are particularly rare, appearing at a high proportion in the population. Genetic analysis into mtDNA show that Indigenous populations of the New World belong to one of four ancestral haplotype lineages (Torroni et al 1992). Likewise Aboriginal populations have comparatively fewer distinct alleles per locus, with a gradient of decreasing genetic diversity as geographical distance from the Bering Strait increased (Wang et al 2007). This north to south reduction in genetic diversity is a probable remnant of the different stages of migration, where a small set of founders migrated into the Americas (Tamm et al 2007, Mulligan et al 2008). Genetic evidence also points to these populations diverging after the initial migration but then remaining relatively in situ, developing into new cultures and languages increasing the genetic differentiation among groups (Hunley and Long 2005, Hunley and Healy 2011), which can be observed in the varied frequencies of SNPs among the First Nation cohorts.

The earliest New World First Nation gene pools were brought about by the series of migrations across the Bering land mass however changes in the frequencies of these gene pools has been significantly altered through unique gene flow phenomena. Pepperell and colleagues (2011 and 2010) traced the patterns of gene flow in *M. tuberculosis* lineages within Aboriginal communities to date and determine coincidental events that

may have affected the migration dynamics of this pathogen. Several findings from these studies can be used to infer how cultural and physical barriers would have also shaped the immunogenetic profiles observed within First Nation cohorts, as similar barriers would have affected tuberculosis transmission into the interior of Canada as well as the hosts carrying it. With the arrival of fur traders and subsequent European immigrants the patterns of social networks and interactions between the different populations changed. During the initial fur trade period, different First Nations as well as European populations lived or at least comingled around trading posts. Marriages between First Nation and European individuals were encouraged as it was seen as beneficial to both parties involved. For example, leaders in both groups thought that marriages between British and French traders to First Nation would assure that economic partnerships would grow and trade goods would be continually available (Bial 2006). However, the arrival of Europeans also impacted the marriage patterns between First Nation populations. For example, Datsanthi, a legendary Dene warrior who had a Chipewyan father and a Cree mother (Abel 2005) although traditionally the Dene and Cree viewed themselves as enemies (Ward 1995). All of these situations led to the introduction of potential new genetic variations into the gene pools of these populations, increasing genetic diversity and possibly improving health, as low genetic diversity has been associated with higher rates of illness (Carrington et al 1999, McNicholl et al 2000).

In contrast to the collaborations noted during the peak of the fur trade, the social barriers and the geographic barriers erected by changes to social policy accounted for diminished gene flow in First Nation populations. Pepperell and colleagues (2010) note that the apparent lack of *Mycobacterium tuberculosis* gene flow following the decline of

the fur trade is explained by the social distance between 19th century homesteaders and First Nation populations. In fact, by the 19th century, many First Nation populations were becoming socially segregated, sometimes forcibly, from non Aboriginal populations through installation of reservations and residential schools (Lux 2001). The geographical barriers that affected gene flow during this period were the relocation to remote areas through the installation of the reserve system. Pepperell and colleagues (2010) note that remote areas acted as a shield for some of the traditional transmission factors for epidemic tuberculosis to spread, however these benefits were lost once regional air travel network expanded in the 1930s. Limiting the contact between different First Nation populations through social and geographic isolation diminished the gene flow in these populations and could also be a factor for the differential SNP frequencies seen among the First Nation cohorts.

Lastly, the effect of genetic drift within the First Nation populations has been observed within genetic profiles, reported in ethnographic documents and has made a lasting impression on the history of the New World. Although not inherently classified as genetic drift, the impact that European pathogens had on the immunologically naive populations of the New World resulted in a bottleneck or reduction in the gene pool. Bottlenecks have serious implications for the health and sustainability of a population as less genetic diversity increases the vulnerability of a population to disease (Carrington et al 1999, McNicholl et al 2000). The bottleneck that occurred after the arrival of Europeans and the subsequent population decline has been recently observed within calibrated mitochondrial sequences and proposes that a significant contraction in population size happened in which the female population was reduced by roughly 50%

(O'Fallon and Fehren-Schmitz 2011). This population reduction would have had a massive effect on the First Nation gene pool at the time and may be a factor in the maintenance of SNP variations within contemporary immunogenetic profiles. O'Fallon and Fehren-Schmitz (2011) also note that the scale of the population contraction was not localized to a particular region or communities that were part of the dataset, but instead the bottleneck would have been widespread and severely impacted most populous regions. This large elimination of genetic variation in the gene pool has also been observed within the bacterial population pool as a bottleneck in *Mycobacterium tuberculosis* occurs within a similar timeframe and results in contemporary tuberculosis lineages spreading from a small founder population (Pepperell et al 2010).

These evolutionary forces have all contributed to the unique immunogenetic profiles that are observed within contemporary First Nation populations. The immune response of First Nation people was once successfully adapted to the pathogen environment, providing a protective Th2 immune response against the high parasitic burden that these populations came into regular contact with. However, with the addition of new pathogen strains and changes to culture resulting from the distinctive historical occurrences faced by First Nations, the genetic variations have potentially become disadvantageous and ultimately led to a possible increased susceptibility to tuberculosis.

6.4 HEALTH IMPLICATIONS OF MANITOBA FIRST NATION IMMUNOGENETIC PROFILES

From a historical standpoint the maintenance of SNP frequencies that support the promotion of a strong Th2 immune response have co-evolved through interactions between a heavily burdened parasitic environment and hosts. However, other disease

complications can arise from an overly promoted Th2 immune response, highlighting the importance of sustaining the balance between Th1 and Th2 pathways. By identifying genetic variations that evoke the promotion of one arm of immunity over the other, an avenue for therapeutic adjustments like tuberculosis vaccine design can be created. The pressing health concerns of contemporary First Nation populations go beyond a varied immune response, and while immunogenetics can play a role, the environmental conditions in which First Nation people live predominates. Ultimately it is the combination of social, environmental and biological factors that will dictate health outcomes.

6.4.1 High Th2 Immune Pathway Disease Complications and Environmental Exacerbations

The Th2 immune response is successful in the containment and elimination of parasitic, helminthic and fungal infections; however it does predispose an individual to increased susceptibilities to infectious bacterial and viral pathogens. A high Th2 immune pathway also may predispose an individual for atopy, allergies and asthma. Allergies are a hypersensitivity to environmental antigens called allergens and the commonest clinical allergy syndrome is atopy-of which complex asthma, rhino-conunctivities and eczema appear in some combined prevalence (Hopkin 2009). These conditions require a skew in the Th1/Th2 balance in favor of the Th2 phenotype as well as particular primers such as house dust mites, pollens, and fungal spores to prime a strong Th2 response (Liu et al 2007). The SNPs frequencies observed in First Nation cohorts suggest that there is a shift towards a promoted Th2 immune response within these populations, and a consequence of this shift may be an increased prevalence of asthma in First Nation populations.

The rates of asthma within First Nation populations have been difficult to clearly define as there are many factors in diagnosis, access to health care and treatment that may be artificially decreasing or under reporting the overall prevalence of this disease. That being said, 12% of Aboriginal children and 11.2% of Aboriginal adults have been diagnosed with asthma, both just below their counterparts in the non Aboriginal Canadian population at 13.4% and 8.4% respectfully (Crighton et al 2010). Notably, asthma and other allergic conditions have been linked to living conditions where there is abundance of indoor and outdoor air pollutants, moulds and tobacco use (Fenton et al 2012). Many of these conditions may already exist on First Nation reserves. Curiously, asthma and allergic conditions are relatively low in First Nation populations despite possessing many elements necessary for the propagation of these conditions.

Housing conditions on First Nation reserves are a serious concern for the health and well being of these populations. Mould, poor ventilation and overcrowding are all factors in acquiring and exacerbating respiratory diseases likes asthma and tuberculosis. Individuals exposed to toxic compounds like allergens, bacterial endotoxins and fungal glucan, which can be common in substandard housing, have an increased risk of developing a chronic low grade inflammatory response, creating an imbalance between pro and anti inflammatory cytokines (Dales et al 1991, Johannessen et al 2005, Lehmann et al 2003). Housing conditions may be continually priming a strong Th2 immune response through the interaction with high concentrations of mould, dust allergens and other household endotoxins, possibly contributing to the immunogenetic profiles detected in the First Nation cohorts. A mould and allergen filled environment would warrant a strong Th2 immune response and therefore could be a selective pressure promoting

genetic variations that are positively adapted and increasing the likelihood of their maintenance at a high frequency in this population.

Especially critical in the discussion of immunological response and associated diseases is the detection of mould in First Nation housing. Mould and fungi allergens are strong inducers of the Th2 immune response and can irritate the respiratory system, increasing the risk of tuberculosis infection and spread. Housing studies on two reserves in Manitoba and one in New Brunswick have found that visible mould in houses is a pressing concern (Larcombe et al 2011, Berghout et al 2005). In the northland Denesuline First Nation Reserve (Dene), Tootinaowaziibeeng Treaty Reserve (Ojibwa), mould was reported in crawlspaces (34% and 12%, respectfully) and on walls, ceilings or floors within main floor rooms (44% and 19%, respectfully) (Larcombe et al 2011). Similar results were reported in houses on the Elsipogtog Reserve (Mi'Kmaq) where 19 of 26 homes analyzed had visible mould on more than 1-2% of the home's floor area (Berghout et al 2005). These findings have many important implications for tuberculosis control, as without an adequate Th1 immune response, *Mycobacterium tuberculosis* will continue to proliferate within these populations.

6.4.2 High Th2 Immune Pathway Consequences for Tuberculosis Therapy

The BCG (Bacille Calmette-Guerin) vaccine has had nearly a century of use, in varying degrees, in many countries, although still remains controversial due to its variable efficacy rates in adults (Zwerling et al 2011). Rates of tuberculosis in 2011 increased to 8.7 million people becoming infected and 1.4 million deaths from this disease (WHO 2012). Clearly, the effectiveness of the BCG vaccine is questionable and new research into designing a tuberculosis vaccine is warranted.

Understanding the complex manner in which immunity is mounted in response to tuberculosis infection allows researchers an avenue for the construction of a vaccine. Moller and Hoal (2010) note that ideally this vaccine design should be personalized for particular populations as different populations respond uniquely to a variety of tuberculosis strains depending on the combinations of genetic variation of the host and the pathogen. A prime example of this has been discussed in relation to the Beijing genotype tuberculosis strain and populations that are increasingly susceptible to these isolates due to SNP variations in TLR2 (Caws et al 2008). Vaccinomics is an approach to personalize or at least provide a population supported vaccine design that takes into account variability in polymorphisms and host immune response induced by the vaccine components (Poland et al 2009). IL-12 DNA vaccinations in mice have provided some protection against tuberculosis infection by establishing a cellular immune response dominated by antigen specific T lymphocytes that produce INF-γ and are cytotoxic towards cells, increasing the expressed activated Th1 cells response from 12 to 40% (Lowrie et al 1999). This is intriguing as it indicates that administration of IL-12 DNA could substantially reduce bacterial numbers in mice with chronic tuberculosis infection, suggesting that the induction of this cytokine, due to its role in the selective promotion of a Th1 immune response, is an important factor in the design of an improved tuberculosis treatment.

6.5 CONCLUSIONS

The relationship between the manner in which immunity is generated and the pathogens that have aided in shaping those mechanisms is one of complexity and antiquity. Underlying many of the nuances that have been associated with differential

susceptibility and/or resistance to infectious disease are genetic mutants like SNPs in promoter regions of key immune receptor genes. Of particular importance for First Nation populations in Canada are the potential variations within immunogenetic profiles that alter the optimal functionality of immune expression to tuberculosis infection. In Canada, Aboriginal populations experience some of the highest incidence rates of tuberculosis, both provincially and nationally, and tuberculosis control within these populations is becoming a countrywide concern (PHAC 2010). The solution to these concerns, however, is not a simple one because tuberculosis epidemiology is significantly influenced by compounding social, biological and environmental factors. To address some of these factors, this research focused on elucidating immunogenetic variations and explored the historic and contemporary implications of these variations on immunity and health.

Based upon the previous identification of cytokine polymorphisms in First Nations conducted by Larcombe and colleagues (2008), the hypothesis for this research stated that the Manitoba First Nation cohorts would maintain a higher frequency of SNPs that could be affecting the manner in which immunity is mounted in response to *Mycobacterium tuberculosis* infection when analyzed against a comparative Caucasian cohort. These SNPs in several key immunoregulatory genes would result in a down regulated immune response to tuberculosis, and could be possibly contributing to the disproportionately high rates of this disease reported in First Nation populations in Manitoba. The primary objectives for this study were threefold: 1) to detect and document the occurrence of SNPs in the promoter regions of IL-12, TLR2 and TLR4 within Dene, Cree and Saulteaux First Nation cohorts as well as a Caucasian cohort; 2) to

investigate how the SNPs may affect the overall immune response to *Mycobacterium tuberculosis*; and 3) to relate how the immunogenetic profile may reflect the unique biocultural and environmental interactions of historical and contemporary Manitoba First Nations. The selection of the genes included in this study were based on their complementary nature to the previous suite of cytokines identified by Larcombe and colleagues (2008) within similar Manitoban First Nation cohorts and additionally, the identification of particular SNPs in these genes could provide a manner to assess the functionality in the recognition of *Mycobacterium tuberculosis* as well as further clarify the fashion in which cytokine expression genotypes can affect the promotion CD4+ pathways in the Th1/Th2 immune balance.

This research has determined that there are statistically significant differences between First Nation and Caucasian cohort SNP frequencies within these genes, as well as significant statistical differences in SNP frequencies between each First Nation cohort. The SNPs detected within both of the TLRs were observed to maintain alleles and genotypes that promoted the high expression of these genes, within all cohorts. These SNPs suggest that within both TLRs, the maintenance of genotypes associated with the adequate expression of these genes to sufficiently recognize invading Mycobacterium pathogens. However, the IL-12 SNP frequencies maintained by the Dene and Cree cohorts seem to favor genotypes associated with a down regulated expression of this gene. A sub optimal IL-12 expression may have serious implications in effectively directing naive T cells into Th1, an immune pathway that is critical for the successful containment and elimination of *Mycobacterium tuberculosis*. These findings combined with the clusters of SNPs previous identified by Larcombe and colleagues (2008) suggest

that Manitoban First Nations possess immunogenetic profiles in which SNPs in key Th1 promotion and maintenance cytokines are functionally down regulated whereas, immunoregulatory Th2 cytokines are promoted through SNPs that have been associated with high expression. The skew towards a Th2 over a Th1 immune pathway has serious implications for increased host susceptibility to tuberculosis, as each of these immune pathways has become specialized: Th1 for bacterial and viral infections and Th2 for parasitic and fungal infections (Abbas and Lichtman 2010). This specialization means that if the "wrong" pathway becomes activated due to altered cytokine expression, a suboptimal immune response can be generated, allowing the invading microbe to proliferate and resulting in overall poor health outcomes.

The emerging Th2 promoted immunogenetic profiles observed within contemporary First Nations did not arise by chance, but are in fact the product of multiple evolutionary pressures that have selected for these genetic variations over generations. The differences in SNP frequencies between the First Nation and Caucasian cohorts and among the First Nation cohorts are a testament to these localized differential selective pressures. In pre contact First Nation populations historical disease environments would have aided in the selection for a strong Th2 type immune response in order for optimal immunity for the high parasitic loads. With the arrival of Europeans to the New World, shifts in culture and the introduction of new pathogens altered the environment that First Nation populations had been successfully adapted to, resulting in a disadvantageous promoted Th2 immunity in an environment that required strong Th1 cytokine expression optimized for high rates of infectious disease. More recently, compounding social determinants of health, increasing co-morbidities, differential access to medical care and

treatment have all begun to significantly exacerbate the immune response to tuberculosis enhancing the down regulated response and contributing to the high rates of this disease reported in Canadian First Nation populations. The solution to these concerning rates is not a simple one, but rather will require a greater understanding of the social, biological and environmental factors that can significantly influence tuberculosis epidemiology. The identification of SNPs in First Nation immunogenetic profiles is a small step in the race to eliminate tuberculosis in these populations and can hopefully one day aid in the treatment of this disease.

6.6 FUTURE DIRECTIONS

The health of Canadian Aboriginal populations is an immense concern for health researchers and front line health providers. Well established social and economic factors influence differential disease resistance and/or susceptibility, and as reported in this study, immunogenetic variability can contribute significantly as well. Of paramount importance to mounting an optimal immune response, SNPs can alter functionality of cytokines affecting T-cell differentiation which can dysregulate the Th1 and Th2 pathways. A skew in favor of one immune pathway over another can strengthen or limit certain population's ability to contain and eliminate pathogens in a timely fashion, often resulting in poorer health outcomes. The results of this study suggest that there is a skew towards the Th2 T-cell differentiation pathway over the Th1 immune pathway in First Nation cohorts. Evidence to support this shift comes from the identification of alleles and genotypes associated with decreased functionality observed within key Th1 regulatory cytokines. However, in order to confirm this shift towards a Th2 promoted immune pathway further examination is required.

There are two areas of potential future research that could additionally aid in the elucidation of the unique immunogenetic profiles of First Nation populations and the manner in which immunity is generated in reaction to *Mycobacterium tuberculosis*. A primary area of research would include further genotyping analysis on particular cytokines that are associated with Th2 promotion and maintenance. IL-4 and IL-5 are two Th2 cytokines that direct the CD4 cells into a Th2 subset, where IL-13 continues to amplify the response (McSorley and Maizels 2012, Dheda et al 2010). An examination of these particular cytokines and the frequencies of the functional expression associated genotypes will provide supplementary support to the premise that First Nation cohorts in Manitoba maintain SNP frequencies that skew naive T-cell differentiation into the Th2 subset over a Th1 and that this could be a factor in the high rates of tuberculosis reported in these populations.

Similar in nature to the examination of the Th2 regulatory cytokines, the second area of potential research should support the investigation of whether these unique immunogenetic profiles exist in other Aboriginal populations and if so, to what degree. The differential SNP frequencies that were observed among the Dene, Cree and Saulteaux First Nation cohorts within this study, demonstrate that there are not only differences in genetic variations among First Nation populations but also that these variations are likely the result of varied historical pathogen, environment and host evolutionary relationships. Additionally, the SNP variation patterns among the First Nation cohorts became more pronounced when geographic location was taken into consideration. The maintenance of SNPs that could be shifting the immune response towards a strong Th2 pathway over a Th1 pathway became more pronounced in the more

northerly orientated First Nation cohort than the southern cohort, with an intermediate First Nation variation pattern being observed within immunogenetics and geography. An extension further north and south into different Aboriginal populations may shed some light on the range of immunogenetic variation observed within these cohorts, the co-evolutionary pressures that affected particular Aboriginal populations and the impact that these immunogenetic profiles may have on current health concerns. Therefore, these proposed avenues of research will not only continue to clarify the distinctive manner in which T-cell differentiation exists within First Nation populations but also explore the implications of functional cytokine driven Th1/Th2 pathways and pressing health concerns of disease that are currently dramatically increasing in Aboriginal populations like diabetes, tuberculosis and other infectious diseases. This research will also provide a means of evaluating the evolutionary pressure and historical pathogens that have helped shape contemporary First Nation immunogenetic profiles.

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APPENDIX A: ETHICS APPROVAL

UNIVERSITY OF MANITOBA | BANNATYNE CAMPUS
Research Ethics Boards

P126 - 770 Bannatyne Avenue
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Tel: (204) 789-3255
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APPROVAL FORM

Principal Investigator: Dr. P. Orr

Ethics Reference Number: H2005:106
Date of Approval: June 8, 2012
Date of Expiry: May 30, 2013

Protocol Title: The Immunogenetic Program of First Nations and susceptibility to Mycobacterium tuberculosis isolates

The following is/are approved for use:

- **Annual Approval**

The above was approved by Dr. John Arnett, Ph.D., C. Psych., Chair, Health Research Ethics Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your submission dated June 6, 2012. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada*.

This approval is valid until the expiry date only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval must be sought from the relevant institution, if required.

Sincerely yours,

John Arnett, PhD., C. Psych.
Chair, Health Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255 / Fax: (204) 789-3414

www.umanitoba.ca/medicine/ethics