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Original Article

High TNFRSF14 and low BTLA are associated with poor prognosis in Follicular Lymphoma and in Diffuse Large B-cell Lymphoma transformation

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The microenvironment influences the behavior of follicular lymphoma (FL) but the specific roles of the immunomodulatory BTLA and TNFRSF14 (HVEM) are unknown. Therefore, we examined their immunohistochemical expression in the intrafollicular, interfollicular and total histological compartments in 106 FL cases (57M/49F; median age 57-years), and in nine relapsed-FL with transformation to DLBCL (tFL). BTLA expression pattern was of follicular T-helper cells (TFH) in the intrafollicular and of T-cells in the interfollicular compartments. The mantle zones were BTLA+ in 35.6% of the cases with similar distribution of IgD. TNFRSF14 expression pattern was of neoplastic B lymphocytes (centroblasts) and "tingible body macrophages". At diagnosis, the averages of total BTLA and TNFRSF14-positive cells were 19.2%±12.4STD (range, 0.6%-58.2%) and 46.7 cells/HPF (1-286.5), respectively. No differences were seen between low-grade vs. high-grade FL but tFL was characterized by low BTLA and high TNFRSF14 expression. High BTLA correlated with good overall survival (OS) (total-BTLA, Hazard Risk=0.479, *P*=0.022) and with high PD-1 and FOXP3+Tregs. High TNFRSF14 correlated with poor OS and progression-free survival (PFS) (total-TNFRSF14, HR=3.9 and 3.2, respectively, P<0.0001), with unfavorable clinical variables and higher risk of transformation (OR=5.3). Multivariate analysis including BTLA, TNFRSF14 and FLIPI showed that TNFRSF14 and FLIPI maintained prognostic value for OS and TNFRSF14 for PFS. In the GSE16131 FL series, high *TNFRSF14* gene expression correlated with worse prognosis and GSEA showed that NFkB pathway was associated with the "High-TNFRSF14/dead-phenotype".
In conclusion, the BTLA-TNFRSF14 immune modulation pathway seems to play a role in the pathobiology and prognosis of FL.

Keywords: Follicular lymphoma, transformed follicular lymphoma, TNFRSF14 (HVEM), BTLA, immune microenvironment

INTRODUCTION

Follicular lymphoma (FL) is the second most common subtype of adult B-cell non-Hodgkin lymphoma (NHL) in Western countries. Most FL patients have incurable disease with a generally indolent course with frequent relapses. The eventual development of resistance to chemotherapy or transformation to diffuse large B-cell lymphoma (tFL to DLBCL) will lead to death from disease. Despite a common underlying genetic abnormality, the clinical course of FL patients is

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heterogeneous. Therefore, it is important to identify the high-risk patients who will experience rapid disease progression.[1-4]

In hematological malignancies the tumor microenvironment plays a critical role in tumorigenesis.[5] In the case of FL, the intimate relationship between the tumor microenvironment and the neoplastic cells implies a dynamic cross-talk in which tumor cells may give and receive instructions through a complex system.[5] Several lines of investigations have highlighted that FL is an immunologically functional disease in which an active interaction between tumor cells and the functional composition of the microenvironment determines the prognosis and response to treatment.[6] Gene expression profiling studies revealed that survival after initial diagnosis of FL could be predicted by biological differences. The "immune-response type 1 signature" included genes encoding T-cell markers and macrophages and was associated with a good prognosis. In contrast, the "immune-response type 2 signature" that included genes expressed in macrophages and dendritic cells associated with a poor prognosis.[7] Immunohistochemical studies were developed to better characterize the FL microenvironment and its influence on the disease outcome. Early studies strongly suggested that a high frequency of CD68+tumor-associated macrophages (TAMs) in FL were associated with a poor prognosis and later confirmed that this poor prognosis was due to M2-like CD163+TAMs.[8,9] Regulatory FOXP3+T lymphyocytes (Tregs) account for 5-10% of CD4+T-cells and have immune suppressive functions.[10] Several studies have shown that a high frequency of Tregs in FL is associated with a good prognosis and a reduced risk of transformation.[1,11] In addition, the architectural pattern of Tregs' infiltration in FL nodes also had an effect: a diffuse pattern was associated with a good prognosis but a follicular or perifollicular pattern was associated with poor prognosis.[11] Programmed cell death protein 1 (PD-1, also known as PDCD1 and CD279) is a member of the CD28 superfamily of membrane receptors that have an important function in the regulation of immune responses and in the tolerance to self-antigens as well as to tumor cells.[12] In our previous study, a high frequency of PD-1-positive cells infiltrating the tumor correlated with favorable survival in patients with FL, independently of the FLIPI; and similar results were obtained by another group.[3,13] Overall, these results suggest that the prognosis of FL depends on several immune cell subsets and pathways working simultaneously rather than being dictated by an individual immune cell subset.[6] A marker related to PD-1 is B- and T-lymphocyte attenuator (BTLA) and they are both expressed by T follicular helper cells (TFH cells).[14] BTLA ligand is the Tumor necrosis factor receptor superfamily member 14 (TNFRSF14, also known as HVEM).[15] BTLA and TNFRSF14 crosstalk regulates inhibition and costimulation of several key players of the immune system: BTLA functions as an inhibitory receptor in T lymphocytes[16] while the ligand TNFRSF14 produces proinflammatory signals via activation of NF-kappaB[17] and it is expressed by antigen presenting cells.[18] Interestingly, TNFRSF14 aberrations in FL increase clinically significant allogeneic T-cell responses[19] and in non-small cell lung cancer TNFRSF14 may contribute to the immune escape.[20]

In this study, we have examined a series of FL patients at diagnosis and after transformation to DLBCL to determine the role of the BTLA and TNFRSF14 immune checkpoint in the progression and outcome of FL.

MATERIALS AND METHODS

Patients and samples

106 diagnostic biopsies of Western FL patients diagnosed in a single institution between 1978 and 2008 were included in the study, reviewed and re-classified according to the WHO classification.[21] The median age of the patients was 57 years (range, 26-88) and the male/female distribution 57/49. The main initial features of the patients are listed in Table 1. FLIPI[4] was retrospectively assessed in 98 patients: low-risk, 45 cases (45.9%); intermediate-risk, 22 (22.4%); and high-risk, 31 (31.6%).

Staging maneuvers were the standard and included patient history and physical examination; blood cell counts and serum biochemistry, including LDH and B2M levels;

Table 1. Main clinical and histopathologic features of 106 patients with FL at diagnosis

Variable	No	(%)
Age≥60 years	47	44.3
Male sex	57	53.8
Histological grade		
Low grade (1,2)	83	78.3
High grade (3)	23	21.7
Architectural pattern		
Follicular	99	93.4
Diffuse	7	6.6
B-symptoms	18	17.3
Poor performance status (ECOG≥2)	15	14.4
Bulky disease (≥10cm)	16	15.4
Ann Arbor Stage IV	65	62.5
Bone marrow involvement	64	61.5
High serum LDH (≥450 IU/L)	22	21.8
High serum β2M (≥3mg/L)	37	38.9
FLIPI		
Good prognosis	45	45.9
Intermediate prognosis	22	22.4
Poor prognosis	31	31.6

ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; β2M, β2-microglobulin; FLIPI, Follicular Lymphoma International Prognostic Index.

computerized tomography scan of chest, abdomen, and pelvis; as well as unilateral bone marrow biopsy. Eighty-three patients received combination chemotherapy consisting on 44 CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) or CHOP-like regimens, 18 FCM (fludarabine, cyclophosphamide and mitoxantrone) or RFCM, and 21 RCHOP or RCHOP-like. In 19 patients the treatment was monotherapy consisting on six with alkylating monotherapy (chlorambucil), four with radiotherapy, one with corticoids, and eight with watch and waiting (patients ≥65 years with no high tumor burden). Twenty-seven patients had received rituximab. Post-therapy restaging consisted of the repetition of the previously abnormal tests and/or biopsies.

Response to therapy was assessed according to conventional criteria. Among the 92 patients with assessable response, 60 (65.2%) achieved a complete response (CR), 23 (25%) a partial response (PR), whereas nine (9.8%) failed to respond to treatment. The median follow-up was 8.13 years, with a range from 0.02 to 23.02 years. The 5, 10 and 15-year OS was 74.9% (95% confidence interval (CI): 83.3%-66.5%), 58.3% (95%CI: 68.3%-48.3%), and 51.6% (95%CI, 62.4%-40.8%), respectively. Twelve patients (11.8%) experienced histological transformation during the follow-up.

The study was approved following the institutional guidelines of the Ethical Committee of Hospital Clinic, Barcelona. Informed consent was provided according to the Declaration of Helsinki.

Immunohistochemical procedures and quantitative assessment

Whole tissue sections from paraffin embedded samples were immunostained using undiluted hybridoma supernatant of anti-BTLA murine monoclonal antibody (AM-005, clone FLO67B, Monoclonal Antibodies Unit, Spanish National Cancer Research Centre, CNIO, Madrid, Spain), anti-TNFRSF14 (Rabbit polyclonal to TNFRSF14 - Aminoterminal end, ab47677, Abcam, Cambridge, UK) and anti-IgD (FLEX Anti-IgD, Code IR517/IS517, Dako Diagnosticos, S.A., Barcelona, Spain). Immunohistochemical staining was automatically performed using a Leica BOND-MAX immunostainer according to manufacturer's instructions (Leica Microsystems, L'Hospitalet de Llobregat, Spain). Eighty-three and 91 cases previously studied for FOXP3 and PD-1 (NAT105) were included in this series. Follicular and interfol·licular BTLA-positive cells were quantified using an automated scanning microscope and computerized digital image analysis system always under pathologist visual supervision as previously described (Ariol SL-50, Leica Biosystems, Barcelona, Spain).[1,3] Briefly, quantification was performed using the gensight multistain assay. Whole tissue sections were automatically scanned at 10x followed by directed high magnification at 200x. Color and shape definition parameters were first assessed and then recalibrated among tissue samples. Positive and negative pixels were counted independently in the follicular and interfol·licular compartments. The follicular compartment included the mantle zone when present. Once the areas of interest were selected the software collected the data automatically. The presence or absence of a BTLA-positive mantle zone (which means that all cells in the mantle were positive) was annotated as a qualitative variable. The number of TNFRSF14-positive cells was quantified as number of positive cells per HPF (400x magnification; Olympus BX51, UPlanFI 40x/0.75; Olympus UK Ltd, Essex, UK). The percentage of BTLA and number of TNFRSF14 positive cells was recorded in the follicular and interfollicular compartments and then the total value was calculated.

Immunofluorescence was performed using biotinylated goat anti-mouse and anti-rabbit IgG antibodies (#BA-9200 and BA-1000, Vector Laboratories, CA, USA), streptavidin, Alexa Fluor 488 and 568 conjugates and ProLong™ Gold antifade mountant with DAPI (#S11223, S11226 and #P36941, Thermo Fisher Scientific K.K., Tokyo, Japan). Zeiss LSM700 laser-scanning confocal microscope was used with linear unmixing and spectral imaging following the manufacurer's instructions (Zeiss, Tokyo, Japan) with combination to Imaris microscopy image analysis software (Bitplane AG, Zurich, Switzerland). Human reactive lymphoid tissue was used to analyze the expression of TNFRSF14 and BTLA with FOXP3 and PD-1 using the double or triple immunofluorescence stainings. In addition, relationship of TNFRSF14 with PAX5 (B-cells), CD21 (FDCs) and MITF (macrophages) was analyzed as well using Bond Polymer Refine Red Detection (#DS9390) and Bond Polymer Refine DAB Detection (#DS9800).

Lymphoid tissue from peyer's patches of small intestine of control BALB/c mice were studied by flow cytometry to quantify the expression frequency of BTLA and TNFRSF14 against the markers of B220 (CD45R), CD11C and FOXP3 as described.[22] Same antibodies were used for direct immunofluorescence in frozen tissue.

Gene expression analysis

The online SurvExpress tool[23] was used to correlate the gene expression levels with the patients' outcome of the previously published series of Dave *et al*. that includes 191 FL cases.[7] Then, the same FL gene expression and clinical features datasets were downloaded from the NCBI, Gene Expression Omnibus (GEO), series GSE16131, platform GPL96 [HG-U133A], Affymetrix human genome U133A array), series matrix file. Gene Set Enrichment Analysis (GSEA) was performed following the Broad Institute software and their instructions.[24,25] To narrow the differences we compared the GSEA between 30 cases of high *TNFRSF14* and dead phenotype vs. 30 cases of low *TNFRSF14* and alive phenotype. The target pathway to test was the NFkB immune inflammatory gene set, M2645, "hinata", homo sapiens, from the Molecular Signatures Database of Broad Institute.

Statistical analysis

The main initial and evolutive variables were analyzed for prognostic significance. BTLA and TNFRSF14-positive-cells were initially quantified and analyzed as a continuous variable and later the best threshold for overall survival was found using Cutoff Finder software.[26] Comparison of means

was performed with independent-samples T Test, and non-parametric tests (independent samples) to automatically compare distributions across groups when required. Categorical data were compared using crosstabs, chi-square, using Fisher's exact test when necessary. Binary logistic regression was performed to calculate the Odds Ratios. Survival analysis was performed using the Kaplan-Meier analysis with Log rank test for univariate analysis; and the Cox Regression (method:enter) for multivariate analysis and also for univariate to calculate the Hazard Ratio. The definition of OS and progression-free survival (PFS) were the standard. Statistical significance was stated a priori at P<0.05. The IBM SPSS software, build 1.0.0.1174, 64-bit edition was used for statistical analysis (IBM Japan, Tokyo).

RESULTS

Clinical and histological features of the patients at diagnosis

The main clinical and histological features of the 106 patients at diagnosis are detailed in the methods section and summarized in Table 1 and Tables 2.1 and 2.2. The histological distribution was low grade (1 and 2) in 83 cases (78.3%), and high grade (3) in 23 (21.7%) cases. The architectural pattern was follicular (i.e. nodular, follicular area>75%) in 99 (93.4%) and predominantly diffuse (follicular area<25%) in seven cases (6.6%). Clinical variables that were associated with unfavorable OS were age (>60 years), a high-grade, the presence of B-symptoms, ECOG≥2, high LDH, high B2M and high-risk FLIPI (P<0.05). Variables that were associated with unfavorable PFS were B-symptoms, ECOG≥2, high LDH, high B2M and high-risk FLIPI (P<0.05). Overall, our patient population was reflective of a commonly observed FL population.

Table 2.1. Overall Survival analysis of the clinicopathological variables in 106 diagnostic cases of FL.

Variable	P value	Hazard Ratio	95% C.I. for HR	
			Lower	Upper
Age >60 y.	0.000193	3.213	1.74	5.936
Sex (male)	0.956	0.984	0.547	1.768
High histological grade	0.001	2.907	1.562	5.413
Diffuse architectural pattern	0.387	0.534	0.129	2.209
B-symptoms	0.002	2.855	1.493	5.463
ECOG≥2	0.000016	4.442	2.256	8.744
Bulky disease (≥10cm)	0.305	0.614	0.241	1.56
Ann Arbor Stage IV	0.635	0.866	0.479	1.566
Bone marrow involvement	0.997	1.001	0.551	1.818
High serum LDH (≥450 IU/L)	0.000144	3.321	1.788	6.168
High serum β2M (≥3mg/L)	0.000202	3.39	1.781	6.455
High risk FLIPI	0.000399	2.962	1.624	5.403

Table 2.2. Progression Free Survival analysis of the clinicopathological variables in 106 diagnostic cases of FL.

Variable	P value	Hazard Ratio	95% C.I. for HR	
			Lower	Upper
Age >60 y.	0.085	1.533	0.942	2.493
Sex (male)	0.627	0.887	0.547	1.439
High histological grade	0.095	1.603	0.921	2.792
Diffuse architectural pattern	0.847	0.905	0.329	2.493
B-symptoms	0.016	2.038	1.143	3.633
ECOG≥2	0.001	2.838	1.559	5.167
Bulky disease (≥10cm)	0.73	1.127	0.572	2.219
Ann Arbor Stage IV	0.408	1.236	0.748	2.043
Bone marrow involvement	0.507	1.184	0.719	1.948
High serum LDH (≥450 IU/L)	0.001	2.501	1.455	4.299
High serum β2M (≥3mg/L)	0.003	2.188	1.302	3.675
High risk FLIPI	0.006	2.052	1.228	3.429

Histopathological features of BTLA-positive cells in FL

BTLA-positive-cells were observed either in the germinal centers of follicular areas and in the interfollicular compartment following a T-cell pattern distribution. In reactive tonsils, triple immunofluorescence confirmed the colocalization of PD-1 and BTLA in the FTH cells located in the germinal centers, while FOXP3 was a specific marker for Tregs (Figure 1G). In addition, BTLA was positive in the mantle and marginal zone area in 36 (35.6%) of the cases following a pattern of the B-cell staining. The mantle zone was also IgD-positive (Figure 1).

The relationship between the percentage of BTLA positive cells with a T-cell distribution in samples at diagnosis and the pathological characteristics of the patients is shown in Table 3.1. The mean proportion of total BTLA in the 101 diagnostic biopsies was 19.2% (STD 12.4%; range, 0.55-57.68%). Overall, the percentiles 25, 50 and 75 were 9.8%, 18.3% and 25.4%, respectively. No differences were found between low- and high-grade follicular lymphoma neither in the total, (intra)follicular nor interfollicular compartments but the transformation to DLBCL was characterized by a striking decrease in the number of BTLA-positive cells (18.3±10.2 vs. 22.1±18.2 vs. 1.9±3.5, respectively; FL vs. tFL *P*=0.000008). FL with a diffuse architectural pattern did not had lower levels of BTLA+cells than those with a follicular pattern (*P*=N.S) (Table 3.1).

Thirty-six percent of the FL samples had a BTLA-positive mantle zone. No correlation was found with the grade of FL (low grade vs. high grade) and the architectural pattern (nodular vs. diffuse), 39.2% vs. 22.7% and 36.8% vs. 16.7%, respectively (*P*= N.S) (Table 3.1).

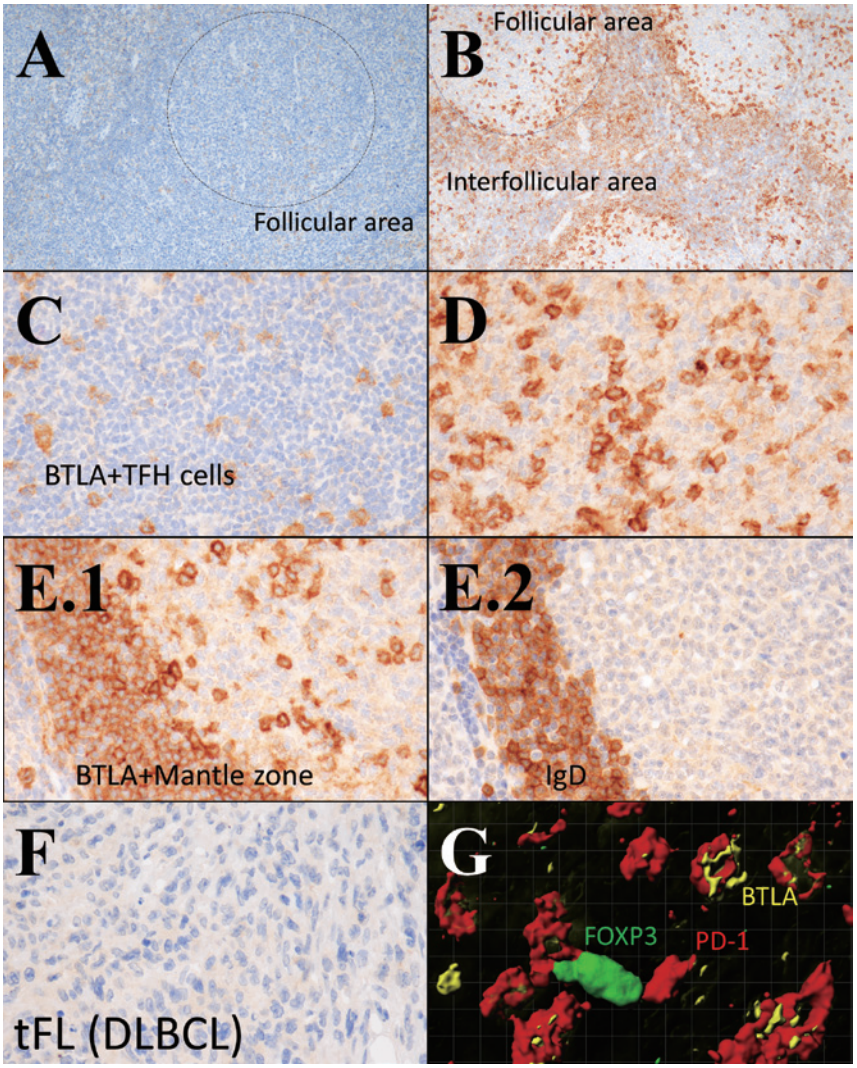

Fig 1. Immunohistochemical expression of BTLA in FL.
A, Low BTLA expression in FL in all compartments. ***B***, High BTLA expression both in the follicular and interfollicular compartments. ***C***, Low BTLA expression in the follicular area, with a follicular T helper cell (TFH) pattern. ***D***, High follicular BTLA expression (+weak staining of B cells, phenomena often seen in the high BTLA expression cases). ***E.1***, BTLA+mantle zone and ***E.2***, same area stained with anti-IgD antibody. ***F***, BTLA expression is very low in transformed FL to Diffuse Large B-cell Lymphoma (tFL). ***G***, Double immunofluorescence combined with peroxidase-DAB-based in reactive tonsil staining using confocal microscopy with volume rendering between BTLA (HRP-DAB-based IHC, yellow), PD-1 (red) and FOXP3 (green). The staining shows that in the germinal centers BTLA is identifying PD-1+follicular T helper cells (FTH).
Original magnification of Figures A and B is 100X, the rest are 400X (Olympus BX53).

Table 3.1. Distribution of BTLA-positive cells in FL at diagnosis

			BTLA-positive cells, %		Mantle
	No (%)	Total	Follicular compartment	Interfollicular compartment	No (%)
Histologic grade					
Grade 1-2	79 (78.2)	18.3±10.2	17.0±10.0	20.1±13.2	31 (39.2)
Grade 3	22 (21.8)	22.1±18.2	18.9±16.2	25.4±23.4	5 (22.7)
DLBCL	9	1.9±3.5	-	-	-
Architectural pattern					
Follicular	95 (94.1)	19.6±12.4	17.8±11.6	21.8±16.1	35 (36.8)
Diffuse	6 (5.9)	11.6±11.1	-	-	1 (16.7)
Total	101 (100)	19.2±12.4	17.4±11.6	21.2±15.9	36 (35.6)

Histopathological features of TNFSF14-positive cells in FL

In reactive tonsil the TNFSF14-positive cells showed a characteristic Golgi staining pattern in the cytoplasm. In the follicular area of reactive tonsil PAX5+lymphocytes also stained positive for TNFRSF14 and were surrounded by CD21+follicular dendritic cells. Macrophages were also MITF+ (Figure 2A).

In FL, TNFRSF14-positive cells had a histological morphology of B-cell centroblasts and the other positive cells had a morphology of macrophages, especially at the stage of transformation to DLBCL. (Figure 2B-D).

The relationship between the number of TNFSF14-positive cells in samples at diagnosis and the pathological characteristics of the patients is shown in Table 3.2. The mean number of total TNFSF14 in the 92 diagnostic biopsies was 46.7 cells/HPF (STD 57.8; range, 1-286). Overall, the percentiles 25, 50 and 75 were 8.0, 33.3 and 60.3, respectively. No differences were found between low- and high-grade follicular lymphoma neither in the total, follicular nor interfollicular compartments but the transformation to DLBCL was characterized by a striking increase in the number of TNFRSF14-positive cells (44.4±56.7 vs. 55.7±62.6 vs. 137.9±91.8, respectively; FL vs. tFL *P*=0.002). FL with a diffuse architectural pattern was characterized by a 29% less numbers of positive cells although it was not statistically significant, only a trend (14.3±11.9 vs. 49.4±59.3, *P*=0.056) (Table 3.2.).

Correlation between BTLA, TNFSF14, FOXP3 and PD-1 markers in FL

A significant positive correlation was found between follicular BTLA and follicular PD-1 expression (Spearman correlation *P*=0.019) as well as with follicular FOXP3 expression (Pearson correlation *P*=0.002). In addition, follicular PD-1 also positively correlated with follicular FOXP3 (Pearson correlation *P*=0.001). Total BTLA also positively correlated with total FOXP3 (Pearson correlation *P*=0.025). Of note, the presence of a BTLA+mantle also positively correlated with follicular FOXP3 (*P*=0.003). Follicular TNFRSF14 did not correlate with follicular PD-1 or follicular FOXP3. Total TNFRSF14 did not correlate with total FOXP3 as well. Total TNFRSF14 and total BTLA didn't correlate either as continuous or as ordinal variable.

In the mice system in peyer's patches, B220+CD11C+cells, present in the mantle/marginal zone also expressed BTLA. Interestingly, by flow cytometry FOXP3+Tregs also could express BTLA and/or HVEM. Of note, one must be cautious when comparing mice and human immune system as results cannot be directly translated. Further analysis will be required in the future.

Relationship between the number of BTLA and TNFSF14-positive cells, clinical features and outcome

The total numbers of BTLA and TNFRSF14-positive cells did not correlate with the outcome when analyzed as a continuous variable, but the cohort was divided in two categorical subsets based on high and low positive numbers (Figures 3-5).

High BTLA expression, either in intrafollicular (≥4.54%) or interfollicular (≥8.05%) areas, and total numbers (≥8.59%), correlated with a favorable OS (Kaplan-Meier, Log Rank *P*=0.005, *P*=0.024 and *P*=0.022, respectively); the Hazard Ratio (HR) was 0.382, 0.478 and 0.479, respectively (Figure 3). The 5- and 15-year OS are shown in Table 4.1. The presence of a BTLA-positive mantle zone also correlated with favorable OS (*P*=0.039 and HR of 0.468). BTLA did not correlate with the PFS. High total BTLA inversely correlated with the presence of B-symptoms and high serum LDH (Table 4.2) (Figure 3). When we analyzed only the cases treated with rituximab (R-CHOP and R-CHOP-like), high total BTLA also associated with a good prognosis of the patients (OS) (Figure 5).

High TNFRSF14, either follicular (≥44), interfollicular (≥32) and total (≥34.9), correlated with a poor OS: Kaplan-Meier, Log Rank *P*=0.000142, *P*=0.000019 and *P*=0.000021, respectively; the HR was 3.393, 3.636 and 3.863, respectively (Figure 4). The 5- and 15-year OS is shown in Table 4.1. TNFRSF14 also correlated with a poor PFS: Kaplan-Meier, Log Rank *P*= 0.000034, *P*=4.407E-08 and *P*= 0.000004, respectively; the HR was 2.911, 4.094 and 3.236, respectively. The 5- and 15-year OS is present at Table 4.1. High total TNFRSF14 positively correlated with B-symptoms, high LDH, high B2M and FLIPI high risk (Table 4.3). TNFRSF14 associated to a higher risk of FL transformation to DLBCL: total TNFRSF14 (cut-off at 42.1%), odds-ratio 5.250 (95% C.I. 1.250-22.058), P=0.024. Of note, BTLA expression did not associate to FL transformation. In the rituximab-treated group, high total TNFRSF14 also associated with a poor OS and PFS (Figure 5).

Both total BTLA and total TNFSF14 expression were included with the FLIPI in a Cox model to identify which one was more important to predict OS. TNFRSF14 and FLIPI retained its prognostic value. When tested for PFS only TNFRSF14 retained prognostic value (Table 5).

Of note, in this series high total FOXP3 (>5%) correlated with a favorable OS (*P*=0.040, HR: 0.476, 95%CI 0.231-0.983) as well as high follicular PD-1 (>6%, *P*=0.001, HR:0.364, 95%CI 0.195-0.677). When total BTLA, total TNFRSF14, total FOXP3 and follicular PD-1 expression as well as FLIPI were included in a Cox model, only TNFRSF14 and FLIPI retained prognostic values.

Gene expression analysis

We used the previously published data of gene expression of FL from the series of Dave to validate our immunohistochemical finding at RNA levels. We could not analyze BTLA because that gene was not present in the Affymetrix chip at that time. High *TNFRSF14* RNA levels were associated with a poor OS (*P*=0.004). Five- and 15-year OS, high vs. medium-low risk: 64.3% (95%CI: 76.7%-51.9%) and 13.4% (26.3%-0.5%) vs. 74.7% (82.7%-66.7%) and 39.4% (51.2%-27.6%). The Hazard Risk was 1.816 (*P*=0.005, 95%CI 1.199-2.751) (Figure 6).

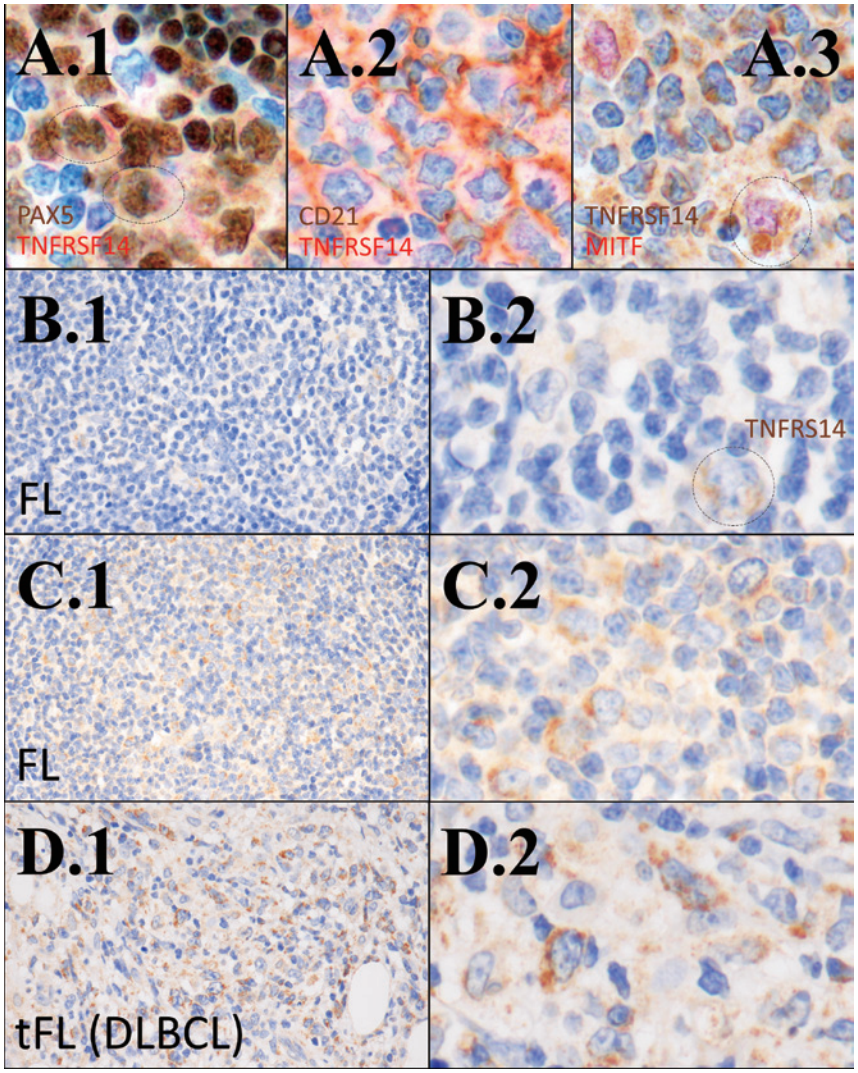

Fig 2. Immunohistochemical expression of TNFRSF14 (HVEM) in FL.
The TNFRSF14 expression was analyzed first in reactive tonsil. ***A.1***, Reactive tonsil, double immunohistochemistry between PAX5 (DAB, brown) and TNFRSF14 (red) shows that neoplastic B lymphocytes (centroblasts) are TNFRSF14 positive. ***A.2***, Double immunohistochemistry between CD21 (DAB, brown) and TNFRSF14 (red) shows how TNFRSF14+B lymphocytes (mainly centroblasts) are surrounded by a network of CD21+follicular dendritic cells (FDC). ***A.3***, MITF+cells with morphology of FCD and macrophage (red color, nuclear staining) are surrounded by TNFRSF14+cells. ***B***, Low frequency of TNFRSF14+cells in FL (***B.1*** 400X, ***B2***, magnified inset). ***C.1***, High frequency of TNFRSF14+cells (***C.2***, inset). ***D.1***, transformed FL to DLBCL (tFL) with high TNFRSF14 expression (***D.2***, inset).

Table 3.2. Distribution of TNFRSF14-positive cells in FL at diagnosis

			TNFRSF14-positive cells, %	
	No (%)	Total	Follicular compartment	Interfollicular compartment
Histologic grade				
Grade 1-2	73 (79.3)	44.4±56.7	60.8±86.2	27.9±40.9
Grade 3	19 (20.7)	55.7±62.6	70.3±89.5	41.1±46.4
DLBCL	9	137.9±91.8	-	-
Architectural pattern				
Follicular	85 (92.4)	49.4±59.3	66.5±88.9	32.2±43.4
Diffuse	7 (7.6)	14.3±11.9	-	-
Total	92 (100)	46.7±57.8	62.8±86.5	30.6±42.2

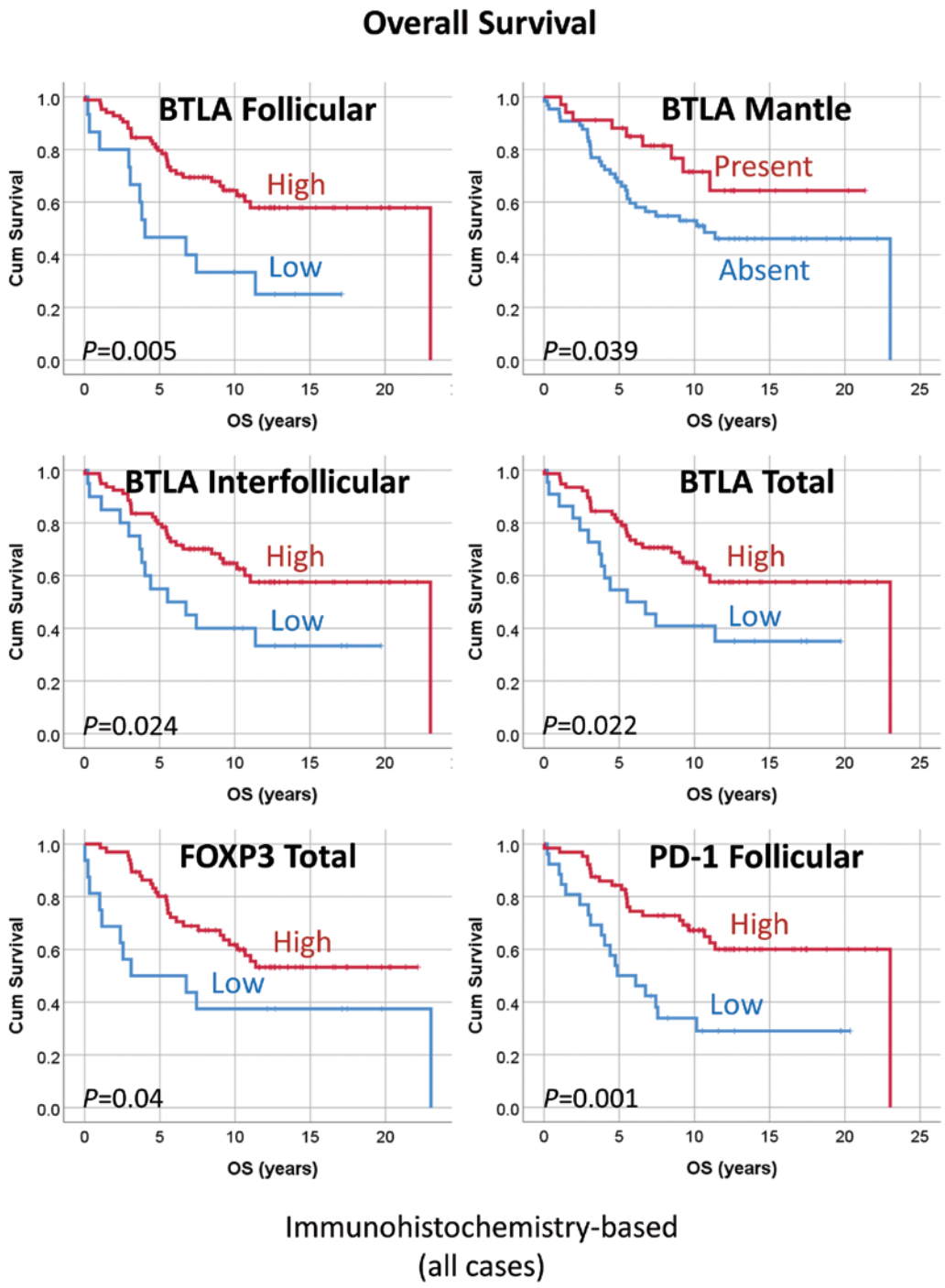

Fig 3. Overall survival of BTLA, FOXP3 and PD-1.
High BTLA expression, either follicular, mantle, interfollicular and total correlated with good OS (P<0.05). Same results were found for total FOXP3 and follicular PD-1. Of note, BTLA did not correlate with the PFS.

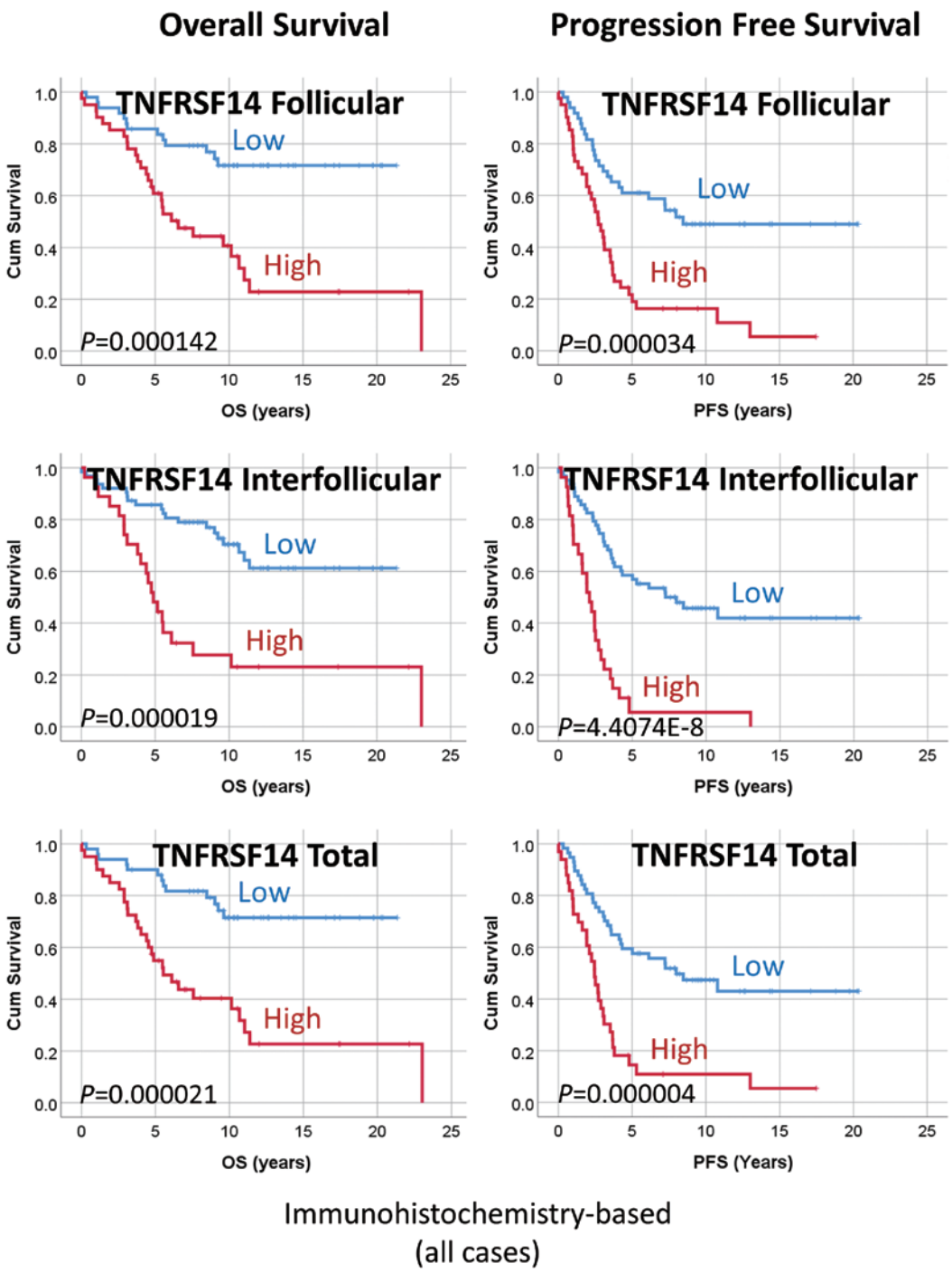

Fig 4. Overall survival and progression free survival of TNFRSF14.
High TNFRSF14 expression associated with poor prognosis in FL either in the follicular, interfollicular and total compartments, for both OS and PFS (P<0.001).

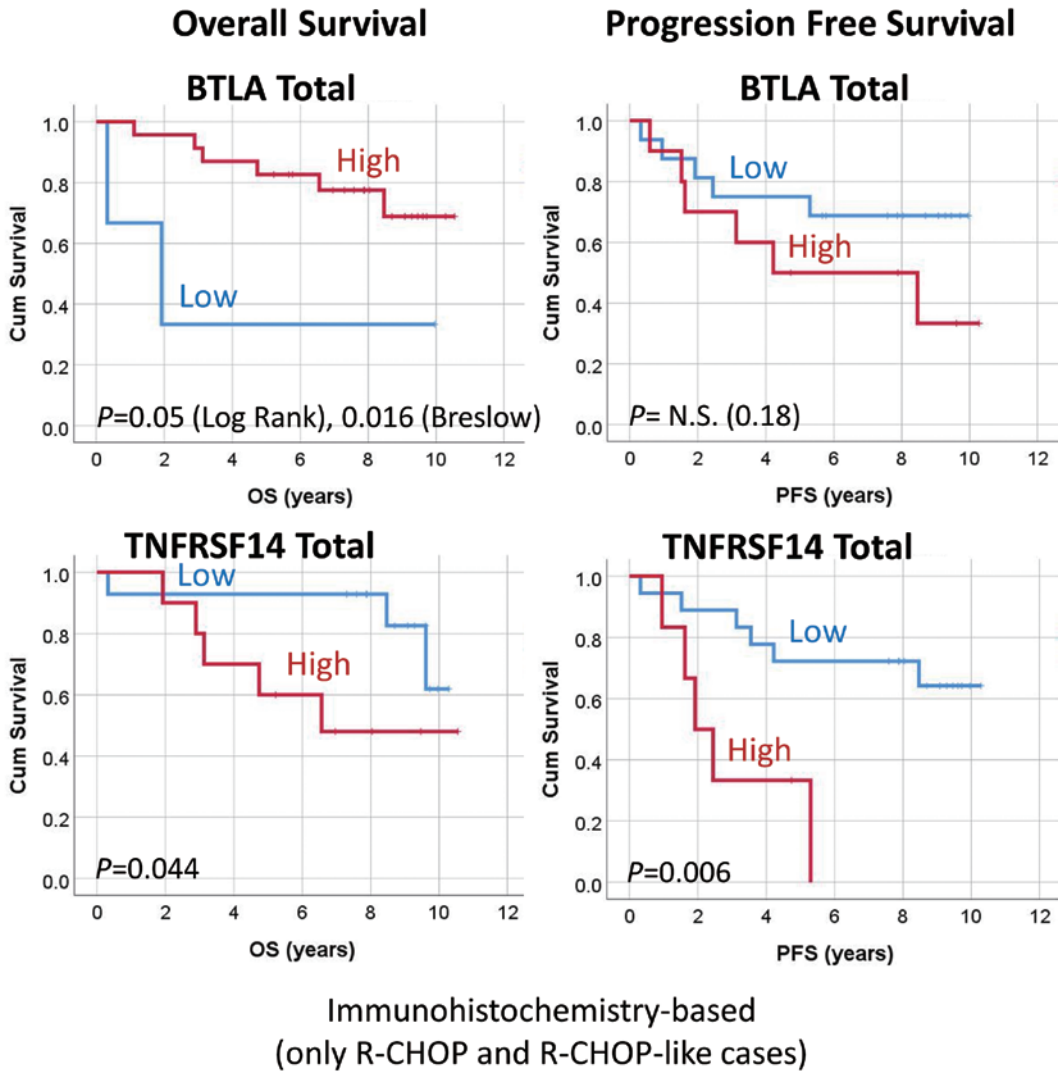

Fig 5. Overall survival and progression free survival of BTLA and TNFRSF14 on the rituximab-treatment cases.
The prognostic relevance of BTLA and TNFRSF14 was maintained when we re-analyzed only the rituximab-treated patients (RCHOP and RCHOP-like).

The GSEA analysis of the NFkB pathway showed an enrichment on the *TNFRSF14* high and dead phenotype (the "high risk" group) (Figure 6). The genes of the core enrichment were *CXCL3, CCL20, CXCL2, IL6, CXCL6, TNFAIP2, IRF1, NFKBIA, IL8* and *IL1B*.

DISCUSSION

In this study, we found that the immune checkpoint genes of BTLA and TNFRSF14 (also known as HVEM) are differentially expressed by cells of the immune microenvironment and by the neoplastic FL B-lymphocytes. We found that the immunohistochemical protein expression correlated with the prognosis of the patients with high BTLA expression being associated with good prognosis and high TNFRSF14 expression with poor outcome, as well as with several clinical variables. In addition, we also found that FL transformation to DLBCL (tTL) was associated with a decrease of BTLA expression but an increase of TNFRSF14.

FL is the second most common subtype of non-Hodgkin lymphoma. It is defined as a lymphoma of follicle center B cells, and virtually always demonstrates a growth pattern that is partially follicular. Germinal centers (GCs) are the site of antibody diversification and affinity maturation and as such are vitally important for humoral immunity.[27] One of the hallmarks of the GC function is the progressive increase of affinity antibodies that is the result of the activation-induced cytidine deaminase (AID)-driven somatic hypermutation (SHM) of the antigen-binding variable regions of immunoglobulin (Ig) genes.[27,28] SHM generates a panel of mutated B cells that are then selected, based on their affinity, to proliferate and differentiate into antibody-secreting plasma cells (PCs) and memory B cells.[27] A mature GC is divided into two compartments with different functions. The centroblasts in the dark zone proliferate in a meshwork of CXCL12-expressing reticular cells, express CXCR4 and AID, and

Table 4.1. Correlation between BTLA and TNFRSF14-positive cells and prognosis

		Overall Survival								Progression free survival							
		5-y	95%CI		15-y	95%CI		HR	*P* value	5-y	95%CI		15-y	95%CI		HR	*P* value
BTLA																	
Follicular	Low	46.7	72.0	21.4	25	47.7	2.3	0.382	0.005	-	-	-	-	-	-	-	0.098
	High	79.7	88.3	71.1	57.9	69.9	45.9			-	-	-	-	-	-		
Interfollicular	Low	55	76.8	33.2	33.3	54.9	11.7	0.478	0.024	-	-	-	-	-	-	-	0.266
	High	79.7	88.5	70.9	57.5	70.0	45.0			-	-	-	-	-	-		
Total	Low	54.5	75.3	33.7	35.1	55.7	14.5	0.479	0.022	-	-	-	-	-	-	-	0.182
	High	80.4	89.2	71.6	57.6	70.3	44.9			-	-	-	-	-	-		
Mantle	Absent	67.7	79.1	56.3	46.1	59.0	33.2	0.468	0.039	-	-	-	-	-	-	-	0.272
	Present	88.1	99.1	77.1	64.4	85.0	43.8			-	-	-	-	-	-		
TNFRSF14																	
Follicular	Low	85.7	95.5	75.9	71.6	84.7	58.5	3.393	0.000142	61	74.7	47.3	48.9	63.4	34.4	2.911	0.000034
	High	60.9	75.8	46.0	22.9	38.8	7.0			21.7	34.4	9.0	5.4	14.8	-4.0		
Interfollicular	Low	85.7	94.3	77.1	61.2	75.5	46.9	3.636	0.000019	58.5	70.7	46.3	42	55.7	28.3	4.094	4.407E-08
	High	48.1	66.9	29.3	23.1	40.0	6.2			5.6	15.4	-4.2	0	0.0	0.0		
Total	Low	90	98.2	81.8	71.5	84.8	58.2	3.863	0.000021	59.4	72.1	46.7	43	57.7	28.3	3.236	0.000004
	High	53.9	69.4	38.4	22.7	38.6	6.8			14.5	26.8	2.2	5.5	14.9	-3.9		

Table 4.2. Correlation between High BTLA Total and the clinicopathological features at FL diagnosis.

Variable	P value	Odds Ratio	95% C.I. for OR	
			Lower	Upper
Age >60 y.	0.443	1.465	0.553	3.884
Sex (male)	0.34	1.588	0.614	4.107
High FL histological grade	0.202	0.502	0.174	1.448
Diffuse FL architectural pattern	0.105	0.25	0.047	1.338
B-symptoms	0.045	0.32	0.105	0.977
ECOG≥2	0.198	0.45	0.133	1.517
Bulky disease (≥10cm)	0.654	0.75	0.213	2.637
Ann Arbor Stage IV	0.207	1.852	0.711	4.826
Bone marrow involvement	0.251	1.75	0.673	4.552
High serum LDH (≥450 IU/L)	0.017	0.28	0.098	0.8
High serum β2M (≥3mg/L)	0.932	1.045	0.383	2.853
FLIPI	0.5	0.819	0.459	1.462

The analysis consisted on binary logistic regression between BTLA Total and the different clinicopathological characteristics. High BTLA Total was defined as a percentage >8.58%, and low as <8.58%. In the logistic regression analysis "low" was considered as the reference.

Table 4.3. Correlation between High TNFRSF14 Total and the clinicopathological features at FL diagnosis.

Variable	P value	Odds Ratio	95% C.I. for OR	
			Lower	Upper
Age >60 y.	0.085	2.1	0.902	4.889
Sex (male)	0.727	1.16	0.505	2.662
High histological grade	0.194	1.971	0.708	5.483
Diffuse architectural pattern	0.999	0	0	-
B-symptoms	0.018	5.222	1.328	20.533
ECOG≥2	0.061	3.339	0.944	11.803
Bulky disease (≥10cm)	0.036	0.187	0.039	0.898
Ann Arbor Stage IV	0.242	1.69	0.702	4.07
Bone marrow involvement	0.357	1.504	0.631	3.582
High serum LDH (≥450 IU/L)	0.057	2.92	0.967	8.816
High serum β2M (≥3mg/L)	0.002	4.327	1.675	11.182
High risk FLIPI	0.005	4.144	1.527	11.245

The analysis consisted on binary logistic regression between TNFRSF14 Total and the different clinicopathological characteristics. High TNFRSF14 Total was defined as a percentage >34.75%, and low as <34.75%. In the logistic regression analysis "low" was considered as the reference.

Table 5. Multivariate analysis between BTLA Total, TNFRSF14 Total and FLIPI.

Variable	P value	Hazard Ratio	95% C.I. for HR	
			Lower	Upper
OS				
High BTLA Total	0.143	0.563	0.261	1.214
High TNFRSF14 Total	0.001	3.605	1.699	7.647
High FLIPI	0.042	2.079	1.026	4.214
PFS				
High BTLA Total	0.547	1.196	0.669	2.138
High TNFRSF14 Total	0.000144	3.118	1.735	5.604
High FLIPI	0.182	1.501	0.827	2.726

undergo Ig SHM.[29] In the light zone there is high quantity of naïve B lymphocytes and follicular dendritic cells, there the higher-affinity B cells (centrocytes) are positively selected thanks to the function of the follicular T helper cells (TFH). TFH cells drive the higher-affinity B cells proliferation and differentiation into the plasma cells.[30] TFH cells are characterized by a gene expression signature distinct from that of other T-cell subsets,[31] and they can be identified by an array of markers including *CXCR5*, *CXCL13*, *PD-1* (*PDCD1*), *ICOS*, *SAP*, *CD200*, *BCL6*, *IL21* and *MAF*.[32] *BTLA* was also identified among the genes specifically expressed in TFH cells.[31] BTLA is a lymphoid receptor that inhibits lymphocyte activation and proliferation on interaction with its ligand, TNFRSF14.[33] BTLA suppresses IL21 production from TFH cells and subsequent humoral immune responses.[34] BTLA is widely express in reactive lymph nodes, except in GC B cells.[33] Among normal B cells, the highest BTLA expression is found in naïve B cells (CD20+, IgD+/CD38-).[33] In our study the expression in reactive tonsils confirmed those finding, BTLA was positive in the GCs with colocalization with PD-1+TFH cells, the B lymphocytes of the mantle zones were also positive and in the interfollicular regions BTLA-positive cells were identified with a pattern similar to PD-1. In FL we found that high BTLA expression in the follicular region was associated with an improved OS. These findings are like those that we had previously reported with PD-1 in FL, focusing on the follicular region.[3] In addition, we found that the presence of a BTLA-positive mantle zone, which also stained with IgD, associated with good prognosis in FL. Interestingly, chronic lymphocytic lymphoma also expresses BTLA[33] and it is derived from CD5+B lymphocytes (B1 cells). We found that BTLA expression in T-cells correlated with PD-1 and FOXP3, two markers associated with good prognosis in FL.[1,3] Functional studies have confirmed the enrichment of TFH cells in the FL microenvironment, which display a specific activation profile characterized by the expression of IL-4 that could sustain FL pathogenesis.[35] Therefore, TFH cells seem to be necessary for the pathogenesis of FL and associates with good clinical evolution. Importantly, as also seen with the PD-1 marker,[3] transformation to DLBCL is associated with a marked reduction of BTLA expression.

TNFRSF14 is the nexus in several signaling pathways and it plays important roles in the immune system, such as T-cell costimulation, regulation of dendritic cell (DC) homeostasis, autoimmune-mediated inflammatory responses, as well as host defense against pathogens.[36] TNFSF14 serves as a bimolecular switch to regulate the host immune response depending on which ligand it engages because TNFRSF14 functions as both a receptor with signal-transducing functions and as a ligand eliciting signaling. As a receptor, TNFRSF14 leads to the activation of NFkB, RELA, AP-1 and AKT pathways that enhances cell proliferation, cytokine production and survival of TNFRSF14-expressing cells.[36,37] Antigen presenting cells (APCs, i.e. dendritic cells, macrophages and B cells) can express TNFRSF14. As a ligand of BTLA on T cells will induce ITIM phosphorylation, recruitment of SHP-1 and SHP-2 that will downregulate the TCR signaling pathway resulting in reduced cellular activation, proliferation, and cytokine production.[36] Therefore, can inhibit the function of CD8+cytotoxic T cells and CD4+T helper cells, including TFH cells.[34,38] In our study the expression of TNFRSF14 had a pattern of APCs including dendritic cells-macrophages and B-cells (centroblasts). We found that a high expression correlated with poor prognosis in FL and with transformation to DLBCL. In addition, GSEA analysis found an enrichment towards the "dead and high *TNFRSF14* phenotype" of the NF-kB pathway. Of note, the NF-kB pathway is related to the pathobiology and chemoresistance in FL and tFL.[39] It has been reported that deletions of 1p36 and TNFRSF14 mutations are associated with worse prognosis of FL, but in that publication the authors did not analyze the protein expression of TNFRSF14[40] and, unfortunately, in our study we have not analyzed the mutational status. TNFRSF14 receptor gene is among the most frequently mutated genes in germinal center lymphomas and loss of TNFRSF14 leads to cell autonomous activation of B cell proliferation and drives the development of GC lymphomas in vivo.[41] TNFRSF14 deficient lymphoma B cells also induce a tumor supportive microenvironment

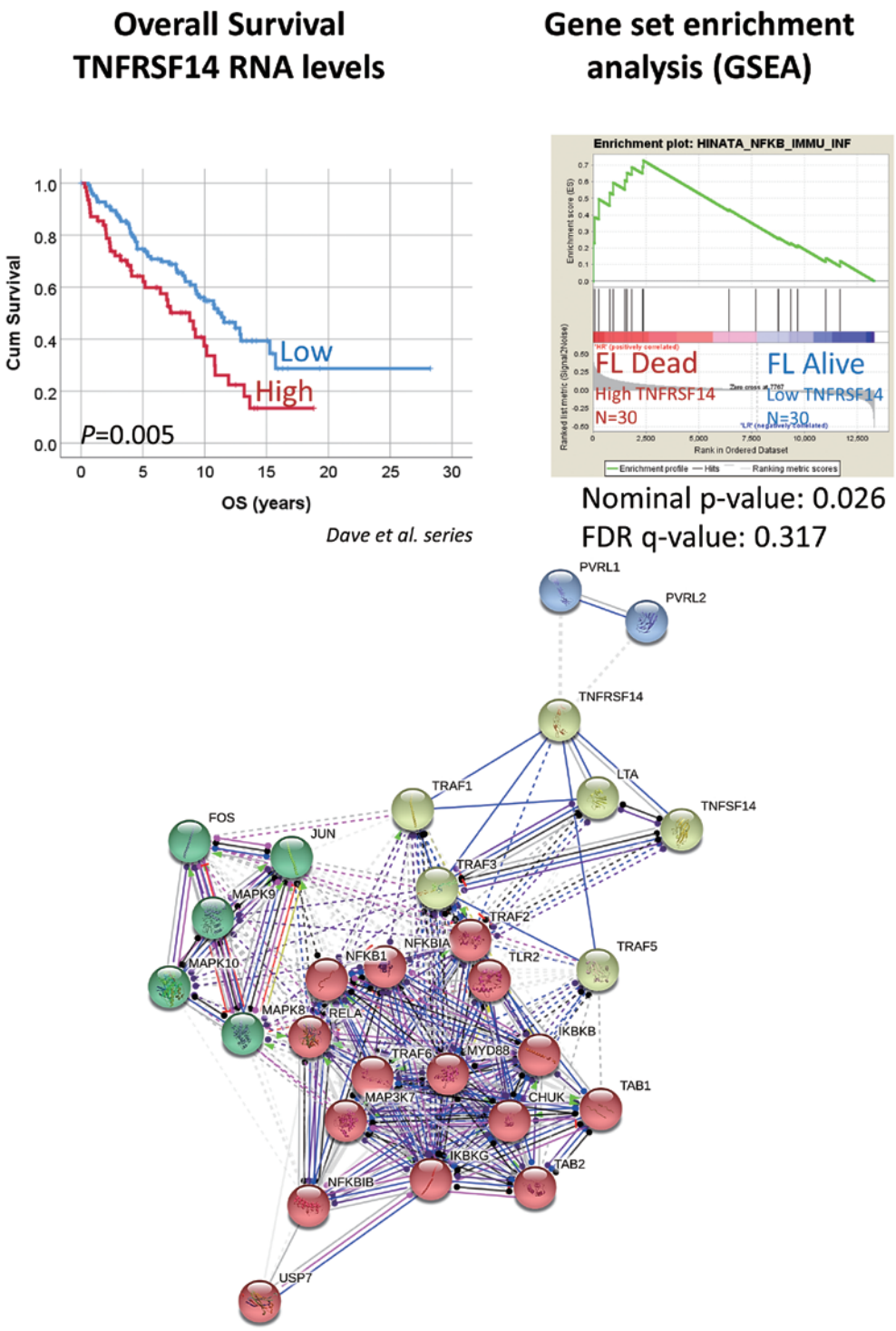

Fig 6. Gene expression analysis of *TNFRSF14* in FL.
At RNA level, high *TNFRSF14* associated with worse OS using the LLMPP FL GSE16131 series. The GSEA plot showed an enrichment of the NFkB pathway towards the FL "dead - high *TNFRSF14* phenotype". Protein-protein interaction analysis of the *TNFRSF14-NFkB* pathway with molecular actions were as follows: green (activation), red (inhibition), blue (binding), pink (posttranslational modification).

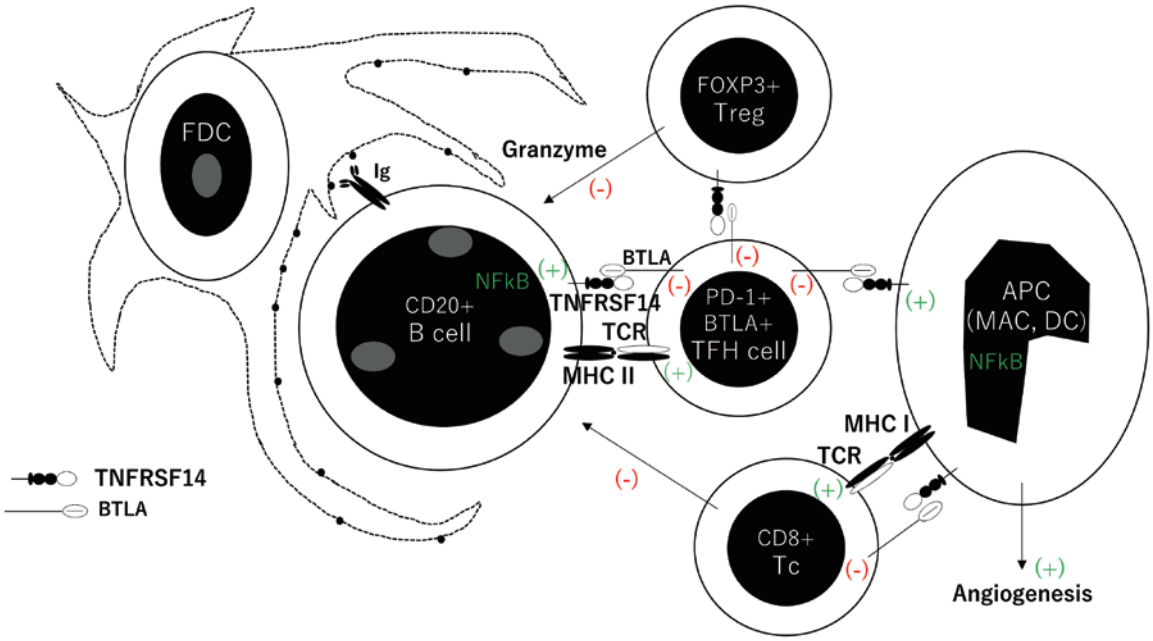

Fig 7. TNFRSF14 (HVEM) and BTLA pathway and the immune microenvironment in FL.
This Figure shows the TNFRSF14 and BTLA expression in the FL microenvironment and the possible effect of the engagement of the ligands and receptors, either activating or inhibiting. Our hypothesis is that the resulting nonequilibrium of factors is what triggers the development and/or progression of FL towards a poor prognosis and transformation to DLBCL. This Figure is based on our experimental results as well as other publications.[16,17,36-38,41-44]

marked by exacerbated lymphoid stroma activation and increased recruitment of T follicular helper (TFH) cells in a FL mice model.[41] These changes result from the disruption of inhibitory cell-cell interactions between the TNFRSF14 and BTLA receptors.[41] In our project the expression of TNFRSF14 was independent of the other components of the microenvironment that we had analyzed: follicular TNFRSF14 did not correlate with follicular PD-1 or follicular FOXP3. Total TNFRSF14 did not correlate with total FOXP3 as well. Total TNFRSF14 and total BTLA didn't correlate either as continuous or as ordinal variable. In a similar way, a mutual exclusive pattern of TNFRSF14 and BTLA expression in human FL samples was also described by Michael Boice *et al*.[41] TNFRSF14 is considered as a tumor suppressor gene but this is when we focus on the effect on BTLA receptor that delivers inhibitory signals. On the other hand, TNFRSF14, which is bidirectional, can also deliver stimulatory signals and therefore could be considered as having a tumor promoting function (Figure 7).[38,42-44] The relationship with deletion, mutation and protein expression remains to be solved in future.

In summary, we found that high BTLA expression is associated with good prognosis, high TNFRSF14 expression with a bad prognosis in FL including a higher risk of transformation, and that tFL expresses low BTLA but high TNFRSF14. These observations suggest that the co-stimulatory and co-inhibitory BTLA-TNFRSF14 pathway plays a relevant role in the pathogenesis of FL.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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