Regulation and characterization of tumor-infiltrating immune cells in breast cancer

Qile Dai[a], Weimiao Wu[a], Amei Amei[b], Xiting Yan[a,c], Lingeng Lu[d,*], Zuoheng Wang[a,*]

[a] Department of Biostatistics, Yale School of Public Health, New Haven, CT, USA

[b] Department of Mathematical Sciences, University of Nevada, Las Vegas, NV, USA

[c] Section of Pulmonary, Critical Care and Sleep Medicine, Yale School of Medicine, New Haven, CT, USA

[d] Department of Chronic Disease Epidemiology, Yale School of Public Health, New Haven, CT, USA

* Corresponding authors at: Department of Biostatistics, Yale School of Public Health, 60 College Street, New Haven, CT 06520, USA.

E-mail address: lingeng.lu@yale.edu (L. Lu); zuoheng.wang@yale.edu (Z. Wang).

Abstract

The effect of immunosuppression blockade therapies depends on the infiltration of effector T cells and other immune cells in tumor. However, it is unclear how molecular pathways regulate the infiltration of immune cells, as well as how the interactions between tumor-infiltrating immune cells and T cell activation affect breast cancer patient survival. CIBERSORT was used to estimate the relative abundance of 22 immune cell types. The association between mRNAs and immune cell abundance were assessed by Spearman correlation analysis. Enriched pathways were identified using MetaCore pathway analysis. The interactions between the T cell activation status and the abundance of tumor-infiltrating immune cells were evaluated using Kaplan-Meier survival and multivariate Cox regression models in a publicly available dataset with 1,081 breast cancer patients. The role of tumor-infiltrating B cells in antitumor immunity, immune response of T cell subsets, and breakdown of $CD4^+$ T cell peripheral tolerance were positively associated with M1 macrophage and $CD8^+$ T cell but negatively associated with M2 macrophage. Abundant plasma cell was associated with prolonged survival (HR = 0.46, 95% CI: 0.32-0.67), and abundant M2 macrophage was associated with shortened survival (HR = 1.78, 95% CI: 1.23-2.60). There exists a significant interaction between the T cell activation status and the resting DC abundance level (p = 0.025). Molecular pathways associated with tumor-infiltrating immune cells provide future directions for developing cancer immunotherapies to control immune cell infiltration, and further influence T cell activation and patient survival in breast cancer.

Keywords: breast cancer; molecular pathway; T cell activation score; tumor-infiltrating immune cell; cancer survival.

1. Introduction

In the past decade, significant efforts have been made to treat breast cancer through rebooting the immune system. Cancer immunotherapies aiming to activate T cells through antibody blockade of immunosuppressive signaling pathways have yielded striking responses but often only in a small subset of patients. One determinant of the effective response to immunosuppression blockade is the sufficient infiltration of T cells in a tumor. Infiltrated effector T cells, after priming and activation in the presence of tumor neoantigens, execute their cytotoxic activities to eliminate malignant cells that express neoantigens. Besides effector T cells, there are other types of infiltrating lymphocytes that also play critical roles in inducing activation of effector T cells and serve as promising biomarkers in the early detection, diagnosis, and treatment of primary breast cancer [1].

Among tumor-infiltrating lymphocytes (TILs), $CD8^+$ T cells and natural killer (NK) cells are involved in the antitumor immune response, whereas regulatory T cells (Tregs) inhibit the functionality of high-avidity $CD8^+$ T cells [2]. Tumor-associated macrophages (TAMs), the prominent components of tumor microenvironment, have two subtypes, M1 and M2. M1 macrophages increase inflammation while M2 macrophages decrease inflammation and regulate tissue repair. In breast cancer patients, TAMs have been found to be associated with shorter disease-free survival and overall survival, mainly attributed to their polarization towards the M2 subtype [3]. Other immune cells such as plasma cells and dendritic cells (DCs) are also associated with breast cancer patient survival [4–6]. Therefore, tumor-infiltrating immune cells, such as $CD8^+$ T cells, NK cells, Tregs, macrophages, plasma cells and DCs, are key instruments in immune response. Further understanding of the molecular pathways associated with the abundance of immune cells provides future directions for developing strategies to control the infiltration of

immune cells, as well as enhances knowledge of the biological functions of immune cells in breast cancer.

Priming and activation of effector $CD8^+$ T cell is initiated and influenced by other immune cells. This process is triggered by neoantigens on antigen presenting cells (APCs) which are newly formed antigens generated by tumor-specific DNA somatic mutations that lead to the change of protein sequences. Neoantigens bind to MHC class I molecules and are then recognized by T-cell receptors (TCRs). In addition to TCR signal, $CD8^+$ T cell activation requires a number of secondary costimulatory signals to effectively destroy malignant cells. However, T cell antitumor immunity is often functionally impaired by tumor microenvironment, raising the hypothesis that alternative immune mechanisms may alter the effect of T cell activation. Two possibilities, if not all but at least, demonstrated the barriers of T cell antitumor immunity. One is that other types of tumor-infiltrating immune cells are directly or indirectly involved in the activation of effector T cells. For example, monocytes, Tregs and DCs were previously reported to be associated with the presentation of costimulatory or suppressive signals, thereby affected the full activation of T cells [7,8]. Without being sufficiently activated, effector T cells lose their capacity of developing further immune responses. Another possibility is that, after T cells being fully activated, the antitumor immune response of T cells could be hampered by immune checkpoints derived from other tumor-infiltrating immune cells or tumor cells. For example, tumor-associated polymorphonuclear neutrophils were speculated to be functionally related to myeloid-derived suppressor cells [9], which are known to inhibit the antitumor immune responses of active T cells [10].

Recently, we reported that high T cell activation score was positively associated with improved survival [11] and that BRCA1, a tumor suppressor involved in DNA damage repair, modified the effect of T cell activation on patient survival in breast cancer [12]. However, it is still unclear how

tumor-infiltrating immune cells interact with T cell activation, and how the infiltration/abundance of immune cells in tumor affects patient survival in breast cancer. In the present study, we sought to identify molecular pathways associated with tumor-infiltrating immune cells, as well as investigate how tumor-infiltrating immune cell composition influences patient survival in breast cancer.

2. Materials and methods

2.1. Study subjects

We obtained the clinical data of 1,081 female patients with primary breast cancer from The Cancer Genome Atlas (TCGA) breast invasive carcinoma study available at TCGA provisional (http://www.cbioportal.org/), accessed in July 2018. The average age at diagnosis was 58.4 (range: 26-90) years old. Among the 1,070 patients with disease stage information, most were diagnosed with breast cancer at an early stage including 181 (16.9%) at stage I and 611 (57.1%) at stage II. The other 278 patients (26.0%) were diagnosed at an advanced stage (III or IV). Among the 1,031 women with a known estrogen receptor (ER) status, 77.0% (n = 794) were ER-positive. Among the 1,028 women with a known progesterone receptor (PR) status, 66.9% (n = 688) were PR-positive. Among the 716 women with a known human epidermal growth factor receptor 2 (HER2) status, 22.5% (n = 161) were HER2-positive. No patients received neoadjuvant treatment.

The normalized RNA sequencing (RNA-seq) data on the patients from the TCGA breast invasive carcinoma study were downloaded from Genomic Data Commons (GDC) data portal (https://portal.gdc.cancer.gov/) in November 2018. The dataset contained gene expression of 60,483 mRNAs. Gene expression and clinicopathologic data were merged, resulting in a set of

1,077 patients included in the study. The average follow-up in the 1,077 patients was 40.9 (range: 0-282.7) months. Within the 10-year follow-up period, 139 patients died.

2.2. Immune cell abundance

We applied CIBERSORT [13] to estimate the relative fractions of immune cell types. CIBERSORT implements a support vector regression algorithm to characterize cell composition of complex tissues from the mRNA gene expression profiles, and has been shown to be effective in tumor samples profiled by either microarray or RNA-Seq [14]. CIBERSORT requires a specialized signature matrix including genes whose expression levels collectively define unique gene expression signatures for each cell type of interest. We used the leukocyte gene signature matrix LM22 to estimate the fractions of 22 immune cell types, including seven T-cell types, naïve and memory B cells, plasma cells, NK cells, and myeloid subsets. For each cell type, the patients were classified into two groups, high and low, with the cutoff value chosen to be the median abundance level.

2.3. Statistical analyses

Spearman correlation analysis was used to identify genes significantly associated with immune cell abundance with an adjusted p-value less than 0.05. Significantly enriched pathways were identified with an adjusted p-value less than 0.05 using MetaCore^{TM} pathway analysis. The p-values were adjusted by the Benjamini-Hochberg (BH) procedure to control false discovery rate (FDR) less than 0.05 [15].

A weighted T cell activation score was calculated for each subject based on 13 genes relevant to the T cell activation status as previously described [11]. We further divided patients into two

groups, activation and exhaustion. Overall survival was the time from surgery until death or the last follow-up. Patients died after 10-year follow-up period were treated as censored at the end of 10 years. The relationships between the abundance levels of immune cells and overall survival were investigated using multivariate Cox proportional hazard models. Patient's age at diagnosis, disease stage, T cell activation status, and ER status were included as covariates in the models to obtain the adjusted hazard ratios (HRs) and their 95% confidence intervals (95% CIs). Interactions between immune cell abundance and T cell activation status were assessed by including their interactions in the Cox regression models. Backward elimination using Akaike information criterion (AIC) was implemented for model selection. The effects of the T cell activation status on patient survival were further assessed in each subgroup stratified by the immune cell abundance levels. Proportional hazards assumption was also examined. Results were considered statistically significant when p-values were less than 0.05. All statistical analyses were performed in R (https://www.r-project.org/).

3. Results

3.1. Abundance of tumor-infiltrating immune cells and pathway analysis

To pursue immunologic molecules that affect the abundance of TILs in breast cancer, we first estimated the relative abundance of tumor-infiltrating immune cells using CIBERSORT, followed by Spearman correlation analysis between transcriptome-wide gene expression and the abundance of $CD8^+$ T cells, Tregs, activated NK cells, plasma cells, M1 macrophages, M2 macrophages, activated DCs, and resting DCs, respectively. The top 500 significant genes were divided into two gene sets based on the direction of the correlations. To understand the biological functions of the

correlated genes, pathway enrichment analysis was performed using MetaCore. The top 5 significant pathways for each of the 8 cell types are displayed in Table 1.

Table 1. Top 5 significant pathways enriched in genes associated with $CD8^+$ T cells, regulatory T cells, activated NK cells, plasma cells, M1 macrophages, M2 macrophages, activated dendritic cells, and resting dendritic cells.

$CD8^+$ T cells					
Positive association	P-value	FDR	**Negative association**	P-value	FDR
Role of tumor-infiltrating B cells in anti-tumor immunity	2.19E-27	<0.001	Neurophysiological process_Dynein-dynactin motor complex in axonal transport in neurons	1.56E-07	<0.001
Breakdown of CD4+ T cell peripheral tolerance in type 1 diabetes mellitus	1.63E-21	<0.001	Development_TGF-beta-dependent induction of EMT via MAPK	6.6E-07	<0.001
Immune response_T cell subsets: cell surface markers	2.31E-19	<0.001	Cytoskeleton remodeling_Integrin outside-in signaling	9.21E-07	<0.001
SLE genetic marker-specific pathways in T cells	4.08E-19	<0.001	Effect of H. pylori infection on gastric epithelial cells motility	4.62E-06	0.001
NK cells in allergic contact dermatitis	8.13E-18	<0.001	TGF-beta 1-mediated induction of EMT in normal and asthmatic airway epithelium	5.41E-06	0.001
Regulatory T cells					
Positive association			**Negative association**		
Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	1.01E-06	0.001	Development_Stimulation of differentiation of mouse embryonic fibroblasts into adipocytes by extracellular factors	2.88E-10	<0.001
Activation of TNF-alpha-dependent pro-tumoral effect in colorectal cancer	2.98E-06	0.001	Ovarian cancer (main signaling cascades)	1.61E-09	<0.001
Immune response_IFN-alpha/beta signaling via PI3K and NF-kB pathways	3.49E-06	0.001	PI3K signaling in gastric cancer	2.01E-09	<0.001
Immune response_Role of PKR in stress-induced antiviral cell response	8.41E-06	0.002	Apoptosis and survival_NGF/ TrkA PI3K-mediated signaling	1.03E-08	<0.001
Apoptosis and survival_Lymphotoxin-beta receptor signaling	1.56E-05	0.003	Development_Thromboxane A2 signaling pathway	2.93E-08	<0.001
Activated NK cells					
Positive association			**Negative association**		
Immune response_Induction of the antigen presentation machinery by IFN-gamma	9.82E-07	0.001	Apoptosis and survival_NGF/ TrkA PI3K-mediated signaling	3.15E-09	<0.001
Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	3.77E-06	0.001	Signal transduction_PKA signaling	6.64E-09	<0.001
Apoptosis and survival_Lymphotoxin-beta receptor signaling	3.77E-06	0.001	Development_Stimulation of differentiation of mouse embryonic fibroblasts into adipocytes by extracellular factors	1.55E-08	<0.001
Ubiquinone metabolism	9.61E-06	0.002	Development_Positive regulation of WNT/Beta-catenin signaling in the cytoplasm	3.24E-08	<0.001
Survival pathways in Prostate Cancer	1.07E-05	0.002	Immune response_IL-3 signaling via ERK and PI3K	8.16E-08	<0.001
Plasma cells					
Positive association			**Negative association**		
Possible regulation of HSF-1/ chaperone pathway in Huntington's disease	1.58E-06	0.001	Cell adhesion_Integrin inside-out signaling in neutrophils	2.17E-19	<0.001
Signal transduction_mTORC1 downstream signaling	1.97E-06	0.001	Inhibition of neutrophil migration by proresolving lipid mediators in COPD	5.5E-19	<0.001
Mechanisms of resistance to EGFR inhibitors in lung cancer	7.83E-05	0.016	Chemokines in inflammation in adipose tissue and liver in obesity, type 2 diabetes and metabolic syndrome X	1.85E-17	<0.001
			Maturation and migration of dendritic cells in skin sensitization	1.91E-17	<0.001
			Rheumatoid arthritis (general schema)	4.37E-17	<0.001
M1 macrophages					
Positive association			**Negative association**		
Role of tumor-infiltrating B cells in anti-tumor immunity	2.09E-30	<0.001			
Immune response_T cell subsets: cell surface markers	3.15E-28	<0.001			
NK cells in allergic contact dermatitis	5.19E-28	<0.001			
Breakdown of CD4+ T cell peripheral tolerance in type 1 diabetes mellitus	3.2E-27	<0.001			
iNKT cell-keratinocyte interactions in allergic contact dermatitis	1.52E-23	<0.001			

M2 macrophages					
Positive association			**Negative association**		
Insulin-dependent stimulation of SREBP-1 in type 2 diabetes in liver	5.13E-07	<0.001	Role of tumor-infiltrating B cells in anti-tumor immunity	7.36E-23	<0.001
LRRK2 in neurons in Parkinson's disease	1.81E-06	0.001	T follicular helper cell dysfunction in SLE	3.05E-20	<0.001
Regulation of lipid metabolism_Regulation of lipid metabolism via LXR, NF-Y and SREBP	4.31E-06	0.001	Immune response_T cell subsets: cell surface markers	8.24E-20	<0.001
Regulation of metabolism_Bile acids regulation of glucose and lipid metabolism via FXR	6.83E-06	0.001	Breakdown of CD4+ T cell peripheral tolerance in type 1 diabetes mellitus	8.62E-19	<0.001
Development_Ligand-independent activation of ESR1 and ESR2	1.04E-05	0.002	SLE genetic marker-specific pathways in T cells	8.48E-18	<0.001
Activated dendritic cells					
Positive association			**Negative association**		
Inhibition of Ephrin receptors in colorectal cancer	3.17E-06	0.002	Breast cancer (general schema)	2.82E-07	<0.001
Beta-catenin-dependent transcription regulation in colorectal cancer	9.69E-06	0.003	Signal transduction_Cyclic AMP signaling	5.72E-05	0.015
Neutrophil chemotaxis in asthma	1.34E-05	0.003	Gamma-secretase regulation of mammary cell development	7.37E-05	0.015
Immune response_IL-17 signaling pathways	1.98E-05	0.004	Stromal-epithelial interaction in Prostate Cancer	9.36E-05	0.015
Inflammatory mechanisms of pancreatic cancerogenesis	4.1E-05	0.006	Signal transduction_mTORC2 downstream signaling	0.000102	0.015
Resting dendritic cells					
Positive association			**Negative association**		
T cell generation in COPD	1.04E-08	<0.001	Cell cycle_The metaphase checkpoint	9.68E-29	<0.001
Role of Langerin+ dermal dendritic cells in contact hypersensitivity	1.21E-06	0.001	Cell cycle_Role of APC in cell cycle regulation	1.17E-20	<0.001
Populations of skin dendritic cells involved in contact hypersensitivity	1.50E-05	0.005	Cell cycle_Spindle assembly and chromosome separation	2.23E-20	<0.001
Common mechanisms of Th17 cell migration	2.70E-05	0.005	Cell cycle_Start of DNA replication in early S phase	3.58E-17	<0.001
Chemokines in inflammation in adipose tissue and liver in obesity, type 2 diabetes and metabolic syndrome X	2.70E-05	0.005	Cell cycle_Chromosome condensation in prometaphase	1.01E-16	<0.001

In genes positively correlated with $CD8^+$ T cells, enriched pathways included the role of tumor-infiltrating B cells in antitumor immunity, breakdown of $CD4^+$ T cell peripheral tolerance, SLE genetic marker-specific pathways in T cells, and NK cells in allergic contact dermatitis. In contrast, in genes negatively correlated with $CD8^+$ T cells, pathway associated with neurophysiological process, TGF-β1-dependent induction of epithelial to mesenchymal transition (EMT), and *Helicobacter pylori* infection were enriched.

For genes positively correlated with Tregs, the top 5 pathways included anti-apoptotic TNFs/NF-kB/Bcl-2, activation of TNF-α-dependent pro-tumoral effect, and IFN-α/β signaling. For genes negatively correlated with Tregs, the top 5 pathways included PI3K signaling, NGF/TrkA PI3K-mediated signaling, and thromboxane A2 signaling.

Induction of the antigen presentation machinery by IFN-γ, anti-apoptotic TNFs/NF-kB/Bcl-2 pathway, and ubiquinone metabolism pathway were positively correlated with activated NK cells. NGF/ TrkA PI3K-mediated signaling, PKA signaling, WNT/β-catenin signaling, and IL-3 signaling pathway were negatively correlated with activated NK cells.

Regulation of HSF-1/ chaperone pathway, mTORC1 downstream signaling, and mechanisms of resistance to EGFR inhibitors pathway were enriched in genes positively correlated with plasma cells. Integrin inside-out signaling in neutrophils, inhibition of neutrophil migration by proresolving lipid mediators, and chemokines in inflammation in adipose tissue pathway were enriched in genes negatively correlated with plasma cells.

For M1 macrophages, pathways associated with antitumor immunity of tumor-infiltrating B cells, immune response of T cell subsets, breakdown of $CD4^+$ T cell peripheral tolerance, and NK cells in allergic contact dermatitis were enriched in genes positively correlated with M1 macrophages. No pathway was significantly enriched in genes negatively correlated with M1 macrophages. For M2 macrophages, genes involved in stimulation of SREBP-1, LRRK2 mutation, and regulation of lipid metabolism were upregulated. The role of tumor-infiltrating B cells in antitumor immunity, T follicular helper cell dysfunction in SLE, and immune response of T cell subsets pathway were enriched in genes negatively correlated with M2 macrophages.

Inhibition of Ephrin receptors, β-catenin-dependent transcription regulation, and IL-17 signaling pathway were positively associated with activated DCs. Pathways negatively associated with activated DCs included breast cancer (general schema), cyclic AMP signaling, and mTORC2 downstream signaling. The role of Langerin+ dermal DCs, populations of skin DCs involved in contact hypersensitivity pathway were positively associated with resting DCs. All of the top 5 pathways negatively associated with resting DCs were related to cell cycle.

3.2. Relationships between mortality and abundance of tumor-infiltrating immune cells

The correlations between the abundance level of each immune cell type and patient mortality were evaluated. After model selection, five infiltrated immune cell types were retained in the multivariate Cox proportional hazard model: plasma cell, M2 macrophage, resting DC, resting memory $CD4^+$ T cell, and neutrophil, adjusted for patient's age of diagnosis, disease stage, ER status, and T cell activation. Among them, the abundance levels of three cell types, plasma cell, M2 macrophage, and resting DC were significantly associated with patient mortality (Table 2).

Table 2. Relationships between overall survival and abundance of plasma cell, M2 macrophage, and resting dendritic cell.

		Death	
Variables	**Number of Patients**	**HR (95% CI)**	***p*-value**
T Cell Activation			
Exhaustion	852	1.00	
Activation	163	0.49 (0.27 - 0.90)	0.021
Plasma Cell			
Low	500	1.00	
High	515	0.49 (0.34 - 0.71)	<0.001
M2 Macrophage			
Low	514	1.00	
High	501	1.74 (1.18 - 2.58)	0.006
Resting Dendritic Cell			
Low	502	1.00	
High	513	0.63 (0.44 - 0.91)	0.012
Resting Memory $CD4^+$ T Cell			
Low	509	1.00	
High	506	1.44 (0.99 - 2.07)	0.051
Neutrophil			
Low	775	1.00	
High	240	1.49 (0.98 - 2.25)	0.061
Age (per 5 years)	1015	1.20 (1.12 - 1.28)	<0.001
ER			
ER-negative	232	1.00	
ER-positive	783	0.40 (0.27 - 0.61)	<0.001

Stage			
Stage I	173	1.00	
Stage II	581	2.05 (1.08 - 3.88)	0.027
Stage III or IV	261	6.73 (3.55 - 12.74)	<0.001

A higher fraction of plasma cells was associated with decreased risk of mortality. The adjusted HR was 0.49 (95% CI: 0.34-0.71) for the high abundance plasma cell group vs. the low abundance group. The 5-year overall survival rate was 0.87 (95% CI: 0.83-0.92) in the high abundance plasma cell group, and 0.78 (95% CI: 0.73-0.84) in the low abundance group (Fig. 1A). A higher fraction of M2 macrophages was associated with increased risk of mortality. The adjusted HR was 1.74 (95% CI: 1.18-2.58) for the high abundance M2 macrophage group vs. the low abundance group. The 5-year overall survival rate was 0.77 (95% CI: 0.71-0.83) in the high abundance M2 macrophage group, and 0.88 (95% CI: 0.84-0.92) in the low abundance group (Fig. 1B). A higher fraction of resting DCs was associated with decreased risk of mortality. The adjusted HR was 0.63 (95% CI: 0.44-0.91) for the high abundance resting DC group vs. the low abundance group. The 5-year overall survival rate was 0.88 (95% CI: 0.84-0.93) in the high abundance resting DC group, and 0.77 (95% CI: 0.71-0.83) in the low abundance group (Fig. 1C). Overall, patients with a high T cell activation score (activation, n = 175) had better overall survival compared to those with a low T cell activation score (exhaustion, n = 906). The adjusted HR was 0.49 (95% CI: 0.27-0.90) for the activation group vs. the exhaustion group.

We also performed analysis stratified by stage. Due to a small sample size in Stage I, none of the immune cell types were significantly associated with patient mortality. In Stage II of 581 patients, only M2 macrophage was significantly associated with patient mortality. In Stage III or IV with 261 patients, plasma cell, resting DC, resting memory $CD4^+$ T Cell and neutrophil were

significantly associated with patient mortality. The direction of association was the same as that in the whole sample, for each of the immune cell types.

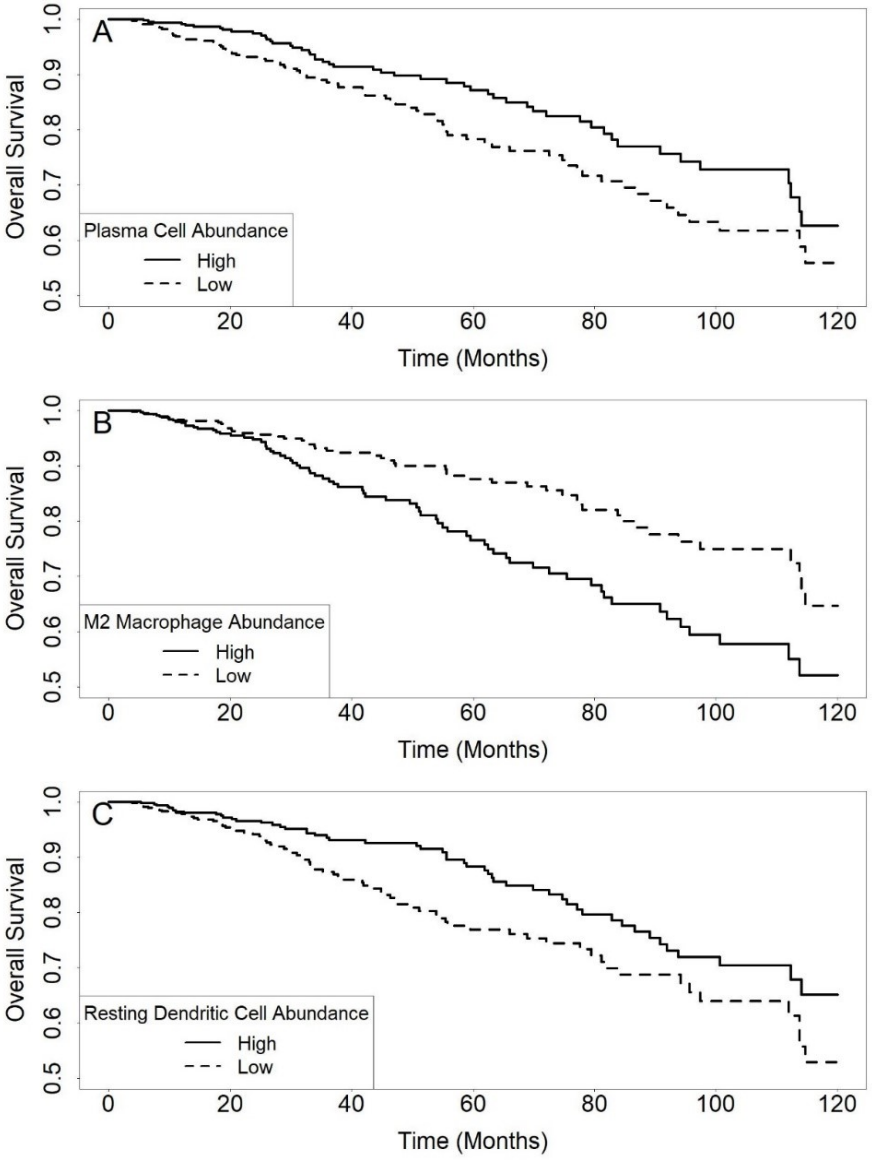

Figure 1. Kaplan-Meier survival curves of breast cancer patients. (**A**) Patients with a high plasma cell abundance level had better overall survival compared to those with a low abundance level (p = 0.011). (**B**) Patients with a high M2 macrophage abundance level had worse overall survival compared to those with a low abundance level (p = 0.001). (**C**) Patients with a high resting dendritic cell abundance level had better overall survival compared to those with a low abundance level (p = 0.007).

3.3. Interaction between T cell activation status and resting dendritic cell abundance in patient survival

Interactions between immune cell abundance and T cell activation status were assessed in the Cox regression model, with adjustment for patient's age of diagnosis, disease stage, and ER status. After model selection, only the interaction between resting DC and T cell activation was significant (p = 0.025, Table 3). The abundance levels of plasma cell and M2 macrophage remained significantly correlated with overall survival. A higher fraction of plasma cells was associated with decreased risk of mortality. The adjusted HR was 0.46 (95% CI: 0.32-0.67) for the high abundance plasma cell group vs. the low abundance group. A higher fraction of M2 macrophages was associated with increased risk of mortality. The adjusted HR was 1.78 (95% CI: 1.23-2.60) for the high abundance M2 macrophage group vs. the low abundance group.

Table 3. Interaction between T cell activation status and resting dendritic cell abundance in the whole sample.

	Death		
Variables	**HR**	**95% CI**	***p*-value**
T Cell Activation × Resting Dendritic Cell	4.74	1.21 - 18.54	0.025
T Cell Activation			
Exhaustion	1.00		
Activation	0.19	0.06 - 0.62	0.006
Plasma Cell			
Low	1.00		
High	0.46	0.32 - 0.67	<0.001
M2 Macrophage			
Low	1.00		
High	1.78	1.23 - 2.60	0.002
Resting Dendritic Cell			
Low	1.00		
High	0.56	0.38 - 0.82	0.003
Age (per 5 years)	1.21	1.13 - 1.29	<0.001
ER			
ER-negative	1.00		
ER-positive	0.41	0.27 - 0.61	<0.001
Stage			
Stage I	1.00		
Stage II	2.00	1.06 - 3.79	0.032

Stage III or IV	6.14	3.26 - 11.55	<0.001

We further investigated the relationship between mortality and T cell activation status stratified by the abundance level of resting DC (Table 4). In the low abundance resting DC group, patients with a high T cell activation score (activation) showed better overall survival compared to those with a low T cell activation score (exhaustion) (Fig. 2A). The 5-year overall survival rate was 0.90 (95% CI: 0.79-1.00) in the activation group, and 0.75 (95% CI: 0.69-0.82) in the exhaustion group. The adjusted HR was 0.24 (95% CI: 0.07-0.78) for activation vs. exhaustion. In contrast, among the patients with a high abundance level of resting DC, there was no significant difference in overall survival between the activation and exhaustion groups (Fig. 2B). The 5-year overall survival rate was 0.91 (95% CI: 0.83-1.00) in the activation group, and 0.88 (95% CI: 0.83-0.93) in the exhaustion group. The adjusted HR was 0.84 (95% CI: 0.42-1.68) for activation vs. exhaustion.

Table 4. Association of T cell activation status and overall survival of breast cancer stratified by the resting dendritic cell abundance level

Stratification		**Death**		
Variable	**Variables**	**HR**	**95% CI**	***p*-value**
Low resting dendritic cell abundance	T Cell Activation			
	Exhaustion	1.00		
	Activation	0.24	0.07 - 0.78	0.019
	Plasma Cell			
	Low	1.00		
	High	0.58	0.36 -0.94	0.028
	M2 Macrophage			
	Low	1.00		
	High	2.18	1.28 - 3.70	0.004
	Age (per 5 years)	1.24	1.12 - 1.36	<0.001
	ER			
	ER-negative	1.00		
	ER-positive	0.44	0.25 - 0.77	0.004
	Stage			
	Stage I	1.00		
	Stage II	2.11	0.81 - 5.47	0.125

	Stage III or IV	6.69	2.59 - 17.29	<0.001
High resting dendritic cell abundance	T Cell Activation			
	Exhaustion	1.00		
	Activation	0.84	0.42 - 1.68	0.617
	Plasma Cell			
	Low	1.00		
	High	0.36	0.20 - 0.67	0.001
	M2 Macrophage			
	Low	1.00		
	High	1.39	0.79 - 2.44	0.249
	Age (per 5 years)	1.19	1.08 - 1.32	0.001
	ER			
	ER-negative	1.00		
	ER-positive	0.34	0.19 - 0.62	<0.001
	Stage			
	Stage I	1.00		
	Stage II	1.91	0.80 - 4.55	0.145
	Stage III or IV	5.59	2.35 - 13.25	<0.001

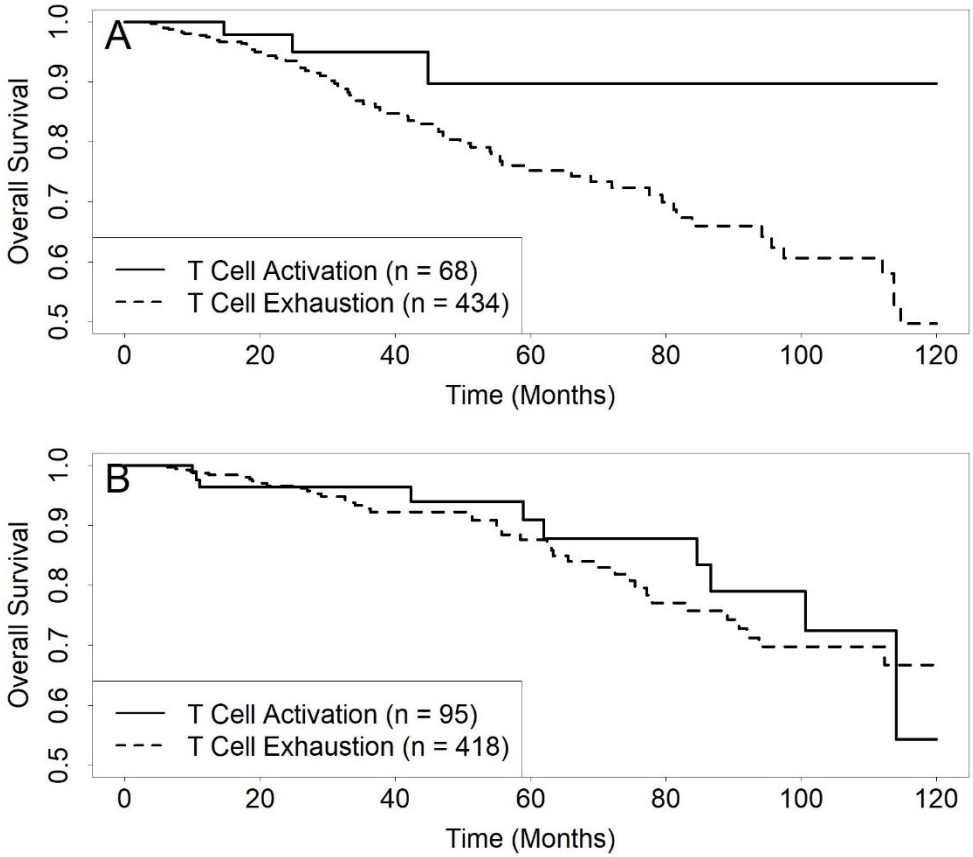

Figure 2. Kaplan-Meier survival curves of breast cancer patients stratified by the abundance level of resting dendritic cell. (**A**) In the subgroup with a low abundance level of resting dendritic cell, patients in the T cell activation group had better overall survival compared to those in the exhaustion group ($p = 0.032$). (**B**) In the subgroup with a high abundance level of resting dendritic cell, there was no significant difference in overall survival between patients in the T cell activation and exhaustion groups ($p = 0.680$).

Finally, we performed pathway enrichment analysis on 1,472 correlated genes shared by the three immune cell types, plasma cell, M2 macrophage and resting DC. Breakdown of $CD4^+$ T cell peripheral tolerance, immune response of T cell subsets, and the role of tumor-infiltrating B cells in antitumor immunity pathways were enriched for these three immune cell types.

4. Discussion

In this study, we identified molecular pathways associated with tumor-infiltrating immune cells and assessed the association between tumor-infiltrating immune cells and patient survival in breast cancer. Pathways associated with immune cell abundance not only provided advanced understanding of the functions of tumor-infiltrating immune cells, but also revealed potential targets for controlling the infiltration of immune cells in breast cancer. We also illuminated that abundant plasma cells were associated with prolonged survival, abundant M2 macrophages were associated with shortened survival, and abundant tumor-infiltrating resting DCs weakened the auspicious impact of T cell activation on survival in breast cancer.

Various strategies to enhance the antitumor immunity of $CD8^+$ T cells have been developed. However, the effective response to the immunosuppression blockade therapies depends on the infiltration of $CD8^+$ T cells in a tumor prior to therapy. Our study identified negative association between the TGF-β1-dependent induction of EMT and $CD8^+$ T cell infiltration. EMT, a process by which fully differentiated epithelial cells transit to mesenchymal stem cells, has been reported

to contribute to tumor development, invasion and metastasis formation [16]. The CXCR3 ligand, CXCL9, which is associated with heavy infiltration of $CD8^+$ T cells, has been demonstrated to abrogate TGF-β1-induced EMT in human alveolar epithelial cells [17]. The inverse correlation between EMT and T cell infiltration was observed in non-small cell lung cancer [18]. In addition, we found that the gastric epithelial cells motility induced by *Helicobacter pylori* infection was negatively associated with $CD8^+$ T cell abundance. These findings suggest that inhibition of TGF-β1 pathway may increase the recruitment of $CD8^+$ T cells.

Tregs play an essential role in maintaining immunological self-tolerance that may hamper effective tumor immunity [19,20]. CD4+Foxp3+ Tregs are the major Treg population in the immune system. We found that Nuclear Factor-κB (NF-κB) was positively correlated with the abundance of Tregs, consistent with the important role of the expression of receptor activator of NF-κB ligand (RNAKL) in the induction of CD4+FOXP3+ Tregs in mammary tumors [21].

NK cells are another effector tumor killer cells that detect and induce apoptosis of susceptible target cells. Activated NK cells function to secrete immunoregulatory cytokines such as IFN-γ and TNF-α [22]. IFN-γ enhances the ability of $CD8^+$ T cells to kill tumor cells [23]. It is expected that anti-apoptotic TNFs/NF-kB/Bcl-2 pathway was positively associated with the abundance of activated NK cells. Our study identified negative association between activated NK cell abundance and PKA signaling. The increased adenosine monophosphate (cAMP) levels mediated by the cAMP-dependent PKA inhibit the ability of NK cells to lyse tumor target cells [24]. We found that anti-apoptotic TNFs/NF-kB/Bcl-2 pathway was positively correlated with both activated NK cells and Tregs, and NGF/ TrkA PI3K-mediated signaling was negatively correlated with both activated NK cells and Tregs, indicating the complicated influences of TNFs/NF-kB/Bcl-2 pathway and NGF/ TrkA PI3K-mediated signaling and in tumor immunity.

Differentiated from B cells, plasma cells are lymphocytes that produce antibodies. Once released into the blood and lymph, the antibody molecules bind to the target antigen and initiate neutralization or destruction. We showed that the abundance of plasma cells was negatively associated with the inflammation in adipose tissue, indicating that obesity might inhibit heavy plasma cell infiltration [25].

Macrophages are a major type of immune cells infiltrating into tumor microenvironment. They are polarized to either M1 or M2 subtypes in the context of the microenvironment, and perform diverse functions such as tissue development and homeostasis, inflammation, pathogen clearance, and wound healing [26]. M1 and M2 macrophage subtypes have opposite effects in cancer progression: M2 macrophages promote tumor growth while M1 macrophages suppress proliferation [27]. Our study identified that the role of tumor-infiltrating B cells in antitumor immunity, immune response of T cell subsets, and breakdown of $CD4^+$ T cell peripheral tolerance pathway were positively associated with M1 macrophages and $CD8^+$ T cells but negatively associated with M2 macrophages, consistent with their opposite effects on tumor progression. We also found positive correlation between M2 macrophage and lipid metabolism, suggesting the involvement of obesity in the activation of M2 macrophages.

DCs perform different functions depending on their maturation state and the environment they encounter. Mature DCs are APCs that digest antigen peptides and present them on the cell surface to prime naïve T cells, inducing cytotoxic T cell responses [28]. However, resting DCs in an immature state are considered tolerogenic due to its inability to present antigens. They maintain immune tolerance by impeding adaptive immune cells from attacking host cells, and have been shown to induce antigen-specific T cell tolerance by failing to present them completely to naïve T cells in animal models [29–33]. We found that breast cancer (general schema) was the most

significant pathway negatively correlated with activated DCs, attenuating the abundance of activated DCs in breast cancer. The mTORC2 downstream signaling pathway was also negatively associated with activated DCs, consistent with the previous finding that the mTORC2 deficiency in DCs enhances their ability of inducing effective immune responses [34]. In contrast, we found that the abundance of resting DCs was negatively associated with the role of APC in cell cycle regulation.

Our results illuminated the prognostic effects of infiltrating plasma cells and M2 Macrophages in breast cancer. The prognostic effect of tumor-infiltrating plasma cells has previously been reported in breast cancer [35], ovarian cancer [36], colorectal cancer [35,37], non-small cell lung cancer [35,38], and gastric cancer [39]. These studies reported improved survival rates in the presence of a high level of plasma cells. Our findings also demonstrated that dense plasma cell infiltration improved overall survival in breast cancer. TAMs generally acquire the M2-like properties [3] and were associated with worse survival in breast cancer patients [40–42]. Our study confirmed that abundant M2 macrophages were associated with shortened overall survival.

Recently, therapeutic antibodies targeting immune checkpoints, including programmed cell death 1 (PD-1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), have yielded unprecedented success in immunotherapy on some breast cancer patients but not others [43], indicating the immunological diversity underlying the responses to the checkpoint immunotherapies. Besides the insufficient infiltration of immune cells, another possible explanation is that even though T cells are activated by therapeutic antibodies, the auspicious effect of T cell activation could be modified by other components of the immune system such as tumor-infiltrating immune cells. We found that in the high abundance group of resting DCs, there was better survival but not statistically significant for the activation vs. exhaustion groups. In contrast,

among the patients with a low level of resting DCs, high T cell activation score was associated with improved survival. It will be of great interest to further explore the conditions that facilitate the maintenance of DCs in an immature or resting state. Treatment therapies can be designed to reduce the abundance of resting DCs or induce the differentiation of resting DCs into mature DCs to ensure the auspicious effect of T cell activation in antitumor immunity.

In conclusion, we identified molecular pathways associated with tumor-infiltrating immune cells and investigated the interaction of tumor-infiltrating immune cells and T cell activation on patient survival to uncover the biological functions of immune cells in breast cancer. Our results provided future directions for the development of effective strategies for regulating immune cell infiltration in cancer immunotherapies.

Funding

This work was supported by the National Institutes of Health [grant number AA023321]; and the National Science Foundation [grant number DMS1916246]. However, the funding body has no role in the design of the study and collection, analysis and interpretation of the data and in writing the manuscript.

Declaration of Competing Interest

The authors have declared no conflicts of interest.

References

[1] R.D. Bense, C. Sotiriou, M.J. Piccart-Gebhart, J.B.A.G. Haanen, M.A.T.M. van Vugt, E.G.E. de Vries, C.P. Schröder, R.S.N. Fehrmann, Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for Disease Outcome in Breast Cancer, Journal of the National Cancer Institute. 109 (2017) djw192. https://doi.org/10.1093/jnci/djw192.

[2] A.M. Ercolini, B.H. Ladle, E.A. Manning, L.W. Pfannenstiel, T.D. Armstrong, J.-P.H. Machiels, J.G. Bieler, L.A. Emens, R.T. Reilly, E.M. Jaffee, Recruitment of latent pools of high-avidity CD8+ T cells to the antitumor immune response, Journal of Experimental Medicine. 201 (2005) 1591–1602. https://doi.org/10.1084/jem.20042167.
[3] A. Mantovani, A. Sica, Macrophages, innate immunity and cancer: balance, tolerance, and diversity, Current Opinion in Immunology. 22 (2010) 231–237. https://doi.org/10.1016/j.coi.2010.01.009.
[4] J. Yeong, J.C.T. Lim, B. Lee, H. Li, N. Chia, C.C.H. Ong, W.K. Lye, T.C. Putti, R. Dent, E. Lim, A.A. Thike, P.H. Tan, J. Iqbal, High Densities of Tumor-Associated Plasma Cells Predict Improved Prognosis in Triple Negative Breast Cancer, Front. Immunol. 9 (2018). https://doi.org/10.3389/fimmu.2018.01209.
[5] B.J. Coventry, P.-L. Lee, D. Gibbs, D.N.J. Hart, Dendritic cell density and activation status in human breast cancer – CD1a, CMRF-44, CMRF-56 and CD-83 expression, British Journal of Cancer. 86 (2002) 546–551. https://doi.org/10.1038/sj.bjc.6600132.
[6] I. Treilleux, Dendritic Cell Infiltration and Prognosis of Early Stage Breast Cancer, Clinical Cancer Research. 10 (2004) 7466–7474. https://doi.org/10.1158/1078-0432.CCR-04-0684.
[7] M.V. Sitkovsky, T regulatory cells: hypoxia-adenosinergic suppression and re-direction of the immune response, Trends in Immunology. 30 (2009) 102–108. https://doi.org/10.1016/j.it.2008.12.002.
[8] S. Gallucci, M. Lolkema, P. Matzinger, Natural adjuvants: Endogenous activators of dendritic cells, Nature Medicine. 5 (1999) 1249–1255. https://doi.org/10.1038/15200.
[9] A.J. Gentles, A.M. Newman, C.L. Liu, S.V. Bratman, W. Feng, D. Kim, V.S. Nair, Y. Xu, A. Khuong, C.D. Hoang, M. Diehn, R.B. West, S.K. Plevritis, A.A. Alizadeh, The prognostic landscape of genes and infiltrating immune cells across human cancers, Nature Medicine. 21 (2015) 938–945. https://doi.org/10.1038/nm.3909.
[10] D.I. Gabrilovich, S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system, Nat. Rev. Immunol. 9 (2009) 162–174. https://doi.org/10.1038/nri2506.
[11] L. Lu, Y. Bai, Z. Wang, Elevated T cell activation score is associated with improved survival of breast cancer, Breast Cancer Research and Treatment. 164 (2017) 689–696. https://doi.org/10.1007/s10549-017-4281-x.
[12] L. Lu, H. Huang, J. Zhou, W. Ma, S. Mackay, Z. Wang, BRCA1 mRNA expression modifies the effect of T cell activation score on patient survival in breast cancer, BMC Cancer. 19 (2019). https://doi.org/10.1186/s12885-019-5595-3.
[13] A.M. Newman, C.L. Liu, M.R. Green, A.J. Gentles, W. Feng, Y. Xu, C.D. Hoang, M. Diehn, A.A. Alizadeh, Robust enumeration of cell subsets from tissue expression profiles, Nat Methods. 12 (2015) 453–457. https://doi.org/10.1038/nmeth.3337.
[14] B. Chen, M.S. Khodadoust, C.L. Liu, A.M. Newman, A.A. Alizadeh, Profiling Tumor Infiltrating Immune Cells with CIBERSORT, in: Humana Press, New York, NY, 2018: pp. 243–259. https://doi.org/10.1007/978-1-4939-7493-1_12.
[15] Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing, Journal of the Royal Statistical Society: Series B (Methodological). 57 (1995) 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.
[16] J.P. Thiery, Epithelial–mesenchymal transitions in tumour progression, Nature Reviews Cancer. 2 (2002) 442–454. https://doi.org/10.1038/nrc822.
[17] S.L. O'Beirne, S.M. Walsh, A. Fabre, C. Reviriego, J.C. Worrell, I.P. Counihan, R.V. Lumsden, J. Cramton-Barnes, J.A. Belperio, S.C. Donnelly, D. Boylan, J. Marchal-Sommé,

R. Kane, M.P. Keane, CXCL9 Regulates TGF-β1-Induced Epithelial to Mesenchymal Transition in Human Alveolar Epithelial Cells, J. Immunol. 195 (2015) 2788–2796. https://doi.org/10.4049/jimmunol.1402008.
[18] Y.K. Chae, S. Chang, T. Ko, J. Anker, S. Agte, W. Iams, W.M. Choi, K. Lee, M. Cruz, Epithelial-mesenchymal transition (EMT) signature is inversely associated with T-cell infiltration in non-small cell lung cancer (NSCLC), Sci Rep. 8 (2018) 2918. https://doi.org/10.1038/s41598-018-21061-1.
[19] S. Sakaguchi, Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses, Annu. Rev. Immunol. 22 (2004) 531–562. https://doi.org/10.1146/annurev.immunol.21.120601.141122.
[20] E.M. Shevach, CD4+ CD25+ suppressor T cells: more questions than answers, Nat. Rev. Immunol. 2 (2002) 389–400. https://doi.org/10.1038/nri821.
[21] W. Tan, W. Zhang, A. Strasner, S. Grivennikov, J.Q. Cheng, R.M. Hoffman, M. Karin, Tumour-infiltrating regulatory T cells stimulate mammary cancer metastasis through RANKL–RANK signalling, Nature. 470 (2011) 548–553. https://doi.org/10.1038/nature09707.
[22] C. Fauriat, E.O. Long, H.-G. Ljunggren, Y.T. Bryceson, Regulation of human NK-cell cytokine and chemokine production by target cell recognition, Blood. 115 (2010) 2167–2176. https://doi.org/10.1182/blood-2009-08-238469.
[23] S. Shao, E. Risch, D. Burner, L. Lu, B. Minev, W. Ma, IFNγ enhances cytotoxic efficiency of the cytotoxic T lymphocytes against human glioma cells, Int. Immunopharmacol. 47 (2017) 159–165. https://doi.org/10.1016/j.intimp.2017.04.003.
[24] A.K. Bariagaber, M.M. Whalen, Decreased adenylyl cyclase and cAMP-Dependent protein kinase activities inhibit the cytotoxic function of human natural killer cells, Human Immunology. 64 (2003) 866–873. https://doi.org/10.1016/S0198-8859(03)00154-X.
[25] J. Palming, B.G. Gabrielsson, E. Jennische, U. Smith, B. Carlsson, L.M.S. Carlsson, M. Lönn, Plasma cells and Fc receptors in human adipose tissue—lipogenic and anti-inflammatory effects of immunoglobulins on adipocytes, Biochemical and Biophysical Research Communications. 343 (2006) 43–48. https://doi.org/10.1016/j.bbrc.2006.02.114.
[26] D. Laoui, K. Movahedi, E.V. Overmeire, J.V. den Bossche, E. Schouppe, C. Mommer, A. Nikolaou, Y. Morias, P.D. Baetselier, J.A.V. Ginderachter, Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions, Int. J. Dev. Biol. 55 (2011) 861–867. https://doi.org/10.1387/ijdb.113371dl.
[27] A. Yuan, Y.-J. Hsiao, H.-Y. Chen, H.-W. Chen, C.-C. Ho, Y.-Y. Chen, Y.-C. Liu, T.-H. Hong, S.-L. Yu, J.J.W. Chen, P.-C. Yang, Opposite Effects of M1 and M2 Macrophage Subtypes on Lung Cancer Progression, Scientific Reports. 5 (2015) 14273. https://doi.org/10.1038/srep14273.
[28] H. Li, S. Shao, J. Cai, D. Burner, L. Lu, Q. Chen, B. Minev, W. Ma, Artificial human antigen-presenting cells are superior to dendritic cells at inducing cytotoxic T-cell responses, Immunology. 152 (2017) 462–471. https://doi.org/10.1111/imm.12783.
[29] H.C. Probst, J. Lagnel, G. Kollias, M. van den Broek, Inducible Transgenic Mice Reveal Resting Dendritic Cells as Potent Inducers of CD8+ T Cell Tolerance, Immunity. 18 (2003) 713–720. https://doi.org/10.1016/S1074-7613(03)00120-1.
[30] P. Guermonprez, J. Valladeau, L. Zitvogel, C. Théry, S. Amigorena, Antigen Presentation and T Cell Stimulation by Dendritic Cells, Annual Review of Immunology. 20 (2002) 621–667. https://doi.org/10.1146/annurev.immunol.20.100301.064828.

[31] C. Scheinecker, R. McHugh, E.M. Shevach, R.N. Germain, Constitutive Presentation of a Natural Tissue Autoantigen Exclusively by Dendritic Cells in the Draining Lymph Node, Journal of Experimental Medicine. 196 (2002) 1079–1090. https://doi.org/10.1084/jem.20020991.
[32] G.T. Belz, G.M.N. Behrens, C.M. Smith, J.F.A.P. Miller, C. Jones, K. Lejon, C.G. Fathman, S.N. Mueller, K. Shortman, F.R. Carbone, W.R. Heath, The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens, J. Exp. Med. 196 (2002) 1099–1104. https://doi.org/10.1084/jem.20020861.
[33] H.C. Probst, K. McCoy, T. Okazaki, T. Honjo, M. van den Broek, Resting dendritic cells induce peripheral CD8 + T cell tolerance through PD-1 and CTLA-4, Nature Immunology. 6 (2005) 280. https://doi.org/10.1038/ni1165.
[34] D. Raïch-Regué, K.P. Fabian, A.R. Watson, R.J. Fecek, W.J. Storkus, A.W. Thomson, Intratumoral delivery of mTORC2-deficient dendritic cells inhibits B16 melanoma growth by promoting CD8+ effector T cell responses, OncoImmunology. 5 (2016) e1146841. https://doi.org/10.1080/2162402X.2016.1146841.
[35] M. Schmidt, B. Hellwig, S. Hammad, A. Othman, M. Lohr, Z. Chen, D. Boehm, S. Gebhard, I. Petry, A. Lebrecht, C. Cadenas, R. Marchan, J.D. Stewart, C. Solbach, L. Holmberg, K. Edlund, H.G. Kultima, A. Rody, A. Berglund, M. Lambe, A. Isaksson, J. Botling, T. Karn, V. Müller, A. Gerhold-Ay, C. Cotarelo, M. Sebastian, R. Kronenwett, H. Bojar, H.-A. Lehr, U. Sahin, H. Koelbl, M. Gehrmann, P. Micke, J. Rahnenführer, J.G. Hengstler, A comprehensive analysis of human gene expression profiles identifies stromal immunoglobulin κ C as a compatible prognostic marker in human solid tumors, Clin. Cancer Res. 18 (2012) 2695–2703. https://doi.org/10.1158/1078-0432.CCR-11-2210.
[36] D.R. Kroeger, K. Milne, B.H. Nelson, Tumor-Infiltrating Plasma Cells Are Associated with Tertiary Lymphoid Structures, Cytolytic T-Cell Responses, and Superior Prognosis in Ovarian Cancer, Clin. Cancer Res. 22 (2016) 3005–3015. https://doi.org/10.1158/1078-0432.CCR-15-2762.
[37] C.H. Richards, K.M. Flegg, C.S.D. Roxburgh, J.J. Going, Z. Mohammed, P.G. Horgan, D.C. McMillan, The relationships between cellular components of the peritumoural inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer, Br. J. Cancer. 106 (2012) 2010–2015. https://doi.org/10.1038/bjc.2012.211.
[38] M. Lohr, K. Edlund, J. Botling, S. Hammad, B. Hellwig, A. Othman, A. Berglund, M. Lambe, L. Holmberg, S. Ekman, M. Bergqvist, F. Pontén, C. Cadenas, R. Marchan, J.G. Hengstler, J. Rahnenführer, P. Micke, The prognostic relevance of tumour-infiltrating plasma cells and immunoglobulin kappa C indicates an important role of the humoral immune response in non-small cell lung cancer, Cancer Letters. 333 (2013) 222–228. https://doi.org/10.1016/j.canlet.2013.01.036.
[39] R. Fristedt, D. Borg, C. Hedner, J. Berntsson, B. Nodin, J. Eberhard, P. Micke, K. Jirström, Prognostic impact of tumour-associated B cells and plasma cells in oesophageal and gastric adenocarcinoma, J Gastrointest Oncol. 7 (2016) 848–859. https://doi.org/10.21037/jgo.2016.11.07.
[40] R.D. Leek, C.E. Lewis, R. Whitehouse, M. Greenall, J. Clarke, A.L. Harris, Association of Macrophage Infiltration with Angiogenesis and Prognosis in Invasive Breast Carcinoma, Cancer Res. 56 (1996) 4625–4629. http://cancerres.aacrjournals.org/content/56/20/4625 (accessed June 21, 2019).

[41] M.J. Campbell, N.Y. Tonlaar, E.R. Garwood, D. Huo, D.H. Moore, A.I. Khramtsov, A. Au, F. Baehner, Y. Chen, D.O. Malaka, A. Lin, O.O. Adeyanju, S. Li, C. Gong, M. McGrath, O.I. Olopade, L.J. Esserman, Proliferating macrophages associated with high grade, hormone receptor negative breast cancer and poor clinical outcome, Breast Cancer Res Treat. 128 (2011) 703–711. https://doi.org/10.1007/s10549-010-1154-y.

[42] Y. Zhang, S. Cheng, M. Zhang, L. Zhen, D. Pang, Q. Zhang, Z. Li, High-Infiltration of Tumor-Associated Macrophages Predicts Unfavorable Clinical Outcome for Node-Negative Breast Cancer, PLOS ONE. 8 (2013) e76147. https://doi.org/10.1371/journal.pone.0076147.

[43] R. Nanda, L.Q.M. Chow, E.C. Dees, R. Berger, S. Gupta, R. Geva, L. Pusztai, K. Pathiraja, G. Aktan, J.D. Cheng, V. Karantza, L. Buisseret, Pembrolizumab in Patients With Advanced Triple-Negative Breast Cancer: Phase Ib KEYNOTE-012 Study., Journal of Clinical Oncology : Official Journal of the American Society of Clinical Oncology. 34 (2016) 2460–2467. https://doi.org/10.1200/JCO.2015.64.8931.