ELL QUANTITY IN NODAL T-FOLLICULAR HELPER CELL LYMPHOMAS AND PERIPHERAL T-CELL LYMPHOMAS, NOT OTHERWISE SPECIFIED AND ITS CORRELATION WITH OVERALL SURVIVAL

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Abstract

Background: The tumor microenvironment (TME) plays an important role in lymphomas,varying in composition and the quantity of specific tumor-infiltrating immune cells (TICs) like FoxP3$^+$ regulatory T-cells (Tregs). The role of FoxP3$^+$ Tregs in T-cell lymphomas (TCL) is complex, and the correlation of FoxP3$^+$ Tregs' quantity and overall survival (OS) remains unclear. Therefore, we aimed to evaluate and compare the quantity of FoxP3$^+$ cells in nodal TCLs and reactive lymph nodes (LN), with a focus on investigating their correlation with OS.

Patients and Methods: Excisional LN biopsies (whole-tissue samples) from 105 lymphoma patients diagnosed with nodal T-follicular helper cell lymphoma (nTFHL), peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS) and composite lymphoma (CL; co-occurrence of nTFHL and diffuse large B-cell lymphoma (DLBCL) or marginal zone lymphoma (MZL)), along with 17 reactive LN biopsies were immunohistochemically stained for FoxP3. Visual scoring was performed by counting FoxP3$^+$ cells under 400x magnification. Different cut-off values (125, 200, 255, and 400 FoxP3$^+$ cells/mm^2) from previous publications and our model were used to categorize patients into high or low quantity groups, and their correlation with OS was calculated. Clinical data were collected from patients' records, and the analyses were conducted retrospectively.

Results: All cases contained FoxP3$^+$ cells in the TME, with a median of 255 (range 4–2037) FoxP3$^+$ cells/mm^2 except for 4 PTCL-NOS cases where FoxP3 was expressed in malignant cells. The quantity of FoxP3$^+$ cells did not significantly differ across the analysed subtypes, except between nTFHL, not otherwise specified (nTFHL-NOS) and CL subtypes. However, their quantity was significantly decreased in nTFHL, follicular type (nTFHL-F) (median of 173 FoxP3$^+$ cells/mm^2), nTFHL, angioimmunoblastic type (nTFHL-AI) pattern 3 (median of 200 FoxP3$^+$ cells/mm^2) and CL (median of 168.5 FoxP3$^+$ cells/mm^2), compared to reactive LNs (median of 431 FoxP3$^+$ cells/mm^2). Univariate analysis revealed a statistically significant difference in OS between groups with high and low FoxP3$^+$ cell quantity with three applied cut-off values. However, the Cox proportional hazards model demonstrated that the FoxP3$^+$ cell quantity is not statistically significantly associated ($p=0.35$) with the risk of death.

Conclusions: Our findings revealed that nodal TCL patients with high FoxP3$^+$ cell quantity had improved OS. However, we did not confirm the Treg value as an independent prognostic marker. Multicentre studies with larger patient cohorts are needed to fully evaluate their prognostic value in these lymphoma subtypes. These results underscore the potential of FoxP3$^+$ cell quantity as an added value in prognosis and highlight the potential use of Treg-stimulating therapies in TCLs.

Tumor microenvironment (TME) plays an important role in the development and progression of cancer, particularly in lymphoma (1). Tumor-infiltrating immune cells (TICs) are a promising resource for the development of new prognostic markers as they are easily detectable. One of these

are regulatory T-cells (Tregs) that express the transcription factor FoxP3 (forkhead box P3) (1, 2). They play an important role in regulating immune responses to self- and nonself-antigens via local immune suppression and may impair the antitumor response by reducing the activity and functions of T-cells and natural killer (NK) cells. Their prognostic value has attracted a substantial interest in the recent decades (2-7). In most solid malignancies there are contradictory data about association of high quantity of $FoxP3^+$ Tregs with decreased or increased survival, depending of the carcinoma type as well as in the different types of lymphoma (2-5). Nevertheless, in specific lymphoma subtypes, increased $FoxP3^+$ Tregs have been associated with conflicting survival outcomes (2-4). The presence of $FoxP3^+$ Tregs within the TME of nodal T cell lymphomas (nTCL) remains unexplored.

The aim of our study was to evaluate and compare the quantity and the prognostic value of $FoxP3^+$ cells in TME among 101 patients diagnosed with nodal T cell follicular helper lymphoma angioimmunoblastic type (nTFHL-AI (patterns 1,2,3)), nodal T cell follicular helper lymphoma, follicular type (nTFHL-F), nodal T cell follicular helper lymphoma not otherwise specified (nTFHL-NOS), peripheral T cell lymphoma not otherwise specified (PTCL-NOS) and composite lymphomas (CL) which were defined as nTFHL-AI with diffuse large B cell lymphoma (DLBCL) or marginal zone lymphoma (MZL), as well as those with reactive LN.

Study was performed on routinely collected histological samples. A total of 101 LN biopsies (whole-tissue samples) obtained from patients diagnosed with nTFHL-AI (n=72), nTFHL-F (n=3), nTFHL-NOS (n=13), PTCL-NOS (n=5), CL, i.e., co-occurrence of nTFHL-AI and DLBCL or MZL (n=8) and 17 reactive LN biopsies were retrieved from the archives of the Institute of Oncology Ljubljana (IOL). All patients were diagnosed between 2007-2022 and underwent treatment at the IOL in accordance with the valid clinical guidelines applicable at the time of diagnosis. Clinical data were obtained at the time of diagnosis or during patients' follow-up and were retrieved retrospectively from their medical records. Automated IHC slide staining for FoxP3 (clone EP340, Epitomics) was performed on 3-4 μm thick whole sections on BenchMark Ultra (Roche Diagnostics, Tucson, Arizon) using the OptiView DAB IHC Detection Kit (Roche, Catal. No. 760-700). Two experienced pathologists (BGK, GG) independently evaluated the IHC staining. They performed visual scoring under 400x magnification, blinded to all clinical data, by counting $FoxP3^+$ cells in the hot-spot area with a higher quantity of $FoxP3^+$ cells. Cells with moderate or strong staining were included in the count, and the result was reported as the number of $FoxP3^+$ cells per square millimetre ($FoxP3^+$ cells/ mm^2).

To determine if there were statistical differences in FoxP3+ cell quantity between the included nodal TCL subtypes and reactive LN, multiple comparisons were performed using Kruskal-Wallis nonparametric test, and pairwise comparisons were performed using Mann-Whitney U test. OS was defined as the time interval from the date of pathological diagnosis until the date of death from any cause. The vital status of the patients was retrieved from the Cancer Registry of the Republic of Slovenia on April 15, 2024. The median survival of the patients was expressed in months. Kaplan-Meier method with log-rank test was used to generate survival curves and to evaluate OS for patient groups with high or low $FoxP3^+$ cell quantity. We used two different cut-off values from previous studies (2,3) (125 and 200 $FoxP3^+$ cells/ mm^2) that investigated the impact of $FoxP3^+$ cells in different TCLs on survival and a median count of our cohort ($FoxP3^+$ cells/ mm^2) to categorize patients into high or low $FoxP3^+$ cell quantity groups. We also attempted to determine an appropriate cut-off value with testing the non-linear effect of data. Confirmation of the non-linear effect would establish that the value at the break of the graph is indeed the appropriate cut-off value. A potentially appropriate value that was suggested from the model (although non-linear effect was insignificant for our data) was also tested. For a variable of interest (Treg value), we built a Cox proportional hazards model using the diagnosis, IPI score (as an ordered variable) and the chosen variable (Treg value as a continuous variable) as independent variables, with OS as the dependent variable. We tested a non-linear association between the Treg value and OS using a 1 restricted cubic spline with 3 nodes. After showing that the non-linear effect was not statistically significant, we updated model to only

include the Treg value as a linear predictor to have a simpler model (the result of the model with the linear predictor remained similar). The proportional hazards assumption was tested using Schoenfeld residuals. Statistically significant result was defined as $p \leq 0.05$. The statistical analyses were conducted using IBM SPSS (v28.0.1.0), GraphPad Prism 9 and R (v4.2.2) with RStudio (v2023.06.0+241) using the packages survival (v3.5-7), rms (v6.6-0), ggplot2 (v3.4.2), blandr (v0.5.1), knitr (v1.42), kableExtra (v1.3.4), and their dependencies.

The analysed patient cohort comprised fifty-six (55%) males and forty-five (45%) females; median age at diagnosis was 69 (range 26–87) years. Most patients (n=93; 92%) presented at advanced stages (Ann Arbor stage III-IV) at diagnosis. Two thirds presented with B-symptoms (n=69; 68%) and a bit more had good Eastern Cooperative Oncology Group (ECOG) performance status (0–1) (n=70; 70%) at diagnosis. Thirteen percent of patients were in the International Prognostic Index (IPI) low-risk group, 22% in low-intermediate, 39% in high-intermediate, and 25% in high-risk. Elevated serum lactate dehydrogenase (LDH) was detected in 53 patients (53%).

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Approximately half of the patients (n=58; 57%) received first-line treatment with chemotherapy, including cyclophosphamide, vincristine, prednisone (COP) or modified COP, or other low-dose treatments. Nineteen (19%) patients received chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) or CHOP-like treatment as first-line treatment. Eighteen patients (18%) received other treatments (radiotherapy, corticosteroids etc.) and the remaining 6 patients did not receive any treatment at all (n=6; 6%). Autologous stem cell transplantation was only undergone by 8 patients as consolidation treatment after the initial therapy.

FoxP3$^+$ cells were detected in the TME of all analysed samples (n=101) in varying numbers, with a median of 255 (range 4–2037) FoxP3$^+$ cells/mm^2. The staining consistently exhibited nuclear localization with high or moderate intensity.

The overall FoxP3$^+$ cell quantity was significantly lower (p=0.01) in the nTCL cohort (median 255, range 4–2037 FoxP3$^+$ cells/mm^2) compared to the reactive LN cohort (median 431, range 199–791 FoxP3$^+$ cells/mm^2). The FoxP3$^+$ cell quantity did not vary across different nTCL subtypes, except between nTFHL-NOS and CL (Kruskal-Wallis Test: p=0.02; Mann-Whitney U Test: p=<0.05). However, FoxP3$^+$ cell quantity showed a significant decrease in nTFHL-F (median 173, range 79–177), nTFHL-AI (median 279, range 4–2037), and CL (median 168.5, range 117–403), compared to reactive LN (median 431, range 199–791 FoxP3$^+$ cells/mm^2). When we subclassified nTFHL-AI into three patterns (pattern 1, 2 and 3), we observed differences compared to reactive LN. The FoxP3$^+$ cell quantity was statistically significantly reduced only in pattern 3 compared to reactive LN (p=<0.01), while pattern 2 nearly reached statistical significance (p=0.06). Conversely, nTFHL-AI pattern 1 had a median FoxP3$^+$ cell quantity of 509 (range 23–1337) FoxP3$^+$ cells/mm^2, which did not differ from reactive LN but was significantly higher (p=<0.05) compared to CL. When comparing patterns, there was an almost statistically significant difference between pattern 1 and 3 (p=0.05).

At the end of follow-up period 76 out of 101 patients died (74.3%), 64 due to lymphoma. The mean OS of analysed nTCL cohort was 37.4 months, the median 24.9 months (range 0.3 – 156.4 months). The 1-year, 2-year and 5-year OS rates were 67.3%, 50.5% and 23.8%. When comparing patients with low and high FoxP3$^+$ cell quantity, determined using different cut-offs (125, 200, 255, or 400 FoxP3$^+$ cells/mm^2), we observed a significant difference in OS between the two groups of patients with the first three cut-offs (log-rank test; p<0.001, p=0.001, p=0.004, and p=0.066). Cox proportional hazards model showed no association between Treg value and risk for death (p=0.35). A decreasing risk is indicated as the Treg value falls towards 400, and then a constant risk at Treg values from 400 onwards. The graph suggests that a value of 400 could be an appropriate cut-off value when confirming the non-linear effect of the data, a confirmation we did not obtain (p=0.44). In the multivariate Cox proportional hazards model, only IPI was confirmed as an independent predictor for the risk of death (p<0.001). The other included variables (diagnosis and Treg value) were not statistically significantly

associated with the risk of death (p=0.92 and p=0.35).

In this study, we assessed FoxP3$^+$ cell quantity in TME across 101 nTCL and in 17 reactive LN and analysed the impact of FoxP3$^+$ cell quantity on OS. A statistically lower FoxP3$^+$ cell quantity in the nTCL cohort compared to the reactive LN cohort was observed. Importantly, we are the first to demonstrate a statistically significant difference in overall survival (OS) among nTCLs based on FoxP3$^+$ cell quantity using various cut-off values obtained from previous publications (3,7). However, the Cox proportional hazards model did not indicate a significant correlation between FoxP3$^+$ cells (Treg value) and the risk of death.

Despite the potential of TICs as prognostic and predictive markers, there is a challenge in setting their cut-off values. The cut-off value serves as the basis for dividing patients into two groups (high *vs.* low quantity) and for evaluating their impact on OS. Differently chosen cut-off values can impact the final outcomes of different research groups and their subsequent comparison. Currently, cut-off values for the number of FoxP3$^+$ cells are not standardized across published articles that study FoxP3$^+$ cells and their impact on OS (2-7), and results are reported using different units, including percentages (%), cells per high-power field (cells/HPF), and cells per square millimetre (cells/mm^2). To enable meaningful cross-study comparisons, it's vital for the scientific community to establish standardized cut-off values and units for TICs.

When comparing patients with low and high FoxP3$^+$ cell quantity, determined using different cut-offs (125, 200, 255 or 400 FoxP3$^+$ cells/mm^2), we observed a significant difference in OS between the two groups in three instances, except when using the cut-off value of 400 FoxP3$^+$ cell/mm^2.

This study revealed a decrease in FoxP3$^+$ cell quantity in nTFHL-F, advance phase of nTFHL-AI (pattern 3) and CL subtypes compared to reactive LN. This marks the first demonstration of such a difference between nTFHL-AI patterns. We illustrated the distinction in OS between groups with low and high FoxP3$^+$ quantity by applying various cut-off values. However, multivariate analysis did not confirm the correlation of Treg value with the risk of death. For future validation of our study's findings, prospective studies with larger sample size, consistent therapy protocols, uniform detection antibodies, and standardized criteria for determining cut-off values are essential. This rigorous approach is crucial to confirm the prognostic value of FoxP3$^+$ cells in the TME of nodal TCLs.

In conclusion, our findings suggest potential refinements in prognostic accuracy and indicate that Treg-supressing drugs, such as cyclosporine, should not be used in nTFHL patients. Instead, the possibility of treating nTFHL with combined therapies, including immunomodulatory drugs that enhance Treg frequency and function, offers a promising strategy. Future efforts should focus on these combined approaches to effectively modulate the TME and improve patient outcomes.

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