Follicular Lymphoma

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The Biology of the Germinal Center

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The immune system requires the production of high affinity antibodies of different subclasses to accomplish its many effector functions. Specific steps in B-cell ontogeny that occur within germinal centers of secondary lymphoid organs create much of the diversity in the immune system. This process also provides the raw material for the genesis of B-cell lymphomas as misdirection of the molecular machinery that regulate these steps can cause chromosomal translocations, prevent apoptosis and promote proliferation of abnormal clones. Many recent avenues of investigation have elucidated that the germinal center is a dynamic microenvironment where B-cells undergo repeated rounds of mutation and selection. Gene expression studies have further shown that malignancies derived from germinal center B-cells elaborate specific gene expression signatures that derive from neoplastic cells as well as elements of the host response such as T-cells and macrophages. This review will examine the current understanding of B-cell development in the germinal center and the key molecules involved in this process. Interactions between lymphoma cells and their cellular partners and models in the growth and development of follicular lymphoma will be presented.

Introduction

The vast majority of B-cell lymphomas derive from germinal center or post–germinal-center B cells due at least in part to the disruption of different phases of normal B-cell development. Just as rearrangement and diversification of immunoglobulin (*Ig*) genes and the integrity of the B-cell antigen receptor are important for B-cell function, deregulation of these steps are important events in B-cell lymphoma pathogenesis. Chromosomal translocations involving the *Ig* loci are typically seen in many B-cell lymphomas, and transformed B cells, like their normal counterparts, rely on signaling through the B-cell antigen receptor for survival. An understanding of normal B-cell development and the germinal center response is therefore essential for consideration of mechanisms governing B-cell lymphomas.

B-Cell Development and the Germinal Center

The first step in B-cell ontogeny that is undertaken by bone marrow–derived B-cell precursors is the recombination of immunoglobulin (*Ig*) heavy (*H*) and light (*L*) chain genes to generate a functional B-cell antigen receptor. This initial step in B-cell ontogeny occurs prior to antigen encounter and is accomplished by the recombination-activating genes *RAG1* and *RAG2*, which target the variable (*V*), diversity (*D*), and joining (*J*) regions of *IgH*, and the *VJ* regions of *IgL*. During the primary immune response low-affinity antibodies of the IgM subclass are produced. Subsequently, driven by antigen stimulation through the B-cell antigen receptor and CD40, naïve B-cells enter the germinal-center microenvironment, where they begin to proliferate and undergo clonal expansion.[1-3] In the germinal center many non-random, single-base changes are introduced by somatic hypermutation (SHM) into the *Ig V* regions that encode the antigen-binding site. This mutational process, which occurs at a rate much higher than in housekeeping genes, results in diversification of the *Ig V* repertoire. Concurrently, the *Ig* constant regions undergo a series of recombinations known as class-switch recombinations (CSR), whereby different subclasses of antibodies (IgG, IgA or IgE) are produced. SHM and CSR occur in the centroblast stage of B-cell maturation and together are responsible for generating high-affinity antibodies of different subclasses that are capable of mediating specific immune responses (**Figure 1**; see Color Figures page 512). Centroblasts that produce high-affinity antibodies either differentiate into plasma cells that are programmed to secrete large quantities of specific antibodies or become memory B cells that are programmed to recognize and re-

spond to antigens on repeat exposure. Those centroblasts that lack high-affinity antibodies or acquire crippling mutations in their *Ig V* regions (non-functional B-cell antigen receptor), or that show autoreactivity are swiftly eliminated by CD95/Fas-mediated apoptosis.[4,5] *Ig V* region genes undergo further diversification through ongoing mutational activity that generates intraclonal heterogeneity. This continued diversification of the *Ig V* regions is considered the hallmark of germinal center-derived B cells.

SHM and CSR require the presence of activation-induced cytidine deaminase (AID), an enzyme that is expressed specifically in germinal-center B cells.[6-9] AID initiates DNA strand-breaks in *Ig V* regions (by RNA-editing or DNA-deamination) that become substrates for a group of low-fidelity DNA polymerases involved in DNA mismatch repair. This inherently error-prone repair mechanism allows for the diversification of *Ig* genes. In *AID*$^{-/-}$ mice SHM and CSR are abrogated, and although they show elevated levels of IgM, other Ig subclasses are absent.[6] Kolar and colleagues have recently identified a novel subpopulation of tonsilar B cells that exhibit features intermediate between those of naïve and germinal-center B cells. These cells express AID, are capable of SHM and CSR, and likely represent a germinal-center founder-cell population that may also be the normal counterpart of mantle cell lymphoma.[10] Disruption of steps in normal B-cell differentiation, including *VDJ* recombination, SHM and CSR, can result in the genesis of B-cell lymphomas. For example, the t(14;18) translocation involving the *IgH* and *BCL2* genes occurs from mistakes in *VDJ* recombination (likely occurring in bone marrow–derived cells) and results in the overexpression of the BCL2 protein that prevents apoptosis. This translocation is the initiating event and genetic hallmark of follicular lymphoma as it is instrumental in generating long-lived clones that are prone to additional molecular defects that ultimately result in lymphoma.[11] A stain for BCL2 protein can effectively discriminate between neoplastic follicles of follicular lymphoma and normal secondary follicles, as the neoplastic follicles express BCL2 protein, whereas normal germinal centers do not (**Figure 2**; see Color Figures page 512). Additional markers that are used for routine diagnosis of follicular lymphoma include CD10 and BCL6: these do not discriminate between normal and neoplastic follicles but are used to ascertain a germinal-center B-cell derivation for the atypical lymphoid cells comprising the lymphoma. Newly characterized germinal center B cell–associated proteins such as HGAL[12,13] and LMO2[14] are also likely to become useful markers in the diagnosis of follicular and other germinal center–derived B-cell lymphomas in the future.

Another byproduct of faulty *VDJ* recombination is the t(11;14) translocation that leads to overexpression of *cyclin D1* and mantle cell lymphoma. By causing DNA strand-breaks SHM and CSR, like faulty *VDJ* recombination, predispose to chromosomal translocations involving the *Ig* genes. To date, more than 33 partners involved in chromosomal translocations have been characterized for *IgH* alone. SHM is likely involved in t(8;14), the defining abnormality in Burkitt lymphoma, which involves the *IgH* and the *c-myc* oncogene.[15,16] SHM and AID activity are not limited to *Ig* genes, but have also been implicated in hypermutations in *BCL6*, *FAS ligand* (*CD95L*), and other proto-oncogenes involved in B-cell lymphomas.[17] Furthermore, CSR participates in aberrant chromosomal translocations involving switch region sequences in plasma-cell myeloma.[18]

Germinal Center Anatomy and Co-stimulatory Signals

Secondary lymphoid organs such as the spleen and lymph nodes provide the critical niches where naïve B cells encounter antigens and produce high-affinity antibodies. This process, however, has been shown to take place in the absence of germinal centers.[19] The germinal-center microenvironment has been traditionally thought to have compartments where specific steps in B-cell development occur. Sinus macrophages take up antigens within minutes of antigen exposure, after which peptides are presented on follicular dendritic cell (FDC) processes. Upon stimulation by antigens (T-cell–dependent), and under the regulation of members of the tumor necrosis factor (TNF) family,[20] 3 or 4 activated B cells migrate into primary lymphoid follicles and undergo rapid clonal expansion of antigen-specific founder B cells. Within 3 days, polarized secondary follicles or germinal centers are formed that display anatomically distinct dark and light zones appreciable on a hematoxylin and eosin–stained histologic section. The dark zone is composed of centroblasts undergoing rapid rounds of proliferation; it is readily highlighted by immunohistologic staining for the proliferaton marker Ki-67 (MIB-1). Numerous admixed tingible body macrophages are also present in this zone. The centroblasts subsequently exit the dark zone (and the cell cycle) to enter the light zone as centrocytes. The light zone possesses antigen-enriched FDC and CD4$^+$ T-cells, where clonal selection based on antibody affinity is thought to occur. The pale-staining light zones show a low proliferative activity with well-developed FDC meshworks on which immune complexes can be visualized (**Figure 3**; see Color Figures page 513).

For continued development and selection of B cells within the germinal center, T-cell help is crucial. T-cell participation primarily occurs through cell-to-cell interactions (T-cell engagement of CD40 on B cells) and T-cell–induced cytokine-mediated signaling (co-stimulation through CD28). Germinal-center T cells are activated helper T cells (CD4$^+$ CD57$^+$ CD25$^-$) that migrate into germinal centers upon activation of the chemokine receptor CXCR5.[21] B cells primarily secrete interleukin (IL)–4. They are thought to originate in paracortical T-cell zones and follicles express IgD, but upon T-cell–dependent maturation, the characteristic follicular CD10$^+$ immunophenotype is acquired. In the absence of T cells, large germinal centers are formed but undergo dramatic spontaneous regression, indicating

that T-cell costimulation is essential for the maintenance and ongoing development of B-cell secondary follicles.[22,23] Similarly, although germinal-center formation is unaffected by the presence of FDC networks,[24] FDCs play an essential role in the formation of memory B cells; this interaction is dependent upon the expression of members of the TNF family.[25] The complex microarchitecture of the germinal center is therefore created not only by distinct stages of B-cell maturation but also by the distribution of immunophenotypically distinct and functionally specialized T, dendritic, and stromal cell subpopulations and their myriad interactions.[26]

Direct visualization of B-cell activity in real time using time-resolved multiphoton microscopy has shown that antigen-stimulated B cells within the germinal center are highly motile and transit intrazonally as well as bidirectionally between the dark and light zones.[27-29] Somewhat unexpectedly, cell divisions were observed in both zones, and B cells were found to reside for only a brief few hours within light zones where occasional stable contacts with T cells were formed despite frequent encounters. In addition, germinal-center B cells were found to have shared trajectories with mantle zone B cells. These elegant *in vivo* observations attest that antigen-stimulated B cells undergo repeated rounds of mutation and selection within the germinal center microenvironment and that they are likely to compete with each other for attention from T cells, which may be a limiting factor.[27-29] This model further emphasizes that germinal center cell dynamics and motility drive competition between B-cell clones for T cell input such that high-affinity antibodies are selected and an optimal immune response ensues. These findings also challenge the view that B-cell maturation occurs in an orderly fashion by transit through discrete compartments of the follicle, where competition of B cells to capture immune complexes displayed on FDC processes drives the germinal-center response.

Regulatory Factors of Germinal-Center Function

Many steps in B-cell differentiation in the germinal center are governed by the expression of transcription factors. Their hierarchic expression pattern and relative abundance provides information regarding normal B-cell development, but can also be exploited in diagnosis and classification of B-cell lymphomas that arise from different stages of B-cell maturation.[30-32] Key among B-cell transcription factors is PAX5, which in turn regulates many B-cell–specific transcription factors and is required for B-cell development from the pro-B to the mature B-cell stage.[33] Its downregulation is controlled by *Blimp1/PRDM1*, a master regulator of plasma cell differentiation.[34,35] The *ets* family transcription factor PU.1[36] and members of the interferon regulatory factor (IRF) family cooperate with *Blimp1/PRDM1* in the regulation of downstream transcription factor BCL6, which is essential for germinal-center formation and maintenance.[37] A 3q27 translocation involving the *BCL6* gene is present in 20% to 40% of diffuse large and 15% of follicular lymphomas. Mutations as well as SHM in the 5′ untranslated regions of *BCL6* are also present in a significant proportion of B-cell lymphomas. *BCL6* expression in germinal-center B cells is inversely correlated with the expression of *IRF4/MUM1*, which induces plasma cell differentiation.[38] *IRF4/MUM1* expression is also required for *AID* expression,[38] indicating that two important functions of the germinal center, SHM and CSR, are also dependent on the hierarchic expression and coordinate regulation of transcription factors. In B-cell lymphomas the expression of transcription factors may not necessarily mirror the pattern in normal germinal-center B cells: for example, *BCL6* and *IRF4/MUM1* are expressed in mutually exclusive subsets of germinal-center B cells, but in a subset of diffuse large B-cell lymphomas, *BCL6* and *IRF4/MUM1* are co-expressed.[39] In addition, the accumulation of new mutations in the 5′ region of the *BCL6* gene is considered to be an important mechanism in the progression of follicular lymphoma to diffuse large B-cell lymphoma.[40] These examples illustrate the important role that transcription factors play in normal germinal center response and how their deregulation leads to aberrant B-cell maturation and the development of lymphoid malignancies.

The Epstein-Barr virus (EBV) is another factor that has been associated with disregulation of germinal-center B cells. EBV is closely linked with a number of lymphoid neoplasms that include Burkitt and classical Hodgkin lymphomas and posttransplant lymphoproliferative disorders. Three independent groups of investigators have recently demonstrated that EBV can transform antigen receptor–deficient germinal-center B cells and enable their escape from apoptosis that is normally observed in B cells with nonfunctional antigen receptors.[41-43] The continued survival of the "rescued" pre-apoptotic B cells allows their proliferation and re-entry into the general circulation. This transforming event brought about by EBV is capable of overriding negative selection in the germinal center. The EBV-encoded latent membrane protein (LMP) 2A resembles a constitutively activated B-cell antigen receptor and is likely to function as the surrogate receptor through which B-cell signaling is triggered.[41-43] This ingenious mechanism of EBV/LMP2A-induced escape of antigen receptor–deficient germinal-center B cells from apoptosis not only offers an intriguing model of lymphomagenesis but also attests to the important role exogenous factors such as viruses play in modulating the germinal-center response.

The cell and developmental stage–specific expression of microRNAs (miRNA) are emerging as important regulators of mammalian cell function, including those of the immune system. Recent investigations by Thai and colleagues[44] have shown that the evolutionarily conserved miR-155, which is generated from the noncoding transcript of the *bic* gene and expressed in a variety of human B-cell lymphomas, is involved in the proliferation and selection of germinal-center B cells. In mice overexpressing miR-155, the antibody response was elevated, whereas it was

markedly reduced in miR-155–deficient mice in comparison with control litter-mates.[44] This topical finding suggests an additional level of complexity imposed by miRNAs on T-cell–dependent antibody response and germinal-center function.

Models of Follicular Lymphoma Pathogenesis

B-cell lymphomas have been shown to arise from all steps along the B-cell differentiation pathway and have been described as "frozen" stages of B-cell maturation. Although B-cell lymphomas show features of their nonmalignant counterparts, they also differ from them in that they usually result from multiple genetic abnormalities that involve their genesis and progression. Gene expression profiling studies have provided a compelling argument that B-cell lymphomas retain at least some characteristics of their cell of origin.[45,46] These studies have allowed the identification of coordinately expressed genes (gene expression signatures) on a genomic scale such that cellular differentiation, transcription factors, signaling pathways, and other regulatory molecules important in normal lymphoid cells and in lymphoid malignancies can be explored. These studies have also identified previously unrecognized signatures associated with prognostic subgroups of lymphomas. For example, diffuse large B-cell lymphomas exhibiting a gene expression signature similar to germinal-center B cells (GCB subtype) were found to be associated with a better overall survival compared with those exhibiting a signature similar to activated peripheral blood B cells (ABC subtype).[45] Furthermore, gene expression profiling studies have introduced an informative platform whereby the contribution of the tumor microenvironment composed of stromal and host-response cells and other contextual factors that are likely to influence tumor development and its biologic behavior can be studied. Gene expression profiling in follicular lymphomas have raised the possibility that survival of patients with this disease may be associated with immune response signatures that are contributed by nonmalignant cells such as T cells and macrophages.[47] Immunohistologic analysis in follicular lymphoma biopsy samples using an anti-CD68 antibody showed that increased infiltrating lymphoma-associated macrophages conferred a worse overall survival.[48] Similarly, tumor-infiltrating lymphocytes expressing CD4 and FoxP3 (as measured by immunohistochemistry) have been shown to correlate with improved survival in patients with follicular lymphoma.[49-51] These studies imply that the tumor microenvironment influences the biologic behavior of follicular lymphoma.

The defining genetic aberration of follicular lymphoma, the t(14;18) translocation, is present in the peripheral blood of a significant proportion of healthy individuals. Although initially thought to occur in resting naïve B cells, recent work by Roulland and colleagues[52] has shown that t(14;18)$^+$ cells in healthy individuals have already undergone CSR (which is indicative of germinal center transit). These t(14;18)$^+$ cells from healthy individuals are therefore similar to follicular lymphoma cells that arise from germinal-center B cells that have undergone SHM and CSR. In 80% of follicular lymphomas, CSR occurs on the productive as well as the nonproductive allele: on the productive allele the t(14;18) translocation disrupts the *Ig V* regions, and the *IgM-IgD* region is deleted; however, on the nonproductive allele this region is spared such that most follicular lymphomas express surface IgM and IgD. This paradoxic expression pattern was also found to be present in healthy individuals carrying t(14;18)$^+$ B cells and has led to the hypothesis that ectopic expression of BCL2 in the germinal center favors extended B-cell survival (presumably for the acquisition of additional genetic changes) and therefore confers a survival advantage on these B cells.[11,52,53] In addition, since the antigen receptor of t(14;18)$^+$ B cells in healthy individuals are capable of responding to antigen stimulation, signaling through the antigen receptor is likely to contribute to neoplastic transformation (**Figure 4**; see Color Figures page 513). This finding sheds new light on the previously held view of long-lived naïve t(14;18)$^+$ B cells in normal individuals and raises the possibility that atypical *BCL2*-rescued germinal-center B cells share a closer relationship with follicular lymphoma than previously recognized.[11,52,53] Since follicular lymphoma cells, unlike normal germinal-center B cells, have been shown to traffic between follicles of affected lymph nodes,[54] the propensity for dissemination of t(14;18)$^+$ B cells to secondary sites may allow the involvement of premalignant niches.[52] Antigen-independent modification of B-cell receptor (BCR) signaling through increased N-glycosylation sites introduced in a lymphoma-specific manner by SHM in the germinal center has also been shown to occur in follicular lymphoma.[55] Recent findings suggest that oligomannose-containing sugar moieties that bind directly to the antigen binding site may have a propensity to modulate signaling in follicular lymphoma B cells.[56] Whether blockade of signaling through the B-cell antigen receptor can be harnessed for therapeutic purposes in follicular lymphoma remains to be seen.

Conclusions

Several new lines of investigation have contributed to our current understanding that the germinal-center microenvironment provides a dynamic niche within which molecular processes are coordinately regulated but retain sufficient plasticity to allow immune diversity. That the error-prone molecular machinery governing germinal-center function is restrained enough in most cases to produce a normal secondary immune response is momentous given its complexity. The retention of some but not all features of different stages of normal germinal-center B-cell maturation by lymphoid malignancies has enhanced the understanding of normal B-cell ontogeny. Gene expression studies have also elucidated previously unrecognized factors relevant to transcriptional regulation, signaling, host re-

sponse, and survival of normal and neoplastic germinal-center B cells, and have contributed to disease models of follicular lymphoma pathogenesis and prognosis. As a result, the events in the germinal center during normal B-cell development as well as the mechanisms that deregulate these events have emerged as important factors in the formulation of disease models of lymphomas and options for targeted therapy for patients with B-cell neoplasia, including follicular lymphoma.

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