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Regulatory B cells producing IL-10 are increased in human tumor draining lymph nodes

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Abstract

The contribution of different immune cell subsets, especially T cells, in anti-tumor immune response is well established. In contrast to T cells, the anti-tumor contribution of B cells has been scarcely investigated. B-cells are often overlooked, even though they are important players in a fully integrated immune response and constitute a substantial fraction of tumor draining lymph nodes (TDLNs) known also as Sentinel Nodes. In this project, samples including TDLNs, non-TDLNs (nTDLNs) and metastatic lymph nodes from 21 patients with oral squamous cell carcinoma were analyzed by flow cytometry. TDLNs were characterized by a significantly higher proportion of B cells compared with nTDLNs ($P = .0127$). TDLNs-associated B cells contained high percentages of naïve B cells, in contrary to nTDLNs which contained significantly higher percentages of memory B cells. Patients having metastases in TDLNs showed a significantly higher presence of immunosuppressive B regulatory cells compared with metastasis-free patients ($P = .0008$). Elevated levels of regulatory B cells in TDLNs were associated with the advancement of the disease. B cells in TDLNs were characterized by significantly higher expression of an immunosuppressive cytokine—IL-10 compared with nTDLNs ($P = .0077$). Our data indicate that B cells in human TDLNs differ from B cells in nTDLNs and exhibit more naïve and immunosuppressive phenotypes. We identified a high accumulation of regulatory B cells within TDLNs which may be a potential obstacle in achieving response to novel cancer immunotherapies (ICIs) in head and neck cancer.

KEYWORDS
B regulatory cells, oral cancer, tumor-draining lymph nodes

Abbreviations: APC, antigen presenting cell; Bregs, B regulatory cells; HLN, healthy lymph node; HNSCC, head and neck squamous cell carcinoma; ICI, immune checkpoint inhibitor; MALT, mucosa-associated lymphoid tissue; nTDLN, non-tumor draining lymph node; OSCC, oral squamous cell carcinoma; TDLN, tumor draining lymph node; TILs, tumor infiltrating lymphocytes; TME, tumor microenvironment.

What's new?

Tumor-draining lymph nodes are becoming increasingly recognized as key players in the modulation of anti-tumor immunity and regulation of response to novel cancer immunotherapies. However, the immunological architecture and roles of tumor-draining lymph nodes remain to be clarified. This study shows that, in oral cancer, B cells in tumor-draining lymph nodes are characterized by more naïve and immunosuppressive phenotypes when compared with B cells in non-tumor-draining lymph nodes. The results also reveal a high accumulation of regulatory B cells within tumor-draining lymph nodes that may represent a potential obstacle in achieving response to novel cancer immunotherapies in oral cancer.

1 | INTRODUCTION

Secondary lymphoid organs include lymph nodes, spleen, Peyer's patches, mucosa-associated lymphoid tissue (MALT), adenoids and tonsils. Their main role is immune surveillance and providing an effective and rapid immune response. Lymph nodes are also the first lymphatic organs to encounter cancer antigens and cancer cells. Spreading of solid tumors to regional tumor draining lymph nodes (TDLNs) is a hallmark of cancer progression and is associated with poor prognosis.

The development of cancer immunotherapy has changed the field of oncology and shifted the focus of researchers from cancer cells to hosts' immune cells. Immune checkpoint inhibitors (ICIs) showed remarkable efficacy across different cancer types and significantly improved survival in some patients, especially in advanced stages of the disease. Still, only a relatively small fraction of patients shows a durable response to ICIs. Thus, international research efforts have focused on finding biomarkers predicting response to this treatment in the blood or tumor microenvironment (TME). However, the results have been unsatisfying so far.

The contribution of different immune cell subsets, especially T cells, in the anti-tumor immune response and response to ICIs is well established. In contrast to T cells, the anti-tumor contribution of B cells has been scarcely investigated. B cells are often overlooked, even though they are important players in a fully integrated immune response and constitute a substantial fraction of TDLNs. TDLNs have also been overlooked in the initial phase of immunotherapy research. Partly due to the fact that they are traditionally removed during the lymph node dissection, which is a part of oncological surgical treatment. Nevertheless, TDLNs have lately been more appreciated, and in recent papers suggested to be primary regulators of anti-tumor immunity and key players in regulating the response to immunotherapy.[1,2]

Furthermore, recent research clearly indicates that B cells are an important source of immunomodulatory cytokines and that they actively participate in the intercellular crosstalk. The use of systemic ICIs agents may also target B cells both in the TME and in the periphery. B cells express PD-1, PD-L1 and CTLA-4 molecules on their surface and the use of ICIs was shown to change their activity and enhance maturation.[3-5] Solid tumors contain significant numbers of infiltrating B cells, suggesting that B cells play an important role in the complex network of cellular interactions in the tumor microenvironment. High infiltration with B cells is often seen as a favorable prognostic factor.[6] However, the role of B cells in anti-tumor immunity is still debated. B cells may play both anti- and protumorigenic roles in cancer progression. On the one hand, B cells in the presence of a tumor rapidly proliferate and begin to produce a wide range of tumor-specific antibodies which initiate a cascade of immunological events leading to cancer cell death.[7] The presence of B cell-rich tertiary lymphoid structures in TME was shown to improve response to immunotherapy and survival in melanoma.[8] On the other hand, there is evidence that tumor-infiltrating B cells directly secret pro-tumorigenic factors and cytokines such as lymphotoxin, IL-8, IL-10 and IL-35.[9-11] These cytokines induce pro-tumorigenic immunosuppression and facilitate metastasis.

Most B cells research focuses on B cells populations within TME and in the peripheral circulation. B cells in TDLNs lack a clear characterization. Therefore, the aim of this project is to provide a detailed characterization of B cells populations with focus on their maturation and presence of B regulatory cells in TDLNs and nTDLNs of patients with oral squamous cell carcinoma (OSCC).

2 | MATERIALS AND METHODS

2.1 | Patient characteristics

Eligible patients enrolled for this study met the following inclusion criteria: (1) diagnosis of primary or recurrent oral squamous cell carcinoma (OSCC), (2) tumor excision with sentinel node assisted elective neck dissection (identification in SPECT-CT, and location confirmed intraoperatively by gamma probe and injection of indocyanine green [ICG] and further visualization with near-infrared light) performed at Karolinska University Hospital, Stockholm, Sweden between March 2019 and June 2020 and (3) willingness to participate in the study. For details regarding the setting of the sentinel node procedure at Karolinska University Hospital, see the paper of Kågedal et al.[12] Exclusion criteria were as follows: (1) systematic autoimmune diseases, (2) synchronous second malignancies and (3) hemo-lymphopoietic malignancies in the past, any other acute or chronic condition that could influence immunological milieu in the lymph nodes.

TDLNs are defined as one or a group of first lymph nodes draining a tumor. In oral cancer, patients have usually one to three TDLNs.

Non-tumor-draining lymph nodes (n-TDLNs) come from the same patient's neck but do not directly drain the tumor. n-TDLNs come usually from neck regions 1 to 3 and are chosen by an experienced pathologist.

2.2 | Sample preparation

The unfixed neck sample after excision were transferred directly to the Pathology Department, where one of the pathologists assessed the samples and divided lymph nodes into halves (all TDLNs and 1-2 n-TDLNs per patient). The lymph nodes after surgical excision were kept in a pre-chilled MACS Tissue Storage Solution (Miltenyi Biotec #130-100-008) and used within 1 h for further analysis. Tumor Dissociation KIT (Miltenyi Biotec #130-095-929) was used to dissociate surgical specimens mechanically and enzymatically. After dissociation, cells were filtered through a 100 µm cell strainer (BD biosciences #352360). Cells were re-suspended in brilliant stain buffer (BD biosciences #563794) and used for downstream analysis.

2.3 | Flow cytometry

Single-cell suspensions with purified cells from surgical specimens were first divided into two equal parts. One was used for direct cell surface markers staining and the other part was used for in-vitro challenge and intracellular IL-10 staining as described under. The surface markers staining included the use of live/dead staining (LIVE/DEAD Fixable Far Red Dead Cell Stain Kit) according to the manufacturer's protocol. Next, samples were incubated with Fc-block for 5 min and consequently following panel of anti-human antibody-conjugates was used: CD19-BB700, CD20-Alexa Fluor 700, CD24-BV421, CD138-PE-CF594, IgM-BV510, IgD-APC-H7, CD27-PE-CY7, CD38-BV711, Kappa light chain-PE and Lambda light chain-FITC. Cells were stained for 20 min at the room temperature in darkness. Staining was followed by two washing steps performed with PBS, 400 g, for 5 min. After two washes cells were resuspended in PBS with 1% paraformaldehyde (HistoLab #02178) and analyzed on LSR FORTESSA (BD Biosciences). Analysis of the flow cytometry data was performed with FlowJo version 10.8.0 (LLC, USA).

The detection of IL-10 was performed following the experiment protocol published by de Masson et al.[13] In summary, cell suspensions were activated in vitro using CpG (0.7 µg/ml) and Recombinant Human CD40 Ligand/TNFSF5 (1 µg/ml) as stimuli in cRPMI for 72 h at 37°C. Leukocyte Activation Cocktail, with BD GolgiPlug was added for the last 4 h of incubation. The following panel of anti-human antibody-conjugates was used: CD19-BB70, CD20-Alexa Fluor 700, CD24-BV421, CD27-PE-CY7, CD38-BV711, IL-10-APC and IL-10-APC Isotype Control. BD Cytofix/Cytoperm solution was used for permeabilization and fixation of cells for detection of intracellular IL-10. Live dead staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit was performed according to the manufacturer's recommendations.

TABLE 1 Summary of the clinical and pathological characteristics of enrolled patients.

Clinical/pathological characteristic	
Age	
Median (range)	66 (23-82)
	N (%)
Sex	
Female	7 (33.3%)
Male	14 (66.7%)
Smoking history	
Yes	7 (33.3%)
No	14 (66.7%)
Tumor localization	
Tounge	17 (81.0%)
Floor of mouth	4 (19.0%)
T-stage	
T1	9 (42.9%)
T2	8 (38.1%)
T3	4 (19.0%)
N-stage	
N0	11 (52.4%)
N1	4 (19.0%)
N2+	6 (28.6%)

2.4 | Statistical analysis

Statistical analyses were performed with GraphPad Prism version 9.0.0 (GraphPad Software, La Jolla, CA). The Kolmogorov-Smirnov normality test was used to determine if data sets were normally distributed, and Mann-Whitney or two-tailed Unpaired *t*-test were chosen, depending on the distribution of the data. Paired *t* test was used to compare paired groups of data. For comparison of more than two groups, one-way ANOVA was performed for normally distributed data and Kruskal-Wallis tests were used for comparisons of more than two groups not meeting the assumption of data normality. $P < .05$ (*) was considered significant, and $P < .01$ (**), $P < .001$ (***), $P < .0001$ (****) were considered highly significant.

3 | RESULTS

3.1 | Patient clinical and pathological characteristics

Twenty-one patients with OSCC were enrolled in the study. There were seven female (33.3%) and 14 male (66.7%) patients with the age range of 23 to 82 and a median age of 66. The summary of clinical and pathological characteristics is shown in Table 1. Patients with known nodal metastases (cN+) were also included. Detailed clinical information is presented in Table S1. A total of 36 TDLNs and

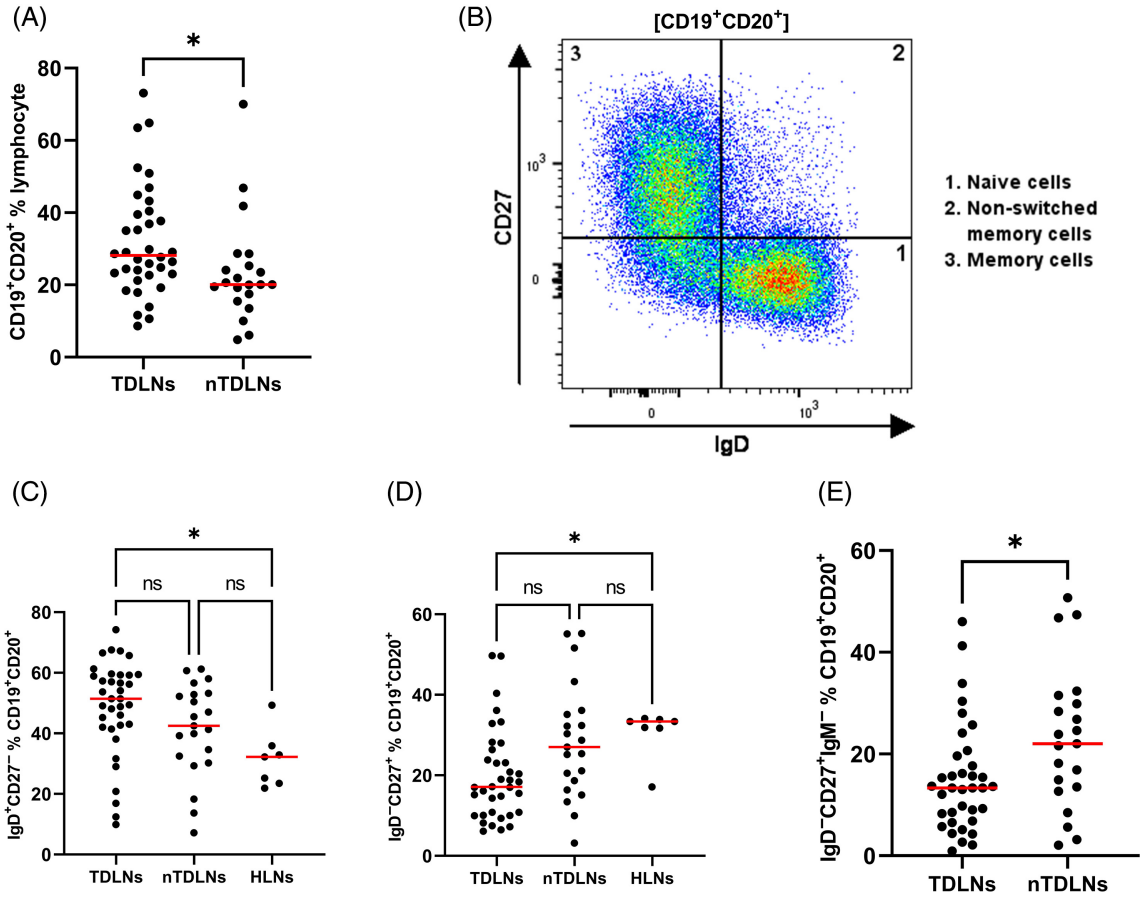


FIGURE 1 (A) Comparison of % of $CD19^+CD20^+$ among viable lymphocytes between TDLNs (n = 36) and nTDLNs (n = 21). (B) The gating strategy for the analysis of naïve B cells and memory B cells based on the expression of CD27 and IgD is delineated by an exemplary dot plot. (C) Percentages of naïve B cells (IgD^+CD27^-) are compared between TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (D) Percentages of memory B cells (IgD^-CD27^+) are compared between TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (E) Percentages of memory switched B cells ($IgD^-CD27^+IgM^-$) are compared between TDLNs (n = 36) and nTDLNs (n = 21).

21 n-TDLNs were investigated. In addition, we analyzed seven neck lymph nodes from patients with benign salivary gland tumors. These lymph nodes are further called as "healthy lymph nodes" (HLNs). Seventeen patients were diagnosed with squamous cell carcinoma of the tongue and four with the squamous cell carcinoma of the floor of the mouth. Ten patients (47.6%) had nodal metastases confirmed in histopathology.

3.2 | Frequencies of B cells in TDLNs and nTDLNS in HNSCC

Single-cell suspensions from 36 TDLNs, 21 n-TDLNs of HNSCC patients and seven HLNs of patients with benign salivary gland tumors were analyzed by flow cytometry. First, doublets were excluded and live cells were identified with LIVE/DEAD Fixable Far-Red stain (Thermofisher, cat# L34973). Lymphocytes were selected based on SSC-A and FSC-A parameters and used for further analysis. B cells were defined as $CD19^+CD20^+$ cells within the lymphocyte gate.

TDLNs were characterized by a significantly higher percentage of B cells among lymphocytes (31.99 ± 15.33%) compared with nTDLNs (23.70 ± 14.51%; $P = .0127$, Figure 1A). The B cells frequencies did not differ significantly between patients with N0 vs N+ stage, between T1 vs T2 vs T3 stages and between lymph nodes with metastases and metastasis-free nodes (data not shown).

3.3 | TDLNs in HNSCC are characterized by high levels of naïve B cells

To investigate major B cell subsets, including naïve and memory B cells, germinal center B cells and transitional B cells, we used following cell surface markers: IgD, CD27, CD38, CD24, IgM and CD138. First, using the CD27, IgD and IgM markers, we discriminated naïve B cells defined as $CD19^+CD20^+CD27^-IgD^+$, non-class-switched/memory B cells as $CD19^+CD20^+CD27^+IgD^+$ and class-switched memory B cells as $CD19^+CD20^+CD27^+IgD^-IgM^-$. The gating strategy for these subsets is outlined in an exemplary lymph node sample (Figure 1B).

TDLNs in HNSCC had higher levels of naïve B cells ($CD19^+CD20^+CD27^-IgD^+$) compared with HLNs (48.64 ± 15.68% vs 31.56 ± 9.44%, $P = .0148$, respectively; Figure 1C). TDLNs had also higher percentage of naïve B cells compared with n-TDLNs (48.64 ± 15.68% vs 41.25 ± 15.27%). However, the observed difference was not statistically significant ($P = .2103$). All studied compartments had

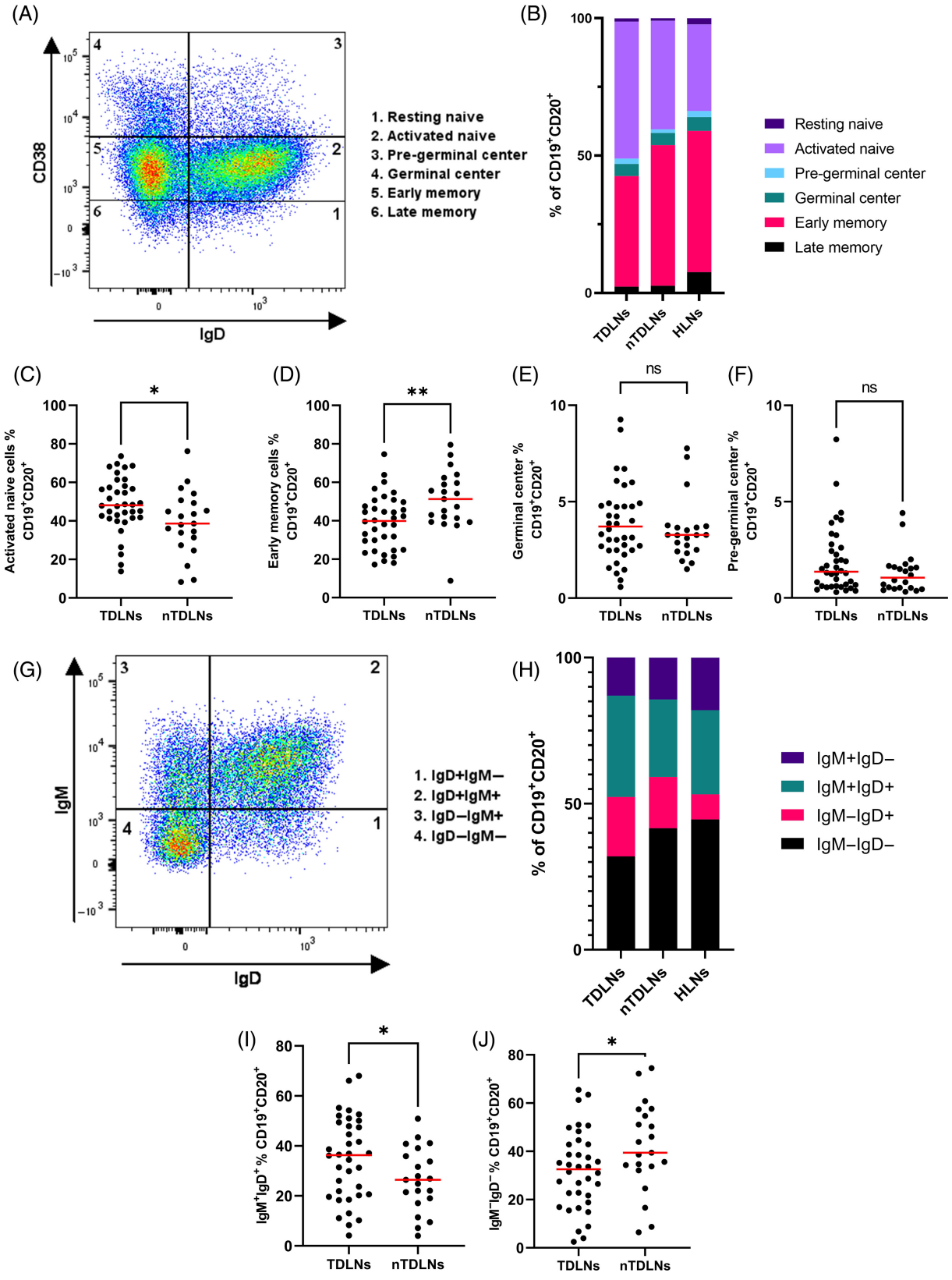


FIGURE 2 Legend on next page.

similar levels of non-class-switched B cells defined as $CD19^+CD20^+CD27^+IgD^+$ with mean percentage between 16% and 21% (data not shown). As anticipated, HLNs were significantly richer in memory B cells compared with TDLNs (30.79 ± 6.11% vs 19.87 ± 11.40, $P = .0265$; Figure 1D). Furthermore, we observed a significant difference in class-switched memory B cells ($CD19^+CD20^+CD27^+IgD^-IgM^-$) between TDLNs and n-TDLNs (15.04 ± 10.62% vs 22.93 ± 13.88%, $P = .0238$; Figure 1E). Patients with N0 vs N+ stage had similar levels of naïve B cells and class-switched memory B cells in their lymph nodes (data not shown).

Another strategy to investigate the maturation stages of B cells in the periphery is based on the expression of CD38 and IgD markers. The exemplary gating strategy is shown in Figure 2A. Based on expression intensity it is possible to define six different stages of B cell maturation: resting naïve, activated naïve, pre-germinal center, germinal center, early memory and late memory cells. Figure 2B summarizes the comparison of mean percentages of every subpopulation between compartments. This gating strategy confirms our findings based on CD27 and IgD expression and shows a similar trend. Namely, TDLNs are significantly richer in activated naïve B cells and have a lower proportion of early memory B cells compared with n-TDLNs (48.63 ± 14.34% vs 39.18 ± 16.63%, $P = .0277$ and 39.23 ± 14.01% vs 50.78 ± 15.58%, $P = .0056$, respectively; Figure 2C, D). There was no significant difference between TDLNs and nTDLNs regarding frequencies of pre-germinal and germinal center B cells (Figure 2E, F). The remaining populations (resting naïve and late memory cells) did not show significant differences between compartments (data not shown).

Analysis of the expression of IgM and IgD confirms our previous findings. The analysis showed that TDLNs contain significantly higher proportion of double-positive IgM^+IgD^+ naïve B cells and a significantly lower fraction of double negative IgM^-IgD^- memory switched B cells compared with nTDLNs (34.69 ± 16.58% vs 26.50 ± 12.72%, $P = .0415$ and 31.98 ± 16.23% vs 41.58 ± 18.43%, $P = .0451$; Figure 2G-J).

3.4 | TDLNs are rich in plasmablasts

Furthermore, other B cell subtypes we investigated were plasmablasts and plasma cells. Plasma cells were defined as $CD19^+CD20^-CD27^+CD38^{++}CD138^+$ cells and plasmablasts as $CD19^+CD20^{+/-}$, $CD27^+$, $CD38^{++}$ and $CD24^-$ cells. The gating strategy for discrimination of plasmablasts is shown in Figure 3A. TDLNs were significantly richer in plasmablasts compared with HLNs (3.66 ± 2.43% vs 1.64 ± 1.47%, $P = .0481$; Figure 3B). TDLNs and nTDLNs had similar percentage of plasmablasts (3.66 ± 2.43% vs 3.09 ± 2.29%, $P = .9238$). The percentage of plasma cells between compartments did not differ significantly. The mean percentage of plasma cells within $CD19^+$ gate equaled in TDLNs 0.65 ± 1.28%, in nTDLNs 0.57 ± 0.83% and in HLNs 2.14 ± 1.88% (Figure 3C, D).

3.5 | B regulatory cells in TDLNs in HNSCC

To date, human B cells with regulatory functions have been essentially defined as $CD24^{high}CD38^{high}$ immature transitional B cell subset producing IL-10. Iwata et al showed also that IL-10 producing Bregs belong to the $CD27^+CD24^{high}$ B cell population. Thus, we used these markers to investigate the frequencies of these subpopulations in the studied compartments. The gating strategy is shown in an exemplary lymph nodes sample (Figures 3A and 4D). Bregs defined as $CD19^+CD20^+CD24^{high}CD38^{high}$ were significantly increased in HLNs compared with TDLNs and nTDLNs (8.35 ± 3.43% vs 5.43 ± 1.91%, $P = .0023$ and vs 4.38 ± 1.53%, $P = <.0001$, respectively; Figure 4A). Frequencies of $CD27^+CD24^{high}$ B cell population did not differ significantly between compartments (Figure 4E, F).

3.6 | Patients with lymph nodes metastases have significantly higher infiltration with Bregs in TDLNs

Patients with N+ stage had significantly higher levels of Bregs ($CD19^+CD20^+CD24^{high}CD38^{high}$) in both TDLNs and n-TDLNs compared with patients without lymph node metastases (6.79 ± 1.78% vs 4.56 ± 1.44%, $P = .0002$; Figure 4B). Lymph nodes containing established nodal metastases were also characterized by a significantly higher percentage of Bregs compared with metastasis-free lymph nodes (6.58 ± 2.13% vs 4.71 ± 1.61%, $P = .0027$; Figure 4C).

3.7 | TDLNs associated B cells express significantly higher levels of IL-10

To further characterize B regulatory cells in lymph nodes of HNSCC patients, we performed an in vitro functional experiment with the intracellular IL-10 staining as described in the Section 2. Shortly, cells were activated with CpG and CD40L agonist for 3 days and treated with Brefeldin A for four h before intracellular staining.

FIGURE 2 (A) The gating strategy for the analysis of resting naïve, activated naïve, pre-germinal center, germinal center, early memory and late memory B cells based on the expression of CD38 and IgD is delineated by an exemplary dot plot. (B) The stacked bar summarizes the mean percentage of the aforementioned populations in TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (C-F) Comparison of the percentage of activated naïve B cells (C), early memory B cells (D), germinal center B cells (E) and pre-germinal center B cells (F) between TDLNs (n = 36) and nTDLNs (n = 21). (G) The gating strategy for the analysis of IgM and IgD positive/negative cells is delineated by an exemplary dot plot. (H) The stacked bar summarizes the mean percentage of $IgM^{+/-}IgD^{+/-}$ populations in TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (I) Percentages of IgM^+IgD^+ are compared between TDLNs (n = 36) and nTDLNs (n = 21). (J) Percentages of IgM^-IgD^- are compared between TDLNs (n = 36) and nTDLNs (n = 21).

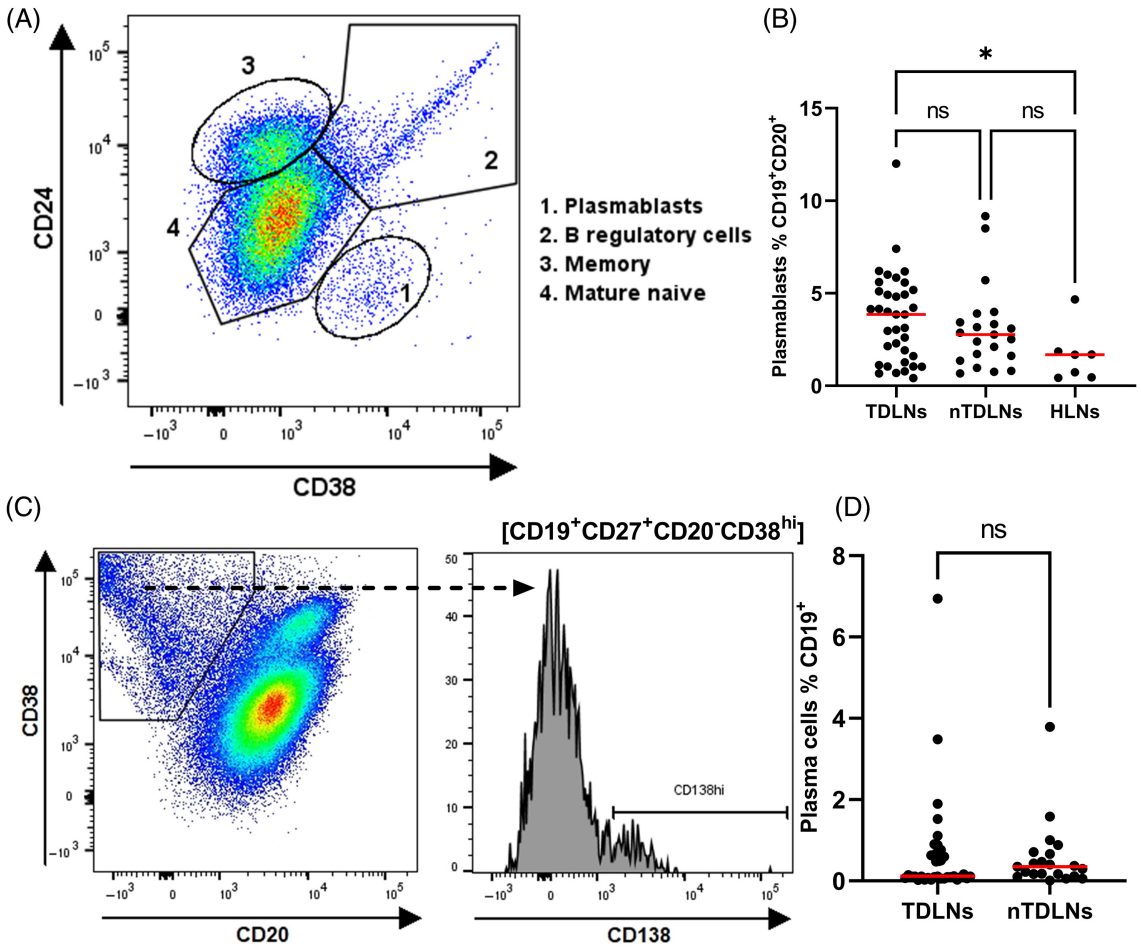


FIGURE 3 (A) The gating strategy for the analysis of plasmablasts, CD24highCD38high B cells, memory and mature naïve B cells based on the expression of CD24 and CD38 is delineated by an exemplary dot plot. (B) Percentages of plasmablasts are compared between TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (C) The gating strategy for the analysis of plasma cells is delineated by an exemplary dot plot and histogram. (D) Percentages of plasma cells are compared between TDLNs (n = 36) and nTDLNs (n = 21).

The intracellular staining with IL-10 after 3 days activation revealed that $CD19^+CD20^+$ B cells in TDLNs expressed significantly higher levels of IL-10 compared with n-TDLNs (25.20 ± 13.85% vs 14.39 ± 7.27%, $P = .0077$; Figure 5A). However, MFI of IL-10 did not differ significantly between compartments (TDLNs: 1477 ± 817.6 vs nTDLNs 1079 ± 529.3, $P = .2084$; Figure 5B). Paired analysis comparing the mean % of IL-10+ cells in TDLNs compared with the mean % of IL-10+ cells in nTDLNs showed a highly significant observation that in the same patient, levels of IL-10 were exclusively higher in the TDLNs compared with nTDLNs ($P = .0020$; Figure 5C).

3.8 | B regulatory cells in TDLNs express high levels of IL-10

We next investigated the expression of IL-10 in three B cell subsets, which were previously described as B regulatory cells: $CD19^+CD20^+CD24^{high}CD38^{high}$, $CD19^+CD20^+CD27^+CD24^{high}$ and $CD19^+CD20^+CD27^+$. IL-10 expression in comparison to an isotype control in an exemplary TDLNs sample is shown in the Figure 5D. The percentage of IL-10 expressing cells and IL-10 MFI within $CD27^+CD24^{high}$ and $CD24^{high}CD38^{high}$ subpopulations were significantly increased compared with the overall $CD19^+CD20^+$ B cell population in TDLNs (25.20 ± 13.85% vs 45.52 ± 18.81%, $P = .0002$ and vs 47.99 ± 16.74%, $P < .0001$; MFI: 1477 ± 817.6 vs 2656 ± 1525, $P = .0065$ vs 2738 ± 1297, $P = .0010$, respectively; Figure 5E, F). Interestingly, $CD27^+$, $CD27^+CD24^{high}$ and $CD24^{high}CD38^{high}$ cells in TDLNs expressed significantly higher levels of IL-10 compared nTDLNs ($P = .0008$, $P = .0066$ and $P = .0166$, respectively; Figure 5G, I).

To extend these findings, we concatenated $IL\text{-}10^+$ cells from TDLNs and n-TDLNs and mapped cells on UMAP composite plots, which revealed a clear localization of most populations. FlowSOM analysis based on CD27, CD24 and CD38 revealed further eight unique clusters in the UMAP space. Figure 6A summarizes the distribution and localization of plotted populations. The analysis revealed

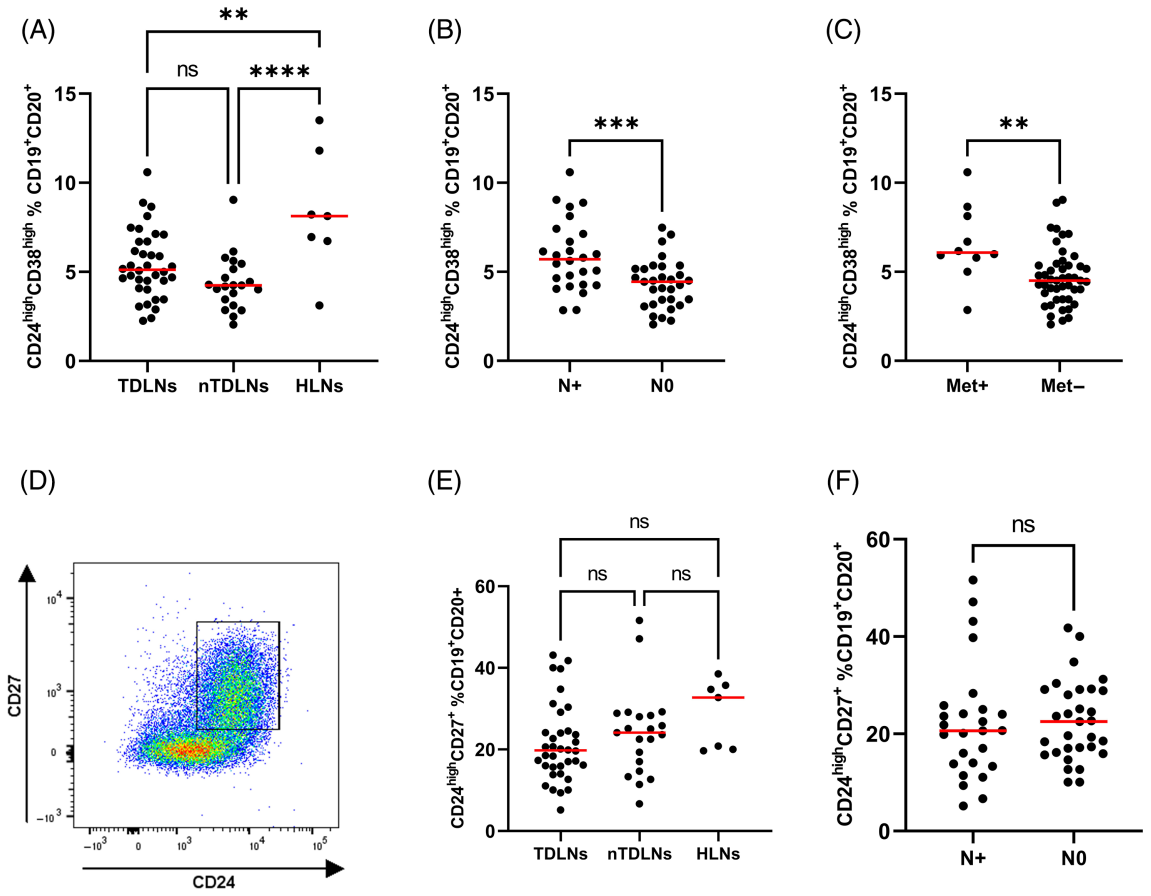


FIGURE 4 (A) Percentages of $CD24^{high}CD38^{high}$ B cells are compared between TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (B) Percentages of $CD24^{high}CD38^{high}$ B cells are compared between lymph nodes from patients with N+ stage (n = 26) and N0 stage (n = 31). (C) Percentages of $CD24^{high}CD38^{high}$ B cells are compared between lymph nodes containing metastases (n = 10) and metastasis-free nodes (n = 47). (D) The gating strategy for the analysis of $CD24^{high}CD27^{+}$ cells is delineated by an exemplary dot plot. (E) Percentages of $CD24^{high}CD27^{+}$ B cells are compared between TDLNs (n = 36), nTDLNs (n = 21) and HLNs (n = 7). (F) Percentages of $CD24^{high}CD27^{+}$ B cells are compared between lymph nodes from patients with N+ stage (n = 26) and N0 stage (n = 31).

that in contrary to nTDLNs, a high fraction of $IL\text{-}10^{+}$ cells in TDLNs is plotted within cluster 2 which contains $CD24^{high}CD38^{high}$ B cells. On the other hand, the majority of IL-10 producing cells in nTDLNs is plotted within cluster 8 which contains $CD27^{high}$ cells (Figure 6B, C).

4 | DISCUSSION

Tumor draining lymph nodes have recently been appreciated as key players in modulation of anti-tumor immunity and in regulation of the response to novel cancer immunotherapies. Since TDLNs have been somewhat ignored in favor of TME and blood, which were extensively investigated, there are still significant gaps in knowledge regarding the immunological architecture and role of human TDLNs. At the same time, the role of B cells in anti-tumor immunity is becoming increasingly apparent and more research is needed to fully understand their role in orchestrating anti-cancer immune responses. To our knowledge, this is the first study investigating B cells populations and B regulatory cells in human TDLNs by multicolor flow cytometry.

The role of B cells in anti-tumor immunity is still debated. B cells may play both anti- and protumorigenic roles in cancer progression. Relatively few studies described B cell populations in tumor microenvironment in human samples.[14,15] B cells in human TDLNs in HNSCC have not been yet investigated in the way we did. In accordance with previous animal studies,[16,17] our data demonstrate that TDLNs are characterized by a significant accumulation of B cells in comparison to nTDLNs. These B cells predominantly consist of naïve B cells suggesting a lymph node hypertrophy and recent expansion of these cells induced by vicinity of the tumor. Lymph node hypertrophy and lymphocyte expansion is also observed in infection and inflammation.[18,19] There, the hyperthrophy is a result of lymphocytes proliferation caused by exposure to foreign or self-antigens by antigen presenting cells (APCs). Similar mechanisms contribute most probably to B cell expansion in TDLNs as showed by Habenicht et al.[20] However, Cochran et al suggested that TDLNs hyperthropy may be associated

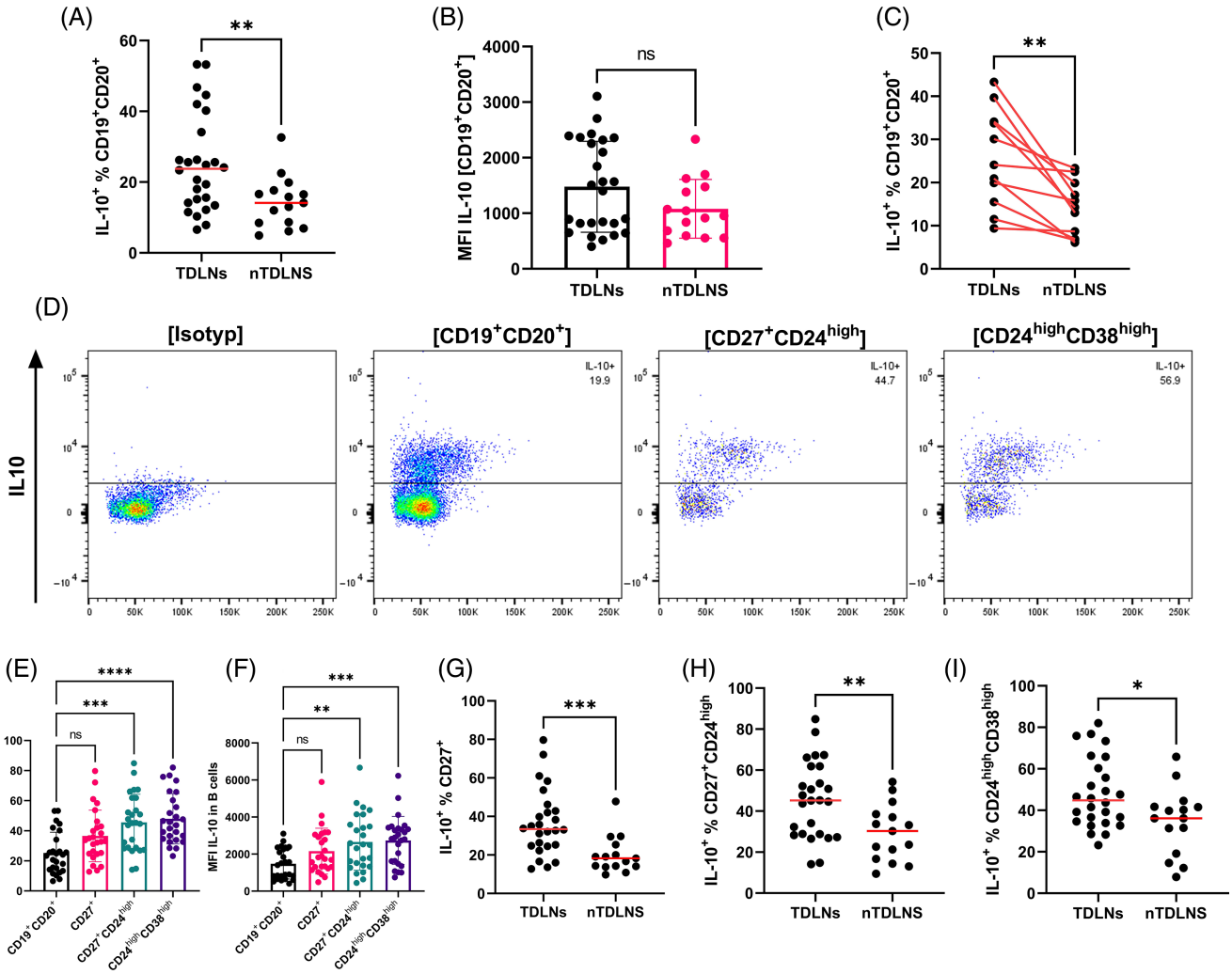
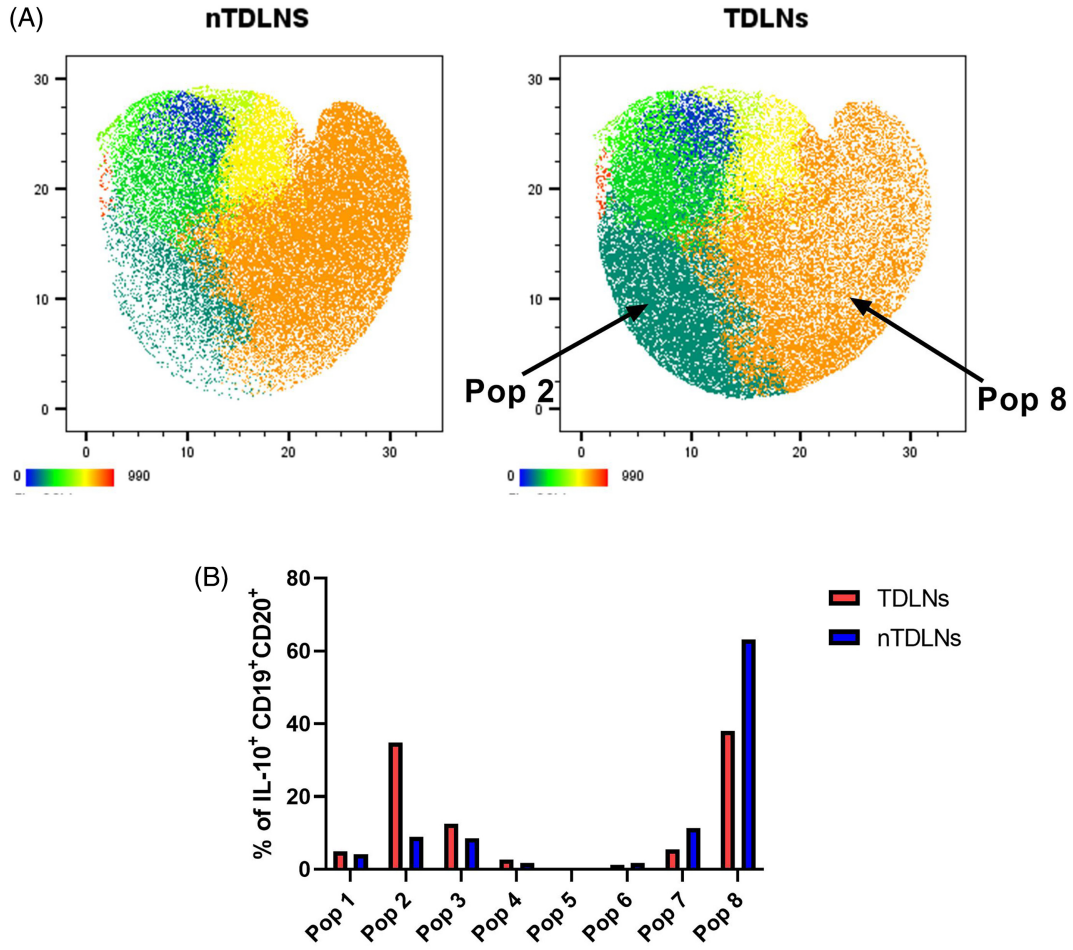


FIGURE 5 (A) Percentages of IL-10 producing $CD19^{+}CD20^{+}$ cells are compared between TDLNs (n = 26 and nTDLNS (n = 15). (B) Bar graphs compares the median fluorescence intensity (MFI) of IL-10 in B cells between TDLNs (n = 26 and nTDLNS (n = 15). (C) Paired scatter plot comparing the mean percentage of $IL\text{-}10^{+}$ B cells in TDLNs (n = 11) and nTDLNs (n = 11) in the same patient. (D) An exemplary flow cytometry data from one lymph node showing $IL\text{-}10^{+}$ cells in $CD19^{+}CD20^{+}$, $CD27^{+}CD24^{high}$, $CD24^{high}CD38^{high}$ in relation to isotype control. (E-F) Percentages of $IL\text{-}10^{+}$ cells within $CD19^{+}CD20^{+}$, $CD27^{+}$, $CD27^{+}CD24^{high}$, $CD24^{high}CD38^{high}$ populations (E) and respective IL-10 MFI within those subpopulations (F). (G) Percentages of IL-10 producing $CD27^{+}$ cells are compared between TDLNs (n = 26) and nTDLNS (n = 15). (H) Percentages of IL-10 producing $CD27^{+}CD24^{high}$ cells are compared between TDLNs (n = 26) and nTDLNS (n = 15). (I) Percentages of IL-10 producing $CD24^{high}CD38^{high}$ cells are compared between TDLNs (n = 26) and nTDLNS (n = 15).

with the suppression of immune system in contrary to inflammation where LN's hyperthrophy is favorable and helps to clear the cause of infection.[21]

Proliferation and expansion of B cell compartment is associated with production of a wide range of tumor-specific antibodies what usually is considered to be a reflection of B cells role in the anti-tumorgenic immune response.[7] These antibodies opsonize tumor cells and initiate an immunological cascade leading to activation of complement, activation of NK cell cytotoxicity and improvement of antigen cross-presentation of tumor antigens. Increased production of tumor specific antibodies is traditionally expressed by high levels of plasmablasts and plasma cells. High levels of plasmablasts and plasma cells were observed within TME of patients with hepatocellular carcinoma[22] and gastro-esophageal adenocarcinoma.[15] Our results, demonstrate significantly elevated levels of plasmablasts in TDLNs compared with lymph nodes from non-cancer patients. Furthermore, tendency towards higher levels of plasmablasts was observed even in nTDLNs when compared with HLNs (did not reach the statistical significance). This finding may imply that the presence of the tumor induces systemic maturation of B cells into antibody-producing cells. Nevertheless, even though production of tumor specific antibodies is believed to reflect anti-tumourgenic function of B cells, some reported that anti-tumor antibodies can facilitate tumor progression by inducing angiogenesis and pro-tumorigenic tissue remodeling.[23,24] Thus, further research is needed to elucidate the role and function of TDLNs derived plasmablasts.



(C)

Population	Markers
1	CD24int CD38high CD27high
2	CD24int CD38high CD27int
3	CD24high CD38int CD27int
4	CD24high CD38high CD27int
5	CD24low CD38high CD27int
6	CD24low CD38int CD27high
7	CD24low CD38low CD27int
8	CD24low CD38int CD27low

FIGURE 6 (A) UMAP plots generated after data concatenation with hierarchical clustering of expression intensity (CD27, CD24 and CD38) derived using FlowSOM. Comparison of FlowSOM-derived cluster pattern of IL-10$^+$ B cells in nTDLNs (left picture) and TDLNs (right picture). Population 2 and 8 are marked with an arrow. (B) A comparison of FlowSOM-derived cluster pattern of B cells in TDLNs (red) and nTDLNs (blue) showed in a bar graph. (C) Table summarizing expression of CD24, CD38 and CD27 in different Flow-SOM derived clusters.

Pro-tumorgenic phenotype of B cells may be expressed in different ways. B cell were shown to secret cytokines that support the progression of the tumor. Cytokines such as lymphotoxin (a factor mediating tumor angiogenesis) or IL-8 (chemoattractant for neutrophils) were shown to be produced by tumor infiltrating B cells.[9,10] The B cells which are specialized in secreting immunosuppressive cytokines are called B regulatory cells (Bregs). Bregs currently lack a clear definition and characteristic markers as known for T regulatory cells. The key factor defining B cell as regulatory is the IL-10 production, less frequently TGF-β.[11] To date, several B cells phenotypes in humans were shown to be increased in IL-10 producing cells. Iwata et al[25] characterized CD19 + CD24hiCD27+ cells as regulatory. In turn, Blair et al,[26] Das et al[27] and Flores-Borja et al[28] proved that CD19 + CD24hiCD38hi cells suppressed T cell responses and exhibited immunosuppressive phenotype. In animal models, B regulatory cells have been proved to have a crucial role in the tumor progression. Bregs were seen to induce conversion of effector T cells into regulatory T cells, what facilitated in turn seeding of distant metastases in

mice breast cancer model.[29] Up to date there are only two animal studies, showing an increased accumulation of Bregs in TDLNs in contrary to nTDLNS.[29,30] Thus, our study is the first one to show by flow cytometric analysis a significant higher accumulation of IL-10 producing B cells derived from TDLNs compared with nTDLNs. Furthermore, $CD19^{+}CD24^{hi}CD38^{hi}$ cells were significantly increased in lymph nodes of patients with more advance stages (N+) and significantly higher level of these cells were seen in lymph nodes containing metastatic cells. $CD19^{+}CD24^{hi}CD27^{+}$ cells did not show similar trend. Both, $CD19^{+}CD24^{hi}CD38^{hi}$ and $CD19^{+}CD24^{hi}CD27^{+}$ populations were characterized in our experiment by significantly higher presence of IL-10 producing cells, what confirms previous results defining these populations as rich in B regulatory cells. Our data suggest that B cells in TDLNs, especially in patients with nodal involvement may support tumor progression by production of immunosuppressive cytokines, which in turn may impair anti-tumor T cell responses.

Interestingly, we found that transitional B cells defined as $CD19^{+}CD20^{+}CD24^{high}CD38^{high}$ cells constitute 8.35% of all B cells in healthy lymph nodes. Transitional B cells account for approximately 4% of all B cells in PBMC of healthy individuals.[31] It is known that in contrary to mice, transitional B cells are present in human lymph nodes,[32] however there are no data available regarding their frequencies, function and phenotypes in HLN. Our understanding of transitional B cells in healthy individuals is still incomplete and need to be further explained to fully understand their role in autoimmune diseases and cancer.

Intratumoral infiltration with B cells is generally considered as a predictor of favorable outcome in variety of solid cancers.[6,33] In HNSCC, influence of B cells on prognosis is still debated. Distel et al proved that tumor-infiltrating B cells are predictor of favorable outcome in early stage HNSCC, in contrast to advanced stages where presence of B cells is associated with poorer prognosis.[34] This clinical observation is in line with previous animal models which showed that B cell depletion prior to tumor inoculation in mice facilitate tumor progression and leads to rapid tumor growth, whereas depletion of B cells in mice with established, advanced tumors leads to improved outcome and improved immunological control of the tumor.[35] All together these findings suggest that B cells may be characterized by a plasticity dependent on the advancement of the disease. In the first phase of disease, B cells may play an anit-tumorgenic role and undergo a shift towards a pro-tumorgenic phenotype with the course of the disease. Our findings, showing increased levels of B regulatory cells in patients with nodal metastases in comparison to patients without nodal involvement further support this hypothesis.

As mentioned in the introduction, B cells express PD-1, PD-L1 and CTLA-4 molecules on their surface and the use of ICIs was shown to change their activity and enhance maturation.[3-5] As presence or absence of PD-L1 on tumor cells turned out to be an unreliable factor predicting response to ICI treatment,[36] there is a need to further identify more reliable and feasible biomarkers. As it is now hypothesized that the ICIs efficacy relies predominantly on the influx of newly primed T cells from TDLNs into tumor microenvironment, rather than on reinvigorating exhausted and fully differentiated T cells infiltrating tumors (TILs),[37] TDLNs have recently attracted increasing attention. However, there are still significant gaps in knowledge regarding cellular architecture of TDLNs in humans and the tumor—TDLNs cross-talk leading eventually to establishing a pre-metastatic niche within a lymph node. Our study provides novel data regarding immunological function of TDLNs and identifies B regulatory cells as potential treatment target which could enhance anti-tumor T cell response in TDLNs.

Our study is not without limitations. The major limitations include the small size of analyzed cohort and lack of similar research on human TDLNs. Furthermore, we provide a characterization of B cells subsets within TDLNs, without looking into their function and interaction with other cells present in the lymph node. This will be made in the coming projects planned by our group.

5 | CONCLUSIONS

Taken together our data indicate that B cells in human TDLNs differ from B cells in nTDLNs and exhibit more naïve and immunosuppressive phenotypes. We identified a high accumulation of regulatory B cells within TDLNs which may be a potential obstacle in achieving response to novel cancer immunotherapies in HNSCC. Elevated levels of regulatory B cells in TDLNs are associated with advancement of the disease. A detailed knowledge of pre-existing antitumor immune response in human TDLNs is needed in order to fully understand the mode of action of ICI agents and potentially to solve tumor and host dependent mechanisms leading to resistance towards ICIs.

AUTHOR CONTRIBUTIONS

Each author has made substantial contributions to the conception and design of the work. Krzysztof Piersiala, Vilma Lagebro and Susanna Kumlien Georén were responsible for the acquisition, analysis and interpretation of data. Eric Hjalmarsson and Pedro Farrajota Neves da Silva were responsible for conceptualization, methodology and data curation. Aeneas Kolev, Magnus Starkhammar, Alexandra Elliot, Linda Marklund and Gregori Margolin were responsible for conceptualization, project administration, enrollment of patients, investigation and writing of the manuscript. Susanna Kumlien Georén, Eva Munck-Wikland, Lars-Olaf Cardell were responsible for supervision. The work reported in the paper has been performed by the authors, unless clearly specified in the text.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article. Further information is available from the corresponding author upon request.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Regional Ethics Committee Approvals: 2015/1650-31/2 and 2019-03518.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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