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Alteration of natural killer cell subset proportion is associated with risk factors of coronary artery disease in aging

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KEYWORDS

Immunosenescence;
NK cell;
Coronary artery disease;
Chronic low-grade inflammation.

ABSTRACT

Currently, the world population is moving to aging society because of decreasing fertility rates and increasing survival in old age. In Thailand, the results of the National Statistics Office showed that the number and proportion of older people increase continuously. Age-related impairment of human immunity involves in many chronic inflammatory diseases such as coronary artery disease (CAD). Thus, age is one of the risk factors for CAD causing the chronic inflammation condition. Natural killer (NK) cell is one of the immune cells that change in aging. Thus, this study aims to investigate the alteration of NK cells and NK cell subsets in aging and their association with the risk factor of CAD. Blood samples from aging (≥ 60 years old) group and who had any risk factors for CAD, were collected. Additionally, blood samples of young group (age < 35 years old) without risk factors associated with CAD were included. Whole blood from these samples were stained with anti-CD3 and anti-CD56 labeled with FITC and PerCP, respectively to determine NK cell subsets by flow cytometry technique. The result showed that the proportion of NK cell subsets (CD56bright/CD56dim) was significantly decreased in aging (p-value < 0.001) and also tended to decrease in aging who had more than or equal to 2 CAD risk factors when compared with aging who had less than 2 CAD risk factors (p-value = 0.051). Thus, this result suggested that alteration of NK cell subsets in aging was associated with the risk factor of CAD and this might be the marker for CAD that needs to be investigated further.

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Introduction

Currently, fertility has decreased and is in contrast with life expectancy. This leads to global aging population growth and progresses to an aging society. In 2015, the proportion of the age population had risen 12%. It is expected to reach 22% in 2050 [1]. In Thailand, the results of the National Statistics Office revealed that the number and proportion of Thai older people increase rapidly and continuously from 6.8% in 1994 to 15.3% in 2014 [2]. Age-associated impairment of the immune system or in terms of immunosenescence leads to the aging immunity becoming more susceptibility to infections [3, 4] and being involved in many low-grade inflammatory diseases. Moreover, Age is one of the risk factors for coronary artery disease (CAD) causing deaths worldwide and especially in developed countries or more than 45% of males global death and 36% in females in 2011[5, 6]. Furthermore, metabolic-related diseases including type 2 diabetes mellitus (T2DM), dyslipidemia and hypertension were accepted to be risk factors of CAD[7-9]. Thus, the immune cells play an important role in these diseases of aging.

Innate and adaptive immunity function together in the immune response. The innate immunity provides the first-line defense against pathogen and drives and regulates the antigen-specific adaptive immunity. Mononuclear phagocytes, neutrophils, and Natural killer (NK) cells are important effector cells in innate immunity. NK cells are bone marrow-derived lymphocytes that characteristic as loss expression of CD3 and positive expression with CD56. Peripheral blood NK cells are present in up to 15% of total lymphocytes in peripheral blood. NK cells can be classified into two subpopulations by the density of CD56 expression [10], CD56dim NK cells and CD56bright NK cells. The major type of NK cell is CD56dim NK cell that is present in about 90% of peripheral blood NK cells involving in cytotoxic activity. These NK cells can produce perforin and granzyme to kill the target cell whereas CD56bright NK cells involve in cytokine production. In the elderly, the NK cells and their subsets were reported to be changed especially the CD56 bright NK cell being declined in advanced age [11]. In addition, the NK cell is also the important immune cell that involves in coronary artery disease.

Previous studies demonstrated that the reduction of the NK cell was associated with CAD patients and related to CAD condition. CD16/CD56 NK cells significantly decreased in CAD patients especially in unstable angina conditions [12, 13]. However, the involvement of the NK cell population changes in aging containing CAD risk factors has still not been reported. This study aimed to investigate the alteration of NK cell populations in the elderly and their association with the risk factors of CAD. We hypothesized that NK cell and their subpopulations would be decreased and these reductions would be involved in chronic inflammation.

Materials and methods

Study populations

Sixty-three aging individual blood samples were collected from Sum Song district, Khon Kaen province including subjects who had aged over or equal 60 years and contained any risk factor associated with CAD (hypertension, dyslipidemia, and diabetes mellitus). Additionally, twenty-seven samples of the young group without the 3 CAD risk factors were included. Subjects who were treated with anti-inflammatory drugs were excluded. All subjects had no diabetic complications, infections, immunological diseases, hematological diseases, malignancies, or inflammatory sickness. This study was approved by the Ethics Committee of Khon Kaen University (HE 622164)

Sample analysis

The blood samples were collected in a fasting state in the morning. WBCs were counted with an automated blood cell counter. Serum triglycerides, total cholesterol, and high-density lipoprotein cholesterol (HDL-C) were determined enzymatically on an automated analyzer system. Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald formula. The blood glucose samples were treated with a hemolytic reagent and glucose was determined enzymatically with glucose dehydrogenase. Blood pressure (BP) was measured to the nearest five

mm Hg with a mercury sphygmomanometer with the subjects in a supine position having rested for five minutes. All processes were operated at AMS Wellness Center, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand.

Flow cytometry of cell surface expression

Cell surface staining was performed using 100 µl of fresh peripheral blood (within 24 hrs.) which was collected in heparin anticoagulant. The blood samples were stained with monoclonal antibodies (mAbs) conjugated with different fluorochromes as follows: anti-CD3-Fluorescein Isothiocyanate (FITC) (UCHT1, BD PharmingenTM, San Diego, CA, USA) and anti-CD56-Peridinin Chlorophyll Protein Complex (PerCP) (HCD56, BioLegend, San Diego, CA, USA) and then were incubated for 15 min at the room temperature in the dark. Red blood cells were lysed using the BD FACSTM lysing solution (BD Biosciences, San Jose, CA, USA) for 15 min before washing with 1X PBS. The samples were analyzed by flow cytometry using the BD FACSCantoTM II flow cytometer (BD Biosciences). The service was provided by Research Instrument Center, Khon Kaen University, Thailand. Isotype antibodies labeled with FITC and PerCP were also used to stain blood samples and used as the negative control for staining. The proportion of NK cell subset was calculated by using the percentage of CD56bright divided by CD56dim NK cell. The setting of flow cytometer was shown in Figure 1.

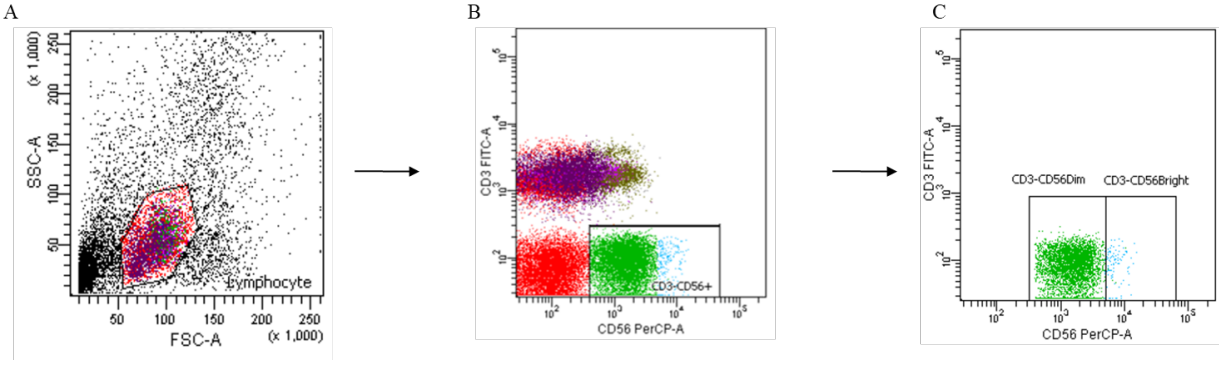

Figure 1 Analysis NK cell subsets by flow cytometer. Whole blood samples were stained with a monoclonal antibody specific to CD3 and CD56 labeled with FITC and PerCP, respectively, and then the stained samples were analyzed by FACSCanto II to determine NK cell subsets. Lymphocyte gate (A) was selected to analyze NK cell population (B) following NK cell subsets based upon the expression of CD56 (C).

Statistical analysis

All data were tested for normal distribution using Kolmogorov-Smirnov test for data set of more than or equal 50 data and Shapiro-Wilk test using for data set of less than 50. Data with normal distribution were explored for significance by Unpaired T-test, whereas non-parametric data were tested by Mann-Whitney U test using the software GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance at p-value < 0.05 was considered.

Results

Clinical characteristics of study subjects

The clinical characteristics of study subjects including total white blood cell count were presented in Table 1. The results showed that body mass index (BMI), systolic and diastolic blood pressure, triglyceride, blood urea nitrogen (BUN), uric acid, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP), and uric acid were significantly increased. In contrast, high-density lipoprotein (HDL) was reduced in aging.

Table 1 Clinical characteristics of study subjects according to total white blood cell counts

Parameters	Young group Mean ± SD	Aging group Mean ± SD	Reference value*	P-value
Number of subjects	27	63		NA
Age (years)	22.9 ± 5.0	69.6 ± 5.4		<0.0001
BMI (kg/m^2)	21.4 ± 2.1	24.1 ± 4.4	18.5 – 24.99	0.0027
FBS (mg/dl)	84.4 ± 6.3	101.8 ± 45.5	70 – 110	0.0466
Total white blood cell count (cell/µl)	6325.0± 973.5	6722.1 ± 2082.2	4600 – 10600	0.5678
Blood pressure				
Systolic (mmHg)	114.7 ± 9.6	138.0 ± 20.5	100 – 140	<0.0001
Diastolic (mmHg)	70.9 ± 6.3	78.7 ± 12.1	60 – 90	0.0007
Lipid profiles				
Total cholesterol (mg/dl)	189.4 ± 19.8	192.8 ± 50.7	127 – 262	0.9181
Triglyceride (mg/dl)	67.3 ± 32.9	159.6 ± 91.1	10 – 200	<0.0001
HDL (mg/dl)	63.6 ± 17.9	38.7 ± 10.7	> 35	<0.0001
LDL (mg/dl)	113.1 ± 27.5	122.0 ± 41.2	10 – 150	0.5978
BUN	12.1 ± 2.8	14.7 ± 5.5	0.5 – 1.5	0.0208
Creatinine	0.9 ± 0.2	0.9 ± 0.3	0.67 - 1.17	0.5720
Uric acid	5.0 ± 1.2	6.0 ± 1.4	2.7 – 7.7	0.0022
AST	21.5 ± 5.2	31.0 ± 16.7	0 – 40	<0.0001
ALT	15.0 ± 5.8	22.9 ± 11.1	0 – 33	0.0007
ALP	54.6 ± 14.6	64.0 ± 14.3	30 – 120	0.0011

Note: * Reference value from Clinical Immunology and Chemistry Unit at Srinagarind Hospital, Khon Kaen, Thailand.

White blood cell count influence by age

To investigate the influence of age on the changing of white blood cell subtype, subpopulation of white blood cells was compared between young and aging group which is presented in Figure 2. The total numbers of white blood cell count and any absolute count of white blood cell subtype did not differ significantly between groups. The result found that only the absolute eosinophil was statistically significant increased in the aging group (*p*-value=0.0022; Figure 2E).

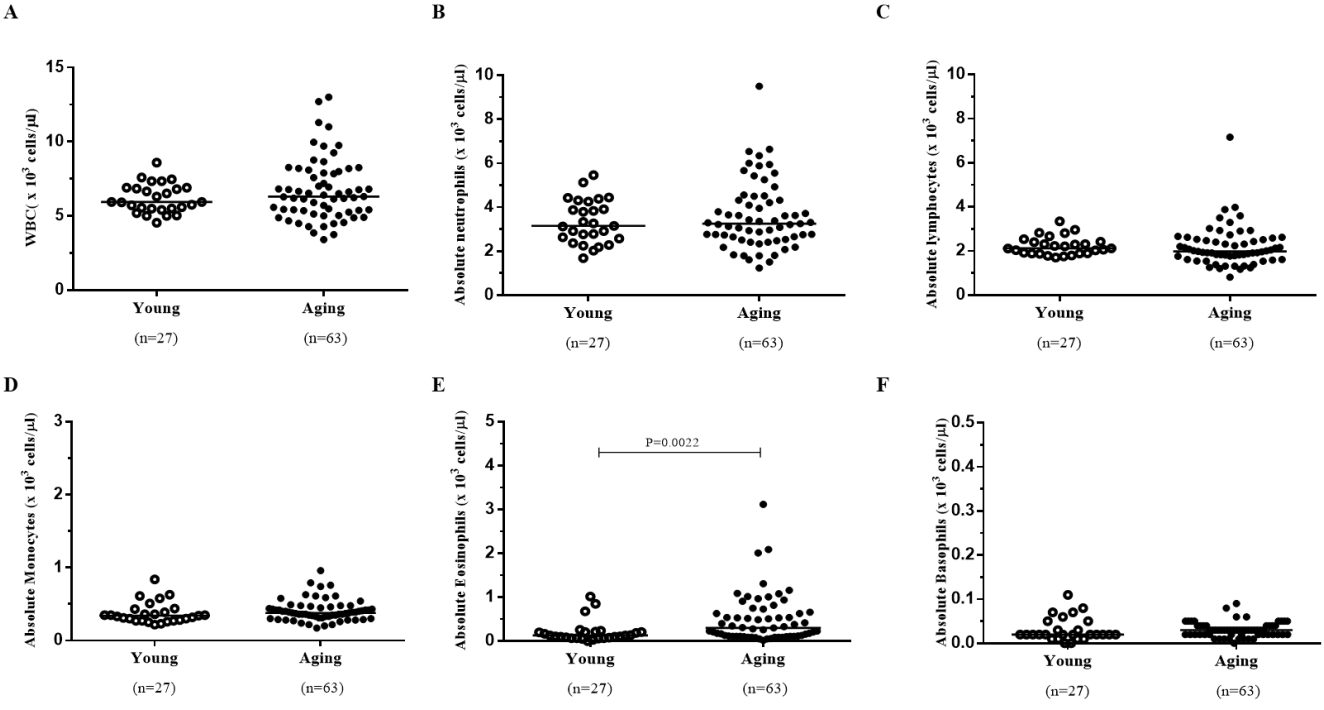

Figure 2 White blood cell count influence by age. Whole blood collected from young and aging individuals were measured for complete blood count at AMS-KKU Excellence Laboratory. Total white blood cell count (A) and the absolute number of each white blood cell types: Neutrophils (B), Lymphocytes (C), Monocytes (D), Eosinophils (E), Basophils (F) was compared between young and aging.

An effect of age on NK cell change

To determine the alteration of NK cells and NK cell subset, whole blood samples from the young and aging group were stained with anti-CD3 and anti-CD56 and detected by flow cytometer. The absolute total NK cell (CD3-CD56+ cell) did not differ significantly between the young and aging group (Figure 3A). The alteration of NK cell subsets found that CD56bright NK cells separated from aging individuals were significantly decreased (p-value<0.0001; Figure 3B.) This was in contrast) with the number of CD56dim NK cells that were slightly increased in the aging group (p-value=0.0446; Figure 3C). Moreover, the proportion of NK cell subsets was also declined in aging (p-value=0.0008; Figure 3D).

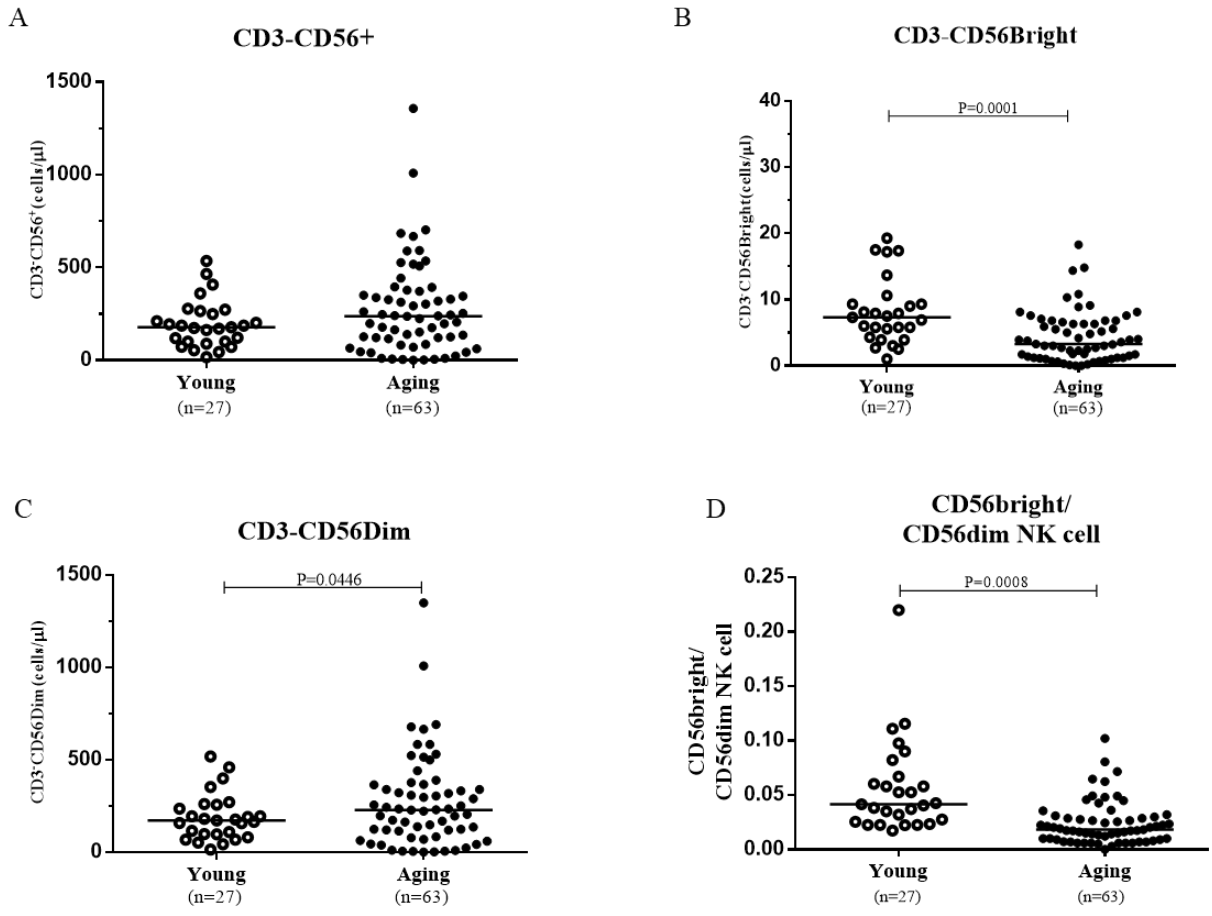

Figure 3 CD3-56+ NK cell and their subset distribution of young group compared with aging. Whole blood from the young and aging group was performed by multicolor staining with antibodies specific to CD and receptor markers and analyzed by FACS Canto II. The absolute number of each population: CD3-CD56+ cell (A), CD3-CD56Bright cell (B), CD3-CD56dim cell (C) was compared between young and aging group. Moreover, NK cell proportion was displayed by CD56bright cell/CD56dim cell ratio (D).

The association of NK cell subset proportion with CAD risk factors

Since hypertension, dyslipidemia, and diabetes mellitus were identified as common manifestation in aging individuals and defined as risk factors of CAD, we divided aging into two groups, aging with less than 2 CAD risk factors (n=30) and aging with more than or equal to 2 CAD risk factors (n=33) to investigate the alteration of NK cell subset influenced by CAD risk factor in aging. The results revealed that the number of overall NK cells and their subsets were not different between two aging groups (data not shown). The proportion of NK cell subsets was decreased in aging with more than or equal to 2 CAD risk factors. (*p*-value=0.0505; Figure 4)

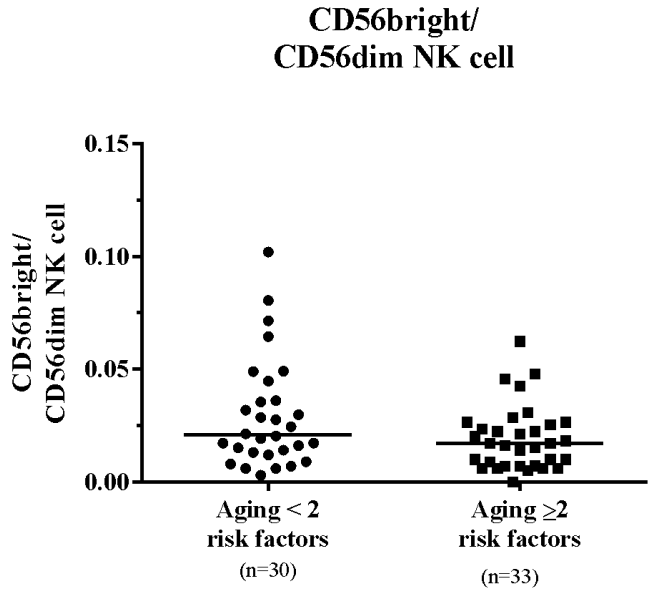

Figure 4 The proportion of NK cell subsets in aging with CAD risk factors. The samples were divided into two groups (aging containing less than 2 CAD risk factors and aging containing more than or equal to 2 CAD risk factors) and analyzed for the proportion of NK cell subsets.

Discussion

Currently, the proportion of aging is rising in several countries around the world. Age is associated with an impairment of the immune system, known as immunosenescence. Generally, aging individuals have the most common manifestation characterized by an elevation of peripheral blood components involving inflammation processes such as acute-phase protein and TNF-α, called chronic low-grade inflammation. These conditions can cause long period tissue damage and involve chronic age-related diseases including type 2 diabetes mellitus, dyslipidemia, and hypertension. Thus, impairment of the immune system in aging involves in many chronic inflammatory diseases such as coronary artery disease. Our study found that age did not influence the alteration of overall white blood cell count and their white blood cell subtypes except in eosinophils that were significantly high in the aging group. However, the previous study by Kubota K et al. suggested that the white blood cell count and platelet count tended to decrease with advancing age [14]. Besides, the previous studies were still a debate about the numbers of neutrophils change with aging. Most studies suggested that with healthy aging there are no changes in their number. Chatta GS et al. indicated that peripheral blood neutrophil and neutrophil precursors in the bone marrow are not lowered in healthy aging individuals[15]. However, the study for eosinophil was still not clear but an elevation of eosinophil in this study may be because the subjects were collected from the area of high prevalence of parasitic infestation[16].

NK cells are important immune cell composition of two subgroups based upon the expression level of CD56 molecule on the cell surface. Thus, there are two groups of NK cells, CD56bright and CD56dim NK cells. To investigate whether the NK cells and their subsets change is influenced by age, we compared the number of overall NK cells and their subsets in the young subject group and aging. Our results demonstrated that although overall NK cells were not changed in old age but both NK cell subsets were affected by age. This was in contrast with the study by Valiathan et al who indicated that the elderly population

presented the highest percentages of peripheral NK cells compared with infant and adulthood [17]. Furthermore, our results also showed that CD56bright NK cells collected from aging individuals were clearly decreased, whereas CD56dim NK cells were slightly high in old age. Interestingly, our study also found that the proportion of NK cell subsets (CD56bright/CD56dim) was reduced in aging. This result was similar to the previous studies showing that NK cells especially CD56bright NK cells were decreased in aging and the proportion of CD56bright/CD56dim NK cells were decreased[11]. Switching of CD56bright NK cells to CD56dim NK cells accumulation may be a marker of immunosenescence process. Moreover, CD94 molecule that involve in cytotoxicity was decreased in both CD56bright NK cells and CD56dim NK cells [18]. These results may cause impairment in NK cell cytotoxicity (NKCC). Thus, the expanding of circulating CD56dim NK cells that occurs in the elderly may be a compensatory mechanism for age-associated decrease in NKCC.

Coronary artery disease (CAD) is a single cause of death in approximately 12% of global death in 2004. Furthermore, age and age-related diseases including type 2 diabetes mellitus, dyslipidemia and hypertension are also risk factors for CAD. To investigate the association of NK cell subset and CAD risk factors, we divided 63 aging individuals into 2 groups based on CAD risk factors including type 2 diabetes mellitus, dyslipidemia, and hypertension, aging with less than 2 CAD risk factors and aging with more than or equal to 2 CAD risk factors. Our results found that overall NK cells and NK cell subsets did not differ in the two age groups. However, the proportion of NK cell (CD56bright/CD56dim) was decreased in aging with more than or equal to 2 CAD risk factors. This finding suggested that a high level of CD56bright/CD56dim ratio might be involved in CAD that needs to be investigated with the high number of sample size and prospective study is needed to determine the biological marker for CAD.

Conclusion

In conclusion, aging is a general phenomenon that has an impact on several immune cells. Our study demonstrates the advancing age is related to CD56dim accumulation and CD56bright depletion. These might be possibly pathogenic involving chronic low-grade inflammation and might be driven to coronary artery disease in advancing age. Thus, our study might suggest that the alteration of NK cell subset in the elderly with CAD risk factor might be a marker for CAD that has to be investigated in the future.

Take home messages

This study determined effects of CAD risk factors; T2DM, dyslipidemia, and hypertension, on NK cell subsets in aging. Our results demonstrated that age and CAD risk factors related to CD56dim alteration and the proportion of CD56bright/CD56dim was reduced. These finding suggested that NK subset alteration might involve CAD progress in aging.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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References

1. United Nations. Department of Economic and Social Affairs. Population Division. World population ageing, 1950-2050. New York: United Nations; 2002; p. 483.
2. National statistical office. The 2014 survey of the older persons in Thailand. Bangkok.
3. Larbi A, Franceschi C, Mazzatti D, Solana R, Wikby A, Pawelec G. Aging of the immune system as a prognostic factor for human longevity. Physiol J 2008; 23: 64-74.

4. Myers CE, Mirza NN, Lustgarten J. Immunity, cancer and aging: lessons from mouse models. Aging Dis 2011; 2(6): 512-23.
5. Ezzati M, Riboli E. Behavioral and dietary risk factors for noncommunicable diseases. N Engl J Med 2013; 369(10): 954-64.
6. Mendis S, Davis S, Norrving B. Organizational update: the world health organization global status report on noncommunicable diseases 2014; one more landmark step in the combat against stroke and vascular disease. Stroke 2015; 46(5): e121-2.
7. World Health Organization. Prevention of cardiovascular disease : guidelines for assessment and management of cardiovascular risk. Geneva: World Health Organization; 2007. p. 86.
8. Mathers C, Fat DM, Boerma JT, World Health Organization. The global burden of disease : 2004 update. Geneva, Switzerland: World Health Organization; 2008. p. 146.
9. World Health Organization. Global status report on noncommunicable diseases. Geneva, Switzerland: World Health Organization.
10. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol 2001; 22(11): 633-40.
11. Chidrawar SM, Khan N, Chan YL, Nayak L, Moss PA. Ageing is associated with a decline in peripheral blood CD56bright NK cells. Immun Ageing 2006; 3: 10.
12. Jabir NR, Firoz CK, Ahmed F, Kamal MA, Hindawi S, Damanhouri GA, et al. Reduction in CD16/CD56 and CD16/CD3/CD56 Natural Killer Cells in Coronary Artery Disease. Immunol Invest 2017; 46(5): 526-35.
13. Backteman K, Ernerudh J, Jonasson L. Natural killer (NK) cell deficit in coronary artery disease: no aberrations in phenotype but sustained reduction of NK cells is associated with low-grade inflammation. Clin Exp Immunol 2014; 175(1): 104-12.
14. Kubota K, Shirakura T, Orui T, Muratani M, Maki T, Tamura J, et al. Changes in the blood cell counts with aging. Nihon Ronen Igakkai Zasshi 1991; 28(4): 509-14.
15. Chatta GS, Andrews RG, Rodger E, Schrag M, Hammond WP, Dale DC. Hematopoietic progenitors and aging: alterations in granulocytic precursors and responsiveness to recombinant human G-CSF, GM-CSF, and IL-3. J Gerontol 1993; 48(5): M207-12.
16. Suwannatrai A, Saichua P, Haswell M. Epidemiology of Opisthorchis viverrini Infection. Adv Parasitol 2018; 101: 41-67.
17. Valiathan R, Ashman M, Asthana D. Effects of Ageing on the Immune System: Infants to Elderly. Scand J Immunol 2016; 83(4): 255-66.
18. Almeida-Oliveira A, Smith-Carvalho M, Porto LC, Cardoso-Oliveira J, Ribeiro Ados S, Falcao RR, et al. Age-related changes in natural killer cell receptors from childhood through old age. Hum Immunol 2011; 72(4): 319-29.