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CD4+/CD57+/CD69+ T lymphocytes and CD14+ dendritic cells accumulate in advanced follicular lymphoma

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ABSTRACT

Follicular lymphoma is the second most frequent non-Hodgkin's lymphoma, accounting for around 20 % of all lymphomas in Western countries. Initially, it behaves indolently, but in time becomes more aggressive and less susceptible to chemotherapy. Multiple features correlate with the survival of the patients and the progression of the disease, such as therapy with rituximab, tumour microenvironment and the intrafollicular proliferation index. Our research was focused on the association of specific components of tumour microenvironment and the tumour behaviour. The presence and the relative percentage of T lymphocytes, follicular dendritic cells, dendritic cells and macrophages was detected by immunohistochemical staining of the antigens specific for certain cell populations. Our results show that T lymphocytes and dendritic cells affect tumour growth, possibly through interactions with tumour cells. Higher patients' ECOG score and the outcome of the disease are associated with the presence of CD14+ dendritic cells in tumour tissue, while the worse overall survival of patients is associated with the increased number of activated helper T lymphocytes that express marker of exhaustion CD57. Taken together, our results suggest that the efficiency of the immune response against follicular lymphoma depends on more than one type of immune cells. Also, we found that the phenotype of these cells, rather than just their number, affects the tumour behaviour and in consequence survival of the patients.

1. Introduction

Follicular lymphoma (FL) is the second most frequent non-Hodgkin's lymphoma (NHL) after diffuse large B-cell lymphoma (DLBCL), accounting for around 20 % of all lymphomas in Western countries. It is composed of germinal centre B lymphocytes packed in follicles that disrupt the normal architecture of lymph nodes. The follicles are usually poorly defined and have reduced or completely absent mantle zones. Neoplastic cells are divided into two groups: small to medium sized centrocytes with angulated, elongated, twisted or cleaved nuclei and scant pale cytoplasm, and large centroblasts with round or oval nuclei and a rim of cytoplasm. Depending on the number of centroblasts, FL is classified into grades 1–3b (Lugano classification) (https://www.ncbi.nlm.nih.gov/books/NBK66057/table/CDR0000062707_1075/). According to World Health Organization (WHO) data, this type of lymphoma appears more often in women, with male-to-female ratio of 1: 1.7 (Swerdlow et al., 2017), although it might depend on the world region as some authors observed male-to-female ratio of 1.2: 1 (Cerhan, 2020). Initially, FL behaves indolently, but in time becomes more aggressive and less susceptible to chemotherapy. As it progresses, it can transform into other types of B-cell lymphomas, most commonly into DLBCL. Although the immunophenotype varies in different grades of tumour, FL cells generally express BCL2 (usually as a consequence of t(14:18) translocation), BCL6 (usually as a consequence of t(3:14) translocation) and HGAL (Swerdlow et al., 2017).

Follicular Lymphoma International Prognostic Index (FLIPI) was

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defined by Solal-Céligny in 2004 in order to separate prognostic index (PI) for mostly indolent FL from PI proposed for aggressive NHL. It is based on five adverse prognostic factors: age (>60 years vs < 60 years), Ann Arbor (AA) stage (III-IV vs I-II), haemoglobin level (<120 g/L vs > 120 g/L), number of nodal areas (>4 vs < 4) and serum lactate dehydrogenase (LDH) level (above normal vs normal or below). According to FLIPI score, patients are divided into three groups: low risk (0–1 adverse factor), intermediate risk (2 adverse factors), and poor risk (>3 adverse factors) (Solal-Céligny et al., 2004). Another scale by which patients' state can be measured is Eastern Clinical Oncology Group (ECOG) performance status (PS), which consists of five grades (1–5) according to the ability of patients to care for themselves and function in day-to-day life. Depending on patients' ECOG status, researchers can observe the effects of various therapies and evaluate their efficiency and toxicity (https://ecog-acrin.org/resources/ecog-performance-status).

Aside from aforementioned factors, other features also correlate with the survival of patients and progression of the disease, such as therapy with rituximab (Buske et al., 2006), tumour microenvironment (TME) (Dave et al., 2004), and proliferation index, determined by the percentage of tumour cells staining for Ki67 protein. It was observed that the proliferation index, rather than the grade of tumour, acts as a better prognostic factor in FL (Wang et al., 2005; Koster et al., 2007). Many researches have described the influence of TME on the development of FL (Dave et al., 2004; Byers et al., 2005; Harjunpää et al., 2006; de Jong et al., 2009; Glas et al., 2005; Glas et al., 2007; Alvaro et al., 2006; Mondello et al., 2021; Tobin et al., 2019). There is plenty of data regarding the possible prognostic role of specific components of the immune system infiltrating the tumour, such as follicular dendritic cells (FDCs), dendritic cells (DCs), macrophages, natural killer cells (NK cells) and various types of T lymphocytes, but there is no definite answer yet, especially in the context of effects of different types of therapies on TME (Dave et al., 2004; Glas et al., 2005; van Oers et al., 2006). Even less is known about the mechanisms behind those prognostic factors and how dysregulation of cellular pathways within cells affects the communication between tumour and non-tumour cells. However, changes in the genes that regulate chromatin modifications (and therefore have a role in the regulation of gene expression) have been observed in FL (reviewed in Green, 2018), indicating a role of epigenetic alterations in the progression of the disease.

This research aims to explore the correlations between the presence of various components of the immune system in tumour mass and survival of the patients diagnosed with FL and propose possible cell interactions leading to the disease development.

2. Materials and methods

2.1. Patients

A total of 64 cases of FL from patients consecutively diagnosed in University Hospital Merkur between 2004 and 2009 were analysed. Only FL samples belonging to grade 1, 2 or 3a were used for this study. The tissue samples were reviewed by three experienced hematopathologists (MD, SG, SD) who confirmed diagnoses according to the criteria of the WHO classification [1].

All samples from previously untreated patients were collected under local ethics committee approval from the Medical School Zagreb.

2.2. Immunohistochemical staining

Formalin fixed, paraffin embedded tissue sections were prepared for immunohistochemical staining. Standard protocols with antibodies most commonly used for selected markers was used.

In brief, immunohistochemical staining was done using 2-μm-thick tissue sections and was performed after heat induced epitope retrieval (HIER) using polymer-based detection systems ImmPRESS (Vector Laboratories) and EnVision (Dako/Agilent) according to the manufacturer's instructions by automated immunostainer (Autostainer Link 48, Dako/Agilent). Monoclonal antibodies listed in Table 1 were used at 1:50 dilution (BCL2 (17/Cell Marque (Rocklin, CA, USA)), BCL6 (PG-B6/Dako/Agilent (Santa Clara, CA, USA)), HGAL (MRQ-49/Cell Marque (Rocklin, CA, USA)), CD57 (NK-1/Cell Marque (Rocklin, CA, USA)), H3K27 (mAbcam 6002/Abcam (Cambridge, UK))), 1:100 dilution (CD21 (1F8/Dako/Agilent (Santa Clara, CA, USA)), CXCL13 (goat polyclonal antibody/R&D Systems (Minneapolis, MN, USA)), CD14 (EPR3653/Cell Marque (Rocklin, CA, USA)), CD68 (PG-M1/Dako/Agilent (Santa Clara, CA, USA)), CD8 (C8/144B/Dako/Agilent (Santa Clara, CA, USA)), FOXP3 (236A/E7/Abcam (Cambridge, UK))) and 1:25 dilution (CD4 (4B12/Dako/Agilent (Santa Clara, CA, USA)) and CD69 (CH11/Novocastra/Leica Biosystems (Buffalo Grove, IL, USA))). Positive controls were samples of non-tumour tonsils. After the staining, samples were analysed using Olympus BX51 microscope at 400x total magnification and DPController/DPManager software was used. Immunohistochemical results were evaluated by 4 independent researchers. The percentage of all markers in tumour tissue was evaluated in comparison to the average percentage of those markers observed in germinative centre of control non-tumour tonsils (n = 20) regarded as a representative tissue of tumour origin. Such evaluation of TME components allowed the assessment of potential specific non-tumour cells accumulation, or lack of those cells, in tumour tissue. Although the percentage of each marker was separately determined as higher or lower than its average percentage in germinative centre of control tonsils, consecutive 2-μm-thick tissue sections were used for immunostaing, allowing comparison of the localization of different markers and therefore assuming subtypes/phenotypes of specific cells.

2.3. Fluorescent in situ hybridisation

Fluorescent *in situ* hybridisation (FISH) was done using 4-μm-thick tissue sections. Sections were deparaffinized, HIER was performed prior to pepsin treatment and finally fluorescent probes were applied. Break apart *BCL2* and *BCL6* FISH probes were used (Abbott Diagnostics, Maidenhead, Berkshire, UK) according to the manufacturer's instructions. Sections were denaturated at 85 °C for five minutes, followed by overnight hybridisation at 37 °C. Next day, slides were washed, mounted with medium containing DAPI and analysed using an Olympus BX51 microscope with different fluorescent filters. A total of 200 nuclei per sample were analysed using a cut-off of 7 %.

2.4. Statistical analysis

After the evaluation of stained and hybridized slides, Chi-square test and Spearman's rho correlation were used to determine the association between the variables. Log-rank tests was used for comparing the overall and event free survival distributions between groups. Statistical analysis

Table 1
Analyzed cell markers and antibodies used for their detection.

Marker	Type of cell	Cut-off value (%)*
BCL2	tumour cells	30
BCL6		30
HGAL		30
H3K27	tumour cells and tumour microenvironment	15
CD21	follicular dendritic cells	30
CXCL13		20
CD14	dendritic cells and macrophages	30
CD68	macrophages	20
CD4	helper T lymphocytes	20
FOXP3	regulatory T lymphocytes	50
CD8	cytotoxic T lymphocytes	5
CD57	mature T lymphocytes and natural killer cells	20
CD69	activated T lymphocytes	40

* Cut-off values were determined in relation to the average percentage of positive cells for specific marker in non-tumour tonsils.

was performed with the STATISTICA software, version 13.0 (StatSoft Inc. Tulsa, OK, USA). The level of significance was set at $p < 0.05$. Multiple testing correction was done using Benjamini and Hochberg (BH) correction and adjusted p-values were reported. BH correction was done in R statistical programming language version 4.1.0.

3. Results

General data about the patients are shown in Table 2. Men predominated over women among the patients (67,7% vs 32,3%). The age span among the patients was between 31 and 81 years of age, with the average age of 56 and median age of 54. Over 90 % of the patients were diagnosed with low grade FL (grades 1/2). In most cases (90,6%), >75 % of the tumour tissue displayed follicular pattern while none of the cases displayed completely diffuse pattern. FLIPI and ECOG scores were available for 52 and 51 patients respectively. Almost 60 % of the patients scored 3 on FLIPI scale, due to three or more adverse factors present. On the other hand, over 80 % of patients scored 0 or 1 on ECOG scale, suggesting the disease generally didn't affect the ability of the patients to care for themselves. Bone marrow infiltration was observed in two thirds of the patients for whom the data was available (42/64). Data on the therapies the patients received and the patients' response to therapy was available for 51 and 48 patients respectively. 68,6% of the patients received therapy with rituximab (R), as well as either cyclophosphamide-hydroxydaunorubicin hydrochloride-vincristine (Oncovin)-prednisone (CHOP) or cyclophosphamide-vincristine sulphate-prednisone (CVP). 32 patients achieved complete remission, while 8 achieved partial remission. The disease relapsed in 2 patients, while 8 patients showed no response to therapy.

3.1. Expression of tumour markers in FL

Tumour cells in FL stained for BCL2, BCL6 and HGAL, but the increased expression of BCL2 and BCL6 did not correlated with those genes' translocations.

All samples contained a high number of tumour cells staining for at least one of the three analysed tumour markers. Most samples contained tumour cells that stained for BCL2 (61/64, 95.3 %). 58 out of 62 samples stained for HGAL, while 58 out of 63 stained for BCL6 (93.5 and 92.1 % respectively) (Fig. 1).

Statistically significant difference was observed in BCL2 expression in relation to tumour grade: in all but one grade 1 (29/30) and all grade 2 samples (29/29) majority of the tumour cells expressed BCL2, while 40 % of grade 3a (2/5) samples contained a low number of BCL2+ cells ($p = 0.016$). Similar statistical significance was observed in HGAL expression in relation to tumour grade: 28 out of 29 grade 1 samples and 28 out of 29 grade 2 samples contained a high number of cells expressing HGAL, while 2 out of 4 grade 3a samples had a low number of HGAL+ cells ($p = 0.016$). The association between tumour grade and BCL6 expression was not found.

Our results showed no association between the presence of *BCL2* or *BCL6* translocations in tumour cells and the expression of their respective protein products. Generally, only one of these two translocations was present among the patients, while only one sample contained both translocations. None of 3a grade samples contained *BCL2* translocation, while it was present in 93 % of grade 1 samples (26/28) and 67 % of grade 2 samples (18/27) ($p < 0.001$). We didn't observe an association between these translocations and the survival of the patients, suggesting that these translocations might induce the development of FL, but they do not drive its progress.

Table 2
General data about the patients.

		N	%
Sex	Male	44	68,7
	Female	20	31,3
Age	≤60	38	59,4
	>60	26	40,6
Grade	1	30	46,9
	2	29	45,3
	3a	5	7,8
Score	>75 % follicular pattern	58	90,6
	25–75 % follicular pattern	5	7,8
	<25 % follicular pattern	1	1,6
FLIPI	1	11	21,2
	2	10	19,2
	3	31	59,6
ECOG	0	29	56,9
	1	14	27,5
	2	7	13,7
	3	1	1,9
Bone marrow infiltration	+	28	43,7
	–	14	21,9
	n.a.	22	34,4
Therapy	R-CHOP/R-CVP	35	68,6
	CHOP/CNOP	5	9,8
	other	11	21,6
Response to therapy	complete remission	32	66,7
	partial remission	8	16,6
	relapse	2	4,2
	no response	6	12,5

n.a. – not available.
FLIPI – Follicular Lymphoma International Prognostic Index; ECOG – Eastern Clinical Oncology Group; R-CHOP – rituximab-cyclophosphamide-hydroxydaunorubicin hydrochloride-vincristine (Oncovin)-prednisone; R-CVP – rituximab-cyclophosphamide-vincristine sulphate-prednisone; CNOP – cyclophosphamide-mitoxantrone-vincristine-prednisone.

3.2. Analysis of microenvironmental cells in FL tumours

We analysed the presence of FDCs, DCs, macrophages and different types of T cells in the FL patients (Fig. 2).

A high number of CD21+ FDCs was detected in 52 out of 63 samples (82.5 %), and only 4 out of 64 samples were rich in cells expressing CXCL13, a cytokine produced by FDCs. Although we didn't find a statistically significant association between the number of CD21+ and CXCL13+ cells, we noticed that all four samples that contained a high number of cells expressing CXCL13 were also rich in CD21+ FDCs.

18 out of 64 (28.1 %) samples were rich in CD14+ DCs. Out of the remaining 46 samples, 21 sample contained a high number of CD14+ macrophages, while the rest of the samples contained a low number of both cell types expressing CD14.

A different subpopulation of macrophages was detected by CD68 staining, and only 2 out of 61 samples were highly infiltrated with that macrophage subpopulation.

The last component of TME we analysed were T cells. 5 samples were rich in CD4+ cells while 21 samples contained a high number of CD8+ cells. 57 out of 64 samples contained a higher number of FOXP3+ cells compared to control tonsils. 9 out of 64 samples contained a high number of cells expressing CD57, 3 of which also contained a high number of CD4+ cells localized in the same areas on consecutive slides. 3 out of remaining 6 samples contained a high number of CD8+ cells localized in the same areas as CD57+ cells on consecutive slides. 12 out of 63 samples contained a high number of cells staining for the marker of activated T lymphocytes CD69.

Statistically significant association of tumour cells expressing BCL2 and CD68+ cell infiltration was observed: samples that contained a high number of BCL2+ tumour cells were infiltrated with a low number of CD68+ macrophages (59/59), while all the samples with a low number of BCL2+ tumour cells were highly infiltrated with CD68+ cells (2/2) ($p = 0.016$). BCL6 expression in tumour cells was statistically significant in relation to CXCL13 expression: samples that contained a high number of BCL6+ tumour cells were infiltrated with a low number of cells expressing CXCL13 (56/58), while 2 out of 5 samples with a low number of BCL6+ tumour cells were rich in cells expressing CXCL13 ($p = 0.016$).

Also, the number of cells expressing CXCL13 positively correlated

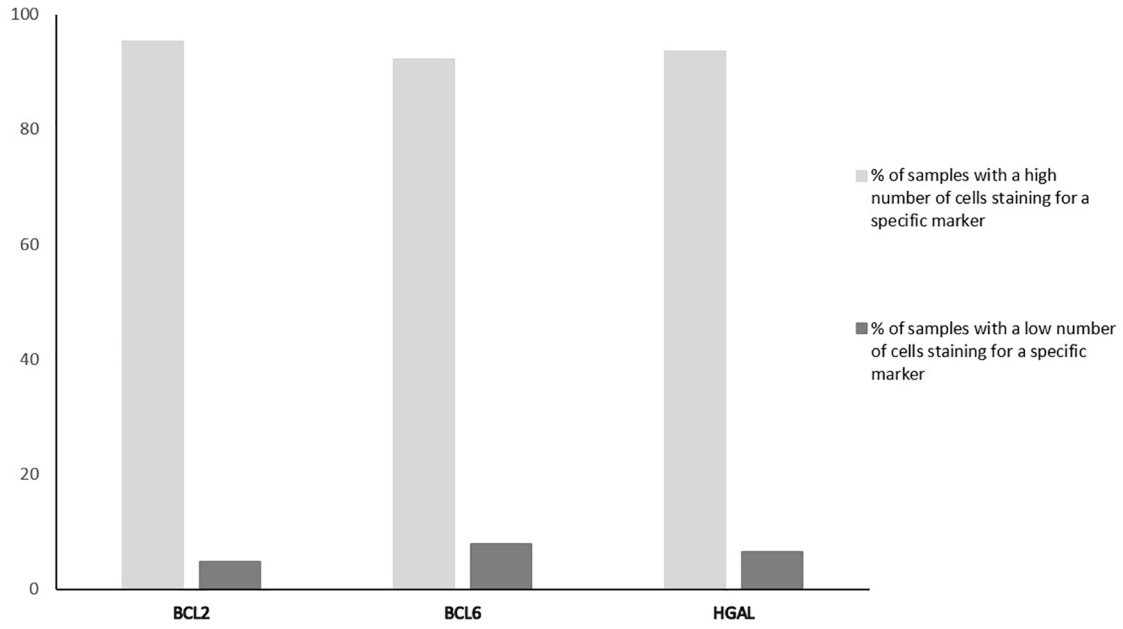

Fig. 1. The percentage of samples with a high number of tumour cells staining for a specific cell marker (light grey) and samples with a low number of tumour cells staining for a specific cell marker (dark grey).

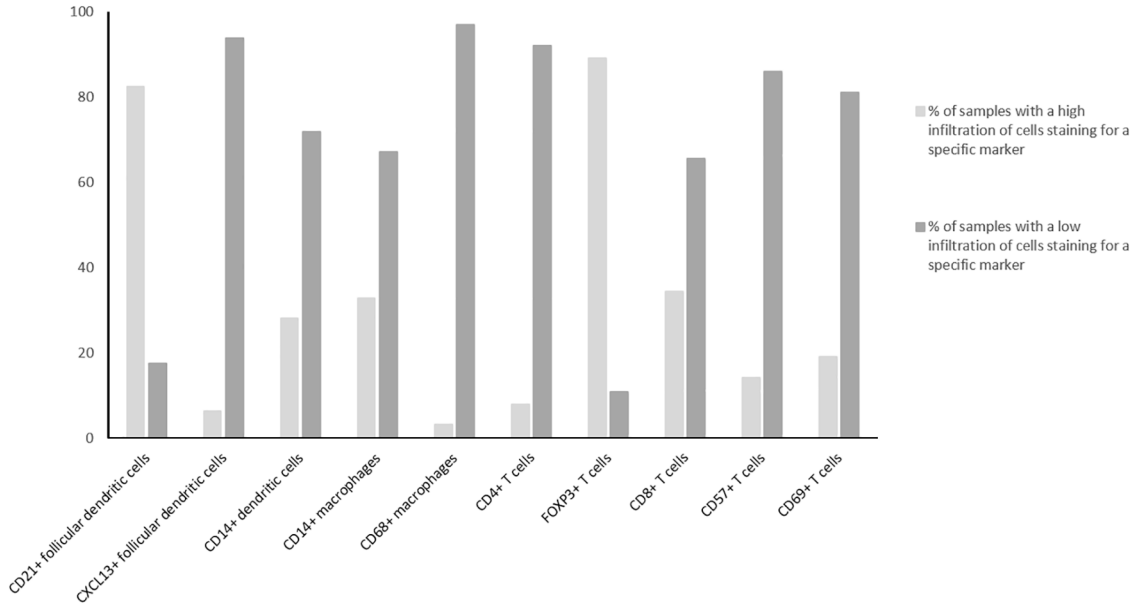

Fig. 2. The percentage of samples with a high (light grey) and low (dark grey) number of cells staining for a specific TME cell marker.

with the number of CD4+ T cells ($p = 0.009$). The higher number of CD4+ lymphocytes positively correlated with the higher expression of marker of exhaustion CD57 ($p = 0.009$) and the higher number of FOXP3+ cells ($p = 0.036$). The number of CD57+ cells also positively correlated with the number of CD68+ macrophages ($p = 0.009$), as well as with the number of cells expressing activation marker CD69 ($p = 0.009$).

3.3. Analysis of FL microenvironment in correlation to disease progression and patients' survival

For evaluation of disease progression, we used histological tumour grades, FLIPI score and ECOG stage. Patients were generally diagnosed with grade 1 (47 %) or grade 2 (45 %), while only 8 % were diagnosed with grade 3a (Table 2). 21 out of 52 (40.4 %) patients for whom the data was available scored 1 or 2 on FLIPI scale, while the remaining patients (59.6 %) scored 3 on FLIPI scale. 43 out of 51 patients scored 0 or 1 on ECOG scale, while the remaining 8 patients for whom the data was available scored 2 or 3 on ECOG scale (Table 2).

We didn't observe a statistically significant association between FLIPI score and the presence of any component of TME. Higher ECOG stage was associated with higher percentage of infiltrating CD14+ DCs ($p = 0.016$). This result suggests that the higher percentage of DCs in tumour tissue is associated with disease progression (Fig. 3).

Average overall survival (OS) of the patients was 54,4 months. For evaluation of patients' survival, we assessed overall and event free

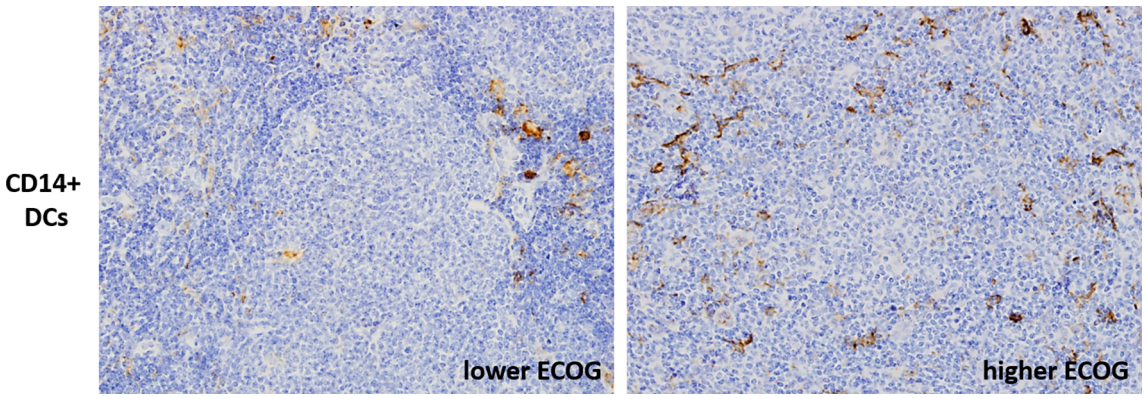

Fig. 3. The presence of a higher number of CD14+ DCs in tumour tissue is associated with higher ECOG stage. FL tumour tissues were immunostained with antibodies specific for CD14. Their presence was detected as brownish staining after the catalytic reaction of peroxidase conjugated on the secondary antibody with 3,3′-Diaminobenzidine (DAB).

periods in relation to specific components of the microenvironment and their simultaneous presence within the tumour tissue. Patients whose samples showed high percentage of CD4+, CD57+ and/or CD69+ cells in tumour tissue had significantly poorer overall survival ($p = 0.022$) (Fig. 4.a). Poorer overall survival was also observed in samples that were infiltrated with CD14+, CD4+ and CD57+ cells simultaneously ($p = 0.017$) (Fig. 4.b). These results suggest poorer survival of patients with higher number of DCs and exhausted helper T lymphocytes infiltrating tumour tissue.

In our study, tumour tissue infiltration with CD4+, CD57+ and CD69+ cells was observed in patients with significantly poorer overall survival (Fig. 5).

We didn't find any association between the score on FLIPI scale and the outcome of the disease, but the outcome of the disease was, as expected, significantly poorer in patients with higher score on ECOG scale ($p = 0.0246$).

4. Discussion

Our research was focused on the association between specific components of tumour microenvironment and the tumour behaviour. We analysed the presence and abundance of different types of T cells, dendritic cells and macrophages in tumour tissue and correlated assessed data with tumour grade and patients' survival. As different types of T cells, macrophages and dendritic cells deliver signals for angiogenesis, adhesion, migration, survival, but could also be cytotoxic, they could differently affect tumour development (Matas-Céspedes et al., 2014). We assessed the presence of each of these cell groups separately and correlated them with tumour characteristics.

Macrophages and DCs play a key role in bridging innate and adaptive immune response by presenting antigens to helper T lymphocytes (Steinman, 2007). The presence of CD14+ DCs in the neoplastic follicles was previously shown to be associated with a shorter period of time needed for FL transformation, indicating that these cells play a role in mediating development/transformation of FL and that their location

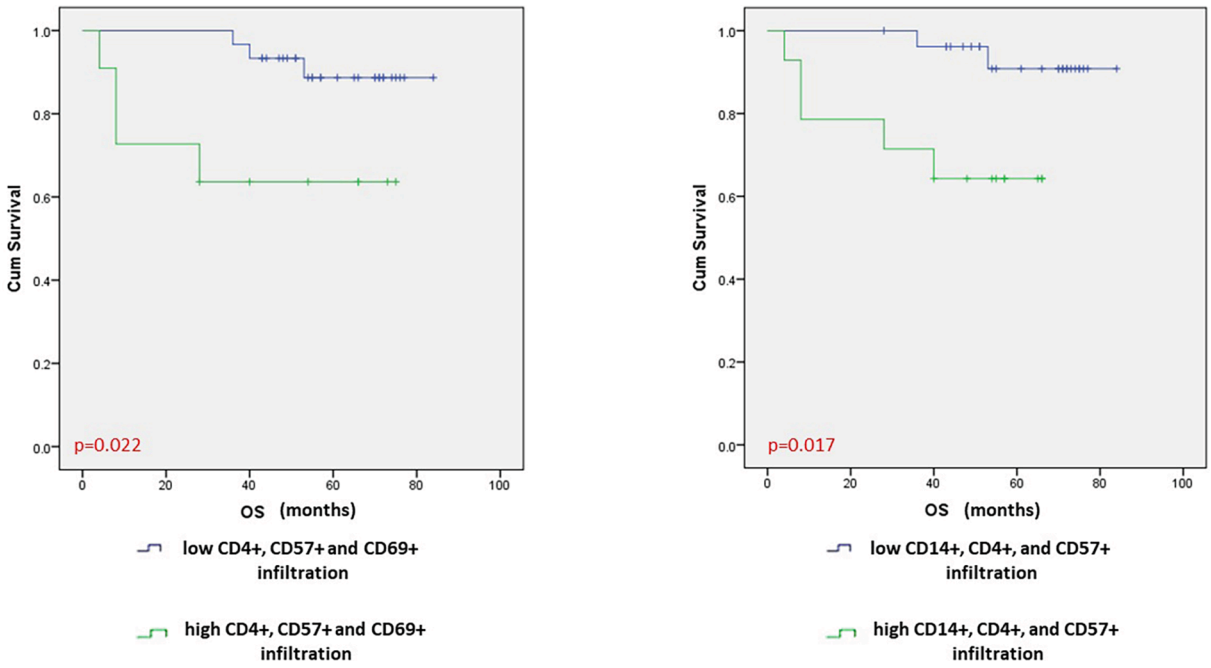

Fig. 4. (a) Patients whose samples showed high percentage of CD4+, CD57+ and CD69+ cells in tumour tissue (activated helper T lymphocytes that show exhausted phenotype) had poorer overall survival (OS). (b) Patients whose samples contained high percentage of CD14+, CD4+ and CD57+ cells in tumour tissue had poorer overall survival.

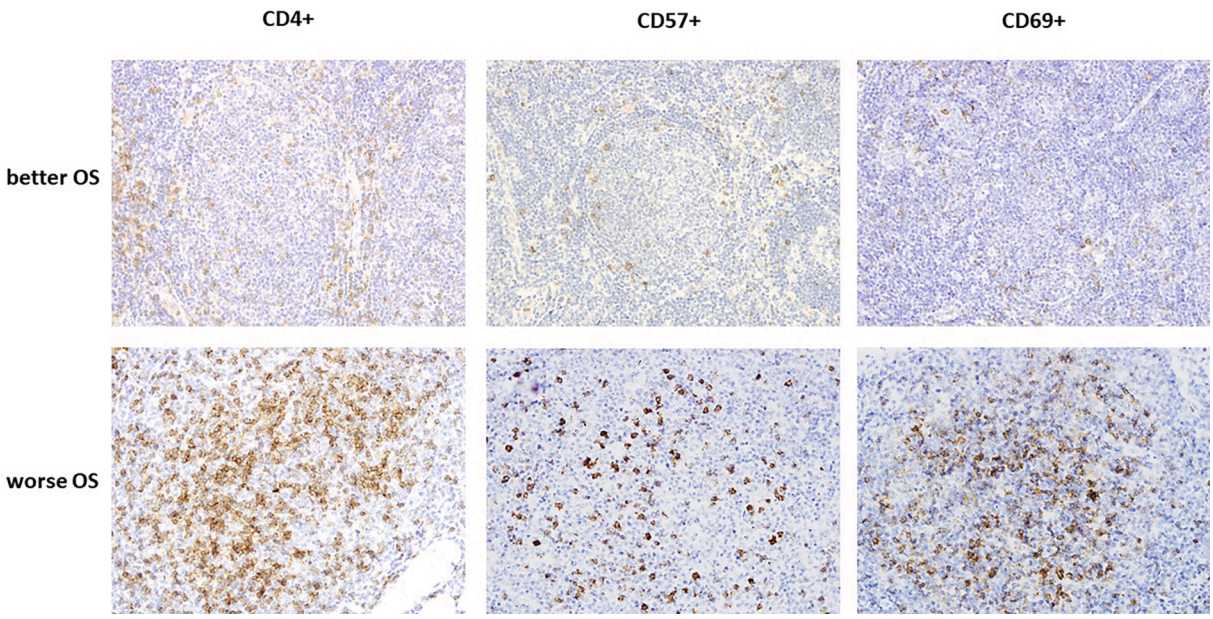

Fig. 5. Overall survival (OS) of patients with FL is dependent on tumour tissue infiltration with CD4+, CD57+ and CD69+ cells. Patients with a higher number of exhausted activated CD4+ lymphocytes had a significantly poorer overall survival. FL tumour tissues were immunostained with antibodies specific for CD4, CD57 and CD69 on consecutive slides. Their presence was detected as brownish staining after the catalytic reaction of peroxidase conjugated on the secondary antibody with DAB.

within the neoplastic follicles, rather than their quantity, promotes tumour development (Smeltzer et al., 2014). Furthermore, CD14 can be found on membranes of not only DCs, but macrophages as well (Ziegler-Heitbrock et al., 2010), which diminishes the specificity of immunohistochemical staining. This obstacle can be overridden by distinguishing DCs from macrophages by their morphological features. In our study, the abundance of DCs in FL correlated with the amount of exhausted CD57+ T cells and NK cells, but also with higher score on ECOG scale. Therefore, this study confirmed previously shown association of DCs in tumour tissue with poorer FL prognosis (Byers et al., 2005; Smeltzer et al., 2014). On the other hand, we didn't observe an effect of macrophages on the patients' survival, regardless of the marker they expressed (CD14/CD68), contrary to multiple other studies (Bingle et al., 2002; Pollard, 2004; Farinha et al., 2005; Alvaro et al., 2006; Taskinen et al., 2007).

In our study, the presence of helper T cells was associated with significantly poorer overall survival. These data are in conflict with previous analyses: a correlation between the infiltration of helper T lymphocytes within the tumour and a better outcome of FL was shown in several studies (de Jong et al., 2009; Mondello et al., 2021; Lee at al., 2006). Mondello et al. observed that favourable outcome was associated with a specific subtype of CD4+ cells – activated, non-exhausted central memory T cells. (Mondello et al. 2021). On the other hand, Yang et al. found that some CD4+ PD1+ cell subtypes are associated with poorer survival (Yang et al., 2019).

The localization of CD4+ T lymphocytes was in one study shown to be different in rapidly transforming FL (within the neoplastic follicles) than in non-transforming patients (in the interfollicular zones) (Glas et al., 2007). All samples in our study had a lower number of CD4+ cell in interfollicular areas compared to control tonsils, but we didn't observe an effect on the patients' survival nor an association with tumour grade.

The presence of CD4+ subtype that has a role in the maintaining immune tolerance and homeostasis (Li et al., 2015), FOXP3+ cells, in FL is known to correlate with better outcome (de Jong et al., 2009; Lee et al., 2006; Carreras et al., 2006; Nelson et al., 2015). However, it remains unclear whether it is the higher number of FOXP3+ Tregs or their dispersion pattern and localization that contributes most to such outcome and how various therapies affect the behaviour of these cells. In our study, the number of FOXP3+ cells in tumour tissue correlated only with the number of CD4+ cells, but didn't affect the outcome of the disease. There was also no statistically significant difference in patient overall or event free survival based on the presence of FOXP3+ cells in tumour tissue or their pattern in lymph nodes affected by FL.

In general, CD4+ cells, as well as other types of T lymphocytes that infiltrate the tumour, regardless of their prognostic potential often display phenotypic and functional features of exhausted T cells. Mediated by various pathways that are regarded as therapeutic targets (reviewed in (Turnis et al., 2015)), T cell exhaustion is one of the means by which tumours escape immune surveillance. The higher infiltration of mature CD57+ T lymphocytes, subpopulation with exhausted phenotype, which reside only in neoplastic follicles, in FL was previously associated with more aggressive clinical behaviour of the tumour and unfavourable outcome (Alvaro et al., 2006; Smeltzer et al., 2014). In this study, we observed a correlation between the percentage of CD57+ cells and CD68+ macrophages infiltrating tumour tissue as well as with the high percentage of helper T cells infiltrating tumour tissue. We observed both better overall and event free survival of patients whose samples had low CD57+ cells infiltration in the tumour tissue, indicating that CD57+ cells could allow tumour progression due to their lack of ability to combat tumour cells.

In our study, we also observed a positive correlation between the number of CD57+ cells and CD69+ cells. Given that cells expressing these markers are localized in the same areas on consecutive slides, we can assume CD57 and CD69 are expressed on the same cells. The higher number of CD57+ CD69+ cells is associated with poorer survival of the patients, indicating that these cells aren't capable of inducing proper immune response. Detection of CD69, the marker of activated T cells, as well as their diffuse pattern within FL has been shown to correlate with better outcome (Nelson et al., 2015), but Glas et al. observed that a high number of activated T lymphocytes appeared in fast-transforming FL, supporting our theory that the activation of immune cells can be overcome by a new set of signals, possibly mediated by tumour cells.

Our results suggest that, although tumour development triggers immune response mediated by helper T cells, activated T lymphocytes quickly become "exhausted" and are not capable of properly responding

to tumour cells. Possible mechanism is that tumour and non-tumour cells' communication might result in constant stimulation of T lymphocytes by DCs and macrophages, which ends in accumulation of dysfunctional, exhausted T cells, no longer able to fulfil their effector function.

5. Conclusion

Our results confirm that the widespread influence of tumour microenvironment on follicular lymphoma which affects the tumour's behaviour and changes physiology of the immune response to tumour cells. Based on the presented data it is evident that tumour microenvironment and tumour cell interaction is highly complex, but that key players in immune response are helper CD4+ T lymphocytes and dendritic cells. Our results suggest that the efficiency of immune response against tumour cells in FL is not dependent on a large number of activated T lymphocytes within tumour tissue, but on their ability to retain their anti-tumour effects.

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Informed consent

Informed consent was obtained from all subjects involved in the study.

Data availability

The data presented in this study are available on request from the corresponding author.

CRediT authorship contribution statement

Paula Gršković: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Suzana Hančić:** Methodology, Writing – review & editing. **Snježana Dotlić:** Methodology, Writing – review & editing. **Maja Matulić:** Writing – review & editing. **Slobodanka Ostojić Kolonić:** Methodology, Writing – review & editing. **Slavko Gašparov:** Methodology, Writing – review & editing. **Mara Dominis:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Petra Korać:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Resources, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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