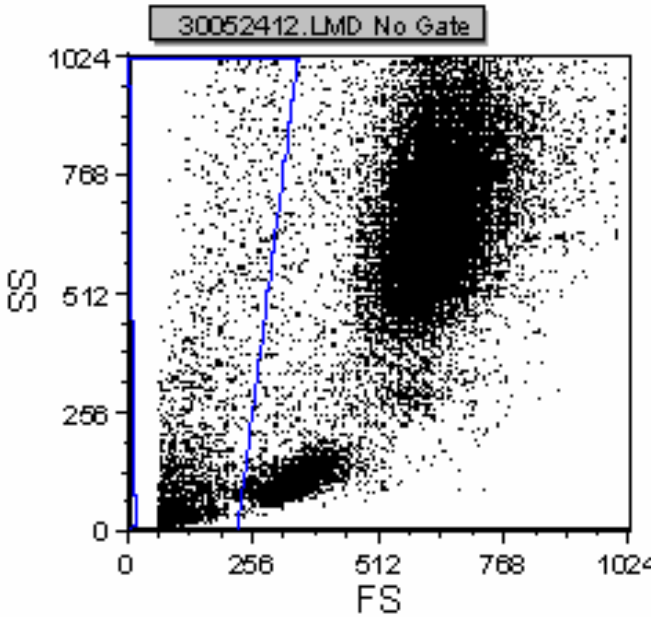
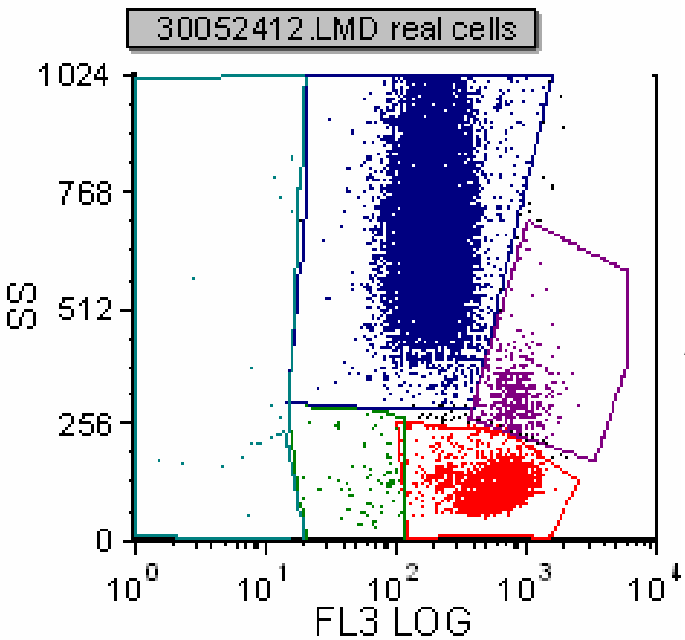
Flowcytometry – Case studies

Flow diagrams from:
http://www.denovosoftware.com/

Normal Blood



The first plot is a forward scatter (size) vs side scatter plot. A gate is drawn to identify and exclude the "debris" which could be nonviable or fragmented cells (low forward scatter), platelets or other small particles.



The second plot (gated on leukocytes) is a side scatter vs CD45 plot (FL3). The vast majority of haematopoietic cells express the surface marker CD45, which is a type I transmembrane protein present on all haematopoietic cells except erythrocytes, that assists in cell activation.

Lymphocytes express low SS and bright CD45 (red). Granulocytes express high SS and bright CD45 (blue). Monocytes express moderate SS and bright CD45 (mauve). Apoptotic cells, erythroid precursors and plasma cells are negative CD45 and variable SS. Blasts are dim to negative CD45 and variable SS (green).

Each population as defined by CD45 and SS is painted in a distinct colour which is carried throughout the entire analysis layout for ease of recognition.

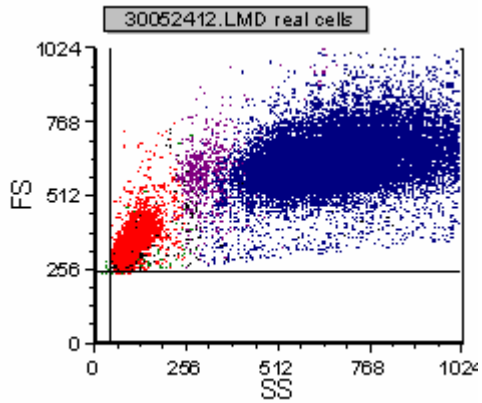
Normal Blood

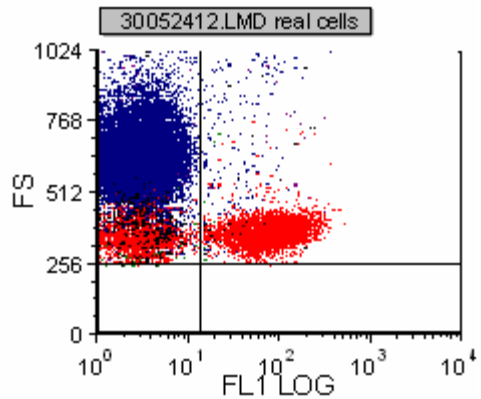
- A systematic approach to analyse a cell population by flow cytometry can look like that:

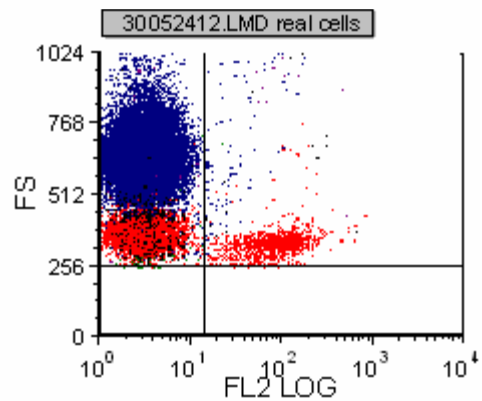
Bone Marrow and Blood 4 Color Panel Configuration:

Tube	Purpose	Markers
1	Characterize B cell malignancy	CD5/CD19/CD45/CD10
2	Identify B cell clonality	kappa/lambda/CD45/CD19+CD20
3	Characterize B cell malignancy	FMC7/CD23/CD45/CD20
4	Identify T cell malignacies	CD7/CD56/CD45/CD3
5	Identify T cell and plasma cell malignancies	CD8/CD38/CD45/CD4
6	myeloid differentiation	CD15/CD117/CD45/CD34
7	myeloid differentiation	CD16/CD13/CD45/HLA-Dr
8	myeloid differentiation	CD11b/Mo2/CD45/CD64

Normal Blood







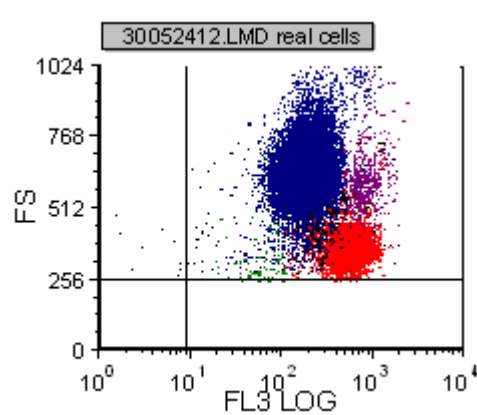
These plots are forward scatter (size) vs each individual marker (fluorescence - FL).

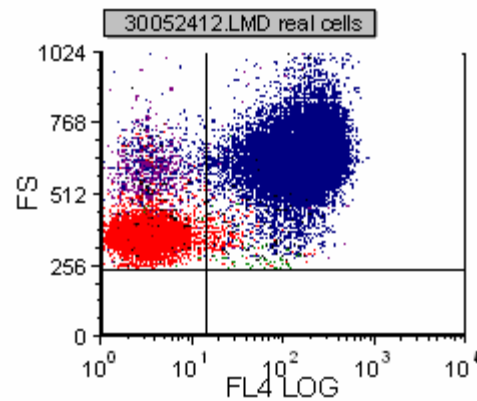
FL1 – CD5
FL2 – CD19
FL3 – CD45
FL4 – CD10

The red lymphocyte population expresses some CD19 (B cell), no CD10 and predominantly CD5 (T cell) expression.

This along with the small size identifies a normal lymphoid population. The granulocyte and monocyte populations are larger (high FS) and negative for CD19 and CD5.

The flow differential shows predominantly granulocytes, a minor lymphocyte and monocyte population and no blasts (dim/negative CD45).





Population	%	Classification
Granulocytes	78.35	NORMAL
Lymphocytes	19.15	NORMAL
Monocytes	1.78	NORMAL
Blasts	0.25	NORMAL
Other	0.10	NORMAL

CD markers

- 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
- monoclonal antibodies are used to identify particular cell antigens
- Cell antigens were initially assessed sequentially, but with advancing technology, several different cell surface molecules can now be labelled simultaneously with monoclonal antibodies carrying fluorochromes to study the co-expression of 2, 3 or 4 antigens

CD markers

- Initially each of the large number of monoclonal antibodies had a unique name, but they were then re-named after the antigenic structure they recognised (cluster designation - CD), and nearly always carry a CD number
- Certain sets of CD markers are used to identify cell lineages, such as the lymphoid (T- and B- cell subsets) and myeloid series
- Others are activation antigens and are not restricted to lineage
- Particular combinations of antigens occur in neoplastic cells, even at minimal involvement of blood or bone marrow

a) T-cell lineage

- T-Cell lineage was the first to be characterized using antigens CD3, CD4, CD8, CD2, CD5 and CD7
- T-cell neoplasms 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
- 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

a) T-cell lineage

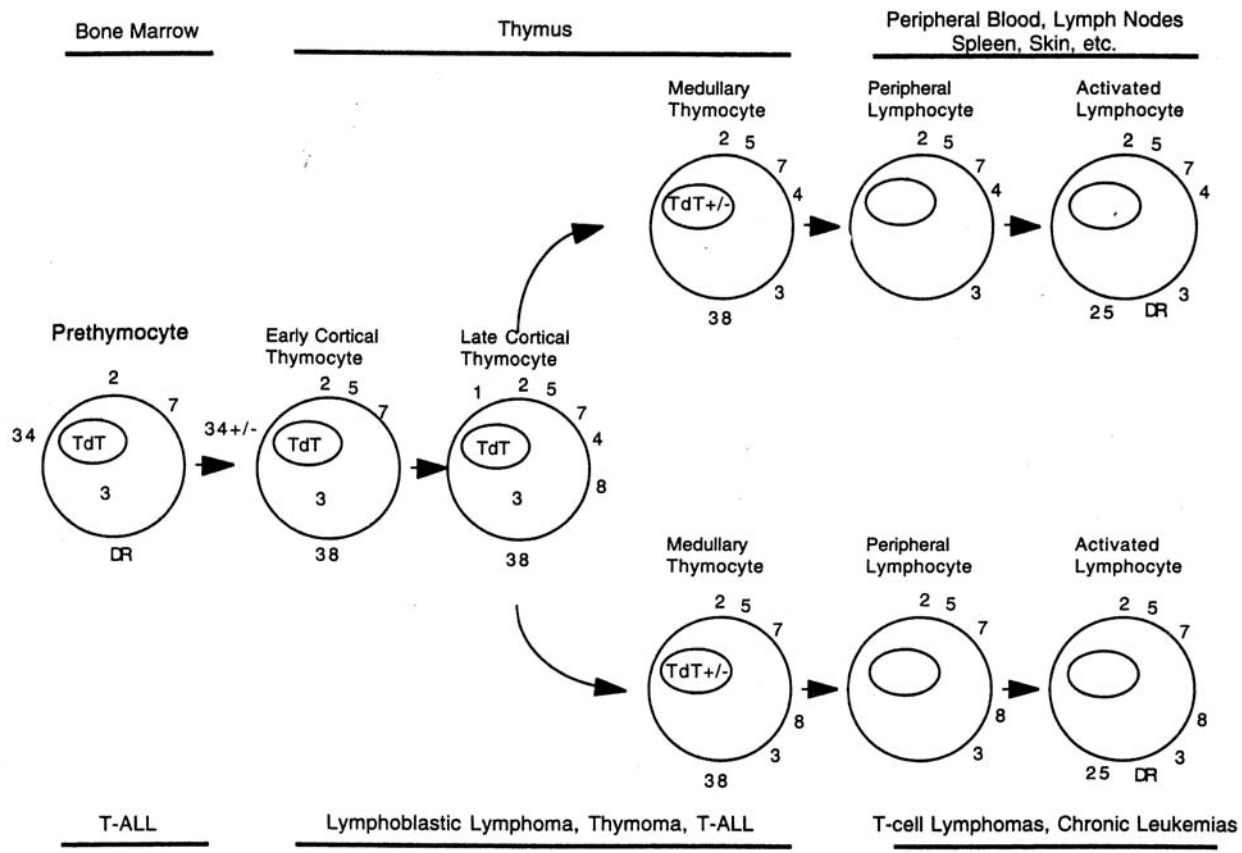
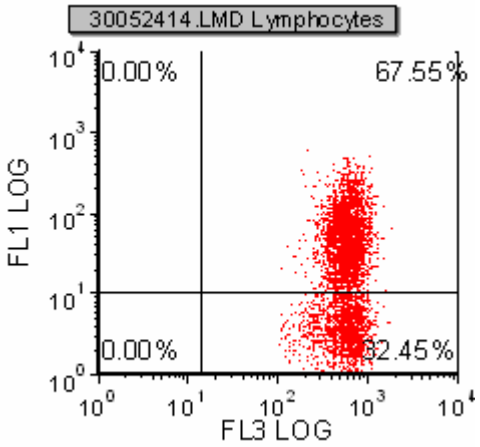
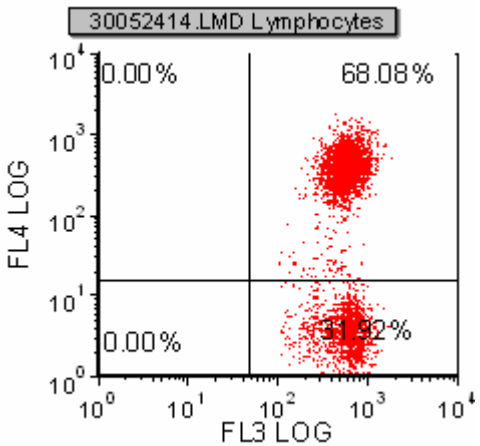
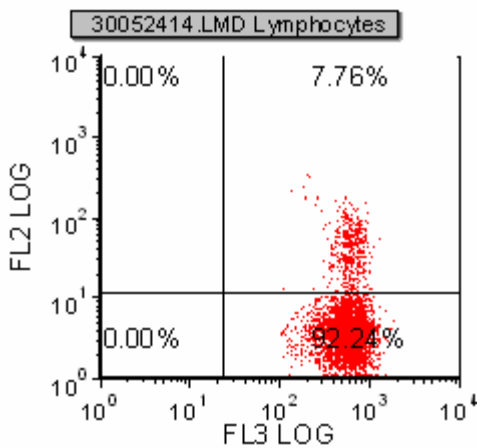


Figure 2. Sequential expression of selected important antigens during T-cell development. Several disorders are shown under the phenotype most often recapitulated.

T-cells







These plots show only the cells in the lymphocyte gate.

FL1 – CD7
FL2 – CD56
FL3 – CD45
FL4 – CD3

CD45 (FL3) is expressed on all lymphocytes.

The pan T cell markers CD3 (FL4) and CD7 (FL1) are expressed in the majority of the normal T-lymphocytes.

CD3 is a lineage specific marker for T cells.

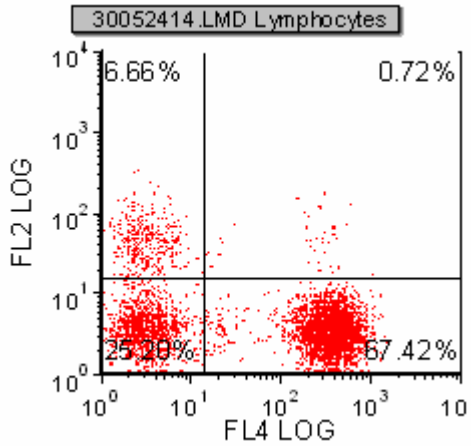
CD7 is expressed on T lymphocytes and natural killer cell (NK) cells.

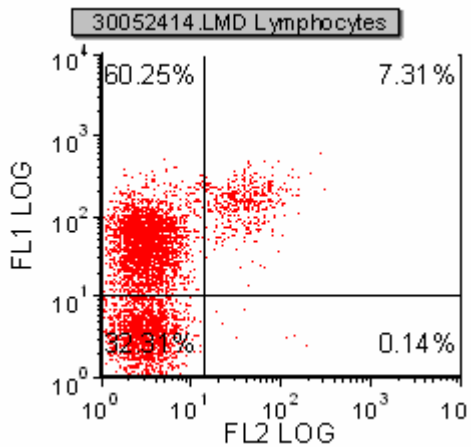
CD56 (FL2) is a NK cell marker.

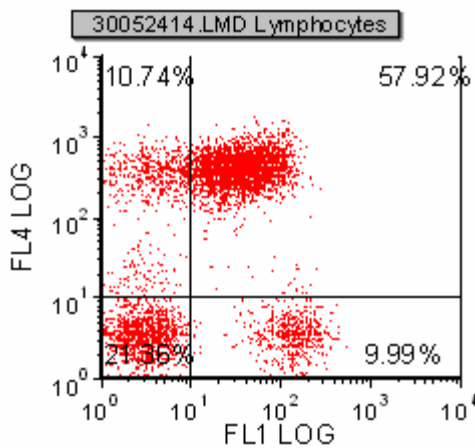
A predominant proportion of the gated lymphocytes expresses the T cell markers CD3 and CD7.

The NK marker CD56 is predominantly negative.

Marker	% of lymphs	
CD7 (T/NK)	67.55	Positive
CD56 (NK)	7.76	Predominantly negative
CD3 (T)	68.08	Positive
CD7+CD56+	7.31	Predominantly negative
CD56+CD3+	0.72	Negative
CD3+CD7+	57.92	Positive







b) B-cell lineage

- B-Cells are identified by the presence of surface Immunoglobulin
- CD19 and CD24 antigen appear early in B-cell development as well as CD10 (or common acute lymphoblastic antigen - CALLA)
- those with cytoplasmic μ heavy chain are precursor B-cells
- CD20, CD21 and CD22 appear in later B-cell differentiation
- Expression of K and λ light chains can be used to establish clonality (clonal malignant cells typically express only one type of light chains)

b) B-cell lineage

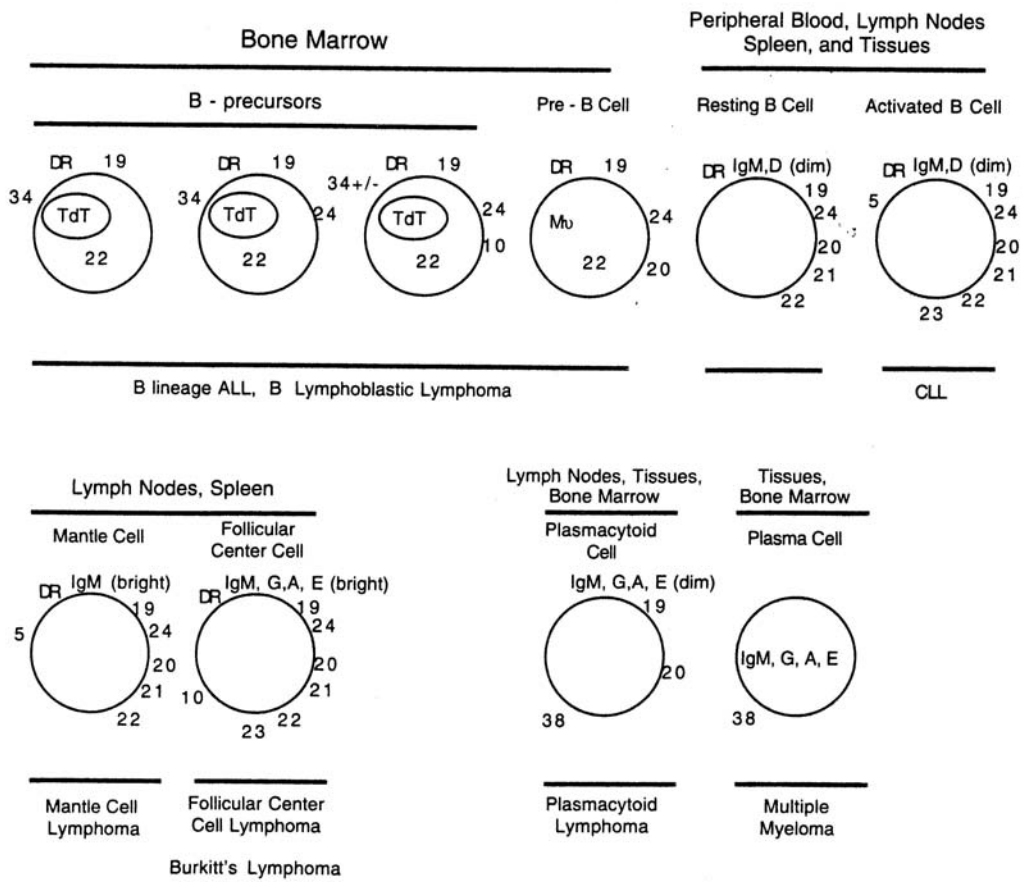
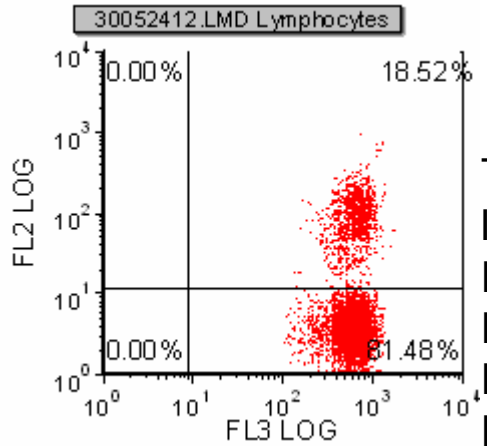
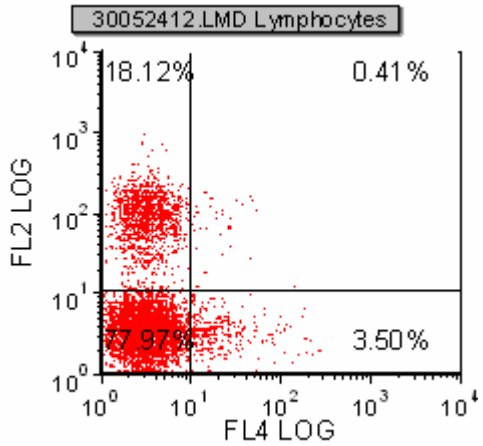


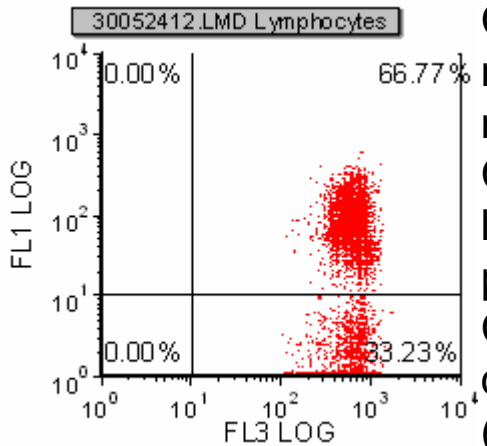
Figure 3. Sequential expression of selected important antigens during B-cell development. Several disorders are shown under the phenotype most often recapitulated The resting and activated B cells express IgM and IgD. The follicular center cell, plasmacytoid cell, and plasma cell express IgM or IgG,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

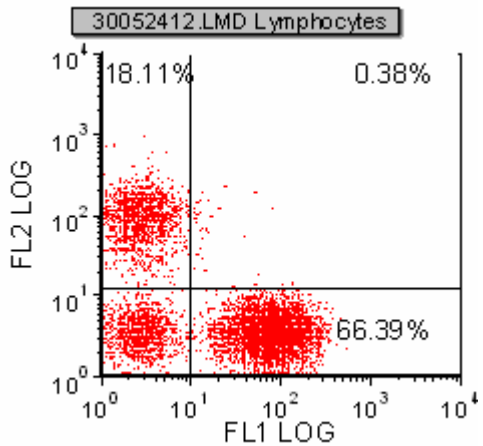
B-cells

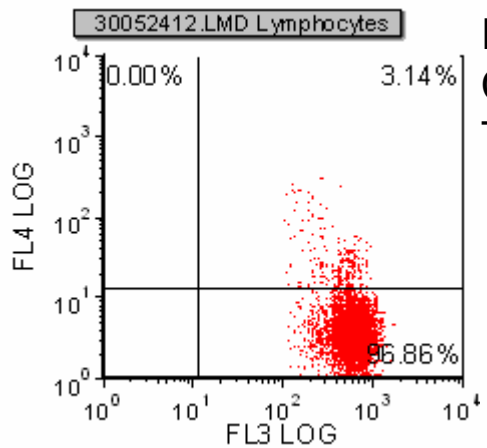




These plots show only the cells in the lymphocyte gate.
FL1 – CD5
FL2 – CD19
FL3 – CD45
FL4 – CD10
CD19 is a pan B cell marker, CD5 is a pan T cell marker and CD10 is a marker present on some malignancies.
CD19 and CD5 expression in normal lymphocyte populations show mutually exclusive populations.
Co-expression of CD19 and CD5 (if present) is diagnostic for chronic lymphocytic leukemia (CLL)



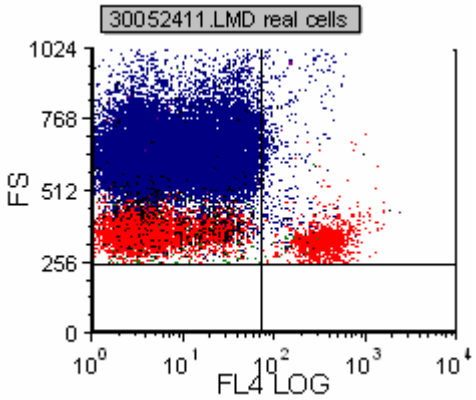


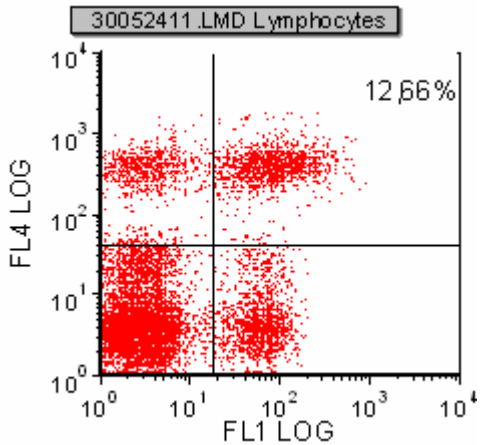


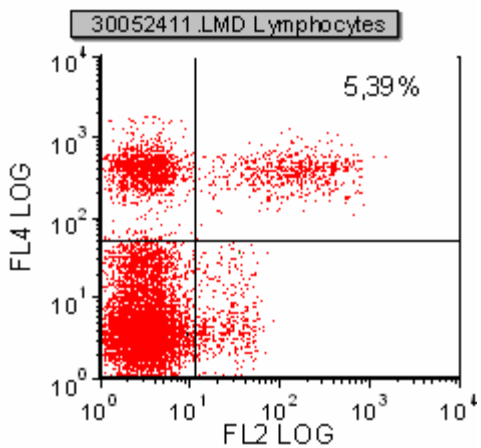
In summary, the lymphocytes express some CD5 and minimal CD19.
CD10 is not expressed.
These represent the residual normal lymphocytes in the sample.

Marker (lymph)	% lymphs	
CD10+	3.14	Negative
CD19+	18.53	Predominantly negative
CD5+	66.77	Positive
CD19+CD5+	0.38	Negative
CD19+CD10+	0.41	Negative

B-cells

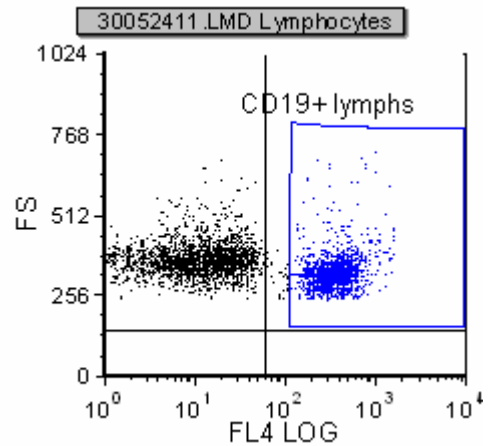


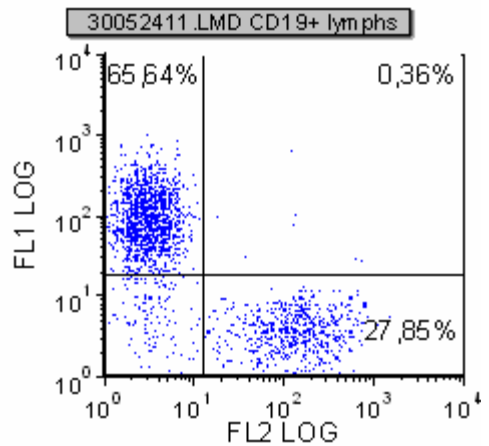




The first plot shows FS versus FL marker
The following plots show only the cells in the lymphocyte gate.
FL1 – κ
FL2 – λ
FL3 – CD45
FL4 – CD19+CD20
The combination CD19+CD20+ (FL4) is a pan B cell marker. There is a portion of Lymphocytes, which expresses CD19+CD20. In this case about about 1/3 of the lymphocytes are B cells, the remainder T-cells.
In addition, a population of lymphocytes express kappa and lambda light chains. An additional gate is placed around these B cells (painted blue) and the kappa (FL1) and lambda (FL2) expression is determined on the cells only in this gate.

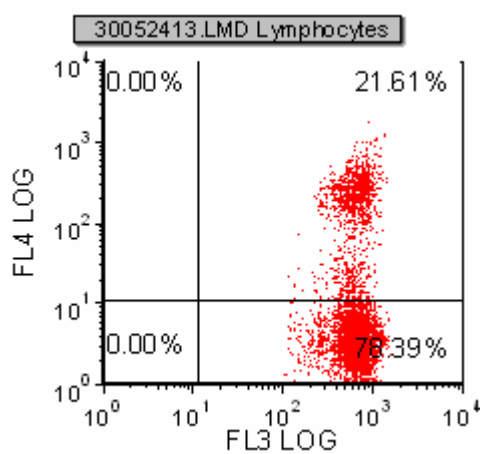
The lymphoid population shows a subset of CD19+CD20+ B lymphocytes which express a normal kappa lambda ratio

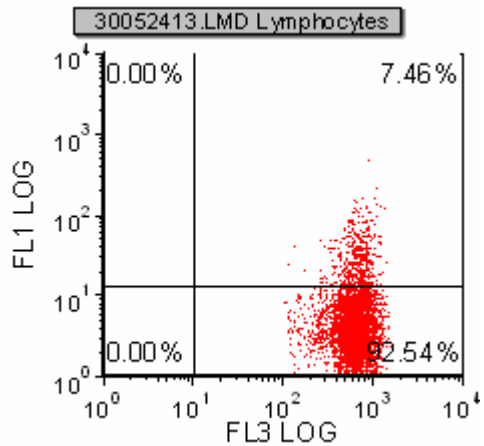


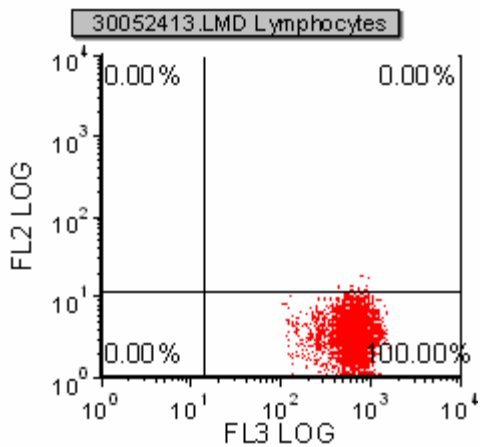


Marker	% of lymphs	
CD19+CD20+	19,88	Positive
CD19+CD20+kappa+	12,66	Predominantly negative
CD19+CD20+lambda+	5,39	Predominantly negative
Kappa/lambda ratio	2,35	Normal

B-cells







These plots show only the cells in the lymphocyte gate.

FL1 – FMC7
FL2 – CD23
FL3 – CD45
FL4 – CD20

CD20, FMC 7 and CD23 are pan B cell markers.
Expression can be used to differentiate B cell neoplasms such as mantle cell lymphoma, prolymphocytic lymphoma and chronic lymphocytic lymphoma

The lymphocytes partially express the B cell markers CD20 and FMC-7 is partially expressed.
CD23 is negative.

Marker	% lymphs	
FMC7+	44.47	Partially expressed
CD20+	21.61	Partially expressed
CD23+	0.00	Negative
CD20+CD23+	1.06	Negative
CD23+FMC7+	0.00	Negative
CD20+FMC7+	15.54	Predominantly negative

Myeloid lineage

- Myeloid lineage cells are characterized by increased side scatter due lysosomal granules in the cytoplasm
- Monocytes have side scatter intermediate between lymphocytes and mature granulocytes
- Blasts have little side scatter, as they contain only very little amount of granules
  - CD34, CD33 and CD117 characterise early myeloid cells
  - CD11b, CD13, CD15, CD65 and MPO characterise more mature myeloid cells
  - Granulocytes carry CD16
  - CD11b, CD14 and CD64, and absence of MPO are characteristic for monocytoid differentiation

Myeloid lineage

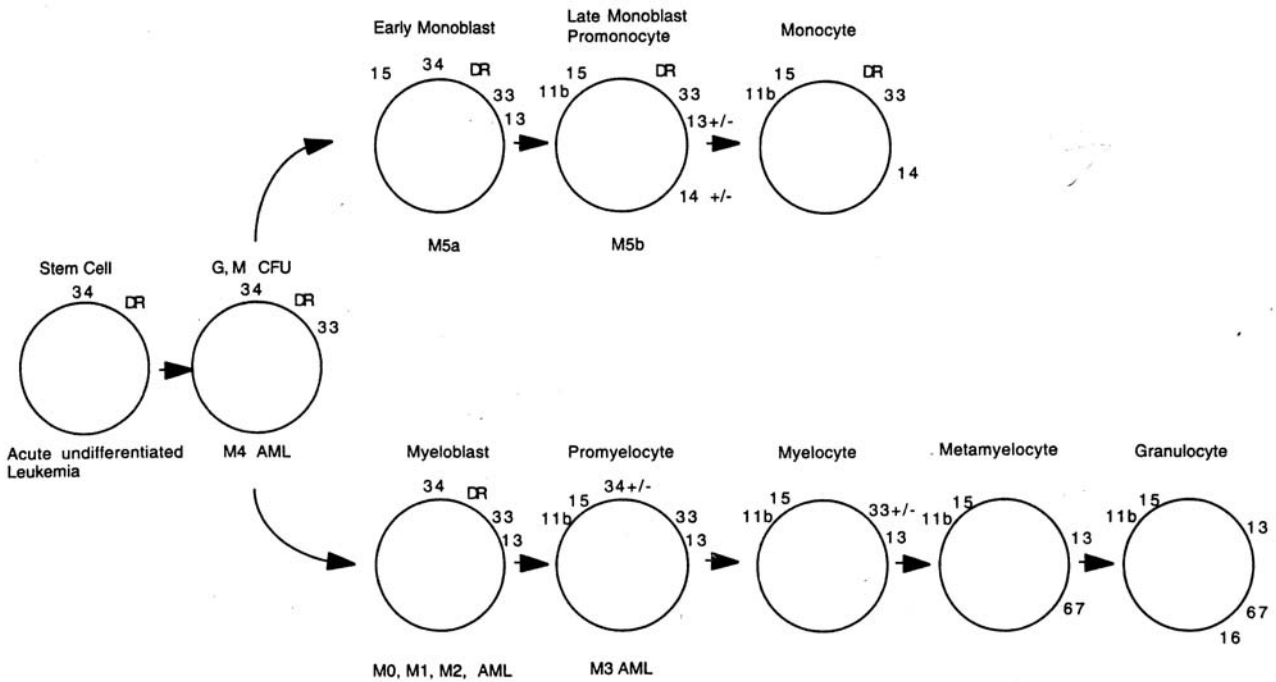
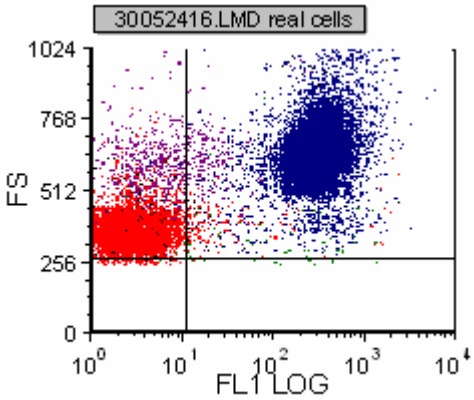
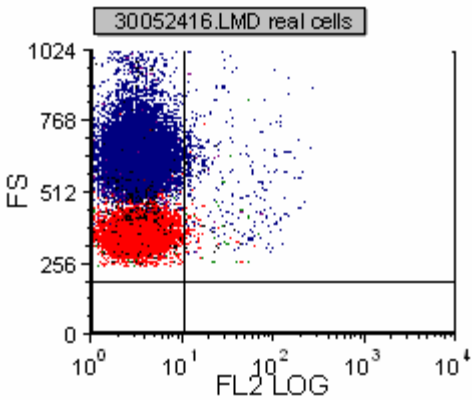
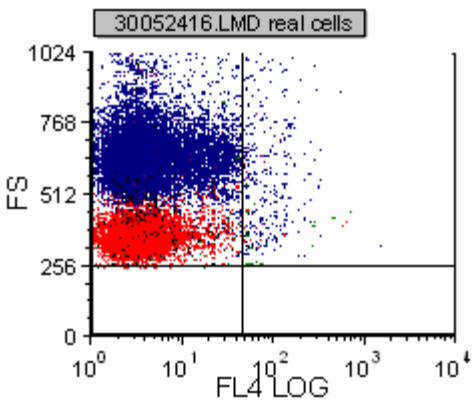


Figure 4. Sequential expression of selected important antigens during myeloid and monocytic cell development. Several disorders are shown under the phenotype most often recapitulated.

Myeloid lineage





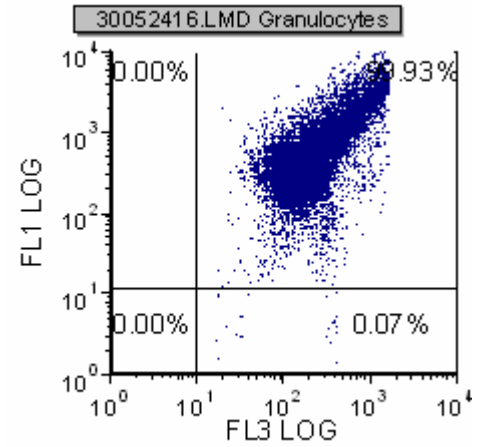


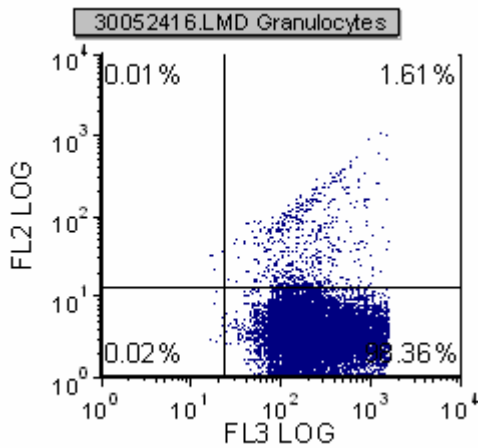
The plots on the L side show forward scatter (size) vs each individual marker (FL). On the right side, only cells in the granuloycte gate are shown.

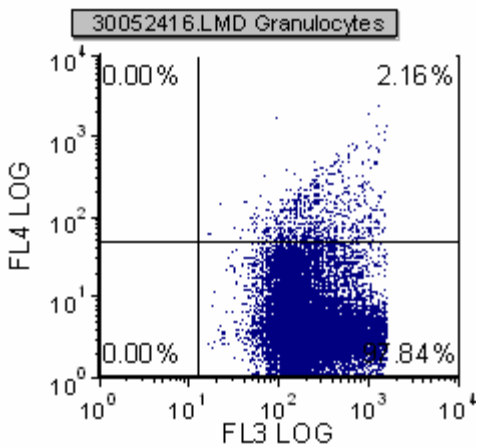
FL1 – CD15
FL2 – CD117
FL3 – CD45
FL4 – CD34

In this tube, different myeloid and maturation markers are used: CD15, CD117, and CD34. The lymphoid population is negative for these markers.
The myeloid population (blue) does hardly express CD117 and CD34, which are markers of myeloid precursors, but expresses mainly the CD15 marker, which marks more mature myeloid cells such as granulocytes.

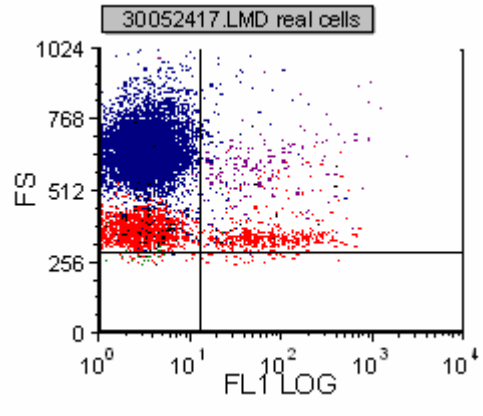
Marker	% granulocytes	
CD34	1.34	Negative
CD15	99.93	Positive
CD117	1.62	Negative

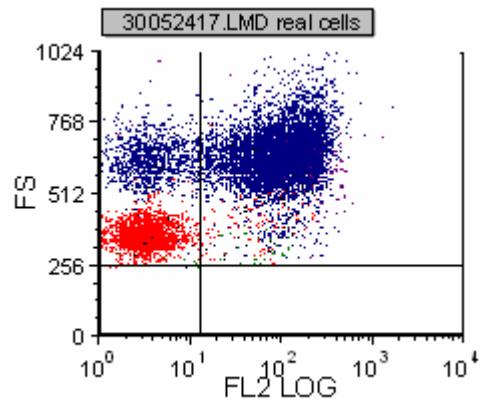


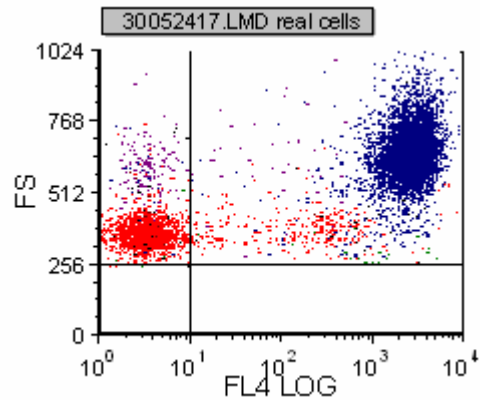




Myeloid lineage







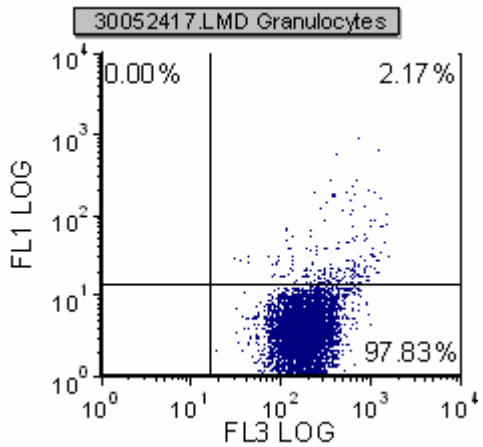
The plots on the L side show forward scatter (size) vs each individual marker (FL). On the right side, only cells in the granuloycte gate are shown.

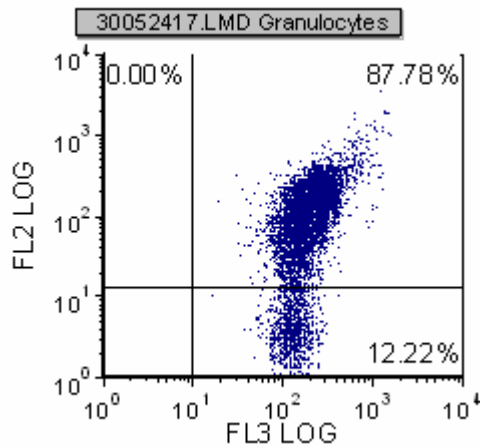
FL1 – HLA-DR
FL2 – CD13
FL3 – CD45
FL4 – CD16

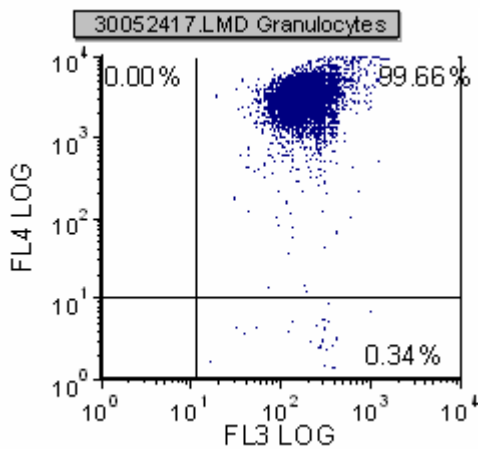
In this tube, different myeloid and maturation markers are used: HLA-Dr, CD13, and CD16. The lymphoid population is negative for these markers, but that the myeloid population (blue) expresses the CD13 and CD16 marker (which marks more mature myeloid cells such as granulocytes).

HLA DR is a marker of immaturity and is negative for the normal granulocytes in this sample.

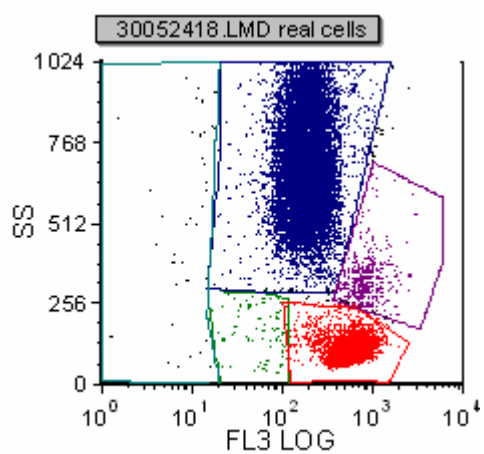
Marker	% of granulocytes	
CD13	87.78	Positive
HLA DR	2.17	negative
CD16	99.66	positive

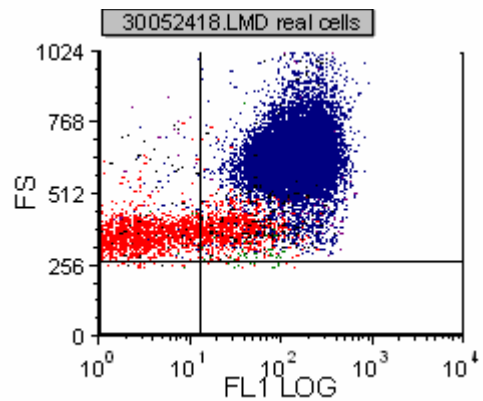


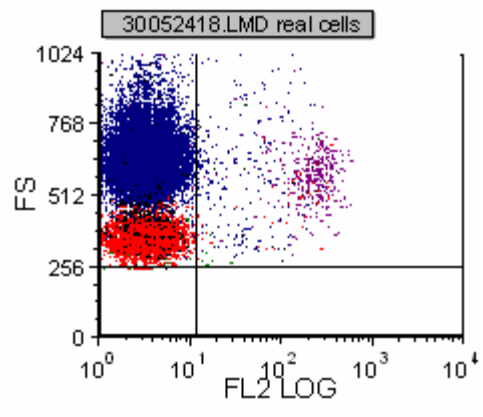




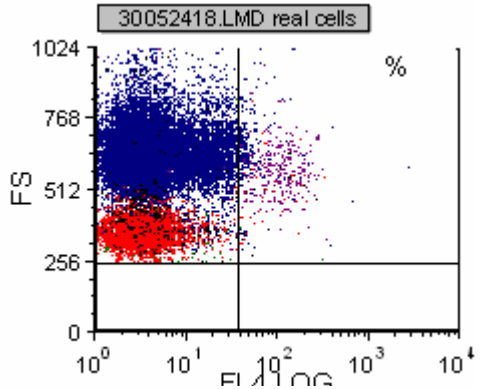
Myeloid lineage

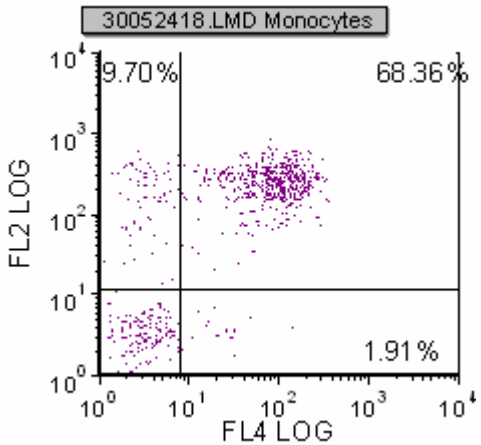






The next five plots are forward scatter (size) vs each individual marker (FL). In this tube, different myeloid/ monocytoid markers are used: CD11b, CD64, and CD14.
FL1 – CD11b
FL2 – CD14
FL3 – CD45
FL4 – CD64
These markers are helpful in assessing or differentiating myeloid/monocytoid cell malignancies.
The lymphoid population is negative for these markers, but that the myeloid population (blue) and monocyte population (purple) expresses CD11b. The CD64 and CD14 are only expressed on the monocyte population.





Normal granulocytes (blue) will normally express CD11b and CD64. CD14 is expressed only on monocytes, but not on granulocytes.

Marker	% of Granulocytes	
CD11b	99.82	Positive
CD64	12.94	Predominantly negative
CD14 (monos)	68.36	Positive

Non Lineage restricted antigens

- **CD34** is present on stem cells, and 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
- **TdT (terminal deoxynucleotidyl transferase)** is a nuclear enyzyme responsible for gene re-arrangement and involved in Immunobglobulin 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; therefore it is a marker of lymphocytic differentiation

Activation 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 at minimal involvement of blood or bone marrow:
  - **Coexpression of CD22 and CD25 is unique to hairy cell leukaemia:** Normal B cells express CD22, and normal T-cells CD25, but never in combination
  - **Coexpression of CD5 and CD23 is unique to CLL**
  - **Aberrant phenotypes are often linked to specific chromosomal translocations,** such as coexpression of B or T-cell markers on myeloid leukaemic blasts, or coexpression of myeloid markers in acute lymphoblastic leukaemia

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

- Anti-glycophorin 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 Ig, 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, HC2 *(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, HC2
Mantle cell lymphoma	Cyclin D1
Plasma cell myeloma	CD38, CD79a, Cy Ig
Lymphoplasmacytoid neoplasm	

T-Lymphoid

Large Granular Lymphocte leukemia	CD4, CD8
T-Prolymphocytic leukaemia	CD7
Adult T-cell lymphoma/leukaemia	CD25
LGL/NK-cell leukaemia/Lymphoma	CD11b, CD16, CD56, CD57