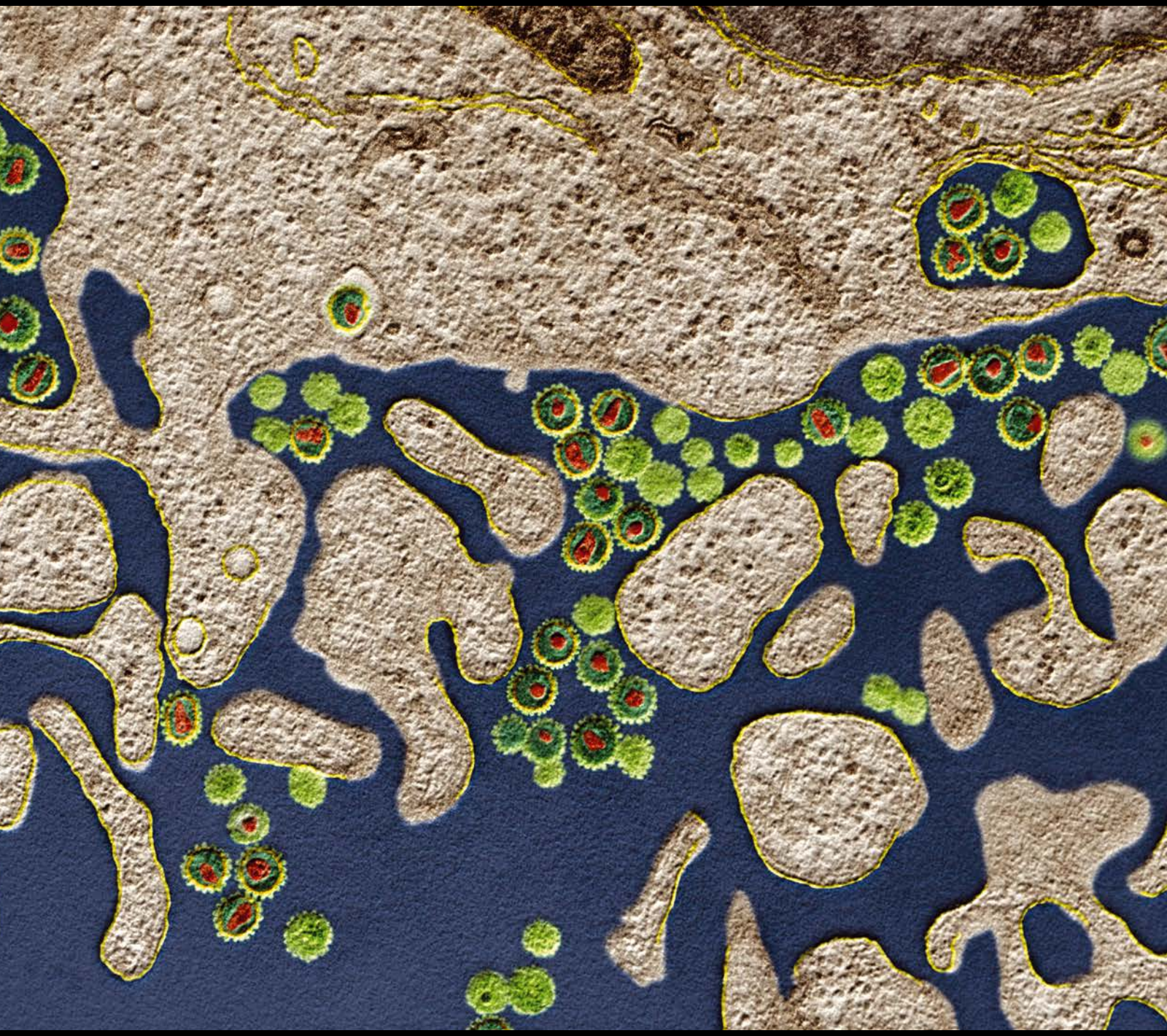
Journal of Immunology Research

Cancer Immunology and Cancer Immunodiagnosis 2016

Guest Editors: Jianying Zhang, Bin Zhang, and Yi Zhang



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Editorial Board

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2017, Article ID 9127382, 1 page
http://dx.doi.org/10.1155/2017/9127382

Editorial

Cancer Immunology and Cancer Immunodiagnosis 2016

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Received 7 November 2016; Accepted 8 November 2016; Published 10 January 2017

Cancer immunology is a branch of immunology that studies interactions between the immune system and cancer cells, which is a growing field of research that aims to identify biomarkers in cancer immunodiagnosis and to discover innovative cancer immunotherapies. The immune response, including the identification and recognition of cancer-specific antigens, is of particular interest in cancer immunology field as knowledge gained drives the development of new vaccines and antibody therapies. Activation of the immune system for therapeutic benefit against cancer has long been a goal in immunooncology. The passive cancer immunotherapy has been well-established for several decades, and continued advances in antibody and T-cell engineering should further enhance their clinical impact in the years to come. In contrast to these passive immunotherapy strategies, the active cancer immunotherapy has been proved elusive. In the context of advances in the understanding of how tolerance, immunity, and immunosuppression regulate antitumor immune responses together with the advent of targeted therapies, these successes suggest that active immunotherapy represents a path to obtain a durable and long-lasting response in cancer patients. The key to cancer immunodiagnosis and immunotherapy is an improved understanding of the immune response during cancer initiation and progression.

According to this background, we have invited investigators to contribute original research articles as well as review articles describing cancer immunodiagnosis and cancer immunotherapy (CICI) and assembled this special issue for updating the recent advances in this field. In this special CICI issue, we have received 23 submitted manuscripts, and 8 manuscripts have been accepted for publication and included in this special issue. For example, a paper of V. Kaewkangsadan et al. has characterized the contribution of T effector/regulatory cells and cytokines to tumor cell death with neoadjuvant chemotherapy (NAC); a paper of L. Qian et al. demonstrated that adoptive cellular immunotherapy (CIT) contributes to improvement of prognosis and inhibition of viral replication in HCV-related HCC patients, without impairment of liver function; a paper of Z. Lu et al. has indicated that p-p70S6K may participate in the invasion and metastasis in the development of esophageal squamous cell carcinoma (ESCC) and downregulation of the expression of p-p70S6K could improve the sensitivity of cells to rapamycin in ESCC; a paper of M. M. Lotem et al. has discussed the adjuvant autologous melanoma vaccine for macroscopic stage III disease. In addition, four review manuscripts were also included in this special issue.

In summary, this special issue covers many important aspects in cancer immunology, including recent advances in the basic and clinical studies relating to cancer immunotherapy. We hope that this special issue can provide valuable information to investigators in the field of CICI and also give the readers a sense of some of the advancements made in this field.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 4757405, 25 pages
http://dx.doi.org/10.1155/2016/4757405

Clinical Study

Crucial Contributions by T Lymphocytes (Effector, Regulatory, and Checkpoint Inhibitor) and Cytokines (TH1, TH2, and TH17) to a Pathological Complete Response Induced by Neoadjuvant Chemotherapy in Women with Breast Cancer

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Received 8 February 2016; Revised 19 August 2016; Accepted 24 August 2016

Academic Editor: Yi Zhang

The tumour microenvironment consists of malignant cells, stroma, and immune cells. Prominent tumour-infiltrating lymphocytes (TILs) in breast cancer are associated with a good prognosis and are predictors of a pathological complete response (pCR) with neoadjuvant chemotherapy (NAC). The contribution of different T effector/regulatory cells and cytokines to tumour cell death with NAC requires further characterisation and was investigated in this study. Breast tumours from 33 women with large and locally advanced breast cancers undergoing NAC were immunohistochemically (intratumoural, stromal) assessed for T cell subsets and cytokine expression using labelled antibodies, employing established semiquantitative methods. Prominent levels of TILs and $CD4^+$, $CD8^+$, and CTLA-4^+ (stromal) T cells and $CD8^+$: $FOXP3^+$ ratios were associated with a significant pCR; no association was seen with $FOXP3^+$, CTLA-4^+ (intratumoural), and PD-1^+ T cells. NAC significantly reduced $CD4^+$, $FOXP3^+$, CTLA-4^+ (stromal) (concurrently blood $FOXP3^+$, CTLA-4^+ Tregs), and PD-1^+ T cells; no reduction was seen with $CD8^+$ and CTLA-4^+ (intratumoural) T cells. High post-NAC tumour levels of $FOXP3^+$ T cells, IL-10, and IL-17 were associated with a failed pCR. Our study has characterised further the contribution of T effector/regulatory cells and cytokines to tumour cell death with NAC.

1. Background

The induction, development, and dissemination of malignant disease in man are complex processes involving a crucial interplay between malignant cells, surrounding stroma and tumour-infiltrating inflammatory and immune cells [1–3]. In a range of human solid tumours, variable numbers of innate and adaptive immune cells have been documented in the tumour microenvironment (tumour cell nests, peritumoural stroma). The distribution and density of the immune cells vary between different histopathological cancer types and amongst cancers of the same type. In general, however, they are present at increased levels compared with nonmalignant tissues [2, 4, 5].

A number of studies have shown that the presence of a prominent lymphocytic infiltrate in tumours is associated with an improved prognosis and good long-term clinical outcome in patients with different types of cancer [2, 4–7]. The presence of tumour-infiltrating lymphocytes (TILs) has been recognised as a biomarker of an antitumour response

in a wide range of solid cancers (breast, bowel, renal, and melanoma) [2, 8]. In breast cancer it has been shown that a prominent TIL presence is associated with an increased incidence of a pathological complete response (pCR) in the tumour following neoadjuvant chemotherapy (NAC) [9–11]. The subsets of T cells ($CD4^+$, $CD8^+$, $FOXP3^+$ (forkhead box protein 3), and PD-1^+ (programmed death molecule 1)) infiltrating breast cancer, however, can have different pathobiological significance and prognostic characteristics and are a matter of continuing debate [2, 5, 12–16]. The interrelationship between NAC and the various subsets is a matter of great scientific and clinical interest. It is, however, not well characterised and is in need of further study to define more precisely its contribution to a possible immune-mediated tumour cell death with NAC [17–20].

We have previously reported that women with large and locally advanced breast cancers (LLABCs) have a significantly increased circulating level of T regulatory cells (Tregs). The % of $FOXP3^+$ Tregs correlated with the pathological response of the LLABCs to subsequent NAC. Following NAC the blood Tregs (%) were significantly reduced in women whose tumours showed a good pathological response. We also documented polarised T helper cell (Th1, Th2, and Th17) profiles in the blood lymphocytes but these were unaltered by NAC [21]. There is evidence that the host anticancer immune response, at both the molecular and cellular levels, varies in different anatomical compartments and that the molecular and cellular changes detected in the blood may not always reflect the situation in the tumour microenvironment [22].

We wished, therefore, to investigate the tumour microenvironment in LLABCs and to establish whether there was a concomitant anticancer immune response, and if the blood immune changes associated with NAC were reflected in comparable changes in the tumour microenvironment. We carried out an immunohistochemical analysis of various lymphocytic immune cells and humoral factors infiltrating LLABCs. We documented the pathological impact of NAC on the tumour microenvironment and the possible contribution of different host immune cells and humoral factors to an immune-mediated tumour cell death and pCR to NAC, a recognised surrogate marker of a beneficial long-term clinical response in breast cancer [23, 24].

2. Materials and Methods

2.1. Patients and Samples. Paraffin-embedded sections of breast tumours from 33 women with L (≥3 cm), LABCs (T3, 4; N1, 2; M0), enrolled in a study of NAC (between 2008 and 2011) were studied [24].

Histological diagnosis was established from ultrasound-guided core biopsies. To minimise tumour heterogeneity and sampling discrepancies, several core biopsies were obtained from each tumour. All tumours prior to NAC had a radioopaque coil inserted. After NAC, wire-guided removal of the residual "tumour" was carried out (in the case of breast conservation) if there was no clinical or radiological evidence of cancer. Operative specimens (wide local excision, mastectomy) had radiological confirmation of the presence of the coil to ensure accurate localisation and histopathological evaluation. Representative tissue sections were used for immunohistochemical evaluation. All pre- and post-NAC specimens were discussed at a multidisciplinary meeting and a consensus was reached about the pathological response and treatment options.

The NAC trial evaluated the effect of the addition of capecitabine (X) to docetaxel (T) preceded by adriamycin and cyclophosphamide (AC). All patients received either 4 courses of AC followed by 4 courses of T ± X or 2 courses of AC followed by 6 courses of T ± X, as per the trial protocol. Pathological responses were assessed in the excised surgical specimens after NAC. Established and previously published grading criteria were used to define histopathological responses in the breast [25, 26]. Good pathological responses were graded 5 (pCR, no residual invasive disease) and 4 (90% loss of invasive disease). Poor pathological responses were graded as 3 (30–90% loss of invasive disease), 2 (<30% loss of invasive disease), and 1 (no loss of tumour cells). The levels of blood $FOXP3^+$ and CTLA-4^+ Tregs from 16 of these 33 patients have been documented in a previous study from our department [21]. An important aim of the work presented was to establish whether the previously documented inhibition of the blood Tregs by NAC occurred concurrently in the breast tumours of the same individuals. Patient cases were randomly selected based on availability of tissue specimens and equability of distribution between compared groups (pCR versus non-pCR).

2.2. Immunohistochemical Assessment. Immunohistochemical (IHC) assessments of immune cell subsets and expression of cytokines were performed in 4 μm tissue sections. Briefly, paraffin-embedded tissue sections were dewaxed and rehydrated using xylene and graded alcohol. Citrate buffer, pH 6.0, at 98°C was added for 20 minutes (mins) for antigen retrieval. After serial blocking, the sections were incubated with the primary monoclonal antibody (MAb) against CD4 (Dako, M7310, clone 4B12), 1 : 80 dilution for 30 mins at room temperature (RT); MAb against CD8 (Dako, M7103, clone C8/144B), 1 : 100 dilution for 30 mins at RT; MAb against FOXP3 (Abcam, ab20034, clone 236A/E7), 20 μg/mL for 30 mins at RT; MAb against CTLA-4 (Santa Cruz Bio, sc-376016, clone F-8), 1 : 300 dilution for 30 mins at RT; MAb against PD-1 (Abcam, ab52587, clone NAT105), 1 : 100 dilution for 30 mins at RT; MAb against interleukin-1 (IL-1) (Abcam, ab8320, clone 11E5), 1 : 150 dilution overnight at 4°C; MAb against IL-2 (Abcam, ab92381, clone EPR2780), 1 : 500 dilution for 30 mins at RT; polyclonal Ab against IL-4 (Abcam, ab9622), 4 μg/mL for 30 mins at RT; polyclonal Ab against IL-10 (Abcam, ab34843), 1 : 400 dilution for 30 mins at RT; polyclonal Ab against IL-17 (Abcam, ab9565), 1 : 100 dilution for 30 mins at RT; polyclonal Ab against interferon-gamma (IFN-γ) (Abcam, ab9657), 4 μg/mL for 30 mins at RT; MAb against transforming growth factor-beta 1 (TGF-β1) (Abcam, ab64715, clone 2Ar2), 12 μg/mL overnight at 4°C; polyclonal Ab against PD-L1 (Abcam, ab58810), 2.5 μg/mL for 15 mins at RT. The Novolink™ polymer detection system,

Leica RE7280-K with polymeric horseradish peroxidase-(HRP-) linker antibody conjugates and diaminobenzidine (DAB) chromogen, was used for enzyme-substrate labelling. Finally, the sections were counterstained with haematoxylin, dehydrated, and mounted in DPX mounting medium. Positive and negative staining controls were carried out with tonsil sections except for CTLA-4 (colon carcinoma sections), IL-1, IL-4, and TGF-β (kidney carcinoma sections), and IL-10 (normal colon sections). Negative staining controls were demonstrated by omitting the primary antibody. Positive and negative controls were simultaneously performed with every IHC staining run.

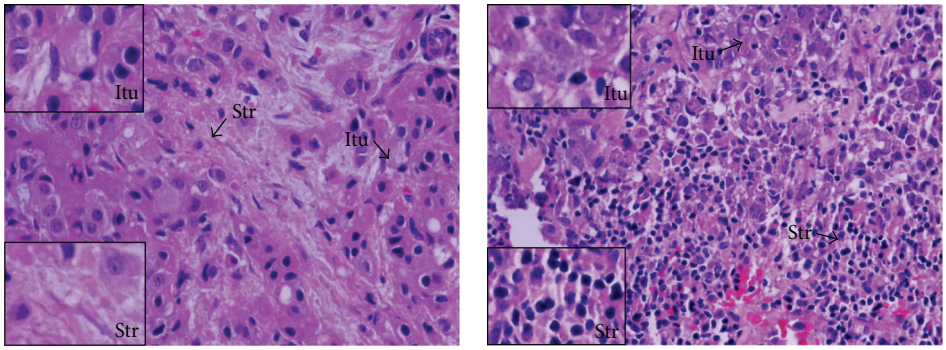
2.3. Semiquantification of IHC Sections. Whole tissue sections were studied rather than microarrays (to minimise sampling bias). Representative high-power fields (HPFs) ×400 magnification are shown for ease and clarity of presentation. To evaluate the presence and extent of specific T cell subsets in the breast tumours, the average numbers of brown membrane/nuclear-stained cells regardless of the intensity were counted in 5 HPFs. Positively stained cells in contact with tumour cells or within the tumour cell nests were defined as "intratumoural" whereas positively stained cells in the interstitial stroma surrounding tumour nests were defined as "stromal." Evaluation of subset infiltrations in post-NAC specimens was undertaken on residual tumour nests and in the case of pCR (complete disappearance of invasive tumour cells in the specimen) in the tumour bed. The latter was characterised histologically as a hyalinised, amorphous area with haemosiderin deposits [27, 28].

To evaluate the presence of IL-1, IL-2, IL-4, IL-10, IL-17, IFN-γ, TGF-β, and PD-L1 the semiquantitative H scoring system was employed using whole tissue sections. The H score was calculated by multiplying the % of positive cells (tumour and immune) by a factor representing the intensity of immune-reactivity (1 for weak, 2 for moderate, and 3 for strong), giving a maximum score of 300. A score of <50 was considered negative and a score of 50–100 was considered weakly positive (1+). A score of 101–200 was regarded as moderately positive (2+) and a score of 201–300 as strongly positive (3+). Negative and 1+ were considered as low expression whereas 2+ and 3+ were considered as high expression.

To evaluate TILs in haematoxylin and eosin (H&E) stained sections, intratumoural lymphocytes (Itu-Ly) were reported as the % of the tumour epithelial nests that contained infiltrating lymphocytes. Stromal lymphocytes (Str-Ly) were defined as the % of tumour stromal areas that contained lymphocytic infiltrates without direct contact with tumour cells. Scores of >60% were considered to be high levels of infiltration, whilst ≤60% were considered to be low levels of infiltration for both Itu-Ly and Str-Ly. Cases were defined as high TILs when Itu-Ly and/or Str-Ly were >60% and as low TILs if both Itu-Ly and Str-Ly were ≤60%. The 60% cut-off point for level of TILs was following previously published studies and the methodological recommendations from the international TILs working group 2014 [9, 29, 30]. All sections were scored without knowledge of the patients' clinical and pathological parameters.

2.4. Phenotypic Analysis of Blood FOXP3$^+$ and CTLA-4$^+$ Tregs. Blood samples were collected before and following completion of NAC. Blood mononuclear cells (BMCs) were collected on Ficoll-Hypaque, washed and made up in RPMI with 10% foetal calf serum (FCS) (Sigma, UK) and antibiotics, and stored at −80°C for further analysis. Whole blood assays were used for documentation of absolute numbers (AbNs). Flow cytometry analysis (Beckman Coulter, FC500) was performed with a panel of MAbs. FOXP3$^+$ Tregs were stained for cell surface markers for 30 mins with 2.5 μL phycoerythrin Texas red conjugate- (ECD-) anti-human CD4, 5 μL phycoerythrin-anti-human CD25, 5 μL allophycocyanin-anti-human CD127; CTLA-4$^+$ Tregs were stained for intracellular CD152. Cell surface markers for CD4 and CD25 were determined by staining for 30 mins with 2.5 μL of ECD anti-human CD4 and 5 μL fluorescein isothiocyanate (FITC) anti-human CD25. The cells were then washed with RPMI and 2% FCS; 2% formaldehyde was used for fixation of BMCs for 10 mins at RT. The BMCs were then washed once in phosphate buffered saline (PBS) containing 2% FCS and twice in PBS/0.5% Tween with 0.05% azide and 3% FCS. 2.5 μL FITC anti-human FOXP3 (intracellular) and 5 μL PE anti-human CTLA-4 (intracellular CD152) were added to the corresponding tubes and incubated for 2 hours at 4°C. The BMC pellet was then washed twice in PBS/0.5% Tween, 0.05% azide, and 3% FCS. The BMCs were resuspended in 400 μL of 0.5% paraformaldehyde fixative solution for FC analysis. Whole blood was used to determine absolute numbers of cells. CD4$^+$ CD25$^+$ Tregs were characterised using 2.5 μL ECD anti-human CD4 and 5 μL of PE anti-human CD25. CTLA-4$^+$ Tregs were characterised using 2.5 μL ECD anti-human CD4, 5 μL of FITC anti-human CD25, and 5 μL PE anti-human CD152 (intracellular CTLA-4). On adding the MAbs to whole blood a gentle vortex was applied for 5 seconds and the FACs tubes were left in the dark for 15 mins at RT. 500 μL of optilyse C solution (Beckman Coulter) was added to induce complete lysis of red blood cells, vortexed, and left for another 15 mins at RT in the dark. 500 μL of PBS was added to the FACS tubes to stop the lysis reaction between the optilyse C and the whole blood. The whole blood mixture was vortexed at RT. 100 μL of Flow Count-fluorosphere beads (Beckman Coulter) was added prior to analysis on the flow-cytometer.

2.5. Statistical Analysis. Statistical analyses were performed with the IBM SPSS statistics software, version 21 (SPSS Inc., Chicago, IL, USA). Where the data did not follow a normal distribution, nonparametric tests (Mann–Whitney U test (between two variables/groups) and Kruskal-Wallis test (amongst three or more variables/groups)) were used to compare the groups based on pathological responses and clinicopathological parameters. Pearson Chi-Square test was performed to compare the binomial data (negative/low versus high) on expression of cytokines between groups. To evaluate and compare the related-sample data between pre-NAC and post-NAC groups, the Related-Samples Wilcoxon Signed Rank test and Related-Samples McNemar test were performed for comparing the number of cell counts and the



(a) (b)

Figure 1: TILs in the sections of LLABCs, using H&E staining, at 400x magnification; (a) low level of lymphocytic infiltration; (b) high level of lymphocytic infiltration. Low level of TILs defined as ≤60% of tumour nests (Itu: intratumoural) and stromal areas (Str: stromal) infiltrated by lymphocytes. High level of TILs defined as >60% of tumour nests and/or stromal areas infiltrated by lymphocytes.

expression of cytokines/PD-L1, respectively. The correlations between TILs and T cell subsets (continuous data) and grade (1–5) of pathological responses (ordinal data) were carried out using Spearman's Correlation Coefficient (rho). A univariate and multivariate (logistic regression) analysis was carried out to establish predictive factors for a pCR with NAC. A probability value (p value) of equal to or less than 0.05 (2-tailed) was considered statistically significant. Based on our previous study with Treg findings and using the N Query Advisor 6.0 analysis software, we established that the minimum number of patients ($n = 7$) in a sample group relating to the pathological response groups was appropriate [21].

3. Results

3.1. Prominent Lymphocytic Infiltration (Intratumoural, Stromal) of LLABCs: Association with a Significant Pathological Complete Response (PCR) in the Tumour following NAC. High levels of TILs were associated with a significant pCR (grade 5 response: no residual invasive disease in the breast cancer) following 8 cycles of NAC. This was seen with both intratumoural ($p = 0.001$) and stromal ($p < 0.001$) TILs and in lymphocyte predominant breast cancers (LPBCs) ($p < 0.001$), irrespective of the tumour microenvironment (Table 1) (Figure 1).

There was a significant positive correlation between pre-NAC intratumoural and stromal TILs (rho = 0.592, $p = 0.016$). There was also a significant positive correlation between post-NAC intratumoural and stromal TILs (rho = 0.693, $p = 0.004$). No significant difference, however, was found between levels of pre-NAC and post-NAC TILs (see Additional File 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/4757405).

3.2. Prominent Intratumoural and Stromal $CD4^+$ and $CD8^+$ T Cell and Stromal CTLA-4^+ T Cell Infiltration in LLABCs: Association with a Significant Pathological Complete Response (PCR) in the Tumour following NAC. Table 2 shows that high levels of intratumoural (tumour cell nests) infiltration by $CD4^+$ and $CD8^+$ T cells were associated with a significant pCR in the breast cancer ($p = 0.023$ and $p = 0.008$, resp.). Infiltration by $FOXP3^+$, CTLA-4^+, and PD-1^+ T cells (much lower level of infiltration) was not associated with a pCR subsequently in LLABCs following NAC.

A prominent level of stromal infiltration by $CD4^+$ and $CD8^+$ T cells was also associated with a pCR following NAC ($p = 0.001$ and $p = 0.002$, resp.). Stromal infiltration by CTLA-4^+ T cells was similarly associated with a pCR ($p = 0.041$) but infiltration by $FOXP3^+$ and PD-1^+ T cells was not (Table 2) (Figures 2, 3, and 4).

3.3. Tumour-Infiltrating $CD8^+ : FOXP3^+$ T Cell Ratio: Significant Association with a Pathological Complete Response (PCR) following NAC. Table 3 documents the significant association between the tumour-infiltrating $CD8^+ : FOXP3^+$ T cell ratio and pathological response. A good pathological response (grades 5 and 4) was seen with intratumoural ($p = 0.027$) and stromal ($p = 0.027$) infiltration ratios. Similar and more significantly pronounced ratios were seen with intratumoural (7.40 versus 1.48, $p = 0.002$) and stromal (5.37 versus 1.67, $p = 0.001$) pCRs (Table 3). Thus the concurrent high level of $CD8^+$ and low level of $FOXP3^+$ T cells are an important factor predisposing to a pCR with NAC in LLABCs.

3.4. Significant Correlations between TILs and Specific Lymphocyte Subsets and Grade of Pathological Response to NAC. Table 4 shows the significant correlations between tumour-infiltrating (intratumoural, stromal) lymphocytes, respectively (TILs: rho = 0.601, $p < 0.001$, and rho = 0.641, $p < 0.001$; $CD4^+$ T cells (stroma): rho = 0.468, $p = 0.006$; $CD8^+$ T cells: rho = 0.446, $p = 0.009$, and rho = 0.471, $p = 0.006$), and grade of pathological response (grade 1 (no pathological response) to grade 5 (pCR)) in the breast cancers following 8 cycles of NAC. Infiltrating intratumoural $CD4^+$ T cells failed to reach statistical significance ($p = 0.073$) (Table 4). There was also a significant correlation between the $CD8^+ : FOXP3^+$ T cell ratios intratumourally (rho = 0.511, $p = 0.002$) and stromally (rho = 0.484, $p = 0.004$) and the grade of response elicited in LLABCs with NAC (Table 4).

Table 1: Levels of tumour-infiltrating lymphocytes (TILs) in women with LLABCs[(1)] and subsequent pathological complete response following NAC[(2)].

TILs	Groups	Low infiltration (n)	High infiltration (n)	Pearson chi-square value (PCR[(3)] versus non-PCR)	p value
Intratumoural	Pathological complete response (PCR, $n = 16$)	6	10	11.890	0.001*
	Nonpathological complete response (non-PCR, $n = 17$)	16	1		
Stromal	Pathological complete response (PCR, $n = 16$)	3	13	16.051	<0.001*
	Nonpathological complete response (non-PCR, $n = 17$)	15	2		
LPBC[(4)]	Pathological complete response (PCR, $n = 16$)	3	13	13.350	<0.001*
	Nonpathological complete response (non-PCR, $n = 17$)	14	3		

[(1)]LLABCs: large and locally advanced breast cancers; [(2)]NAC: neoadjuvant chemotherapy; [(3)]PCR (grade 5): no residual invasive disease; [(4)]LPBC: lymphocyte-predominant breast cancer; *statistically significant.

Table 2: Levels of tumour-infiltrating T cell subsets in women with LLABCs[(1)] and subsequent pathological complete response following NAC[(2)].

T cell subsets	Groups	Intratumoural Median (range)[(3)]	p value[(4)] (PCR[(5)] versus non-PCR)	Stromal Median (range)[(3)]	p value[(4)] (PCR versus non-PCR)
$CD4^+$	Pathological complete response (PCR, $n = 16$)	45.2 (1.6–171.0)	0.023*	43.4 (1.0–242.0)	0.001*
	Nonpathological complete response (non-PCR, $n = 17$)	5.8 (0.6–166.2)		10.4 (1.0–113.0)	
$CD8^+$	Pathological complete response (PCR, $n = 16$)	40.6 (5.2–202.4)	0.008*	75.5 (5.6–201.6)	0.002*
	Nonpathological complete response (non-PCR, $n = 17$)	12.8 (0.4–99.2)		12.2 (1.8–110.0)	
$FOXP3^+$	Pathological complete response (PCR, $n = 16$)	6.3 (0.4–96.8)	0.958	12.5 (0.8–110.6)	0.363
	Nonpathological complete response (non-PCR, $n = 17$)	5.4 (0.8–45.6)		10.8 (0.8–44.8)	
$CTLA\text{-}4^+$	Pathological complete response (PCR, $n = 16$)	0.5 (0.0–4.0)	0.068	1.4 (0.0–10.0)	0.041*
	Nonpathological complete response (non-PCR, $n = 17$)	0.4 (0.0–2.2)		0.4 (0.0–2.2)	
$PD\text{-}1^+$	Pathological complete response (PCR, $n = 6$)	2.6 (0.0–57.4)	0.118	1.9 (0.4–81.2)	0.093
	Nonpathological complete response (non-PCR, $n = 10$)	0.5 (0.0–3.2)		0.9 (0.0–3.6)	

[(1)]LLABCs: large and locally advanced breast cancers; [(2)]NAC: neoadjuvant chemotherapy; [(3)]average cell count per 400x high-power field (core biopsies of breast cancers); [(4)]Mann–Whitney U test; [(5)]PCR (grade 5): no residual invasive disease; *statistically significant.

Table 3: Tumour-infiltrating $CD8^+$: $FOXP3^+$ T cell ratio in LLABCs[(1)] and subsequent pathological response to NAC[(2)].

T cell subsets (n = 33)	Groups	Pre-NAC intratumoural Median (range)[(3)]	p value[(4)] (GPR versus PRR, PCR versus non-PCR)	Pre-NAC stromal Median (range)[(3)]	p value[(4)] (GPR versus PRR, PCR versus non-PCR)
$CD8^+$: $FOXP3^+$ T cell ratio	Good pathological response (GPR, n = 21)[(5)]	3.26 (0.18–45.00)	0.027*	4.67 (0.53–23.29)	0.027*
	Poor pathological response (PPR, n = 12)[(6)]	1.37 (0.67–6.04)		1.81 (0.10–6.78)	
	Pathological complete response (PCR, n = 16)[(7)]	7.40 (0.27–45.00)	0.002*	5.37 (1.08–23.29)	0.001*
	Nonpathological complete response (non-PCR, n = 17)	1.48 (0.18–6.04)		1.67 (0.10–6.78)	

[(1)]LLABCs: large and locally advanced breast cancers; [(2)]NAC: neoadjuvant chemotherapy; [(3)]$CD8^+$ T cell/$FOXP3^+$ Treg ratio; [(4)]Mann–Whitney U test; [(5)]GPR (good pathological response, grades 5 and 4): no residual invasive disease, >90% loss of invasive disease, respectively; [(6)]PPR (poor pathological response, grades 3, 2, and 1): 30–90% loss of invasive disease, <30% loss of invasive disease, and no loss of tumour cells, respectively; [(7)]PCR (pathological complete response, grade 5); *statistically significant.

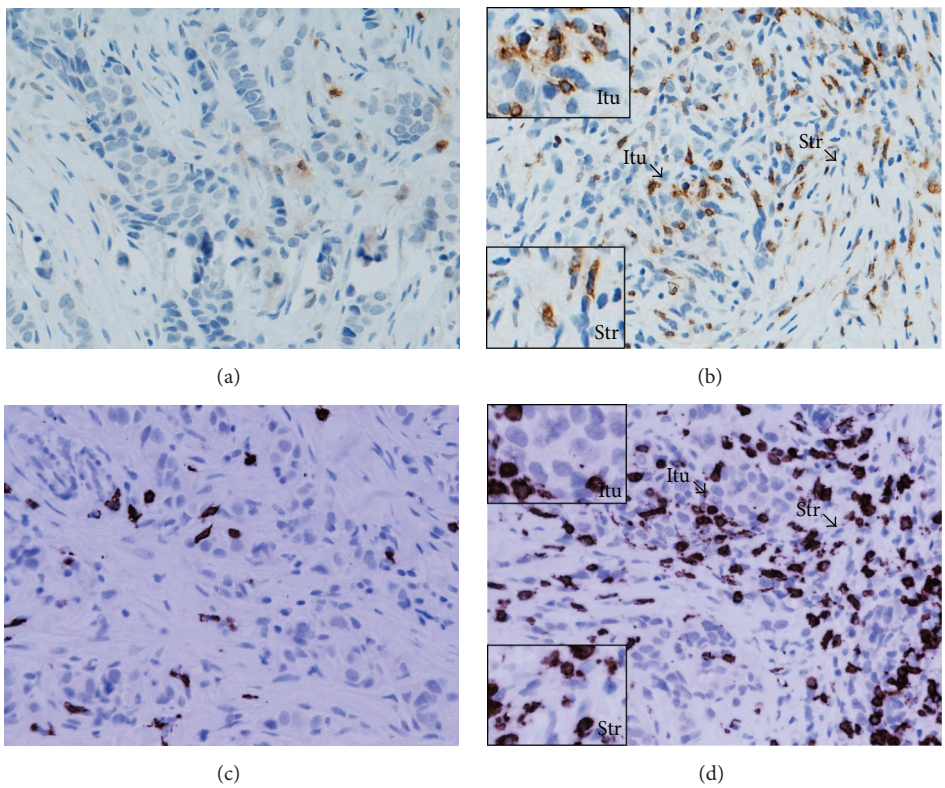


Figure 2: $CD4^+$ (a, b) and $CD8^+$ (c, d) T lymphocytes in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to CD4 (Dako, M7310) at a 1 : 80 dilution for 30 mins at RT and MAbs to CD8 (Dako, M7103) at a 1 : 100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a, c) Low level of $CD4^+$ and $CD8^+$ T cell infiltration; (b, d) high level of $CD4^+$ and $CD8^+$ T cell infiltration. The average number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal) per HPF was counted.

Table 4: Correlations between tumour-infiltrating lymphocytes (TILs) and specific T cell subsets and grade of pathological response to NAC[(1)] (Spearman's correlation coefficient (rho)) in women with LLABCs[(2)].

Lymphocytes (n = 33)	Groups	Grade of pathological response[(3)]	
		Correlation coefficient	p value (2-tailed)
TILs	Intratumoural infiltration	0.601	<0.001*
	Stromal infiltration	0.641	<0.001*
$CD4^+$ T cells	Intratumoural infiltration	0.316	0.073
	Stromal infiltration	0.468	0.006*
$CD8^+$ T cells	Intratumoural infiltration	0.446	0.009*
	Stromal infiltration	0.471	0.006*
$CD8^+$: $FOXP3^+$ T cell ratio	Intratumoural infiltration	0.511	0.002*
	Stromal infiltration	0.484	0.004*

[(1)]NAC: neoadjuvant chemotherapy; [(2)]LLABCs: large and locally advanced breast cancers; [(3)]pathological responses were graded from grades 1 to 5 (grade 1 (no loss of tumour cells), grade 2 (<30% loss of invasive disease), grade 3 (30–90% loss of invasive disease), grade 4 (>90% loss of invasive disease), and grade 5 (complete pathological response, no residual invasive disease)); *statistically significant.

3.5. Infiltration by T Cell Subsets in LLABCs: Significant Subset Reductions ($CD4^+$, $FOXP3^+$, CTLA-4^+, and PD-1^+ T cells but Not $CD8^+$ T Cells) following NAC. Various lymphocyte subsets ($CD4^+$, $CD8^+$, $FOXP3^+$, CTLA-4^+, and PD-1^+ T cells) were documented infiltrating LLABCs (Table 5). The most prominent infiltration was by $CD4^+$ and $CD8^+$ T cells, there being a threefold increase for $CD4^+$ T cells and a twofold increase for $CD8^+$ T cells in the peritumoural stroma compared with the intratumoural (tumour cell nests) compartment (45.6 [6.8–242.0] versus 15.4 [2.6–171.0] and 43.6 [1.8–201.6] versus 20.2 [3.4–202.4]), respectively (Table 5) (Figure 2). There was a smaller but still prominent infiltration

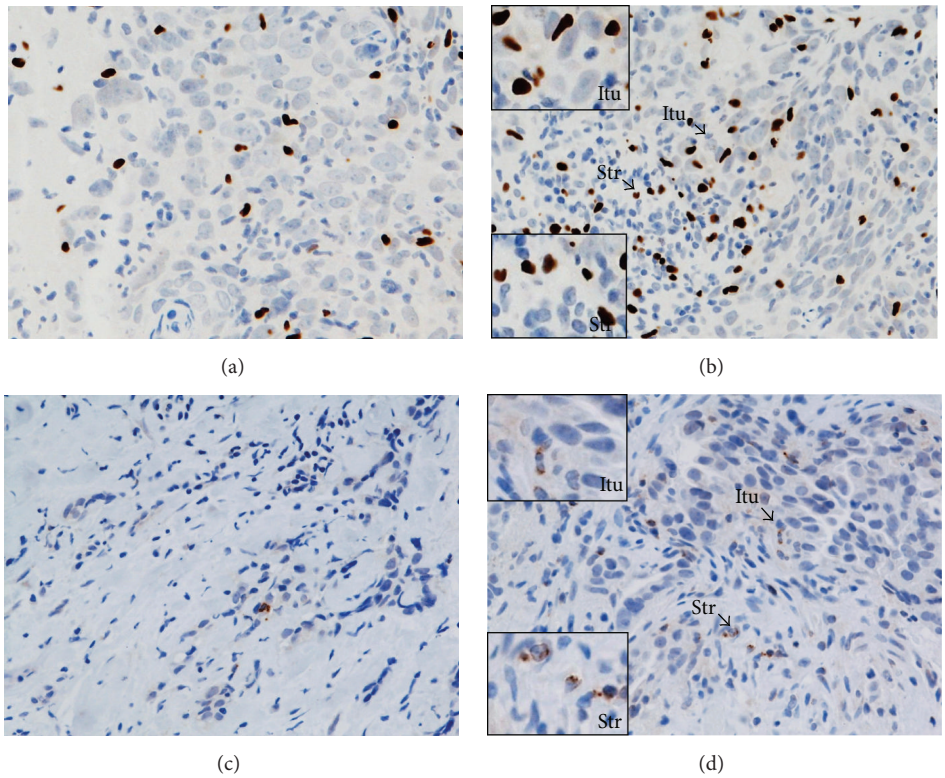


Figure 3: FOXP3$^+$ (a, b) and CTLA-4$^+$ (c, d) Tregs in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to FOXP3 (Abcam, ab20034) at a concentration of 20 μg/mL for 30 mins at RT and MAbs to CTLA-4 (Santa Cruz Bio, sc-376016) at a 1 : 300 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a, c) Low level of FOXP3$^+$, CTLA-4$^+$ Treg infiltration; (b, d) high level of FOXP3$^+$ and CTLA-4$^+$ Treg infiltration. The average number of brown nuclear-stained (FOXP3), membrane-stained (CTLA-4) cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal) per HPF was counted.

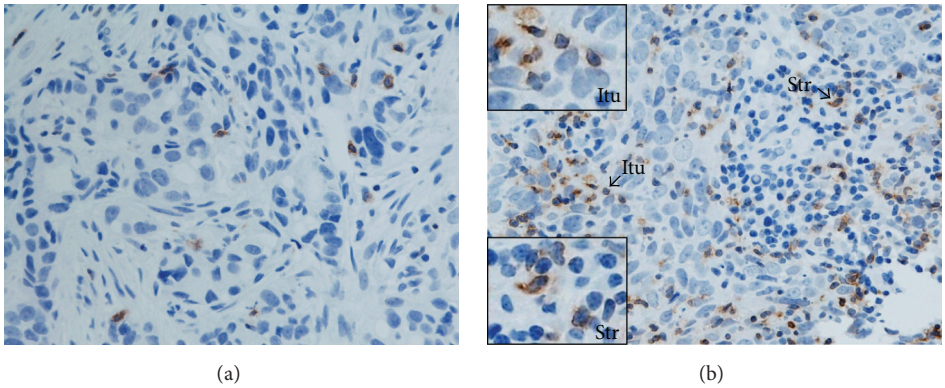


Figure 4: PD-1$^+$ T cells in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to PD-1 (Abcam, ab52587) at a 1 : 100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a) Low level of PD-1$^+$ T cell infiltration; (b) high level of PD-1$^+$ T cell infiltration. The average number of brown membrane-stained cells, regardless of intensity, in contact with tumour cells or within tumour cell nests (Itu: intratumoural) and in the interstitial stroma (Str: stromal) per HPF was counted.

Table 5: T cell subsets infiltrating tumours (intratumoural, stromal) in women with LLABCs[(1)]: significant reduction with NAC[(2)].

T cell subsets (n = 16)	Groups	Pre-NAC Median (range)[(3)]	Post-NAC Median (range)[(3)]	p value[(4)] Pre-versus post-NAC
$CD4^+$	Intratumoural infiltration	15.4 (2.6–171.0)	3.0 (0.0–71.6)	0.010*
	Stromal infiltration	45.6 (6.8–242.0)	6.3 (1.2–236.0)	0.006*
$CD8^+$	Intratumoural infiltration	20.2 (3.4–202.4)	10.3 (0.0–83.6)	0.278
	Stromal infiltration	43.6 (1.8–201.6)	27.1 (1.6–144.6)	0.326
$FOXP3^+$	Intratumoural infiltration	14.8 (2.4–96.8)	0.7 (0.0–22.2)	0.001*
	Stromal Infiltration	15.9 (2.2–110.6)	1.4 (0.4–28.4)	0.001*
CTLA-4^+	Intratumoural infiltration	0.4 (0.0–4.0)	0.1 (0.0–1.2)	0.060
	Stromal infiltration	0.6 (0.2–10.0)	0.1 (0.0–5.2)	0.029*
PD-1^+	Intratumoural infiltration	0.7 (0.0–57.4)	0.0 (0.0–0.6)	0.005*
	Stromal infiltration	1.5 (0.0–81.2)	0.0 (0.0–4.0)	0.016*

[(1)]LLABCs: large and locally advanced breast cancers; [(2)]NAC: neoadjuvant chemotherapy; [(3)]average cell count per 400x high-power field; [(4)]Wilcoxon signed rank test; *statistically significant.

Table 6: Blood[(1)] and tumour-infiltrating $FOXP3^+$ and CTLA-4^+ T cells in women with LLABCs[(2)]: significant reduction with NAC[(3)].

T cell subsets (n = 16)	Groups	Pre-NAC Median (range)[(4)]	Post-NAC Median (range)	p value[(5)] Pre- versus post-NAC
$FOXP3^+$	Intratumoural Infiltrating	14.8 (2.4–96.8)	0.7 (0–22.2)	0.001*
	Stromal Infiltrating	15.9 (2.2–110.6)	1.4 (0.4–28.4)	0.001*
	% Circulating	1.54 (0.62–3.40)	0.81 (0.25–1.85)	0.001*
	AbN circulating[(6)]	170 (107–427)	159 (35–230)	0.001*
CTLA-4^+	Intratumoural Infiltrating	0.4 (0.0–4.0)	0.1 (0.0–1.2)	0.060
	Stromal infiltrating	0.6 (0.2–10.0)	0.1 (0.0–5.2)	0.029*
	% circulating	1.31 (0.05–3.24)	0.72 (0.10–1.71)	0.017*
	AbN circulating	15 (5–19)	6 (2–15)	0.001*

[(1)]Blood: data previously published (Verma et al., 2013 [21]); [(2)]LLABCs: large and locally advanced breast cancers; [(3)]NAC: neoadjuvant chemotherapy; [(4)]average cell count per 400x high-power field; [(5)]Wilcoxon signed rank test; [(6)]AbN: absolute number (cells/mm^3); *statistically significant.

by $FOXP3^+$ T cells, comparable in both compartments (15.9 [2.2–110.6] versus 14.8 [12.4–96.8]). CTLA-4^+ T cells, on the other hand, were present in low numbers in both the stromal and intratumoural compartments (Table 5) (Figure 3). Similarly, the infiltration by PD-1^+ T cells was low, albeit there was a wide range of values in both the intra- and peritumoural compartments (Table 5) (Figure 4).

Eight cycles of NAC induced a substantial and significant reduction in various T lymphocyte subsets. There was a significant reduction in both the intratumoural (p = 0.010) and stromal (p = 0.006) $CD4^+$ T cells. There was, however, no significant reduction in intratumoural (p = 0.278) or stromal (p = 0.326) $CD8^+$ T cell infiltration after NAC, albeit there was some reduction in the level of infiltration in both compartments (Table 5). Following 8 cycles of NAC there was a significant reduction in both the intratumoural (p = 0.001) and stromal (p = 0.001) $FOXP3^+$ T cells. There was also a significant reduction of stromal (p = 0.029) CTLA-4^+ T cells. Although the intratumoural CTLA-4^+ T cells were reduced as well, this just failed to reach statistical significance (p = 0.060) (Table 5). NAC significantly reduced intratumoural and stromal PD-1^+ T cells (p = 0.005 and p = 0.016, resp.) (Table 5). Thus 8 cycles of NAC significantly reduced all the above T lymphocyte subsets, apart from $CD8^+$ T cells, infiltrating LLABCs.

3.6. Significant Concurrent Reduction of $FOXP3^+$ and CTLA-4^+ T Cells in the Blood and Tumours in Women with LLABCs Undergoing NAC. There was a significant reduction of $FOXP3^+$ T cells in the blood (% [p = 0.001], absolute numbers (AbNs) [p = 0.001]) and breast cancers (intratumoural [p = 0.001], stromal [p = 0.001]) following 8 cycles of NAC in the same cohort of 16 patients (Table 6). There was also a significant reduction of CTLA-4^+ T cells in the blood (% [p = 0.017], AbNs [p = 0.001]) and breast cancers (stromal [p = 0.029]) following 8 cycles of NAC in the same cohort of 16 patients. The intratumoural infiltration just failed to reach statistical significance (p = 0.060) (Table 6). The reduction in the tumour was at least 10-fold for $FOXP3^+$ and 4-fold for CTLA-4^+. This was more pronounced than the reduction seen in blood (twofold for $FOXP3^+$ and CTLA-4^+ %).

The blood $FOXP3^+$ and CTLA-4^+ T cell results were from a much larger cohort of patients and have been published by us previously [21].

There was a positive correlation between post-NAC % of blood $FOXP3^+$ T cells and post-NAC intratumoural $FOXP3^+$

T cells (rho = 0.687, $p = 0.003$). There was also a nonsignificant trend for a correlation between pre-NAC AbNs of blood and post-NAC intratumoural FOXP3$^+$ T cells (rho = 0.470, $p = 0.066$). There were no correlations demonstrated for CTLA-4$^+$ T cells (see Additional Files 2 and 3).

3.7. FOXP3$^+$/CTLA-4$^+$ T Cell Profile (Blood, Tumour-Infiltrating) and Pathological Response to NAC. At diagnosis and prior to NAC, there were no significant differences in the levels of circulating (%, AbNs) and tumour-infiltrating T cells (FOXP3$^+$, CTLA-4$^+$) and the subsequent different NAC response groups (good pathological response versus poor pathological response and pCR versus non-pCR) (see Additional File 4).

After NAC, however, there was a significantly higher % of blood FOXP3$^+$ T cells and significantly higher levels of intratumoural (tumour cell nests) FOXP3$^+$ T cells in those women whose tumours had a poor pathological response to 8 cycles of NAC ($p = 0.001$ and $p = 0.016$, resp.) or failed to demonstrate a pCR ($p = 0.007$ and $p < 0.001$, resp.) (Table 7). In the case of CTLA-4$^+$ T cells, higher blood levels of AbNs were significantly associated with a poor pathological response to 8 cycles of NAC ($p = 0.008$) (Table 7).

3.8. Cytokine (TH1, TH2, and TH17) Profiles in the Tumour Microenvironment: Association with NAC-Induced PCR. Table 8 documents the expression of various cytokines in the tumour microenvironment in women with LLABCs, prior to and following 8 cycles of NAC. There was no significant association with a pCR following NAC and the expression *in situ* of Th1 (IL-2, IFN-γ) cytokines (Figure 5). There was, however, a significant association with a failed pCR following NAC and the expression *in situ* of the immunosuppressive Th2 cytokine IL-10 ($p = 0.039$) (Figure 6). Expression *in situ* of the Th17 cytokine IL-17 was also significantly associated with a poor pathological response and failure to achieve a pCR ($p = 0.013$) (Table 8) (Figure 7). There was a nonsignificant association between the *in situ* expression of the immunosuppressive cytokine TGF-β and a pCR in the breast cancer following NAC ($p = 0.062$) (Figure 7).

NAC had no significant effect on the expression *in situ* of the Th1 (IL-2, IFN-γ), Th2 (IL-10), and Th17 (IL-17) cytokines in the tumour microenvironment. The expression of the Th2 cytokine IL-4, however, was significantly altered following NAC ($p = 0.016$) (see Additional File 5). There was a high level of expression of IL-4 (87.5% (14 out of 16)) in the pre-NAC specimens. Following NAC, 43.8% (7 out of 16) of tumour samples showed alteration in the level of expression of IL-4. 50% (7 out of 14) of the tumour samples showing a high level of expression before NAC were altered to a low/negative level of expression of IL-4 after NAC. None of the cases studied (0%) changed to a high level of expression. A nonsignificant reduction of *in situ* IL-2 expression was also seen after NAC, being reduced from 11 out of 16 (68.8%) in pre-NAC specimens to 5 out of 16 (31.3%) in post-NAC specimens ($p = 0.070$).

3.9. Clinicopathological Characteristics and T Lymphocytic Subsets (CD4$^+$, CD8$^+$, and FOXP3$^+$) Infiltrating LLABCs. Table 9 documents a range of clinical features, NAC regimens, and tumour characteristics of the patients studied. There was a significant association of T lymphocyte subsets (CD4$^+$, CD8$^+$, and FOXP3$^+$) with tumour grade: infiltration by CD4$^+$ and CD8$^+$ T cells, intratumoural ($p = 0.026$ and $p = 0.038$, resp.) and stromal ($p = 0.004$ and $p = 0.032$, resp.), and stromal infiltration by FOXP3$^+$ T cells ($p = 0.018$). High levels of tumour infiltration by these three T cell subsets were significantly associated with high grade (3) tumours. There was no obvious association with the other pathological and clinical parameters in the small patient ($n = 33$) sample.

Univariate analysis showed the following predictive factors for pCR: TILs ($p = 0.001$), tumour grade ($p = 0.005$), and oestrogen receptor (ER) status ($p = 0.049$) (Table 10). Multivariate analysis, however, showed that TILs were the only independent predictor of a pCR in the 33 patients studied with LLABCs undergoing NAC (Table 10).

4. Discussion

The presence of a high level of TILs in various human solid cancers, including breast cancer, has been shown to be a reliable prognostic indicator and associated with an improved clinical outcome [4, 6–8, 31, 32]. Studies of specific T cell subsets, however, have produced variable results in different tumour types [2].

The association of TILs and different lymphocyte subsets in breast cancer patients undergoing NAC and contributing to tumour cell death has generated clinical and scientific interest. Demaria et al. (2001) first showed that, following NAC with paclitaxel, the presence of TILs following treatment correlated with the pathological response elicited in the breast cancer to NAC [33]. Several studies have subsequently shown TILs to be important predictors of a pathological response, in particular a pCR [9, 11, 34]. In fact, Denkert et al. (2010) showed TILs to be an independent predictor for a pCR in women undergoing NAC [9]. Our study, with a much smaller cohort of patients, also showed high levels of TILs to be an independent predictive factor (multivariate analysis) for a pCR, a recognised surrogate marker of a good outcome in breast cancer following NAC [23, 24]. West et al. (2011) reported that the presence of TILs in breast cancer was a good predictor of a pCR in patients with ER −ve tumours who had received an anthracycline-based NAC regimen [35]. Ono et al. (2012) demonstrated that TILs correlated with response to NAC (anthracycline ± taxane-based) in triple −ve breast cancer [34]. Dieci et al. (2014) also demonstrated that high levels of TILs (stromal and intratumoural) in the residual breast tumour following NAC in triple −ve cancers were significantly associated with a better disease-free survival (DFS) and overall survival (OS) [29]. Lee et al. (2013) showed TILs to be associated with a better prognosis in axillary lymph node (ALN) +ve, ER −ve, and HER2 −ve subtypes following NAC (anthracycline ± taxane-based) [36]. In our study, the NAC regimen consisted of cyclophosphamide, the anthracycline doxorubicin, and the taxane docetaxel ±

Table 7: Blood[(1)] and tumour-infiltrating FOXP3$^+$ and CTLA-4$^+$ T cells (post-NAC) in women with LLABCs[(2)] and pathological response elicited in tumours following NAC[(3)].

T cell subsets	Groups (n = 16)	Intratumoural Median (range)[(4)]	p value	Stromal Median (range)	p value	% blood Median (range)	p value	AbN blood Median (range)[(5)]	p value[(6)]
FOXP3$^+$	GPR (n = 9)[(7)]	0.0 (0.0–2.4)	0.016*	0.8 (0.4–7.4)	0.252	0.53 (0.25–0.90)	0.001*	166 (35–230)	0.470
	PPR (n = 7)[(8)]	2.2 (0.6–22.2)		1.4 (1.0–28.4)		1.18 (0.80–1.85)		157 (118–168)	
	PCR (n = 6)[(9)]	0.0 (0.0–0.0)	<0.001*	1.3 (0.4–7.4)	0.635	0.35 (0.25–0.90)	0.007*	173 (49–230)	0.313
	Non PCR (n = 10)	1.8 (0.6–22.2)		1.4 (0.4–28.4)		1.15 (0.53–1.85)		158 (35–177)	
CTLA-4$^+$	GPR (n = 9)	0.0 (0.0–1.2)	0.114	0.0 (0.0–1.2)	0.299	0.58 (0.10–1.71)	0.299	5 (2–7)	0.008*
	PPR (n = 7)	0.4 (0.0–1.2)		0.4 (0.0–5.2)		0.89 (0.37–1.69)		7 (6–15)	
	PCR (n = 6)	0.0 (0.0–1.0)	0.118	0.0 (0.0–0.2)	0.181	0.55 (0.10–1.25)	0.220	5.5 (2–7)	0.181
	Non PCR (n = 10)	0.3 (0.0–1.2)		0.3 (0.0–5.2)		0.77 (0.37–1.71)		6.5 (4–15)	

[(1)]Blood: data previously published (Verma et al., 2013 [21]); [(2)]LLABCs: large and locally advanced breast cancers; [(3)]NAC: neoadjuvant chemotherapy; [(4)]average cell count per 400x high-power field; [(5)]AbN: absolute number (cells/mm^3); [(6)]Mann–Whitney U test; [(7)]GPR (good pathological response, grades 5 and 4): no residual invasive disease, >90% loss of invasive disease, respectively; [(8)]PPR (poor pathological response, grades 3, 2, and 1): 30–90% loss of invasive disease, <30% loss of invasive disease, and no loss of tumour cells, respectively; [(9)]PCR (pathological complete response, grade 5): no residual invasive disease; *statistically significant.

Table 8: Expression of cytokines and PD-L1[1] in LLABCs[2] (pre-NAC and post-NAC[3]) and pathological complete response elicited in tumours following NAC.

Cytokines and PD-L1 (n = 16)		Pre-NAC				Post-NAC			
		Low/negative expression (n)	High expression (n)	Pearson chi-square value (PCR[4] versus non-PCR)	p value	Low/negative expression (n)	High expression (n)	Pearson chi-square value (PCR versus non-PCR)	p value
IL-1	PCR (n = 6)	1	5	0.950	0.330	3	3	0.640	0.424
	Non PCR (n = 10)	4	6			3	7		
IL-2	PCR (n = 6)	2	4	0.019	0.889	3	3	1.571	0.210
	Non PCR (n = 10)	3	7			8	2		
IFN-γ	PCR (n = 6)	0	6	1.371	0.242	2	4	0.71	0.790
	Non PCR (n = 10)	2	8			4	6		
IL-4	PCR (n = 6)	1	5	0.152	0.696	4	2	0.423	0.515
	Non PCR (n = 10)	1	9			5	5		
IL-10	PCR (n = 6)	2	4	0.071	0.790	5	1	4.267	0.039*
	Non PCR (n = 10)	4	6			3	7		
IL-17	PCR (n = 6)	2	4	0.019	0.889	5	1	6.112	0.013*
	Non PCR (n = 10)	3	7			2	8		
TGF-β[5]	PCR (n = 6)	4	2	0.423	0.515	4	2	3.484	0.062
	Non PCR (n = 10)	5	5			2	8		
PD-L1	PCR (n = 6)	3	3	0.640	0.424	4	2	0.019	0.889
	Non PCR (n = 10)	3	7			7	3		

[1]PD-L1: programmed death ligand 1; [2]LLABCs: large and locally advanced breast cancers; [3]NAC: neoadjuvant chemotherapy; [4]PCR (pathological complete response, grade 5: no residual invasive disease); [5]TGF-β: scored as negative or positive; *statistically significant.

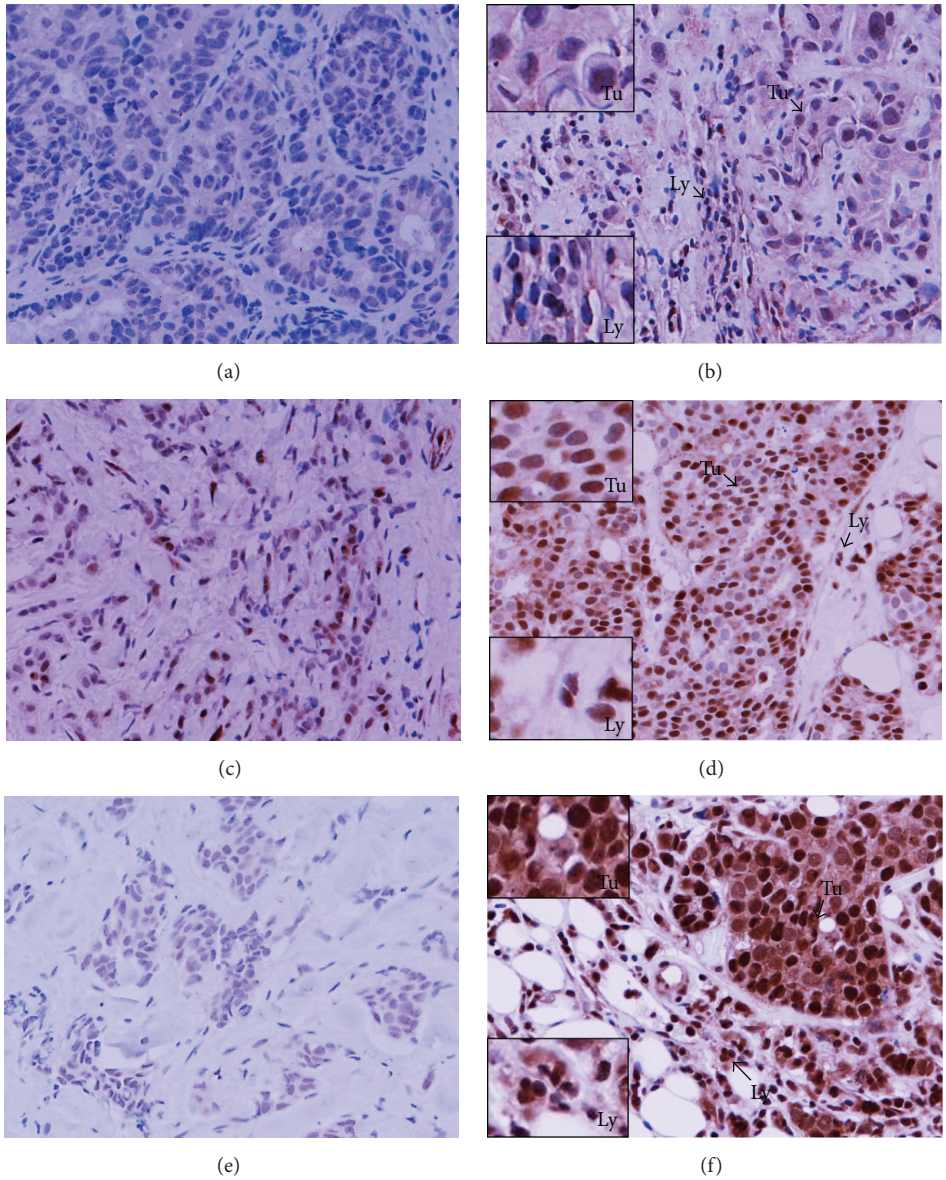


Figure 5: IL-1 (a, b), IL-2 (c, d), and IFN-γ (e, f) expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with MAbs to IL-1 (Abcam, ab8320) at a 1 : 150 dilution overnight at 4°C and MAbs to IL-2 (Abcam, ab92381) at a 1 : 500 dilution for 30 mins at RT and polyclonal Abs to IFN-γ (Abcam, ab9657) at a concentration of 4 μg/mL for 30 mins at RT, respectively. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a, c, e) Low level of expression; (b, d, f) high level of expression. The *H* score (% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) × intensity of staining (1 to 3)) was used to assess the level of expression; low was ≤100 and high was >100. Scoring performed on whole tissue section (>10 HPFs); Tu: tumour and Ly: lymphocyte.

capecitabine. Sixty-seven percent of the tumour specimens studied, however, were ER +ve and only 9% were triple −ve [24]. We showed a significant correlation between high levels of TILs (intratumoural, stromal) and the subsequent pathological grade of response (5–1) in LLABCs after 8 cycles of NAC, a finding not previously reported.

Although the impact of TILs in breast cancer, with or without NAC, has been documented in a large cohort of patients, the contribution of the various TIL subsets is inadequately studied and data for several of the subsets is poorly documented. There is a paucity of published data regarding $CD4^+$ T cells infiltrating breast tumours. Droesser et al. (2012) found that they were not a prognostic indicator [37]. Heys et al. (2012) reported low levels of $CD4^+$ T cells to be significantly associated with a better response to NAC [38].

In a range of human solid cancers (colorectal, ovarian, oesophageal, lung, breast, and pancreas) the presence of high levels of tumour-infiltrating $CD8^+$ T cells (and $CD45RO^+$

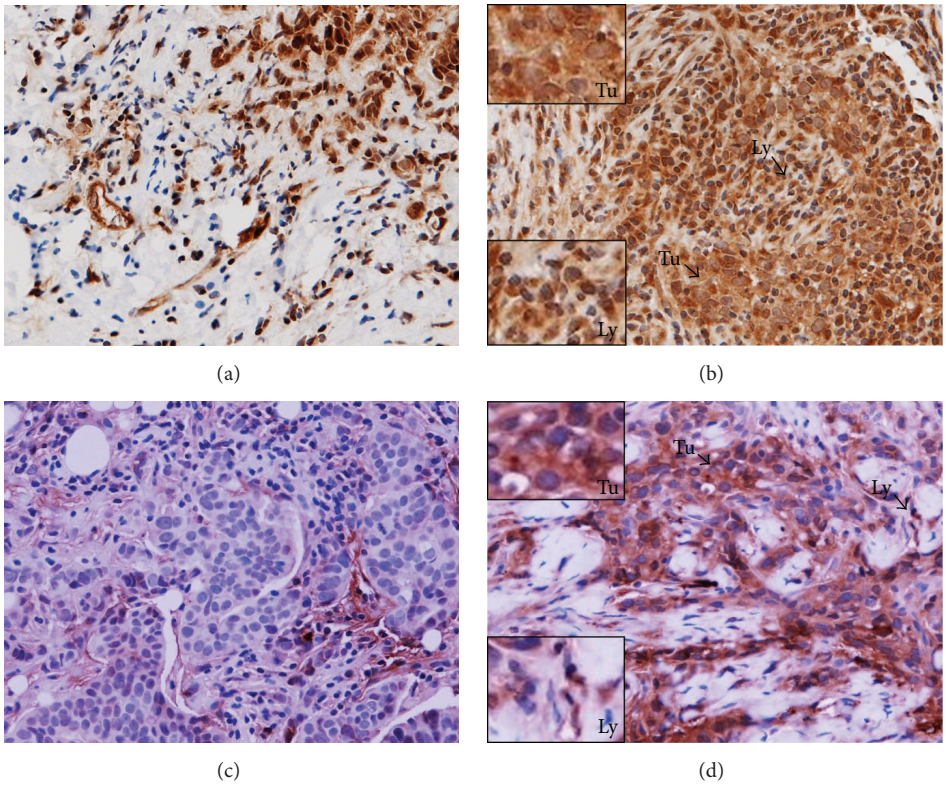


Figure 6: IL-4 (a, b) and IL-10 (c, d) expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-4 (Abcam, ab9622) at a concentration of 4 μg/mL for 30 mins at RT and polyclonal Abs to IL-10 (Abcam, ab34843) at a 1 : 400 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a, c) Low level of expression; (b, d) high level of expression. The *H* score (% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) × intensity of staining (1 to 3)) was used to assess the level of expression; low was ≤100 and high was >100. Scoring performed on whole tissue section (>10 HPFs); Tu: tumour and Ly: lymphocyte.

memory T cells) was associated with a favourable prognosis [1, 4]. Mahmoud et al. (2011) documented $CD8^+$ T cells in tumour cell nests and stroma. High $CD8^+$ T cell counts were independently associated with longer breast cancer-specific survival [15]. Matkowski et al. (2009), however, showed that a high level of $CD8^+$ T cells in breast tumours was associated with high tumour grade, ER negativity, expression of HER2, metastatic spread to ALNs, and a poor prognosis [39]. A small number of studies have documented the relevance of tumour-infiltrating $CD8^+$ T cells with NAC. Two studies found that high levels of $CD8^+$ T cells in breast cancer were associated with a pCR following NAC [27]. In HER2 overexpressing breast cancers, a high $CD8^+ : FOXP3^+$ T cell ratio was associated with a pCR and an improved DFS and OS [40].

Our study demonstrated infiltration by both $CD4^+$ and $CD8^+$ T cells, with a predominance in the peritumoural stroma compared with tumour cell nests. This profile and compartmentalisation of effector T cells in breast cancer are not well characterised in the literature. Degnim et al. (2014) documented the pattern of infiltration by $CD4^+$ and $CD8^+$ T cells in normal human breast lobules [41]. $CD4^+$ T cells were comparable (median, interquartile range) with the intratumoural levels documented in our patients. $CD8^+$ T cells, however, were more prominent and 2.5-fold higher than the intratumoural levels documented in our patients. Thus, in breast cancer there is a reduction of the normal $CD8^+ : CD4^+$ T cell ratio due to lower levels of $CD8^+$ T cell infiltration. Following NAC there was a significant reduction in both the intratumoural and stromal $CD4^+$ T cells but not $CD8^+$ T cells, albeit there was some reduction in $CD8^+$ T cell levels.

In our study high levels of $CD4^+$ and $CD8^+$ T cells, intratumourally and stromally, in LLABCs were associated with a subsequent pCR following NAC. These findings are in agreement with recently published data [27, 42–44]. We also established that a high $CD8^+ : FOXP3^+$ T cell ratio in LLABCs prior to NAC was associated with a subsequent pCR. Ladoire et al. (2011) documented similar findings in a HER2 overexpressing breast cancer subset. The majority of the tumours in our study, however, (as in breast cancer in general), were HER2 −ve. Our study also demonstrated a significant correlation between tumour-infiltrating $CD4^+$ and $CD8^+$ T cells, $CD8^+ : FOXP3^+$ T cell ratio and the pathological grade of response (5–1) elicited with NAC. To the

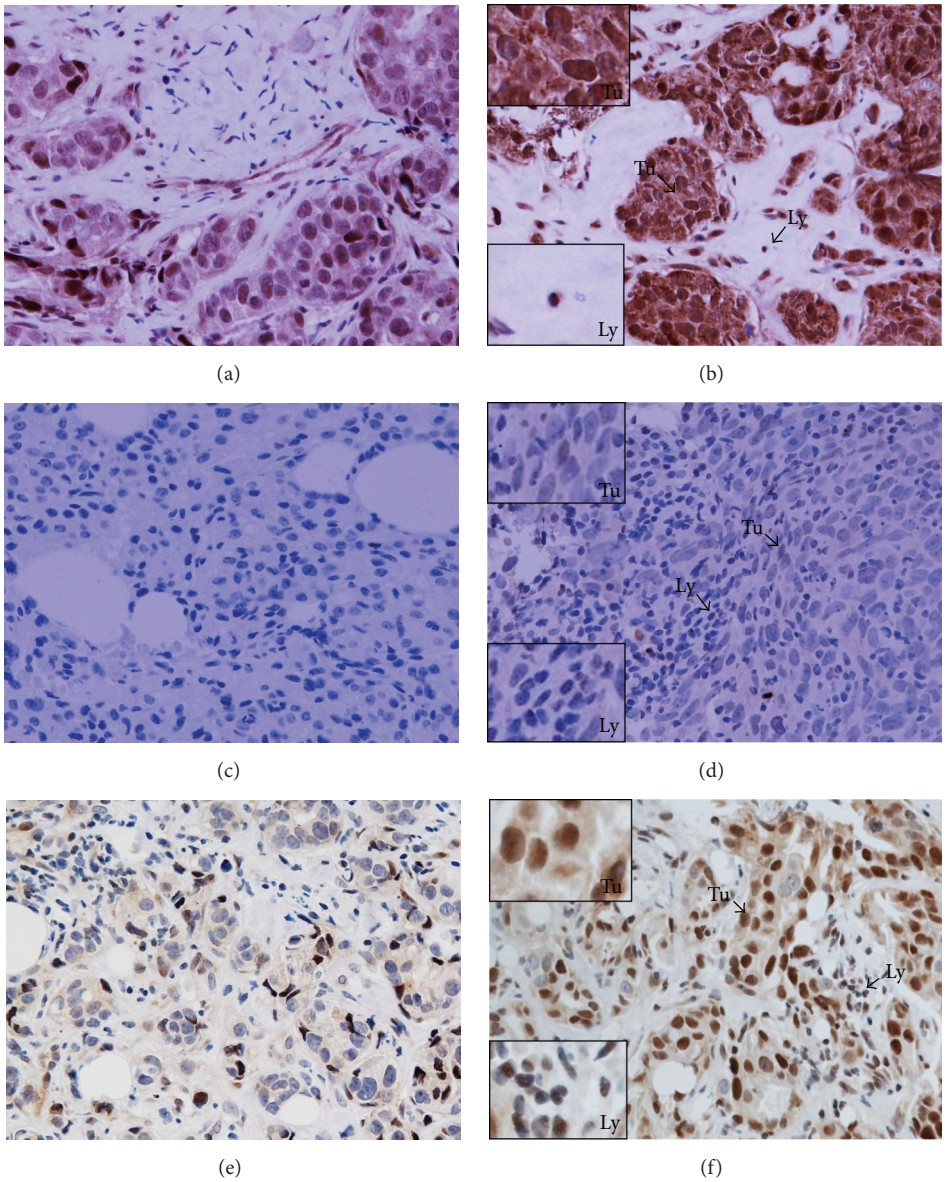


Figure 7: IL-17 (a, b), TGF-β (c, d), and PD-L1 (e, f) expression in the sections of LLABCs, using IHC staining, at 400x magnification. Briefly, heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with polyclonal Abs to IL-17 (Abcam, ab9565) at a 1 : 100 dilution for 30 mins at RT, MAbs to TGF-β (Abcam, ab64715) at a concentration of 12 μg/mL overnight at 4°C, and polyclonal Abs to PDL1 (Abcam, ab58810) at a concentration of 2.5 μg/mL for 15 mins at RT, respectively. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualize the staining. The sections were counterstained with haematoxylin. (a, c, e) Low level of expression; (b, d, f) high level of expression. The *H* score (% of positive cells (brown membrane/cytoplasmic-stained tumour and immune cells) × intensity of staining (1 to 3)) was used to assess the level of expression; low was ≤100 and high was >100. Scoring performed on whole tissue section (>10 HPFs); Tu: tumour and Ly: lymphocyte.

best of our knowledge, such findings have not been previously published. Moreover, we have recently documented a significant correlation between high levels of stromal infiltration by natural killer (NK) cells and pathological grade of response in LLABCs [20]. Thus, our findings suggest that various adaptive and innate lymphocyte subsets appear to play an important role in facilitating an effective anticancer response associated with NAC, in women with LLABCs. Functional assays need to be carried out on isolated T cell subsets to define more precisely these roles.

$CD4^+$ T cells include different Th cell subsets (Th1, Th2, and Th17) secreting a wide range of pro- and anti-inflammatory cytokines (Il-2, IFN-ϒ, IL-4, IL-5, IL-10, and IL-17), as well as natural and inducible $CD4^+CD25^+$ $FOXP3^+$ Tregs. These subsets show a degree of plasticity (Th17 cells secreting the Th1 cytokine IFN-ϒ; transformation of Tregs

Table 9: Clinical and pathological parameters of patients ($n = 33$) studied and the presence of pre-NAC[1] tumour-infiltrating $CD4^+$ and $CD8^+$ and $FOXP3^+$ T cells.

Groups	N	$CD4^+$ T cells		$CD8^+$ T cells		$FOXP3^+$ T cells	
		Intratumoural Median (range)[2] [p value[3]]	Stromal Median (range) [p value]	Intratumoural Median (range) [p value]	Stromal Median (range) [p value]	Intratumoural Median (range) [p value]	Stromal Median (range) [p value]
Age (years)							
<50	14	16.9 (1.4–166.2)	20.0 (1.0–162.2)	24.1 (0.8–97.4)	17.7 (1.8–110.0)	5.6 (0.4–26.2)	12.6 (0.8–26.8)
≥50	19	8.8 (0.6–171.0)	16.8 (1.0–242.0)	14.4 (0.4–202.4)	26.2 (2.0–201.6)	4.8 (0.8–96.8)	11.2 (0.8–110.6)
		[0.653]	[0.957]	[1.000]	[0.397]	[0.957]	[0.900]
BMI[4] (kg/m^2)							
≤30	20	7.6 (0.6–166.2)	16.4 (1.0–190.4)	20.3 (0.4–202.4)	22.8 (2.0–127.2)	6.3 (0.8–96.8)	14.3 (0.8–110.6)
>30	13	12.4 (1.4–171.0)	19.2 (1.0–242.0)	14.4 (0.8–197.2)	22.4 (1.8–201.6)	4.8 (0.4–60.4)	6.6 (0.8–27.8)
		[0.524]	[0.842]	[0.899]	[0.730]	[0.703]	[0.118]
Menopausal							
Pre	16	39.4 (1.4–171.0)	33.0 (1.0–242.0)	31.7 (0.8–197.2)	41.8 (1.8–201.6)	9.1 (0.4–60.4)	14.7 (0.8–27.8)
Post	17	6.4 (0.6–158.4)	15.8 (1.0–190.4)	12.8 (0.4–202.4)	22.4 (2.0–127.2)	4.6 (0.8–96.8)	6.6 (0.8–110.6)
		[0.191]	[0.204]	[0.157]	[0.958]	[0.326]	[0.423]
Tumour size							
<40 mm	18	7.6 (1.4–171.0)	17.3 (1.0–242.0)	20.8 (0.8–202.4)	22.8 (1.8–201.6)	4.4 (0.8–77.0)	9.3 (0.8–110.6)
≥40 mm	15	12.4 (0.6–129.0)	16.8 (1.0–162.2)	19.4 (0.4–99.2)	22.4 (3.4–114.0)	11.2 (0.4–96.8)	14.2 (0.8–44.8)
		[0.708]	[0.929]	[0.817]	[0.901]	[0.190]	[0.486]
Nodal status							
Negative	10	13.1 (3.6–171.0)	54.1 (9.0–242.0)	13.9 (3.4–202.4)	43.6 (1.8–201.6)	8.8 (2.4–77.0)	12.7 (3.0–110.6)
Positive	23	8.8 (0.6–166.2)	16.8 (1.0–162.2)	21.2 (0.4–112.6)	18.4 (2.0–118.8)	5.6 (0.4–96.8)	10.0 (0.8–32.0)
		[0.475]	[0.144]	[0.576]	[0.603]	[0.144]	[0.221]
Tumour grade							
1 (low)	2	47.6 (17.0–78.2)	106.5 (104–109)	64.0 (28.8–99.2)	88.0 (87.2–88.8)	32.5 (19.4–45.6)	35.8 (26.8–44.8)
2 (moderate)	13	4.8 (1.4–166.2)	10.4 (1.0–113.0)	10.4 (0.8–78.4)	11.2 (2.6–110.0)	4.2 (0.4–26.2)	5.2 (0.8–21.8)
3 (high)	18	22.7 (0.6–171.0)	37.5 (2.6–242.0)	31.9 (0.4–202.4)	70.5 (1.8–199.8)	6.3 (0.8–96.8)	14.0 (0.8–110.6)
		[0.026[5]*]	[0.004*]	[0.038*]	[0.032*]	[0.109]	[0.018*]
ER[6] status							
Negative	11	16.8 (5.8–158.4)	43.0 (5.2–190.4)	29.6 (1.0–202.4)	65.2 (2.0–127.2)	5.6 (0.8–96.8)	11.2 (0.8–110.6)
Positive	22	5.8 (0.6–171.0)	14.5 (1.0–242.0)	13.7 (0.4–197.2)	18.9 (1.8–201.6)	5.5 (0.4–60.4)	11.2 (0.8–44.8)
		[0.105]	[0.089]	[0.281]	[0.440]	[0.721]	[0.866]
HER-2 status							
Negative	23	12.4 (0.6–171.0)	16.0 (1.0–242.0)	19.4 (0.4–202.4)	26.2 (2.6–201.6)	7.4 (0.4–96.8)	11.6 (0.8–110.6)
Positive	10	7.6 (3.6–66.8)	20.0 (5.2–119.2)	14.3 (1.0–97.4)	15.0 (1.8–86.4)	3.6 (0.8–11.6)	9.3 (0.8–17.4)
		[0.658]	[0.658]	[0.428]	[0.133]	[0.114]	[0.221]
NAC regimen							
AC-TX[7]	16	12.8 (1.4–171.0)	20.3 (1.0–242.0)	25.4 (0.8–197.2)	47.8 (2.6–201.6)	7.2 (0.4–60.4)	12.7 (0.8–44.8)
AC-T	17	8.8 (0.6–166.2)	15.8 (2.6–190.4)	13.4 (0.4–202.4)	19.4 (1.8–127.2)	4.8 (0.8–96.8)	10.8 (0.8–110.6)
		[0.873]	[0.901]	[0.326]	[0.657]	[0.929]	[0.817]

[1]NAC: neoadjuvant chemotherapy; [2]average cell count per 400x high-power field; [3]Mann–Whitney U test; [4]BMI: body mass index (≤30: nonobese, >30: obese); [5]Kruskal-Wallis test; [6]ER: oestrogen receptor; [7]AC-TX: doxorubicin, cyclophosphamide, taxotere, and Xeloda® (capecitabine), respectively; *statistically significant.

Table 10: Univariate and multivariate (logistic regression) analyses of clinicopathological parameters as predictive factors for pathological complete response to NAC[(1)] in LLABCs[(2)] ($n = 33$).

Parameters	Univariate analysis			Multivariate analysis		
	OR[(3)]	95% CI[(4)]	p value	OR	95% CI	p value
TILs[(5)]: high (LPBC[(6)]) versus low	20.22	3.45–118.65	0.001*	11.17	1.41–88.49	0.022*
Age: <50 versus ≥50	0.68	0.17–2.71	0.579	NA	NA	NA
Tumour size: <40 mm versus ≥40 mm	1.14	0.29–4.51	0.849	NA	NA	NA
Tumour grade: 3 versus 1/2	10.4	2.03–53.20	0.005*	2.99	0.33–27.00	0.328
ER[(7)] status: negative versus positive	4.67	0.96–22.79	0.049*	1.01	0.11–9.63	0.994
HER-2 status: positive versus negative	1.95	0.43–8.83	0.386	NA	NA	NA
NAC regimen: AC-TX[(8)] versus AC-T	3.06	0.74–12.63	0.123	NA	NA	NA

[(1)]NAC: neoadjuvant chemotherapy; [(2)]LLABCs: large and locally advanced breast cancers; [(3)]OR: odds ratio; [(4)]CI: confidence interval; [(5)]TILs: tumour-infiltrating lymphocytes; [(6)]LPBC: lymphocyte-predominant breast cancer; [(7)]ER: oestrogen receptor; [(8)]AC-TX: doxorubicin, cyclophosphamide, taxotere, and Xeloda (capecitabine), respectively; *statistically significant; NA: not applicable.

into Th1 and Th17 subsets) [45]. This complex profile makes it difficult to attribute precisely the contribution of each subset or combination of $CD4^+$ Th cell subsets to a pCR with NAC. The lack of association with pCR of $FOXP3^+$ T cells (putative Tregs) suggests an important role for the Th subsets. $CD8^+$ T cells also consist of different subsets, namely, naive, memory, and activated $CD8^+$ cytotoxic T lymphocytes (CTLs). $CD8^+$ T suppressor cells, lacking expression of CD28 but expressing CD122 and FOXP3, have also been described. This is a highly restricted and weak suppressor cell subset [46].

Interest has focused on the possible contribution of $FOXP3^+$ TILs to prognosis and pathological responses in breast cancer induced by NAC and is a matter of continuing debate [13, 28, 40, 47]. Bates et al. (2006) studied normal breast tissue (reduction mammoplasties) and found very low levels of infiltration by $FOXP3^+$ T cells [48]. High levels of $FOXP3^+$ T cells in breast tumours have been reported in both ductal carcinoma *in situ* (DCIS) and in much higher levels in invasive breast cancer [14, 48, 49]. Our study showed a 45-fold higher level of $FOXP3^+$ T cells in the LLABC specimens (median, interquartile range), compared with normal breast tissue [48]. High levels of $FOXP3^+$ T cells have been found to be significantly increased in HER2 +ve breast cancers [13, 50]. In our study, $FOXP3^+$ T cells were also prominent in HER2 −ve cancers (major phenotype in breast cancer).

Tregs ($FOXP3^+$) play an important role in the control of autoimmunity, maintenance of transplantation tolerance and suppression of anticancer immune responses. FOXP3 is a transcription factor required for the generation of $CD4^+$ $CD25^+$ Tregs and is a key marker for identifying such cells. Tregs in peripheral tissues are a mixture of natural and induced $FOXP3^+$ Tregs. Induced $FOXP3^+$ Tregs have a more heterogeneous phenotype (some cells lack CD25) and are induced by TGF-β and IL-10 [51]. In the breast cancer tissue sections studied, there was *in situ* expression of IL-10 and TGF-β and therefore the likely presence of induced $FOXP3^+$ Tregs. It was not possible, however, to distinguish between the two Treg types. Both, on the other hand, contribute to inhibition of immune responses. The contribution by $CD8^+FOXP3^+$ Tregs is likely to be minimal as they are a small subset with weak immune suppressive activity [46].

Tregs are generated in the early phase of the adaptive immune response and IL-2 is central to their development and survival. They suppress the function of a wide range of immune cells ($CD4^+$ and $CD8^+$ T cells, NK and NK T cells, and dendritic cells (DCs)) [52, 53]. As a substantial number of human $CD4^+$ T cells transiently express $FOXP3^+$ during activation but not necessarily acquisition of regulatory function caution has been expressed about its uniqueness as a marker for Tregs [51].

Increased levels of $FOXP3^+$ Tregs have been documented in blood, lymph nodes, and infiltrating various human tumours [2, 14, 21, 54–56]. In many, a high level of $FOXP3^+$ T cell infiltration was shown to be associated with an unfavourable clinical outcome [2, 14, 48]. In some cancers (colorectal, ovarian, bladder, head, and neck) high levels of tumour-infiltrating $FOXP3^+$ T cells were found to be associated with an improved prognosis [2, 57]. Bates et al. (2006) reported that the presence of $FOXP3^+$ T cells identified breast cancer patients at high risk of relapse [48]. Gobert et al. (2009) found regulatory T cells to be selectively activated in lymphoid infiltrates in breast cancers, leading to a poor prognosis [58]. Demir et al. (2013) stated that intratumoural $FOXP3^+$ T cells were prognostic factors in LLABCs [28]. Mahmoud et al. (2011), however, did not demonstrate any relationship to clinical outcome with tumour-infiltrating $FOXP3^+$ Tregs in breast cancers [16]. Paradoxically, high levels of $FOXP3^+$ Tregs in ER −ve breast cancers (less common type of breast cancer) were shown to be associated with a good clinical outcome [59].

Oda et al. (2012) documented that high levels of tumour $FOX3^+$ T cells prior to NAC were associated with high pCR rates [27]. In a cohort of patients with HER2 +ve cancers there was a better OS and DFS if the breast cancer cells themselves expressed $FOXP3^+$, possibly acting as a tumour suppressor gene [60]. In our study, the majority of breast samples were HER2 −ve and in only one specimen was $FOXP3^+$ expressed in the breast cancer cells. Lui et al. (2012) reported that decreased stromal $FOXP3^+$ Tregs after NAC were associated with a pCR, whilst intratumoural reduction after NAC was an independent prognostic predictor of OS [47]. High levels of $FOXP3^+$ Treg infiltration after NAC, however, correlated

with enhanced rates of pCR in another study [28]. Our results are in agreement with the published findings regarding $CD8^+$: $FOXP3^+$ T cell profiles and the post-NAC reduction of $FOXP3^+$ T cells and pCR (surrogate marker of improved survival). Our findings, however, do not agree with the data reporting a beneficial response to NAC with high levels of $FOXP3^+$ cell infiltration before and after NAC. The reasons for this discrepancy are not clear.

CTLA-4 (CD152) is a coinhibitory receptor molecule expressed on activated T cells and Tregs that negatively regulates T cell interaction with B7-1 (CD80)/B7-2 (CD86) ligand binding sites competing with CD28 which upregulates T cell activation [61, 62]. There is little expression on inactive or naive Tregs [63, 64]. CTLA-4 inhibits the interaction of CD28 receptors on $CD4^+$ and $CD8^+$ T cells with CD80/86 ligands on DCs and reduces IL-2 production, IL-2 receptor expression, and cell cycle progression of activated T lymphocytes, resulting in inhibition of activated DCs and generation of $CD4^+$ Th subsets and $CD8^+$ CTLs [65, 66]. Thus CTLA-4 is an important immune checkpoint inhibitor of both $CD4^+$ and $CD8^+$ T effector cells preventing inappropriate and prolonged T cell activation and resultant tissue damage. In breast cancer there is increased expression of CTLA-4, compared with normal breast tissue [66]. Increased mRNA levels of CTLA-4 were shown to be associated with ALN metastases and more advanced tumour stage [66, 67]. We had previously demonstrated high levels of CTLA-4^+ cells in the blood of women with LLABCs [21]. In our current study there was a wide range of levels of CTLA-4^+ cells infiltrating the LLABCs but overall, the levels were low.

We demonstrated a significant reduction of $FOXP3^+$ (intratumoural, stromal) and CTLA-4^+ T cells (stromal) in tumours following 8 cycles of NAC. The FOXP3 findings are in agreement with published data [28, 44, 47]. The CTLA-4^+ T cell findings have not been previously reported. We also showed a concurrent significant reduction of Tregs ($FOXP3^+$, CTLA-4^+) in the blood of the same cohort of patients [21]. There was, moreover, a positive correlation between the post-NAC % of blood $FOXP3^+$ Tregs and post-NAC intratumoural infiltration by $FOXP3^+$ T cells. Thus, the significant reduction in the circulating levels of Tregs in women with LLABCs undergoing NAC was associated with a substantial and significant concomitant reduction of $FOXP3^+$ and CTLA-4^+ T cells infiltrating the breast tumours. After NAC there was a significantly higher % of blood $FOXP3^+$ Tregs and significantly higher level of intratumoural (tumour cell nests) $FOXP3^+$ T cells in patients whose tumours had a poor pathological response and failed to demonstrate a pCR. To the best of our knowledge, these various findings have not been previously published. Our results highlight the importance of regulatory suppressor mechanisms in the circulation and tumour environment in inducing immune-mediated tumour cell death with NAC.

PD-1 (CD279) is a transmembrane receptor and a member of the CD28 family and is expressed on activated T cells and other lymphocytes (Tregs, NK cells, and B cells) [68–70]. When interacting with PD-L1 and PD-L2 in a coinhibitory pathway in peripheral tissues it dampens down activated T cells (cytotoxic activity, proliferation, and cytokine production) maintaining peripheral T cell tolerance and preventing autoimmunity [71]. The PD-1 pathway is one of the immune checkpoints exploited by cancer cells to escape anticancer immune defenses [72]. PD-L1 is expressed on different lymphoid cells, is upregulated in various normal cells in inflammation, and is expressed in many human cancers. It has been shown to correlate with tumour size, grade, metastatic spread, and reduced levels of tumour-infiltrating $CD8^+$ T cells [73–75]. High levels of PD-1^+ cells have been shown to have a significant correlation with reduced patient survival [76]. In our study, although there was a wide range in both the intra- and peritumoural stromal compartments, the infiltration in general was low. A significant reduction of both intratumoural and stromal infiltration by PD-1^+ T lymphocytes was seen following 8 cycles of NAC. The level of infiltration in LLABCs, however, was not associated with a subsequent pCR following NAC. There is a lack of data in the literature about the effect of NAC on the PD-1^+ T cell subsets infiltrating LLABCs. We believe this to be a newly reported finding.

In various human cancers malignant cells and host infiltrating cells express and secrete a range of Th1, Th2, and Th17 cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-17, and IFN-γ) and TGF-β. These cytokines modulate and suppress the *in situ* anticancer immune responses, enhancing tumour cell growth and progression, and propensity to metastasize [77–83]. In our study the semiquantitative method used did not discriminate between the tumour-infiltrating immune and inflammatory cells and the malignant cells nor quantify precisely the contribution of the various host immune and inflammatory cells to the cytokine levels in the tumour microenvironment.

In the tumour microenvironment Th1, Th2, and Th17 cytokines, as well as TGF-β, play an important role in modulating *in situ* innate and adaptive immune mechanisms [84]. The Th1 cytokines IL-2 and IFN-Υ enhance CTL-and NK cell-mediated regression of cancer cells. IFN-Υ can either promote or suppress Treg activity depending on the cytokine environment. IL-2 also has a key role in controlling Treg function in the periphery [51]. The Th2 cytokines IL-4 and IL-10 suppress the generation of CTLs and Th1 cells and recruit tumour entry of Tregs [1, 53]. Moreover, IL-4 has been shown to both increase and inhibit Treg function. It can enhance FOXP3 expression and suppressor activity of Tregs and conversely can inhibit TGF-β induced Treg development [85, 86]. Th1 and Th2 cytokine expression in tumours has a variable effect on patient outcomes in a range of human cancers, including breast cancer [2]. The role of IL-17 is not well defined. Some animal studies suggest it promotes tumour growth and angiogenesis [87, 88]. Yamazaki et al. (2008) have shown that IL-17 promotes the recruitment of Tregs to sites of IL-17 mediated inflammation [89]. Others have suggested an increased generation of CTLs and an enhanced tumour rejection [90, 91]. Contradictory results have been demonstrated in a range of human tumours, including breast cancer [2]. In one study in breast cancer, the level of Th17 cells was shown to be increased and associated with an improved prognosis [81]. TGF-β expression is usually

upregulated in human cancers. It induces production of FOXP3$^+$ Tregs and has strong immunosuppressive effects, inhibiting the generation and activity of innate (DCs, NK cells) and adaptive (CD4$^+$ and CD8$^+$ T cells) immunity [53, 84]. TGF-β can promote an epithelial to mesenchymal transition, resulting in enhanced tumour cell mobility, local invasion, and formation of metastases [92]. An inflammatory environment, not infrequent in tumours, can induce the transformation of FOXP3$^+$ Tregs into FOXP3$^-$ effector cells producing IFN-Υ [93]. IL-6 can also induce FOXP3$^+$ Treg loss and transformation to a Th17 phenotype and function [94]. This is further evidence of the plasticity of the different CD4$^+$ T cell effector-regulator subsets. The interplay between the different T cell profiles in human cancers is complex, the outcomes variable, and in need of further careful study.

The effect of NAC on Th1, Th2, and TH17 cytokine production in tumours is poorly documented. In our study, pre-NAC levels of expression were not associated with a pCR following NAC. IL-4 was significantly reduced in the tumour microenvironment following NAC. A similar but nonsignificant trend was seen with the *in situ* expression of IL-2 ($p = 0.070$). Post-NAC expression of IL-10 and IL-17, however, showed a significant association with failure to achieve a pCR. There was a similar trend for the *in situ* presence of TGF-β ($p = 0.062$). These various findings with NAC have not previously been reported.

High tumour grade is known to be associated with a NAC-induced pCR in breast cancer. High tumour grade was shown to be significantly associated with tumour infiltration (intratumoural, stromal) by CD4$^+$ and CD8$^+$ T cells and stromal FOXP3$^+$ T cells, which may have contributed to the NAC-induced pCR. There was no significant association with any of the clinical or other pathological parameters studied. This may be due to the relatively small number of specimens studied. In a multivariate analysis, a high TIL level was a significant independent predictor of a pCR with NAC and is in agreement with published data.

Most chemotherapeutic agents inhibit aspects of innate and adaptive immunity. Some, however, can enhance anticancer immunity and activate immune-mediated tumour cell death [19, 95–97]. Chemotherapy can induce cancer cell stress/damage resulting in the release of "danger" signals (e.g., heat shock proteins) and immunogenic tumour-associated antigens (TAAs). The former activate innate immune cells, whilst the latter are taken up by DCs leading to the release of proinflammatory cytokines and the generation of anticancer CTL responses. Anthracyclines, in particular, induce tumour cell damage and exposure of calreticulin and other endoplasmic reticulum proteins, secretion of ATP, and release of the high-mobility group box 1 (HMGB1) molecules. These interact with receptors on DCs, stimulating uptake and presentation of TAAs to naive T cells [18, 98–100].

The NAC combination (anthracycline, cyclophosphamide, and taxane ± capecitabine) used in our trial is known to have immunomodulatory effects. Doxorubicin has been shown to enhance the generation of antigen-specific CD8$^+$ T cells and promote tumour infiltration by activated IFN-γ producing CD8$^+$ T cells [69, 101]. *In vitro*, doxorubicin increased antigen-specific CD4$^+$ Th1 responses by inducing expression of CD40L and 4-1BB on CD4$^+$ T cells [69]. Cyclophosphamide inhibits the generation and function of FOXP3$^+$ Tregs in humans with various cancers [97, 102]. Taxanes have been shown to have immune stimulatory effects against tumours [95, 103]. In patients with advanced breast cancer, docetaxel therapy was associated with an increase in serum IFN-γ, IL-2, and IL-6 levels and enhancement of circulating NK cell activity [95]. Capecitabine is enzymatically converted to 5-fluorouracil (5-FU) on ingestion. 5-FU is known to increase the expression of TAAs on tumour cells and to enhance antibody-dependent cell-mediated cytotoxicity [104]. In mice, 5-FU induced depletion of immunosuppressive myeloid-derived suppressor cells and enhanced production of IFN-γ by tumour-infiltrating CD8$^+$ T cells [105].

The NAC combination used in our study differentially preserved the tumour-infiltrating CD8$^+$ T cell population but significantly reduced both the circulating and tumour-infiltrating FOXP3$^+$, CTLA-4$^+$ (stromal), and immune checkpoint PD-1$^+$ T cells, thereby preventing the secretion of inhibitory cytokines (IL-4, IL-10, and TGF-β) and disrupting the PD-1/PD-L1 pathway. The restoration of immune anticancer effector mechanisms is likely to lead to an enhancement of immune-mediated tumour cell death. Moreover, the significant correlation of high CD8$^+$ T cells and CD8$^+$: FOXP3$^+$ T cell ratio with pCR (and hence DFS and OS) suggests a close association between high levels of CD8$^+$ T cells/CTLs and the concomitant depletion of Tregs. Dysfunctional CD8$^+$ T cell responses as a result of excessive and prolonged stimulation and continuous inappropriate signal activation result in T cell exhaustion and loss of effector and memory function. This persists even after removal of Tregs [106]. The close interrelationship between a pCR in LLABCs and the concomitant immune changes induced by NAC suggests that immune-mediated cell death may be a crucial component of NAC-associated tumour cell destruction and removal. A better understanding of this complex relationship in human cancer, in particular, the factors preventing optimal delivery of immune-mediated tumour cell death, is essential for devising more effective chemotherapeutic strategies in the management of cancer.

5. Conclusions

Our study has confirmed previously published findings and documented novel findings, further establishing that the immune microenvironment is a key contributing factor in achieving a better pathological response with NAC. The level of TILs and CD4$^+$ and CD8$^+$ T cell subsets in LLABCs, which were well demonstrated with the IHC techniques used, could be clinically useful to further define women with LLABCs who may benefit from NAC. These biological markers can be readily determined from histopathological examination of breast tumour biopsies (using H&E and IHC) before commencing therapy. They may supplement other clinical parameters in establishing optimal treatment, as well as prognostic prediction, for individual women with LLABCs suitable for NAC.

Abbreviations

5-FU: 5-Fluorouracil
A: Adriamycin
AbN: Absolute number
ALN: Axillary lymph node
C: Cyclophosphamide
CD: Cluster of differentiation
CTL: Cytotoxic T lymphocyte
CTLA-4: Cytotoxic T lymphocyte antigen 4
DAB: Diaminobenzidine
DFS: Disease-free survival
DC: Dendritic cell
DCIS: Ductal carcinoma *in situ*
ER: Oestrogen receptor
FOXP3: Forkhead box protein 3
HER2: Human epidermal growth factor receptor 2
HMGB1: High-mobility group box 1
HPF: High-power field
HRP: Horseradish peroxidase
H&E: Haematoxylin and eosin
IHC: Immunohistochemistry
IL: Interleukin
IFN-γ: Interferon-gamma
Itu-Ly: Intratumoural lymphocyte
LLABC: Large locally advanced breast cancer
MAb: Monoclonal antibody
MHC: Major histocompatibility complex
NAC: Neoadjuvant chemotherapy
NK: Natural killer
OS: Overall survival
pCR: Pathological complete response
PD-1: Programmed death 1
PD-L1: Programmed death ligand 1
RT: Room temperature
Str-Ly: Stromal lymphocyte
T: Docetaxel
TAA: Tumour-associated antigen
Th: T helper
Treg: T regulatory cell
TCR: T cell receptor
TGF-β: Transforming growth factor-beta
TIL: Tumour-infiltrating lymphocyte
X: Capecitabine.

Ethical Approval

The study was given approval by the Leicestershire, Northamptonshire & Rutland Research Ethics Committee 1: Reference no. 07/H0406/260, favourable opinion 24/01/2008. The study registration is ISRCTN00407556.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

The authors contributed as follows: conception and design: Viriya Kaewkangsadan, Chandan Verma, Jennifer M. Eremin, Gerard Cowley, and Oleg Eremin; data acquisition: Viriya Kaewkangsadan, Chandan Verma, Jennifer M. Eremin, Gerard Cowley, and Oleg Eremin; data analysis and interpretation: Viriya Kaewkangsadan, Chandan Verma, Jennifer M. Eremin, Gerard Cowley, Mohammed Ilyas, and Oleg Eremin; laboratory assays: Viriya Kaewkangsadan, Chandan Verma, and Gerard Cowley; writing of manuscript: Viriya Kaewkangsadan, Chandan Verma, Jennifer M. Eremin, and Oleg Eremin; review of and final approval of manuscript: Viriya Kaewkangsadan, Chandan Verma, Jennifer M. Eremin, Gerard Cowley, Mohammed Ilyas, and Oleg Eremin.

Acknowledgments

The authors wish to acknowledge Mr. Christopher Nolan (Academic Unit of Clinical Oncology, City Hospital, University of Nottingham) for his advice and help with the IHC assays. The clinical trial, from which patients' tissue specimens and blood samples were collected for the study, was supported by educational grants from Sanofi-Aventis, UK, Roche, UK, and Chughai, UK. The authors wish to acknowledge the financial support provided for this study by a grant from the Nottinghamshire, Derbyshire and Lincolnshire Research Alliance, and Candles Charity.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 7828916, 9 pages
http://dx.doi.org/10.1155/2016/7828916

Research Article

Downregulation of p70S6K Enhances Cell Sensitivity to Rapamycin in Esophageal Squamous Cell Carcinoma

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Received 10 January 2016; Accepted 13 July 2016

Academic Editor: Kurt Blaser

It has been demonstrated that mTOR/p70S6K pathway was abnormally activated in many cancers and rapamycin and its analogs can restrain tumor growth through inhibiting this pathway, but some tumors including esophageal squamous cell carcinoma (ESCC) appear to be insensitive to rapamycin in recent studies. In the present study, we explored the measures to improve the sensitivity of ESCC cells to rapamycin and identified the clinical significance of the expression of phosphorylated p70S6K (p-p70S6K). The results showed that, after downregulating the expression of p70S6K and p-p70S6K by p70S6K siRNA, the inhibitory effects of rapamycin on cell proliferation, cell cycle, and tumor growth were significantly enhanced *in vitro* and *in vivo*. Furthermore, p-p70S6K had strong positive expression in ESCC tissues and its expression was closely related to lymph node metastasis and the TNM staging. These results indicated that p-p70S6K may participate in the invasion and metastasis in the development of ESCC and downregulation of the expression of p-p70S6K could improve the sensitivity of cells to rapamycin in ESCC.

1. Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most common and aggressive malignant tumors in China [1]. Although therapeutic strategies have been improved, the prognosis of ESCC patients is still poor because of the clinicopathological characteristics of ESCC such as rapid clinical progress, lymph node metastasis, local recurrence, and resistance to chemotherapeutic drugs [2, 3], which impel us to further explore the molecular mechanism in carcinogenesis and progression and treatment strategy of ESCC.

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase and can be activated by insulin, nutrients, and growth factors [4–6]. mTOR kinase exists in two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 consists of Raptor, Lst8, FKBP38, Deptor, PRAS40, and mTOR; mTORC2 complex is built up by Rictor, Lst8, Sin1, Deptor, Protor, and mTOR. In mammals, mTORC1 plays key roles in ribosome biogenesis and cap-dependent mRNA translation through p70S6K, which is a major downstream effector of mTORC1. The abnormal activation of mTOR pathway has been demonstrated in many cancers [6–8]; the activated mTORC1 promotes the phosphorylation of p70S6K (p-p70S6K) and the releasing of phosphorylated eukaryotic elongation initiation factor 4E (eIF4E) binding protein 1 (4EBP1) from eIF4E, which ultimately result in enhanced translation of genes that are required for cell growth. Accumulating evidences have demonstrated that mTOR and its downstream effectors such as p70S6K and 4EBP1 have central roles not only in cell growth but also in tumor invasion and metastasis [6–8]. Therefore, mTOR pathway has been recognized as an important and attractive therapeutic target for cancer therapy [9, 10]. It has been shown that the inhibitors of mTOR such as rapamycin (Rapa), temsirolimus (CCI-779), and everolimus (RAD001) can reduce tumor cell size and inhibit cell proliferation by inhibiting mTOR pathway, which have been studied both preclinically and clinically for treating a variety of tumor types [11–15]. But recently, it is increasingly recognized that rapamycin and its

analogs (rapalogs) are not sufficient to achieve abroad and robust anticancer effects; some tumors are even not sensitive or have resistance to them [16–18]. To explore the methods to improve the sensitivity of ESCC cells to rapamycin, in the present study, the expression of p70S6K and p-p70S6K in EC9706 cells was downregulated by p70S6K siRNA; the changes of cell sensitivity to rapamycin in cell proliferation, cell cycle, and tumor growth were investigated *in vitro* and *in vivo*. Moreover, the expression and clinicopathological significance of p-p70S6K were analyzed in tissues of 35 ESCC patients. This study explores the method to improve the sensitivity of cells to rapamycin and provides a diagnosis target for ESCC patients.

2. Materials and Methods

2.1. Cell Culture, Animal Treatment, and Patient Tissue Samples. Human ESCC cell lines EC9706, ECa109, and EC1 were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI/1640 medium with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere consisting of 5% CO_2, as described in our previous study [19]. Male athymic BALB/c nude mice (Slack King of Experimental Animals Co. Ltd., Wuhan, China) at 4 to 6 weeks of age and 18 to 22 g of weight were used in this study. All the animals were housed in independently ventilated cages (IVC) at a temperature of 25-26°C and a relative humidity of ~50%, lit 12 hours/day. All animal studies were carried out in compliance with the Guide for the Care and Use of Laboratory Animals of Henan Province, China. The data of patient tissue samples were described as in our previous paper [20].

2.2. Western Blot. ESCC cells were collected (or tissues of xenografts ground with liquid nitrogen in mortar) and lysed in protein lysis buffer and then centrifuged at 14,000 rpm for 15 minutes at 4°C and the supernatant was the total protein extracts. The protein concentration was determined by Bradford method [21]. Equivalent amounts of protein (30 μg) were separated by SDS-PAGE and electrotransferred to a PVDF membrane by a semidry transferor. After incubated for 1 hour at RT in blocking buffer (5% skimmed milk in PBS-T containing 0.05% Tween 20), the membranes were incubated with indicated primary antibodies: anti-p70S6K, anti-p-p70S6K, and anti-GAPDH (Santa Cruz Biotechnology, USA) of 1 : 400 diluted in 2% skimmed milk in PBS-T, respectively, at 4°C overnight, followed by incubating with the appropriate HRP-conjugated secondary antibodies of 1 : 8000. Finally, the bands of specific proteins on the membranes were visualized with chemiluminescent substrate (Santa Cruz Biotechnology, USA) according to manufacturer's instructions. Between the incubations of every step described above, the membranes were rinsed three times with PBS-T.

2.3. Semiquantitative RT-PCR. After EC9706 cells transfected with p70S6K siRNA or negative control siRNA (Santa Cruz Biotechnology, USA) were cultured for different time, the expression of p70S6K mRNA was detected by RT-PCR. In brief, total RNA of EC9706 cells was extracted with Trizol reagent (Invitrogen, Carlsbad, USA) and reversely transcribed to cDNA using AMV First Strand DNA Synthesis Kit (Biotech Company, Shanghai, China). The PCR amplification mixture (25 μL) consisted of 0.5 μL cDNA mixture, 0.5 U Taq DNA polymerase, 2.5 μL 10x PCR buffer, 2.5 mM dNTP mixture, and 50 pM of each of sense and antisense primers. The sequence of primers for p70S6K (204 bp) is forward primer 5′-ATG CTG CTT CTC GTC TGG-3′ and reverse primer 5′-TTG AGT CAT CTG GGC TGT-3′ and for GAPDH (internal control, 570 bp) is forward primer 5′-CAA GGT CAT CCA TGA CAA CTT TG-3′ and reverse primer 5′-GTC CAC CAC CCT GTT GCT GTA G-3′. The PCR conditions were as described previously [19]. The amplified products were subjected to electrophoresis on 1% agarose gel containing 0.2 μg/μL ethidium bromide and visualized under UV light.

2.4. Cell Proliferation and Cell Cycle Phase Analysis. Cell proliferation was determined by Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, China) according to manufacturer's instructions. Briefly, EC9706 cells transfected with p70S6K siRNA or negative control siRNA for 24 hours were harvested and seeded in a 96-well flat-bottomed plate (5×10^3 cells/well) and cultured at 37°C for 24 hours. Subsequently, these cells were treated with rapamycin (Sigma Aldrich, USA) at different concentrations for 48 h. After 10 μL CCK-8 reagent was added to each well, cells were incubated at 37°C for 4 hours and the absorbance was finally determined at 450 nm using a microplate reader (Bio-Rad Laboratories, USA). Each treatment group was assayed in triplicate for each group.

Cell cycle phase analysis was conducted by flow cytometry. Briefly, cells transfected with p70S6K siRNA for 24 hours were treated with 100 nM rapamycin for 48 hours. Cells were harvested and fixed in 70% cold ethanol and kept at 4°C. After cells were further incubated with RNase (50 μg/mL) for 30 minutes at 37°C, 5 μL propidium iodide (50 μg/mL) was added to cell suspension and continued to incubate at RT for 30 minutes in the dark before analysis, and then the cell cycle phase was analyzed by flow cytometry. Cells with negative control siRNA were used as controls [22].

2.5. Xenograft Studies. Twenty athymic mice were divided into two groups of 10 mice each and were subcutaneously inoculated with EC9706 cells transfected with p70S6K siRNA or negative control siRNA for 24 hours, respectively. Briefly, cells with p70S6K siRNA or control siRNA were harvested, washed, and resuspended in PBS at 2×10^7 cells/mL. A cell resuspension of 200 μL (4×10^6 cells) was inoculated s.c. into the left flank of athymic mice. For tumor growth analysis, the tumor size was measured every other day with a sliding caliper, and the tumor volume was defined as (longest diameter) × (shortest diameter)^2/2. Further, tumor-bearing animals of the two groups were randomly subdivided into 2 subgroups of 5 animals each, respectively, and drug treatment was initiated when tumor volume reached 60–100 mm^3 [23].

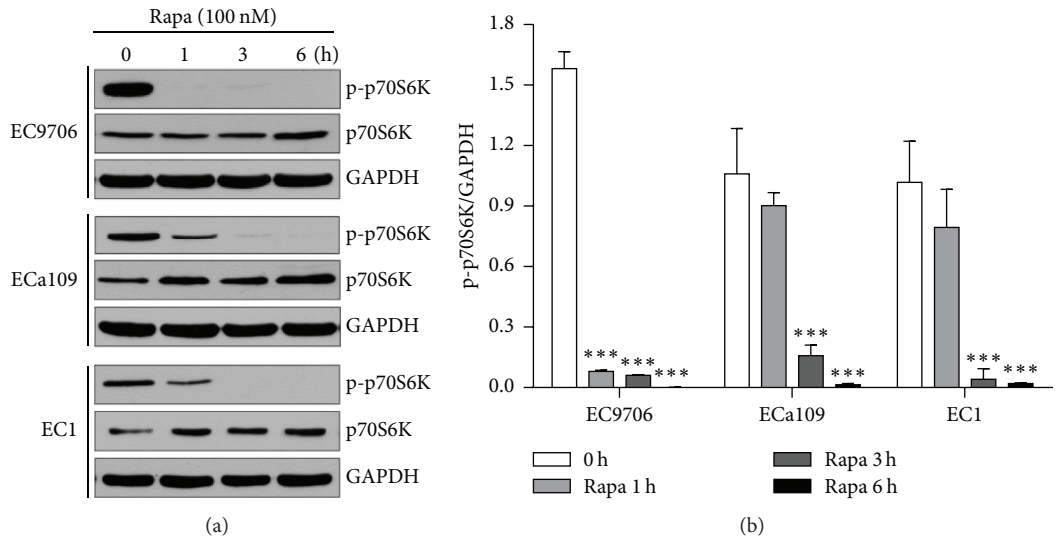


Figure 1: Effects of rapamycin on protein expressions of p70S6K/p-p70S6K in ESCC cell lines. (a) Antibodies to p-p70S6K and p70S6K, respectively. (b) Semiquantitative values from three independently repeated experiments, which were statistically analyzed by densitometry using software Image J (NIH, USA), are expressed as means ± SD. $^{***}P < 0.001$ compared to untreated cells. GAPDH was used as loading control.

The treatment schedule was that the groups with p70S6K siRNA or negative control siRNA were injected i.p. with rapamycin (50 mg/kg) or PBS as controls, respectively, every other day for two and a half weeks. After the treatment was over, tumor-bearing mice were sacrificed and the tumors were removed, weighted, and then stored in liquid nitrogen till for protein analysis. All procedures were conducted in a laminar-flow biosafety hood. Inhibition rate = [(tumor volume of control group − tumor volume of experimental group)/tumor volume of control group] × 100%.

2.6. Immunohistochemical Analysis. The expressions of p-p70S6K in 35 tissues were measured by immunohistochemistry and the protocols in detail were described as in our previous study [20]. The anti-p-p70S6K antibody was used at a dilution of 1 : 200 and the evaluation of immunohistochemical results was performed by a pathologist without knowledge of the clinical and pathologic characteristics of these patients. The tumor cells in slides were scored according to the intensity (I), distribution (D), and pattern (P) reported by Dong et al. [24]: I score: 0, negative; 1, weak; 2, moderate; and 3, strong; D score (%): 0, negative; 1, 10–50%; 2, 51–90%; and 3, >90%; P score: 0, no staining; 1, sporadic positive staining; 2, focal positive staining; and 3, diffuse positive staining. The total scores of each tissue = $I \times D \times P$, and the 0 score was negative and ≥1 score was positive. The relationship between the expression levels of p-p70S6K and differentiation degree, depth of infiltration, lymph node metastasis and TNM stage, and the expression relevance of p-p70S6K with mTOR (data of mTOR expression has been reported in our previous study [19]) were analyzed, respectively.

2.7. Statistical Analysis. The results of all experiments were analyzed by standard Chi-square test or one-way analysis of variance where it was appropriate, using SPSS 16.0 (SPSS, Chicago, USA). All summary statistics were expressed as mean ± SD. In all statistical analyses, $P < 0.05$ was considered statistically significant.

2.8. Study Ethics Approval. The study was approved by the Ethics Committee of Zhengzhou University, Henan, China.

3. Results

3.1. Protein Expression of p70S6K/p-p70S6K and Effect of Rapamycin on Them in ESCC Cell Lines. After EC9706, ECa109, and EC1 cells were exposed to 100 nM of rapamycin for different time (0, 1, 3, and 6 h), respectively, the protein expressions of p-p70S6K and p70S6K were analyzed by Western blot. The results showed that p-p70S6K had obvious expression in the three cell lines, which had the highest expression level in EC9706 cells. After cells were treated with rapamycin, the expression of p-p70S6K was obviously reduced in the three ESCC cell lines, especially in EC9706 cells, and rapamycin obviously inhibited the expression of p-p70S6K at a short time (1 hour) compared to that in the other two ESCC cell lines ($P < 0.001$, Figure 1).

3.2. p70S6K siRNA Downregulated the Expression of p70S6K mRNA and Protein in EC9706 Cells. To detect the interfering efficiency of p70S6K siRNA to the expression of p70S6K in EC9706 cells, the levels of p70S6K mRNA and protein in cells transfected with p70S6K siRNA for different times were measured by RT-PCR and Western blot. As shown in Figures 2(a) and 2(b), the expression levels of p70S6K mRNA decreased markedly in a time dependent manner. Compared to cells transfected with negative control siRNA, the inhibition rates of p70S6K siRNA on the expression of

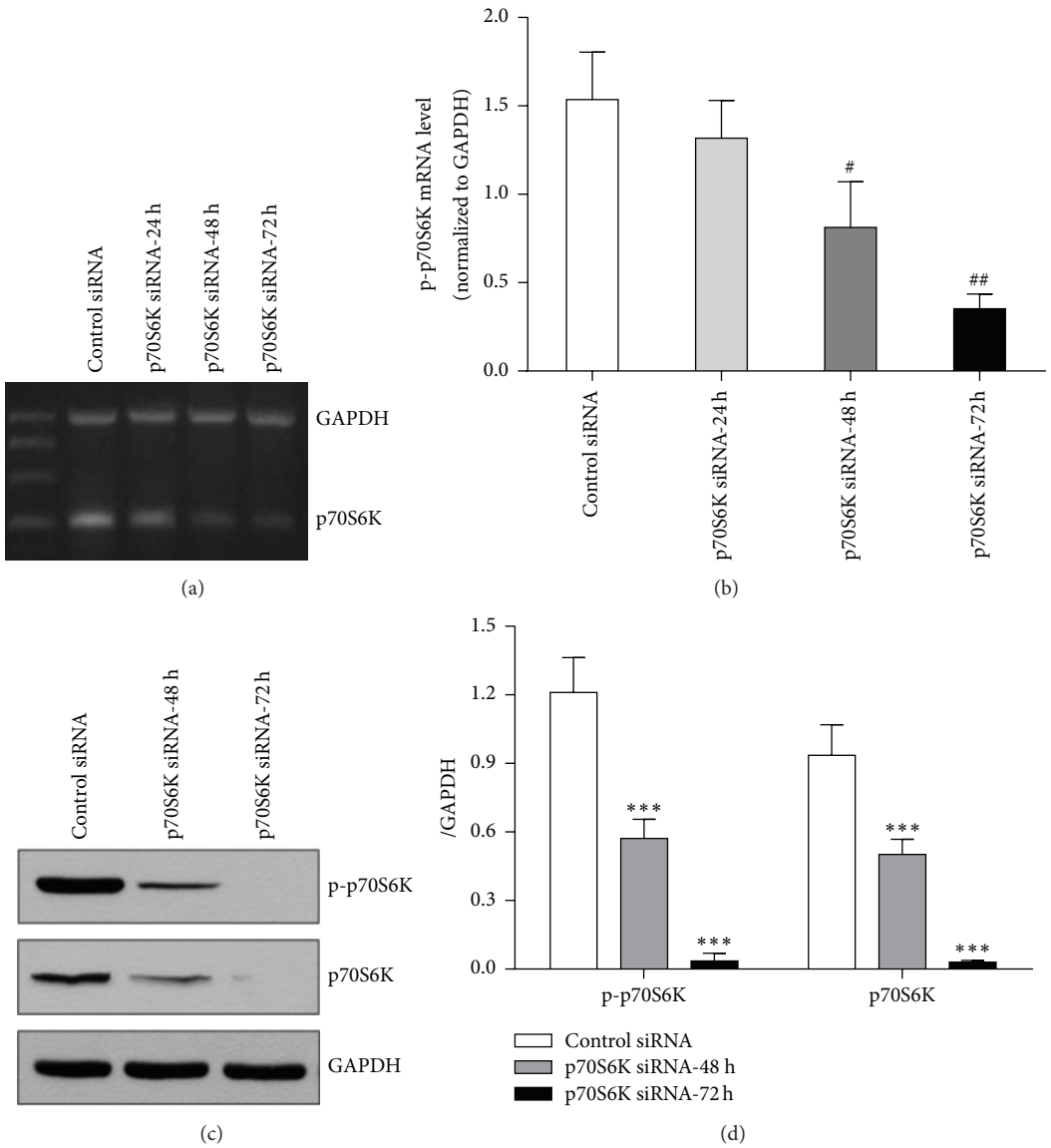


Figure 2: Interference efficiency of p70S6K siRNA on mRNA and protein of p70S6K in EC9706 cells. (a) Expression of p70S6K mRNA. (b) Semiquantitative values of p70S6K mRNA normalized to GAPDH mRNA. (c) Expressions of p70S6K/p-p70S6K protein. (d) Semiquantitative values of p-p70S6K/p70S6K protein normalized to GAPDH. Results were from three independently repeated experiments analyzed by using Image J (NIH, USA); data were expressed as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ for mRNA results, and $^{***}P < 0.001$ for protein expression, compared to those of EC9706 cells treated with negative control siRNA.

p70S6K mRNA were 14.76%, 43.75%, and 79.00% at 24, 48, and 72 hours, respectively. Moreover, the protein expression of p-p70S6K and p70S6K decreased significantly after cells were transfected with p70S6K siRNA and the decreasing ratios were 72.0% and 92.73% for p-p70S6K and 59.86% and 85.52% for p70S6K at 48 and 72 hours, respectively, compared to cells with negative control siRNA (Figures 2(c) and 2(d)). The results above showed that p70S6K siRNA could efficiently downregulate the expressions of p70S6K and p-p70S6K.

3.3. p70S6K siRNA Increased the Inhibition Effects of Rapamycin on Cell Proliferation and Cell Cycle of EC9706 Cells. The results of cell proliferation showed that, in cells with negative control siRNA, rapamycin inhibited cell proliferation at low concentration (≤100 nM), while the inhibition effects receded along with the increase of rapamycin concentration (≥100 nM). And, compared to untreated cells, the inhibition rates were 23.20%, 25.47%, 31.73%, 28.26%, 23.77%, and 19.79% at 20, 50, 100, 200, 500, and 1000 nM of rapamycin, respectively. But after cells were transfected with p70S6K siRNA, the inhibition effects of rapamycin on cell proliferation were enhanced, and compared to untreated cells, the inhibition rates were 25.48%, 51.33%, 64.55%, 86.00%, 89.03%, and 97.30%, respectively. Furthermore, beginning with 50 nM, the inhibition effects of the same concentration of rapamycin on proliferation of cells

Table 1: Effects of rapamycin alone or combined with p70S6K siRNA on the growth of ESCC xenografts in nude mice ($n = 5$).

Groups	Animal weight (g)	Volume before therapy (mm^3)	Volume after therapy (mm^3)	Inhibition rate (%)
Control	18.80 ± 1.92	82.68 ± 11.94	1907.86 ± 326.00	0
Rapamycin	20.07 ± 1.50	63.12 ± 23.18	546.62 ± 94.53	70.35*
p70S6K siRNA	19.54 ± 0.63	87.99 ± 9.08	1658.72 ± 159.58	13.06
siRNA + Rapa	20.12 ± 1.28	61.14 ± 15.42	67.03 ± 33.88*	96.49*

* $P < 0.05$, compared with control group.

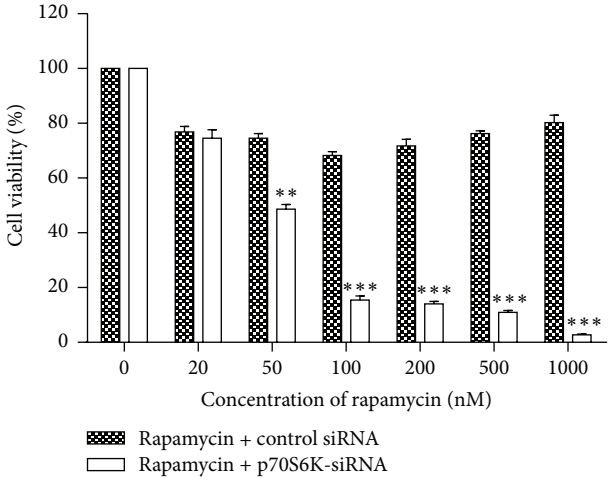


Figure 3: Effects of rapamycin on cell proliferation. Cells transfected with p70S6K siRNA or negative control siRNA for 24 hours were treated with rapamycin at different concentrations for 48 hours and cell proliferation was detected with CCK-8 kit. Data pooled from three independent experiments were expressed as mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared to cells with negative control siRNA treated with rapamycin at the same concentration.

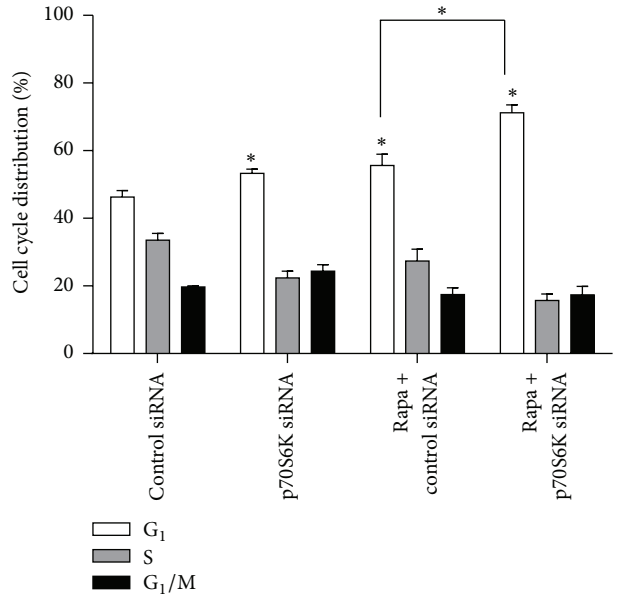


Figure 4: Effects of rapamycin on cell cycle phase. EC9706 cells were transfected with p70S6K siRNA or negative control siRNA for 24 hours and then treated with 100 nmol/L rapamycin for 48 hours. Cell cycle phase was analyzed by flow cytometry. $^{*}P < 0.05$, compared to cells with negative control siRNA.

with p70S6K siRNA increased obviously compared to that with control siRNA ($P < 0.01$ or $P < 0.001$, Figure 3). The results above showed that the inhibition effects of rapamycin on cell proliferation became strong after cells were transfected with p70S6K siRNA.

The results of cell cycle analysis showed that rapamycin and p70S6K siRNA alone retarded cells to G_0/G_1 phase and the ratios of cells in G_0/G_1 phase were 57.87% and 53.82%, respectively, which had significant difference compared to control cells (ratio in G_0/G_1: 46.09%; $P < 0.05$). But when cells were treated with rapamycin combined with p70S6K siRNA, the ratio of cells in G_0/G_1 phase obviously increased and reached 73.73% (Figure 4). The results above indicate that p70S6K siRNA could promote the inhibition effects of rapamycin on the cell cycle phase of ESCC cells.

3.4. p70S6K siRNA Enhanced the Inhibition Effects of Rapamycin on Xenografts Growth of EC9706 Cells. The effects of p70S6K siRNA on cell sensitivity to rapamycin *in vivo* were investigated by xenografts experiment. As seen from the curves of tumor growth (Figure 5(a)), the growth of tumors in every experimental group was slower than that in PBS group. p70S6K siRNA alone had a relative smaller effect on tumor growth (inhibition rate 13.06%; Table 1), rapamycin significantly inhibited the growth of xenografts, and the inhibition rate reached 70.35% ($P < 0.05$), while rapamycin combined with p70S6K siRNA had the strongest inhibitory effect (inhibition rate: 96.49%; $P < 0.001$), and the tumor growth nearly stopped (Figures 5(a) and 5(b)). The Western blot results in xenografts showed that the expression of p-p70S6K in rapamycin + p70S6K siRNA group was lower than that in rapamycin or p70S6K siRNA group (Figures 5(c) and 5(d)), which maybe explain the reason that p70S6K siRNA enhanced the inhibition effects of rapamycin on tumor growth *in vivo*.

3.5. Expression and Analysis of Clinical Significance of p-p70S6K in ESCC Tissues. The immunohistochemistry results of p-p70S6K in 35 ESCC tissues showed that p-p70S6K was mainly expressed in the cell nucleolus (Figure 6). The positive expression rates of p-p70S6K were 33.3% (5/15), 73.3% (11/15), and 74.3% (26/35) in normal esophageal, dysplasia, and cancer tissues, respectively, which had a significant statistical

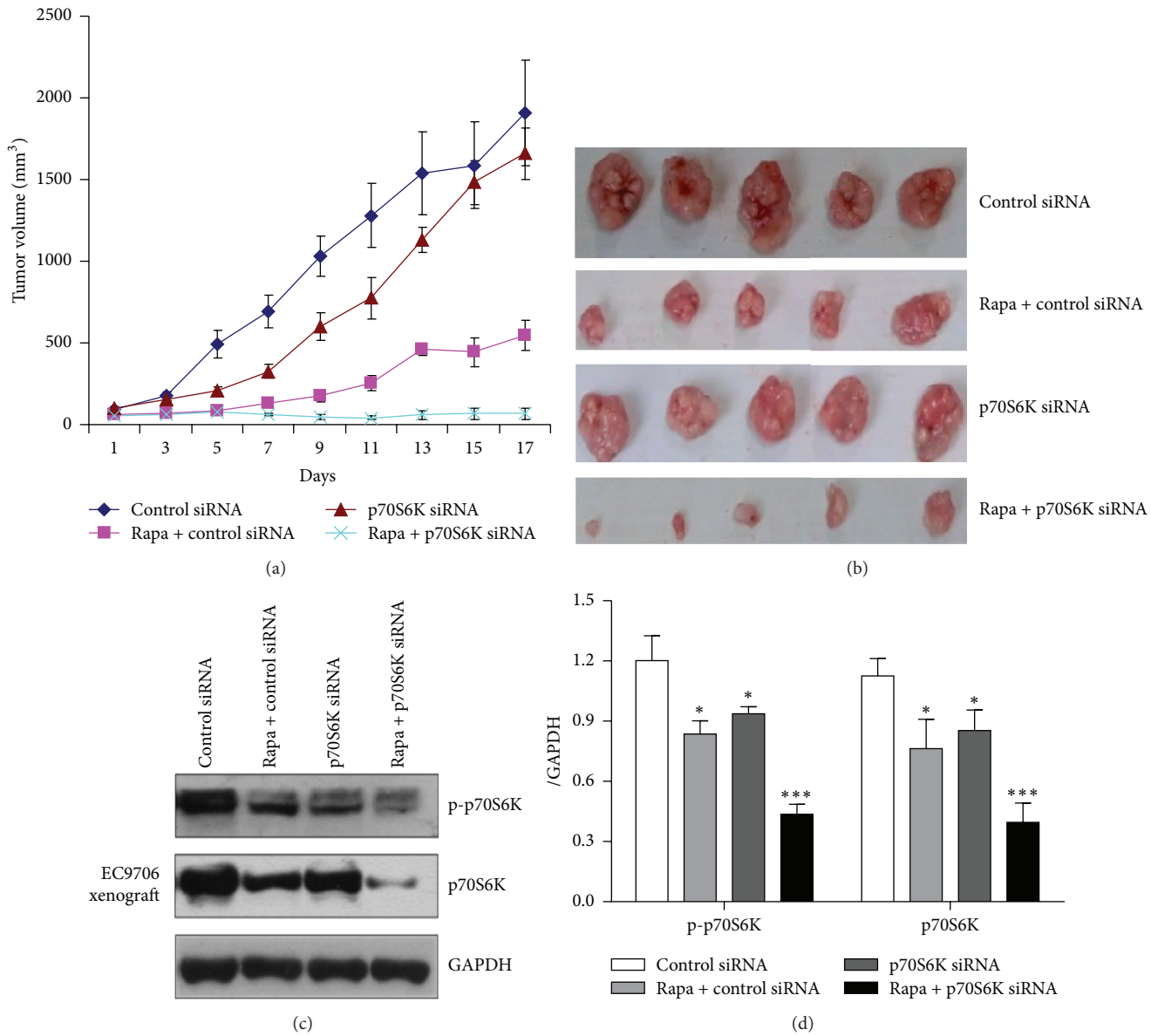
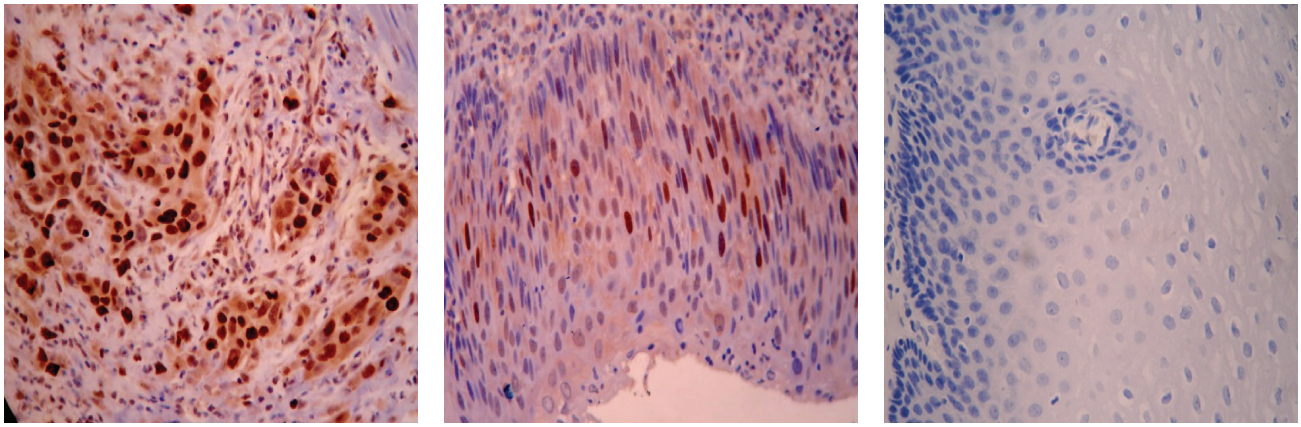


Figure 5: Tumor regression observed in EC9706 xenografts treated with different ways and the protein expressions of p70S6K and p-p70S6K in xenografts of each group. (a) Tumor volumes from the xenografts of each group were assessed every other day, and the results were expressed as means ± SE (mm^3). The tumor growth of each treated group became slow, in which group treated with p70S6K siRNA combined with rapamycin was the slowest. (b) Tumors from the xenografts treated with different ways for two and a half weeks. (c) The protein expression of p70S6K and p-p70S6K in EC9706 xenografts analyzed by Western blot. (d) Semiquantitative values from three independently repeated experiments, which were statistically analyzed by densitometry using software Image J (NIH, USA), are expressed as means ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to control group. GAPDH was used as loading control.

difference among them ($P < 0.05$; Tables 2 and 3). The expression of p-p70S6K was not related to the histologic type and the depth of infiltration (both $P > 0.05$) but closely related to lymph node metastasis and the TNM stage (both $P < 0.05$). Furthermore, there were 19 tissues with positive expression of p-p70S6K in 22 tissues with positive expression of mTOR, while there were 6 tissues with negative expression of p-p70S6K in 13 tissues with negative expression of mTOR, indicating a positive correlation of the expression level between p-p70S6K and mTOR ($P < 0.05$; Table 4). The results above indicate that p-p70S6K might participate in metastasis and invasion of ESCC and could look as a diagnosis target of ESCC patient.

4. Discussion

Rapamycin, a macrolide antibiotic discovered from the bacterium *Streptomyces hygroscopicus*, was the first identified mTOR inhibitor and its anticancer effects were disclosed for the first time in 2002 [25], while rapamycin was not used in



(a) (b) (c)

Figure 6: Expression of p-p70S6K in human normal esophageal and ESCC tissues by immunohistochemical analysis (×400). (a) Positive expression of p-p70S6K in ESCC tissues. (b) Moderate positive expression of p-p70S6K in dysplasia tissues of the esophagus (×400). (c) Negative expression of p-p70S6K in normal tissues of the esophagus (×400).

Table 2: Expressions of p-p70S6K protein in different tissues.

Tissue type	p-p70S6K				P
	n	−	+	Positive rate (%)	
Normal	15	10	5	33.3	
Dysplasia	15	4	11	73.3	0.015
Cancer	35	9	26	74.3	

Table 3: Clinical significance of p-p70S6K protein expression.

Pathological features	n	p-p70S6K	
		Positive n (%)	P
Histology classification			
I	9	7 (77.8)	
II	14	10 (71.4)	0.942
III	12	9 (75.0)	
Depth of infiltration			
Mucosa	7	4 (57.1)	
Muscle layer	15	11 (73.3)	0.404
Fiber membrane	13	11 (84.6)	
Lymph node metastasis			
No	19	11 (57.9)	0.016
Yes	16	15 (93.8)	
TNM phase			
I, II	13	7 (53.8)	0.033
III, IV	22	19 (86.4)	

Table 4: Correlation of the expression level of p-p70S6K and mTOR in ESCC tissues.

p-p70S6K	n	mTOR		P
		+	−	
+	26	19	7	0.034
−	9	3	6	

clinic but only in basic study about mTOR pathway because of its some drawbacks such as low aqueous solubility, poor oral bioavailability, and systemic toxicity [10]. CCI-779 and RAD001, the derivatives of rapamycin, have been proved by the FDA for treating some tumors such as advanced-stage renal cell carcinoma, pancreatic tumor, and hormone-receptor-positive advanced breast cancer [14, 15, 26], and RAD001 is also currently being tested as a single agent or in combination with additional therapies for the treatment of various cancer types [27]. However, although many papers have reported the antitumor effects of rapamycin and its derivatives in preclinical models of human tumors *in vitro* and *in vivo*, the efficacy of them as broad-based monotherapy for the treatment of cancer patients has not been as promising as initially expected because of their poor proapoptotic activity, not targeting all mTORC1 outputs, and the existence of multiple negative feedback regulatory loops [17, 26]. It has been shown that small interfering RNA (siRNA) and short hairpin RNA (shRNA) can effectively downregulate gene expression, modulating or selectively blocking the biological processes regulated by the target genes, and thus have been widely used in cancer research [28]. ESCC is a common cancer in China and had high mortality rate; we have conducted some previous studies about the mTOR pathway in ESCC and demonstrated the activation of mTOR pathway in ESCC [19, 20, 22]. Moreover, we found that some ESCC cell lines were not sensitive to rapamycin and even had resistance to rapamycin (paper in publishing by Disease of Esophagus). For exploring the method to improve the sensitivity of ESCC cells to rapalogs, we investigated the effects of rapamycin combined with RNA interference on ESCC *in vitro* and *in vivo* in the present study. Furthermore, the expression of p-p70S6K in tissues of clinical ESCC patients and clinic significance were analyzed for finding a diagnosis target of ESCC patients.

In this study, we showed that p-p70S6K had obvious expression and rapamycin suppressed the expression of

p-p70S6K and promoted the expression of p70S6K in the three cell lines, while, in EC9706 cells, p-p70S6K had the highest expression and the inhibition effect of rapamycin on it was the strongest. Thus, we chose EC9706 cell line to explore the methods to improve the sensitivity of cells to rapamycin in the following experiments. Now that aberrant activation of mTOR/p70S6K pathway plays an important role in tumorigenesis and phosphorylated p70S6K by mTOR has higher activity to promote translation than p70S6K [6, 9], we speculated the combination of mTOR inhibitor and p70S6K siRNA could inhibit mTOR/p70S6K pathway at the most extent and thus inhibit tumor growth better. To verify our hypothesis, p70S6K siRNA was used in the present study for interfering with the expression of p70S6K, and then the changes of cell sensitivity to rapamycin were investigated in ESCC cells and xenografts. The results demonstrated that p70S6K siRNA downregulated the expression of p70S6K at mRNA and protein levels efficiently. When cells transfected with p70S6K siRNA were treated with rapamycin, the proportions of cells at G_0/G_1 phase increased significantly. Also, the inhibition effects of rapamycin on cell proliferation became strong and cells obtained again sensitivity to rapamycin of high concentration (>100 nM) after cells were transfected with p70S6K siRNA. The results above indicated that p70S6K siRNA improved the sensitivity of cells to rapamycin on cell proliferation and cell cycle *in vitro*. Moreover, the results of *in vivo* experiment further revealed that rapamycin had much stronger inhibition effects on the growth of tumors from cells transfected with p70S6K siRNA than that with control siRNA. Moreover, the Western blot results in xenografts tissues showed that the expressions of p70S6K and p-p70S6K were the lowest in rapamycin + p70S6K siRNA group, which might conform our speculation in molecular mechanism. Above all, inhibiting the expressions of p70S6K and p-p70S6K by siRNA remarkably improved the sensitivity of EC9706 cells to rapamycin both *in vitro* and *in vivo*.

To explore the roles of p-p70S6K, the key factor of mTOR pathway, in the progression of ESCC, we investigated the protein expression of p-p70S6K in ESCC tissues and analyzed its clinical significance and correlation with mTOR. Our results showed that p-p70S6K had a higher positive expression in ESCC tissues than that in atypical hyperplasia and normal esophageal mucosa tissues. Moreover, the expression of p-p70S6K was closely related to lymph node metastasis and the TNM stage of ESCC. We also confirmed that p-p70S6K had positive correlation with the expression of mTOR. Our results above suggest that both mTOR and p-p70S6K have higher expression in malignant type of ESCC tumors and may participate in the invasion and metastasis of ESCC, and p-p70S6K can be looked at as a target for evaluating malignancy grade of ESCC.

In conclusion, we propose that mTOR/p70S6K pathway has a central role in the progression and development of ESCC, and the expression of p-p70S6K would be of importance in clinical diagnosis of ESCC. In addition, based on our *in vitro* and *in vivo* results, rapamycin combined with p70S6K siRNA would be a suitable molecular therapeutic strategy for ESCC patients.

Competing Interests

The authors declare that there is no conflict of interests regarding this paper.

Acknowledgments

The study was supported by grants from the National Natural Sciences Foundation of China (no. 30901778 and no. 81430085), Fund for Science and Technology Research Projects of Science and Technology Department of Henan Province (no. 112300410055 and no. 162300410122), and Fund for Graduate Education and Research Special Fund Project of Zhengzhou University.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 8605134, 6 pages
http://dx.doi.org/10.1155/2016/8605134

Review Article

Immune Regulation and Antitumor Effect of TIM-1

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Received 18 February 2016; Revised 10 April 2016; Accepted 28 April 2016

Academic Editor: Bin Zhang

T cells play an important role in antitumor immunity, and the T cell immunoglobulin domain and the mucin domain protein-1 (TIM-1) on its surface, as a costimulatory molecule, has a strong regulatory effect on T cells. TIM-1 can regulate and enhance type 1 immune response of tumor association. Therefore, TIM-1 costimulatory pathways may be a promising therapeutic target in future tumor immunotherapy. This review describes the immune regulation and antitumor effect of TIM-1.

1. Introduction

Immune suppression is an important factor for immune evasion of tumor. Generally, the immune systems of tumor patients often have excessive inhibitory functions, which are induced by regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), or the secretion of immunosuppressive cytokines, such as tumor growth factor-β (TGF-β) and interleukin-10 (IL-10). These conditions constitute an extremely favorable microenvironment for tumor progression [1–4]. Therefore, it is important to find novel targets for reversing immunosuppression microenvironment.

The identification of new classes of costimulatory molecules provides new exciting opportunities for inducing and enhancing effective endogenous immune response to cancer. TIM-1, a key member and costimulatory molecule in the T cell immunoglobulin mucin (TIM) family, is expressed on the surface of T cells. It can promote the activation and proliferation of T cells and the secretion of cytokines, which play critical roles in tumor immunity [5–9]. Our preliminary studies have shown that TIM-1 may be a novel candidate tumor therapeutic costimulatory molecule, because it may directly enhance the functions of $CD8^+$ T cells and/or NK cells, as well as altering the tumor microenvironment for more effective antitumor immune response (data not shown). This review tries to describe how TIM-1 regulates immune function and takes part in antitumor immune responses and illustrates the mechanism of immune regulation.

2. Structure and Basic Function of TIM-1

In human, there are three members (TIM-1, TIM-3, and TIM-4) located in the human chromosome 5q33.2 region. In mouse, the TIM family consists of eight members (TIMs 1–8) located in the 11B1.1 region of chromosome. The human and mouse TIM family genes are highly homologous [8, 10]. Like other TIM members, TIM-1 is similar in structure to the type 1 membrane protein, which consists of an N-terminal Cys-rich immunoglobulin variable- (IgV-) like domain, a mucin-like domain, a transmembrane domain, and an intracellular tail [11, 12]. The intracellular tail of TIM-1 contains tyrosine phosphorylation motifs that are involved in transmembrane signal [8, 13–15].

The expression of human TIM-1 was first detected in damaged kidney and named human kidney injury molecule-1 (KIM-1) [16–19]. Previous studies have indicated that *in vivo* TIM-1 gene mutations in human and mouse are associated with some allergic diseases [8, 20]. Abnormal expression of TIM-1 is related to some autoimmune diseases [21–27]. In recent years, study found that TIM-1 is mainly expressed on the surfaces of $CD4^+$ T cells, $CD8^+$ T cells, NK cells,

macrophages, DCs, B cells, and mast cells [28]. Moreover, it is also found that TIM-1 is expressed in lymphoid tissues [8, 29] and confirmed that TIM-1 can promote the production of cytokines and enhance the antigen induced immune response of T cells [30–35]. Therefore, TIM-1 may be a potential costimulatory molecule to enhance antitumor immune response [8, 23, 35–38].

3. Immune Regulation of TIM-1

TIM-1 is a highly efficient costimulatory molecule, which can enhance the formation of CD3-TCR with agonistic anti-TIM-1 antibody involved in the activation of T cells [7, 8, 37, 39]. The main ligands of TIM-1 are TIM-4 and phosphatidylserine (PS) [36, 40, 41]. TIM-4 is expressed on the surface of antigen presenting cells (APCs) such as macrophages and dendritic cells, working as an endogenous ligand of TIM-1 [5, 42, 43]. TIM-4 can promote T cell activation, proliferation, and cytokine production by binding to TIM-1, which mediates the positive regulation of T cells and triggers the immune response with costimulatory effect [30, 40]. PS is another important ligand of TIM-1 and can activate NKT cells by binding to TIM-1 on the surface of NKT cells [12, 44, 45]. In addition, P-selectin and S-selectin are also potential ligands for TIM-1 and may play roles in inflammation and autoimmune diseases. This signal pathway is closely related to the migration of Th1 and Th17 cells in blood vessels [38, 46].

The biological function of TIM-1 mainly depends on lymphocytes. TIM-1 in $CD4^+$ T cells can upregulate the activation signal of T cells by interacting with T cell receptor (TCR), which promotes the synergistic effect of TIM-1 [8, 47]. In immune regulation, the positive and negative regulation of TIM-1 are essential for the maintenance of immune homeostasis. The immune regulation of TIM-1 mainly depends on its ligands [8]. It has been reported that agonistic TIM-1 mAbs (clone 3B3 and clone 1H8.2) augment T cell-mediated immune responses, whereas an antagonistic antibody inhibits immune responses through regulatory B cells [48]. Agonistic TIM-1 monoclonal antibody can promote the proliferation of $CD8^+$ T cells *in vitro* and enhance their biological function [49]. The different effects of agonistic and antagonistic TIM-1 mAbs *in vivo* may be due to the fact that different TIM-1 mAbs deliver qualitatively and quantitatively different signals to T cells and B cells. The TIM-1 signaling on B cells is important in maintaining normal homeostasis of the immune system and preventing systemic autoimmunity [50, 51]. In $CD4^+$ T cells, the TIM-1 molecules bound with agonistic TIM-1 mAbs [39] or other agonistic ligands can produce a strong costimulation signal to activate T cells, promote the differentiation and proliferation of T cells *in vivo*, activate the production of cytokines, and enhance the antigen induced immune response of T cells [30–34]. Previous studies have found that the inhibition of TIM-1 signal of $CD4^+$ T cell can reduce the level of white blood cells and the production of inflammatory mediators, which can reduce the tissue damage caused by excessive inflammatory reactions [30, 35, 52, 53].

The negative regulation of immune function of TIM-1 in B cells plays a key role in preventing immune rejection [51, 54]. The inhibition of TIM-1-Fc signaling inhibits the differentiation and function of $CD4^+$ T cells and further reduces chronic rejection reactions [55]. Zhang et al. have found that the suppression of the TIM-1 signal in $CD4^+$ T cells can inhibit the activity of macrophages and reduce the injury of transplanted liver in a mouse model [56]. TIM-1 is also a key molecule in the regulation of immune rejection of allogeneic transplantation [49], and functional deficiency of TIM-1 is also one of the mechanisms of autoimmune diseases [50]. The expressions of TIM-3 and TIM-1 on the surface of mouse mast cells promote the secretion of IL-13, IL-6, and IL-4, indicating that mast cells also regulate immune function through TIM members [57]. Study also found that the inhibition of TIM-1 signal can reduce infiltration of T cells into allergic skin tissues and tissues of autoimmune diseases [38], and deficiency of TIM-1 reduces the incidence of allergic asthma in a mouse model [58]. Therefore, TIM-1 may also be related to the molecular mechanism of allergic diseases.

4. TIM-1 for Cancer Immunity

Type 1 immune response, mediated by Th1 cells, cytotoxic T lymphocytes (CTLs), NK cells, NKT cells, and gamma delta T cells, is considered as a critical component of cell-mediated immunity against tumor. $CD8^+$ T cells are important T cell subsets in specific immune response. They are the final effector cells to kill tumor and inhibit tumor progression *in vivo*, which are widely used in tumor adoptive immunotherapy [59, 60]. In human, the presence of Th1 cells and CTLs in tumor can be a favorable prognostic indicator [61]. However, many tumor infiltrating Th1 and $CD8^+$ T cells are in a status of nonresponsiveness due to local and systemic mechanisms of immune suppression in cancer patients as well as in tumor-bearing mice and even play a protective role for tumor [62, 63]. The lack of costimulation of type 1 lymphocytes is the major mechanism underlying tumor-induced immune tolerance [64, 65]. Thus, agonistic antibodies against costimulatory receptors such as 4-1BB and CD40 have shown promising antitumor effects in various preclinical tumor models, which are evaluated in clinical trials. The costimulation signal plays an important role in $CD8^+$ T cells [64]. In the model of acute renal injury induced by cisplatin, blocking of TIM-1 signal can significantly reduce the number of $CD8^+$ T cells and inhibit the secretion of IFN-γ, indicating that TIM-1 costimulation signal can enhance the effect of $CD8^+$ T cells [66].

In the TIM family, to date, it has been confirmed that TIM-3 is related to tumor [67, 68] and found that the expression of TIM-3 has an important influence on tumor microenvironment [69, 70]. However, we still have a lot of unknowns regarding the effects of tumor immunity of TIM-1. There are only a few articles that can be retrieved, which are about antitumor effect of TIM-1 [5, 6], but it has been determined that TIM-1 can promote the proliferation and differentiation of T cells by binding to different agonistic ligands [15, 30, 40, 71]. A study has demonstrated that TIM-1 tyrosine phosphorylation can recruit the PI3K adaptors p85, which stimulates the activation and function of T cells [15].

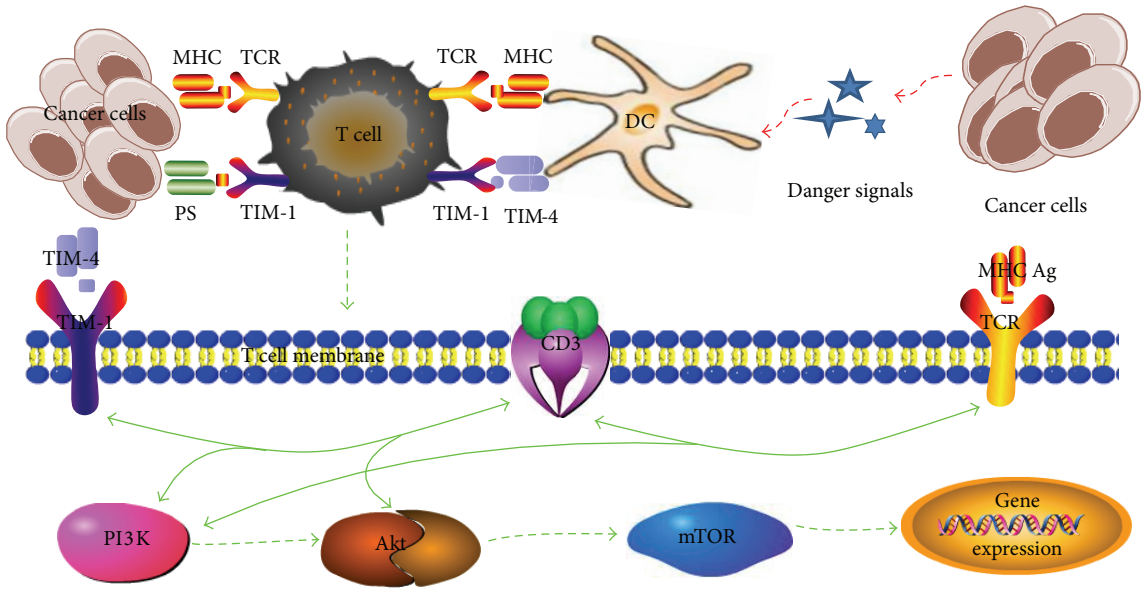

Figure 1: Tumor cells release signals, which are received by dendritic cells (DCs). Tumor antigens are processed to MHC antigens and then presented to the T cell receptor (TCR) for activation. TIM-4 (or phosphatidylserine) on DCs binds to TIM-1 on T cells to form the CD3-TCR complex, which participates in TCR-mediated T cell activation and initiates the intracellular PI3K signal pathway. PI3K signal pathway consists of the interaction between TIM-1 and ligands, tyrosine phosphorylation of the intracellular region of TIM-1, the recruitment of PI3K, the activation of Akt by PI3K, and the activation of mTOR by Akt. Activated mTOR can regulate the biological functions of T cells.

In tumor microenvironment, the effector cells, such as $CD8^+$ T cells, directly participate in immune response and can enhance antigen recognition, proliferation, and differentiation of other effector cells.

Ligation of the transmembrane protein TIM-1 can costimulate T cell activation by the PI3K signaling pathway. Agonistic antibodies to TIM-1 are also capable of inducing T cell activation without additional stimuli; PI3K is an important factor in mediating TIM-1 signaling [15]. It has been known that the PI3K/Akt/mTOR signaling pathway plays a crucial role in the regulation of cell growth, proliferation, and metabolism. The immune cells and tumor cells compete for energy. The activation of some signaling molecules closely related to energy metabolism regulates T cell activation, differentiation, and function and further enhances the antigen recognition, proliferation, and the differentiation of T cells. So far, PI3K/Akt/mTOR signaling pathway is a target of tumor therapy [72–77].

The transcription factor T-bet/Eomes is involved in the regulation of $CD8^+$ T cell function and induces the differentiation of $CD8^+$ T cells to effector and central memory T cells [78, 79]. The expression level of TIM-1 and T-bet/Eomes has important effects on regulating the biological function of T cells, and the expression of T-bet is closely related to the prognosis of tumor patients [24, 80]. We have analyzed 152 cases of gastric cancer patients and found that the expression of T-bet is closely related to the survival of tumor patients. The number of T-bet positive T cells in tumor tissues has a significant effect on the prognosis of the patients [81]. T-bet/Eomes, which stimulates the activation and differentiation of $CD8^+$ T cells, is significantly upregulated in the tumor of the third day after radiofrequency ablation (RFA), and the expression level of TIM-1 in infiltrating $CD8^+$ T cells is significantly upregulated. In T-bet/Eomes double knockout tumor model mice, it has been found that the expression of TIM-1 is very low in infiltrating $CD8^+$ T cells stimulated by tumor antigen, and in wild type mice it is significantly upregulated (data not shown). At present, TIM-1 is considered to improve the secretion of some cytokines such as IL-4 and IFN-γ [82]. Type 1 immune response of TIM-1-mediated T cell activation is associated with tumor immunity through transcription factor T-bet/Eomes [71, 83] and the PI3K signal pathway [15] (Figure 1).

5. Prospect

We speculate that TIM-1, a new costimulatory candidate molecule for tumor treatment, not only directly enhances the antitumor effect of $CD8^+$ T cells and NK cells but also changes the tumor microenvironment to induce more effective antitumor immune response. As a target molecule, it may have a good application prospect in clinical cancer research. In addition, agonistic anti-TIM-1 monoclonal antibody or other ligands can enhance the function of T cells [39, 82], increase $CD8^+$ T cells and NK cells, reduce MDSC in tumor tissues, and inhibit tumor growth (data not shown). It is important to define the mode of action and determine whether $CD8^+$ T cells and NK cells mediate the antitumor effect of agonistic TIM-1 mAbs *in vivo*. These may provide a theoretical basis to construct a new tumor therapy model of TIM-1 signal interference.

Competing Interests

There are no potential competing interests to disclose.

Acknowledgments

This work was supported by grants from the National Key Technology R&D Program (no. 2015BAI12B12) and the National Natural Science Foundation of China (nos. 81171653, 31428005, 31570877, and 31570908).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 7128702, 6 pages
http://dx.doi.org/10.1155/2016/7128702

Review Article

Function of G-Protein-Coupled Estrogen Receptor-1 in Reproductive System Tumors

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Received 27 January 2016; Revised 8 April 2016; Accepted 8 May 2016

Academic Editor: Bin Zhang

The G-protein-coupled estrogen receptor-1 (GPER-1), also known as GPR30, is a novel estrogen receptor mediating estrogen receptor signaling in multiple cell types. The progress of estrogen-related cancer is promoted by GPER-1 activation through mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase (PI3K), and phospholipase C (PLC) signaling pathways. However, this promoting effect of GPER-1 is nonclassic estrogen receptor (ER) dependent manner. In addition, clinical evidences revealed that GPER-1 is associated with estrogen resistance in estrogen-related cancer patients. These give a hint that GPER-1 may be a novel therapeutic target for the estrogen-related cancers. However, preclinical studies also found that GPER-1 activation of its special agonist G-1 inhibits cancer cell proliferation. This review aims to summarize the characteristics and complex functions of GPER-1 in cancers.

1. Introduction

Estrogen is an important hormone in human beings, especially in females. It plays several important physiological and pathological roles in not only reproductive system but also other systems. Estrogen disorder results in various diseases, such as endometrial diseases, skeletal diseases, and reproductive system tumors. Increasing attention has been paid to revealing of the functions of estrogen in physiological and pathological conditions. Estrogen receptors (ER) α and β, the two well established nuclear estrogen receptors, have different physiological functions depending upon their various distributions [1]. Meanwhile, the activity of ERβ is opposed to ERα in many systems. Lots of evidences show that estrogen induces the proliferation of cancer cells in breast, uterus, and ovarian cancer through ERα. On the contrary, activation of ERβ can reverse this effect. Notably, a novel transmembrane estrogen receptor, known as G-couple estrogen receptor (GPER), was found [2]. ER are involved in the initiation, migration, and progression of estrogen-related multiorgan cancers, such as breast cancer, ovarian cancer, prostate cancer, testicular cancer, liver cancer, and lung cancer as well [3]. Although increasing studied are focused on the roles of GPER-1 in different types of cancers, the functions of GPER-1 in cancers remain unclear yet. Characteristics and functions of reproductive system cancer will be summarized and discussed in the present review.

2. The Characteristics of GPER-1

2.1. The Structure and Distribution of GPER-1. G-protein-coupled estrogen receptor-1 (GPER-1), a seven-transmembrane-domain receptor localized in cell surface, was first identified in 1996 [2]. GPER-1 is detected broadly in numerous human tissues, such as breast, prostate, ovary, placenta, subcutaneous adipose, visceral adipose, arteries, vessels, heart, liver, lung and intestine tissues. GPER-1 is a member of GPCR superfamily, which is structurally unrelated to the classical ERα and ERβ. There are four transcriptional variants encoding 375 amino acids composing seven transmembrane proteins [4].

Classical GPCR are cell membrane proteins which bind their ligands at cell surface. But GPER-1 binding domain exists inside the plasma membranes and the endoplasmic reticulum [5–8]. The biological functions of GPER-1 might be associated with cell types and its location. Estradiol, the

major type of estrogen, binds to GPER-1 with a high affinity to GPER-1 while the other two isoforms, estrone and estriol, have very low binding affinities [5, 9]. Furthermore, numbers of environmental estrogen bind to GPER-1 and activate the downstream signaling pathways, such as bisphenol A, genistein, and nonylphenol [10]. GPER-1-specific compound 1 (G-1) is a specific agonist of GPER-1, which has no function of ERα and ERβ and was identified using virtual and biomolecular screening in 2006 [11]. G-1 has been widely used as a target tool to evaluate the function of GPER-1 in different cells and disease models.

2.2. GPER-1 Signaling Pathways. GPER-1 mediates both genomic and nongenomic response with its ligands. To date, GPER-1 signaling pathways have not been fully elucidated yet. The binding ligands of GPER-1, such as estrogen, G-1, tamoxifen, and ICI182,780, cross the plasma membrane and bind to the GPER-1 on endoplasmic reticulum where they activate its β and γ subunits and subsequently activate both Src and adenylyl cyclase (AC) leading to the intracellular cAMP production. The phosphorylation of Src induces matrix metalloproteinase (MMP) production, which cleaves pro-heparan-bound epidermal growth factor (pro-HB-EGF) releasing free HB-EGF. HB-EGF binds to the EGFR leading to activation of multiple molecules such as Ras, PI3K, AKT, and Erk1/2. The downstream signal of PI3K and AKT results in several nuclear receptors activation which is closely related the proliferation and migration of cancer cell. GPER-1 also binds to the G-couple protein α subunit and activates the phospholipase C (PLC), AC, and CAMP. Activated PLC results in inositol triphosphate (IP3) production, which further binds to its receptor and leads to intracellular calcium mobilization.

3. Functions of GPER-1 in Reproductive System Tumors

3.1. Breast Cancer. Breast cancer is the most common and deadly cancer in females worldwide [12]. Breast cancer is generally classified into estrogen receptor positive (ER+) and ER negative (ER−) [13]. In clinical practices, the endocrine treatments such as tamoxifen and aromatase are recommended in the ER positive breast cancers, while there is no benefit in the ER negative cancers [14]. GPER-1 is widely expressed in both of these breast cancer types and the primary breast cancers. Recent clinical study results showed that the expression of GPER-1 might correlate with clinical and pathological poor outcome biomarkers [15]. Other results also showed that the expression of GPER-1 was inversely correlated with the ER expression. Coexpression of GPER-1 and ER was found in almost 24% patients with inflammatory breast cancer, while 19% only express ER and 46% only express GPER-1 [16].

The GPER-1 mRNA levels were significant higher in ER positive breast cancer cells compared to ER negative cancer cells, and the expression of GPER-1 depends on ERα mRNA level. Interestingly, GPER-1 preformed a different proliferation manner in ER positive MCF-7 breast cancer cell line [17]. G-1 enhanced migration of MCF-7 breast cancer cells by activating ERK1/2 and EGFR signaling pathway, which is tremendously attenuated by G15 [18]. The other evidences also approved that GPER-1 is an initiator of tamoxifen resistance in breast cancers [19–21]. The promotion roles of GPER-1 in cancer cells proliferation and migration may be correlated with the autolysis of calpain 1 [22], cleavage of cyclin E [18], or the expression of target gene. There are also some studies which found that GPER-1 inhibits the growth of ER positive MCF-7 cells, which is probably through G-couple β and γ subunits activating without CAMP signal activation [21–24]. However, combination treatment with G-1 and Her2 antibody Trastuzumab exerted an additive growth inhibitory effect on breast cancer cells [25]. Thus, GPER-1 inhibits ER positive breast cancers proliferation which is a potential target for ER positive breast cancers and drug-resistant breast cancer.

ER negative breast cancer cells are more aggressive than ER positive cancer cells. Deficiency of ER in breast cancer is correlated with poor response to endocrine therapy [26]. In ER negative breast cancers, GPER-1 stimulates the ERK1/2 through the EGFR/MAPK signal cascade, inducing target gene like c-fos expression, which is involved in the progressing of breast malignancies [27–29]. Estrogen and antiestrogens can also promote the production of the early growth response-1 (Egr-1), connective tissue growth factor (CTGF), and insulin-like growth factor 1 (IGF-1) through the GPER-1 [28, 30, 31]. GRP30 activation stimulated breast cancer cells migration through CTGH, CXC receptor (CXCR1), and notch pathways [28]. Furthermore, GPER-1 agonist G-1 promoted inflammation in breast cancers [32]. GPER-1 was reported to affect the deformation of breast glandular structure inducing the malignant transformation of breast tissue [33]. GPER-1 can also induce expression of cancer-associated fibroblasts (CAFs) in tumor microenvironment [34, 35]. On the contrary, a recent study showed that activation of GPER-1 by G-1 resulted in G2/M-phase arrest and induction of mitochondrial-related apoptosis [36]. The other studies also proved that G-1 treatment suppressed the growth of SKBr3 cancer cells and increased the survival rate by inducing the ERK1/2 signal activation [36, 37].

15–20% of breast cancers are included in triple negative breast cancers (TNBC), characterized by lack of ERα, progesterone receptor (PR), and EGFR2 (Her-2). A higher rate of recurrence and aggressive biological features were found in younger females [38, 39]. GPER-1 expression was found in majority of TNBCs patients [40]. In the GPER knockdown mice model, the proliferation of TNBCs, the activation of EGFR, and c-fos expression were reduced [41]. These findings suggest that GPER plays a key role in putative mechanism for TNBCs and GPER might be a therapeutic target for TNBCs.

Paradoxical debates still exist on the functions of GPER-1 in breast cancers. SNPs of GPER-1, histone acetylation, and transcription factor recruitment were significantly associated with tumor size and histological grading [42, 43]. The different results of GPER-1 in breast cancer were summarized in Table 1.

3.2. Ovarian Tumors. Estrogens play a crucial role in the development of ovarian cancers. GPER RNA as well as GPER-1 protein presents in both primary and malignant

Table 1: The effect of GPER-1 in reproductive system tumors.

Cancer types	ER positive breast cancer	ER negative breast cancer	Triple negative breast cancer	Ovarian cancer	Testicular cancer	Prostate cancer
Proliferation	+24, 31 −17, 25, 52	+28, 31 −23, 36, 37	+40, 41	+47, 48, 49, 50 −51, 52	+55, 56, 57	−61, 62
Migration and metastasis	+18, 21, 22, 31, 15, 32	+27, 28, 29	+41	+48, 49	/	/
Poor survival	+20, 16	−36	+38, 39, 40	+46	/	/
Drug resistance	+19, 20, 21	+26	/	/	/	/

ovarian tumor tissues [44]. The expression of GPER-1 was significantly increased in ovarian carcinomas compared to pericarcinomatous tissues impendent with the expression of EGFR, ERα, and ERβ [45]. Further investigation showed that the expression of GPER-1 was associated with lower survival rates [46]. Estrogen and G-1 induce ovarian cancer cell growth responses via EGFR-MAPK signaling pathways. This procedure required coexpression of ERα [44, 47]. Furthermore, GPER-1 promoted the migration and invasion of ovarian cancer cells OVCAR5 which is characterized by negative ERα and positive GPER by increasing the expression MMP-9 [48, 49]. Atrazine, one of the most common pesticide contaminants, promoted ovarian cancer cells proliferation via induction of Erk and expression of estrogen target gene through GPER-1 pathway [50]. But other studies results showed that G-1 suppressed proliferation and induced apoptosis of human ovarian cancer cells probably through inhibition of cell cycle progression in G2/M-phase in ovarian carcinomas [51, 52].

3.3. Testicular Cancers. GPER-1 has been shown to be involved in a variety of hormone-dependent cancers. It is well understood that estrogens play a critical role in pathological germ cell proliferation in testicular germ cell tumors. GPER-1 seems to be involved in modulating the growth of estrogen dependent testicular cancer cells [53]. Estrogen induces the high expression of GPER-1 correlated with low levels of ERβ in human testicular carcinoma in situ and seminomas [53, 54]. Bisphenol A, a common environmental estrogen, can also promote the proliferation of testicular seminomas cells through GPER-1 [55]. The above findings suggested that GPER-1 may be a potential therapeutic target [56, 57].

3.4. Prostate Cancer. Estrogen has an efficacy for advanced prostate cancer (PC) via the mediation of the classical estrogen receptors [58]. The effects of ER on PC growth and metastases have different mechanisms in different cellular microenvironments [59]. The expression of GPER-1 is higher in the preneoplastic lesions and normal areas of benign prostate than the basal epithelial cells [60]. G-1, the selectively activating GPER-1, inhibited the growth of multiple PC cells in vitro and in vivo through Erk1/2 and c-jun/c-fos signaling pathways, which indicates that the G-1 may be a new option for PC through targeting GPER-1 [61]. G-1 inhibited castration-resistant phase but had no effect on androgen-sensitive tumors. The antitumor effect of G-1 on CR tumors was related to necrosis (approximately 65%) accompanied with neutrophils infiltration. G-1 can also upregulate neutrophil-related chemokines and inflammation-mediated cytokines in the CR tumors. In one word, GPER-1 is an androgen-repressed target. The antitumor effect of G-1 was neutrophil-infiltration-associated necrosis [62].

4. GPER-1 in Other Tumors

Overexpression of GPER-1 was detected in various reproductive system cancers. Studies showed that the activation of GPER-1 signaling pathways leads to tumor. There are other studies which proved that GPER-1 induced proliferation, differentiation, and drug resistance of lung cancers [63, 64], thyroid cancers [65], bladder cancers [66], and oral squamous carcinomas [67]. More studies to reveal the functions and mechanisms of GPER-1 in the other system cancers are warranted.

5. Conclusion

GPER-1 activation by estrogen induces nongenomic signaling pathways and regulates certain gene transcriptions. Majority of the study results addressed that activation of GPER-1 by estrogen and G-1 results in the downstream signals and target genes activation, which promotes the proliferation, migration, and invasion of cancer cells. And this effect is in nonclassical ER expression dependent manner in most cancers except for ovarian cancers. It is interesting that several other studies showed that G-1, the special agonist of GPER-1, promoted the expression of GPER-1 and inhibited the proliferation of ER negative breast cancer cells, ovarian cancer cells, and prostate cancer cells. The opposite effects of GPER-1 in cancer cells may be associated with the epigenetic of GPER-1, such as the SNPs and histone acetylation. The different cell types, tumor microenvironment, and hormonal level may also affect the functions of GPER-1. Controversies still exist on the GPER-1 localization and related signaling pathways, in particular the potential action as proapoptotic mediator. Since the function and mechanisms of GPER-1 are still unclear, more researches and clinical studies are strongly warranted to clarify the different function and mechanisms in different cancer types and conditions.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This work is supported by the National Nature Science Foundation of China (no. 81471534).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 8941260, 14 pages
http://dx.doi.org/10.1155/2016/8941260

Review Article

Effector, Memory, and Dysfunctional CD8$^+$ T Cell Fates in the Antitumor Immune Response

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Received 26 February 2016; Accepted 28 April 2016

Academic Editor: Yi Zhang

The adaptive immune system plays a pivotal role in the host's ability to mount an effective, antigen-specific immune response against tumors. CD8$^+$ tumor-infiltrating lymphocytes (TILs) mediate tumor rejection through recognition of tumor antigens and direct killing of transformed cells. In growing tumors, TILs are often functionally impaired as a result of interaction with, or signals from, transformed cells and the tumor microenvironment. These interactions and signals can lead to transcriptional, functional, and phenotypic changes in TILs that diminish the host's ability to eradicate the tumor. In addition to effector and memory CD8$^+$ T cells, populations described as exhausted, anergic, senescent, and regulatory CD8$^+$ T cells have been observed in clinical and basic studies of antitumor immune responses. In the context of antitumor immunity, these CD8$^+$ T cell subsets remain poorly characterized in terms of fate-specific biomarkers and transcription factor profiles. Here we discuss the current characterization of CD8$^+$ T cell fates in antitumor immune responses and discuss recent insights into how signals in the tumor microenvironment influence TIL transcriptional networks to promote CD8$^+$ T cell dysfunction.

1. Introduction

Decades of research have resulted in substantial insights into the role of the adaptive immune system, including CD8$^+$ T cells, in antitumor responses. In 1977, Fortner and Kripke demonstrated that tumor-challenged lymphocytes from irradiated donor mice were unreactive against syngeneic UV-induced tumors *in vitro* whereas tumor-challenged lymphocytes from nonirradiated mice rejected the same tumor. This finding implied that irradiation induced dysfunction of tumor-specific lymphocytes, which failed to reject the tumor [1]. In the mid-1980s, Rosenberg and colleagues defined tumor-infiltrating lymphocytes (TILs) as a subset of highly cytotoxic lymphocytes isolated from tumor-bearing patients that exhibited objective responses following adoptive transfer in human cancer patients [2, 3]. Further studies in athymic nude and SCID mice revealed that T cell deficiency correlates with a higher frequency of both spontaneous and chemically induced cancer, indicating a role for T cells in cancer immunosurveillance [4, 5]. In a study by Shankaran et al., the authors concluded that both lymphocytes and IFNγ were critical in antitumor immunity, suggesting a critical role for CD8$^+$ T cells in antitumor immune responses [6]. Shortly after, Dudley et al. showed that a clonal repopulation of CD8$^+$ TILs was responsible for tumor regression in patients with metastatic melanoma following lymphodepletion [7]. These studies highlighted a major role for CD8$^+$ TILs in antitumor immune responses, supporting the use of tumor-specific CD8$^+$ T cells in adoptive immunotherapy.

Clinical studies have shown a positive correlation between the frequency of CD8$^+$ TILs and cancer-free survival in patients with breast, lung, melanoma, colorectal, and brain cancer [8–12]. Current immunotherapies involve enhancing the activity of antigen-specific CD8$^+$ TILs through cytokine treatment, immune checkpoint blockade, chimeric antigen receptor therapy, and adoptive T cell transfer (ACT) [13]. Despite some clinical success, ACT experiments in both humans and mice have shown that initial tumor regression often yields to uncontrolled relapse [14, 15]. This suggests that the initial T cell response incompletely eliminates tumor cells and that, upon regrowth, tumor-specific T cells become unable to control the tumor. This finding has been supported

Table 1: Classification of human $CD8^+$ T cell fates based on surface markers, transcription profiles, and observed phenotype.

$CD8^+$ T cell fate	Surface marker profile	Transcription profile	Phenotype
Effector [18–22]	(i) $KLRG1^+$ (ii) $CD43^+$ (iii) $CD62L^-$ (iv) $CD69^+$ (v) $CD95^+$ (vi) $CD137^+$	(i) T-bet^{hi}/$Eomes^{hi}$ (ii) Blimp-1 (iii) Runx3 (iv) Stat4/Stat5 (v) Id2	(i) Direct cytotoxicity against transformed and virus-infected cells (ii) Mediate cytotoxicity through Fas/FasL and granzyme/perforin
Central memory [23–28]	(i) $CCR7^+$ (ii) $CD44^+$ (iii) $CD45RO^+$ (iv) $CD62L^+$ (v) $CD122^+$ (vi) $CD127^+$ (vii) $IL15R^+$	(i) T-bet^{lo}/$Eomes^{hi}$ (ii) Bcl6 (iii) Tcf1 (iv) Stat3 (v) Id3 (vi) WNT-β-catenin	(i) Less differentiated (ii) Residing in lymph nodes, spleen, bone marrow, and blood (iii) No immediate effector function (iv) Differentiating into T_{EFF} upon antigen rechallenge (v) Self-renewal capacity (vi) IL-7/IL-15 dependence
Effector memory [23–28]	(i) $CCR7^-$ (ii) $CD44^+$ (iii) $CD45RO^+$ (iv) $CD62L^-$ (v) $CD127^+$ (vi) $KLRG1^+$	(i) T-bet^{int}/$Eomes^{int}$ (ii) Blimp-1/Bcl-6	(i) Found in both lymphoid and peripheral tissues (ii) Rapidly release effector molecules (iii) Highly cytotoxic (iv) Intermediate differentiation stage (v) Rapidly differentiate into T_{EFF} upon antigen rechallenge
Exhausted [29–33]	(i) $CD45RO^+$ (ii) $CD57^+$ (iii) $CD95^+$ (iv) PD-1^+ (v) CTLA-4^+ (vi) Tim-3^+ (vii) Lag-3^+ (viii) $BTLA^+$	(i) NFAT (ii) T-bet^{lo}/$Eomes^{hi}$ (iii) Blimp-1 (iv) BATF (v) FoxP1	(i) Reduced proliferation (ii) Decreased cytokine production (iii) Reduced cytotoxicity (iv) Reduced IFNγ and IL-2 secretion (v) Eventual cell death
Anergic/tolerant [34–41]	(i) Lag-3^+ (ii) PD-1^+	(i) NFAT (ii) NF-kB/RelA (iii) Ikaros (iv) Egr1/Egr2	(i) Reduced IL-2 secretion (ii) Reduced proliferation
Senescent/regulatory [42–44]	(i) $KLRG1^+$ (ii) $CD28^-$ (iii) $CD57^+$	(i) FoxP3	(i) Cell-cycle arrest (ii) Immunosuppressive

in human patients as analysis of tumor-infiltrated lymph nodes (TILN) in late-stage melanoma patients revealed an aberrant tumor-specific T cell phenotype as compared to the phenotype observed in circulating effector, memory, and naïve T cells [16]. A separate study in late-stage melanoma patients found that a fraction of circulating antigen-specific $CD8^+$ T cells are functionally impaired, supporting the coexistence of multiple T cell fates in the antitumor immune response [17].

There is no universally accepted classification system of $CD8^+$ T cell fates in the context of antitumor immunity. Classifying $CD8^+$ T cell subsets is challenging due to lack of fate-specific biomarkers, unclear subset distinction, and disparity between cancer types. However, at least six subsets of $CD8^+$ T cell fates have been defined in both cancer patients and experimental models. These include effector T cells, memory T cells, exhausted T cells, anergic T cells, regulatory T cells, and senescent T cells. The following sections highlight the current view of $CD8^+$ T cell fates in the context of the antitumor immune response, including the transcriptional regulation of cell fate determination.

2. Characterization of $CD8^+$ T Cell Fate in the Antitumor Immune Response

2.1. Effector $CD8^+$ T Cells. Naïve $CD8^+$ T cells differentiate into effector T cells (T_{EFF}) upon TCR engagement with antigen and costimulation by an antigen-presenting cell (APC). In antitumor responses, robust $CD8^+$ T cell priming occurs primarily in tumor-draining lymph nodes (TDLNs). Activation and differentiation of effector $CD8^+$ T cells can also occur directly in the tumor by tissue-resident, cross-presenting APCs as well as tumor cells themselves [45–48]. T_{EFF} are identified based on the expression of surface markers such as CD25, CD69, CD95, CD137, and KLRG-1 [18–20] (Table 1 and Figure 1). Terminally differentiated T_{EFF} are IL-2 dependent and highly cytotoxic, rapidly expressing high levels of IFNγ, TNFα, perforin, and granzymes following activation [21, 22]. Tumor antigen-specific T_{EFF} that efficiently invade primary tumor lesions are termed TILs. TILs recognize and lyse tumor cells both *in vitro* and *in vivo*; however *in vivo* antitumor T cell responses are variable, owing to disparity in T cell activation, cytokine signaling, and immunosuppressive mechanisms

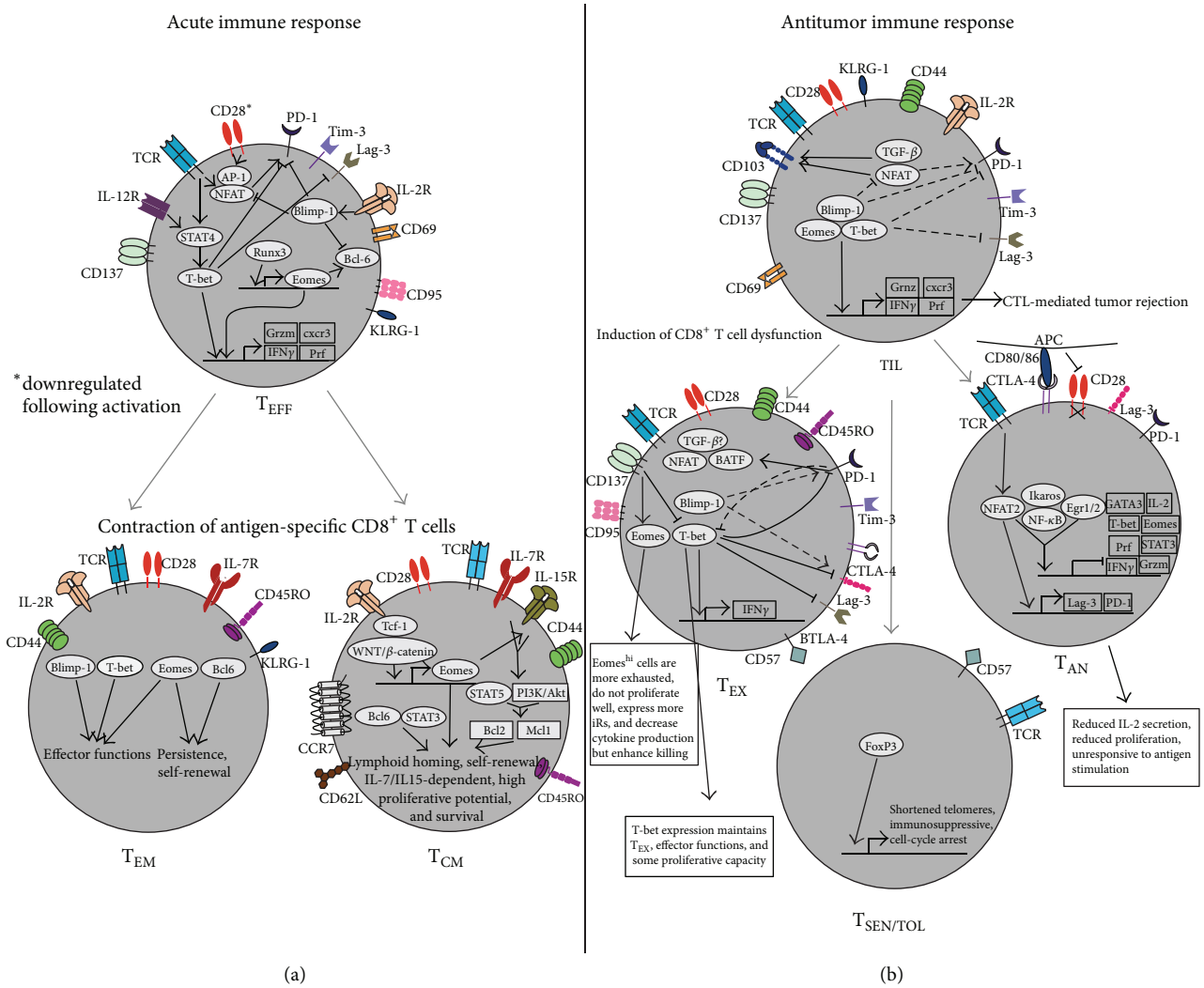

FIGURE 1: Characterization of CD8$^+$ T cell fates in acute and antitumor immune responses. (a) In an acute immune response, CD8$^+$ T cell priming induces cytotoxic T_{EFF} regulated by the transcription factors T-bet, Runx3, Eomes, Blimp-1, and NFAT and the cytokines IL-2 and IL-12. Following antigen clearance, T_{EFF} contract into T_{EM} and T_{CM}. T_{EM} are regulated by different levels of T-bet/Eomes and Blimp-1/Bcl-6. T_{CM} have higher levels of Eomes and Bcl-6 as compared to T_{EM} and are influenced by expression of Tcf-1, WNT/β-catenin, STAT3, and STAT5, which cooperate to maintain a persistent population of T_{CM} with high proliferative potential. IL-7 and IL-15 maintain homeostatic proliferation of CD8$^+$ memory T cells. (b) Tumor antigen primed T_{EFF} traffic to tumors as TILs. T-bet and Blimp-1 cooperate to repress iR expression and, with Eomes, promote CTL-mediated tumor rejection. NFAT and TGF-β promote tumor cell lysis through CD103 expression. Dysfunctional TIL can become T_{EX}, T_{AN}, or $T_{SEN/TOL}$. High T-bet expression maintains functional T_{EX} whereas high Eomes expression promotes severe exhaustion. There is complex interplay between T-bet, Blimp-1, and iRs in T_{EX}. T_{AN} result from insufficient costimulation through CD28. Unbalanced NFAT signaling induces anergy-inducing genes and, along with Ikaros, Egr1/2, and NF-κB, inhibits effector molecule expression. $T_{SEN/TOL}$ lack CD28 expression and may be regulated by FoxP3.

between tumor types [49–52]. T_{EFF} likely represent the majority of the TIL population in well-controlled tumors and are responsible for positive clinical responses, as adoptive transfer experiments using autologous T_{EFF} derived from CD8$^+$ TILs successfully eradicate tumors in cancer patients [3, 7, 9, 53, 54]. In acute immune responses, T_{EFF} are short-lived and undergo apoptosis upon elimination of antigen [55]. However, tumor load or prime-boost cancer vaccines can chronically stimulate CD8$^+$ T cells, leading to phenotypic changes and functional impairment. The switch from a highly active CD8$^+$ TIL population to chronically stimulated CD8$^+$ T cells favors the tumor over the host immune response and can ultimately lead to immune escape (Figure 1) [56]. The dysfunctional CD8$^+$ T cell fates that are induced by uncontrolled tumor load are discussed in detail below.

2.2. Memory CD8$^+$ T Cells. In several types of acute infectious challenges, T_{EFF} undergo a rapid, apoptosis-induced contractile phase following antigen clearance. After resolution of acute infection, a small subset of antigen-experienced

$CD8^+$ T cells remains as memory $CD8^+$ T cells [57–60]. It should be noted that adaptive "memory" implies the absence of antigen, a condition that is not often met in an antitumor immune response. For the remainder of this discussion, we will continue to refer to these cells as memory $CD8^+$ T cells, though they may more appropriately be characterized as "persistent" $CD8^+$ T cells in the context of the antitumor immune response.

Memory $CD8^+$ T cells were subdivided in 1999 into two broad subsets, central memory (T_{CM}) and effector memory (T_{EM}), distinguished by the relative expression of two homing molecules, CD62L and CCR7 [23–25]. T_{EM} have a phenotype more similar to that of effector cells, characterized by a loss of CCR7 expression and intermediate to no CD62L expression. These cells exhibit rapid effector function, readily differentiating into T_{EFF} that secrete high amounts of IFNγ and are highly cytotoxic [26]. In contrast, T_{CM} are less differentiated, have increased proliferative potential and greater self-renewal capability, can produce high amounts of IL-2, and acquire effector functions less rapidly. Upon secondary antigen challenge, both subsets give rise to progeny that differentiate into T_{EFF} [27, 28, 56]. Subsets of tumor-specific T_{EM} and T_{CM} have been identified in breast and colorectal cancer patients [61–64]. Similarly, studies in both mice and humans have demonstrated that memory $CD8^+$ T cells develop *in vivo* following adoptive transfer, maintain effector capabilities, and mediate tumor regression [65, 66].

2.3. Exhausted $CD8^+$ T Cells. Exhausted T cells (T_{EX}) are defined as a persistent T cell population with low IL-2 and IFNγ production, reduced cytotoxic activity, reduced proliferative potential, and eventual deletion of the population of antigen-specific T cells [29, 75]. T cell exhaustion is observed in the context of uncontrolled viral infection and cancer, and investigators believe that chronic antigen exposure drives $CD8^+$ T cells to an exhausted fate [29, 75, 76]. A number of inhibitory receptors (iRs) are upregulated on T_{EX}, indicating a role for these receptors in the attenuation of T cell function. In healthy individuals, iRs on $CD8^+$ T cells promote self-tolerance and prevent autoimmunity by competing for costimulatory receptor ligands, attenuating positive TCR signaling, and/or inducing immunosuppressive genes. In the context of an antitumor immune response, elevated expression of multiple iRs promotes $CD8^+$ T cell exhaustion and immune evasion. Some of these receptors include PD-1, CTLA-4, TIM-3, LAG-3, CD160, BTLA, TIGIT, and 2B4 [29–33] (Table 1).

Early experimental evidence for $CD8^+$ T cell exhaustion in antitumor immunity was observed in a transgenic mastocytoma cell line overexpressing programmed death-ligand 1 (PD-L1). This cell line resisted TCR-mediated cell lysis *in vitro* and was more tumorigenic and invasive *in vivo* [77]. In the same year, Dong et al. demonstrated that PD-L1 was expressed in lung, ovary, and colon cancers as well as melanomas [78]. Further studies revealed that T_{EX} expressed high levels of PD-1 in Hodgkin's lymphoma, melanoma, hepatocellular carcinoma, and gastric cancer patients [79–82]. CTLA-4 is another T cell-specific iR known to be upregulated in exhausted T cells [31]. In the early 2000s, investigators began testing anti-CTLA-4 antibodies for their ability to reverse T cell dysfunction in cancer patients. In 2011, Ipilimumab became the first FDA-approved immune checkpoint inhibitor, approved for the use in patients with late-stage metastatic melanoma [83, 84]. A more recent study showed that dual blockade of CTLA-4 and PD-1 corresponded with reversal of T cell exhaustion, characterized by increase cytokine release, suppression of Tregs, and upregulation of signaling molecules associated with activation. Dual blockade led to tumor rejection in murine models of ovarian and colon carcinoma [85]. An elegant study from Baitsch et al. revealed a marked distinction between $CD8^+$ T cell fates in patients with metastatic melanoma. While circulating tumor-specific T cells exhibited normal effector function, TILs isolated from the tumor-draining lymph node (TDLN) showed a markedly exhausted phenotype, characterized by decreased IFNγ expression and upregulation of CTLA-4 and Lag-3. The investigators concluded that T_{EFF} and T_{EX} coexist in patients with metastatic melanoma, supporting the coexistence of multiple $CD8^+$ T cell fates in antitumor immune responses. The study further highlights the complexity of the tumor microenvironment as a largely immunosuppressive environment and suggests that tumor-specific expression of ligands for T cell iRs promotes immune evasion [16]. The discovery that T cell exhaustion could be reversed *in vitro* (removal from immunosuppressive environment) and *in vivo* (immune checkpoint blockade) has prompted the rapid development of other immune checkpoint inhibitors as novel immunotherapies [86, 87]. Detailed reviews of FDA-approved and clinical trial immune checkpoint inhibitors have been described elsewhere [88–90].

Though iRs are classically used to identify T_{EX} *in vivo*, many of these receptors are upregulated following T cell activation. Legat et al. showed that PD-1, CTLA-4, and LAG-3 were upregulated upon T cell activation in the antitumor response. In contrast to the study by Baitsch et al., the authors demonstrate that $CD8^+$ T cells isolated from both metastatic and nonmetastatic lymph nodes in melanoma patients exhibit increased expression of iRs and decreased cytokine production [91]. PD-1 expression was found to identify patient-specific, tumor-reactive TILs in a number of human tumors. Expression of the iRs PD-1, LAG-3, and TIM-3 correlated with antigen-experienced $CD8^+$ TILs that recognized and lysed autologous tumor cell lines [92]. In line with this idea, Duraiswamy et al. showed that $CD8^+PD\text{-}1^{hi}$ T cells from healthy donors exhibit a distinct transcriptional profile as compared to $CD8^+PD\text{-}1^{hi}$ T cells in HIV-infected patients. In healthy donors, PD-1 expression correlated with a T_{EM} phenotype as opposed to terminal T_{EFF} [93]. Thus, canonical identification of T_{EX} by iR expression does not always correlate with T cell dysfunction. The correlation of iR expression and $CD8^+$ T cell exhaustion needs to be further investigated and may depend on activation state, quantity of expression, coexpression of multiple receptors, and strength of the inhibitory signal. Indeed, studies have shown that iR expression and signal strength influence $CD8^+$ T cell fate towards an exhausted phenotype in infectious disease and cancer [94, 95].

$CD8^+$ T cell exhaustion represents a distinct but reversible T cell fate in the context of antitumor immune responses. At least some iRs are expressed on activated T_{EFF}, and it remains incompletely defined to what extent individual iRs contribute to the functional impairment of $CD8^+$ T cells observed in cancer as opposed to serving as phenotypic markers of exhaustion. Anticancer activity of iR-blocking antibodies in mice and humans supports at least a partial direct role for these receptors in T cell dysfunction [89, 96]. Continued work in this area will help determine which iRs best identify exhausted T cells and are most amenable to therapeutic targeting. Similarly, further insight into iR signaling may allow targeting of specific downstream molecules.

2.4. Anergic $CD8^+$ T Cells. One of the pivotal obstacles in immunotherapy is overcoming tolerance. Central tolerance deletes self-reactive T cells with high avidity TCRs for self-antigen. Self-antigen-specific T cells that escape the thymus are often tolerized in the periphery, through either deletion or induction of anergy [97]. Because tumor antigens are often nonmutated self-antigens, these two processes significantly impair the host's ability to mount an effective antitumor immune response [98]. Anergy refers to a hyporesponsive state of impaired IL-2 production and proliferation, resulting from inefficient costimulation and/or high coinhibitory signaling or from partial or chronic TCR stimulation [99]. In antitumor immune responses, the scarcity of circulating tumor-specific T cells, most of which express low-avidity TCRs, impedes the recognition and destruction of tumor cells [49]. Nevertheless, tumor antigen-specific TILs can be found at high numbers in many cancer types, though often unable to control the tumor [100]. Anergy is usually characterized *in vitro*, but anergy induction *in vivo* promotes what is referred to as T cell tolerance [101]. It is difficult to accurately identify anergic/tolerant T cells (T_{AN} and T_{TOL}, resp.) in *in vivo* cancer models due to a lack of distinctive biomarkers. However, multiple studies suggest that immunosuppressive mechanisms in the tumor microenvironment are capable of promoting an anergic phenotype. Cancer cells and tumor-associated APCs can express high levels of coinhibitory molecules, and both APCs and cancer cells directly activate $CD8^+$ T cells *in vivo* [47, 102]. A combination of $CD8^+$ T cells priming with strong coinhibitory signaling might promote T cell anergy in the tumor microenvironment. Studies have validated expression of B7 family members on myeloid dendritic cells, tumor-associated macrophages, and cancer cells. These studies also showed that blockade of inhibitory B7 molecules reduced tumor growth *in vivo* [102–104].

The transcriptional network that promotes $CD8^+$ T cell anergy is complex and many of the transcription factors that promote an anergic phenotype also promote T cell exhaustion. Still, evidence suggests that these two $CD8^+$ T cell fates are distinct in antitumor immune responses [99, 105]. In a model of chronic LCMV infection, it was shown that gene expression profiles from $CD8^+$ T_{AN} and T_{EX} were significantly different, suggesting a functional difference between the two subsets (see below) [106]. From a temporal standpoint, the development of T cell anergy is believed to occur before or in the early stages of tumor progression. For one, anergy induction in the thymus or periphery renders it unlikely that a significant number of tumor-reactive $CD8^+$ T cells exist in circulation even before a tumor is established [97]. Along these lines, a study by Staveley-O'Carroll et al. suggests that T cells are rendered anergic in the early stages of tumor progression [107]. On the other hand, $CD8^+$ T cell exhaustion is an eventual state of T cell dysfunction that occurs in progressive stages and varies depending on the context and abundance of antigen [29, 31, 105]. Collectively, these studies imply that $CD8^+$ T cell anergy occurs before or in the early stages of tumorigenesis whereas exhaustion is a gradual state of T cell dysfunction. Further analysis of dysfunctional $CD8^+$ T cells in multiple stages of tumor development and different tumor types will help further delineate the role of T_{AN} and T_{EX} in antitumor immune responses.

2.5. Senescent/Regulatory $CD8^+$ T Cells. Senescent T cells (T_{SEN}) are defined by loss of CD28 expression, permanent cell-cycle arrest, and shortened telomere length. It is well known that T_{SEN} have implications in human ageing, but their role in cancer is less clear [42]. Interestingly, $CD8^+$ T cells displaying a senescent phenotype ($CD8^+CD28^-$) have been associated with suppressor function *in vitro* [108, 109], indicating a potential immunosuppressive role in antitumor immune responses. Similarly, populations of regulatory $CD8^+$ T cells have been identified in head and neck and lung cancer, marked by lack of CD28 expression [43, 110]. Thus, $CD8^+CD28^-$ T cells may comprise a heterogeneous population, containing both senescent and/or regulatory $CD8^+$ T cells. A comprehensive study by Filaci et al. revealed that $CD8^+CD28^-$ regulatory T cells (T_{REG}) are present in metastatic lymph nodes in a number of cancers. This study concluded that $CD8^+CD28^-$ T_{REG} reduced T_{EFF} proliferation and cytolytic capacity via IL-10 secretion [111]. However, this study did not identify this population of $CD8^+CD28^-$ cells as senescent, but instead as a regulatory T cell population, similar to but phenotypically distinct from $CD4^+FoxP3^+$ T_{REG}. Thus CD28 expression alone may not distinguish between $CD8^+$ T_{SEN} and T_{REG}. Montes et al. demonstrated that tumor cell lines could induce properties characteristic of $CD8^+CD28^-$ regulatory/senescent T cells, including shortened telomeres and immunosuppressive activity. Importantly, the study showed that inhibition of T_{EFF} proliferation was contact-dependent [112]. The same group then demonstrated that $CD8^+CD27^-CD28^-$ T_{SEN} could similarly be induced by soluble factors and that this phenotype is inhibited by exogenous IL-7 [113]. It remains to be determined whether these populations represent distinct T cell fates or comprise a single $CD8^+$ T cell subset and how the context of tumor control and tumor type contribute to the differentiation/maintenance of $CD8^+$ T_{SEN} and T_{REG}. One study demonstrated that $CD8^+CD28^-$ expression identifies a T cell subset that recognizes and responds to HPV-induced cervical cancer, suggesting that CD28 may not serve as a reliable biomarker for $CD8^+$ T_{SEN}/T_{REG} [114]. In line with this idea, CD57 was found to be a marker of replicative senescent T cells in a model of HIV infection, regardless of CD28 expression [44]. A recent study illuminated the impact of senescent $CD8^+$ T cells in patients with late-stage lung cancer.

The $CD8^+$ T cell population in patients was consistent with an immunosenescent phenotype, based on CD28 and CD57 expression, before the onset of chemotherapy. Following chemotherapy, the proportion of senescent and terminally differentiated $CD8^+CD28^-CD57^+$ cells was significantly increased in stage IV lung cancer patients as compared to the healthy controls. Similarly, the population of naïve and memory $CD8^+CD28^+CD57^-$ T cells was decreased in the same patients as compared to healthy controls. These findings suggest that the number of $CD8^+CD28^-CD57^+$ T_{SEN} cells correlates with disease stage in late-stage lung cancer patients, offering a role for $CD8^+$ T_{SEN} in antitumor immune responses [115]. Further phenotypic and functional analysis of $CD8^+$ T_{SEN} and T_{REG} is needed to characterize these cells as individual $CD8^+$ T cell fates.

3. Which Subset Promotes Optimal Antitumor Immune Responses?

There is conflicting evidence as to which subset of $CD8^+$ T cell promotes superior antitumor immunity. Adoptive T cell transfer of T_{EFF} promotes robust responses, but these cells often exhibit reduced persistence *in vivo* [7, 9, 54]. Initial antitumor responses often yield to tumor recurrence and the population of antigen-specific T cells becomes functionally impaired [15]. Gattinoni et al. found that more differentiated T_{EFF} were increasingly cytotoxic *in vitro* but exhibited impaired proliferative capacity and antitumor activity *in vivo* [67]. Still, multiple studies have shown that transfer of highly active T_{EFF} leads to tumor rejection in both humans and mice [3, 52, 53, 68]. One study showed that terminal T_{EFF} cultured *in vitro* transitioned into a smaller population of T_{EM} that promoted tumor regression and persisted for 2 months after transfer in patients with metastatic melanoma [69]. Both T_{CM} and T_{EM} from human breast cancer patients selectively homed to and rejected tumors in NOD/SCID mice with breast cancer, suggesting that both memory subsets can promote antitumor activity *in vivo* [61]. In a murine model of melanoma, *in vitro*-generated T_{CM} exhibited robust expansion and rejected tumors *in vivo* whereas T_{EM} did not [70]. Wu et al. demonstrated that TCR-transgenic T_{CM} displayed both an effector and memory phenotype and possessed superior antitumor activity as compared to TCR-transgenic T_{EFF} [71]. These studies suggest increased efficacy of less-differentiated T_{CM} in adoptive cell transfer therapy. The ability to promote the development of functional T cell memory *in vitro* and *in vivo* may provide a mechanism to enhance $CD8^+$ T cell-mediated antitumor immune responses.

More recently, two additional subsets of memory T cells have been identified: tissue-resident memory T cells (T_{RM}) and T memory stem cells (T_{SCM}) [8, 57, 72–74]. Djenidi et al. identified a subset of TILs that correlated with increased survival in patients with non-small-cell lung carcinoma (NSCLC). The authors characterized these cells as T_{RM} based on surface expression of CD8, CD103, PD-1, and Tim-3 [8]. T_{RM} are a relatively recently defined subset, and it remains to be determined to what degree these cells represent a distinct memory T cell subset, although emerging evidence suggests that they are transcriptionally, phenotypically, and functionally different from T_{EM} and T_{CM} [74]. Gattinoni et al. characterized a population of T_{SCM} based on expression of surface markers distinctive of both naïve ($CD45RO^-$, $CCR7^+$, $CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$, and IL-7Rα^+) and memory ($CD95^+$, IL-2Rβ^+, $CXCR3^+$, and LFA-1^+) $CD8^+$ T cells. This cell population proliferated more efficiently and elicited better antitumor immune responses as compared to T_{CM}, suggesting that the T_{SCM} population might yield more objective antitumor responses owing to its less-differentiated state and increased proliferative potential [72]. These studies offer a more complex view of T cell memory subsets, where multiple stages of memory T cell differentiation correlate with phenotypic and functional changes.

4. Transcriptional Regulation of $CD8^+$ T Cell Fate Decision in Antitumor Immune Responses

It is well known that $CD8^+$ T cell fate in the tumor microenvironment is influenced by multiple factors including the nature of antigen stimulation/$CD8^+$ T cell priming, soluble and cell-surface immunomodulatory ligands, and nutrient and oxygen availability [116–119]. $CD8^+$ T cell dysfunction is likely caused by a combination of immunosuppressive mechanisms. It is unclear how all of these factors regulate the transcriptional profile of dysfunctional $CD8^+$ TILs. In this section, we will discuss transcriptional changes that promote the differentiation of different $CD8^+$ T cell fates in antitumor immune responses.

4.1. Anergic/Tolerant $CD8^+$ T Cells. Few studies have demonstrated that T_{AN} or T_{TOL} $CD8^+$ T cells persist at a relevant level in cancer but it stands to reason that these cells could play a significant role in mediating immune evasion. Tolerant or anergic $CD8^+$ T cells that would otherwise respond to a tumor-specific antigen (TSA) or tumor-associated antigen (TAA) would be unable to trigger an effective immune response against transformed cells.

Transcriptional networks in anergic $CD4^+$ T cells have been studied both *in vitro* and *in vivo*. Strong TCR stimulation in the absence of sufficient costimulatory signaling via CD28 leads to activation of NFAT (nuclear factor of activated T cells) without activation of AP-1 (activator protein 1). The absence of NFAT/AP-1 heterodimerization allows NFAT homodimerization and promotes the expression of anergy-inducing genes including Egr2, Ikaros, and members of both the E2F transcription factors and the E3 ubiquitin ligase family. Many of these anergy-inducing genes then repress critical effector molecules including IL-2, IFNγ, and TNFα [99, 120, 121]. Few studies have attempted to elucidate the transcriptional network in $CD8^+$ T_{AN} and the anergy-inducing genes that promote tolerance remain relatively uncharacterized. In an *in vivo* anergy induction model, Srinivasan and Frauwirth demonstrated a defect in calcium signaling in $CD8^+$ T cells, which resulted in translocation of NFAT2 but not NFAT1 to the nucleus. This suggests a signaling network whereby NFAT isoforms become activated in response to different concentrations of intracellular calcium and NFAT2 regulates expression of anergy-inducing genes [34] (Figure 1). In primary culture,

Ikaros haploinsufficient $CD8^+$ T cells produced autocrine IL-2 and differentiated into IFNγ-secreting CTL without the addition of exogenous IL-2. These cells exhibited enhanced efficacy against B16 melanoma tumors *in vivo* as compared to WT cells, suggesting a role for Ikaros in maintaining tolerant $CD8^+$ T cells [35]. Genetic ablation of the E3 ubiquitin ligase Cbl-b was shown to prevent induction of anergy in TCR-transgenic $CD8^+$ T cells *in vivo* [36]. Similarly, blockade of the iR Lag-3 was shown to rescue tolerant $CD8^+$ T cells in a self-tolerance and tumor model. Upon Lag-3 blockade, $CD8^+$ T cells exhibited restored effector function and accumulated at greater numbers in tumor tissue [37]. In line with this idea, an intricate study by Schietinger et al. compared gene signature profiles between naïve, memory, tolerant, rescued, and retolerized $CD8^+$ T cells. Lag-3 was found to be significantly upregulated in tolerant $CD8^+$ T cells. Similar to $CD4^+$ T cells, Egr1/Egr2 were downregulated in rescued and memory $CD8^+$ T cells. Effector genes such as Infg, Prf1, and Gzmm were found to be upregulated in rescued and memory $CD8^+$ T cells as were the transcription factors Tbx21, Eomes, Gata3, and Stat3 as well as multiple chemokine and cytokine molecules. Gene signature profiling also revealed significant differences in genes regulating chromatin modification in tolerant versus retolerized $CD8^+$ T cells, implying that epigenetic changes are critical in $CD8^+$ T cell fate decision [38].

One study implicated the iR PD-1 in the induction of $CD8^+$ T cell anergy *in vivo* [39]. PD-1 is known to inhibit T cell function through different mechanisms, including negative signaling upon TCR engagement through phosphatase recruitment [122]. NFAT promotes PD-1 expression in early activated $CD8^+$ T cells and unbalanced NFAT signaling may therefore contribute to T cell anergy through PD-1 expression [123] (Figure 1). Thus, interplay between transcription factors and iRs promotes various states of $CD8^+$ T cell dysfunction including exhaustion and tolerance.

The NF-κB transcription factor family is known to regulate T cell-specific gene expression and NF-κB is necessary to mediate $CD8^+$ T cell tumor rejection *in vivo* [40]. One study showed that T cells from tumor-bearing mice exhibited decreased IFNγ production that correlated with expression of distinct NF-κB/Rel isoforms, suggesting that NF-κB signaling influences T cell effector function in antitumor immune responses [41]. A recent study by Clavijo and Frauwirth supports these findings, as they found that T_{AN} exhibit impaired NF-κB activation in a model of T cell tolerance [124]. Further studies are needed to facilitate accurate characterization of $CD8^+$ T_{AN}/T_{TOL} and elucidate their role in antitumor immune responses.

4.2. Senescent/Regulatory $CD8^+$ T Cells. There is little known concerning the transcriptional networks involved in $CD8^+$ T_{SEN}/T_{REG} in the context of antitumor immune responses, yet studies suggest that tumors are capable of inducing a T_{SEN}/T_{REG} phenotype both *in vitro* and *in vivo* [43, 110, 112]. $CD8^+CD28^-$ T_{REG} were found to express higher levels of FoxP3 mRNA in patients with lung cancer, suggesting the existence of a regulatory $CD8^+CD28^-$ population in cancer patients, possibly regulated by the expression of FoxP3 [43]. Similarly, two studies identified a $CD8^+FoxP3^+$ subset of T_{REG} in patients with colorectal and prostate cancer, suggesting that FoxP3 can be expressed in $CD8^+$ T cells and promote an immunosuppressive phenotype in cancer patients [125, 126]. Another study highlighted similarities between $CD8^+Foxp3^+$ T cells and $CD4^+$ $Foxp3^+$ T cells in terms of phenotypic markers and lack of effector molecules but found that the $CD8^+$ subset does not possess potent suppressive activity [127]. Currently, whether T_{SEN} and T_{REG} are two distinct T cell fates or represent a mutual phenotype remains to be determined. Ramello et al. offered a potential mechanism by which tumor-induced $CD8^+$ T_{SEN} promote tumorigenesis by influencing monocyte and macrophage secretion of proinflammatory cytokines and angiogenic factors. $CD8^+$ T_{SEN} increased monocyte/macrophage-specific production of IL-1β, TNF, and IL-6, MMP-9, VEGF-A, and IL-8 in a contact-dependent manner. Importantly, this proinflammatory phenotype was found to be dependent on Tim-3 and CD40L as blocking antibodies against these receptors reduced production of many of the proinflammatory factors [128]. This study does not identify transcription factors involved in $CD8^+$ T_{SEN} signaling but implies that costimulatory/coinhibitory receptors play a role in promoting this fate. The authors did not characterize the suppressive activity of the $CD8^+$ T_{SEN} on other T cells, and so it is unknown whether this subset of cells was functionally distinct from $CD8^+$ T_{REG}. The characterization of the transcription factors that regulate these phenotypes will help advance our understanding of the role of $CD8^+$ T_{SEN}/T_{REG} in antitumor immune responses.

4.3. Exhausted $CD8^+$ T Cells. $CD8^+$ T_{EX} represent the most commonly identified subset of dysfunctional T cells in antitumor immune responses. Expression of cell fate-influencing transcription factors in exhausted $CD8^+$ T cells has been investigated in models of chronic viral infection to a greater degree than in cancer models. Though few studies have examined the transcriptional profile of $CD8^+$ T_{EX} in cancer, crosstalk between iRs and transcription factors is indicated in promoting this fate. Persistent antigenic stimulation and inflammation are characteristics of both chronic viral infection and cancer, and, thus, transcriptional programming of exhaustion in the two disease states may be similar [16, 29].

Both T-bet and Eomes are known to be important in antitumor immune responses, consistent with their role as mediators of effector function in $CD8^+$ T cells [129, 130]. T-bet expression was found to correlate with increased cancer-free survival in human colorectal cancer patients [10]. Studies in mice have identified multiple roles for T-bet and Eomes in antitumor immune responses, including controlling $CD8^+$ T cell number, trafficking, effector function, and memory recall responses [129, 131]. One study demonstrated that exhausted $CD8^+$ TILs express low levels of both T-bet and Eomes. PD-1, PD-L1, and CTLA-4 antagonism increased levels of both T-bet and Eomes and restored effector function [85]. Similarly, Berrien-Elliott et al. showed that blockade of CTLA-4, PD-1, and LAG-3 increased T-bet but not Eomes expression in $CD8^+$ T cells. Reexpression of T-bet was required for IFNγ production and cytotoxic activity against FBL leukemia in mice [132]. This study suggests a feedback loop between T-bet and PD-1, as T-bet is known to repress PD-1 expression

and maintain $CD8^+$ T_{EX} in chronic infection [133]. Our lab has shown that T-bet and Eomes are coexpressed with iRs PD-1 and LAG-3 as well as costimulatory receptors 4-1BB and OX40 in exhausted $CD8^+$ TILs in a murine lymphoma model. Agonistic ligation of 4-1BB was associated with increased Eomes, decreased T-bet expression, and delayed tumor growth [134]. One study found that T-bet expression was decreased in $CD8^+$ T cells in a model of chronic LCMV infection. Overexpression of T-bet in P14 cells repressed PD-1, Lag-3, CD160, and BTLA [133]. In another study of chronic viral infection, $CD8^+$ T_{EX} consisted of a majority of $Eomes^{hi}PD\text{-}1^{hi}$ population and a much smaller, but highly proliferative, $T\text{-}bet^{hi}PD\text{-}1^{int}$ population. This study suggests a dynamic conversion from $T\text{-}bet^{hi}$ to $Eomes^{hi}$ virus-specific $CD8^+$ T cells during a state of persistent antigen challenge and that these two populations cooperate to control viral infection [135]. Buggert et al. compared T-bet and Eomes expression between patients with acute viral infection (CMV) and chronic viral infection (HIV). Similar to the previous studies, HIV patients displayed an exhaustive $CD8^+$ T cell profile characterized by high Eomes expression and low T-bet expression. This population of cells displayed elevated expression of multiple iRs [136]. These studies imply a heterogeneic population of antigen-specific $CD8^+$ T cells in chronic viral infection and cancer, where $CD8^+$ T cells eventually display an exhaustive phenotype characterized by high Eomes and low T-bet expression. These studies suggest that T-bet and Eomes have distinct roles in CTL-mediated antitumor immune responses. Whereas T-bet promotes terminal differentiation in acute immune responses, it maintains effector functions in $CD8^+$ T_{EX}. On the other hand, high Eomes expression correlates with severe $CD8^+$ T cell exhaustion. The above studies suggest complex interplay between iRs and T-bet and Eomes in exhausted $CD8^+$ T cells and differential costimulatory/coinhibitory receptor signaling likely influences their expression as well as $CD8^+$ T cell fate (Figure 1).

Like T-bet, Blimp-1 promotes the differentiation of $CD8^+$ T_{EFF} while repressing transition into a central memory phenotype [137]. In a model of acute viral infection, Blimp-1 was shown to repress the expression of PD-1 both directly and indirectly by interfering with NFAT binding to the PD-1 promoter [138]. NFAT regulates the expression of PD-1 and Tim-3 and thus may contribute to $CD8^+$ T cell exhaustion in chronic viral infection and cancer [139]. As mentioned earlier, disproportionate NFAT signaling is implicated in the induction of $CD8^+$ T cell anergy, offering a potential role for this transcription factor in promoting more than one state of $CD8^+$ T cell dysfunction in antitumor responses [140]. Blimp-1 may therefore prevent T cell dysfunction in early activated T cells through repression of both PD-1 and NFAT. In line with this idea, Blimp-1 was identified as a key regulator of $CD8^+$ TIL effector function in advanced lung cancer patients. Blocking of miR-23a correlated with upregulation of Blimp-1, reacquisition of effector function, and delayed tumor progression [141]. The role of Blimp-1 in $CD8^+$ T cells during chronic viral infection differs greatly from a well-controlled infectious challenge. $PD\text{-}1^{hi}$ $CD8^+$ T cells had 2 to 3 times more Blimp-1 expression than $PD\text{-}1^{int/lo}$ $CD8^+$ T cells. Similarly, iR^{hi} (PD-1, LAG-3, 2B4, and CD160) cells all had higher levels of Blimp-1 expression as compared to iR^{lo} $CD8^+$ T cells. Blimp-1 expression correlated with a higher number of coexpressed iRs on a per cell basis. Importantly, conditional deletion of Blimp-1 was unable to rescue $CD8^+$ T_{EX} because Blimp-1 induces granzyme B expression and cytotoxic activity [142]. Thus, Blimp-1 is important in promoting critical effector functions in acute immune responses but correlates with markers of $CD8^+$ T cell exhaustion in chronic viral infection and possibly cancer.

Recent studies have implicated basic leucine zipper transcription factor (BATF) in $CD8^+$ T cell exhaustion. BATF was shown to drive T-bet and Blimp-1 expression while inhibiting granzyme B and IFNγ in early effector $CD8^+$ T cells. Thus, BATF promotes expression of transcription factors involved in effector differentiation but prevents effector molecule expression, suggesting that BATF may impede progression to an exhausted phenotype [143]. However, PD-1 expression was found to upregulate expression of BATF in HIV-specific $CD8^+$ T cells, which inhibited T cell function. Signaling through PD-1 upregulated BATF expression, which in turn decreased T cell effector function through reduced proliferation and IL-2 production [144]. Thus, iRs may suppress $CD8^+$ T cell-mediated antitumor immunity twofold, through diminished TCR signaling as well as regulation of context-specific transcription factors that influence $CD8^+$ T cell fate.

TGF-β is an immunosuppressive cytokine that is released by $CD4^+$ T_{regs} and APCs in the tumor microenvironment and directly inhibits CTL-mediated antitumor immune responses [119, 145–148]. Inhibition of $CD8^+$ T cell function involves the formation of Smad (mothers against decapentaplegic homolog) transcription factor complexes. High-affinity DNA-binding is achieved by Smad interaction with coregulatory molecules such as FoxP1 (forkhead box). FoxP1 is upregulated in $CD8^+$ TIL in the tumor microenvironment and necessary for TGF-β-mediated suppression of TIL, preventing rejection of ovarian tumors *in vivo* [149, 150]. Recent studies suggest that there may also be an antitumor effect of TGF-β signaling in CTL-mediated antitumor immunity. The TGF-β downstream molecules Smad2/3 and NFAT-1 were shown to promote CD103 expression on $CD8^+$ TIL, an integrin that binds E-cadherin on tumor cells and induces cell lysis through granule exocytosis [151, 152]. In a separate study, TGF-β was shown to repress KLRG1 expression in $CD8^+$ T cells *in vitro*. KLRG1 is an iR specific for E-cadherin and therefore inhibits CTL-mediated responses against E-cadherin expressing cells. TGF-β-deficient $CD8^+$ T cells exhibited higher KLRG1 expression *in vivo*, suggesting that TGF-β may promote CTL-mediated tumor rejection through reciprocal regulation of KLRG1 and CD103 [153] (Figure 1). In line with this idea, Quatromoni et al. demonstrated that early blockade of TGF-β signaling prevented expansion of $CD8^+$ TIL and negatively correlated with tumor volume, implying that some level of TGF-β signaling may be critical in generating CTL-mediated tumor rejection [154]. Conflicting evidence concerning the role of TGF-β signaling on $CD8^+$ TILs highlights the need for more in-depth investigation. Studies

have demonstrated both antitumor and protumorigenic roles for Smad transcription factors [155–158]. Therapies that aim to block TGF-β signaling in the tumor microenvironment are of high interest and have generated favorable responses in clinical trials, yet the importance of TGF-β signaling on $CD8^+$ TIL in the tumor microenvironment remains to be determined [159, 160].

5. Conclusion

One of the current foci in the field of immunology is delineating the function of the adaptive immune system in antitumor responses. While cytotoxic $CD8^+$ T lymphocytes are capable of recognizing and directly lysing transformed cells, $CD8^+$ tumor-infiltrating lymphocytes often display dysfunctional properties *in vivo*. Reasons for $CD8^+$ T cell impairment remain incompletely understood, but recent studies have identified multiple states of $CD8^+$ T cell dysfunction in cancer patients as well as experimental models. These subsets include exhausted, anergic/tolerant, and regulatory/senescent $CD8^+$ T cells. The current characterization of these dynamic fates in terms of surface marker profile and transcription factor expression is not sufficient to clearly delineate distinct $CD8^+$ T cell fates. Transcription factors and inhibitory receptors exhibit multiple levels of crosstalk and feedback signaling both in early activated T_{EFF} cells and in the context of persistent antigenic stimulation, leading to diverse $CD8^+$ T cell fates. Many of the key transcription factors that promote an effector phenotype also promote iR expression, perhaps maintaining an equilibrium between effector function and autoimmunity. In the context of antitumor immunity, increased iR expression limits CTL-mediated tumor rejection by promoting $CD8^+$ T cell dysfunction. Novel immunotherapies that target multiple iRs may reverse the transcriptional network that regulates $CD8^+$ T cell dysfunction and promote the adoption of effector and memory fates associated with active antitumor immunity.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 8121985, 12 pages
http://dx.doi.org/10.1155/2016/8121985

Clinical Study

Adjuvant Autologous Melanoma Vaccine for Macroscopic Stage III Disease: Survival, Biomarkers, and Improved Response to CTLA-4 Blockade

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Received 11 January 2016; Revised 30 March 2016; Accepted 17 April 2016

Academic Editor: Yi Zhang

Background. There is not yet an agreed adjuvant treatment for melanoma patients with American Joint Committee on Cancer stages III B and C. We report administration of an autologous melanoma vaccine to prevent disease recurrence. *Patients and Methods*. 126 patients received eight doses of irradiated autologous melanoma cells conjugated to dinitrophenyl and mixed with BCG. Delayed type hypersensitivity (DTH) response to unmodified melanoma cells was determined on the vaccine days 5 and 8. Gene expression analysis was performed on 35 tumors from patients with good or poor survival. *Results*. Median overall survival was 88 months with a 5-year survival of 54%. Patients attaining a strong DTH response had a significantly better ($p = 0.0001$) 5-year overall survival of 75% compared with 44% in patients without a strong response. Gene expression array linked a 50-gene signature to prognosis, including a cluster of four cancer testis antigens: CTAG2 (NY-ESO-2), MAGEA1, SSX1, and SSX4. Thirty-five patients, who received an autologous vaccine, followed by ipilimumab for progressive disease, had a significantly improved 3-year survival of 46% compared with 19% in nonvaccinated patients treated with ipilimumab alone ($p = 0.007$). *Conclusion*. Improved survival in patients attaining a strong DTH and increased response rate with subsequent ipilimumab suggests that the autologous vaccine confers protective immunity.

In loving memory of Olga Drize, Ph.D.

1. Introduction

The treatment of metastatic melanoma has been revolutionized in the last three years, with the FDA registration of Yervoy™, a monoclonal antibody blocking lymphocyte regulatory receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and shortly afterward, the entry to the clinic of Zelboraf, a small molecule inhibitor of mutated B-RAF.

From being an incurable disease, stage IV melanoma has become an illness in which prolonged and even complete responses can be envisioned. However, while the prospects have improved for stage IV disease, for patients with American Joint Committee on Cancer (AJCC) stage III disease no new treatment options have been developed and validated since the approval of interferon α (IFNα) almost two decades ago [1–7]. A pegylated formulation did not offer improved tolerability and treatment was discontinued due to toxicity. Both the EORTC trials and the ECOG pooled analysis [8] showed that AJCC stage III patients with macroscopic lymph node involvement derived the smallest survival benefit, if any, from IFNα.

In this situation, melanoma vaccines could have been an alternative to IFNα, since they could induce a tumor-specific immune response to inhibit micrometastases at a stage when the suppressive effects of an advanced tumor are not yet an obstacle [9]. In the few controlled clinical trials reported to date, vaccinated patients did not experience a survival benefit [10]. This fact, together with the paucity of objective responses to active immunization in stage IV melanoma patients using different vaccination strategies, rendered a general impression of the futility of cancer vaccines [11, 12].

The resurgence of interest in cancer vaccination was the result of several clinical trials demonstrating that a component of active immunization could improve clinical outcome of immunotherapy protocols. Examples include the addition of a peptide vaccine to the administration of high dose interleukin-2 (IL-2) [13] and the use of a GMCSF-secreting tumor vaccine in combination with CTLA-4 blockade for metastatic prostate cancer [14]. These trials led to the increasing understanding that cancer immunotherapy is a multifaceted strategy and that a single treatment modality would not suffice. Noting that individuals exhibit heterogeneity of tumor antigens [15], the use of autologous tumor as a basis for vaccination can provide antigen authenticity. The unique expression profile of normal and mutated proteins in the patient's tumor cells is presented in conjunction with their own major histocompatibility complex (MHC) molecules, and this combination is necessary to induce antigen-specific reactive lymphocytes [16]. The coadministration of Bacillus Calmette-Guérin (BCG), a widely used immunological adjuvant, with the autologous vaccine, has previously been shown to enhance response to autologous vaccination protocols [17, 18].

In this paper we report our experience with an autologous melanoma vaccine as an adjuvant therapy for melanoma patients in the advanced categories of AJCC stage III disease: macroscopic lymph node involvement and resectable in-transit metastases. We demonstrate that clinical immune response (delayed type hypersensitivity, DTH) is linked to improved survival. Furthermore, using this vaccination protocol, molecular analysis of the melanoma showed that cancer testis antigens (CTAs), which are generally considered targets of immune response, are predictive of survival. Interestingly, patients who had previously received the melanoma cell vaccine had improved overall survival when treated with ipilimumab immunotherapy for metastatic disease.

2. Materials and Methods

2.1. Patients. This prospective phase II, single-institution, single-arm study included patients with operable AJCC stages IIIB and C melanoma. Clinical staging was based on palpable lymph nodes and/or satellites prior to surgery. Since we did not have pathological data on ulceration of the primary melanoma for all patients, AJCC stage IIIB was defined for any T (tumor) with pathological N1b, N2b, and N2c. Stage IIIC was defined as any T with pathological N3 [19].

2.2. Outcomes. The aim of the study was to document overall and disease-free survival (primary outcome) and to correlate skin reactivity to the autologous tumor of patients that were treated with the autologous melanoma cell vaccine with survival (secondary outcome).

2.3. Inclusion and Exclusion Criteria. To participate, patients had to undergo complete removal of their metastatic disease and have a normal CT scan within 30 days prior to vaccination. Additional inclusion criteria included an age of 18 years or older, normal liver and renal function tests, baseline lactate dehydrogenase (LDH) value below the laboratory upper limit of normal, and ability to provide informed consent. Exclusion criteria included primary ocular melanoma. Fertile patients were requested to use adequate anticonceptive measures throughout the study period.

Screening procedures included baseline blood analyses (hematological, chemical) and computed tomography (CT) and/or magnetic resonance imaging (MRI) of the entire body, including the brain, every 4 months in the first 2 years and then every 6 months for 10 years. The study was not sponsored and was conducted following approval by the institutional ethics committee; written informed consent was obtained from all patients. Seventy-five patients from this group were included in an immune monitoring study reported earlier [16].

2.4. Melanoma Cell Lines. For the preparation of the autologous vaccine, melanoma cell lines were established from resected metastases. All patients gave their informed consent to receive the vaccine. The melanoma cell lines were established and cultured as described [18]. Briefly, cells were extracted mechanically from fresh and sterile tumor specimens, frozen, and stored in liquid nitrogen in a medium containing 2.5% human albumin and 20% DMSO until needed. To assure melanocytic progeny, the expressions of S100, MART-1, and gp100 were determined by immunostaining using polyclonal rabbit anti-S100, monoclonal A-103, and HMB-45 Abs, respectively (Dako). Positive staining of more than 50% of cells with at least one of these antibodies was required. MHC class I-related chain A (MICA) expression was determined by flow cytometry of melanoma cell lines using anti-human MICA-APC (Allophycocyanin), R&D systems, Minneapolis, MN, USA.

Cell lines were routinely tested for mycoplasma contamination by EZ-PCR (Biological Laboratories, Beth Haemek, Israel), according to the manufacturer's instructions. Tumor

cultures that were found contaminated were incubated in the presence of 10 mg/mL Ciproxin 200 (Bayer) for two weeks, with change of medium every three days. The cells were retested after treatment and were used only after being found mycoplasma-free.

2.5. Vaccine Preparation and Vaccination Procedure. Melanoma cell lines were expanded to the required number necessary for preparation of at least 8 vaccine doses of $10–25 \times 10^6$ cells each and cryopreserved at −70°C. On the day of treatment, one dose of cells was thawed, washed, and irradiated to a dose of 230 Gy. At this stage, cells were still viable. Conjugation of melanoma cells with DNP was performed as described [20], leading to death of the cells (as determined by trypan blue exclusion). BCG (Statens Serum Institut, Denmark) was added to the vaccine prior to injection, diluted to 1 : 50 for the first three vaccine doses and up to 1 : 500 for the following doses, to avoid overreactivity at the injection site. BCG was reported to trigger dendritic cell maturation and to aid in diverting the CD4 T cell response towards a Th1 phenotype [21]. The vaccination procedure was carried out as described [18]. Briefly, patients were sensitized to DNP, to enhance the response to the vaccine, by applying 0.1 mL of 2% DNP dissolved in acetone-corn oil (Sigma) topically to the inner aspect of the arm ten days prior to injection of the first vaccine. Cyclophosphamide, 300 mg/m^2 per dose, was given 4 days preceding the first and second vaccines. This practice was based on the observation that cyclophosphamide prior to vaccination can reduce the proportion of T regulatory cells and enhance tumor-specific immune response [22–25]. Generally, vaccines were injected in 3 adjacent sites on the upper arm or thigh, avoiding limbs with dissected lymphatic basins. Each patient received eight vaccine doses, at three-week intervals.

Patients were evaluated periodically every 3 months and had a total body CT scan performed every 6 months, or as required according to their symptoms.

2.6. DTH Reaction. Evaluation of DTH to autologous melanoma cells was performed on the vaccine days 5 and 8, by intradermal injection of $1–3 \times 10^6$ irradiated (170 Gy) autologous melanoma cells. The DTH response was measured 48 h after injection. Erythema diameter of 5 mm and less was arbitrarily defined as negative; 5–10 mm weak; 10–15 mm positive; and ≥15 mm strong positive DTH.

2.7. Gene Expression and Connectivity Map Analysis (C-MAP). Thirty-five melanoma cell lines that were retrieved from 35 vaccinated patients were selected for gene expression analysis based on retrospective survival data of the donors. Gene expression profiling was performed using an assay based on a collection of cellular genomic signatures that produced a pattern-matching tool and formulation-based deduction of a wider expression profile. One thousand transcripts were identified, from which the remainder of the transcriptome could be computationally inferred. These 1,000 "landmark" transcripts were measured on Luminex beads, as part of the Connectivity Map (C-MAP) project (unpublished, R. Narayan, Broad Institute of Harvard University and MIT, Cambridge, MA). Cultures underwent a median of 4 passages (range 2–13) and mRNA was extracted from melanoma cell microcultures harvested at 90% to 100% confluence, produced in a synchronous way, under identical conditions of growth, in four replicates. Initial analysis was conducted with a bioconductor- (R-) based test (SSAT), which applies a Cox-regression analysis followed by a second test based on rank statistics. The analysis determines the best cut-off value that separates patients into those with favorable versus worse survival time.

2.8. Quantitative Real Time PCR (qRT-PCR). Applied Biosystems premade and custom primer probes designed with NCBI Primer–Blast Tool were used (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). RNA was normalized to GAPDH-RNA content using ABI 7500 SDS software, v1.2.2 (Applied Biosystems Inc., Foster City, CA). Positive and negative controls, as well as samples with no DNA, were included in every qRT-PCR experiment. PCR reactions were performed using ABI qRT-PCR thermocycler (7500 Real Time PCR System, Applied Biosystems Inc., Foster City, CA). The qRT-PCR program was run for 45 cycles, following an initial incubation at 95°C, 10 min. Each cycle consisted of 95°C × 15 sec and 60°C × 1 min.

B-RAF genotype was determined using Cobas® 4800 (Roche).

2.9. Patients Undergoing Ipilimumab Treatment. During the autologous vaccine study period, ipilimumab (Yervoy, BMS) was administered in our institution to patients with metastatic melanoma in the framework of BMS studies CA184-004, 024, and 025. Since its FDA approval as a standard second-line treatment, ipilimumab was given after a one- or two-dose course of DTIC. Patients from the autologous vaccine study which later developed nonresectable metastatic disease were among a larger group recruited for these protocols. Survival data is reported for all patients getting ipilimumab during 2007–2014.

2.10. Statistical Method. The comparison of survival curves between groups was carried out using the Kaplan-Meier Survival analysis with the log rank test. All tests applied were two-tailed, and a p value of 5% or less was considered statistically significant.

3. Results

3.1. Study Patients. Melanoma metastases were obtained from 159 eligible patients. From 33 patients (20%) we could not generate the number of cells required for the treatment. A total of 126 patients (55% male; median age, 59 years) with postoperative AJCC stages IIIB and C (45% stage IIIB; 55% stage IIIC) were enrolled. For patient characteristics see Table 1. Twenty-four patients (19%) presented with enlarged lymph nodes (LNs) at the time of diagnosis of the primary melanoma; 11 (9%) had unknown primary lesion; 22 (17%) had metastases *in transit*. Nineteen patients (15%)

Table 1: Patients characteristics.

Patients	126
Age, median (range)	59 (15–86)
Gender	
Male	69 (55%)
Female	57 (45%)
Primary melanoma	
Breslow	Median 3.05 mm (0.2–20)
Cutaneous	76 at least
Acral	10
Mucosal	0
Unknown	11
Ulcerated	29/58 documented
AJCC stage	
IIIB	57 (45%)
IIIC	69 (55%)
Satellites	22 (17%)
Number of involved LNs, mean (range)	2.4 (0–17)
Adjuvant radiotherapy	56 (44%)

had noninvolved sentinel LN but developed macroscopic disease later, in the same lymphatic basin. Forty-two patients (33%) had not undergone a sentinel LN biopsy and developed macroscopic LN involvement. The mean number of involved lymph nodes was 2.4, ranging from 0 (satellites) to 17. Fifty-six patients (44%) had undergone radiotherapy in addition to the surgical procedure, which was added when more than three nodes were involved or in cases of extracapsular invasion by melanoma cells.

3.2. Vaccine Safety. No grade 3-4 adverse events (CTCAE V4) were encountered among the 126 vaccinated patients. In all patients, an erythematous nodule developed at the vaccination site and resolved in the course of 3–6 months leaving a depressed scar.

3.3. Patient Survival Correlates with Intensity of Evolving DTH Response to Unmodified Melanoma Cells. The OS and disease-free survival (DFS) of participating patients were measured from the day of the first vaccine until the current analysis was performed. A total of 126 patients were included, with a median follow-up of 44.5 months (range 8–189 months). OS survival data was available for all 126 patients and DFS data was available for 107 patients. OS and DFS were analyzed for DTH < 15 mm (weak/positive DTH) versus DTH > 15 mm (strong positive DTH). Overall, for the whole cohort, the 5-year OS was 54% and DFS was 34%. There was no difference between OS of stage IIIB and IIIC patients ($p = 0.182$). Of 119 patients with recorded DTH response, 48 patients (40%) attained strong positive DTH (DTH > 15 mm), whereas 71 (60%) had a weak DTH response (<15 mm). The patients with strong DTH response had a 5-year OS of 75% and DFS of 47%. In contrast, patients with weak DTH had a significantly lower 5-year OS of 44% ($p < 0.0001$) and DFS of 26% ($p = 0.27$). Using the Kaplan-Meier analysis and the log rank test, the single parameter that most strongly correlated with OS and DFS in a univariate analysis was the DTH response (Figure 1). In Table 3, OS and DFS are compared between patients attaining DTH responses of 10 and 15 mm and patients who did not develop such response. For patients who attained strong positive DTH (>15 mm), the 5-year overall survival hazard ratio (HR) was 0.24 (95% CI 0.1–0.53; $p < 0.001$). The HR for 5-year disease recurrence was 0.4 (95% CI 0.1–0.83, $p = 0.015$ Pearson's chi square test), but in a longer follow-up, the protection from recurrence decreased to a HR of 0.63 (95% CI 0.3–1.32; $p = 0.2$). Age, gender, and depth of invasion of the primary melanoma had no impact on survival. In a survival analysis done for DTH cut-off of 10 mm, a similar trend was noted with a smaller p value (0.003) for improved 5-year OS in patients attaining a DTH response of >10 mm (64%) versus 32% in patients with DTH <10 mm. DFS was similar in the two groups ($p = 0.36$). Thus, the acquisition of powerful skin reactivity against nonmodified autologous melanoma cells, which reflects the development of specific cell mediated immunity, correlates favorably with survival, supporting previous results by us and by others, for example, [16, 18, 26].

For 56 patients in whom more than 3 involved lymph nodes were removed, radiation therapy was added to the resected lymphatic basin. Even though the patients requiring this added treatment belonged to a grave prognostic group, radiotherapy may enhance the immune response of the patients [27]. Indeed, the rate of strong DTH response in patients receiving radiotherapy was 42%. Five-year OS of these patients was 58% compared to 33% of those who received radiotherapy and had a weak DTH response ($p = 0.024$).

3.4. Cancer Testis Antigen mRNA Expression in Melanoma Cells Correlates with Improved OS. The C-MAP project was based on a collection of cellular genomic signatures to drugs, disease states, and cancer, in order to produce a pattern-matching tool and a formulation-based deduction of a wider expression profile. One thousand transcripts were identified from which the remainder of the transcriptome could be computationally inferred. These 1,000 "landmark" transcripts were measured on Luminex beads (unpublished, R. Narayan, Broad Institute of Harvard University and MIT, Cambridge, MA).

Thirty-five tumor samples, representing distinct subclasses of poor and good responders, were selected for C-MAP analysis: (1) eighteen poor responding patients with a median OS of 19 months (range 8–34), all of whom failed to develop strong skin reactivity to their autologous tumor, and (2) seventeen good responding patients with median OS of 105 months (range 46–194), 12 of whom also developed strong skin reactivity (DTH data missing for one). Figure 2 shows the hierarchical clustering of 50 genes expressed on patients' melanoma cells, which yield a significantly improved or worsened HR for survival. Several genes of interest are listed in Table 4. Cancer testis antigens CTAG2 (NY-ESO-2), MAGEA1, SSX1, and SSX4 clustered together in the hierarchical diagram (depicted in a circle in Figure 2). High

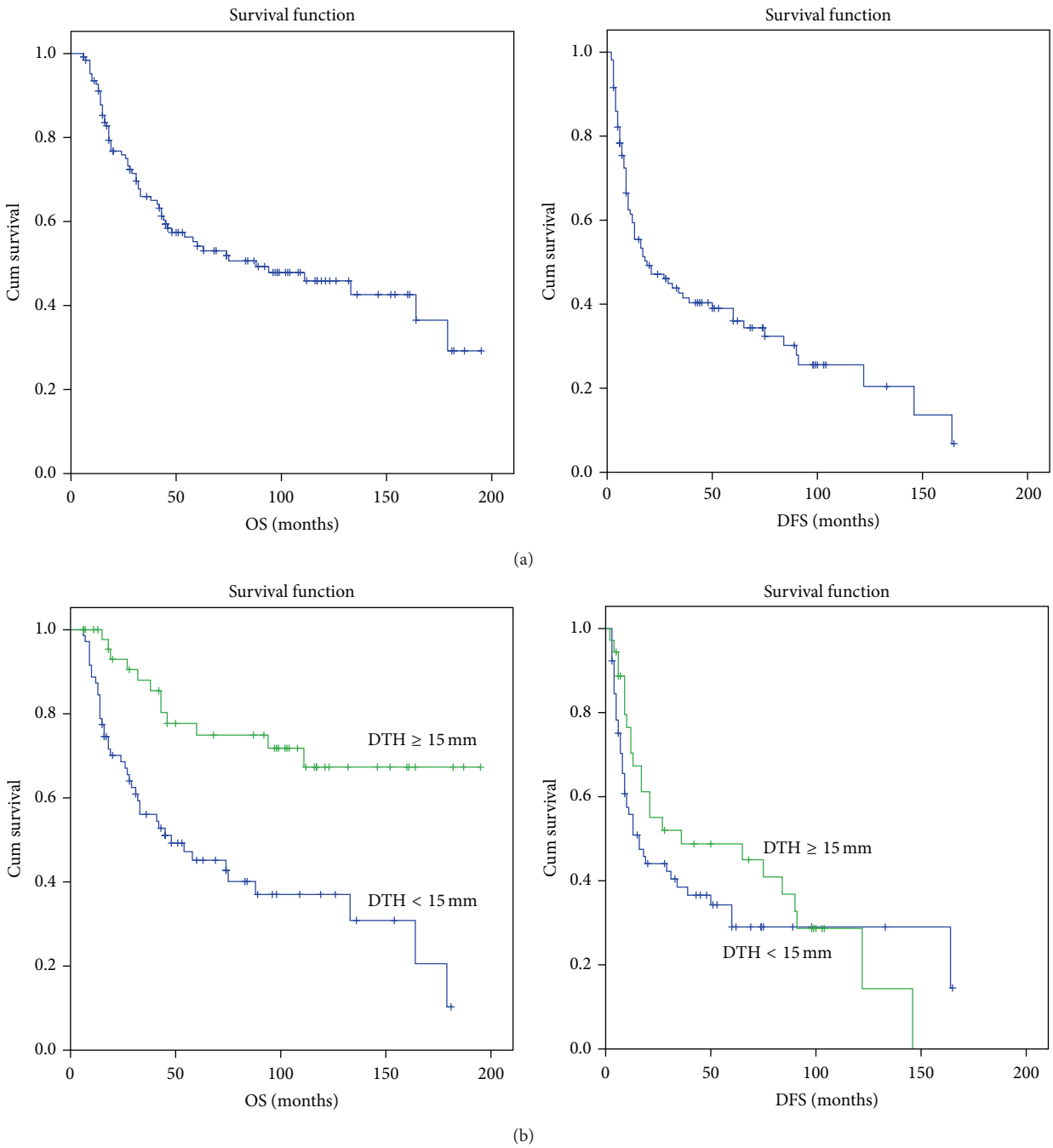

Figure 1: Kaplan-Meier survival curves of 126 melanoma patients with AJCC stages III B and C disease. (a) Survival data of all patients undergoing autologous vaccination. (b) Correlation of survival with delayed type hypersensitivity (DTH) response to unmodified melanoma attained following vaccination. OS: overall survival; DFS: disease free survival.

expression of each of these CTA genes was associated with a reduced risk of death. As CTA genes are coexpressed, we performed a principal component analysis (PCA) of the genes to stratify the patients, using the "princomp" function in R. We found the first principal component (PC1) to explain more than 80% of the variance when expression values from the 35 samples corresponding to all 51 probe sets from CTA genes in the C-MAP array were used (Figure 3(a)). To validate the predictive data extracted from C-MAP, we used qPCR results obtained from 21 melanoma line samples for MAGE-A1, SSX1, SSX4, and NY-ESO-1 (CTAG1B). Similar to the in silico data, a PCA analysis based on these four genes was able to explain 75% of the variance. In both analyses, stratifying the patients into low/intermediate and high expression based on PC1 values, we show worse outcome for low CTA expressors (below quartile 1) compared to high

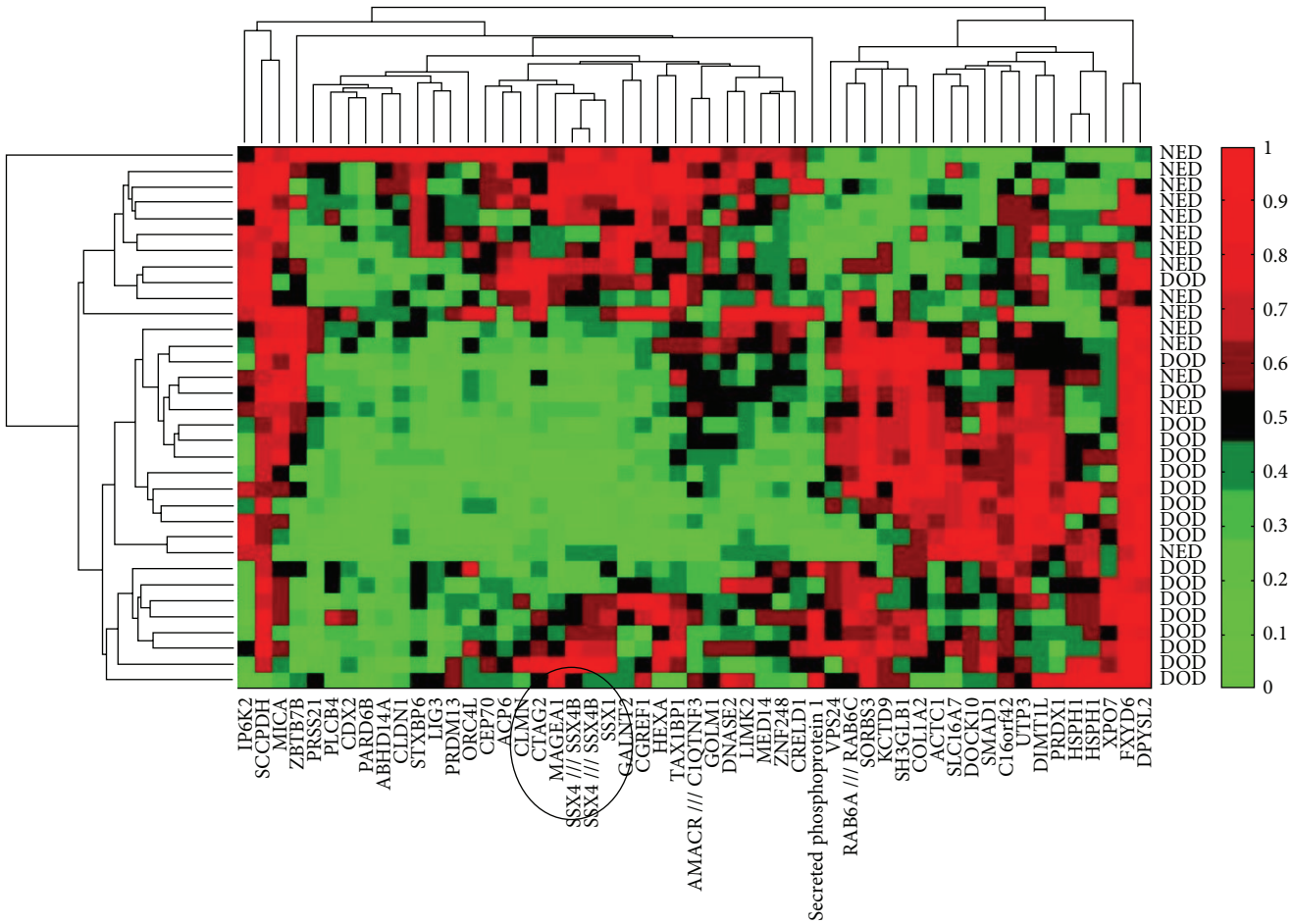

Figure 2: Hierarchical clustering gene expression of a 50-gene signature showing strongest association with prognosis in 35 stage III melanoma patients. Cancer testis antigens CTAG2, MAGEA1, SSX1, and SSX4 are circled in a cluster. NED: no evidence of disease at time of analysis; DOD: died of metastatic disease.

expressors (above quartile 4), $p = 0.02$ (Figure 3). A similar analysis performed using an unrelated melanoma cohort (GSE19234 [28]), which never had an autologous vaccine, did not reveal an association between CTA gene expression and survival (not shown), suggesting that CTA gene expression is not a prognostic factor by itself but becomes one in the context of autologous vaccination.

MICA (MHC class I related chain A), another gene which correlated with a reduced risk of death and for which there is an antibody for flow cytometry, was used to validate the expression data (Figure 4). Out of 11 samples analyzed, 10 yielded MICA protein expression (by flow cytometry) concordant with the gene expression value. In 8 samples the data was predictive of patient's current status, whether alive, no evidence of disease (NED), or died of disease (DOD).

3.5. B-RAF Status and Survival. B-RAF status was determined for 32 patients of the 35 that were analyzed for gene expression. Eleven patients (33%) harbored the V600E mutation. The median survival for patients with V600E mutation was 50 months compared with 45 months for the wild type (WT) group ($p = 0.9$). Since most patients in the series who had recurrent disease died before 2012, none of them were treated with B-RAF inhibitors when they developed metastases, and consequently differences in survival could not be attributed to better treatment options. Four out of 11 patients with V600E mutations had strong DTH response (36%), compared with seven out of 21 (33.3%) in the WT group ($p = 0.86$).

3.6. Patients Who Received Melanoma Vaccine Had Improved Survival following Ipilimumab Treatment for Advanced Disease. Thirty-five patients who received melanoma cell vaccine and developed nonresectable metastatic disease were referred to ipilimumab treatment in BMS studies CA 184-004, 024, and 025 and later as a standard second-line treatment. These patients were compared with a nonselected concurrent group of other 35 patients, who never received the vaccine (Table 2). The majority, 62 patients (89%), received ipilimumab at a dose of 3 mg/kg. Response Evaluation Criteria in Solid Tumors (RECIST) were used to define response to treatment. Complete response was achieved in six patients (8.6%), partial response in 14 (20%), and stable disease in 7 (10%). Median OS of the group was 14 months (95% CI

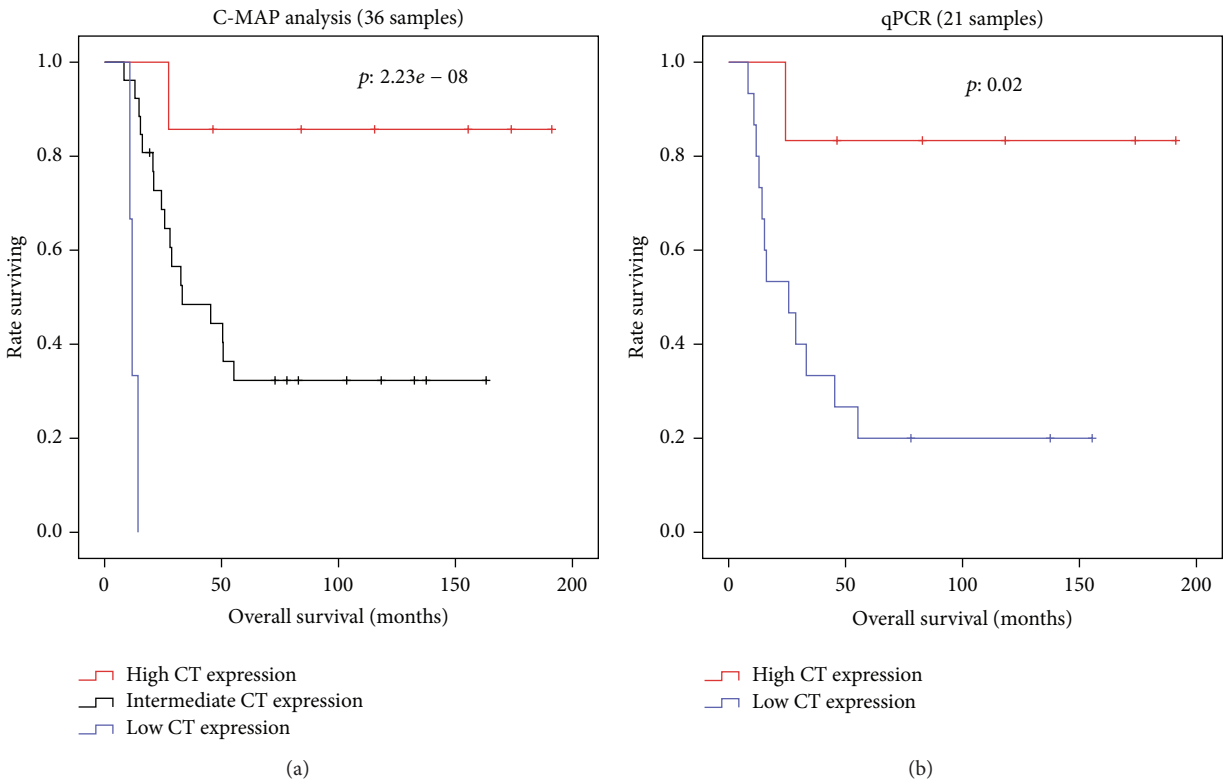

Figure 3: Overall survival curves stratifying the patients according to integrated cancer testis antigen genes expression. As CTA genes are coexpressed, we performed a principal component analysis (PCA) of the genes to stratify the patients into low/intermediate and high expression based on first principal component (PC1) values. (a) PC1 was determined in 35 melanoma lines based on all 51 probe sets from CTA genes in the C-MAP array. (b) PC1 was determined in 21 melanoma lines based on qPCR data generated for CTAs MAGE-A1, SSX1, SSX4, and NY-ESO-1 (CTAG1B).

5.7–22 months). Multivariate analysis revealed that the single parameter that correlated with an improved OS time was previous treatment with melanoma cell vaccine. In the group of 35 vaccinated patients, median OS was 31 months with a 3-year survival of 46%, compared with a median OS of 9 months and 3-year survival of only 19% in the nonvaccinated patients, ($p = 0.007$, Pearson's chi square test). The results from this retrospective cohort of patients suggest that response rate and survival are improved when ipilimumab treatment succeeds autologous melanoma vaccine.

4. Discussion

In this phase II study we administered a vaccine composed of the autologous tumor given to melanoma patients as a postoperative adjuvant for AJCC stage III disease following resection of macro metastases. The study was single-armed, as it was initiated prior to the registration of IFNα as standard of care for the adjuvant treatment of melanoma stages IIB and III. In an initial cohort, which included patients of worse prognoses (stages IIIB, IIIC, and IV) we observed an unexpected good survival rate with this vaccine [18]. In view of the reduced toxicity of the vaccination regimen, we did not offer patients a high dose interferon arm after its registration. On the other hand, the inclusion of an observation arm was ethically questionable. Thus, we opted to continue with the protocol in its single-arm design, to record survival rates in adjuvant stage IIIB and C patients. Eventually, these patients were the less likely ones to derive benefit from adjuvant IFNα therapy, since treatment with the standard of care IFNα-2b or PEG-IFN did not yield any survival advantage for them [29]. The projected 5-year OS for these patients was estimated at 40% and the DFS at 30%, as shown in the Kaplan-Meier curves generated in a meta-analysis from EORTC trials 18952 and 18992 of patients with AJCC stage III-N2. In our group of patients with the same stage disease, the 5-year OS reached 54%, with a median OS of 88 months and a 5-year DFS of 34%; these were achieved with no major adverse effects. In another series, the Nordic study, the best median survival, 72 months, was derived from intermediate-high doses of IFNα-2b for one year. These results, worse than those we observed with the autologous vaccine, were generated in a group of patients in which 35% had earlier stage disease (IIIA).

Notably, in our study population, patients with a strong positive skin reaction to their unmodified melanoma had much better prognosis, with a 5-year OS of 75% and DFS of 44%. The link between immune parameters that attest to the development of antitumor response and improved survival has been previously observed [17, 30–36]. DTH is a crude measurement, but an easy test to apply on all patients.

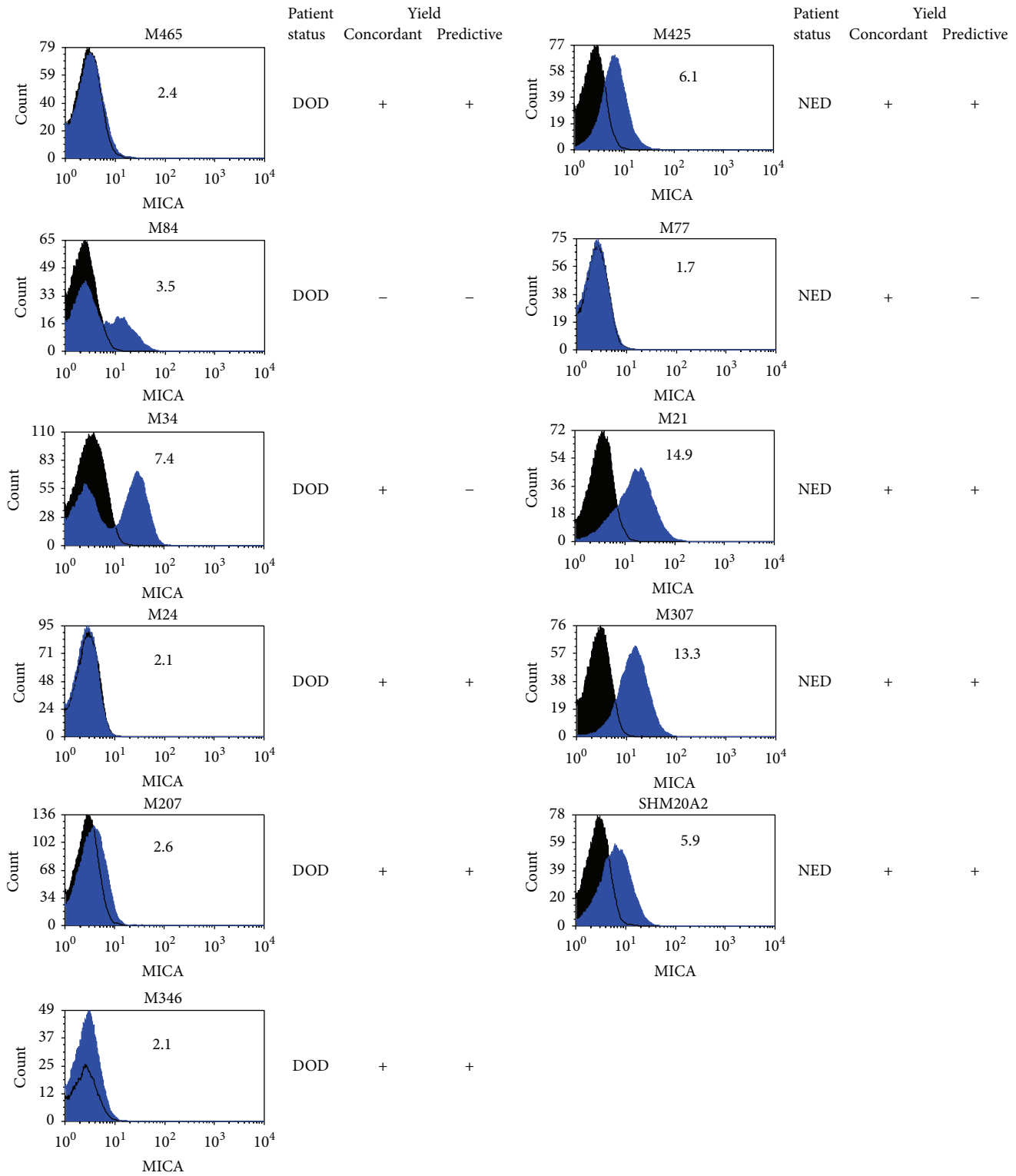

Figure 4: Flow cytometry analysis of MICA surface expression on melanoma cell lines. The number in the histogram is the Luminex 1000 expression value of MICA. Black histogram: background staining with isotype control Ab. Blue histogram: MICA staining with anti-MICA specific Ab.

Table 2: Clinical data and ipilimumab treatment results of 70 patients with AJCC stage IV melanoma who received or did not receive melanoma vaccine.

Prior vaccination	Yes		No		p^*
	n	%	n	%	
Patient number	35	50	35	50	
M stage					
M1A	8	23	3	9	
M1B	8	23	10	28	*0.3*
M1C	19	54	22	63	
Dose					
3 mg/kg	30	86	32	91	*0.45*
10 mg/kg	5	14	3	9	
Reinduction	4	12	3	9	
Treatment stopped for toxicity	5	14	2	6	
Objective response					
CR	3	9	3	9	
PR	10	29	4	11	*0.03***
S	6	17	1	3	
P	16	46	27	77	
Survival data					
Median OS (months)	31 (3–47)		9 (2–50)		*0.007*
3-year survival rate	46%		19%		

CR: complete response, PR: partial response, S: stable disease, and P: progression.
*Pearson chi square (two-sided) test.
**p value for progression (P) versus any benefit (CR, PR, and S).

Several vaccine clinical trials included a DTH test as part of their clinical evaluation and reported a positive correlation between DTH response and longer survival [37, 38]. Biopsies taken from skin injection sites revealed vaccine-induced antigen-specific T cells [39, 40]. We previously demonstrated effective antimelanoma CD4 T cell activity associated with improved survival in a cohort included in the present study [16]. Thus, a positive DTH reaction could be indicative of the emergence of antimelanoma immunity, and the improved survival of patients attaining strong DTH would attest to the vaccine's protective effects.

Since autologous tumor tissue is often not available for vaccine preparation, defined antigens, consisting of short or long peptides, are used as a substitution [41–43]. The enthusiasm for the use of defined antigens decreased when successful generation of antigen-specific T cells failed to protect against tumor progression [11, 44]. Loss of MHC and impaired peptide presentation by the melanoma were among the reasons for vaccine failure, but another limitation is that many tumor-progeny antigens are essentially self-antigens that evoke weak responses [45]. Unlike wildtype proteins, mutations have the potential to generate neoantigens which are better targets. The significance of mutation-derived neoantigens was illustrated when adoptively transferred tumor-infiltrating lymphocytes that destroyed melanoma in patients were surveyed and found to target mutated epitopes [46]. Furthermore, recent data has clearly demonstrated that these mutations are, in large part, the functional targets of immune checkpoint blockade [47]. It may not take long until "mutanome" libraries are generated for melanoma patients. But until the hurdles of preparing a totally individualized vaccine are overcome, the best source of mutated antigens, as we have previously shown, is still the autologous tumor and, ideally, those tumors that express both MHC class I and class II [16].

Another important component of melanoma cell vaccines, as reflected by our data, are a class of antigens known as cancer testis antigens. CTAs are products of several multigene families, many of which map to chromosome X, that have arisen through chromosomal duplications and were initially identified through immunologic assays [48]. When associated with disease outcome, CTAs sometimes confer worse prognosis [49], but when protective immune responses are recorded, CTAs are dominant targets. For example, rising antibody responses to CTA NY-ESO-1 (CTAG1B) were recorded following shrinkage of melanoma in a patient with abscopal tumor response [50], and among patients treated with ipilimumab, seropositivity to NY-ESO-1 with associated CD8 T cell response correlated with 77% clinical benefit [51].

Using a Luminex-based method to generate a gene expression array and qPCR validation, we showed increased expression of MAGEA1, CTAG1, CTAG2, SSX1, and SSX4 in patients with improved survival. We suggest that these patients' prolonged survival is attributed to the melanoma immunogenicity potentiated by the CTAs and augmented by the vaccination procedure.

Lastly, our data implies that patients who had been immunized against the autologous tumor prior to receiving ipilimumab survived longer than patients who did not receive an autologous melanoma vaccine. The precise mechanism of action of ipilimumab is not completely clear. CTLA-4 receptor blockade prevents inhibition of activated effector lymphocytes and selective increase in the ratio between T effectors and regulatory T cells within the tumor [52]. In patients with preexisting immune response, the removal of inhibitory signals boosts antitumor activity [51], leading to the hypothesis that prior vaccination might induce in a subset of patients a population of specific antitumor cytotoxic T cells and a potent immune response following immune checkpoint blockade. In this respect, it was encouraging to note that in a cohort of vaccinated patients that had received ipilimumab, the 3-year survival was 46%, compared with 19% for the nonvaccinated patients ($p = 0.007$).

5. Conclusions

In this noncontrolled phase II study we have shown that adjuvant treatment with autologous melanoma vaccine yields overall and disease-free survival rates that are not inferior to those obtained with interferon alpha. In a subgroup of patients who attained a strong positive skin response to unmodified autologous tumor, survival rates were exceptionally good, with a 5-year OS of 75%. Increased expression of CTAs by the tumor correlated with improved survival. Lastly, improved survival time following ipilimumab treatment was observed for patients who had previously been

Table 3: Patients survival data.

	Number (%)	1 year (%)	2 years (%)	5 years (%)	Median (mo, 95% CI)	p value
Overall survival						
All*	126	93	77	54	88 (40–137)	
DTH ≥ 15 mm	48 (40)	100	93	75	Not reached	
DTH < 15 mm	71 (60)	89	70	44	45 (13–20)	**<0001**
DTH ≥ 10 mm	75 (63)	97	83	64	181 (75–287)	
DTH < 10 mm	44 (37)	87	66	32	41 (23–59)	**0.003**
Disease-free survival						
All**	107	58	45	34	18 (5–31)	
DTH ≥ 15 mm	36 (36)	74	53	47	36 (0.00–87)	
DTH < 15 mm	65 (64)	55	42	26	15 (7–23)	**0.27**
Adjuvant radiotherapy Overall survival						
All	56	90	69	41	43 (27–59)	**0.055**
DTH ≥ 15 mm	22 (42)	100	87	58	111	
DTH < 15 mm	31 (58)	84	56	33	31 (20–42)	**0.024**

*DTH data was available for 119 of 126 patients.
**DTH data was available for 101 of 107 patients with recorded disease-free survival.

Table 4: Selected genes expressed on melanoma cells which correlate with overall survival. The genes were depicted by Maxstat-package utilizing a Cox-regression analysis followed by rank statistics to determine the best cut-off value which separates patients into favorable versus unfavorable survival groups. ↑ = higher expression correlates with prolonged survival time; ↓ = higher expression correlates with decreased survival time.

Gene	↑/↓	Full name	Maxstat cut point	Maxstat p value	CoxPH hazard ratio	CoxPH p value
MICA	↑	MHC class I-related chain A (NKG2D ligand)	7.8	0.018	0.5	0.015
CTAG2	↑	Cancer testis antigen 2 (LAGE-1, NY-ESO-2)	5.9	0.014	0.58	0.015
SSX4 /// SSX4B	↑	Synovial sarcoma, X breakpoint 4	3.9	0.008	0.58	0.017
TGFA	↑	Transforming growth factor α	4.08	0.011	0.66	0.018
KIR3DX1	↑	Killer cell Ig-like receptor	4	0.023	0.56	0.02
MAGEA1	↑	Melanoma antigen family A, 1	5.52	0.042	0.69	0.028
SSX1	↑	Synovial sarcoma, X breakpoint 1	3.28	0.035	0.63	0.043
SMAD1	↓	SMAD family member 1	5.5	0.046	2.58	0.0002
HSPH1	↓	Heat shock 105 kDa/110 kDa protein 1	10.7	0.008	1.9	0.007

vaccinated. Thus, we suggest that autologous melanoma vaccine induces protective immunity and may offer leverage for other immunotherapies.

Disclosure

The funding sources had no involvement in the study design or in its performance.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors wish to acknowledge the devoted technical work of Anna Kuznetz, Inna Ben David, and Yael Gelfand. This work was supported by research grants from Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF), Israel Cancer Association, Deutsche Forschungsgemeinschaft (DFG), Rosetrees Trust, and the Perlstein Family Fund. Professor Ali O. Gure was supported by TUBITAK Grant no. 112S023.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 6837241, 8 pages
http://dx.doi.org/10.1155/2016/6837241

Clinical Study

Dual Effects of Cellular Immunotherapy in Inhibition of Virus Replication and Prolongation of Survival in HCV-Positive Hepatocellular Carcinoma Patients

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Received 19 November 2015; Revised 14 January 2016; Accepted 14 February 2016

Academic Editor: Kurt Blaser

Immune cells play an important role in the development and progression of hepatitis C virus (HCV) and hepatocellular carcinoma (HCC). We conducted a retrospective study to evaluate the influence of adoptive cellular immunotherapy (CIT) on viral load and progression-free survival (PFS) for HCC patients infected with HCV. Patients ($n = 104$) were divided into a control group (conventional therapy, $n = 73$) and study group (combination of CIT and conventional therapy, $n = 31$). Autologous mononuclear cells were induced into natural killer, $\gamma\delta$T, and cytokine-induced killer cells and infused intravenously to study group patients. More patients had shown viral load decrease or were stable in study group (100% versus 75%) ($p = 0.014$). The median PFS of the study group and control group was 16 and 10 months, respectively ($p = 0.0041$), and only CIT was an independent prognostic factor for PFS (hazard ratio, 0.422; $p = 0.005$). Three patients developed transient moderate fever after infusion, and there were no significant differences in alanine aminotransferase and aspartate aminotransferase levels before and after treatment in both groups. Our results show that CIT contributes to improvement of prognosis and inhibition of viral replication in HCV-related HCC patients, without impairment of liver function.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, with 749 000 new cases diagnosed in 2008, and the third-ranking cause of cancer-related deaths, contributing to 695 000 cases annually. HCC is mainly caused by hepatitis B virus (HBV) and hepatitis C virus (HVC) infection. It has been estimated that over 185 million people are chronically affected with HCV worldwide. Furthermore, chronic hepatitis C patients show a higher probability of developing liver cirrhosis and HCC, which represents a significant public health problem [1, 2].

Accordingly, virus elimination or control is important for reducing the rate of tumor occurrence in hepatitis C patients [3, 4], which may also contribute to protecting liver function and decreasing the risk of recurrence in the context of HCC. Currently, the combination of pegylated interferon-α and ribavirin is the recommended treatment for chronic hepatitis C; however, this therapy has frequent and numerous side effects, especially for those in the decompensation stage of liver cirrhosis and HCC [5]. The introduction of novel nucleotide analogues such as sofosbuvir has brought about a new treatment era for HCV patients; however, these drugs remain very expensive and do not appear to be suitable for hepatitis C patients with HCC [6, 7]. Thus, at present, there is essentially no effective drug available for HCC patients with HCV for controlling virus replication. Furthermore, few studies focused on the relationship between the HCV load and outcome of HCC patients partly due to limited option of drugs suitable for hepatitis C virus control in HCC, although it is well confirmed that control of HBV contributes to decreasing recurrence of HCC.

The intrahepatic immune system is likely to play a key role in determining the outcome of HCV infection, because of its potential for viral clearance [8]. The hepatic lymphocyte repertoire is characterized by high CD8/CD4 T cell ratios and large numbers of gamma delta T ($\gamma\delta$T) cells and natural killer (NK) cells. Persistence of HCV is generally considered to be due to qualitative and/or quantitative inadequacies in these cells, which influences the immune response [9]. Many studies have also demonstrated impaired T cell activity in HCV-infected patients, and viral persistence has been attributed to defective T cell immunity [8]. Recently, the role of innate immunity in determining the outcome of HCV infection and in regulating and maintaining specific immune responses has received increasing attention [10, 11]. For example, Corado et al. [12] showed that spontaneous NK cytotoxicity was fourfold lower in HCV patients than in controls and suggested that altered NK cell function may be a significant contributing factor to the chronicity of HCV infection. In fact, the clearance or control of HCV depends on the synergistic effect of various immunocytes.

Most of the previous work on immunity against HCV has focused on the generation of hepatic HCV-specific T cell lines *in vitro* [13]; however, recent studies have highlighted essential roles for NK, $\gamma\delta$T, and cytokine-induced killer (CIK) cells. These cells not only show direct antitumor or antivirus effects but are also required for the optimal priming and cytotoxic function of specific T cells [14–16]. In addition to the similar antitumor and antivirus effects of these three kinds of innate immune cells, they also have synergistic effects and are influenced differently by the intrahepatic environment and HCV virus. For example, intrahepatic $\gamma\delta$T and T cell activation could be directly induced by the HCV/E2 particle through CD81 triggering. By contrast, NK cells might be inhibited by the HCV/E2 particle [17]. Therefore, this combination could be useful to overcome immune resistance in various aspects.

Indeed, in a preliminary study, we observed that the hepatitis C viral load declined in three patients diagnosed with HCC that received a combination of cellular immunotherapy (CIT) with NK, $\gamma\delta$T, and CIK cells along with conventional radiofrequency ablation (RFA) for HCC [18]. Although liver dysfunction was not observed in our previous study and autologous CIT have been reported to be well-tolerant, the potential side effect merits consideration. Therefore, in this retrospective study, we investigated whether the combination of NK, $\gamma\delta$T, and CIK cells might inhibit HCV replication in HCC patients and the specific effects of this treatment on progression-free survival (PFS). In addition, we carefully monitored the change in liver function and other events after infusion.

2. Materials and Methods

2.1. Patients and Study Design. The HCC patients infected with HCV that were hospitalized from January 2010 to March 2015 in the Cancer Center of the First Hospital of Jilin University were retrospectively analyzed in the study. The inclusion criteria were as follows: (1) diagnosis of HCC by biopsy/imaging; (2) presence of HCV infection confirmed by Real-Time PCR Hepatitis C Virus RNA Diagnostic Kit; (3) patients with newly diagnosed or recurrent HCC; (4) Eastern Cooperative Oncology Group (ECOG) performance status ≥ 3; (5) obtaining complete remission (CR), partial remission (PR), or stable disease (SD) after conventional therapy (surgery, RFA, transcatheter arterial chemoembolization [TACE]); (6) giving CIT after conventional therapy and within three months, in case of patients receiving CIT. The exclusion criteria were as follows: (1) interferon and/or ribavirin use during the study period; (2) concomitant infection with human immunodeficiency virus or HBV; (3) use of sorafenib (see Figure 1). Patients were divided into the study group (CIT combined with conventional therapy) and control group (conventional therapy alone).

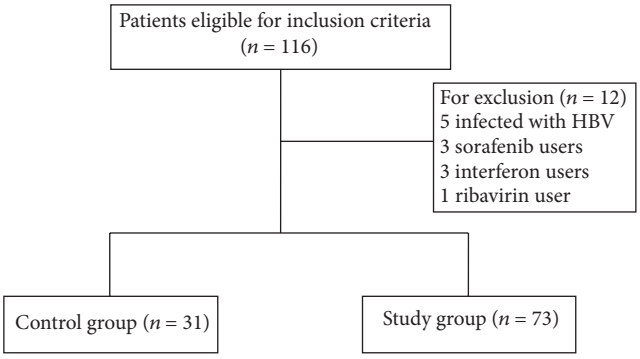

Figure 1: Flowchart for inclusion and exclusion details.

The baseline characteristics, change of viral load, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were collected and compared between groups. We defined AST1/ALT1 as the first point of detection before treatment and AST2/ALT2 as the second point of detection after treatment. Thus, the change of liver function was measured according to deviation in these values (denoted as "AST2-ASL1" or "ALT2-ALT1"). PFS was defined from the time of conventional therapy (RFA, TACE, or surgery) to progression. The data of viral load and ALT/AST used for comparison were selected from the measurements taken close to these two points of time used for PFS determination. The primary and secondary endpoints of this study were viral load change and PFS, respectively.

The combination of conventional treatment and CIT for HCC patients was an observational study in our hospital, and the study design was previously reviewed and approved by the Ethics Committee of the First Hospital of Jilin University, and informed consent was obtained from each patient.

2.2. Immunocytes Preparation and Infusion. Autologous peripheral blood mononuclear cells (PBMCs) (1–1.5 × 10^9 cells) were obtained from HCC patients by apheresis using COBE SPECTRA™ (Gambro BCT, Inc., Lakewood, CO) (D0). After collection, PBMCs were split into two 50 mL centrifuge tubes that were spun for 10 min at 3000 rpm. The supernatant was discarded, and the cell pellets were resuspended in 30 mL of phosphate buffered saline (PBS) and placed on top of a 15 mL Hypaque (Amersham Biosciences) in a 50 mL sterile tube.

Lymphocytes were isolated from PBMCs by means of Ficoll-Hypaque density centrifugation (Ficoll separation) to yield ~1.5×10^9 ($1.1–1.8 \times 10^9$) cells and were then separated into three pools to induce NK, $\gamma\delta$T, and CIK cells through the use of different cytokines as previously described [18]. All procedures for preparing the autologous immune cells were carried out under good manufacturing practice conditions, approved by the Jilin Provincial Center for Sanitation Inspection and Test (China, certificate ID A20090047).

Before administration, immunocytes stained with specific fluorescence-conjugated monoclonal antibodies (mAbs) (BD Biosciences, San Diego, CA, USA) were identified via four-color flow cytometry performed on a FACSCalibur system (BD Biosciences, San Diego, CA, USA) [18]. NK cells were then incubated for 15 min with CD3-PerCP, CD69-PE, and CD56-APC. CIK cells were then incubated with CD3-PerCP, CD4-FITC, CD8-PE, and CD56-APC. Finally, $\gamma\delta$T cells were incubated with Vγ9-FITC and CD3-APC.

The release criteria for the cultured cells were as follows: (i) cell viability > 90%; (ii) no contamination of mycoplasma, endotoxin, bacteria, or fungi, as determined by PCR, performed 24 h before and on the day of product release; (iii) the number of the cells around 1-2 $\times 10^9$ per infusion; (iv) the percentage of NK and $\gamma\delta$T cells each >50%, as detected by flow cytometry.

The initial transfusion began 14 days after apheresis. One course of CIT was accomplished during D14–D17 after the apheresis, including infusion of NK, CIK, and $\gamma\delta$T cells. Each patient received 8 infusions (2 infusions per day) in one course of CIT.

2.3. HCV RNA Determination. The HCV viral load in serum was detected by the Real-Time PCR Hepatitis C Virus RNA Diagnostic Kit (Shanghai Haoyuan Biotech Co, Ltd.). The change in viral load was measured by subtracting the before-treatment value from the after-treatment value, with the HCV RNA values transformed to the log scale. For further statistical comparison, patients were classified into different categories according to the degree of change in HCV RNA as follows: increase over 1 log, increase between 0.5 and 1 log, increase between 0 and 0.5 log, decrease over 1 log, decrease between 0.5 and 1 log, and decrease between 0 and 0.5 log. If the change was between 0 and 0.5 log in either direction, the viral load was regarded as stable. Otherwise, the change was regarded as a meaningful change, that is, representing an actual decrease or increase.

2.4. Adverse Events. We evaluated the adverse events by monitoring and talking to patients during the infusion. In general, previous reports have shown that transfusion of immune cells may cause fever, rash, or arthralgia. Thus, the evaluation included all of the aforementioned symptoms in addition to any other main complaints from the patients. Given that immunocytes may suppress HCV replication through killing infected hepatocytes, which could lead to the release of hepatic transaminase, ALT and AST levels were detected before and after CIT. The adverse events were evaluated according to Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Table 1: The baseline characteristics of patients.

Characteristics	Control group *n* (%)	Study group *n* (%)	χ^2	*p* value
Age (y)			0.029	0.866
≤65	39 (53%)	16 (52%)		
>65	34	15		
Gender (M/F)			0.006	0.937
Male	43 (59%)	18 (58%)		
Female	30	13		
BCLC staging			4.964	0.174
0	10	2		
A	42 (58%)	14 (45%)		
B	17	10		
C	4	5		
Conventional therapy			4.935	0.085
RFA	57 (78%)	24 (77%)		
TACE	6	6		
Surgery	10	1		

2.5. Statistical Analysis. The chi-square test was used to compare baseline clinical characteristics, and the nonparametric Mann-Whitney test was used for comparing the difference in HCV RNA and ALT/AST levels in the two groups. The Spearman test was used to explore the correlation between a change in viral load and PFS. PFS analysis was conducted using the Kaplan-Meier and Cox proportional hazards models (SPSS 17.0, Chicago, IL). A two-tailed *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Clinical Characteristics of the Patients. The general clinical characteristics of the patients are summarized in Table 1. A total of 104 eligible patients were included in the study: 73 in the control group and 31 in study group. The records of the viral loads for 8 patients were incomplete (3 in the control group, 5 in the study group); thus, these data were regarded as missing values in the statistical analysis.

The median age was 65 years (range, 46–82). The majority of the patients were male (61/104, 59%), with stage A HCC (56/104, 54%) according to Barcelona Clinic Liver Cancer (BCLC) staging system. The conventional therapies were mainly determined according to the Chinese Guideline on HCC published in 2009. For all patients, routine clinical examinations and evaluations were completed within 4 days after hospitalization. The strategy of treatment was determined by multiple disciplinary team (MDT) in our hospital. Usually, RFA therapy was used for patients who had single or two tumors less than 3 cm diameter without blood vessel invasion and metastasis. Patients who had tumors less than 4 and tumor diameter less than 5 cm with or without vessel invasion (most with classification A and partly with BCLC stage B) received surgery. TACE treatment was chosen for patients who had more progressive stage with the Child-Pugh

Table 2: The comparison of baseline and the change of ASL/ALT level.

	Control group (IU/mL) median (range)	Study group (IU/mL) median (range)	p value
AST1	56 (20–313)	49 (21–102)	0.215
ALT1	56 (15–232)	46 (14–176)	0.087
AST2-AST1	3 (−264–283)	−3 (−43–42)	0.139
ALT2-ALT1	0 (−211–376)	−3 (−94–81)	0.443

Note: AST1/ALT1 as the first point of detection before treatment and AST2/ALT2 as the second point of detection after treatment.
The change of liver function was measured according to deviation in these values (denoted as "AST2-ASL1" or "ALT2-ALT1"). ALT: alanine aminotransferase; AST: aspartate aminotransferase.

score A or B. Doxorubicin Hydrochloride (20–50 mg) and Lipiodol (5–10 mL) were used for TACE treatment.

The baseline characteristics, including age, gender, BCLC stage, and initial therapy, were well-balanced between the study and control groups, with no significant differences according to the chi-square test, indicating the suitability of these groups for further analysis (Table 1). In addition, for the study and control group, the median AST level was 49 IU/mL (range, 21–102 IU/mL) and 56 IU/mL (range, 20–313 IU/mL), indicating no significant difference ($p = 0.215$) (Table 2). For the study group, the median number of CIT courses was 3 (range, 1–11).

3.2. Quality of Cultured Immunocytes. The immunocytes were induced and expanded successfully in all patients. The viability of the immunocytes was found to exceed 95%. Mycoplasma, endotoxin, bacteria, and fungi were detected to confirm no contamination. The percentages of CIK (CD56+CD3+), NK (CD56+CD3+), and $\gamma\delta$T (Vγ9+) cells before and after induction were 4.39% (1.5–8%) versus 46.32% (27–50%), 10.35% (5.1–12.6%) versus 95.28% (70.1–99.6%), and 4.72% (2.61–11.2%) versus 90.64% (60.5–97.9%), respectively. Representative results from one patient in the study group are shown in Figure 2. The cytotoxicity of expanded immunocytes to cancer cell line (HepG2) was detected by LDH release assay *in vitro* as described in our previous study [18].

3.3. Decline in Viral Load. In the study group, 4 of 28 patients showed a decrease in viral load greater than 1 log, whereas no patient had an increase greater than 1 log. By contrast, in the control group, 5 of 68 patients showed a decrease in HCV load greater than 1 log, while 9 patients showed an increase greater than 1 log (Table 3). Thus, considering a change of ±0.5 log as stable, overall, 17/68 (25%) of the patients showed an increase in viral load, 37/68 (54%) were stable, and 14/68 (21%) showed a decrease in the control group. In contrast, no patient in the study group showed an increase in viral load, 20/28 (71%) were stable, and 8/28 (29%) showed a decrease (Table 4). There was a significant difference between the two groups according to the nonparametric Mann-Whitney test ($p = 0.014$).

Furthermore, we considered the possibility that there might be a difference in the reaction to immunotherapy between sexes, given that gender has been shown to be significant predictive factor of the efficacy of interferon treatment in chronic hepatitis C patients. Therefore, we also compared the change in viral load between male and female patients in the study group. Among the patients who showed a viral load decrease in the study group, 5/13 (38.4%) were female and only 3/18 (16.7%) were male ($p = 0.028$).

3.4. Prolongation of PFS. The deadline of follow-up was November 2015 and the median follow-up duration was 10 months (1–42 months), with 18 censored data entries (5 in the study group and 13 in the control group). The study group showed better PFS, with a median PFS of 16 months compared to 10 months for the control group ($p = 0.004$; Figure 3). Using the log-rank statistical test (Mantel-Cox) regression model, with CIT and staging as individual predictors of PFS, only CIT emerged as an independent prognostic factor for PFS (hazard ratio, 0.422; $p = 0.005$). We also evaluated the relationship between viral load change and PFS. The change of viral load for patients receiving CIT therapy was assigned to "increase," "stable," and "decrease" subgroups as defined previously. There was no significant correlation between viral load change and PFS using the Spearman test ($p = 0.453$).

3.5. Adverse Events. About half of patients underwent mild adverse events (grades 1-2) of ALT and AST in both groups, with mainly distribution of grade 1 (about 40%). Only 2.7% patients had grade 3 adverse events in control group when evaluating AST. It appears to be no obvious difference in the two groups. More detailed data were shown in Table 5. For study and control group, the median ALT level after treatment was 46 IU/mL (range, 14–176 IU/mL) and 56 IU/mL (range, 15–232 IU/mL), respectively, which was not significantly different ($p = 0.087$), respectively. There were no significant differences in the change of AST (median change −3 versus 3, $p = 0.139$) and ALT (median change −3 versus 0, $p = 0.443$) between the two groups (Table 2).

Only three patients (3/31, 9.7%) developed moderate fever after infusion, with temperature ranging from 38.2 to 38.5°C (grade 1), and they all recovered within one hour after receiving oral nonsteroidal anti-inflammatory drugs.

4. Discussion

Simultaneous infection of chronic hepatitis C increases the rate of progression and imposes a great challenge for the treatment of patients with HCC. Chronic HCV infection attenuates both the innate and adaptive immune responses, thereby reducing the likelihood of viral clearance as well as the degree of immune-mediated liver injury to allow for coexistence of both the virus and host. HCV thus outpaces the rate of host immune responses and its elimination requires activation of all aspects of immunity. Furthermore, interaction between immune cells is also critical for the control of cancer *in vivo* as well, so that it will have more chance to

Table 3: The effects of CIT on levels of virus load in HCC.

	Decrease		Stable		Increase	
	↓>1 log	↓0.5–1 log	↓0–0.5 log	↑0–0.5 log	↑0.5–1 log	↑1 log
Control group (n = 68)	5 (7%)	9 (13%)	18 (26%)	19 (28%)	8 (12%)	9 (13%)
Study group (n = 28)	4 (14%)	4 (14%)	9 (32%)	11 (39%)	0 (0%)	0 (0%)

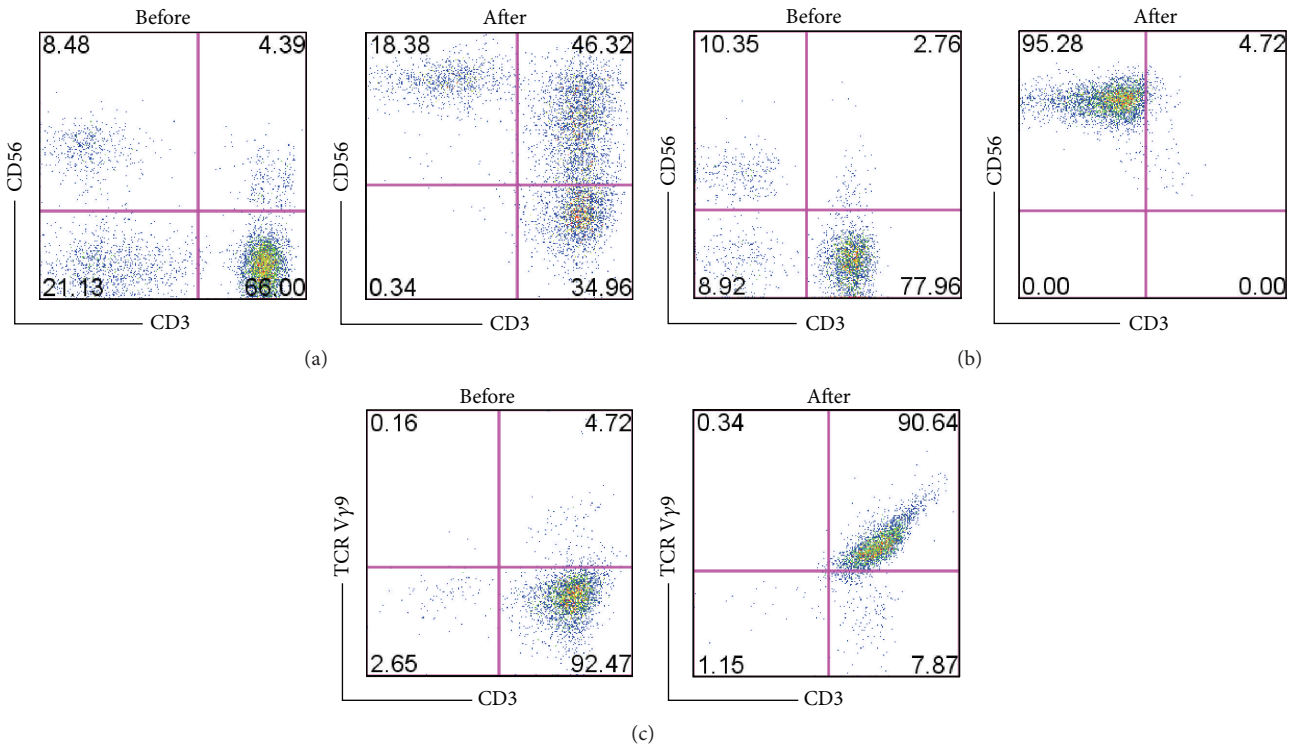

Figure 2: (a) The percentage of CIK cells including (CD3+ CD56+), (CD3+ CD56−), and (CD3− CD56+) before and after induction and CD4+ and CD8+ before and after induction in one of the patients. (b) The percentage of NK cells (CD3+ CD56−) before and after induction and the activated NK (CD56+ CD69+) before and after induction in one of the patients. (c) The percentage of $\gamma\delta$T before and after induction in one of the patients.

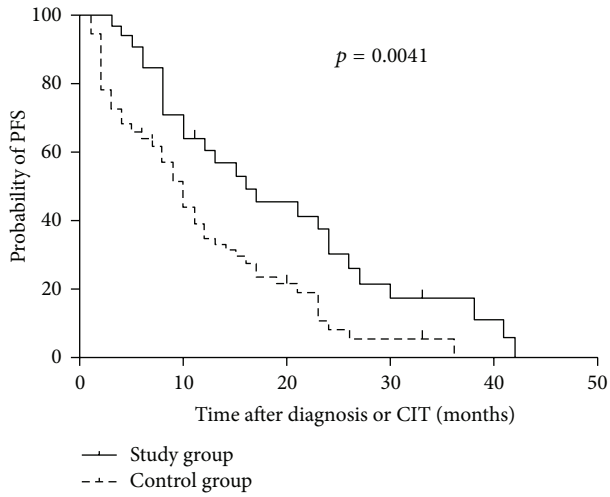

Figure 3: Progression-free survival (PFS) in study and control group. The median PFS in study (n = 31) and control group (n = 73) was 16 and 10 months, respectively (p = 0.0041).

Table 4: The effects of CIT on distribution of virus load in HCC.

	Decrease	Stable	Increase	χ^2	p value
Control group (n = 68)	14 (21%)	37 (54%)	17 (25%)	8.519	0.014
Study group (n = 28)	8 (29%)	20 (71%)	0 (0%)		

Table 5: The distribution of adverse events of ASL2/ALT2 in the two groups.

	Grade 1	Grade 2	Grade 3
AST2 in control (n = 73)	29 (39.7%)	14 (19.2%)	2 (2.7%)
AST2 in study (n = 31)	15 (48.4%)	3 (9.6%)	0 (0.0%)
ALT2 in control (n = 73)	30 (41.1%)	9 (12.3%)	0 (0.0%)
ALT2 in study (n = 31)	8 (25.8%)	2 (6.5%)	0 (0.0%)

progress in a patient with an immune system compromised by HCV. Therefore, it is a rational and novel ideal for application of multiple kinds of immunocytes rather than single kind of immunocytes. The results of this retrospective study also showed the significance of this regimen over previous studies on CIT with single type of cells. Previous study showed that CIT with CIK cells alone could prolong PFS in HCC patients [19] but failed to decrease the virus load [20]. Our study showed not only survival benefit of the CIT with three kinds of immunocytes, but also its function for virus control, owing to the synergistic potential of these immune cell types.

In order to control confounding factors to examine the antivirus effect of CIT alone as much as possible, we excluded patients that were coinfected with HBV and HIV because of their potential interaction and defective immunity. Sorafenib users were also excluded because of its evident antitumor and possible anti-HCV effect [21, 22]. Thus, CIT was the sole intervention for viral replication in our study. To evaluate the actual change in viral load for each patient, we adopted the definition of viral load change criteria in other studies. That is, the definition of virus load change in responder patients is defined as experiencing more than a 0.5 log viral load reduction [23]. Minor change and no changes are defined as 0.5–1.0 log reduction and <0.5 log reduction [24]. In our study, we also considered that a change of over 0.5 log was meaningful, since the normal error range is ±0.45 log according to the instruction of the Hepatitis C Virus RNA Diagnostic Kit (Real-Time PCR). The overall viral load decreased in the range of 0.5–2 log, showing good potential of this regimen, since CIT could prevent the increase of viral load during the course of disease in HCC patients with HCV who have no other option for virus control. At the same time, the ideal end-point of HCV treatment is to acquire a sustained virological response. Therefore, there is a relatively big gap of this standard comparing with the ideal end-point. Nevertheless, the moderate virus clearance effects observed suggest that an intrahepatic resistance environment also plays an important role in the performance of CIT.

To further determine the clinical significance of moderate suppression of HCV replication through CIT, such as decreased recurrence of cancer or prolonged survival, survival curves were plotted, and the study group showed better PFS. Previous studies have also shown that CIK cells alone can prolong PFS or overall survival for HCC directly through its antitumor effect [25–28]. We speculate that CIT might have a double impact on virus replication and provide a direct PFS benefit, given that the decrease of virus titer does not appear to contribute to the prolongation of PFS. However, this effect might be due to the insufficient decline in viral load for effective comparison or small sample size; therefore, this association warrants further study.

Our present strategy also showed good efficacy when comparing with other immunotherapies, such as vaccines, and use of immunomodulatory antibodies. A phase I clinical trial using a vaccine in which monocyte-derived dendritic cells were loaded and activated *ex vivo* with lipopeptides also failed to influence viral load [29]. This suggested that some patients may have a reaction to current vaccines and the antivirus effect is still limited. The programmed cell death-1 (PD-1) pathway plays an important role in T cell exhaustion and dysfunction; thus the PD-1 antibody was tested as a candidate immunomodulatory antibody in chimpanzees with HCV. A significant reduction in HCV viremia was observed in one of three treated animals. However, viremia rebounded in the responder animals when the antibody treatment was discontinued [30]. This study suggests that although a clear antivirus effect was observed, only a subsample of subjects might show a reaction, and the effect is transient. According to our results, the combination of CIT showed a moderate and long-lasting antivirus effect and also showed antitumor potential.

We also tried to explore factors that may have an influence on treatment efficacy and found the tendency of female patients achieving better virus control though no significant difference was obtained. In the group showing a decrease of HCV RNA, 5 of 8 patients were female. Although given the small sample size this finding cannot lead to a definitive conclusion, the general trend is certainly worthy of further exploration. It is well established that females usually have a good response to interferon-γ (IFN-γ) treatment, which may imply that CIT could function via IFN-γ. One female patient (72 years) who received 11 courses of CIT showed a decline in HCV RNA levels of over 2 log (from 2.3×10^7 to 1.97×10^5) within 34 months. This may suggest that the number of treatment courses could have a positive effect on the degree of HCV decline. However, the effect of HCV genotype on CIT response remains to be determined, because different genotypes show various responses to interferon. For example, IFN-γ is effective in approximately half of patients chronically infected with genotypes 2 and 3 but is much less effective in patients infected with genotypes 1 and 4 [31, 32].

Liver injury secondary to HCV infection is considered to be immune-mediated and not to result from the direct cytopathic effects of the virus [33]. Thus, efforts to enhance host antiviral immunity may theoretically act to promote liver injury. However, in our study, the liver function was found

to be stable after CIT, suggesting that it may not be directly involved in liver damage.

Transient fever was the only severe side effect observed in patients, which was readily relieved by nonsteroidal anti-inflammatory drugs. In fact, our results showed that immunotherapy may ameliorate some symptoms: patients reported an increased appetite, improved sleep, gained body weight, and pain relief.

Taken together, our data demonstrate that a combination of innate immune cells could suppress virus replication and preserve the liver function of HCC patients with HCV infection. Simultaneously, the immune cells could perform their antitumor function and prolong the PFS of these patients. Thus, this study provides evidence that the CIT is safe and effective in the treatment of HCC with HCV, highlighting the importance and need to perform a prospective study for this treatment.

Conflict of Interests

The authors declare that there is no conflict of interests related to publishing this paper.

Acknowledgments

This work was supported by Jilin Provincial Science and Technology Department Grant (20111807, 20140414014GH, and 20150101176), the Key Project of Science and Technology Research of the Ministry of Education (311015), the Bethune Program B (2012202) of the Jilin University, the Platform Construction Project of Development and Reform Commission of Jilin Province (2014N147), National Major Scientific and Technological Special Project (2013ZX09102032), and National Major Scientific and Technological Special Project (no. 2013ZX10004609-004).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2016, Article ID 3850839, 11 pages
http://dx.doi.org/10.1155/2016/3850839

Review Article

Chimeric Antigen Receptor-Modified T Cells for Solid Tumors: Challenges and Prospects

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Received 20 October 2015; Revised 18 December 2015; Accepted 28 January 2016

Academic Editor: Kurt Blaser

Recent studies have highlighted the successes of chimeric antigen receptor-modified T- (CART-) cell-based therapy for B-cell malignancies, and early phase clinical trials have been launched in recent years. The few published clinical studies of CART cells in solid tumors have addressed safety and feasibility, but the clinical outcome data are limited. Although antitumor effects were confirmed *in vitro* and in animal models, CART-cell-based therapy still faces several challenges when directed towards solid tumors, and it has been difficult to achieve the desired outcomes in clinical practice. Many studies have struggled to improve the clinical responses to and benefits of CART-cell treatment of solid tumors. In this review, the status quo of CART cells and their clinical applications for solid tumors will be summarized first. Importantly, we will suggest improvements that could increase the therapeutic effectiveness of CART cells for solid tumors and their future clinical applications. These interventions will make treatment with CART cells an effective and routine therapy for solid tumors.

1. Introduction

Recently, chimeric antigen receptor-modified T- (CART-) cell-based therapy, an innovative approach to tumor treatment, was demonstrated to potentially exhibit MHC-independent antitumor effects. These cells could directly recognize tumor cells by genetic modification to express a chimeric antigen receptor (CAR), and they were activated to exhibit a durable persistence *in vivo* through the T-cell activation endodomain with costimulatory signaling molecules [1, 2]. After two decades of preclinical research and clinical trials, the safety and feasibility of CART-cell-based therapy have been confirmed, and unprecedented clinical results have been obtained in hematological malignancies [3–5]. For example, several groups have reported clinical trials with anti-CD19 CART cells in which favorable clinical efficacy resulted from the specific recognition and eradication of CD19-positive tumor cells [3, 4, 6]. These clinical studies indicate that CART-cell therapy can produce clinical responses in patients with advanced hematological malignancies.

The clinical studies of CART cells for solid tumors have begun recently. Up to date, eleven studies of CART-cell therapy for solid tumors have been conducted in the past decade (Table 1), and thirty-five clinical trials for various solid tumors are listed at ClinicalTrials.gov (http://www.clinicaltrials.gov) (Figure 1). The registered numbers of clinical trials increase annually, and a range of tumor antigens, including CEA, mesothelin, HER2, and GD2, are being targeted for various solid tumors.

In preclinical studies, antitumor efficacy of CART cells has been confirmed *in vitro* and in animal experiments; however, the clinical outcomes in recent studies of CART cells treating solid tumors remain marginal, even though the safety and feasibility have been established [7–9]. Recently, several studies have attempted to search efficient approaches to improve the effectiveness of CART cells for solid tumors. In this review, we discuss the main challenges that impede the development of favorable clinical responses in solid tumors, and we suggest improvements for future clinical applications of CART cells.

2. A Concise History of the Clinical Applications of CART Cells in Solid Tumors

CAR redirected T-cell-based therapy has emerged as a promising strategy for malignant diseases since the first

Table 1: Recent published clinical studies on CART cells specific for solid tumor antigens.

Antigen	CAR	Gene transfer	Cancer	Case number	Clinical outcome	Time	Reference
HER2	ScFv-CD28-CD3ζ	Retrovirus	HER2-positive sarcoma	19	1 PR, 4 SD	2015	[15]
CEA	ScFv-CD28-CD3ζ	Retrovirus	CEA+ liver metastases	8	1 SD, 5 DOD	2015	[17]
Mesothelin	ScFv-4-1BB-TCRζ	Electrotransfer	Mesothelioma	2	1 PR, 1 SD	2014	[14]
Mesothelin	ScFv-4-1BB-TCRζ	Electrotransfer	Mesothelioma	1	1 PR	2013	[16]
CAIX	ScFv-FcεRIγ	Retrovirus	CAIX+ metastatic RCC	12	NED	2013	[8]
GD2	ScFv-CD3ζ	Retrovirus	Neuroblastoma	19	3 CR, 1 PR	2011	[5]
ERBB2*	ScFv-CD28-4-1BB-CD3ζ	Gamma-retrovirus	Colon cancer	1	Dead	2010	[13]
GD2	ScFv-CD3ζ	Retrovirus	Neuroblastoma	11	1 CR, 2 SD, 2 tumor necrosis	2008	[12]
CD171	ScFv-CD3ζ	Electrotransfer	Neuroblastoma	10	1 PR	2007	[9]
FR	ScFv-FcεRIγ	Retrovirus	Ovarian cancer	8	NED	2006	[11]
CAIX	ScFv-FcεRIγ	Retrovirus	CAIX+ metastatic RCC	3	NED	2006	[7]

CAIX: carboxy-anhydrase-IX; CEA: carcinoembryonic antigen; CR: complete response; DOD: dead of disease; FR: folate receptor; HER2: human epidermal growth factor receptor 2; NED: no evidence of disease; PR: partial response; RCC: renal cell carcinoma; ScFv: single chain fragment of variable region antibody; SD: stable disease.
*HER2/neu.

report by Gross et al. in 1989 [10]. In the past two decades, several studies have demonstrated encouraging clinical outcomes in patients with B-cell malignancies that are treated by CART cells, and the results from these studies indicated that CART cells could produce clinical responses in other types of cancer [3, 4, 6]. Theoretically, CART-cell therapy could be curative for solid tumors if the genetically modified T cells encountered the tumor cells *in vivo*. Accordingly, the development of CART cells for solid tumors is imperative in the clinic. Nevertheless, there are few reports of successful clinical studies of solid tumors that are treated with CART cells.

Thus far, CART-cell-based therapy has been tested against several types of solid tumors, including ovarian cancer, neuroblastoma, colon cancer, and mesothelioma (Table 1) [11–14]. In the first clinical study, three patients with metastatic renal cell carcinoma who were administered CART cells specific for CAIX developed liver toxicity [7]. And a further trial of 12 patients treated with anti-CAIX CART cells is still ongoing to assess the safety of the cells [8]. Further initial reports demonstrated encouraging outcomes in 30 patients with neuroblastoma treated with CART-GD2 cells [5, 12]. A clinical study of neuroblastoma from another center used CD171-specific CART cells and indicated some evidence of antitumor efficacy [9]. Importantly, these studies show that CART-cell therapy is safe for patients with advanced solid tumors, but the use of first-generation CART cells and their limited survival may account for the lack of a spectacular clinical response.

To enhance the persistence of CART cells and improve the clinical outcome in solid tumors, costimulators, such as CD28, 4-1BB, and OX40, were integrated into the fusion CAR protein [13, 15, 16]. In one case report, a patient with colon cancer that metastasized to the lungs and liver, who received conditioning lymphodepletion and was treated with 10^{10} third-generation ERBB2-specific CD28.4-1BB.ζ-CART cells combined with IL-2, developed acute respiratory distress syndrome and died five days after the treatment [13]. In another study, sarcoma patients treated with up to $10^8/m^2$ second-generation CART cells encoding a HER2.CD28.ζ-CAR without conditioning chemotherapy or administration of IL-2 experienced no toxicity, but the antitumor effect was limited [15]. Several other clinical studies with CEA- and mesothelin-specific second-generation CART cells for solid tumors have been reported recently, and the safety and efficacy of this cell-based therapy have been confirmed [14, 16, 17].

Taken together, the clinical experience with CART-cell therapy for solid tumors suggests that several factors, including the tumor antigens, costimulatory molecules, CART-cell development process, and conditioning therapies, likely contributed to the different clinical outcomes. Thus, several urgent issues need to be resolved to improve the safety and clinical responses of CART cells for patients with solid tumors.

3. Potential Challenges for CART-Cell Treatment of Solid Tumors in the Clinic

Although CART-cell-based therapy has been shown to be a potential treatment strategy for few solid tumors [14, 15, 17], the challenges to this strategy that affect safety and clinical outcomes should be addressed. The current critical issues are discussed in the following.

3.1. The Screening of Solid Tumor Target Antigens. Preclinical studies on CART cells that are specific for many different tumor antigens expressed on solid tumors have been conducted and have shown antitumor effects [18, 19]. To date, numerous potential solid tumor target antigens have been explored for CART-cell-based therapy (Table 2), but unfortunately, few antigens are uniquely specific for solid

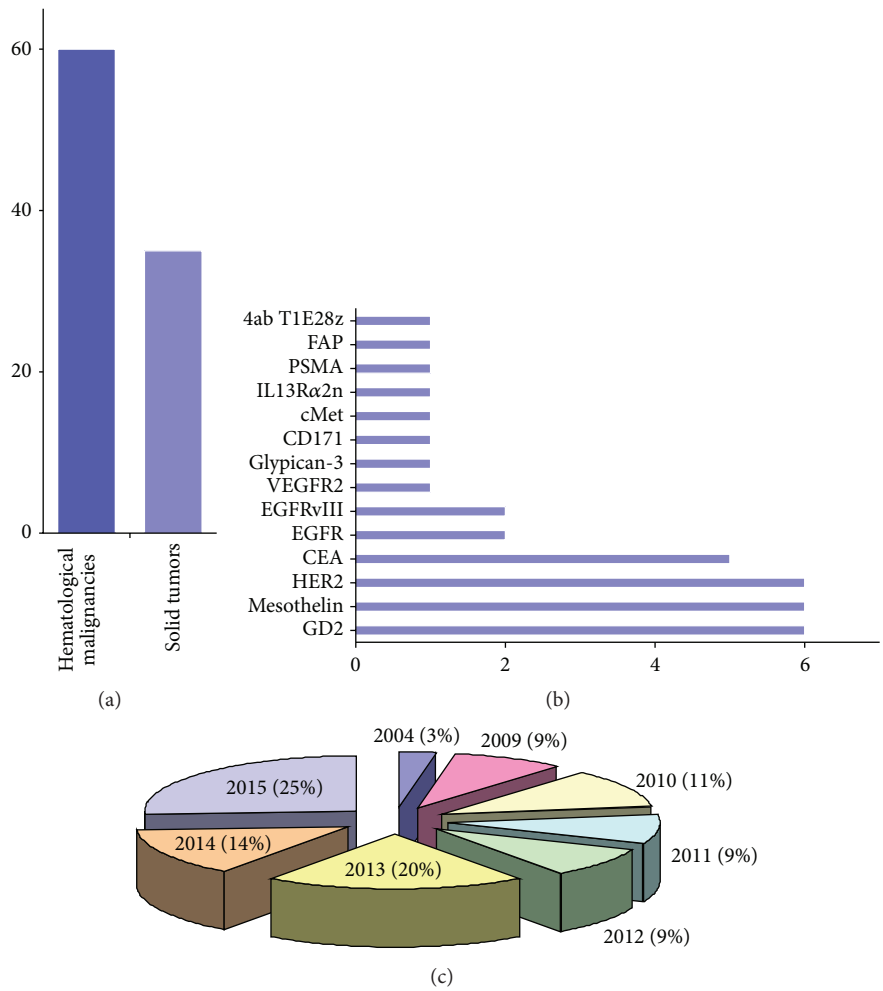

Figure 1: Current status of clinical trials of chimeric antigen receptor-modified T (CART) cells in malignancies. These data were searched on 15 June, 2015, from the website ClinicalTrials.gov (http://www.clinicaltrials.gov). The key phrases "chimeric antigen receptor-modified T cells", "chimeric antigen receptor", "CART", and "CAR" were used. (a) Comparison of the number of registered CART-cell trials for solid tumors and hematological malignancies on the ClinicalTrials.gov website. (b) The registered solid tumor targets for CART cells on the ClinicalTrials.gov website. EGFR: epidermal growth factor receptor; FAP: fibroblast activation protein; PSMA: prostate-specific membrane antigen; VEGFR2: vascular endothelial growth factor receptor 2. (c) Proportion of annual registered numbers of CART cells in solid tumors on the ClinicalTrials.gov website.

tumors. A major concern of CART cells in solid tumor treatments is ensuring the effective elimination of tumor cells while avoiding the off-tumor/on-target toxicity that caused when these T cells attack healthy tissues. Experience indicates several principles that should be observed to overcome this problem: (1) preferred selection of specific tumor antigens and (2) selection of tumor targets based on their expression level and frequency on tumor and normal tissues [20, 21]. The density of tumor antigen expression can also affect the selection of CART-cell targets [22].

In addition, it is well known that tumor-associated antigens can be divided into two groups, including mutated antigens (also called neoantigens) and "self-" antigens such as tissue/lineage antigens, developmental antigens, and overexpressed antigens [23]. Most of the recent studies have indicated that cancer immunotherapies have remained focused on recognizing "self-" antigens; however, only few immunotherapies target neoantigens [24, 25]. Neoantigens, short 8 to 12 amino acid peptides that are known to be created by cancer cell genomes mutations, can be rapidly identified by high-throughput next-generation sequencing (NGS) in several cancers, including melanoma, ovarian cancer, and cholangiocarcinoma [19–26]. In contrast to "self-" antigens that are expressed on tumor and normal cells, neoantigens are only found in tumor cells, showing accurate specific targets for cancer immunotherapy to reduce the risk for autoimmune disease, for example, a splice variant of the epidermal growth factor receptor (EGFRvIII) [26, 27]. Based on their specific features, recent clinical evidence has confirmed that neoantigens are the best potential targets for adoptive T-cell therapy with least possible toxicity [28]. Therefore, it is reasonable that a strategy using CART cells that specifically target neoantigens is the best potential therapeutic treatment for cancers without severe target-mediated toxicity.

Table 2: Potential solid tumor targets for CART cell-based therapy.

Antigen	Cancer
CD44v7/8	Cervical carcinoma
DNAM-1	Prostate carcinoma
EGP-40	Colorectal cancer
EpCAM	Prostate cancer
FBP	Ovarian cancer
FR	Rhabdomyosarcoma
GD3	Melanoma
VEGFR2	Tumor neovasculature
LMP-1	PVR and nectin-2 expressing solid tumors
MUC1	Breast, ovary
PSCA	Melanoma, synovial cell sarcoma

DNAM-1: DNAX accessory molecule-1; EGP-40: epithelial glycoprotein-40; EpCAM: epithelial cell adhesion molecule; FBP: folate-binding protein; LMP-1: latent membrane protein 1; MUC1: mucin 1; PSCA: prostate stem cell antigen.

3.2. Optimizing the Affinity of the CAR. The affinity of CAR is also important for its antitumor effect and target-mediated toxicity. The relationship among the CAR affinity and density and tumor antigen density could impact the effector function of CART cells. Low affinity was more effective than high-affinity CAR under conditions when the levels of CAR were limiting, whereas no significant difference was observed on the variance of CAR affinity on conditions of high levels of CAR expression [29]. In addition, high-affinity CAR did not increase the activity of T cells against target tumor cells compared with low CAR affinity, and the high-affinity CAR distinguished less well between tumor cells with high or low levels of antigen expression, whereas low affinity CAR showed negligible responses to tumor antigens expressed at low or undetectable levels, but they were highly reactive to the tumor cells that overexpressed antigen [30, 31]. A recent study on the sensitivity of CAR to EGFR density indicated that CAR with reduced affinity could render CART cells able to distinguish tumor from normal tissues, and their antitumor effects were decreased along with the reduced density of EGFR [32]. On the basis of the careful conclusions from previous studies on solid tumors, it is possible to select the reduced affinity of CAR to avoid the off-tumor/on-target toxicity when target antigens are overexpressed on tumor cells and expressed at low levels in normal tissues. However, for highly specific tumor antigens, high-affinity CAR should be considered to prevent tumor escape when tumor cells express a low level of antigens.

3.3. The Source of the Single-Chain Fragment of the Variable Region Antibody (scFv). Most existing studies have derived the scFv components of the chimeric receptor from mouse monoclonal antibodies [11, 33]. Although this construct only contains the variable regions of the mouse monoclonal antibody, a human anti-mouse antibody by the recipient could, after cell infusion, block the interaction between CAR and the target tumor antigen to inhibit the antitumor effect of the CART cells. The use of humanized scFvs or scFvs derived from human monoclonal antibodies for CAR will solve this issue. Advances in biotechnology will expand the prospects for humanized scFvs for CART-cell-based therapy for solid tumors.

3.4. Costimulatory Molecules. To improve the expansion of CART cells in solid tumors, costimulatory molecules, including CD28 and 4-1BB, have also been incorporated in the CAR gene by several groups, with increased persistence *in vivo* [14, 15, 17]. Recent studies indicated that CD28 can accelerate T-cell expansion, leading to T-cell exhaustion and reduced cell persistence compared with the 4-1BB domain [34]. Additionally, it has been reported that 4-1BB is superior to CD28 costimulation because 4-1BB preferentially promotes the expansion of memory T cells, whereas CD28 expands naïve T cells [35]. However, other studies showed that there was no any clear superiority for either CD28- or 4-1BB-based CART cells. For example, no significantly different cytotoxicity *in vitro* and *in vivo* was observed on CART cells with either a CD28 or 4-1BB costimulator, although CD28-based CART cells produced higher IL-2, IL-6, and IFN-gamma levels [36]. Other studies showed that the expansion and antitumor cytotoxicity by CD28- and 4-1BB-based CART cells were similar [37]. In addition, Hombach et al. demonstrated that CD28-CART cells were superior to CD28-OX40-CART cells because the CD28-OX40 super-costimulation increased activation-induced cell death (AICD) and reduced the cells' antitumor function [38]. In contrast, some studies indicated that the CAR gene containing two costimulators, such as CD28 and 4-1BB, yielded improved T-cell survival and cytotoxicity compared with a single co-stimulator [37, 39]. After careful consideration, these studies indicate that the choice of costimulatory molecules affects the therapeutic response, but it remains unclear whether any costimulatory molecule is superior to another [40, 41]. Therefore, more attempts to develop CAR with different costimulatory molecules are urgently needed to further explore the therapeutic outcomes *in vitro* and *in vivo*.

Here, some suggestions for the choice of costimulatory molecules will be delineated for solid tumors. For solid tumors, the migration to the tumor sites is a prerequisite for CART cells to play an antitumor efficacy. Once breaking through the tumor microenvironment and making contact with target cells, CART cells need to undergo rapid expansion to have an antitumor function, while avoiding inhibition by the tumor environment. In clinical trials, for example, CD28 was associated with faster expansion than 4-1BB costimulation, and multiple cycles of infusion could overcome the shorter persistence of CD28-based CART cells in solid tumors.

3.5. The Optimal Processing of T Cells Specific for Solid Tumors. The response of solid tumors to CART cells in clinical studies has been limited [7–9]. These suboptimal outcomes could reflect the use of first-generation CART cells with a low ability to persist. Costimulation by integrating CD28 or 4-1BB into CAR molecules can improve the persistence of CART cells *in vivo* [42–44]. Moreover, the differentiation states (e.g., naïve

T cells) and replicative frequencies of T cells could be key to achieving better clinical outcomes [45–47]. Previous studies have indicated that the stimulus and cytokine environment in the cell culture process can determine the T-cell differentiation state. For example, IL-7, IL-15, and IL-21 could slow T-cell differentiation [48, 49], whereas activation by soluble anti-CD3 and CD28 monoclonal antibodies achieved optimal T-cell differentiation [50]. Activation by soluble anti-CD3 and CD28 monoclonal antibodies in the presence of IL-15 and IL-21 enhanced T cells with a naïve phenotype and with a lower proportion of $CD4^+CD25^+CD127^-$ expression [47].

Trafficking to and accumulating in the tumor sites are prerequisites for CART cells to play an antitumor efficacy, particularly for solid tumors. Nevertheless, CART cells cannot easily contact with target tumor cells due to the tumor microenvironment, resulting in the inability of these infused cells to fully activate and proliferate. T-cell migration to tumor sites requires integrins, chemokines, and chemokine receptors [51, 52]. However, cell culture *in vitro* and genetic modification could cause the loss of chemokine receptors, possibly resulting in CART cells being unable to localize accurately to the tumor tissues [53]. In previous studies, chemokine receptors, such as CXCR2 and CCR4, were genetically modified to be expressed on T cells to enhance their homing and antitumor activity [54, 55]. Therefore, the forced expression of integrins, chemokines, and chemokine receptors on CART cells could improve their migration ability and promote their antitumor activity.

3.6. Preconditioning Therapy. Immunotherapy is a promising and efficient approach to cancer treatment. Basic research and clinical studies indicate that only a fraction of patients achieve durable clinical responses after immunotherapy. The immune system is highly important for maintaining a balance between protection from tumor development and the promotion of tumor growth, whereas tumor cells can escape the immune system leading to cancer progression that is facilitated by the tumor microenvironment when the balance is destroyed [56, 57]. The microenvironment of solid tumors has been reported to interfere with the desired clinical outcome through multiple networks of cellular interactions, which could create immune tolerance and negate immunotherapies, including CART-cell-based therapy. The tumor microenvironment is extremely complex and contributes to tumorigenesis and metastasis by limiting immune responses to cancer cells and preventing the eradication of tumors [58]. Interference with immune cell infiltration, activation, and proliferation in the tumor microenvironment can ultimately facilitate tumor development, metastasis, and resistance to therapy. Therefore, strategies to counteract the tumor microenvironment and to enhance antitumor effects are urgently needed.

Immunosuppressive cells (e.g., regulatory T lymphocytes, Tregs) can be induced to accumulate in tumor site by the tumor microenvironment, playing an essential role in tumorigenesis [59]. Preconditioning therapy to remove Tregs can effectively enhance the antitumor effects of CART cells for solid tumors. Fortunately, chemotherapy can make the tumor microenvironment highly permissive for antitumor immunity [60]. Chemotherapeutic agents, such as cyclophosphamide, docetaxel, and pemetrexed, could impair Treg function and enhance the host's immunity in clinical studies [61–63]. Other strategies have been explored to reduce Tregs. For example, denileukin diftitox, an IL-2-diphtheria toxin fusion protein, directly killed Tregs through selective targeting of CD25 in preclinical cancer models [64]. A high-dose of IL-2 could downregulate the level of Tregs, at least in the periphery [65].

In addition, previous studies demonstrated that lymphodepleting chemotherapy preconditioning could enhance the antitumor efficacy of tumor-infiltrating lymphocytes [66]. Lymphodepletion creates an appropriate "lymphoid space" for the proliferation of adoptive infused immune cells. Additionally, lymphodepleting conditioning can improve the expansion and persistence of CART cells in solid tumor patients.

To the best of our knowledge, radiotherapy commonly induces tumor cell death through cell stress by altering cellular survival, and by apoptosis pathways and cell cycle regulatory mechanisms [67]. However, preclinical studies have also indicated that radiotherapy can make tumor cells more immunogenic by several mechanisms [68–70]. First, radiotherapy can make the tumor microenvironment more susceptible to attack by immune cells [71]. Second, tumor antigen expression is increased after local treatment by radiotherapy [72]. Third, radiotherapy could induce intratumoral dendritic cells expressing chemokines that attract immune cells into tumor sites [73, 74]. Finally, Fas, ICAM-1, and NKG2D ligands were upregulated on tumor cells after radiotherapy [75–77]. Based on this information, radiotherapy could play a role in enhancing adaptive antitumor effects, in addition to promoting the regression of tumors. Therefore, the antisolid tumor effects of CART cells could be enhanced by radiotherapy.

4. Strategy of CART Cells Specific for Tumor Stroma

Immunotherapy aims to improve the clinical antitumor response of cancer patients. Nevertheless, for many immunotherapies, the tumor microenvironment is the major barrier to an antitumor response [78]. Tumor stroma, a composition of the tumor microenvironment, could support tumor growth and resistance to therapy by the following mechanisms [67, 79–84]: (1) blocking therapeutic agents that attack tumor cells; (2) producing growth factors, chemokines, and matrix that could support tumor growth, invasion, and angiogenesis; (3) expressing inhibitory surface molecules such as programmed death-1 ligand (PD-L1) and PD-L2, producing factors to attract Tregs, myeloid-derived suppressor cells, and macrophages, and secreting factors to regulate T-cell functions to create an immunosuppressive milieu to inhibit immune cell function; and (4) the mechanisms of tumorigenesis that are supported by stroma coexisting among a variety of stromal cell types.

The most recent clinical studies see CART cells as attacking tumor cells. However, there can be limitations to the use of

CART cells that are specific for solid tumors as, for example, tumor stroma, which could create bias towards an undesirable clinical response, compared with the considerable success in the treatment of hematologic malignancies. CART cells might not activate and proliferate well due to the tumor stroma inhibiting immune cells from making contact with tumor cells. Therefore, a strategy to disrupt the tumor stroma could improve the antitumor function of immunotherapy. CART cells that are specific for tumor stroma could promote the treatment of a broad spectrum of solid tumors.

To date, four attempts using CART cells that are specific for the fibroblast activation protein (FAP) that is highly expressed in cancer-associated stroma cells have been reported in animal models [85–88]. Antitumor activity was observed after CART-cell administration in these studies, although adverse events, such as on-target toxicity, were also observed because FAP is also expressed on normal tissues, including pancreas, lung, and bone marrow [85, 88]. The on-target/off-tumor toxicity occurred because the scFv that targeted mouse stroma caused the CART cells to attack normal mouse stroma cells [85, 88]. In contrast, other studies employing CART cells derived from human T cells and a scFv that targeted mouse or mouse/human stroma had no adverse events [86, 87]. More importantly, the antitumor efficacy of the endogenous immune cell antitumor response was augmented by the CART-cell infusion; the CART cells lost their antitumor effect in immunodeficient mice [88].

Based on the data from the preclinical studies, CART cells that target tumor stroma could be candidates for solid tumor treatment in the future. However, several issues should be addressed before their clinical application: (1) the selection of the tumor stroma cell antigen; (2) the development of protocols to augment the antitumor effect for CART cells by combination with other immunotherapies, such as CART cells that are specific for tumor cells; and (3) the concern for potential adverse events such as on-target/off-tumor toxicity.

5. Novel Concept of CAR Design for the Precision Treatment in Solid Tumors

Tumor antigens are important to activate CART cells to induce immune activity against tumor cells. Nevertheless, solid tumor cells typically express highly heterogeneous tumor-associated antigens, rendering them able to escape detection by the immune system [89]. Only few antigens are tumor-specific for the treatment of solid tumors using CART cells. Although recent clinical studies indicated that CART cells were safe and feasible for solid tumors, on-target/off-tumor toxicity remains the main concern impacting their clinical application. Accordingly, novel concepts of CAR design for solid tumor precision treatment have been explored to enhance the on-tumor specificity. Recently, several studies indicated that bispecific CAR design could improve the tumor cell specificity and limit the target-mediated toxicity of CART cells. Contrary to conventional CART cells that only target a single antigen, bispecific CART cells can recognize multiple antigens by expressing two CARs on genetically modified T cells. For example, in preclinical models, T cells expressing two CAR molecules specific for PSMA and PMCA specifically targeted prostate cancer cells, and they were only activated in the presence of both antigens, not by either alone [90]. Another concept of bispecific CAR design uses a negative signal to enhance the tumor specificity: in one example a cytotoxic T lymphocyte antigen-4- (CTLA-4-) or programmed death-1- (PD-1-) based antigen-specific inhibitory CAR (iCAR) was designed to preemptively constrain T cells' responses [91]. These T cells selectively limited their cytokine secretion, cytotoxicity, and proliferation in response to normal tissues on which the iCAR was present. Bispecific CART cells, expressing a CAR and an iCAR specific for an antigen present on normal tissues, could avoid a CART-cell-mediated attack on normal tissues, consequently enhancing tumor specificity [91, 92]. In addition, the tandem CAR (TanCAR) design, which is also a bispecific CAR, can recognize each antigen and improve the activation and effective function when it encounters both antigens simultaneously using a single CAR molecule with two antigen recognition moieties that are joined in tandem [93]. The novel concept of CAR design to genetically modify T cells to target multiple tumor antigens could avoid the risk of immune escape [94]. This approach can also protect normal tissues by increasing the tumor specificity of CART cells. Ultimately, we must optimize the testing of bispecific CART cells to ensure their safety and efficacy before their clinical application for solid tumors.

In addition, to reverse on-target/off-tumor toxicity, several attempts to encode suicide genes in CART cells have shown that this adverse event can be irreversibly prevented through the selective destruction of the infused genetically modified T cells [95–97]. The addition of suicide genes to CART cells could ensure their safety for solid tumor treatments, avoiding unwanted and severe adverse events and increasing on-tumor specificity.

The precision treatment for solid tumors is improving more rapidly due to advances in biotechnology development (Figure 2). Recent advances in CART-cell-based therapy are currently being translated from the laboratory to the clinic. Novel concepts of CAR design could ensure the clinical application of CART cells for solid tumors with enhanced tumor specificity. Coupled with individual and diversified interventions (such as chemotherapies and vaccines), the precision of CART cells could provide great promise for the treatment of solid malignant patients in the future.

6. Combinatorial CART-Cell Therapy to Improve Clinical Benefit in Solid Tumors

The ultimate goal of cancer therapy is to be curative, including CART-cell immunotherapy. However, for solid tumors, the microenvironment is the major barrier to treatment with immunotherapy. It is necessary to develop a potent product to prevent the suppressive function of the solid tumor microenvironment to enhance the antitumor activity of CART-cell

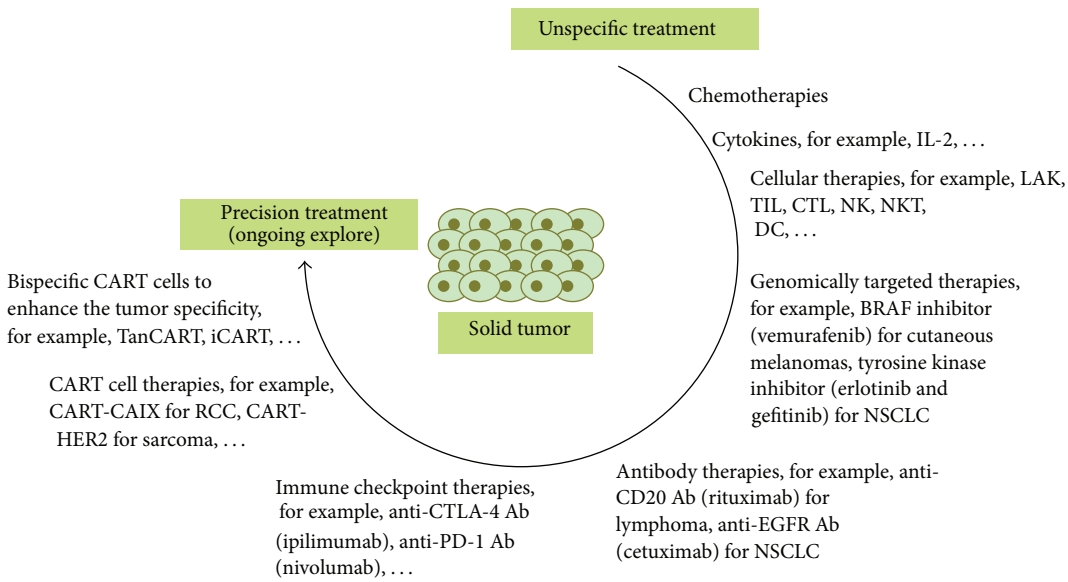

Figure 2: Development of precision treatment for solid tumor. Ab: antibody; CTL: cytotoxic T cells; CTLA-4: cytotoxic T lymphocyte-associated antigen-4; DC: dendritic cells; iCART: inhibitory signal-based antigen-specific CART cells; IL-2: interleukin-2; LAK: lymphokine-activated killer cells; NK: natural killer cells; NKT: natural killer T cells; NSCLC: non-small-cell lung cancer; PD-1: programmed death-1; TanCART: tandem CART cells; TIL: tumor-infiltrating lymphocytes.

therapy. To the best of our knowledge, it is well known that solid tumors can create a complex microenvironment to defend against an attack from the immune system. For example, the antitumor effect of T cells can be inhibited by expressing PD-1 when it interacts with its ligands PD-L1 and PD-L2 that are expressed on tumor cells and/or stroma cells [98, 99]. Most *in vitro* and preclinical data have indicated that the blockade of the interaction between PD-1 and PD-L1 or PD-L2 provides a potentially promising approach for cancer immunotherapy by improving the response of T cells [99, 100]. Several clinical studies of anti-PD-1 monoclonal antibody have demonstrated the safety and activity for patients with advanced solid tumors, such as melanoma, non-small-cell lung cancer, and renal cell cancer [101, 102]. In addition, a phase I clinical study showed evidence for an antitumor effect of anti-PD-L1 antibody against advanced solid tumors [103]. It is promising that two antibodies against PD-1 (pembrolizumab and nivolumab) have been approved by the U.S. Food and Drug Administration in 2014 [58]. Therefore, exploration of CART cells combined with PD-1/PD-L1-specific antibodies is expected to increase the antitumor effect in solid tumors.

Several negative regulators other than PD-1 have been identified and reported to inhibit the response of T cells to attack against tumors, for example, CTLA-4, T-cell immunoglobulin and mucin-containing protein 3 (TIM-3), lymphocyte-activated gene-3 (LAG-3), T-cell immunoreceptor with Ig and ITIM domains (TIGIT), B and T lymphocyte attenuator (BTLA), and V-domain Ig suppressor of T-cell activation (VISTA) [104]. The continued development of CART-cell therapy combined with inhibitors of these negative regulators could improve their clinical benefit in solid tumors.

7. CART Cells as a Primary Strategy for Treating Solid Tumors

Due to economic and medical technological factors, most cancer patients are diagnosed at an advanced disease stage. The strategies for treating patients with advanced solid malignant diseases mainly include surgery, chemotherapy, radiotherapy, targeted therapy, and supportive care, but cancers generally relapse or become refractory, denying patients their best opportunity for treatment. Recent studies indicated that adoptive cell transfer treatments can stimulate and improve the function of the immune system and overcome chemotherapy resistance [105, 106]. Nevertheless, patients are often first treated by traditional approaches rather than by the adoptive transfer of immune cells.

CART-cell treatment as a primary strategy needs to be implemented urgently to increase the therapeutic benefit for patients with solid tumors. Although experience with the adoptive transfer of CART cells to treat solid tumors remains limited, technological improvements will enhance clinical responses in the future. Several tasks should be addressed, including (1) careful screening of patients to ensure that they have the specific tumor target to reduce the risk of on-target/off-tumor adverse event; (2) suggesting CART-cell therapy as a primary strategy for patients and clinical researchers, alone or in combination with other therapies; (3) establishing the benefit of using CART cells as a first treatment; (4) monitoring and resolving the toxicities in these strategies; and (5) analyzing the clinical response compared with other therapies. In addition, to improve the clinical response and standardize the procedures, large-scale, controlled, grouped, and multiple-center clinical trials are of particular importance to implement. On this basis,

the treatment of solid tumors by CART cells as a primary strategy can be extended.

8. Conclusions and Perspectives

Efforts to treat solid tumors with CART cells are ongoing. Considering the recent studies together, treatment with CART cells has been shown to be safe and is thus potential promising for the treatment of solid tumors. However, none of these CART-cell-based strategies has been superior to the existing options, and a number of the challenges and limitations mentioned above must be resolved to ensure better patient benefit and to extend this treatment approach. Based on previous studies, the safety and clinical responses are still the main exploring focuses in the future. CART cells combined with other therapies, such as chemotherapy, radiotherapy, and PD-1/PD-L1 antibodies, will also be relevant. The best clinical responses can be achieved through careful preparation of patients, CART cells and doses, preconditioning regimens, and follow-up treatments. In addition, CART cells will likely be commercialized to increase their convenience and flexibility for patients with solid tumors, or even other malignancies, using streamlined, centralized, and large-scale generation of CART cells from uniform cell sources. These interventions will make treatment with CART cells an effective and routine therapy for solid tumors. In conclusion, although more work is needed to meet the challenges, treatment with CART cells has a significant potential to improve clinical responses in solid tumors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yelei Guo and Yao Wang equally contributed to the work.

Acknowledgments

This work was supported by Nursery Innovation Fund (Grant no. 15KMM50), Science and Technology Planning Project of Beijing City (Grant no. Z151100003915076 to Weidong Han), and National Natural Science Foundation of China (no. 31270820 and no. 81230061 to Weidong Han).

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