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Review Article

Molecular Pathogenesis of Follicular Lymphoma

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t(14;18) translocation has been recognized as a genetic hallmark of follicular lymphoma (FL), but it is now known that additional genetic aberrations are required for the development of FL. With recent advances in the technology for DNA analysis, recurrent gene aberrations such as *TNFRSF14*, *EPHA7*, *EZH2*, *CREBBP*, *EP300*, *MLL2* and *MEF2B* have been identified. A few t(14;18)-positive B cells can be detected in healthy individuals, and these B cells are reported to have their own biological features that are closely associated with the pathogenesis of FL. On the other hand, FL is characterized by a unique microenvironment. Further understanding of the pathogenesis of FL is expected to contribute to the development of novel treatment approaches for this disease. 〔*J Clin Exp Hematop 54(1): 23-30, 2014*〕

Keywords: follicular lymphoma, pathogenesis, microenvironment

INTRODUCTION

Follicular lymphoma (FL) is one of the most common subtypes of indolent lymphoma. FL patients have been considered as incurable by standard chemotherapy approaches, but novel chemotherapeutic drugs, biological modifiers or allogeneic stem cell transplantation are expected to improve the clinical course of the disease. In recent years, there have been significant advances in our knowledge of genetic aberrations in FL, and characteristic biological properties during the development and maintenance of the disease have also been unveiled. Here, we review the current understanding of these molecular and biological aspects of FL.

GENETIC ABNORMALITIES

The t(14;18)(q32;q21) translocation is a genetic hallmark of FL and is observed in 85-90% of FL cases.[1,2] This chromosomal translocation places the *BCL2* gene under the control of immunoglobulin heavy-chain (*IGH*) enhancer,[2] and sequence analysis of the breakpoint region indicates that this translocation is generated by an error during physiological VDJ rearrangement of the *IGH* gene.[3-5] This genetic aberration results in the constitutive expression of *BCL2* and confers a survival advantage to B cells, which plays an essential part in the pathogenesis of FL. However, t(14;18) alone is insufficient for the development of FL, as healthy individuals often harbor a small population of t(14;18)-positive B cells.[6-9] It is now recognized that various genetic abnormalities participate in the generation of FL, and this is assumed to constitute a major reason for the clinical heterogeneity of the disease (Table 1).[10]

Cytogenetic analyses of FL samples have revealed that they contain 4 to 6 additional genomic alterations on average, and 6q-, trisomy 7, trisomy 12, der (18) and +X are frequently observed.[11-13] Recently, chromosome comparative genomic hybridization (CGH) and array CGH have been carried out by several researchers, and various gene alterations have been

Table 1. Genetic alterations in follicular lymphoma at diagnosis (adapted from Ref. 10)

Gene	Frequency (%)
BCL2	85% (translocation)
	96% (mutation)
MLL2	89%
EPHA7	70%
BCL6	47% (mutation)
	6-14% (translocation)
TNFRSF14	18-46%
CREBBP	33%
MEF2B	15%
EP300	9%
EZH2	7%
TNFAIP3/A20	2-26%
FAS	6%
TP53	<5%

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shown to be related to tumor progression and histological transformation. The deletion of 6q25-27 has been shown to be strongly correlated with poor prognosis,[14] and another study showed that the deletion of 1p36.22-p36.33 and 6q21-q24.3 is significantly correlated with shorter survival.[15] Recently, *TNFRSF14* was identified as a target gene at the 1p36.32 locus in FL.[16,17]

Deletions affecting chromosome 6q have been reported as a common gene abnormality shared by many B-cell malignancies, and some of the target genes, such as *BLIMP1* and *TNFAIP3/A20*, are found in this region. Oricchio *et al*. identified ephrin receptor A7 (*EPHA7*) as a tumor suppressor in FL by combining array CGH and RNAi-deletion library screening.[18] *EPHA7* was shown to be targeted by epigenetic silencing, in addition to genomic deletions, and *in vitro* treatment of human lymphoma cells with 5'aza-2'deoxycytidine caused re-expression of the gene. They also demonstrated that knockdown of the *EPHA7* gene accelerated lymphoma development of the Vav-Bcl2 transgenic FL mouse model.[19]

Morin *et al*. found recurrent somatic mutations affecting the polycomb-group oncogene *EZH2* in FL and germinal center B-cell subtype diffuse large B-cell lymphoma samples.[20] EZH2 is a catalytic component of the polycomb repressive complex 2 (PRC2), which is responsible for trimethylating Lys27 of histone H3 (H3K27) and has a role in repressing transcription at the targeted loci. EZH2 is physiologically upregulated in normal germinal center (GC) B cells and targets some key cell cycle-related tumor suppressor genes. In a recent mouse experiment, it was shown that mutant *EZH2* alleles led to increased H3K27 methylation and inhibited egress of B cells from GC, and conditional expression of mutant *EZH2* accelerated lymphomagenesis in cooperation with *BCL2*.[21]

Recurrent mutations in other epigenome-related genes have been reported in FL. Two highly related histone and non-histone acetyltransferases (HATs), *CREBBP* and *EP300*, were recently shown to be frequently altered in FL and diffuse large B-cell lymphoma.[22] They work as transcriptional coactivators for a large number of DNA-binding transcription factors in multiple signaling and developmental pathways. Structural alterations of these two genes did not coexist in FL and their inactivation is assumed to be functionally equivalent. It is hypothesized that mutant CREBBP and EP300 proteins are deficient in acetylating Bcl-6 and p53, which results in constitutive activation of the former and decreased activity of the latter protein.

Morin *et al*. reported that two histone-modifying genes, *MLL2* and *MEF2B*, were somatically mutated in FL.[23] The prevalence of *MLL2* mutations is as high as the frequency of the t(14;18)(q32;q21) translocation, and is assumed to play a central role in the tumorigenesis of FL.

The recent rapid progress in the techniques for comprehensive genomic analysis has revealed some characteristic genomic alterations in FL, in a set of molecules that are related to epigenetic modification. These findings are of significant importance for our understanding of the pathogenesis of FL. It is assumed that normal germinal center B cells are strictly controlled by a specific epigenetic program, and alterations in this epigenetic pattern might result in developmental arrest and/or deregulated proliferation in this specific B-cell stage.

CLONAL DEVELOPMENT OF FL CELLS

t(14:18) is generally considered to be the first hit of the oncogenic cascade leading to FL, based on the frequency of this genetic aberration. This translocation occurs following a double-strand break at the *IGH* locus on chromosome 14, which is attributed to defective RAG-mediated VDJ recombination. As VDJ recombination occurs at an early B-cell developmental stage in the bone marrow, t(14;18) is believed to occur similarly in the bone marrow, but not to prevent further differentiation.

Interestingly, t(14;18)-positive circulating B cells can be detected in the blood of 50-70% of healthy individuals who are not prone to developing FL, at an average rate of 0.1-10 cells per million.[24,25] The frequency of cells carrying the translocation appears to increase with age, smoking and pesticide exposure, and such cells may be less common in ethnic groups with a lower incidence of FL.[7,26] It had been hypothesized that t(14;18) is carried by resting naive B cells, but the oncogenic potential of this translocation was considered to be limited in them because of the fact that they physiologically express Bcl-2.

A series of 25 cases demonstrating abnormal Bcl-2 expression in follicle centers associated with fairly preserved tissue architecture and residual reactive GC, a lesion termed "follicular lymphoma *in situ* (FLIS)", was first described in 2002.[27] The cells expressing Bcl-2 exhibited a propensity to be confined to the GC, without evidence of disseminated disease in most patients. These cases were mostly identified as an incidental finding in a reactive lymph node, and in a recent report, an extended follow-up for many of the cases was described.[28] Of the 21 cases with no other evidence of lymphoma at diagnosis, only one (5%) developed FL, supporting the idea that FLIS is the tissue counterpart of FL-like B cells in the peripheral blood of healthy individuals. A recent case report described the presence of identical *BCL2/IGH* fusion gene sequences in a mesenteric lymph node with a FLIS pattern and in peripheral blood B cells, without disease progression after 2 years of observation.[29] Therefore, one explanation for FLIS pathogenesis is that circulating t(14;18)-positive B cells seed individual GC and proliferate in an antigen-dependent manner. Indeed, FLIS lymph nodes typically show follicular hyperplasia in most of the specimens.

Roulland *et al*. reported that most of the peripheral

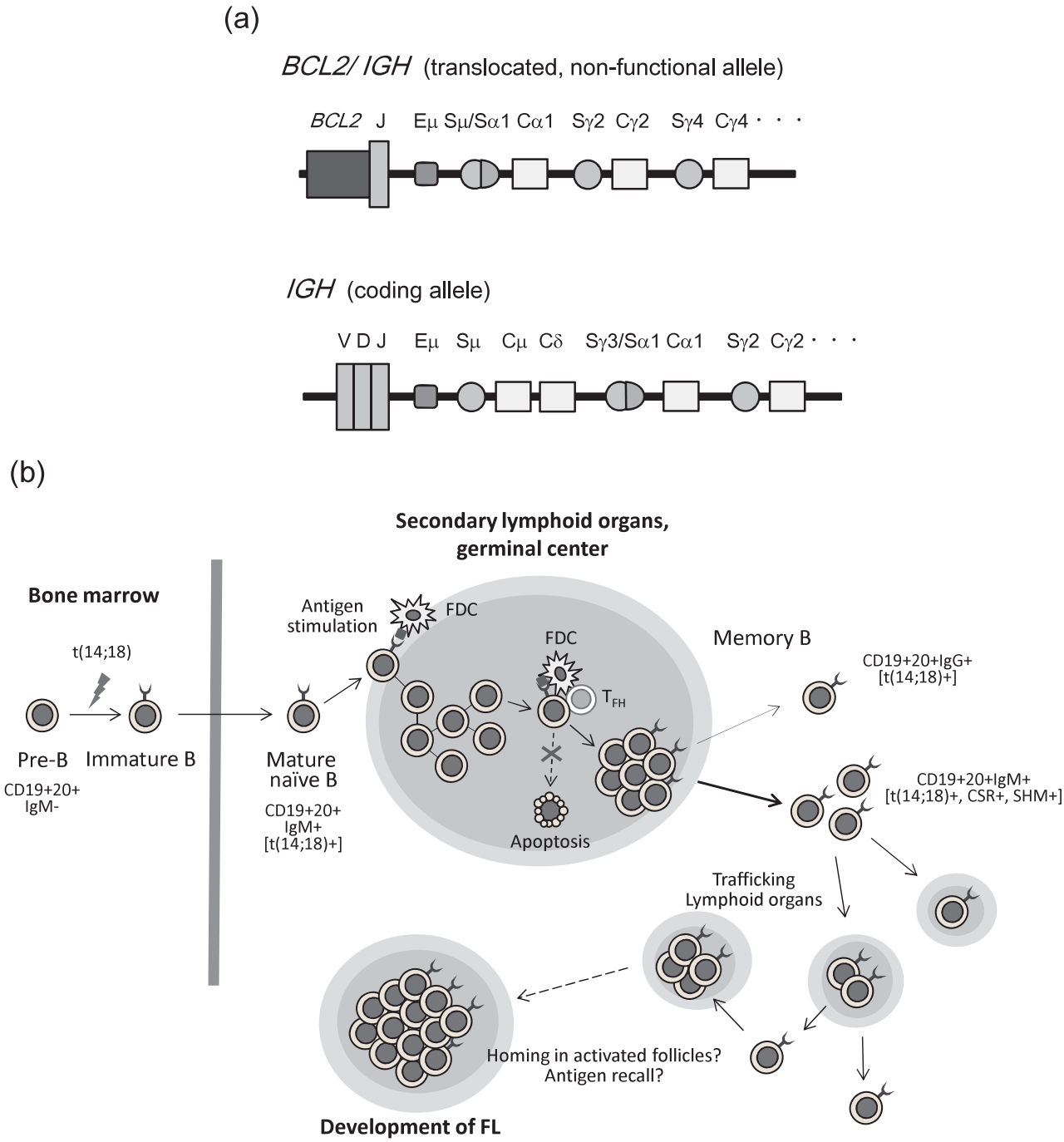

Fig. 1. The *IGH* gene configurations in follicular lymphoma (FL) and current understanding of the pathogenesis of FL. (***1a***) A schematic illustration of an example of the *IGH* gene configurations in FL (*cf.* Ref. 30-33). In most cases, CSR occurs in the translocated non-functional *IGH* allele (*upper panel*). In contrast, the Cμ region is usually spared from deletion in the coding *IGH* allele, while "downstream switching" is observed at high frequency (*lower panel*). Only the gene orders are shown and relative distances are not to scale. (***1b***) Current understanding of the pathogenesis of FL (adapted from Ref. 37). t(14;18) translocation occurs during early B-cell development in the bone marrow, and this aberration does not apparently interrupt the process of maturation of B cells. Naive t(14;18)-positive B cells are activated upon antigen stimulation in secondary lymphoid organs, and overexpression of Bcl-2 probably allows them to escape from apoptosis even when they carry a low-affinity receptor. Most t(14;18)-positive B cells then exist as IgM memory B cells, and they are thought to move to lymphoid organs in search of appropriate signals for further maturation. Repeated stimulation of these t(14;18)-positive B cells over years ultimately leads to FL development.

t(14;18)-positive B cells in healthy individuals are not naive B cells but instead are GC-experienced memory B cells.[30] These cells predominantly showed a $CD27^+$ memory B-cell phenotype. In addition, the $IgD^+/CD27^+$ subset (IgM memory) exhibited a significantly higher rate of translocation than $IgD^-/CD27^+$ memory B cells (switched memory). By long-distance PCR analysis, they showed that most of the *IGH* genes in the t(14;18)-positive B cells were class-switched, and interestingly, class switch recombination (CSR) occurred mostly in the translocated non-functional *IGH* allele but not in the productive allele. This is in sharp contrast with the features of the peripheral IgM memory B-cell subset, which entirely lacks CSR on both the productive and the non-productive alleles. In a previous report on FL, it was shown that CSR also occurs on the productive allele, but "downstream switching" (e.g., Sγ to Sα) occurs at an unusually high frequency and the Cμ region is spared from deletion.[31] Despite CSR occurring on both alleles in more than 80% of cases, in fact, most FL cells express IgM and only a minority express IgG, IgA or no Ig.[31-33] This "allele paradox" indicates the presence of selective pressure in favor of IgM expression on the t(14;18)-positive B-cell population that is at the same time permanently driven to switch (Fig. 1a). During this process, further genetic alterations may be added by the effect of activation-induced cytidine deaminase (AID), a DNA editing enzyme that is responsible for both CSR and somatic hypermutation (SHM). On the basis of these experiments, t(14;18)-positive B cells in healthy individuals are suggested to constitute more advanced precursors of the FL pathogenesis than previously thought.

Karyotypic data and examinations of SHM in the variable regions of the *IGH* gene have shown extensive intraclonal heterogeneity in FL B cells. It has long been assumed that transformation of FL to a more aggressive lymphoma reflects the emergence of an aggressive subclone of cells from the FL population. However, recent cytogenetic, CGH and single nucleotide polymorphism (SNP) data suggest a more immature common progenitor cell (CPC), and FL and transformed FL might arise by divergent evolution from CPC. In an analysis of the SHM pattern of 18 sequential FL/transformed FL samples, the existence of a putative CPC was suggested in 12 (67%) of the cases.[34] One donor/recipient paired sample in which FL developed after hematopoietic stem cell transplantation was also analyzed, and the presence of CPC was similarly suggested. The remaining 6 sequential cases (33%) were considered to have been transformed by direct evolution. An array CGH analysis investigating sequential biopsied FL samples showed that, in the majority of the cases (27 of 39), 1 or more of the copy number alterations (CNAs) present in the initial sample were absent in a later sample and new CNAs were acquired, indicating the presence of CPC.[35] In the remaining 12 cases, the CNA profiles were either unchanged or displayed additional CNAs in the late samples, being compatible with direct progression from the initial sample to the later relapse.

Recently, hierarchy in somatic mutations arising during the development and progression of FL has been analyzed by whole exome sequencing of subpopulations.[36] Cell-surface CD20 expression level is known to be heterogeneous in FL cells, and CD20-high ($CD20^h$) and CD20-intermediate ($CD20^{int}$) subpopulations of tumor B cells as well as tumor-infiltrating T cells (patient-matched normal cells) were sorted from FL tumor samples. By an exome-sequencing strategy, 877 coding somatic nucleotide variants (cSNVs) of missense and nonsense mutations were identified in 569 unique genes, and their distribution of mutation frequencies within individual cases showed patterns that were indicative of subclonal representation of the majority of cSNVs. Notably, only a minority of mutations were present at comparable frequencies within each subpopulation (mean = 27.2%, range = 3.8%-63.6 %), and the clonal diversity between $CD20^{hi}$ and $CD20^{int}$ subpopulations increased along with the total number of mutations. In 2 patients, sequentially biopsied samples were compared at diagnosis and relapse. Whereas the relapse sample maintained the majority of mutations identified in the diagnostic specimen in one patient, the other showed a low concordance rate of mutations between the sequentially biopsied samples. Somatic mutations were divided into 3 categories of a primary founder mutation, secondary driver mutations and tertiary mutations. In the proposed model for FL tumorigenesis, t(14;18) was a founder mutation, as has generally been considered. *CREBBP* was highlighted as a secondary genetic event, reflecting the high frequency and uniform representation of mutations between subpopulations, as well as their maintained presence between diagnosis and relapses. In contrast to previous reports, *MLL2* and *TNFRSF14* were categorized as tertiary genetic events, indicated by their variable mutational representation between subpopulations and their loss at relapse.

In summary, t(14;18)-positive B cells in healthy individuals are suggested to be mostly IgM memory B cells that are repeatedly stimulated in the GC. These premalignant B cells are rescued from apoptosis by Bcl-2 expression, expand in the GC and are released into the bloodstream, possibly in search of adequate niches in which the follicular microenvironment might support maintenance and proliferation in the context of antigenic stimulation.[37] This context of maturation arrest confers a high propensity for genomic instability, and acquisition of a broad diversity of genetic changes ultimately results in full malignant transformation into FL (Fig. 1b).

MICROENVIRONMENT

As with normal follicular germinal centers, FL tissue contains varying proportions of non-malignant immune cells. Malignant B-cell proliferation and survival are strongly de-

pendent on a combination of external stimuli delivered by the microenvironment within specific niches in invaded lymph nodes and bone marrow (Fig. 2). The importance of the microenvironment in FL is further highlighted by the fact that researchers have been unable to propagate FL cell lines, and even short-term growth *in vitro* requires survival signals derived from feeder cells or cytokines.

By using gene expression profiles of 191 FL biopsy specimens at diagnosis, Dave *et al*. demonstrated that tumor-infiltrating immune cell expression signatures independently predict clinical outcome.[38] They separately analyzed gene expression of malignant B cells and non-malignant immune cells of FL tumor tissues, and reported that T-cell-related gene expression, such as *CD7*, *CD8B1*, *LEF1* and *STAT4*, and macrophage-related gene expression, such as *ACTN1* and *BAFF*, was related to a favorable outcome (immune response 1). On the other hand, macrophage and dendritic cell (DC)-related gene expression, such as *TLR5*, *FcγR-1* and *SEPT10*, was related to an unfavorable outcome (immune response 2). However, distinct cellular subsets cannot be easily attributed to one of these immune-response signatures, as illustrated by numerous immunohistochemical studies that attempted to clarify the intricate relationship between non-neoplastic immune cells in FL, but resulted in highly contradictory results.

The number of tumor-infiltrating FOXP3-positive regulatory T cells (Tregs) in tumor samples was suggested to be a predictor of survival in patients with FL, and the number decreases during large cell transformation. Tregs play a crucial role in the suppression of T-cell-mediated autoimmunity, and are also known to participate in the evasion of anti-tumor immunity. Carreras *et al*. reported that a higher Treg number in FL tissues was associated with a better response to therapy and improved OS.[39] In other studies, the architectural pattern of FOXP3-positive cells was suggested as a predictor of a better outcome in FL.[40-42] PD-1 is a member of the CD28 family of membrane receptors that have an important function

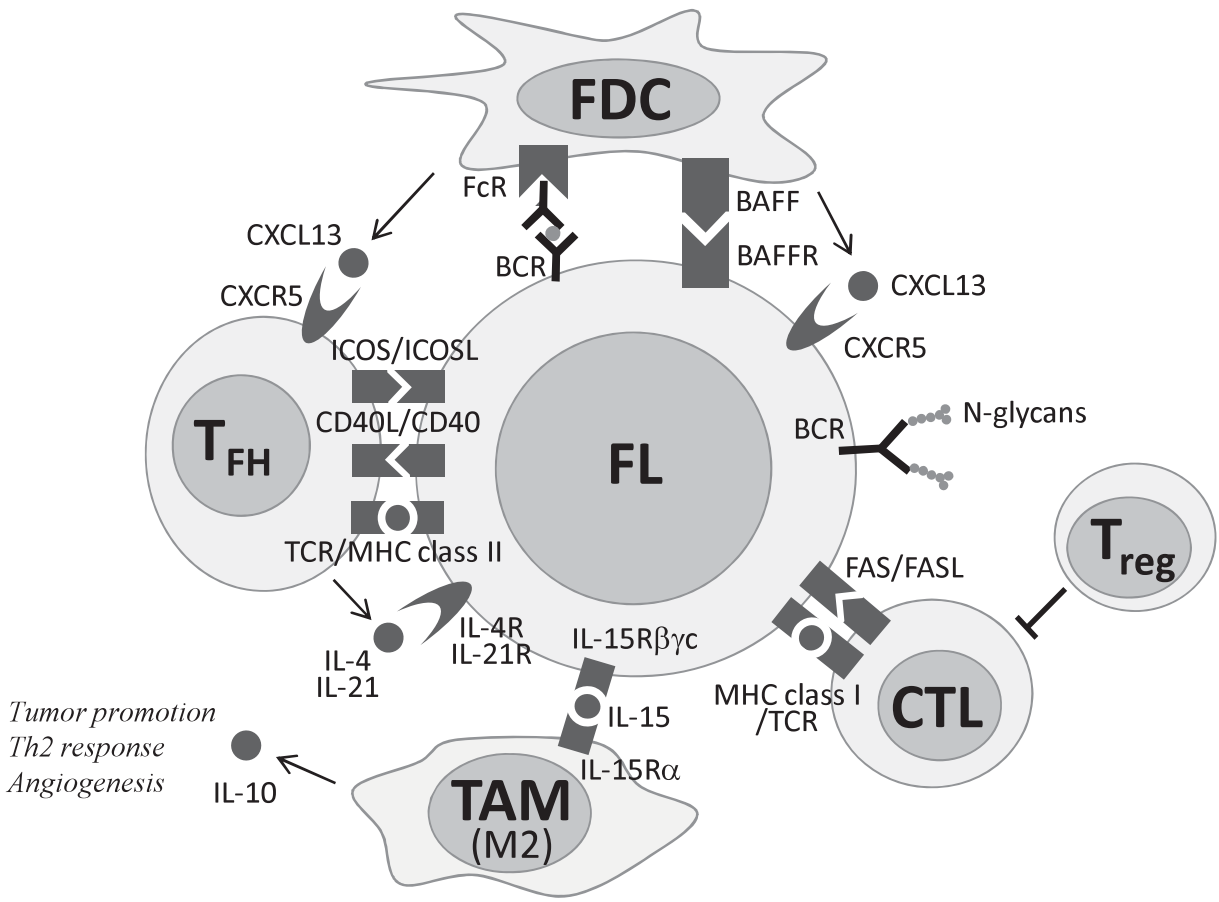

Fig. 2. A model of the follicular lymphoma (FL) microenvironment (reviewed in Ref. 10). Tumor cell survival and growth are supported by a variety of cells, such as follicular helper T cells (T_{FH}), follicular dendritic cells (FDC) and M2-polarized tumor-associated macrophages (TAM). B-cell receptor (BCR) signaling occurs by stimulation of the innate immune system through N-glycans, or by specific antigens expressed on professional antigen-presenting cells such as FDC.

as regulators attenuating T-cell activation and promoters of T-cell tolerance. A high level of PD-1-positive cells was reported as a predictor of a favorable outcome of FL patients independently of FLIPI, whereas a marked reduction was observed in transformation.[43] A study using a tissue microarray of 70 biopsied FL samples showed similar results, by using computerized image analysis and separating cells inside and outside the follicles.[42] On the other hand, high numbers of $CD68^+$ or $CD163^+$ tumor-associated macrophages (TAM) were shown to be linked to a poor prognosis in FL patients treated by chemotherapy without rituximab.[44-48]

$CD4^+$ T cells in the follicles, called follicular helper T cells (T_{FH}), constitute a distinct lineage of helper T 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. Through transcriptomic and flow cytometry analyses, Pagnault *et al*. reported that the FL microenvironment was characterized by strong enrichment in T_{FH}, and these T_{FH} cells expressed IL-4 at very high levels.[49] They also performed gene expression profiling of purified B-cell and non-B-cell compartments obtained from FL and reactive lymph nodes, and an IL-4-centered pathway was demonstrated. The phospho-STAT6-positive tumor B cells and IL-4-expressing T_{FH} cells were considered to create an IL-4-dependent T_{FH}-B-cell axis in the tumor microenvironment.

In FL tissues, malignant B cells are admixed with heterogeneous lymphoid-like stromal cells. Guilloton *et al*. reported that bone marrow-mesenchymal stromal cells (MSCs) obtained from patients with FL displayed a signature of being enriched for genes associated with a lymphoid-like commitment, especially CCL2.[50] CCL2 is one of the most frequently observed chemokines in a variety of solid cancers, where it exhibits multifaceted activities by targeting both tumor cells and infiltrating myeloid cells. A high level of CCL2 is detected within the FL-cell niche, suggesting bidirectional crosstalk between malignant B cells and stromal cells. CCL2 is induced in normal MSCs by coculture with malignant B cells, and the priming of macrophages with IFN-γ was shown to mediate STAT1-dependent induction of CCR2, associated with an increased migration response to CCL2. These results highlight the complex role of FL stromal cells that promote tumor B-cell growth and orchestrate the FL cell niche.

The variable regions of the *IGH* genes (*IGHV*) of FL tumor cells carry acceptor sequence motifs for N-glycan addition. These sequence motifs are introduced by SHM and are characteristic only of FL and some other GC tumors, but very infrequent in normal B cells and other B-cell malignancies.[51] The frequency of motifs in *IGHV* sequences was calculated to be 79% (55 of 70) in FL, compared with 9% (7 of 75%) in normal memory B cells. These motifs are also known to be found in the immunoglobulin light chain (*IGL*) gene of FL cells, and the overall incidence in FL may be up to 100%.[52] These glycans terminate at high-mannose regions, a feature usually observed only in immature glycoproteins in the endoplasmic reticulum. Mannnosylated B-cell receptor (BCR) was identified at the surface of primary FL cells, and interestingly, recombinant lectin domains of the mannose receptor or DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) bound to FL cells *via* mannosylated BCR *in vitro*, which resulted in BCR-associated increase in intracellular Ca^{2+}.[53] Mannose receptor and DC-SIGN are physiologically expressed in the macrophages and DCs, respectively. This unique interaction reveals a potential route for the microenvironmental support of FL cells.

FUTURE PERSPECTIVES

FL is among the most extensively analyzed hematological malignancies, and research on FL has progressed together with the advancement of biological experimental techniques. The accumulation of knowledge on FL has enabled us to explain the gross outline of the disease's development process. A greater understanding of its pathogenesis should lead to novel approaches for treating the disease, and it is highly expected that the disease will be better controlled, or even prevented, in future.

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