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TNFR2 but not TNFR1 is the main TNFR expressed by B and T lymphocytes in breast cancer draining lymph nodes

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is a key cytokine in inflammation and a driving force for leukocyte migration and recruitment. However, it may exert contrasting effects on the immune responses depend on its differential binding to its receptors (TNFR1 or TNFR2). The expression of TNF receptors by lymphocytes in the tumor draining lymph nodes (TDLNs) has not been thoroughly investigated. Herein, the expression of TNFRs on lymphocytes in the breast TDLNs was assessed. 40 axillary LN samples were obtained from breast cancer patients. Mononuclear cells were isolated using Ficoll-Hypaque gradient centrifugation and stained with anti-CD3, CD4, CD8, CD19, TNFR1 and TNFR2 and subjected to flow cytometry. Results showed that TNFR2 was detected on unstimulated B or T cells in the breast TDLNs while TNFR1 was nearly absent on these cells. Short or long term activation did not increase the expression of TNFR1. The percentage of $TNFR2^+$ cells was significantly higher in $CD4^+$ compared to $CD19^+$ or $CD8^+$ cells. No significant association was observed between the percentage of TNFR2 expressing T cells and prognostic indicators. However, the percentage of $TNFR2^+$ B cells in the metastatic LNs had negative associations with tumor grade and the number of involved LNs ($P = 0.009$ and $P = 0.04$, respectively). Collectively, TNFR2 was the main TNFR expressed by unstimulated B or T lymphocytes in the breast TDLNs and the frequency of $TNFR2^+$ B cells was connected to good prognosticators. The effects of TNFR2 expression by lymphocytes of breast TDLNs on their functions requires more functional studies.

1. Introduction

Cytokines, as the major mediators of inflammation and immune responses, have important roles in the orchestrating of the immune responses in the tumor microenvironment and tumor draining lymph nodes (TDLNs) [1]. Tumor necrosis factor alpha (TNF-α) is a pleotropic cytokine with diverse and opposing functions including cell survival, proliferation, differentiation and cell death [2]. The complexity of TNF-α, somehow originates from having two forms–transmembrane (mTNF-α) and soluble (sTNF-α), and two receptors– TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2)– each of which has several distinct functions [3].

TNFR1 (CD120a) and TNFR2 (CD120b) are 55 and 75 kDa type I transmembrane proteins belonging to the large family of TNF receptor superfamily. The extracellular domains of TNFR1 & 2 which bind to TNF-α share extreme homology [4]; however, their intracellular domains are not homologue, causing activation of different signaling pathways and distinct functions [5]. TNFRs can have soluble isoforms as they can be cleaved by TACE (TNF-α converting enzyme) from the cell surface before or after ligand binding. The soluble form is still able to interact with its ligands acting as a decoy mechanism to remove the ligand and counter balance the TNF-induced effects [6,7]. It has been reported that TNFR1 have ubiquitous expression on most cell types except erythrocytes [5]; however, there are studies which showed that TNFR1 was near absent or expressed at very low level on unstimulated B or T lymphocytes [8,9]. TNFR1 binds to both soluble and transmembrane forms of TNF-α. TNF-α-TNFR1 interaction results in the activation of the pathways mediated by its cytoplasmic death-domain and promoting several effects including inflammation, cytotoxicity and apoptosis [6]. In comparison to TNFR1, TNFR2 is expressed on limited cell types such as lymphocytes, endothelial cells, neurons [3] and hematopoietic cells [10]. TNFR2 is preferentially stimulated by mTNF-α, however it can also bind to the soluble form of this cytokine [11]. As the death-domain motifs are absent in the intracellular portion of

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TNFR2, it is not capable of directly inducing programmed cell death [3]. mTNF-α /TNFR2 interaction can promote lymphocyte activation and proliferation but the exact signaling pathways and biological functions of the receptor has not been completely understood [12]. It was shown that TNFR2 have a co-stimulatory functions in both B and T cells and induce the production of both inflammatory and suppressive cytokines [8,13,14]. Besides, it is expressed by regulatory T cells (Tregs). Data on the effects of TNFR2 stimulation on Foxp3 expression and suppressive activities of Tregs are controversial and both promoting and inhibitory effects have been reported [6,15–18].

There are limited data on the expression of TNFRs by the lymphocytes in TDLNs, therefore in the present study the expression of TNFRs on lymphocytes of breast TDLNs was assessed and its associations with disease parameters were investigated.

2. Materials and methods

2.1. Patients

Axillary Lymph Node (LN) samples were taken from 40 breast cancer patients (one LN from each patient) who underwent axillary lymph node dissection (ALND) or modified radical mastectomy (MRM). The collaborating pathologist randomly select one dissected axillary LN while sentinel LNs and small axillary LNs were excluded because they should totally undergo pathological examination. Breast cancer was confirmed in all patients according to pathological reports. Patients who received chemo- or radiotherapy at any time before surgery were excluded from our study. Patients were informed about aims and process of the study and all signed written informed consents. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences. A fresh part of each LN was obtained and transferred to complete culture medium [RPMI-1640 with 10% FBS and 1% Penicillin/Streptomycin (CM10), all from Gibco, Life Technologies, USA] and a part, remained for routine pathological examination.

2.2. Preparation of mononuclear cells

To obtain homogenous cell suspension, fresh LNs were mechanically crushed into very small pieces in complete culture medium and filtered through a 40 μm cell strainer (SPL LIFE SCIENCES, South Korea). In order to isolate mononuclear cells, cell suspension was centrifuged over a Ficoll-Hypaque (Lymphedex, inno-train Diagnostik GmbH, Germany) gradient. After that, cells were re-suspended in a complete culture medium. To assess the expression of TNFR1 and TNFR2, unstimulated cells were used. However, for the evaluation of the effect of stimulation on TNFR1 expression, lymphocytes of three samples were stimulated with Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma-Aldrich, Germany) and Ionomycine (1 μg/ml, Sigma-Aldrich) for 5 h and in two samples with Phytohemagglutinin (PHA) (1 μl/ml and 2 μl/ml, Gibco) for 24 h.

2.3. Flow cytometry analysis

2.3.1. Antibodies

We used Fluorescein isothiocyanate (FITC)–conjugated anti-human CD3 (Clone: UCHT1), FITC anti-human CD16 (Clone: 3G8), Phycoerythrin (PE)–conjugated anti-human TNFR2 (CD120b, Clone: 3G7A02), PE anti-human TNFR1 (CD120a, Clone: W15099A), PerCP/Cy5.5 anti-human CD19 (Clone: HIB19), PerCP/Cy5.5 anti-human CD4 (Clone: RPA-T4), PerCP/Cy5.5 anti-human CD8a (Clone: HIT8a) and PerCP anti-human CD3 (Clone: SK7) antibodies and their associated isotype controls, all purchased from Biolegend, USA. FITC anti-human CD14 (Clone: MQP6) and its isotype control was obtained from BD Biosciences, USA.

2.3.2. Cell staining

Unstimulated or stimulated cells were washed and re-suspended in staining buffer (PBS containing 2% FBS) and surface staining was done using anti-TNFR1, anti-TNFR2, anti-CD3, anti-CD4, anti-CD8 and anti-CD19 or their respective isotype controls. After 30 min of incubation, cells were washed with staining buffer, re-suspended in PBS and subjected to flow cytometry (Four-color FACSCalibur, BD Biosciences, USA).

For the detection of TNFR1 on leukocytes of human peripheral blood, 3 samples were prepared in EDTA. In each sample, appropriate amounts of anti-TNFR1, anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD16 and anti-CD14 antibodies or their associated isotype control antibodies were added and incubated for 30 min at room temperature. After that, 1 ml of FACS lysing solution (1X, BD Biosciences, USA) was added into each tube. Tubes were incubated for 10–12 min at room temperature. Then, they were centrifuged and supernatant was discarded. Finally, cells were twice washed and acquired on flow cytometer.

2.3.3. Flow cytometry data analysis

For the analysis of TDLNs, lymphocytes were gated according to their forward and side scatters. Next, $CD19^+$, $CD4^+$, $CD8^+$ and $CD3^+$ cells were determined in lymphocytes gate and the expression of TNFR1 & 2 were assessed in these gates.

For the analysis of peripheral blood samples, granulocytes, monocytes and lymphocytes were gated based on their forward and side scatters. $CD14^+$ cells were determined in monocytes gate and $CD4^+$, $CD8^+$, $CD19^+$ and $CD16^+$ cells were gated in lymphocytes population. The frequencies of TNFR1 expressing cells were assessed in granulocytes, $CD14^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD16^+$ gates.

2.4. Statistical analysis

Results were analyzed using SPSS (version 16, SPSS Inc, USA). Nonparametric Mann–Whitney *U* and Kruskal–Wallis H tests were applied to compare cell subsets in two or multiple groups, respectively. Dunn's post-test was used to compare all pairs in multiple groups. Correlations between the frequency of different lymphocyte subsets and the tumor size or the number of involved lymph nodes were determined using Spearman rank's correlation test. P values less than 0.05 were considered as statistically significant. GraphPad Prism 6 software (GraphPad Software, Inc., USA) was used for preparing the Graphs.

3. Results

3.1. Clinico-pathological characteristics of breast cancer patients

40 patients with pathologically confirmed breast cancer were enrolled in this study. The mean age of patients was 50.8 ± 11.9 years (31–79). Among the examined LNs, 18 (45%) of them were involved by the tumor (metastatic lymph nodes, MLNs) while 22 (55%) of them were tumor free (non-metastatic lymph nodes, nMLNs). All patients except 3, were node positive with at least one metastatic lymph node. Invasive ductal carcinoma was the most common tumor type among patients with the frequency of 80% (32 out of 40 cases). For TNM staging, patients were categorized according to 7th edition of AJCC (American Joint Committee on cancer Classification and stage group). 50% of the patients were in stage II, and 45% were in stage III. More details about clinical and pathological characteristics of the patients are classified in Table 1.

3.2. TNFR2 expression by B and T lymphocytes in the TDLNs of breast cancer patients

The percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$ and $CD19^+$ lymphocytes and their TNFR2 expressing subsets were assessed

Table 1
Clinico-pathological characteristics of breast cancer patients.

Characteristics	Value
Age (years)	50.8 ± 11.9 (31–79)
Lymph Node (LN) Status	
N0 (Free LNs)	3 (7.5%)
N1 (1–3 involved LNs)	19 (47.5%)
N2 (4–9 involved LNs)	10 (25%)
N3 (> 9 involved LNs)	8 (20%)
Tumor Size (greatest dimension, cm)	
T1 (≤2)	15 (37.5%)
T2 (2-5)	22 (55%)
Tx (Unknown)	3 (7.5%)
Stage	
II	20 (50%)
III	18 (45%)
Unknown	2 (5%)
Histological Grade	
Well differentiated (I)	3 (7.5%)
Moderately differentiated (II)	27 (67.5%)
Poorly differentiated (III)	7 (17.5%)
Unknown	3 (7.5%)
Tumor Type	
Invasive ductal carcinoma (IDC)	32 (80%)
IDC with Medullary features (IDC + M)	4 (7.5%)
Others (Lobular carcinoma, Metaplastic carcinoma)	2 (5%)
Unknown	3 (7.5%)
Her2 Expression	
Positive	7 (17.5%)
Negative	26 (65%)
Equivocal	5 (12.5%)
Unknown	2 (5%)
ER Expression	
Positive	32 (80%)
Negative	6 (15%)
Unknown	2 (5%)
PR Expression	
Positive	29 (72.5%)
Negative	9 (22.5%)
Unknown	2 (5%)
Lymph Nodes Characteristic	
MLNs	18 (45%)
nMLNs	22 (55%)

MLN: Metastatic Lymph Node, nMLN: non-Metastatic Lymph Node, ER: Estrogen Receptor, PR: Progesterone Receptor, Her2: Human Epidermal Growth Factor Receptor 2.

Table 2
Percentages of B or T cells in lymphocyte gate and the percentages of TNFR2 expressing cells in $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$ and $CD19^+$ gates in the TDLNs of breast cancer patients.

Cell subset	Min	Max	Median	Mean ± SD
$CD3^+$	26.4	80.7	60.1	58.5 ± 12.3
$CD4^+$	18.1	73	49.9	48.7 ± 12.4
$CD8^+$	4.9	14.8	9.4	9.6 ± 2.9
$CD8^+CD3^+$	4.8	14.6	9.1	9.4 ± 2.9
$CD19^+$	14.5	63.5	34.7	35.1 ± 11
$TNFR2^+$ cells (in lymphocytes gate)	25	69.2	41.8	43.1 ± 9.2
$CD3^+TNFR2^+$	20.9	62	45.9	45.9 ± 10.3
$CD4^+TNFR2^+$	25.6	71.9	48.2	49 ± 9.9
$CD8^+TNFR2^+$	13.6	68.3	40.6	42.2 ± 13.2
$CD3^+CD8^+TNFR2^+$	12.6	68	40.1	41.6 ± 13.4
$CD19^+TNFR2^+$	10.2	62.8	36.9	38.2 ± 11.4

TDLN: Tumor Draining Lymph Node, SD: Standard Deviation.

(Table 2, Fig. 1). Comparison of the frequencies of $TNFR2^+$ T and B cells showed that the frequency of $TNFR2^+$ cells was significantly higher among $CD4^+$ T cells in comparison with $CD19^+$ and $CD8^+$ lymphocytes (P = 0.0002 and P = 0.030, respectively, Fig. 2).

3.3. TNFR2 expressing B or T cell subsets in the MLNs and nMLNs of breast cancer patients

The percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$ and $CD19^+$ lymphocytes were determined in the breast TDLNs. Analysis revealed that the frequency of $CD19^+$ cells had a reverse correlation with the percentages of $CD4^+$ and $CD3^+$ cells (R = −0.8, P < 0.001). In addition, $CD4^+$ and $CD3^+$ lymphocytes were significantly higher in the nMLNs (P = 0.021 and P = 0.040, respectively, Fig. 3A). There were no significant changes in the frequencies of $CD8^+$, $CD8^+CD3^+$ and $CD19^+$ lymphocytes in the MLNs compared to nMLNs. More analysis revealed that $CD4^+/CD8^+$ T cell ratio was significantly lower in the MLNs (P = 0.022) while $CD19^+/CD4^+$ cell ratio had a trend toward increase in these nodes (P = 0.069.). Next, the frequencies of TNFR2 expressing subsets were determined in the lymphocytes' gate which showed no significant differences in the MLNs and nMLNs (Fig. 3B).

3.4. TNFR2 expressing B or T cells in different breast cancer stages

Analysis of the frequencies of different cell subsets in patients with stage II and III showed that $CD4^+$ and $CD3^+$ T cells have significant higher frequencies in stage II as compared with stage III (P = 0.024 and P = 0.013, respectively, Fig. 4A), while the frequencies of $CD8^+$,$CD8^+CD3^+$ and $CD19^+$ lymphocytes have no significant changes. Both $CD19^+/CD8^+$ and $CD19^+/CD4^+$ cell ratios were significantly higher in stage III (P = 0.041 and P = 0.033, respectively). The percentages of $CD3^+TNFR2^+$, $CD4^+TNFR2^+$, $CD8^+TNFR2^+$, $CD8^+CD3^+TNFR2^+$ and $CD19^+TNFR2^+$ were not significantly different in patients with stage II in comparison with stage III (Fig. 4B).

3.5. TNFR2 expressing lymphocyte subsets and tumor size or grade

Analysis revealed that neither the frequencies of lymphocyte subsets nor the percentages of their TNFR2 expressing subpopulations were significantly different in the patients with tumor sizes ≤2 cm and patients with tumor sizes > 2 cm (data not shown).

Likewise, the percentages of T / B cells or their $TNFR2^+$ subsets did not show significant associations with tumor grade (Data not shown). $CD19^+/CD8^+$ cell ratio was higher in grade III compared to grade I + II however, the difference did not reach the statistical significance (P = 0.053). Moreover, the percentage of $CD19^+TNFR2^+$ cells showed a non-significant increasing trend in patients with grade I + II of the tumor. However, when MLNs and nMLNs were separately considered, the percentage of $TNFR2^+$B cells was significantly higher in the MLNs of patients with grade I + II (P = 0.009, Fig. 5). The frequencies of other TNFR2 expressing subsets showed no significant association with tumor grade in the MLNs and nMLNs.

3.6. Association of the TNFR2 expressing lymphocytes subsets with the number of involved LNs

There were only 3 node negative patients in our study, therefore, the analysis was focused on node positive ones with different numbers of involved LNs (from N1 to N3). The $CD4^+$ and $CD3^+$ lymphocytes frequencies significantly decreased in TDLNs of patients with > 9 involved LNs (N3) compared to patients with 1–3 involved LNs (N1) (P = 0.007, Fig. 6A). Besides, $CD19^+/CD4^+$ cell ratio was significantly higher in N3 group in comparison with N1 (P = 0.007). There were no significant differences in the percentages of $CD3^+TNFR2^+$, $CD4^+$TNFR2, $CD8^+TNFR2^+$, $CD8^+CD3^+TNFR2^+$ and $CD19^+TNFR2^+$ lymphocytes between N1, N2 and N3 group (Fig. 6B).

Moreover, the percentages of $CD3^+$ and $CD4^+$ populations reversely correlated with the number of involved LNs (R= −0.3, P = 0.04, and R= −0.3, P = 0.02, respectively). In the MLNs, a significant negative correlation was seen between the percentage of $CD19^+TNFR2^+$ subset and the number of involved LNs (R = −0.5,

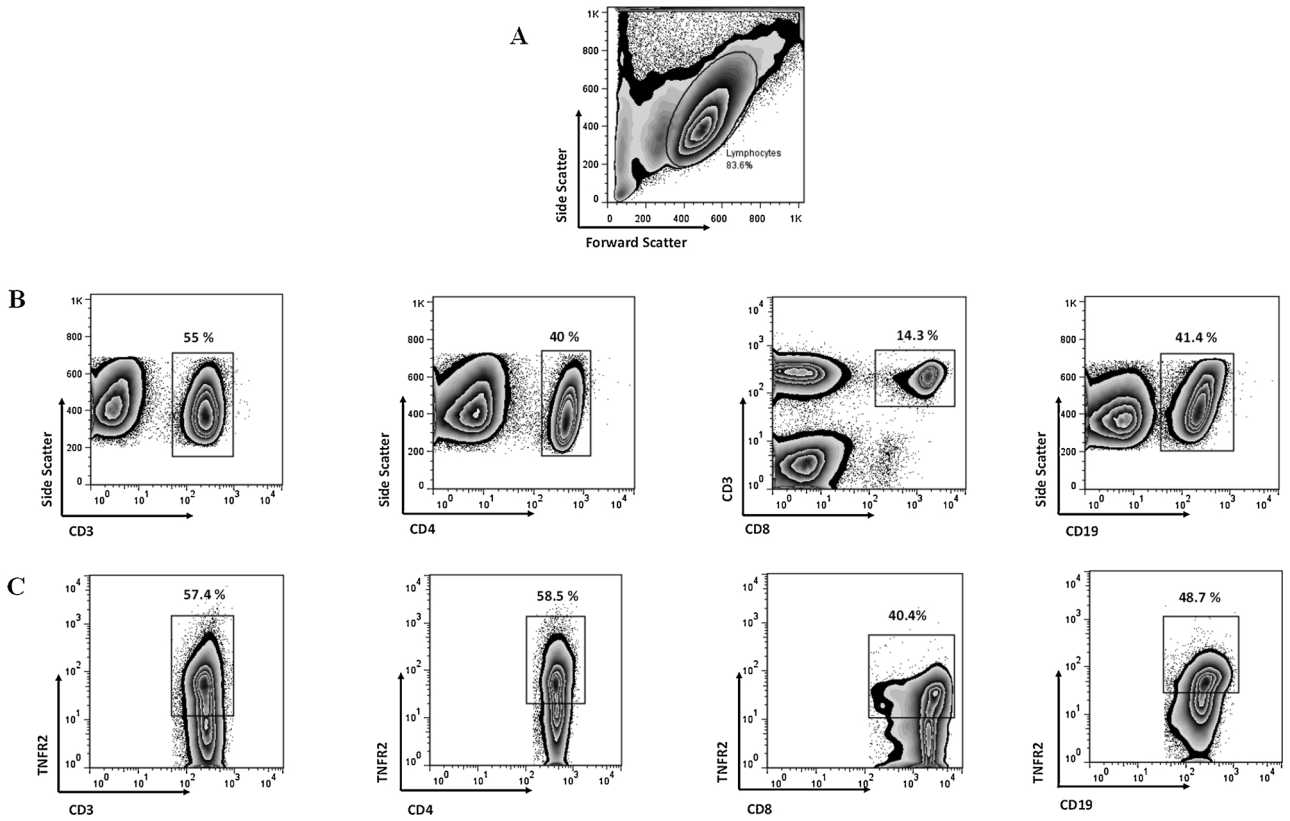

Fig. 1. Flow cytometry evaluation of the TNFR2 expression on lymphocytes subsets. **A.** Lymphocytes were gated according to their forward and side scatters. **B.** $CD3^+$, $CD4^+$, $CD3^+CD8^+$ and $CD19^+$ cells were determined in the lymphocytes gate. **C.** The frequency of $TNFR2^+$ cells was assessed in each lymphocyte subset.

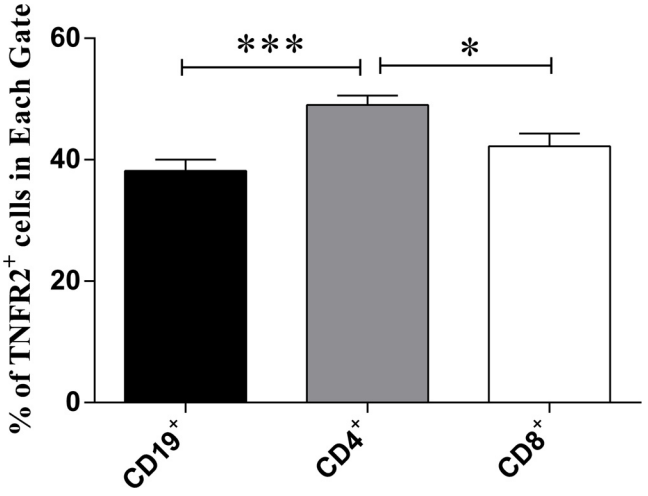

Fig. 2. Comparison of the frequencies of $TNFR2^+$ cells in $CD19^+$, $CD4^+$ and $CD8^+$ gate. Data are shown as mean ± SEM, * P value < 0.05, *** P value < 0.001.

P = 0.04).

3.7. TNFR2 expressing lymphocyte subsets in patients with different ER/PR or Her2 expression statues

Results of the present study indicated that there were no significant associations between the frequencies of T and B lymphocytes or their $TNFR2^+$ subsets and estrogen receptor (ER)/progesterone receptor (PR) or Her2 expression by tumors. However, the percentages of $CD19^+$ cells was non-significantly higher in the TDLNs of Her2– patients (P = 0.082).

3.8. Assessment of the TNFR1 expression by B and T lymphocytes in the breast tumor draining LNs

TNFR1 expression was evaluated in the TDLNs of 10 breast cancer patients (7 nMLNs and 3 MLNs). It was shown that TNFR1 expressed at very low levels on $CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ T cells in the TDLNs (Fig. 7). Data revealed that the maximum percentage of TNFR1 expressing lymphocytes was about 0.6% (0.2 ± 0.2). In addition, the frequencies of TNFR1 expressing $CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ cells were 0.4 ± 0.4, 0.2 ± 0.3, ≤0.1, 0.4 ± 0.4, respectively.

To see whether stimulation can up-regulate the expression of TNFR1 on lymphocytes, in 3 samples, cells were stimulated with PMA/I for 5 h. This stimulation did not change the expression level of TNFR1 (Fig. 8A & B). Likewise, TNFR1 was not up-regulated on the surface of lymphocytes after 24 h of stimulation with PHA (Fig. 8C & D).

3.9. Expression of TNFR1 by different leukocyte subsets of normal peripheral blood

The expression of TNFR1 on lymphocytes, monocytes and granulocytes of peripheral blood of 3 normal individuals was assessed (Fig. 9). Data showed that TNFR1 expressed on almost all granulocytes in peripheral blood (≥ 98.8%) and on more than half of $CD14^+$monocytes (≥ 60.5%). Similar to the results mentioned for breast TDLNs, TNFR1 expression was still low on lymphocytic subsets in the peripheral blood, as the percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD16^+$ lymphocytes expressing TNFR1 were ≤ 0.7%, ≤ 1.1%, ≤ 1.5%, ≤ 4.5% and ≤ 6.1%, respectively.

4. Discussion

It has been shown that immune contexture of the tumors have strong association with prognostic indicators and patient's survival and

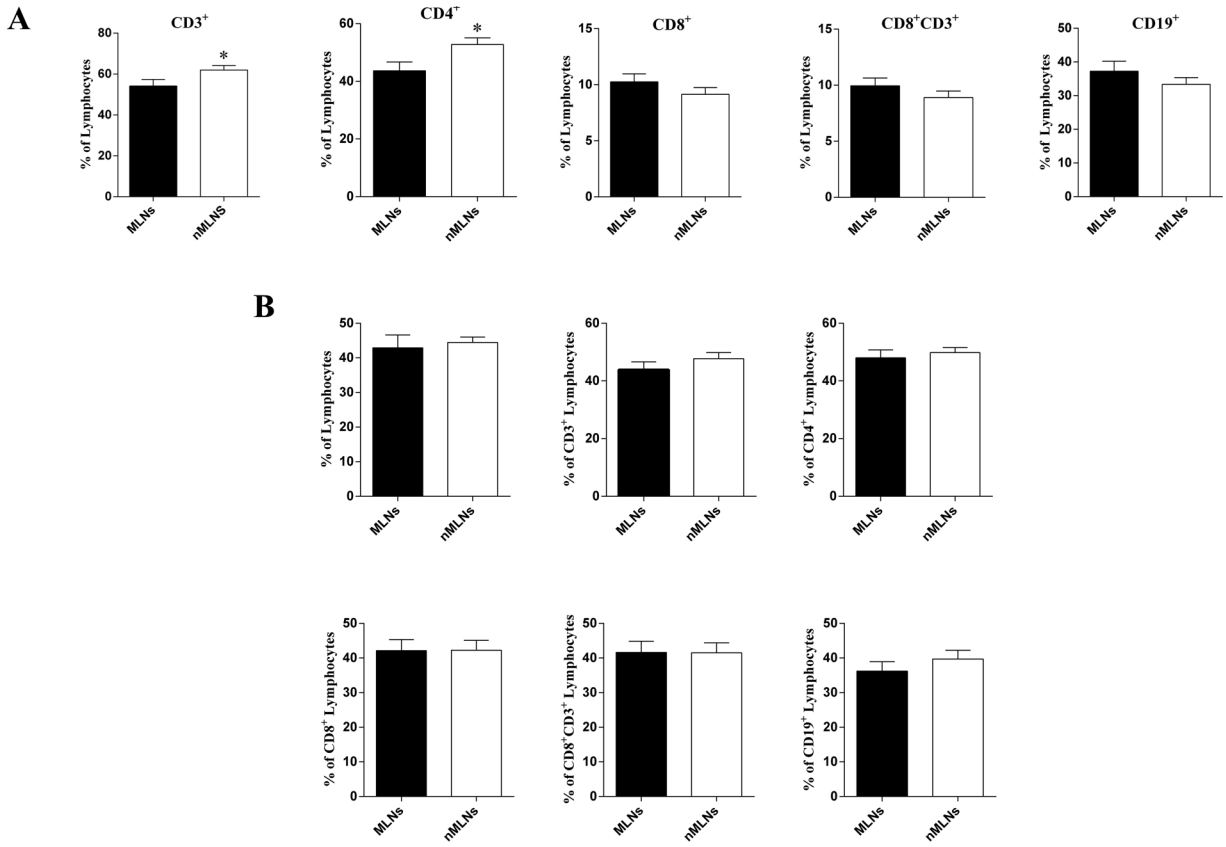

Fig. 3. Comparison of the frequencies of **A.** $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$, $CD19^+$ cells and **B.** their TNFR2 expressing subsets in metastatic (MLNs) and non-metastatic lymph nodes (nMLNs) of breast cancer patients. Data are shown as mean ± SEM. * P value < 0.05.

even can be used as an independent prognosticator [19,20]. As mentioned before, cytokines have critical role in orchestrating the immune responses against tumor which can be used or targeted to improve cancer therapy [21]. One of these cytokines, TNF-α, has been shown to have complex effects in the tumor microenvironment [6]. In this study, we investigated the association between the expression of TNF receptors (TNFR1 & 2) by lymphocytes of the breast TDLNs and breast cancer parameters.

Our study revealed that the frequencies of $CD3^+$ and $CD4^+$ T cells were higher in the nMLNs and had negative associations with poor prognostic indicators such as higher stage or more involved LNs. It can be concluded that the percentage of $CD4^+$ and $CD3^+$ T cells might be used as a prognostic indicator. This conclusion is supported by another study which showed that the frequency of $CD4^+$ T cells in the TDLNs of breast cancer patients, had positive association with patients' survival, even independent of nodal invasion [20]. Besides, other studies showed the association of the frequencies of $CD4^+$ or $CD8^+$ T cells with cancer parameters. The percentage of $CD3^+$ T cells in breast TDLNs have been reported to have negative correlation with tumor size [22]. It has been reported that the $CD8^+/CD19^+$ ratio in tumor infiltrating lymphocytes of medullary carcinoma showed reverse correlation with Her2 expression and presence of LN involvement [23]. In our study $CD19^+/CD8^+$ ratio had positive association with two poor prognostic markers, higher stage and higher tumor grade.

Our analysis showed that all lymphocytes subsets expressed TNFR2 and $CD4^+$ T cells were the major lymphocyte subset which expressed TNFR2. To date, there is limited data on the expression of TNFR1 or 2 by the lymphocyte subsets in the TDLNs or peripheral blood of cancer patients. However, the expression of TNFRs by the peripheral blood lymphocytes of normal donors was investigated which showed contrasting results. In one study, the TNFR2 expression was assessed in lymphocytes of peripheral blood and bronco-alveolar lavage fluid of sarcoidosis patients and healthy subjects. The study revealed that more than 80% of lymphocytes expressed TNFR2 [24]. In contrast, it was reported in another study that 3.8 ± 2.2% and 8.2 ± 5.5% of unstimulated peripheral blood $CD4^+$ and $CD8^+$ lymphocytes expressed TNFR2, respectively. Upon 24 h of stimulation with anti-CD3 antibody, the percentages of $CD4^+TNFR2^+$ or $CD8^+TNFR2^+$ lymphocytes increased to 46.5 ± 13.3% and 35.2 ± 12.6%, respectively [9]. The mean percentages of TNFR2 expressing $CD4^+$ and $CD8^+$ cells in activated state in the mentioned study, are close to the mean percentages of $TNFR2^+CD4^+$ and $CD8^+$ cells in our investigation. Similarly, another study showed that TNFR2 was expressed on less than 10% of unstimulated peripheral blood $CD19^+$ B cells, but the frequency of B cells expressed TNFR2 increased to about 70% of B cells following 3 days activation with TLR-9 agonist [8]. These studies indicates that TNFR2 expression increases following activation in both B and T cells. Therefore, higher percentage of $TNFR2^+$ cells in breast TDLNs in comparison with those reported for lymphocytes in normal peripheral blood, might be due to higher activation states of lymphocytes in the TDLNs in comparison with normal peripheral lymphocytes. To further address this issue, the expression of TNFR2 on lymphocytes of draining LNs of other cancers, reactive nodes of non-cancerous patients and other lymphoid tissues such as tonsil or spleen should be investigated.

In this study, no significant association was observed between the percentages of TNFR2 expressing T cells and breast cancer prognosticators; however, higher percentages of $TNFR2^+$ B cells were associated to lower tumor grade and less involved LNs and both associations were significant in the MLNs. This might imply that TNFR2 expressing B cells may have positive role in the TDLNs, this assumption, however should be validated with larger sample size and functional studies. It should not be ignored that different subpopulations of regulatory or effector B or T cells have been reported to express TNFR2 and the ligation of this receptor by TNF-α can have differential effects on

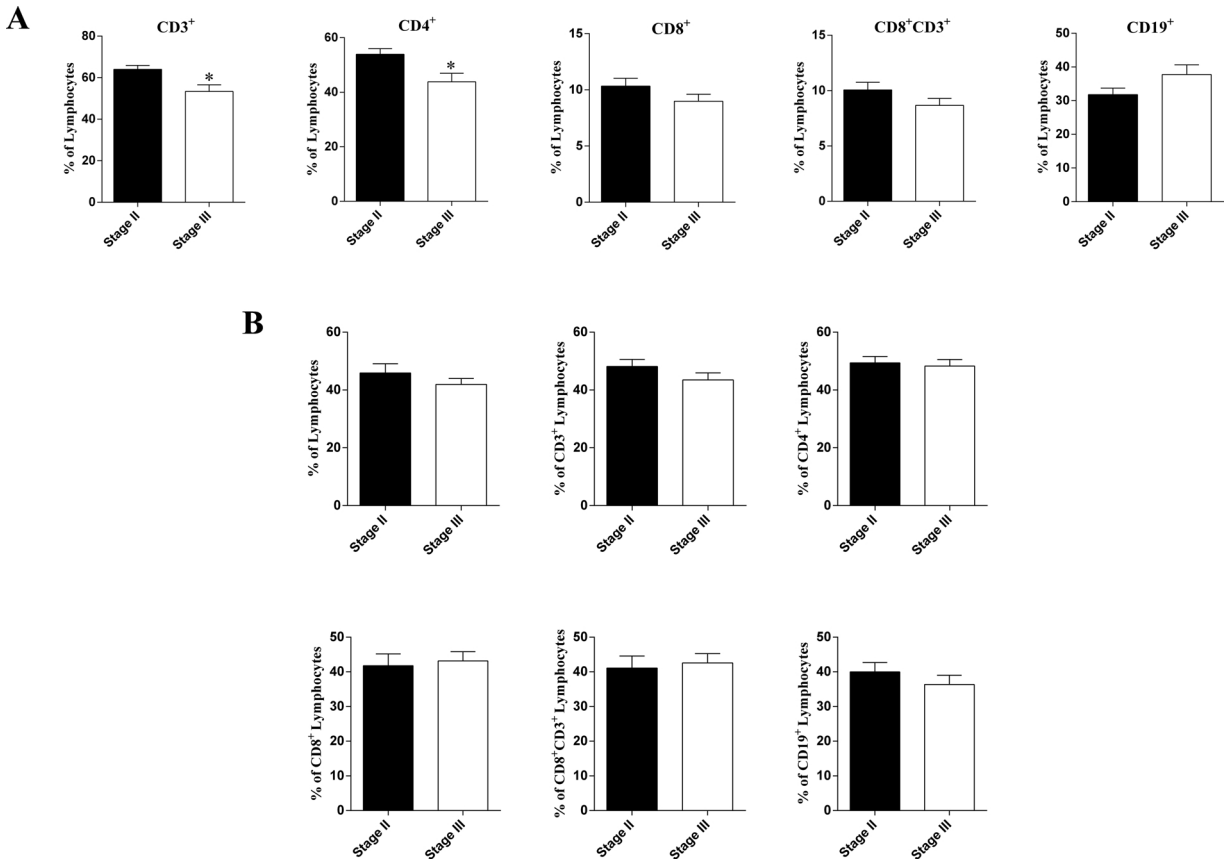

Fig. 4. Comparison of the frequencies of **A.** $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$, $CD19^+$ cells and **B.** their TNFR2 expressing subsets in the TDLNs of breast cancer patients with stage II and stage III. Data are shown as mean ± SEM. * P value < 0.05.

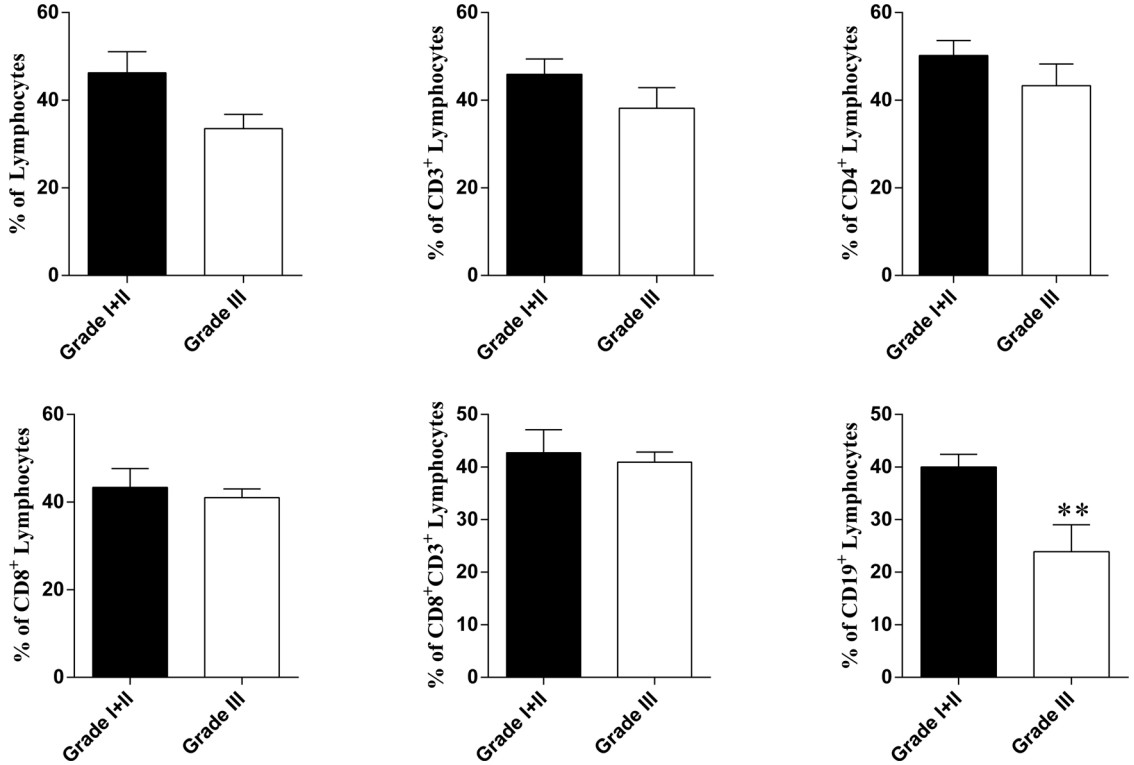

Fig. 5. Comparison of the frequencies of TNFR2 expressing cells in $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$, $CD19^+$ gate in metastatic lymph nodes (MLNs) of breast cancer patients according to tumor grade. Data are shown as mean ± SEM. ** P value < 0.01.

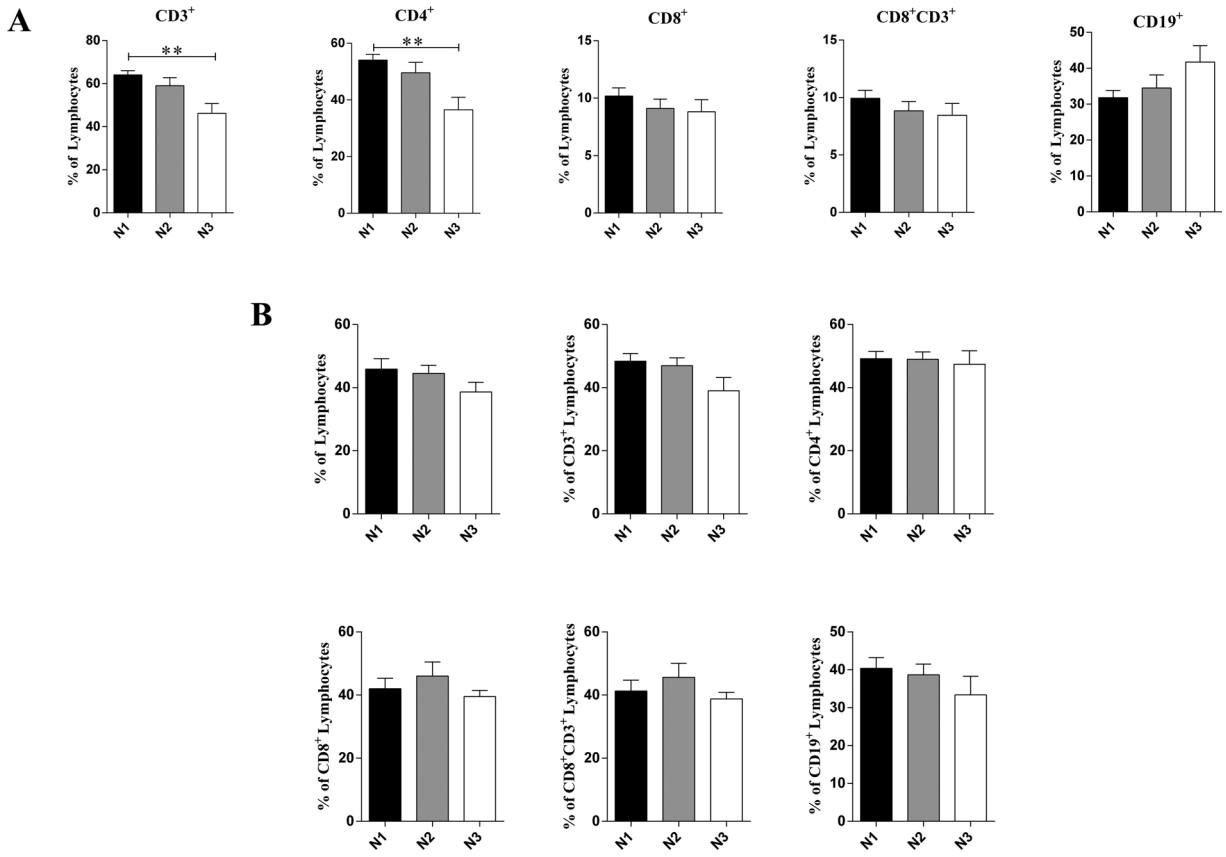

Fig. 6. Comparison of the frequencies of **A.** $CD3^+$, $CD4^+$, $CD8^+$, $CD8^+CD3^+$, $CD19^+$ cells and **B.** their TNFR2 expressing subsets in TDLNs of breast cancer patients according to nodal status (N1: 1–3 involved LNs, N2: 4–9 involved LNs and N3: > 9 involved LNs). Data are shown as mean ± SEM. ** P value < 0.01.

these cells. A recent study showed that $TNFR2^+$ B cells may or may not be able to produce IL-10, while the majority of IL-10 producing B cells in the peripheral blood of normal individuals were $TNFR2^+$. When $TNFR2^+$ B cells were stimulated with CpG and a variant of TNF-α, which mimicked the mTNF-α, the cells produced both IL-10 and IL-6 [8]. The role of TNF-α in induction and expansion of $IL\text{-}10^+$ B cells have been shown in another study as well [25]. Therefore TNF-α can have opposing effects on B cell cytokine production and either pro-inflammatory or regulatory cytokines can be induced in B cells upon TNFR2 stimulation. In addition, TNFR2 expression by both $FoxP3^+$ Tregs and $FoxP3^-$ non-Tregs has been shown [26,27]. TNFR2 signaling has co-stimulatory effects on effector $CD4^+$ T cells and enhance IFN-γ production [14]. On the other hand, both positive and negative effects of TNFR2 stimulation on the suppressive function of Treg cells have been shown [6]. Therefore, assessment of the expression of FoxP3, effector or regulatory molecules by $TNFR2^+$ B and/or T cells will help gain more complete insight to the significance of TNFR2 expression by lymphocytes subsets in breast TDLNs.

Although it was frequently mentioned in the literature that TNFR1 is expressed on almost all cell types, results of our study showed that TNFR1 was not expressed on the lymphocytes of TDLNs of breast cancer patients. Short or long time stimulations with poly clonal activator did not induce its expression. In line with this finding, there are studies showing that TNFR1 is expressed at very low level on unstimulated peripheral blood lymphocytes and its expression is relatively up-regulated only following long-time activation. It was shown in a study that tumor infiltrating lymphocytes including $CD3^+$, $CD4^+$, $CD8^+$ and $CD56^+$ cells derived from melanoma, colorectal and lung cancer patients did not express the 55 KDa receptor (TNFR1), while expressed the 75KDa one [28]. In another study, 1.8 ± 2.6% of peripheral blood $CD4^+$ lymphocytes expressed TNFR1 in unstimulated condition. When the cells were stimulated with plate coated anti-CD3 antibody for 24 h, the mean frequency of $TNFR1^+$ $CD4^+$ cells was still lower than 10% (9.3 ± 10.8%). The percentage of TNFR1 expressing $CD8^+$ lymphocytes was 2.1 ± 2.3 and 7.7 ± 6.7 in unstimulated and stimulated cells, respectively [9]. A study done in 2018, demonstrated that a minor

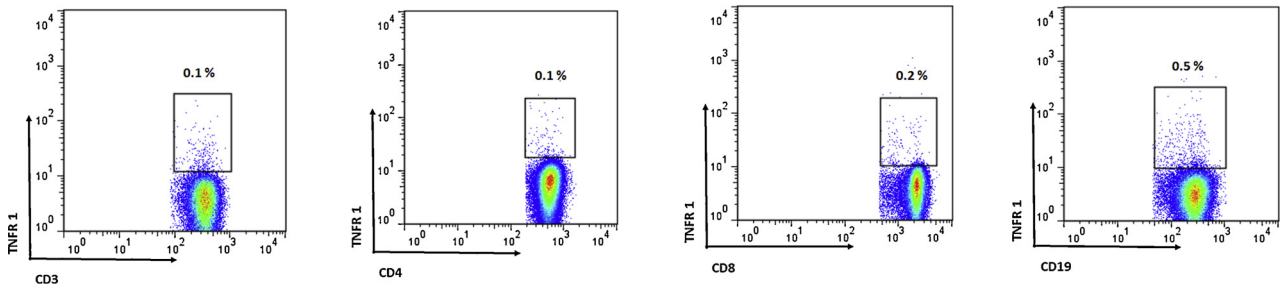

Fig. 7. Assessment of the TNFR1 expression by lymphocytes subsets in the TDLNs of breast cancer patients. $CD3^+$, $CD4^+$, $CD3^+CD8^+$ and $CD19^+$ cells were determined in the lymphocyte gate. The frequency of $TNFR1^+$ cells were assessed in each lymphocyte subset.

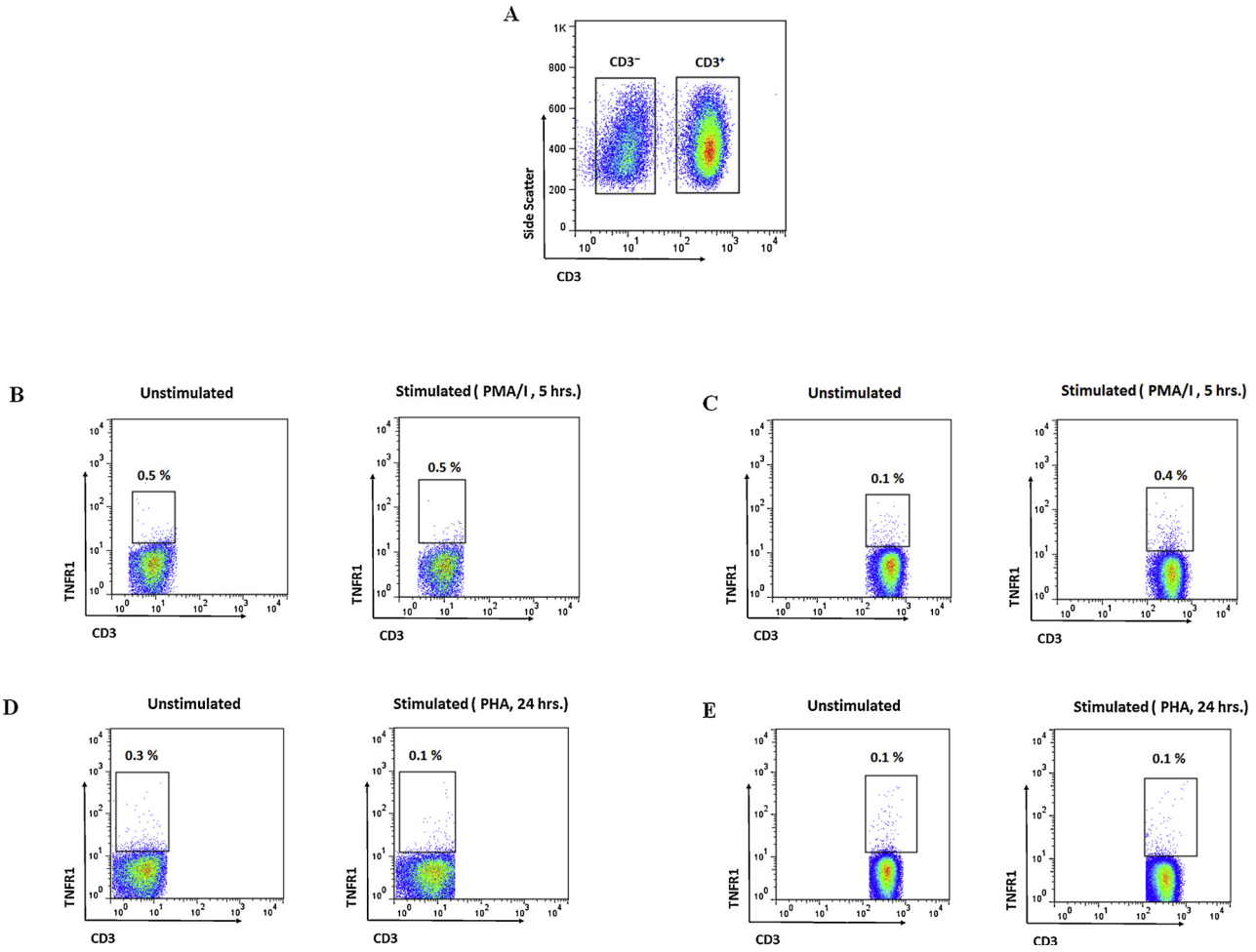

Fig. 8. Assessment of the TNFR1 expression by $CD3^-$ and $CD3^+$ lymphocytes from TDLNs of breast cancer patients. Lymphocytes were stimulated with PMA/I for 5 h or with PHA for 24 h and after staining for CD3 and TNFR1 were subjected to flow cytometry. **A.** $CD3^+$ and $CD3^-$ cells were gated and the frequency of TNFR1 expressing cells were determined in **B & D**. $CD3^-$ and **C & E.** $CD3^+$ cells. (Part **A** is representative of gating strategy in a PMA/I stimulated sample, the same strategy was followed for PHA stimulated samples.).

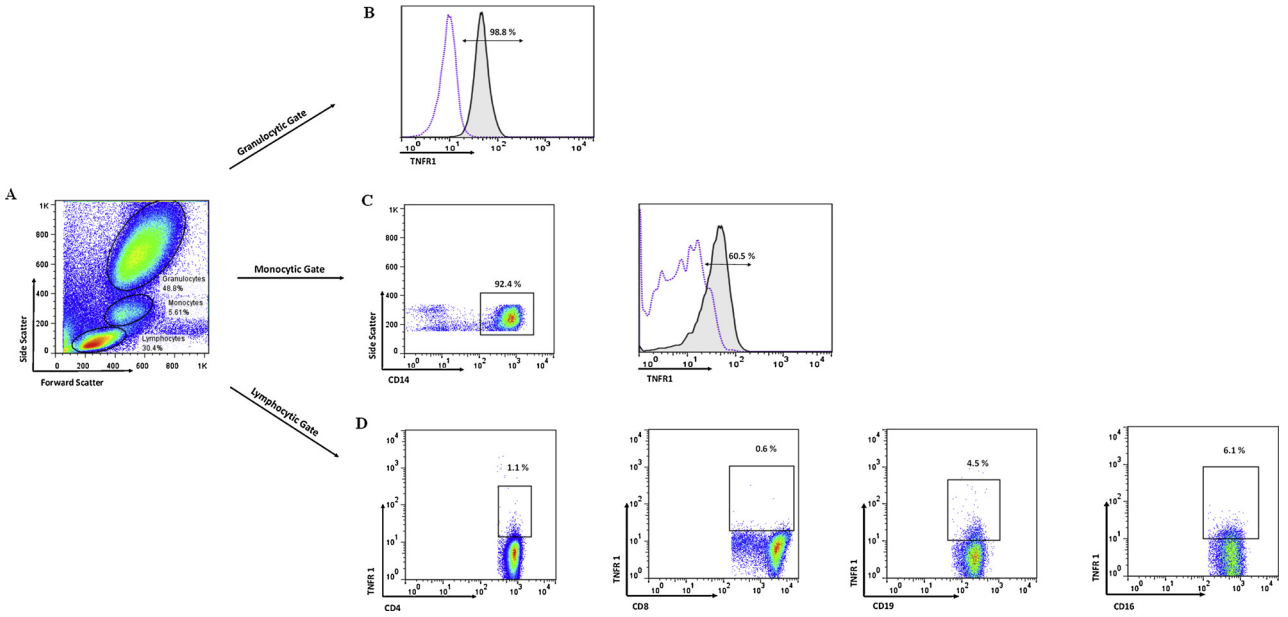

Fig. 9. Flow cytometry evaluation of the TNFR1 expression by leukocytes of normal peripheral blood. **A.** Granulocytes, monocytes and lymphocytes were gated according to their forward and side scatters. TNFR1 expression was assessed in **B.** granulocytes, **C.** $CD14^+$ monocytes, and **C.** in $CD4^+$, $CD8^+$, $CD19^+$ and $CD16^+$ lymphocytes. In part B and C dotted plots are representative of isotype controls.

fraction of B cells isolated from peripheral blood of normal individuals expressed TNFR1. When the cells were stimulated with CpG for 3 and 5 days, the frequencies of $TNFR1^+CD19^+$ lymphocytes remained low [8].

Assessment of TNFR1 expression on leukocyte subsets in normal peripheral blood revealed its expression on almost all granulocytes and more than half of monocytes. In addition, a minor fraction of B cells and $CD16^+$ cells was found to express TNFR1. Controversial data exists regarding the expression of TNFR1 on NK cells of normal peripheral blood ranging from < 3% to up to 29 ± 3.4% [29]. Variable expressions of TNFR1 by peripheral blood monocytes has been reported. Gane et al. demonstrated that the expression of TNFR1 could be observed on 1% to > 90% of monocytes in peripheral blood of normal individuals [30]. The observed difference between the expression of TNFR1 on B cells of the TDLNs and peripheral blood remains unexplained and needs further evaluation.

5. Conclusion

Collectively, results of this study suggested that TNFR2 was the main TNF receptor expressed by B and T lymphocytes in the breast TDLNs and therefore is responsible for the effects of TNF-α on these cells. There were no significant associations between the frequencies of TNFR2 expressing B or T cells when all TDLNs are considered. However, the percentage of $TNFR2^+$ B cells in MLNs was negatively associated with tumor grade and the number of involved LNs. Our results, however requires further phenotypical and functional studies and should be confirmed with larger sample size.

Conflict of interest

No conflict of interest was declared.

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References

[1] M.F. Fransen, R. Arens, C.J. Melief, Local targets for immune therapy to cancer: tumor draining lymph nodes and tumor microenvironment, Int. J. Cancer 132 (2013) 1971–1976.
[2] X. Wang, Y. Lin, Tumor necrosis factor and cancer, buddies or foes? 1, Acta Pharmacol. Sin. 29 (2008) 1275–1288.
[3] G.D. Kalliolias, L.B. Ivashkiv, TNF biology, pathogenic mechanisms and emerging therapeutic strategies, Nat. Rev. Rheumatol. 12 (49) (2016).
[4] L. Puimège, C. Libert, F. Van Hauwermeiren, Regulation and dysregulation of tumor necrosis factor receptor-1, Cytokine Growth Factor Rev. 25 (2014) 285–300.
[5] H.T. Idriss, J.H. Naismith, TNFα and the TNF receptor superfamily: structure--function relationship (s), Microsc. Res. Technol. 50 (2000) 184–195.
[6] X. Chen, J.J. Oppenheim, Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity, FEBS Lett. 585 (2011) 3611–3618.
[7] G. Eissner, W. Kolch, P. Scheurich, Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system, Cytokine Growth Factor Rev. 15 (2004) 353–366.
[8] O. Ticha, L. Moos, H. Wajant, I. Bekeredjian-Ding, Expression of tumor necrosis factor receptor 2 characterizes Tlr9-driven formation of interleukin-10-producing B cells, Front. Immunol. 8 (2018) 1951.
[9] L.M. de Oliveira Pinto, S. Garcia, H. Lecoeur, C. Rapp, M.L. Gougeon, Increased sensitivity of T lymphocytes to tumor necrosis factor receptor 1 (TNFR1)- and TNFR2-mediated apoptosis in HIV infection: relation to expression of Bcl-2 and active caspase-8 and caspase-3, Blood 99 (2002) 1666–1675.
[10] F. Balkwill, Tumour necrosis factor and cancer, Nat. Rev. Cancer 9 (2009) 361.
[11] M. Grell, E. Douni, H. Wajant, M. Löhden, M. Clauss, B. Maxeiner, et al., The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor, Cell 83 (1995) 793–802.
[12] Y. Qu, G. Zhao, H. Li, Forward and reverse signaling mediated by transmembrane tumor necrosis factor-alpha and TNF receptor 2: potential roles in an immunosuppressive tumor microenvironment, Front. Immunol. 8 (1675) (2017).
[13] R.M. Aspalter, M.M. Eibl, H.M. Wolf, Regulation of TCR-mediated T cell activation by TNF-RII, J. Leukoc. Biol. 74 (2003) 572–582.
[14] X. Chen, Y. Nie, H. Xiao, Z. Bian, A.J. Scarzello, N.-Y. Song, et al., TNFR2 expression by CD4 effector T cells is required to induce full-fledged experimental colitis, Sci. Rep. 6 (32834) (2016).
[15] X. Chen, J.J. Subleski, H. Kopf, O.Z. Howard, D.N. Männel, J.J. Oppenheim, Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse CD4+ CD25+ FoxP3+ T regulatory cells: applicability to tumor-infiltrating T regulatory cells, J. Immunol. 180 (2008) 6467–6471.
[16] M. Nagar, J. Jacob-Hirsch, H. Vernitsky, Y. Berkun, S. Ben-Horin, N. Amariglio, et al., TNF activates a NF-kappaB-regulated cellular program in human CD45RA- regulatory T cells that modulates their suppressive function, J. Immunol. 184 (2010) 3570–3581.
[17] X. Valencia, G. Stephens, R. Goldbach-Mansky, M. Wilson, E.M. Shevach, P.E. Lipsky, TNF downmodulates the function of human CD4+CD25hi T-regulatory cells, Blood 108 (2006) 253–261.
[18] Q. Zhang, F. Cui, L. Fang, J. Hong, B. Zheng, J.Z. Zhang, TNF-alpha impairs differentiation and function of TGF-beta-induced Treg cells in autoimmune diseases through Akt and Smad3 signaling pathway, J. Mol. Cell Biol. 5 (2013) 85–98.
[19] T.L. Whiteside, Immune responses to cancer: are they potential biomarkers of prognosis? Front. Oncol. 3 (107) (2013).
[20] H.E. Kohrt, N. Nouri, K. Nowels, D. Johnson, S. Holmes, P.P. Lee, Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer, PLoS Med. 2 (2005) e284.
[21] S. Lee, K. Margolin, Cytokines in cancer immunotherapy, Cancers 3 (2011) 3856–3893.
[22] Z. Faghih, S. Rezaeifard, A. Safaei, A. Ghaderi, N. Erfani, IL-17 and IL-4 producing CD8+ T cells in tumor draining lymph nodes of breast cancer patients: positive association with tumor progression, Iran. J. Immunol. 10 (2013) 193–204.
[23] K.H.J. Lim, P.U. Telisinghe, M.S. Abdullah, R. Ramasamy, Possible significance of differences in proportions of cytotoxic T cells and B-lineage cells in the tumour-infiltrating lymphocytes of typical and atypical medullary carcinomas of the breast, Cancer Immun. Archive 10 (3) (2010).
[24] R. Kieszko, P. Krawczyk, S. Chocholska, A. Bojarska-Junak, O. Jankowska, A. Król, et al., Tumor necrosis factor receptors (TNFRs) on T lymphocytes and soluble TNFRs in different clinical courses of sarcoidosis, Respir. Med. 101 (2007) 645–654.
[25] T. Schioppa, R. Moore, R.G. Thompson, E.C. Rosser, H. Kulbe, S. Nedospasov, et al., B regulatory cells and the tumor-promoting actions of TNF-alpha during squamous carcinogenesis, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 10662–10667.
[26] D.X. Nguyen, M.R. Ehrenstein, Anti-TNF drives regulatory T cell expansion by paradoxically promoting membrane TNF–TNF-RII binding in rheumatoid arthritis, J. Exp. Med. 213 (2016) 1241–1253.
[27] G.S. Williams, B. Mistry, S. Guillard, J.C. Ulrichsen, A.M. Sandercock, J. Wang, et al., Phenotypic screening reveals TNFR2 as a promising target for cancer immunotherapy, Oncotarget 7 (68278) (2016).
[28] L. Trentin, R. Zambello, P. Bulian, A. Cerutti, C. Enthammer, M. Cassatella, et al., Tumour-infiltrating lymphocytes bear the 75 kDa tumour necrosis factor receptor, Br. J. Cancer 71 (240) (1995).
[29] W. Almishri, T. Santodomingo-Garzon, T. Le, D. Stack, C.H. Mody, M.G. Swain, TNFalpha augments cytokine-induced NK cell IFNgamma production through TNFR2, J. Innate Immun. 8 (2016) 617–629.
[30] J.M. Gane, R.A. Stockley, E. Sapey, TNF-alpha autocrine feedback loops in human monocytes: the pro- and anti-inflammatory roles of the TNF-alpha receptors support the concept of selective TNFR1 blockade in vivo, J. Immunol. Res. 2016 (2016) 1079851.