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

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## Key Points

- Follicular lymphoma-like cells found in healthy individuals accumulate within germinal centers in reactive lymphoid tissues.
- Follicular lymphoma-like cells are nonproliferating cells in situ and in vitro.

**Follicular lymphoma (FL) is a B-cell neoplasm resulting from the transformation of germinal center (GC) B cells. Although t(14;18) and ectopic B-cell lymphoma 2 (BCL2) expression constitute the genetic hallmark of FL, t(14;18)$^{pos}$ B cells bearing genotypic and phenotypic features of FL cells can be found in the blood of most healthy individuals. Nevertheless, the localization of these FL-like cells (FLLCs) in nonmalignant GC-rich tissues and the functional consequences of BCL2 overexpression have not been evaluated thus far. Among 85 reactive lymph node (RLN) samples, 14% were found to contain high levels of t(14;18) by quantitative polymerase chain reaction. In t(14;18)$^{hi}$ RLNs, CD20$^{pos}$BCL2$^{pos}$CD10$^{pos}$ FLLCs consistently accumulated within the GC, essentially as nonproliferative CXCR4$^{neg}$ centrocytes. Moreover, they displayed a reduced response to proliferative stimuli in vitro. Altogether, our findings provide new insights into in situ FLLC functional properties and suggest that these cells have not acquired the ultimate genetic events leading to FL transformation. (*Blood*. 2014;123(22):3462-3465)**

## Introduction

Follicular lymphoma (FL) is the most frequent indolent lymphoma and results from the malignant transformation of germinal center (GC) B cells.[1] 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.[2] Interestingly, transgenic overexpression of *BCL2* results in a delay in cell cycle progression both in vitro and in mouse models.[3-5] 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).[6,7] The frequency of t(14;18)$^{pos}$ circulating B cells increases with age and exposure to pesticides,[8-11] 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). Existence of t(14;18)$^{pos}$ precursor lymphoma-initiating cells has been recently supported by 2 reports of clonally related FL arising in both the donor and the recipient after allogeneic hematopoietic stem cell transplantation.[12,13] Similarly, a clonal relationship between FL and FLLCs was reported by molecular backtracking in 3 FL cases for which prediagnostic blood samples were available.[14] However, the connection between FLLCs and progression to overt FL remains elusive.

To further explore the in situ FLLC evolution, we identified a series of healthy individuals with GC-rich reactive lymph nodes (RLNs) displaying high levels of t(14;18), allowing us to visualize and characterize FLLCs in situ and to perform functional in vitro studies. We demonstrated that FLLCs are nonproliferating cells with specific enrichment within the GC.

## Study design

For details, see supplemental Methods available on the *Blood* Web site.

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**Table 1. Distribution of $CD20^{pos}BCL2^{pos}CD10^{pos}$ triple positive cells in the GC of RLNs**

| Sample* | Triple positive cells/$10^5$ cells† | Triple positive cells in GC | GC surface (%)‡ | Enrichment coefficient in GC§ |
|---|---|---|---|---|
| RLN1 | 39 | 15 (38.5%) | 6.0 | 6.41 |
| RLN2 | 18 | 7 (39%) | 11.3 | 3.45 |
| RLN3 | 15 | 5 (33.3%) | 6.1 | 5.45 |
| RLN4 | 48 | 35 (72.9%) | 11 | 6.63 |
| RLN5 | 53 | 3 (5.7%) | 5.7 | 1 |
| RLN9 | 4 | 0 | 8.3 | 0 |
| RLN10 | 8 | 0 | 4.9 | 0 |
| RLN11 | 5 | 0 | 31.7 | 0 |

*RLN 1-5 and RLN 9-11 samples correspond to $t(14;18)^{hi}$ and $t(14;18)^{neg}$ LNs, respectively. The frequency of t(14;18) was determined by qPCR on dissociated RLN cells.

†Number of $CD20^{pos}BCL2^{pos}CD10^{pos}$ triple positive cells visualized by immunohistofluorescence. For each sample, between 1 and 3 large (2-5 $mm^2$) representative areas including GC were chosen (corresponding to $7 \times 10^4$ to $2 \times 10^5$ cells per sample) for counting.

‡Percentage of the analyzed area occupied by GC zones (defined as areas of $CD20^{pos}CD10^{pos}Bcl2^{neg}$ cells).

§Enrichment coefficient = (% triple positive cells in GC)/(% GC surface).

### Samples

Lymph node samples were collected from individuals recruited under institutional review board approval and the informed consent process according to the Declaration of Helsinki. Among them, hyperplastic RLNs from 85 individuals with nonmalignant diseases were both preserved as formalin-fixed paraffin-embedded tissues and rapidly dissociated and stored as frozen viable cells. When indicated, $CD20^{pos}$ B lymphocytes were purified using magnetic beads, whereas $CD20^{pos}CD10^{pos}CXCR4^{pos}$ $Hoechst^{pos}$ proliferating centroblasts and $CD20^{pos}CD10^{pos}CXCR4^{neg}Hoechst^{neg}$ nonproliferating centrocytes were sorted on a FACSAria cell sorter (BD Biosciences).

### Quantitative polymerase chain reaction and fluctuation polymerase chain reaction

Genomic DNA was extracted using the All Prep kit (Qiagen). The absolute quantification of t(14;18) was performed by real-time quantitative polymerase chain reaction (qPCR) for $BCL2/J_H$ and *GAPDH*.[9] Standard curves were generated using serial dilutions of cloned *BCL2/JH* and *GAPDH* PCR products. As reported,[13] the sensitivity of this technique is about $4 \times 10^{-5}$. The $\chi^2$ test was used for analyzing differences between groups (GraphPad Prism software). Semiquantitative fluctuation PCR was performed on cell-sorted centroblasts and centrocytes, as previously described.[10] The sensitivity of this technique is about $10^{-6}$.

### Immunohistochemical and immunohistofluorescence studies

Deparaffinized tissue sections were used for double immunohistochemical staining (PAX5/BCL2) and triple immunohistofluorescence staining (CD20/BCL2/CD10).

### Proliferation assay

$CD20^{pos}$ purified B cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and stimulated as previously described.[15] After 4 days of culture, highly proliferative viable $CFSE^{lo}DAPI^{neg}$ and nonproliferative viable $CFSE^{hi}$-$DAPI^{neg}$ B cells were sorted before quantification of t(14;18) by qPCR.

## Results and discussion

In humans, circulating FLLCs have been characterized at the phenotypic and molecular levels,[9,10] but their in situ distribution within nonmalignant tissues with follicular hyperplasia and the functional consequences of endogenous BCL2 overexpression have not been evaluated.

To characterize FLLCs in situ, $BCL2/J_H$ 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 ± 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 $t(14;18)^{neg}$ 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 $PAX\text{-}5^{pos}BCL2^{pos}$ B cells within the GC in $t(14;18)^{hi}$ RLNs, whereas such cells were essentially undetectable in the GC from $t(14;18)^{neg}$ samples (supplemental Figure 1). These GC $PAX\text{-}5^{pos}BCL2^{pos}$ cells visualized inside the GC could comprise $IgD^{neg}$ 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 $t(14;18)^{neg}$ RLNs. Interestingly, these cells were visualized both inside and outside the GC in $t(14;18)^{hi}$ RLNs. By contrast, in control $t(14;18)^{neg}$ 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

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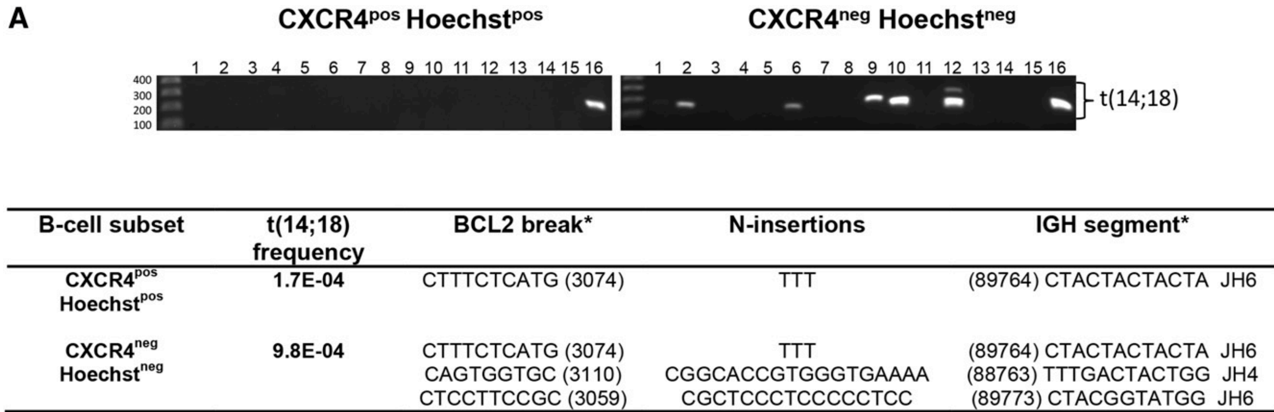


| B-cell subset | t(14;18) frequency | BCL2 break* | N-insertions | IGH segment* |
|---|---|---|---|---|
| CXCR4pos Hoechstpos | 1.7E-04 | CTTTCTCATG (3074) | TTT | (89764) CTACTACTACTA JH6 |
| CXCR4neg Hoechstneg | 9.8E-04 | CTTTCTCATG (3074) | TTT | (89764) CTACTACTACTA JH6 |
| | | CAGTGGTGC (3110) | CGGCACCGTGGGTGAAAA | (88763) TTTGACTACTGG JH4 |
| | | CTCCTTCCGC (3059) | CGCTCCCTCCCCCTCC | (89773) CTACGGTATGG JH6 |

* BCL2 and JH breakpoints are numbered according to accession number M14745 (BCL2) and X97051 (IGH)

B

Day 0

LN suspension

CD19pos B cells

1/400

CD19

Stimulation

Day 4

1/800

CFSE lo CFSE hi

CFSElo NQ

CFSEhi 1/300

CFSE

BCL2/IGH

GAPDH

D4 CFSElo

D4 CD19pos

D4 CFSEhi

D0 CD19pos

D4 CFSEhi

D4 CFSElo

D0 CD19pos

D4 CD19pos

Ct

Log [CO]

**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$^{lo}$DAPI$^{neg}$ and nonproliferative viable CFSE$^{hi}$DAPI$^{neg}$ 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$^{hi}$ population was above the CFSE$^{lo}$ cell population, where it remained below the quantification threshold of 1/25 000 cells. NQ, not quantifiable.

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contained a lower frequency of t(14;18)$^{pos}$ B cells than their nonproliferative 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 in situ, purified B cells from 3 t(14;18)$^{hi}$ RLNs were stained with CFSE and stimulated in vitro during 4 days using an optimal cocktail to activate both naive and memory B cells.[15] Highly proliferative (CFSE$^{lo}$) and nonproliferative (CFSE$^{hi}$) B cells were then sorted, and the frequency of the $BCL2/J_H$ translocation was determined (Figure 1B). As shown in supplemental Table 2, t(14;18)$^{pos}$ cells were systematically found in CFSE$^{hi}$ nonproliferative cells, whereas they could not be detected in CFSE$^{lo}$ 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.[3-5]

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)$^{pos}$ 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.

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## Authorship

Contribution: J.T., C. Menard, and S.R. designed and performed research and analyzed data; N.M., C. Monvoisin, and L.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.

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