Systemic immune dynamics in cancer

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CHAPTER 3

Comprehensive Analysis of the Systemic Immune Landscape Across Breast Cancer Subtypes and Disease Stages

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Abstract

Breast cancer is a systemic disease, yet the impact of tumor molecular subtype and disease stage on the systemic immune landscape, remains poorly understood. In this study, we comprehensively analyzed the systemic immune landscape in a large cohort of breast cancer patients, encompassing all molecular subtypes and disease stages, alongside a control group of healthy donors. Using multi-parameter flow cytometry, we assessed the abundance, phenotype, and activation status of diverse innate and adaptive immune cell populations across peripheral blood samples from 355 breast cancer patients and 65 healthy donors. Analyzing all blood samples immediately after collection enabled analysis of often overlooked, but highly abundant granulocyte populations, including neutrophils and eosinophils. Our findings reveal that early-stage breast cancer patients exhibit increased cell counts of neutrophils, classical monocytes, and CD1c$^-$ DCs compared to healthy donors. In late-stage breast cancer patients, we observed elevated counts of neutrophils, classical monocytes, and non-classical monocytes compared to healthy donors. Additionally, reductions were observed in memory B cells, plasmablast-like cells, conventional CD4 T cells, and regulatory T cells. Notably, distinct molecular subtypes were associated with specific changes in the immune landscape, with the most significant changes observed in the triple-negative subtype. In conclusion, our data indicate that the systemic immune landscape undergoes more profound alterations in metastatic breast cancer than non-metastatic cases, with disease stage exerting a greater influence on systemic immune composition than tumor subtype.

Introduction

Breast cancer can be considered a systemic disease, but the influence of breast cancer on the systemic immune landscape, especially in relation to tumor molecular subtype and disease stage, is not well understood. Breast cancer accounts for nearly a quarter of all cancer diagnoses and necessitates complex treatment strategies, which frequently result in side effects that cause physical and emotional suffering for those who are affected and their loved ones[1]. Breast cancer is classified into three main subtypes, based on hormone receptor (HR) and human epidermal growth factor receptor 2 (HER2) expression: HR+ tumors (~70%), HER2+ tumors (15-20%), and triple-negative breast cancer (TNBC, ~15%)[2]. Each subtype exhibits distinct molecular signatures and clinical behaviors[3]. Despite advancements in treatment tailored to molecular subtypes and other clinical parameters, breast cancer continues to be the leading cause of cancer-related mortality among women worldwide[1], underscoring the urgent need for innovative therapeutic approaches.

Although immune checkpoint inhibitors (ICI) have transformed the treatment of various cancer types, their efficacy in breast cancer has been relatively modest. TNBC is considered the most immunogenic subtype of breast cancer, characterized by higher levels of tumor infiltrating lymphocytes (TILs), a higher tumor mutational burden, and increased expression of programmed death-ligand 1 (PD-L1) compared to the other breast cancer subtypes[4]. However, even in TNBC, only a minority of patients benefits from current immunotherapeutic strategies. This limited response to ICI can be partly attributed to the inherently low immunogenicity of many breast tumors. In addition, a significant contributing factor is tumor-associated immune suppression, which enables cancer cells to evade local immune responses[5-10]. Tumor-associated immune suppression often extends beyond the tumor microenvironment (TME)[11-13], leading to an impact on the systemic immune system of the host. This systemic effect can manifest as altered immune cell populations and functions throughout the body, weakening overall immune defense and contributing to disease progression. Most studies have highlighted the impact of breast tumor subtypes on the local immune microenvironment, but our understanding of the systemic immune landscape across different molecular subtypes and disease stages remains limited. While some studies have provided valuable insights into the impact of cancer on peripheral immune cells[14,15], several questions remain open for exploration. For instance, previous studies rely on PBMCs, excluding granulocytes and thereby omitting a significant portion of myeloid cells. The complex interplay between tumor stage, molecular subtypes, and systemic immune alterations remains poorly understood, yet is of critical importance for guiding the development of novel immunotherapeutic approaches tailored to individual patients.

Our goal is to study how tumor stage and molecular subtype impact the systemic immune landscape in patients with breast cancer. Therefore, we conducted a comprehensive characterization of the circulating immune landscape in a large cohort of breast cancer patients spanning different molecular subtypes and disease stages, alongside a matched healthy donor (HD) control group. Employing multi-parameter flow cytometry analysis, we assessed the abundance, phenotype, and activation status of various innate and adaptive immune cell populations from over 400 fresh peripheral blood samples. This enabled us to generate detailed quantitative and phenotypic data on circulating granulocyte subsets, dendritic cells (DCs), monocytes, T cells, B cells, and natural killer (NK) cells, shedding light on the intricate interplay between breast cancer and systemic immune profile. This dataset is unique because of its large, well-defined patient cohorts, the inclusion of age- and BMI-matched healthy controls, and the incorporation of neutrophils, eosinophils, and basophils on this large scale, enabling a comprehensive and integrative approach to analysis.

We show that changes in the systemic immune landscape are most pronounced in patients with late-stage breast cancer and characterized by a general increase in the myeloid lineage and a decrease in the lymphoid lineage, especially in the metastatic setting, indicating that disease stage is a critical factor influencing the immunological profile of breast cancer patients. Furthermore, specific molecular subtypes notably induce distinct alterations in

the immune landscape of breast cancer patients. Our findings suggest that the most significant differences in the systemic immune landscape between the three subtypes and HDs, are observed in the TNBC subtype. These data provide a valuable resource on the circulatory immune landscape of breast cancer patients compared to HDs, informing future pre-clinical and clinical research and paving the way for innovative, stage- and subtype-specific immunomodulatory treatment approaches.

Material and Methods

Human blood samples

Fresh blood samples from 53 healthy women (healthy donors, HD) were obtained after approval by the local medical ethical committee (NCT03819829). Additionally, fresh blood samples from 12 healthy women were obtained anonymously from the Dutch national blood transfusion service (Sanquin Blood supply, Amsterdam, The Netherlands). In our cohort of patients with breast cancer, blood samples were obtained from patients enrolled in either a clinical trial or biobank protocol, after approval by the local medical ethical committee and/or institutional review board of the Netherlands Cancer Institute. 185 patients were enrolled in a biobanking protocol of the Netherlands Cancer Institute (CFMPB450); 59 patients were included in the BELLINI trial[16] (NCT03815890); 91 patients were included in the Triple B trial[17] (NCT01898117); 10 patients were included in the MIMOSA trial[18] (NCT04307329). Where blood was obtained in the context of a clinical trial, only baseline blood samples were included in the analysis for this study. Basic clinical parameters were retrieved from the electronic patient records by qualified medical staff.

We included 121 patients with HR+ breast cancer (ER >10%, PR+/- and HER2 negative), of which 33 had stage I disease, 53 had stage II, 15 stage III and 20 patients had stage IV disease. Furthermore, we included 67 patients with HER2+ breast cancer (either score 3 for HER2 using immunohistochemistry (IHC) or positive at in situ hybridization [CISH or FISH]) in case of score 2 on IHC) were included, of which 16 had stage I disease, 17 stage II, 17 stage III and 17 patients had stage IV disease. Additionally, we included 167 patients with TNBC (histologically confirmed ER < 10% of positive tumor cells using IHC; HER2: either score 0 or 1 for HER2 at IHC with no amplification detected by in situ hybridization [CISH or FISH] in case of score 2 on IHC) of which 17 had stage I disease, 40 had stage II, 17 had stage III and 93 patients had stage IV disease (Figure 1a).

In this study, all patients with early-stage disease (stage I-III) were treatment naïve at the time of blood donation. In the late-stage disease (stage IV) setting, blood from patients with mTNBC was taken before any treatment for metastatic disease. Patients with HR+ tumors and HER2+ tumors did receive prior treatment for metastatic disease (Supplementary Table 1). For the treated patients from all subtypes, a washout period of at least 3 weeks was maintained between the last drug administration and the blood draw.

All study protocols were conducted in accordance with the ICH Harmonised Tripartite Guideline for Good Clinical Practice and the principles of the Declaration of Helsinki. All patients and HDs provided written informed consent before enrolment.

Flow cytometry

Blood samples were processed and analyzed within 24 hours after blood draw. All samples were processed uniformly, by the same team and within the same laboratory. Peripheral blood was collected in EDTA vacutainers (BD) and subjected to red blood cell lysis (lysis buffer: dH2O, NH4Cl, NaHCCO3, EDTA). Cells were resuspended in PBS containing 0.5% BSA and 2mM EDTA and counted using the NucleoCounter NC-200 automated cell counter (Chemometec). To obtain white blood cell (WBC) counts per mL of blood, the total amount of post lysis cells was divided by the volume (mL) of blood obtained from the patient (~10 mL).

For the labeling of surface antigens, cells underwent an initial incubation with human FcR Blocking Reagent (diluted 1:100 Miltenyi) for 15 minutes at 4°C, followed by a 30-minute incubation with fluorochrome-conjugated antibodies at 4°C, shielded from light. For intracellular staining, cells were fixed in Fixation/Permeabilization solution 1X (Foxp3/Transcription Factor Staining Buffer Set, eBioscience) at 4°C for 30 minutes, then stained with fluorochrome-conjugated antibodies in Permeabilization buffer 1X (eBioscience) for 30 minutes at room temperature. Viability was determined by staining with either 7AAD staining solution (diluted 1:10; eBioscience) or Zombie Red Fixable Viability Kit (diluted 1:800, BioLegend).

Data acquisition of all samples was performed on the same LSRII SORP flow cytometer (BD Biosciences) operated with Diva software. To make the performance of this machine as constant over time as possible, CS&T beads (BD) were used to optimize general performance and Sphero 8 peaks Rainbow Calibration particles (BD) were used to adjust PMT voltages if necessary. Additionally, single stained compensation controls are taken along for each experiment. Flow data analysis was conducted using FlowJo software (version 10). Flow cytometry antibody details are provided in Supplementary Table 2 and gating strategies are illustrated in Supplementary Figures 1a (Myeloid panel gating), 1b (B and NK cell panel gating), and 1c (T cell panel gating).

Data analysis and statistics

GraphPad Prism (version 10.1.2) software was used for statistical analysis and graphing of the flow cytometry data. Kruskal-Wallis test was applied when comparing multiple groups, followed by Dunn's test to obtain adjusted p-values corrected for the number of groups in the graph (not the number of immune cell populations). PCAs and heatmaps were generated using Qlucore software (version 3.8). Missing values were imputed by mean values from

the sample group. Correlation between neutrophil counts and time of blood draw was performed in R (version 4.3.2) using linear modeling function. Corrected p-values <0.05 were considered significant and are depicted in the graphs using asterixes: * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

Results

Breast cancer alters the systemic immune landscape

To gain insights into the impact of breast cancer on the circulatory immune compartment at early or late disease stages, we conducted high-dimensional flow cytometry on 420 fresh peripheral blood samples. We developed an analysis pipeline specifically tailored for fresh blood samples[19]. This pipeline employs a panel of 50 antibodies distributed across a myeloid panel, a B- and NK cell panel, and a T cell panel (Supplementary Figure 1a-c). This robust approach enables a comprehensive analysis of the systemic immune landscape, including granulocytes, which are typically lost in standard peripheral blood mononuclear cell (PBMC)-based analyses. We profiled samples of patients without distant metastases (stage I-III, referred to as early-stage, n=225) and patients with distant metastases (stage IV, referred to as late-stage, n=130) (Figure 1a). From the patients with early-stage breast cancer, 101 patients had HR+ disease, 50 patients had HER2+ disease and 74 patients had TNBC (Figure 1a). From the patients with late-stage breast cancer, 20 patients had HR+ disease, 17 patients had HER2+ disease and 93 patients had TNBC (Figure 1a). As a control group, we profiled age-, sex- and BMI-matched healthy donors (HDs, n=65) (Figure 1a). Age and BMI of breast cancer patients and HDs are visualized in Supplementary Figure 2 a, b. Given that neutrophil release from the bone marrow follows a circadian rhythm[20], we tested for correlations between neutrophil counts and time of blood draw using a linear model. No statistically significant correlations were found, except in the early-stage TNBC group, where a weak correlation was observed (r=0.0999, Supplementary Figure 2c). The very low rho-value (<10%) suggests minimal variance explained by blood draw time, so we chose not to adjust for it in our dataset.

To explore the flow cytometry data of the three antibody panels in an unbiased manner, we performed a principal component analysis (PCA). By taking the first three principal components into account, we could explain 77% of the variance in the data. When plotting these three principal components, we observed that the HDs cluster away from all breast cancer groups, and that the early-stage breast cancer groups clustered away from the late-stage breast cancer groups (Figure 1b). Moreover, disease stage seemed to have a dominant impact on the systemic immune landscape over tumor subtype (Figure 1c). Hierarchical clustering of 18 major immune populations analysed, confirmed our PCA analysis with HDs blood profile separating from breast cancer patient blood profiles (Figure 1d).

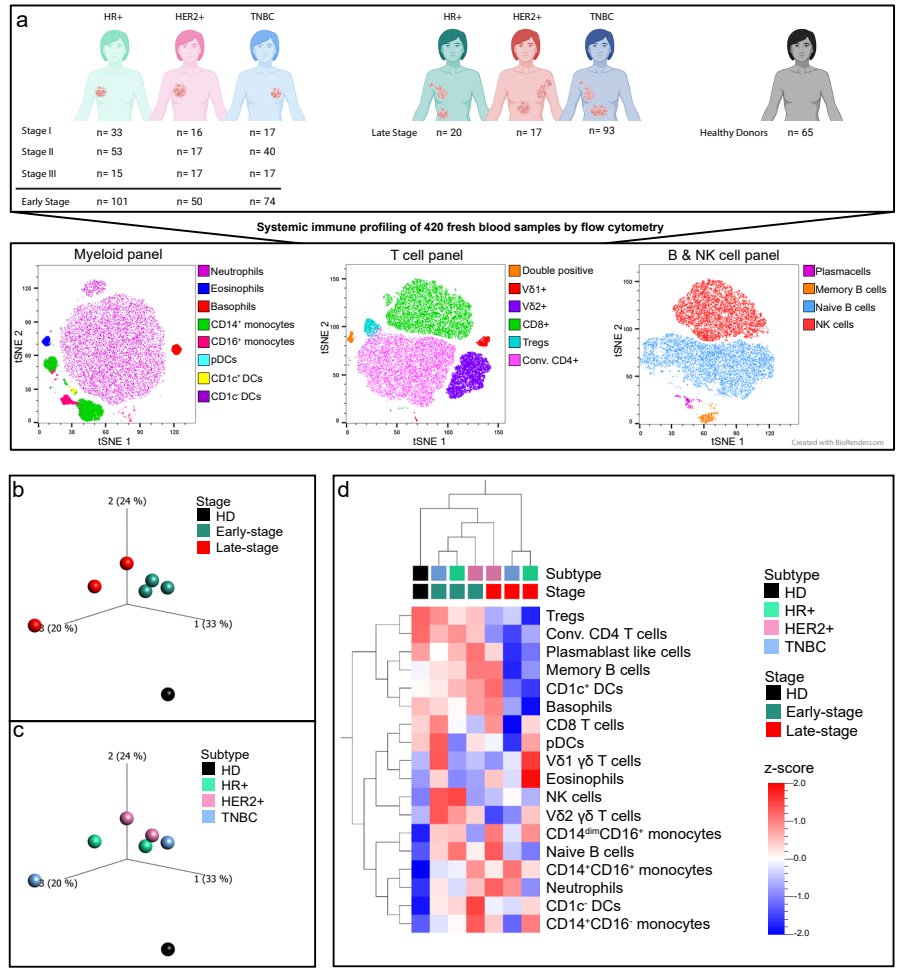


Figure 1. Breast cancer alters the systemic immune landscape. (a) *Graphical summary of included human blood samples and the systemic immune cell populations that were assessed immediately after blood collection using flow cytometry.* ***(b, c)*** *Principal component analysis was conducted on the Log_2-transformed median cell counts per mL of blood from major immune cell populations (see d), measured by flow cytometry in fresh blood samples. The results were colored by disease stage discriminating patients with early-stage breast cancer (n=225), late-stage breast cancer (n=130), and healthy donors (HD) (n=65)* ***(b)****, and by tumor subtype in discriminating between patients with a HR+ tumor (n=121), a HER2+ tumor (n=67) or a triple negative tumor (n=167), and healthy donors (n=65)* ***(c)****.* ***(d)*** *Heatmap based on the Log_2-transformed median cell counts per mL blood, visualizing the major immune cell populations, as assessed by flow cytometry in fresh blood samples from patients with early-stage breast cancer, late-stage breast cancer, across different breast cancer subtypes, and healthy donors. Hierarchical clustering was performed on the immune cell populations and on tumor subtype and disease stage. The color scale represents row Z-scores, ranging from -2 to 2.*

Breast cancer associated alterations to the systemic immune landscape are disease stage dependent

Next, we investigated which immune cell populations are driving the clustering patterns (Figure 1b, d) and how disease stage is impacting the circulating immune composition. We found both neutrophils and classical monocytes to be significantly increased in patients with early- and late-stage breast cancer compared to HDs (Figure 2a, b). Additionally, we observed a statistically significant increase in the number of circulating non-classical monocytes in patients with late-stage breast cancer compared to HDs (Figure 2b). Though no significant difference was observed between patients with early-stage and patients with late-stage breast cancer when all subtypes are grouped together in neutrophil and (non-) classical monocyte counts, we did observe an increasing trend, suggesting that neutrophil and (non-)classical monocyte numbers are being increasingly dysregulated as disease progresses (Figure 2a, b). Furthermore, we found CD1c$^+$ DCs to be reduced in the late-stage group compared to the early-stage group and an increase in CD1c$^-$ DCs in the early-stage group compared to the HDs (Figure 2c).

Within the circulating lymphoid compartment we found a decrease in memory B cells and plasmablast-like cells in patients with late-stage disease compared to HDs and patients with early-stage disease (Figure 2d). Similarly, we observed that the cell counts of CD8+ T cells, conventional CD4+ T cells, Tregs and Vδ2 γδ-T cells were reduced in patients with late-stage disease when compared to patients with early-stage disease (Figure 2e). Additionally, conventional CD4+ T cell and Treg counts were decreased in patients with late-stage disease when compared to HDs (Figure 2e). Together these data indicate that breast cancer impacts the systemic immune landscape in a disease stage dependent manner.

Systemic immune landscape of healthy donors and patients with early-stage breast cancer across different molecular subtypes

Our finding that disease stage is associated with multiple differences in the systemic immune landscape (Figure 2) raises the question of whether these alterations differ per breast cancer subtype. Therefore, we first sought to explore the influence of molecular subtype within the patients with early-stage disease and HDs. We observed that the increase in neutrophils, classical monocytes and CD1c$^-$ DCs is restricted to patients with early-stage HR+ tumors (Figure 3a-c). Conversely, the increase in non-classical monocytes was only found to be statistically significant in patients with early-stage TNBC compared to HDs (Figure 3b).

When evaluating the influence of molecular subtype in patients with early-stage breast on the circulating lymphoid compartment, we found that Vδ2 γδ-T cell counts were statistically significantly elevated in patients with TNBC compared to HDs (Figure 3e). Furthermore, we observed a reduced plasmablast-like cell count among patients with TNBC compared to patients with HR+ tumors (Figure 3d). Conversely, we found NK cell counts to

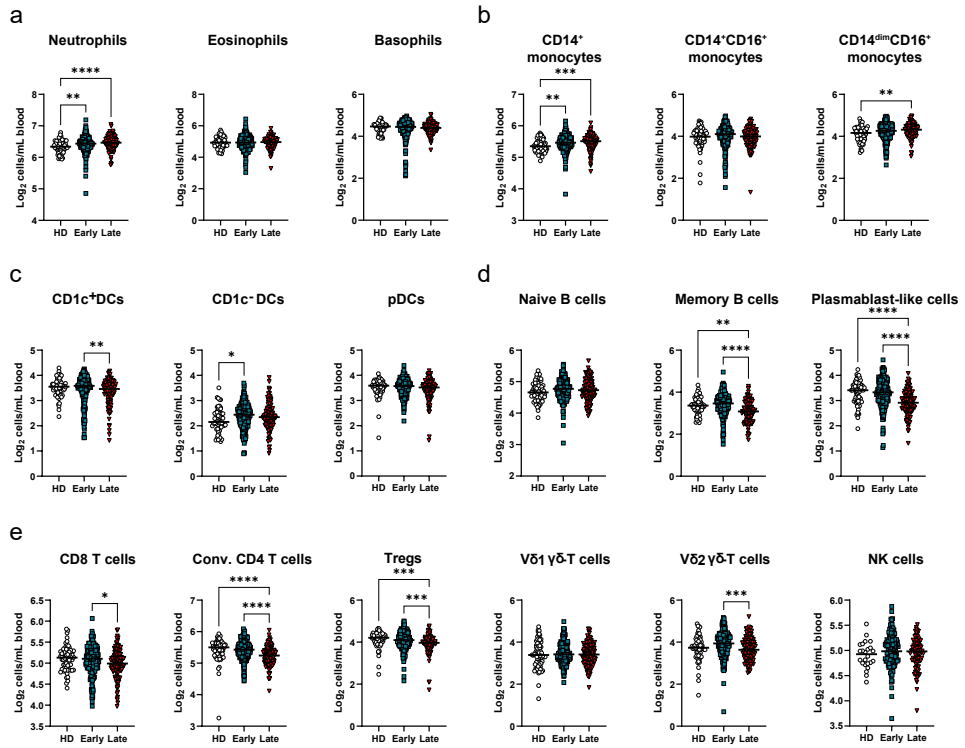


Figure 2. Breast cancer stage impacts the circulating immune composition. *Log$_2$-transformed cell counts per mL blood of major systemic immune cell populations measured by flow cytometry in patients with stage I-III breast cancer (Early) (n=225), stage IV breast cancer (Late) (n=130), and healthy donors (HD) (n=65), visualizing **(a)** granulocytes, **(b)** monocyte populations, **(c)** DC subsets, **(d)** B cell subpopulations and **(e)** different conventional and unconventional T cell subpopulations and NK cells. P-values for **(a-e)** were computed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test.*

be increased in patients with TNBC compared to patients with HR+ tumors (Figure 3e). No other statistically significant differences between the molecular subtypes were observed, suggesting that disease stage had a stronger influence on the systemic immune landscape (Figure 2) than the molecular subtype in patients with early-stage breast cancer (Figure 3).

Systemic immune landscape of healthy donors and patients with late-stage breast cancer across different molecular subtypes

Next, we investigated which differences in the systemic immune landscape of advanced breast cancer patients were associated with a certain molecular subtype. To achieve this, we took the immune profiles of patients with late-stage breast cancer and compared the

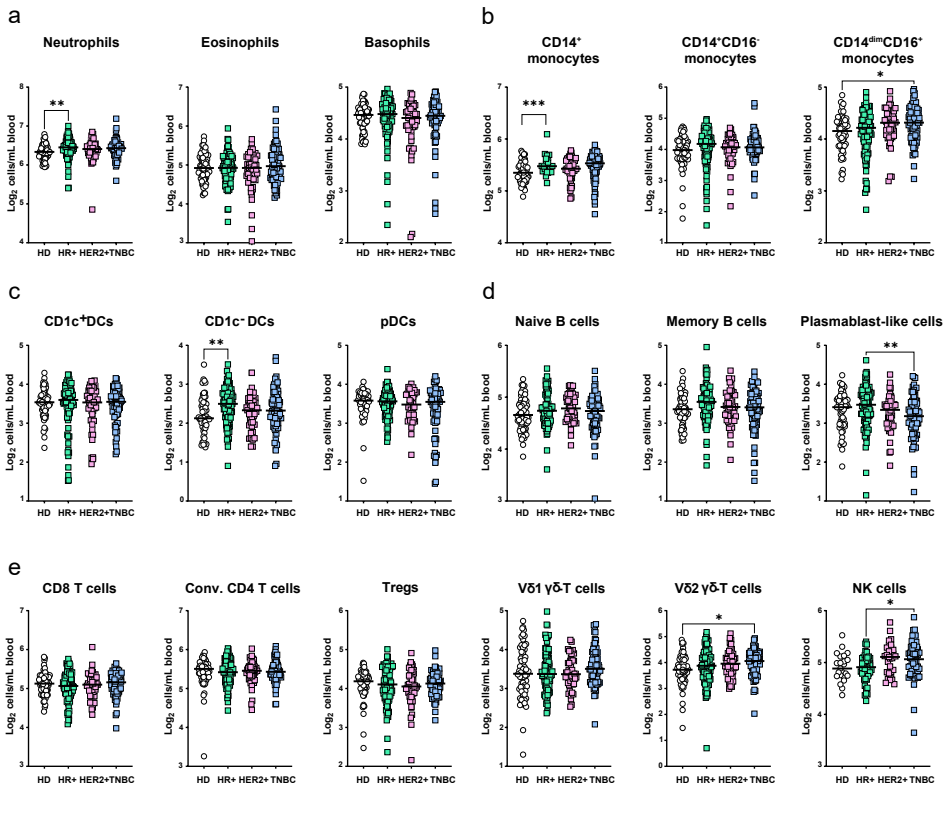


Figure 3. Systemic immune landscape of patients with early-stage breast cancer across different molecular subtypes. *Log_2-transformed cell counts per mL blood of major systemic immune cell populations measured by flow cytometry in patients with early-stage breast cancer with a HR+ tumor (n=101), a HER2+ tumor (n=50) or a triple negative tumor (n=74), and healthy donors (n=65), visualizing **(a)** granulocytes, **(b)** monocyte populations, **(c)** DC subsets, **(d)** B cell subpopulations and **(e)** different conventional and unconventional T cell subpopulations and NK cells. Adjusted p-values for **(a-e)** were computed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test.*

three molecular subtypes to each other and to the immune profiles of HDs. When subdividing late-stage patients based on the molecular subtype of their tumor, we observed an imbalance in the n-number of patients per group (Figure 1a). However, our results confirm that the systemic increase in neutrophils observed in patients with late-stage disease compared to HDs (Figure 2) is present in both patients with HR+ breast cancer and those with TNBC, while this was not observed for HER2+ stage 4 disease (Figure 4a). The systemic increase in classical monocytes in late-stage patients compared to HDs (Figure 2) was predominantly driven by patients with TNBC (Figure 4b). In contrast, the observed increase

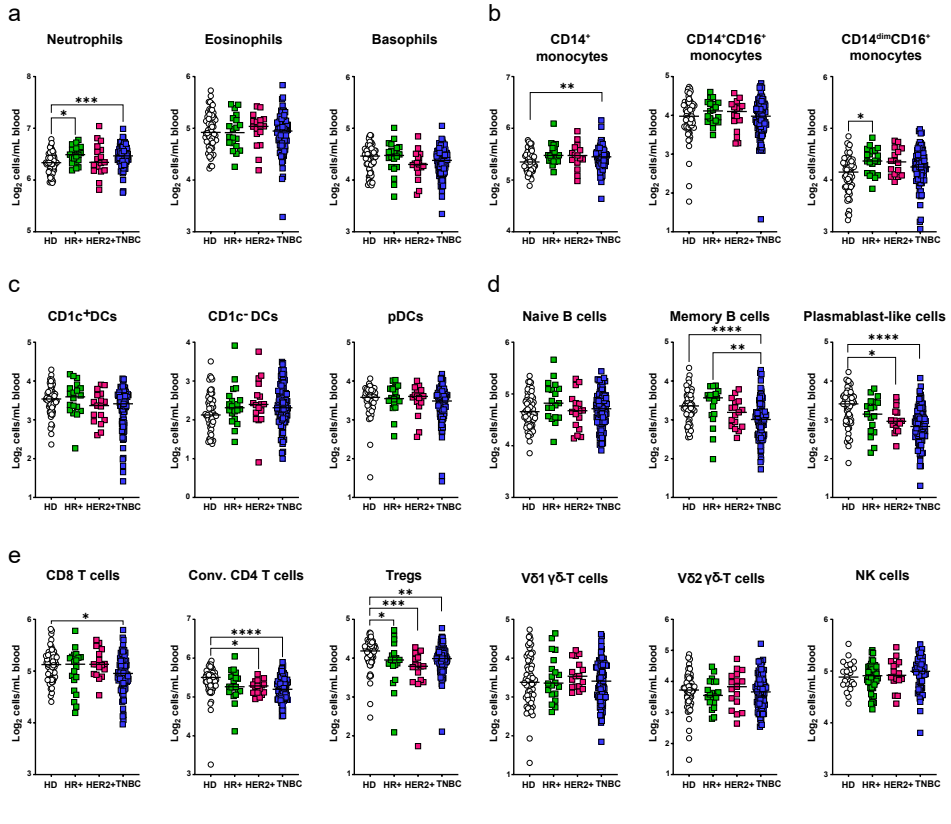


Figure 4. Systemic immune landscape of patients with late-stage breast cancer across different molecular subtypes. *Log_2-transformed cell counts per mL blood of major systemic immune cell populations measured by flow cytometry in patients with late-stage breast cancer with a HR+ tumor (n=20), a HER2+ tumor (n=17) or a triple negative tumor (n=93), and healthy donors (n=65), visualizing **(a)** granulocytes, **(b)** monocyte populations, **(c)** DC subsets, **(d)** B cell subpopulations and **(e)** different conventional and unconventional T cell subpopulations and NK cells. Adjusted p-values for **(a-e)** were computed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test.*

in non-classical monocyte counts in patients with late-stage breast cancer compared to HDs (Figure 2) was found to be attributed to patients with a HR+ tumor (Figure 4b). These data indicate that the alterations detected in the circulating immune compartment exhibit varying degrees of penetration across the three molecular subtypes. Among the myeloid cell populations, eosinophils, basophils, $CD14^+CD16^+$ monocytes and the DC subsets remained unaffected in abundance across the different breast cancer subtypes (Figure 4a-c).

Within the lymphoid compartment, plasmablast-like cells were profoundly reduced in patients with mTNBC and HER2+ tumors compared to HDs (Figure 4d). Similarly, circulating

memory B cells were also found to be reduced in patients with mTNBC tumors. These data suggest that late-stage TNBC tumors, and to a lesser extent late-stage HER2+ tumors have a profound effect on the B cell compartment. When comparing the T cell subset abundances across molecular subtypes and HDs, we observed that patients with late-stage TNBC had reduced counts of CD8 T cells, conventional CD4 T cells and Tregs, patients with HER2+ tumors had reduced counts of conventional CD4 T cells and Tregs, and patients with HR+ tumors had reduced counts of Tregs (Figure 4e). These data indicate that circulating T cell abundances are most affected in patients with TNBC. Beyond the intrinsic effects of this tumor subtype on the systemic immune environment, this observation may also be attributable to a treatment history in the (neo)adjuvant setting with chemotherapeutic agents by a substantial proportion of late-stage TNBC patients. The observation that CD8 T cell counts are reduced in patients with TNBC compared to HDs was previously masked by other molecular subtypes, that did not show this decrease compared to HDs (Figure 4e). Apart from memory B cells (Figure 4d), no significant differences between the molecular subtypes were observed, suggesting once more that disease stage (Figure 2) has a stronger influence on circulating immune composition than the molecular subtype (Figure 4).

Breast cancer influences T cell phenotype and cytokine production in a tumor subtype- and disease stage-specific manner.

Given the observed decrease in total counts of CD8+, conventional CD4+, and regulatory T cells in patients with metastatic disease compared to those with non-metastatic disease — and for conventional CD4+ and regulatory T cells also compared to HDs— (Figure 2), we sought to investigate the phenotype and differentiation state of circulating T lymphocytes by flow cytometry (Supplementary Figure 1c) in relation to tumor subtype and disease stage. We observed a lower fraction of PD-1^{+} CD8+ T cells and PD-1^{+} conventional CD4+ T cells in patients with TNBC compared to HDs (Figure 5a), suggestive of altered systemic T cell activation. Furthermore, in patients with early-stage breast cancer, we observed a lower proportion of CTLA-4 expressing conventional CD4+ T cells in TNBC patients compared to patients with HR+ breast cancer (Figure 5a). When testing for differences in T cell phenotype in late-stage patients across molecular subtypes and HDs, we observed that patients with advanced HER2+ breast cancer had a lower frequency of CLTA-4+ and PD-1+ CD8+ T cells compared to HDs (Figure 5b), which was not yet observed in early disease stage (Figure 5a). Additionally, we found the frequency of PD-1+ conventional CD4 T cells to be reduced in patients with HER2+ advanced breast cancer compared to TNBC and HDs (Figure 5b).

Flow cytometry-based analysis of the T cell differentiation state (naïve T cells being CCR7+CD45RA+, central memory T cells (CM) being CCR7+CD45RA-, effector memory T cells (EM) being CCR7-CD45RA- and effector T cells (T eff) being CCR7-CD45RA+) (Supplementary Figure 1c), revealed a notable degree of heterogeneity in the T cell differentiation state,

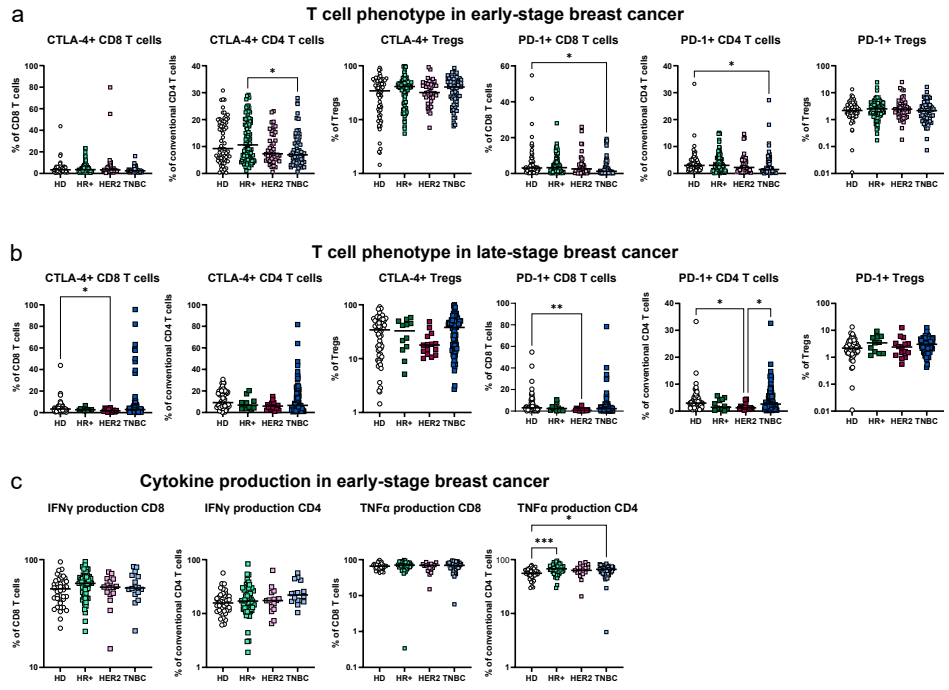


Figure 5. T cell phenotype and cytokine production in patients with early- and late-stage breast cancer across different molecular subtypes. (a) *Phenotypic characterization of circulating T cells at early disease stage, visualizing CTLA-4+ and PD-1+ CD8 T cells, conventional CD4 and regulatory T cells. Frequencies were determined using flow cytometry on fresh blood samples of patients with a HR+ tumor (n=20), a HER2+ tumor (n=17) or a triple negative tumor (n=93), and healthy donors (n=65).* ***(b)*** *CTLA-4 and PD-1 expression of CD8 T cells, conventional CD4 T cells and regulatory T cells. Frequencies of CTLA-4 and PD-1 positivity were determined using flow cytometry on fresh blood samples of patients with a HR+ tumor (n=20), a HER2+ tumor (n=17) or a triple negative tumor (n=93), and healthy donors (n=65).* ***(c)*** *Ex vivo production of cytokines IFNγ and TNFα by CD8 and conventional CD4 T cells. Stimulated fresh blood samples of patients with early-stage disease with a HR+ tumor (n=54), a HER2+ tumor (n=16) or a triple negative tumor (n=16) and healthy donors (n=41). Adjusted p-values for* ***(a-c)*** *were computed by effectuating the Kruskal-Wallis test followed by Dunn's multiple comparisons test.*

which appeared largely unaffected by the subtype of breast cancer at early-stage (Supplementary Figure 3a) or late-stage (Supplementary Figure 3b). Next, we investigated whether the capacity of T cells to produce cytokines IFNγ and TNFα following *ex vivo* stimulation with PMA-ionomycin for three hours, was altered in a breast cancer subtype dependent manner (Supplementary Figure 1c). Due to sample processing limitations, we confined this part of our analysis to patients with early-stage disease. The ability to produce IFNγ by CD8+ and conventional CD4+ T cells upon stimulation was not affected by the

presence of a tumor of any subtype (HR+, HER2+ and TNBC) (Figure 5c), suggesting that T cells from patients with early-stage breast cancer retained similar potential to produce this cytokine *ex vivo*. However, when analyzing T cells' ability to produce TNFα upon stimulation, we observed that CD4 T cells of breast cancer patients produced more of this cytokine compared to HDs. This increase in TNFα was statistically significant in patients with HR+ and TNBC subtypes, and showed a trend toward significance in HER2+ patients (Figure 5c). No differences were observed in cytokine production between the molecular breast cancer subtypes. Together these data indicate that T cell phenotype and functionality is modestly altered across the molecular subtypes in early and late stages of disease compared to HDs.

Discussion

In recent years, it has become increasingly clear that solid tumors impact the immune system in ways that extend far beyond the tumor microenvironment[12,13]. However, the influence of tumors on the systemic immune landscape, particularly in relation to (breast) cancer subtype and disease stage, remains poorly understood. This study aimed to investigate the changes in the circulating immune landscape across different disease stages and molecular subtypes of breast cancer. We utilized multi-parameter flow cytometry to comprehensively assess the abundance, phenotype, and activation states of both lymphoid and myeloid immune populations from freshly collected peripheral blood samples. Pre-clinical evidence indicates a critical role for neutrophils in disease progression[21-23]; however, these fragile and short-lived cells are often overlooked due to their inability to be stored. By analyzing fresh blood samples, we successfully captured the full complexity of the immune landscape, including all granulocyte populations.

Our data indicate that the systemic immune landscape in patients with breast cancer differs significantly from that of HDs, with more pronounced immune cell abnormalities in late-stage compared to early-stage disease. In metastatic breast cancer, we observed a general trend of the innate immune compartment expansion and adaptive immune compartment reduction relative to HDs. These findings highlight disease stage as a critical determinant of the circulating immunological profile in breast cancer, consistent with the expectation that more advanced, disseminated disease exerts a greater impact on the immune system. Moreover, we established that certain changes in the systemic immune landscape of breast cancer patients within early- and late-stage disease associated with a particular molecular subtype. For example, circulating CD8 T cells are specifically decreased in patients with mTNBC compared to HDs, but this was not observed in patients with late-stage HR+ or HER2+ breast cancer. Similarly, the systemic increase in neutrophils observed in late-stage breast cancer is seen only in HR+ and triple-negative subtypes, while neutrophil levels in late-stage HER2+ breast cancer closely resemble those observed in HDs. Notably,

the most profound immune dysregulation was observed in patients with TNBC, highlighting the subtype-specific nature of immune cell perturbations in breast cancer. Furthermore, we observed that disease stage seems a more dominant factor than molecular subtype in shaping the circulating immune landscape in patients with breast cancer.

The precise mechanisms by which breast tumors of different molecular subtypes differentially impact the systemic immune landscape are yet to be fully elucidated. Each breast cancer subtype is characterized by unique genetic mutations, copy number variations, and gene expression profiles, which can directly or indirectly lead to distinct patterns of cytokine and chemokine release[24-26]. These variations in cytokine and chemokine profiles may contribute to subtype-specific immune alterations. Additionally, epigenetic reprogramming of cancer cells—such as changes in DNA methylation and histone modifications—can further influence immune cell function and gene expression, leading to systemic immune changes. We hypothesize that these factors are crucial for understanding the differential immune responses observed among the various breast cancer subtypes.

Apart from tumor molecular subtype and disease stage, other factors could influence the systemic immune profile. It is important to acknowledge that the patients included in this study closely reflect those encountered in clinical practice, meaning that a proportion of patients with metastatic disease had received prior treatment for their primary tumor. It has previously been shown that treatment with chemotherapeutics impacts the circulating immune compartment for longer than the three-week washout period that was used in this study[27,28]. We would therefore like to emphasize that the observed differences regarding patients with late-stage disease are not necessarily purely tumor driven, but can be a result of multiple combined factors, including treatment history, tumor grade or histological subtype. In addition to treatment history, the genetic make-up of the tumors may have a strong additive effect on the systemic immune landscape[29-31], as described above. Though some driver mutations (e.g. mutations in *TP53* or *PIK3CA)* are more prevalent within a specific breast cancer subtype, they are not exclusively found in just one subtype[24,32,33]. If specific mutations influence the immune profile in blood and are present across different subtypes and stages, these tumor mutations may mask potential differences driven by disease subtype and stage. Since we do not have data on tumor mutations, further research is needed to investigate the relation between tumor-genotype/immuno-phenotype.

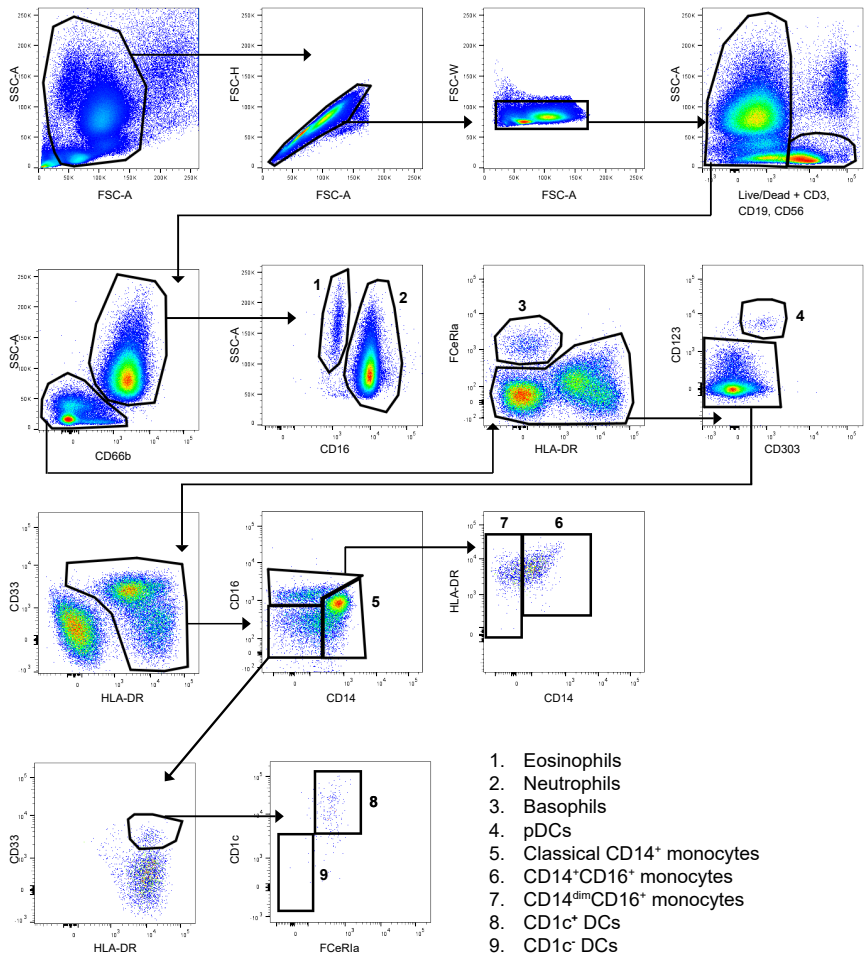
Finally, we would like to discuss the potential clinical significance of our findings. Clinical research has demonstrated that an elevated neutrophil-to-lymphocyte ratio (NLR) as well as an reduced lymphocyte-to-monocyte ratio (LMR) is associated with worse disease prognosis and diminished therapeutic response across various cancer types, including breast cancer[34-40]. Since we did not observe a concordant increase in lymphocyte counts with the increased numbers of classical monocytes and neutrophils, our findings suggests that patients with breast cancer exhibit a skewed immune profile, characterized by an increased

dominance of myeloid over lymphoid cells. This myeloid-skewed systemic immune landscape may leave patients less equipped to mount an effective immune response, potentially leading to poorer clinical outcomes than they would have experienced with a more balanced immune profile. Restoring the NLR and LMR to ratio's similar to those observed in HDs may represent a promising therapeutic strategy, potentially enhancing the efficacy of immunotherapy when administered either right after or in combination with it. Turning to the clinical implications of lymphoid perturbations, tumor-infiltrating B cells and plasma cells have shown considerable predictive and prognostic value in various cancers, particularly in the context of both conventional therapies and immune checkpoint inhibitors[41,42]. Others have shown in a small set of matched tumor-blood samples, that the decrease in memory B cells in the blood contrasts with an increase in class-switched memory B cells within the tumor[43]. Whether the observed systemic reduction in memory B cells and plasmablast-like cells is associated with an aberrant TME and altered patient outcomes remains to be determined and warrants further investigation. CD4 T cells, particularly T helper cells, are essential for orchestrating a robust immune response, as they facilitate the activation and differentiation of various immune cells, including cytotoxic T cells and B cells, which are crucial for effective tumor clearance[16,44,45].

Overall, our data show that patients with late-stage disease have more of the cell types that associate with poor clinical outcome like neutrophils and monocytes[37,39,40,46-48], and less of favorable immune cell types like cytotoxic T cells and T helper cells[49-51]. Given that the systemic immune profile of breast cancer patients appears to become increasingly dysregulated as the disease progresses, it is important to consider initiating immune modulatory strategies before metastatic spread occurs. Indeed, across cancer types, increased response rates are observed with neoadjuvant immune checkpoint blockade, when compared to immune checkpoint blockade administered in the more advanced disease setting[52-54], suggesting that earlier intervention may harness a more functional immune system to achieve better therapeutic outcomes. Lastly, we propose that developing therapeutic strategies aimed at normalizing the systemic immune landscape may hold potential to enhance treatment efficacy and improve overall outcomes for patients.

Supplementary Figures

A



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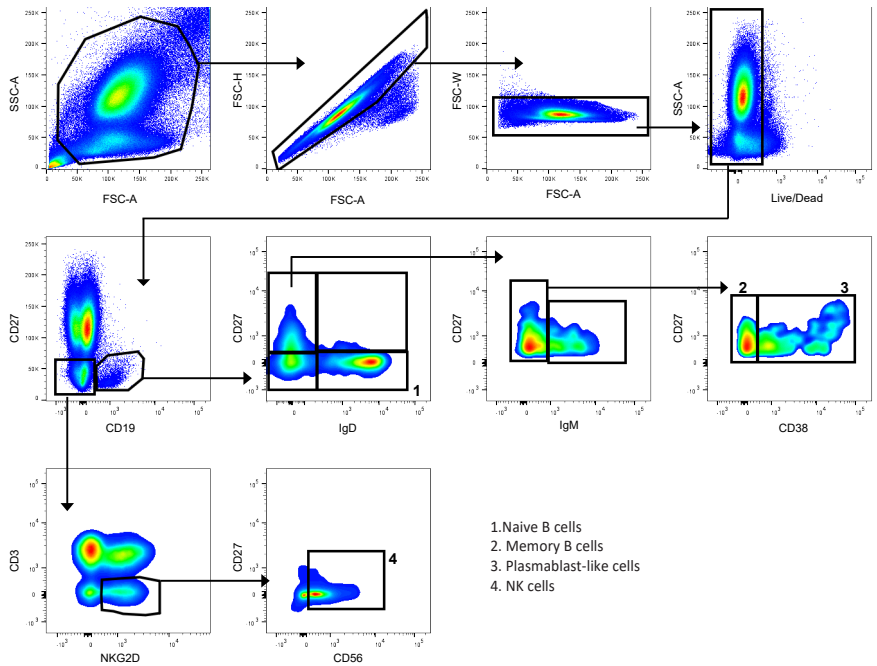

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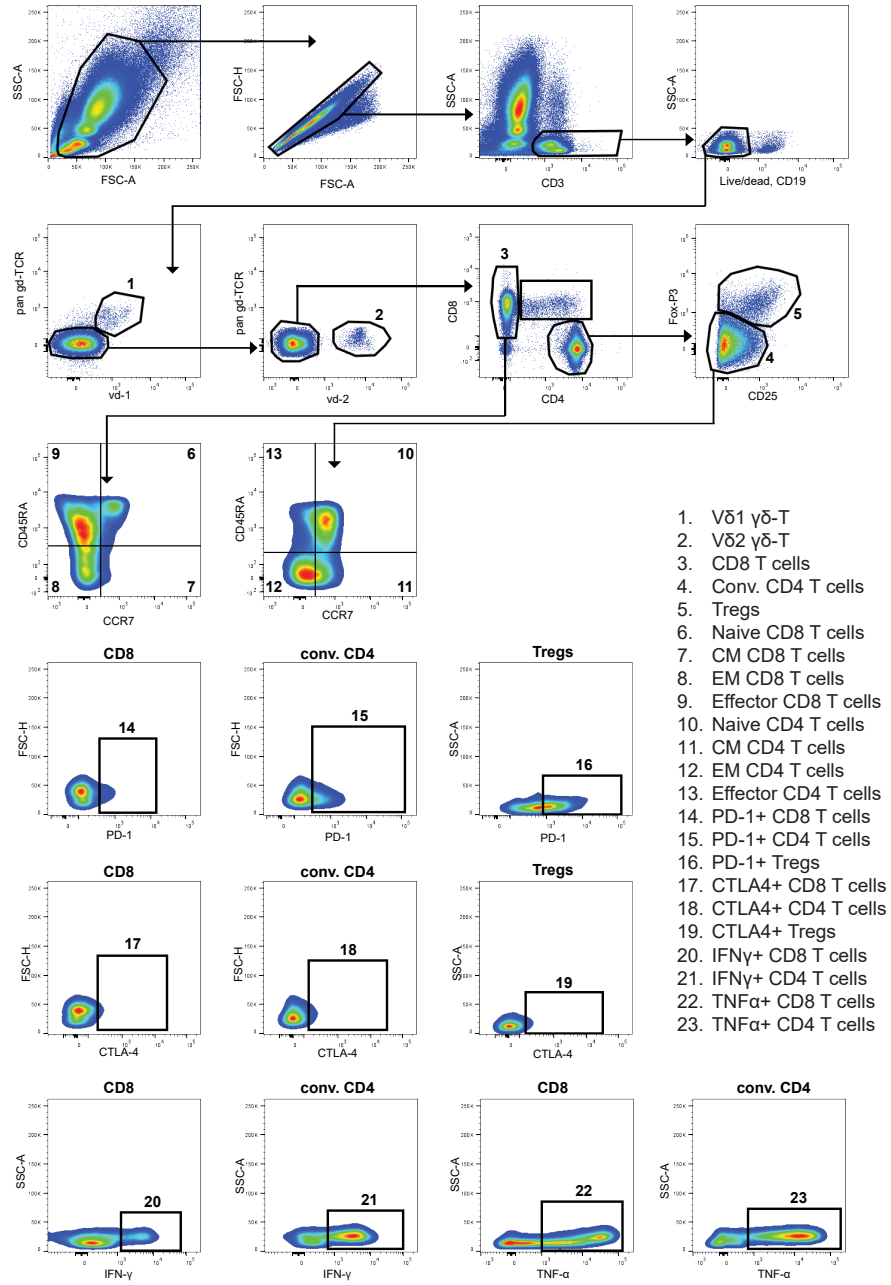
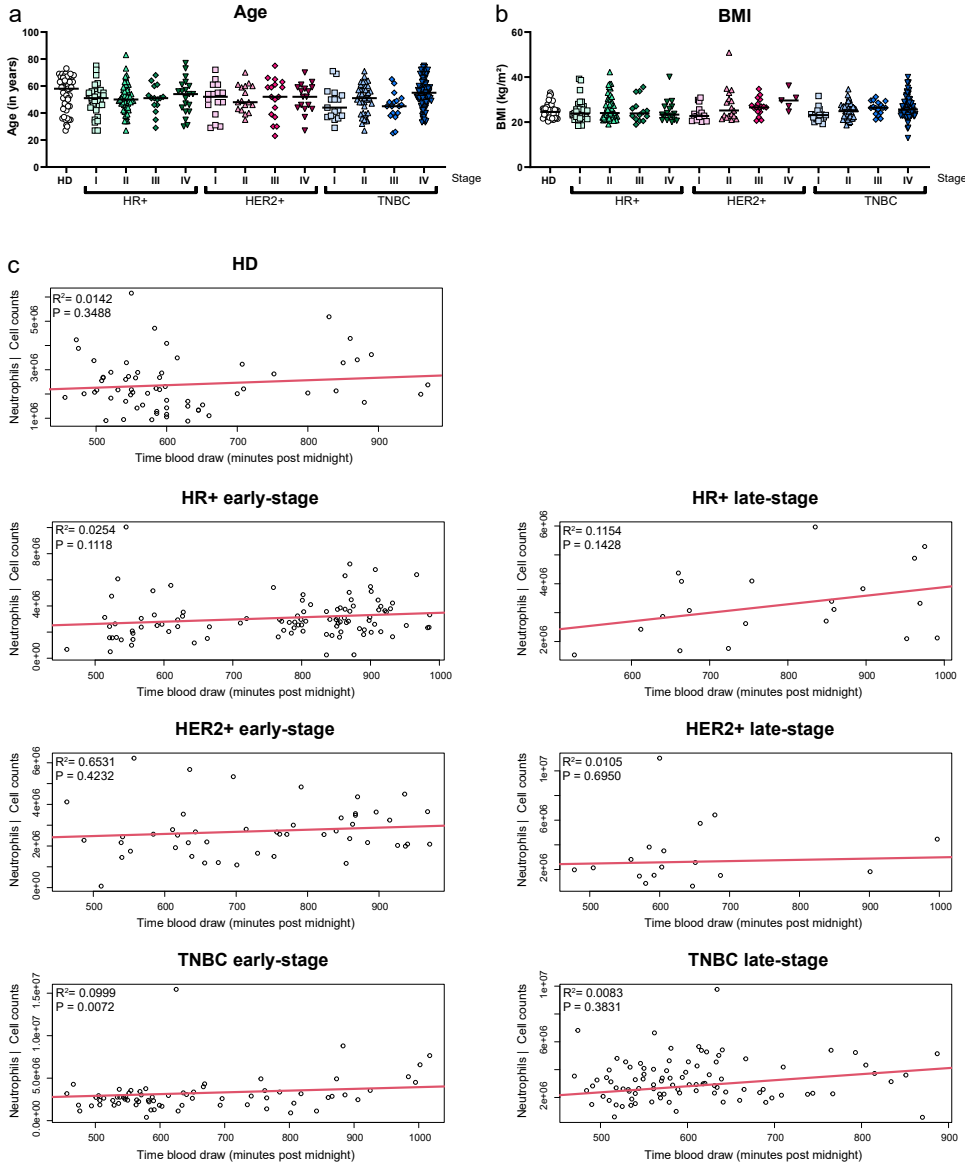
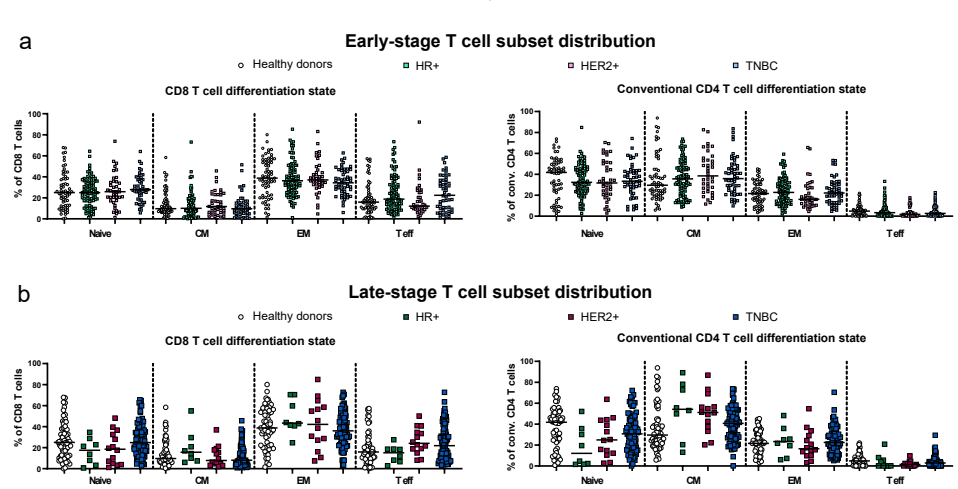


Supplementary Figure 1. Gating strategies for flow cytometry analysis of peripheral blood immune populations. (a) *Myeloid panel gating strategy identifying eosinophils (lineage$^-$, high side scatter, CD66b$^+$ CD16$^-$), neutrophils (lineage$^-$, high side scatter, CD66b$^+$ CD16+), basophils (lineage$^-$, FcεRIα$^+$, HLA-DR$^-$), plasmacytoid DCs (pDCs) (lineage$^-$, HLA-DR$^+$, CD303$^+$, CD123$^+$), Classical monocytes (lineage$^-$, HLA-DR$^+$, CD33$^+$, CD14$^+$, CD16$^-$), Transitional monocytes (lineage$^-$, HLA-DR$^+$, CD33$^+$, CD14$^+$, CD16$^+$), Non-classical monocytes (lineage$^-$, HLA-DR$^+$, CD33$^+$, CD14^{dim}, CD16$^+$), CD1c$^+$ DCs (lineage$^-$, HLA-DR$^+$, CD33$^+$, CD14$^-$, CD16$^-$, CD1c$^+$, FcεRIα$^+$) and CD1c$^-$ DCs (lineage$^-$, HLA-DR$^+$, CD33$^+$, CD14$^-$, CD16$^-$, CD1c$^-$, FcεRIα$^-$). **(b)** Gating strategy to identify B cell subsets identifying naive B cells (CD19$^+$, CD27$^-$, IgD$^+$), switched memory B cells (CD19$^+$, CD27$^+$, IgD$^-$, IgM$^-$, CD38$^-$), and plasmablasts-like cells (CD19$^+$, CD27$^+$, IgD$^-$, IgM$^-$, CD38$^+$). Gating strategy to identify NK cells (CD19$^-$, CD3$^-$, NKG2D$^+$, CD56$^+$). **(c)** T cell panel gating strategy identifying Vδ1 γδ T cells (CD3$^+$, Vδ1$^+$, pan γδ TCR$^+$), Vδ2 γδ T cells (CD3$^+$, Vδ2$^+$), CD8 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^+$, CD4$^-$), conventional CD4 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^-$, CD4$^+$, FoxP3$^-$), Tregs (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^-$, CD4$^+$, FoxP3$^+$, CD25^{hi}). Differentiation states were obtained as follows for both the conventional CD4 T cells and CD8 T cells: naïve T cells (CD45RA$^+$, CCR7$^+$), central memory (CM) T cells (CD45RA$^-$, CCR7$^+$), effector memory (EM) T cells (CD45RA$^-$, CCR7$^-$), effector T cells (CD45RA$^+$, CCR7$^-$). Additional phenotypic markers were gated according to the population names. Cytokine production was measured after PMA-ionomycin stimulation. Gating strategy identifying IFNγ$^+$ CD8 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^+$, CD4$^-$, IFNγ$^+$), TNFα$^+$ CD8 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^+$, CD4$^-$, TNFα$^+$), IFNγ$^+$ conventional CD4 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^-$, CD4$^+$, FoxP3$^-$, IFNγ$^+$), TNFα$^+$ conventional CD4 T cells (CD3$^+$, Vδ1$^-$, pan γδ TCR$^-$, Vδ2$^-$, CD8$^-$, CD4$^+$, FoxP3$^-$, TNFα$^+$).*



Supplementary Figure 2. Clinical parameters and time of blood draw of patients with early- and late-stage breast cancer across different molecular subtypes. (a) *Age distribution and* ***(b)*** *Body Mass Index (kg/length in m²) distribution in patients with breast cancer separated by disease stage and tumor subtype, and in healthy donors. Adjusted p-values for* ***(a)*** *and* ***(b)*** *were computed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test.* ***(c)*** *Correlation analysis between neutrophil cell counts per mL blood and the time of day the blood was taken. R^2 and P values are provided in the top-left corner of each graph.*



Supplementary Figure 3. T cell differentiation state in patients with early- and late-stage breast cancer across different molecular subtypes. *T cell differentiation state based on surface marker expression of CD45RA and CCR7 as determined by flow cytometry (see Supplementary Figure 1c), comparing proportions within conventional CD4+ and CD8+ T cells for* ***(a)*** *early-stage breast cancer patients with HR+ tumors (n=101), HER2+ tumors (n=50), triple negative tumors (n=74) and healthy donors (n=65) and* ***(b)*** *late-stage breast cancer patients with HR+ tumors (n=20), HER2+ tumors (n=17), triple negative tumors (n=93) and healthy donors (n=65). CM = central memory, EM = effector memory and T eff = effector T cells. Adjusted p-values for were computed with the Kruskal-Wallis test followed by Dunn's multiple comparisons test.*

Supplementary Tables

Supplementary Table 1: *Treatment history at the time of blood donation for patients with late-stage disease. Chemotherapy for mHER2+ BC: taxane, for 1 pt platinum agent. Dual anti-HER2: trastuzumab and pertuzumab. T-DM1 is Trastuzumab-Emtansine.*

	Treatment for metastatic disease
Late-stage HR+ *n=20*	n = 11 (55%) treatment-naïve for M1-diseaes n = 7 (35%) aromatase inhibitor n = 1 (5%) anti-hormonal therapy (e.g. tamoxifen) n = 1 (5%) oestrogen receptor antagonist (e.g. fulvestrant)
Late-stage HER2+ *n=17*	n = 6 (30%) chemo-naïve for M1-disease n = 11 (70%) chemotherapy + dual anti-HER2 blockade n = 7 (41%) T-DM1 as second line n = 4 (24%) chemo + trastuzumab as third line n = 1 (6%) Tyrosine kinase inhibitor
Late-stage TNBC *n=93*	Treatment-naïve for M1-disease

Supplementary Table 2: *List of antibodies used for flow cytometry.*

Human flow cytometry antibodies

Antigen	Fluorochrome	Clone	Dilution	Company	Catalogue number
CD3	PE Cy5	UCHT1	1:200	BD Bioscience	555334
CD4	BV421	RPA-T4	1:100	BD Bioscience	562424
CD8	BUV805	SK1	1:200	BD Bioscience	612754
Pan γδ TCR	PE	11F2	1:100	BD Bioscience	555717
vδ1	FITC	TS8.2	1:100	Thermofisher	TCR2730
vδ2	BUV395	B6	1:100	BD Bioscience	748582
FoxP3	PE Cy5.5	FJK-16s	1:50	Thermofisher	35-5773-82
CCR7	APC R700	150503	1:50	BD Bioscience	565868
CD45RA	BUV737	HI100	1:400	BD Bioscience	612846
CD25	AF647	BC96	1:100	BioLegend	302618
PD-1	APC Cy7	EH12.2H7	1:100	BioLegend	329922
CTLA-4	PE CF594	BNI3	1:200	BD Bioscience	562742
IL-17	PerCP Cy5.5	N49-653	1:50	BD Bioscience	560799
IFNγ	BV785	4S.B3	1:200	BioLegend	502542
TNFα	PE Cy7	Mab11	1:400	BioLegend	502930
CD27	BV786	L128	1:100	BD Bioscience	563327
TIGIT	PerCP Cy5.5	A151536	1:100	BioLegend	372718
Ki-67	PE Cy7	B56	1:50	BD Bioscience	561283
CTLA-4	PE CF594	PE/Dazzle594	1:200	BioLegend	369616
CD19	PE Cy5	HIB19	1:200	BD Bioscience	555414
CD3	BUV496	UCHT1	1:100	BD Bioscience	612940
CD56	PE Cy5	B159	1:100	BD Bioscience	555517
CD161	PE Cy5	DX12	1:100	BD Bioscience	551138
HLA-DR	BUV661	G46-6	1:100	BD Bioscience	612980
CD14	BUV737	M5E2	1:100	BD Bioscience	612763
CD16	BUV496	3G8	1:100	BD Bioscience	612944
CD16	AF700	3G8	1:200	BioLegend	302026
CD11b	BV421	ICRF44	1:200	BioLegend	301324
CD11c	BV785	3.9	1:100	BioLegend	301644
cKIT/CD117	PE Cy5.5	104D2	1:400	Thermofisher	CD11718
CD1c	PE Cy7	L161	1:100	BioLegend	331516
CD141	BV711	1A4	1:100	BD Bioscience	563155
CD123	PE	6H6	1:200	BioLegend	396604
CD66b	PerCP-Cy5.5	G10F5	1:200	BD Bioscience	562254
CD66b	AF647	G10F5	1:200	BD Bioscience	561645
CD33	PerCP Cy5.5	WM53	1:100	BioLegend	303414
CD303	APC vio770	REA693	1:100	Miltenyi Biotech	130-114-178
CD41a	BUV395	HIP8	1:400	BD Bioscience	740295
FcεRIα	PE Dazzle 594	AER-37(CRA-1)	1:200	BioLegend	334634
CD34	FITC	581	1:100	BD Bioscience	555821
CD19	BUV395	SJ25C1	1:50	BD Bioscience	563549
IgD	APC	IA6-2	1:100	BD Bioscience	561303
CD20	BUV805	2H7	1:200	BD Bioscience	612905
CD27	PE	M-T271	1:200	BD Bioscience	555441
CD10	AF700	HI10a	1:200	BD Bioscience	563509
CD24	BB515	ML5	1:200	BD Bioscience	564521
IgM	APC Cy7	MHM-88	1:100	BioLegend	314520
CD38	BUV737	HIT2	1:400	BD Bioscience	741837
CD5	PE Dazzle 594	L17F12	1:400	BioLegend	364012
CD1d	BV786	42.1	1:200	BD Bioscience	743608
CD138	BV711	MI15	1:200	BioLegend	563184

Conflicts of interest

N.A.M.B, H.G., V.G., E.C., C.K., M.D., I.N., R.C.A.M.G., M.d.G., R.V., M.C.L., E.L. have no conflicts of interest to declare. H.M.O reports funding to the institute from Roche in order to perform the Triple B study and an advisory board fee from Novartis, Pfizer, Gilead, AstraZeneca and Daiichi Sankyo outside the submitted work. M.K. reports funding to the institute from BMS, Roche/ Genentech, AZ, and an advisory role/speakers fee for Alderaan, BMS, Domain Therapeutics, Gilead, Roche, MSD, and Daiichi Sankyo, outside the submitted work. K.E.d.V. reports research funding from Roche/ Genentech and is consultant for Macomics, outside the scope of this work.

Authors contribution

N.A.M.B. analyzed and interpreted data and wrote the manuscript with K.E.d.V.. H.G. designed flow cytometry panels, which N.A.M.B. modified. N.A.M.B., H.G., E.C., C.K. and M.D. processed the fresh blood samples and applied compensation and gating strategy to the flow cytometry data. N.A.M.B., V.G., I.N., R.J.G., M.d.G., R.V., M.C.L., E.L., H.M.O. and M.K. wrote a biobank protocol, asked patients for informed consent to participate in this study and/or were coordinating/supervising clinical trials of which the baseline samples were used. K.E.d.V. and M.K. conceived the project, gave critical input throughout the analysis and supervised the study. All authors edited and approved the manuscript.

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