

Deep immunophenotypic dissection and clinical impact of T cells in the follicular lymphoma microenvironment

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Abstract

Follicular lymphoma (FL) is an indolent B-cell lymphoma with a heterogeneous disease course, and patients may not require immediate treatment upon diagnosis. Scrutiny of its microenvironment may provide key insights into lymphomagenesis and enhancement of therapeutic options. We analyzed the T-cell composition of a large, well-annotated follicular hyperplasia (FH; N=43) cohort utilizing standardized high dimensionality flow cytometry (>150,000 cells analyzed/sample) and a novel reproducible analytical pipeline leading to identification of even minor T-cell subsets. This baseline reference set was compared to prospectively collected FL samples (N=91) from untreated patients (FL-UT) and patients with relapsed/refractory disease (FL-RR). Compared to FH, both FL-UT and FL-RR specimens exhibited depletion of CD4⁺ and CD8⁺ naïve subsets and were characterized by an immune suppressive microenvironment enriched in specific inhibitory T cells, along with exhausted memory T cells overexpressing varying combinations of immune checkpoint receptors. FL specimens showed enrichment of T-follicular regulatory cells (TFR) and two highly suppressive regulatory T-cell (T-reg) populations expressing TIGIT and CTLA4 (TC) and PD1, TIGIT, CTLA4, and TIM3 (PTCTi). FL-UT cases with either increased T-reg TC or increased T-follicular helper cells (TFH) showed reduced time to first treatment (≤ 3 months). Our study suggests that changes in the balance between TFR, T-reg and TFH may lead to greater tumor growth and identifies factors that are associated with earlier time to treatment in FL-UT. We also identified specific combinations of immune checkpoint receptors that may be used to target specific inhibitory T-cell subsets and regulatory cells in FL to increase anti-tumor immune response.

Introduction

Follicular lymphoma (FL) is the most common type of indolent non-Hodgkin lymphoma.¹ Despite improvements in therapy, FL remains incurable with standard therapy, and most patients have multiple relapses.² The disease course is heterogeneous, with a subset of patients having early progression and transformation to aggressive B-cell lymphomas, while the others have indolent disease and do not require immediate treatment. Several markers and indices have been studied in pursuit of better prognostication of FL, though these are not routinely used to guide management.³⁻⁷ Therefore, improvements in prognostic and therapeutic approaches are needed.

Prior studies have suggested that the non-malignant immune cells present in the FL tumor microenvironment (TME) may predict survival and patient outcome.⁸⁻¹⁶ The

neoplastic B cells in FL are dependent on the dynamic bidirectional communication with their TME for their proliferation and survival.^{15,17-19} Dissection of TME composition provides valuable insight into lymphomagenesis, better prognostication, and enhancement of therapeutic options. However, FL TME has been partially studied employing only a limited number of samples. Comprehensive and large scaled studies characterizing FL TME utilizing a robust, reproducible approach for analyzing the multidimensional data are currently lacking. Furthermore, studies elucidating the dynamics of TME change during lymphoma treatment and in relapse settings are crucial.

It is also essential to have extensive repositories of normal/reactive tissue to serve as a basis for future discoveries and translational cancer research. Reactive lymph nodes (LN) reveal structural and functional differences depending on their site of drainage.²⁰ Thus, large scale studies describing

and defining the T-cell repertoire in normal reactive LN are required. To address this, we created a highly detailed T-cell subset library of reactive LN from a wide range of sites. Since LN with follicular hyperplasia (FH) are physiologically the closest comparator group for FL, a cohort of FH cases was analyzed as the baseline reference set. The second aim of the study was to compare this baseline reference set with tumor-infiltrating T-cell subsets in FL, including untreated patients (FL-UT) and patients with relapsed/refractory disease (FL-RR), to construe FL TME and its evolution with treatment. Flow cytometry is a powerful and reproducible method for single-cell analysis. Thus, we utilized a robust standardized high dimensionality flow cytometry approach along with a novel reproducible analytical pipeline.

Methods

Case selection and retrospective clinical data collection

A cohort of LN samples displaying immunophenotype consistent with FH, defined as at least 10% of B cells showing germinal center B-cell phenotype with intermediate CD10 and CD38 expression, was obtained from the Memorial Sloan Kettering Cancer Center flow cytometry laboratory database. In cases with concurrent histologic specimens, FH was further verified by morphology. A second cohort of FL cases was obtained through integrated flow cytometric and morphologic analysis which was independently reverified by PG, MR, and A.Dogan according to the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues (2017).¹ The neoplastic B cells were identified by flow cytometry as a discrete B-cell population with an aberrant immunophenotype including dim CD19, CD10, dim/negative CD38, dim CD20, dim/intermediate CD45 and light chain restriction. Clinical data was retrospectively collected for all FL patients including basic demographic data, clinical characteristics, treatment, and outcome data. The Institutional Review Board of Memorial Sloan Kettering Cancer Center approved the study.

Antibodies

Two panels (16 antigens/9 colors; *Online Supplementary Table S1*) were utilized for the initial characterization and quantification of the B cells, T cells, and natural killer (NK) cells by flow cytometry. In addition, two research panels (23 antigens/18 colors) were developed for this study. Research panel I was developed to identify the T-cell subsets (CD4, CD8 and CD4/CD8 double-negative T cells), their activation state (naïve, central memory, effector memory, effector, T-memory stem cells), and their immune checkpoint expression (TIGIT, TIM3, PD-1, CD96, LAG3, CTLA4, CD73). Research panel II was used to further characterize the CD4⁺ subsets, identified through panel I, with chemokine receptors CXCR5, CXCR3, CCR6, and CCR10. Each panel was validated by performing Fluorescence Minus One, single-stain, and precision tests. Samples were

acquired on BD LSR Fortessa X-20 flow cytometer with 18 detectors and BD FACSCanto flow cytometer (for details see the *Online Supplementary Appendix*). Immunohistochemistry (IHC) studies (*Online Supplementary Table S2*) were performed for diagnostic purposes, and additional stains were performed to study the topographic distribution of various T-cell subsets on ten FH, eight FL-UT and five FL-RR excisional LN specimens. In addition, multiplex immunofluorescence (MxIF) assay was performed targeting CD3, CD20, CD4, FoxP3, PD-1 and BCL-6 antigens (*Online Supplementary Table S3*) on three excisional specimens each of FH, FL-UT and FL-RR cases.

Analytical pipeline and T-cell subsets

Research panel I was analyzed using a new analytical pipeline that was specifically designed to reduce human bias during cluster identification (*Online Supplementary Figure S1*). Briefly, T cells were identified using CD3 and scatter parameters and used as input for the analytical pipeline. To minimize technical variation in sample acquisition over time we used the R-based algorithm GaussNorm.²¹ To avoid computational artifacts due to misreading of high-density regions, the following channels were excluded from the normalization: BV480-CCR7, APC-CTLA4, BUV395-TIGIT, and BV605-PD1. Eight thousand CD3⁺ events from each sample were concatenated, and the file generated (containing both FH and FL specimens) was used to identify the T-cell subsets by combining a dimensionality reduction technique (UMAP)²² with an unsupervised clustering algorithm (FlowSOM)²³ using the OMIQ software from Dotmatics. Expert manual sub-clustering was performed to refine phenotypes that showed excessive heterogeneity. Utilizing data from all the 134 samples (43 FH and 91 FL) we identified 13 CD4⁺ and six CD8⁺ T-cell subsets through this pipeline (Figure 1). Each population was characterized using a previously well-described phenotype reflecting physiological functions.^{24,25} Their detailed phenotypes are illustrated in Figure 1C.

The phenotype observed in the TFR, TFH and T-reg populations with research panel I, was reproduced through manual gating using the common markers in research panel II for further characterization.

Results

Cohort clinicopathologic characteristics (Table 1)

Forty-three FH and 91 FL cases including 54 FL-UT and 37 FL-RR were obtained between January 2019 and December 2020. Patient characteristics are summarized in Table 1. In the FL-UT cohort most of the cases (43 of 54) were diagnosed as grade 1-2, with nine grade 3A and two grade 3B FL. At a median follow-up of 2.4 years, 36 patients had initiated treatment for FL at a median of 31 days (interquartile range, 21-89) after the biopsy, and ten patients experienced disease progression within 24 months of first therapy initiation (POD24). The median progression-free

survival (PFS) from the time of front-line therapy in treated patients was 2.25 years. Transformation to diffuse large B-cell lymphoma (DLBCL) was seen in four patients. In the FL-RR cohort grade 1-2 was observed in 31, grade

3A in five, and grade 3B FL in one of the 37 samples. Prior to biopsy, FL-RR cohort patients had received a median of one (range, 1-6) prior lines of therapy, including anti-CD20 monoclonal antibody-containing therapy in 34 patients and

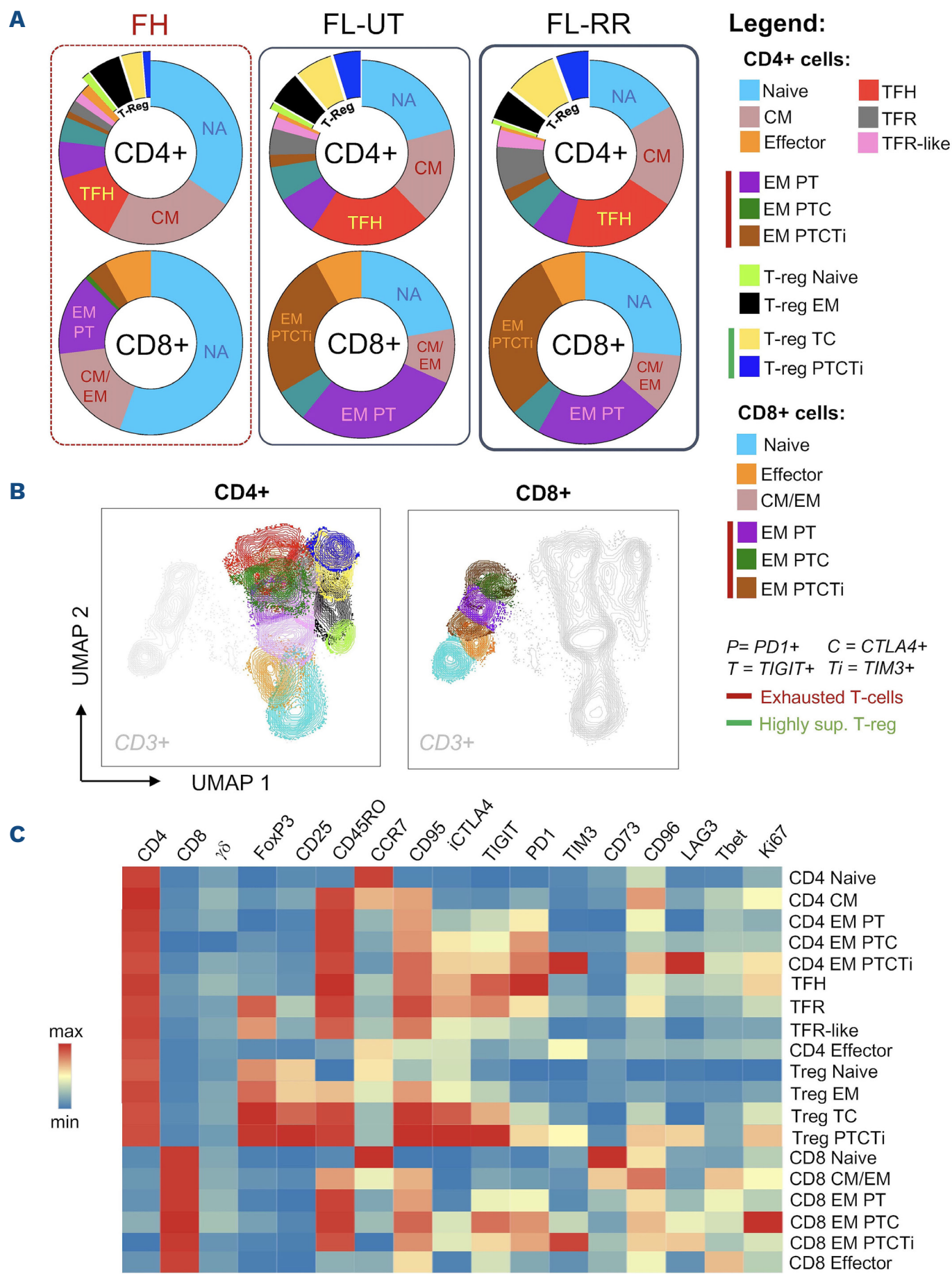


Figure 1. Distribution and immunophenotyping of CD3⁺ T cells in reactive follicular hyperplasia, untreated follicular lymphoma and relapsed/refractory follicular lymphoma lymph node specimens. Using the pipeline illustrated in *Online Supplementary Figure S1*, 13 CD4⁺ and 6 CD8⁺ T-cell subsets were identified. (A) Distribution of CD4⁺ and CD8⁺ T-cell subsets in follicular hyperplasia (FH), untreated follicular lymphoma (FL-UT) and relapsed/refractory follicular lymphoma (FL-RR). (B) Uniform Manifold Approximation and Projection (UMAP) of CD4⁺ and CD8⁺ T-cell subsets in FH, FL-UT and FL-RR. (C) Heatmap generated using the mean surface marker expression in each cluster.

Table 1. Patient clinicopathologic characteristics.

Characteristics at time of biopsy	Follicular hyperplasia	Follicular lymphoma	
		Untreated	Relapsed/refractory
N of cases	43	54	37
Age in years at biopsy, median (range)	56 (12-86)	65 (33-89)	56 (37-79)
Sex: F/M	31/12	32/22	14/23
Anatomic site			
Nodal, N (%)	43 (100)	53 (98)	32 (86)
Axillary	19	6	5
Cervical	13	14	3
Head	0	3	3
Inguinal	2	16	10
Retroperitoneal	1	7	3
Thoracic	4	0	2
Tonsil	2	1	1
Other	2	6	5
Spleen, N (%)	0 (0)	1 (2)	0 (0)
Extranodal, N (%)	0(0)	0 (0)	4 (11)
Diagnosis, N (%)			
FL grade 1-2	-	43 (80)	31 (84)
FL grade 3A	-	9 (17)	5 (14)
FL grade 3B	-	2 (4)	1 (3)
Stage at biopsy, N (%)			
1-2	-	8 (17)	2 (6)
3-4	-	40 (83)	33 (94)
FLIPI at sampling, median (range)	-	2 (0-5)	2 (0-5)
0-1, N (%)	-	10 (21)	10 (29)
2, N (%)	-	20 (42)	13 (37)
3-5, N (%)	-	18 (38)	12 (34)
History of aggressive lymphoma prior to biopsy, N	-	0	9
N of prior lines of therapy, median (range)	-	0 (0)	1 (1-6)
1	-	-	8
2	-	-	1
3	-	-	0
4	-	-	2
>4	-	-	3
POD24 after initial therapy, N (%)	-	-	17 (49)
Prior anti-CD20, N (%)	-	-	34 (97)
Prior chemotherapy, N (%)	-	-	28 (80)
Anthracycline-containing, N (%)	-	-	21 (60)
Bendamustine-containing, N (%)	-	-	10 (29)
Post-biopsy outcomes	Follicular hyperplasia	Follicular lymphoma	
		Untreated N=48	Relapsed/refractory N=35
Treated after biopsy, N (%)	-	36 (75)	34 (97)
Median time in days to treatment (range)	-	32 (6-591)	30 (9-556)
Treated within 6 months of sample, N (%)	-	29 (60)	30 (86)
Treated more than 6 months after sample, N (%)	-	7 (15)	4 (11)
Treatment regimen, N (%)	-		
Radiation only	-	6 (17)	5 (15)
Rituximab monotherapy	-	5 (14)	6 (18)
R- or O-chemotherapy	-	24 (67)	9 (26)
Bendamustine	-	8 (22)	5 (15)
CHOP	-	15 (42)	3 (9)
Other	-	1 (3)	1 (3)

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Post-biopsy outcomes	Follicular hyperplasia	Follicular lymphoma	
		Untreated N=48	Relapsed/refractory N=35
R- or O-lenalidomide	-	1 (3)	8 (24)
Other	-	0 (0)	6 (18)
POD after treatment, N (%)	-	13 (36)	20 (59)
Progression within 24 months (POD24), N (%)	-	10 (28)	16 (47)
Median follow-up in years (range)	-	3.1 (0.5-3.7)	3.1 (0.4-3.9)
Transformation during follow-up, N (%)	-	4 (8.3)	3 (8.6)
Death during follow-up, N (%)	-	4 (8.3)	5 (14)

F: female; M: male; FL: follicular lymphoma; FLIPI: Follicular lymphoma international prognostic index; POD24: progression of disease within 24 months of initial treatment; R-: rituximab; O-: obinutuzumab.

chemotherapy in 28 patients, with anthracycline-containing regimens in 21 patients. POD24 had occurred in 49% of patients. Transformation to DLBCL, with treatment and complete remission of aggressive disease, had previously occurred in nine patients. The median time from last treatment to biopsy time was 1.8 years (range, 0.002-12.5) with seven patients receiving treatment within last 1 year of sampling. All but one patient (N=34) in the FL-RR cohort received subsequent therapy, a median of 30 days (IQR, 20-59) after biopsy. The median PFS after this new line of treatment initiation was 10.1 months, with 16 patients having POD within 24 months of treatment initiation and three having subsequent transformation after biopsy.

Characterization of the T-cell repertoire in follicular hyperplasia lymphnode specimens

To create a baseline, we first analyzed the T-cell subset distribution in the FH cohort (Figure 1). Naïve cells (CD45RO⁺CCR7⁺) were the most represented subsets in both CD4 and CD8 compartments (28.2% and 6.2% of CD3⁺ T cells, respectively), followed by memory cells not expressing any inhibitory immune checkpoint receptors namely CD4⁺ central memory (CM; CD45RO⁺CCR7^{dim+}), and a distinct CD8⁺ subset showing phenotypic overlap between central memory and effector memory T cells (CD45RO⁺, CCR7^{dim+/-}, CD95⁺, CD-73^{dim+}) designated as CM/EM. In the CD4 compartment, high numbers of T-follicular helper cells (TFH) expressing bright PD1 along with TIGIT and CTLA4 were also noted. While the T-follicular regulatory cells (TFR) expressing FoxP3, dim/intermediate PD1, and dim/negative CD25 were rare. Most of the regulatory T cells (T-reg; CD4⁺FoxP3⁺CD25⁺) showed a non-inhibitory phenotype, characterized by a dim/negative expression of CTLA-4 and CD45RO (T-reg naïve and T-reg EM). A small subset of FH patients (N=9) had a prior history of chemo/radiotherapy for other non-FL cancers. Comparison of the T-cell repertoire between patients with no history of prior chemo/radiotherapy and patients with prior therapy revealed no significant differences except for two small CD4⁺ subsets (naïve T-reg and EM T-reg; $P\leq0.05$; *Online Supplementary Figure S2*). No significant differences were observed on evaluating the T-cell subset distribution

among the different sites in FH LN (*Online Supplementary Figure S3*).

Follicular lymphoma tumor microenvironment shows significant difference from follicular hyperplasia T-cell repertoire (Table 2)

From the analysis performed on the initial characterization flow cytometry panel (*Online Supplementary Table S4*) we observed a significant decrease in the number of NK cells, T cells and as expected non-neoplastic B cells while the B:T ratio was significantly higher in both the FL cohorts, in comparison to FH specimens (*Online Supplementary Figure S4*).

In contrast to FH specimens, FL specimens revealed a significant depletion of naïve T cells, in both CD4 and CD8 subsets (Figure 1A).

Follicular lymphoma specimens show an immune suppressive microenvironment

FL specimens were enriched in two subsets of activated T-reg: one expressing TIGIT, CTLA4 (T-reg TC; $P=0.001$ for both FL-UT and FL-RR) and the other expressing PD1, TIGIT, CTLA4, and TIM3 (T-reg PTCTi; $P=0.001$ for both FL-UT and FL-RR; Figure 2A-D). T-reg subsets expressing various immune checkpoint receptors have previously shown to be highly suppressive.²⁶⁻²⁹ With the expansion of these highly suppressive T-reg subsets (T-reg TC and T-reg PTCTi) a concomitant reduction in CD4 effector cells was noted in the FL specimens (Figure 1A; Table 2). This was further accentuated in the FL-RR cohort with CD4 effector cells (*Online Supplementary Figure S5*).

In addition, the FL samples also revealed an expansion in CD4⁺ populations known to be responsible for germinal center formation, maturation, and maintenance, namely the TFH and TFR. Both these subsets were present at a higher frequency in the FL samples (FL-UT; $P=0.001$ for both TFH and TFR and FL-RR; $P=0.002$ for TFH and 0.001 for TFR) compared to FH samples (Figure 2A-D).

Both T-reg and TFR populations show phenotypic overlap with the expression of CD45RO, FoxP3, CTLA4, and TIGIT; however, were distinguished based on the differential

Table 2. T-cell repertoire of the follicular hyperplasia and follicular lymphoma cohorts.

Population name			Immunophenotype	Median (count/1,000)			Adjusted pairwise <i>P</i>		
				FH	FL-UT	FL-RR	FH vs. FL-UT	FH vs. FL-RR	FL-UT vs. FL-RR
CD4 ⁺	Naïve		CD45RO ⁻ , CCR7 ⁺	296.445	165.89	78.352	0.001 ↓	0.001 ↓	NS
	Memory	CM	CD45RO ⁺ , CCR7 ⁺ , CD95 ⁺	200.922	127.411	128.259	0.002 ↓	0.003 ↓	NS
		EM PT	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , TIGIT ^{dim} , PD1 ^{dim}	39.289	47.593	41.832	NS	NS	NS
		EM PTC	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , TIGIT ⁺ , PD1 ⁺ , CTLA4 ⁺	25.973	39.788	34.659	NS	NS	NS
		EM PTCTi	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , TIGIT ⁺ , PD1 ⁺ , CTLA4 ⁺ , TIM3 ⁺	5.368	10.676	9.146	0.001 ↑	0.002 ↑	NS
	TFH		CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ⁺ , TIGIT ⁺ , PD1 ^{bright}	59.724	148.957	137.775	0.001 ↑	0.002 ↑	NS
	TFR		FoxP3 ⁺ , CD25 ^{dim/-} , CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ⁺ , TIGIT ⁺ , PD1 ⁺ , CXCR5 ⁺	10.605	32.946	48.33	0.001 ↑	0.001 ↑	0.018 ↑
	TFR-like		FoxP3 ⁺ , CD25 ⁻ , CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ^{dim} , TIGIT ^{dim} , PD1 ⁻	12.812	15.505	15.195	NS	NS	NS
	Effector		CD45RO ⁻ , CCR7 ⁻ , CD95 ^{dim} , CD96 ⁺ , TIM3 ⁺	13.389	4.691	3.536	0.001 ↓	0.001 ↓	NS
	T-reg	Naïve	FoxP3 ⁺ , CD25, CD45RO ⁻ , CCR7 ⁻ , CD95 ⁻	8.755	6.156	4.95	NS	0.001 ↓	0.04 ↓
		EM	FoxP3 ⁺ , CD25 ^{dim} , CD45RO ^{dim} , CCR7 ⁻ , CD95 ^{dim} , CTLA4 ^{dim} , TIGIT ^{dim}	44.577	40.958	35.595	NS	NS	NS
		TC	FoxP3 ⁺ , CD25 ⁺ , CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ⁺ , TIGIT ⁺ , PD1 ^{dim}	21.584	48.132	51.255	0.001 ↑	0.001 ↑	NS
		PTCTi	FoxP3 ⁺ , CD25 ⁺ , CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ⁺ , TIGIT ⁺ , PD1 ⁺ , TIM3 ⁺	5.018	22.977	30.144	0.001 ↑	0.001 ↑	NS
CD8 ⁺	Naïve		CD45RO ⁻ , CCR7 ⁺ , CD73 ⁺	63.242	24.299	31.259	0.001 ↓	NS	NS
	Memory	CM/EM	CD45RO ⁺ , CCR7 ^{dim+/-} , CD95 ⁺ , CD73 ^{dim}	21.53	14.263	17.02	NS	NS	NS
		EM PT	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CD96, TIGIT ^{dim} , PD1 ^{dim}	21.095	43.211	52.553	0.001 ↑	0.001 ↑	NS
		EM PTC	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , CTLA4 ⁺ , TIGIT ⁺ , PD1 ⁺ , Ki67 [±]	1.06	6.924	10.375	0.001 ↑	0.001 ↑	NS
		EM PTCTi	CD45RO ⁺ , CCR7 ⁻ , CD95 ⁺ , TIGIT ⁺ , PD1 ⁺ , TIM3 ⁺ , CTLA4 ⁺	3.456	30.669	37.726	0.001 ↑	0.001 ↑	NS
	Effector		CD45RO ⁻ , CCR7 ⁻ , CD95 ⁺ , TIGIT ^{dim} , Tbet ^{dim}	8.339	10.397	12.209	NS	NS	NS

CM: central memory; EM: effector memory; FH: follicular hyperplasia; FL-RR: Follicular lymphoma relapsed/refractory; FL-UT: follicular lymphoma untreated; TFH: T-follicular helper cells; TFR: T-follicular regulatory cells; PT: PD1⁺TIGIT⁺; PTC: PD1⁺TIGIT⁺CTLA-4⁺; PTCTi: PD1⁺TIGIT⁺CTAL-4⁺TIM3⁺; TC: TIGIT⁺CTLA-4⁺; NS: not significant.

expression of CD25 (brighter on T-reg; Figures 1C and 2C) and CXCR5 evaluated on research panel II (predominantly positive on TFR; Figure 2D; *Online Supplementary Figure S6*).

Follicular lymphoma T-cell microenvironment shows an expansion of exhausted memory T cells

In addition to the expansion of the inhibitory subsets of T-reg and TFR, FL specimens showed a marked increase in specific exhausted T-cell subsets (Figure 3). The concentration of CD4 EM expressing PD1, TIGIT, CTLA4 and TIM3 (EM PTCTi) was 2-fold higher in both FL cohorts in comparison to FH specimens (Figure 3A). However, the fre-

quency of CD4 EM expressing PD1, TIGIT (EM PT) and PD1, TIGIT, CTLA4 (EM PTC) did not change among the cohorts. In contrast, CD8 EM were characterized by the expansion of double-positive PD1, TIGIT cells (EM PT), triple-positive PD1, TIGIT, CTLA4 cells (CD8 EM PTC) and cells expressing bright TIM3 in addition to the other three inhibitory receptors (EM PTCTi) (Figure 3B).

Alterations in follicular lymphoma microenvironment in patients with relapsed/refractory disease in comparison to untreated patients

An expansion of TFH and TFR populations was observed

in both FL-UT and FL-RR cases. However, the FL-RR cases revealed a significantly greater expansion of the TFR compared to FL-UT specimens (*Online Supplementary Figure S7*; $P=0.018$). In contrast, naïve T-reg, known to be resistant to apoptosis induction and less immunosuppressive than memory T-reg, were reduced in FL-RR in comparison to both FH and FL-UT samples (*Online Supplementary Figure S7*; $P=0.04$).

Topographic evaluation of T-cell microenvironment of follicular hyperplasia, untreated follicular lymphoma, relapsed/refractory follicular lymphoma

The spatial distribution of the various T-cell subsets was examined using MxIF assay and immunohistochemistry (IHC) studies. In the FH samples, MxIF studies revealed the presence of CD3⁺CD4⁺PD1⁺BCL6⁺ TFH cells within the germinal centers, polarized predominantly to the light zone (Figure 4A). Corroborating these findings, the IHC studies also revealed

CD4⁺ PD1^{brightly+} TFH cells (*Online Supplementary Figure S8*) in a similar distribution, that were also positive for ICOS, CTLA4, and TIGIT. Few CD3⁺CD4⁺FoxP3⁺PD1⁺ T-reg (additionally positive for CD25 by IHC) were evenly distributed in the sinusoids and medullary cords. Rare cases revealed increased T-reg cells. On further investigation, these were found to have recent histories of carcinoma (lung and breast). Similar to FH LN specimens, these populations were visualized on FL LN tissue sections utilizing MxIF and IHC. The TFH cells were seen within the neoplastic follicles. Cells expressing CD3, CD4, FoxP3, PD1^{dim}, BCL6 and ICOS were identified as TFR cells and were seen both within and surrounding the neoplastic follicles (Figure 4A; *Online Supplementary Figure S8*). These studies also confirmed the presence of a higher number of TFR cells in FL-RR specimens (Figure 4A; *Online Supplementary Figure S8*). The images were further analyzed using the image analysis platform HALO and the percentage of TFH and TFR as % of

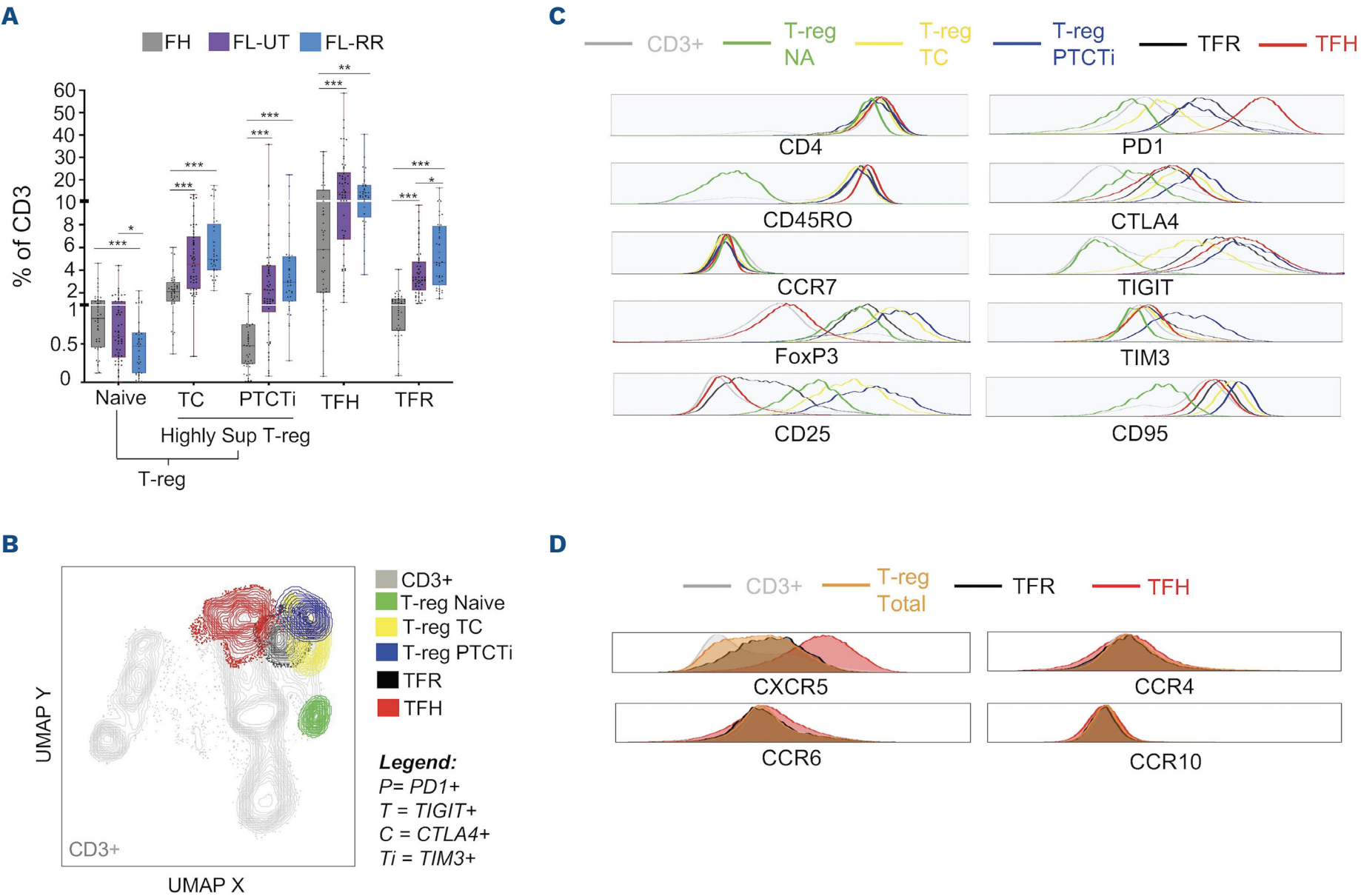


Figure 2. Distribution of T-regulatory cells (naïve and highly suppressive), T-follicular helper cells and T-follicular regulatory cells in follicular hyperplasia, untreated follicular lymphoma and relapsed/refractory follicular lymphoma. (A) Histograms showing percentage of T-regulatory cells (T-reg), T-follicular helper cells (TFH) and T-follicular regulatory cells (TFR) subsets in the CD3⁺ compartment. (B) Uniform Manifold Approximation and Projection (UMAP) of T-reg, TFH and TFR. (C) Phenotype comparison between the T-reg subpopulations, TFR, TFH and the total CD3⁺ T-cell population. (D) Additional characterization of total T-reg, TFR and TFH using research panel II and the gate strategy indicated in *Online Supplementary Figure S5*. *** $P \leq 0.001$; ** $P \leq 0.002$; * $P \leq 0.05$.

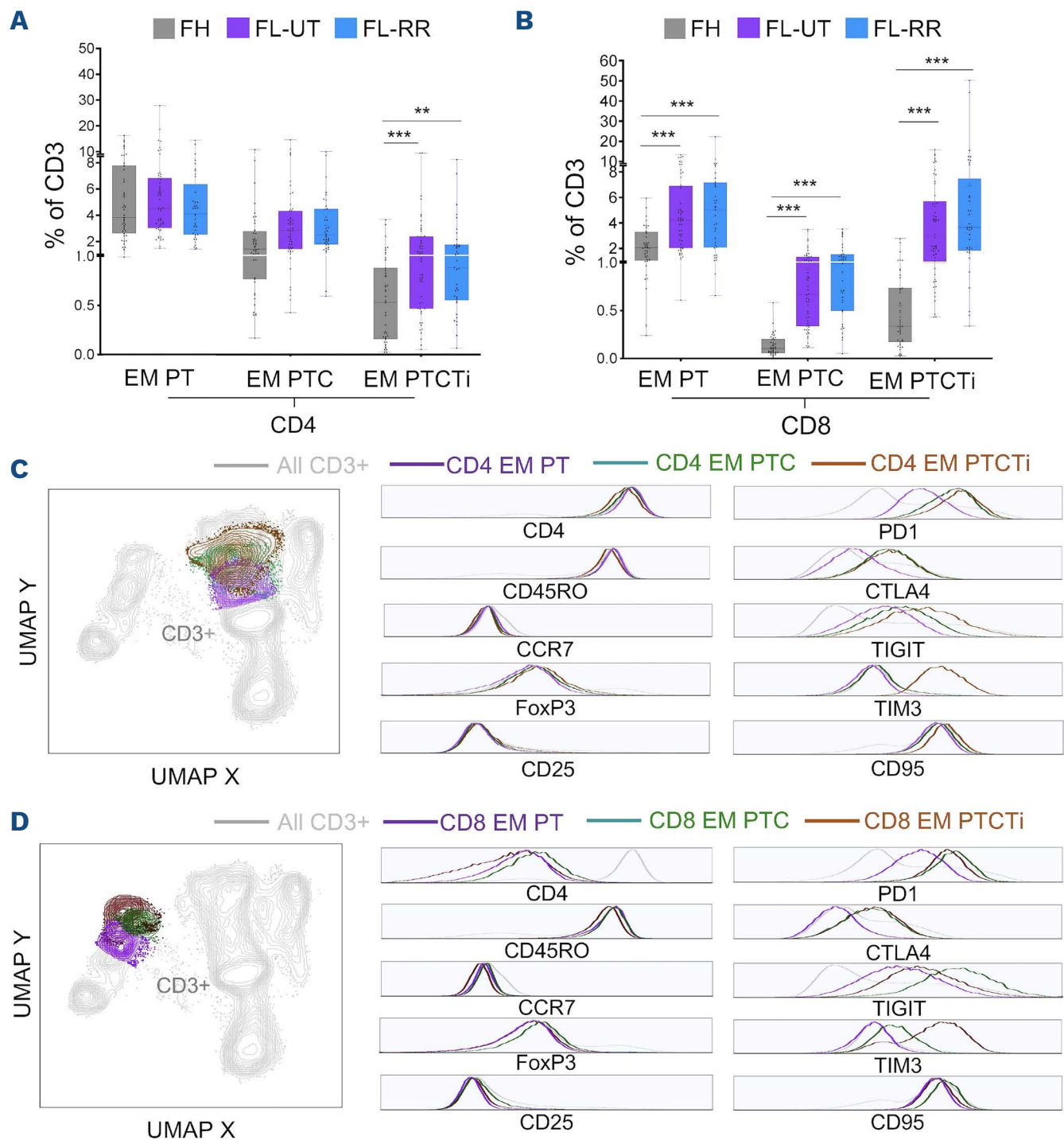


Figure 3. Distribution of CD3⁺ subpopulations expressing inhibitory immune checkpoint receptors in CD4 and CD8 compartments. (A, B) Distribution of effector memory CD4⁺ and CD8⁺ subpopulations expressing the inhibitory immune checkpoint receptors PD1 and TIGIT (EM PT); PD1, TIGIT and CTLA-4 (EM PTC); PD1, TIGIT, CTLA-4 and TIM3 (EM PTCTi). (C, D) Uniform Manifold Approximation and Projection (UMAP) of various inhibitory immune checkpoint receptors expressing CD4⁺ and CD8⁺ subpopulations (on the left) and their phenotype comparison (on the right). *** $P \leq 0.001$; ** $P \leq 0.002$

total CD3⁺ T cells was calculated. On performing linear regression analysis for these two subpopulations evaluated by MxIF versus flow cytometry, a strong correlation was found for both the populations, with a coefficient of determination (R^2) equal to 0.93 for TFR and 0.8 for TFH subpopulations (Figure 4B).

Immune suppressive microenvironment in follicular lymphoma is associated with earlier initiation of therapy in untreated follicular lymphoma

The association between these immune suppressive populations (T-reg TC, T-reg PTCTi, TFR) and TFH and various pathologic and clinical parameters was examined. First, we analyzed the time to first treatment in FL-UT cohort

as a discrete clinically defined time point; ≤ 3 months, >3 months and patients never receiving any treatment during follow-up. This analysis revealed that the presence of a higher percentage of T-reg TC cells and TFH cells were associated with earlier initiation of therapy in the FL-UT cohort (Figure 5A, B), while T-reg PTCTi and TFR cells did not show an association with time to first treatment (Online Supplementary Figure S9). After adjusting for age, stage and FLIPI scores these results remained significant for the T-reg TC population but not for TFH population (Online Supplementary Tables S5, S6). Employing a different approach, we performed maximally selected rank statistics on the FL-UT cases and estimated optimal cut-off thresholds for T-reg TC and

TFH percentages (2.7% and 4.79% of WBC, respectively) (*Online Supplementary Figure S10*). Utilizing these cut-off thresholds, the time to event analysis revealed that patients with T-reg TC and TFH percentages above the cut-off thresholds had significantly shorter time to first treatment (Figure 5C, D). On a multivariate analysis,

confounding factors of age, stage and FLIPI scores did not change the significance or magnitude of results for both T-reg TC and TFH populations (*Online Supplementary Tables S7, S8*). There was no association between the frequency of T-reg, TFH, or TFR cells and other clinicopathologic parameters

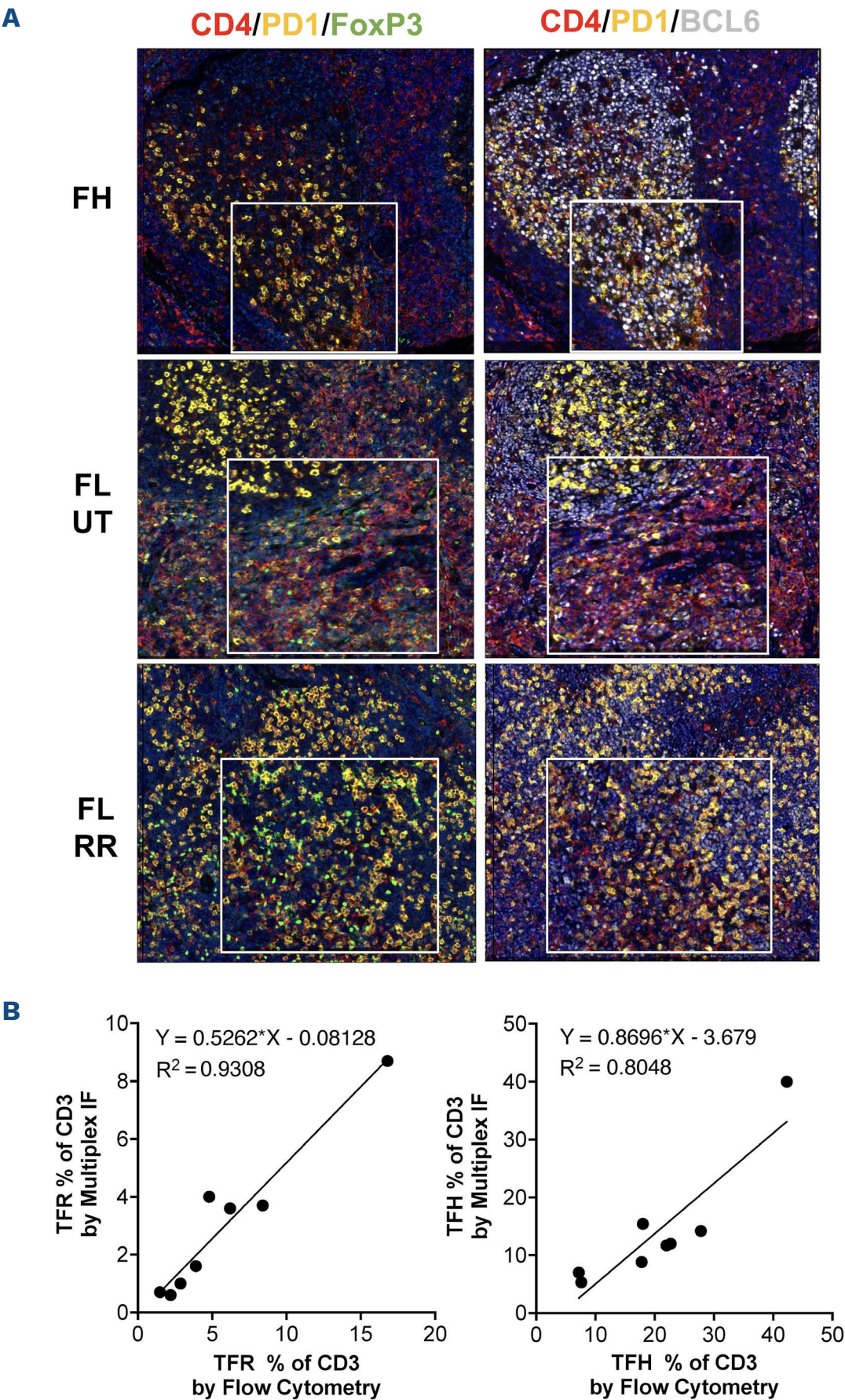


Figure 4. Multiplex immunofluorescence assay using antibodies against CD4, PD1, FoxP3 and BCL6 and its comparison to flow cytometric evaluation on follicular hyperplasia, untreated follicular lymphoma and relapsed/refractory follicular lymphoma specimens. (A) Multiplex immunofluorescence (MxIF) images demonstrating CD4⁺ PD1^(dim)+ FoxP3⁺ T-follicular regulatory cells (TFR) cells and CD4⁺ BCL6⁺ PD1⁺ T-follicular helper cells (TFH) cells on follicular hyperplasia (FH), untreated follicular lymphoma (FL-UT) and relapsed/refractory follicular lymphoma (FL-RR) specimens. (B) Linear regression analysis for TFR and TFH subpopulations as evaluated by MxIF versus flow cytometry.

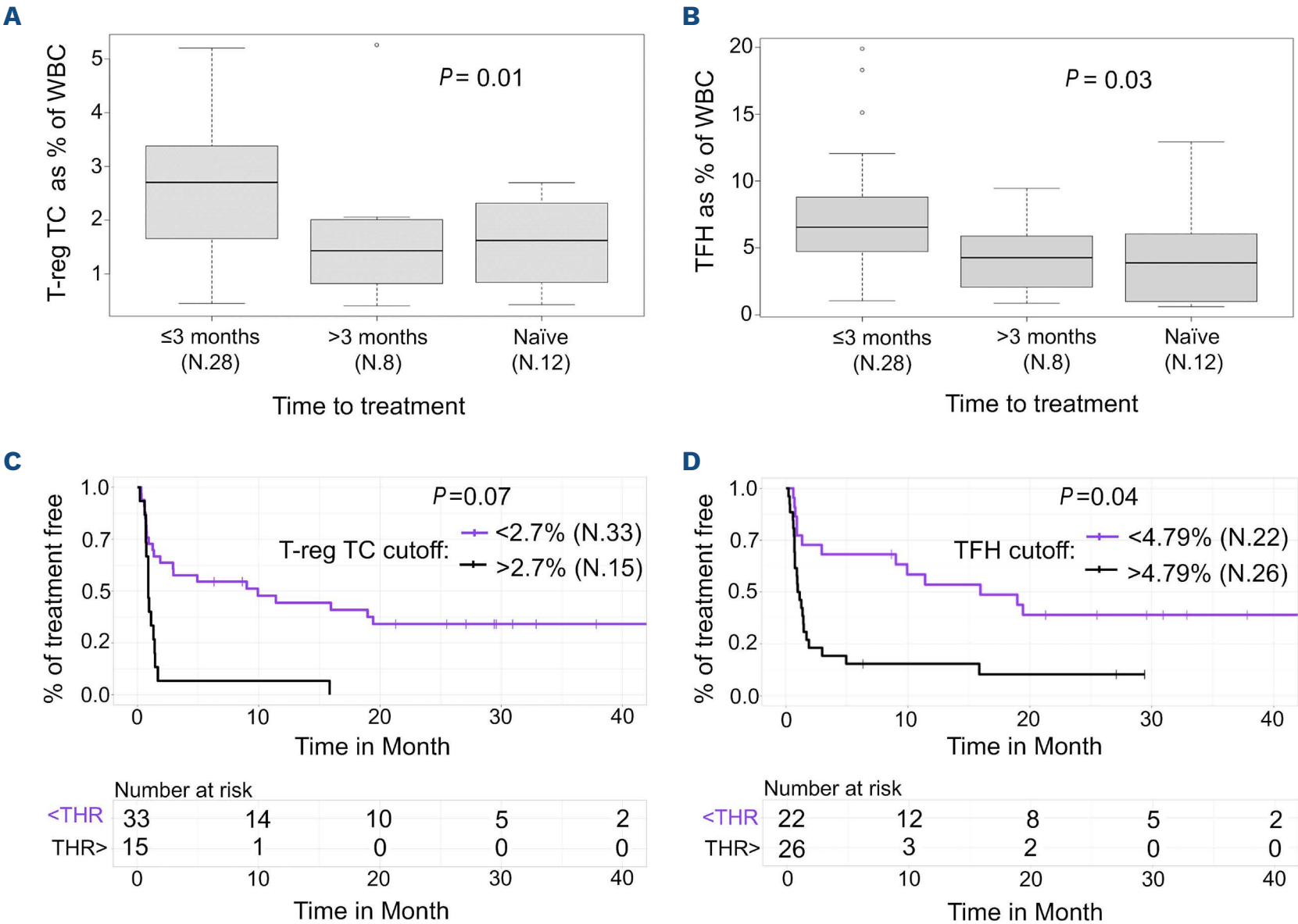


Figure 5. Correlation of T-regulatory cells and T-follicular helper cells with earlier initiation of the therapy in untreated follicular lymphoma cohort. (A, B) T-regulatory cells (T-reg) TC and T-follicular helper cells (TFH) represented as percent of white blood cell (WBC) plotted against time to first treatment (≤ 3 months, N=28; > 3 months, N=8; treatment naïve, N=12). (C) Kaplan Meier curve illustrating time to first treatment of the 2 cohorts based on optimal cutoff threshold for T-reg TC percentage. (D) Kaplan Meier curve illustrating time to first treatment of the 2 cohorts based on optimal cut-off threshold for TFH percentage. TC: TIGIT⁺CTLA4⁺.

namely the tumor grade (grade 1-2 vs. grade 3A/B), lymphoma stage, anatomic site, patient age ($>/< 60$ years), or FLIPI (low/intermediate vs. high) (*Online Supplementary Figure S11*) in both the FL cohorts.

Unsupervised hierarchical clustering based on T-cell subsets distinguished follicular hyperplasia specimens from follicular lymphoma specimens and identified a unique subset of follicular lymphoma cases

To further explore the distribution of various T-cell subsets in an unbiased manner, we took an orthogonal approach and performed unsupervised hierarchical clustering based on T-cell subsets as a percentage of WBC. This revealed three distinct clusters (*Figure 6*). Clusters #1 and #2 were enriched in FL specimens without clear separation between FL-UT and FL-RR specimens. However, FH specimens clustered separately as cluster #3 with 10 FL-UT being admixed in this cluster. The T-cell subsets driving the unsupervised hierarchal clustering were TFH, TFR, T-reg TC, and T-reg PTCTi, which were increased in clusters #1 and #2 (*Online*

Supplementary Figure S12), and CD4 naïve, CD4 CM, and CD4 effector cells, which were decreased in clusters #1 and #2 in comparison to cluster #3. A higher proportion of T-reg TC and TFR in cluster #2 (*Online Supplementary Figure S13*) distinguished it from cluster #1. Cluster #3 was enriched in FH specimens and showed an admixture of ten FL-UT cases (FH-like FL cases). These “FH-like FL” cases had significantly fewer TFH, T-reg TC and T-reg PTCTi in comparison to other FL-UT cases present in clusters #1 and #2 (*Online Supplementary Figure S14*). The pathologic features including the extent of involvement of the specimen (complete vs. partial involvement), anatomic site, and tumor grade of these “FH-like FL” cases showed no significant differences compared to cluster #1 and #2 FL-UT cases. However, of the eight “FH-like FL” cases having available clinical data, only two initiated therapy within 6 months from diagnosis (including 1 with stage 1 disease who received radiotherapy), as opposed to 26 of 40 patients with FL-UT in the other two clusters. They also showed higher proportion of stage 1 disease (3 of 8 vs. 2 of 40) and

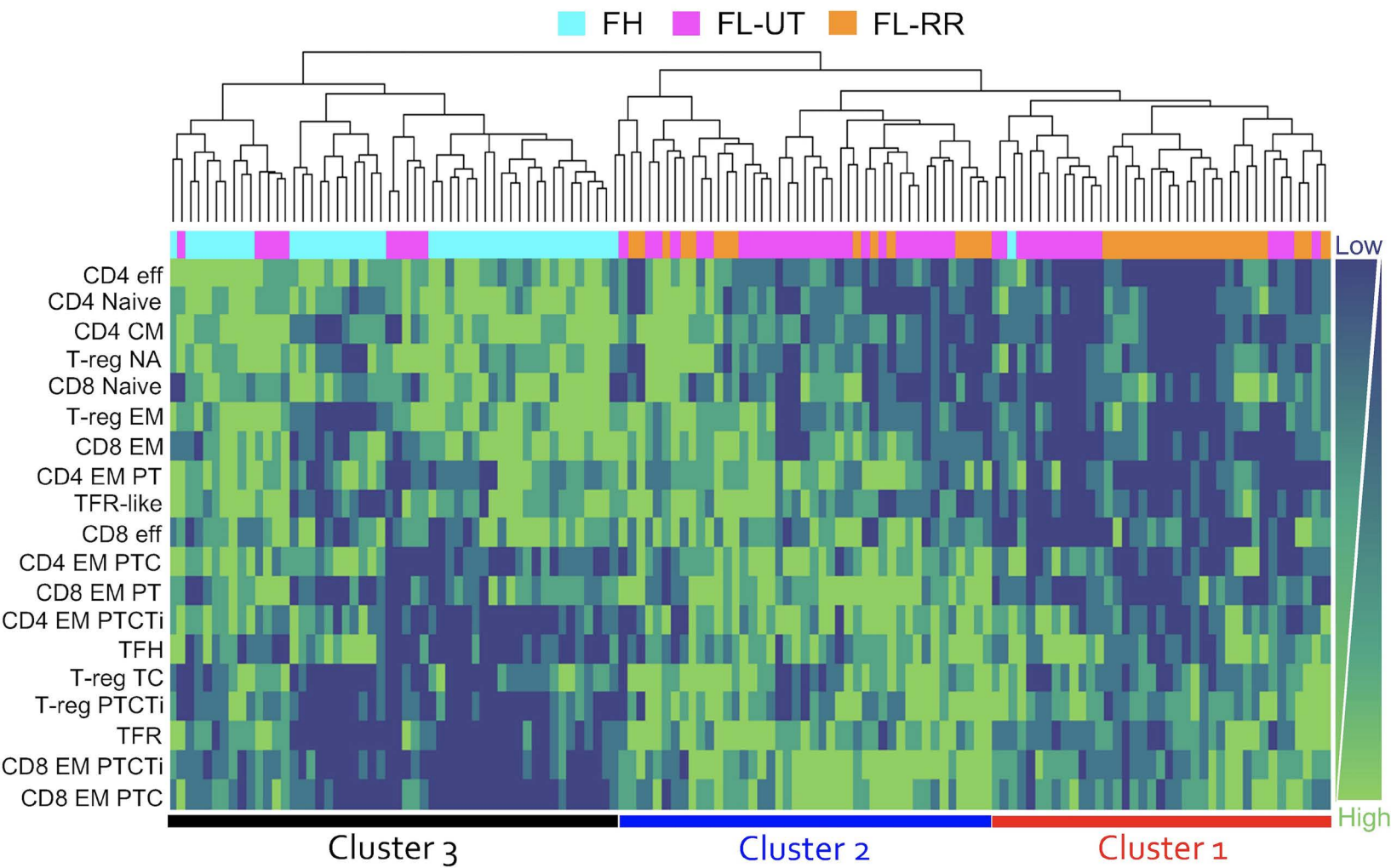


Figure 6. Unsupervised hierarchical clustering of T-cell subsets as a percentage of white blood cell count showing follicular lymphoma cases clustering together in clusters #1 and #2 while follicular hyperplasia cases being enriched in cluster #3. FH: follicular hyperplasia; FL-RR: relapsed/refractory lymphoma; T-reg: regulatory T cell; FL-UT: follicular lymphoma untreated; TFH: T-follicular helper cells; TFR: T-follicular regulatory cells; PT: PD1⁺TIGIT⁺; PTC: PD1⁺TIGIT⁺CTLA-4⁺; PTCTi: PD1⁺TIGIT⁺CTAL-4⁺TIM3⁺; TC: TIGIT⁺CTLA-4⁺; CM: central memory; EM: effector memory.

no patients had a FILPI score of 4-5 (*Online Supplementary Table S9*). Although, there was no significant difference in overall survival, the time to first treatment of the “FH-like FL” cases showed a trend towards longer treatment-free time intervals compared to FL-UT cases in clusters #1 and #2 (*Online Supplementary Figure S15*).

Discussion

This study is the first to combine computational algorithms with single-cell analysis to comprehensively characterize the T-cell composition of a large, well-annotated FH reference set from different anatomic sites. Using this extensive library as a baseline, we were able to examine the T-cell microenvironment of FL specimens, including untreated and previously treated relapsed/ refractory samples. This has important clinical implications as recent studies have highlighted the critical role of TME in the regulation of immune response and the efficacy of anti-tumor therapies. While blocking antibodies against immune checkpoint receptors have not shown much clinical efficacy in FL, these therapies alone or in combination with other therapies, have shown beneficial effect on other lympho-

ma subtypes.³⁰⁻³² Given the development of T-cell-based therapies in FL, including chimeric antigen receptor T cells and bispecific T-cell engaging antibodies, there is greater interest in understanding T-cell microenvironment which may be associated with outcome.³³⁻³⁵ In our study FL specimens revealed a significant depletion of both CD4 and CD8 naïve T cells along with depletion of CD4 CM and CD4 effector cells. Yang *et al.*³⁶ reported a significantly lower number of naïve T cells in FL specimens in comparison to tonsils and the frequency of naïve T cells correlated with improved patient survival. They also reported that activated effector memory cells and naïve T cells were highly represented in tonsil tissue. Another study indicated that a lack of intrafollicular activated CD4 CM T cells predicted early clinical failure in newly diagnosed FL patients.³⁷ We observed that FL specimens were characterized by an immune suppressive microenvironment enriched in inhibitory cells and exhausted memory cells. Two distinct CD4 inhibitory subsets were expanded in FL specimens: T-reg and TFR. T-reg repress the immune response targeting a wide range of immune cells, including lymphocytes, macrophages, and dendritic cells.³⁸ They are comprised of many cell states with specialized regulatory functions.

Studies have shown that intra-tumoral T-reg are overrepresented in B-cell lymphomas and suppress proliferation and cytokine production of infiltrating CD4⁺CD25⁻ T cells.³⁹ Here, we observed the expansion of two highly suppressive T-reg phenotypes: T-reg TC expressing TIGIT and CTLA4 and T-reg PTCTi expressing PD1, TICIT, CTLA4 and TIM3. Several studies reveal that TIGIT⁺ T-reg can specifically suppress the pro-inflammatory Th1 and Th17 (but not Th2) immune response, while T-reg expressing TIM3 are specialized tissue-resident T-reg that accumulate in FL tissue in the early stages and promote neoplastic growth.^{40,41} In keeping with these studies, our analysis revealed that a higher percentage of T-reg TC was associated with earlier initiation of therapy in previously untreated patients.

TFR is a distinct subset of CD4 T-helper cells that express FoxP3, acquire a follicular phenotype, and migrate into germinal centers of the LN, where they exert their specific regulatory role by modulating TFH number and functioning.⁴²⁻⁴⁴ It has been postulated that the balance between TFH and TFR helps shape the humoral response, with TFH promoting the formation and maintenance of germinal centers in secondary lymphoid organs and TFR inhibiting B-cell and TFH activation.⁴²⁻⁴⁴ Both TFH and TFR populations were expanded in FL cases. Our data shows that in FL-UT cases, a higher percentage of TFH was associated with an earlier initiation of therapy. While both TFH and TFR compartments were expanded in FL, the FL-RR cases showed a further expansion of TFR compartment in comparison to FL-UT cases. This likely reflects a more immunosuppressive environment suggesting that a disequilibrium favoring TFR in the TFH/TFR balance may lead to tumor re-growth and possibly therapy-resistant disease in FL.

To further explore the clinical impact, we pursued optimal cut-off points for T-reg TC and TFH (2 subsets which were seen associated with earlier time to first treatment). These were 2.75% and 4.79% T-reg TC and TFH, respectively. At these cut-off percentages, the treatment naïve cases showed clear separation into cohorts requiring early treatment. However, our cohort of untreated patients with complete clinical data was comprised of 48 cases with limited clinical follow-up, limiting conclusions. Further expansion and longer-term follow-up may facilitate the creation of a more comprehensive predictive model including other T-cell subsets.

In line with prior studies,^{15, 45-47} we also observed a marked expansion of exhausted memory T cells in FL, characterized by varying combinations of PD1, TIGIT, CTLA4 and TIM3. While effector memory cells co-expressing PD1, TIGIT and PD1, TIGIT, CTLA4, TIM3 were equally distributed and expanded in both CD4⁺ and CD8⁺ compartments, triple positive PD1, TIGIT, TIM3 and PD1, TIGIT, CTLA4 were specifically expanded only in the CD8 and CD4 compartments, respectively. Therefore, co-targeting the inhibitory receptors PD1 and TIM3, may be an effective strategy in FL to reprimed the CD8 effector functions and, at the

same time, to promote the Th1 pro-inflammatory response by blocking Th1-specific T-reg.

Another unique finding of the current study is the clusters identified by unsupervised hierarchical clustering based on T-cell subsets. Despite being purely based on T-cell microenvironment, the clustering accurately differentiated FL from FH cases (clusters #1 and #2). Cluster #3, though enriched in FH cases, had ten FL-UT cases we describe as “FH-like FL” cases. These “FH-like FL” cases were indistinguishable pathologically from the other FL-UT cases. However, on a closer review of their clinical characteristics, they showed a trend towards longer treatment-free time intervals compared to other FL-UT cases, identifying a potentially more indolent or early group of FL cases with a less disturbed microenvironment.

In summary, FL specimens showed an immune suppressive microenvironment enriched in activated T-reg, TFR, and exhausted memory T cells. This immune suppressive microenvironment was found to be associated with an earlier initiation of therapy in newly diagnosed patients. Furthermore, our data indicate that the altered balance between TFH and TFR occurs in patients with recurrent disease, potentially reflecting a more tumor-prone or therapy-resistant environment. We also identified a specific combination of immune checkpoint receptors that may be used to target specific inhibitory T-cell subsets and regulatory cells in FL in an effort to increase anti-tumor immune response.

Our study provides an extensive, well-characterized library of the FH microenvironment and a robust framework for further investigations with larger study cohorts with longer follow-ups that may be helpful in creating a TME prognostic index for FL. Given that our study utilizes flow cytometry instead of sequencing-based techniques, it might be easily implemented in multiple centers with shorter processing time and lower cost.

Disclosures

MZ is on the advisory board in Leica biosystems. MR is on the scientific advisory board in Auron Pharmaceutical for which he received equity support; he receives research funding from Celularity, Roche-Genentech, Beat AML and NGM; travel fund from BD Biosciences; and does compensate advising for Syros. PG has received research support from Paige.AI. GS provided consultancy and/or received honoraria from AbbVie, Allogene, Beigene, BMS/Celgene, Debiopharm, Epizyme, Genentech/Roche, Genmab, Incyte, Ipsen, Janssen, Kite-Gilead, Loxo, Miltneiy, MorphoSys, Novartis, Rapt, Regeneron, Takeda, and Velosbio; and was also funded in part through the NIH/NCI Cancer Center support grant P30 CA008748. A.Dogan received honoraria and consulting or advisory role fees from Roche, Corvus Pharmaceuticals, Physicians' Education Resource, Seattle Genetics, Peerview Institute, Pharmacyclics, Celgene, Novartis, Loxo, Takeda, and EUSAPharma; and research grants from National Cancer Institute and Roche-Genentech.

Contributions

SED, JB and PG identified patients. PG, MR and ADogan reviewed pathology. SED performed flow cytometric analysis and plotted figures. SED, PG, MZ, MR and ADogan analyzed and reviewed the flow cytometric data. DQ collected clinical data and reviewed it with GS. SB and LB performed multiplex immunofluorescence assay. SB, LB and PG analyzed and reviewed the multiplex immunofluorescence assay. VS and ADerkach performed statistical analysis. ADogan. and MR supervised the project. SED, PG and DQ wrote and all authors provided critical review and revision of each man-

uscript draft and provided approval for submission of the final draft.

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Data-sharing statement

Original data can be shared upon request by contacting the corresponding author.

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