RESEARCH ARTICLE

Editorial Process: Submission:09/28/2024 Acceptance:05/09/2025

The Presence of Non-Exhausted Senescent T Cells in Breast Cancer Patients

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Abstract

Objective: This study aimed to investigate the presence of cancer-associated senescent T cells in breast cancer patients and the impact of the progression of age on their senescent phenotypes. **Methods:** The exhausted T cell lineage was excluded from the analysis of T cells by using PD1 marker. The percentages of CD28- cells were used to determine the senescent phenotype, in which the CD57 expression was used to further characterize their phenotypes. The flow cytometry was used on CytoFLEX flow cytometer and analysed with CytExpert analysis software. **Results:** In this study, both senescent and non-exhausted senescent T cells were significantly increased in breast cancer patients. However, non-exhausted T cells could demonstrate more significant proportion of senescent T cells, and these phenotypes in CD8+ T cells were increased in breast cancer patients, which were 53.03% and 37.25% ($p<0.001$). Moreover, CD28-CD57- cells of non-exhausted CD8+ T cells were increased irrespective of age, while the increase in CD28-CD57+ cells was positively correlated with the progression of age ($r=0.353$, $p=0.015$). In addition, the predominant CD28-CD57- cells were attenuated after 52 years of age. **Conclusion:** The presence of non-exhausted senescent T cells seemed to be a concern regarding immune dysfunction in breast cancer patients. These phenotypes were presented in both young-age and old-age breast cancer, but the terminal phenotype seemed to be abundant in the elderly.

Keywords: T cell senescence- breast cancer- CD28- T cell

Asian Pac J Cancer Prev, **26 (5)**, 1633-1640

Introduction

The impairment of T cell responses is widely recognized in promoting carcinogenesis according to the aberration of immune-surveillance and inadequate immune responses [1, 2]. The T cell dysfunctions, such as T cell anergy, exhaustion and senescence, are the important mechanisms of this failure [3, 4]. The senescent T cells are terminally differentiated T cells, which are pronounced in the elderly, and also found as dysfunctional T cells in many cancers [5]. However, the senescent T cells seem to be intertwined with exhausted T cells in many previous studies [3, 6, 7]. Recently, exhausted T cells have been found to demonstrate different pathogenesis, in which chronic antigen stimulation is the main mechanism [6, 8], and these T cells express immune checkpoint molecules [9]. The senescent T cells enrich of DNA damage levels, and express the senescent phenotypes [10]. In previous in-vitro studies, peripheral T cells became senescent after being co-cultured with various types of cancer cell-lines or tumour-derived suppressive immune cells, and these senescent T cells showed an increased in DNA damage markers [11-13]. These entities of T cells are distinctive in terms of phenotypes and pathogenesis in cancer, therefore, the differentiation of these two entities in cancer patients would point out the significance of senescent T cells in breast cancer patients.

The presence of senescent T cells has been demonstrated in patients with various types of cancer [5]. Furthermore, the presence of these cells has been linked with poorer prognosis and poor treatment outcomes [14, 15]. In breast cancer, peripheral T cell population changes were described in previous studies which include a decrease in cytotoxic T cell population, an increase in regulatory T cells, and an increase in senescent T cells [16, 17]. The presence of these features in peripheral T cells emphasizes the systemic effects of breast cancer cells on immune suppression. In a previous study, the presence of senescent T cells was not only associated with breast cancer patients, but also coincided with the presence of decreased TCRζ expression and increased apoptotic markers of those T cells [16]. Moreover, in another study, the decrease of

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peripheral CD3+CD28+ immune cells, where senescent T cells became expanded, was found in patients with metastatic and lymph node positive breast cancer [15, 14]. As a result, the presence of T cell senescence in breast cancer was found to have a negative impact on the treatment outcomes and overall prognosis.

Although the presence of T cell senescence has been reported in breast cancer patients, the correlation with age, which is the most influential factor of senescence, has not been clarified [18, 16, 19]. In addition, the differentiation with exhausted T cell lineage has not been reported. In this study, we used PD1 to characterize exhausted T cells, while CD28 and CD57 were used to characterize the proportion of senescent T cells in breast cancer patients and healthy controls. Moreover, we stratified age-groups to determine the effects of age on the presence of those senescent T cells.

Materials and Methods

Patient samples

A total of 47 breast cancer patients and 41 healthy women were recruited in this study from December 2020 to September 2021. The recruitment and sample retrieval were performed before all therapeutic modalities were given. The control group was defined as the presence of negative annual mammographic results or surgical patients with non-cancerous pathological results, in which the numbers of participants were 18 and 23 respectively. Both groups were stratified every ten years of age from 30 to 69 years old. All medical history records were assessed, and patients with ductal carcinoma in-situ, any autoimmune diseases, immunodeficiency, and previous chemotherapy were excluded from this study. Then, pathological results were analysed after blood sample retrieval. All clinical assessments were conducted according to the principles of the Declaration of Helsinki. The Institutional Review Board of Faculty of Medicine, Chulalongkorn University approved the study. (No.789/2563 Date 30 November 2020) Written informed consent was obtained from all individual participants.

PBMC isolation

An EDTA tube was used to collect venepuncture blood sample. After that, the acquired fresh blood was diluted by RPMI media in the ratio of 1:1. Then, each sample was gently layered on Lymphoprep™ (Stemcell, USA) in conical tubes and centrifuged at 500g for 30 minutes with brake off. The interface peripheral mononuclear cell (PBMC) layer was collected and placed in conical tubes containing 5-ml warm RPMI and centrifuged at 500g for 10 minutes, and repeated twice. Trypan blue was used to determinate cell number and 1x106 cells were resuspended in 100 µl PBS for antibody staining.

Antibody staining and flow cytometric analysis

Apoptosis in PBMC samples was assessed by Zombie Aqua (Biolegend, USA) staining. After incubating for 15 minute, centrifugation was performed for each samples while supernatant was discarded. The samples were incubated with the fluorophore conjugated antibodies including phycoerythrin-cyanin 5 labelled antihuman CD3, phycoerythrin-cyanin7 labelled antihuman CD57, Alexa Fluor®647 antihuman CD45, Alexa Fluor®700 antihuman CD279(PD1), allophycocyanin-cyanine7 labelled antihuman CD28, brilliant violet 421 labelled antihuman CD4 and brilliant violet 650 labelled antihuman CD8 for 15 minutes at 4˚C in the dark. All antibodies were purchased from Biolegend, USA. All samples including fluorescence minus one (FMO) were analysed on CytoFLEX flow cytometer (Beckman Coulter, CA) with CytExpert analysis software (Beckman Coulter, CA). Viable leukocytes were gated in dot plots obtained from side scatter and forward scatter plots and were used to the analysis. The sequence of gating strategy was shown in a Supplement Figure 1.

Statistical analysis

All statistical analysis was performed by under IBM SPSS version 22 (Armonk, NY, USA). Unpaired t-test and Pearson's correlation were used to evaluate statistical significances. The linear regression model was also computed. The statistical significance was considered when p-value <0.05.

Results

Patient characteristics

In this study, there were 47 breast cancer patients and 41 healthy volunteers. The mean age was 50.74 years old in breast cancer group and 49.34 years old in control group. In both groups, participants were classified in to 4 age-groups, which were 30-39, 40-49, 50-59 and 60-69 years old. In cancer group, there were 10, 11, 15, and 11 participants in each age-group respectively. In healthy group, there were 10, 11, 10, and 10 participants in each group respectively. The body mass index and menopausal status, as well as the clinicopathological characteristics of breast cancer patients were shown in Table 1.

The proportions of senescent T cells in breast cancer patients

There was no different in the percentages of CD4+ T cells, CD8+ T cells, and their ratios between groups as shown in Table 2. The analysis of senescent phenotypes was demonstrated as percentages of each phenotype in CD3+ or CD4+ or CD8+ T cells. After analysis of these phenotypes in CD3+ cells, the proportions of CD28- , CD28-CD57-, and CD28-CD57+ cells were found significantly increased in the cancer group as shown in Table 2. The further analysis of these phenotypes in T cell subgroups was performed, and CD8+ T cells showed a significantly increased in proportion of CD28- cells in cancer group, but the proportions of CD28-CD57-, and CD28-CD57+ cells were not significantly different between groups. To enrich these senescent phenotypes, exhausted T cells, which were characterized by PD1+, were excluded from the further analysis, and these senescent phenotypes were re-evaluated in non-exhausted T cells or PD1- cells.

Table 1. Baseline Characteristics

	Cancer (47)	Control (41)
Age	50.74	49.34
BMI	23.64	22.39
Menopausal status		
Pre-menopause	24 (51%)	19 (46%)
Post-menopause	23 (49%)	22 (54%)
Stage		
I	18 (38%)	
II	18 (38%)	
III	6 (13%)	
IV	5 (11%)	
Grade		
1	9 (19%)	
2	18 (38%)	
3	20 (43%)	
ER		
Positive	35 (74%)	
Negative	12 (26%)	
PR		
Positive	26 (55%)	
Negative	21 (45%)	
HER2		
Positive	12 (26%)	
Negative	35 (74%)	

Table 2. Mean T Cell Proportions and Mean Percentages of Senescent T Cells in All T Cells in Cancer Group and Control Group.

Phenotypes	Percentages of cells Mean ± SEM.		p-value (88)
	cancer	control	
CD3+CD4+	49.84 ± 2.29	55.59 ± 1.88	0.059
CD3+CD8+	37.31 ± 1.99	33.33 ± 1.58	0.128
CD4+/CD8+ ratio	1.62 ± 0.13	1.94 ± 0.16	0.123
CD28-			
CD3+*	28.29 ± 2.35	20.18 ± 1.58	0.001
CD4+	10.30 ± 1.52	7.45 ±1.18	0.149
CD8+*	40.19 ± 2.60	32.23 ± 2.52	0.032
CD28-CD57-			
CD3+*	14.52 ± 1.35	10.85 ± 0.80	0.027
CD4+	5.65 ± 0.85	4.32 ± 0.68	0.115
CD8+	19.74 ± 1.70	16.03 ± 1.44	0.106
CD28-CD57+			
CD3+*	13.73 ± 1.49	9.33 ± 1.15	0.024
CD4+	5.20 ± 1.02	3.13 ± 0.81	0.065
CD8+	20.45 ± 1.91	16.21 ± 1.99	0.127

The presence of non-exhausted senescent CD8+ T cells in breast cancer patients

Although there was an increase in the proportions of PD1+ T cells, it was not statistically significant as shown in Table 3. The senescent phenotypes were also re-investigated in non-exhausted CD3+, CD4+, and CD8+ T cells, where PD1 was negative. The analyses of senescent phenotypes were demonstrated as percentages of each phenotype in CD3+PD1- or CD4+PD1- or CD8+PD1- T cells. Again, there was a significant increase in these senescent phenotypes in CD3+ T cells including CD28-, CD28-CD57-, and CD28-CD57+ cells in breast cancer group as shown in Table 3. In CD4+ and CD8+ T cells, the non-exhausted CD8+ T cells were significantly increased the proportions of CD28-, CD28-CD57-, and CD28-CD57+ cells in the breast cancer group. However, these increases were marginal in CD4+ T cells as shown in Table 3. As a result, PD1- cells could enrich these senescent phenotypes in both CD4+ and CD8+ T cells, and a significant proportion of non-exhausted senescent CD8+ T cells were demonstrated in both CD28-CD57-, and CD28-CD57+ cells as shown in Figure 1. These findings seemed to extrapolate that the increase in these senescent phenotypes of T cells was more significant in

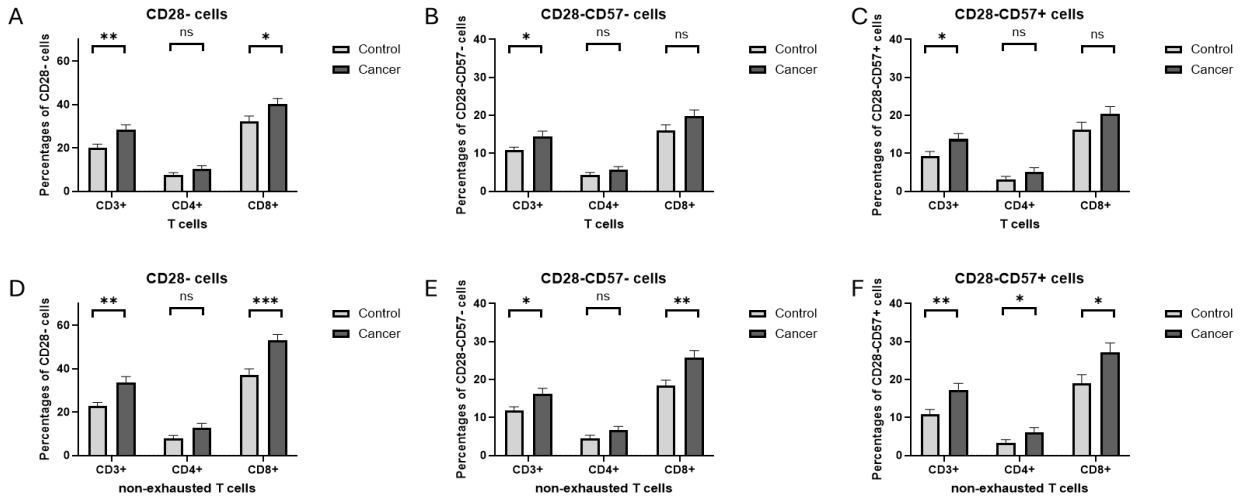

Figure 1. Shows Bars Representing Mean Percentages and SEM of Senescent T Cells in All T Cells (A-C) and Non-Exhausted T Cells (D-F) in Cancer Group (Dark Grey) and Control Group (Light Grey). The asterisk represented p-value (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$) and ns represented non-significant p-value.

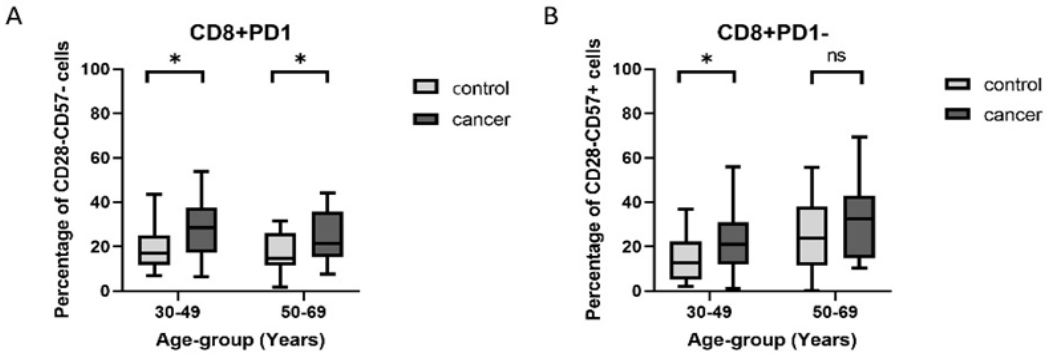

Figure 2. Shows Box Plots Representing Means and Ranges of the Percentages of CD28-CD57- Cells in CD8+PD1- cells (A) and the percentages of CD28-CD57+ cells in CD8+PD1- cells (B) in cancer group (dark grey) and control group (light grey) with age stratification.

Table 3. Mean Percentages of Exhausted T Cells and Mean Percentages of Senescent T Cells in Non-Exhausted T Cells in Cancer Group and Control Group.

Phenotypes	Percentages of cells Mean ± SEM.		p-value (88)
	cancer	control	
CD3+PD1+	19.88 ± 2.39	14.25 ± 1.74	0.059
CD8+PD1+	20.15 ± 2.48	14.08 ± 1.87	0.066
CD4+PD1+	19.29 ± 2.39	13.36 ± 1.64	0.05
CD28-			
CD3+PD1-*	33.76 ± 2.59	22.72 ± 1.76	0.001
CD4+PD1-	12.79 ± 2.02	7.79 ± 1.37	0.05
CD8+PD1-*	53.03 ± 2.79	37.25 ± 2.65	<0.001
CD28-CD57-			
CD3+PD1-*	16.27 ± 1.44	11.92 ± 0.94	0.016
CD4+PD1-	6.64 ± 1.02	4.54 ± 0.78	0.128
CD8+PD1-*	25.81 ± 1.83	18.44 ± 1.41	0.003
CD28-CD57+			
CD3+PD1-*	17.49 ± 1.76	10.80 ± 1.34	0.004
CD4+PD1-*	6.15 ± 1.21	3.25 ± 0.92	0.044
CD8+PD1-*	27.23 ± 2.38	19.01 ± 2.28	0.015

CD8+ subgroup.

The increase in CD28-CD57- cells is irrespective of age, while the increase in CD28-CD57+ cells is significant in the young age-group

The non-exhausted CD8+ T cells were further analysed between two age-groups, which were 30-49 years old and 50-69 years old, to demonstrate how the progression of age affected these senescent cells. In CD28-CD57- cells, the percentages of these cells were significantly higher in breast cancer participants in both age-groups as shown in Figure 2. The mean proportion of this phenotype was 28.37 percent in 30-49 years old group, but this percentage was lower in 50-69 years old group, which was 23.74. In CD28-CD57+ cells, an increased proportion of these cells were observed in the 30-49 years-old breast cancer group, although an slight increase without statistical significance was observed in the 50-69 years old breast cancer group as shown in Figure 2. In cancer participants, the mean proportion of this phenotype was 22.39 in 30-49 years old group, and further increased to 31.13 in 50-69 years old group. Therefore, the increase in CD28-CD57- cells were significant in cancer group irrespective of age, while the increases in the CD28-CD57- and CD28-CD57+ cells in breast cancer participants seemed to show an inverse

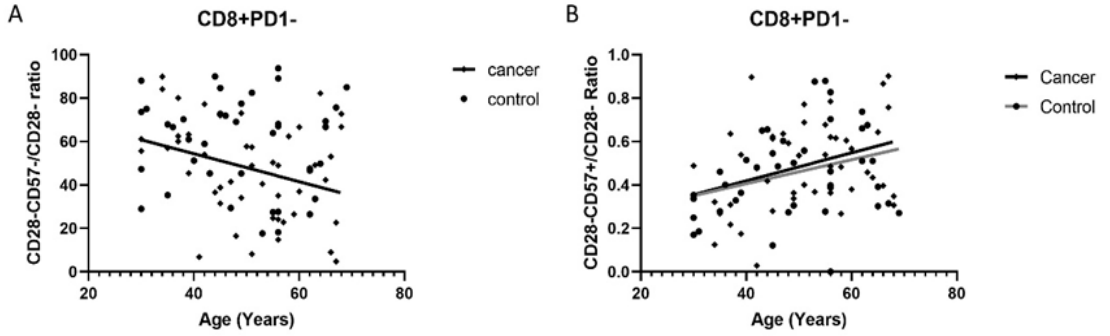

Figure 3. Shows Plots and Linear Regression Models of the Ratio of CD28-CD57-/CD28- Cells (A) and the ratio of CD28-CD57+/CD28- cells (B) with the progression of age in cancer group (dark grey/square) and control group (light grey/circle). The linear regression model of CD28-CD57-/CD28- cell ratio was Y = -0.6442*X + 80.16 (R^2 = 0.101, p = 0.024) in cancer group, while this was not statistically significant in control group (A). The linear regression models of CD28-CD57+/CD28- cell ratio with age were Y = 0.006444*X + 0.1609 (R2 = 0.124 ,p = 0.015) in cancer group, and Y = 0.005520*X + 0.1847 (R^2 = 0.098, p = 0.047) in control group.

relationship with age.

The increase in CD28-CD57- cells is attenuated with the progression of age and is predominant before 52 years of age

The proportional changes of CD28-CD57-, and CD28-CD57+ phenotypes in these senescent T cells with the progression of age were also investigated. In correlation with age, the ratio of CD28-CD57+/CD28- non-exhausted T cells depicted a positive correlation with age in both cancer group and control group ($r = 0.353$, $p = 0.015$ and $r = 0.313$ $p = 0.047$, respectively) as shown in Figure 3. However, only the ratio of CD28-CD57-/CD28- cells was correlated with age in cancer group ($r = -0.318$, $p = 0.023$), but not in the control group ($r = -0.122$, $p = 0.449$). To further analyse these directions, a linear regression model was calculated as the CD28-CD57+/CD28- ratios with progression of age in both cancer and control groups, and these models were fit with linear regression models as shown in Figure 3. The equation was used to determine the specific age at which the CD28-CD57+ population became predominant or gain a ratio of 0.5. As a result, the CD28-CD57+ cells were predominated after 57.1 years old in control group and after 52.6 years old in cancer group.

Discussion

In this study, the senescent T cells were enriched after those cells were differentiated from the exhausted T cell lineage, which expressed PD1 molecule. Moreover, the significance of non-exhausted CD8+ T cells was also determined in breast cancer patients. This was supported by previous studies demonstrating the senescent T cells as one of the cancer associated dysfunctional T lymphocytes due to their distinctive pathological features [11, 12, 5]. Moreover, Martinez-Zamudio et al. reported that PD1 expressed CD8+ T cells showed a very small proportion of key senescent features and did not share the expression profiles with the terminally differentiated T lymphocytes [20]. This finding could explain the worsened clinical outcomes, where the presence of both senescent CD8+ T cells and exhausted T cells was found in cancer patients [21], and the treatment targeting both phenotypes could demonstrated synergistic effects [22].

The senescent T cells were detected in both infiltrating immune cells and circulating cells [4]. In previous studies, the cancer associated senescent T cells seemed to be more abundant in circulating T cells than infiltrating T cells, and the abundance of these senescent cells could determine poor clinical outcomes [5, 23]. According to previous literatures, the change to senescent immune cells was observed from the co-culture of lymphocytes with tumour derived immune cells [24, 12]. In addition, these changes were also found in non-metastatic axillary lymph node in breast cancer patients [25]. These features emphasized the systemic effects of cancer cells that led peripheral immune cells to adopt senescent phenotypes, and the levels of these effects seemed to determine the cancer prognosis. Furthermore, these changes appear to occur via a non-contact fashion, in which the association with many soluble factors had been described [24, 26]. The capture of senescent phenotypes in this study could demonstrate the systemic effects of breast cancer cells, which seemed to be abundant in peripheral T cells.

An increase in CD28- T cells were found in patients with lung, head and neck, multiple myeloma, and breast cancer [3, 27, 18, 28]. Moreover, the presence of this phenotype may correlate with the worsening prognosis [29, 4]. This finding is similar to our study, which also demonstrated the increase in senescent T cells and non-exhausted senescent T cells among these groups of patients. Furthermore, we also found that CD28- T cells were pronounced in CD8+ T cells more than CD4+ T cells. Additionally, a marginally increase in senescent CD4+ T cells in surface marker changes was also noted in our study. The presence of senescent phenotypes might be different in CD4+ T cells, which were found to impair the downstream signalling pathways, such as T cell receptor signalling and mTOR pathway [30, 31]. As a result, additional markers were needed to detect senescence related signalling pathway defects.

The senescent T cells are the chronological changes of T cell differentiation [31]. There are several markers to determine this entity, such as telomere length, impairment of cell cycle, and surface markers [32, 33]. The challenging point is to identify early senescent cells, where proliferation capability or rejuvenation possibility still remains. In this study, we used CD28- as a marker of senescent T cells and their expression of CD57 as chronological changes. In previous studies, CD28-CD57+ T cells were recognized as terminally differentiated T cells, therefore, the proliferative capability was defective in these cells [34, 29]. The CD28-CD57- T cells had not been well described regarding their functional profiles, but there was a study showed proliferative capability of CD28-CD57- T cells [34]. The increase in CD28-CD57- T cells that was irrespective of age in breast cancer patients, pointed out the importance of senescent phenotypes in these patients, and this finding seemed to allow the possibility of immune rejuvenation in breast cancer patients. Although the capture of these early senescent T cells was demonstrated in the patients, the functional assays would more clarify specific subgroups of these T cells, and this warrants further studies.

Aging is the main factor related to senescent T cell expansion since the thymic involution, but the T cell homeostasis could attenuate this phenomenon [35, 36]. These lead to the pronounced senescent T cells after 65 years-old [37, 38]. This occurrence pointed out the influence of the progression of age on T cell senescence phenotypes. In our study, we could determine these effects in both cancer and control groups, and we also demonstrated the presence of premature T cell senescence in breast cancer patients, which were the increase in the proportion of non-exhausted CD8+CD28- cells and proportional change of CD28-CD57- cells and CD28-CD57+ cells. These premature changes could extrapolate as the effects of breast cancer cells on peripheral T cells. Moreover, we found that even though CD28-CD57- cells increase regardless of age, their proportions gradually decreased as age progression. These seemed to show the different

phenotypic changes regarding natural aging. In the young age-group, the senescent phenotype seemed to be early senescent phenotypes, while the change to terminally differentiated phenotype was more abundant in the old-age group. A further study regarding the rejuvenation capability seemed to be more interesting in below 50 year-old patients, while the senescent phenotypic changes in the old-age group seemed not to be reversible.

The control group in this study was the participants with negative mammographic results and the non-cancerous pathological results. Most common pathological results were fibroadenoma and fibrocystic disease. Of these, one participant had usual duct hyperplasia. These pathologies had not been found the association with breast cancer and breast cancer risk [39]. In both groups, the mean age was 55 years old in mammography group, whereas the other group was 44 years old. The proportions of non-exhausted CD8+ CD28- cells were 40.99% and 29.37% in mammography and pathology group respectively (p=0.04), while the proportions of non-exhausted CD8+ CD28-CD57- cells were 19.05% and 17.69%, respectively (p=0.67), and the proportions of non-exhausted CD8+ CD28-CD57+ cells were 21.95% and 11.68%, respectively (p=0.02). As we know, the annual mammography is recommended in women above 40 years old, hence the mammography group included older participants in this study. This resulted in the increased proportion of senescent CD8+ cells except the CD28-CD57- cells, and this seemed due to the impact of old age, rather than the benign pathology. Although some benign pathologies of breast could demonstrate the infiltrating immune cells, these findings did not related to the subsequent breast cancer risk [40].

The limitation of our study is the limited ranges of age-groups and sample size. However, a high incidence of breast cancer was reported within this selected range of ages, where the changes of senescent immune profiles were expected [41].

In conclusion, the presence of non-exhausted senescent T cells in breast cancer patients could emphasize the importance of this entity in breast cancer patients. In this study, both CD28-CD57- and CD28-CD57+ phenotypes of CD8+ T cells were increased in breast cancer patients. In these senescent cells, the increase of CD28-CD57- T cells were observed, irrespective of age, and the proportion of CD28-CD57+ T cells were increased with the advancement of age. Moreover, the effect of CD28-CD57- cell expansion was attenuated after 52 years of age. This could be a future implication of immunotherapy targeting immune senescence or immune cell rejuvenation in breast cancer patients especially in young age-group patients.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

Availability of data

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Scientific Body Approval

This study is a component of Sikrit Denariyakoon PhD thesis.

Funding

The research was funded by grants from Graduate School, Chulalongkorn University and Faculty of Medicine, Chulalongkorn University and the National Science and Technology Development Agency (FDA-CO-2561-8477-TH).

Ethical Committee Approval

This study has been approved by The Institutional Review Board of Faculty of Medicine, Chulalongkorn University approved the study (No.789/2563 Date 30 November 2020).

Conflict of Interest

The authors have no conflicts of interest to declare.

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