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ORIGINAL RESEARCH

Stromal B Lymphocytes Affecting Prognosis in Triple-Negative Breast Cancer by Opal/TSA Multiplexed Immunofluorescence

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Objective: Immune cells play a key role in tumor microenvironment. The purpose of this study was to investigate the infiltration and clinical indication of immune cells including their combined prognostic value in microenvironment of triple negative breast cancer.

Methods: We investigated 100 patients with triple negative breast cancer by Opal/Tyramide Signal Amplification multispectral immunofluorescence between 2003 and 2017 at Zhejiang Provincial people's Hospital. Intratumoral and stromal immune cells of triple negative breast cancer were classified and quantitatively analyzed. Survival outcomes were compared using the Kaplan–Meier method and further analyzed with multivariate analysis.

Results: Infiltration level of stromal B lymphocytes, stromal and intratumoral CD8+ T cells, stromal CD4+ T cells, stromal PD-L1 and intratumoral tumor associated macrophages 2 cells were shown as independent factors affecting disease-free survival and overall survival in univariate analysis. Stromal B lymphocytes, T stage, N stage and pathological type were independent predictive factors for both DFS and OS in multivariate analysis. We firstly found that patients with B lymphocytes-enriched subtypes have a better prognosis than those with T lymphocytes-enriched subtypes and tumor-associated macrophage-enriched subtypes.

Conclusion: The present study identified a bunch of immune targets and subtypes, which could be exploited in future combined immunotherapy/chemotherapy strategies for triple negative breast cancer patients.

Keywords: triple negative breast cancer, B lymphocytes, T lymphocytes, tumor associated macrophages, multispectral immunofluorescence, prognosis

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer (BC) that does not express estrogen (ER), progesterone receptors (PR) and human epidermal growth factor receptor type 2 (HER2). It is noted for its power to impact younger females, metastasize early in spite of optimal adjuvant therapy and bring a miserable prognosis.[1] Nowadays, with limited targeted therapy options, standard of care for TNBC remains chemotherapy. Although TNBC is the subtype with the most complete response to chemotherapy (22%), the metastasis and recurrence rate of TNBC patients was still higher than non-TNBCs.[2]

Recently, immunotherapy has become a new choice for tumor treatment which has the advantages of high specificity and little side effects.[3] Programmed death receptor-1 (PD-1) and programmed death ligand 1 (PD-L1) play an important role in the activation of T lymphocytes. PD-1:PD-L1 signal pathway mediates immune escape and immune tolerance in breast cancer and other tumors. Immunotherapeutics targeting the inhibitory receptors (IRs) PD-1 or PD-L1 have made

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substantial clinical progress in multiple cancer such as non–small-cell lung cancer.[4] In 2019, the Food and Drug Administration (FDA) of the United States has approved PD-L1 monoclonal antibody (atezolizumab) combined with chemotherapy as the first-line treatment of unresectable locally advanced or metastatic PD-L1 positive TNBC which can gain survival benefits. Moreover, only a small percentage of TNBC patients may experience a dramatic response to Immune Checkpoint Blocking (ICB).[5,6] It has been found that tumor infiltrating immune cells can predict the efficacy of immunotherapy with PD-1/PD-L1 inhibitors in patients with TNBC. For example, Schmid et al showed that patients with high expression of tumor infiltrating immune cells had greater clinical benefits with PD-1 treatment (12.6m VS 6.6m).[7] The complexity and heterogeneity of tumor infiltrating lymphocytosis (TILs) level was known to cause poor response to therapies and different effect on individuals in TNBC.[8] Therefore, it is important to detect the relevant immune targets in TNBC in order to further identify the potential beneficiary population and guide the accurate clinical use of drugs.

Both intratumoral and stromal immune cells are important in the development and progression of cancer which is also an important factor in the tumor microenvironment. Moreover, it is previously reported that besides CD4+, CD8+ and FOXP3+ TILs, other cells from immune system such as tumor associated macrophages (TAMs), dendritic cells (DCs), tumor associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells and B lymphocytes also exert important impact on TNBC progression.[9–12] Previous immunohistochemical studies reported[13–15] that tumor infiltrating immune cells is related to prognosis in patients with TNBC. However, its clinical value had not yet been deeply explored with the Opal/Tyramide Signal Amplification (TSA) multispectral immunofluorescence (mIF) in formalin fixed paraffin-embedded tissue sections (FFPE) of TNBC.

At present, the main methods to study TILs in tumor microenvironment are immunohistochemistry or immunofluorescence which have some shortcomings (such as one or two cell subtypes can be evaluated at the same time, antibody from different species is needed, and the fluorescence signal is easy to be lost and so on), and the information obtained is only semi-quantitative and will be greatly affected by the different observers. The recently developed Opal/TSA multi-label staining technique overcomes the above limitations and can stain up to 10 indexes at the same time. The quantitative analysis can accurately detect the content, localization and interaction of various cells and related factors in the tumor microenvironment, and obtain the phenotypes of various cell subsets. Some experts have described the use of Opal/TSA multi-labeling immunofluorescence technique for in-depth analysis of lung cancer tumor microenvironment in authoritative journals.[16]. Based on these backgrounds, we designed the current study to comprehensive explore the immune portrait of TNBC to the prognosis among these patients.

Materials and Methods

Patients with TNBC Samples Clinical Specimens

This study was approved by the institutional review board of Zhejiang Provincial people's Hospital (Ethical approval number: 20230808123500057828 4) which complies with the Declaration of Helsinki. Written ethics consent was obtained from the Institutional Ethical Review Board of the Zhejiang Provincial People's Hospital (ZJPPH) before the samples were analyzed. One hundred patients with TNBC from the Department of Breast Surgery of Zhejiang Provincial people's Hospital from March 2003 to May 2017 were enrolled. The major eligibility criteria were as follows: histopathologically confirmed TNBC; no preoperative chemotherapy, radiotherapy, endocrine therapy, immunotherapy and other anti-cancer treatments except operation; no combined tumors, no autoimmune diseases and infectious diseases; and no recent history of trauma. Written informed consents were signed by all patients. We collected the clinical data of all patients including age at the time of diagnosis, tumor size, number of positive lymph nodes, stage, histological grade, pathological type and so on. Follow-up began at the end of treatment, and ended on July 26 2023, which lasted for at least 5 years.

Opal/TSA Multispectral Immunofluorescence

Change of tumor immunoprofiling and its prognostic role were explored by multiplex immunofluorescence (mIF) based on tyramide coupled to a fluorophore (PerkinElmer, Waltham, MA, USA). FFPE tissues from TNBC patients were obtained from the Department of Anatomical Pathology, Division of Pathology, ZJPPH.

Each 5 mm slide obtained from the FFPE was put through six sequential rounds of staining. After heated at 65 °C for 2hr or overnight in the oven, all slides were deparaffinized and tissues were fixed with 10% formalin prior to antigen retrieval in heated Antigen Retrieval Buffer for 18 min in Retriever microwave. Tissues were then blocked with a protein block (Dako, X0909) for 10 min before an 1h incubation at room temperature with primary antibodies, followed by application of a polymeric horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (anti-rabbit EnVision, Dako cat. K400211) for 10 min at room temperature. All antibodies and their dilutions are listed in (Supplementary Table S1). Each HRP-conjugated polymer mediated the covalent binding of a different fluorophore (Opal-520, Opal-570, Opal-620, Opal-650, and Opal-690) using tyramide signal amplification (TSA) buffer (PerkinElmer, Waltham, MA, USA) at 1:50 dilution. Each antibody was coupled with an appropriate fluorophore-conjugated TSA of specific spectrum based on the protein cellular localisation, to prevent potential signal crossover of colocalised targets. This covalent reaction was followed by additional antigen retrieval in heated Antigen Retrieval Buffer for 18 min to remove bound antibodies before the next step in the sequence. Slides were rinsed with washing buffer after each step. After all six sequential reactions, sections were counterstained with DAPI (Thermo Fisher Scientific, P36935) at 1:10 dilution and mounted with Vecta shield fluorescence mounting medium (Vector Labs, Burlingame, CA).

Slides were imaged using a Vectra 3.0 pathology imaging system microscope (PerkinElmer, Waltham, MA, USA). Images analyses and cell phenotyping were performed with Inform Cell Analysis software (PerkinElmer). A total of 1–9 images were obtained from the same slide, and at least five images were selected randomly and quantified separately from tumor parenchyma and stroma by two experienced pathologists who were blind to the prognosis. The criteria is the selected part that must include tumors, exclude normal breast tissue and ductal carcinoma in situ. Also they manually examine all images to remove staining artifacts and damaged tissue areas later.

Spectral Unmixing and Phenotyping

A spectral library containing the emitting spectral peaks of all fluorophores (DAPI, FITC, Cy3, Texas Red and Cy5) was created with the Image Analysis software (Perkin Elmer) (Supplementary Table S2), using multispectral images obtained from single stained slides for each marker and associated fluorophore. The spectral library was then used to separate each multispectral image cube into its individual components (spectral unmixing) allowing for the colour-based identification of all six markers of interest in a single image using the inform image analysis software. All spectrally unmixed and segmented images were subsequently subjected to a proprietary inform active learning phenotyping algorithm. This allows for the individual identification of each DAPI-stained cell according to their pattern of fluorophore expression and nuclear/cell morphological features, associating their phenotype. Cells were phenotyped into different classes according to our markers of interest as follows (Supplementary Table S2): cancer cells (CK+), CD8+ T cells (CD8+), CD4+ T cells (CD4+), T regulatory cells (Treg) (FoxP3+, co-expressing CD4), tumor associated macrophages 1 (TAM1 cells (CD68+)), tumor associated macrophages 2 (TAM2 cells (CD68+, co-expressing CD163), Natural killer cell (NK, CD56+), B lymphocytes (CD20+), polymorphonuclear myeloid—derived suppressor cells pMDSC (CD33+CD11b+HLA-DR-CD15+), mononuclear myeloid—derived suppressor cells (mMDSC) (CD33+CD11b +HLA-DR- CD15-), PD-1(PD-1+), PD-L1(PD-L1+) and other cells (stromal cells without any immune marker or CK expression were classified as other cells (only DAPI+)). All phenotyping and subsequent quantifications were performed blinded to the sample identity and clinical outcomes.

Acquisition of Public Datasets

The Affymetrix microarray GSE135565[17] taken in platform GPL570[HG-U133_Plus_2] and GSE31519[18] in platform GPL96 [HG-U133A] were acquired from the public database Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/). These two studies contained a total of 84 and 67 patients with triple negative breast cancer enrolled for gene expression profiling and follow-up of overall survival respectively. We combined the two datasets and conducted analyses as a whole.

Bioinformatic and Statistical Analyses

The baseline characteristics of patients were summarized by descriptive statistics and frequency tables. Overall survival (OS) was defined as time from the date of surgery to the date of death or the last follow-up (censored). Disease-free survival (DFS) was defined as time from the date of surgery to the date of local any recurrence as the first site of recurrence (radiologic or pathologic).

An algorithm, CIBERSORT, was used to deconvolve the relative purity of 18 immune cell subsets in bulk tumors with the default parameters.[19] The percentage of each immune cell was equal to the number of immune cells to the total number of cells in each field with five fields, and then average was obtained. The same method is used to calculate the percentages of tumor cells and other cells. Heatmap is generated using MeV software 4.6.0 (Python software). Data from the immune cell expressions are presented as means and standard errors, generated using GraphPad Prism software version 8 (GraphPad Software, 2023).

Using X-Tile software version 3.6.1 (Yale University), we found the best cutoff of relative infiltration of 18 immune cell in public dataset and 9 immune cells in our own study for univariate survival analysis. According to the cut-off value, it can be divided into low and high infiltration group. Then, univariate Kaplan–Meier survival analysis taking Log rank test on survival curves was performed with those cut-offs.

All analyses were performed by using SPSS (Windows version 21.0). Univariable and multivariable Cox proportional hazard models were used to assess the hazard ratios (HRs) with a confidence interval (CI) of 95%. All statistical tests were two-sided, and the significance level was set at $P < 0.05$.

Results

Patient and Sample Characteristics

A total of one hundred TNBC patients were enrolled from March 2003 to May 2017 at ZJPPH. Their ages ranged from 25 to 89 years old with a median age of 49 years old. According to the clinical staging criteria of AJCC breast cancer in 2008, there were 21 cases of stage I, 59 cases of stage II and 20 cases of stage III. All the postoperative specimens were pathologically diagnosed by pathologists, including 89 cases of invasive ductal carcinoma and 11 cases of other pathological diagnosis such as medullary carcinoma, mucinous carcinoma and metaplastic carcinoma. Baseline characteristics of the eligible TNBC patients are summarized in Table 1.

Table 1 Clinical Characteristics of the Triple Negative Breast Cancer Patients Included in the Study of Our Cohort

Patient Cohort	Group	N(%)	All
Age(year)	<49 ≥49	52(52%) 48(48%)	100
T stage	T1 T2 T3	39(39%) 47(47%) 14(14%)	100
N Stage	0 1 2 3	61(61%) 20(20%) 12(12%) 7(7%)	100
AJCC 8th Stage	I II III	21(21%) 59(59%) 20(20%)	100
Histological grade	1 2 3	5(5%) 58(58%) 37(37%)	100
Pathological type	Infiltrating ductal carcinoma Other	89(89%) 11(11%)	100

Intratumoral and Stromal Immune Cells in TNBC

We used Opal/TSA multi-labeling immunofluorescence technique to detect the expression of immune cell-related markers in TNBC microenvironment, and combined with Inform software image processing software, we classified the immune cells in different positions of the microenvironment of TNBC patients, and identified the number of immune cells in tumor and stroma. The quantification of the different markers (percentage of each immune cell) was shown in Supplementary Table S3.

Intratumoral and Stromal Immune Cells Predict Survival in TNBC

The long-term prognostic value of immune cells was examined in 100 TNBC patients. At the time of July 26 2023, the median follow-up time was 102.23 months (range: 4.47–150.53 months) with 3 patients lost follow-up. Twenty-nine (29.0%) patients had experienced treatment failure, of which 13 (13.0%) patients were diagnosed with local recurrence, the other 16 (16.0%) with distant metastasis, and no patient was considered having experienced both local-regional and distant metastasis.

The median OS of the entire cohort was 102.23 months. The 1- and 2-year OS rate was 88.7% (95% CI: 0.824–0.95) and 76.3% (95% CI: 0.679–0.824), respectively. The median DFS was 99.07 months. The 1- and 2-year DFS rate was 87.6% (95% CI: 0.811–0.941) and 75.3% (95% CI: 0.667–0.839), respectively.

TNBC patients were stratified into low or high infiltration groups based upon the cutoff percentages for each immune cell subpopulation. Among them, total CD8+ T cells, total CD4+ T cells and total B lymphocyte were positively correlated with DFS while total TAM2 was negatively correlated with DFS (P<0.05). Total CD8+ T cells, total CD8+ T cells and total B lymphocyte were also positively correlated with OS and total TAM2 was negatively correlated with OS (P<0.05). KM plots are shown in Supplementary Figure S1.

Moreover, it shows that patients with higher infiltration level of intratumoral CD8+ T cells, stromal CD8+ T cells, stromal CD4+ T cells and stromal B lymphocytes and lower infiltration level of intratumoral TAM2 and stromal PD-L1 had significantly longer DFS and OS than the others in univariate analysis (P<0.05). Univariate Kaplan–Meier survival analysis of the triple negative breast cancer patients of our cohort was shown in Supplementary Table S4. KM plots are shown in Figure 1 and Supplementary Figure S2.

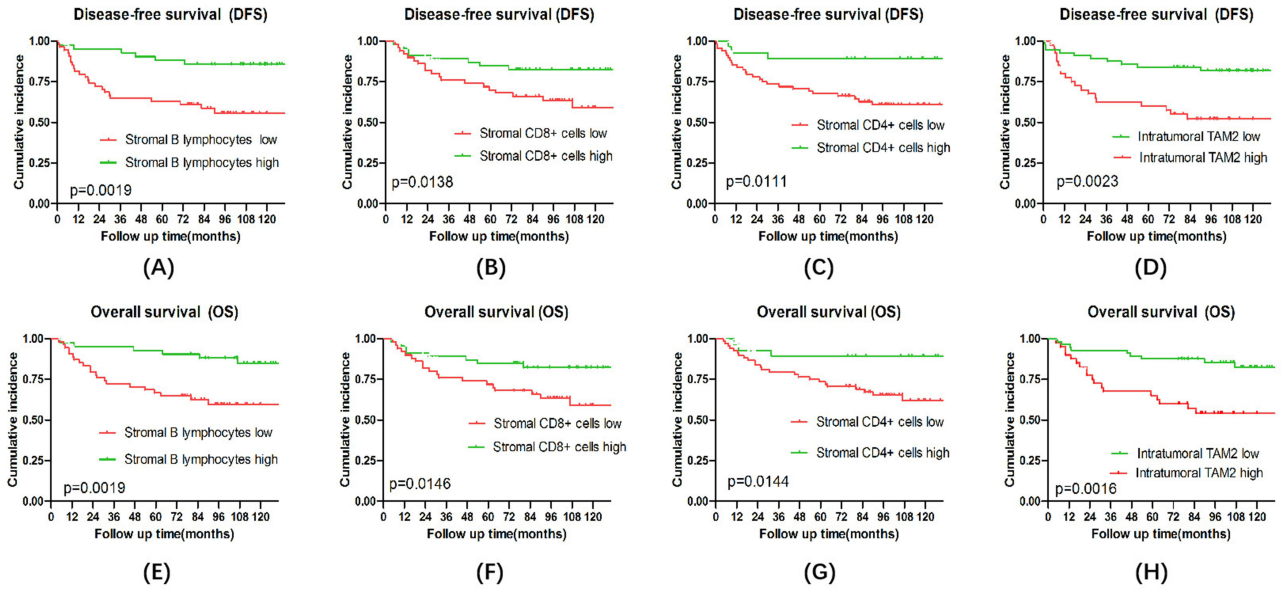

Figure 1 Intratumoral and stromal immune cells predict long-term survivors in formalin-fixed paraffin-embedded tissue sections (FFPE) of TNBC patient of our cohort. (**A**–**H**) Kaplan–Meier curves showed that patients with higher infiltration level of stromal B lymphocytes, stromal CD8+ T cells, stromal CD4+ T cells and lower Intratumoral tumor associated intratumoral 2 (TAM2) had significantly better survival than the others in univariate analysis (P<0.05).

The infiltration level of the intratumoral and stromal immune cells, age, T stage, N stage, AJCC stage, pathological type and histological grade was then taken into multivariate Cox regression modeling. The results of multivariate analysis show that the infiltration level of stromal B lymphocytes, T stage, N stage and pathological type were independent factors affecting both DFS and OS in TNBC patients (P<0.05) (Table 2).

Analyses of Public Datasets

Univariate Kaplan–Meier (KM) survival analysis taking Log rank test on survival curves was performed on relative infiltration of 18 immune cell with optimised cut-off found by X-Tile software (Supplementary Table S5). Among them, B lymphocytes, T cells CD4 memory resting, T cells CD4 memory activated, T cells follicular helper, T cells regulatory (Tregs), T cells, Macrophages M0, Macrophages M2, Macrophages were found to be independent prognostic factors for overall survival (P<0.05). Moreover, it shows that patients with higher infiltration level of B lymphocytes, higher infiltration level of T cells CD4 memory resting, lower infiltration level of T cells CD4 memory activated, higher infiltration level of T cells follicular helper, lower infiltration level of T cells regulatory (Tregs), higher infiltration level of T cells, lower infiltration level of Macrophages M0, lower infiltration level of Macrophages M2, lower infiltration level of Macrophages, had significantly longer overall survival than the others (P<0.05). This is in consistent with our data, and KM plots are shown in Supplementary Figure S3.

In order to identify immune phenotyping of patients, we conducted hierarchical analyses on infiltration level of different samples. As shown in Supplementary Figure S4, different samples were clustered into four groups. Cluster 1 showed infiltration of more tumor-associated macrophages but less B lymphocytes and T lymphocytes which was identified Macrophages-enriched subtypes. Cluster 2 showed infiltration of more T lymphocytes but less tumor associated macrophages and B lymphocytes which was identified T lymphocytes-enriched subtypes, Cluster 3 showed rather balanced infiltration of three types of cells. Cluster 4 showed infiltration of more tumor-associated macrophages and T lymphocytes but less B lymphocytes which was identified macrophages and T lymphocytes -enriched subtypes. Further, although it did not show significant difference of four clusters in KM plot, cluster 2 (T lymphocytes-enriched subtypes) had significantly longer OS than cluster 1 (macrophages-enriched subtypes) (P=0.024). KM plots are shown in Figure 2A and B.

Immune Phenotyping

Inspired by the results of public database, we also performed immune phenotyping on our cohort. As shown in Figure 3, different samples were clustered into four groups. Representative images of different cluster after multiplexed immunofluorescence (mIF) staining in formalin fixed paraffin-embedded tissue sections (FFPE) of triple negative breast cancer

Table 2 Multivariate Analysis of Overall Survival (OS) and Disease-Free Survival (DFS) in Patients with Triple Negative Breast Cancer of Our Cohort

Characteristic	DFS			OS		
	HR	95% CI	P	HR	95% CI	P
Age at diagnosis, <49 vs ≥49	0.723	0.299–1.751	0.473	0.700	0.278–1.761	0.448
T stage, T1 vs.T2-3	11.403	1.423–91.380	**0.022**	10.425	1.317–82.485	**0.026**
N stage, N0 vs N1-3	4.410	1.700–11.439	**0.002**	5.213	1.907–14.248	**0.001**
Stage, I vs II–III	0.174	0.013–2.319	0.186	0.384	0.020–7.372	0.526
Grading, Grade I vs grade II–III	0.264	0.050–1.401	0.118	0.428	0.068–2.712	0.368
Pathology, Invasive ductal carcinoma vs others	6.819	1.899–24.488	**0.003**	7.681	2.131–27.680	**0.002**
Stromal CD8+ T cells low vs high	1.734	0.690–4.359	0.242	1.574	0.584–4.241	0.370
Intratumoral CD8+T cells, low vs high	0.431	0.229–2.359	0.231	0.451	0.241–2.539	0.430
Stromal CD4+ T cells, low vs high	0.421	0.116–1.535	0.190	0.447	0.118–1.688	0.235
Stromal B lymphocytes, low vs high	0.150	0.042–0.533	**0.003**	0.163	0.045–0.593	**0.006**
Intratumoral TAM2, low vs high	2.013	0.808–5.015	0.133	1.896	0.749–4.803	0.177

Note: Bold number included in the table means p<0.05.
Abbreviations: DFS, Disease-free survival; OS, Overall survival; TAM, Tumor-associated macrophage.

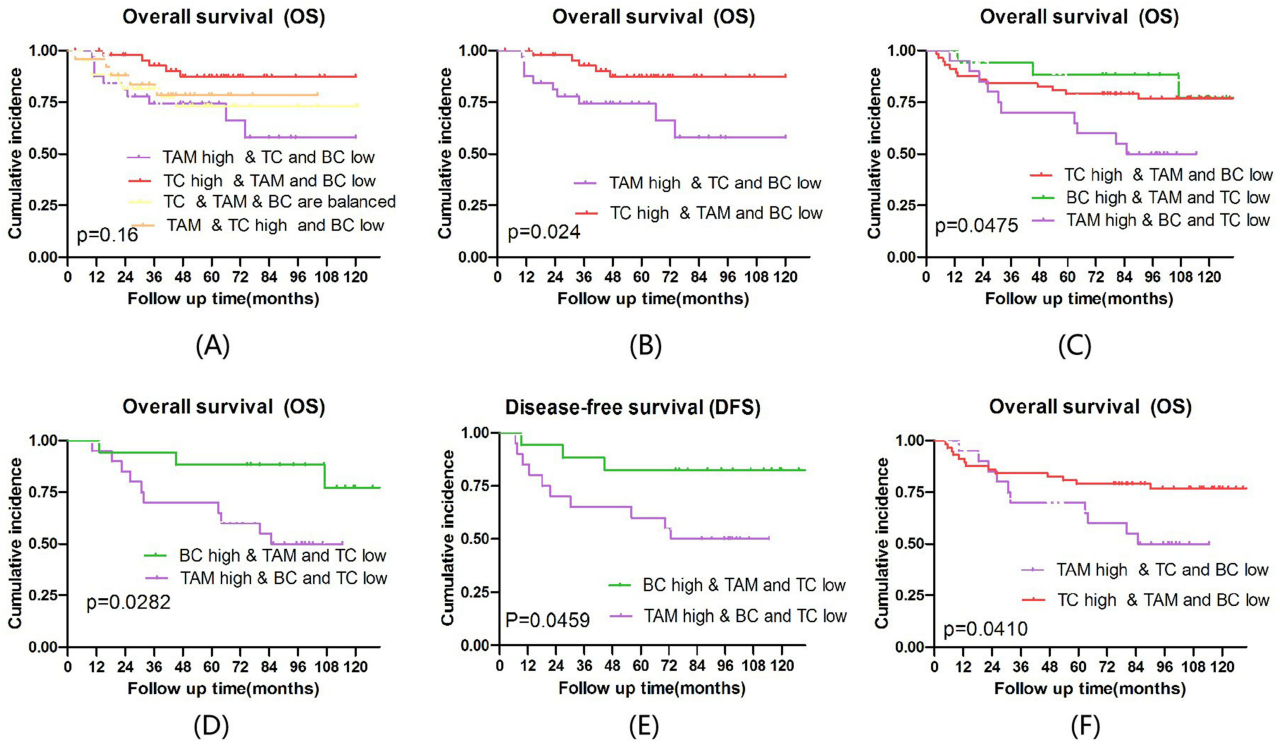

Figure 2 KM plot of different clusters in TNBC patients in public datasets and on our cohort. Tumor associated macrophages (TAMs), T cell (T lymphocytes), B cell (B lymphocytes). (**A**) KM plot of clusters 1–4 for OS in public datasets. It did not show significant difference of four clusters in KM plot (P>0.05). (**B**) KM plot of clusters 1 vs.2 for OS in public datasets. Cluster 2 (T lymphocytes-enriched subtypes) had significantly longer OS than Cluster 1 (Tumor associated macrophages-enriched subtypes) (P<0.05). (**C**) KM plot of clusters 1–3 for OS on our cohort. Significant difference of OS was observed between three clusters in KM plot (P<0.05). (**D** and **E**) KM plot of clusters 2 vs 3 for OS and DFS on our cohort. Cluster 2 (B lymphocytes-enriched subtypes) had significantly longer OS and DFS than Cluster 3 (Tumor associated macrophages-enriched subtypes) did (P<0.05). (**F**) KM plot of clusters 1 vs.3 for OS on our cohort. Cluster 1 (T lymphocytes-enriched subtypes) had significantly longer OS than Cluster 3 (Tumor associated macrophages-enriched subtypes) did (P<0.05).

are shown in Figure 4. Cluster 1 showed infiltration of more T lymphocytes but less tumor-associated macrophages and B lymphocytes which was identified T lymphocytes-enriched subtypes. Cluster 2 showed infiltration of more B lymphocytes but less tumor-associated macrophages and T lymphocytes which was identified B lymphocytes-enriched subtypes. Cluster 3 showed infiltration of more tumor-associated macrophages but less B lymphocytes and T lymphocytes which was identified macrophages -enriched subtypes. There were only two samples in Cluster 4, which was considered not meaningful. Furthermore, significant difference of OS was observed between three clusters rather than DFS. Cluster 1 (T lymphocytes-enriched subtypes) had significantly longer OS than Cluster 3 (macrophages -enriched subtypes), although there was no significance in DFS between two subtypes. On the other hand, Cluster 2 (B lymphocytes-enriched subtypes) had significantly longer OS and DFS than Cluster 3 (macrophages -enriched subtypes) (Figure 2C, D, E and F).

Discussion

Immune cells play an important in the development and progression of cancer. Immune cells are divided into intratumoral and stromal immune cells. Intratumoral immune cells are distributed within cancer cell nests and directly infiltrating tumor cells whenever stromal immune cells are distributed within the tumor stroma.

The traditional method for the determination of TILs is semi-quantitative by hematoxylin-eosin staining or monochromatic immunohistochemistry. This method is subjective and reproducible, and lacks the ability to characterize cell subtypes at the same time. The recently developed Opal/TSA multi-label staining technique can reflect the content, location and interaction of various immune cells in tumors and stroma in the tumor microenvironment. This method removes the first antibody + second antibody + HRP complex by microwave antigen repair, and avoids the cross reaction among the antibodies which can be used to detect as many as 10 markers at the same time. It can greatly reduce the

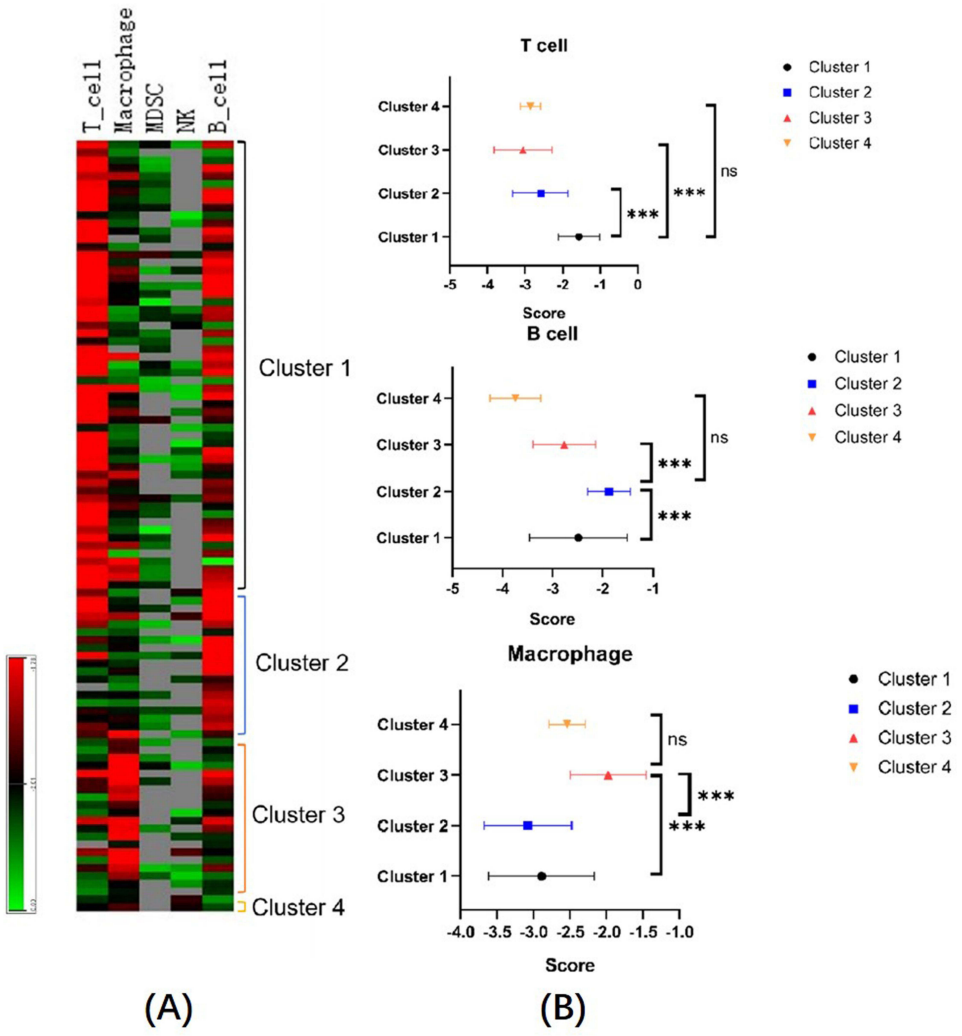

Figure 3 Hierarchical analyses of infiltration level of different immune cell types and different samples on our cohort. (**A**) Clustering of TNBC microenvironment phenotypes based on the estimated numbers of 5 immune cell (T cell, tumor associated Macrophage, myeloid derived suppressor cell (MDSC), natural killer (NK) and B cell) subsets calculated by MeV software. (**B**) Signature scores of T cell, B cell, and macrophage among clusters. ***p<0.001, ns means nonsense.

amount of sample and antibody with the concentration of the first antibody is 1 to 5 of the conventional concentration compared with the conventional method. A number of experts have presented their study in authoritative journals that the use of Opal/TSA multi-labeling immunofluorescence technology for in-depth analysis of tumor microenvironment has more outstanding advantages compared with traditional detection methods.[16]

Several studies have interrogated the immune landscape of TNBC,[20] but most have exclusively focused on a single immune subset[21] or on tumor-associated plasma cells.[22] Our study not only detects single immune cell, but also provides a comprehensive description of the immune environment in FFPE of TNBC by Opal/TSA mIF technique. We further analyzed clinicopathological features and survival of patients in TNBC. In univariate analysis of TNBC, infiltration level of stromal B lymphocytes, intratumoral CD8+ T cells, stromal CD8+ T cells, stromal CD4+ T cells, and intratumoral TAM2 were found to be significant factors related with DFS and OS. In subsequent multivariate survival analysis, stromal B lymphocytes, T stage, N stage and pathological type were independent predictive factors for both DFS and OS. We firstly found that patients with B lymphocytes-enriched subtypes have a better prognosis than other subtypes.

CD8+ T cells can kill tumor cells by mediating target cell apoptosis and cell lysis. Previous studies[23,24] have shown that intratumoral CD8+ T cells play an important role in predicting the prognosis of patients than stromal CD8+ T cells in BC and TNBC. Intratumoral CD8+ T cells have similar prognostic effects in different stages of TNBC patients.[23–27] In

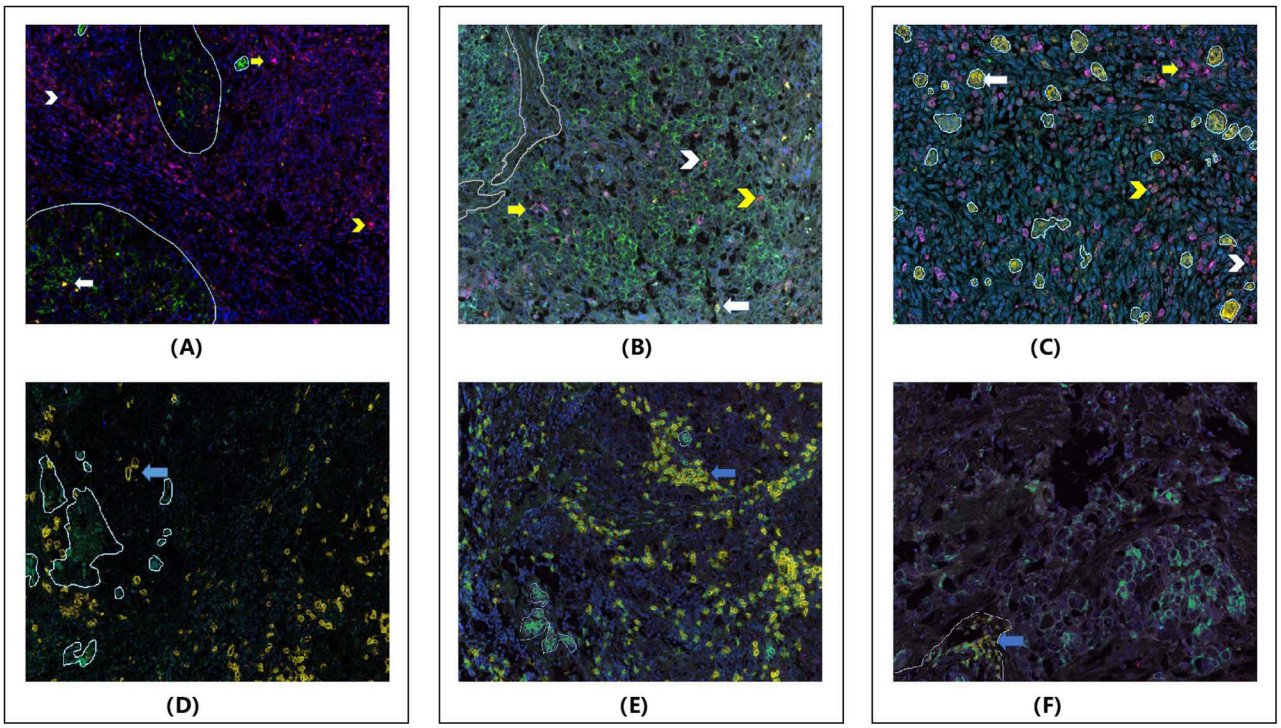

Figure 4 Representative images of different clusters after multiplexed immunofluorescence(mIF) staining in formalin fixed paraffin-embedded tissue sections(FFPE) of triple negative breast cancer of our cohort. (× 200). This information suggests that different subtypes of triple negative breast cancer can be characterized by variations in lymphocyte and macrophage infiltration. In the images, the light turquoise circle in (**A, C, D** and **E**) represents the tumor (Except for the tumor, it's stroma), while the gray circle in (**B** and **F**) represents the stroma (Except for the stroma, it's tumor). The various arrows indicate different cell types: White long arrow: Tumor-associated macrophages 1 (TAM1) - indicated in yellow; Yellow V-shape arrow: CD4+ T cells - indicated in pink; Yellow long arrow: Tumor-associated macrophages 2 (TAM2) - indicated in purple; White V-shape arrow: CD8+ T cells- indicated in red; Blue arrow: B lymphocytes - indicated in yellow. Based on the clusters identified: Cluster 1 (images **A** and **D**): T lymphocytes-enriched subtypes. These patients have higher infiltration of T lymphocytes but lower levels of tumor-associated macrophages and B lymphocytes. Cluster 2 (images **B** and **E**): B lymphocytes-enriched subtypes. These patients have higher infiltration of B lymphocytes but lower levels of T lymphocytes and tumor-associated macrophages. Cluster 3 (images **C** and **F**): Macrophage-enriched subtypes. These patients have higher infiltration of tumor-associated macrophages but lower levels of T lymphocytes and B lymphocytes.

TNBC, the presence of intratumoral CD8+ T cells reduced the risk of mortality by 28%, and stromal CD8+ T cells reduced the risk by 21%.[23] We observed that a high level of both intratumoral and stromal CD8+ T cells showed improved prognosis in TNBC in univariate analyses for CD8+ T cells is the main effector cell of immune response in TNBC. CD4+ T cells can present tumor-associated antigens to the corresponding effector cells and play an anti-tumor effect. CD4+ T cellsT have shown improved survival although previous study in BC is not yet come to reach a consensus.[28] Although the roles of CD4+ T cellsT are very complicated, We found that high density of stromal CD4+ T cellsT was positively correlated with DFS and OS in TNBC in univariate analyses and it is necessary to conduct more prospective studies to confirm its prognostic value in TNBC.

Iglesia MD et al revealed that B lymphocytes may play important functional roles in comprehensive anti-tumor immune responses in BC.[29] In TNBC, elevated B lymphocytes assemble within stromal clusters which are activated by B cell receptors. Tumor-infiltrating B lymphocytes are associated with favorable prognosis in TNBC which is clonally expanded, IgG isotype-biased humoral immunity after assemble in clusters, undergoing B-cell receptor–driven activation, proliferation, and isotype switching.[30] A few studies have shown that B lymphocytes are correlated to a positive prognosis in BC.[31–33] Kuroda H et al also reported that high densities of stromal B lymphocytes were related to favorable prognosis in TNBC.[20] Our study demonstrated that high density of stromal B lymphocytes was significantly related to favorable prognosis in both DFS and OS in univariate and multivariate analysis. Genetic studies also support these findings, which show a significant association between high densities of B lymphocytes and favorable prognosis in TNBCs.[34,35] Robert J. Harris implicates considerable biological and associated prognostic heterogeneity that extends to the tumor microenvironment in TNBC, despite the poor prognosis and aggressive nature of TNBC. In a proportion of

individuals, expansive and active B lymphocyte cancer infiltrates provide a degree of antitumor activity, which may confer a survival benefit and may be exploited with immunotherapies.[30] B cells are not only antibody producers but also participate in immune regulating and potent antigen-presenting in which the CD40L/CD40 signaling pathway is playing an important role. In vitro and in vivo, CD40-activated B cells can induce specific T-cell responses. CD40-activated B cell-based cancer immunotherapy was proved to have antitumor immunity in preclinical animal cancer models. Clinical trials indicate that B cell-based immunotherapy is safe and with little toxicity although there are only few clinical studies about B cell-based cancer vaccines.[36]

TAMs can be divided into two subcategories, M1 type macrophages (TAM1) which play a role against tumor cells, and M2 type macrophages (TAM2), which are involved in the inhibition of inflammation, angiogenesis and tumor progression.[37] Tumor cells can transform M1 into M2 type macrophages and play a role in promoting tumor occurrence and development. Medrek et al showed that[38] TAM2 in TNBC/basal-like breast cancer is more common than that in hormone receptor-positive breast cancer. They also reported that TAM2 is closely related to TNBC/basal-like carcinoma, high grade and tumor. TAMs can hinder the effective tumor immunity circulation by reducing activation of CD8+ T cells, limiting antigen presentation and supporting tumor cell survival, angiogenesis and metastasis.[39] Previous studies have shown that TAMs are associated with a worse prognosis in BC and TNBC.[40–42] We found that a high level of intratumoral TAM2 rather than TAM1 was correlated to worse prognosis.

PD-L1 is an important immunosuppressive molecule, which plays an important role in regulating the immune process. Some studies have found that the expression of PD-L1 in TNBC is significantly higher than that in non-TNBC patients.[43] The increased expression of PD-1 and PD-L1 in TNBC creates conditions for tumor cells to escape the recognition and killing of the immune system. Antibodies targeting to block PD-1/PD-L1 pathway can reverse tumor immune escape and thus play an anti-tumor role. TILs can predict the efficacy of PD-1/PD-L1 inhibitors in TNBC patients.[7] Joe Yeong et al also found that immunotherapy can be optimized by measuring drug targets in tumor tissue.[22] A meta analysis showed that PD-L1 overexpression was associated with OS shortening (HR = 1.76, 95% CI 1.09–2.82; p = 0.02), and a higher level of PD-L1 expression was found in TNBC. The meta-analysis also studied the subtypes and found that PD-L1 was a significant predictor of OS in basal breast cancer (HR:2.60, CI: 1.016–6.652, p = 0.046).[44] Our study showed that the infiltration level of PD-L1 in tumor stroma was negatively correlated with PFS and OS in TNBC patients.

Recent results suggest that immune cells may be a major predictive biomarker for immune checkpoint therapy,[1] but it is not reliable to use a single immune biomarker to predict the effect on any drug. Most TNBC patients do not get pathological complete remission and eventually relapse, which suggests that the immune system may play a role in a specific subtype of TNBC. Kim et al identified "immune subtypes" of TNBC in multiple clinical datasets, and macrophage-enriched subtypes (MES) show varying reactions to immune checkpoint blockade (ICB), while neutrophil-enriched (NES) displays resistance to ICB.[45] Inspired by their study, our study showed that patients with B lymphocytes-enriched subtypes have a better prognosis than those patients with T lymphocytes -enriched subtypes and with macrophages-enriched subtypes. This prognostic immunophenotype made us stratify TNBC outcome and is helpful in finding standard immunotherapy for patients.

Our study has certain limitations. As a complex disease, there is not only intratumoral and stromal immune cells in TNBC but also other factors such as the cell surface markers or internal genetic changes of immune cells which may also affect the prognosis of TNBC patients. Also it is necessary to increase the sample size to continue further verification. We will also further sequence to identify more in-depth molecular mechanisms which can guide the direction of biomarker for immune checkpoint therapy strategies for TNBC patients.

Conclusions

Together, our study is one of the most comprehensive analyses in immune landscapes of TNBC patients. Our findings not only identify a bunch of immune targets but also prognostic immunophenotype which could be exploited in future combined immunotherapy/chemotherapy strategies for TNBC patients.

Ethics Statement

The study was reviewed and approved by Institutional Ethical Review Board of the Zhejiang Provincial People's Hospital.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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