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Follicular Lymphoma cell niche:

Identification of a preeminent IL-4-dependent T_{FH}- B cell axis

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Running title: T_{FH}-derived IL-4 in Follicular Lymphoma

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ABSTRACT

Follicular lymphoma (FL) B cells contract tight connections with their microenvironment, which governs the pathogenesis and progression of the disease. Indeed, specific immune response gene signatures, obtained on whole biopsy samples, have been associated with patient survival. In this study we performed gene expression profiling of purified B-cell and non-B cell compartments obtained from FL and reactive lymph nodes. We identified 677 nonredundant genes defining the FL **interface** and involving 26 FL-specific functional networks. This approach highlighted an IL-4-centered pathway associated with an activation of STAT6 that favors overexpression of IL-4-target genes. In addition, FL microenvironment was characterized by a strong enrichment in follicular helper T cells (T_{FH}), as demonstrated through transcriptomic and flow cytometry analyses. The majority of phospho-STAT6^{POS} B cells were located at the vicinity of cells expressing the PD-1 T_{FH} marker. Moreover, purified FL-derived T_{FH} , expressed *IL4* at very high levels compared to purified tonsil-derived T_{FH} or non- T_{FH} microenvironment. Altogether, our study demonstrated that tumor-infiltrating T_{FH} specifically express functional IL-4 in FL, creating an IL-4-dependent T_{FH} -B cell axis. This crosstalk could sustain FL pathogenesis and represent a new potential therapeutic target.

Keywords: follicular lymphoma, follicular helper T cells, microenvironment, IL-4

INTRODUCTION

Follicular lymphoma (FL) is the most common indolent non-Hodgkin's lymphoma and remains virtually incurable. FL tumors arise from germinal center (GC) B cells and are characterized by the balanced chromosomal translocation t(14;18) leading to the deregulation of *BCL2* expression.¹ Occurring in about 85% of FL cases, this *BCL2-JH* translocation was also detected in a small proportion of circulating atypical B cells in healthy individuals, that represent potential premalignant intermediates of the FL pathogenesis.² Therefore, translocated *BCL2* gene is not sufficient *per se* to drive FL development, thus predicting the existence of additional genetic and/or environmental factors crucial for lymphomagenesis and clinical behavior. Several studies argue for FL as a disease under the dependency of strong interactions between tumor B cells and their microenvironment. Both stromal cells and CD40L signal efficiently promoted the survival of FL B-cells *in vitro*.^{3,4} In addition, within invaded lymph nodes (LN) and bone marrow, malignant B cells are found admixed with specialized stromal cells, including

follicular dendritic cells, and CD4^{pos} T cells, thus mimicking the normal GC organization.¹ Interestingly, CD4^{pos} T cells in the follicles, called follicular helper T cells (T_{FH}), were recently suggested to constitute a distinct lineage of T helper cells that arises independently of Th1, Th2, and Th17 effector subsets, and is required for normal B-cell selection and differentiation into long-lived plasma cells.⁵⁻⁷ However, no studies have addressed to date the role of T_{FH} in FL pathogenesis. The cellular composition of the non-malignant infiltrate was described as related to FL prognosis but these studies have often generated conflicting results. High numbers of CD7^{pos}, CD4^{pos}, Foxp3^{pos}, and PD-1^{pos} T-cells were successively reported to predict a longer survival in FL patients.⁸⁻¹¹ Moreover, the spatial distribution, rather than a numerical change, of non-malignant cells seems to be important, since a perifollicular location of Foxp3^{pos} T cells was associated with a better prognosis⁸ whereas FL with rapid transformation showed a predominantly intrafollicular CD4^{pos} pattern of expression.¹² However, these results remain controversial and a range of commonly used T-cell markers fail to identify a significant association with overall survival.^{8,13} Other studies reported that a reactive environment infiltrated by a low number of CD8^{pos} T cells¹⁴ or a high content of CD68^{pos} or CD163^{pos} macrophages^{9,13} or mast cells¹⁵ was associated with adverse clinicobiologic manifestations and unfavorable outcome in FL patients that were treated with conventional chemotherapy. Of note, the predictive value of microenvironment cell populations in FL is highly dependent on specific treatment protocols.^{16,17} To draw a more complete picture of the influence of the microenvironment in FL, microarray analyses were performed and allowed to identify two specific molecular signatures associated with patient's outcome independently of classical clinical features.¹⁸ One was associated with a favorable outcome and corresponded to T cell- and monocyte-restricted genes; whereas the other one involved genes expressed by activated macrophages and dendritic cells and was associated with an adverse prognosis. Subsequent papers confirmed that the clinical behavior in FL is determined essentially by the gene expression profile of the microenvironment rather than by inherent properties of tumor cells themselves.^{19,20} However, the specific contribution of the non-malignant compartment has never been addressed in these retrospective studies. To better understand the host-tumor interaction in FL, we performed comprehensive gene expression analyses on purified B-cell and non-B cell compartments of tumor and reactive LN. This study enables to explore molecular crosstalk between tumor cells and tumor-infiltrating immune cells by identifying a specific list of genes involved in **the FL**

interface. Among this list, we identified an IL-4-centered pathway and demonstrated that T_{FH} are expanded within invaded LN and are the major IL-4 producing cells in FL.

MATERIALS and METHODS

Patients and Samples

All tissues used for this study came from subjects recruited under institutional review board approval and informed consent process according to the Declaration of Helsinki. Samples were obtained either from freshly isolated LN from 29 patients with *de novo* FL at diagnosis, and 11 patients with reactive non-malignant disease (NEG) considered as normal counterpart, or from 11 human tonsils (TONS) collected from children undergoing routine tonsillectomy. All FL showed a predominantly follicular growth pattern, CD10 expression, and were classified into grades 1, 2, or 3a according to the WHO diagnostic criteria. Patients with FL grade 3b or with disease in relapse after treatment, as well as transformed FL were excluded. Clinical characteristics of FL patients are listed in supplementary Table S1. Tissues were rapidly dissociated after collection and flushed using syringe and needle. Cell suspensions were then filtered and washed by centrifugation to obtain the unselected cell fraction.

Flow cytometry characterization of B and non-B subpopulations

Flow cytometry analyses of cell suspensions obtained after mechanically dissociation were performed on a Cytomics FC500 (Beckman Coulter, Miami, FL) or a FACSAria (Becton Dickinson, Franklin Lakes, NJ) flow cytometer using several cocktails of mAbs (Supplementary Table S2). Percentages of tumor B cells among CD19^{pos} B cells were determined as CD19^{pos} cells with a restricted expression of kappa or lambda light chain **after subtraction of the expected percentage of normal B cells expressing this isotype.**

Cell sorting

Purification of B (CD19^{pos}) and non-B (CD19^{neg}CD22^{neg}) fractions was performed using a two-step magnetic bead cell-sorting. First, CD19^{pos} B lymphocytes were obtained by positive selection using CD19 microbeads (StemCell Technologies, Vancouver, Canada). Residual B cells were then eliminated from the unbound fraction by a second round of depletion using CD22 microbeads (Miltenyi Biotec, Gladach, Germany), obtaining therefore the CD19^{neg}CD22^{neg} microenvironment compartment (Env). Purity of each fraction was assessed by flow cytometry on CD20 expression.

CD3^{pos}CD4^{pos}CXCR5^{hi}ICOS^{hi}CD25^{neg} T_{FH} and T_{FH}-depleted/B cell-depleted cell compartment (Env nonT_{FH}) were purified using a FACSAria cell sorter from tonsils and FL lymph nodes.

RNA extraction

Total RNA was extracted using AllPrepTM ARN/ADN mini kit (Qiagen, Valencia, CA), as recommended by the manufacturer including DNase I treatment. RNA purity and integrity was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent, Santa Clara, CA) and the 2100 Expert software. All samples used for microarray studies displayed a RNA Integrity number (RIN) of at least 6.7 (mean: 9.2, range: 6.7-10).

Microarray hybridization

Microarray analyses were performed either on CD19^{pos} fractions of 16 FL (FL_B) and 5 non-malignant (NEG_B) LN, or CD19^{neg}CD22^{neg} microenvironment fractions (8 FL_Env and 6 NEG_Env). Biotinylated cRNA were amplified according to the small sample labeling protocol and hybridized on the GeneChip HG-U133 Plus 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA), according to the manufacturer's instruction. Data analyses were performed using the ArrayAssist[®] software (Stratagene, La Jolla, CA, USA) (see supplementary data).

Real-time quantitative PCR (RQ-PCR)

Microarray results were confirmed by RQ-PCR on a set of 19 FL_Env, 8 NEG_Env, and 6 CD19^{neg}CD22^{neg} fractions obtained from tonsils (TONS_Env). Reverse transcription was performed on 1 µg of total RNA using the Superscript II reverse transcriptase **and random hexamers** (Invitrogen). On-demand gene expression assays and the Taqman Universal master mix on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) were used for RQ-PCR. *ABL* was determined as the appropriate internal housekeeping gene. For each sample, the Ct value for the gene of interest was determined, normalized to its respective value of *ABL* and compared to the value obtained for NEG_Env, using the Δ Ct method. **For *IL4* mRNA expression, the median of FL_Env expression, evaluated by RQ-PCR, was 11.1 compared to NEG_Env (assigned to 1). We classified the FL cases according to *IL4* low ($IL4^lo < 10$) or high ($IL4^hi > 12$) relative expression.** Statistical analyses were performed with Prism software (GraphPad software Inc, San Diego, CA) using a Mann-Whitney non-parametric U-test

(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). In addition, *GCET2/HGAL* was explored on purified FL_B treated or not with IL-4 (R&D Systems, 10 ng/ml) whereas *IL4* was quantified on purified T_{FH} versus Env non T_{FH} obtained from both tonsils and FL.

Immunohistochemistry

Tonsils (n=3), reactive lymph nodes with follicular hyperplasia (n=7) and follicular lymphoma (n=12) biopsies were analyzed as previously described.²¹ Briefly, after appropriate antigen retrieval with microwave heating in high pH Target Retrieval Solution (DakoCytomation, Glostrup, Denmark), deparaffinized tissue sections were stained with anti-Phospho-STAT6/Tyr641 Ab (polyclonal rabbit, dilution 1/30, Cell Signaling Technology, Danvers, MA), using an indirect immunoperoxidase method (ImmPRESS anti-rabbit, Vector Laboratories, Burlingame) and diaminobenzidine (DAB, Vector Laboratories) as chromogen. For double immunostaining, slides were first incubated with anti-CD20 (clone L26, Dako), anti-CD5 (clone 4C7, Novocastra, Newcastle, UK) or anti-PD1 (clone NAT, Abcam, Cambridge, UK) mAb and labelled using the alkaline phosphatase – conjugated ABC procedure (Vector Laboratories). Naphtol phosphate fast red was used as chromogen (Sigma, Saint Louis, MO). Then, slides were incubated with anti-phospho-STAT6/Tyr641 and detected as described above. External positive controls included nodular sclerosis classical Hodgkin lymphomas as well as paraffin embedded blocks of L428 Hodgkin lymphoma-derived and MedB1 primary mediastinal B-cell lymphoma-derived cell lines. Images were captured with a Zeiss Axioskop2 microscope (Zeiss, Oberkochen, Germany) and Neofluar 100x/0.1 NA optical lenses (Zeiss). Photographs were taken with a DP70 Olympus camera (Olympus, Tokyo, Japan). Image acquisition was performed with Olympus DP Controller 2002, and images were processed with Adobe Photoshop v7.0 (Adobe Systems, San Jose, CA).

RESULTS

Validation of our cell sorting method

To validate our approach of cell separation, we analyzed by flow cytometry the different compartments before and after cell purification. Malignant B cells were evaluated on the basis of kappa/lambda (K/L) staining on CD19^{pos} cells and, before cell sorting, represented more than 75% of the total cell count and 90% of B lymphocytes [median: 96.5%; range: 75-100]. After the two-step purification, B cell purity was at least 92% of

the CD19-sorted fractions [median: 97%; range: 92-100], while the non-B-cell fractions were contaminated by less than 12% of CD20^{pos} B cells [median: 0.7%; range: 0-12]. To further explore our cell purification method, we demonstrated, using tonsils samples, the unbiased enrichment of T cell subsets (Supplementary Figure S1), NK cells (CD3^{neg}CD16^{pos}CD56^{pos}), myeloid (Lin^{neg}HLA-DR^{pos}CD11c^{pos}) and plasmacytoid dendritic (Lin^{neg}HLA-DR^{pos}CD123^{pos}) cells in non-B cell fractions, whereas the kappa/lambda ratio was preserved in B cells (Supplementary Figure S1).

Microarray analysis of the FL **specific interface**

We used a microarray approach to characterize gene expression patterns in different purified subpopulations of 16 FL (16 FL_B and 8 FL_Env) and 6 non-malignant (NEG) LN (5 NEG_B and 6 NEG_Env). These 35 Samples were labeled and hybridized onto Affymetrix U133 Plus 2.0 oligonucleotide arrays. Raw data were normalized using GC-RMA method and filtered to select probesets (PS) with standard deviation to mean ratio superior to 80%. This method reduces the panel to 8779 PS showing the highest expression variation. Unsupervised analyses of either B-cell compartments (n=21) or non-B cell compartments (n=14) allowed to differentiate FL from NEG samples (Figure 1A). To determine the gene expression pattern specific to each cell compartment implicated in the host-tumor **crosstalk**, a step-wise statistical approach was conducted on the 35 samples by combining a SAM method and an asymptotic unpaired Mann-Whitney non-parametric test. Based on this approach, we defined four different PS lists (Figure 1B, Left Panel): 1) 886 PS differentially expressed between FL and normal B cells, representing the FL-specific B signature; 2) 897 PS differentially expressed between FL and normal non-B microenvironment, representing the FL-specific microenvironment signature; 3) 4127 PS distinguishing B and non-B FL samples (FL interface); and 4) 2308 PS distinguishing B and non-B reactive samples (normal interface). In order to define the specific host-tumor interface, which contains genes specifically implicated in the crosstalk between tumor B cells and their microenvironment, we established two ways to select relevant PS. We first subtracted the normal interface signature (2308 PS) from the FL interface signature (4127 PS) ending therefore with a 2206 PS signature called FL specific interface-1. The second way consisted to sum the FL-specific B PS to the FL-specific microenvironment PS, allowing the definition of the FL specific interface-2 involving 1698 PS (Figure 1B, Middle Panel). Finally, the **overlap merging** between these two previous genelists

involved 788 highly specific FL-interface PS corresponding to 677 non-redundant genes and characterizing what we further designed as the FL tumor-microenvironment **specific interface** (Figure 1B, Right Panel). Among this list, 219 PS were up-regulated in FL B cells and 569 PS up-regulated in FL non-B cell compartment (Table S3).

IL-4 is upregulated in FL and promotes malignant B-cell activation

Within the 788-PS list defining the FL **specific interface**, we identified, using Ingenuity pathway analysis, 26 FL-specific functional networks, including an IL-4 centered pathway, a cell growth and proliferation pathway, and an immune response pathway (Table S4). We pointed out that cell growth, T-lymphocyte proliferation and T-lymphocyte activation were altered in the FL microenvironment, with upregulation of *BATF*, *IFNRRAR2*, *TNFRSF9*, *TRAF1*, *CCL4*, *IL10*, *IL15*, *IL4*, *TNFRSF4*, or *TNFRSF18* gene expression. In addition, a Gene Ontology analysis of the 788-PS list confirmed a statistically significant overrepresentation of genes involved in the biological processes of lymphocyte proliferation (GO: 0042100, $P=0.00066$) or lymphocyte activation (GO:0046649, $P=0.00016$), especially T-cell activation (GO:0042110, $P=0.00011$). According to the Onto-Tools pathway impact analysis, *IL4* was present in 6 out of the top 20 functional pathways relevant for the 788-PS list description. Moreover, we performed hierarchical clustering analysis based on the previously described immune-response 1 and 2 signatures.¹⁸ Of interest, the immune-response 1 segregated the FL_Env from the NEG_Env indicating a preferential T-cell signature in the FL cases (Figure S2). To further explore the T-cell implication in FL tumorigenesis and specifically through the IL-4 centered pathway, we first validated by RQ-PCR the overexpression of *IL4* mRNA in FL_Env, compared to NEG_Env (Figure 2A). Similarly, *IL4* was expressed at a very low level in non-B cell fraction from inflamed benign tonsils. The result confirmed the recently described higher levels of IL-4 protein in FL compared to benign follicular hyperplasia.²² In this previous study, activation of STAT6 was poorly detectable, probably due to the use of whole tissue lysates. Given that FL B cells, like normal GC-derived B cells, strongly expressed CD124, the IL-4 receptor alpha (Figure 2B), we decided to investigate the expression of activated phospho-STAT6 *in situ* by immunohistochemistry in malignant *versus* normal B cells. In reactive tonsils and lymph nodes (n=10), only exceptional single cells positive for P-STAT6 (1 or 2 per section in magnification x 200) (Figure 2C, panel 1) were observed. These cells were located in the interfollicular area or in the mantle zone of follicles and were not observed within

GC. In only one case of reactive lymph node, a cluster of P-STAT6^{pos} cells was found in the paracortex. By contrast, a high number of P-STAT6 positive cells was evidenced in all FL cases with interpretable staining (n=10), and were mainly located within the neoplastic follicles. These P-STAT6 positive cells consisted of either multiple scattered cells or small aggregates with a tendency of distribution at the periphery of malignant follicles (Figure 2C, panels 2 and 3). Double staining revealed that the P-STAT6 positive cells were CD20^{pos} B lymphocytes (Figure 2C, insert in panel 3). Of note, the level of STAT6 mRNA was not different between FL_B and NEG_B compartments (data not shown). Since IL-13 was not overexpressed in FL non-B cell compartments (data not shown), this phosphorylation of STAT6 is more likely to result from IL-4 than from IL-13. The overexpression of IL-4 within FL microenvironment was thus associated to a strong activation of malignant B cells. In agreement, we found that both *IL4I1* and *HOXC4*, two previously described IL-4 target genes,^{21,23} belonged to the 886-PS FL_B list (Supplementary Figure S3A). Similarly, *GCET2/HGAL*, another gene reported as induced by IL-4 in normal B cells and in some malignant B-cell lines,²⁴ was significantly upregulated in FL_B compartment (Supplementary Figure S3B) even if it was excluded from the microarray analysis by the initial SD-based filtering. Interestingly, we demonstrated that *GCET2/HGAL* expression was also increased by IL-4 in purified primary B cells obtained from FL patients (Figure 2D).

IL-4 is predominantly produced by FL-derived T_{FH}

IL-4 is widely recognized as a Th2 cytokine in the periphery even if recent studies have suggested that, within reactive LN, T_{FH} are the main IL-4-secreting T cells, in agreement with the critical role of this cytokine for antibody production.^{7,25-27} In order to identify the IL-4 producing cells in FL context, we first compared by RQ-PCR, in the microenvironment of FL *versus* nonmalignant samples, the expression of *BCL6*, *GATA3*, and *TBX21/TBET* transcription factors, which are involved in the commitment to T_{FH}, Th2, and Th1 differentiation respectively. Both, the *BCL6* expression and the *GATA3/TBX21* ratio were increased in FL tumors, suggesting a simultaneous enrichment in T_{FH} and Th2- polarized CD4^{pos} T cells. In addition, *IL10*, the second prototypic Th2 cytokine, was also overexpressed in FL_Env (Figure S4A). Since T_{FH} also express low levels of *GATA3*,²⁷ we decided to further explore the potential contribution of T_{FH} to FL pathogenesis through the synthesis of IL-4. First, a Gene Set Enrichment Analysis (GSEA) approach was used in order to specifically assess the

over-representation of previously described T_{FH} genes within the FL microenvironment. Given the recent demonstration in both mice and human that CXCR5 and ICOS are the two most relevant T_{FH} phenotypic markers, we defined our T_{FH} signature based on the datasets previously published by Rasheed *et al.*, where CXCR5^{hi}ICOS^{hi}CD4^{pos} T cells were isolated from tonsils and compared, using Affymetrix U133A/B microarrays, to purified non-T_{FH}, naïve T cells, central memory T cells, and effector memory T cells.²⁸ We underlined 326 PS significantly upregulated in T_{FH} cells compared to the other CD4^{pos} T-cell subpopulations and corresponding to 267 non-redundant genes. Having ranked the microenvironment cell samples according to FL *versus* NEG distinction, we found that FL_Env were significantly enriched in genes specifically overexpressed in T_{FH} cells ($P < 0.01$ using both SNR-based ranking and FC-based ranking) (Figure S4B). Interestingly, when we performed an unsupervised hierarchical clustering restricted to the 226 PS of the T_{FH} signature belonging to our initial dataset of 8879 PS, we were able to segregate FL cases from non-tumoral tissues (data not shown), indicating that the T_{FH} signature was sufficient by itself to distinguish the FL microenvironment from the non-malignant microenvironment. The cluster of coordinately regulated T_{FH}-associated genes was underlined in Figure 1A. **Moreover, unsupervised analysis based on T_{FH} signature revealed the clustering of FL_Env according to their *IL4* mRNA expression level (Figure 3), providing additional evidence that IL-4 could be secreted by the T_{FH} compartment. Furthermore, we also found by GSEA analysis a significant enrichment of the T_{FH} signature in FL with high expression of *IL4*^{hi} ($P < 0.001$; data not shown).** In agreement, we validated by RQ-PCR the overexpression of *CXCL13*, *CXCR5*, and *PDCD1*, three canonical human T_{FH} markers, in FL_Env compared to NEG_Env (Figure S4C). In order to take into account the inflammatory process that develops within FL microenvironment, we decided to explore also the non-B cell compartment obtained from chronically activated tonsils. In fact, substantial differences were seen in the degree of GC compartmentalization between human lymph node GC and human tonsil GC, including an accumulation of GC T cells in the latter one.²⁹ Interestingly, the level of expression of *CXCL13*, *CXCR5*, and *PDCD1* in tonsil microenvironment was also upregulated compared to normal LN in similar **extent** than FL LN. We then quantified by flow cytometry the percentage of CD4^{pos}CD25^{neg} T cells coexpressing high levels of CXCR5 and ICOS T_{FH} markers (Figure 4A) and found a significant expansion of the T_{FH} cell compartment, in both FL tumors and tonsils compared to normal LN (Figure 4B). **Of note, the two reactive LN with higher percentages of T_{FH} corresponded to samples with**

a strong follicular hyperplasia. FL microenvironment is thus characterized, like samples with strong GC activation, by a high infiltration of T_{FH} cells. Within FL biopsies, P-STAT6^{pos} B cells were found in close contact to CD5^{pos} T cells and most of them were located at the vicinity of cells expressing the PD-1 T_{FH} marker (Figure 4C). Previously, it had been very difficult to identify IL-4 producing cells *in situ*²⁵ and T_{FH} are highly susceptible to apoptosis *in vitro*.²⁸ To definitively determine the IL-4 producing cells, we thus decided to purify the T_{FH} and the non-B non-T_{FH} cell subsets in FL and tonsils and to screen these cell compartments for *IL4* mRNA. *IL4* was highly produced by the T_{FH} fraction issued from FL compared to the other non-malignant cells of the microenvironment, whereas tonsil-derived T_{FH} expressed a low but significant level of *IL4* mRNA (Figure 4D). In addition, when considering more specifically the non-T_{FH} CD4^{pos}CXCR5^{neg}ICOS^{neg} T cell subset, we confirmed that T_{FH} are the predominant *IL4* producing CD4^{pos} T cells in FL (data not shown). Collectively, these results indicated that FL-infiltrating T_{FH} specifically produced very high levels of IL-4.

DISCUSSION

Follicular lymphoma represents a paradigm of microenvironment involvement in cancer behavior. Indeed, as previously described, the aggressiveness of the disease and the prognosis were carried by molecular features of non-malignant cells.¹⁸ Various studies have demonstrated the potential for using gene expression profiles for the assessment of tumor specific pathways. In order to explore the cellular crosstalk between lymphoma cells and their microenvironment, previous studies developed, in few biopsy samples, cell sorting experiments allowing the purification of both a CD19^{pos} malignant and a CD19^{neg} non-malignant subpopulations in order to validate some specific findings obtained by microarray on global tissues.¹⁸ To characterize the FL cell niche and molecular features that sustain tumor B-cell growth within a specific microenvironment, we carried out an exhaustive gene profiling study based on a validated purification method separating systematically fresh tumor cell suspension into CD19^{pos} and CD19^{neg}CD22^{neg} compartments and we compared *de novo* FL to non-malignant LN.

Various genelists were generated leading to the definition of the FL interface as the specific host-tumor interface, including an IL-4-centered signature. We were able to identify by microarray the overexpression of *IL4* RNA in FL, demonstrating the better sensibility of our purification-based microarray approach compared to previous studies, ruling out the idea that for IL-4 no correlation may exist in FL between gene expression

and protein levels.²³ Of interest, although the size of our FL cohort and our study design did not allow the exploration of patient's prognosis in regard to gene profile, we found that the immune-response 1 described by Dave *et al.* could segregate the FL from the non-malignant microenvironment indicating a preferential T-cell signature among the FL cases.¹⁸ In addition, our FL specific interface showed a preferential expression of the CXCL13 chemokine in FL. Therefore both findings together suggest that the FL microenvironment retain features of the germinal center light zone.³⁰ Whereas we detected, as previously described,³¹ an upregulation of *IL4R* mRNA in purified FL cells compared to normal CD19^{pos} B cells and CD19^{pos}CD10^{pos} GC B cells (data not shown), CD124 was similarly expressed at the cell surface in FL cells and tonsil-derived GC B cells, indicating that the major tumor-associated event in the IL-4 pathway lies in the microenvironment. We detected significant *in vivo* activation of STAT6 in malignant B cells in contrast to normal GC B cells, in agreement with a functional IL-4-dependent signaling. Strikingly, STAT6-associated signal has been associated with other B-cell malignancies. In primary mediastinal large B-cell lymphoma, STAT6 is constitutively activated in both cell lines and primary samples³² whereas Hodgkin Reed-Sternberg cells produce autocrine IL-13 resulting in STAT6 activation in classical Hodgkin lymphoma.³³ However, even if IL-4 overexpression was already described in whole FL biopsies,²² we reinforced the belief that a paracrine secretion of IL-4 by tumor microenvironment could be involved in lymphomagenesis. STAT6 is a critical signaling molecule for IL-4-mediated protection of spontaneous and Fas-induced cell death in mice primary B cells.³⁴ Moreover, even if few data are available on the direct effect of IL-4 on FL cells,³⁵ in particular due to the lack of appropriate cell lines, STAT6 knockdown induces apoptosis in Hodgkin lymphoma cell lines³⁶ and IL-4 increases cell proliferation of GC-like diffuse large B-cell lymphomas (DLBCL) through STAT6 phosphorylation.²⁴ A high proportion of genes overexpressed in FL B cells compared to normal CD19^{pos} B cells, including *MME/CD10*, *BCL6*, and *MTA3*, corresponded in fact to GC-associated genes (data not shown). However, our approach, that compares purified FL cells, representing GC-derived neoplastic B cells, with normal B cells including both GC and non-GC derived B cells, is a suboptimal approach to characterize specifically FL B-cell features. One could therefore hypothesize that, since FL B cells gene profiling showed important molecular features of normal GC B cells and given that FL could evolve into GC-like DLBCL,³⁷ the association of IL-4 overexpression and STAT6 activation could favor malignant B cell survival and proliferation. In addition, as

previously described for GC-like DLBCL lines, IL-4 increased the expression of *GCET2/HGAL* in FL cells, and, given the role of HGAL in modulating GC B-cell motility,³⁸ could thus contribute to the confinement of malignant B cells to the GC. Moreover, IL-4 drives macrophages toward a M2 phenotype endowed with immunoregulatory and proangiogenic properties, and several studies have indicated that tumors could educate recruited monocytes to exhibit a M2-like phenotype³⁹ **potentially** implicated in the adverse prognostic value of a high number of tumor-associated macrophages in FL.¹³ Interestingly, the IL-4-specific target gene *IL4I1* was recently found as specifically expressed by malignant B cells in GC-derived B-cell lymphomas including FL, whereas no expression was observed in tumor cells of T/NK neoplasms, mantle cell lymphomas, or marginal zone lymphomas.²² **Moreover, a significant correlation ($P=0.036$) was found between high *IL4* mRNA expression and a low FLIPI score. However, a large cohort will be necessary to explore the prognostic value of IL-4/*IL4I1* target gene overexpression in FL.**

Besides IL-4, several other cytokines were found to be overexpressed in FL_Env. In particular, we confirmed herein our previous results describing the induction of inflammatory cytokines, including *TNF* and *IFNG*, in FL microenvironment³ in agreement with the former detection of these two factors in FL-invaded LN using *in situ* hybridization or PCR experiments.^{40,41} The reason of the discrepancy between these data and the study of Calvo K. *et al* that reported a decrease of TNF- α and IFN- γ proteins in FL whole biopsies²² is not clear. Of course, gene expression does not always correlate with protein expression but, since elevated levels of circulating TNF- α have been found in FL and DLBCL patients,⁴² another possible explanation could be the higher sensitivity of our purification-based method. In agreement, both *FASLG* and *GZMA* belonged to the 897-PS genelist defining the FL microenvironment, suggesting an activated T-cell signature in this disease.

Numerous studies have described several years ago that IL-4 is produced by the Th2 T-cell subset and promotes the Th2 polarization. In agreement with the overexpression of *IL4* in FL microenvironment, we reported an increased *GATA3/ TBET* ratio in this disease, a finding generally interpreted as a bias in the Th1/Th2 balance. This result fit well with previous data showing that the few tumor-reactive effector T cells within FL tumors display a Th2 functional profile after *in vitro* stimulation.⁴³ In addition, a mouse model of T-dependent lymphomagenesis has been recently developed where repeated infusions of idiotype-specific Th2 cells support the development of B-cell lymphoma,

which could be conversely eradicated by Th1 cells.⁴⁴ Finally, we detected a significant upregulation of *IL10* expression, another emblematic Th2 cytokine, within FL microenvironment. Interestingly, SNP in the *IL10* promoter were associated with increased risk for both DLBCL and FL.⁴⁵ However, the segregation of helper T cells into unique highly specialized subsets has been recently challenged by several studies suggesting a common inductive history with intermediate stages exhibiting higher functional plasticity than previously believed.⁴⁶ Gene expression profiling and analyses of knock-out mice models have first suggested that T_{FH} cells constitute a separate Th cell lineage with distinct developmental programming and specific effector functions, like providing help to B cells for their differentiation, into Ig-secreting cells.⁴⁷ In addition, recent findings suggest that T_{FH} represent the normal counterpart of angioimmunoblastic T-cell lymphomas.^{47,48} Moreover, recent papers suggest that IL-4 secretion by lymphoid cells is highly related to T_{FH} cells in mice after helminth infection and in human inflamed tonsils^{25-27,47}. We here demonstrate for the first time that chronically activated tumor-infiltrating CXCR5^{hi}ICOS^{hi} T_{FH} express high levels of IL-4 in FL suggesting major therapeutic implications.

IL-4 dependent STAT6 activation was reported to modulate the behavior of another very important CD4^{pos} T-cell subset; *i.e* regulatory T cells (Treg). However, whereas initial studies clearly demonstrated that STAT6 directly represses TGFβ1-mediated *FOXP3* transcription and overrules inducible Treg differentiation,⁴⁹ exogenous IL-4 conversely maintains *FOXP3* level in natural Treg and promotes their proliferation.⁵⁰ In agreement, we found no correlation between the number of T_{FH} and the number of Treg within FL LN (data not shown).

In summary, we propose a new sensitive and specific gene profiling approach allowing the identification of genes involved in the crosstalk between malignant cells and their non-malignant microenvironment. Using this original and fully validated method, we revealed that FL microenvironment, along with P-STAT6 activated FL B cells, is characterized by a strong infiltration by T_{FH} cells displaying a specific activation profile characterized by the expression of IL-4. These findings could be very useful to better understand lymphoma biology and to develop effective targeted therapies in this still incurable disease.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

Supplementary information is available at the Leukemia’s website.

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FIGURE LEGENDS

Figure 1: Microarray analysis and « FL specific interface » definition.

(A) Cluster diagrams of B and non-B cell compartments in FL and non-malignant lymph nodes. Hierarchical clustering of the CD19^{pos} compartments (Left Panel) and CD19^{neg}CD22^{neg} compartments (Right Panel) issued from FL lymph nodes (FL_B or FL_Env) and non-malignant lymph nodes (NEG_B or NEG_Env). The relative level of gene expression is depicted according to the shown color scale. To the right of the dendrogram, the positioning of the genes making up the T_{FH} signature. (B) Schematic approach to identify host-tumor interface gene expression. Left Panel: Microarray signature overlapping of the four B and non-B, follicular and non-malignant compartments to define the FL interface (4127 PS), the normal interface (2308 PS), FL-specific B signature (886 PS) and FL-specific non-B environment signature (897 PS). Middle Panel: FL specific interface 1 is defined as the FL interface cleared of probesets overlapping with the normal interface; interface 2 is defined as the addition of the FL-specific B and FL-specific non-B signatures. Right Panel: The so called “FL specific interface” defined the most relevant genes involved in the FL and microenvironment interface, represented by the probesets which overlapped between FL-specific interface 1 and interface 2.

Figure 2: Specific expression of functional IL-4 and PSTAT6 in FL microenvironment.

(A) Gene expression of *IL4* in non-B cell fractions. RQ-PCR quantification of *IL4* in CD19^{neg}CD22^{neg} fractions obtained from FL lymph nodes (FL_Env), non-malignant lymph nodes (NEG_Env) and tonsils (TONS_Env). The arbitrary value of 1 was assigned to the median expression of NEG_Env. (B) CD124 membrane expression on B cells. Expression of CD124 on CD19^{pos} B cells from FL lymph nodes (FL_B) and non-malignant lymph nodes (NEG_B), as well as CD19^{pos}CD10^{pos} germinal center B cells from tonsils (TONS_GC B) was analyzed as the ratio of mean fluorescence intensity (rMFI). (C) Immunohistochemical staining of Phospho STAT6: 1/ a single P-STAT6^{pos} cell with nuclear staining (brown) is observed within the GC of a reactive tonsil with follicular hyperplasia (arrow), 2 and 3/ a significant number of P-STAT6^{pos} cells are present in neoplastic follicles in FL, consisting of clusters predominating at the periphery of follicles (C2, see details in insert) or scattered positive cells (C3); as shown in insert

(C3), P-STAT6^{pos} cells (brown) are CD20^{pos} (red) B cells. Original magnifications: x100 (C2), x200 (C1, C3), x400 (inserts). (D) Gene expression of *GCET2/HGAL* in B cells. RQ-PCR quantification of *GCET2/HGAL* in purified CD19^{pos} fractions from FL lymph nodes (FL_B) cultured with (IL-4) or without (CTL) IL-4 for 2 days. The arbitrary value of 1 was assigned to the median expression of unstimulated (CTL) FL_B. (* $P < 0.05$, *** $P < 0.001$).

Figure 3: Classification of FL_Env displaying various *IL4* expressions based on the T_{FH} signature.

Hierarchical clustering of FL_Env was performed on the selected genes making up the T_{FH} signature. The FL_Env cases were assigned to IL4^{low} (*IL4^{lo}*) or IL4^{high} (*IL4^{hi}*) expression according to their relative mRNA expression compared to NEG_Env (see Materials and Methods).

Figure 4: Specific expression of IL-4 by FL-infiltrating T_{FH}.

(A) Strategy for flow cytometry analysis and cell sorting of T_{FH} cells. T_{FH} are defined as CXCR5^{hi}ICOS^{hi} cells within CD4^{pos}CD25^{neg} T cells. (B) Quantification of the T_{FH} cell compartment. Enumeration by flow cytometry of CXCR5^{hi}ICOS^{hi}CD25^{neg} cells within CD4^{pos} T cells in FL lymph nodes, non-malignant (NEG) lymph nodes, and tonsils (TONS). (C) Double staining of P-STAT6^{pos} cells in FL samples. 1/ P-STAT6^{pos} cells (brown) are in close contact with CD5^{pos} T cells (red), 2/ numerous PD1^{pos} cells (brown) within a neoplastic follicle are seen among P-STAT6^{pos} (red) cells; insert illustrates P-STAT6^{pos} cells at the vicinity of PD1^{pos} cells. Original magnification, x200 (C2), x400 (C1, insert). (D) Gene expression of *IL4* in T_{FH} versus nonT_{FH} microenvironment. RQ-PCR quantification of *IL4* in T_{FH} and T_{FH}-depleted B cell-depleted cell compartment (Env non T_{FH}) cell sorted from FL lymph nodes (7 FL_T_{FH} and 4 FL_Env nonT_{FH}) and tonsils (7 TONS_T_{FH} and 4 TONS_Env nonT_{FH}). The arbitrary value of 1 was assigned to the median expression obtained for 4 CD19^{neg}CD22^{neg} fractions from FL lymph nodes. Bar represents the median. (** $P < 0.01$; *** $P < 0.001$)

SUPPLEMENTARY MATERIALS AND METHODS

Data Analyses of Microarrays

Expression signal values and P -values were obtained for each probeset (PS) using the ArrayAssist® software (Stratagene, La Jolla, CA, USA) by the Robust Multichip Averaging algorithm using GC content of probes in normalization (GC-RMA). Background was discarded by selecting the PS with a standard deviation to mean ratio superior to 0.8. A hierarchical clustering algorithm was used to group genes on the basis of similarity and data visualization was carried out with Cluster and Treeview (Eisen softwares, Stanford, CA). Supervised analyses included two approaches : 1) Significance Analysis of Microarrays (SAM) software, using 500 permutations, a fold change (FC) >2 or <0.5 and an false rate discovery (FDR) of 5% for comparison of tumor and reactive B compartments or FDR of 1% for comparison of B *versus* non-B compartments; 2) Unpaired Mann-Whitney non parametric test carried out with ArrayAssist® software and selection of PS with a $|FC|>2$ and a P -value less than 0.01. Generated gene lists were then crossed to retain only overlapping PS. Networks and canonical pathways were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) or by Gene Ontology analysis and the Onto-Tools impact pathway analysis (<http://vortex.cs.wayne.edu/Projects.html>).¹

Raw data (Affymetrix U133A CEL files) from 15 samples corresponding to 5 CD4^{pos} T-cell subpopulations purified from tonsils (CXCR5^{hi}ICOS^{hi} T_{FH}, and CXCR5^{neg}ICOS^{neg/lo} nonT_{FH}) or peripheral blood (CXCR5^{neg}CD45RA^{neg} CCR7^{neg} effector memory T cells, CXCR5^{neg}CD45RA^{neg}CCR7^{pos} central memory T cells, and CXCR5^{neg}CD45RA^{pos} naïve T cells) were downloaded from ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>, Experiment # E-MEXP-750)². Data were normalized using MAS 5.0 (R software V2.8.1) and only the 11719 PS with a "present" call in at least 3 samples were retained for further analyses. The T_{FH} signature was defined as the list of PS upregulated in T_{FH} compared to all the other T-cell subsets using the Partek Genomic Suite software ($|FC|>2$ and $P<0.05$ using a Mann-Whitney test). Gene Set Enrichment Analysis (GSEA) was then performed as previously described³ to assess the overexpression of T_{FH}-related gene sets in the FL_Env *versus* NEG_Env samples. Non redundant gene datasets were generated and two different statistics were used to rank the genes: signal-to-noise ratio (SNR) and FC of expression. A P -value was calculated for a

weighted enrichment score (ES) by using a sample-based permutation test procedure including 1000 permutations.

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SUPPLEMENTARY FIGURES LEGENDS

Figure S1: Validation of cell sorting procedure by flow cytometry characterization of B and non-B subpopulations.

(A) CD20 expression on B and non-B cell fractions. CD20 expression analyzed by flow cytometry in tonsil-derived CD19^{pos} (Left Panel) or CD19^{neg}CD22^{neg} (Right Panel) fractions. (B) Isotype expression on B cells. Flow cytometry expression of kappa isotype on CD20^{pos} B cells or CD20^{pos}CD10^{pos} germinal center B cells obtained from tonsils. Analysis were performed before (dark panel) or after (grey panel) CD19^{pos} cell sorting showing that both unseparated cell suspensions and purified B cells present similar K/L cell repartition. Bars represent the mean +/- SD of 3 independent experiments. (C) Flow cytometry analysis of tonsil-derived CD3^{pos} T subpopulations. Percentages of CD4^{pos}, CD8^{pos}, TCR $\gamma\delta$ ^{pos}, CD4^{pos}CD25^{pos}Foxp3^{pos} (Treg) and CD4^{pos}CXCR5^{hi}ICOS^{hi}CD25^{neg} (T_{FH}) cells were defined within CD3^{pos} lymphocytes. Analysis were performed before (dark panel) or after (grey panel) CD19^{neg}CD22^{neg} cell sorting. Bars represent the mean +/- SD of 5 independent experiments.

Figure S2: Immune Response signature in FL microenvironment.

Hierarchical clustering of non-B cells compartments purified from FL (FL_Env) and non-malignant (NEG_Env) lymph nodes based on IR1 signature according to Dave *et al.*¹⁸

Figure S3: Overexpression of IL-4 induced genes in FL_B cells.

Expression of *IL4I1* (A, left panel), *HOXC4* (A, right panel) and *GCET2/HGAL* (B) as detected on Affymetrix microarrays in purified CD19^{pos} B cells isolated from FL (FL_B) versus nonmalignant lymph nodes (NEG_B). (** $P < 0.01$).

Figure S4: T_{FH} signature in FL microenvironment.

(A) Expression of the transcription factors involved in helper T cell commitment. RQ-PCR quantification of *BCL6*, *GATA3*, *TBX21* and *IL10* in CD19^{neg}CD22^{neg} fractions obtained from FL lymph nodes (FL_Env), non-malignant lymph nodes (NEG_Env) and tonsils (TONS_Env). The arbitrary value of 1 was assigned to the median expression of NEG_Env. *GATA3* and *TBX21* expression are shown as the ratio of 2^{-DDCT} obtained for *GATA3* and *TBX21*. (B) GSEA Enrichment Score (ES) curves for the T_{FH} signature in ENV-samples. Vertical black lines indicate the position of each of the 267 non-

redundant genes comprising the T_{FH} signature and the green curves represent the running sum of the weighted ES. Left panel: Comparison of FL_Env and NEG_Env using the SNR statistic to rank the genes. Right Panel: Comparison of FL_Env and NEG_Env using the fold change between the average of the 8 FL_Env versus the average of the 6 NEG_Env to rank the genes. (C) Validation of the expression of T_{FH} genes. RQ-PCR quantification of *CXCL13*, *CXCR5*, and *PDCD1* in CD19^{neg}CD22^{neg} fractions obtained from FL lymph nodes (FL_Env), non-malignant lymph nodes (NEG_Env) and tonsils (TONS_Env). The arbitrary value of 1 was assigned to the median expression of NEG_Env. (**P*<0.05; ***P*<.01; ****P*<0.001)

Table S1: FL patient characteristics

		Bioclinical characteristics (n=29)
Sex ratio	M/F	15/14
Age (n)	≤ 60ys	13
	> 60ys	16
Tumor grade (n)	1	17
	2	11
	3a	1
FLIPI risk group* (n)	Low	9
	Intermediate	10
	High	10
Tumor B cells among CD19^{pos} cells before cell sorting (%)	Median	96.5
	Range	76-100
BCL2 protein expression** (n)		27
IgH-BCL2 gene fusion*** (n)		20
t(3;14) (n)		1

* Follicular Lymphoma International Prognostic Index (FLIPI) values were classified as low (0-1), intermediate (2) or high (3 to 5).

** BCL2 protein expression was analyzed by flow cytometry and/or immunohistochemistry.

*** BCL2 rearrangements were determined by PCR according to Biomed2 recommendations¹ or by FISH analysis

Référence :

1. van Dongen JJ, Langerak AW, Bruggemann M, Evans PA, Hummel M, Lavender FL, *et al.* Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. *Leukemia* 2003 Dec; **17**(12): 2257-2317.

Table S2: Antibodies for flow cytometry analyses

Anti-human monoclonal antibody	Conjugation*	Supplier**
CD3	FITC	BC
CD3	ECD	BC
CD4	PC5	BC
CD4	FITC	BC
CD8	PC7	BC
CD10	PC5	BC
CD11c	APC	BD
CD16	FITC	BC
CD19	PC7	BC
CD19	PB	EB
CD20	FITC	BC
CD20	PC7	BC
CD20	VB	M
CD25	PC5	BC
CD25	APC	BD
CD56	PE	BC
CD123	PE	BD
CD124	PE	BC
CXCR5	PE	RD
FOXP3	PE	EB
$\gamma\delta$	PE	BD
HLA-DR	ECD	BC
ICOS	BIOT	EB
LIN	FITC	BD
Kappa light chain	FITC	D
Lambda light chain	PE	D

* FITC: fluorescein isothiocyanate; PC5: phycoerythrin cyanin 5; PC7: phycoerythrin cyanin 7; APC: allophycocyanin; ECD: energy-coupled-dye; VB: Vioblue; PB: pacific blue; BIOT: biotinylated monoclonal antibody (revealed by streptavidin-PC5 or PC7 from Beckman Coulter)

** D: Dako (Glostrup, Denmark); BC: Beckman Coulter (Fullerton, CA); BD: BD Bioscience (San Diego, CA); EB: eBioscience (San Diego, CA); M: Miltenyi Biotec (Gladach, Germany); RD: R&D Systems (Abingdon, UK).