Exploring The Contribution of Innate Immune Cells to Breast Cancer Immunotherapy

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Abstract

Breast cancer is the leading type of cancer in women. About 10% to 15% of breast cancers are triple-negative breast cancer (TNBC), a subtype with the worst prognosis. Due to the lack of estrogen, progesterone, and HER2 receptor expression, chemotherapies have been the standard of care for decades. Immunotherapy has emerged as promising for TNBC treatment. In 2020, the Food and Drug Administration (FDA) granted approval to Pembrolizumab in combination with chemotherapy for patients with advanced triple-negative breast cancer. However, only a subgroup of advanced TNBC patients lived longer than those whose tumors have a PD-L1 Combined Positive Score of at least 10 (CPS≥10). There is still an unmet medical need to provide alternative treatment for the rest of patients.

Interestingly, a few of the patients at UCSD Moores Cancer Center had excellent responses to Pembrolizumab despite low CPS scores (termed Elite Responders). There may be an alternative immune response mechanism and/or crosstalk happening between the innate and adaptive immune systems that contributed to this unexpected excellent response, especially in Natural Killer Cells and Macrophages. Our procedure used ACDBio RNAscope Multiplex Fluorescence v2 method to spatially analyze innate immune cells (Natural Killer cells and macrophages) and adaptive immune cells (T cells) in the tumor microenvironment. Our data demonstrated increased tumor infiltration of innate immune cells (Macrophages and Natural Killer cells) in the Elite Responders. This conclusion indicated the joint effort of two immune systems (innate and adaptive) which eventually led to increased survival.

Keywords: Triple Negative Breast Cancer (TNBC), Natural Killer cells (NKs), Immune Checkpoint Blockades (ICB), Programmed Death-Ligand 1 (PD-L1), Programmed Cell Death Protein 1 (PD1), Tumor Microenvironment (TME)

Background

Triple-negative breast cancer (TNBC) accounts for 10% to 15% of all breast cancers. It is an aggressive type of breast cancer that does not express Estrogen Receptors (ER), Progesterone Receptors (PR), nor normal Human Epidermal growth factor Receptor type 2 (HER2).[1] TNBC patients have the poorest prognosis due to the lack of targeted therapeutics. TNBC patients have a lower 5-year survival rate (62.1% and 80.8% for TNBC and non-TNBC patients), and a higher mortality rate (42.2% in TNBC vs 28% in non-TNBC subtypes, respectively).[2,3] TNBC has high risk of relapse as commonly seen in women younger than age 40, who are of African-American descent, or who have a BRCA1 mutation. Visceral metastasis formation is frequent and involves the lung, bone, and brain. Chemotherapy is the current standard-of-care treatment of this disease. Although TNBCs are highly sensitive to chemotherapy, the frequent occurrence of relapse enforces the search and evaluation of novel therapeutic approaches, such as immunotherapy.

Breakthrough Treatment Immunotherapy

In 2020, the Food and Drug Administration (FDA) approved the combination therapy of Pembrolizumab with chemotherapy for patients with advanced Triple-Negative Breast cancer whose tumors had relatively high levels of the PD-L1 protein—a PD-L1 Combined Positive Score of at least 10 (CPS≥10). CPS is essentially a measure of the extent to which cells in a tumor produce PD-L1, the immune checkpoint protein that Pembrolizumab targets.[4]

Pembrolizumab is a high-affinity, highly selective antibody against PD-1. The programmed death receptor 1 (PD-1) is an inhibitory immune checkpoint receptor expressed on activated T cells, B cells, and Natural Killer cells. PD-L1, a PD-1 ligand, is an immunosuppressive signal. By blocking immune checkpoints, Pembrolizumab and other immune checkpoint inhibitors unleash the immune system against cancer cells. However, Pembrolizumab is not a silver bullet for advanced TNBC because only 50% of the patients express PD-L1.

The human immune system consists of several major types of lymphocytes including T cells, B cells, Macrophages, and NK cells. T cells and B cells are members of the adaptive immune system that is triggered by the specific detection of antigen-presenting cells/pathogenic antigens in the blood, after the innate immune system fails to remove an infection. Macrophage and NK cells belong to the innate immune system, targeting nonspecific foreign antigens and their chemical properties in the body.[5]

NK cells are the third largest lymphocyte in the blood. NK cells can eliminate tumor cells without antigen-specific cell surface receptors, making them ideal in killing pathogen infected cells. NK cells use a different mechanism to fight cancers. They are primed to fight any unnaturally altered cells. It is because of this search and destroy mode of

action that NK cells are often referred to as the "first line of defense" with regards to the adaptive immune system.[6] NK cells cross-talking among immune cells also plays a regulatory role in mediating the anti-tumor adaptive immunity of T and B cells.[7]

Tumor associated macrophages (TAMs) are long-living cells that not only play a role in phagocytosis but are also involved in antigen presentation to T cells.[8] TAMs can be typically divided into M1 (proinflammatory, tumor killing) and M2 (anti-inflammatory, tumor promoting) types. TNBC releases soluble factors such as CSF-1 and extracellular vesicles (Evs) that promote macrophage differentiation into M1 which led to a better prognosis.[9]

Study Rationale

Current TNBC immunotherapies target the PD-L1 and PD-1 axis, which harness the adaptive immune system to fight cancer. There have been limited studies on the innate immune system in TNBC. A few patients at UCSD Moores Cancer Center displayed excellent responses to Pembrolizumab despite low CPS scores, and they lived for a considerably longer time than the regular TNBC patients, indicating alternative response mechanisms and/or contributions from other immune cells.

Materials, Methods, and Objectives

In this study, we analyzed cancer samples from the Elite Responders to explore the roles of Macrophages, Natural Killer cells and T cells in tumor microenvironments using target-specific probes and the RNAscope Multiplex Fluorescent Assay V2 (Figure 1).

Samples

Formalin-Fixed Paraffin Embedded (FFPE) Triple Negative Breast cancer biopsies from patients who received Pembrolizumab alone or in combination with chemotherapy were retrieved from the archives of UCSD Moores Cancer Center biorepository with institutional review board approval (IRB# 15-0348). Of the 12 samples studied, 6 were from Elite Responders who received Pembrolizumab longer than 12 months, 4 were from patients who didn't respond to Pembrolizumab, and 1 was from a patient with high PD-L1 and responded to Pembrolizumab. 8-10 serial sections were collected from each tissue block.

Assay, fluorophores, and RNA probes

Serial sections of slides were subjected to gene expression analysis by applying RNAscope Multiplex Fluorescence Assay V2 (Part ID: 323100, 323120) to visualize 4 targets simultaneously on FFPE tissues (Figure 1). These included cell specific markers for tumor cells (Pan-CK), Natural Killer cells (NCR1), Macrophages (CD68) and T cells (CD3). Different fluorophores were assigned to the four channels in accordance with the ACD Vivid dyes (Part ID: 323271, 323272 and 323273) and Akoya

Biosciences Opal™ Polaris 780 dye (FP1501001KT) of that channel. They emitted a different color upon light excitation (520 nm green, 570 nm red, 650 nm purple, and 780 nm cyan). ACD RNA probes included Hs-panCK (Part ID: 404751), Hs-NCR1-C2 (Part ID: 312651-C2), HsCD68-C3 (Part ID: 560591-C3), Hs-CD3G-C4 (Part ID: 586341-C4), Hs-CD3D-C4(Part ID: 599391-C4), and Hs-CD3E-C4 (Part ID: 553971-C4).

Imaging and Quantification

The slides were scanned with a Zeiss Axioscan Z.1 epifluorescence slide scanner with a Colibri 7 light source and a 20x (0.8 NA) dry objective. The images were analyzed with QuPath to count the total number of cells in a field of view based on DAPI staining, tumor cells, NKs, Macrophages and T cells were reported as percentage of total number of cells normalized by the number of Pan-CK positive cells.

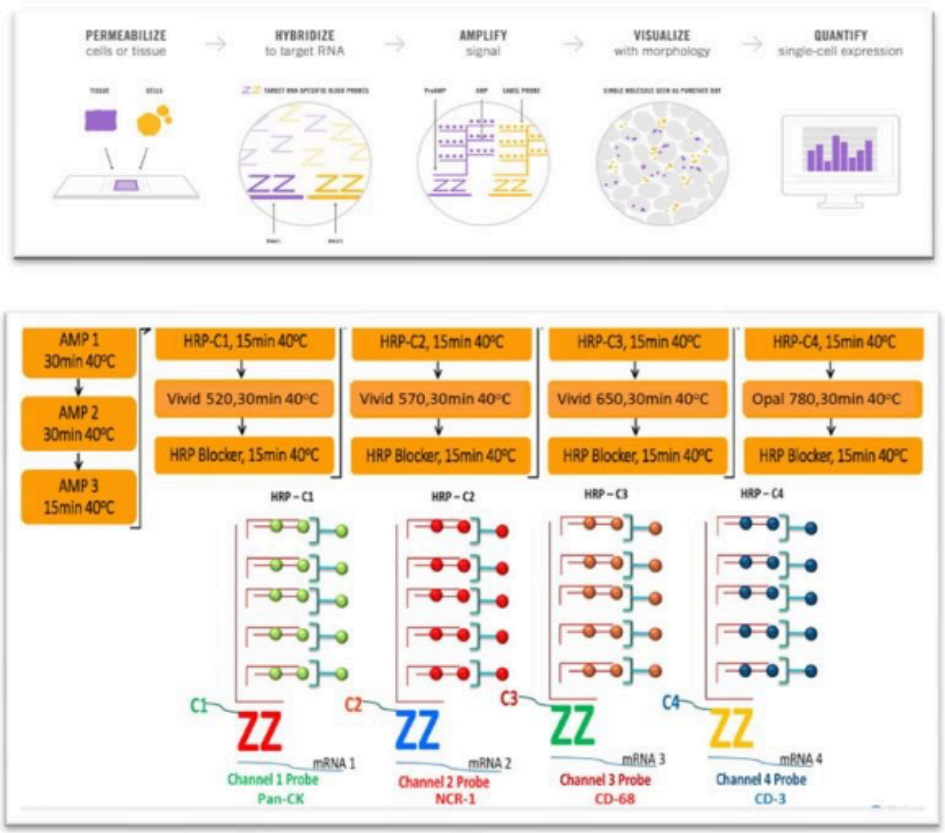

FIGURE 1. Workflow of RNAscope Multiplex Fluorescent V2 assay.

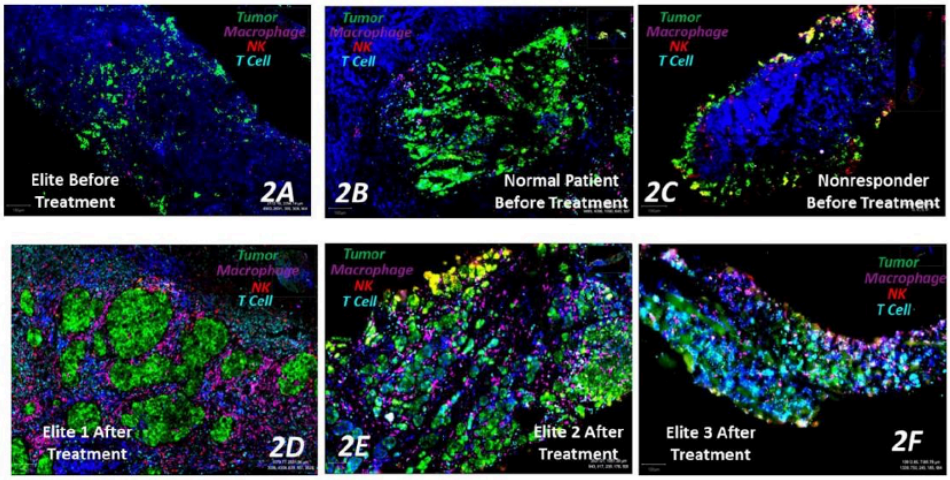

FIGURE 2. Infiltration of Macrophages, NK and T cells in Triple Negative Breast Cancer tumor microenvironment visualized by RNAscope Multiplex Fluorescent V2 assay. A combination of specific marker probes: tumor cells (Pan-CK, green), NK cells (NCR1, red), Macrophages (CD68, purple) and T cells (CD3, cyan) allowed for detection in TNBC samples. Before Pembrolizumab treatment, Elite Responder (2A), Normal Responder (2B) and Non-Responder (2C) expressed low levels of Macrophages and NKs. Normal Responder has a higher level of T cells than Elite Responder and Non-Responder. After Pembrolizumab treatment, Elite responders demonstrated significant infiltration of macrophages, NKs and T cells in the TME (2D, 2E, 2F).

%	NonResponder Before Treatment	Normal Responder Before Treatment	Elite Before Treatment	Elite After Treatment
Macrophage	0.24	0.01	0.18	2.6
NK cells	0.021	0.038	0.01	0.17
CD3+ T Cell	0.018	1.1	0.01	5.9

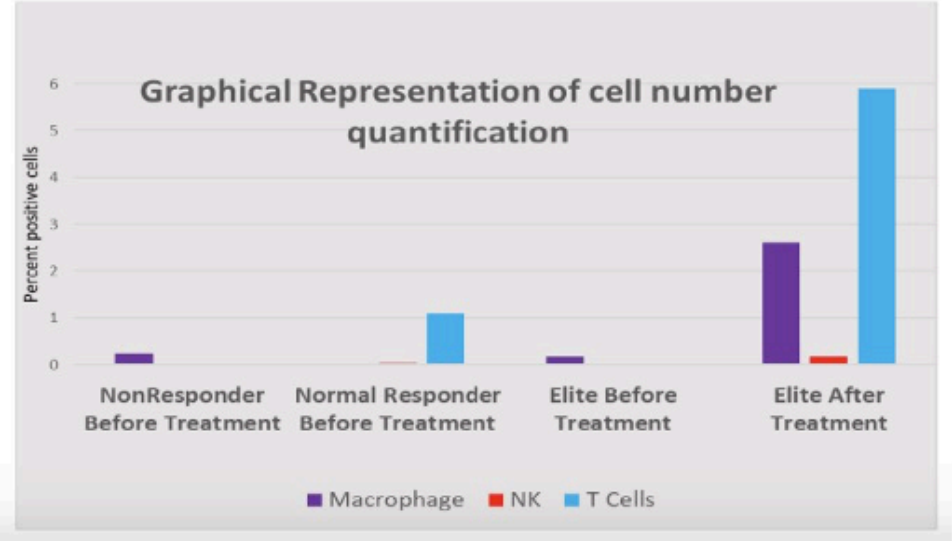

FIGURE 3. Quantification of Macrophages, NKs and T cells in Triple Negative Breast Cancer tumor microenvironment. Total number of cells in a field of view based on DAPI staining, macrophages, NKs, and T cells were reported as percentage of total number of cells normalized to number of Pan-CK positive cells.

Results

RNAscope Multiplex Fluorescence assay revealed expression of all four targets (PanCK, NKs, Macrophages and T cells) with high sensitivity and specificity at single cell level. We were able to successfully visualize spatial relationship between innate and adaptive immune cells in TME when assessing response to Pembrolizumab.

Before the start of Pembrolizumab, samples collected from Elite Responder (Fig. 2A, n=1), Normal Responder with high PD-L1 (Fig. 2B, n=1) and non-responder (Fig. 2C, n=4) displayed low levels of NKs and Macrophages (Fig. 3). However, the Normal Responder displayed infiltration of T cells in TME (co-localization of Pan-CK and CD3, data not shown). This patient eventually benefited from Pembrolizumab, which is expected.

After the start of Pembrolizumab, Elite Responders (Fig. 2D, 2E, 2F, n=5) displayed significant increase of all three cell populations. While Macrophages tend to overlap with T cells and NKs in the TME and adjacent areas indicating a potential crosstalk between innate and adaptive immune cells, NKs were also observed in tumors (co-localization of Pan-CK and NCR-1, data not shown).

The trend is confirmed by cell number quantification in tumor areas selected by the pathologist, analyzed by QuPath (version 0.3.2) and reported as percentage of cell count normalized by Pan-CK positive cell count. Elite responders have an average of 2.6% Macrophages, 5.9% T cells, and 0.17% NKs, a significant increase compared to the Elite Responder before the start of therapy (0.18% macrophage, 0.01% T cells, and 0.01% NKs). Due to the small sample size and lack of access to historical tissue blocks as the true control, we would like to carefully report the observation as a trend.

Conclusions and Discussions

Adding the immunotherapy drug Pembrolizumab to chemotherapy helped some advanced Triple Negative Breast Cancer patients live longer, but only for those with high levels of PD-L1 proteins (CPS≥10). However, more than half of the TNBC patients have PD-L1 combined positive scores of less than 10. Our study identified a few Elite Responders to Pembrolizumab who have low CPS scores, and spatial analysis of immune cell infiltration of the tumor microenvironment showed significant involvement of the innate immune system after the start of Pembrolizumab. These findings demonstrate that Elite Responders may use a harmonious mechanism to build antitumor immunity, harnessing both the innate and adaptive immune systems to fight TNBC. Our findings echo with recent reports that even if the adaptive immune system is compromised[10] or the function of T cells cannot be fully recovered by PD-1 inhibitors under specific circumstances,[11] PD-1/PD-L1 antagonisms can still increase antitumor efficacy.

Tumors are infiltrated by different populations of immune cells including Macrophages. Tumor-associated macrophages (TAMs) are heterogeneous with M1 (proinflammatory) and M2 (pro-tumor growth) populations. There have been recent reports that M1 dominating TNBC creates a proinflammatory environment with increased infiltration of T lymphocytes and NK cells and a better prognosis.[12,13] Based on our observations of Macrophage involvement in Elite Responders and other reports that anti-PD-1 or PD-L1 immune checkpoint blockade induces M1 Macrophage polarization/repolarization,[14,15] leading to enhanced antineoplastic effect, we propose that future studies should focus on the role of M1 subpopulation of Macrophages and NKs in TNBC.

In conclusion, this study established a quick, sensitive, and practical methodology of using Multiplex RNAscope to spatially characterize four protein/gene markers and build a digital profile of

immune cells (innate and adaptive) in the tumor microenvironment, which will help to identify TNBC patients who are more likely to benefit from Pembrolizumab. It will be used to molecularly guide the Region of Interest (ROI) selection for the GeoMx Digital Spatial Profiler (DSP), which will perform highplex RNA profiling in cancer patients.

Another implication of my finding is that it gives new hope to the other 50% of the advanced TNBC patients with low CPS scores, where addition of Macrophages and NK cells to their treatment regimen may lead to longer survival. Both are potential targets for future therapeutic development.

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