Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Interleukin (IL)-21 and IL-21 receptor expression in peripheral T and B cells of patients with breast cancer

Elham Babaeinia[1,2], Atri Ghods[2], Reza Rasolmali[3], Abdoul-Rasoul Talei[4], Fereshteh Mehdipour[2,*], Abbas Ghaderi[1,2,*]

[1] *Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[2] *Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[3] *Department of Pathology, Shiraz Central Hospital, Shiraz, Iran*
[4] *Breast Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

Article Info

Article history:

Received: August 21, 2023
Accepted: February 26, 2024
Published: April 30, 2024

Use your device to scan and read the article online

Abstract

IL-21 is a cytokine with versatile antitumor and pro-tumorigenic activities. It is mainly produced by $CD4^+$ T cells and B cells are one of its pivotal targets. In this study, we assessed and compared the expression of IL-21 by $CD4^+$ T cells and the IL-21 receptor (IL-21R) on B cells in the peripheral blood of women with breast cancer and healthy individuals. Blood samples were taken from both patients and controls. Mononuclear cells were seperated using Ficoll-Hypaque density gradient centrifugation. These isolated cells were then stained with either anti-CD19/anti-IL-21R or anti-CD4/anti-IL-21 antibodies and analyzed using flow cytometry. The results showed that there was no significant difference in the percentage of $IL\text{-}21R^+$ B cells and $IL\text{-}21^+CD4^+$ T cells between patients and controls. However, the percentage of $CD4^+$ T cells decreased significantly in patients with breast cancer (P=0.003). This decline was observed from the early stage and before lymph node (LN) involvement. In comparison to the control group, $IL\text{-}21R^+$ B cells were relatively lower in patients with stages I+II and those with fewer than 4 involved LNs. The intensity of IL-21 expression in T cells was associated with HER2 expression (P=0.029). Furthermore, we found that the majority of $IL\text{-}21R^+$ B cells exhibited a naïve phenotype and most of $IL\text{-}21^+CD4^+$ T cells did not produce IFN-γ or IL-17. In conclusion, breast cancer from the early stages leads to a significant reduction in the proportion of peripheral CD4+ T cells. However, we did not find a significant change in IL-21 and its receptor expression during disease progression.

Keywords: IL-21, IL-21 receptor, Lymphocytes, Peripheral blood, Breast cancer.

1. Introduction

Breast cancer is the most common cancer among women in both developed and developing countries. Developing new immunotherapies requires an understanding of the interaction between the immune system and tumors [1]. Furthermore, cancer is a systemic disorder and the cellular components and concentration of soluble factors change in the peripheral blood during cancer progression [2]. One of these soluble factors is cytokines which play an important role in immune activation and regulation [3]. Interleukin (IL)-21 belongs to the common gamma chain cytokine family [3] and is primarily produced by NKT and activated $CD4^+$ T cells including T helper (Th)1, Th17 and T follicular helper (Tfh) cells [4]. IL-21 exerts its effects by binding to the IL-21 receptor (IL-21R) which is predominantly expressed by B, T, NK and myeloid cells. Among these, B cells have the highest level of IL-21R expression [5].

IL-21 has both positive and negative effects on B cells depending on the presence or absence of other (co)stimulatory signals [6]. IL-21, along with anti-IgM, and anti-CD40, enhances the proliferation of human and murine B cells. In contrast, IL-21 inhibits the proliferation of B cells stimulated with anti-IgM and IL-4 [6, 7]. .Furthermore, IL-21, in combination with anti-B cell receptor (BCR), promotes Granzyme B production in B cells. However, CD40 stimulation blocks Granzyme B production in B cells and further enhances antibody production [8]. Similar to its various effects on B cells, IL-21 shapes T cells' responses in different ways including promoting Th17 generation [9], inhibiting T regulatory (Treg) cell expansion [10], or inducing IL-10 production by T regulatory (Tr)1 cells [11].

Opposing roles have been reported for IL-21 in cancer immunity. Overexpression of IL-21 in intestinal tissues has been associated with a poor prognosis in patients with ulcerative colitis (UC) or UC-associated colorectal cancer (CRC) due to its inhibitory effects on Tregs [12, 13]. Additionally, IL-21 induces regulatory γδ T cells and its presence in fresh breast cancer tissues has been linked to a poor prognosis [14]. On the other hand, the absence of IL-21 reduces T cell infiltration, activation and memory formation but increases the accumulation of myeloid-

* Corresponding author.
E-mail address: mehdipourf@sums.ac.ir (F. Mehdipour), ghaderia@sums.ac.ir (A. Ghaderi).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.4.3

derived suppressor cells (MDSCs) in an IL-21R$^{-/-}$ mouse model of hepatocellular carcinoma (HCC).[15] Moreover, IL-21 attenuates the proliferation, invasion and migration of A549 cells [16].

Given the important role of IL-21 in cellular and humoral immunity, we investigated the expressions of IL-21 and IL-21R in peripheral CD4^{+}T and B cells, respectively, and determined their changes during the course of breast cancer.

2. Materials and methods

2.1. Subjects

We collected blood samples from 53 and 41 women with pathologically confirmed breast cancer for the assessment of IL-21R^{+} B and IL-21^{+}CD4^{+} T cells, respectively. In addition, 23 age- and sex-matched healthy controls were included (Table 1). Blood samples were collected in tubes containing heparin as an anticoagulant. Patients did not have a previous history of chemo- or radiotherapy. Written informed consents were obtained from all patients and controls, and the study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethics code: IR.SUMS.REC.1399.1127). Clinicopathological characteristics of the patients are presented in Table 2.

2.2. Isolation and activation of mononuclear cells:

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood using density gradient centrifugation over Ficoll-Hypaque (Lymphodex,inno-train Diagnostik

Table 1. The age information of study groups.

Study group	Age (years)	
	Median (min-max)	Mean± SD
Patients (IL-21R) (n=53)	46 (29-74)	47.4±10.4
Patients (IL-21) (n=41)	45.5 (29-73)	46.8±10.5
Control (n=23)	47 (34-64)	47.4±8.3

Table 2. Clinico-pathological characteristics of breast cancer patients.

Characteristics	Value
Nodal Status	
N0 (Free LNs)	27 (59.9%)
N1 (1-3 involved LNs)	18 (34%)
N2 (4-9 involved LNs)	4 (7.5%)
N3 (>9 involved LNs)	4 (7.5%)
Tumor Size (greatest dimension, cm)	
T1 (≤2)	29 (54.7%)
T2 (2-5)	21 (39.6%)
T3	1 (1.9%)
Tx (Unknown)	2 (3.8%)
Stage*	
I	16 (30.2%)
II	27 (50.9%)
III	8 (15.1%)
Unknown	2 (3.8%)
Histological Grade	
Well differentiated (I)	3 (5.7%)
Moderately differentiated (II)	42 (79.2%)
Poorly differentiated (III)	6 (11.3%)
Unknown	2 (3.8%)
Tumor Type	
Infiltrating ductal carcinoma (IDC)	46 (86.8%)
IDC with medullary features (IDC+M)	5 (9.4%)
Others (Lobular carcinoma, Metaplastic Carcinoma) Unknown	2 (3.8%)
HER2 Expression	
Positive	8 (15.1%)
Negative	29 (54.7%)
Equivocal	6 (11.3%)
Unknown	10 (18.9%)
ER Expression	
Positive	32 (60.4%)
Negative	11 (20.8%)
Unknown	10 (18.9%)
PR Expression	
Positive	25 (47.2%)
Negative	16 (30.2%)
Unknown	12 (22.6%)

ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2, * according to the American Joint Committee on Cancer Classification and Stage Group (AJCC, 7th edition).

GmbH, Germany). The cells were then resuspended in complete culture medium (RPMI-1640 containing 10% FBS and 1% Penicillin/ Streptomycin, all from Gibco, Life Technologies, USA) and added to a cell culture flask and were placed in the incubator overnight to exclude monocytes and macrophages.

The expression of IL-21R on B cells was investigated in unstimulated lymphocytes. For the assessment of IL-21 expression by $CD4^+$T cells, lymphocytes (2×10^6 cells/ml) were activated with PMA (50 ng/ml, Sigma, Germany) and Ionomycine (1µg/ml, Sigma) in the presence of Brefeldin (1µl/ml, Biolegend, USA) for 6 hours.

2.3. Surface and intracellular staining

We washed lymphocytes with staining buffer (PBS+ 10% FBS) and stained them with APC-conjugated anti-CD19 (clone: HIB19), PE-conjugated anti-IL-21R (Clone: 17A12) and/or PE Mouse IgG1, κ isotype control antibodies (Clone: MOPC-21, all from Biolegend).

To assess IL-21 production by $CD4^+$ T cells, stimulated cells were harvested, washed with staining buffer and after fixation with 1% Paraformaldehyde (Sigma-Aldrich) and permeabilization with 1X Wash buffer (Biolegend), stained with PerCP-cy 5.5 anti-CD-4 (Clone: RPA-T4) and PE-conjugated anti-IL-21 and/or PE Mouse IgG1, κ isotype control antibodies, all purchased from Biolegend. After surface or intracellular staining, cells were washed and subjected to flow cytometry.

2.4. Flow cytometry data acquisition and analysis

Data was acquired on a four-color FACSCalibur flow cytometer (BD Biosciences, USA). The Flow cytometry data was analyzed using FlowJo software (Version 10.1, Ashland, OR, USA). First lymphocytes were gated based on their forward and side scatters. Then, $CD19^+$ and $CD4^+$ cells were gated as B and T lymphocytes, respectively. The frequencies of IL-21R-expressing B cells and IL-21 expressing T cells were assessed using fluorescence minus one (FMO) tubes. The Geometric Mean Fluorescence Intensity (gMFI) of IL-21 was used as a standard for its per cell production. The gMFIs were normalized using negative populations in each test.

2.5. Statistical analysis

We utilized SPSS 16 software (SPSS GmbH Software, Germany) and conducted non-parametric tests such as Mann-Whitney U, Kruskal-Wallis H, Dunn's posttest and Spearman's ranks correlation to compare the frequencies of various cell subsets in two or more groups, compare all pairs in multiple groups and determine their correlation with the tumor size or the number of involved LNs, respectively. We considered P values < 0.05 as significant and created graphs using GraphPad Prism 6 software (Inc: San Diego CA, USA).

3. Results

3.1. IL-21R-expressing B cells and IL-21$^+$CD4$^+$ T cells in peripheral blood of patients and controls

We assessed the frequencies of $CD19^+$ B and $CD4^+$ T cells and determined the percentage of IL-21R- and IL-21-expressing cells in B and $CD4^+$ T cells, respectively (Figure 1A & Table 3). The results showed that the frequency of B cells was not significantly different in the peripheral blood of patients and controls, but the frequency of $CD4^+$ T cells was significantly lower in patients compared with controls (P=0.003). We found no significant difference in the frequencies of IL-21R$^+$ B and IL-21$^+$CD4$^+$ T cells in patients and controls (Figure 1B).

3.2. Comparison of the frequencies of IL-21R$^+$ B cells and IL-21$^+$ CD4$^+$ T cells in the peripheral blood of patients with different breast cancer stages

There were no significant differences in the frequencies of the mentioned cell subsets among patients with different stages of breast cancer. Additionally, no significant differences were found between controls and patients with stages I, II, and III (Figure 2A). When comparing patients in stages I+II and stage III with controls, the frequency of $CD4^+$ T cells was significantly lower in patients with stages I+II and slightly lower in stage III compared to controls (P=0.031 and 0.063, respectively, Figure 2B). Interestingly, the percentage of IL-21R+ B cells showed a relative decrease in stages I+II compared to controls while this trend was not observed in stage III (Figure 2B).

3.3. Association of the frequencies of IL-21R$^+$ B cells and IL-21$^+$ CD4$^+$ T cells with lymph node involvement in patients with breast cancer

The analysis revealed that lymph node involvement did

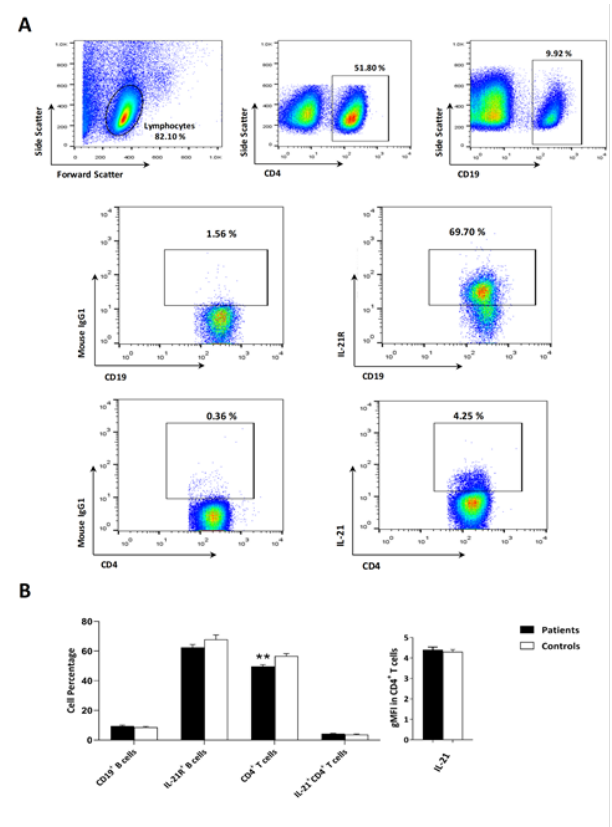

Fig. 1. A. Representative flow cytometry plots show IL-21R$^+$CD19$^+$ B cells and IL-21$^+$CD4$^+$T cells in the peripheral blood of patients with breast cancer and healthy controls. Lymphocytes were gated based on their forward and side scatters followed by determining $CD4^+$ and $CD19^+$ cells within the lymphocyte gate. The frequency of IL-21R$^+$ and IL-21$^+$ expressing cells was assessed in $CD19^+$ B and $CD4^+$ T cell gates, respectively using FMO. **B.** Comparison of the frequencies of $CD19^+$, IL-21R$^+$CD19$^+$, $CD4^+$, and IL-21$^+$CD4$^+$ cells, as well as gMFI of IL-21 in $CD4^+$ T cells in the peripheral blood of patients with breast cancer and healthy individuals. Data are presented as Mean ± SEM. ** P value < 0.01. FMO: Florescence Minus One, gMFI: geometric mean fluorescence intensity, SEM: standard error of the mean.

Table 3. The percentages of B, CD4$^+$ T, IL-21R-expressing B cells and IL-21$^+$CD4$^+$ T cells in the peripheral blood of patients with breast cancer and controls.

Cell subsets	Min		Max		Median		Mean±SD	
	C	Pt	C	Pt	C	Pt	C	Pt
CD19$^+$ cells (in lymphocytes' gate)	4.2	4.1	16.7	30.1	8.3	9.2	8.6±2.8	9.6±4.2
CD4$^+$ cells (in lymphocytes' gate)	48.6	37.1	78.3	67.6	54.7	48.5	56.7±7.2	49.6±7.8
IL-21R$^+$ cells (in B cells' gate)	31.9	23.5	89	88.1	68.6	65.1	67.8±14.9	62.5±14.3
IL-21$^+$CD4$^+$ cells (in CD4+ T cells' gate)	1	9	8.1	11	3.7	4.3	3.8±1.8	4.3±2.6

C: control, Pt: Patients.

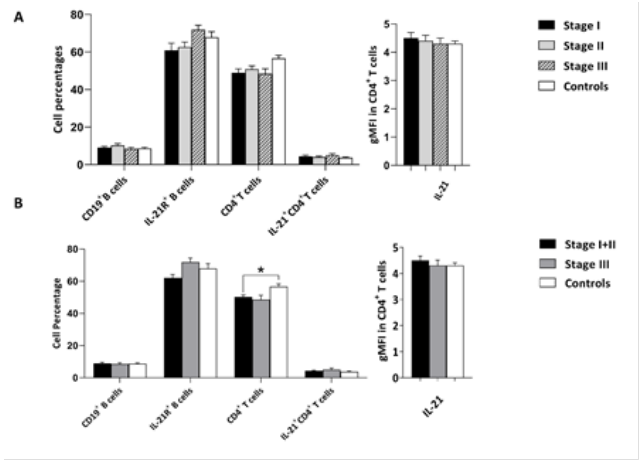

Fig. 2. Comparison of the frequencies of CD19$^+$, IL-21R$^+$CD19$^+$, CD4$^+$ and IL-21$^+$CD4$^+$ cells, as well as gMFI of IL-21 in CD4$^+$ T cells in the peripheral blood of patients with **A.** stage I, II and III with controls and **B.** in stages I+II and III with healthy women. Data are shown as Mean ± SEM. * P value < 0.05, gMFI: geometric mean fluorescence intensity, SEM: standard error of the mean.

not significantly affect the frequencies of B cells, CD4$^+$ T cells, IL-21R$^+$ B and IL-21$^+$CD4$^+$ T cells in the peripheral blood of patients with breast cancer. In addition, there were no significant differences in the frequencies of B, IL-21R$^+$ B and IL-21$^+$ CD4$^+$ T cells between patients with and without LN involvement (LN+ and LN–, respectively) compared with controls. However, the frequency of CD4$^+$ T cells decreased in both LN– and LN+ patients compared with controls (P=0.030 and P=0.023, respectively, Figure 3A).

Moreover, the frequencies of B, IL-21R$^+$ B, CD4$^+$ and IL-21$^+$CD4$^+$ T cells did not show any significant differences between patients with different nodal statuses (N0 to N3) and controls (Figure 3B). However, the frequency of IL-21R$^+$ B cells showed a trend toward reduction in N0+N1 patients compared to N2+N3. In addition, compared to controls, the frequencies of CD19$^+$ B, IL-21R$^+$ B and IL-21$^+$ CD4$^+$ T cells were not significantly different in (N0+N1) or N2+N3 whereas CD4$^+$ T cell frequency decreased in both N0+N1 and N2+N3 groups, although the P value in the latter was not significant (P=0.025 and P=0.056, respectively, Figure 3C). Furthermore, there was a nonsignificant direct correlation between the frequency of IL-21R$^+$ B cells and the number of involved LNs (R=0.2 and P=0.086).

3.4. The relationship between the frequencies of IL-21R$^+$ B cells and IL-21$^+$CD4$^+$ T cells and tumor size and grade in patients with breast cancer

There were no significant differences in the frequencies of B cells, IL-21R$^+$ B cells, CD4$^+$ cells, and IL-21$^+$CD4$^+$ T cells in patients with tumor size≤2cm (T1) and patients with tumor size>2 cm (T2). Among the analyzed cell subsets only the frequency of CD4$^+$ T cells was significantly decreased in T1 and showed a trend toward reduction in T2 patients compared with controls (P=0.030 and P=0.091, respectively, Figure 4A).

The percentages of B cells, IL-21R$^+$ B cells, CD4$^+$ cells, and IL-21$^+$CD4$^+$ T cells did not show a significant association with tumor grade. In addition, the frequencies of these cells did not significantly differ in patients with grade I or II compared to controls, except for the frequency of CD4$^+$ T cells which was significantly lower in patients with grade II tumors in comparison with controls (P=0.011, Figure 4B).

3.5. Association between the frequencies of IL-21R$^+$ B cells and IL-21$^+$CD4$^+$ T cells and the status of ER, PR and HER2 expression

The analysis revealed that frequencies of B cells, IL-21R$^+$ B cells and IL-21$^+$CD4$^+$ T cells did not show a si-

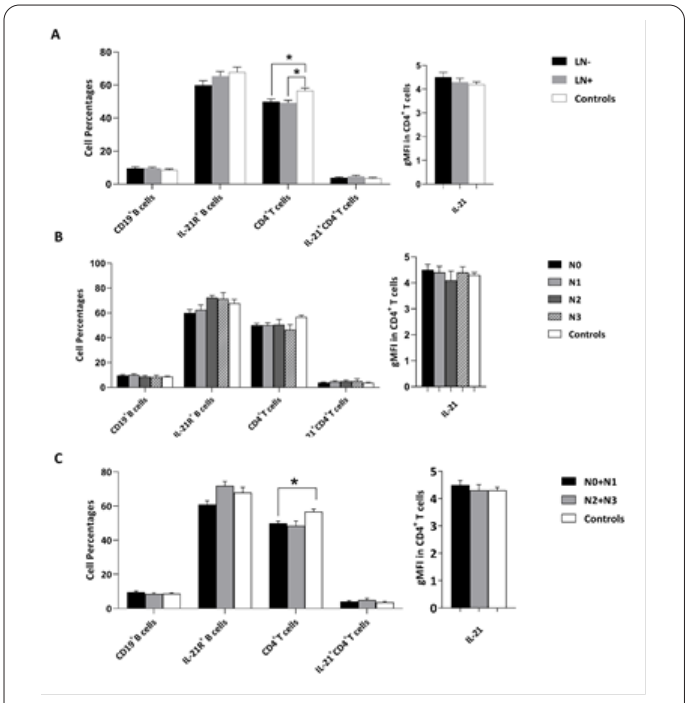

Fig. 3. A. The frequencies of CD19$^+$, IL-21R$^+$CD19$^+$, CD4$^+$ and IL-21$^+$CD4$^+$ cells as well as gMFI of IL-21 in CD4$^+$ T cells in the peripheral blood of patients with and without LN involvement (LN+ and LN–, respectively) were compared with controls. **B.** The frequencies of the aforementioned subsets were compared in patients with different nodal statuses and healthy controls. **C.** The same comparisons were done between N0+N1(less than 4 involved LNs), N2+N3 (with more than 4 involved LNs) and control groups. Data are shown as Mean ± SEM. * P value < 0.05. . ** P value < 0.01. gMFI: geometric mean fluorescent intensity, SEM: standard error of the mean.

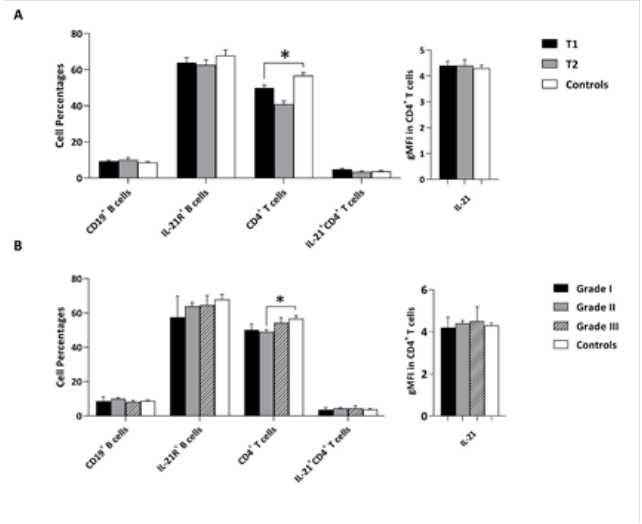

Fig. 4. Comparison of the frequencies of $CD19^+$, $IL\text{-}21R^+CD19^+$, $CD4^+$ and $IL\text{-}21^+CD4^+$ cells, as well as gMFI of IL-21 in $CD4^+$ T cells in the peripheral blood of patients **A.** with tumor size ≤ 2cm (T1) and tumor size > 2cm (T2), and healthy individuals. **B.** with different tumor grades compared with controls. Data are shown as Mean ± SEM. * P value < 0.05, gMFI: geometric mean fluorescent intensity, SEM: standard error of the mean.

gnificant association with the ER/PR status of the tumor. However, the percentage of $CD4^+$ T cells was significantly lower in patients with ER+ or PR+ tumors compared to controls (P=0.006 and P=0.023, respectively, Figure 5A&B). It should be noted that the decrease in $CD4^+$ cell frequency was observed in ER– or PR– patients compared to controls however the P values did not reach statistical significance (P=0.1 and P=0.062, respectively, Figure 5A&B). In addition, frequencies of B cells, $IL\text{-}21R^+$ B cells and $IL\text{-}21^+CD4^+$ T cells did not show significant differences in patients with HER2+ compared to HER2– tumors. However, the gMFI of IL-21 in $CD4^+$ T cells was higher in the HER2+ compared to the HER2– group and controls, however in the latter the P value just bordered statistical significance (P=0.029 and P=0.051, respectively, Figure 6A). Moreover, the frequency of $CD4^+$ T cells significantly decreased in both HER2+ and HER2– groups compared to controls (P=0.015 and P=0.024, respectively, Figure 6A). When HER2+ patients with ER+ or PR+ tumors were compared with HER2– patients with ER+ or PR+ tumors, the same results were obtained and only the gMFI of IL-21 in $CD4^+$ T cells was significantly higher in the HER2+ compared to the HER2– group (P=0.029, Figure 6B).

3.6. Phenotypic characterization of $IL\text{-}21R^+CD19^+$ B cells and $IL\text{-}21^+CD4^+$ T cells

We assessed the expression of CD27, the surface marker for memory and activated B cells, in $IL\text{-}21R^+$ B cells from 10 samples (5 from patients and 5 from controls), (Figure 7A). The analysis revealed that 12.2±7% and 7.5±2.6% of $IL\text{-}21R^+$ B cells expressed CD27 in the peripheral blood of patients and controls, respectively. Thus, the majority of $IL\text{-}21R^+$ B cells did not express CD27 and exhibited a naïve phenotype.

We also evaluated the expression of IFN-γ (Th1 cytokine) and IL-17 (Th17 cytokine) in $IL\text{-}21^+CD4^+$ T cells in the peripheral blood of 5 patients and 5 controls (Figure 7B). Our analysis revealed that 27.4±10.7% and 18.4±6.6% of $IL\text{-}21^+CD4^+$ T cells produced IFN-γ in patients and controls, respectively. In addition, 11.9±4.5% and 7.4±2.6% of $IL\text{-}21^+CD4^+$ T cells expressed IL-17 in patients and controls, respectively. Therefore, the majority of $IL\text{-}21^+CD4^+$ T cells were not categorized into Th1 and Th17 cell subsets in the peripheral blood of patients and controls.

4. Discussion

Re-educating and redirecting immune responses against tumors through immunotherapeutic approaches have shown promising results [17]. Numerous studies

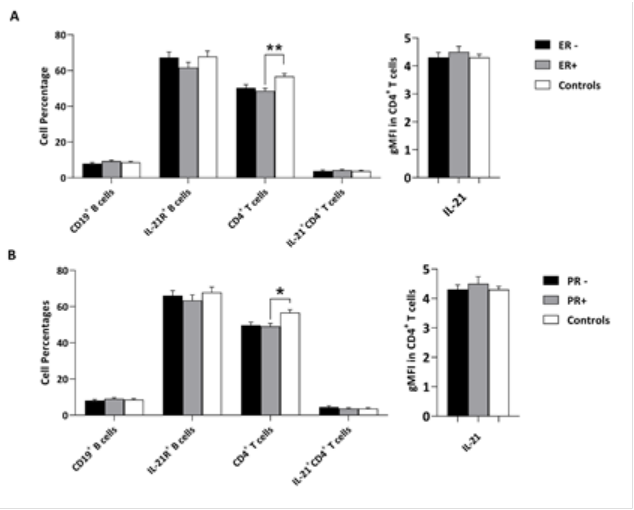

Fig. 5. Comparison of the frequencies of $CD19^+$, $IL\text{-}21R^+CD19^+$, $CD4^+$ and $IL\text{-}21^+CD4^+$ cells, as well as the gMFI of IL-21 in $CD4^+$ T cells in the peripheral blood of patients according to **A.** ER, and **B.** PR expression compared to controls. Data are shown as Mean ± SEM. * P value < 0.05. ** P value < 0.01. ER: estrogen receptor, PR: progesterone receptor, gMFI: geometric mean fluorescent intensity, SEM: standard error of the mean.

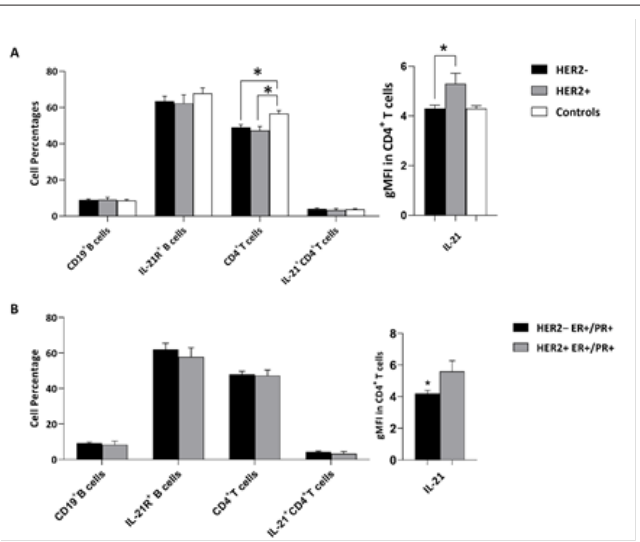

Fig. 6. Comparison of the frequencies of CD19+, IL-21R+CD19+, CD4+ and IL-21+CD4+ cells, as well as the gMFI of IL-21 in CD4+ T cells in the peripheral blood of patients with HER2+ and HER2– tumors with healthy controls. **B.** the same comparisons were conducted between HER2+ patients with ER+ and/or PR+ and HER2– patients with ER+ and/or PR+ tumors. Data is presented as Mean ± SEM. * P value < 0.05. gMFI: geometric mean fluorescent intensity, SEM: standard error of the mean.

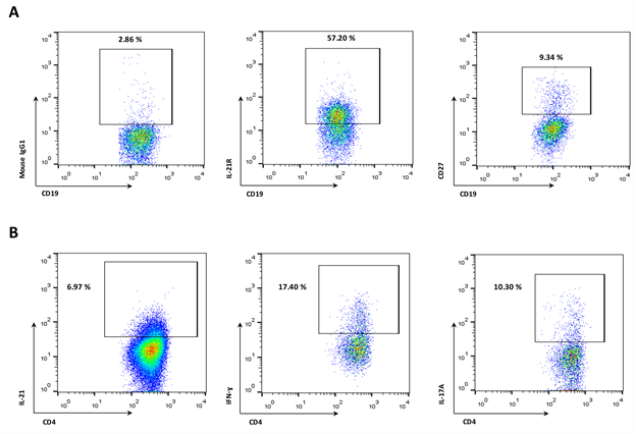

Fig. 7. A. Representative flow cytometry plots show the assessment of CD27 expression by IL-21R$^+$ B cells in the peripheral blood of patients and healthy individuals. IL-21R$^+$ B cells were identified and the percentage of CD27-expressing cells was determined within the CD19$^+$IL-21R$^+$ population. **B.** Representative flow cytometry plots display the assessment of IFN-γ and IL-17 expression by IL-21$^+$CD4$^+$ T cells derived from the peripheral blood of patients and healthy individuals. IL-21$^+$ cells were determined within the CD4$^+$ gate and the frequencies of IFN-γ- and IL-17-expressing cells were assessed within the CD4$^+$IL-21$^+$ gate.

have focused on determining immune profiles in the tumor site, tumor-draining lymph nodes and peripheral blood of cancer patients to increase knowledge about the molecules and pathways involved in tumor suppression or progression [18, 19]. In this context, we investigated changes in the expressions of IL-21 and its receptor in CD4$^+$ T and CD19$^+$ B cells in the peripheral blood of patients with breast cancer during cancer progression and compared them with healthy individuals.

Our results revealed that breast cancer development causes a significant reduction in the frequency of CD4$^+$ T cells in the peripheral blood. This reduction appears to occur from the beginning of breast cancer, as a decreased frequency of CD4$^+$ T cells was observed in T1, LN– and lower stages of breast cancer when compared with controls [20]. It is suggested that CD4$^+$ lymphopenia and especially severe CD4$^+$ lymphopenia (CD4$^+$ T cells count < 200 cells/µl), can be a better prognostic factor compared to CD8$^+$, CD19$^+$, and CD56$^+$ lymphopenia for predicting patients' survival in breast cancer. This may be attributed to the critical role of CD4$^+$ T cells in anti-tumor immunity [21].

In contrast to the decreased frequency of CD4$^+$ T cells in patients with breast cancer, the frequency of CD19$^+$B cells did not show a significant difference in patients compared to healthy controls. Similar to this study, it has been reported that in patients with various solid malignancies such as colon cancer, malignant melanoma, pancreatic and prostate cancer, the frequency of peripheral B cells did not change compared to healthy controls [22]. Another study showed that the frequency of CD19$^+$ B cells was not different in the peripheral blood of patients with breast cancer and their age-matched healthy controls [23]. In contrast to these studies, a group showed that the frequency of CD19$^+$ B cells- the majority of which were memory B cells- increased in patients with breast cancer [24].

This study revealed that the frequency of peripheral IL-21R$^+$CD19$^+$ B cells was not different between patients and healthy controls. However, an interesting pattern emerged in the frequency of B cells expressing IL-21R during cancer progression. The frequency of these cells decreased in the peripheral blood of patients with no or fewer than 4 involved LNs and in the early stages of breast cancer. But the frequency gradually increased with breast cancer progression to stage III and involvement of more than 4 LNs. Indeed the reasons behind the decrease in peripheral IL-21R-expressing B cells with cancer initiation needs more investigation. However, several explanations can be proposed. Phenotypic characterization revealed that the majority of IL-21R$^+$ B cells were naïve and not active or memory B cells. It can be suggested that the decrease in the frequency of IL-21R$^+$ B cells in the peripheral blood may be a reflection of decreased naïve B cells at the onset of cancer. At the onset of cancer, the immune system is still intact and naïve B cells are recruited to the tumor site and TDLNs. Supporting this idea, Carpenter et al. demonstrated that the frequency of memory B cells decreased in the peripheral blood of patients with advanced melanoma and other solid tumors like breast, glioma and pancreatic cancer compared to healthy individuals. Additionally, the ratio of naïve to memory B cells was significantly higher in the peripheral blood of patients compared to controls [25].

Furthermore, this study showed that the frequency of IL-21$^+$CD4$^+$ T cells was not significantly different between patients and controls and their percentage was almost equal to what was seen in the draining LNs [26]. It has been shown that the frequency of IL-21$^+$CD4$^+$ T cells in the breast tumor-draining LNs did not show an association with disease parameters and was only slightly higher in the higher tumor grade. However, the gMFI of IL-21 was associated with a lower stage and fewer LNs involvement. In contrast, in peripheral blood, the gMFI of IL-21 in CD4$^+$ T cells was positively associated with HER2 expression - a poor prognostic factor in breast cancer.

Phenotyping of IL-21$^+$CD4$^+$ T cells revealed that the majority of IL-21-expressing CD4$^+$ T cells were not Th1 or Th17, indicating other sources of IL-21 in the peripheral blood. Previous studies have identified Th17, Tfh and Th1 cells as the main producers of IL-21 [4]. However, Zhao et al. recently introduced a subset of CD4$^+$ tumor infiltrating lymphocytes (TILs) that are influenced by inflammation in head and neck cancer and produce IL-21. Further analysis showed that most of these cells are Foxp3$^-$ with approximately 50% producing IFN-γ but rarely IL-4 and IL-17 [27]. The frequency of IL-21$^+$CD4$^+$ T cells was higher in tumors than adjacent normal tissues in patients with head and neck squamous cell carcinoma (HNSCC) and was associated with lower survival rates [27].

5. Conclusion

This study revealed that most of B cells expressed IL-21R while only a small portion of CD4$^+$ T cells produced IL-21 in the peripheral blood of both control subjects and patients with breast cancer. The percentage of CD4$^+$ T cells decreased in patients compared to controls and this decline was not influenced by LN involvement or cancer stage. The expression of IL-21 and IL-21R in peripheral T and B lymphocytes remained relatively stable throughout the progression of breast cancer. Ultimately, the findings of this study should be validated with a larger sample size and more functional and phenotypical analyses.

Acknowledgment
This work was financially supported by Shiraz University of Medical Sciences (Grant No. 21217), and Shiraz Institute for Cancer Research (Grant No. ICR-100-508). This study was a part of MSc project of Elham Babaeinia, Department of Immunology, Shiraz University of Medical Sciences.

Conflict of Interests
The authors have no conflicts of interest.

Consent for publications
All authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Ethics code: IR.SUMS.REC.1399.1127).

Informed Consent
Written informed consents were obtained from all patients and controls.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Author contributions
Conception and design of the study: Abbas Ghaderi and Fereshteh Mehdipour. Selection of patients and pathological examination: Abdol-Rasoul Talei and Reza Rasolmali. Lab work and data collection: Elham Babaeinia. Analysis of the data and interpretation of the results: Elham Babaeinia, Atri Ghods and Fereshteh Mehdipour. Writing – original draft: Elham Babaeinia. Writing – review & editing: Abbas Ghaderi and Fereshteh Mehdipour. Approval of the final manuscript: all authors.

References

1. Salemme V, Centonze G, Cavallo F, Defilippi P, Conti L (2021) The crosstalk between tumor cells and the immune microenvironment in breast cancer: implications for immunotherapy. Front Oncol 11:289. doi: 10.3389/fonc.2021.610303
2. Paul D (2020) The systemic hallmarks of cancer. J Cancer Metastasis Treat 6 doi: 10.20517/2394-4722.2020.63
3. Sudan R (2020) Cytokines in Cancer Immunotherapy. Systems and Synthetic Immunology: Springer 255-269.
4. Spolski R, Leonard WJ (2008) Interleukin-21: basic biology and implications for cancer and autoimmunity. Annu Rev Immunol 26:57-79. doi: 10.1146/annurev.immunol.26.021607.090316
5. Yuan MJ, Wang T (2016) Advances of the interleukin-21 signaling pathway in immunity and angiogenesis. Biomed repo. 5(1):3-6. doi: 10.3892/br.2016.665
6. Konforte D, Simard N, Paige CJ (2009) IL-21: an executor of B cell fate. J Immunol. 182(4):1781-1787. doi: 10.4049/jimmunol.0803009
7. Ozaki K, Spolski R, Ettinger R, Kim H-P, Wang G, Qi C-F, et al. (2004) Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. J Immunol. 173(9):5361-5371. doi: 10.4049/jimmunol.173.9.5361
8. Hagn M, Sontheimer K, Dahlke K, Brueggemann S, Kaltenmeier C, Beyer T, et al. (2012) Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help. Immunol cell biol. 90(4):457-467. doi: 10.1038/icb.2011.64
9. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. (2007) IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat immunol. 8(9):967-974. doi: 10.1038/ni1488
10. Schmitz I, Schneider C, Fröhlich A, Frebel H, Christ D, Leonard WJ, et al. (2013) IL-21 restricts virus-driven Treg cell expansion in chronic LCMV infection. PLoS Pathog 9(5):e1003362. doi: 10.1371/journal.ppat.1003362
11. Pot C, Jin H, Awasthi A, Liu SM, Lai C-Y, Madan R, et al. (2009) Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells. J Immunol 183(2):797-801. doi: 10.4049/jimmunol.0901233
12. De Simone V, Ronchetti G, Franzè E, Colantoni A, Ortenzi A, Fantini MC, et al. (2015) Interleukin-21 sustains inflammatory signals that contribute to sporadic colon tumorigenesis. Oncotarget 6(12):9908. doi: 10.18632/oncotarget.3532
13. Stolfi C, Rizzo A, Franzè E, Rotondi A, Fantini MC, Sarra M, et al. (2011) Involvement of interleukin-21 in the regulation of colitis-associated colon cancer. J Exp Med 208(11):2279-2290. doi: 10.1084/jem.20111106
14. Chabab G, Barjon C, Abdellaoui N, Salvador-Prince L, Dejou C, Michaud HA, et al. (2020) Identification of a regulatory Vδ1 gamma delta T cell subpopulation expressing CD73 in human breast cancer. J leukoc biol 107(6):1057-1067. doi: 10.1002/JLB.3MA0420-278RR.
15. Zheng X, Zhou Y, Yi X, Chen C, Wen C, Ye G, et al. (2018) IL-21 receptor signaling is essential for control of hepatocellular carcinoma growth and immunological memory for tumor challenge. Oncoimmunology 7(12):e1500673. doi: 10.1080/2162402X.2018.1500673
16. Xue D, Yang P, Wei Q, Li X, Lin L, Lin T (2019) IL-21/IL-21R inhibit tumor growth and invasion in non-small cell lung cancer cells via suppressing Wnt/β-catenin signaling and PD-L1 expression. Int J Mol Med 44(5):1697-1706. doi: 10.3892/ijmm.2019.4354
17. Ngiow SF, Young A (2020) Re-education of the tumor microenvironment with targeted therapies and immunotherapies. Front Immunol 11:1633. doi: 10.3389/fimmu.2020.01633
18. du Bois H, Heim TA, Lund AW. (2021) Tumor-draining lymph nodes: At the crossroads of metastasis and immunity. Sci Immunol 6(63):eabg3551. doi: 10.1126/sciimmunol.abg3551
19. Liu J-Y, Yang G-F, Chen F-F, Peng C-W. (2019) Evaluating the prognostic significance of tumor-infiltrating lymphocytes in solid tumor: practice of a standardized method from the International Immuno-Oncology Biomarkers Working Group. Cancer Manag Res 11:6815. doi: 10.2147/CMAR.S201538
20. Ray-Coquard I, Cropet C, Van Glabbeke M, Sebban C, Le Cesne A, Judson I, et al. (2009) Lymphopenia as a prognostic factor for overall survival in advanced carcinomas, sarcomas, and lymphomas. Cancer Res 69(13):5383-5391. doi: 10.1158/0008-5472.CAN-08-3845
21. Trédan O, Manuel M, Clapisson G, Bachelot T, Chabaud S, Bardin-dit-Courageot C, et al. (2013) Patients with metastatic breast cancer leading to CD4+ T cell lymphopaenia have poor outcome. Eur J cancer. 49(7):1673-1682. doi: 10.1016/j.ejca.2012.11.028
22. Zirakzadeh AA, Marits P, Sherif A, Winqvist O (2013) Multiplex B cell characterization in blood, lymph nodes, and tumors from patients with malignancies. J Immunol 190(11):5847-5855. doi: 10.4049/jimmunol.1203279
23. Foulds GA, Vadakekolathu J, Abdel-Fatah T, Nagarajan D, Reeder S, Johnson C, et al. (2018) Immune-phenotyping and trans-

criptomic profiling of peripheral blood mononuclear cells from patients with breast cancer: identification of a 3 gene signature which predicts relapse of triple negative breast cancer. Front Immunol 9:2028. doi: 10.3389/fimmu.2018.02028

24. Tsuda B, Miyamoto A, Yokoyama K, Ogiya R, Oshitanai R, Terao M, et al. (2018) B-cell populations are expanded in breast cancer patients compared with healthy controls. Breast Cancer 25(3):284-291. doi: 10.1007/s12282-017-0824-6
25. Carpenter EL, Mick R, Rech AJ, Beatty GL, Colligon TA, Rosenfeld MR, et al. (2009) Collapse of the CD27+ B-cell compartment associated with systemic plasmacytosis in patients with advanced melanoma and other cancers. Clin Cancer Res 15(13):4277-4287. doi: 10.1158/1078-0432.CCR-09-0537
26. Balouchi-Anaraki S, Mohammadsadeghi S, Norouzian M, Rasolmali R, Talei A-R, Mehdipour F, et al. (2022) Expression of Interleukin-21 and Interleukin-21 receptor in lymphocytes derived from tumor-draining lymph nodes of breast cancer. Breast Dis 41(1):373-382. doi: 10.3233/BD-220013
27. Zhao Y, Zhang Z, Lei W, Wei Y, Ma R, Wen Y, et al. (2021) IL-21 Is an Accomplice of PD-L1 in the Induction of PD-1-Dependent Treg Generation in Head and Neck Cancer. Front Oncol 11:1601. doi: 10.3389/fonc.2021.648293