1 **TITLE: Diagnostic Significance of Intratumoral CD8+ Tumor Infiltrating Lymphocytes in Medullary Carcinoma.**

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ABSTRACT

Invasive ductal carcinomas of breast with marked stromal lymphocytic infiltration have come to be classified as lymphocyte predominant breast cancer (LPBC) because it obtains high pathological complete response rates with neoadjuvant chemotherapy. Medullary carcinoma (MC), which is independent from LPBC, is a rare histological subtype of invasive breast carcinoma accompanied by abundant lymphoplasmacytic infiltration as LPBC. Although MC shows marked cellular and structural atypia, it usually has a favorable outcome. It is occasionally difficult to distinguish MC from LPBC because both subtypes have nonspecific morphological features according to the present diagnostic criteria. Herein, we adopted multiplexed fluorescent immunohistochemistry to perform quantitative and simultaneous analyses of tumor infiltrating lymphocytes (TILs) considering their spatial distribution and examined focal immune reaction differences between MC and LPBC.

We found that CD8+ TILs are predominant in the intratumoral region while CD4+ TILs are less common in MC. In non-luminal type cancers, the numbers of stromal and intratumoral CD8+ TILs were significantly higher in MC than in LPBC. Stratified analyses by CD4+ TIL subsets showed robust infiltration of intratumoral CD8+ TILs in non-luminal type MC even in suppressive environments, such a low T helper 1 (Th1) / regulatory T cell (Treg) ratio.

Our results suggest that extensive intratumoral CD8+ TIL infiltration might well be a promising biomarker for distinguishing MC from LPBC, especially in non-luminal type cancers. Intratumoral CD8+ TILs and non-luminal intrinsic subtypes may serve as diagnostic characteristics allowing reliable histological criteria to be established for reproducibly diagnosing MC.

HIGHLIGHTS

- Both MC and LPBC accompany with abundant TILs and have favorable prognosis.
- There is still difficulty to distinguish MC from LPBC.
- Extensive intratumoral CD8+ TIL infiltration even in immuno-suppressive environments is the character of non-Luminal type of MC.
- Suitable diagnostic criteria for MC might prevent overtreatment.

1 **1.INTRODUCTION**

2 Breast cancers are the most common malignancies in middle-aged women.
3 Neoadjuvant chemotherapy may be chosen for breast cancer considering intrinsic subtype
4 of the cancer, presence of lymph node metastasis, or progress of the tumor in local site
5 because pathological complete response by neoadjuvant chemotherapy is associated with
6 favorable survival time [1].

7 It is well known that local immune reactions modify the biological behaviors of
8 cancers in various organs. As for breast cancer, the existence of tumor infiltrating
9 lymphocytes (TILs) are correlates with good outcomes and favorable therapeutic effects,
10 particularly in non-luminal type cancers that are negative for estrogen receptor (ER)
11 expression [2-5]. Breast cancers with marked stromal lymphocytic infiltration showing a
12 high pathological complete response rate to neoadjuvant chemotherapy consequently
13 have come to be classified as lymphocyte predominant breast cancer (LPBC) [6-9].

14 However, confusingly, there is a special histological subtype of breast cancer called
15 medullary carcinoma (MC) which also accompanies with marked lymphocytic infiltration
16 as LPBC. MC of the breast is a rare histologic type accounting for approximately 5% of
17 all breast cancers [10,11]. The latest diagnostic criteria for MC from the World Health
18 Organization (WHO) [12] are simplified and recommend the inclusion of all three types
19 of carcinoma showing MC-type morphology (classical MC which completely conforms
20 to Ridolfi's criteria [13] , atypical MCs not meeting all of Ridolfi's criteria, and invasive
21 carcinoma with medullary features) in the "carcinoma with medullary features" category,
22 because difficulty remains in obtaining reproducible and accurate diagnostic results for
23 this infrequent histological subtype.

24 MC is associated with abundant lymphoplasmacytic infiltration in the cancer

stroma and into cancer cell nests, like LPBC, though MC is recognized as being independent from LPBC because of its characteristic biological behaviors [6]. Although MCs show high-grade cancer cell morphology that is equivalent to Grade 3 of conventional invasive ductal carcinomas, MC is characterized by having a more favorable prognosis [14-16].

Among the various immune cells functioning in the cancer microenvironment, CD8+ T-cells, representative TILs that infiltrate or surround cancer cell nests, have been shown to affect the biological behaviors of cancers [17,18]. High levels of CD8+ TIL infiltration are well known to be associated with more favorable outcomes for patients with various types of cancer, and scoring of CD8+ TILs is thought to be a promising standard for evaluating the malignant potential of sporadic cancers [19,20]. Prior studies found that the majority of TILs in MCs are mature T-cells comprised mainly of CD8+ TILs, suggesting the existence of a cytotoxic immune reaction against malignant cells [21-23].

CD8+ T-cell functions are coordinated by CD4+ T-cell subsets. CD4+ T-cells comprise a variety of functional subsets including T helper 1 (Th1) cells, Th2 cells, induced regulatory T-cells (Tregs), regulatory type 1 cells (Tr1), Th17 cells, follicular helper T-cells (Tfh), Th22 cells, and Th9 cells [24,25]. Each of these CD4+ T-cell subsets mediates immune reactions via its inherent characteristic cytokine production. Among these subsets, Th1 and Treg are representative CD4+ subsets that control CD8+ T-cell functions. Th1 cells activate CD8+ T-cells via interferon-gamma production and Tregs suppress CD8+ T cell functions by producing TGF-beta and IL-10. CD4+ TILs also infiltrate MC [15,21,22], though histological studies have not generally examined CD4+ T-cell subsets, the exception being a rather small number of investigations focusing on

1 the significance of the Tregs infiltrating MCs [15,26].

2 Hematoxylin and eosin (H&E) stained specimens are commonly evaluated to
3 determine LPBC [7,9,27]. According to the International TILs Working Group, it is
4 recommended that TILs of breast cancers be evaluated by determining the percentage of
5 stromal TILs occupying an area [6]. Evaluation of TILs by H&E staining requires
6 extensive experience, and a simpler yet reliable rating system for TILs is thus required.
7 In this study, we adopted quantitative and simultaneous immunohistochemical analyses
8 employing multiplexed fluorescent immunohistochemistry to evaluate multiple types of
9 TILs including CD8+ cytotoxic T-lymphocytes and subsets of CD4+ T-lymphocytes
10 considering their spatial distribution. We explored unusual immunological features of
11 MCs as compared with LPBC to develop a diagnostic standard for MC based on
12 immunological features. Our goal was to resolve the diagnostic difficulties associated
13 with this rare histological subtype.

14

15 **2.Materials and Methods**

16 **2.1. Patients**

17 With approval from institutional ethics committee, breast cancer tissue specimens of
18 typical medullary carcinoma (TMC), atypical medullar carcinoma (AMC), invasive
19 carcinoma of no special type with medullary features (NSTM), and LPBC, obtained at
20 Juntendo University Hospital between 1997 and 2015, were studied retrospectively. The
21 sample size was not statistically determined because of the exploratory nature of this
22 study. TMC, AMC and NSTM were defined according to the criteria of the WHO
23 Histologic Classification Tumors of the Breast 4th edition [12]. Invasive ductal carcinoma
24 with prominent lymphocyte infiltration involving more than 50% of stromal lymphocytic

infiltration was defined as LPBC. For NSTM and LPBC, Grade 3 (high-grade) cancers according to the Scarff-Bloom-Richardson grading system [28], with a high Ki67 labeling index (≧30%), were collected selectively to match their morphological features with those of MC.TMC and AMC were categorized as MC [29], while NSTM were grouped into the LPBC category in this study. HE-stained specimens were independently read by an experienced pathologist (E. S. from Tokyo Medical University) and cases showing diagnostic agreement were enrolled in the cohort.

2.2. Multiplexed Fluorescent Immunohistochemistry

Multiplexed fluorescent immunohistochemistry was performed by the Tyramide Signal Amplification (TSA) method using an Opal IHC kit (PerkinElmer, Waltham, MA) according to the manufacturer's instructions. Tissue sections, 3 micrometers in thickness, were cut from formalin-fixed paraffin-embedded (FFPE) tumor specimens and then baked at 60℃ onto adhesive glass slides for 30 min before deparaffinization. The primary antibodies used were anti-human CD4 (clone 4B12, DAKO, Glostrup, Denmark, working concentration (WC) 10 μg/ml), anti-human CD8 (clone C8/144b, DAKO, WC 12 μg/ml), anti-human FoxP3 (clone 236A/E7, Abcam, Cambridge, UK, WC 8 μg/ml), anti-human T-bet (Santa Cruz Biotechnology, Dallas TX, WC 8 μg/ml), and cytokeratin (clone AE1/ AE3, DAKO, WC 15 μg/ml). Tris-EDTA (pH9) buffer was used for microwave heating before CD4, Foxp3 and T-bet labeling, while AR6 buffer (PerkinElmer) was adopted for CD8 and cytokeratin. Opal 520, 650, 540, 570 and 690 fluorophores were used for labeling of CD4, CD8, Foxp3, T-bet, and cytokeratin, respectively. AR6 buffer and opal fluorophores are components of the OPAL IHC labeling kit (PerkinElmer). A horseradish peroxidase labeled secondary detection system (EnVision plus, DAKO) was employed as a catalyst for fluorophore-conjugated tyramide.

Microwave heating was performed for primary antigen unmasking and for antibody removal after each fluorescent labeling.

2.3. Image Analysis and Quantification

Multiplexed fluorescent labeled images of three randomly selected fields (669x500 micrometer each) were captured with an automated imaging system (Vectra ver. 3.0 PerkinElmer). An image analyzing software program (InForm, PerkinElmer) was used to segment cancer tissue into cancer cell nests (intratumoral) and the framework (stromal) region, and to detect immune cells with specific phenotypes. Training sessions for tissue segmentation and phenotype recognition were repeated until the algorithm reached the level of confidence recommended by the program supplier (at least 90% accuracy) before performing the final evaluation. Infiltrating immune cells were quantified using an analytic software program (Spotfire, TIBCO, Palo Alto, CA), and then calculated per area.

2.4. Statistical analysis

As the TIL count distributions were skewed rightward, the Mann-Whitney U test was employed for statistical analysis to compare the numbers of TILs between MC and LPBC patients. Statistical analyses were performed employing SPSS Statistics ver.22 (IBM, Armonk, NY). A p-value less than 0.05 was considered to indicate a statistically significant difference.

3. RESULTS

Patient characteristics are summarized in Table 1. The breast cancer patients in this cohort were all women, including 24 with MC and 17 with LPBC. No patients had synchronous distant metastasis at the time of diagnosis, while approximately 17 % of

patients in both subgroups had axillary lymph node metastasis. Metachronous distant metastasis was found in 2 of the 24 MC patients with the triple negative breast cancer (TNBC) phenotype during observation following surgery and adjuvant chemotherapy, and both died due to their cancers. As for intrinsic subtypes, luminal type cancer accounted for 30-40% of both MC and LPBC, and non-luminal type cancers were predominant.

3.1. Distributions of CD4+ and CD8+ TILs in MC and LPBC

Representative images are shown on Figure 1. Multiplex fluorescent immunohistochemistry allowed us to simultaneously examine cytokeratin expressions on cancer cells along with CD4+ and CD8+ lymphocyte infiltration and to evaluate TILs infiltrating the intratumoral and stromal regions independently in the same field.

Although the difference was not considered to be statistically significant between MC and LPBC in the numbers of CD4+ and CD8+ TILs in either the intratumoral or the stromal region (Figure 2), CD4+ TILs were revealed to predominantly infiltrate the stromal area in both MC and LPBC (Figure 3A, 3B), while CD8+ TILs infiltrated mainly the intratumoral area in MC (Figure 3C). Such a distribution of intratumoral CD8+ TILs is not observed in LPBC (Figure 3D), and selective CD8+ TIL infiltration of the intratumoral region is thus suggested to be characteristic of MC. We next aimed to perform detailed examinations of CD8+ TILs in these cancers, stratified according to their intrinsic subtypes.

3.2. TIL distribution differences among ER status

Because the presented cohort includes few MC cases, since MC is a rare histological type, we simply classified MC and LPBC into two subgroups based on the presence of ER expression. ER+ cases, including those with ER+/HER2+ tumors were

1 classified into the luminal type, whereas ER- cases were grouped into the non-luminal
2 type consisting of TNBC and HER2 type cancers.
3 Our findings revealed the number of stromal and intratumoral CD8+ TILs to be
4 significantly higher in MC than in LPBC in non-luminal type cancer (Figure 4A, 4B),
5 while the absence of any significant difference between MC and LPBC in CD8+ TILs
6 was confirmed for luminal type cancer (Figure 4C,4D). Regarding CD4+ TILs, there
7 was no difference between MC and LPBC in either the luminal or the non-luminal
8 subtype (data not shown). However, the intratumoral CD8/ CD4 ratio was significantly
9 higher in MC than in LPBC in the non-luminal subgroup (Figure 4F). Although the
10 higher intratumoral CD8/ CD4 ratio in non-luminal type MC might simply reflect a
11 high frequency of intratumoral CD8+ TIL, considering the likelihood of a high
12 frequency of intratumoral CD8+ TILs being highly characteristic of non-luminal MC,
13 and that CD8+ TILs might by modulated by CD4+ TILs, we analyzed the CD4+ TIL
14 subset in non-luminal breast cancer specimens.

15 **3.3. The balance between CD4+ TIL subsets regulates intratumoral CD8+ TILs**

16 We established a sub-cohort composed of non-luminal breast cancer cases to
17 evaluate CD4+ T-cell subset infiltration. Based on their transcription factor expressions,
18 Th1 cells were defined as CD4+ T-bet + and Treg as CD4+ Foxp3+. Th1, Treg, and
19 CD8+ TIL infiltrations were simultaneously evaluated by multispectral imaging of both
20 the intratumoral and the stromal region (Figure 5). T-bet expressions by CD8+ TILs
21 (Figure 5C) and by CD4+ TILs (Figure 5A) were accurately detected with sufficient
22 reproducibility by the well-trained cell phenotyping software program (Supplementary
23 Fig.S1). Statistical analysis showed that there were no significant differences in the
24 numbers of infiltrating Th1 and Treg cells between MC and LPBC (Supplementary

1 Fig.S2 A-D). We additionally evaluated the Th1/Treg ratio, but the differences did not
2 reach statistical significance such that this parameter could not be used to characterize
3 these two groups. (Supplementary Fig.S2 E, F) Next, we stratified non-luminal cancers
4 into two groups according to the median value of the Th1/Treg ratio and examined
5 CD8+ TIL infiltration accordingly. The difference in intratumoral CD8+ TIL infiltration
6 between MC and LPBC was statistically significant in the subgroup with lower stromal
7 and intratumoral Th1/Treg ratios (Treg predominant), while there was no difference in
8 intratumoral CD8+ TIL infiltration in the subgroup with a high Th1/Treg (Th1
9 predominant) ratio (Figure 6).
10
11 **4. DISCUSSION**
12 In this study, we revealed that intratumoral CD8+ TILs to show more marked
13 infiltration than CD4+ TILs in MC. Several studies have evaluated stromal and
14 intratumoral TILs separately [6]. As to breast cancer studies, Mahmoud et al. reported the
15 prognostic significance of stromal CD8+ TILs [30], whereas Liu et al. indicated the
16 significance of intratumoral CD8+ TILs as a predictor of favorable outcomes [31].
17 Furthermore, Loi and Denkert et al. described both stromal and intratumoral CD8+ TILs
18 as being associated with favorable outcomes [2,8]. A lthough our results do not allow
19 definitive conclusions to be drawn, the predominance of CD8+ TILs in the intratumoral
20 region was suggested to be characteristic of MC. Regarding stromal TILs, CD4+
21 predominated over CD8+ TILs in both MC and LPBC. It would be immunologically
22 consistent for stromal CD4+ TILs to regulate or activate intratumoral CD8+ TILs that
23 directly damage target cancer cells.
24 We also conducted analyses taking ER status into consideration. In ER- non-

luminal type cancers, we found that the number of CD8+ TILs to be significantly higher in both the stromal and the intratumoral region in MC than in LPBC, whereas such differences were not detected in ER+ luminal type cancers. Previous reports also indicated that more marked TIL infiltration is observed in TNBC, which is of the non-luminal type, than in luminal type cancers [2]. The inhibitory immunologic features of luminal type cancers, including suppressive gene expressions involving HLA class I, CD3+ and/or CD8+, have also been reported [32,33]. Furthermore, non-luminal type cancers, including MC and TNBC, are reportedly associated with genomic instability or mutations of TP53, BRCA1 and PI3CA genes [34,35]. Such characteristic phenotypes of non-luminal cancer might be related to the production of immunogenic neoantigens, and might have been the source of the high frequency of intratumoral CD8+ TIL infiltration in non-luminal MC.

We found the intratumoral CD8/CD4 ratio to be significantly higher in MC than in LPBC in the non-luminal subgroup. The CD8/CD4 ratio can be regarded as reflecting the balance between direct cell damage and indirect control. We analyzed CD4+ TIL subsets among non-luminal type cancers to assess the significance of the CD8/CD4 ratio in detail. In a variety of CD4+ T-cell subsets, we focused on Th1 and Treg cells that directly modulate the cytotoxic functions of CD8+ T-cells. The number of infiltrating Th1 or Treg cells, as well as the Th1/Treg ratio, did not differ significantly between MC and LPBC. However, as to cancers with a lower Th1/Treg ratio, the number of intratumoral CD8+ TIL was significantly higher in MC than in LPBC. Because a lower Th1/Treg ratio reflects the dominance of suppression by Treg, though the mechanism underlying the induction of the characteristically robust intratumoral infiltration of CD8+ TILs in MC was not clarified by our analyses, our results raise the possibility that intratumoral CD8+

TILs robustly infiltrate MC, despite an environment favoring suppression.

Diagnostic criteria and therapeutic guidelines for MC remain controversial. It is assumed that a considerable proportion of conventional high grade invasive ductal carcinomas would be classified as MC because of the rarity of MC and its ambiguous morphological diagnostic criteria, and this might lead to unstable clinical data. MC is morphologically defined in the present diagnostic criteria, and intrinsic subtype is not considered when diagnosing MC. Our results suggest that focal immunological reactions, especially modulatory functions involving CD4+ lymphocyte subsets, differ between MC of the luminal and non-luminal types. More reproducible and stable diagnostic criteria for MC allowing precise diagnosis and thereby avoidance of overtreatment might be established by considering intrinsic subtype and intratumoral CD8+ lymphocyte infiltration of the cancer in addition to morphological features.

We could not perform survival rate analyses in this study because most cases survived throughout the observation period, the exceptions being two advanced cases with metachronous distant metastasis. Not a few patients in this study who did not receive chemotherapy, for various social or personal reasons, survived without recurrence. This high survival rate is probably because cancers with favorable outcomes, such as MC and LPBC, were collected in this study. A comparison between MC and conventional invasive ductal carcinoma is required to complete survival analyses.

In this study, we demonstrated that marked intratumoral CD8+ lymphocyte infiltration is a characteristic finding of MC. Such marked intratumoral CD8+ lymphocyte infiltration is preserved even when the balance of the stromal CD4+ lymphocyte subset favors immunosuppression. It might be appropriate to define invasive breast carcinomas with abundant intratumoral CD8+ lymphocyte infiltration

accompanying medullary morphological features as medullary carcinoma. Suitable diagnostic criteria for medullary carcinoma are requisite to preventing overtreatment for this special histological subtype with favorable outcomes. The significance of intratumoral CD8+ lymphocyte infiltration in medullary carcinoma is a significant issue which awaits extensive verification.

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1 **FIGURE LEGENDS**

2 **Table 1**

3 Summary of patient characteristics

4 **Figure 1**

5 Representative histological images

6 (A, B) H&E staining of MC. (C) Immunohistochemistry staining of CD8 in MC. (D)
7 Multiplex fluorescent combination of CD8 (Red) and CD4 (Green) in MC. (E, F) H&E
8 staining of LPBC. (G) Immunohistochemistry staining of CD8 in LPBC. (H) CD8 (Red)
9 and CD4 (Green) labeling of LPBC. In the fluorescent images, cancer cells are also
10 labeled with cytokeratin (Yellow) to distinguish the intratumoral from the stromal
11 regions.

12 **Figure 2**

13 Comparison of TIL infiltration between MC and LPBC

14 There were no significant differences between the two histological types in the number
15 of (A) stromal CD8+ TILs, (B) stromal CD4+ TILs, (C) intratumoral CD8+ TILs, or
16 (D) intratumoral CD4+ TILs. The number of each cell type per field was counted
17 (cells/0.25 mm^2).

18 **Figure 3**

19 Quantification of CD8+ and CD4+ TILs

20 (A, B) CD4+ TILs are predominant, as compared to CD8+ TILs, in the stroma of both
21 MC and LPBC. (C, D) CD8+ TILs are predominant in the intratumoral region in MC,
22 while no significant differences in the proportions of CD4+ and CD8+ TILs are seen in
23 the intratumoral region of LPBC. The number of each cell type per field was counted
24 (cells/0.25 mm^2).

1 **Figure 4**

2 Stratified analyses based on intrinsic subtypes

3 In non-luminal type cancers, there were significantly more (A) stromal CD8+ TILs and
4 (B) intratumoral CD8+ TILs in MC than in LPBC. In luminal type cancers, such
5 differences in (C) stromal CD8+ TILs and (D) intratumoral CD8+ TILs were not
6 observed. (E) No CD8/CD4 ratio disproportion in stromal TILs was detected in the two
7 histological subtypes. (F) The intratumoral CD8/CD4 ratio was significantly higher in
8 MC than in LPBC for the non-luminal subtype.

9 **Figure 5**

10 Representative multiplex fluorescent labeling pictures including T-bet and Foxp3
11 expressions (A) Th1 cells are detectable with the CD4 (Green) and T-bet (Blue)
12 combination, (B) Treg are detected as CD4+ (Green) and Foxp3+ (Orange) are double-
13 positive cells. (C)The majority of CD8+ TILs (Red) in the intratumoral region also
14 show T-bet (Blue) expression. Cancer cells are labeled with cytokeratin (Yellow) to
15 distinguish the intratumoral from the stromal regions. High resolution images
16 corresponding to each image are also shown.

17 **Figure 6**

18 Stratification of non-luminal cancers by the stromal Th1/Treg ratio.

19 In the subgroup with high (A) stromal and (C) intratumoral Th1/Treg ratios, there was
20 no difference in the number of intratumoral CD8+ TILs between MC and LPBC,
21 whereas in the subgroup with low (B) stromal and (D) intratumoral Th1/Treg ratios, the
22 proportion of intratumoral CD8+ TILs was significantly higher in MC than in LPBC.
23 The number of each cell type per field was counted (cells/0.25 mm^2).
24

1 **Supplementary Figure 1**

2 Procedures for spatial segmentation, phenotype detection and quantification of TILs by
3 image analyzing software programs (A) A spectral composite image includes multiplex
4 fluorescent signals of CD8 (red), CD4 (green), T-bet (blue), Foxp3 (orange), and
5 cytokeratin (yellow). (B) Intratumoral regions with cytokeratin+ signals and stromal
6 regions without cytokeratin signals are segmented. (C) Each of the target immune cell
7 types, including CD8+, CD4+/T-bet+, Th1 cells and CD4+ Foxp3+ Tregs, was
8 classified into the corresponding phenotype and labeled with dots of different colors.
9 (D) The dots were finally quantified independently in each segmented region, as shown.
10 The number of each cell type per field was counted (cells/0.25 mm^2).

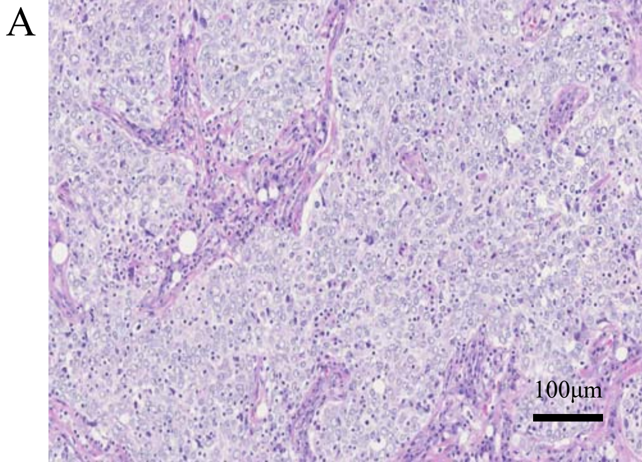
11 **Supplementary Figure 2**

12 Th1, Treg, and Th1/Treg ratios in non-luminal type cancers.
13 There was no significant difference between MC and LPBC in the numbers of (A)
14 stromal Th1, (B) intratumoral Th1, (C) stromal Treg, (D) intratumoral Treg, (E) the
15 stromal Th1/Treg ratio, or (F) the intratumoral Th1/Treg ratio. Note that the
16 intratumoral Th1 and Treg counts are much lower in number than the stromal Th1 and
17 Treg counts. The number of each cell type per field was counted (cells/0.25 mm^2).

		ALL	Range	MC	LPBC
Number of patients		41		24	17
				TMC 16	NSTM 4
				AMC 8	
Median age (Y)		60	(36-88)	60.5	58
Median follow up period (days)		1835	(255-5979)	1964	1255
pT					
T1		16		8	8
T2		23		15	8
T3		1		0	1
Unknown*		1		1	0
pN					
N0		34		20	14
N1		7		4	3
pStage					
I		15		8	7
II		25		15	10
Unknown*		1		1	0
subtype					
Luminal [	HER2-	10		6	4
	HER2+	5		2	3
non-Luminal [	HER2+	9		6	3
	TN**	17		10	7
Ki-67(%)					
<30		2		2	0
30≦		39		22	17
Recurrence					
yes		2		2	0
no		39		22	17
Surgical procedure					
Mastectomy		19		11	8
Partial mastectomy		22		13	9
Adjuvant systemic therapy					
CT***		20		10	10
HT****		6		4	2
CT+HT		5		2	3
none		10		8	2

*state after tumorectomy **Triple Negative ***Chemo therapy ****Hormonal therapy

Figure1

A


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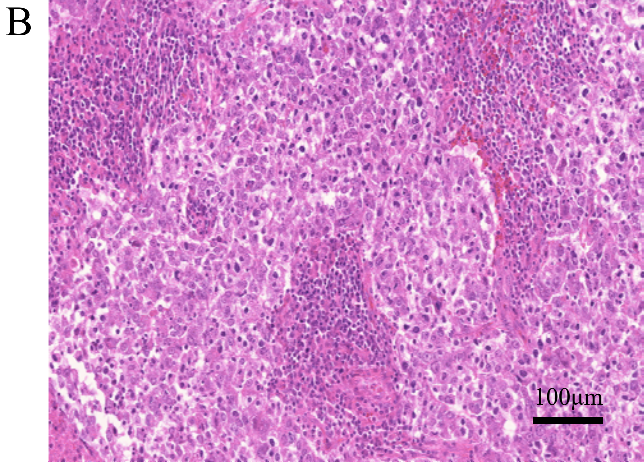

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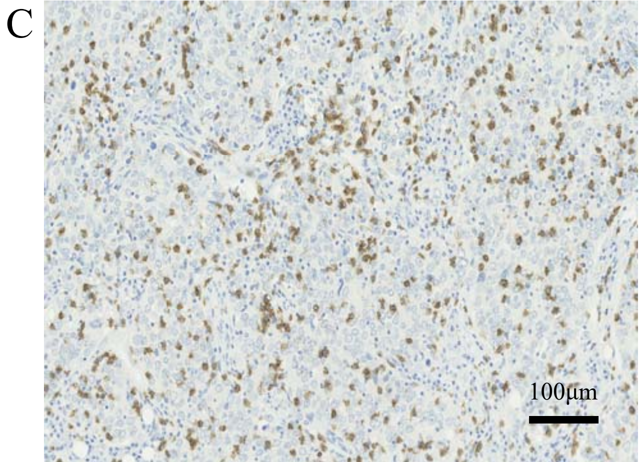

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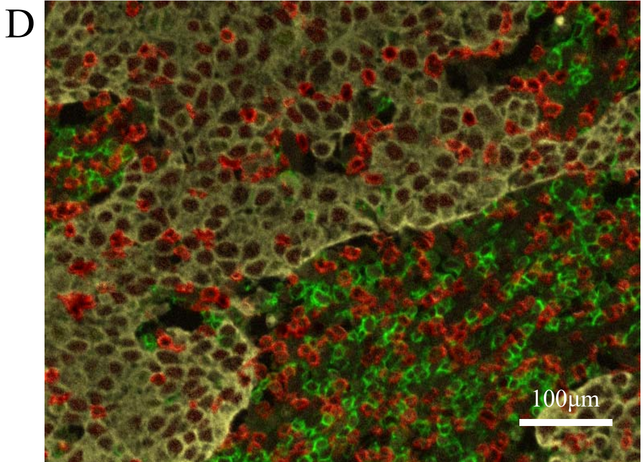

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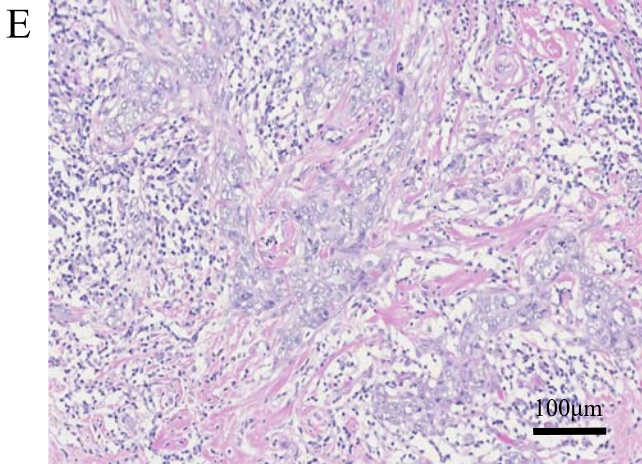

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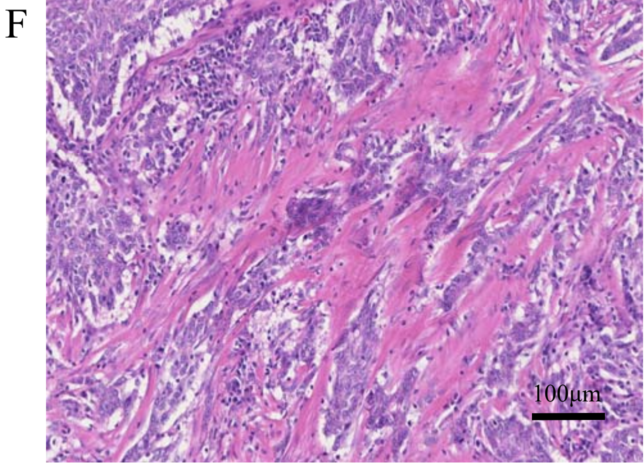

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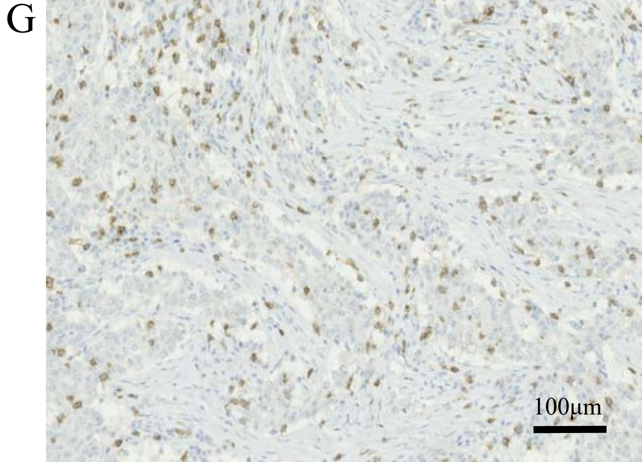

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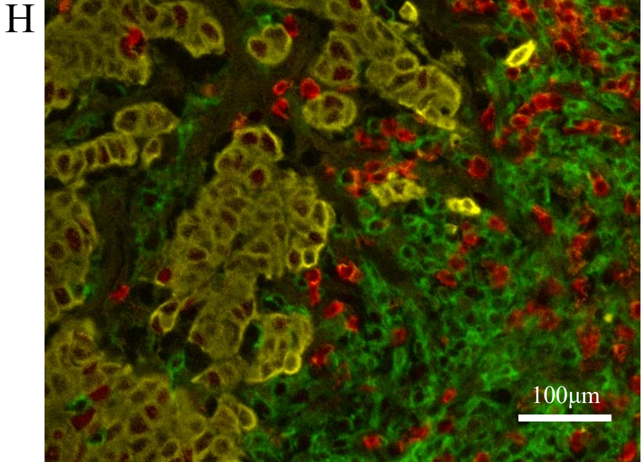

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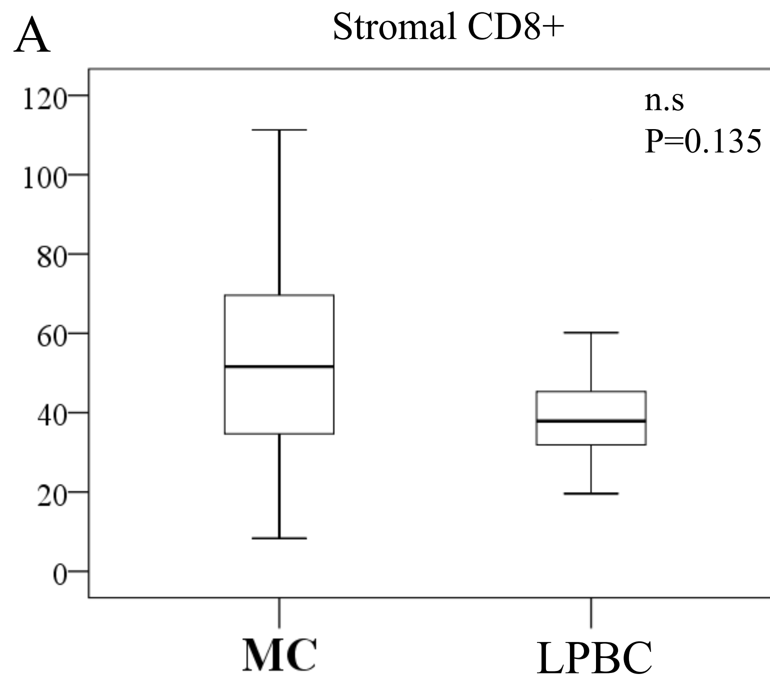
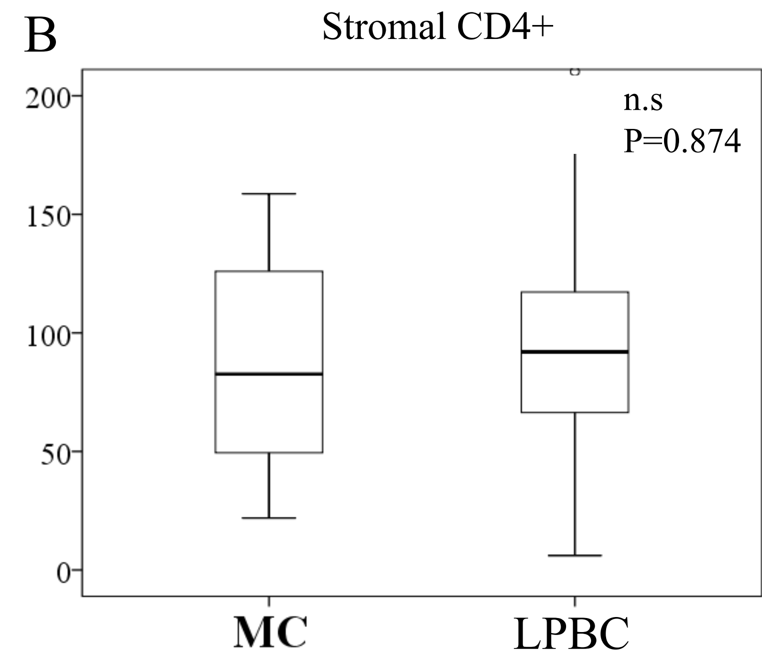
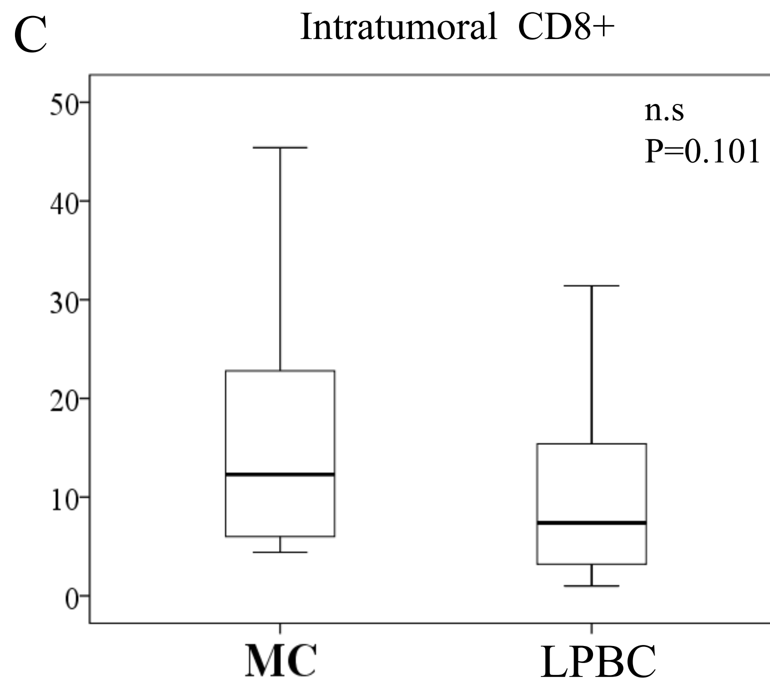






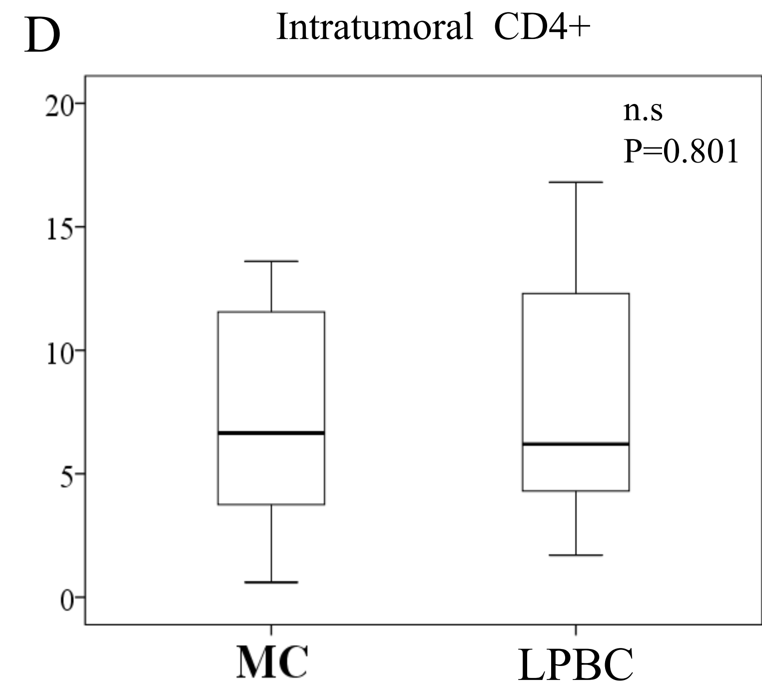
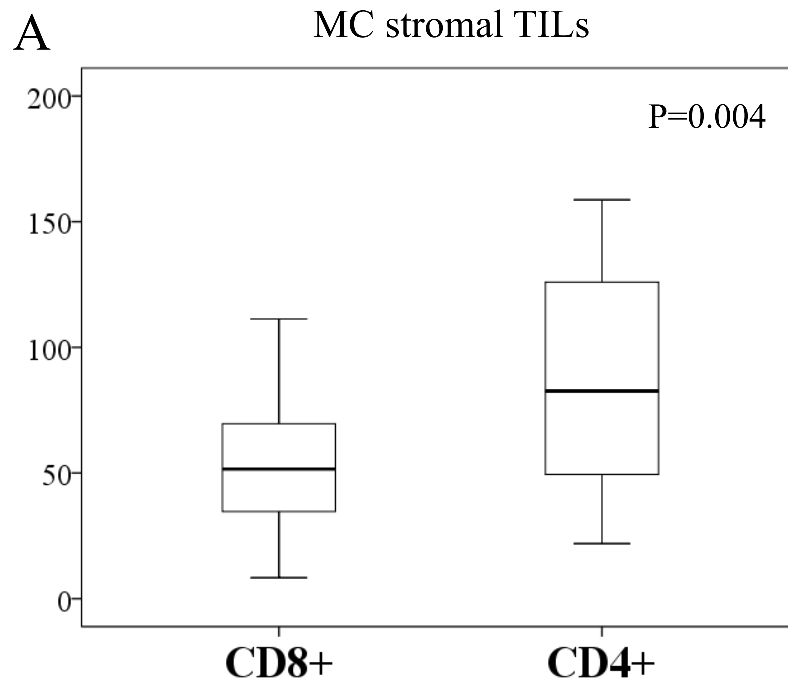
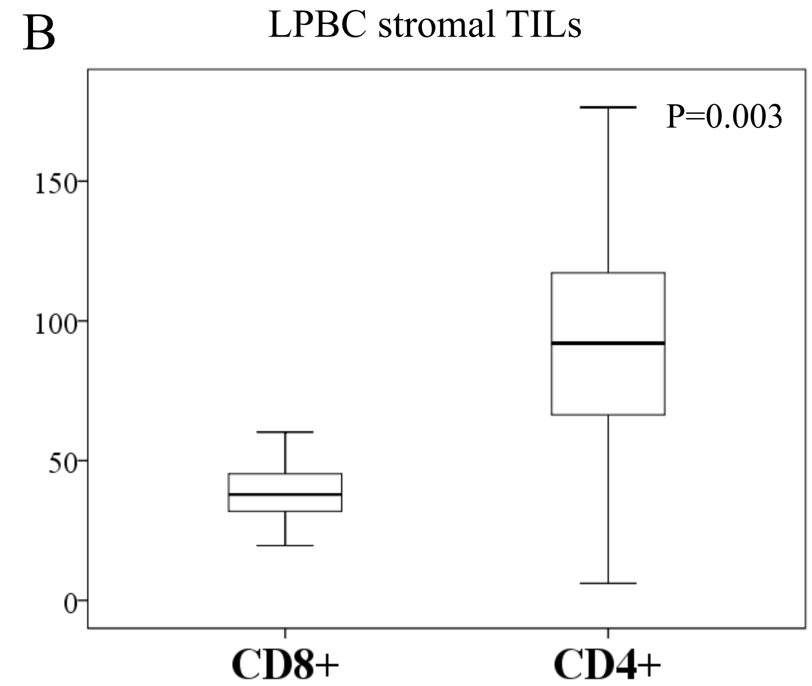
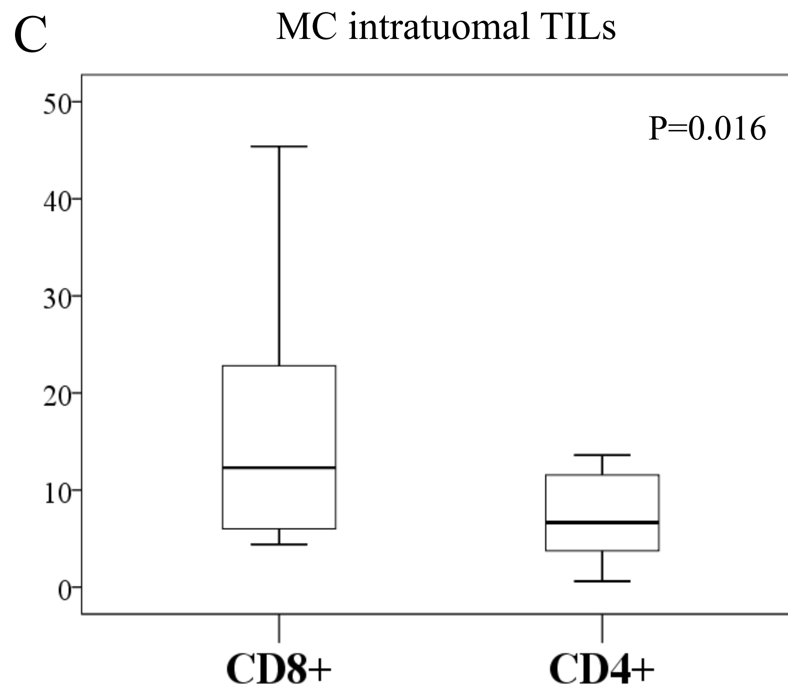


Figure3







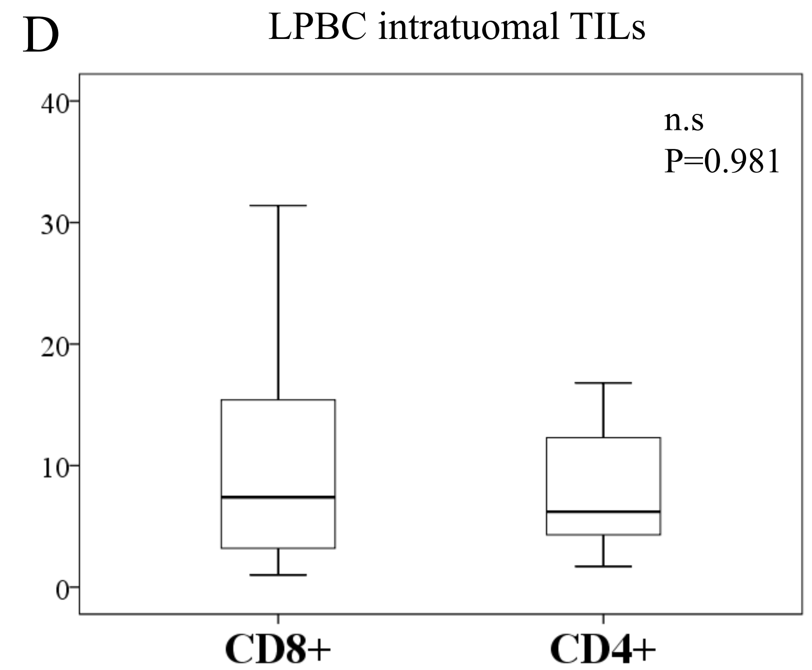
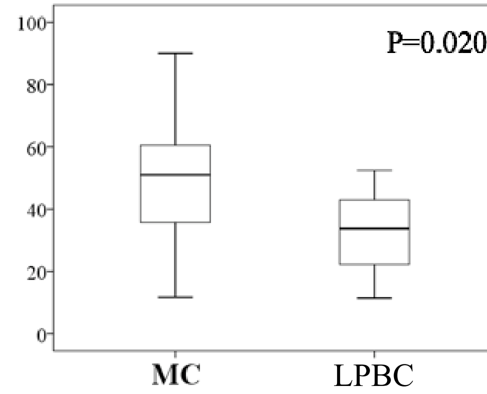


Figure 4

A Stromal CD8+ non-Luminal



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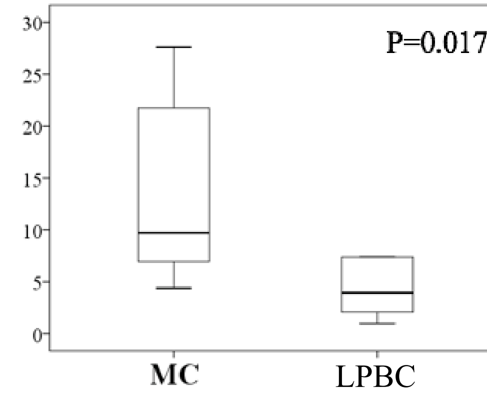
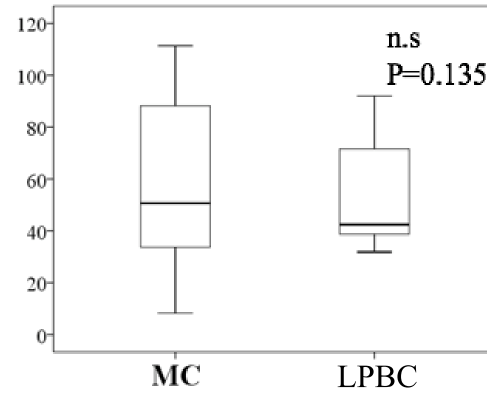
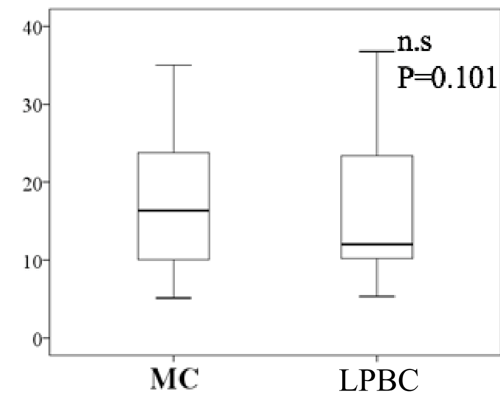


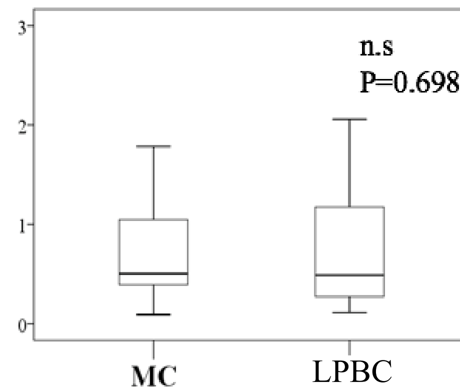
C Stromal CD8+ Luminal



D Intratumoral CD8+ Luminal



E Stromal CD8+/CD4+ non-Luminal



F Intratumoral CD8+/CD4+ non-Luminal

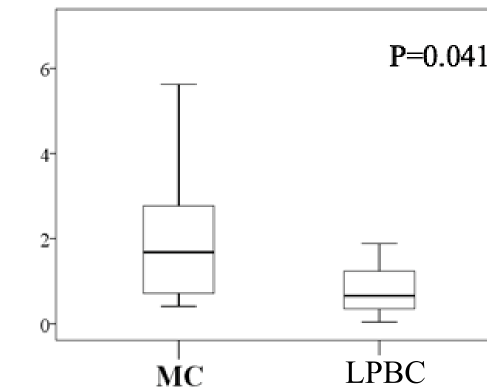
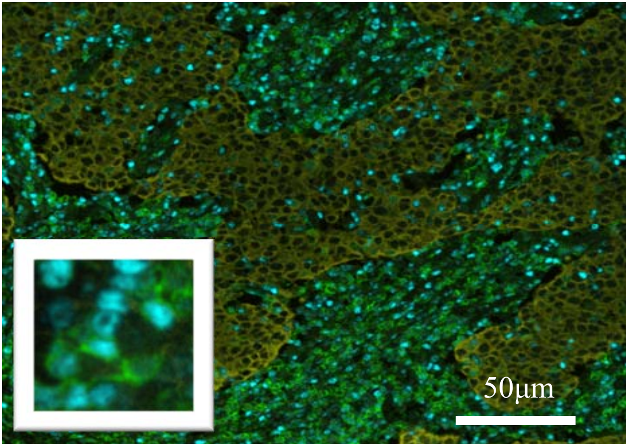
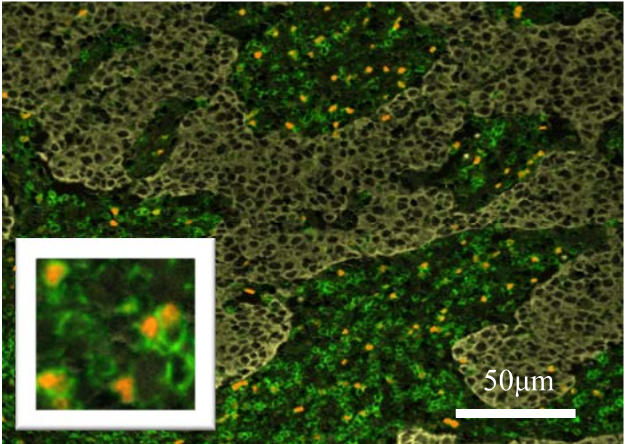


Figure 5

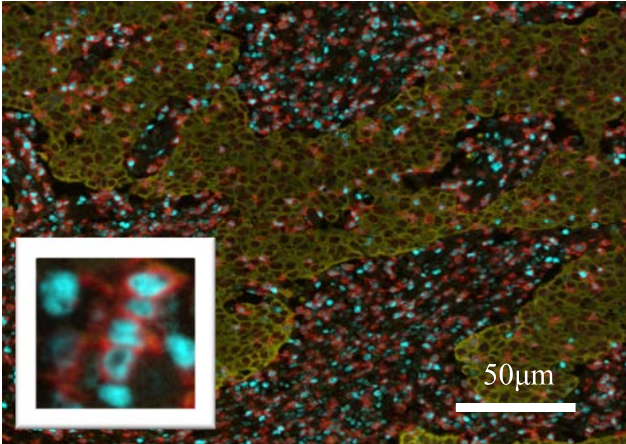
A Th1(CD4+/T-bet+)



B Treg(CD4+/Foxp3+)



C (CD8+/T-bet+)



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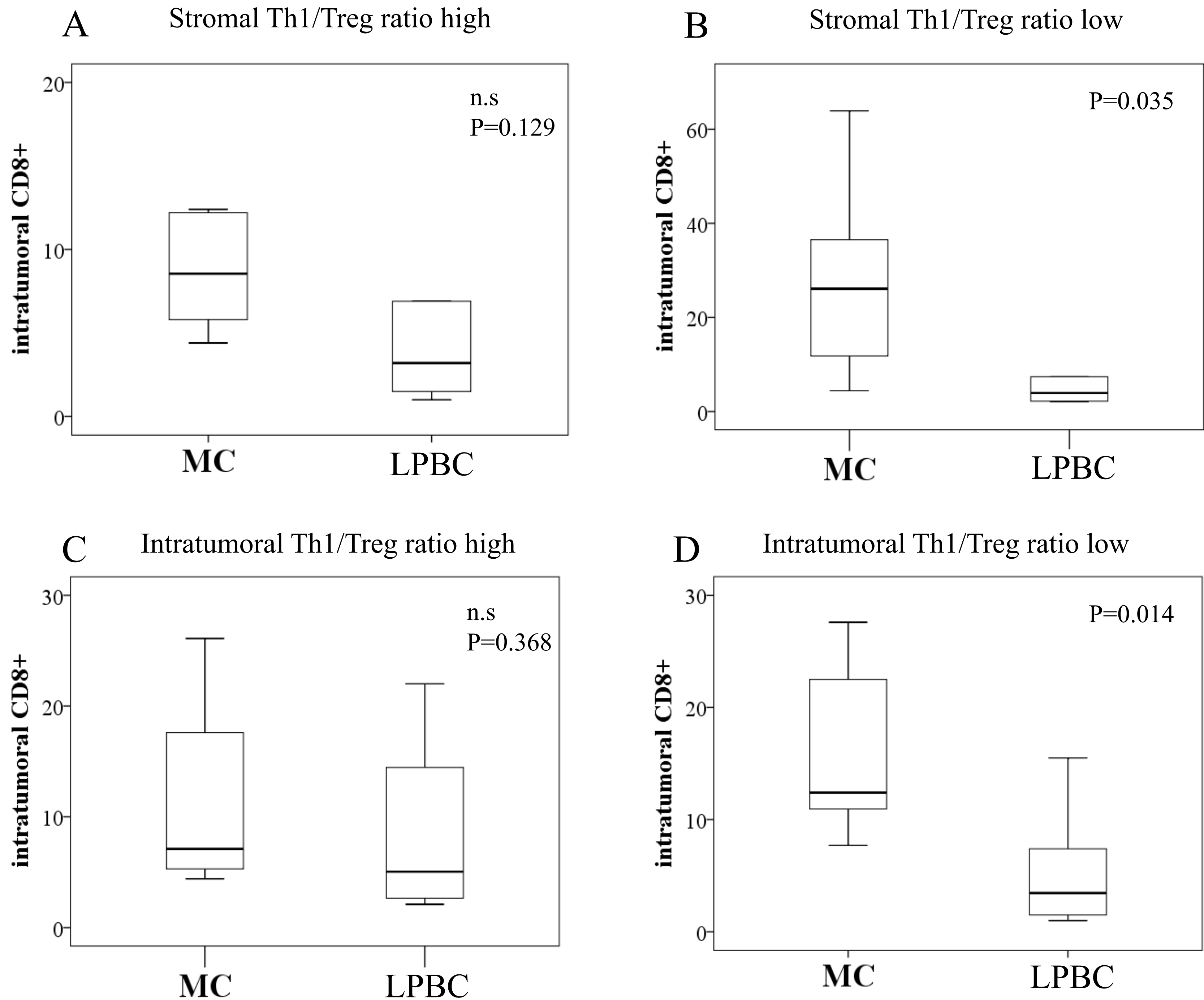
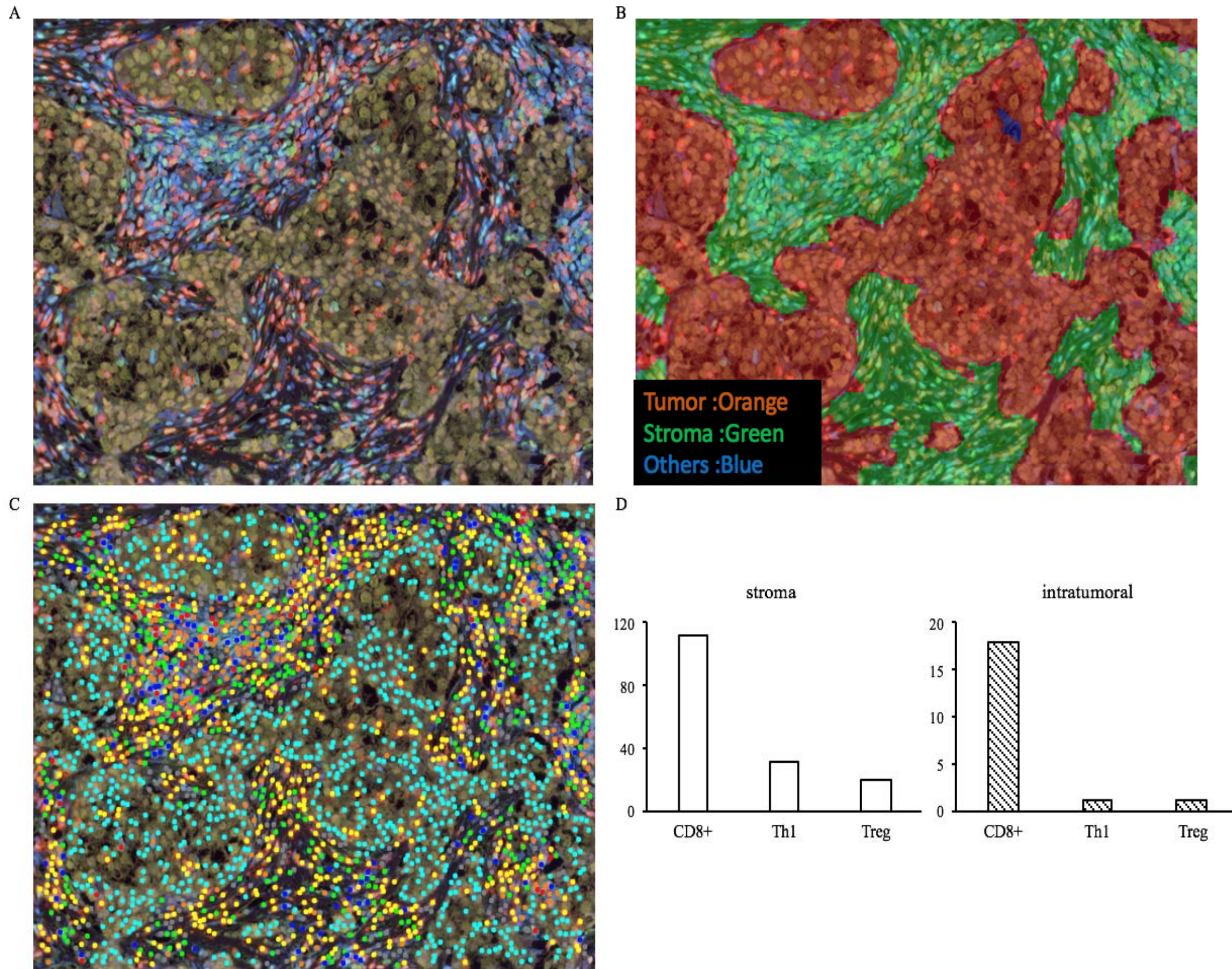


Supplementary Fig.S1



Supplementary Fig.S2