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NK Cells and Their Role in Disease and Cancer Treatment

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By

Alexander P. Sung

Dr. Dorothy Hudig/Dissertation Advisor

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We recommend that the dissertation
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ALEXANDER P. SUNG

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Dorothy Hudig Ph.D
Advisor

Subhash Verma Ph.D
Committee Member

Kenneth Hunter Sc.D
Committee Member

Gretchen Korver M.D./Ph.D
Committee Member

Julie Smith-Gagen Ph.D
Graduate School Representative

David W. Zeh, Ph.D., Dean
Graduate School

May, 2022

Abstract

Natural killer (NK) cells are a crucial part of the human immune system. They play an important role in fighting and controlling viral infections as well as killing cells that have transformed into tumors. NK cells are involved in the pathogenesis of a number of diseases and in this thesis we will explore NK cells and their role in disease and cancer treatment. First, we will discuss antibody dependent cell mediated cytotoxicity (ADCC) and how deficiencies in ADCC play a role in human disease. We will also cover improvements we have made to quantifying and measuring ADCC through an improved chromium 51 assay. Then we will look at chronic fatigue syndrome (CFS) and the role innate immunity and impairment of NK cells function contribute to the disease. We report the results of our research on patients with CFS and compare their innate immunity to their healthy family members. Finally, we will discuss NK cells and their role in cancer treatment as well as ongoing work involving development of an “off the shelf” NK cell therapy for cancer treatment through CRISPR/Cas9 gene editing and deletion of MHC I. The last chapter provides my insights into potential research directions for the study of human NK cells.

Dedication

To my loving mother Quenny my sincerest thanks. None of this would have been possible without your support.

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I would like to give my foremost acknowledgement to my research mentor Dorothy Hudig. She brought me up since I was an undergrad and gave me the skills and confidence to become a researcher. It has been my absolute pleasure to work with and learn from her for the past eight years. Thank you.

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Introduction

NK cell overview and role in the immune system

The immune system is divided into two general components. There is the innate immune system and the adaptive immune system. The innate immune system, which is the first line of defense, excels at pattern recognition of foreign invaders or host cells that have been compromised in some way such as through viral infection or conversion to cancer. The adaptive immune system excels at specific targeting of bacteria, viruses, and other harmful elements (1). In this dissertation, I asked how impaired innate immunity could contribute to Chronic Fatigue Syndrome (CFS) and explore ways innate immunity could be harnessed for anti-tumor immunotherapies.

Natural killer (NK) cells are a part of the innate immune system and display significant cytotoxic activity against cancer cells and cells that have been virally infected. NK cells have two major mechanisms in which they can help protect the body against foreign invaders. First, there is natural killer activity which is based on recognition of activation ligands induced upon viral infection of cells or associated with normal cell to tumor conversion (2). Then there is antibody dependent cell mediated cytotoxicity (ADCC) which can directly target harmful cells through coating with antibodies that are directed towards viral proteins in the membranes of infected cells. NK cells were first characterized in the 70s and were initially shown to be a different cell type which had the properties of both innate immunity and some antibody-based targeting similar to that of the adaptive immune system (3–5).

ADCC is not only a crucial mechanism for natural innate immunity against virally infected cells, it also plays a role in the mechanism of many immunotherapies against tumor cells, with rituximab being one such example (6–9). ADCC can be broadly broken up into two phases. The first phase involves recognition of antibodies that have been bound to a target cell. The second phase involves cytotoxic lysing of the antibody-coated target cell by the NK cell. The main receptor used by NK cells to recognize bound antibody is the Fc gamma receptor CD16A. This activating receptor recognizes the Fc portion of bound IgG and can in turn signal the NK cell to perform lysis. It was discovered in the 1990s that signaling for the CD16A receptor was similar to that of cytotoxic T cells. Activation of the CD16A surface transmembrane portion subsequently activates intracellular signaling via the CD3 Zeta receptor which activates intracellular signaling cascades via phosphokinase activity and calcium influx to begin the process of lysis (10, 11).

Within and in addition to the interaction between CD16A and the target bound antibody there are a number of factors that influence ADCC efficacy. Firstly, there are allelic variations in the CD16A receptor. Polymorphisms have been identified in the CD16A receptor which confer a lower or higher affinity for the Fc portion of bound antibody. At amino acid position 158 in the CD16A receptor there can be a valine or a phenylalanine. Presence of a valine produces a receptor which binds more tightly to antibodies leading to a higher efficacy of ADCC. Receptors that have a phenylalanine have a lower affinity for bound IgG leading to lower levels of ADCC (12, 13).

On the other side of the interaction there can also be differences in the structure of antibodies leading to differences in binding affinity. It has been shown repeatedly and confirmed through observations in our own experiments that glycosylation and fucosylation of IgG can have a dramatic effect on antibody affinity and killing capacity. In general, unmodified antibodies such as rituximab (an anti-B cell monoclonal antibody used for immunotherapy of lymphomas and leukemias) (14) have posttranslational fucosylation added by the antibody-producing cell. This Fc-fucosylation lowers the affinity of the Fc for CD16A receptors. Next generation glycoengineered antibodies such as obinutuzumab have no fucosylation and a higher affinity for receptors (15–18).

In addition to the primary interaction of CD16A with the Fc region of antibody, activation of NK cells is regulated by a host of inhibitory and activating receptors such as killer immunoglobulin-like receptor (KIR) and natural killer group 2 (NKG2), and natural cytotoxicity receptor (NKp) families of receptors. Many of these receptors including those in the KIR family and NKG2A are involved in recognition of healthy “self” cells through interactions with MHC I. Interactions with a self MHC I causes inhibition of the NK cells so that they do not kill healthy cells. Viruses often down-regulate MHC I proteins to avoid detection by cytotoxic T cells. Interactions with a target cell lacking MHC I will not have this inhibition of killing leading to NK cell mediated lysis of the targeted cell. For example, NKG2A binds HLA-E and the KIR2DL family of receptors binds HLA-C. Sensing of this binding causes inhibition of NK cell killing. There are also a host of activating receptors such as NKP 30, 44, 46 which detect cells that have been infected

with viruses and can cause activation of NK cells. Another example is NKG2D which can detect stress ligands such as MICA and MICB which produce an activation signal for NK cells (19, 20). It is the balance between activating and inhibitory signals that each NK cell receives that determines what and when it will kill another cell.

As previously discussed, ADCC is a vital component of the body's defense mechanism against virally infected cells and cancer cells. Measurement of ADCC has been crucial in developing new cancer treatments and understanding the body's ability to fight viral infections. The gold standard for measurement of ADCC has been the chromium 51 assay. In this assay a target tumor cell is labeled with radioactive chromium 51 which integrates into the cytoplasm. The target cells are then incubated with effector NK cells and target specific antibodies, which allows the effector NK cells to target the tumor cells and kill them. When the NK cells kill the tumor targets the chromium 51 which had bound to proteins in the cytoplasm leaks from the cells. By measuring the amount of radiation that has leaked out the percentage of cells that have been lysed through ADCC can be determined (21).

Work done by our lab has provided some significant improvements to the original chromium 51 assay eliminating the need for NK cell isolation and allowing for a smaller volumes of patient samples to be used. These improvements were reported in my first paper titled "An improved method to quantify human NK cell-mediated antibody-dependent cell- mediated cytotoxicity (ADCC) per IgG FcR-positive NK cell without purification of NK cells" detailed in chapter 2 of this dissertation. There are

three essential parts to a chromium assay, the lymphocyte effector, the antibody, and the tumor target. We have made improvements to all three components to develop an improved assay. On the effector side through the use of flow cytometry and procedural improvements we have eliminated the need for isolation of NK cells from peripheral blood mononuclear cells (PBMCs). The antibody used in out assay is the next generation of glycoengineered antibodies (lacking fucose) with increased affinity for the NK Fc receptor allowing for tighter binding and detection of killing at lower concentrations of antibody. For the target side our assay uses a tumor target cell Daudi which is poorly recognized by NK cells in the absence of antibodies, so that the ADCC killing is easy to detect over a low background NK (22).

NK cells play a vital role in the prevention of disease and are involved in a variety of health conditions. Impairment in NK cell numbers or function has been described as a component of a number of immunodeficiencies such as severe combined immunodeficiency (SCID), combined variable immunodeficiency (CVID), Chediak-Higashi, hyper IgE syndrome, and bare lymphocyte syndrome to name a few (23). A number of clinical sequelae can present as a result of the impairment of the NK cells. Increased susceptibility to a variety of viral infections such as CMV, Varicella, EBV among others is frequently seen in conditions with impaired NK cells. Presence or absence of certain KIR alleles can be associated with increased or decreased chances for certain health conditions. For example, certain KIR alleles in the KIR2D family are associated with increased ability to clear hepatitis B infection and delayed progression of HIV while

others in the KIR2D family are associated with increased chance of developing autoimmune conditions such as scleroderma, diabetes, and psoriasis (23–25). NK cells also seem to play a prominent role in pregnancy as up to 70% of immune cells found in the decidua are NK cells. Certain KIR phenotypes are associated with preeclampsia and new research shows that education of NK cells in the uterus has a large influence on pregnancy outcomes (26–28).

Chronic Fatigue Syndrome

Another disease where NK cells are involved and has also been a focus of our lab is Chronic Fatigue Syndrome/Myalgic Encephalomyelitis (CFS/ME). Some studies estimate the prevalence of CFS as high as 2 to 3 percent in the primary care setting with the majority of patients being middle aged women. There are a wide variety of societal guidelines and clinical definitions for CFS. The one most commonly used in the US is the 1994 CDC Fukuda criteria. These criteria define chronic fatigue syndrome as unexplained fatigue of new onset with limited alleviation by rest with fatigue lasting greater than six months. To meet the definition, a patient must also experience 4 of the following symptoms: subjective memory impairment, tender lymph nodes, muscle pain, joint pain, headache, unrefreshing sleep, post exertional malaise. CFS is a disease of exclusion with the presence of any other complicating disease that could explain the fatigue being exclusion criteria from diagnosis. Prognosis for the condition has largely poor outcomes with roughly 5% of people making a full recovery to pre-disease states and only between 20 and 50% of adults showing any improvement over the medium term of 12

to 39 months. Treatment for the disease is extremely limited with varying degrees of efficacy and mainly involve therapeutic lifestyle changes such as improving sleep hygiene and exercise therapy (29, 30).

Our understanding of chronic fatigue syndrome and its pathophysiology is in its infancy. The disease is receiving more attention now because similarity with "long-haul" Covid (31, 32). A number of potential influencing and contributing factors have been identified for CFS. Many of them revolve around infection and immune system dysregulation. There also seems to be a genetic predisposition for the disease with a relative risk increase of 2.7 times for those that have a first degree relative with chronic fatigue syndrome (29). Some studies have shown a connection between severe infections and development of CFS with 11% of people developing CFS after severe infection with one of several pathogens including EBV, Giardia, parvovirus B 19, and Q fever (33). A number of immune system changes have also been identified including a shift of the proportion of T1 and T2 helper T cells with a skew towards an increase in T2 helper cells (34, 35). Mitochondrial abnormalities have also been identified which results in impairment of oxidative phosphorylation (36, 37).

Of interest, especially to our lab, there is a widely reported link between CFS and innate immune dysfunction. Several studies have identified deficiencies in NK cell mediated immunity in people with chronic fatigue syndrome. There is a correlation between the severity of disease and the degree of NK cell dysfunction (38–40). In the quest for a biomarker for CFS, our lab was the first to explore deficiencies in NK cell

mediated ADCC in patients with CFS as well as their healthy family members. This is detailed in chapter 3 of this dissertation titled “Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) in Familial Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)”. Though our research showed that patients who have chronic fatigue syndrome have a statistically reduced capacity for ADCC, we also discovered that family members who do not have CFS also have statistically reduced capacity for ADCC as compared to non-related healthy controls. This new information suggests that though there is some genetic predisposition for reduced innate immune capacity there is some other factor that produces conversion from a higher risk state for CFS to actual development of the disease.

NK Cells in Immunotherapies.

Over the last several decades, a hot bed of research in the field of NK cell study has been around the use of NK cells in cancer therapies. As previously discussed, NK cells have natural anti-tumor capabilities and have a high cytotoxic capacity as well as a blend of the pattern recognition seen in innate immunity and more targeted recognition seen in adaptive immunity. A variety of different avenues are being pursued for development of NK cell based therapies. Broadly they can be broken down into two categories. There is enhancement of native NK cell function and there is modification of the NK cells themselves.

In the realm of enhancement of native NK cell function, a number of methods have been studied to enhance the natural killer and/or ADCC efficacy and capacity. At

the most basic level, NK cell targeting of tumors can be done with monoclonal antibodies such as the previously discussed rituximab and others that support an ADCC component like trastuzumab, anti-GD2 and cetuximab (41, 42). The next evolution of this monoclonal antibody-directed immunotherapy and a hot area of research has been bi-specific antibodies and single chain variable fragment targeting. With bispecific antibodies, they are engineered to have one part of the structure be anti CD16A to bind the NK cell activating receptor and the other part of the antibody to bind a tumor specific antigen. This intracellular crosslinking also creates an extremely strong immunological synapse and allows for increased cytotoxicity of the tumor targets by NK cells. In an effort to strengthen the immunological synapse some antibodies have even been engineered with only a single engineered protein chain containing both the light and heavy variable regions of the antibody that binds the cellular antigens and thereby reduces the size of antibody (43, 44). This design also simplifies and facilitates pharmaceutical production.

Another big area of interest has been immune checkpoint inhibitors for NK cells. There are a number of inhibitory receptors as previously discussed for NK cell cytotoxicity and blockade of KIRs and the NKG2 family of receptors is being explored as a therapeutic option to increase cytotoxicity. Others have gone in the direction of reducing CD16A cleavage by metalloproteases which can temporarily disable NK cell capacity for ADCC. This genetic modification along with targeting of other more

established immune checkpoint inhibitors such as anti PD-1 are also being explored (42, 45, 46).

Of special interest to me is the modification of the NK cells themselves. On this side of the research in the field there has been significant effort devoted to adoptive transfer of NK cells and genetic engineering of NK cells. Several studies have investigated improved methods to expand NK cells *ex vivo* and *in vivo* with different cytokine mixes many of which include IL-2. Addition of IL-12, IL-15, and IL-18 have also shown promising results in expansion of NK cells with features of "memory" cells (43, 47). Clinical trials have been run with adoptive transfer of these expanded and often additionally activated NK cells for cancer treatment, with promising results in both solid and liquid tumors (44, 45, 47). Another forefront in NK cell modification for cancer treatment has been genetic engineering and production of chimeric antigen receptor (CAR) natural killer cells (CAR-NK). These cells which have been genetically modified to express receptors which can bind tumor antigens have shown extremely promising results in clinical trials (48, 49). The T cell version of this technology has already made its way onto the market and is in use in clinical practice (50). There have also been clinical trials utilizing the tumor NK cell line NK92 which possesses higher proliferation capacity then primary NK cells (50–52).

My particular area of interest within NK cell modification has been production of an off the shelf primary or tumor NK cell line which can evade host immune system detection. One difficulty with modification of NK cells is that human beings recognize

the cells of others as foreign via differences in MHC I proteins, differences that occur in all pairs except monozygotic (identical) twins. Autologous cells from the patient themselves must be harvested and purified then modified, which can yield a low initial number of cells which require massive, costly expansion. Off the shelf non-autologous solutions suffer from a short half-life in the blood as they have non-self MHC I proteins, will be immunogenic and thus will be targeted by the host immune system. We have proposed genetic engineering of NK cells which lack all their MHC Is, the crucial molecules for T cell recognition of foreign cells. An ongoing project to explore this concept was funded by an American Association of Immunologists Fellowship that is detailed in chapter 4 of this dissertation titled “Development of Immunologically Undetectable and Highly Cytotoxic Natural Killer Cell Lines Suitable for Transfusion and Treatment of Multiple Cancer Types” with a goal to develop a non-immunogenic off the shelf NK cell line and explore its applications for cancer therapy.

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Chapter 2

An improved method to quantify human NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) per IgG FcR-positive NK cell without purification of NK cells

Abstract

Natural killer (NK) lymphocyte ADCC supports anti-viral protection and monoclonal antibody (mAb) anti-tumor therapies. To predict *in vivo* ADCC therapeutic responses of different individuals, measurement of both ADCC cellular lytic capacity and their NK cellular receptor recognition of antibodies on 'target' cells is needed, using clinically available amounts of blood. Twenty ml of blood provides sufficient peripheral blood mononuclear cells (PBMCs) for the new assay for lytic capacity described here and for an antibody EC_{50} assay for Fc-receptor recognition. For the lytic capacity assay, we employed flow cytometry to quantify the CD16A IgG Fc-receptor positive NK effector cells from PBMCs to avoid loss of NKs during isolation. Targets were ^{51}Cr-labeled Daudi B cells pretreated with excess obinutuzumab type 2 anti-CD20 mAb and washed; remaining free mAb was insufficient to convert B cells in the PBMCs into 'targets'. We calculated: the percentage Daudis killed at a 1:1 ratio of CD16A-positive NK cells to Daudis (CX1:1); lytic slopes; and $ADCC_{50}$ lytic units. Among 27 donors, we detected wide ranges in CX1:1 (16-73% targets killed) and in lytic slopes. Slope variations prevented application of lytic units. We recommend CX1:1 to compare individuals' ADCC capacity. CX1:1 was similar for purified NK cells *vs.* PBMCs and independent of CD16A V & F genotypes and antibody EC_{50}s. With high mAb bound onto targets and the high affinity of obinutuzumab Fc for CD16A, CX1:1 measurements discern ADCC lytic capacity rather than antibody recognition. This assay allows ADCC to be quantified without NK cell isolation and avoids distortion associated with lytic units.

***Abbreviations used: ADCC,** antibody-dependent cell-mediated cytotoxicity; **CD16A,** cluster of differentiation designation for the Fc-gamma type III low affinity receptor for IgG, found on NK cells; **CD20,** cluster of differentiation designation for a B cell surface protein; **CX1:1,** the % cytotoxicity mediated at a 1:1 E:T ratio of CD16A cells to Daudi cells pretreated with antibody; **EC_{50}**, the "effective concentration" of antibody in the ADCC assay that will support 50% of maximal lysis that is caused by saturating antibody concentrations; **E:T,** effector to target ratio; **F and V alleles of CD16A**, alleles that encode phenylalanine (F) or valine (V) at amino acid position 158 of the protein; ***FCGR3A*,** the gene that encodes CD16A that binds human IgG 1&3 antibodies; **Fc,** the invariable, heavy-chain, crystallizable fragment of immunoglobulins; **FcR,** immunoglobulin Fc-region receptor; **KIR,** killer-cell immunoglobulin-like receptor; **LU_{50},** the number of effector cells required to kill 50% of the 10^4 target cells in the experimental assay wells, expressed as the number of LU_{50}'s per 10^6 effector cells; **mAb,** monoclonal antibody; **NK,** natural killer cell; **PBMC**, peripheral blood mononuclear cell.

4. Introduction

Antibody dependent cell-mediated cytotoxicity (ADCC) is an important mechanism used by natural killer (NK) lymphocytes to target virally infected cells, reviewed (Gunn and Alter, 2016), and is a major effector mechanism operating in many antibody-based tumor immunotherapies (Alderson and Sondel, 2011; Boross and Leusen, 2012; Busfield et al., 2014). Patients with low ADCC have a higher risk of developing lesions due to herpes virus infection (Moraru et al., 2015) Lower ADCC may also contribute to poor responses to additional chronic viruses. Patients with low ADCC may have lowered responses to monoclonal antibody (mAb) tumor immunotherapy (Monteverde et al., 2015). For example, rituximab (Rituxan^{R}) is a pharmaceutical mAb used to treat several B cell tumors and elicits widely variable individual therapeutic responses (Cartron et al., 2011). Even the most positive clinical trials reported only a 73% response rate to rituximab with CD20-postive tumors (Colombat et al., 2001) with others reporting much lower response rates (Pan et al., 2002; Conconi et al., 2003; Younes et al., 2003), indicating that some patients fail to respond. The non-responders included many patients with the favorable CD16A (IgG Fc-receptor) AA158 V/V genotype that binds IgG optimally (Liu et al., 2016), raising the possibility that low ADCC lytic activity as well as low CD16A recognition of antibody Fc could contribute to poor outcomes. If this is so, patients could be screened for ADCC lytic activity before starting therapy (Battella et al., 2016), and potentially nonresponsive patients could be recommended for another treatment or be treated to increase their ADCC.

NK cell-mediated ADCC has two stages: first, cellular recognition of antibody-coated cells and second, cytotoxicity after recognition. ADCC assays for recognition of antibodies have characterized differences between AA158 F (phe) and V (val) CD16A receptor-bearing NK cells (Hatjiharissi et al., 2007; Treon, 2010) and have been used to determine the therapeutic significance of individual differences in antibody recognition for anti-cancer immunotherapies (Cartron et al., 2002; Cartron, 2009; Dahal et al., 2015). It has been a major challenge to create a quantitative assay for cytotoxic lytic capacity, particularly for an assay suitable to compare the ADCC of different donors, as variations in lytic slopes complicate use of lytic units. Donor differences in NK cell inhibitory receptors and low clinically available amounts of blood add to the challenges. Here we describe an improved assay for comparing ADCC lytic activity based on the CX1:1 values (efficiency of ADCC at a 1:1 CD16pos NK cell to antibody-coated target cell ratio). The assay utilizes Daudi 'target' cells that lack the MHC class 1 proteins that serve as ligands for most of the NK cell inhibitory receptors. The assay has the capability to identify lower ADCC activity (in addition to antibody recognition) as a risk in diseased *vs.* healthy patients and has the potential to predict patient responses to monoclonal anti-tumor therapies.

The new assay for lytic capacity, illustrated in the graphical abstract of this paper, has four aspects that promote measurement of ADCC. *One*, pretreatment of the Daudi B tumor cells with antibody (and washing away unbound antibody) *before* addition of the Daudi cells to the assay allows B cells in the peripheral blood mononuclear cells (PBMCs)

to go un-recognized as 'target' cells. Customarily all components (PBMCs, labeled target cells, and antibody) are put into the assay together. However, anti-CD20 antibody will bind to both healthy B cells and radiolabeled Daudi cells, creating "cold" target competition to interfere with killing of the Daudi cells. The new assay allows use of PBMCs without the problem of 'cold target' competition. *Two*, the anti-CD20 mAb, obinutuzumab (Gagez and Cartron, 2014), has advantages. Obinutuzumab is genetically engineered in the Fc-region for high affinity to the CD16A FcRs of NK cells, and is also non-fucosylated, so that NK cells bind better to this mAb. Thus obinutuzumab supports better ADCC than rituximab (Awasthi et al., 2015; Liu et al., 2015; Herting et al., 2016). As a type 2 monoclonal antibody, obinutuzumab remains on the surface of B cells unlike type 1 mAbs like rituximab which are internalized (Beers et al., 2010). A substitution from valine (V) to phenylalanine (F) at amino acid number 158 in the NK cell IgG Fc-receptor CD16A creates alleles encoding different affinity receptors, with the V/V genotype having the best recognition of antibody on cells to support better ADCC activity at low antibody concentrations (Wu et al., 1997; Dall'Ozzo et al., 2004; Hatjiharissi et al., 2007). However, at saturating obinutuzumab concentrations on target cells no difference in ADCC was observed between V/V and F/F CD16A genotypes in the assay reported here. Thus avidity due to persistent type 2 antibody and the high affinity of obinutuzumab for CD16A were dominant over the differences in CD16A receptor-Fc affinity conferred by the V & F alleles. In addition, we used a non-fucosylated version of obinutuzumab that further increased its interaction with CD16A. *Three,* the Daudi B cell tumor line lacks MHC 1 (Seong et al., 1988). This lack of MHC 1 prevents KIR

interactions and their negative regulation of ADCC that contributes to variation among people (Binyamin et al., 2008; Borgerding et al., 2010; Terszowski et al., 2014). NKG2A and CD85j also bind to MHC 1 molecules to down-regulate ADCC (Braud et al., 1998; Roberti et al., 2015), so that it is likely that the new assay with MHC 1-negative target cells measures the near maximal ADCC lytic capacity of individuals. *Four,* the new assay allows for accurate measurement of the NK cells in ADCC without the need for NK cell isolation. We used TruCount^{R} beads with flow cytometry to measure exactly how many CD16A- positive NK cells present in the PBMC samples used in the ADCC assays (Nicholson et al., 1997). By eliminating the need for NK cell isolation, our assay is suitable for clinically available amounts of blood.

Here we describe our improved assay that can quantify human ADCC regardless of CD16A FcR affinity and KIR regulation and that does so without isolation of NK cells. This assay detected wide variations in ADCC between healthy human subjects. We recommend use of the CX1:1 measurement to compare ADCC lytic capability of healthy human subjects and patients.

2. Materials and Methods

2.1 Blood Donors and Whole Blood Collection

Twenty-seven healthy peripheral blood donors at the Bateman Horne Center (BHC, Salt Lake City, UT) participated as subjects for evaluation of the ADCC ^{51}Cr assays. Supplement Table 1 provides their gender, ages, CD16A genotypes, as well as the ADCC values we determined in this study. Healthy was defined as HIV-negative, clear of overt infections at the time of blood donation, and without diagnoses of chronic fatigue syndrome (CFS). Thirteen healthy donors were unrelated and 14 healthy donors belonged to five different multigenerational families that had two or more CFS patients. (This research was funded in part by an NIH grant to characterize ADCC of CFS patients.) The blood samples were collected with heparin at the Bateman Horne Center and shipped overnight to Reno, NV. The CX1:1 and EC_{50} values (Figs. 1-3) were determined from 20 ml of these blood samples. Two additional blood samples from healthy donors were collected at the University of Nevada, Reno School of Medicine (UNR SoM) with EDTA as the anticoagulant and the isolated PBMCs frozen in liquid N_2 until use; these cells were used only to compare ADCC by purified NK cells *vs.* PBMCs (Fig. 4). The use of human subjects was approved by IBR protocols issued to both the Bateman Horne Center and Drs. Barao and Hudig at the University of Nevada, Reno. Informed consent was obtained after the possible consequences of the studies were explained. De-identified frozen PBMCs, collected from citrated blood, were purchased from Stem Cell

Technologies (Canada) in order to have sufficient cells to evaluate the potential of frozen PBMCs as internal standards and used only for this purpose (Fig. 5).

2.2 PBMC Isolation, Culture and Storage

PBMCs were isolated at UNR from blood from the BHC or UNR from 20 ml whole blood by ficoll-hypaque density gradient centrifugation (Boyum et al., 1991). The PBMCs were resuspended into complete assay media, 90% Dulbecco' s complete media with high (4.5 g/L) glucose with L-glutamine (Corning), 10% Fetal calf serum (Atlanta Biologicals), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). PBMCs from BHC donors were cultured overnight at 1-2 x 10^6 cells/ml. Frozen PBMCs, prepared at UNR or purchased (from Stem Cell Technologies), were used only to compare ADCC by purified NK cells *vs.* PBMCs and to evaluate frozen PBMCs as internal standards. The frozen cells were thawed and cultured overnight before use which restores ADCC activity to frozen cells (Mata et al., 2014). One lot of fetal calf serum was frozen and used for all the experiments to reduce intra-experimental variation. One lot of 75 mm tissue culture flasks (Biolite, Thermo Scientific) was used throughout the experiments.

2.3 Target Cell Line and Antibodies for ADCC

The Daudi lymphoma cell line (Patarroyo et al., 1980) was obtained from the ATCC (Manassas, VA, catalog # CCL-213) and cultured with RPMI media (per ATCC

instructions) at 5.5% CO_2 and 37^0C. Passages 10-80 were used as targets cells in the assays at $1x10^4$ cells/well. The non-fucosylated anti CD-20 monoclonal antibody obinutuzumab (brand name Gazyva^R; also reported as GA101) (Mossner et al., 2010; Bologna et al., 2011; Niederfellner et al., 2011) was obtained from Roche Innovation Center, Zurich, Switzerland, and used to trigger ADCC in the assay. Obinutuzumab was produced in CHO cells that lacked the ability to fucosylate antibodies (Mossner et al., 2010).

2.4 ^{51}Chromium Release ADCC and NK Assays

4 hr assay ADCC and NK assays. Daudi targets were labeled for 1 hour with 0.5 mCi $Na^{51}CrO_4$ (Perkin Elmer, Waltham, MA), cultured in assay media, complete media supplemented with 10 mM hepes (Sigma-Aldrich). The Daudi cells used as targets for ADCC were then pretreated with 1 ug/ml of obinutuzumab antibody for 0.5 hour at room temperature then washed 5 times to remove excess antibody and chromium. One µg/ml of obinutuzumab was determined to be saturating for Daudi cells using flow cytometry with FITC-mouse IgG2a anti-human Fc mAb (clone HP6017, BioLegend, San Diego, CA). We tested for residual antibody in the supernatant of the last wash to confirm that it was too low to support ADCC when added to ^{51}Cr-labeled Daudi cells with PBMCs. Daudi cells used as targets for NK were handled similarly but without added antibody. The cell concentrations were determined by hemacytometer counting of >600 Daudi target cells. PBMCs were evaluated by flow cytometry to determine the

CD16Apos NK cell concentrations. PBMCs were diluted 2-fold in quadruplicate in V-bottom plates (Costar 3894, 96 well) in 0.1 ml to create six CD16A NK effector to target cell ratios that were obtained from the flow cytometric TruCounts. Daudi cells (1 $x10^4$ in 0.1 ml), (with or without mAb) were added per well, for ADCC or NK activities, respectively. Plates were spun for 3 minutes with low acceleration at 1000 rpm and incubated for 4 hours at 5.5% CO_2 and 37^0C. After incubation, plates were spun down for 10 minutes at 1000 rpm and 0.1 ml of supernatant was taken for analysis in a Packard Cobre II gamma counter. Percent specific release was calculated using the formula = [(Experimental counts – Spontaneous Release)/(Max – Spontaneous Release)] x 100. Spontaneous release was the leak rate of targets with no effectors and the Max was the radioactivity released by targets lysed with 1% SDS. Spontaneous release values were less than 25%.

NK activity towards the Daudi cells was monitored separately using 6 dilutions of PBMCs in quadruplicate in the microplate wells with the number of CD3negCD56pos cells as the denominator for activity per cell.

A second sampling of ADCC at 6 hours was made to verify that ADCC was complete at 4 hours. After the collection of supernatants at 4 hrs, the microtiter plates were returned for an additional 2 hours of incubation, 0.1 ml of assay media was added to each well, the plates were centrifuged again, and 0.1 ml of supernatant was collected from each well and counted separately. The ^{51}Cr released at 6 hours for individual wells was calculated as follows: (0.5*4 hr cpm released – (0.5*machine background)) + 6 hr cpm

released) and these 6 hr values were used to calculate the % specific release using the equation indicated above.

CX1:1, lytic slopes, and LU_{50} per 10^6 CD16A-positive NK cells. Data analyses from 27 donors are illustrated in Fig. 1 and were determined as follows. Values for individual wells were plotted as y = % specific ^{51}Cr release *vs.* x= the $\log_{10}$ of the Trucount^R effector cell to Daudi target cell ratio. The effector cells were the CD16Apos NK cells for ADCC and CD56pos NK cells for NK activity. The linear range of cytotoxicity *vs.* E:T usually spanned all 6 E:T cell dilutions and was used to calculate y =mx+b, where b = the y intercept = CX1:1 (because 10^0=1) and the lytic slope m is the increase in the % of dead Daudi cells per 10-fold increase in effector cells. The P values for linearity were <0.05, with R^2 values >0.8. To calculate one lytic $unit_{50}$ (LU_{50}) (Pross et al., 1981; Pross et al., 1986) as the number of effector cells needed to kill 50% of the target cells (5,000 cells), we solved the equation ((50-b)/m) = x, so the antilog of x is one LU_{50}. For comparison of ADCC and NK activities, we expressed cytotoxicity as the number of lytic $unit_{50}$ per million effector cells.

EC_{50}. The effective concentration of antibody needed to support 50% of maximal ADCC (EC_{50}, (Chung et al., 2014) is a measurement of NK cell receptor engagement of antibody on the target cells. The final obinutuzumab antibody concentrations in the wells were in seven 4-fold dilutions starting with 0.25 µg/ml as the highest concentration. The PBMCs were at a high ratio to Daudi cells (20-30:1) in order to ensure a substantial maximal

lysis (between 30-65%); wells were in triplicate or duplicate depending on the cells available from each donor. We had insufficient cells for EC_{50} determinations for two donors.

2.5 Flow cytometric analyses. ***Instrumentation and software.*** The instrument was a BD Biosciences Special Order Research Product (SORP) LSR II analytical flow cytometer with a High Throughput Sampler (HTS) unit for antibody-labeled samples in plates. The LSR has four lasers (405 nm, 488 nm, 561 nm and 640 nm) and 18 fluorescent detectors. The data were gated and analyzed using FlowJo versions 9 and 10 (FlowJo, LLC, Ashland, OR).

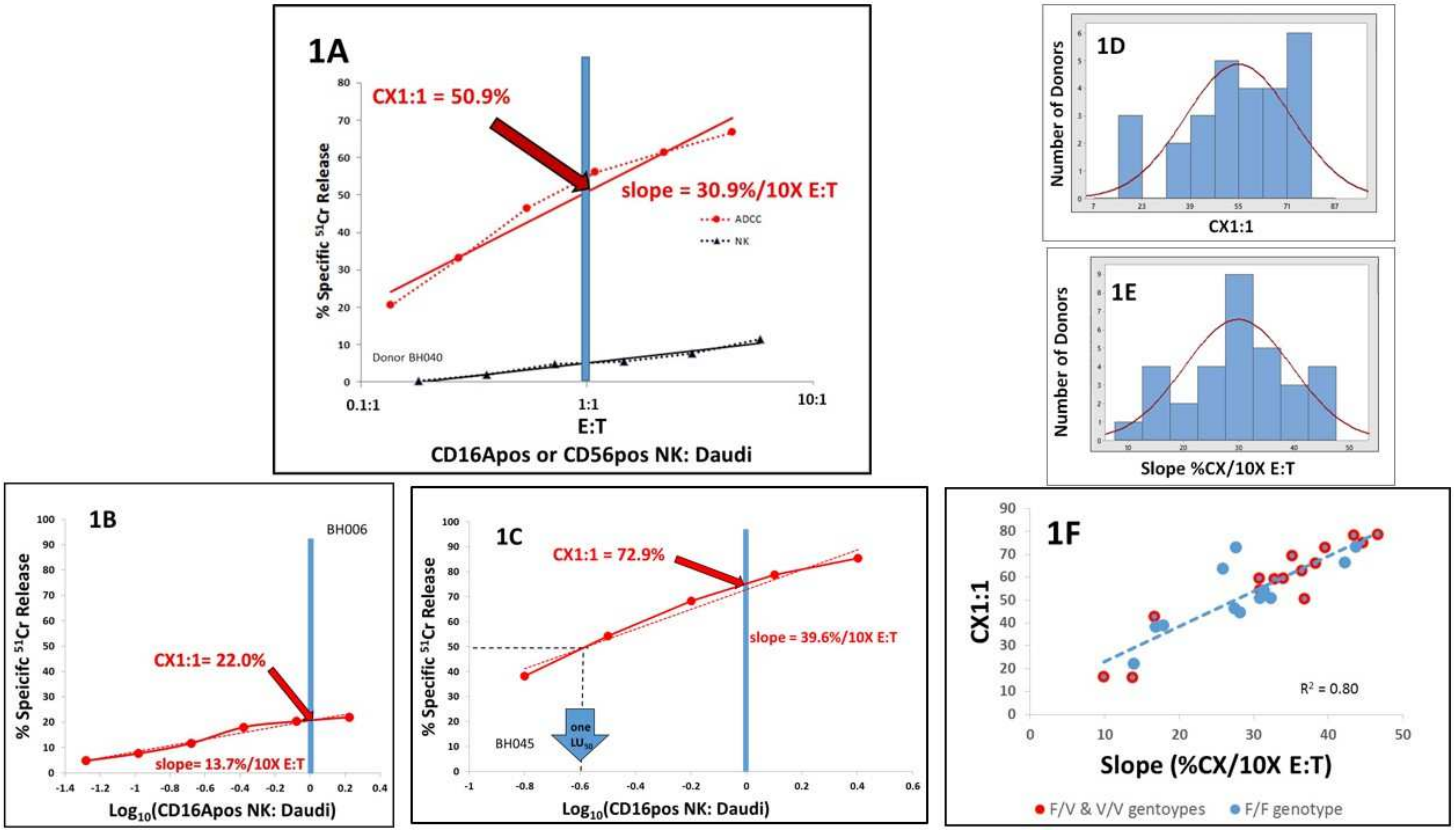

Figure 1. CX1:1 measurement of NK cell mediated-ADCC. ADCC was directed towards Daudi tumor cells that were pretreated with obinutuzumab (Gazyva^{R}), a type 2 anti-CD20 monoclonal antibody and then washed; NK was directed towards Daudi cells without antibody. CX1:1 values (% dead cells at a 1:1 E:T) were calculated from linear regressions ADCC *vs.* the log10 CD16A E:T as illustrated in **A-C**. Symbols represent average values of 4 wells; standard deviations were usually less than 2%. Blue vertical lines mark the 1:1 ratio of CD16Apos NK cells: Daudi cells. Red arrows indicate the CX1:1 values. Determination of one ADCC lytic unit$_{50}$, the number of CD16Apos NK cells required to kill 50% of the 10^4 Daudi target cells per well, is illustrated in **C**. LU$_{50}$ values

are provided in the legends to illustrate how inappropriate comparison by LU_{50} is when the slopes of cytotoxicity differ greatly (in **B** the LU_{50} is off scale). **A. ADCC and NK activities of a representative donor.** The X axis indicates the log10 of the E:T ratios for either the CD16Apos cells (for ADCC) or the CD56pos NK cells (for NK activity); the CD16Apos NK cells were 75.3% of the CD56pos NK cells. The solid red line of ADCC indicates the linear regression of 24 wells, 4 per E:T, that determined the CX1:1 and the slope of cooperative killing. This CX1:1 was near the median among the donors. In this case, one ADCC LU_{50} was 9,364 CD16Apos NK cells. ADCC R^2 was 0.96. NK activity is shown in black and was negligible compared to the ADCC. **B. ADCC of a donor with a low CX1:1**. The slope of cytotoxicity was also low. One LU_{50} (>1 $x10^6$ CD16Apos NK cells) is off the scale. $R^2 = 0.90$. **C. ADCC of a donor with a high CX1:1**. The blue arrow indicates the log_{10} of the E:T for one lytic $unit_{50}$ (2,643 cells for this donor). $R^2 = 0.98$. **D. Frequency distribution of CX1:1 values of 27 donors.** The range was 4.8 fold, with CX1:1s from 16.2 to 78.4%, a median of 59.2%, and an average +/- standard deviation of 54.9 +/- 17.7%. The P=0.097 for a fit to a normal distribution, and was too high for a statistical significance at a $P < 0.05$. A bimodal distribution might become discernable with a larger set of donors. **E. Frequency distribution of slopes of cytotoxicities.** The range of slopes was 4.7 fold, from 9.9 to 46.7% cytotoxicity per 10-fold increase in E:T, with a median slope of 30.9% and an average of 29.8+/- 9.8%. The P>>0.1 indicates a normal distribution**. F. Positive correlation between CX1:1 and the slope of cytotoxicity.** The Pearson coefficient of 0.895 indicates a strong linear correlation; the R^2 value of 0.80 indicates that a few donors deviated from the linear relationship that is

illustrated by the dashed line. Fifteen donors with F/V & V/V CD16A genotypes are indicated with red symbols, while blue symbols indicate the 12 donors homozygous F/F for the lower affinity FcR allele. Note that many F/F donors have high CX1:1 and slopes of cytotoxicity.

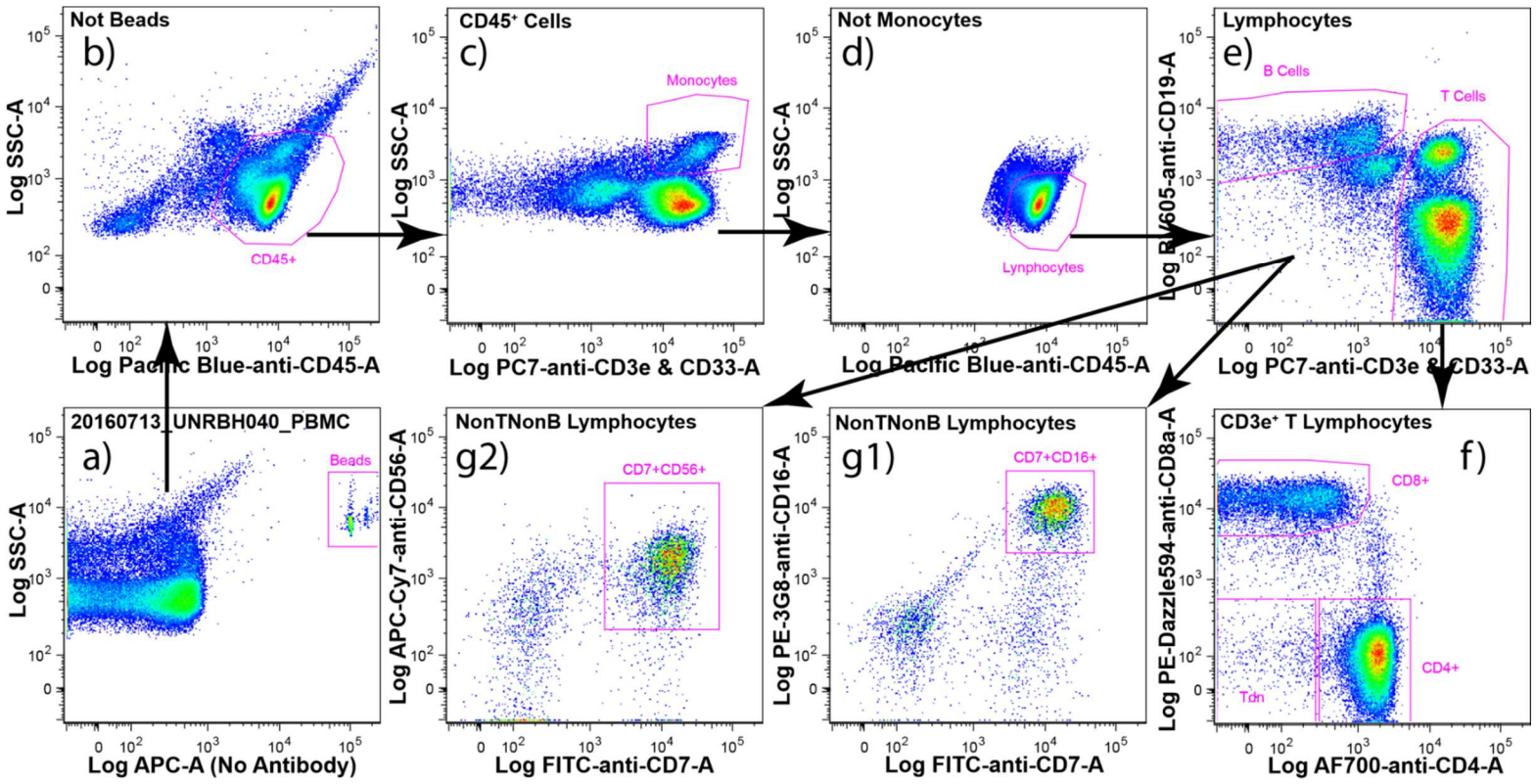

Figure 2. Flow cytometric determination of the CD16Apositive NK cells in the PBMCs used for the ADCC assays. It was critical to differentiate the CD16Apos, CD7pos NK cells from CD16AposCD7neg monocytes, so that the CD16a positive NK cell counts could serve as the denominator to calculate the CX1:1 values. PBMCs were placed in TruCount^{R} tubes containing fluorescent count beads (indicated in **a**) and labeled with the 9 fluorescent mAbs listed in the methods. Immediate flow cytometry of unwashed labeled cells and beads ensured accurate ADCC effector cell counts. The progressive gating of cells is indicated with letters that start in the lower left corner with (**a**) and end with CD16Apositive NK cells (**g1**). Cells of donor BH040 of Fig 1A are illustrated.

2.6 Isolation of NK cells for comparison of ADCC by PBMCs and by purified NK cells. We used the Stem Cell Technologies EasySep Human NK Isolation Kit, a magnetic bead based negative selection method, to bind and deplete B & T cells and monocytes from PBMCs. All the antibodies in this kit are mouse IgG1 an isotype that does not bind to human CD16A (unlike mouse IgG2a that binds to human CD16A). The control of unseparated PBMCs was also transferred into the EDTA-containing RoboSep Buffer for a similar duration as the bead-treatment for NK cell isolation, centrifuged and replaced into ADCC assay buffer to ensure equal potential loss of activity due to cell handling. The isolated NK cells were >90% CD56pos lymphocytes.

2.7 CD16A AA 158 V and F genotypes. The F/F, V/F and V/V genotypes at AA158 of CD16A were determined by PCR and DNA sequence analysis at the Frederick National Laboratory for Cancer Research, Frederick, M.D., by Stephen K. Anderson, Ph.D. Amplicons specific for the CD16A gene (and excluding the CD16B gene) were generated with forward and reverse PCR primers, (5' to 3') for CD16 (TCCTACTTCTGCAGGGGGCTTGT) and (CCAACTCAACTTCCCAGTGTGATTG), respectively. The amplicons were directly sequenced using Sanger methodology to determine the genotypes (Wu et al., 1997). The F/F genotype was also distinguished from V/F & V/V genotypes by flow cytometry using the MEM-154 clone of anti-CD16 mAb (PE-tagged, Pierce Chemical Co, Rockford, IL) to identify CD16A F/F donors. MEM154 reacts only with the AA 158 V genotype of CD16A protein but not the AA158 F genotype (Bottcher et al., 2005). MEM154 also reacts with CD16B (that has only the valine form); CD16B is

present on neutrophils but not on NK cells. Genotypes determined by PCR-DNA sequencing and by flow cytometry were concordant: MEM154 reacted with V/V and V/F genotypes and was non-reactive with F/F genotypes *with PBMCs*. However, the approach was suitable only with PBMCs; when whole blood was labeled, the neutrophils absorbed most of the MEM-154 mAb so that the V/V and V/F NK cells of whole blood labeled poorly with MEM154.

2.8 Statistical analyses. We used the Excel Analysis Tool Pack for Student's t-test, best fit for linear regressions, and other statistical calculations. We used Minitab^{R} for the Ryan-Joiner frequency distributions, for Pearson correlation coefficients, and for the whisker box graphics. To assess reproducibility of the ADCC assay for duplicate frozen samples, cultured on different days, for donors SC4328 and SC0980, we applied hierarchical linear regression to evaluate the similarity of the duplicates vs. the two different donors with an SAS statistical program.

3. Results

3.1 Overall Strategy.

Our strategy has two critical innovative features: 1) quantification of the CD16A-positive NK cells within the PBMCs to provide the cytotoxic effector cell denominator for ADCC per FcR-positive cell; and 2) pretreatment of target cells (with anti-CD20 mAb) to label only the B cell tumor targets for killing and not the normal B cells within the PBMCs. Flow cytometry with TruCount^{R} beads allowed us to determine the exact numbers of CD16A cells within the PBMC suspensions used in the ADCC assays instead of purifying NK cells. The large number of CD16A cells counted by flow cytometry allowed for more reliable assessments of cell concentrations than by manual counts by hemacytometer. Trucount^{R} samples were prepared without cell washes to remove unbound antibodies and thus avoided biases due to selective losses of different cells when PBMCs are washed to remove unbound fluorescent mAbs.

The pretreatment of the ADCC Daudi targets cells was critical and required careful selection of the monoclonal antibody. Obinutuzumab is a type 2 anti-CD20 mAb, which means it is cleared more slowly from B cell surfaces than type 1 anti-CD20 mAbs, which we verified after 4 hrs of incubation for the Daudi cells (unpublished results). Under these conditions, we found that a type 1 rituximab-like antibody was cleared from the Daudi cells and was unsuitable for these assays. To compare ADCC of different donors we assayed killing at multiple E:T ratios and then calculated their CX@1:1, lytic slope, and ADCC LU_{50} as measurements to compare ADCC. CX@1:1 provides an indication of

the % of CD16A cells that are killers and lytic slope indicates the cooperativity among CD16A cells to kill targets. The ADCC LU_{50} values only approximate lytic activities.

3.2 Daudi cells, pretreated with obinutuzumab anti-CD20, are killed by NK cell-mediated ADCC with cytotoxicities at 1:1 (CX1:1) and cytotoxic slopes that differed among donors.

Daudi cells pretreated with obinutuzumab were suitable for ADCC assays (Fig 1A-C). There was little NK lysis of the ^{51}Cr-Daudi cells without antibody (black line, Fig. 1A) and substantial ADCC of the cells with antibody (red lines). We used PBMCs in the assay and determined the effectors as FcR-positive NK cells, as non-monocyte (CD33neg), non-T cell (CD3neg), and CD16Apos cells, with effector cell numbers obtained by using TruCount^{R} beads in the differential cell count (Fig. 2). Individuals' %CD16Apos of CD56posCD7posCD3neg NK cells ranged from 36 to 100% with an average 92.2 +/- 19.8%, values that are similar but slightly higher than data reported by Jordan *et al.* (Angelo et al., 2015) and similar to the average reported by Hsieh *et al. (Hsieh et al., 2017)*. These differential cell counts determined the number of CD16Apos NK effectors in the initial PBMC cell suspensions that were serially diluted to generate the E:T ratios. In Figs. 1A-C, the ADCC data are plotted as the $\log_{10}$ of the ratios of CD16Apos NK cells: Daudi cells (the effector: target ratios, E:T), *vs.* % specific ^{51}Cr release. The actual E:T ratios are indicated for clarity in Fig 1A and the $\log_{10}$ values that were used for the linear

equations illustrated in Figs 1B&C. Representative subjects with low and high ADCC, are illustrated in Figs. 1B&C, respectively.

CX1:1. When the E:T is 1:1, the $\log_{10}$ of the E:T is zero, and, at this E:T, the % cytotoxicity is the intercept "b" of the linear equation "y = **m**x + **b**" for the ADCC. The cytotoxicity at the 1:1 E:T ratio will be referred to as CX1:1. For the donor in Fig 1A, CX1:1 was 50.9%, meaning 50.9% of the targets were killed. Across all donors, the minimum and maximum CX1:1s observed were 16.2% and 78.4%, a variation of 4.8 fold. Between 4 and 6 hours, there was little additional killing (not illustrated), which indicates that there was only the typical single first round of ADCC with little additional serial killing. Thus approximately 49% of the donor's FcR-positive NK cells in Fig.1A were ineffective ADCC killers – even when given more time to kill. This single round of ADCC activity, "one and done", was finished at 4 hrs and applied to all donors evaluated. This phenomenon was first reported in 1977 (Cordier et al., 1977) and later well delineated by Georgio Trinchieri and colleagues (Perussia et al., 1979; Perussia et al., 1984; Trinchieri, 1989). CX1:1 values varied widely (Fig.1D), and by as much as 29% between different donors assayed on the same day. Thus the overnight culture conditions and the Daudi cell preparation were kept constant and the differences are fully attributable to the donors' cells.

Slopes of cytotoxic cell cooperativity. The lytic slope **m** of the ADCC in Fig.1A was 28.17% per 10-fold increase (one $\log_{10}$) in CD16A NK effectors. This slope indicates that even when there was a 10-fold increase in CD16Apos NK cells per Daudi, the killing was

increased by only an additional 28.17%. Across all the donors (Fig.1E), the variation of slopes was 4.7 fold (between the minimum and maximum values of 9.9% and 46.7% per 10-fold increase). There was a linear relationship between CX1:1 and the slopes of cytotoxicity (Fig.1F), though the R^2 value of 0.80 indicates that there is considerable variation from strict linearity. As indicated later (Fig.4), the slopes were similar for ADCC mediated by PBMCs and by purified NK cells and, on this basis, we propose that the lytic slopes represent effector cell cytotoxic cooperativity for killing the Daudi cells and are little influenced by the presence of CD16Apos-monocytes in the PBMCs.

Comparison of donors by lytic units (LU_{50} per 1 million CD16A cells). Application of lytic units is invalid because similar (parallel) slopes are needed for comparison of cytotoxicity by lytic units (Pross et al., 1986). Small slopes exaggerate the lytic units, as indicated by comparisons of the lytic units calculated for the ADCC of Figs.1B&C. LU_{50}s for all donors ranged more than 15,000-fold, from 0.04 to 669.5 LU_{50} per 1 million CD16A cells.

3.3 CX1:1 measures lytic function (as opposed to FcR recognition).

CX1:1 values vary independently of EC_{50}, a measurement of FcR recognition. EC_{50} values are the concentrations of soluble antibody in the ADCC assay needed to support 50% of maximal ADCC. EC_{50} values remain constant over a range of E:T values for a single donor's cells. EC_{50}'s reflect cellular recognition of antibody bound to 'target' cells,

and are dependent on the affinity of cellular FcRs for the antibody used. In our experiments, the EC_{50}'s were higher for the donors with the lower affinity CD16A F/F genotype FcRs compared to the donors with V/V & V/F genotypes that have the higher affinity V allele genotype (Fig.3A, P= 0.07). Similar EC_{50} results with PBMCs and different B cell tumors were previously indicated with a type 2 anti-CD20 mAb and also yielded an insignificant P value (Chung et al., 2014) and with obinutuzumab (Herter et al., 2013). In contrast, the CX1:1 values varied independently of these alleles (Fig.3B, P=0.43). Importantly, the CX1:1 values were completely independent of the EC_{50}'s (Fig.3C) with a statistically insignificant Pearson correlation of -0.21. Thus, it is clear that the CX1:1 values (with obinutuzumab) measure ADCC that is dependent on variables other than the cellular recognition of antibodies bound to CD20-positive cells.

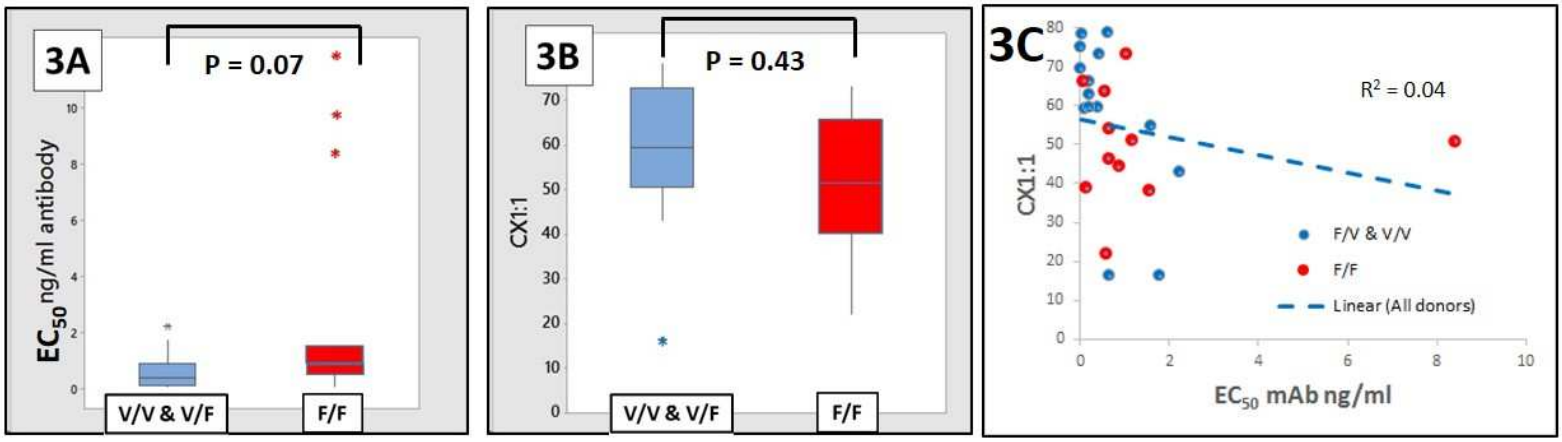

Figure 3. CX1:1 and antibody EC_{50} values vary independently of CD16A AA158 F and V alleles while EC_{50} values are higher for donors with the F/F genotype. There were 12 F/F (low affinity CD16A FcR) donors and 15 combined V/F & V/V donors. **A. The EC_{50} values** (antibody concentration required to support 50% of maximal lysis at any E:T) were greater for the donors homozygous F/F for CD16A (red, 2.6 +/- 3.9 ng/ml) than the V/F & V/V donors (blue, 0.64 +/- 0.70 ng/ml) though the P value of 0.07 was below 0.05 significance. The need for more antibody for F/F donors is consistent with the CD16A F allele affinity for IgG1 and with previous reports. **B. Similar CX1:1 values for F/F *vs.* V/F & V/V CD16A genotypes.** Average CX1:1 values were 51.8 +/- 15.4% for F/F donors and

57.4 +/- 19.5% for V/F & V/V donors. **C. Lack of correlation between CX1:1 and EC_{50} values.** Each symbol represents a single donor with the F/F donor indicted with red symbols. The Pearson correlation coefficient was low, -0.214, which, together with the extremely low R^2 for distribution of the values (0.04), indicates independent variability. Thus CX1:1 measures ADCC requirements other than cellular recognition of antibodies.

3.4 Purified NK cells and unfractionated PBMCs have similar ADCC in the improved assay

To determine if there was any difference in ADCC between unfractionated PBMCs and purified NK cells, we separated and concurrently assayed purified NK cells and PBMCs (treated with identical buffers and washing conditions) from the same donor. Our results (Fig. 4) show similar ADCC by each: the CX1:1 was 47.5% for isolated NK cells and 44.8% for PBMCs, respectively; the lytic slope was 33.9% and 37.6%/10X E:T for NK cells and PBMC, respectively. The NK cells had slightly more activity than the PBMCs. However, for a second donor with lower overall ADCC, the purified NK cells had slightly less activity than the PBMCs (not illustrated).

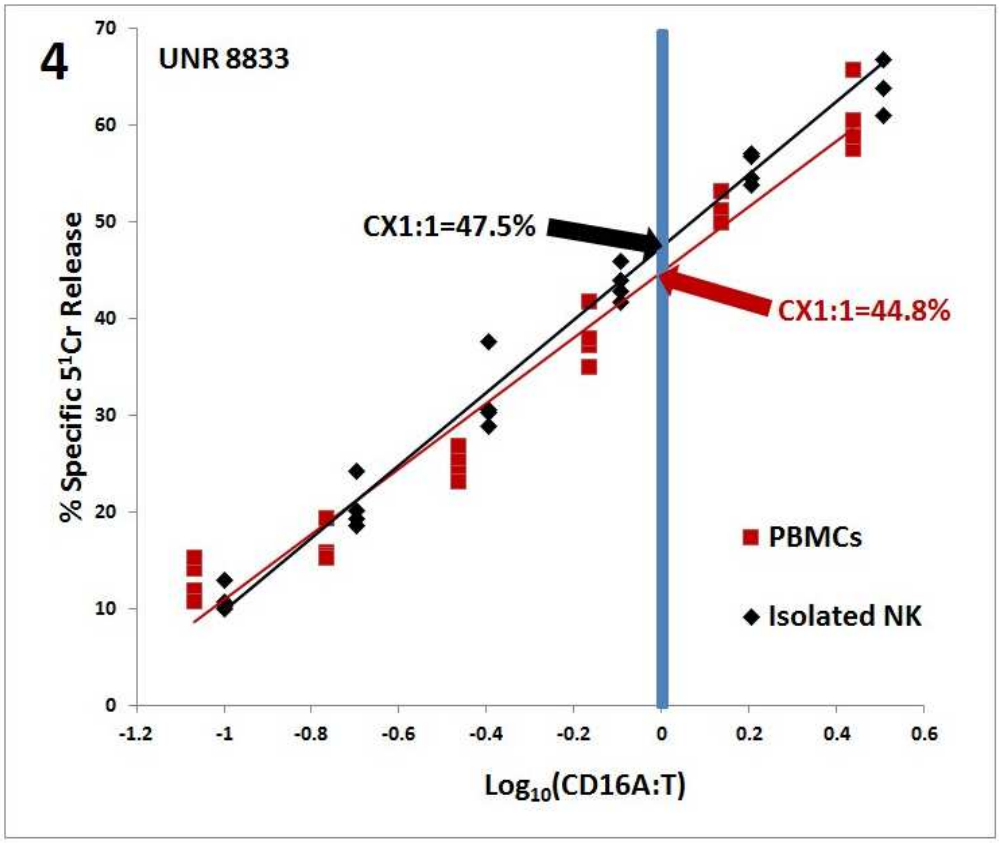

Figure 4. Similar ADCC CX1:1s and Lytic Slopes of Purified NK cells *vs.* PBMCs. CX1:1 values are illustrated by the arrows with the blue line marking the 1:1 E:T. The lytic slope for the NK cells was 37.6% per 10-fold increase in E:T and 33.9% for the PBMCs. R^2 values were >0.95. A duplicate experiment with cells of another donor, UNR 2119, also showed similarity between NK cells and PBMCs, but with lower activity of the isolated NK cells.

3.5 Reproducibility of CX1:1 using frozen PBMC standards. An internal standard of one stock of frozen PBMCs could indicate day-to-day variability in the assay. Thawed PBMCs require overnight culture to restore their ADCC activity (Mata et al., 2014). To evaluate assay reproducibility, we used commercial frozen PBMCs to obtain identical frozen vials (because of our IRB restrictions on the amounts of blood we were able to draw). We used PBMCs from two different donors. Figures 5A&B illustrate the two assays done on different days for each donor and the variability of their CX1:1 values compared to that of the BHC donors. Inserts mark the two values over Fig. 1D of the BHC donors. It is clear that duplicates from one donor are much more similar than random sampling from the BHC donors. The intra-donor variability is less than the inter-donor variability and far less than would be expected from samples taken at random from the BHC set. Statistical analyses were favored by the fortunate differences in CX1:1 of the two donors. The random effects model indicates the variance between persons (0.1760 +/- std error 0.2891) was larger than the variance for the repeats for individual donors (i.e., 0.04753 +/- 0.05020). These samples were two by two and neither comparison indicated statistically significance variances ($p = 0.2713$ and $p= 0.1719$) respectively.

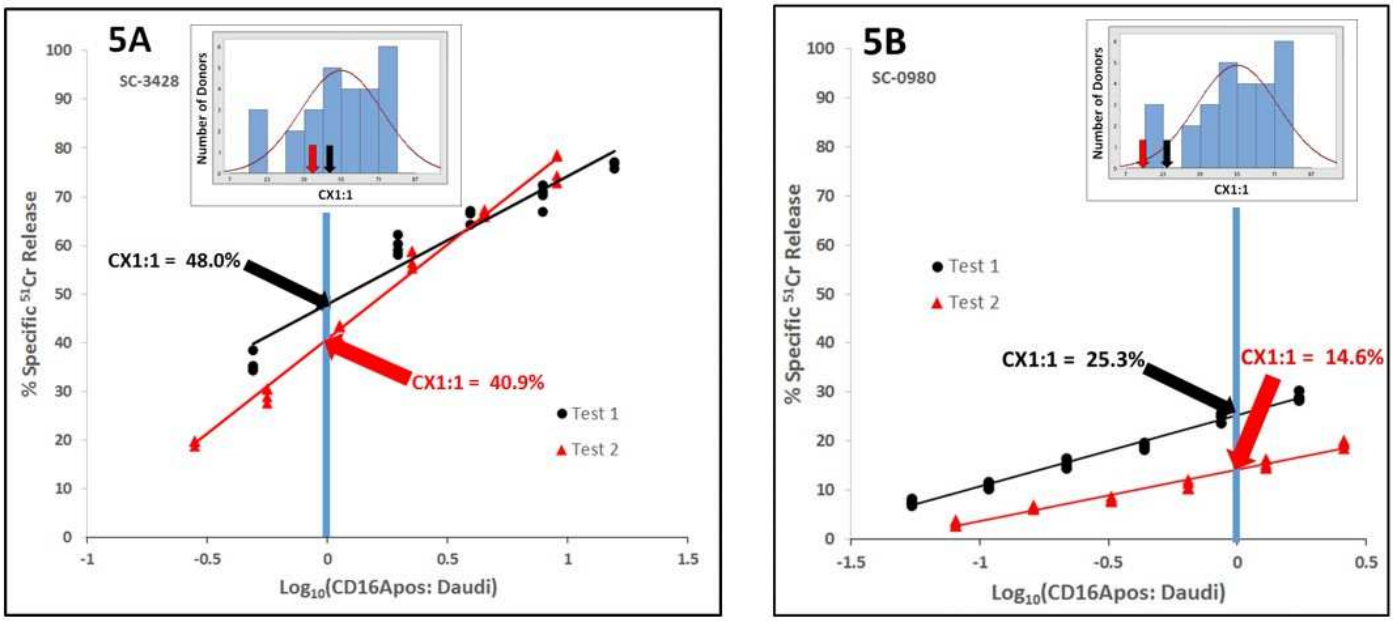

Figure 5. Frozen PBMCs as standards for reproducibility of ADCC. Aliquots of frozen PBMCs were thawed and cultured on two different days and assayed to evaluate reproducibility. PBMCs from two different donors were assessed, illustrated in **A** (donor SC-3428) and **B** (donor SC-0980). Inserts in the figures place arrows for the donor's two CX1:1 values on the CX1:1 intra-donor distribution of the BHC donors (figure 1D) in order to facilitate comparison of the 2 intra-donor values with the 27 BHC inter-donor values. Even though the inter-donor differences between SC-3428 and SC-0980 are greater than the intra-donor differences determined on separate days, statistical analysis, discussed in the results, indicated that even the larger intra-donor differences were statistically insignificant.

4. Discussion

W**e describe an improved ADCC assay that will allow comparison of NK cells' ability to *kill* rather than to *recognize* antibody-bound cells.** There are 3 key aspects: 1) independence from recognition due to the affinities of CD16A V&F allelic recognition of the antibodies bound to targets and from donor-variable KIR recognition; 2) economy of time and cost by use of PBMCs rather than purified NK cells; and 3) a requirement for only ~10 ml of blood so that 20 ml of patient blood can be used for both lytic capacity and EC_{50} assays. The new assay for lytic capacity is independent of CD16A FcR allelic V *vs.* F affinity. Most likely, cellular avidity overwhelmed the effects of single-receptor affinity because we used saturating amounts of a high affinity anti-CD20 antibody and there are ~24,600 molecules of CD20 per B cell ("Density of Common Human Surface Antigens", BD Biosciences pamphlet) and thousands of molecules of CD16A per mature human NK cell (Montaldo et al., 2013), which ensures multiple FcR engagements per NK cell. The type 2 anti-CD20 nature of obinutuzumab also reduced clearance of the mAb from the pretreated target cells during the assay. Lack of MHC 1 on Daudi cells obviated KIR engagements as well as preventing inhibitory engagements by NKG2A and CD85j NK receptors that also recognize MHC class 1 molecules. Application of flow cytometry with TruCounts made it possible to determine ADCC per CD16Apos NK cell without purification of NK cells so that cell losses during NK cell purification could be minimized. Thus, we were able to save time, reduce monetary costs, avoid NK cell purification, and prevent loss of cytotoxicity activity that will occur over time (Castillo de Febres and Kohl, 1983; Son et al., 1996). With only 15-20 ml of whole blood, we performed 3 assays:

CX1:1 ADCC for lytic capacity, EC_{50} ADCC for recognition of antibody, and concurrent NK to the Daudis (consistently negligible). With freshly isolated PBMCs, we detected a 5-fold range in CX1:1 lytic activities among donors.

The CX1:1 values address several issues. With major differences in lytic slope, it was inappropriate to compare donors' ADCC by lytic units. For this reason, we recommend CX1:1 values for comparison. The CX1:1 values measure cytotoxic efficiency and incorporate the variations in the slopes (because they are also proportional to the lytic slopes). In our ADCC assay, the NK cells finished killing by 4 hours with little more killing at 6 hrs. Most likely, they killed only once, eliminating only a single target cell, rather than progressing on to kill additional cells. This mode is consistent with reports of "one and done" ADCC as early as Giorgio Trinchieri's reports from the 1980's and recently substantiated by very low frequencies of ADCC serial killing observed by time-lapse cinematography (Romain et al., 2014). The lack of serial ADCC, in contrast to serial NK, means that the CX1:1 actually represents the final fraction of CD16A-receptor positive cells that can kill. Thus the CX1:1 may be a very important variable to affect clinical efficacy of anti-cancer mAb's that support ADCC to tumor cells. CX1:1 variations may also contribute to viral resistance. Since MHC 1 is down regulated by several DNA viruses (reviewed, (Hewitt and Dugan, 2004; Seliger et al., 2006), the CX1:1 monitored with the MHC 1-negative Daudi cells may have physiological relevance. It will also be enlightening to determine if higher CX1:1 values are associated with FcR-gamma chain

negative “memory” NK cells that are highly responsive after FcR engagement (Zhang et al., 2013; Lee et al., 2015; Schlums et al., 2015). The CX1:1 values were always well below 100% of the CD16A-positive NK cells which is very curious since these receptor-positive cells were highly perforin-positive indicating that some apparently “armed” cells were disengaged from killing.

We present additional information and discuss potential refinements that are relevant to this ADCC assay. We have found that this assay is suitable for resting NK cells but not for cytokine-activated NK cells (Siegel et al., 1987; Romee et al., 2012; Leong et al., 2014). Cytokine-activated NK cells killed Daudi cells so well in the absence of antibodies that ADCC was negligible (unpublished results). For perspective, it has yet to be determined if CX1:1 fluctuates for healthy donors from week to week or month to month, or during temporary illness. Refinements that we recommend for future applications are to use freshly drawn blood cells *without* overnight culture (to avoid tissue culture variables). We manually counted >600 radioactive target cells with a hemacytometer and recommend use of an automated method to count >10,000 ^{51}Cr Daudi cells to thereby increase the accuracy of the E:T ratios. It is possible that a frozen stock of vials of calcein AM-labeled Daudi cells (Chung et al., 2017), pretreated with mAb and washed could be used for multiple experiments, thus avoiding Daudi culture variations and permitting cytometric TruCounts^{R} of non-radioactive Daudi cells.

The CX1:1 concept may have additional applications. It may be desirable to evaluate NK and ADCC for a single donor towards different targets cells. If there are differences in cytotoxic slopes with different ADCC and/or NK targets and if there is negligible serial killing, then the different CX1:1 values could provide an indication of the proportion of effectors capable of lysing each particular target. This CX1:1 evaluation would be of potential use with transformed cells of the donor origin, to include the natural MHC 1-KIR and other interactions.

CX1:1 measurement of ADCC lytic capacity has supported novel results in a clinical study and has potential for additional applications. Our immediate application that funded this assay development, was to compare ADCC of chronic fatigue syndrome (CFS) patients, CFS family members and healthy donors. We were able to distinguish statistically significant CX1:1 differences between members of 5 CFS families *vs.* unrelated healthy donors that were independent of CD16A F and V genotypes (manuscript in preparation). We are also interested in determining what cellular properties affect CX1:1. As part of the current studies (manuscript in preparation), we did not see any correlation between CX1:1 values and intra-donor differences in perforin and cell surface CD16A. We hope that other investigators will apply CX1:1 measurement to query contributions of lytic capacity to the protective ADCC of HIV-positive non-progressors to AIDS (Kulkarni et al., 2017). In addition, there is a need to evaluate individual ADCC cellular lytic capacity, as well as production of anti-HIV

antibodies to support ADCC (Konstantinus et al., 2016; Brown et al., 2017; Shete et al., 2017) to provide insights into anti-HIV vaccine efficacy. Good CX1:1 may contribute to the resistance of individuals who are chronically infected with *Herpes simplex* but have low incidences of lesions (Moraru et al., 2015). CX1:1 measurements may also be suitable to predict therapeutic responsiveness to mAb anti-tumor immunotherapies. The CX1:1 values will also be useful to monitor therapeutic NK cellular activation for ADCC to increase further the *in vivo* cytotoxicity mediated by ADCC (Ochoa et al., 2017) that has already been optimized by engineering mAb's for higher FcR affinity (Repp et al., 2011; Kellner et al., 2014; Saxena and Wu, 2016).

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Conflicts of interest.

None. The authors lack financial or personal interests that would influence this report.

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Supplement Table 1. Human Subjects							
BH code no.[a]	**Yrs of age**	**Gender**	**CD16A genotype[b]**	**CX1:1 (% dead)**	**slope %CX/ 10X E:T**	**EC50 (mAb ng/ml)**	**ADCC LU50 per 1 M CD16Apos cells**
1	61	F	V/F	42.8	16.7	2.24	37.1
2	56	F	V/F	16.2	13.7	0.67	0.3
5	61	M	V/F	16.3	9.9	1.79	37.1
6	56	F	F/F	22.0	14.0	0.57	0.3
15	58	F	V/F	50.5	36.9	NA[c]	102.9
16	58	M	F/F	72.8	27.6	NA	669.5
31	25	F	F/F	46.3	27.5	0.65	73.2
32	59	F	F/F	38.1	16.9	1.56	19.8
33	57	F	F/F	50.9	32.3	1.18	106.7
34	65	F	F/F	38.8	17.9	0.15	23.8
35	62	F	F/F	66.3	42.3	0.08	243.2
38	52	M	F/F	54.2	31.5	0.65	135.7
40	43	F	F/F	50.9	30.9	8.43	106.7
41	41	F	F/F	63.7	25.9	0.54	338.6
42	69	M	V/F or V/V	62.7	36.5	0.22	223.6
43	35	M	V/F or V/V	59.2	32.8	0.11	190.5
44	21	F	F/F	44.4	28.2	0.87	63.1
45	28	F	V/F or V/V	72.9	39.6	0.45	378.4
46	23	M	V/F or V/V	54.5	30.9	1.59	139.4
47	22	M	V/F or V/V	59.3	34.0	0.40	188.3
48	30	M	F/F	73.3	43.8	1.03	170.5
49	32	F	V/F	78.2	43.5	0.04	222.4
50	38	F	V/F	78.4	46.7	0.63	203.2
51	51	F	V/V	75.0	44.6	0.03	181.6
52	23	M	V/F or V/V	65.9	38.3	0.21	259.7
53	40	F	V/F or V/V	59.5	30.8	0.22	203.3
54	53	F	V/F or V/V	69.4	35.2	0.04	355.7
min	21.0			16.2	9.9	0.03	0.34
max	69.0			78.4	46.7	8.43	669.47
median	51.0			59.2	31.5	0.57	170.45
average	45.1			54.9	30.7	0.97	173.13
std dev	15.4			17.7	10.3	1.66	145.43

Fold range				4.8	4.7	254	1941
[a]Kinship. Thirteen healthy donors were unrelated. Fourteen healthy donors were members of 5 families in which there were CFS patients.							
[b]Genotypes determined by flow cytometry could be distinguished only as F/F and either V/V *or* V/F.							
[c]Insufficient cells for EC_{50} measurements.							

Chapter 3

Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) in Familial Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

Abstract

Background: Chronic fatigue syndrome (CFS) is an illness of unknown origin that may have familial risks. Low natural killer (NK) lymphocyte activity was proposed as a risk for familial CFS in 1998. Since then, there have been many studies of NK lymphocytes in CFS in general populations but few in familial CFS. Antibody-dependent cell-mediated cytotoxicity (ADCC) by NK lymphocytes helps control viral infections. ADCC is affected by variant CD16A receptors for antibody that are genetically encoded by *FCGR3A*. **Methods**: This report characterizes ADCC effector NK cell numbers, ADCC activities, and *FCGR3A* variants of five families each with 2-5 CFS patients, their family members without CFS and unrelated controls. The patients met the Fukuda diagnostic criteria. We determined: CD16Apositive blood NK cell counts; EC50s for NK cell recognition of antibody; ADCC lytic capacity; *FCGR3A* alleles encoding CD16A variants, ROC tests for biomarkers, and synergistic risks. **Results:** CFS patients *and* their family members had fewer CD16Apositive NK cells, required more antibody, and had ADCC that was lower than the unrelated controls. CFS family members were predominantly genetically CD16A F/F s for the variant with low affinity for antibodies. ROC tests indicated unsuitability of ADCC as a biomarker for CFS because of the low ADCC of family members without CFS. Familial synergistic risk *vs.* controls was evident for the combination of CD16Apositive NK cell counts with ADCC capacity. **Conclusions:** Low ADCC may be a risk factor for familial CFS. Furthermore, characterization of familial CFS represents an opportunity to identify pathogenic mechanisms of CFS.

Keywords: Chronic Fatigue Syndrome, ADCC, antibody-dependent cell-mediated cytotoxicity; NK, CD16A, family studies

Abbreviations: AA, amino acid; **ADCC**, antibody-dependent cell-mediated cytotoxicity; **AUC**, area under the curve; **CD,** cluster of differentiation; **CD16A**, IgG Fc-receptor of NK cells; **CFS**, chronic fatigue syndrome; **CX1:1**, the % cells killed at a 1:1 ratio of CD16Apositive NK cells to Daudi 'target' cells; **CX-slope**, linear slope of cytotoxicity with increased killer cells; **E:T**, effector to target cell ratio; **EC50**, the effective concentration of antibody required for 50% of maximal ADCC; **FcR,** cellular receptor for the Fc region of immunoglobulin (antibody); ***FCGR3A,*** the gene encoding CD16A; **KIR**, killer cell immunoglobulin-like receptor; **ME**, myalgic encephalomyelitis; **NK**, natural killer lymphocyte; **PBMC,** peripheral blood mononuclear cells; **ROC,** receiver-operating characteristic plot; **UHC**, unrelated healthy control subject.

Introduction

Myalgic encephalomyelitis (ME)/chronic fatigue syndrome (CFS) is disease of unknown etiology. Its nomenclature is still unresolved [1] despite concerted efforts [2]. 'ME' and 'CFS' are often used interchangeably; CFS will be used in this article. The disease is identified by debilitating chronic fatigue and diagnostic criteria, well-defined by the Centers for Disease Control USA in 1994 [3] and further delineated by the National Academy of Medicine USA in 2015 [1]. CFS affects 800,000 to 2.5 million adults in the USA and ~0.4% of the population worldwide [2]. Symptoms include severe fatigue for more than six months, long-lasting post-exertional malaise, un-refreshing sleep, 'brain fog' in the form of loss of memory and/or lessened ability to think, and chronic pain [3]. There are no known causes for most cases; however, CFS-like pathology can follow severe viral or bacterial infections [4]. CFS is receiving renewed attention as a distinct disease [5, 6] with high costs to society [7].

Subgroups of CFS patients [8, 9], have been proposed based on symptoms, candidate etiologies and potential disease-promoting mechanisms [10]. Familial CFS, defined by the occurrence of two or more CFS patients who are first-degree relatives within a family, represents a subgroup of CFS. Familial CFS was first reported in a 1998 study of natural killer (NK) lymphocytes of one family with 8 CFS patients [11]. A 2001 report found that 6/25 unrelated CFS patients (24%) had first-degree relatives with CFS [12]. Of these six patients, two had two other CFS-affected family members and four had one

other CFS-affected family member (personal communication from author [12] Nor Zainal, Ph.D.) Both 1998 and 2001 reports used the Fukuda CDC 1994 diagnostic criteria for CFS. Two Studies of concordant twins add further evidence for familial CFS, reviewed [12]. The family described in 1998 with CFS patients with impaired NK activity also had non-CFS first degree relatives with low NK activity. NK activity has been reported to be low in many but not all studies of NK lymphocytes in non-familial CFS (reviewed, [13]). To our knowledge, the study of NK-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) reported here is the first study of immunity in familial CFS since 1998.

NK cells use both 'natural cytotoxicity' and ADCC to kill virally infected cells and tumor cells. 'Natural cytotoxicity' involves recognition of stress or other ligands present in 'target' cells and occurs in the absence of antibodies (reviewed, [14]). ADCC occurs only when specific antibodies are bound to infected cells or tumor cells. The NK cell receptor required for ADCC is CD16A which binds to antibodies and is present on most but not all NK cells. As a result of different recognition systems for natural cytotoxicity and ADCC, it is reasonable to postulate that ADCC activity could be altered without affecting natural cytotoxic activity. There are two common allelic variants of the gene *FCGR3A* that encode single amino acid differences in CD16A, with either phenylalanine (F) or valine (V) at AA158, that could affect ADCC. The V158 CD16A has twice the affinity for IgG antibody and higher cellular expression than the F158 CD16A [15, 16]. We

postulated that low ADCC and the homozygous F/F form of CD16A could be familial CFS risk factors.

ADCC is an attractive consideration for CFS because NK cell-mediated ADCC helps control chronic herpes viral infections. Consideration of viral etiologies for CFS began in 1984 [17]. Viruses that have been proposed include the chronic herpes viruses Epstein Barr virus [18], human cytomegalovirus [19], herpes zoster [20], human herpes virus 6 (HHV6) [21], and a different DNA virus, parvovirus B19 [22]. The fatigue of CFS resembles the fatigue induced by gamma interferon during viral infections [23]. Elevated gamma interferon is detectable in the blood of patients with severe CFS [24]. In the face of rigorous research attempts and the high incidences of herpes viruses in the general population, proving that herpes viral infections are universally linked with CFS disease has been challenging and the issue remains unresolved [25].

The pilot study of ADCC in familial CFS reported here encompasses *in vivo* availability of NK cells that can mediate ADCC, ADCC functions assayed *in vitro,* and genetics of CD16A. We addressed: a) counts of NK cells with CD16A receptors; b) EC50s for the amount of antibody required for ADCC; c) NK cell ADCC capacity; and d) *FCGR3A* alleles. Two methods were developed: TruCount® bead enumeration of CD16A-positive(pos) NK cells and an assay to detect ADCC cytotoxic capacity regardless of *FCGR3A* genotypes [26].

The report focuses on five CFS families. The common genetic backgrounds within each family promote detection of specific alleles that might be preferentially inherited by CFS patients compared to their non-CFS siblings. Familial environments may direct environmentally stimulated subgroups of NK cells [27] and a background of similar NK development could favor detection of changes in ADCC specific to the CFS patients.

This study reports several novel observations. (1) CD16Apos NK cell counts of both CFS patients *and* their family members were lower than those of unrelated healthy controls. (2) There was lower ADCC for CFS patients compared to unrelated controls. (3) There was *also* lower ADCC of the family members without CFS compared to unrelated controls. (4) CFS family members (with or without the disease) were more likely to have a combination of low ADCC activity with low CD16A NK cell counts versus the unrealted controls. (5) The CFS families were predominantly *FCGR3A* homozygous for CD16A F/F. Based on these observations, we suggest that low ADCC may be a risk factor for the familial form of CFS.

Methods

CFS patients, family members and unrelated healthy donors. Five families were selected from many families afflicted with CFS. The patients were diagnosed at the Bateman Horne Center in Salt Lake City, UT, and met the Fukuda criteria [3] when first

diagnosed. Selection was for families with several CFS patients and unaffected siblings of patients. Selection was also influenced by family members' geographic availability to donate blood. The families had a total of 13 CFS patients with 2 to 5 CFS patients per family. Figure 1 illustrates the family pedigrees; participants in the study are indicated by their CD16A genotypes. Eleven CFS patients and 22 family members without CFS participated. Sixteen of the participating family members were first degree relatives of the patients and had 50% of all genes in common with a CFS patient in the family. Four non-CFS family members were the 2nd degree relatives and shared 25% genes with a CFS patient. The remaining two non-CFS family members were fathers of children included in the study and unrelated to the patients. Sixteen unrelated healthy control donors were matched by race, sex and age to the CFS patients. Healthy was defined as HIV-negative, without overt infections at the time of blood donation, and without CFS. All participants were Caucasian.

Research with human subjects was approved by institutional review boards for the Bateman Horne Center and for the University of Nevada, Reno School of Medicine, IRB #2014B016. Written informed consent was obtained from the blood donors.

Questionnaires. Patients and their family members without CFS answered the Rand-36 [28] (Rand Health Care, Santa Monica, CA) and Fibromyalgia Impact [29] (American Academy of Family Physicians, Leakwood, KS) questionnaires within weeks before blood

donation. The timing of the questionnaires assured that symptoms reported were current with the evaluations of ADCC immunity.

Preparation of peripheral blood mononuclear cells (PBMCs).

Blood was drawn in Salt Lake City between 8-10 AM. Most overnight shipments to Reno, NV, contained blood from multiple CFS family members and two unrelated healthy controls. Blood samples were coded in Salt Lake City. The blood CD16A NK cell counts, EC50 assays, ADCC, and CD16A genotypes were run as coded samples and decoded after completion of the experiments.

For ADCC assays, PBMCs were isolated from 24 ml of blood by ficoll-hypaque density gradient centrifugation. The PBMCs were cultured overnight to ensure ADCC activity [30], at 1-2 x 10^6 cells/ml in complete assay media, 90% Dulbecco' s media containing 4.5 g/L glucose and L-glutamine (Corning), 10% fetal calf serum (Atlanta Biologicals), 10 mM hepes (Sigma-Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Sigma-Aldrich). Culture and assay conditions were standardized using one lot of fetal calf serum and one lot of tissue culture flasks (Biolite, Thermo Scientific).

TruCounts® of CD16Apos NK cells

CD16Apos NK cells per µl blood

Fifty µl aliquots of blood were labeled on arrival with a panel of antibodies designed for no-wash analyses with TruCount® beads (Becton Dickenson no. 340334 [31]), The antibody panel contained FITC-anti-**CD3e** (clone UCHT1); PerCP-anti-**CD16A** (3G8); PacBlue anti-**CD45** (clone HI30); and FITC-anti-**CD91** (2MR-alpha), all purchased from BioLegend (San Diego, CA) except for anti-CD91 from Becton Dickenson. The flow cytometric gating is illustrated in supplement Figure S1. The ADCC effector cells were CD3negCD16posCD45posCD91neg.

CD16Apos NK cells per µl PBMCs

Fifty µl aliquots of PBMCs were labeled with a similar panel of antibodies in tubes with TruCount® beads, with the substitution of PE-Cy7 anti-**CD33** (clone P67.6) for CD91 and inclusion of FITC-anti-**CD7** (clone CD7-6B7) and APC-Cy7 anti-**CD56** (clone HCD56). The NK ADCC effector cells were CD3negCD7posCD16posCD33negCD45posCD56variable. Anti-CD7 was critical to discriminate CD16AposCD7pos***CD56neg*** NK cells [32] from CD16Apos***CD56neg*** monocytes (that are all CD7neg and largely CD33pos) [33].

Cytometry for TruCounts®

Cells were analyzed with a BD Biosciences Special Order Research Product LSR II analytical flow cytometer. Analyses were with FlowJo software (FlowJo, LLC, Ashland, OR) to determine the CD16Apos NK cells and TruCount® beads in order to calculate the number of CD16Apos NK cells in the solutions [26].

ADCC assays

EC50 assay for antibody recognition by NK cells

The EC50s, effective concentrations of antibody needed for 50% of maximal ADCC [34], were determined with 4-fold dilutions of obinutuzumab that ranged from 0.04 to 625 ng/ml in the ^{51}Cr assays described below. ADCC was determined at 4 hours, with duplicate or triplicate wells for each antibody concentration. The yields of PBMCs supported EC50 determinations for most, but not all, donors.

ADCC cell capacity and cooperativity

The ADCC assay has three critical features. 1) MHC class I-negative Daudi cells were used as 'targets' to avoid inter-donor variations caused by MHC I-dependent KIR inhibition The Daudi cells from the ATCC (Manassas, VA, catalog # CCL-213) were routinely tested and negative for mycoplasma. 2) A type 2 anti-CD20 monoclonal antibody obinutuzumab [35] was selected because it is poorly cleared from the membranes of B cells compared to type 1 anti-CD20 antibodies. Non-fucosylated obinutuzumab was obtained from the Roche Innovation Center, Zurich, Switzerland. 3) The persistence of the bound antibody on washed Daudi cells allowed use of unfractionated CD16A NK cells within PBMCs as effectors and avoided killing of normal B cells in PBMCs, thereby maximizing usage of the patients' cells [26].

ADCC was measured by ^{51}Cr-release [26]. Daudi cells were labeled with $Na^{51}CrO_4$ (Perkin Elmer, Waltham, MA), pretreated with 1 μg/ml obinutuzumab for 0.5 hour at room temperature and washed 5 times to remove unbound antibody and unincorporated chromium. The PBMC solutions containing the CD16A NK cells were diluted 2-fold serially in V-bottom plates (Costar 3894, 96 well) in 0.1 ml to create six CD16Apos NK:Daudi target cell (effector:target, E:T) ratios, 4 wells per ratio. Daudi cells, with or without mAb, 1 x 10^4 per well in 0.1 ml, were added, for ADCC or NK background activities, respectively. Plates were centrifuged for 3 minutes at 1000 rpm and incubated for 4 hours at 5% CO_2 and 37^{o}C. After incubation, plates were centrifuged for 10 minutes at 1200 rpm and 0.1 ml of cell-free supernatants were removed and counted in a Packard Cobre II gamma counter. The percent specific release (SR) was calculated using the formula

% SR = [*(Experimental counts – Spontaneous Release)/(Max – Spontaneous Release)]* x 100.

Spontaneous release was the leak rate of targets without PBMCs and the Max was the radioactivity released by targets lysed with 1% SDS. There was negligible 'natural cytotoxicity' in the absence of antibody for all donors.

We report separately the lytic slopes (CX-slope) and ADCC capacities used to compare patients with the two other groups of donors. This distinction was necessary because there were intra-donor differences in lytic slopes. These differences prevented application of lytic units to compare ADCC activities. CX1:1 [26] measurement of ADCC

capacity is the % of the targets killed at a CD16Apos NK cells to Daudi ratio of 1:1. It was calculated as follows. Percentages of ADCC from the assay were plotted as linear cytotoxicity with y = % specific ^{51}Cr release *vs.* x = the $\log_{10}$ of the 6 CD16Apos NK cells (in the PBMCs) to Daudi ratios. The linear cytotoxicity was used to determine y = mx + b, with the CX-lytic slope = m, x = $\log_{10}$ of the E:T and b = the y intercept. The P values for linear fit were <0.05, with R^2s >0.8. To determine CX1:1, y was calculated with x =1 and $\log_{10}(1) = 0$.

FCGR3A Genotyping

Genotyping by DNA sequences. The *FCGR3A* genotypes encoding CD16A at AA158 were determined by PCR and DNA sequence analysis at the Frederick National Laboratory for Cancer Research, Frederick, MD, by Stephen K. Anderson, Ph.D. Amplicons specific for the *FCGR3A* gene and that exclude the *FCGR3B* gene (which encodes neutrophil CD16B) were generated with forward and reverse PCR primers, (5' to 3') for *FCGR3A* (TCCTACTTCTGCAGGGGGCTTGT) and (CCAACTCAACTTCCCAGTGTGATTG), respectively. The amplicons were directly sequenced using Sanger methodology.

Genotyping by flow cytometry. This genotyping was done for all donors and was the sole method used for a few of the control donors. The homozygous F/F genotype was distinguished from F/V & V/V genotypes using the MEM-154 clone of anti-CD16 mAb (PE-labeled, Pierce Chemical Co, Rockford, IL). MEM154 reacts with the CD16A 158 V

but not the 158 F [36). PBMCs were labeled with: FITC-anti-**CD3e** (cloneOKT3); PE-anti-**CD16A** (3G8) or PE anti-**CD16A 158V selective**-(MEM154); BV605-anti-**CD19** (HIB19.11); PacBlue anti-**CD45** (HI30); FITC-anti-**CD91** (2MR-alpha) and APC-Cy7-anti-**CD56** (HCD56), purchased from BioLegend (San Diego, CA) with the exception of MEM154. CD16A F/F cells were negative with clone MEM154 and positive with clone 3G8.

Health and Safety.

BSL level 2 laboratory safety procedures were maintained throughout the experiments.

Statistical analyses

Cytotoxic activities were determined with the Excel Analysis Tool Pack, using best fit for linear regressions. Student's t-tests were used to compare the different groups of subjects. Excel and GraphPad Prism 7 (San Diego, CA) were used for illustrations. The UCSF website http://www.sample-size.net/sample-size-proportions/ was used to determine the 95% confidence limits for Table 4 (calculation: 'CI for Proportion'). The quadrants for the analyses of synergy were divided by the median values of the variables CX1:1 or CX-slope *vs.* CD16Apos NK cell counts. Logistic regression using interaction terms was applied for comparisons of distributions in quadrants. Results were expressed as the odds of being in the low-low quadrant for CFS patients & family members *vs.* unrelated healthy controls, determined with SAS software (version 9.2

Institute Inc., Cary, NC, USA). Biomarkers were evaluated using receiver operating characteristics (ROC) analyses [37] with SAS version 9.4. P values were determined by maximum likelihood tests for difference of AUCs from 0.5. For Table 4, P values for the frequencies of CD16A F/F parents were determined using the Appendix to Chapter 5: Exact Binomial Probability Calculator available at http://vassarstats.net/textbook/ch5apx.html. Unavailability of the flow cytometer impacted data collection for a few donors. As a result, there are more measurements of CX-slopes available for statistical comparison than CX1:1s, as the CX1:1s are dependent on TruCount®s.

Results

Study participants

The study participants were divided into 3 groups: CFS patients; their family members without CFS; and unrelated healthy controls. The pedigrees of the 5 families are illustrated in Figure 1. Each family had 2 to 5 CFS patients. Eleven of the 13 patients (85%) participated. Six of the 13 patients (46%) were second-generation CFS and four participated, so 36% of the CFS patients in the study were children of CFS patients. The participating CFS patients were 82% female, with a mean age of 23.4 years at first diagnosis and mean duration of illness of 22 years. Twenty-two of 27 (81%) of the eligible family members without CFS participated. The participants are indicated by the

FCGR3 genotypes in Figure 1. The controls matched the CFS patients by gender and age. Table 1 indicates the age and gender distributions of the three groups. The patients and controls were gender-matched and predominantly female as is the disease CFS but there were only slightly more women than men among the non-CFS family members. Overall, the three groups share dominant gender, average age, and Caucasian race.

Table 1. Characteristics of the Three Study Populations

Characteristic	CFS Cases		Family members w/o CFS		Unrelated healthy controls	
Number of participants	11		22		16	
Number of families	5		5		16	
Age in years, mean +/- SD	45.5 +/- 21.3		46.2 +/- 14.7		42.8 +/- 18.6	
Sex	**Number**	**%**	**Number**	**%**	**Number**	**%**
Female	9	81.8	12	54.5	12	75.0
Male	2	18.2	10	45.5	4	25.0

The CFS patients and their non-CFS family members differed in the symptoms used to diagnose CFS (Table 2). Information excerpted from Rand36 Questionnaires, Fibromyalgia Impact Questionnaires, and Bateman Horne Center medical histories indicates that the patients were 'moderately' affected. They were able to walk and lift groceries. All experienced post-exertional malaise. The differences between the patients and their non-CFS family members were significant ($P>0.05$) for all the CFS diagnostic symptoms.

Table 2A. Characteristics of the CFS Patients

Donor number[e]	Bateman Horne Center (BHC) Patient History[a]					Rand36[b] Qs re Fatigue; 1 worse >3			Rand36[b] (5 worse>1)	Postexertional Malaise (PEM)[c]		BHC History[a]		Fibromyalgia Impact Questionnaire[d]				DNA Sequence
	Family #	Gender	Age[f]	Age of first diagnosis	Duration of CFS, yrs	Q5[g] Lifting groceries	Q7[h] Climbing stairs	Q11[i] Difficulty, walk 1 block	General health Q36[j]	pem_onset[k]: 1 minutes; 2 hours; 3 one day after	pem_duration[l]: 1 = 1 day; 2=several days; 3 = at least a week	Fibro-myalgia	Head-aches	Unrefreshed sleep[m] (100% worst)	Memory[n] (100% worst)	Muscle pain[o] (100% worst)	Balance[p] (100% worst)	CD16A Genotype
3	10	M	23	15	8	2	2	3	4	3	3	Yes	No	55	19	51.0	14	F/F
4	10	M	26	6	20	3	3	3	2	1	2	No	No	14	7	14.0	5	F/F
13	28	F	61	31	30	2	3	3	5	2	2	Yes	No	71	16	37.0	21	F/F
14	28	F	86	53	33	3	3	3	4	2	1	No	Yes	0	56	0.0	64	V/F
20	20	F	30	11	19	2	2	3	5	3	2	No	Yes	82	85	47.0	65	F/F
21	20	F	60	33	27	2	3	3	4	2	1	No	Yes	46	21	64.0	62	F/F
23	20	F	27	6	21	2	2	3	5	2	2	Yes	Yes	88	100	54.0	62	F/F
25	22	F	50	18	32	1	1	1	5	1	2	Yes	Yes	100	98	77.0	94	F/F
27	22	F	21	11	10	2	2	2	4	1	2	Yes	Yes	30	20	70.0	0	F/F
36	3	F	57	24	33	2	2	3	5	2	2	Yes	Yes	100	82	58.0	70	F/F
37	3	F	60	49	11	2	2	2	5	2	1	Yes	Yes	91	94	74.0	73	F/F
Average			46	23.4	22.2	2.1**	2.3**	2.6*	4.4**	1.9	1.8			61.5*	54.4*	49.6**	48.2**	
std deviation			21	16.4	9.5	0.5	0.6	0.7	0.9	0.7	0.6			35.2	38.1	24.4	31.9	
median			50	18	21	2	2	3	5	2	2	64%[α]	73%[β]	71	56	54	62	91% F/F
minimum			21	6	8	1	1	1	2	1	1			0	7	0	0	
maximum			86	53	33	3	3	3	5	3	3			100	100	77	94	

Table 2B. Characteristics of the Family Members without CFS

Number of donors	Family #	% F	Agef			Rand36[b] Qs re Fatigue; 1 worse >3			Rand36[b] (5 worse>1)			BHC History[a]		Fibromyalgia Impact Questionnaire[d]				DNA Sequence
						Q5[g] Lifting groceries	Q7[h] Climbing stairs	Q11[i] Difficulty, walk 1 block	General health Q36[j] (5 worse>1)			Fibro-myalgia	Head-aches	Unrefreshed sleep[m] (100% worst)	Memory[n] (100% worst)	Muscle pain[o] (100% worst)	Balance[p] (100% worst)	CD16A Genotype
22	3, 10, 20, 22, 28	55		NA	NA					NA	NA							
Average			46			2.9	2.9	3.0	2.3					35.5	25.3	10.0	1.9	
std deviation			15			0.4	0.3	0.2	1.3					31.4	25.8	11.5	3.4	
median			47			3.0	3.0	3.0	2.0			0%	23%	22.5	17.0	7.0	0.0	82% F/F
minimum			21			2.0	2.0	2.0	1.0					0.0	0.0	0.0	0	
maximum			69			3.0	3.0	3.0	5.0					100.0	92.0	50.0	9	

*P<0.05 & **P<0.01 by T-test; [α]P<0.05 by Fisher exact test; [β]P<0.05 by Chi square.

[a]BHC data from patient records

[b]Rand36 questionnaire

[c]The Canadian Consensus Criteria defines Post-Exertional Malaise (PEM) as an inappropriate loss of physical and mental stamina, rapid muscular and cognitive fatigability.

[d]Fibromyalgia Impact Questionnaire

[e]Numbers assigned at site of ADCC assays.

[f]Age at time of blood donation

[g]Difficulty lifting or carrying groceries. 1, Yes, limited a lot; 2, Yes, limited a little; 3, No, not limited at all

[h]Climbing one flight of stairs. 1, Yes, limited a lot ; 2, Yes, limited a little; 3, No, not limited at all

[i]Walking one block. 1, Yes, limited a lot ; 2, Yes, limited a little; 3, No, not limited at all

[j]My health is excellent. 1, Definitely true; 2, Mostly true; 3, Don't know; 4, Mostly false; 5, Definitely false

[k]Does PEM happen …1, minutes after exertion; 2, hours after exertion; 3, a day or more after exertion; 4, not at all

[l]Duration How long does it take you to recover from PEM? 1, minutes after exertion; 2, hours after exertion; 3, a day or more after exertion; 4, not at all

[j]My health is excellent. 1, Definitely true; 2, Mostly true; 3, Don't know; 4, Mostly false; 5, Definitely false

[k]Does PEM happen …1, minutes after exertion; 2, hours after exertion; 3, a day or more after exertion; 4, not at all

[l]Duration How long does it take you to recover from PEM? 1, minutes after exertion; 2, hours after exertion; 3, a day or more after exertion; 4, not at all

[m]Please rate the quality of your sleep. Slider scale, 0-100: Awoke well rested *vs.* Awoke very tired
[n]Please rate your level of memory problems. Slider scale, 0 -100: Good memory vs. Very poor memory
[o]Please rate your level of pain. Slider scale, 0 -100: No pain *vs.* Unbearable pain
[p]Please rate your level of balance problems. Slider scale, 0 -100: No imbalance *vs.* Severe imbalance

Comparisons of CD16Apos NK blood cell counts

An individual's *in vivo* ADCC will be affected by the number of available CD16Apos NK cells and by the activity of the individual's killer cells. Only the CD16Apos set of NK cells mediates ADCC and most of these CD16Apos NK cells are located in blood [38]. Tissue NK cells lack CD16A (38). In this section we compare CD16Apos NK blood cell counts and in the following sections we compare the ADCC functions of these cells.

Counts of CD16Apos NK cells per ul of blood for the 3 groups of donors are illustrated in Figure 2A. The counts were lower (74%) for CFS patients *vs*. unrelated healthy controls. However, the counts were indistinguishable between CFS patients and their family members without CFS, 74% and 78% of controls, respectively. These results are suggestive of a family trait rather than a feature specific for CFS patients. The lower counts of each of the two family groups were statistically insignificant compared to the controls but, when the family members were combined, the CD16Apos NK cells were 77% of controls and the family-wide difference was statistically significant $P < 0.05$ by one-tailed analysis. Within each family, the CD16Apos NK cell counts of CFS patients and non-CFS family members were evenly distributed (high and low) (supplement Figure

S2A), also consistent with a trait that affected all family members rather than CFS patients preferentially. Three of four families evaluated had low CD16Apos NK counts. One family (#3 which included four 2nd degree relatives) was similar to the unrelated healthy controls. Thus, there is a difference in CD16Apos NK ADCC cell counts between several CFS families and healthy controls. The difference is of unknown cause though it is compatible with genetic inheritance.

EC50 NK cell recognition of antibodies

ADCC function is affected by an initial step of NK cell recognition of antibodies and by a subsequent step, the extent of killing that occurs after recognition. EC50s measure the first step. EC50s are the concentrations of antibody needed to support 50% of the maximal ADCC that the lymphocytes can effect. The lower the EC50, the better the NK cells recognize antibody bound to target cells.

High EC50s were required by the CFS patients and by their family members (Figure 2B). Average EC50s were 4.0 +/- 4.0 and 2.6 +/- 3.8 ng/ml obinutuzumab, respectively. The average unrelated control EC50 was 0.39 +/- 0.35 ng/ml ($P<0.05$ for differences from the CFS groups). The EC50s of the CFS family members were bimodal, with a lower mode greater than the unimodal unrelated controls.

Interpretation of the high EC50s is complicated by differences in *FCGR3A* genotypes between the families and the controls. To assess if high EC50s are CFS-associated, it is

appropriate to compare EC50s of donors with the same *FCGR3A* genotypes, since the *FCGR3A* V allele encodes high affinity CD16A which is associated with low EC50s. The CFS family members were 82% CD16A F/F while 80.2% of the unrelated controls had a V allele, data presented later in the results. Only three unrelated controls were F/F, with average EC50s of 0.6 ng/ml, but there were too few unrelated donors to support inter-group statistical comparisons of only F/F genotypes. The high EC50s were common to both CFS patients and non-CFS family members and, as a consequence, disqualify EC50s as a CFS-specific biomarker.

ADCC capacities

ADCC capacity was evaluated using CX1:1and CX-slopes that are illustrated in supplement Figure S3. In contrast to the EC50s, these assessments of ADCC capacity were made with excess concentrations of antibody (625 ng/ml), a concentration that saturated both low and high affinity CD16A receptors. Both CX1:1 and CX-slope are unaffected by CD16A genotypes under these experimental conditions [26]. CX1:1 measures the percentage of 'target' cells that are killed at a 1:1 ratio of CD16Apos NK cells to target cells. It measures net capacity: not every cell with CD16A receptors will kill while a few cells are capable of killing more than one target. CX-slope indicates cellular cooperativity when several lymphocytes attack a single target cell.

CX1:1 and CX-slopes were significantly lower for the CFS patients compared to controls (Figure 2C&D). These measurements were lower for both the patients and their non-CFS family members compared to controls: 66% & 82% of controls for CX1:1 and 71% & 78% for CX-slopes, respectively. When all the family members were combined into one group, there was statistically significant lower ADCC compared to the unrelated healthy controls ($P<0.01$). When each family was considered separately, the lower ADCC occurred in all five families (supplement Figure S2B & C). The CFS patients ran the gamut from high to low ADCC activities within each family. In aggregate, the data are consistent with low ADCC as a potential risk factor for CFS families.

ROC assessment of biomarker potential

We used receiver operating characteristic (ROC) plots to test the validity of low ADCC as a biomarker for CFS. The CFS patients were the true positives. The true negatives were either: non-CFS family members; unrelated controls; or these two groups combined to represent all donors without CFS. Figure 3 illustrates the ROCs. Table 3 indicates the areas under the curves (AUCs). For both CX1:1 and CX-slope tests, AUCs were large and test validity was good for unrelated healthy controls (Ps = 0.06 & 0.02, respectively). However, the AUCs were also greater than 0.5 for the family members without CFS. These AUCs indicate that ADCC is unsuitable as a CFS biomarker when applied to family members without CFS: some of these family members would test false positive for CFS. Ps for test validity for this group of true negatives were insignificant, 0.29 & 0.92 for CX1:1 and CX-slope, respectively). When all donors without CFS were combined into a

true negative group with a larger sample number, the ROC test indicated non-validity. Overall, the ROC tests indicate that low ADCC may be a risk factor but is unsuitable as a diagnostic biomarker for CFS, particularly for close relatives of CFS patients.

Table 3. ROC Assessments of Low ADCC as a Biomarker for CFS

True *vs*. False Test Groups	ADCC	Area Under Curve	P
CFS patients *vs.* Family w/o CFS	CX1:1	0.69	0.29
CFS patients *vs.* All Donors w/o CFS	CX1:1	0.72	0.09
CFS patients vs. Unrelated Healthy Donors	CX1:1	0.76	**0.06**
CFS patients *vs.* Family w/o CFS	CX-slope	0.45	0.92
CFS patients *vs.* All Donors w/o CFS	CX-slope	0.69	0.08
CFS patients vs. Unrelated Healthy Donors	CX-slope	0.77	**0.02**

Synergistic risk of low ADCC plus low CD16Apos NK cell numbers

The combination of low CD16Apos NK blood cell counts plus low ADCC capacity could represent familial synergistic risk for CFS. Quadrant analyses offer a simple means to determine if a group of subjects is over-represented within the quadrant with combined low activities for two variables compared to the other 3 quadrants. First, we assessed if ADCC and the cell counts varied independently, which would affect synergy. Even though there were positive correlations of the CX1:1s and the CX-slopes with the cell counts (not illustrated), the R^2s for linearity were low, consistent with independence. Figure 4A indicates that CFS patients and family members were over-represented in the low-low quadrant for low CX1:1 combined with low CD16Apos NK blood cell counts.

These group of all family members together was 24-fold more likely than unrelated healthy donors to be in this low-low quadrant (P = 0.02). All family members together were also 12.5-fold more likely than controls to be in the low-low quadrant for CX-slope *vs.* cell counts (Figure 4B, P<0.05). Overall, the quadrant analyses indicate that synergistic risks may exist for all members of a family that includes multiple CFS patients.

FCGR3A genetics

To qualify the *FCGR3A* F/F genotype as a risk factor for CFS, this genotype would have to be significantly greater for patients or families than what would occur randomly. Qualification is problematic because the F allele has high frequency. The Utah population of this family study is of non-Finnish northern European descent [39] with an expected 41.9% F/F frequency [40]. The frequency of F/F was 91% for the CFS patients and 82% for the family members without CFS, while the unrelated healthy controls were 18.8% F/F (Table 4). Interpretation of these data is impacted by the fact that F/F by F/F parents will produce only F/F offspring. This scenario of homozygous F/F parents applied to the families of this study (Figure 1). To make matters more challenging to interpret, 3 parents were deceased. The genotypes of the 7 living parents were 5 F/F and 2 F/V. The deceased parents were either F/F or F/V, and not V/V as determined from the genes inherited by their progeny. Two estimates (for the highest and lowest possible F/F frequencies) were used to assess the significance of the parental F/F

homozygosity families, see Table 4. The highest estimate of 8 (80%) F/F parents was statistically significantly different from northern European controls (P = 0.02). The lowest estimate of 5 (50%) F/F parents was insignificant (P = 0.42). These results indicate that F/F homozygosity *could* affect CFS families but may not. The results are simply inconclusive.

Table 4. Frequencies of CD16A F/F homozygosity

Populations	Number donors			% F/F	95% confidence limits[+]
	F/F	F/V or V/V	All		
CFS patients	10	1	11	**90.9**	58.7-99.8%
Non-CFS family members	18	4	22	**81.8**	59.7 -94.8%
Unrelated healthy donors	3	13	16	**18.8**	4-45.6%
CFS family parents, highest possible %F/F#	8	2	10	**80[@]**	44.4-97.5%
CFS family parents, lowest possible %F/F#	5	5	10	**50.0**	18.7-81.3%
Reference Group: Northern European origin^	385	534	919	**41.9**	38.7-45.2%

+Binomial "exact" calculations of the confidence intervals for proportions

#Ten CFS family parents: living 5 F/F, 2 F/V, and 0 V/V; 3 deceased.

[@]P <0.05, for 8 or more F/F, calculated with 0.419 as the probability that a parent will be F/F.

^From reference (40); the *FCGR3A* allele encoding V158 CD16A had a frequency of 0.363.

Discussion

This pilot report provides five findings that indicate that low ADCC may be of importance in familial CFS. (1) *The CD16Apos NK cell blood counts were lower for all family members vs. unrelated healthy controls*. Family members, both patients and non-CFS, had ~75% of cytotoxic cells compared to the unrelated healthy controls. (2) *Patients and family members required high amounts of antibody (EC50s) for NK cell*

recognition of cells bound with antibodies. This finding suggests that family members might need to produce more antibodies than controls in order to control infections. (3) *ADCC capacity of CFS patients and their family members without CFS was lower than that of unrelated controls.* The modest reduction, to ~75% of unrelated control ADCC, may be of unexpected importance because NK cells lose their CD16A receptors during killing. After only a few rounds of killing [41), the NK cells' ability to mediate ADCC is lost. Re-synthesis of receptors is insufficient to rapidly replenish a depleted supply of CD16Apos NK cells. (4) *Synergistic combinations of low CD16A NK cell numbers and low ADCC occurred in the CFS families.* Simplistically, a multiplier combination of two reductions, ~0.75 times ~0.75, could potentially decrease the ADCC component of immunity for some individuals to ~0.56 of controls. (5) *Parents in the CFS-affected families had a high incidence of homozygous low affinity CD16A F/F antibody receptors.* The low sample number contributed to a lack of statistical significance. However, if familial CFS were to have multiple genetic risks, CD16A F/F homozygosity would be a candidate for future investigation.

The findings above resemble and contrast with previous studies. The similar low ADCC of the patients and their unaffected family members resembles observations reported by Dr. Paul Levine *et al.* [11]. Their study monitored antibody-independent natural killing towards K562 tumor cells. They reported a lack of difference in NK activities (P=0.38) for 8 family members with CFS compared to 12 unaffected family members.

The rank order of NK activity was lowest for CFS patients, intermediate for non-CFS family members and greatest for controls. We observed the same rankings for ADCC. For perspective, the low ADCC may apply only to familial CFS. An early report found low ADCC in unrelated CFS patients [42]. However, the ADCC of unrelated CFS patients in Stockholm was lower than healthy controls [43] but statistically insignificant. The investigators of the Scandinavian study used CD107A expression by effector NK lymphocytes rather than cytotoxicity to monitor ADCC, which is a less sensitive way to monitor ADCC and may have affected detection of low ADCC. The issue of low ADCC in unrelated CFS patients (and their family members) warrants further attention. Thus, the present report considerably extends research concerning the role of ADCC in CFS, including consideration of FCGR3A genetics for the first time, and raises new questions.

The synergistic ADCC risks applied to all the CFS family members. Individuals with combined risks could have prolonged clearance times for viral infections while cytotoxic T cells may exert secondary control of viral infections. As observed with type 1A autoimmune diabetes [44], multiple immunological risk factors can predispose individuals to a disease without causing the disease.

This report has several limitations: numbers of families and donors, inclusion of only familial CFS (with the exclusion of families with only one case of CFS), only a single blood

sample per donor; one race; one geographic location; and CFS patients with only moderate rather than severe disease. The effects of low ADCC in CFS for other locations, races and in extreme CFS disease are un-addressed. The continuation of research presents opportunities to address these limitations and to benefit the patients by guiding therapies. Identification of CFS patients with low NK and/or low ADCC may indicate those patients most likely to respond to immunomodulatory therapies such as poly(I:C) [45] that promotes NK cell cytotoxic functions. In summary, this report supports a role for low ADCC as a risk factor for familial CFS and suggests aspects of immunology that may also apply to other subgroups of CFS patients.

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Data availability

Additional clinical information may be obtained upon request.

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Competing interests

The authors lack financial and non-financial competing interests in this study.

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Geolocation (Reno, NV and Salt Lake City, UT, USA)

Figure 1

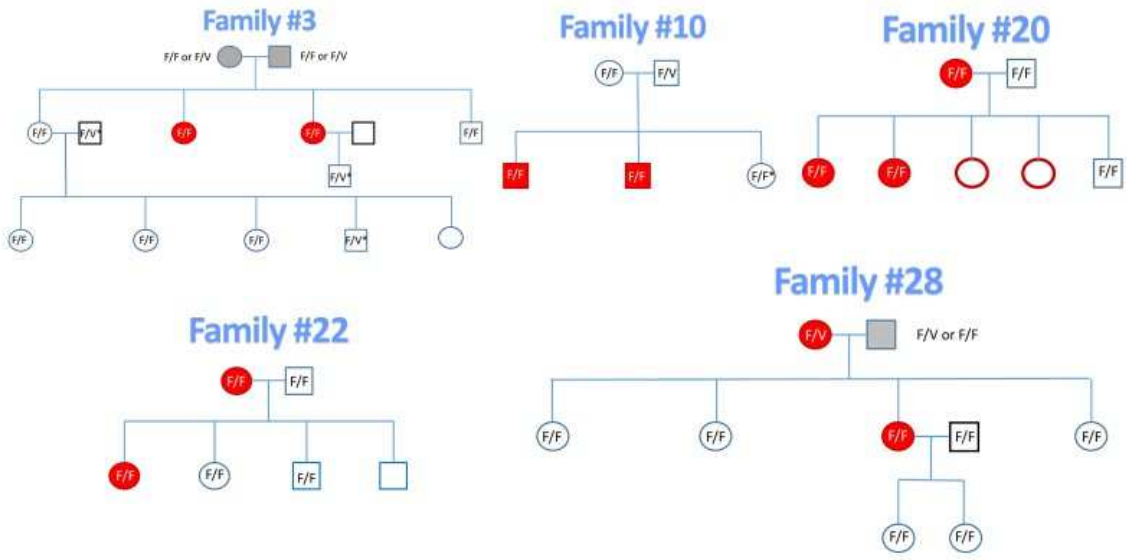


Figure 1. CFS family pedigrees. Red symbols indicate CFS patients and closed symbols indicate the 11 patients who participated. Study participants are indicated by their CD16A genotypes. The deceased parents are indicated with gray symbols. The 3 fathers of the 3rd generation were unrelated to the patients and are indicated with black bold squares. The genotypes marked F/V* were determined by flow cytometry and allelic inheritance.

Figure 2

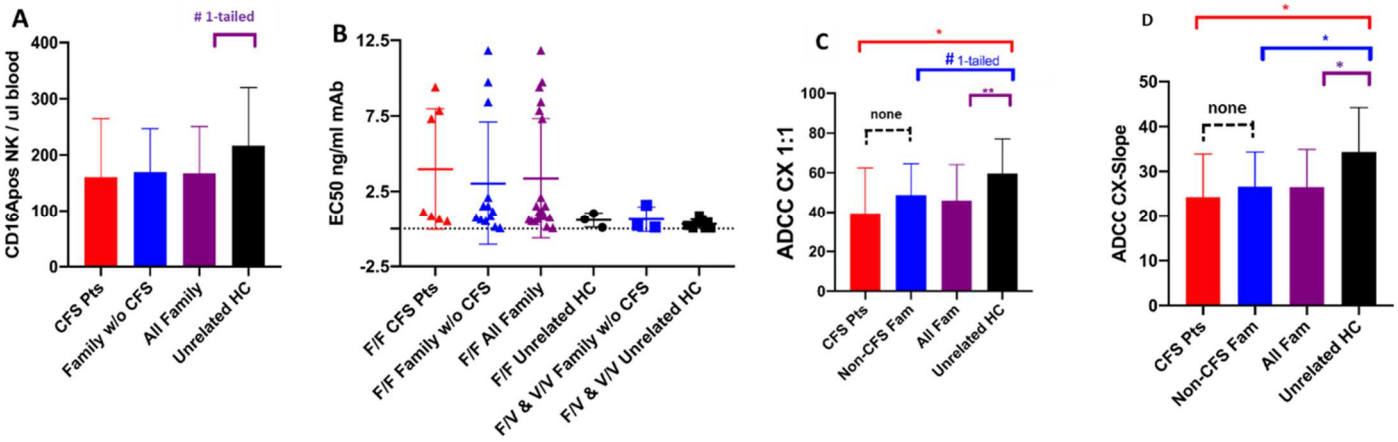


Figure 2. ADCC by CFS patients, family members without CFS, and unrelated healthy controls. Mean values with standard deviations are indicated. Single asterisks above the brackets indicate Ps<0.05, two asterisks Ps <0.01, for two-tailed T-tests. A hashtag indicates P<0.05 for one-tailed T tests. Unbracketed mean values indicate the absence of statistical significance compared with other means within the data set. **A. CD16Apos NK cells per ul blood. B. EC50s (effective concentrations of antibody to support 50% of maximal lysis).** CD16A F/F donors are represented separately from the F/V and V/V donors in order to compare genotypes with and without high affinity CD16A V receptors. The statistically significant differences (P<0.05) between CFS patients or CFS family members and controls, are not illustrated because of biases in comparisons of predominantly F/F groups with a predominantly F/V control group. **C. ADCC CX1:1s (Cells killed by ADCC at a CD16Apos NK to target cell ratio of 1:1).** The CX1:1 data are from families #3, 10 & 28. **D. ADCC CX-slopes.** CX-slopes are the % increase in dead cells per 10-fold increase in CD16A NK cells. The data are from families #3, 10, 20, 22 & 28.

Figure 3

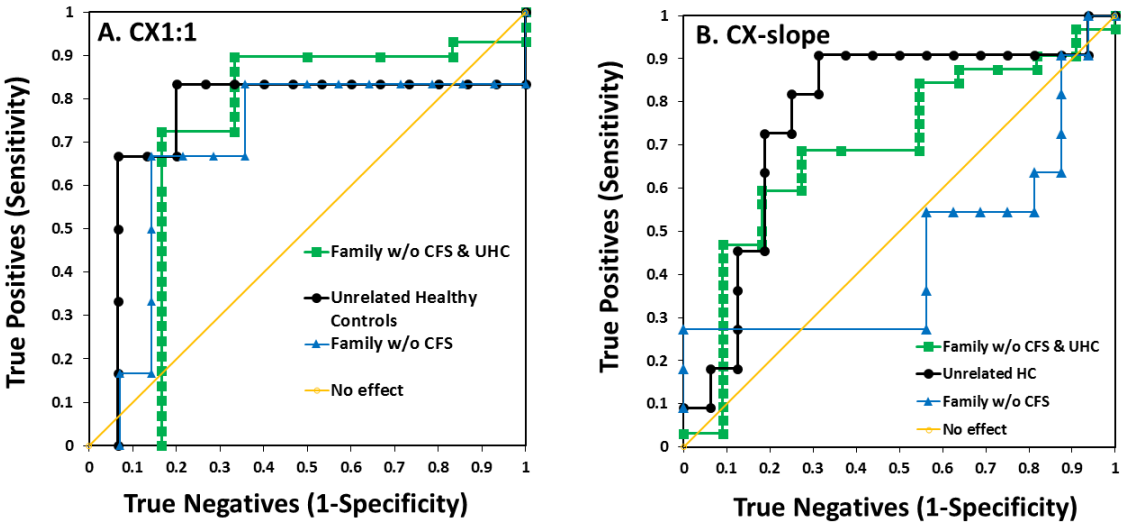


Figure 3. ROC tests of ADCC to diagnose CFS. The areas under the curve (AUC) indicate suitability of a test. For the ROC analyses, the true positives were defined as the CFS patients. The true negatives were defined as either: 1) family members without CFS; 2) the unrelated healthy controls; or 3) all donors without CFS, the unrelated donors and the family members without CFS combined. The diagonal line marks an AUC of 0.5 that indicates a test without the ability to distinguish true positives from true negatives.

Figure 4

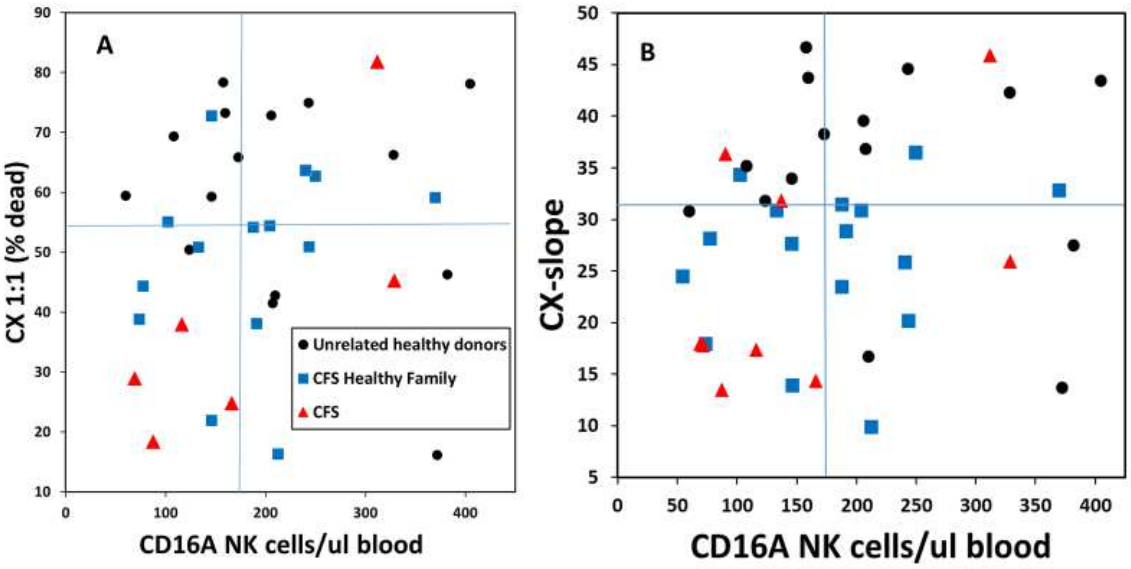
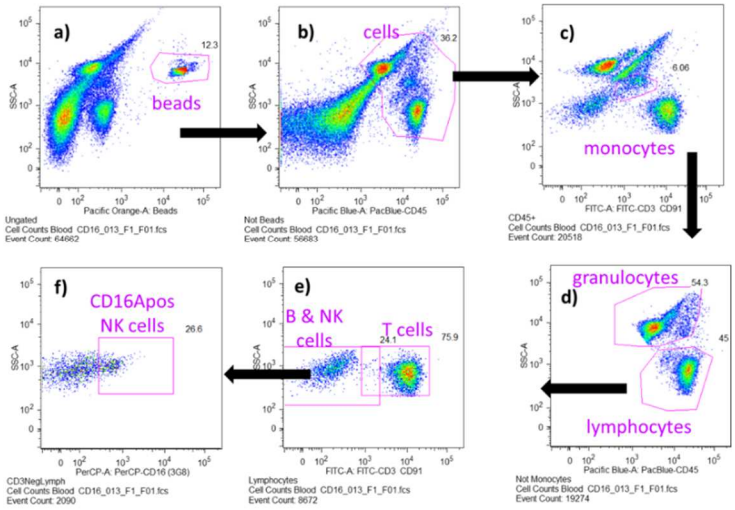
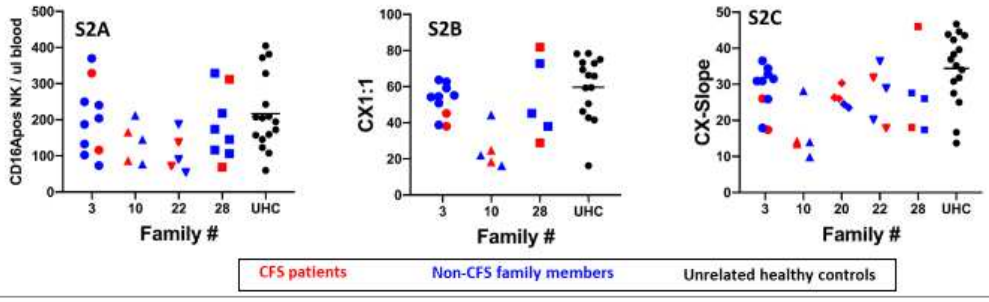


Figure 4. Assessment of synergistic risks for CFS. Synergy was evaluated for low CD16A NK effector cell counts combined with low ADCC capacities. CFS family members, with CFS and without CFS, were compared to unrelated controls. Division of the quadrants was based on the median values of each variable. **A. CX1:1.** Data are from families #3, 10 & 28. **B. CX-slopes.** Data are from families #3, 10, 22 & 28. Ps <0.05 for family members in the low-low quadrants.

Supporting Information

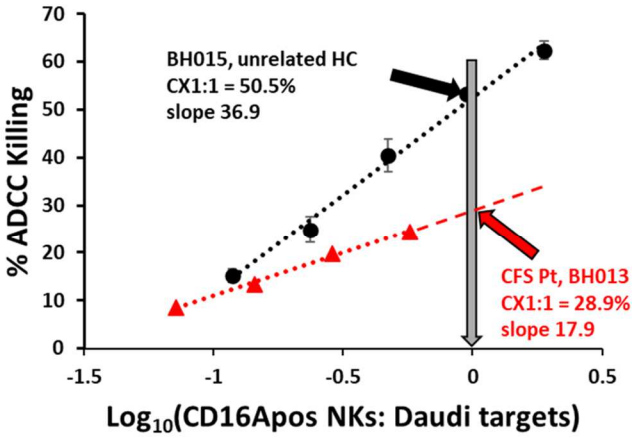
Supplemental Figure S1. TruCount^{R} determination of CD16Apos NK cells per ul blood. Sequential gating with FlowJo software: a) bead; b) intact cells; c) gating out of CD91pos monocytes; d) lymphocytes; e) CD3neg B & NK cells; f) CD16Apos NK cells.





Supplemental Figure S2. Distribution of ADCC values among CFS and family members without CFS within each family. Each family is indicated by different symbols. Red symbols indicate CFS patients and blue non-CFS family members. Black symbols indicate the unrelated healthy donors with bars for the mean values. **A. CD16A NK cell counts per µl of blood.** The one-tailed P values for each *vs.* the UHCs were 0.34, 0.06, 0.02 and 0.23 for families 3, 10, 22 and 28, respectively. **B. CX1:1.** The one-tailed P values for each family *vs.* the unrelated healthy controls were 0.11, <0.001 and 0.26 for families 3, 10 and 28, respectively. **C. CX-slopes.** The one-tailed P values for each family *vs.* the unrelated healthy controls were 0.05, 0.001, 0.04, 0.07 and 0.09, for families 3, 10, 20, 22 and 28, respectively.

Supplemental Figure S3. Linear ADCC is illustrate for one unrelated healthy control and one CFS patient. Black and red arrows indicate the killing at the CD16Apos NK cell to Daudi targets with antibody ratio of 1:1, The $\log_{10}$ value for the 1:1 ratio is indicated by the gray arrow. Cytotoxic activity in the absence of antibody was negligible and is not illustrated.



Chapter 4

Development of Immunologically Undetectable and Highly Cytotoxic Natural Killer Cell Lines Suitable for Transfusion and Treatment of Multiple Cancer Types

(A proposal funded by the American Association of Immunologists, with Addition of a Research Progress Update)

American Association of Immunologists Proposal

Research Plan

Current cancer treatments such as chemotherapy and radiation have a myriad of unwanted side effects due to their low specificity for selectively killing tumor cells (1–3). New treatments, such as monoclonal antibodies and chimeric antigen receptor T cells, are more specific for tumor cells but are susceptible to antigen loss and can only target specific tumor types based on their tumor antigens (4, 5). Here we propose the development of transfusable universally tolerated natural killer (NK) cell lines (U-NKs) that will be tolerated by the host immune system and effective against multiple tumor types. The NK cell lines will be highly cytotoxic and able to recognize tumor-associated ligands expressed by a variety of tumors which are absent from healthy cells. To create these cell lines, we will perform gene editing to knock out MHC class I expression in order to prevent host immune recognition of our universal NK cells (6, 7). Human cells are notoriously difficult in terms of gene editing and common techniques like viral vectors introduce viral DNA which integrates into the host genome (8). Improvements are needed in both the protein delivery method and editing system for human cells. We will use a new method known as iTop which takes advantage of natural endocytic mechanisms to allow for efficient delivery of editing proteins into cells without viral integration (9). We will couple this delivery method with CRSIPR/Cas9 genome-editing to create knockouts of MHC I in our universal cell lines (10).

Specific aim 1. To create a transfusable tumor-derived NK cell line that would escape recognition by the recipient patients' T cells. To develop a NK cell line suitable for transfusion, cells must be able to evade host immunity. Recognition of foreign cells by the host is promoted by T-cell recognition of non-self MHC class I molecules (11, 12). Beta-2-microglobulin (B2M) is an integral part of all MHC class I molecules and its deletion will prevent MHC-I expression and T cell recognition (7). We will: a) delete b2M from NK-92s using CRISPR-Cas9 to create a new U-NK92 cell line and b) measure loss of recognition by T cells as lower mixed leukocyte responses (MLRs) (13). When the MLRs are substantially lower, we will conclude that elimination of b2M promoted tolerance of the U-NK92.

Aim 1a. Deletion of beta-2-microglobulin from the NK92 cell line. We will use the CRISPR/Cas9 genome editing system combined with a new intracellular protein delivery method known as iTop. iTop takes advantage of natural endocytic mechanisms to allow for efficient delivery of CAS9 proteins and sgRNAs into human cells without the need for a viral vector(9). Viral DNA integrated into the host genome is likely to make the edited cells into targets for host NK cells.

For editing our single guide RNA (sgRNA) used to target the beta-2-microglobulin gene is (AGAGATATATCTGGTCAAGG) and was chosen using the Broad Institute sgRNA creation tool and the NCBI sequence for the b2M gene (NC_000015.10). For delivery of the gene editing components, we will use the iTOP delivery which takes advantage of natural endocytic mechanisms, reducing stress put on the cell and increasing efficiency. This

method has 4 components: A compound to initiate endocytosis (GABA); Na^+ to create a large osmolar transmembrane gradient; osmoprotectants to prevent lysing of the cells and protect protein integrity (glycine + glycerol); and our sgRNA with Cas9 proteins (Thermo Fisher) to be delivered into the cell. Our sgRNA and Cas9 proteins will have a nuclear localization signal to tag for transport into the nucleus after they have been endocytosed. After treatment, cells will be cultured for several days, and then MHCI negative cells will be selected.

Aim 1b. Test, select for, and culture successfully edited cells and measure their recognition by T cells. After cells have been edited and cultured we will test for the successful edits using flow cytometry and FACS (fluorescent activated cell sorting) to isolate cells which do not have MHC class I molecules. Once these beta-2-microglobulin negative cells are cultured, we can test for T cell evasion of our edited cells using mixed lymphocyte reaction (MLR) assays.

To test for MHC-I negative cells will use the PE-tagged anti-human β2-microglobulin monoclonal antibody (clone 2M2, BioLegend). Cells that are negative will be sorted using FACS and cultured. Cultured cells will then be tested for T cell evasion using a CFSE-based MLR assay. Test NK cells will be treated with mitomycin c to prevent cell division, then exposed to T cells labeled with CFSE from a healthy donor. When labeled T cells react to foreign cells they will divide, creating dimmer CFSE progeny. We anticipate responses to standard NK92s but not our U-NK92s. At the end of aim 1, we will have generated a U-NK92 cell line that is capable of evasion of host T cells. As a tumor itself,

the U-NK92 must be irradiated before transfer to patients and will survive for a short amount of time because it cannot divide after irradiation.

Specific aim 2. To create a non-tumor, primary U-NK cell line with maximal potential to kill multiple tumors by selecting NK cells which lack inhibitory receptors, deleting b2M from these cells and evaluating their ability to kill multiple tumors. In contrast to the U-NK92 of aim 1, a primary U-NK cell line derived from a healthy donor could be transfused into a patient without risk of causing a secondary tumor and would survive for weeks because it retains the ability to divide (14). NK cells in the body are highly diverse, with differential expression of multiple inhibitory receptors such as KIRs and NKG2A (15). Our new U-NK cell line will be highly cytotoxic, lack b2M, and also lack inhibitory receptors so that it can kill tumors with inhibitory ligands. We will: a) select by FACS sorting NK cells without inhibitory KIRs and NKG2A (16–18); b) delete b2M and measure NK cytotoxicity to tumor cells (19).

2a. Select for primary NK cells lacking inhibitory receptors (to prevent inhibition of killing by ligands on tumor targets) and then delete their beta-2-microglobulin (so that they escape cytotoxic T cells of recipient cancer patients). Humans have a diverse pool of NK lymphocytes in circulation with variable expression of inhibitory receptors such as KIRs and NKG2A (15). To ensure that our primary NK cell line has maximum killing capacity we will select for cells that don't have the inhibitory receptors KIR2DL1 C2, KIR2DL2 C1, KIR2DL3 C1, KIR2DL4, NKG2A using the anti-human antibodies, clones HP-MA4, DX27, mAb 33, and 16A11, respectively (BioLegend). This selection will prevent

KIR based inhibition of killing when NK cells interact with inhibitory ligands on tumor targets. Once these cells have been sorted by FACS, they will be cultured for several days with IL-2 to induce cell proliferation. This new cell line will then be edited to remove b2M identically to the cells in aim 1.

2b. Test for tolerance of new primary NK cell lines when exposed to peripheral blood lymphocytes of different donors and cytotoxic efficacy against tumor cells.

Once the edited cell lines have been successfully cultured, we will test for tolerance using peripheral blood mononuclear cells (PBMC's) obtained from healthy donors. We will also test our new cell line for cytotoxicity to multiple tumors. Our U-NK cells will be tested against 4 tumor cell lines: Daudi (Burkitt's lymphoma MHC I neg), Raji (Burkitt's lymphoma MHC I pos & able to bind to KIRs), AU565 (Her2 pos adenocarcinoma), and HT-29 (colorectal carcinoma). We will use the chromium 51 killing assay to determine both tolerance and killing efficacy of our newly edited primary NK cell line(19). For evasion of recognition, our edited U-NK cell line and control NK cells will be labeled with Cr51 which integrates into the cell cytoplasm. Labeled cells will then be exposed to PBMC's from a healthy donor. Chromium will be leaked from the cytoplasm to the outside as cells are lysed and this can be measured. Then when radioactivity values are compared to cells that have been artificially lysed, we can determine what percentage of unedited and edited NK cells were recognized as foreign by host immune cells and have been killed.

Another ^{51}Cr assay will be run to determine our cell lines' capacity to kill different types of tumor cells. We will use the four types of tumor cells stated above and in this experiment the tumor cells will be labeled with ^{51}Cr and our primary U-NK cell line will serve as the effector. Measuring the amount of ^{51}Cr leaked from lysed tumor cells killed by our U-NK cell lines will indicate how effective our cell lines are at killing different types of tumors. Once our U-NK cell line has demonstrated high tolerance by the host immune system and efficacy in killing different types of tumors we will conclude that we have successfully created an immunologically tolerated and highly cytotoxic natural killer cell line suitable for treatment of multiple cancer types.

Research Progress Update to the American Association of Immunologists Proposal

Progress on the project has been slow but not discouraging. We lost a significant amount of time due to lab closures because of the COVID-19 pandemic but were able to begin serious work in late 2021. We were able to successfully culture and establish our NK92 tumor cell line as well as produce our lentivirus. We selected two sgRNAs which we successfully reproduced in larger quantities and have moved onto the transfection stage. Initial cell viability of lentivirus incubated NK92 cells was good but we suspect that CRISPR editing may have stressed the cells and necessitated closer monitoring of the NK-92 cell culture or different cell culture parameters. As a result of this, our first attempt at transfection was unsuccessful but will provide an excellent basis for repeat trials in the future. We are confident based on similar research with gene editing and

NK-92 cells as well as attempts at deleting MHC I in other cell types that our current method is viable for deletion of MHC I in the NK-92 cell line (20,21,22).

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Chapter 5

Perspectives and Conclusions

NK cell based cancer therapies

My current feelings are that NK cell based cancer therapies, though finally catching on, are still an under-researched area in cancer treatment. Technologies tend to trickle down from areas of intense research once they have been partially or fully developed to adjacent areas. Just like how many of the technologies for our modern cars were developed in Formula One racing first, then eventually trickled down into production cars, many of the technologies now being apply to NK cells were first developed for T cells. An example of this would be CAR NK cells which were based on chimeric antigen receptor research in the T cell field. Because NK cells make up a relatively small *in vivo* proportion of the total lymphocytes and were discovered later, T cells have been the larger and more established area of research. Logically if there are less of a cell type in the body to take advantage of for treatments they should be less effective overall as a treatment. Research thus would naturally be focused on the cell type that is more predominant and theoretically should have a higher total capacity to facilitate treatment.

However, with recent advancements in *ex vivo* and *in vivo* expansion and our ever improving genetic engineering technology, I think that the foremost quality that should be evaluated in terms of potential as a cancer treatment should be the raw

cytotoxic capacity of the cell type. As our expansion technology becomes better and better, it should be possible to produce any number of a certain cell type for transfusion and thus the important part in developing treatments will not be the availability or number of the cells used for treatment but their efficacy in killing tumors per cell. This is why I think NK cells with their large capacity for cytotoxicity given the amount of granzymes and perforin are going to be the future for cancer treatment.

Chronic Fatigue Syndrome

There exists a significant amount of stigma around chronic fatigue syndrome. Though I think this stigma is a significant hindrance to patients who have the disease, I can understand from a provider's standpoint why the stigma exists. I think there are several contributing factors including CFS being a diagnosis of exclusion, symptoms being often vague and subjective, and our lack of understanding of the pathophysiology of the disease. CFS has developed a similar reputation to that of fibromyalgia where, when people have nonspecific symptoms, they are just assigned this sometimes inaccurate diagnosis of CFS. This difficulty in diagnosis can lead to patients having problems finding appropriate care and may inject some bias on the part of the provider, perhaps chalking up symptoms to over exaggeration, psychological manifestation, or some other cause.

It is my personal viewpoint that chronic fatigue syndrome in its current state may be more than one disease and be several different diseases lumped together. Since the diagnostic criteria are only based on presence of subjective symptoms, we cannot

be sure that we are not putting different diseases in the same bin. I think that those patients who truly have chronic fatigue syndrome are the ones who have the well-established deficiencies in immune system function. Though this immune system impairment may or may not be the cause of the disease, there is a clear pathological problem that is present. Some people who may be experiencing similar symptoms to people that have this innate immune deficiency may also be classified as having chronic fatigue syndrome though they may have a different disease entirely. I think that mixing together these different groups is not beneficial for either patient or provider making it more difficult to diagnose and treat the patients. Perhaps it would be beneficial if in the work up for chronic fatigue syndrome patients were divided into Class A and Class B with those that have these immune dysfunctions documented on work up being put in one class and those that do not being put in another.

Maybe one way to go in the future would be patients who have chronic fatigue syndrome with documented immune deficiencies could receive treatments beyond supportive care and these treatments could be developed specifically for this group of patients to target the immune deficiencies. Treatments targeted towards improving the host immune system such as adoptive transfer of expanded lymphocytes to make up for the shortcomings of the host system or even genetic engineering to improve cytotoxic efficacy of the patients' lymphocytes could be tried. The only thing that is certain is that we have a limited understanding of the disease and even more limited number of effective treatments.

The Institute of Medicine has made a push to rename CFS as systemic exertion intolerance disease (SEID) with new looser definitions on who can qualify for diagnosis. I personally feel this is a step in the wrong direction. For the price of a more inclusive definition the group of people who have true pathologic innate immune dysfunction will further be buried in a larger group of people who have similar symptoms but do not have the same immune problems. This will further muddy diagnosis and treatment option and will make research more difficult as it will be more difficult to find and study those with the aforementioned immune dysfunction. Perhaps the renaming of the disease is an effort reduce stigma and allow for a restart with a new name and an opportunity for new branding. I however am skeptical that this will work as even some of the biggest and most well-funded rebranding efforts have not been successful. We are all aware that Meta is just Facebook and Alphabet is Google. I think a renaming of CFS to SEID can only further add to confusion around the diagnosis and treatment of the disease.

Appendix

Titles and Abstracts of Other, Co-Authored Publications

1. Natural Killer (NK) Cell Expression of CD2 as a Predictor of Serial Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).

Jennifer J-J. Tang, **Alexander P. Sung,** Michael J. Guglielmo, Lydia Navarrete-Galvan, Doug Redelman, Julie Smith-Gagen and Dorothy Hudig. Antibodies (Basel). 2020 Oct 16;9(4):E54. doi: 10.3390/antib9040054. PMID: 33081115

Abstract: NK cell ADCC supports monoclonal antibody anti-tumor therapies. We investigated serial ADCC and whether it could be predicted by NK phenotypes, including expression of CD16A, CD2 and perforin. CD16A, the NK receptor for antibodies, has AA158 valine or phenylalanine variants with different affinities for IgG.CD2, a costimulatory protein, associates with CD16A and can augment CD16A-signaling. Pore-forming perforin is essential for rapid NK-mediated killing. NK cells were monitored for their ADCC serial killing frequency (KF). KF is the average number of target cells killed per cell by a cytotoxic cell population. KF comparisons were made at 1:4 CD16pos NK effector:target ratios. ADCC was toward Daudi cells labeled with 51Cr and obinutuzumab anti-CD20 antibody. CD16A genotypes were determined by DNA sequencing. CD2, CD16A, and perforin expression was monitored by flow cytometry. Serial killing KFs varied two-fold among 24 donors and were independent of CD16A genotypes and perforin levels. However, high percentages of CD2pos of the CD16Apos NK cells and high levels of CD16A were associated with high KFs. ROC analysis indicated

that the %CD2pos of CD16Apos NK cells can predict KFs. In conclusion, the extent of serial ADCC varies significantly among donors and appears predictable by the CD2posCD16Apos NK phenotype.

2. Inclusion of Family Members without ME/CFS in Research Studies Promotes Discovery of Biomarkers Specific for ME/CFS.

Tokunaga, K, **Sung AP**, Tang JJ, Guglielmo MJ, Redelman D, Smith-Gagen J, Bateman L, Hudig D. Work 66: 327–337, 2020. (special issue on CFS, June 2020) doi: 10.3233/WOR-203177. PMID: 32568152

BACKGROUND: The search for a biomarker specific for ME/CFS (myalgic encephalomyelitis/chronic fatigue syndrome) has been long, arduous and, to date, unsuccessful. Researchers need to consider their expenditures on each new candidate biomarker. In a previous study of antibody-dependent cell-mediated cytotoxicity (ADCC) by natural killer lymphocytes, we found lower ADCC for ME/CFS patients vs. unrelated donors but ruled against low ADCC as a biomarker because of similar ADCC for patients vs. their family members without ME/CFS.

OBJECTIVE: We applied inclusion of family members without ME/CFS, from families with multiple CFS patients, as a second non-ME/CFS control group in order to re-examine inflammation in ME/CFS.

METHOD: Total and CD16A-positive 'non-classical' anti-inflammatory monocytes were monitored.

RESULTS: Non-classical monocytes were elevated for patients vs. unrelated healthy donors but these differences were insignificant between patients vs. unaffected family members.

CONCLUSIONS: Inclusion of family members ruled against biomarker considerations for the monocytes characterized. These pilot findings for the non-classical monocytes are novel in the field of ME/CFS. We recommend that occupational therapists advocate and explain to family members without ME/CFS the need for the family members' participation as a second set of controls in pilot studies to rapidly eliminate false biomarkers, optimize patient participation, and save researchers' labor.