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Abstract

Feline invasive mammary carcinomas are characterized by their high clinical aggressiveness, rare expression of hormone receptors, and pathological resemblance to human breast cancer, especially triple-negative breast cancer (negative to estrogen receptor, progesterone receptor, and epidermal growth factor receptor type 2). Recent gene expression studies of triple-negative breast cancers have highlighted their heterogeneity and the importance of immune responses in their biology and prognostic assessment. Indeed, regulatory T cells may play a crucial role in producing an immunesuppressed microenvironment, notably in triple-negative breast cancers. Feline invasive mammary carcinomas arise spontaneously in immune-competent animals, in which we hypothesized that the immune tumor microenvironment also plays a role. The aims of this study were to determine the quantity and prognostic value of forkhead box protein P3-positive peritumoral and intratumoral regulatory T cells in feline invasive mammary carcinomas, and to identify an immunesuppressed subgroup of triple-negative basal-like feline invasive mammary carcinomas. One hundred and eighty female cats with feline invasive mammary carcinomas, treated by surgery only, with 2-year follow-up post-mastectomy, were included in this study. Forkhead box protein P3, estrogen receptor, progesterone receptor, Ki-67, epidermal growth factor receptor type 2, and cytokeratin 14 expression were assessed by automated immunohistochemistry. Peritumoral regulatory T cells were over 300 times more abundant than intratumoral regulatory T cells in feline invasive mammary carcinomas. Peritumoral and intratumoral regulatory T cells were associated with shorter disease-free interval and overall survival in both triple-negative (ER-, PR-, HER2-, N = 123 out of 180) and luminal (ER+ and/or PR+, N = 57) feline invasive mammary carcinomas. In feline triple-negative basal-like (CK14+) mammary carcinomas, a regulatory T-cellenriched subgroup was associated with significantly poorer disease-free interval, overall survival, and cancer-specific survival than regulatory T-cell-poor triple-negative basal-like feline invasive mammary carcinomas. High regulatory T-cell numbers had strong and negative prognostic value in feline invasive mammary carcinomas, especially in the triplenegative basal-like subgroup, which might contain a "basal-like immune-suppressed" subtype, as described in triplenegative breast cancer. Cats with feline invasive mammary carcinomas may thus be interesting spontaneous animal models to investigate new strategies of cancer immunotherapy in an immune-suppressed tumor microenvironment.

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Introduction

Mammary carcinomas that spontaneously arise in female cats are characterized by their high frequency,^{1,2} clinical aggressiveness,^{3–8} and poor therapeutic responses.⁹ Feline mammary carcinomas (FMCs) are considered to be mostly triple-negative, that is, negative to estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2)^{7,10–16} and basal-like (positive to basal cytokeratins).¹⁷

Cats with FMCs represent one of the rare immunocompetent animal models for human breast cancer. This is fundamental, as the immune tumor microenvironment is crucial in subtyping triple-negative breast cancer and might also serve as a therapeutic target. Triple-negative basal-like breast cancers have been subdivided into basal-like immune-suppressed (BLIS) and basal-like immune-activated (BLIA) carcinomas by gene expression analysis,¹⁸ or equivalent subtypes, such as the C2 (basallike enriched, immune-suppressed) and C3 (basal-like enriched, adaptive immune response) clusters according to Jézéquel et al.,^{19,20} or the immunomodulatory subtype according to the study by Lehmann et al.^{21,22}

Among immune cells of the tumor microenvironment, regulatory T cells (Tregs) play a critical role in immune tolerance and deficiency of anti-tumor immunity,^{23–25} and Tregs identified by FoxP3 immunohistochemistry on paraffin-embedded archival samples have been associated with poor prognosis in breast cancer patients.^{26–30} The transcription factor FoxP3 (forkhead box protein P3), critical for the development and function of Tregs, is considered to be the most specific marker of CD4+ CD25+ Tregs by immunohistochemistry, in humans^{31–33} and in cats.^{34,35}

The objectives of our study were (1) to quantify intratumoral and peritumoral FoxP3+ Tregs in FMCs, (2) to assess their relationships with other clinical and pathological features, (3) to investigate their prognostic significance in FMCs, and (4) to better characterize triple-negative basal-like FMCs, that is, to identify a BLIS phenotype.

Materials and methods

Animals and inclusion criteria

This retrospective study included 180 cats with invasive mammary carcinomas, which have been previously described.³⁶ Female cats were eligible for inclusion if they had an invasive mammary carcinoma, if they were

treated solely by surgery, and if follow-up was available for at least 2 years post-mastectomy.

Owners' written consent was obtained prior to inclusion. Approval was obtained from the local animal welfare and ethics committee "Comité d'Ethique en Recherche clinique et épidémiologique Vétérinaire d'Oniris" (CERVO), of the Nantes Atlantic College of Veterinary Medicine, Food Science and Engineering (Oniris, France).

Recorded epidemiologic and clinical data included age at diagnosis, breed, medical and reproductive history, the location of the mammary carcinoma, and clinical stage according to the modified World Health Organization staging system,^{37,38} as previously described.³⁶ Distant metastasis assessment, performed by veterinary practitioners using medical imaging (thoracic radiography and/ or abdominal ultrasound), was recorded as M0 (absence of distant metastasis at diagnosis), M1 (presence of distant metastases), or MX (absence of information because owners declined medical imaging for financial reasons). Follow-up methodology is described in Supplementary Methods.

Histopathology

After 1–4 days of formalin fixation, samples were embedded in paraffin blocks and were cut into 3-μmthick sections for hematoxylin–eosin–saffron (HES) staining. In the case of multifocal (within a given mammary gland) or multicentric (within different mammary glands) invasive mammary carcinomas, the largest one was selected for analysis. Recorded histological data were the histological types, lymphovascular invasion (LVI), histological grade according to the Elston and Ellis grading system for human breast cancer,³⁹ which has been validated in FMCs,⁴⁰ presence/absence of central necrosis or squamous differentiation, and tumorassociated lymphohistiocytic inflammation, as previously described.³⁶

The pathologic tumor (pT) size was measured on histological sections as the greatest tumor diameter, in millimeters. This was achieved on a single slide for tumors of <25 mm (inner dimension of a histological cassette), or on two adjacent slides for tumors of 25-50 mm in diameter. Tumors greater than 50 mm in diameter could not be precisely measured.

The pathologic nodal (pN) stage was first assessed on HES-stained slides and designated pN+ for the presence of metastatic cells within the draining lymph node, whatever their numbers. Thus, a pN+ nodal stage could correspond to isolated tumor cells (<0.2 mm in diameter), micro-metastases (0.2-2.0 mm in diameter), or macrometastases (>2.0 mm in diameter). When the lymph node was free of metastatic cells on HES-stained slides (N = 24 cases), pancytokeratin immunohistochemistry was performed (4 out of 24 cases were then reclassified as pN+). Absence of nodal metastatic cells was referred to as pN0. The nodal stage was pNX when the lymph node had not been sampled for histopathology.

Immunohistochemistry

Automated immunohistochemistry (Benchmark XT, Ventana Medical Systems, Roche Diagnostics) was used as previously described^{36,40} and further detailed in Supplementary Methods, to detect p63 (myoepithelial marker that differentiates invasive from in situ breast carcinomas),^{41,42} cytokeratins (for isolated tumor cell detection in draining lymph nodes), LMO2 (lymphatic endothelial cell marker in cats, used to confirm LVI in uncertain cases), ER-alpha, PR, HER2, the proliferation marker Ki-67, cytokeratin 14 (CK14), and FoxP3.

The thresholds for positivity were 10% for ER and PR as previously reported for canine,^{43–45} feline,³⁶ and human⁴⁶ mammary carcinomas, 20% for Ki-67,⁴⁷ and 15% for CK14. HER2 was scored according to recommendations for breast cancer.⁴⁸

The 180 invasive FMCs were classified as luminal (ER+ and/or PR+, any HER2 score) or triple-negative (ER-, PR-, HER2 score 0 to 2+),^{10,17,49} and triple-negative basal-like carcinomas were defined as ER-, PR-, HER2-, and CK14+.

Nuclear FoxP3 expression in lymphocytes located within mammary carcinomas allowed identifying intratumoral Tregs, which were counted in 10 representative high-power fields (HPFs; at 400 × magnification), separately as Tregs in direct contact with tumor cells (IT^{contact} Tregs, surrounded by carcinoma cells, Figure 1(a)) and Tregs of the intratumoral stroma (IT^{stroma} Tregs, surrounded by collagen, cancer-associated fibroblasts, neocapillaries, and other inflammatory cells, Figure 1(a)). Peritumoral Tregs located at the invasive edge of FMCs (Figure 1(b)) were counted in one representative HPF. Nuclear FoxP3 expression in neoplastic cells was only observed in two cases, concerned a very low proportion of cancer cells, and was not further investigated.

Statistical analyses

Statistical analyses were conducted using the MedCalc[®] statistical software (Ostend, Belgium). Chi-squared tests (for discontinuous variables) and one-way analyses of variance (for continuous variables) were used to



Figure 1. FoxP3 expression in tumor-infiltrating lymphocytes of feline invasive mammary carcinomas: (a) Intratumoral FoxP3+ regulatory T cells were counted separately in direct contact with tumor cells (within circle, IT^{contact} Tregs) and in the intratumoral stroma (within square, IT^{stroma} Tregs). (b) Peritumoral FoxP3+ regulatory T cells. The dotted line represents the borders of the mammary carcinoma. Peroxidase-DAB revelation system. Original magnification $400 \times$. Bar = $20 \,\mu$ m.

compare the clinicopathologic characteristics of Tregenriched and Treg-poor FMCs. The Kaplan–Meier method and log-rank tests were used in univariate survival analyses, and Cox proportional hazard models for multivariate survival analyses. The results are reported using the hazard ratio (HR), its 95% confidence interval (95% CI), and the p-value of each covariate. The prognostic cutoffs for Treg numbers were determined by receiver operating characteristic curve analysis calculated for 2-year cancer-specific survival. A p-value of < 0.05 was considered significant.

Results

Patient characteristics

The cohort comprised 180 previously described³⁶ female cats with invasive mammary carcinoma. Briefly,

the mean age at diagnosis was 11.1 ± 2.7 years, most of the patients were European (short- or long-haired) domestic cats (154 out of 180, 85%), and most were intact females (N = 112, 62%). Twenty-six FMCs (14%) were multiple. The pT size was ≥ 20 mm in 95 cases (53%). Fifty-six percent of the patients (101 out of 180) had a positive pN stage (pN+), and 8 out of 180 (4%) had distant metastases (M1) at diagnosis.

Only 49 out of 180 FMCs (27%) were ER positive, 13 out of 180 (7%) were PR positive, and none of the FMCs overexpressed HER2. Fifty-seven FMCs (32%) were luminal, of which 8 were luminal-A (Ki-67 index of <20%), and 49 were luminal-B (Ki-67 \ge 20%), and 123 out of 180 FMCs (68%) were triple-negative, of which 93 (76%) were triple-negative basal-like (CK14+) FMCs.

Numbers of FoxP3+ Tregs

The mean IT^{contact} Treg numbers within FMCs were $6 \pm 11/\text{mm}^2$ (median: 1.3; range: 0–100). Most of the carcinomas (148 out of 180, 82%) contained at least 1 IT^{contact} Treg. At a threshold of ≥ 2 IT^{contact} Tregs/mm², 81 FMCs (45%) were considered rich in IT^{contact} Tregs, including 31 out of 57 luminal FMCs (54%), and 50 out of 123 triple-negative FMCs (41%).

The mean IT^{stroma} Treg numbers within FMCs were $7 \pm 9/\text{mm}^2$ (median: 3.6; range: 0–68). Most of the carcinomas (165 out of 180, 92%) were infiltrated with IT^{stroma} Tregs. At a threshold of ≥ 6 IT^{stroma} Tregs/mm², 64 FMCs (36%) were considered rich in IT^{stroma} Tregs, including 26 out of 57 luminal FMCs (47%) and 38 out of 123 triple-negative FMCs (31%).

The mean peritumoral Treg numbers around FMCs were $1064 \pm 800/\text{mm}^2$ (median: 926; range: 0–4307). Most of the carcinomas (175 out of 180, 97.2%) contained peritumoral Tregs. At a threshold of \geq 575/mm², 126 FMCs (70%) were considered rich in peritumoral Tregs, including 43 out of 57 luminal FMCs (75%), and 83 out of 123 triple-negative FMCs (67%). Overall, peritumoral Tregs were 344 times more numerous than intratumoral Tregs in the FMCs studied.

Associations between Treg numbers and clinicopathological parameters

High numbers of intratumoral Tregs were associated with tumor-associated inflammation and squamous differentiation (Table 1). FMCs with squamous differentiation contained in average $8 \pm 14 \text{ IT}^{\text{contact}}$ Tregs/mm² and $9 \pm 12 \text{ IT}^{\text{stroma}}$ Tregs/mm², whereas FMCs without squamous differentiation contained on average only $4 \pm 7 \text{ IT}^{\text{contact}}$ Tregs/mm² and $5 \pm 6 \text{ IT}^{\text{stroma}}$ Tregs/mm². There was also a positive association between IT^{stroma} Tregs and LVI (Table 1).

High numbers of peritumoral Tregs were positively correlated with (1) the histological grade (Table 1); Grade I–II FMCs were surrounded by 906 \pm 719 peritumoral Tregs/mm², compared to 1237 ± 848 around Grade III FMCs; (2) LVI; LVI+ FMCs were surrounded by 1193 ± 805 peritumoral Tregs/mm², compared to 877 ± 748 around LVI– FMCs; (3) tumorassociated inflammation; FMCs with moderate to severe tumor-associated inflammation had 1237 ± 834 peritumoral Tregs/mm², compared to only 834 ± 691 around FMCs with absent to mild inflammation; (4) the pT size; $FMCs \ge 20 \text{ mm}$ in diameter had 1179 ± 791 peritumoral Tregs/mm², compared to 935 ± 791 around smaller FMCs; (5) the pN stage; pN+ FMCs were surrounded by 1222 ± 877 peritumoral Tregs/mm², compared to 933 ± 656 around pNX FMCs, and 782 ± 680 around pN0 FMCs; and (6) the clinical stages; there were 863 ± 647 peritumoral Tregs/mm² around Stage I–II FMCs, versus 1193 ± 863 around Stage III–IV FMCs.

Tregs in different localizations were also correlated with each other (Table 1 for Tregs considered as categorical variables). There was a positive correlation between peritumoral Tregs and IT^{contact} Tregs ($R^2 = 0.031$, p = 0.0179), between peritumoral Tregs and IT^{stroma} Tregs ($R^2 = 0.087$, p < 0.001), and between IT^{contact} and IT^{stroma} Tregs ($R^2 = 0.262$, p < 0.0001).

Prognostic value of IT^{contact} Tregs

By multivariate survival analysis, high numbers of $IT^{contact}$ Tregs were associated with a shorter disease-free interval (HR = 1.45, p = 0.049) and overall survival (HR = 1.41, p = 0.035), independently of tumor size and distant metastasis, and a shorter cancer-specific survival (HR = 1.44, p = 0.049) with pT, distant metastasis and PR as the covariates (Table 2).

The prognostic value of IT^{contact} Tregs was not observed in luminal FMCs, but was significant in triple-negative FMCs (Table 3).

Prognostic value of IT^{stroma} Tregs

By multivariate survival analysis, IT^{stroma} Tregs were associated with a shorter disease-free interval (HR = 1.49, p = 0.045), overall survival (HR = 1.42, p = 0.031), and specific survival (HR = 1.45, p = 0.044), independently of tumor size and distant metastasis (Table 4).

IT^{stroma} Tregs were also associated with an unfavorable outcome in the luminal subcohort, however not in triple-negative FMCs (Table 3).

Prognostic value of peritumoral Tregs

High numbers of peritumoral Tregs were associated with an unfavorable outcome in the 180 cats, in terms

$IT^{contact}$ Tregs (≥ 2 vs $<2/mm^2$)		Р	Odds ratio	95% CI
Tumor-associated inflammation	Absent to mild (N = 77)	0.005	1.00	-
	Moderate to severe (N = 103)		2.48	1.34-4.49
Squamous differentiation	Absent (N = 97)	0.014	1.00	-
	Present (N = 83)		2.20	1.21-4.01
IT ^{stroma} Tregs (≥6 vs <6/mm ²)		Ρ	Odds ratio	95% CI
Tumor-associated inflammation	Absent to mild $(N = 77)$	< 0.001	1.00	_
	Moderate to severe $(N = 103)$		5.52	2.67-11.42
Squamous differentiation	Absent (N = 97)	0.002	1.00	_
- 1	Present $(N = 83)$		2.83	1.51-5.32
Lymphovascular invasion	LVI– (N = 70)	0.032	1.00	_
, 1	LVI+(N=II0)		2.15	1.12-4.14
IT ^{contact} Tregs	$<2/mm^{2}$ (N = 99)	<0.001	1.00	_
-	$\geq 2/\text{mm}^2(N=81)$		15.14	6.97–32.90
Peritumoral Tregs (≥575 vs <575/mm²)		Р	Odds ratio	95% CI
Histological grade	Grade I–II (N = 92)	0.019	1.00	_
	Grade III (N = 88)		2.30	1.19-4.45
Lymphovascular invasion	LVI-(N = 70)	0.002	1.00	_
, ,	LVI+(N = 110)		2.90	1.50-5.59
Tumor-associated inflammation	Absent to mild $(N = 77)$	<0.001	1.00	_
	Moderate to severe ($N = 103$)		3.68	1.88–7.19
Pathologic tumor size	pT <20 mm (N = 85)	0.009	1.00	_
0	$pT \ge 20 \text{ mm} (N = 95)$		2.50	1.30-4.82
Pathologic nodal stage	$_{\rm P}N0 \ ({\rm N}=20)$	0.006	1.00	_
	PNX(N = 59)		1.57	0.57-4.36
	PN+(N=101)		3.81	1.40-10.35
Clinical stage	Stage I–II (N = 68)	0.003	1.00	-
	Stage III–IV (N = 112)		2.50	1.29–4.83
IT ^{contact} Tregs	$<2/mm^{2}(N = 99)$	0.004	1.00	-
	$\geq 2/mm^2$ (N = 81)		2.86	1.43–5.71
IT ^{stroma} Tregs	$< 6/mm^{2}(N = 116)$	<0.001	1.00	—
	≥ 6/mm² (N = 64)		8.63	3.22-23.10

Table 1. Associations between Treg numbers and clinicopathological features of the 180 feline mammary carcinomas.

CI: confidence interval; LVI: lymphovascular invasion; Treg: regulatory T cell.

Table 2.	Prognostic value of intratumoral	Tregs in direct contact with tumor cell	s (IT ^{contact} Tregs).
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Disease-free interval	Categories		ЦR	95% CI
	Categories	Ρ	TIIX	75% CI
IT ^{contact} Tregs	\geq 2 vs <2/mm ²	0.0489	1.45	1.00-2.15
Pathologic tumor size	pT ≥ 20 vs <20 mm	0.0200	1.57	1.08-2.29
Distant metastasis	M0 vs MI	<0.0001	0.19	0.09-0.41
	MX vs MI	<0.0001	0.17	0.08–0.36
Overall survival	Categories	Р	HR	95% CI
IT ^{contact} Tregs	\geq 2 vs <2/mm ²	0.0347	1.41	1.03–1.95
Pathologic tumor size	pT ≥ 20 vs <20 mm	0.0003	1.79	1.31-2.45
Distant metastasis	M0 vs M1	0.0068	0.35	0.17-0.75
	MX vs MI	0.0027	0.32	0.15–0.67
Cancer-specific survival	Categories	Р	HR	95% CI
IT ^{contact} Tregs	\geq 2 vs <2/mm ²	0.0495	1.44	1.00-2.08
Pathologic tumor size	pT ≥ 20 vs <20 mm	0.0014	1.81	1.26-2.60
Distant metastasis	M0 vs M1	0.0066	0.35	0.16-0.74
	MX vs MI	0.0007	0.27	0.13-0.57
Progesterone receptor	PR+ vs PR-	0.0177	0.25	0.08–0.78

HR: hazard ratio; CI: confidence interval; PR: progesterone receptor; Treg: regulatory T cell.

Multivariate survival analyses (180 cases).

		Luminal FMCs (N = 57)			Triple-negative FMCs (N = 123)		
Disease-free interval		Р	HR	95% CI	Р	HR	95% CI
IT ^{contact} Tregs IT ^{stroma} Tregs Peritumoral Tregs	$ \ge 2 \text{ vs } <2/\text{mm}^2 \ge 6 \text{ vs } <6/\text{mm}^2 \ge 575 \text{ vs } <575/\text{mm}^2 $	NS 0.037 0.053		_ 0.95–3.57 1.04–4.43	0.014 NS 0.030	1.12 - 1.71	1.04–1.20 _ 1.07–2.72
Overall survival		Р	HR	95% CI	Р	HR	95% CI
IT ^{contact} Tregs IT ^{stroma} Tregs Peritumoral Tregs	$\geq 2 \text{ vs } <2/\text{mm}^2$ $\geq 6 \text{ vs } <6/\text{mm}^2$ $\geq 575 \text{ vs } <575/\text{mm}^2$	NS 0.016 0.008	 1.88 2.29	 1.05–3.35 1.31–4.00	NS NS 0.018	_ _ I.60	_ _ 1.10–2.32
Cancer-specific survival		Р	HR	95% CI	Ρ	HR	95% CI
IT ^{contact} Tregs IT ^{stroma} Tregs Peritumoral Tregs	$ \ge 2 \text{ vs } <2/\text{mm}^2 \ge 6 \text{ vs } <6/\text{mm}^2 \ge 575 \text{ vs } <575/\text{mm}^2 $	NS 0.015 0.049	 2.08 2.02	 1.08–3.98 1.06–3.83	0.044 NS 0.011	1.08 _ 1.82	1.01–1.17 1.19–2.79

Table 3. Treg prognostic value in luminal and triple-negative FMCs.

FMC: feline invasive mammary carcinoma; NS: not significant; HR: hazard ratio; CI: confidence interval. Univariate survival analyses; Treg: regulatory T cell.

Table 4. Prognostic value of Tregs of the intratumoral stroma (IT^{stroma} Tregs).

Disease-free interval	Categories	Р	HR	95% CI
IT ^{stroma} Tregs	\geq 6 vs < 6/mm ²	0.0450	1.49	1.01-2.19
Pathologic tumor size	$pT \ge 20 \text{ vs} < 20 \text{ mm}$	0.0335	1.50	1.03-2.19
Distant metastasis	M0 vs M1	0.0001	0.20	0.09-0.43
	MX vs MI	<0.0001	0.17	0.08–0.36
Overall survival	Categories	Р	HR	95% CI
IT ^{stroma} Tregs	\geq 6 vs < 6/mm ²	0.0307	1.42	1.04-1.96
Pathologic tumor size	pT ≥ 20 vs <20 mm	0.0009	1.70	1.24-2.31
Distant metastasis	M0 vs MI	0.0066	0.35	0.16-0.74
	MX vs MI	0.0016	0.30	0.14–0.63
Cancer-specific survival	Categories	Р	HR	95% CI
IT ^{stroma} Tregs	\geq 6 vs < 6/mm ²	0.0443	1.45	1.01–2.07
Pathologic tumor size	pT ≥ 20 vs <20 mm	0.0007	1.85	1.30–2.64
Distant metastasis	M0 vs MI	0.0041	0.33	0.15-0.70
	MX vs MI	0.0002	0.24	0.11-0.51

HR: hazard ratio; CI: confidence interval; Treg: regulatory T cell.

Multivariate survival analyses (180 cases).

of disease-free interval (HR = 1.77, 95% CI: 1.22-2.58; p = 0.005; Figure 2(a)), overall survival (HR = 1.82, 95% CI: 1.33-2.48; p = 0.0004; Figure 2(b)), and cancer-specific survival (HR = 1.90, 95% CI: 1.33-2.72; p = 0.001; Figure 2(c)).

By multivariate survival analysis, peritumoral Tregs were associated with a shorter disease-free interval (HR = 1.71, p = 0.013) independently of pN (Table 5), poorer overall survival (HR = 1.58, p = 0.013) independently of pT, pN, and distant metastasis, and shorter specific survival (HR = 1.57, p = 0.034) after adjustment for pT, pN, M, and PR (Table 5).

Peritumoral Treg prognostic value was confirmed in both luminal and triple-negative FMCs (Table 3).

Peritumoral and intratumoral Tregs in triple-negative basal-like (TNBL) FMCs

Among the 93 TNBL FMCs, 39 (42%) were rich in IT^{contact} Tregs, 32 (34%) were rich in IT^{stroma} Tregs, and 63 (68%) were rich in peritumoral Tregs. In TNBL FMCs, IT^{stroma} Tregs were not significantly associated with outcomes, whereas IT^{contact} Tregs were associated with shorter disease-free interval and poorer overall



Figure 2. Kaplan–Meier survival curves of 180 cats according to peritumoral FoxP3+ regulatory T cells in their invasive mammary carcinomas: (a) disease-free interval, (b) overall survival, and (c) cancer-specific survival.

survival, independently of clinical stages (Table 6), and poorer cancer-specific survival after adjustment for pT, pN, and M (Table 6, Model 1).

Peritumoral Tregs surrounding TNBL FMCs were also associated with shorter specific survival by multivariate analysis, independently of distant metastasis (Table 6, Model 2), indicating that local immune suppression played a significant role in TNBL FMCs.

Discussion

The first objective of our study was to assess the numbers of FMC-associated Tregs. In this study, 148 FMCs (82%) contained at least 1 IT^{contact} Treg, and 165 FMCs (92%) contained at least 1 IT^{stroma} Treg; in human breast cancer, Liu et al.⁵⁰ reported that 45% (1475 out of 3277) of breast cancers contained at least 1 $IT^{contact}$ Treg, and 75% (2458 out of 3277) contained at least 1 IT^{stroma} Treg, identified by FoxP3 positivity: there are more feline than human mammary carcinomas that contain tumor-infiltrating Tregs.

The mean intratumoral Treg numbers within FMCs were $6 \pm 11 \text{ IT}^{\text{contact}}$ Tregs/mm², and $7 \pm 9 \text{ IT}^{\text{stroma}}$ Tregs/mm², for a mean total of 12 ± 18 intratumoral Tregs/mm². By comparison, Sun et al. reported a mean density of 72 IT^{contact} Tregs/mm² in 208 breast cancers:⁵¹ IT^{contact} Tregs appear more numerous in human than in feline mammary cancers. Expressed per HPF (400 ×), there were $1.7 \pm 3.3 \text{ IT}^{\text{contact}}$ Tregs/HPF and $2.1 \pm 2.9 \text{ IT}^{\text{stroma}}$ Tregs/HPF for a mean total of 3.9 ± 2.9 intratumoral Tregs/HPF within FMCs. By comparison in breast cancer, intratumoral Treg numbers are in the range of 5.5^{52} to 13.7^{53} per HPF: human breast cancers contain more intratumoral Tregs than FMCs.

However, the mean Treg numbers around FMCs were $1064 \pm 800/\text{mm}^2$, corresponding to 318 ± 241 peritumoral Tregs/HPF. By comparison in breast cancer, peritumoral Treg numbers are in the range of 6.4^{54} to 43.3^{53} per HPF: peritumoral Tregs are much more abundant in FMCs than in breast cancers.

In this study, the ratio of intratumoral to peritumoral Tregs was 1:344, compared to 1:1.7⁵⁴ and 1:3.2⁵³ in breast cancers: in the cat as in human breast cancer, peritumoral Tregs are more abundant than intratumoral Tregs, but FMCs contain much more peritumoral Tregs than intratumoral Tregs.

The second objective of this study was to correlate Treg numbers with the clinicopathological characteristics of FMCs. Higher peritumoral Treg infiltration was associated with larger pT size, as reported for intratumoral Treg numbers in breast cancer,²⁷ and with nodal metastasis, as described in breast cancer for intratumoral Tregs.^{27,50,55} Thus, in both feline and human mammary carcinomas, increasing numbers of Tregs were associated with advanced clinical stage.^{50,51} In this study, peritumoral Tregs around FMCs were positively associated with a higher histological grade, as reported in breast cancer, for intratumoral^{50,51,53-58} and peritumoral Tregs.^{54,56} Finally, in FMCs, Treg numbers were positively correlated with tumor-associated inflammation, visible on HES-stained sections, which was associated with poor prognosis in cats of the present study and in the veterinary literature.⁵⁹ Taking into account the fact that larger and/or higher stage carcinomas contained higher Treg numbers, our results suggest that tumor enlargement in cats is accompanied by an increase in Treg-enriched inflammation. This would suggest that larger FMCs were diagnosed in the "immune escape" phase of cancer immunoediting,^{60–62}

Disease-free interval	Categories	D	HR	95% CI
	6	Г		
Peritumoral Tregs	<575 vs ≥ 575/mm²	0.0132	0.58	0.38–0.89
Pathologic nodal stage	pN+ vs pN0	0.0189	2.12	1.14–3.95
	pN+ vs pNX	0.1985	1.31	0.87–1.98
Overall survival	Categories	Ρ	HR	95% CI
Peritumoral Tregs	<575 vs ≥ 575/mm ²	0.0128	0.63	0.44-0.91
Pathologic tumor size	$pT \ge 20 \text{ vs} < 20 \text{ mm}$	0.0096	1.52	1.11–2.09
Pathologic nodal stage	pN+ vs pN0	0.0152	1.88	1.13-3.12
5 5	pN+ vs pNX	0.0148	1.56	1.09-2.22
Distant metastasis	MI vs M0	0.0146	2.57	1.21–5.47
	MI vs MX	0.0030	3.10	1.47–6.51
Cancer-specific survival	Categories	Ρ	HR	95% CI
Peritumoral Tregs	$<$ 575 vs \geq 575/mm ²	0.0344	0.64	0.42–0.96
Pathologic tumor size $pT \ge 20 \text{ vs} < 20 \text{ mm}$		0.0233	1.53	1.06-2.20
Pathologic nodal stage	pN+ vs pN0	0.0070	2.29	1.26-4.19
6 6	pN+ vs pNX	0.0644	1.49	0.98-2.27
Distant metastasis	MI vs M0	0.0143	2.62	1.22-5.63
	MI vs MX	0.0007	3.76	1.76-8.03
Progesterone receptor	PR+ vs PR-	0.0272	0.27	0.08–0.86

Table 5. Prognostic value of peritumoral Tregs.

HR: hazard ratio; CI: confidence interval; PR: progesterone receptor; Treg: regulatory T cell. Multivariate survival analyses (180 cases).

Table 6. Treg prognostic value in triple-negative basal-like FMCs.

Disease-free interval		Р	HR	95% CI
IT ^{contact} Tregs	\ge 2 vs <2/mm ²	0.0007	1.14	1.06–1.22
Clinical stage	Stage I–II vs Stage III–IV	0.0028	0.43	0.25–0.75
Overall survival		Р	HR	95% CI
IT ^{contact} Tregs	≥ 2 vs <2/mm²	0.0032	1.12	1.04–1.20
Clinical stage	Stage I–II vs Stage III–IV	<0.0001	0.38	0.24–0.61
Cancer-specific survival—Mode	el I	Р	HR	95% CI
IT ^{contact} Tregs	≥ 2 vs <2/mm ²	0.0060	1.11	1.03–1.19
Pathologic tumor size	≥ 20 vs <20 mm	0.0440	1.68	1.02–2.79
Pathologic nodal stage	pN+ vs pN0–pNX	0.000 I	2.76	1.65–4.61
Distant metastasis	MI vs M0–MX	0.0394	2.72	1.05–7.03
Cancer-specific survival—Mode	el 2	Р	HR	95% CI
Peritumoral Tregs	≥ 575 vs <575/mm²	0.0490	1.72	1.01–2.95
Distant metastasis	MI vs M0–MX	0.0154	3.20	1.25–8.15

FMC: feline invasive mammary carcinomas; HR: hazard ratio; CI: confidence interval.

Multivariate survival analyses (93 cases).

in which local immune suppression surpasses antitumoral immunity.

The third objective of this study was to assess Treg prognostic significance in FMCs. Intratumoral Tregs were associated with a worse outcome in terms of disease-free interval, overall survival and cancer-specific survival by multivariate survival analyses in the FMCs studied. IT^{stroma} Tregs were similarly associated with unfavorable outcome in the subcohort of luminal FMCs. In human breast cancer, the presence of intratumoral Tregs, especially in luminal breast cancers, is also associated with poorer prognosis.^{53,55,56,63}

However, peritumoral Tregs had a stronger prognostic value than intratumoral Tregs in cats of this study. Peritumoral Tregs were associated with shorter diseasefree interval, overall survival, and cancer-specific survival by multivariate analyses, including in luminal FMCs considered alone, as reported in breast cancer, and in ER+ breast cancer in particular.⁵⁶

Specifically, in triple-negative FMCs, our results showed that both peritumoral and IT^{contact} Tregs were associated with poorer disease-free interval and cancerspecific survival, whereas in human breast cancer, higher numbers of FoxP3+ Tregs have been associated with a prolonged relapse-free interval⁶⁴ and better overall survival.⁶⁵ Two hypotheses might contribute to explain this discrepancy. First, women with triple-negative breast cancer receive chemotherapy, contrary to the present cats, and Tregs in breast cancer have been associated with better responses to neoadjuvant chemotherapy.^{66,67} Second, the immune microenvironment of triple-negative mammary carcinomas might not be identical between queens and women. In cats, there are no screening programs: all mammary carcinomas were diagnosed because they were symptomatic (visible or palpable), thus at a late stage. Later stages were associated with higher Treg numbers, suggesting that most FMCs might be diagnosed in the "immune escape" phase of cancer immunoediting. In women, breast cancer screening programs allow for early detection, and it is likely that most breast cancers are diagnosed in the "equilibrium" phase of cancer immunoediting, as high numbers of Tregs have been associated with high numbers of CD8+ cytotoxic T cells,⁶⁴ which have favorable prognostic influence in breast cancer.^{68–70} We attempted to identify CD8+ cytotoxic T cells in FMCs, but the commercially available monoclonal antibodies that we tested (clones vpg9, C8/1779R, and SP57) either do not cross-react with feline CD8, or do not work on formalin-fixed, paraffin-embedded tissues.

The last objective of this study was to investigate the prognostic significance of Tregs in the subgroup of TNBL FMCs, because TNBL breast cancers have been subdivided into BLIS and BLIA carcinomas or equivalent subtypes by gene expression analysis.¹⁸⁻²² We found that TNBL FMCs enriched in either peritumoral or IT^{contact} Tregs were associated with poorer prognosis, a finding in agreement with the shorter diseasefree survival and cancer-specific survival reported for BLIS breast cancers compared to BLIA breast cancers.¹⁸ In this study, most TNBL FMCs (63 out of 93, 68%) were enriched in peritumoral Tregs: most TNBL FMCs seem to be diagnosed in the "immune escape" phase of cancer immunoediting, and these carcinomas might correspond to BLIS breast cancers, in which the immune microenvironment is protumoral, and in which immunosuppressive Tregs may play a major role.

Conclusion

FoxP3+ Tregs were abundant in feline invasive mammary carcinomas, especially in peritumoral areas, were associated with advanced stage at diagnosis, and had negative prognostic value, especially in the triplenegative basal-like subgroup, which may contain a Treg-enriched "BLIS" subtype. Female cats with FMCs may thus be interesting spontaneous animal models to investigate new strategies of cancer immunotherapy that target Tregs in an immune-suppressed tumor microenvironment.

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Author contributions

Conceptualization, project administration, and supervision: J.A., M.C., and F.N. Format analysis, investigation, methodology: E.D., L.S., D.L., and F.N. Funding acquisition: J.A., M.C., and F.N. Writing, original draft preparation: E.D. and F.N. Writing, review, and editing: L.S., J.A., D.L., M.C., and F.N.

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Availability of data and materials

The datasets used and analyzed during this study are available from the corresponding author on reasonable request.

Supplemental material

Supplemental material for this article is available online.

References

- 1. Hayes AA and Mooney S. Feline mammary tumors. Vet Clin North Am Small Anim Pract 1985; 15: 513–520.
- 2. Misdorp W and Weijer K. Animal model of human disease: breast cancer. *Am J Pathol* 1980; 98: 573–576.
- Castagnaro M, Casalone C, Bozzetta E, et al. Tumour grading and the one-year post-surgical prognosis in feline mammary carcinomas. *J Comp Pathol* 1998; 119(3): 263–275.
- Castagnaro M, Casalone C, Ru G, et al. Argyrophilic nucleolar organiser regions (AgNORs) count as indicator of post-surgical prognosis in feline mammary carcinomas. *Res Vet Sci* 1998; 64(2): 97–100.
- Castagnaro M, De Maria R, Bozzetta E, et al. Ki-67 index as indicator of the post-surgical prognosis in feline mammary carcinomas. *Res Vet Sci* 1998; 65(3): 223–226.
- Ito T, Kadosawa T, Mochizuki M, et al. Prognosis of malignant mammary tumor in 53 cats. J Vet Med Sci 1996; 58(8): 723–726.
- Millanta F, Calandrella M, Citi S, et al. Overexpression of HER-2 in feline invasive mammary carcinomas: an immunohistochemical survey and evaluation of its prognostic potential. *Vet Pathol* 2005; 42(1): 30–34.
- Seixas F, Palmeira C, Pires MA, et al. Grade is an independent prognostic factor for feline mammary carcinomas: a clinicopathological and survival analysis. *Vet J* 2011; 187(1): 65–71.
- Gimenez F, Hecht S, Craig LE, et al. Early detection, aggressive therapy: optimizing the management of feline mammary masses. J Feline Med Surg 2010; 12(3): 214–224.
- Brunetti B, Asproni P, Beha G, et al. Molecular phenotype in mammary tumours of queens: correlation between primary tumour and lymph node metastasis. *J Comp Pathol* 2013; 148(2–3): 206–213.
- 11. Burrai GP, Mohammed SI, Miller MA, et al. Spontaneous feline mammary intraepithelial lesions as a model for human estrogen receptor- and progesterone receptornegative breast lesions. *BMC Cancer* 2010; 10: 156.
- Caliari D, Zappulli V, Rasotto R, et al. Triple-negative vimentin-positive heterogeneous feline mammary carcinomas as a potential comparative model for breast cancer. *BMC Vet Res* 2014; 10: 185.
- De Maria R, Olivero M, Iussich S, et al. Spontaneous feline mammary carcinoma is a model of HER2 overexpressing poor prognosis human breast cancer. *Cancer Res* 2005; 65(3): 907–912.

- Millanta F, Calandrella M, Vannozzi I, et al. Steroid hormone receptors in normal, dysplastic and neoplastic feline mammary tissues and their prognostic significance. *Vet Rec* 2006; 158(24): 821–824.
- Rasotto R, Caliari D, Castagnaro M, et al. An immunohistochemical study of HER-2 expression in feline mammary tumours. J Comp Pathol 2011; 144(2–3): 170–179.
- Soares M, Correia J, Rodrigues P, et al. Feline HER2 protein expression levels and gene status in feline mammary carcinoma: optimization of immunohistochemistry (IHC) and in situ hybridization (ISH) Techniques. *Microsc Microanal* 2013; 19(4): 876–882.
- Wiese DA, Thaiwong T, Yuzbasiyan-Gurkan V, et al. Feline mammary basal-like adenocarcinomas: a potential model for human triple-negative breast cancer (TNBC) with basal-like subtype. *BMC Cancer* 2013; 13: 403.
- Burstein MD, Tsimelzon A, Poage GM, et al. Comprehensive genomic analysis identifies novel subtypes and targets of triple-negative breast cancer. *Clin Cancer Res* 2015; 21(7): 1688–1698.
- Jézéquel P, Loussouarn D, Guerin-Charbonnel C, et al. Gene-expression molecular subtyping of triple-negative breast cancer tumours: importance of immune response. *Breast Cancer Res* 2015; 17: 43.
- Jézéquel P, Kerdraon O, Hondermarck H, et al. Identification of three subtypes of triple-negative breast cancer with potential therapeutic implications. *Breast Cancer Res* 2019; 21(1): 65.
- Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest* 2011; 121(7): 2750–2767.
- Lehmann BD, Jovanovic B, Chen X, et al. Refinement of triple-negative breast cancer molecular subtypes: implications for neoadjuvant chemotherapy selection. *PLoS ONE* 2016; 11(6): e0157368.
- DiPaolo RJ, Glass DD, Bijwaard KE, et al. CD4+CD25+ T cells prevent the development of organspecific autoimmune disease by inhibiting the differentiation of autoreactive effector T cells. *J Immunol* 2005; 175(11): 7135–7142.
- Josefowicz SZ and Rudensky A. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 2009; 30(5): 616–625.
- Kryczek I, Liu R, Wang G, et al. FoxP3 defines regulatory T cells in human tumor and autoimmune disease. *Cancer Res* 2009; 69(9): 3995–4000.
- Bohling SD and Allison KH. Immunosuppressive regulatory T cells are associated with aggressive breast cancer phenotypes: a potential therapeutic target. *Mod Pathol* 2008; 21(12): 1527–1532.
- Maeda N, Yoshimura K, Yamamoto S, et al. Expression of B7-H3, a potential factor of tumor immune evasion in combination with the number of regulatory T cells, affects against recurrence-free survival in breast cancer patients. *Ann Surg Oncol* 2014; 21(Suppl. 4): S546–S554.
- Shou J, Zhang Z, Lai Y, et al. Worse outcome in breast cancer with higher tumor-infiltrating FoxP3+ Tregs: a systematic review and meta-analysis. *BMC Cancer* 2016; 16: 687.

- 29. Xu Y, Lan S and Zheng Q. Prognostic significance of infiltrating immune cell subtypes in invasive ductal carcinoma of the breast. *Tumori* 2018; 104(3): 196–201.
- 30. Yan M, Jene N, Byrne D, et al. Recruitment of regulatory T cells is correlated with hypoxia-induced CXCR4 expression, and is associated with poor prognosis in basal-like breast cancers. *Breast Cancer Res* 2011; 13(2): R47.
- Fontenot JD, Gavin MA and Rudensky AY. FoxP3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003; 4: 330–336.
- Hori S, Nomura T and Sakaguchi S. Control of regulatory T cell development by the transcription factor FoxP3. *Science* 2003; 299: 1057–1061.
- Khattri R, Cox T, Yasayko SA, et al. An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 2003; 4: 337–342.
- Lankford S, Petty C, LaVoy A, et al. Cloning of feline FoxP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol* 2008; 122(1–2): 159–166.
- Petty CS, Tompkins MB and Tompkins WA. Transforming growth factor-beta/transforming growth factorbetaRII signaling may regulate CD4+CD25+ T-regulatory cell homeostasis and suppressor function in feline AIDS lentivirus infection. J Acquir Immune Defic Syndr 2008; 47(2): 148–160.
- Dagher E, Abadie J, Loussouarn D, et al. Bcl-2 expression and prognostic significance in feline invasive mammary carcinomas: a retrospective observational study. BMC Vet Res 2019; 15(1): 25.
- Morris J. Mammary tumors in the cat: size matters, so early intervention saves lives. *J Feline Med Surg* 2013; 15: 391–400.
- Owen LN. TNM classification of tumours in domestic animals (ed LN Owen). Geneva: World Health Organization, http://www.who.int/iris/handle/10665/68618 (1980, accessed 5 July 2019).
- Elston CW and Ellis IO. Pathological prognostic factors in breast cancer: I—the value of histological grade in breast cancer: experience from a large study with longterm follow-up (Republished from Elston CW and Ellis IO. *Histopathology* 1991; 19; 403–410). *Histopathology* 2002; 41: 151–153.
- Dagher E, Abadie J, Loussouarn D, et al. Feline invasive mammary carcinomas: prognostic value of histological grading. *Vet Pathol* 2019; 56(5): 660–670.
- Moriya T, Kanomata N, Kozuka Y, et al. Usefulness of immunohistochemistry for differential diagnosis between benign and malignant breast lesions. *Breast Cancer* 2009; 16(3): 173–178.
- 42. Shamloula MM, El-Shorbagy SH and Saied EM. P63 and cytokeratin8/18 expression in breast, atypical ductal hyperplasia, ductal carcinoma in situ and invasive duct carcinoma. J Egypt Natl Canc Inst 2007; 19(3): 202–210.
- Gama A, Alves A and Schmitt F. Identification of molecular phenotypes in canine mammary carcinomas with clinical implications: application of the human classification. *Virchows Arch* 2008; 453(2): 123–132.
- Guil-Luna S, Sanchez-Cespedes R, Millan Y, et al. Aglepristone decreases proliferation in progesterone receptor-

positive canine mammary carcinomas. J Vet Intern Med 2011; 25(3): 518–523.

- 45. Nguyen F, Peña L, Ibisch C, et al. Canine invasive mammary carcinomas as models of human breast cancer part 1: natural history and prognostic factors. *Breast Cancer Res Treat* 2018; 167: 635–648.
- Yi M, Huo L, Koenig KB, et al. Which threshold for ER positivity? A retrospective study based on 9639 patients. *Ann Oncol* 2014; 25(5): 1004–1011.
- 47. Ono M, Tsuda H, Yunokawa M, et al. Prognostic impact of Ki-67 labeling indices with 3 different cutoff values, histological grade, and nuclear grade in hormone-receptor-positive, HER2-negative, node-negative invasive breast cancers. *Breast Cancer* 2015; 22(2): 141–152.
- Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol 2013; 31: 3997–4013.
- Soares M, Correia J, Peleteiro MC, et al. St Gallen molecular subtypes in feline mammary carcinoma and paired metastases-disease progression and clinical implications from a 3-year follow-up study. *Tumour Biol* 2016; 37(3): 4053–4064.
- 50. Liu S, Foulkes WD, Leung S, et al. Prognostic significance of FoxP3+ tumor-infiltrating lymphocytes in breast cancer depends on estrogen receptor and human epidermal growth factor receptor-2 expression status and concurrent cytotoxic T-cell infiltration. *Breast Cancer Res* 2014; 16(5): 432.
- Sun S, Fei X, Mao Y, et al. PD-1(+) immune cell infiltration inversely correlates with survival of operable breast cancer patients. *Cancer Immunol Immunother* 2014; 63(4): 395–406.
- Oda N, Shimazu K, Naoi Y, et al. Intratumoral regulatory T cells as an independent predictive factor for pathological complete response to neoadjuvant paclitaxel followed by 5-FU/epirubicin/cyclophosphamide in breast cancer patients. *Breast Cancer Res Treat* 2012; 136(1): 107–116.
- 53. Liu F, Lang R, Zhao J, et al. CD8⁺ cytotoxic T cell and FoxP3⁺ regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat* 2011; 130(2): 645–655.
- Kim S, Lee A, Lim W, et al. Zonal difference and prognostic significance of FoxP3 regulatory T cell infiltration in breast cancer. *J Breast Cancer* 2014; 17(1): 8–17.
- 55. Bates GJ, Fox SB, Han C, et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. J Clin Oncol 2006; 24(34): 5373–5380.
- 56. Gobert M, Treilleux I, Bendriss-Vermare N, et al. Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Res* 2009; 69(5): 2000–2009.
- 57. Seo AN, Lee HJ, Kim EJ, et al. Tumour-infiltrating CD8+ lymphocytes as an independent predictive factor for pathological complete response to primary systemic therapy in breast cancer. *Br J Cancer* 2013; 109(10): 2705–2713.

- Takenaka M, Seki N, Toh U, et al. FoxP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. *Mol Clin Oncol* 2013; 1(4): 625–632.
- Weijer K and Hart AA. Prognostic factors in feline mammary carcinoma. J Natl Cancer Inst 1983; 70(4): 709–716.
- Block MS and Markovic SN. The tumor/immune interface: clinical evidence of cancer immunosurveillance, immunoediting and immunosubversion. *Am J Immunol* 2009; 5: 29–40.
- Dunn GP, Old LJ and Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004; 21(2): 137–148.
- Dunn GP, Old LJ and Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004; 22: 329–360.
- Qian F, Qingping Y, Linquan W, et al. High tumorinfiltrating FoxP3+ T cells predict poor survival in estrogen receptor-positive breast cancer: a meta-analysis. *Eur J Surg Oncol* 2017; 43(7): 1258–1264.
- 64. West NR, Kost SE, Martin SD, et al. Tumour-infiltrating FoxP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in

oestrogen receptor-negative breast cancer. Br J Cancer 2013; 108(1): 155–162.

- 65. Lee S, Cho EY, Park YH, et al. Prognostic impact of FoxP3 expression in triple-negative breast cancer. *Acta Oncol* 2013; 52(1): 73–81.
- 66. De Kruijf EM, van Nes JG, Sajet A, et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010; 16(4): 1272–1280.
- Lee HJ, Seo JY, Ahn JH, et al. Tumor-associated lymphocytes predict response to neoadjuvant chemotherapy in breast cancer patients. *J Breast Cancer* 2013; 16(1): 32–39.
- Baker K, Lachapelle J, Zlobec I, et al. Prognostic significance of CD8+ T lymphocytes in breast cancer depends upon both oestrogen receptor status and histological grade. *Histopathology* 2011; 58(7): 1107–1116.
- Mahmoud SM, Paish EC, Powe DG, et al. Tumour-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 2011; 29: 1949–1955.
- West NR, Milne K, Truong PT, et al. Tumour-infiltrating lymphocytes predict response to anthracycline-based chemotherapy in oestrogen receptor-negative breast cancer. *Breast Cancer Res* 2011; 13: R126.