University Of Nevada, Reno

CELLULAR IMMUNE CHANGES in THE PERIPHERAL BLOOD of SUBJECTS CHRONICALLY INFECTED with XMRV

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Science/Environmental Toxicology

By

Deborah L.S. Goetz

Dr. Mark Walker/Dissertation Advisor

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DEBORAH L.S. GOETZ

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Mark Walker, Ph.D., Advisor

Judy Mikovits, Ph.D., Committee Member

Frank Ruscetti, Ph.D., Committee Member

Glenn Miller, Ph.D., Committee Member

Kenneth Hunter, Sc.D,, Graduate School Representative

Marsha H. Read, Ph. D., Associate Dean, Graduate School

December, 2010

Abstract:

Retroviruses chronically alter the regulation of the innate immune system causing immunosuppression. Recently, evidence of a new human retrovirus, Xenotropic murine leukemia virus-related virus, XMRV, was detected in the blood of >67% of 101 Chronic Fatigue Syndrome (CFS) patients tested and shown to be infectious. CFS is a debilitating disease characterized by innate immune system activation and low natural killer (NK) cell activity. We hypothesized that XMRV infection plays a role in the pathogenesis of CFS through the dysregulation of the innate immune and/or the adaptive immune responses. We addressed this hypothesis by phenotypic analysis of leukocyte subsets by multiplex flow cytometry in CFS patients infected with XMRV versus uninfected controls. While there was no significant difference in total cellularity and CD45+ leukocytes between XMRV-infected CFS patients and XMRV negative controls, we found significant reductions in the percentage of CD45+ lymphocytes, CD45+ CD3- lymphocytes, CD45+ CD3- CD19- CD56+ NK cells and CD19+ B cells. Moreover, the NK phenotype in XMRV+ patients was altered, with 80% of XMRV+ patients having a significantly reduced $CD56^{DIM}$ population. Within this $CD56^{DIM}$ population present in XMRV+ patients, only 40% express CD16. XMRV infected patients showed a important increase in CD3+ CD56+ NKT cells with a NK to NKT ratio that was 55% higher than XMRV- healthy controls. The B cells present in the peripheral blood of XMRV+ CFS patients contained a remarkable CD20+, CD23+ mature B cells. The myeloid cell compartment

showed an increased population of activated antigen presenting cells. These data suggest a chronic innate immune activation in XMRV-infected subjects which cause innate and adaptive immune deficiency, similar to those seen in other human retroviral infections. These data also suggest that the immunological defects seen in some CFS patients could be attributed to XMRV infection either directly though XMRV infection of specific leukocyte subsets or indirectly through the dysregulation of cytokines and chemokines.

DEDICATION:

To my grandchildren –

Reach outside yourself.

Never settle for the status quo.

Never give up and never surrender!

Never, ever, forget that I love you very much!

and

To Lauren, Andrea and all who suffer -

This small work is really for you. Don't give up – help is on the way!

WITH DEEP GRATITUDE:

To all of my family, friends and colleagues who have endured this with me, thank you! I deeply appreciate all that you have contributed to this process. I could not have done this without you! I especially wish to thank:

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To You be all the glory, honor and praise!

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Thank you for your support, encouragement, patience and love.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

INTRODUCTION:

Retroviruses, from the family of Retroviridae, are RNA viruses that infect humans and animals. The distinguishing characteristic of retroviruses is that replication occurs through a proviral DNA and the virion contains a lipid bilayer envelope which surrounds a core containing a helical nucleocapsid. Retroviruses were first discovered in animals (1). An avian sarcoma virus, later called the Rous sarcoma virus, which causes tumors in chickens, was the first retrovirus to be characterized (2-3). The first mammalian retrovirus, murine leukemia virus, was characterized forty years later (4), and the first primate retrovirus was found in 1970(5). Isolation of the first human retrovirus, however, did not occur until 1980 when Ruscetti et al. (6) discovered IL-2, a growth factor critical immune system cells, T and Natural Killer (NK) cells. The discovery of IL-2 allowed for human leukemic cells to be cultured and allowed for isolation of human retroviruses from the cultured cells (6). This critical discovery opened the door for future human retroviral characterization. Shortly thereafter, Poiesz and Ruscetti went on to characterize the human T cell leukemia virus (HTLV) (7). An estimated 20 million people are infected with the HTLV-1 virus, and it is considered endemic to the Caribbean, Southern Japan, Africa, South America and the Pacific Islands. HTLV is the causive agent for adult T cell leukemia/lymphoma, but also tropic spastic paraparesis /HTLV associated myopathy (TSP/HAM) and is associated with a host of other inflammatory

disorders (8). A second human T cell leukemia virus was characterized in 1982 from cultures taken from a patient diagnosed with hairy cell leukemia (HTLV-II)(9).

Xenotropic murine leukemia virus-related virus (XMRV) was first detected in 2006 when viral sequences were found in stromal tissues surrounding tumor bearing prostate tissue(10). A single nucleotide polymorphism of the ribonuclease (RNase) L, R462Q, was suggested to be a susceptibility factor for prostate cancer(11). Ribonucleases are crucial to the maturation of all RNA molecules and play a critical role in the viral defense(12). RNase L is an interferon induced nuclease that destroys viral RNA when activated. The R462Q RNase polymorphism results in a reduced ability to degrade viral RNA. Urisman et al. investigated hereditary prostate cancer, when the R462Q variant was identified. The R462Q variant was associated with only 10% of prostate cancer samples. They then explored the potential association of the R462Q variant with viral infection (10). They found the presence of retroviral sequences in patients homozygous for the R462Q polymorphism. The gammaretroviral sequences found were closely related to murine leukemia virus (MLV), a virus known to cause leukemia, sarcoma and neurological disease in animals, yet distinct (10). XMRV has become the third human retrovirus discovered following HTLV and HIV.

The process of characterization of XMRV and the investigation into disease association follows a similar path as that of the HIV infection in AIDS patients, and prior to that, the discovery of HTLV infection in TSP/HAM patients. Some postulate that without the groundbreaking research, particularly the discovery of IL-2 and the ability to culture T cells, prior to and leading up to the characterization of HIV, the causative agent of AIDS would not have been discovered (7, 13-14). HIV researchers began with clinical observations of immune depression suggestive of viral interaction, particularly herpesviruses and retroviruses. Their initial research detected the activity of reverse transcriptase, a DNA polymerase enzyme that transcribes single stranded RNA into double stranded DNA. The activity of the reverse transcriptase enzyme was similar to that found in the newly characterized HTLV virus, narrowing their research to retroviruses, which eventually led to the identification of HIV(15-17). In order for their discovery to be accepted by the scientific community as the primary etiological component of AIDS, further in-depth characterization of the virus was needed to establish a definitive link between the observed disease and the newly discovered virus. The viral characterization led to development of the first diagnostic tests and the prevention of transmission of HIV virus in blood products. Discovery of biomarkers and prognostic components began shortly after viral discovery through observation of the immune system response to infection (16-17). One may observe from the history of the discovery and characterization of the HTLV and HIV viruses that acceptance of disease causation was not easily won.

Among its many functions, the immune system protects the body from microbial invasion. It does this through the interaction of the different cell types within the two main sub-systems, the innate and adaptive immune systems. In peripheral blood, the leukocytes, sometimes known as white blood cells, can be broadly divided into three categories: lymphocytes (15 to 40% of all leukocytes), including NK, T, and B cells; granulocytes (50 to 60% of all leukocytes), including neutrophils, eosinophils, basophils and basophils; and monocytes which include macrophages and dendritic cells (about 5 to15%of all leukocytes)(18-19). The innate immune system consists of cell types that respond within minutes to microbial attack. Macrophages, monocytes and granulocytes can respond first, engulfing the pathogen and signaling other innate cell types to the area of invasion through cytokines including and chemokines. The pattern recognition Toll-like receptors on the innate immune cell types can also stimulate production of pro-inflammatory cytokines and chemokines, causing recruitment of other cell types including dendritic cells and natural killer (NK) cells. Dendritic cells respond to the chemokine trail, taking up antigens through phagocytosis or macropinocytosis for presentation to, and initiation of the adaptive immune system lymphocytes in lymphatic tissues. NK cells also respond and are activated by the increase in cytokines and chemokines and directly kill virally infected cells as well as cancerous cells.

The cellular component of the innate immune system consists of NK cells, dendritic cells, macrophages, monocytes and granulocytes. They respond first to

pathogenic attack killing the pathogen and infected cells and spewing activating and inflammatory cytokines and chemokines that summon other immune system cells to the attack. The innate immune system has evolved specifically as the first line of defense against microbial infection. Natural killer cells are large, granular lymphocytes comprising 6 to 14% of all peripheral blood lymphocytes (3, 20-22). NK cells bridge the innate and adaptive immune systems by providing primary cytotoxic and cytostatic functions while interacting and collaborating with adaptive immune system lymphocytes. They also mediate the activation and regulatory effects on other cell types and inflammatory responses (3, 20, 23-24). NK cells are abundant in the blood, spleen and liver, but are also found in the lymph nodes. They also migrate to sites of inflammation and are found in tumor-bearing tissues. NK cells are armed with the cytolytic molecules perforin and granzymes and a host of stimulatory and inhibitory receptors including the killer cell immunoglobulin-like receptors (KIRs), CD94/NKG2 receptors, NKG2D, NKP and LIT receptors. Yet, even though armed, NK cells are activated through type 1 interferons, IFNα and IFNβ, or through pro-inflammatory cytokines including IL-12, IL-15 and IL-18 (20, 25-27) .

The antiviral effects of NK cells are well documented. Primary NK cell responses including IFNγ production and cytotoxicity occur within hours to days of a viral infection. Targeting mainly virally infected cells and tumor cells, NK cells elicit spontaneous cytotoxic activity, predominantly through a delicate balance of the activating and inhibiting signals transmitted by the KIRs and other activating

receptors generating perforin/granzymes mediated killing (28). NK cells also kill through inflammatory cytokine secretion, antibody dependent cellular cytotoxcity (ADCC) through the FcγRIII (CD16) receptor and death receptor interaction (29-31). Yet, NK cells are sensitive to cytokine and chemokine levels in order to maintain the delicate balance of cytotoxicity. Two chemokines, MIP1α and MIP1β, attract NK cells to the site of inflammation. Dendritic cells and other cell types increase levels of antiviral IFNα and IFNβ as well as IL-12 and IL-15. These, in turn, activate NK cells, adding to the inflammatory state. Activated T cells also add to the state of inflammation through their added production of IL-2, which further activates NK cells.

Viral pathogens have evolved mechanisms to evade NK cytotoxicity. The human cytomegalovirus (HCMV) evades cytotoxicity through production of peptides, UL-40, that are overexpressed on human leukocyte antigen (HLA-E) which activates inhibitory receptors NKG2A (32-33). Retroviruses have evolved mechanisms to evade NK killing by taking advantage of the KIR and HLA polymorphisms. For example, HIV infected individuals expressing the Bw4 epitope on the HLA-B alleles may have a delayed onset of AIDS, and AIDS progression is even slower for those that also have a polymorphism of the KIR3DL1 receptor together with the Bw4 epitope (34-35). HIV encodes a protein known as NEF that decreases the expression of HLA-A, HLA-B and NKG2D activating receptor, thus increasing the opportunity for inhibitory KIR receptor

engagement on NK cells (36-38). As a result, NK cells are sensitive to receptor dysregulation mediated by pathogens and specifically viral infection (39-41).

NK cells are difficult to characterize phenotypically and are defined as much by what they lack as by what they express on the cell surface. The lack of the T cell receptor, CD3, distinguishes NK cells from T and NKT cells. Similar to T cells, however, NK cells express CD2, a cell adhesion molecule also known as LFA-2, CD161, a killer cell lectin like receptor also known as NKR-P1A and CD57, and a neuronal adhesion molecule also known as HNK-1. Further, NK cells also share the IL-2β/γ receptor, CD122, with other lymphocytes, particularly T cells (20, 22, 24, 28, 42).

Neural cell or neuronal adhesion molecule, NCAM also known as CD56, is a type 1 transmembrane glycoprotein of the Ig superfamily. It is dominantly expressed by neural and muscle cells as well as NK cells and a small subset of T cells. CD56 is involved in adhesion, migration, growth, differentiation and other cellular functions (43-47). The level of CD56 expression, however, is indicative of distinct subpopulations of NK cells, and is considered the “gold standard” for NK cells (26, 42). The two best-defined subpopulations among healthy individuals are the CD3- $CD56^{DIM}$ and the CD3- $CD56^{BRIGHT}$ subsets. The majority of NK cells in peripheral blood are the CD3- $CD56^{DIM}$ subset, which express high levels of the FcγRIII/CD16 molecule. This subset is characterized by their ability to rapidly mediate cytotoxicity through the expression of perforin as well as target opsonized cells (cells covered by antibodies) via the low affinity

FcyRIII receptor through ADCC. The CD3- $CD56^{BRIGHT}$ subset comprises only 5 to 10% of healthy NK cells and is characterized by their immunoregulatory functions, particularly in secondary lymphoid organs, by their ability to secrete large amounts of many cytokines, including IFNγ and TGFβ. This subset does not normally express CD16 and largely lacks perforin and killer inhibitory receptors (26, 42, 48-49).

Several days following the pathogenic attack, the adaptive immune system is up regulated. Unlike the innate immune system cells, adaptive immune system cells mediate their response against specific antigens and generate immunological memory. There are several subpopulations of adaptive immune cells where the distinction of which arm of the immune system they belong to is less clear, and a few of these bridge both immune systems. These would include γδ T cells, NKT cells, B-1 cells and marginal zone B cells (50). The characteristics and/or function of these subpopulations are more closely aligned with the innate immune system, and the γδ T cells and NKT cells have functions which bridge the innate and adaptive immune system, particularly with regard to activation and immunoregulation. The NKT cells, for example, consist of a several subpopulations (51-52) which modulate the innate immune system by activating NK and dendritic cells through cytokine production but also suppress innate immune cell activity and development (50-51, 53). NKT cells are produce explosive amounts of cytokines, particularly IL-4 and IFNγ, and can become cytotoxic and release perforin and granzymes similarly to NK cells .

NKT cells can also express many of the same surface markers as NK cells including CD16 and CD161. While NKTs have similarities to NK cells, they are true T cells. They have the CD3 T cell receptor and develop in the thymus and periphery. They do not, however, elicit immunological memory. Their function may be modulated by the presence or absence of CD1d, and like T and NK cells, they require IL-2, IL-12, IL-18, and IFNα and/or IFNβ for development and activation (51-53).

The B cell population may also be altered by viral pathogens. Along with T cells, B cells are a critical component of the adaptive immune system, yet also bridge the innate with the adaptive immune system by their antigen presenting capability. The principal functions of activated B cells are to generate a humoral immune response against pathogens through the generation of antibodies against antigen. These antibodies may elicit ADCC by NK cells, present antigen to T cells, and after such antigen presentation develop a memory B cell population. B cells undergo significant alteration in their development taking them from the pro-B stage through the memory B stage, including the development of antibody producing plasma cells. B cells undergo functional rearrangement of the immunoglobulin-like B cell receptor during early development stages to recognize “self”. During clonal expansion, activated B cells undergo a directed somatic hypermutation of the B cell receptor allowing for selection of B cells with a greater capacity to recognize and bind specific antigen. (54) Finally, with increasing exposure to specific antigen during an immune

response, B cells undergo a process of refining their ability to bind specific antigen known as affinity maturation (54-55). These development stages are characterized by upregulation and downregulation of cell surface receptors including CD5, CD19, CD20, CD21, CD22, CD23, CD24, CD27, CD40, CD72 and CD79 (54, 56).

Retroviruses have evolved mechanisms to either evade the humoral response or subvert B cell function or development, increasing pathogenesis. HIV infected patients and/or AIDS patients, for example, suffer from hypergammaglobulinemia, polyclonal B cell hyperactivation, elevated serum levels of autoantibodies, expanded areas within lymphoid tissues for B cell activation, and alteration of B cell activation and differentiation markers (57-62). Further, studies have shown that HIV infected individuals have B cell subpopulations not seen in healthy individuals. Four subpopulations predominate including plasmablasts, immature transitional B cells, activated mature B cells and exhausted memory B cells (60, 63-65).

Current thought is that myeloid cells are immune system cells that have developed from a common myeloid progenitor in contrast to lymphocytes that have developed from a common lymphoid progenitor (66-68). The myeloid cell compartment is highly diverse and includes monocytes, macrophages, granulocytes as well as dendritic cells . Monocytes have the unique ability to enter the bloodstream and move quickly to sites of infection and differentiate into either macrophages or mDCs, thus replenishing these populations (66, 69).

Both monocytes and macrophages engulf pathogens and similar to neutrophils, macrophages will subject the pathogen to cytotoxic granules. Monocytes that have migrated to the site of infection can differentiate into myeloid derived dendritic cells (mDCs). Immature mDCs are found in mucosal membranes and the bloodstream, sampling the surrounding environment for antigenic particles produced by viruses and other pathogens using endocytosis and macropinocytosis (66, 70).

Once the antigen is recognized as “non-self” by an immature dendritic cell (DC), a critical functional change or activation occurs. This functional change bridges the innate immune system response with the adaptive immune system. The activated immature DC undergoes a maturation process as it travels via the lymphatic system to a lymph node becoming the immune system’s premier antigen presenting cell. Here, the mature DC presents the antigen to T and B cells, thus activating the adaptive immune system. Yet, in the course of antigen acquisition, maturation and antigen presentation, the DC can become infected by retroviruses, spreading the infection from the periphery to lymph nodes, infecting other immune system cells, particularly T and B cells as well as other DCs and NK cells (70).

Granulocytes are characterized by granules in their cytoplasm and generally include neutrophils, basophils, and eosinophils. Mast cells also contain granules in their cytoplasms and are also considered to be granulocytes. Neutrophils are the most abundant of the granulocytes and compose about 50 to

60% of leukocytes circulating in the in the bloodstream. During pathogenic invasion, neutrophils leave the bloodstream to enter the site of infection, all within as little as thirty minutes. Neutrophils rapidly engulf microorganisms that are coated with proteins that are part of the complement system, and pathogens that have been opsonized by antibodies (68). The engulfed pathogen is encased in phagosomes and granules containing reactive oxygen compounds, including nitric oxide, superoxide, defensins, cationic proteins, lysozymes, lactoferrin and acid hydrolases, are released. In most cases, this kills the pathogen (71) . Neutrophils do not return to the blood, but rapidly die (72). Eosinophils are also found predominantly in the blood, and also have granules containing reactive oxygen compounds. But, unlike neutrophils, eosinophils predominantly protect against parasites (73-74). Basophils are the least abundant in the blood, and will release histamines from their granules adding to the inflammatory response against pathogens. Mast cells, like basophils, release histamines, and are mediators of allergic reactions (75).

In the transmission of HIV, viral entry is a subject of much debate. Entry points could arise on any cell with a CCR5 receptor, predominantly T cells including memory T cells expressing CD4, macrophages, dendritic cells and microglia. Macrophages and monocytes likely play a pivotal role in transmitting the infection to the T cell population (76). Current findings suggest that HIV moves through intercellular spaces in the epithelium and thus makes initial contact with Langerhans cells and CD4 T cells in the underlying mucosa (77).

Innate immune activation through the secretion of cytokines, activation and infection of DCs as well as the activation of T and B cells accelerate the deleterious effects of acute HIV infection (70) . Direct viral infection of immune cells and bystander effects of the virus including the escalation of cytokine production by both innate and adaptive immune cells leads to apoptosis of T and B cells (78). As the virus continues its rampage through the immune system, changes in NK cell subsets contribute to the increased secretion of pro-inflammatory cytokines (38, 79). The $CD56^{DIM}$ subset is reduced and there is a significant increase in a dysfunctional CD56- subset. This CD56- CD16+ population displays reduced cytotoxicity and IFNγ production but an increase production in MIP1-β (80-82). NK cell mediated killing is diminished by the downregulation of HLA-A and HLA-B class 1 molecules. This can further skew the NK repertoire towards cells with HLA-C specific inhibitory KIRs (83-84). The premier antigen presenting cells, DCs, are activated. The activated DCs carry the virus to lymph nodes further adding to the infection of T and B cell populations and resulting apoptosis. Activated T cells expressing the CCR5 and CD4 receptors are marked for infection by HIV. The CD21 receptor on B cells also binds the virus and continues the spread (70, 85). B cells are not depleted during early infection but B cell responses are altered probably due to other cell types being destroyed during acute infection. These cell types are important for development of the germinal centers of the lymph nodes (86). The activation and further immune suppression allow for viral coinfections that further alter the

immune response (78, 87). Thus this chronic immune stimulation that occurs leads to immune exhaustion and immunosuppression (70, 88-89).

Like HIV, the HTLV virus also targets CD4+ T cells for infection as well as CD8 T cells and appears to do this through the ubiquitious glucose transporter 1, neuropilin 1 and surface heparin sulfate proteoglycan surface receptors (90) . But, unlike HIV, virion particles are not detected by current techniques in plasma outside of the cell (91). Transmission appears to occur primarily through direct cell-to-cell contact through a viral synapse triggered by the viral protein TAX, and many times involves transfer of virions from an antigen presenting dendritic cell and a T cell (trans-infection), or later in the infection cycle by newly made virions from an infected dendritic cell to a T cell (cis-infection) (92). Among others, the TAX protein promotes cell proliferation of the T lymphocytes thus causing viral replication. Researchers hypothesize that the upregulation of viral promoters and the viral replication through sustained cell proliferation allow for immune activation and for cell selection favoring immune evasion. Further, sustained cell proliferation could cause DNA damage and growth promoting mutations to occur allowing for the development of T cell lymphoma.

Recently, researchers have found that retroviral envelope (Env) proteins not only have a mechanical function but also suppress the immune system through an immunosuppressive domain. They demonstrate that this immunosuppressive domain directly affects NK and CD8 T cellular responses in vivo (93).

In a similar manner to HIV researchers, researchers at the Whittemore Peterson Institute for Neuro-Immune Diseases (WPI) noted the immune depression and an RNase L defect in CFS patients, suggesting a retrovirus (94). Although retroviruses are not widespread, further research revealed an association between the recently identified XMRV virus and CFS patients. The prevalence of XMRV is not known. Further, there are no quantitative assays for XMRV and sensitivity varies among assays thus requiring identification by multiple methods. Low copy number, particularly in unactivated cells, further complicates viral detection. WPI used several methods for XMRV detection: detection of antibodies to viral proteins in peripheral blood, detection of the viral proteins by Western blot, detection of viral RNA by RT-PCR, and by viral transmission of XMRV to LNCaP cells. WPI recently reported isolation of infectious and transmissible XMRV from the blood of CFS patients. XMRV nucleic acids were detected in the PBMC of 68 of 101 (67%) CFS patients, whereas only 8 of 218 (3.7%) regional, healthy controls contained XMRV DNA. Furthermore, WPI researchers demonstrated that infectious virus was transmitted from activated primary PBMC, as well as from purified B and T cell cultures and plasma derived from CFS patients by establishing a secondary infection in uninfected primary lymphocytes (94).

CFS is a complex and poorly understood disease of unknown etiology affecting 17 million people worldwide. CFS is commonly characterized by innate immune defects, chronic immune activation and dysregulation, often leading to

neurological abnormalities (Reviewed in (95)). CFS also involves other biological systems such as the musculoskeletal, gastrointestinal and endocrine systems (96-98). Although several common symptoms are primarily reported and predominate, they may differ among individuals, are often intermittent and can persist for years, frequently resulting in substantial disability (99). Some of the most commonly reported physical symptoms include muscle weakness and pain, tender or swollen lymph nodes and chronic flu-like symptoms (100). Memory and concentration impairment, blurred vision, dizziness and sleep abnormalities represent some of the cognitive symptoms typically observed while immunological symptoms often manifest themselves through viral reactivation, decreased natural killer cell function and susceptibility to opportunistic infections (101-104).

While biological and immune observations related to CFS have been made, there are no specific biological markers of disease. Diagnosis of CFS occurs through the observation of clinical symptoms. The actual case definition for the diagnosis has been a subject of debate among CFS clinicians, but the most widely accepted criteria was developed by the Center for Disease Control (105). CFS is first defined by the specific onset of a persistent or relapsing fatigue not brought on by exertion and not alleviated by sleep. Indeed, CFS is characterized by unrefreshing sleep. Further, four or more of the following symptoms must have persisted or recurred for six months or more and not predate the fatigue: short term memory impairment and/or a severe inability to

concentrate that disrupts personal, occupational, educational and social activities, sore throat, tenderness of lymph nodes, muscle and joint pain without swelling or redness, headaches and post exertional malaise. In addition, other physical and psychiatric diseases must be eliminated in order to arrive at a final diagnosis. As stated, the cause of CFS is unknown, but bacterial and viral infection have been implicated, as well as autoimmunity, inflammation of the nervous system, in addition to stress and environmental factors (106).

Because of the dearth of biological markers of disease in CFS, the clinical and research focus has been on psychological causes and remedies, leaving CFS patients and their families with feelings of hopelessness for definitive treatment and a cure. A pervasive attitude among many CFS patients is that they feel locked into a cycle of distrust of the medical community and yet an unrelenting attraction toward allopathic and/or homeopathic treatments for relief. Further, the impact of the disease upon family members and friends can be devastating and can lead to separation, divorce and isolation.

Many biochemical observations associated with CFS suggest an underlying innate immune involvement and chronic infection. CFS patients often display antiviral enzyme RNase L dysfunction and dysregulation of inflammatory cytokines and chemokines (107-108). Pathogens commonly associated with CFS such as parvovirus B19 (109), which effect macrophage function (110) and enteroviruses (96) which are known to inhibit RNase L function (111), underscore the importance of the innate immune response in CFS.

The dysfunction of the NK cell compartment in CFS has been widely noted, including decreased NK cell activity (101, 103, 112-113). In a previous study, Maher et al.(2005) found low perforin levels and depressed NK cell activity in a group of 30 CFS patients (114). Levine et al.(1998) suggested the low NK cell activity seen in CFS patients may be the result of a shift in NK cell populations, which would cause an increase in less active cells, a decrease in NK cell modulating cytokines or the presence of inhibitory factors (112). Imbalances in circulating T and B cell populations, as well as low NK cytotoxicity, have been reported in CFS patients (115).

Cytokines and chemokines play important roles in controlling the homoeostasis of the immune system. NK cells are also affected by the cytokines produced by other immune cells. For instance, IFNα, IL-12, IL-15 and IL-2 are involved in NK cell activation and maturation. IFNα and IL-12, produced early in a viral infection, are critical for stimulation of the cytotoxicity, the proliferation and the IFNγ production of NK cells, activating both STAT1 and STAT4 intercellular signaling. IFNα induces NK cell cytotoxicity and IL-12 is critical for IFNγ production; IL-15 has been found to sustain NK cell accumulation in the presence of IFNs (25, 116). Furthermore, the macrophage inflammatory chemokines MIP1α and MIP1β are generated by NK cells and can block virus binding and infection (25).

We hypothesized that chronic XMRV infection creates an underlying immune deficiency leading to either direct or indirect effects on cells of the innate

immune response, particularly NK cells. To test this hypothesis we profiled the immune cells in peripheral blood in XMRV infected CFS patients using multi-parameter flow cytometry for phenotypic analysis and cytokine release using multiplex plasma proteomic analysis of 26 cytokines and chemokines in a suspension ELISA on a Luminex platform. Herein, we detail defects in the immune profiles of CFS patients infected with XMRV (XMRV-POSITIVE patients) including an overall reduction in the lymphocyte compartment in the 65 subjects as well as a significant reduction in CD45+ CD3- CD19- CD56+ NK cells particularly the $CD56^{DIM}$ subpopulation. We further demonstrate aberrant cytokine and chemokine production with a decrease in type I interferon that correlates with XMRV infection. These novel findings identify patterns or profiles of XMRV infection as biomarkers which could be used to develop clinical diagnostic tests.

Chapter 2

EXPERIMENTAL DESIGN, MATERIALS AND METHODS

2.1: Experimental Design:

We hypothesized that chronic XMRV infection creates an underlying immune deficiency leading to either direct or indirect effects on cells of the innate immune response, particularly NK cells. To test this hypothesis I profiled the immune cells in peripheral blood in XMRV-Positive patients and XMRV-Negative healthy controls using multi-parameter flow cytometry for phenotypic analysis. My data was also correlated these data with concurrent cytokine release data generated by Dr. Vincent Lombardi of the WPI using multiplex plasma proteomic analysis of 26 cytokines and chemokines in a suspension ELISA on a Luminex platform. The corollary goal of this research was to determine a phenotypic and cytokine “signature” that could be used as a biomarker for further testing and diagnosis.

We obtained whole blood samples from over 60 individuals that had been diagnosed with CFS by a physician according to the criteria stipulated in the CDC Fukuda Criteria and the Canadian Consensus Criteria (CCC). In addition, we obtained whole blood from healthy individuals to use as a control, together with standards and controls for each experiment, as well as published levels of immune system subsets from current literature for verification. Each patient and control sample was assigned a randomized number by the WPI Laboratory

Administrator before being processed in order to insure patient confidentiality as well as reduce selection bias.

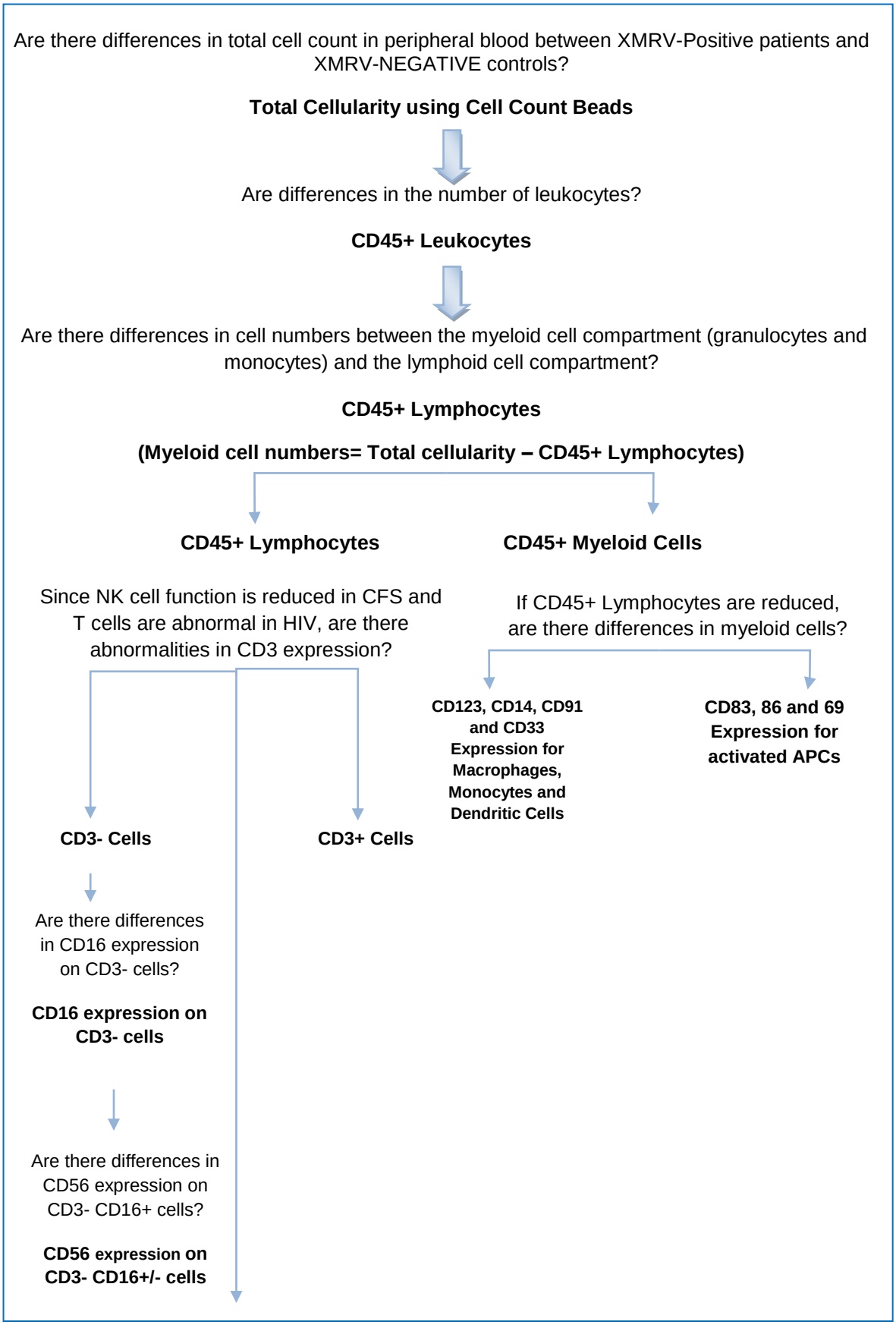
As part of a larger study being funded by the WPI, the following experiments were performed on each sample: 1) Phenotypic analysis by flow cytometry; 2). Cytokine analysis by Luminex; 3) XMRV detection by at least two of the four methods outlined in Lombardi et al. (94): RT-PCR, viral antibody, viral protein expression in PBMC or viral transmission to LNCaP cells.

Each sample was divided into two major portions. One portion was centrifuged and the plasma separated from the cells for cytokine analysis by multiplex plasma proteomic ELISA on a Luminex platform, and, later, for verification of XMRV infection by transmission of the virus to cultured LnCAP cells, and viral antibody detection. The peripheral blood mononuclear cells (PBMCs) were separated using Ficoll density centrifugation. A portion of the PBMCs was frozen for later RT-PCR processing for XMRV detection. The remaining PBMCs were cultured in media for later XMRV detection. The second major portion of whole blood was used for phenotypic analysis and labeled with the monoclonal antibodies selected for cell population differentiation.

Flow cytometry is used by cell and molecular biologists for the detection of particles or cells, and specifically, in our case, the receptors on a cell. The technique is based upon the Coulter principle where cells or particles pulled through a sheath or orifice along with an electric impulse, or in our case, a laser, will be impeded in proportion to the size of the cell or particle. Fluorochromes

are bound to antibodies specific for a receptor on a cell. A combination of up to five antibodies is prepared. Each combination is selected to delineate one or more cell populations of interest. The combination of antibodies is then incubated with the cells, and each specific antibody binds with the receptor specific for that antibody.

In preparation for this experiment, specific combinations of monoclonal antibodies were made for the detection of the cell populations including CD3+ T cells including NKT-like cells, NK cells and specific CD56 and CD16 expression, B cells and specific CD20 and CD23 expression, and myeloid derived cells. The results from one population led to investigation of another population which led to exploration of another population and so on (Fig. 2.1). The antibodies were pre-tested on human cells for optimal fluorescence with minimal spillover and to predetermine the optimal settings and parameters on the FC500 flow cytometer. Upon receipt of each patient and each control sample, the whole blood was aliquoted into labeling tubes with the various combinations of antibodies, incubated and prepared for flow cytometric measurement. Positive and negative bead controls, and in most cases, cultured cells of known phenotype were prepared and analyzed with each sample processed as controls.


Are there differences in total cell count in peripheral blood between XMRV-Positive patients and XMRV-NEGATIVE controls?
Total Cellularity using Cell Count Beads
Are differences in the number of leukocytes?
CD45+ Leukocytes
Are there differences in cell numbers between the myeloid cell compartment (granulocytes and monocytes) and the lymphoid cell compartment?
CD45+ Lymphocytes
(Myeloid cell numbers= Total cellularity – CD45+ Lymphocytes)
CD45+ Lymphocytes
CD45+ Myeloid Cells
Since NK cell function is reduced in CFS and T cells are abnormal in HIV, are there abnormalities in CD3 expression?
If CD45+ Lymphocytes are reduced, are there differences in myeloid cells?
CD123, CD14, CD91 and CD33 Expression for Macrophages, Monocytes and Dendritic Cells
CD83, 86 and 69 Expression for activated APCs
CD3- Cells
CD3+ Cells
Are there differences in CD16 expression on CD3- cells?
CD16 expression on CD3- cells
Are there differences in CD56 expression on CD3- CD16+ cells?
CD56 expression on CD3- CD16+/- cells

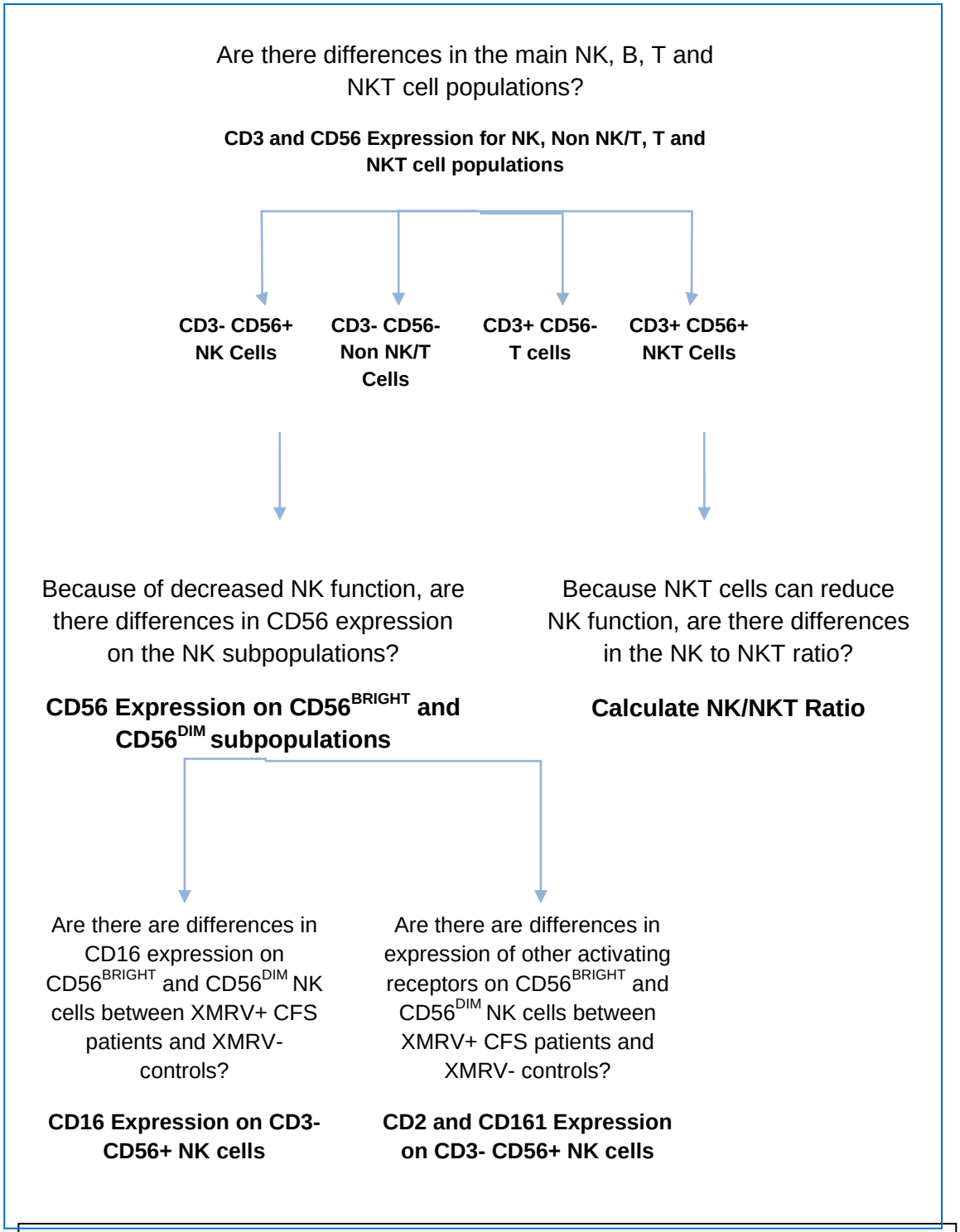


Figure 2.1: Pathway and leading questions for Phenotyping of Peripheral blood in XMRV POSITIVE CFS patients and XMRV NEGATIVE healthy controls.

The cells are then suspended in a stream of fluid, and forced through a hydrodynamically focused sheath which aligns each cell to pass singly before a laser. As the cell passes in front of a laser, the light is scattered and detected on the forward scatter detector that is in line or parallel with the laser, and on a side scatter detector that is usually perpendicular to the laser. The data from these two detectors can give a relative measure of the volume of the cell and the inner complexity of the cell based upon the roughness of the cell membrane, the nucleus and sometimes cytoplasmic granules. The laser also excites the fluorochrome on the antibody that is bound to the receptor on the cell. The excited fluorochrome then emits a photon that is detected on one of many fluorescent detectors. As with the forward and side scatter detectors, the level of brightness or intensity is determined resulting in an image of positive and negative values. The data generated from the fluorescent detectors can give a relative measure of the percentage of cells with that specific receptor. Obtaining the mean fluorescent intensity or the median fluorescent intensity can also provide a relative measure of the percentage of cells with the specific receptor or be an indication of the number of receptors on the cells.

The flow cytometry data was analyzed by FlowJo software (Treestar) and differences between the quantity and spatial distribution of “hits” which is representative of the cells was used and compared to healthy controls. Mean fluorescent intensity was another parameter used for comparison. Mean fluorescent intensity or MFI is the intensity of the fluorescence that can be

generated by either numerous cells expressing the particular receptor that the antibody is bound to, or the number of receptors per cell. When MFI is used, a histogram showing the MFI is generated from a representative XMRV-Negative healthy control. Similarly, histograms are generated from XMRV-Positive CFS samples then overlayed onto the histogram representing the healthy control as a way of comparison. In addition, published reference values from healthy controls are used (18-19). Additional analysis of the data would be done comparing XMRV-Positive versus XMRV-Negative samples following XMRV detection analysis.

2.2: Materials and Methods

Patient and Healthy Control Samples:

Fresh whole blood samples used for the flow cytometry immune profiling were obtained directly from patients and controls and analyzed within 6 hours of blood draw. Sixty-three CFS patient and eight healthy control whole blood samples were drawn and analyzed for immuno-phenotype. Written permission was obtained from each patient and control prior to the blood draw, and samples were acquired in accordance with University of Nevada, Reno IRB Numbers B0910-019 and B0809-037. Plasma from the CFS patients may have been part of the cytokine and chemokine profiling studies described herein. Of the 63 patients, 40 were female and 23 were male, all with ages ranging from 19-75 (Table 2.1).

Table 1: Study Population Characteristics

WPI ID	Male/Female	Age	XMRV status
WPI-3-100	Female	56	positive
WPI-3-101	Female	32	positive
WPI-3-102	Female	48	positive
WPI-3-103	Female	62	positive
WPI-3-104	Female	58	positive
WPI-3-105	Female	68	positive
WPI-3-106	Female	62	positive
WPI-3-107	Male	54	positive
WPI-3-108	Female	52	positive
WPI-3-109	Female	73	positive
WPI-3-110	Male	71	positive
WPI-3-111	Male	61	positive
WPI-3-112	Female	68	positive
WPI-3-113	Female	64	positive
WPI-3-114	Male	48	positive
WPI-3-115	Female	56	positive
WPI-3-116	Female	57	positive
WPI-3-117	Female	48	positive
WPI-3-118	Female	61	positive
WPI-3-119	Female	69	positive
WPI-3-120	Female	61	positive
WPI-3-121	Female	56	positive
WPI-3-122	Male	66	positive
WPI-3-123	Male	51	positive
WPI-3-124	Female	53	positive
WPI-3-125	Female	50	positive
WPI-3-126	Male	44	positive
WPI-3-127	Male	37	positive
WPI-3-128	Female	45	positive
WPI-3-129	Male	62	positive
WPI-3-130	Male	59	positive
WPI-3-131	Female	59	positive
WPI-3-132	Male	64	positive
WPI-3-133	Male	31	positive
WPI-3-134	Female	60	positive
WPI-3-135	Male	22	positive
WPI-3-136	Female	59	positive
WPI-3-137	Female	53	positive

Table 1: Study Population Characteristics

WPI ID	Male/Female	Age	XMRV status
WPI-3-138	Female	26	positive
WPI-3-139	Female	52	positive
WPI-3-140	Male	NP	positive
WPI-3-141	Male	28	positive
WPI-3-142	Female	55	positive
WPI-3-143	Male	47	positive
WPI-3-144	Female	54	positive
WPI-3-145	Female	56	positive
WPI-3-146	Male	19	positive
WPI-3-147	Female	28	positive
WPI-3-148	Female	56	positive
WPI-3-149	Female	54	positive
WPI-3-150	Female	38	positive
WPI-3-151	Male	39	positive
WPI-3-153	Female	61	positive
WPI-3-155	Male	52	positive
WPI-3-156	Female	59	positive
WPI-3-157	Male	NP	positive
WPI-3-158	Female	65	positive
WPI-3-159	Male	58	positive
WPI-3-160	Female	18	negative
WPI-3-161	Male	29	negative
WPI-3-162	Female	56	positive
WPI-3-163	Male	NP	positive
WPI-3-164	Female	43	negative
WPI-3-165	Female	24	positive
WPI-3-166	Female	NP	negative
WPI-3-167	Male	43	positive
WPI-3-168	Male	52	positive
WPI-3-169	Female	61	positive
WPI-3-170	Female	66	positive
WPI-3-171	Female	52	positive
WPI-3-172	Male	NP	positive
WPI-3-173	Female	51	negative
WPI-3-174	Female	NP	positive
WPI-3-175	Female	35	negative
WPI-3-176	Male	62	negative
WPI-3-177	Female	47	negative
WPI 3-1282	Male	Deceased	positive

Collection and Storage of Blood plasma:

Ten milliliters of peripheral blood was drawn from subjects using standardized phlebotomy procedures. Handling and processing was similar for all patients. Blood samples were collected into green-capped vacutainers containing the anti-coagulant, sodium heparin (Becton Dickinson).

Cell Counts and Viability:

Media to culture cells consisted of RPMI, Fetal Bovine Serum, and Penicillin/Streptomycin (Invitrogen, Camarillo, CA). Cultured cells were counted every 2-3 days using a Countess^{TM} automated cell counter (Invitrogen) to determine proliferation and viability. Approximately 20 µL of cell suspension were stained with the dye, Trypan Blue, on a counting slide. The Countess was then set to determine live cells from dead cells and optically counted them.

Cell counts were also obtained by flow cytometry using Cell Count Fluorospheres (Beckman Coulter, Fullerton, CA). The method is based upon a known volume and concentration of fluorospheres mixed with an identical volume of cell culture. The flow cytometer counts the fluorospheres along with the cells. The fluorospheres contain a dye that when excited with a 488nm laser, emits between 525nm to 700nm. Using FlowJo software to optically display the counts, the emission of the fluorospheres displays a unique pattern that is then gated on, and the number of events are collected within this gate. Then, using the assayed concentration provided with each vial of fluorospheres, the total number of cells can be determined.

Cell labeling, Flow Cytometry and Analysis:

Appropriate combinations of fluorochrome conjugated anti-human monoclonal antibodies (Table 2.2) were used to label 50 µL of whole blood or from cultured cells using approximately 1 x 10^6 of isolated PBMCs per tube. After a 25-minute incubation at room temperature in the dark, 1.00 mL of FACSLYSE buffer was added to each whole blood tube and incubated for an additional 10 minutes. All samples were then washed with PBS and resuspended in 600 µL of fixative solution (95% PBS plus 5% formalin). In some cases, intracellular cytokine labeling for Perforin and Granzymes was also done in accordance with Intraprep (Beckman Coulter) kit optimized protocol.

Table 2.2: Monoclonal Antibodies Used for Immune Phenotyping

Monoclonal Antibody	Fluorochrome	Clone	Supplier	Product No.
CD2	PE	39C1.5	Beckman Coulter	IM0443U
CD3	FITC	UCHT1	Beckman Coulter	6604623
CD3	PerCP	UCHT1	Biolegend	300428
CD3	PC5	UCHT1	Beckman Coulter	IM26354
CD3	PC5.5	UCHT1	Biolegend	300430
CD3	PC7	UCHT1	Beckman Coulter	6607100
CD4	FITC	OKT4	Biolegend	317408
CD4	APC	OKT4	Biolegend	317416
CD14	ECD	RM052	Beckman Coulter	IM2707U
CD16	PE	3G8	Beckman Coulter	IM1238U
CD16	PC7	3G8	Beckman Coulter	IM2708U
CD19	FITC	J3-119	Beckman Coulter	IM1284U
CD19	PE-Texas Red	J3-119	Beckman Coulter	IM2708U
CD19	PC5	HIB19	Biolegend	302210
CD33	PC5	906	Beckman Coulter	IM2647U
CD45	FITC	J.33	Beckman Coulter	IM0782U
CD45	PC7	J.33	Beckman Coulter	IM35448U
CD56	PE	N901	Beckman Coulter	IM2073
CD56	APC	B159	BD Biosciences	555518
CD56	PC5	N901	Beckman Coulter	IM2654U
CD56	PC7	HCD56	Biolegend	304628
CD69	PC7	FN50	Biolegend	310911
CD83	PC5	HB15a	Beckman Coulter	IM3240U
CD86	FITC	2331 (FUN-1)	BD Biosciences	555657
CD91	FITC	A2MR-α2	BD Biosciences	550496
CD122	PE	CF1	Beckman Coulter	6604931
CD123	PE	55DCL4 107D2	Beckman Coulter	1932535
CD161	PE	191B3	Beckman Coulter	IM3450
CD244	PE	C1.7	Beckman Coulter	IM1608

Flow cytometric acquisition was done using a FC500 flow cytometer (Beckman Coulter, Fullerton, CA) with MXP software. Software compensation and cell population analysis was performed using FlowJo software (Treestar, San Carlos, CA). Eight XMRV-Negative healthy donors (four male and four female) and reference values based on a healthy population (provided by Becton Dickinson) were used as normal baselines. All events were collected through a live gate, and compensated for fluorochrome spillover using FlowJo software compensation. Gating was drawn consistently among all samples.

Determination of XMRV Infection:

All patients tested positive for XMRV infection according to at least two of the methods detailed (PCR, viral antibody, viral protein expression in PBMC or viral transmission to LNCaP cells) in Lombardi et al (94). Briefly, nucleic acids from PBMCs were isolated and assayed for XMRV gag sequences by nested polymerase chain reaction (PCR). Further, western blot methods have been developed and an intracellular cell staining method is being developed that utilize antibodies with novel specificities including SFFV gp 55 Env, MuLV p30 Gag, goat antisera to whole NZB xenotropic MuLV, and purified Rauscher MuLV gp70, p10 and p30. Lymphocytes were activated with PHA and IL-2 and were cultured to generate an infectious XMRV, then co-cultured with the prostate cell line LNCaP resulting in transmission of XMRV to the cell line. Finally, a virus isolation spinning protocol was used with XMRV-Positive patient plasma to infect different cell types in vitro.

Statistical Analysis:

The approximate total number of cells was determined by cell counts and the volume of whole blood or cell culture used. These were consistent between patients and controls. Percentages of lymphocyte populations were derived from FlowJo analysis. The raw percentages were used to prepare tables and graphs using GraphPad Prism (Santa Maria, CA) and/or Microsoft Excel (Bellevue, WA). Statistical analysis was also performed utilizing Prism. The main statistical methods used to determine p values and statistical significance included the unpaired t test with Welch's correction, and in some cases, one or two way analysis of variance with Bonferroni posttests as appropriate. Since the data were near normally distributed and contained unpaired values and no equal variances, the unpaired t test with Welch's correction was used to compared values between patients and controls. In a few cases of skewed, non-normal data, the Mann Whitney t test was used and the variability of the data was so noted. Verification of the fit of the statistical method was also performed using SigmaStat (SigmaStat Software, San Jose, CA). For general comparison of XMRV-Positive to XMRV-NEGATIVE subjects, a mean was established for control using the actual XMRV-NEGATIVE subjects together with averages from the cited literature (3, 18-20, 42, 65). The XMRV-Positive percentages were compared to the control mean, and the percentage of difference was reported, with variations noted.

Chapter 3

RESULTS

3.1: Profile of circulating leukocytes in XMRV-Positive patients.

Critical imbalances of both the number and function of leukocytes occur in the peripheral blood of patients infected in HIV-1 and HTLV-1 (70). Similarly, in CFS patients, significantly fewer $CD3^{+}CD25^{-}$ T cells and significantly more $CD20^{+}CD5^{+}$ B cells, a subset associated with auto-antibodies have been found (117). Significantly fewer $CD56^{+}$ NK cells with reduced activity were also observed in CFS (reviewed in (103)). This has been associated with low levels of intracellular perforin (114). Because of the similarities of immune responses seen with other retroviruses, we hypothesized that XMRV dysregulates the innate and/or adaptive immune response and may play a role in the pathogenesis of CFS.

To address this hypothesis, a phenotypic analysis of major and minor circulating leukocyte subsets was conducted in XMRV-infected (XMRV Positive or XMRV+) CFS patients concurrently with cytokine profiling studies and viral detection studies by PCR. Whole blood was obtained from CFS patients and healthy controls for the phenotypic and cytokine studies. Each sample was tested for XMRV (tested at least two different time points by two or more different assays discussed in Lombardi et al. (2009) and briefly discussed in Methods (94)). All of the CFS patients used tested positive for XMRV, but only 3.9% of

the healthy controls tested positive for XMRV. Fresh whole blood samples were labeled with combinations of monoclonal antibodies and cell count beads were added, and analyzed by flow cytometry. The results from the blood samples from XMRV-Positive CFS patients were compared to the XMRV- (XMRV Negative or XMRV-) healthy control donors and the published reference values of healthy donors.

I first compared the total cellularity between the XMRV Positive patients and the XMRV Negative controls using cell count fluorospheres. Using FlowJo software, the cell count beads were identified on the dot plots, gated and the total cell counts were calculated, resulting in no significant difference in cell counts between the XMRV Positive patients and XMRV Negative patients.

The total CD45+ leukocyte population was, then, identified (Fig. 3.1A). The total percentage of CD45+ leukocytes, comprising the lymphocyte, monocyte and granulocyte populations, in peripheral blood was found to not vary between XMRV Positive patients and XMRV Negative healthy controls.

Because the total cellularity and the CD45+ leukocyte population were unchanged between XMRV Positive patients and XMRV Negative controls, I then looked at the CD45+ lymphocyte population.

XMRV Negative Subject

XMRV Positive Patients

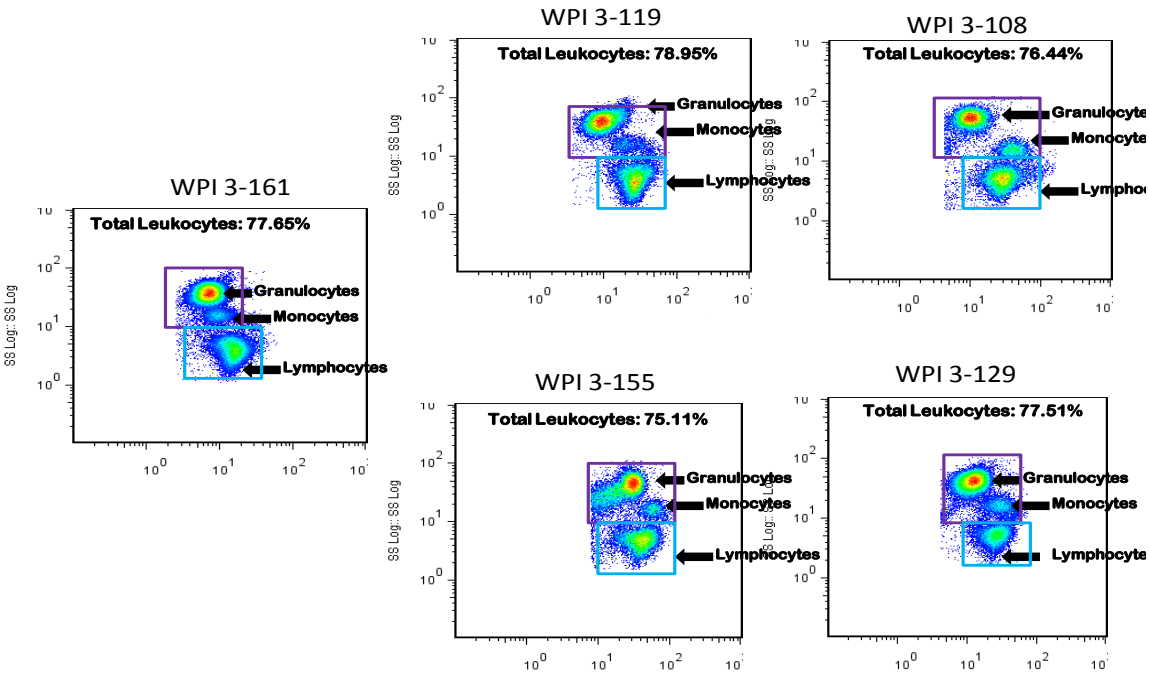


Figure 3.1A: CD45+ Total Leukocytes. Dot plot representation show of flow cytometry analysis of leukocyte populations from whole blood for a representative XMRV Negative healthy control and 4 patients of 63 XMRV Positive CFS patients. The three CD45+ populations are lymphocytes, monocytes and granuloctyes. Leukocytes were gated from CD45+ cells and reported as percentages of total cells. The total percentage of CD45+ leukocytes in peripheral blood and the total cellularity was not different between XMRV Positive CFS patients and XMRV Negative healthy controls.

The granulocyte, monocyte and lymphocyte populations were gated from the CD45+ leukocytes. From the lymphocyte gate, four major cell populations were examined using CD3 and CD56. They were obtained the NK cell population (CD3- CD56+), the NKT cell population (CD3+ CD56+), the T cell population (CD3+ CD56-), and a group containing the B cells (CD3- CD56-) **(**Fig 3.1B**).**

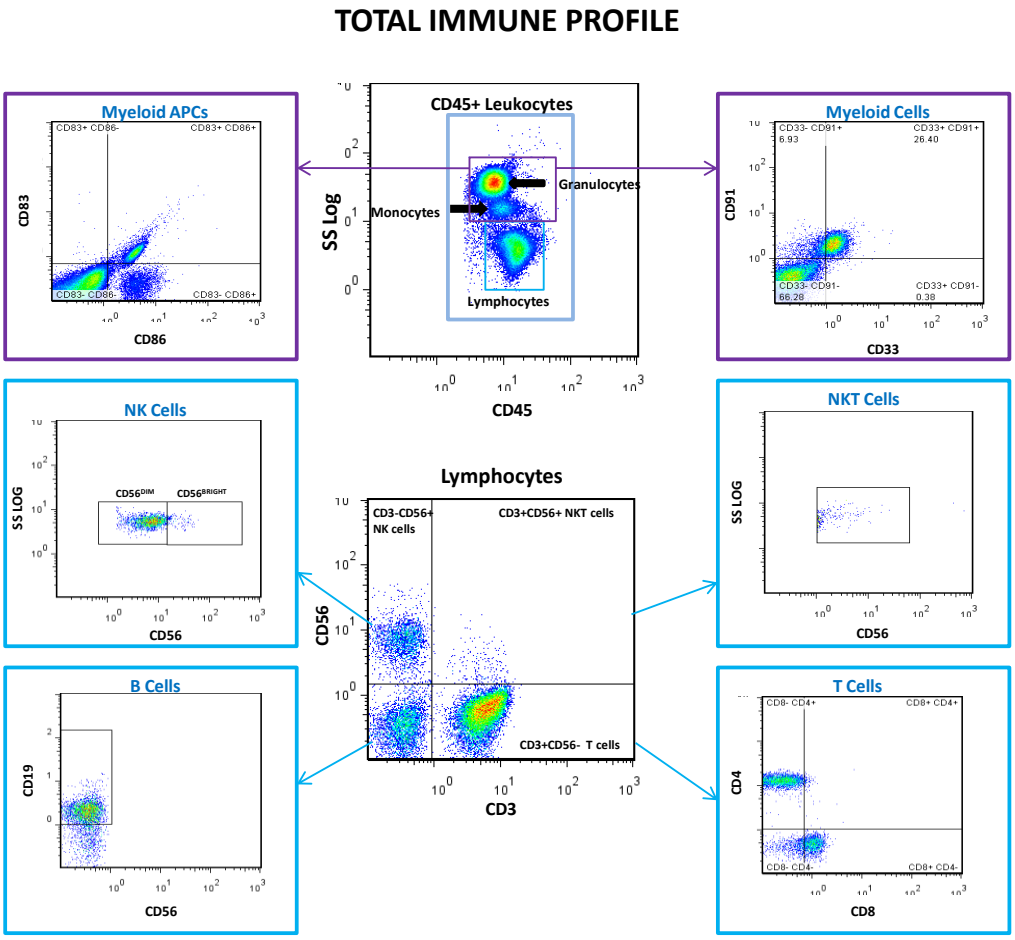


Figure 3.1B: Total Immune Profile. Multi-parameter flow cytometry was used to generate this schematic of a representative of the 63 XMRV Positive CFS patients. Whole blood was labeled with monoclonal antibodies and analyzed by flow cytometry. CD45+ leukocytes were first separated, then either the lymphocyte population or the granulocyte/monocyte groups were gated out. From the lymphocyte gate the four major cell populations were examined using CD3 and CD56: the NK cell population (CD3- CD56+), the NKT cell population (CD3+ CD56+), the T cell population (CD3+ CD56-), and a group containing the B cells (CD3- CD56-). Dendritic cells were derived from the CD45+ leukocyte population. Myeloid cells were gated out from the granulocyte/monocyte groups. While the cellularity of the individual populations varies, the overall cellularity for the XMRV Positive patients and XMRV Negative controls does not vary.

3.2: XMRV Positive patients have a lower percentage of lymphocytes.

From the CD45+ leukocytes, the total lymphocyte population was gated and the percentage of lymphocytes was obtained for each subject. The percentage of lymphocytes from the peripheral blood of XMRV Positive patients was compared with the mean of the XMRV Negative subjects. All but three of the XMRV Positive patients showed a lower percentage of CD45+ lymphocytes than the mean of the XMRV Negative controls, and 69% of XMRV Positive patients showed a three-fold decrease in CD45+ lymphocytes as compared to XMRV Negative controls. Thus, the overall mean percentage of the CD45+ lymphocyte population for XMRV Positive patients was significantly lower than the XMRV Negative healthy controls (Fig.3.2A, p value = 0.009123).

T and NK numbers and phenotypes are altered in patients infected by retroviruses and in CFS patients. Because the percentage of CD45+ lymphocytes are reduced in XMRV Positive patients, I then asked if there are differences in CD3 expression.

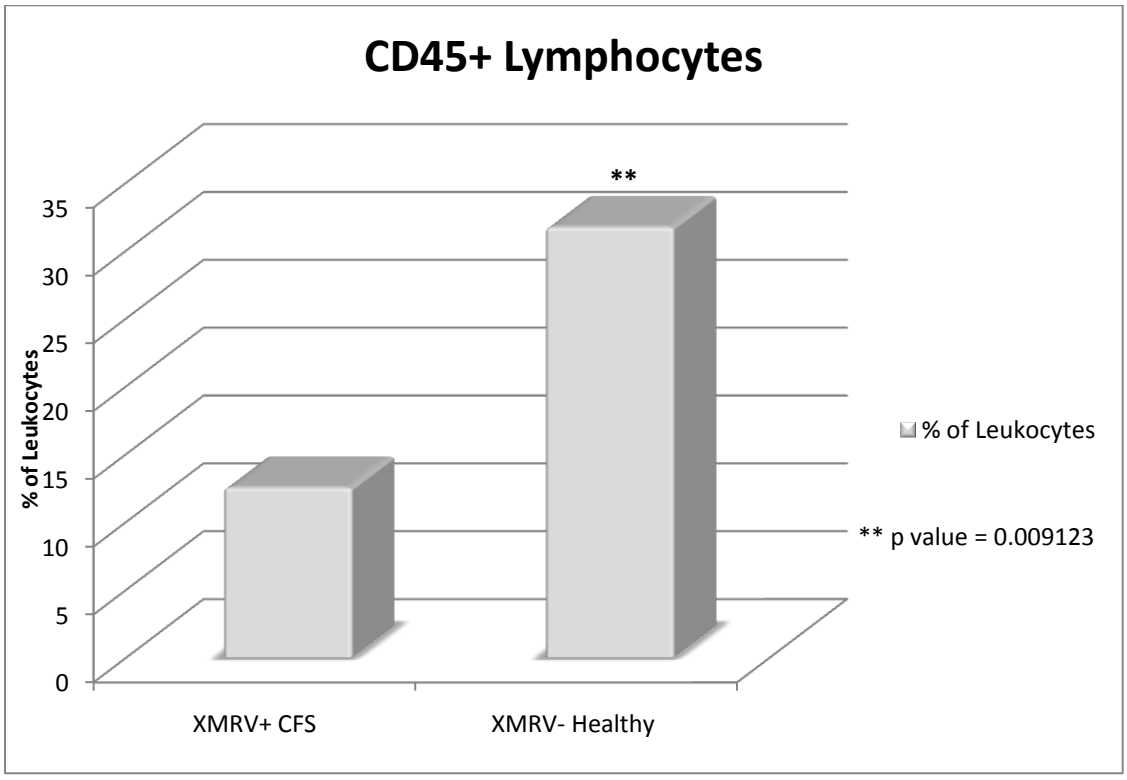


Figure 3.2A: CD45+ Lymphocytes are reduced in XMRV Positive patients. The percentages of CD45+ lymphocytes from total CD45+ leukocytes from whole blood samples of XMRV+ patients (n=63) and XMRV- controls (n=8) were compared. Statistical analysis using unpaired t test with Welch's correction yielded a p value of the significance of the the difference as 0.009123.

To address which population or populations of lymphocytes were reduced, the lymphocyte population was then separated by expression of CD3. The T cell co-receptor, CD3, is expressed on T and NKT cells, but not on NK and B cells. The CD45+ CD3+ lymphocyte population was largely unchanged, but the CD45+ CD3- lymphocytes comprised primarily of NK and B cells in peripheral blood were also significantly reduced in XMRV Positive patients **(**Fig.3.2B, p value = 0.0029).

To verify this finding, I then looked at the expression of CD3 on cells gated for CD56 expression. Using CD3- CD56+ and CD3- CD56- cells, the CD45+ CD3- population was significantly reduced in the XMRV Positive subjects. Using these populations, 85% of XMRV Positive patients have fewer CD45+ CD3- lymphocytes than XMRV Negative controls (Fig. 3.2C, p value = 0.0045).

Because of the significant decrease in CD3- lymphocytes, I next asked if this decrease was in NK cells or B cells or both. In addition, while there did not seem to be a difference in CD3+ lymphocytes, the literature has suggested that T cell numbers are reduced in retroviral infections. To investigate this further, I looked at CD3 expression in conjunction with CD56 expression.

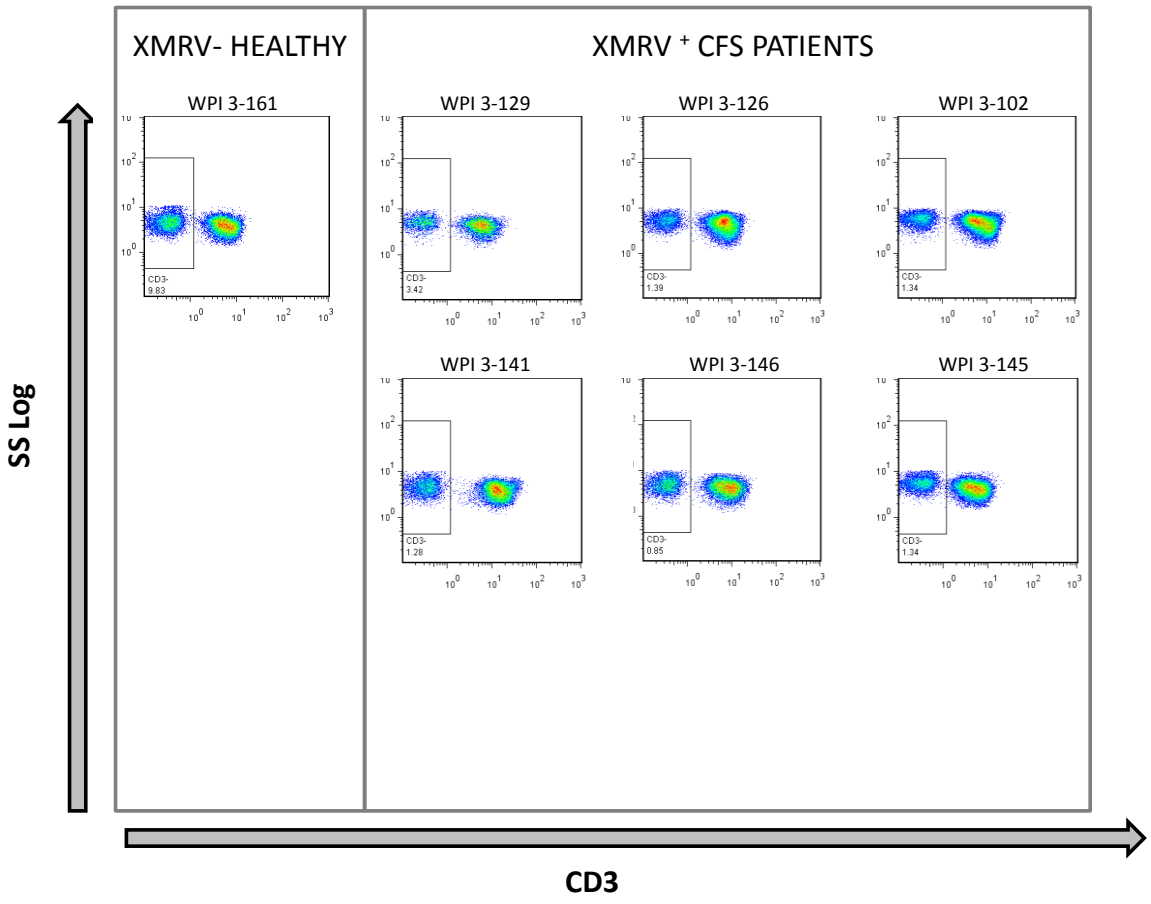
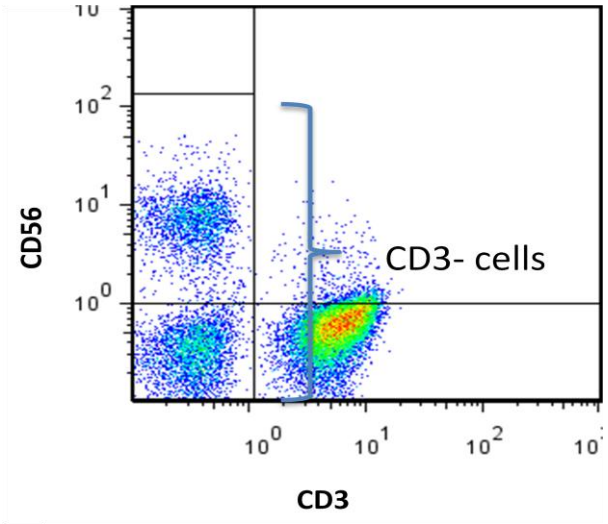


Figure 3.2B: CD45+ CD3- lymphocytes are reduced. Dot plot representations of CD3 expression on CD45+ lymphocytes from the peripheral blood of six representative XMRV+ patients are compared with one representative XMRV- healthy control. Statistical analysis using Unpaired t test with Welch's correction yielded a p value representative of the significance of the difference as 0.0029.



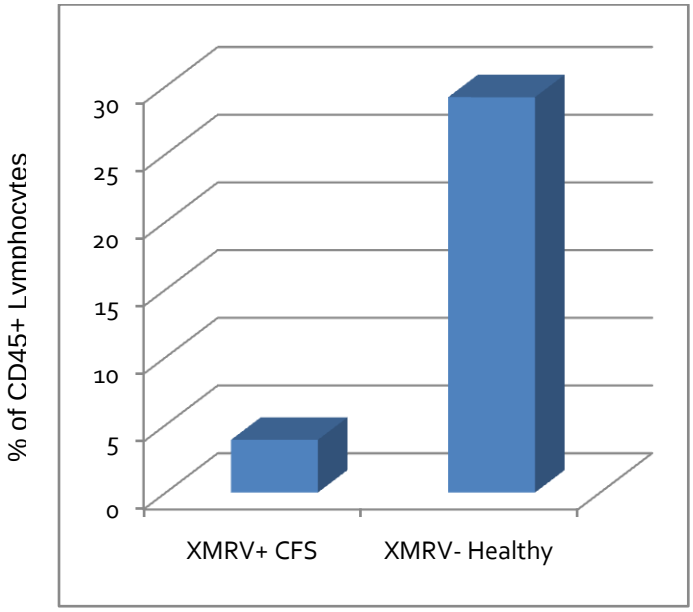


Figure 3.2C: CD45+ CD3- lymphocytes are reduced from CD3- CD56+ and CD3- CD56- lymphocytes. Dot plot representations of CD3 expression on CD45+ lymphocytes gated for CD56 from the peripheral blood of a representative XMRV Negative healthy control (top). All XMRV Positive patients are compared with all XMRV Negative healthy controls. Statistical analysis using unpaired t test with Welch's correction yielded a p value illustrating the significance of the difference as 0.0045 (bottom).

3.3: NKT cells are significantly increased and NK cells, particularly the $CD56^{DIM}$ populations, are significantly reduced in XMRV Positive patients.

CD3 and CD56 expression are used as indicators of the major lymphocyte populations. Four major lymphocyte populations were generally identified by flow cytometry: CD3+ CD56- T lymphocytes, CD3- CD56+ NK cells, a unique population of cytotoxic T lymphocytes known as cytokine-induced killer (CIK) cells or NK-likeT cells (NKT) with a phenotype of CD3+ CD56+, and CD3- CD56- lymphocytes which include B cells **(**Fig 3.3A**).**

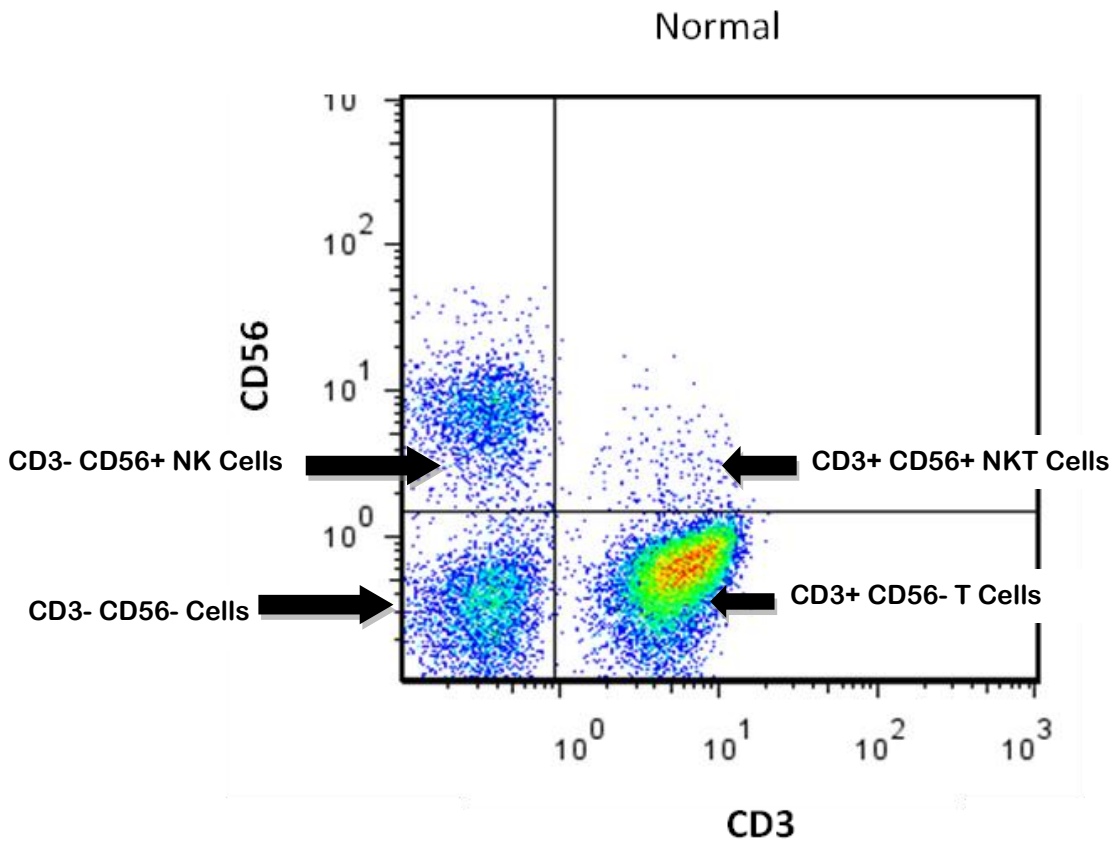


Figure 3.3A: CD3 and CD56 expression on a representative XMRV Negative control. Dot plot representation of CD3 and CD56 expression on CD45+ lymphocytes from the peripheral blood of a representative XMRV- healthy control. Four major populations are loosely identified: CD3- CD56+ NK cells, CD3- CD56- cells which contain B cells, CD3+ CD56- T cells and CD3+ CD56+ cells termed as NKT cells. The CD3 and CD56 expression of this representative control is consistent with controls tested (n=8) and with observations reported in other studies (2,4,18-19).

The percentage of CD3+ CD56+ NKT cells increased in XMRV Positive patients (Figure 3.3B; p value = 0.0041). To further illustrate the difference in CD3+ CD56+ NKT cells, the mean fluorescent intensity was used for CD56 expression on CD3+ cells. A significant increase in MFI was observed in XMRV Positive patients (Figure 3.3C).

Conversely, a significant reduction in CD56 expression in the CD3- CD56+ NK population was observed in the XMRV Positive patients with 93% of XMRV-Positive patients significantly below the mean of the XMRV-Negative controls **(**Fig. 3.3B; p value=0.0054**)**.

I next asked if the reduction in CD56 expression in the NK population from XMRV Positive patients is represented equally in the $CD56^{BRIGHT}$ and $CD56^{DIM}$ subpopulations or unequally, and are there differences in the two subpopulations between XMRV Positive patients and XMRV Negative patients.

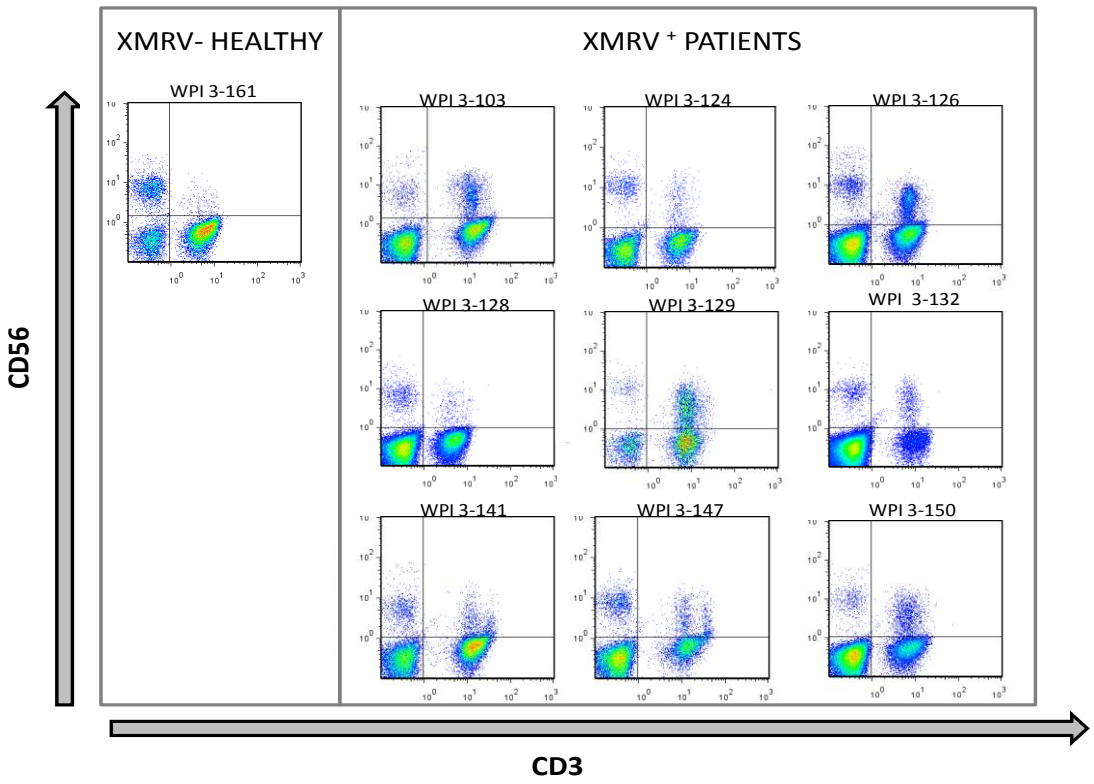


Figure 3.3B: CD3 and CD56 expression on the NK, Non NK/T, T and NKT populations: CD3+ CD56+ NKT cells are significantly increased while CD3- CD56+ NK cells are significantly reduced. Dot plot representations of CD3 and CD56 expression on CD45+ lymphocytes from the peripheral blood of nine representative XMRV+ patients are compared with one representative XMRV- healthy control. Statistical analysis with unpaired t test with Welch's correction yielded a p value evaluating the significance of the difference between XMRV Positive patients and XMRV Negative controls for CD3+ CD56+ NKT cells as 0.0041, and for CD3- CD56+ NK cells as 0.0054.

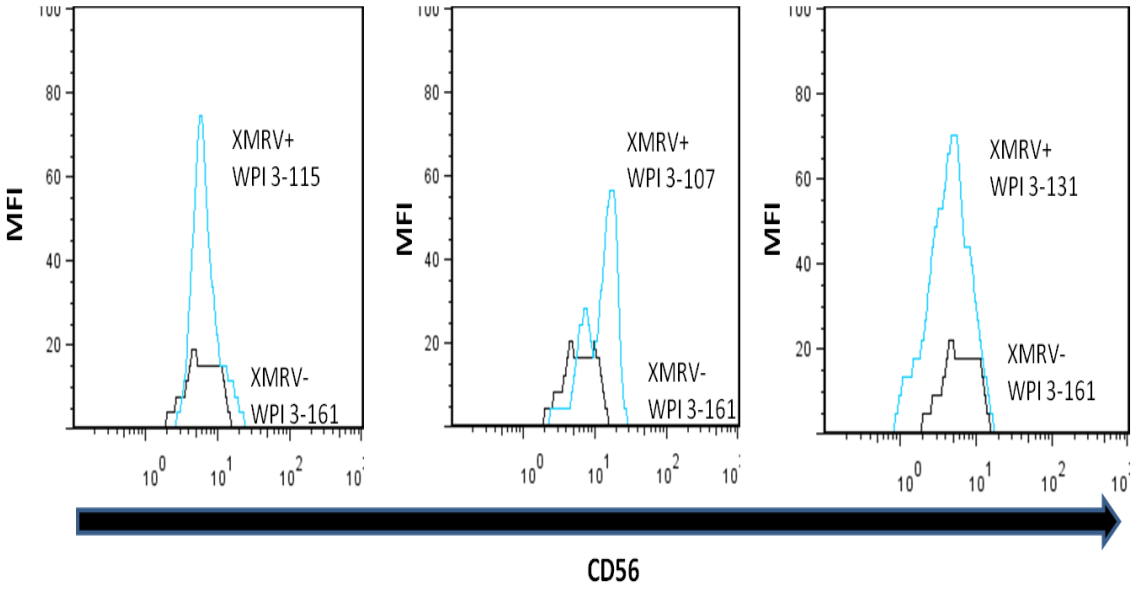


Figure 3.3C: CD3+ CD56+ NKT cells are increased in XMRV Positive Patients: Histograms of the MFI of one representative XMRV Negative healthy control overlayed on histograms of the MFI of three representative XMRV Positive patients. Statistical analysis with unpaired t test with Welch's correction yielded a p value illustrating the difference in the MFI values as 0.0021.

Both subsets, and particularly the $CD56^{DIM}$ subset of NK cells, are reduced in XMRV Positive patients compared to XMRV Negative controls. The intensity of the CD56 expression was examined by gating on the $CD56^{BRIGHT}$ and $CD56^{DIM}$ subpopulations (Fig 3.3D).

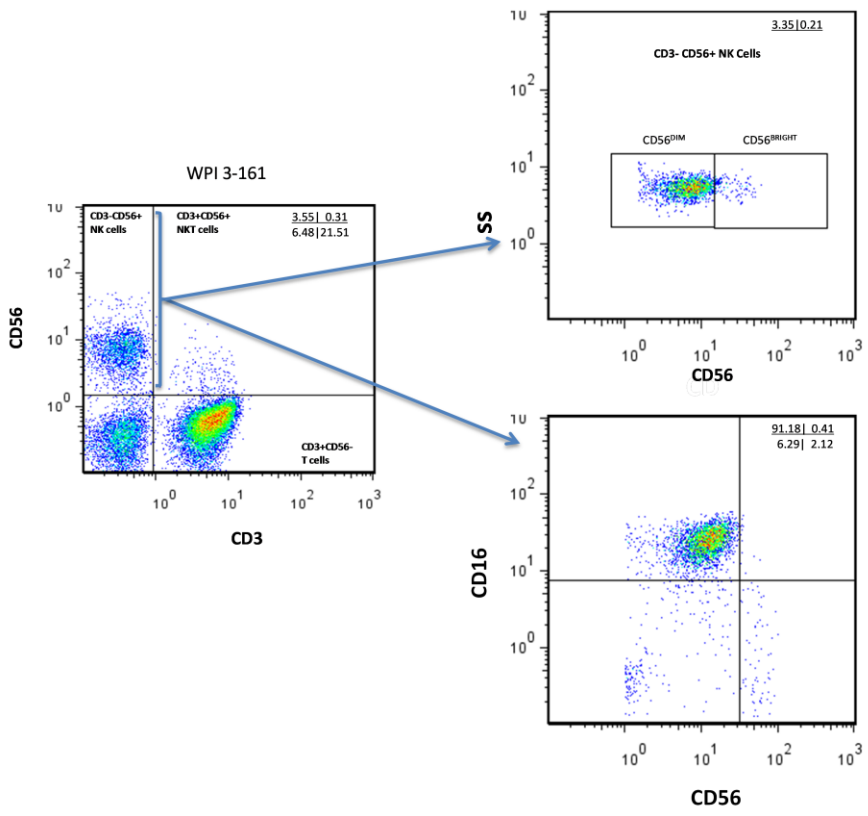


Figure 3.3D: CD56 Expression on NK cells is reduced. Dot plot representation of CD3 and CD56 expression on CD45+ lymphocytes from a representative XMRV- control. The CD3- CD56+ lymphocytes are gated, then CD56 expression is examined in detail (upper right). Then CD16 expression was examined on the CD3- CD56+ NK cell population (lower right).

The XMRV Negative subjects were compared with the XMRV Positive patients by overlaying the CD56+ populations from the XMRV Positive samples on to the CD56+ population of each of the XMRV Negative controls. A plot of one representative XMRV Negative subject overlayed onto several XMRV Positive patient samples is illustrated. (Fig. 3.3E, F) The MFI between the XMRV Negative and XMRV Positive samples remained relatively the same, but the percentage of CD3- lymphocytes expressing CD56 was significantly reduced (Fig 3.3E, F; p value = 0.0025).

CD16 expression is important to the further definition of the NK subpopulations, particularly the $CD56^{BRIGHT}$ CD16- and $CD56^{DIM}$ CD16+ subpopulations. To first explore and verify my findings, I looked at CD16 expression on CD3- cells, then investigated CD56 expression on these cells. Following this, I looked at CD16 expression on the CD3- CD56+ NK cells.

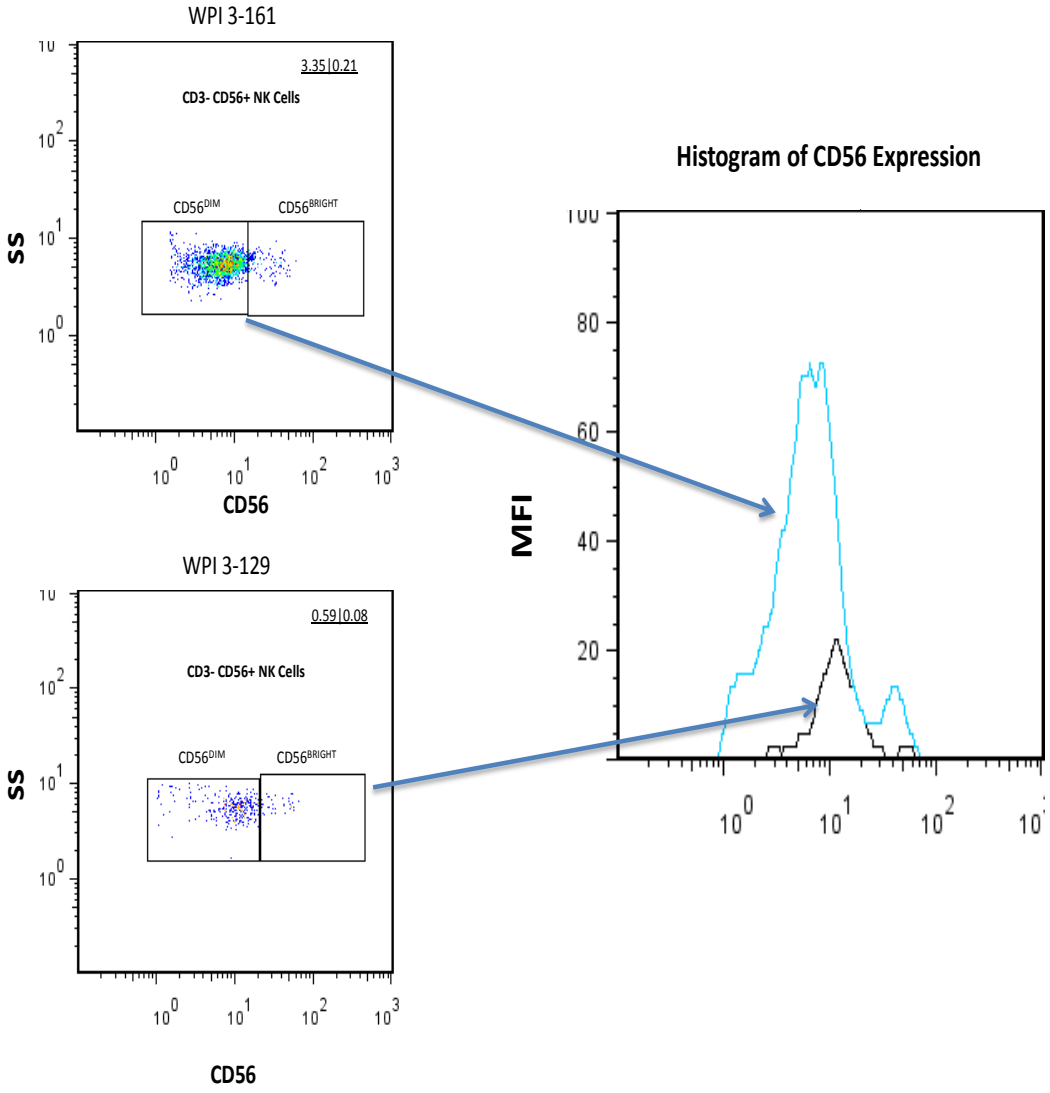


Figure 3.3E: NK cells, particularly the CD56^{DIM} populations, are significantly reduced in XMRV Positive patients. Dot blot representations of flow cytometry analysis are shown. CD56^{BRIGHT} and CD56^{DIM} subpopulations were gated and histograms of CD56+ expression from a XMRV+ patient were compared against a representative XMRV- subject.

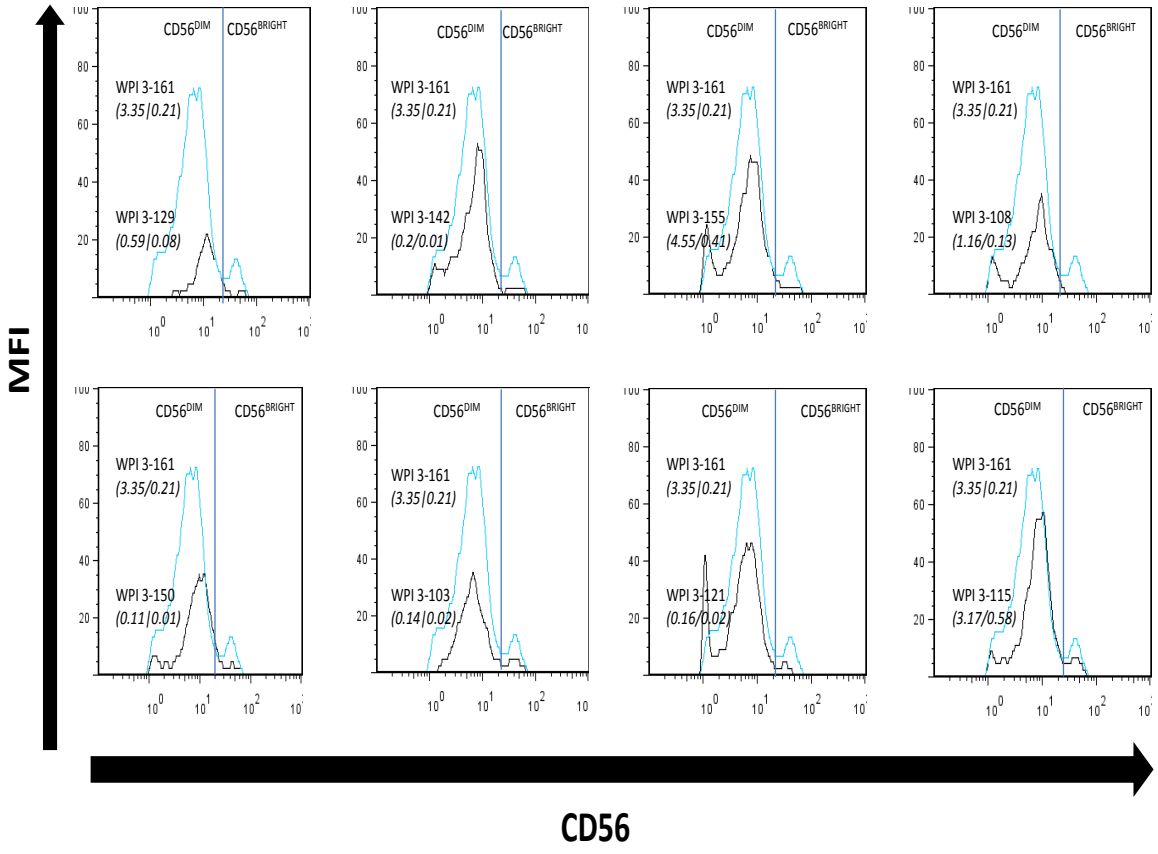


Figure 3.3F: NK cells, particularly the CD56^{DIM} populations, are significantly reduced in XMRV+ patients. Histograms of CD56 expression from of eight representative XMRV+ patients were compared against a representative XMRV- subject. Statistical analysis with unpaired t test with Welch's correction yielded a p value evaluating the significance of the differences as 0.0025.

Similar results were seen when the XMRV Positive patients were compared with other XMRV Negative controls from this study (data not shown). This reduction in the percentage of CD56+ NK cells is represented mainly in the significant reduction of the $CD56^{DIM}$ subpopulation (Fig. 3.3F, p value =0.0025)**.** A similar reduction in the $CD56^{BRIGHT}$ subpopulation is observed, but this reduction may be a reflection of the overall lower percentages of lymphocytes seen in XMRV-Positive patients.

CD16 expression on NK cells is considered an indicator of function through mediation of antibody dependent cellular cytotoxicity (ADCC). Infection of NK cells by retroviruses such as HIV and HTLV have been shown to alter CD3- CD16+ NK cell populations (40). The expression of CD16 on CD3- cells was examined from XMRV Positive patients. Initial observation showed no significant differences in CD16 expression although a greater variability in the expression of CD16 between $CD16^{LOW}$ and $CD16^{HIGH}$ subsets was noted. The significance of these subsets is unknown**.**

CD56 expression was then examined on the CD3- CD16+ population. A histogram was generated for each XMRV Positive patient and XMRV Negative control. The histograms from three representative XMRV Positive patient samples were overlayed by a representative XMRV Negative control sample as shown in Figure 3.3G. A significant reduction in CD56 expression was observed in the XMRV Positive patient samples when compared with the XMRV Negative controls on this CD3- CD16+ subpopulation (p value = 0.0081). This process

was repeated with other XMRV Positive patient samples and XMRV Negative control samples with the same result. These data, together with the reduction in $CD56^{DIM}$ subpopulation, indicate a reduction in the cytotoxic CD3- $CD56^{DIM}$ CD16+ NK subset.

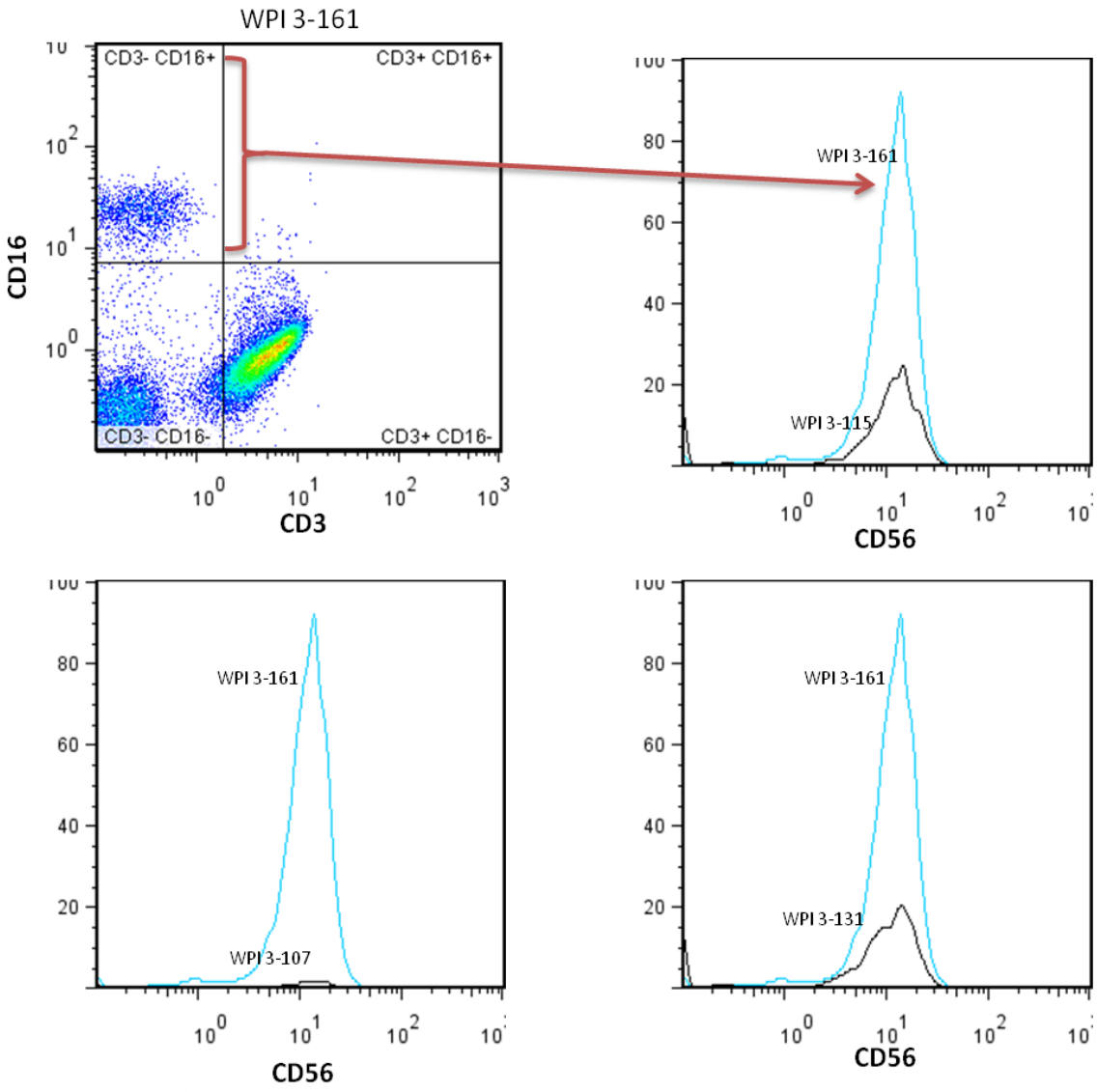


Figure 3.3G: CD16 expression on CD3- cells is reduced in XMRV+ patients. Histograms were generated from dot blot representations of three representative XMRV+ patients were compared against a representative XMRV- healthy subject. Statistical analysis using Unpaired t test with Welch's correction yielded a p value = 0.0081.

The $CD56^{DIM}$ subpopulations in normal individuals typically express high levels of CD16 whereas $CD56^{BRIGHT}$ subpopulations do not express CD16 or if at all, only at low levels (26, 118). In order to confirm CD16 expression on the CD56 subpopulations, the CD3- CD56+ NK cell population was examined. The $CD56^{BRIGHT}$ and $CD56^{DIM}$ subpopulations were first gated, with the subpopulations then gated for CD16 expression. The relationship of CD16 expression to the CD56+ subpopulations was also examined **(**Fig. 3.3H**).**

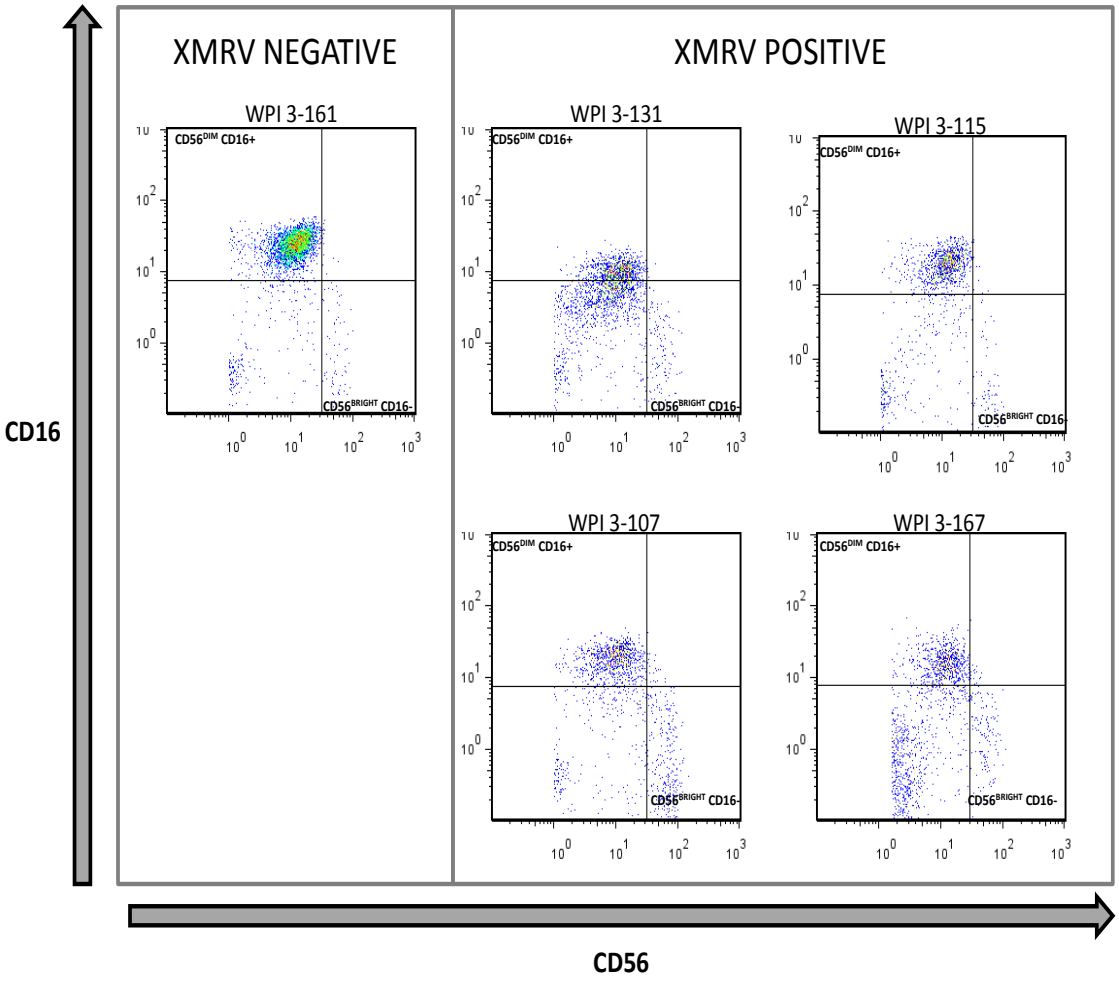


Figure 3.3H: CD16 and CD56 expression on lymphocytes is reduced in XMRV Positive patients. Dot blot representations of flow cytometry analysis of four representative XMRV+ patients were compared against a representative XMRV- subject. Statistical analysis using unpaired t test with Welch's correction yielded a p value evaluating the significance of the difference at 0.033.

A decrease in CD16 expression on CD56+ NK cells from XMRV Positive patients was observed as compared to the XMRV Negative healthy controls, particularly the $CD56^{DIM}$ subpopulation (p value = 0.033). The $CD56^{BRIGHT}$ CD16- was also reduced, although not significantly. Phenotypic expression changes were also observed in other NK cell surface markers. Expression of CD122, the IL-2Rβ/γ receptor, was also decreased, but showed a wider variation than CD56 expression (data not shown). Moreover CD2 and CD161 also showed significant decreases in expression in XMRV Positive patients (Fig. 3.3I). CD2 and CD161 are activation markers on NK cells. Based upon these data and in addition to the previously reported loss in NK numbers, these data show an imbalance in the NK subsets in XMRV Positive patients and explain the decrease NK cytotoxic activity.

These data show a significant increase in NKT cells and a significant decrease in NK cells in XMRV Positive Patients as compared to XMRV Negative controls. With this significant alteration in the lymphocyte population, I asked if there could be a difference in B cells as well.

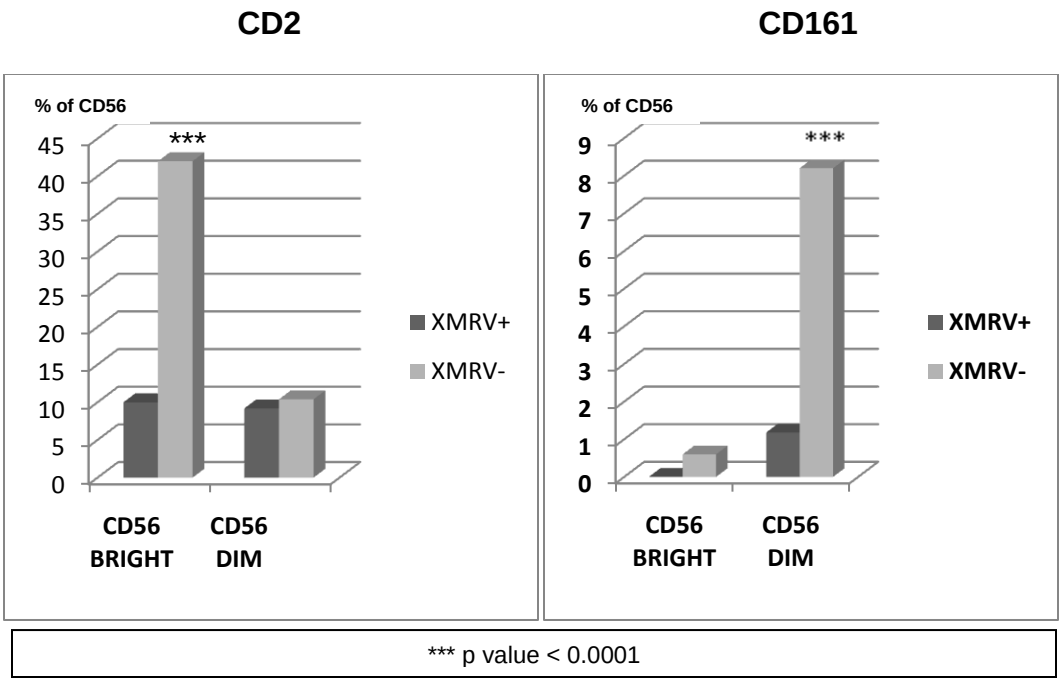


Figure 3.3l CD2 and CD161 expression on NK cells are reduced in XMRV Positive patients. Bar graph representation of CD56+ NK cell subsets expressing CD2 (expressed as percentage of lymphocytes) and bar graph representation of CD56+ NK cell subsets expressing CD161 (expressed as mean fluorescent intensity) from the peripheral blood flow cytometry analysis of 63 XMRV+ subjects as compared to 6 XMRV- healthy subjects. Statistical analysis by one way analysis of variance yielded statistically significant results indicated by ***.

3.4 The percentage of CD19+ B cells is reduced in XMRV Positive patients.

CFS patients suffer from immunoglobulin deficiency which prompted me to investigate the differences in phenotypic expression of the B cell compartment. From the CD3- CD56- cells, the CD19+ B cells were examined. CD19 is a immunoglobulin superfamily member molecule that is present on most precursor B cells and mature B cells except plasma cells and is associated with B cell development. CD19 associates with the antigen receptor to regulate the threshold for antigen dependent receptor stimulation. The CD19+ B cells from representative XMRV Positive patients were compared with those of the representative XMRV Negative controls and a significant reduction in the percentage of CD19+ B cells was observed. (Fig 3.4A, B; p value= 0.043).

The common B cell developmental markers CD5, CD20, CD22 and CD23 were then examined. CD5 is a surface molecule found on IgM secreting immature and mature B cells and T cells. CD5 regulates the stimulating signals generated by the B cell receptor such that only very strong stimuli activates the receptor and not normal tissue proteins (119). The B lymphocyte antigen, CD20, is found on the surface of most B cells and is involved in development and differentiation of mature B cells, but is not typically seen on antibody producing plasma cells (119-120). Moreover, the antileukemic drug, Rituximab (Rituxan^{TM}), targets CD20, reducing the B cell ability to activate and proliferate. Rituxan has been given to CFS patients in a preliminary case series with positive results (121). CD22, like CD5, is found on mature B cells and somewhat on immature B cells and is a regulatory protein that prevents overactivation of the B cell

response. As a immunoglobulin superfamily member, CD22 functions as an inhibitory receptor for the B cell receptor (122). CD23 is the FcεRII low affinity receptor of IgE and is mainly found on mature B cells and is involved in inflammatory responses. Increases in IgE expression have been know to upregulate the cellular expression of CD23 (123). While small numbers of CD20+ CD23+ mature B cells can seen in healthy individuals, significant numbers of CD20+ CD23+ mature B cells are often seen in HIV-infected individuals (63, 65).

In contrast to the reduced numbers of CD19+ B cells, we hypothesized that we would see an increase in highly activated B cells, especially CD20+ CD23+ mature B cells as seen in HIV infected patients. Using the CD19+ B cells from XMRV Positive CFS patients, CD20 expression was much higher in XMRV-Positive subjects. Further the increased CD20 expression was coupled with CD23 expression as evidenced by the increase in intensity in CD23 expression on CD3- CD19+ CD20+ B cells **(**Fig. 3.4C, D; p value=0.038**).** Clearly, activated mature B cells account for a large part of the B cell compartment within XMRV Positive individuals.

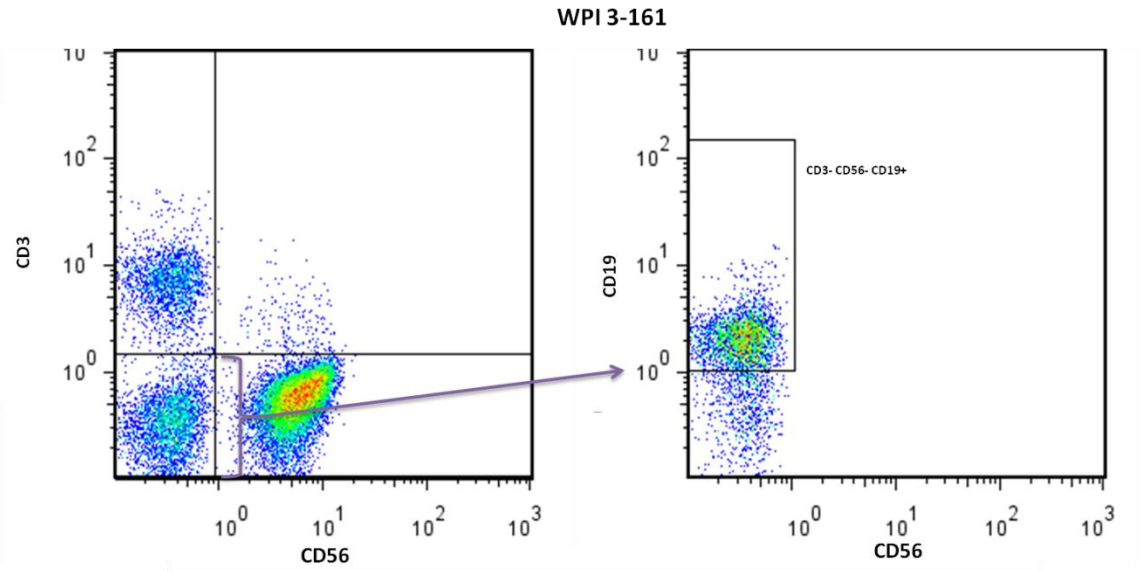


Figure 3.4A: The percentage of CD19+ B cells is reduced in XMRV+ patients. Dot blot representations of flow cytometry analysis are shown. (A) CD3- CD56- gated cell populations were used to determine CD19 expression. CD3- CD56- CD19+ B cells from representative XMRV+ subjects were compared with a representative XMRV- healthy subject.

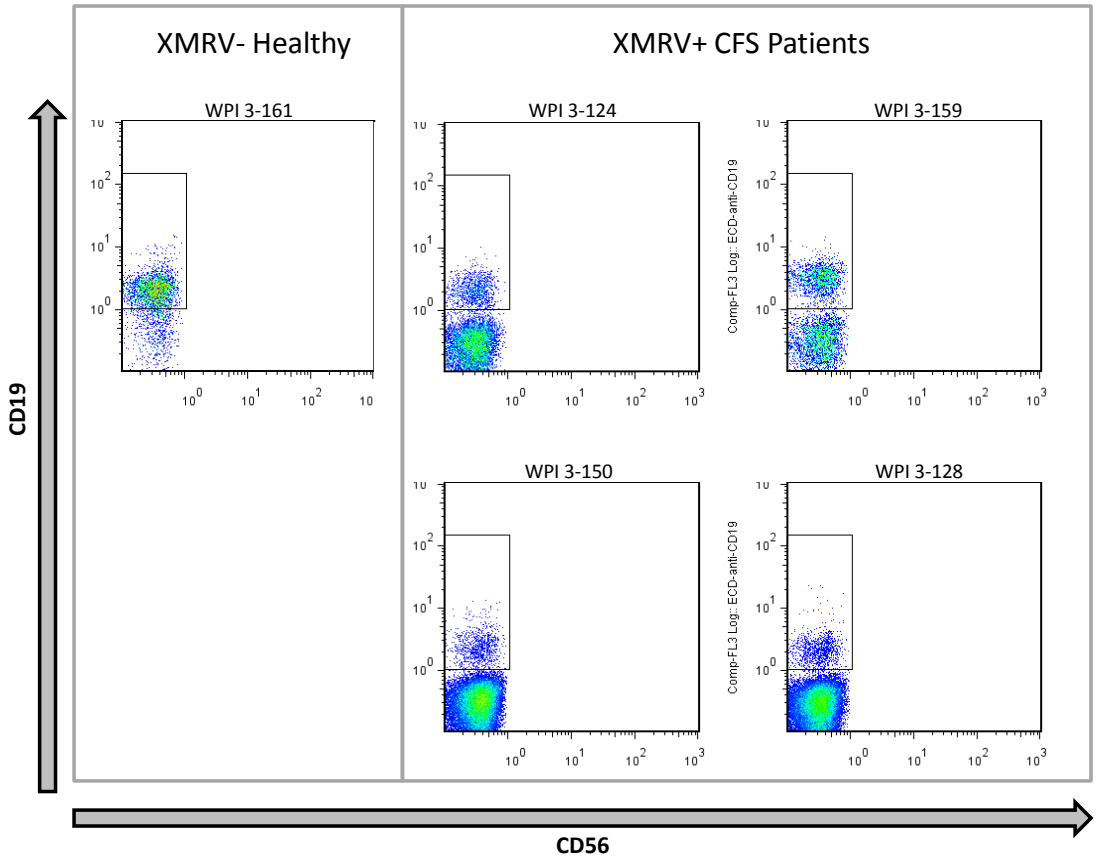


Figure 3.4B: The percentage of CD19+ B cells is reduced in XMRV+ patients. Dot blot representations of flow cytometry analysis are shown. CD3- CD56- CD19+ B cells from representative XMRV+ subjects were compared with a representative XMRV- subject. Statistical analysis using Unpaired t test with Welch's correction yielded a p value=0.043.

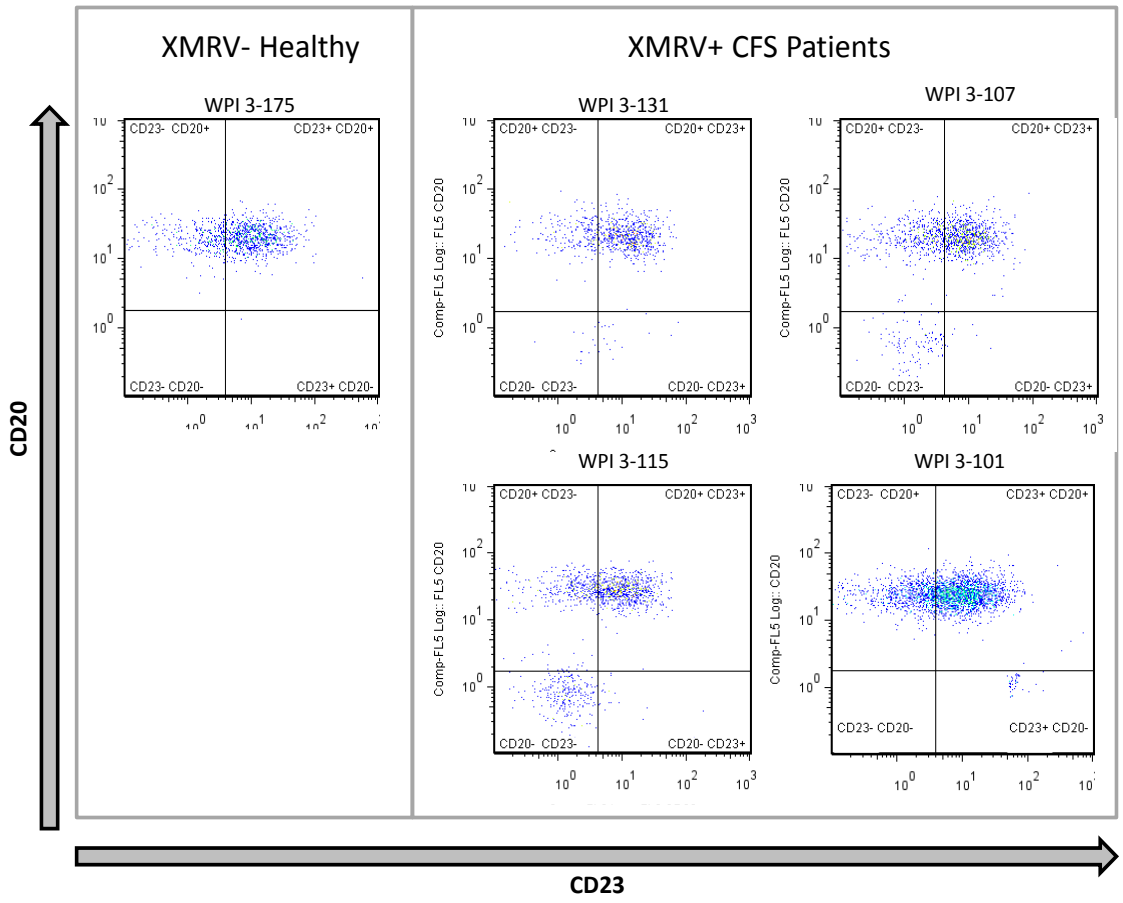


Figure 3.4C: The percentage of CD3- CD56- CD19+ CD20+ CD23+ B cells is increased in XMRV+ patients. Dot blot representations of flow cytometry analysis are shown. From the CD3- CD56- CD19+ B cell populations, CD20 and CD23 expression was determined and compared between the representative XMRV+ patients and a representative XMRV- subject.

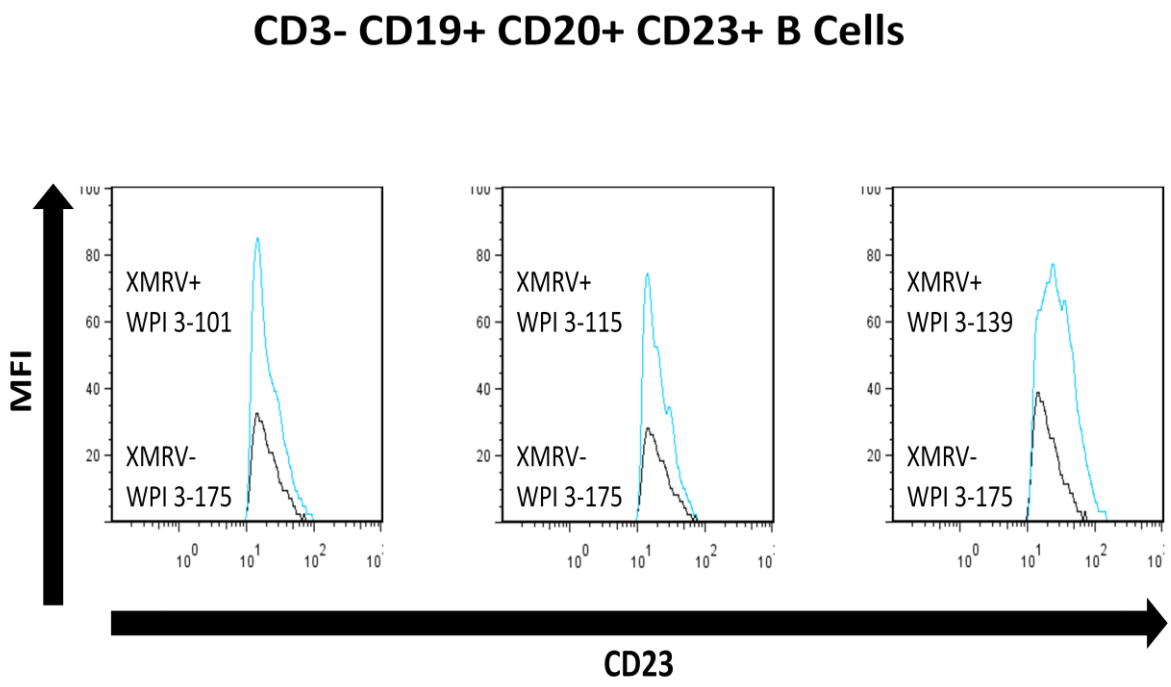


Figure 3.4D: The MFI of CD3- CD19+ CD20+ CD23+ B cells is increased in XMRV+ subjects: Histograms generated from dot plot representations of CD23+ expression on CD3- CD19+ CD20+ B cells from three representative XMRV+ patients (WPI 3-101, WPI 3-115 and WPI 3-139) are overlayed onto a representative XMRV- control (WPI 3-175). Statistical analysis by unpaired t test with Welch's correction yielded a p value indicating significance of the difference as 0.038.

HIV and HTLV are known to produce lymphomas (7). Further, within the CFS population, lymphomas may be more common than the U.S. population (epidemiological study in progress). The WPI typically cultures lymphocytes from patient samples. Concurrent with the phenotypic studies, lymphocytes derived from the whole blood of XMRV-infected patients were cultured in complete media supplemented with IL-4. Three of these cultured samples developed into immortalized cell lines. Using the mantle cell lymphoma cell line, Mino, as a positive control, these cell lines have been extensively assayed for XMRV infection and phenotyped at least three separate time points. Each of the XMRV-Positive cell lines were CD45+ CD3- CD56- CD19+ CD5+ HLA-DR+ B cell lines. All three lines were CD20+, and two were CD23+ (Table 3.4). CD20 expression with CD23 expression indicates the potential for mantle cell lymphoma whereas CD20+ CD23+ expression indicates the potential for chronic lymphocytic leukemia. One cell line, WPI 3-282, comes from a patient who died of mantle cell lymphoma. The phenotype of WPI 3-282 was positive for CD20 but not CD23. Moreover, the cell lines produce infectious XMRV. The phenotype of the cell lines displays strong similarity to the aberrant B cell compartment in XMRV-Positive patients.

The Epstein Barr virus (EBV) has also been known to alter the phenotype of the B cell compartment in peripheral blood (124). EBV is highly infectious and presents as mononucleosis. Many CFS patients have tested positive for EBV. In order to determine if the changes seen in the B cell compartment are a result of

XMRV infection rather than EBV infection, we tested the XMRV-Positive cell lines and found them to be negative for active EBV expression. Moreover, the cell lines are unique as determined by small tandem repeat (STR) DNA analysis (Rachel Bagni , NCI, via personal communication in September 2010). Thus, it appears that the changes seen in the B cell compartment in these XMRV-Positive cell lines as well as the XMRV-Positive patients is the result of XMRV, and not EBV.

At this point, we have seen a substantial change in the lymphocyte populations in the peripheral blood of XMRV Positive patients from XMRV Negative healthy controls. Therefore, if the lymphocyte compartment is reduced as evidenced by the decrease in CD45+ lymphocytes, what differences are there in the myeloid cell compartment in the peripheral blood of XMRV Positive patients and XMRV Negative controls?

Table 3.4: Phenotype of 3 Cell lines derived from XMRV-Positive patients

Marker	Mino	WPI 3-282	WPI 3-134	WPI 3-143
CD5	+	+	+	+
CD23	-	-	+	+
CD19	+	+	+	+
CD20	+	+	+	+
FMC7	+	+	-	-
CD3	-	-	-	-
CD4	-	-	-	-
CD7	+	-	-	-
CD8	-	-	-	-
CD10	-	-	-	-
CD38	+	+	+	+
CD45	+	+	+	+
CD56	-	-	-	-
CD122	-	-	-	-
HLA-DR	+	+	+	+
Lambda	+	+	-	-
Kappa	+	+	+	+

3.5: Expression of activated myeloid derived cells in XMRV Positive patients.

The results of the phenotypic analysis in the XMRV-Positive patients has shown a decrease in lymphocytes as evidenced by the decrease in the NK and B cells but with the same overall cellularity as XMRV-Negative healthy controls. We then asked if the myeloid cell compartment could be replacing the decrease in the lymphoid compartment.

We therefore explored the overall myeloid cell compartment in the XMRV-Positive patient samples. There are many different receptors with which antibodies could be used to further delineate the myriad myeloid populations. Monoclonal antibodies to CD14 and CD123 were first used. CD14, a co-receptor for the recognition of bacterial lipopolysaccharide, is expressed mainly on the monocyte-macrophage lineage. Once activated CD14+ monocytes can differentiate into a number of cell types including macrophages and dendritic cells (125-127). CD123 is the IL-3 receptor and is found in high levels on activated myeloid cells, particularly dendritic cells and granulocytes, but at low levels on monocytes. The granulocyte and monocyte populations were first identified and gated (Fig.3.1A,B)**.** Using the CD14 and CD123 antibodies, a variety of myeloid derived cell types were observed (Fig.3.5A)**.**

CD14 and CD123 expression is shown in Figure 3.5B from a representative XMRV Negative healthy control and three representative XMRV Positive CFS patients. The myeloid populations varied in XMRV Positive

patients. To further differentiate these populations, monoclonal antibodies for CD33 and CD91 were used. CD33 is a receptor expressed predominantly on cells of myeloid origin to characterize them from those of lymphoid origin. CD33 may serve a regulatory role in myeloid maturation, particularly dendritic cell development (128). CD91 is also expressed in peripheral blood on cells of myeloid origin, predominately macrophages. CD91 is a low density lipoprotein (LRP1) and forms a receptor involved in endocytosis. Interestingly, the MFI for CD33 expression on the monocyte/macrophage population, CD14+ CD123- was reduced. The sample size for this analysis were to small for reliable statistical assessment.

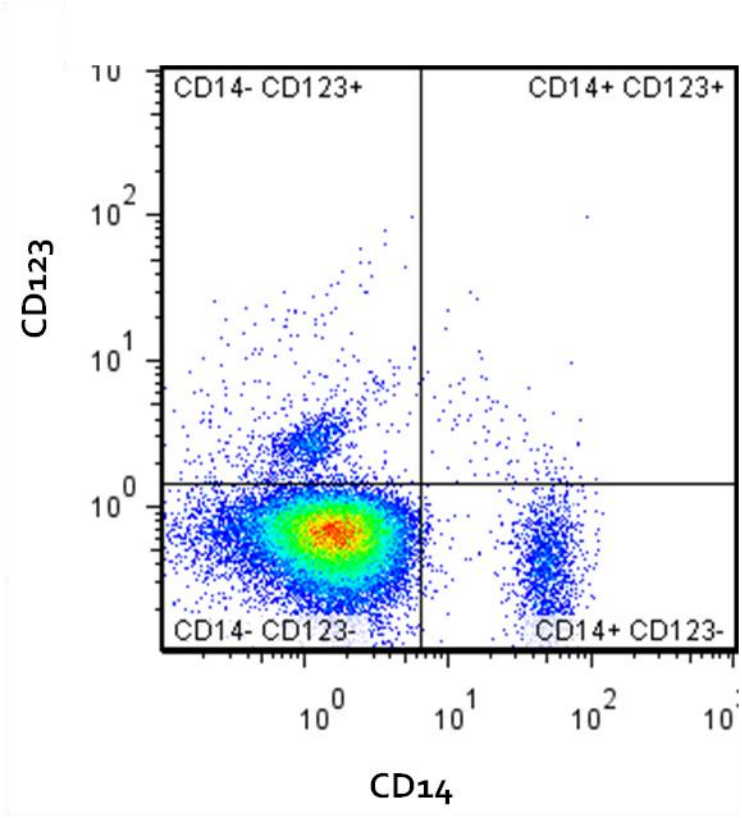


Figure 3.5A: Expression of activated myeloid derived cells in XMRV Negative healthy control. Dot blot representations of flow cytometry analysis are shown. Granulocyte and monocyte populations were gated from CD45+ leukocytes, then CD123 and CD14 expression were used to examine myeloid populations.

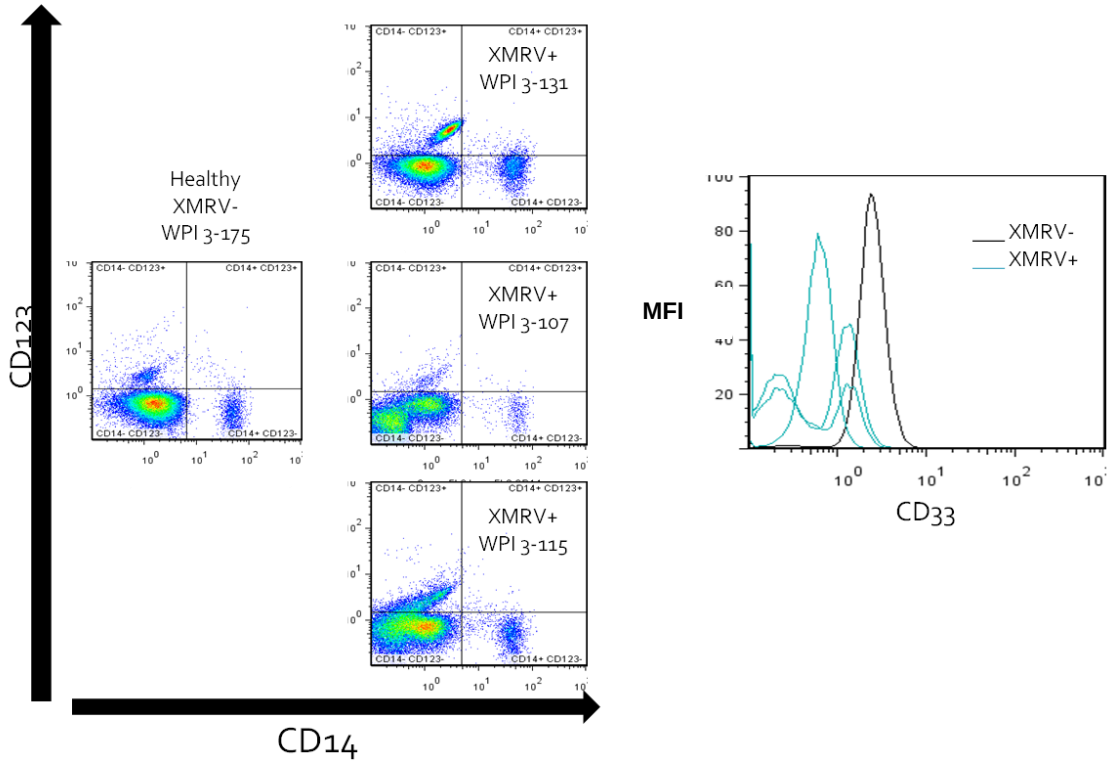


Figure 3.5B: Expression of CD14 and CD123 on myeloid derived cells. Reduced expression of CD33 in XMRV Positive patients: Dot blot representations of flow cytometry analysis are shown. Granulocyte and monocyte populations were gated from CD45+ leukocytes, then CD123 and CD14 expression were used to examine myeloid populations. A representative XMRV Negative healthy control is shown (Left). Three representative XMRV Positive patients are shown (Middle). Histograms of MFI were taken from XMRV Negative control samples and overlayed onto XMRV Positive patient samples. Histograms for XMRV Positive and XMRV Negative subjects is shown (Right). XMRV Positive patients show a reduced expression of CD33 (No statistical assessment).

3.6: Expression of CD69 on CD83+ CD86+ cells in XMRV Positive patients may be associated with an increase in antigen presenting cells.

Because the focus of this study has been on inflammatory responses in XMRV-Positive patients, the activation markers CD83, CD86 and CD69 on CD14 negative myeloid cells were used. Within the myeloid compartment, progenitors can develop into myeloid dendritic cells (129). CD83 is a glycoprotein strongly associated with mature dendritic cells and may play a role in inducing immune responses (129). CD86, or B7.2, is a receptor that acts as co-receptor in T-cell activation and is strongly upregulated in the antigen presenting cell (APC) maturation process. CD69 is another activation marker associated with early activation of leukocytes, particularly APCs. Therefore, the association of CD83 expression with CD86 in the myeloid cells from XMRV-Positive patients was explored. Interestingly, a population of CD83+ CD86+ cells in the representative XMRV-Positive samples was observed. This subpopulation was then isolated. Surprisingly, greater than 94% of the cells were CD69+ as well (Fig 3.6A). This could suggest that an activated APC compartment is present in the peripheral blood of XMRV-Positive patients.

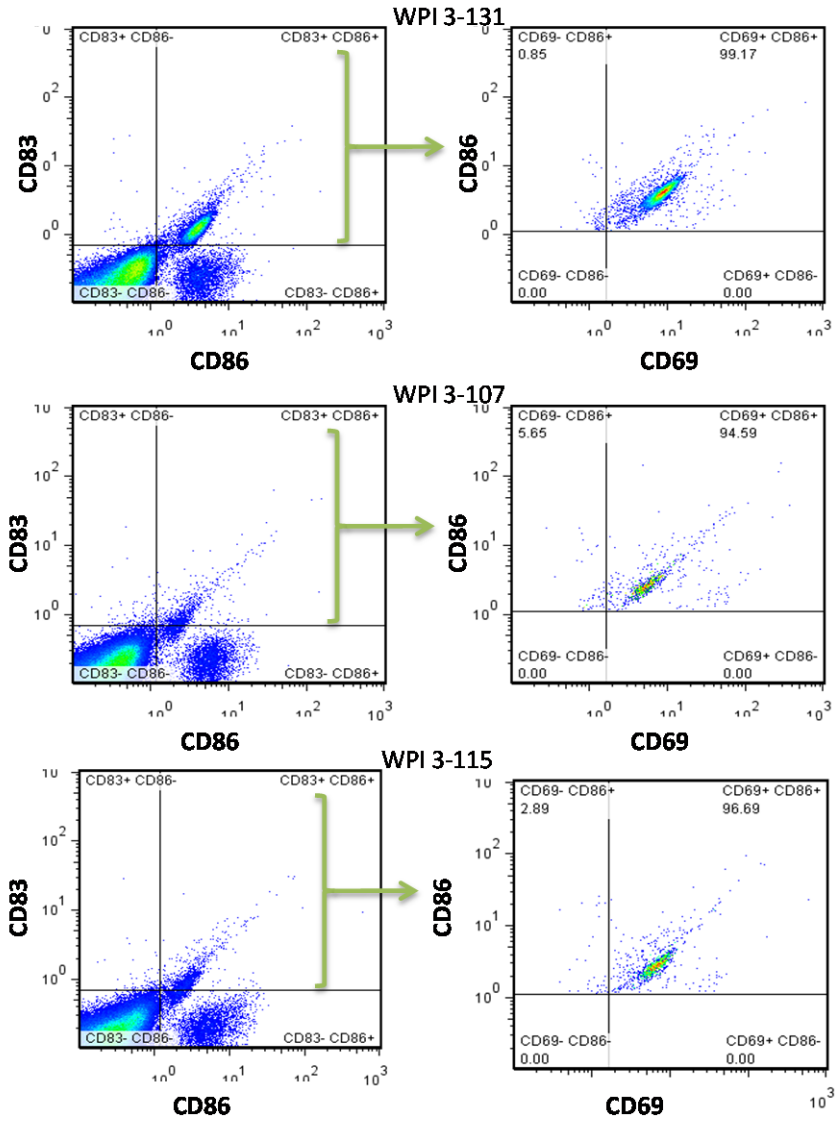


Figure 3.6A: Expression of CD69 on CD83+ CD86+ cells in XMRV Positive patients may be associated with an increase in antigen presenting cells. Dot blot representations of flow cytometry analysis are shown. CD83 and CD86 expression is shown on leukocytes. From the CD83+ CD86+ group, CD69 expression was determined. Representative XMRV+ patients are shown.

3.7 Summary of Results:

In the peripheral blood of XMRV Positive patients, we have observed decreases in several lymphocyte populations with increases in the NKT population, activated B lymphocytes, myeloid derived antigen presenting cells as well as significant dysregulation in inflammatory cytokines and chemokines from the concurrent cytokine profiling study conducted by Dr. Vincent Lombardi (data not shown). These data plus previous data showing a decrease in NK cell functional subsets suggest that XMRV Positive CFS patients have a profound dysregulation in the immune response.

Are there differences in total cell count in peripheral blood between XMRV-Positive patients and XMRV-NEGATIVE controls?

Total Cellularity using Cell Count Beads

No difference in total cellularity

Are differences in the number of leukocytes?

CD45+ Leukocytes

No difference in CD45+ Leukocytes

Are there differences in cell numbers between the myeloid cell compartment (granulocytes and monocytes) and the lymphoid cell compartment?

CD45+ Lymphocytes

(Myeloid cell numbers= Total cellularity – CD45+ Lymphocytes)

CD45+ Lymphocytes are reduced

Since NK cell function is reduced in CFS and T cells are abnormal in HIV, are there abnormalities in CD3 expression?

CD3- Cells

CD3- Cells are

Are there differences in CD16 expression on CD3- cells?

CD16 expression on CD3- cells

CD16 expression is reduced

Are there differences in CD56 expression on CD3- CD16+ cells?

CD56 expression on CD3- CD16+/- cells

CD56 expression is reduced

CD3+ Cells

If CD45+ Lymphocytes are reduced, are there differences in myeloid cells?

CD123, CD14, CD91 and CD33 Expression for Macrophages, Monocytes and Dendritic Cells

CD123- CD14+ CD33+ cells are reduced; Myeloid cell expression is dysregulated

CD83, 86 and 69 Expression for activated APCs

CD69 expression on CD83+CD86+ cells

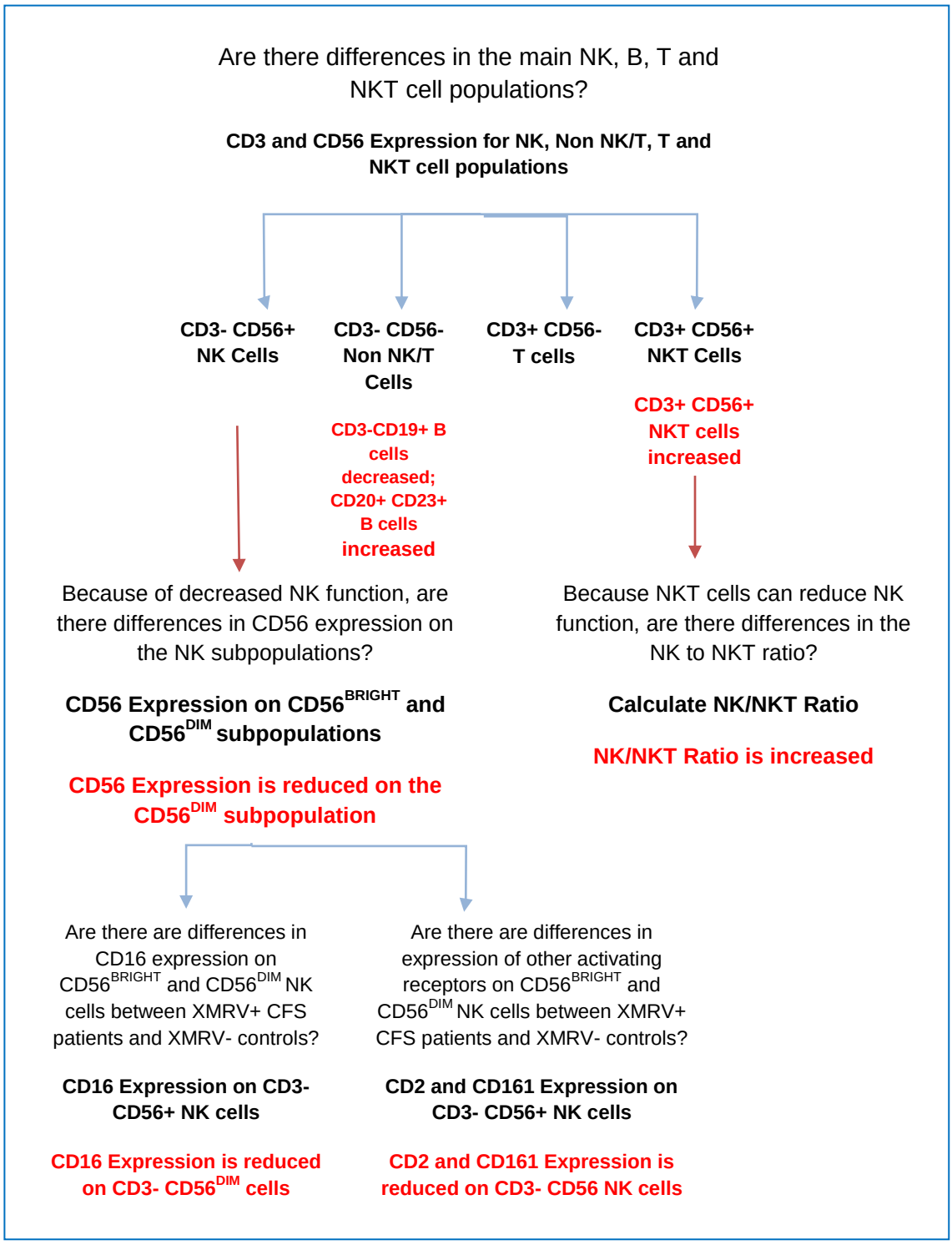


Figure 3.7A: Pathway and leading questions for Phenotyping of Peripheral blood in XMRV POSITIVE CFS patients and XMRV NEGATIVE healthy controls with Results: Results are shown in Red.

	XMRV+ vs. XMRV-
Total Cellularity	No Difference
CD_{45}+ Leukocytes	No Difference
CD_{45}+ Lymphocytes	Reduced in XMRV+
CD_{45}+ CD_3- Lymphocytes	Reduced in XMRV+
CD_3+ CD_{56}+ NKT cells	Increased in XMRV+
CD_3- CD_{56}+ NK cells	Reduced in XMRV+
CD_{56}^{DIM} NK subpopulation	Reduced in XMRV+
CD_{56}^{DIM} CD_{16}+ NK subpopulation	Reduced in XMRV+
CD_{19}+ B cells	Reduced in XMRV+
CD_{19}+ CD_{20}+ CD_{23}+ B subpopulation	Increased in XMRV+
CD_{33}+ CD_{14}+ CD_{123}- myeloid Population	Reduced in XMRV+
Activated APCs	Increased in XMRV+

Figure 3.7B: Summary of Results of Phenotyping of Peripheral blood in XMRV POSITIVE CFS patients and XMRV NEGATIVE healthy controls with Results

CHAPTER 4

DISCUSSION OF RESULTS, FUTURE DIRECTIONS, AND CONCLUSIONS

4.1 Discussion of Results

Over the past two decades, numerous immunological abnormalities have been described in CFS (130). These abnormalities include but are not limited to: an alteration in cytokine profile, a decreased function of NK cells, a presence of auto-antibodies, reduced antigenic responses of T cells, abnormal activation of T lymphocyte subsets, a decrease in CD16 that could lead to a decrease in antibody-dependent cell-mediated cytotoxicity, and increased numbers of CD8+ cytotoxic T lymphocytes and CD38 and HLA-DR activation markers (103, 115, 117, 131-132). In addition to decreased NK function, a significant expansion of $CD26^+$ activated T cells in CFS subjects have been reported (103). This multifunctional molecule plays a major role in the regulation, development, maturation and migration of Th1 and NK cells as well as in B cell immunoglobulin switching (133). Moreover, abnormal expression of $CD26^+$ is found in autoimmune diseases (134). Clinical features of CFS include immunological, neurological and autoimmune abnormalities, all of which are known to be associated with HTLV and HIV infection and are consistent with a chronic untreated retrovirus infection (135-136). Prior to the detection of evidence of a new human retrovirus, XMRV, in more than two-thirds of 101 CFS patients studied by Lombardi et al., and the very recent study by Lo et al., retroviruses had not been considered a factor in CFS pathophysiology (137).

In this study, multi-parameter flow cytometry was used in profiling immune cells in 63 XMRV Positive patients respectively, to address the hypothesis that XMRV infection could cause chronic inflammation and immune deficiency. Chronic inflammatory and immune deficiency conditions could result in an inability to mount an effective immune response to opportunistic infections and the immunologic abnormalities similar to those seen in CFS.

This study demonstrates that PBMCs phenotyped from XMRV Positive CFS patients, while possessing the same level of cellularity as normal controls, show a dysregulation of the immune response characterized by the increase in NKT cells and an increased NK to NKT ratio, expansion of peripheral CD20+ CD23+ mature B cells, activation of myeloid derived APCs marked by mature CD83+ CD86+ CD69+ APCs, and a decrease in CD33 expression on CD14+ CD123- monocytoid cells. This study also demonstrated a concomitant decrease of CD3-CD19-CD56+ NK cells and CD19+ B cells. Interestingly, we also saw similar changes in phenotype in XMRV Positive infected controls (n=4, Fig 4.2A).

NKT cells have a varied effect exerting an immunostimulatory or, in other cases, an immunosuppressive effect and may cause a dysregulation of the innate immune system (52-53, 138). The results presented here suggest that XMRV exerts a direct or indirect effect on the NKT cells from the peripheral blood of XMRV-infected CFS patients which may suppress NK cells, particularly $CD56^{DIM}$ CD16+ NK cells. Moreover, the dysregulation of the myeloid cell compartment may also be an effect of the increase in NKT cells.

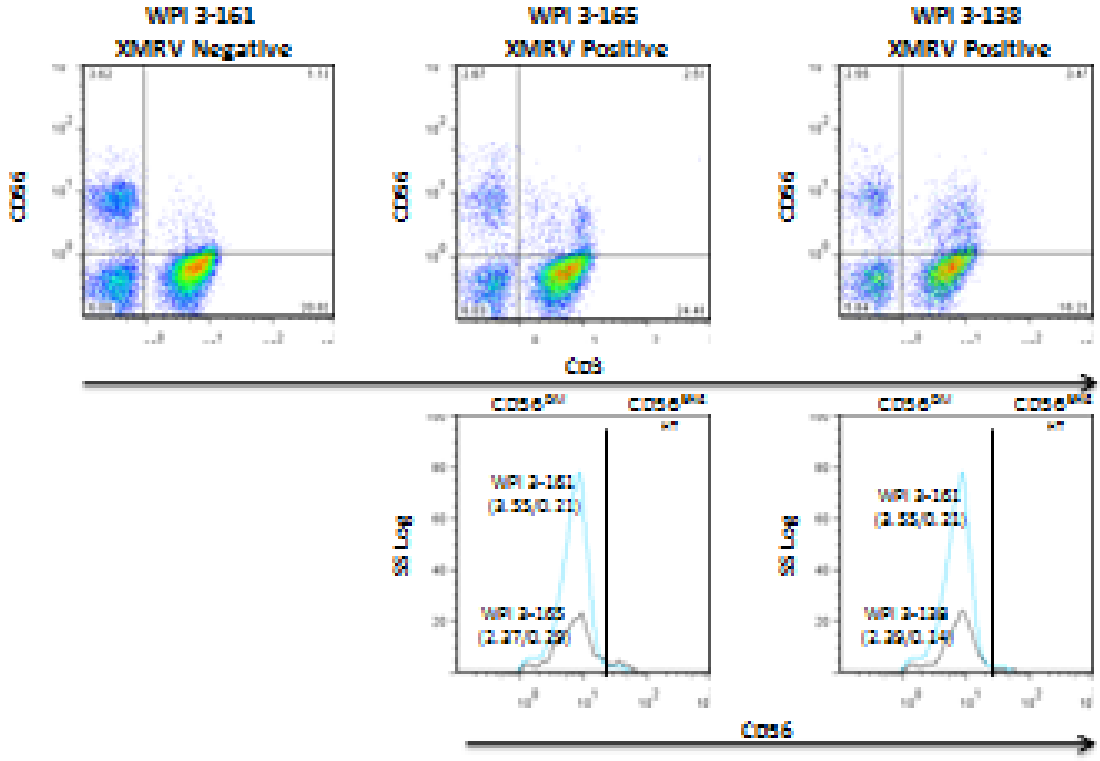


Figure 4.2a: CD56 Expression comparison between XMRV Negative Control and XMRV Positive Controls: Dot plot representations of CD3 and CD56 expression on CD45+ lymphocytes from the peripheral blood of nine representative XMRV+ CFS patients are compared with one representative XMRV- healthy control.

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Our observations of activation of APCs and the presence of B cell CD20+ CD23+ subpopulations in XMRV Positive individuals, which are not normally present to any substantial degree in the peripheral blood of XMRV Negative individuals, is similar to many of the myeloid and B cell defects described in HIV associated disease including increased expression of activation markers CD80 and CD86. The B cell defects described in HIV, for example, include increased production of auto-antibodies and an increase in the frequency of B cell malignancies. These can result from an expansion or contraction of one or several of these B cell subpopulations (65). APCs play a role in the increased pathogenesis of HIV by the uptake and transfer of the antigenic virus from antigen presenting cells to lymphocyte populations, thus causing a functional impairment of T cells in spite of an activated T cell phenotype (129, 139-140). These alterations may cause depletion and loss of function by T cells from the dysregulated pro-apoptotic or suppressive cytokines induced by chronically activated innate immune cells (70). The activation of myeloid and lymphoid APCs, as evidenced by the mature CD20+ CD23+ B cell subpopulation, as seen in XMRV Positive CFS patients, suggest a similar retroviral cycle of chronic innate immune activation leading to an immune dysregulation and chronic immunosuppression.

Dr. Lombardi's cytokine profile observed high levels of pro-inflammatory cytokines and chemokines in XMRV Positive CFS patients. These may explain some of the manifestations such as fatigue and flu-like symptoms; additionally it

may influence NK activity as we have shown herein and as have been previously described (25, 116). Moreover, the most significant defects shown herein are in the NK compartment and in particular the significant decrease in $CD56^{DIM}$ NK cells (Fig.3.3E). In HIV, viral replication can cause chronic NK activation and even outstrip NK proliferation and function causing depletion of NK cell cytotoxicity and increased percentages of an exhausted, dysfunctional CD56-CD16+ NK cell subset (141). HIV and HTLV viruses infect T lymphocytes altering their phenotype and function and can cause reduction in T cell numbers and tumorgenesis. In addition, other cell types including B cells, dendritic cells and NK cells can become infected altering their function and adding to the inflammatory state of the immune system. In HIV, the infected NK cells have lower CD56 expression and can also have decreased activity, including decreased perforin, causing low cytotoxicity. HIV infection of NK cells can also elicit significant or chronic immune activation through cytokine secretion (39, 84, 142). WPI showed that B and T cells can become infected by XMRV ex vivo (94). We hypothesized that XMRV infects both innate and adaptive immune cell types ex vivo, particularly NK cells, and may play a role in aberrant phenotypic expression and production of cytokines and chemokines in a manner similar to what is seen in XMRV-Positive CFS patients. Our initial results found that XMRV does infect isolated NK cells, yet dysregulated phenotype and cytokine production appear to stem from indirect infectivity of PBMCs rather than direct infectivity (data not shown). The NK phenotypic alterations and cytokine production from XMRV-positive patients display dysregulation similar to that seen

in HIV and HTLV and suggest a retrovirally induced innate immune system dysfunction leading to an immune and adaptive immune system suppression.

At this time, little is known about XMRV infection in humans. This study is the first to address changes to immune phenotypes in individuals with chronic XMRV infection. Currently, the level of XMRV replication in infected individuals, the cell types infected, and the resultant pathogenesis of this infection are poorly understood. Considerable knowledge generated during decades of basic research studying both human and non-human retroviruses, the closely related X-MLVs and other members of their genus (gammaretroviruses), has provided important clues about XMRV biology. MLVs can replicate to a high level in vivo, establishing high viral burdens, causing immune disorders through direct infection and proliferative disorders through site selective DNA genome integration.

Both human and animal retroviruses can infect the central nervous system (CNS). These are associated with neurological disease directly through infection of immune cells which traffic to the brain and indirectly through increases in proinflammatory cytokines and chemokines. In the absence of detectable brain inflammation, these indirect effects are known as bystander effects and cause chronic retroviral replication of immune cells (143). Similarly HTLV-1 is the etiologic agent of a neurological syndrome known as HTLV-associated-myelopathy (HAM); a progressive myelopathy characterized by inflammation, principally in the thoracic region of the spinal cord. Clinical characteristics of

HAM share features of patients with relapsing and remitting multiple sclerosis (MS) and those with CFS (144). While the mechanism of pathogenesis of HAM is not known, virus-host immunological interactions have been suggested to play a role in the pathogenesis of this disorder (145).

Infection with HIV results in dysregulation of the cytokine profile in vivo and in vitro. During the course of HIV-1 infection, secretion of Th1 cytokines such as IL-2 and antiviral IFNγ is generally decreased, whereas production of Th2 cytokines, IL-4, IL-10, pro-inflammatory cytokines IL-1, IL-6, IL-8 and TNFα, is increased. Such abnormal cytokine production contributes to the pathogenesis of the disease by impairing cell-mediated immunity. Similar activation of proinflammatory cytokines and chemokines occur in animal models of gammaretrovirus induced neurological disease when activated microglial cells and differential cytokine responses map to differences in MLV and HIV envelope proteins (146-148).

The significant changes demonstrated in the myeloid compartment including phenotypes, are suggestive of activation of APCs. Dendritic cells (DCs) are potent APCs that are central in immune responses against viral infections. DCs are located at the sites of viral entry including mucosal membranes and peripheral blood. Although the functional characteristics of the two blood DC subsets, myeloid DCs (myDCs) and plasmacytoid DCs (pDCs), are distinct, both subsets are capable of taking up antigen, migrating to lymphoid organs and presenting antigen to T cells (70). In addition to their role in adaptive immunity,

pDCs are potent mediators of innate immune responses. Many viruses infect DCs to facilitate their transmission, including the retroviruses HIV-1 and mouse mammary tumor virus (129). Some viruses do this directly, acting as "hitchhikers" or hijacking the trafficking properties of DCs to facilitate their transport from the periphery to lymph nodes where they infect target cells. Viral infection of DCs can also indirectly facilitate spread by impairing the ability of DCs to mount an appropriate immune response. For many viruses, infection of DCs affects the host's ability to mount an appropriate immune response by interfering with DC development, maturation, function and/or viability, including a reduction of type 1 interferons (70). A number of aspects of HTLV-1 induced immunosuppression are consistent with alterations in DC function. Adaptive immunity is impaired in asymptomatic HTLV-1 infected individuals (136). Taken together, these findings indicate that innate immune responses play a central role in chronic immune activation, which is a driver of HIV-1 mediated disease.

The data presented herein support the hypothesis that XMRV, like HIV and HTLV, chronically activates the innate immune response causing a dysregulation in the adaptive immune system. This can occur through direct XMRV infection of specific leukocyte subsets, and/or through dysregulation of cytokine and/or chemokine production. In addition, like chronic diseases associated with HTLV-1 and HIV, the data presented suggest that XMRV does contribute to the chronic immune stimulation observed in some CFS patients and may contribute to the immunopathogenesis of CFS. The results presented here

are the first to show phenotypic changes in patients infected with XMRV. The unique increase in the NKT populations coupled with the decrease in NK populations may produce a viable biomarker of XMRV pathogenesis similar to the CD4+ to CD8+ T cell ratio. Moreover, the CD19+ CD20+ CD23+ B cell subset may also prove to be a useful biomarker for XMRV infection. XMRV, like HIV and HTLV, is a variation of the classical retroviral theme of chronic innate immune stimulation leading to innate and adaptive immune system immunosuppression.

4.2 Future Directions:

The association of XMRV with CFS patients was discovered in 2009 by the WPI. This discovery has come under intense scrutiny including three recent studies that reported negative results (149-151). Recently, the U.S. Food and Drug Administration (FDA) and the National Cancer Institute (NCI) collaborated and found a similar association of MLV related viruses and CFS, using similar methods to the original WPI research. These studies have paved the way for future research of XMRV and other MLV related viruses and their association with neuroimmune diseases like CFS.

The study presented herein is also a basic overview of the immune response in XMRV Positive patients, and helps define directions for future study. There are, however, deficiencies in this phenotypic study. Preeminent among them are the low number of XMRV-Negative healthy controls (n=8), the low number of XMRV-Positive healthy controls (n=4), and the lack of XMRV-Negative patients. The phenotypic study used fresh, whole blood samples rather than frozen PBMCs from a repository. Patients consented to the study during doctor visits for physical issues, and their blood samples were obtained at that time. Healthy control samples were much more difficult to obtain. During the two year time period for the study, only a few healthy control samples were obtained, and four were XMRV-Positive.

This study of phenotype and cytokine production should be continued. The subject samples should come from several different sources, and health

status should be blinded to the researcher conducting the study, and XMRV determination should be done concurrently but by a different researcher who is also blinded. The number of subjects should be great enough for power to all four groups - XMRV Positive patients, XMRV Negative patients, XMRV Positive healthy controls and XMRV Negative healthy controls.

Further phenotypic and functional characterization should be done of the the NKT populations, particularly Type I and Type II NKT subpopulations. The myeloid compartment, particularly dendritic cells, macrophages and monocytes also need further characterization. These results, together with the NK phenotypic results presented here, will give a clearer picture of the interrelationship of NKT cells to the innate immune system and determine if the NK to NKT ratio is a viable biomarker for XMRV infection for detection and lead to therapeutic targets for treatment.

The B cell compartment should be further characterized, particularly to determine any alteration in B cell development. HIV alters B cell development producing immature, activated mature, exhausted memory and short lived plasmablasts (63, 65). Our research suggests that XMRV-Positive patients have lower B cell numbers, but an expansion of mature CD20+ CD23+ B cells when compared to XMRV-Negative healthy subjects. This subset of B cells should be fully characterized on each XMRV Positive patient and compared with XMRV Negative controls to determine is sensitivity as a biomarker of XMRV infection. A study of antibody production would also shed further light on the B cell

subpopulations. Further characterization of the B cell compartment may provide therapeutic targets and treatment options, such as the use of Rituximab (Rituxan^{TM}) against aberrant CD20+ B cell populations.

The CD3- CD56+ alterations were our original observation and, as a result, this population was the original starting point for this study. Although the CD3+ CD56- population was largely unchanged in XMRV Positive individuals, the T cell compartment should be examined. In particular, CD4+ and CD8+ T cells should be phenotyped, as well as the unique T cell populations, especially γδ T cells and T regulatory cells (Tregs) due to their regulatory functions on the innate immune system.

The entry for XMRV is thought to be the XPR-1 receptor, a ubiquitous receptor on many cell types (152-153). Lombardi et al.(2009) identified B and T cell infection by XMRV, and this study identified NK cell infection by XMRV. Further studies on the infectivity of XMRV in immune cells need to be continued to address stimulation requirements for replication, as well as phenotypic and functional changes. The low copy numbers found in the nucleic acid of PBMCs suggest that the periphery is not the reservoir for XMRV. Similar analysis of immune cells from other lymphatic tissues and other tissues such as placental, mammary and potentially glial tissues may help in determining the viral reservoir.

4.3 Conclusion

The 2006 discovery of XMRV in prostate cancer patients and the 2009 discovery of an association of XMRV with CFS have pointed to the potential public health hazard of this retrovirus. Current detection methods are difficult due to low viral copy number and the viral reservoir is unknown. Additional biomarkers are needed to determine infection, disease progression and potential treatment targets. We hypothesized that chronic XMRV infection creates an underlying immune deficiency leading to either direct or indirect effects on cells of the innate immune response, particularly NK cells. Our research found a decrease in lymphocytes which appear to be expressed as a reduction in NK cells and aberrant expression of NK subpopulations in XMRV-Positive CFS patients when compared to XMRV-Negative healthy controls. We also found an overall increase in NKT cells, a reduction in CD19+ B cells yet we observed the expression of a mature CD20+ CD23+ B cell subpopulation. The decrease in lymphocytes suggests an increase in the myeloid compartment as evidenced by the activated myeloid derived cells suggesting an increase in antigen presenting cells. Similar alterations in phenotype have been seen in HIV and HTLV infected patients. The results from this research suggest an activation of the innate immune system typical of retroviruses leading to innate and adaptive immunosuppression, thus confirming and expanding our original hypothesis. This research is the first to show phenotypic changes in XMRV Positive patients

suggesting an immune response to XMRV and may guide future research towards the development of biomarkers and treatment targets.

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