Acute Leukemias of Ambiguous Origin

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Key Words: Acute leukemia; Immunophenotype; Lineage; Myeloid; Lymphoid; Mixed phenotype acute leukemia; Undifferentiated acute leukemia

ABSTRACT

Objectives: This session of the Society for Hematopathology/European Association for Haematopathology Workshop focused on acute leukemias of ambiguous origin.

Methods: We provide an overview of mixed-phenotype acute leukemia (MPAL) as recognized in the current World Health Organization classification and summarize diagnostic criteria for major categories of MPAL: B/myeloid, T/myeloid, B/T, and B/T/myeloid.

Results: Most MPAL cases submitted were B/myeloid and T/myeloid MPAL, the most frequent types, but three cases of B/T MPAL were also submitted, and examples of all categories are illustrated. We emphasize that a comprehensive approach to immunophenotyping is required to accurately establish the diagnosis of MPAL. Flow cytometry immunophenotyping using a large panel of antibodies is needed as well as confirmatory immunohistochemical analysis and cytochemistry studies for myeloperoxidase and nonspecific esterase. We discuss technical issues in determining blast lineage and possible pitfalls in MPAL diagnosis. In particular, rare cases of B-acute lymphoblastic leukemia (B-ALL) can express myeloperoxidase but are otherwise consistent with B-ALL and should be treated as such. Last, we review the differential diagnosis between acute undifferentiated leukemia and acute myeloid leukemia with minimal differentiation.

Conclusions: There was an agreement that diagnosis of MPAL can be challenging, especially if applied flow cytometry panels are not comprehensive enough.

What Is the Definition of Acute Leukemia of Ambiguous Lineage?

In the 2008 World Health Organization (WHO) classification, 1 acute leukemias of ambiguous lineage were defined as leukemias that show no clear evidence of differentiation along a single lineage. The recognition of acute leukemia of ambiguous lineage requires extensive multiparametric flow cytometry (FCM) immunophenotyping disclosing the specific features of these diseases Figure 1. In extremely rare cases of acute undifferentiated leukemia (AUL), although markers of all lineages have been investigated, no significant expression is detected on blast cells except for CD34 and/or HLA-DR (present in most of these cases). A second, slightly more frequent occurrence is that of blast cells labeling with several monoclonal antibodies, recognizing antigens normally expressed on different lineages. Formerly called biphenotypic acute leukemias (BALs), these neoplasms have been renamed mixed-phenotype acute leukemia (MPAL). On the basis of associated cytogenetic anomalies, MPAL can be subdivided according to the presence of the Philadelphia chromosome into subgroups: t(9;22)/BCR-ABL1, t(v;11q23)/MLL, or not otherwise specified. All other unusual immunophenotypes, including early natural killer (NK) leukemias, constitute the remainder of leukemias of ambiguous origin.

Introduction to MPAL

The morphologic features of the blasts in most cases of MPAL are mostly uninformative, and therefore the
diagnosis of MPAL relies exclusively on immunophenotypic features. MPAL cells appear most often as morphologically undifferentiated, but sometimes the neoplastic cells may display more lymphoblastic or myeloblastic cytology. In some cases, two types of blasts with a distinctive size and morphology point to the different, extremely rare occurrence of a bilineal or biclonal leukemia. These neoplasms can also be recognized by FCM analysis as two cell subsets with different immunophenotypes.

MPAL can be identified using the recommended panel of the European LeukemiaNet or other comprehensive combinations. Importantly, several markers specific for the myelo-monocytic lineage as well as for the B- and T-lymphoid lineages must be tested, excluding a restrictive strategy of quick orientation followed by selected lineage-specific markers. Of note, as detailed below, at least two key cytoplasmic (c) markers must be investigated: myeloperoxidase (MPO) for myeloid lineage and cCD3 for T lineage. Recommendations were first published by the European Group for the Immunological Characterization of Leukemias (EGIL), with a scoring system identifying BAL as cases with more than two immunophenotypic points in more than one lineage. This proposal relied on 26 antibodies to perform a proper score, yet published reports seldom applied such extensive panels.

In the 2008 WHO classification, the name of these leukemias and their immunophenotypic definition (as above) were changed. MPO stands as the most robust marker that will identify myeloid engagement. Recent publications have redefined the threshold for MPO positivity, and yet most MPAL cases with a myeloid component will express MPO broadly in most blasts. An alternative, for MPAL with a strong monocytic differentiation, is represented by (very rare) cases in which blasts will be positive for nonspecific esterase and/or express at least two of the following antigens: CD14, CD11c, CD36, CD64, or cytoplasmic lysozyme. Determination of the engagement of blast cells in the B-lymphoid lineage will rely on the expression of surface CD19. If the latter is bright, demonstration of expression of one other B-lineage–associated marker (eg, cCD79a, surface or cCD22, or CD10) will be required. If CD19 expression is weak, however, at least two other B-lineage markers need to be expressed by the blasts. Assessment of T-lineage engagement relies on the demonstration of cCD3 expression as mentioned above. Of note, the fluorochrome used for this determination should yield strong fluorescence, for instance, phycoerythrin or allophycocyanin. The immunophenotypic strategy to explore these cells involves the use of two monoclonal antibodies directed to CD3 (usually anti-ε chain) conjugated to different fluorochromes. Cells are first incubated without manipulation, ideally using whole bone marrow (BM). After incubation, permeabilizing reagents will allow both lysis of erythrocytes and intracytoplasmic labeling with the second anti-CD3 antibody. Analysis by FCM will then identify normal residual T lymphocytes as costained with both antibodies, while T-lineage blast cells lacking surface CD3 will be stained with the second anti-CD3 only.

What Possible Combinations of MPAL Occur and What Are Their Frequencies?

All possible combinations of MPAL can be observed, including B/myeloid, T/myeloid, B/T, or even rare B/T/myeloid. In a series of 100 cases of MPAL published by the EGIL, B/myeloid cases were most frequent, representing 59% of all MPAL cases. The frequencies of T/myeloid, B/T, or B/T/myeloid were 35%, 4%, and 2%, respectively. The blast cells in MPAL show a specific gene expression pattern, as illustrated by a microarray study of acute leukemias performed at St Jude Children’s Research Hospital, where 35 childhood MPAL cases segregated in a specific cluster between B-acute lymphoblastic leukemia (B-ALL), T-acute lymphoblastic leukemia (T-ALL), and acute myeloid leukemia (AML).

How Are Patients With MPAL Managed Clinically?

The clinical management of MPAL cases is problematic. Often, an initial course of corticosteroids is attempted, followed by an acute lymphoblastic leukemia (ALL)–like approach. In the absence of response to corticosteroids, most clinicians will switch to an AML-like approach with alkylating agents. However, patients who fared well in the literature were clearly those who could benefit from allogeneic stem cell transplantation.

It is important to identify MPAL cases and not misdiagnose these tumors as ALL or AML by using immunophenotypic panels that are not sufficiently comprehensive. Indeed,
some refractory cases of acute leukemia, with poor response to therapy, may represent undetected cases of MPAL that were incorrectly assigned to a single lineage. Using an FCM intracytoplasmic orientation panel combining MPO, cCD3, and CD19 (possibly with cCD79a, cCD22, or CD10) could be a good approach to detect such cases early in the process of immunophenotyping a neoplasm. It is important to point out that immunohistochemistry (IHC) on BM biopsy sections also can be useful to confirm B, T, or myeloid differentiation by using antibodies specific for PAX5, CD3, and MPO and/or lysozyme, respectively. Immunohistochemical analysis may be of great help in cases with two or more different populations with specific distributions in BM or other tissue biopsy specimens since it allows direct visualization of cell location.

**Discussion of Workshop Cases**

There were 32 cases included in this workshop session. Several cases submitted initially to this session did not completely meet the criteria for MPAL and were moved to other sessions, although the moving in some cases was debated by members of the review panel, revealing the need for more robust criteria and greater consensus on definitions. The workshop cases are discussed in the subtypes to which they were allocated. The summary of these cases (below) illustrates the heterogeneity of MPAL presentation. The following discussion also describes the characteristics of MPAL cases, technical issues involved in MPAL diagnosis, and groups of cases that can be linked by some common characteristics or diagnostic issues.

**Typical Examples of MPAL**

**MPAL B/Myeloid**

Typical examples of MPAL with populations of blasts showing B lineage and myeloid differentiation are illustrated in **Image 11** and **Image 21**. Immunophenotypes of all cases classified as B/myeloid MPAL are given in **Table 11**. Expression of the B-cell marker CD19 was moderate to strong in cases 10, 208, 285, 323, and 328 and weak/partial in all other cases. B-cell lineage was most often corroborated by expression of cCD7α (demonstrated by FCM, IHC, or both), cCD22 (by FCM), and/or PAX5 (by IHC), whereas CD10 was positive in three cases (cases 10, 208, and 214). MPO expression was variable: most blast cells were MPO positive in four cases (cases 10, 76, 285, and 323), but other cases showed only subpopulations of MPO-positive cells. Cases 208, 328, and 214 represented the most common cytogenetic variant of B/myeloid MPAL, associated with t(9;22)(q34;q11.2)/BCR-ABL1. Three cases had highly hyperdiploid and/or a near-tetraploid karyotype, and one case showed hypodiploidy. Highly hyperdiploid and/or near-tetraploid karyotypes are rare in AML and ALL, and have been reported previously as common in MPAL and BAL classified according to the EGIL system.3,7,11 In the case series collected for this workshop, the highly hyperdiploid and/or near-tetraploid karyotype was also common (five [16%] of 32 cases: three B/myeloid, one T/myeloid, and one B/T).

One of the near-tetraploid cases (case 285) carried also the t(12;17)(p13;q11.2) involving the ZNF384/CIZ (chromosome 12p13) and TAF15 (chromosome 17q11.2) genes. The t(12;17)(p13;q11) has been described as a rare but recurrent abnormality, predominantly observed in B-ALL cases that usually have an early pre–B immunophenotype and showing aberrant expression of the myeloid antigens CD13 and/or CD33. The t(12;17) is less common in AML, and reported cases often showed aberrant expression of CD19.12-16 In a recent case report, a pro–B/B-I ALL, which switched to an AML at relapse, was described.17 Case 285 is the first case with this translocation in which the WHO criteria for MPAL are fulfilled. Another chromosomal abnormality hitherto not described in MPAL, inv(3)(q12q26.2), was detected in case 283. Inv(3)(q21q26.2) or t(3;3)(q21;q26.2) displays an inversion or homologous reciprocal translocation that leads to juxtaposition of the ecotropic viral integration site 1 (EVI1) gene with the ribophorin 1 (RP11) gene. AML cases carrying this abnormality are classified as one of the categories of AML with recurrent cytogenetic abnormalities in the WHO classification and most commonly have a myeloid or myelomonocytic immunophenotype and poor prognosis.18,19 An MPAL phenotype has not been reported previously in a leukemia with inv(3) or t(3;3).

**MPAL T/Myeloid**

The characteristics of MPAL cases with T and myeloid lineage markers are illustrated in **Image 31**, **Image 41**, and **Table 21**. Most T/myeloid MPALs were characterized by two populations of blasts: one with a T-precursor immunophenotype (usually coexpressing some myeloid markers but negative for MPO) and the other fulfilling MPAL criteria. In two cases (case 139 and 200), the myeloid lineage showed a monocytic differentiation pattern. In two patients (cases 200 and 402), different immunophenotypes were observed in a lymph node biopsy specimen and the BM. In case 200, BM showed an immunophenotype suggestive of AML with monocytic differentiation, but in the lymph node two aberrant populations were detected: one with cCD3 positivity and one with a myelomonocytic immunophenotype. Cytogenetic analysis also showed two different clonal populations,
one with 46,XY,der(12)t(11;12)(q21;p11.2) and the other with 45,XY,dic(12;18)(p11.2;p11.2). In case 402, a lymph node biopsy specimen showed a T-precursor immunophenotype, whereas in the BM, a population of blasts fulfilling the criteria of MPAL was detected.

MPAL B/T

MPAL cases that express both B- and T-lineage markers are rare (<5% of MPALs). Seven examples (cases 312, 359, and 390) were submitted to the workshop Table 3 and Image 5. In two cases, a population of blasts was detected that coexpressed CD19 (strong) and cCD3. B-cell lineage was supported by CD22 expression in one case and CD79a in the other cases. CD7 was expressed in one of the cases, and no other T-cell–associated markers were seen in the other. One of these cases had a normal karyotype, and one was hyperdiploid. The third MPAL B/T case had trisomy 11 and two populations of blasts: one with an immunophenotype of B-precursor ALL (EGIL B-II) and one of T-ALL with early T-cell precursor immunophenotype (EGIL T-I).

MPAL B/T/Myeloid

Case 268 was the only case of acute leukemia that expressed markers of all three lineages fulfilling criteria for B/T/myeloid MPAL. The patient was a 58-year-old man with two separate leukemic cell populations. One
population corresponded to an AML and was positive for CD7, CD4 (dim), CD11b, CD13, CD33, CD41, MPO, and HLA-DR, and the other population corresponded to MPAL with a B/T immunophenotype positive for TdT, CD19, CD20, CD10, cCD79a, CD22, PAX5, CD33 (dim), cCD3, and CD5 (dim) and negative for MPO Image 61 and Image 71. Conventional cytogenetics showed a normal karyotype, and BCR-ABL1 was negative. Another case fulfilling criteria for T/myeloid MPAL and also showing weak CD19 expression was case 356 (Table 2). The patient was a 14-year-old girl in whom both a lymph node biopsy specimen and a BM showed blasts positive for cCD3 and MPO with weak expression of CD19 and cCD79a. However, other B-cell markers were not found and the criteria for B-lineage differentiation were not fulfilled.

**MLL Rearrangement and Immunophenotype Switch**

Cases 180, 256, 370, and 392 were examples of MPAL associated with **MLL** rearrangement Table 4. MPAL with **MLL** rearrangement is considered a separate entity in the WHO classification, with a frequency of approximately 10% of adult and 12% to 18% of pediatric MPAL cases. Most cases of MPAL with **MLL** rearrangement are B/myeloid. Case 256 illustrated a rare occurrence of a T/myeloid MPAL (Table 4). The other three cases demonstrated an immunophenotype switch, previously reported in **MLL** rearranged leukemias.22-27

**MLL** rearrangement juxtaposes the amino-terminus of the histone methyltransferase **MLL** with a variety of different fusion partners. To date, more than 70 fusion partners of the **MLL** gene have been characterized.28 Deregulation of **HOX** expression is high in stem cells and early precursors and needs to be downregulated for maturation, a continuous ectopic **HOX** expression will create a basis for the development of abnormal preleukemic precursors that may progress to various forms of acute leukemia.28,29 In cases 180 and 370 submitted to this session, the primary diagnosis was AML with t(9;11)(p22;q23), but the relapse presented as ALL/lymphoma with an EGIL B-I immunophenotype (CD10 negative). In the third patient (case 392), a therapy-related myeloid neoplasm presented as a t(4;11)(q21;q23) B-ALL/lymphoma with an EGIL B-I immunophenotype, whereas AML with monocytic differentiation was seen at first relapse and B/myeloid MPAL at second relapse. The
## Table 1
Summary of Cases of MPAL, B/Myeloid

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/ Sex</th>
<th>Immunophenotype</th>
<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/ Comments</th>
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<tbody>
<tr>
<td>208</td>
<td>36, F</td>
<td>Two populations of blasts (75% of BM cells in total): B lymphoblastic (39%) CD19+, CD10+, CD13+, CD34+, cCD79a, HLA-DR+, TdT+ with variable expression of cCD22 IgM, negative for CD4, CD11b, CD14, CD15, CD64, MPO</td>
<td>t(9;22)(q34;q11.2); BCR-ABL1 (by FISH)</td>
<td>MPAL (B/ myeloid), with t(9;22) (q34;q11.2)</td>
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<td>Myelo(mon)blastic (36%) with the following immunophenotype: CD4+, CD13+, CD15+, CD19+, CD33+, CD34+, CD64+ HLA-DR+, MPO+ with variable expression of CD10, CD11b, CD79a, and TdT and negative for cCD22 and IgM</td>
<td>e1a2 BCR-ABL1 by RT-PCR</td>
<td></td>
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<tr>
<td>328</td>
<td>35, M</td>
<td>FCM: 13% blasts CD34+, CD117s+, CD33s+, CD13s+, CD15s+, CD19+, CD22s+, cCD79a dim+, CD25s+, TdT+, MPOs+, CD10–, CD66c–, CD2–, CD5–, CD7–, CD56–</td>
<td>46,XY,t(9;22)(q34;q11.2) [15] and 46,idem, del(20)(q11.2q13.3)[5]</td>
<td>MPAL (B/ myeloid), with t(9;22) (q34;q11.2)</td>
</tr>
<tr>
<td>214</td>
<td>63, F</td>
<td>Two populations of blasts (60% of BM cells in total): B-lymphoblastic: CD19+, CD10+, CD34+, CD33+, HLA-DR+, and CD38+, TdT+, PAX5+, CD45 dim</td>
<td>45,XX,del(7)(p15), t(9;22)(q34;q11.2), −16 [19/46,XX]</td>
<td>MPAL (B/ myeloid), (q34;q11.2)</td>
</tr>
<tr>
<td>285</td>
<td>41, F</td>
<td>One population of blasts: CD19+, cCD79a+, MPO+, CD33+, CD34+, HLA-DR+, CD38+, and with partial expression of CD13 and CD15</td>
<td>82-84,XXX−,−1,−2,−3,−4,−7,−8,−9,−10, t(12;17)(p13;q11.2)x2, der(12)t(12;17)(p13;q11.2),−13,−15,−16,−17,−20,−21,+22[cp7]/46,XX[13]</td>
<td>MPAL NOS (B/ myeloid)</td>
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<tr>
<td></td>
<td></td>
<td>By IHC: PAX5+, CD19+, TdT+, MPO+ (30%), CD117−, CD20−</td>
<td>e1a2 BCR-ABL1 by RT-PCR</td>
<td></td>
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<tr>
<td>283</td>
<td>40, M</td>
<td>One blast population 40% CD2–, surface CD3–, cCD3–, CD4–, CD5–, CD7+, CD8–, CD10–, CD11bs+, CD11cs+, CD13+, CD14+, CD15s+, CD16–, CD19s+, CD20–, CD22s+, CD32s+, CD33+, CD34+, CD45+, CD56–, CD64–, CD79as+, CD117+, HLA-DR+, MPOs+ (6%-8%), TdT+</td>
<td>46,XY;inv(3)q21;26.2, inv(9) [20]</td>
<td>MPAL NOS, B/ myeloid</td>
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<td>323</td>
<td>90, F</td>
<td>One population of blasts: CD19 dim, CD14 dim, CD15+, CD45 dim, sCD22 dim, CD56 dim, CD71 dim, CD79a+, and CD34+, ICD13–, or CD20– or CD33–, cCD79a+, cdCD3–</td>
<td>35,XX,−3,−4,−5,−7,−8,−9,−13,−15,−16,−17,−20[9]</td>
<td>MPAL NOS, B/ myeloid</td>
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<td></td>
<td></td>
<td>By IHC: MPO+, PAX5+, TdT+, CD79a/+−, and negative for lysozyme, CD10, and CD20</td>
<td>46,XX,7t(5;6)q23;24[1]</td>
<td>*With hypodiploidy</td>
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<tr>
<td>10</td>
<td>5, F</td>
<td>One population of blasts: CD45 dim−, low SSC, CD10+, CD19+, CD22+, partial CD13+, partial CD33+, CD34+, cCD79a+, nTdT+, HLA-DR, MPO+, cCD3–, CD15−, and CD117−</td>
<td>63-64&lt;3n&gt;,XXX,add(1)p13,−2,−3,−7,−10,+13,−14,−15,−19,−20,−21,+21[cp6]/63-65&lt;3n&gt;,[dem],add(2)p23,−10,+[mar][cp2]/46,XX[26]</td>
<td>MPAL NOS, B/ myeloid</td>
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<tr>
<td></td>
<td></td>
<td>FISH for t(9;22) and MLL: neg</td>
<td>46,XX[10]</td>
<td>*With near tetraploidy</td>
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<tr>
<td>76</td>
<td>26, M</td>
<td>One population of blasts: BM FCM: CD34+, CD33+, HLA-DR+, CD7+, MPO+, cCD79a+, cCD22 dim+, CD33 dim−, sCD5 and sCD19+, TdT−, CD2−, CD4−, CD8−, and mCD3−</td>
<td>80-85&lt;4n&gt;,XXY,Y,a(11)p36,−2,add(2)q(q31)x2,−3,add(3)q12–21;2x2,−6,−7,der(7)t(7;8)p15;q13,−8,−9,−9,add(12)p11.2 x2,−12,−13,−15,−16,−17,−17,−18,3-9[cp9]/46,XY[16]</td>
<td>MPAL NOS, B/ myeloid</td>
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<td></td>
<td></td>
<td>Lymph node IHC: CD34+, CD33+, CD99+, CD5+, CD56+ weak, CD7+, MPO+, CD79a+, PAX5− negative for cCD3, CD4, CD8, CD2, CD10, CD123, CD117, and TdT</td>
<td>46,XX; negative for BCR-ABL1 gene fusion and MLL rearrangement by FISH</td>
<td>*Associated with near tetraploidy</td>
</tr>
<tr>
<td>206</td>
<td>51, F</td>
<td>Two populations of blasts (90% of BM cells in total): main population: CD4s+, CD7 dim, CD13s+, CD14+, CD15+, CD36+, CD38+, CD45 dim, CD58+, CD117− dim, HLA-DRs+, MPOs+, cCD3–, CD5–, CD8–, CD19–, CD20–, CD22−, CD34−, CD56−, CD61−, nTdT−, cCD79a−</td>
<td>46,XX; negative for BCR-ABL1 gene fusion and MLL rearrangement by FISH</td>
<td>MPAL NOS, B/ myeloid</td>
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</tbody>
</table>
Table II (cont)
Summary of Cases of MPAL, B/Myeloid

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/ Sex</th>
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<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/ Comments</th>
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<tbody>
<tr>
<td>206</td>
<td>51, F</td>
<td>Minor population (5%-7%): CD19+, CD22 s–/bright, CD34+, CD38+, CD45 dim+, CD68+, nTdT+, cCD79a+, CD10–, CD14–, CD33–, cCD3–, MPO–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>365</td>
<td>41, M</td>
<td>Blasts 42% of BM cells: CD34+, CD33 dim+, CD38s+, MPO dim+ (18%), CD7+, HLA-DR dim+, CD117+, CD64 dim+, CD11bs+, CD19s+ (30%), CD79a dim+, nTdT–, cCD3–, CD15–, CD13–, CD20–, CD14–</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>45,XY,–7 [20]</td>
<td>MPAL, NOS, B/myeloid</td>
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</tbody>
</table>

BM, bone marrow; c, cytoplasmic; FCM, flow cytometry; FISH, fluorescence in situ hybridization; IgM, immunoglobulin M; IHC, immunohistochemistry; MPAL, mixed-phenotype acute leukemia; MPO, myeloperoxidase; neg, negative; NOS, not otherwise specified; RT-PCR, reverse transcriptase polymerase chain reaction; SSC, side scatter; WHO, World Health Organization.

Image 3 Example of T/myeloid mixed-phenotype acute leukemia showing in both bone marrow smears (A, May-Grünwald-Giemsa) and biopsy sections (B, H&E) presence of small myeloperoxidase (MPO)–negative blasts and approximately 25% larger MPO+ blasts. Positivity for both cytoplasmic CD3 (C) and MPO (D) is illustrated in the lymph node sections from the same patient. See Table 4 for details and Image 4 for flow cytometry findings. (Case 279, courtesy of R. E. Alexander, MD, and M. Czader, MD, PhD.)
**Image 4** Flow cytometry immunophenotypic findings in case 279: bilineal case with clearly different immunophenotypes on lymphoblasts (cyan dots, cCD3+, CD7+, CD2+, TdT+) and myeloblasts (dark blue dots, CD33+, CD15+, CD117+, MPO+). FITC, fluorescein isothiocyanate; MPO, myeloperoxidase. Red, neutrophils; green, monocytes; pink, lymphocytes; light blue, lymphoblasts; dark blue, myeloid blasts.

**MLL-AF4** fusion has been reported in therapy-related ALL, after treatment with either topoisomerase II inhibitors or alkylating agents, and seems to be associated with a worse clinical outcome.30,31

What Are Some of the Important Technical and Interpretation Issues in the Diagnosis of MPAL?

**MPO**

The issue of MPO expression in the diagnosis of MPAL has been discussed in the literature.32-34 Previous French-American-British group guidelines, based on cytochemistry, used 3% of MPO-positive blasts in BM smears as sufficient to call a leukemia MPO positive.35,36 A threshold of 10% expression has been used by the EGIL group.3 However, discordant cases are found when both methods are compared,6,37 leading to the conclusion that the 10% threshold may be conservative but not very sensitive. It should be emphasized that MPO expression can be a difficult test to establish by FCM immunophenotypic analysis since differences in permeabilization reagents and various antibodies have been reported.38-40 Thus, MPO expression in leukemic blasts has to be compared with internal negative and positive controls. In a study comparing MPO expression detected by FCM and cytochemistry in cases of AML and ALL, a 13% threshold was found to be relevant using an isotype control as a background reference (sensitivity, 95.1%; specificity, 91.7%).5 If residual normal lymphocytes were used as reference, a threshold of 28% had to be applied, yielding an improved 97.4% sensitivity and 96.1% specificity in distinguishing between ALL and AML. The WHO criteria for MPAL do not indicate any lower limit for MPO expression,1 but it seems reasonable to use published thresholds.3,5 Since MPAL cases often show more than one population of blasts, MPO could be present in only a minor population, which has to be identified as the myeloid component. Another issue is that AML with minimal differentiation and no MPO expression could be involved in MPAL. Therefore, as stated in the WHO criteria, when there are two or more distinct populations of leukemic cells, one with coexpression of a number of myeloid markers, no MPO and no lymphoid markers also can be accepted to define the myeloid component of MPAL.1

Rarely, cases of otherwise typical B-ALL/lymphoma can express MPO by FCM or IHC (case 259) or least often by cytochemistry.32-34,41 These cases are best classified and treated as B-ALL/lymphoma. In contrast, most MPAL cases show expression of other myeloid-associated markers and also are characterized by the presence of two or more
**Table 2**

Summary of Cases of MPAL, T/Myeloid

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/Sex</th>
<th>Immunophenotype</th>
<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/Comments</th>
</tr>
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<tbody>
<tr>
<td>279</td>
<td>13, M</td>
<td>Two populations of blasts: T lymphoblasts: CD2+, CD7+, CD5+, cCD3+, nTdT+, CD38+</td>
<td>45-46,XY,add(1)(p22), del(17)(q21)</td>
<td>MPAL, NOS; T/myeloid</td>
</tr>
<tr>
<td>92</td>
<td>17, F</td>
<td>Two populations of blasts: 90% cCD3+, nTdT+, CD34+, CD13+, CD117+, 10% cMPO+</td>
<td>46,XX</td>
<td>MPAL, NOS; T/myeloid</td>
</tr>
<tr>
<td>225</td>
<td>7, M</td>
<td>Two populations: all CD34 and cCD3, subpopulation also CD117+ and MPO+ by FCM and IHC</td>
<td>46,XY</td>
<td>MPAL, T/myeloid, NOS</td>
</tr>
<tr>
<td>139</td>
<td>80, M</td>
<td>Two populations: 17% blast gate CD4+/7+/11b+/13+/33+/45+/64+ and cCD3 (partial), 50% monocytic gate: CD4+/11b+/13+/14+/33+/64+/1H: lysozyme+</td>
<td>High hyperdiploid karyotype: trisomies for chromosomes 8, 11, and 12 and an extra chromosome X. In addition, five of these abnormal cells had trisomies for chromosomes 10 and 21.</td>
<td>*Relapse as AML MPAL, NOS T/myeloid (bilineal) Monocytic differentiation and hyperdiploidy MPAL, NOS T/myeloid</td>
</tr>
<tr>
<td>200</td>
<td>58, M</td>
<td>BM: FCM CD33 dim, CD7 dim, CD4 dim, CD38+, CD11c+, CD11b+, CD19 dim+, CD4+, CD5+, CD13+, CD20–, CD68 (KP1+), MPO+, CD11c+, IHC: lysozyme+, CD34+</td>
<td>46,XY, del(12)11q12, –del(19)q11.2q13.1</td>
<td>MPAL, T/myeloid</td>
</tr>
<tr>
<td>402</td>
<td>10, F</td>
<td>Lymph node FCM: 74%, CD7+, CD5 dim, CD2+, CD34+, CD117+, CD11b+, CD3+, CD10, CD19 dim, CD33, CD34+</td>
<td>45,XY, del(5)(p15)</td>
<td>MPAL, NOS T/myeloid</td>
</tr>
<tr>
<td>259</td>
<td>14, F</td>
<td>BM FCM: 7%-8%, CD7+, CD34+, cCD3+, MPOs+</td>
<td>BM aspirate: complex karyotype.</td>
<td>*T-LBL in the lymph node, MPAL in BM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MPAL, NOS T/myeloid</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; BM, bone marrow; c, cytoplasmic; FCM, flow cytometry; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; MPAL, mixed-phenotype acute leukemia; MPO, myeloperoxidase; NOS, not otherwise specified; SSC, side scatter; T-LBL, T-lymphoblastic; WHO, World Health Organization.

subpopulations of blasts with slightly different immunophenotypes. In some cases of MPAL, MPO may be expressed only in a small subset of blasts, with or without lymphoid markers (biphenotypic or bilineal presentation).

**Cytoplasmic CD3**

Cytoplasmic CD3 (cCD3) is considered the most specific marker for the T-cell lineage and has been used to diagnose T-cell malignancies since the 1980s. FCM immunophenotyping methods to detect cCD3 were established in the 1990s when permeabilization reagents became available. As in the case of MPO, expression of cCD3 in leukemic blasts has to be determined in comparison with internal negative and positive controls (B cells, monocytes, granulocytes, and normal T lymphocytes, respectively). At least a fraction of blasts should express cCD3 at the level of least a fraction of blasts should express cCD3 at the level of...
**Table 3**

Summary of Cases of MPAL, B/T

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/Sex</th>
<th>Immunophenotype</th>
<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/ *Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>390</td>
<td>82, M</td>
<td>One population of blasts: cCD3+, CD7+, CD19+, CD22h, CD13+, CD117+, CD15s+, CD5–, CD11b–, CD14–, CD33–, CD56–, CD61–, CD79a–, CD235a–, MPO–</td>
<td>46,XY, FISH negative for:5p15.2 (D5S23), 5q31 (EGR1), 7q31 (D7S486), 8cen (D8Z2), 11q23(MLL), 13q14 (D13S319), 13q34 (LAMP1), and 20q12 (D20S108)</td>
<td>MPAL, NOS, B/T</td>
</tr>
<tr>
<td>359</td>
<td>44, M</td>
<td>One population of blasts: CD34+, CD19+, CD33+, CD56+, HLA-DR+, CD49d+, cCD79a+, CD123s+, CD45 dim, CD38 bright, cCD3+, CD1–, CD2–, mCD3–, CD4–, CD6–, CD7–, CD8–, CD10–, CD13–, CD14–, CD15–, CD20–, CD22–, CD25–, CD36–, CD41–, CD64–, CD66c–, CD81–, CD117–, TdT–, slg–, clgM–, MPO–</td>
<td>50,XY,dup(1)(p22p36.1),+4,+10,–15,+21,+22,+mar(12)[5].,del(11)[q12],add(19)[q13.1][3]; diploid male karyotype 46,XY[5] Negative for all recurrent translocations, negative for mutations in IDH1, IDH2, JAK2, KIT, KRAS, NPM1, NRAS, FLT3 (including ITD), and CEBPA</td>
<td>MPAL, NOS, B/T</td>
</tr>
<tr>
<td>312</td>
<td>11, F</td>
<td>Two blast populations: 1: CD45 dim+, CD19+, CD20-/dim+, CD10+, TdT+, CD34 dim+, CD117–, MPO–, CD13–, CD33–, CD38+, CD79a, PAX5 2: cCD3+, mCD3–, CD7+, CD34+, CD117+, HLA-DR+, CD38+, CD19+, CD20–, CD10–, TdT–, MPO–, CD13+, CD33–</td>
<td>47,XX,+11[19]/46.XX[1]; FISH negative for monosomy 7, BCR-ABL1, ET6-RUNX1, MLL rearrangement</td>
<td>MPAL, NOS, B/T</td>
</tr>
</tbody>
</table>

c, cytoplasmic; FISH, fluorescence in situ hybridization; MPAL, mixed-phenotype acute leukemia; MPO, myeloperoxidase; NOS, not otherwise specified; WHO, World Health Organization.

Image 5 Example of rare B/T mixed-phenotype acute leukemia with medium- to large-sized blasts: (A), May-Grünwald-Giemsa) bone marrow smear and (B), H&E) bone marrow biopsy specimen. By flow cytometry, blasts were positive for cCD3 (T lineage, C) and CD19, CD10 and CD79a (B lineage, C and D). See Table 3 for details. (Case 390, courtesy of H. Ayyad, MD, and colleagues.)
normal T cells, and weak expression in a minor fraction of blasts is insufficient to diagnose MPAL. Many AML cases show aberrant expression of other T-cell–associated markers, such as CD2, CD4, CD5, or CD7, or NK-cell–associated markers, such as CD56. Thus, the correct interpretation of cCD3 staining is important for final diagnosis. If a BM biopsy specimen is available, the expression of cCD3 may be confirmed by IHC. In case 261, positive for CD2, CD4, and CD56, review of the FCM files revealed no sufficient cCD3 expression, and no confirmatory IHC could be performed. Thus, considering the presence of a complex karyotype, a consensus diagnosis of AML with myelodysplasia-related changes was rendered Table 5. Similarly, in case 349 (Table 5), expression of cCD3 was very weak and could not be confirmed by IHC. Also, in case 303 (Table 2), heterogeneous expression of cCD3 was not confirmed by IHC, although a fraction of blasts that showed adequately high expression to qualify as MPAL was observed by FCM, together with CD5 and CD7. No cCD3 expression was seen at relapse in this patient.

### CD19 and Other B-Cell Markers

The WHO classification provides no definite threshold for the size of the population expressing CD19 that is...
Flow cytometry immunophenotypic findings in case 268: CD45 dim (blast) area contains complex populations of normal B-cell precursors, myeloid blasts, and an abnormal B/T population coexpressing cytoplasmic CD3 and surface CD19. A, Apparently homogeneous blast (Bermudes) cell population. B, B/T compartment. C, Myeloid M0 blasts. D, Hematogones. FITC, fluorescein isothiocyanate. Red, neutrophils; green, monocytes; pink, lymphocytes; light blue, blasts; dark blue, CD34+ cells; yellow, hematogones. (Case 268, courtesy of S. A. Ely, MD, and colleagues.)
<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/sex</th>
<th>Immunophenotype</th>
<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/*Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>256</td>
<td>15, F</td>
<td>Lymph node: FCM: 37% blasts, CD3 dim, CD5 dim, CD7+, CD33+, CD34+, CD79a+, TdT+, CD11s+, MPO+, IHC: CD3 (weak), CD34, TdT, MPO (weak), CD20, lysozyme –, NOTCH1 negative, suggesting the absence of activating alterations in this pathway. BM 30% blasts not available for review.</td>
<td>FISH on the lymph node, break-apart probe MLL rearrangement+, BCR-ABL1 negative on blood</td>
<td>MPAL, T/myeloid, with t(11; q23); MLL rearranged</td>
</tr>
<tr>
<td>180</td>
<td>63, M</td>
<td>Initial: CD34–, cMPO–, CD117–, CD45+, HLA–</td>
<td>t(9;11)p22;q23</td>
<td>Acute leukemia with recurrent cytogenetic abnormality, t(9;11)(p22;q23)</td>
</tr>
<tr>
<td>370</td>
<td>9, M</td>
<td>2005 BM: FCM, 74% blasts CD33+, CD15+, CD4+, CD16+, CD56+, HLA-DR+, CD11b. The blasts were negative for CD13 and CD14, B and T markers.</td>
<td>BM 2005: 46, XY, add(12)(p11.2)</td>
<td>Acute leukemia with MLL rearrangement with cryptic variant t(9;11)(p22;q23), MLL-T3-MLL fusion with evolving new clone of IGH rearrangement presenting as (1) acute myeloid leukemia in 2005, (2) first relapse as B-acute lymphoblastic leukemia in 2006, and (3) second relapse as extramedullary (vertebral mass) involvement by B-acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>392</td>
<td>68, M</td>
<td>Previous therapy for B-lymphoma. Diagnosis: FCM: CD45 dim, HLA-DR+, CD19 bright, CD22+, CD38+, CD45s+, CD10–, IHC: TdT+, PAX5+, and CD79a+, negative: CD10, CD34, CD117, CD22, CD68, lysozyme, and BCL6</td>
<td>Diagnosis: 46, XY, t(4;11)</td>
<td>Therapy-related myeloid neoplasm, with t(4;11), MLL rearranged, presenting as B-lymphoblastic leukemia with relapse as acute myeloid leukemia (monocytic differentiation) and second relapse as MPAL/B/myeloid with t(11; q23); MLL rearranged (bilineal)</td>
</tr>
</tbody>
</table>

**Table 4**

Summary of Cases of MPAL With MLL Rearrangement

- B-LBL, B-lymphoblastic; BM, bone marrow; c, cytoplasmic; FCM, flow cytometry; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; MLL, mixed-lineage leukemia; MPAL, mixed-phenotype acute leukemia; MPO, myeloperoxidase; WHO, World Health Organization.
necessary to define B-lineage involvement. In two cases of bilineal B/myeloid MPAL (cases 206 and 365), only minor CD19+ populations were detected (5%-7% and 12.5% in cases 206 and 365, respectively). Since B-cell lineage was confirmed by other markers (Table 1), criteria were met for the diagnosis of MPAL. The intensity of CD19 expression by blasts should be compared with that expected by normal B cells and, if comparable, considered bright. It has to be stressed that in cases in which the whole blast cell population strongly expresses CD19, with the immunophenotype otherwise consistent with B-ALL/lymphoma, the presence of MPO expression as a sole aberrant marker should not dissuade therapy using a B-ALL regimen, and patient prognosis does not seem to differ from other patients with B-ALL.

**What Criteria Are Helpful in the Differential Diagnosis of AUL vs AML With Minimal Differentiation?**

Five cases submitted to this session (cases 199, 250, 261, 349, and 396) illustrated classification difficulties between AUL and AML with minimal differentiation (Table 5). True AUL is very rare, and these neoplasms should not express markers of B, T, or myeloid lineage and should not fulfill criteria for B- or T-ALL, AML with minimal differentiation, or MPAL. Nevertheless, AUL cases can be positive for CD34, CD38, HLA-DR, and/or TdT. Importantly, there may be cases that cannot be adequately classified due to insufficient immunophenotyping data or discordant expression of various markers, rendering definitive classification impossible. The latter cases should be designated as acute

### Table 5

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Patient Age, y/ Sex</th>
<th>Immunophenotype</th>
<th>Cytogenetics/Molecular Genetics</th>
<th>Consensus WHO Diagnosis/ *Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>199</td>
<td>89, F</td>
<td>FCM, one blast population CD34+, CD117+/−, CD13, CD33 dim, HLA-DR, TdT, CD11b dim, CD4, CD7s dim, and cCD22 dim, negative for CD3, CD19, CD20, CD10, CD2, CD5, and MPO. IHC: PAX5s and CD79as</td>
<td>46,XX</td>
<td>AUL (consensus not reached)</td>
</tr>
<tr>
<td>250</td>
<td>47, F</td>
<td>FCM, one blast population (86%): CD7+, CD33+, CD34+, CD38+, HLA-DR+</td>
<td>92:97&lt;4n&gt;, XXXX, +4,+4,+6,−7,t(10;11)(p12;13q14;21) x2,−13,i(I17) q(10),+20(qp4)/46,XX[16]</td>
<td>FISH for BCR-ABL1 negative, four signals each for BCR and ABL1; FISH for PML-RARA negative, four signals each for PML and RARA</td>
</tr>
<tr>
<td>356</td>
<td>38, M</td>
<td>FCM, one blast population (96%): CD34+, HLA-DR+, CD7+, CD33 dim, CD38s dim, CD9s dim, CD52, CD58, CD81, CD49b, CD184s dim</td>
<td>Insufficient</td>
<td>Molecular: Negative for b3a2, b2a2, and e1a2 transcripts of bcr-abl/t(9;22)(q34;q11), the short and long forms of PML-RARA/t(15;17)(q22;q21), the A and D forms of CBFB-MYH11/inv(16), AML1-ETO/8;21(q22;q22), E2A-PBX-1/t(1;19)(q23;p13), MLL-AF4/t(4;11)(q12;q23), and TEL-AML1/t(12;21)(p12;q22)</td>
</tr>
<tr>
<td>349</td>
<td>7 mo, M</td>
<td>FCM: one population (skull mass and BM) cCD3 dim, CD13 dim, CD33, CD34, CD56 bright, and CD117. Negative for CD1a, CD2, mCD3, CD3, CD4, CD7, CD8, CD11b, CD11c, CD14, CD16, CD41, CD64, CD117, and MPO</td>
<td>46,XY</td>
<td>AML with minimal differentiation</td>
</tr>
<tr>
<td>261</td>
<td>37, F</td>
<td>FCM one population (82%) CD2+, CD4+, CD13+, CD33+, CD34+ dim, CD56+, CD6, and MPO</td>
<td>46,XX,inv(21)(p13q22.1)[8]/45-46,sl,add(6)ip22),<a href="q10">8</a>[2],del(11)[(t(11;11)(p15,q13),del(12)[(p11.2)[2],add(1)[p13],[del(17)[p13][4]),del(18)[q21.1)(2][cp10]/46XX[2]</td>
<td>AML with myelodysplasia related changes (complex karyotype)</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukemia; AUL, acute unclassifiable leukemia; BM, bone marrow; c, cytoplasmic; FCM, flow cytometry; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; MPO, myeloperoxidase; WHO, World Health Organization.
unclassifiable leukemia, which is different from AUL.\textsuperscript{46} The diagnosis of acute unclassifiable leukemia could be applied to case 199, for which the review panel could not reach a consensus. In three other cases (Table 5), the panel consensus diagnosis was AML with minimal differentiation due to the expression of CD33 with no expression of B- or T-lineage–associated markers.

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References


18. Haferlach C, Bacher U, Haferlach T, et al. The inv(3) (q21q26)/t(3;3)(q21;q26) is frequently accompanied by alterations of the RUNX1, KRAS and NRAS and NFI genes and mediates adverse prognosis both in MDS and in AML: a study in 39 cases of MDS or AML. Leukemia. 2011;25:874-877.


