Arabpour M, et al. Iran J Immunol. 2019; 16(2):108-116.
https://doi.org/10.22034/iji.2019.80254.
Iranian Journal of Immunology (IJI)
iji@sums.ac.ir

ORIGINAL ARTICLE

Correlation of 4-1BBL+ B Cells in Tumor Draining Lymph Nodes with Pathological Characteristics of Breast Cancer

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ABSTRACT

Background: B cells can increase the expression of granzyme B in $CD8^+$ T cells through 4-1BBL/4-1BB interaction and promote anti-tumor immunity. **Objective:** To investigate the expression of 4-1BBL on B cells in the breast tumor draining lymph nodes (TDLNs) and its association with disease parameters. **Methods:** Using Ficoll-Hypaque gradient centrifugation, mononuclear cells were isolated from axillary lymph nodes of 42 patients. Cells received 4 hours of PMA/Ionomycin stimulation, *in vitro*. Both unstimulated and stimulated cells were stained with anti–CD19 and anti–4-1BBL antibodies and subjected to flow cytometry. **Results:** 4-1BBL expression was detected on 2.8 ± 1.7% of unstimulated B cells, while 27.4 ± 11.9% of B cells expressed this co-stimulatory molecule following stimulation. In steady state, the percentage of 4-$1BBL^+$ B cells was not associated with cancer characteristics. However, in patients with invasive ductal carcinoma, the percentage of 4-1BBL expressing B cells in stimulated condition had a decreasing trend in grade III, compared to grade II+I. In addition, significantly higher frequency of 4-$1BBL^+$ B cells was seen in the TDLNs of ER+ or PR+ compared with ER– or PR– patients (p=0.021 and p=0.015, respectively). No significant associations were observed between the frequency of 4-$1BBL^+$ B cells and the number of involved LNs, Her2 expression or disease stage. **Conclusions:** The frequency of 4-$1BBL^+$ B cells significantly increased following a short time activation, and showed relative and significant associations with tumor grade and estrogen receptor status, respectively. More investigations are required to evaluate the potential of 4-$1BBL^+$ B cells for use in immunotherapy.

Received: 2019-01-09, Revised: 2019-03-12, Accepted: 2019-04-16.
Citation: Arabpour M, Ghods A, Shariat M, Talei AR, Mehdipour F, Ghaderi A. Correlation of 4-1BBL+ B Cells in Tumor Draining Lymph Nodes with Pathological Characteristics of Breast Cancer. *Iran J Immunol.* 2019; 16(2):108-116. doi: 10.22034/iji.2019.80254.

Keywords: 4-1BBL, Breast Cancer, B Cells, Tumor Draining Lymph Nodes

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INTRODUCTION

Breast cancer is known as the most prevalent cancer among women with an annual increasing incidence (1). Therefore, this condition is considered as a health problem worldwide and many researchers are searching for new approaches for better breast cancer diagnosis and treatment. It has been revealed that during breast cancer progression, immune cells and responses undergo significant changes both in peripheral and local components (2). In the previous studies in our center, the immune profile of the tumor draining lymph nodes (TDLNs) both in B and T cell compartments was significantly associated with breast cancer parameters (3,4). A major constituent of tumor draining lymph nodes are B cells which are primarily considered as antibody producing cells (5). Their protective role, however, is not limited to the production of tumor specific antibodies, as they influence T cell responses by expressing polarizing cytokines and/or co-stimulatory molecules (5-7). For instance, B cells are capable of cross-presenting antigens to $CD8^+$ T cells, improving their survival through CD27-CD70 and enhancing their expression of granzyme B via 4-1BBL (on B cells) / 4-1BB (on T cells) interaction (8-10). 4-1BBL or CD137L is a member of the TNF superfamily, expressed by antigen presenting cells, including B cells (11). The 4-1BBL receptor or 4-1BB (CD137) belongs to the TNF receptor superfamily, expressed by activated T cells, NK cells and endothelial cells (12). The interaction between 4-1BB and its ligand prepare a co-stimulatory signal for the activation and growth of NK and $CD8^+$ T cells (12). It has been demonstrated that 4-1BB signaling could enhance $CD8^+$ T cell effector as well as long term memory responses and rendered effector T cells, resistant to suppression by $CD4^+CD25^+FoxP3^+$ T regulatory cells (Tregs). These effects offered a potent anti-tumor immunity in mouse models of cervical and lung cancer (13). Lee *et al.* showed that, by presenting endogenous antigens, a subset of mouse and human $4\text{-}1BBL^+$ B cells induced the expression of granzyme B in $CD8^+CD28^-$ T cells. They showed that $4\text{-}1BBL^+$ B cell played a part in preventing melanoma progression in mice (9). On the other hand, another study showed that regulatory B cells (Breg) in a mouse model of breast cancer, expressed a low level of 4-1BBL and enhanced lung metastasis while *in-vitro* B cell stimulation was able to up-regulate 4-1BBL on B cells and improve antitumor responses (14).
Accordingly, 4-1BBL can be considered as one of the most valuable targets for inducing specific anti-tumor responses. The present objective was to assess 4-1BBL expression by B cells in the TDLNs of breast cancer patients and its association with clinico-pathological parameters of the disease.

MATERIALS AND METHODS

Patients. Fresh axillary LNs samples were obtained from 42 women with breast cancer (Table 1) undergoing surgery to remove their tumor mass. Patients had no history of either chemotherapy or radiotherapy, and agreed to participate in the investigation by signing informed consents. Our study was approved by the Ethics Committee of Shiraz University of Medical Sciences.
Isolation and activation of mononuclear cells. Fresh LNs were mechanically crushed into very small pieces in complete culture medium (RPMI 1640 containing 10% FBS and 1% penicillin / streptomycin, all purchased from Gibco, Life Technologies, USA) and filtered through a 40 μm cell strainer (SPL LIFE SCIENCES, South Korea) to provide a

homogenous cell suspension. In order to isolate mononuclear cells, cell suspension was centrifuged over a Ficoll-Hypaque (Lymphedex, inno-train Diagnostik GmbH, Germany) gradient. Mononuclear cells were re-suspended in complete culture medium at a concentration of 2×10^6 cells/ml, and were stimulated for 4 hours with 50 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (both from Sigma-Aldrich, Germany).

Flow cytometric analysis. Surface staining of 4-1BBL and CD19 markers was performed for both stimulated and unstimulated cells. Cells were washed and re-suspended in staining buffer (PBS containing 2% FBS) and stained with allophycocyanin (APC)–conjugated anti–human 4-1BBL (CD137L, Clone: 5F4) and Percpcy5.5–conjugated anti–human CD19 (Clone: HIB19) antibodies or their isotype controls (all from Biolegend, USA). Following 30 minutes of incubation, cells were washed with staining buffer, re-suspended in PBS and acquired on four color FACSCalibur flow cytometer (BD Biosciences, USA). Flow cytometric data were analyzed using FlowJo software (version 7.6.2, USA). Lymphocytes were gated based on their forward and side scatters; ultimately, cells expressing CD19 were gated as B cells and the expression of 4-1BBL was assessed in this gate.

Statistical analysis. Nonparametric Mann–Whitney U test was used to compare two groups and Kruskal-Wallis H and Dann's post-tests were applied to compare three or more groups. The Spearman rank's correlation test was used to determine the correlation between the frequency of B cells and the number of metastatic LNs or tumor size. P-values <0.05 were considered as statistically significant. SPSS (version 16, SPSS Inc, USA) was used for data analysis and graphs were prepared through the use of GraphPad Prism 6 software (GraphPad Software, Inc., USA).

RESULTS

Clinico-pathological characteristics of the patients with breast cancer.

Table 1 shows the details of the clinical and pathological parameters in 42 patients with breast cancer enrolled in our study. The average age of the patients was 49.7 ± 13.5 (29–79) years. 17 (40.5%) lymph nodes were involved (metastatic LNs, MLNs), while 25 (59.5%) were not involved by the tumor (non-metastatic, nMLNs). The most common type of tumor was invasive ductal carcinoma (34 out of 42 cases (81%)). According to the AJCC (7th edition), 2 patients were in stage I (4.8%), 21 patients in stage II (50%) and 18 patients in Stage III (42.9%).

4-1BBL$^+$ B cells in MLNs and nMLNs of breast cancer patients.

The percentage of 4-1BBL$^+$ B cells was determined in unstimulated and stimulated mononuclear cells (Figure 1, Table 2). Data analysis showed that 2.8 ± 1.7% of unstimulated B cells expressed 4-1BBL, while this co-stimulatory molecule was detected on 27.4 ± 11.9% of stimulated B cells (Table 2), indicating that short-term activation resulted in the significant up-regulation of 4-1BBL on B cells ($p<0.0001$, Figure 2). The percentage of B cells, expressing 4-1BBL, was not significantly different concerning MLNs and nMLNs, either in steady state or after stimulation (Figure 3).

Frequency of 4-1BBL$^+$ B cells and the number of involved LNs.

Statistical analysis showed that the percentage of 4-1BBL$^+$ B cells was not significantly different in patients categorized as N0-N3 (data not shown).

Table 1. Clinico-pathological characteristics of the breast cancer patients.

Characteristics	Value
Age (years)	49.7 ± 13.5 (29-79)
Lymph node (LN) status	
N0 (Free LNs)	5 (11.9%)
N1 (1-3 involved LNs)	19 (45.2%)
N2 (4-9 involved LNs)	12 (28.6%)
N3(>9 involved LNs)	6 (14.3%)
Tumor size (greatest dimension,cm)	
T1 (≤2)	19 (45.2%)
T2 (2-5)	21 (50%)
Tx (Unknown)	2 (4.8%)
Stage	
I	2 (4.8%)
II	21 (50%)
III	18 (42.9%)
Unknown	1 (2.4%)
Histological grade	
Well differentiated (I)	3 (7.1%)
Moderately differentiated (II)	28 (66.7%)
Poorly differentiated (III)	9 (21.4%)
Unknown	2 (4.8%)
Tumor type	
Infiltrating ductal carcinoma (IDC)	34 (81%)
Infiltrating ductal carcinoma with medullary feature (IDC+M)	5 (11.9%)
Lobular carcinoma	1 (2.4%)
Unknown	2 (4.8%)
Her2 expression	
Positive	6 (14.3%)
Negative	28 (66.7%)
Equivocal	5 (11.9%)
Unknown	3 (7.1%)
ER expression	
Positive	29 (69.1 %)
Negative	10 (23.8%)
Unknown	3 (7.1%)
PR expression	
Positive	30 (71.4%)
Negative	9 (21.4%)
Unknown	3 (7.1%)
Lymph nodes characteristic	
MLNs	17 (40.5%)
nMLNs	25 (59.5%)

MLN: metastatic lymph node, nMLN: non-metastatic lymph node, ER: Estrogen receptor, PR: Progesterone receptor.

Moreover, no significant correlation was observed between the frequency of 4-1BBL$^+$ B cells and the number of involved LNs.

Frequency of 4-1BBL+ B cells and tumor size, grade and breast cancer stages.

The frequency of 4-1BBL$^+$ B cells did not show significant associations with tumor size or grade and disease stage (data not shown). However, in patients with IDC, the percentage of 4-1BBL expressing B cells in stimulated state had a decreasing trend in grade III as compared with grade II+I. (p=0.092, Figure 4).

Table 2. The frequency of 4-1BBL expressing B cells in the TDLNs of the breast cancer patients.

B cell subsets	Min	Max	Median	Mean ± SD
$CD19^+$ cells	8.5	58.9	33.9	34.5 ± 12.7
$4\text{-}1BBL^+$ cells (in B cell gate) (unst)	0.4	7.2	2.3	2.8 ± 1.7
$4\text{-}1BBL^+$ cells (in B cell gate) (sti)	7.6	51.7	28.9	27.4 ± 11.9

unst: unstimulated, sti: stimulated, TDLNs: tumor draining lymph nodes.

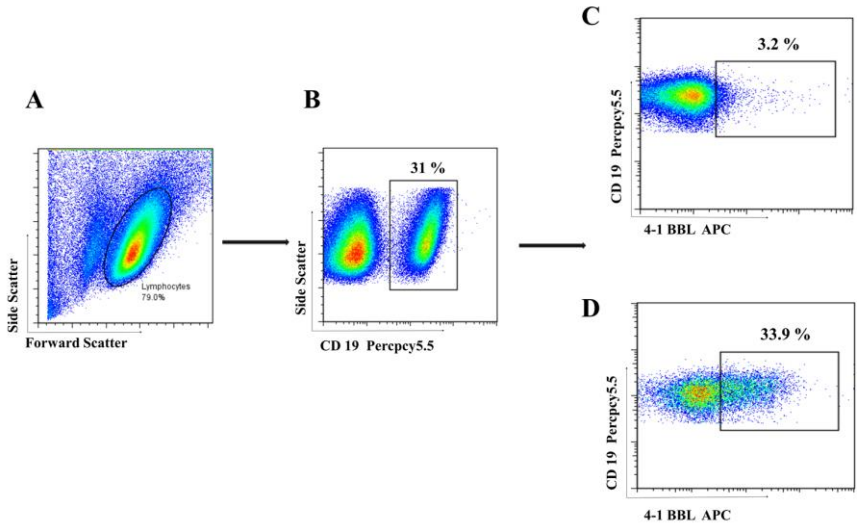

Figure 1. Flow cytometry analysis of 4-1BBL expressing B cells in the TDLNs of breast cancer patients. (A) Lymphocytes were gated based on their forward and side scatters, **(B)** B cells were gated as cells expressing CD19. **(C)** The percentage of $4\text{-}1BBL^+$ cells were determined in $CD19^+$ gate in unstimulated state. **(D)** The frequency of 4-1BBL expressing cells were assessed in B cells after 4 hours stimulation with PMA/Ionomycin.

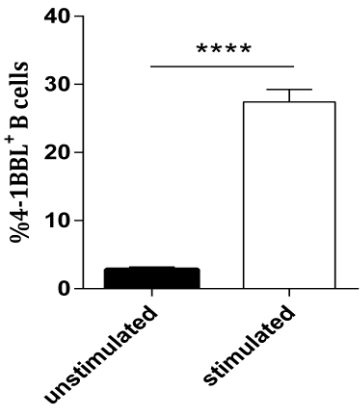

Figure 2. Comparison of the frequencies of $4\text{-}1BBL^+$ B cells before and after stimulation. Percentages of B cells expressing 4-1BBL were compared in unstimulated and stimulated samples. The frequency of $4\text{-}1BBL^+$ cells was assessed in B cell gate. Data are shown as mean ± SEM. **** p-value<0.0001.

Correlation of 4-1BBL+ B cells with age in breast cancer patients.
Analysis showed that the frequency of 4-1BBL+ B cells was not correlated with the age of breast cancer patients. Further assessed was this correlation in patients with stage II and III separately, where it was found that in stage II, the frequency of 4-1BBL+ B cells in stimulated condition had a significant reverse correlation with patients' age (R=–0.5, p=0.013); however, such correlation was not observed in stage III.

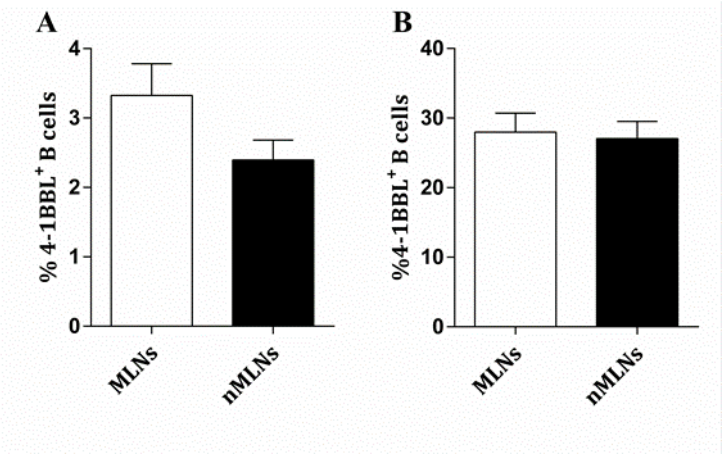

Figure 3. Comparison of the frequency of 4-1BBL+ B cells in MLNs and nMLNs of breast cancer patients (A) in steady state and (B) after 4 hours of stimulation with PMA/Ionomycin. B cells were gated and the frequency of 4-1BBL+ cells was assessed in this gate. Data are shown as mean ± SEM. MLN: metastatic lymph node, nMLN: non-metastatic lymph node.

Frequency of 4-1BBL+ B cells and Her2, ER and PR expression.
We did not observe any significant association between the frequency of 4-1BBL+ B cells and Her2 expression in breast cancer patients. However, following stimulation, the percentage of 4-1BBL+ B cells was significantly higher in TDLNs of ER+ than in ER– patients (p=0.021), and in PR+ patients compared to PR– ones (p=0.015, Figure 5).

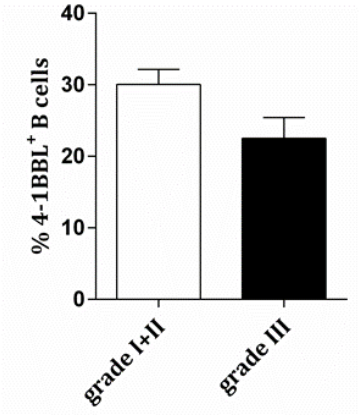

Figure 4. Association between the frequency of 4-1BBL+ B cells in the TDLNs of patients with IDC and tumor grade. 4-1BBL expression was assessed in B cells after 4 hours of stimulation with PMA/Ionomycin. CD19+ cells were gated and the frequency of 4-1BBL+ cells assessed in this gate. Data are shown as mean ± SEM. IDC: invasive ductal carcinoma.

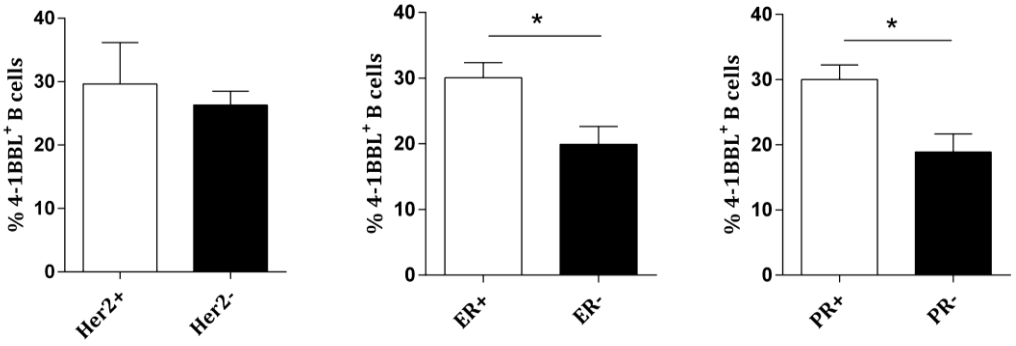

Figure 5. Association between the frequency of 4-1BBL$^+$ B cells in TDLNs of breast cancer patients with Her2, ER and PR expression. 4-1BBL expression was assessed in B cells after 4 hours of stimulation with PMA/Ionomycin. B cells were gated and the frequency of 4-1BBL$^+$ cells was assessed in this gate. Data are shown as mean ± SEM.* p-value<0.05.

DISCUSSION

It is currently well accepted that B cells are able to enhance the survival and function of T cells. It is proposed that B cells are required for proper activation and anti-tumor responses of both CD8$^+$ and CD4$^+$ T cells (15). In mouse models of melanoma and breast cancer, B cell depletion resulted in reduced T cells activity and increased tumor progression (14,16). 4-1BB–4-1BBL axis is known as a potent stimulator of cell mediated immunity as it has been shown to induce and augment both Th1 and Tc1 responses (17). It has further been demonstrated that via 4-1BBL, B cells can induce granzyme B or IFN-γ in CD8$^+$ T cells and enhance their anti-tumor activity (9,18). In the present research, it was shown that a minor fraction of TDLN B cells expressed 4-1BBL in unstimulated state, while short term activation resulted in a significant up-regulation of this molecule on B cells. This may indicate that B cells are ready to rapidly up-regulate 4-1BBL following proper stimulation. Bodogai and her co-workers demonstrated that regulatory B cells in breast tumor bearing mice had decreased levels of 4-1BBL expression and played a part in promoting lung metastasis. On the other hand, they showed that *in vitro* activation of B cells resulted in the up-regulation of 4-1BBL, and adoptive transfer of the activated B cells was able to efficiently improve anti-tumor activity of CD8$^+$ T cells via 4-1BBL/4-1BB axis and prevent lung metastasis (14). In addition, a recent study illustrated that B cells which constitutively expressed CD40L along with other co-stimulatory molecules such as 4-1BBL, CD70 or OX40L were able to improve anti-tumor immunity in a mouse model of B16 melanoma. Mechanistically, they showed that B cells expressing 4-1BBL alone or with CD40L and CD70 could serve as efficient antigen presenting cells and were capable of inducing IFN-γ responses in tumor specific CD8$^+$ T cells (18). As mentioned above, our study showed that 4-1BBL could be up-regulated on B cells following activation, hence it can be proposed that B cells can be extracted from the TDLNs of breast cancer patients, activated *in vitro*, to up-regulate 4-1BBL, and then transferred back to the patients to boost their anti-tumor immunity. The efficacy of the adoptive transfer of activated TDLNs B cells in enhancing anti-tumor immune responses has been shown in experimental models (19,20). Upregulation of 4-1BBL on B cells, not

only enhances T cells' specific response to tumor, but also augments the proliferation and antibody production in B cells via reverse signaling (21).

Another finding of this study was that the up-regulation level of 4-1BBL on B cells was not consistent among all samples. Neither was any difference observed between the percentage of 4-1BBL$^+$ B cells in MLNs and nMLNs; it can be concluded that the ability of B cells to up-regulate 4-1BBL is not influenced by the presence of tumor in LNs. The idea that different B cells have different abilities to upregulate 4-1BBL should be considered when using *in vitro* stimulated B cells for immunotherapy, since a stimulation protocol may not result in the same efficiency in all samples. On the other hand, the frequency of 4-1BBL expressing B cells showed a relative negative association with tumor grade. Unpublished data of our lab showed that the frequency of PD-L1 expressing B cells from breast TDLNs was positively associated with tumor grade. In addition, the percentage of 4-1BBL$^+$ B cells showed positive associations with ER/PR expression by breast tumors. Our current investigation showed that, the frequency of CD25$^+$FoxP3$^+$ Treg cells was higher in ER–/PR– patients (22), and our unpublished data revealed a negative relationship between the frequency of PD-L1$^+$ B cells and ER/PR expression by breast tumors. Because PD-L1$^+$ B cells are considered as regulatory B cells and 4-1BBL has been shown to be downregulated on regulatory B cells, the opposite association of 4-1BBL$^+$ and PD-L1$^+$ B cells with tumor grade and ER/PR expression may further support the fact that the immune profile of B cells is influenced by tumor characteristics. Furthermore, the opposite association of 4-1BBL+ and regulatory B or T cells with ER/PR expression may suggest that ER+/PR+ tumors may favor or induce a more effector immune microenvironment compared with ER–/PR– ones. This idea, however, requires additional studies with larger sample size along with functional investigations. Taken together, this descriptive study may propose a positive role for 4-1BBL$^+$ B cells in breast TDLNs; it is of note that we need to elucidate the functional relationship between 4-1BBL$^+$ B cells and CD4$^+$ or CD8$^+$ T cells in the draining lymph nodes of breast cancer.

In Conclusion, we showed that B cells can significantly increase the expression of the co-stimulatory molecule, 4-1BBL, following short-time stimulation. The higher frequency of 4-1BBL$^+$ B cells in breast TDLNs was related to two tumor characteristics, positive estrogen/progesterone receptor and lower grade. More studies are required to reveal the interplay between activated 4-1BBL expressing B cells and CD8$^+$ or CD4$^+$ T cells and their possible application in immunotherapies against breast cancer.

ACKNOWLEDGEMENTS

This work was financially supported by Shiraz University of Medical Sciences (Grant No. 95-01-01-12565) and Shiraz Institute for Cancer Research (Grant No. ICR-100-508). This study was a part of MSc project of Mohsen Arabpour, Department of Immunology, Shiraz University of Medical Sciences.

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