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Research Article

Cancer Immunology Research

Validation of Intratumoral T-bet$^+$ Lymphoid Cells as Predictors of Disease-Free Survival in Breast Cancer

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Abstract

We previously observed T-bet$^+$ lymphocytes to be associated with a good prognosis in a cohort of women with familial breast cancer. To validate this finding, we evaluated lymphocyte T-bet expression in an independent unselected prospectively accrued series of women with lymph node–negative breast carcinoma. T-bet and clinicopathologic data were available for 614 women. Hormone receptors, HER2, Ki-67, CK5, EGFR, p53, and T-bet status were determined using IHC and/or biochemical methods. Tumors were assigned to luminal A, luminal B, HER2, and basal subtypes based on the expression of IHC markers. Multiple cutpoints were examined in a univariate penalized Cox model to stratify tumors into T-bet$^{+/high}$ and T-bet$^{-/low}$. Fisher exact test was used to analyze T-bet associations with clinicopathologic variables, IHC markers, and molecular subtype. Survival analyses were by the Cox proportional hazards model. All tests were two sided. A test with a P value < 0.05 was considered statistically significant. T-bet$^{+/high}$ tumor status was significantly associated with large tumor size, high grade, hormone receptor negativity, CK5, EGFR and p53 positivity, high Ki-67, and basal subtype. With a median follow-up of 96.5 months, T-bet$^{-/low}$ tumor status was associated with a reduced disease-free survival compared with T-bet$^{+/high}$ tumor status in multivariate analysis ($P = 0.0027$; relative risk $= 5.62$; 95% confidence intervals, 1.48–50.19). Despite being associated with adverse clinicopathologic characteristics, T-bet$^+$ tumor-infiltrating lymphoid cells are associated with a favorable outcome. This supports their role in Th1-mediated antitumor activity and may provide insight for the development of new therapeutic strategies. *Cancer Immunol Res; 4(1); 41–48. ©2015 AACR.*

Introduction

Breast cancer is a complex and heterogeneous disease with significant disparity in clinical outcomes still being seen, despite improvements in disease classification using tumor-related prognostic markers. More recently, attention has been placed on the components of the tumor microenvironment, including lymphocytic infiltration, whose interaction with the tumor can strongly influence the patient's long-term outcome. The interplay between the immune system and cancer is not straightforward: tumor cells can induce an inflammatory microenvironment that is essential for tumor growth (1); yet, components of the immune system play an essential role in both innate and adaptive immunity and are involved in tumor immune surveillance (2, 3).

Early studies that examined the relationship between lymphocytic infiltration in tumors and outcome in breast cancer showed conflicting results, with some reporting a favorable association with outcome and a dense inflammatory infiltrate (4) and others identifying an adverse association (5). More recent results from large cohorts have demonstrated a good prognosis in patients whose breast cancers showed a marked lymphocytic infiltrate as well as an association with high response rates by such tumors to neoadjuvant therapy (4, 6–12). The apparently contradictory findings may reflect the diverse functional roles of the individual components of lymphocytic subtypes (13). While recognition of the individual immune cell subsets that consistently mediate favorable effects remains elusive, the current consensus is that CD4$^+$ Th1 and CD8$^+$ T cells are among the players that can generate effective although potentially attenuated antitumor responses while CD4$^+$ Th2 cells and CD4$^+$ Tregs are among the cells that can suppress antitumor immunity and can promote tumor progression (14–16).

T-bet (T-box transcription factor 21), an immune cell–specific member of the T-box family of transcription factors, is expressed in multiple cells of the innate and adaptive immune system [including dendritic cells, natural killer (NK) cells, CD4$^+$ and CD8$^+$ effector cells, B cells, and a subset of regulatory T cells], and its expression is required for the survival, development, and proper functioning of immune cells (17–23). In disease states, T-bet plays an important role in infectious and inflammatory

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conditions as well as in tumor progression: in the absence of T-bet, susceptibility to metastases from melanoma was shown to be increased because of impaired NK-cell function and survival *in vivo* (24). Similarly, in a murine model of lung adenocarcinoma, T-bet deficiency led to a marked induction of tumor load (25). More recently, T-bet has been shown to be required for promoting blockade-induced $CD8^+$ T-cell effector responses sufficient to eradicate disseminated leukemia in an animal model (26). Furthermore, high numbers of T-bet^+ intratumoral lymphoid cells have been found to correlate with improved outcome in gastric cancer (27) and in high-grade cervical intraepithelial neoplasia (28).

In a cohort of women with familial breast cancer from the Ontario site of the Breast Cancer Family Registry (29), studied for expression of chemokine CXCL10 and tumoral lymphocytic infiltration, we observed that T-bet^+ lymphocytes were associated with the basal molecular subtype as well as with morphologic features characteristic of such tumors, including high-grade, p53 expression, ER negativity, CK5 positivity, and EGFR positivity (30). Despite the association with an aggressive phenotype, T-bet positivity was associated with a good prognosis. To confirm this finding in an independent unselected hospital-based cohort, we assessed the presence of T-bet^+ immune cells in a large cohort of axillary node negative (ANN) breast cancer patients and determined the relationship between T-bet positivity and IHC biomarkers, clinicopathologic characteristics, molecular subtypes (luminal A, luminal B, HER2, and basal), and patient outcome. We hypothesized that intratumoral T-bet^+ lymphoid cells would correlate with the basal subtype and would be associated with a more favorable outcome.

Materials and Methods

Patient cohort and clinical follow-up

The patient cohort comprised a prospectively accrued consecutive series of 1,561 women with lymph node–negative, invasive breast carcinoma enrolled at eight Toronto hospitals from September 1987 to October 1996, as previously described (31, 32). This included 887 women on whom paraffin-embedded tissue blocks were available for use in the construction of tissue microarrays (TMA). The characteristics of the whole cohort and TMA cohort have been reported previously (33). Written informed consent was obtained from all study participants.

We followed women in the cohort for recurrence and death. Disease-free survival (DFS) was taken as the time between diagnosis and the confirmation of non-breast recurrence. All patients were monitored for death whether or not they experienced disease recurrence. Using clinical follow-up data, patient status on January 10, 2002, determined DFS time and censoring status. Follow-up data were monitored for an additional 6 months to confirm patient status at the termination date. Excluding the patients lost to follow-up or with distant recurrence, the minimum follow-up time was 56 months after surgery and the median follow-up time was 100 months. Approval of the study protocol was obtained from the Research Ethics Boards of Mount Sinai Hospital (#01-0313-U; Toronto, Ontario, Canada) and the University Health Network (#02-0881-C; Toronto, Ontario, Canada).

Hormone receptor status, tissue microarray construction, and IHC staining

Estrogen receptor (ER) and progesterone receptor (PgR) status were determined biochemically at the time of surgery by ligand-binding assays of frozen tissue, which was the standard approach used at the time, and by IHC detection on the TMA. Formalin-fixed paraffin-embedded tumor blocks were available for 887 patients. Areas of invasive carcinoma were selected from an H&E-stained section of each tumor, and two 0.6-mm cores of tissue were taken from the corresponding areas of the paraffin block. The selected donor cores were embedded in a recipient paraffin block, and 4-μm sections were cut and immunohistochemically stained for ER, PgR, HER2, Ki-67, CK5, EGFR, p53, and T-bet, under the conditions described in Table 1. Microwave antigen retrieval was carried out in a Micromed T/T Mega Microwave Processing Lab Station (ESBE Scientific). Sections were developed with diaminobenzidine tetrahydrochloride and counterstained in Mayer's hematoxylin.

Except for Ki-67 and T-bet, each of the IHC-stained sections was scored using the Allred scoring method (34). Nuclear staining was assessed for ER, PgR, and p53. Strong complete membrane staining was assessed for HER2. Membranous and/or cytoplasmic membrane staining was scored for CK5 and EGFR. The Ki-67 labeling index was determined based on the percentage of positive tumor nuclei, regardless of intensity of staining. In all, 50 nuclei were counted and therefore tissue microarray core samples with <50 tumor cells were deemed unsatisfactory, resulting in exclusion of 220 cases. Absolute counts of T-bet^+ lymphoid cells were conducted, and these were categorized as intratumoral [when within the epithelial nests or within close proximity (the distance between positive lymphocyte and tumor nest is equal to or less than the size of one tumor cell)] or peritumoral (at a distance from the epithelial nests).

The raw score data were processed using a TMA deconvoluter software program into a format suitable for statistical analysis (35). As two cores from each tumor were assessed, the larger of the two values was chosen for use in statistical analysis to minimize the effect of false negatives on the array. For ER, PgR, HER2, and p53, cutpoints to define positivity were based on previous

Table 1. Summary of antibodies and conditions of use

Antibody	Clone	Dilution	Source	Pretreatment
ER	6F11	1/75	Vector	Tris buffer (pH 9.0)
PgR	PgR 1294	1/1,000	DAKO	Tris buffer (pH 9.0)
p53	D.07	1/400	ID Lab	Tris buffer (pH 9.0)
CK5	XM26	1/400	Vector	Tris buffer (pH 9.0)
HER2	CB11 TAB250 (cocktail)	1/300	Novocastra	Pepsin 10 minutes at 37°C
		1/300	Zymed	
EGFR	31G7	1/25	Zymed	Pepsin 10 minutes at 37°C
Ki67	MIB1	1/300	DAKO	Citrate buffer (pH 6.0)
T-bet	4B10	1/100	Santa Cruz Biotechnology	Citrate buffer (pH 6.0)

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validation studies (34, 36–39). For CK5 and EGFR, the cutpoint for positivity was arbitrarily specified as ≥4. Ki-67 was dichotomized into Ki-67 high (Ki-67 labeling index ≥ 14%) or Ki-67 low (Ki67 labeling index < 14%; ref. 40). For T-bet, following the examination of multiple cutpoints, an absolute count of 30 positive intratumoral lymphoid cells (within or within close proximity of the epithelial cell nests) was used as the cutoff for positivity (T-bet$^{+/high}$). Tumors with lower levels or absence of T-bet^{+} intratumoral lymphoid cells were considered as T-bet$^{-/low}$. Interpretable scores were obtained in 618 tumors. In four cases, clinicopathologic characteristics were unavailable, resulting in the inclusion of 614 patients in the final statistical analyses.

Definitions of intrinsic subtypes

Tumors from each group were assigned to molecular subtypes based on previous publications (41–44). Tumors that were positive for HER2 protein overexpression were assigned to the HER2 subtype. Tumors that were negative for HER2 but positive for ER were assigned to the luminal subtype. Tumors that were negative for HER2 and ER, and positive for CK5 or EGFR, were assigned to the basal subtype. The luminal subtype was subsequently subdivided into luminal A and luminal B based on PgR, p53, and Ki-67 labeling index. Tumors that had a Ki-67 labeling index ≥14% and were negative for PgR or positive for p53 were assigned to the luminal B subtype (44).

Statistical analysis

Fisher exact test was used to analyze the T-bet marker associations with clinicopathologic variables, IHC markers (markers used to define molecular subtype), and molecular subtype. Clinicopathologic variables used in analyses represent traditional and/or known prognostic factors for node-negative breast cancer and were chosen based on the literature and on previous prognostic modeling we conducted in this cohort (31–33, 44). Analyses of the association of DFS with T-bet marker status were conducted by the univariate Cox proportional hazards (PH) model (45) with Kaplan–Meier survival curves. To evaluate the additional and independent prognostic contribution of T-bet, a multivariate DFS analysis was carried out controlling for prognostic clinicopathologic factors including HER2 status by the Cox PH model. Relative risks (RR) for each factor were estimated by the HR in the Cox PH model. To deal with the low event rate in the T-bet$^{+/high}$ group, we applied Firth's penalized regression method for the Cox PH model with the penalized likelihood ratio (LR) test (46). All tests were two sided. The cutpoint of T-bet = 30 was chosen by examination of multiple cutpoints (10, 15, 20, 25, 30) in a univariate penalized Cox model. Adjustment of the minimum P value for multiple testing of five correlated tests (47) yielded a strict T-bet significance test criterion of P value < 0.016 for strong family-wise type I error control. A further evaluation of the association between DFS and a three-level categorization (absent, low count, high count) was performed and reported in Supplementary Tables S1 and S2, with RRs and P values for the five cutpoints examined. It is evident in the three-level KM plots (Supplementary Fig. S1A and S1B) that the absent and low count groups are indistinguishable no matter which of the cutpoints is used to define the high- versus the low-count group, and the RRs comparing absent versus high and low versus high are remarkably similar (Supplementary Tables S1 and S2). Further division of the low-count group according to present/absent did not improve discrimination further.

A P value < 0.05 was applied for tests of all other factors. Statistical analyses of associations were performed using SAS 9.1 software (SAS Institute, Inc.). Survival curves were plotted using R statistical software, version 2.15.0 (http://www.r-project.org/).

Results

Frequency and localization of T-bet^{+} lymphoid cells

T-bet^{+} lymphoid cells were distributed among defined tumor compartments as intratumoral [when within the epithelial nests or within close proximity (the distance between positive lymphocyte and tumor nest is equal to or less than the size of one tumor cell)] or peritumoral (at a distance from the epithelial nests; Fig. 1). Intratumoral T-bet^{+} cells were more numerous than were peritumoral cells. The mean, median, and range of intratumoral T-bet^{+} lymphoid cells were 7.4, 0.0, and

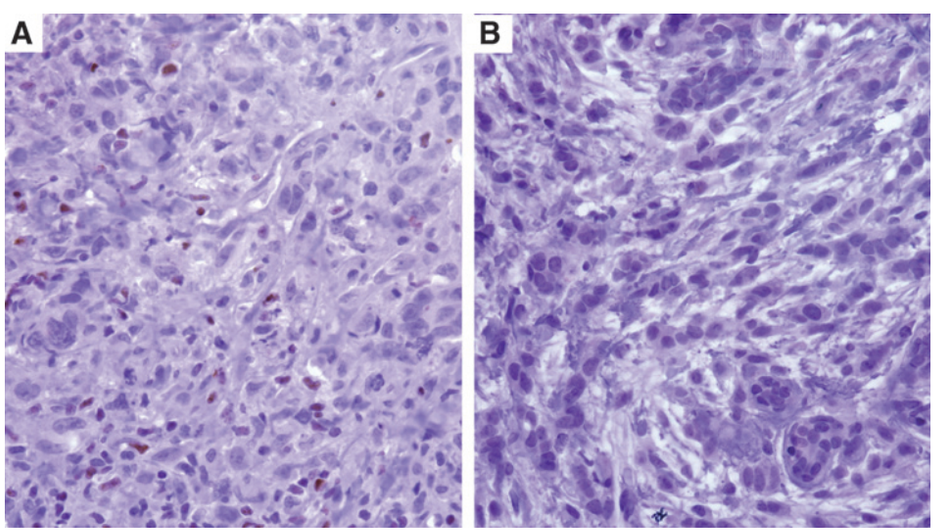

Figure 1.
Intratumoral T-bet^{+} lymphoid cells in invasive carcinoma. A, T-bet$^{+/high}$ (×40 magnification). B, T-bet$^{-/low}$ (×40 magnification).

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0 to 220, respectively, and the mean, median, and range of peritumoral T-bet$^+$ lymphoid cells were 2.6, 0.0, and 0 to 33, respectively. Intratumoral T-bet$^{+/high}$ lymphoid cells (≥30) were seen in 48 tumors, whereas 566 tumors (92.2%) had low or absent T-bet$^+$ lymphoid cells. We performed analyses for intratumoral T-bet$^+$ cells because this localization proved to be prognostically relevant in our previous study (30).

Clinicopathologic and biologic parameters of intratumoral T-bet$^+$ lymphoid cells

Patients with tumors showing high levels of intratumoral T-bet$^+$ lymphoid cells (T-bet$^{+/high}$) were more likely to have larger tumors ($P = 0.0045$), to have higher-grade tumors ($P < 0.0001$), and to have received chemotherapy ($P < 0.0001$; Table 2). These patients were also more likely to have ER-negative/equivocal tumors and PgR-negative/equivocal tumors ($P < 0.0001$; based on biochemical data, Table 2). Menopausal status and lymphovascular invasion did not differ significantly between the patients with T-bet$^{+/high}$ and T-bet$^{-/low}$ tumors (Table 2).

Table 2. Association between clinical characteristics and T-bet$^+$

Characteristic	T-bet$^{-/low}$ ($n = 566$) Number (%)	T-bet$^{+/high}$ ($n = 48$) Number (%)	P[a]
Number of recurrences	76 (13.4)	1 (2.1)	
Menopausal status			
Pre	199 (35.1)	22 (45.8)	0.3168
Peri	31 (5.5)	1 (2.1)	
Post	336 (59.4)	25 (52.1)	
Lymphatic invasion[b]			
Yes	83 (14.7)	5 (10.4)	0.5236
No	482 (85.3)	43 (89.6)	
Tumor size			
≤0.5 cm	24 (4.2)	1 (2.1)	0.0045
>0.5 to 1 cm	114 (20.1)	1 (2.1)	
>1 to 2 cm	238 (42.1)	23 (47.9)	
>2 to 5 cm	171 (30.2)	22 (45.8)	
>5 cm	19 (3.4)	1 (2.1)	
ER[c]			
Positive	361 (63.8)	13 (27.1)	<0.0001
Negative/equivocal	120 (21.2)	34 (70.8)	
ND[d]	85 (15.0)	1 (2.1)	
Progesterone receptor[c]			
Positive	323 (57.1)	15 (31.3)	<0.0001
Negative/equivocal	158 (27.9)	32 (66.7)	
ND[d]	85 (15.0)	1 (2.0)	
Histologic grade			
1	159 (28.1)	6 (12.5)	<0.0001
2	209 (36.9)	7 (14.6)	
3	159 (28.1)	30 (62.5)	
ND[d]	39 (6.9)	5 (10.4)	
Adjuvant treatment[b]			
Hormonal	264 (46.7)	11 (22.9)	<0.0001
Chemotherapy	85 (15.0)	22 (45.8)	
Both	24 (4.3)	0 (0.0)	
None	192 (34.0)	15 (31.3)	
Age, years			
Mean	55.23	51.95	0.0656
SD	11.76	12.88	
Minimum	25.49	22.43	
Maximum	75.82	74.18	
SEM	0.49	1.86	

[a]Fisher exact test; ND groups were not used in testing.
[b]One patient without data.
[c]From pathology reports.
[d]Unknown, not done, or missing.

IHC biomarkers of intratumoral T-bet$^+$ lymphoid cells

Intratumoral T-bet$^{+/high}$ lymphoid cell infiltration was positively associated with CK5 expression ($P < 0.0001$), and negatively associated with both ER and PgR expression ($P < 0.0001$; Table 3). Tumors with high levels of T-bet$^+$ cells were more likely to be positive for p53, EGFR, and Ki-67 ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively; Table 3). HER2 status did not differ significantly between the two groups (Table 3).

Molecular subtypes of intratumoral T-bet$^+$ lymphoid cells

In the 48 tumors with high amounts of T-bet$^+$ lymphoid cells, a molecular phenotype was assignable for 38 tumors (Table 4). Twenty-four (63.2%) of these were basal, 3 (7.9%) were HER2 overexpressing, 8 (21.0%) were luminal A, and 3 (7.9%) were luminal B. In comparison with tumors with low or absent T-bet$^+$ intratumoral lymphoid cells, T-bet$^{+/high}$ tumors were more likely to belong to the basal molecular subtype (63.2% vs. 12.0%; $P < 0.0001$). Of basal tumors, 32% were T-bet$^{+/high}$, compared with 6.5% of HER2$^+$ tumors, 2.8% of luminal A tumors, and 5.8% of luminal B tumors.

Prognostic relevance of intratumoral T-bet$^+$ lymphoid cells

Univariate analysis. When intratumoral T-bet status was considered alone throughout the entire follow-up period (minimum: 56.1 months; median: 96.5 months), intratumoral T-bet$^{-/low}$ was associated with reduced DFS (LR test $P = 0.0133$, RR = 4.72, 95% CI, 1.30–41.54; Table 5; Fig. 2).

T-bet$^+$ lymphoid cells did not demonstrate prognostic significance when stratified according to molecular subtype or adjuvant therapy group (systemic adjuvant therapy vs. none) due to the small number of events in each subgroup.

Multivariate analysis. The above association was retained when intratumoral T-bet status was assessed in a multivariate model for long-term follow-up that included traditional clinicopathologic factors and HER2, (LR test $P = 0.0027$, RR = 5.62, 95% CI, 1.48–50.19; Table 5).

Discussion

In this prospectively accrued cohort of women with lymph node–negative breast cancer, we have demonstrated that intratumoral T-bet$^+$ lymphoid cells are significantly associated with a good prognosis. This is despite being associated with adverse clinicopathologic features, including larger tumor size, higher histologic grade, hormone receptor negativity, and the basal phenotype. The data from this independent cohort of women unselected for family history add to the findings from our previous study with a cohort of women with familial breast cancer which had examined expression of the chemokine CXCL10 and tumoral lymphocytic infiltration (30). The familial cohort included tumors from patients with a strong family history of breast cancer, a significant proportion of whom carry a germline BRCA mutation. The cohort, representing incident breast cancer cases identified from population-based cancer registries, included large numbers of high-grade (54%) and basal-type (25%) tumors compared with 33% and 16%, respectively, in our current cohort. Furthermore, 46% were associated with lymph node metastases at diagnosis, in contrast with the current cohort in which patients were selected based on lymph node–negative status alone. The ANN cohort has many strengths for prognostic biomarker

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Table 3. Association between T-bet$^+$ and IHC markers

Marker[a]	T-bet$^{-/low}$ ($n = 566$)[a] Number (%)	T-bet$^{+/high}$ ($n = 48$)[a] Number (%)	P[b]
HER2			
Negative	500 (92.1)	44 (93.6)	1.0000
Positive	43 (7.9)	3 (6.4)	
ER			
Negative	129 (25.7)	30 (73.2)	<0.0001
Positive	373 (74.3)	11 (26.8)	
PgR			
Negative	217 (41.6)	34 (81.0)	<0.0001
Positive	305 (58.4)	8 (19.0)	
p53			
Negative	417 (77.5)	24 (50.0)	0.0001
Positive	121 (22.5)	24 (50.0)	
EGFR			
Negative	489 (94.6)	30 (65.2)	<0.0001
Positive	28 (5.4)	16 (34.8)	
CK5			
Negative	459 (85.8)	21 (44.7)	<0.0001
Positive	76 (14.2)	26 (55.3)	
Ki-67			
<14%	181 (40.9)	2 (4.8)	<0.0001
≥14%	262 (59.1)	40 (95.2)	

[a]IHC marker data are not available for some tumors.
[b]By Fisher exact test.

validation: its multi-institutional nature allows generalizability of the study population with regard to patient ethnicity, disease severity, differences in treatment, and variations in follow-up and endpoint assessments. Furthermore, the prospective accrual of patients in this cohort has the advantage of decreasing potential biases inherent in many tissue microarray studies. Thus, this current study provides important validation data for the use of T-bet as a prognostic marker in early-stage breast cancer.

Many studies have examined the prognostic effect of inflammation in breast cancer. The methods have varied considerably, with some studies assessing lymphoid infiltrates without subtype specification and other studies examining a specific subtype of the immune cell infiltrate using IHC markers. More recently, the prognostic relevance of tumor-infiltrating lymphocytes has been examined in two large prospectively accrued cohorts. In the first, the prognostic effect of tumor-infiltrating lymphocytes was assessed according to molecular subtype and type of chemotherapy in over 2,000 women with lymph node–positive breast cancer who were enrolled in the BIG 02-98 adjuvant phase III trial (12). This study found that incremental increases in lymphocytic infiltration (whether intratumoral or stromal) resulted in improved outcome in patients who had hormone receptor and HER2$^-$ disease only, regardless of chemotherapeutic regimen. Benefit (stromal lymphocytic infiltration only) was also seen in women with HER2$^+$ disease in just one of the treatment arms (anthracycline-only). In a follow-up study from the same group (48) using tumor tissue from over 900 women enrolled in the prospective FinHER trial, a decreased distant disease recurrence rate was seen in women with increasing numbers of stromal lymphocytes in the triple-negative breast cancer group only. The strength of the effect was similar to what was seen in the earlier study.

The association of a lymphoid rich stroma with basal-like cancers is well recognized. Although this class is generally associated with a poor prognosis, outcome data suggest that it is a heterogeneous group of tumors and we and others have demonstrated that a subset of patients with basal-like breast cancer can expect to show long-term survival (33). Furthermore, medullary carcinomas cluster with the basal group, and this rare subset has been reported to be associated with a better prognosis than that of other grade 3 carcinomas (49, 50). Although one of the definitional criteria for a diagnosis of medullary carcinoma is a moderate or marked lymphoid infiltrate in the stroma between tumor nests, Rakha and colleagues (11) found no statistically significant outcome difference in patients with grade 3 medullary carcinomas and those with grade 3 ductal carcinomas with prominent inflammation, suggesting that the presence of the inflammatory infiltrate confers an improved prognosis rather than the diagnosis of medullary carcinoma per se and that this prominent inflammatory component may play an important role in determining outcome in basal-like tumors. In our study, high numbers of T-bet$^+$ lymphoid cells were associated with the basal subtype; however, we were not able to detect a survival difference within this subgroup when analyzed separately, due to small numbers in this subanalysis. However, in a similar analysis of the basal subtype performed in our previous study (30), we found the association of higher T-bet expression with improved outcome to be of borderline significance (data not shown).

Traditionally, CD8$^+$ cytotoxic T cells have been considered to be the key component in mounting an effective antitumor immune response and higher numbers have been associated with better patient survival (9, 51). However, CD4$^+$ T cells have been shown to be necessary for full functioning of CD8$^+$ cytotoxicity *in vivo* (52, 53). Furthermore, CD4$^+$ T cells influence innate immunity by helping to shape the character and magnitude of the inflammatory response (54). Many breast cancer investigations before this have focused on the effects of CD4$^+$ Tregs (55); however, increasing evidence suggests that the development of Th1 adaptive immunity is associated with improved outcome in various cancer types (56, 57), and T-bet, as the master regulator of Th1 cell differentiation, plays a pivotal role (21, 58). T-bet has been associated with better clinical outcomes in colorectal carcinoma (59) and gastric carcinoma (27); nevertheless, in breast cancer T-bet has rarely been studied. Ladoire and colleagues (60) examined T-bet expression in intratumoral lymphoid structures in women with HER2$^+$ breast cancer who had been given prior treatment with neoadjuvant trastuzumab as well as anthracycline or taxanes. In 102 women, better recurrence-free survival was seen in those women who were treated with trastuzumab and taxanes who had T-bet$^+$ cells in peritumoral lymphoid structures after chemotherapy. A correlation with T-bet$^+$ cells following therapy was not seen with pathologic complete response to therapy. This study highlighted the importance, not only of the host's immune

Table 4. Association between T-bet$^+$ and intrinsic subgroups defined by IHC markers[a]

Subgroup	T-bet$^{-/low}$ ($n = 566$)[b] Number (%)	T-bet$^{+/high}$ ($n = 48$)[b] Number (%)	P[c]
Basal	51 (12.0)	24 (63.2)	<0.0001
HER2	43 (10.1)	3 (7.9)	
Luminal A	282 (66.4)	8 (21.0)	
Luminal B	49 (11.5)	3 (7.9)	
Unassigned	141	10	

[a]Test was performed without the "Unassigned" group.
[b]Subgroup data are not available for some tumors due to unavailability of IHC markers data.
[c]By Fisher exact test.

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Table 5. Results of DFS analysis by Cox proportional hazards model

	Univariate		Multivariate[a]	
Prognostic factor	**RR (95% CI)**	***P***	**RR (95% CI)**	***P***
T-bet				
Low vs. high	4.72[a] (1.30[a]–41.54[a])	0.0133[b]	5.62 (1.48–50.19)	0.0027[b]
HER2				
Positive vs. negative	2.30 (1.35–3.93)	0.0023	0.92 (0.40–1.86)	0.8209
Menopausal status				
Pre/peri vs. post	1.28 (0.88–1.87)	0.2022	0.84 (0.36–1.94)	0.6824
ER				
Negative/equivocal vs. ND/positive	1.26 (0.83–1.91)	0.2858	1.14 (0.62–2.032)	0.6661
Tumor size				
2–5 cm vs. <2 cm	2.16 (1.45–3.20)	0.0001	2.01 (1.20–3.45)	0.0102
>5 cm vs. <2 cm	3.46 (1.68–7.11)	0.0007	2.64 (1.00–6.02)	0.0332
Histologic grade				
Grade 2–3 vs. grade 1	3.48 (1.94–6.24)	<0.0001	3.78 (1.67–10.44)	0.0044
ND vs. grade 1	3.51 (1.67–7.38)	0.0010	5.08 (1.79–15.87)	0.0033
Lymphatic invasion				
Present vs. absent	2.12 (1.36–3.31)	0.0010	2.38 (1.36–4.06)	0.0020
Age at diagnosis, years				
Linear	0.88 (0.74–1.04)	0.1317	0.94 (0.67–1.34)	0.7334
Quadratic	0.93 (0.81–1.06)	0.2615	0.99 (0.83–1.17)	0.9177
Adjuvant treatment				
Hormonal vs. none	0.55 (0.36–0.82)	0.0038	0.51 (0.29–0.86)	0.0132
Chemotherapy vs. none	0.94 (0.58–1.52)	0.8087	0.60 (0.29–1.17)	0.1415

[a]Firth penalized regression.
[b]From the likelihood ratio test; all other *P* values are from the Wald test.

response in breast cancer, but also of the interaction between certain chemotherapeutic regimens and the immune system. The role the immune system plays in response to trastuzumab is well described: trastuzumab activates the host's immune system through antibody-dependent cellular toxicity (ADCC; ref. 61), which leads to Th1 activation and production of IFNγ that has been implicated in control of cancer growth (62). Evidence also suggests that taxanes could exert an immunostimulatory effect against breast cancer (63–66) by inducing a Th1 response. Paclitaxel has been shown to stimulate the secretion by macrophages of proinflammatory and Th1 cytokines such as IL1b or IL12 (67, 68). Carson and colleagues (65) showed that in 227 breast cancer patients, T-cell activation was significantly higher in patients receiving taxanes compared with non–taxane-containing regimens. In contrast, anthracyclines primarily activate $CD8^+$ T cells rather than $CD4^+$ T cells to produce IFNγ, as shown in mouse models (69).

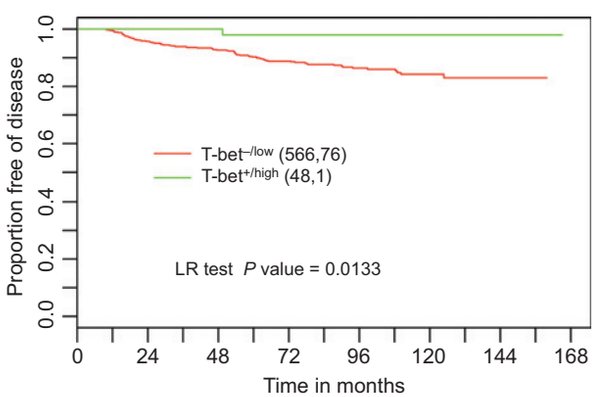

Figure 2.
Kaplan–Meier DFS curves stratified by $T\text{-bet}^+$ status showing a longer DFS in patients with $T\text{-bet}^{+/high}$ tumors.

In this study, we based analyses on intratumoral rather than peritumoral $T\text{-bet}^+$ lymphoid cells for two reasons. First, because of our prior findings of the prognostic significance of intratumoral $T\text{-bet}^+$ lymphoid cells in the Breast Cancer Family Registry, Ontario site (30), and second, because we used TMAs in this study. TMAs preferentially include areas rich in tumor cells, and peritumoral areas may not be represented consistently, thereby introducing a potential bias in peritumoral $T\text{-bet}^+$ lymphoid cell counts.

In conclusion, intratumoral $T\text{-bet}^+$ lymphoid cells in breast cancer are associated with adverse pathologic features, including the basal subtype. Nevertheless, their presence confers a favorable outcome which is independent of traditional clinicopathologic parameters and HER2 status. The potential use of T-bet as a prognostic marker in breast cancer needs to be evaluated in additional larger cohorts, particularly those rich in basal-like or $HER2^+$ breast cancer. Understanding the mechanisms mediating these immunologic responses and biomarkers of such responses may help in better tailoring specific therapies including combinations of agents for women with breast cancer. Furthermore, modulation of T-bet expression has the potential to become a powerful therapeutic target for the treatment of cancer in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.M. Mulligan, I.L. Andrulis
Development of methodology: A.M. Mulligan, I.L. Andrulis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Mulligan, I.L. Andrulis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Mulligan, D. Pinnaduwage, S.B. Bull
Writing, review, and/or revision of the manuscript: A.M. Mulligan, D. Pinnaduwage, S. Tchatchou, S.B. Bull, I.L. Andrulis
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