Reduction of $CD16^{-}CD56^{bright}$ NK cell subset Precedes NK Cell Dysfunction in Prostate Cancer

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by Author

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Reduction of $CD16^{-}CD56^{bright}$ NK cell subset Precedes NK Cell Dysfunction in Prostate Cancer

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Introduction: Natural cytotoxicity, mediated by natural killer (NK) cells is known to play an important role in inhibition and elimination of malignant tumor cells. In order to investigate the immunoregulatory role of NK cells and potential as a diagnostic tool, NK cell activity of prostate cancer (PCa) patients were analyzed with particular focus on NK cell subset distribution.

Materials and Methods: The study was based on a prospective cross-sectional analysis of 51 patients initially diagnosed with PCa and 54 healthy controls. NK cell activity and NK cell subset distribution patterns were analyzed. NK cell activity was represented by levels of IFN-γ after stimulation of peripheral blood with proinflammatory cytokines. For distribution of NK cell subsets, PBMCs were stained with FITC-anti-CD3, Alexa Fluor® 647-anti-CD16, and PE-anti-CD56 monoclonal antibodies. Then, $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ cells gated on $CD56^{+}CD3^{-}$ cells were analyzed by LSRII flow cytometer.

Results: Significant associations between NK cell-related factors and clinicopathological data were evident. NK cell activity was significantly lower in patients compared to controls (430.9 pg/ml vs. 975.2 pg/ml; $p < 0.001$). Moreover, patients with higher stages tended to show greater reduction in NK cell activity (stages II-IV: 546.1 pg/ml, 427.8 pg/ml, and 194.5 pg/ml; p for trend = 0.001). The proportion of $CD56^{bright}$ cells was lower in patients (2.48 % vs. 4.13 %; $p<0.001$). According to stage progression, $CD56^{bright}$ cells tended to gradually reduce (2.74 %, 2.21 %, and 1.72 %; p for trend < 0.001) in relation to $CD56^{dim}$ cells, confirmed by an increase in $CD56^{dim}$ to $CD56^{bright}$ cell ratio (p for trend = 0.001). The sensitivity and specificity of NK cell activity regarding PCa detection were 72% and 74%, respectively (best cut-off value at 530.9 pg/ml, AUC = 0.786 ± 0.051).

Conclusions: Results of the present study implicate that reduction of $CD56^{bright}$ cells may precede NK cell dysfunction, leading to impaired cytolytic activity against PCa cells. These observations may explain one of the mechanisms behind immunoregulatory function of NK cells, and lend further support to potential immunotherapeutic strategies for tumor.

Key words : Cytotoxicity; Immunity; Killer cell, Natural; Prostate cancer

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I. INTRODUCTION

Natural killer (NK) cells serve a major role in innate and adaptive immune responses against tumor transformation or pathogen-infected cells.[1,2] Without prior sensitization or class I MHC restriction, NK cells exert natural cytotoxic activity in eliminating malignant cells.[3,4] Furthermore, NK cells stimulate the adaptive immune response by secreting proinflammatory cytokines such as IFN-γ to counteract the escape mechanisms promoted by tumor cells.[5,6] Much progress has been made in understanding the biology of NK cells regarding cytolytic activity and subset distribution; nonetheless, considering the importance of NK cells in tumor control and elimination of blood-borne metastases, further clarification remains to be determined.

NK cells are defined phenotypically by their expression of CD56 and lack of

CD3 expression.[7] According to surface membrane densities of CD56 and CD16, NK cells are classified into $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ subsets which possess distinct distribution and function.[8] The majority of NK cells are $CD56^{dim}$ cells, which mainly exert potent cytotoxicity.[9] $CD56^{dim}$ NK cells, constituting approximately 90% of total NK cells, recognize tumor cells by antibody-dependent cellular toxicity and exert strong cytotoxicity.[9] In contrast, $CD56^{bright}$ cells constitutes approximately 10% of total NK cells, mediate low cytotoxicity, but upon activation by monokines, acquire greater cytolytic activity than $CD56^{dim}$ cells by releasing proinflammatory cytokines such as IFN-γ.[10,11] The level of IFN-γ secretion, i.e., NK cell activity, is generally associated with oncologic outcome of cancers, which implies the essential role of differential NK cell subset expression in immune regulation of tumor cells.[12] NK cell activity has been shown to serve an important role in immune surveillance and elimination of tumor cells.[13,14] Both *in vivo* and *in vitro* studies have shown that low NK cell activity leads to high incidences of tumor occurrence and metastasis, and that the degree of impairment correlates with invasiveness of malignancy.[15,16] On the contrary, high NK cell activity has been shown to correlate with good oncological prognosis.[3,17]

Prostate cancer (PCa) is the most common solid organ malignancy and the second most common cause of cancer-related death among men in industrialized nations.[18] The exact cause of PCa is not yet clearly defined, although several mechanisms have been proposed; including hereditary, environmental factors such as diet or occupational exposure, age-related remodeling, and immune response.[19] Regarding immune response, suppression of host cell-mediated immune activity, and imbalance of lymphocyte

distribution have been observed; however, the role of NK cell dysfunction is not yet fully understood.

There is accumulating evidence that impaired immune response is a crucial factor in the pathogenesis of prostate cancer (PCa).[18,19] Recent developments of novel drugs based on immune response, namely, Sipuleucel-T (Provenge®), Prostvac-VF®, or Ipilimumab (Yervoy®), and their survival benefit in castration-resistant PCa, lend further support to the notion that immune response partly has role in development and progression of PCa. Moreover, such observations suggest that PCa has rationale targets for immunotherapy. NK cell dysfunction has been noticed in PCa along with a wide variety of tumors.[20-22] Despite several proposed mechanisms including reduced number, immunosuppressive cytokines, activating and inhibitory receptor repertoire imbalance, the pathophysiology of NK cell dysfunction in the development and progression of PCa is not fully understood.[8] Regarding the underlying role of NK cell activity in tumor regulation, harnessing the mechanisms behind NK cell biology is clearly an important component for a successful immunotherapy against PCa.

Prostate-specific antigen (PSA) is the most widely used serum marker that has revolutionized early detection and management of PCa.[23] Although PSA, as an independent variable, is the best predictor of PCa, the relative lack of cancer-specificity, without upper or lower threshold value, is a major drawback which may lead to unnecessary risks and costs.[24] Various factors are associated with false-elevations of PSA in noncancerous conditions, namely, benign prostatic hyperplasia, infection, trauma, or age, which not only cause unnecessary biopsies that lead to potential complications, but often mask

aggressive forms of cancers that may lead to substantial harm.[23] Various attempts have been made to overcome these limitations, such as the utilizations of PSA density, percentage of free PSA, PSA velocity, age-specific PSA ranges, complex PSA, and novel genetic-based, serologic, and urinary biomarkers. However, none of these have clearly outweighed diagnostic benefits against drawbacks and achieved satisfactory results applicable to everyday clinical practice.

To address these issues, the level of NK cell activity and the distributions of $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets were analyzed between PCa patients and healthy controls. Our findings indicate that immunoregulation in PCa is impaired due to a reduction in NK cell activity preceded by redistribution of NK cell subsets. Moreover, NK cell activity itself may be applied as a supportive diagnostic marker to patients whose PSA is indefinitely correlated to clinical findings.

II. MATERIALS AND METHODS

1. Patients and healthy controls

A prospective cross-sectional analysis involved 51 patients with newly diagnosed pathological PCa and 54 age-matched healthy controls from March to December, 2012. None of the patients had received prior treatment for PCa, were known of immunological or other malignant conditions, and were free of active infection or inflammation as shown by C-reactive protein < 1.0 mg/L and white blood cell count < 10,000 cells/ul (Table 1). All controls were also free from inflammatory conditions without prior exposure to immunosuppressive agents. Independent approval was obtained from Yonsei University Ethics

Committee (4-2011-0660), with blood samples collected from all individuals after obtaining informed consent.

2. Measurement of NK cell activity

The cytotoxic activity of NK cells were determined by NK Vue Kit® (ATgen, Sungnam, Korea). Whole blood was collected by BD Vacutainer® heparin^{N1} tubes. 1 ml of whole blood was incubated at 37℃, 7.5% CO_2 with the indicated dose of recombinant IL-2 and 1 ml of RPMI 1640 media. The cell free supernatants were then harvested and IFN-γ levels were determined according to the manufacturer's protocols.

3. Measurement of NK cell subset distribution

A. Preparation of PBMCs

Three milliliters of heparinized venous blood was obtained and cell surface markers were analyzed within 5 hours of collection. PBMCs were isolated by density gradient centrifugation using CPT® cell preparation tubes (BD Vacutainer®) at 1600g for 20 min at 20℃. The collected PBMCs (1-2 x 10^6 cells/ml) were washed and resuspended in 10% fetal bovine serum (FBS) + phosphate buffered saline (PBS).

B. Antibody staining

For the expression of CD3, CD16, and CD56 on NK cells, PBMCs were stained with FITC-anti-CD3, Alexa Fluor® 647-anti-CD16, and PE-anti-CD56 fluorochrome-conjugated monoclonal antibodies, all obtained from BD Biosciences. After staining for 30 min at 4℃, cells were washed extensively

Table 1. Demographic data of clinicopathological characteristics of patients with PCa and healthy controls. All values are given as means ± SD.

	Patients	**Controls**	***p*-value**
Clinical characteristics			
n	51	54	NS
Age (years)	63.7 ± 6.9	61.6 ± 5.8	0.092
BMI	24.4±2.9	24.1 ± 2.1	0.705
Preoperative PSA (ng/ml)	16.9 ± 23.5	1.1 ± 0.9	<0.001
Prostate volume (gm)	34.2 ± 10.4	28.8 ± 10.9	0.02
WBC (cells/μl)	6463 ± 1670	6278 ± 1315	0.874
% Lymphocyte	31.4 ± 7.3	33.2 ± 5.2	0.153
% Neutrophil	57.9 ± 9.1	55.6 ± 8.1	0.269
C-reactive protein	<1.0	<1.0	NS
Tumor charactertistics			
Extracapsular extension	27 (53%)		NS
SV invasion	9 (16%)		NS
LN metastasis	8 (15%)		NS
TNM stage			NS
II	18 (36%)		
III	24 (47%)		
IV	9 (17%)		
Pathologic Gleason score			NS
6	10 (19%)		
7	25 (49%)		
8	8 (17%)		
9	8 (15%)		

and fixed in 1% paraformaldehyde-PBS until assessment.

C. Flow cytometry

To determine the total percentage of NK cells gated on a $CD3^{-}CD56^{+}$ cell population, at least 10,000 target cells were acquired by LSRII flow cytometry (BD Biosciences). For the distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cells, $CD16^{+}CD56^{dim}$ NK cells and $CD16^{-}CD56^{bright}$ NK cells gated on $CD3^{-}CD56^{+}$ cells were presented as the percentage of total NK cells. For each sample, data were further analyzed by FlowJo 8.1.1.1 (Tree Star, Inc, Ashland, OR, USA).

4. Prostate-specific antigen (PSA) detection

PSA levels of all patients and healthy controls enrolled in the study were detected by a photometric method using the Cobas C601 analyzer (ROCHE Diagnostic, USA).

5. Tumor stage classification

PCa staging was determined according to the 7^{th} American Joint Committee on Cancer (AJCC) TNM system. Stage distribution and pathological characteristics of the patients are shown in Table 1. All classifications were confirmed by a single pathologist.

6. Statistical analysis

Data are presented as mean ± standard deviation. Statistical analyses were performed using Student's *t*-tests when comparing unpaired two group non-parametric data, and Kruskal-Wallis tests with Bonferroni post-hoc

correction when comparing more than two groups. The accuracy of NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ ratio in detecting PCa was determined by the Receiver Operating Characteristic (ROC) and the corresponding area under a curve (AUC). The sensitivity and specificity were calculated at the best cut-off value of each ROC curve. Correlation analysis was used to evaluate the association between clinicopathological variables and measurements of NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ ratio. Findings were considered significant when *p*-values were less than 0.05.

III. RESULTS

1. Demographic data of investigated subjects

All patients and controls were investigated clinically and pathologically with respect to factors shown in Table 1. Neither group of subjects showed significant differences in age, BMI, WBC count, % lymphocyte, % neutrophil, and C-reactive protein.

2. Frequency of total NK cells and distribution of $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets

Representative flow cytometric data shows the distribution of total NK cell population represented as $CD3^{-}CD56^{+}$ cells (Fig. 1A) and two major subsets, $CD16^{+}CD56^{dim}$ and $CD16^{-}CD56^{bright}$ expressed as a percentage of total NK cells (Fig. 1B). Circulating frequencies of total NK cell population did not differ between patients and controls, or between stage groups (Fig. 2A; Table 2).

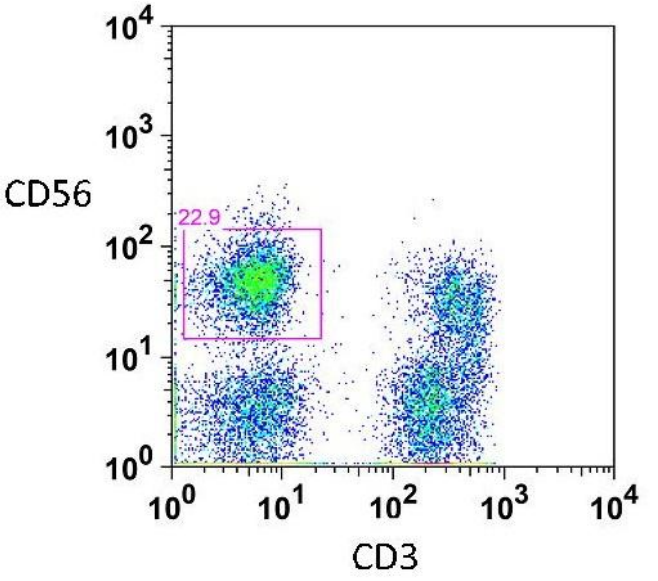

Fig. 1(A) Representative flow cytometric data of the distribution of total NK cell population represented by $CD3^{-}CD56^{+}$ cells

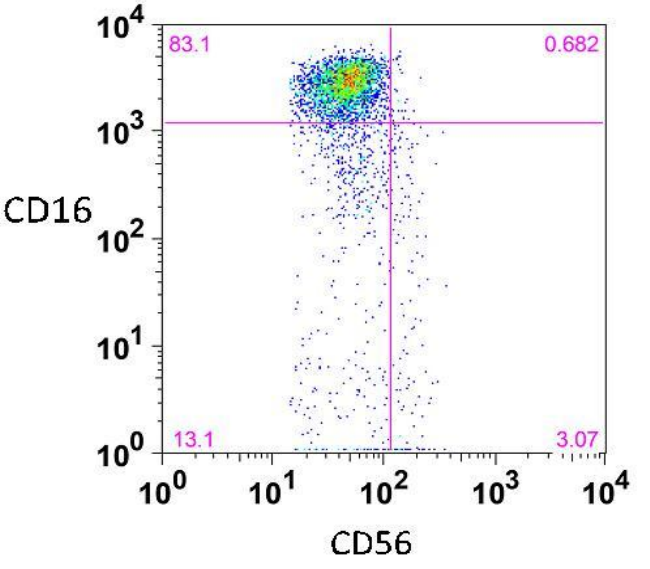

Fig. 1(B) Representative flow cytometric data of two major NK cell subsets detected in peripheral blood. Left, upper box: $CD16^{+}CD56^{dim}$ NK cell subset. Right, lower box: $CD16^{-}CD56^{bright}$ NK cell subset

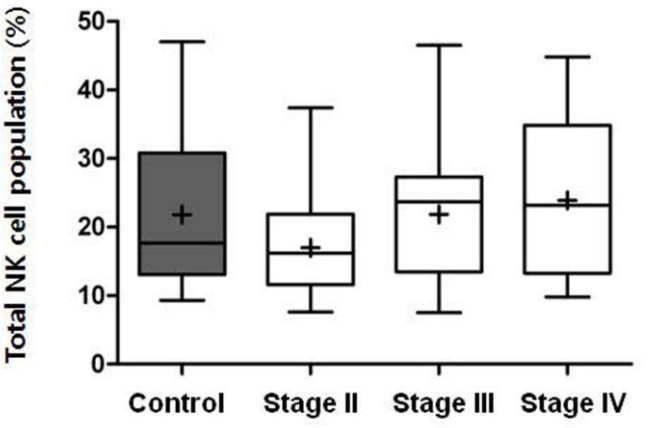

Fig. 2(A) Boxplot diagrams showing flow cytometric distribution results of total NK cell population % between controls and patients, grouped according to cancer stage.

However, a preferential decrease in frequency of $CD56^{bright}$ cells was noted in patients compared to controls. Moreover, $CD56^{bright}$ cells tended to gradually reduce according to tumor stage progression (*p* for trend < *0.001*) (Fig. 2B; Table 2). In order to evaluate the relative distributional shift between the two phenotypes, $CD56^{dim}$ to $CD56^{bright}$ NK cell ratio was calculated. A significantly higher ratio was observed in patients compared to controls. According to tumor progression, a gradual increase of the ratio was observed (*p* for trend = *0.001*), implying a significant reduction of $CD56^{bright}$ cells in relation to alteration of $CD56^{dim}$ cells (Fig 2C; Table2).

3. NK cell activity

NK cell activity was measured in culture supernatants derived from patients prior to treatment. Results obtained in studies on NK cell activity are presented

in Fig. 3 and Table 2. As indicated, patients showed significantly lower NK cell activity than controls. According to stage progression, those with higher stages tended to show greater reduction of NK cell activity (*p* for trend < *0.001*).

Table 2. Comparisons of NK cell activity, % total NK cell population, distribution of $CD56^{dim}$ and $CD56^{bright}$ subsets, and $CD56^{dim}$ to $CD56^{bright}$ ratio between patients and controls. All data represented as mean ± S.D.

	NK cell activity (pg/ml)	**Population (%)**	**$CD56^{dim}$ (%)**	**$CD56^{bright}$ (%)**	**ratio**
Patients	430.9±455.6	20.19±9.16	85.36±8.52	2.36±1.04	41.83±15.74
stage II	546.1±564.2	16.97±7.53	83.5±7.62	2.74±1.3	35.79±13.71
stage III	427.8±403.2	21.84±9.02	86.19±9.47	2.21±0.81	43.63±15.56
stage IV	194.5±208.9	23.88±13.12	88.02±7.11	1.72±0.44	54.44±16.45
Controls	975.2±507.4	21.79±10.65	88.91±4.61	3.76±1.78	30.29±17.96
***p*-value**	<0.001	0.595	0.103	<0.001	<0.001

4. Analysis by ROC curves

ROC curves and the best cut-off values were used to calculate the sensitivity and specificity of each NK cell-related parameter (Table 3). The sensitivity and specificity of NK cell activity levels with respect to PCa detection were 72% and 74%, whereas $CD56^{dim}$ to $CD56^{bright}$ cell ratio showed a sensitivity of 66% and a specificity of 71% (Fig. 4A). In further analysis, the sensitivity and specificity of NK cell activity were determined according to PSA values

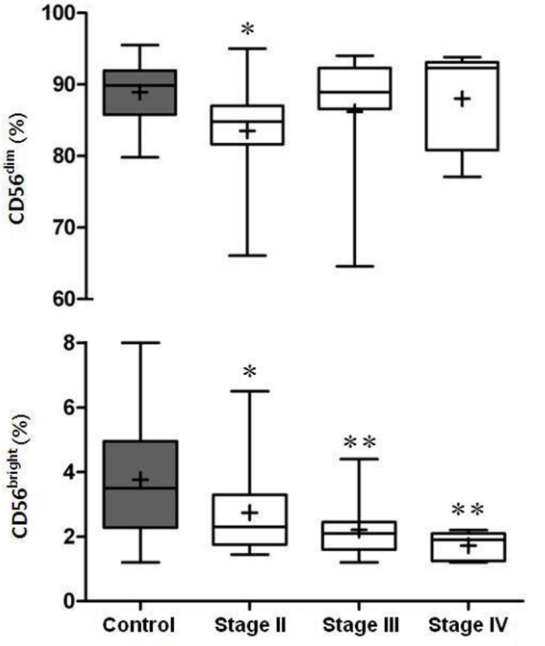

Fig. 2(B) Boxplot diagrams showing flow cytometric distribution results of $CD56^{dim}$ and $CD56^{bright}$ subset distributions within total NK cells between controls and patients. * $p < 0.05$, ** $p < 0.01$ in relation to controls

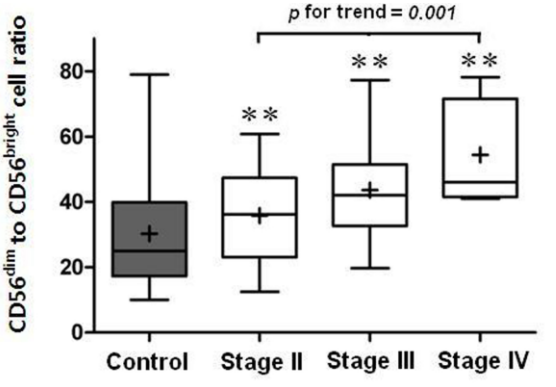

Fig. 2C Boxplot diagrams showing results of $CD56^{dim}$-to-$CD56^{bright}$ ratio measurements between controls and patients. ** $p < 0.01$ in relation to controls

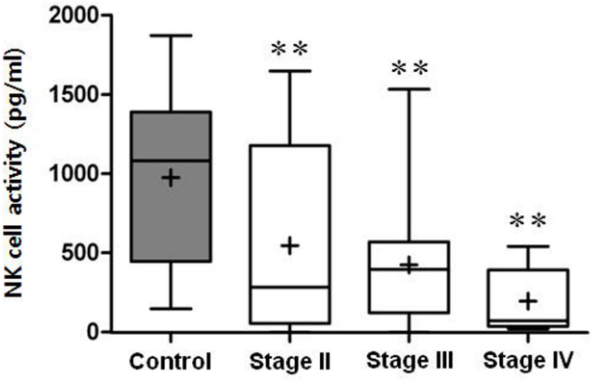

Fig. 3 Boxplot diagram comparing NK cell activity between controls and patients grouped according to cancer stage. ** $p < 0.01$ in relation to controls

grouped as; 4 to 10 ng/ml, i.e., critical diagnostic grey zone, and greater than 10 ng/ml. At a set specificity of 74%, NK cell activity for PSA values within the grey zone showed higher sensitivity (73% vs. 71%) and AUC (0.82 ± 0.06 vs. 0.76 ± 0.07) than for PSA values greater than 10 ng/ml (Fig. 4B).

5. NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ cell ratio according to clinicopathological variables

NK cell activity showed significant negative correlations with PSA ($r = -0.41$), and with tumor stage ($r = -0.51$) ($p < 0.001$). Oppositely, $CD56^{dim}$ to $CD56^{bright}$ cell ratio showed positive correlations between and PSA ($r = 0.44$), and tumor stage ($r = 0.44$) ($p < 0.001$). Between NK activity and $CD56^{dim}$ to $CD56^{bright}$ cell ratio, a negative correlation was observed ($r = -0.28$, $p = 0.01$).

Table 3. Comparisons of sensitivity and specificity of NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ ratio to detect PCa. The sensitivity of NK cell activity for corresponding PSA range is set at 74% specificity.

Diagnostic test	**AUC**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Cut-off value**
NK cell activity	0.79±0.05	72%	74%	530.9 pg/ml
PSA 4-10 ng/ml	0.82±0.06	73%	74%	
PSA > 10 ng/ml	0.76±0.07	70%	74%	
$CD56^{dim}$ to $CD56^{bright}$ ratio	0.72±0.06	66%	71%	35.5

AUC = area under the curve

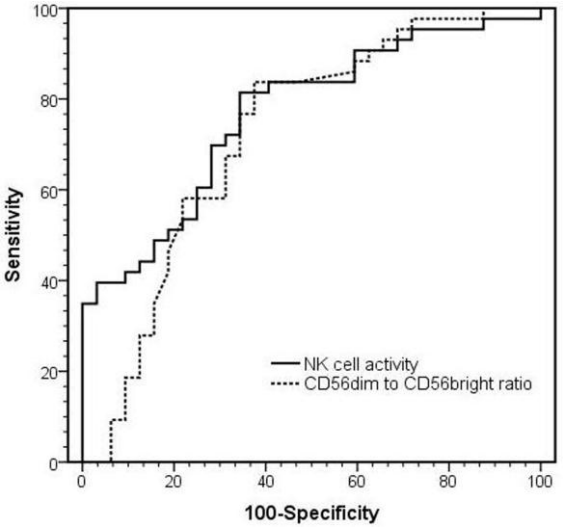

Fig. 4A ROC curves comparing the performances of NK cell activity and $CD56^{dim}$-to-$CD56^{bright}$ ratio measurements. (AUC = Area Under the Curve)

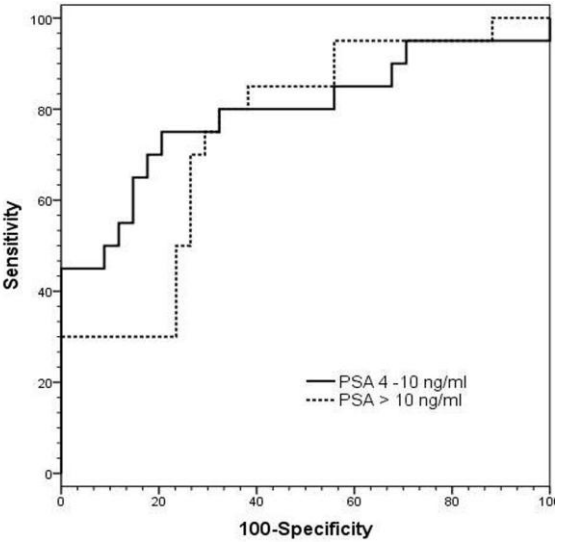

Fig. 4B ROC curves comparing the performances of NK cell activity measurement according to PSA grouped as; 4 to 10 ng/ml and greater than 10 ng/ml. (AUC = Area Under the Curve)

However, there were no significant correlations noted between NK cell activity and total NK cell population, for the overall population or for the subgroups, i.e., controls and patients, and according to cancer stage category. In respect to other clinicopathological variates (e.g., Gleason score, extracapsular extension, seminal vesicle invasion, lymph node metastasis, prostate and tumor volume), analyses of NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ ratio failed to demonstrate significant correlations (Table 4). NK activity and $CD56^{dim}$ to $CD56^{bright}$ ratio was compared between controls and patients grouped according to clinicopathological variables (Table 5). Although $CD56^{dim}$ to $CD56^{bright}$ ratio failed to discriminate patients with Gleason score <7 and those without

Table 4. Correlation of NK cell activity and $CD56^{dim}$ to $CD56^{bright}$ ratio between clinicopathological variables.

Variable	**NK cell activity**		**$CD56^{dim}$ to $CD56^{bright}$ ratio**	
	r	***p***	***r***	***p***
NK cell activity	*NS*	*NS*	*-0.28*	*0.01*
PSA	*-0.41*	*<0.001*	*0.44*	*<0.001*
Stage	*-0.51*	*<0.001*	*0.44*	*<0.001*
Extracapsular extension	*-0.12*	*0.43*	*0.28*	*0.06*
Seminal vesicle invasion	*-0.26*	*0.08*	*0.24*	*0.11*
Lymph node metastasis	*-0.18*	*0.24*	*0.27*	*0.08*
Gleason score	*-0.08*	*0.58*	*0.14*	*0.36*
Tumor volume	*0.01*	*0.99*	*0.07*	*0.67*
Prostate volume†	*-0.22*	*0.06*	*0.13*	*0.26*
Age†	*-0.18*	*0.12*	*0.19*	*0.09*
Body mass index†	*-0.19*	*0.08*	*0.24*	*0.03*

extracapsular extension, all other subgroups were distinguishable from controls by NK activity and $CD56^{dim}$ to $CD56^{bright}$ ratio. Analysis in-between patient subgroups revealed significantly higher $CD56^{dim}$ to $CD56^{bright}$ ratio in patients with pathologically confirmed LN metastasis ($p = 0.043$; data not shown).

IV. DISCUSSION

The aim of the present study was to clarify the role of NK cells in immune response against prostate cancer (PCa). Several mechanisms of PCa

Table 5. Comparisons of NK activity and $CD56^{dim}$ to $CD56^{bright}$ ratio between controls and patients grouped according to clinicopathological variables.

Variable	Mean ± S.D		*P* *	
	NK activity (pg/ml)	**$CD56^{dim}$ to $CD56^{bright}$ ratio**	**NK activity (pg/ml)**	**$CD56^{dim}$ to $CD56^{bright}$ ratio**
Controls	975.2±507.4	30.3±17.9		
Patients				
PSA (ng/ml)				
4-10	373.1±468	38.9±12.6	*<0.001*	*0.007*
≥10	498.3±427	45.9±19.0	*0.002*	*0.004*
Gleason score				
<7	540.1±625.2	34.1±10.7	*0.041*	*0.174*
≥7	411.9±403.1	43.8±16.3	*<0.001*	*<0.001*
SV invasion				
(-)	487.4±467.1	39.8±14.1	*<0.001*	*0.002*
(+)	166.5±205.6	54.5±21.2	*<0.001*	*0.019*
ECE				
(-)	545.8±547.4	35.7±13.4	*0.004*	*0.097*
(+)	365.3±366.3	46.4±16.1	*<0.001*	*<0.001*
LN metastasis				
(-)	478.1±472.4	40.2±15.1	*0.001*	*0.003*
(+)	217.3±214.6	50.9±15.9	*<0.001*	*0.011*

ECE=Extracapsular extension

SV=Seminal vesicle

development and progression have been proposed, e.g., age-related tissue remodeling, hormonal, metabolic alterations, and immune response.[9,25] Regarding immune response, there are accumulating evidences that host cell-mediated immune activity is suppressed in PCa,[18,26-29] and that different lymphocyte populations are involved in its occurrence and progression.[25] However, there is limited information regarding the functional role of NK cells in the immune response to PCa. To address this issue, we investigated levels of IFN-γ, i.e., NK cell activity, and distribution of NK cell subsets in PBMCs of PCa patients. The results of our study indicate that impaired NK cell activity is presumably preceded by a reduction of $CD56^{bright}$ NK cells, and that the level of NK cell activity could be potentially utilized as a supportive diagnostic marker to PSA.

1. Preferential reduction of $CD56^{bright}$ NK cells in PCa patients

NK cells are functionally classified into $CD56^{dim}$ and $CD56^{bright}$ NK subsets according to surface expressions of CD56 and CD16. $CD16^{+}CD56^{dim}$ NK cells are effector cells with high quantities of cytolytic granules which express potent cytotoxicity against tumor cells.[6,30] $CD16^{-}CD56^{bright}$ NK cells release proinflammatory cytokines such as IFN-γ upon activation, which drives cellular and molecular inflammatory mechanisms that regulate tumor initiation, immunoevasion, survival, and outgrowth.[31,32] Interesting discoveries on the importance of $CD56^{bright}$ NK cells have been made in recent years that it constitutes the majority of NK cells in lymphoid tissues and that it is not just a minor subpopulation among total NK cells, but are immature precursors of $CD56^{dim}$ NK cells.[33] This article focuses on this particular subset, regarding its

importance in regulation of NK cell-mediated adaptive response mechanisms against tumor cells.

Investigation on distributional patterns of NK cell subsets revealed that the proportion of $CD56^{bright}$ NK cells of patients was lower than that of controls. Moreover, a tendency of gradual reduction of $CD56^{bright}$ NK cells was observed according to tumor progression, i.e., extracapsular extension, regional node or adjacent organ metastasis. The significant reduction of $CD56^{bright}$ NK cells in relation to $CD56^{dim}$ NK cells was confirmed by an upward shift of the $CD56^{dim}$ to $CD56^{bright}$ NK cell ratio. Previous studies on various tumor-bearing hosts have reported distinct interrelationships between $CD56^{dim}$ and $CD56^{bright}$ NK subsets. In contrast to our results, reduction of $CD56^{dim}$ NK cells without alteration of $CD56^{bright}$ NK cells were noted in gastric and esophageal cancers.[34] On the other hand, reduction of $CD56^{bright}$ NK cells observed in breast, head, and neck cancers and equal distribution of $CD56^{dim}$ cells in PCa were observations that are consistent to the present study.[10,25] The latter results imply that alteration of the $CD56^{bright}$ NK subset may be associated with immune response against various tumors.

2. Alteration of $CD56^{bright}$ NK cells as a response mechanism to tumor microenvironment

The significance of our study was to primarily observe a preferential reduction of $CD56^{bright}$ NK cells without alteration of $CD56^{dim}$ NK cells. Although the underlying cause has not yet been clearly defined, three possible explanations can be raised with regard to the 1) maturation process, 2) recruitment process, and 3) effect of regulatory cytokines on NK cells.

As mentioned, $CD56^{bright}$ NK cells are accepted as precursor cells of $CD56^{dim}$ NK cells, i.e., each subset represents distinct maturation stages of NK cells.[35] Possibly, an excessive demand for effector cells in response to tumor may have provocated a rapid peripheral transition of immature $CD56^{bright}$ NK cells into $CD56^{dim}$ NK cells. A similar explanation has been proposed for reduction of $CD56^{bright}$ cells in patients with head and neck cancers.[10] However, this presupposes a concomitant increase of $CD56^{dim}$ NK cells, which was not observed in the present study.

An alternative explanation without demonstration, is that peripheral $CD56^{bright}$ NK cells may have been recruited to lymphoid tissue sites, e.g., metastatic LNs, in order to acquire cytotoxicity. This postulation was raised by previous observations that $CD56^{bright}$ NK cells preferentially accumulate in the T cell area of LNs through selective expressions of CCR7 and CD62L, until being activated to produce abundant proinflammatory cytokines.[10,31,33] Moreover, the observation that $CD56^{bright}$ NK cells isolated from human LNs become strongly cytotoxic upon stimulation by IL-2, suggests NK cells that are recruited to LNs might represent an immature pool of effector cells.[36]

A significantly higher $CD56^{dim}$ to $CD56^{bright}$ NK cell ratio in patients with pathologically confirmed LN metastasis was observed in our study, implying that these circulating cells may have been recruited to pathologic or secondary LNs in response to tumor. This is of relevance because draining LNs are usually the primary metastatic sites of cancers and $CD56^{bright}$ NK cells are the primary subset found in LNs that obviously counteracts metastatic cells at these sites.[37] To confirm this issue, it would be interesting to examine whether $CD56^{bright}$ NK cells are excessively accumulated in metastatic LN specimens of patients who

receive radical prostatectomy.

Immunoregulatory cytokines may have a role in preferential reduction of peripheral $CD56^{bright}$ NK cells in PCa. An *in vitro* study demonstrated that culture of peripheral NK cells with transforming growth factor-β (TGF-β) induces transition of $CD16^{+}CD56^{dim}$ NK cells into $CD16^{-}CD56^{bright}$ NK cells.[38] Although data not shown, level of peripheral TGF-β was observed to be significantly lower in our patients compared to controls. This implies that the down-regulation of TGF-β may have compromised the transition of $CD56^{dim}$ NK cells into $CD56^{bright}$ NK cells.

3. NK cell dysfunction as a consequence to reduction of $CD56^{bright}$ NK cells

NK cell activity was investigated to determine the influence reduction of $CD56^{bright}$ NK cells has on cytolytic activity against PCa tumor cells. Parallel to observations with $CD56^{bright}$ NK cells, NK cell activity was observed to be lower in patients along with a tendency to gradually decrease according to stage progression. These findings are consistent to previous reports that NK cell activity is compromised in a broad spectrum of tumors, including those of hematological origin and solid tumors such as bladder, esophageal, gastric, and laryngeal carcinomas.[5,12,15,39-41] Several mechanisms of compromised NK cell activity in tumor microenvironment have been proposed, such as decreased number of tumor-infiltrating NK cells,[34] increased NK cell surface receptors for immune suppressor factors,[42] and inactivation of NK effector cells.[15]

Correlations observed between NK cell activity and the distribution of $CD56^{bright}$ NK cells may be of direct relevance to suggest an additional mechanism that weak NK cell activity, i.e., reduced secretion of IFN-γ, is a

consequence to reduced $CD56^{bright}$ NK cells. The major cytokines secreted by $CD56^{bright}$ NK cells are tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, IL-10, IL-13, and IFN-γ.[7] Focusing on IFN-γ, it has been reported that NK cells, particular the $CD56^{bright}$ NK cell subset is one of major sources of this cytokine.[33] Secretion of IFN-γ seems to directly depend on $CD56^{bright}$ NK cells, as observed in *in vitro* studies that $CD56^{bright}$ NK cells preferentially proliferate in coculture with immature dendritic cells and lipopolysaccharides to produce IFN-γ.[43] Also, stimulation of $CD56^{bright}$ NK cells with transduced carcinoma cells resulted with enhanced ability to produce IFN-γ and gain high cytotoxicity.[9] Further to this notion, *in vivo* studies have observed production of IFN-γ by tonsillar $CD56^{bright}$ NK cells before maturation into effector cells.[36] Reversely, reduction of $CD56^{bright}$ NK cells was observed to induce impaired secretion of IFN-γ in patients with allergic rhinitis.[44]

As of tumors, IFN-γ stimulates the adaptive immune response in the polarization of T-helper type 1 cells to counteract the escape mechanisms promoted by tumor cells.[5] In practice, the level of IFN-γ is generally accepted to predict oncologic outcome, suggesting the importance of this particular subset of NK cells in adaptive immunity.[12] Our findings that NK cell activity did not correlate to total NK cell population, implies that cytotoxicity exerted by NK cells, does not merely depend on the number of NK cells. This lends further support to the notion that the activity of $CD56^{bright}$ NK cells on cytokine secretion, relative to that of $CD56^{dim}$ NK cells, is the crucial factor that regulates NK cell activity.

Considering these supportive findings, we prefer to hypothesize that reduction of $CD56^{bright}$ NK cells is a potential mechanism involved in reduced

secretion of IFN-γ, i.e., low NK cell activity, which leads to impaired cytotoxicity against PCa cells.

4. NK cell activity, a supportive diagnostic marker to PSA

The area under a curve (AUC) for NK cell-related parameters in diagnosing PCa, implies that NK cell activity may serve as a supportive marker to PSA. It is clear that PSA provides the highest diagnostic value for PCa and that its application to clinical practice has revolutionized management of this disease.[45] However, the major drawback is its relative lack of specificity, especially in the critical diagnostic grey zone of 4 to 10 ng/ml where only 25% of biopsied patients will demonstrate cancer.[23,46] False-elevations in noncancerous conditions not only cause unnecessary biopsies that lead to potential complications, but often mask aggressive forms of cancer that may lead to substantial harm.[19,24] Although this ongoing clinical challenge has aroused scientific challenges to evaluate novel methods of PCa screening, none have clearly outweighed diagnostic benefits against drawbacks.[19] The American Urological Association best practice guideline for PSA screening has recommended screening to be offered in all healthy men over the age of 40. However, due to such limitations, in 2012 the United States Preventive Service Task Force accumulated solid evidences based on prospective randomized-controlled studies to recommend against routine PSA screening.

This paper does not seek to settle these concerns, but raise the possibility that NK cell-related factors such as NK cell activity may be utilized in combination with PSA to provide additional diagnostic value for preventing underdiagnosis and overdiagnosis; as in our results, especially for those within

the diagnostic grey zone.

There are several limitations of this study that are worth mentioning. First, the current study was based on healthy controls versus patients diagnosed of PCa due to elevated PSA on routine health examinations. Therefore, the absence of PCa patients with normal PSA (< 4 ng/ml) was the major limitation which hampered a direct comparison of diagnostic yield between PSA and NK cell activity. Although this group of patients would most probably present clinically insignificant cancer and represent candidates for active surveillance, clearly an extended population study is needed to confirm our preliminary findings and to assess economic impact.

Second, although PCa patients were age-matched to healthy controls, and there were no differences in CRP or WBC counts confirming the absence of active infection or immunosuppression, the possibility of disparities in immune response may have existed. To confirm the absence of confounders related to immunoregulation that may affect the level of NK cell activity or the distribution in NK cell subsets, additional investigation with surrogate markers should have been performed.

Third, the indirect assay based on IFN-γ measurements after stimulation of the peripheral blood with IL-2 could be considered insufficient, and rather a direct evidence such as NK cell killing assay to confirm alterations in NK cell cytotoxicity is needed. However, the scopes of the current study was to utilize and validate the usefulness of a measurement that is reproducible, simple, less time-consuming, and less costly which is applicable to everyday clinical practice. The conventional widely-used measurements, such as the ^{51}Cr release assay, has limitations regarding quantification and standardization as it utilizes

K-562 cell lines as target cells and PBMCs as effector cells. The NK Vue Kit used in this study is a measurement of indirectly stimulated IFN-γ levels; however, by monitoring IFN- γ levels after stimulation of the peripheral blood with IL-2, it may be assumed that this may provide an actual quantitation of NK cell cytotoxicity exerted on tumor cells. Indeed, the cytokines secreted by NK cells, most importantly IFN-γ, is known to be generally associated with oncologic outcome of cancers, which is the main scope of our study.

Fourth, NK cell activity was characterized in this study as the level of IFN-γ secretion in the supernatant following *ex vivo* activation of the total blood by IL-2. As IL-2 could also activate the secretion of IFN-γ by T cells, there is potential that the diminution of IFN-γ was not specific to NK cells, and intracellular flow cytometric staining or purification of NK cells from total blood should have performed. However, in a set of our unpublished data which utilized intracellular staining protocols, IL-2 was observed to mainly stimulate IFN-γ from NK cells, and not from T cells. Moreover, the concentration of IL-2 in the NK Vue kit was set at 8 ng/ml; a cut-off concentration that was insufficient to stimulate IFN-γ from T cells, but only from NK cells.

Further studies regarding activating and inhibitory receptor profiles and degranulation assays in a prospective well-designed, randomized control trial are warranted to confirm our results and utilize the biology of NK cell as a immunotherapeutic strategy against PCa development and progression.

V. CONCLUSION

The present study provided novel findings that the $CD56^{bright}$ NK cell subset is not only a minor subpopulation among total NK cells, but serves an important

role in adaptive response against PCa cells. This notion lends further support that longitudinal studies regarding NK cell immunosurveillance clearly deserves additional research, which could potentially lead to novel immunotherapeutic strategies for enhancing oncological outcomes of PCa.

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ABSTRACT

전립선암에서 관찰되는 자연살해세포 활성장애 기전으로써 $CD16^{-}CD56^{bright}$ subset의 감소

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Ⅰ. 서론

자연살해세포에 의한 세포독성은 종양세포의 억제와 제거 기전에 있어 주요한 역할을 한다. 본 연구는 전립선암의 발생 및 진행의 면역요인에 있어서 자연살해세포의 역할에 대해 알아보기 위해 전립선암 환자의 말초혈액에서 자연살해세포의 subset에 초점을 맞춰 자연살해세포 활성도를 분석하고, 전립선암 진단 표지자로써 이들의 가치에 대해 알아보았다.

Ⅱ. 재료 및 방법

전립선암으로 확진되어 수술을 앞둔 51명의 환자들과 나이를

보정한 건강대조군 54명을 대상으로 자연살해세포 활성도와 자연살해세포 subset의 분포를 전향적으로 분석하였다. 자연살해세포 활성도는 말초혈액을 IL-2로 자극시킨 후 배양액의 상층액에서 검출된 IFN-γ치를 측정값으로 사용하였다. 자연살해세포 subset 분포를 분석하기 위해 말초혈액을 단일 클론 형광색소 항체로 염색 후 세포분석기를 이용하여 측정하였으며, 자연살해세포를 구성하는 $CD56^{+}CD3^{-}$ 세포 중 $CD16^{+}CD56^{dim}$ subset과 $CD16^{-}CD56^{bright}$ subset의 세포수를 측정하여 전체 자연살해세포 수 중에서 차지하는 비율로써 분석하였다.

III. 결과

자연살해세포 활성도와 $CD56^{bright}$ subset 세포수는 대조군에 비해 환자군에서 유의하게 낮았다(430.9 pg/ml 대 975.2 pg/ml, 2.3% 대 3.8%; $p < 0.001$). 암병기가 증가할수록 두 인자 모두 감소하는 유의한 경향이 관찰되었다 (p for trend $= 0.001$). $CD56^{dim}$ 대 $CD56^{bright}$ subset 비율은 환자군에서 유의하게 높았으며 (41.8 대 30.3; $p < 0.001$) 병기가 높을수록 증가하는 유의한 경향을 보임으로써 (p for trend = 0.001), $CD56^{dim}$ subset 세포수의 변화에 비해 $CD56^{bright}$ subset 세포수가 유의하게 상대적으로 감소했음을 확인할 수 있었다. 전립선암 진단에 있어서 자연살해세포 활성도의 예민도와 특이도는 각각 72%와 74%로 나타났다.

Ⅳ. 결론

CD56^{bright} subset의 세포수 감소는 자연살해세포 활성장애에 선행하는 것으로 생각되며, 이로 인하여 전립선암 세포에 대한 세포독성장애가 발생하는 것으로 보인다. 본 관찰 결과는 전립선암의 미세종양환경에서 자연살해세포 독성장애의 기전을 제시하며, 향후 면역기반 치료의 개발에 응용될 수 있으리라 기대한다.

핵심되는 말 : 세포독성; 자연살해세포; 전립선암

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