Published OnlineFirst September 18, 2019; DOI: 10.1158/1055-9965.EPI-19-0469

Research Article

Cancer Epidemiology, Biomarkers & Prevention

Immune Profiles of Tumor Microenvironment and Clinical Prognosis among Women with Triple-Negative Breast Cancer

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Abstract

Background: The impact of the immune landscape of the microenvironment on cancer progression is not well understood for triple-negative breast cancer (TNBC). We, therefore, aimed to examine the association of immune cell enrichment scores as a proxy for immune profiles of tumor microenvironment with TNBC prognosis.

Methods: We included 76 patients with TNBC diagnosed between 2008 to 2016 in West China Hospital and 158 patients with TNBC from The Cancer Genome Atlas. On the basis of transcriptome data, we calculated the overall ImmuneScore and type-specific enrichment scores for 34 types of immune cells, using xCell, a gene signature–based method. HRs of recurrence-free survival (RFS) and overall survival (OS) were calculated by Cox proportional hazards models.

Results: During the median follow-up time of 2.8 (0.1–9.8) years, 42 patients had a recurrence, and 34 patients died. The overall ImmuneScore and most immune cell enrichment scores were relatively higher in tumors than normal tissues. A higher enrichment score of plasma cells was associated with favorable RFS [HR 0.45; 95% confidence interval (CI), 0.27–0.73] and OS (HR 0.32; 95% CI, 0.17–0.61). The score of $CD4^+$ central memory T cell (Tcm) was negatively associated with RFS (HR 1.52; 95% CI, 1.17–1.97). Besides, $CD4^+$ Tcm enrichment score was higher in invasive tumors that were not ductal/lobular carcinoma (OR 1.59; 95% CI, 1.06–2.37).

Conclusions: Our findings suggest that plasma cells and $CD4^+$ Tcm in the tumor microenvironment may play a role in the subsequent progression of TNBC.

Impact: This study provides evidence of the role of immune cells in TNBC progression that may have clinical utility.

Introduction

Breast cancers are very heterogeneous, with different molecular subtypes. Triple-negative breast cancer (TNBC) is characterized by lacking estrogen receptor (ER) and progesterone receptor (PR) expression and HER2 amplification. Previously, breast cancer was thought to be not immunogenic cancer, but the IMpassion 130 study revealed the clinical efficacy of immunotherapy in metastatic TNBC (1). In the context of a strong desire to maximize the potential of immunotherapy and understand the molecular mode of antitumor immunity, increasing attention was paid to the immune microenvironment (2).

The components of the immune microenvironment consist of some innate and adaptive immune cell subpopulations, endothelial cells, fibroblasts, adipocytes, and other stromal cells (3). Tumor-infiltrating lymphocytes (TIL) and tumor-associated macrophages (TAM) were the most studied immune cell in breast cancer (4–6). TILs mainly comprise of $CD8^+$ T lymphocytes, $CD4^+$ T helper cells, $CD4^+$ T regulatory cells (Tregs), natural killer cells (NK), and B cells, while TAMs include M1 (classically activated) and M2 (alternatively activated). These immune cells show paradox effects on prognosis, either antitumor or tumor-promoting effects (4, 7, 8). However, other immune cells were not fully elucidated. Furthermore, the profile and functionality of immune cell infiltration differ by breast cancer subtype (4, 5, 9). Therefore, understanding the unique infiltration profile in the microenvironment, and defining the prognostic role of each immune cell on TNBC, may help elucidate how the immune microenvironment contributes to the progression of TNBC.

Several studies have examined TILs, including $CD8^+$ T cells and Tregs, and survival in TNBC (9–14). Recently, CIBERSORT, an *in silico* analysis based on gene expression signature, was applied to estimate the fraction of 22 types of immune cells in breast cancer. The profile of immune cell types frequently differed per breast cancer subtype and even in the subtypes of TNBC (15, 16), and was associated with mutant allele tumor heterogeneity of breast cancer (17). Moreover, a recent study with fewer endpoint events suggested that a higher fraction of resting NK cells may be associated with worse prognosis among patients with ER-negative/HER2-negative breast cancer (16). However, the prognostic roles of some other types of immune cells in the tumor

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Cancer Epidemiol Biomarkers Prev 2019;28:1977–85

doi: 10.1158/1055-9965.EPI-19-0469

Published OnlineFirst September 18, 2019; DOI: 10.1158/1055-9965.EPI-19-0469

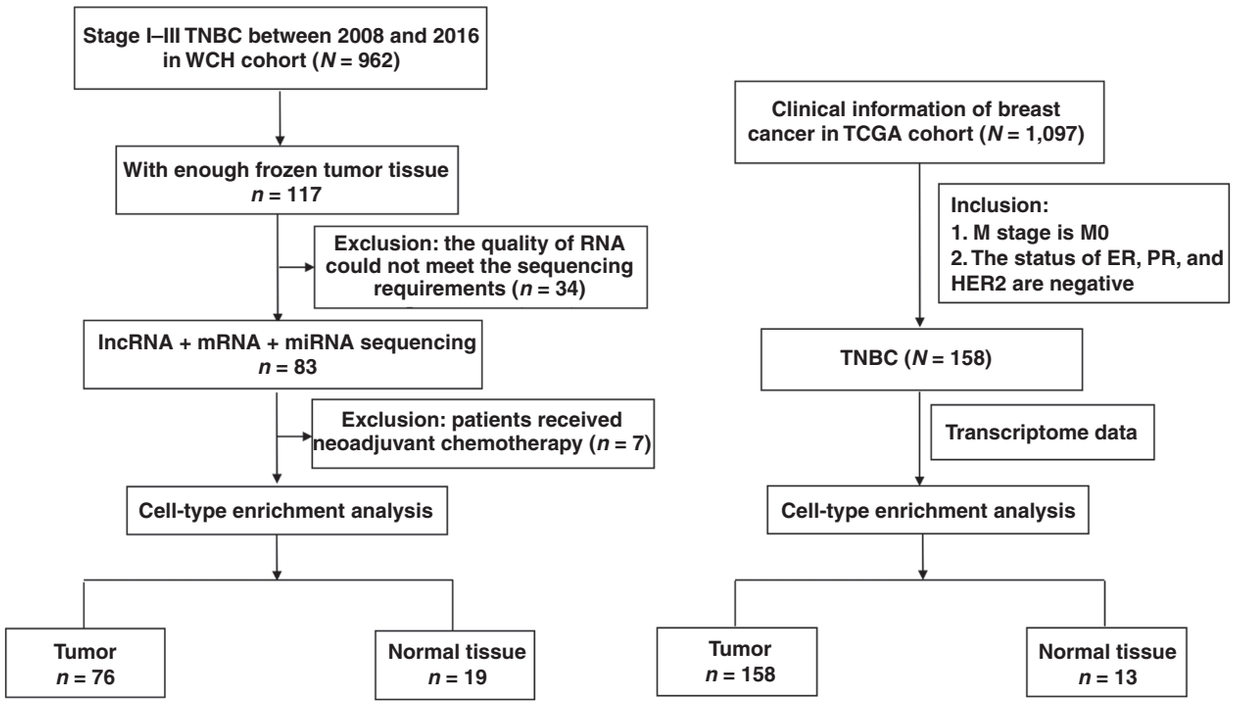

Figure 1.
Flowchart of the study design and patient selection.

microenvironment are not well studied specifically in TNBC, such as TAM, B cells, DCs, and granulocytes (9, 18–22).

In this study, leveraging 234 tumor samples from the West China Hospital (WCH, Sichuan, China) cohort and The Cancer Genome Atlas (TCGA), we aimed to examine the association of overall and cell type–specific immune enrichment scores with risks of recurrence-free and overall survival among patients with TNBC.

Materials and Methods

Patients and samples

All patients pathologically diagnosed with breast cancer were prospectively registered in the Breast Cancer Information Management System at the WCH (Sichuan, China) beginning in 2008. A total of 117 patients diagnosed with stage I–III TNBC during 2008–2016 and donated frozen tumor tissues were included. The status of ER and PR by IHC was identified following the Guidelines for Testing of ER and PR in Breast Cancer (23). The status of HER2 by IHC and FISH scoring was evaluated according to the Guidelines for HER2 Detection in Breast Cancer (24).

Clinical information of 1,097 patients with breast cancer in the TCGA project (TCGA-BRCA) was downloaded from GDC Data Portal in November 2018. TCGA, a community resource project, is a large-scale, collaborative effort led by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) to map the genomic and epigenomic changes. On the basis of the information of tumor stage, ER, PR status by IHC, and HER2 status by IHC and *in situ* hybridization outcome, we included patients who were M0 stage, with negative ER, PR, and HER2 expression. A total of 158 cases of primary TNBC in TCGA were included.

Diagram of the study design and flow of patients was shown in Fig. 1. On the basis of the WCH and TCGA cohorts, 275 patients were included. Thirty-four cases from WCH were excluded because the RNA quality could not meet the sequencing requirements. Seven patients from WCH who underwent neoadjuvant chemotherapy were also excluded. Finally, further analyses were performed on 234 patients with primary TNBC with tumor tissues. Among them, the adjacent normal tissue of 32 patients was applied for further analyses, including 19 cases from the WCH cohort, and 13 cases from the TCGA cohort.

RNA sequencing

In the WCH cohort, a total amount of 3 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) following the manufacturer's recommendations. After cluster generation, the library preparations were sequenced on an Illumina Hiseq4000 platform, and 150 bp paired-end reads were generated. Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. In this step, 14.1 ± 1.3G clean data was obtained for each sample. Next, the RNA-Seq reads were mapped to the human genome (Hg19, GRCh37) and transcriptome (gencode version 19) using Bowtie v2.0.6 and TopHat v2.0.9. HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. Then, Fragments Per KilobaseMillion (FPKM) of each gene was calculated for further analysis. The FPKM calculation normalizes read

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Figure 2.

The cellular landscape of tumor immune microenvironment in TNBC. The heatmap represents cell type enrichment score of each immune cell type for all samples. DC, dendritic cell; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; Tcm, central memory T cell; Tem, effective memory T cell; Tgd cell, gamma delta T cell.

count by dividing it by the gene length and the total number of reads mapped to protein-coding genes. A total of 55,765 genes were profiled.

In the TCGA cohort, RNA sequencing was performed on the Illumina HiSeq 2000 platform (25), and the HTSeq-FPKM data were downloaded from GDC Data Portal, including 158 tumors and 13 normal tissues. Data in the GDC Data Portal has been harmonized using GDC Bioinformatics Pipelines. According to the GDC mRNA quantification analysis pipeline, the FPKM values are generated by first aligning reads to the GRCh38 reference genome and then by quantifying the mapped reads. To facilitate harmonization across samples, all RNA-Seq reads are treated as unstranded during analyses. A total of 53,143 genes were profiled.

Cell-type enrichment analysis

To determine the profile of tumor immune microenvironment, we applied a computational method, xCell, to estimate the cell type enrichment score (26). xCell is a method for cell-type enrichment analysis using single-sample gene set enrichment analysis (ssGSEA), and it employs a spillover compensation technique to reduce dependencies between closely related cell types. xCell uses a set of 10,808 genes for scoring. Missing values in a sample are treated as missing genes (the xCell tool requires the intersection of at least 5,000 genes). For the WCH cohort, three genes (*PCDHGB5*, *BAHCC1*, *CD24*) were not identified, whereas 79 genes were not available in the TCGA cohort. Briefly, the ssGSEA scores are calculated for 489 gene signatures, and scores of all signatures corresponding to a cell type are averaged. The result is a matrix (A) with 64 rows and N columns. Then, each element in the scores matrix (Aij) is transformed using sequencing-based parameter and formula. At last, spillover compensation is performed using linear least squares (26). The enrichment score of 64 cell types, including 34 types of immune cells, 30 types of stroma and other cells, was obtained for each sample. According to the characteristics of cell development and differentiation, these 34 types of immune cells can be classified into 9 categories, including CD4 T-cell subpopulations, CD8 T-cell subpopulations, gamma delta T cells (Tgd cells), NK cells, NKT, B-cell subpopulations, monocyte/macrophage subpopulations, dendritic cell (DC) subpopulations, and granulocyte subpopulations (27–30). An overall score of immune cells, ImmuneScore, for each sample was also generated. The overall and cell type–specific enrichment score of each cell type was normalized as Z-score in WCH or TCGA cohort separately for further analyses.

Outcomes

We used recurrence-free survival (RFS) as the primary outcome, while overall survival (OS) as the secondary outcome. RFS was defined as the time from the date of surgery to the date of confirmed tumor recurrence, metastasis, and death, or the date of the last follow-up visit for recurrence-free patients. OS was defined as the interval between the date of surgery and the date of patient death, or last follow-up.

In the WCH cohort, patients were followed (through physical examination, blood tests, X-ray mammography, CT scan of head, chest, and abdomen, and bone scintigraphy) every 3 months within the first 2 years after surgery, and then every 6 months from 3 to 5 years after diagnosis, and every year thereafter. Follow-up on survival status was available until August 25, 2018 in the WCH cohort, whereas April 29, 2015 in the TCGA cohort.

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Table 1. Immune cell enrichment score between tumor and normal breast tissues of patients with triple-negative breast cancer

	Tumor ($n = 234$) Mean ± SD	Normal ($n = 32$) Mean ± SD	*P*
ImmuneScore	0.081 ± 0.997	−0.592 ± 0.800	**<0.001**
CD4 T-cell subpopulations			
$CD4^+$ T cells	0.020 ± 1.040	−0.148 ± 0.602	0.372
$CD4^+$ naïve T cells	0.009 ± 1.029	−0.067 ± 0.748	0.688
$CD4^+$ memory T cells	0.035 ± 1.025	−0.253 ± 0.739	0.127
$CD4^+$ Tcm	−0.112 ± 0.942	0.815 ± 1.035	**<0.001**
$CD4^+$ Tem	0.055 ± 1.037	−0.399 ± 0.500	**<0.001**
Tregs	0.087 ± 0.993	−0.639 ± 0.788	**<0.001**
Th1 cells	0.123 ± 0.968	−0.899 ± 0.726	**<0.001**
Th2 cells	0.099 ± 0.976	−0.723 ± 0.858	**<0.001**
CD8 T-cell subpopulations			
$CD8^+$ T cells	0.056 ± 1.011	−0.412 ± 0.795	**0.012**
$CD8^+$ naïve T cells	0.033 ± 1.026	−0.242 ± 0.733	0.065
$CD8^+$ Tcm	0.059 ± 1.027	−0.434 ± 0.609	**<0.001**
$CD8^+$ Tem	0.068 ± 1.032	−0.499 ± 0.470	**<0.001**
Tgd cells	−0.013 ± 0.975	0.098 ± 1.167	0.553
NKT	−0.053 ± 0.980	0.388 ± 1.056	**0.019**
NK cells	0.002 ± 1.006	−0.017 ± 0.957	0.918
B-cell subpopulations			
Pro B cells	0.073 ± 1.010	−0.536 ± 0.720	**0.001**
B cells	0.068 ± 1.001	−0.495 ± 0.776	**0.001**
Naïve B cells	0.018 ± 1.004	−0.129 ± 0.957	0.436
Memory B cells	0.027 ± 1.021	−0.200 ± 0.796	0.227
Class-switched memory B cells	0.054 ± 0.994	−0.398 ± 0.948	**0.016**
Plasma cells	0.031 ± 1.013	−0.227 ± 0.859	0.171
Monocyte/macrophage subpopulations			
Monocytes	0.025 ± 1.026	−0.186 ± 0.751	0.261
Macrophages	0.095 ± 1.019	−0.619 ± 0.505	**<0.001**
Macrophages M1	0.099 ± 1.009	−0.721 ± 0.512	**<0.001**
Macrophages M2	−0.022 ± 1.036	0.166 ± 0.646	0.318
DC subpopulations			
DCs	0.082 ± 1.001	−0.598 ± 0.748	**<0.001**
Activated DCs	0.073 ± 1.016	−0.535 ± 0.647	**<0.001**
Conventional DCs	0.047 ± 1.028	−0.344 ± 0.660	**0.037**
Plasmacytoid DCs	0.080 ± 1.032	−0.585 ± 0.323	**<0.001**
Immature DCs	0.010 ± 1.003	−0.072 ± 0.972	0.662
Granulocyte subpopulations			
Neutrophils	−0.057 ± 0.789	0.414 ± 1.907	0.177
Eosinophils	0.118 ± 1.019	−0.129 ± 0.836	0.435
Mast cells	−0.036 ± 0.973	0.262 ± 1.147	0.114
Basophils	−0.051 ± 0.990	0.373 ± 0.989	**0.024**

NOTE: Bold indicates statistical significance.

Abbreviations: DC, dendritic cell; Tem, effector memory T cell; Tgd cell, gamma delta T cell.

Statistical analysis

We first compared the baseline characteristics between patients in WCH and TCGA cohorts, using one-way ANOVA test for age at diagnosis and χ^2 test (or Fisher exact test if necessary) for other variables. The heatmap was generated according to the Z-score of cell-type enrichment score and ImmuneScore. The overall and cell-type–specific enrichment scores in tumor samples and normal breast tissue samples were compared by *t* test.

The associations of overall and cell-type–specific enrichment score with RFS and OS were evaluated using the Cox proportional hazards model. The Cox proportional hazards assumption was assessed on the basis of Schoenfeld residuals and were not violated. Each score was evaluated by three models for estimating the *P* value and HR with a 95% confidence interval (CI). In model A, HRs were adjusted for demographic factors, including age at diagnosis, menopausal status (premenopause, postmenopause, or unknown), and race (Asian, Black, White, or unknown). In model B, HRs were further adjusted for pathologic factors, including histologic type (invasive ductal/lobular carcinoma and other carcinomas), T stage (T1 and T2+), and nodal status (negative and positive). In model C, HRs were additionally adjusted for treatment modes, including surgery (simple mastectomy, modified radical mastectomy, lumpectomy, or other) and radiotherapy (yes, no, or unknown). Next, the meta-analysis was conducted to pool the HR across the two cohorts, using a random-effects model with the estimate of heterogeneity, and to calculate the summary HR estimates with 95% CI. Kaplan–Meier survival analysis and log-rank test were further conducted for the identified immune cell enrichment scores. We also evaluated whether the association between the identified immune scores and survival differs across different tumor characteristics by model C.

To highlight potential biological mechanisms mediating the prognosis, the logistic regression model was applied to assess the relationship between tumor characteristics and identified immune cell scores that are associated with survival. ORs were adjusted for demographic factors and then pooled using a random-effects model.

False discovery rate (FDR) was calculated to help correct for multiple testing. All analyses were performed in R (Version 3.4.0), Stata (Version 14) and SPSS (Version 23).

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Table 2. Associations of selected immune cell enrichment scores with recurrence-free and overall survival among patients with triple-negative breast cancer

	Model A[a]			Model B[b]			Model C[c]		
	HR (95% CI)	*P*	FDR	HR (95% CI)	*P*	FDR	HR (95% CI)	*P*	FDR
Recurrence-free survival									
$CD4^+$ Tcm									
WCH	1.45 (0.85–2.45)	0.170	0.662	1.51 (0.82–2.79)	0.187	0.798	1.68 (0.87–3.24)	0.122	0.625
TCGA	1.30 (1.01–1.66)	0.042	0.250	1.52 (1.14–2.02)	0.004	0.070	1.64 (1.21–2.22)	0.001	0.035
Meta-analysis	1.33 (1.06–1.66)	0.014	0.222	1.52 (1.17–1.97)	0.002	**0.035**	1.65 (1.25–2.17)	<0.001	**0.009**
I^2, $P_{heterogeneity}$	*I^2 = 0.0%, P = 0.714*			*I^2 = 75.0%, P = 0.985*			*I^2 = 75.0%, P = 0.948*		
Plasma cells									
WCH	0.42 (0.20–0.91)	0.027	0.379	0.43 (0.20–0.89)	0.023	0.403	0.42 (0.20–0.90)	0.026	0.499
TCGA	0.47 (0.25–0.92)	0.027	0.245	0.37 (0.18–0.75)	0.006	0.070	0.34 (0.16–0.73)	0.006	0.093
Meta-analysis	0.45 (0.27–0.73)	0.001	**0.035**	0.40 (0.24–0.67)	<0.001	**0.018**	0.38 (0.22–0.65)	<0.001	**0.009**
I^2, $P_{heterogeneity}$	*I^2 = 0.0%, P = 0.825*			*I^2 = 0.0%, P = 0.775*			*I^2 = 0.0%, P = 0.698*		
Overall survival									
Plasma cells									
WCH	0.18 (0.05–0.65)	0.009	0.245	0.19 (0.06–0.68)	0.010	0.350	0.20 (0.05–0.75)	0.017	0.595
TCGA	0.49 (0.25–0.98)	0.043	0.298	0.39 (0.18–0.81)	0.010	0.210	0.36 (0.16–0.80)	0.012	0.163
Meta-analysis	0.35 (0.14–0.88)	0.026	0.460	0.32 (0.17–0.61)	<0.001	**0.018**	0.31 (0.16–0.62)	0.001	**0.035**
I^2, $P_{heterogeneity}$	*I^2 = 45.2%, P = 0.177*			*I^2 = 0.0%, P = 0.324*			*I^2 = 0.0%, P = 0.465*		

NOTE: Italicized text indicates that I^2 statistic and $P_{heterogeneity}$ were between-study heterogeneity in the meta-analysis. Bold indicates statistical significance.
[a]Adjusted for age at diagnosis, menopause status (premenopause, postmenopause, or unknown), and race (Asian, Black, White, or unknown).
[b]Additionally adjusted for histologic type (invasive ductal/lobular carcinoma and other carcinoma), T stage (T1 and T2+), and nodal status (negative and positive).
[c]Additionally adjusted for surgery (simple mastectomy, modified radical mastectomy, lumpectomy, or other) and radiotherapy (yes, no, or unknown).

Ethical statement

This study was approved by the Clinical Test and Biomedical Ethics Committee of West China Hospital, Sichuan University (Sichuan, China; reference number 2012-130 and 2017-243). Consent forms have been obtained from participants in WCH (Sichuan, China). For participants in the TCGA cohort, specimens were obtained from patients with appropriate consent from institutional review boards at each tissue source site.

Data availability

RNA sequence data of WCH cohort has been deposited in the Sequence Read Archive (SRA accession: PRJNA553096).

Results

The basic characteristics of patients with TNBC

During the median follow-up time of 2.8 (0.1–9.8) years, 42 (17.9%) patients had a recurrence, and 34 (14.5%) patients died. Compared with patients in the TCGA cohort, TNBC patients in the WCH cohort were more likely to be young and premenopausal at diagnosis and undergo simple mastectomy and less likely to receive radiotherapy (Supplementary Table S1). There was no difference in histologic type, T stage, N stage, and TNM stage between patients in two cohorts. Of note, patients in the WCH cohort were all Asians.

The immune landscape of tumor microenvironment in TNBC

The heatmap of overall and type-specific enrichment scores was illustrated to identify the immune landscape of TNBCs (Fig. 2). The overall ImmuneScore in tumors was higher than that in normal breast tissues (Table 1). Compared with normal breast tissues, the majority of T-cell enrichment score was relatively higher in tumor tissues, except for CD4/8-naïve T cells, $CD4^+$ memory T cells, Tgd cells, and NKT cells. Most DC and B-cell enrichment scores were higher in tumors, except for immature DCs, plasma cells, naïve, and memory B cells. The enrichment score of most monocyte/macrophages and granulocyte subsets were nonsignificant between tumor and normal breast tissues, except for macrophage M1 and basophils.

Immune cell enrichment score and patient prognosis

A higher enrichment score of plasma cells was associated with significantly favorable RFS (pooled HR 0.45; 95% CI, 0.27–0.73; FDR = 0.035) after adjustment for demographic factors in model A (Table 2). Similar pooled HRs were yielded when further adjusting for tumor characteristics (model B) and treatment modes (model C). The $CD4^+$ central memory T-cell (Tcm) enrichment score was significantly associated with RFS in model B (pooled HR 1.52; 95% CI, 1.17–1.97; FDR = 0.035). Similar HR was yielded in model C. Such associations for the enrichment score of plasma cells and $CD4^+$ Tcm were similar between the WCH and TCGA cohorts. The RFS curve for high and low enrichment scores of plasma cells and $CD4^+$ Tcm was shown in Fig. 3A and B. A higher enrichment score of macrophages M2 was suggested for worse RFS (Supplementary Table S2).

A higher enrichment score of plasma cells was significantly associated with favorable OS (pooled HR 0.32; 95% CI, 0.17–0.61; FDR = 0.018) after adjustment for demographic and tumor factors in model B (Table 2). When further adjusting for treatment modes (model C), the association between plasma cell score and OS remained significant and similar. The OS curve for high and low enrichment scores of plasma cells was shown in Fig. 3C. The higher enrichment scores of $CD4^+$ Tcm, macrophages M2, immature DCs, and eosinophils were suggested for worse OS, whereas a higher $CD8^+$ Tcm score was suggested for better OS (Supplementary Table S3).

The overall ImmuneScore and the enrichment scores of the rest immune cells were neither associated with RFS nor OS (all P values and FDRs for the pooled HRs > 0.05). Several cell enrichment scores were suggested to be linked with RFS or OS in a certain cohort, but the relationship was not significant after correction by FDR (Supplementary Tables S2 and S3). Most cell enrichment scores did not show statistical heterogeneity across cohorts, except for Th1 cells, Th2 cells, $CD8^+$-naïve T cells, $CD8^+$ T cells, pro B cells, B cells, memory B

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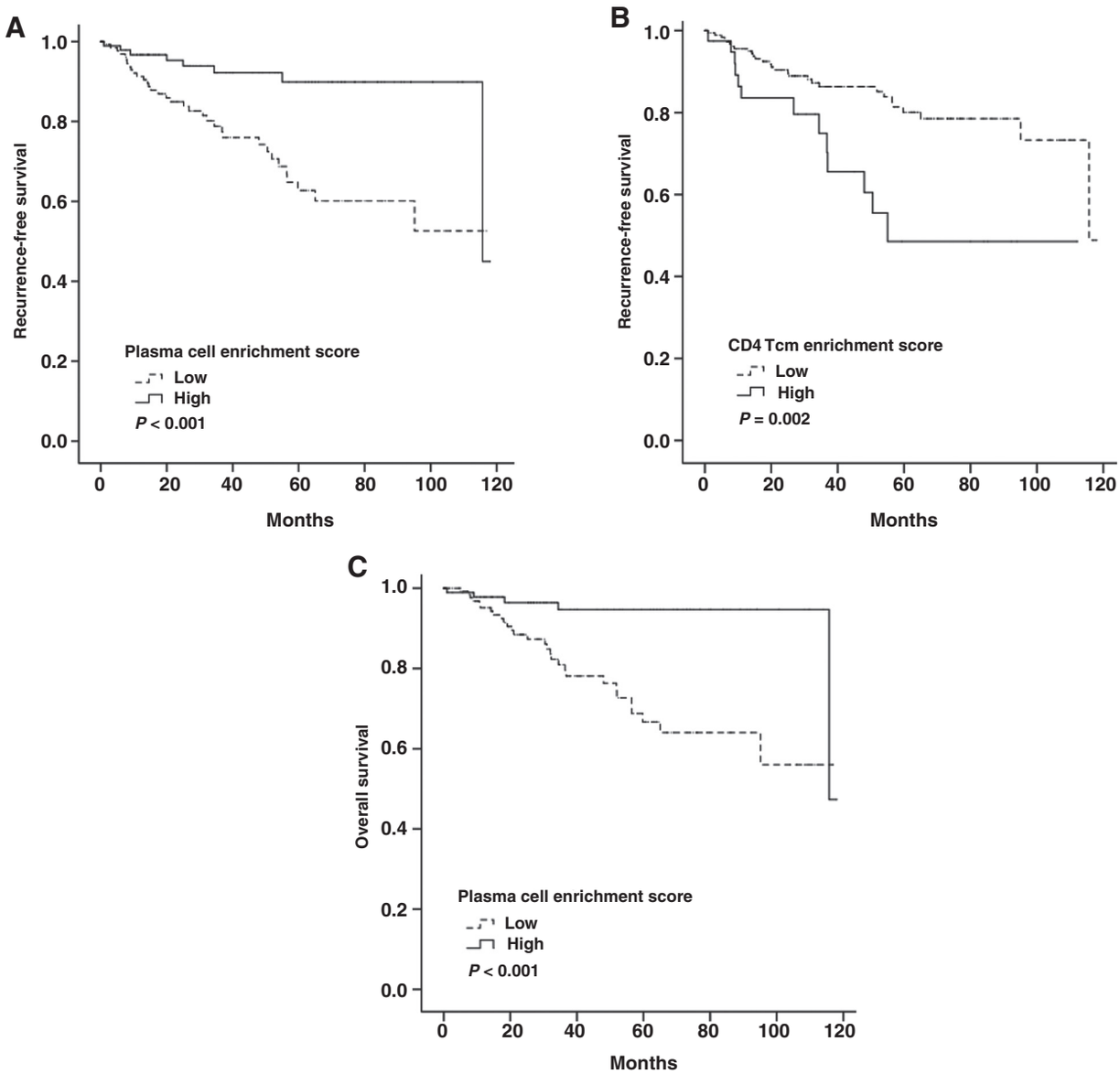

Figure 3.

The survival curve for enrichment scores of identified immune cells. Kaplan–Meier survival analysis and log-rank test were conducted for the identified immune cell enrichment scores. **A** and **B,** Recurrence-free survival for enrichment scores of plasma cells and $CD4^+$ central memory T cells (Tcm). **C,** Overall survival for plasma cell enrichment scores.

cells, class-switched memory B cells, monocytes, macrophages M1, plasmacytoid DCs, and eosinophils, as well as the overall ImmuneScore.

Given the wide CIs, the associations of plasma cells/$CD4^+$ Tcm enrichment score with survival were not clearly modified by tumor characteristics, including the T stage, nodal status, and TNM stage (Supplementary Table S4).

Immune cell enrichment score and tumor characteristics

Compared with invasive ductal/lobular carcinoma, $CD4^+$ Tcm enrichment score was higher in other histologic types of breast cancer (OR 1.59; 95% CI, 1.06–2.37; Table 3). Enrichment score of plasma cells was not significantly associated with any studied tumor characteristics.

Discussion

To the best of our knowledge, this is the first study to comprehensively and systematically assess the association of immune cells in the tumor microenvironment with TNBC prognosis using *in silico* approach. With pooled data of 234 patients with TNBC from the WCH and TCGA cohorts, we identified scores of plasma cells and $CD4^+$ Tcm were associated with patient survival. Of note, such associations were independent of tumor characteristics

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Table 3. Associations of immune cell enrichment scores of tumor tissue with tumor characteristics among patients with triple-negative breast cancer

	WCH	TCGA	Meta-analysis		
	OR (95% CI)[a]	OR (95% CI)[b]	OR (95% CI)	*P*	I^2, $P_{heterogeneity}$
Plasma cells					
T stage (T2+ vs. T1)	0.79 (0.46–1.33)	0.92 (0.65–1.30)	0.88 (0.66–1.18)	0.384	*I^2 = 0.0%, P = 0.638*
Nodal status (positive vs. negative)	1.14 (0.73–1.78)	1.09 (0.77–1.54)	1.11 (0.84–1.46)	0.460	*I^2 = 0.0%, P = 0.876*
Histologic type (other vs. IDC/ILC)	0.62 (0.15–2.53)	0.51 (0.20–1.29)	0.54 (0.25–1.18)	0.122	*I^2 = 0.0%, P = 0.821*
Histologic grade (III vs. II)	0.98 (0.50–1.93)	—			
Ki67 (≥20% vs. <20%)	1.30 (0.59–2.85)	—			
$CD4^+$ Tcm					
T stage (T2+ vs. T1)	1.13 (0.61–2.10)	0.77 (0.53–1.11)	0.86 (0.61–1.20)	0.376	*I^2 = 8.2%, P = 0.297*
Nodal status (positive vs. negative)	1.47 (0.87–2.48)	0.69 (0.38–1.25)	1.02 (0.49–2.14)	0.956	*I^2 = 71.4%, P = 0.062*
Histologic type (other vs. IDC/ILC)	0.88 (0.21–3.73)	1.67 (1.10–2.54)	1.59 (1.06–2.37)	**0.024**	*I^2 = 0.0%, P = 0.402*
Histologic grade (III vs. II)	2.95 (0.84–10.38)	—			
Ki67 (≥20% vs. <20%)	0.80 (0.36–1.80)	—			

NOTE: Italicized text indicates that I^2 statistic and $P_{heterogeneity}$ were between-study heterogeneity in the meta-analysis. Bold indicates statistical significance.
[a]Adjusted by age at diagnosis and menopause status.
[b]Adjusted by age at diagnosis, menopause status, and race.

and treatment modes and were largely consistent between two cohorts. Moreover, $CD4^+$ Tcm enrichment score was lower in invasive ductal/lobular carcinoma than other histologic types.

Our study found that the overall ImmuneScore and most immune cell enrichment scores were higher in tumors than in normal breast tissues. However, the higher overall ImmuneScore was not significantly associated with RFS and OS. This may suggest, although the immune profile is different between tumor and normal breast tissue in patients with TNBC, the complexity of tumor-induced inflammation and different roles of immune cell types may restrain the use of overall immune status as a prognostic biomarker. Thus, analysis of cell type–specific score may further shed light on the players that are most relevant to TNBC prognosis.

Bense and colleagues characterized 22 immune cell types using CIBERSORT algorithm, based on smaller sample size (119 and 95 in DFS and OS analyses, respectively) with fewer endpoint events (19 and 27 in DFS and OS analyses, respectively). They reported that the score of resting NK cells (HR 18.91; 95% CI, 3.05–117.14) was significantly associated with worse DFS in patients with ER-negative/HER2-negative breast cancer (16). A weaker association was found for the activated NK cells (HR 1.63, 95% CI, 0.65–4.12). Although we did not differentiate the resting and activated NK cells, we did not observe such association between NK cells and TNBC prognosis (HR 0.75; 95% CI, 0.44–1.29 for RFS and HR 0.84; 95% CI, 0.50–1.42 for OS, respectively). Of note, ER-negative/HER2-negative breast cancer may not be identical to TNBC given the unknown status of PR.

Plasma cells are derived from B cells. Previous studies showed tumor-infiltrating plasma cells were linked with a contradictory prognostic role in breast cancer (31–34). In this study, a higher enrichment score of plasma cells was significantly associated with favorable RFS (HR 0.45; 95% CI, 0.27–0.73) and OS (HR 0.32; 95% CI, 0.17–0.61) in patients with TNBC. Bense and colleagues also showed that the plasma cell fraction might be related to better DFS (HR 0.38; 95% CI, 0.14–1.08) and OS (HR 0.41; 95% CI, 0.17–1.01) in ER-negative/HER2-negative breast cancer (16). This correlation between favorable prognosis and plasma cells may relate to the protective roles in antitumor immunity, such as antigen spreading, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity (35). One study in ovarian cancer showed that plasma cells were an integral component of $CD8^+$ tumor-infiltrating lymphocyte responses, thus associated with superior prognosis (36).

Memory T cells have a critically important role in host defense for infection and tumor immunity. Several studies found that high density of $CD45RO^+$ memory T cells was associated with an improved prognosis in gastric, esophageal, and colorectal cancer (37–39), while the result in renal cell carcinoma showed in an opposite way (40). Nonetheless, the subtypes of memory T cells were not well distinguished in these studies, and the prognostic role of $CD4^+$ Tcm was not well characterized. Our findings revealed that a high $CD4^+$ Tcm enrichment score was associated with worse RFS (HR 1.52; 95% CI, 1.17–1.97) of patients withTNBC. The association of $CD4^+$ Tcm enrichment score with poor OS was also suggested (HR 1.61; 95% CI, 1.08–2.40). The reason for this negative prognostic role of $CD4^+$ Tcm in patients with TNBC is unknown, complex inhibitory mechanisms may be involved, including apoptosis, cytokine inhibition, angiogenesis, and other regulatory functions on TILs (40). Besides, we found $CD4^+$ Tcm enrichment score was higher in TNBC that was not invasive ductal/lobular carcinoma in histology. Compared with invasive ductal/lobular carcinoma, other histologic types of TNBC, including metaplastic, medullary, and another unspecified carcinoma, were associated with worse RFS and OS in our data (HR 1.99, 95% CI, 1.20–3.30 and HR 1.84, 95% CI, 1.02–3.30, respectively, Supplementary Table S5). One explanation is that TNBC of origins other than ductal/lobular carcinoma may induce differential infiltration of immune cells, such as $CD4^+$ Tcm cells, in the tumor microenvironment. It is also not implausible that local differential inflammation and immune dysregulation in the first-place lead to rare origins of TNBC and therefore worse prognosis. However, due to small numbers, the association of $CD4^+$ Tcm enrichment score with each histologic type and its possible impact on TNBC biology deserves further attention in future studies.

One merit of this study is that we applied a recently developed *in silico* method, xCell. This method can calculate 34 types of cell-type–specific enrichment score for nine categories of immune cells, which encompasses more types of immune cells than existing algorithms and employs a spillover compensation technique to reduce dependencies between closely related cell types. xCell is shown to outperform extensive *in silico* analyses (including CIBERSORT), as well as cytometry immunophenotyping (26). This allows us to evaluate tumor microenvironment

Published OnlineFirst September 18, 2019; DOI: 10.1158/1055-9965.EPI-19-0469

comprehensively. Moreover, the prognostic roles of plasma cells and $CD4^+$ Tcm enrichment score have been mutually confirmed by both WCH and TCGA cohorts. These factors strengthen the power of this study.

Several caveats for our findings should be noted. First, although this study analyzed the immune environment in TNBC pooled from the WCH and TCGA cohorts, the overall sample size was still relatively small, and the median follow-up time was not long enough. Second, there were some heterogeneities in the cellular composition of the several immune cells between the two cohorts. In addition to ethnic difference, there may be potential differences in sample collection, storage, and the process of RNA sequencing and data analyzing. However, we found largely similar associations between the two cohorts. There was no statistical heterogeneity for the prognostic effect of most immune cell enrichment scores across the two cohorts. Moreover, the differences between cohorts are well accounted for the random-effects model. Third, this study only used xCell algorithm to analyze the immune profiling but did not compare with the direct staining or perform the individual marker analysis. However, the validity of the xCell method is partially done using two independent cohorts, in which the FACS fractions of the cell subsets by cytometry immune profiling and the calculated scores by xCell were significantly correlated in most cell subsets (26). Fourth, several genes were not available for xCell scoring in both cohorts. However, most of the nonidentified genes are involved in the gene signatures of stroma cells, including 55 genes of the TCGA cohort and three genes of the WCH cohort. The other nonidentified 24 genes of the TCGA cohort are involved in 64-gene signatures referring to 23 types of immune cells. Forty-four gene signatures have only one gene missing, and 20 gene signatures have 2 to 3 genes missing. The proportion of missing genes in a single gene signature is very small, with a median missing ratio of 3.1% (0.8%–18.1%). Therefore, although these missing genes may cause some inaccuracies in the estimated immune cell scores, the effect should be very small based on the low proportion of missing genes in a single gene signature. Finally, our approach uses gene expression assessed at a single point in time for each woman, and because of this, we are only provided with a biologic snapshot to investigate these immune cells. This approach does not allow us to make temporal or functional claims. The contribution of this article, then, is to demonstrate that some immune cells are related to the prognosis of TNBC, and to provide the guidance that the plasma cells and $CD4^+$ Tcm look most interesting to pursue. Future research is needed to confirm these associations, understand the precise mechanism, and determine whether intervention through these immune cells could be effective for intervention.

In summary, our findings suggest that plasma cells and $CD4^+$ Tcm in the tumor environment may play a role in the subsequent progression of TNBC. Our findings may provide new markers to predict TNBC prognosis. If confirmed in functional studies, our findings may open up new avenues to novel targets of immune therapy in TNBC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: D. Lu, Y.-P. Wang, H. Bu, H. Zheng
Development of methodology: D. Lu, Y. Bai
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-P. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Deng, D. Lu, Y. Bai
Writing, review, and/or revision of the manuscript: L. Deng, D. Lu, Y.-P. Wang, H. Zheng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Bu
Study supervision: D. Lu, Y.-P. Wang, H. Bu, H. Zheng

Acknowledgments

The authors thank TCGA for providing the clinical and RNA-sequencing data. This work was supported by the key program of Science & Technology Department of Sichuan Province (grant number: 2017SZ0005, to H. Zheng), by China Postdoctoral Science Foundation (grant number: 2018M643498, to L. Deng), and by Post-Doctor Research Project, West China Hospital, Sichuan University (grant number: 2019HXBH015, to L. Deng).

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Received April 25, 2019; revised August 15, 2019; accepted September 11, 2019; published first September 18, 2019.

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Published OnlineFirst September 18, 2019; DOI: 10.1158/1055-9965.EPI-19-0469

Cancer Epidemiology, Biomarkers & Prevention

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Cancer Epidemiol Biomarkers Prev 2019;28:1977-1985. Published OnlineFirst September 18, 2019.