Pattern of CD14⁺ Follicular Dendritic Cells and PD1⁺ T Cells Independently Predicts Time to Transformation in Follicular Lymphoma

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Abstract

Purpose: Transformation of follicular lymphoma is a critical event associated with a poor prognosis. The role of the tumor microenvironment in previous transformation studies has yielded conflicting results.

Experimental Design: To define cell subtypes associated with transformation, we examined tissue specimens at diagnosis from patients with follicular lymphoma that later transformed and, using immunohistochemistry (IHC), stained for CD68, CD11c, CD21, CXCL13, FOXP3, PD1, and CD14. Cell content and the pattern of expression were evaluated. Those identified as significantly associated with time to transformation (TTT) and overall survival (OS) were further characterized by flow cytometry and multicolor IHC.

Results: Of note, 58 patients were analyzed with median TTT of 4.7 years. The pattern of PD1⁺ and CD14⁺ cells rather than the quantity of cells was predictive of clinical outcomes. On multivariate analysis, including the follicular lymphoma international prognostic index score, CD14⁺ cells localized in the follicle were associated with a shorter TTT (HR, 3.0; \( P = 0.004 \)). PD1⁺ cells with diffuse staining were associated with a shorter TTT (HR, 1.9; \( P = 0.045 \)) and inferior OS (HR, 2.5; \( P = 0.012 \)). Multicolor IHC and flow cytometry identified CD14⁺ cells as follicular dendritic cells (FDC), whereas PD1⁺ cells represented two separate populations, Tfh and exhausted T cells.

Conclusion: These results identify the presence of PD1⁺ T cells and CD14⁺ FDC as independent predictors of transformation in follicular lymphoma. Clin Cancer Res; 20(11); 2862–72. ©2014 AACR.

Introduction

Follicular lymphoma is the second most common type of non-Hodgkin lymphoma (NHL). With a median overall survival (OS) of nearly 10 years, follicular lymphoma is classically thought of an indolent lymphoma that exhibits periods of disease remission and stability punctuated by intermittent relapses (1). However, the disease course is often heterogeneous with some patients undergoing histologic transformation to an aggressive lymphoma, most often diffuse large B-cell lymphoma (DLBCL). Histologic transformation is often associated with rapid progression, refractoriness to treatment, and an overall dismal prognosis (1–4). The incidence of transformation is variable ranging from 10% to 60% in different studies. The difference in incidence is largely due to differences in follow-up, biopsy confirmation, and inconsistent definitions of transformation (1, 3–8). The largest cohort reported an annual incidence of 3% (1). Prognostic tools using clinical and laboratory factors have been developed such as the follicular lymphoma international prognostic index (FLIPI) score that can predict risk of transformation at diagnosis (3, 9).

Recent studies have demonstrated the prominent role the tumor microenvironment plays in disease severity and outcomes in follicular lymphoma (10). Gene expression profiling from the Leukemia/Lymphoma Molecular Profiling Project identified the nonmalignant microenvironment immune cells rather than the tumor cells as predictive of clinical outcomes and behavior. One expression signature, immune response-1, seemed to be derived from reactive T cells and was associated with a favorable outcome. The other expression profile, immune response-2, included genes preferentially expressed by macrophages and dendritic cells (DC) that were associated with inferior survival (11). Immunohistochemical studies of the microenvironment have identified multiple immune subsets of interest (FOXP3⁺, PD1⁺, and CD4⁺/CD8⁺ ratio) that correlate with divergent outcomes (12–16). However, these studies have often analyzed a few different immunohistochemical markers at a
Translational Relevance

This study identifies two independent architectural biomarkers associated with a shorter time to transformation (TTT) in follicular lymphoma. CD14+ intratumoral cells localized to the follicle were associated with a shorter TTT; whereas, PD1+ cells present in a diffuse pattern had a shorter TTT compared with a follicular pattern. Multicolor immunohistochemistry (IHC) and flow cytometry identified CD14+ cells as follicular dendritic cells (FDC). These FDCs were shown to promote B-cell growth. Two separate populations of PD1+ cells were identified; with PD1+/TIM3+ exhausted T cells associated with an inferior survival and shorter TTT. This is the first study to report an association of intratumoral CD14+ cells and clinical outcomes in follicular lymphoma. In addition, it provides additional understanding of the various PD1+ subsets found throughout the microenvironment. Overall these results further the understanding of the tumor microenvironment and will aid in the design and use of immunotherapies.

time and many of the studies have led to contradictory results (12, 17).

The association of an unfavorable outcome with genes expressed by macrophages and DCs has led to increased interest in these immune subsets in follicular lymphoma patients. Farinha and colleagues previously described that CD68+ macrophages or lymphoma-associated macrophages (LAM) were correlated with inferior survival in their cohort (18), though this effect was demonstrated to be overcome with treatment with rituximab (19). CD14+ monocytes that are also HLA-DRlow have been shown to have immunosuppressive effects in various clinical conditions and several solids tumors (20–23). Lin and colleagues (24) recently described the role of CD14+ monocytes in patients with B-cell NHL. They showed that increased levels of CD14+ HLA-DRlow monocytes in the peripheral blood were associated with more advanced and aggressive disease and a shorter time to progression. Though these studies suggest an association of CD14+ cells with inferior outcomes, they were based on peripheral blood and not tumor tissue. In addition, various other factors in the microenvironment such as PD1 expression have been identified as potentially affecting clinical outcomes in follicular lymphoma (13, 25). Recent studies have also demonstrated that the location of microenvironment cells with respect to the neoplastic follicle rather than the total cell quantity is predictive of clinical outcomes in follicular lymphoma (17).

We hypothesized that intratumoral cells expressing CD14 or PD1 would be associated with a shorter time to transformation (TTT) in patients with follicular lymphoma. To this end, we studied the clinical correlation between the prevalence and distribution of various components of the tumor microenvironment, including CD14+ cells, CD68+ macrophages, FOXP3+ and PD1+ cells, and the TTT and OS in a retrospective cohort of patients with transformed follicular lymphoma. Beyond identifying these cells of interest, we also attempted to better characterize and identify the underlying immune cell type through multicolor IHC and flow cytometry.

Patients and Methods

Patients

Patients with follicular lymphoma that later transformed to DLBCL were identified from the Mayo Clinic Lymphoma Database. All samples were from the time of diagnosis and confirmed by a hematopathologist (A.L. Feldman) to be follicular lymphoma. Transformation was confirmed by biopsy and all histologies at transformation were consistent with DLBCL. Of note, 58 patients with tissue available at diagnosis were identified and included in this analysis. Clinical characteristics, including age, sex, presence of B symptoms, stage, grade, and laboratory parameters were collected at time of diagnosis. The various components of the FLIPI score [age >60, stage III or IV, Hgb <12 mg/dL, >4 nodal areas involved and LDH (lactate dehydrogenase) > upper limit normal] were collected to calculate the FLIPI score for each patient. This study was approved through the Mayo Clinic Institutional Review Board.

Immunohistochemistry

Paraffin-embedded tissue was obtained from the Mayo Clinic Tissue Registry and serial 5-µm sections were used for IHC. The tissue was deparaffinized with three changes of xylene and cleared through graded series of ethanol. Endogenous peroxidase was quenched by incubation in 50% methanol/H2O2 and after rinsing with tap water, all sections were pretreated for 30 minutes with 50 mmol/L EDTA using a steamer and cooled for additional 5 minutes. All staining was done automatically on DAKO Autostainer using the following antibodies to CD11c (Leica Microsystems 5D11), CD14 (Cell Marque EPR 3653), CD21 (DAKO 1F8), CD68 (DAKO PG-M1), CXCL13 (R&D Systems 53610), FOXP3 (Abcam 236AE/7), and PD1 (Abcam NAT). The sections were viewed with an Olympus BXFA51 microscope and pictures taken with an Olympus DP71 camera. Slides were characterized by the pattern of expression in relationship to the neoplastic follicle. The follicular pattern was defined as a majority of cells localized to the follicle or perifollicular area, whereas in the diffuse pattern a majority of positive cells were not confined to the follicle. Quantity and intensity of immunohistochemical stain were analyzed using a 0 to 3 scale. Assessment of IHC was done independently by two physicians (I.P. Smeltzer and J.M. Jones) with >90% concordance. Because identifying a single antigen in isolation through IHC does not identify the underlying immune cell that is represented, coexpression of multiple antigens was visualized using a novel method devised by Glass and colleagues (26). The resulting images captured were overlaid and each antigen assigned a color using Adobe Photoshop CS2 (Adobe Systems, Inc.). To differentiate CD14+ cells from macrophages and follicular DCs...
(FDC), slides initially positive for CD14 were costained with CD68 and CD163 as well as CD21 and CD23. To differentiate different PD1+ cells such as exhausted effector T cells and T follicular helper cells (TFH), cells, PD1+ cells were costained with CD3, CD19, CXCR5, and TIM3.

**Flow cytometry**

Cells were stained with fluorochrome-conjugated antibodies to human ICAM (R&D Systems; clone BBIG-l1), CD3 (clone HIT3a), CD19 (clone 4G7), PD1 (clone EH12.1), CD14 (clone M5E2), CD163 (clone GH1/61), CD21 (clone B-ly4), CD273 (clone M1H18), CD274 (clone M1H1), TIM3 (clone 344823), and CD23 (clone EBVCS-5; all obtained from BD Biosciences) and analyzed by flow cytometry and the data were analyzed using CellQuest software (Becton Dickinson).

**Cell culture**

FDC-enriched fractions were isolated as described by Schrieber and colleagues (27) with some modifications. Briefly, tonsils obtained from routine tonsillectomy were cut into small pieces and incubated for 1 hour at 37°C with some modifications. Cell culture software (Becton Dickinson).

**Results**

There were 58 patients with follicular lymphoma that later transformed to DLBCL. Patients presented with clinical symptoms compatible with transformation, including rapidly progressive lymphadenopathy, increasing serum LDH, or new constitutional symptoms. The diagnosis of transformation to large cell lymphoma, however, was confirmed by histologic analysis in all cases. The median age at diagnosis was 64 years and 24 (41%) were male. Other clinical baseline characteristics are summarized in Table 1. The majority (72%) of patients had advanced-stage disease and all patients were either follicular grade 1 or 2 NHL. The FLIPI scores at diagnosis were: 18 (31%) low risk, 27 (47%) intermediate risk, and 12 (21%) high risk. Of note, 42 (72%) patients were initially observed, 9 (15%) were treated with a combination of cyclophosphamide, vincristine, and prednisone, and 5 (9%) received an anthracycline combination. Only 2 patients received rituximab. The median TTT was 4.7 years (range, 0.4–20 years). The median estimated follow-up for still alive patients (n = 15) was 22.5 years, whereas the median OS for the entire cohort was 9.2 years. Gender, stage, absolute lymphocyte count, presence of constitutional symptoms, and grade did not correlate with TTT or OS. The FLIPI score was predictive of the rate of transformation to DLBCL (P = 0.01) and a lower FLIPI score was associated with improved median OS (P = 0.04).

**Relationship between CD14+ cells and TTT**

Paraffin tissue specimens were analyzed by the pattern of location and cell content as described in Materials and Methods. No relationship with TTT or OS was seen with the number or distribution of cells expressing FOXP3, CXCL3, CD21, CD68, or CD11c. The quantity of CD14+ cells was also not associated with TTT or OS; however, the location of CD14+ cells was predictive of TTT. As referenced in Materials and Methods, CD14+ cells were categorized on the basis of the pattern of location, follicular (n = 13) and nonfollicular (n = 41) (Fig. 1A). Patients with CD14+ cells localized to the follicle had a median TTT of 3.8 years compared with 5.9 years for those with a nonfollicular staining pattern (P = 0.027; Fig. 1B). The location of CD14+ cells was not associated with a significant difference in OS (P = 0.66; Fig. 1C).

**Relationship between PD1+ cells and TTT**

Similar to CD14+ cells, the relative quantity of PD1+ cells was not associated with either OS or TTT; however, the location of PD1+ cell was predictive of clinical outcomes. Thirty-eight patients had a follicular pattern of PD1+ cells and 19 a diffuse pattern (Fig. 2A). Patients with PD1+ cells localized to the follicle had a median TTT of 6.1 years compared with 3.6 years for those with a diffuse pattern (Fig. 2B; P = 0.033). The median OS for patients with PD1+ cells localized to the follicle was 9.7 versus 4.6 years for those with a diffuse distribution of PD1+ cells (Fig. 2C; P = 0.009). Categorizing the samples by early (<1 year) versus late (>5 years) transformation confirmed that early transformation exclusively involved patients with a diffuse pattern of PD1+ cell expression, whereas late transformation predominantly involved patients with a follicular pattern (Fig. 2D). Of note, the pattern of PD1+ and CD14+ cells did not correlate with each other (P = 0.36). Instead, the
Inclusion of PD1+ and CD14+ seemed to have an inverse relationship, as patients with PD1+ cells localized to the follicle had superior outcomes, whereas those with CD14+ cells localized to follicle had inferior outcomes. Patients with both follicular PD1+ and diffuse CD14+ cells had a delay in TTT (6.4 vs. 3.2 years.) compared with patients with follicular CD14+ cells and diffuse PD1+ cells (P = 0.01). Previous studies have identified the FLIPI score, anemia, and an elevated LDH as associated with transformation (28). The FLIPI score, which incorporates anemia and LDH, was included in a multivariate analysis and the pattern of PD1+ [HR, 1.9; 95% confidence interval (CI), 1.0–3.5; P = 0.045] and CD14+ (HR, 3.0; 95% CI, 1.5–6.1; P = 0.004) cells remained significantly associated with inferior TTT. After accounting for the FLIPI score, the diffuse pattern of PD1+ cells remained significantly associated with an inferior OS (HR, 2.5; 95% CI, 1.2–4.8; P = 0.012), whereas the pattern of CD14 cells was not (HR, 1.4; 95% CI, 0.6–2.9; P = 0.37).

Identification of PD1+ Immune cells

As different immune cells can express PD1 when evaluated by IHC, one cannot reliably identify the underlying immune cell of interest by using single immunohistochemical staining. Multicolor IHC was, therefore, undertaken to better identify which immune cells are represented by PD1+ staining. PD1+ cells are thought to be primarily T cells but it can also be expressed on activated B cells (29). Multicolor IHC illustrated that PD1+ cells in our sample primarily expressed CD3 and did not colocalize with the B-cell marker CD19 (Fig. 3A). To further analyze other markers of coexpression, flow cytometry was performed on follicular lymphoma samples with antibodies to CD3, CD19, and PD1. Again, CD3 was predominantly coexpressed with PD1 (Fig. 3B) with low levels of PD1 expression on B cells that was similar when compared with normal hyperplastic tonsils (data not shown).

Previous studies have identified PD1 on TFH cells (30). TFH cells express CXCR5 and we and others have previously demonstrated that PD1+ cells in follicular lymphoma express CXCR5 (31, 32). However, given the divergent outcomes based on the pattern of localization of PD1+ cells, we wondered whether these different areas (follicular vs. interfollicular) represented two separate populations. In addition to TFH, PD1 expression has also been described on exhausted T cells (32). As these exhausted cells characteristically express TIM3, this can act as a marker for the presence of exhausted T cells (32). Flow cytometry on follicular samples confirmed coexpression of CXCR5 and PD1 and also identified a population of PD1+ cells that coexpress TIM3 (Fig. 3C). There was little TIM3 expression on cells outside of the lymphocyte gate, suggesting that TIM3 is not expressed on other immune subsets such as DCs. Multicolor IHC with CD3, PD1, and TIM3 confirmed that PD1+ cells in the follicle were positive for CD3 but completely negative for TIM3; however, PD1+ cells in the interfollicular space did colocalize with TIM3 (Fig. 3D). Cells in the follicle stained positive for both CXCR5 and PD1 (Fig. 3E). These results confirm that PD1+ cells in the follicle are TIM3 negative and do not represent exhausted T cells, and given their location and the findings of IHC and flow cytometry it seems that these cells are primarily TFH cells. Thus, the improved clinical outcomes associated with the follicular pattern of PD1+ cells such as delay in TTT and improved survival are related to the maintained presence of TFH cells. PD1+ cells outside of the follicle are a distinct cell type compared with follicular PD1+ cells. These PD1+TIM3+ cells that are outside the follicle seem to represent exhausted T cells and are associated with inferior clinical outcomes.

Identification of CD14+ cells

CD14 is often associated with monocytes; however, CD14 is not exclusively expressed on monocytic cells and can be seen on other cell types (33). We analyzed our CD14+ cell population using multicolor flow cytometry and IHC to various monocytic, macrophage, and DC markers to better characterize the underlying cell type. Flow cytometry demonstrated minimal coexpression of CD14 with either CD68 or CD163 (Fig. 4A). However, a substantial portion of CD14+ cells coexpressed CD21 and CD23, which are markers typically associated with FDCs. Multicolor IHC demonstrated that CD21 and CD23 were coexpressed on nearly all CD14+ cells and were absent on
CD68\(^+\) and CD163\(^+\) cells (Fig. 4B and C). These results confirm the flow cytometry findings and suggest that rather than representing monocytes or macrophages, these CD14\(^+\) cells are FDCs.

**Follicular DCs promote malignant cell viability**

The prognostic role of FDC in follicular lymphoma is unclear. Our results suggest an association of inferior outcomes with an increase in CD14\(^+\) cells that localize to the follicle and in which immunophenotype is consistent with FDC. To understand how increased FDCs affect follicular lymphoma, we cocultured B cells from patients with follicular lymphoma with FDC and assessed for changes in cell viability compared with untreated follicular lymphoma B cells. After 48 hours, the cell viability of B cells doubled, 42\% (±1.7) versus 19\% (±2.2), with the presence of FDC compared with controls (Fig. 5A; \(P = 0.003\)). These results demonstrate that FDCs prolong the survival of B cells in follicular lymphoma. Given their shared localization the neoplastic follicle, CD14\(^+\) FDC may prolong survival of the tumor cells in follicular lymphoma. Though promotion of viability is not directly related to transformation, any mechanism that promotes malignant B-cell survival may well promote transformation. Because it is difficult to use fresh patient tissue to show proliferation or intracellular signaling, we chose promotion of malignant B-cell viability as evidence that FDCs support malignant B-cell growth.

**Sequential biopsies**

A potential limitation of our study is the long interval between the immunologic findings on diagnostic biopsy and the date of transformation in some of the patients. It could be argued that the biopsies were taken at a fixed time point (diagnosis), but that the changes in the microenvironment continue to evolve over time. To address this further, we analyzed CD14\(^+\) and PD1\(^+\) cell expression on samples from 6 patients in which serial biopsies were available from diagnosis to transformation. On average there were three serial biopsies per patient, the pathology varied from hyperplasia to follicular lymphoma and the time range encompassed was from 3 to 16 years. The intensity of PD1 or CD14 cell staining did not change in serial biopsies as patients progressed to transformation. However, serial biopsies confirmed that the pattern of CD14\(^+\) cells transitioned from a nonfollicular to follicular pattern as the time of transformation approached. Similarly, the pattern of PD1 expression changed from a follicular to a diffuse pattern before transformation (Fig. 5B and C). These serial biopsies demonstrate an evolution in the pattern of CD14 and PD1 expression as patients approach transformation.

**Discussion**

This study demonstrates that the pattern of CD14\(^+\) and PD1\(^+\) cells is predictive of the TTT in patients with follicular lymphoma. Patient samples in which CD14\(^+\) cells localized
to the follicle were associated with a shorter TTT than those that did not. Conversely, patient samples in which PD1+ cells were no longer localized to the follicle were associated with a shorter TTT and inferior survival. These results were independent of each other and remained statistically significant after accounting for clinical factors such as the FLIPI score.

This is the first report of an association of CD14+ cells predicting outcomes in patients with follicular lymphoma. Rather than the quantity of CD14+ cells, it was their location within the tumor and microenvironment that was predictive of outcome. CD14 is a lipopolysaccharide-binding protein that can act as receptor for endotoxin that is often associated with monocytes (34). Lymphoma-associated monocytes have been associated with poor survival in follicular lymphoma (18, 35), and initially it was assumed the CD14+ cells represented intratumoral monocytes. However, further analysis through flow cytometry and multicolor IHC confirmed that the CD14+ cells were distinct from monocytes or macrophages and represented FDC. Though FDC are typically identified by CD21, CD23, and CD35 surface markers they have also previously been associated with CD14 as well (36). CD14+ monocytes are thought to be immunosuppressive and their presence is associated with more aggressive tumors in NHL (24); however, the role of CD14+ cells that are representative of a FDC phenotype is unknown.

Previous gene expression profiles of follicular lymphoma patients identified that an increase in genes expressed by DCs was associated with poor OS (11). A recent study assessed the prognostic role of FDC in follicular lymphoma based on the extent of FDCs (37). They found no association with clinical outcomes; however, this study focused on all FDCs using a pan-FDC antibody in which, as in our study, the predictive component was limited to CD14+ cells. Similarly, in this study no association with clinical outcomes was seen when the more common FDC marker CD21+ was compared. This suggests that the CD14+ FDC of interest might represent a unique subset of FDC. To clarify the role of FDC in follicular lymphoma, we cocultured FDC and malignant B cells and demonstrated the presence of FDC led to increased B-cell viability. Others have also found that FDCs promote the proliferation and prevent apoptosis of transformed malignant B cells (38, 39). Though there are limitations in these studies as tonsil-derived FDCs were used and viability rather than transformation was measured, these results confirm these previous findings and provide a biologic rationale for the observation that CD14+ FDCs localized to the follicle are associated with a shorter TTT.

In addition to CD14+ FDC, PD1+ cells were found to be strongly correlated with clinical outcomes. Patients whose biopsies demonstrated PD1+ cells that localized to the follicle had a significantly prolonged TTT and OS compared with those with a diffuse pattern of PD1+ cells.

Figure 2. The PD1+ cell pattern and TTT and OS. A, the follicular and diffuse pattern of PD1+ cell expression. The follicular pattern of PD1+ cells is associated with prolonged TTT (B; 6.1 vs. 3.6 years, \( P = 0.03 \)) and OS (C; 9.7 vs. 4.6 years, \( P = 0.009 \)) compared with the diffuse pattern. D, all patients with early transformation (<1 year) had diffuse staining of PD1+ cells compared with the follicular pattern predominating amongst late transformation (>5 years).
The role of PD1 in solid tumors is well recognized with some solid tumors expressing PD-L1 and this ligand for PD1 being associated with a poor prognosis possibly through a decrease in tumor immunosurveillance (40, 41). Previous analyses of PD1+ cells in follicular lymphoma have yielded conflicting results. Two studies supported that increased levels of PD1+ cells were associated with superior outcomes, including a decreased risk of transformation (13, 25). However, additional studies have not confirmed the correlation between improved outcome and an increased number of PD1+ cells (42). In fact, some studies have found that increased PD1+ cells are associated with an inferior survival in follicular lymphoma (15). Though this study found no association between the TTT and the quantity of PD1+ cell content, it, like other microenvironment analyses, found an association between the location within the microenvironment and patient outcomes (14, 17, 43). When reviewing the previous analyses that did find a positive association of PD1+ cells and survival, in one study >90% of PD1+ cells were localized to the follicle and in the other the results were accentuated when the analysis was limited to the follicular subset (25). Therefore, our observation of superior clinical outcomes when PD1+ cells are localized to the follicle seems to be in agreement with these previous studies.

The retrospective and subjective nature of all of these studies, this study included, can affect their reproducibility and concordances with other studies. In addition, analysis restricted to a single marker by IHC may identify a heterogeneous population of different underlying immune cells that share a similar marker. To clarify this, multicolor IHC and flow cytometry were used in this study to identify PD1+ cells and showed that PD1+ cells in the follicle are primarily TFH cells, whereas those that are outside of the follicle are TIM3+ exhausted T cells. These results are consistent with other findings that PD1 is expressed on TFH cells; however, they also illustrate that not all PD1+ cells are the same. The prognosis of TFH cells in follicular lymphoma is currently unknown. However, our results suggest that superior clinical outcomes, including a prolonged TTT that are associated with follicular PD1+ cells, are attributable to TFH cells.
In contrast, we have previously demonstrated that exhausted T cells identified as TIM3\(^+\) cells are associated with inferior prognosis in follicular lymphoma (32). This study demonstrates that the PD1\(^+\) cells that reside outside the follicle represent TIM3\(^+\) exhausted T cells and the presence of these PD1\(^+\)TIM3\(^+\) cells is associated with poor survival and shorter TTT. One possible explanation is that a subset of T cells function to suppress transformation and with T-cell exhaustion this suppression is lost, thereby allowing for transformation.

This study did not find an association with increased quantity of CD68\(^+\) macrophages or FOXP3\(^+\) cells and clinical outcomes as others have previously reported (12, 18). This could be related to sample size, variation in treatment received as well as difference in technique and interpretation of IHC. However, follow-up studies of the significance of increased CD68\(^+\) macrophages have been contradictory (19, 43, 44). Similarly, further studies have not confirmed the association of increased FOXP3\(^+\) cells and superior outcomes in follicular lymphoma (17, 44); in fact, Carreras and colleagues noted that after adjusting for the presence of PD1\(^+\) cells, FOXP3 was no longer associated with improved survival (13). In addition, this study involved a majority of patients treated before the rituximab era. Thus, our microenvironment findings do not reflect the potential effects of rituximab treatment and previous studies have demonstrated that the introduction of rituximab can affect microenvironment associations (19).

Furthermore, previous studies have documented a constant annual rate of transformation rather than a dynamic rate of transformation, as seen in this study, which may suggest an evolving change in the microenvironment. The microenvironment factors identified in our analysis, therefore, likely contribute to transformation but may not be comprehensive. It is conceivable that the interactions and effects of PD1 and CD14 cells with other immune cells and/or the tumor itself lead to a more stepwise development of transformation.

This study identifies two independent factors of the follicular lymphoma microenvironment that are associated with a shorter TTT. In both instances, it is the pattern of location rather than quantity of PD1\(^+\) and CD14\(^+\) cells that is predictive of clinical outcomes. This study is the first to report an association between CD14\(^+\) FDC and clinical outcomes in follicular lymphoma. The study also provides additional understanding to the complex interaction of PD1 in follicular lymphoma. We find that PD1\(^+\) TFH cells are associated with superior clinical outcomes, whereas

Figure 4. CD14\(^+\) cells consistent with FDC. A, flow cytometry demonstrates coexpression primarily on CD21 and 23 and not CD68 and 163 (n = 3). B, multicolor IHC of CD14 (blue), CD68 (red), and CD163 (orange) demonstrating no colocalization. C, cells staining for CD14 (blue) colocalize to identical cells staining positive for CD21 (yellow) and CD 23 (green) exemplified on magnified insets.
PD1⁺TIM3⁺ exhausted T cells outside the follicle are associated with an inferior survival and a shorter TTT. Additional prospective studies are warranted to confirm these findings and determine their prognostic importance relative to other previously identified predictive factors in the microenvironment. Further understanding of these interactions could be useful in designing therapies that modulate the immune microenvironment in follicular lymphoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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