Immunophenotyping of lymphoproliferative disorders: state of the art

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Summary
Immunophenotyping was introduced into diagnostic pathology over 30 years ago to assist in the diagnosis and classification of lymphoproliferative disorders. Today the role of immunophenotyping has been expanded beyond this to include the detection of markers of prognosis, determination of disease phenotypes associated with specific chromosomal abnormalities, detection of targets for immunotherapy and to monitor residual disease. Immunoperoxidase detection methods remain the most popular in histopathology, whilst flow cytometry is most commonly applied for haematological samples. The range of monoclonal antibodies available, including those which work in routinely performed tissue specimens, continues to increase. This is in part a result of gene expression studies identifying precise genetic signatures for certain lymphoproliferative disorders and the generation of new protein markers to gene products of upregulated genes. This review summarises the current status and applications of immunophenotyping in the assessment of many of the lymphoid malignancies.

Key words: Phenotype, immunohistochemistry, flow cytometry, lymphoproliferative, lymphoma.

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INTRODUCTION
Immunophenotyping characterises cellular antigen expression by their ability to bind antibodies. This has an essential role in the diagnosis and classification of lymphoproliferative disorders. Although immunophenotyping has been available since the 1970s, it is still an evolving technique. Both new antibodies, which are being added on an almost weekly basis, and new applications are appearing. The recent development of gene expression profiling, or microarray studies, has shown that some disorders have specific genetic signatures. Monoclonal antibodies are now being developed to the products of those genes that are upregulated in neoplastic disorders, and novel immunophenotypical markers are thereby being created. These new antibodies will enable further refinement of the classification of lymphoproliferative disorders and help us gain important prognostic information by identifying biologically distinct subgroups. In addition, new therapeutic targets may be identified.

The applications of immunophenotyping therefore have expanded beyond diagnosis and disease classification to predicting prognosis, detecting therapeutic targets and disease monitoring. Phenotypical profiles of neoplastic lymphoid cells are increasingly being used as surrogate markers of genetic abnormalities related to malignant transformation. Phenotyping is also being applied to assess whether a neoplastic cell expresses a specific target antigen for immunotherapy (i.e., monoclonal antibodies bound to cytotoxic agents). During and after treatment of a lymphoid malignancy, minimal residual disease activity may be assessed by following the expression of a particular abnormal immunophenotypical profile. These novel applications highlight the increasing importance of immunophenotyping in the analysis of lymphoproliferative disorders.

Immunophenotyping of lymphoproliferative disorders, be it by flow cytometry or immunohistochemistry, therefore has a number of roles including:

1. Determining the B- or T-cell lineage of an abnormal lymphoid population.
2. Determining the clonality of lymphoid proliferations, especially those of B-cell origin.
3. Determining whether lymphoid cells have a phenotype associated with a specific disorder or a particular chromosomal abnormality.
4. Establishing the phenotype of neoplastic lymphoid cells which may be useful for monitoring residual disease.
5. Assessing the expression of specific molecules which may be targets for immunotherapy (e.g., CD20).
6. Assessing cell proliferation and turnover rate.
7. Detection of markers which may assist in determining prognosis.

In this review, some general comments will be made on immunophenotyping methods, followed by a more extensive discussion of those lymphoproliferative disorders with distinctive phenotypical features. We have not set out to give an exhaustive list of data on the immunophenotype of all lymphoproliferative disorders or all methods available for detecting antigen expression. Data are presented on some of the newer antibodies that have been shown, at least in initial studies, to have prognostic significance or to be useful targets for immunotherapy. We accept that, in this rapidly evolving field, some conflicting data are emerging, especially in regard to the prognostic significance of some novel immunophenotypical profiles.
IMMUNOPHENOTYPING METHODS

Immunophenotyping was first described in 1941 by Albert Coons, who demonstrated the use of a fluorescence labelled antibody to localise cellular antigens in tissue sections. This immuno-fluorescence technique began to be used in leukaemia diagnosis in the 1970s, but was not universally applicable to routine diagnostic laboratories as they required fluorescence microscopy or large flow cytometers. These drawbacks were largely overcome by the development of immunoenzymatic techniques using a number of different enzyme labels, such as acid phosphatase, horseradish peroxidase and alkaline phosphatase. Immunoperoxidase techniques were most applicable to tissue samples, but due to the high levels of endogenous peroxidase in haemopoietic cells (especially erythroid and myeloid cells) they were not as suitable for blood and bone marrow samples. Immunoalkaline phosphatase techniques were developed and found to be preferable for use with these peroxidase-rich samples. Immunoperoxidase procedures became widely used on tissue samples and remain the method of choice for immunohistology. With further developments in flow cytometry and the availability of bench-top flow cytometers applicable to routine laboratories, fluorescence labelling has largely replaced immunoenzymatic techniques using a number of different enzyme labels, such as acid phosphatase, horseradish peroxidase and alkaline phosphatase. These drawbacks were largely overcome by the development of immunoenzymatic techniques using a number of different enzyme labels, such as acid phosphatase, horseradish peroxidase and alkaline phosphatase.

Flow cytometry

Flow cytometry is widely used to assist in the diagnosis, classification, detection and monitoring of minimal residual disease in the majority of lymphoproliferative disorders. This method allows the rapid and simultaneous analysis of multiple cell parameters, including cell size, complexity, and both surface membrane and intracellular antigens, on large numbers of fresh viable cells (Table 1). The major advantage of flow cytometry is that a number of cellular antigens can be analysed simultaneously on a cell population using multiple monoclonal antibodies labelled with fluorochromes, each of which emits light at a different wavelength. The large range of antibodies available makes flow cytometry the method of choice for establishing the phenotype of lymphoid malignancies and detecting clonality of B-cell disorders.

Flow cytometry requires cells to be in suspension and can be performed on a wide range of specimens including peripheral blood, bone marrow aspirate, fine needle aspirate (FNA) and fluid samples. Cell suspensions can also be prepared from cells extracted from solid tissue biopsies (e.g., lymph nodes) and analysed by flow cytometry. The mechanical dissociation of cells is done by teasing out cells from fresh unfixed tissue using a scalpel and forceps, needle and syringe and wire mesh, or using an automated device (e.g., Medimachine, BD Biosciences, USA; DakoCytomation, Denmark). Enzymatic digestion using a proteolytic enzyme (e.g., pepsin or trypsin) can be used to assist with separation of cells from fibrotic samples. Red cell lysis can be used to remove contaminating red cells that may interfere with the flow cytometric analysis; this does not denature or destroy cellular antigens. Density gradient centrifugation (e.g., Ficoll-Hypaque) can also be used to remove red cells and cell debris from specimens. This method also concentrates the cells of interest. As flow cytometers are now readily available, are simple to use and can be used to analyse all sample types, flow cytometry is becoming the method of choice for the phenotypical analysis of lymphoproliferative disorders.

However, there remains a major drawback of flow cytometry: the inability to directly assess cellular morphology and to correlate this with antigen detection. Therefore, it is critical that the cell sample to be analysed by flow cytometry is assessed morphologically (i.e., cell smear or cytocentrifuge preparation of the extracted cells). This not only ensures that it morphologically resembles the initial specimen and that there are sufficient intact cells, but also guides antibody selection. Typically, many nodal large cell lymphomas, fibrotic lesions (e.g., mediastinal large B-cell lymphoma) and Hodgkin lymphomas do not yield diagnostic samples suitable for flow cytometry due to the relatively low numbers of viable malignant cells extracted compared with the reactive surrounding cells.

As morphology cannot be used to ‘isolate’ the cell of interest, other parameters must be used. Identification of the cell population of interest by flow cytometry is

<table>
<thead>
<tr>
<th>Table 1 Comparison of immunohistochemistry and flow cytometry</th>
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<tr>
<td><strong>Immunohistochemistry</strong></td>
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<tr>
<td>Morphological correlation</td>
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<tr>
<td>Automation</td>
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<tr>
<td>Specimen types</td>
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<tr>
<td>Antibodies</td>
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<tr>
<td>Turn-around time</td>
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<tr>
<td>Permanent record</td>
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<tr>
<td>Simultaneous detection of multiple antigens</td>
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<td>T/B cell clonality</td>
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<tr>
<td>Interpretation</td>
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<td>Minimal residual disease assessment and rare cell analysis</td>
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performed by ‘gating’. This electronically selects the population of interest based on cell parameters, such as forward light scatter which separates cells depending on their size, and side scatter which correlates with internal cell complexity (including nuclear configuration and cytoplasmic granularity). Cell populations can also be ‘gated’ by their ability to bind a particular antibody. CD45 (leucocyte common antigen) is most commonly used in this regard to gate lymphoid cell populations (Fig. 1). Differential intensity of expression of this leucocyte common antigen together with forward and/or side scatter can isolate distinct lymphoid cell subpopulations (e.g., lymphoblasts with weak CD45 expression; hairy cell leukaemia with strong CD45). Lineage-specific antibodies, such as CD19 for B cells or CD3 for T cells, may also be used in gating strategies. Isotype controls should be included for all analyses. These are negative controls which ensure that there is no non-specific binding of the primary antibody to the cell population of interest. Positive and negative quadrants are set from the negative isotype control. Most samples analysed will contain some negative cells (i.e., normal cells that do not express the antigen of interest) which also act as internal negative controls.

Interpretation of the data obtained by flow cytometry is quantitative. The percentage of the cells of interest (i.e., gated events) positive for a particular antigen is provided together with the fluorescence intensity (mean channel of fluorescence). The latter is proportional to the density of

Fig. 1 Flow cytometry of lymphoblasts in ALL. (A) (B) Variable intensity of CD45 expression on CD45/side scatter gating (boxes). (C) Dual cytoplasmic CD79a and nuclear TdT in a precursor B-lymphoblastic leukaemia. (D) Cytoplasmic CD3 (and lack of myeloperoxidase) in a case of precursor T-lymphoblastic leukaemia.
antigen expression by the cell. In most analyses there is clear separation of positive from negative cells based on fluorescence intensity (Fig. 1). However, with some neoplastic lymphoid cells, where antigens are only weakly expressed, there is no clear cut-off between cells in the positive and negative quadrants (Fig. 2). In this situation, interpretation is based on the percentage of cells in the positive quadrant. An arbitrary cut-off level is used to define positive expression; this is usually 20% of cells.

Flow cytometry is being increasingly used for lymph node analysis but has still not gained routine acceptance. This is despite the ability to rapidly assess the cell sample (i.e., within hours), determine clonality by light chain restriction, and simultaneously detect multiple antigens on a cell (e.g., CD5 expression by B cells). These are some of the specific areas where flow cytometry has advantages over immunohistochemistry. Other areas where flow cytometry has advantages but remains under-utilised are the analysis of fine needle aspirate samples (e.g., of lymph nodes) to assess a possible lymphoproliferative disorder, and bone marrow staging of lymphoma. For FNA samples, sufficient cells can be aspirated to assess clonality of B cells, and a basic panel of markers may be examined. This technique is useful as an initial screening test for a lymphoproliferative disorder, with a follow-up tissue biopsy to confirm.5–7 In marrow staging of lymphoproliferative disorders, flow cytometry may be more sensitive than histology in detecting low levels of clonal cells, but may miss cases where there are small focal paratrabeular infiltrates. The clinical relevance of small numbers of clonal cells in the bone marrow detected by flow cytometry alone is yet to be determined.8–10

Fig. 2 Flow cytometry of chronic lymphocytic leukaemia demonstrating the expression of the prognostic markers CD38 and ZAP70. (A) Expression of CD5 by the CD19-positive B cells, a gating strategy to isolate the CLL cells. (B) 78% of the cells are B cells that are CD38 positive. (C)(D) ZAP70 on two cases of CLL. In (C) the CLL cells (identified with cytoplasmic CD79a) are predominantly ZAP70 negative, whereas in (D) the majority (64%) are ZAP70 positive.
**Immunohistochemistry**

Immunohistochemistry (IH) is an integral part of routine diagnostic histopathology and remains the phenotyping method of choice for tissue biopsies. IH is generally performed on routinely processed, formalin-fixed, paraffin-embedded tissue. The major advantage of IH over flow cytometry is that morphology and the antigen label (precipitated chromogenic substrate) can be visualised simultaneously by light microscopy, enabling accurate identification of the cells or region of interest (Table 1). Automated systems are available that can fully automate immunostaining for formalin-fixed, paraffin-embedded tissues, frozen sections, cytospins, cell smears and FNA. Some will perform the entire process from dewaxing paraffin-embedded tissues, performing antigen retrieval, application of the antibody and the chromogenic substrate through to nuclear counter-staining. These high-throughput devices can be programmed to optimise tissue pre-treatment and staining conditions for all types of samples and antibodies, with some having batch processing facilities.

The majority of immunohistochemistry performed on tissue sections uses horseradish peroxidase as the enzyme label, although some laboratories prefer alkaline phosphatase. The immunological methods available include indirect antibody staining, unlabelled antibody bridge (e.g., peroxidase-anti-peroxidase), avidin (or streptavidin)-biotin antibody staining, unlabelled antibody bridge (e.g., peroxidase, autacolyse, water bath heating, or pressure cooking and use of buffers (e.g., citrate, EDTA) at pH 6.0–9.5. The choice of antigen retrieval methods varies from laboratory to laboratory, in large part due to differences in tissue preparation, and also by antibody clone. Hence, no advice can be given as to the optimal methodology to be used for a particular antibody.

Currently there is an extensive range of antibodies to lymphoid-associated antigens that can be used in routinely processed tissue but this still remains more limited than for fresh cells (Table 2). This is most relevant in the assessment of clonality, as light chains of immunoglobulin are not as readily detected by IH on fixed material as on fresh cells. Other limitations of immunohistochemistry in the assessment of lymphoproliferative disorders are (Table 1):  

1. The inability to assess more than one antigen on the same cell (i.e., ‘multiple staining’).

**Table 2** Antibodies of value for phenotyping lymphoproliferative disorders by flow cytometry (or fresh cell analysis) or immunohistochemistry on fixed tissue*

<table>
<thead>
<tr>
<th>Disorder/cell type</th>
<th>Fresh cells (e.g., flow cytometry, cell smears or frozen sections)</th>
<th>Fixed tissue (IH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-cell malignancies/Pan-B-cell antigens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precursor B-cells</td>
<td>CD19, CD20, CD22, CD79b, Igk, Igδ, Igk, Igδ</td>
<td>CD20, CD45RA, CD75, CD79a, PAX5, MUM1</td>
</tr>
<tr>
<td>Chronic lymphocytic leukaemia</td>
<td>CD16, CD10, CD19, CD20, CD45, CD58, TdT, NG2, Myelod markers</td>
<td>CD10, CD34, TdT</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>CD5, CD23, CD38, CD52, ZAP70</td>
<td>CD5, CD23, p53</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>CD10c, CD25, CD103</td>
<td>CD11c, DBA-44</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>CD5, CD10</td>
<td>CD10, CD21, BCL2, BCL6</td>
</tr>
<tr>
<td>Burkitt’s lymphoma</td>
<td>CD10, TdT</td>
<td>CD5, CD10, CD30, BCL2, BCL6, MUM1</td>
</tr>
<tr>
<td>T-cell malignancies/Pan-T-cell antigens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precursor T cells</td>
<td>CD2, CD3, CD4, CD5, CD7, CD8, 2β TCR, 2α TCR</td>
<td>CD2, CD3, CD4, CD5, CD7, CD8, CD43, CD45RA</td>
</tr>
<tr>
<td>T-prolymphocytic leukaemia</td>
<td>CD1a, TdT, Myelod markers</td>
<td>CD34, TdT</td>
</tr>
<tr>
<td>T-large granular lymphocytic leukaemia</td>
<td>CD16, CD56, CD57</td>
<td>TIA-1, granzyme B</td>
</tr>
<tr>
<td>Sézary syndrome/Mycosis fungoides</td>
<td>CD26</td>
<td></td>
</tr>
<tr>
<td>Adult T-cell leukaemia/lymphoma</td>
<td>CD23, HLA-DR</td>
<td>CD30</td>
</tr>
<tr>
<td>Anaplastic large cell lymphoma</td>
<td>TCR 2β, TCR 3β, CD16, CD56</td>
<td>CD15, CD30, CD45, ALK, TIA-1, perforin, EMA, granzyme B</td>
</tr>
<tr>
<td>Hepatosplenic T-cell lymphoma</td>
<td>TCR 2β, TCR 3β</td>
<td>TIA-1</td>
</tr>
<tr>
<td>Natural killer cell antigens (and natural killer cell malignancies)</td>
<td>CD2, CD7, CD8, CD16, CD56, CD57</td>
<td>CD2, CD57</td>
</tr>
</tbody>
</table>

*Pan-T, -B and natural killer cell antigens are listed as well as those antibodies of particular value for individual lymphoproliferative disorders.

†CD52 is not diagnostically useful but identifies a potentially useful therapeutic target.
2. Slow turnaround time as immunohistochemistry is performed only after initial morphological assessment of the tissue sections.
3. Only limited analysis is possible on small samples.

New techniques are being developed in IH and include the analysis of tissue arrays and use of antibody arrays. Tissue arrays are a high throughput approach to the study of antigen expression in tissue biopsies. The arrayed tissue biopsies are stained using identical immunohistochemical methods to those used for routine diagnostic testing. This array-based technology can be used to rapidly evaluate the diagnostic and prognostic value of newly produced antibodies. This approach is rapidly generating vast amounts of information about the utility of these new antibodies in diagnostic pathology, some examples of which will be given in this review.

Antibody arrays or solid-phase cytometry technology have also been developed. These are arrays of antibodies immobilised onto a solid phase (e.g., cellulose membrane or glass slide) with cell capture assessed by immunocytochemical staining or light transmission. The phenotype of cells bound to the arrayed antibodies correlates with surface antigen expression. This methodology enables large numbers of cellular antigens to be assessed simultaneously in a single analysis. Both of these new techniques have potential for routine immunophenotyping but have yet to be incorporated into routine laboratory practice.

THE PHENOTYPE OF LYMPHOPROLIFERATIVE DISORDERS

Phenotyping is a routine component in the assessment of the majority of lymphoproliferative disorders. To date, no single antibody has been shown to be specific for a malignant lymphoid cell, so patterns of antigen expression are used for the immunophenotypical classification of lymphoproliferative disorders. Hence, panels of antibodies are used to determine the cell lineage, stage of differentiation and, especially for B cells, clonality. Using this approach, a number of the lymphoproliferative disorders exhibit characteristic phenotypes or signatures which assist in disease classification. The optimal number and range of antibodies to evaluate lymphoproliferative disorders is a matter of debate. Table 2 lists many of the most useful antibodies currently available. For most analyses, a limited primary panel of antibodies should be used. This would include pan-B (e.g., CD19, CD20, CD79a) and pan-T (e.g., CD3, CD5) cell antibodies. If the morphology suggests a primary panel of antibodies should be used. This would include pan-B (e.g., CD19, CD20, CD79a) and pan-T (e.g., CD3, CD5) cell antibodies. If the morphology suggests a specific lymphoproliferative disorder with a characteristic phenotype (e.g., hairy cell leukaemia) then these antibodies (e.g., CD11c, CD25, CD103) should be included (Table 2).

B-cell lymphoproliferative disorders are identified by the expression of B-cell-associated antigens and light chain (κ or λ) restriction, indicating clonality. The precise disease type may also be determined from the pattern of antigen expression. The immunophenotypical diagnosis of T-cell lymphoproliferative disorders is more difficult than for the B-cell malignancies as T-cell clonality cannot be as readily established. However, many T-cell malignancies show atypical T-cell phenotypes, such as aberrant loss of an expected T-cell antigen (typically CD5 or CD7), and loss of or co-expression of CD4 and CD8 antigens. Detection of aberrant T-cell phenotypes can be used as an indicator of neoplasia. For some B- and T-cell disorders, additional non-lineage-associated antibodies may be used to predict use of immunotherapy (e.g., CD20), determining proliferation rate (e.g., MIB1) or prognosis, or recognition of antigens of use in residual disease monitoring during and following treatment.

Initially, antibodies used in routine phenotyping were to cell surface antigens expressed by normal lymphoid cells, either during ontogeny or following activation, and those recognising heavy and light chains of immunoglobulin (Ig). More recently antibodies to gene products of chromosomal translocations have been introduced into routine phenotyping, such as antibodies to cyclin-D1 from rearrangement of the CCND1 (BCLI) gene as a result of t(11;14) in mantle cell lymphoma. The protein products of other genes activated by chromosomal rearrangements also have a role as markers of either lineage (e.g., PAX5 [B-cell-specific activator protein; BSAP] for B cells) or maturation stage (e.g., BCL6 for germinal centre). Newer antibodies to molecules whose genes are upregulated are also being incorporated into phenotyping studies (e.g., FOXP1 and HGAL) for lymphoid malignancies. These antibodies as well as lineage-specific or lineage-associated antibodies will be discussed in relation to particular lymphoid malignancies.

Lymphoblastic leukaemia/lymphoma

Lymphoblastic leukaemias and lymphomas (ALL/LBL) are neoplasms derived from progenitor lymphoid cells or lymphoblasts of B or T lineage, and are overlapping presentations of the same disease entity. They are classified according to phenotype, with both the cell lineage (i.e., B or T) and the stage of differentiation at which maturation arrest occurred being relevant. The lymphoblasts are characterised by expression of terminal deoxynucleotidyl transferase (TdT) within the nucleus. The B or T lineage of ALL/LBL is based on the expression of at least cytoplasmic CD79a for B-cell and cytoplasmic CD3 for T-cell disease. In 1995 the European Group for the Immunological Characterisation of Leukaemias (EGIL) proposed a classification system that divided precursor B-ALL and precursor T-ALL each into subgroups based on cell phenotype. This phenotypical subclassification provides useful prognostic information. B-lineage ALL is more common than T-ALL, accounting for 85–90% of paediatric, and 75% of adult cases of ALL. In contrast, T-LBL is more common than B-LBL, comprising 85–90% of cases.

Minimal residual disease (MRD) detection is becoming increasingly important in the management of ALL. Several studies have shown that combinations of antibodies and multi-parameter flow cytometry has a sensitivity of 10⁻⁴ for MRD assessment, which is comparable with molecular methods. Clinical use of this approach necessitates extensive phenotypical analysis at diagnosis to identify novel phenotypical combinations expressed on the leukemic cells but not on normal haemopoietic cells.

Phenotyping is also performed in ALL/LBL to detect potential immunotherapeutic targets, such as CD19, CD20, CD22, CD33 and CD52. A prerequisite for antibody
therapy is the presence of the target antigen on at least 20–30% of the blast cells. To date, most experience has been with CD20 (rituximab) in precursor B-LBL. The role of immunophenotyping in lymphoblastic neoplasms therefore is not restricted to diagnosis and it can be used to:

1. Confirm the neoplastic cells are primitive lymphoid cells and establish the B- or T-cell lineage of the lymphoblasts.
2. Determine the stage of differentiation of the lymphoblast and hence the precise classification of the ALL/LBL.
3. Identify markers which may have prognostic significance.
4. Identify unique antigens or patterns of antigen expression that could be used to monitor residual disease following therapy.
5. Assess the expression of molecules that may be targets for immunotherapy.

The phenotypical characteristics of B and T lymphoblastic leukaemias and lymphomas will be discussed. Most of the data presented have been established by flow cytometric analysis of peripheral blood and/or bone marrow. However, IH can also be used to detect many of the antigens required to assess these disorders (Table 2).

**Precursor B-lymphoblastic leukaemia/lymphoma**

The lymphoblasts in precursor B-ALL/LBL are CD19 and cytoplasmic CD79a positive, CD22 positive (initially in the cytoplasm and later on the cell surface), express TdT within the nucleus, and are HLA-DR positive. They have variable expression of CD34 and weak CD45 expression (Fig. 1). Precursor B-ALL/LBL can be further subclassified based on the degree of B cell differentiation and expression of CD10, CD20 and cytoplasmic or surface IgM (heavy chains of IgM) (Table 3). The three subtypes are:

1. Pro-B-ALL, the earliest stage in which the neoplastic cells are negative for CD10, CD20 and cytoplasmic (cy) IgM.
2. Common ALL, the intermediate stage, where the cells express CD10, are negative for cy IgM and have variable CD20 expression (a pan-B-cell antigen expressed slightly later in B-cell development than CD19, CD22 and CD79a).
3. Pre-B-ALL, which is derived from the most mature precursor B cell, with neoplastic cells positive for CD10, CD20 and cy IgM but negative for surface IgM.

Other antigens of note in B-cell ALL/LBL are CD45 and antibodies to myeloid associated antigens. CD45, the leucocyte common antigen, is characteristically expressed more weakly on lymphoblasts than normal lymphocytes, and the intensity can vary between ALL/LBL cases. On flow cytometry, this weak CD45 expression together with low side scatter are useful characteristics for gating the lymphoblasts (Fig. 1). As ALL cases are commonly analysed with a panel of antibodies, aberrant expression of myeloid antigens (e.g., CD33) may be detected. Approximately one-third of B-ALL aberrantly express myeloid associated antigens (e.g., CD13 or CD33). This is particularly seen in cases with MLL gene rearrangements. These MLL rearranged B-ALL cases can also be identified by reactivity with antibody NG2 (which recognises chondroitin sulfate proteoglycan neuron-glial antigen) with a specificity of 90–100%. Specific B-ALL/LBL phenotypes are also seen in association with other recurrent cytogenetic abnormalities, many of which are of diagnostic and prognostic significance (Table 4). Examples include the pre-B-ALL phenotype with the t(1;19) and common ALL phenotype in BCR/ABL t(9;22).

Recent additions to antibody panels have indicated that CD1d and CD58 may be valuable as prognostic markers or for monitoring residual B-ALL. CD1d is expressed by 15% of B-ALL and these positive cases have been reported to have a poorer prognosis. CD1d positivity has been detected in infant leukaemia, leukaemias with a Pro-B phenotype and mixed-lineage leukaemia with the MLL/AF4 gene rearrangement. It is unclear whether CD1d expression is an independent poor prognostic marker, or its expression is associated with B-ALL subgroups with poor prognosis. Detection of CD1d expression may also be useful to predict immunotherapeutic response to glycolipids. As alpha-galactosylceramide is able to be presented via CD1d to CD1d-restricted T cells with cytotoxic potential, CD1d may prove to be a useful therapeutic target.

CD58, a member of the Ig superfamily, is over-expressed in B-ALL blasts when compared with normal precursor B cells. This was first uncovered by gene expression analysis. Recent studies have suggested that CD58 expression is a powerful tool for disease monitoring and detection of MRD; however, this has not gained widespread acceptance. Gene expression profiling has also shown distinct patterns of gene expression in biologically distinct subgroups of ALL. These genetic studies are likely to identify additional novel biological groups not predicted by conventional morphological, phenotypical and cytogenetic variables. Production of antibodies to gene products of the upregulated genes may provide further new diagnostic tools, add significant prognostic information and may be able to be used to identify novel therapeutic targets.

**Precursor T-lymphoblastic leukaemia/lymphoma**

T-ALL/LBL are malignancies derived from precursor T cells or T lymphoblasts. They are TdT positive, express cytoplasmic CD3 and surface CD7, and have variable

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**Table 3 Immunophenotype of B-lymphoblastic leukaemia/lymphoma (based on the EGIL Classification)**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Pro-B-ALL</th>
<th>Common ALL</th>
<th>Pre-B-ALL</th>
</tr>
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<tbody>
<tr>
<td>TdT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cy CD79a</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD19</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD22</td>
<td>+/−</td>
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<td>CD10</td>
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<tr>
<td>CD20</td>
<td>−</td>
<td>+/−</td>
<td>+</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD34</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cy IgM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sm IgM</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
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</table>

ALL, acute lymphoblastic leukaemia; Cy, cytoplasmic; Sm, surface membrane.
expression of other T-cell-associated antigens and CD45. CD3 is lineage specific and is expressed within the cytoplasm early in T-cell development. CD7 is the earliest T-cell-associated antigen to be expressed on the cell surface in T-cell differentiation. CD1 and CD5 are expressed later, followed by surface membrane CD3. T-ALL can be classified therefore according to the stage of intra-thymic T-cell differentiation by antigen expression, as follows (Table 5):

1. Immature thymocyte T-ALL (early T-ALL) is derived from the earliest T lymphoblast and does not express CD1a or surface CD3. It may co-express CD34 and myeloid-associated antigens (e.g., CD33).
2. Common thymocyte T-ALL (intermediate, thymic or cortical T-ALL) is defined by expression of CD1a, with frequent co-expression of CD4 and CD8 antigens. Some cases may be surface CD3 positive.
3. Mature T-ALL is CD1a negative, expresses either CD4 or CD8 and is surface CD3 positive.

This phenotypical subclassification is important, as there is evidence that the immunological subtype is the most important prognostic factor in T-ALL. The best disease-free survival is seen in common thymocyte T-ALL. Up to a quarter of T-ALL cases have aberrant myeloid antigen expression (e.g., CD13, CD33, CD117), the significance of which is uncertain. A small number of T-ALL cases express CD10 or cytoplasmic CD79a. BCL6 has been reported to be positive in a significant number of T-ALL cases expressing the anti-apoptotic gene occurs in approximately 5–10% of childhood and 30% of adult T-ALL cases and is associated with a favourable prognosis. Other significant genes identified are LYL1 seen in immature thymocyte T-ALL, HOX11L2 in T-ALL of common thymocyte type, and TAL1 in mature T-ALL. The prognostic significance of these genomic features is yet to be determined and at present, phenotyping for the gene products is not routinely performed.

### Table 4 Phenotype/genotype correlations for paediatric precursor B-lymphoblastic leukaemia with non-random cytogenetic abnormalities

<table>
<thead>
<tr>
<th>Cytogenetic abnormality</th>
<th>Genetic alteration</th>
<th>Immunophenotype</th>
<th>Frequency</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(12;21)(p13;q22)</td>
<td>TEL/AML1</td>
<td>CD10+/TdT+/CD19+/CD20−/~CD34+. Commonly CD33+ CD13+ (or both)</td>
<td>Up to 25%</td>
<td>Good</td>
</tr>
<tr>
<td>t(1;19)(q23;p13.3)</td>
<td>PBX1/E2A</td>
<td>Most commonly Pre-B-ALL (CD10+/CD19+/CD20+). May have common ALL phenotype.</td>
<td>25% of Pre-B-ALL</td>
<td>Poor or standard risk</td>
</tr>
<tr>
<td>t(9;22)(q34;q11.2)</td>
<td>BCR/ABL</td>
<td>Common ALL phenotype (CD10+/CD19+/CD20−/~TdT+). CD33+ and/or CD13+.</td>
<td>3-4%</td>
<td>Poor</td>
</tr>
<tr>
<td>t(4;11)(q21;q23)</td>
<td>AF4/MLL</td>
<td>Pro-B-ALL (CD10−/CD19+/CD20−) and TdT−. Also CD34+, CD65+ and NG2+.</td>
<td>2-3%</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Phenotypical assessment of minimal residual disease assessment for T-ALL can be performed on either blood or bone marrow as there is comparable dissemination of the leukaemic cells which are of thymic origin. TdT can be used in combination with one or more T-cell antigens known to be expressed by the leukaemic cell (e.g., CD7 or CD2). The presence of a TdT-positive T cell in blood or bone marrow is indicative of leukaemia, as such double positive cells normally only reside in the thymus.

Microarray gene expression analysis of T-ALL/LBL has provided greater insight into the biological heterogeneity of the disease and revealed clinically relevant molecular subtypes. Gene expression profiling has shown that overexpression of the HOX1I gene occurs in approximately 5–10% of childhood and 30% of adult T-ALL cases and is associated with a favourable prognosis. Other significant genes identified are LYL1 seen in immature thymocyte T-ALL, HOX11L2 in T-ALL of common thymocyte type, and TAL1 in mature T-ALL. The prognostic significance of these genomic features is yet to be determined and at present, phenotyping for the gene products is not routinely performed.

### Table 5 Immunophenotype of T-lymphoblastic leukaemia/lymphoma subtypes

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immature thymocyte T-ALL</th>
<th>Common thymocyte T-ALL</th>
<th>Mature T-ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cy CD3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD5</td>
<td>4−/−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD1a</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Sm CD3</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>CD4+/− CD8−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>CD4+ or CD8+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukaemia; Cy, cytoplasmic; Sm, surface membrane.

### MATURE B-CELL NEOPLASMS

Mature B-cell malignancies are clonal expansions of mature B cells that are, by definition, TdT negative. They arise from B cells at different stages of differentiation which may be pre- or post-antigenic stimulation in the germinal centre. As B-cell neoplasms arise from the clonal expansion of a B-cell population they express only one light chain, either Igk or Igλ. Flow cytometry of fresh cells is particularly valuable for the detection of light chain restriction or an overwhelming majority of one light chain. In some cases where there are residual normal B cells present, a skewed Igk or Igλ ratio (i.e., Igk/Igλ > 6:1 or Igk/Igλ < 1:1) may be indicative of a clonal B-cell disorder. Many mature B-cell malignancies have a characteristic immunophenotype that can easily be distinguished by immunohistochemistry or multiparameter flow cytometry (Table 6). Phenotyping can also be used to identify aberrant antigen expression by B cells or down-regulation of immunoglobulin, both of which may be helpful in identifying a B-cell malignancy. The phenotypes of the most common mature B-cell neoplasms are presented.

**Chronic lymphocytic leukaemia/Small lymphocytic lymphoma**

Chronic lymphocytic leukaemia (CLL) and small lymphocytic lymphoma (SLL), generally regarded as the blood and
tissue phases of the same disease, have the same phenotype, being CD5-positive B-cell disorders. The neoplastic B-cells express pan-B-cell antigens with variable intensity, surface Ig heavy chains (Igμ) and show light chain restriction. Specifically, CD20, CD22 and Igμ are weakly expressed whilst CD79b is generally very weak or negative.44 CLL cells are characteristically positive for both CD5 and CD23 antigens (by flow cytometry and IH). However, neither CD5 nor CD23 alone is sufficient to diagnose CLL/SLL as these antigens can also be detected on other B-cell malignancies. Specifically, CD5 is expressed by mantle cell lymphoma, however, CD23 is generally negative in this disorder. The phenotypical features (expression of CD5 and CD23, absence of CD79b, weak Igμ/m) form the basis of the CLL scoring system (Table 7).45,46 It is of note that this scoring system was developed for cellular assessment by flow cytometry and is less applicable for IH. When CLL/SLL cases are analysed by IH the neoplastic cells can be shown to express CD5, CD20 and CD23 antigens; however, clonality (i.e., light chain restriction) and intensity of expression of Igμ cannot usually be determined by IH. Up to 35% of cases of CLL have been reported to express (or lack) at least one aberrant marker and this may be associated with more aggressive disease.47 These lack of CD5, although this is uncommon, or CD23. Less commonly, CLL can express other B, T or myeloid-associated antigens, such as FMC7, CD11c, CD79b, CD2, CD7, CD10, CD13 or CD33.48–50

Recent studies have shown that there are two subtypes of CLL based on mutational status of Ig genes. The majority of CLL cases are derived from a B cell that has undergone somatic hypermutation of the Ig genes. A smaller number are derived from pre-follicular B cells (i.e., lack somatic hypermutation) and have more aggressive behaviour with a poorer prognosis. There is close (but not complete) correlation between mutational status and phenotypical expression of ZAP70, and to a lesser extent CD38 antigen.48–50 A number of studies have now demonstrated that immunophenotyping can provide prognostic information in CLL, based on expression of ZAP70 and CD38 antigens.51 expression is also reported to be associated with poor prognosis disease.

ZAP70 protein is an intracellular tyrosine kinase essential for T-cell signalling, but not found in normal B cells. Current data indicate that ZAP70 expression by more than 20% CLL cells is reliably associated with a poorer prognosis. At present, flow cytometry for ZAP70 remains to be standardised, in part because of the need for membrane permeabilisation to access this intracellular molecule and also the difficulty in clear separation of positive from negative cells (Fig. 2). Few antibodies are available that enable assessment by IH on fixed material.

Many reports have described the poor prognostic significance of CD38 expression in CLL.49,51,52 CD38 expression can be readily detected on B cells by multiparameter flow cytometry on cells with the CLL phenotype (CD5, CD23 positive B cells); hence it is commonly used as a prognostic marker. Although there is debate as to the level of antigen expression that should be defined as positive, with investigators using different criteria, a cut-off of more than 30% positive cells is commonly used. CD38 has weak prognostic significance in univariate analysis, and

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**Table 6** Immunophenotype of mature B-cell lymphoproliferative disorders

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CLL</th>
<th>B-PLL</th>
<th>HCL</th>
<th>SLVL</th>
<th>MCL</th>
<th>FL</th>
<th>DLBCL</th>
<th>BL</th>
<th>MALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5</td>
<td>−/−</td>
<td>−/−</td>
<td>−</td>
<td>−/−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>CD19</td>
<td>−−</td>
<td>+−</td>
<td>−+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD79b</td>
<td>−</td>
<td>−/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sm Igμ</td>
<td>−</td>
<td>−/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BCL2</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>BCL6</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+ (GC)</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

**Table 7** CLL scoring system from Moreau et al.46

<table>
<thead>
<tr>
<th>Antibody</th>
<th>CLL</th>
<th>Score*</th>
<th>Other B-cell neoplasms</th>
<th>Score*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5</td>
<td>+</td>
<td>1</td>
<td>−/−</td>
<td>0</td>
</tr>
<tr>
<td>CD23</td>
<td>+</td>
<td>1</td>
<td>−/−</td>
<td>0</td>
</tr>
<tr>
<td>FMC7</td>
<td>−</td>
<td>1</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>CD79b /Sm Igμ</td>
<td>Weak</td>
<td>1</td>
<td>Strong</td>
<td>0</td>
</tr>
<tr>
<td>CD22</td>
<td>−</td>
<td>1</td>
<td>Strong</td>
<td>0</td>
</tr>
</tbody>
</table>

*CLL usually scores 4–5, with other B-cell lymphoproliferative disorders scoring 0–2.
†Except mantle cell lymphoma.
CLL, chronic lymphocytic leukaemia.
does not improve predictive power of ZAP70 or Ig gene mutational status in multivariate analysis. However, the antibody is readily available and flow cytometry simple to perform and detect positivity (Fig. 2). The predictive value of CD38 expression can be increased by quantitative measurement of CD38 antigen density (antibody binding capacity) rather than as the percentage of CLL cells expressing the antigen. The antibody binding capacity of CD38 closely correlates with the relative median fluorescence intensity on flow cytometry. CD38 has the disadvantage of altered expression over the course of the disease.

Mutations in the p53 gene are also reported to be associated with poor prognosis in CLL, as well as in several other lymphoproliferative disorders (e.g., diffuse large B-cell lymphoma and mantle cell lymphoma). Mutations and over-expression of p53 are most commonly evaluated by fluorescence in situ hybridisation (FISH), but flow cytometry has also been studied using monoclonal antibodies specific for the N-terminus, Bp53 and DO-1, and the central region of the p53 protein, DO-11.53

The combination of ZAP70 expression, CD38 antigen density, p53 function and the concentration of serum factors (e.g., soluble CD23), is likely to provide extremely accurate prognostic information in future studies.52

Another antibody that may have prognostic value in CLL is MUM1. MUM1 (multiple myeloma oncogene 1) is a proto-oncogene that is deregulated as a result of t(6;14)(p25;q32) in multiple myeloma, and is also expressed in a variety of malignant lymphomas. The translocation juxtaposes the Ig heavy-chain (IgH) locus to MUM1/IRF4 gene, a member of the interferon regulatory factor (IRF) family. CLL with expression of MUM1/IRF4, indicating post-germinal centre origin, has been shown in one study to have a more favourable clinical course. However, in another it was shown that MUM1 expression was an independent unfavourable prognostic factor in CLL/SLL.55

Therefore, more work is needed to clarify whether MUM1 expression in CLL is a useful prognostic marker.

Another antibody of importance in the phenotypical assessment of CLL is CD52. The CD52 antigen is a glycoprotein anchored on the cell membrane of mature B and T lymphocytes, monocytes and eosinophils. CD52 is the antigenic target of the monoclonal antibody Campath-1H (alemtuzamab), which is used primarily in refractory and relapsed CLL. Phenotypical detection of the antigen is important if this is to be a target for immunotherapy.

Immunophenotyping can also be used to monitor residual disease following aggressive therapy for CLL. Approaches that have been used to detect small numbers of residual CLL cells include:

1. Flow cytometric quantitation of CD5, CD23 dual-positive B cells expressing the Ig light chain known to be expressed by the neoplastic CLL clone.
2. Sensitive, quantitative flow cytometry utilising the weak CD79b expression of B cells.56 For example, multi-parameter flow cytometry for co-expression of CD19/CD5/CD20/CD79b can differentiate CLL cells from normal B cells and detect one CLL cell in 10^9 or 10^5.57
3. Determining CD22 and CD81 expression by CD19-gated B cells that express CD5, as there is differential intensity of expression of these antigens compared with normal B cells. This combination is currently being assessed by flow cytometry for its value in detecting MRD following aggressive therapy.58

**B-cell prolymphocytic leukaemia**

B-cell prolymphocytic leukaemia (B-PLL) differs from CLL by its morphology, phenotype and more aggressive clinical course. Prolymphocytes of B-cell origin are large, round cells with abundant basophilic cytoplasm and a single central nucleolus. In contrast to CLL, B-PLL cells have high levels of surface IgM expression and pan-B cell markers (e.g., CD20, CD22), including CD79b. B-PLL is also positive for the antibody FMC7, now known to be a weak antibody of the CD20 cluster. B-PLL cells are commonly weakly CD5 positive and generally CD23 negative. CD11c, CD25 and CD103, expressed by hairy cells, are not expressed.

**Hairy cell leukaemia and related disorders**

**Hairy cell leukaemia** Hairy cell leukaemia (HCL) is a disorder of mature B cells that has a unique phenotype. The cells strongly express pan-B-cell antigens (e.g., CD19, CD20, CD22) and surface membrane IgM. Most distinctively they are positive for CD11c, CD25, CD103 and HC2 (Table 6).60-62 CD11c, normally expressed on neutrophils and monocytes, is rare on B-cell disorders other than HCL. However, CD103, the human mucosal lymphocyte antigen, is the most specific marker of HCL (Table 6). CD52 is usually positive in HCL, leading to suggestions that Campath-1H may be a useful therapeutic agent.62

Other markers that have recently been shown to be useful in the diagnosis of HCL are CD123 (antibody to the α-chain of the human interleukin-3 receptor), TIA-1 and annexin A1.60 TIA-1 has been shown to be expressed by the majority of HCL cases and none of 94 other B-cell lymphoproliferative disorders tested, thereby suggesting that TIA-1 expression may be a useful confirmatory marker for HCL.64 By gene expression profiling it has been shown that annexin A1 (ANXA1) is upregulated in HCL. Immunohistochemical staining of 500 B-cell tumours with an anti-ANXA1 monoclonal antibody has demonstrated that ANXA1 protein expression is specific for HCL. Further, ANXA1 can distinguish HCL from splenic lymphoma with villous lymphocytes (SLVL) and variant hairy cell leukaemia.65

Immunohistochemistry can be performed on paraffin-embedded biopsies, including decalcified bone marrow trephines to phenotype HCL. To date, the most useful antibodies for diagnosis have been the pan-B-cell marker CD20, DBA-44 and TRAcP (monoclonal antibody 9C5), an antibody that recognises tartrate-resistant acid phosphatase.66,67 DBA-44, a monoclonal antibody that recognises a membrane antigen expressed by a subpopulation of B-lymphoid cells has proved a useful marker of HCL but is not specific. It is also positive in SLVL and therefore is not helpful in differentiating between these two entities. Recently, a CD11c antibody that can be used in paraffin-embedded material and decalcified bone marrow trephines has been produced (personal observation) (Fig. 3). This should be of particular value in combination with CD20 for the diagnosis and monitoring of HCL when the trephine biopsy is the only available diagnostic material.68

Flow cytometry is particularly useful for identifying hairy cells. On flow cytometry hairy cells have characteristic light
scatter properties, due to the fine cytoplasmic projections giving increased side scatter, and stronger CD45 expression (mean cell fluorescence) than normal lymphocytes and monocytes (Fig. 3). These unique characteristics provide a sensitive way to 'gate' hairy cells, both at diagnosis and when monitoring residual disease following therapy.

Further, multi-parameter analysis enables B cells that co-express CD11c, CD25 and CD103 to be identified even when only present in small numbers (e.g., less than 1% of lymphoid cells). Therefore, flow cytometry is the immunophenotypical method of choice for the diagnosis and monitoring of HCL, for the following reasons:

Fig. 3 CD11c expression by hairy cell leukaemia using different immunophenotyping methods. (A) Flow cytometry showing the distinctive strong CD45 expression used to gate the cells and dual CD11c/CD19 positivity. (B) CD11c by immuno-alkaline phosphatase (APAAP) staining of peripheral blood. (C) CD11c positivity (immunoperoxidase stain) in formalin-fixed decalcified bone marrow trephine biopsy.
1. It has specific CD45 and side scatter properties.
2. There are limited specific antibodies of diagnostic utility that can be used by IH.
3. CD103 is the most specific marker of HCL and can be used in combination with CD11c and CD25 for simultaneous assessment by flow cytometry.
4. Flow cytometric immunophenotyping of peripheral blood or bone marrow is capable of detecting low levels of malignant cells, even when the patient is leukopenic.

Hairy cell leukaemia variant
HCL variant is a rare B-cell disorder which accounts for 10% of HCL cases. 71,72 The morphology of the cells is intermediate between prolymphocytes and hairy cells, having a prominent nucleolus and cytoplasmic projections. The immunophenotype is similar to HCL. The cells are mature B cells that express pan-B-cell antigens (e.g., CD19, CD20, CD22), although more weakly than classical HCL, and CD11c. In contrast to typical HCL, the neoplastic cells show weak expression or are negative for CD103 and are negative for CD25, CD123 and HC2 antigens. 73

Splenic marginal zone lymphoma
Splenic marginal zone lymphoma is an indolent lymphoproliferative disease with marked splenomegaly, and is frequently accompanied by circulating villous lymphocytes. Hence it is also known as splenic lymphoma with villous lymphocytes (SLVL) and can be difficult to distinguish from HCL. 74,75 Immunophenotypically, the tumour cells have a mature B-cell phenotype with moderately strong surface IgM and IgD. They express pan-B-cell antigens (e.g., CD19, CD20, CD22) and have moderate intensity CD79b. SLVL cases may be CD11c and are usually DBA-44 positive, but are negative for HCL-associated antigens CD25 and CD103. Other markers that are positive are CD24 and FMC7, which help to distinguish SLVL from other splenic lymphomas. In addition, SLVL are generally, but not universally, negative for CD5, CD10, CD23 and CD38, which are often expressed by other B-cell lymphomas, and HC2 and CD123 expressed by HCL. 76 DBA-44 can be of value in paraffin-embedded biopsies as its positivity in SLVL allows it to be distinguished from CLL but not HCL. 66

In 1994, Matutes et al. proposed a scoring system to distinguish HCL from variant HCL and SLVL. 76 This approach, which has not been widely adopted, considered the reactivity with the four most common phenotypical markers of HCL (CD11c, CD25, HC2 and CD103). Reactivity with each gives 1 point and 0 points if negative. Based on this system, 98% of HCL have scores of 3 or 4. In contrast 88% of HCL variant and 77% of SLVL score a maximum of 2. 76

Mantle cell lymphoma
The characteristic phenotype of mantle cell lymphoma (MCL) is expression of CD5 with strong pan-B-cell antigens and surface membrane IgM (Fig 4). MCL has similar intensity of expression of CD5 to CLL but the CD20 and CD79b expression are significantly stronger. Cyclin-D1 is expressed as a consequence of the BCL1-IgH fusion resulting from the t(11;14). At present, cyclin-D1 can only be detected by IH; technical difficulties have resulted in there being no satisfactory reproducible method for detecting cyclin-D1 expression by flow cytometry. However, for the direct detection of t(11;14)(q13;q32) FISH analysis has higher sensitivity than IH and is the method of choice. Other useful markers to distinguish MCL from CLL (which is also CD5 positive) are the absence of CD23 and positivity with FMC7. These phenotypical differences result in a ‘CLL score’ for MCL of below 3 in 96% of cases. 77 MCL is also negative for CD10, enabling it to be distinguished from follicular lymphoma.

MCL has a relatively aggressive clinical course compared with other small cell B-cell lymphomas. CD5-negative MCL cases have been reported and these may have a more indolent clinical course. 78 The blastoid variant behaves in an even more aggressive fashion. These blastoid cases are also CD5 and cyclin-D1 positive and can express CD10.

Follicular lymphoma
The phenotype of follicular lymphoma (FL) reflects that of normal germinal centre B cells. The FL tumour cells are characterised by expression of CD10, BCL2, BCL6, pan-B-cell markers (CD19 and strong CD20), CD45RA, CD75 and relatively strong surface membrane IgM. CD23 may be expressed on a proportion of cells and Igδ and Igη may be positive. FL has tight meshworks of follicular dendritic cells which are CD21 positive, as seen on IH. The neoplastic cells consistently lack CD5 and CD43 antigens.

CD10, BCL2 and BCL6 are the phenotypical hallmarks of germinal centre B cells from which FL originates. CD10, although characteristic of FL, is also present on Burkitt’s lymphoma and some cases of diffuse large B-cell lymphoma. Normal B-cell progenitors or haematogones, and precursor B-ALL/LBL are also CD10 positive, but these immature B cells can be distinguished by their expression of TdT and lack of surface IgM and light chain restriction.

The t(14;18)(q32;q21) chromosomal translocation, characteristic of FL, results in over-expression of BCL2 as the translocation places the BCL2 gene under the control of the IgH gene. BCL2, which is expressed intracellularly, can be detected by IH and flow cytometry and distinguishes FL cells from their non-neoplastic counterparts in reactive germinal centres. Approximately 10% of FL are reported to be BCL2 negative by standard IH, and the majority of these cases lack the t(14;18) translocation. However, some cases with a t(14;18) have a mutation in the translocated BCL2 gene, resulting in an amino acid substitution in the region of the epitope recognised by the BCL2 antibody. 79 Therefore, negative BCL2 by IH or flow cytometry does not exclude a diagnosis of FL. Paediatric FL, in contrast to adult FL, is generally BCL2 negative, suggesting that dysregulated BCL2 expression does not play a significant pathogenetic role in most of these cases. 80

Immunophenotypical detection of BCL6 is important to distinguish between FL, reactive lymphoid hyperplasia and other low-grade B-cell lymphomas. 81 BCL6, a transcriptional repressor required for germinal centre formation, is over-expressed in the majority of FL. This expression is largely independent of chromosome 3q27 rearrangements. 82,83 It is of note that BCL6 is over-expressed in the majority of FL cases without t(14;18) but which have t(3;14)(q27;q32), implying a role for BCL6 in the
pathogenesis of these cases. Therefore, BCL6 is one of the most useful confirmatory immunophenotypical markers for the detection of FL. However, there remains poor standardisation for its detection by IH and it is rarely performed by flow cytometry.

The human germinal centre-associated lymphoma (HGAL) protein has recently been shown to be expressed in the majority of FL, when tested by immunohistochemistry on tissue microarrays.84 HGAL protein is normally expressed in the cytoplasm of germinal centre lymphocytes and in lymphomas derived from germinal centre cells. Antibodies to HGAL protein are not yet being used routinely in diagnostic pathology but may prove to be another useful phenotypical marker for FL.

**Diffuse large B-cell lymphoma**

Diffuse large B-cell lymphoma (DLBCL) includes all malignant lymphomas characterised by the large size of the neoplastic cells, B-cell origin, aggressive clinical course, and the need for high dose cytotoxic chemotherapy regimens. The phenotype of DLBCL is variable, reflecting the heterogeneous nature of this group of tumours.

DLBCL cases express a number of pan-B-cell antigens including CD19, CD20, CD22 and CD79b. However, any one of these may not be expressed in a specific case. Surface membrane IgM is detectable in up to 75% of cases in addition to light chain restriction. CD10 is positive in up to 50% of cases. CD5 expression is seen in approximately 10% of DLBCL, and has been shown to be associated with a higher International Prognostic Index (IPI) score at diagnosis, and a significantly shorter overall survival.85 However, CD23 is rarely expressed (approximately 15% of cases). BCL2 protein is expressed in 30–50% of cases, usually in non-germinal centre type DLBCL, and generally associated with a poorer prognosis. CD21 expression may be associated with improved prognosis disease, independent of IPI score.86 BCL6 protein is expressed in a high percentage of cases of DLBCL. The BCL6 gene on 3q27 is rearranged as a result of chromosomal translocations, for example t(3;14), in about one-third of cases of DLBCL. This disrupts the normal transcriptional regulation of the
gene leading to constitutive over-expression of BCL6. In addition, about three-quarters of cases of DLBCL display multiple somatic mutations in the 5' non-coding region of BCL6, which occur independently of chromosomal translocations and appear to be due to the IgV-associated somatic hypermutation process. Cyclin-D1 is negative in DLBCL, providing a useful marker for discriminating this from blastoid variant of mantle cell lymphoma.

Gene expression profiling has demonstrated the extreme genetic heterogeneity of DLBCL. Data from cDNA microarray analysis have shown that DLBCL can be divided into three prognostically distinct disease subgroups based on gene expression profiles. 88

1. Germinal centre (GC)-like DLBCL which expresses genes characteristic of normal germinal centre B cells (i.e., those encoding CD10 and BCL6 antigens) and has variable MUM1 expression.

2. Activated peripheral B-cell (ABC)-like DLBCL which expresses a subset of the genes that are characteristic of plasma cells, particularly those encoding endoplasmic reticulum and golgi proteins involved in secretion. ABC-like DLBCL have lower BCL6 gene expression than GC-like DLBCL and express IRF-4. These cases do not express CD10 and are usually BCL6 antigen negative and may express MUM1.

3. ‘Type 3’ which expresses CD5.

Immunohistochemical analysis of tissue microarrays has shown some success in discriminating between the GC and non-GC (ABC and ‘type 3’) subtypes of DLBCL. 89 The authors suggest that GC and non-GC subtypes of DLBCL can be determined and predict prognosis using antibodies CD10, BCL6 and MUM1 and immunohistochemistry; however, this remains to be verified by other groups. Expression of GC-like phenotypical features (BCL6 and CD10) correlated with genotype and were reported to be associated with better overall survival. In contrast, a non-GC phenotype, including expression of MUM1, cyclin-D2 and BCL2 were associated with a poorer prognosis and worse overall survival. If these phenotypical features are confirmed, these may be useful for determining DLBCL subtypes and predicting survival, without the need for cDNA microarray. This could be of significant clinical benefit as the 5-year overall survival for the GC-like DLBCL group is reported as 76%, compared with only 34% for the non-GC DLBCL group (p<0.001). 89

A further marker that may prove useful in subclassifying DLBCL is HGAL protein. Gene expression profiling has shown that DLBCL cases with high levels of HGAL mRNA are associated with significantly longer overall survival. IH staining of tissue microarrays of DLBCL shows that HGAL protein expression correlates with expression of GC-associated proteins BCL6 and CD10. HGAL protein is also negative when MUM1 and BCL2 antigens associated with a non-GC phenotype, are expressed. This HGAL protein expression pattern suggests that the antibody may have a role in identifying GC-type DLBCL. 84 It is of note that HGAL is not specific for GC-like DLBCL as it is also expressed by the majority of follicular, Burkitt’s and mediastinal large B-cell lymphomas. 90

FOXP1, a forkhead transcription factor, is another molecule recently shown in gene expression studies to be of value in identifying DLBCL subgroups. This has been confirmed by IH analysis on tissue microarrays. 89 FOXP1 is more commonly detected in non-GC type (MUM1 and BCL2 positive) than GC-like DLBCL. Its expression has predictive value independent of the IPI subgrouping, with FOXP1-negative patients having better overall survival. 91 It is likely that FOXP1 expression will become a standard part of the phenotypical workup of DLBCL.

Other useful prognostic information in DLBCL can be obtained by assessing the infiltrating T cells in DLBCL. CD4-positive T H cells that comprise 20% or more of cells within the tumour are reported to be associated with prolonged overall survival and decreased likelihood of relapse. 92 Another report showed increased overall survival when T cells comprised 20% or more of tumour cells, and there was a CD4 to CD8 ratio > 2. 93

Mediastinal large B-cell lymphoma

Mediastinal large B-cell lymphoma (MLBCL) is a distinct subtype of DLBCL seen predominantly in young females. It has a B-cell phenotype (CD20, CD79a, PAX5 positive), expresses CD30, BCL2 and usually BCL6 with variable expression of surface Igms. MUM1/IRF4 is variably positive and the tumour is negative for CD21 and CD10. 94 The majority of cases (approximately 70%) are also CD23 positive, in contrast to other DLBCL cases which are rarely positive. 95 Microarray studies have shown that MLBCL has a distinct gene expression profile which differs from that of DLBCL and shares some features with classic Hodgkin lymphoma. 96 IH has confirmed that the gene products of some of the upregulated genes can be detected in MLBCL by IH (e.g., STAT1 and TNF receptor-associated factor-1). Preliminary data suggest that a number of cell signalling molecules, including Syk, BLNK and PLC-gamma2 (absent from Reed-Sternberg cells) are also expressed in the majority of MLBCL. 97 Some of these proteins (e.g., nuclear phosphorylated STAT6 and activated c-Rel) can be used to distinguish MLBCL from both DLBCL and classical Hodgkin lymphoma. 98, 99 However, these antibodies are not yet routinely available for diagnostic IH analysis.

Burkitt’s lymphomalleukaemia

Burkitt’s lymphoma is a malignancy derived from follicle centre B cells and translocations involving MYC are a constant feature. The malignant cells express surface membrane Ig, pan-B cell antigens (CD19, CD20, CD79a), CD10 and BCL6 and show light chain restriction. CD45 is more strongly expressed than on lymphoblasts of ALL/LBL and they are both CD34 and TdT negative. They are negative for CD23 and BCL2 and have variable MUM1 expression. Occasional CD5-positive cases have been reported. These must be distinguished from the blastoid variant of mantle cell lymphoma, which is usually possible by demonstrating rearrangement of MYC or IH for c-MYC. 100, 101

Burkitt’s lymphoma characteristically has a very high cell proliferation rate. Immunological assessment of cell proliferation with MIB1 (Ki-67) characteristically shows positivity approaching 100% of Burkitt’s lymphoma cells.
This is higher than in other follicle centre-derived tumours and DLBCL, and can therefore be used to assist in diagnosis. This proliferation index can be performed by IH on fixed or fresh tissue, or by flow cytometry.102,103

Extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue

Extranodal marginal zone B-cell lymphomas of mucosa-associated lymphoid tissue, or MALT-type lymphomas, occur in a number of anatomical sites and are comprised of mature B cells.104 These express pan-B-cell antigens (CD19, CD20, Igμ and commonly Igδ, and show light chain restriction. The tumour shows CD21 and CD35 positivity and lacks expression of CD5, CD10 and CD23. There is no unique phenotypical marker of MALT-type lymphomas, however, they are associated with chromosomal translocations t(11;18)(q21;q21), t(1;14)(p22;q32) and t(14;18)(q32;q21). High cytoplasmic expression of MALT1 and BCL10 is seen in those MALT-lymphoma cases with the t(14;18)(q32;q21).105 Despite the phenotypical and morphological features of MALT-type lymphoma, the diagnosis can still be challenging as monoclonal B cells can also be seen in Helicobacter pylori-associated gastritis without lymphoma.106

MATURE T-CELL LYMPHOPROLIFERATIVE DISORDERS

Mature T-cell lymphoproliferative disorders are uncommon, making up approximately 10% of all lymphoproliferative disorders, and can be very challenging diagnoses in haematopathology. Unlike the more common B-cell disorders, in which clonality is often readily discernible by surface immunoglobulin light chain restriction, there is no specific immunophenotypical signature that is diagnostic of a clonal mature T-cell population. The definitive demonstration of T-cell clonality is dependent on molecular biological techniques using PCR or Southern blotting.

Mature T-cell disorders are TdT negative, and as such are also referred to as post-thymic T malignancies. Antibodies to T-cell-associated antigens that are useful in establishing the T-cell origin of lymphoproliferations include CD3, CD5, CD4, CD7, CD8 and those to the T-cell receptors (TCR). CD43 and CD45RO are also used for the immunohistochemical determination of the T-cell nature of a proliferation. Immunophenotypical criteria that are helpful in the diagnosis of T-cell neoplasms include:107,108

1. Aberrant loss of one or more pan-T-cell antigen relative to normal T-cell populations (typically CD5 or CD7).
2. T-cell subset antigen restriction (i.e., restricted CD4 or CD8) or predominance of either CD4 or CD8 expressing T cells.
3. Dual-positive or dual-negative CD4 and CD8 expression.
4. Loss of or reduced expression of CD45 (by flow cytometry).
5. Restricted γδ T-cell receptor (rather than αβ TCR) expression.
6. Restricted Vβ expression. Flow cytometry using antibodies to the Vβ repertoire antigens can be used as a screening test for T-cell monoclonality; however, as this is expensive it is rarely performed routinely.109,110
7. Expression of additional or aberrant antigens not expressed by normal T cells (e.g., CD30, CD20).

In the following sections we will discuss those mature T-cell lymphoproliferative disorders with characteristic

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<tr>
<th>Antibody</th>
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T-PLL, T-cell prolymphocytic leukaemia; T-LGL, T-cell large granular lymphocytic leukaemia; ATLL, adult T-cell leukaemia/lymphoma; ALCL, anaplastic large cell lymphoma; U, not known.
phenotypes (Table 8). The large group of peripheral T-cell lymphomas, unspecified, which have variable expression of pan-T-cell antigens, will not be discussed.

**T-cell prolymphocytic leukaemia**

T-cell prolymphocytic leukaemia (T-PLL) is characterised by small mature T lymphocytes that express pan-T-cell antigens CD2, CD3 and CD5 and have characteristically strong expression of CD7 (Fig. 5). The majority (approximately 70%) of cases express CD4, whilst dual CD4 and CD8 expression (20% of cases), or CD8 only cases (10%) are occasionally seen. The vast majority of T-PLL patients express membrane z/β TCR, however, case reports of γδ T-PLL exist. T-PLL cells do not express HLA-DR or CD25 antigens. CD52 is expressed, and good remissions have been documented with CD52 immunotherapy with Campath-1H. Immunostaining for the oncoprotein T-cell leukemia-1 (TCL-1) may aid diagnosis in rare cases of extra-medullary T-PLL, where it is positive in 60–70% of cases.

**T-cell large granular lymphocytic leukaemia**

A lymphocytosis of large granular lymphocytes can be of T-cell or natural killer (NK)-cell origin. T-cell large granular lymphocytic leukaemia (T-LGL) is a clonal disorder of mature post-thymic T-cells that express CD3 and other pan-T-cell antigens (CD2, CD5, CD7) and CD8. Rare variant forms may be CD4-positive, co-express CD4 and CD8 antigens or express the γδ T-cell receptor. They commonly express NK-cell associated antigens CD16 and CD57, but rarely CD56. The T-LGL cells are TIA-1 (a cytotoxic granule-associated protein) and granzyme positive. CD26, a T-cell activation antigen and binding protein for adenine deaminase (ADA), has been shown in flow cytometric analyses to identify cases with more aggressive clinical behaviour and shorter survival. Therefore, CD26 can be applied to identify these poor prognostic T-LGL and to identify cases which may not respond to deoxycoformicin, a potent inhibitor of ADA. Research phenotyping studies and gene

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![Fig. 5 T-cell prolymphocytic leukaemia. (A) Strong CD7 expression by APPAP immunocytochemical staining. (B) Dual CD4 and CD8 expression by the neoplastic cells using flow cytometry.](image-url)
expression analyses have demonstrated T-LGL leukaemias to be derived from activated effector cytotoxic T lymphocytes.\textsuperscript{118,119}

An aggressive variant of T-LGL has been described which presents with fever and hepatosplenomegaly and is rapidly fatal. These cases are CD2 and CD3 positive, usually express HLA-DR and have aberrant CD56 expression. CD5, CD7 and CD8 are variably expressed and CD16 is negative. These cases are usually neoplasms of γδ T cells. Other T-LGL leukaemia variants have been reported to be derived from γδ T-LGL.

\textit{Sézary syndrome and mycosis fungoides}

Sézary syndrome and mycosis fungoides are mature post-thymic T-cell malignancies with cutaneous involvement and which can have leukaemic spread. The malignant cells express the pan-T-cell-associated antigens CD2, CD3, CD5 and CD45RO and are usually of helper (CD4) phenotype. The majority of cases are negative for CD7, CD8 and CD25. Aberrant T-cell phenotypes are common; for example, CD3 and CD4 expression levels can be lower than on normal T cells. This provides a useful means for monitoring disease, especially leukaemic involvement. Sézary cells usually lack CD26, which is expressed by the majority of normal T cells.\textsuperscript{120} The phenotype CD4+/CD7−/CD26− seen in Sézary cells has been successfully used to detect and monitor minimal circulating disease. The specificity of this phenotype can be further increased by detecting a restricted TCR Vβ repertoire.\textsuperscript{121}

\textit{Adult T-cell leukaemia/lymphoma}

Adult T-cell leukaemia/lymphoma (ATLL) is an aggressive peripheral T-cell leukaemia/lymphoma associated with clonal HTLV-1 integration. Characteristically, the tumour cells have an activated T-helper phenotype expressing pan-T-cell-associated antigens (CD2, CD3 and CD5), CD4, CD25, CD95 (FAS) and HLA-DR. They are usually negative for CD7, CD8 and CD56 antigens and for cytotoxic-associated molecules granzyme and TIA-1.\textsuperscript{122} Variant ATLL phenotypes (e.g., CD4/CD8+ or dual CD4+/CD8+) have been described, as have rare CD30 positive cases with anaplastic large cell lymphoma morphological features. These are ALK negative and do not express TIA-1 or granzyme B.\textsuperscript{123}

\begin{figure}[h]
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\caption{Anaplastic large cell lymphoma. (A) Anaplastic large cell lymphoma in a bone marrow aspirate (Romanowsky stain). (B) Bone marrow trephine (H&E stain). (C) Lymphoma cells are CD3 positive and (D) CD30 positive (immunoperoxidase stain).}
\end{figure}
Anaplastic large cell lymphoma

Anaplastic large cell lymphoma (ALCL) is a peripheral T-cell lymphoma that expresses pan-T-cell antigens (e.g., CD3), CD30 antigen, anaplastic large cell lymphoma kinase (ALK), epithelial membrane antigen, CD45, and is frequently CD4 positive (Fig. 6). Most cases express cytotoxic granule-associated proteins TIA-1, granzyme B and perforin, but are CD8 negative. CD15, expressed by Hodgkin lymphoma, is negative. ALK-negative cases are also seen, usually in older patients, and these are associated with poorer prognosis. In contrast, ALK-positive ALCL is the most common paediatric mature T-cell malignancy, and usually has a good prognosis. ALCL is most commonly diagnosed on tissue biopsies, including bone marrow trephine biopsies, with phenotyping performed by IH (Fig. 6). Flow cytometry can give false negative results as the neoplastic cells may comprise only a small proportion of the population analysed.

Hepatosplenic T-cell lymphoma

Hepatosplenic T-cell lymphoma is a rare clonal T-cell proliferation derived from cytotoxic T cells and usually of γδ T-cell type. It involves the sinusoids of the liver and spleen and can have blood involvement. Phenotypically, the neoplastic cells, being of T-cell origin, express CD3, CD45 and CD45RO. Unlike the majority of other T-cell lymphoproliferative disorders, the γδ TCR receptor is usually expressed and not the αβ T-cell receptor (Fig. 7). Characteristically, the neoplastic cells lack both CD4 and CD8 antigens, although in approximately
15% of the cells express CD8. Virtually all cases are positive for TIA-1 but approximately 50% lack perforin and granzyme B, indicating a non-activated cytolytic T-cell phenotype. The neoplastic cells are commonly CD2 and CD16 positive and may express CD56.

NATURAL KILLER CELL LYMPHOPROLIFERATIVE DISORDERS

Lymphoproliferative disorders derived from natural killer (NK) cells are uncommon. These may have an acute clinical presentation with systemic symptoms or be a chronic disorder and have minimal symptoms. The best characterised NK-cell disorders are the nasal and nasal-type NK lymphomas, the indolent NK-LGL leukemias and aggressive NK-cell leukemias. There is some overlap in the antigenic profile with T cells, as NK cells contain cytoplasmic CD3 epsilon (CD3ε) chain and can express CD2, CD7 and CD8 antigens. However, they are negative for surface membrane CD3 and CD5, do not express CD4 or CD8, and are usually CD57 negative. The most common NK lymphoproliferation is NK-LGL lymphocytosis. In this disorder the cells have the typical NK-cell phenotype expressing CD16, and commonly CD8, whilst lacking CD3.

Aggressive NK-cell leukaemia and extranodal NK/T-cell lymphoma, nasal type

Aggressive natural killer cell leukaemia (ANKL), a rare form of LGL leukaemia with systemic proliferation of NK cells, and nasal type NK/T cell lymphoma have identical phenotypes. The neoplastic cells express NK-cell antigens CD16 and CD56, but CD57 is usually negative. ‘Surrogate’ NK-cell markers CD2 and CD7 are usually positive. Both CD4 and CD8 positive cases have been reported. Cytoplasmic CD3ε is expressed but not surface membrane CD3 or the T-cell receptor. CD11b and HLA-DR are commonly positive and other myeloid-associated antigens may be expressed (e.g., CD13, CD33).

CONCLUSION

The lymphoproliferative disorders are a heterogeneous group of disorders and phenotyping has an essential role in both the diagnosis and classification of these diseases. This is specifically to determine the lymphoid cell lineage (B, T or NK cell) of the tumour, the stage of cell differentiation, clonality (especially of B cells) and to identify those disorders with characteristic disease-associated phenotypes. Recently the role of phenotyping has expanded to include the demonstration of markers of prognostic significance, detect molecules which may be used as targets for immunotherapy and to identify antigen combinations that may be used to monitor therapy. Results of gene expression analyses have identified genetic signatures for specific subsets of diseases. The production of monoclonal antibodies to the products of upregulated genes is increasing the repertoire of antibodies available. These are already having impact in the phenotypical identification of prognostically distinct subgroups of these disorders. This should further enhance the capability of phenotyping in future. Optimisation of immunophenotyping methods is also occurring. Most significant are the increasing range of antibodies that can be applied to routinely processed tissue biopsies, the use of flow cytometry for analysing cells extracted from fresh tissue and the ability to use tissue arrays for the rapid assessment of new antibodies.

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