Natural Killer Cells

ATGen Canada Inc.
Innovation in Immunodiagnostics
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Natural Killer Cells – Overview of Role and Function

Natural killer (NK) cells are large granular lymphocytes with a characteristic morphology which play a role in innate immunity. NK cells are classified as group 1 innate lymphoid cells (ILCs) (Spits and DiSanto, 2011, Campbell and Hasegawa, 2013), are involved in the early defense against infection and tumors and may be involved in autoimmunity and hypersensitivity reactions (Bryceson et al 2011). Recruient of NK cells to the site of inflammation, infection or transformation is a first step in the response to infection and tumors (Bryceson et al, 2011) and occurs prior to the initiation of an adaptive immune response against infection or tumor (Langers et al, 2012). The mechanisms by which NK cells defend the body are via targeted cell death and release of chemokines and cytokines (innate immune system), as well as by helping other immune cells in their targeted cell elimination (adaptive immune system) (Bryceson et al 2011), thus linking innate and cellular immunities. NK cells regulate immune responses via their role in enhancing antigen-specific T-cell responses, in the regulatory crosstalk network with dendritic cells and neutrophils (Campbell and Hasegawa, 2013) and by mediating major histocompatibility (MHC)-independent and antibody-dependent cellular cytoxicity (Yu, Freud and Caligiuri, 2013) and may even be subject to developmental NK cell education and memory (Campbell and Hasegawa, 2013; Yu, Freud and Caligiuri, 2013).

NK cells can be identified from other lymphocytes by their expression of the clusters of differentiation markers (CD markers) surface antigens CD56 and the absence of expression of CD3 (CD3 is found on T cell lymphocytes) (Caligiuri 2008). NK cells are part of the hematopoietic system and are derived from CD34 positive hematopoietic progenitor cells (Caligiuri 2008). It appears that both T cells and NK cells may have evolved from a common early cytolytic effector cell (Caligiuri 2008, Rabson, Roitt & Delves, 2005). NK cells represent 5-20% of peripheral blood mononuclear cells and are also found in liver, spleen, peritoneal cavity, lung, bone marrow, placenta or uterine mucosa (Langers et al, 2012, Campbell and Hasegawa, 2013). NK cells are short-lived – at any one time, there are likely more than 2 billion circulating in an adult (Blum and Pabst, 2007).

How do NK cells work?

NK cells have activating and inhibitory surface receptors (see Figure 1). The activating receptors recognize structures on glycoproteins on the surface of virally infected cells or on tumor cells. These activating receptors bring the target cell close to the NK cell to allow the NK cells to act on these target cells. (Rabson, Roitt & Delves, 2005). In humans, the major activation receptors include NKp46, NKp30 and NKp44 (called natural cytotoxicity receptors or NCRs) (Bottino et al, 2005, Moretta & Moretta, 2004), CD16 (FcγRIIIA) and NKG2D (Langers et al, 2012). Therefore, NK cell phenotype is often expressed in scientific terms as $CD3^-$ $CD56^+NKp46^+$NK cells (Bryceson et al 2011), or alternatively $CD16^+CD56^+CD3^-$ NK cells (Langers et al, 2012), although these populations are heterogeneous in their phenotype (ie. density of surface antigens) and in functionality – see discussion below.

The ligands for NK cell activating receptors can also be found on non-infected normal cells. The NK cells do not kill these normal cells due to the presence of the inhibitory surface receptor (killer-cell immunoglobulin-like receptors – KIR), which recognizes MHC class I glycoproteins found on the surface of all nucleated cells. The inhibitory receptors thus override the signals from the activating receptors. The activating receptors will cause the NK cell to kill a target cell in the absence of an MHC class I surface protein, proteins usually not expressed in virally infected or tumor cells. In fact, in breast cancer, around 60% of metastatic tumors lack MHC class I. (Rabson, Roitt & Delves, 2005)

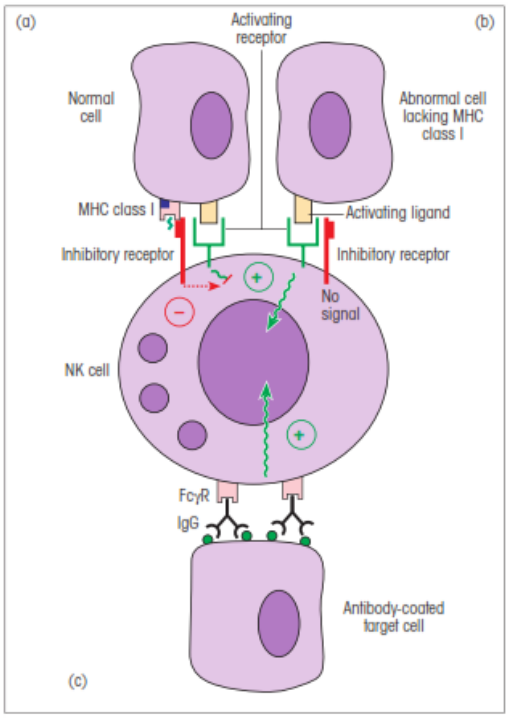

Figure 1: Natural killer (NK) cells. Natural killer cells possess both activating and inhibitory receptors. Engagement of an activating receptor by its ligand sends a stimulatory signal into the NK cell but this is normally subverted by a signal transmitted upon recognition of major histocompatibility complex (MHC) class I molecules by an inhibitory receptor (a). Any nucleated cell lacking class I is deemed abnormal and is killed following unimpeded transmission of the activation signal (b). Natural killer cells usually possess several different inhibitory and activating receptors and it is the balance of signals from these that determines whether the cell becomes activated. Like several other cell types, NK cells can utilize their Fcγ receptors to mediate antibody-dependent cellular cytotoxicity (ADCC) on target cells coated with antibody (c).

Figure taken from (Rabson, Roitt & Delves, 2005)

Dynamic multiple receptor-ligand interactions are involved in NK cell activation by target cells (Bryceson et al 2011). NK cell activation can also be induced by cytokines, including interleukin (IL)-2, IL-12, IL-15, and IL-18 (Moretta et al, 2008; Cooper et al, 2004; Ferlazzo et al, 2004). Cytokines such as IL-2 and IFN-γ are released by T-cells (T-helper and cytotoxic T cells respectively) in cell-mediated innate immunity ie. control of intracellular infections (Rabson, Roitt & Delves, 2005). These cytokines also can help activate macrophages to allow them to kill intracellular parasites like viruses (Rabson, Roitt & Delves, 2005).

Once activated, NK cells also release several types of cytokines, such as IL-1, TNF, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ) and transforming growth factor beta (TGF-β). (Rabson, Roitt & Delves, 2005)

NK cells do not have antigen-specific receptors, however, the Fcγ region of immunoglobulin G (IgG) antibodies attached to the surface of some target cells can result in antibody-dependent cellular cytotoxicity (ADCC) via recognition of these antibodies by the FCγ receptor on NK cells CD16 (Rabson, Roitt & Delves, 2005, Langers et al, 2012) Activation of the Fc receptor CD16

on NK cells can induce NK cells to bind the tumor cell coated with antibodies and subsequent degranulation (Bryceson et al 2011, Langers et al, 2012).

Following activation of the NK cell, there is a polarization of granules from the NK cell to the target cell, followed by release of the contents into the extracellular space and subsequent death of the target cell (see Figure 2). In human cells, one of these granule contents is a cytolysin (perforin) which creates a pore in the target cell outer membrane, allowing the passage of granzymes (serine protease enzymes) and other cytotoxic factors from the granules to pass through and cause cell death (or "apoptosis") by attacking the DNA within the target cell. (Rabson, Roitt & Delves, 2005) The release of perforin and granzymes is the fastest and most powerful manner of tumor cell lysis (Langers et al, 2012).

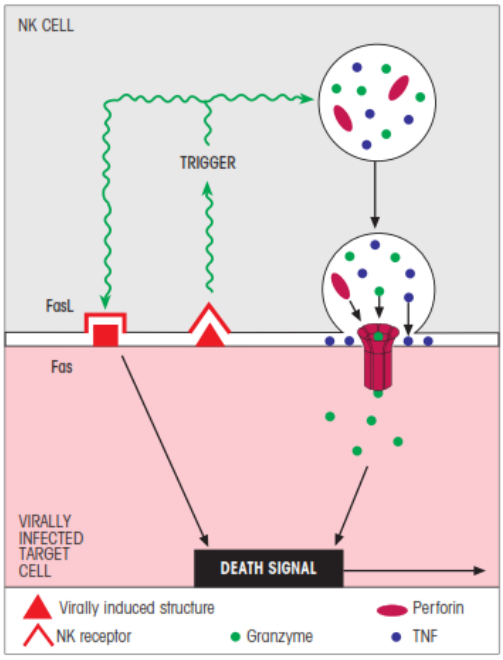

Figure 2: Extracellular killing of virally infected cell by natural killer (NK) cell. Binding of the NK receptors to the surface of the virally infected cell triggers the extracellular release of perforin molecules from the granules. These polymerize to form transmembrane channels which may facilitate lysis of the target by permitting entry of granzymes, tumor necrosis factor (TNF) and other potentially cytotoxic factors derived from the granules. Engagement of the NK receptor also activates a parallel killing mechanism mediated through the binding of the FasL (Fas-ligand) on the effector to the target cell Fas receptor thereby delivering a signal for apoptosis.

Figure taken from (Rabson, Roitt & Delves, 2005)

An alternate mechanism in which NK cells are involved in destruction of tumor cells is by death receptor mediated apoptosis, a slower less efficient method than perforin/granzyme-mediated cytotoxicity (Langers et al, 2012). NK cell granules also contain ligands for Fas receptors found on target cell surfaces, such as ligands from the TNF family (Langers et al, 2012). The Fas receptor-ligand interaction can induce apoptosis (Rabson, Roitt & Delves, 2005). In addition,

expression on NK cells of the ligand tumor necrosis factor-related apoptosis-inducing ligand, or TRAIL can control effectiveness of cytoxicity since this ligand can interact with tumor necrosis factor (TNF) receptors on target cells to induce cell death (Rabson, Roitt & Delves, 2005; Langers et al, 2012).

NK cells can also eliminate tumor metastases via secretion of cytokines such as IFN-γ, TNF-α, GM-CSF, IL-10 or IL-13, all of which have anti-tumor effects (Trincheri, 1995). IFN-γ enhances NK cell cytotoxicity via several manners (see Langers et al, 2012) and plays a role in the stimulation of dendritic cells, contributing indirectly in tumor control by aiding in the initiation and maintenance, via crosstalk with dendritic cells, of T cell mediated antitumor responses (Langers et al, 2012). In this collaborative way, NK cells amplify the immune response to tumors.

The maturation and activation of dentritic cells, macrophages and T cells is boosted by NK cells upon priming by various soluble factors (for example, 1L-15, type I IFN, 1-12, IL-18; Figure 3, red arrows) (Vivier et al, 2008). This is accomplished through a combination of cell surface receptors and cytokines. NK cells also have an additional regulatory function – they can kill immature dendritic cells, hyperactivated macrophages and CD4+ T cells (see Figure 3, blue arrows; Vivier et al 2008)

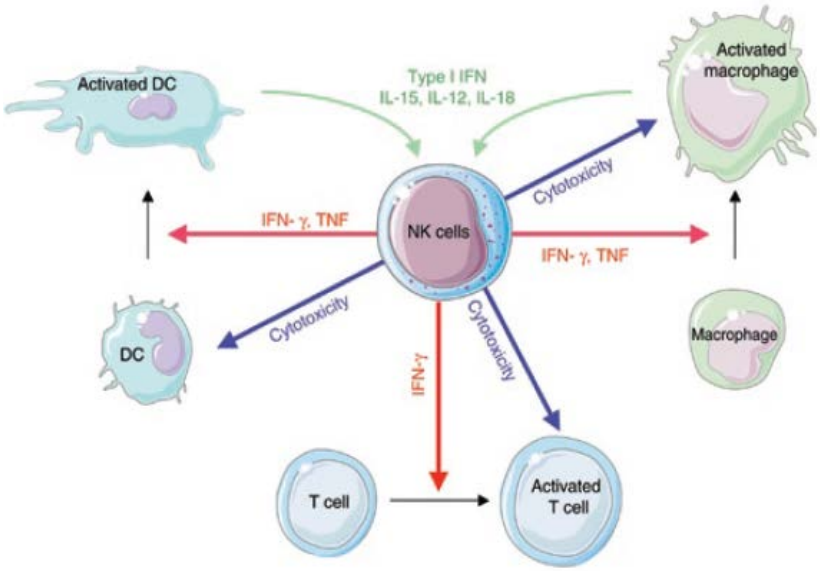

Figure 3: Regulation of immune responses by NK cells.

Figure taken from Vivier et al, 2008

The induction of NK cell responses depends on the strength of the activating stimuli. In order of strength of activating stimuli, weak stimuli will cause signals for adhesion of NK cells via leukocyte functional antigen (LFA)-1, whereas stronger stimuli will cause induction of chemokines (such as macrophage inflammatory proteins - MIP), followed by degranulation and production of cytokines such as tumor necrosis factor (TNF)-α and IFN-γ (Bryceson et al 2011).

The two main functions of NK cells, cytokine production and cytolytic activity, have been assigned to distinct NK cell population subsets, the NK $CD56^{bright}$ and NK $CD56^{dim}$ subsets, as described below in Table 1 and Figure 4. It is thought that the bright subtype is developmentally an earlier stage of the dim subtype in NK cell maturation (Yu, Freud and Caligiuri, 2013).

Table 1: Properties of NK cell subsets*

	NK $CD56^{bright}$	**NK $CD56^{dim}$**
Properties	• High density surface expression of CD56 • May express CD16 • Less mature than dim subset	• More abundant population than bright subset • Expression of CD16
Location	• Blood (only 10% of NK cells are bright subset) • Lymph nodes • Tonsils	• Blood • Bone marrow • Spleen
Function	• Immunoregulatory role • Do not readily produce cytokines in response to target cell recognition • Produce primarily IFN-γ and TNF-α in response to IL-1, IL-2, IL-12, IL-15 and IL-18 or engagement of CD16 or NKG2D (usually IL-12 + one other signal) • Express low levels of perforin • Cannot spontaneously kill tumor cell targets (lower cytotoxicity)	• Effector role with simultaneous, transient cytokine-mediated immunoregulatory role • Produce IFN-γ in response to cytokines • Produce ample amounts of cytokines in response to target cell recognition • Have higher levels of perforin expression • Potent cytolytic activity - can spontaneously lyse susceptible tumor cell targets
	• Begin producing IFN-γ later than $CD56^{dim}$ cells i.e. >16h	• Produce IFN-γ 2-4h after receptor-mediated or cytokine activation • Express CD107a (measure of cytolytic function) at 4h • Receptor-mediated IFN-γ production is transient and decreases by 16h • Cytokine-mediated IFN-γ production increases and persists over time • Presence of preformed IFN-γ mRNA

*Information taken from Caligiuri 2008, De Maria et al 2011 and Bryceson et al 2011

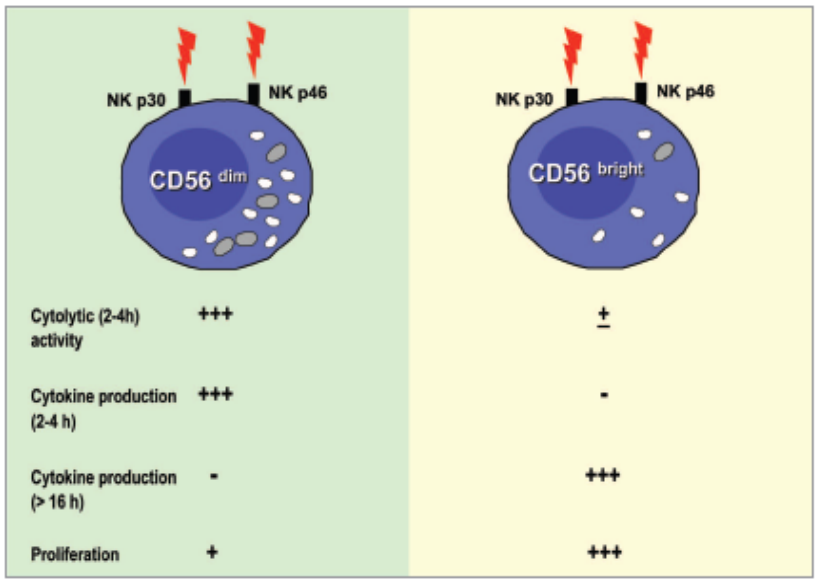

Figure 4: Functional capabilities of CD56^{dim} and CD56^{bright} human NK cell subsets

Figure taken from DeMaria & Moretta, 2011

Although CD56^{bright} cells are more efficient at cytokine production, early cytokine production can also be attributed to CD56^{dim} cells due to their ability to rapidly secrete cytokines (Campbell and Hasegawa, 2013). The CD56^{bright} NK cells which normally are weak cytotoxic cells (see above), can be induced to increase their cytotoxic potential by stimulation with cytokines such as IL-2 or IL-12 (Poli et al 2008). In addition, in humans, NK cells are one of the most important sources of the released cytokine IFN-γ (Poli et al, 2008), confirming the work reported by Thornton and coworkers (2001) describing that in the mouse, the major IFN-γ secreting-cells after in vivo IL-2 administration are NK cells.

How do we measure for NK cell function?

A number of different assays are available for measuring numbers of NK cells, NK cell cytotoxicity and NK function. Flow cytometry is commonly used to separate the different cells from whole blood or peripheral blood mononuclear cells, based on cell phenotype, and can be used to quantify NK cell numbers. However, this method is unable to detect the actual function of the NK cells. The following assays are able to measure the activity of NK cells.

a) Cytotoxicity assays currently used

^{51}Chromium Assay

Since 1968, the 'gold standard' assay for NK cell activity has been the Chromium 51 (^{51}Cr) release assay, used in research to determine the cytolytic activity of effector cell populations (Valiathan et al 2012). Figure 7 below depicts the process for measuring cytoxicity using the Chromium 51 assay. Whole blood is taken from subjects and incubated with Chromium 51 (^{51}Cr)-labeled target cells in various cell ratios (effector to target cell) in multiwell plates. ^{51}Cr (as radiolabelled sodium chromate) binds to intracellular proteins of cells grown in culture. These target cells are usually from the K562 cell line (erythroleukemia cell line) or other cell lines that NK cells (the effector cells) recognize as being foreign. After incubation, washing and centrifuugation, the ^{51}Cr released from lysed cells is counted using a gamma counter. The percent lysis is measured as the amount of cell-bound ^{51}Cr vs free ^{51}Cr (i.e representative of lysed cells). In this way, the ^{51}Cr assay measures target cell killing capacity of NK cells (Natural Killer Cell Cytoxicity or NKCC).

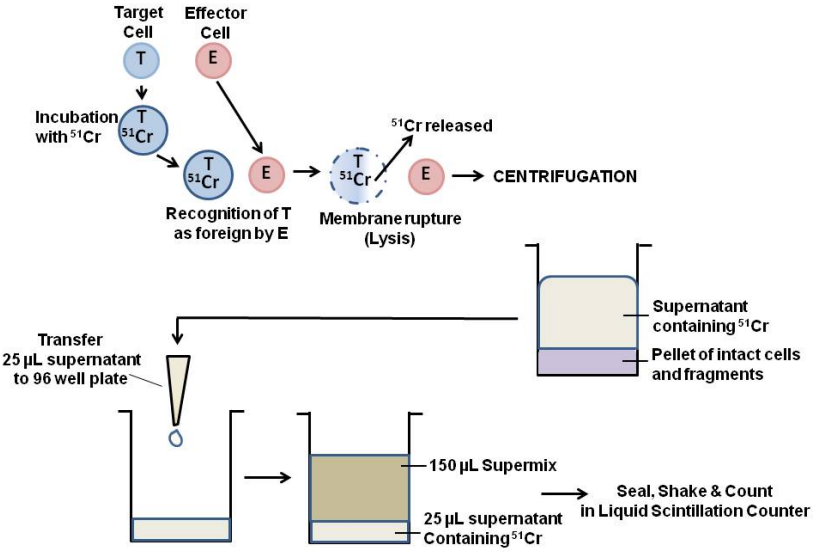

Figure 5: Procedure for measuring NKCC using the ^{51}Cr assay.

Figure taken from http://www.perkinelmer.ca/en-ca/Resources/TechnicalResources/ApplicationSupportKnowledgebase/radiometric/chromium51.xhl

The chromium release assay has many limitations, such as: (1) hazardous radioactivity; (2) high cost; (3) short half-life; (4) increased staff requirements for radiation safety training and licensing; and (5) disposal of radioactive waste (Valiathan et al 2012). In addition, inter-laboratory variability of this assay is a further limitation.

Flow Cytometry

Alternative methods to the ^{51}Cr release assay have been proposed. One such method is using flow cytometry and fluorescent dyes (Valiathan et al 2012). Dioctadecyloxacarbocyanine perchlorate (DiO), a fluorescent green dye, is used to stain plasma membranes of the target cells, K562 cell line. Propidium iodide (PI), a red fluorescent dye, is used to bind DNA (it cannot cross membranes and as such can only bind if a cell is lysed and the membrane is permeable). Intact (unlysed) target cells exhibit green fluorescence. Targets killed by effector cells exhibit both green and red fluorescence. The assay uses NK cells isolated using flow cytometry from peripheral blood mononuclear cells isolated from whole blood. This method does not distinguish between the NK cell subsets as the flow cytometer isolates $CD45^{+}CD3^{-}CD16^{+}CD56^{+}$ cells (Valiathan et al 2012).

b) NKVue test kit

NKVue is an vitro diagnostic test kit which uses a quantitative sandwich enzyme immunoassay technique (Sandwich ELISA). NK cell activity in vitro cannot be measured without stimulating NK cells since NK cells do not secrete effector cytokines ex vivo in the absence of appropriate activating stimuli. Therefore, NKVue employs the principle of stimulation of whole blood with a proprietary stabilized stimulatory cytokine and measurement of the release of IFN-γ from NK cells using Sandwich ELISA. A monoclonal antibody (immunocapture antibody) against an active NK cell secreted antigen (IFN-γ) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any active-NK-cell-secreted-antigen (IFN-γ) present is bound by the immobilized capture antibody. After washing away any unbound substances, a specific active-NK-cell-secreted-antigen monoclonal antibody conjugate (antibody-enzyme reagent) is added to the wells. The detector antibody in this reagent binds to a different epitope of the IFN-γ antigen immobilized by the capture antibody. The detector antibody is labelled with biotin, which binds to a streptavidin tagged horseradish peroxidase (HRP) enzyme label.

Following a wash to remove any unbound antibody-enzyme reagent, a chromogenic substrate solution containing 3,3',5,5'-Tetramethylbenzidine (B) is added to the wells and color develops in proportion to the amount of active-NK-cell-secreted-antigen bound in the initial step. The

color development is stopped with sulfuric acid and absorbance is measured at 450nm (see Figures 6 and 7).

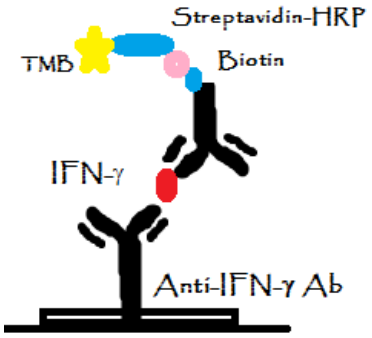

Figure 6: NK Vue Kit Sandwich ELISA

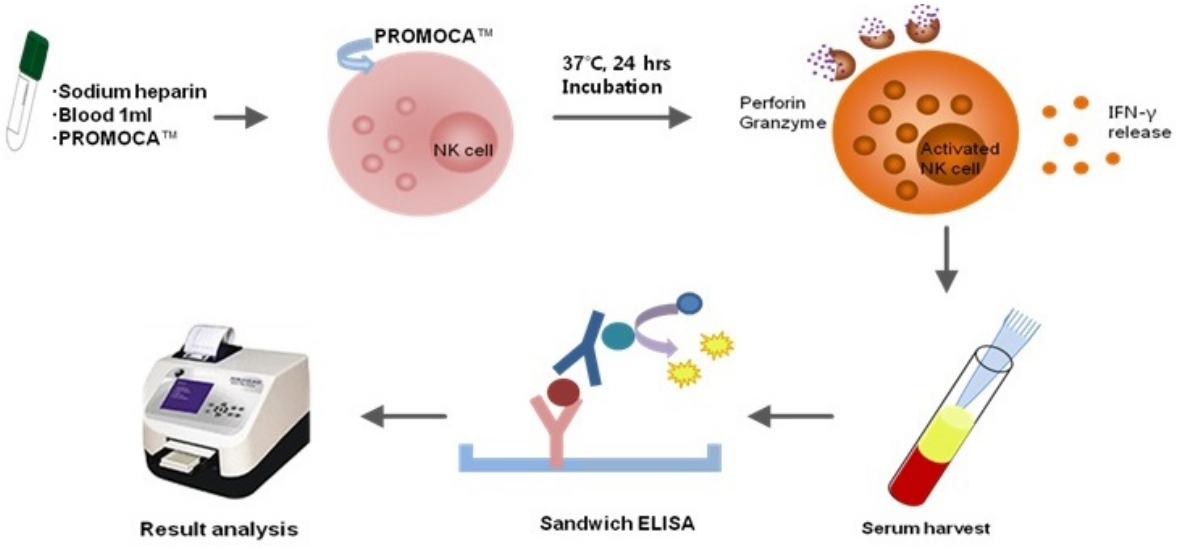

Figure 7: NK Vue Kit Procedure

The principle of NKVue – the release of IFN-γ following cytokine stimulation – is to measure the cytotoxic potential of the whole NK cell population, including both $CD56^{bright}$ and $CD56^{dim}$ (mature) subsets as well as the ability of these cells to amplify the immune response to tumors. Both of these NK cells subsets release IFN-γ following cytokine stimulation. In vivo, it is the $CD56^{dim}$ subset that is responsible for cytoxocity whereas the $CD56^{bright}$ subset is not cytoxic. As described above, $CD56^{bright}$ cells can be induced to become cytotoxic in response to cytokine

stimulation. Both subsets release IFN-γ in response to cytokine stimulation. Therefore, the advantage of the NKVue is that, in comparison to the chromium 51 release assay, which only measures the cytoxic potential of NK cells isolated from whole blood (i.e. the $CD56^{dim}$ subset), NKVue measures the immune potential of both NK cell subsets. The amount of IFN-γ released is indicative of both the cytotoxic ability of the $CD56^{dim}$ subset and the immunomodulation by this cytokine, released from both subsets, to stimulate dendritic cells, and aid in the T-cell mediated antitumor response. **Therefore, NKVue is a true measure of NK cell activity, which includes both innate immune cytotoxicity and immunomodulation of the adaptive immune response.**

How is the function of NK cells different in different populations?

NK cell activity is a stable trait in individuals, and healthy individuals' low, medium or high NK cell activity remains unchanged over the years unless disease occurs (Whiteside et al, 1990). The activity of NK cells can differ in many different populations and can be influenced by lifestyle such as sleep, exercise, stress, diet and by infection or disease. It has been well-established that decreased NK cell activity (as measured with the traditional assay methods) is found in patients with a variety of solid tumors and large tumor burdens, and that this may be associated with development of distant metastases (Whiteside et al, 1990).

NK Cells and Cancer

Natural killer cells of the innate immune system orchestrate, with the adaptive immune system, the eradication of malignant cells (Gutkin and Sherwin, 2013). The cells of the innate immune system are the first to detect the emergence of neoplastic cells (Gutkin and Sherwin, 2013).

Incidence of Cancer

In Canada, cancer is the leading cause of death and is responsible for approximately 30% of all deaths. An estimated 187,600 new cases of cancer and 75,500 deaths from cancer will occur in Canada in 2013. The vast majority (88%) of Canadians who develop cancer are over the age of 50. At the beginning of 2009, there were about 838,724 Canadians living with a cancer that had been diagnosed in the previous 10 years. About 2 in 5 Canadians will develop cancer in their

lifetimes and 1 in 4 will die of the disease. More than half (about 52%) of all new cases will be lung, breast, colorectal and prostate cancers. (Canadian Cancer Society, http://www.cancer.ca, accessed January 2014, see Figure 8 below).

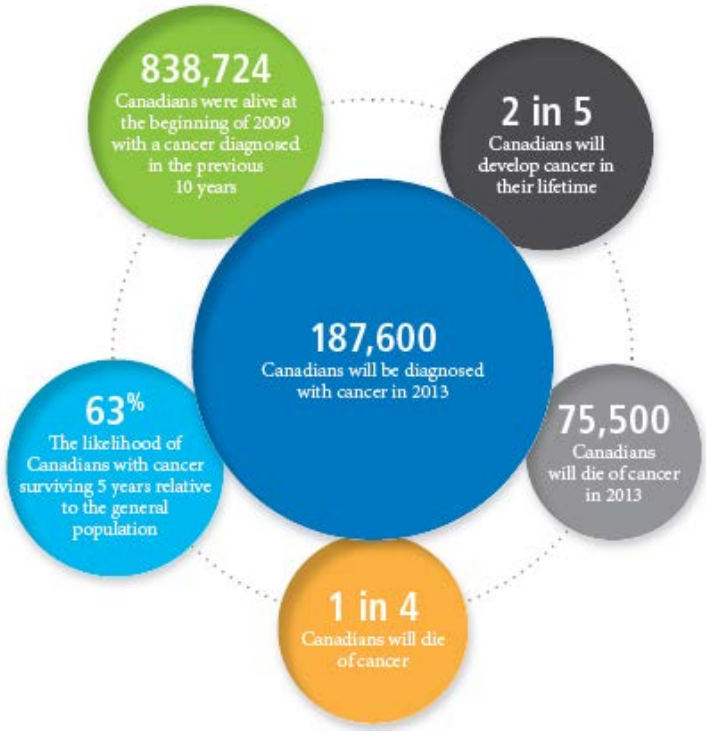

Figure 8: Incidence of cancer in Canada

A number of cancers have been linked to aging, After age 65, the US National Cancer Institute has found that there are 10 times more cases of cancer than in a younger population, with cancer incidence increasing after age 40 (Curado et al, 2008). The most common cancers in the elderly are prostate, breast, colon, pancreatic, bladder, stomach, lung and rectal cancers (Camous et al, 2012). Apart from the merely probabilistic considerations (i.e., the longer a person lives, the higher are the chances of developing a tumor), two additional causes have been ascribed as being responsible factors: a general weakening of the immune system, and the eventual accumulation and long term effects of pollutants and toxic compounds in the organism (Camous et al., 2012).

Immunosurveillance of cancer

The importance of natural cytotoxicity in immune surveillance against cancer (a term coined by Burnet in 1970) has been highlighted by a number of initial studies on mice melanoma models where the in vivo activation of NK cells was crucial for the anti-tumor response (Talmadge et al.

1980; Gorelik et al 1982). Recently, the concept of immunosurveillance has been expanded to include immunoediting as an important mechanism for the development of cancer (Dunn 2004). It was suggested that cancer immunoediting comprises three phases: elimination, equilibrium and escape. Elimination represents the classical concept of immunosurveillance. Equilibrium and escape include the interaction and cross signaling between the immune effectors, the tumor cells, and the effectors of the connective tissue in the tumor microenvironment, which may result in the generation of tumors capable of causing gradual inactivation or death of the immune effector cells.

It has been shown that tumor cells can express molecules on their surface and release mediators (cytokines, soluble domains of receptors and other relevant membrane proteins) that allow their evasion from NK cell immunosurveillance (Zitvogel et al. 2006). Well characterized mechanisms include increased inhibition of NK cell cytotoxicity (by increasing the levels of tumor surface inhibitory ligands, leading to NK cell anergy; LeMaoult 2005; Levy 2008; Urosevic 2008; Farnault et al. 2012) and impaired activation of NK cells (by downregulating NK cell surface activating receptors or release of immunosuppressive factors such as IL-10 or TGF-β; Farnault et al. 2012, Salazar-Onfray 2007; Castriconi 2003). Additional mechanisms of tumor evasion of NK cell immunosurveillance have been described by Jewett and Tseng (2011; see Table 2 below).

Table 2: Mechanism of immune evasion by tumors (table taken from Jewett and Tseng, 2011)

Defect	Mechanism
Loss of T cell Recognition	Decreased MHC class I expression/shedding of class I; decreased TCR zeta chain expression, expression of co-stimulatory molecules (B7H1) on tumor cells
Altered NK cell recognition	Decreased expression of NK receptor (NKG2D) ligands, increased MHC class I expression, decreased expression of adhesion molecules, CD16 and zeta chain
Loss of NK cell function	Induction of anergy in NK cells. Activation of upstream transcription factors (NFκB) in differentiated tumors and tumor cell production of inhibitory factors (IL-10, IL-6, IL-1β, PGE2, GM-CSF, IL-8). Decreased IFN-γ secretion by the NK cells when co-cultured with increased NFκB function in tumors
Enhanced tumor cell survival/resistance to killing	Expression of anti-apoptotic molecules by tumor cells via activation of upstream transcription factors (c-Myc, AP-1, NFκB, STAT3)
Increased NK and T cell apoptosis	TNF-α induced apoptosis, Fas ligand expression by tumor cells and membranous vesicles; Fas-mediated apoptosis of responding T cells, expression of DF3 and Muc1 in tumor cells
Inhibition of macrophage/DC maturation and function	Tumor cell production of inhibitory cytokines (VEGF, IL-6, GM-CSF) and activation of STAT3-mediated transcription in DCs.
Inhibition of T cell chemotaxis to tumor microenvironment	Constitutive STAT3 activation in tumor cells, decreased expression of T cell chemotactic factors (RANTES, IP-10)
Increased recruitment or function of CD14+HLADR-monocytes,Tumor-associated Macrophages, MDSCs, Cancer Associated Fibroblasts, MSCs, Tregs and DCregs	Suppression of cytotoxic immune effectors

NK cell immunoediting was described recently by Balsamo et al (2012), in melanoma cells, where NK cells control the early phases of tumor growth together with other immune cells (see Figure 9). NK cells may be unable to completely eliminate tumor cells in lesions that have grown beyond a defined size or have limited NK cell infiltration. Upon interaction with other immune cells and exposure to inflammatory cytokines, NK cells produce IFN-γ, which, in turn, induces HLA-l upregulation on melanoma cells, the immunoediting phase. In the late phase of tumor proliferation of melanoma cells, the escape phase, these cells become NK cell-resistant and this results in further decreased NK/melanoma cells ratios. In these conditions, killing of melanoma cells is further limited (Balsamo et al 2012).

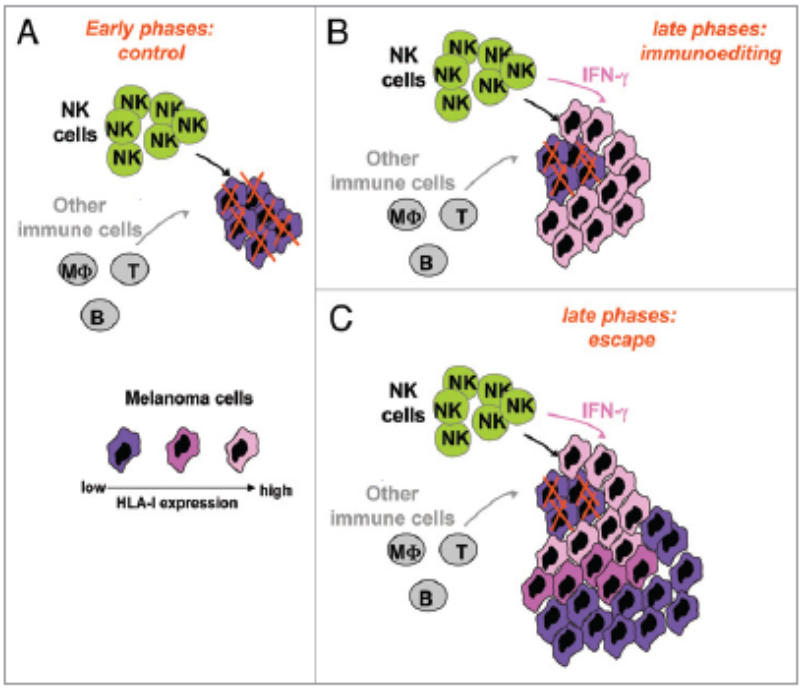

Figure 9: Mechanisms of immunoediting of melanoma cells by NK cells

Figure taken from Balsamo et al 2012.

Further evidence of tumor escape from NK cell immunosurveillance was shown in prostate cancer by Ray et al (2012). Inhibition of granzyme B by its natural inhibitor, PI-9, produced by cells from certain prostate cancer cell lines, protects these cells from NK-mediated apoptosis. This data suggests that *in vivo* overexpression of PI-9 by prostate cancer cells may protect these tumors from innate immunosurveillance. Indeed, these authors showed greater expression of PI-9 mRNA in low grade prostate tumors taken from prostate biopsies compared to benign control, with variable expression seen in higher grade tumors, suggesting that PI-9 function is needed for early progression of prostate cancer.

Interestingly, chemotherapy can also disrupt potentially competent immunosurveillance mechanisms. In a study analyzing the effect of 28 frequently used chemotherapeutic agents on the capacity of NK cells to kill tumor cells, it was found that most used drugs quantitatively decreased NK cell counts (Markasz et al. 2007). However, whereas some inhibited NK cell activity (Vinblastine, Paclitaxel, Docetaxel, Cladribine, Chlorambucil, Bortezomib, MG-132), others had no effect (Bevacizumab, Bleomycin. Doxorubicin, Epirubicin, Etoposide, 5-FU, Hydroxyurea, Streptozocin, 6-Mercaptopurine).

Families with high incidences of cancer (more than 3 siblings with cancer) have shown reduced NK cell activities compared to control families as evidenced in Figure 10 below (Strayer et al 1984). A similar result was also described in familial melanoma families (Hersey et al 1979).

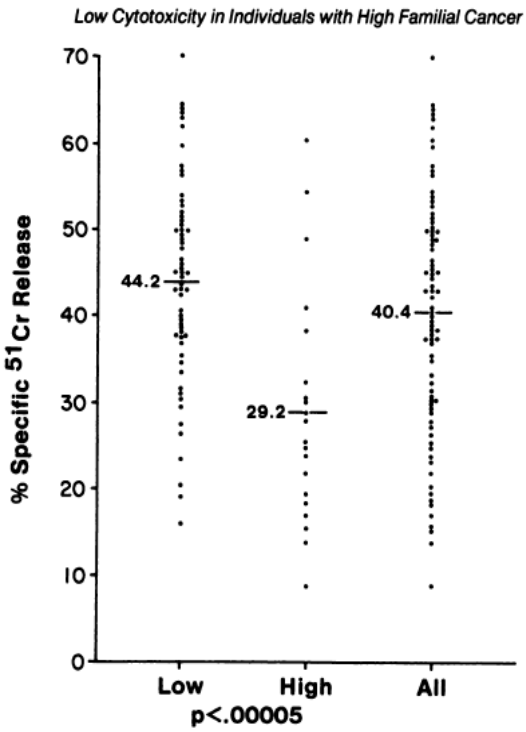

Figure 10: NK cell-mediated cytotoxicity for individuals with low and high familial incidences of cancer

Figure taken from Strayer et al, 1984

In a recent study in lung cancer, PBMCs harvested from patients and healthy controls were tested ex vivo for their ability to kill tumor target cells and produce IFNγ. PBMCs of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) patients had a reduced ability to kill K562 myelogenous leukemia cells in a standard 51Cr release assay (Al Omar et al. 2011). Additionally, the percentage of NK cells stimulated to produce IFNγ, by ex vivo treaent with IL-12 or IL-18, was reduced in PBMC samples from patients compared to healthy donors. These data suggest that NK cell effector functions, including cytotoxic activity and cytokine production, could be related to the detection of tumors and could likely be associated with the rate of growth, and the metastatic potential of lung cancer in humans.

In hepatic tumor immunosurveillance, it appears that compared to other organs, the liver displays an augmented cytolytic activity of NK cells (Subleski et al. 2009). Tumor-surveillance functions of NK cells (i.e., reduced levels of IFNγ production and cytotoxicity) are often suppressed in precancerous fibrotic and cirrhotic as well as cancerous tumor-containing livers (Cai et al. 2008). On the other hand, a higher density of total intratumoral $CD56^{+}$NK cells has been shown to correlate with long survival rates in hepatocellular carcinoma patients (Chew et al. 2012).

In Gardner's syndrome, a condition that predisposes one to colorectal cancer, 50% of patients have shown some form of NK cell activity deficiencies in ^{51}CR assays, with all advanced cases presenting such a decline in NK cell activity (Warren et al, 1985). NK cell activity deficiencies have also been reported in prostate, colorectal and breast cancers, discussed in detail below.

Finally, a recent epidemiological study on humans stressing the relationship between NK cell activity and cancer was conducted by Imai and coworkers (2000). Their study comprised 3625 Japanese men and women, mostly over the age of 40, which were followed for 11 years for cancer incidence. The authors found that medium and high cytotoxic activity of peripheral-blood lymphocytes was associated with reduced cancer risk when compared to those with low activity (41% and 37% lower, respectively). Regardless of the relatively modest sample size and the significant variations observed in cytotoxic activity among individuals (eventually ascribed mostly to lifestyle), this study suggests that NK cell cytotoxicity does play a part in preventing the development of cancer in humans (Imai et al. 2000). In addition, a subset of this cohort was investigated to determine the phenotype of their Natural Killer Complex gene region (12p13.2-p12.3) (Hayashi et al, 2006). The haplotypes of the NKG2D gene, which triggers cell-mediated cytotoxicity in NK cells, was found to be significantly associated with natural cytotoxic activity in individuals - the haplotype HNK1/HNK1 (high cytotoxic activity-related alleles) revealed a decreased risk of cancer (odds ratio, 0.471; 95% confidence interval, 0.233-0.952) compared with LNK1/LNK1 (low cytotoxic activity-related alleles) (Hayashi et al, 2006). Those with LNK1/LNK1 made up one-third of the control population studied and more than 40% of the cancer cases. Therefore, NKG2D haplotyping may in part identify individuals who are genetically predisposed to have low or high natural cytotoxic activity, which in turn reveals an increased or decreased risk of cancer development (Hayashi et al, 2006).

Therefore, evaluation of NK cell activity plays an important role in both immunosurveillance of cancer as well as in potential prognosis of severity and recurrence of this disease. Indeed, several authors have suggested that NK cell activity is associated with oncological prognosis, tumor incidence, metastasis, infiltration and invasiveness (Lin et al 2010, Takeuchi et al, 2001, Schleypen et al 2006, Vivier et al 2012).

Prostate Cancer

Incidence in Canada

Prostate cancer is the most common cancer among Canadian men, excluding non-melanoma skin cancers, and the third leading cause of death in men from cancer (Canadian Cancer Society, 2013b). In 2013, 23,600 men will be diagnosed with prostate cancer and 3,900 men will die of the disease, an incidence rate that has increased likely due to the use of the PSA test for early detection (Canadian Cancer Society, 2013b).

Provincial Prostate Cancer Statistics 2012:

Location	Estimated Prostate Cancer Cases (% of Population)	Estimated Prostate Cancer Deaths (% of Population)
Nationally	26,500 (0.075%)	4,000 (0.023%)
AB	2,500	370
BC	3,700	530
MN	750	180
NB	860	100
NL	490	70
NS	920	120
ON	10,900	1,500
PE	160	25
QC	5,400	830
SK	880	210

Table adapted from Canadian Cancer Society 2012

Prostate cancer and NK cells

In prostate cancer patients, NK cells appear to play a significant role in tumor cell immunosurveillance. In a murine model, Jachetti et al (2013) demonstrated the ability of NK cells to be involved in both lymphokine-activated cytotoxicity and dendritic cell mediated cytotoxicity.

Early pilot studies had shown that natural killer cell activity as measured using the ^{51}Cr assay was significantly different in patients with advanced stages of prostate cancer (stage D) but not in patients with localized prostate cancer (stages B and C) (Marumo et al, 1989). Lahat et al (1989) demonstrated a significant decrease in natural killer cell activity with increasing prostate cancer disease spread (stages A through D). In 1992, Kastelan and coworkers demonstrated that in prostate cancer patients in stages D0/D1 and D2 during progression of the disease, lower NK cell activity was seen. Interestingly, in patients experiencing a stable D2 stage, normal NK cell activity was seen (Kastelan et al 1992). The same group also confirmed the reduction in NK cell activity in D1+D2 stages of prostate cancer (Tarle et al 1993). In addition, they showed that such levels, as well as PSA, were correlated to cancer stage. More recent work by the same group (Kastelan et al 1997) showed that NK cell activity was significantly different between healthy controls and patients with local tumor or those with disseminated disease, as well as between healthy controls and patients responding to therapy and those not responding to therapy. The changes in NK cell activity were related to both metastatic extension of disease and tumor response to therapy, as well as to PSA levels (Kastelan et al 1997).

Of interest, in the case of prostate cancer, the hormonal milieu does not seem to affect NK cell activity. Levels of cortisol were not correlated with NK cell activity (Tarle et al 1993), nor was NK cell activity affected by treaent with pharmacological agents used (estradiol, cyproterone acetate, diesethylstibestrol and flutamide)(Kastelan et al 1992).

Therefore, NK cell activity reduction seems, according to these authors, linked to later stages of disease and the presence of circulating tumor cells. On the contrary, the presence of stable disease appears to be linked to normal levels of NK cell activity, highlighting the role of NK cells in immunosurveillance of cancer cells (Kastelan et al 1992).

In a recent study by Koo et al (2013), NK cell activity as measured using the NK Vue kit (release of IFN-γ subsequent to Promoca® stimulation) was significantly lower in prostate cancer patients compared to controls. Furthermore, patients with advanced stages of cancer progression showed a greater reduction in NK cell activity (Figure 11), confirming the earlier work of the Kastelan group (1992, 1997). Interestingly, a gradual decrease in the frequency of $CD56^{bright}$ subset of NK cells (leading to a significantly higher $CD56^{dim}$ to $CD56^{bright}$ ratio) was shown to clearly

correlate with cancer progression (no change was seen in the $CD56^{dim}$ subset of NK cells) (Figure 12).

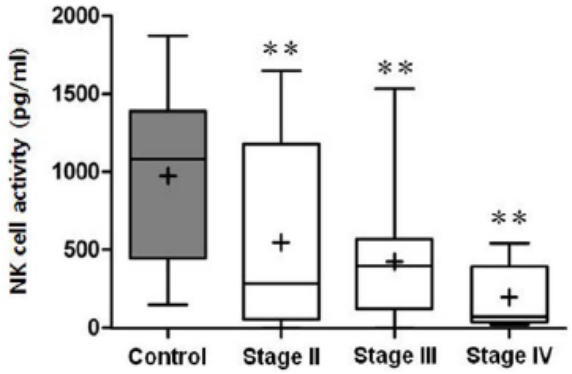

Figure 11: NK cell activity in controls and prostate cancer patients.

**p<0.01 vs controls

Figure taken from Koo et al, 2013

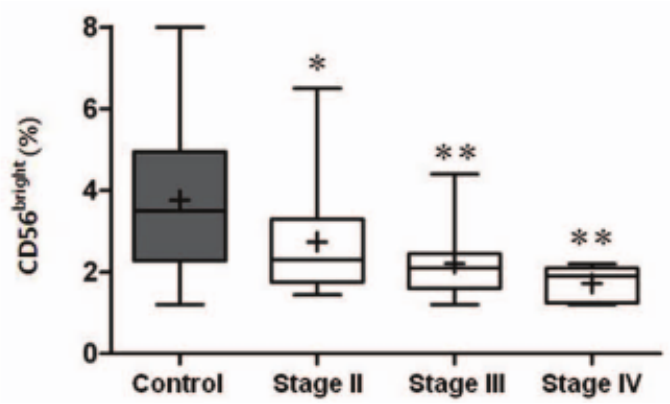

Figure 12: Flow cytometric distribution results of the $CD56^{bright}$ subset of NK cells in controls and prostate cancer patients.

*p<0.05 **p<0.01 vs controls

Figure taken from Koo et al, 2013

Furthermore, according to these authors, NK cell activity may serve as a supportive marker for PSA in diagnosing prostate cancer, particularly in the diagnostic grey-zone (PSA from 4 to 10 ng/mL). Using correlation statistics, the Koo et al study (2013) demonstrated that the lower the activity of NK cells, the higher the PSA values, the more advanced the cancer stage and the higher the $CD56^{dim}$ to $CD56^{bright}$ ratio was in prostate cancer patients. In addition, the lower the numbers of $CD56^{bright}$ cells, the higher the PSA values and the more likely it was to find pathologically confirmed metastasis (Koo et al 2013). Although Sotosek et al (2011) did not see a difference in the frequency of the two NK cell subtypes in prostate cancer patients compared to controls, they did see a negative correlation between the frequency of NK cells and PSA in cases

of prostate cancer as well as a significantly lower cytotoxic activity in these patients. Koo and coworkers not only confirmed that NK cell activity was reduced in prostate cancer, but that such reduction was linked to both disease stage and grade. This data indicates that impaired NK cell activity is likely preceded by a reduction in the $CD56^{bright}$ cells. This data, along with the data from the Kastelan group (Kastelan et al 1992, 1997, Tarle et al 1993) demonstrates that NK cell activity might add significant value to the use of the PSA test in assessing the risk of disease progression in patients with prostate cancer.

Therefore, assessment of NK cell activity, in combination with current diagnostic measures for prostate cancer, might prove useful in predicting which patients may be at a greater risk of progression or metastasis. As suggested by the Koo et al (2013) data, NK cell activity may also prove to be an asset in the identification of patients at higher risk for prostate cancer despite showing relatively normal PSA levels.

Colorectal Cancer

Incidence in Canada

In Canada, 1 in 13 men and 1 in 15 women will be diagnosed with colorectal cancer (CRC) annually. This amounts to approximately 23,900 (about 13,200 males and 10,600 female) new Canadian cases of CRC. (Ellison and Wilkins, 2012). Colorectal cancer is the third most common cancer (13% of all cancers) (Ellison and Wilkins, 2012). Overall, incidence rates of CRC have declined steadily (by about 0.8% per year) since 2000, though remained relatively stable in males and declined slightly in females. The mortality rates on average have declined significantly (2.6% per year in males and 1.8% per year in females) over more than a decade, likely due to screening efforts.

There is an increasing number of individuals surviving CRC as a result of early detection and better treaents available. Based on individuals alive in Canada as of January 1, 2009 there were approximately: 32,610 (18,129 males 14,481 females) alive for 2 years that were diagnosed in 2007; 67,219 (36,860 males and 30,359 females) alive for 5 years that were diagnosed in 2004; and 105,194 (56,648 males and 48,546 females) alive for 10 years that were diagnosed in 1999.

(Ellison and Wilkins, 2012) Although there are more people being diagnosed with the disease, the actual mortality rate dropped by about 2% per year between 2001 and 2009. (Canadian Cancer Society 2012)

The incidence of CRC in Canada is highest for both males and females residing in Newfoundland & Labrador. High rates are also reported for females residing in Nova Scotia and Prince Edward Island. The lowest rate of incidence for both males and females is in British Columbia (see table below; Canadian Cancer Society 2012)

Provincial CRC Statistics 2012:

Location	Estimated CRC Cases			Estimated CRC Deaths		
	Total (% of population)	Male (% of population	Female (% of population	Total (% of population	Male (% of population	Female(% of population
Nationally	23,900 (6.8%)	13,200 (7.6%)	10,600 (6.0%)	9,200 (2.6%)	5,000 (2.9%)	4,200 (2.4%)
AB	2,010	1,150	860	700	400	300
BC	2,900	1,600	1,300	1,180	650	530
MN	920	510	410	340	180	160
NB	600	350	250	210	110	100
NL	530	310	220	240	140	100
NS	880	480	400	360	200	160
ON	8,700	4,800	3,900	3,350	1,850	1,500
PE	115	60	55	50	25	25
QC	6,300	3,500	2,800	2,450	1,,300	1,150
SK	740	410	330	270	150	120

(Table prepared using statistics from Ellison and Wilkins, 2012)

Since approximately 2007, provincial health authorities began to initiate CRC screening programs and as of this date all provinces have either commenced or are in the process of planning and implementing population based screening programs (Ellison and Wilkins, 2012).

The most common current screening tests for CRC include: FOBT (fecal occult blood testing), FIT (Fecal Immunochemical Test), and colonoscopy. FOBT allows detection before onset of symptoms, resulting in mortality reductions of 15-33% (Xu et al, 2011). However, FOBT screening does not reduce CRC mortality to the rate predicated by randomized controlled trials due to poor patient compliance. Screening with a fecal occult blood or immunochemical test (FOBT or FIT) is recommended every two years for people between the ages of 50-74 years and

who are of average risk; however, colonoscopy may be appropriate in certain circumstances (Ellison and Wilkins, 2012).

Colonoscopy is the 'gold standard' for CRC screening. However, it is expensive, invasive, and not always easily accessed. Many patients are unwilling to undergo a colonoscopy, with only 29% of those at risk seeking a colonoscopy in the last 10 years (Xu et al, 2011). Less than 10% of average risk persons for CRC undergoing colonoscopy have advanced adenomas (Xu et al, 2011). Colonoscopy is arguably overkill for primary CRC screening.

CRC and NK cells

In terms of alternate screening tools for CRC, Xu et al. (2011) have shown that gene expression related to NK cells in peripheral blood could be informative in identifying CRC patients. These atuthors analyzed blood from 119 CRC patients and 101 colonoscopy-negative control samples and observed 327 differentially expressed genes (DEG), mostly associated with immune cell activation and trafficking. Using this approach, the natural killer cell score showed 96% specificity to discriminate colonoscopy-negative controls from CRC patients, suggesting the possibility of a quite robust biological story behind the NK cell score.

Xu et al. (2011) also showed that NK cell signaling and cytotoxicity-associated genes appeared to undergo major changes in CRC peripheral blood samples. These changes were more pronounced in the advanced stages of the disease (see Figure 13). A summarizing score of the expression of ten genes related to NK cells revealed a marked heterogeneity within the CRC Stage IV group, suggesting possible further stratification of the patients. Statistically significant differences were mainly observed between controls and CRC Stages II–IV and between CRC Stage I and CRC Stages II and IV (p values < 0.05).

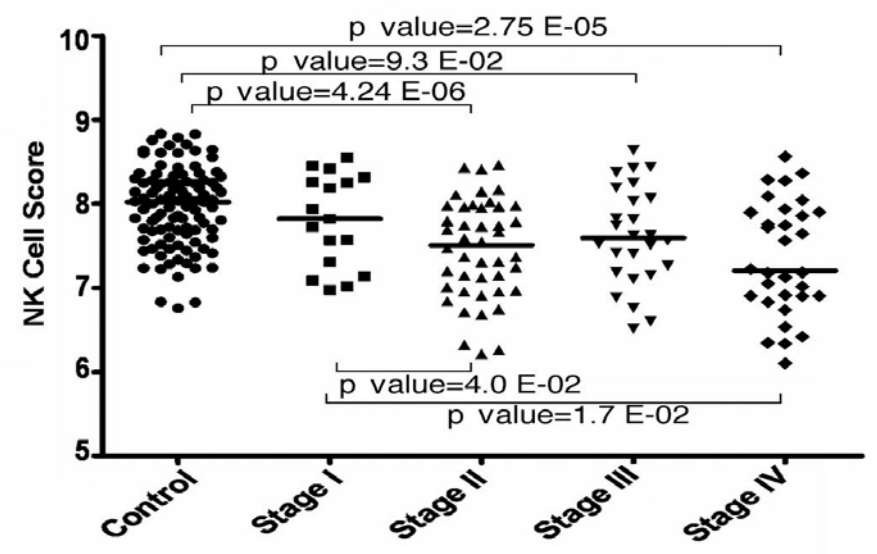

Figure 13: NK cell score in colonoscopy-negative control (CNC) and colorectal cancer (CRC) patient blood samples, with the distribution for CRC samples according to the cancer stage.

Figure taken from Xu et al, 2011

The Xu et al (2011) study shows the potential of transcriptomics in peripheral blood to discover biomarkers and provides new insight on the immune response in CRC. These results also showed that the expression analysis of genes like those related to NK cells should allow the stratification of patients with colorectal cancer, thus enhancing possibilities for personalized medicine.

In addition to changes in gene expression, it has been shown that the activity of natural killer cells is impaired in CRC patients. Analysis of PBMC in colorectal patients through a simplified technique based on immunofluorescence rather than ^{51}CR to measure NK cell activity (Kim et al, 2013) showed that CRC patients presented lower NK cell activity levels compared to that of controls. This study also demonstrated that tumor reduction through surgery led to no change in NK cell activity (similar low activity was seen in CRC compared to healthy control, pre- and post-operatively) and showed that CRC was significantly associated with cytolytic activity, percentage of $CD56^+$ NK cells and percentage of lymphocytes. These findings suggest that defective cytolytic activity of circulating blood immune cells might be associated with phenotypic changes and that a combination of examination of phenotypic change with cytolytic activity can be used to functionally discriminate CRC patients from healthy individuals (Kim et al 2013).

Unlike Xu et al. (2011), Kondo, et al. (2003) found that decreases in NK cell activity did not necessarily correspond to tumor stage. However, in curatively operated stage I-III diseases,

preoperative NK cell activity of 20% or less correlated with poor survival. Lower activity was also associated with metachronous distant metastases but not with local recurrences and more than half the stage III patients with attenuated NK cell activity developed metastases (Kondo et al 2003).

In addition, attenuated NK cell activity was a significant predictor of distant metastasis following curative surgery for CRC (Kondo et al 2003). Preoperative NK cell activity had significant prognostic value in curatively operated CRC patients, particularly for the development of metachronous distant metastasis in stage III patients (Kondo et al 2003).

Qui et al (2009) evaluated the prognostic significance of preoperative T-lymphocyte subsets and natural killer cells and their correlation with other prognostic factors in patients with colorectal cancer who underwent surgical treaent. The numbers of T-lymphocytes and NK cells in the peripheral blood of patients with CRC were counted by flow cytometry, and the correlations between indicators of cellular immunity and clinico-pathological characteristics and the prognosis of patients after surgical treaent were analyzed by univariate and multivariate analyses. In the multivariate regression analysis, all potential prognostic factors essentially reflected TNM stage, and tumor resection, NK lymphocyte counts, and peripheral CD3+ and CD4+/CD8+ counts were significant ($p < 0.05$) independent prognostic indicators of overall survival; patients with higher CD3+, CD4+/CD8+, and NK levels had longer overall survival rates than those with lower CD3+, CD4+/CD8+, and NK levels (Qui et al 2009). Measurement of cellular immunity in the peripheral blood of patients with colorectal cancer allowed Qui and coworkers to identify associations between immune status and clinical outcomes.

Nüssler et al (2007) demonstrated that the presence of colorectal cancer lead to a reduction of NK cell activity in cases where metastatic diseases were present either at time of diagnosis or within a year after diagnosis. Of note in this trial, in these patients who progressed to metastatic disease despite curative treaent, such a reduction in the NK cell activity was noticed despite increased percentages of NK cells in the blood, this pointing to a profound disturbance in NK cell function. Such results raised interesting questions about NK cell activity as a potential marker for metastatic disease or risk of progression.

Researchers have suggested that a blood-based test will have much greater compliance than the most common screening standards (FOBT/FIT and colonoscopy) hence increasing screening compliance. The reduction in NK cell activity may thus be useful as an indicator of immune status of the CRC patient and help to determine risk of progression.

Breast Cancer

Incidence in Canada

Breast cancer is the most common cancer among Canadian women and is the second leading cause of cancer death for women. Breast cancer also occurs in men, but is uncommon. Estimates for 2013 indicate that 23,800 new cases of breast cancer will be diagnosed, with 5,100 deaths, representing 14% of all cancer deaths for women in 2013. Therefore, on average, 65 women will be diagnosed with breast cancer daily, and 14 of those will die daily. Two hundred men will be diagnosed with breast cancer annually; 60 will die from breast cancer annually. (Canadian Cancer Society 2013a).

Provincial Breast Cancer Statistics 2012:

Location	Estimated Breast Cancer Cases			Estimated Breast Cancer Deaths		
	Total (% of population)	Male (% of population*	Female (% of population	Total (% of population	Male (% of population*	Female (% of population
Nationally	22,900 (0.065%)	200 (0.001%)	22,700 (0.13%)	5,200 (0.029%)	55 (0.0003%)	5,145 (0.029%)
AB			1,950			390
BC			3,000			630
MN			800			210
NB			550			110
NL			300			90
NS			740			160
ON			9,100			2,000
PE			95			30
QC			5,500			1350
SK			690			160

*available only at national level; Table data taken from Canadian Cancer Society 2013a and Canadian Breast Cancer Foundation 2013

Breast cancer rates in Canada rose steadily through the 1990's and into the early 2000's. The increases were due in large measure to the introduction of mammography and breast cancer screening programs. The use of HRT (hormone replacement therapy) therapy in post-menopausal was also a contributing factor to increased rates. Decreases in breast cancer rates

coincided with a significant reduction in HRT therapy among post-menopausal women once the link between HRT therapy and breast cancer rates was publicized. Breast cancer rates continue to fall due to aggressive screening and improved treaents (Canadian Cancer Society 2013a).

Screening, when used systematically, will help to find breast cancer before obvious symptoms have developed, early detection increases the chance of successful treaent, and longer term survival. Screening is recommended for women who are 50-69 years of age. Mammography is the most reliable method of detecting breast cancer early, and the current screening standard in Canada (Canadian Cancer Society 2013c).

Women who are between 50-69 years of age should have a screening mammography every 2 years (Canadian Cancer Society 2013c). Women who are between 40-49 years of age are not currently screened by mammography. There is some literature that suggests this group could benefit from screening mammography yearly as breast cancer behaves differently in younger women (Canadian Cancer Society 2013c). There is recent clinical evidence in a Canadian cohort of close to 90,000 women, however, to suggest that mammography may not be of greater benefit in reducing breast cancer mortality in women aged 40-59 years, relative to usual care or regular physical breast examination (Miller et al., 2014). Though tumour sizes were larger and more likely node positive in the control arm, 22% of the small and non-palpable cancers in the mammography arm were attributed to over-diagnosis (i.e., the likelihood that the cancer would not become clinically apparent during the woman's lifetime; Miller et al, 2014). The authors concluded that "annual mammography does not result in a reduction in breast cancer specific mortality… [and] the value of mammography screening should be reassessed." (Miller et al, 2014).

Breast Cancer and NK cells

Significant amounts of data have been collected supporting a role for immunosurveillance in regulating breast cancer metastasis and their further growth (see Slaney et al. 2013). White et al (1982) showed that NK cell activity is reduced in malignant but not benign breast disease. The degree of effect on NK cell activity, however, has been suggested to be related to the hormonal status of breast cancer patients (White et al, 1982), since a significant decrease was seen in

premenopausal but not in posenopausal breast cancer patients (see Figure 14). White and coworkers (1982) also showed that NK cell activity in the control group depended on the time in the menstrual cycle that blood was taken with lower NK cell activity seen in the first half of the menstrual cycle.

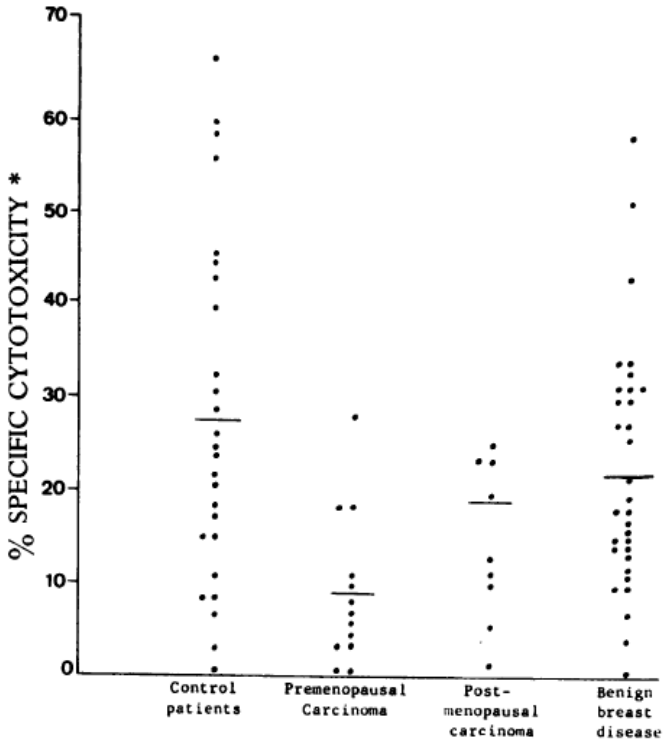

Figure 14: NK Activity in patients with benign and malignant breast disease and controls.

Figure taken from White et al, 1982

Piroozmand and Hassan (2010) showed that NK cell activity correlated with various stages of breast cancer tumor, pre- and post-surgery (see Figure 15). Additional evidence for the role of innate immunosurveillance comes from a study where NK cells were measured and characterized in peripheral blood and primary tumors in patients with advanced breast cancer; NK cells from peripheral blood were found to have impaired functionality and this was even more marked in tumor-infiltrating NK cells (Mamessier et al. 2011).

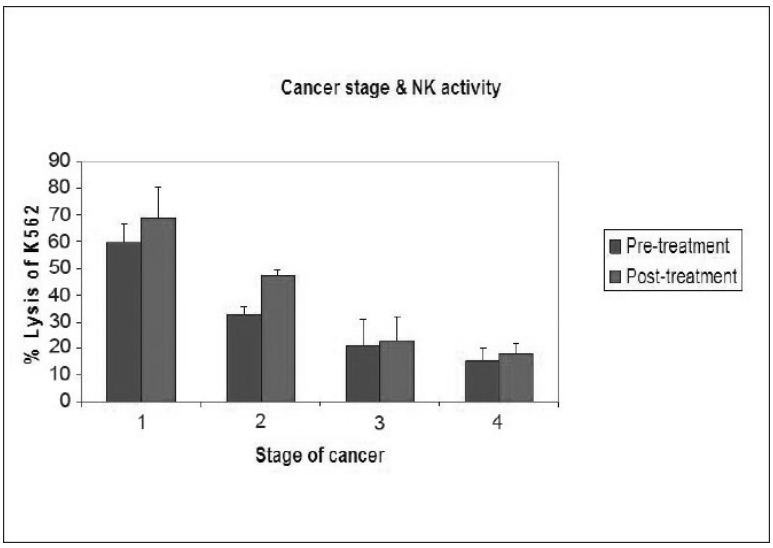

Figure 15: Correlation between NK cell activity and various stages of tumor pre- and post-surgery

Figure taken from Piroozmand and Hassan, 2010

Dewan and coworkers (2009) showed that NK cell activity was substantially lower in breast cancer patients compared to controls and that HER2-negative breast cancer patients had lower activity compared to HER2-positive patients These authors suggested that NK cell activity may play a role in progression of breast cancer. Ascierto et al (2013) also demonstrated that the presence of molecular signatures indicative of NK cells was predictive of relapse free survival in breast cancer. Presence and overexpression of 8 known markers of NK activation such as NKP46, NKG2D, among others could be linked to both relapse free survival at 120 months, and to overall survival risk function at 100 months of follow-up. Therefore, NK cell activity is significantly reduced in breast cancer patients, and may be indicative of disease progression and recurrence.

The elderly

A progressive decline in immune function with age, or "immunosenescence", has been described and supported by studies, both clinical and epidemiologic. All types of immune cells are affected in ageing, however, T-cell activity is usually used as a biomarker for estimating the degree of immunosenescence (Camous et al, 2012). Immunosenescence is characterized by changes in the thymus, decreases in T-cell and B-cell populations, and deregulation of the T-helper cell system (DelaRosa et al, 2006, Kaszubowska et al 2011). The status of the elderly immune system however, due to continuous stimulation over time by the innate immune system and antigenic load, has been described as "inflammaging", characterized by increased serum

levels of cytokines (Franceschi and Bonafe, 2003; Kaszubowska et al 2011). This pro-inflammatory status appears to relate to many diseases of the elderly, which have an inflammatory component (arteriosclerosis, dementia, osteoporosis, cancer) (Sansoni et al 2008).

However, the innate immune system (in comparison to the adaptive immune system), appears to be better preserved when looking at age-related decline in immune function (Sansoni et al, 2008). The level and function of NK cells in aging depends largely on the aged population being studied. In a study looking at biomarkers of immune function in healthy males aged 20-75 years, in Austria, Spain and France (Vasson et al 2013), regional differences in these biomarkers were found and ascribed to differences in diet, lifestyle, environment, genetic and socio-economic factor. Compared to young controls, the percentage of NK cells (of total leukocyte cells in the blood samples) was higher in Spain than in either France or Austria (in any age group), with increase in numbers seen in both Spain and France after age 60 (Vasson et al, 2013).

The increase in NK cells seen in the elderly compared to young controls, appears to be due to an increase in the cytotoxic $CD56^{dim}$ (mature NK) cells and a decrease in the $CD56^{bright}$ (less mature) subpopulations (Almeida-Oliveira et al, 2011; Sansoni et al 1993). However, in the elderly, activated NK cells appear to secrete less IFN-γ (Almeida-Oliveira et al, 2011). Therefore, although there is an in increase in the percent of mature NK cells in the blood of healthy elderly subjects, this is associated with an NK cell activity that is well preserved (ie. the increased absolute numbers of NK cells each secreting less cytokine) (Solana and Mariani, 2000).

However, in a study in centenarians, the increase in NK cells was reflective of an increase in cytotoxicity (Sansoni et al, 1993). Elevated NK cell activity in the elderly correlated well with well-preserved endocrine functions and muscle mass (Mariani et al, 1999) whereas low NK activity in the elderly was predictive of morbidity and mortality (Levy et al, 1991). Low NK cell activity has been associated with increased risk of infections and associated morbidities in immunologically normal elderly subject with an impaired performance status (Ogata et al, 2001). Well-preserved NK cell activity in the elderly can thus be interpreted as a factor in longevity

since this activity is substantially decreased in non-healthy and frail elderly (Sansoni et al 2008, DelaRosa et al, 2006). Therefore, preserved NK cell cytotoxicity should be considered a biomarker of healthy ageing and longevity whereas low NK cell activity is a predictor of morbidity and mortality due to infections (DelaRosa et al 2006).

Autoimmune disorders

Multiple Sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system characterized by demyelination, thought to be mediated by autoreactive T-cells (Lunemann et al 2011). In MS patients with relapsing-remitting type MS, NK cells have been shown to have an impaired ability to produce IFN-γ and to proliferate in response to IL-12, suggesting that the NK $CD56^{bright}$ cell subset is primarily lacking in MS patients, this seen with no differences in NK cell cytotoxicity compared to healthy controls. (Lunemann et al 2011). Treaent of MS patients with daclizumab (monoclonal antibody which blocks the Tac epitope on T-cells) caused a gradual decline in circulating T-cells and an expansion of $CD56^{bright}$ NK cells. The expansion of $CD56^{bright}$ NK cells correlated with inhibition of contrast-enhancing lesions on a brain MRI (measure of brain inflammation) and with increased NK cytotoxicity in treated patients (Bielekova et al 2006). Therefore, NK cells may play a regulatory role in modulating demyelination, via cytokine production and cytotoxicity, by affecting the autoreactive T-cell activation and survival (Mayo et al 2012).

Rheumatoid arthritis (RA) is an autoimmune disorder characterized by inflammatory synovitis, bone erosion and rheumatoid factor (Aramaki et al 2009). Compared to control subjects, NK cell cytotoxicity on a per-cell basis was reduced in patients with RA, likely due to the diminished expression of NK cell activating receptors found in these patients (Aramaki et al 2009). Therefore, it is likely that lower NK cell activity has an effect on the immune regulation of autoreactive T-cells and B cells which induce autoimmune disorders such as RA (Aramaki et al 2009).

Systemic Lupus Erythromatosus (SLE) is a heterogenous autoimmune disorder, characterized by numerous immune abnormalities (Erkeller-Yusel et al 1993; Hervier et al 2011). In patients with active SLE, NK cell numbers were shown to be decreased, however no change in the relative

amounts of $NK56^{dim}$ and $NK56^{bright}$ subsets was seen, and a lower NK cell cytotoxicity was seen in these patients compared to healthy controls (Hervier et al 2011).

Chronic fatigue

Chronic Fatigue Syndrome (CFS) is a syndrome associated with immunologic abnormalities whose etiology is not well understood (Patarca-Montero R, et al. 2001). Immune system abnormalities have been found in CFS patients, although none has emerged as a diagnostic marker, likely due to the heterogeneity of the disease (Whiteside and Friberg, 1998, Patarca-Montero R, et al. 2001). The most common marker, reported in a significant amount CFS cases, is diminished natural killer cell function (Whiteside and Friberg, 1998, Patarca-Montero R, et al. 2001). Low NK cell activity has been found in CFS patients with normal absolute NK cell numbers (Whiteside and Friberg, 1998).

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