# Natural Killer Cells Generate Memory-type Responses to Human Cytomegalovirus-infected Fibroblasts

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## Disclaimer

The work presented within this thesis has been conducted in full by Nicholas Newhook, and published within the European Journal of Immunology under the title “Natural Killer Cells Generate Memory-type responses to Human Cytomegalovirus-infected Fibroblasts” on May 23rd, 2017 [1].

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## Abstract

Natural killer (NK) cells are cytotoxic lymphocytes that selectively respond against abnormal cells. Human cytomegalovirus (HCMV) infection causes expansion of $NKG2C^+CD57^+$ NK cells in vivo and $NKG2C^+$ NK cells proliferate when cultured with HCMV-infected cells. This raises the possibility of an NK-cell subset selectively responding against a specific pathogen and accruing memory. To test this possibility, we compared proliferation, natural cytotoxicity and interferon-γ (IFN-γ) production of NK cells from HCMV-seropositive and HCMV-seronegative individuals co-cultured with HCMV-infected or uninfected MRC-5 cells. There was no significant difference in proliferation of NK cells from HCMV-seropositive or seronegative individuals against uninfected MRC-5 cells, but significantly more NK cells from the HCMV-seropositive groups proliferated in response to HCMV-infected MRC-5 cells. Natural cytotoxicity of NK cells against K562 cells increased following co-culture with HCMV-infected versus uninfected MRC-5 cells only for the HCMV-seropositive group. After co-culture with HCMV-infected MRC-5 cells, proliferating NK cells from HCMV-seropositive donors selectively produced IFN-γ when re-exposed to HCMV-infected MRC-5 cells. Both $NKG2C^+$ and $NKG2C^-$ NK cells proliferated in co-culture with HCMV-infected MRC-5 cells, with the fraction of proliferating $NKG2C^+$ NK cells directly correlating with the circulating $NKG2C^+$ fraction. These data illustrate an at least partly NKG2C-independent human NK-cell memory-type response against HCMV.

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## Abbreviations

| | |
|---|---|
| ADCC | Antibody dependent cellular cytotoxicity |
| AML | acute myeloid leukemia |
| APCs | antigen presenting cells |
| °C | degrees Celsius |
| CHS | contact hypersensitivity |
| CIML | cytokine-induced memory-like |
| CMV | Cytomegalovirus |
| DMEM | Dulbecco's modified eagle medium |
| DTH | Delayed-type hypersensitivity |
| EBV | Epstein Barr Virus |
| FITC | Fluorescein isothiocyanate |
| HCl | hydrochloric acid |
| HCMV | Human cytomegalovirus |
| HCV | Hepatitis C virus |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HIV | human immunodeficiency virus |
| HLA | human leukocyte antigen |
| HLA-C | human leukocyte antigen C |
| IFNγ | interferon gamma |
| Ig | immunoglobulin |
| IL | interleukin |

| | |
|---|---|
| ITAM | immunoreceptor tyrosine-based activating motif |
| ITIM | immunoreceptor tyrosine-based inhibitory motif |
| IU | international units |
| k | kilo |
| kb | kilobase |
| KIR | killer cell immunoglobulin-like receptor |
| l | litre(s) |
| M | molar |
| MCMV | murine cytomegalovirus |
| MHC-I | major histocompatibility complex class I |
| n | nano |
| NaAc | Sodium acetate |
| NaCl | Sodium chloride |
| NCR | natual cytotoxicity receptor |
| NK | natural killer |
| PBMC | peripheral blood mononuclear cell(s) |
| PBS | phosphate-buffered saline |
| PE | phycoerythrin |
| PerCP | peridinyll-chlorophyll protein |
| PFA | paraformaldehyde |
| pH | potential of hydrogen |
| P/S | penicillin/streptomycin |

| | |
|---|---|
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| RPMI 1640 | Roswell Park Memorial Institute medium |
| SCID | severe combined immunodeficiency |
| SIV | Simian immunodeficiency virus |
| SHIV | simian-human immunodeficiency virus |
| TNF | tumour necrosis factor |
| Tris | tris(hydroxymethyl)aminomethane |
| μ | micro ($10^{-6}$) |
| UV | ultraviolet |
| VLP | Virus-like particle |
| VSV | vesicular stomatitis virus |
| ζ | zeta |

# Chapter 1: Introduction

## 1.1 Natural Killer Cells

Natural Killer cells (NK cells) are bone-marrow derived granular lymphocytes comprising 5-25% of peripheral blood mononuclear cells (PBMC). NK cells are sentinels of the innate immune system that provide rapid protection against virally infected and neoplastic cells, controlling their dissemination through cytotoxic and proinflamatory cytokine responses without prior sensitization [2]. The importance of NK cells is most clearly established in viral infection as illustrated in individuals with NK cell deficiencies, who have elevated susceptibility to herpes infections and their conferred morbidities [3-6]. Initially, NK cells were believed to be a homogeneous cell population working in concert with cytotoxic T-cells by recognizing and lysing cells with aberrant major histocompatibility complex class I (MHC-I) expression. Advances in NK cell biology have since revealed their heterogeneous nature in both function and phenotype, including specialized populations with immunoregulatory roles as well as long lived NK cells with immunological memory [7, 8].

Host protection from virus infection and cancer is achieved through several essential NK cell effector functions. First, NK cells produce an array of chemokines and cytokines that conduct host immune responses through chemotaxis and activation of other immune cells. This function endows NK cells with the ability to tune up or dampen

impending and ongoing immune responses, as illustrated by their promotion of type 1 $CD4^+$ T-cell priming, and conversely, by their capacity to kill activated dendritic cells [9, 10]. Interferon gamma (IFNγ) represents the prototypical NK cell cytokine, which is released upon NK cell activation [11]. This multifaceted messenger protein is essential in the early stages of infection for its induction of MHC-I on antigen presenting cells (APCs), activation of phagocytes to control obligate intracellular pathogens, as well as directly inhibiting viral replication and proliferation of malignant transformed cells [12-14]. NK cells also provide host protection through cytotoxic intervention using perforin and granzyme B sequestered within cytoplasmic granules. Upon interaction with a target cell, NK cells form an immunological synapse and release their stored perforin, which oligomerizes to form pores in the target cells membrane. These perforin channels disrupt the osmotic polarity of the cell membrane resulting in cell lysis, or serve as portals for the passive diffusion of granzyme B, which initiates apoptosis of the target cell [15].

Unlike B and T cells, NK cells lack the recombination machinery necessary for generation of clonal antigen receptors, and, therefore, are believed to lack antigen specificity. Instead, NK cell control is mediated through a sophisticated arsenal of germline encoded inhibitory and activating receptors that ligate receptors expressed on healthy and aberrant cells [16, 17]. Inhibitory NK cell receptors are characterized by the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within their long cytoplasmic tails, that when engaged, recruit phosphatases that act to dampen the cell's state of

activation [18, 19]. In contrast, activating receptors generally have short cytoplasmic tails free of ITIM's, but have a positively charged amino acid, namely arginine or lysine, within their transmembrane region. When phosphorylated, these residues act as docking sites for signaling adaptor molecules bearing immunoreceptor tyrosine-based activating motifs (ITAM's) such as DAP10 and DAP12, which initiate cascades involved in activation [18, 19]. NK cell activation is determined by an integration of these signals upon interaction with a target cell, acting as an excitatory rheostat for appropriate initiation of effector functions.

Major histocompatibility complex (MHC) class 1 proteins act as ligands for many inhibitory NK cell receptors. This receptor ligand interaction acts as a failsafe against NK cell mediated-autoimmunity toward healthy tissues, as MHC class I molecules are ubiquitously expressed on the surface of nucleated cells. However, when MHC class I expression declines, such as in viral infection and cancer, the inhibitory threshold is reduced, thereby increasing the likelihood of NK cell detection and killing [20-24]. In this model, activating receptors also recognize ubiquitously expressed proteins on the cell surface, but inhibitory signals act as the determinants of the response. NK cell recognition in the context of aberrant MHC class I expression is termed "missing self" detection (figure 1)[19, 25]. This contrasts with "induced self" detection where activating receptors dictate effector function by recognizing ligands expressed on stressed cells (figure 1a-c)[26-28]. Although these models differ, it is important to note that they are not mutually exclusive.

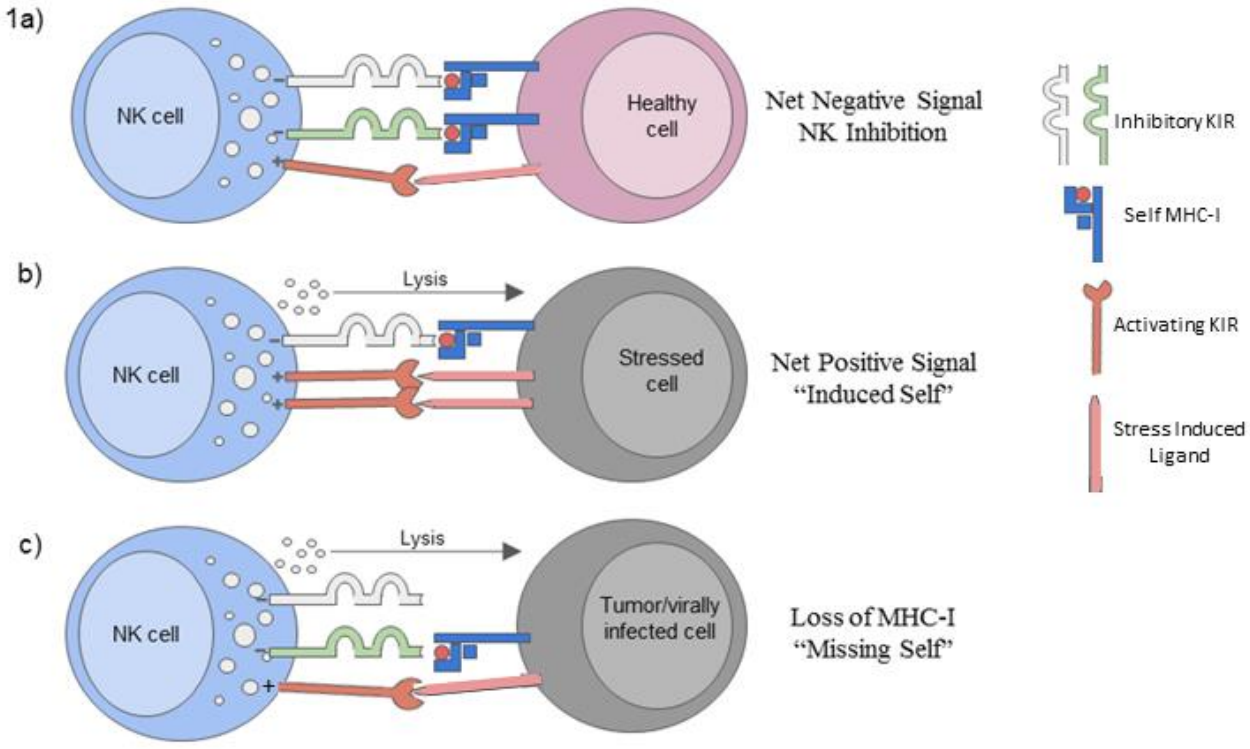


**Figure 1.0: Mechanisms of NK cell target detection and self-tolerance.** NK cells are tolerant of healthy cells, as the strength of excitatory input is dampened by inhibitory NK cell receptor engagements with self MHC-I (a). When host cells become stressed, such as in viral and bacterial infection or caner, NK cell activating receptors recognize induced ligands on the cell surface resulting in a net positive signal and subsequent NK cell attack (b). Viral infection and cancer often downregulate MHC-I to evade detection by cytotoxic T-cells. NK cells can be activated by these cells because they are no longer kept in check by inhibitory receptor engagement with self-MHC-I (c).

Besides recognition of stress induced ligands and loss of MHC-I, NK cells can be activated by antibody opsonized targets. Appropriately coined antibody dependent cell-mediated cytotoxicity (ADCC), NK cells span the gap between innate and adaptive immunity with the ability to recognize antibodies bound to targets through the low affinity Fc receptors FcγRIIIa and FcγRIIc (CD16) [29, 30]. Like other excitatory NK cell receptors, CD16 lacks intracellular activating motifs and instead relies on ITAM containing adaptor proteins, namely CD3ζ or FcεRIγ [31, 32]. Upon ligation with antibodies, CD16 becomes co-localized with either of these two adaptor proteins which then initiates potent excitatory signaling cascades that end in cytokine production and cytotoxic granule release [31, 32]. It is important to note that NK cell-mediated ADCC cannot be triggered by low threshold antibody stimulation, but requires crosslinking of multiple CD16 molecules to overcome basal NK cell inhibitory signaling [33]. Crosslinking and the low affinity of the receptor likely provide failsafe against inappropriate NK cell activation that may otherwise occur due to ubiquity of antibodies *in vivo*.

### 1.2 NK cell Education and Tolerance

NK cell functional development relies on interactions between NK cell inhibitory receptors and MHC class I expressed on bone marrow stromal cells [34]. These interactions define the basal inhibitory threshold of each NK cell and ensure self-tolerance [35]. This process of "NK cell education", is indispensable due to the stochastic nature of NK cell

receptor acquisition, which gives rise to thousands of functionally different NK cell subsets [35, 36].

A selective process in NK cell maturation involving inhibitory NK receptors was first suggested with the observation that all NK cells collected from two unrelated donors expressed at least one inhibitory receptor for self MHC class I [37]. NK cell subsets void of self-MHC class I specific inhibitory receptors have since been observed [38, 39], however, these cells are not self-reactive, are generally unresponsive to excitatory receptor engagement *in vitro*, and fail to target MHC class I deficient targets [34, 38, 40]. Similar observations have been made in studies using NK cells carrying inhibitory receptors with mutated ITIM motifs, which conserve inhibitory receptor interactions with MHC class I, but interrupt downstream inhibitory signaling. When introduced during development, such mutations render NK cells hyporeactive, but paradoxically, inhibitory receptor blockade in mature NK cells enhances responsive potential [40]. Together, these data strongly suggest inhibitory NK cell receptor interactions with self MHC-I augment NK cell responsiveness and ensure self-tolerance. However, the molecular mechanism governing NK cell education is largely unknown.

While poorly understood, three hypotheses exist that address the mechanism of NK cell education and self-tolerance: the "licensing model"; the "disarming model"; the "rheostat model" (figure 1.1). The licensing model postulates that NK cells are initially unresponsive, but become "licensed" to function following inhibitory receptor engagement

with self MHC-I (figure 1.1a)[40]. In support of this model, transgenic mice that have complete expression of foreign MHC-I develop NK cells that reject self-bone marrow and lymphocytes. This autoreactivity is proposed to be facilitated by NK cells lacking inhibitory receptors for self MHC-I, but express those that recognize the introduced protein, resulting in inappropriate licensing and a subsequent breech in NK cell self-tolerance [41]. This finding suggests that NK inhibitory receptor interactions may play an instructive role, rather than inhibitory, in NK cell education. It is important to note that "self" bone marrow was only rejected in this study following ex vivo culture, and therefore results may reflect an artifact of experimental design, rather than NK cell licensing [41].

In contrast to the licensing model, the disarming model suggests that NK cells are initially responsive, but when left uninhibited are rendered anergic by chronic stimulation from normal cells (figure 1.1b)[42]. This model of NK cell education is supported by studies using transgenic mice with mosaic expression of non-self MHC-I. Such mice express the foreign protein on a fraction of their hematopoietic cells, but curiously fail to reject self-bone marrow like those with complete expression [41]. This finding indicates that effective NK cell education requires ubiquitous expression of self MHC-I, supporting an inhibitory role of these interactions as predicted by the disarming model [34, 41, 43]. However, SHIP-1 phosphatases, which are important in NK cell inhibitory signaling, are seemingly dispensable for NK cell education questioning the role of inhibitory interactions, and thus the disarming model [44].

Alternatively, the rheostat hypothesis theorizes that NK cell responsive potential is bestowed quantitatively and differentially based on the frequency and affinity of inhibitory receptor engagements during development (figure 1.1c)[43, 45]. In support of this theory, NK cells bearing multiple inhibitory receptors for self MHC class I respond more frequently and elicit stronger effector functions than those bearing a single self-inhibitory receptor [46]. Further studies have found that NK cell education is also influenced by the affinity of these inhibitory interactions, with stronger binding leading to heightened responsiveness [47]. Evidently, it seems NK cell responsive potential may be bestowed on a continuum where NK cells can be "tuned up" to counter chronic inhibitory stimulation from self, or "tuned down" when self-inhibition is weak. This process likely fine tunes immature NK cells to their optimal responsive potential, while simultaneously insuring self-tolerance [45].

Although each model is independently supported, it is important to note these hypotheses are not mutually exclusive, and NK cell education is likely achieved by combination of these mechanisms. Additionally, recent evidence suggests that NK cells education may not be permanently endowed during NK cell development, but rather reflect a dynamic process. For instance, multiple recent studies have found that mature NK cells can be educationally "re-programmed" following exposure to novel HLA environments [48, 49].

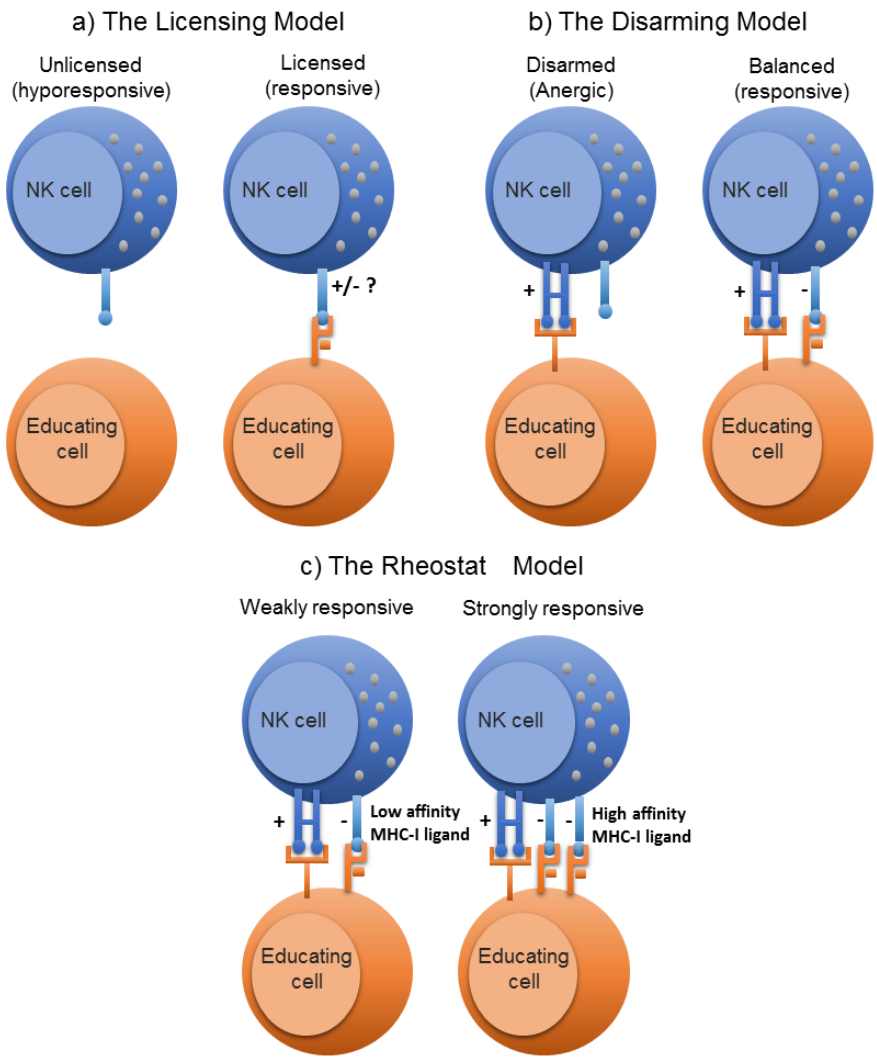


**Figure 1.1: Hypothesized mechanisms of NK cell education.** The licensing model postulates that NK cells are initially unresponsive, but become "licensed" to function following inhibitory receptor engagement with self MHC-I (a). In the disarming model, NK cells are initially responsive, but when left uninhibited are rendered anergic by chronic stimulation from normal cells (b). The rheostat hypothesis theorizes that NK cell responsive potential is bestowed quantitatively and differentially based on the frequency and affinity of inhibitory receptor engagements during development (c)

### 1.3 NK cell Receptors

#### 1.3.1 Ly49 Receptors

The highly-studied c-type lectin-like Ly49 proteins represent a large NK cell receptor family in mice. These proteins have both inhibitory and activating isoforms which respectively signal through ITIMs in their cytoplasmic tail, or ITAM-containing adaptor proteins [50, 51]. Inhibitory isoforms generally ligate with murine MHC class I proteins, while activating isoform ligands remain mostly unidentified [52]. However, some excitatory Ly49 have been shown to bind MHC-I in the presence of viral chaperones, as well as MHC-I-like viral gene products [53-55]. Recognition of cognate H-2 requires the presence of a bound peptide within the groove of the MHC protein [56-58]. Although these interactions are peptide dependent, they are generally not peptide-specific, as crystal structures demonstrate that Ly49 receptor-binding residues are distal to the peptide-binding groove of MHC-I[59]. One exception is inhibitory Ly49C, which has been shown to provide differing protective capacities based on the peptide bound to its cognate H-2 ligand [60].

As consequence of binding MHC-I, one of the most highly polymorphic loci in mammals, Ly49 receptors have evolved into an extensively polymorphic receptor system [61]. The close proximity of Ly49 gene loci results in inheritance of variable haplotypes, often including receptors for MHC-I alleles absent in a given animal [19]. Adding to this

complexity, the Ly49 repertoire seems to be initially stochastically generated giving rise to variegated expression patterns between strains and between individual NK cells [62-64]. However, the functional repertoire seems to be shaped by MHC-I dependent educational processes which silence potentially autoreactive clones and disfavor those with multiple self-specific Ly49 receptors [65]. In a separate process, the density of Ly49 proteins on NK cells is altered to an optimal amount based on the level of MHC-I expression in the animal [66, 67]. This mechanism is believed to occur posttranscriptionally through ligand mediated downmodulation [62].

### 1.3.2 Killer-cell immunoglobulin-like receptors (KIR)

In primates the Ly49 family of receptors is absent except for a single residual pseudogene within the Ly49 gene cluster in humans [68]. However, these proteins seem to have been replaced by the structurally distinct killer immunoglobulin-like receptors (KIR) through an exquisite example of convergent evolution [69]. Supporting this theory are striking similarities between the two protein families, including the presence of inhibitory and excitatory isoforms with common ITIM and ITAM signaling. Additionally, both inhibitory KIR and Ly49 proteins recognize MHC-1 proteins and are involved in NK cell education, while activating isoforms bind ligands indicative of host cell aberration [69]. Evolution of both gene sets has also taken remarkably similar routes including multiple duplication episodes and common descent of excitatory isoforms from inhibitory counterparts [70].

Although functionally and evolutionarily similar, KIR are structurally distinct from Ly49 receptors as type 1 glycoproteins of the immunoglobulin superfamily. As this suggests, KIR share structural features with immunoglobulins in the form of two to three immunoglobulin folds [71]. These domains are extracellular and denoted in KIR nomenclature as KIR2D or KIR3D. Alleles are further characterized by possession of long or short cytoplasmic tails as denoted in their classification as an L(long) or an S(short), which proceeds the number of extracellular domains (eg. KIR2DL or KIR2DS). Long cytoplasmic tails are the product of ITIM motifs and characterize inhibitory KIR, while excitatory forms are short and rely on ITAM containing adaptor proteins [72]. KIR specifically bind HLA-A, -B, and -C, and have been shown to interact with the peptide binding region of these proteins when a peptide is bound [73-75]. Evidently, KIR demonstrate peptide selectivity by recognizing broad amino acid motifs, which contrasts with T-cell receptors, which recognize specific epitopes [76].

Like Ly49, KIR are highly polymorphic and polygenic with 14 to 17 receptors and hundreds of distinct alleles [77]. KIR repertoires are inherited as two differing haplotypes termed group A and group B. Group A haplotypes show little variation in gene content between individuals and are dominated by inhibitory receptors, while group B haplotypes are highly variable and contain multiple stimulatory KIR. Receptors within haplotype A provide high affinity recognition of MHC-I molecules, and those within group B contribute diversity to the NK cell repertoire [78]. Interestingly, group B haplotypes have been

associated with elevated protection in several diseases including cancer and human immunodeficiency virus (HIV) infection, illustrating their importance in host defense [79-83]. Expression of KIR is initially stochastic producing subsets of NK cells with variable, but overlapping KIR phenotypes. The initial KIR repertoire is then condensed through educational processes to generate a functional NK cell pool. This expression system creates an arsenal of unique NK cells and increases recognition of a broad array of target cells [39, 84].

### 1.3.3 NKG2 Receptors

Another important group of receptors in NK cell function are the highly conserved NKG2 proteins. This family of transmembrane receptors structurally belong to the c-type lectin-like superfamily and consists of seven members: NKG2A; B; C; D; E; F; H [85]. Isotypes exist as inhibitory or excitatory forms in an analogous manner to KIR and Ly49 and have an integral role in NK cell cytotoxic responses [86]. Of these, five species (NKG2A, B, C, E and H) have been shown to form disulfide linked heterodimers with CD94, which has a short cytoplasmic region involved in signal transduction [87]. Dimeric CD94/NKG2 recognizes the widely distributed, but scantily expressed non-classical MHC-I protein HLA-E. HLA-E serves the specialized role of presenting leader peptide sequences trimmed from classical MHC-I during their expression. Therefore, NKG2 proteins allow for NK cells to examine the health of cells by indirectly detecting aberrations in classical MHC-I expression [85, 88].

### 1.4 Adaptive Characteristics of NK cells

Immunological memory refers to the immune system's ability to mount responses of greater magnitude and with faster kinetics upon reencounter with the same antigen. This immune attribute conventionally defines responses mediated by T and B cells, which carry somatically recombined antigen receptors and that following priming, generate long lived, phenotypically distinct memory cells with enhanced function [89]. In contrast, NK cells favor a binary mode of activation governed by a finite number of germline encoded receptors that recognize a restricted panel of ligands expressed on healthy and stressed cells. Evidently, there is no commonly accepted mechanism to endow NK cells with the capacity for either selective adaptation to antigen exposure or for immunological memory [16, 17]. This concept, however, is becoming rapidly challenged by evidence of NK cell subpopulations with extended life spans and augmented recall responses. Current research has elucidated NK cell memory-like responses through three main forms of challenge: hapten based contact sensitization; cytokine induced memory; viral infection [90-92].

NK cell antigenic memory was first demonstrated by T and B-cell deficient mice exhibiting hapten-based contact hypersensitivities (CHS) [93]. CHS reactions are a form of delayed-type hypersensitivity (DTH) that occurs when the epithelial surface is exposed to compounds known as haptens. Haptens bind and chemically alter self-proteins allowing for their detection by the adaptive immune system and a subsequent development of hapten-specific memory cells. Upon reencounter with the same hapten the secondary

immune response presents as redness and swelling of the exposed area [94]. Considered a classic example of adaptive memory, it came as a surprise when Von Adrian *et al.* discovered such responses to 2,4-dinitrofluorobenzene and oxazolone in T and B-cell deficient mice. Incredibly, these responses were hapten specific, long lasting (>4 weeks), and conferred on naïve mice by adoptive transfer of hepatic NK cells from exposed mice [95]. Research from the same group also demonstrated NK mediated DTH responses to virus like particles (VLP) of human immunodeficiency virus, influenza and vesicular stomatitis virus [93]. Antibody blockade experiments revealed that DTH responses from both forms of challenge rely on expression of chemokine receptor CXCR6 on liver resident NK cells [96]. However, CXCR6 is also expressed on splenic and unsensitized hepatic NK cells, which fail to mount specific immune responses to haptens and VLPs [96]. The mechanism underlying NK cell mediated CHS responses in mice remains unsolved, but demands elucidation in hopes of similar discovery in humans. Translation of NK cell based DTH responses into human models would undoubtedly lead to advances in vaccination and adoptive NK cell therapies.

In addition to CHS responses, NK cells gain memory-like features when preactivated with a cocktail of interleukin-12 (IL-12), IL-15 and IL-18. This phenomenon was first defined by Yokoyama and colleagues after adoptively transferring cytokine stimulated murine NK cells into T and B cell deficient syngeneic mice [97]. These preactivated NK cells were phenotypically identical to untreated NK cells, but persisted up

to three weeks later, and produced IFNγ at higher frequencies upon restimulation with cytokines or activating receptor engagement [97]. This ability to retain memory of past cytokine activation has also been demonstrated in human NK cells, which upon stimulation with IL-12, IL-15 and IL-18 develop similar enhancement [98, 99]. However, unlike mice, human CIML (cytokine-induced memory-like) NK cells display elevated cytotoxic potential, specifically against leukemia cell lines and human AML (acute myeloid leukemia) blasts *in vitro* [99]. These findings have since been translated into phase I clinical trials in which cytokine preactivated NK cells were adoptively transferred into patients combating AML. Therein, transplanted NK cells proliferated, exhibited heightened anti-leukemic function *in vitro*, and in four cases led to complete remission [100]. Despite their use in humans, the molecular mechanism governing memory-like attributes of these cells still requires elucidation.

Although NK cells gain memory-like aspects following various forms of challenge, identification of NK cell subpopulations endowed with antigen specific receptors remain elusive and provocative, with one exception. Infection of mice with MCMV serves as the gold standard for NK cell virus interaction, representing the only definitive example of NK-mediated antigenic memory in any species. In C57BL/6 mice, circulating NK cells carry the activating C-type lectin Ly49H, which directly binds the MCMV virulence factor M157 expressed on infected cells [101-103]. Recognition of M157 drives an NK cell response strikingly like those mediated by T-cells with all hallmarks of adaptive immunity

including clonal expansion upon antigen recognition, contraction following clearance, and establishment of a memory population [104, 105]. Cells from the memory pool are detectable as late as 70 days post infection, and display enhanced effector function following secondary expansion ex vivo. Furthermore, these cells confer protection against MCMV re-challenge, but not from heterologous infections, defining them as bona-fide MCMV specific memory cells [106-108].

Recognition of M157 by murine NK cells is achieved due to homologies between M157 and Ly49H's native ligand H-2. Therefore, M157 likely functions as an MCMV evasion protein derived to attenuate the anti-MCMV NK cell response by binding inhibitory Ly49. However, this strategy was likely evolutionarily countered by the emergence of excitatory Ly49 isoforms that also recognize H-2 [103-106]. Underlining the importance of the M157 antigen in this response are experiments using mutant MCMV lacking M157, or variants recognized by inhibitory Ly49, which fail to preferentially expand Ly49H+ NK cells and protect mice from infection [106, 109]. Taken together, murine NK cells can remember previous exposure with a pathogen. However, proof of antigenic recognition and memory in human NK cells remains a challenge.

In primates, NK cell-mediated adaptive responses have proved challenging to unmask, likely due to ethical and economic constraints attached to their experimentation. Nevertheless, headway on this topic has been made. For example, human NK cells have been shown to lyse influenza infected cells through the recognition of hemagglutinin (HA)

by the activating receptor NKp46 in a glycosylation dependent fashion [110-112]. In one independent study, healthy volunteers vaccinated with formulations of the seasonal influenza vaccine elicited augmented IFNγ production by $NKp46^{+}$ NK cells upon viral restimulation for up to six months post vaccination [113]. This finding is evocative of NK cell antigenic memory in mice, however, clonal expansion and development of a discrete memory population is yet to be described. It is possible that the NK cell memory pool resides outside the peripheral blood compartment evading the scope of researchers.

Besides recognition of HA, one study has shown that human NK cells preferentially kill autologous B cells pulsed with peptides derived from Epstein Barr virus (EBV) and CMV, but only when collected from EBV and CMV exposed donors akin to adaptive memory [114]. While intriguing, the mechanism governing these peptide specific NK cell responses could not be determined and the results are yet to be replicated. However, comparable results have been shown in simian immunodeficiency virus (SIV) and simian-human immunodeficiency virus (SHIV) infected macaques [115]. Hepatic and splenic NK cells from infected animals rapidly eliminate gag and env-pulsed autologous dendritic cells in a NKG2-dependent fashion [112]. Of particular interest, macaques also develop NK cell memory responses following Ad26-vaccination, which can be elicited up to 5 years post vaccination [115]. These findings indicate that primate NK cells can elicit robust antigen specific memory responses following infection and vaccination.

Reminiscent of Ly49H+ NK cells in MCMV, human cytomegalovirus (HCMV) infection is associated with the durable expansion of an NK cell subpopulation found within the functionally mature $CD56^{dim}$ NK cell fraction [116]. These cells express high levels of the activating c-type lectin NKG2C, and express the terminal differentiation marker CD57, marking $NKG2C^+CD57^+$ NK cells as a mature population with distinct phenotype and function [116, 117]. $NKG2C^+CD57^+$ NK cells have been shown to expand in vivo during acute HCMV infection and during HCMV reactivation post solid organ and hematopoietic stem cell transplantation [118-121]. This response yields an $NKG2C^+$ memory population that remains long term (>1 year) and when transplanted from seropositive donors elicits enhanced effector function upon secondary HCMV exposure [120, 122]. Curiously, these results are only seen in $HCMV^+$ recipients with active or subclinical expression of HCMV antigens [120, 122]. Furthermore, HCMV infected fibroblasts have been shown to expand $NKG2C^+CD57^+$ NK cells *in vitro*, but only in HCMV seropositive donors [123]. Failure to generate $NKG2C^+CD57^+$ NK cells in seronegative donors *in vitro* illustrates an intrinsic difference between this system and $NKG2C^+$ NK expansion *in vivo*.

Although HCMV infection is clearly integral in the expansion of memory-like $NKG2C^+CD57^+$ NK, there is little evidence of the driving mechanism. In *in vitro* experiments $NKG2C^+CD57^+$ NK expansion following stimulation with HCMV-infected fibroblasts is abrogated by NKG2C blockade using monoclonal antibodies [123, 124]. This

implicates that this receptor helps mediate proliferation of memory-like NK cells, but the mechanistic significance of its presence remains unclear. One possibility lies in the NKG2 ligand HLA-E, which presents leader peptides from classical MHC class I molecules to NK cells [88]. NK cells can be activated by subtle differences in HLA-E bound peptide, thus, it is conceivable that HCMV peptides may be recognized by NKG2C via HLA-E presentation to promote preferential expansion of $NKG2C^+$ cells [125-127]. In support of this theory, certain HCMV derived peptides loaded on HLA-E promote degranulation of NK cells bearing NKG2C, provided its inhibitory counterpart NKG2A is absent [127, 128]. This finding is in-line with the phenotype of $NKG2C^+CD57^+$ NK cells, which lack co-expression of NKG2A [117]. Alternatively, it is possible that NKG2C may be working akin to Ly49H in MCMV and directly recognizes an unknown viral protein expressed on the surface of infected cells.

While *in vitro* proliferation can be interpreted as a secondary response of $NKG2C^+$ NK cells initially activated by HCMV *in vivo*, the mechanistic basis for selective expansion of this NK cell subset and adaptive memory is unclear. To further investigate the possibility that proliferation of NK cells from HCMV-seropositive subjects during co-culture with HCMV-infected fibroblasts represents immunological memory, we compared responses of NK cells from HCMV-seropositive and seronegative individuals to co-culture with HCMV-infected cells in the absence of exogenous cytokines. If memory NK cells are in fact generated during HCMV infection, NK cells from seropositive donors will deliver

more robust proliferative responses following exposure to HCMV-infected cells compared to those collected from seronegative donors. Additionally, enhanced proliferation should be coupled with augmented function, such as elevated cytotoxicity and selective cytokine production by responding cells, to fully recapitulate the classical elements of immunological memory. Therefore, following *in vitro* HCMV-expansion we investigated the phenotype and cytokine production of responding and non-responding cells, as well as changes in NK cell cytotoxic potential towards an MHC-I bare cell line. Our data are consistent with an adaptive NK cell response to HCMV, but raise questions as to a primary role for NKG2C in this response.

# Chapter 2: Materials and Methods

## 2.1. Subjects and sample processing

This study received ethical approval from the Newfoundland and Labrador Health Research Ethics Authority. Whole blood was collected with informed consent from apparently healthy donors by forearm venipuncture into acid-citrate dextrose containing vacutainers. Whole blood was initially centrifuged for 10 min at 1000*g* and plasma collected to determine HCMV serostatus. The packed cellular whole blood fraction was then diluted with phosphate buffered saline (PBS) to twice its original volume and PBMC isolated by density gradient centrifugation using Ficoll hypaque (GE Healthcare Biosciences, Mississauga, ON, Canada). T cells were depleted using EasySep $CD3^+$ selection kits (STEMCELL Technologies Inc, Vancouver, BC, Canada) before co-culture with HCMV-infected cells.

## 2.2. HCMV Lysate ELISA

HCMV lysate was generated by infecting 1 x $10^7$ human MRC-5 fibroblasts with AD169-HCMV at an MOI of 0.5 and harvested after 3 days of propagation. Infected cells were mechanically removed from the plate using a sterile scraper, pelleted via centrifugation and lysed in 1mL of lysis buffer. The lysate was then diluted 1/1000 in bicarbonate coating buffer, and 100µL was added to each well of a Immunlon-2 ELISA plate (VWR Scientific, Mississauga, Ontario, Canada) and incubated overnight at 4°c.

Plasma samples were thawed, diluted 1/500 with PBS, and added to the lysate coated plate before a 90 minute incubation. In sequence, the plate was washed and developed with goat-antihuman IgG-horseradish peroxidase conjugate (Jackson ImmunoResearch Labs, West Grove Pennsylvania, USA) followed by tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, Missouri, USA). Colour was developed for 30 minutes in the dark at room temperature, and stopped using 1N $H_2SO_4$. Optical density was read at 450nm using a BioTek Synergy HT fluorometer. HCMV positives were identified based on relative minimum fluorescence detection limit of diluted plasma from a clinically confirmed $HCMV^+$ standard control. Negative control wells followed the same protocol, but the lysate was generated in the absence of AD169.

### 2.3. NK co-culture with HCMV-infected MRC-5 cells

MRC-5 cells were a kind gift from Dr. K. Hirasawa, Division of BioMedical Sciences, Faculty of Medicine, Memorial University of Newfoundland. The HCMV lab strain AD169 was obtained through the NIH AIDS Reagent Program, AIDS Program, NIAID, and NIH from Dr. Karen Biron [129]. $2.5 \times 10^5$ MRC-5 fibroblasts were seeded into 24 well plates, and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% non-inactivated fetal calf serum (FCS), 100 μg/mL streptomycin, 100 IU/mL penicillin, 2 mM L-glutamine, and 1% sodium pyruvate (all from Invitrogen, Carlsbad, CA, USA). Once confluent, MRC-5 cells were infected with the AD169 at

multiplicity of infection (MOI) 0.025 for 1 hour, or left untreated. Cells were then incubated at 37°C in a 5% $CO_2$ humidified incubator and monitored daily for development of cytopathic effects (3-4 days). T-cell depleted PBMC from HCMV-seropositive and seronegative donors ($3.0 \times 10^6$) were stained with 5 μM CFSE as per manufacturer's (Invitrogen, Carlsbad, CA, USA) protocol and placed into culture with HCMV-infected, or uninfected, MRC-5 cells (figure 2.0a). Cells were then co-cultured for 14 days in RPMI-1640 supplemented with 10% FCS, 100 μg/mL streptomycin, 100 IU/mL penicillin, 2 mM L-glutamine, 10 mM HEPES buffer, and $2 \times 10^{-5}$ M 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA, USA). Medium was partially replaced each 2-3 days. Following co-culture proliferation was analyzed by flow cytometry using CFSE fluorescence intensity dilution (figure 2.0b). To assess production of IFN-γ by cultured proliferating and non-proliferating NK from HCMV-seropositive individuals, cells were cultured as described and on day 14, transferred to 24 well plates containing MRC-5 cells newly infected with HCMV as described above. After 1 hour, brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) was added to 10 μg/mL. The cells were kept in co-culture a further 4 hours and IFN-γ production by proliferating and non-proliferating NK measured by intracellular flow cytometry (figure 2.0c).

## 2.4. Antibodies and Flow Cytometry

The proportion of NKG2C$^+$ CD57$^+$ NK in subjects' circulating PBMC was measured by flow cytometry using the following fluorochrome-conjugated monoclonal antibodies: anti-CD3-peridinyll chlorophyll protein (PerCP), clone BW264/56 (Miltenyi, San Diego, CA, USA;); anti-CD56-fluorescein isothiocyanate (FITC), clone MEM188 (eBioscience, San Diego, CA, USA); anti-CD57-phycoerythrin (PE), clone HNK-1 (Biolegend, San Diego, CA, USA); anti-NKG2C-allophycocyanin (APC), clone 134591 (R&D Systems, Minneapolis, MN, USA). PBMC were washed twice in flow cytometry buffer (0.2% sodium azide, 0.5% FCS, 5 mM EDTA in PBS), stained, washed again and fixed with 1% paraformaldehyde in PBS. To measure proliferation of NKG2C$^+$ and NKG2C$^-$ NK cells following co culture with HCMV-infected MRC-5 cells, co-cultured cells were washed twice with flow cytometry buffer and stained with anti-CD3-PerCP, anti-CD56-PE, anti-NKG2C-APC and eFluor 780 (eBioscience) viability marker. Viable lymphocytes were identified, CD3$^+$ cells excluded and CD56$^+$ cells gated for analysis. Proliferation of NKG2C$^+$ and NKG2C$^-$ CD56$^+$ was assessed by CFSE dilution. Anti-IFN-γ APC (clone 4S.B3) from eBioscience was used to analyze IFN-γ-producing cells. Data were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

## 2.5. NK Cytotoxicity Assay

The co-culture procedure for cytotoxicity assays was as described above without CFSE staining. Changes in NK cytotoxicity following HCMV co-culture were assessed against K562 target cells. K562 were labeled with 100 µCi $Na_2{}^{51}CrO_4$ (MP Biomedicals, Santa Ana, CA, USA) for 90 minutes at 37°C. Cells were removed from co-culture with HCMV-infected and uninfected cells, numbers normalized and maximizing the use of recovered cells, tested in duplicate at high, medium and low effector to target (E:T) ratios against 5 x $10^3$ K562 target cells in a total volume of 300 µL RPMI-1640 supplemented as above. Maximum release wells contained K562 cells in 1 N HCl, while minimum release wells contained K562 cells in medium alone. The plate was incubated at 37°C for 5 hours in a 5% $CO_2$ humidity controlled incubator and radioactivity released into supernatants measured using a Wallac Wizard 1470 gamma counter. Percent specific lysis was calculated as test $^{51}Cr$ release – spontaneous $^{51}Cr$ release/maximum $^{51}Cr$ release – spontaneous $^{51}Cr$ release x 100.

## 2.6. Statistical Analysis

All statistical analysis was performed using Graphpad Prism software, version 5.01. All data sets were first assessed for normality by Kolmogorov-Smirnov test. Differences between the responses of HCMV-seropositive and seronegative groups and

between the percentages of $CFSE^{hi}$ and $CFSE^{lo}$ NK cells producing IFN-γ in response to HCMV-infected cells were assessed by non-parametric two-tailed Mann-Whitney testing. The Wilcoxon signed rank test for paired samples was used to test the significance of increased NK cytotoxicity following co-culture with HCMV-infected cells. Correlations were assessed using Spearman's Rank Correlation Coefficient. P values < 0.05 were considered significant.

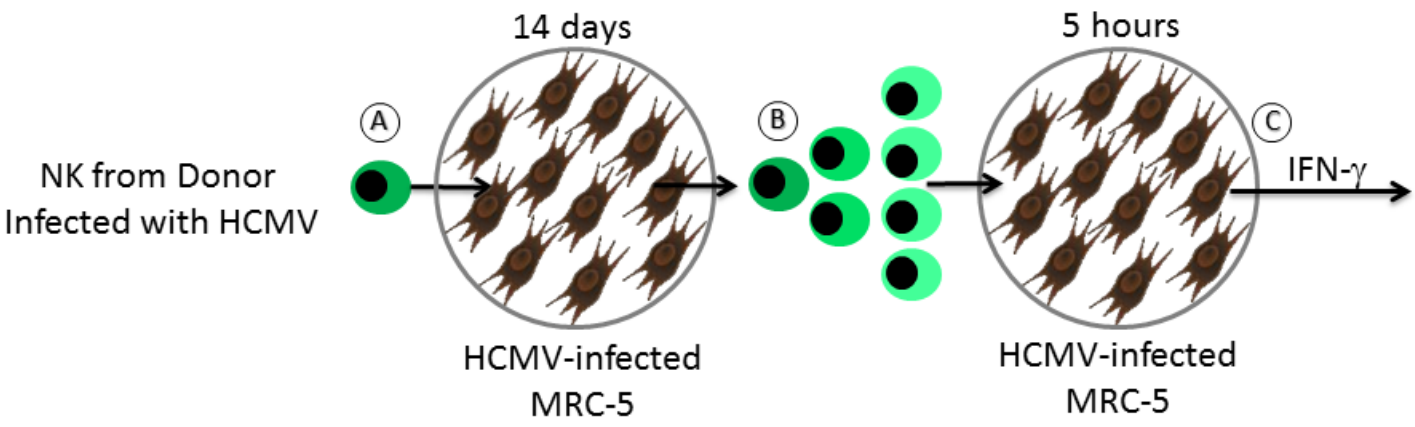


**Figure 2.0. HCMV co-culture to assess HCMV specific NK cells.** T-cell depleted PBMC from HCMV-seropositive and negative donors are stained with CFSE and co-cultured with HCMV infected MRC-5 for 14 days (A). Proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ and $NKG2C^{-}$ cells is then assessed by CFSE fluorescence intensity dilution (B), and co-cultured cells transferred to a secondary culture of HCMV infected MRC-5 for five hours to assess IFNy production by proliferated and non-proliferated NK cells (C). All cultures are run in duplicate and in parallel with uninfected co-cultures as a control.

## Chapter 3: Results

HCMV infection has been strongly linked in independent studies to the expansion and maintenance of NKG2C$^{+}$CD57$^{+}$ NK cells, which expand in response to HCMV reactivation, and exhibit increased activity following a secondary HCMV event. These observations are reminiscent of HCMV specific NK responses seen in mice, but there is no evidence for specific recall. To elucidate HCMV specific NK memory we compared NK expansion, phenotype, and function between HCMV seropositive and seronegative donors following in vitro stimulation with HCMV infected fibroblasts.

### 3.1. Selective proliferation of NK cells from HCMV-seropositive donors.

To test whether HCMV infection in vivo sensitizes NK cells to respond to HCMV-infected cells in vitro, we co-cultured freshly-isolated NK cells from 19 HCMV-seropositive and 11 seronegative individuals with uninfected or HCMV-infected MRC-5 fibroblasts and measured NK cell proliferation over 14 days. Proliferation of CD3$^{-}$CD56$^{+}$ NKG2C$^{+}$ and NKG2C$^{-}$ cells was assessed on day 14 of culture by carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensity dilution as shown (Figure 3.1.0). Representative flow plots collected from a HCMV-seropositive and seronegative donor are shown in figure 3.1.1 and figure 3.1.2, respectively. Proliferation of NK cells cultured with uninfected MRC-5 cells ranged from 0.8% to 26.3%, with no significant difference between HCMV-seropositive and seronegative donors; mean % proliferating NK = 10.90,

95% confidence interval (95% C.I.) 5.77-16.03 versus 7.88, 95% C.I. 4.90-10.85, p = 0.25 (Figure 3.1.4). In response to HCMV co-culture NK cell proliferation ranged from 1.67% to 48.32% in HCMV-seropositive donors, and 0.91% to 19.54% in the seronegative group. Curiously, only cells collected from HCMV-seropositive donors consistently elicited greater NK cell proliferative responses towards HCMV-infected MRC-5 compared to the control. Conversely, responses from HCMV-seronegative donors were often greater when co-cultured with uninfected MRC-5 (Figure 3.1.3), likely due to viral cytolysis and a temporal decrease in stimulation. After subtracting NK cell proliferation in response to co-culture with uninfected MRC-5 fibroblasts, we observed significantly more proliferation of NK cells from HCMV-seropositive donors; median % proliferating NK = 7.70, interquartile range (IQR) 6.23-20.82 versus 0.80, IQR 0.00-4.60, p < 0.0001(Figure 3.1.5). These data suggest that host infection with HCMV sensitizes NK cells to proliferate in vitro when exposed to HCMV-infected cells.

Due to the use of magnetic separation for the depletion of T-cells in our system, which has a maximum effective removal of 99%, few cultures contained small numbers of contaminating T-cells. To account for the possibility of these T-cells affecting NK cell proliferation we correlated the relative proportion of CD3+ cells post HCMV co-culture with total NK cell proliferation. We found no significant relationship between the proportions of contaminating T-cells and HCMV-induced NK cell proliferation; r=-0.29, $p > 0.05$ (figure 3.1.6).

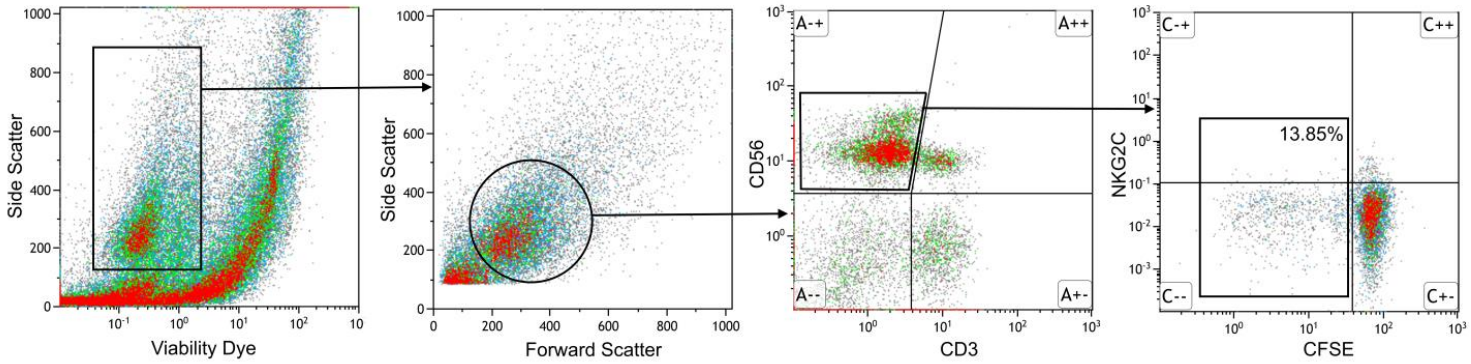


**Figure 3.1.0 Gating strategy to assess NK-cell proliferation in response to HCMV-infected MRC-5 cells.** Proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ and $NKG2C^{-}$ cells was assessed by CFSE fluorescence intensity dilution following 14 day HCMV-co-culture without exogenous cytokines. Representative flow cytometry plots from left to right above show dead cell exclusion, lymphocyte inclusion, gating on $CD56^{+}CD3^{-}$ cells and representation of proliferating $NKG2C^{+}$ and $NKG2C^{-}$ cells.

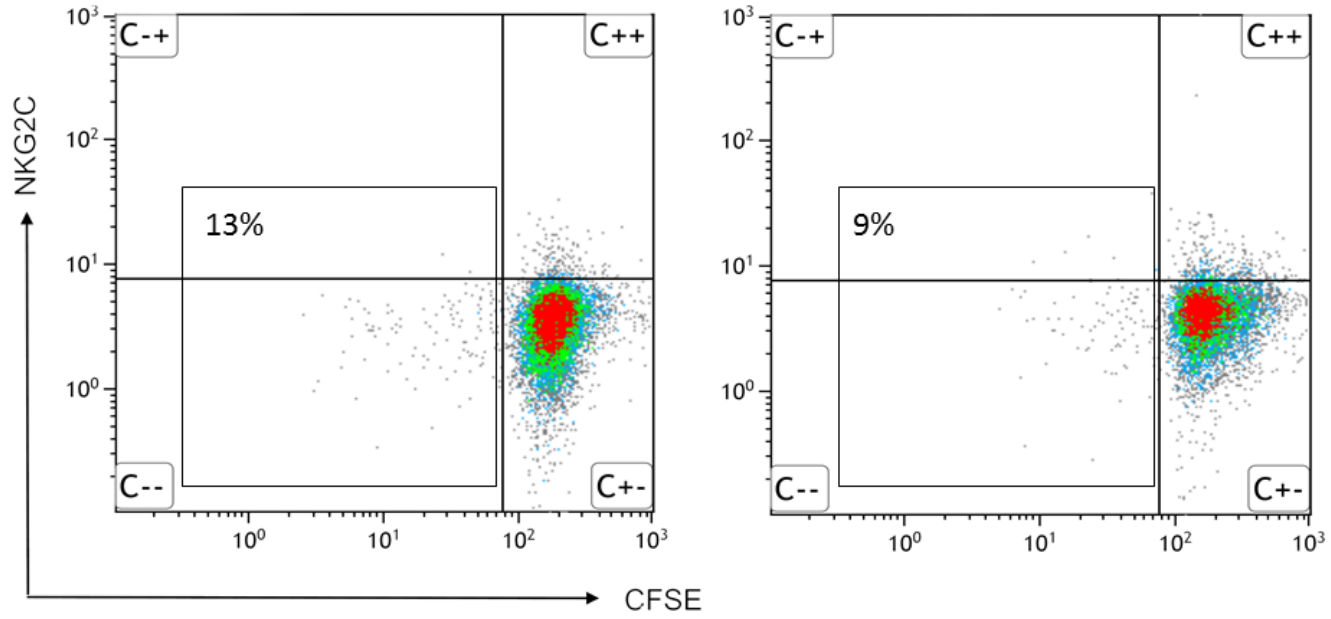


**Figure 3.1.1 NK cell proliferation of an HCMV-seronegative donor in response to HCMV-infected MRC-5.** Following 14 day co-culture, proliferation of $CD3^-CD56^+$ $NKG2C^+$ and $NKG2C^-$ NK cells was assessed. Proliferated $CFSE^{low}$ NK cells in the uninfected (left) and HCMV-infected (right) co-cultures are highlighted within the black squares, and annotated numbers represent relative proportions of proliferating NK cells.

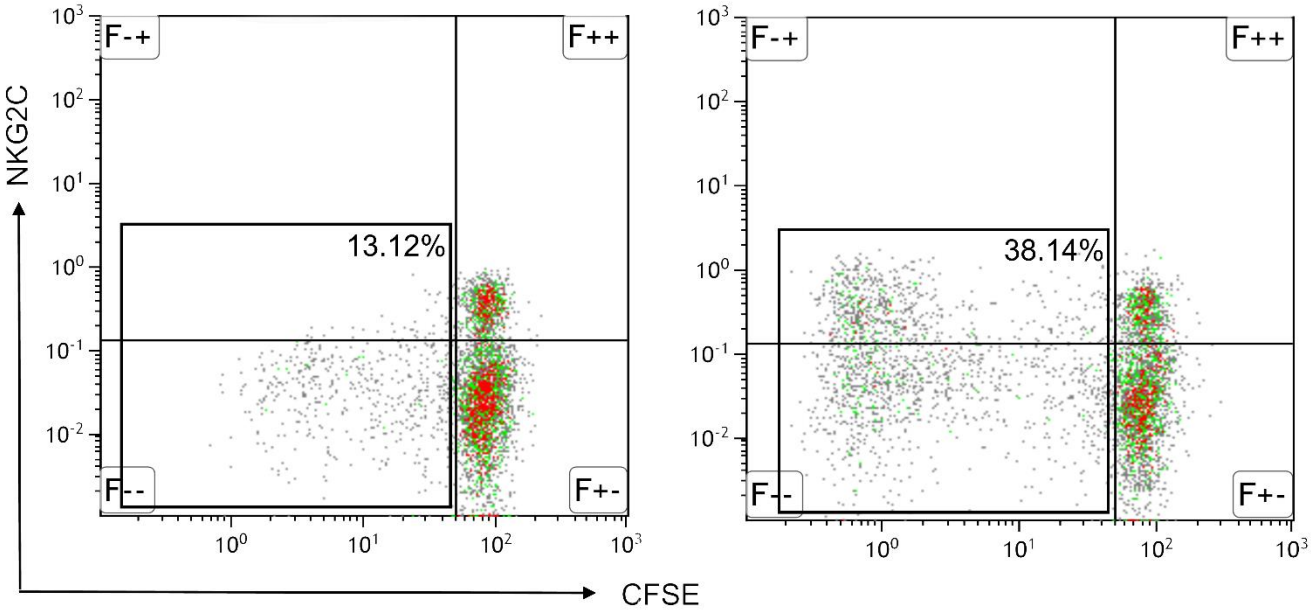


**Figure 3.1.2 NK cell proliferation of an HCMV-seropositive donor in response to HCMV-infected MRC-5.** Following 14 days co-culture, proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ and $NKG2C^{-}$ cells was assessed. $CFSE^{low}$ NK cells proliferated in uninfected (left) and infected (right) co-cultures are highlighted within the black squares, and annotated numbers represent relative proportions of proliferating NK cells.

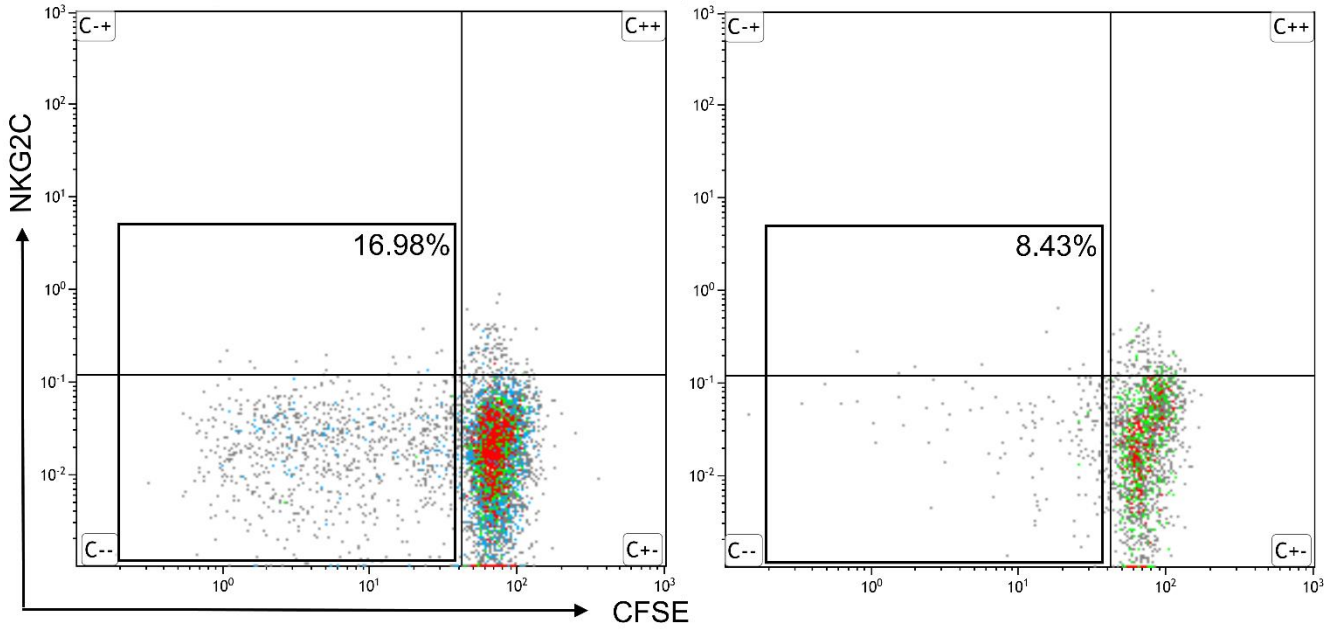


**Figure 3.1.3 HCMV-seronegative donor preferentially expanding against uninfected MRC-5.** After 14 days co-culture, proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ and $NKG2C^{-}$ cells is assessed by CFSE fluorescence intensity dilution. Proliferated $CFSE^{low}$ NK cells in the uninfected (left) and infected (right) co-cultures are highlighted within the black squares, and annotated numbers represent relative proportions of proliferating NK cells.

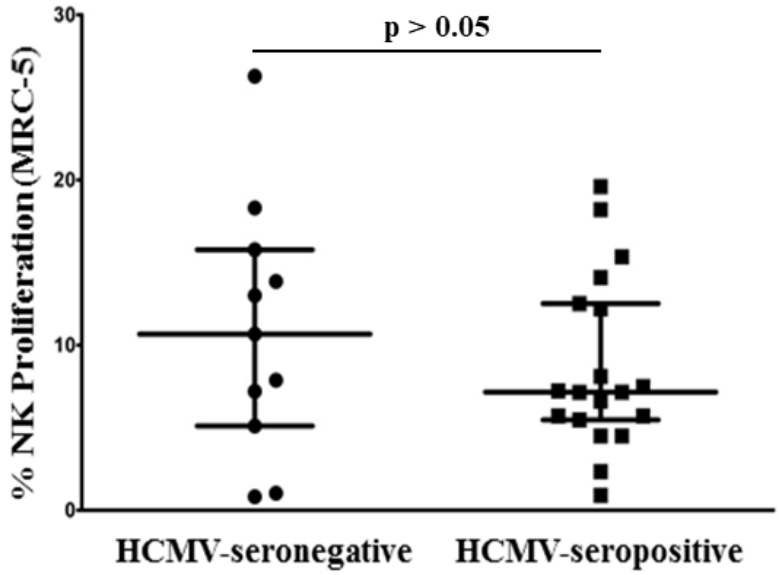


**Figure 3.1.4 NK-cell proliferation in response to MRC-5 cells.** Proliferation of NK cells from 19 different HCMV-seropositive (right) and 11 seronegative individuals (left) in response to co-culture with healthy MRC-5 cells. Horizontal lines bisecting the groups represent mean values for the groups with 95% confidence intervals shown above and below (mean % proliferating NK = 10.90, 95% C.I. = 5.77-16.03 versus 7.88, 95% C.I. 4.90-10.85). Significance between means are shown above bars spanning the groups compared (p = 0.25) (Students *t*-test). Data are pooled from 5 experiments with 3 to 5 donor samples per experiment.

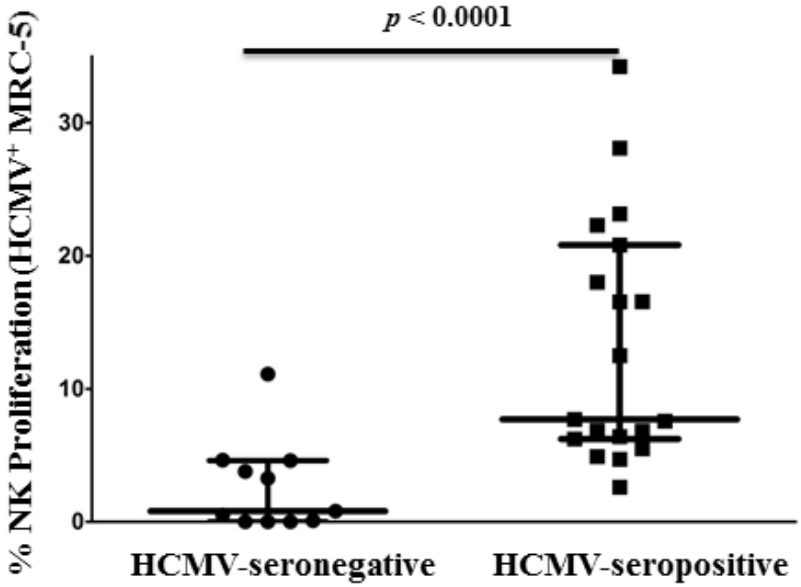


**Figure 3.1.5 NK-cell proliferation in response to HCMV-infected MRC-5 cells.** Proliferation of NK cells from 19 different HCMV-seropositive and 11 seronegative individuals in response to co-culture with uninfected MRC-5 cells was subtracted for each donor to derive net specific NK cell proliferation against HCMV infected MRC-5 cells. Horizontal lines bisecting the groups represent median values for the groups with interquartile range shown above and below compared (median % proliferating NK = 7.70, IQR 6.23-20.82 versus 0.80, IQR 0.00-4.60). Significant differences between medians are shown above bars spanning the groups (p = 0.0002) (Mann–Whitney test). Data are pooled from five experiments with three to five donor samples per experiment.

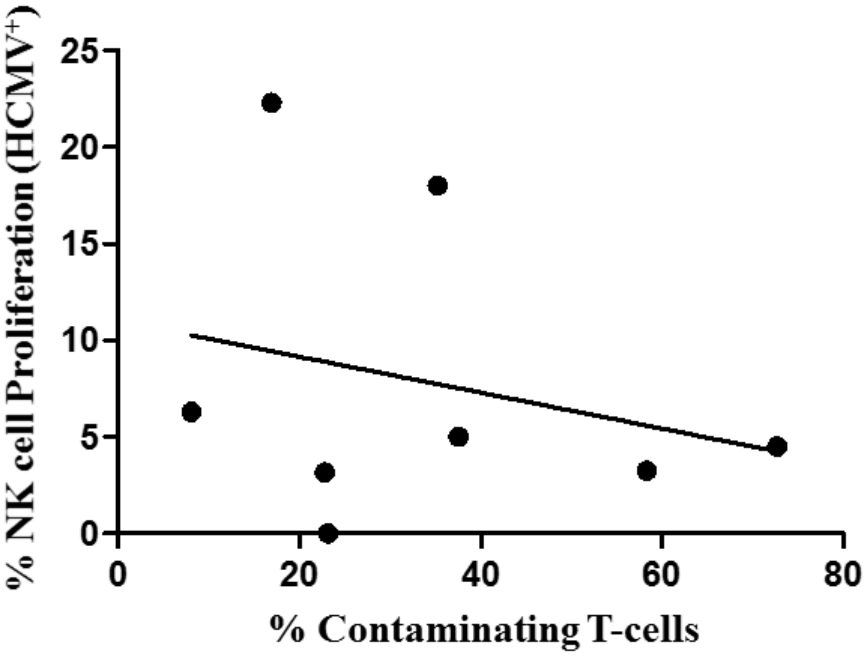


**Figure 3.1.6 Effect of contaminating T-cells on NK cell expansion following HCMV-co-culture.** Following 14 day HCMV co-culture, HCMV-seropositive donor cultures were quantified for contaminating fractions of $CD3^+$ cells. Relative proportions of T-cells were then correlated with relative proportions of expanding NK cells by spearman correlation to determine potential effect of HCMV-specific T-cells on NK cell expansion ( $r = -0.286$, $p = 0.501$).

### 3.2 Selective functional enhancement of NK cells from HCMV-seropositive donors.

Adaptive immunity involves the selective expansion of antigen-specific cell populations with concurrent acquisition of enhanced effector functions. To determine whether the selective proliferation of NK cells from HCMV-seropositive individuals co-cultured with HCMV-infected fibroblasts was reflected in enhanced function, non CFSE-labeled NK cell co-cultures were set up in parallel for 7 HCMV-seropositive and 7 seronegative individuals and direct cytotoxicity against K562 target cells was measured on day 14 of co-culture. Differences in cytotoxicity between NK cells cultured with uninfected versus HCMV-infected fibroblasts were somewhat variable, with lysis of K562 cells by NK cells from the majority of HCMV-seronegative individuals falling, by up to 27%. However, for two donors, K562 lysis increased by 9 and 27% respectively (Figure 3.2.0).

In aggregate, there was no statistically significant change in killing of K562 cells for the HCMV-seronegative group following co-culture with HCMV-infected fibroblasts versus co-culture with uninfected fibroblasts; $p > 0.05$ (Wilcoxon signed rank test) (Figure 3.2.0). In contrast, NK cells from HCMV-seropositive donors consistently increased their cytotoxicity against K562 cells following co-culture with HCMV-infected fibroblasts versus co-culture with uninfected fibroblasts (Figure 3.2.1). In 6 of 7 individuals tested, NK cell cytotoxicity increased by 4-46%, with NK cells from one donor showing a 9% decrease in cytotoxicity (Figure 3.2.1). Overall, killing of K562 by NK cells from HCMV-

seropositive individuals following co-culture with HCMV-infected cells was significantly increased over killing by NK cells co-cultured with uninfected cells; $p < 0.046$ (Figure 3.2.1). These data suggest that host infection with HCMV sensitizes NK cells to acquire enhanced cytotoxic function when exposed to HCMV-infected cells in vitro. Thus, NK cells from HCMV-infected individuals selectively demonstrate attributes of memory lymphocytes when exposed to HCMV-infected cells in vitro and tested for cytotoxic function.

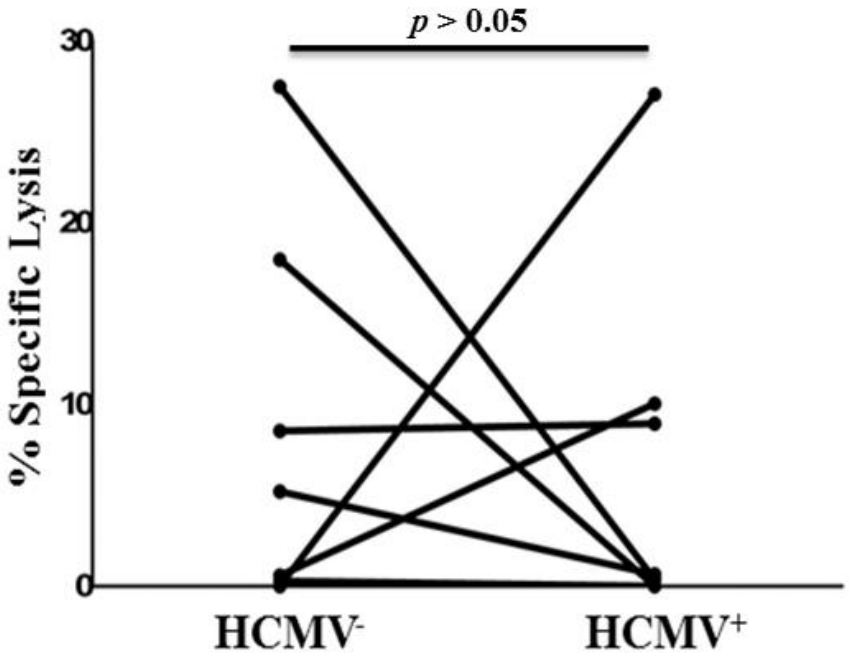


**Figure 3.2.0 Effect of HCMV co-culture on natural cytotoxicity in HCMV-seronegative donors.** Co-cultured cells from 7 HCMV-seronegative were collected, counted and tested at equal E:T ratios for killing of K562 cells after 14 days co-culture with either HCMV-infected or uninfected MRC-5 cells. Lines joining percent specific lysis values show the direction of change in cytotoxicity for each individual following co-culture with HCMV-infected (right column) or uninfected MRC-5 cells (left column). The significant difference in percent lysis between co-culture with uninfected versus HCMV-infected MRC-5 for the HCMV-seronegative donors is shown above the bar spanning the group values ($p = 0.0813$) (Wilcoxon signed rank test).

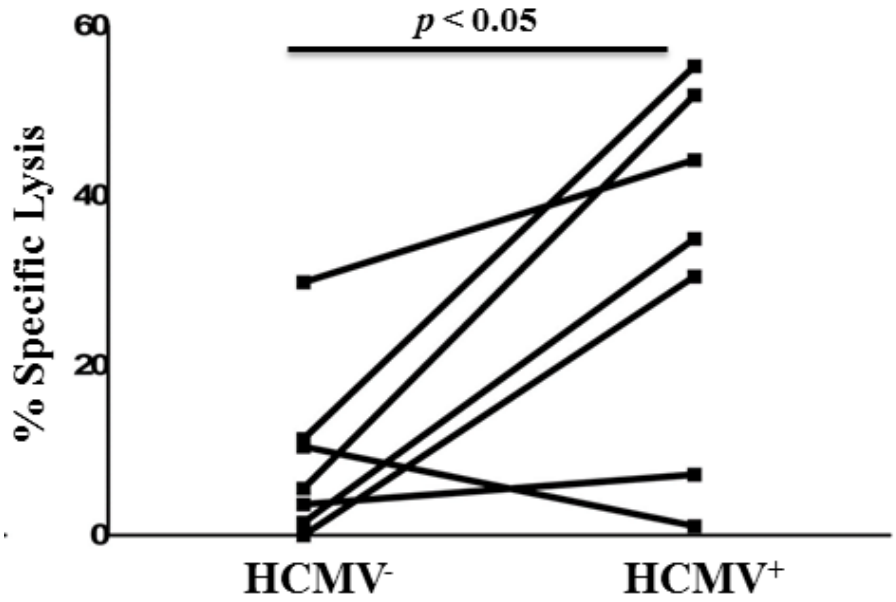


**Figure 3.2.1 Effect of HCMV co-culture on natural cytotoxicity in HCMV-seropositive donors.** Co-cultured NK cells from 7 HCMV-seropositive individuals were collected, counted and tested at equal E:T ratios for killing of K562 cells after 14 days co-culture with either HCMV-infected or uninfected MRC-5 cells. Lines joining percent specific lysis values show the direction of change in cytotoxicity for each individual following co-culture with HCMV-infected or uninfected MRC-5 cells. The significant difference in percent lysis between co-culture with uninfected versus HCMV-infected MRC-5 for the HCMV-seropositive donors is shown above the bar spanning the group values ($p = 0.047$) (Wilcoxon signed rank test).

### 3.3 NK cells proliferating in vitro in response to HCMV-infected MRC-5 selectively recognize HCMV-infected cells

To test whether NK cells proliferating in vitro in response to HCMV-infected MRC-5 cells selectively recognized HCMV-infected cells compared to the non-proliferating NK cells, we re-exposed NK cells co-cultured with HCMV-infected MRC-5 cells for 14 days to freshly infected HCMV-infected cells for 5 hours. Production of interferon-gamma (IFN-γ) was compared between NK cells that had undergone proliferation and those that had not proliferated (Figure. 3.3.0). In 8/8 cases tested, we observed selective IFN-γ production by NK cells that had undergone proliferation in response to in vitro exposure to HCMV-infected MRC-5 cells. A representative example is shown (Figure 3.3.1). Overall, a significantly higher fraction of the NK cells that had undergone proliferation produced IFN-γ when re-exposed to HCMV-infected MRC-5 cells (median % proliferating NK cells producing IFN-γ = 17.74, IQR 10.95-35.74 versus 3.30, IQR 1.86-4.81, p = 0.0002) (Mann-Whitney test). For 6 HCMV-seropositive individuals tested, no NK cells produced IFN-γ when freshly-isolated.

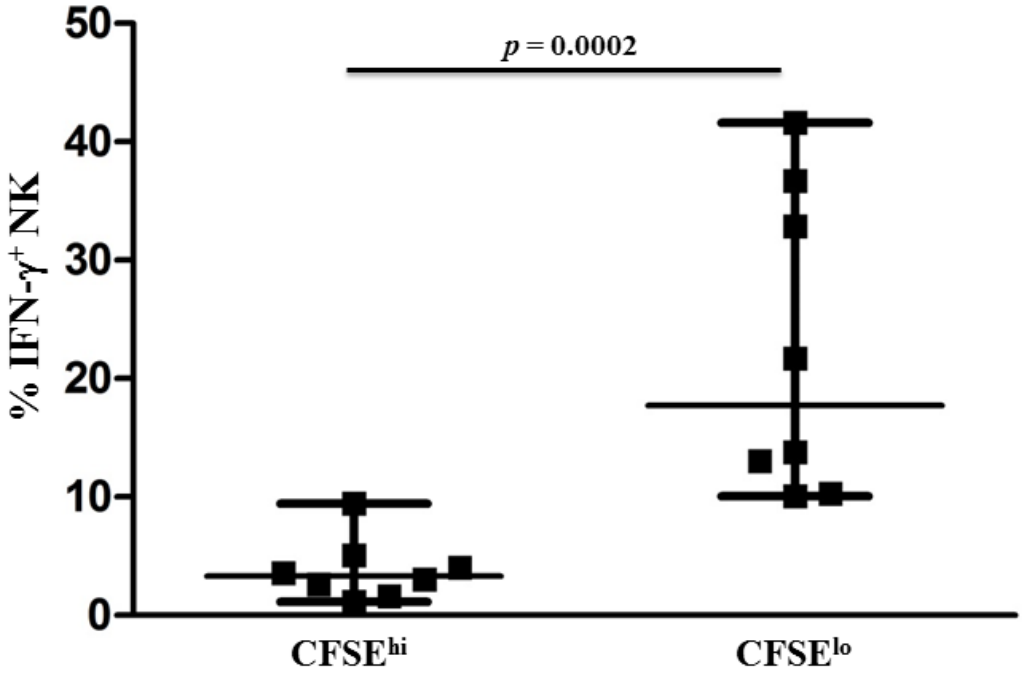


**Figure 3.3.0 Effect of re-exposure to HCMV-infected MRC-5 cells on proliferated NK-cell IFN-γ production.** Cells from 8 HCMV-seropositive individuals were collected after 14 days HCMV co-culture and transferred to freshly infected MRC-5 cells for 5 hours, after which IFN-γ production by proliferating ($CFSE^{lo}$) and non-proliferating ($CFSE^{hi}$) NK cells was assessed. Horizontal lines bisecting the groups represent median values for the groups with interquartile range shown above and below (median % proliferating NK cells producing IFN-γ = 17.74, IQR 10.95-35.74 versus 3.30, IQR 1.86-4.81). Significant differences between medians are shown above bars spanning the groups compared (Mann-Whitney test) ($p = 0.0002$).

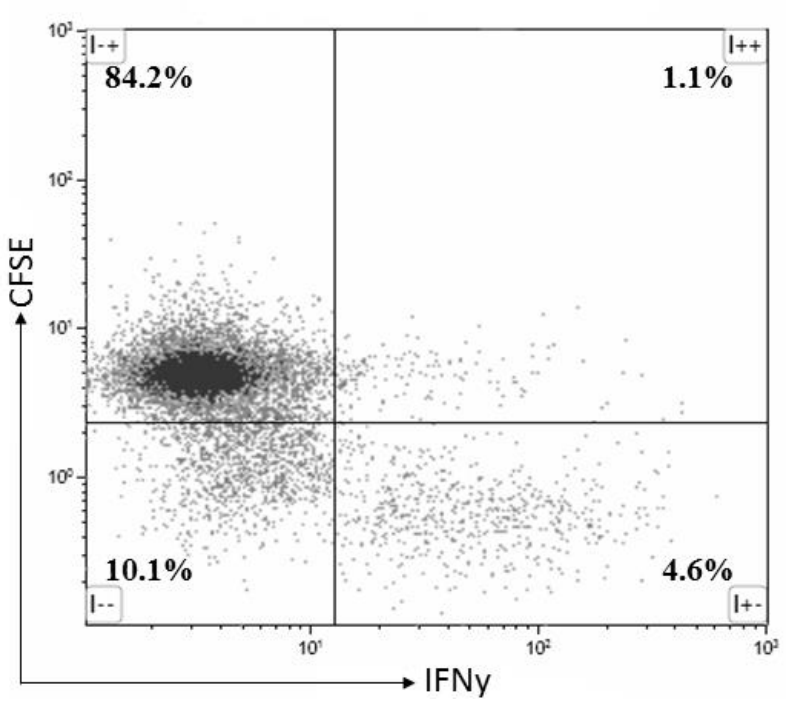


**Figure 3.3.1 IFN-γ production by proliferating and non-proliferating NK cells following re-exposure to HCMV-infected cells.** Cells from HCMV-seropositive individuals were collected after HCMV co-culture and re-exposed to freshly infected MRC-5 cells, after which IFN-γ production by proliferating ($CFSE^{lo}$) and non-proliferating ($CFSE^{hi}$) NK cells was assessed. General gating strategy was as described in figure 1A with the addition of anti-IFN-γ. Relative percentages of gated cells are annotated within each quadrant of the plot.

### 3.4 NKG2C$^+$ and NKG2C$^-$ NK proliferate in response to HCMV-infected fibroblasts in vitro.

Since previous reports of NK cell proliferation in response to HCMV-infected cells in vitro focused on NKG2C$^+$ NK cells from HCMV-seropositive donors, we measured the fraction of proliferating NK cells from HCMV-infected donors that expressed NKG2C in our system. In 14 HCMV-seropositive subjects tested, the percentage of proliferating NK cells expressing NKG2C ranged from 0-83% (Figure 3.4.0). With two exceptions, the majority of NK cells proliferating in response to HCMV-infected cells did not express NKG2C in our in vitro system. An anecdotal example of an HCMV-seropositive donor eliciting little NKG2C$^+$ proliferation is depicted in figure 3.4.1, and an example with most responding cells expressing NKG2C in figure 3.4.2.

As the individual with the highest fraction of proliferating NK cells expressing NKG2C also had the highest fraction of circulating NK cells expressing NKG2C, we evaluated the correlation between circulating and proliferating NK cell fractions expressing NKG2C. Figure 3.4.3 shows the significant correlation observed, $r = 0.67$, $p = 0.009$ (Spearman's rank correlation co-efficient). This indicates that expansion of NKG2C$^+$ NK cells in response to HCMV-infected cells in vitro occurs proportionately to the size of the starting NKG2C$^+$ NK cell population. There was no significant correlation between the fraction of circulating NK cells expressing NKG2C and the total percentage of NK cells proliferating in vitro in response to HCMV-infected cells. Together, these data suggest

that despite the in vivo accumulation of $CD57^{+}NKG2C^{+}$ NK cells associated with HCMV infection, NKG2C is not necessary for selective in vitro proliferation of NK cells from HCMV-seropositive subjects in response to HCMV-infected cells.

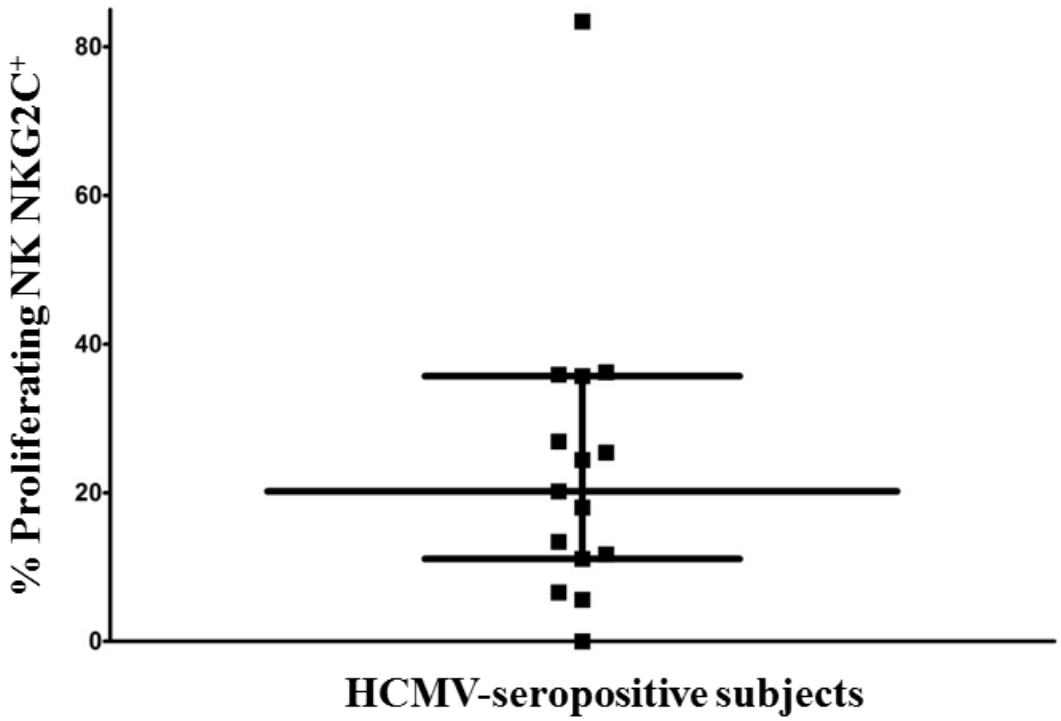


**Figure 3.4.0 Effect of HCMV co-culture on the proliferation of NKG2C+ NK cells.** NK cells from HCMV-seropositive donors were harvested from 14-day HCMV-co-culture, and proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ cells was assessed by flow cytometry. Depicted are the relative fractions of proliferating $NKG2C^{+}$ NK cells for 14 HCMV-seropositive subjects with a horizontal bar bisecting the group representing the median and interquartile range shown above and below (Median 21.7, IQR 11.79-38.82).

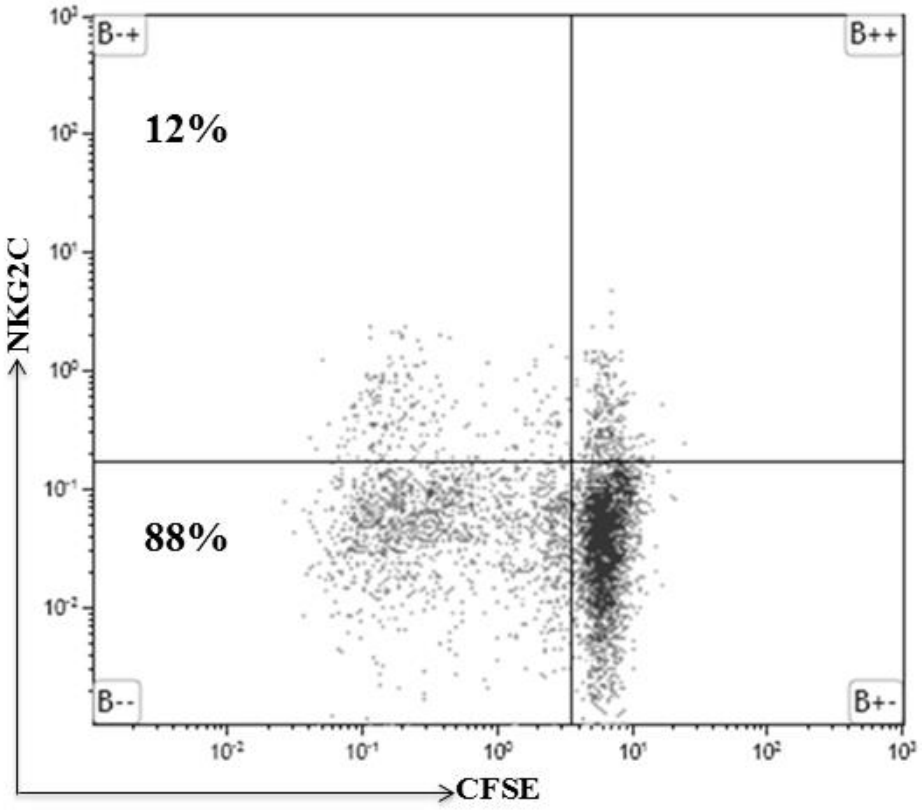


**Figure 3.4.1 Preferential expansion of NKG2C$^-$ NK cells in response to HCMV infected MRC-5.** Proliferation of CD3$^-$CD56$^+$ NKG2C$^+$ cells was assessed following 14 day HCMV-co-culture. Depicted above is a representative example showing HCMV-seropositive individual with low (12%) proportions of NKG2C$^+$ cells among NK cells proliferating in response to HCMV-infected MRC-5 cells.

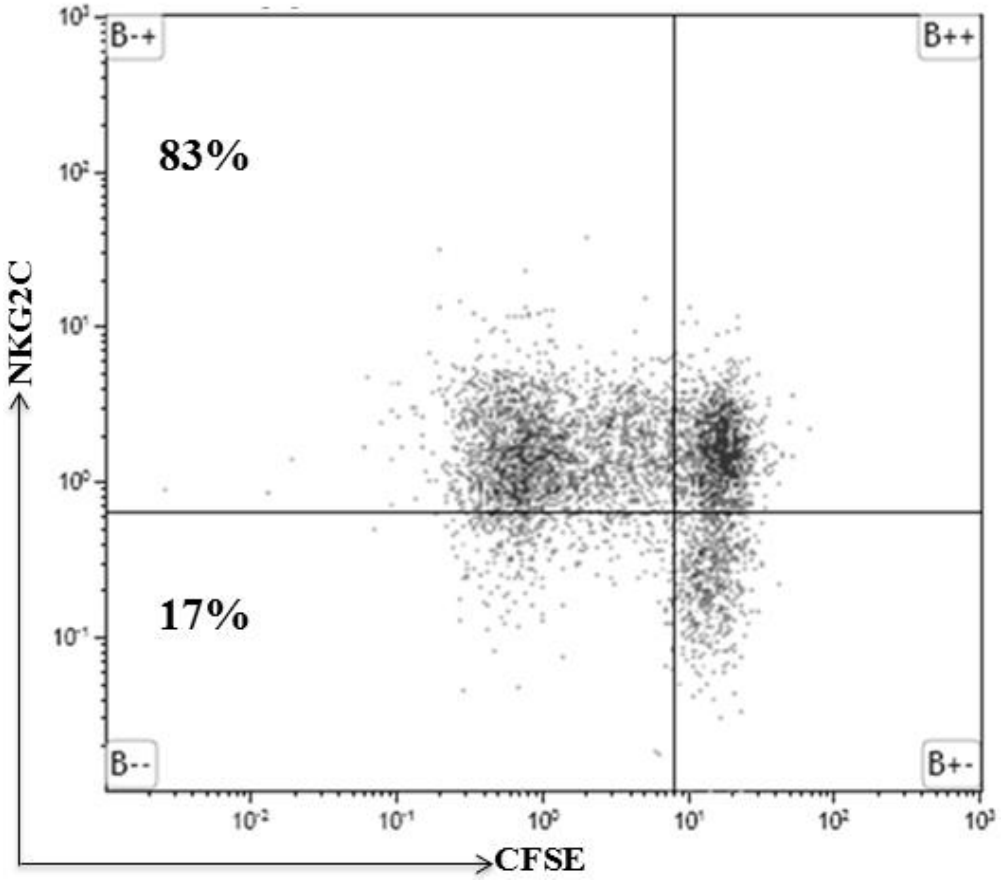


**Figure 3.4.2 Preferential expansion of NKG2C+ NK cells in response to HCMV infected MRC-5.** Proliferation of $CD3^{-}CD56^{+}$ $NKG2C^{+}$ cells was assessed following 14 day HCMV-co-culture. Depicted above is an example showing a donor with high (83%) proportions of $NKG2C^{+}$ cells among NK cells proliferating in response to HCMV-infected MRC-5 cells.

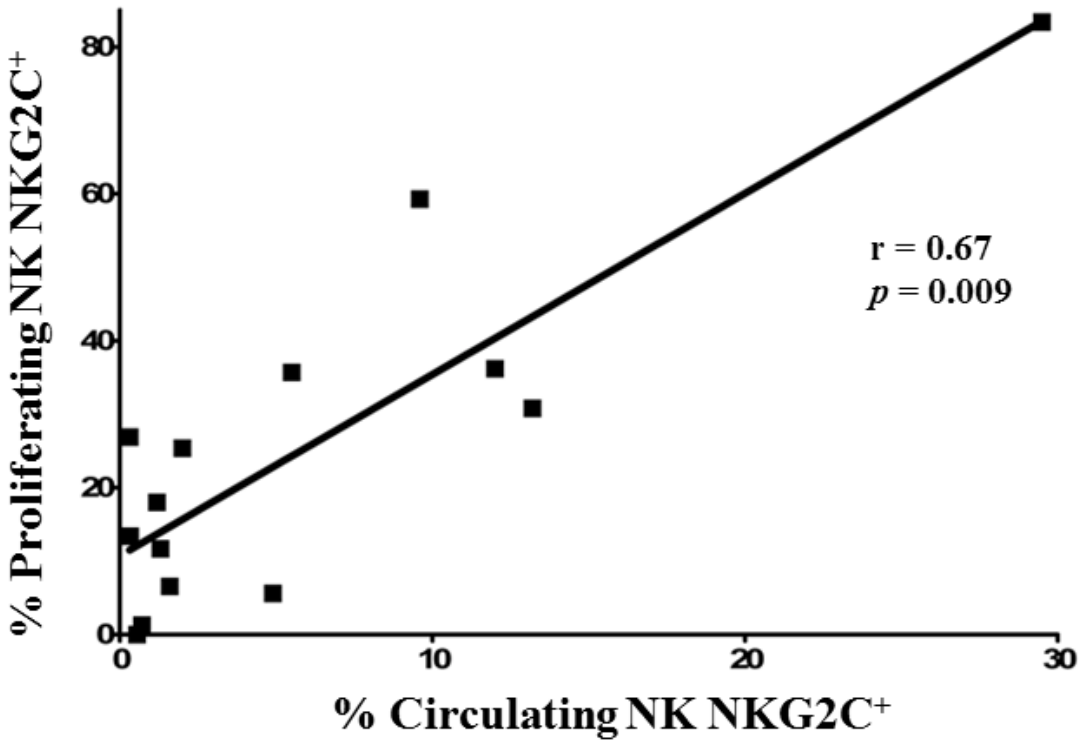


**Figure 3.4.3 Correlation between fractions of NKG2C$^+$ NK cells in peripheral blood and in NK cells proliferating in response to HCMV-infected cells.** NK cells from HCMV-seropositive donors were harvested from 14-day HCMV-co-culture, and proliferation of CD3$^-$CD56$^+$ NKG2C$^+$ cells was assessed by flow cytometry. Relative proportion of proliferating NKG2C$^+$ NK cells was correlated with relative in vivo fractions of NKG2C$^+$ NK cells measured prior to co-culture. The correlation coefficient (r) and probability of significant correlation (Spearman's rank correlation co-efficient) are shown within the plot frame.

# Chapter 4: Discussion

## 4.1 Research summary

By comparing the selective proliferation of NK cells from HCMV-seropositive versus seronegative individuals in response to HCMV-infected or uninfected fibroblasts, we found evidence of a memory-type NK cell response against HCMV-infected cells. A significantly higher percentage of NK cells from HCMV-seropositive subjects proliferated against HCMV-infected cells and NK cells from HCMV-seropositive subjects were significantly more likely to increase their cytotoxicity against K562 target cells following co-culture with HCMV-infected cells. In addition, a significantly greater fraction of the NK proliferating than non-proliferating in response to HCMV-infected cells in vitro produced IFN-γ when re-exposed to HCMV-infected cells. These features characterize classical secondary immune responses detected in vitro following in vivo priming of the immune system through infection, vaccination or other forms of antigenic exposure. While our findings are consistent with previous observations of selective expansion of an $NKG2C^+$ NK cell subset in individuals infected with HCMV, the in vitro proliferative response against HCMV-infected cells we observed was predominantly independent of NKG2C expression. The in vivo selective expansion of $NKG2C^+$ NK cells in HCMV-infected individuals is paralleled in the in vitro expansion systems used by several groups showing that this subset does proliferate in response to HCMV-infected cells and responds robustly against HCMV-infected cells via antibody-dependent cytokine responses in vitro

[123, 130]. Since the NKG2C subset rarely expands to substantial levels in HCMV-seronegative individuals, it was not feasible to compare proliferation of NKG2C$^+$ NK cells from HCMV-seronegative subjects in response to HCMV-infected cells in vitro. However, we did not observe a significant correlation between the overall extent of NK cell proliferation against HCMV-infected cells and the percentage of circulating NK cells expressing NKG2C in HCMV-seropositive responders. Despite the clearly selective expansion of NKG2C$^+$ NK cells associated with HCMV infection in vivo, our in vitro system, which recapitulated the classical elements of immune memory, indicates that NK cells can respond robustly against HCMV-infected cells independently of NKG2C expression.

**4.2 Comparison with the literature**

Our findings do not necessarily conflict with previous studies focused on the NKG2C$^+$ NK cell subset, but likely reflect application of different in vitro co-culture conditions and choice of subjects. We depleted T cells and added no exogenous cytokines to co-cultures, which potentially limits NK cell expansion. Several previous studies described marked increases in NKG2C$^+$ NK cells, to the point where they outnumbered the NKG2C$^-$ NK cell population by day 10-12 of co-culture [123, 124]. This rarely occurred in our system, possibly due to the lack of exogenous interleukin (IL)-2, as expansion of NKG2C$^+$CD57$^+$ NK has been linked to monocyte produced IL-

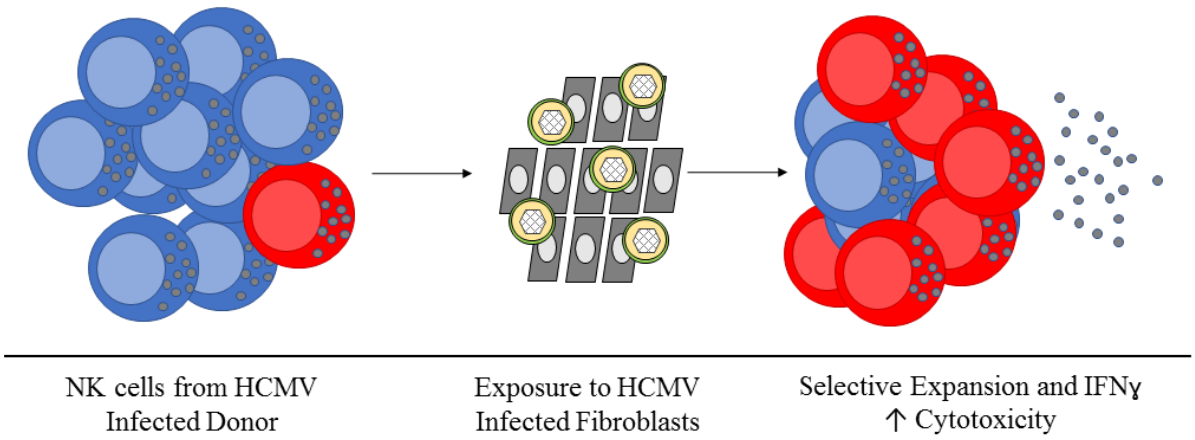


**Figure 4.0 NK cell resposes of HCMV-seropositive donors following HCMV co-culture.** After 14 day exposure to HCMV infected MRC-5 fibroblasts, NK cells from HCMV-seropositive donors selectively expand and elicit elevated cytotoxicity against MHC-I deficient targets. In addition, significantly greater fractions of proliferated NK produce IFN-γ when re-exposed to HCMV-infected cells compared to bystander NK. These features characterise classical secondary immune responses, but are recapitulated by NK cells using our system.

12, which sensitizes NKG2C$^{+}$CD57$^{+}$ NK cells to IL-2 by inducing CD25 expression [124]. This phenomenon paired with HLA-E engagement on infected cells by NKG2C without its inhibitory counterpart NKG2A, a hallmark of this NK cell population, may selectively expand NKG2C$^{+}$CD57$^{+}$ NK cells *in vitro* [131]. In this respect, previous studies have found that HLA-E expression on infected fibroblasts contributed to NKG2C$^{+}$CD57$^{+}$ NK expansion in vitro [123, 124]. While antibody blockade of NKG2C or its HLA-E ligand reduced NK cell proliferation in vitro, the molecular basis for specific NKG2C/HLA-E interactions contributing to the NK cell response against HCMV infection remains unspecified [123, 124].

The NKG2 ligand, HLA-E, presents leader peptides from classical MHC class I molecules to NK cells [132, 133]. Subtle differences in HLA-E bound peptides can lead to NK cell activation, thus, it is conceivable that HCMV peptides are recognized by NKG2C via HLA-E presentation, thereby promoting preferential expansion of NKG2C$^{+}$ cells [125, 126, 134, 135]. Certain HCMV derived peptides loaded on HLA-E do promote degranulation of NK cells bearing NKG2C, in the absence of its inhibitory counterpart NKG2A, consistent with the predominant phenotype of functionally mature NKG2C$^{+}$CD57$^{+}$ NK cells [116, 117, 127, 136]. The cytokine environment and possibly the HLA-E/NKG2C stimulatory axis may be subdued in our in vitro system compared to others. In addition, our subjects were mostly young to middle-aged healthy adults with relatively low frequencies of NKG2C$^{+}$ NK cells. Most cases of pronounced NKG2C$^{+}$ NK

cell expansion in vivo occur against a backdrop of genetic, infectious or transplant-related immunodeficiency [118, 120, 137, 138]. Selection of NK cells for in vitro culture from individuals with high circulating frequencies of $CD57^{+}NKG2C^{+}$ NK cells, whether acquired acutely or through protracted processes, may skew results to represent a terminal rather than evolving process of NK cell adaptation to HCMV, similar to the accumulation of terminally differentiated $CD28^{-}CD57^{+}$ $CD8^{+}$ T cells specific for HCMV in old elderly and human immunodeficiency virus-infected subjects [138, 139]. In this context, the subject with the highest $NKG2C^{+}$ NK cell response in our system was the oldest individual in our study and also had the highest circulating $NKG2C^{+}$ NK cell frequency.

The scarcity of robust NKG2C responses in our systems conforms with previous studies, which reported robust $NKG2C^{+}CD57^{+}$ proliferation in only half of donors tested [123, 124]. Variable responses between HCMV-seropositive donors may be explained by the distribution of differentially expressed killer-cell immunoglobulin-like receptors (KIR) on human NK cells. Inhibitory KIR are highly polymorphic and stochastically expressed, which generates many NK subsets with differing inhibitory thresholds that likely affect subset expansion in our system. With regards to $NKG2C^{+}CD57^{+}$ NK, there is evidence that this subset expresses inhibitory KIR for self MHC-I, which may lower inhibitory signaling when MHC-I alleles expressed on MRC-5 differ from the host [117].

Perhaps the strongest evidence for an NKG2C-independent component or stage in the NK cell response to HCMV is HCMV infection-associated maturation of NK cells from NKG2C$^{null}$ individuals. Adaptive cells in the NK cell populations of HCMV-infected individuals have been defined by exclusive or additive loss of FcεRγ1, SYK kinase and Ewing's sarcoma's/FLI1-activated transcript 2 (EAT-2) adaptor proteins, or the transcription factor promyelocytic leukaemia zinc finger protein (PLZF) [140, 141]. Absence of these proteins actually signifies NK cell acquisition of adaptive qualities such as superior proliferation upon engagement of intracellular tyrosine activation motif (ITAM) coupled receptors and enhanced cytokine responses [140-142]. There is significant overlap between the population of CD57$^{+}$NKG2C$^{+}$ and NK cells lacking these proteins, as illustrated in figure 4.1, which may relate to their robust in vivo and in vitro expansion in certain subjects with elevated fractions [140]. Individuals lacking a functional NKG2C gene undergo HCMV driven NK cell maturation, illustrated by analogous expansion of CD57$^{+}$NKG2A$^{-}$ NK cells and the aforementioned HCMV-associated adaptive NK cells [143, 144]. Conversely, one study did find HCMV-related NK maturation to be delayed in NKG2C$^{null}$ children and there is evidence of NK cell compensation for NKG2C deficiency by up-regulation of CD2 [145, 146]. Thus, even if NKG2C is at least partially dispensable for the NK cell response to HCMV infection, it appears to confer a selective advantage reflected by in vivo and in vitro enrichment for NKG2C$^{+}$ NK cells.

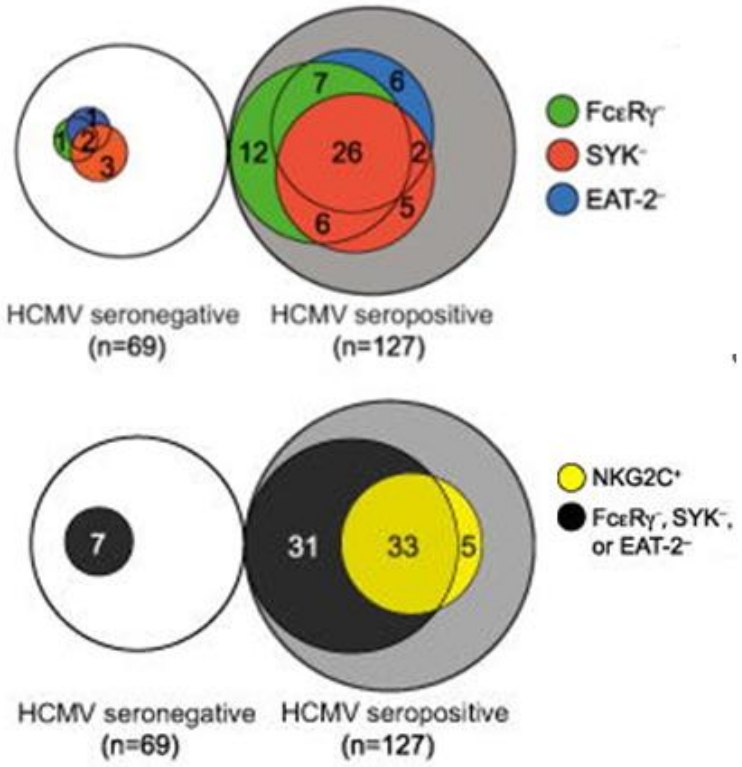


**Figure 4.1 Overlap of NK cell fractions with HCMV driven protein deficiencies.** HCMV has been shown to drive additive or exclusive loss of the adaptor proteins FcεRγ1, SYK kinase, and EAT-2, as well as the transcription factor PLZF. Such deficiencies are associated with the acquisition of adaptive qualities in NK cells, and have significant overlap with the $CD57^{+}NKG2C^{+}$ NK cell population. This may relate to the durable expansion of $NKG2C^{+}$ NK cells in persons with HCMV. *Figure adapted from "Cytomegalovirus infection drives adaptive epigenetic diversification of NK cells with altered signaling and effector function", Schlum et al.*[137].

### 4.3 Research limitations

Despite careful attention to detail and planning during the execution of this study, there are, as with all studies, some limitations that dampen the ability to draw firm conclusions regarding NK cell adaptive responses using our system. Firstly, we used magnetic separation for T-cell depletion, a cost effective and efficient method of bulk removal. However, the maximum effective depletion lies just below 100%, therefore T-cell contamination can be unavoidable without further intervention. This presents the possibility of HCMV-specific T-cells from seropositive donors affecting NK cell proliferation and function through the cytokine responses in vitro. Reducing the seriousness of this issue, few cultures contained detectable levels of T-cell contamination, and the proportions of T-cells in cultures that did had no measurable effect on NK cell proliferation as illustrated by figure 3.6. Nonetheless, this issue should not be disregarded, and requires further experimentation to draw firm conclusions regarding these adaptive NK cell responses.

A second limitation stems from the use of the HCMV lab strain AD169, which has been extensively passaged, and consequently has suffered substantial deletions throughout its genome. This includes a 15kb deletion containing 19 open reading frames within HCMV's long unique region ($U_L/b'$) otherwise present in clinical isolates [147, 148]. Absence of these genes allow AD169 to be permissive in human fibroblasts cell lines, but retards replication in endothelial and epithelial cells, the primary sites of replication in vivo [149, 150]. These genes are essential for the persistence of HCMV in the host, and as a result AD169 does not replicate in vivo, even in individuals undergoing immunosuppressant therapy [151-154]. Evidently, some researchers question the validity

of AD169 as a model virus for HCMV. While this could be problematic when studying the characteristics of HCMV, our work is geared toward elucidation of adaptive NK cell responses using AD169 as a tool. However, the genetic abnormalities of AD169 may relate to the limited NKG2C$^{+}$ NK cell responses *in vitro*. It is conceivable that these cells may recognize a viral ligand expressed by clinical strains of HCMV, but deleted from the highly passaged AD169.

Another potential limitation stems from the potential confounding variable of education/HLA background of the NK cell donors. It is important to note that preferential expansion of NK cells from specific donors could be altered based on their individual KIR phenotypes by lacking inhibitory receptors or possessing activating receptors for HLA expressed or downregulated on HCMV-infected MRC-5. Evidently, to fully interpret and analyze our findings it would be important to asses donor KIR profiles and the expression of HLA and other KIR ligands on MRC-5 cells with and without infection.

### 4.4 Future Directions

Future directions for this research should center around understanding the underlying mechanism of these adaptive NK cell responses. For example, are these memory like responses driven by an HCMV induced ligand, or by recognition of a viral antigen by a currently undefined NK cell receptor, or perhaps by simple selection process. Uncovering such mechanisms would redefine NK cells as an immune cell, and would undoubtedly present opportunity for exploitation in vaccine development and treatment of cancer.

While we found variable responses from NKG2C$^+$ NK cells, this population provides some selective advantage as reflected by in vivo and in vitro enrichment. Therefore, defining the precise phenotype of these cells and comparing them with those eliciting memory-like responses using our system may be useful in identifying receptors involved. However, fractions of NKG2C$^+$CD57$^+$ NK cells are generally very small in healthy individuals, thus acquiring a reliable sample size for accurate flow cytometry is difficult. To circumvent this issue, we would look to donors coinfected with HIV and HCMV, as such donors have exaggerated fractions of NKG2C$^+$CD57$^+$ NK cells, sometimes dominating 75% of the NK cell compartment. This anomaly is presumably a compensatory mechanism to control HCMV infection in the setting of compromised adaptive immunity [138].

Because KIR profiles are implicated in antiviral NK cell responses, and this is the closest receptor family to Ly49 in mice, initial phenotyping should focus on this system. The first step in this process would be to define donors with the largest NKG2C$^+$CD57$^+$ NK cell fractions by flow cytometry. Once these individuals are selected, each donor would be genotyped for their specific KIR profile using commercially available PCR based typing kits. Using this information, individual fluor panels using anti-KIR antibodies would be tailored to each donor, and their NK cells analyzed by flow cytometry. These data would then be used to determine trends in KIR related to HCMV induced expansion of NKG2C$^+$CD57$^+$ NK cells, which could then be compared to the responding cells in our system. Additionally, because KIR are involved in NK cell education further genotyping of donor MHC-I loci, the putative ligand of KIR, could be used to determine the

educational state of these cells. This may be useful considering uneducated NK cells have been found more effective at viral clearance of MCMV in mice [44].

A second future direction would be to address the limitations regarding the use of AD169 as a model virus of HCMV. This would be accomplished by repeating co-cultures using a clinical strain of HCMV such as Toledo, FIX, PH, or TR, which have been passaged to a limited extent in the laboratory and therefore have complete HCMV genomes [148]. However, clinical strains are notoriously difficult to grow in vitro due to limited replication in fibroblasts, and limited cytopathic effect. If these viruses prove unproductive it would be possible to control for the deleted $U_L/b'$ region in AD169 by using fresh isolates of the HCMV strain Merlin. While Merlin is a laboratory strain like HCMV, the viral DNA was promptly inserted into a bacterial artificial chromosome (BAC) upon its clinical isolation. Evidently, Merlin can be stably cloned in e. coli and transfected to produce low passage virus with little genetic variation from wild-type HCMV, including an intact $U_L/b'$ region [155].

## 4.5 Concluding Remarks

Selectively augmented proliferation, cytotoxicity and IFN-γ production by NK cells from HCMV-seropositive donors in response to HCMV-infected cells in vitro are hallmarks of a secondary immune response. Despite the pronounced expansion of $NKG2C^{+}$ NK cells in association with HCMV infection in vivo and in response to HCMV-infected cells in other in vitro systems, we observed predominantly NKG2C-independent NK cell responses in vitro. This, coupled with studies of $NKG2C^{null}$ individuals, suggests that selective NK cell responses against HCMV can be mounted independently of NKG2C, but evolve under extreme conditions or over protracted periods towards $NKG2C^{+}$ dominance.

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