The Prognostic Impact of CD163-Positive Macrophages in Follicular Lymphoma: A Study from the BC Cancer Agency and the Lymphoma Study Association

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Abstract

Purpose: We aimed to assess the prognostic significance of follicular lymphoma–associated macrophages in the era of rituximab treatment and maintenance.

Experimental Design: We applied immunohistochemistry for CD68 and CD163 to two large tissue microarrays (TMA). The first TMA included samples from 186 patients from the BC Cancer Agency (BCCA) who had been treated with first-line systemic treatment including rituximab, cyclophosphamide, vincristine, and prednisone. The second contained 395 samples from PRIMA trial patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, and randomized to rituximab and prednisone. The second contained 395 samples from PRIMA trial patients treated with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone, and randomized to rituximab maintenance or observation. Macrophage infiltration was assessed using Aperio image analysis. Each of the two cohorts was randomly split into training/validation sets.

Results: An increased CD163-positive pixel count was predictive of adverse outcome in the BCCA dataset (5-year progression-free survival (PFS) 38% vs. 72%, respectively, P = 0.004 in the training cohort and 5-year PFS 29% vs. 61%, respectively, P = 0.004 in the validation cohort). In the PRIMA trial, an increased CD163 pixel count was associated with favorable outcome (5-year PFS 60% vs. 44%, respectively, P = 0.011 in the training cohort and 5-year PFS 55% vs. 37%, respectively, P = 0.030 in the validation cohort).

Conclusions: CD163-positive macrophages predict outcome in follicular lymphoma, but their prognostic impact is highly dependent on treatment received. Clin Cancer Res; 21(15); 3428–35.

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Introduction

Follicular lymphoma is the most common indolent lymphoma subtype and is clinically characterized by prolonged survival with median survival times in excess of 10 years (1). Patient outcomes are, however, heterogeneous, and a nonnegligible proportion of patients are at risk of early progression and/or transformation (secondary development of diffuse large B-cell lymphoma), putting them at risk for adverse outcome. Treatment decisions are currently almost exclusively guided by clinical characteristics and range from watchful waiting to anti-CD20 directed immunotherapy alone or in combination with chemotherapy (2).

Despite the presence of highly recurrent genetic alterations such as the t(14;18)(q32;q21) translocation or mutations in epigenetic modifiers, malignant follicular lymphoma cells fail to thrive in vitro, suggesting that the tumor microenvironment plays a crucial role in their expansion and survival (3). The landmark study of the Lymphoma/Leukemia Molecular Profiling Project established that gene expression signatures derived from nonmalignant tumor-infiltrating cells influence patients’ outcomes (4). The correlation of macrophage and T-cell counts with prognosis is an area of intensive translational investigation. However, for tumor-associated macrophages (TAM) in particular, the results

References

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

In follicular lymphoma, the tumor microenvironment has been shown to influence patient outcomes, but the association of tumor-infiltrating macrophages with survival is controversial. Here, we report the correlation of CD163, a marker of alternatively polarized macrophages, with outcome in two large datasets. We show that increased CD163+ macrophages are associated with poor survival in patients treated with R-CVP, and with favorable outcome in patients treated with R-CHOP/R-maintenance. Our findings are of translational relevance as they illustrate that the tumor microenvironment modulates response to therapy and patient outcomes. Furthermore, we demonstrate, for the first time in patient data, the existence of a positive interaction between chemotherapy (doxorubicin) and rituximab, mirroring data from preclinical animal models. In the context of heterogeneous patient outcomes in follicular lymphoma, our findings inform on the rational use of immunochemotherapy in this disease.

are apparently contradictory as their presence correlated with adverse outcome in some studies (5–10) but not in others (11). It is increasingly recognized that the prognostic effect of TAMs is modulated by treatment, and several studies suggest a positive interaction between rituximab and tumor-suppressive functions of macrophages (7, 11, 12).

The monocyte and macrophage lineage is characterized by considerable functional heterogeneity and plasticity, accounting for the implication of these cells in biologic processes as diverse as inflammation, infection, or cancer (13, 14). TAMs adopt a broad spectrum of functional states that may evolve and fluctuate between the extremes of antitumoral M1-type and protumoral M2-type phenotypes (13, 14). In follicular lymphoma, stromal cells recruit monocytes via the secretion of CCL2 and TAMs, in cooperation with stromal cells, increase the proliferation of B-cell cell lines, and prolong the survival of primary follicular lymphoma cells in culture (15). Further, CD36-purified TAMs overexpress IL15, stimulating follicular lymphoma cell survival in combination with CD40 activation (16). CD163, a surface marker that is predominantly found on M2-skewed macrophages (17), has been associated with poor prognosis and increased angiogenic sprouting in follicular lymphoma (10).

We designed this study to ask the question whether the M2-restricted macrophage marker CD163 would provide better prognostic value than CD68 and whether that effect would be consistent across two different patient cohorts. We tried to overcome limitations from prior studies by evaluating outcome correlations in two large datasets with relative treatment uniformity and by using sensitive, state of the art image analysis.

Subjects and Methods

Patients

We constructed a first tissue microarray (TMA) from 1.5 mm duplicate cores using the samples from 186 patients treated between 2004 and 2009 with rituximab, cyclophosphamide, vincristine, and prednisone (R-CVP) at the BC Cancer Agency (BCCA). The median time from date of biopsy (study sample) to initiation of systemic treatment was 1.84 months (interquartile range, 5.57 months). Twelve patients of 186 (7%) had received local therapy (surgery and/or radiotherapy) before R-CVP. From 2006 onwards, rituximab was given as maintenance treatment (once every 12 weeks for 2 years) for patients achieving at least a partial response after R-CVP. The second TMA was built from 1.0 mm triplicate cores using samples from patients who participated in PRIMA, an international randomized phase III trial that assigned patients responding to first-line therapy to rituximab maintenance (every 8 weeks for 2 years) or observation (18). For PRIMA trial patients, we restricted the analysis to those patients receiving rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) as first-line treatment (93% of the population represented on the TMA) in order to ensure treatment homogeneity. The PRIMA trial required the study sample to be taken within 4 months before study registration.

Immunohistochemistry, scoring, and immunofluorescence

Both TMAs were stained in Vancouver with mouse monoclonal antibodies against CD68 (clone KP1; Dako) and CD163 (clone 10D6; Novocastra) on a Ventana BenchMark XT automated slide staining system. Image analysis was performed on an Aperio ScanScope XT using the Positive Pixel Count algorithm with a color saturation threshold (CST) set at 0.1. All slides were reviewed by pathologists (K. Tan and A. Vawda) to exclude nonspecific staining and in rare cases, the CST was increased to 0.15 to reduce background staining. The percentage of positive pixels was determined as described in Tan and colleagues (19) and averaged across all cores from each sample. In addition, visual scoring was performed on the BCCA TMA by estimating percentages of CD68- and CD163-positive cells. Scores were reported in discrete categories (0%, 1%, 5%, 10%, 20%, and 30%).

Double immunofluorescence was performed on a 4-μm formalin-fixed paraffin-embedded section after heat-induced epitope retrieval using an AlexaFluor-647-labeled mouse anti-human CD68 antibody (clone KP1; Santa Cruz) and an unlabeled rabbit anti-human CD163 antibody (clone K20-T; Abnova) in combination with a secondary goat anti-rabbit Cy3 antibody. The immunofluorescent image was acquired using a CoolSnap HQ digital camera on an inverted microscope (IX70 Olympus) in combination with a DeltaVision RT imaging system (Applied Precision). The image was scanned in stacks, deconvoluted, and projected onto a single plane using SoftWoRx (Applied Precision).

Statistical analysis

As, on average, the extent of macrophage infiltration is low in follicular lymphoma and point estimates can be imprecise on TMAs, we required each valid patient sample to have at least two measurements and the relative standard error to be less than 33.33%. Correlation between Aperio and manual scoring was assessed using the Pearson correlation coefficient, whereas the correlation between CD68 and CD163 positivity was determined using Spearman correlation as visual inspection suggested that the underlying assumptions of linear regression were not met. For survival analysis, both the BCCA and the PRIMA cohorts were randomly split into training/validation sets in 1:1 ratios. As the distributions of CD68 and CD163 expression did not reveal natural cutpoints, we used the X-tile software (version 3.6.1; Yale University, New Haven, CT) to bisect these distributions at values that gave the maximum χ2 values of the log-rank test (20).
Thresholds of CD68 and CD163 expression were defined using progression-free survival (PFS) in each of the training cohorts. They were then locked and carried forward into the validation cohorts for PFS and the training/validation cohorts for overall survival (OS) analysis. Times to event [relapse or death from any cause (PFS) or death from any cause (OS)] were calculated from the date of the first dose of R-CVP for BCCA patients, and from date of registration for PRIMA patients. Associations of clinical characteristics with patient cohorts were evaluated using the χ² test, and the distributions of CD163 staining in risk categories were compared using the Mann–Whitney or the Kruskal–Wallis tests. P < 0.05 was considered significant.

Results

Patient cohorts

The study overview is shown in Supplementary Fig. S1. After quality assessment, 180 of 186 (97%) and 335 of 436 (77%) patient samples were deemed evaluable for the assessment of either CD68 or CD163 in the BCMA and PRIMA datasets, respectively. The two cohorts differed in several clinical characteristics, including age, stage, performance status, lactate dehydrogenase (LDH), and hemoglobin levels, but after repartition of patients into FLIPI risk categories, the study populations were not significantly different between BCMA and PRIMA patients (P = 0.329; Table 1). The median follow-up of living patients was 70.7 and 55.2 months for the BCMA and the PRIMA cohorts, respectively. Rituximab maintenance was intended for 150 of 180 BCMA patients (83%) and randomly administered (according to the study design) to 141 of 335 PRIMA patients (42%).

Macrophage staining

Aperio scoring of macrophage infiltration was well correlated with manual assessment of percentage of stained cells (R = 0.827 and P < 0.001 for CD68; R = 0.907 and P < 0.001 for CD163; Supplementary Fig. S2). The extent of macrophage infiltration of the tumor was low, as assessed by a median pixel positivity of 3.87% and 2.61% for CD68 and 1.07% and 0.98% for CD163, in the BCMA and PRIMA cohorts, respectively (Supplementary Fig. S3). On average, CD68 stained more pixels than CD163 (P < 0.001 in both datasets; Supplementary Fig. S4A). The distribution of CD163 staining was superimposable in the two cohorts (P = 0.222), but significantly different for CD68 (P < 0.001; Supplementary Fig. S5). CD68 and CD163 staining were not significantly correlated (R = 0.157 and P = 0.057 for the BCMA cohort, and R = 0.112 and P = 0.068 for the PRIMA cohort; Supplementary Fig. S6). By immunofluorescence, CD163-positive cells costained positive for CD68 (Supplementary Fig. S4B). These cells were mostly located in inter-follicular areas, whereas scattered CD68-positive/CD163-negative cells tended to be found inside follicles.

Survival analysis in the BCMA cohort

CD68 positivity was weakly associated with PFS in the training cohort, but a significant association could not be found in the validation cohort (Supplementary Fig. S7). High positive pixel counts for CD163 on the other hand were significantly associated with poor PFS and OS in both the training and validation cohorts (Fig. 1). Using the optimal cutoff of 3.97% for CD163, 5-year PFS was 38% versus 72% in the training cohort (P = 0.004), and 29% versus 61% in the validation cohort (P = 0.004). The CD163-positive pixel count was not significantly associated with clinical risk factors (Table 2). The adverse prognostic impact of CD163+ TAMs remained significant in a Cox multivariate regression model when CD163 was used as a continuous variable and after adjusting for the FLIPI index and use of rituximab maintenance by intention to treat (HR 1.12 for PFS, P = 0.022; Table 3).
determination using X-tile revealed an optimal cutoff of 0.16% for CD163 in the PRIMA data. Based on this threshold, a high CD163-positive pixel count was indeed associated with favorable PFS in the training cohort (5-year PFS 60% vs. 44% and $P = 0.011$), and this association remained true in the validation cohort (5-year PFS 55% vs. 37% and $P = 0.030$). A high CD163-positive pixel count was associated with age > 60 years ($P = 0.018$), female gender ($P = 0.026$), low LDH ($P = 0.022$), and ≤ 4 nodal sites ($P < 0.001$). The CD163-positive pixel count was not associated with the FLIPI index (Table 2).

We then performed a multivariate Cox regression analysis in which CD163 was introduced as a continuous variable in order to avoid overfitting of the model and relying on predefined cutpoints (Table 4). The analysis was stratified by randomization into either the rituximab or observation arms. High CD163 remained significantly associated with favorable outcome when adjusted for the FLIPI in those patients assigned to the rituximab arm (HR 0.73 for PFS, $P = 0.015$), but not in those patients randomized to the observation arm (HR 1.06 for PFS, $P = 0.322$). These latter results confirm the association between a high CD163-positive pixel count and favorable outcome in the rituximab arm of the PRIMA trial only. They suggest that CD163\textsuperscript{+} TAMs modulate the efficacy of rituximab, supporting the hypothesis of a favorable interaction between TAMs and response to maintenance rituximab.

**Discussion**

Here, we investigated the correlation of TAMs with outcome using modern image analysis. We first analyzed a single institution experience with uniform therapy (BCCA cohort, R-CVP) and then compared the findings with those from a prospective, randomized phase III clinical trial (PRIMA, R-CHOP). Computer-assisted scoring has recently been proposed to be a more reliable means for enumeration of microenvironment cell populations than traditional manual scoring (21). We showed that most diagnostic follicular lymphoma samples are infiltrated by few macrophages. Increased staining for CD163 was associated with poor PFS and OS in the BCCA cohort and favorable PFS in the PRIMA cohort. On the other hand, CD68 staining cells did not predict outcome in either cohort.

Of the two markers that were used in the present study, CD163 gave us much cleaner and stronger staining than CD68, as described by our group and by others (19, 22, 23), whereas CD68 has more nonspecific background signal and stains some nonhistiocytic cellular elements (24). These considerations may
Table 2. Association of CD163 with clinical characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>BCCA</th>
<th>PRIMA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% CD163-positive pixels count, mean</td>
<td>% CD163-positive pixels count, mean</td>
</tr>
<tr>
<td>Age at induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>1.87 NS 1.52</td>
<td>0.018</td>
</tr>
<tr>
<td>&gt;60</td>
<td>2.10 NS 2.32</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>2.05 NS 1.92</td>
<td>0.026</td>
</tr>
<tr>
<td>Male</td>
<td>1.95 NS 1.66</td>
<td></td>
</tr>
<tr>
<td>Ann Arbor stage*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>2.46 NS 2.21</td>
<td>NS</td>
</tr>
<tr>
<td>III-IV</td>
<td>1.90 1.75</td>
<td></td>
</tr>
<tr>
<td>ECOG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.53 1.73</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.39 1.91</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.40 1.78</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.68 NS NA NS</td>
<td></td>
</tr>
<tr>
<td>LDH&lt;ULN</td>
<td>1.81 1.84</td>
<td></td>
</tr>
<tr>
<td>&gt;ULN</td>
<td>2.93 NS 1.66 0.022</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin&lt;12 g/dL</td>
<td>2.77 2.04</td>
<td></td>
</tr>
<tr>
<td>&gt;12 g/dL</td>
<td>1.85 NS 1.72 NS</td>
<td></td>
</tr>
<tr>
<td>Nodal areas*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤4</td>
<td>2.26 2.45</td>
<td></td>
</tr>
<tr>
<td>&gt;4</td>
<td>1.82 NS 1.42 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>FLIPIf</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low risk</td>
<td>2.28 2.19</td>
<td></td>
</tr>
<tr>
<td>Intermediate risk</td>
<td>1.66 1.56</td>
<td></td>
</tr>
<tr>
<td>High risk</td>
<td>2.00 NS 1.78 NS</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: ECOG, Eastern Cooperative Oncology Group performance status; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

Within the PRIMA trial, the randomization also offered an unbiased, controlled comparison of outcome modulation by CD163 and use of maintenance rituximab versus observation. Elevated CD163 staining was significantly associated with favorable PFS only in the rituximab maintenance arm, suggesting that macrophages predicted poor event-free survival in the cyclophosphamide, doxorubicin, etoposide, prednisolone and interferon (CHVP-I) arm but not in the rituximab plus CHVP-I arm (7), and the study by Taskinen and colleagues in which elevated CD163 staining was correlated with age > 60 years, the female gender, low levels of LDH, ≤ 4 nodal areas but not the FLIPI index. The Cox multivariate regression analysis shows, however, that the differential prognostic impact of TAMs on PFS is independent of established risk factors and potential confounders. It is noteworthy that one of the main differences in terms of treatment resides in the administration of doxorubicin as part of the R-CHOP regimen that was uniformly given in those PRIMA patients that were evaluated in this study, whereas BCCA patients were invariably managed without an anthracycline as part of their first-line therapy. In an allograft mouse model, depletion of macrophages reduced the efficacy of doxorubicin, but not daunorubicin, and conversely, prior macrophage activation enhanced the efficacy of doxorubicin (25, 27). Recent experimental data have also indicated that antihuman CD163 are able to modulate the differentiation and function of cells involved in innate immunity toward a tumoricidal phenotype (28). These findings, in combination with our data, suggest that doxorubicin contributes to better outcome in those R-CHOP patients whose samples harbor elevated numbers of macrophages.

Table 3. Cox multivariate regression analysis in the BCCA cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLIPI index</td>
<td>1.12 1.02-1.23</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>FLIPI risk group</td>
<td>1.46 1.02-2.07</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>Rituximab maintenance</td>
<td>0.83 0.44-1.58</td>
<td>0.573</td>
<td></td>
</tr>
<tr>
<td>FLIPI individual factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD163-positive pixel count*</td>
<td>1.12 1.01-1.24</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>1.60 0.91-2.82</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>1.74 0.77-3.93</td>
<td>0.182</td>
<td></td>
</tr>
<tr>
<td>LDH &gt; ULN</td>
<td>0.78 0.36-1.69</td>
<td>0.527</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin &lt;120 g/L</td>
<td>2.09 0.90-4.84</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
<td>&gt;4 nodal sites</td>
<td>0.88 0.48-1.59</td>
<td>0.663</td>
<td></td>
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<tr>
<td>Rituximab maintenance</td>
<td>0.80 0.42-1.54</td>
<td>0.505</td>
<td></td>
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</tbody>
</table>

Abbreviations: CI, confidence interval; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

*continuous variable.
rational marker to identify those TAMs that participate in rituximab-mediated antitumor responses (32).

In conclusion, our findings lend texture to a rich but highly conflicted literature on tumor microenvironment markers in follicular lymphoma, and the interpretation needs to be nuanced. In the absence of doxorubicin as part of primary immunochemotherapy, increased numbers of CD163\(^+\) TAMs correlate with worse outcome, and this negative effect is not compensated by maintenance rituximab. However, when doxorubicin is given, as it was in the R-CHOP–treated PRIMA trial patients, maintenance rituximab emerges as important, and its inclusion reverses the negative influence of CD163\(^+\) TAMs. Although the development of a robust biomarker is highly desirable for follicular lymphoma patients in the precision medicine era, the apparently opposite

Table 4. Cox multivariate regression analysis in the PRIMA cohort

<table>
<thead>
<tr>
<th>Variable</th>
<th>Rituximab arm: PFS(^a)</th>
<th>Observation arm: PFS(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
</tr>
<tr>
<td>FLIPI index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD163-positive pixel count(^c)</td>
<td>0.73</td>
<td>0.57–0.94</td>
</tr>
<tr>
<td>FLIPI risk group</td>
<td>2.58</td>
<td>1.45–4.58</td>
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<tr>
<td>FLIPI individual factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD163-positive pixel count(^c)</td>
<td>0.77</td>
<td>0.58–1.01</td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>1.58</td>
<td>0.75–3.33</td>
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<tr>
<td>Stage III/IV</td>
<td>0.85</td>
<td>0.09–8.27</td>
</tr>
<tr>
<td>LDH &gt; ULN</td>
<td>2.21</td>
<td>1.08–4.52</td>
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<tr>
<td>Hemoglobin &lt;120 g/L</td>
<td>0.96</td>
<td>0.43–2.18</td>
</tr>
<tr>
<td>&gt;4 nodal sites</td>
<td>3.90</td>
<td>1.55–13.24</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; FLIPI, Follicular Lymphoma International Prognostic Index; ULN, upper limit of normal.

\(^a\)This analysis was performed using data from all 113 patients who were evaluable for CD163 staining and randomized to the rituximab arm.

\(^b\)This analysis was performed using data from all 147 patients who were evaluable for CD163 staining and randomized to the observation arm.

\(^c\)Continuous variable.
results from the BCCA and the PRIMA studies suggest that prognostication is highly dependent on patient characteristics and/or treatment. Furthermore, as the threshold that most significantly distinguished the outcomes of those BCCA cases with high from those cases with low CD163+ positivity did not validate in the PRIMA cohort, we do not presently recommend staining for CD163 in routine clinical evaluation. Future studies are warranted in well-annotated external patient populations and should also extend to cohorts of patients treated with the nowadays more commonly used bendamustine–rituximab regimen, or new agents such as imids or kinase inhibitors.

Disclosure of Potential Conflicts of Interest
L.H. Sehn reports receiving speakers bureau honoraria from Agen, Celgene, Gilead, Janssen, Lundbeck, Pfizer, Roche/Terumo, and Seattle Genetics. G. Salles reports receiving a commercial research grant from Roche, and is a consultant/advisory board member for Celgene, Gilead, Janssen, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Xerri, K. Tan, A. Vawda, E.A. Chavez, J.M. Connors
Study supervision: R.D. Gascoyne, G. Salles

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