Review

Clin Ter 2012; 163 (1):47-57

Diagnosis and classification of B-cell non-Hodgkin lymphomas. The role of multiparameter flow cytometry

G. Carulli[1], A. Marini[2]

[1]Division of Hematology and Section of Flow Cytometry, Department of Oncology, Transplants and New Technologies in Medicine, University of Pisa; [2]Laboratory of Clinical Pathology, Versilia Hospital, Lido di Camaiore, Italy

Abstract

Immunophenotyping is a routine method to evaluate B-cell non-Hodgkin lymphomas. Flow cytometry plays a complementary role in diagnosis and classification of these types of lymphomas, since combination of morphologic, immunophenotypic and genotypic features is needed to correctly classifying each disease entity. Multiparameter flow cytometry, which is now carried out with routine combinations of six to eight monoclonal antibodies, allows identifying even small lymphomatous cell populations on the basis of aberrant B-cell marker expression and clonality. The immunophenotypic patterns obtained by multiparameter flow cytometry are useful to correctly diagnose most of cases of specific subtypes of B-cell non-Hodgkin lymphomas and to discover peculiar clinical presentations, such as discordant and composite lymphomas. Immunophenotypic variability, however, characterizes B-cell lymphomas. Therefore, flow cytometry should always be used in combination with other techniques to correctly classify each disease entity. Finally, multiparameter flow cytometry is characterized by high sensitivity in detecting residual disease. ***Clin Ter 2012; 163(1):47-57***

Key words: *B-cell non-Hodgkin lymphomas, flow cytometry, immunophenotyping*

Introduction

In the last years, flow cytometric immunophenotyping (FCI) has confirmed its usefulness in the diagnostic approach of hematological malignancies. Significant improvements in instrumentation and availability of an expanded range of monoclonal antibodies (MoAbs) and fluorochromes has led to more accurate phenotyping of cells, leading to enhanced identification of abnormal populations (1). Multiparameter flow cytometry (MFC) is now carried out with routine antibodies panels expanded up to 8 fluorescences, but cytometers that allow 9-10-color immunophenotypic analyses are available (2).

In 2006 a panel of experts met in Bethesda and formulated consensus recommendations for FCI in hematological malignancies. In particular, a practical approach was delineated, based on the clinical presentation (3).

The World Health Organization (WHO) classification for tumors of the hematopoietic and lymphoid tissues identifies pathologic entities on the basis of morphologic, phenotypic, and genotypic features that are characteristic of each disease entity. Neoplasms of mature lymphoid cells include B-cell chronic lymphocytic leukemia (B-CLL), B-cell prolymphocytic leukemia (B-PLL) and the other diseases that are classified as B-cell non-Hodgkin lymphomas (B-NHLs) (4).

Flow cytometry is a diagnostic tool able to identify lineage-associated antigens belonging to the B lineage (such as membrane B-cell markers and surface immunoglobulins). The 2008 WHO classification confirms that FCI studies are indispensable for the diagnosis of mature B-cell lymphoid neoplasms through the identification of phenotypically abnormal cells belonging to the B-cell lineage and recognition of phenotypes characteristic of separate disease entities.

This review is focused on the uses and current status of MFC in the evaluation of B-NHLs. The most useful markers (generally identified as CD antigens) will be reviewed, with particular reference to their aberrant expression by lymphomatous lymphocytes. The capacity of FCI to identify specific pathologic patterns will be analyzed and the most recent opinions about a possible correlation between phenotypic behaviors and specific disease entities will be discussed. Finally, the merits and limitations of MFC will be analyzed, since its usefulness has to be compared with the properties of the other diagnostic techniques, such as morphology, immunohistochemistry, karyotyping, FISH, gene rearrangement assays.

B-cell-associated molecules with wide expression

B-lymphocytes are detected by using sensitive surface markers, such as CD19, CD20 and CD22. These molecu-

Correspondence: Dr Giovanni Carulli, Division of Hematology, Santa Chiara Hospital, Via Roma 67, 56126 Pisa, Italy. Tel: +39.050.992.731. Fax: +39.050.993378. E-mail: giovannicarulli@alice.it

les are also detected on the surface of pathologic cells in B-NHLs, but the pattern of their expression is generally different from the normal counterpart. In addition, significant differences can be found among the different types of B-NHLs, and variations in fluorescence intensity can be useful in the differential diagnosis.

CD19 is the most useful diagnostic marker and an initial gate including the CD19+ population is a preliminary step to identify both normal and pathologic B-cell subpopulations. Diminished expression of CD19, in terms of surface molecule density, characterizes about 30% of B-NHLs and is a very frequent finding in follicular lymphomas (FL), accounting for about 80% of cases without any relationship with the histological grading (5). Some studies carried out by immunohistochemistry have shown that occasional B-NHLs, generally belonging to the diffuse large B-cell lymphoma subtype (DLBCL), may lack CD19 expression. This phenomenon seems to be more frequent in T-cell rich B-NHLs (6).

CD20 is characteristically dimly expressed, or may even be absent, in B-CLL/SLL (small lymphocytic lymphoma), while it is expressed with higher fluorescence intensity by the other B-NHLs, with the highest expression in hairy cell leukemia (HCL) (7). It has been demonstrated that CD20 mutations can occur in about 20% of cases because of a C-terminal deletion (8).

CD22 in another molecule expressed on cell surface by most of mature normal B-lymphocytes. In B-NHLs, the highest intensity of CD22 is found in HCL and B-PLL. Instead, B-CLL is characterized by very low levels of this markers or even by its absence.

B-lymphocytes express a multimeric receptor complex, termed B-cell receptor (BCR) and classified as CD79. It is composed of two domains, CD79a and CD79b; the former is the cytoplasmic domain of BCR and its recognition is made after cell membrane permeabilization, while the latter is the external domain and is labeled by routine methods. BCR has a key-role in antigen recognition and acts together with surface IgM and IgD (9). CD79a is an early B-cell marker, being found both in precursor B-cell neoplasias and in chronic lymphoproliferative diseases. CD79b expression has been shown useful in improving the diagnostic accuracy in distinguishing B-CLL from mantle cell lymphoma (MCL) or other B-NHLs that sometimes may mimic B-CLL because of unexpected CD5-positivity (10, 11). In fact, surface levels of CD79b are very low or even undetectable in most cases B-CLL and normally expressed in the majority of other B-LNHs. Some exceptions have been reported, because some cases of B-CLL show high CD79b levels (10) and occasional cases of HCL are characterized by relatively low surface density of this molecule (12).

Therefore, combinations of two or more B-cell-associated markers allow identifying pathologic cell clones with great accuracy, because most of B-NHLs show pattern of surface marker expression that improves differential diagnosis. Thus, as far as pan B-cell molecules are concerned, typical B-CLL is CD19+, CD20-or dim, CD22- or dim, CD79b- or dim; MCL generally is CD19+, CD20+^{bright}, CD22+, CD79b+; FL generally is CD19+^{dim}, CD20+^{bright}, CD22+^{bright}, CD79b+ (Fig. 1).

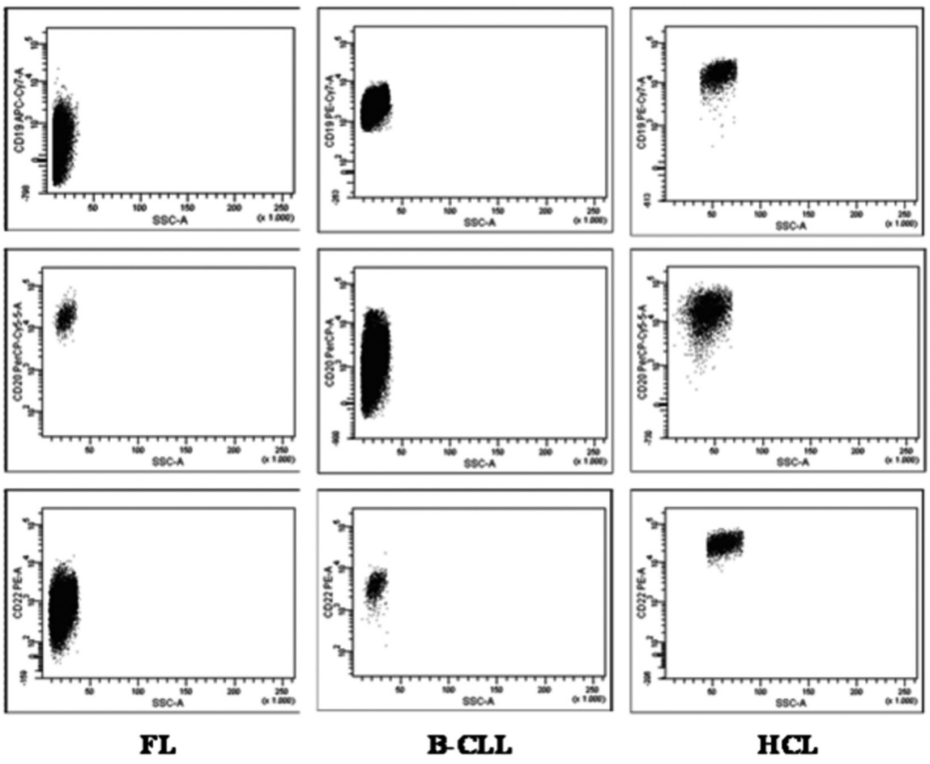

Fig. 1. Different pattern of expression of B-cell markers in follicular lymphoma (FL), B-cell chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL). From the top: CD19, CD20, CD22.

CD5-positive B-NHLs

The CD5 molecule is a marker acquired by T-lymphocytes during thymic maturation. It is not specific of the T-lineage because a small normal B-lymphocyte subset, circulating in peripheral blood and residing in fetal and adult lymphoid tissue, expresses this molecule.

Some B-NHLs are characterized by aberrant CD5 expression. It is the case of B-CLL, which is typically CD5+, and MCL, which expresses this molecule in most cases (13). CD5 expression, however, is not restricted to these two types of B-NHLs. In fact, cases of marginal zone lymphoma (MZL), FL and DLBCL can express CD5, with a frequency that is not negligible.

The incidence of CD5 molecule expression has been reported to be approximately 20-25% in some large series of splenic MZL diagnosed on the basis of cytological and immunophenotypic studies of peripheral blood (14, 15). In the few cases of splenic MZL with blood and spleen analyses previously reported, cells were CD5+ in the blood and bone marrow but were CD5- on spleen sections (16). A modulation of CD5 expression according to the involved compartment could account for these discordant results between blood and spleen, since CD5 expression can depend on the micro-environment. These CD5-positive splenic MZL cases have many similarities with classical CD5-negative splenic MZL cases, including their clinical presentation, cytological, morphological and immunological features, cytogenetics and molecular profile, and are often characterized by higher lymphocytosis (17). This suggests that they could arise from a common B cell of the marginal compartment differing only by CD5 expression.

Cases of de novo CD5+ DLBCL, which are not a result of the transformation of B-CLL and MCL, have been reported since 1995 (18). Recent studies show that this group of DLBCL has heterogeneous morphological features (monomorphic, giant cell-rich, polymorphic, immunoblastic), high recurrence of central nervous system involvement, frequent expression of bcl-2 protein, no derivation from germinal center (19).

FL can also express CD5 (20, 21) with a frequency of about 1% (22) and some cases of DLBCL derived from a FL are characterized by this feature (23).

CD10+ NHLs

The CD10 molecule is generally considered as typical of FL. It is a zinc metallopeptidase expressed in many normal tissue cell types, including early lymphoid progenitors and normal germinal center B cells. CD10 expression has been used as a diagnostic marker not only of germinal center-derived B cell lymphomas, but also of germinal center B-cell-like DLBL and Burkitt lymphoma (BL). Aberrant expression of CD10 has also been observed in a variety of non-germinal center B-cell lymphomas, such as HCL, B-CLL/SLL, lymphoplasmacytic lymphoma (LPL), MZL, and MCL. This aberrant expression, when associated with other immunophenotypic anomalies, might be responsible for erroneous diagnoses. For example, peculiar cases of CD10+/CD5- MCL have been described (24), even with cytomorphology arranged in a nodular proliferation pattern with residual follicular dendritic network, closely mimicking low-grade FL (25). In such cases, further criteria should be used to make diagnosis, such as FISH for the specific translocations that characterize MCL and FL.

CD103+ B-NHLs

The molecule CD103, also termed mucosa lymphocyte antigen-MLA, is expressed on almost all intestinal intraepithelial lymphocytes, in about 40% of lamina propria T-lymphocytes in the intestine (26), in the majority of intraepithelial lymphocytes in extra-intestinal sites (27) and by 0.5-5% T-lymphocytes in peripheral blood and peripheral lymphoid tissues. CD103 is also expressed by a subset of dendritic cells (28). Among the B-NHLs, HCL is characterized by the peculiar co-expression of CD103, CD25 and CD11c (29). Thus, MFC assays with a MoAb combinations including this triplet allow easy detection of this subtype of lymphoproliferative disease both in peripheral blood and bone marrow samples. Variant cases of HCL lacking CD103 can be found (30). In such cases, diagnosis has to be confirmed by more specific tests, such as immunohistochemistry for annexin A1 (31). Other phenotypic variant cases can show co-expression of only CD103 and CD11c, lacking CD25. The differential diagnosis includes atypical HCL and CD103+ MZL (32). Annexin A1 has to be tested also in such cases.

CD23+ B-NHLs

CD23 is a low-affinity IgE Fc receptor and a marker of activated B cells. It is expressed very weakly or is absent in resting mature peripheral blood and lymphoid tissue B cells (33). Among CD5+ B-cell lymphoproliferative disorders, expression of the CD23 antigen is useful for distinguishing B-CLL/SLL from MCL. The majority of SLL/B-CLL cases are CD23+, whereas MCL cases usually are CD23–. However, CD23 positivity can be observed in a subset of MCL, and this finding can lead to diagnostic confusion. The frequency of CD23 positivity in MCL is uncertain. In studies carried out by flow cytometry and 2- or 3-color methods, aimed to compare B-CLL/SLL and MCL, an overlapping CD23 staining has been found in up to 45% of cases (34). Thus, although CD23 intensity often is brighter in B-CLL than in MCL, its evaluation to differentiate these two types of B-NHLs seems to be useful only when its expression is negative.

Positivity for CD23 can be found in several cases of FL. A recent report, carried out by flow cytometry in lymph node samples, showed CD23 expression in 70% of cases. This feature was more frequent in cases of grade 1 and 2 and in inguinal lymph nodes, and correlated with a better prognosis (35).

CD23 expression is a frequent finding also in splenic MZL, with up to 50% positive cases in cases with co-expression of CD5 (36). This finding may cause difficulties in the differential diagnosis with B-CLL and additional criteria, such as splenic histology, should be used to this aim.

Other useful molecules

Other markers can be used in the MoAb panels when typing B-NHLs. CD200 (OX2), a transmembrane glycoprotein with immunosuppressive functions, expressed on T-lymphocytes, dendritic cells, several solid tissues and weakly expressed by normal B-cells, can be used to improve diagnosis in B-NHLs. In fact, its overexpression seems to be a constant feature in B-CLL and in HCL, as demonstrated by both flow cytometry (37, 38) and immunohistochemistry (39). Therefore, its use may be useful in differential diagnosis with MCL, CD5+ MZL, variant subtypes of CD103+ lymphomas.

CD43 is another molecule with interesting biological characteristics. It is often considered as a T-cell lineage-associated antigen, but can also be expressed by activated normal B-cells. Among B-NHLs, its expression shows no restriction since it is positive in about 65% B-CLL and MCL (40). However, CD43 can be useful in distinguishing B-CLL/SLL from CD5+ MZL and from CD5+ FL.

The monoclonal antibody FMC7, originally described in 1981, is frequently used in immunophenotypic analysis of B-NLHs, although it has never been clusterized. It recognizes an epitope of CD20, but cannot be used in alternative to this antigen. The epitope recognized by FMC7 is very sensitive to membrane cholesterol amounts and FMC7 expression will be different in cells with the same amount of CD20 molecules, if the cholesterol level varies (41). In addition, FMC7 seems to recognize a glycosylated and multimeric CD20 complex (CD20 is usually a non-glycosylated molecule), which is not shown by all B-NHLs (42).

The FCM7 MoAb is generally used in the differential diagnosis of B-CLL from the other B-NHLs. A significant correlation between CD20 and FMC7 expression has been found in typical B-CLL, in which low levels of both markers are very frequent. In the other B-NHLs both FMC7 and CD20 are detected with higher levels and it has been hypothesized that the particular conformation of CD20 that is recognized by FMC7 is manifested only by cells with high CD20 levels (43).

CD38 is a membrane glycoprotein and is expressed on all pre-B lymphocytes and all plasma cells. During B-cell differentiation, this antigen is expressed in early stages, is lost during the intermediate stages of maturation, and reappears during the final stages of maturation. Measuring CD38 expression in B-CLL provides useful pieces of information about prognosis, since CD38 expression on more than 20-30% of the neoplastic cells seems to be associated with an unfavorable prognosis (44, 45).

ZAP-70 (zeta-associated protein with molecular weight of 70 kD) is a molecule used to transmit a signal from the T-cell receptor to downstream pathways. Most B cells lack ZAP-70 and instead use a related tyrosine kinase, Syk, for signal transduction. A subset of B-CLL with ZAP-70 expression is characterized by unmutated IgVH gene and unfavorable prognosis. The simultaneous assessment of CD38 and ZAP-70 is able to identify different risk classes among B-CLL patients (45). ZAP-70 is an intracellular molecule and for its detection a cell permeabilization method has to be used.

CD45 expression

The common leukocyte antigen, termed CD45, is a family of glycoproteins with phospho-tyrosine phosphatase activity expressed on all cells of hematopoietic origin, except immature nucleated red cells and erythrocytes. CD45 seems to play an important role in the regulation of cell differentiation. Various isoforms of this molecule (CD45RA, CD45RB, CD45RC, CD45RO) can be recognized by specific MoAbs, and the common part of the CD45 molecule is recognized by MoAbs used in routine flow cytometric analysis. CD45 expression varies during B-cell ontogeny (46). B-cell precursors (hematogones) show relatively low levels of CD45, while mature B-cells show higher surface CD45 density (47). It has been found that lymphocytes from typical B-CLL show very low levels of CD45 and that HCL lymphocytes are characterized by the highest CD45 density among the various subtypes of low-grade B-cell NHL (48-50). Lymphocytes from MCL display slightly higher levels of CD45 when compared with B-CLL lymphocytes, and intermediate values of CD45 expression are observed in the other NHL subtypes, such as LPL, MZL and FL (50). Measuring CD45 expression on lymphomatous lymphocytes could be useful to improve diagnosis in atypical cases. In fact, both the absence of CD23 in B-CLL and the presence of CD23 in MCL are characterized by B very high levels of CD45 expression, with a behavior that is clearly different from both typical CLL and typical MCL: these findings should suggest the usefulness of further diagnostic tools (such as cytogenetics and/or PCR for bcl-1/JH translocation) in such cases. Similarly, B-CLL with atypical morphology, which has a negative prognostic impact (51), shows higher CD45 expression than typical CLL cases, suggesting a different maturational level of such neoplastic cells (50).

Clonality assessment

Neoplastic mature B-lymphoid cells can be distinguished from normal cells also by the identification of immunoglobulin light chain class restriction. In contrast to most normal and reactive populations, neoplasms of mature B cells usually represent a single clone of cells that express only one class of immunoglobulin light chain, i.e. K or λ. Therefore, the so-called "K/λ analysis" is one of the technical steps routinely used in B-NLH immunophenotyping. A normal B-cell population shows a K/λ ratio with a very wide range, and K/λ ratios ranging 0.5:1 to 3:1 are generally used in most laboratories. On the contrary, a lymphomatous B-cell population show restriction for either K or λ light chain.

Although often used as a surrogate marker of clonality, light chain class restriction has been reported in nonclonal reactive B-cell populations, such as nonclonal proliferations in tonsillar specimens during childhood and in multicentric Castleman disease (52). Moreover, B-cell clonality has been described in HCV infection, both by PCR assays (53) and by flow cytometry, even in absence of documented B-NHL (54). Therefore, it should not be assumed that immunoglobulin light chain class restriction is synonymous with monoclonality or is by itself diagnostic of neoplasia. Therefore, the

results of FCI should be interpreted in conjunction with other clinical, morphologic, and sometimes genotypic data.

Identification of a large relatively pure population of light chain–restricted B cells is fairly straightforward using FCI, and is usually reflected in an abnormal kappa-lambda ratio. However, evaluation of the K/λ ratio may fail to identify smaller clonal populations admixed with reactive polyclonal B cells. A more sensitive approach for the detection of light chain restriction is the separate evaluation of populations of cells that have a distinct phenotype and/or light scatter characteristics. For example, a different CD19 and/or CD20 expression can be used to differentiate pathologic lymphocytes from the normal population, and K/λ analysis carried out separately is able to detect clonality with more precision. This approach can be used, for example, for the diagnosis of lymphoid neoplasms that have a large number of accompanying reactive cells, as seen in MZL.

Interpretation of staining for K and λ immunoglobulin light chains can be made more difficult by the presence of nonspecific staining. Nonspecific (cytophilic) binding of antibodies can occur through association with Fc receptors and adherence of antibody to damaged or dying cells. Binding of antibodies to non–B cells can be excluded by evaluating only cells that express one or more B-lineage–associated antigens. Adequate technical procedures have to be carried out to minimize nonspecific staining and to avoid false-negative results. Samples have to undergo washings to remove plasma (which is rich of immunoglobulins and "captures" anti-K and anti-λ antibodies) and incubation with blocking reagents, such as heat-inactivated fetal calf serum. Nonspecific staining is frequently encountered in HCL. An apparent negative test can be due to deletion of the immunoglobulin epitope recognized by the antibody (55). Both nonspecific staining and apparent negative results can be minimize by using $F(ab')_2$ polyclonal antibodies directed to K and λ light chains: the lack of the Fc fragment avoids a nonspecific binding to cells, and the polyclonal nature of the antibodies facilitates the binding to several immunoglobulin epitopes.

Clonality assessment by means of K/λ ratio is facilitated by the different behavior of immunoglobulin expression in several B-NHLs in terms of cell surface density. In many cases the surface immunoglobulin density is higher than the normal counterpart and neoplastic lymphocytes can be detected easier. In other instances, typically in B-CLL, the number of surface immunoglobulins is very low and the fluorescence intensity obtained is lower than that observed in normal lymphocytes and in the other B-NHLs (Fig. 2). This phenomenon is responsible for the apparent lack of surface immunoglobulins in several cases of B-CLL and for the impossibility of determining K/λ ratio, and is probably due to a very low expression of all components of the membrane B-cell–receptor complex that includes immunoglobulins, CD20, CD22, and CD79b.

Cases of B-NHLs really lacking K or λ chains because of an absent immunoglobulin expression can be found, with a frequency of about 3% (56), and it has been shown that cases with more than 25% B cells lacking surface immunoglobulins all represented lymphoma (55).

There is some evidence for cases with apparent dual light-chain expression (i.e. simultaneous K and λ chain expression) (56). In the presence of dual light-chain expression, however, a K/λ ratio will likely be a polytypic pattern, depending on the percent dual expressing cell population. However, the percentages of K+/CD19+ plus λ+/CD19+ B cells will exceed the total percent CD19+ B cells. In such cases, if the sum of K+/CD19+ plus λ+/CD19+ lymphocytes is 10% greater than the total percent of CD19+ B-cells, or if the dual light-chain expressing B-cell population comprises more than 10% of the total number of mature cells, clonality is likely and a PCR study for IgH gene rearrangement should be performed.

The interesting phenomenon of dual light-chain expression can be explained by an unusual pattern of immunoglobulin gene rearrangement, which can also occur in normal subjects during B-cell ontogeny (57, 58) and needs a careful evaluation of cytograms and histograms.

Extending immunophenotyping to plasma cells

Some B-NHLs are characterized by plasma cells involvement. It is the case of LPL and Waldenström Disease. LPL is a low-grade B-cell malignancy exhibiting a cytological spectrum of plasmacytic differentiation ranging from small lymphocytes to true plasma cells, and including cells with features intermediate between these two, referred to as plasmacytoid lymphocytes. LPL characteristically

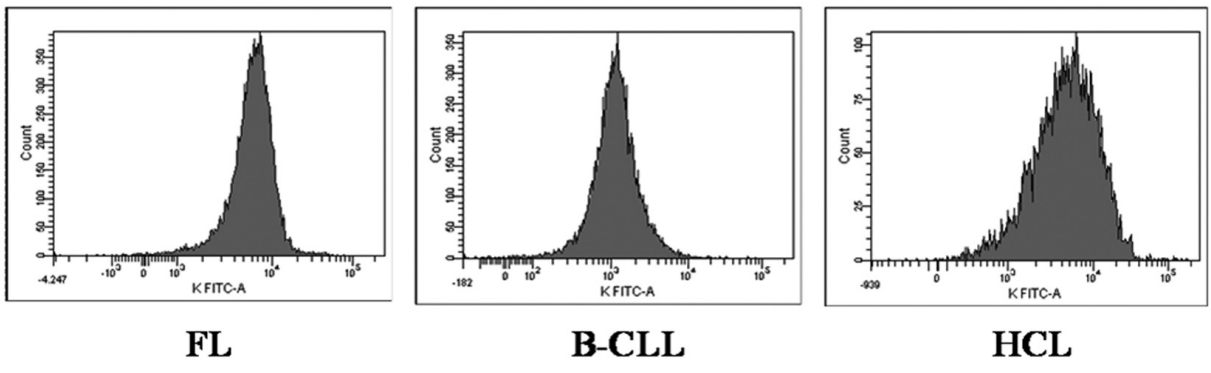

Fig. 2. Different immunoglobulin light chain expression in follicular lymphoma (FL), B-cell chronic lymphocytic leukemia (B-CLL) and hairy cell leukemia (HCL).

has an associated monoclonal serum immunoglobulin M (IgM) paraprotein (sometimes an IgG or IgA monoclonal component), although neither the paraprotein isotype nor the quantity reliably distinguishes this type of B-NHL from other lymphoma types. In a subset of LPL patients, the paraproteinemia is associated with distinctive clinical findings, such as serum hyperviscosity, cryoglobulinemia, autoimmune phenomena such as peripheral neuropathy, and amyloidosis. This latter type of lymphoma is called Waldenström Disease.

In these B-NHLs both lymphocytes and plasma cells show clonality (60, 61). When studying the plasma cell population, however, permeabilization techniques have to be carried out to detect immunoglobulin light and/or heavy chains, because in such cells they are located into the cytoplasm. MFC with at least 6 fluorescence is able to perform a simultaneous evaluation of both pathologic cell populations (61). Immunophenotyping appears of great usefulness not only in the diagnostic phase, but also in the evaluation of response to therapy, since a different sensitivity towards therapy can be shown by lymphocytes and plasma cells.

Multiparameter flow cytometry in composite, discordant, biclonal and collision lymphomas

"Composite lymphomas" are particular clinical entities characterized by the occurrence of two or more morphologically and immunophenotypically distinct lymphoma clones in a single anatomical site, i.e. within a single organ or tissue (62). Such cases have to be differentiated from the so-called "discordant lymphomas", in which two distinct lymphomas involve different anatomical sites. MFC is very useful in such cases, and its diagnostic role appears of particular relevance in composite lymphoma, since the simultaneous staining with several monoclonal antibodies allows a clear separation of the two pathologic clones in most cases. This is particularly true when a different clonality is found (Fig. 3). In alternative, a different expression of B-cell markers provides similar results.

A third peculiar behavior is represented by biclonal B-NHLs. In these cases a single lymphoma, with coherent morphology and monomorphous B-cell-associated markers behavior, shows biclonality, with two distinct cell populations after K/λ analysis. In such cases, confirmation by PCR assays for IgH rearrangement is needed to detect the two distinct patterns. Rare case can show more than two different clones within an apparently single case of lymphoma (63).

The overall incidence of composite and biclonal B-NHLs might be estimated about 5% and is more frequent in HCL, DLBCL and atypical B-CLL (63).

Another peculiar occurrence is represented by a multidirectional pathways of B-NLHs, with the rare occurrence of "collision lymphomas" (64). In such conditions two different clones of an identical type of B-NHL are found together with other B-cell diseases, such as multiple myeloma. MFC is a powerful tool to differentiate different B-cell clones (for example, by means of K/λ analysis), in association with morphology and immunohistochemistry.

The existence of the above peculiar presentations of B-NHLs emphasizes the necessity of using wide MoAb panels and of studying the immunophenotypic profile in more than one anatomical site. In fact, immunophenotypic discrepancies between two different anatomical sites in the same patients can occur in up to 22% of cases (65). In some cases, there are concordant morphological features and discordant immunophenotype, but in other cases both morphology and immunophenotype are different.

The different combinations of B-NHLs so far reported in the literature include FL and B-CLL/SLL, SLL and DLBCL, MCL and MZL, FL, and MCL, FL and DLBCL (62, 66). Possible hypotheses for the occurrence of such clinical presentations of B-NHLs include clonal selection, genomic instability and genetic predisposition, a common precursor cell.

Planning diagnostic panels

Flow cytometers currently available for diagnostic purposes are equipped with 2 or 3 lasers. Depending on number and type of lasers and on the dedicated software, it is possible perform 6 to 8 fluorescence analyses. Therefore, the current MoAb panels are more complex than in the past and allow detecting pathologic B-cell populations with more precision and sensitivity. The diagnostic power of MFC is particularly evident when very small pathologic cell populations have to be investigated in minimal residual disease setting after aggressive chemotherapy and stem cell transplantation.

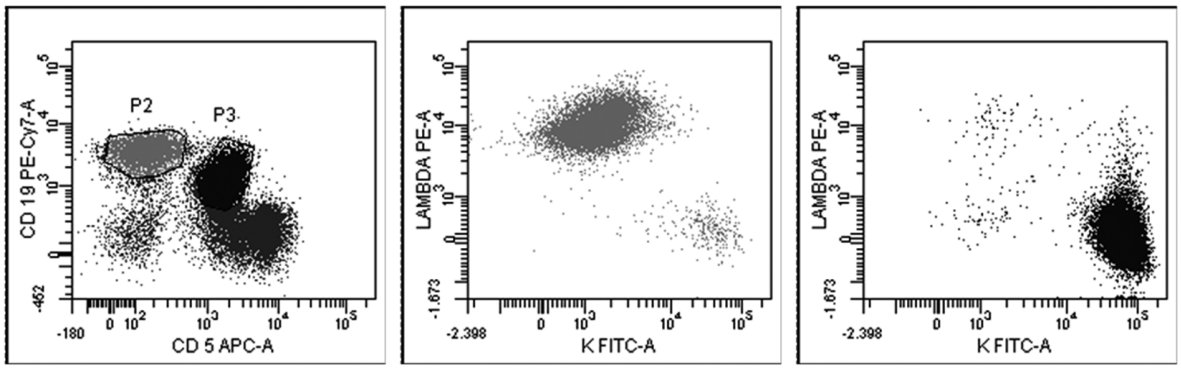

Fig. 3. A case of composite lymphoma (bone marrow aspirate). The population P2 (CD19+CD5-) shows restriction for surface lambda chain (grey), while the population P3 (CD19+CD5+) displays restriction for surface K chain (black).

Table 1. Example of a diagnostic panel with 6 to 8 fluorescences.

	FITC	PE	PerCP-Cy5.5	PE-Cy.7	APC	APC-Cy.7	Am-Cyan	Pacific Blue
Tube 1	CD38	CD5	CD3	CD19	CD16/56	CD8	CD45	CD8
Tube 2	FMC7	CD22	CD20	CD5	CD23	CD19	CD45	
Tube 3	K	Lambda	CD20	CD5	CD10	CD19	CD45	
Tube 4	CD103	CD200	CD20	CD25	CD11c	CD19	CD45	
Tube 5	CD5	ZAP-70	CD3	CD19	CD38	CD45		

The proposed MoAb panel is designed for cytometers equipped with three lasers (405, 488 and 633 nm). Tube 5 can be used to obtain a simultaneous staining of CD38 and ZAP-70 in B-CLL cases at diagnosis

When several MoAbs are assembled together to organize the current diagnostic panels, a limited number of tubes have to be prepared, thus reducing sample processing costs.

Different MoAb panels are organized in every single laboratory, depending on experience of the observers, typology of instrumentation, availability of laboratory reagents, analysis technique. An example of a 7- and 8-color MoAb panel that can be used by a FacCanto II cytometer equipped with three lasers is shown in Table 1.

The proposed panel, which uses only 4 tubes, is able to: analyze T, B and NK populations in the same tube, identifying an eventual CD19+/CD5+ B-cell population and its eventual CD38 expression; detect an eventual aberrant expression of CD4 or CD8 by B-cell, as can occur in some B-NHLs (67); measure co-expression of several B-cell-associated markers and detect two or more B-cell subsets; measure K/λanalysis of different B-cell populations identified by separate gates; detecting B-cells with HCL-like phenotype; detect hematogones, which percentage can be calculated separately from more mature normal B-cells and from pathologic lymphocytes.

The proposed panel is only an example among several possible way to assemble the diagnostic reagent. Depending on sample characteristics and/or specific necessities, additional panels can be organized. For example, in B-CLL additional tubes can be prepared for a better determination of CD38 expression, for ZAP-70 expression, for a more extensive analysis of cell phenotype (CD43, CD79b, etc.).

Specific MoAb panels have to be organized in order to detect minimal residual disease. This is particularly true in the case of B-CLL. It has been demonstrated that a MoAb combination containing CD5, CD19, CD20, CD38, CD81, CD22, CD79b and CD43, analyzed using a sequential gating strategy, is able to detect minimal residual disease between 0.1-0.01% with low inter-laboratory variation and false detection rates. Experienced operators demonstrated an accuracy of 95.7% (specificity 98.8%, sensitivity 91.1%). MFC showed close correlation and 95% concordance with RQ-ASO IgH-PCR for detection of B-CLL above 0.01% (68).

Immunophenotype and diagnosis of B-cell lymphomas

Immunophenotyping is only one of the diagnostic step in a case of B-NHLs. Morphology, immunohistochemistry, conventional genetics and molecular genetics has to be used together in order to establish a correct diagnosis. Although several cases of B-NHLs display a highly suggestive phenotypic profile, many B-cell lymphomas cannot be fully subclassified by peripheral blood bone marrow immunophenotyping alone because no singular disease entity–specific antigen expressed on the cell surface has been identified.

In the past, there has been a clear tendency to subclassify B-cell lymphomas based on either peripheral blood or bone marrow immunophenotyping alone or in combination with bone marrow histopathology.

As far as the role of FCI in the classification of mature B-cell lymphoid neoplasms is concerned, most of investigators consider 4 broad groups as determined by their expression of CD5 and CD10: CD5+/CD10-, CD5-/CD10+, CD5+/CD10+, and CD5-/CD10-. For each group, additional flow cytometric data in combination with the morphology can narrow down the diagnostic possibilities and direct the use of additional ancillary studies (1).

B-cell lymphoid neoplasms positive for CD5 and negative for CD10 include most patients with CLL/SLL and MCL, some patients with B-PLL, up to 20-25% of cases of MZL, some cases of DLBCL and FL, and possibly some patients with LPL. In addition, it should be remembered that CD5 is expressed on a population of normal B cells and therefore it should not be used in isolation to establish the presence of a neoplasm.

DLBCL and FL represent the most frequent CD10+, CD5- mature B-cell lymphoid neoplasms, followed by BL. CD10+ HCL is uncommon but can be easily overlooked if the appropriate antibody combinations are not included in an initial flow cytometric screening panel. CD10 expression in other types of lymphoma is unusual with only a few reports of CD10+ LPL, and very rare CD10+ MZL and MCL. It should also be remembered that CD10 is also expressed by normal follicular center B cells, precursor B-lymphoblastic leukemia/lymphomas, subsets of mature T cells, precursor T-cell lymphoblasts, neutrophils, and some non-hematolymphoid cells.

Mature B-cell neoplasms lacking expression of CD5 and CD10 represent a diverse group that includes DLBCL, MZL, HCL, LPL, CD10- FL, and CD5- MCL. Further classification usually requires correlation with morphology and often additional ancillary studies.

Mature B-cell lymphoid neoplasms expressing both CD5 and CD10 are uncommon. This group includes several different subtypes of lymphoma (in order of incidence): DLBCL, FL, MCL, CLL/SLL, BL, and rare individual reports of other mature B-cell malignancies.

Because of an evident overlap of membrane marker expression in a significant percentage of cases, in the past two

scoring systems were proposed, with the aim to differentiate the most frequent B-NHLs on the basis of the expression of selected markers (69-72). These scoring systems included the MoAb FMC7, which was considered as a very sensitive marker for distinguishing B-CLL from the other B-NHLs.

In a more recent paper, Morice et al (73) evaluated the effectiveness of peripheral blood and bone marrow immunophenotyping in predicting the histologic B-cell lymphoma type in 252 patients, using a four-color method. The surface density of immunoglobulins and CD20 was a fundamental element of histogram and cytogram evaluation. In a second step, the fluorescence intensity of CD5 and CD23 were considered as important variables, and the pattern of their expression was taken into special consideration. CD10 and CD103 staining were additional variables and, for CD103, dual CD11c/CD22 expression was checked for.

Seven immunophenotypic patterns were detected: prototypic B-CLL, prototypic MCL, CD5+, nonspecific, partial CD5+, CD10+, prototypic HCL, B-NHL non specific (CD5-, CD10- and CD103-). CD5+ accounted for 69% of cases: prototypic B-CLL and MCL patterns yielded 96% e 99% specificity values respectively, and 82% and 56% sensitivity values, respectively. The positive predictive value of CD5+ nonspecific and partial CD5+ pattern was 88% and 42%, respectively. Among 175 CD5+ cases, 55 were clustered into these two latter patterns. Both the B-CLL and the MCL phenotypes had highly predictive value only when all expected features are exhibited. Indeed, the CD5+ nonspecific category contained nearly equivalent numbers of B-CLL and MCL cases, without any possibility of distinguishing between the two possibilities because of overlapping features. Cases with partial CD5+ pattern showed nonspecific immunophenotype, compatible con all B-NLHs.

Table 2 summarizes the various relevant immunophenotypic patterns that can be detected by MFC. Some patterns are highly suggestive for specific subtypes of B-NHLs, but in a non-negligible percentage of cases diagnosis must be established by a combination of parameters that can be detected by other techniques, such as morphology, immunohistochemistry, FISH and PCR assays for specific translocations.

It is interesting to notice that some immunophenotypic patterns are incompatible with specific B-NHLs subtypes. For example: CD5-/CD10+/CD23+ or CD5+/CD10+/CD23- and B-CLL; CD5+/CD10-/CD23+ and FL; CD5+/CD10-/CD23- and BL; CD5+/CD10+/CD23- or CD5-/CD10+/CD23+ and B-PLL; CD5+/CD10+/CD23- and MZL (74).

Conclusions

The laboratory diagnosis of non B-NHls is usually accompanied by immunologic evaluation by immunohistochemistry and/or multiparameter flow cytometry. Other techniques, such as conventional karyotyping, FISH, gene rearrangement studies for the IgH gene and/or specific translocations are often necessary. It should be clear that flow cytometry and other ancillary techniques serve an adjunctive role in the diagnosis and classification of hematopoietic neoplasms.. Although MFC offers many advantages in comparison to immunohistochemistry in the diagnosis and classification of B-NHLs, it can also be used as a complementary rather than a competitive technique for this role. Despite its widespread use, however, proper use of antibodies and interpretation of flow cytometric data can be challenging not only for individuals with limited experience but also for flow technologists and pathologists well versed in this technique.

Table 2. Immunophenotypic patterns detected by multiparameter flow cytometry and correlation with B-NHLs subtypes.

IMMUNOPHENOTYIC PATTERN	Most probable diagnosis	Differential diagnosis	Additional diagnostic information
CD5+, CD20^{dim},sIg^{dim},CD23+,CD200+, CD22^{dim},FMC7-,CD10-	B-CLL (>80% of cases)	MZL, LPL, DLBCL	Morphology, cyIg (LPL)
CD5+,CD20^{int},CD22^{int},CD23-/+, FMC7+/-, sIg+,CD200-,CD10-	**MCL**	**Other CD5+ B-NHLs**	**Morphology, cyclin-D1 [BCL-1/JH, t(11;14)]**
CD5+,CD20^{bright},CD22+,CD23-/^{het}, sIg$^{dim/int}$,FMC7+/-,	Nonspecific	B-CLL, MCL, B-PLL, other B-NHLs	Morphology, CD200, cyclin-D1, CD103
CD5+^{het},CD20+,CD23-/+,CD200-,sIg+, CD22+	Nonspecific	Various B-NHLs	Morphology, t(1;14), t(14;18)/MALT-1, t(11;18),
CD10+,CD5-,CD19^{dim},CD20+,CD22+, sIg+	FL	DLBCL, BL, LPL	Morphology, BCL-2, c-MYC (BL), t(14;18), CD43 (BL)
CD10+,CD5+,CD20+,sIg+,CD19$^{+/dim}$	Rare cases of FL, MCL, BL	Rare cases of HCL	Morphology, BLC-2, c-MYC, t(14;18), cyclin-D1, CD103
CD103+,CD11c+,CD25+,CD19+, CD20^{bright},CD22+,CD200+, sIg+	HCL		Morphology, Annexin-A1
CD103+,CD11c+,CD25-,CD20+, CD22+,CD19+,CD200-,sIg+	HCL variant, MZL		Morphology, Annexin-A1, t(1;14), t(14;18)/MALT-1, t(11;18)
CD5-,CD10-,CD19+,CD20+,CD22+, sIg+,CD103-,CD200-,CD11c+/-	Nonspecific	DLBCL, rare cases of MCL, FL, MZL, HCL	Morphology, Annexin-A1, BCL-2, cyclin-D1, t(14;18)/MALT-1

Dim: low florescence levels; bright: high fluorescence levels; int: intermediate fluorescence levels; het: heterogeneous fluorescence distribution; +/-: combination of various possible patterns of fluorescence (negative, positive, heterogeneous); sIg: surface immunoglobulins and/or immunoglobulin light chain (either K or λ).

Several well-done papers published in the past, reporting data obtained with 3- or 4-color flow cytometry, showed a good correlation with data obtained with bone marrow trephine biopsies (76-79). A significant percentage of cases, however, showed discordant results between histology and immunophenotyping, suggesting the necessity of performing PCR assays and other genetic tests to establish a correct diagnosis. It is possible that flow cytometric panels with 6-8 colors might improve sensitivity and specificity of flow cytometry in this setting (Carulli G, unpublished).

Flow cytometry shows high sensitivity and appears to be of particular usefulness in the detection of residual disease in some instances, such as B-CLL. The application of new MoAb panels including more sensitive combinations, aimed to detect antigenic patterns highly suggestive of lymphoid neoplasia, is possible with the more advanced cytometers now available. The combination of clonality and the simultaneous expression of several cell markers, giving rise to prototypic immunophenotypic patterns, seems to be able to improve accuracy of flow cytometry.

The ability of MFC to detect not only surface but also intracellular and intranuclear molecules such as receptors, enzymes, nucleic acid and protein products of specific genes is likely to lead to novel diagnostic and prognostic applications of this technique for several possible future uses in lymphoma management. Such applications might range from detection of novel specific surface, intracellular, or intranuclear protein products relevant in diagnosis, classification, or prognostication, to evaluating enzyme activities for cell kinetics and tumor cell metabolism and response to drugs.

References

1. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood 2008; 111:3941-67
2. Wood B. 9-color and 10-color flow cytometry in the clinical laboratory. Arch Pathol Lab Med 2006; 130:680-90
3. Davis BH, Holden JT, Bene MC, et al. 2006 Bethesda International Consensus recommendations on the flow cytometric immunophenotypic analysis of hematolymphoid neoplasia: medical indications. Cytometry B Clin Cytom 2007; 72B: S5-S13
4. Jaffe ES. The 2008 WHO classification of lymphomas: implications for clinical practice and translational research. Hematology Am Soc Hematol Educ Program 2009; 523-31
5. Yang W, Agrawal N, Patel J, et al. Diminished expression of CD19 in B-cell lymphomas. Cytometry Part B 2005; 63B: 28-35
6. Masir N, Marafioti T, Jones M, et al. Loss of CD19 expression in B-cell neoplasms. Histopathology 2006; 48:239-46
7. Huh YO, Keating MJ, Saffer HL, et al. Higher levels of surface CD20 expression on circulating lymphocytes compared with bone marrow and lymph nodes in B-Cell chronic lymphocytic leukemia. Am J Clin Pathol 2001; 116:437-43
8. Terui Y, Mishima Y, Sugimura N, et al. Identification of CD20 C-terminal deletion mutations associated with loss of CD20 expression in non-Hodgkin's lymphoma. Clin Cancer Res 2009; 15:2523-30
9. Geisberger R, Lamers M, Achatz G. The riddle of the dual expression of IgM and IgD. Immunol 2006; 118:429-37
10. McCarron KF, Hammel JP, His ED. Usefulness of CD79b expression in the diagnosis of B-cell chronic lymphoproliferative disorders. Am J Clin Pathol 2000; 113:805-13
11. D'Arena G, Musto P, Cascavilla N, et al. Quantitative flow cytometry for the differential diagnosis of leukemic B-cell chronic lymphoproliferative disorders. Am J Hematol 2000; 64:275-81
12. Zomas AP, Matutes E, Morilla R, et al. Expression of the immunoglobulin-associated protein B29 in B cell disorders with the monoclonal antibody SN8 (CD79b). Leukemia 1996; 10:1966-70
13. Dunphy CH. Mature B-cell neoplasms. In: Dunphy CH (ed). Integrated hematopathology. American Society for Clinical Pathology Press, 2010;136-43
14. Matutes E, Morilla R, Owusu-Ankomah K, et al. The immunophenotype of splenic lymphoma with villous lymphocytes and its relevance to the differential diagnosis with other B-cell disorders. Blood. 1994; 83:1558-62
15. Troussard X, Valensi F, Duchayne E, et al. Splenic lymphoma with villous lymphocytes: clinical presentation, biology and prognostic factors in a series of 100 patients. Groupe Francais d'Hématologie Cellulaire (GFHC). Br J Haematol 1996; 93:731-6
16. Giannouli S, Paterakis G, Ziakas PD, et al. Related splenic marginal zone lymphomas with peripheral CD5 expression. Haematol 2004; 89:113-14
17. Baseggio L, Traverse-Glehen A, Petinataud F, et al.. CD5 expression identifies a subset of splenic marginal zone lymphomas with higher lymphocytosis: a clinico-pathological, cytogenetic and molecular study of 24 cases. Haematol 2010; 95:604-12
18. Matolcsy A, Chadburn A, Knowles DM. De novo CD5-positive and Richter's syndrome-associated diffuse large B cell lymphomas are genotypically distinct. Am J Pathol 1995; 147:207-26
19. Yamaguchi M, Nakamura N, Suzuki R, et al. De novo CD5+ diffuse large B-cell lymphoma: results of a detailed clinicopathological review in 120 patients. Haematologica 2008; 93:1195-202
20. Tiesinga JJ, Wu CD, Inghirami G. CD5+ follicle center lymphoma: immunophenotyping detects a unique subset of "floral" follicular lymphoma. Am J Clin Pathol 2000; 114: 912-21
21. Dong HY, Gorczyca W, Liu Z, et al. B-cell lymphomas with coexpression of CD5 and CD10. Am J Clin Pathol 2003; 119: 218-30
22. Barry TS, Jaffe ES, Kingma DW, et al. CD5+ follicular lymphoma: a clinicopathologic study of three cases. Am J Clin Pathol 2002; 118:589-98
23. Manazza AD, Bonello L, Pagano M, et al. Follicular origin of a subset of CD5+ diffuse large B-cell lymphomas. Am J Clin Pathol 2005; 124:182-90
24. Morice WG, Hodnefield JM, Kurtin PJ, et al. An unusual case of leukemic mantle cell lymphoma with a blastoid component showing loss of CD5 and aberrant expression of CD10. Am J Clin Pathol 2004; 122:122-7
25. Zapata M, Budnick SD, Bordoni R, et al. An uncommon case of de novo CD10+ CD5- mantle cell lymphoma mimics follicle center B cell lymphoma. Int J Clin Exp Pathol 2010; 3: 430-6
26. Junker Y, Bode H, Wahnschaffe U, et al. Comparative analysis of mononuclear cells isolated from mucosal lymphoid follicles of the human ileum and colon. Clin Exp Immunol 2009; 156:232-7

27. Nestle FO, Di Meglio P, Qin ZJ, et al. Skin immune sentinels in health and disease. Nat Rev Immunol 2009; 9:679-91
28. Del Rio ML, Bernhardt G, Rodriguez-Barbosa JI, et al. Development and functional specialization of CD103+ dendritic cells. Immunol Rev 2010; 234:268-81
29. Wanko SO, De Castro C. Hairy Cell Leukemia: an elusive but treatable disease. The Oncologist 2006; 11:780-9
30. Chen Y-H, Tallman MS, Goolsby C, et al. Immunophenotypic Variations in Hairy Cell Leukemia. Am J Clin Pathol 2006; 125:251-9
31. Falini B, Tiacci E, Basso K, et al. Simple diagnostic assay for hairy cell leukaemia by immunocytochemical detection of annexin A1 (ANXA1). Lancet 2004; 363:1869-70
32. Dong HY, Weisberger J, Liu Z, et al. Immunophenotypic analysis of CD103+ B-lymphoproliferative disorders: hairy cell leukemia and its mimics. Am J Clin Pathol 2009; 131: 586-95
33. Chan MA, Gigliotti NM, Matangkasombut P, et al. CD23-mediated cell signaling in human B cells differs from signaling in cells of the monocytic lineage. Clin Immunol 2010; 137:330-6
34. Gong JZ, Lagoo AS, Peters D, et al. Value of CD23 determination by flow cytometry in differentiating mantle cell lymphoma from chronic lymphocytic leukemia/small lymphocytic lymphoma. Am J Clin Pathol 2001; 116:893-7
35. Olteanu H, Fenske TS, Harrington AM, et al. CD23 expression in follicular lymphoma: clinicopathologic correlations. Am J Clin Pathol 2011; 135:46-53
36. Kojima M, Sato E, Oshimi K, et al. Characteristics of CD5-positive splenic marginal zone lymphoma with leukemic manifestation; clinical, flow cytometry, and histopathological findings of 11 cases. J Clin Exp Hematopathol 2010; 50: 107-12
37. Palumbo GA, Parrinello N, Fargione G, et al. CD200 expression may help in differential diagnosis between mantle celle lymphoma and B-cell chronic lymphocytic leukemia. Leuk Res 2009; 33:1212-6
38. Brunetti L, Di Noto R, Abate G, et al. CD200/OX2, a cell surface molecule with immuno-regulatory function, is consistently expressed on hairy cell leukaemia neoplastic cells. Br J Haematol 2009; 145:665-7
39. Dorfman DA, Shahsafaei A. CD200 (OX-2 membrane glycoprotein) expression in b cell-derived neoplasms. Am J Clin Pathol 2010; 134:726-33
40. Contos MJ, Kornstein MJ, Innes DJ, et al. The utility of CD20 and CD43 in subclassification of low-grade B-cell lymphoma on paraffin sections. Mod Pathol 1992; 5:631-3
41. Deans JP, Polyak MJ. FMC7 is an epitope of CD20. Blood 2008; 111:2942
42. Serke S, Schwaner I, Yordanova M, et al. Monoclonal antibody FMC7 detects a conformational epitope on the CD20 molecule: evidence from phenotyping after Rituxan therapy and transfectant cell analyses. Cytometry 2001; 46:98-104
43. Delgado J, Matutes E, Morilla AM, et al. Diagnostic significance of CD20 and FMC7 expression in B-cell disorders. Am J Clin Pathol 2003; 120:750-9
44. Del Poeta G, Maurillo L, Venditti A et al. Clinical significance of CD38 expression in chronic lymphocytic leukemia. Blood 2001; 98:2633-9
45. Shanafelt TD. Predicting clinical outcome in CLL: how and why. Hematology Am Soc Hematol Educ Program 2009: 421-9
46. Shah VO, Civin CI, Loken MR. Flow cytometric analysis of human bone marrow. IV. Differential quantitative expression of T-200 common leukocyte antigen during normal hemopoiesis. J Immunol 1988; 140:1861-7
47. Caldwell C-W, Patterson W-P. Relationship between CD45 antigen expression and putative stages of differentiation in B-cell malignancies. Am J Hematol 1991; 36:111-5
48. Hendricks A, Bossuyt X. Quantification of the leukocyte common antigen (CD45) in mature B-cell malignancies. Cytometry 2001; 46:336-9
49. Maljaei SH, Asvadi-e-Kermani I, Eivazi-e-Ziaei J, et al. Usefulness of CD45 density in the diagnosis of B-cell chronic lymphoproliferative disorders. Indian J Med Sci 2005; 59: 187-94
50. Carulli G, Cannizzo E, Zucca A, et al. CD45 expression in low-grade B-cell non-Hodgkin's lymphomas. Leuk Res 2007; 263-7
51. Vallespì T, Monserrat E, Sanz MA. Chronic lymphocytic leukaemia: prognostic value of lymphocyte morphological subtypes: a multivariate analysis in 146 patients. Br J Haematol 1991; 77:478–85.
52. Kroft SH. Monoclones, monotypes, and neoplasia pitfalls in lymphoma diagnosis. Am J Clin Pathol 2004; 121:457-9
53. Vallat L, Benhamou Y, Gutierrez M, et al. Clonal B cell populations in the blood and liver of patients with chronic hepatitis C virus infection. Arthritis Rheum 2004; 50:3668-78
54. Ohtsubo K, Sata M, Kawaguchi T, et al. Characterization of the light chain-restricted clonal B cells in peripheral blood of HCV-positive patients. Int J Hematol 2009; 89:452-9
55. Li S, Eshleman JR, Borowitz MJ. Lack of surface immunoglobulin light chain expression by flow cytometric immunophenotyping can help diagnose peripheral B-cell lymphoma. Am J Clin Pathol 2002; 118:229-34
56. Kaleem Z, Zehnbauer BA, White G, et al. Lack of expression of surface immunoglobulin light chains in B-cell non-Hodgkin lymphomas. Am J Clin Pathol 2000; 113:399-405
57. Xu D. Dual surface immunoglobulin light-chain expression in B-cell lymphoproliferative disorders. Arch Pathol Lab Med 2006; 130:853-6
58. Liu D, Jenab J, Rosenberg N. Kappa and lambda rearrangement occur simultaneously in transformed pre-B cells. J Immunol 1997; 159:6061-9
59. Pauza ME, Rehmann JA, LeBien TW. Unusual patterns of immunoglobulin gene rearrangement and expression during human B cell ontogeny: human B cells can simultaneously express cell surface kappa and lambda light chains. J Exp Med 1993; 178:139-49
60. Konoplev S, Medeiros LJ, Bueso-Ramos CE, et al. Immunophenotypic profile of lymphoplasmacytic lymphoma/Waldenström macroglobulinemia. Am J Clin Pathol 2005; 124:414-20
61. Morice WG, Chen D, Kurtin PJ, et al. Novel immunophenotypic features of marrow lymphoplasmacytic lymphoma and correlation with Waldenström's macroglobulinemia. Mod Pathol 2009; 22:807-16
62. Mokhtar NM. Review Article. Composite Lymphoma. Journal of the Egyptian Nat Cancer Inst 2007; 19:171-5
63. Sanchez M-L, Almeida J, Gonzales D, et al. Incidence and clinicobiologic characteristics of leukemic B-cell chronic lymphoproliferative disorders with more than one B-cell clone. Blood 2003; 102:2994-3002
64. Wang HY, Karandikar N, Payne D, et al. A 3-way collision tumor of the upper respiratory tract: A composite of 2 immunophenotypically distinct mantle cell lymphomas and a plasmacytoma. Hum Pathol 2008; 39:781-7

65. Onciu M, Berrak SG, Medeiros LJ, et al. Discrepancies in the immunophenotype of lymphoma cells in samples obtained simultaneously from different anatomic sites. Am J Clin Pathol 2002; 117:644-50
66. Cannizzo E, Sogani AR, Ferry JA, et al. Carcinoma and multiple lymphomas in one patient: establishing the diagnoses and analyzing risk factors. J Hematopathol 2009; 2:163-70
67. Carulli G, Stacchini A, Marini A, et al. Aberrant expression of CD8 in B-cell non-Hodgkin lymphoma. A multicenter study of 951 bone marrow samples with lymphomatous infiltration. Am J Clin Pathol 2009; 132:186-90
68. Rawstron AC, Villamor N, Ritgen M, et al. International standardized approach for flow cytometric residual disease monitoring in chronic lymphocytic leukaemia. Leukemia 2007; 21:956-64
69. Matutes E, Owusu-Ankomah K, Morilla R, et al. The immunological profile of B cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 1994; 8:1640-5
70. Moreau EJ, Matutes E, A'Hern RP, et al. Improvement of the chronic lymphocytic leukemia scoring system with the monoclonal antibody SN8 (CD79b). Am J Clin Pathol 1997; 108:378-82
71. Matutes E, Polliack A. Morphological and immunophenotypic features of chronic lymphocytic leukemia. Rev Clin Exp Hematol. 2000; 4:22-47
72. Cro L, Guffanti A, Colombi M, et al. Diagnostic role and prognostic significance of a simplified immunophenotypic classification of mature B cell chronic lymphoid leukemias. Leukemia 2003; 17:125-32
73. Morice WG, Kurtin PJ, Hodnefield JM, et al. Predictive value of blood and bone marrow flow cytometry in B-cell lymphoma classification: comparative analysis of flow cytometry and tissue biopsy in 252 patients. Mayo Clin Proc 2008; 83:776-85
74. Kaleem Z. Flow cytometric analysis of lymphomas. Current status and usefulness. Arch Pathol Lab Med 2006; 130: 1850-8
75. Gomyo H, Shimoyama M, Minagawa K, et al. Morphologic, flow cytometric and cytogenetic evaluation of bone marrow involvement in B-cell lymphoma. Haematologica 2003; 88: 1358-65
76. Sah SP, Matutes E, Wotherspoon AC, et al. A comparison of flow cytometry, bone marrow biopsy, and bone marrow aspirates in the detection of lymphoid infiltration in B cell disorders. J Clin Pathol 2003; 56:129-32
77. Stacchini A, Demurtas A, Godio L, et al. Flow cytometry in the bone marrow staging of mature B-cell neoplasms. Cytometry 2003; 54B:10-8
78. Carulli G, Canigiani S, Volpini M, et al. Bone marrow infiltration in B-cell non-Hodgkin's lymphomas: comparison-between flow cytometry and bone marrow biopsy. Rec Prog Med 2005; 96:284-90
79. Carulli G, Orciuolo E, Cannizzo E, et al. Combination of morphology, flow cytometry and PCR assay to detect bone marrow infiltration in B-cell non-Hodgkin's lymphomas. Clin Ter 2010; 161:253-8