Immunophenotyping in hematological malignancies

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Unilab-Lg, Hematobiology
Blood
Marrow
Lymph node
CSF

Dissociation
Red cell lysis

Incubation with
fluorescence-tagged
antibodies
(w/ or w/o
permeabilization)

Dot plot

Red fluorescence

Green fluorescence

phycoerythrin

fluorescein

cytometer
« Gating » and « dot plots »

- granulocytes
- monocytes
- blasts
- lymphocytes
- CD8+ T cells
- CD4+ T cells
- B/NK cells
<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Abbreviation</th>
<th>Excitation max (nm)</th>
<th>Emission max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cascade blue</td>
<td></td>
<td>380, 401</td>
<td>419</td>
</tr>
<tr>
<td>Cascade yellow</td>
<td></td>
<td>399</td>
<td>549</td>
</tr>
<tr>
<td>Pacific blue</td>
<td></td>
<td>410</td>
<td>455</td>
</tr>
<tr>
<td>Alexa 488*</td>
<td></td>
<td>495</td>
<td>519</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate*</td>
<td>FITC</td>
<td>494</td>
<td>519</td>
</tr>
<tr>
<td>Phycoerythrin*</td>
<td>PE</td>
<td>496, 546</td>
<td>578</td>
</tr>
<tr>
<td>Texas red*</td>
<td>ECD</td>
<td>595</td>
<td>615</td>
</tr>
<tr>
<td>PE-cyanine 5*</td>
<td>PC5/PE-Cy5</td>
<td>496, 546</td>
<td>667</td>
</tr>
<tr>
<td>PE-cyanine 5.5*</td>
<td>PC5.5/PE-Cy5.5</td>
<td>495, 564</td>
<td>696</td>
</tr>
<tr>
<td>PE-cyanine 7*</td>
<td>PC7/PE-Cy7</td>
<td>495, 564</td>
<td>767</td>
</tr>
<tr>
<td>Peridinin-chlorophyll*</td>
<td>PerCP</td>
<td>482</td>
<td>678</td>
</tr>
<tr>
<td>PerCP-cyanine 5.5</td>
<td>PerCP-Cy5.5</td>
<td>482</td>
<td>678</td>
</tr>
<tr>
<td>Allophycocyanin*</td>
<td>APC</td>
<td>650</td>
<td>660</td>
</tr>
<tr>
<td>APC-cyanine 7</td>
<td>APC-Cy7</td>
<td>650</td>
<td>785</td>
</tr>
</tbody>
</table>

What is your favourite colour?

In clinical flow cytometry (2021): standard = 8-12 colour combinations
Main indications for immunophenotyping in haematological malignancies

- Acute leukaemias
- Chronic lymphoproliferative disorders (B/T)
- Plasma cell disorders
- Minimal residual disease (ALL, AML, MM, CLL)
ACUTE LEUKAEMIAS
Acute leukaemias
Flow chart

1. Is the abnormal cell population of a precursor cell type?

2. What is the lineage specificity?
   i.e., T, B, myeloid, mixed-type or undifferentiated

3. Is there aberrant antigen expression?
   « difference from normal »
   Further assessment of minimal residual disease
# Acute leukaemias: Identification of precursor cells

<table>
<thead>
<tr>
<th>Precursor cell antigens</th>
<th>Normal expression</th>
<th>Hematological malignancy expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>Hematopoietic stem cells, Myeloid, B and T precursors</td>
<td>AML (70%), MDS blasts (50-100%), B-ALL (65-80%), T-ALL (30-50%)</td>
</tr>
<tr>
<td>CD117</td>
<td>Immature myeloid cells, Mast cells, Some plasma cells</td>
<td>AML (60-70%), Mastocytosis, Multiple myeloma</td>
</tr>
<tr>
<td>TdT</td>
<td>Lymphoid precursors (B and T), Very primitive myeloid precursors</td>
<td>ALL (90%), Undifferentiated AML</td>
</tr>
<tr>
<td>CD1a</td>
<td>Cortical thymocytes, Immature dendritic cells</td>
<td>T-ALL (40-60%, indicative of cortical phenotype)</td>
</tr>
<tr>
<td>CD45</td>
<td>All leucocytes, brighter on lymphocytes and monocytes</td>
<td>Dim expression on precursor cells</td>
</tr>
</tbody>
</table>
### Requirements for lineage assignments (WHO)

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Relevant antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid</td>
<td>• Myeloperoxidase (MPO) or &lt;br&gt;• Monocytic antigens (two of CD11c, CD14, CD64, lysozyme)</td>
</tr>
<tr>
<td>T-lineage</td>
<td>Cytoplasmic CD3 (cCD3)</td>
</tr>
<tr>
<td>B-lineage</td>
<td>• Strong CD19 + one of cCD79a/cCD22/CD10 or &lt;br&gt;• Weak CD19 + two of cCD79a/cCD22/CD10</td>
</tr>
</tbody>
</table>
Acute leukaemias of ambiguous lineage

• < 5% of AL, poor prognosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute undifferentiated leukemia</td>
<td>No expression of <strong>specific</strong> myeloid or lymphoid markers</td>
</tr>
<tr>
<td></td>
<td>Often CD34+, HLA-DR+, CD38+</td>
</tr>
<tr>
<td></td>
<td>Sometimes TdT+, CD7+</td>
</tr>
<tr>
<td>Mixed phenotype acute leukaemia (MPAL)</td>
<td>Co-expression of specific lymphoid and myeloid markers (mostly B/myeloid, T/myeloid)</td>
</tr>
</tbody>
</table>
## Acute leukaemias: aberrant expression – « lineage infidelity »

<table>
<thead>
<tr>
<th></th>
<th>AML</th>
<th>B-ALL</th>
<th>T-ALL</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
<td>CD13, CD14, CD15, CD33, CD65</td>
<td>CD13, CD33</td>
</tr>
<tr>
<td>B</td>
<td>TdT, CD19</td>
<td></td>
<td>CD79a</td>
</tr>
<tr>
<td>T</td>
<td>TdT, CD7, CD2, CD4</td>
<td>CD4</td>
<td></td>
</tr>
<tr>
<td>NK</td>
<td>CD56</td>
<td>CD56</td>
<td>CD56</td>
</tr>
</tbody>
</table>

Specific phenotype of tumor cells ≠ normal blasts
CHRONIC LYMPROLIFERATIVE DISEASES
B-cell chronic lymphoproliferative diseases

• Identification of a B-cell clone
  – Clonality: skewing of kappa/lambda Ig light chain expression
  – Weak or absent Ig light chain expression
  – Weak or absent markers expressed by normal B cells: CD79a, CD22, CD20
The Catovsky-Matutes score and differential diagnosis of B-CLPD

<table>
<thead>
<tr>
<th>Markers</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CD5</td>
<td>Negative</td>
</tr>
<tr>
<td>CD23</td>
<td>Negative</td>
</tr>
<tr>
<td>FMC7 (CD20 epitope)</td>
<td>Positive</td>
</tr>
<tr>
<td>CD79a</td>
<td>Positive</td>
</tr>
<tr>
<td>Kappa or lambda</td>
<td>Moderate/bright</td>
</tr>
</tbody>
</table>

Score = 4-5 → **CLL/MBL**  
Score = 3 → atypical **CLL/MBL**  
Score = 0-2  
→ differential diagnosis of CD5+ **LPD**: → MCL, SMZL, B-PLL  
→ differential diagnosis of CD10+ **LPD**: → FL, DLBCL, BL, B-ALL  
→ **CD11c+, CD103+, CD25+, CD123+:** → **HCL**
CLL, SLL and monoclonal B cell lymphocytosis

• B cell reference range: 100-500 polyclonal B cells/µl
• CLL = > 5000 monoclonal B cells/µl
• < 5000 monoclonal B cells
  – With node/spleen involvement = SLL
  – Without node/spleen involvement = MBL
    • < 500/µl: low count MBL, no progression to CLL
    • > 500/µl: high count MBL, 1% progression to CLL/year
Identification of clonal T CLPD

- Inbalance of the CD4/CD8 ratio >10 or <0.1
- CD4+CD8+ or CD4-CD8- T cells
- Inbalance of the TCR Vβ repertoire
- Loss of normal T cell markers: CD5, CD7

Differential diagnosis

- CD4+CD8-
- CD4-CD8+
- CD4-CD8-
- CD4+CD8+

Identification of clonal T cell disorders.

Clonality markers

TCR Vβ analysis

CD3+CD4+CD7-
NK cells proliferative disorders.
Clonality.

- Killer-cell Immunoglobulin-like Receptors (KIR):
  - NK cells
  - Some T CD8+ subsets
- Clustered to the CD158 family, 14 isoforms
- Indicative of clonality:
  - Restricted expression of a single KIR isoform
PLASMA CELL DISORDERS
Plasma cell disorders

Normal plasmocytes
Clonal plasmocytes
Residual normal plasmocytes and progression from MGUS to MM

Perez-Persona et al., Blood. 2007;110:2586-2592
MINIMAL RESIDUAL DISEASE
Immunophenotyping and MRD

MRD: disease load not identifiable by standard methods (morphology)
General principles for MRD quantitation by immunophenotyping

• Target disease ~ unique immunophenotype, at least two aberrant markers for discrimination from normal cells
• High sensitivity → large number of cells analyzed
  – « rough estimate » = minimum cluster of 40 cells with a well-defined aberrant phenotype
  – $1 \times 10^{-4}$ sensitivity → $400,000$ cells to analyze
  – $1 \times 10^{-5}$ sensitivity → $4 \times 10^6$ cells to analyze

• Main applications of MRD analysis by flow cytometry
  – B- and T-ALL: independent risk factor for relapse, recommended in clinical practice
  – AML, MM and CLL: used in clinical trials
    • AML: optimal sampling intervals?, frequent clonal evolution
    • MM: focal disease, no current guidelines to change therapy
    • CLL: prognostic value of MRD is therapy dependent
B-ALL and MRD

UKALL Flow MRD Group, Irving et al., Haematologica 2009
MM and MRD

HR, 0.21; 95% CI, 0.12 to 0.36; P < .001

PFS (%)

0 12 24 36 48
Time From MRD Assessment After Consolidation (months)

Undetectable MRD, median PFS: not reached
Persistent MRD, median PFS: 36 months

Paiva et al., JCO 2020;38:784
REPORTING PHENOTYPIC DATA
Flow cytometric data reporting

- **Patient information:** indication, previous FCM data, other lab results (WBC, differential)
- **Sample information:** sample type, anticoagulant, date collected/received
- **Sample preparation:** **antibodies used**, cell viability
- **Data analysis:**
  - Overall information on normal cells (B/T cells, CD4:CD8 ratio, NK, monocytes, granulocytes)
  - If present, % abnormal cells compared to a **defined** population (total leucocytes, total lymphocytes...)
  - Marker distribution on abnormal cells: +, −, partial; fluorescence intensity if relevant (dim, bright, heterogeneous, homogeneous)
  - **List of % positive cells for each marker tested:** irrelevant, misleading!
- **Interpretation:**
  - Differential diagnosis according to WHO defined subtypes
  - A definite diagnosis requires integration with relevant pathology/molecular biology/cytogenetic data

Recommendations of the Bethesda Consensus Conference, Wood et al., Cytometry, 2007, 72B-S14
References

• Minimal residual disease:
    Paiva B. et al., JCO 2020;38:784
    Stephens D., Blood 2019; 133:386
  – AML: Schuurhuis G.J. et al., Blood 2018; 131-1275