Introduction

Follicular lymphoma (FL) is the most frequent indolent lymphoma and results from the malignant transformation of germinal center (GC) B cells. FL pathogenesis is a protracted, multistep oncogenic process, in which the first genetic hit is ascribed to the t(14;18) translocation that results from the illegitimate joining between the BCL2 proto-oncogene and the IGH locus. Although t(14;18) is the molecular hallmark of FL, t(14;18)pos B cells are found in the blood of most healthy individuals at a low frequency, indicating that ectopic expression of the antiapoptotic B-cell lymphoma 2 (BCL2) protein is not sufficient for tumor progression. Interestingly, transgenic overexpression of BCL2 results in a delay in cell cycle progression both in vitro and in mouse models. However, such an antiproliferative effect of BCL2 has never been evaluated in naturally occurring human t(14;18)pos B cells.

FL pathogenesis requires additional oncogenic events that are supposed to be acquired in the GC on exposure to the off-target effects of activation-induced cytidine deaminase, the key enzyme of antigen-driven antibody diversification. Such oncogenic events could already be detected in early FL lesions including FL in situ (FLIS). The frequency of t(14;18)pos circulating B cells increases with age and exposure to pesticides, reflecting the persistence and expansion of clonal populations of memory B cells bearing genotypic and phenotypic features of FL cells and called FL-like cells (FLLCs).

Study design

For details, see supplemental Methods available on the Blood Web site.
To characterize FLLCs in situ, BCL2/JH translocation was quantified by qPCR on 85 consecutive RLNs. Twelve samples (14%) were found to be positive (supplemental Table 1), with a frequency ranging from 4 to 66 $\times$ 10$^{-5}$, ie, 1 in 25 000 to 1 in 1 500 cells, within samples containing 38.7 $\pm$ 8% CD19$^{pos}$ B cells (data not shown). The prevalence of these t(14;18)$^{hi}$ samples was independent of gender but showed a significant correlation with age, reaching 28% in donors $>50$ years. A frequency of circulating FLLCs $>1$ in 25 000 mononuclear cells was previously reported in 4.6% of healthy adults, and these t(14;18)$^{hi}$ donors reached 9.2% $>50$ years, in agreement with our age-related prevalence increase.11,16 Overall, we identified 12 RLN with a frequency of FLLCs suitable for in situ visualization and/or functional studies and focused on 8 of them (RLN 1-8) for further analysis. We also selected 3 t(14;18)$^{pos}$ RLNs (RLN 9-11) as controls.

To visualize FLLCs in situ we first performed immunohistochemical staining for the pan-B-cell transcription factor PAX-5 and for BCL2, that is specifically down-regulated in normal GC B cells, except in the presence of the t(14;18) translocation.17 This approach revealed variable amounts of isolated PAX-5$^{pos}$BCL2$^{pos}$ B cells within the GC in t(14;18)$^{hi}$ RLNs, whereas such cells were essentially undetectable in the GC from t(14;18)$^{pos}$ samples (supplemental Figure 1). These GC PAX-5$^{pos}$BCL2$^{pos}$ cells visualized inside the GC could comprise IgD$^{pos}$ cells, corresponding to bona fide FLLCs, but also some IgD$^{pos}$ small lymphocytes invaginating from the mantle zone (data not shown). Moreover, because BCL2 is normally expressed in naive and memory B cells, such a strategy could not evaluate the presence of FLLCs outside the GC. We thus focused on CD10, a classical GC B-cell marker, and quantified cells coexpressing CD20, BCL2, and CD10 in 5 t(14;18)$^{hi}$ and 3 negative RLNs by immunohistofluorescence (Table 1; supplemental Figure 2). In fact, FLLCs have been suggested to resemble FL cells in retaining an atypical CD10$^{pos}$BCL2$^{pos}$ frozen GC-like phenotype outside the GC.2 We consistently detected more CD20$^{pos}$BCL2$^{pos}$CD10$^{pos}$ triple positive cells in t(14;18)$^{hi}$ than in t(14;18)$^{pos}$ RLNs. Interestingly, these cells were visualized both inside and outside the GC in t(14;18)$^{hi}$ RLNs. By contrast, in control t(14;18)$^{pos}$ samples, the few triple positive cells were exclusively located outside the GC and might correspond to rare CD27$^{pos}$BCL2$^{pos}$CD10$^{pos}$ memory B cells previously described in peripheral blood.9,18 However, the number of t(14;18)$^{pos}$ cells outside the GC was higher in t(14;18)$^{hi}$ than in negative RLNs and probably included post-GC FLLCs. In agreement, we detected by long-range PCR that the majority of t(14;18)$^{hi}$ RLNs displayed a class switch recombination to $\gamma$ on the translocated allele (supplemental Figure 4). Most importantly, when normalizing the percentage of triple positive cells within GC to the surface occupied by the GC in corresponding samples, we pinpointed in 5 of 6 t(14;18)$^{hi}$ samples a clear enrichment (3.5- to 6.5-fold) of CD20$^{pos}$BCL2$^{pos}$CD10$^{pos}$ within the GC. This enrichment strongly suggests a preferential homing/retention into GC.

Although FLLCs accumulate within the GC of t(14;18)$^{hi}$ RLNs, they presented as scattered cells without proliferation foci. In addition, high t(14;18) frequency was not correlated to high GC expansion, making it unlikely that resident FLLCs contributed significantly to follicular hyperplasia. These observations raised the question of the proliferation potential of FLLCs within the hyperproliferative GC environment. To address this issue in the absence of any specific marker allowing the sorting of FLLCs, we first purified from t(14;18)$^{hi}$ RLN proliferative centroblasts vs nonproliferative centrocytes based on their differential membrane expression of CXCR4$^{19}$ and staining with Hoechst dye (supplemental Figure 3). Interestingly, CD20$^{pos}$CD10$^{pos}$CXCR4$^{pos}$Hoechst$^{pos}$ centroblasts...
Figure 1. FLLCs are nonproliferative cells. (A) Distribution of the t(14;18) pos cells within proliferative CD20 pos CD10 pos CXCR4 pos Hoechst pos centroblasts vs CD20 pos CD10 pos CXCR4 neg Hoechst neg nonproliferative centrocytes cell sorted from a t(14;18) hi RLN7 sample. Genomic DNA from each GC subsets was tested using a 2-step fluctuation nested PCR assay consisting of 16 replicates. BCL2/IGH translocations were sequenced. Multiple size bands revealed oligoclonality. (B) CFSE-labeled CD20 pos purified B cells from RLN4 were stimulated for 4 days. Highly proliferative viable CFSE lo DAPIneg and nonproliferative viable CFSE hi DAPIneg B cells were then sorted before quantification of t(14;18) by qPCR. Standard curves were generated from cloned BCL2/IGH (red) and GAPDH (green) PCR products, and it was demonstrated that detection of the BCL2/IGH transcript from the CFSE lo population was above the CFSE hi cell population, where it remained below the quantification threshold of 1/25 000 cells. NQ, not quantifiable.

<table>
<thead>
<tr>
<th>B-cell subset</th>
<th>t(14;18) frequency</th>
<th>BCL2 break*</th>
<th>N-insertions</th>
<th>IGH segment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR4 pos Hoechst pos</td>
<td>1.7E-04</td>
<td>CTTTCTCATG (3074)</td>
<td>TTT</td>
<td>(89764) CTACTACTA JH6</td>
</tr>
<tr>
<td>CXCR4 neg Hoechst neg</td>
<td>9.8E-04</td>
<td>CAGTGGTGCG (3110)</td>
<td>CGGCACCGTGTTGAAA</td>
<td>(88763) TTTGACTACTGG JH4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CTCCTCCGC (3059)</td>
<td>CGCTCCCTCCCCCTCC</td>
<td>(89773) CTACCGTATGG JH6</td>
</tr>
</tbody>
</table>

* BCL2 and JH breakpoints are numbered according to accession number M14745 (BCL2) and X97051 (IGH)
contained a lower frequency of t(14;18)hi B cells than their non-proliferative centrocyte counterparts as determined by sensitive fluctuation PCR (Figure 1A). In addition, different B-cell clones could be detected in centrocytes, including the unique one detected in centroblasts. To better understand the lack of proliferation of FLLCs could be detected in centrocytes, including the unique one detected in proliferative cells, whereas they could not be detected in CFSElo proliferative cells. Altogether, these data demonstrate that human nonmalignant B cells with naturally occurring t(14;18) accumulate within a poorly proliferative B-cell compartment and support the hypothesis of an antiproliferative effect of BCL2, as previously shown in mouse and human BCL2-transgenic B cells.1,5

In conclusion, our in situ visualization of FLLCs in nonmalignant RLNs with follicular hyperplasia demonstrates for the first time that FLLCs are not randomly distributed but display preferential homing within the GC, a property shared with FL and FLIS cells that accumulate as poorly proliferative centrocytes in the early stage of the disease. FLIS have been previously proposed as the in situ counterpart of circulating FLLCs,20 but we demonstrate here that such t(14;18)hi B cells could accumulate within the GC even in the absence of classical FLIS lesions characterized as homogeneous follicles exhibiting strong CD20, CD10, BCL6, and BCL2 positivity.21 Scattered GC FLLCs thus potentially represent an earlier precursor stage in FL pathogenesis. Altogether, our findings provide new insights into the dynamics of FLLC progression and the connection between FLLC and FL.

Acknowledgments

The authors thank Patrick Tas, Céline Pangault, and the Centre de Ressources Biologiques–Santé of Rennes’ hospital for providing high-quality samples and Geserende Caron for cell sorting.

This work was supported by research grants from the Institut National du Cancer (INCa libre PL06-10 and Programmes d’Actions Intégrées de Recherche Lymphome 2008-019), the Ligue Contre le Cancer (Equipe Labellisée 2013), and institutional grants from Institut National de la Santé et de la Recherche Médicale and Centre National de la Recherche Scientifique.

Authorship

Contribution: J.T., C. Menard, and S.R. designed and performed research and analyzed data; N.M., C. Monvoisin, and I.C. performed research; B.N. contributed to the study design; P.G. contributed to the study design, analyzed data, and wrote the paper; and C.S. and K.T. designed and supervised research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Claudine Schiff, Centre d’Immunologie de Marseille-Luminy, Case 906, F-13288 Marseille Cedex 09, France; e-mail: schiff@cml.univ-mrs.fr; and Karin Tarte, INSERM U917, Faculté de Médecine, 2 Avenue du Pr Léon Bernard, F-35043 Rennes, France; e-mail: karin.tarte@univ-rennes1.fr.

References

Human t(14;18)positive germinal center B cells: a new step in follicular lymphoma pathogenesis?

Julie Tellier, Cedric Menard, Sandrine Roulland, Nadine Martin, Céline Monvoisin, Lionel Chasson, Bertrand Nadel, Philippe Gaulard, Claudine Schiff and Karin Tarte