The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 15 2012

Tumor-Infiltrating γδ T Lymphocytes Predict Clinical Outcome in Human Breast Cancer FREE

Chunling Ma; ... et. al

J Immunol (2012) 189 (10): 5029–5036.
https://doi.org/10.4049/jimmunol.1201892

Tumor-Infiltrating γδ T Lymphocytes Predict Clinical Outcome in Human Breast Cancer

Chunling Ma,*,†,[1] Qunyuan Zhang,‡,[1] Jian Ye,* Fang Wang,*,§ Yanping Zhang,¶ Eric Wevers,* Theresa Schwartz,¶ Pamela Hunborg,¶ Mark A. Varvares,‖ Daniel F. Hoft,* Eddy C. Hsueh,¶ and Guangyong Peng*

Understanding and dissecting the role of different subsets of regulatory tumor-infiltrating lymphocytes (TILs) in the immunopathogenesis of individual cancer is a challenge for anti-tumor immunotherapy. High levels of γδ regulatory T cells have been discovered in breast TILs. However, the clinical relevance of these intratumoral γδ T cells is unknown. In this study, γδ T cell populations were analyzed by performing immunohistochemical staining in primary breast cancer tissues from patients with different stages of cancer progression. Retrospective multivariate analyses of the correlations between γδ T cell levels and other prognostic factors and clinical outcomes were completed. We found that γδ T cell infiltration and accumulation in breast tumor sites was a general feature in breast cancer patients. Intratumoral γδ T cell numbers were positively correlated with advanced tumor stages, HER2 expression status, and high lymph node metastasis but inversely correlated with relapse-free survival and overall survival of breast cancer patients. Multivariate and univariate analyses of tumor-infiltrating γδ T cells and other prognostic factors further suggested that intratumoral γδ T cells represented the most significant independent prognostic factor for assessing severity of breast cancer compared with the other known factors. Intratumoral γδ T cells were positively correlated with FOXP3$^+$ cells and CD4$^+$ T cells but negatively correlated with CD8$^+$ T cells in breast cancer tissues. These findings suggest that intratumoral γδ T cells may serve as a valuable and independent prognostic biomarker, as well as a potential therapeutic target for human breast cancer. ***The Journal of Immunology*****, 2012, 189: 5029–5036.**

Increasing evidence suggests that immunotherapy is a promising approach to treating patients with invasive and metastatic breast cancers (1–3). However, the immunosuppressive microenvironments induced by different types of regulatory T cells (Tregs) in breast cancer present major barriers to successful anti-tumor immunotherapy (1, 4–6). Emerging studies are showing elevated levels of CD4$^+$CD25$^+$ Tregs among the total T cell populations isolated from tumor tissues or peripheral blood in patients with various cancers, including breast cancer (4, 7). Importantly, several studies have demonstrated a correlation between intratumoral Tregs and tumor pathogenesis (8–10). Furthermore, more recent studies have also shown that quantification of tumor-infiltrating FOXP3$^+$ Tregs is a novel marker for identifying high-risk breast cancer patients and is valuable for assessing disease prognosis and progression (7, 11, 12). We recently observed that tumor-infiltrating γδ1 T cells, which exist in the tumor microenvironment of breast cancer patients, had potent suppressive activity toward conventional T cells both in vitro and in vivo (6). Understanding the role of different subsets of regulatory tumor-infiltrating lymphocytes (TILs) in the immunopathogenesis of individual cancer is critical for anti-tumor immunotherapy (13–16).

γδ T cells not only serve as sentinels in the innate system but also act as a bridge between innate and adaptive immune responses, performing multiple functions (17–20). There are two major subsets of human γδ T cells, Vδ1 and Vγ9Vδ2 T cells. Vδ1 T cells are the predominant subset found at mucosal surfaces and in epithelial tissues (17, 18, 21). Human Vδ1 T cells share certain characteristics with murine γδ intraepithelial lymphocytes and may recognize either MHC class I-related chain A or B, which are induced on epithelial cells and tumor cells by stress or structural damage (22–25). Vγ9Vδ2 (also known as Vγ2Vδ2) T cells dominate in the peripheral blood and lymph nodes and respond to microbial infections by recognizing small nonpeptide molecules (21, 22, 26, 27). The roles of human Vγ9Vδ2 T cells in mediating immunity against microbial pathogens and tumors have been well described (28). Several clinical trials focusing on the activation of Vγ9Vδ2 T cells as a cancer treatment in patients with renal cell carcinoma, non-Hodgkin's lymphoma, or multiple myeloma and prostate cancer have shown promising results (29–33). Recent studies from mouse tumor models have demonstrated that γδ T cells within the tumor microenvironment were involved in the induction of tumor-specific immune tolerance (34–36). However, little is known about negative

*Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, MO 63104; †Department of Immunology and Microbiology, Shandong Medical College, Linyi 276000, People's Republic of China; ‡Department of Genetics, Washington University School of Medicine, St. Louis, MO 63108; §Department of Laboratory Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, People's Republic of China; ¶Department of Surgery, Saint Louis University School of Medicine, St. Louis, MO 63110; and ‖Department of Otolaryngology–Head and Neck Surgery, Saint Louis University School of Medicine, St. Louis, MO 63110

[1]C.M. and Q.Z. contributed equally to this work.

Received for publication July 9, 2012. Accepted for publication September 13, 2012.

This work was supported by grants from the American Cancer Society (RSG-10-160-01-LIB to G.P.) and the National Institutes of Health (AI097852 and AI094478 to G.P.).

C.M., E.C.H., and G.P. conceived and designed the study; Y.Z., T.S., P.H., M.A.V., and E.C.H. provided study materials or patients; C.M., T.S., P.H., D.F.H., E.C.H., and G.P. collected and assembled data; C.M., Q.Z., J.Y., F.W., Y.Z., E.W., M.A.V., D.F.H., E.C.H., and G.P. analyzed and interpreted data; and C.M., Q.Z., D.F.H., E.C.H., and G.P. wrote the manuscript.

Address correspondence and reprint requests to Dr. Eddy C. Hsueh or Dr. Guangyong Peng, Department of Surgery, Saint Louis University School of Medicine, 3635 Vista Avenue, St. Louis, MO 63110 (E.C.H.) or Department of Internal Medicine, Saint Louis University School of Medicine, 1100 S. Grand Boulevard, St. Louis, MO 63104 (G.P.). E-mail addresses: hsuehec@slu.edu (E.C.H.) and gpeng@slu.edu (G.P.)

Abbreviations used in this article: CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; OS, overall survival; RFS, relapse-free survival; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.

regulation by γδ T cells in human disease, especially in anti-tumor immunity in cancer patients. We recently analyzed cell populations in TILs isolated from human breast tumors and identified high percentages of γδ1 Tregs existing in the tumor microenvironment (6). We observed that these breast tumor-derived γδ1 Tregs possessed a broad suppressive function that affected $CD4^+$, $CD8^+$, and γδ2 T cells and blocked the maturation and activity of dendritic cells (6). In addition, this new subset of Tregs has further been confirmed in patients by more recent studies from other groups (37–39). Although we observed that suppressive γδ1 T cells were enriched in TILs of breast cancer patients, the function of such Tregs in the context of tumor immune tolerance and immunopathogenesis is unclear.

In the current study, we performed immunohistochemical staining of γδ T cells in tumor tissues and paired normal breast tissues from patients with different stages of primary breast cancers undergoing surgery and retrospectively analyzed the correlation between the γδ T cell levels with tumor stages, metastasis characteristics, prognostic factors, and clinical outcome of patients. We also analyzed the correlations between γδ T cell levels and other TILs, including $CD4^+$, $CD8^+$, and $FOXP3^+$ T cells. We observed that patients with a high proportion of γδ T cells had advanced cancer stages and high lymph node metastasis. Importantly, high numbers of γδ T cells in breast cancer tissues were correlated with poor survival and high risks of relapse. These data clearly suggest that γδ T cells constitute a dominant population existing in the breast tumor suppressive microenvironment that is significantly and negatively correlated with clinical outcome.

Materials and Methods

Patients and samples collection

Tumor samples were obtained from breast cancer patients treated at Saint Louis University Department of Surgery from 2004 to 2010 who gave informed consent for enrollment in a prospective tumor procurement protocol approved by the Saint Louis University Institutional Review Board. A total of 81 tumor tissues from different stages of identified primary breast cancer was collected for this study. Whenever feasible without interfering with histopathologic analysis for ongoing clinical decision making, paired fresh tumor tissues and normal breast tissues were obtained perioperatively and snap-frozen in liquid nitrogen ($n = 46$). For patients from whom fresh tissues were not obtained, paraffin blocks of tumor tissues were obtained for analysis ($n = 35$). Clinical data of patients were also collected for analysis. In addition, 26 fresh-frozen melanoma tumor tissues (metastatic cutaneous melanoma) were also collected as controls for this study.

Immunohistochemical staining

The cell populations of γδ, $CD4^+$, and $CD8^+$ T cells and $FOXP3^+$ cells in cancer and normal tissues were determined using immunohistochemical staining. The frozen or paraffin-embedded sections were stained with a panel of the first specific mAbs against human CD4, CD8, TCR-γδ, and FOXP3. After washing, sections were incubated with biotin-labeled secondary Ab streptavidin–HRP solutions, following the procedure of the Histostain-Plus 3rd Gen IHC Detection Kit (Invitrogen). For frozen section staining, the mouse anti-human γδ TCR (clone B1.1), FOXP3 (clone 236A/E7), CD4 (clone RPA-T4), and CD8 (clone RPA-T8) (eBioscience, San Diego, CA) mAbs were used at diluted concentrations of 1:50, 1:50, 1:100, and 1:100, respectively. For paraffin-embedded tumor sections, FOXP3 (clone 236A/E7), CD4 (clone BC/1F6), and CD8 (clone 144B) (Abcam, Boston, MA) mAbs were used at a diluted concentration of 1:50. Controls were performed by incubating slides with the isotype control Ab instead of primary Abs or second Ab alone. Normal breast tissues and tumor tissues from melanoma patients served as controls. The positive cells were counted and analyzed microscopically.

Quantification method

Expressions of $CD4^+$, $CD8^+$, and γδ T cells and $FOXP3^+$ cells in tissues were evaluated manually using a computerized image system composed of a Leica ICC50 camera system equipped on a Leica DM750 microscope (North Central Instruments, Minneapolis, MN). Photographs were obtained from 20 randomly selected areas within the tumor tissues of 10 cancer nest areas and 10 cancer stroma areas at a high-power magnification (×400). Ten fields (magnification, ×400) of each tumor tissue section, including both cancer nest and stroma areas, were counted and summed and the means of positive cell numbers per field reported. In addition, the results were further confirmed by directly counting positive cells microscopically. The counting was performed by three independent investigators (C.M., J.Y., and F.W.) who had no previous knowledge of the clinical backgrounds of patients, and the results were averaged.

Statistical analysis

Given that there was no clinically defined cutoff points for the numbers of TILs ($CD4^+$, $CD8^+$, γδ, and $FOXP3^+$ T cells) in the tumor tissues, the median expression of each TIL (9 for γδ T cells, 16 for $CD4^+$ T cells, 12 for $FOXP3^+$ cells, and 13 for $CD8^+$ T cells) in breast cancer tissues was used as a cutoff point to define the TIL-high and TIL-low groups. Pearson's χ^2 test was used prospectively to analyze the correlations between the cell number of each TIL and clinical features, including age, nodal status, tumor size, tumor stage, estrogen receptor (ER) status, epidermal growth factor receptor 2 (HER2) positivity, relapse-free survival (RFS), and overall survival (OS). OS was determined from the date of surgery to the date of death by any cause or to the date of the last follow-up. RFS was measured as the length of time from surgery to the date of relapse. For all categorical predictors (including the cell numbers dichotomized by medians), the log-rank test was used to perform univariate survival association analyses for OS and RFS. Survival and relapse-free probability and cumulative hazard associated with prognostic factors for OS and RFS were estimated by the Kaplan–Meier method, and hazard ratios were estimated

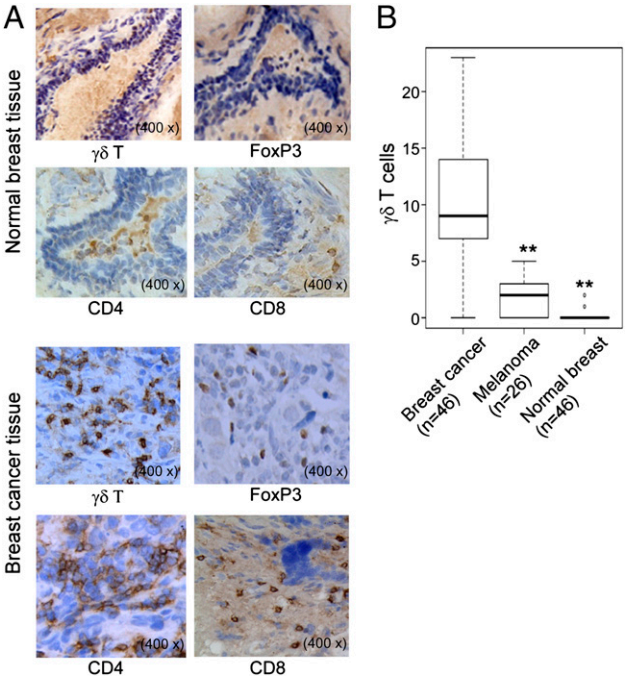

FIGURE 1. Accumulation of γδ T cells in breast cancer but not in normal breast tissues. (**A**) Immunohistochemical staining of γδ, $CD4^+$, and $CD8^+$ T cells and $FOXP3^+$ cells in normal breast and cancer tissues. Few γδ, $CD4^+$, and $CD8^+$ T cells and $FOXP3^+$ cells were observed in normal breast tissues. However, high numbers of γδ, $CD4^+$, and $CD8^+$ T cells and $FOXP3^+$ cells were detected in breast cancer tissues. Frozen or paraffin-embedded tissue sections were immunohistochemically stained to detect the indicated cells. (**B**) Significantly increased numbers of γδ T cells existed in breast cancer tissues compared with normal breast tissues and melanoma tumor tissues. Frozen sections from breast tumor samples and controls of paired normal breast tissues ($n = 46$) and melanoma tissues ($n = 26$) were immunohistochemically stained to detect γδ T cells. Number of γδ T cells shown is the average numbers per high field (original magnification ×400) in each tissue sample. The median number of γδ T cells in each group is shown as a horizontal line. Significance was determined by paired (breast cancer versus normal breast tissues) or unpaired (breast cancer versus melanoma tissues) t test. $^{**}p < 0.01$ (compared with γδ T cells in the breast cancer tissues).

Table I. Comparison of γδ T cell and FOXP3$^+$ cell positive incidence among normal breast and tumor tissues

	γδ T Cells				FOXP3$^+$ Cells			
Samples	Cases	+	−	*p*	Cases	+	−	*p*
Normal breast	46	2 (4.35%)	44 (95.65%)		46	7 (15.22%)	39 (84.78%)	
Breast tumor	46	43 (93.48%)	3 (6.52%)	2.20×10^{-16}	81	79 (97.53%)	2 (2.47%)	2.24×10^{-16}

Evaluated by χ^2 test.

by a Cox proportional hazard regression model. Data processing and statistical analyses were performed using SAS 9.1 and R 2.13.0. Statistical significance was defined as $\alpha < 0.05$ (two-tailed).

Results

Prevalence of γδ T cells in situ in breast cancer tissues

We have recently demonstrated that high percentages of γδ1 Tregs existed in breast TILs (6). This novel finding prompted us to investigate the functional role of tumor-infiltrating γδ T cells in the pathogenesis of human breast cancer. We first determined whether γδ T cells were prevalent in situ in breast tumor sites. Given that the commercially available anti-human γδ TCR Ab was only suitable for frozen sections, we performed immunohistochemical staining to detect γδ T cells in 46 freshly frozen breast cancer sections and patient-paired normal breast tissues (Fig. 1A). In normal breast tissues, very few samples had detectable γδ T cells (2 of 46 breast tissues). In contrast, significantly increased numbers of γδ T cells were detected in breast tumor tissues (43 of 46 tumor samples; median, 9; range, 0–23) (Fig. 1B and Table I). In addition, we investigated the existence of γδ T cells in melanoma tumor tissues (as a tumor type control). However, γδ T cell numbers in melanoma tissues were much lower than those in breast cancer tissues, consistent with our previous finding that low percentages of γδ T cells exist in melanoma TILs (6) (Fig. 1B). These results strongly indicate that γδ T cell development in TILs was a unique feature in breast cancer patients. In parallel experiments, we analyzed the other key TILs, including CD4$^+$, CD8$^+$, and FOXP3$^+$ T cells, in 81 tumor (frozen and paraffin-embedded) tissues from different stages of breast cancer (7, 11, 40) (Fig. 1A). We found that very high percentages of CD4$^+$ and CD8$^+$ T cells and FOXP3$^+$ cells also existed in breast cancer tissues compared with those in paired-normal breast tissues (Table I and data not shown).

Correlations of intratumoral γδ T cells with clinicopathological parameters of breast cancer patients

To investigate further the clinical significance of γδ T cells in human breast cancer, the cancer clinicopathological factors of breast cancer patients were analyzed relative to the levels of the intratumoral γδ T cells. In addition, cancer-specific survival rates for patients were analyzed in correlation with γδ T cells and other immune cells (CD4$^+$, CD8$^+$, FOXP3$^+$ T cells). As shown in Table II, γδ T cell numbers were positively correlated with higher tumor stages ($p = 1.19 \times 10^{-6}$), positive lymph node status ($p = 9.94 \times 10^{-6}$), and HER2 expression ($p = 0.002$). In contrast, γδ T cell infiltration was inversely correlated with RFS ($p = 1.27 \times 10^{-5}$) and OS ($p = 3.97 \times 10^{-6}$) of breast cancer patients.

Table II. Correlations between γδ, CD4$^+$, and CD8$^+$ T cell and FOXP3$^+$ cell expression and clinicopathologic characteristics in breast cancer patients

	γδ T (*n* = 46)			FOXP3 (*n* = 81)			CD4 (*n* = 81)			CD8 (*n* = 81)		
Parameter	≤9	>9	*p*	≤12	>12	*p*	≤16	>16	*p*	≤13	>13	*p*
Age (y)												
<60	12	11		23	18		23	18		14	25	
≥60	11	12	0.878	21	18	0.982	24	15	0.789	21	20	0.247
Tumor stage												
I	17	0		24	6		27	3		7	23	
II	6	10		18	12		15	15		15	15	
III	1	12	**1.19×10^{-6}**	3	18	**1.69×10^{-5}**	6	15	**2.75×10^{-5}**	13	8	**0.015**
Tumor size (cm)												
<2.1	15	8		27	14		30	11		15	24	
≥2.1	9	14	0.101	17	22	0.075	17	22	**0.014**	20	21	0.481
Nodal status												
Negative	21	4		30	12		32	10		11	31	
Positive	3	18	**9.94×10^{-6}**	15	24	**0.006**	16	23	**0.003**	24	15	**2.84×10^{-3}**
ER status												
Negative	8	3		12	11		11	12		7	16	
Positive	16	19	0.223	33	25	0.890	37	21	0.285	28	30	0.225
HER2												
High	2	12		10	12		13	9		11	11	
Low	21	7		29	14		28	15		15	28	
Negative	2	2	**0.002**	3	7	**0.049**	2	8	**0.033**	6	4	0.249
RFS												
No recurrence	24	8		39	16		38	17		12	35	
Recurrence	0	14	**1.27×10^{-5}**	3	14	**3.04×10^{-4}**	7	10	**0.073**	15	19	**2.67×10^{-5}**
OS												
Alive	24	7		40	16		37	19		17	39	
Died	0	15	**3.97×10^{-6}**	5	20	**4.89×10^{-5}**	11	14	0.105	18	7	**1.14×10^{-3}**

Evaluated by χ^2 test. Boldface indicates the significance of the *p* value.

In addition, numbers of tumor-infiltrating $CD4^+$ T cells and $FOXP3^+$ cells in breast cancer were also positively correlated with tumor stages and lymph nodal status but negatively correlated with RFS and OS. However, $CD8^+$ T cell numbers were negatively correlated with high tumor stages and positive lymph node status and positively correlated with clinical outcomes of RFS and OS (Table II). These results further confirmed the different effects mediated by TILs in tumor immunity and in pathogenesis of breast cancer. Notably, among these four subsets of tumor-infiltrating T cells, γδ T cells were shown to have the most significant correlation with the pathological factors and clinical outcomes in breast cancer patients.

Correlations of γδ T cells with $CD4^+$ and $CD8^+$ T cells and with $FOXP3^+$ cells in breast cancer TILs

Tumor-infiltrating $FOXP3^+$ T cells have been shown to be an important biomarker for assessing disease prognosis and progression of breast cancer (7, 11, 40). Our current studies observed that breast tumor-infiltrating γδ T cells were also negatively correlated with clinical outcomes. Therefore, we further investigated the correlation between tumor-infiltrating γδ T cells and $FOXP3^+$ cells in breast cancer patients. Box plot and linear correlation analysis demonstrated that there was a significant correlation between intratumoral γδ T cells and $FOXP3^+$ cells in breast cancer tissues ($p = 7.72 \times 10^{-5}$, $r = 0.549$) (Fig. 2A, 2B). In addition, we investigated the correlations between tumor-infiltrating γδ T cells and $CD4^+$ as well as $CD8^+$ T cells in breast cancer patients. There was a positive correlation between γδ T cells and $CD4^+$ T cells but a negative correlation between γδ T cells and $CD8^+$ T cells ($p = 4.59 \times 10^{-3}$, $r = 0.411$ and $p = 7.36 \times 10^{-4}$, $r = -0.48$, respectively) (Fig. 2C, 2D). These results collectively suggested that both $FOXP3^+$ and γδ T cells are important negative regulatory components of TILs in breast cancer patients, and the increase and activation of $CD8^+$ T cells is an important strategy for anti-tumor immunity.

Intratumoral γδ T cells are an independent prognostic factor for assessing disease severity of breast cancer

Because intratumoral γδ T cells were inversely associated with OS and RFS (Table II), we further performed univariate Cox proportional hazard regression analyses of the relationships between γδ T cell levels, other prognostic factors, and clinical outcomes in our patient cohort. As shown in Table III, lymph node status, tumor stage, tumor-infiltrating γδ, $CD4^+$ and $CD8^+$ T cells, and $FOXP3^+$ cells were all significant factors for the prediction of breast cancer outcomes. Importantly, level of intratumoral γδ T cells was the most significant risk factor among all these factors, with a hazard ratio (HR) of 41.69 [95% confidence interval (CI), 5.4–321.96, $p = 4.79 \times 10^{-8}$] for patient RFS and an HR of 44.73 (95% CI, 5.79–345.22, $p = 1.51 \times 10^{-8}$) for patient OS. In addition, we performed multivariate Cox regression analyses by including six predictor variables (lymph node status, tumor stage, intratumoral γδ, $CD4^+$ and $CD8^+$ T cells, and $FOXP3^+$ cells) that were significant in univariate analyses of both OS and RFS. The multivariate analyses confirmed that γδ T cell level had independent effects on both OS and RFS. As shown in Table IV, γδ T cell level was still significant ($p = 0.0004$ for RFS and $p = 0.0201$ for OS) after adjustment for the other five predictors, with an HR of 34.68 for RFS and 3.34 for OS. Besides γδ T cells, only $CD8^+$ T cells and tumor stage maintained significance in the multivariate analysis of RFS, indicating that γδ T cell level might be an important driver predictor among these factors (Table IV). It is noteworthy that high numbers of tumor-infiltrating γδ T cells predicted poor OS and RFS in breast cancer patients. On the basis of the median of γδ T cell numbers in breast cancer tissues, patients were divided into two groups (γδ T cells ≤9, and γδ T cells >9). Kaplan–Meier analyses further demonstrated that the 5-y OS and RFS probabilities both were 100% in cancer patients with γδ T cells ≤9 but only ∼35% (OS) and 30% (RFS) in cancer patients with γδ T cells >9 (Fig. 3A). These results collectively suggest that intratumoral γδ T cell level is an independent prognostic factor for prediction of breast cancer outcome.

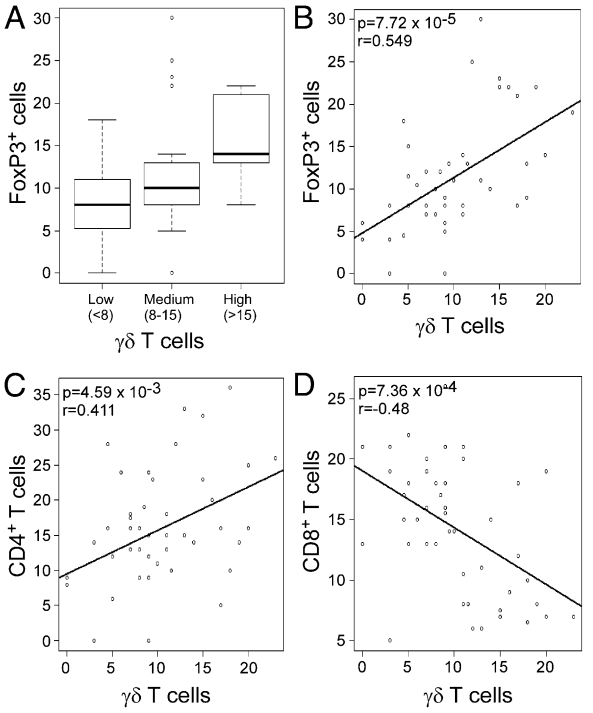

FIGURE 2. Correlations between γδ T cells and $CD4^+$ T cells, $CD8^+$ T cells, and $FOXP3^+$ cells in breast cancer TILs. (**A** and **B**) Box plot (A) and scatter diagram (B) analyses showing positive correlation between γδ T cells and $FOXP3^+$ cells in breast cancer TILs. The median number of γδ T cells in each group is shown as a horizontal line in (A). (**C**) Scatter diagram showing positive correlation between γδ T cells and $CD4^+$ T cells in breast cancer TILs. (**D**) Scatter diagram showing negative correlation between γδ T cells and $CD8^+$ T cells in breast cancer TILs. Different types of TILs in frozen sections of breast tumor samples ($n = 46$) were immunohistochemically determined as described for Fig. 1.

Given that ER-negative breast cancer patients have a worse prognosis than ER-positive patients, we next determined whether γδ T cells had a different prognostic value for clinical outcomes in breast cancer patients with different ER expression status. As expected, intratumoral γδ T cells were still the most significant independent risk factor for breast cancer outcomes compared with the other factors in ER-positive cancer patients. High number of intratumoral γδ T cells had the highest HR for OS (HR = 28.11, 95% CI 3.62–218.08, $p = 2.04 \times 10^{-6}$) and RFS (HR = 26.45, 95% CI 3.41–205.07, $p = 4.26 \times 10^{-6}$) in ER-positive breast cancer patients (Table V). Furthermore, ER-positive patients with higher numbers of γδ T cells (>9) had lower 5-y RFS (30%) and OS (25%). In contrast, the probabilities of 5-y RFS and OS were 100% for ER-positive patients containing low γδ T cells (≤9) (Fig. 3B). In addition, we observed similar results in ER-negative breast cancer patients (data not shown). HER2 expression in tumor cells is another important prognostic factor for breast cancer outcomes. Our results demonstrated a significant correlation between γδ T cell numbers and HER2 expression in breast cancer patients ($p = 0.002$) (Table II). We found that HER2-positive cancer patients containing high γδ T cells (>9) also had significantly higher HRs for OS (HR = 35.79, 95% CI 4.51–283.87, $p =$

Table III. Univariate analyses of factors associated with RFS and OS in breast cancer patients (n = 81)

Variables	RFS			OS		
	HR	95% CI	p	HR	95% CI	p
CD4 (>16)	3.92	1.48–10.36	0.005	2.61	1.17–5.82	0.018
CD8 (>13)	0.062	0.01–0.47	6.71E-05	0.32	0.13–0.79	0.007
γδ T (>9)	41.69	5.40–321.96	4.79E-08	44.73	5.79–345.22	1.51E-08
FOXP3 (>12)	9.68	2.77–33.82	2.49E-05	6.86	2.56–18.37	1.13E-05
ER status (positive)	1.26	0.41–3.87	0.679	1.71	0.64–4.59	0.260
HER2 (positive)	0.18	0.054–0.57	0.012	0.359	0.10–1.28	0.155
Stage (III versus I + II)	11.94	4.31–33.07	1.53E-06	6.72	2.88–15.71	1.65E-05
Nodal (positive)	16.71	3.76–74.26	1.35E-06	6.97	2.58–18.79	1.15E-05
Size (>2.1 cm)	2.19	0.83–5.78	0.108	3.435	1.42–8.22	0.003

Results obtained using the Cox proportional hazard regression model.

1.28×10^{-6}) and RFS (HR = 39.39, 95% CI 4.99–310.65, $p = 5.42 \times 10^{-7}$) (Table VI), thus had significantly shorter 5-y survival rates for RFS (30%) and OS (40%) (Fig. 3C). Because of the small sample size for HER2-negative cancer patients in the current study, statistical analyses of γδ T cells were not performed in these patients. Notably, our current study further confirmed that level of FOXP3$^+$ T cells was another significant biomarker for prediction of breast cancer progression and clinical outcome based on univariate analysis and Kaplan–Meier survival results, consist with findings from other groups (Tables III, V, and VI and Fig. 4) (7, 11, 40). However, our results suggested that intratumoral γδ T cells might be a more valuable and significant factor than FOXP3 expression for the prediction of the risk and prognosis of breast cancer.

Prognostic value of γδ T cells for the risk of breast cancer development

In addition to analyzing retrospectively the associations between tumor-infiltrating γδ T cell levels, other prognostic factors, and OS and RFS in breast cancer patients, we further determined the prognostic significance of intratumoral γδ T cells for the prediction of cancer development during the follow-up period. As shown in Fig. 5A, there were significantly increased cumulative HRs with increasing follow-up years for mortality and relapse in the cancer patients containing high levels of tumor-infiltrating γδ T cells (γδ T cells >9). In contrast, cancer patients containing low levels of tumor-infiltrating γδ T cells (≤9) had 100% OS and RFS throughout the entire 5-y follow-up (cumulative hazard is 0). Although the level of tumor-infiltrating FOXP3$^+$ T cells is also an important prognostic factor for the prediction of breast cancer development, cancer patients containing low levels of tumor-infiltrating FOXP3$^+$ cells (≤12) still may die or relapse with a cumulative hazard of 0.2 throughout the entire 5-y follow-up (Fig. 5B). These results clearly suggest that intratumoral γδ T cells are a novel clinical biomarker for identifying the risk for late relapse and survival of breast cancer patients.

Discussion

Dissecting the functional role of different subsets of TILs in the tumor suppressive microenvironment is critical for the development of effective strategies for anti-tumor immunotherapy. Recent studies have shown a high frequency of γδ1 T cells among TILs or circulating PBMCs from cancer patients with renal carcinoma tumors, acute leukemia, and squamous cell carcinoma of the head and neck (41–44). Furthermore, we demonstrated that high percentages of γδ1 Tregs with potent suppressive function existed in breast cancer TILs (6). However, the importance of these γδ T cells for clinical outcomes has not been determined. In the current study, we identified that γδ T cells constituted a dominant population existing in the breast cancer suppressive microenvironment during breast cancer progression. Importantly, we further showed that level of tumor-infiltrating γδ T cells was negatively correlated with clinical outcomes and was a novel and independent prognostic factor in human breast cancer. These studies clearly suggest that the development of effective strategies targeting γδ Tregs is essential for breast cancer immunotherapy.

Although negative regulation by γδ T cells in mouse tumor models has been documented (36, 45), little is known about the role of such cells in tumor immunity in cancer patients. In the current study, we explored the clinical significance of γδ T cells in the pathogenesis of breast cancer. First, our studies clearly showed that intratumoral γδ T cell numbers were positively correlated with advanced tumor stages and high lymph node metastasis but were inversely correlated with RFS and OS of breast cancer patients. Second, multivariate and univariate analyses of intratumoral γδ T cells and other prognostic factors further demonstrated that intratumoral γδ T cells were the most significant independent risk factor for breast cancer among all other known factors associated with patient OS and RFS. Third, it is important that high numbers of tumor-infiltrating γδ T cells not only predict poor OS and RFS in breast cancer patients but also have prognostic significance for identifying a high risk for late relapse and poor survival of cancer

Table IV. Multivariate analyses of HRs with RFS and OS in breast cancer patients (n = 81)

Variables	RFS			OS		
	HR	95% CI	p	HR	95% CI	p
γδ T (>9)	34.68	4.79–250.88	0.0004	3.34	1.21–9.25	0.020
FOXP3 (>12)	3.08	0.49–19.17	0.228	3.05	0.95–9.77	0.061
CD4 (>16)	2.31	0.66–8.07	0.189	1.20	0.47–3.09	0.702
CD8 (>13)	0.04	0.01–0.35	0.004	0.47	0.18–1.27	0.137
Stage (III versus I + II)	5.24	1.25–22.02	0.023	2.17	0.83–5.67	0.114
Nodal (positive)	2.36	0.32–17.59	0.404	1.99	0.54–7.31	0.301

Results obtained using the Cox proportional hazard regression model.

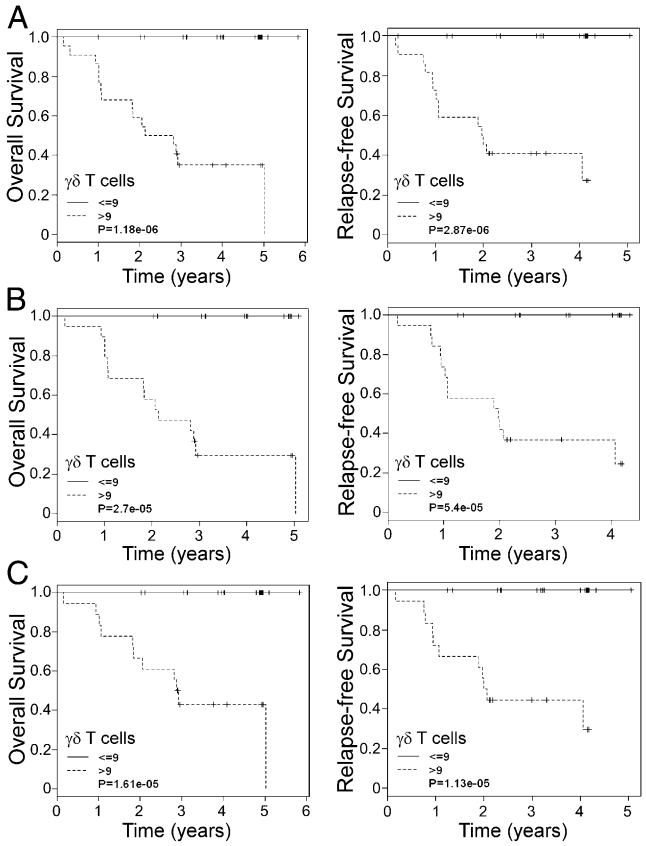

FIGURE 3. High numbers of tumor-infiltrating γδ T cells predict poor overall survival and shorter relapse-free survival in breast cancer patients. Kaplan–Meier curves for overall survival and relapse-free survival stratified by the high and low numbers of γδ T cells in all breast cancer patients (**A**), ER-positive patients (**B**), and HER2-postive patients (**C**). The median expression of γδ T cells (i.e., 9) in breast cancer tissues was used as a cutoff point to define γδ T cell-high and γδ T cell-low groups. Forty-six breast cancer patients in total were analyzed. The *p* values were calculated with use of the log-rank test.

patients during cancer development. These studies collectively suggest that breast tumor-infiltrating γδ T cells play a significant role in breast cancer progression and pathogenesis and may serve as a valuable and independent prognostic biomarker for human breast cancer. Because the available anti-human γδ T cell Ab is only suitable for frozen-section studies, the current study was limited to small numbers of frozen breast tumor tissues at different stages of cancer progression. Our future studies should expand the breast cancer sample size to confirm further the functional role of intratumoral γδ T cells in the pathogenesis of breast cancer. Furthermore, it remains unclear whether the presence of γδ Tregs is a primary driver of the pathogenesis of breast cancer. Mechanistic studies using suitable animal models are needed firmly to establish the role of γδ T cells in human breast cancer development. In addition, we will extend the current studies of breast cancer to other types of cancers, such as prostate cancer, to determine whether the tumor-infiltrating γδ Tregs are a unique feature only for human breast cancer.

It has become clear that an immunosuppressive microenvironment mediated by tumor-infiltrating Tregs is a major obstacle to the success of immunotherapy against breast cancer (4–6). $FOXP3^+$ Tregs have been shown to be an important marker for assessing disease prognosis and progression of breast cancer (7, 11, 40). In the current studies, we also demonstrated that $FOXP3^+$ T cells were a significant biomarker for prediction of clinical outcomes in breast cancer that was negatively correlated with RFS and OS of cancer patients. Given that that γδ Tregs constituted a dominant population existing in the breast tumor suppressive microenvironment that was also significantly negatively associated with clinical outcome, we further investigated the relationship between tumor-infiltrating γδ T cells and presence of other infiltrating immune cells, especially $FOXP3^+$ T cells. We have previously shown that breast tumor-derived γδ Tregs do not express CD25 and FOXP3 markers, which are typically expressed by $CD4^+$ Tregs (6). Our current studies further showed that the intratumoral γδ T cells were positively correlated with $FOXP3^+$ cells in breast cancer tissues. Importantly, our results demonstrated that intratumoral γδ T cells were more significantly correlated with poor outcome than $FOXP3^+$ cells. These results strongly suggest that both $FOXP3^+$ Tregs and γδ T cells play critical roles in the immune pathogenesis of human breast cancer. In addition, novel immunologic approaches targeting both γδ T cells and $FOXP3^+$ Tregs in breast tumor microenvironments are urgently needed.

To augment the success of immunotherapy against breast cancer, one challenge is how to identify the origin and mechanisms governing the increase of different types of Tregs in cancer patients. Recent studies suggest that there are several potential sources of Tregs that exist in tumor sites (13–16, 46). One key mechanism responsible for accumulation of Tregs within the tumor microenvironment is preferential recruitment of these Tregs. Studies of Hodgkin's lymphoma and ovarian cancer have shown that tumor microenvironmental CCL22 derived from cancer cells specifically recruits CCR4-positive $CD4^+$ Tregs to tumor sites (10, 47). Our current and previous studies have shown that increased numbers of γδ T cells were only observed in breast tumor tissues and not in

Table V. Univariate analyses of factors associated with RFS and OS in ER-positive breast cancer patients (n = 58)

	RFS			OS		
Variables	HR	95% CI	*p*	HR	95% CI	*p*
CD4 (>16)	3.66	1.22–10.98	0.022	2.67	1.07–6.59	0.035
CD8 (>13)	0.06	0.008–0.47	0.0001	0.15	0.04–0.49	0.0002
γδ T (>9)	26.45	3.41–205.07	4.26E-06	28.11	3.62–218.08	2.04E-06
FOXP3 (>12)	10.33	2.28–46.76	0.0002	5.33	1.93–14.73	0.0004
HER2 (positive)	0.42	0.05–3.38	0.471	0.64	0.08–5.03	0.688
Stage (III versus I + II)	10.55	3.19–34.81	5.24E-5	5.76	2.27–14.62	0.0002
Nodal (positive)	21.82	2.801–170.01	2.07E-05	10.27	2.96–35.61	7.58E-06
Size (>2.1 cm)	3.29	1.01–10.74	0.037	4.26	1.54–11.81	0.002

Results obtained using the Cox proportional hazard regression model.

Table VI. Univariate analyses of factors associated with RFS and OS in HER2-positive breast cancer patients (n = 64)

Variables	RFS			OS		
	HR	95% CI	p	HR	95% CI	p
CD4 (>16)	2.95	0.95–9.21	0.067	3.25	1.22–8.67	0.017
CD8 (>13)	0.09	0.01–0.67	0.001	0.28	0.09–0.87	0.016
γδ T (>9)	39.39	4.99–310.65	5.42E-07	35.79	4.51–283.87	1.28E-06
FOXP3 (>12)	6.67	1.79–24.70	0.001	9.10	2.60–31.81	4.29E-05
ER status (positive)	4.02	0.52–31.17	0.106	3.24	0.73–14.28	0.073
Stage (III versus I + II)	13.77	4.06–46.67	1.85E-05	13.53	4.53–40.47	2.05E-06
Nodal (positive)	NA	NA	6.85E-08	14.84	3.36–65.43	3.44E-06
Size (>2.1 cm)	2.93	0.88–9.77	0.068	4.23	1.38–13.04	0.006

Results obtained using the Cox proportional hazard regression model. All patients with negative nodal status had no recurrence and were alive during the follow-up period, and the software could not perform the analysis. NA, No analysis.

normal breast tissues, suggesting the recruitment and expansion of γδ T cells by breast tumor microenvironments (6). Our future studies will focus on the identification of mechanisms responsible for the accumulation of γδ T cells in breast tumor microenvironments mediated by tumor cells and/or tumor-derived stromal and immune cells. Another challenge is the understanding of the immunosuppressive mechanisms used by these tumor-derived γδ Tregs. Our previous studies have shown that γδ Tregs are functionally distinct from naturally occurring $CD4^+CD25^+$ Tregs. γδ Treg-mediated immune suppression is through unknown soluble factor(s), which is independent of IL-10 and/or TGF-β, in contrast to the cell–cell contact-dependent suppressive mechanism of $CD4^+CD25^+$ Tregs (6). Importantly, we recently demonstrated that human TLR8 signaling completely reversed the suppressive functions of naturally occurring $CD4^+CD25^+$ Tregs and tumor-derived $CD4^+$, $CD8^+$, and γδ Tregs (6, 48, 49). Once we obtain a better understanding of the mechanisms for the immunosuppression and accumulation of γδ T cells in breast cancer, we can develop combined novel strategies to block trafficking and recruitment of γδ Tregs and to reverse the immune suppression mediated by γδ Tregs, which would augment the anti-tumor immune responses in breast cancer immunotherapy.

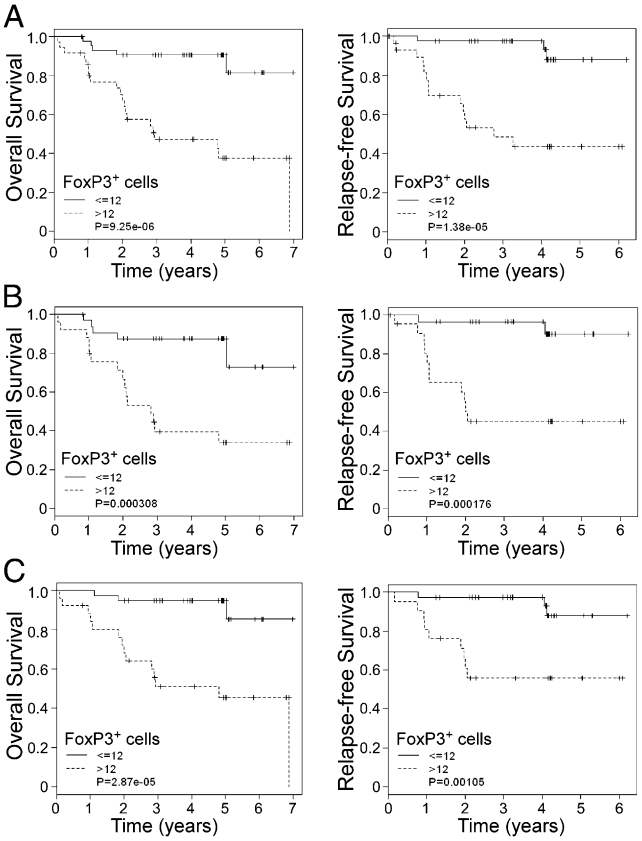

FIGURE 4. Kaplan–Meier analyses of overall survival and relapse-free survival stratified for high and low numbers of tumor-infiltrating $FOXP3^+$ cells in breast cancer patients. Kaplan–Meier curve for overall survival and relapse-free survival stratified by the median number of $FOXP3^+$ cells in all breast cancer patients (**A**), ER-positive patients (**B**), and HER2-postive patients (**C**). The median expression of $FOXP3^+$ cells (i.e., 12) in breast cancer tissues was used as a cutoff point and to define $FOXP3^+$ cell-high and $FOXP3^+$ cell-low groups. A total of 81 breast cancer patients was analyzed. The p values were calculated with use of the log-rank test.

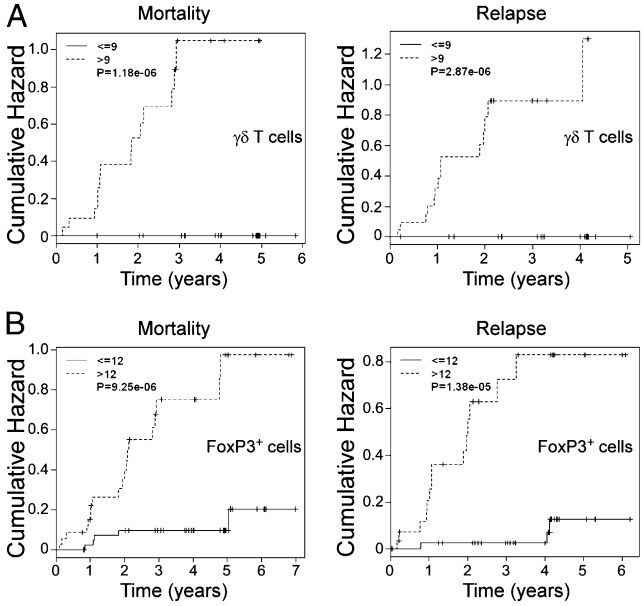

FIGURE 5. Prognostic values of γδ T cells and $FOXP3^+$ cells for the risks of breast cancer mortality and relapse in all breast cancer patients during the follow-up period. An increasing annual HR for mortality and relapse per year in the breast cancer patients who have high numbers of tumor-infiltrating γδ T cells [(**A**), n = 46] and $FOXP3^+$ cells [(**B**), n = 81] compared with those who have low numbers of these two subsets of TILs throughout the entire follow-up period.

Acknowledgments

We thank Dr. William S.M. Wold and Jacqueline Spencer (Department of Molecular Microbiology and Immunology, Saint Louis University) for providing the microtome for tissue sections. We also thank Dr. Edward S. Bolesta (Department of Pathology, Saint Louis University) for providing paraffin-embedded specimens of breast cancer.

Disclosures

The authors have no financial conflicts of interest.

References

1. Emens, L. A., R. T. Reilly, and E. M. Jaffee. 2005. Breast cancer vaccines: maximizing cancer treatment by tapping into host immunity. *Endocr. Relat. Cancer* 12: 1–17.
2. Zhou, J., and Y. Zhong. 2004. Breast cancer immunotherapy. *Cell. Mol. Immunol.* 1: 247–255.
3. Hudis, C. A. 2003. Current status and future directions in breast cancer therapy. *Clin. Breast Cancer* 4(Suppl 2): S70–S75.
4. Liyanage, U. K., T. T. Moore, H. G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein, P. S. Goedegebuure, and D. C. Linehan. 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J. Immunol.* 169: 2756–2761.
5. Perez, S. A., M. V. Karamouzis, D. V. Skarlos, A. Ardavanis, N. N. Sotiriadou, E. G. Iliopoulou, M. L. Salagianni, G. Orphanos, C. N. Baxevanis, G. Rigatos, and M. Papamichail. 2007. CD4+CD25+ regulatory T-cell frequency in HER-2/neu (HER)-positive and HER-negative advanced-stage breast cancer patients. *Clin. Cancer Res.* 13: 2714–2721.
6. Peng, G., H. Y. Wang, W. Peng, Y. Kiniwa, K. H. Seo, and R. F. Wang. 2007. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. *Immunity* 27: 334–348.
7. Bates, G. J., S. B. Fox, C. Han, R. D. Leek, J. F. Garcia, A. L. Harris, and A. H. Banham. 2006. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J. Clin. Oncol.* 24: 5373–5380.
8. Siddiqui, S. A., X. Frigola, S. Bonne-Annee, M. Mercader, S. M. Kuntz, A. E. Krambeck, S. Sengupta, H. Dong, J. C. Cheville, C. M. Lohse, et al. 2007. Tumor-infiltrating Foxp3-CD4+CD25+ T cells predict poor survival in renal cell carcinoma. *Clin. Cancer Res.* 13: 2075–2081.
9. Shen, Z., S. Zhou, Y. Wang, R. L. Li, C. Zhong, C. Liang, and Y. Sun. 2010. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. *J. Cancer Res. Clin. Oncol.* 136: 1585–1595.
10. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
11. Merlo, A., P. Casalini, M. L. Carcangiu, C. Malventano, T. Triulzi, S. Mènard, E. Tagliabue, and A. Balsari. 2009. FOXP3 expression and overall survival in breast cancer. *J. Clin. Oncol.* 27: 1746–1752.
12. Ghebeh, H., E. Barhoush, A. Tulbah, N. Elkum, T. Al-Tweigeri, and S. Dermime. 2008. FOXP3+ Tregs and B7-H1+/PD-1+ T lymphocytes co-infiltrate the tumor tissues of high-risk breast cancer patients: Implication for immunotherapy. *BMC Cancer* 8: 57.
13. Curiel, T. J. 2007. Tregs and rethinking cancer immunotherapy. *J. Clin. Invest.* 117: 1167–1174.
14. Zou, W. 2006. Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 6: 295–307.
15. Knutson, K. L., M. L. Disis, and L. G. Salazar. 2007. CD4 regulatory T cells in human cancer pathogenesis. *Cancer Immunol. Immunother.* 56: 271–285.
16. Wang, H. Y., and R. F. Wang. 2007. Regulatory T cells and cancer. *Curr. Opin. Immunol.* 19: 217–223.
17. Carding, S. R., and P. J. Egan. 2002. Gammadelta T cells: functional plasticity and heterogeneity. *Nat. Rev. Immunol.* 2: 336–345.
18. Hayday, A., and R. Tigelaar. 2003. Immunoregulation in the tissues by gammadelta T cells. *Nat. Rev. Immunol.* 3: 233–242.
19. Maeda, Y., P. Reddy, K. P. Lowler, C. Liu, D. K. Bishop, and J. L. Ferrara. 2005. Critical role of host gammadelta T cells in experimental acute graft-versus-host disease. *Blood* 106: 749–755.
20. Brandes, M., K. Willimann, and B. Moser. 2005. Professional antigen-presentation function by human gammadelta T cells. *Science* 309: 264–268.
21. Pang, D. J., J. F. Neves, N. Sumaria, and D. J. Pennington. 2012. Understanding the complexity of γδ T-cell subsets in mouse and human. *Immunology* 136: 283–290.
22. Hayday, A. C. 2009. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* 31: 184–196.
23. Hayday, A. C. 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol.* 18: 975–1026.
24. Groh, V., A. Steinle, S. Bauer, and T. Spies. 1998. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* 279: 1737–1740.
25. Witherden, D. A., and W. L. Havran. 2011. Molecular aspects of epithelial γδ T cell regulation. *Trends Immunol.* 32: 265–271.
26. Bukowski, J. F., C. T. Morita, and M. B. Brenner. 1999. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity* 11: 57–65.
27. Constant, P., F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville, and J. J. Fournié. 1994. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* 264: 267–270.
28. Spencer, C. T., G. Abate, A. Blazevic, and D. F. Hoft. 2008. Only a subset of phosphoantigen-responsive gamma9delta2 T cells mediate protective tuberculosis immunity. *J. Immunol.* 181: 4471–4484.
29. Viey, E., G. Fromont, B. Escudier, Y. Morel, S. Da Rocha, S. Chouaib, and A. Caignard. 2005. Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. *J. Immunol.* 174: 1338–1347.
30. Kobayashi, H., Y. Tanaka, J. Yagi, Y. Osaka, H. Nakazawa, T. Uchiyama, N. Minato, and H. Toma. 2007. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. *Cancer Immunol. Immunother.* 56: 469–476.
31. Wilhelm, M., V. Kunzmann, S. Eckstein, P. Reimer, F. Weissinger, T. Ruediger, and H. P. Tony. 2003. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. *Blood* 102: 200–206.
32. Mattarollo, S. R., T. Kenna, M. Nieda, and A. J. Nicol. 2007. Chemotherapy and zoledronate sensitize solid tumour cells to Vgamma9Vdelta2 T cell cytotoxicity. *Cancer Immunol. Immunother.* 56: 1285–1297.
33. Dieli, F., D. Vermijlen, F. Fulfaro, N. Caccamo, S. Meraviglia, G. Cicero, A. Roberts, S. Buccheri, M. D'Asaro, N. Gebbia, et al. 2007. Targeting human gammadelta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 67: 7450–7457.
34. Kapp, J. A., L. M. Kapp, K. C. McKenna, and J. P. Lake. 2004. gammadelta T-cell clones from intestinal intraepithelial lymphocytes inhibit development of CTL responses ex vivo. *Immunology* 111: 155–164.
35. Ke, Y., L. M. Kapp, and J. A. Kapp. 2003. Inhibition of tumor rejection by gammadelta T cells and IL-10. *Cell. Immunol.* 221: 107–114.
36. Seo, N., Y. Tokura, M. Takigawa, and K. Egawa. 1999. Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J. Immunol.* 163: 242–249.
37. Bhagat, G., A. J. Naiyer, J. G. Shah, J. Harper, B. Jabri, T. C. Wang, P. H. Green, and J. S. Manavalan. 2008. Small intestinal CD8+TCRgammadelta+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. *J. Clin. Invest.* 118: 281–293.
38. Li, X., N. Kang, X. Zhang, X. Dong, W. Wei, L. Cui, D. Ba, and W. He. 2011. Generation of human regulatory gammadelta T cells by TCRgammadelta stimulation in the presence of TGF-beta and their involvement in the pathogenesis of systemic lupus erythematosus. *J. Immunol.* 186: 6693–6700.
39. Merims, S., P. Dokouhaki, B. Joe, and L. Zhang. 2011. Human Vδ1-T cells regulate immune responses by targeting autologous immature dendritic cells. *Hum. Immunol.* 72: 32–36.
40. Mahmoud, S. M., E. C. Paish, D. G. Powe, R. D. Macmillan, A. H. Lee, I. O. Ellis, and A. R. Green. 2011. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Res. Treat.* 127: 99–108.
41. Aswald, J. M., X. H. Wang, S. Aswald, A. Lutynski, M. D. Minden, H. A. Messner, and A. Keating. 2006. Flow cytometric assessment of autologous gammadelta T cells in patients with acute myeloid leukemia: potential effector cells for immunotherapy? *Cytometry B Clin. Cytom.* 70: 379–390.
42. Bas, M., H. Bier, K. Schirlau, U. Friebe-Hoffmann, K. Scheckenbach, V. Balz, T. L. Whiteside, and T. K. Hoffmann. 2006. Gamma-delta T-cells in patients with squamous cell carcinoma of the head and neck. *Oral Oncol.* 42: 691–697.
43. Meeh, P. F., M. King, R. L. O'Brien, S. Muga, P. Buckhalts, R. Neuberg, and L. S. Lamb, Jr. 2006. Characterization of the gammadelta T cell response to acute leukemia. *Cancer Immunol. Immunother.* 55: 1072–1080.
44. Kowalczyk, D., W. Skorupski, Z. Kwias, and J. Nowak. 1997. Flow cytometric analysis of tumour-infiltrating lymphocytes in patients with renal cell carcinoma. *Br. J. Urol.* 80: 543–547.
45. Kapp, J. A., L. M. Kapp, and K. C. McKenna. 2004. Gammadelta T cells play an essential role in several forms of tolerance. *Immunol. Res.* 29: 93–102.
46. Gajewski, T. F. 2007. The expanding universe of regulatory T cell subsets in cancer. *Immunity* 27: 185–187.
47. Ishida, T., T. Ishii, A. Inagaki, H. Yano, H. Komatsu, S. Iida, H. Inagaki, and R. Ueda. 2006. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res.* 66: 5716–5722.
48. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R. F. Wang. 2005. Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science* 309: 1380–1384.
49. Kiniwa, Y., Y. Miyahara, H. Y. Wang, W. Peng, G. Peng, T. M. Wheeler, T. C. Thompson, L. J. Old, and R. F. Wang. 2007. CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer. *Clin. Cancer Res.* 13: 6947–6958.