Chapter Title: **Innate Immune Changes in the Peripheral Blood of Chronic Fatigue Syndrome Patients: Risk Factors for Disease Progression and Management**

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ABSTRACT
Chronic Fatigue Syndrome (CFS) is recognized by the WHO as an alternative name for Myalgic Encephalomyelitis, which has been classified as a disease of the central nervous system since 1969. The concept that chronic microbial infection drives constant activation of the innate immune system through alterations in the production of innate immune cells and accompanied by abnormal production of pro-inflammatory cytokines and chemokines, and that this leads to progressive immune deficiency seen in many CFS patients has only recently been appreciated. In investigating the distribution of immune cells in the peripheral blood of a cohort of CFS patients who have an antibody recognizing the SFFV envelope protein, we discovered profound alterations in the number and types of cells, particularly in the cells regulating the innate immune system. These changes included chronic activation of monocytes and dendritic cells, and a marked increase in NKT cells and decrease in NK cells. The cytokines in plasma from these CFS patients was assayed in a multiplex platform, and one of us published findings showing signatures of infection; that is, significantly high levels of many pro-inflammatory mediators such as IL-12, MCP-1, IL-8, IP-10, IL-6, TNF-α, and IL-1β while being low in the critical antiviral cytokine IFN-α. In expanding these results, we found that subsets of these CFS patients had increased TGF-β and others had increased IL-9. We will discuss these and other published results that suggest that chronic stimulation of the innate immune system is a component of the development and progression of disease in many CFS patients. In chronic diseases the resulting immunodeficiency allows activation and replication of many secondary pathogens. Thus CFS patients can share complex pathogenic complications with patients with HIV AIDS and HTLV-1 associated myelopathy. In many CFS patient populations, the presence of several concomitant infections, from Borrelia burgdorferi to reactivated exogenous and endogenous viruses, chronically dysregulating the immune system, is a major risk factor in the development of pathology. Other risk factors include alterations in microbiota regulation, mitochondrial toxicity, cognitive dysfunction and impaired methylation pathways. These factors can also increase the risk of others diseases, including cancer, in some CFS patients. These results have important implications for the management of many people with this diagnosis. We will review in this chapter the use of anti-inflammatories, anti-virals and other therapies, as well as discussing how repurposing drugs holds promise in the treatment of patients displaying the immune abnormalities identified in these CFS patients.

INTRODUCTION

Chronic fatigue syndrome (CFS) is recognized by the WHO as an alternative name for Myalgic Encephalomyelitis (ME) which has been classified as a disease of the central nervous system since 1969 [2]. In current practice, however, CFS is a diagnosis afforded to people who present with fatigue of varying severity with or without additional minor symptoms, whose cause is not revealed via the use of rudimentary tests [3,4]. The use of diagnostic criteria entirely based on the presence of non- specific symptoms routinely leads to the recruitment of study populations where patients display excessive heterogeneity from a clinical and pathophysiological perspective. It is now clear that a diagnosis of CFS does not represent a single illness with a unitary pathogenesis and pathophysiology [3,4,6]. Given our appreciation of the considerable clinical and biological heterogeneity of CFS patient populations, we present data from a cohort of patients who satisfied the requirements of the international consensus criteria for the diagnosis of CFS, all of whom have an antibody recognizing the SFFV envelope protein.

Many biochemical observations associated with CFS suggest an underlying innate immune dysregulation leading to chronic infections as the primary mechanism of pathogenesis of the disease [2]. CFS patients often display antiviral enzyme RNase L dysfunction and dysregulation of inflammatory cytokines and chemokines [7,8]. Pathogens commonly associated with CFS such as parvovirus B19 [9], which effect macrophage function [10] and enteroviruses [11] which are known to inhibit RNase L function [12] underscore the importance of the innate immune response in CFS.

The innate immune system, whose cellular components consist of several cell types including Natural Killer (NK) cells, dendritic cells, and macrophages, evolved specifically as the front line of host defense against microbial infection (Fig. 1).

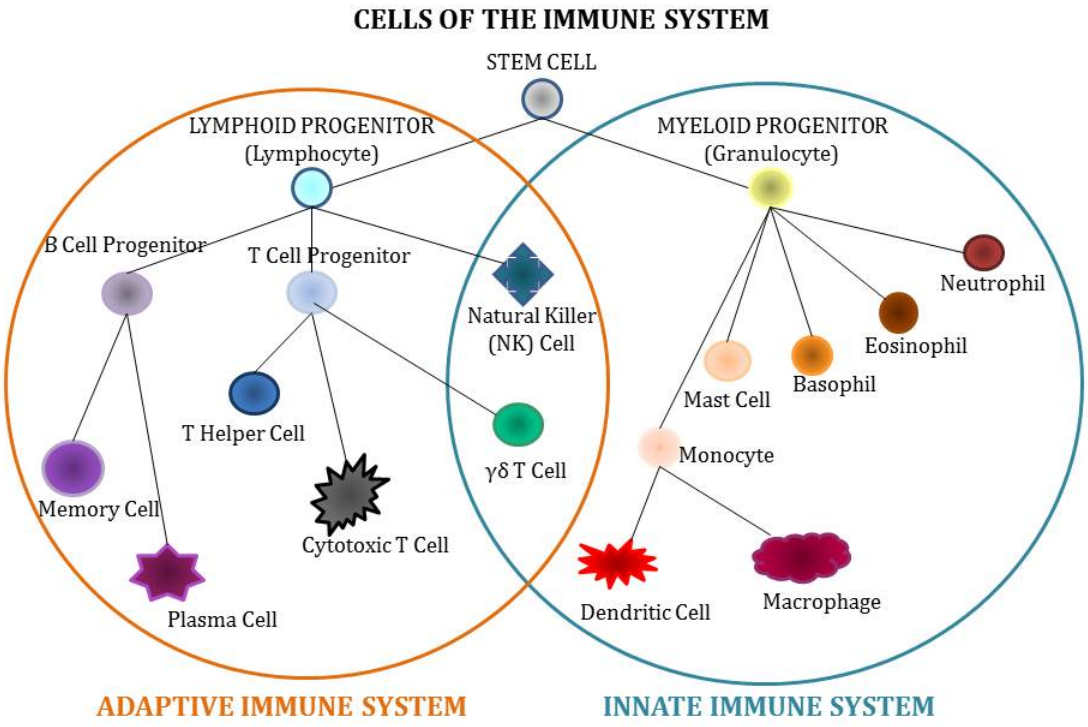

FIGURE 1: Cellular components of the Immune System: The Immune System consists of the Innate Immune System and the Adaptive Immune System. The Innate Immune System responds first to microbial invasion, and consists neutrophils, eosinophils, basophils, mast cells, macrophages, dendritic cells. and NK cells. The dendritic cells, NK cells and γδ T cells are thought to bridge the innate and adaptive immune systems.

NK cells are large, granular lymphocytes comprising 6 to 14% of all peripheral blood lymphocytes [13-16]. Like most lymphoid innate immune cells, NK cells bridge the innate and adaptive immune systems by providing primary cytotoxic and cytostatic functions for the innate immune system while interacting and collaborating with adaptive immune system lymphocytes, as well as mediating the activation and regulatory effects on other cell types and inflammatory responses [13,14,17,18]. The antiviral and anti-tumor effects of NK cells have been well documented [19]. Primary NK cell responses including IFN-γ 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 killer immunoglobulin receptors and other activating receptors generating perforin/granzyme mediated killing [20]. NK cells also kill through inflammatory cytokine secretion, antibody dependent cellular cytotoxicity through the Fcγ receptor III (CD16) and death receptor interaction [21-23]. As a result, NK cells are sensitive to receptor dysregulation mediated by pathogens and specifically viral infection [24-26].

The dysfunction of the NK cell compartment in many patients with CFS has been widely noted, including decreased NK cell activity [27-30]. In a previous study, Maher et al. found low perforin levels and depressed NK cell activity in a group of 30

CFS patients [31]. Levine et al. 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, or perhaps a decrease in NK cell modulating cytokines or the presence of inhibitory factors is responsible [29].

Our hypothesis is that development of disease in this CFS patient cohort is fueled by inflammation and infection, creating an underlying immune deficiency leading to either direct or indirect effects on cells of the innate immune response. To test this hypothesis, a profile of the immune cells in peripheral blood of CFS patients showing reactivity against the spleen focus forming viral (SFFV) envelope protein was developed. Herein, we detail defects in the immune profiles of these CFS patients with an antibody to SFFV Env, including an overall reduction in the lymphocyte compartment as well as a significant reduction in CD45+ CD3- CD19- CD56+ NK cells particularly the $CD56^{DIM}$ subpopulation. A highly significant aberrant cytokine and chemokine production with a decrease in type I interferon was found in this subset of CFS patients with antibody to SFFV Env.

Profile of circulating leukocytes in SFFV Env antibody positive CFS individuals.

Critical imbalances in both the number and function of leukocytes occur in the peripheral blood of patients infected with known human exogenous retroviruses associated with disease, HIV-1 and HTLV-1[32]. Similarly, in many CFS patients, significantly fewer $CD3^{+}CD25^{-}$ T cells and significantly more $CD20^{+}CD5^{+}$ B cells, a subset associated with auto-antibodies, have been found [33]. Significantly fewer $CD56^{+}$ NK cells with reduced activity were also observed in many other CFS patients [28]. Several CFS patient populations have been associated with lower levels of intracellular perforin [31]. Therefore, the status of other lymphoid and myeloid cells of the innate immune response in peripheral blood of a cohort of CFS patients expressing an antibody to SFFV Env protein was examined.

A detailed phenotypic analysis of major and minor circulating leukocyte subsets in this cohort of CFS patients was conducted [1]. We obtained whole blood from patients and healthy controls for the phenotype and cytokine studies. All of the CFS patients used and 3% of the healthy controls tested positive for SFFV antibody. Blood samples were labeled with combinations of monoclonal antibodies and analyzed the results by flow cytometry. The results were compared to normal donors and the published reference values of healthy donors. The total percentage of CD45+ leukocytes was not significantly different between SFFV Env antibody expressing patient cells and normal controls.

We then gated on either the lymphocyte population or the granulocyte/monocyte groups. From the lymphocyte gate, we examined four major cell populations using CD3 and CD56 and 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 2). It is important to note that CD56 is not exclusively an NK marker for class 1 lymphoid initiating cells (rare in the blood) and other non-lymphoid express CD56.

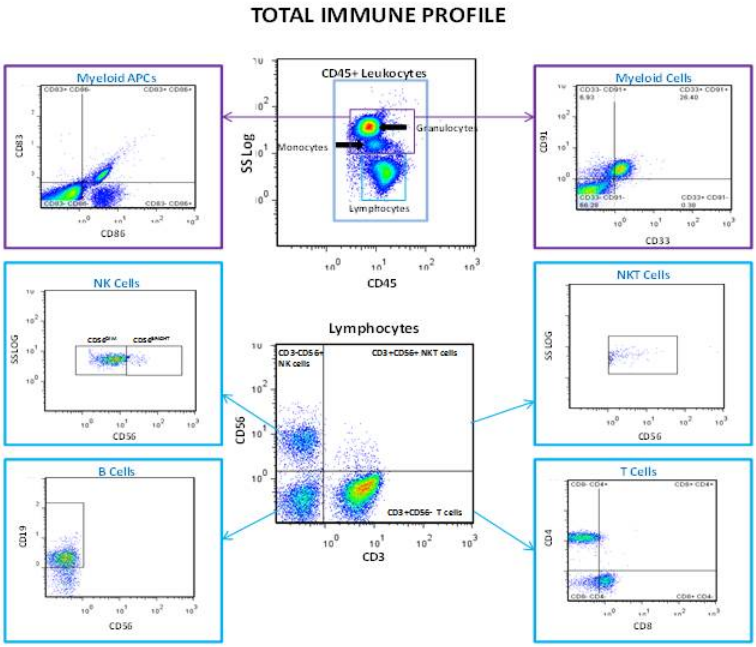

FIGURE 2: Total Immune Profile: Multi-parameter flow cytometry was used to generate this schematic of a representative whole blood sample of the SFFV Env Antibody Positive ME/CFS patients. CD45+ leukocytes were first separated, then either the lymphocyte population or the granulocyte/monocyte groups 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.

SFFV Env Antibody positive CFS patients have lower lymphocyte percentages.

From the CD45+ leukocytes, the total lymphocyte population was gated and the percentage of lymphocytes was obtained for each subject. We compared the percentage of lymphocytes from the peripheral blood of these CFS patients with the mean of the healthy subjects. All but three of the SFFV Env Antibody positive CFS patients showed a lower percentage of CD45+ lymphocytes than the mean of the healthy controls, and 69% of these CFS patients showed a three-fold decrease in CD45+ lymphocytes as compared to controls (Fig. 3; p value<0.0091**)**. The lymphocytes were then separated by CD3 expression. The CD45+ CD3- lymphocytes were also significantly reduced in these CFS patients (p value= 0.0029**),** in contrast to only a slight reduction in CD45+ CD3+ T cells (data not shown).

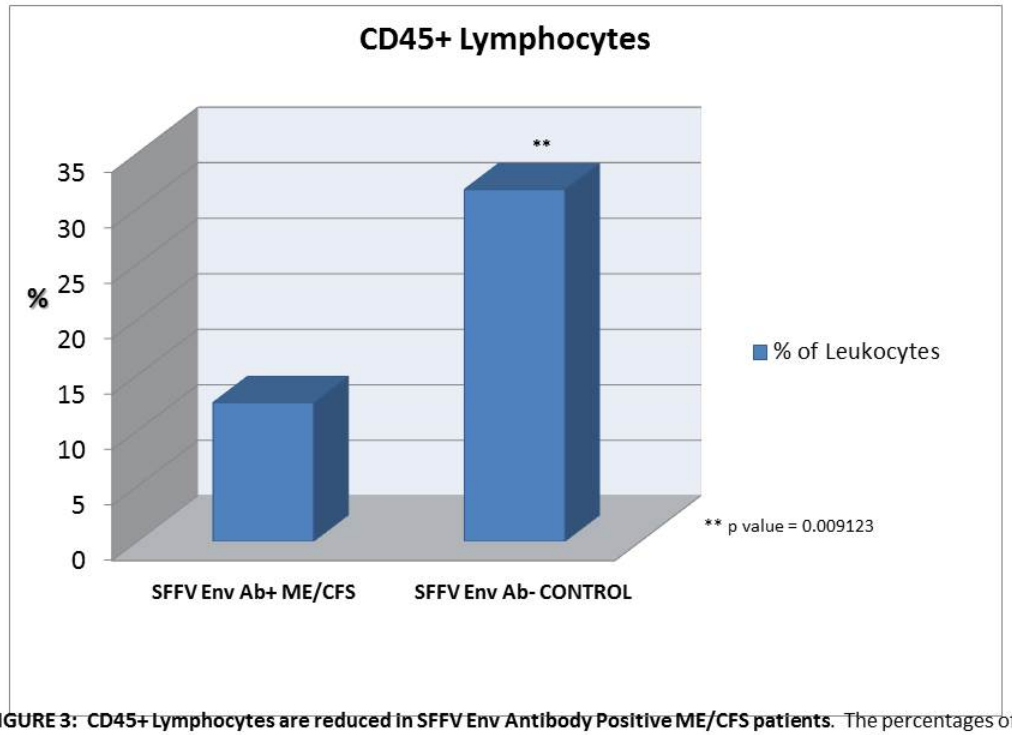

FIGURE 3: CD45+ Lymphocytes are reduced in SFFV Env Antibody Positive ME/CFS patients. The percentages of CD45+ lymphocytes from total CD45+ leukocytes from whole blood samples of SFFV Env Ab+ ME/CFS patients and SFFV Env Ab- controls (n=8) were compared. P value =0.009123 (Statistical analysis using unpaired t test with Welch's correction .)

NKT cells are markedly increased and NK cells, particularly the $CD56^{DIM}$ cells, are significantly reduced in SFFV Env Antibody positive CFS patients.

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[34-38]. The level of CD56 expression, however, is indicative of distinct subpopulations of NK cells [39,40]. 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 the 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 antibody-opsonized cells through the low affinity FcyRIII receptor. The CD3- $CD56^{BRIGHT}$ subset comprises only 5 to 10% of healthy NK cells and is characterized by the immunoregulatory functions particularly in secondary lymphoid organs by secreting large amounts of many cytokines, including IFN-γ and TGF-β. This subset does not normally express CD16 and largely lacks perforin and killer inhibitory receptors [39-42].

CD3 and CD56 expression are used as indicators of the major lymphocyte populations. CD3+ CD56- T lymphocytes, CD3+ CD56+ NK-like-T cells (NKT) also

known as Cytokine-induced killer (CIK) cells are a unique population of cytotoxic T lymphocytes (CTL) with the characteristic CD3+CD56+ phenotype. Both the CD3+CD56+ and the CD3- CD56+ NK cells were identified by flow cytometry **(**Fig. 4**).**

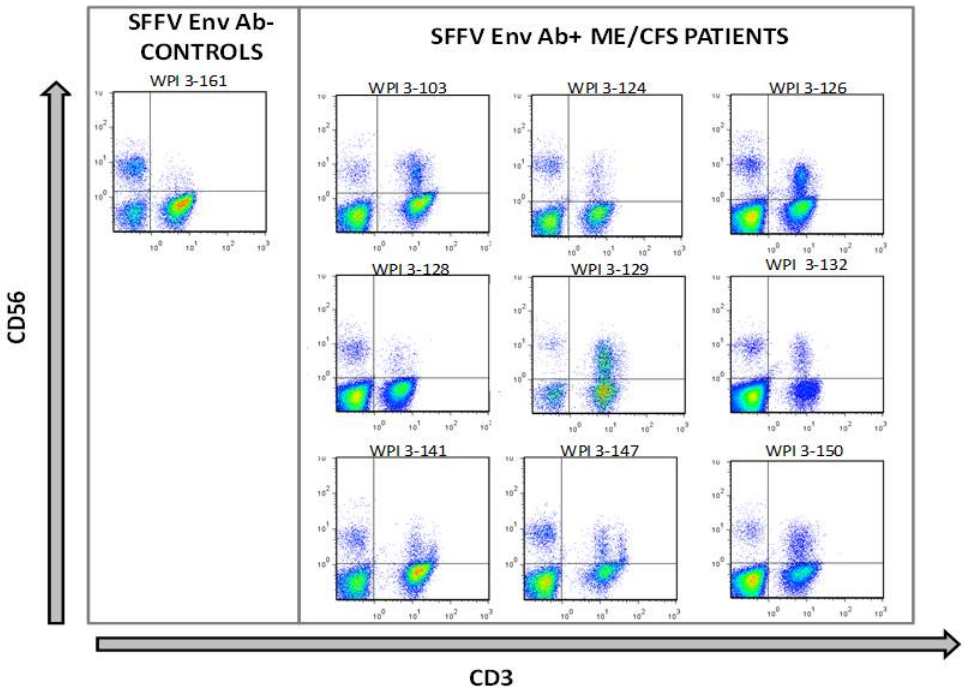

FIGURE 4: CD3 and CD56 Expression on the NK, Non NK/T, T and NKT populations: CD3+ CD56+ NKT cells are significantly increased while CD3- CD56+ NK cells are significantly reduced. Dot plot representations of CD3 and CD56 expression on CD45+ lymphocytes from the peripheral blood of 9 representative SFFV Env Ab+ ME/CFS patients are compared with one representative SFFV Env Ab- control. P Value=0.0054 (Statistical analysis with unpaired t test with Welch's correction)

Both subsets were dysregulated in SFFV Env Antibody positive CFS patients studied compared to healthy controls. We observed for the first time a marked increase in CD3+CD56+ (NKT) cells in some SFFV positive CFS patients and a significant reduction in CD56 expression in the CD3- CD56+ NK population in this CFS patient population **(**Fig. 4; p value=0.0054**)** with 93% of the CFS subjects significantly below the mean of the negative controls. Thus, we confirm studies showing reduction of NK cells in other CFS patient populations and extend these findings to SFFV Env antibody expressing CFS patients.

Next, the intensity of the CD56 expression was examined by gating on the $CD56^{BRIGHT}$ and $CD56^{DIM}$ subpopulations. We then compared a representative SFFV Env antibody negative healthy subject with several representative SFFV Env antibody positive CFS patients by overlaying the CD56+ populations from the SFFV Env antibody positive samples on to the CD56+ populations from the representative control. The mean fluorescent intensity (MFI) between the negative controls and CFS positive samples remained relatively the same, but the percentage of CD3- lymphocytes expressing CD56 was significantly reduced (Fig 5). Similar results were seen when the SFFV Env antibody positive CFS patients were compared with additional normal controls (data not shown). This reduction in the percentage of CD56+ NK cells is represented mainly in the significant reduction of the $CD56^{DIM}$

subpopulation (p value =0.0025). We also saw a reduction in the CD56^{BRIGHT} subpopulation, but this reduction may be a reflection of the overall lower percentages of lymphocytes seen in SFFV Env antibody positive CFS patients.

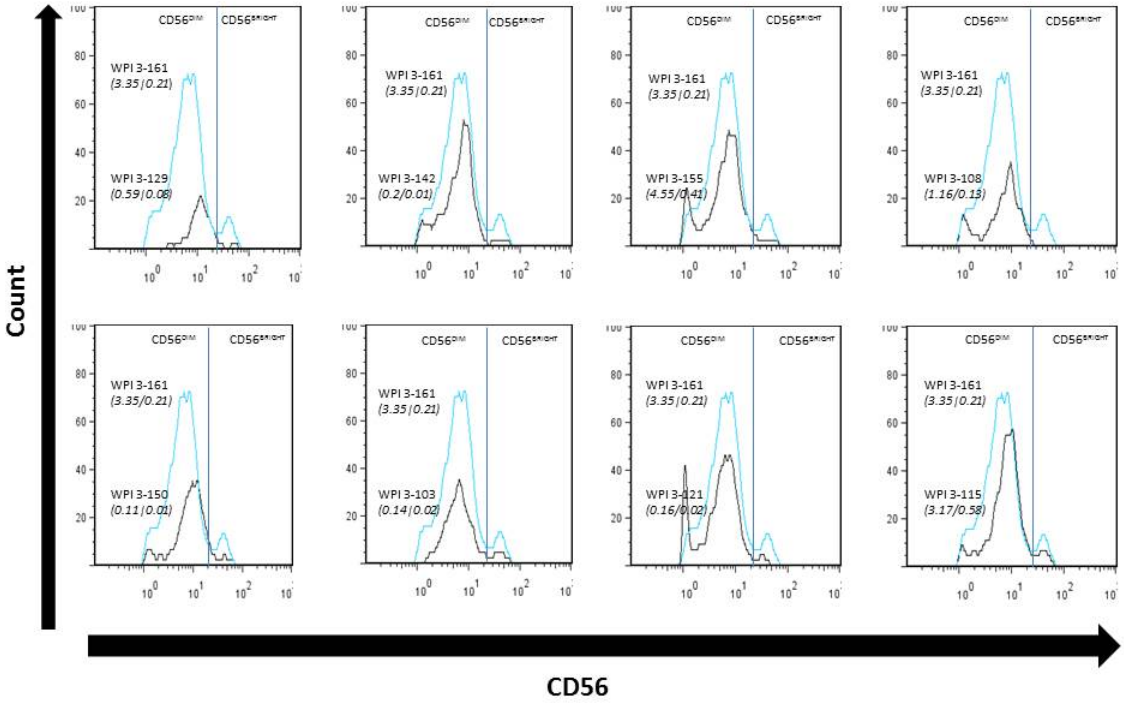

FIGURE 5: NK cells, particularly the CD56^{DIM} populations, are significantly reduced in SFFV Env Ab+ ME/CFS patients. Histograms of CD56 expression from of eight representative SFFV Env Ab+ patients were compared against a representative SFFV Env Ab- control . P value=0.0025 (Statistical analysis with unpaired t test with Welch's correction)

CD16 expression on NK cells is considered an indicator of function through mediation of antibody dependent cellular cytotoxicity. Infection of NK cells by retroviruses such as HIV and HTLV have been shown to alter CD3- CD16+ NK cell populations [25]. We looked at the expression of CD16 on CD3- cells from SFFV Env antibody positive CFS patients, and initially observed no significant differences in CD16 expression although we did note a greater variability in the expression of CD16 between CD16^{LOW} and CD16^{HIGH} subsets. The significance of these subsets is unknown.

We then overlayed the CD3- CD56+ population from these CFS patients onto the same population from a representative SSFV Env antibody negative healthy control. A significant reduction in CD16 expression was observed (Fig. 6). The CD56^{DIM} subpopulations in normal individuals typically express high levels of CD16 whereas CD56^{BRIGHT} subpopulations do not express CD16 or express at low levels [40,43]. In order to determine CD16 expression on the CD56 subpopulations, we next looked at the CD3- CD56+ NK cell population, gated on the CD56^{BRIGHT} and CD56^{DIM} subpopulations, then gated on CD16 for each subpopulation.

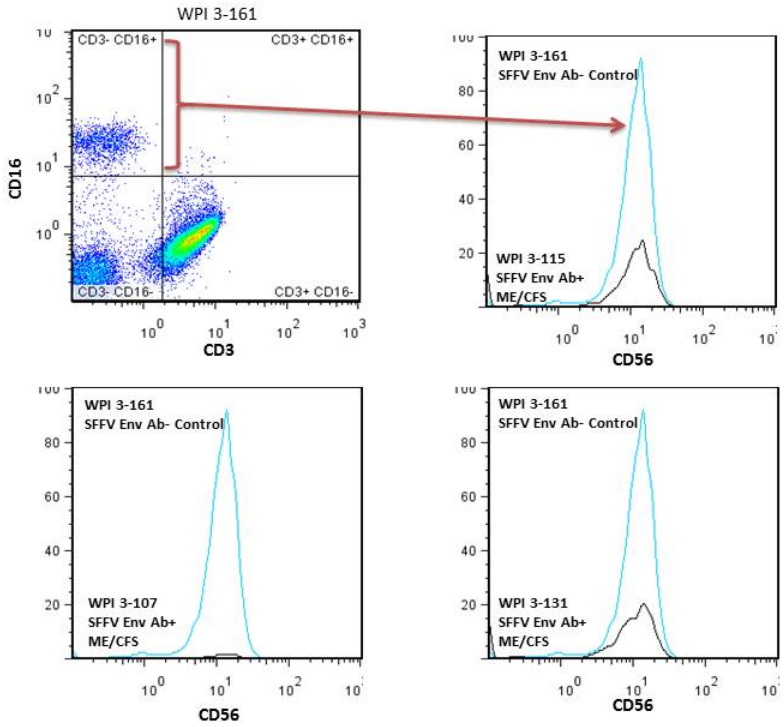

FIGURE 6: CD16 expression on CD3- cells is reduced in SFFV Env Antibody Positive ME/CFS patients. Histograms were generated from dot blot representations of three representative SFFV Env Ab+ patients were compared against a representative SFFV Env Ab- control. P value = 0.0081. (Statistical analysis using Unpaired t test with Welch's correction)

We also looked at the relationship of CD16 expression to the CD56+ subpopulations (Fig. 7). We observed a decrease in CD16 expression on CD56+ NK cells from SFFV Env antibody positive CFS patients as compared to the negative controls, particularly the $CD56^{DIM}$ subpopulation (p value = 0.033.). The $CD56^{BRIGHT}$ CD16-was also reduced, although not significantly. Other NK cell surface markers were examined, and phenotypic changes in expression were found. CD122 expression, the IL-2Rβ/γ, 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 these CFS patients (data not shown). In terms of the previously reported loss in NK numbers, these data suggest an imbalance in the NK subsets in this cohort of CFS patients.

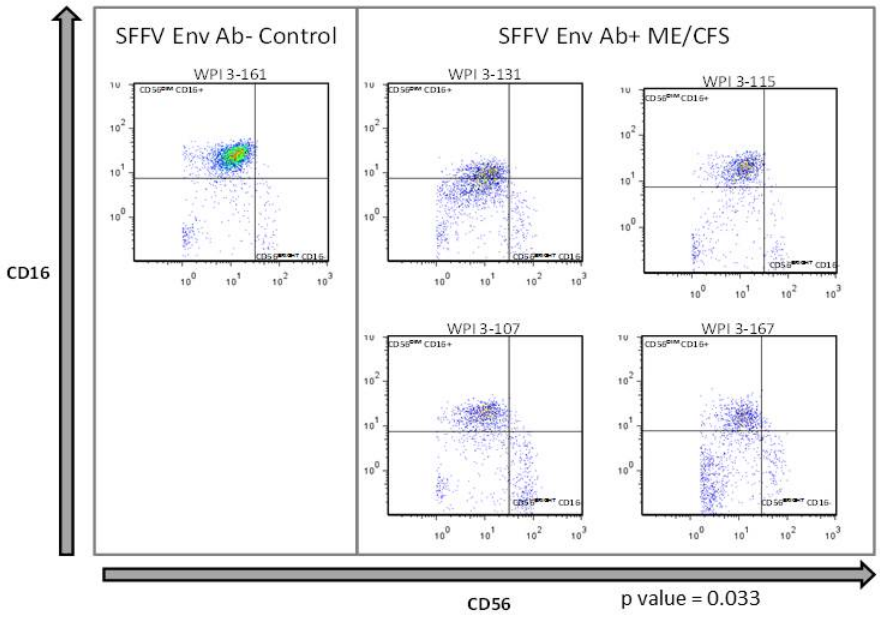

FIGURE 7: CD16 and CD56 expression on lymphocytes is reduced in SFFV Env Antibody Positive ME/CFS patients. Dot blot representations of flow cytometry analysis of four representative SFFV Env Ab+ patients were compared against a representative SFFV Env Ab- control. P value = 0.033. (Statistical analysis using unpaired t test with Welch's correction)

The percentage of CD19+ B cells is reduced in SFFV ENV antibody positive CFS patients.

From the CD3- CD56- cells, we then gated on the CD19+ B cells. CD19 is a cell surface molecule that is present on all B cells except most mature plasma cells, early developing B cells and associates with the antigen receptor to decrease the threshold for antigen dependent receptor stimulation. The CD19+ B cells from these CFS patients were compared with those of a representative healthy control and a significant reduction in the percentage of CD19+ B cells was observed (Fig 8; p value= 0.043).

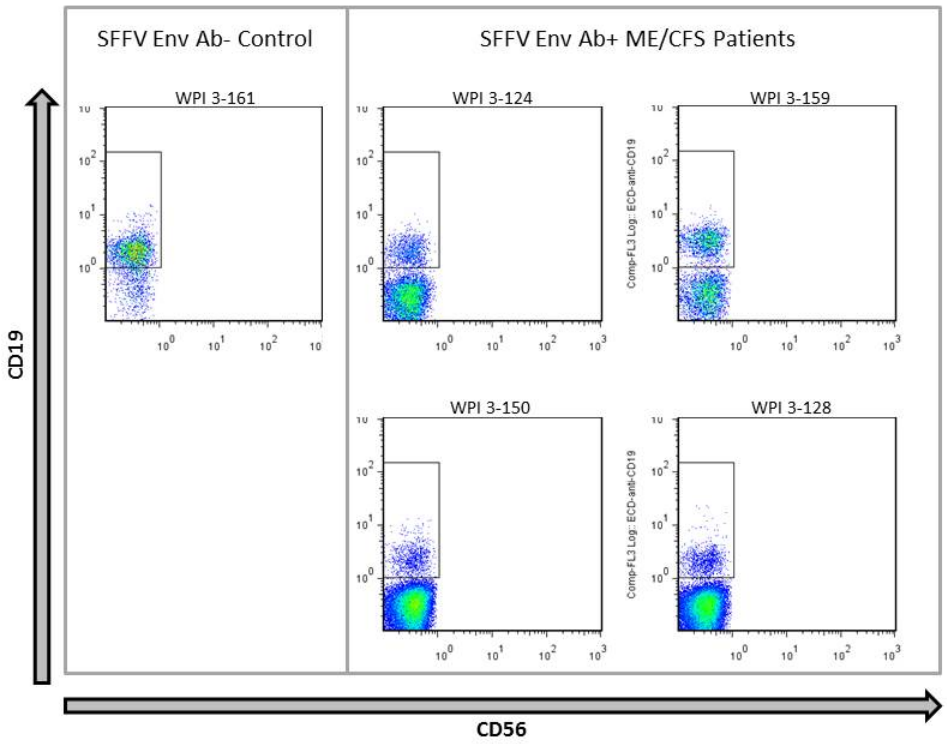

FIGURE 8: The percentage of CD19+ B cells is reduced in SFFV Env Antibody Positive ME/CFS patients. Dot blot representations of flow cytometry analysis are shown. CD3- CD56- CD19+ B cells from representative SFFV Env Ab+ patients were compared with a representative SFFV Env Ab- control. P Value = 0.043. (Statistical analysis using Unpaired t test with Welch's correction)

We then looked at common B cell developmental markers CD5, CD20, CD22 and CD23. Using the CD19+ B cells from SFFV Env antibody positive CFS patients, we observed a high CD20 expression coupled with CD23 expression (Fig. 9). 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 to plasma cells [44]. CD23 is the FcεRII receptor of IgE and is mainly found on mature B cells and is involved in inflammatory responses [45]. Significant numbers of CD20+CD23+ mature B cells are rarely found circulating in the peripheral blood of healthy individuals but are often seen in HIV-infected individuals treated with highly active antiretroviral therapy (HAART) [46]. Significantly, CD20 cells are targeted by the humanized monoclonal antibody Rituximab, which has shown some clinical benefit in a small trial of CFS patients in Norway [47]. It is important to note again the heterogeneity of patients with the diagnosis of CFS. Given that serious adverse events such as the development of progressive multifocal leukoencephalopathy (PML) through reactivation of the JC virus can occur with B cell depletion and immune suppressive strategies such as Rituximab, immune profiling of patient populations revealing increased numbers of the target cells could predict responders and predicting benefit. As profiling technologies are being used in CFS, it is clear that different patient cohorts with the diagnosis of CFS have patients without this increase in CD20+ B cells and thus would not be candidates for Rituximab therapy [48].

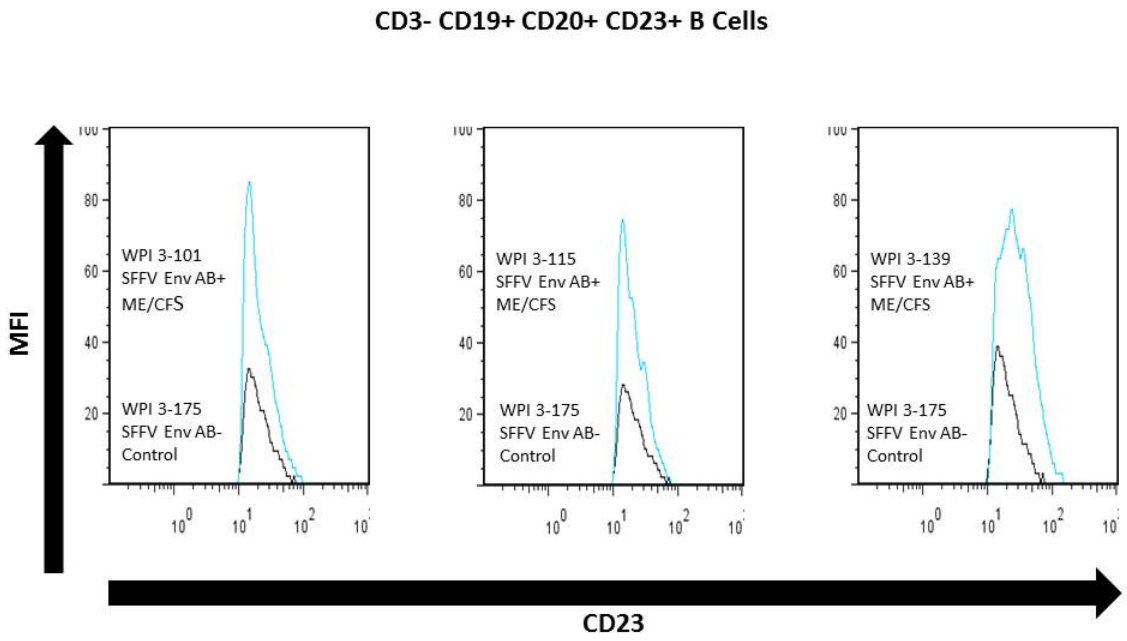

FIGURE 9: The MFI of CD3- CD19+ CD20+ CD23+ B cells is increased in SFFV Env Antibody Positive ME/CFS patients: Histograms generated from dot plot representations of CD23+ expression on CD3- CD19+ CD20+ B cells from three representative SFFV Env Ab+ patients (WPI 3-101, WPI 3-115 and WPI 3-139) are overlayed onto a representative SFFV Env Ab- control (WPI 3-175). P value = 0.038 (Statistical analysis by unpaired t test with Welch's correction)

Increased activation of myeloid derived cells in SFFV Env antibody positive CFS patients.

We also wanted to explore the myeloid cell compartment in the SFFV Env antibody positive CFS patients. CD14, a co-receptor for the recognition of bacterial lipopolysaccharide, is expressed mainly on the monocyte-macrophage lineage. CD14+ monocytes can differentiate into a number of cell types including macrophages and dendritic cells [49-51]. CD123 is the IL-3 receptor, and it is found in high levels on plasmacytoid dendritic cells (PDC) and granulocytes, but at low levels on monocytes and myeloid dendritic cells. We first gated on all the granulocyte and monocyte groups of cells first shown in Fig. 2. Using these two antibodies we observed a variety of myeloid derived cell types.

Representative plots from the SFFV Env antibody positive CFS patients are shown in Fig. 10. We saw widely variable differences in myeloid populations in SFFV Env antibody positive CFS patients. CD33 and CD91 were next used to further differentiate the cell types. CD33 is a receptor expressed mainly on cells of myeloid origin, and CD91 is also expressed in peripheral blood on cells of myeloid origin, predominately macrophages. For each of the CD123-CD14 subpopulations, we looked at the CD33-CD91 expression. Again, we observed substantial populations of monocytes and macrophages, though not significantly different from normal observed ranges reported in the literature.

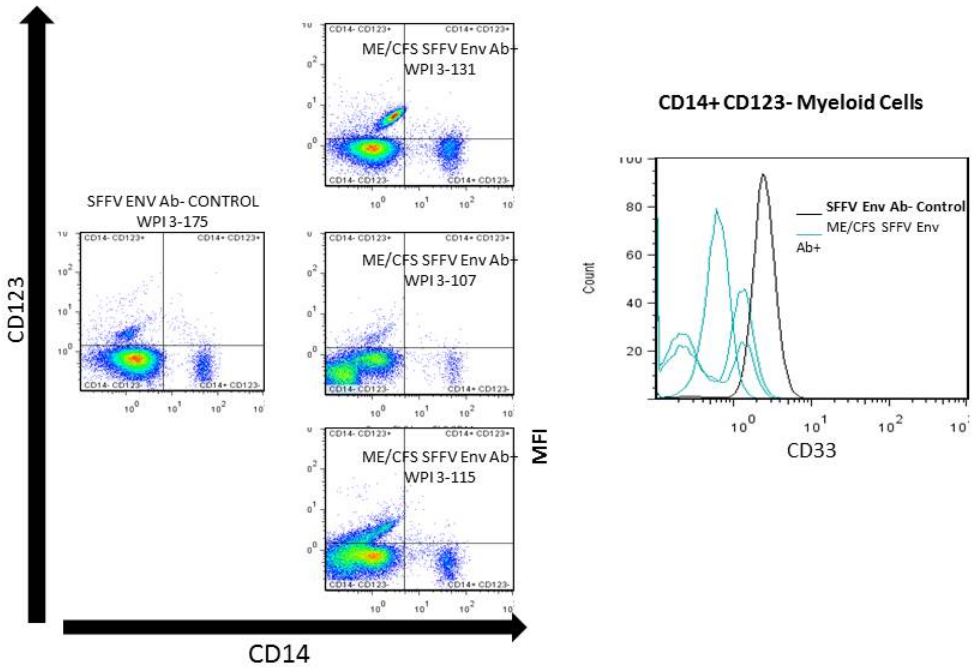

FIGURE 10: Expression of CD14 and CD123 on myeloid derived cells. Reduced expression of CD33 in SFFV Env Antibody Positive ME/CFS patients: Dot blot representations of flow cytometry analysis are shown. Granulocyte and monocyte populations were gated from CD45+ leukocytes, then CD123 and CD14 expression were used to examine myeloid populations. A representative SFFV Env Ab- control is shown (Left). Three representative SFFV Env Ab+ patients are shown (Middle). Histograms of MFI were taken from SFFV Env Ab- control samples and overlayed onto SFFV Env Ab+ patient samples. Histograms for SFFV Env Ab+ and SFFV Env Ab- controls are shown (Right). SFFV Env Ab+ patients show a reduced expression of CD33 (No statistical assessment).

Expression of CD69 on CD83+ CD86+ cells in SFFV Env antibody positive CFS patients is associated with an activated, mature dendritic cell compartment.

Within the myeloid compartment, progenitors can develop into myeloid dendritic cells[52]. Because the focus of our study has been on inflammatory responses in SFFV Env antibody positive CFS patients, we chose to use the activation markers CD83, CD86 and CD69 on CD14 negative myeloid cells (also negative for B and T cell markers). CD83 is a glycoprotein strongly associated with mature dendritic cells, and it may play a role in inducing immune responses [52]. CD86, a receptor that acts as co-receptor in T-cell activation, is strongly upregulated in the DC maturation process. CD69 is another activation markers associated with early activation of leukocytes. Therefore, we explored the association of the mature DC marker CD83 expression with CD86 in the CFS patients. We observed a population of CD83+ CD86+ cells in the SFFV Env antibody positive CFS samples. We gated this subpopulation and found that greater than 94% of the cells were CD69+ as well (Fig 11). This suggests that highly activated mature DC cell compartment is present in the peripheral blood of these SFFV Env antibody positive CFS patients. Prior to initiating the immune profiling studies, we had frequently observed this highly activated population in these patient cells by light microscopy and demonstrated ex vivo that these activated PDC were dysfunctional in that they did not express IFNα (data not shown). Similar PDC defects were published by one of us previously studying PDC from HTLV-1 infected ATL and HAM/TSP patients [53]. These data

prompted us to hypothesize signatures of infection and disease in heterogeneous diseases such as CFS.

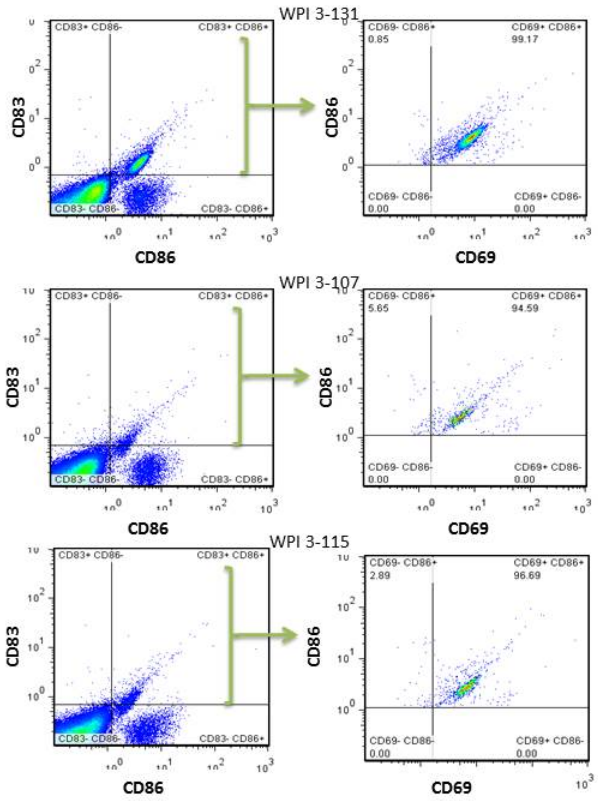

FIGURE 11: Expression of CD69 on CD83+ CD86+ cells in SFFV Env Antibody Positive ME/CFS patients may be associated with highly activated mature DC cell compartment . Dot blot representations of flow cytometry analysis are shown. CD83 and CD86 expression is shown on leukocytes. From the CD83+ CD86+ group, CD69 expression was determined. Representative SFFV Env Ab+ patients are shown.

Cytokines and chemokines are dysregulated in SFFV Env antibody positive CFS patients.

We and others had demonstrated chronic immune cell dysfunction and activation in CFS patients [28], and our demonstration for the first time of activated DC and B cells and defective PDC in the peripheral blood of SFFV Env antibody positive CFS patients suggests an ongoing inflammatory process. We had previously examined protein patterns of cytokines and chemokines in the peripheral blood of a larger cohort of CFS patients, who had antibodies to SFFV-Env. Cytokines and chemokines play important roles in controlling the homoeostasis of the immune system and their dysregulation exacerbates many disease processes [54]. NK cells are also affected by the cytokines produced by other immune cells. For instance, IFN-α, IL-12, IL-15 and IL-2 are involved in NK cell activation and maturation. IFN-α and IL-12, produced early in a viral infection, are critical for stimulation of the cytotoxicity, the proliferation and the IFN-γ production of NK cells, activating both STAT1 and STAT4 intercellular signaling. IFN-α induces NK cell cytotoxicity and IL-12 is critical for IFN-γ production; IL-15 has been found to sustain NK cell accumulation in the presence of IFNs [55,56]. Furthermore, the macrophage inflammatory chemokines MIP-1α and MIP-1β generated by NK cells can block virus

binding and infection [55]. Interestingly, MIP-1α and MIP-1β are also produced by activated macrophages, which we observed in this cohort of CFS patients expressing SFFV Env antibody.

In an attempt to delineate a signature of infection or disease in SFFV Env antibody positive CFS patients compared to SFFV Env antibody negative healthy control populations, the concentrations of 26 different cytokines and chemokines were evaluated in a multiplexed assay using xMap technology. Analyses were made using the plasma samples of healthy control subjects and from CFS patients. Of the 26 cytokines and chemokines assayed, 18 were differentially expressed as determined by log transformed Students- t test at the 99% confidence level; 11 were up-regulated, 8 were down-regulated (Table 1).

CYTOKINES/ CHEMOKINES	Patient N = 118	Control N=138	P value	FUNCTION IN INFLAMMATION
IL-8	1045	13	<0.0001	RNase L and CMV activated
MIP-1α	763	91	0.0062	Elevated in Neurodegenerative disease
MIP-1β	1985	164	<0.0001	Elevated in Neurodegenerative disease
IL-6	336	29	<0.0001	Stimulates chronic inflammation
TNF-α	148	13	<0.0001	Stimulates chronic inflammation
IL1β	500	56	<0.0001	Stimulates chronic inflammation
IP-10	98	32	<0.0001	Interferon response protein
IFN-α	35	60	<0.0001	Stimulates macrophages and NK cells to elicit an anti-viral response
IL-13	28	86	<0.0001	Inhibits inflammatory cytokine production
IL-7	160	60	<0.0001	Stimulates proliferation of B and T lymphocytes and NK cells

Mean values in pg/ml: Red notes up regulation, Blue notes down regulation

Interestingly, those up-regulated included the inflammatory cytokines IL-6, TNF-α, IL-1β and IL-12, as well as the inflammatory chemokines IL-8, MIP-1α and MIP-1β which act as chemo-attractants in response to inflammatory conditions and have also shown to be involved in neurodegeneration caused by xenotropic murine leukemia viruses [57]. Moreover, we found highly significant down regulation of IFN-α, produced by lymphocytes in response to viral infection, and IL-13, which mediates and can suppress responses from inflammatory cells. IFN-α exhibits potent antiviral activity in vitro and plays a major role in the early defense against viruses. As PDC are the immune cells that produce most of the IFN-α in humans, our observation of defective PDC in our SFFV Env antibody positive cohort of CFS patients is supported by these data. A possible mechanism for the observed

downregulation of IFN-α in SFFV Env antibody Positive CFS patients may be that IL-8 is upregulated. Matshushima and colleagues have found that IL-8 can inhibit the antiviral activity of IFN-α significantly [58]. An alternative but not mutually exclusive explanation could involve IFN-α driven apoptosis of PDCs and the fact that low glutathione seen in patients with chronic inflammation and immune activation might interfere with the maturation of naive DCs. It is also perhaps worth noting that PDCs become insensitive to the presence of chronic retroviral infection resulting in the blockage in IFN-α production [59].

Increasing the number of cytokines tested to 51 in the plasmas of SFFV-Env antibody positive CFS patients revealed that a subset of patients had elevated levels of TGF-β and IL-9. TGF-β has profound effects of all cell members of innate and adaptive immunity (Fig. 12), and IL-9 through stimulation of mast cells and basophils is a prime mediator of response to allergens. Through stimulation of eosinophils it is an important mediator of the response to parasite infections. This is a Th17 profile that had previously been detected in another CFS cohort [60]. The high levels of IL-6 and TGF-β would be expected to drive the differentiation of naive T cells towards the Th17 phenotype. It is also interesting that the high IL-1β could drive IL-17 secretion by Tregs in these patients [2,61]. CFS patient populations with high levels of TGF-β were first identified by Shoemaker. It is worth noting that opposite results were found in a study by Broderick in 2010 [62], further emphasizing the immunological heterogeneity in different cohorts despite patients carrying the same diagnosis. Shoemaker uses the acronym CIRS or chronic inflammatory response syndrome and found elevated TGF-β1 to be a common finding. He pioneered the use of small molecule, AT1 inhibitor Losartan, to lower TGF-β1and slow progression of vascular symptoms in Marfan's Syndrome. Marfan's and Ehlers Danlos Syndromes appear to be over-represented in the CFS patient group.

Though a detailed discussion of anti-inflammatory molecules with potential efficacy against TGF-β and this TH17 profile is beyond the scope of this chapter, it is worth noting an excellent review of Herbal TGF-β inhibitors [63] and the rapidly growing literature to support the medicinal use of the Cannabis plant for a wide range of inflammatory conditions. It has been observed that both THC and CBD cannabinoids decrease the Th17 phenotype by down regulating IL-17 and IL-6 and upregulating IL-10 [64]. The American Academy of Neurology just endorsed the use of medical Cannabis for MS and HIV patients, who share the inflammatory profile with these CFS patients [65].

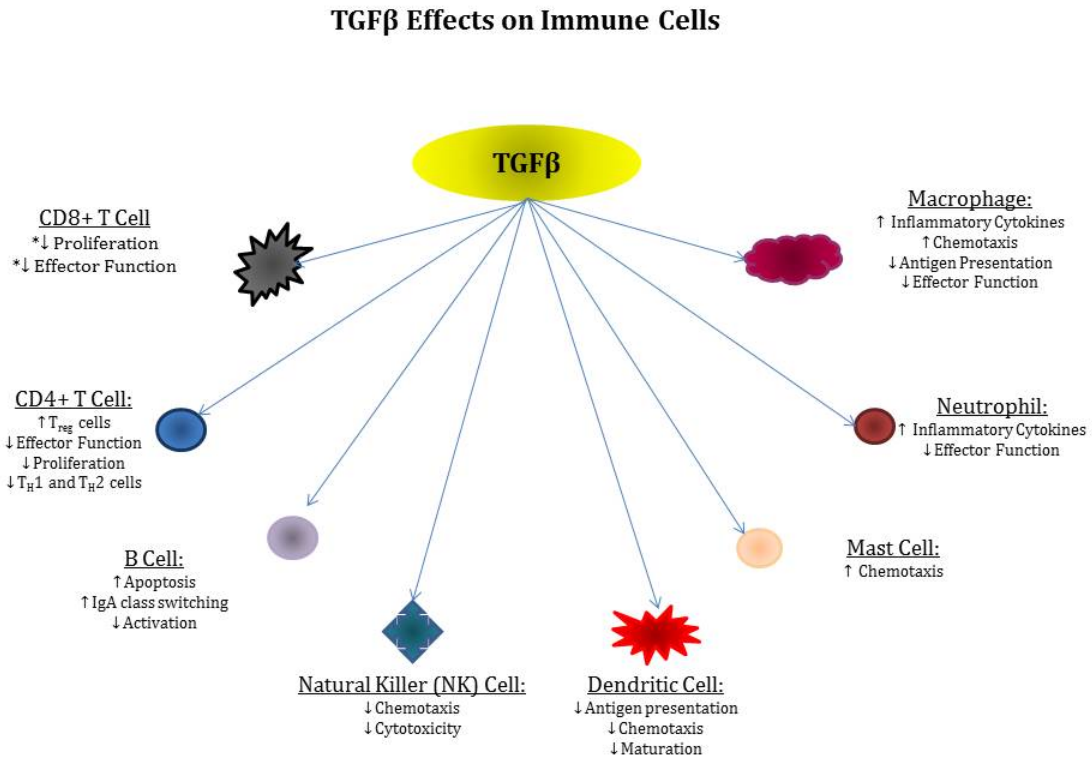

FIGURE 12: TGFβ Effects in Immune Cells: TGFβ elicits profound effects on all cell members of the Innate and Adaptive Immune Systems.

The identification of a new subgroup of CFS patients with antibodies to SFFV Env and elevated levels of IL9 suggests a critical role for mast cell dysfunction in CFS. Mast cells are critical in the innate immune response to allergens and parasites. Mast cells are activated not only by IgE, but also by cytokines, environmental, food, infection, drug and stress triggers, leading to secretion of multiple mediators. The symptom profile and comorbidities associated with diseases such as CFS and fibromyalgia have been well characterized by the Theorarides laboratory, which recently published an excellent review on the spectrum of mast cell activation disorders [66]. Their pioneering work has demonstrated that more than one hundred triggers for mast cells could be released, many without degranulation, including GM-CSF, which is also upregulated in the serum of CFS patients in our studies. Importantly, when considering risk factors for CFS, it should be noted that mast cells are the most exquisitely sensitive sensors of the body stimulated by mercury, aluminum, arsenic and many other heavy metals and mitochondrial DNA, which can be measured in the blood of patients with neuroimmune diseases including CFS [67]. Mitochondrial DNA stimulates several cell types in addition to mast cells and is highly toxic to neurons. Mitochondrial DNA also stimulates the DNA methylation machinery contributing to aberrant methylation. In addition, free degraded mitochrondrial DNA outside of the cells can act as a damage-associated molecular pattern (DAMP) molecule which stimulates a proinflammatory immune response, and it has been suggested to be a trigger for neurogeneration. This chronic

upregulation of toll-like receptors could make a quite independent contribution to chronic inflammation and immune activation.

Neuroinflammation is central to CFS. Environmental toxins such as aluminum, arsenic, mercury result in the disruption of both the blood brain barrier and tight endothelial junctions in the gut resulting in mast cell activation disorders. These toxins also disrupt the DNA methylation machinery, as microbial DNA contains unmethylated CpG that results in activation-dependent DNA methylation. This aberrant DNA methylation leads to silencing of key transcription factors. Recent data suggests a critical role for mTOR signaling in the DNA methylation of critical transcription factors key to immune function Could this hypermethylation be a response to chronic pathogen stimulation [68]?

A key transcriptional regulator for expression of perforin and granzyme, key defects in the NK and cytotoxic T cells seen in many CFS patients is T-bet. T-bet is essential for the development of many experimental autoimmune diseases where pathogenic CD8+ T cells are thought to play a role [69,70]. T-bet promotes effector differentiation and function [71-73]. T-bet binds to the promoter regions of both the perforin and granzyme B genes in mice and humans [73]. T-bet expression in HIV/AIDS correlates with disease progression, as elite controllers demonstrate higher levels of granzyme and perforin than rapid progressors to AIDS. It has been shown that there is not a global immune defect but rather aberrant regulation of T-bet and other key transcription factors such PTEN, which regulates mTOR [74]. We propose hypermethylation of PTEN and T-bet as a mechanism of immune dysfunction in the CFS patients expressing an antibody to SFFV Env. Low PTEN leads to high mTOR and activated mast cells, all of which are part of the innate immune dysfunction seen in this CFS patient population. It has been suggested that mTOR sits at the crossroads of plasticity, memory and disease [75]. mTOR is also a sensor of mitochondrial function, and its chronic activation leads to hyperpolarization of mitochondrial membranes and the collapse of the electrochemical gradient across the inner mitochondrial membrane [76]. This predisposes to necrotic cell death and would go some way to explaining the observation of cell-free DNA and mitochondrial dysfunction in a cohort of patients afforded a diagnosis of CFS Activation of mTOR also occurs in an environment of chronic oxidative stress [76].

T-bet is exquisitely regulated by DNA methylation [77]. As we have previously shown, HIV infection results in aberrant methylation of cellular genes through the upregulation of the maintenance DNA methyltransferase, DNMT1 [78]. As with other immune abnormalities observed in HIV/AIDS, aberrant methylation occurred in both uninfected cells and those infected with defective proviruses [79].

As similar defects in perforin and granzyme expression in cytotoxic T cells and NK cells in CFS patients in our studies were observed, we hypothesized that aberrant methylation of T-bet may be one mechanism which could be easily diagnosed and treated with DNA methylation modulating therapeutic strategies in combination with anti-retroviral and cytokine therapy such as Viread and IFN-β respectively. A combination therapy using AZT and IFN-α has demonstrated beneficial results in Adult T Cell Leukemia (ATL) an extremely aggressive and previously untreatable cancer caused by HTLV-1 [80] such that in a subset of ATL patients, it is considered front line therapy. A recent clinical study on ATL showed that a shift in cytokine

production away from a Treg /Th2 phenotype toward a Th1 phenotype that occurred 30 days after treatment with arsenic/IFN-α/AZT may play an role in restoring an immuno-competent micro-environment, which enhances the eradication of ATL cells and the prevention of opportunistic infections [81]. This hypothesis is supported by the fact that HAART does not restore T-bet, perforin or Granzyme B in HIV/AIDS patients. Rapamycin and the flavonoid luteolin are inhibitors of m-TOR and modulators of DNA methylation that have shown benefit in HIV/AIDs, HAM/TSP as well as some patients with CFS.

In the peripheral blood of this CFS patient population, we have observed decreases in several lymphoid populations and marked increases in NKT cell numbers with substantial activation and maturation of DC and B cells as well as significant dysregulation in inflammatory cytokines and chemokines. These data plus previous data showing a decrease in NK cell functional subsets suggest that CFS subjects have a profound dysregulation in the innate immune response. Since all these innate immune cells have effector and suppressive functions on the immune response to pathogens, allergens and auto-antigens mediated by a complex mixture of cytokines and chemokines, further studies will be needed to determine the context in which the immune cells and the humoral mediators interact.

In this study, we have also demonstrated that PBMC isolated from SFFV Env antibody positive (thought previously to be evidence of XMRV infection and discussed below) CFS patients *ex vivo*, while possessing the same level of cellularity as normal controls, show a dysregulation of the immune response characterized by hyper-activation of myeloid cells, marked by mature CD83+, CD86+ CD69+ dendritic cells, CD91+ monocytoid cells, expansion of peripheral CD20+, CD23+ mature B cells and NKT cells with a concomitant decrease of CD3-CD19-CD56+ NK cells and total CD19+ B cells. Plasma profiling of cytokines and chemokines in these patients confirmed hyperactivation, showing highly significantly upregulated levels of proinflammatory molecules such a IL-6, IL-8, TNF-α, MIP-1α, β and MCP-1 and downregulation of IL13 and IFN-α. Some subsets have elevated TGF-β and others IL-9.

Our observations of hyperactivation of myeloid cells and the presence of B-cell CD20+CD23+ subpopulations in SFFV Env antibody positive individuals, which are not normally present to any substantial degree in the peripheral blood of uninfected 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, increased production of auto-antibodies and an increase in the frequency of B-cell malignancies. Such alterations, together with the immune evasion mechanisms of the virus, help to explain the deficiencies in antibody responses against HIV and other pathogens in HIV-infected individuals.

The observed high levels of pro-inflammatory cytokines and chemokines in CFS patients may explain some of the manifestations such as fatigue and flu-like symptoms and influence NK activity as we have shown herein and as have been previously described [55,56]. Further, the most significant defects shown herein are in the NK compartment and in particular the significant increase in NKT cells and concomitant decrease in $CD56^{DIM}$ NK cells. 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[82]. Chronically depleted glutathione following chronic immune activation would also lead to a decrease in NK activity seen in many CFS patients as would compromised ATP [2,83]. Interestingly, NK cells can become long-living reservoirs for HIV [84]. When this occurs, phenotypic changes in NK cell subsets can occur particularly in CD56 expression [20]. In HIV, the infected NK cells have lower CD56 expression and can also have decreased activity, including decreased perforin causing low cytotoxicity, or elicit significant or chronic immune activation through cytokine secretion [24,85-88]. NK and dendritic cells have been shown to be dysregulated in several chronic diseases including CFS. Similar to regulatory (suppressor) T cells and effector T cells, most innate immune cells have alternate functional states as M1 and M2 macrophages, effector and tolerized dendritic cells, invariant and non-invariant NKT, myeloid suppressor cells, mast cells, neutrophils and eosinophils. It will be important to ascertain the functional status of these cell types in CFS.

An excellent review[2] discusses the similarities of immune dysfunction in CFS and multiple sclerosis, an autoimmune disease also associated with expression of retroviruses including MSRV and HERV-W. Therefore, as an example, we will discuss HTLV-1 and its' causative MS-like disease HAM/TSP[88]. Several studies have assessed the characteristics of innate immune cells from persons infected HTLV-1. It has been demonstrated that the frequency of iNKT, NK, and dendritic cells in the peripheral blood of HAM/TSP and ATL patients is decreased [89,90]. One excellent study [91] showed that *in vitro* stimulation of peripheral blood cells with α-galactosyl-ceramide led to an increase in the iNKT cell number and a subsequent decrease in the HTLV-1–infected T-cell number in samples from viral carriers but not HAM/TSP or ATL patients [91]. Also, In HAM/TSP patients, iNKT cells with anti-HTLV-1 activity were depleted. Also, in these patients, both plasma interferon-α and PDCs the greatest producers of type 1 interferon in the body, are decreased *in vivo* [89,90]. We have found that PDC infected with HTLV-1 *in vitro* do not produce IFN-α (data not shown). These results suggest that iNKT and PDC cells contribute to the immune defense against HTLV-1, and iNKT- and PDC cell depletion play an important role in the pathogenesis of HAM/TSP and ATL.

PDC and iNKT cells are unique cells derived from lymphoid progenitors that regulate the immune response to microbes, cancers, and autoimmunity. They represent a very small number of the total number of cells in the peripheral blood. How can they have such profound effects on these many disease systems?
The answer to this is based in the contextual regulation of the multiple effector functions of activated, PDC and iNKT cells and their rapid production of large amounts of many cytokines. The distinct mechanisms of iNKT cell activation can partially control the resulting effector functions. Rapid production of cytokines — including IFN-γ, TNF-α, IL2, IL3, IL4, IL5, IL9, IL10, IL13, IL17, IL21 and $GMCSF_{57}$ — is a major outcome of iNKT cell activation [92]. As previously noted, PDC cells are the largest producers of type 1 IFN plus high levels of IL-12, IL10 etc. [93]. These cell types can also assist in cross presentation of antigens, which is important in the rapidity and magnitude of the antibody response. Besides the differences in how PDC and iNKT cells are activated, multiple phenotypically distinct subsets have been

identified. In addition, as PDC and iNKT cells orchestrate immune responses through their influence on other cell types, their localization and interactions with other cell types critically regulate the outcome of activation. It is interesting to note that a similar picture regarding expansion in NKT occurs in animal gammaretroviruses [94].

Reactivity to SFFV ENV: Viral, Cellular, Biomarker or Red Herring?

Serum from these CFS patients contained a reactivity presumably to an antibody, which recognized the spleen focus forming virus (SFFV) envelope protein in assay in which the SFFV Env is expressed on the surface of cells. This is a conformational epitope. Reactivity was specifically competed by a purified monoclonal antibody, 7C10, to SFFV Env, which recognizes all known polytropic and xenotropic gamma-retroviruses tested to date but does not recognize beta-retroviruses [95]. Antibody binding in CFS patients serum/plasma was NOT competed by antibodies to other human retroviruses, HTLV and HIV or by antibodies to endogenous beta retroviruses such as HERV K [96].

A recent paper examining 7C10 in gut biopsies from CFS patients claimed to show reactivity to 7C10 by proteins expressed the dendritic cells in the gut [96]. The authors suggested that the reactivity seen was a cross reactivity with HERVK but experiments done by us failed to show any reactivity to HERVK Env, which is expressed in significant levels in breast cancer cell lines such as MCF. This was to be expected since HERV K is a beta-retrovirus, which are not recognized by 7C10, confirming the original characterization of the monoclonal antibody [95].

What could this reactivity to SFFV ENV be recognizing in the human population? The first possibility is that the cross reactivity of antibody to a retroviral protein and to a cellular protein may be important in a disease process. For example, a recently identified cross-reactive antibody response between HTLV-1-p24-(gag) and peroxiredoxin-1 (PrX-1) could potentially contribute to the pathogenesis of HTLV-1 associated neurological disease via molecular mimicry [97]. Recent data from the same lab determined that the immunoreactive epitopes contained branched oligomannose and suggested that immunoreactivity to oligomannose on the infecting and host antigens contributes to molecular mimicry and may be important in the pathogenesis of HTLV-1 associated neurological disease [97]. This type of molecular mimicry has been implicated in the pathogenesis of neurological diseases such as Sydenham's chorea (SC), Guillain-Barre Syndrome (GBS) and multiple sclerosis (MS) [88, 97-100]. An emerging concept is that 'structural mimicry' (based upon the three-dimensional structure of molecules) rather than sequence homology is biologically important [101].

The second possibility is SFFV Env reactivity is to a cross reacting viral epitope created as mentioned above by natural pseudotyping or by recombination. This envelope would then have pathogenic properties similar to those described for the envelope of XMRV-like viruses [102]. The importance of minor changes in the envelope cannot be overemphasized. In animal models, it has been shown that two amino acids changes in the friend Mulv envelope changes the disease pathology from an erythroid leukemia to a paralytic neurodegenerative disease [103]. In addition, it has recently been discovered that a broadly reactive antibody-binding

protein (Protein M) from human mycoplasma binds with high affinity to all types of human and nonhuman immunoglobulin G, predominantly through attachment to the conserved portions of the variable region of the κ and λ light chains. Protein M blocks antibody-antigen union, likely because its large C-terminal domain extends over the antibody-combining site, and this blocks entry to large antigens [104]. Since some CFS patients can have high levels of mycoplasma the antibody response observed could be an underestimate.

A third possibility is that this cross-reactivity is a meaningless red herring. However, we consider this the least likely possibility given that the immune abnormalities detailed in this chapter of this specific patient population are characteristic of CFS patients in other studies with evidence of novel human retroviral infections [105-107] and who demonstrated a clinical response to the immune modulator Ampligen in a retrospective study of more than 300 patients. Conversely, in a multi-center study of an CFS patient population which did not share the immune abnormalities described herein, and had no evidence of novel human retroviral infection, the percent of CFS patients with an antibody to SFFV Env was the same as the healthy control population of 3-6%, suggesting that this antibody activity is at the very least a biomarker for a subgroup of CFS patients who may be more likely to respond to antiretroviral therapies and immune modulating therapies [108].

Retroviruses Revisited

At the time these studies were being conducted, little was known about XMRV infection in humans. The available data was consistent with the hypothesis that XMRV, like HIV and HTLV, dysregulated the innate and/or adaptive immune response either directly through infection of specific leukocyte subsets, or through dysregulation of cytokine and/or chemokine production. Since that time, the origin of XMRV thought to be involved in human diseases such as prostate cancer (PCa) [109] and CFS [108], was shown to be generated in a laboratory experiment through recombination. In this study, it was demonstrated that XMRV originated by recombination between two distinct endogenous MLVs [110].

At this time the preponderance of the data suggest that XMRV has no natural history of infection in man. The conclusion was reached that the recombination events were so rare that XMRV-like viruses were not a threat to humans [110]. However, recent published data are at odds with this and may help explain the antibody footprints of retroviral infection in this CFS population. To consider these data in the context of a retrovirus infection, it is useful to briefly review basic biology of retroviruses.

Retroviruses are a family of single-stranded RNA viruses having a helical envelope (which binds to a cellular receptor allowing entry) and containing an enzyme (reverse transcriptase) that allows for a reversal of the flow of genetic information, from RNA to DNA rather than the usual DNA to RNA. The newly transcribed viral DNA is now incorporated into the genome of the host (by the viral integrase). It is now called a provirus since it is the template for the production of new virions using the cell's transcriptional machinery. The provirus can persist indefinitely in the DNA of the host. The singular biology of retroviruses results in two powerful mechanisms

that could have profound effects on the development of human disease. First, its robust ability to undergo recombination allows the development of novel retroviruses even from two viruses that are replication defective, and it can capture and overexpress cellular genes such as oncogenes [111]. Second, phenotypic intermixing of viral components (pseudotyping) in the cell can alter the characteristics of the resulting viruses without recombination [112].

Although the chance of recombination events necessary to produce XMRV is exceedingly small, recent publications and the data presented in this chapter suggest that retroviral mechanism of pathogenesis in CFS should be revisited. First, while recombination to make an identical XMRV probably cannot occur, identification of other recombinant XMRV-like retroviruses can and has occurred. In 2010, the Owens lab reported that another xenotransplanted tumor cell line harbors a retrovirus sharing a 93% homology to XMRV. The virus was originally designated XMRV-2. When published, it was called B4rv not XMRV-2 [102]. However, the authors make conclusions so important that it stated the following:

"In summary, the studies described here have identified a second independent xenograft-derived MLV retrovirus designated B4rv that, like XMRV, has acquired the capability of infecting human tumor cells in vitro. Moreover, we provide novel evidence that infection of tumor cells with either XMRV or B4rv results in tumors that are larger and which exhibit multiple changes consistent with disruption of tumor vascular maturation including decreased perivascular cell coverage, and increased hemorrhage (importantly, it was demonstrated that these pathogenic effects of these viruses could be mediated by the viral envelope alone.) Although it is extremely unlikely that XMRV or B4rv have, or could infect humans, results herein raise the possibility that additional XMRV-like viruses may exist, or could evolve, that contain gene sequences that impact tumor pathogenesis, and of greatest concern might also acquire the ability to infect humans."

Furthermore, a recent publication demonstrated *in vitro* generation of multiple replication-competent retroviruses through recombination between preXMRV-1 and preXMRV-2 in human cells in ten days [113]. These novel retroviruses occurred during transfection showing that potentially pathogenic XMRV-like viruses can be made with greater frequency in the laboratory and easily infect human cells. *In vivo,* a replication competent helper virus that could co-package preXMRV-1 and pre XMRV-2 would be needed to allow recombination to occur during reverse transcription. These recombinants would be subjected to greater selection pressures and take longer to evolve.

It was been claimed recently that natural pseudotyping can occur between the viral components of XMRV and HIV *in vitro* [114]. The importance of this observation is that XMRV pseudotyped HIV-1 and not HIV-1 infects primary female lower genital epithelial cells. Thus, since in chronic diseases, viruses seldom travel alone, natural pseudotyping could expand the host range and pathogenic consequences of HIV. For XMRV-like viruses, an XMRV envelope with the pathogenic potential described by Murgai et al., [102] which evolved to enter man could have pathogenic consequences by pseudotyping a virus competent in replicating in humans.

Finally, Dr. Lipkin, in a public conference call with the Centers of Disease Control (CDC)(September 10, 2013) stated "We have found retroviral sequences in 85% of the sample pools. Again, it is very difficult to know whether or not this is clinically significant or not. And given the previous experience with retroviruses in chronic fatigue, I am going to be very clear in telling you I am reporting them in Professor Montoya's samples, neither he nor we have concluded there is a relationship to disease". Given the current climate this is certainly the politically correct position to take. Whether, it is the scientifically correct position remains open for future developments. Thus, the concept that XMRV-like viruses cannot be a threat to humans is premature and given the recent appreciation of the ease of recombination of animal and human endogenous retroviruses in the laboratory, man-made genetic elements and vectors including those in vaccines and genetically modified foods and the explosion of diseases associated with retroviral expression such as CFS, MS, ALS should be explored as a risk factor *in vivo* in the development and progression of these diseases.

Risk Factors for Disease Progression and Management

The safety of GM foods depends upon an assumption, that the DNA and protein in food is completely degraded by the digestive process into its component amino and nucleic acids, so the genetic sequence ingested doesn't matter. A growing body of literature is finding that this is not true, especially in patients with inflammatory diseases. In fact, it has been demonstrated that the presence of foreign DNA in human plasma is not unusual. The amount of DNA of foreign origin (plant, virus, bacteria) has been demonstrated to be higher in patients with IBD and other inflammatory diseases than in controls, and whole foreign gene fragments have been found in the human circulation [115]. Plant derived recombinant DNA has also been demonstrated in the terminal GI tract, in gut endothelium and in solid tissues of farm animals fed Roundup ready crops [116,117]. These foreign DNA particles stimulate the DNA methylation machinery leading to aberrant methylation patterns of cellular genes and increase the possibility of re-expression of latent retroviruses, as has been demonstrated in HIV infected individuals [118] and patients in the CFS population characterized in this chapter.

Cauliflower mosaic virus (CaMV) is a DNA virus that reproduces by reverse transcription. Its promoter fragment has been used extensively in GMO crops to activate transgenes. It has been documented in the tissues of trout fed GM soybeans. The promoter fragment was demonstrated in leukocytes, head, kidney and muscle of GM soy fed fish, but not when non-GM feed was used [119]. CaMV exists in nature, but for the purposes of GM food, it has been altered in ways that may increase risk. Variants of CaMV promoter, 35S, have been used in 54 of 86 approved transgenic plants in the U.S, including Roundup-Ready soy beans. In 2012, decades after CaMV 35S was known to be capable of harming other species [120], Podevin and du Jarden published a paper "Possible consequences of the overlap between the CaMV 35S promoter regions in plant transformation vectors used and the viral gene VI in transgenic plants". They concluded that the presence of segments of Gene VI "might result in unintended phenotypic changes". Gene VI encodes for a protein, P6, which is a transactivator of cellular RNA, inhibitor of cellular defense mechanisms and

inhibitor of RNA silencing. Numerous proteins produced in this setting could be toxins or allergens for humans and other animals, including essential insects. In addition, many examples of viral synergism and transcomplemetation, in which a viral protein expressed from a transgene support the infection of a virus, are presented in the literature, including transcomplementation involving species that are widely divergent phylogenetically, increasing the chances for recombination events to generate novel viruses or unintentional infection of new host species [121]. The risks of GMO for Immune deficient patients such as SFFV Env antibody positive CFS patients and the more than 10 million Americans expressing antibodies to SFFV should be considered at risk for methylation defects and aberrant expression of endogenous elements contributing to disease.

Co-infections and Disease Management

Our data support the hypothesis that as in some patient populations of retroviral associated neuroinflammatory diseases, HIV AIDS, HAM/TSP and MS, the resulting immune deficiencies described in these CFS patients allows activation and replication of many pathogens such that single pathogens never seem to travel alone. Thus, many patients with a diagnosis of CFS share complex pathogenic complications as in patients with HIV AIDS and. In many CFS patient populations, the presence of several concomitant infections, from mold to mycobacterium to reactivated exogenous and endogenous viruses, chronically dysregulating the immune system is a major risk factor in the development of pathology. Other risk factors include the damage from a distance in the brain caused by the constant presence of these mediators, alterations in microbiota regulation, in mitochondrial toxicity, and in methylation pathways. The good news is that a quarter century of knowledge in treatment strategies for concomitant infections, reactivated endogenous viruses and recently introduced environmental toxins afford new treatment strategies for CFS, a disease for which there is currently no approved treatment. It is appreciated that the time to market for the development of a new drug takes at least two decades. CFS in its most severe form reaches a level of relentless suffering that has made suicide a common cause of death, so waiting for scientific consensus and new drug development is simply not a clinical option.

The available data suggest numerous risks for disease progression as well as strategies for diagnosis, treatment and prevention for patient populations at risk of rapid disease progression. As a group, CFS patients tend to be prone to serious adverse events from even the gentlest of therapies. It is rare for any single intervention to have a major impact on the course of the disease. Knowledge of immune and biochemical abnormalities characterized in this patient population enable the physician to better assess the risk benefit ratio of various therapeutics. Combining therapies in a trial and error fashion is generally needed for a therapeutic response, and relapses are common following a period of better health and functioning. It is best to start with low doses and work up to a therapeutic dose, except where this is contraindicated, e.g. antimicrobials. Avoidance of triggers is often required for improvement, but limiting and isolating. Clear progression is seen in most patients over time.

Clinical success has been reported in small numbers of CFS patients with both aggressive immunostimulating and immunosuppressive therapies, e.g. Ampligen and Rituxan, which are both in clinical trials. In the context of the patient population with antibodies to SFFV Env, a retrospective study of more than 300 CFS patients with chronic active herpes virus infections and immune deficiencies seen in the SFFV anti-body positive population in this study revealed that the 30% demonstrated to be positive for SFFV Env were those who showed overall quality of life benefit (data not shown). This represents a clinical conundrum. Is the clinical presentation of a given individual due to persistent immune activation from an underlying latent retroviral infection or dysregulation of endogenous elements through hypomethlation. Should Immune modulation therapies, anti-inflammatories, methylation modulating drugs be used in combination with anti-retroviral therapy?

From a clinical point of view, anti-retrovirals may be useful, whether we are treating an exogenous retrovirus, an activated HERV or possibly even to prevent retrotransposition and the consequent damage to genomic DNA. There has been so little invested in scientific exploration of CFS that one must look to the literature concerning related diseases for answers. There is so much overlap with HIV and HAM/TSP, as outlined above, that it has been suggested CFS be renamed non-HIV AIDS. MS is another much studied disease with an extensive literature of similar aberrant immune deficiencies, aberrant methylation patterns and expression of endogenous retroviruses such as HERV-W and to inform which are detailed in a recent review paper that convincingly discusses the similarities and differences, "Myalgic encephalomyelitis/chronic fatigue syndrome and encephalomyelitis disseminata/multiple sclerosis show remarkable levels of similarity in phenomenology and neuroimmune characteristics" by Morris and Maes. There is also much overlap with HTLV/HAM/TSP. AZT in combination with IFN-α has been investigated for HTLV associated disease including T cell lymphoma [80].

MSRV, a nearly replication competent endogenous gamma retrovirus, HERV-W, has been shown to be associated with MS. MSRV produces a neuropathogenic viral envelope protein [122]. It has also been demonstrated to increase MSRV env copy load correlating with severity of illness.

Diagnosis with HIV/AIDS and treatment with HAART has been associated with remission of preexisting MS. There is a current clinical trial investigating the use of an integrase inhibitor, Isentress, for MS. A note of caution that Isentress should not be used as a monotherapy in SFFV antibody positive CFS patients, as unintegrated DNA can simulate expression of type I IFN pathway which our data suggests is defective in these patients. In these patients combination therapy of RT inhibitors and Isentress is recommended. The HIV/AIDS literature clearly demonstrates that even after control of the virus with HAART to the point where patients are not infectious and not at risk for AIDS defining complications, they have immunological abnormalities, display gut microbial translocation, develop coagulopathies and suffer increased all cause morbidity and mortality. There is much ongoing research concerned with persistent inflammation and premature aging in HIV/AIDS and

other diseases, such as diabetes, coronary artery disease and inflammatory bowel diseases, that may ultimately bear fruit for may CFS patients [123].

Reverse transcriptase inhibitors are the class of drugs most likely to be useful in modifying the disease process in the CFS patient population characterized here. AZT has been shown to be active against every studied retrovirus and has the additional advantage of crossing the blood brain barrier [124].

Extrapolating from the available evidence, neuroinflammatory diseases, including these CFS patients, that may be associated with retroviruses, which produce viral particles, whether endogenous or exogenous, suggest the possibility that HIV protease inhibitors might have an effect on progression. Andrew Mason has found evidence of a beta retrovirus related to MMTV (HBRV) in patients with primary biliary cirrhosis, a comorbid inflammatory disease seen in some SFFV Env antibody positive patients. In these patients Mason et al have found that Truvada and Kaletra is an efficacious treatment for these extremely treatment refractory patients [125]. At the time of this writing, Truvada has been deemed safe enough by the WHO to have been recently recommended as prophylaxis against HIV for people who are at risk of acquiring HIV. Tenofovir is a very safe drug with proper monitoring. However, it is possible that CFS patients with impaired mitochondrial function are more likely to experience a decline in renal function from Tenofovir, so more frequent monitoring than is done with HIV or healthy patients is in order.

Although scientific investigation into the role of retroviruses in the pathophysiology CFS is currently not being investigated, the risk to possible benefit ratio of a trial is low for patients fitting the profile described in this chapter. In particular, the reverse transcriptase inhibitors, Viread and Zidovidine, and integrase inhibitor, Isentress, have had some apparent clinical benefit in a number of patients.

Additional co-morbid infections in SFFV Env antibody positive patients.

CFS is clinically indistinguishable from chronic or treatment resistant Lyme Disease. Despite recent recognition that the incidence of Lyme Disease is much higher than previously acknowledged, available diagnostic testing is woefully inadequate. This has unfortunately resulted in the widespread use of empirical antibiotics in clinical practice for patients not proven to have a tick borne disease.

The use of indiscriminate antibiotics in a population of patients already suffering from dysbiosis and gut translocation is contraindicated. The popular myth that a prolonged “herx” is a positive clinical sign is incorrect. The term “herx” is short for Jarisch Herxheimer reaction, seen when secondary syphilis is treated with penicillin; it is accompanied by a rash and resolves in days. In the author’s opinion, this error has been responsible for considerable iatrogenic injury. Rather, a prolonged exacerbation of symptoms is a sign of a cytokine storm, indicating worsening of the disease state and, unless it resolves quickly, the treatment strategy should be reevaluated.

Tick borne diseases are clearly an emerging epidemic and no doubt contributing to the explosion of chronic complex inflammatory illnesses we are seeing. Antibiotics should of course not be withheld from patients with an untreated active infection, but rapid improvement is expected when an antibiotic is given in

therapeutic doses to treat a susceptible organism. A chronic exacerbation of an inflammatory state should not be the result. The evidence for efficacy of long-term antibiotics in this setting is lacking. However, trials of combination antiviral and antimicrobial therapy in the setting of immune deficiencies and chronic infection is warranted. The evidence for serious and persisting alterations in gut flora from even short courses of antibiotics is irrefutable. Antibiotic induced intestinal dysbiosis is known to cause damage that allows transmucosal penetration of pathogenic and commensal bacteria. The effects of long term antibiotics are largely unknown. The risk always needs to be weighed against any possible benefit.

Patients being evaluated for tick borne diseases should also be evaluated for not only Borrelia burgdorferi, but other species of Borrelia (STARI), ehrlichiosis, bartenellosis and babesiosis. Babesia microti, a malaria-like parasite which infects erythrocytes, is generally a self-limited infection in an immunocompetent human host, but can be difficult to eradicate in these multiply infected patients, similar to what is seen with this organism in AIDS. Rocky Mountain Spotted Fever, Q fever and tularemia are also in the differential. Emerging tick borne viral pathogens are of growing concern, e.g. Powassan virus, which has a 10% fatality rate.

Concurrent evidence of infection with various species of Mycoplasma, including M. fermentans and penetrans, are seen in many CFS patients including SFFV Env antibody CFS patients, as is found in other immunocompromised hosts, such as HIV/AIDS patients. AIDS related Mycoplasma species are believed to be a cofactor for AIDS progression [126].

Herpes virus reactivation and chronic expression is another frequent comorbidity in the many with a diagnosis of CFS including the SFFV Env antibody positive CFS patients**.** Several groups reported success treating CFS patients who have high EBV and HHV-6 titers with Valganciclovir. Clinical benefit including improved cognition and decreased fatigue in some patients, but without change in titers is reported [127,128] As Valganciclovir is not known to be active against EBV or HHV-6, one could speculate that it is immunomodulating for reasons other than direct antiviral activity. Valganciclovir can cause myelosuppression, as well as central nervous system effects such as insomnia, paresthesis and neuropathic pain, so it needs to be closely monitored [127]. Improvement has also been reported in CFS with Valacyclovir, in patients without evidence of CMV infection [128]. Clinical success has been reported with other antivirals as well, Acyclovir and Famciclovir, especially in patients with active HSV1/2 or shingles.

There are quite a few small randomized placebo controlled and crossover studies of Amantadine for MS fatigue suggesting possible efficacy, mechanism unknown, and there are anecdotal reports of benefit in CFS [129,130]. There has also been clinical benefit reported with herbal antivirals, such as Artemesinin and other Artemesia derivatives, which have broad spectrum activity against viruses [131].

CONCLUSION

Technologies like multiplex immune profiling, next generation sequencing (NGS) have afforded investigators the opportunity to look at complex chronic diseases such as CFS identifying new disease susceptibilities, therapeutic targets and drug

repurposing opportunities not previously appreciated. As the data innate immune deficiencies detailed in this chapter suggested underlying genetic defects in the nuclear genes regulating mitochondrial function methylation pathways and vitamin D receptor pathways. Though much more work needs to be done, targeting these pathways has shown promise for clinical benefit.

Recent advances in genomic technologies have identified more than one thousand nuclear genes that regulate mitochondrial function. We used an NGS diagnostic strategy to examine nuclear genes associated with a bedridden SFFV Env antibody positive CFS patient. The results revealed an abnormal autosomal dominant variant was found in the SCN4A gene, which is likely a pathological mutation identifying an underlying channelopathy. Similar pathological mutations found in two other patients with a similar diagnosis were found to be clinically responsive to the drug acetazolamide (Diamox), which has been used for decades in multiple conditions including altitude sickness and epileptic seizures.

Similarly, folate and one-carbon metabolism are linked to chronic disease risk through their integral role in DNA synthesis and methylation. Variation in one-carbon metabolism genes, particularly MTHFR, has been associated with risk of a number of diseases including many with a CFS diagnosis. The critical enzyme methionine synthase, required to convert 5-MTHF to THF and homocysteine to methionine, requires the active coenzyme methylcobalamin. This reaction is necessary for the normal function of every cell in the body. Adequate levels of Vitamin B12 are essential for normal nervous system function and deficiency can produce all of the symptoms of CFS. The reaction catalyzed by methionine synthase produces methyl groups necessary for proper methylation of DNA and proteins, DNA synthesis and repair, production of neurotransmitters and the essential antioxidant glutathione, normal function of the transsulfuration pathway. The other form of B12 that occurs naturally in the human body is adenosylcobalamin, which needs to be converted to methylcobalamin to catalyze this reaction. The most readily available form, cyanocobalamin leaves trace amounts of cyanide and should be avoided in these chemically sensitive patients.

Supplementation with folates can mask B12 deficiency. Folate and B12 are so intimately bound in a biochemical process essential for normal cell function that supplementation of the active metabolites in this patient population seems a given. As noted above, 5-MTHF requires some clinical finesse to optimize the dose, but most patients tolerate even large doses of methylcobalamin, sublingually or by subcutaneous injection. In a patient population with malabsorption of various causes, such as bacterial overgrowth, achlorhydria and gastric atrophy with poor production of intrinsic factor, injections should be considered.

The role of vitamin D in maintaining immunity is becoming appreciated. The concept of the vitamin D axis underlines the complexity of the biological events controlled by biologically active vitamin D (1,25(OH)(2)D3), its two binding proteins that are the vitamin D receptor (VDR) and the vitamin D-binding protein-derived macrophage activating factor (GcMAF) [132]. Both Vitamin D and GcMAF have shown clinical efficacy in CFS patients including the population characterized in this chapter. As VDR receptor polymorphisms are being increasingly implicated in the pathogenesis of a

number of diseases. The association is strongest with MS and Type 1 diabetes, and HIV AIDS but many other diseases are under investigation [133]. GcMAF and Vitamin D have also both shown efficacy in those populations. Because GcMAF is a human blood product, there are concerns about its' manufacture and safety. However, given it efficacy in small studies further research is warranted into VDR receptor polymorphisms, which may predict efficacy or resistance to GcMAF.

Thus, drug repositioning holds promise for bringing potential new therapies in an expedient manner to CFS treatment.

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