Long-term Follow-up After Interstitial Laser Thermotherapy of Breast Cancer

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Abstract. *Aim: To review the effect of immunological changes induced by interstitial laser thermotherapy (ILT) on long-term outcome of patients with breast cancer. Patients and Methods: Twenty-four patients with invasive breast cancer were treated with ILT followed by standard surgical excision. Immunohistological reactions on immunocompetent cells were performed on specimens obtained before and after ILT. Follow-up time was 116 (range=91-136) months. Results: Significant prognostic factors were histologically-positive axillary lymph nodes and Ki67 positivity. ILT increased cytotoxic T ($CD8^+$) lymphocytes within the tumor and mature dendritic cells ($CD83^+$) and reduced the number of T-regulatory cells (T_{reg}) $CD25^+$/Forkhead box $p3^+$ ($FOXP3^+$) lymphocytes in regional lymph nodes. These changes did not correlate with prognosis. The number of $CD8^+$ cells within the tumor, both before and after treatment, was significantly higher in patients with recurrence than in those without recurrence ($p<0.01$ and $p<0.05$, respectively). Patients with recurrent disease had a lower number of $CD57^+$ cells in tumor-free lymph nodes than did patients without recurrence ($p<0.05$). Conclusion: ILT did not have any long-term adverse effects. The clinical impact of the supposedly favourable immune changes after ILT should be examined in a larger patient population.*

Minimally- invasive techniques such as laser thermotherapy, radiofrequency ablation, cryotherapy and high-intensity focused ultrasound are used in the treatment of breast cancer, but there is no currently established optimum technique. Advantages with these methods include minimal trauma, less immunosuppression than with standard surgical resection and possibly the induction of a favourable anti-tumor immune response (1-5). Local recurrence after local destruction is relatively frequent and another concern is the risk of inducing metastatic spread. This has been described to occur, although rarely, after cryotherapy and radiofrequency ablation (6, 7). Laser-induced thermotherapy does not appear to carry this risk but investigations are limited (7).

Interstitial laser thermotherapy (ILT) is attractive as a local destruction method since it gives precise control of the lesion size and, most importantly, provides a unique source of tumor antigens for induction of anti-tumor immunity. We showed in experimental studies that ILT can induce immunity that eradicates minimal residual disease and prevents metastatic spread and that it has a distant bystander (abscopal) effect, *i.e.* that it gives pronounced suppression of the growth of a simultaneous untreated tumor (8-11). Isbert *et al.* confirmed some of these findings, showing an enhanced cellular immune response and a distant bystander effect after laser-induced thermotherapy (12). In the clinical setting, we have shown a distant bystander effect after laser thermotherapy in a patient with malignant melanoma (13).

We previously reported on 24 women with breast cancer who were treated with ILT followed by surgical resection about two weeks later. Immunohistological reactions were performed on specimens obtained before (core needle biopsy) and after (excised specimen) ILT and evaluation was based on pre- and post-treatment differences for each individual patient. ILT increased $CD8^+$ cytotoxic T-cells within the tumor, $CD68^+$ macrophages within the tumor and at the tumor border, and mature dendritic $CD83^+$ cells at the tumor border and lowered the number of $CD25^+$ Forkhead box $p3^+$ ($FOXP3^+$) T-regulatory (T_{reg}) lymphocytes in regional lymph nodes (14, 15).

Mature dendritic cells ($CD83^+$) and cytotoxic T-lymphocytes ($CD8^+$) have been shown to be associated with a favourable prognosis when found in tumors (16, 17), whereas others such as T_{reg} cells have been shown to

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indicate poor prognosis (18). In most human tumor types, infiltration of tumor-associated macrophages (M2 polarized macrophages) is an unfavourable prognostic indicator. However, M1 polarized macrophages are cells of acute inflammation reacting to foreign antigens and thereby cells with potential anti-tumor effects (19, 20).

The aim of the present study was to examine if the ILT-induced changes of immunocompetent cells varied with long-term outcome of our patients with breast cancer.

Materials and Methods

Patients. Between 2001-2005, 24 women were treated with ILT for breast cancer, as previously described (14, 15). Patients with newly diagnosed breast cancer were selected from a multi-disciplinary conference, and the treatment plan included a curative operation, usually partial mastectomy. Pre-treatment diagnostic work-up within a week before treatment included pulmonary x-ray, liver function tests and a core biopsy of the tumor (18- or 16-gauge), for histological diagnosis, receptor status and immunohistochemistry of immunocompetent cells.

ILT-induced necrosis of invasive cancer was 33% (range=0-100%) and was complete in three patients. The intention was to treat patients with tumors smaller than 1.5-2 cm but tumors were larger than demonstrated by preoperative ultrasound and mammography, which was probably a major reason why the percentage tumor necrosis was small in many patients (14). ILT was well- tolerated, with the only complication being small skin necrosis in two patients. The post-operative course after surgical resection was unremarkable.

Tumor and treatment characteristics are summarized in Table I and a summary of the operations that were performed after ILT is given in Table II.

The study was approved by the local Ethics Committee (LU 488-97) and informed verbal and written consent was obtained in all patients.

Design. In the evaluation of ILT-induced changes of immunocompetent cells, 17 women that underwent radical surgical excision after non-radical ILT were studied. Seven women were excluded from the immunological analysis because of radical ILT treatment (n=3) and low necrosis volume (≤1%) after ILT (n=4). ILT was performed at a steady-state temperature of 48˚C for 30 min in the outpatient clinic. Standard surgical excision was performed 12 (range=6-23) days after ILT. Immunohistological reactions were performed on core needle biopsies prior to treatment and on the excised specimens. The ILT-induced effects on immunocompetent cells were calculated as the difference between pre-and postoperative values for each patient.

Immunohistological methods and analysis. Immunocompetent cells (CD1a, CD4, CD8, CD20, CD57, CD68, CD83, CD94, granzyme B, CD25 and FOXP3) were determined within and at the tumor border. The immunohistological analysis of the lymph nodes was performed after ILT only and included CD1a, CD57, CD83, CD94, granzyme B, CD25 and FOXP3. Methods for tissue sampling, immunohistochemistry and data analysis have been described in detail elsewhere (15) and are summarized herein.

For single immunohistochemistry, the reactions were performed in a TechMate 500 (Ventana BioTech Systems, Tucson, AZ, USA) automatic immunostainer with Dako ChemMate Detection kit peroxidase/DAB+ (Dako A/S, Glostrup, Denmark), giving a brown colour. They were performed with antibodies against CD1a, an antigen characterizing one type of immature dendritic cell (diluted 1:50; Dako, Glostrup, Denmark); CD4, a T-helper cell antigen (1:100; Novocastra, Newcastle, UK); CD8, a T-cytotoxic cell antigen (1:20; Dako); CD20, a B-cell antigen (1:2,000; Dako); CD25, interleukin-2 receptor (1:100; Novocastra); CD57, a natural killer (NK) cell antigen (1:100; Novocastra); CD68, a macrophage antigen (1:20; Dako); CD83, a mature dendritic cell antigen (1:20; Novocastra); CD94, an NK cell antigen (1:20; Immunotech, Marseille, France); granzyme-B, an antigen for perforin and granzyme B present in some cytotoxic T-cells and NK cells (1:50; Dako); FOXP3, an antigen for forkhead/winged helix family of transcription factors (1:100, 236A/E7; eBioscience, San Diego, CA, USA). The background staining was carried out with Mayer's haematoxylin.

Double staining immunohistochemical reactions were performed for CD25FOXP3. The double staining was performed in a DakoCytomation Autostainer (Dako, Glostrup, Denmark). We used Envision G/2 Doublestain System (1:100 for both antibodies, Dako code K5361) where DAB, brown colour was used for FOXP3 and permanent red was used for CD25.

Photographs of the immunohistological reactions were taken of vital tumor at the tumor border and in the interior of the tumors using a standard light microscope and a ×10 objective (Bx-60; Olympus, Tokyo, Japan). The median number of photographs taken of the core biopsies was 4 (range=1-10) at the border of the tumor and 9 (range=1-36) within the tumor. The number of photographs taken of the vital tumor after ILT was 19 (range=3-42) at the edge and 17 (range=2-46) within. For computerized digital analysis, images were scanned and captured with a three-colour charge-coupled device in order to facilitate interpretation and quantification of findings with Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). This software was used for counting the cells for all antibodies except $CD68^+$ and $CD25^+$ $FOXP3^+$ cells. These were marked as the others but then counted manually (15).

Tumor markers. Herceptin-2 (HER2), cytokeratin 5/6 (CK5/6, diluted 1:100; Dako), epidermal growth factor receptor (EGFR, kit 760-500; Ventana, Tucson, AZ, USA) and Ki67 (diluted 1:50; Dako) were determined on a section from the resected primary tumor. For

Table I. *Clinical characteristics (n=24). Values are means (range).*

Tumor size, mm	
Ultrasound	14 (5-35)
Microscopic	23 (7-60)
Pathological diagnosis DC/LC/DLC	15/8/1
Lymph node metastases, Y/N	8/16
Histological grading I/II/III	3/17/2[a]
ILT-induced tumor necrosis, %	33% (0-100%)
Age (years)	61 (39-84)

DC: Ductal carcinoma, LC: lobular carcinoma, DLC: mixed invasive ductal carcinoma and lobular carcinoma; ILT: interstitial laser thermotherapy. [a]Histological grading was not possible in two patients because of tumor radically treated with ILT and lack of material for core biopsy.

Table II. *Operation type (n=24).*

	Primary operation	n	Re-operation	n
T1N0 (n=12)	PM and SNB	11	Re-PM	1
	Mastectomy	1	Mastectomy	1
T1N1 (n=4)	PM and SNB+axillary dissection	4	Re-PM	1
	Mastectomy	2		
T2N0 (n=3)	PM and SNB	2		
	Mastectomy	1		
T2N1 (n=2)	PM and SNB+axillary dissection	2	Mastectomy	1
T2N2/T3N0/T3N3 (n=1/1/1)	Mastectomy and axillary dissection	2	Mastectomy	1
	PM and SNB+axillary dissection	1		

PM: Partial mastectomy, SNB: sentinel node biopsy. All patients with PM were given radiotherapy to remaining breast tissue postoperatively.

detection of HER2, we used PATHWAY anti-HER2/neu (clone 4B5) (Ventana) with Ventana NIEW DAB Detection Kit.

The antibody against oestrogen receptor alpha was Dako clone 1D5 (diluted 1:35) and that against progesterone receptor was Dako clone PgR 636 (1:150). For these two antibodies, the Dako DAB kit K5001 was used.

For HER2, reaction in more than 10% of cells (2+ or more) was considered positive. For CK5/6 and EGFR, more than 5% of cells with positive reaction was considered positive. For Ki67, over 10% per 200 cells was considered positive and these counts were carried out in hot spots (21).

Follow-up. The median follow-up time after ILT was 116 (range=91-136) months. Follow-up adhered to the standard protocol established for southern Sweden and included postoperative clinical check up 2 weeks after surgery and then clinical examination and mammography, and in some cases ultrasound, every year for 3 years. After that, patients younger than 75 years of age entered the standard screening protocol. Information after scheduled routine follow-up was obtained from hospital records and the civil register. No patient was lost to follow-up.

Statistical methods. Differences between groups were tested with Students *t*-test. A *p*-value of <0.05 was considered significant. Values reported are means±SEM.

Results

In women with pT1-3 tumors, only three were radically treated with ILT. The patients operated with partial mastectomy (PM) received radiotherapy after surgery, all patients with oestrogen-positive tumors received adjuvant anti-oestrogen treatment. Following PM and re-resection in seven patients due to narrow margins, no patient had local recurrence. All patients had tumors with histological grade I and II except two patients, one with a pT3N3 and one with a pT2N2 tumor, who had histological grade III tumors (Table II). All patients had tumors that were negative for CK5/6 and EGFR. Five patients had HER2-positive tumors and 10 were Ki67 positive (Table III). No patient has had a local recurrence.

Table III. *Receptor status.*

Receptor	Patients without recurrence (n=19)	Patients developing metastatic disease (n=5)
ER+	18	3
PgR+	11	3
HER2+	3	2
Ki67+	5	5
TNBC	1	0

ER^+: Oestrogen receptor-positive; PgR^+: progesterone receptor-positive; $HER2^+$: herceptin receptor-positive; $Ki67^+$: Ki67-positive; TNBC: triple-negative breast cancer. All tumors were negative for CK5/6 and epidermal growth factor receptor.

Prognosis and stage of disease. Sixteen patients had pN0 status and none developed local recurrence or metastatic breast cancer during follow-up. Eight patients had pN 1-3 and five of them developed distant metastasis. The disease-free survival for patients with tumor stages I, II and III were 100% (n=12), 90% (n=10) and 0% (n=2), respectively. Eighteen patients were still alive at the time of analysis.

Relapse-free patients. Nineteen patients were relapse-free at a median of 116 (range=91-139) months after surgery. All of them but one had oestrogen-positive tumor. Twelve relapse-free patients had pT1N0 tumor, two had pT1N1, three had pT2N0, one had pT2N1 and pT3N0 tumor, respectively. Eleven had invasive ductal cancer (DC), seven had invasive lobular cancer (LC) and one had mixed invasive ductal carcinoma and lobular carcinoma (DLC). Two patients died of causes not related to cancer. One patient died of metastatic pancreatic cancer six years after breast cancer surgery.

Patients with relapse. Five patients developed metastatic disease. Four patients had DC and one had LC. The median tumor necrosis in ILT was 19% (range=0-60%).

Two patients had pT1N1 tumor, one refused adjuvant treatment and developed bone metastases and but was still alive 9 years after treatment. The other developed metastases in the liver and lungs and died one year after surgery. One patient with pT2N1 tumor died 16 months after surgery with liver metastases. All patients with recurrent disease had $Ki67^+$ tumors (Table III).

One patient with pT2N2 tumor received radiotherapy of the thoracic and axillary regions and anti-oestrogen therapy and chemotherapy. She was alive at the time of writing but had experienced relapse with positive supra- and infraclavicular lymph nodes, and has received further chemotherapy at intervals.

One patient with pT3N3 died within a year with liver metastases. She had a HER2 and Ki67-positive tumor and received only radiotherapy postoperatively.

Prognosis and immunocompetent cells.
Tumor: In order to assess possible long-term effects of ILT, we compared the differences in number of immunocompetent cells, analysed separately at the tumor border and within the tumor, before and about 2 weeks after ILT, in patients with and without recurrent disease. We examined immature dendritic cells ($CD1a^+$), mature dendritic cells ($CD83^+$), T-helper cells ($CD4^+$), cytotoxic T-cells ($CD8^+$), granzyme B, NK cells ($CD57^+$, $CD94^+$), macrophages ($CD68^+$), B-lymphocytes ($CD20^+$) and Tregs ($CD25^+FOXP3^+$) and found no significant relationships with recurrence when all or patients with stage II-III disease only were included (data not shown).

The mean number of CD8+ cells within tumor in the core biopsy obtained before ILT was 12±3.2 in patients without recurrence and 34±7.9 in patients with recurrence ($p<0.01$). In the resected specimens, the mean number of CD8+ cells was 24±6.3 in patients without recurrence and 64±27.1 in patients with recurrence ($p<0.05$).

Regional lymph nodes: Patients with recurrence of disease had a lower number of $CD57^+$ cells in tumor-free lymph nodes than patients without recurrence ($p<0.05$). Numbers of $CD1a^+$, $CD83^+$, $CD94^+$, granzyme B and $CD25^+FOXP3^+$-positive cells in the lymph nodes were similar in patients with and without recurrence.

Discussion

The most important prognostic factor for our patients was the presence of histologically positive axillary lymph nodes.

A number of reports have demonstrated that intratumoral immune cell responses predict patient prognosis. T-Cells need to be activated for effective immune responses, for instance by so called' danger signals, such as interferon-γ, and an acute inflammatory reaction induced by cell death (22-24). ILT induces such changes and also provides a source of tumor antigens, which may increase adaptive tumor immunogenicity (10-13, 25). In the present group of patients, we found that ILT resulted in increased infiltration of $CD8^+$ lymphocytes within the tumor, increased numbers of $CD83^+$ dendritic cells at the tumor border and lower numbers of $CD25^+FOXP3^+$ lymphocytes in regional lymph nodes (15). These presumably favourable effects of ILT on immune cells did not correlate with prognosis in our small group of patients with stage II-III disease, perhaps due to the small number of patients.

A high number of cytotoxic T-lymphocytes in the tumor has been shown to be a positive prognostic factor in various cancer forms (26). It was, therefore, unexpected to find that an increased number of $CD8^+$ cells, both before and after treatment, correlated with recurrence. However, similar findings have been reported before. In patients with breast cancer stage I-III, Sheu *et al.* showed that a high percentage of $CD8^+$ tumor-infiltrating lymphocytes correlated with cancer progression and was a sign of poor prognosis (27). Matkowski *et al.* reported that a high expression of $CD4^+$ and $CD8^+$ correlated with cancer-positive lymph nodes and poor prognosis in patients with breast cancer (28). Nakano *et al.* showed that a high infiltration of $CD8^+$ cells correlated with shorter survival in patients with renal cell carcinoma (29). They also showed a positive correlation between tumor grade and infiltration of $CD8^+$ cells, suggesting that tumor grade, and the proliferative activity of tumor cells, was a stronger prognostic factor than lymphocytes. In a large cohort of breast cancer patients, Mahmoud *et al.* described a weak correlation between CD8 infiltration and tumor grade and a positive correlation between CD8 infiltration and cancer-specific survival (30). Thus, in the latter study the net result of CD8 anti-tumor activity was strong enough to counterbalance the tumor proliferative activity. In a recent article, CD8 cells were found to be a favourable prognostic sign for some subgroups of breast cancer (17). The interplay between CD8 cells and Treg cells is also of importance. Previous studies evaluating the prognostic value of Treg cells have given conflicting findings (17, 18, 31, 32). Other mechanisms of immune escape and tolerance may also influence outcome (24).

It has been reported that NK cell infiltration of regional lymph nodes lacks correlation with prognosis in oral and gastric cancer (33, 34), whereas it has been shown to correlate with prognosis in gastric and lung cancer (35, 36). Our results with a larger number of CD57 cells in lymph nodes from patients without than with recurrent disease suggest a role for NK cell activity.

In contrast to our findings, Iwamoto *et al.* found that an increased number of CD83 cells at the tumor border was a positive prognostic factor in breast cancer (16). Dendritic cells have a key role in tumor immune response but the tumor microenvironment can also transform them into having immunosuppressive phenotype (37, 38). In our patients, we

showed that lymph nodes with cancer had low counts of CD83 cells, both when compared to lymph nodes in patients without lymph node metastases and when compared to negative lymph nodes in patients with positive nodes (15). These results agree with those of Poindexter *et al*., who found a trend towards lower numbers of CD83 in sentinel nodes with cancer than in tumor-free sentinel nodes (39).

Conclusion

ILT seems to be a safe method from a long-term perspective. Immunostimulation is a promising feature of ILT but demonstration of its possible clinical significance awaits evaluation in a larger patient population.

Conflicts of Interest

K-G Tranberg is co-founder and shareholder of Clinical Laserthermia Systems AB. The prototype system that was used in this article was a research protype and is not for commercial use. Clinical Laserthermia Systems AB is in the process of developing the prototype system into a user-friendly commercial version. None of the other authors have financial interests in the equipment or any competing materials.

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References

1 Roubidoux MA, Yang W and Stafford RJ: Image-guided ablation in breast cancer treatment. Tech Vasc Interventional Rad *17*: 49-54, 2014.

2 Fornage BD and Hwang RF: Current status of imaging-guided percutaneous ablation of breast cancer. AJR *203*: 442-448, 2014.

3 Nguyen T, Hattery E and Khatri VP: Radiofrequency ablation and breast cancer: a review. Gland Surgery *3*: 128-135, 2014.

4 Wu F, Wang ZB, Lu P, Xu ZL, Chen WZ, Zhu H and Jin CB: Activated anti-tumor immunity in cancer patients after high-intensity focused ultrasound ablation. Ultrasound Med Biol *30*: 1217-1222, 2004.

5 Zerbini A, Pilli M, Penna A, Pelosi G, Schianchi C, Molinari A, Schivazappa S, Zibera C, Fagnoni FF, Ferrari C and Missale G: Radiofrequency thermal ablation of hepatocellular carcinoma liver nodules can activate and enhance tumor-specific T-cell responses. Cancer Res *66*: 1139-1146, 2006.

6 Angonese C, Baldan A, Cillo U, D'Alessandro A, De Antoni M, De Giorgio M, Masotto A, Marino D, Massani M, Mazzucco M, Miola E, Neri D, Paccagnella D, Pivetta G, Tommasi L, Tremolada F, Tufano A, Zanus G. and Farinati F: Complications of radiofrequency thermal ablation in hepatocellular carcinoma: What about "explosive" spread? Gut *55*: 435-436, 2006.

7 Schuld J, Richter S, Oberkircher LW, Seeland U, Debnar-Daumler KI, Rauch J, Menger MD, Schilling MK and Kollmar O: Evidence for tumor cell spread during local hepatic ablation of colorectal liver metastases. J Surg Res *178*: 268-279, 2012.

8 Möller PH, Ivarsson K, Stenram U, Radnell M and Tranberg K-G: Interstitial laser thermotherapy of an adenocarcinoma transplanted into rat liver. Eur J Surg *63*: 867-870, 1997.

9 Möller PH, Ivarsson K, Stenram U, Radnell M and Tranberg K-G: Comparison between interstitial laser thermotherapy and excision of an adenocarcinoma transplanted into rat liver. Br J Cancer *77*: 1884-1892, 1998.

10 Tranberg K-G, Myllymäki L, Möller PH, Ivarsson K and Sjögren HO: Interstitial laser thermotherapy of a rat liver adenocarcinoma. J X-ray Science Technol *10*: 177-185, 2002.

11 Ivarsson K, Myllymaki L, Jansner K, Stenram U and Tranberg KG: Resistance to tumor challenge after tumor laser thermotherapy is associated with a cellular immune response. Br J Cancer *93*: 435-440, 2005.

12 Isbert C, Ritz JP, Roggan A, Schuppan D, Ruhl M, Buhr HJ and Germer CT: Enhancement of the immune response to residual intrahepatic tumor tissue by laser-induced thermotherapy (LITT) compared to hepatic resection. Lasers Surg Med *35*: 284-292, 2004.

13 Tranberg K-G, Ivarsson K, Willner J, Håkansson L and Stenram U: Induction of a distant anti-tumor effect by interstitial laser thermotherapy (ILT) in a patient with malignant melanoma. *In*: Proceedings of the Second International Conference on Tumor Microenvironment: Progression, Therapy and Prevention, Baden, Austria. Witz A (ed.) p 49. 2002.

14 Haraldsdóttir KH, Ivarsson K, Götberg S, Ingvar C, Stenram U and Tranberg K-G: Interstitial laser thermotherapy (ILT) of breast cancer. Eur J Surg Oncol *34*: 739-745, 2008.

15 Haraldsdóttir KH, Ivarsson K, Jansner K, Stenram U and Tranberg K-G: Changes in immunocompetent cells after interstitial laser thermotherapy of breast cancer. Cancer Immunol Immunother *60*: 847-856, 2011.

16 Iwamoto M, Shinohara H, Miyamoto A, Okuzawa M, Mabuchi H, Nohara T, Gon G, Toyoda M and Tanigawa N: Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. Int J Cancer *104*: 92-97, 2003.

17 Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JMS, Mahmoud SMA, Rakha E, Ellis IO, Liu S, Gao D and Nielsen TO: Association between CD8+ T-cell infiltration and breast cancer survival in 12439 patients. Ann Oncol *25*: 1536-1543, 2014.

18 Bates G, Fox S, Han C, Leek RD, Garcia JF, Harris AL and Banham AH: 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, 2006.

19 Wyckoff J, Wang Y, Lin EY, Li J-F, Goswami S, Stanley ER, Segall JE, Pollard JW and Condeelis J: Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res *67*: 2649-2656, 2007.

20 Ghesquiére B, Wong BW, Kuchnio A and Carmeliet P: Metabolism of stromal and immune cells in health and disease. Nature *511*: 167-176, 2014.

21 Romero Q, Bendahl P-O, Klintman M, Loman N, Ingvar C, Rydén L, Rose C, Grabau D and Borgquist S: Ki67 proliferation in core biopsies versus surgical samples – a model for neo-adjuvant breast cancer studies. BMC Cancer *11*: 341, 2011.

22 Mantovani A, Romero P, Palucka AK and Marincola FM: Tumor immunity: effector response to tumor and role of the microenvironment. Lancet *371*: 771-783, 2008.
23 Shi Y, Zheng W and Rock KL: Cell injury releases endogenous adjuvants that stimulate cytotoxic T-cell responses. Proc Natl Acad Sci USA *97*: 14590-14595, 2000.
24 Schreiber RD, Old LJ and Smyth MJ: Cancer Immunoediting: Integrating immunity's roles in cancer suppression and promotion. Science *331*: 1565-1570, 2011.
25 Tranberg K-G, Ivarsson K, Sjögren H and Stenram U: Laser thermotherapy of rat liver carcinoma increases anti-tumor lymphocyte responsiveness in tumor-draining and systemic lymph nodes and in spleen. J HPB *10(Suppl 1)*: 10, 2008.
26 Gooden MJM, de Bock GH, Leffers N, Daemen T and Nijman HW: The prognostic influence of tumor-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer *105*: 93-103, 2011.
27 Sheu BC, Kuo WH, Chen RJ, Huang SC, Chang KJ and Chow SN: Clinical significance of tumor-infiltrating lymphocytes in neoplastic progression and lymph node metastasis of human breast cancer. Breast *17*: 604-610, 2008.
28 Matkowski R, Gisterek I, Halon A, Lacko A, Szewczyk K, Staszek U, Pudelko M, Szynglarewicz B, Szelachowska J, Zolnierek A and Kornafel J: The prognostic role of tumor-infiltrating CD4 and CD8 T lymphocytes in breast cancer. Anticancer Res *29*: 2445-2452, 2009.
29 Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, Suzuki Y, Shintaku I, Nagura H and Ohtani H: Proliferative activity of intratumoral $CD8^+$ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of anti-tumor immunity. Cancer Res *61*: 5132-5136, 2001.
30 Mahmoud SMA, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AHS, Ellis IO and Green AR: Tumor-infiltrating $CD8^+$ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol *29*: 1949-55, 2011.
31 Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Lee AHS, Ellis IO, Green AR. An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. Breast Cancer Res Treat *127(1)*: 99-108, 2011.
32 West NR, Kost SE, Martin SD, Milne K, Deleeuw RJ, Nelson BH and Watson PH: Tumor-infiltrating FOXP3(+) lymphocytes are associated with cytotoxic immune responses and good clinical outcome in oestrogen receptor-negative breast cancer. Br J Cancer *108(1):* 155-162, 2013.
33 Sakakura K, Chikamatsu K, Sakurai T, Takahashi K, Murata T, Oriuchi N and Furuya N: Infiltration of dendritic cells and NK cells into the sentinel lymph node in oral cavity cancer. Oral Oncology *41*: 89-96, 2005.
34 Ishigami S, Natsugoe S, Uenosono Y, Hata Y, Nakajo A, Miyazono F, Matsumoto M, Hokita S and Aikou T: Infiltration of anti-tumor immunocytes into the sentinel node in gastric cancer. J Gastrointest Surg *7*: 735-739, 2003.
35 Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, Aridome K, Hokita S and Aikou T: Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer *88*: 577-583, 2000.
36 Villegas FR, Coca S, Villarrubia VG, Jiménez R, Chillón MJ, Jareño J, Zuil M and Callol L: Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer *35*: 23-28, 2002.
37 Palucka K, Coussens LM and O'Shaughnessy J: Dendritic cells, inflammation, and breast cancer. Cancer J *19(6)*: 511-516, 2013.
38 da Cunha A, Michelin MA and Murta EF: Pattern response of dendritic cells in the tumor microenvironment and breast cancer. World J Clin Oncol *5(3)*: 495-502, 2014.
39 Poindexter NJ, Sahin A, Hunt KK and Grimm EA: Analysis of dendritic cells in tumor-free and tumor-containing sentinel lymph nodes from patients with breast cancer. Breast Cancer Res *6(4)*: R408-415, 2004.

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