Review

Flow-cytometric immunophenotyping of normal and malignant lymphocytes

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Abstract

During the past two decades, flow-cytometric immunophenotyping of lymphocytes has evolved from a research technique into a routine laboratory diagnostic test. Extensive studies in healthy individuals resulted in detailed age-related reference values for different lymphocyte subpopulations in peripheral blood. This is an important tool for the diagnosis of hematological and immunological disorders. Similar, albeit less detailed, information is now available for other lymphoid organs, e.g., normal bone marrow, lymph nodes, tonsils, thymus and spleen. Flow-cytometric immunophenotyping forms the basis of modern classification of acute and chronic leukemias and is increasingly applied for initial diagnostic work-up of non-Hodgkin’s lymphomas. Finally, with multiparameter flow cytometry, it is now possible to identify routinely and reliably low numbers of leukemia and lymphoma cells (minimal residual disease).

Keywords: bone marrow; CD markers; flow cytometry; leukemias; lymph node; lymphocyte; lymphomas; minimal residual disease; peripheral blood.

Introduction

During the past two decades, immunological marker analysis (immunophenotyping) of lymphocytes has rapidly evolved from a research technique into a routine laboratory diagnostic test. This was largely owing to the development of modern bench-top flow cytometers as well as the availability of many different monoclonal antibodies (McAbs) that mainly detect membrane-bound antigens, as well as a limited number of intracellular antigens. Flow cytometry requires single-cell suspensions, which are easily obtained from peripheral blood (PB) samples and bone marrow (BM) aspirates. However, single-cell suspensions can also be prepared from lymph node biopsies and fine needle aspirates, BM trephine biopsies and biopsies from other lymphoid organs. However, in many laboratories immunohistochemistry is preferred for immunophenotyping of lymphoid tissue biopsies, because this technique allows a combined interpretation with histomorphologic and immunophenotypic information.

The aim of this review is to provide an up-to-date summary of the current diagnostic applications of flow-cytometric immunophenotyping of lymphocytes. The information on normal lymphocyte subsets is presented as a normal template for clinical samples. Aberrant features of lymphoid differentiation form the basis of flow-cytometric immunophenotyping for the diagnosis and follow-up of lymphoid malignancies, which is also discussed in more detail.

Flow cytometry for immunophenotyping of lymphocytes

Current routine fluorescence-activated flow cytometers typically consist of an argon-ion laser light source, which provides monochromatic light at 488 nm. Individual cells flow through the laser beam, refract the light and, if present, fluorochromes are excited and emit fluorescence light. The refracted light is measured at two different angles, giving the so-called scatter characteristics. In the forward direction of the laser beam, the forward scatter (FSC) is measured, and at 90°, the sideward scatter (SSC) is recorded. The FSC signal is primarily dependent on the volume of the particles or cells, whereas the SSC signal provides a measure of internal organization, cytoplasmic granularity, nuclear density, and external cell structure. Most currently used single-laser flow cytometers allow the simultaneous detection of at least three fluorochromes. Fluorescein isothiocyanate (FITC), phycoerythrin (PE), and peridinin chlorophyll protein (PerCP) are most commonly used, but other fluorochromes, including Alexa dyes and the tandem fluorochromes PE-Cy5, PerCP-Cy5, and PE-Cy7, can be used as well. Guidelines for optimal choice of fluorochromes, such as preference of the brightest fluorochromes (e.g., PE) for weakly expressed antigens, have been reported (1–3).

Guidelines for optimal choice of fluorochromes, such as preference of the brightest fluorochromes (e.g., PE) for weakly expressed antigens, have been reported (1–3). Flow cytometers equipped with a second or even third laser are increasingly introduced into immunological and hematological diagnostic laboratories, allowing the inclusion of additional fluorochromes, e.g., APC, APC-
Cy7, Pacific Blue, and Cascade yellow, implying that at least six (up to eight or more) different fluorochromes can be applied simultaneously (4, 5). It should be noted that the use of multiple fluorochromes requires an appropriate instrument set-up to compensate for spectral spillover. Finally, if tandem fluorochromes are applied, the photosensitive degradation of these molecules should be considered. Careful selection of fluorochromes and antibodies can, however, limit the risk of false-positive and false-negative results.

The amount of information obtained during measurement is extensive, and sophisticated software programs are required for fast and easy data processing and analysis. In routine lymphocyte immunophenotyping, typically at least 5000–10,000 cells are evaluated, and for each cell, generally at least five different parameters are measured and stored. For immunophenotyping of lymphoid cells, initial gating on light scatter characteristics is frequently used. The gated cells can subsequently be analyzed for various fluorescence parameters. For instance, the combination of low SSC and weak to intermediate expression of the CD45 molecule can identify a unique blast cell region to set the gate on most immature lymphoid cells (6). It is clear that modern flow cytometry allows multiparameter analysis of various cell populations.

Leukocyte antigens and the CD nomenclature

A large proportion of the McAbs against leukocyte antigens has been grouped into antibody clusters based on their reactivity with identical molecules. Each antibody cluster has its own code, the so-called CD (cluster of differentiation) code. As a consequence of the CD nomenclature, the molecules and epitopes recognized by the clustered antibodies have been defined as CD molecules, CD antigens, and CD epitopes. During eight Human Leukocyte Differentiation Antigen (HLDA) Conferences (Paris, 1982; Boston, 1984; Oxford, 1987; Vienna, 1989; Boston, 1993; Kobe, 1996; Harrogate, 2000; Adelaide 2004), 339 CD codes have been established (summarized in refs. 7–9).

Table 1 provides detailed information about clustered and non-clustered antibodies, which are frequently used for immunophenotyping of normal and malignant lymphocytes. This information concerns the function of the recognized antigen and its molecular mass, as well as the reactivity of the antibodies with leukocytes. For each cluster a few typical McAb examples are given.

Precursor markers

Hematopoietic stem cells generally express the CD34 antigen and some of them also express the CD117 antigen, which is the receptor for the stem cell factor (c-kit). CD117 expression is characteristic for myeloid precursors, while in the lymphoid compartment, CD117 expression is restricted to a small natural killer (NK)-cell subset and early T-cell precursors (10). Terminal deoxynucleotidyl transferase (TdT) is a nuclear enzyme that is expressed in all immature lymphoid cells (immature precursor B-cells and immature cortical thymocytes) and a very small fraction of precursor myeloid cells (11). Other markers that are expressed by stem cells are CD90 and CD133 (12, 13). CD90 is expressed on some early T- and B-lymphocytes and a small subset of CD34+ cells in bone marrow; it is absent on human thymocytes (14). CD133 (AC133, prominin-1) is rapidly downregulated when human stem cells differentiate into phenotypically restricted cells. Within the hematopoietic system, CD133 can be found on non-committed CD34+ cells, as well as the majority of CD34+ cells committed to the granulocytic/monocytic pathway (15).

B-cell markers

The CD19, CD22, and CD72 antigens are pan-B-cell markers expressed on the cell surface membrane of both immature and mature differentiation stages (16, 17). The CD79a and CD79b molecules (also known as mb-1 and B29, or Igα and Igβ, respectively), are signal-transducing elements associated with immunoglobulin (Ig) molecules (18). Expression of the CD79 molecules is only found in B-lineage cells: during precursor B-cell differentiation, both CD79 chains are expressed in the cytoplasm (CyCD79), whereas in the more mature differentiation stages, the CD79 chains are associated with surface membrane-bound Ig (SmIg) molecules (SmIg-CD79 complex). CyCD79a is one of the earliest B-lineage markers found in B-cell ontogeny, preceding surface expression of CD19 (19, 20). Characterization of the various B-cell differentiation stages can be based on the expression of B-cell antigens (CD10, CD20, CD21, CD23, and CD37), as well as the various expression patterns of Ig chains (16, 17, 20, 21). The characteristic feature of the pre-B-cell stage is the weak cytoplasmic expression of Igμ heavy chains in the absence of normal Ig light chains, but in the presence of Vpre-B (CD179a) and λ5 (CD179b) surrogate light chains (pre-B Cylgμ): these surrogate light chains are already found in the cytoplasm in earlier differentiation stages (20, 21). In some of the pre-B-cells, the incomplete pre-B Ig complex is also weakly expressed on the cell surface membrane (pre-B Igμ-CD79 or pre-B-complex); however, this faint expression is not easily detectable by flow cytometry (18). Naïve immature B-cells in BM express SmIgα chains and are CD27-, while the vast majority of early immunocompetent B-cells in PB are SmIgμδ+. Positive B-cell selection in germinal centers is associated with somatic hypermutation of the Ig genes and Ig isotype switching, and most B-cells then become SmIgα, SmIgδ, or SmIgε and CD27+. Plasma cells express high levels of cytoplasmic Ig (Cyig) and are often positive for the CD38 antigen and the CD138 (syndecan-1) molecule.

T-cell markers

Virtually all T-lineage cells express the CD2 antigen and the CD7 antigen (22, 23). Moreover, the T-cell specific transcription factor TCF1 can be detected in the
Table 1  Detailed information concerning antigens suited for immunophenotyping studies of lymphoid cells.

<table>
<thead>
<tr>
<th>CD no.*</th>
<th>Antigen name(s)/function</th>
<th>Molecular mass (Mr), kDa</th>
<th>Reactivity with hematopoietic cells</th>
<th>Typical McAb examples (no complete listing)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Precursor markers</strong></td>
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<tr>
<td>CD34</td>
<td>Precursor antigen</td>
<td>gp105–120</td>
<td>Lymphoid and myeloid progenitor cells</td>
<td>HPCA-1/My10, HPCA-2/8G12, BI-3C5</td>
</tr>
<tr>
<td>CD90</td>
<td>Thy-1 antigen</td>
<td>gp18</td>
<td>Some early T and B lymphocytes and a small subset of CD34&lt;sup&gt;+&lt;/sup&gt; cells in bone marrow</td>
<td>F15-42-1, 5E10</td>
</tr>
<tr>
<td>CD117</td>
<td>SCFR (stem cell factor receptor); c-kit; SLF (Steel factor) receptor</td>
<td>gp145</td>
<td>Hematopoietic progenitor cells, most colony forming cells, and mast cells</td>
<td>17F11, YB5.B8, 104D2</td>
</tr>
<tr>
<td>CD133</td>
<td>PROM1, prominin1</td>
<td>gp97</td>
<td>Primitive hematopoietic stem cells, CD34&lt;sup&gt;+&lt;/sup&gt; cells committed to the granulocytic/monocytic pathway</td>
<td>AC133</td>
</tr>
<tr>
<td>–</td>
<td>TdT/function in Ig and TCR gene rearrangement (insertion of nucleotides at junction sites)</td>
<td>p58</td>
<td>Immature lymphoid cells, small fraction of myeloid precursor cells, virtually all ALL, and some AML</td>
<td>Conventional antisera and HTdT-1 McAb</td>
</tr>
<tr>
<td><strong>B-cell markers</strong></td>
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<tr>
<td>CD10</td>
<td>Common ALL antigen (CALLA)/neutral endopeptidase (enkephalinase)</td>
<td>gp100</td>
<td>Subset of precursor-B-cells, subset of B-lymphocytes (follicular center cells), subset of cortical thymocytes, granulocytes</td>
<td>J5, VIL-A1, BA-3</td>
</tr>
<tr>
<td>CD19</td>
<td>pan-B-cell antigen/function in B-cell activation; associates with CD21 antigen (CR2)</td>
<td>gp90</td>
<td>Precursor B-cells and B-lymphocytes</td>
<td>Leu-12, B4, HD37</td>
</tr>
<tr>
<td>CD20</td>
<td>B-cell antigen/function in B-cell activation</td>
<td>p35</td>
<td>Subpopulation of precursor-B-cells, all B-lymphocytes, follicular dendritic reticulum cells</td>
<td>Leu-16, B1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L26 detects intracellular epitope (CD20-Cy Ab)</td>
</tr>
<tr>
<td>CD20</td>
<td>Mature B-cell antigen, FMC7 antibody detects a conformational epitope on the CD20 molecule</td>
<td>gp105</td>
<td>B-lymphocytes (all B-lymphocytes positive for FMC7 are always CD20 positive)</td>
<td>FMC7</td>
</tr>
<tr>
<td>CD21</td>
<td>B-cell antigen/CRI (C3d receptor); EBV receptor</td>
<td>gp140</td>
<td>Subpopulations of B-lymphocytes (e.g., follicular mantle cells), follicular dendritic reticulum cells, subset of thymocytes</td>
<td>OKB7, B2</td>
</tr>
<tr>
<td>CD22</td>
<td>B-cell antigen/function in B-cell adhesion and B-cell activation</td>
<td>gp135</td>
<td>Precursor B-cells and B-lymphocytes</td>
<td>Leu-14/SHCL-1, RFB4, HD39</td>
</tr>
<tr>
<td>CD23</td>
<td>B-cell antigen/FcRll (low-affinity Fc receptor for IgE); two types of FcRll exist that differ in their cytoplasmic domain (FcRlla and FcRllb)</td>
<td>gp45</td>
<td>FcRlla is expressed by a subpopulation of B-lymphocytes (e.g., follicular mantle cells) and B-CLL cells; FcRllb is expressed by subsets of B-lymphocytes, monocytes, eosinophils, dendritic cells</td>
<td>Leu-20/EBVCS-5, Tü1</td>
</tr>
<tr>
<td>CD37</td>
<td>B-cell antigen (tetraspan molecule)</td>
<td>gp40–52</td>
<td>B-lymphocytes; weak expression on T-cells, monocytes and granulocytes</td>
<td>RFB7, Y29/55</td>
</tr>
<tr>
<td>CD72</td>
<td>B-cell antigen/ligand for CD6 mb-1; Igα (disulfide linked to CD79b and associated with Smlg)/signal transduction from Smlg to cytoplasm</td>
<td>gp43/39</td>
<td>Precursor B-cells and B-lymphocytes</td>
<td>J3-109</td>
</tr>
<tr>
<td>CD79a</td>
<td>gp32–33</td>
<td>Precursor B-cells (cytoplastic expression; CyCD79a) and Smlg&lt;sup&gt;+&lt;/sup&gt; B-cells (membrane expression; SmCD79a)</td>
<td>HM57 detects intracellular epitopes of CD79a (CD79a-Cy Ab)</td>
<td></td>
</tr>
<tr>
<td>CD79b</td>
<td>gp37–39</td>
<td>Precursor B-cells (cytoplastic expression; CyCD79b) and Smlg&lt;sup&gt;+&lt;/sup&gt; B-cells (membrane expression; SmCD79b)</td>
<td>B29/123 detects intracellular epitope of CD79b (CD79b-Cy Ab)</td>
<td></td>
</tr>
<tr>
<td><strong>CD138</strong></td>
<td>Plasma cell antigen</td>
<td>gp20</td>
<td>Plasma cells and multiple myeloma</td>
<td>B-B4</td>
</tr>
<tr>
<td><strong>CD179a</strong></td>
<td>VpreB, proximal part of the surrogate light chain</td>
<td>gp18</td>
<td>Precursor-B-cells</td>
<td>HSL96</td>
</tr>
<tr>
<td><strong>CD179b</strong></td>
<td>λ chain, distal part of the surrogate light chain</td>
<td>gp22</td>
<td>Proteins that bind to the surrogate light chain</td>
<td>HSL11</td>
</tr>
<tr>
<td>CD no.</td>
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<tr>
<td>–</td>
<td>Pre-B CyIg (weak cytoplasmic expression of IgM chain)</td>
<td>gp70</td>
<td>Pre-B-cells; only μ heavy chains weakly expressed in the cytoplasm (no mature Ig light chains)</td>
<td>Selected anti-μ antisera</td>
</tr>
<tr>
<td>–</td>
<td>SmIg (surface membrane immunoglobulin); IgM, IgD, IgG, IgA, IgE</td>
<td>Depends on Ig class</td>
<td>SmIg-positive cells; each B-cell clone expresses only one type of Ig light chain (κ or λ), but may express multiple IgH chains</td>
<td>Conventional antisera and McAb</td>
</tr>
<tr>
<td>–</td>
<td>CyIg (cytoplasmic immunoglobulin)</td>
<td>Depends on Ig class</td>
<td>CyIg positive cells (immunoblasts, immunocytes, and plasma cells)</td>
<td>Conventional antisera and McAb</td>
</tr>
</tbody>
</table>

**T-cell markers**

| CD1    | T6 antigen; common thymocyte antigen/MHC-like protein; can associate with β2-microglobulin | CD1a: gp49 | Cortical thymocytes, Langerhans cells, subpopulations of dendritic cells and B-lymphocytes | OKT6, NA1/34 |
|        | T11 antigen; SRBC receptor (E rosette receptor); LFA-2/receptor for T-cell activation; ligand for CD58 (LFA-3) | CD1b: gp45, CD1c: gp43 | All T-cells, most NK-cells; three different antigenic epitopes are known, of which one is the SRBC binding site | 7C4/160/4G9, 7C6/162/3B10 |
| CD2    | T3 antigen (associated with TCR/ signal transduction from TCR to cytoplasm) | gp50 | Immature T-cells (cytoplasmic expression; CyCD3) and mature T-cells (membrane expression) | Leu-4/SK7, OKT3, UCHT1, VIT-3 |
| CD4    | T4 antigen/involved in MHC class II-restricted antigen recognition; HIV receptor | gp59 | Subset of cortical thymocytes, helper/inducer T-lymphocytes, subpopulation of monocytes and macrophages; some AML | Leu-3a, OKT4 |
| CD5    | T1 antigen/function in T-cell proliferation; ligand for CD72 antigen on B-lymphocytes | gp67 | Thymocytes and mature T-lymphocytes, subpopulation of B-lymphocytes; B-CLL | Leu-1, T1 |
| CD6    | T12 antigen/related to CD5 antigen | gp120 | Thymocytes and mature T-lymphocytes, subpopulation of B-lymphocytes; B-CLL | OKT17, T12 |
| CD7    | Tp41 antigen/Fc receptor for IgM (FcµRI) | gp41 | Almost all T-cells, NK-cells, sub-population of immature myeloid cells; some AML | Leu-9, 3A1, WT1 |
| CD8    | T8 antigen; the CD8 molecule consists of two disulfide-linked chains: α-β homodimer or α-β heterodimer/involved in MHC class I-restricted antigen recognition | CD8-α: gp32, CD8-β: gp32 | Subpopulation of cortical thymocytes, cytotoxic/suppressor T-lymphocytes, subpopulation of NK-cells | Most CD8 antibodies detect CD8-α chain: Leu-2a, OKT8 |
| –      | TCRαβ (classical TCR; TCR2) | gp80 (44/40) | TCRαβ is expressed by the majority of mature CD3<sup>+</sup> T-cells | WT31, BMA031 |
| –      | TCRαβ chains | gp40 | Intracellular TCRαβ chains in most cortical thymocytes and many T-ALL | βF1 |
| –      | TCRγδ (alternative TCR; TCR1) | gp75 (44/42 or 55/42) | TCRγδ is expressed by a minority of mature CD3<sup>+</sup> T-cells | anti-TCRγδ<sub>1</sub>-1, TCRδ1 |
| –      | Vβ domains | gp10 | Subpopulation of thymocytes and T-cells | Large panel of 20–25 Vβ McAb, recognizing 65–75% of blood T-lymphocytes |
| –      | Vδ domains | gp10 | Subpopulation of thymocytes and T-cells | Vδ<sub>1</sub> (R9.12), Vδ<sub>2</sub> (Immu389, BB3) |
| –      | Vγ domains | gp10 | Subpopulation of thymocytes and T-cells | Vγ<sub>2</sub>/3/4 (23D12), Vγ<sub>3</sub>/35 (65.3), Vγ<sub>8</sub> (R4.5) |
| –      | TCF1, T-cell-specific transcription factor | p27 | Thymocytes, activated T-cells | Vγ<sub>9</sub> (Ti-γA, Immu360) |

**NK-cell markers**

| CD56   | NCAM; PI-linked and transmembrane forms human natural killer cell antigen | gp120,140,180 | NK-cells, some T-lymphocytes (neuroectodermal cells) | Leu-19/My31, NKH-1 |
| CD57   | gp110 | Subpopulation of NK-cells, subpopulation of T-lymphocytes, some B-cells | Leu-7/HNK-1 |
(Table 1 continued)

<table>
<thead>
<tr>
<th>CD no.*</th>
<th>Antigen name(s)/function</th>
<th>Molecular mass (M&lt;sub&gt;m&lt;/sub&gt;), kDa</th>
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</tr>
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<tbody>
<tr>
<td>CD94</td>
<td>Kp43, HLA class I inhibitory receptor for different HLA-A, -B or -C alleles</td>
<td>gp43</td>
<td>NK-cells, minor subset of T-lymphocytes</td>
<td>Z199, Z270, NKH-3, HP-3B1</td>
</tr>
<tr>
<td>CD158</td>
<td>KIR family, inhibitory NK-cell receptors specific for different HLA class I allotypes</td>
<td>Depends on KIR type</td>
<td>NK-cells, minor subset of T-lymphocytes</td>
<td>EB6, HP-3E4, GL183, CH-L DX9, Z27, Q66, Q241</td>
</tr>
<tr>
<td>CD161</td>
<td>Killer cell lectin-like receptor subfamily B, member 1, NKR-P1A</td>
<td>p25</td>
<td>Most NK cells, subsets of thymocytes and fetal liver T-cells, subsets of mature CD3&lt;sup&gt;+&lt;/sup&gt; T-cells, particularly CD1d-reactive NKT cells</td>
<td>DX12, 191B8</td>
</tr>
<tr>
<td>CD335</td>
<td>NCR1, Nkp46, natural cytotoxicity receptor</td>
<td>p34</td>
<td>NK-cells</td>
<td>BAB281</td>
</tr>
<tr>
<td>CD336</td>
<td>NCR2, Nkp44, natural cytotoxicity receptor</td>
<td>p31</td>
<td>Activated NK-cells</td>
<td>Z231</td>
</tr>
<tr>
<td>CD337</td>
<td>NCR3, Nkp30, natural cytotoxicity receptor</td>
<td>p22</td>
<td>NK-cells</td>
<td>Z25</td>
</tr>
</tbody>
</table>

Non-lineage restricted markers

| CD9     | p24 antigen (tetraspan molecule)/induction of aggregation of platelets | p24 | Subpopulation of precursor-B-cells, subpopulation of B-lymphocytes (follicular center cells), monocytes, megakaryocytes, platelets, eosinophils, basophils | BA-2 |
| CD11b   | p170 antigen, integrin αM, complement component receptor 3α | gp170 | Neutrophils, monocytes, natural killer cells, and a subset of lymphocytes (CD8<sup>+</sup> T-cells, memory B-cells) | ICRF44, D12, CLB-mon-gran/1 |
| CD11c   | p150,95 antigen (integrin αX chain); associated with CD18 antigen/adhesion molecule; CR4 (C3bi, C3dg receptor) | gp150 | Monocytes, granulocytes, subpopulations of lymphocytes (e.g., HCL-like cells in the spleen and NK-cells); no membrane expression in LAD-1 patients | Leu-M5/SHCL3 |
| CD16    | F<sub>c</sub>γRIII (low-affinity Fc receptor for IgG); F<sub>c</sub>γRIIIA (transmembrane form) and F<sub>c</sub>γRIIIB (P1-linked form) | gp50–65 | Neutrophilic granulocytes, monocytes (weak), macrophages (weak), NK-cells; F<sub>c</sub>γRIIIb is absent on granulocytes in patients with PNH | Leu-11b, 5D2, CLB-FcR-gran/1; these antibodies recognize both F<sub>c</sub>γRIIIA and F<sub>c</sub>γRIIIB |
| CD24    | B-cell-granulocytic antigen; P1-linked protein on granulocytes | gp42 | Subpopulation of (precursor) B-cells, granulocytes; absent on granulocytes in patients with PNH | BA-1, VIB-C5 |
| CD25    | Tac antigen/α chain of the IL2 receptor (low-affinity IL2R); high-affinity IL2R when associated with β chain (CD122 antigen) and/ or γ chain | gp55 | Activated T-cells, activated B-lymphocytes, activated macrophages; HCL | 2A3, ACT-1 |
| CD27    | TNF receptor superfamily, member 7/receptor for CD70 antigen | p120 (55/55) | Mature T-cells, activated T-cells, memory B-lymphocytes, and NK cells | L128, OKT18A, 1A4CD27 |
| CD38    | T10 antigen | gp45 | Activated T- and B-cells, precursor cells (e.g., thymocytes), subpopulations of B-cells (e.g., follicular center cells), plasma cells | Leu-17/HB7, OKT10 |
| CD103   | HML-1 (human mucosal lymphocyte 1 integrin); αE chain, which is associated with β7 chain | gp150,25 | Mucosa-associated T-lymphocytes (especially intra-epithelial CD8<sup>+</sup> T-cells), 2–6% of blood lymphocytes; part of mucosal T-NHL (not other peripheral T-NHL) and HCL | B-ly7 |
|        | Granzyme B | p27 | Cytotoxic T-lymphocytes, lymphokine-activated killer (LAK), and NK cells | CLB-B11, GrB-7, MCA1645 |
Together these Vb normal and malignant T-cells with TCR most against the protein products of V gene segments of TCR, TCR membranes in association with the T-cell receptor. T-cells express CD3 molecules on their cell surface plasma (CyCD3) of immature T-cells, whereas mature the expression of the CD79 antigen during B-cell differ-

sion (25). In addition, antibodies against most V domains of TCRγδ molecules have become available (see Table 1).

**Natural killer cell markers**

NK-cells are positive for the pan-T-cell marker CD7 and often also for the CD2-antigen, but they are negative for the TCR-CD3 complex. Most NK-cells are positive for the CD16 and the CD56 antigens, and some NK-cells express the CD57 antigen (26). Expression of the CD8 antigen can be found on 5–15% of NK-cells. Recently, several human NK-cell receptors have been identified that trigger the process of natural cytotoxicity. These so-called “natural cytotoxicity receptors” (NCR) include Nkp46 (CD335), Nkp44 (CD336) and Nkp30 (CD337) proteins, which mediate cytosis of allogeneic cells or tumor cells and induce cytokine production (27, 28). Moreover, NK-cells differentially express various inhibitory receptors (“killing inhibitory receptors”, KIR) specific for HLA class I molecules, e.g., CD94, the CD158 family (at least 12 members), and CD161, which prevent cytotoxicity against autologous HLA class I-positive cells (29). NCRs are exclusively expressed by NK-cells, while other NK-markers are not NK-cell specific. For example, T-lymphocytes, especially CD8+ T-lymphocytes, can also be positive for CD16, CD56, and CD57 molecules, as well as for KIRs (27, 29). Finally, the cytolytic granules of NK-cells and cytotoxic CD8+ T-cells contain several cytotoxic proteins, including perforins, granzymes, and TIA1 (T-cell “restricted” intracellular antigen).

**Immunophenotyping of normal peripheral blood lymphocytes**

Immunophenotyping of PB lymphocytes is an important tool in the diagnosis of immunological and hematological disorders such as immunodeficiencies, lymphoproliferative disorders and infectious diseases. Age-related reference values for major PB lymphocyte subsets are now available (30–32; Figure 1 and Table 2). The relative and absolute sizes of lymphocyte subsets are particularly dynamic during childhood because of maturation and expansion of the immune system in the first years of life. The absolute numbers of B-cells (as defined by CD19 expression) increases two-fold immediately after birth, and subsequently decreases 6.5-fold from 2 years to adulthood (Figure 1). This decrease mainly involves immuno-
**Figure 1** Reference values for main lymphocyte subpopulations in peripheral blood in neonates, children, and adults. The absolute values are indicated from the 5th to the 95th percentile. The age range of the childhood age groups and the number of individuals per age group are indicated. NCB, neonatal cord blood. Based on the original data by Comans-Bitter et al. (31).

The absolute size of the CD3⁺CD4⁺ T-lymphocyte subpopulation follows the same pattern as the total CD3⁺ population, but the CD3⁺CD8⁺ T-lymphocytes remain stable from birth up to 2 years of age, followed by a gradual three-fold decrease toward adult levels (31). The absolute number of CD3⁺ T-cells in adults decreases with aging: this involves both CD4⁺ and CD8⁺ subsets, so the CD4/CD8 ratio remains stable (30). Interestingly, in adults there are differences related to gender, with significantly lower absolute values of CD4⁺ T-cells (particularly CD45RO⁺) in females (30). Approximately 1–2% of CD4⁺ T-cells in PB show high CD25 expression and represent a so-called T-cell regulatory population that plays a key role in the maintenance of self-tolerance, thus preventing severe or even fatal immunopathology (35, 36). The vast majority of PB T-lymphocytes belong to the TCRαβ lineage, while TCRγδ⁺ T-cells generally comprise only 5–15% of PB T-cells. A small subset of TCRαβ⁺ T-cells, generally expressing Vα24 and Vβ11 (TRAV10 and TRBV25-1) according to the IMGT (International ImMunoGene-
cells largely correspond to CD22 brightCD45 bright cells CD45RO–, with variable expression of CD57 (39). 

4. This proportion and ratio further increase with age CD4/CD8 ratio in BM is 0.5 from the first year of life onwards (40).

Table 2 Relative frequency of lymphoid subsets in blood and lymphoid tissues.

<table>
<thead>
<tr>
<th></th>
<th>Peripheral blooda</th>
<th>Lymph nodeb</th>
<th>Tonsilsb</th>
<th>Splenda</th>
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<tr>
<td></td>
<td>Children 2–5 years</td>
<td>Adults 5–15 years</td>
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<tr>
<td>CD19+ B-lymphocytes</td>
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<tr>
<td>NK-cells</td>
<td>10%</td>
<td>13%</td>
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<td>1%</td>
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</table>

a For details see Comans-Bitter et al. (31). b Based on data from Battaglia et al. (42). c Average values obtained for adult patients, for details see Bergler et al. (44). d Based on data from Colovai et al. (45).

Immunophenotyping of normal bone marrow

B-lineage cells constitute the major part of the lymphoid compartment of normal BM in children and approximately one-third in adults (40). More than half of these cells in children are B-cell precursors expressing CD10, while in adult BM, mature CD10+CD20+ B-cells predominate (40). Within the CD19+ B-cells, at least four sequential maturation stages can be discriminated based on CD10 and CD20 expression. These four differentiation stages form a continuous staining pattern in a CD10/CD20 plot as a result of the stepwise loss of CD10 and the gradual gain of CD20 during maturation (Figure 2A) (41). Plasma cells, when present, reveal a minor CD10−/CD20− population. Adding TdT to the CD10/CD20 combination of McAb allows better discrimination between the immature CD10bright cells that are TdT+ and the more mature CD10− cells that are TdT−. The most immature B-cell precursors (TdT+CD10+ cells) largely correspond to the CD34+CD22−CD45dim cells. CD45 expression increases during maturation, followed by increased CD22 expression. Mature CD10−CD20+ B-cells largely correspond to CD22brightCD45bright cells in the CD22/CD45 dot plot. When present, CD22− CD19−CD34+ pro-B-cells can be identified as a minor population in addition to the earlier described four stages of B-cell differentiation (20, 41). In a standardized setting, with a calibrated flow cytometer and using the same McAb clones, the staining patterns for all precursor B-cell subsets described are quite stable. Shifts in the relative sizes of the subpopulations or a different location in the plot may therefore indicate (ab)normal variations or aberrations in the B-cell development.

T-cells constitute 10–20% of normal lymphoid cells in BM of children, and their proportion gradually increases to 20–40% in adult BM (40). In contrast to PB, the majority of BM T-cells are CD8+, and the average CD4/CD8 ratio in BM is 0.5 from the first year of life onwards (40).

Immunophenotyping of normal lymph nodes and other lymphoid tissues

Large-scale studies of lymphocyte subpopulations in normal lymph nodes are limited owing to ethical constraints. Most information concerns either reactive or neoplastic lymph nodes. Nevertheless, a few studies have generated a good impression of lymphocyte subsets in normal lymph nodes. In contrast to PB, B-cells constitute nearly half of the lymphocytes (approx. 40%), and consequently the T-cell predominance is less obvious (approx. 55%) (42). Up to one-third of lymph node B-cells coexpress the CD5 antigen; they are mainly located within the follicular mantle zone (33). Follicular center B-cells may weakly express the CD10 antigen. Frequencies of these CD10+ B-cells, generally showing bright CD20 expression, are frequently increased in cases of reactive lymphoid follicular hyperplasia (43). Among CD3+ cells, the predominance of CD4+ cells is much more prominent than in PB, with an average CD4/CD8 ratio above 4. The frequency of NK-cells in the lymph nodes is very low (approx. 1%) (42).

More knowledge has been gained about normal lymphocyte subsets in human tonsils. This is typically a B-cell organ, where CD19+ B-cells comprise the majority of lymphocytes. However, the proportion of B-cells declines from approximately 70% in early childhood to approximately 50% in adults (44). The remaining lymphocytes are CD3+ T-cells. Similarly to normal lymph nodes, CD4+ T-cells predominate in normal tonsils, with an average CD4/CD8 ratio above 4. This proportion and ratio further increase with aging. Naive CD45RA+ T-cells increase during childhood and adolescence, and gradually decrease thereafter. In contrast, CD45RO+ T-cells show an opposite trend. Tonsillar CD3+/CD56+ NK-cells constitute a minor population at all ages (44).
Figure 2. (A) Normal B-cell development in bone marrow (BM). A CD19 gate was used to select B-lineage cells. Within the CD19+ B-cells, at least four subpopulations can be identified. The most immature B-cell precursors (pro-B-cells) are CD34+ /TdT+ /CD10−. The pre-B-I-cells are CD10bright and TdT+ (indicated as black dots). Pre-B-II-cells show CyIg expression, most of them are negative for TdT and many are CD20+. Mature B-cells express SmIg+, CD20 and are CD10−. (B) Flow-cytometric immunophenotyping of BM cells from a precursor-B-ALL patient at diagnosis. The precursor-B-ALL cells expressed CD19, CD10, CD22 on the cell surface and TdT intracellularly, but they did not produce IgM protein and were CD20-negative. Such an immunophenotype is typical for common ALL. In contrast to normal B-cell precursors, the ALL cells showed aberrant absence of CD45 and overexpression of CD10. Based on such a leukemia-associated immunophenotype, it is possible to detect minimal residual disease. The remaining few normal B-cell precursors are represented by gray dots.

CD19+ B-lymphocytes also form the largest subset in normal human spleen, constituting approximately 55% of cells, while CD3+ T-cells are slightly less prevalent (40% on average) (45). These proportions can be reversed in reactive, non-malignant spleens. The CD4/CD8 ratios and percentage of NK-cells are similar to those found for PB lymphocyte subsets. Less than 10% of B-cells in adult spleen coexpress CD5 (33). A typical characteristic of the spleen is the presence of a substantial population (approx. 5%) of early plasma cells showing the CD19+ CD38+ CD20−CD138− immunophenotype and positivity for cytoplasmic Ig light chains (45).

Information about normal lymphoid subsets in human thymus was obtained by studying thymic lobes from children undergoing major cardiac surgery (46). The majority of the thymic lymphoid population (>75% after the first month of life) consist of CD4- /CD8+ double-positive thymocytes. A significant proportion of these cells already expresses the CD3/TCRαβ complex. Immature double-negative CD4+ /CD8+ thymocytic precursors, and more mature CD4+ and CD8+ single-positive thymocytic subsets each constitute approximately 5%. TCRγδ+ T-lineage cells, B-lineage cells (both immature and mature), and NK-lineage cells are all detectable in childhood thymuses, albeit at very low frequencies (<0.5%) (46).

Immunophenotyping of lymphoid malignancies at diagnosis

Modern classification systems for lymphoid malignancies incorporate information yielded by cytomorphology and/or histopathology, cytochemistry, immunophenotyping and cytogenetics. Such a combined diagnostic approach forms the basis of the current WHO classification of tumors of hematopoietic and lymphoid tissues (47, 48). For decades, cytomorphology and cytochemistry have been used for the initial diagnosis of leukemia, while the initial diagnosis of non-Hodgkin’s lymphomas (NHL) relies on histopathologic investigation. However, cytomorphology and/or histopathology are generally insufficient to identify biologically and clinically relevant subsets within the main leukemia/lymphoma categories. Immunophenotyping plays an important role in establishing the diagnosis and classifying lymphoid malignancies. Immunophenotyping is a basic investigation, which precisely defines the lineage and stage of differentiation of malignantly transformed hematopoietic cells. Immunophenotyping can also reveal phenotypes highly suggestive of certain chromosome aberrations, but to fully identify such aberrations, cytogenetic and/or molecular studies are required.

Several groups have reported guidelines and antibody panels for the immunophenotyping of lymphoid malignancies (1–3, 49, 50). The most conventional approach consists of two phases (49). The first (screening) phase focuses on the rapid and easy identification of the presence of a lymphoid malignancy and its lineage. In the second (classification) phase, the antibody panel provides more detailed information on the classification of the malignancy (see ref. 49 for detailed antibody panels). A third panel
may contain various antibodies selected to address specific clinical and diagnostic research questions. The antibody panels selected should include appropriate negative controls for both surface and intracellular staining. These negative controls may include isotype-matched control antibodies, but nowadays cell populations that are negative for a particular antibody but are present in the sample are used as negative reference as well. In the past, positivity for a specific antigen was frequently concluded when 20–30% of the target cells (e.g., malignant cells) were expressing this antigen. In multiparameter cytometry, it is possible to precisely delineate the malignant lymphoid population. If it is clear that the neoplastic population exhibits heterogeneous expression of a particular marker, it is more appropriate to provide the percentage of malignant lymphoid cells positive for the antigen involved (51). Some reports emphasize that antigen expression on the leukemic cells should preferably be reported in quantitative units (molecule equivalents of soluble fluorochrome) and the coefficient of variation (2). This is particularly important when expression of a marker is dim, i.e., displaying an expression level that is only slightly shifted compared to negative cells. In acute leukemias, residual normal lymphocytes can serve as an additional negative control and for studying fluorescence intensity (52).

**Immunophenotyping at diagnosis of acute lymphoblastic leukemia**

Immunophenotyping forms the basis of current classification of acute lymphoblastic leukemia (ALL), distinguishing at least seven ALL subtypes (summarized in refs. 48, 53). As indicated in Figure 3, this concerns four types of precursor B-ALL and at least three types of T-ALL. Virtually all ALL cases are TdT-positive, with precursor B-ALL blasts expressing CD79 in their cytoplasm (CyCD79) and all T-ALL showing CyCD3 expression (7).

**Precursor-B-ALL** Precursor B-ALL cells generally display positivity for TdT and CyCD79, but also for HLA-DR, CD19 and usually for CD22. Precursor B-ALL can be further divided based on the expression of CD10, weak cytoplasmic expression of IgM heavy chains (pre-B CyIgM), and weak surface expression of IgM without normal Ig light chains (pre-B SmIgM). Based on these markers, it is possible to discriminate between pro-B-ALL (CD10+/CyIgM–), common ALL (CD10+/CyIgM–), pre-B-ALL (CD10+/CyIgM+/SmIgM–), and transitional pre-B-ALL (CD10+/CyIgM+/SmIgM+) (Figures 2B and 3) (7, 17, 54). For many years “B-ALL” has been regarded as a rare subset of B-lineage ALL. However, substantial evidence has shown that the term “B-ALL” is a misnomer and that it represents a leukemic variant of Burkitt’s lymphoma. Comparably to Burkitt’s lymphoma, “B-ALL” is characterized by chromosome translocations involving c-MYC and Ig genes and has typical features of mature (post-)follicular B-cells such as somatic hypermutation (55). This is in line with the WHO classification, which recognizes Burkitt’s lymphoma/leukemia as a single disease (see later) (48).

More detailed analysis using antibodies specific to components of the surrogate light chain, i.e., Vpre-B (CD179a) and λ5 (CD179b), allows further subdivision of precursor B-ALL, but this is not routinely exploited in clinical practice (21). Furthermore, certain immunophenotypic features distinguish precursor B-ALL with particular chromosomal translocations, which might be of significant prognostic value (reviewed in 56). For instance, high-risk ALL cases with rearrangements of the mixed lineage leukemia (MLL) gene (11q23) typically have a pro-B-ALL phenotype with ectopic expression of the NG2 antigen and/or cross-lineage expression of the myeloid markers CD15 and CD65 (57, 58). Flow cytometry with more sophisticated quantitation of antigen expression can discriminate t(4;11)-positive cases from other MLL-rearranged cases (59). High-hyperdiploid ALL is frequently associated with CD10+ overexpression, CD66c positivity and the absence of surface CD45 (56). The t(1;19) seems to be restricted to CD10+/CD19−/CD34− precursor B-ALL, especially pre-B-ALL (60). The t(12;21) that occurs in more than 25% of pediatric precursor-B-ALL is characterizedly CD66c-negative, CD9-negative or partly -positive, CD20-negative or partly -positive, and frequently positive for myeloid antigens CD13 and/or CD33 (56). Moreover, quantitative flowcytometric analyses showed that t(12;21)+ ALL cells display a higher intensity of CD10 and HLA-DR expression, together with lower levels of CD20, CD45, CD135 and CD34, which can be used for identification of t(12;21)+ cases with a sensitivity of 86% and a specificity of 100% (61). The most frequent aberration in adult ALL, t(9;22), is significantly linked with CD25 and CD66c positivity; expression of CD13 and/or CD33 is frequent (62, 63).

**T-ALL** Virtually all T-ALL cells are positive for CyCD3, as well as for CD2, CD7 and T-cell specific transcription factor TCF1 (7, 22). Based on expression of CD1a and membrane-bound CD3 (SmCD3), T-ALL can be subdivided into three major groups (7, 17, 54). Immature T-ALL cells are negative for both CD1a and SmCD3 and either concern the rarely occurring prothymocytic T-ALL (pro-T-ALL; CyCD3+/CD7−) or the immature thymocytic T-ALL (pre-T-ALL; CyCD3+ and CD2− and/or CD5− and/or CD8−) (17, 54). A significant proportion of immature T-ALL cells do not express cytoplasmic TCR β chains (CyTCRβ), corresponding to non-restricted thymic precursors, and frequently co-express cross-lineage or precursor markers such as CD13, CD33, CD56, and CD34 (64). The common thymocytic T-ALL (intermediate or cortical T-ALL) cells are positive for CD1a, frequently double-positive for CD4/CD8 and partly for SmCD3, and therefore can be divided further into SmCD3− and SmCD3+ types. Mature T-ALL cells are defined as negative for CD1a and positive for SmCD3 (Figure 3) (7, 17).

Based on the presence of SmCD3 and the type of TCR expressed, T-ALL can also be divided into SmCD3− T-ALL (~65%), SmCD3+/TCRαβ+ T-ALL
Figure 3  Hypothetical scheme of lymphoid differentiation. The expression of relevant immunological markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and non-Hodgkin lymphomas (NHL) and indicate where these malignancies can be located according to their maturation arrest. Abbreviations used: AILT, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma (of T-cell type); ALL, acute lymphoblastic leukemia; ATLL, adult T-cell leukemia lymphoma; AUL, acute undifferentiated leukemia; BL, Burkitt’s lymphoma; CLL, chronic lymphocytic leukemia; CTCL, cutaneous T-cell lymphoma; CTLL, cutaneous T-cell leukemia lymphoma (mycosis fungoides/Sézary syndrome); DLBCL, diffuse large B-cell lymphoma; EATL, enteropathy-associated T-cell lymphoma; FL, follicular lymphoma; follic., follicular; HCL, hairy cell leukemia; HTLV, human T-cell leukemia virus; LGL, large granular lymphocyte; LBL, lymphoblastic lymphoma; LPL, lymphoplasmacytic lymphoma; MALT, mucosa-associated tissue lymphoma; MCL, mantle cell lymphoma; mut., mutated; NHL, non-Hodgkin’s lymphoma; PLL, prolymphocytic leukemia; SMZL, splenic marginal zone lymphoma with villous lymphocytes; trans., transitional. For further references see (7).

Immunophenotyping at diagnosis of mature B-cell leukemias

B-cell malignancies are clonal expansions of B-cells characterized by expression of only one type of Ig light chain (κ or λ). The Igκ/Igλ ratio of a malignant B-cell population therefore deviates from the normal ratio characteristic for reactive polyclonal B-cells (normal Igκ/Igλ ratio 1.4; range, 0.8–2.4). Therefore, immunophenotyping is highly suitable for the detection of clonal Igκ B-cells (7). In addition, the differentiation stage of the malignant cell population can be further delineated by investigation of the type of Ig heavy-chain molecule expressed and the presence of certain differentiation markers (7, 17).

Currently, four groups of chronic B-cell leukemias can be distinguished, namely the most frequently occurring B-cell chronic lymphocytic leukemia (B-CLL) representing 85–90% of chronic B-cell leukemias, rare B-cell prolymphocytic leukemia (B-PLL) (<5%), the “hairy cell” leukemia (HCL) (5–10%) and the uncommon “hairy cell variant” leukemia (HCLv) (Table 3).

B-cell chronic lymphocytic leukemia  The typical B-CLL cell is relatively small, usually slightly bigger than normal lymphocytes. The most prevalent type of Ig heavy chain expressed is Igκ, followed by Igλ (9–10%). SmCD3–/TCRγδ– T-ALL (~10%). SmCD3– T-ALL can be further characterized based on monoclonal antibodies specific to particular Vβ, Vγ, or Vδ gene segments (65).
of B-CLL without somatic hypermutation of Ig genes, which is associated with more aggressive biology and poorer outcome (67). The lack of somatic mutations in this high-risk subset of B-CLL correlates with flow cytometric positivity for ZAP-70 and to lesser extent with CD38 positivity (68).

**B-cell prolymphocytic leukemia** When mature B-cell leukemia is characterized by the presence of more than 55% of prolymphocytes (medium-sized, round lymphoid cells with one large, centrally located nucleus) it is classified as B-PLL (48). Immunophenotypically, this rare type of leukemia shows stronger SmIg expression compared to B-CLL, strong positivity of CD20 molecule, and no expression of CD5, CD6 or CD23. HCL cells are also positive for CD11c, CD25, CD103 and HC2, and negative for CD24 (69, 70).

**Hairy cell leukemia and HCLv** The cells of such leukemias have mostly an oval to kidney-shaped nucleus, abundant cytoplasm and a cell membrane with fine, irregular projections (“hairy cell”) (48, 70). This characteristic morphology influences light scatter properties in flow cytometric analysis, which is different from the normal lymphocyte scatter pattern and resembles the monocytic scatter pattern. The immunophenotype of HCL cells is rather unique, showing strong expression of SmIg, CD20 and CD22. HCL cells are also positive for CD11c, CD25, CD103 and HC2, and negative for CD24 (69, 70). CD25 and HC2 antigens are not present on HCLv cells.

**Immunophenotyping at diagnosis of B-cell non-Hodgkin’s lymphomas**

Flow cytometry immunophenotyping is increasingly used for diagnosis of B-NHL (reviewed in 71). Similarly to mature B-cell leukemias, it relies on either light chain restriction, the absence of normal antigens (e.g., absence of light chain expression, CD20 negativity) and the presence of antigens normally absent on mature B-cells (e.g., CD2 positivity) (71). However, in the majority of cases the initial diagnosis of B-NHL relies on cytomorphology and immunohistochemistry. Nevertheless, flow cytometry has greater sensitivity, reduced interpretive subjectivity, is more optimal for multiple antigen stainings and quantification of antigen expression, and is also faster than immunohistochemistry (71). Therefore, analysis of tissue suspensions by flow cytometry using an extended panel of McAbs is increasing in importance. In an extensive study of 373 lymphoid tissue specimens, the finding of a clonal lymphoma population by flow cytometry was always consistent with a final diagnosis of NHL (72). However, in 11% of NHL cases, flow cytometry could not show an aberrant lymphoid population.

**Reasons for these discrepancies included partial tissue involvement by the NHL, T-cell-rich or lymphohistiocytic-rich B-NHL with a small population of monoclonal B-cells, marked tumor sclerosis or poor tumor preservation (72).**

Flow-cytometric immunophenotyping is particularly attractive for B-NHL categories that enter relatively frequently into a leukemic phase. In such cases, it is of clinical importance to distinguish between classical B-CLL and leukemic presentation of the B-NHL, such as mantle cell lymphoma (MCL), follicular lymphoma (FL) or splenic marginal zone lymphoma with villous lymphocytes (SMZL). Nevertheless, it should be noted that in almost every type of B-NHL, larger or smaller populations of malignant cells might be detectable in PB and/or BM. It is still a subject of extensive debate as to whether flow cytometry of fine needle biopsies would become a standard diagnostic test in B-NHL (73).

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**Table 3** Immunophenotypic characteristics of chronic B-cell leukemias and leukemic B-NHL.

<table>
<thead>
<tr>
<th>Marker</th>
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<th>Leukemic B-NHL</th>
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<tbody>
<tr>
<td></td>
<td>B-CLL</td>
<td>B-PLL</td>
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<tr>
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<td>HC2</td>
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</table>

Symbols: –, <10% of the leukemias are positive; ±, 10–25% of the leukemias are positive; +, 25–75% of the leukemias are positive; ++, >75% of the leukemias are positive; w, weak antigen expression; s, strong antigen expression. B-CLL, B-cell chronic lymphocytic leukemia; B-PLL, B-cell prolymphocytic leukemia; HCL, hairy cell leukemia; HCLv, HCL variant; SMZL, splenic marginal zone lymphoma with villous lymphocytes; MCL, mantle cell lymphoma; FL, follicular lymphoma.
Figure 4 (A) Polyclonal B-cells in reactive lymph node, which show a normal distribution of Igκ and Igλ light chains. A distinct subset of CD10+ B-cells could be identified, suggestive of follicular hyperplasia (indicated as black dots). Such cells also show decreased Ig expression, strong CD38 positivity and weak BCL2 expression. (B) Flow cytometric analysis of a follicular lymphoma (FL) patient. The FL population is characterized by monoclonal Igκ expression, strong CD10 positivity and BCL2 overexpression (black dots). The remaining normal B-cells are represented by gray dots.

Mantle cell lymphoma MCL typically consists of small- to medium-sized cells with scarce cytoplasm and an irregular and/or cleaved nucleus. MCL is frequently disseminated to BM and PB. The immunophenotypic hallmarks of MCL are CD5+, strong CD20+, CD10−, CD23−, moderately strong SmIg+/κ+ and positivity for cyclin D1 (69, 74). The latter is caused by the translocation t(11;14) characteristic for MCL. The combination of strong CD20 positivity and a lack of CD23 expression is an important feature differentiating MCL from B-CLL (75).

Follicular lymphoma FL quickly progresses into a leukemic phase. The immunophenotype of FL cells is distinguishable by quite strong SmIg expression (mostly IgM) and frequently by weak expression of the CD10 antigen. Sometimes FL displays positivity for CD5 or CD23 (48, 69). In virtually all FL cells, overexpression of BCL2 protein is found. Flow-cytometric investigation of intracellular BCL2 can clearly distinguish between (weak) expression in normal reactive B-cells and overexpression in FL cells caused by t(14;18), through which the BCL2 gene is coupled to the IGH gene (Figure 4) (73).

Burkitt’s lymphoma/leukemia The immunophenotype of Burkitt’s lymphoma/leukemia is of mature B-lymphocytes with SmIgM expression and quite strong CD10 expression (7, 48). Furthermore, virtually all Burkitt’s lymphomas and leukemias (“B-ALL”) show overexpression of c-MYC protein resulting from t(8;14) or one of the rarely occurring variant translocations t(2;8) or t(8;22), which transpose the c-MYC gene on chromosome 8 in proximity to one of the Ig genes. Unfortunately, the antibodies for flow-cytometric c-MYC detection are not yet optimally developed.

Diffuse large B-cell lymphoma (DLBCL) Diffuse, large B-cell lymphomas encompass a group of tumors with heterogeneous morphological, immunophenotypic, cytogenetic, molecular and clinical features (76). DLBCL cells do not undergo leukemic dissemination as quickly as MCL or FL, but frequently present as an extramedullary tumor mass. There is no specific DLBCL immunophenotype. Large neoplastic B-cells have strikingly higher FSC compared to normal lymphocytes (71). Pan-B-cell markers (CD19, CD20, CD22 and CD79a) are variably expressed and, despite their B-cell origin, a subset of these lymphomas do not demonstrate SmIg or CyIg expression (48). A proportion of cases express CD5 or CD10. In 20–30% of patients, the BCL2 gene is involved via t(14;18), in 25–40% the BCL6 gene is found to be translocated or somatically mutated, and in 5–15% of cases translocations of the c-MYC gene on chromosome 8 to one of the Ig genes can be demonstrated (77, 78).

Splenic marginal zone lymphoma with villous lymphocytes SMZL is composed of small to medium-sized lymphocytes with round nuclei and quite abundant cytoplasm with small projections, mostly on one side of the cell. The immunophenotype of SMZL is characterized by normal or strong SmIg expression (sometimes also by CyIg expression in a proportion of cells) (48, 79). Similarly to HCL, SMZL can show positivity to CD11c and CD24, but it is usually negative for CD25 and CD103 (69). The majority of SMZL cells are also negative for CD5 and CD23. Although the immunophenotype of SMZL is not fully specific, together with the clinical picture and cytomorphology, it provides sufficient diagnostic information.
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type and nodal marginal zone B-cell lymphoma Mucosa-associated lymphoid tissue (MALT) lymphomas are in fact extranodal presentations of marginal zone lymphomas, as they also involve lymph nodes and spleen. The cytomorphologic presentation of the cells is heterogeneous (48). The spreading of lymphoma cells into PB and BM is not frequent. When this occurs, the number of lymphoma cells is usually low. There are no immunophenotypic features specific for MALT lymphomas and other marginal zone lymphomas. The cells are usually CD20−, CD79a+, CD5− (distinction from MCL and B-CLL), CD10− (distinction from FL), CD23+, CD11cweak and negative for cyclin D1 (distinction from MCL) (48). Some lymphoma cells can be CyIg+.

Lymphoplasmacytic lymphoma The tumors of lymphoplasmacytic lymphoma consist of small lymphocytes with some maturation into “plasmacytoid” lymphocytes (immunocytes) and plasma cells (48). The BM can be massively involved, while the number of lymphoma cells in PB is very small. A high concentration of IgM paraprotein can be frequently found in the serum (Waldenström’s macroglobulinemia). Immunophenotypic analysis shows strong monotypic surface Ig (IgMδ−), frequently with simultaneous CyIg and expression of CD19 and CD20, but not CD5, CD10 or CD23. A high proportion of cases also show positivity for CD25 (80).

Multiple myeloma Multiple myeloma is primarily localized in BM and consists of monoclonal CyIg+ plasma cells (without detectable SmIg expression). The monoclonal CyIg+ plasma cells in this disease are positive for CD38 and CD138, but in principal are negative for all other B-cell markers, including CD79. Malignant plasma cells frequently express CD56 and/or CD117, whereas CD45 expression is frequently lost (81, 82).

Immunophenotyping at diagnosis of mature T-cell and NK-cell malignancies Mature (TdT− and CD1a−) T-cell malignancies are also known as peripheral or post-thymic CD3+ T-cell malignancies, most frequently characterized by expression of TCRβγ molecules. The expression of TCRγδ is rarely observed and mostly concerns T-cell large granular lymphocyte (T-LGL) leukemias and rare subtypes of T-NHL. On rare occasions, TCR molecules on peripheral T-cell malignancies cannot be detected. For further immunological classification, the CD4/CD8 immunophenotype should be taken into account. Intracellular granules containing cytotoxic proteins are a typical finding for CD8+ T-cell malignancies. In contrast to IgB− B-cell malignancies, the currently available immunological markers generally do not allow precise identification of malignant mature T-cells from normal T-lymphocytes. Therefore, it is generally not possible to determine whether suspected T-cells represent reactive cells or clonal leukemic cells. Sometimes the loss of a marker (the absence of
The clonality of suspected T-cell proliferations can only be proven at the DNA (or RNA) level through analysis of the TCR gene configuration by Southern blotting or polymerase chain reaction (PCR) analyses (85, 86). Although only TCR gene analysis can provide definitive proof or exclusion of the clonal origin of the suspected T-cell population, the application of Vβ, Vγ and Vδ antibodies can give a good insight into the TCRαβ and TCRγδ repertoire, particularly if this repertoire is abnormal (25, 65).

NK-cell malignancies are frequently included together with the group of T-cell malignancies and they are in principal positive for cytokytic proteins, but commonly negative for CD8. Moreover, NK-cells and cytotoxic T-cells differentially express various KIRs specific for HLA class I molecules (29). The monotypic expression of KIRs or the absence of detectable expression of any of the KIR antigens might serve as a surrogate “clonality” marker at the protein level (29, 87–89). In addition, NCR proteins, including Nkp46 (CD335), Nkp44 (CD336) and Nkp30 (CD337), can be used to further support the NK-cell origin (27, 89).

T-cell prolymphocytic leukemia T-PLL is a very aggressive T-cell leukemia, frequently with high leukocyte counts, in PB. The majority of T-PLL cells (± 70%) have the CD3+/CD4+/CD8– immunophenotype (48, 90). Similar to other CD4+/CD8– T-cell leukemias, T-PLL cells display strong CD7 expression and are usually negative or very faintly positive for CD25 (90).

Adult T-cell leukemia/lymphoma (ATLL) Such T-cell leukemias are typical in Japan and Caribbean areas and are associated with the endemic occurrence of human T-cell leukemia virus type 1 (HTLV-1) in these regions. The leukemic cells are highly pleomorphic, medium-sized to large lymphoid cells. ATLL generally has a CD4+/CD8– immunophenotype and shows strong positivity for CD25 (48).

Sézary syndrome Sézary syndrome represents a rare leukemic variant of mycosis fungoides, the most frequently occurring T-cell lymphoma of the skin. This syndrome is characterized by the presence of erythroderma, lymphadenopathy and neoplastic T-cells in PB (48, 91). The nuclei of malignant T-cells have a characteristic cerebriform morphology. Similar to ATLL, leukemic T-cells generally have a CD4+/CD8– immunophenotype, but they are CD25+ (91).

Large granular lymphocyte leukemias LGL leukemias form a separate heterogeneous group of chronic T-cell proliferations. The two main subgroups are CD3+ LGL (T-LGL), accounting for 85–90% of LGL leukemias, and CD3– LGL (NK-LGL), constituting the remaining 10–15% (92).

In most cases the diagnosis of LGL leukemia can be made based on the following clinical and laboratory guidelines: 1) lymphocytosis of > 2×109/L, or immunophenotypic or molecular evidence of expansion of a discrete LGL population persisting for 6 months or longer without an obvious cause; 2) morphology of the PB lymphocytes (LGL cytomorphology is not essential, but can support the diagnosis); 3) neutropenia or any other cytopenia in the absence of heavy BM infiltration; and 4) predominance of a specific discrete T-cell subset by membrane marker analysis (e.g., CD8, CD16, CD56, CD57, and HLA-DR) (92, 93).

The vast majority of T-LGL leukemias belong to the αβ-lineage and express a CD16+, CD57+, CD45RA+, CD122+, CD25– phenotype, while positivity for CD56 is observed in a minority of T-LGL patients (Figure 5) (94). They are usually CD4+/CD8–, but sometimes coexpress CD4 and CD8, and rarely the CD4+/CD8– immunophenotype can be observed (48, 94). The rarely occurring TCRγδ+ T-LGL leukemias are predominantly positive for CD2, CD3, CD5, CD7, CD8, and CD57 (95, 96). CD16, CD56, CD11b and CD11c are variably expressed. Half of these cases show the Vγ9/Vδ2 immunophenotype and one-third are positive for Vδ1 and Vγ other than Vγ9 (96). Interestingly, all evaluated Vγ9/Vδ2+ T-LGL cells show the typical antigen-selected motifs in the Vδ2–Jδ1 junctional region (96, 97). This seems to reflect the spectrum of normal TCRγδ+ T-cells in PB.

NK-LGL, defined according to the WHO classification as an aggressive NK-cell leukemia, is occasionally CD8+, but typically CD4+/CD8– (48, 92). Virtually all NK-LGL cells have a characteristic CD16+/CD56+/CD94+/CD45RA+/CD122+/CD25– immunophenotype, whereas only a minority are positive for CD57 (94, 98).

Hepatosplenic T-cell lymphoma Hepatosplenic T-cell lymphoma is a systemic γδ T-cell proliferation characterized by a cytotoxic immunophenotype (CD16+/TIA1+ and frequently CD8–/CD56+) (48, 95, 99). This rare malignancy typically involves liver, spleen and BM and is associated with a very aggressive clinical course and generally unfavorable outcome (99).

Immunophenotyping for minimal residual disease detection in lymphoid malignancies

Thanks to developments in diagnostic tests for hematopoietic malignancies during the past two decades, it is now possible to reliably identify low numbers of malignant cells during and after therapy, i.e., to detect minimal residual disease (MRD) (100). The two main techniques currently applied for MRD detection are multiparameter flow cytometry and real-time quantitative PCR (101–103). Several studies have demonstrated that the results of MRD monitoring in hematopoietic malignancies significantly correlate with clinical outcome (100). In ALL, MRD detection is useful for evaluation of early treatment response in both front-line and relapse treatment protocols (104–109). This has resulted in MRD-based stratification of ALL treatment protocols (104, 105). In contrast, the value of MRD information in chronic lymphoid malignancies relies on monitoring over a clinically rel-
evant disease-specific time-span, with the possibility of attuning treatment based on MRD results. Further studies are required to define the disease-specific “MRD windows” (required sensitivity and time-span) for clinically reliable MRD monitoring in CLL and NHL. The detection of MRD might be particularly valuable in more aggressive disorders, which can be cured with intensive treatment approaches, including BM transplant (110, 111). For example, the development of novel treatment protocols with the inclusion of McAbs aims to achieve an MRD-negative status in all CLL and NHL patients (112). Furthermore, MRD techniques can be applied for several other specific aims, such as detection of minimal central nervous system involvement in ALL, early diagnosis of leukemia/lymphoma in patients with unexplained cytopenias, improved staging of lymphomas, and the detection of malignant cells in autologous grafts (reviewed in 113).

Aberrant immunophenotypes as targets for MRD detection

Acute and chronic leukemias can be regarded as malignant counterparts of cells in immature and more mature stages of hematopoiesis, respectively (1, 16, 17). Most ALL cells indeed have immunophenotypes comparable to normal immature lymphoid cells and CLL/NHL cells resemble mature B- or T-lymphocytes. This implies that the presence of normal hematopoietic cells limits the immunophenotypic detection of leukemic cells (113–115). Despite this inherent limitation for the detection of malignant cells, MRD detection is still possible because hematopoietic malignancies display aberrant or unusual antigen expression or clonal patterns of Ig or TCR protein expression (summarized in 113).

Aberrant or unusual immunophenotypes are the result of cross-lineage antigen expression, maturational asynchronous expression of antigens, antigen overexpression, the absence of antigen expression, and/or ectopic antigen expression (114, 115). The latter refers to the expression of particular antigens on cells outside their normal breeding sites or homing areas, or to the expression of antigens that are normally only expressed on non-hematopoietic cells.

Using leukemia-specific immunophenotypes, flow-cytometric MRD detection can frequently reach sensitivities of $10^{-3}$–$10^{-4}$ (i.e., one malignant cell per 1000 or 10,000 normal cells). However, it should be taken into account that false signals, which may have a minimal impact during immunophenotyping of leukemia at diagnosis, may be a source of major errors when studying MRD (116). It should be noted that routine flow-cytometric MRD detection requires both high technical skills and expert experience.

Immunophenotypic MRD detection in ALL

MRD in precursor-B-ALL cannot be detected by simple monitoring of CD10+ /TdT+ B-cell precursors, because such cells are present not only in low amounts in normal BM (generally <10% of mononuclear cells) and PB (generally <0.4%), but also occur in BM at high frequencies after chemotherapy courses (117, 118). Only atypical features of blast cells like CD10 overexpression on precursor-B-ALL cells or molecular clonality analysis can support the discrimination between normal and malignant precursor-B-cells. Other aberrant immunological characteristics applied to distinguish precursor B-ALL blasts from normal early B-lineage cells include cross-lineage expression of T-lineage antigens (e.g., CD5, CD7) or myeloid antigens (CD13, CD15, CD33, CD66c), maturational asynchronous expression of antigens (e.g., CD20 on CD45− cells), a lack of expression of CD45 (particularly the isoform CD45RA), and antigen overexpression (e.g., CD10) (summarized in 113). The finding of NG2 expression on the surface membrane of precursor B-ALL cells with the MLL gene rearrangement represents an example of a true ectopic antigen expression (58). Studies with cDNA microarrays, analyzing expression levels of thousands of genes, might further aid the discovery of new leukemia-specific markers (119, 120). For example, based on microarray studies, the CD58 antigen was shown to be significantly overexpressed on precursor-B-ALL blasts in contrast to normal B-cell precursors in normal and post-chemotherapy regenerating BM (119, 121). Multiparameter investigation of normal BM B-lineage precursors allowed the establishment of templates for normal B-cell development, whereas malignant precursor-B lymphoblasts frequently display aberrant immunophenotypic features and thereby fall into so-called “empty spaces” outside the normal B-lineage pathways (4, 122, 123). Thus, current four-color flow-cytometric investigations allow the detection of leukemia-associated phenotypes in approximately 85–95% of precursor B-ALL patients (4, 104, 124–127).

Interestingly, changes in quantitative antigen expression, such as downmodulation of CD10 and CD34 and upmodulation of CD19 and CD20, can be observed on residual BM blasts during the early phases of precursor B-ALL treatment (128, 129). Such shifts of intensity or coefficient of variation of the distribution of one or more markers cannot be explained by a change in maturation stage, but rather as a treatment-induced modulation (130). Therefore, use of strict gating procedures, based on the exact immunophenotype of the blast cells at diagnosis, should be avoided for the analysis of MRD (129, 130).

Another important pitfall of MRD detection in precursor B-ALL is the occurrence of immunophenotypic shifts during the course of the disease (128, 129, 131). Clear differences in immunological marker expression are found between diagnosis and relapse in up to two-thirds of precursor-B-ALL patients (131). However, at least one leukemia-specific marker combination is retained by the leukemic cells at relapse in at least 80% of patients (101). This implies that at least two marker combinations per patient should be used for immunophenotypic MRD monitoring. Furthermore, immunophenotypic shifts can also be observed during the early phase of treatment (128, 129). These drug-induced shifts seem to be related to the induction of cell death (129).
In T-ALL, it is possible to routinely detect MRD in 90% of cases by simple flow-cytometric T-cell marker/TdT stainings (132, 133). This is owing to the fact that in healthy individuals the combination of a T-cell marker and TdT expression is found in the cortical thymus, but this immunophenotype is absent or rare in extrathymic locations such as BM or PB. Moreover, if T-cell marker/TdT cells are found in BM or PB, they only express CD2 and/or CD7, and no other T-cell antigens such as CD3, CD4, or CD8. T-cell marker/TdT combinations are stable throughout the disease course and are retained at relapse in the vast majority of T-ALL cases (131). Flow-cytometric analysis, based on cross-lineage myeloid antigen expression, asynchronous antigen expression (e.g., CD34+/CD3-), and antigen overexpression (e.g., CD7+/-, CD99+), can also be used for MRD detection in T-ALL (132-134). In particular, the combination of CD99/T-cell markers can be applied for MRD detection in the vast majority of T-ALL cases, and is probably as effective as the classical T-cell marker/TdT combination (134). Similarly to precursor-B-ALL, multiparameter flow cytometry in T-ALL reveals “empty spaces” outside the templates for the normal T-cell compartment in BM and PB (133). Together, the various leukemia-associated immunophenotypes can be employed for MRD detection in all T-ALL (132, 133).

**Immunophenotypic MRD detection in chronic B-cell leukemias, B-NHL and multiple myeloma**

Detection of residual B-CLL by analysis of Ig light-chain restriction has limited sensitivity owing to the background of the normal B-cell population in BM, PB, and lymph nodes. Analysis of Igk and Igλ expression within the B-cell population via triple stainings, using CD19, CD20, CD22, or CD37 as pan-B-cell antigens in BM and PB, allows single Ig light-chain detection of clonal B-cells down to only 1–5%.

The use of additional antigens in quadruple stainings allows MRD detection at levels of approximately 10⁻²–10⁻³. Such additional markers should concern antigens that are normally only expressed on a subpopulation of B-cells. Examples include the CD103 antigen on HCL cells and the CD5 antigen on B-CLL and MCL cells (summarized in 113). The sensitivity of detection might be further increased by using differences in expression levels between normal and malignant B-cells, e.g., bright CD19 expression or increased CD5 expression in B-CLL patients (135). Application of several quadruple stainings (e.g., CD19/CD5/CD20/CD79b, CD19/CD5/CD38/CD79, CD22/CD23/CD19/CD5, Smlgκ/Smlgλ/CD19/CD5 and CD19/CD5/CD38/CD20) can reveal leukemia-specific immunophenotypes in virtually all B-CLL patients, potentially reaching sensitivities of 10⁻²⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻~-~-arkanowski et al.: Immunophenotyping of normal and malignant lymphocytes 791

Markers in quadruple stainings. For example, BCL2/B-cell antigen/Ig light-chain quadruple stainings may be employed for MRD detection in patients with FL (138).

**Immunophenotypic MRD detection in chronic T-cell leukemias and T-NHL**

The relatively high frequencies of T-lymphocytes and NK-cells in normal BM and PB samples and T-lymphocytes in lymph nodes hamper the immunophenotypic detectability of mature leukemic T-cells and T-NHL cells. Even the expression of the less common CD3⁺/CD4⁻/CD8⁻, CD3⁺/CD4⁺/CD8⁻ or TCRγδ⁺ phenotypes, as well as antigen loss (absence of particular T-cell antigens), generally results in a limited sensitivity of only 10⁻². Vγ and Vδ antibodies might be useful for detection and monitoring of malignant (clonal) TCRγδ⁺ T-cells (65), but the presence of normal TCRγδ⁺ T-lymphocytes might interfere with this application. The application of Vβ, Vγ, and Vδ antibodies in well-chosen quadruple stainings can result in sensitivity levels of approximately 10⁻² (potentially 10⁻³) (83). For example, CD3 can be used for life gating together with CD4 (or CD8), followed by analysis with a specific Vβ antibody, and an additional leukemia-associated antigen (e.g., CD7 in T-PLL, CD25 in ATLL, CD16 in T-LGL, etc.). The resulting sensitivities of approximately 10⁻² do not allow true MRD detection, but may be suitable for monitoring of T-cell leukemia patients during treatment or predicting the possible outgrowth of a dominant subclone in the case of oligoclonal T-cell proliferations (25, 83, 84).

**Conclusions and future perspectives**

Multiparameter flow-cytometric immunophenotyping of lymphocytes has become an indispensable technology in immunology and hematology. Extensive studies in healthy children and adults have resulted in detailed age-related reference values for the major lymphocyte subpopulations in PB. This is an important tool for the diagnosis of immunological and hematological disorders such as immunodeficiencies, lymphoproliferative disorders, and infectious diseases. Similar, albeit less detailed, information has
accumulated concerning other lymphoid tissues, such as BM, lymph nodes, tonsils, thymus and spleen. Flow-cytometric immunophenotyping forms the basis of modern prognostic classification systems of acute and chronic leukemias and is also increasingly applied for initial diagnostic work-up of NHL. Finally, multiparameter flow cytometry allows reliable detection of MRD in most lymphoid malignancies, thereby providing a better insight into treatment effectiveness. This has resulted in MRD-based treatment interventions, particularly in childhood ALL.

Flow-cytometric immunophenotyping is the sole technique that fulfills the requirements of high speed, broad applicability at diagnosis and during follow-up of immunological and hematological disorders with accurate focusing on the cell population of interest, using membrane-bound and intracellular proteins as targets. The innovative steps involved concern the development of novel antibodies, novel flow-cytometry software, and novel immunostaining protocols for six or more colors. This requires a multidisciplinary translational research approach using cutting edge technologies and biological data arising from genomic research, which can be addressed most successfully via close collaboration between industry and academia. These are the aims of the recently established EuroFlow consortium, which consists of two small-medium enterprises (SME) and eight diagnostic research groups, regarded as experts in the fields of flow cytometric and molecular diagnostics.

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