RESEARCH ARTICLE

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Impaired Granularity in T cell Subsets but not in B cell Favors the Carcinogenesis of the Breast: A Preliminary Study in Indonesian Women Cohort

Ika Nurlaila[1]*, Wulyo Rajabto[2], Febby Nurdiya Ningsih[1], Erlin Listiyaningsih[3], Udani Sari Ratih[1]

Abstract

Objective: The progress made in cancer immunology has led to the development of innovative therapeutic strategies. However, despite these advances, the superficial characteristics of immune cells have been frequently overlooked: This oversight may be attributed to a limited understanding of the intricate relationships between immune cells and their microenvironment. This study seeks to address this limitation by comprehensively examining cell size and granularity in breast cancer (BC) patients and healthy donors (HD). **Methods:** Peripheral blood mononuclear cell (PBMC) samples were isolated from BC patients and HD. We examined the size (FSC-A%rCV) and granularity (SSC-A%rCV) of immune cell subsets in both patient groups and HD using flowcytometry. **Results:** Despite the absence of statistically significant variations in cell size between BC and HD, visual examination reveals noticeable discrepancies. There is a substantial decrease in granularity in CD8 and CD4 T-cell populations in BC compared to HD which is not observed in B cells. **Conclusion:** Our analysis shows that while the size of immune cells may not be significantly altered in breast cancer patients compared to healthy donors, a closer examination of cell granularity reveals a distinct pattern. Specifically, the T-cell populations, including CD8 and CD4 cells, exhibit a substantial decrease in granularity in BC compared to HD. In contrast, B cells remain unaffected, suggesting that the granularity of T cells is uniquely susceptible to perturbations in breast cancer. This observation highlights the importance of considering cell granularity as a critical aspect of immune cell function, particularly in the context of cancer development.

Keywords: Breast cancer- granularity- cell size- immunosurveillance- B cell- T cell

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Introduction

It is widely accepted that the immune system plays a crucial role in carcinogenesis, a complex process that arises from the cumulative impact of multiple cellular insults at various biological levels which ultimately lead to a transformative cascade of events [1]. The intricate interplay between immune cells and cancer cells gives rise to a complex interdependent relationship, wherein immune cell populations adapt and evolve in tandem with their tumour counterparts. This dual sides-evolvement enables cancer cells to thrive, culminating in the preparation of a premetastatic niche that facilitates tumour expansion and dissemination [2].

In this study, we made an effort to elucidate the complexities of breast cancer (BC) in Indonesian women. BC is the most prevalent type of solid cancer in the country with a staggering 65,858 new cases reported in 2020 alone [3]. The concept of "form follows function" suggests that the physical structure of a cell can reveal its purpose. In this context, the granularity of a lymphocyte serves as a reliable indicator of its state and function [4]. In addition, the size of the immune cells, we hypothesized, may exhibit altered sizes as they undergo changes to accommodate the incorporation of granules carrying antitumor properties. Given the scarcity of studies on cell size and granularity in the context of carcinogenesis in BC, we chose to investigate both parameters as key variables in our study. Despite the relative dearth of research on this matter, we learned that the dynamic interplay between cancer cells and immune cells is crucial to understanding the outcome of their coexistence. Therefore, in the present study we aimed to evaluate these superficial changes in sizes and granularity of the two key components in adaptive immunity: T cells that represent cellular immunity, and B cells that represent humoral immunity

*[1]Research Center for Vaccine and Drugs, The National Research and Innovation Agency (BRIN), South Tangerang 15310, Indonesia. [2]Hematology-Medical Oncology, Department of Internal Medicine, Dr. Cipto Mangunkusumo Hospital/Faculty of Medicine Universitas Indonesia, Jakarta, 10340, Indonesia. [3]Faculty of Medicine, Universitas Muhammadiyah Prof. Dr. HAMKA, Jakarta, 12130, Indonesia. *For Correspondence: ika.nurlaila@brin.go.id*

using flowcytometry.

Materials and Methods

Informed Consent

Our study earned approval from the Health Research Ethics Committee, the National General Hospital Dr. Cipto Mangunkusumo-Faculty of Medicine, University of Indonesia with ethical clearance No. KET-734/UN2.F1/ETIK/PPM.00.02/2023. Prior to blood sampling, written informed consent was obtained from each participant. The study population comprised 18-year-old or older BC patients who were HIV-negative, HBV-negative, and HCV-negative. A matched control group of women without BC and with no history of HIV, HBV, or HCV infection was also recruited. Whole blood samples (12-24 ml) were collected from each participant, using heparinized tubes.

Peripheral blood mononuclear cell (PBMC) isolation

To isolate PBMC, the whole blood was first diluted in 1% antibiotic (Penicillin-Streptomycin, Himedia)-containing RPMI-1640 (Gibco)—next is referred to as wash media— in a final ratio of 1:1. This mix was then overlaid onto Ficoll solution (Ficoll Paque Premium) with a final ratio of 3:1 (diluted blood:Ficoll) prior to gradient centrifugation at a velocity of 450g for 45 minutes without brake. The interphase (buffy layer) was then transferred into a fresh tube using a sterile Pasteur pipette and washed using wash media as much as 5x approximate volume of the transferred buffy layer. This buffy layer was centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded. After the last wash, the pellet was resuspended in the remaining wash media. A minimum of 200,000 cells were then counted using a haemocytometer and were placed into 5 ml FACS tube. The remaining cells were diluted in 10% dimethylsulfoxide (DMSO, Glentham)-containing fetal bovine serum (FBS, Himedia)—referred to as freezing media—and placed into cryovials. The cells that were put into 5 ml FACS tubes were washed again in a 2 ml MACS buffer (PBS pH 7.5 supplemented with 2mM EDTA and 0.5% BSA). The tubes were then centrifuged at 1200 rpm for 5 minutes, RT. The supernatant was discarded. After this step, the cells were stained as detailed in the following sub chapter (Immune cell subset assay).

Immune cell subset assay

After washing with MACS buffer, the cells were resuspended in the remaining buffer then stained with 3 µl of 7-AAD staining solution (BD Biosciences) and incubated on ice for 10-15 mins. Next, each tube was added with antibody mix as listed in Table 1. All the antibodies were purchased from BD Biosciences. The tubes were incubated on ice for 30-45 minutes. Washing with 2ml MACS buffer was again done. Finally, each tube was added with 100 µl MACS buffer and ready to be run immediately on the flowcytometry machine (BD FACS MelodyTM).

Statistics Analysis

To investigate potential disparities between two distinct groups, BC and healthy donors (HD), a one-tailed Mann-Whitney U test was conducted using GraphPad Prism 8.0.2. The statistical analysis was designed with a significance threshold $\alpha = 0.90$ and a confidence interval of $p < 0.01$. As a non-parametric test, it was employed to examine the distributional differences between the two groups. If only the calculated p-value falls below 0.01, the null hypothesis is rejected thus significant difference is observed.

Results

Demographic of the Participants

The demographic profiles of all participants enrolled in this study are displayed in Table 2. The participants' ages were calculated by subtracting their birthdates from the dates on which they provided informed consent and underwent venipuncture. The table provides an overview of the vital statistics for both BC and HD groups. The BC data also includes information on the treatments received by the patients. Our study has ensured that the participants recruited for this research exhibit a homogeneous demographic profile with regard to age as shown in Figure 1. The BC patients had an age range of 38.6 to 56.3 years, whereas the HD's fell within an age range of 29.7 to 53.6 years. To further corroborate

Table 1. The List of Antibodies Mix Used for FACS Analysis

Antibody	Flurochrome	Volume/reaction
CD4	FITC	4 µl
CD8	APC.Cy7	5 µl
CD3	APC	5 µl
CD19	BV510	4 µl
Fc blocking solution		3 µl
Total volume/reaction	21 µl	

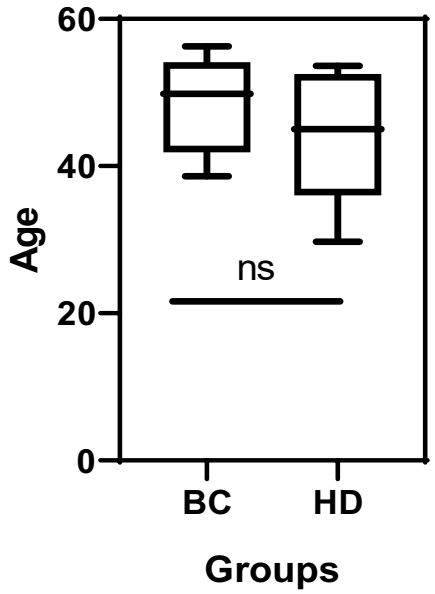

Figure 1. Comparison of Age Distributions between BC and HD Using an Unpaired Two-Tailed t-test ($\alpha = 0.05$)

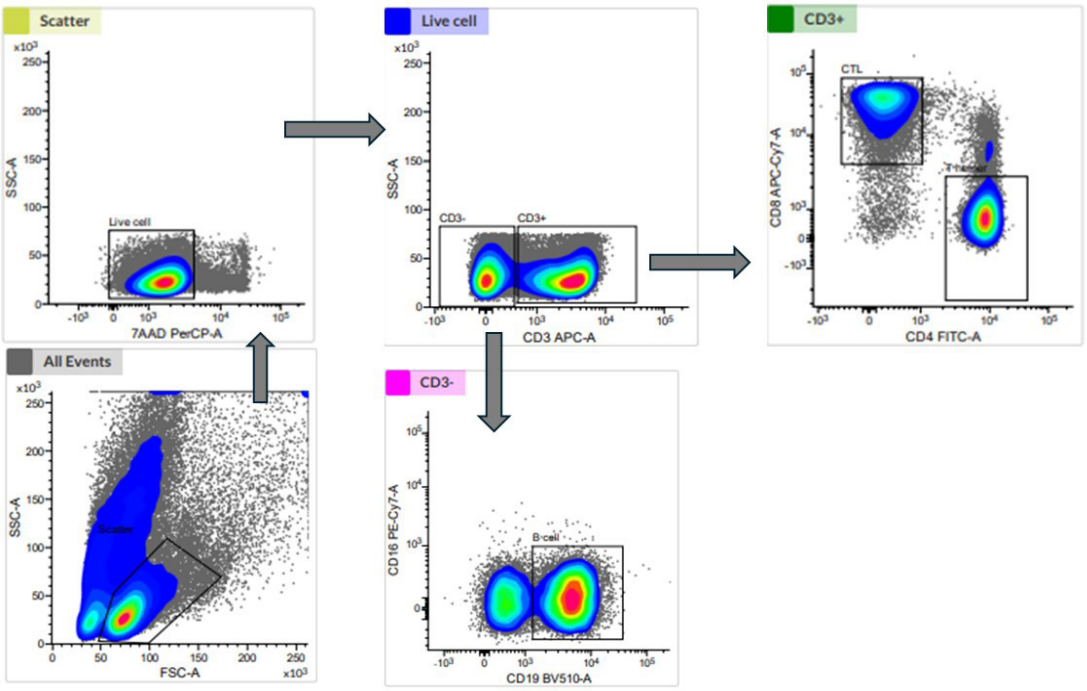

Figure 2. Gating Strategy to Identify and Enumerate the Two Major Immune Cells in Adaptive Immunity being T and B Cell Subsets.

Table 2. Demographic and Clinical Characteristics of Breast Cancer Patients and Healthy Donors

HD	Age	BC	Age	Cancer position	Treatment	
HD-01	41.6	BC-01	51.8	Sinistra and Dextra	Chemotherapy	Mastectomy (surgery)
HD-02	53.6	BC-02	54.4	Sinistra and Dextra	Chemotherapy	Hormonal
HD-03	48.4	BC-03	38.6	Sinistra	Chemotherapy	Chemotherapy
HD-04	35.1	BC-04	53.2	Dextra	Chemotherapy	Mastectomy (surgery)
HD-05	38.8	BC-05	42.3	Sinistra and Dextra	Chemotherapy	Hormonal
HD-06	53.1	BC-06	56.3	Sinistra	Chemotherapy	Radiotherapy
HD-07	29.7	BC-07	47.9	Sinistra	Chemotherapy	Lumpectomy (surgery)
HD-08	50.7	BC-08	41.7	Sinistra	Chemotherapy	Radiotherapy

the lack of significant age-related differences between the two groups, a two-sample t-test was employed to assess whether the means of the two distributions were statistically equivalent. The results of this analysis revealed no significant difference (p value=0.28, significance at α <0.05) in age between the BC patients and the HD, thereby providing evidence that aging does not confound our findings.

Three out of eight (37.5%) show tumors in both breasts. Meanwhile, five patients (62.5%) had unilateral tumors, either on the left or right side. All patients (100%) underwent chemotherapy as their primary treatment. For one individual, chemotherapy was her sole treatment throughout her entire BC therapy journey. The other patients received additional therapies alongside chemotherapy. Two out of eight patients (25%) also received hormonal therapy, while three patients (37.5%) underwent surgical procedures, including lumpectomies or mastectomies. Additionally, one patient (12.5%) included radiotherapy among their treatment options.

Gating Strategy

To start our analysis, we commenced by employing a gating strategy, utilizing the X-axis (FSC-A) and Y-axis (SSC-A) to gate out non-viable cells from further examination. This initial step allowed us to exclude any compromised cells, ensuring the integrity of our subsequent analysis. Next, we utilized a viability dye (7-AAD) to effectively eliminate dead cells from consideration, thereby focusing on live lymphocytes. We then proceeded to dissect these live cells based on the expression of CD3, which serves as a marker for activated T lymphocytes. This permitted us to isolate CD8+ and CD4+ T-cell subsets from the CD3+ population, while the CD3- subset was categorized as non-T cells. Notably, within the CD3- subset, cells expressing CD19 were identified as B cells. Ultimately, our gating strategy yielded distinct populations of CD8+, CD4+ T-cells and B cells, enabling us to precisely define the immune cell composition as shown in Figure 2.

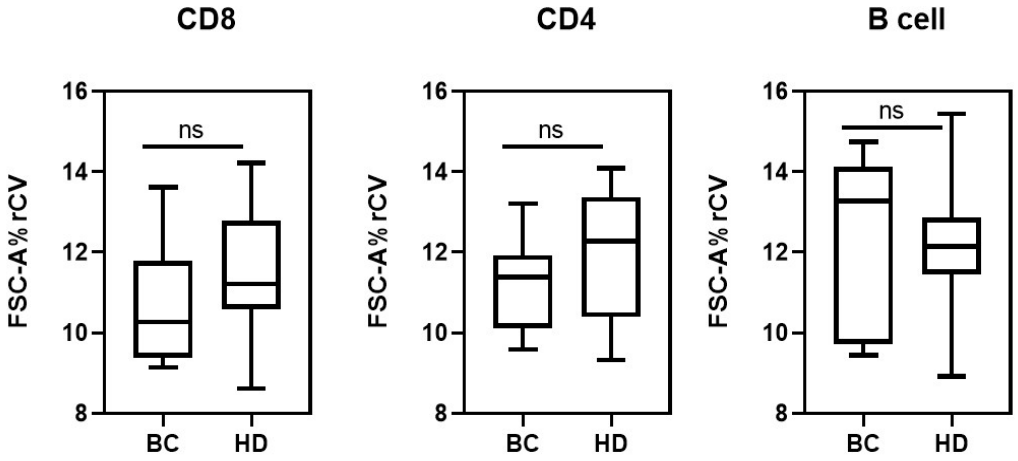

Figure 3. FCS-A%rCV Demonstrates Statistically Insignificant Difference in Cell Size of Each Immune Cell Subset between BC and HD

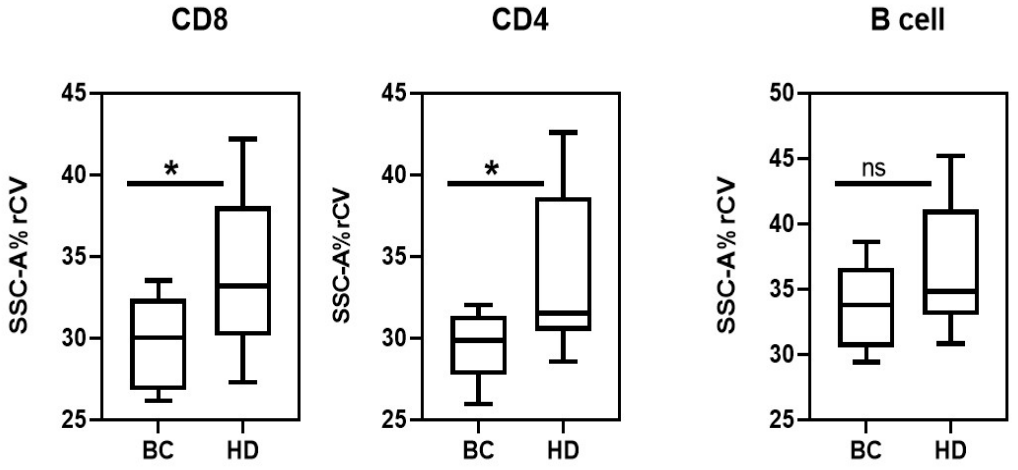

Figure 4. SSC-A%rCV Depicts Significant Reduction in Granularity of Both CTL and T Helper but not B Cell in BC

Immune Cell Subset Disparity

In order to assess both cell size (FSC) and granularity (SSC) of immune cells, we opted for using robust coefficient of variation (A%rCV) values instead of median values. This approach is more suitable as A%rCV is less susceptible to the influence of extreme values, which can significantly affect the median. Moreover, A%rCV is a dimensionless value, allowing it to capture the overall variation among immune cell subsets in a more comprehensive manner than median values. In choosing A%rCV over median values, we aimed to minimize the impact of outliers on our analysis. By using a dimensionless value, we can provide a more nuanced understanding of their characteristics. Notably, our hypotheses regarding cell size parameter were not statistically supported, as evident in Figure 3.

A comparison of the coefficient of variation in forward scatter (FSC-A%rCV) for CD8, CD4, and B cell subsets demonstrates statistically insignificant differences, as evidenced by p-values of 0.1115, 0.2209, and 0.3131, respectively. Although these findings may not be statistically significant, a visual examination of the data still displays observable differences.

Notably, the size of CD8 and CD4 cells tends to be smaller in BC compared to HD. Conversely, the size of B cells in HD is smaller than that in BC. This contrast between the two groups suggests that immune cell subsets may play distinct roles in these distinct pathologies.

Our analysis proceeded to investigate the intracellular realm, focusing on the granularity of each immune cell subset. Within the cytoplasm, minute particles known as granules are present, which can be visualized as distinct, discrete spots when examined under a microscope. Our results demonstrate that, compared to HD, T lymphocytes in BC exhibit a significant granularity reduction (Figure 4).

The analysis of CD8 T cells in patients with breast cancer (BC) and healthy donors (HD) reveals significant differences in median granularity values. Specifically, the granularity values for CD8 T cells in BC patients were 30.03, compared to 33.19 in HD, resulting in a statistically significant difference (p-value = 0.0415, $\alpha < 0.05$ or $\alpha < 0.1$). Similarly, CD4 T cells demonstrated lower granularity in BC compared to HD, with median SSC-A%rCV values of 29.84 and 31.51, respectively (p-value

= 0.0379, $\alpha < 0.05$ or $\alpha < 0.1$). In contrast, B cell subset shows no significant difference in granularity between BC and HD groups, despite a trend towards lower granularity in BC (median values of 33.78 and 34.81, respectively; p-value = 0.3282, $\alpha < 0.05$ or $\alpha < 0.1$).

Discussion

The concept of immunosurveillance where the two arms of immune system do their respective job to assure that foreign matter-expressing abnormally-grown cells are killed is widely acknowledged and supported with strong evidences over the decades. But one might still query why people are still vulnerable to cancer [5]. We hypothesized that BC group showed impaired equilibrium which lead to the weakened anticancer immunity properties against cancer cells. Superficially, this might be represented by the smaller size of each immune cell subset and reduced granularity.

In order to gauge this perturbed equilibrium, we employed flow cytometry. The fundamental principle of flow cytometry is built around the precise analysis of individual cells as they flow through a narrow stream in a single file, intersecting a perpendicular laser beam. When the laser beam encounters a cell, it scatters light in both forward and lateral directions, generating forward-scattered light (FSC) and side-scattered light (SSC), respectively. These signals provide valuable information about cell size and granularity [6, 7].

The cells with higher granularity (SSChigh) display a heightened propensity towards terminal differentiation, indicating that they are poised to execute immediate effector functions, such as cell-mediated cytotoxicity and the production of pro-inflammatory cytokines. In contrast, cells with lower granularity (SSClow) indicate their immature state although they can still respond toward antigen exposure [4].

Our results showed that the size of immune cell subsets, including CD8, CD4, and B cells, did not significantly differ between BC and healthy donors (HD). However, we observed a reduction in granularity of CD8 and CD4 T cells in BC , indicating a potential impairment in their ability to perform effector functions. The granularity of B cells remained unchanged between the two groups. These granules are a hallmark of intracellular structure and play a crucial role in cellular function. Frequently, these granules serve as storage vessels for secreted substances, playing a crucial role in the cell's physiological functions [8, 9].

The activation of T lymphocytes is a complex process involving multiple factors, including specific binding of the T cell receptor-CD3 complex to processed tumor antigen-derived peptides bound to major histocompatibility complex (MHC) class I molecules for CD8 T cells and MHC class II molecules for CD4 T cells, accompanied by costimulatory signals from CD8 and CD28 [10, 11]. Upon activation, the microtubule-organizing center (MTOC) rapidly polarizes the traffic of preformed secretory granules towards the presynaptic membrane. These granules then fuse with the plasma membrane at the immunological synapse, releasing their contents, such as perforin and granzymes, which ultimately lead to tumor elimination. Defects in proteins controlling intracellular trafficking and granule fusion can impair exocytosis, reducing the ability of T lymphocytes to kill target cells [12].

Within the immune system CD8 T cells exhibit a remarkable ability to identify and eliminate transformed cells, in this context is cancer cells. This process of cell elimination occurs at a highly coordinated interface, designated as the immunological synapse, where CTL orchestrate the controlled release of cytotoxic granules, thereby eliminating the targeted cells [8]. In contrast, CD4 T cells, conventionally, have been linked to helper functions. However, pioneering research in the 1970s showed that CD4 T cells can exhibit cytotoxic activity in the context of allograft rejection [13, 14]. A transcriptomic study has identified CD4 T cell clusters displaying cytotoxic phenotypes similar to those of CD8 T cells. Furthermore, it was shown that these cytotoxic CD4 T cells exhibit a direct, contact-dependent, and granzyme-mediated killing activity against tumors, albeit with slower kinetics compared to traditional cytotoxic lymphocytes [15].

Whilst the cellular mediated cancer immunity is driven by the aforementioned CD8 and CD4 T cell subsets, the humoral responses is sculted by B cell subset, a specific type of immune cell that produces antibodies in response to the detection of a particular antigen, in this context is cancer-specific or cancer-associated antigens [16].

The crosstalk between B and T lymphocytes has been recently postulated in the progression of cancer. Some studies showed that the immune response of T cells against adenocarcinoma tumor cells is significantly enhanced in the absence of B cells, whereas the presence of B cells has been suggested to suppress the cytotoxic effects of T lymphocytes on tumor immunity. This complex intercellular communication has significant implications for our understanding of the intricate relationships between immune cells and their role in tumorigenesis [17].

In contrast to the pronounced perturbation in CD4+ and CD8+ T cell granularity, our findings indicate that B cell granularity remains consistent across both groups, implying a relative resilience of this immune subset to the carcinogenic process. This observation can also be viewed as a reflection of the relatively static nature of humoral immunity in response to carcinogenesis, as compared to the dynamic and adaptive responses exhibited by T cells.

The concept of "form follows function" posits that the morphology of a cell is influenced by the specific functions it performs [18]. As our analysis showed a significant difference in granularity between CD4 and CD8+ T cells in breast cancer (BC) patients compared to healthy donors (HD), we initially thought that this difference would be accompanied by perturbation in cell size, particularly among CD4 and CD8 T cells, as well as B cells, given the altered functional requirements in cancer contexts. However, our analysis does not support this hypothesis as we figured out that cell sizes of CD4, CD8, and B cells remain unchanged between BC and HD patients, implying that functional differences are established before any observable changes in cell morphology occur.

Notwithstanding our findings, a more comprehensive

understanding of metabolic granularity patterns in T and B cells is essential to elucidate the mechanisms underlying the optimal state of granularity, which is pivotal not only for cancer prevention but also for the development of effective curative strategies. The intricate interplay between metabolic pathways and immune cell function is a crucial aspect of immune homeostasis, and uncovering the nuances of this relationship will be instrumental in designing novel therapeutic approaches that capitalize on the immune system's capacity for cancer surveillance and elimination.

We, next, thought that, as we also catalouged the treatments the patients have received or undergone and this variable is absolutely what distinguished them from HD who never encounter anticancer modalities. We figured out that the top three most reduced granularity are seen in BC01, BC04 and BC07 with SSC-A%rCV 26.16%, 26,19% and 28.58%, respectively. What differed them from other patients in the context of treatment are that they underwent surgery (mastectomy or lumpectomy) during their therapy courses, not only chemotherapy. The removal of these tissues are abductively thought to play a role in suppressing the granularity but this warrants more investigation. Learned that the treatments were designed to eliminate the cancers cells, it is abductively expected that BC groups showed a higher intensity in granularity as a study by Kim, Lee and Kim reported in 2009. They investigated the morphological and cellular responses of Ba/F3 cells to doxorubicin (DOX) treatment. DOX is a widely employed chemotherapeutic agent [19]. Following 24-hour exposure to 100 nmol/l DOX, flow cytometry revealed a significant increase in granularity but no change in cell size.

However, the profile of increased granularity in patients who were treated with chemotherapy was not observable in our study. Despite receiving chemotherapy, the BC group did not exhibit enhanced granularity, indicating that the cancer progression outcompeted the treatment. This lack of enhancement suggests that the therapy failed to strengthen the quality and quantity of granules in immune cells, which are typically tasked with countering cancer progression. Instead, the reduction in granules favored the advancement of cancer, underscoring the treatment's limited effectiveness

Overall, these findings suggest that the adaptive immune arm is impaired in BC patients, which may contribute to disease progression. The reduced granularity of both T cell subsets may hinder their ability to eliminate cancer cells effectively. Furthermore, defects in proteins controlling intracellular trafficking and granule fusion can impair exocytosis, reducing the ability of T lymphocytes to kill target cells. In contrast, HD exhibits a robust and effective immune response, enabling them to overcome potentially deleterious abnormal growth. The ability of HD to mount an enhanced immune response highlights the complex interplay between genetic and environmental influences that shape an individual's susceptibility to cancer.

Limitations

Our findings are tempered by several limitations. The relatively small sample size of BC patients and HD may not accurately represent the broader population. Furthermore, the study's focus on immune cell granularity in BC patients and HD omits the examination of other cancer types and healthy individuals, thereby limiting the scope and potential for generalizability. Additionally, the reliance on a specific type of flow cytometry analysis may overlook confounding variables or aspects of immune cell function, while the analysis of only two T cell subsets may overlook other crucial immune players.

Moreover, the analysis did not consider potential confounding factors such as age, sex, or treatment history that may influence the results. The use of median values instead of mean values to describe the data may also be susceptible to being influenced by extreme values. Finally, the study's findings may not be generalizable to other types of cancer or populations with different genetic backgrounds. Despite these limitations, the study provides valuable insights into the relationship between immune cell granularity and breast cancer susceptibility, and future studies should aim to address these limitations by expanding the sample size, investigating a broader range of cancer types and populations, and incorporating additional immune cell subsets and potential confounding variables into the analysis.

Author Contribution Statement

IN conceived the central research hypothesis, developed the theoretical foundation, conducted flow cytometry experiments, and drafted and edited the manuscript. WR were accountable for securing informed consent from patients, diagnosing eligibility, and verifying that patients meet the inclusion criteria. FNN performed sample preparation, analysis and formatted the manuscript. EL managed ethical clearance for the study and sponsored a comprehensive training on flow cytometry (BD Melody) techniques prior to the commencement of the experiment. USR performed venipuncture for healthy donors.

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General

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Funding Statement

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Approval

This study was approved by the Health Research Ethics Committee at the National General Hospital Dr. Cipto Mangunkusumo-Faculty of Medicine, University of Indonesia, with ethical clearance number KET-734/UN2.F1/ETIK/PPM.00.02/2023

Conflict of Interest

The authors declare that there is no conflict of interest throughout the process of this submission.

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