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

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Running title:	*IN SITU* ANALYSIS OF FOLLICULAR LYMPHOMA-LIKE CELLS
Scientific heading:	LYMPHOID NEOPLASIAS
Text word count	1458
Abstract word count	148
Reference count	20
Figure count	1
Table count	1
Supplemental Figure	4
Supplemental Tables	2

Key Points

1. Follicular Lymphoma-like cells found in healthy individuals accumulate within germinal centers in reactive lymphoid tissues
2. Follicular Lymphoma-like cells are non-proliferating cells *in situ* and *in vitro*

Abstract

Follicular Lymphoma (FL) is a B-cell neoplasm resulting from the transformation of germinal center (GC) B cells. Although t(14;18) and ectopic 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 (FLLC) in non-malignant GC-rich tissues and the functional consequences of BCL2 overexpression have not been evaluated so far. Among 85 reactive lymph node (RLN) samples, 14% were found to contain high levels of t(14;18) by quantitative PCR. In t(14;18)^{hi} RLN, CD20^{pos}BCL2^{pos}CD10^{pos} FLLC consistently accumulated within GC, essentially as non-proliferative CXCR4^{neg} centrocytes. Moreover, they displayed a reduced response to proliferative stimuli *in vitro*. Altogether, our findings provide new insights into the *in situ* FLLC functional properties and suggest that these cells have not acquired the ultimate genetic events leading to FL transformation.

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, multi-step 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 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 anti-proliferative 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 upon exposure to the off-target effects of activation-induced cytidine deaminase (AID), the key enzyme of antigen-driven antibody diversification. Such oncogenic events could be already 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 (FLLC). Existence of t(14;18)^{pos} precursor lymphoma-initiating cells has been recently supported by two reports of clonally related FL arising in both the donor and the recipient after allogeneic hematopoietic stem cell transplantation[12,13]. However, the connection between FLLC 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 (RLN) displaying high levels of t(14;18), allowing us to visualize and characterize FLLC *in situ* and to perform functional *in vitro* studies. We could demonstrate that FLLC are non-proliferating cells with specific enrichment within GC.

Study design

(For details see supplemental file)

Samples

Lymph node samples were collected from individuals recruited under institutional review board approval and informed consent process according to the Declaration of Helsinki. Among them, hyperplastic RLN from 85 individuals with non-malignant 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 $CD20^{pos}CD10^{pos}CXCR4^{neg}Hoechst^{neg}$ non-proliferating centrocytes were sorted on a FACSAria cell sorter (BD Biosciences).

Q-PCR and fluctuation PCR

Genomic DNA was extracted using All Prep kit (Qiagen). The absolute quantification of t(14;18) was performed by real-time quantitative PCR for *BCL2/J_H* and *GAPDH*[10]. 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 $4x10^{-5}$. Chi-square test was used for analyzing differences between groups (GraphPad Prism software). Semi-quantitative fluctuation PCR was performed on cell-sorted centroblasts and centrocytes, as previously described[10]. The sensitivity of this technique is about 10^{-6}.

Immunohistochemical (IHC) and immunohistofluorescence (IHF) studies

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

Proliferation assay

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

Results and Discussion

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

In order to characterize FLLC *in situ*, *BCL2/J_H* translocation was quantified by Q-PCR on 85 consecutive RLN. Twelve samples (14%) were found positive (Table S1) with a frequency ranging from 4 to 66 x10^{-5}, *i.e.* 1 in 25 000 to 1 in 1 500 cells comprising 38.7 ± 8% $CD19^{pos}$ B cells (not shown). The prevalence of these $t(14;18)^{hi}$ samples was independent on gender but showed a significant correlation with age, reaching 28% in donors older than 50 years. A frequency of circulating FLLC above 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% above 50 years, in agreement with our age-related prevalence increase[11,15]. Overall, we identified 12 RLN with a frequency of FLLC suitable for *in situ* visualization and/or functional studies and focused on 8 of them (RLN 1-8) for further analyzes. We also selected 3 $t(14;18)^{neg}$ RLN (RLN 9-11) as controls.

To visualize FLLC *in situ* we first performed IHC staining for the pan-B-cell transcription factor PAX-5 and BCL2 that is specifically downregulated in normal GC B cells, except in the presence of the t(14;18) translocation[16]. This approach revealed variable amounts of isolated $PAX\text{-}5^{pos}BCL2^{pos}$ B cells within GC in $t(14;18)^{hi}$ RLN whereas such cells were essentially undetectable in GC from $t(14;18)^{neg}$ samples (Figure S1). Since BCL2 is expressed in naive and memory B cells, this strategy could not evaluate the presence of FLLC outside GC. Moreover, $PAX\text{-}5^{pos}BCL2^{pos}$ cells highlighted inside GC could comprise IgD^{neg} centrocytes, corresponding to *bona fide* FLLC, but also some IgD^{pos} small lymphocytes invaginating from mantle zone (data not shown). 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 RLN by IHF (Table 1 and Figure S2). In fact, FLLC have been suggested to resemble FL cells in retaining an atypical $CD10^{pos}BCL2^{pos}$ frozen GC-like phenotype outside GC[2]. We consistently detected more $CD20^{pos}BCL2^{pos}CD10^{pos}$ triple positive cells in $t(14;18)^{hi}$ than in $t(14;18)^{neg}$ RLN. Interestingly, these cells were visualized both

inside and outside GC in t(14;18)^{hi} RLN. By contrast, in control t(14;18)^{neg} samples, the few triple positive cells were exclusively located outside GC and might correspond to rare CD27^{pos}BCL2^{pos}CD10^{pos} memory B cells previously described in peripheral blood[17, 9]. However, the number of t(14;18)^{pos} cells outside GC was higher in t(14;18)^{hi} than in negative RLN and included probably post-GC FLLC. In agreement, we detected only BCL2/Sγ unlike BCL2/Sμ by long-range PCR in all t(14;18)^{hi} RLN tested (Figure S4). Most importantly, when normalizing the percentage of triple positive cells within GC to the surface occupied by GC in corresponding samples, we pinpointed in 5 out of 6 t(14;18)^{hi} samples a clear enrichment (3.5 to 6.5 fold) of CD20^{pos}BCL2^{pos}CD10^{pos} within GC. This enrichment strongly suggests a preferential homing/retention into GC.

Although FLLC accumulate within GC of t(14;18)^{hi} RLN, 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 FLLC contributed significantly to follicular hyperplasia. These observations raised the question of the proliferation potential of FLLC within the hyperproliferative GC environment. To address this issue in the absence of any specific marker allowing the sorting of FLLC, we first purified from a t(14;18)^{hi} RLN proliferative centroblasts *versus* non-proliferative centrocytes based on their differential membrane expression of CXCR4[18] and staining with Hoechst dye (Figure S3). Interestingly, CD20^{pos}CD10^{pos} CXCR4^{pos}Hoechst^{pos} centroblasts contained a lower frequency of t(14;18)^{pos} 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 FLLC *in situ*, purified B cells from 3 t(14;18)^{hi} RLN were stained with CFSE and stimulated *in vitro* during 4 days using an optimal cocktail to activate both naive and memory B cells[14]. Highly proliferative (CFSE^{lo}) and non-proliferative (CFSE^{hi}) B cells were then sorted and the frequency of the *BCL2/J$_H$* translocation was determined (Figure 1B). As shown in Table S2, t(14;18)^{pos} cells were systematically found in CFSE^{hi} non-proliferative B cells whereas they could not be detected in CFSE^{lo} proliferative cells. Altogether these data demonstrate that human non-malignant B cells with naturally occurring t(14;18) accumulate within a poorly proliferative B-cell compartment and, in agreement with the data previously obtained

in mouse and human BCL2-transgenic B cells[3-5], support the hypothesis of an anti-proliferative effect of BCL2.

In conclusion, our *in situ* visualization of FLLC in non-malignant RLN with follicular hyperplasia demonstrates for the first time that FLLC are not randomly distributed but display preferential homing within GC, a property shared with FL 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 FLLC[19] but we demonstrated here that such t(14;18)^{pos} B cells could accumulate within GC even in the absence of classical FLIS lesions characterized as homogeneous follicles exhibiting strong CD20, CD10, BCL6, and BCL2 positivity[20]. Scattered GC FLLC cells 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.

Acknowledgements
This work was supported by research grants from the Institut National du Cancer (INCa libre PL06-10 and PAIR Lymphome 2008-019), the Ligue Contre le Cancer (Equipe Labellisée 2013) and institutional grants from INSERM (Institut National de la Santé et de la Recherche Médicale) and CNRS (Centre National de la Recherche Scientifique).
We thank Patrick Tas, Céline Pangault, and the Centre de Ressources Biologiques (CRB) - Santé of Rennes' hospital for providing us with high quality samples and Gersende Caron for cell sorting.

Authorship contributions
JT, CMe, and SR designed and performed research, and analyzed data
NM, CMo, LC performed research
BN contributed to the study design
PG contributed to study design, analyzed data, and wrote the paper
CS and KT designed and supervised research, analyzed data, and wrote the paper

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

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Table 1. Distribution of $CD20^{pos}BCL2^{pos}CD10^{pos}$ triple positive cells in GC of reactive lymph nodes

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 $t(14;18)^{neg}$ LN, respectively. The frequency of t(14;18) was determined by Q-PCR 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 7.10^4 to 2.10^5 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).

Figure Legends

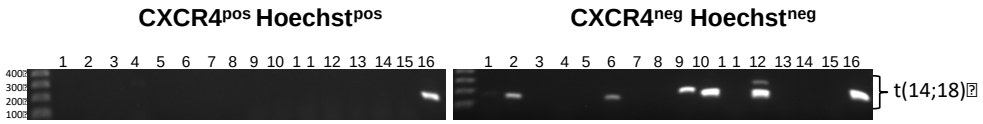
Figure 1: FLLC are non proliferative cells

(**A**) Distribution of the t(14;18)^{pos} cells within proliferative CD20^{pos}CD10^{pos} CXCR4^{pos}Hoechst^{pos} centroblasts *versus* CD20^{pos}CD10^{pos}CXCR4^{neg}Hoechst^{neg} non-proliferative centrocytes cell-sorted from t(14;18)^{hi} RLN7 sample. Genomic DNA from each GC subsets was tested using a two-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 non proliferative viable CFSE^{hi}DAPI^{neg} B cells were then sorted before quantification of t(14;18) by Q-PCR. Standard curves were generated from cloned *BCL2/IGH* (red) and *GAPDH* (green) PCR products and demonstrated that detection of the *BCL2/IGH* transcript from CFSE^{hi} population was above the CFSE^{lo} cell population where it remained below the quantification threshold of 1/25000 cells. NQ: not quantifiable.

Figure 1

A



B-cell subset	Nb of positive PCR/total replicates	BCL2 break*	N-insertions	IGH segment*	t(14;18) frequency
CXCR4^{pos} Hoechst^{pos}	1+/16	CTTTCTCATG (3074)	TTT	(89764) CTACTACTACTA JH6	**1.7E-04**
CXCR4^{neg} Hoechst^{neg}	4+/16	CTCCTTCCGC (3059)	CGCTCCCTCCCCCTCC	(89773) CTACGGTATGG JH6	**9.8E-04**
	1+/16	CAGTGGTGC (3110)	CGGCACCGTGGGTGAAAA	(88763) TTTGACTACTGG JH4	
	1+/16	CTTTCTCATG (3074)	TTT	(89764) CTACTACTACTA JH6	

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

B