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Indolent lymphoma: follicular lymphoma and the microenvironment—insights from the microscope

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Follicular lymphomas (FLs) are neoplasms of germinal center (GC) B cells, which retain many of the morphologic, immunophenotypic, genetic, and functional features of normal GC B cells. Both normal GCs and neoplastic follicles of FL also contain non-neoplastic cells (microenvironment) that influence and are influenced by the GC and FL B cells and are likely important for tumor cell survival. Many insights into the nature of the GC/FL microenvironment have come from morphologic and immunophenotypic analysis, both before and after the discoveries from gene expression profiling. This chapter reviews what we have learned from the microscope and highlights the pitfalls involved in trying to enumerate cells in the microenvironment for clinical prognostication.

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Learning Objective

- To perceive the strong interconnections revealed by microscopic observation between immune system and stromal cells in the microenvironment and neoplastic cells in the development and progression of FL

Introduction

Follicular lymphomas (FLs) are neoplasms of germinal center (GC) B cells, which retain many of the morphologic, immunophenotypic, genetic, and functional features of normal GC B cells. FL (FL) is defined in the fourth edition of the World Health Organization (WHO) classification[1] as "a neoplasm composed of follicle centre (germinal centre) B-cells (typically both centrocytes and centroblasts), which usually has at least a partially follicular pattern. Lymphomas composed of centrocytes and centroblasts with an entirely diffuse pattern in the sampled tissue may be included in this category." The neoplastic follicles in FL contain, not only neoplastic GC B cells, but also non-neoplastic T cells, macrophages, and dendritic cells that comprise the FL microenvironment. Many of the components of the FL microenvironment mimic those present in normal GCs, but there are important differences that may contribute to tumor cell survival. This chapter begins with a summary of what morphology and immunophenotyping can tell us about the GC reaction, both the B-cell component and the microenvironment, and then provides a discussion of similar issues in FL.

Microenvironment of the GC reaction

The GC reaction occurs within a few days of primary antigen exposure and with subsequent antigen challenges. Each GC is formed from between 3 and 10 naive B cells, and ultimately contains ~10 000-15 000 B cells; Therefore,]mt]10 generations are required to form a GC.[2,3] This reaction is highly dependent on the GC microenvironment, which includes follicular dendritic cells (FDCs), macrophages, and T cells of both the follicular helper cell (T_{FH}) and regulatory type (Treg).

FDCs and macrophages

FDC are large cells with nuclei that are similar in size to centroblast nuclei, but have delicate nuclear membranes and central, small, eosinophilic nucleoli. FDCs are often binucleate and the 2 nuclei are typically apposed to one another, with flattening of the adjacent nuclear membranes. The long processes are not usually visible on routine sections. FDC processes have surface complement receptors (CD21) and Fc receptors (CD23) and bind both free antigen and antigen–antibody complexes for presentation to B cells. In GCs, the FDCs express both CD21 and CD23, whereas in primary follicles and mantle zones, typically only CD21 is detected. Macrophages present in the GC ($CD68^+$) are typically phagocytic (tingible body macrophages), containing apoptotic debris from B cells that have failed to reexpress a surface Ig molecule (SIg), also known as the BCR, after undergoing somatic hypermutation (SHM) of the Ig variable region genes (*IGV*) (see Centroblasts below). The exact role of macrophages in the GC has not been extensively studied, but evidence suggests that they both facilitate and regulate GC development.[45,6]

GC T cells

GC T cells appear as small, mature lymphocytes, predominantly in the light zone. The majority of GC T cells are T_{FH} cells ($CD3^+CD4^+CD57^+PD1^+CXCL13^+CXCR5^+BCL6^+$), which are important for selection of B cells for entry into and proliferation within the GC. Follicular Treg cells ($Foxp3^+Blimp\text{-}1^+CD4^+CXCR5_{high}PD\text{-}1^{high}$) exert negative regulatory effects on both immunoreactive T cells and B cells and are required to shut off the GC reaction and prevent excessive immune responses and autoimmunity; these are present in the GC, but in smaller numbers.[7,89]

GC B cells

B cells home to lymph nodes via receptors on the endothelial cells of the high endothelial venules[10] and within the lymph node via interactions with stromal cells.[11] B cells that have encountered antigen on dendritic cells, macrophages, or in soluble form[12] and have high-affinity BCRs (SIg) are selected by antigen-specific $CD4^+$ T_{FH} cells for entry into the GC,[13] which is formed by expansion of selected clones within the FDC meshwork.[2,14] The small lymphocytes (naive B cells) of the primary follicle are pushed aside to form the mantle zone.

Centroblasts

The proliferating GC B cells, known as centroblasts, are large cells with vesicular nuclei, 1-3 prominent, peripheral nucleoli, and a

narrow rim of basophilic cytoplasm. Centroblasts express SIg at low levels[2,15,16] and also switch off the gene that encodes the BCL2 protein; therefore, they and their progeny are susceptible to death through apoptosis.[17] BCL6 protein is expressed.[18,19] Centroblasts undergo SHM of the *IGV* genes, which alters the antigen affinity of the Ig that will be produced by the cell.[20,21] Somatic mutation is mediated by activation-induced cytosine deaminase (AID),[22] and increased expression of AID protein can be demonstrated in the dark zone of human GCs.[23] SHM thus results in marked intraclonal diversity of antibody-combining sites in a population of cells derived from only a few precursors.[3] Also in the GC, the *BCL6* gene undergoes SHM of the 5′ noncoding promoter region at a lower frequency than is seen in the *IGV* genes.[24-26] The functional significance of this SHM is not known.

Centrocytes

When centroblasts stop dividing, they become medium-sized cells with irregular nuclei, inconspicuous nucleoli, and scant cytoplasm called centrocytes, which accumulate among the centroblasts and then migrate to the opposite pole of the GC, resulting in polarization of the GC into a "dark zone," which contains centroblasts and closely packed centrocytes, and a "light zone," which contains centrocytes, a high concentration of FDCs, and numerous T cells. GC cells in the light zone express antigens associated with activation, most of which are involved with interaction with T cells, including CD23, CD71, CD40, and CD86,[27-32] as well as antigens associated with adhesion to FDCs, including CD11a/18 and CD29/49d.[33,34] MYC is transiently expressed in the light zone, where it is important for reentry into the dark zone[35] As shown by *in vivo* imaging and flow cytometry, expression of CXCR4 and CD83 can be used to distinguish between cells in the dark zone (CXCR4^{high}CD83^{low}) and those in the light zone (CXCR4^{low}CD83^{high}).[23]

Centrocytes express a BCR/SIg that has an altered antibody-combining site because of the somatic mutations in the *IGV* region.[3] Centrocytes with *IGV* gene mutations that have resulted in decreased affinity for antigen rapidly die by apoptosis (programmed cell death); the prominent "starry sky" pattern of phagocytic macrophages seen in GCs at this stage is a result of the apoptosis of centrocytes. Centrocytes with *IGV* gene mutations that have resulted in increased affinity process the antigen and present it to CD4$^+$ T$_{FH}$ cells in the light zone of the GC. T$_{FH}$ cells (CD3$^+$CD4$^+$CD57$^+$PD1$^+$CXCL13$^+$CXCR5$^+$BCL6$^+$) are present in large numbers in the light zone and express CD40 ligand (CD40L), which can engage CD40 on the B cell. Both ligation of the BCR by antigen and ligation of CD40 on the surfaces of GC B cells "rescues" them from apoptosis.[14-16,36-38] As shown by *in vivo* imaging and flow cytometry, the selected centrocytes reenter the dark zone, where clonal expansion occurs; cells may cycle between the dark and light zones multiple times.[23,39] Therefore, both entry into the GC and clonal expansion within the GC are mediated by the affinity of the BCR for antigen and its effective presentation to CD4$^+$ T$_{FH}$ cells.

Centrocytes then go on to become either Ig-secreting plasma cells or memory B cells. Interaction with surface molecules expressed by FDCs, such as CD23, are important in directing differentiation of the centrocytes into plasma cells and stimulating class switching from IgM to IgG or IgA.[14,40] Interaction with the numerous T$_{FH}$ cells present in the light zone, through the CD40-CD40 ligand, appears to be important in the generation of memory B cells.[2,14] In addition, both antigen-receptor ligation and CD40 ligation switch off *BCL6* mRNA production and BCL6 protein expression.[41] Through the mechanisms of *IGV* region mutation and class switching, the GC reaction gives rise to the better-fitting IgG or IgA antibody of the late primary or secondary immune response,[42] as well as B-cell memory.

Microenvironment of FL

FL B cells

FL B cells are predominantly centrocytes, with a minor population of centroblasts in most cases; therefore, they resemble the light zone of the GC. Perhaps not surprisingly, the gene expression profile of FL is similar to that of the light zone, with up-regulation of pathways associated with cellular activation, and is distinct from that of the dark zone, which is characterized by up-regulation of cell-cycle-associated genes.[23] Neoplastic B cells express many of the antigens that are found on normal GC B cells and are associated with interactions with both T cells and dendritic cells, such as costimulatory molecules CD80 and CD86 and CD40[43,44] and CD70.[45] These molecules facilitate interaction with T cells and, in the case of CD70, may result in conversion of T cells to a Treg phenotype.[45]

Like normal GC B cells, FL B cells have undergone SHM of the *IGV* genes and almost always express SIg. The lymphoma cells have undergone Ig class switch in 20%–50% of the cases.[46] The expression of SIg appears to be important for FL cell survival, because the administration of anti-idiotype antibody can result in tumor regression. An important property of the SIg produced by FL is the acquisition during SHM of sequence motifs for the addition of unusual mannosylated glycans not seen in normal B cells to the mutated *IGV* regions (for review, see Stevenson and Stevenson[47]). These glycans bind to lectins (mannose-binding lectin and DC-SIGN) on dendritic cells and macrophages of the innate immune system, allowing them to survive in the GC environment in the absence of cognate antigen. Consistent with this observation is the old observation that FDCs in FL do not have surface deposits of Ig on their processes, in contrast to FDCs of normal GCs.[48] Neoplastic follicles contain many elements of the GC microenvironment.[49]

Non-neoplastic B cells

Neoplastic follicles usually lack mantle zones, but in some cases, partial or complete mantle zones composed of non-neoplastic naive B cells may be present around all or some of the follicles.[48] This phenomenon may reflect colonization of preexisting follicles by the neoplastic B cells[50] or it could be the result of some kind of dynamic interaction between the neoplastic and normal B cells. The fact that the majority of the neoplastic follicles lack mantle zones suggests that they are true *de novo* neoplastic structures and not simply reactive GCs "hijacked" by the neoplastic B cells.

FDCs

Nodular meshworks of FDCs invest the neoplastic follicles, as demonstrated by monoclonal antibodies to CD21 or CD23.[46] Expression of CD21 and CD23 is variable in FL and FDC in some FL may express one and not the other. In diffuse areas of FLs, FDCs are absent, suggesting either a loss of reliance of the neoplastic B cells on this aspect of the microenvironment or a loss of ability to recruit FDCs. FDCs are present in FL follicles even in extranodal sites[46] and BM, suggesting that they are recruited by the neoplastic cells, rather being than the result of involvement of preexisting follicles.

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FL-associated T cells

Neoplastic follicles also contain T cells, which are usually less numerous than in reactive follicles and are randomly distributed, in contrast to the concentration in the light zone that characterizes reactive follicles.[51,52] T cells in neoplastic follicles are predominantly CD4^{+}, similar to those in GCs, and are more numerous, with a higher CD4:CD8 ratio in low-grade (Grades 1-2) than in Grade 3 FL.[52] T_{FH} and Treg T cells are also present. Although PD1^{+} T_{FH} cells in FL appear to be similar to those in tonsil GCs,[53] Treg cells are more numerous in FL than in reactive GCs.[9] Treg cells are more numerous in Grades 1-2 FL than in Grade 3 FL, are more numerous in follicular than diffuse areas, and are decreased in areas of transformation to diffuse large B-cell lymphoma.[54]

FL-associated macrophages

CD68^{+} histiocytes/macrophages are also present in FL, but are less conspicuous than in reactive GCs and are typically not phagocytic, correlating with the relatively low proliferation fraction and lack of apoptosis in FL compared with GC. Macrophages in FL and other cancers are thought to show M2 polarization, a phenotype associated with tissue remodeling and tumor progression.[5556] This phenotype can be associated with up-regulated mannose receptors and could facilitate survival of neoplastic B cells via binding of the glycated VH regions of the BCR.[47]

Like normal B cells, the neoplastic cells (as well as the precursor cells) of FL may circulate and home to preexisting follicles. This may take the form of so-called "FL *in situ*/intrafollicular neoplasia"[57]or partial nodal involvement by FL.[50] This phenomenon likely reflects the ability of preexisting reactive GCs to be entered by newly antigen-reactive B cells.[58] Interestingly, FL B cells within preexisting GCs often exhibit stronger staining for BCL2 than the typical neoplastic follicles of the same lymphoma (personal observation).

FL microenvironment and prognosis

After the landmark study from the Lymphoma/Leukemia/Molecular Profiling Project demonstrated the prognostic importance of genes expressed by cells of the FL microenvironment,[59] numerous immunohistochemical studies addressed the challenge of enumerating T cells, macrophages, and other cells of the microenvironment to assess their impact on prognosis. The results have been highly variable.

Lymphoma-associated macrophages

Increased numbers of lymphoma-associated macrophages was found to predict worse survival in patients treated with chemotherapy,[60,61] but was associated with improved survival in patients treated with rituximab immunochemotherapy.[61,62] Other studies have found no impact of the number of lymphoma-associated macrophages on outcome.[63,64]

Lymphoma-associated T cells

The number and distribution of Foxp3^{+} Treg cells and T_{FH} cells have been reported to predict clinical prognosis in some,[54,65-67] but not all,[64] studies (see Table 2 in Rimsza and Jaramillo[68]) Even in those studies that found an effect, the results were inconsistent, with some finding that absolute numbers of Treg cells were important[54] and others[63,69,70] that the pattern (follicular, perifollicular, diffuse) was important in predicting outcome. The impact on outcome has also varied, with some studies finding that increased Treg cells were associated with a good prognosis[54,63] and others finding an association with advanced disease or a poor prognosis.[69,70] Those investigators who found that increased Treg cells are associated with negative clinical features interpreted these results to suggest that Tregs are primarily affecting immunoreactive T cells that might have tumor-suppressive effects,[70] whereas those who found that increased Treg cells are beneficial propose that they are exerting primarily repressive effects on the neoplastic B cells.[54]

Why the discrepancies?

There are likely several reasons for the discordant results among studies. These include the use of different patient populations and different treatment regimens (particularly the use of rituximab). Probably equally, if not more, important is the difference in staining results among laboratories and the difference among pathologists in interpreting the results, particularly when enumerating stained cells in tissue sections. One study confirmed this variability and suggested that automated microscopy analysis may be more reproducible and accurate.[71] Other problems include variability in the choice of cutoff numbers of cells to define "low" versus "high" levels of immune cells and the consideration of pattern (where the immune cells are vis a vis the neoplastic cells). Unfortunately, until and unless these issues can be resolved, the utility of such testing in clinical practice remains dubious.

Summary

Neoplastic follicles of FL recapitulate many features of the normal GC, including elements of the microenvironment, such as FDCs, macrophages, and T cells. Understanding the similarities and differences between normal and neoplastic follicles, including elements of the microenvironment, has led to insights into lymphoma development and progression. Understanding the relationship of the neoplastic cells to the microenvironment (ie, which cells are needed for lymphoma growth and which are involved in regulating lymphoma growth) will be crucial to developing therapies aimed at the microenvironment. Techniques for enumerating such cells will be of limited value in lymphoma management until these issues are understood and better standardization of immunohistochemistry is possible.

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