Adaptive NK cells:
Their development, role against viral infection and cytotoxicity

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Immunology

in the

OFFICE OF GRADUATE STUDIES

of the
UNIVERSITY OF CALIFORNIA

DAVIS

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2023

Natural killer (NK) cells are cytotoxic innate lymphoid cells that protect the host from pathogens. NK cells were classically considered part of the innate immune system, participating as a first-line defense against tumor and virus infected cells. However, recent studies have discovered a subset of NK cells with adaptive immune features. This novel subset of NK cells, characterized by downregulation of signaling molecules FcRγ and expression of NKG2C receptor on cell surface, demonstrated adaptive immune features such as clonal expansion, long-term persistence, and altered effector functions.

While recent research has highlighted the significance of adaptive NK cells, there is still limited understanding about how this adaptive NK cell pool is generated. Epidemiological analyses have indicated an association between the presence of adaptive NK cells in humans and seropositivity for human cytomegalovirus (HCMV). However, it has been demonstrated that not all HCMV seropositive individuals have adaptive NK cells, and the co-infection of humans with other viruses makes it difficult to discern the precise contribution of HCMV infection to the development and expansion of the adaptive NK cell pool. In Chapter 2, I investigated the impact of primary CMV infection on the generation of adaptive NK cell pool using rhesus macaque as an animal model. I found that a subgroup of rhesus macaques with naturally acquired rhesus cytomegalovirus (RhCMV) infection possessed FcRγ-deficient NK cells that resemble phenotypic and functional characteristics of human adaptive NK cells. These cells were not detected in specific pathogen free (SPF) animals, however, experimental RhCMV infection of the SPF animals led to the induction of FcRγ-deficient NK cells in a strain-specific manner. Serological analysis of non-SPF animals indicated that subclinical infections by other common viruses can contribute to the expansion of this adaptive NK cell pool.

NK cells have been shown to be a potential mediator during viral infections, including HIV. While their exact role during the course of HIV infection requires further investigation, recent research has provided insights regarding the impact of HIV infection on NK cell population. Studies have shown that HIV infection alters the distribution and function of NK cell subpopulations, particularly leading to the expansion of unusual CD56neg NK cells. In Chapter 3, I examined the CD56neg NK cell population in HIV-viremic patients, which was reported to be hypo-functional. I found that the majority of CD56neg NK cells found in HIV patients were deficient in the signaling adaptor FcRγ and were specialized for antibody-dependent effector functions. These FcRγ-CD56neg NK cells shared characteristics similar to the adaptive NK cells and were hypo-responsive to tumor or cytokine stimulation but highly responsive to HIV-infected cells in the presence of anti-HIV antibodies. This study suggests that despite the previous understanding of CD56neg NK cells as dysfunctional in HIV patients, they may be cytotoxically active during HIV infection and contribute during disease progression.

NK cells mediate their cytotoxicity by releasing lytic granules containing granzymes and perforin. Granzymes, expressed in both NK cells and cytotoxic T cells, are a family of serine proteases, which play a crucial role during the elimination target cells by inducing apoptosis. NK cells release several different types of granzymes during their degranulation, thus the killing of a target cell is likely a result of a combined action of different granzymes. Granzyme B (GrB) is the most abundant and well-characterized granzyme, and they were shown to cleave and activate caspases and other cellular proteins that regulate apoptosis. Although numerous studies have revealed the mechanism of GrB-induced cytotoxicity, it is still uncertain whether NK cell cytotoxicity absolutely requires the presence of GrB. In Chapter 4, we generated NK cells that lack the expression of GrB and perforin using CRISPR Cas9 to knockout their genes. These GrB

KO and perforin KO NK cells showed stable expression of activating surface receptors, however, GrB KO NK cells showed reduced expression of GrH and GrM compared to control KO. Importantly, I demonstrated that absence of GrB does not affect cytotoxicity of NK cells, while absence of perforin completely disabled NK cell cytotoxicity. These results imply that the role of GrB could be substituted by other types of granzymes. Furthermore, I propose using CRISPR Cas9 to generate specific knockouts of granzymes in human NK cells as a promising model for delineating the specific roles of each granzyme during the NK cell cytotoxicity.

Acknowledgement

I would like to express my sincere gratitude to all those who have contributed to the completion of this dissertation. First and foremost, I would like to thank my mentor Dr Sungjin Kim for his valuable guidance and support. His insightful comments, constructive feedback, and timely suggestions have been the driving force behind this work. Likewise, I would like to extend my gratitude to my committee members, Dr. Chris Miller and Dr. Dennis Hartigan O'Connor, who graciously devoted time to evaluate my research progress and shared their expertise. Next, I would like to thank every previous members of Kim lab, Dr. Jeannine Scott, Dr. Tianxiang Zhang, Dr. Tae Hyung Lee, Suyeon Hong, Helena Chang, Peter Park, and Weiru Liu for their training, valuable insights, and hand-on assistance. Lastly, I would like to express my thanks to my parents for their love and support.

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List of Abbreviations

g-NK	FcRγ-deficient NK
CMV	Cytomegalovirus
PBMC	Peripheral blood mononuclear cell
NKR	NK cell receptor
KIR	killer immunoglobulin-like receptors
NCR	Natural cytotoxicity receptor
MFI	Median fluorescence intensity
ITAM	Immunoreceptor tyrosine-based activation motif
SPF	Specific pathogen free
RhCMV	Rhesus cytomegalovirus
HIV	Human immunodeficiency virus
ART	Antiretroviral therapy

1. Research Background and Chapters Overview

1.1 Introduction to NK cells

Natural Killer (NK) cells are effector lymphocytes that play a crucial role in controlling both tumor growth and viral infections [1]. Initially described as granular lymphocytes with natural cytotoxicity against tumor cells, NK cells were later recognized as a distinct lymphocyte lineage with cytotoxicity and cytokine-producing effector functions [2-4]. Unlike T and B lymphocytes, which require prior exposure to specific antigens for activation, NK cells can be activated rapidly in response to signals from pathogens. NK cells mediate their effects by recognizing and killing target cells and producing immunoregulatory cytokines, such as IFN-g and TNF-a, which enhance the innate immune response and promote the subsequent adaptive immune response [5, 6].

NK cell activation is tightly regulated by range of germline-encoded inhibitory and activating receptors [7, 8]. Activation receptors, such as NKG2D and natural cytotoxicity receptors (NCR), recognize stress ligands or viral-associated molecules that are upregulated on the surface of target cells, enabling direct recognition and activation of NK cells [9, 10]. On the other hand, inhibitory receptors, such as killer immunoglobulin-like receptors (KIRs) and NKG2A, ensures self-tolerance while allowing robust response against virally infected cells or tumor cells that have downregulated major histocompatibility complex class I (MHC I) molecules [11, 12]. This interaction between inhibitory receptors and self-MHC molecules plays a crucial role during NK cell development, promoting their education and fine tuning the level of responsiveness [13, 14]. Additionally, NK cells can detect target-bound IgG antibodies via the CD16 receptor, triggering antibody-dependent cell-mediated cytotoxicity (ADCC) against the target cells [15]. This interaction leads to phosphorylation of the immunoreceptor tyrosine-based

activation motif (ITAM) domains of FcRγ and CD3z adaptor molecules associated with CD16, initiating a signaling cascade that results in killing of the antibody-coated target cell [16].

1.2 Adaptive memory-like NK cells

NK cells have a limited repertoire of germline-encoded pathogen-recognition receptors, lacking immunological memory [8]. Therefore, NK cells were regarded to be part of innate immune system, lacking adaptive immune features of T cells and B cells characterized by enhanced response upon rechallenge to pathogen/antigens. However, emerging studies have shown that NK cells can have adaptive memory-like features, such as clonal expansion, enhanced effector function, and longevity.

1.2.1 Adaptive NK cells in mice

First evidence of adaptive immune response from NK cells was observed in mice, where mice deficient in T cells and B cells developed antigen-specific memory to haptens [17]. In addition, cytokine-induced memory-like properties of NK cells were observed when mice NK cells became highly proliferative and robustly produced IFN-g following pre-activation with a combination of IL-12, IL-15, and IL-18 [18]. The first evidence of adaptive NK cells in relation to viral infection was also reported in mice infected with mouse cytomegalovirus (MCMV). In 2009, Sun and et al demonstrated that a subset of NK cells expressing the activating receptor Ly49H underwent clonal expansion following acute MCMV infection [19]. These Ly49H+ NK cells exhibited superior degranulation and cytokine production capabilities compared to naïve NK cells upon restimulation. This effect was dependent on the interaction between m157 glycoprotein expressed by MCMV-infected cells and the Ly49H receptor on NK cells [19].

1.2.2 Adaptive NK cells in human

Similar to the findings in mice infected with MCMV, recent studies have identified presence of adaptive NK cells in human in response to human cytomegalovirus (HCMV) infection. HCMV is a highly prevalent herpesvirus, with up to 70-90% of adults worldwide estimated to be infected [20]. HCMV can establish lifelong persistence in a healthy host, characterized by the presence of latent viral genomes that intermittently reactivate to produce infectious virus [21, 22]. While asymptomatic in healthy individuals, HCMV infection can lead to severe complications in individuals with weakened immunity, such as transplant recipients or those with HIV/AIDS [23]. Furthermore, individuals lacking NK cells may suffer from symptomatic and recurrent HCMV infection, highlighting the crucial role of NK cells in controlling this viral infection [24, 25].

In 2004, Gumá et al. published a study showing that healthy HCMV seropositive individuals possess high proportion of NKG2C+ NK cell population [26]. Further research demonstrated that NKG2C+ NK cells expand in response to HCMV-infected fibroblasts and are highly specific to the virus, with CD57+NKG2C+ NK cells expanding early after HCMV infection *in vivo* [27-29]. Multiple studies have suggested that HLA-E, a nonclassical human leukocyte antigens (HLA) molecule capable of binding to NKG2C and presenting peptides to NK cells, contributes to the expansion of NKG2C+ NK cells [30, 31]. In-depth characterization of NKG2C+ NK subsets in HCMV seropositive individuals has revealed altered expression of various NK receptors, including reduced levels of NKp46, NKp30, siglec-7, and CD161, acquisition of late differentiation markers CD57 and ILT2, and increased levels of CD2 and KIRs, some of which correlate with functional alterations [29, 32-34]. Consequently, the

NKG2C+ NK subsets developed upon HCMV infection were regarded as human adaptive NK cells.

While NKG2C+ NK cells have been shown to exhibit some adaptive immune features, the extent to which NKG2C can serve as a marker to distinguish adaptive NK cells is still unclear and requires further investigation [33, 35]. Notably, NKG2C- NK cells with phenotypic similarities to adaptive NKG2C+ NK cells have been observed in HCMV seropositive individuals [33], and in HSCT recipients undergoing acute HCMV infection, subsets of NKG2C- NK cells develop rapidly and display hallmarks of clonal expansion [35]. Moreover, there is currently no evidence of NKG2C receptor triggering NK cell effector functions against HCMV-infected cells, underscoring the need for further research in this area.

During our investigating NK cell responsiveness to tumor target and CD16 stimulation, our lab identified a novel subset of NK cells in healthy individuals that are deficient for FcRγ but express CD3ζ normally, the two signaling adaptor molecules associated with Fc receptor CD16 [36-38]. These FcRγ-deficient NK cells, termed g-NK cells, constituted as much as 90% of circulating NK cells, and displayed enhanced response upon CD16 stimulation but responded poorly towards tumor cells [36]. Further investigation revealed that the presence of g-NK cells is strongly associated with prior HCMV exposure [38]. Multiple transcription factors and signaling protein deficiencies were identified in g-NK cell population, including tyrosine kinase SYK deficiency that is associated with epigenetic modifications [37, 39]. Similar to NKG2C+ NK cell populations, phenotypic analyses of g-NK cells revealed low levels of both NKp46 and NKp30 and skewed expression of NK cell receptors, suggesting clonal expansion [36, 38]. The presence of g-NK cells persisted over time in healthy HCMV seropositive individuals, indicating longevity and self-renewal [38]. Importantly, g-NK cells showed robust effector response and

expansion capabilities when stimulated with HCMV infected target cells in the presence of HCMV seropositive antibody [37, 38]. This enhanced response and expansion were also observed from stimulation with flu-infected cells with presence of flu seropositive antibody, highlighting potent ADCC capability of g-NK cells. Upon conducting an in-depth analysis of population intersection between g-NK and NKG2C+ NK cells in healthy HCMV seropositive individuals, g-NK cells were shown to overlap significantly with NKG2C+ NK cells, and in most cases, encompassing the NKG2C+ NK cell population [38, 39]. Additional studies have demonstrated that the effector function of NKG2C+ NK cells is dependent to CD16 and antibody [40, 41]. These data indicate that the FcRγ deficiency can be used to identify adaptive NK cell population, providing a more accurate characterization of their enhanced ADCC functional capability.

Despite the intriguing adaptive immune features of g-NK cells, little is known about how this memory-like g-NK cell pool is induced and shaped within individuals. Epidemiological analyses have associated the presence of g-NK cells with HCMV seropositivity, but their specific role in the induction and expansion of g-NK cells has been difficult to determine due to natural exposure to numerous microbes and environmental agents, including other viruses. Moreover, g-NK cells have only been detectable in a subgroup of HCMV seropositive individuals, further complicating the puzzle. A controlled animal model is necessary to investigate the causal relationship between HCMV infection and the development of g-NK cells, as this would allow for the examination of primary CMV infection without the confounding influence of other viral infections. Attempts to induce FcRγ-deficient NK cells in mice through experimental infection with MCMV have been unsuccessful, leaving no current mouse model available for the study of g-NK cells [37].

1.3 Using rhesus macaque to study adaptive NK cells

Rhesus macaque is widely used non-human primate model in biomedical research, including studies of the immune system and infectious diseases [42]. Rhesus macaque share significant genetic and physiological characteristics with humans, providing an opportunity to conduct studies in a species that closely mimics human biology while still being maintained under laboratory conditions [43]. Rhesus cytomegalovirus (RhCMV) shares many biological and immunological characteristics with HCMV, including the ability to establish latent infection in host cells and to induce immune responses [44]. RhCMV infection, like HCMV, is endemic in both captive and wild populations of macaques, with seroprevalence rates reaching close to 100% by the age of one year [45, 46]. Importantly, availability of specific pathogen free (SPF) macaques, which the animals are bred separately to be free of multiple viruses including RhCMV, enables controlled examination of primary RhCMV infection without complications from other viral infections [47].

1.4 Role of Adaptive NK cells during HIV-1 infection

Human immunodeficiency virus (HIV) is a retrovirus that infects and weakens the immune system of an individual. HIV-1, the most common and widespread strain, primarily targets CD4+ T cells in the body, which can lead to the development of acquired immunodeficiency syndrome (AIDS) over time [48]. Once the virus enters the body, it begins to replicate inside the CD4+ T cells, gradually weakening the immune system and leaving the individual vulnerable to infections and illnesses. Although there is no cure for HIV-1, antiretroviral therapy (ART) can suppress the virus and prevent it from damaging the immune system, allowing people with HIV-1 to maintain good health.

Numerous studies have highlighted the significant role of NK cells as potential mediators of protection against HIV-1. During the acute phase HIV-1 infection, NK cells are among the first immune populations to expand and may be directly involved in preventing virus replication and disease [49, 50]. Moreover, examination of NK cells from HIV-1 positive slow progressors have linked beneficial outcomes with specific KIR present on NK cells and the human leukocyte antigen-class I molecules (HLA) on target cells [51-53]. Specifically, activating NK cell KIR receptor, KIR3DS1, in the presence or absence of its ligand HLA-Bw4, is associated with slower disease progression and increased resistance to HIV-1 infection [51]. Furthermore, HIV vaccine study has identified the critical importance of as a pathway to protective vaccine responses, primarily mediated by NK cells via non-neutralizing antibodies [54]. These ADCC responses were associated with specific immunoglobulin subclasses and glycan modifications that directly impact the efficiency of NK cell responses [55, 56].

Better understanding of adaptive NK cells has led to investigation of their involvement during HIV infection and disease progression. Since CMV co-infection is near universal in HIV infected cohorts, CMV reactivation may have a strong influence on shaping adaptive NK cell repertoire during chronic HIV infection. As expected, NK cells lacking FcRγ was shown to be expanded in chronically infected HIV patients with enhanced ADCC activity, resembling the adaptive g-NK cell population [57]. Furthermore, the presence of NKG2C+CD57+ NK cells inversely correlated with HIV viral load and better early response to ART, while patients with NKG2C gene deletion showed higher HIV susceptibility and disease progression [58-60]. These investigations shed light on the potential involvement of adaptive NK cells in HIV pathogenesis and treatment, offering possible therapeutic strategies for HIV-infected individuals.

1.5 Role of granzymes during NK cell cytotoxicity

Cellular cytotoxicity, the ability to kill other cells, is a crucial effector mechanism employed by NK cells and cytotoxic T cells to combat cancer and virus-infected cells [61]. NK cells utilize two distinct pathways to exert their cytotoxic activity [62]. The first pathway involves releasing cytotoxic granules containing perforin and granzymes. The second pathway involves expressing TRAIL and/or Fas ligand (FasL) on their surface, which engages TRAIL-R1/-R2 or CD95/Fas, respectively, on the surface of target cells, inducing death receptor-mediated apoptosis. While granule-mediated apoptosis is a fast process, death receptor-mediated cytotoxicity requires more time.

Granzymes are a family of closely related serine proteases that are expressed in cytotoxic T cells and NK cells. The family of human granzymes consists of 5 members: granzyme A, granzyme B, granzyme H, granzyme K, and granzyme M [62]. NK cells release several different granzymes during their degranulation, so that the killing of a target cell is likely a result of a combined action of different granzymes. The process of granule-mediated cytotoxicity begins with the adhesion of the NK cell to the target cell via integrins, leading to the formation of the immunologic synapse [63]. Upon receiving signals from activation receptors, NK cell releases cytotoxic granules into the space between the NK cell and the target cell. These granules enter the target cell through pores created by perforin and activate the apoptotic pathway. As a result, granule-mediated apoptosis is a fast and effective process that eliminates the target cell within a few minutes.

Granzyme B is a major component of NK cell granules, and extensive research has elucidated the mechanisms of granzyme B-induced cytotoxicity. Granzyme B was shown to promote target cell death through various pathways [64]. One mechanism involves the direct

activation of caspases, where granzyme B cleaves and activates caspase-3 and caspase-7, leading to caspase-mediated degradation of numerous protein substrates and rapid apoptosis [65]. Additionally, granzyme B can facilitate apoptosis through mitochondrial outer membrane permeabilization, which is initiated by the proteolysis of BH3-only protein Bid [66]. This process leads to the oligomerization of mitochondrial membranes, facilitating the release of cytochrome c into the cytosol and the subsequent activation of the caspase cascade.

While the majority of research on granzymes has focused on granzyme B, studies have recently begun to uncover the potential roles of the other granzymes. For instance, Granzyme K has been shown to process similar substrates as Granzyme A, and Granzyme M has been demonstrated to promote caspase-independent cell death [67, 68]. Additionally, Granzyme H, which is present only in humans and not in mice, has been found to interfere with viral replication in Adenovirus-infected cells [69]. Overall, granzymes are a critical component of the NK cell response to viral infections and malignancies, and understanding the mechanisms by which granzymes induce apoptosis in target cells is essential for developing novel therapies for diseases.

1.6 Research Objective and Chapter Overview

Our understanding of adaptive NK cells has advanced considerably since their initial discovery, yet many questions remain regarding their origins and potential roles in disease progression. In my thesis work, I explored the following question: (i) Is CMV infection alone sufficient to induce the development of adaptive NK cells, or are other factors or infections also necessary for their generation? (ii) What role does adaptive NK cells play during disease progression, particularly in the context of HIV infection? (iii) What roles do granzymes play during NK cell mediated cytotoxicity and can their role be interchanged?

Although the presence of adaptive NK cells has been linked to HCMV seropositivity, the mechanism of how these adaptive NK cells are induced and shaped within individuals is not well understood. Due to natural exposures to numerous microbes and environmental factors, it has been challenging to determine the specific effect of primary HCMV infection. However, by utilizing rhesus macaque as an animal model, I was able to examine the causal relationship between CMV infection and adaptive NK cells. In Chapter 2, I present the findings from the experimental RhCMV infection of SPF animals, demonstrating the induction of FcRγ-deficient NK cells in a strain-specific manner.

HIV infection has been shown to change the distribution and functions of NK cell subpopulations, with a notable expansion of the functionally impaired CD56neg NK cells. In Chapter 3, I examined CD56neg NK cell population from acute HIV patients and found that majority of them are FcRγ-deficient NK cells. My findings reveal that FcRγ-deficient CD56neg NK cells exhibit phenotypic and functional characteristics that closely resemble those of the adaptive NK cell population. Importantly, I demonstrate that FcRγ-deficient NK cells present in CD56neg NK cells exhibit an enhanced capability for ADCC response, which contradicts previous beliefs that these NK cells are functionally impaired.

Granzymes play a pivotal role during NK cell-mediated cytotoxicity against target cells. Upon activation, human NK cells are capable of releasing five different types of granzymes, which trigger apoptosis in the target cells. In order to elucidate the distinct role of each type of granzyme in NK cell-mediated cytotoxicity, I employed CRISPR Cas9 technology to create granzyme B knockout NK cells. Chapter 4 of my study involved an investigation into the cytotoxic analysis of the granzyme B knockout NK cells, which was assessed by evaluating their ability to induce apoptosis in tumor target cells. My findings demonstrate that the absence of

granzyme B does not impact the cytotoxicity of NK cells, suggesting that granzymes may have an interchangeable function in the process of NK cell-mediated cytotoxicity.

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2. FcRγ⁻ NK cell induction by specific CMV and expansion by subclinical viral infections in rhesus macaques

2.1 Abstract

Long-lived 'memory-like' NK cells, characterized by FcRγ-deficiency and enhanced responsiveness to antibody-bound virus-infected cells, have been found in certain human cytomegalovirus (HCMV)-seropositive individuals. Because humans are exposed to numerous microbes and environmental agents, specific relationships between HCMV and FcRγ-deficient NK cells (also known as g-NK cells) have been challenging to define. Here, we show that a subgroup of rhesus cytomegalovirus (RhCMV)-seropositive macaques possesses FcRγ-deficient NK cells that stably persist and display phenotype resembling human FcRγ-deficient NK cells. Moreover, these macaque NK cells resembled human FcRγ-deficient NK cells with respect to functional characteristics, including enhanced responsiveness to RhCMV-infected target in an antibody-dependent manner and hypo-responsiveness to tumor and cytokine stimulation. These cells were not detected in specific-pathogen-free (SPF) macaques free of RhCMV and six other viruses; however, experimental infection of SPF animals with RhCMV strain UCD59, but not RhCMV strain 68-1 or SIV, led to induction of FcRγ-deficient NK cells. In non-SPF macaques, co-infection by RhCMV with other common viruses was associated with higher frequencies of FcRγ-deficient NK cells. These results support a causal role for specific cytomegalovirus strain(s) in the induction of FcRγ-deficient NK cells, and suggest that co-infection by other viruses further expands this memory-like NK cell pool.

2.2 Introduction

Although NK cells have been traditionally considered part of the innate immune system, several recent studies have revealed subsets of NK cells with adaptive immune features, including antigen-specific memory responses [1-8]. However, since NK cells express only germline-encoded receptors, they presumably cannot mount antigen-specific memory responses to diverse targets through direct recognition.

We discovered a distinct subset of human NK cells that displays adaptive immune features, including clonal-like expansion and long-term persistence [9-11]. This novel NK cell subset, named g-NK cells, is characterized by deficiency in FcRγ, a signaling adaptor normally associated with the IgG Fc receptor CD16, either as a homodimer or as a heterodimer with another adaptor CD3ζ [12]. The deficiency appears to result from DNA hypermethylation in the FcRγ gene, along with other epigenetic modifications [13], which are associated with altered expression of multiple proteins, including SYK tyrosine kinase. Importantly, human g-NK cells exhibit enhanced abilities to produce cytokines and expand in number upon interaction with cells infected with viruses (e.g., HCMV and influenza virus) in the presence of virus-specific antibodies via the action of CD16 [10]. Additionally, we have determined g-NK cells can constitute as much as 85% of circulating NK cells, thereby surpassing the number of circulating memory $CD8^+$ T cells in certain individuals [9, 11]. Therefore, g-NK cells have heightened potential for protective immune responses to a broad spectrum of viral infections through antibody-dependent memory-like effector functions [10, 14-17].

Despite their important adaptive immune features, little is known about how this memory-like NK cell pool is induced and shaped within individuals. To date, g-NK cells have been detected at a wide range of frequencies in approximately one-third of healthy people [9, 11, 14, 16, 18, 19].

Epidemiological analyses indicate that the presence of these cells is associated with seropositivity for HCMV [8, 9, 14]. However, because humans are naturally exposed to numerous microbes and environmental agents, including other viruses, and because g-NK cells were detectable in only a subgroup of HCMV-seropositive individuals, it has been challenging to determine the specific role HCMV might play in the induction and/or expansion of g-NK cells. Interestingly, HCMV-seropositivity has also been associated with 'adaptive' NKG2C^{+} NK cells in a manner similar to g-NK cells [20-24]. Establishing whether there is a causal relationship between HCMV infection and g-NK cell development will be an important step toward delineating the specific factors and processes by which this memory-like NK cell pool is induced and shaped through expansion. However, a recent attempt to induce FcRγ-deficient NK cells in mice by experimental infection with murine CMV was unsuccessful [10], leaving no current mouse model available for the study of g-NK cells.

In this study, we found that a subgroup of non-SPF rhesus macaques with naturally acquired RhCMV infection possessed FcRγ-deficient NK cells (hereafter called FcRγ^{-} NK cells) that displayed phenotypic and functional characteristics resembling human g-NK cells. Importantly, these cells were not detected in SPF animals. However, experimental RhCMV infection of SPF animals led to the induction of FcRγ^{-} NK cells in a strain-specific manner. Serological analysis of non-SPF animals revealed that subclinical infections by other common viruses can contribute to the expansion of this adaptive, memory-like NK cell pool.

2.3 Material and Methods

2.3.1 Ethics Statement

All studies conducted at the California National Primate Research Center (CNPRC) were approved in advance by the University of California Davis (UC Davis) Institutional Animal Care and Use Committee (approval numbers 16779, and 17880). UC Davis has an Animal Welfare Assurance on file with the National Institutes of Health Office of Laboratory Animal Welfare, and is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All animals had visual and auditory access to other macaques 24 hours per day, and were fed a balanced commercial macaque chow (Purina Mills, Gray Summit, MO) twice daily with fresh produce twice weekly, and free access to water 24 hours per day. Supplemental food was provided when clinically indicated. Environmental enrichment was provided daily, including manipulanda (forage boards, mirrors, puzzle feeders) and novel foodstuffs. When immobilization was necessary, animals were administered 10 mg/kg body weight ketamine-HCl (Parke-Davis, Morris Plains, NJ). The Guidelines for Humane Euthanasia of Animals on Projects (GHEAP) at the CNPRC were followed by the clinical veterinary staff to determine if euthanasia was indicated before the planned endpoint. GHEAP provides guidelines for selecting an endpoint that reduces animal pain and/or distress, while still meeting research objectives. All possible efforts were made to minimize pain and discomfort, including those associated with SIV-associated disease. SIV infection of susceptible macaques can produce a progressive fatal immunodeficiency disease characterized by hematologic abnormalities, lymphocyte depletion, diarrhea, weight loss and cachexia, and infection with opportunistic pathogens. Analgesics were given to minimize pain and discomfort at the discretion of the CNPRC veterinary staff and nutritional supplements were administered as necessary.

2.3.2 Rhesus Macaques and Viral Infections

SPF cohort of rhesus macaques (*Macaca mulatta*) were maintained as free of RhCMV, SFV, BV, RRV, SIV, SRV and STLV. For RhCMV infection study, a group of animals (n = 7, age of 3 – 4 yrs, median age 3 yrs, male and female) was subcutaneously inoculated with 10^3 PFU of $RhCMV_{UCD59}$. Another group of animals (n = 12, age of 3 − 7 yrs, median age 3.5 yrs, all female) was used in a SIV vaccine study. Animals received either $RhCMV_{68\text{-}1}$ or $RhCMV_{68\text{-}1}$-derived RhCMV/SIV recombinant viruses, including $RhCMV_{68\text{-}1Gag}$, $RhCMV68_{\text{-}1RTN}$ and $RhCMV_{68\text{-}1Env}$ (**49**) by a combination of subcutaneous inoculation (10^4 PFU) and oral exposure (10^5 PFU). Vaccination consisted of an initial priming at week 0 and two boosts at weeks 12 and 24. Starting on week 36, all animals received weekly low dose SIV challenges by the intravaginal route with SIV_{mac251} at a dose of 5×10^3 $TCID_{50}$ (0.54 ng/mL p27) until infected. Challenge SIV_{mac251} stock was kindly provided by N. Miller (NIH, Bethesda, MD) and Quality Biologics Inc. (Gaithersburg, MD). Blood samples collected from all animals were processed for plasma and peripheral blood mononuclear cells (PBMC) by Accu-Paque gradient centrifugation (Accurate Chemical & Scientific Corp., Westbury, NY) and cryopreserved at -80°C (plasma) or in liquid nitrogen (PBMC) for subsequent assessments.

2.3.3 Serology Screening

Plasma IgG to a panel of specific pathogenic agents (SIV, SRV, STLV, BV, RhCMV, RRV, SFV) was measured on the Luminex (Austin, Tx) liquid bead based array platform using multiplex microbead bead reagents obtained from Charles River Laboratories (Wilmington, MA). By measuring the spectral properties of the beads and amount of associated R-phycoerythrin (R-PE), the median fluorescence intensity (MFI) for each specific antigen was determined. The sample

MFI was compared against a previously validated known positive/negative cutoff value to qualitatively determine the presence or absence of antibody.

2.3.4 Antibodies and Reagents

The following antibodies were purchased from the indicated manufacturers and used for flow cytometry; BD Bioscience: anti-CD3ε (SP34-2); Miltenyi Biotec: anti-NKG2A (REA110); MBL: anti-FcεR1γ (1D6); Beckman Coulter: anti-NKp46 (BAB281); Biolegend: anti-CD3ζ (6B10.2), anti-CD8 (RPA-T8), anti-CD16 (3G8), anti-GrB (GB11), anti-NKp30 (P30-15), anti-PECAM (WM59), anti-CD49f (GoH3), anti-CD2 (RPA-2.10), anti-SYK (4D10.2), anti-TNF-α (Mab11) and anti-IFN-γ (B27).

2.3.5 Flow Cytometric Analysis of Rhesus NK cells

Rhesus PBMCs and cell suspensions prepared from tissues were stained for flow cytometric analysis using fluorochrome-conjugated antibodies as previously described [10]. Briefly, cell surface markers were stained with Abs and then fixed in 1.5% formaldehyde. To stain intracellular markers, samples were treated with permeabilization buffer containing 0.1% saponin, followed by staining of intracellular proteins. Analysis of PBMC samples collected from SPF animals with experimental viral infection was performed in a retrospective manner, while analysis of PBMC samples collected from non-SPF animals was performed in both retrospective and prospective manners.

2.3.6 Functional Analysis of Rhesus NK cells

Telomerized rhesus fibroblast (Telo-RF) [25] were cultured in 96-well plates, infected with $RhCMV_{68\text{-}1}$ (MOI = 1) for 2 hours, and then washed with PBS to remove unattached virus. At 3-4 days post infection, macaque PBMCs were added to the culture and incubated overnight in the

presence of recombinant human IL-2 (10 U/ml). Six hours prior to analysis, 1µl autologous plasma was added along with Brefeldin A for cytokine analysis. For tumor and cytokine stimulation assays, macaque PBMCs were co-cultured for 12 hrs with human tumor target cells (K562 or 721.221) at a ratio of 10:1 (E:T) or 24 hrs with cytokines (human IL-12, IL-15 and IL-18). Six hours prior to analysis, Brefeldin A was added for analysis of cytokine expression. For CD16 crosslinking assays, macaque PBMCs were stimulated for 6 hrs with plate-bound anti-CD16 (3G8) antibody along with Brefeldin A (**9**). Following stimulation, NK cells were identified as $CD3\varepsilon^{-}$ $CD14^{-}CD20^{-/dim}NKG2A^{+}$ cell population.

2.3.7 Statistics

Statistical analysis and graphing were conducted with Prism software (GraphPad Software, Inc., San Diego, CA). Wilcoxon matched-pairs signed-rank test was used for comparison of the frequency difference during NK cell functional assays. Nonparametric Mann-Whitney and chi-square tests were used for the difference in frequency and incidence of $Fc R\gamma^{-}$ NK cells between indicated groups, respectively. Correlation analyses were performed by Spearman's rank tests. Differences were considered significant when $p< 0.05$.

2.4 Results and Discussion

2.4.1 Identification and long-term persistence of FcRγ-NK cells in rhesus macaques

To explore a non-human primate model for the study of g-NK cells, we examined the expression of CD16-associated FcRγ and CD3ζ adaptors in NK cells from peripheral blood samples of rhesus macaques. Since previous studies have shown that the majority of macaque NK cells express NKG2A [5, 26, 27], we initially used this marker to identify NK cells as $CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}$ and $NKG2A^{+}$. Using this gating strategy, we observed that all

$CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}NKG2A^{+}$ cells expressed CD3ζ in all macaque subjects examined, but several macaques (e.g. RM$^{\#}$663) harbored a distinct subset that was deficient in FcRγ (**Fig. 2.1A**). Throughout this investigation, we used anti-FcRγ mAb (1D6), which allowed for much sharper resolution between these macaque $FcR\gamma^{-}$ NK cells and FcRγ-expressing conventional NK cells compared to the polyclonal antibodies used in previous studies [9-11, 28, 29].

Because many g-NK cells in humans do not express NKG2A [9, 17], gating strategies that utilize NKG2A might not include the entire $FcR\gamma^{-}$ NK cell population in macaques. Therefore, we considered alternative markers to identify all NK cells in rhesus macaques. Since all human NK cells express CD3ζ [11], and because this adaptor appeared to be expressed exclusively by NK cells and T cells in both humans and macaques (**Fig. 2.1A** and data not shown), we examined FcRγ expression in the $CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}CD3\zeta^{+}$ cell population to determine the frequency of $FcR\gamma^{-}$ NK cells among total circulating NK cells in macaques. Indeed, this gating strategy revealed that some animals (e.g. RM$^{\#}$716 and $^{\#}$700) had readily detectable, albeit often at low frequency, $FcR\gamma^{-}$ NK cells that expressed little or low levels of NKG2A in addition to $FcR\gamma^{-}$ NK cells that expressed high levels of NKG2A (**Fig. 2.1B**). The majority of these $CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}CD3\zeta^{+}NKG2A^{-/dim}$ $FcR\gamma^{-}$ cells expressed NK cell-associated markers CD8 and CD16 (**30**), and the effector molecule granzyme B (**Fig. 2.1B** and Supplemental Fig. 1), indicating that $NKG2A^{-/dim}$ NK cells that are also FcRγ-deficient exist in certain macaques. Although the $CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}CD3\zeta^{+}$ gating strategy was more inclusive of $FcR\gamma^{-}$ NK cells, the gated population appeared to contain a small population of cells that did not express NK cell-associated proteins, including CD16 (Supplemental Fig. 1). Therefore, we modified the gating strategy to include CD16 expression to more accurately assess the frequency of $FcR\gamma^{-}$ NK cells with a final profile defined as $CD3\varepsilon^{-}CD14^{-}CD20^{-/dim}CD3\zeta^{+}CD16^{+}$ $FcR\gamma^{-}$ NK cells. The resulting

analysis of a large cohort of non-SPF macaques (*n*=128) using this gating strategy showed that approximately one-third of the macaques had a distinct population of FcRγ⁻ NK cells (**Fig. 2.1C**). The incidence and frequencies of FcRγ⁻ NK cells did not show notable association with age or the gender of the animal subjects (**Fig. 2.1C** and data not shown). These data demonstrate that a subgroup of rhesus macaques possesses FcRγ⁻ NK cells, similar to the observed incidence of g-NK cells in humans [11, 14, 19].

Longitudinal studies showed that the frequencies of circulating FcRγ⁻ NK cells were nearly constant over a period of 4 to 6 months (**Fig. 2.1D**). In contrast, macaques that initially had no detectable FcRγ⁻ NK cells showed no appearance of such cells during this period. These data indicate that the size of FcRγ⁻ NK cell pool is maintained at a relatively stable level.

2.4.2 Phenotypic characteristics of FcRγ-NK cells

It has been shown that several cell surface markers and intracellular proteins, such as NKp46 and SYK, are differentially expressed between human g-NK and conventional NK cells [9-11]. Consistent with this phenotypic difference in human NK cells, macaque FcRγ⁻ NK cells showed lower levels of both NKp30 and NKp46 compared to conventional NK cells (**Fig. 2.2A**). Also resembling human NK cell populations [10], the expression of adhesion molecules PECAM and CD49F was lower, while CD2 was higher in FcRγ⁻ NK cells compared to conventional NK cells (**Fig. 2.2A**).

Moreover, while macaque conventional NK cells expressed SYK at uniform levels, FcRγ⁻ NK cells showed variable levels of SYK among different animals; i.e., FcRγ⁻ NK cells displayed near complete or partial SYK-deficiency in some animals, while displaying near normal SYK expression in other animals (**Fig. 2.2B**). Overall, FcRγ⁻ NK cells showed reduced, yet heterogeneous, expression of SYK, and SYK-deficient NK cells were found in approximately one

quarter of the animals examined. Taken together, these data show that macaque FcRγ⁻ NK cells and human g-NK cells have several phenotypic characteristics in common.

Analysis of tissue specimens showed that these cells were present in spleen, lungs, liver, and bone marrow in macaques that had FcRγ⁻ NK cells in peripheral blood (**Fig. 2.2C**). In contrast, macaques that lacked FcRγ⁻ NK cells in peripheral blood consistently lacked detectable FcRγ⁻ NK cells in these tissues. Interestingly, compared to peripheral blood, the relative frequency of FcRγ⁻ NK cells was higher in lung, lower in spleen and liver, and comparable in bone marrow (**Fig. 2.2D**). SYK-deficient NK cells were also present in these tissues from macaques with SYK-deficient NK cells in peripheral blood (**Fig. 2.2E**). These data demonstrated that FcRγ⁻ NK cells, as well as SYK-deficient NK cells, were widely distributed in the body at variable frequencies depending on the tissues. Longitudinal studies showed that the frequencies of SYK-deficient NK cells were also nearly constant during the follow-up period (**Fig. 2.2F**), again resembling the persistent presence of SYK-deficient NK cells observed in humans [10].

2.4.3 Enhanced antibody-dependent activation of FcRγ-NK cells against virus-infected cells

One of the defining features of human g-NK cells is their enhanced ability to respond to virus-infected target cells in the presence of virus-specific antibodies [9, 10, 14-16]. To determine whether macaque FcRγ⁻ NK cells also exhibit this functional property, we examined NK cell intracellular cytokine expression by flow cytometry after stimulation with rhesus fibroblasts (Telo-RF) that were either RhCMV- or mock-infected in the presence or absence of RhCMV-seropositive plasma. In response to mock-infected target cells, both FcRγ⁻ and conventional NK cells revealed little or no production of TNF-α regardless of the presence of seropositive plasma (**Fig. 2.3A**).

Similarly, neither FcRγ⁻ nor conventional NK cells produced appreciable amounts of TNF-α in response to RhCMV-infected cells in the absence of RhCMV-seropositive plasma. However, the addition of seropositive plasma led to a dramatic increase in frequency of TNF-α expressing FcRγ⁻ NK cells at levels significantly higher than that of TNF-α expressing conventional NK cells ($p<0.01$) (**Fig. 2.3A**). In contrast, the addition of RhCMV seronegative plasma did not elicit these effects (data not shown). Additionally, significantly higher frequencies of IFN-γ expressing cells were observed within the FcRγ⁻ NK cell subset compared to conventional NK cells when both RhCMV-infected cells and seropositive plasma were present (**Fig. 2.3B**). These results showed that, compared to conventional NK cells, FcRγ⁻ NK cells exhibited enhanced responses to RhCMV-infected cells in an antibody-dependent manner. However, macaque FcRγ⁻ NK cells did not show higher cytokine production compared to conventional NK cells when stimulated with immobilized anti-human CD16 mAb (3G8) (**Fig. 2.3C**).

It has been shown that human g-NK cells respond poorly to IL-12 and IL-18 co-stimulation (**14**), consistent with the observed lower levels of transcripts encoding IL-12Rb2 and IL-18RAP receptor subunits determined via transcriptome analyses[10, 14]. Similarly, treatment of macaque FcRγ⁻ NK cells with these cytokines resulted in much lower frequencies of IFN-γ-expressing cells compared to conventional NK cells ($p<0.05$) (**Fig. 2.3D**). Finally, we have previously shown that human g-NK cells respond poorly to certain tumor cells in the absence of tumor cell-specific antibodies (**9**). To compare the functional responsiveness of macaque FcRγ⁻ NK cells and conventional NK cells to tumor targets, we examined cytokine expression following stimulation with human K562 or 721.221 tumor cells. Exposure of FcRγ⁻ NK cells to either target cell line resulted in lower frequencies of TNF-α and IFN-γ-expressing cells compared to conventional NK

cells ($p<0.05$) (**Fig. 2.3E** and 2.**3F**). These data indicate that FcRγ⁻ NK cells are hypo-responsive to stimuli other than antibody-dependent stimulation.

2.4.4 Induction of FcRγ -NK cells following experimental infection with a specific strain of RhCMV

Considering the association between HCMV serostatus and g-NK cells [9, 10, 14], we hypothesized that RhCMV infection may play a role in the induction of FcRγ⁻ NK cells in macaques. To explore this possibility, we first determined whether FcRγ⁻ NK cells are present in SPF macaques that were raised in an outdoor environment free from RhCMV as well as six other viruses that include simian foamy virus (SFV), herpes B virus (BV) rhesus monkey rhadinovirus (RRV), type D simian retrovirus (SRV), simian immunodeficiency virus (SIV) and simian T-lymphotropic virus (STLV). These SPF animals were confirmed seronegative for all seven viruses, and ranged in age from 2 to 13 years, with the median age of 5 years. Importantly, analysis of peripheral blood samples from these SPF macaques showed that none had detectable FcRγ⁻ NK cells, and all NK cells maintained uniformly high levels of NKG2A and SYK (**Fig. 2.4A** and 2.**4B**). Thus, these data suggest that regardless of age, the presence of FcRγ⁻ NK cells in rhesus macaques requires infection with at least one of these seven viruses.

We next sought to examine the role of RhCMV infection in the induction of FcRγ⁻ NK cells through retrospective analysis of PBMC samples that had been collected from SPF animals (n=7) pre- and post-infection with $RhCMV_{UCD59}$, a strain originally isolated from a non-SPF macaque [30]. While the analysis of baseline samples of uninfected SPF animals showed no detectable FcRγ⁻ NK cells initially, at 20 weeks post-infection, a noticeable subset of NK cells that was deficient for FcRγ was observed in several animals, albeit at low frequencies (**Fig. 2.4C** and 2.**4E**), providing evidence for a role of RhCMV infection in the induction of FcRγ⁻ NK

cells. We also conducted retrospective analysis of PBMCs collected from a group of SPF animals (*n*=6) that had been experimentally infected with a mixture of three variants of $RhCMV_{68\text{-}1}$, a prototypic fibroblast-tropic strain of RhCMV that has been widely used for experimental infection of macaques, including vaccine studies [31-33]. Intriguingly, FcRγ^{-} NK cells were not detected in any of the PBMC samples collected at 2, 20, or 32 weeks post-infection with these $RhCMV_{68\text{-}1}$ variants (**Fig. 2.4D** and 2.**4E**). In addition, we analyzed PBMCs collected from a separate group of SPF macaques (*n*=6) that were infected with $RhCMV_{68\text{-}1}$ alone. Again, no animals infected with $RhCMV_{68\text{-}1}$ revealed detectable FcRγ^{-} NK cells for up to 32 weeks post-infection (**Fig. 2.4E**). As part of a vaccine study, these animals were challenged with SIV starting from 36 weeks after infection with $RhCMV_{68\text{-}1}$ or $RhCMV_{68\text{-}1}$ variants, and the subsequent analysis of PBMCs collected between 22 - 34 weeks after SIV infection showed no evidence of FcRγ^{-} NK cells (**Fig. 2.4D** and 2.**4E**). Taken together, these results indicate that unlike $RhCMV_{UCD59}$, infection with $RhCMV_{68\text{-}1}$, $RhCMV_{68\text{-}1}$ variants or SIV did not induce FcRγ^{-} NK cells during the indicated time periods.

Importantly, the RhCMV strain-specific effect we observed was not due to a difference in the ability of these viruses to induce a global expansion of NK cells. Indeed, infection with $RhCMV_{68\text{-}1}$, $RhCMV_{68\text{-}1}$ variants, and SIV led to the expansion of NK cells at levels comparable to those observed following infection with $RhCMV_{UCD59}$ (**Fig. 2.4F**). Moreover, infection with $RhCMV_{68\text{-}1}$ and $RhCMV_{68\text{-}1}$ variants elicited RhCMV-specific IgG at concentrations comparable to those observed following infection with $RhCMV_{UCD59}$ (**Fig. 2.4G**), likely reflecting productive long-term infection by these strains.

2.4.5 Assocation of FcRγ -NK cells with natural infection by RhCMV and other viruses

Considering the strain-specific effect observed with experimental RhCMV infection, we examined the potential relationship between natural RhCMV infection and FcRγ$^-$ NK cells in non-SPF macaques. Serological analysis of plasma samples for RhCMV-specific antibodies showed that the majority of non-SPF macaques were seropositive for RhCMV (**Fig. 2.5A**). Among the RhCMV-seropositive macaques examined, approximately one-third had detectable FcRγ$^-$ NK cells in their peripheral blood, while this subset was detected in only one RhCMV-seronegative macaque. Both the incidence and frequency of FcRγ$^-$ NK cells were significantly higher in the RhCMV-seropositive group than in the RhCMV-seronegative group ($p<0.01$) (**Fig. 2.5A**), indicating the association of FcRγ$^-$ NK cells with naturally acquired RhCMV infection.

Since the frequencies of FcRγ$^-$ NK cells in the SPF macaques experimentally infected with $RhCMV_{UCD59}$ were generally lower than those detected in non-SPF RhCMV-seropositive macaques (**Fig. 2. 4C** and **Fig. 2.5A**), and since non-SPF animals are exposed to potentially many other viruses, microbes, or environmental agents, we hypothesized that these additional exposures might contribute to the higher frequencies of FcRγ$^-$ NK cells inF these animals. To investigate this possibility, we assessed the levels of plasma antibodies specific for each of the six other viruses known to be absent in SPF macaques. Serologic results showed that, similar to RhCMV, the majority of macaques were seropositive for SFV. Approximately one half of the animals were seropositive for BV and RRV, only a small fraction of macaques was seropositive for SRV and/or STLV, and none were seropositive for SIV (**Fig. 2.5A** and data not shown). Thus, SFV, BV and RRV were also relatively common in our non-SPF cohorts. Interestingly, FcRγ$^-$ NK cells were detected in a subgroup of SFV-seropositive macaques, but not in SFV-seronegative macaques, indicating a positive correlation between prior SFV infection and the presence of FcRγ$^-$ NK cells

($p<0.01$). In addition, the incidence of FcRγ$^-$ NK cells was slightly higher in the BV-seropositive group than in the BV-seronegative group ($p<0.05$), with the seropositive group showing significantly higher frequencies of FcRγ$^-$ NK cells ($p<0.05$). In contrast, neither the incidence nor frequency of FcRγ$^-$ NK cells differed between the RRV-seropositive vs. -seronegative animals. Potential associations with SRV or STLV could not be reliably evaluated due to the limited number of animals that were seropositive for these viruses. The frequencies of FcRγ$^-$ NK cells showed positive correlation with plasma IgG titers against RhCMV, SFV and BV (**Fig. 2.5B**). These data indicate that infection with SFV and/or BV, as well as RhCMV, but not RRV, may be associated with higher incidence and frequency of FcRγ$^-$ NK cells in non-SPF macaques.

To explore the possibility that co-infection of RhCMV with SFV and/or BV might influence the incidence and frequency of FcRγ$^-$ NK cells, we categorized the animals in our cohorts according to their serological status for each of these three viruses. Notably, all macaques with FcRγ$^-$ NK cells, except for one, were seropositive for both RhCMV and SFV, regardless of BV-seropositivity (**Fig. 2.5C** and Supplemental Fig. 2.2). Thus, the data did not allow for further dissection of potential impact of RhCMV and SFV on the incidence and frequency of FcRγ$^-$ NK cells. The single exception among the animals with FcRγ$^-$ NK cells was an individual that was seropositive for both SFV and RRV (Supplemental Fig. 2.2); however, none of the animals that were seropositive for either SFV or RRV and seronegative for RhCMV had detectable FcRγ$^-$ NK cells (Supplemental Fig. 2.2). Importantly, although our epidemiologic analysis did not reveal specific association with a particular virus, there was a trend toward higher frequencies of FcRγ$^-$ NK cells in the animals that were seropositive for multiple viruses (**Fig. 2.5C**). For instance, all animals with relatively high frequencies (>20%) of FcRγ$^-$ NK cells were seropositive for BV as well as RhCMV and SFV, indicating that these animals had been exposed to all three viruses.

Taken together, these data indicate that co-infection with RhCMV and other common viruses, such as SFV and BV, corelates with higher frequencies of FcRγ⁻ NK cells in non-SPF animals.

2.5 Conclusion

Here, we demonstrate that a subgroup of non-SPF macaques with RhCMV sero-positivity possess FcRγ⁻ NK cells that resemble human g-NK cells with adaptive features. Macaque FcRγ⁻ NK cells showed phenotypic characteristics similar to g-NK cells, including reduced expression of natural cytotoxicity receptors, NKp30 and NKp46 [11]. In addition, FcRγ⁻ NK cells displayed long-term persistence in macaques. Furthermore, consistent with functional characteristics of g-NK cells, macaque FcRγ⁻ NK cells were hypo-responsive to tumor and cytokine stimulation but were highly responsive to RhCMV-infected cells in an antibody-dependent manner. We speculate that FcRγ⁻ NK cells may play a specialized role in responding to conditions of chronic, reactivated, or recurrent viral infection where pathogen-specific antibodies are available.

Importantly, we observed that SPF animals free of RhCMV and six other viruses did not have detectable FcRγ⁻ NK cells, indicating that one or more viral infection is required for the induction of these cells. The appearance of FcRγ⁻ NK cells in SPF macaques following $RhCMV_{UCD59}$ infection provides strong support for a causal relationship between RhCMV infection and the induction of FcRγ⁻ NK cells, and presumably an analogous causal relationship between HCMV and g-NK cells in humans. The lack of detectable FcRγ⁻ NK cells in SPF macaques following infection with $RhCMV_{68\text{-}1}$ and its variants suggests that there is an RhCMV strain-specific effect. The observation that $RhCMV_{UCD59}$ and $RhCMV_{68\text{-}1}$ strains were able to elicit comparably high levels of RhCMV-specific IgG suggests that both strains resulted in productive long-term infection. Consistent with this, it has been shown that $RhCMV_{68\text{-}1}$ can disseminate and

persist in many tissues throughout the body following experimental infection [31, 33, 34]. Thus, it is unlikely that the appearance of FcRγ⁻ NK cells following $RhCMV_{UCD59}$ infection is simply due to expansion of pre-existing FcRγ⁻ NK cells that might be present at very low frequencies in these SPF macaques before experimental viral infection. However, considering that one of RhCMV-seronegative macaques had readily detectable FcRγ⁻ NK cells, our data do not exclude the possibility that infection with virus(es) other than RhCMV can also lead to the induction of this NK cell subset. It is also possible that this animal was indeed infected with RhCMV but serological testing did not detect RhCMV-specific antibodies, similar to the reported lack of serological detection of HCMV infection in certain individuals [9, 35]. Nonetheless, our retrospective study provides evidence that infection with a specific RhCMV strain is sufficient to induce FcRγ⁻ NK cells, albeit at low frequencies, in the absence of naturally infecting RhCMV and six other viruses in SPF macaques.

The RhCMV strain-specific effect observed in this study provides a potential explanation for why FcRγ⁻ NK cells have been detected in only certain cytomegalovirus-seropositive macaques and humans. Additionally, differences between strains of RhCMV may also account for the apparent lack of association between FcRγ⁻ NK cells and CMV reactivation following hematopoietic stem cell transplantation in rhesus macaques [36]. Nucleotide sequence analysis indicates that the main difference between $RhCMV_{UCD59}$ and $RhCMV_{68\text{-}1}$ lies in the UL/b' region that contains several genes involved in regulating host cell tropism and immune evasion [30, 37, 38], suggesting these genes may be important in the induction of FcRγ⁻ NK cells. Prospective infection studies utilizing SPF macaques and RhCMV strains with variations in these genes will be instrumental for delineating viral factors and cellular processes that lead to the induction of FcRγ⁻ NK cells.

Concerning the wide variation in observed frequencies of macaque FcRγ⁻ NK cells, our results from non-SPF macaques suggest that co-infection of RhCMV with other viruses, such as SFV and BV, can lead to further expansion of these cells. In this regard, we have previously demonstrated that g-NK cells preferentially expand over conventional NK cells *in vitro* in response to influenza virus-infected cells, as well as HCMV-infected cells, in a manner dependent on virus-specific antibodies [10], supporting the idea that persistent or recurrent infection by various viruses may drive antibody-dependent expansion of FcRγ⁻ NK cells *in vivo*. Two recent studies have reported that FcRγ⁻ NK cells are present in the majority of macaques from two specific cohorts [28, 29] at what appears to be generally higher frequencies than we observed in our cohort. It is unclear whether the apparent discrepancy is due to the use of different antibodies (monoclonal vs. polyclonal) for FcRγ detection or to a difference in housing environment and/or genetic make-up of macaques. Along this line, the expression of NKp46 was not altered when polyclonal anti-FcRγ was used [29], in contrast to the reduced expression of this activation receptor on FcRγ⁻ NK cells in our study. Nonetheless, these studies suggested that FcRγ⁻ NK cells can expand in non-SPF macaques following SIV infection, further supporting the role of other viral infections in the expansion of these cells.

Experimental infection of SPF macaques with viruses establishing persistent infection, in combination with RhCMV variants, will be useful for determining the potential contributions of these viruses to the expansion of FcRγ⁻ NK cells. These infection models will be also useful for determining the kinetics of FcRγ⁻ NK cell expansion [19], as well as for evaluating the roles of viral genes [39-45], such as those that are involved in modulating NKG2C-mediated activation of ‘adaptive’ NK cells [46, 47]. Based on our observations from the experimental RhCMV infection studies, and the incidence of FcRγ⁻ NK cells in non-SPF macaques, we propose a two-step model

(induction and expansion) in which the FcRγ⁻ NK cell population is initially induced by infection with a strain of CMV that bears, as of yet, undefined properties, perhaps leading to epigenetic changes in a small number of NK cells. Subsequent superinfection or reactivation of CMV, and/or recurrent infection by other pathogens where specific antibodies are present could then drive expansion of these FcRγ⁻ NK cells, thereby shaping the antibody-dependent memory-like NK cell pool in an ongoing manner.

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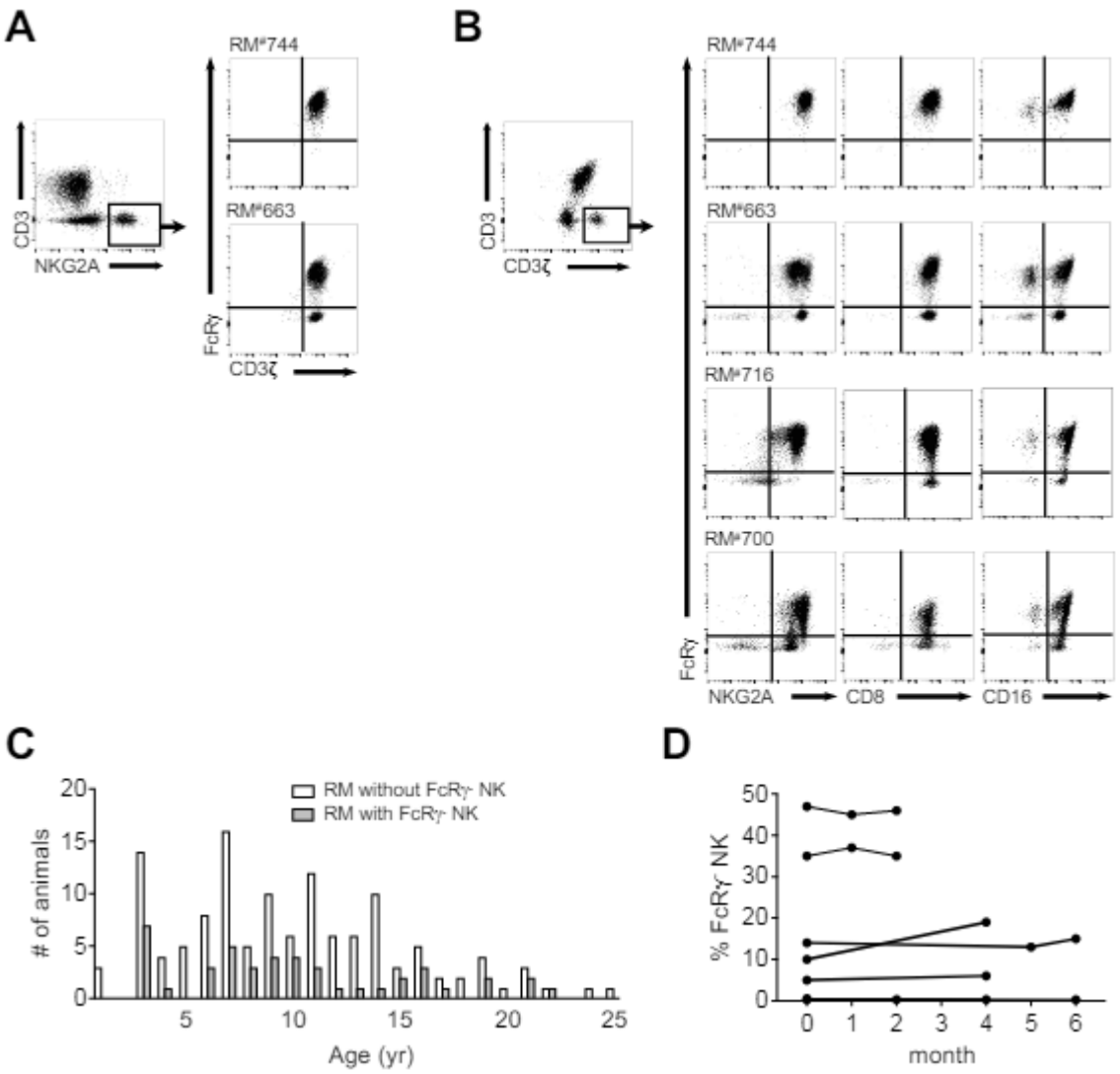

Figure 2.1 FcRγ- NK cells are present in a subgroup of rhesus macaques

Macaque PBMCs were analyzed by flow cytometry for the presence of FcRγ$^-$ NK cells. Live lymphocytes were gated as CD3ε$^-$CD14$^-$CD20$^{-/dim}$NKG2A$^+$(**A**) or CD3ε$^-$CD14$^-$CD20$^{-/dim}$CD3ζ$^+$(**B**). Dot plots show presence or absence of FcRγ$^-$ NK cells along with expression of indicated markers from several representative macaques. (**C**) PBMCs from individual macaques (*n*=128) were analyzed to determine the presence of FcRγ$^-$ NK cells among the CD3ε$^-$CD14$^-$CD20$^{-/dim}$CD3ζ$^+$CD16$^+$ NK cell population for each sample. Bar graph shows the number of surveyed animals with (grey bar) and without (white bar) FcRγ$^-$ NK cells at specified ages. (**D**)

Longitudinal monitoring of FcRγ$^-$ NK cell subset. Shown are frequencies of FcRγ$^-$ NK cell subsets among the total NK cell population in PBMC samples collected for up to 6 months from 7 individual macaques.

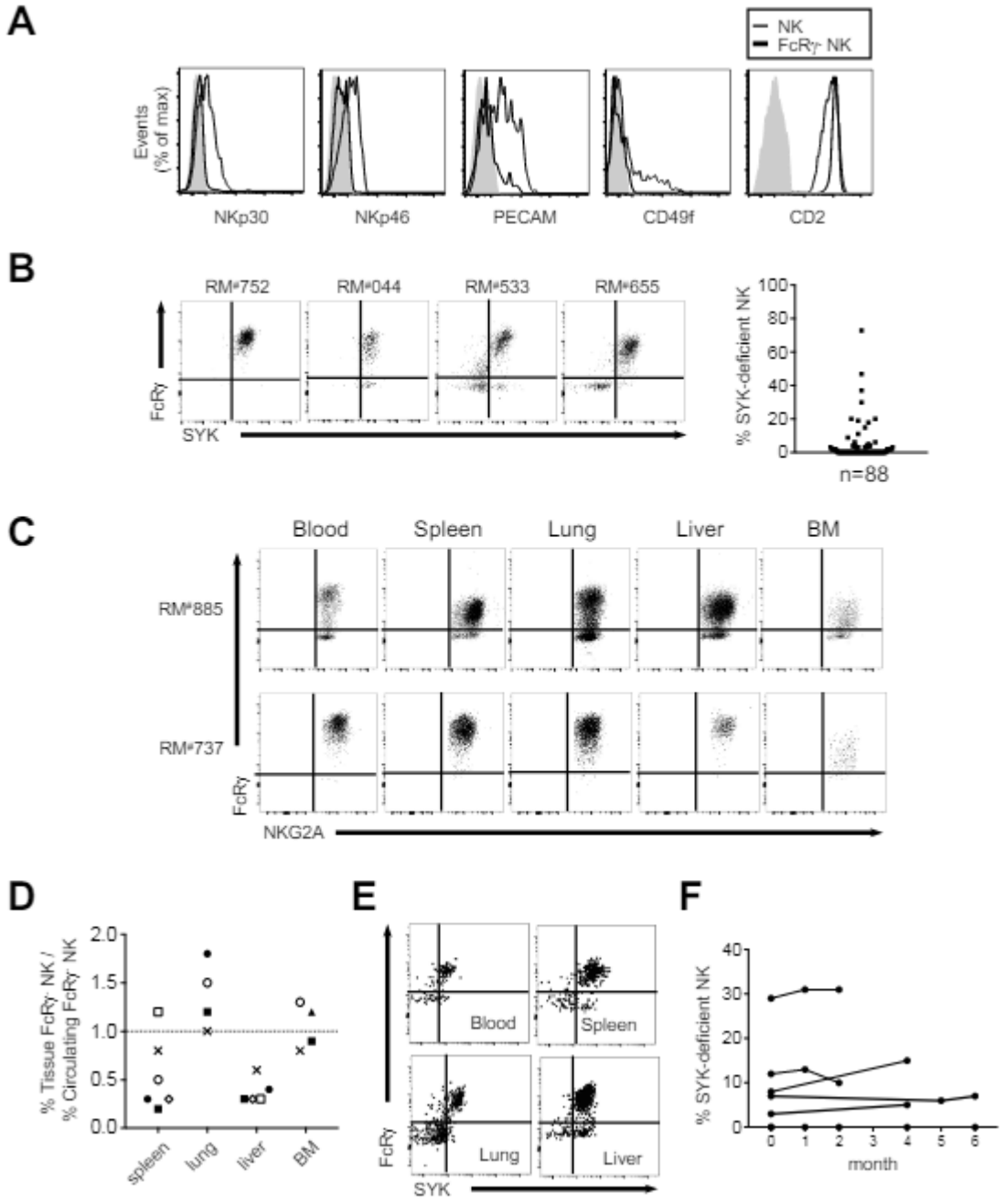

Figure 2.2 Phenotypic properties, tissue distribution and long-term persistence of SYK-deficient NK cells

Figure 2.2 Phenotypic properties, tissue distribution and long-term persistence of SYK-deficient NK cells

(**A**) Histograms show expression of indicated surface markers on FcRγ$^-$ NK cells (bold line), conventional NK cells (thin line), and staining controls (grey shaded) in the CD3ε$^-$CD14$^-$CD20$^-$

$^{/dim}$CD3ζ^{+}CD16^{+} NK cell population. Data are representative of seven macaque PBMC samples. (**B**) Expression of FcRγ and SYK in the CD3ε^{-}CD14^{-}CD20$^{-/dim}$CD3ζ^{+}CD16^{+} NK cell population from PBMCs of four representative macaques. Dot graph shows the frequencies of SYK-deficient cells among the NK cell population in PBMCs, with each data point representing an individual animal (*n*=88). (**C**) FcRγ and NKG2A expression of NK cell population in PBMCs, spleen, lung, liver, and bone marrow (BM) from two representative macaques. (**D**) Comparative ratio of frequencies of tissue-resident FcRγ^{-} NK cells to circulating FcRγ^{-} NK cells in each animal. Each symbol represents data points from the same macaque. (**E**) FcRγ and SYK expression in NK cells from peripheral blood, spleen, lung, and liver samples from a representative macaque. (**F**) Longitudinal monitoring of the SYK-deficient NK cell subset. Shown are frequencies of SYK-deficient NK cells among the total NK cell population in PBMC samples collected for up to 6 months from 7 individual macaques.

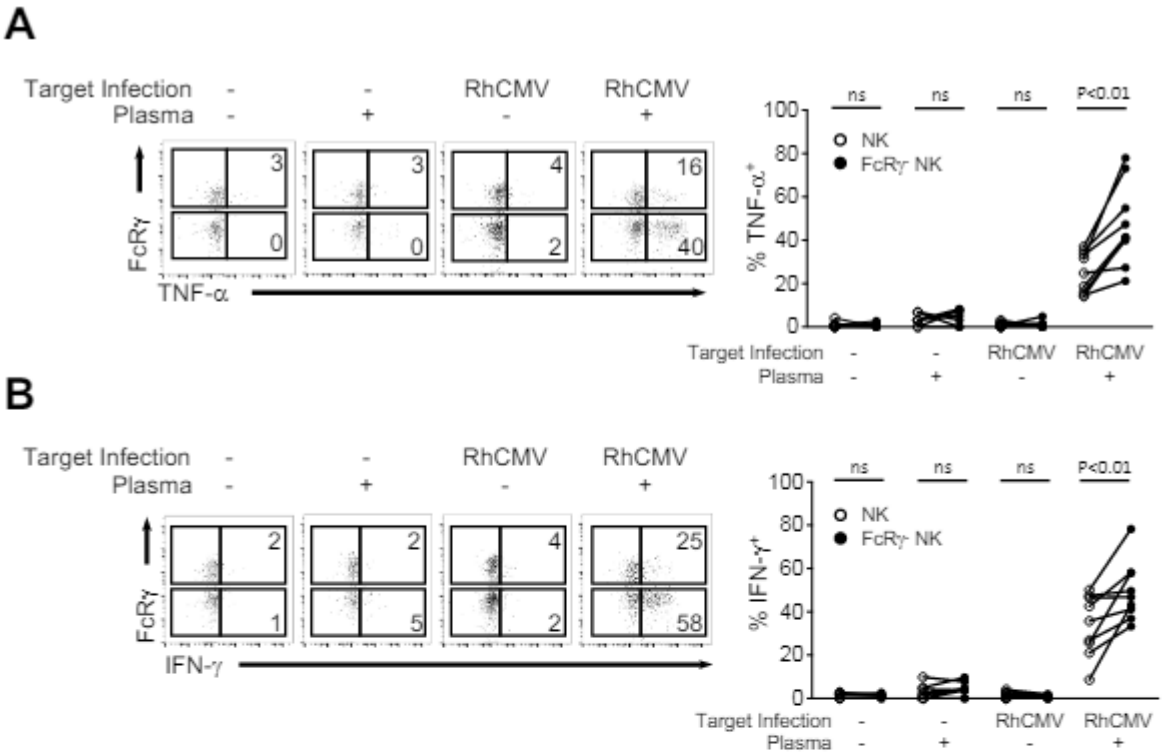
A
Target Infection - - RhCMV RhCMV
Plasma - + - +
FcRγ
TNF-α
3
0
3
0
4
2
16
40
ns
ns
ns
P<0.01
% TNF-α+
NK
FcRγ- NK
B
Target Infection - - RhCMV RhCMV
Plasma - + - +
FcRγ
IFN-γ
2
1
2
5
4
2
25
58
% IFN-γ+
C
CD16 crosslinking
D
IL-12 + IL-18
P<0.05
E
Target : K562
F
Target : 721.221

Figure 2.3 FcRγ$^-$ NK cells show enhanced effector function against virus-infected target cells in the presence of virus-specific antibodies.

(**A and B**) Macaque PBMCs were co-cultured with mock- or RhCMV-infected cells in the presence or absence of RhCMV-specific plasma as indicated. Induction of TNF-α (**A**) and IFN-γ (**B**) by conventional NK and FcRγ$^-$ NK cells were assessed by flow cytometry. Numbers represent the relative percentage of TNF-α$^+$ or IFN-γ$^+$ NK cells among each NK cell subset. Line graphs on the right show the percentages of responding conventional NK (○) or FcRγ$^-$ NK (●) cells. Circles connected by a line designate the same macaque sample. (**C-F**) Percentages of conventional NK (○) or FcRγ$^-$ NK (●) cells that expressed TNF-α or IFN-γ following stimulation with CD16 crosslinking (**C**), IL-12 and IL-18 (**D**), K562 (**E**) or 721.221 (**F**) tumor target cells. Statistical analyses were performed using nonparametric Mann-Whitney tests. ns, not significant.

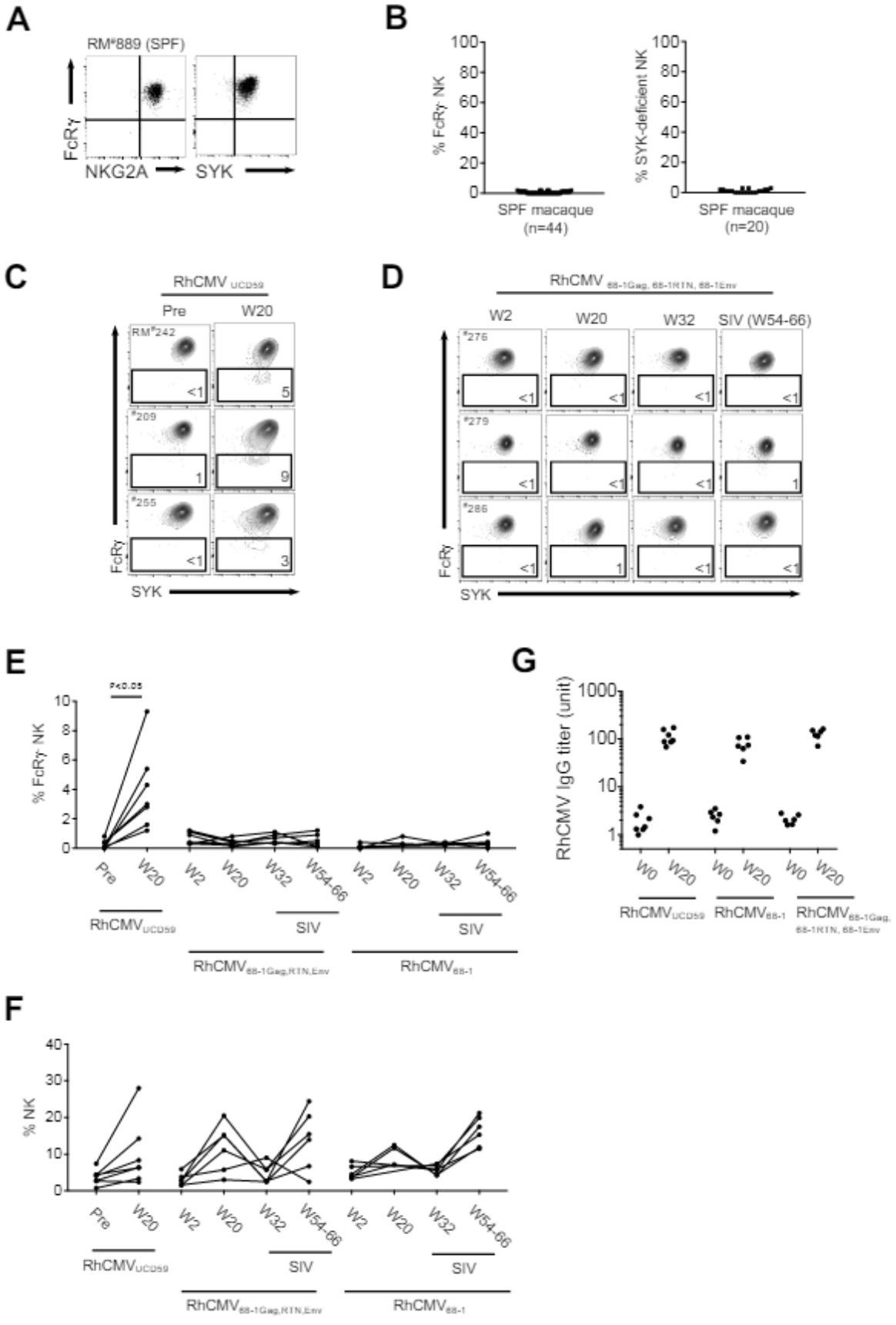
A
RM#889 (SPF)
FcRγ
NKG2A
SYK
B
% FcRγ- NK
% SYK-deficient NK
SPF macaque (n=44)
SPF macaque (n=20)
C
RhCMV UCD59
Pre
W20
RM#242
#209
#255
FcRγ
SYK
D
RhCMV 68-1Gag, 68-1RTN, 68-1Env
W2
W20
W32
SIV (W54-66)
#276
#279
#286
E
P<0.05
% FcRγ- NK
RhCMV UCD59
SIV
RhCMV 68-1Gag,RTN,Env
RhCMV 68-1
G
RhCMV IgG titer (unit)
W0
W20
RhCMV UCD59
RhCMV 68-1
RhCMV 68-1Gag, 68-1RTN, 68-1Env
F
% NK
RhCMV UCD59
SIV
RhCMV 68-1Gag,RTN,Env
RhCMV 68-1

Figure 2.4. FcRγ$^-$ NK cells are induced following primary infection with a specific RhCMV strain.

(**A**) Expression of NKG2A and SYK with respect to FcRγ in the CD3ε$^-$CD14$^-$CD20$^-$ $^{/dim}$CD3ζ$^+$CD16$^+$ NK cell population from a representative SPF macaque. **(B)** Frequencies of FcRγ$^-$ and SYK-deficient NK cells in PBMCs of SPF macaques. (**C**) FcRγ and SYK expression in NK cells from PBMC samples from a representative SPF macaque before (W0) and 20 weeks (W20) after RhCMV$_{UCD59}$ infection. (**D and E**) FcRγ and SYK expression in NK cells from PBMC samples from a representative SPF macaque at 2 weeks (W2), 20 weeks (W20) and 32 weeks (W32) after infection with RhCMV$_{68\text{-}1}$ (**D**) or a combination of RhCMV$_{68\text{-}1Gag}$, RhCMV$_{68\text{-}1RTN}$, and RhCMV$_{68\text{-}1Env}$ (**E**). These macaques were subsequently challenged with SIV at 36 weeks after initial RhCMV infection. Dot plots show flow cytometric analysis of FcRγ and SYK expression in NK cells obtained before (W32) and between 17-24 weeks post-SIV infection. (**F**) NK cell frequencies are shown before and after infection by indicated strains of RhCMV and SIV. (**G**) Plasma IgG titers for RhCMV from macaques before (W0) and 20 weeks after infection by indicated strains of RhCMV.

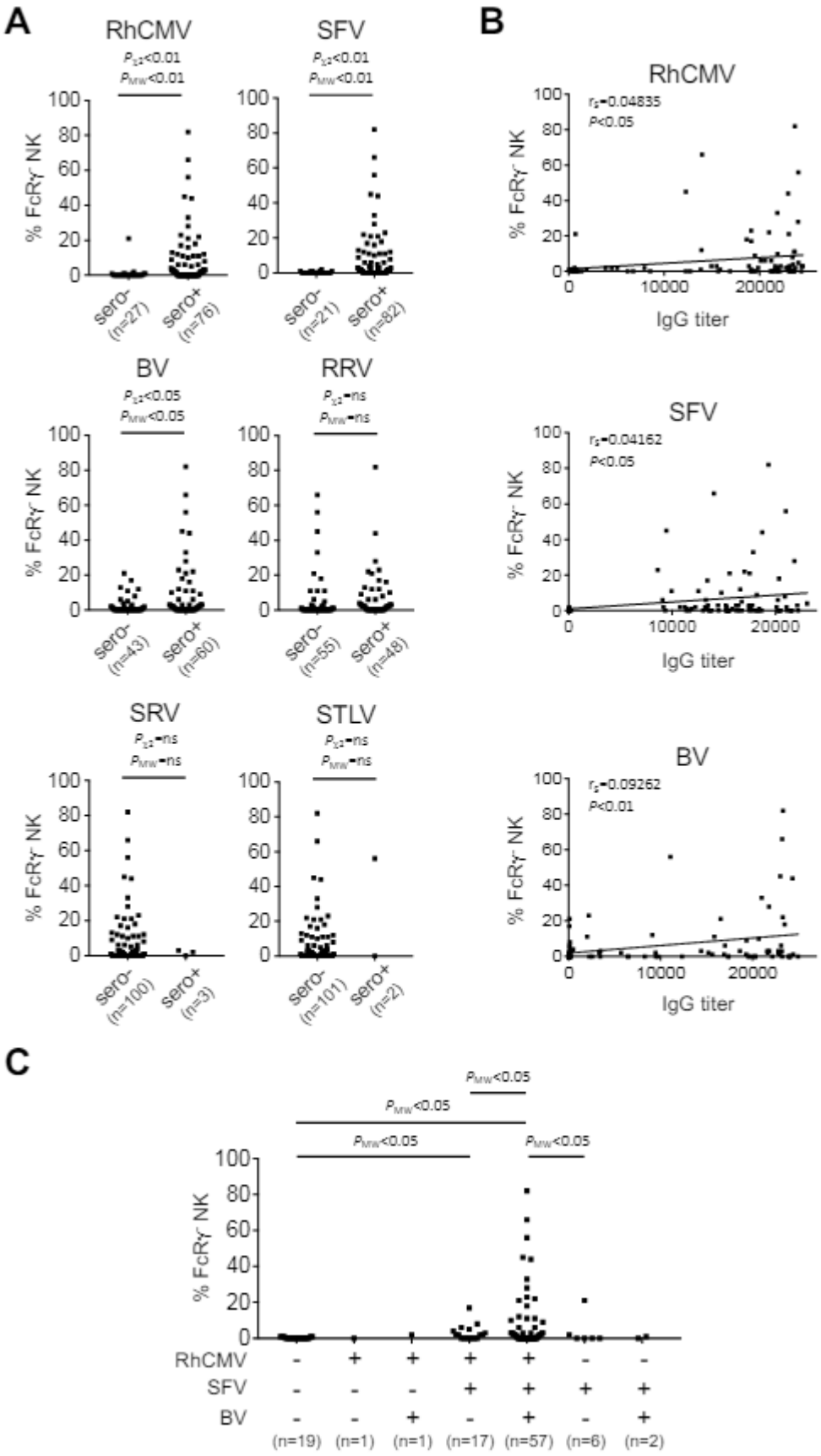
A
RhCMV
SFV
BV
RRV
SRV
STLV
% FcRγ⁻ NK
sero- (n=27)
sero+ (n=76)
sero- (n=21)
sero+ (n=82)
sero- (n=43)
sero+ (n=60)
sero- (n=55)
sero+ (n=48)
sero- (n=100)
sero+ (n=3)
sero- (n=101)
sero+ (n=2)
B
RhCMV
SFV
BV
IgG titer
C
RhCMV
SFV
BV
(n=19)
(n=1)
(n=1)
(n=17)
(n=57)
(n=6)
(n=2)

Figure 2.5. Association of FcRγ$^-$ NK cells with infection by RhCMV, SFV and BV

(A) Frequencies of FcRγ$^-$ NK cells within individual non-SPF macaques are grouped according to IgG serostatus for infection of RhCMV, SFV, BV, RRV, SRV and STLV. (**B**) FcRγ$^-$ NK cell frequencies were plotted according to plasma IgG titers for each animal for indicated viruses. (**C**) Frequencies of FcRγ$^-$ NK cells within individual macaques grouped according to their IgG serological statuses with respect to 3 common viruses (RhCMV, SFV and BV). Statistical analyses for the incidence and frequencies of FcRγ$^-$ NK cells were performed using chi-square tests ($P_{\chi 2}$) and nonparametric Mann-Whitney tests (P_{MW}), respectively. Correlation analyses were performed using Spearman's rank test. ns, not significant.

3. Adaptive g-NK cells exhibit potent antibody-dependent functional activities and comprise major population of unconventional CD56neg NK cells in HIV-viremic patients

3.1 Abstract

Individuals chronically infected with certain viruses, including HIV-1 possess abnormally high numbers of unconventional CD56neg NK cells with impaired natural cytotoxicity. However, little is known about molecular mechanisms underlying this functional impairment and how the CD56neg NK cell pool is formed. Here, we show that the majority of CD56neg NK cells in HIV-viremic patients are deficient of signaling adaptor FcRγ and specialized for antibody-dependent effector functions. With respect to phenotypic and functional characteristics, these CD56neg FcRγ-deficient NK cells were nearly identical to the adaptive-like g-NK cells, as well as CD56pos FcRγ-deficient NK cells, were hypo-responsive to tumor or cytokine stimulation, but highly responsive to HIV-infected cells in the presence of anti-HIV antibodies. These results suggest, despite the previous understanding of dysfunctionality of CD56neg NK cells in HIV patients, CD56neg NK cells may be cytotoxically active during HIV infection and may play a role in disease progression.

3.2 Introduction

NK cells play a crucial role in combating acute viral infection. Generally identified as CD3-CD14-CD19-CD56+ subset of lymphocytes in human, NK cells are capable of directly identifying and destroying virally infected cells. Recently, a novel subset of adaptive NK cells has been identified, characterized by clonal-like expansion and long-term persistence [1-3]. These adaptive NK cells, termed g-NK cells, have reduced expression of signaling adapter

molecule FcRγ and high levels of activation receptor NKG2C [2, 4]. Importantly, these g-NK cells show a promising potential for antiviral response due to their enhanced function of CD16, an Fc receptor that can bind to the Fc portion of multiple subclasses of IgG [3]. However, these adaptive NK cells exhibit defective expression of NCRs, NKp30 and NKp46, resulting in a poor response against tumors [2].

While the role of NK cells during the course of HIV infection has yet to be fully understood, recent research has shed light on the impact of HIV infection on NK cell population and function. HIV infection was shown to change the distribution and functions of NK cell subpopulations, most notably expansion of CD56neg NK cell population [5, 6]. In healthy individuals, the CD56neg NK cell subset encompasses only a small proportion of the NK cell compartment in peripheral blood [7]. However, in chronic HIV patients, CD56neg NK cells may constitute up to half of peripheral NK cells [8, 9]. These CD56neg NK cells, also observed in during HCV infection, exhibited lower levels of NCRs, NKp30 and NKp46, resulting in significantly lower cytolytic activity and ability to secrete cytokines against tumor stimulation [6, 10]. The expansion of CD56neg NK cells was shown to be associated with high HIV viral loads, and successful suppression of HIV replication by ART resulted in considerable restoration of CD56pos NK cell reservoir [7, 9, 11].

Current consensus regarding CD56neg NK cells found in HIV patients is that they are functionally impaired. These cells have demonstrated poor responsiveness to tumor and cytokine stimulation in various studies [7, 9]. Additionally, phenotypic analysis of CD56neg NK cells revealed lower levels of granzyme B and perforin expression, suggesting an exhausted state [7, 12]. However, current studies have only shown functional responses against tumor and cytokine

stimulation, neglecting examination of CD16 activation, another major mechanism of NK cell activation.

In this study, we examined CD56neg NK cells from PBMCs collected from individuals with acute HIV infection. Our analysis revealed that the majority of CD56neg NK cells lacked FcRγ expression. These FcRγ-deficient CD56neg NK cells demonstrated strong activation in response to CD16 crosslinking stimulation, while exhibiting poor responsiveness to tumor and cytokine stimulation. The phenotypic characteristics of these FcRγ-deficient NK cells within the CD56neg NK cell population resembled those of adaptive g-NK cells observed in healthy HCMV seropositive individuals. Remarkably, FcRγ-deficient CD56neg NK cells show robust activation against HIV-infected target cells in the presence of HIV seropositive antibody. These findings indicate that CD56neg NK cells, previously regarded to be dysfunctional in HIV patients, may exhibit cytotoxic activity and potentially contribute to the progression of the disease during the course of HIV infection.

3.3 Material and Methods

3.3.1 Human subjects and blood samples

PBMCs from pre-ART HIV-acute patients were obtained from Dr. David Asmuth at University of California Davis Medical Center

3.3.2 Flow cytometric analysis of NK cells

For analysis of cell surface markers, perforin, SYK, GrB, IFN-g, TNF-a, and CD107a expression, PBMCs or cultured cells were stained as previously described in Chapter 2.

3.3.3 Functional assays of NK cells

For CD16 crosslinking assays, PBMCs from HIV+ patients were stimulated for 6hrs with plate-bound anti-CD16 (3G8) antibody along with Brefeldin A. For tumor and cytokine stimulation assays, PBMCs from HIV+ patients were co-cultured for 12 hrs with K562 tumor cells at a ratio of 10:1 (E:T) or 24 hrs with cytokines (IL-12, IL-15, and IL-18). Six hours prior to analysis, Brefeldin A was added. For HIV-infected target cell assay, cultured CEM.NKR in 96-well plate were spin-infected with HIV (IIIF strain, MOI = 1) at 800g for 2 hours and washed with PBS then transferred into CO2. PBMCs obtained from HIV+ patients were added to infected cells 2 days post infection, along with Brefeldin A, anti-CD107a and with/without HIV seropositive/seronegative plasma. Cells were harvested 6hrs post coincubation and stained for further analysis.

3.3.4 Statistics

The Wilcoxon matched-pairs signed-rank test was used for comparison of the frequency changes in functional assays and phenotypic analysis. Differences were considered significant when $p < 0.05$ (GraphPad Prism).

3.4 Result

3.4.1 Majority of CD56neg NK cell population in ART-naïve HIV patients are FcRγ-deficient

We analyzed PBMCs from pre-ART HIV+ patients (MSM n=41, HIV+ n=35) as an attempt to examine the prevalence of FcRγ-deficient NK cells in the HIV patients. Comparing to the HIV- control group (n=19) and healthy CMV seropositive HIV- group (n=101), both HIV+ patient groups showed a notably higher frequency of FcRγ-deficient NK cells when NK cells

were selected as the CD3-CD14-CD19-CD56+ population (Fig 2.1A). Since a large proportion of NK cells from HIV+ patients have downregulated expression of CD56, this analysis of NK cells may have overlooked CD56neg NK cell population [7]. To evaluate NK cells from HIV+ patients irrespective of their CD56 expression level, CD7 and/or NKp80 could be utilized as an alternative gating strategy instead of CD56 [13, 14]. Our analysis of NK cells gated with either CD3-CD14-CD19-CD7+ or CD3-CD14-CD19-NKp80+ gating strategy revealed that FcRγ-deficient NK cell population was skewed towards CD56neg NK cell population (Fig 2.1B and 2.1C). The proportion of FcRγ-deficient NK cells in CD56neg NK cell population was consistent among all the HIV patients we analyzed, with an average of 70% of CD56neg NK cells being FcRγ-deficient (Fig 2.1D).

3.4.2 CD56neg NK cells show functional capability upon CD16 activation

We proceeded to assess the functional ability of CD56neg NK cells in the HIV+ patients. When stimulated with K562 tumor cells, CD56neg NK cells showed significantly lower expression of CD107a, TNF-α, and IFN-γ compared to CD56pos NK cell population (Fig 2.2A). Stimulation with cytokines (IL-2, IL-12, IL-18) also resulted in lower production of IFN-γ from CD56neg NK cells compared to CD56pos NK cells (Fig 2.2B). These results were consistent with earlier reports of impaired CD56neg NK cells [7, 12]. Given that CD16 is another significant pathway for activating NK cells, we proceeded to assess the responsiveness of CD56neg NK cells via a CD16 crosslinking assay [15]. Interestingly, unlike the results obtained from tumor and cytokine stimulations, both CD56neg and CD56pos NK cell populations exhibited similar levels of CD107a, TNF-α, and IFN-γ expression upon CD16 activation (Fig 2.2C).

3.4.3 FcRγ-deficient CD56neg NK cells show enhanced response to CD16 crosslinking

In a previous report, we demonstrated that FcRγ-deficient g-NK cells exhibit heightened reactivity in response to CD16 crosslinking [3]. Given that a significant proportion of CD56neg NK cells lack FcRγ, we sought to investigate whether the increased responsiveness of CD56neg NK cells to CD16 crosslinking is largely attributed to the high proportion of FcRγ-deficient NK cells within this population. In order to examine which subpopulation of NK cells are responding to the simulation, we divided NK cell population in to four quadrants in respect to their expression of FcRγ and CD56 (Q1 : FcRγ-expressing CD56neg, Q2 : FcRγ-expressing CD56pos, Q3 : FcRγ-deficient CD56pos, Q4 : FcRγ-deficient CD56neg) and compared activation status in each quadrants. Our analysis revealed that FcRγ-deficient populations of NK cells regardless of their CD56 expression status (Q3 and Q4) exhibited higher responses to CD16 crosslinking compared to FcRγ-expressing populations (Q1 and Q2) (Fig 3.3A). Using the 4 quadrants analysis, we revisited our results from K562 and cytokine stimulation. While FcRγ-deficient CD56neg NK cells (Q3) were still shown to be nonresponsive to K562 and cytokine stimulation, FcRγ-expressing CD56neg NK cells (Q1) showed substantial activation from these stimuli, with levels comparable to FcRγ-expressing CD56pos NK cells (Q2) (Fig 3.3B and 3.3C).

3.4.4 Phenotypic characteristics of FcRγ-deficient CD56neg NK cells resembles FcRγ-deficient CD56pos NK cells

g-NK cells from healthy individuals show unique phenotype compared to conventional NK cells, such as lower level of NCRs and signaling molecule [2]. Using the 4 quadrants analysis, we examined phenotypic characteristics of each NK cell subpopulation in HIV patient. Regardless of CD56 expression, both FcRγ-deficient CD56neg (Q3) and FcRγ-deficient

CD56pos (Q4) NK cell population showed significantly lower expression level of both NKp30 and NKp46 on their surface (Fig 3.4A). Among FcRγ-expressing NK cell populations, FcRγ-expressing CD56neg NK cell (Q1) subset showed slightly lower expression of both NKp30 and NKp46 compared to CD56pos FcRγ-expressing CD56pos NK cells (Q2), although the differences were not statistically significant. CD16 expression level were higher in both groups of FcRγ-deficient NK cells (Q3 and Q4) compared to FcRγ-expressing NK cell population (Q1 and Q2) though the difference was not statistically significant (Fig 3.4A).

In addition to surface receptors, we analyzed expressions of adaptive NK cell markers and intracellular molecules (Fig 3.4B). While the expression of NKG2A was observed from both FcRγ-expressing CD56neg (Q1) and FcRγ-expressing CD56pos (Q2) NK cells, the expression was nearly nonexistence in both FcRγ-deficient NK cell population (Q3 and Q4). Expression of NKG2C and CD57 were highest in FcRγ-deficient CD56pos NK cells (Q3) compared to other subpopulation, although the expression level varied among individuals. Analysis of intracellular molecules revealed lower expression level of perforin and granzyme B in FcRγ-deficient CD56neg NK cell population (Fig 3.4C).

3.4.5 FcRγ-deficient NK cells display robust functional capabilities against HIV infected target cells in presence of antibody

To investigate the potential biological impact of CD56neg NK cells during HIV infection, we evaluated their reactivity towards HIV-infected target cells. PBMCs from HIV+ patients were incubated with HIV-infected CEM.NKR cells and production of IFN-γ, TNF- α and CD107a expression were measured from the four quadrants of NK cells. Our result showed that none of the subpopulation of NK cells responded to HIV-infected cells (Fig 3.5). However,

addition of HIV-seropositive plasma induced robust production of the cytokines and CD107a from both FcRγ-deficient CD56pos (Q2) and FcRγ-deficient CD56neg (Q3) population, which was not observed from addition of HIV-seronegative plasma.

3.5 Conclusion

Here, we showed that majority of CD56neg NK cells in HIV patients are composed of FcRγ-deficient NK cells. These FcRγ-deficient NK cells among the CD56neg NK cell population showed observable activation capability through CD16 stimulation. Furthermore, FcRγ-deficient CD56neg NK cells displayed robust antibody dependent activation against HIV-infected target cells, further supporting functional capability of this NK cell population. Our analysis indicates that FcRγ-deficient NK cells found in CD56neg NK cell population closely resemble adaptive g-NK cell population in terms of phenotypic and functional characteristics.

We demonstrate that the CD56neg NK cell population is functionally active through CD16 activation, which contradicts previous reports of hypo-functionality of this population. The earlier studies assessed the functional capacity of CD56neg NK cells solely on tumor and cytokine stimulation [5, 7, 9, 16]. However, because CD56neg NK cells are primarily composed of FcRγ-deficient NK cells, the reported dysfunctions of CD56neg NK cells are likely reflecting the functional characteristics of g-NK cells, which have been shown to have a poor response to tumor and cytokine stimulation [2]. Indeed, our analysis showed that FcRγ-expressing CD56neg NK cells were responding to K562 and cytokine stimulation, albeit the response was lower than CD56pos NK cell (Fig 3B and 3C). Furthermore, previous studies mostly employed a negative gating strategy (CD3-CD14-CD19-) to identify NK cells, which may have resulted in contamination of non-NK cells and skewed the analysis of CD56neg NK cell population [7, 9]. Similar results have been reported in recent studies that employed CD7 or NKp80 as a gating

strategy for NK cells, suggesting that CD56neg NK cells are not entirely dysfunctional [14, 17]. Still, variations in the activation and phenotype levels among CD56neg NK cells from our analysis suggest a possibility that some portion of these cells may be dysfunctional or exhausted [17].

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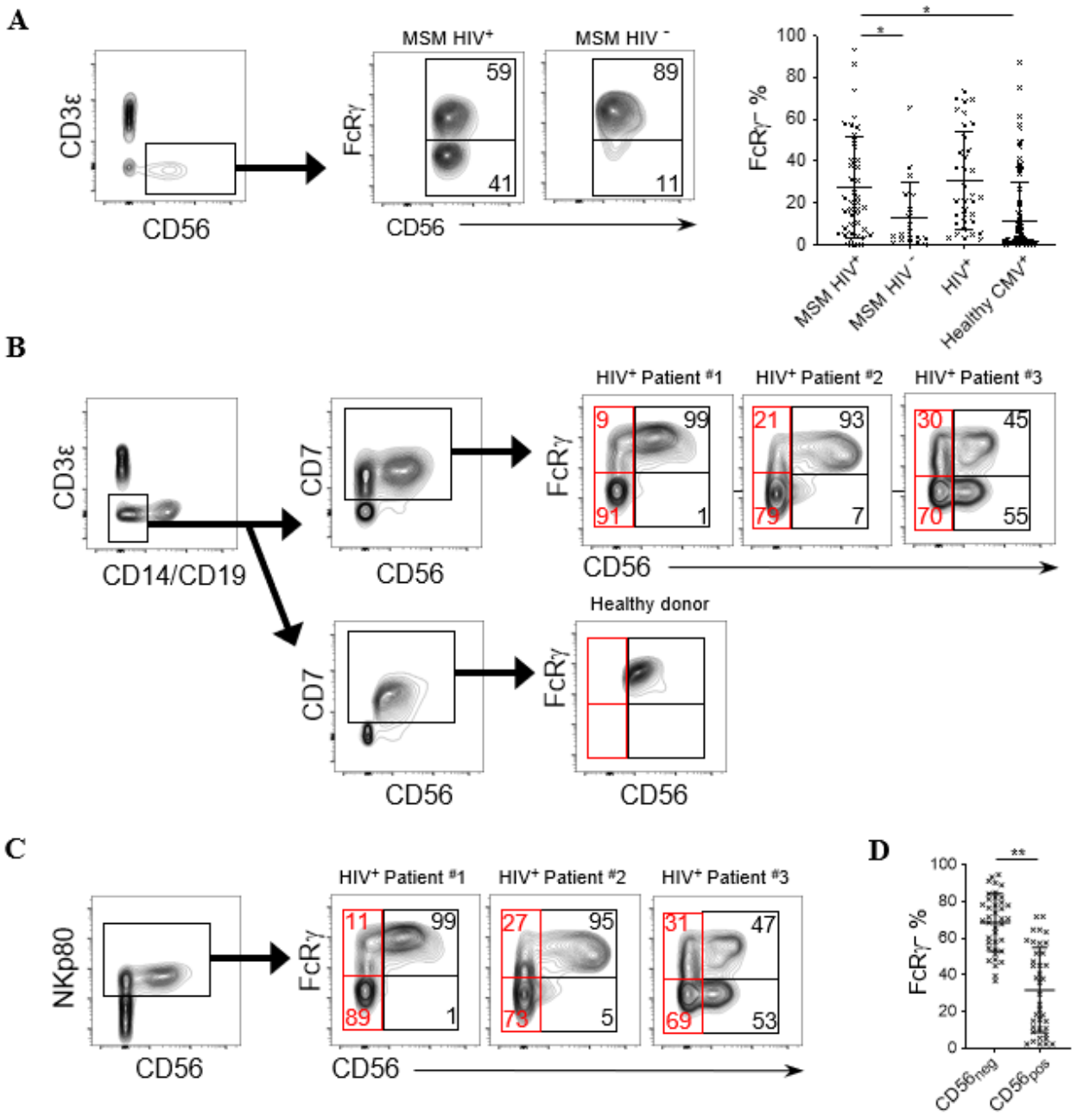


Figure 3.1. The majority of CD56neg NK cells in ART-naïve HIV patients are FcRγ-deficient

PBMCs from HIV+ acute patients were analyzed by flow cytometry for the presence of FcRγ-deficient NK cells using (A) CD14-CD19-CD3-CD56+, (B) CD14-CD19-CD3-CD7+, and (C) CD14-CD19-CD3-NKp80+ NK cell gating strategies. Contour plots show presence of FcRγ-

deficient NK cells in representative HIV+ patients and healthy donor. Relative frequencies of FcRγ expression are indicated among CD56neg (red box) and CD56pos (black block) populations. (D) Frequencies of FcRγ-deficient NK cells within CD56neg and CD56pos NK cells gated using CD3-CD14-CD19-CD7+ gating strategy. All the following analysis of NK cells in this study used this gating strategy.

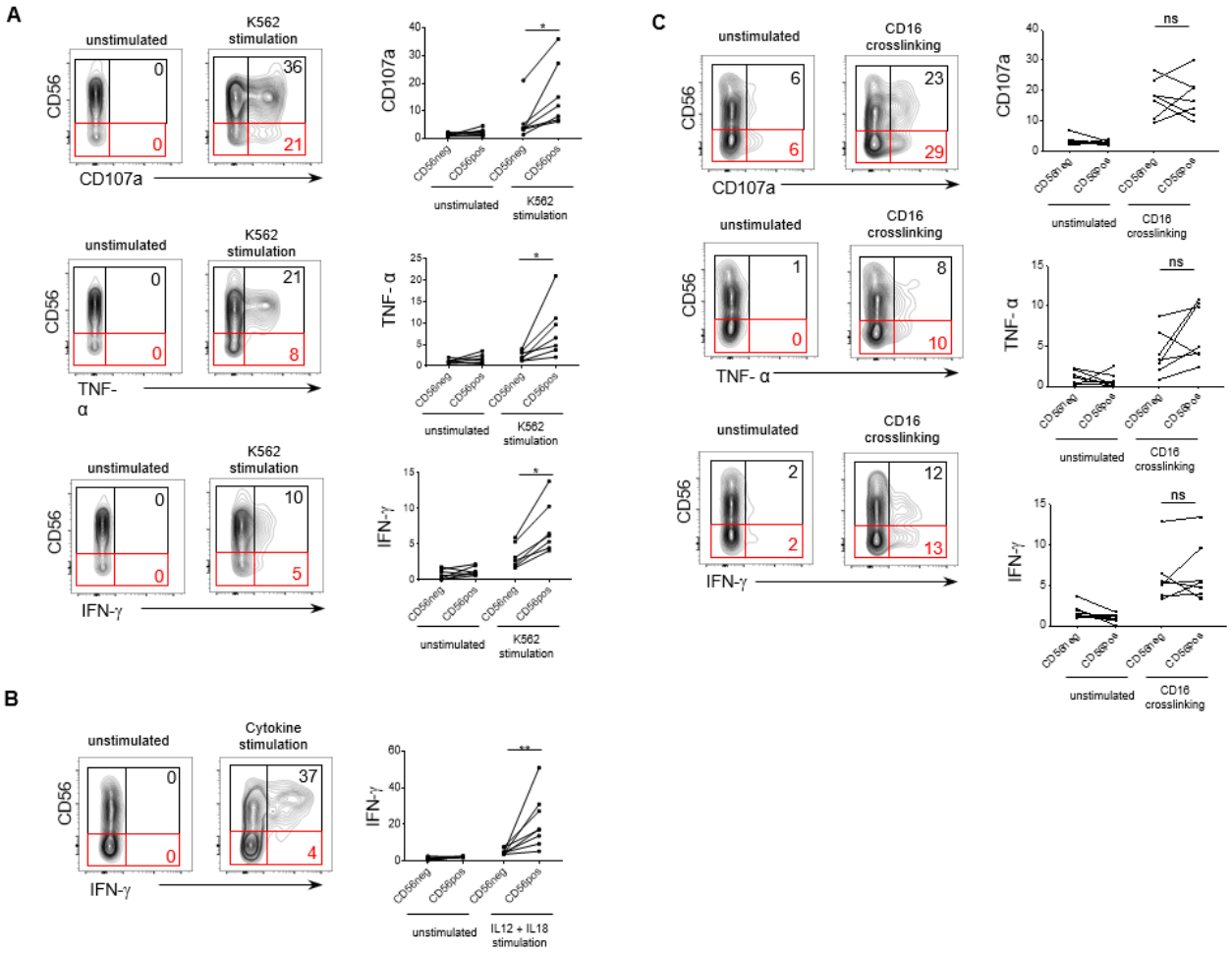
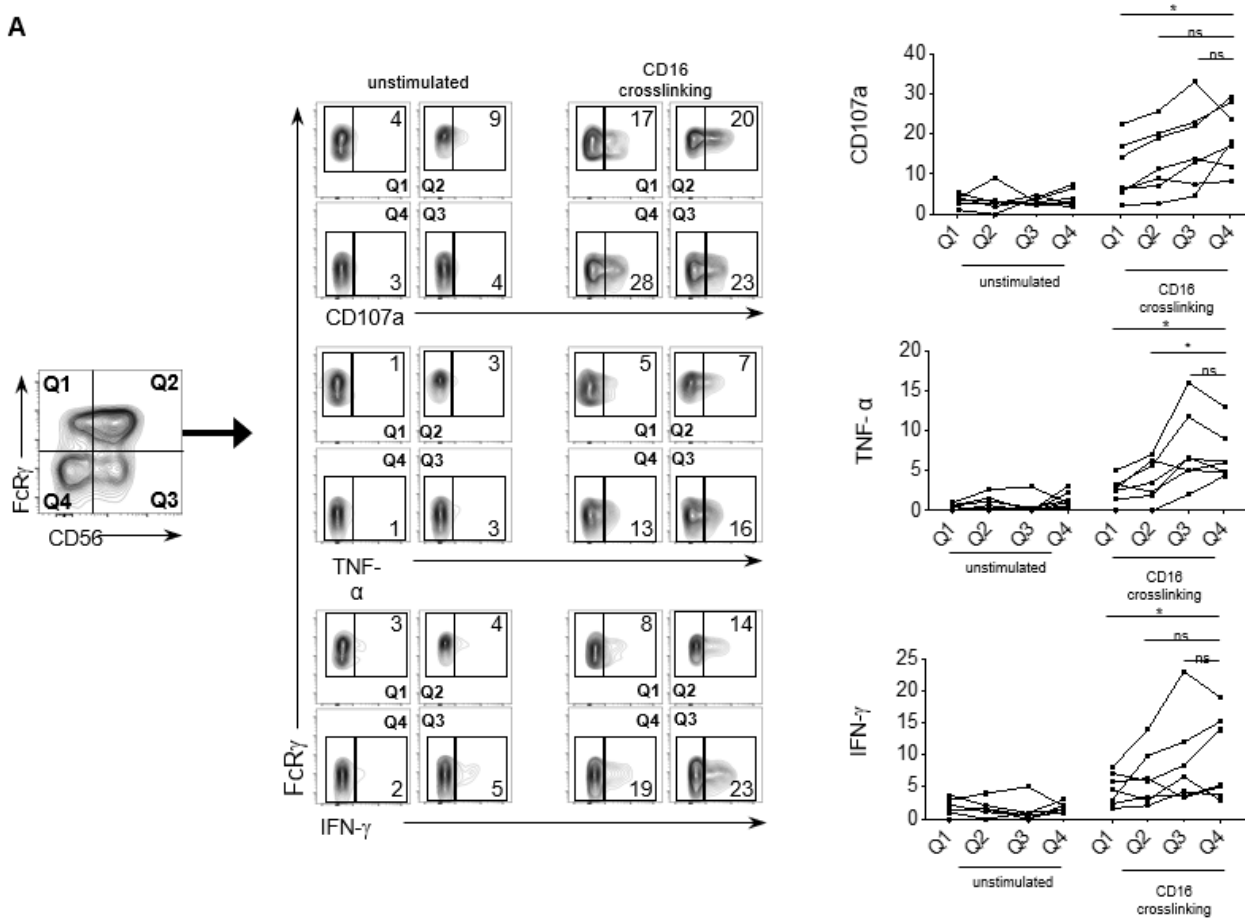


Figure 3.2. CD56neg NK cells show functional capability upon CD16 activation

Dot plots from one representative HIV+ patients depicting relative percentages of CD56neg (red box) and CD56pos (black box) NK cells that produced CD107a, IFN-g, and TNF-a following incubation with K562 tumor cells (A), combination of cytokines (B), and response to immobilized anti-CD16 cross-linking (C). Line graphs show percentages of CD56neg or CD56pos NK cells that expressed CD107a, IFN-g, and TNF-a following indicated stimulation from multiple HIV+ individuals (n=6). Dots connected by a line represent data obtained from the same donor sample.


A
unstimulated
CD16 crosslinking
CD107a
TNF-α
IFN-γ
FcRγ
CD56
Q1
Q2
Q3
Q4
4
9
17
20
3
4
28
23
1
3
5
7
1
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CD107a
TNF- α
IFN-γ
unstimulated
CD16 crosslinking
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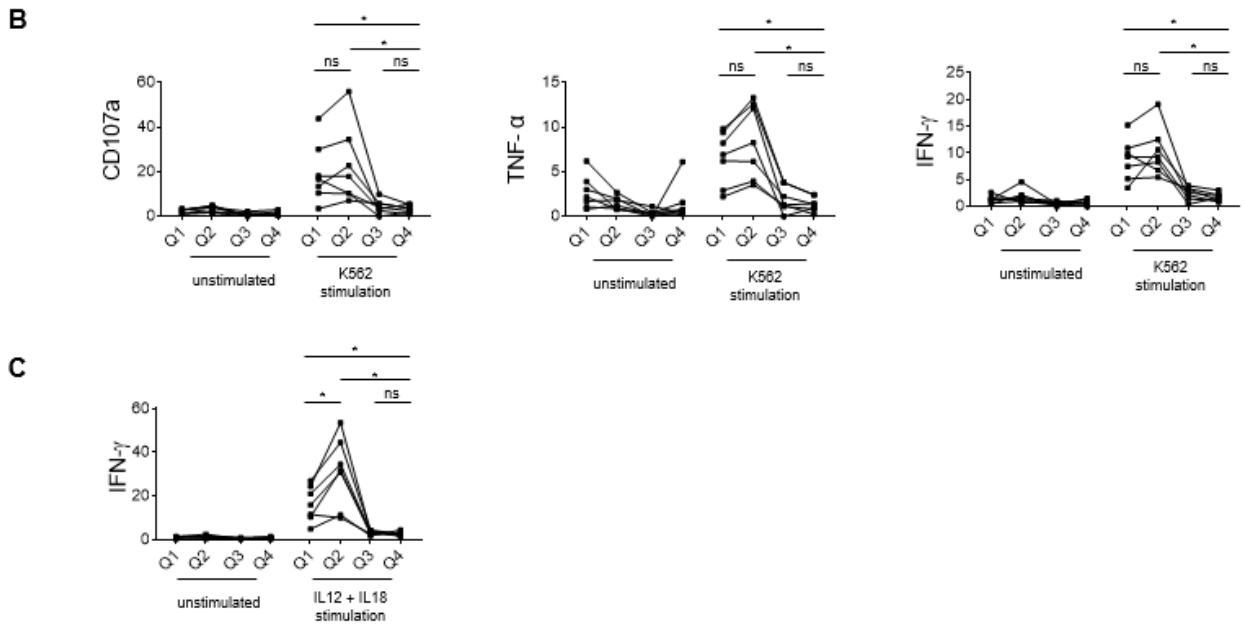

Figure 3.3. FcRγ-deficient CD56neg NK cells show robust activation upon CD16 stimulation

CD14-CD19-CD3-CD7+ NK cell from was divided into 4 quadrants in respect to their FcR□ and CD56 expression (Q1 : FcRγ+CD56neg, Q2 : FcRγ+CD56pos, Q3 : FcRγ-CD56pos, Q4 : FcRγ-CD56neg). (A) Dot plots from one representative HIV+ patients showing expression of CD107a, IFN-g and TNF-a from indicated quadrants in response to immobilized anti-CD16 cross-linking. Line graphs show percentages of CD107a, TNF- α, and INF-γ following CD16 crosslinking (A), K562 tumor (B) and cytokine stimulation (C) from indicated quadrants of NK cells from multiple HIV+ patients (n=6). Dots connected by a line represent data obtained from the same donor.

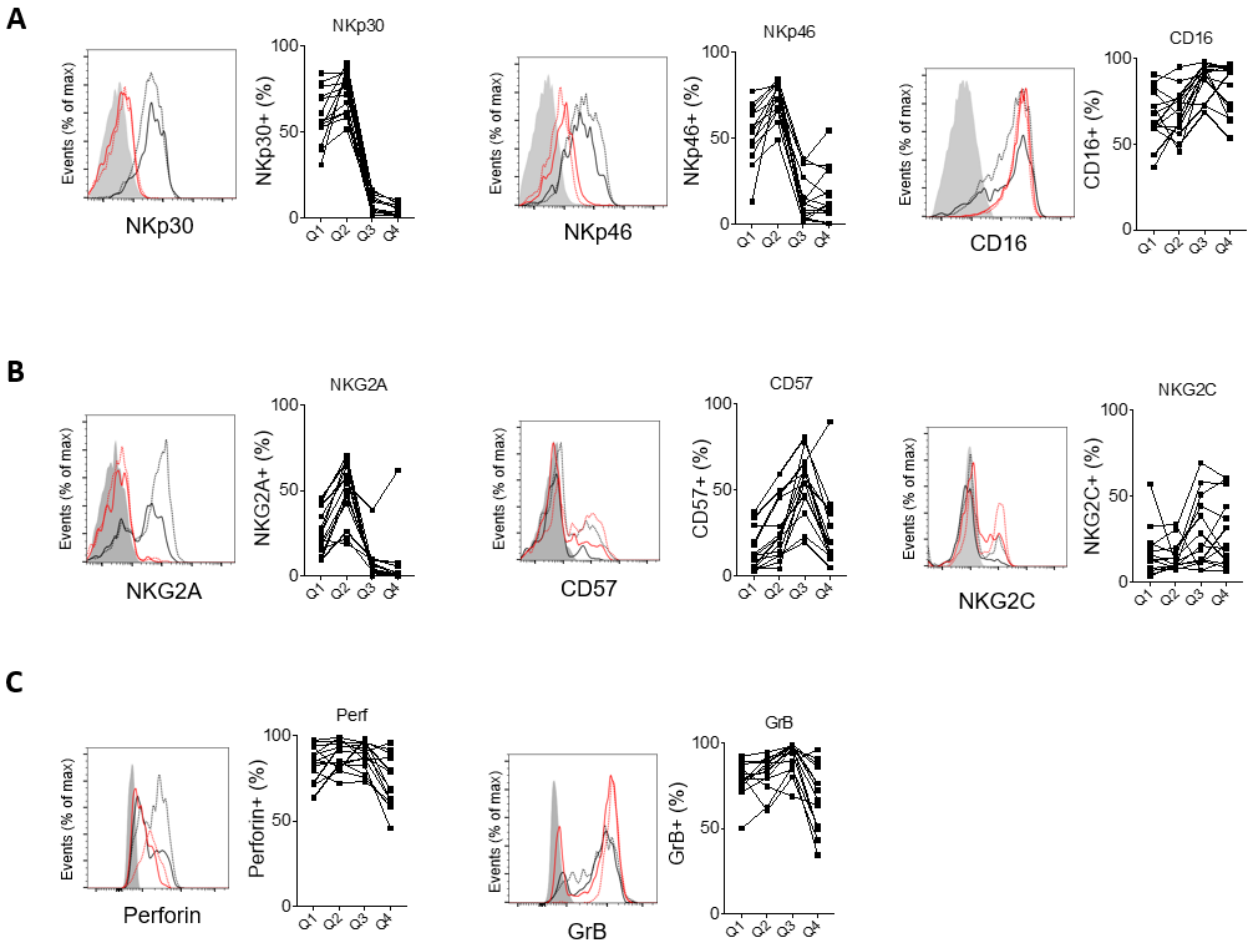

Fig 3.4. Phenotypic characteristics of FcRγ-deficient CD56neg NK cells resembles FcRγ-deficient CD56pos NK cells

Histograms show expression of cell surface markers (A), adaptive NK cell markers (B), and intracellular markers (C) on FcRγ+CD56neg (Q1, black solid line), FcRγ+CD56pos (Q2, black dotted line), FcRγ-CD56pos (Q3, red dotted line), and FcRγ-CD56neg (Q4: red solid line) NK cell population compared to control staining (shaded peak). Line graphs show percentage of indicated markers expressed from each quadrant from multiple HIV+ individuals (n=8). Dots connected by a line represent data obtained from the same donor.

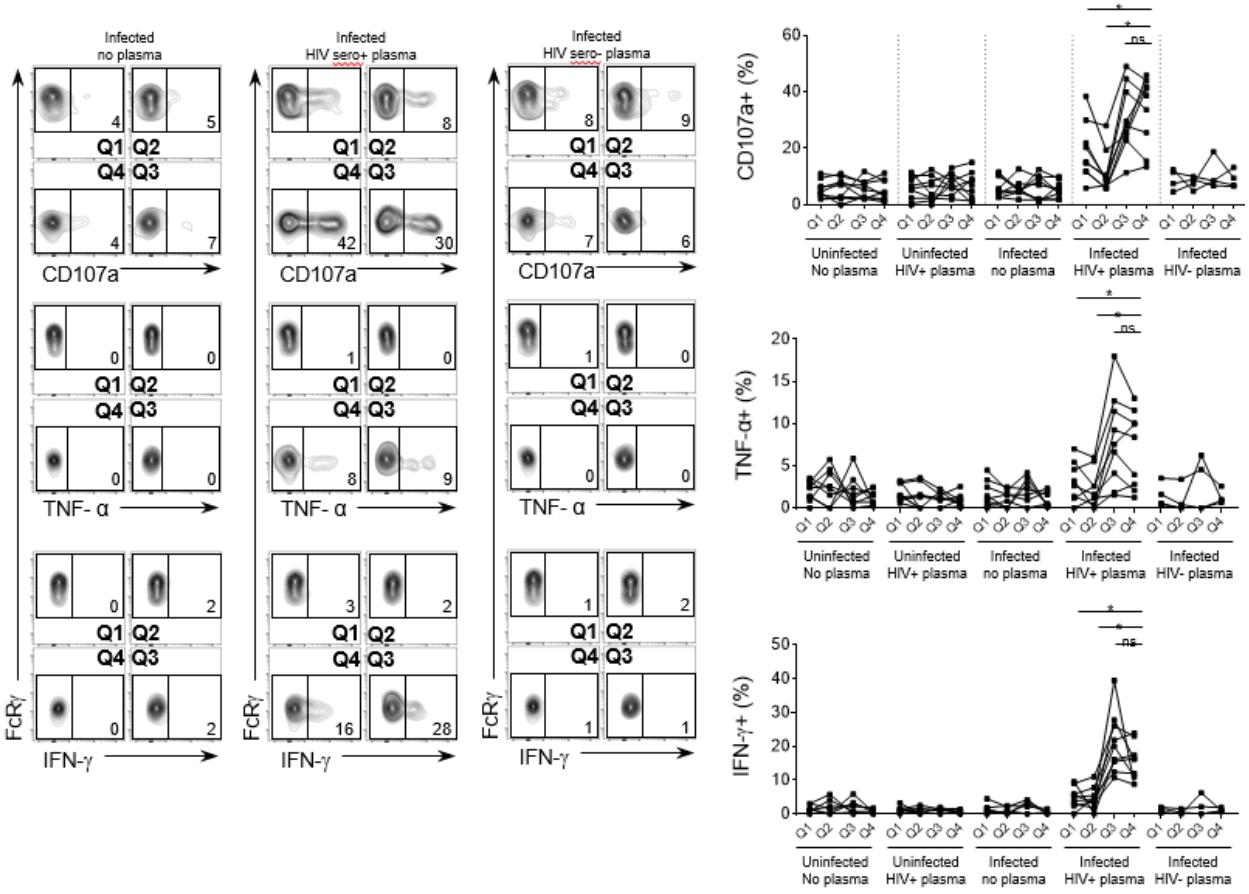

Fig 3.5. CD56neg NK cells show enhanced functional capability against HIV-infected target cells in presence of seropositive plasma

PBMCs were cultured with mock- or HIV-infected CEM.NKR cells in the presence or absence of HIV seropositive or seronegative plasma. Dot plots from one representative HIV+ patient's sample show production of CD107a, TNF-α and IFN-γ by indicated quadrants of NK cells (Q1 : FcRγ+CD56neg, Q2 : FcRγ+CD56pos, Q3 : FcRγ-CD56pos, Q4 : FcRγ-CD56neg) following the incubation. Line graphs show percentage of CD107a and indicated cytokines expressed from each quadrants from multiple HIV+ patients (n=8). Dots connected by a line represents data obtained from the same donor.

4. Role of Granzyme B during NK cell cytotoxicity

4.1 Abstract

Natural killer (NK) cells use lytic granules containing granzymes and perforin to perform cytotoxicity against cancer or virus-infected cells. Multiple types of granzymes are released by activated NK cells, which transport into target cells through pores created by the perforin and trigger apoptotic pathways. Granzyme B is the most abundant granzyme which extensive studies delineated its crucial role during NK cell mediated cytotoxicity. However, it remains unclear whether NK cell cytotoxicity is dependent on granzyme B to eliminate target cells. In this study, we used CRISPR Cas9 to generate NK cells that lack the expression of granzyme B and perforin. These cells retained stable expression of activating surface receptors, but granzyme B knockout cells exhibited lower levels of granzyme H and granzyme M expression compared to control knockout cells. Importantly, we demonstrated that absence of granzyme did not affect cytotoxicity of NK cells against tumor, while absence of perforin completely disabled NK cell cytotoxicity. These results imply that the cytotoxic role of granzyme B could be substituted by other types of granzymes.

4.2 Introduction

Natural killer (NK) cells are cytotoxic innate lymphocytes that can lyse cancer or virus infected cells [1]. NK cells mediate their cytotoxicity by releasing lytic granules containing granzymes and perforin. Granzymes, expressed in both NK cells and cytotoxic T cells, are a family of serine proteases, which play a crucial role during the process of killing target cells by inducing apoptosis [2]. When NK cells are activated, granzymes transport into target cells through pores created by the protein perforin. Once inside the target cell, granzymes can trigger apoptotic pathways by cleaving specific substrates, such as caspases, which are crucial for the

execution of apoptosis. Additionally, granzymes have been associated with other functions, such as regulating inflammation and the immune response, as well as modulating viral replication [3].

Five different types of granzymes (granzyme A, B, H, K, and M) are discovered in humans. NK cells release several different granzymes during their degranulation, thus the killing of a target cell is likely a result of a combined action of different granzymes [3]. Granzyme A (GrA) and Granzyme B (GrB) are the most abundant and well-characterized granzyme, and they were shown to cleave and activate caspases and other cellular proteins that regulate apoptosis [3]. Studies with GrB KO mice have shown that cytotoxicity is impaired in the absence of GrB, and even more so when both GrA and GrB are absent [4-7]. While the remaining granzymes have not been studied as extensively as GrA and GrB, studies have begun to uncover their potential roles. Granzyme K was shown to process similar substrates as GrA [8], and granzyme M was shown to promote a caspase-independent mechanism of cell death [9]. Granzyme H, which is only found in humans and not in mice, was shown to interfere with viral replication in Adenovirus infected cells [10].

Although numerous studies have revealed the mechanism of GrB-induced cytotoxicity, it is still uncertain whether GrB is absolutely required for NK cell cytotoxicity. Most *in vitro* studies examining the cytotoxicity of granzymes have been conducted using purified granzymes, which may skew the results since any types of serine protease can be potentially toxic after intracellular delivery [11]. Additionally, there are differences in the substrate specificity of human and mice GrB, thus applying findings from mice GrB studies to human GrB could be misleading [12, 13]. An ideal *in vitro* approach to investigate the cytotoxicity would involve utilizing NK cells with specific granzyme knockouts and evaluating their cytotoxicity against target cells.

In this study, we generated human NK cells that lack the expression of GrB and perforin using CRISPR Cas9 to knockout their genes. These GrB KO and perforin KO NK cells showed stable expression of activating surface receptors, however, GrB KO NK cells showed reduced expression of GrH and GrM compared to control KO. Importantly, we demonstrated that absence of GrB does not affect cytotoxicity of NK cells, while absence of perforin completely disabled NK cell cytotoxicity. These results imply that the role of GrB could be substituted by other types of granzymes. Furthermore, we propose using CRISPR Cas9 to generate specific knockouts of granzymes in human NK cells as a promising model for delineating the specific roles of each granzyme during NK cell cytotoxicity.

4.3 Material and Methods

4.3.1 Electroporation of Cas9/gRNA ribonucleoprotein

NK cells were isolated as CD3-CD14-CD19-CD56+ population from cryopreserved PBMCs from healthy human donors using Aria sorter. Isolated NK cells were mixed with feeder cells (irradiated PBMCs and RPMI 8866 cells) to obtain total number of 2 x 10^6 cells for optimal electrophoresis condition. For the knockout of GrB or perforin genes, two target-specific modified sgRNA (Table 1) were used together to maximize the chances of obtaining knockout cells, using ‘Knockout Guide Design’ tool provided by Synthego. sgRNA and Cas9 2NLS nuclease were pre-assembled at a 9:1 ratio in nucleofector solution provided within the human NK cell nucleofector kit (Lonza). 0.5 nmol RNP complex was electroporated into 2M expanded NK cells using the Nucleofector Device (Lonza) program “X-1”. Pulsed cells were incubated in 37C incubator with serum-free RPMI1640 for 2hrs and further expanded as stated below.

4.3.2 Expansion of electroporated NK cells

Pulsed cells were resuspended with NK cell culture medium (RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin-streptomycin, 5% human AB serum, 500 U/mL IL-2, sodium pyruvate, 1X MEM non-essential amino acids, and 10ug/mL phytohemagglutinin-L) and cultured in U-bottom 96w plate containing 50 cells per well. Seven days post incubation, each wells were screened for their knockout efficiency by flowcytometry analysis, and wells containing higher than 90% KO efficiency were transferred into 48w plates for further analysis.

4.3.3 Flow cytometry

Cells were stained for flow cytometry analysis as described in Chapter 2.

4.3.4 Cytotoxicity assay

Target cells (K562, 721.221, and Raji) were stained with 10mM of Calcein AM in serum-free RPMI1640 for 2 hours and washed. Stained target cells were plated into U-bottom 96w plate along with electroporated NK cells (E:T 10:1) for 4 hours. The amount of target cell apoptosis was measured by examining RFU from the supernatant (Ex/Em 485/530nm).

4.3.5 Statistical analysis

All statistical analyses were performed with Graphpad Prism. The Wilcoxon matched pairs signed rank test was used for all assays. Differences were considered significant when $p < 0.05$.

4.4 Result

4.4.1 CRISPR KO of granzyme B and perforin

GrB or perforin knockout cells were generated using CRISPR Cas9 electrophoresis as mentioned in the method section. After expanding the treated NK cells for 10 days, we validated the success of the gene knockout by staining for GrB and perforin expression. Our flow cytometry analysis revealed that the control KO NK cells displayed full expression of both GrB and perforin, whereas the GrB KO and perforin KO NK cells exhibited a significant reduction in GrB or perforin expression, respectively, with a knockout efficiency exceeding 90% (Fig 1).

4.4.2 KO of granzyme B effects the expression of granzyme H and granzyme M

To validate the preservation of functional surface receptors following electroporation, we examined the expression of NKp30, NKp46, and CD16 on the surface of expanded NK cells. Although we noticed slight variations in the expression levels of surface receptors among individual sets of expanded NK cells, there was no significant differences in the expression levels of these receptors between with control KO, GrB KO, and perforin KO (Fig 2A and 2B). Subsequently, we investigated the potential impact of GrB or perforin KO on the expression of other granzymes. As expected, all control KO and perforin KO expanded NK cells exhibited full GrB expression, whereas GrB KO NK cells showed less than 5% GrB expression. Perforin staining demonstrated complete downregulation in perforin KO NK cells, while more than 90% of control KO and GrB KO NK cells expressed perforin. All expanded NK cells regardless of their KO status fully expressed granzyme A. The expression of granzyme K varied among different sets of expanded NK cells, although no significant differences were detected between control KO, GrB KO, and perforin KO NK cells. We observed significantly lower expression levels of granzyme H and granzyme M in GrB KO NK cells compared to control KO and

perforin KO NK cells. However, the expression levels varied substantially among individual sets of expanded NK cells, particularly for granzyme H.

4.4.3 NK cells show cytotoxicity against tumor cells without granzyme B

To investigate the impact of GrB or perforin KO on NK cell cytotoxicity, we co-cultured calcein AM stained target tumor cells (K562, 721.221 and Raji) with the expanded NK cells. We quantified the level of tumor cell lysis by measuring the fluorescence intensity of hydrolyzed calcein AM released into the supernatant, which is proportional to the extent of NK cell-mediated cytotoxicity [14]. To serve as negative and positive controls, we measured the fluorescence intensity in wells that contained only the calcein AM stained target cells (representing spontaneous lysis) and wells with the stained target cells lysed by 0.1% detergent (representing total lysis).

The fluorescence intensity in the supernatant of wells containing tumor cells incubated with control KO NK cells was more than 50% of the intensity observed in total lysis, indicating that NK cells without any knockout were capable of killing roughly half of the target tumor cells. In wells with GrB KO NK cells, the intensity was similar to that of the control KO NK cells, suggesting that the absence of GrB did not affect the NK cell's cytotoxicity. Conversely, wells containing perforin KO NK cells showed no signs of cytotoxicity, as the fluorescence level was equivalent to that of the spontaneous lysis, highlighting the necessity of perforin for NK cell cytotoxicity. Since NK cells can recognize Raji cells through the CD16 receptor in the presence of rituximab, we investigated the effect of adding rituximab on NK cell cytotoxicity. Since NK cells are capable of recognizing Raji cells via the CD16 receptor when rituximab antibody is present, we examined the impact of rituximab on NK cell cytotoxicity for our assay condition. The addition of rituximab substantially increased the cytotoxicity of control NK cells by

approximately 30% (Fig 3C). However, the increase in cytotoxicity was not significant in GrB NK cells, leading to a significant difference in cytotoxicity between control KO and GrB NK cells against Raji cells when rituximab was present. On the other hand, perforin NK cells demonstrated no cytotoxicity even with the presence of rituximab.

4.5 Conclusion

In this study, we utilized the CRISPR Cas9 technique to knockout expression of either GrB or perforin from expanded NK cells. Both GrB and perforin KO NK cells retained stable expression of key functional surface receptors, while GrB KO NK cells displayed decrease in GrH and GrM expression level. Intriguingly, killing assay against tumor target cells indicated that GrB KO NK cells exhibited similar levels of cytotoxicity as control knockout cells, while perforin KO NK cells displayed no cytotoxicity. Our findings suggest that the absence of GrB during NK cell cytotoxicity may be compensated by other types of granzymes, although the specific granzyme that takes on this role remains unknown. Nonetheless, our study presents a promising approach to investigate the function of each granzyme further by potentially using the CRISPR Cas9 technique to knock out specific granzymes.

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14. Wang, X.M., et al., *A new microcellular cytotoxicity test based on calcein AM release.* Hum Immunol, 1993. **37**(4): p. 264-70.

Gene	Genecode Transcript	Guide RNA Sequence
GZMB	ENST00000216341 (primary) (-)	CCUUCAGGGGAGAUCAUCGG
GZMB	ENST00000216341 (primary) (-)	UAAGAUAAGCCAUGUAGGGG
PRF1	ENST00000373209 (primary) (-)	GAUGCCCAGGAGGAGCAGAC
PRF1	ENST00000373209 (primary) (-)	GCAGGAGAAGGAUGCCCAGG

Table 4.1. Sequence of guide RNA used for CRISPR Cas9 experiment to knockout GrB and perforin

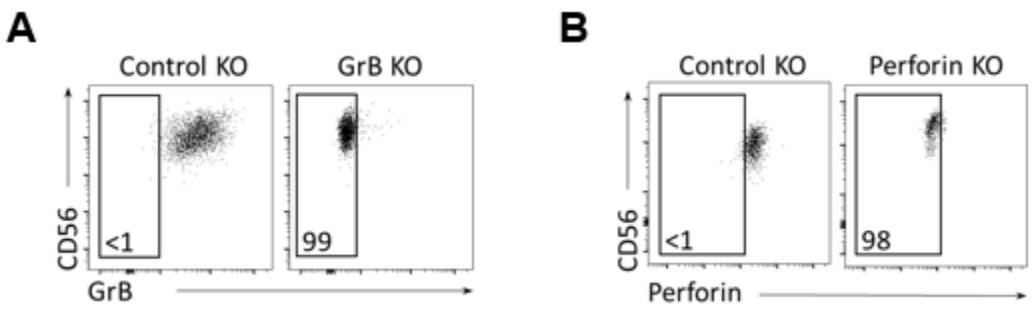
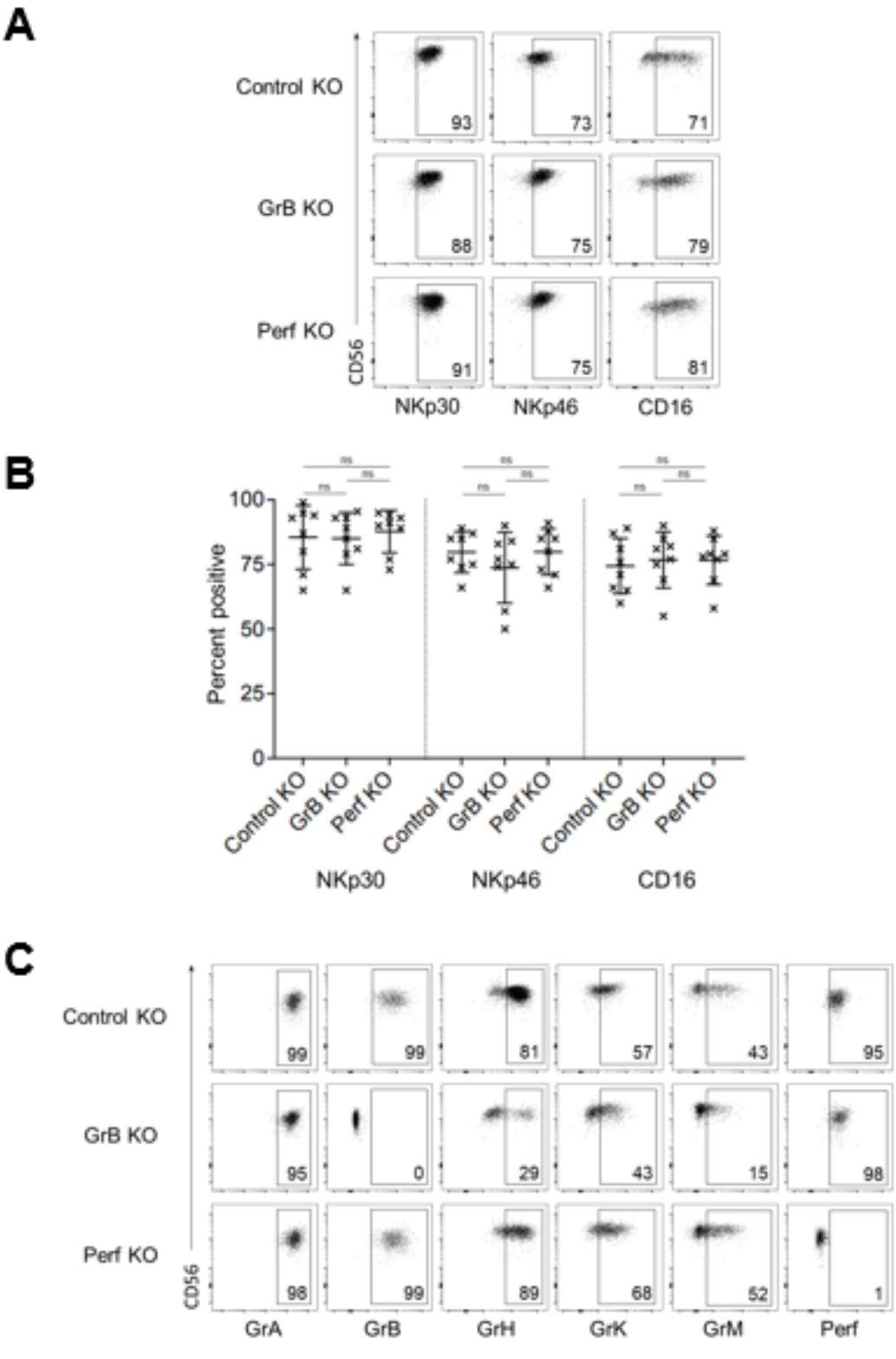


Figure 4.1 Successful knockout of granzyme B and perforin using CRISPR Cas9

Dot plots show expression of granzyme B (A) and perforin (B) in enriched NK cells expanded for 7 days after electroporation with non-targeting CRISPR Cas9 ribonucleoprotein (Control KO), granzyme-B targeting (GrB KO), or perforin targeting (Perforin KO) siRNA. The numbers in the boxes indicate the percentage of granzyme B or perforin negative cells among the total expanded NK cells under each condition. The data are representative of three independent experiments using expanded NK cells from four donors.


A
Control KO
GrB KO
Perf KO
CD56
93
73
71
88
75
79
91
75
81
NKp30
NKp46
CD16
B
Percent positive
100
75
50
25
0
ns
Control KO
GrB KO
Perf KO
NKp30
NKp46
CD16
C
Control KO
GrB KO
Perf KO
CD56
99
99
81
57
43
95
95
0
29
43
15
98
98
99
89
68
52
1
GrA
GrB
GrH
GrK
GrM
Perf

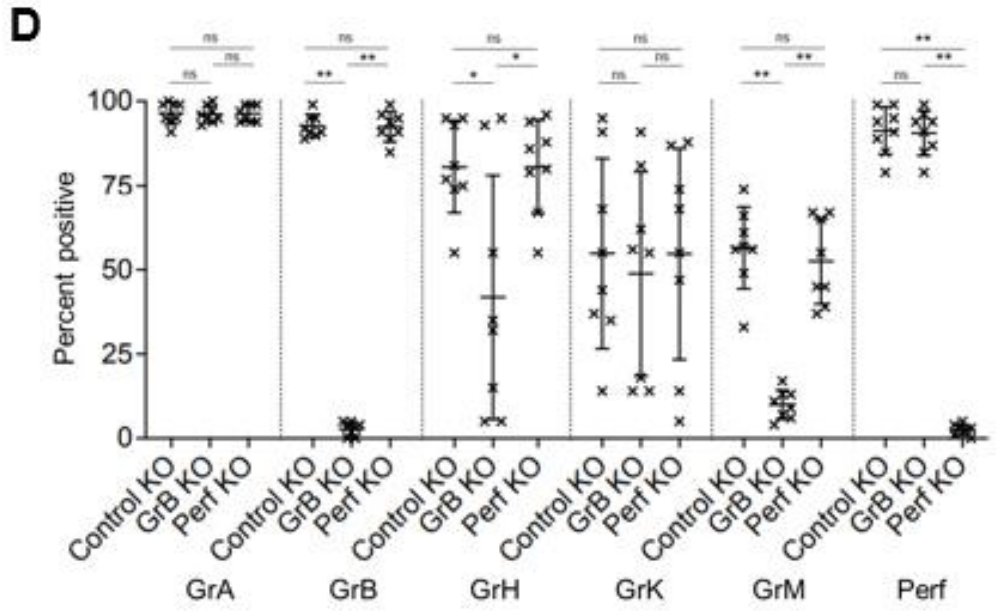


Figure 4.2 Phenotypic analysis of expanded knockout NK cells

Dot plots show expression of surface receptors (A) and intracellular granzymes and perforin (C) from representative control KO, granzyme B KO, and perforin KO expanded NK cells. The numbers in the boxes indicate the percentage of expanded NK cells expressing indicated markers. Dot graph show frequencies of expanded NK cells that are positive of indicated surface receptors (B) and intracellular granzymes and perforin (D). 8 individual sets of expanded NK cells were analyzed for each knockout conditions.

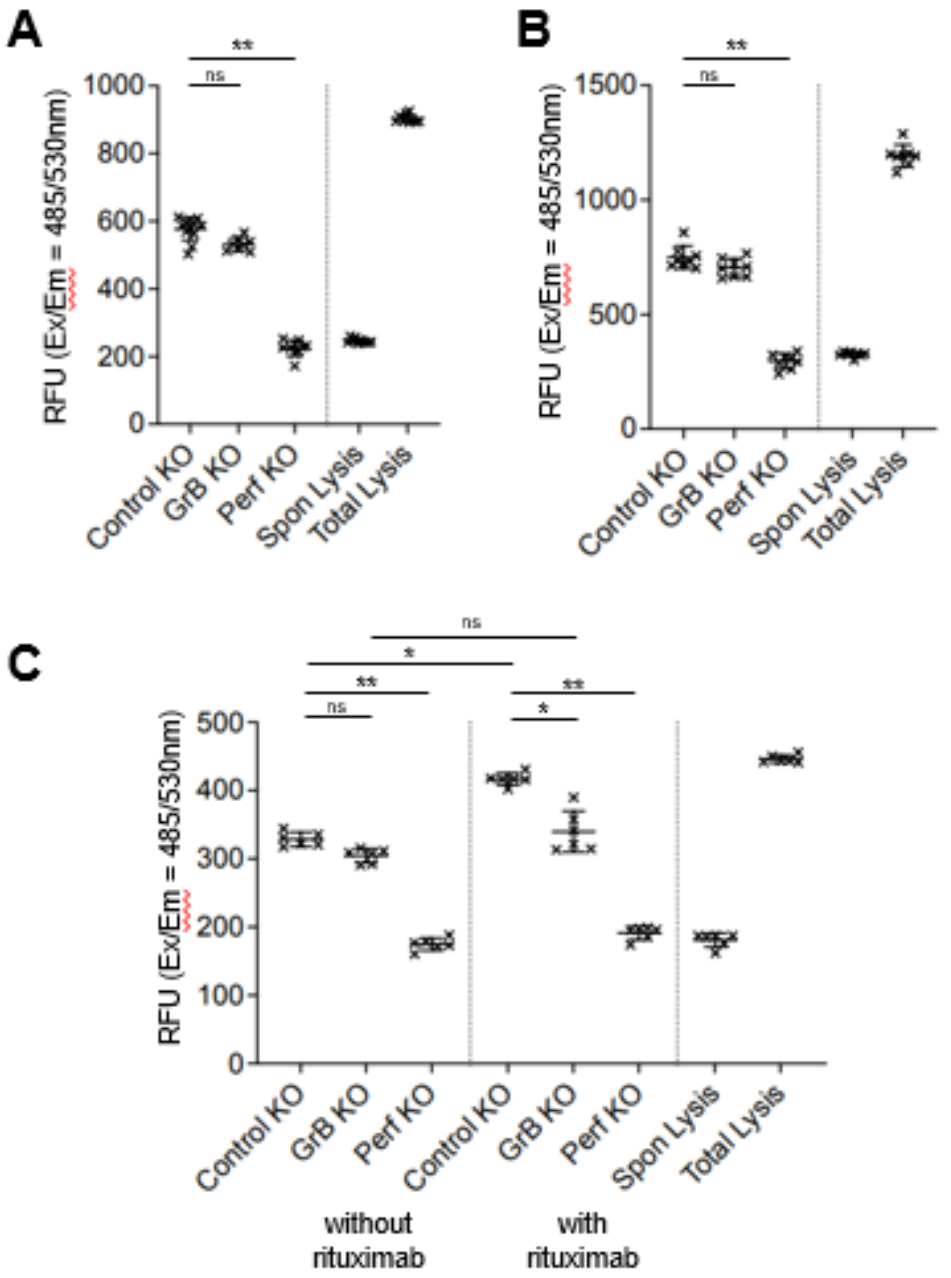


Figure 4.3 NK cell cytotoxicity is not affected by absence of granzyme B

The dot graphs show the fluorescence levels (Ex/Em 485/530nm) measured from the supernatant of wells that contain expanded NK cells with the indicated knockout, which were incubated with K562 (A), 721.221 (B), Raji (C) target cells or the absence of target cells (spon lysis).