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Down-Regulation of Natural Killer Cell Activation in Response to Influenza Virus in Older Adults

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by

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ABSTRACT

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Yu Jing
Eastern Virginia Medical School and Old Dominion University, 2007
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Immune senescence contributes to influenza-associated high mortality and morbidity and reduced vaccine efficacy in elderly people. Type I T cell (Th1)-mediated immunity plays a significant role in immune responses to influenza infection and vaccination. Natural killer (NK) cells secrete significant amount of IFN-γ, a hallmark Th1 cytokine, in response to influenza infection. How aging influences human NK cell IFN-γ production in response to influenza virus has not been well documented. In this study we employed human peripheral blood mononuclear cells (PBMC) and performed intracellular cytokine staining and flow cytometry primarily to investigate how aging influences NK cell activation with respect to IFN-γ production in response to influenza virus. We have found that NK cell IFN-γ production, mediated by both soluble factors and cell-cell contact factors is down-regulated in elderly subjects compared to young subjects in response to influenza virus. As for soluble factors, IFN-α and IFN-γ are proven to be important in inducing NK cell to produce IFN-γ. The frequencies of IFN-α-producing plasmacytoid dendritic cells (pDC) and IFN-γ-producing T cells in PBMC are lower in the elderly subjects than in the young subjects. Further more, pDC from the young subjects produce more IFN-α on a per-cell basis and mediate NK cells to produce more

IFN-γ than pDC from the elderly subjects. As for cell-cell contact factors, the expressions of NKp44 and NKp46, two natural cytotoxicity receptors on NK cell surface specifically recognizing influenza hemagglutinin (HA) expressed on antigen presenting cells, are up-regulated upon influenza infection of PBMC, but display higher expression levels in older subjects than young subjects. Our data have demonstrated that aging-related numerical and/or functional impairment in pDC and T cells results in lower production of IFN-α and IFN-γ, and consequently contributes to the down-regulation of IFN-γ production in NK cells in response to influenza virus in older adults. How aging affects NK cell IFN-γ production through influencing cell-cell contact regulation between NK cells and antigen presenting cells remains to be elusive. It's our strong belief that our study and the related findings will help enrich our knowledge about how aging affects innate as well as adaptive immune system in response to influenza virus, and help build the fundamentals for developing more effective prophylactic and therapeutic approaches to fulfill the long-term goal of reducing influenza morbidity and mortality and improving the quality of life for the elderly.

This work is dedicated to my grandmother and to the memory of my grandfather for their love and affection throughout my life.

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I. INTRODUCTION

Influenza virus is highly contagious and among those pathogens with greatest potential to cause life-threatening epidemic and pandemic outbreaks (1). Influenza-related morbidity and mortality increase with age, particularly in people over 65 years old, making influenza and its most common complication, pneumonia among the top 5 leading causes of death for this age group (2, 3). Vaccination can effectively prevent the occurrence and severity of the disease (2, 4, 5). However, the vaccine efficacy is significantly lower in old people than in their young counterparts (3, 4, 6-12). The cause for the age-related increase in mortality and morbidity and reduction in vaccine efficacy is related to immune senescence (3, 4, 13). Investigations on how the immune responses to influenza virus are impaired in older adults will provide the fundamental for developing more effective prophylactic and therapeutic approaches for older people. Natural killer (NK) cells are the major lymphocytes in innate immune system and play a significant role in influenza-induced host defense by eliciting cytotoxicity and secreting IFN-γ (14). How aging influences NK cell activation, particularly with respect to their function of IFN-γ production in response to influenza virus has not been well documented. The *overall objective* of this study is to investigate the age-related changes and their potential causal factors in NK cell activation determined as the frequency of IFN-γ producing NK cells in response to influenza virus in older subjects.

I.1 Overview of Influenza Virus

Influenza viruses are members of the family of orthomyxoviridae with single stranded, negative sensed segmented RNA genome. Based on antigenic differences in viral nuclear protein (NP), influenza viruses are classified into A, B and C three types (15). Influenza A and B are the two types known to be associated with diseases in human and animal (15).

The journal model for this dissertation is *Journal of Immunology*.

- *Epidemiology of influenza in humans*

Influenza is a serious public health problem. According to Center for Disease Control, each year in the United States, 5% to 20% of the population gets influenza, over 200,000 people are hospitalized, and about 36,000 people die from influenza (16-19). Influenza is associated with high morbidity (over 50%) and mortality in people 65 years and older (over 70%). The high morbidity rate is also found in children less than one year old (as high as 70%) (1, 20). Majority of influenza occurs between late fall and early spring, indicating influenza is a seasonal disease (21).

Influenza viruses continuously undergo antigenic variation by two means: antigenic drift and shift (1). Antigenic drift involves minor changes in the envelope glycoproteins hemagglutinin (HA) and neuraminidase (NA) due to mutation, deletion or other changes in genes encoding these proteins during viral replication. Antigenic shift involves major changes in these molecules resulted from reassortment of viral genomic segments when more than one strain of influenza virus co-infect a host cell (1, 22). According to the antigenic diversity of HA and NA, there have been identified 16 HA subtypes and 9 NA subtypes of influenza A viruses (23). Changes in antigenecity of HA and NA allow influenza viruses to evade from host immunity and significantly increases the chance for infection (1).

Influenza virus is associated pandemic outbreak (24). Important influenza pandemic outbreaks in human history documented since 1889 include those in 1889 (H2N?), 1899 (H3N8), 1918 (H1N1), 1957 (H2N2), 1968 (H3N2) and 1977 (H1N1) (25). Three explanations have been addressed for the formation of a pandemic strain (1). Antigenic shift could facilitate generation of a new high-virulence strain. Direct transfer of a high-virulence avian strain to human could occur or a human strain that caused an epidemic many years ago could re-emerge (1, 26). Direct transfer of a high-virulence strain from avian animals to human has been confirmed by the influenza epidemics that took place in Hong Kong during 1997-1998 (27). Influenza transmission is through the aerosol route. Avian and domestic animals are the important hosts and reservoirs of influenza viruses

(1). Close contact with these animals may increase the chance for co-infection as well as direct transmission (1, 28).

- *Replication of influenza virus*

Influenza A genome contains 8 negative sensed RNA segments encoding for 10 proteins: polymerase B2 (PB2), PB1, polymerase A (PA), hemaglutinin (HA), NP, neuraminidase (NA), matrix proteins 1 and 2 (M1 and M2), and non-structural proteins 1 and 2 (NS1 and NS2) (15). The HA residing on the viral envelope mediates attachment of the virus to target cell via binding to sialic acid residues on glycoproteins of host cell membrane (29). After binding, influenza virion enters the cell by endocytosis and arrives in acidic endosome, where HA is cleaved and M2 ion channel is activated. Establishment of acidic environment in the virion induces alteration in M1/RNP and M1/HA interactions and conformational change in the HA, consequently activating fusion of viral envelope and endosomal membrane (15, 30). The nucleocapsid is released to the cytosol and transferred to the nuclear, in which viral replication takes place. Making use of m7G cap from cellular mRNA, the three RNA polymerases PB2, PB1 and PA function in concord to transcribe the viral genome into positive sense mRNA and cRNA, the former used for protein translation and the latter used as the template for generating new viral genome. Newly generated NP protein migrates to nuclear and associates with newly synthesized vRNA to form nucleocapsid. The accumulation of nucleocapsid in cytosol initiates the assembly (31, 32). Being facilitated by NA protein, the assembled virions are released from the cell via budding process and spread to other cells (31, 33).

- *Influenza viral-host interaction*

Like many other viruses, influenza virus encodes proteins that interact with cellular components and counteract the host anti-viral activity in favor of its own replication. Influenza NS1 protein can modulate viral RNA transcription and replication, and inhibit nuclear export of the host polyadenylated mRNA (34), mRNA splicing (35-37), and apoptosis (38). Further more, it is a potent inhibitor for IFN-α production and IFN-α

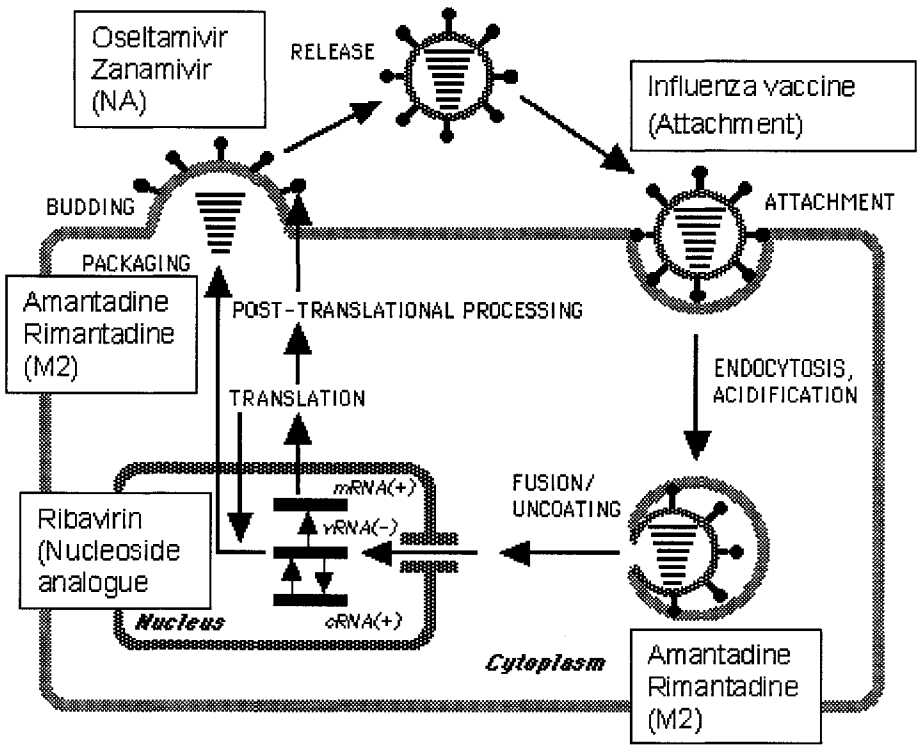


Figure 1. Replication of influenza virus and therapeutic approaches. (Modified, original figure from Microbiology @ Leicester: Virology: Orthomyxoviruses or http://www-micro.msb.le.ac.uk/3035/Orthomyxoviruses.html)

effector function (39). Particularly, NS1 has been known to be able to bind to dsRNA and inhibit dsRNA-induced IFN-α gene transcription (40, 41). It is also able to interfere with 2-5(A) synthetase/RNaseL as well as PKR activation pathways (42-45) to inhibit antiviral activity of IFN-α. A recent study has shown genes affected by NS1 are not restricted to IFN-α. Those coding for human macrophage inflammatory protein 1b (MIP1b), interleukin-12 p35 (IL-12 p35), IL-23 p19, RANTES, IL-8, and CCR7 are down-regulated by NS1, underlying NS1 may "specifically suppress DC maturation, migration, and NK and T-cell stimulatory activity" (46). In addition to NS1, influenza HA has been newly revealed to selectively suppress interleukin 12 p35 transcription in murine bone marrow-derived dendritic cells (47). IL-12 production is under the detectable level in human PBMC stimulated with influenza virus alone (48, 49). Whether NS1 and/or HA protein significantly contribute to the cessation of IL-12 production in human PBMC remains unknown.

- *Pathogenesis of influenza virus*

Influenza virus infects and exclusively replicates in epithelial cells of the respiratory tract and causes most significant pathology in the lower respiratory tract (1). Release of viral particles from the apical surface of the cells restricts systemic spread of the virus but facilitates accumulation of virus in the lumen of the respiratory tract for transmission through air ways (1). Local inflammation and edema are associated with infected tissues. Ciliated epithelial cells can be vacuolated, lose their cilia and become desquamated. Viral antigen can be detected in epithelial cells and mononuclear leukocytes. In the case of severe primary viral pneumonia, mononuclear leukocyte infiltration, capillary dilation and thrombosis are predominant and viral antigens can be detected in type 1 and type 2 alveolar cells and macrophages (50). Influenza A and B can cause the same spectrum of diseases. Primary viral pneumonia, combined viral-bacterial pneumonia and bacterial pneumonia following an influenza infection are three severe conditions associated with influenza. Complications are most often in the elderly (1)

- *Treatment of influenza*

Influenza can be treated or prevented by chemical drugs specifically targeting M2 ion channel (amantadine and rimantadine), NA protein (Zanamivir and Oseltamivir) or viral RNA replication (ribavirin,(51, 52). Distinct from chemical drugs, influenza vaccination has been proved to be the most cost-effective method in reduction of influenza-related mortality and morbidity (53, 54). Because of the antigentic drift, the strains used for generating the vaccine have to be determined every year. The vaccine contains three influenza strains, one A (H3N2) strain, one A (H1N1) strain, and one B strain, each representing one of the three groups of viruses circulating among people in a given year (1, 55).

There are two types of licensed vaccines. The first is the inactivated or split trivalent influenza vaccine (TIV). It contains killed virus and are effective for both young and elderly subjects. The TIV is highly recommended to people at high risk of influenza, including children 6-59 months, people 50 years and older, pregnant women and people

with chronic medical conditions (16, 56). Though effective, the TIV efficacy is significantly lower in elderly subjects than that in the young, making the elderly people less effectively protected by the vaccination (3, 12). Immune senescence is believed to contribute to the low vaccine efficacy in the older adults (13). The second type of influenza vaccine is a live attenuated influenza vaccine (LAIV) or FluMist. FluMist is recommended to healthy people between 5-49 years old and has been shown more effective to stimulate immune responses in healthy young adults or even high-risk subjects, such as children diagnosed with asthma than TIV (16, 57, 58).

I.2 Significance of Influenza Virus in Aging

Influenza/pneumonia is the fifth leading cause of death for people 65 years and older (59). As recorded for the United States in year 2000, influenza/pneumonia claimed 60,261 lives aged 65 years and older, which constituted 0.173% of the total population and 3.3% of elderly of the same age range in the country (59). Influenza infection is associated with high mortality and morbidity rates in the elderly. However, the vaccine efficacy is significantly lower in older people than that in the young. The lower vaccine efficacy is manifested by age-associated decline in intact humoral response to influenza vaccination and low protective rate of the vaccines. In particular, seroconversion, measured as fold of increments in sera antibody titer upon vaccination, and seroprotection, measured as certain dilution factor of the sera antibody, rates are significantly lower in older subjects than those in the young (8, 60). More over, in contrast to 70-90% protective rate in young adults, there is only 30-40% of influenza infection that can be prevented by vaccination in the elderly (3, 8-11). With the unprecedented enlargement of the body of aged population, it is believed influenza will have a significant impact on the quality of life for the elderly.

I.3 Overview of Aging

Aging is defined as "a process that converts healthy adults into frail ones with diminished reserves in most physiologic systems and an exponentially increasing vulnerability to

most diseases and to death" (59). Aging has been viewed as a distinct variable from diseases. Richard Miller has proposed that slowing-down of aging alone will produce longer life expectation than curing any individual or combinations of the diseases that are leading causes of death for the elderly (Figure 2) (59, 61). According to R. Miller, a question yet to be answered is "which age-related changes can be considered a part of normal aging". A study to examine aging effect on a particular event by comparing healthy young and "healthy" elderly subjects most time has to be in compliance with the fact that elderly are rarely completely "disease-free" and any disease could be a potential "confounding" factor for normal aging (59). On the other hand, if the elderly subjects whose health status is strictly comparable to that of their younger counterparts are used in a study, how much these extremely healthy subjects would represent the typical elderly population will be questionable.

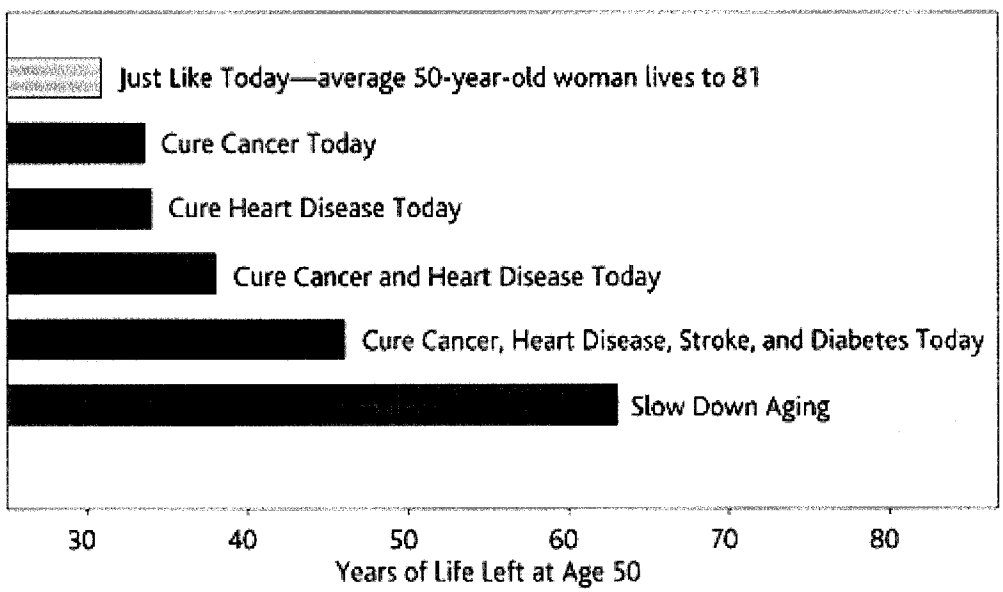


Figure 2. Life expectancy at age of 50 by disease categories. (Source: R.A. Miller/ University of Michigan)

- *Epidemiology of aging*

Aging of human population has formed a global trend. The growing elderly population is not only caused by "general increase in the overall population size" but also more influenced by increased longevity of older people accompanied by declining birth rate following the birth boom in 1940's and 1950's (59). According to US Census Bureau, actual or projected total US population are 76.1 million in 1900, 152.3 million in 1950, 276.1 million in 2000 and 403.7 million in 2050, with 4.1%, 8.2%, 12.6% and 20.3% in each of these years accordingly being people 65 years and older. In 1900, only 39% male and 43% female could live till 65 years with average life expectancy of 32-49 years. By 1997, the percentages have been increased to 77% for male and 86 % for female with average life expectancy of 67-80 years (59).

Aging is associated with greater mortality and morbidity. A wide range of diseases such as cancer, cardiovascular and neurodegenerative disorders have a significant age-dependent onset (62). In 2000, heart diseases and cancer has claimed nearly one million older lives in the United States, which accounts for over 50% of total death in older people in the year. For over two decades, heart diseases, sequentially followed by cancer, stroke, chronic obstructive pulmonary diseases (COPD), pneumonia/influenza, diabetes mellitus and Alzheimer's diseases have been the leading causes of death for people over 65 years of age (59). With the rapid increase in the aging population, it is important for us to gain better understanding about the aging mechanisms. This provides the fundamentals for developing more effective prophylactic and therapeutic approaches for diseases associated with advanced aging.

- *Biology of aging*

Aging, although not a disease, like many diseases is a genetically controlled, environmentally influenced process. Studies on caloric restriction (CR) or a single gene mutation that help produce long-lived animals in yeasts, nematodes, fruit flies and rodents have evidenced close relationships between activation of *daf16* gene, lower level

of insulin and insulin-like growth factor 1 (IGF-1), increased DNA repairing ability and postponement of aging (59, 63, 64). Caloric restricted mice and Ames dwarf (AD) mice, the latter carrying a mutation at Prop1 gene on chromosome 11 leading to their growth retardation live 30-50% longer and share lower body temperature and lower level of IGF-1 than the control mice (59, 63). Single gene mutation in certain *daf* or *age* genes that has produced longevity in fruit fly *Drosophila melanogaster* and soil nematode *Caenorhabditis elegans* is found to be associated with activation of *daf16* gene whose regulatory factor daf2 is homologous to insulin and IGF-1 receptors in mammals (65, 66). Long-lived yeasts and worms have shown stronger resistance to cellular damage caused by ultraviolet, oxidizing agents and overheating and IGF-1 has been reported to play a significant role in mediating DNA repair (59, 67). These evidences suggest the aging process may be fundamentally related to cellular repair system.

I.4 Immunology of Aging

Aging of the immune system or immune senescence, leads to immune dysfunction. Immune senescence has been proposed by Walford to account for three major causes of disease in old age: increased autoimmunity, higher occurrence of cancer and increased susceptibility to infectious diseases (6, 68). Changes in both innate and adaptive immune systems are found with aging.

- *Innate immune system in aging*

In the innate immune system, TLRs are a family of pattern recognition receptors binding to pathogen-associated molecular patterns (PAMPs) and serve as a sentinel to induce cytokine production (69, 70). TLRs are widely and differentially expressed in antigen presenting cells as well as lymphocytes in human PBMC. Human and mouse studies have indicated myeloid DC (mDC) and monocytes express TLR 1, 2, 4, 5, 6 and 10 either at mRNA level or at protein level and mainly respond to bacterial stimuli by producing TNF-alpha, IL-6 and IL-12. Plasmacytoid DC (pDC) preferentially express TLR7 and 9 proteins, which confers their ability to respond to viruses and CpG by producing large

amount of type I IFNs (71, 72). TNF-a, IL-12 and IFN-α are able to stimulate Th1 cell-mediated immune response while IL-6 is able to stimulate B cell development and differentiation into plasma cells to produce antibody (73, 74). Characteristics of mammalian TLRs are summarized in Table I. Renshaw and colleagues reported the expression of TLR 1-9 in APC of aged mice is down-regulated compared with young mice. Consistently, declined TLR expressions contribute to less production of cytokines TNF-alpha and IL-6 in macrophages stimulated by TLR ligands (75), suggesting TLR pathway is affected by aging process. Among cytokines and their producing cells, type I IFNs and pDC have been drawn special attention and being investigated prosperously in recent years (76-78). However, studies on their age-related changes are far from sufficient even after pDC was identified as the most potent type I IFN producing cells (79-81).

In the innate immune system, NK cells are the major type of cells derived from lymphoid lineage and represent 5-15% of total human PBMC (82-84). Two distinct subtypes of NK cells can be identified based on their differential expressions of cell surface antigen CD56, and CD16. $CD56^{bright}CD16^{dim}$ NK cells preferentially secrete high levels of IFN-γ and $CD56^{bright}CD16^{dim}$ NK cells primarily execute cytotoxicity and secrete relatively lower levels of IFN-γ upon activation (84, 85). It is known in human peripheral blood NK cell frequency increases and their per-cell-based cytotoxic activity declines with age (86-90). Details of phenotypic and functional properties of NK cells will be discussed in session I.6.

- *Antigen presentation in aging*

Dendritic cells, monocytes are the professional antigen presenting cells (APC) that link the innate and adaptive immune systems. Dendritic cells, existing in peripheral blood at a frequency of less than 1%, include two major subsets, myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) (94). Resting human mDC expresses high level of CD11c, an unconventional MHC class I-like molecule and low level of CD123, IL-3 receptor alpha chain. On the opposite, resting human pDC expresses high level of

Table I. Characteristics of mammalian Toll-like receptors (69, 72, 91-93)

TLRs	Dimerization	Location	Ligands	Human-restriction	Expressing cell
1	heterodimer with TLR2	cell surface	triacyl lipoprotein	no	macrophage, monocytes, mDC
2	heterodimer with TLR1 or 6	cell surface	bacterial lipoprotein, peptidoglycan; viral glycoprotein;fungi	no	macrophage, monocytes, mDC, T
3	homodimer	cytoplasm	dsRNA	no	NK, T
4	homodimer	cell surface	lipopolysaccharide (LPS)	no	macrophage, monocyte, mDC
5	homodimer	cell surface	falgellin	no	macrophage, monocytes, mDC, epithelial cell, T
6	heterodimer with TLR2	cell surface	diacyl lipoprotein	no	macrophage, monocytes, mDC, T
7	homodimer	cytoplasm	ssRNA	no	pDC, NK
8	homodimer	cytoplasm	ssRNA	no	NK, TREG
9	homodimer	cytoplasm	non-methylated CpG	no	pDC, B, T
10	unknown	unknown	unknown	no	macrophage, epithelial cell
11	unknown	unknown	uropathogenic bacteria, profilin-like protein in protozoa	yes	mDC

CD123 but no CD11c (95, 96). Monocytes comprise about 5-10% of total human PBMC and express cell surface marker CD14, a co-receptor along with Toll-like receptor (TLR) 4 for bacterial lipopolysacchrides (97). Antigen presentation involves formation of complex of endogenous or exogenous antigens to be presented with MHC class I or class II molecules. The majority of protein-derived antigens are short (8-11 amino acids) peptides (98). Some of these peptides are derived from newly generated proteins, forming complexes with MHC class I molecules and presented to CD8+ T cells. Others

are derived from exogenous proteins, forming complexes with MHC class II molecules and presented to CD4+ T cells or forming complexes with MHC class I molecules and cross primed to CD8+ T cells (98-101). In addition to peptides, new forms of protein-originated antigens have been recently identified. These include the intact proteins and the complexes formed by peptides and heat shock proteins, both being found to be involved in MHC class II-restricted presentation and/or MHC class I-related cross-priming (99, 102-105). Evidence has been shown that aged mice alveolar macrophages are less efficient in antigen presentation than young cells, indicating antigen presentation is potentially affected by aging (106, 107).

- *Adaptive immune system in aging*

Beyond the innate immune system, the adaptive immune system, in which T and B lymphocytes are major cell types, is characterized with specificity, memory and tolerance in eliciting immune responses. Although age-associated changes in the number of total peripheral T lymphocytes have not been convinced, numerous functional impairments in T cells have been displayed with advanced aging. First, loss of CD28 in aged T cells significantly reduce T cell proliferative ability though expression of the early activation signals, such as CD69, is well preserved (108-111). Second, aged T cells are known to be deficient of calcium (112). Calcium is critical for many cellular events. Calcium deficiency can lead to failure of activation of important enzymes, such as MAPK and MEK involved in signal transduction pathways and consequently affect cytokine production, cell proliferation, and cytotoxicity (113-116). Third, aging influences T cell transcriptional factors directly. NF-kB and AP-1 have been found to be inactivated in aged T cells (117). IL-2 is critical for T cell proliferation and able to stimulate NK cell differentiation and activation (81, 118). Down-regulation of IL-2 expression is largely accounted for by the impairment of transcriptional factors in aged T cells (119).

T cells are highly heterogeneous and aging influences the frequency and function of different T cell subtypes. Type 1 (Th1) and type 2 (Th2) T helper cells are the two major subtypes of CD4+ T cells, the former stimulating cell-mediated immunity and producing

IFN-γ, IL-2, and the latter stimulating humoral immunity and producing IL-4, IL-5 and IL-10 (98) . Productions of Th1 cytokines and Th2 cytokines often counteract each other (98). There appears to be a shift in predominance of helper T cell responses from type 1 to type 2 with advanced aging (81, 120, 121). Diversity in clonal expansion is a critical feature of T cell activation. However, restricted TCR repertoire has been found to be associated with CD8+ T cells (122-124). T cells can also be classified into naive versus memory T cells. It is known the frequencies of both CD4+ and CD8+ naive T cells in human peripheral blood decrease with age (125). Diminishing of the naive cells is due to thymic involution (125), which contribute to increased susceptibility to new pathogens in older subjects. Accumulated memory T cells may impair the long term T cell activation (79, 126, 127). Naive/memory T cell ratio declines with age with CD8+ more rapidly than CD4+ T cells (128-130). Interestingly, the frequencies of different subtypes of T cells do not change with age consistently at least for CD8+ T cells. Gupta and Hong have reported in human peripheral blood the frequencies of naive (CD45RA+CCR7+) and/or central memory T (TCM, CD45RO+CCR7+) CD8+ cells are decreased with age, whereas those of effector memory cells (TEM, CD45RO+CCR7-) and effector (TEMRA, CD45RA+CCR7-) CD8+ T cells are increased with age (128, 131). Besides conventional T cells, T regulatory (TREG) cells are also influenced by aging. TREGs express Foxp3 transcriptional repressor and play an immuno-suppressive role in maintaining immunological tolerance and homeostasis (132). It has been found TREG number in human peripheral blood increases with age, which may lead to the imbalance of TREG homeostasis and predispose the aged individuals to higher risk of autoimmune diseases (133-135).

In humoral immune system, age-associated changes are indicated to be associated with B cells in multiple aspects. With respect to proliferative responsiveness to signals, reduced RNA synthesis and DNA replication upon stimulation with immunoglobulin (sIg), polyclonal activator staphylococcus aureus Cowan I (SAC), CD20 or CD40 have been detected in aged human B cells (136). With respect to B cell genesis, studies have shown increased longevity is associated with decreased number of de novo B cells (137, 138) but no change has been observed in the number of peripheral blood B cells in mouse and

human (120, 139). Further more, Studies in human and mice also show aged mature B cells exhibit altered B cell receptor (BCR) repertoire, particularly in V region (140). Antibody production in majority of B cells requires the assistance of CD4+ T cells. T cells interact with B cells and induce hypermutation in immunoglobulin genes in B cells. The age-related dysfunction of T cells in conjunction with the intrinsic impairment of B cells significantly affects the production of antibodies in the elderly(141, 142). This is reflected by more restricted antibody repertoire in the elderly than that in young people. The malfunction of humoral immune system results in the impaired primary and secondary humoral responses to vaccination in older people (143, 144). For instance, the peak titer of antibody in older subjects tends to be lower and occurs later than in younger subjects (120).

I.5 Overview of Immune Responses to Influenza Virus

Influenza virus induces humoral as well as cell-mediated immune responses. Humoral immunity plays a crucial role by providing protective antibodies to neutralize infectious virion. Specific antibodies to HA, NA, NP, M1 and M2 have been detected in human or mouse in response to influenza infection but only HA and NA antibodies (IgM, IgG and IgA) have been proven to possess primary protective function (1, 145). Influenza-induced antibody can not provide life long protection against the disease. Possible reasons include a diminution of protective antibody with time and the rapid emergence of new viruses with unprecedented antigenic epitopes in HA (1, 146). CD4+ type 2 T (Th2) cells mediate antibody production via physical contact with B cells and cytokine secretion to stimulate B cell development and influence Ig subclass switching. IL-4, IL-5, IL-6 and IL-10 are Th2 cytokines detected in response to influenza stimulation (147, 148). IL-4 has been found to stimulate the production of IgG1 and IgE in mice (149).

Cell mediated immune responses (CMI) to influenza virus involve cytotoxicity and Th1 cytokine production (150). Cytotoxicity is elicited by T cells and NK cells. MHC class I-restricted CD8+ cytotoxic T cells have been found to recognize influenza HA, M, NP and PB2-derived antigens (151, 152). Recent data indicate that T cell cytotoxicity is not restricted to CD8+ T cells but extends to CD4+ T cells. MHC class II-restricted porferin-

mediated cytotoxicity by CD4+ T cells have been identified towards influenza virus and other viruses (153, 154). NK cell-mediated cytotoxicity involves a variety of NK cell surface receptors and will be discussed in session I.7. Type I T helper (Th1) cells play an important role in mediating the responses to influenza infection and vaccination through secreting cytokines such as IL-2 and IFN-γ (7, 150). In addition to T cells, NK cells secrete large amount of IFN-γ in response to influenza stimulation (155, 156). Th1 cell and NK cell functions have been shown to be significantly modulated by antigen presenting cells and/or their secreted cytokines (157-160), which will be addressed in detail in session I.6. Upstream to cytokine production, TLRs have been proven to significantly contribute to immune activation in response to influenza virus. Toll-like receptors (TLR) serve as the important sentinels for pathogen in the innate system and largely mediate Th1 immunity (161). Influenza virus contains a single stranded RNA genome and produces dsRNA intermediate during its replication, thus is able to activate TLR3, 7 and 8 constitutively expressed or induced in NK cells, pDC and/or monocytes (162-164). Some activation may require an additional factor, such as IFN-γ to facilitate (165).

I.6 Significance of Th1 Immunity Induced by Influenza Stimulation in Aging

Influenza infection and vaccination induce humoral and cell-mediated immune responses (CMI). CMI is believed to play an important role in conjunction with antibody response for complete clearance of influenza infection and largely determines the vaccine efficacy (166). Age-associated impairment in CMI has been suggested to account for the increased susceptibility to influenza in the elderly (150). Immune modulation via Th1 and pro-Th1 cytokines is one important aspect of influenza virus-induced CMI because these cytokines function to stimulate T cell proliferation, display effector anti-viral activity, modulate other cell function and influence antibody subclass profile (167). IL-2 and IFN-γ are two well defined Th1 cytokines. IL-2 is secreted by activated T cells and has been shown to be indispensable in stimulating both Th1 and cytotoxic T cell proliferation, differentiation, and activating NK cells (168, 169). In addition, IL-2 enhances B-T cell interaction so as to contribute to antibody generation (170). IL-2

production has been shown to be down-regulated or remain at a comparable level in older subjects compared to young control in response to influenza infection and vaccination, depending on specific experimental conditions, indicating influenza-related down-regulation of Th1 immunity with aging is not solely attributed to the age-related changes in IL-2 production (171-175). IFN-γ is a hallmark Th1 cytokine and secreted by activated T cells and NK cells upon influenza stimulation. Besides an effector cytokine delivering direct anti-viral effect to viral infected cells, IFN-γ is a critical immune modulator and influences other immune cell function and antibody subclass profiling (176). For instance, IFN-γ has been known as the primary macrophage-activating factor that up-regulates MHC class I and II molecules to stimulate antigen presentation and influence expression of other cytokines, chemokines to favor cellular antiviral activities(176-178). In addition, it is able to up-regulate TLRs in macrophage and epithelial cells (179). As a Th1 cytokine, IFN-γ stimulates Ig class switching in favor of IgG2a and IgG3 in mouse (180, 181) and IgG1 in human (182, 183) in vitro and/or in vivo (184). In human, systemic levels of IgG1 and IgG3 are important for complement fixation and Ab-dependent cellular cytotoxicity (185). IgG is the dominant Ig subclass stimulated by trivalent influenza vaccine and IgG1 is the most prominent IgG isotype (182, 186). IFN-γ production has been shown to be down-regulated in older subjects in response to influenza infection and vaccination (173, 187). Our recent data has indicated that Th1 response determined as the frequency of IFN-γ secreting CD4+ Th1 and cytotoxic influenza-specific memory T cells (ISMT) declines with age and correlates with antibody response to influenza vaccination(174, 188). Consistent with our finding, a new observation has indicated Th1 but not antibody response correlated with the outcome of influenza vaccination (189). These evidences have suggested CMI, particularly Th1 immunity, contributes to reducing the morbidity and mortality of influenza in the elderly and capable of influencing vaccine effectiveness.

The production of IFN-γ can be modulated by pro-Th1 cytokines secreted by antigen presenting cells. For instance, IFN-α/β alone or synergistically with IL-18 is able to induce type I T cell and NK cell activations (190). One prominent role of IL-18 is to act together with IFN-α/β, IL-12 and/or IL-23 to activate Th1 and NK cells to induce IFN-γ

production and NK cytotoxicity (157, 191-193). Although IL-12 and IL-23 are not induced in human PBMC stimulated with influenza virus, IFN-α/β is abundantly produced by pDC and monocytes in response to influenza infection (48, 49, 194, 195). In general, pDC is the predominant cell type in production of type I IFNs responsive to viral infection or CpG via TLR-7 or TLR9 pathway (196, 197). However, the accurate comparison in the amount of type I IFN production responsive to influenza infection between pDC and monocytes has not been documented. In addition to direct antiviral effect and induction of IFN-γ in T cells and NK cells, type I IFN and pDC has been recently shown to mediate antibody production (198, 199). Although Type I IFNs and pDC are demonstrated to play a significant role in antiviral immunity, how important they are in influenza-induced immunity and how relevant they are to age-associated decline in IFN-γ production in response to influenza stimulation in human remains to be investigated.

I.7 Significance of NK Cell Activation Induced by Influenza Stimulation

As we described earlier in session I.6, Th1 immunity is important in response to influenza stimulation and Th1 response determined as the frequency of IFN-γ secreting CD4+ T cells is declined in elderly subjects. NK cells secrete IFN-γ in response to influenza stimulation therefore participate in influenza-induced Th1 immunity. However, how IFN-γ production in NK cells is maintained in older people in response to influenza infection remains unknown. Our study will be focused on investigating the activation, specifically the IFN-γ production in human peripheral NK cells in response to influenza virus and attempts to unravel the important regulatory factors that contribute to the age-associated changes in NK cell activation in response to influenza virus.

- *Overview of NK cells*

NK cells are the major lymphocytes in the innate immune system. Capable of eliciting cytotoxicity to virus-infected or tumor cells via MHC class I molecule-dependent or independent mechanisms, NK cells play a crucial role in controlling viral load at early

stages of infection and performing immune surveillance on tumorigenesis (82, 200). NK cell function is mediated by a spectrum of cell surface receptors (201, 202). NK surface receptors can be classified into two categories, activating receptors and inhibitory receptors. Activating receptors include natural cytotoxicity receptors (NCRs, NKp30, NKp44 and NKp46), NKG2 family of receptors (NKG2 A/B, C, D, E and F), CD16, CD244, CD161, and CD226 (DNAM-1) and CD96. NCRs recognize viral or tumor molecules and trigger lysis in abnormal cells (200). NKG2 receptors form homodimers or heterodimers with CD94 and generate either activating (NKG2C or D) or inhibitory (NKG2A) effect through binding to MHC I chain-related proteins (203, 204). CD16 mediates direct and/or antibody-dependent cell-mediated cytotoxicity (ADCC) (205). CD244 displays MHC class I-independent activating or inhibitory effects depending upon stimuli (206). CD161 recognizes non-peptide antigens and is found to be co-expressed with T cell receptors (TCR) on natural killer T (NKT) cells (207). CD226 and CD96 have been shown to recognize poliovirus receptor (PVR) and suggested to mediate cytotoxicity and cell adhesion (208). NK cytotoxicity can be prevented by NK inhibitory receptors Ly49, KIR and NKG2A/CD94 in a MHC-I-dependent manner (209, 210). NK cells express TLR 3 which enable them to recognize dsRNA and TLR7/8, which enable them to recognize ssRNA (211, 212). Activated NK cells produce IFN-γ, display cytotoxicity and mediate immune regulation. In addition to IFN-γ, NK cells secrete GM-CSF, which is known to be capable to induce monocyte differentiation into mDC or macrophages in the presence or absence of IL-4, respectively in vitro (213, 214).

- *NK cell activation in response to influenza virus*

NK cell activation involves cytotoxicity, IFN-γ production and cell proliferation and is regulated by cell-cell contact factors and soluble cytokines, the latter through STAT-participated signal transduction pathways with STAT1 mediating cytotoxicity and STAT4 inducing IFN-γ production (215, 216).

NK receptors mediate cytotoxicity via MHC class I-dependent or independent mechanism. In an MHC class I-independent manner, intact influenza viral protein HA

cross-presented or attached to the surface of infected cells, has been found to be recognized by natural cytotoxicity receptors NKp44 and NKp46 via HA binding to the sialic acid residue attached to galactose on the NCR receptors, supporting a significant role of receptor-mediated cell-cell contact regulation in the NK cell activity specifically in response to influenza stimulation (217-219).

NK cells comprise 2 major differentiation subsets differed by surface expressions of CD56 and CD16 (84). $CD56^{dim}CD16^{bright}$ NK cells preferentially execute cytotoxicity and $CD56^{dim}CD16^{bright}$ NK cells secrete IFN-γ (84). In spite of functional polarity displayed by subsets of NK cells, IFN-γ production has been shown to correlate with the lytic function in human peripheral NK cells (220), suggesting concordance in activity between the two NK subsets. As we described earlier, NK cell IFN-γ production can be modulated by type I interferons via STAT4 signal transduction pathways (216). In fact, NK cells not only produce IFN-γ but are influenced by IFN-γ for their activation. Human peripheral NK cells exist in mature or immature status with mature cells being the majority and able to produce IFN-γ. IL13+ immature NK cells have been shown to be able to differentiate into IL-13- cells in the presence of IL-2 and further mature into IFN-γ producing cells by IFN-γ (221). He and colleagues has postulated "T-cell dependent production of IFN-γ by NK cells in response to influenza A virus" in a IL-2 dependent manner, supporting the role of IFN-γ in NK cell differentiation and activation (222). Thus, both type I interferons and IFN-γ are playing a significant role in activating NK cells in response to influenza infection. Unfortunately, the importance of these soluble factors in influenza-related immunity in an aging context remains unclear.

NK cell response to influenza stimulation is not restricted to infection but extends to vaccination. Skoner and Mysliwska and colleagues have reported NK cytotoxicity can be augmented during the acute phase and convalescent phase post vaccination with either inactivated or live vaccine (155, 156). In addition, higher NK activity post vaccination have been shown to correlate with higher HA antibody titer and better protection against clinical infection (155, 156, 223). These evidences indicate NK cells may not only be

important in defending against acute infection but also participate in preventing influenza post vaccination.

NK cell frequency in peripheral blood increases with age but cytotoxic activity decreases in the elderly in response to influenza infection and vaccination (87-90). IFN-γ production is one of the most important activities of NK cells. How aging influences NK cell activation, particularly IFN-γ production has far less been documented. In this study, we have compared the frequency of IFN-γ producing NK cells in PBMC in response to influenza infection between young and older subjects and investigated how aging influences the regulatory components of NK activation and consequently contribute to the reduced IFN-γ production in NK cells in response to influenza virus in the elderly.

I.8 Hypothesis and Specific Aims

As described earlier NK cell activation manifested by IFN-γ production in response to influenza virus is regulated via soluble factors and cell-cell contact mechanism. Specifically, pro-Th1/Th1 cytokines IFN-α and IFN-γ along with NK cytotoxic receptors (NCRs) and/or viral protein HA involved in cell-cell recognition between NK cells and antigen presenting cells may play an important role in regulating NK cell activation. How aging influences the regulatory factors for NK activity and leads to altered IFN-γ production in NK cells in the elderly in response to influenza virus have been the focus of this study. Components potentially contributing to NK cell IFN-γ production in response to influenza virus involved in current study are depicted in Figure 3.

- *Hypothesis*

We hypothesize the impairment in soluble factors, such as the pro-Th1/Th1 cytokines secreted from pDC and T cells, and/or the defect in expression of cell surface proteins involved in cell-cell contact between NK cells and monocytes contributes to the down-regulated NK cell activation in response to influenza virus in older subjects.

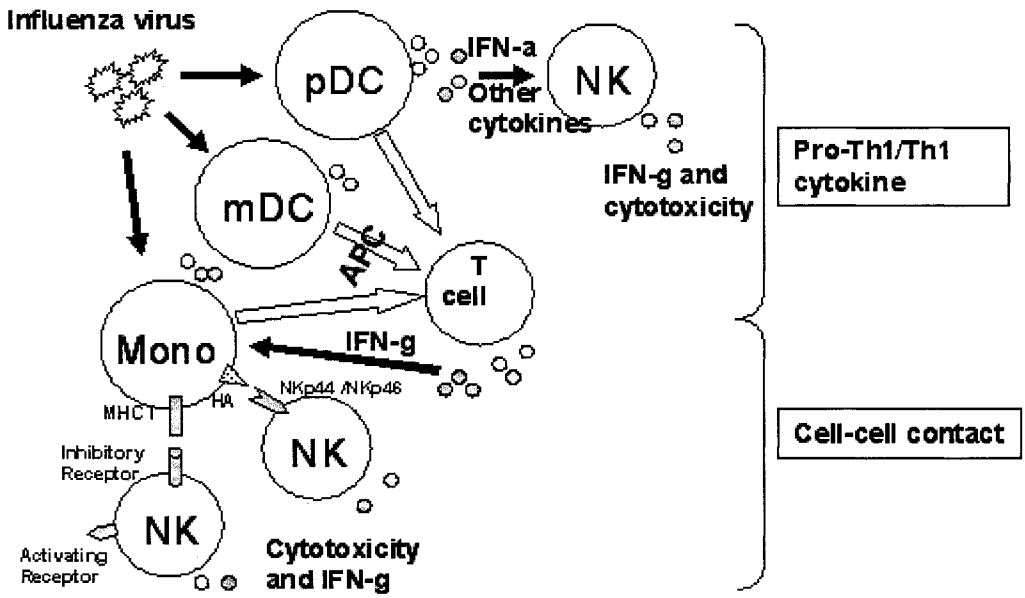


Figure 3. NK cell IFN- γ production and cytotoxicity in response to influenza virus is regulated by proTh1/Th1 cytokines as well as cell-cell contact

Influenza virus infects human PBMC and triggers IFN- α production in monocytes and dendritic cells (pDC and mDC). IFN-α in conjunction with other cytokines, such as IL-18, induces NK cell to generate IFN-γ. At the same time, NK cytotoxicity and IFN-γ production can be induced by physical contact of NK cells with viral infected monocytes via NK receptor-MHC I or viral HA protein recognition. Further more, IFN-γ secreted from T cells may activate monocytes and indirectly augment NK cell activities. Grey circles represent IFN-α or IFN-γ and white circles represent other soluble factors.

- *Specific aims*

To test this hypothesis, the following two specific aims have been addressed:

1. To examine to what extent the impairment in soluble factors, particularly the pro-Th1 cytokines secreted by pDC and T cells play a role in the reduced IFN-γ production in NK cells in response to influenza virus in older subjects.

2. To examine to what extent the reduced IFN-γ secretion by NK cells in older subjects is due to changes in the expression of cell surface receptors/proteins involved in cell-cell contact regulation in NK cell activation in response to influenza virus.

II. MATERIALS AND METHODS

II.1 Experimental Design

The following schematic graph (Figure 4) describes the experimental design of this study. The study was focused on investigating the factors regulating and contributing to the down-regulation of NK cell activation, specifically IFN-γ production in NK cells in older subjects compared to young subjects in response to influenza virus. To achieve Specific Aim1, we propose to confirm the roles of IFN-α and IFN-γ in regulating NK cell activation in response to influenza virus followed by investigating how aging influences cytokine-producing cells pDC and T cells and consequently influences NK cell IFN-g production. To achieve Specific Aim 2, we propose to confirm the roles of cell-cell contact between NK cells and antigen presenting cells in regulating NK cell activation in response to influenza virus followed by examining how aging influences components involved in the cell-cell contact regulation.

II.2 Human Subjects and Cells

- *Human subjects and peripheral blood mononuclear cells (PBMC)*

Healthy young (21-45 years old) and healthy older (60 years and older) volunteers were recruited to this study upon proper consenting. All documents related to recruiting human subjects for this study were approved by Institutional Board Review (IRB) at Eastern Virginia Medical School. PBMC were processed at room temperature (RT) from heparinized whole blood. Briefly, the whole blood was centrifuged at 320 g for 10 min. Buffy coat (PBMC) were aspirated and laid onto a Ficoll reagent, histopaque 1077 (Sigma-Aldrich, St. Louis, MO) followed by centrifugation at 650 g for 30 min. After Ficolling, PBMC were washed twice with RPMI media (Invitrogen, Carlsbad, CA) by centrifugation at 500 g and 320 g sequentially for 10 min each time, counted by automatic counter machine (Coulter $A^c \cdot T$, Beckman Coulter, Miami, FL) and re-suspended in CTL media (RPMI1640 media containing 10% FBS, 2 mM of L-Glutamine,

100 U of penicillin, 100 μg/ml of streptomycin and 55 nM of 2-mercaptoethanol) at a concentration of 1 million PBMC per ml for downstream experiments.

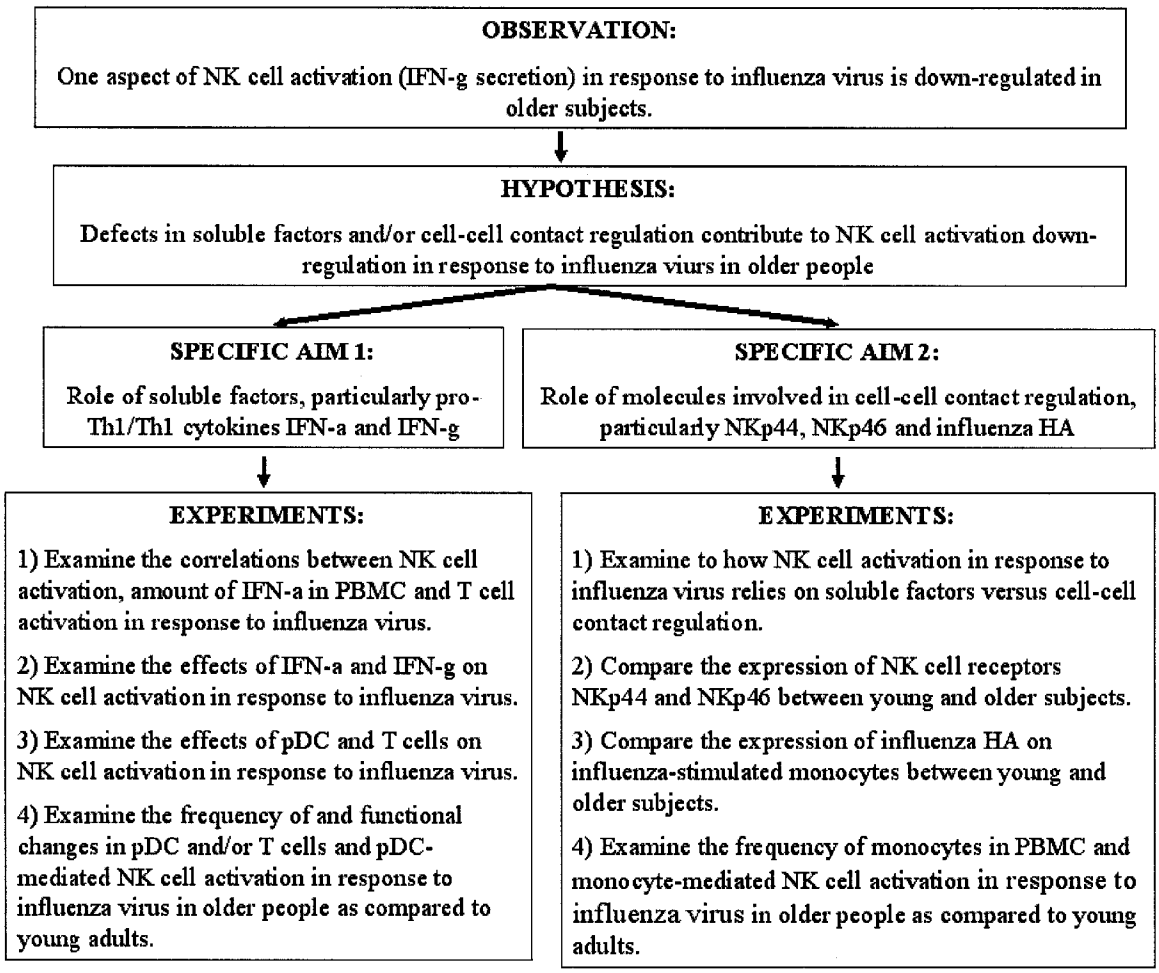


Figure 4. Experimental design of the current study

II.3 Influenza Virus

- *Propagation of influenza virus*

The influenza virus used in this study was A/Sydney/05/97 (H3N2, Bio-safety level II, seed virus from Center for Disease Control, Atlanta, GA). The A(H3N2) subtype emerged in 1968 and is present in influenza trivalent vaccine (TIV) (16, 224). The virus

was propagated in embryonic chicken eggs by our lab staff. Briefly, fertilized chicken eggs (CBT Farms, Chester Town, MA) were incubated at 37°C for 10 days. The viability of the embryos was checked with candle in dark. At room temperature, 0.1 ml of 1:1000-diluted seed virus in diluent buffer (1x PBS, pH 7.2 containing 100U/ml penicillin, 100μg/ml streptomysin and 50μg/ml gentamycin) was injected to the egg from ethanol (70%)-sprayed top of the egg with 1-ml tuberculin syringe attached to a 22 gauge-one-inch needle under sterile conditions. Injection site was sealed with glue and the eggs were incubated at 37°C for 48 more hours before being harvested. For harvesting allantoid fluid, ethanol-sprayed top of eggs was broken and allantoid fluid was collected with a sterile pipette. Allantoic fluid containing A/Sydney virus was filtered via 0.4 um-pore size nitrocellulose membrane and was aliquoted into working size and stored at the -80°C until use. The aliquots were used only once to minimize freeze-thaw cycle. All procedures were conducted under the Guidelines for Bio-safety as detailed in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) published by the CDC and by the Office of Safety at the Eastern Virginia Medical School.

- *Titration of influenza virus*

The viral stock contained 10^7 tissue culture infectious dose 50 ($TCID_{50}$)/ml of live viruses as tested in Madin-Darby canine kidney (MDCK) cells and 1024 hemagglutinin unit (HAU)/50ul as tested with chicken red blood cells. For procedures to determine $TCID_{50}$, please refer to Karber's method described in the reference(225). To determine HAU, fifty microliter of 1:20 diluted viral stock in PBS (pH 7.4, GIBCO/Invitrogen, Carlsbad, CA) was placed in the first column of V-bottom 96-well plate (in duplicates and serially diluted at 1:2 ratio for the remaining columns with each well in the columns containing 50 μl of the diluted virus. Fifty microliter of 0.5% chicken red blood cells (CRBCs) was added to each well and the plate was incubated at room temperature for 45 min. Agglutination was evaluated and using the criteria described earlier(11). HAU was calculated according to the formula HAU/50 $\mu l = 10x2^n$, where n is the highest column number in the plate where agglutination occurs.

II.4 Assays and Specific Methods

- *Live cell staining, intracellular cytokine staining and flow cytometry*

For cell surface antigen only staining, 1 million or less live cells were co-stained with fluorescent-conjugated antibodies against cell surface antigens in 1% BSA-PBS at a volume of 26 μl per well in 96-well U-bottom tissue culture (TC) plate at RT for 20 min followed by 2 times of washing with 1%BSA-PBS. The cells were then re-suspended in 200 μl of 1%BSA-PBS, placed on ice and ready for flow cytometry analysis (FACSCalibur, BD Biosciences, San Diego, CA). For analysis, forward scanner (FSC) and side scanner (SSC) were used to gate PBMC or specific cell types together with specific antigens. A total 20,000 to 200,000 events per sample, depending on the relative proportion of cells of interest were saved for analysis (CellQuest 3.3 software, BD Biosciences, San Diego, CA). For intracellular cytokine staining, cells were fixed with 1% paraformaldehyde-PBS, permeablized with permeablization buffer (Becton Dickinson, San Diego, CA) followed by one time washing with 150 μl of 1%BSA-PBS. Then the cells were treated the same way as for live cell staining.

- *Detection of cell activation by FastImmune (FI) assay*

FastImmune assay consists of 2 consecutive procedures, culture set-up followed by intracellular cytokine staining and flow cytometry. To set up cultures, one million PBMC, pDC-, T cell- or NK cell-deducted PBMC, or different amount (see corresponding figure description in result session) of selectively isolated cells were stimulated with or without 1 μl (10^4 $TCID_{50}$) of influenza virus A/Sydney in 150 μl of cytotoxic T lymphocytes (CTL) media in 96-well U-bottom TC plate at 37°C overnight. Brefeldin A, a reagent that interferes with protein trafficking so that newly generated cytokines can be trapped inside the cell for intracellular cytokine staining (BFA, Sigma-Aldrich, St. Louis, MO) was added to each well at 5μg/ml with total culturing volume brought up to 180 ul (226, 227). The culture was incubated at 37°C for 3 more hours after addition of BFA and supernatants were removed from the cells or saved when

needed, before the cells were fixed and analyzed by intracellular cytokine staining and flow cytometry. The total culture incubation time was 20-24 hours in this assay. To detect NK and T cell activation, anti-CD3 (FITC), CD56/16 (PE), CD69 (PerCP), and IFN-γ (APC) antibodies were used in intracellular cytokine staining. To detect pDC activation, anti-IFN-α2 (PE, Chromaprobe, Maryland Heights, MO), CD69 (PerCP) and CD123 (APC) antibodies (BD Biosciences, San Diego, CA) were used in the staining. Flow cytometry was used to determine the frequency of cytokine secreting cells as well as the per-cell-based geometric mean intensity of fluorescent. In some experiments to detect early production of cytokines, BFA was added 4 hours posterior to stimulation of cells with A/Sydney and the culture was continued at 37°C overnight followed by intracellular cytokine staining and flow cytometry analysis. When needed, IFN-α neutralizing antibody (polyclonal, Biosource, Miami, FL), IFN-α/β receptor neutralizing antibody (monoclonal, Cellsciences, Inc, Vanton, MA), IFN-γ neutralizing antibody (monoclonal, R&D Systems, Minneapolis, MN), rIFN-α2 (PBL Biomedical Laboratories, Piscatawaym NJ), rIFN-γ (Invitrogen, Carlsbad, CA) or CpG (ODN2216 at a final concentration of 6 μg/ml, MWG-Biotech, high Point, NC), rIL-12 (p70, PharMingen, San Diego, CA) was added to the culture to examine their effects on A/Sydney-induced NK or T cell activation.

- *Measurement of pDC, monocyte, and T cell frequencies in PBMC*

Live cell staining and flow cytometry were used to examine frequencies of various cell types in PBMC. For measuring pDC frequency, 1 million PBMC were co-stained with human lineage markers (including anti-CD3, CD14, CD16, CD19, CD 20 and CD56, fluorescein isothocyanate, or FITC-labeled antibodies), CD123 (phycoerythrin, or PE-labeled), HLA-DR (peridinin chlorophyll protein, or PerCP-labeled) and CD11c (allophycocyanin, or APC-labeled) antibodies (BD Biosciences, San Diego, CA) followed by 2 washes. For measuring monocyte and T cell frequencies, 1 million PBMC were co-stained with anti-human CD14 (FITC), CD3 (PE), CD8 (PerCP), and CD4 (APC) followed by 2 washes. Following staining, flow cytometry was used to determine the frequency of each cell type.

- *Isolation of pDC*

Plasmacytoid DC were isolated by using BDCA4 cell isolation kit (Miltenyi Biotec, Auburn, CA). Briefly, every up to 10^8 PBMC were incubated with 300 μl of MACS buffer (0.5%BSA and 2mM EDTA in PBS, pH7.2), 100 μl of FcR blocking reagent and 100 μl of anti-BDCA4 magnetic micro-beads at 4°C for 15min followed by one time wash with 7.5 ml of MACS buffer. The cells were then separated by running through MS column placed in a magnetic field. BDCA4+ cells (pDC) retained in the column were eluted after magnetic field was removed. Isolated pDC and pDC(-) PBMC were re-suspended in CTL media and counted by automatic counter machine (Coulter $A^c \cdot T$, Beckman Coulter, Miami, FL). A portion of isolated cells were stained with anti-lineage marker (FITC), CD123 (PE), HLA-DR (PerCP) and CD11c (APC) antibodies (BD Biosciences, San Diego, CA) followed by flow cytometry to examine the purity. The purity ranged from 70% to over 95% depending on the amount of starting PBMC and donor. More PBMC was associated with higher purity. Because pDC exist in PBMC at a low frequency (0.1-0.5%) and the amount of PBMC obtained from each individual was limited, in some experiments when relatively larger amount of pDC was needed, PBMC from 2 to 8 subjects, depending on the availability of subjects, in young or old group were pooled for pDC isolation. The pooled PBMC comprises equivalent amount of PBMC from each subject of the same age group.

- *Isolation of monocytes*

Monocytes were isolated by using anti-CD14 micro-beads (Miltenyi Biotec, Auburn, CA). Briefly, up to 10^7 PBMC were incubated with 80 μl of MACS buffer and 20 μl of anti-CD14 micro-beads at 4°C for 15min followed by one time wash with 1.5 ml of MACS buffer. The cells were then separated by running through MS column (Miltenyi Biotec, Auburn, CA) placed in magnetic field. CD14+ cells (monocytes) retained in the column were eluted after magnetic field was removed. Isolated cells were re-suspended in CTL media and counted by automatic counter machine (Coulter $A^c \cdot T$, Beckman Coulter, Miami, FL). A portion of cells were stained with anti-CD14 (FITC, BD Biosciences, San

Diego, CA) and CD56 (APC, Miltenyi Biotec, Auburn, CA) antibodies followed by flow cytometry to examine the purity

- *Isolation NK cells and T cells*

NK cells were isolated by using anti-CD56 magnetic micro-beads or NK cell isolation kit II (Miltenyi Biotec, Auburn, CA). By using anti-CD56 micro-beads, up to 10^7 PBMC were incubated with 20 μl of anti-CD56 micro-beads and 80 μl of MACS buffer at 4°C for 15min followed by one time of wash with 1.5 ml of MACS. The cells were then separated by running through MS column placed in magnetic field. CD56+ cells (NK) retained in the column were eluted after magnetic field was removed. CD56+ NK cells and CD56(-) PBMC were re-suspended in CTL media and counted by automatic counter machine (Coulter $A^c \cdot T$, Beckman Coulter, Miami, FL). A portion of isolated cells were stained with anti-CD14 (FITC, BD Biosciences, San Diego, CA) and CD56 (APC, Miltenyi Biotec, Auburn, CA) antibodies followed by flow cytometry to examine the purity. By using NK cell isolation kit, up to 10^7 PBMC were incubated with 10 μl of biotin-conjμgated antibodies against CD3, CD4, CD14, CD15, CD19, CD123 and glycophorin and 40 μl of MACS buffer (0.5%BSA and 2mM EDTA in PBS, pH7.2) at 4°C for 10 min followed by incubation with 20 μl of anti-biotin micro-beads and 30 μl of MACS buffer at 4°C for 15min. The cells were then washed once with 7.5 ml of MACS buffer and applied to LS column for separation. Positively labeled un-related cells were retained in the column and untouched NK cells were enriched in the eluted buffer. Isolated cells were re-suspended in CTL media and counted by automatic counter machine. A portion of isolated cells were stained with anti-CD3 (FITC), CD56/16 (PE), and CD14 (APC) antibodies (BD Biosciences, San Diego, CA) followed by flow cytometry to examine the purity. T cell isolation procedure was similar to that of NK isolation by using anti-CD56 micro-beads except that anti-CD3 instead of CD56 micro-beads was used. Anti-CD3 (FITC), CD56/16 (PE), and CD14 (APC) antibodies were used for staining to examine the purity.

- *Quantification of IFN-α by ELISA*

Supernatant from FastImmune PBMC or influenza virus-stimulated pDC were examined for IFN-α levels using human IFN-α ELISA kit (Biosource, Camarillo, CA). Briefly, supernatant at 1:25 dilution and standards were incubated in 96-well plate wells coated with capturing antibodies recognizing multi-subspecies of IFN-α including subtypes A, D, K, G, C, J but not B2, F and H. Then detecting antibody (secondary antibody), anti-secondary antibody conjμgated to horseradish peroxidase (HRP) and substrate tetramethyl-benzidine (TMB) were used sequentially for the rest of the assay following manufacturer's instruction. The plates were read at 450 nm by PowerReports$_x$ ELISA reader and analyzed with KC4 (version 3.0) software (Bio-Tek Instruments, Inc., Winooski, VT).

- *Stimulation of pDC of young and older subjects*

To compare IFN-α level secreted by pDC between young and older people, 0.05 million pDC isolated from pooled PBMC from 2-6 young subjects or 4-8 older subjects were stimulated with 1 μl of influenza virus in 96-well U-bottom plate at 37°C in the absence of BFA. Supernatants were collected the next day and examined for IFN-α level by ELISA.

- *Examination of effect of supernatant on NK cells*

One million PBMC were stimulated with or without 1 μl of influenza virus under regular FastImmune (FI) assay culture set-up conditions except for in the absence of BFA. Supernatants from cultured wells were collected the next day and utilized to stimulate autologous NK cells isolated from PBMC. Each well containing 0.2 million isolated NK cells in 20 μl of CTL media in conjunction with 130 μl of cultured supernatant was incubated at 37°C overnight before BFA was added to the culture. All other conditions were same as in regular FI assay.

- *Transwell experiment*

Three million CD56 (-) PBMC were placed in regular 24-well plate and stimulated with or without 20 μl of influenza virus in 700 μl of CTL media. CD56+ NK cells were placed in transwell (Fisher Scientific, Pittsburgh, PA) at 0.2 million per well in 50 μl of CTL media. As a control, equal amount of NK cells were mixed with CD56 (-) PBMC in a regular well. The plate was incubated at 37°C overnight followed by regular FI procedures for intracellular cytokine staining and flow cytometry to detect IFN-γ secretion by CD56+ NK cells. Anti-IFN-γ (FITC, BD Biosciences, San Diego, CA), CD69 (PE, BD Biosciences, San Diego, CA) and CD56 (APC, Miltenyi Biotec, Auburn, CA) antibodies were used in the staining.

- *Examination of NKp44 and NKp46 expressions on NK cells in PBMC*

One million resting PBMC or PBMC stimulated with or without 2 μl of influenza virus in regular FI procedure but in the absence of BFA were examined for NKp44 and NKp46 expressions by live cell, indirect staining. Briefly, PBMC were stained with mouse-anti-human NKp44 antibody (R&D Systems, Minneapolis, MN) followed by secondary antibody, goat-anti-mouse IgG (FITC, Jackson Immuno Research, West Grove, PA), staining. After blocking with normal mouse serum, the cells were co-stained with anti-human NKp46 (PE, R&D Systems, Minneapolis, MN), CD3 (PerCP) and CD19 (APC) antibodies (BD Biosciences, San Diego, CA) followed by two washes. Flow cytometry was used to determine the intensity of fluorescent level of NKp44 and NKp46 on NK cells identified by excluding CD3+, CD19+ lymphocytes and morphologically different monocytes from PBMC.

- *Examination of influenza HA expression on monocytes in PBMC*

One million resting PBMC or PBMC stimulated with or without 1 μl of influenza virus as in regular FI procedure but in the absence of BFA were examined for HA expression on monocytes by live cell, indirect staining. Briefly, PBMC were stained with mouse anti-

HA (for H3 subtype of influenza A virus only, Chemicon International, Inc, Temecula, CA) followed by secondary antibody staining with goat-anti-mouse IgG (FITC, Jackson Immuno Research, West Grove, PA). After blocking with normal mouse serum, the cells were co-stained with anti- CD69 (PerCP) and CD14 (APC) antibodies (BD Biosciences, San Diego, CA) followed by two times of wash. Flowcyotmetry was used to determine the intensity of fluorescent level of HA and CD69 on CD14+ monocytes.

- *Experiment of differential effect of young or aged pDC on NK cell activation*

Plasmacytoid DC of young and older subjects, namely pDC(Y) and pDC(O) respectively, were isolated from pooled PBMC from 2-3 donors. One million PBMC of each young or older subject or 1 million pDC(-) pooled PBMC were stimulated with 1 μl of influenza virus with or without addition of 0.01 million pDC(Y) or pDC(O). That equal amount of pDC(Y) and pDC(O) was added to the culture was verified by measuring the frequency of pDC by live cell staining and flow cytometry with pDC identification antibodies described earlier. Other conditions for culture set-up and detection of NK cell activation were same with those in regular FI procedures. Three independent experiments involving a total of 8 young and 8 older subjects were performed.

- *Crossover experiment of monocytes between young and older subjects*

CD14+ monocytes of young and older subjects, namely Mono(Y) and Mono(O), respectively, were generated by pooling equal amount of PBMC from 2-3 individual. One million CD14(-) PBMC of each young or older subject were stimulated with 1 μl of influenza virus with or without addition of 0.1 million CD14+ Mono(Y) or pDC(O). Equal amount of Mono(Y) and Mono(O) added to the culture was verified by measuring the frequency of added CD14+ monocytes using live cell staining and flow cytometry with anti-CD14 antibody. Other conditions for culture set-up and detection of NK cell IFN-γ production were as same as in regular FI procedures. Four independent experiments involving a total of 10 young and 10 older subjects were performed.

II.5 Statistical Analysis

All statistical analysis was done by using SPSS software. Two-tailed non-parametric Mann-Whitney test or Sign test was used to compare differences between groups when variables were not suggested to follow normal distribution by normality test or sample size was relatively small. Two-tailed t test or ANOVA was used when sample size was relatively big and normality test suggested normal distribution.

III. RESULTS

III.1 Down-regulation of NK Cell Activation in PBMC in Response to Influenza Virus in Older People

- *NK cell activation determined as IFN-γ production in response to influenza virus was down-regulated in older subjects*

Knowing NK cells secrete a significant amount of IFN-γ in response to influenza virus, we first wanted to know whether there was a difference in IFN-γ production NK cells in influenza virus-stimulated PBMC between young and older people. We used an activation priming marker CD69 in conjunction with IFN-γ to monitor NK cell activation. By comparing IFN-γ secretion between 18 healthy young and 25 healthy older subjects by FastImmune (FI) assay, we found upon influenza viral stimulation of PBMC, both the frequency of CD69+IFN-γ+ NK cells and the per-cell-based intensity of IFN-γ were significantly less in older subjects than those in the young subjects ($p<0.001$, Figure 5A, B and C). The frequency of CD69+IFN-γ+ NK cells and per cell-based IFN-γ intensity among total NK cells was 14.9±2.2% (mean±s.e.m.) and 320±156 (mean±s.d.), respectively in the young subjects and 2.8±1.8% (mean±s.e.m.) 232±101 (mean±s.d.), respectively in the older subjects (Figure 5B left). Significant lower frequency of activated NK cells was also found in older subjects when total lymphocytes instead of NK cells were used as the denominator (0.85±0.17% in the young and 0.24±0.13% in the old, mean±s.e.m., $p=0.002$, Figure 5B right), indicating the aging-related up-regulation of overall NK cell frequency in lymphocytes (6.4±3.2% in the young and 12.0±3.2% in the old, mean+s.d., $p=0.001$) was not adequate to compensate the reduction in number of NK cells secreting IFN-γ in response to influenza virus in older people (S. Figure 1). We could not find significant difference in CD69 intensity in either CD69+IFN-γ+ or CD69+IFN-γ- NK cells (S. Figure 2A) or in CD69+IFN-γ- NK cell frequency (S. Figure 2A) in total NK cells between young and older subjects, indicating early signal transduction event in NK cell activation in influenza-virus stimulated PBMC is relatively well preserved with aging.

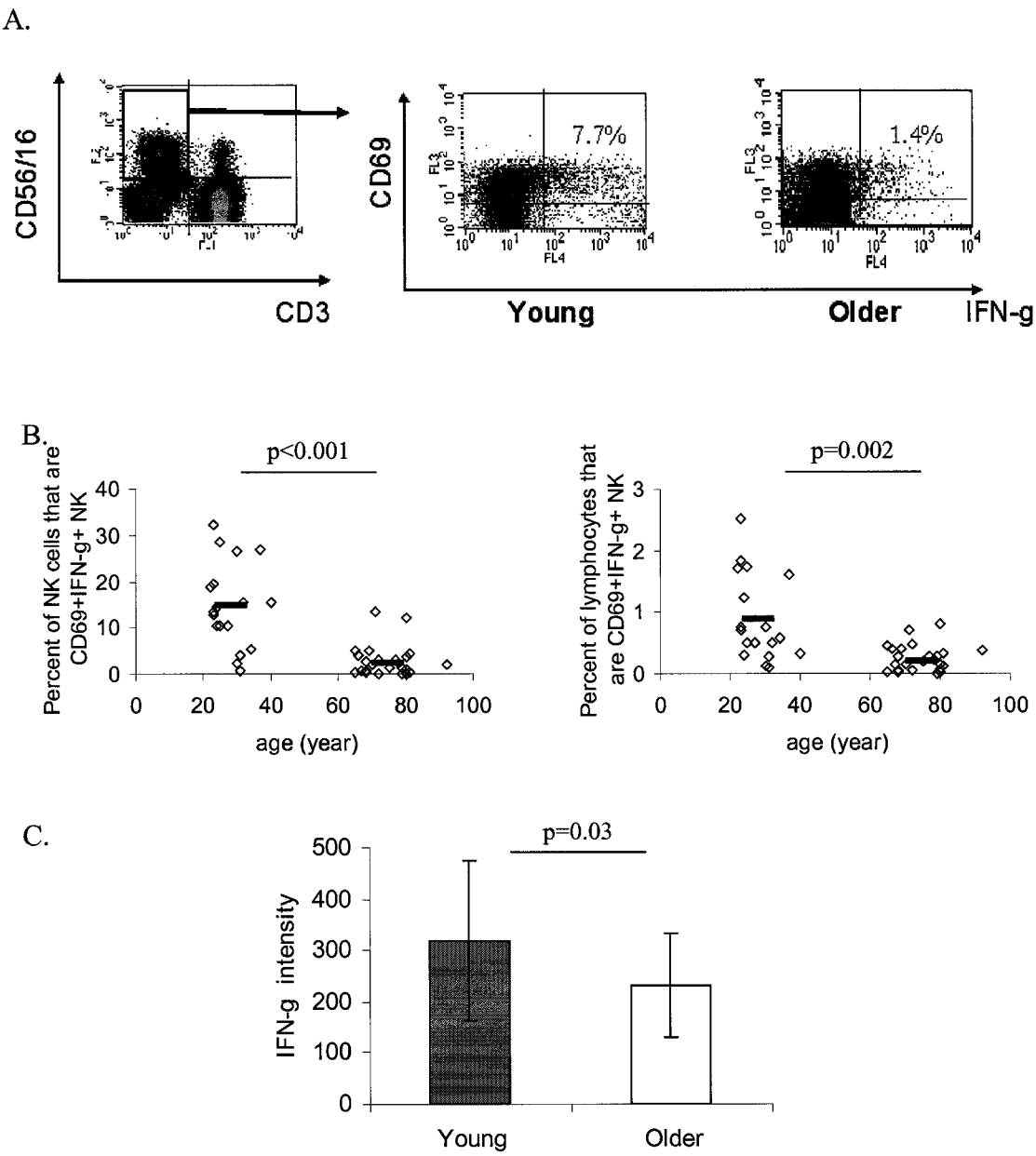


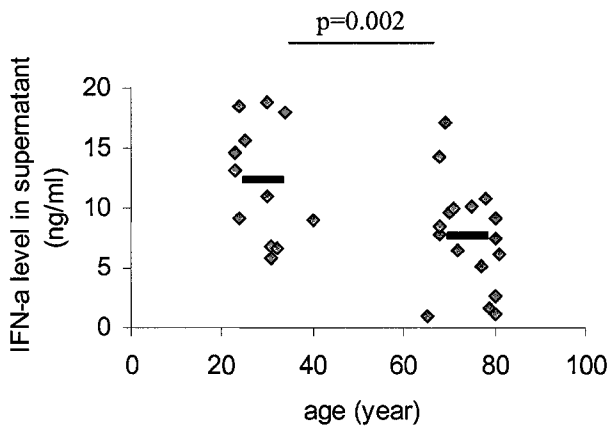
Figure 5. Down-regulation of IFN-γ production in NK cells in PBMC in response to influenza virus in older subjects

One million PBMC from each of 15 healthy young and 25 healthy older subjects were stimulated with 1 μl of influenza virus in 96-well plate overnight. FastImmune assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. A, CD56+or16+ NK cells were gated out of lymphocytes and analyzed for CD69 and IFN-γ expression by flow cytometry. An example of expression of CD69 and IFN-γ in NK cells in a young and an older subject was presented. B, comparison of CD69+IFN-γ+ NK cell frequency in total NK cells (left graph in panel B) and lymphocytes (right graph in panel B) between young and older subjects. Horizontal bars represent the mean. C, Average intensity of IFN-γ in CD69+IFN-γ+ NK cells in young and older subjects. Error bars represent standard deviation.

- *Down-regulated NK cell IFN-γ production in response to influenza virus in older subjects was associated with reduced T cell activation and IFN-α secretion in PBMC*

We next wanted to examine whether down-regulated NK cell activation in older subjects in response to influenza stimulation was associated with changes in other factors in PBMC. We examined a subset of the same samples studied in Figure 4 for supernatant level of IFN-α by ELISA and T cell (CD3+) activation by flow cytometry and found both IFN-α levels in supernatant and the frequency of CD69+IFN-γ+ T cells were significantly reduced in older subjects compared to the young subjects (Figure 6, $p=0.002$ and $p<0.001$ for IFN-α level and activated T cell frequency, respectively). The supernatant IFN-α levels and the frequency of CD69+IFN-γ+ T cells among total T cells were 12.3 ± 4.8 ng/ml (mean+s.d.) and $1.42\pm0.36\%$ (mean+s.e.m.), respectively, in the young subjects, compared to 7.5 ± 4.5 ng/ml (mean+s.d.) and $0.30\pm0.26\%$ (mean+s.e.m.), respectively, in the older people. Then we calculated the correlation coefficients, which measure the linear relationship between frequency of activated NK cells, supernatant IFN-α level and frequency of activated T cells. We found the frequency of CD69+IFN-γ+ NK cells in total NK cells moderately correlated with IFN-α level in supernatant ($r=0.56$) and highly correlated with the frequency of CD69+IFN-γ+ T cells in total T cells ($r=0.86$) (Table II), indicating down-regulated NK cell IFN-γ production in response to influenza virus in older subjects was associated, though not necessarily in a causal/effect relationship, with reduced T cell activation and IFN-α secretion in PBMC. The fact that poor correlation between supernatant IFN-α level and frequency of IFN-γ+ T cells ($r=0.35$) indicates the relationship between these two variables is at least not linear in our experimental system (Table II).

A.



B.

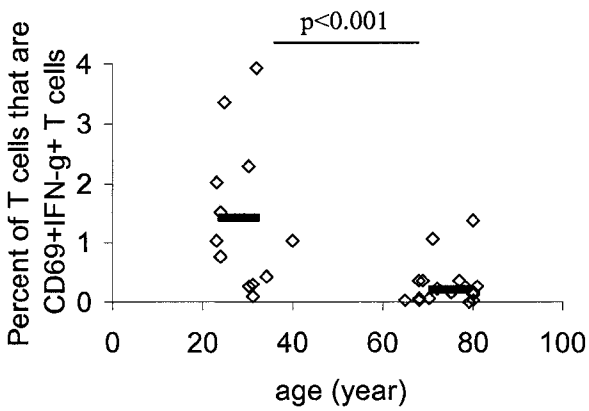


Figure 6. Reduced level of IFN-α and lower frequency of IFN-γ-producing T cells in PBMC in response to influenza virus in older subjects

A subset of the same samples studied in Figure 4 (One million PBMC from 12 healthy young and 18 healthy older subjects stimulated with 1 μl of influenza virus) was analyzed for T cell IFN-γ production and IFN-α secretion. Supernatants saved from the same wells that were examined for NK and T cell activation were analyzed for IFN-α by ELISA. A, supernatant IFN-α level in young and older subjects. B, frequency of CD69+IFN-γ+ T cells in total T cells in young and older subjects. T cells were identified by CD3+ antibody. Horizontal bars represent the mean.

Table II. Correlation between frequency of activated NK cells*, supernatant IFN-α level, and frequency of activated T cells* in young and older subjects

Age groups and variables to analyze	Young and old		Young only		Old only	
	IFN-α level	Frequency of activated T cells	IFN-α level	Frequency of activated T cells	IFN-α level	Frequency of activated T cells
Frequency of activated NK cells	0.56	0.86	0.49	0.77	0.32	0.95
IFN-α level		0.35		0.09		0.27

* Frequencies of activated NK cells or T cells are expressed as the percent of total NK or T cells that are CD69+IFN-γ+.

III.2 IFN-α and IFN-γ Regulated NK Cell IFN-γ Production in Response to Influenza Virus

- *Neutralizing antibodies against IFN-α or IFN-α receptor down-regulated NK cell activation in response to influenza virus*

To find whether down-regulation of IFN-γ production in NK cells was a potential outcome of down-regulation of IFN-α secretion in PBMC upon influenza viral stimulation, we first asked how important IFN-α was in regulating NK cell IFN-γ production in response to influenza virus. By performing FI assay with adding anti-IFN-α or anti-IFN-α/β receptor neutralizing antibodies to PBMC from healthy young subjects, we observed NK cell activation determined as the frequency of CD69+IFN-γ+ NK cells in total NK cells were down-regulated in a dose-dependent manner with the increased dose of either antibody (Figure 7). There was no significant difference in NK cell activation between the treatments without antibody and with the control antibody containing equivalent amount of IgG to that in the well bearing highest neutralizing unit of antibody. Our data clearly indicates the importance of IFN-α in mediating NK cell IFN-γ production specifically in response to influenza virus, supporting that age-related

changes in IFN-α production, if any, bears the potential to contribute to the age-related impairment in NK cell IFN-γ production in response to influenza virus.

A.

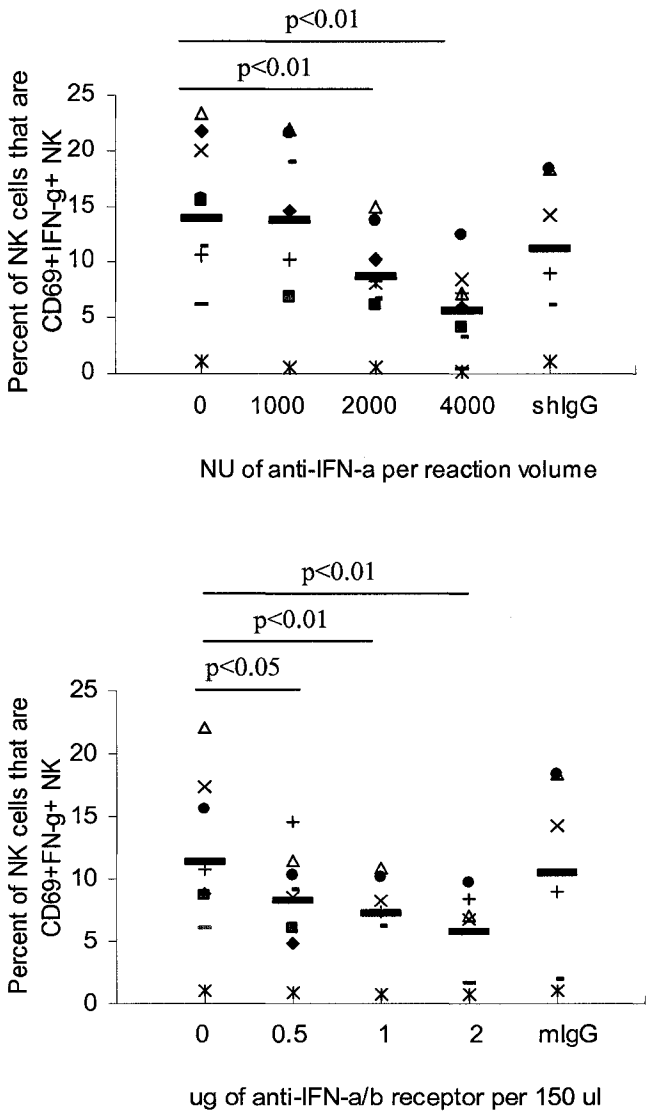


Figure 7. Neutralizing antibodies against IFN-α or IFN-α/β receptor down-regulated NK cell activation in response to influenza virus in PBMC

One million PBMC from 9 healthy young subjects were stimulated with 1 μl of influenza virus in the presence of different doses of IFN-α or IFN-α receptor netralizing antibodies. FastImmune assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. A, with anti-IFN-α antibody. B, with anti-IFN-α receptor antibody. Horizontal bars represent the median.

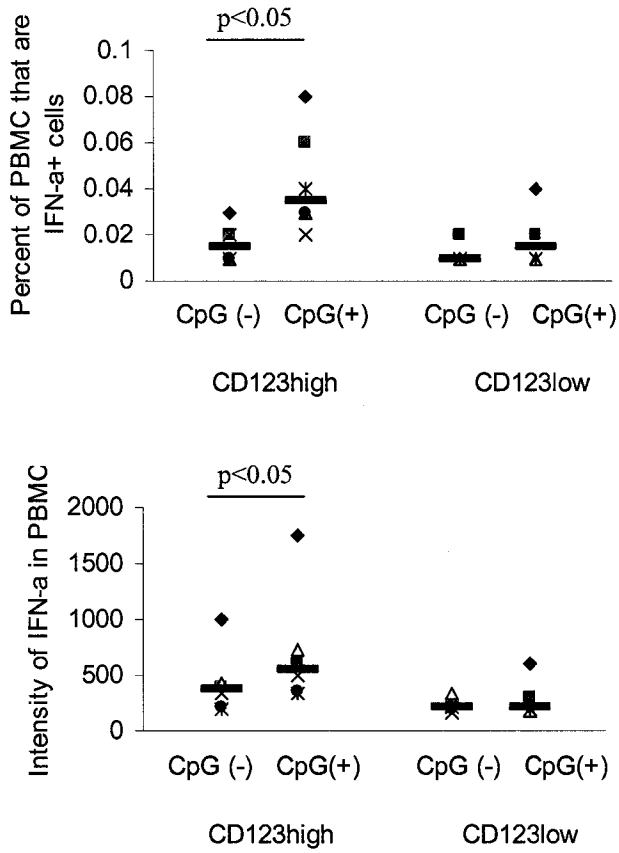
- *CpG up-regulated NK cell activation in response to influenza virus*

To further confirm the role of IFN-α in up-regulating NK cell IFN-γ prodution in response to influenza virus, we used CpG ODN2216, a TLR-9 agonist that induces IFN-α production in pDC to examine whether up-regulation of IFN-α would enable NK cells in PBMC to produce more IFN-γ in response to influenza virus. By performing regular FI assay with or without addition of CpG to influenza viral stimulated PBMC from healthy young subjects, we found in the presence of CpG both the frequency of IFN-α+ cells and per-cell-based IFN-α intensity in CD123hi (a manifest of pDC) but not CD123low (related to monocytes and mDC) cells were significantly increased ($p<0.05$, Figure 8A). At the same time the frequency of activated NK cells was up-regulated ($p<0.05$, Figure 8B). This result, consistent with that in Figure 7, confirms the significant role of IFN-α in mediating NK cell IFN-γ production in PBMC in response to influenza virus.

- *Effects of anti-IFN-γ neutralizing antibody on NK cell IFN-γ production in PBMC in response to influenza virus*

In addition to IFN-α, we also wanted to examine the role of IFN-γ in NK cell activation to influenza stimulation. Similarly, we performed regular FI assay, in which different doses of anti-IFN-γ neutralizing antibody were added to influenza viral stimulated PBMC from healthy young subjects, and monitored frequency of IFN-γ producing NK cells. At concentrations of 0, 0.25, and 0.5 μg of antibody per 150 μl of reaction volume, there was a trend of the dose-dependent reduction in the frequency of activated NK cells, but it didn't reach the significant level of α=0.05 (Figure 9). It should be noted that an antibody dose-dependent down-regulation of NK cell activation occurred in all three subjects with higher (3.3-7.1%) baseline (in the absence of antibody) levels but not in subjects with relatively low (0.1-0.7%) baseline levels of NK cell activation. This seemed to suggest that baseline level of IFN-γ lower than certain limit may not respond effectively to the neutralizing antibody. Increasing sample size and dividing subjects based on their baseline responsive level to influenza virus may help clarify the results. In conclusion, the importance of the role of IFN-γ in regulating NK cell IFN-γ production

A.



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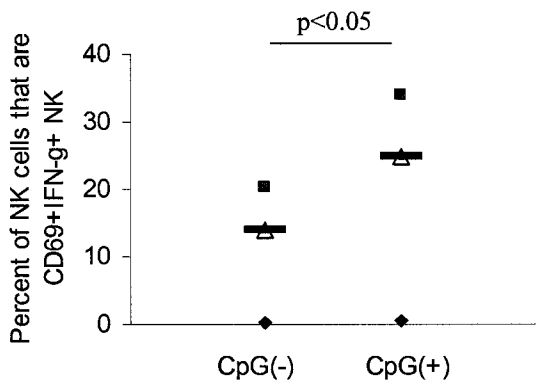


Figure 8. Effects of CpG on production of IFN-α and NK IFN-γ in PBMC

One million PBMC from 6 (A) or 3 (B, the 3 subjects in B were also participating in A) healthy young subjects were stimulated with 1 μl of influenza virus in the presence of 6 ng/ml of CpG ODN2216. CpG was added 4 hours after initiation of influenza infection. FastImmune assay and flow cytometry was used to determine the intensity of IFN-α, frequency of IFN-α+CD123hi or low cells and CD69+IFN-γ+ NK cells. A, IFN-α+ cell frequency and IFN-α intensity in CD123hi or low cells in the presence or absence of CpG. B, activated NK cell frequency in the presence or absence of CpG. Horizontal bars represent the median.

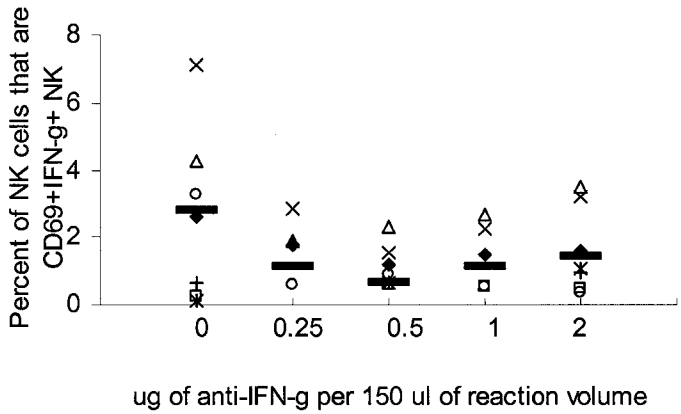


Figure 9. Effects of neutralizing antibody against IFN-γ on NK cell IFN-γ production in response to influenza virus in PBMC

One million PBMC from 6 healthy young subjects were stimulated with 1 μl of influenza virus in the presence of different doses of IFN-γ neutralizing antibody. FastImmune assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. Horizontal bars represent the median.

could not be verified by the usage of anti-IFN-γ neutralizing antibody.

- *IFN-γ facilitated monocytes to activate NK cells in response to influenza virus*

To clarify the role of IFN-γ in NK cell activation regulation, we decided to use isolated cells to avoid actions taken at one time by too many regulatory factors in PBMC in response to influenza stimulation. Because rIFN-γ alone or in conjunction with influenza virus could not activate NK cells to produce IFN-γ while monocytes are able to be activated by IFN-γ and capable to produce NK-activating cytokines, such as IFN-α, IL-18, and IL-15, a cytokine that shares similarity with IL-2 in stimulating NK activation, we isolated monocytes and NK cells from resting PBMC from healthy young subjects by using CD14+ micro-beads and untouched CD56/16 NK cell isolation kit, respectively and examined the effect of rIFN-γ on NK cell activation to influenza viral stimulation in the presence or absence of monocytes. In the presence of monocytes, influenza virus in conjunction with rIFN-γ effectively activated NK cells and induced NK cell IFN-γ production in a rIFN-γ dose-dependent manner (Figure 10A and B). These results confirmed the important role of IFN-γ in regulating NK cell IFN-γ production in response

to influenza virus and provided the possibility that the age-related down-regulation in NK IFN-γ production might be due to age-related reduction, if any, in IFN-γ production prior to activation of NK cells in PBMC.

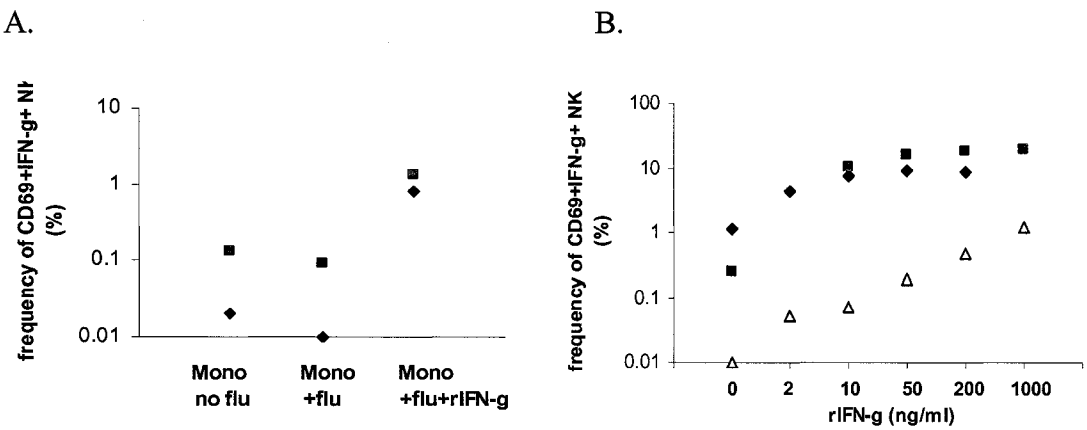


Figure 10. Recombinant IFN-γ positively regulated NK cell activation in the presence of monocytes in response to influenza virus

One fifth million isolated monocytes (by CD14 beads) and 0.2 million autologous NK cells (including both CD56+ or CD16+ NK but untouched) from 2 or 3 healthy young subjects were stimulated with 1 μl of influenza virus in the presence or absence of different doses of rIFN-γ. FI assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. A. NK cell activation in the presence or absence of influenza virus or 100 ng/ml of rIFN-γ. B, NK cell activation to influenza virus in the presence of different doses of rIFN-γ.

III.3 Plasmacytoid DC and T cell Regulated NK Cell Activation in Response to Influenza Virus

We have shown previously that both IFN-α and IFN-γ play an important role in influenza virus stimulated NK cell IFN-γ production. Next we wanted to explore the role of major cell types producing these cytokines. Plasmacytoid DC secrete significant amount of IFN-α in response to influenza stimulation (228). We decided to remove pDC from resting PBMC from healthy young subjects by using BDCA4 micro-beads and examine how reduction of pDC influence NK cell activation in response to influenza virus by performing FI assay. BDCA4 is a cell adhesion molecule and known to be exclusively expressed on pDC in resting PBMC. We found removal of pDC from PBMC

significantly down-regulated NK cell activation determined as the frequency of CD69+IFN-γ+ NK cells over total NK cells and adding removed pDC back to the pDC-reduced PBMC tended to restore NK cell activation though the restoration did not reach the significant level at α=0.05 (Figure 11A, left). In fact, pDC dose-dependent increment of NK cell activation was observed, though the number of subjects tested was limited (Figure 11A, right), supporting that pDC could be important in NK cell activation regulation.

In addition to pDC we wanted to know how T cells influence NK cell activation for T cell is the only cell type, in addition to NK cells to secrete significant amount of IFN-γ upon activation. We decided to remove T cells from resting PBMC from healthy young subjects by using CD3 micro-beads and examine how reduction of T cells influence NK cell activation in response to influenza virus by performing FI assay. We could not find significant difference in NK cell activation by influenza virus between PBMC and T cell-reduced PBMC. This could be due to some non-specific of the isolation procedure effect on NK cells or other cells regulating NK activity. Instead of this, when the removed T cells were added back to the T cell-reduced PBMC, there was a T cell dose-dependent increment in NK cell activation (Figure 11B), indicating T cells were playing a significant role in mediating NK cell activation in response to influenza virus.

III.4 Aging Affected pDC and T Cell Frequencies and Cytokine Productions as well as pDC-mediated NK Cell IFN-γ Production

- *Plasmacytoid DC frequency in PBMC and ability to secrete IFN-α were down-regulated in older subjects*

In previous experiments we confirmed IFN-α was a critical mediator for NK cell activation in response to influenza virus (Figures 7 and 8) and IFN-α levels were significantly reduced in PBMC in response to influenza stimulation in older subjects (Figure 6A). IFN-α in PBMC in response to influenza virus was significantly contributed by pDC therefore it was of importance to know how aging influence pDC with respect to

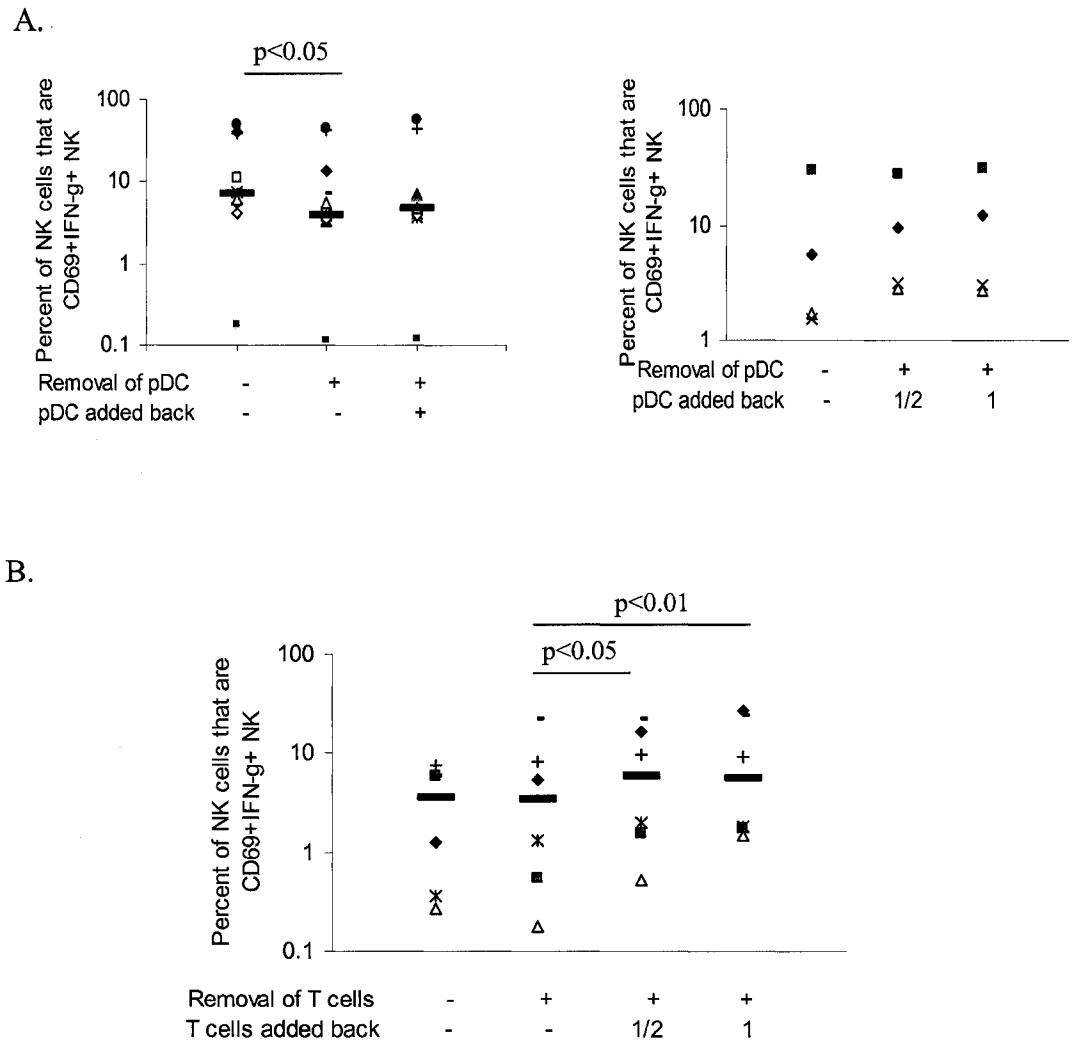


Figure 11. Effects of pDC or T cells on NK cell IFN-γ production in response to influenza virus in PBMC

A. Effect of pDC. One million PBMC or pDC-removed PBMC from 12 healthy young subjects were stimulated with 1 μl of influenza virus with or without addition of isolated pDC. FastImmune assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. Left, effect of reducing pDC or adding pDC back to pDC-removed PBMC. Right, pDC dose-dependent effect. One part of pDC dose was comparable to pDC amount isolated from 1 million PBMC. B. Effect of T cells. Half million T-cell reduced PBMC from 4 healthy young subjects were stimulated with 1 μl of influenza virus with or without addition of isolated T cells. FastImmune assay and flow cytometry was used to determine the frequency of CD69+IFN-γ+ NK cells. One part of T cell dose was comparable to T cell amount isolated from 1 million PBMC. Horizontal bars represent the median.

its availability and function. To examine whether there was a difference in pDC frequency in PBMC between young and older people, we performed live cell staining on PBMC from 52 healthy young and 75 healthy older subjects. It was observed that the frequency of pDC in PBMC in older subjects was significantly lower than that in the young subjects ($p<0.05$, Figure 12). The median of pDC frequency in the young subjects was 0.14%, which was in contrast to the median, 0.10%, in older people. The absolute median value of pDC frequency was 0.04% different between young and older subjects, which reflected a 29% numerical deduction in the older subjects relative to the young. Further, we wanted to examine whether there was an intrinsic functional defect in pDC from older adults. We isolated pDC from pooled PBMC from young or older subjects, stimulated them with influenza virus and monitored IFN-α secretion in supernatant by ELISA. Significantly lower level of IFN-α secretion was found in older subjects (2.9-15.6, median=7.6 ng/ml, 37.6±13.4% of reduction, mean±SD) than that in the young control (5.7-19.2, median=10.9 ng/ml, $p<0.05$, Figure 13). These results suggests that down-regulation of both the frequency and intrinsic function of pDC leads to reduced production of IFN-α and contributes to NK cell activation down-regulation in response to influenza virus in older people.

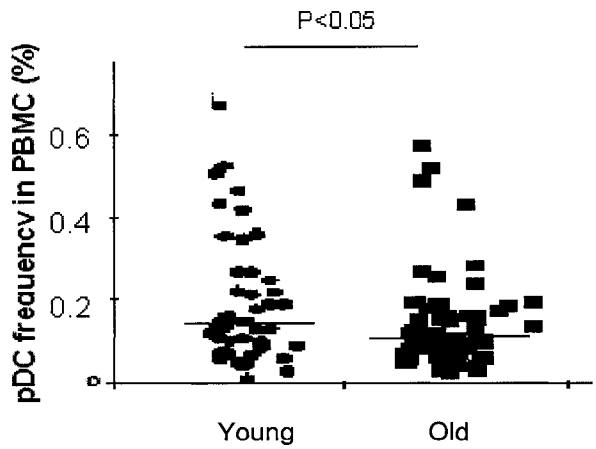


Figure 12. Comparison of frequency of pDC in PBMC between young and older people

Plasmacytoid DC frequency in PBMC between 52 healthy young and 75 healthy older adults was compared. PBMC were co-stained with anti-lineage marker (FITC), CD123(PE), HLA-DR(PerCP) and CD11c(APC) antibodies and identified as cells characterized as CD123+HLA-DR+ and lineage marker-CD11c- by flow cytometry.

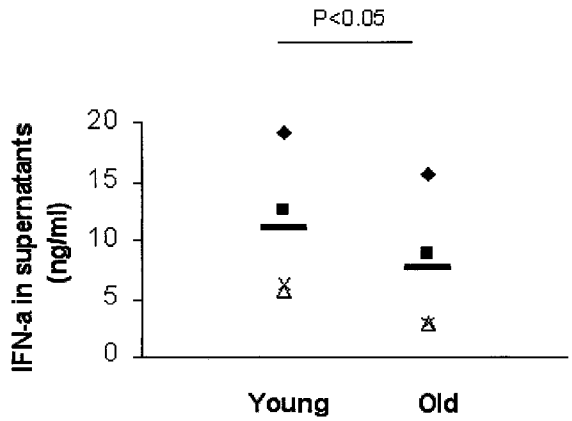


Figure 13. Plasmacytoid DC isolated from older subjects secreted less IFN-α upon influenza infection

Plasmacytoid DC were isolated by using BDCA-4 magnetic beads from pooled PBMC of young (2-6 donors per pool) or older (4-8 donors per pool) volunteers. Isolated pDC were infected with 1 μl of influenza virus in 96-well plate. Supernatant was collected the next day and examined for IFN-α by ELISA. Data from 5 independent experiments involving 22 young and 30 older subjects are shown. Elongated bars represent the median.

- *T cell frequency and function decrease with age*

The age-related changes in T cell function have been one of the major focuses in the field of immunology and aging. Data from our early research showed T cell, particularly CD4+ and CD8+ memory T cell responses to influenza infection and vaccination declined in elderly people (174). This is consistent with our current data that NK cell activation correlated with T cell activation and both were down-regulated with in older people (Figure 1 and 2B, and II). Here we simply compared the quantities of PBMC, T cells and monocytes in human peripheral blood between 12 healthy young and 23 healthy older subjects and observed PBMC yield, pan T cell, CD4+ T cell and CD8+ T cell quantities in blood were significantly lower in older subjects than those in the young people (Table III). On the other hand, significant differences in numbers of CD14+ monocytes between the two age groups was not detected (Table III).

Table III. Comparison of PBMC, T cell and monocyte numbers* in human peripheral blood between young and older subjects

		T cells			Monocytes
	PBMC (million/ml)	CD3+ (million/ml)	CD4+CD3+ (million/ml)	CD8+CD3+ (million/ml)	CD14+ (million/ml)
Young (n=12)	1.25 + 0.43	0.84+0.30	0.57+0.21	0.23+0.076	0.059+0.047
Old (n=23)	0.85 + 0.30	0.49+0.23	0.36+0.17	0.13+0.099	0.044+0.029
P value (2-tail t- test)	0.003	0.0006	0.0038	0.0031	0.2693
P value (2-tail Mann-Whitney test)				<0.001	

* PBMC number was calculated by dividing total amount of PBMC obtained after blood processing by total volume of blood from which PBMC were isolated. CD3, CD4, CD8 positive T cells or CD14 positive monocyte numbers were caluculated by multiplying PBMC number by frequency of each cell type in PBMC. The frequency of each cell type in PBMC was determined by live cell staining of resting PBMC and flow cytometry.

- *Plasmacytoid DC from young subjects better facilitated NK cell activation than pDC from older subjects in response to influenza virus*

Previous experiments in this study suggested that pDC were important in activating NK cells in response to influenza stimulation. We asked whether pDC from young or older people differentially mediate NK cell activation by influenza virus. We performed FI assay, in which equal amounts of pDC isolated from PBMC pool from either young or older subjects were added to individual PBMC followed by stimulation with influenza virus. We found pDC isolated from young adults better functioned significantly than pDC isolated from older subjects in stimulating NK cell activation in both young and older subjects ($p<0.05$, Figure 12). The percent of NK cells that were activated in the presence of young or aged pDC in the young subjects ranged from 0.4-5.2% with median=2.1% and 0.4-4.9% with median=1.3%, respectively. Percent of NK cells that were activated in the presence of young or aged pDC in the older subjects ranged from

0.1-6.2% with median=1.1% and 0.03-2.8% with median=0.6%, respectively. We didn't observe an up-regulation of NK cell activation when additional pDC, whether young or aged were added to PBMC as compared to that without additional pDC. There was a trend, though not having reached the significant level that NK cell activation was lower in older subjects than that in the young when PBMC from young and older subjects were treated the same way (Figure 14). It was surprising that the difference in NK cell activation in influenza stimulated PBMC between young and older subjects was not as significant as we observed before (Figure 5B). Beside differences due to donor variation and experimental error, this could be a consequence of smaller sample size and/or smaller age difference between young and older subjects in current study than that in the previous (43 subjects with age range of 23-40 years, mean=29 years for the young and 65-81 years, mean=74 years for the older in previous study compared with 17 subjects with age range of 21-44, mean=33 year for the young and 60-84 years, mean=69 years for the older subjects in current study). In general, these results are consistent with what we have observed in the earlier part of this study, together indicating down-regulated pDC function contributes to down-regulated NK cell activation in response to influenza virus in older subjects.

III. 5 Soluble Factors vs. Cell-cell Contact in Regulating NK Cell IFN-γ Production

- *NK cells were activated by soluble factors in the absence of contact with other cells*

To investigate to what extent soluble factors vs. cell-cell contact participates in mediating NK cell IFN-γ production in response to influenza stimulation we first asked whether NK cells were activated by soluble factors alone. To exclude the influence of cellular contact, we used supernatants generated from influenza virus –stimulated PBMC to stimulate autologous NK cells isolated from PBMC by using CD56 micro-beads. As shown in Figure 15, both CD69 intensity in NK cells and the frequency of IFN-γ producing NK cells in the presence of influenza virus-stimulated supernatant (9.3 to 16.6, median =11.7 for CD69 intensity, and 0.14-20%, median=4.5% for IFN-γ+ NK frequency) were

significantly higher ($p<0.05$) than those in the presence of non-stimulated supernatant (6.0 to 8.0, median=7.1 for CD69 intensity, and 0-0.7%, median=0.08% for IFN-γ+ NK frequency) or media control (5.7 to 7.7, median=6.8 for CD69 intensity, and 0.07-1.7%, median=0.17% for IFN-γ+ NK frequency, Figure 15A and B). This clearly indicated NK cells were able to be activated by soluble factors only without cell-cell contact between NK and other cells, particularly antigen presenting cells in PBMC in response to influenza virus.

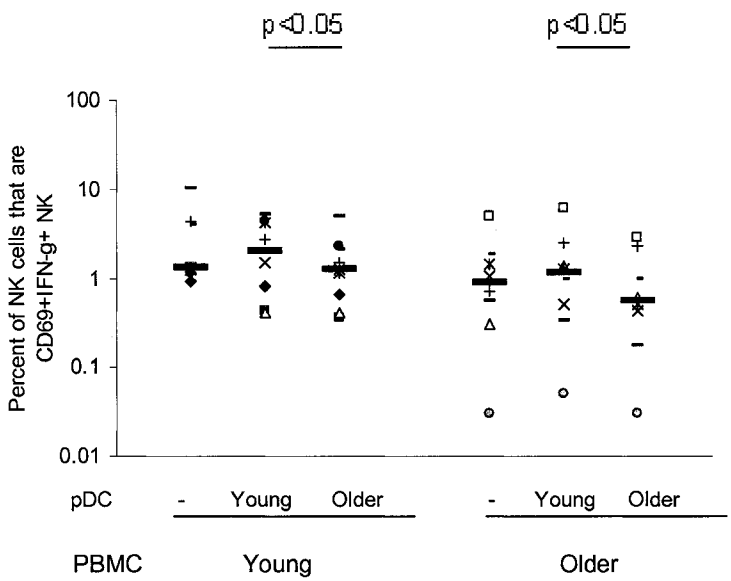


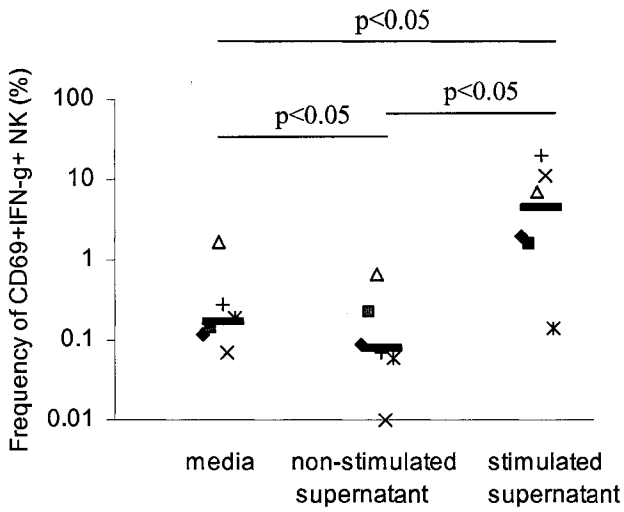
Figure 14. Differential effects of pDC from young or older subjects on NK cell activation in response to influenza virus

Plasmacytoid DC were isolated by using BDCA-4 magnetic beads from pooled PBMC from either young (2-3 donors per pool) or older (2-3 donors per pool) volunteers.). One a hundredth million isolated pDC were co-cultured with 1 million individual PBMC in the presence of 1 μl of influenza virus in 96-well plate. FI assay and flow cytometry was used to determine the frequency of activated NK cells. The graph represents data from 4 independent experiments involving 9 young and 8 older subjects. Horizontal bars represent the median.

- *Cell-cell contact contributes to NK cell activation in response to influenza viral stimulation*

Results from Figure 13 suggested that NK cells are activated in the absence of contact

A.



B.

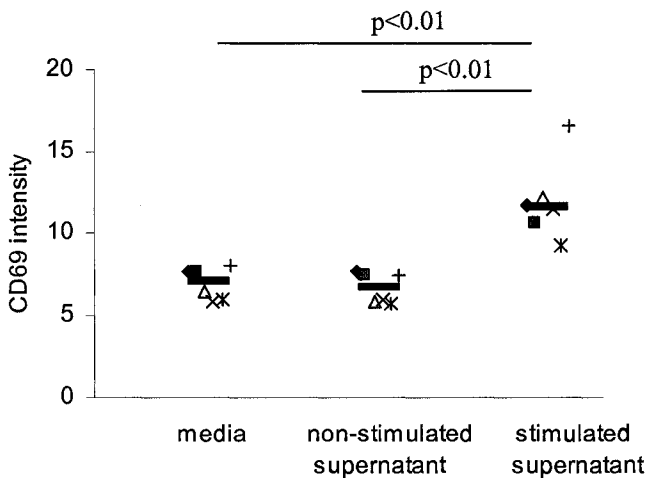


Figure 15. NK cell activation by supernatant generated from influenza virus-stimulated PBMC

One million PBMC were stimulated with 1 μl of influenza virus as did in regular FI assay except for no addition of BFA. Supernatant was collected the next day and used to stimulate NK cells isolated by CD56 micro-beads without addition of influenza virus. Supernatant and NK cells were donor-matched. A regular FI assay and flow cytometry was used to detect NK cell activation manifested by IFN-γ secretion in CD69+ cells. A, frequency of CD69+IFN-γ+ NK cells. B, CD69 intensity in NK cells. Six healthy young subjects were recruited to this study. Horizontal bars represent the median.

with other cells. On the other hand, in the environment of PBMC, contact with other, particularly antigen presenting cells may provide NK cell a better opportunity to be

activated to a higher level than that by soluble factors alone. To examine whether cellular contact plays a significant role in NK cell activation, we performed a transwell experiment, in which isolated CD56+ NK cells were placed in transwells and prevented from contact with CD56-removed PBMC placed in the regular well and stimulated with influenza virus. Soluble factors can freely transport between the transwells and regular wells through semi-permeable membranes. It was observed when cell-cell contact was prevented, NK cell activation was significantly down-regulated, reflected by a reduction in both the percentage of CD69+IFN-γ+ NK cells and CD69 intensity in overall NK cells compared to the contact control (Figure 14). In contrast to 2-3.9% of NK cells activated by contact with CD56(-) PBMC, only 0.05-0.3% of NK cells were activated without contact ($p<0.05$). The intensity of CD69 was 5.3-11.6 with contact and 4.8-9.2 without contact ($p<0.05$). These results clearly indicated that cell-cell contact regulation is playing a vital role in activating NK cells in response to influenza stimulation.

III.6 Influences of aging on expression of receptor or ligand involved in cell-cell recognition between NK cells and antigen presenting cells

- *Differential expressions of NKp44 and NKp46 receptors on NK cells in PBMC between young and older subjects*

NK cells recognize and elicit cytotoxicity towards viral infected antigen presenting cells, particularly monocytes via conventional and unconventional ways. The conventional way involves interaction between NK inhibitory receptors and MHC I molecule on monocytes and an unconventional way involves recognizing viral proteins, HA protein for influenza virus, expressed on infected APC by NK activating receptors NKp44 and NKp46. Since cell-cell contact significantly influences IFN-γ production in NK cells in PBMC in response to influenza virus (Figure 16), it is of interest to examine how aging influences the expression of the activating receptors specifically recognizing influenza HA protein. The expression pattern of NKp44 and NKp46 was different in NK cells. The former didn't show distinct NKp44 positive or negative cell populations, therefore was analyzed only the overall intensity on NK cells. The latter exhibited distinct NKp46

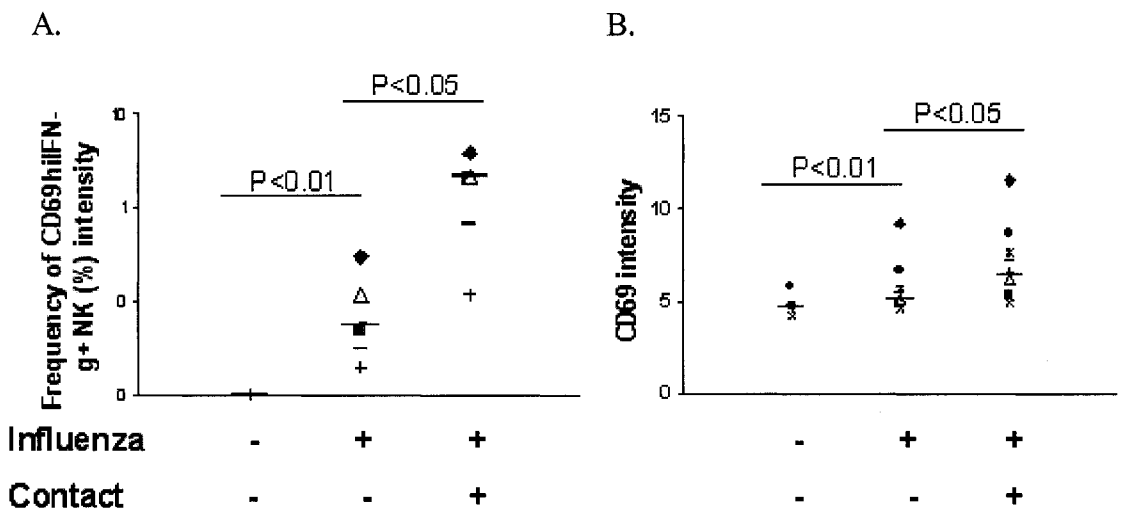


Figure 16. Down-regulation of NK cell activation in response to influenza virus without cellular contact

One million CD56-reduced PBMC were stimulated with 20 μl of influenza virus. 0.2 million CD56+ NK cells isolated from PBMC was placed in transwell or mixed with CD56-reduced PBMC in regular well as the control. FI assay and flow cytometry was used to detect NK cell activation manifested by IFN-γ secretion in CD69+ cells. A, frequency of CD69+IFN-γ+ NK cells. B, CD69 intensity in overall NK cells. Elongated bars represent the median.

positive or negative cell populations, therefore we analyzed both the intensity of NKp46+ NK cells and the frequency of NKp46+ cells in total NK cell populations. We observed that both the NKp44 and NKp46 expression were significantly increased by influenza viral stimulation, reflected by the increased intensity of NKp44 and NKp46 in both age groups ($p<0.05$, 0.01 or 0.001, Figure 17A) but not the frequency of NKp46+ NK cells, although the frequency of NKp46+ NK cells increased in both mock-stimulated and influenza virus-stimulated PBMC as compared to resting PBMC (Figure 17B). The values of the measurements are listed in Table IV. Opposite to that lower frequency of IFN-γ producing NK cell frequency was detected in the elderly than young subjects, higher intensity of NKp44 on resting NK, media (mock)-stimulated NK and influenza-stimulated NK cells (Figure 17A, upper plot) and higher frequency NKp46+ NK cells after influenza stimulation (Figure 17B, lower plot) were found in the older subjects than the young subjects. Considering older people possessed higher frequency of total NK cells in lymphocytes than young subjects (S. Figure 1), it was not surprising that the

frequency of NKp46+ NK cells in lymphocytes, whether under resting, mock-stimulated or influenza-stimulated conditions, was higher in the older subjects than that in the young (Figure 17B). These results indicated that NKp44 and NKp46 might not be the contributing factors for down-regulated NK cell activation with respect to IFN-γ production in older people, but a valid conclusion can not be drawn unless functional studies show that aging does not affect the functions of these receptors.

- *Differential expression of influenza HA protein on monocytes in influenza virus infected PBMC between young and older subjects*

NKp44 and NKp46 recognize influenza HA protein expressed on monocytes. We wonder whether there is differential expression of HA on monocytes between young and older subjects upon influenza viral infection. As expected, HA expression on CD14 monocytes was significantly elevated in response to influenza infection as compared to the mock (media) infection in both young and older subjects (Figure 18A, left) with HA intensity ranging from 43-55 (median=49) and 50-104 (median=69) for mock-stimulation and influenza-stimulation, respectively, in the young subjects and 36-65 (median=52) and 54-114 (median=66) for mock- and influenza-stimulation, respectively, in the older subjects. Consistently, CD69 expression on the monocytes was increased from 13.1-26.8 (median=18.8) to 19.4-48.6 (median=25.7) after influenza viral stimulation in the young subjects and from 13.9-26.5(median=18.4) to 22.2-50.6 (median=36.5) after influenza viral stimulation in the older subjects, confirming monocytes were activated by influenza virus. However, we could not detect differences in either the absolute values of HA intensity, CD69 expression on monocytes (Figure 18A) or the fold increase in them in response to influenza viral infection (Figure 18B) between young and older subjects.

A.

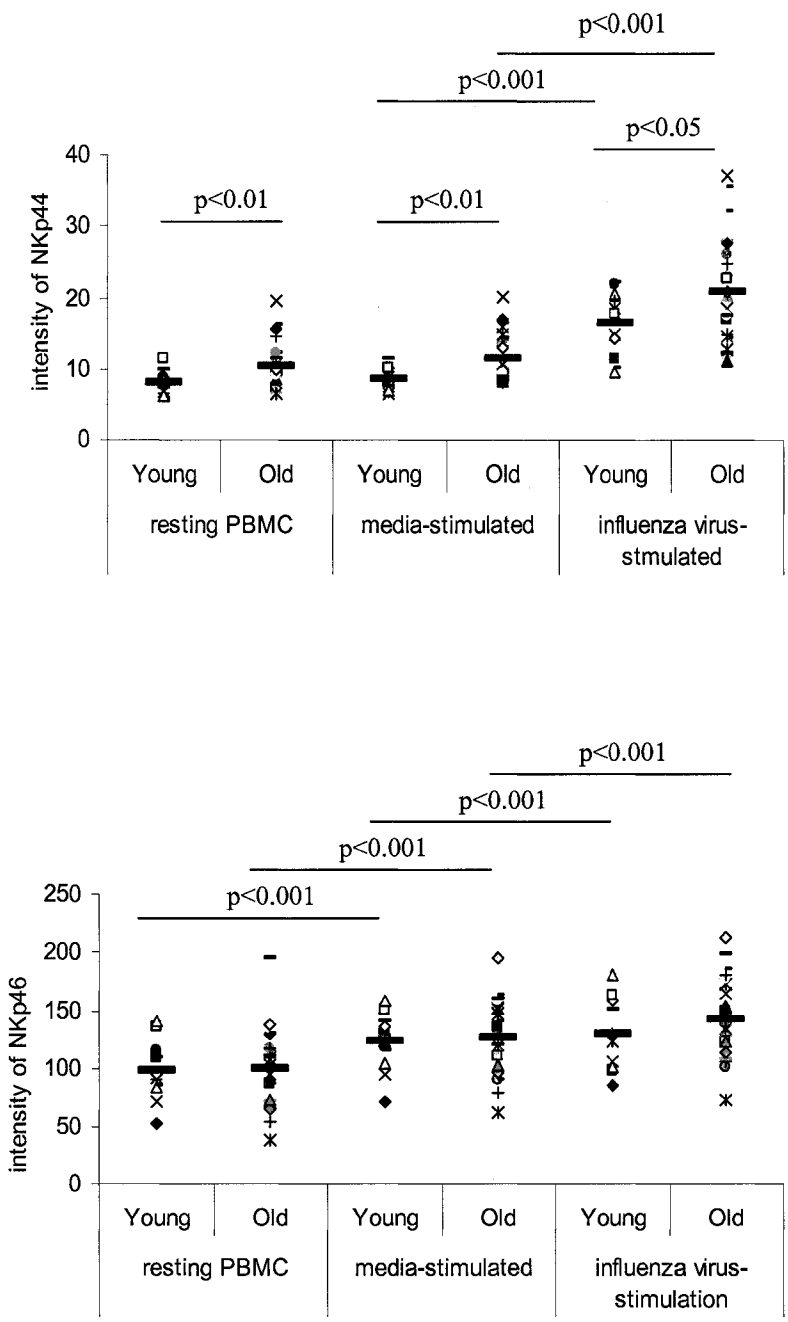


Figure 17. Comparison of NKp44 and NKp46 expression between young and older subjects

Resting, media (mock)-activated or influenza-activated PBMC from 12 healthy young and 24 healthy older subjects were measured by live cell staining and flow cytometry for NKp44 and NKp46 expression. The stimulation procedure followed regular FI culture set-up procedures except for using 2μl of influenza virus and adding no BFA. The cells were cultured for 18—20 hours before being stained. A, intensity of NKp44 (top) or NKp46 (bottom). B, Frequency of NKp46+ NK cells in overall NK cells (top) or in overall lymphocytes (bottom). Horizontal bars represent the mean.

B.

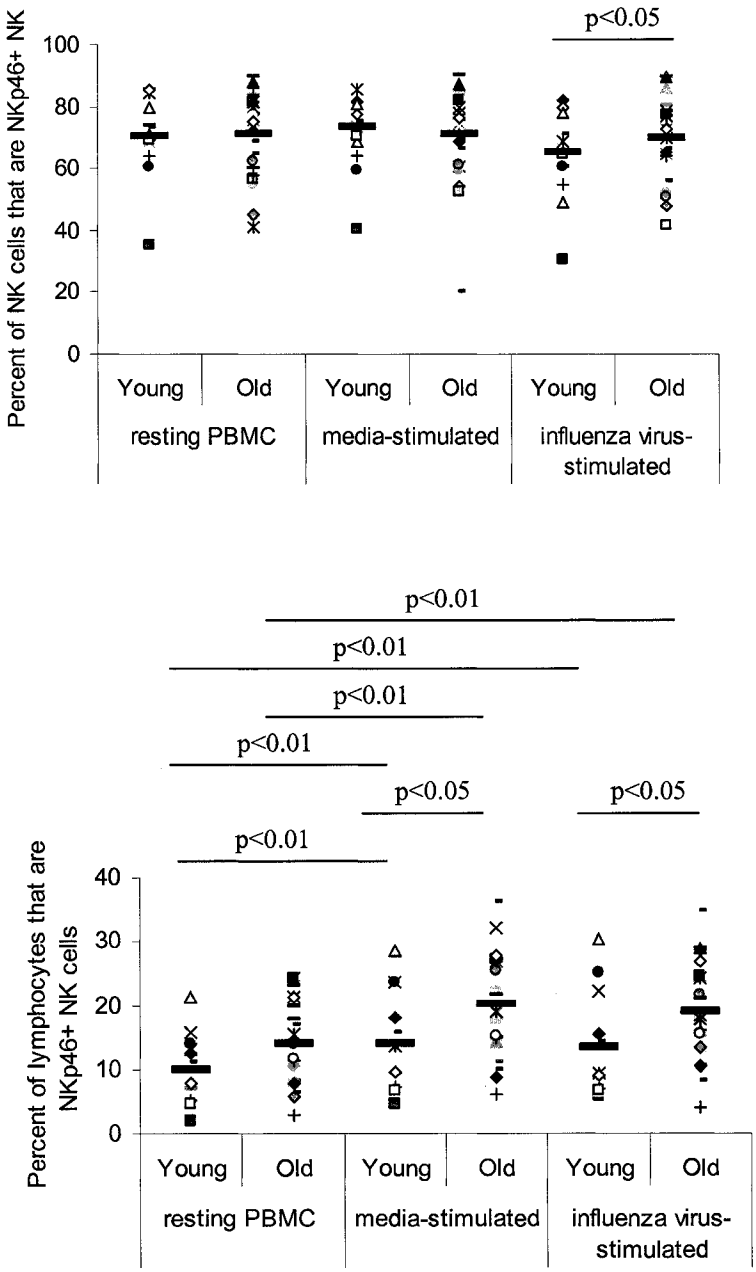


Figure 17 (cont.). Comparison of NKp44 and NKp46 expression between young and older subjects

Resting, media (mock)-activated or influenza-activated PBMC from 12 healthy young and 24 healthy older subjects were measured by live cell staining and flow cytometry for NKp44 and NKp46 expression. The stimulation procedure followed regular FI culture set-up procedures except for using 2μl of influenza virus and adding no BFA. The cells were cultured for 18—20 hours before being stained. A, intensity of NKp44 (top) or NKp46 (bottom). B, Frequency of NKp46+ NK cells in overall NK cells (top) or in overall lymphocytes (bottom). Horizontal bars represent the mean.

Table IV. Expressions of NKp44 and NKp46 on NK cells in PBMC *

Activation Status	Intensity of NKp44		Intensity of NKp46		NKp46+ NK% in NK		NKp46+ NK% in inlymphocytes	
	young	old	young	old	young	old	young	old
resting PBMC	8.1(1.6)	10.6(3.1)	99(26)	100(34)	70(13)	71(13)	10(5.6)	14(6.7)
media-stimualted	8.5(1.5)	11.6(3.1)	124(24)	127(32)	74(12)	71(15)	14(7.9)	20(7.8)
influenza virus-stimulated	16.6(4.4)	21.0(7.3)	130(29)	144(34)	66(14)	70(13)	13(8.3)	19(7.4)

* Values are expressed in the format of mean (s.d.).

III.7 Examination on Monocyte-mediated NK Cell Activation in Response to Influenza Virus

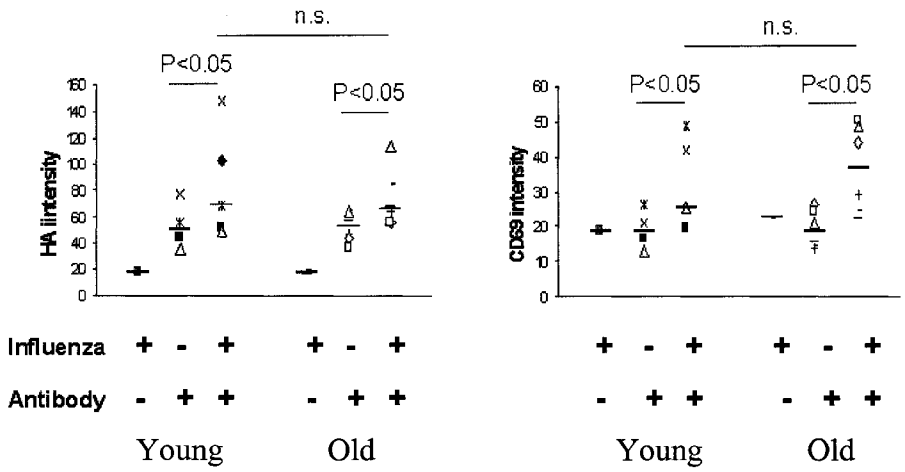
- *Comparison in frequency of monocytes between young and older subjects*

Monocytes play an important role in cell-cell contact regulation of NK cell activation(229). We wanted to know whether the frequency of monocytes in PBMC was different between young and older subjects. As indicated in Table III, there was no significant difference in the frequency of CD14+ monocytes between the two age groups, indicating the aging effect on monocyte-mediated NK cell activation, if any, was not a result from age-related numerical deduction of monocyte.

- *Examination of monocyte-mediated NK cell activation in young and older adults*

Monocytes regulate NK cell activation via secreting cytokines and cell-cell contact mechanism in response to influenza virus; therefore this interaction can be a potential contributor for NK cell activation down-regulation in older people. It is of interest to know how monoyctes isolated from young or older people differentially mediate NK cell activation in response to influenza virus. We performed FI assays, in which equal amounts of CD14+ monocytes generated by pooling equal amount of monocytes isolated from individual donors in young or older subjects were added to moncyte-depleted,

A.



B.

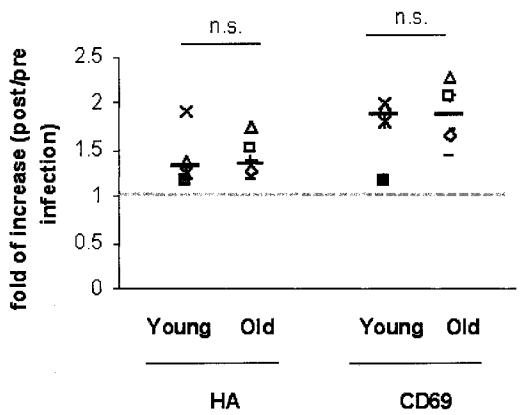


Figure 18. Comparison of influenza HA and CD69 expression on monocytes between young and older subjects

Media (mock)- or influenza-activated PBMC from 12 healthy young and 24 healthy older subjects were measured by live cell staining and flow cytometry for HA and CD69 expression on Cd14+ monocytes. Regular FI culture set-up procedures were followed except for not having BFA added into the culture. One μl of influenza virus was used for infection. The cells were cultured for 14-16 hours before staining was initiated. A, intensity of HA (left) or CD69 (right). B, fold of increase in HA and CD69 intensity upon influenza stimulation. Horizontal bars represent the median.

namely Mono(-) PBMC and infected by influenza virus. We could not detect a significant difference in NK cell activation in Mono(-) PBMC from either young or old subjects between addition of non-aged (young) monocytes and aged (older) monocytes (Figure 19), although there was a trend of lower NK cell activation in older subjects

between addition of two kinds of monocytes (p=0.08, Figure 19). The percent of NK cells that were activated in the presence of young and aged monocytes in the young subjects ranged from 0.8-18.2% with median=10.1% and 0.6-24.3% with median=4.2%, respectively. Percent of NK cells that were activated in the presence of young or aged monocytes in the older subjects ranged from 0.5-11.7% with median=2.6% and 0.2-6.7% with median=1.2%. We didn't observed an up-regulation of NK cell activation when additional monocytes, whether young or aged were added to Mono(-) PBMC as compared to that without additional monocytes. There was a trend, though not having reached the significant level that NK cell activation was lower in older subjects than that in the young when Mono(-) PBMC from young and older subjects were treated the same way (Figure 19). There seemed to be an influenza-unrelated activation due to monocyte isolation procedure in influenza virus-stimulated Mono(-) PBMC for NK cell activation was significantly elevated in Mono(-) PBMC, regardless the age groups, compared to that in the untouched PBMC (Figure 17). From these data, we could not draw a conclusion on how monocytes from young and older subjects differentially mediated NK cell activation while it seemed the difference between young and aged monocyte in activating NK cells with lower activation by aged monocytes was more obvious in older Mono(-), PBMC than that in young Mono(-) PBMC. Whether this is a valid conclusion remains to be further investigated.

III.8 Summary of Results

- *Specific Aim 1*

1. NK cell activation measured as the frequency of CD69+IFN-γ+ NK cells as well as the intensity of IFN-γ in CD69+IFN-γ+ NK cells in response to influenza viral stimulation in PBMC is reduced in older subjects as compared to the young subjects.

2. Down-regulated NK cell activation correlated with down-regulated IFN-α level and T cell activation in response to influenza viral stimulation in PBMC in older subjects as compared to the young subjects.

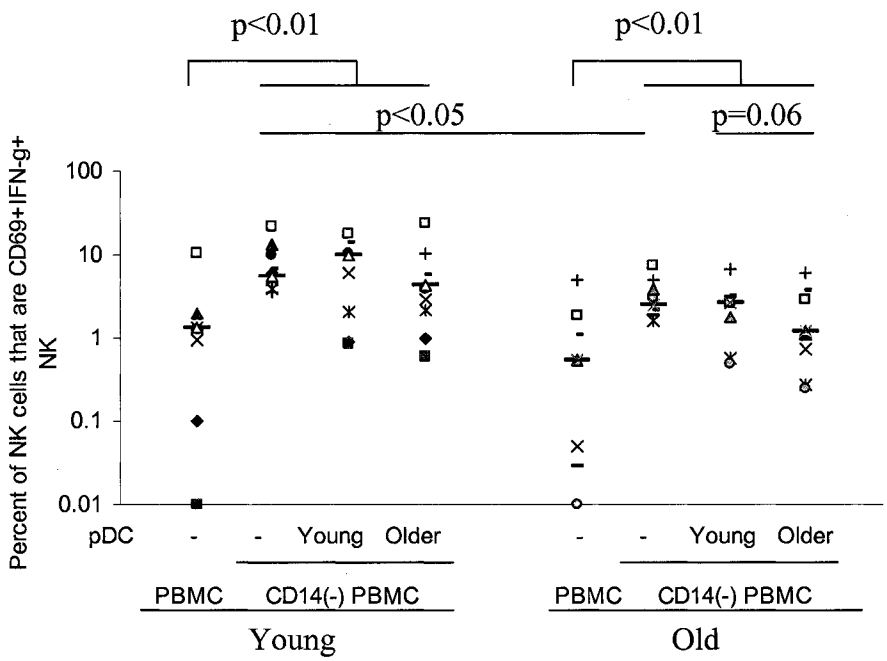


Figure 19. Effect of monocytes from young or older subjects on NK cell activation in response to influenza virus

Monocytes were isolated by using CD14 magnetic beads from individual PBMC and pooled for either young (2-3 donors per pool) or aged (2-3 donors per pool) monocytes for study. One a hundredth million isolated pDC were co-cultured with 1 million individual PBMC in the presence of 1 μl of influenza virus in 96-well plate. FastImmune assay and flow cytometry was used to determine the frequency of activated NK cells. The graph represents data from 4 independent experiments involving 9 young and 8 older subjects. Elongated bars represent the median.

3. Both IFN-α and IFN-γ play positive roles in stimulating NK cells to produce IFN-γ in response to influenza virus as demonstrated by effects of neutralizing antibodies against IFN-α/IFN-α/β receptor or effects of rIFN-γ.

4. Plasmacytoid DC and T cells play a positive role in stimulating NK IFN-γ production as demonstrated by pDC-depletion assays.

5. Lower frequencies of pDC and CD3+ T cells in PBMC were observed in older subjects. Lower levels of IFN-α secreted by purified pDC and lower frequency of IFN-γ-producing

CD3+ T cells in PBMC in response to influenza virus were detected in older subjects than young subjects.

6. Plasmacytoid DC isolated from young subjects better facilitated NK cell activation than pDC isolated from older subjects in response to influenza viral stimulation in PBMC.

- *Specific Aim 2*

1. Supernatants generated from influenza virus-activated PBMC in the absence of cell-cell contact stimulated NK cells to produce IFN-γ. Preventing NK cell contact with other cells by transwell significantly down-regulates NK cell to produce IFN-γ.

2. Expression of NK cell activating receptors NKp44 and NKp46 on NK cells in PBMC were elevated in response to influenza stimulation in both young and older subjects. Surprisingly, expression levels of both receptors were higher in older subjects than that in the young whether PBMC were at resting, mock-stimulated or influenza-stimulated status.

3. No significant difference in expression levels of influenza HA on monocytes upon influenza infection was observed between young and older subjects.

4. No significant difference in frequency of monocytes in PBMC was observed between young and older subjects. Monocytes isolated from young subjects tended to better facilitate NK cell activation than monocytes isolated from older subjects in response to influenza virus in older subjects.

IV. DISCUSSION AND CONCLUSIONS

IV.1 Down-regulated Natural Killer (NK) Cell Activation in Response to Influenza Virus in Older Subjects

Influenza is a disease associated with high mortality and morbidity in elderly population (2-4). Natural killer (NK) cells play a critical role in controlling viral load at early stages of immune defense by eliciting cytotoxicity and secreting cytokines, particularly IFN- γ (82). NK cells respond to influenza infection and vaccination (85, 155, 156). Our study demonstrates a significant aging-related down-regulation of IFN-γ production by NK cells in response to influenza virus (Figure 5).

The aging-related impairment in NK cell IFN-γ production is manifested by a lower frequency of CD69+IFN-γ+ NK cells (Figure 3B) in influenza virus-infected PBMC from the elderly subjects than that from the young subjects. The frequencies of CD69+IFN-γ+ NK cells were calculated in both total NK cells and total lymphocytes. The frequencies calculated using these two different denominators were highly correlated with the correlation coefficients of 0.90, 0.74 and 0.85 in the young, elderly and combined groups, respectively. We deem the necessity of calculating CD69+IFN-γ+ NK frequencies in both total NK cells and total lymphocytes lies in two aspects. First, NK cells are phenotypically and functionally heterogeneous with $CD56^{dim}CD16^{bright}$ subset primarily executing cytotoxicity and secreting small amount of IFN-γ, and $CD56^{bright}CD16^{dim}$ subset being the primary IFN-γ-secreting cells (82, 84). Using total NK cell number as the denominator can not guarantee the age-related decline in the frequency of CD69+IFN-γ+ NK cells is due to the decline of the IFN-γ-producing NK cell number but not the increase of the non-IFN-γ-producing NK cell number. In fact, an age-related increase in peripheral NK cell number has been found in both $CD56^{dim}CD16^{bright}$ and $CD56^{bright}CD16^{dim}$ NK subsets with higher increased number of $CD16^{bright}$ cells than $CD56^{bright}$ cells (86, 230, 231). Unfortunately, we were not able to differentiate these two subsets in our experiment due to the same fluorescent shared by CD16 and CD56 antibodies. Second, ignoring NK subsets, the age-related increase in the number of total

NK cells bears the potential to balance the age-related decrease in the frequency of IFN-γ + NK cells calculated in total NK cells. Calculating the frequencies of IFN-γ -producing NK cells in total lymphocytes becomes necessary in determining whether lower proportion of IFN-γ+ NK cells in total NK cells can be a fair reflection of less IFN-γ -producing NK cells in peripheral blood in elderly subjects than young subjects. As shown in Figure 5B, whether total NK cell number or total lymphocyte number was used as the denominator, the frequency of CD69+IFN-γ+ NK cells in response to influenza stimulation is lower in the elderly subjects than that in the young subjects, suggesting the lower frequency of CD69+IFN-γ+ NK cells results from the reduction in the number of NK subsets that are capable of producing IFN-γ.

In addition to numerical decline, the aging-related impairment of NK cell IFN- γ production in response to influenza virus was reflected by the significantly lower IFN- γ intensity in NK cells in the elderly subjects than that in the young subjects (Figure 5C). The lower IFN-γ intensity indicates NK cells, as a heterogeneous entity in PBMC, are functionally impaired in IFN-γ production in response to influenza virus in the elderly subjects compared to the young. It should be noted that this lower IFN-γ intensity can be a consequence of the reduced IFN-γ production in NK cells in a per-cell basis and/or the relative increase in the proportion of $CD16^{high}$ over $CD56^{high}$ cells in the total IFN-γ+ NK cells with no change in the per-cell based IFN-γ production in a single NK cell. Thus, no conclusion regarding truly functional impairment in per-cell based IFN-γ production in NK cells in the elderly subjects compared to the young can be drawn without differentiating $CD56^{high}$ versus $CD16^{high}$ NK cell subsets, though NK cell as a whole does display aging-related functional defect in IFN-γ production. The aging-related down-regulation of IFN-γ production by NK cells in response to influenza virus is not related to the impairment in signal transduction during the early stage of NK activation for no significant difference in the frequency or intensity of CD69+ NK cells has been detected between young and elderly subjects. This suggests early signal transduction during NK cell activation in PBMC in response to influenza infection is likely well preserved with advanced aging.

In summary, our data demonstrates the significantly down-regulated human NK cell IFN-γ production in PBMC from elderly subjects compared to young subjects in response to influenza infection. This aging-related down-regulation of IFN-γ production in NK cells is manifested by the reduction in the frequencies of CD69+IFN-γ + NK cells in total NK cells as well as in total lymphocytes, and in the amount of IFN-γ produced by total NK cells, but not in the early signal transduction reflected by CD69 expression. NK cell activation involves three activities: proliferation, cytotoxicity, cytokine, particularly IFN-γ production (230). Evidence has shown that purified NK cells from the elderly subjects proliferate to the less extent responsive to IL-2 than NK cells from the young subjects while the IL-2 levels in response to influenza virus have been shown to be lowered with advanced aging under many, if not all experimental conditions (171, 172, 175, 230). NK cytotoxicity is also known to be affected by aging. Thus, age-related changes have been detected in all three facets of NK activation in response to influenza virus, suggesting NK cells could be one of the major targets of the aging process that contributes to the age-related impairment in immune defense against influenza.

IV.2 IFN-α, pDC and Down-regulated NK Cell IFN-γ Production in Response to Influenza Virus in Older Subjects

IFN-α is a family of cytokines comprising 13 different functional subtypes (232, 233). IFN- α have been recognized as a pro-Th1 cytokine that stimulates NK and T cell cytotoxicity and IFN-γ production synergistically with IL-18 (216, 234). In addition, type I interferons including IFN-α and IFN-β, have been considered important in enhancing humoral immunity, particularly by providing the help for plasma cell differentiation (199, 235, 236). NK cell IFN-γ production in response to influenza virus has been shown down-regulated in the elderly subjects compared to the young subjects in earlier session of our study (Figure 5). How important IFN-α is involved in influenza-induced NK cell IFN-γ production in the aging context has not been addressed previously. Our data have suggested IFN-α is a significant contributor participating in the aging-related down-regulation of NK cell IFN-γ production in response to influenza virus by providing the following evidences.

First, in the presence of IFN-α or IFN-α/β receptor neutralizing antibodies, the frequency of IFN-γ producing NK cells considerably decreased compared to the control, indicating IFN-α is a significant mediator for NK cell IFN-γ production in influenza virus-stimulated PBMC (Figure 7). The importance of IFN-α in this scenario is further verified by the use of CpG ODN2216, and the depletion of pDC, one of the major sources of IFN-α in PBMC (237). The CpG is known to specifically induce IFN-α production in pDC though activating TLR-9 (238). Our data has verified that CpG ODN2216 specifically induces IFN-α production in CD123high (pDC) cells but not CD123low (monocytes) cells (Figure 8A). The frequency of IFN-γ -producing NK cells in influenza-stimulated PBMC increases in parallel with the elevated IFN-α production in the presence of the CpG ODN2116 while decreases by pDC depletion (Figure 8B and 11A), confirming the regulatory role of IFN-α in stimulating NK IFN-γ production in response to influenza virus. We have realized that the inhibitory effect of pDC depletion on NK IFN-γ production is not as strong as the neutralization of IFN-α or its receptors, and replacing the depleted pDC back to the culture only created a trend but not the significant level of restoration of the frequency of IFN-γ-producing NK cells (Figure 7 and 8A). This is likely due to the effect of IFN-α produced by the remaining monocytes and possibly pDC in PBMC after pDC depletion. We have also tried to examine the effect of rIFN-α on NK IFN-γ production in a dose dependent manner but have not detected any effect. This is unlikely due to the mismatched IFN-α subtype used in our experiment because IFN-α2, the subtype we used is known to be a functioning subtype in PBMC in response to influenza virus and was detected by IFN-α2 specific antibody in our assays (ref, Figure 8A). Possible reasons could be that endogenous IFN-α level had been saturated before rIFN-α was added and/or there needs another soluble factor working in conjunction with IFN-α2 to optimize the effect.

Second, we have found IFN-α level in the supernatant of activated PBMC is significantly reduced in the elderly subjects compared to the young subjects in response to influenza viral stimulation, which coincides with the lower frequency of IFN-γ-producing NK cells described earlier (Figure 6A and 5). The correlation between IFN-α level and the

frequency of IFN-γ-producing NK cells from these samples is moderate (r=0.56) but statistically significant ($p<0.05$, Table II). Moreover, we have found about 30% reduction in the average frequency of pDC in PBMC and 38±13.4% (mean+SD) reduction in the amount of IFN-α produced by purified pDC stimulated with influenza virus in the elderly subjects compared to the young subjects, suggesting that both numerical and functional impairments are present in pDC in response to influenza infection in the elderly. Figure 14 further suggests the existence of intrinsic defects in pDC in older subjects by showing higher frequency of IFN-γ+ NK cells in the presence of pDC isolated from the young subjects than those isolated from the older subjects in response to the influenza virus infection. That pDC secrete higher amount of IFN-α than monocyte in a per-cell basis while IFN-α+ cell number are comparable between CD123hi (manifest of pDC) and CD123low (majority monocytes) cells (data not shown) suggests that pDC is more productive in IFN-α generation than monocytes in response to influenza infection. Considering the significant role of IFN-α in activating NK cells, it is not surprising that the aging-associated impairments in pDC will significantly contribute to the down-regulation of NK IFN-γ production in response to influenza virus in the elderly subjects.

IV.3 IFN-γ, T cells and Down-regulated NK Cell Activation in Response to Influenza Virus in Older Subjects

In addition to its role as an effector cytokine, IFN-γ plays a regulatory role in mediating Th1 immune response, promoting NK cell differentiation and influencing antibody production (176, 184). IFN-γ is secreted by CD4+ type I helper T cells, CD8+ cytotoxic T cells and NK cells in response to influenza infection (174, 239). Our preliminary kinetic study on CD69 expression in PBMC suggests that T cell activation precedes NK cell activation in response to influenza virus, generating the possibility that IFN-γ produced by T cells influences NK cell IFN-γ production in response to influenza virus (S. Figure 3).

The role of IFN-γ in regulating NK IFN-γ production specifically in response to influenza virus is verified in our study. First, our data indicates a trend that the frequency of IFN-γ -producing NK cells declines in the presence of neutralizing antibodies against IFN-γ, though the decline does not reach significant level (Figure 9). In fact, the trend of the decline is obvious in the subjects with higher baseline levels of IFN-γ+ NK cell frequency than those with relatively lower baseline levels (Figure 9), indicating the responsiveness to the neutralizing antibody may require certain baseline limit. Second and more importantly, in response to influenza virus purified (CD56+ and/or CD16+) NK cells can not be activated to produce IFN-γ in the presence of monocytes unless rIFN-γ is added, indicating the importance of pre-existing IFN-γ in inducing NK cell IFN-γ production (Figure 10). Moreover, purified NK cells can not be activated to produce IFN-γ in the presence of combination of influenza virus and rIFN-γ (data not shown), indicating IFN-γ mediates NK cell activation via acting on monocytes. He and Romagnani have reported previously that the production of IFN-γ in CD56bright NK cells is mediated by CD3+ or CD4+ T cells in an IL-2-dependent manner (159, 222). Monocytes are not known to be a source of IL-2 but IL-15, a cytokine that share similarity with IL-2 and capable to activate NK cells via a NK-monocyte contact-dependent mechanism (240, 241). It will be of interest to see whether IFN-γ is able to induce IL-15 secretion in monocytes and further mediate NK IFN-γ production in response to influenza virus. Last, we have tried to deplete CD3+ T cells from PBMC but have not found the difference in NK cell IFN-γ production between CD3+ T cell-depleted and non-depleted PBMC. Interestingly, when isolated T cells were added back to the CD3(-) PBMC, there exhibits a T cell amount-dependent increase in NK IFN-γ production in response to influenza stimulation (Figure 11B), supporting the role of T cells in positively influencing NK IFN-γ production. We think the unresponsiveness of NK IFN-γ production to CD3+ T cell depletion might be due to the non-specific activation in T(-) PBMC caused by the isolation procedure.

Taken together, our data suggest a positive role of existing IFN-γ on NK cell IFN-γ production prior to NK activation. The source of the earlier generated IFN-γ is likely the activated memory T cells. Importantly, down-regulation of IFN-γ production in CD4+

Th1 and CD8+ cytotoxic T cells in response to influenza infection and vaccination with advanced aging has been documented (81, 174, 188, 242). Consistent with the literature, our data shows that IFN-γ production determined as the frequency of IFN-γ + CD3+ T cells in PBMC stimulated with influenza virus is reduced and correlates with the decreased frequency of IFN-γ-producing NK cells in the elderly subjects compared to the young subjects (Figure 5B, 6B and II). Along with the decreased IFN-γ+ T cell frequency, a lower frequency of total peripheral T cells is found in the elderly subjects than in the young subjects (Table III), indicating both numerical and functional impairment of IFN-γ producing T cells is associated with aging in response to influenza virus. Considering the significant role of IFN-γ in mediating NK cell IFN-γ production, we believe the age-associated down-regulation of T cell IFN-γ production contribute to down-regulated NK cell IFN-γ production in older people in response to influenza virus.

IV.4 Cell-cell Contact Regulation in NK Cell Activation in Response to Influenza Virus in an Aging Context

Cell-cell contact between NK cells and antigen presenting cells, particularly dendritic cells and monocytes has been suggested a required condition for different aspects of NK cell activation (229). For instance, in human ex vivo experiments, preventing the contact between monocytes or monocyte-derived mDC can reduce NK cell IFN-γ secretion, cytotoxicity and CD69 expression and preventing the contact between pDC and NK cells can lower CD69 expression on NK cells without affecting NK IFN-γ secretion and cytotoxicity in response to influenza virus (194, 239). Consistent with these findings, our data shows a significant reduction in both the frequency of IFN-γ+ NK cells and the CD69 intensity in NK cells in response to influenza virus when cell-cell contact between NK cells and NK(-) PBMC is prevented compared to when the contact remains available (Figure 16). On the other hand, our data shows purified CD56high NK cells can be activated by supernatant generated from influenza-stimulated PBMC without the presence of contacting with other cell types, suggesting cellular contact between NK cells and antigen presenting cells (APC) may not be an essential condition, but possibly required for the optimal level of NK cell activation (Figure 15 and 16).

The mechanism of cellular contact between NK cells and APC to mediate NK activation could lie in the reciprocal interactions between NK cells and APC, that is, cellular contact is required for full activation of APC and conversely fully activated APC secrete optimal levels of cytokines to promote NK activation (194). NK receptors or other molecular elements that participate in this cell-cell contact regulation are not clear yet. But evidence has shown that NK natural cytotoxic receptors (NCR) NKp44 and NKp46 specifically recognize influenza HA and sendai virus hemaglutinin-neuraminidase (HN), both viruses inducing cytokine as well as NK- monocyte-derived macrophages contact-dependent NK cell activations (229). NKp44 and NKp46 are known to be exclusively expressed on NK cells and mediate NK cytotoxicity (202, 217). Our data shows that influenza virus enhances the expressions of these two receptors on NK cells (Figure 17A and B). Without knowing the role of these receptors in mediating NK cell IFN-γ secretion, we started with examining how aging influences the expressions of the receptors by comparing their expression levels on NK cells before and after influenza stimulation between the young and the older subjects. Our data shows whether at resting, mock- or influenza-stimulated status, NKp44 intensity is higher in the older subjects than that in the young (Figure 17A). NKp46 intensity level is comparable between the young and the old subjects independent of the treatments while the frequency of NKp46+ NK cells in lymphocytes is significantly higher in the older subjects than that in the young, leading to a numerical increase in NKp46+ NK cells with advanced aging (Figure 17A and B). The higher frequency or expression level of NKp44 and NKp46 on NK cells from the elderly subjects than the young subjects can hardly explain the aging-related impairment in NK cell IFN-γ production in response to the influenza virus. But there are a few possibilities that might help understand this contradiction. First, a frequency change does not always positively correlates with a functional change. We did not perform functional analysis on the NCR receptors, therefore that the receptor-positive NK cell frequency increases as a compensatory mechanism in response to the declined function of the receptors remains possible. Second, the time when the measurements (12-14 hours post viral stimulation) were done may not be the optimal time point to detect the difference in the receptor expression between the two age groups. It is reported that

influenza virus infects and the viral replication continues for 4-6 hours before being halted in human peripheral monocytes (243). It is possible that the lower receptor intensity in the NK cells from the young subjects than those from the older subjects at the time of the detection results from the kinetic difference in the receptor expression between the young and the elderly subjects, or more specifically is caused by the earlier declining of the elevated receptor expression on the surface of NK cells in response to influenza virus in the young subjects than in the older subjects. Future studies addressing these possibilities may help clarify the observation.

To examine whether monocytes from young or older people function differentially in stimulating NK cell to produce IFN-γ in response to influenza, we have examined NK cells in monocyte-depleted PBMC with restoration of monocytes isolated from either the young or the elderly subjects. Unfortunately, we could not detect the significant differences in NK cell IFN-γ production between young and old monocyte-mediated conditions in either the young or the elderly group (Figure 19). In spite of this, there was a trend that the young monocytes facilitate a better NK cell activation than the aged monocytes, particularly for the older subjects (Figure 19). Increasing sample size in future studies might help generate more significant data and better delineate the role of monocytes in regulating NK cell activation in an aging context.

It is interesting that in the transwell experiment when CD56+ NK cell was in transwell and CD56(-) PBMC were in the regular well, not only the IFN-γ production of NK cells in the transwell was down-regulated but also that of the T cells in the regular well (data not shown). This is consistent with our previous observation that NK cell activation highly correlates with T cell activation in PBMC in response to influenza stimulation (Figure 4 and Table II). A simple explanation would be that preventing cell-cell contact down regulate NK cell IFN-γ production, which in turn affect monocyte activation and consequently lead to down-regulated T cell activation. If this was true, a reciprocal regulation between NK cell and T cell activations by IFN-γ autocrine/paracrine asecretions could be established through the link of monocytes.

In summary, cell-cell contact regulation between NK cells and APC, particularly moncytes, monocyte-derived mDC and macrophages have been suggested as an important part of the requirements for optimal NK cell activation. Our data has confirmed that both cell-cell contact between NK cells and APC and soluble factors play a significant role in inducing NK cell IFN-γ production. How cell-cell contact functions alone and cooperates with soluble factors to activate NK cells remains to be elusive.

IV.5 Potential Roles of Other Soluble Factors in Aging-related Down-regulation of NK Cell IFN-γ Production in Elderly Subjects in Response to Influenza Virus

Our study has suggested that aging-related reduction in pDC IFN-α and T cell IFN-γ productions contribute to the impairment in NK cell IFN-γ production in PBMC in response to influenza virus for the elderly adults. The fact that no significant NK cell IFN-γ production was detected when isolated CD56+NK cells were stimulated with IFN-α alone, IFN-γ alone, influenza alone or any combination of the three stimuli (data not shown) indicates other factors are required for inducing the NK cell activity in response to influenza. Correlation analysis in the combined young and elderly subjects reveals moderate correlations between the supernatant IFN-α level and the frequency of IFN-γ-producing NK cells (r=0.56), and weak correlation between the IFN-α level and the frequency of IFN-γ-producing T cells (r=0.35), further supporting that multiple factors are involved in NK and T cell activation in PBMC and weaken the linear relationship between the supernatant IFN-α level and the frequencies of IFN-γ+ NK or T cells (Table II).

Besides IFN-α and IFN-γ, other soluble factors that potentially display the age-related changes and able to influence NK IFN-γ production in an aging context include, but are not restricted to IFN-β, IL-18, TNF-α and IL-2 (74, 234). IFN-β is one of the major type I IFNs that shares the similarities with IFN-α in antiviral effect and inducing NK cells and T cells to produce IFN-γ (232). IL-18 is known to be able to induce NK and T cell cytotoxicity and IFN-γ production synergistically with IFN-α/β, IL-12 and/or TNF-α, among which, IL-12 is not known to be significantly produced in human PBMC in

response to influenza virus (48, 157, 193, 244, 245). Our previous data has shown PBMC from elderly subjects secrete lower level of TNF-α in supernatant than young subjects at 3 hours but not 18 hours post influenza A stimulation, indicating a potential role of TNF-α at early stage of immune reactions in contributing to the age-related NK activation down-regulation (174). Surprisingly, in the same study no significant difference in IL-2 level between young and old subjects were detected, indicating IL-2 level may not be necessarily an essential factor for the age-related impairment of NK IFN-γ production in our experimental system, although IL-2 levels have been shown to be down-regulated with advanced aging in response to influenza stimulation under many other conditions (118, 119, 172, 175, 225, 246). Information about the age-related changes in the productions of IFN-b and IL-18 are very limited up to date, therefore their contribution to NK activation including IFN-γ production in the aging context remains unclear.

In addition to external factors, internal defects of NK cells with aging should be taken into consideration when comparisons in NK activation are made between different age groups. Literatures have shown that purified peripheral NK cells from elderly subjects display lower CD69 expression and proliferation ability in response to IL-2 than those from young subjects, indicating intrinsic impairment in NK cells do exist with advanced aging (230). On the other hand, studies on the expressions of cytokine receptors on NK cells have not suggested a difference in IL-2 receptor expression whether on resting or IL-2-stimulated NK cells between young and elderly subjects(247). Moreover, Plett and others even reports that higher level of IFN-α/β receptors with comparable IFN-α/b/receptor binding activity is found on NK cells from aged mice than young mice. But this elevated IFN-α/β receptor expression in the aged mice may be associated with enhanced apoptosis of NK cells induced by IFN-α. Apparently more studies are needed for obtaining a clear picture of how NK cell functions are influenced by aging. Future investigations on both the intrinsic and extrinsic factors related to NK activation will provide more valuable insights into the aging-related changes on NK cell activation in responses to influenza virus.

IV.6 Conclusions

In this study we have compared IFN-γ production in NK cells in influenza virus-stimulated PBMC between young and elderly subjects and investigated soluble factors, particularly IFN-α and IFN-γ, and cell-cell contact between NK cells and other cells in PBMC in mediating the NK cell IFN-γ production in an aging context. Based on our data, we conclude:

1. Interferon-alpha (IFN-α) and IFN-γ are important regulatory cytokines in stimulating NK cells to produce IFN-γ in PBMC in response to influenza virus. Aging-related numerical and functional impairments in pDC and T cells lead to the reduced production of IFN-α and IFN-γ, which contribute to the down-regulated NK cell IFN-γ production in the elderly subjects compared to the young subjects.

2. Cell-cell contact regulation between NK cells and APC in PBMC may be important in activating NK cells to the full extent in conjunction with soluble factors in response to influenza virus. How cell-cell contact regulates NK cell activation and what age-related differences in components involved in the cell-cell contact regulation contribute to the impaired NK cell activation in older people remain to be elucidated.

IV.7 Significance of the Study

Immune senescence leads to increased mortality and morbidity and decreased vaccine efficacy associated with influenza with advanced aging. As a critical component of the innate immune system, NK cells play an important role in defending viral infection by eliciting cytotoxicity and secreting IFN-γ. Our study has focused on delineating the aging-related changes in NK cell IFN-γ production in response to influenza virus and revealing potential causal factors for these changes. We have concluded that IFN-α and IFN-γ are important mediators for influenza-induced NK cell IFN-γ production, and aging-related impairments in IFN-α production by pDC and IFN-γ production by T cells contribute to the down-regulated NK cell IFN-γ production in the elderly subjects

compared to the young subjects. We have also suggested that the cell-cell contact between NK cells and monocytes is important in activating NK cells to produce IFN-γ to the full extent. To our knowledge, this is the first systemic investigation about the aging effect on NK cell IFN-γ production in response to influenza virus. Our data supports that NK activity can be one of the major targets of the aging process that leads to the immune dysfunction in the elderly people in response to influenza virus.

The significance of NK cells in influenza-induced immune response extends from the innate immunity to the adaptive immunity via the production of IFN-γ, the manifest cytokine of Th1 lymphocytes by NK cells. Previous studies have suggested Th1 but not Th2 lymphocytes prevent lethal dose of influenza virus or promote the recovery in normal or aging-accelerated mice, and Th1 but not antibody response correlates with the outcome of influenza vaccination in human (174, 189, 248, 249). These evidences suggest that Th1 immunity is an important part of anti-influenza immune responses. Our earlier work has shown an age-related impairment in IFN-γ production in influenza-specific CD4+ Th1 cells and cytotoxic T cells in older adults. Since T cells and NK cells are the predominant source of IFN-γ, our current study completes the picture that the deficiency of IFN-γ production in elderly adults in response to influenza virus is attributed not only to the T cells but also to the NK cells. Moreover, our study has confirmed the importance of IFN-α and IFN-γ in mediating NK IFN-γ production specifically to influenza virus and pointed out that the aging-related impairments in the productions of these interferons by pDC and CD3+ T cells contribute to the aging-related down-regulations in NK cell IFN-γ production. Therefore, our study has provided valuable information on how Th1 immune response is impaired in the elderly people in response to influenza virus.

NK cell activities, particularly cytotoxicity have been documented to be responsive to influenza vaccination with live-attenuated, inactivated trivalent split vaccines or inactivated viral particles in human, indicating NK cells are significantly involved in vaccination-induced defending mechanism against influenza (155, 156, 223). However, information about NK cell IFN-γ production in response to influenza vaccine is very

limited if not completely missing. We deem monitoring NK cell IFN-γ production should be as equivalently important as monitoring NK cytotoxcicity for a vaccination study. Although not directly related to a vaccine study our finding is valuable to be used as a reference at the starting point for a comparative influenza vaccine study between young and elderly subjects.

Our study utilizes human blood as the experimental material, therefore bypasses the possible discrepancies in information translation of the findings from animals to human. It's our strong belief that our findings will bring valuable insights on how aging influences innate immune system and Th1 immunity and help establish the fundamentals for developing more effective prophylactic and therapeutic approaches for elderly people.

IV.8 Restrictions of the Study

Similar to many other studies, our study is restricted in certain ways. First, influenza is a localized but not systemic infection (1). Using influenza-stimulated PBMC is a mimic of a systemic infection. However, this restriction is hard to be avoided due to the limited tissue types to be obtained from human subjects. Second, we did not consider the survival factors for the elderly subjects in our study. Aging is commonly associated with a survival issue. If the selection pressure for the survival is unrelated to the variable to be investigated, survival factor will not be important. Otherwise, it will be affecting the validity of the age-related change because the "change" could be a simply "unchanged" fact that is survived from other age-related mortality. Unfortunately, in most occasions it is hardly to know whether the selection pressure for the survival is related to the variable to be investigated or not. Longitudinal study, which is more cost-inefficient, would be a solution to avoid the influence of the survival factor. Third, it is common in human studies that sample availability restricts sample size, duplication possibility, repeating possibility. These restrictions may increase the chance of getting data with bigger variations and un-conclusive results.

IV.9 Future Directions

We would like to continue to investigate soluble factors in influenza-induced Th1 immunity in an aging context. We would differentiate subgroups of NK cells, particularly differing CD56high versus CD56low cells so that the aging-related absolute and relative increase of the frequencies of IFN-γ-producing NK subsets and decrease in the per-cell-based IFN-γ intensity will be clarified. Further more, questions as what is the mechanism by which cell-cell contact regulates NK cell activation; how relatively important pDC versus monocytes are in producing IFN-α; which subtypes of IFN-α are important; whether IFN-b participates in aging-related changes in immune regulation; how IFN-α contributes to the impaired T cell activation in the elderly; how IFN-γ influences monocytes and consequently influences NK cell activation; and how NK cells influence T cell function in response to influenza virus would be of great interest to be investigated.

We would also like to investigate the relationship between Th1 response and antibody response in influenza-induced immunity. Generating protective antibody is one of the primary goals of the vaccination. Knowing how cytokines influence antibody production in young and elderly subjects in response to influenza infection and vaccination will be of great value for providing the fundamentals for developing more effective prophylactic and therapeutic approaches for human.

Lastly, we would like to evaluate the potential of IL-12 family of cytokines in enhancing influenza-induced human Th1 immunity in the elderly subjects. Interleukin 12 (IL-12) and IL-23 are not produced in human PBMC stimulated with influenza while exogenous IL-12 significantly enhances IFN-γ production in human PBMC either alone or synergistically with influenza virus (48, 49, and data not shown). Whether IL-12 family of cytokines can be developed into an adjuvant for influenza vaccines in the elderly people is of interest to be evaluated.

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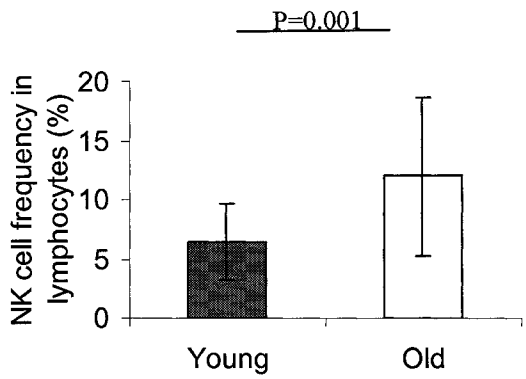
VI. APPENDICES

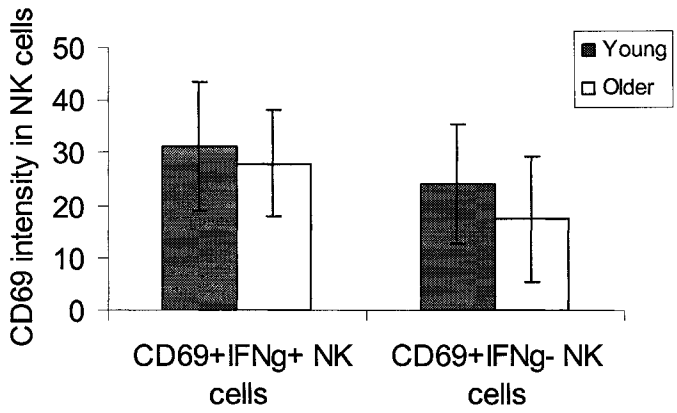
VI.1 Supplemented Figures (S. Figures)



S. Figure 1. Frequencies of total NK cells in total lymphocytes in young and elderly subjects.

One million PBMC from each of 15 healthy young and 25 healthy older subjects were stained for CD56+or16+CD3- NK cells and NK cell frequencies were determined by flow cytometry. Columns represent the mean and error bars represent the standard deviation.

A.



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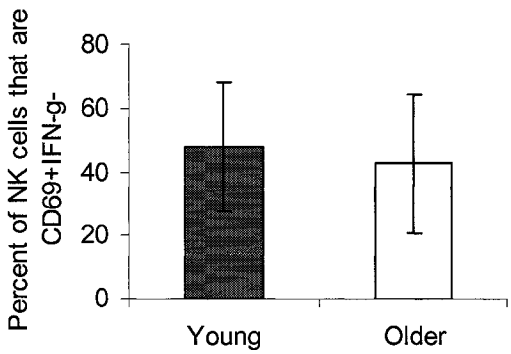
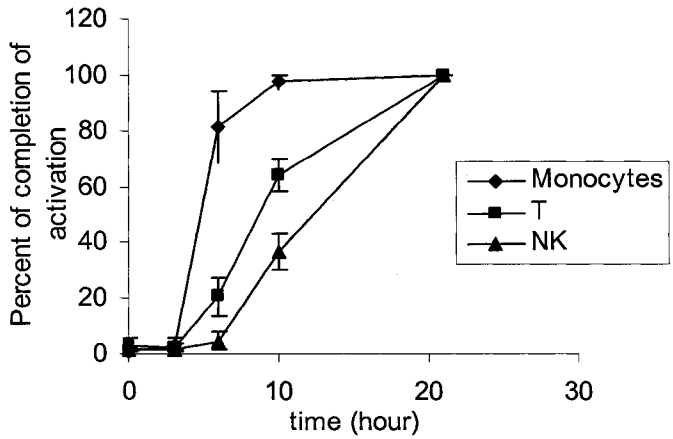


S. Figure 2. Expression of CD69 on NK cells in PBMC from young and elderly subjects.

One million PBMC from each of 15 healthy young and 25 healthy older subjects were stimulated with 1 μl of influenza virus in 96-well plate overnight. FastImmune assay and flow cytometry was used to determine the expression of CD69 and IFN-γ in NK cells. A, CD56+or16+ NK cells were gated out of lymphocytes and analyzed for CD69 intensity on CD69+IFN-γ+ or CD69+IFN-γ- NK cells by flow cytometry. Columns represent the mean and error bars represent the standard deviation. B, comparison of CD69+IFN-γ- NK cell frequency in total NK cells between young and older subjects. Columns represent the mean and error bars represent the standard deviation.



S. Figure 3. Sequential activation of monocytes, T cells and NK cells in PBMC in response to influenza infection

One million PBMC from each of 3 healthy young subjects were stimulated with 1 μl of influenza virus in 96-well plate overnight followed by staining with anti-human CD3(FITC), CD56/16(PE), CD69 (PerCP) and CD14 (APC) antibodies. Frequencies of CD69-expressing cells in each cell type were determined by flow cytometry. The percent of completion of activation determined as the ratio of frequency of CD69+ cells at each time point over the frequency of CD69+ cells at 21-hour time point are shown.