Principles of Immunophenotyping

Dr Marc Heller and Professor Barbara Bain

Flow Cytometry

Definitions I

- Flow Cytometry is based on the manufacturing of highly specific monoclonal antibodies, and the use of laser technology, by the use of which the surface of a cell is interrogated by a laser beam
- Upon entering the interrogation chamber, the cell's light scattering properties and fluorescent properties are assessed

Flow Cytometry

Definitions II

- Depending on the type and stage of maturation of a cell, different antigens may be expressed on the surface, in the cytoplasm and in the nucleus of a cell
- Ability to identify particular antigens depends on the specificity and affinity of the antibody, hence the use of monoclonal antibodies

Definitions III

- Monoclonal antibodies designed to recognise an antigenic structure on a cell, are labelled with a fluorochrome, and bind to cell surface antigens
- The flow cytometer allows classification of cells according to their light scattering characteristics, and the intensity of their fluorescence caused by the fluorochrome-carrying antibody bound to the cell

Definitions IV

- Light scattering properties describe physical characteristics of the cell such as size and cytoplasmic granularity
- It allows clustering of cell populations based in measurements of physical characteristics

Flow Cytometry Blood

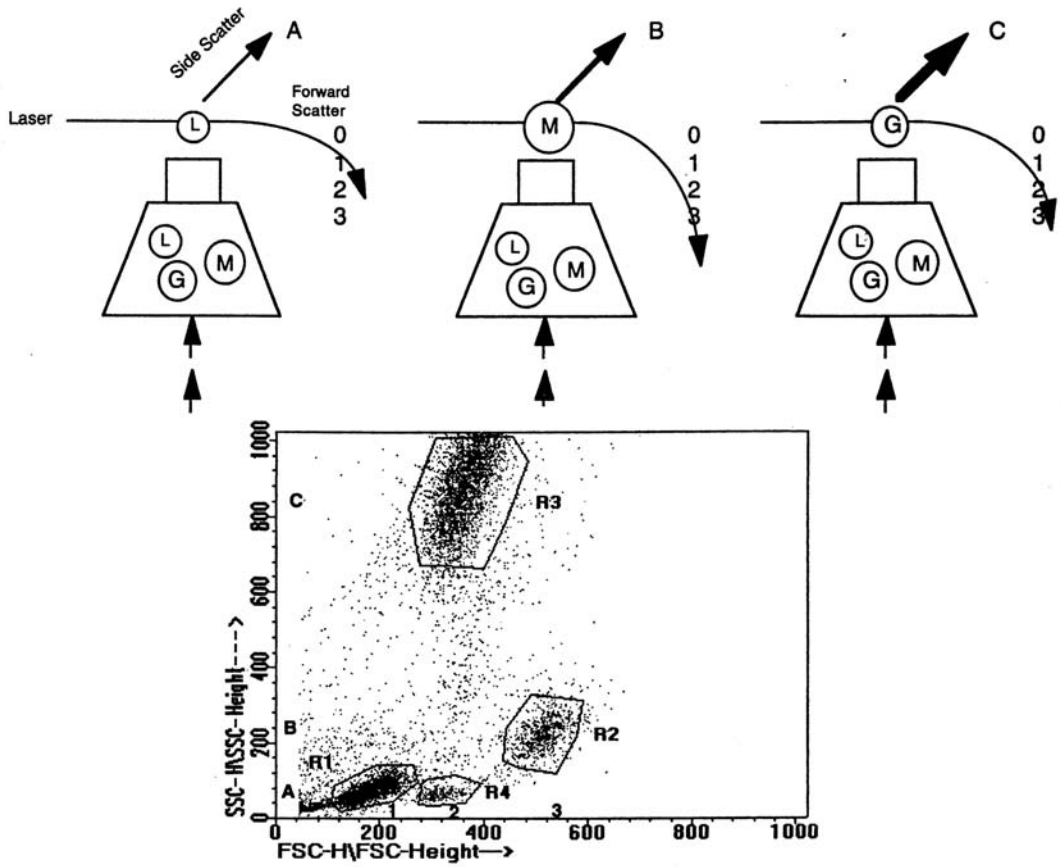

Figure 1. The upper three figures show a mixture of lymphocytes (L), monocytes (M), and granulocytes (G) entering the flow cell. Cell size is proportional to forward scatter (0, 1, 2, 3) and cytoplasmic complexity is proportional to side scatter (A, B, C). The lower figure is a forward-side scatter plot with the arbitrary values of forward scatter (1, 2, 3) and side scatter (A, B, C) superimposed. Clusters apparent are R1-lymphocytes (1, A), R2-monocytes (3, B), R3-granulocytes (2, C), and R4-, a cluster of abnormal cells with increased size but lymphocyte-like cytoplasm.

Definitions V

- Individual cell fluorescence values are detected by fluorescent detectors with different wavelength filters matched to the fluochromes coupled to different monoclonal antibodies
- Cell surface antigens were initially assessed in an isolated fashion, but with advancing technology, multiple different cell surface molecules can now be assessed simultaneously

Monoclonal antibodies

- Initially each of the large number of monoclonal antibodies had a unique designation, which was confusing
- Groups of monoclonal antibodies were then re-named after the antigenic structure they recognised (cluster designation - CD), and nearly always carry a CD number
- It is important to know that antibodies with the same CD number do not always recognise exactly the same epitope of the antigen they are designed to recognise

Problems and Pitfalls

- Flow cytometry has the disadvantage that the immunophenotype obtained is not related to a morphological picture of the cells examined
- This is especially important when the number of cells for examination is small
- Gating techniques are used in order to select the correct subgroup of cells for analysis, and it is essential that these cells form the neoplastic clone (gating can be based on the light scattering characteristics indicating the size of a cell)

Advantages

- Flow cytometry is a rapid procedure
- Multiple monoclonal antibodies can be used simultaneously to study the co-expression of 2, 3 or 4 antigens
- The amount of antigen expressed on a particular cell can be quantified
- therefore, minor abnormal cell populations can be detected by showing a atypical combination of antigens (as in the detection of minimal residual disease)

Lineage definitions
a) T-cell lineage

- T-Cell lineage was first to be characterized, by antigens CD3, CD4, CD8, CD2, CD5 and CD7
- T-cell neoplasm were divided into those with thymic phenotype (T-ALL, lymphoblastic lymphoma), and more mature or peripheral T-cell neoplasms such as Sézary cell leukaemia or other mature T-cell leukaemias

Lineage definitions
a) T-cell lineage

- NONE of the specific antigens can be used as indicators of clonality
- A good indicator of malignancy is the presence of an aberrant phenotype such as loss of antigens, inappropriate combinations or additions of antigens

Lineage definitions
a) T-cell lineage

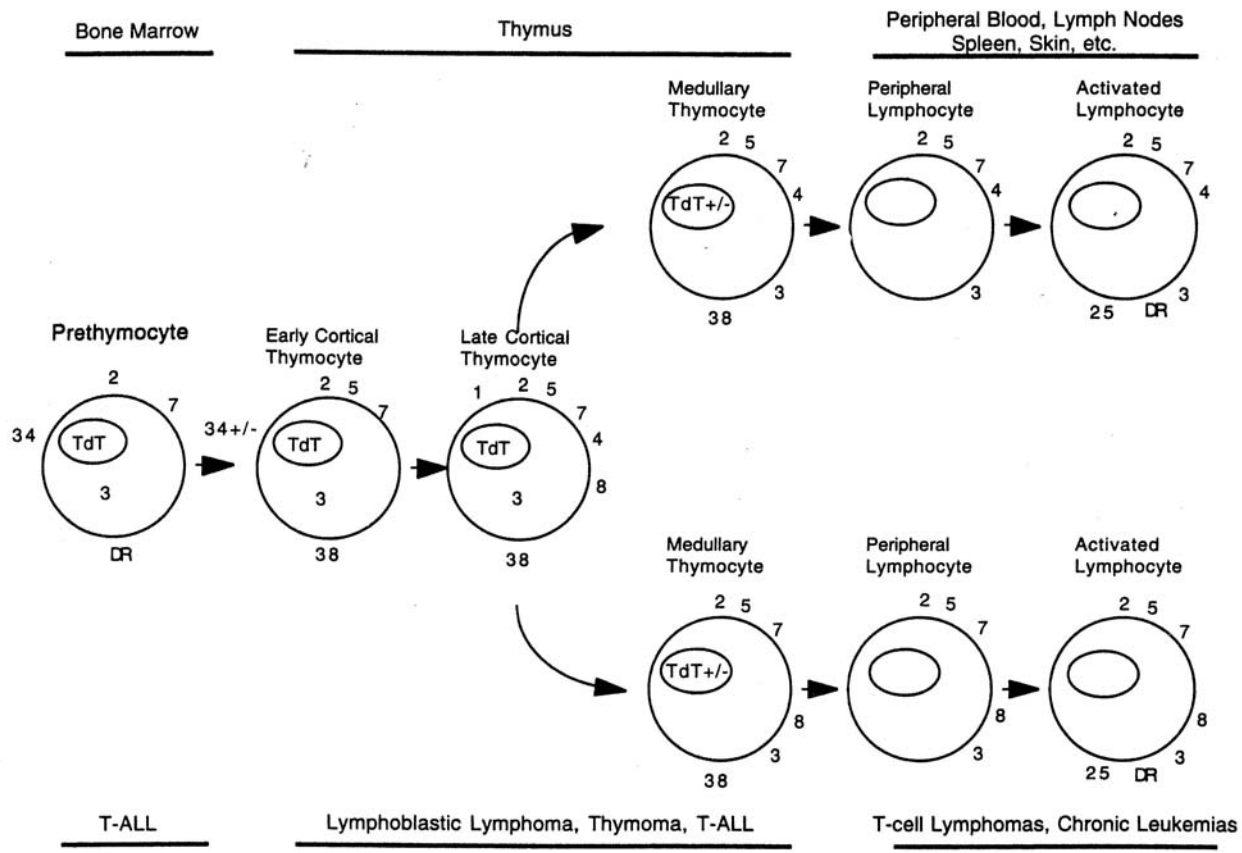

Figure 2. Sequential expression of selected important antigens during T-cell development. Several disorders are shown under the phenotype most often recapitulated.

Lineage definitions
b) B-cell lineage

- B cells were initially identified by the presence of surface immunoglobulin, and those with cytoplasmic μ heavy chain as precursor cells (pre-B cells)
- CD19 and CD24 antigen appear early in B-cell development as well as CD10 or common acute lymphoblastic leukaemia antigen (CALLA)
- CD20, CD21 and CD22 appear in later B-cell differentiation

Lineage definitions
b) B-cell lineage

- If the neoplasm expresses κ and λ light chains, these can be used to establish clonality, as clonal malignant cells express only one type of light chain
- In this case , flow cytometry allows the assessment of clonality as well as of lineage, which can be used to differentiate low grade neoplasms such as CLL form reactive B-cell proliferations

Lineage definitions
b) B-cell lineage

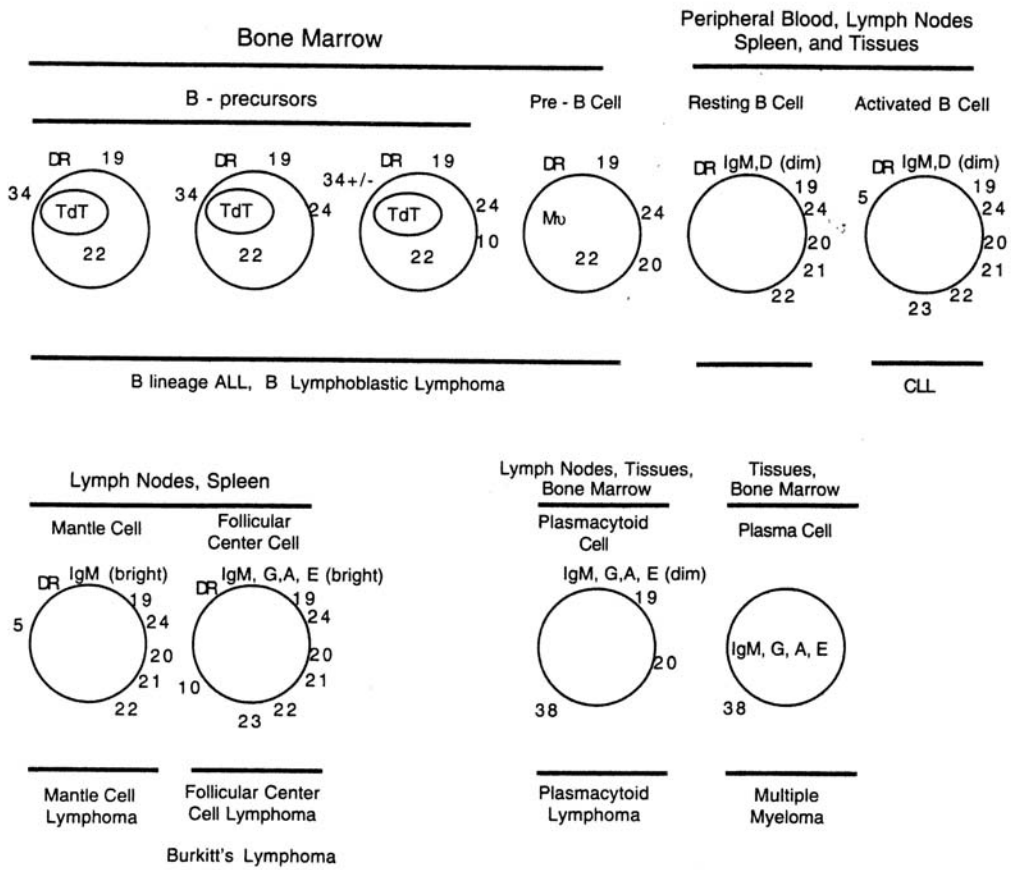

Figure 3. Sequential expression of selected important antigens during B-cell development. Several disorders are shown under the phenotype most often recapitulated The resting and activated B cells express IgM and IgD. The follicular center cell, plasmacytoid cell, and plasma cell express IgM or IgG, IgA, or IgE.

Lineage definitions
c) Myeloid lineage

- Myeloid lineage cells are characterized by increased side scatter due to the presence of lysosomal granules in the cytoplasm
- Blasts have little side scatter, as they contain only very little amount of granules

Lineage definitions
c) Myeloid lineage

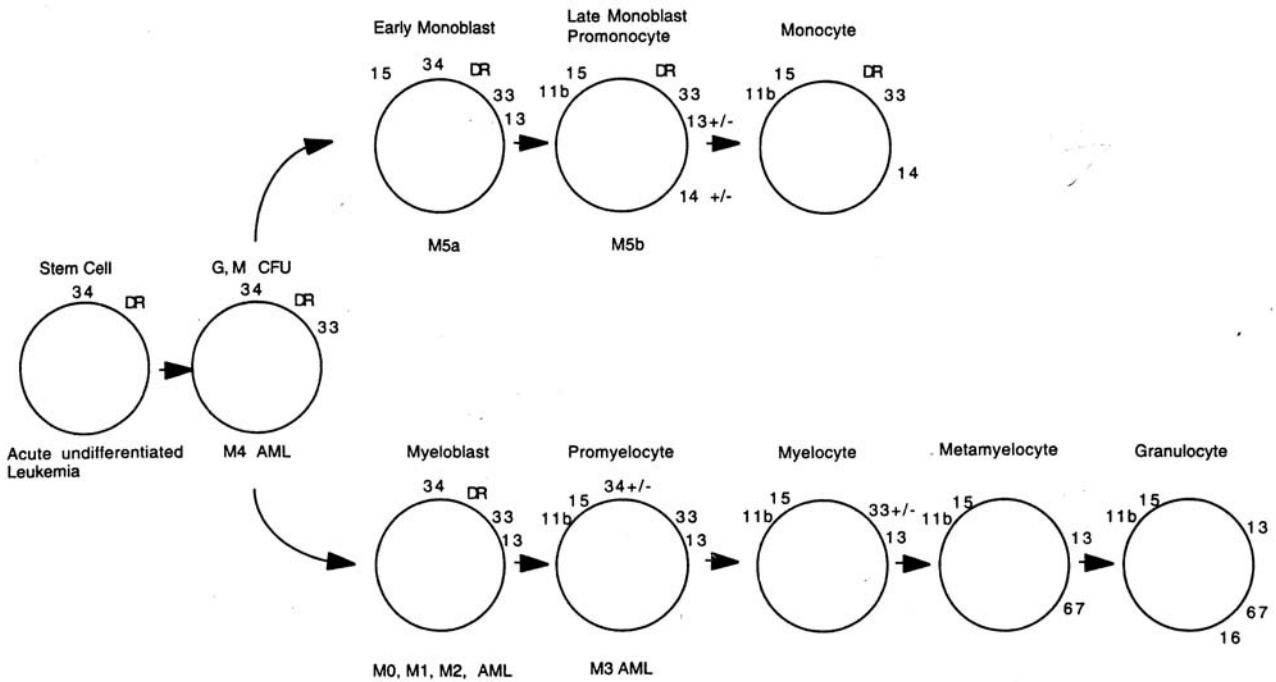

Figure 4. Sequential expression of selected important antigens during myeloid and monocytic cell development. Several disorders are shown under the phenotype most often recapitulated.

Non-lineage-restricted antigens

- **CD34** is present on stem cells, and can be seen on lymphoid, myeloid and monocytic precursors as well as leukaemic blasts
- **HLA-DR** is present on early T cells and early myeloid cells, activated T cells and monocytes, and most B cells

Non Lineage restricted antigens

- **TdT (terminal deoxynucleotidyl transferase)** is a nuclear enyzyme responsible for gene re-arrangement and involved in Immunoglobulin heavy chain gene re-arrangement
- Ig gene re-arrangement marks the transition form the precursor to the naive B cell, which then carries surface immunoglobulin
- It is therefore a marker of lymphocytic differentiation

Terminal deoxynucleotidyl transferase

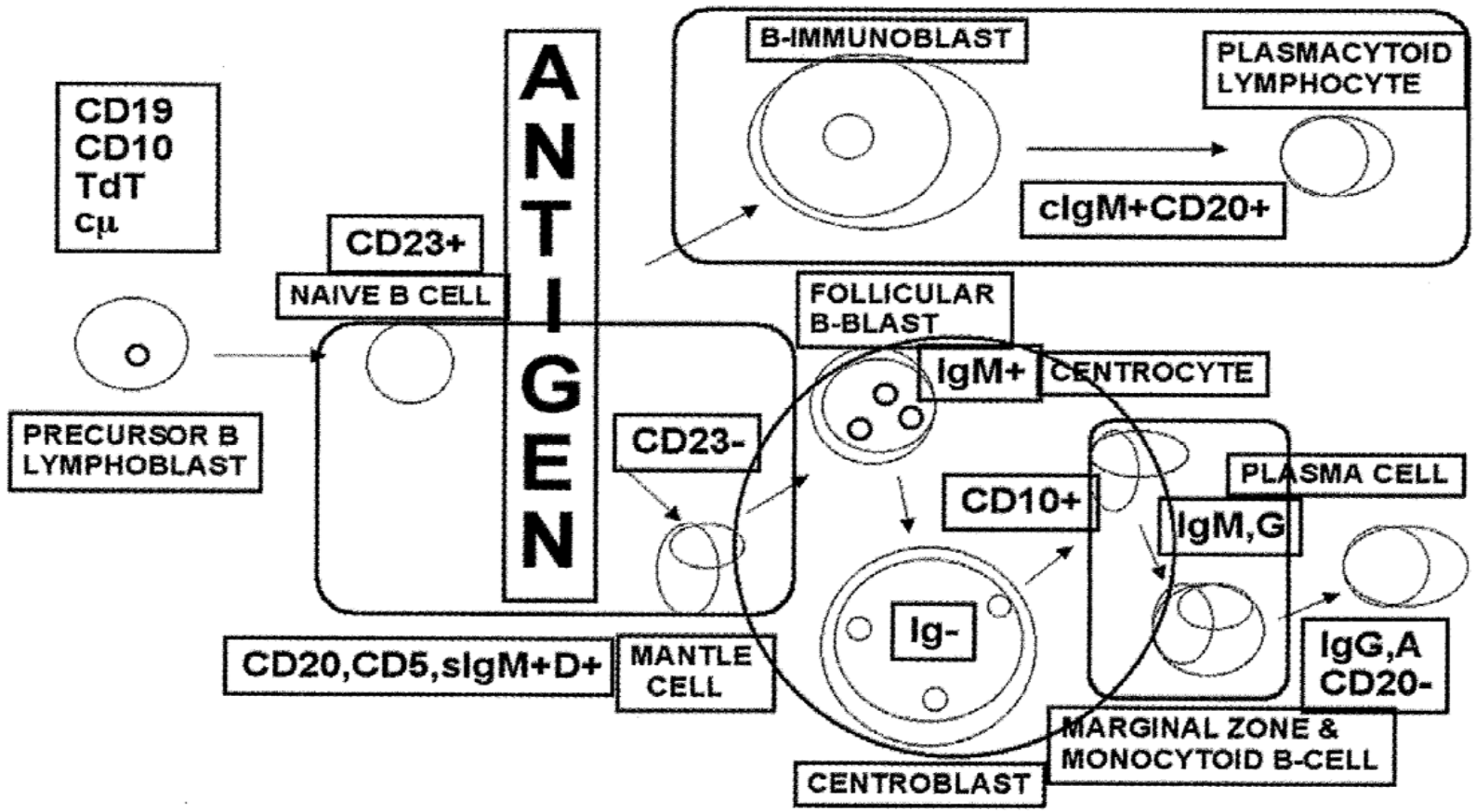

Fig. 6.03 Scheme of B-cell differentiation, showing changes in antigen expressio at various stages.

Non-lineage-restricted antigens

- **CD23 on B cells** and **CD25 on T cells** are activation antigens
- **CD56 and CD57** are found on natural killer cells

Multiparameter analysis

- Particular combinations of antigens allows the identification of neoplasms, even with minimal involvement of blood or bone marrow
- **Coexpression of CD22 and CD25 is unique to hairy cell leukaemia:** Normal B cells express CD22, and some normal T-cells CD25, but never in combination

Multiparameter analysis

- **Aberrant phenotypes are often linked to specific chromosomal translocations,** such as co-expression of B- or T- cell markers on myeloid leukaemic blasts, or co-expression of myeloid markers in acute lymphoblastic leukaemia

Bone marrow analysis

- For leukaemia diagnosis, mostly bone marrow is used, which contains different cells with overlapping forward scatter characteristics such as monocytes, blasts and metamyelocytes
- All bone marrow cells, as they progressively mature, express CD45, and they can be separated in flow cytometry on the basis of the amount of CD45 expressed

Bone marrow analysis

- Combination of CD45 and side scatter allows separation of lineages according to granularity

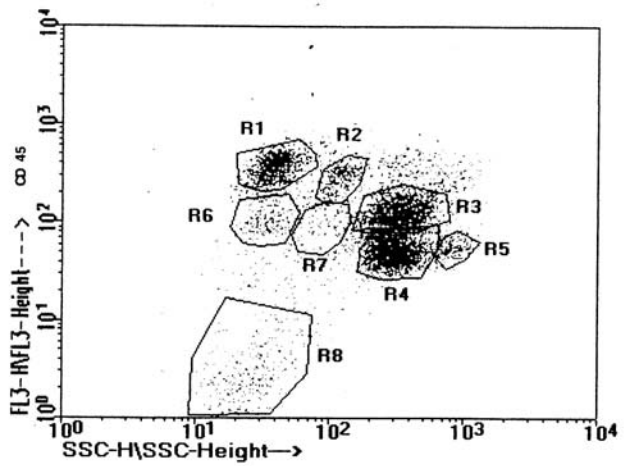

Figure 5. Normal marrow on a CD45-side scatter plot. Populations present are: R1, lymphocytes; R2, monocytes; R3, granulocytes; R4, myelocytes and metamyelocytes; R5, promyelocytes; R6, lymphoblasts; R7, myeloblasts; R8, nucleated erythroids. A few eosinophils are seen above R3.

Selected antibody panels in suspected acute leukaemia

- Most of the immunophenotypic markers are not lineage specific, and therefore it is always indicated to use a combination of antibodies
- Good lineage specificity include:

- CD79a and CD79b for the B-cell lineage
- CD3 for the T-cell lineage
- MPO for myeloid cells

- Poor lineage specificity include:

- TdT, HLA-DR, CD7, CD10

Selected antibody panels in suspected acute leukaemia

- **Primary panel**

- Myeloid differentiation	CD117, CD13, CD33, anti-MPO, CD65
- B-lymphoid differentiation	CD19, CD22, CD79a
- T-lymphoid differentiation	CD2, CD3, anti-TCRαβ, anti-TCRγδ
- Immature cells	Anti-TdT, CD34, HLA-DR

Selected antibody panels in suspected acute leukaemia

- **Secondary panel**

1) Myeloid differentiation

- Antiglycophorin for erythroid differentiation
- CD41, CD61 for megakaryocte differentiation
- CD14 for monocytic differentiation
- CD11b for granulocytic or monocytic differentiation

Selected antibody panels in suspected acute leukaemia

- **Secondary panel**

2) <u>B-lineage differentiation</u>

- CD10
- Cytoplasmic μ, surface Ig

3) <u>T-lineage differentiation</u>

- CD1a
- CD4, CD5
- CD8

Selected antibody panels in suspected chronic lymphoproliferative disorders

- **Primary panel**

- To establish lineage — pan-B marker: CD79a, CD19, CD24; pan-T marker: CD2, CD3
- To establish clonality — anti-κ, anti-λ

Selected antibody panels in suspected chronic lymphoproliferative disorders

- **Primary panel**

- To differentiate between CLL and other less common B-/T-cell disorders:

  CD5, CD23 *(positive in CLL)*
  CD22, CD79b, FMC7 *(weak or negative in CLL)*

Selected antibody panels in suspected chronic lymphoproliferative disorders

- **Secondary panel**

- To further differentiate B-cell disorders:
  CD10 *(follicular lymphoma)*
  CD11c, CD25, CD103, CD123 *(Hairy cell leukaemia)*
  Cyclin D1 *(Mantle cell lymphoma)*
  CD38, CD79a, CD138, Cy Ig *(Plasma cell or lymphoplasmacytoid neoplasm)*

Selected antibody panels in suspected chronic lymphoproliferative disorders

- **Secondary panel**

- To further differentiate T-cell disorders:
  CD4, CD8 *(large granular lymphocyte leukaemia)*
  CD7 *(T-PLL)*
  CD25 *(ATLL)*
  CD11b, CD16, CD56, CD57 *(large granular lymphocyte/NK-cell leukaemia/lymphoma)*

Selected antibody panels in suspected chronic lymphoproliferative disorders

- **Secondary panel**

- Anti-Terminal deoxynucleotidyl transferase (TDT) *(lymphoblast versus mature lymphocyte)*
- CD20 *(mature lymphocyte)*

Summary acute leukaemia

Primary panel

Immature TdT, CD34, CD117

Myeloid	B-Lymphoid	T-lymphoid
CD117	CD19	CD2
CD13	CD22	CD3
CD33	CD79a	TCRαβ
MPO		TCRγδ
CD65		

Secondary panel

CD41	CD10	CD1a
CD61	cytoplasmic Ig	CD4
CD14		CD5
CD11b		CD8

Summary chronic lymphoproliferative disorder

Primary panel

B-Lymphoid	T-lymphoid
CD79a	CD2
CD19	CD3
CD24	

Clonality

anti-κ

anti-λ

CLL

CD5

CD23

Summary chronic lymphoproliferative disorder

Secondary panel

B-Lymphoid	
Follicular lymphoma	CD10
Hairy cell leukaemia	CD11c, CD25, CD103, CD123
Mantle cell lymphoma	Cyclin D1
Plasma cell myeloma/ lymphoplasmacytoid neoplasm	CD38, CD79a, Cy Ig
T-Lymphoid	
Large granular lymphocyte leukaemia	CD4, CD8
T-prolymphocytic leukaemia	CD7
Adult T-cell lymphoma/leukaemia	CD25
LGL/NK-cell leukaemia/lymphoma	CD11b, CD16, CD56, CD57