Received: 28 May 2018 | Revised: 11 July 2018 | Accepted: 12 July 2018

DOI: 10.1002/jcla.22640

RESEARCH ARTICLE

WILEY

# Association of neutrophil-to-lymphocyte ratio and natural killer cell activity revealed by measurement of interferon-gamma levels in a healthy population

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**Background**: While a method of assaying natural killer (NK) cell activity by measuring the amount of interferon (IFN)-γ released from NK cells has been proposed, no data are available about the factors that influence IFN-γ levels related to NK cell activity. NLR has recently been reported to be a predictor of several diseases. In the present study, we investigated the pre-analytical variables for NK cell activity using measurements of IFN-γ and the relationship between NLR and NK cell activity.

**Methods**: The NK cell activity was assessed with the measurement of IFN-γ after stimulation with an NK cell-specific stimulant (NK Vue™, ATgen, Sungnam, Korea). One hundred and six adult volunteers were recruited and analysis of their complete blood count data and serum C-reactive protein was done. Blood sample from 59 of the participants was also used for analysis of lymphocyte subpopulations.

**Result**: Natural killer cell activity varied widely (range, 44.2-1775.6 pg/mL). NK cell activity was higher in females than in males ($P = 0.014$). NK cell activity decreased with increasing NLR ($P = 0.004$, $r = -0.32$) but NK cell activity showed no significant association with NK cell count or other lymphocyte subpopulations. NK cell activity levels according to CRP quartile were significantly different ($P = 0.025$).

**Conclusion**: We have observed that NK cell activity when assessed by IFN-γ level measurement was negatively correlating with NLR. This result can be helpful in interpreting or predicting NK cell activity in the clinical environment.

KEYWORDS

immunoregulation, interferon gamma, neutrophil-to-lymphocyte ratio, NK cell, NK cell activity

## 1 | INTRODUCTION

Natural killer (NK) cells play a crucial role in the innate immune system. NK cells have cytotoxic activity via both direct and antibody-mediated cellular mechanisms.[1] Activated NK cells release cytotoxic granules and secrete cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α; these cytokines play an immunoregulatory role as they activate NK cells and further promote cytokine secretion.[2] NK cell activity is related to several types of cancer, tumor stage, and survival rates.[3-5] Patients with impaired NK cell activity commonly experience a relapse of cancer and have problems with defenses against viral infections.[6,7] In this context, assessment of NK cell activity is of interest in the clinical environment.

The gold standard method for analyzing the cytotoxic activity of NK cells is the Chromium 51 ($^{51}Cr$) release assay (CRA). However, this assay carries a risk of radiation exposure, and the procedures involved are time-consuming and expensive.[8,9] Therefore, several alternative methods have been developed; flow cytometric NK cell cytotoxicity assay using many different fluorescent dyes [i.e., Calcein AM (acetoxymethyl), DIOC18, CFSE] and CD107a degranulation assay.[10] These assays are done in multiple steps, such

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as cell culture and staining, and requires a large amount of whole blood.[8]

Recently, a simple method for determining NK cell activity that uses quantitative sandwich enzyme-linked immunosorbent assay (ELISA) to measure released IFN-γ released from NK cells exposed to a specific recombinant cytokine (NK Vue™, ATgen, Sungnam, Korea). The advantages of this method are that it would measure the true NK cell activity with simple and quick methods.[11,12] However, this method relies on measurement of IFN-γ, which is not secreted by only NK cells. Investigation on factors determining IFN-γ secretion levels would be valuable data in interpreting IFN-γ secretion levels.

There has been substantial evidence demonstrating that cancer-associated inflammation is a major determinant of outcome in cancer patients.[13] Spiegel and colleagues have demonstrated that neutrophils inhibit natural killer (NK) cell functions, and lead to a significant increase in survival time of cancer cells.[14] Neutrophils protect cancer cells from rapid intraluminal clearance by inhibition of NK cells, and proved with in vitro functional assays that NK cells with response to neutrophils have decreased intracellular cytokine production. Therefore, Neutrophil-to-lymphocyte ratio (NLR) has been reported to be an independent prognostic value in patients with a variety of cancers.[13,15] NLR itself is a useful marker, and is simple to derive from routine laboratory studies, as the complete blood count (CBC). Its relationship with systematic inflammation [16] has been demonstrated in various disorders, ranging from myocardial infarction and coronary diseases[17] to diabetes mellitus, hypertension, metabolic syndrome,[18] or life style habit-related conditions,[19] and in various malignancies.[20-23]

C-reactive protein (CRP) is an acute phase reactant, and reflects inflammation.[24,25] Association of increased levels of CRP and risk of developing cancer also was suggested in a study done to a general population,[26,27] and the results are suggestive of positive correlation of CRP levels and cancer risk. As CRP provides a link between the innate and adaptive immune systems via activation of complements and interaction with Fcγ receptors,[28] relationship between CRP and NK cell activity can be expected.

There is currently a lack of data regarding the association between NK cell activity and NLR or CRP. Accordingly, in the present study, we investigated that their relationship. Furthermore, data on pre-analytical variables for NK cell activity as assayed by measuring IFN-γ levels are scarce. In order to fill this gap in previous research, we also tried to identify the factors that predict NK cell activity measurements.

## 2 | SUBJECTS AND METHODS

Healthy subjects over the age of 20 were recruited in this study. All participants were not on any medical therapies, and had no history of malignancies or infectious diseases. Peripheral blood samples were collected for analysis of NK cell activity, serum c-reactive protein (CRP) levels, CBC, and lymphocyte subsets. Blood sampling was done at 13:00-13:30 to avoid variation due to circadian rhythms. This study was approved by the Institutional Review Board of Dong-A University Hospital. Informed consent forms were signed by all participants.

Complete blood count count was performed using an XE-2100 (Sysmex, Kobe, Japan) hematology analyzer. Absolute neutrophil and lymphocyte count were analyzed, and NLR was calculated. Calculation of NLR was done from the absolute values of neutrophils and lymphocytes from CBC results.[29] Neutropenia, lymphopenic or leukopenic subjects were to be excluded from enrollment, as the study targeted healthy subjects. CRP was measured with TBA-200FR (Toshiba Medical Systems, Tokyo, Japan). Lymphocyte subset analysis was done by flow cytometric methods with the Navios flow cytometer (Beckman Coulter, Miami, FL). The percentages of CD3+, CD3+/4+, CD3+/8+, CD19+, and CD3-/16+/56+ cells were analyzed using Navios software (Beckman Coulter) and the total cell counts were obtained.

Natural kille cell activity measurement was performed with the NK Vue™ kit (ATgen, Sungnam, Korea) following the manufacturer's instructions. This method detectes IFN-γ with a qualtitative sandwich enzyme-linked immunosorbent assay (ELISA) from NK cells when exposed to a specific recombinant cytokine, Promoca™ (ATgen), and this quantification result is used as a surrogate marker for NK cell activity in the peripheral blood of the subject.[11,12] One milliliter of whole blood was collected directly into a manufacturer-provided Promoca™ tube and the tube was gently inverted 3 times. The tubes were incubated for 24 hours in a 37°C chamber. Optical density was measured using a CODA microplate processor (Biorad, Hercules, CA). Seven standards in duplicate were used to create standard curves. The OD values were then entered into the NK Vue™ software; the test results were calculated to pg/mL.

All variables influencing NK cell activity were subjected to regression analysis. Kruskal-Wallis test was used to compare the differences of NK cell activity between lymphocyte quartiles, NLR quartiles, and CRP quartiles. MedCalc Software (MedCalc Software bvba, Ostend, Belguim) was used for data analysis. *P*-values < 0.05 were considered statistically significant with two-tailed tests.

## 3 | RESULTS

### 3.1 | Demographic data and distributions of IFN-γ levels

The demographic data of the subjects are shown in Table 1. NK cell activity showed a wide range, from 44.20 to 1775.60 pg/mL, and did not follow a normal distribution. The median NK cell activity was 1237.70 pg/mL [95% confidence interval (CI), 1031.60-1325.47] (Figure 1).

### 3.2 | Effects of age and gender on NK cell activity

Natural killer cell activity by age is presented in Figure 2. No significant difference was found based on age ($r = -0.077$, $P = 0.429$). The

**TABLE 1** Demographic and laboratory characteristics of the study participants

| Characteristic | Total | Male | Female |
|---|---|---|---|
| Age (y), mean ± SD | 37.87 ± 10.71 | 42.85 ± 10.71 | 34.85 ± 9.52 |
| 20-40 (n, %) | 62 (58.49) | 14 (35.00) | 48 (72.73) |
| 41-60 (n, %) | 43 (40.57) | 25 (62.50) | 18 (27.27) |
| ≥60 (n, %) | 1 (0.94) | 1 (2.50) | 0 |
| CBC | | | |
| WBC, total (/μL) | 6142.23 ± 1399.40 | 6297.15 ± 1556.75 | 5945.00 ± 1298.31 |
| Neutrophil (/μL) | 3419.57 ± 1122.52 | 3517.35 ± 1270.98 | 3360.31 ± 1028.00 |
| Lymphocyte (/μL) | 2131.82 ± 545.10 | 2161.05 ± 593.49 | 2114.10 ± 517.50 |
| Neutrophil differential count (%) | 54.97 ± 8.35 | 55.01 ± 7.42 | 54.94 ± 8.93 |
| Lymphocyte differential count (%) | 35.24 ± 7.22 | 34.90 ± 7.40 | 35.45 ± 7.15 |
| Neutrophil-to-lymphocyte ratio | 1.69 ± 0.70 | 1.72 ± 0.79 | 1.67 ± 0.64 |
| CRP (mg/dL) | 0.14 ± 0.29 | 0.11 ± 0.15 | 0.15 ± 0.34 |

Data are presented as mean ± standard deviation.
CBC, complete blood count; CRP, C-reactive protein; WBC, white blood cell.

median values were 1309.30 pg/mL (95% CI, 1130.53-1453.41) in female subjects and 1047.55 pg/mL (95% CI, 732.63-1243.39) in male subjects, showing statistical difference ($P$ = 0.014, Figure 1 middle and bottom).

### 3.3 | Assocation of NLR, neutrophils, lymphocytes, and NK cell counts and NK cell activity

Although statistically significant, the count of WBC and neutrophils had small effect on NK cell activity ($P$-values 0.026, 0.028, respectively, and $r$-values −0.214, −0.212, respectively, Figure 3 top). Lymphocyte count had no statistically significant effect on NK cell activity, but lymphocyte proportion were better correlated with NK cell activity compared to lymphocyte count ($P$ = 0.002, $r$ = 0.298, Figure 3 middle). Neutrophil-to-lymphocyte ratio (NLR) also was correlated with NK cell activity ($P$ = 0.004, $r$ = −0.320, Figure 3 bottom). When comparing NK cell activity according to NLR quartiles, it was significantly different according to NLR quartile; it decreased with increasing NLR quartile ($P$ < 0.001, Figure 4 top). Median NK cell activity was 1426.80 pg/mL in the first NLR quartile (≤1.21, n = 27), 1371.05 in the second quartile (1.22-1.56, n = 26), 1010.10 in the third quartile (1.58-1.95, n = 27), and 823.90 in the fourth quartile (≥1.96, n = 26). Furthermore, we have found no significant relationship between lymphocyte subpopulations and NK cell activity (Table S1).

### 3.4 | Relationship between CRP and NK cell activity

No statistical significance between NK cell activity and the levels of CRP was reached ($P$ = 0.29, $r$ = −0.100). The median NK cell activity was 1146.50 pg/mL in the first CRP quartile (≤0.03 mg/dL, n = 33), 1235.25 pg/mL in the second quartile (0.04-0.05 mg/dL, n = 23), 755.13 pg/mL in the third quartile (0.06-0.1 mg/dL, n = 25), and 345.23 pg/mL in the fourth quartile (0.11-2.25 mg/dL, n = 25). NK cell activity was higher in the second quartile than in other quartiles and it gradually decreased in the third and fourth quartiles. Overall, NK cell activity levels according to CRP quartile were significantly different ($P$ = 0.025, Figure 4 bottom).

## 4 | DISCUSSION

We have presented data regarding the relationship of various clinical parameters that influence NK cell activity measured by IFN-γ. The method we used in this study has advantages compared to conventional methods in measuring NK cell activity, yet more data regarding the performance of this method is to be accumulated before broad clinical use. We identified a novel finding in this study; that NLR has a negative association with NK cell activity as assayed by IFN-γ levels, which has not been previously reported. During this study, we have observed a large variation among healthy subjects in NK cell activity assessed by IFN-γ secretion. It would be interesting to evaluate the levels of IFN-γ as NK cell activity in various clinical conditions.

Previous research regarding the development of this assay[12] presented data regarding geometric mean and standard deviation of NKA as a function of gender and age group of volunteers. This study expanded this finding with more detail on other laboratory parameters. We have observed that NK cell activity decreased with an increase in NLR quartile ($P$ < 0.001) and NLR value ($P$ = 0.004). Our finding suggests that a high NLR might be related to low IFN-γ secretion. It was previously suggested that the decreased secretion of IFN-γ was due to the inhibitory effects of

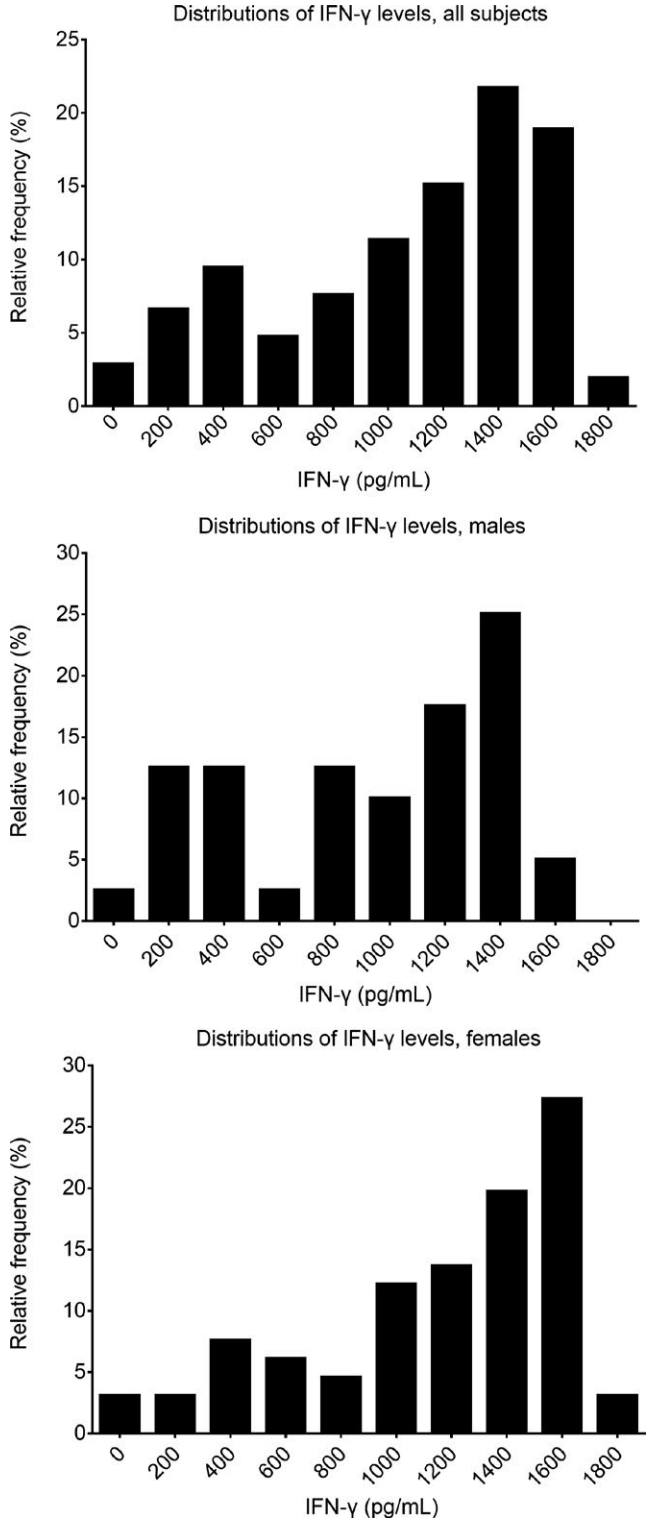


**FIGURE 1** Distributions of IFN-γ levels as NK cell activity in all subjects (top), females (middle), and males (bottom). NK cell values were normally distributed for males but were not normally distributed for females or overall subjects

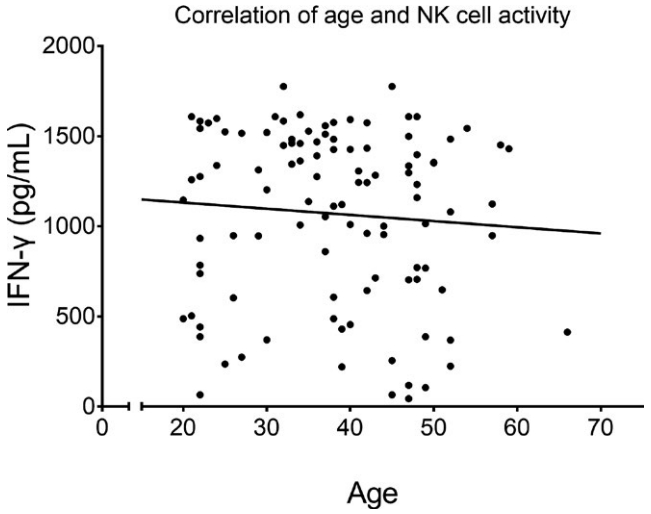


**FIGURE 2** Distributions of IFN-γ levels as NK cell activity according to age. Plot of regression analysis between log NK cell activity and age. Age was not correlated with NK cell activity ($r = -0.077$, $P = 0.429$)

neutrophil-derived molecules. Several reports have shown that neutrophil-derived molecules inhibit NK cell activity and the survival of NK cells.[30-33] This study demonstrated that the relative ratio of neutrophils to lymphocytes has effect on NK cell activity. NLR is an independent prognostic value in cancer patients and has correlation with clinical outcome[13,15]; this may be due to the fact that mature neutrophil subset has immunosuppressive functions.[34-36] The advantageous part of our observation is that NLR is easy to use, and has been sought as a useful tool[37] and has been shown to have association with various disorders.[16-23,29,37,38] It is easy to apply from simple CBC results has made it much investigated, along with other variables that can be derived from simple laboratory studies[39-44] are increasingly being of interest, due to their ease to issue in clinical practice. However, it should be noted that these variables can differ in ethnicities, underlying general condition, medication status, nutritional status, etc. We suggest that the relationship of NLR to NK cell activity be limited to healthy Asian individuals, as this study`s subjects.

We have also observed that gender was one of the factors that influenced NK cell activity. Significantly greater NK cell activity was observed in females. Although the gender difference is not fully understood, one possibility may be related to gender-related differences in the immune system. Hirokawa et al reported that a decline in T cell proliferation and decreased cytokine production are related to age and are gender-specific. A slower decline in immune system function was observed in females than in males.[45] It could also be the case that the results of the present study were influenced by differences in the age distribution among the two genders. The majority of males enrolled in the present study were 41-60 years old, whereas the majority of females were 20-40 years old. This may have also contributed to the results of our study being unable to find age influencing NK cell activity. For the precise evaluation of gender differences in NK cell activity, a larger number of participants must be examined, including more elderly subjects.

The method we used to evaluate NK cell activity relies on detecting IFN-γ secreted by NK cells. Lee et al have analyzed the specificity of the effect of PROMOCA™ on NK cells. Results showed that

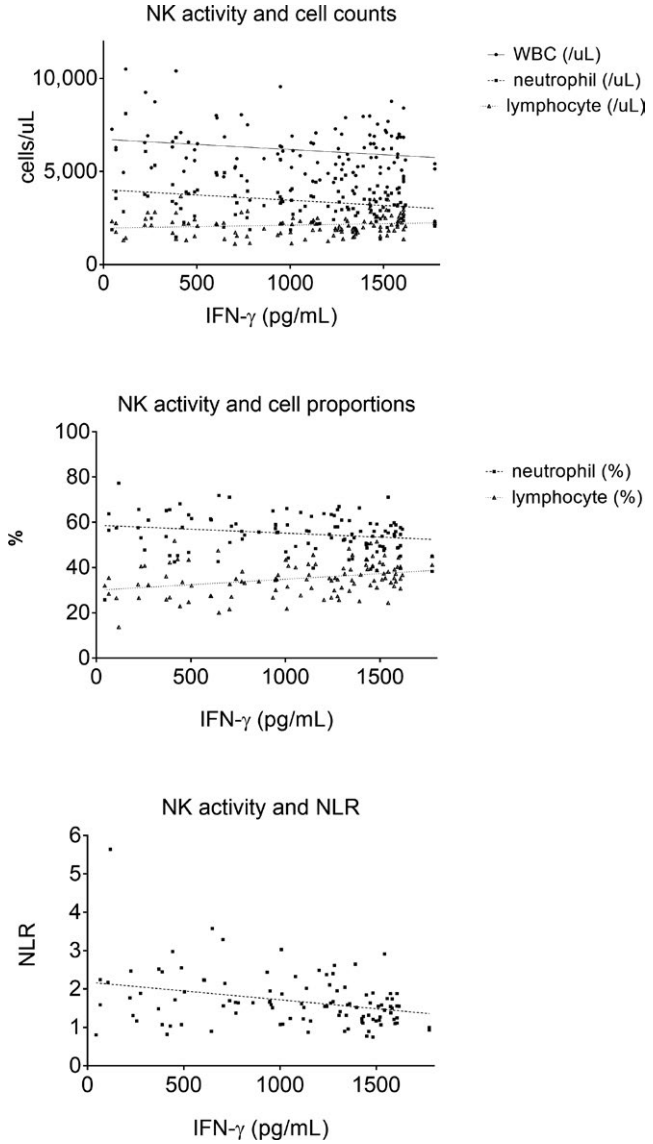


**FIGURE 3** Plots of regression analysis examining the relationships between IFN-γ levels with WBC, neutrophil and lymphocyte counts (top), IFN-γ levels with neutrophil and lymphocyte proportions (middle), and IFN-γ levels with neutrophil-to-lymphocyte ratio (NLR). WBC and neutrophils count were negatively correlated with NK cell activity (top graph, *P*-values 0.026, 0.028, respectively, and *r*-values −0.214, −0.212, respectively). Lymphocyte proportion showed positive correlation with NK cell activity ($P = 0.002$, $r = 0.298$, middle graph). Neutrophil-to-lymphocyte ratio showed negative correlation with NK cell activity ($P = 0.004$, $r = -0.320$)

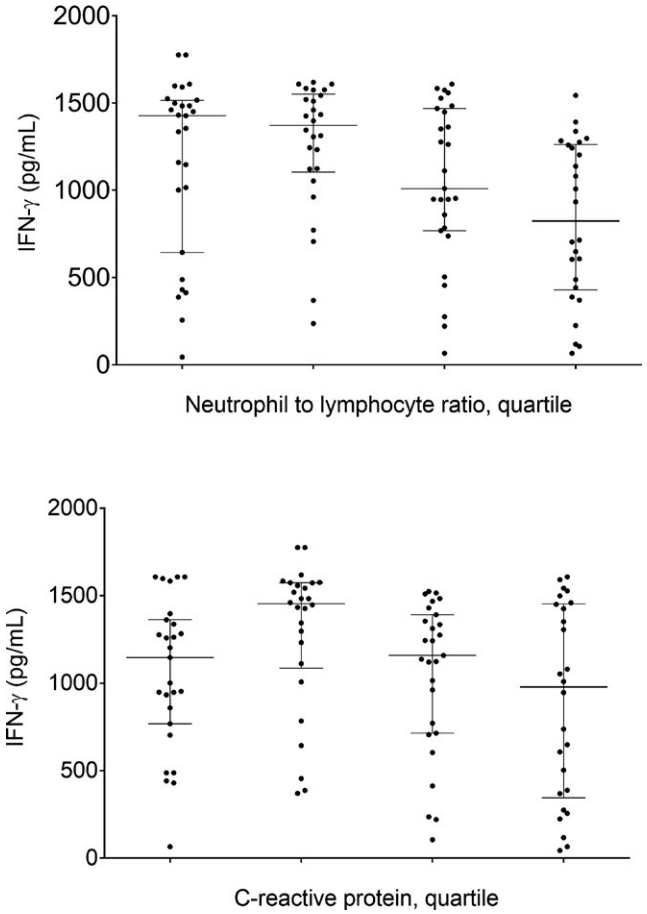


**FIGURE 4** Distribution of levels of NK cell activity in different neutrophil-to-lymphocyte ratio (NLR) quartiles and C-reactive protein (CRP) levels. NK cell activity according to NLR quartile was statistically significant ($P < 0.001$, the values of each quartile are Q1: 0.75-1.21, n = 27; Q2: 1.22-1.56, n = 26; Q3: 1.58-1.95, n = 27; Q4: 1.96-5.64, n = 26, respectively). Horizontal lines indicate medians and 1st/3rd interquartile intervals (top graph). Increased CRP levels after the second quantile group was suggestive of decreased NK cell activity (bottom graph)

50% of IFN-γ+ cells were CD3-CD56+ NK cells and around 30% of IFN-γ+ cells were CD3+ cells or NKT cells. CD19+ cells and CD14+ monocytes were negative for IFN-γ staining.[12] This speculates that T cell portions in subjects can interfere the results done with the NK Vue™ kit. However, CD3+, CD3+/4+, and CD3+/8+ cellular subpopulations were not related to NK cell activity in the present study (data not shown). There was a report that quantification of total NK cells or NK cell subpopulations could not substitute for an assessment of NK cell activity, as all NK cells present in peripheral blood do not mediate cytotoxic effects.[9] Our group also evaluated NK cell function through cytolytic behavior,[46] and found no correlation between the NK cell count and the NK cell activity; this finding was also observed in this study through assessment by measuring IFN-γ. Factors influencing NK cell activity measurement through IFN-γ is a relatively unknown field and further evaluation with consideration to lymphocyte subsets would be required to fully understand its influence on IFN-γ secretion after NK cell stimulation.

Unlike NLR showing direct significant association with NK cell activity in our study, the levels of CRP showed a negative correlation with NK cell activity but were not statistically significant. In the elderly, the number of $CD56^{bright}$ NK cells negatively correlated with the levels of CRP,[47] there were no report of association between NK cell activity and the level of CRP. As this is the first study to evaluate CRP levels and NK cell activity measured by IFN-γ assays, we divided our subjects into quartiles. We observed that NK cell activity levels according to CRP quartile were significantly different. We

found higher NK cell activity in subjects with CRP ≤0.05 mg/dL, and NK cell activity significantly decreased with increasing CRP. Though this finding is of some interest, we could not explain why the NK cell activity of subject with CRP levels ≤0.03 mg/dL (first quartile) was lower than subjects with levels from 0.04 to 0.05 mg/dL (second quartile). Furthermore, most CRP results were within the normal range and the variance of that range was small. This result itself cannot be conclusive, and to evaluate the relationship between CRP and NK cell activity, a well-chosen population having a wide range of CRP levels will be required.

In summary, the results of the present study suggest that the NLR has a negative association with NK cell activity. This result reflects the possibility that normal, physiological neutrophils may inhibit NK cell activity. Also, as lymphocyte counts did not have effect on NK cell activity, the absentness of correlation between lymphocyte subsets and NK cell activity further solidifies our observation regarding the relationship with neutrophil counts and NK cell activity, which was presented as the correlation of NLR and NK cell activity. Further studies are required to clarify the clinical impact of NLR with NK cell activity; however, as NLR is easily calculated from CBC results, the clinical impact of this observation has much potential. We hope that more evaluation of a larger population, as well as evaluation of subjects with various underlying morbidities will further reveal the utility of NLR in predicting NK cell activity.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Kim B-R, Chun S, Cho D, Kim K-H. Association of neutrophil-to-lymphocyte ratio and natural killer cell activity revealed by measurement of interferon-gamma levels in a healthy population. *J Clin Lab Anal*. 2019;33:e22640. https://doi.org/10.1002/jcla.22640