Original Research

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The Frequency and the Significance of TNF-α$^+$CD19$^+$ B-cell Population in Lymph Nodes of Patients with Squamous Cell Carcinoma of Head and Neck

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Abstract

Objective: B-cells can contribute to the suppression or progression of the tumor growth through the secretion of various cytokines. In this study, the relevance of B-cell cytokine production to the outcome variables in patients having squamous cell carcinoma (SCC) of the head and neck was studied.

Materials and Methods: Thirty-six draining lymph nodes from untreated patients with tongue and larynx SCC were stimulated with CpG, recombinant CD40L and phorbol-12-myristate-13-acetate/ionomycin and analyzed by flow cytometry for the frequency of B-cell subsets based on interleukin (IL)-10 and tumor necrosis factor-alpha (TNF-α) cytokine production.

Results: It was indicated that patients with uninvolved lymph nodes and low stage of the disease had an increased percentage of TNF-α$^+$CD19$^+$ B-cells (p=0.006 and p=0.041, respectively), whereas the frequency of IL-10$^+$CD19$^+$ B-cells showed an increasing trend in tumor-draining lymph nodes (TDLNs) of patients with grade 2+3 compared to grade 1 (p=0.054).

Conclusion: Collectively, TNF-α-producing B-cells in TDLNs has been reported to be associated with good prognosticators in patients with SCC of head and neck that might have a positive role in immunity to SCC of head and neck.

Keywords: B-cells, IL-10, SCC of the head and neck TNF-α, tumor draining lymph nodes

Introduction

Squamous cell carcinoma (SCC) of the head and neck represents a heterogeneous group of tumors characterized by variations in the tumor microenvironment and immune landscape (1-3). Despite advancements in surgical, chemotherapy, and radiotherapy treatments, patients diagnosed with SCC of the head and neck continue to face a challenging prognosis mainly due to high recurrence rates (4). The unfavorable prognosis observed in these cases might be attributed to the immunomodulatory effects of SCC of the head and neck, which have been the subject of several studies. However, the available data on the specific types of immune cells and their prognostic implications in immune cell infiltrates remain controversial (5-8).

The tumor microenvironment of SCC of the head and neck is a complex interplay of immune and non-immune cells, along with mucosa-associated lymphoid tissues (9). Among the immune cell populations that infiltrate the tumor microenvironment, cytotoxic CD8$^+$ T-cells exhibit predominant presence, followed by CD4$^+$ helper T-cells, CD19$^+$ B-cells, and limited numbers of natural killer cells (10). The intricate interactions between these immune cell populations, as well as their activation status, can influence the development and progression of tumors and metastasis (10).

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Lymph nodes, specialized structures for T-cell and B-cell immune responses, play a crucial role in the surveillance and regulation of immune activities (11). The presence of tumor cells within lymph nodes indicates potential evasion from immune surveillance. Previous studies have explored the association between the "immunomorphology" of SCC of the head and neck draining lymph nodes and disease parameters and outcomes, revealing intriguing findings such as a correlation between increased germinal centers and higher nodal metastasis (12-15).

In the study by Norouzian et al. (16), significant changes were identified in the composition of B-cell subpopulations with regulatory phenotypes in the tumor-draining lymph nodes (TDLNs) of head and neck SCC during tumor development. Building upon these insights, we aimed to investigate the impact of cancer progression on B-cell function by examining the association between B-cell characteristics and disease parameters and outcomes.

In this study, particular attention was given to investigating the presence and role of interleukin (IL)-10 and tumor necrosis factor (TNF) cytokines within the context of SCC of the head and neck. IL-10 is an anti-inflammatory cytokine known for its immunosuppressive properties, modulating the immune response by inhibiting the production of pro-inflammatory cytokines and dampening immune cell activity (17). We aimed to understand the potential role of IL-10-producing cells in the tumor microenvironment and their impact on disease progression.

Conversely, TNF-α is a pro-inflammatory cytokine involved in immune responses and inflammation (18). Its role in cancer is complex, as it can exhibit both pro-tumor and anti-tumor effects depending on the circumstances (19). Given the heterogeneity of SCC of the head and neck and the intricate interplay between tumor cells and the immune system, we chose to investigate TNF-α-producing cells to gain insights into its potential role in disease progression, immune evasion, or anti-tumor immune responses.

By examining the presence and activity of IL-10 and TNF-α-producing cells, we also aimed to unravel valuable insights into the immune landscape, cellular interactions, and their associations with disease parameters and outcomes in SCC of the head and neck.

Materials and Methods

Patients

Thirty-six lymph nodes were taken from 15 untreated patients with laryngeal SCC and 21 patients with tongue SCC who underwent surgical resection. Disease stage was determined in accordance with the 7th edition of American Joint Committee on Cancer Classification and stage group. Patients' characteristics are summarized in Table 1. The protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S664, date: 04.11.2017) and written informed consent was obtained for participation in the study.

Cell Preparations and Culture Conditions

Lymph node mononuclear cells were purified using standardized density gradient technique (Lymphedex, Inno-Train Diagnostik, Germany) and activated in culture medium, RPMI 1640 containing 10% FBS and 1%

Table 1. Clinico-pathological characteristics of HNSCC patients enrolled in the study of cytokine profile of B-cells

Characteristics	Value
Age (years)	61.5 ± 16.4 (27-88)
Gender	
Male	25 (69.4%)
Female	11 (30.6%)
Tumor type	
Larynx	15 (41.7%)
Tongue	21 (58.3%)
Lymph node	
N0	21 (58.3%)
N1	4 (11.1%)
N2	10 (27.8%)
N3	1 (2.8%)
Tumor size	
T1	4 (11.1%)
T2	12 (33.3%)
T3	13 (36.1%)
T4	7 (19.4%)
Stage	
I	4 (11.1%)
II	7 (19.4%)
III	9 (25%)
IV	16 (44.4%)
Histological grade	
Well differentiated (I)	18 (50%)
Moderately differentiated (II)	14 (38.9%)
Poorly differentiated (III)	3 (8.3%)
Unknown	1 (2.8%)
Perineural/lymphovascular invasion	
Positive	8 (22.2%)
Negative	28 (77.8%)
Lymph nodes characteristic	
MLNs	12 (33.3%)
nMLNs	22 (61.1%)
Not determined	2 (5.6%)

MLN: Metastatic lymph node, nMLN: Non-metastatic lymph node, HNSCC: Head and neck squamous cell carcinoma

penicillin/streptomycin (all from Gibco, Life Technologies, USA) containing 10 µg/mL CpG, (Invivogen, USA) and 200 ng/mL recombinant CD40L (R&D System, USA) and after 4 hours, phorbol-12-myristate-13-acetate (50 ng/mL) (Sigma-Alderich, Germany), ionomycine (1 µg/mL) (Sigma-Alderich, Germany) and berefeldin A (1 µL/mL) (BD Bioscience, USA) were added for an additional 6 hours.

Flow Cytometry

After activation and washing with staining buffer, cells were incubated with PerCP-Cy5.5-conjugated anti-CD19-antibody (clone: HIB19; Biolegend). Then cells were fixed and resuspended in perm/wash buffer and incubated with allophycocyanin-conjugated anti-IL-10 (clone: JES3-19F1; BD biosciences) and phycoerythrin-conjugated anti-TNF-α (MAB11; BD biosciences, USA) antibodies. Data were analyzed using FACSCalibur flow cytometer (BD biosciences, USA) and the FlowJo software (version 7.6.2, Ashland, San Diego CA, USA), respectively.

Statistical Analysis

Comparisons of quantitative variables between two and three or more groups were analyzed by the Mann-Whitney U test and the Kruskal-Wallis test, respectively, followed by the Dunn's post-hoc test. Correlations were determined using the Spearman's rank test. All statistical analyses were conducted using SPSS 16 software and p-value <0.05 was considered significant.

Results

Demographical, Clinical and Pathological Characteristics of SCC of the Head and Neck Patients

A total of 36 patients including 11 untreated women and 25 men (mean age: 61.2 ± 15.8 years) were enrolled. According to pathological reports, 12 LNs (33.3%) were involved by the tumor [lymph nodes (LNs), metastatic LNs (MLNs) and 22 (61.1%) were not involved nonmetastatic LNs, nMLNs]. Considering tumour-node-metastasis staging, 4 patients (11.1%) were in stage I, 7 (19.4%) were in stage II, 9 (25%) were in stage III, and 16 (44.4%) patients had distant metastasis (stage IV). Data of the patients are detailed in Table 1.

Associations Between B-cell Cytokine Profile and LN Involvement

The frequencies of cytokine-producing B lymphocytes and the expression intensity of their cytokines were assessed in TDLNs of patients with SCC of the head and neck (Figure 1, Table 2). The percentages of IL-10$^+$ and TNF-α$^+$ B-cells and the geometric mean fluorescence intensity (gMFI) of these cytokines did not show significant differences between metastatic and non-metastatic LNs of patients with SCC of the head and neck. However, when patients with tongue SCC and laryngeal SCC were analyzed separately, TNF-α-secreting B-cells had higher frequency in nMLNs of patients with tongue SCC compared to those of MLNs (p=0.006, Figure 2). Furthermore, our results showed higher percentage of TNF-α- secreting B-cells in TDLNs of patients with tongue SCC who did not have any metastatic LN (LN$^-$) in comparison with those with at least one metastatic LN (LN$^+$) (p=0.041, Figure 3).

Table 2. Percentages of cytokine producing B-cells in the TDLNs of HNSCC patients

Cell subset	Minimum	Maximum	Median	Mean ± SD
B-cell subsets				
CD19$^+$TNF-α$^+$	35.4	93	73.7	71.4 ± 15.3
CD19$^+$IL-10$^+$	0.3	8.2	0.8	1.2 ± 1.3

TDLN: Tumor draining lymph node, SD: Standard deviation, HNSCC: Head and neck squamous cell carcinoma, TNF-α: Tumor necrosis factor alpha, IL-10: Interleukin-10

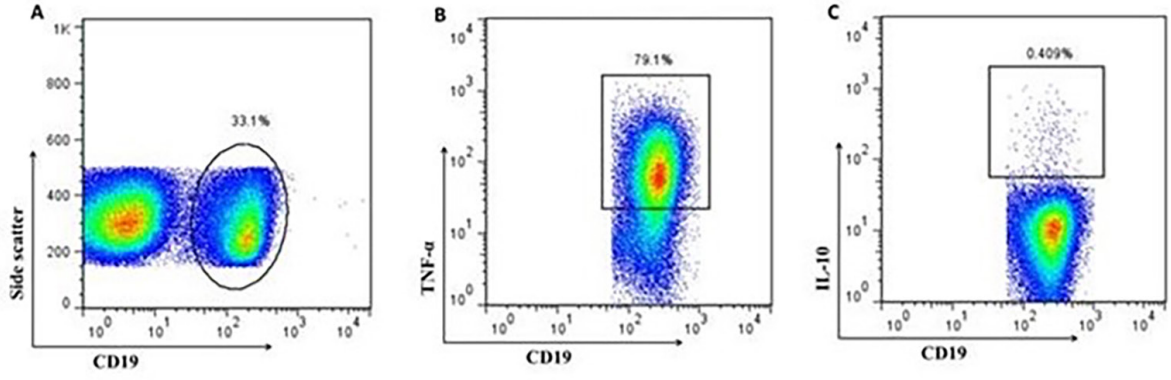

Figure 1. Cytokine-secreting B-cells from draining lymph nodes of patients with SCC of the head and neck. Flow cytometry dot plots show the percentages of A) CD19$^+$ B-cells, B) TNF-α$^+$CD19$^+$ and C) IL-10$^+$CD19$^+$ B-cells.
SCC: Squamous cell carcinoma, TNF-α$^+$: Tumor necrosis factor alpha, IL-10$^+$: Interleukin-10

Associations Between B-cell Cytokine Response and Tumor Stage

Comparing the frequency of cytokine-secreting B-cells and the gMFI of their cytokines among the draining LNs of patients with low and advanced stages showed that the frequency of TNF-α- secreting B-cells in low stages (I+II) was significantly higher than those with advanced stages (III+IV) (p=0.041) (Figure 4).

Relationship Between B-cell Cytokine Response and Tumor Grade or Size and Perineural/Lympho-Vascular Invasion

No significant differences were found between the frequencies of cytokine-secreting B-cells and the gMFI of their cytokines among draining LNs of patients with SCC of the head and neck with different tumor grades; however, it was found that the percentage of IL-10- secreting B-cells was slightly higher in TDLNs of patients with grade II+III (p=0.054, Figure 5).

Additionally, no association was observed between the percentage of cytokine-secreting B-cells and other tumor histopathological characteristics such as tumor size and perineural/lympho-vascular tumor invasion.

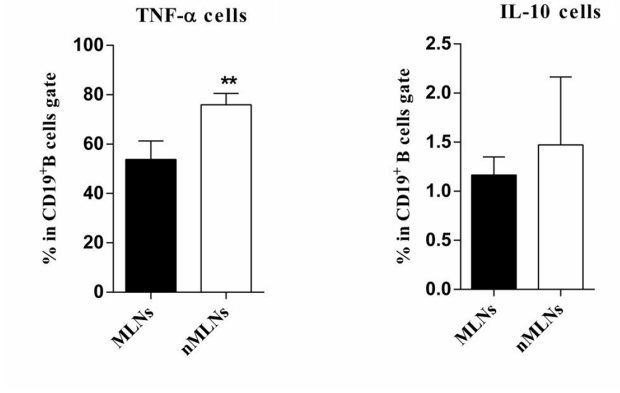

Figure 2. Bar graph shows the comparison of (A) the percentages of B-cells based on cytokine production and (B) the gMFI of their cytokines in metastatic and non-metastatic lymph nodes of tongue SCC patients. Data are shown as mean ± SEM. **: p=0.006.
TNF-α: Tumor necrosis factor alpha, IL-10: Interleukin-10, MLNs: Metastatic lymph nodes, nMLNs: Non-metastatic metastatic lymph nodes, SCC: Squamous cell carcinoma, SEM: Standard error of mean

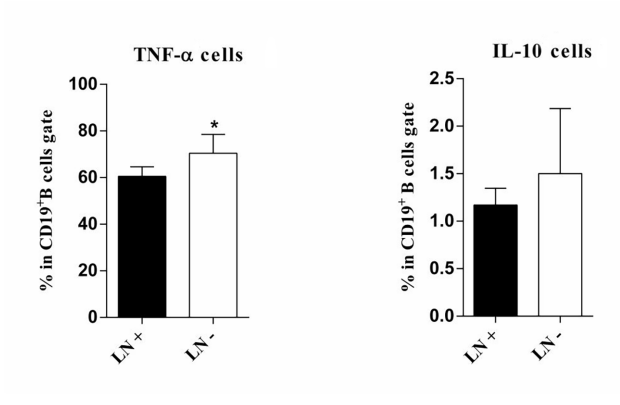

Figure 3. Bar graph shows the comparison of (A) the percentages of B-cells based on cytokine production and (B) the gMFI of their cytokines in involved lymph node (LN^+) and uninvolved lymph node (LN^-) of tongue SCC patients. Data are shown as the mean ± SEM. *: p=0.041.
TNF-α: Tumor necrosis factor alpha, IL-10: Interleukin-10, gMFI: Geometric mean fluorescence intensity, SCC: Squamous cell carcinoma, SEM: Standard error of mean

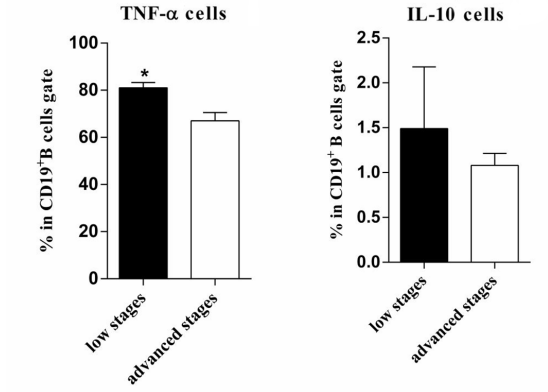

Figure 4. Bar graph shows the comparison of (A) the percentages of B-cells based on cytokine production and (B) the gMFI of their cytokines between SCC of the head and neck patients' groups with low stages and advanced stages of disease. Data are shown as the mean ± SEM. *: p=0.041.
TNF-α: Tumor necrosis factor alpha, IL-10: Interleukin-10, gMFI: Geometric mean fluorescence intensity, SCC: Squamous cell carcinoma, SEM: Standard error of mean

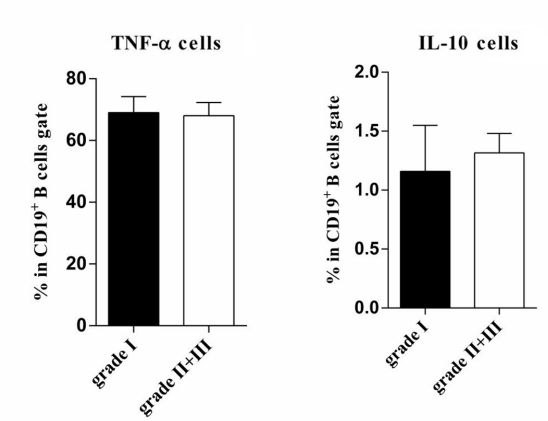

Figure 5. Bar graph shows the comparison of (A) the percentages of B-cells based on cytokine production and (B) the gMFI of their cytokines between SCC of the head and neck patients' groups with grade I and grade II+III. Data are shown as the mean ± SEM, p=0.054.
TNF-α: Tumor necrosis factor alpha, IL-10: Interleukin-10, gMFI: Geometric mean fluorescence intensity, SCC: Squamous cell carcinoma, SEM: Standard error of mean

Discussion

It is recently appreciated that B-cells contribute significantly to shape the immune response in the tumor microenvironment (20). Tertiary lymphoid structures (TLSs) within tumor milieu composed of T-cell and B-cell areas in which B-cell can promote anti-tumor immunity by antibody dependent cytotoxicity and presenting antigen to activate T-cells (21). On the other hand, B-cells located at the site of tumor or TLSs are reported to have regulatory functions and suppress anti-inflammatory response through production of inhibitory cytokines (22-24).

Various subpopulations of B-cells have the ability to produce a wide range of cytokines which can have the potential to exert both, T-cell-promoting and inhibiting activities (25,26). In a study, murine B-cells producing regulatory cytokine such as IL-10 are shown to affect the balance of Th1/Th2 response through inducing IL-4 and down regulating IL-12 production in dendritic cells (27). Moreover, in a study of mice infected with *Toxoplasma gondii*, B-cells increased Th1 response against this pathogen by producing TNF-α (28). Another study showed that B-cells production of IL-2 was important in the development of a protective Th2 memory response against *H. polygyrus* (29). Biopsies from patients suffering from hepatocellular carcinoma and breast cancer demonstrated that higher IFN-γ- and TNF-α- secreting B-cells and $CD8^+$ T-cells infiltrates were linked with improved outcome, suggesting the significance of tumor-infiltrating B-cells producing inflammatory cytokines in promoting cytotoxic T-cell immunity (30,31).

The present study revealed negative associations of the frequency of TNF-α-producing B-cells with lymph node involvement and advanced disease stage, whereas the frequency of IL-10-producing B-cells positively correlated with higher tumor grade. On the other hand, a previous study showed that tumor invasion of draining LNs was associated with a decrease in regulatory $FOXP3^+$ T-cells in patients with head and neck SCC (32).

The results of this study were similar to those of study that was published by Mehdipour et al. (33), showing the association of the lower frequency of TNF^{hi} B-cells with LN involvement in breast cancer-draining LNs. However, in contrast, they reported reverse correlations between the percentages of TNF-α-producing B-cells and regulatory T-cells in nMLNs were found, while the rate of regulatory T-cells were found to show associated with poor prognostic factors. This may suggest that prognostic value of TNF-α-producing B-cell and Treg cell and their interaction in the draining LNs may be associated with different immunological pathways.

Considering the similar association of TNF-expressing B-cells and $CD4^+$ regulatory T-cells with LN involvement, it can be hypothesized that in the chronic inflammation, seen in SCC of the head and neck, TNF may promote antitumor immunity by boosting Tregs via TNFR2.

It seems that the presence of a higher number of TNF-α B producing cells and a tendency of IL-10 producing cells in certain conditions may have associated with progression and severity of the disease.

TNF-α B producing cells, were associated with the absence of LN metastasis and earlier stages of the disease. This could suggest that these cells could have a protective effect, potentially by inhibiting the invasion of cancer cells to the lymph nodes and contributing to a less advanced disease stage.

IL-10 is known for its anti-inflammatory properties and its role in regulating immune responses. It's possible that a higher presence of IL-10 producing cells could contribute to a more favorable immune environment, potentially affecting disease progression.

However, it's important to note that the significance of these findings may vary depending on individual cases, and further research is needed to fully understand the implications. These observations could potentially guide future studies and contribute to the development of targeted therapies or diagnostic markers.

Conclusion

The present study has shown negative associations between the percentage of B-cells that produce TNF-α with the indicators of unfavorable prognosis (LN involvement and advanced stage of disease). These findings indicate that TNF-α production by B-cells may have a potential role in promoting anti-tumor responses. However, more functional studies are needed to elucidate the role of TNF produced by B lymphocytes in immunity against SCC of the head and neck.

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Ethics

Ethics Committee Approval: The protocol was approved by the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S664, date: 04.11.2017).

Informed Consent: Written informed consent was obtained for participation in the study.

Authorship Contributions

Surgical and Medical Practices: M.N., F.M., M.J.A., B.K., A.G., Concept: M.N., F.M., M.J.A., B.K., A.G., Design: M.N., F.M., M.J.A., B.K., A.G., Data Collection or Processing: M.N., F.M., M.J.A., B.K., A.G., Analysis or Interpretation: M.N., F.M., M.J.A., B.K., A.G., Literature Search: M.N., F.M., M.J.A., B.K., A.G., Writing: M.N., F.M., M.J.A., B.K., A.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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