original reports

# Progression of Disease Within 24 Months in Follicular Lymphoma Is Associated With Reduced Intratumoral Immune Infiltration

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abstract

**PURPOSE** Understanding the immunobiology of the 15% to 30% of patients with follicular lymphoma (FL) who experience progression of disease within 24 months (POD24) remains a priority. Solid tumors with low levels of intratumoral immune infiltration have inferior outcomes. It is unknown whether a similar relationship exists between POD24 in FL.

**PATIENTS AND METHODS** Digital gene expression using a custom code set—five immune effector, six immune checkpoint, one macrophage molecules—was applied to a discovery cohort of patients with early- and advanced-stage FL (n = 132). T-cell receptor repertoire analysis, flow cytometry, multispectral immunofluorescence, and next-generation sequencing were performed. The immune infiltration profile was validated in two independent cohorts of patients with advanced-stage FL requiring systemic treatment (n = 138, rituximab plus cyclophosphamide, vincristine, prednisone; n = 45, rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone), with the latter selected to permit comparison of patients experiencing a POD24 event with those having no progression at 5 years or more.

**RESULTS** Immune molecules showed distinct clustering, characterized by either high or low expression regardless of categorization as an immune effector, immune checkpoint, or macrophage molecule. Low programmed death-ligand 2 (PD-L2) was the most sensitive/specific marker to segregate patients with adverse outcomes; therefore, PD-L2 expression was chosen to distinguish immune infiltration$^{HI}$ (ie, high PD-L2) FL biopsies from immune infiltration$^{LO}$ (ie, low PD-L2) tumors. Immune infiltration$^{HI}$ tissues were highly infiltrated with macrophages and expanded populations of T-cell clones. Of note, the immune infiltration$^{LO}$ subset of patients with FL was enriched for POD24 events (odds ratio [OR], 4.32; c-statistic, 0.81; $P = .001$), validated in the independent cohorts (rituximab plus cyclophosphamide, vincristine, prednisone: OR, 2.95; c-statistic, 0.75; $P = .011$; and rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone: OR, 7.09; c-statistic, 0.88; $P = .011$). Mutations were equally proportioned across tissues, which indicated that degree of immune infiltration is capturing aspects of FL biology distinct from its mutational profile.

**CONCLUSION** Assessment of immune-infiltration by PD-L2 expression is a promising tool with which to help identify patients who are at risk for POD24.

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**Data Supplement**

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## INTRODUCTION

Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma.[1] FL is typified by a prolonged, relapsing-remitting course, and median overall survival may extend beyond 18 years.[2,3] The FL International Prognostic Index (FLIPI) is useful for comparing outcomes in clinical trials,[4] but fails to specifically predict the 15% to 30% of patients who experience an early relapse, which is associated with shorter survival.[5,6]

An accepted measure of high-risk patients who experience an aggressive course is defined by progression of disease within 24 months of diagnosis (POD24). POD24 has predicted overall survival of 26% to 50% by 5 years.[6] A pooled analysis of more than 5,000 patients from 13 randomized trials confirmed POD24 as an early clinical end point of poor survival in FL.[7] A pretherapy clinicogenetic risk model was specifically designed to sensitively predict POD24, termed the POD24 prognostic index (POD24-PI), using a modified definition of POD24 for which the risk-defining event was POD24 after first-line treatment initiation.[8] POD24-PI remains untested outside of the originally published populations, and there are unanswered questions regarding the underlying tumor microenvironment (TME) in those who are at high risk of a POD24 event. Better understanding

of the immunobiologic factors that constitute high-risk FL at diagnosis has been identified as a top priority.[9]

The Immune Survival Score (ISS) demonstrated differential expression of specific gene expression signatures that represent intratumoral immune cells in patients with good or poor outcomes.[10] A notable feature of that study was that it included patients with early- and advanced-stage FL, some of whom were initially treated with observation alone. The study population therefore reflected the clinical heterogeneity of FL and, hence, was more likely to represent the breadth of the intratumoral immune response. Although influential, this 2004 study was before the rituximab era, did not test for POD24, and did not take into consideration potentially actionable immune molecules, such as those involved in the programmed cell death 1 (PD-1) axis.

In some solid tumors, low levels of immune infiltration—for example, as measured by T-cell and myeloid cell infiltration—convey inferior outcomes to conventional therapy compared with high immune infiltration (immune infiltration$^{HI}$).[11-13] Furthermore, clinical responses to PD-1 axis blockade occur most often in patients with an immune infiltration$^{HI}$ intratumoral immunophenotype.[14] This suggests that PD-1 axis blockade reverses the local immunosuppression that has developed as an adaption to counteract antitumor immunity within the TME.[15] The biologic importance of the TME in FL pathogenesis is well established[16-19]; however, characterization of FL into immune infiltration$^{HI}$ versus immune infiltration$^{LO}$ phenotypes and the relationship between immune infiltration and POD24 has not been previously investigated.

## PATIENTS AND METHODS

### Patient Populations

Patient characteristics are described in the Data Supplement. The discovery cohort (n = 132 patients) was identified from a prospectively maintained clinical lymphoma database (Data Supplement) containing patients at the Princess Alexandra Hospital (PAH) and included patients with early- and advanced-stage disease. The latter received treatment or observation as per published criteria.[20,21] PAH is a metropolitan hospital with a catchment of approximately one million. All 198 consecutive patients with available tissue and a new diagnosis of FL between January 2001 and December 2015 were assessed for eligibility. Eligible patients were age 18 years or older with newly diagnosed histologic grade I to IIIa FL with available formalin-fixed paraffin-embedded tissue from a pretreatment diagnostic biopsy. Median follow-up was 6.67 years.

The British Columbia Cancer Agency (BCCA) cohort consisted of 138 patients with FL from a population-based registry diagnosed between 2004 and 2009 with symptomatic advanced-stage grade FL I to IIIa that required treatment with rituximab plus cyclophosphamide, vincristine, and prednisolone immunochemotherapy.[22] The second validation cohort consisted of 45 patients with symptomatic advanced-stage grade FL I to IIIa treated with rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisolone from the German Low Grade Lymphoma Study Group 2000 (GLSG2000) trial.[23] These patients were recruited between 2000 and 2010 and were specifically selected, including the availability of tissue, to permit a comparison of the 29 patients who experienced a POD24 event with 16 who experienced no progression within 5 years.[23] Validation cohorts were included in the original POD24-PI description.[8] This study was approved by the relevant institutional regulatory boards in concordance with the Declaration of Helsinki.

### Sequencing, Gene Expression, and Multispectral Immunofluorescence Imaging

A targeted sequencing panel of 11 genes was used to identify FL-relevant mutations.[22,24,25] Gene expression used a NanoString custom code set of 12 clinically pertinent immune effector (CD137, CD4, CD7, CD8A, TNF$\alpha$), immune checkpoint (PD-1, PD-L1, PD-L2, TIM3, LAG3, FOXP3), and macrophage (CD68) molecules (Data Supplement).[26,27] We performed high-throughput T-cell receptor (TCR) repertoire sequencing as published (Adaptive Biotechnologies, Seattle, WA).[28] Multispectral immunofluorescence (MIF) used the Opal Multiplex Assay (PerkinElmer, Waltham, MA) on a tissue microarray.[29] The Data Supplement provides additional details.

### Statistics

In the discovery cohort, POD24 is defined as primary-refractory disease (less than partial response), progression, transformation, or relapse within 24 months after diagnosis. Deaths not a result of FL were excluded. In the validation cohorts, time was from the initiation of therapy. There were 19 individuals from the original GLSG2000 cohort who were not evaluable for POD24 that were excluded from the analysis (six deceased and 13 lost to follow-up). Categorical data were compared with Fisher's exact or $\chi^2$ test, when appropriate, and continuous data using two-tailed paired *t* tests. POD24-PI was calculated as originally described.[8] We performed hierarchical clustering with Euclidean distances and visualized using R statistical software (https://www.r-project.org/). Additional details on statistical methods are provided in the Data Supplement.

## RESULTS

### Immune Infiltration Shows Distinct Clustering in FL Samples

A heat map was made using nonhierarchical clustering of immune effector, immune checkpoint, and macrophage gene expression in the discovery cohort (Fig 1). This showed distinct clustering of FL samples, which was characterized by either high or low molecule expression, irrespective of their designation as an immune effector,

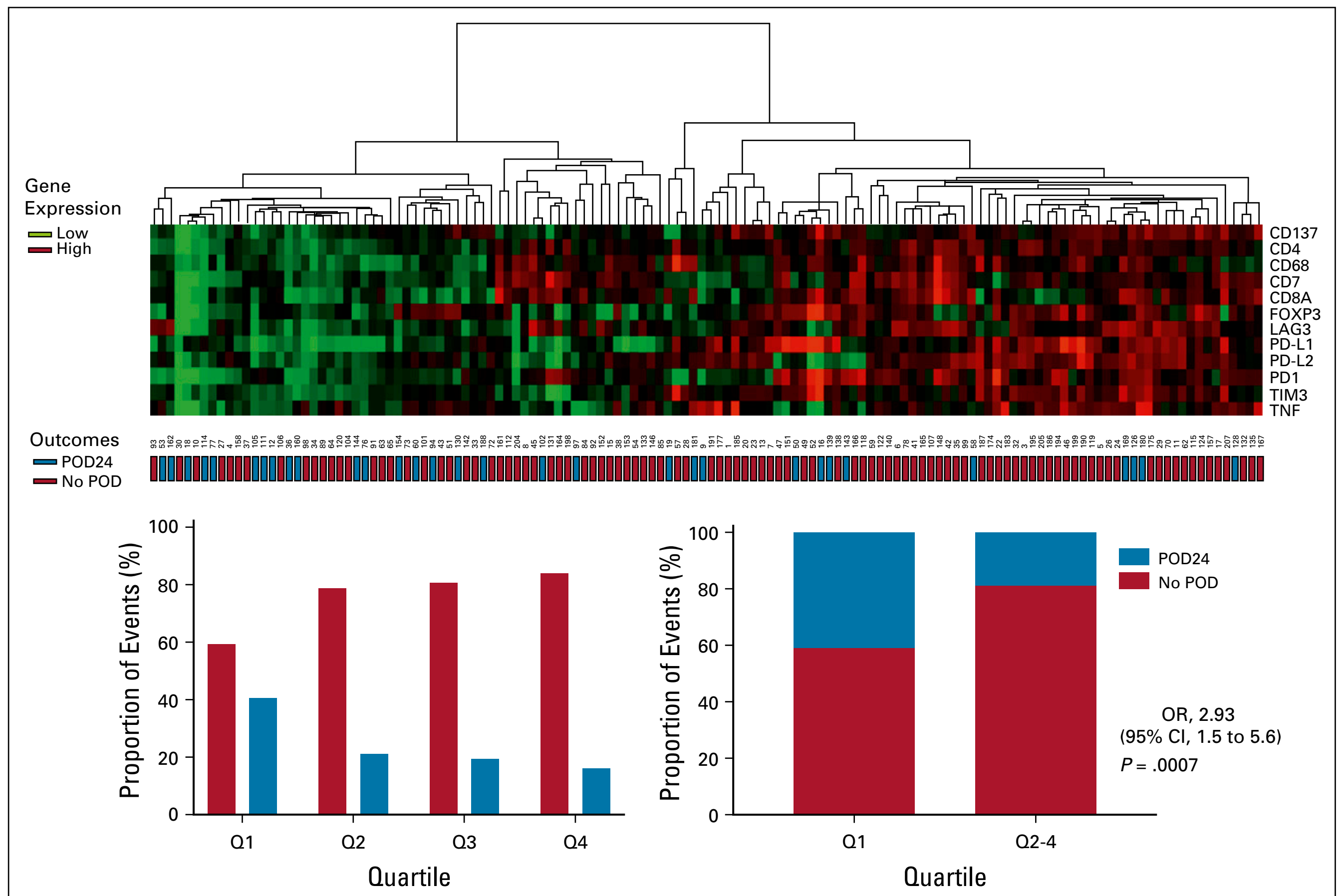


**FIG 1.** Unsupervised hierarchical clustering identifies immune infiltration$^{HI}$ and immune infiltration$^{LO}$ follicular lymphoma (FL) tissues. Immune effector, immune checkpoint, and macrophage gene expression in the FL tissue discovery cohort (n = 132) was digitally quantified by NanoString. Green denotes low and red indicates high gene expression. Genes were categorized as follows: immune effector (CD137, CD4, CD7, CD8A, TNFα); immune checkpoint (PD-1, PD-L1, PD-L2, TIM3, LAG3, FOXP3); and macrophage (CD68) molecules. POD, progression of disease; POD24, progression of disease within 24 months.

immune checkpoint, or macrophage marker. We then assessed individual genes from the targeted immune panel to dichotomize patients into high- and low-risk subsets for adverse outcomes—defined as progression, relapse, transformation, or death from any cause—over the 6.5-year period of follow-up for each immune gene in the discovery cohort. Use of this continuous variable enabled the identification of a binary cutoff. We applied a regressive partitioning model (Data Supplement).[27] The optimum cutoff point was calculated for each marker individually. Four immune markers—PD-L2, TNFα, CD4, and CD68—had significant hazard ratios ($P < .05$; Table 1) using unadjusted *P* values. Of note, all four biomarkers, regardless of their designation as immune effectors (TNFα, CD4), checkpoints (PD-L2), or macrophage markers (CD68), were associated with a poor outcome in the group with low expression. After adjustment for multiple genes, two markers—the immune checkpoint PD-L2 and the immune effector TNFα—had significant corrected *P* values. The most significant adverse immune marker, also showing the best specificity and sensitivity, was PD-L2 (cutoff normalized count, 139.0; median count, 237; 25th to 75th percentile, 128 to 348).

### Tissues With High Levels of Immune Infiltration Have a Distinct Immunobiology

We chose PD-L2 gene expression to compare the immunobiology of tissues that were dichotomized into immune infiltration$^{HI}$ and immune infiltration$^{LO}$ nodes. PD-L2 is an immune checkpoint molecule that shows broad dynamic range and that we expected to be present within malignant and nonmalignant cells.[29] To confirm this, we quantified the relative distribution of PD-L2 molecules between B cells and non–B cells in seven fresh deaggregated FL diagnostic nodes. CD20$^{+}$ and non-CD20$^{+}$ cells were sorted using fluorescence-activated cell sorting and PD-L2 was quantified using quantitative polymerase chain reaction. This showed that PD-L2 gene expression was distributed in both cell populations, but that the proportion of PD-L2 was higher in non-CD20$^{+}$ cells relative to CD20$^{+}$ cells within the node ($P = .015$; Fig 2A). Using the PD-L2 cutoff as defined by the partitioning model previously, we observed

**TABLE 1.** Targeted Immune Panel Used to Dichotomize Patients Into High- and Low-Risk Subsets for Adverse Outcomes

| Gene | Immune Category | High Risk, No. (%) | Digital Gene Expression Cut Point | Total Adverse Events, No. | $P$ | $P_{corrected}$ | HR | 95% CI | Sp | Sn |
|---|---|---|---|---|---|---|---|---|---|---|
| PD-L2 | Checkpoint | 35 (26.5) | 139 | 29 | $2.1 \times 10^{-5}$ | $2.5 \times 10^{-4}$ | 0.30 | 0.18 to 0.49 | 83.7 | 45.7 |
| TNF | Effector | 21 (15.9) | 245 | 15 | .002 | .024 | 0.40 | 0.23 to 0.73 | 78.5 | 40.0 |
| CD68 | Macrophage | 39 (29.5) | 1,624 | 27 | .008 | .096 | 0.52 | 0.32 to 0.84 | 80.9 | 36.8 |
| CD4 | Effector | 24 (18.2) | 1,722 | 18 | .014 | .168 | 0.51 | 0.27 to 0.98 | 79.1 | 41.2 |
| LAG3 | Checkpoint | 24 (18.2) | 170 | 17 | .056 | .672 | 0.48 | 0.25 to 0.92 | 79.6 | 41.7 |
| CD137 | Effector | 27 (20.5) | 418 | 14 | .057 | .684 | 0.59 | 0.29 to 1.04 | 79.8 | 43.5 |
| PD-L1 | Checkpoint | 59 (44.7) | 65 | 36 | .071 | .852 | 0.94 | 0.58 to 1.52 | 57.3 | 51.2 |
| CD7 | Effector | 28 (21.2) | 651 | 18 | .078 | .936 | 0.57 | 0.30 to 1.06 | 37.0 | 79.0 |
| PD-1 | Checkpoint | 28 (21.2) | 155 | 17 | .079 | .948 | 0.61 | 0.32 to 1.16 | 81.3 | 32.3 |
| TIM3 | Checkpoint | 27 (20.5) | 275 | 18 | .091 | 1.00 | 0.70 | 0.39 to 1.26 | 78.8 | 39.1 |
| FOXP3 | Checkpoint | 34 (25.8) | 117 | 12 | .204 | 1.00 | 0.87 | 0.43 to 1.37 | 78.2 | 33.3 |
| CD8A | Effector | 46 (34.5) | 1,348 | 25 | .281 | 1.00 | 0.77 | 0.47 to 1.28 | 69.7 | 51.7 |

NOTE. Digital gene expression profiling was performed on 132 patients from the Princess Alexandra Hospital cohort. Immune gene markers were dichotomized into high- and low-risk subsets using a regressive partitioning model. Of note, all four significant ($P < .05$) biomarkers—regardless of their designation as immune effectors, checkpoints, or tumor-associated macrophage markers—were associated with differences in adverse outcomes (combined total events of progression, relapse, transformation, or death from any cause) in the group with low expression.

Abbreviations: HR, hazard ratio; Sn, sensitivity; Sp, specificity.

significantly lower expression of immune effector, immune checkpoint, and macrophage molecules in the immune infiltration$^{LO}$ phenotype compared with immune infiltration$^{HI}$ (Fig 2B). In contrast, housekeeper genes did not show this, which indicates that clustering was not reflecting tissue RNA quality or quantity. To compare the expression of a range of immune molecules across tissues, we stratified FL tissues by the top and bottom quartiles of PD-L2 gene expression. This highlighted that, in these tissues, immune molecule expression correlated with each other, with clear grouping of tissues into those expressions that were either immune infiltration$^{HI}$ or immune infiltration$^{LO}$ (Fig 2C). To establish whether this was also true of T-cell protein expression, we analyzed flow cytometry that was performed on the diagnostic biopsy. This demonstrated a higher proportion of CD3$^{+}$ T cells (mean, 41% *v* 30%; $P$ = .016) in immune infiltration$^{HI}$ versus immune infiltration$^{LO}$ tumors (Fig 2D).

### TME Contains Abundant Clonally Expanded T-Cell Populations and PD-L1–Expressing Macrophages in Immune Infiltration$^{HI}$ FL Tissues

We performed TCR repertoire sequencing on FL tissues (Fig 3A). This demonstrated an increase in productive clonality—a measure of repertoire unevenness as a result of clonal expansions—among the immune infiltration$^{HI}$ tissue subset compared with the immune infiltration$^{LO}$ subset (0.0065 *v* 0.0032; $P$ = .037). Next, to examine protein expression in situ, we performed MIF using representative markers of immune effectors, immune checkpoints, and macrophages on a separate cohort of 21 FL tissue microarray tissues (Ochsner Health System, New Orleans, LA). Samples were stratified by PD-L2 NanoString gene expression, and samples with the highest quartile (n = 6; 6 of 6 immune infiltration$^{HI}$) and lowest quartile (n = 5; 5 of 5 immune infiltration$^{LO}$) of PD-L2 were stained for expression of CD8 and PD-L1. Quantitative analysis used a computer-learning algorithm (Data Supplement). These analyses (Figs 3B and 3C) demonstrated differences between immune infiltration$^{HI}$ and immune infiltration$^{LO}$ tumors in the density of CD8 and PD-L1 (both $P < .02$). CD68 was used to localize macrophages. A high proportion of PD-L1 staining (percent total fluorescence units) was on CD68$^{+}$ macrophages (median, 31.5%; range, 15.7% to 55.7%) and this was higher in immune infiltration$^{HI}$ versus immune infiltration$^{LO}$ tumors (Fig 3D; 41.7% *v* 22.8%; $P$ = .032).

### Immune Infiltration and POD24

POD24 occurred in 24.4% of patients in the PAH cohort and was a powerful predictor of 5-year mortality (55.3% *v* 90.8%; hazard ratio, 0.24; $P < .001$). POD24 rarely occurred in those with localized disease compared with those with advanced-stage disease (8.1% *v* 28.7%; $P$ = .009). Using PD-L2 level as defined by the partitioning model previously as a marker of immune infiltration, levels were compared between POD24+/−ve groupings. This showed that immune infiltration$^{LO}$ FL tissues were enriched in POD24 events (Fig 4A). Findings were consistent in the subset of 79 patients with symptomatic advanced-stage FL (Fig 4B and Data Supplement). To validate these findings, we compared results with two independent populations—the BCCA and GLSG2000 cohorts—using regression partitioning (Fig 4A, 4B, and Data Supplement). For patients in the discovery cohort, 45.7% with low PD-L2 had POD24 versus 16.3% with high PD-L2 (odds ratio [OR], 4.32; 95% CI, 1.81 to 9.67; c-statistic, 0.81; $P$ = .001). For

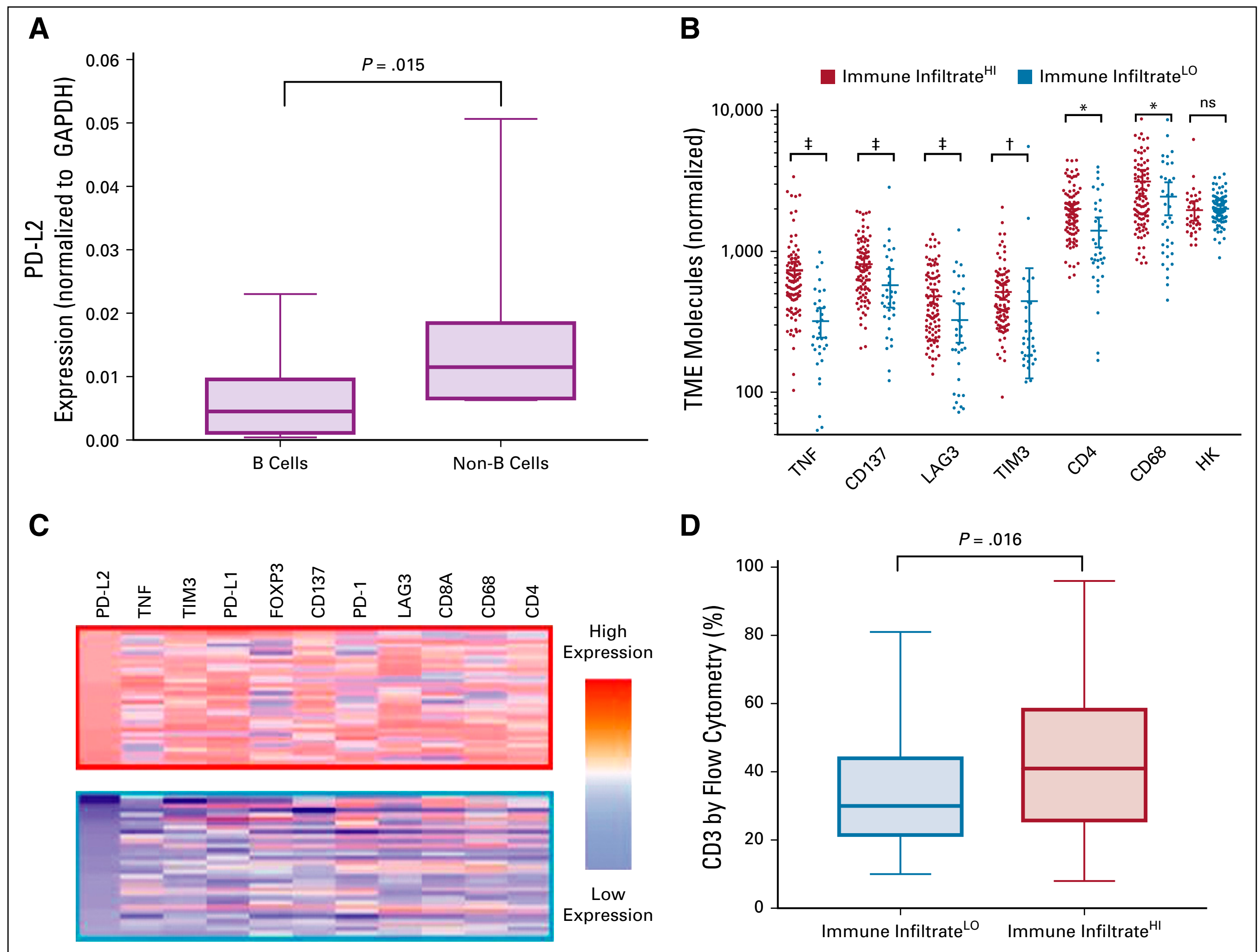


**FIG 2.** Immune infiltration$^{HI}$ and immune infiltration$^{LO}$ follicular lymphoma (FL) tissues show distinct gene and protein expression patterns. (A) Fluorescence-activated cell sorting was used to sort cells from deaggregated FL tissues and PD-L2 was quantified by quantitative polymerase chain reaction, normalized to GAPDH and adjusted for the relative proportion of CD20$^+$ to non-CD20$^+$ cells. Histogram with mean and 95% CIs are shown. The effect size (ie, the measure of the magnitude of the increase in PD-L2 in non-B cells), as conveyed by the median of differences for the samples, is 0.007122. (B) Dichotomizing FL samples as immune infiltration$^{HI}$ or immune infiltration$^{LO}$ on the basis of PD-L2 gene expression showed significantly higher gene expression of multiple immune molecules (effector, checkpoint, and macrophages). (C) Stratification of FL tissues by the top and bottom quartiles of PD-L2 expression highlighted the clustering of immune genes. (D) PD-L2 is used to stratify between immune infiltration$^{HI}$ and immune infiltration$^{LO}$ tissues in which flow cytometry was performed at diagnosis on fresh deaggregated FL tissues. HK, housekeeping genes; ns, not significant. (*) $P$ = .05 to .01; (†) $P$ = .001 to .0001; (‡) $P$ < .0001.

the BCCA validation cohort, POD24 was observed in 46.7% of low PD-L2 versus 24.0% of high PD-L2 (OR, 2.95; 95% CI, 1.23 to 6.97; c-statistic, 0.75; $P$ = .011), and for the GLSG2000 cohort, values for PD-L2 low and high were 54.2% versus 14.3% (OR, 7.09; 95% CI, 1.77 to 26.27; c-statistic, 0.88; $P$ = .011), respectively. The GLSG2000 validation cohort was specifically restricted to high-risk patients—that is, a POD24 event had occurred—and low-risk patients—no POD event within 5 years. Taken together, the results are consistent with low PD-L2 identifying a subset of patients with FL who are enriched for POD24.

PD-L2 expression was not significantly different between early- versus advanced-stage disease ($P$ = .56). POD24 events were rare in early-stage FL (two of 27) and there was no difference in POD24 events between high and low PD-L2 subsets ($P$ = .34; OR, 5.25; 95% CI, 0.22 to 103.3).

We next tested the proportion of POD24 events occurring in the PAH discovery cohort stratified by FLIPI and ISS (Data Supplement). This showed that patients with high-risk FLIPI (score, 3 to 5) were enriched in POD24 events (33.9% with high-risk FLIPI *v* 15.8% with low-risk FLIPI; OR, 3.1; 95% CI, 1.5 to 6.19; $P$ = .0033), whereas no significant enrichment was observed by ISS (OR, 2.3; 95% CI, 0.93 to 5.37; $P$ = .068). The overlap between low PD-L2 and both high-risk FLIPI and high-risk ISS was relatively modest (Data Supplement). We next constructed

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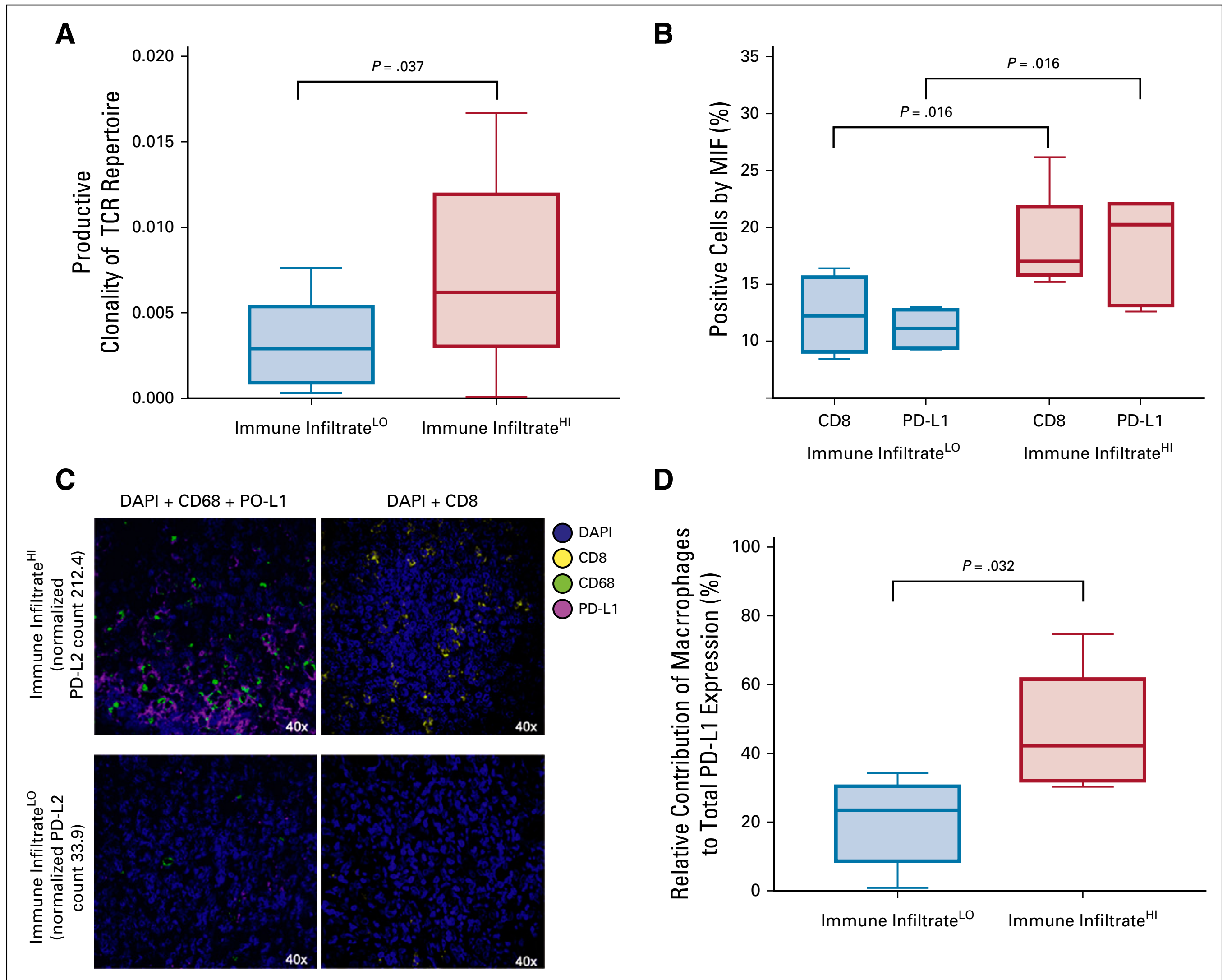


**FIG 3.** (A) Immune-infiltration$^{HI}$ tumors demonstrate a higher productive clonality of the TCR repertoire in the FL tumor microenvironment. Clonality describes the degree to which one or a few clones dominate the repertoire. (B) Quantitative MIF staining demonstrates increased protein expression of key immune-effectors (CD8) and immune-checkpoints (PD-L1) in immune-infiltration$^{HI}$ tumors. (C) Quantitative multispectral immunofluorescence (MIF) staining demonstrates increased protein expression of key immune effectors (CD8), immune checkpoints (PD-L1), and macrophages (CD68) in immune infiltration$^{HI}$ tumors. MIF images demonstrating increased immune infiltrate and intensity of immune effectors, checkpoints, and macrophage molecules in immune infiltration$^{HI}$ tumors. (D) Quantitatively, PD-L1 expression is upregulated on macrophages in immune infiltration$^{HI}$ tumors.

integrated prognostic models (Data Supplement). This demonstrated that combining immune infiltration with FLIPI or ISS led to a modest increase in specificity—for example, compared with immune infiltration alone, the specificity of a combined FLIPI–PD-L2 score to correctly identify POD24 increased from 83.7% to 90%.

## Mutational Profile Is Similar Between Immune Infiltration$^{HI}$ and Immune Infiltration$^{LO}$ Tissues

To investigate potential associations between immune infiltration$^{HI}$ and immune infiltration$^{LO}$ phenotypes across a range of relevant genetic aberrations, we compared the proportions of mutations in *BCL2, KMT2D, EZH2, ARID1A, MEF2B, TNFRSF14, EP300, TP53, FOXO1, CREBBP,* and *CARD11* in immune infiltration$^{HI}$ and immune infiltration$^{LO}$ nodes. Mutations were detected in equal proportions (Fig 5), which was consistent with the mutational profile not influencing the presence of immune infiltration$^{HI}$ or immune infiltration$^{LO}$ FL phenotypes.

## DISCUSSION

The host intratumoral immune response is critical to the pathogenesis and outcome of FL.[10,16-19,30] We have found that expression of immune checkpoint and immune effector molecules showed distinct clustering of FL samples, characterized by either high or low immune infiltration, regardless of their categorization as an immune effector,

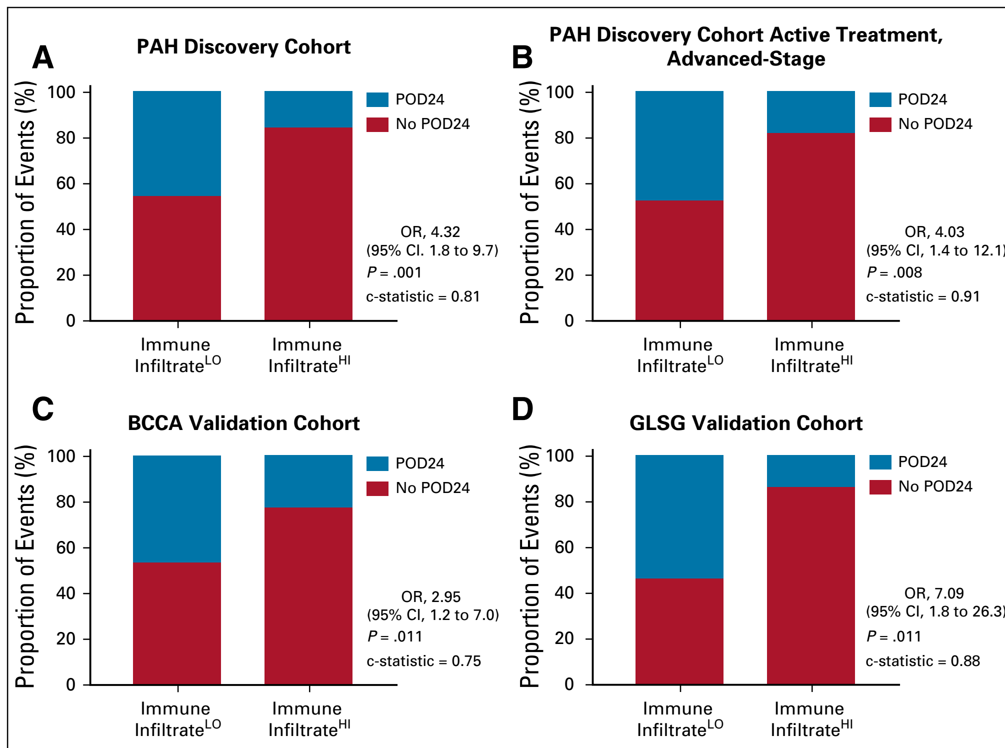


**FIG 4.** Low immune infiltration cases are enriched in progression of disease within 24 months (POD24) events. The proportion of POD24 events occurring in the (A) Princess Alexandra Hospital (PAH) discovery cohort (B) patients in the PAH discovery cohort with active treatment, advanced stage (C) British Columbia Cancer Agency (BCCA) validation cohort and (D) German Low Grade Lymphoma Study Group 2000 (GLSG2000) validation cohort were stratified by the degree of immune infiltration. OR, odds ratio.

immune checkpoint, or macrophage molecule. Low PD-L2 was the most sensitive/specific immune molecule to segregate patients into those with or without a combined end point of progression, relapse, transformation, or death. Therefore, high PD-L2 expression was chosen to distinguish immune infiltration$^{HI}$ from low PD-L2 expressing immune infiltration$^{LO}$ FL tumors. Using a variety of techniques, immune infiltration$^{HI}$ hot and immune infiltration$^{LO}$ cold phenotypes were demonstrated to have a distinct underlying immunobiology, particularly with regard to infiltration by expanded populations of clonal T cells and PD-L1–expressing macrophages. The outcome of FL remains highly heterogeneous, and there is increasing emphasis on early predictors of outcomes, such as POD24. There is limited understanding of the relationship between the intratumoral immune microenvironment and POD24 in FL. Using a discovery/validation approach, we demonstrated that low PD-L2 expression, as a marker of low immune infiltration, is enriched in early events—that is, related to POD24—and is indicative of a more aggressive immunobiology. Of interest, the immune infiltration$^{LO}$ subset of patients with FL was markedly more enriched for POD24 compared with patients with high-risk FLIPI. There was only modest overlap between immune infiltration$^{LO}$ FL and high-risk FLIPI groupings, indicating that PD-L2 expression captures a different subgroup of patients.

We have previously shown that the TCR repertoire is related to prognosis in diffuse large B-cell lymphoma.[28] Of interest, in the current study, T cells within immune infiltration$^{LO}$

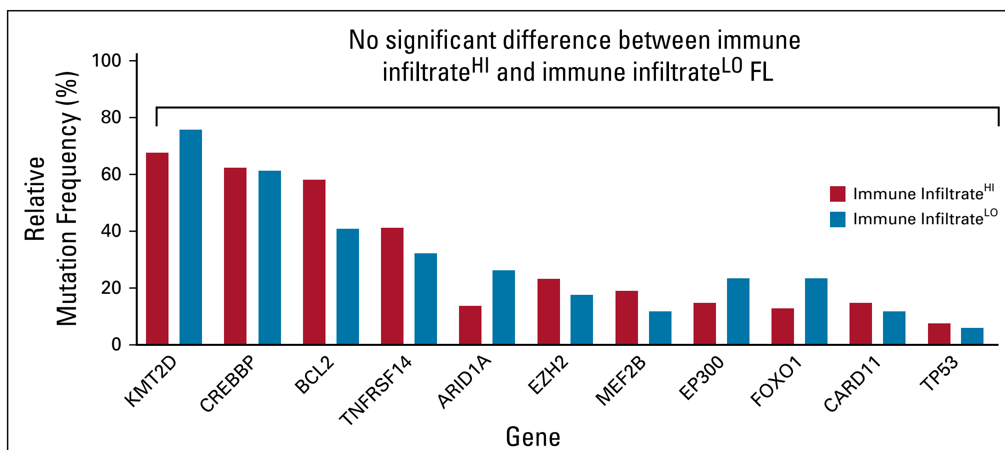


**FIG 5.** Distribution of gene mutations in immune infiltration$^{HI}$ and immune infiltration$^{LO}$ follicular lymphoma lymph nodes. Relative frequency of FL relevant mutations stratified by immune infiltration$^{HI}$ and immune infiltration$^{LO}$ gene expression. No significant differences were seen for any mutation.

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tumors exhibited decreased TCR clonality. One explanation is that this may reflect reduced T-cell clonal expansion in response to reduced neoantigen production or an impaired ability to present antigen; however, this remains to be formally tested. Additional studies are required to compare the TCR repertoire with tumor mutational burden—for example, by whole-genome sequencing—and to identify the antigen specificity of individual expanded T-cell clones to better assess the mechanistic implications of the T-cell expansions in FL.

In the discovery cohort (early- and advanced-stage disease), the POD24 risk defining event was calculated as time since diagnosis, as per the original definition.[6] Of importance, findings in the discovery cohort were validated in advanced-stage patients who were treated in a uniform manner (rituximab plus cyclophosphamide, vincristine, and prednisolone for BCCA; and rituximab plus cyclophosphamide, vincristine, doxorubicin, and prednisolone for GLSG2000) in whom the POD24 risk defining event was calculated as time since initiation of therapy. Taken together, immune infiltration seems to apply to the original and modified definitions of POD24.

Our data indicate that patients with biopsies showing reduced immune infiltration are more likely to experience POD24 events. Although specificity was high, this was at the expense of sensitivity, which indicates that immune infiltration fails to capture a subset of patients who will develop POD24. This makes it a valuable predictor in certain clinical situations—for example, when testing poorly tolerated intensive regimens—but with the proviso that some patients who might potentially benefit from treatment intensification will be missed. Additional improvements, including the integration of additional biomarkers, are needed to capture these patients. Furthermore, POD24 only reflects events that occur over a specific time period. Clinicogenetic scores that reflect a different aspect of tumor biology can predict the risk of progression after induction immunochemotherapy,[22] which (as well as POD24) is a relevant end point regarding treatment efficacy and overall survival.[31] Of note, the frequency of mutations in genes that are known to be commonly mutated in FL was no different between immune infiltration$^{HI}$ and immune infiltration$^{LO}$ tissues, indicating that the degree of immune infiltration is capturing aspects of FL biology that are distinct from its mutational profile. Huet et al[32] identified a 23-gene signature characteristic of B-cell centroblasts that was prognostic independently of FLIPI which underlines that, in addition to the TME, tumor B-cell biology contributes to the clinical aggressiveness of FL. Frontline treatment options for FL are expanding,[33] hence additional refinements in the dichotomization of immune infiltration, along with combination with other clinicogenetic and gene expression tissue markers, as well as cell-free circulating DNA and functional imaging,[34-37] should be prioritized as part of collaborative efforts to develop integrated pretherapy models that sensitively and accurately predict both POD24 and risk of relapse/progression in FL so as to maximize clinical utility.[38,39]

Until now, studies that characterize the degree of immune infiltration have been largely restricted to solid cancers. Those with immune infiltration$^{HI}$ tumors have better outcomes with conventional therapy and checkpoint blockade.[12,14] Of interest, similar to our findings in FL, low PD-L2 expression is superior to PD-L1 as a measure of immune infiltration that has been demonstrated to be adversely associated with outcomes in metastatic melanoma treated with conventional—that is, non–checkpoint blockade—therapy.[40] Furthermore, studies in melanoma and other solid cancer subtypes have demonstrated that PD-L2 is a marker of adaptive immune resistance that outperforms PD-L1 as a measure of antitumor immunity and interferon gamma signaling.[41,42] We have recently demonstrated that PD-L1 and PD-L2 are expressed on macrophages in Hodgkin lymphoma and diffuse large B-cell lymphoma, where they are functionally active in impairing antilymphoma effector immunity.[29] In the current study, using a combination of gene expression, flow cytometry, and MIF, we show that in FL—a setting in which gene amplification of PD-1 ligands rarely occurs[43,44]—both PD-L1 and PD-L2 are predominantly expressed in nonmalignant cells. Although checkpoint ligands seem to be an informative strategy by which to stratify immune infiltration, the findings of this study should be seen as a reflection of the importance of the degree of immune infiltration to the immunobiology of FL, rather than as a definitive statement of the importance of a particular immune biomarker—or a particular technique—over another to characterize immune infiltration in FL tissues. Choice of PD-L2 gene expression was determined on the basis of disease outcome rather than immune parameters. Additional studies are required to explore the immunobiology of FL in more depth, and it is necessary also to validate approaches that are more accessible than PD-L2 gene expression that might be applicable to the diagnostic laboratory, such as immunohistochemistry.

The majority of patients with FL do not experience a response to checkpoint blockade.[45] It remains to be tested whether segregating FL by immune infiltration helps predict immunotherapy responsiveness, and whether conversion of immune infiltration$^{LO}$ into immune infiltration$^{HI}$ tumors sensitizes FL to immunotherapies as proposed in solid tumors.[46] Understanding the differential mechanisms of resistance that are operative in immune infiltration$^{HI}$ and immune infiltration$^{LO}$ FL will be critical.

In summary, we demonstrate that FL can be characterized into immune infiltration$^{HI}$ or immune infiltration$^{LO}$ immunophenotypes. Assessment of immune infiltration seems to be a promising tool with which to help identify patients who are at risk for POD24.

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## REFERENCES


1. Morton LM, Wang SS, Devesa SS, et al: Lymphoma incidence patterns by WHO subtype in the United States, 1992-2001. Blood 107:265-276, 2006
2. Gandhi MK, Marcus RE: Follicular lymphoma: Time for a re-think? Blood Rev 19:165-178, 2005
3. Tan D, Horning SJ, Hoppe RT, et al: Improvements in observed and relative survival in follicular grade 1-2 lymphoma during 4 decades: The Stanford University experience. Blood 122:981-987, 2013
4. Solal-Céligny P, Roy P, Colombat P, et al: Follicular lymphoma international prognostic index. Blood 104:1258-1265, 2004
5. Shi Q, Flowers CR, Hiddemann W, et al: Thirty-month complete response as a surrogate end point in first-line follicular lymphoma therapy: An individual patient-level analysis of multiple randomized trials. J Clin Oncol 35:552-560, 2017
6. Casulo C, Byrtek M, Dawson KL, et al: Early relapse of follicular lymphoma after rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone defines patients at high risk for death: An analysis from the National LymphoCare Study. J Clin Oncol 33:2516-2522, 2015
7. Casulo C, Le-Rademacher J, Dixon J, et al: Validation of POD24 as a robust early clinical endpoint of poor survival in follicular lymphoma: Results from the Follicular Lymphoma Analysis of Surrogacy Hypothesis (FLASH) investigation using individual data from 5,453 patients on 13 clinical trials. Blood 130:412, 2017 (suppl 1)
8. Jurinovic V, Kridel R, Staiger AM, et al: Clinicogenetic risk models predict early progression of follicular lymphoma after first-line immunochemotherapy. Blood 128:1112-1120, 2016
9. Maddocks K, Barr PM, Cheson BD, et al: Recommendations for clinical trial development in follicular lymphoma. J Natl Cancer Inst 109:djw255, 2016
10. Dave SS, Wright G, Tan B, et al: Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. N Engl J Med 351:2159-2169, 2004
11. Chen DS, Mellman I: Elements of cancer immunity and the cancer-immune set point. Nature 541:321-330, 2017
12. Naito Y, Saito K, Shiiba K, et al: CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. Cancer Res 58:3491-3494, 1998
13. Li J, Byrne KT, Yan F, et al: Tumor cell-intrinsic factors underlie heterogeneity of immune cell infiltration and response to immunotherapy. Immunity 49:178-193.e7, 2018
14. Tumeh PC, Harview CL, Yearley JH, et al: PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 515:568-571, 2014
15. Taube JM, Anders RA, Young GD, et al: Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. Sci Transl Med 4:127ra37, 2012
16. Fowler NH, Cheah CY, Gascoyne RD, et al: Role of the tumor microenvironment in mature B-cell lymphoid malignancies. Haematologica 101:531-540, 2016

17. Yang ZZ, Grote DM, Ziesmer SC, et al: IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. J Clin Invest 122:1271-1282, 2012
18. Kiaii S, Clear AJ, Ramsay AG, et al: Follicular lymphoma cells induce changes in T-cell gene expression and function: Potential impact on survival and risk of transformation. J Clin Oncol 31:2654-2661, 2013
19. Hellmuth JC, Louissaint A Jr, Szczepanowski M, et al: Duodenal-type and nodal follicular lymphomas differ by their immune microenvironment rather than their mutation profiles. Blood 132:1695-1702, 2018
20. Brice P, Bastion Y, Lepage E, et al: Comparison in low-tumor-burden follicular lymphomas between an initial no-treatment policy, prednimustine, or interferon alfa: A randomized study from the Groupe d'Etude des Lymphomes Folliculaires. Groupe d'Etude des Lymphomes de l'Adulte. J Clin Oncol 15:1110-1117, 1997
21. Nastoupil LJ, Sinha R, Byrtek M, et al: Outcomes following watchful waiting for stage II-IV follicular lymphoma patients in the modern era. Br J Haematol 172: 724-734, 2016
22. Pastore A, Jurinovic V, Kridel R, et al: Integration of gene mutations in risk prognostication for patients receiving first-line immunochemotherapy for follicular lymphoma: A retrospective analysis of a prospective clinical trial and validation in a population-based registry. Lancet Oncol 16:1111-1122, 2015
23. Hiddemann W, Kneba M, Dreyling M, et al: Frontline therapy with rituximab added to the combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) significantly improves the outcome for patients with advanced-stage follicular lymphoma compared with therapy with CHOP alone: Results of a prospective randomized study of the German Low-Grade Lymphoma Study Group. Blood 106:3725-3732, 2005
24. Kridel R, Sehn LH, Gascoyne RD: Pathogenesis of follicular lymphoma. J Clin Invest 122:3424-3431, 2012
25. Okosun J, Bödör C, Wang J, et al: Integrated genomic analysis identifies recurrent mutations and evolution patterns driving the initiation and progression of follicular lymphoma. Nat Genet 46:176-181, 2014
26. Jones K, Wockner L, Brennan RM, et al: The impact of HLA class I and EBV latency-II antigen-specific CD8(+) T cells on the pathogenesis of EBV(+) Hodgkin lymphoma. Clin Exp Immunol 183:206-220, 2016
27. Keane C, Vari F, Hertzberg M, et al: Ratios of T-cell immune effectors and checkpoint molecules as prognostic biomarkers in diffuse large B-cell lymphoma: A population-based study. Lancet Haematol 2:e445-e455, 2015
28. Keane C, Gould C, Jones K, et al: The T-cell receptor repertoire influences the tumor microenvironment and is associated with survival in aggressive B-cell lymphoma. Clin Cancer Res 23:1820-1828, 2017
29. Vari F, Arpon D, Keane C, et al: Immune evasion via PD-1/PD-L1 on NK cells and monocyte/macrophages is more prominent in Hodgkin lymphoma than DLBCL. Blood 131:1809-1819, 2018
30. Wahlin BE, Aggarwal M, Montes-Moreno S, et al: A unifying microenvironment model in follicular lymphoma: Outcome is predicted by programmed death-1–positive, regulatory, cytotoxic, and helper T cells and macrophages. Clin Cancer Res 16:637-650, 2010
31. López-Guillermo A: A novel clinicogenetic prognostic score for follicular lymphoma. Lancet Oncol 16:1011-1012, 2015
32. Huet S, Tesson B, Jais JP, et al: A gene-expression profiling score for prediction of outcome in patients with follicular lymphoma: A retrospective training and validation analysis in three international cohorts. Lancet Oncol 19:549-561, 2018
33. Cheah CY, Fowler NH: Chemotherapy-free treatment of follicular lymphoma: We have the ingredients, now for some recipes. Oncology (Williston Park) 29: 769-772, 772, 2015
34. Bachy E, Maurer MJ, Habermann TM, et al: A simplified scoring system in de novo follicular lymphoma treated initially with immunochemotherapy. Blood 132: 49-58, 2018
35. Cottereau AS, Versari A, Luminari S, et al: Prognostic model for high-tumor-burden follicular lymphoma integrating baseline and end-induction PET: A LYSA/FIL study. Blood 131:2449-2453, 2018
36. Kurtz DM, Green MR, Bratman SV, et al: Noninvasive monitoring of diffuse large B-cell lymphoma by immunoglobulin high-throughput sequencing. Blood 125: 3679-3687, 2015
37. Trotman J, Barrington SF, Belada D, et al: Prognostic value of end-of-induction PET response after first-line immunochemotherapy for follicular lymphoma (GALLIUM): Secondary analysis of a randomised, phase 3 trial. Lancet Oncol 19:1530-1542, 2018
38. Weigert O, Weinstock DM: The promises and challenges of using gene mutations for patient stratification in follicular lymphoma. Blood 130:1491-1498, 2017
39. Leonard JP, Nastoupil LJ, Flowers CR: Where to start? Upfront therapy for follicular lymphoma in 2018. Hematology (Am Soc Hematol Educ Program) 2018: 185-188, 2018
40. Obeid JM, Erdag G, Smolkin ME, et al: PD-L1, PD-L2 and PD-1 expression in metastatic melanoma: Correlation with tumor-infiltrating immune cells and clinical outcome. OncoImmunology 5:e1235107, 2016
41. Danilova L, Wang H, Sunshine J, et al: Association of PD-1/PD-L axis expression with cytolytic activity, mutational load, and prognosis in melanoma and other solid tumors. Proc Natl Acad Sci USA 113:E7769-E7777, 2016
42. Ribas A: Adaptive immune resistance: How cancer protects from immune attack. Cancer Discov 5:915-919, 2015
43. Andorsky DJ, Yamada RE, Said J, et al: Programmed death ligand 1 is expressed by non-Hodgkin lymphomas and inhibits the activity of tumor-associated T cells. Clin Cancer Res 17:4232-4244, 2011
44. Laurent C, Baron M, Amara N, et al: Impact of expert pathologic review of lymphoma diagnosis: Study of patients from the French Lymphopath Network. J Clin Oncol 35:2008-2017, 2017
45. Xu-Monette ZY, Zhou J, Young KH: PD-1 expression and clinical PD-1 blockade in B-cell lymphomas. Blood 131:68-83, 2018
46. Trujillo JA, Sweis RF, Bao R, et al: T cell-inflamed versus non-T cell-inflamed tumors: A conceptual framework for cancer immunotherapy drug development and combination therapy selection. Cancer Immunol Res 6:990-1000, 2018

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

**Progression of Disease Within 24 Months in Follicular Lymphoma Is Associated With Reduced Intratumoral Immune Infiltration**

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