Blood and bone marrow differentiation by flowcytometry

## Workshop 4.1 MB&C26 meeting, UCLL

Depreter Barbara, clinical biologist PharmD PhD

Department of Laboratory Medicine

AZ Delta Roeselare

General

- Unique properties of flow cytometry
  - Multiparametric
  - Rapid analysis of large number of cells
  - Information at single cell level

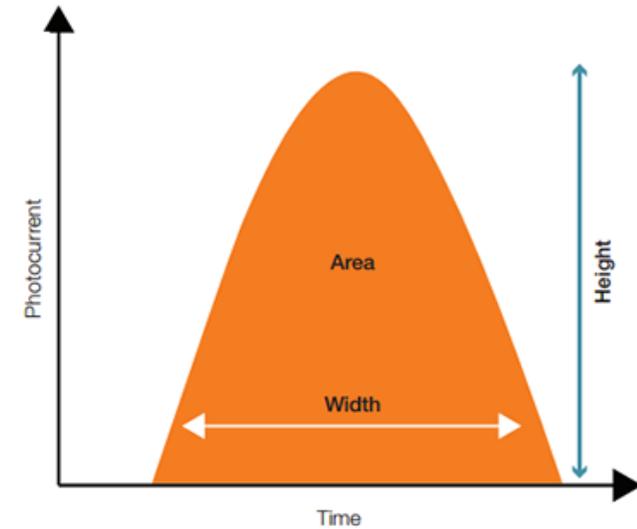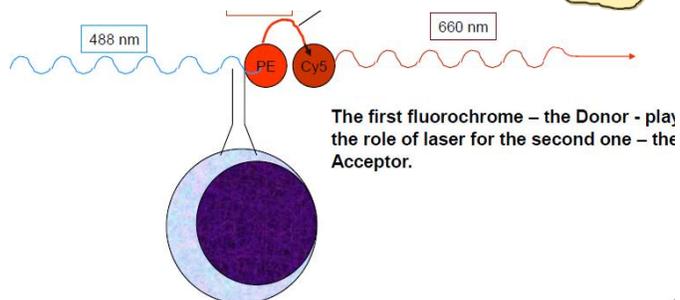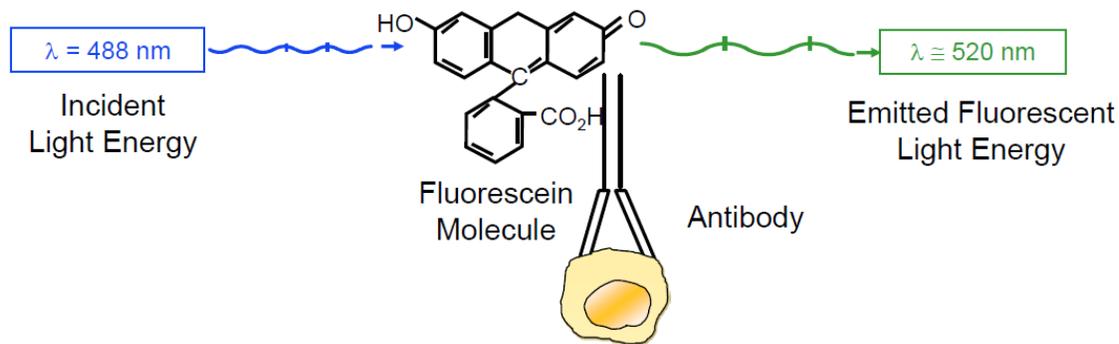- What Can a Flow Cytometer Tell Us About a Cell?

General

- Scatter parameters

  - Forward Scatter (FSC) = diffracted light
    - Detected <u>along</u> axis of incident light
    - ~ relative size (cell surface area)
  - Side Scatter (SSC)=reflected and refracted light
    - Detected <u>at 90°</u> to the laser beam
   - ~ relative granularity (internal complexity)
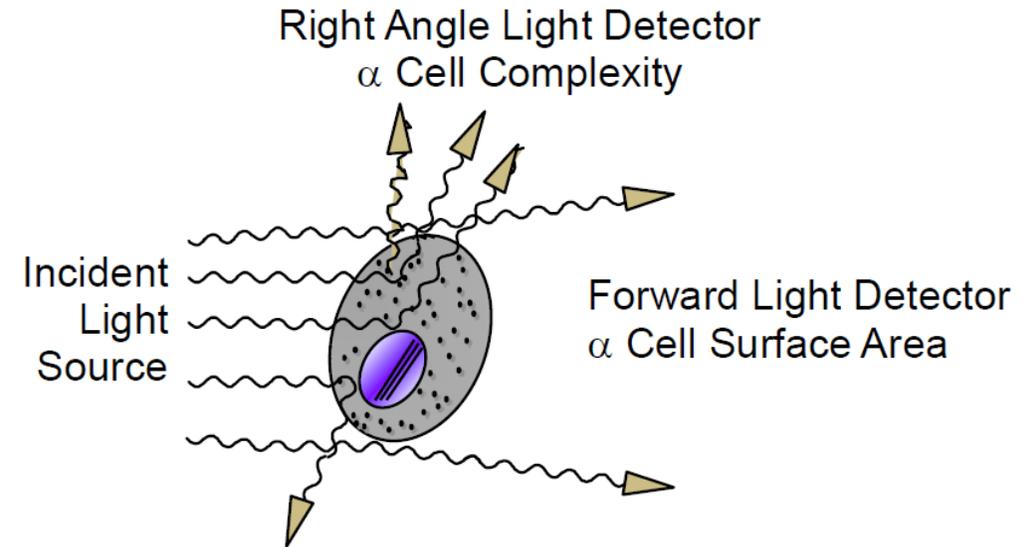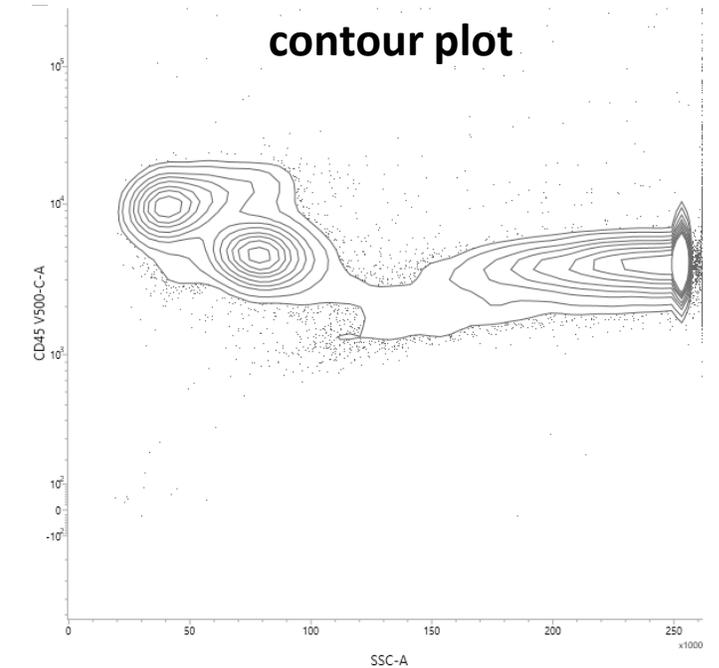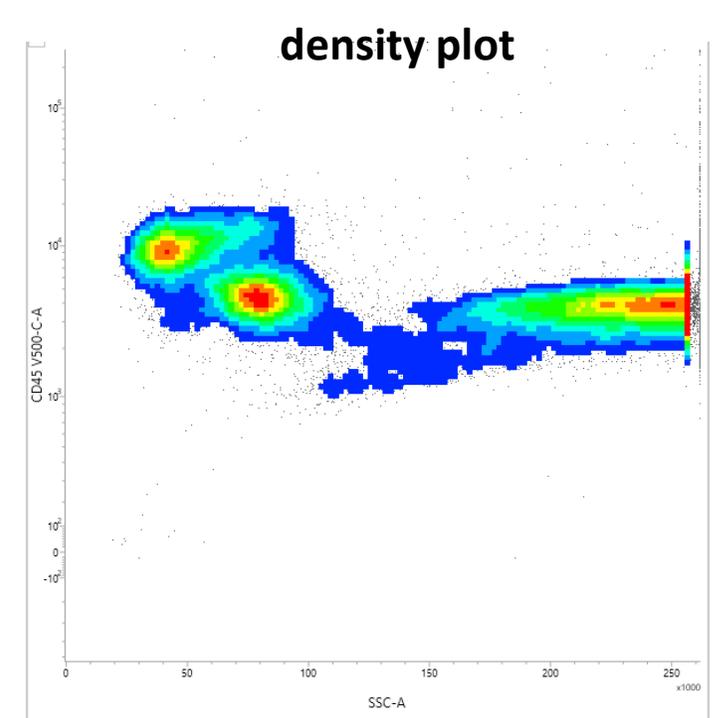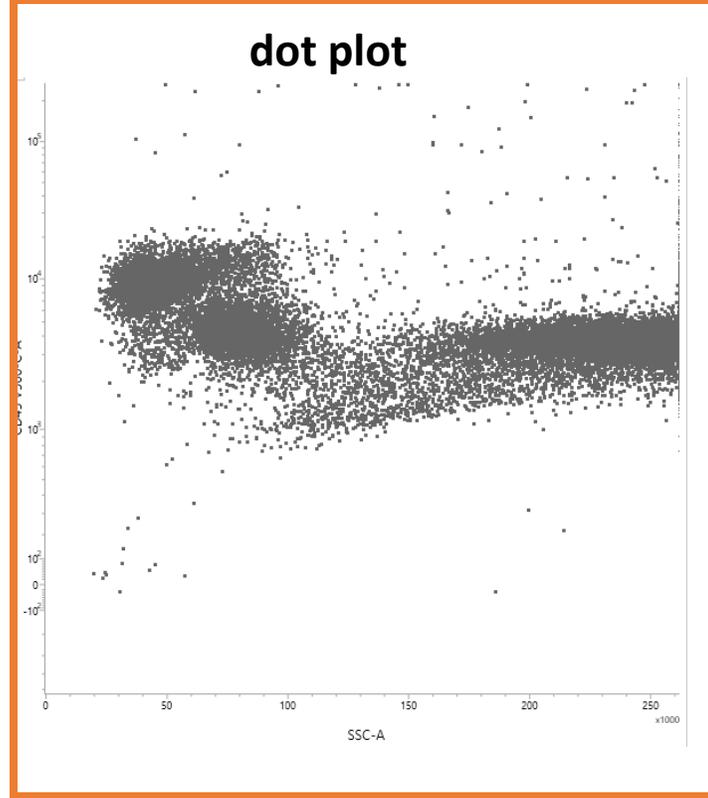
General

- Fluorescence parameters

  - mAb-fluorochrome binds cluster of differentiation (CD) antigen on the cell → fluorochrome absorbs energy from the laser and releases the absorbed energy (decreased[1]

  - Tandem fluorochromes: bleedthrough emission and decoupling

λ = 488 nm

Incident Light Energy

HO

O

C

CO₂H

Fluorescein Molecule

Antibody

λ ≅ 520 nm

Emitted Fluorescent Light Energy

488 nm

PE Cy5

660 nm

The first fluorochrome – the Donor - plays the role of laser for the second one – the Acceptor.

Photocurrent

Area

Height

Width

Time

Signal intensity: area (A) or height (H)

Time that the cell spends in the laser: width (W)

General

- Plot types

dot plot

density plot

contour plot

histogram
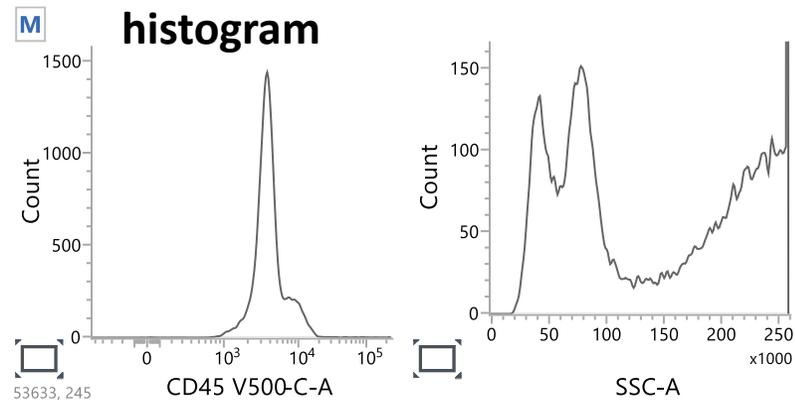
General

- Gating
  - Cleaning data: removal of debris (air, clogs, small particles) and aggregated cells
  - Setting upper and lower limits on the type and amount of throughpassing cells using electric windows (gates): judge fluorescence intensity based on a cut-off for positivity
    - Solid cut-offs i.e. 1E3
    - Negative reference populations (for example lysed red-cells fraction)
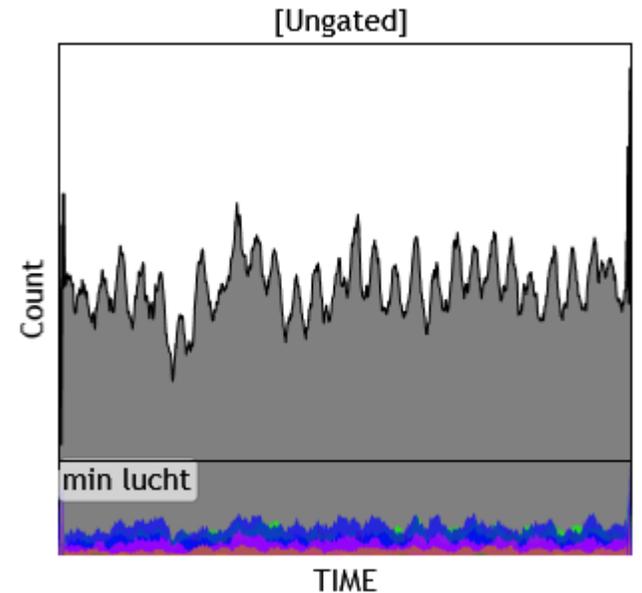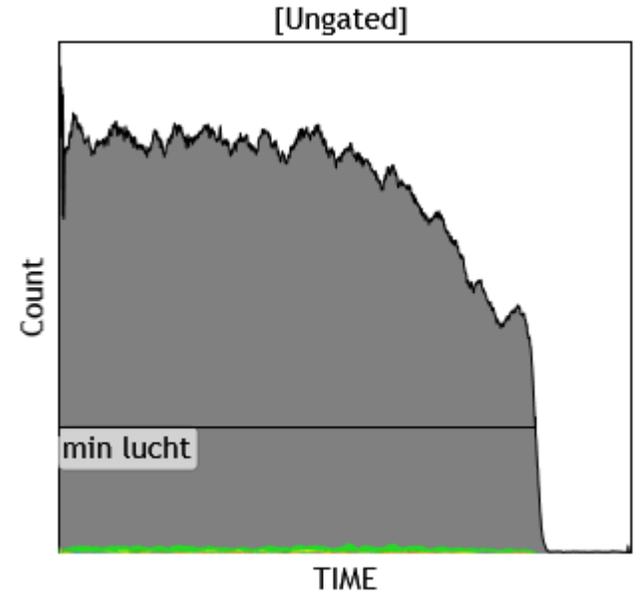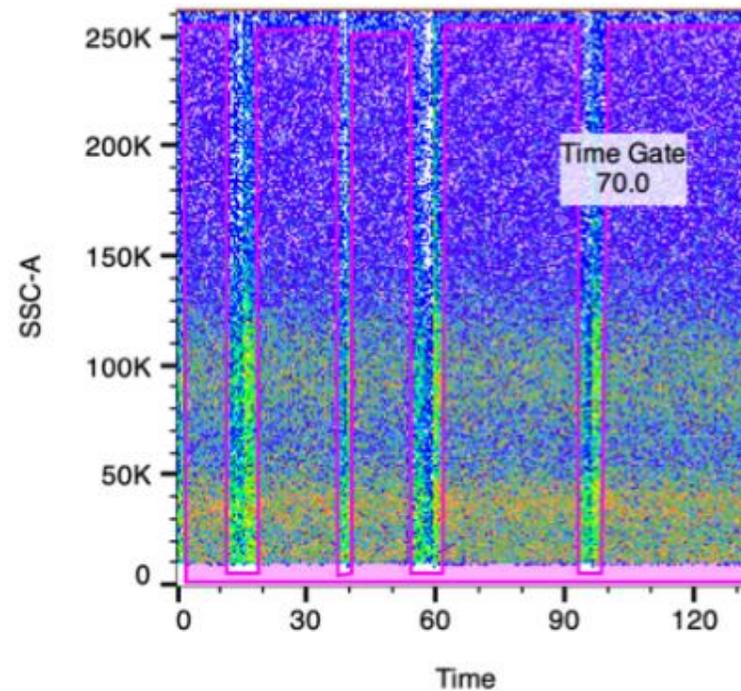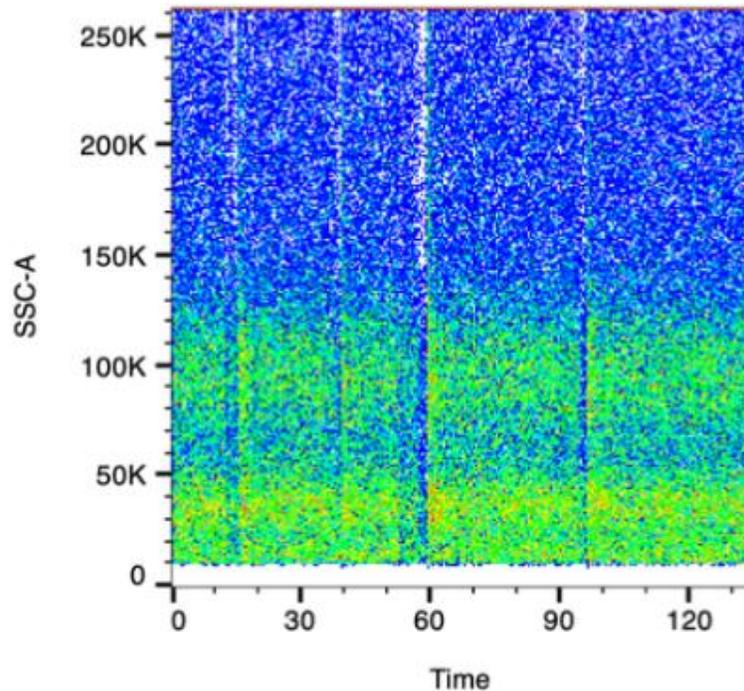   - Fluorescence-minus one (FMO) controls
    - Isotype controls

  → Select only a certain population for analysis

Flow cytometry analysis

- Standard analysis starts with
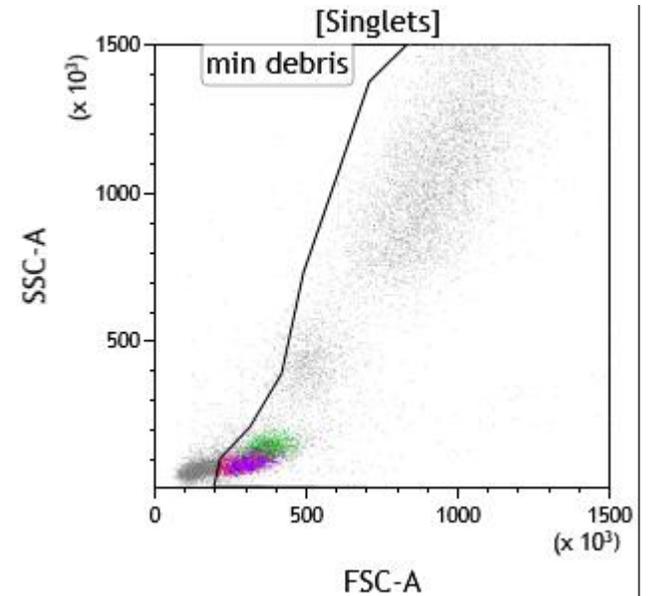
1. Time plot
   - Microbubbels, clogs and air
   - Acquisition issues → eliminate spikes

Flow cytometry analysis

- Standard analysis starts with

1. Time plot

2. Removal of debris and platelets

Flow cytometry analysis
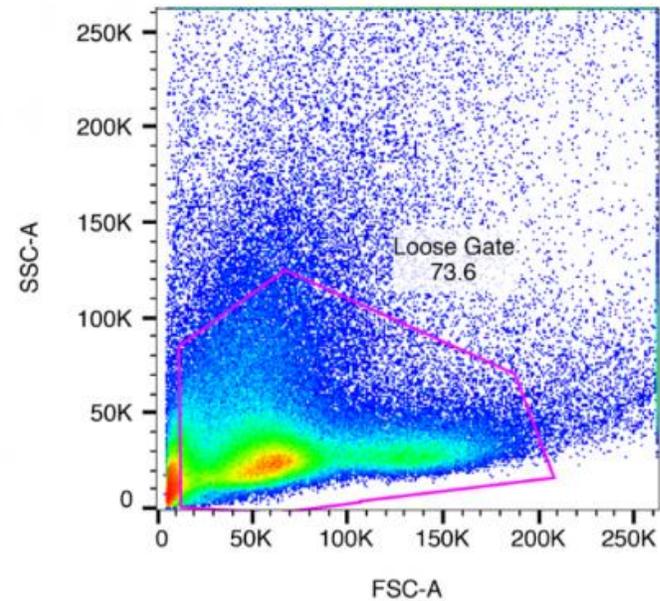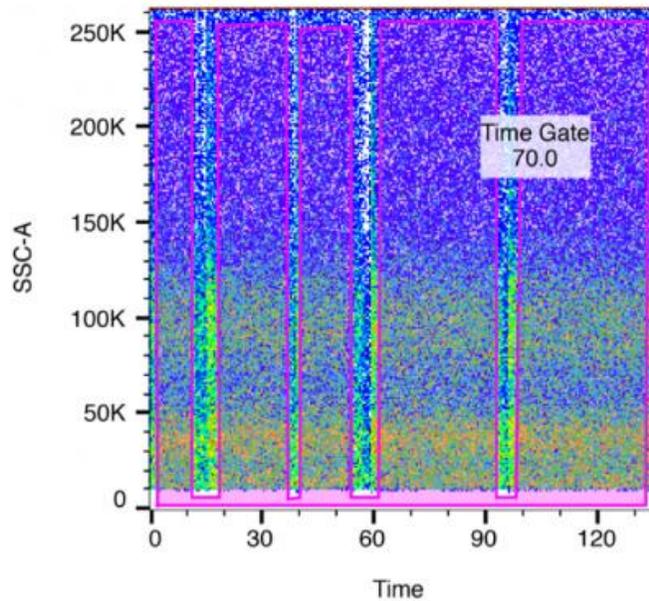
- FSC-A x FSC-H
- SSC-H x SSC-W
- FSC-H x FSC-W

- Standard analysis starts with

1. Time plot

2. Removal of debris and platelets

3. Removal of doublets
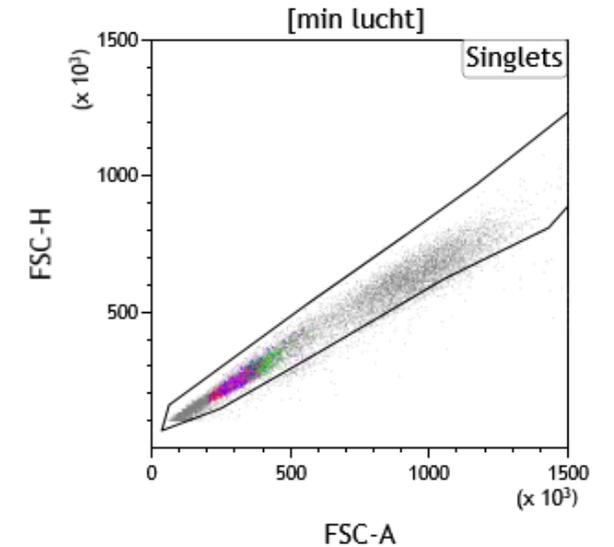
Matrix

- WBC differentiation by flow cytometry as reliable as by standard cytology, already with 5-color FCM (Faucher et al 2007, Bjornsson et al 2008)
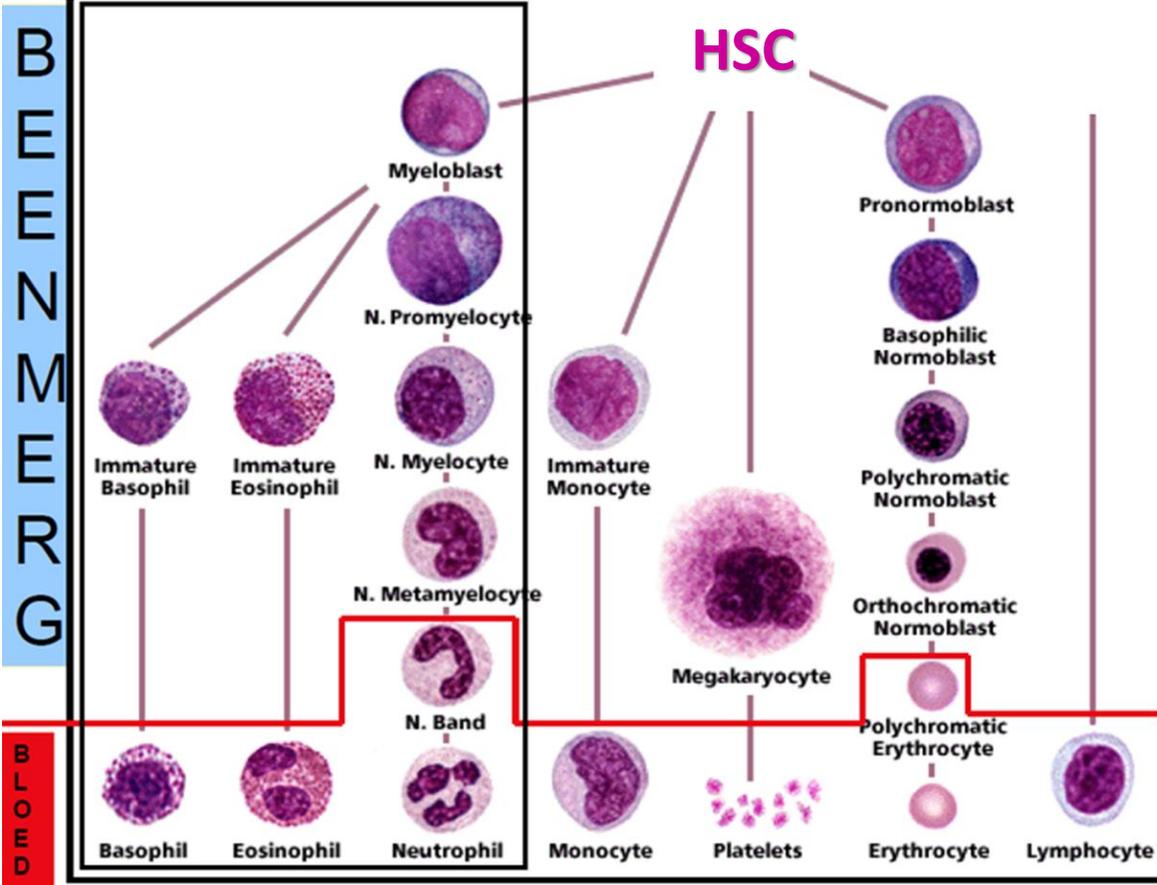
Matrix

- Whole blood
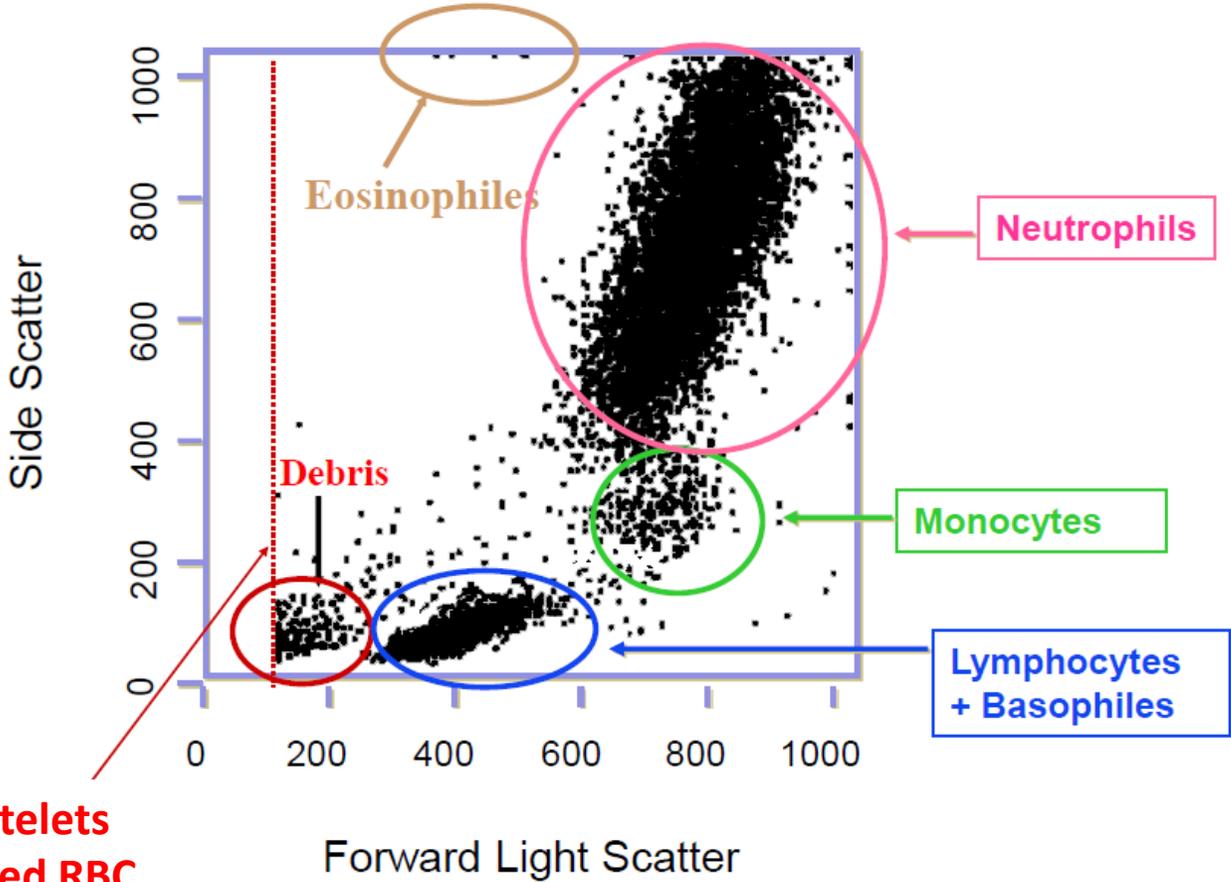
Matrix

- Bone marrow: assess hemodilution
  - First pull aspirate (2 mL)
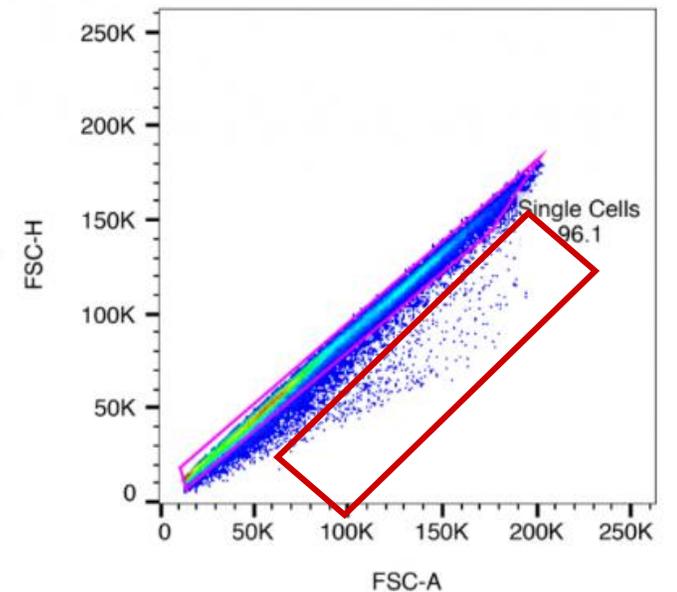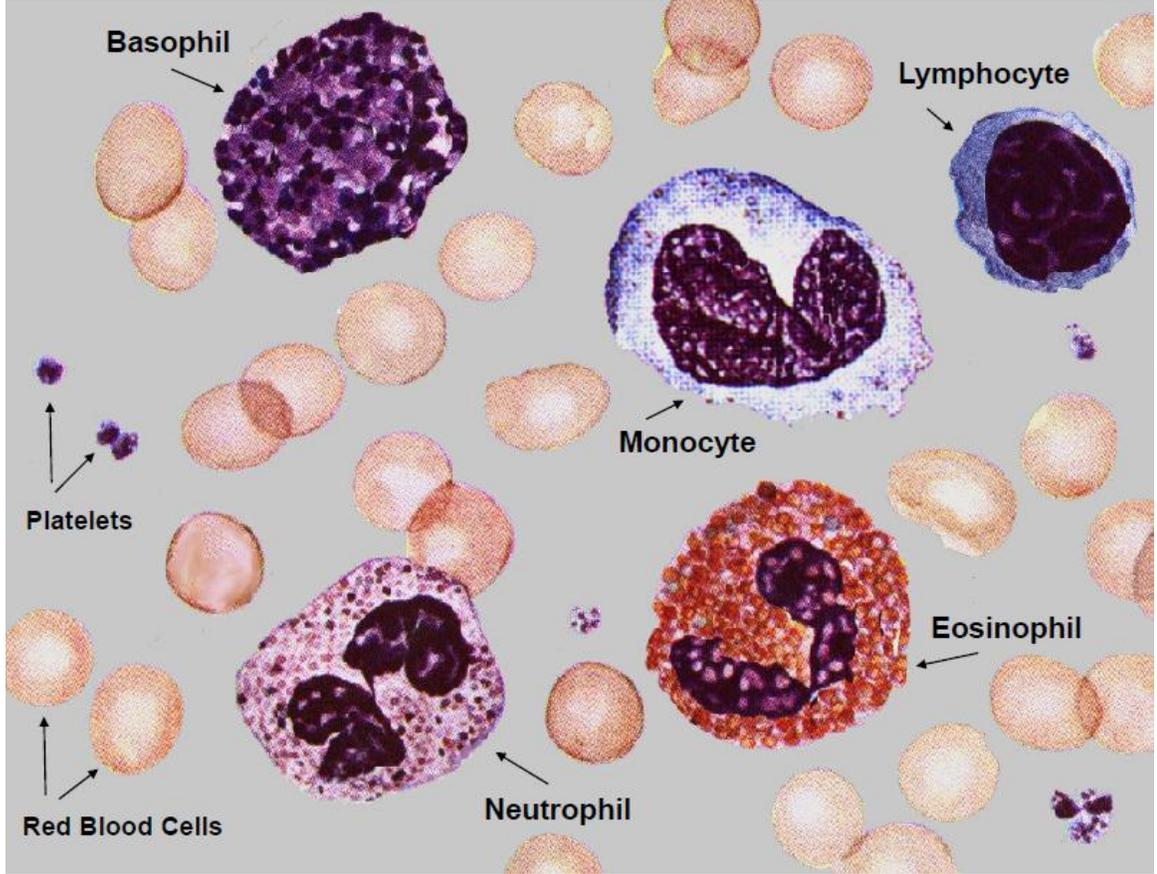 - Importance: follow-up and staging, detection of measurable residual disease (MRD), MDS (maturation-associated abnormalities)
    - (Predicted) BM purity
    - Quantification of BM-restricted nucleated cells: mast cells, plasma cells, pDCs and/or NRBCs

BM pull 1

3 mL

Bone Marrow aspiration

Formula for detecting hemodilution	Disease measured	Additional requirements
Bone marrow purity = [1-(erythrocytes BM/erythrocytes PB) x (leukocytes PB/leukocytes BM)] x 100%	MDS	Matched PB
PB contamination index = −3.052 + 0.065 x (%CD10+ neutrophils of granulocytes) −0.609 x (%CD34+)−2.008 x (%plasma cells)	MM (not used post induction)	CD10, CD38, CD138, CD34 positive cells and plasma cells
Normalized blast count = (80% / % dim CD16) x blast count	MDS/ AML	CD16 (maturing neutrophils)
Predicted bone marrow purity = [1 - (Lymphocytes FCM / Lymphocytes PB) x (Leukocytes PB / Leukocytes FCM)] x 100%	AML/MDS	Matched PB
Suggested blood contamination if mast cell population (CD117$^+$) $\leqslant$ 0.002%	MM	CD117 positive mast cells
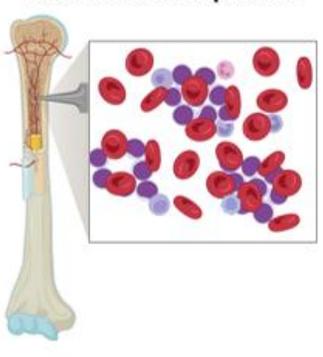
Normal cell populations

- Whole blood: lymphocyte subset analysis
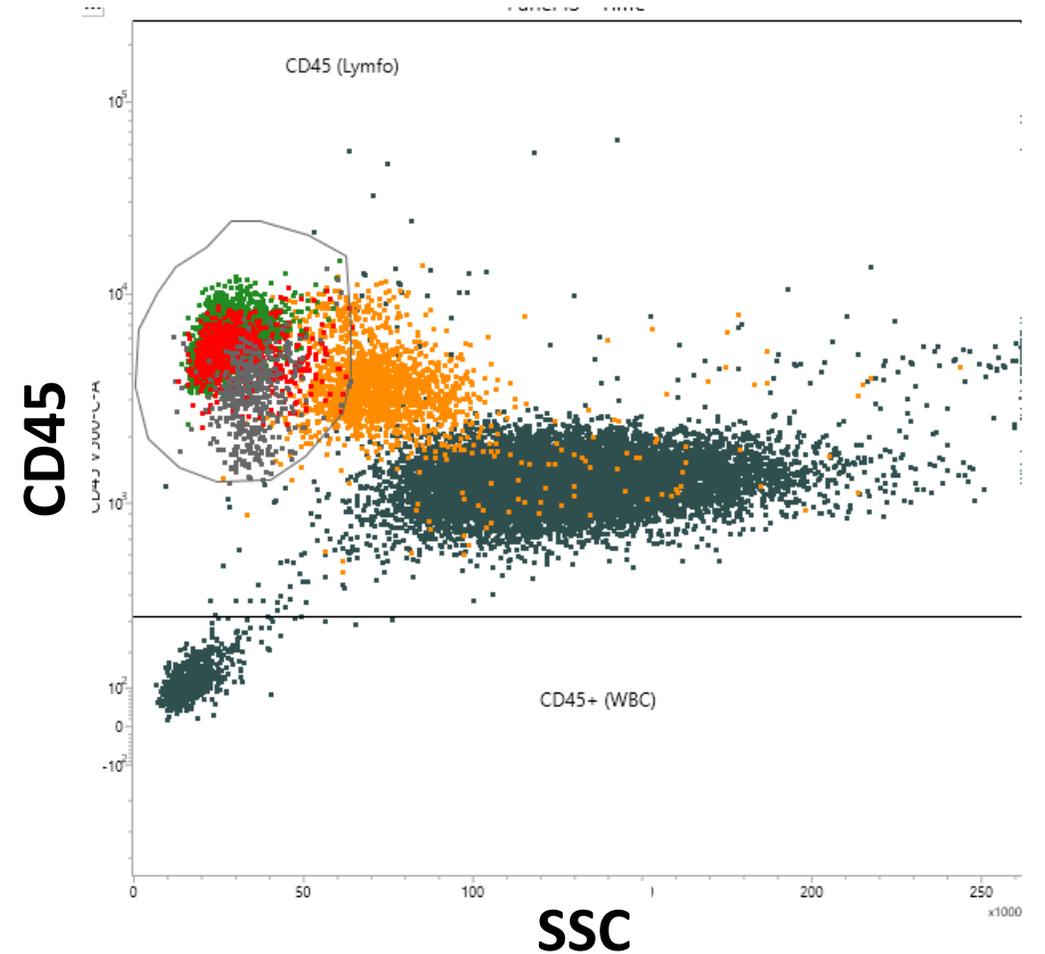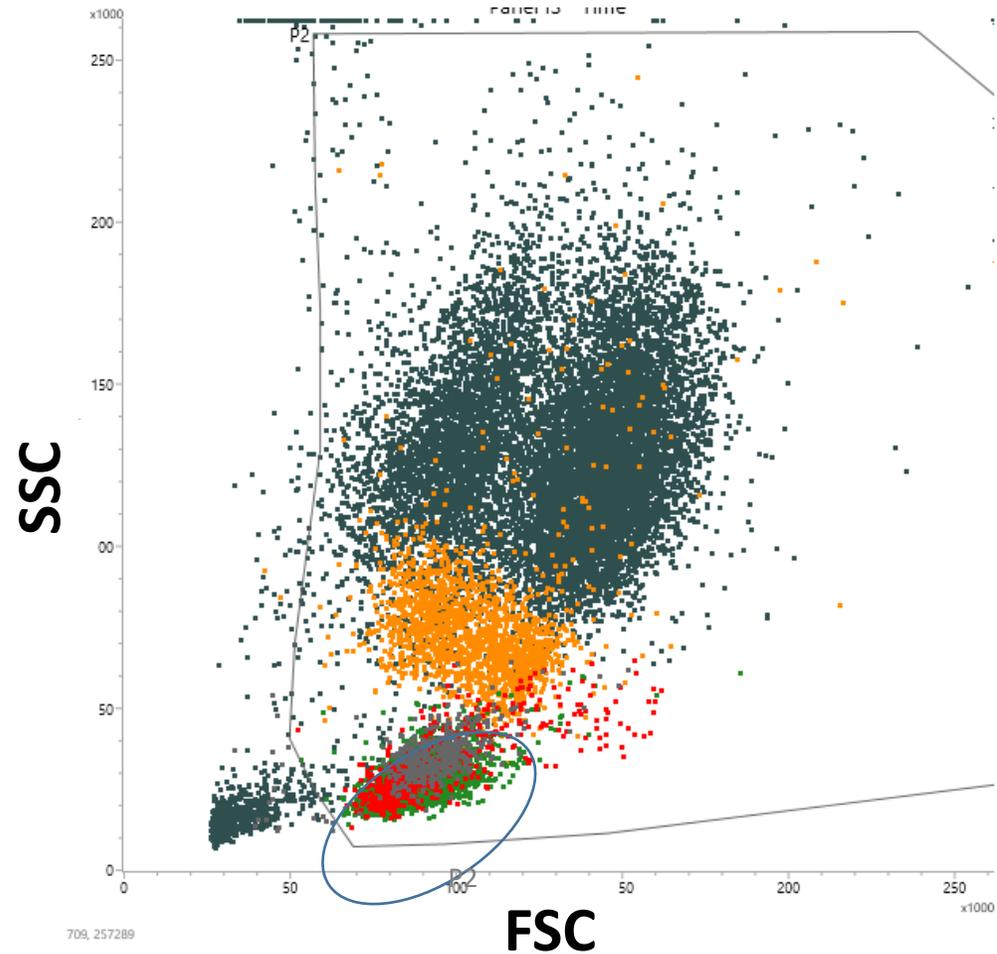
Normal cell populations

- Whole blood: lymphocyte subset analysis
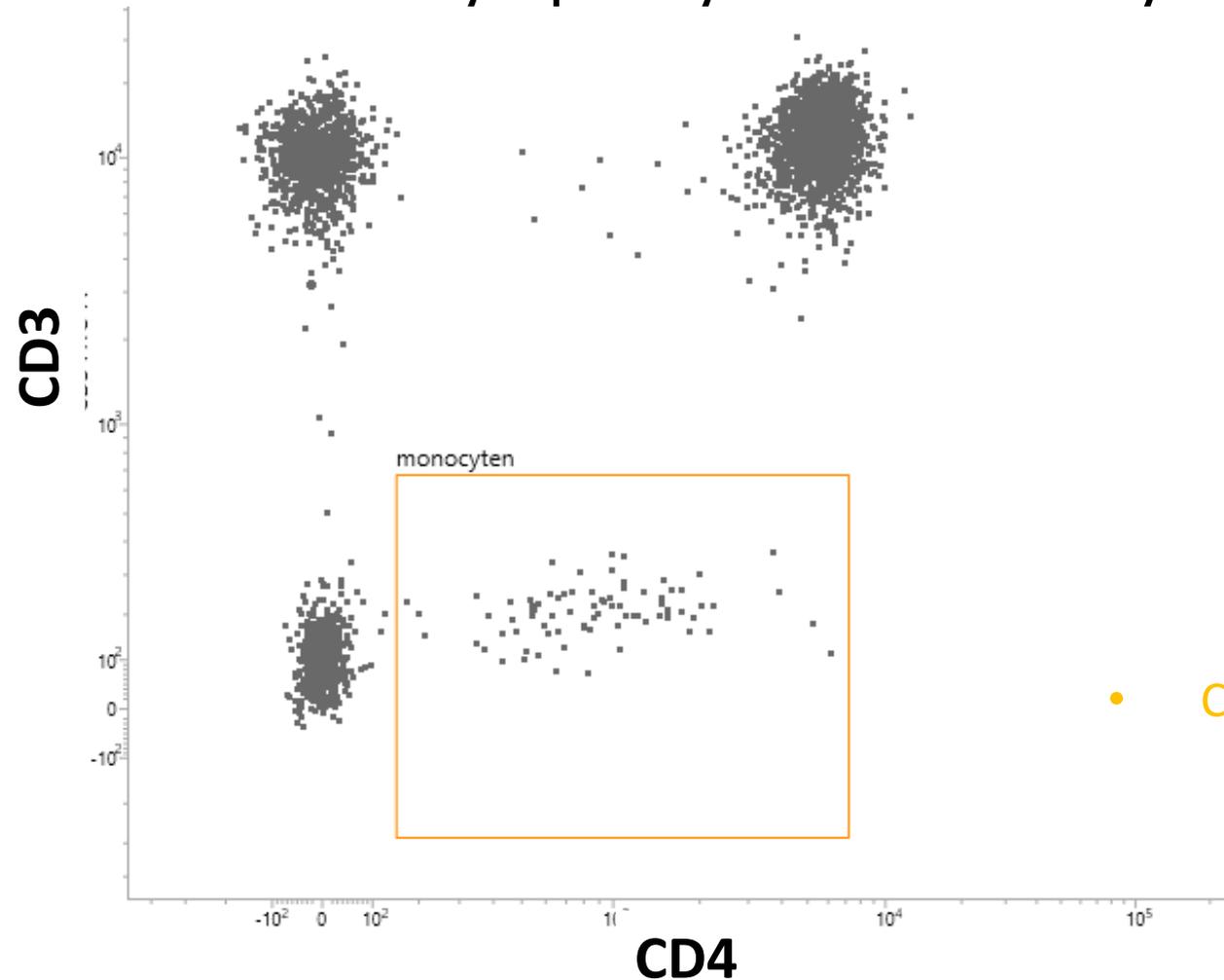
- CD4 = exclude **monocytic** interference

Normal cell populations

- Whole blood: lymphocyte subset analysis

- CD19 = B cell lineage
- CD3 = T cell lineage
- CD16+56 = NK marker
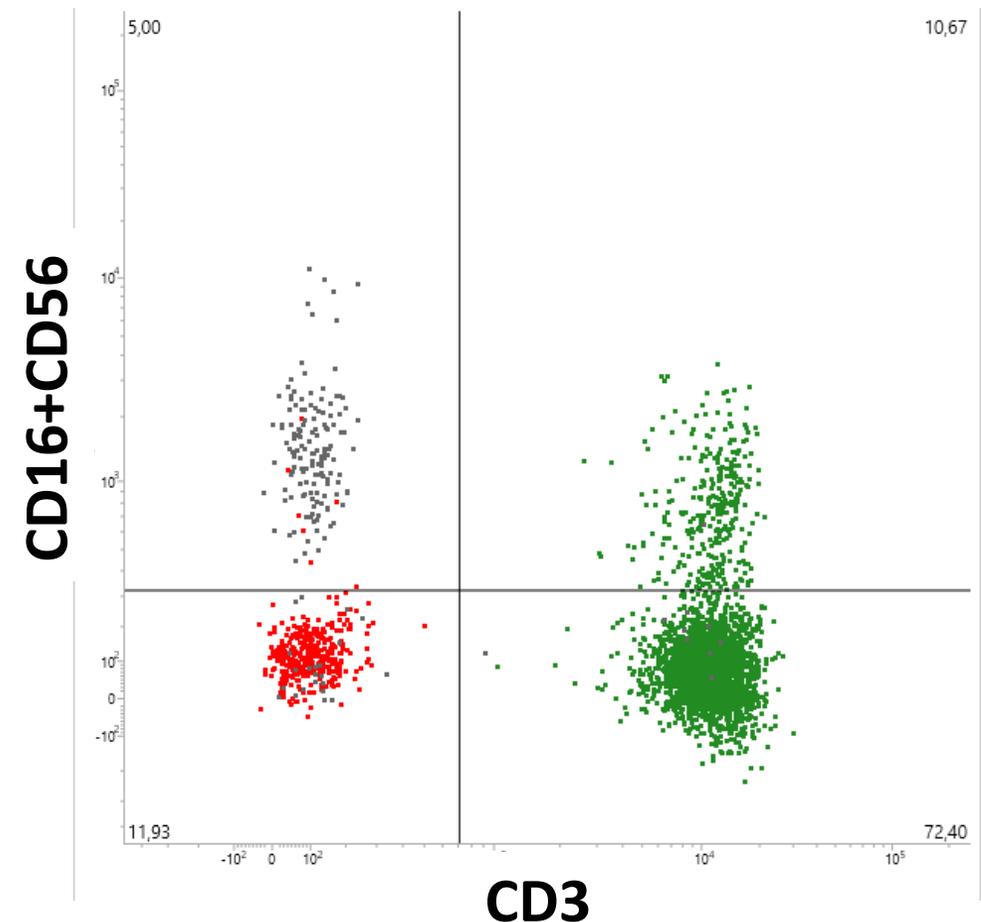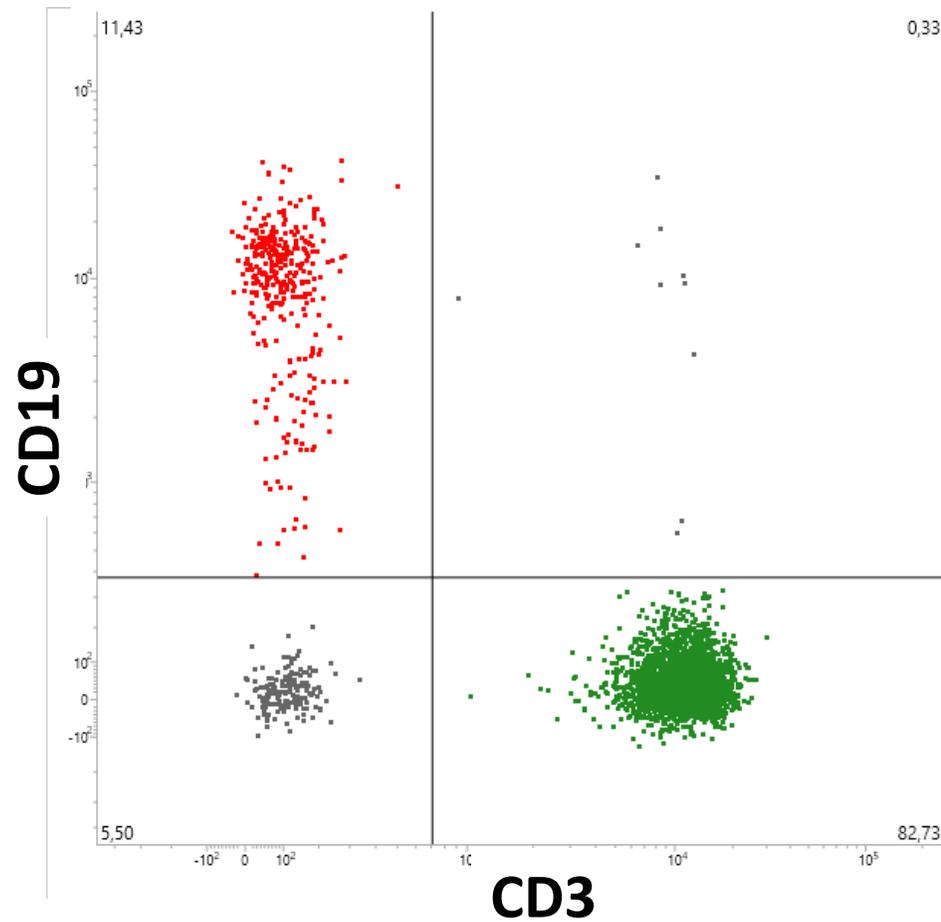
Normal cell populations

- B-cells (bone marrow, BM)
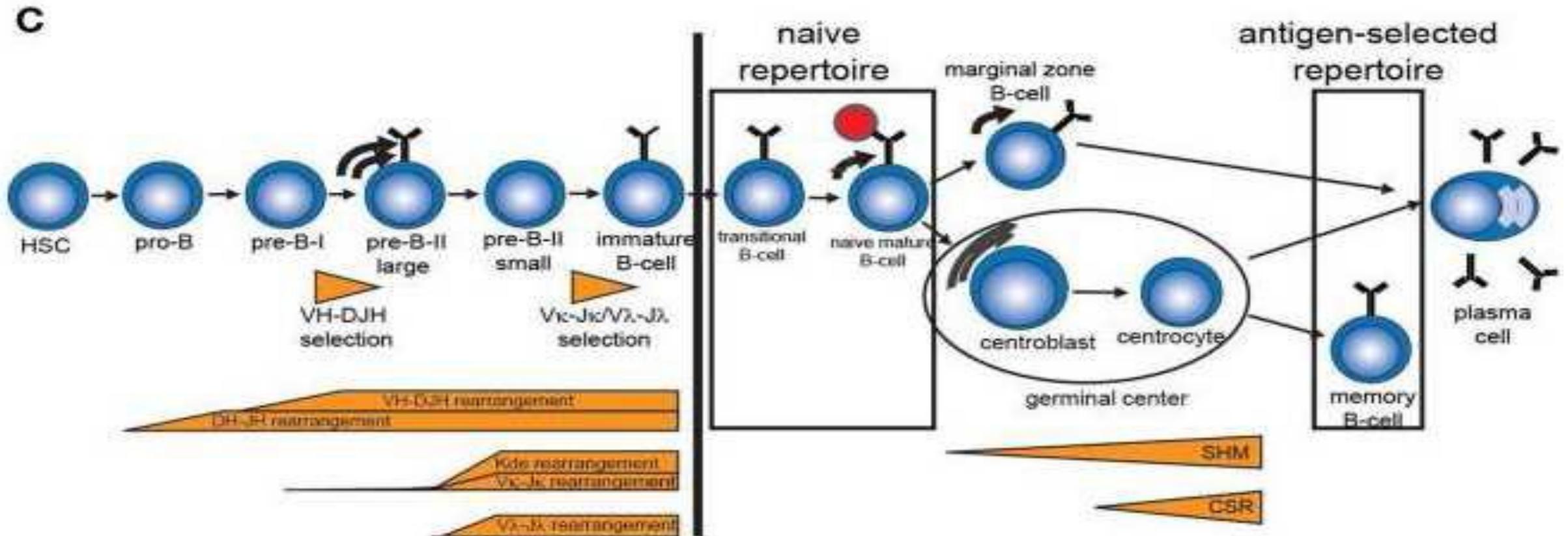
Normal cell populations

- Immature B-cells (BM): CD45(weak)/SSClow and CD10 vs CD20
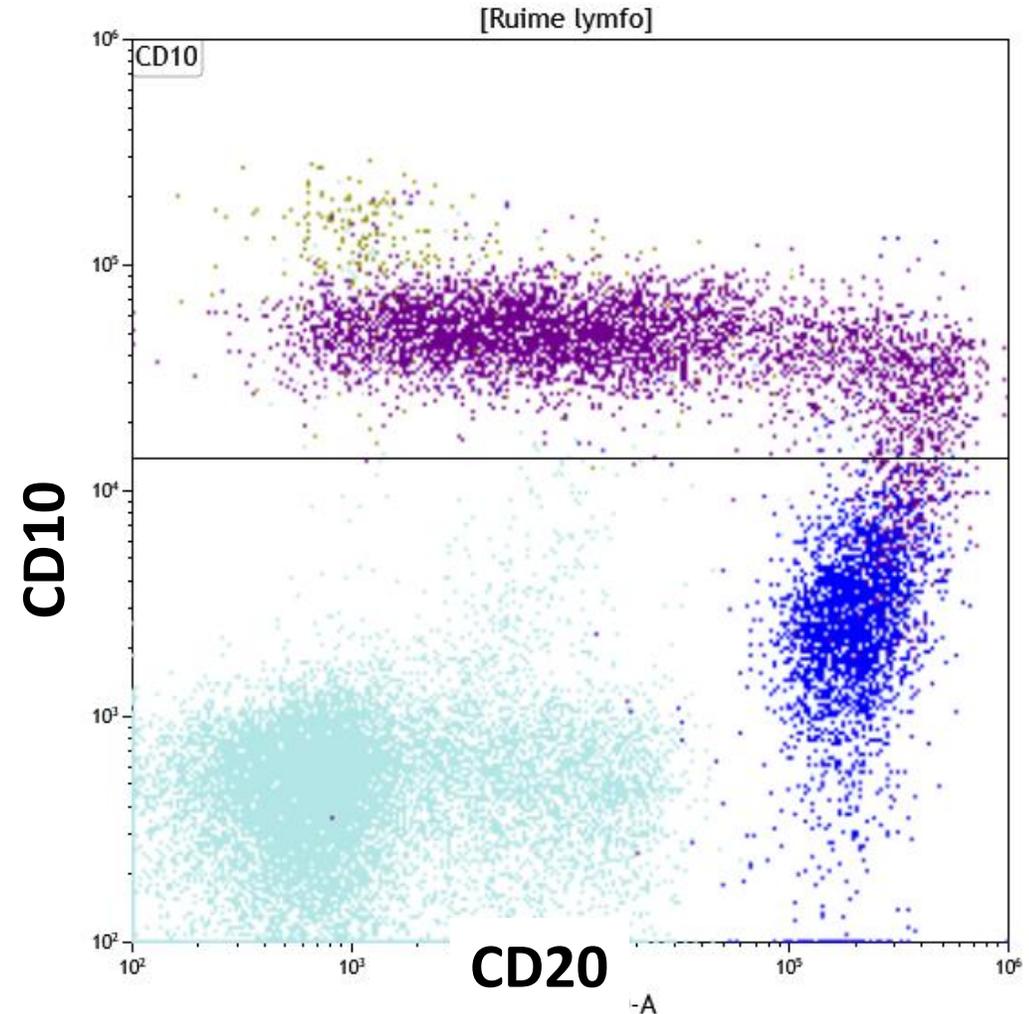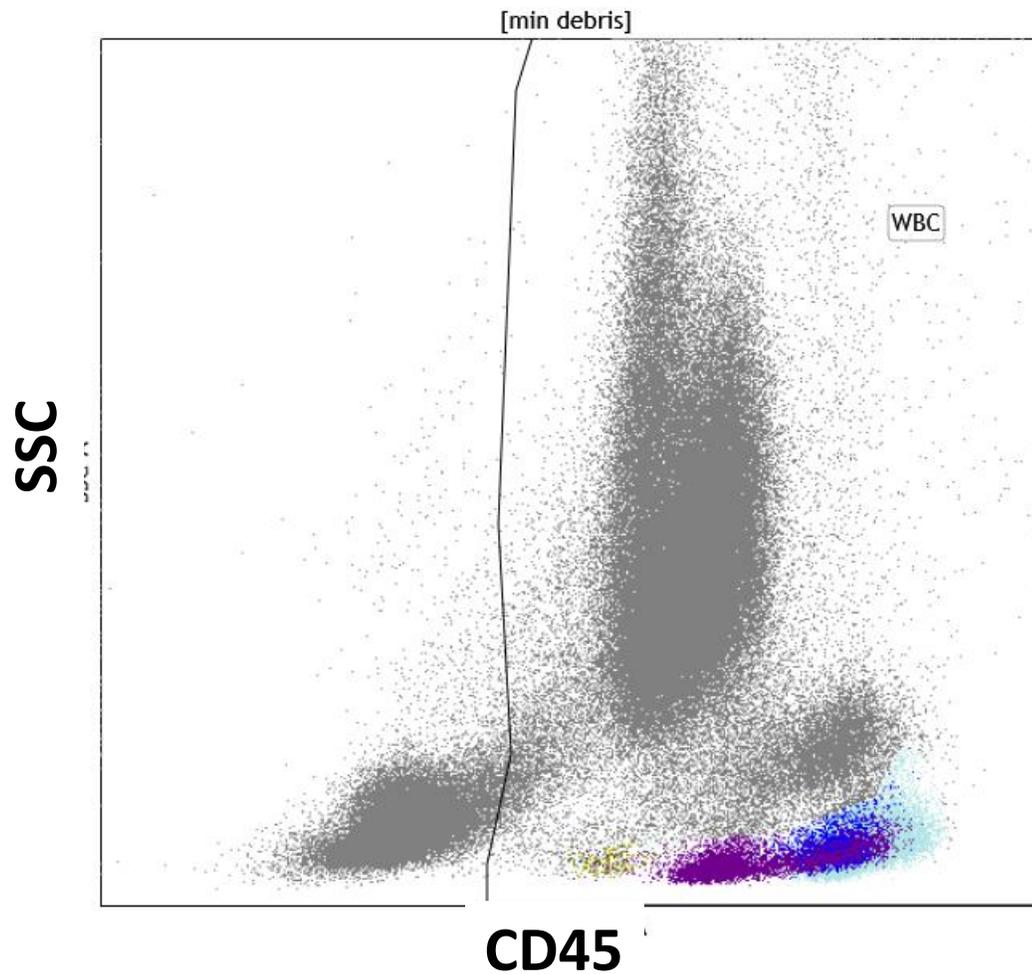
Normal cell populations

- Immature B-cells (hematogones)
  - Type I hematogonen (pre-B-I): CD10++, CD20-, CD34+, CD45+weak
  - Type II hematogonen (pre-B-II) **CD10+**, CD20+zwak, CD34-, CD45+weak
  - type III hematogonen (immature/transitional): **CD10+zwak**, CD20+, CD34-, CD45+

- Mature B-cells in PB (10-30%): CD20+/CD22+/CD34-/CD10-/CD45strong/ SSChigher
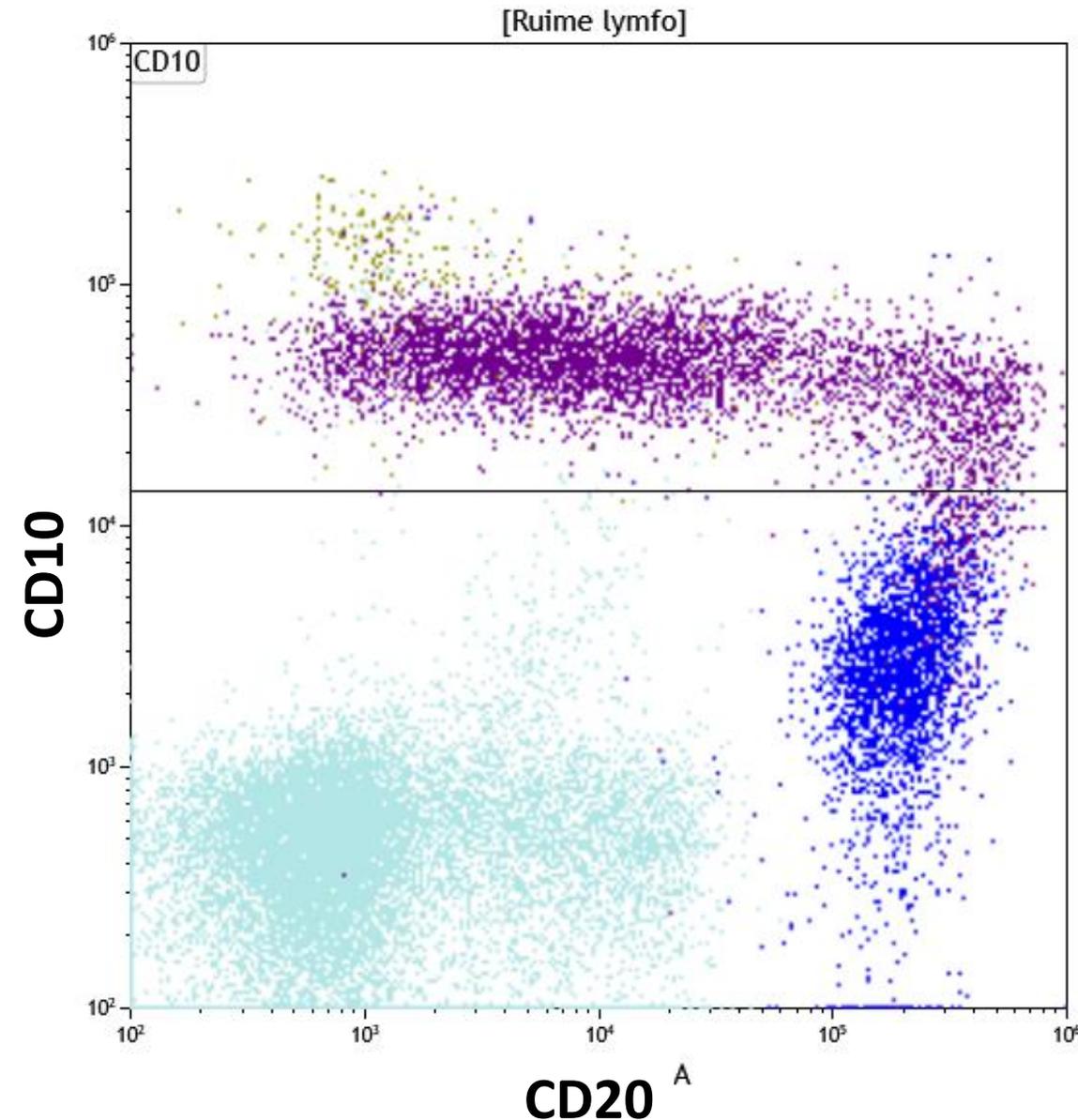
Normal cell populations

- (advanced) Very early B-cell precursors (VEBP): CD34+/CD22+zw/**CD19-**/CD24-/CD20-

  Start with "rough B-cell gate": CD22+(weak)/CD24+/SSClow
  - Type I: CD38+/CD10-/CD22+z-zw: +/- 1.3% (range 0.1-4.9%)
  - Type II: CD38+/CD10+zw/CD22+zw: +/- 3.3% (range 0.3-12.1%)
  ! may interfere in MRD B-ALL analysis if **anti-CD19** targeted therapy
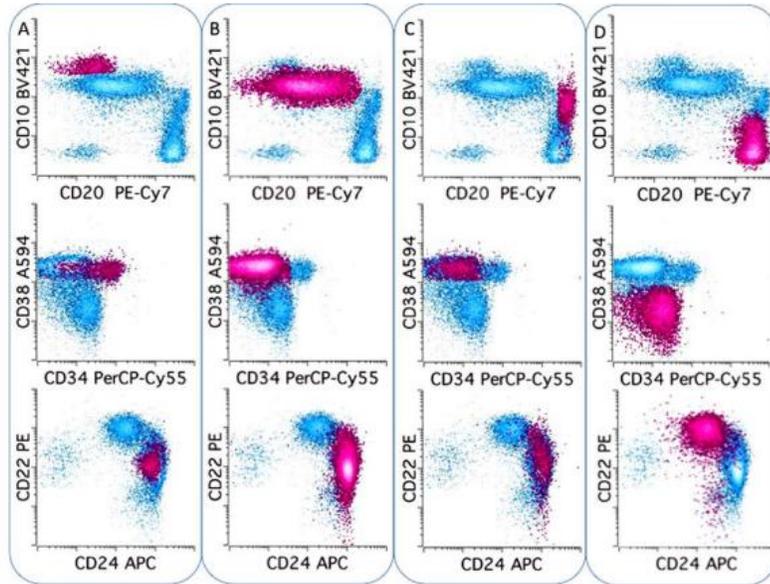
FIG. 2. CD19-positive B cell precursors and mature B cell populations are present in the rough B cell gate. All plots show the "rough B cell gate". Different stages of B cell maturation are highlighted in panels A–D. Where described, the population of interest is in magenta; all in blue. In panel A, early hematogones expressing bright CD10 with CD34 and CD38, strong CD24, and decreased CD22 w highlighted. Panel B highlights intermediate stage hematogones expressing CD10 with variable CD20 without CD34 with CD38, s decreased CD22. Panel C highlights immature pre-naive B cells expressing low CD10 with strong CD20, expression of CD38, s decreased CD22 without CD34. Finally, mature B cells are highlighted in panel D and show expression of strong CD22 and CD20 CD24 and without CD10, CD34, or CD38. Note, no CD38 bright plasma cells are present in the rough B cell gate. See electronic figures. [Color figure can be viewed at wileyonlinelibrary.com]
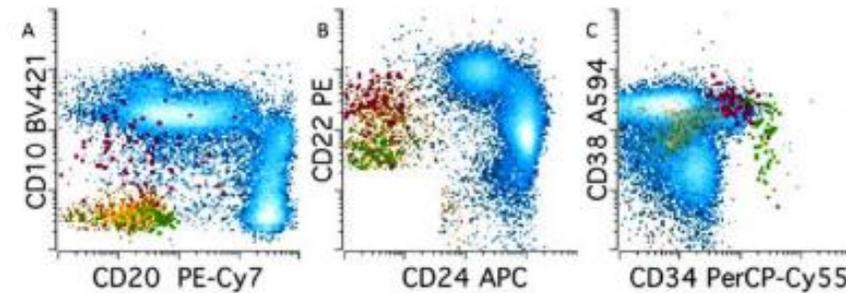
FIG. 3. CD19-negative progenitor populations. All plots show cells in the "rough B cell gate". In addition to the known CD19 positive B cell populations outlined in Figure 2, the "rough B cell gate" contains other progenitor populations. Two such progenitor populations expressing CD22 without CD19 or CD24 deserve mention. The first (population A, highlighted and colored green) expresses CD34 (bright) and CD38 (intermediate) with low CD22 without CD10 or CD20. The second (population B highlighted in purple) expresses CD34, CD38 at the level of hematogones, and CD22 with variable CD10 and low, variable CD20. Also present is a population (colored yellow), which is predominantly positive for CD22 without CD24 (with a smaller subset positive for CD24 without CD22) and is negative for CD10 and CD34. These cells are not interpreted to be B cells or progenitor cells. The specific lineage of this population is not clear. See electronic version for color

Cherian, S., et al. (2018)

Normal cell populations
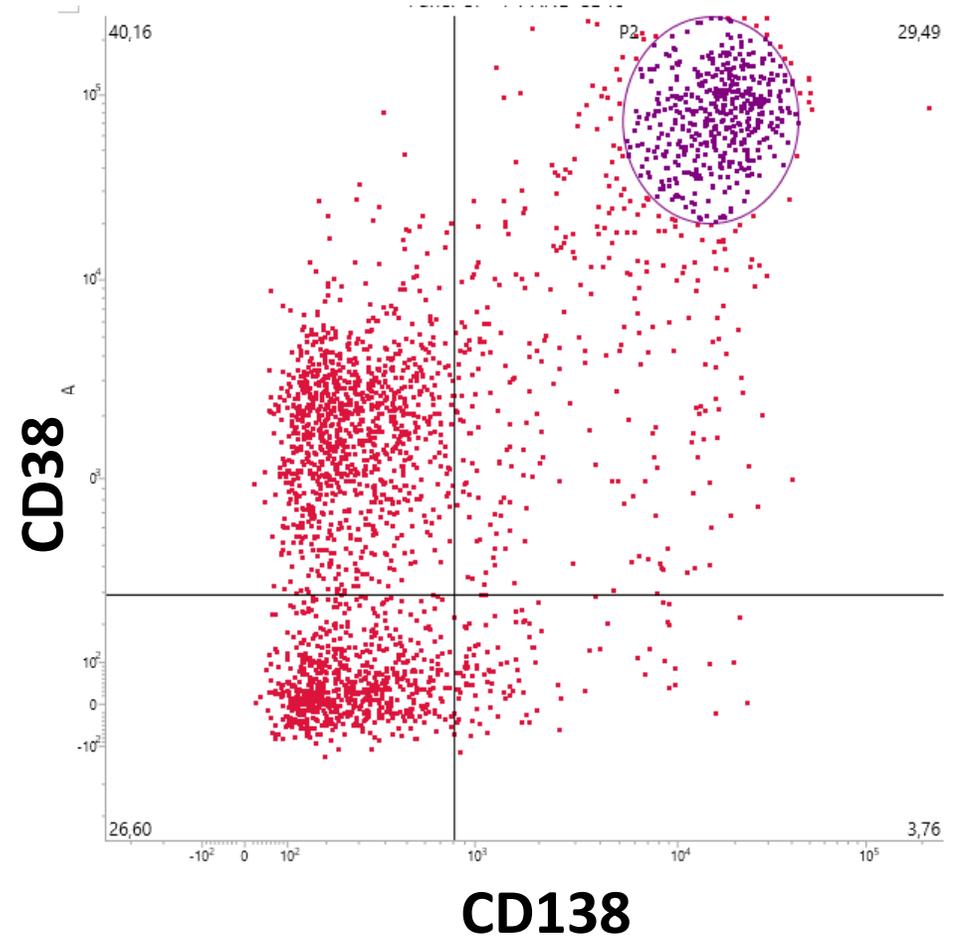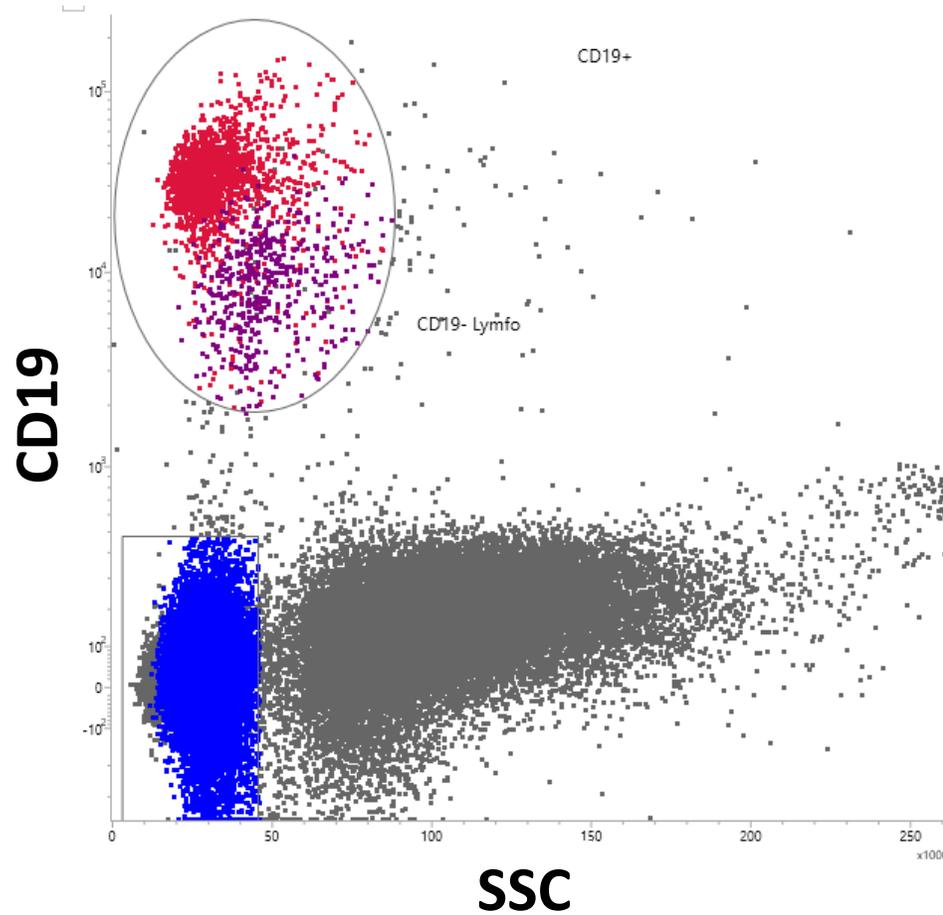
- Plasmacells

! CD138 (*SDC1*) decreases rapidly after sampling

Normal cell populations
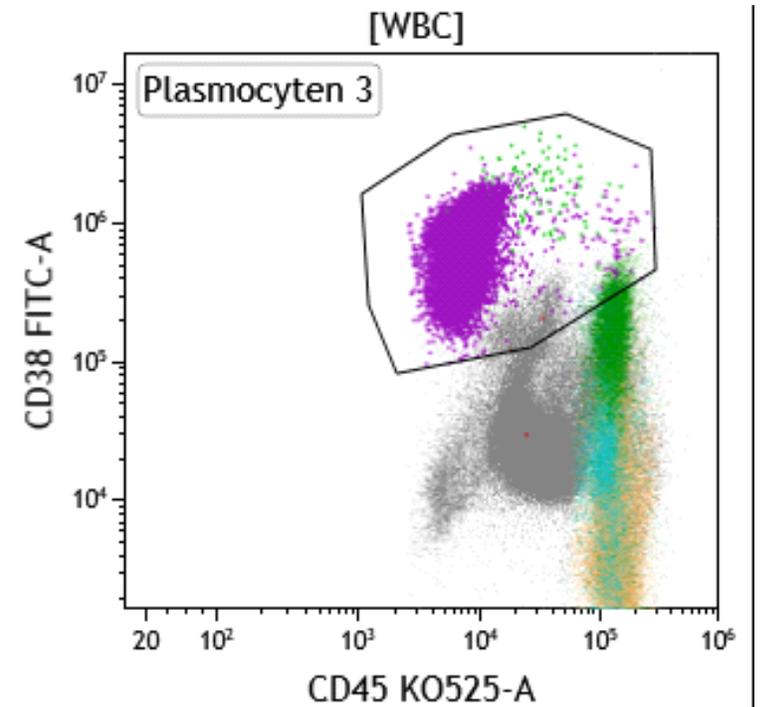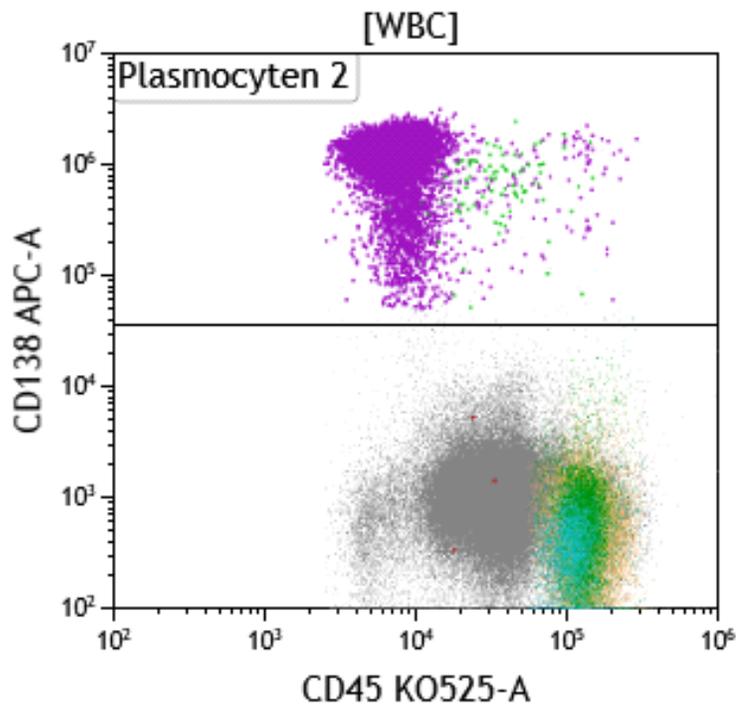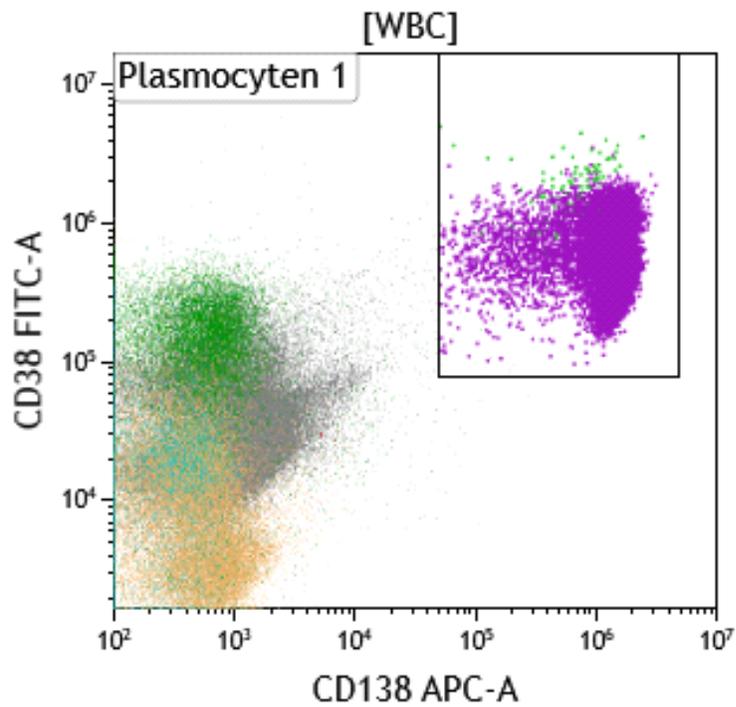
- Plasmacells

Normal cell populations
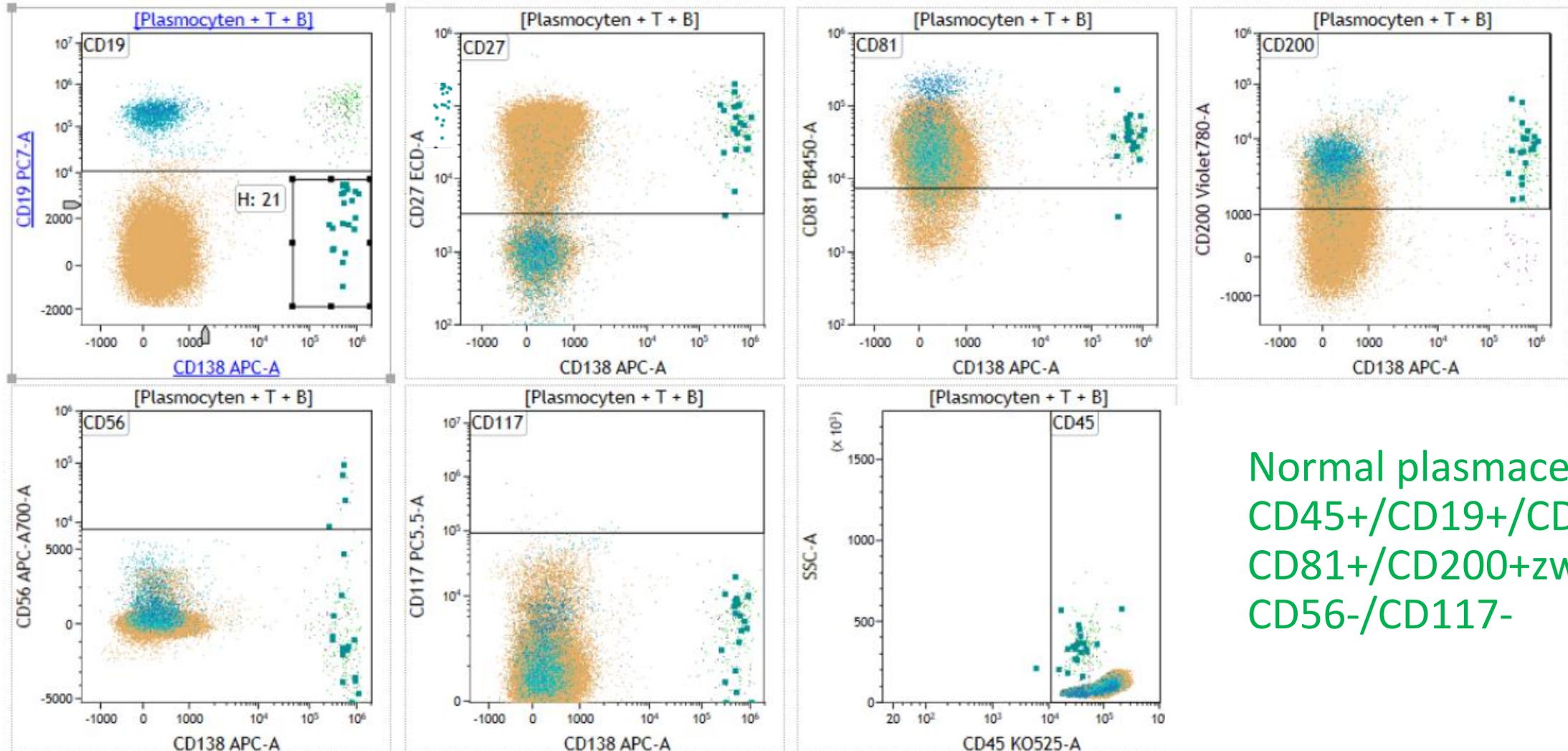
Normal plasmacells:
CD45+/CD19+/CD27++/
CD81+/CD200+zw/
CD56-/CD117-

Malignant cell populations
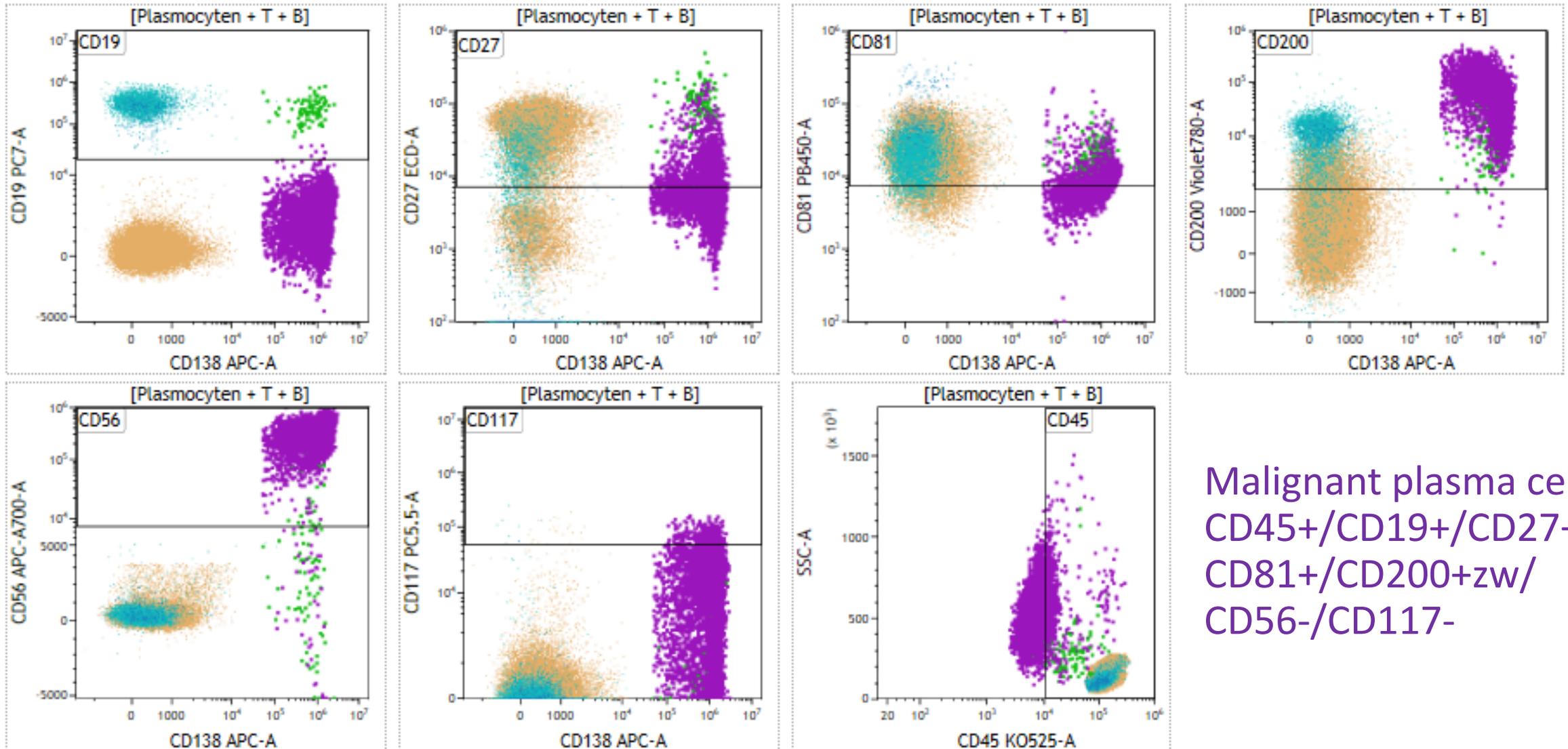
Malignant plasma cells:
CD45+/CD19+/CD27++/
CD81+/CD200+zw/
CD56-/CD117-

Malignant cell populations

- ## How to differentiate between normal and neoplastic B cells
  1. Relative increased percentage
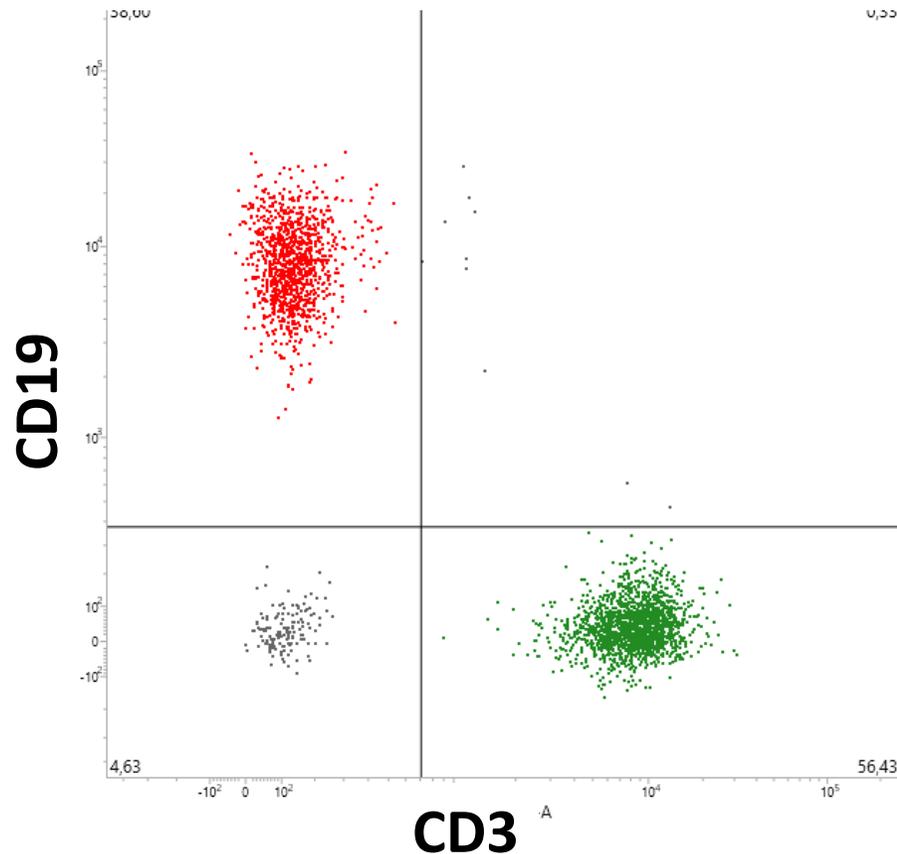
Malignant cell populations

- ## How to differentiate between normal and malignant B cells
  1. Relative increased percentage
  2. Monoclonality
     - Ig light chain class restriction
       - 60-75% kappa
       - 25-40% lambda
       - Cut off ƙ/ƛ ratio +- [0.8 2.1]
     - Ig heavy chain class restriction
       - 80-85% IgM and IgD
       - 12% IgG
       - 3-8% IgA
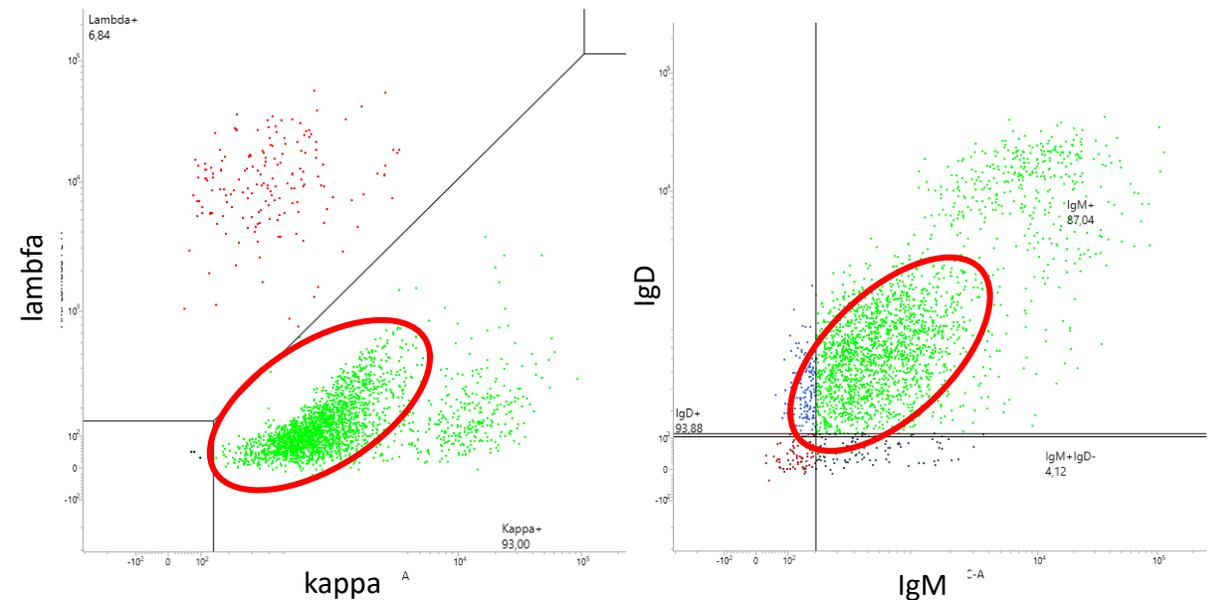
! Biclonal populations
! multiple heavy chain isotypes (e.g. HCL)

Malignant cell populations

- ## How to differentiate between normal and malignant B cells
    1. Relative increased percentage
    2. Monoclonality
    3. Aberrant marker expression
        - CD5
        - CD23
        - FMC7
        - CD79b
        - CD43
        - CD81

        - CD200
        - CD38
        - CD138
        - CD25
        - CD11c
        - CD103

        - CD180
        - CD305
        - ROR1
        - CD10
        - CD22
        - CD45

Malignant cell populations

- ## How to differentiate between normal and malignant B cells

  1. Relative increased percentage
  2. Monoclonality
  3. Aberrant marker expression: **normal B-cells**

     - CD5-
     - CD23-/weak+
     - FMC7+
     - CD79b+
     - CD43-
     - CD81+

     - CD200+weak
     - CD38-/weak+
     - CD138-
     - CD25-/weak+
     - CD11c-
     - CD103-

     - CD180+
     - CD305 (LAIR1)+(weak)
     - ROR1-
     - CD10-
     - CD22+
     - CD45++

Malignant cell populations

- ## How to differentiate between normal and malignant B cells
  1. Relative increased percentage
  2. Monoclonality
  3. Aberrant marker expression
     - CD5
     - CD23
     - FMC7
     - CD79b
     - CD43
     - CD81
     - CD200
     - CD38
     - CD138
     - CD25
     - CD11c
     - CD103
     - CD180
     - CD305
     - ROR1
     - CD10
     - CD22
     - CD45

**Example: CLL → ERIC &
ESCCA harmonization project
reproducible diagnosis of CLL**

Reproducible diagnosis of chronic lymphocytic leukemia by flow cytometry: An European Research Initiative on CLL (ERIC) & European Society for Clinical Cell Analysis (ESCCA) Harmonisation project

Malignant cell populations

- ## How to differentiate between normal and malignant B cells
  1. Relative increased percentage
  2. Monoclonality
  3. Aberrant marker expression in **CLL**

  - CD5+
  - CD23+
  - FMC7
  - CD79b weak+/-
  - CD43+
  - CD81 weak+/-

  - CD200weak+
  - CD38
  - CD138
  - CD25
  - CD11c
  - CD103

  - CD180
  - CD305
  - ROR1+
  - CD10-
  - CD22
  - CD45

Example: CLL → ERIC & ESCCA harmonization project reproducible diagnosis of CLL:
- 5 required markers: CD19, weak CD20, weak light chain restriction, CD5 and CD23
- 6 recommended markers: CD79b/CD43/CD81/CD200/CD10/**ROR1**
= score /8 or /10
- 4 not recommended markers: Individual lab preference: CD22/FMC7/CD45/CD38

Normal cell populations
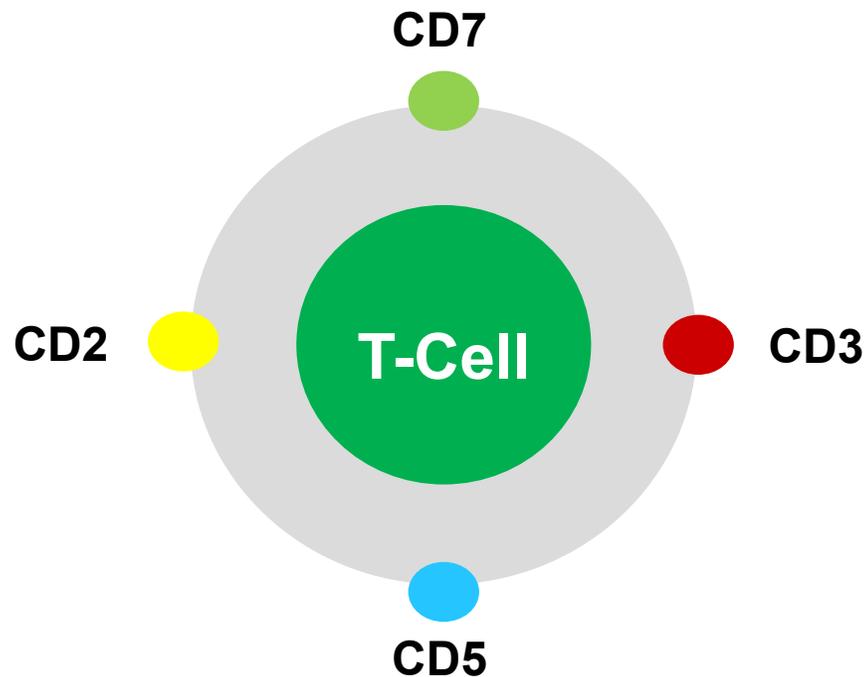
- T-cells and NK-cells

CD3+ T cells (70-90%)

1. **TCRγδ+ T-cells (5%)**
   - CD4-/CD8-
   - Decreased expression of CD5 and CD7
   - Ethnic/genetic and environmental factors
   - Extramedullary tissue

Normal cell populations

- TCRγδ+ T-cells (CD4-/CD8-/CD3++)
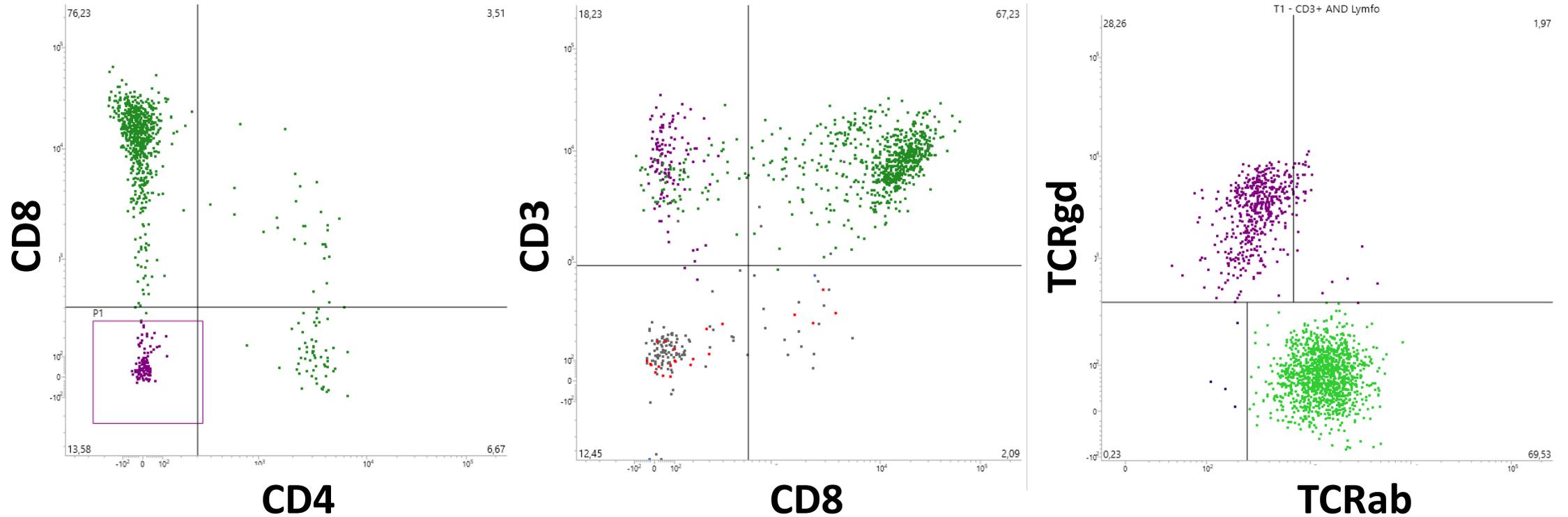
Normal cell populations

- T-cells and NK-cells
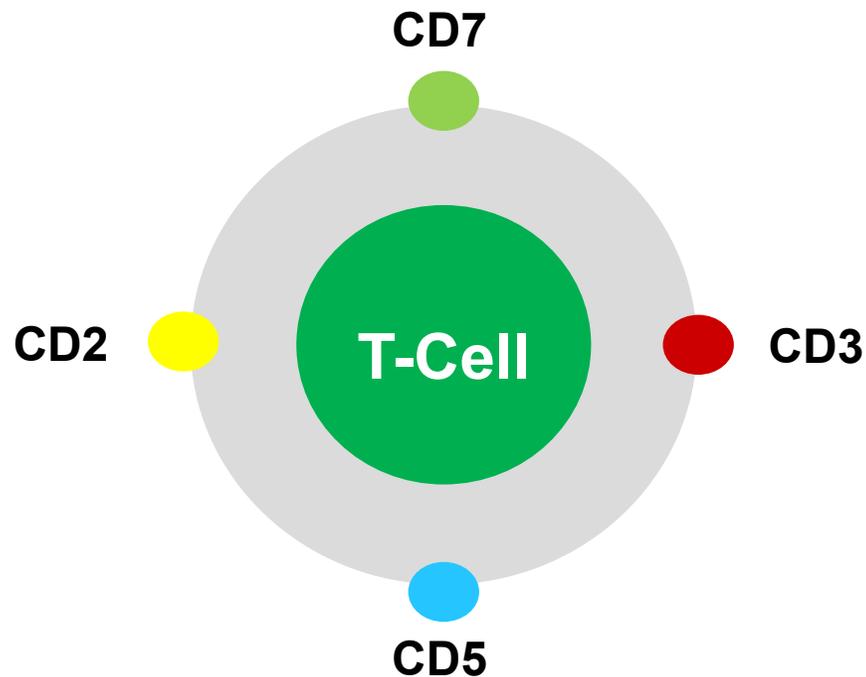
CD3+ T cells (70-90%)

2. **TCRαβ+ (95%)**
   - Helper T-cells: CD4+/CD8-
   - Cytotoxic T-cells: CD4-/CD8+
     →CD4/CD8 ratio (PB): 1.3 – 3.6

   - CD4+/CD8+ double positive: normal thymus, thymic hyperplasia and thymoma (CD3/CD1a) dd (cortical) T-ALL and mature T-cell neoplasms

Normal cell populations

- T-cells and NK-cells

CD3- NK-cells: most cases: CD5-/CD7+/CD2+/CD94+/CD38+

- Immature (10%): CD56(bright+)/CD16/CD57(dim+)/NKp46(bright+)

- Mature (90%): CD56(dim+)/CD16/CD57(bright+)/NKp46(dim+)

- CD8 expressed on 40% NK cells (more cytolytic than CD8- counterpart)

CD3+ NK-like T-cellen: CD5±/CD7+/CD2+

- <u>CD4+</u> >> CD4-CD8- >> CD8+

- CD16+/CD56+/CD57+

(advanced) cyCD3+/CD3- NK-cells:

- May interfere with T-ALL MRD analysis

- Link between Notch signaling and the generation of intracellular CD3epsilon

De Smedt M, et al. Blood. 2007.

Malignant cell populations

- How to differentiate between normal and neoplastic T- cells
    1. CD4/CD8 ratio
        - Shifts
        - Increased double positive or double negative populations
        ! DD reactive conditions

Malignant cell populations
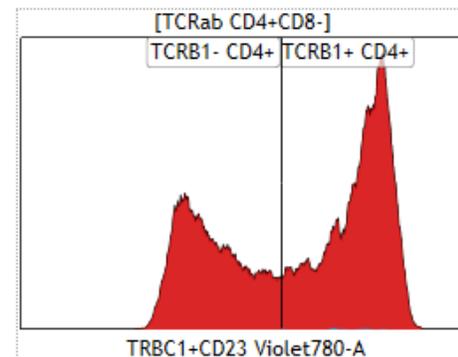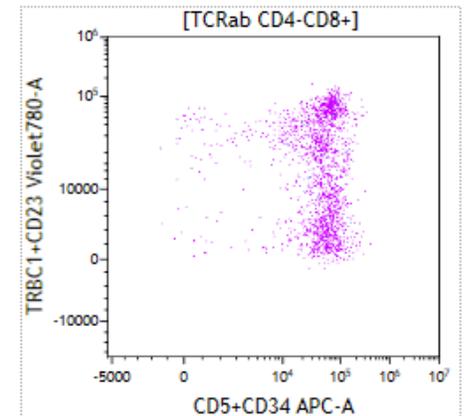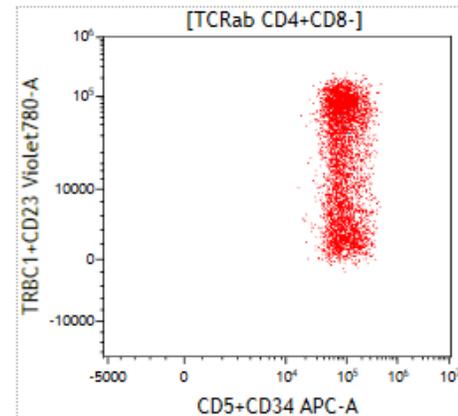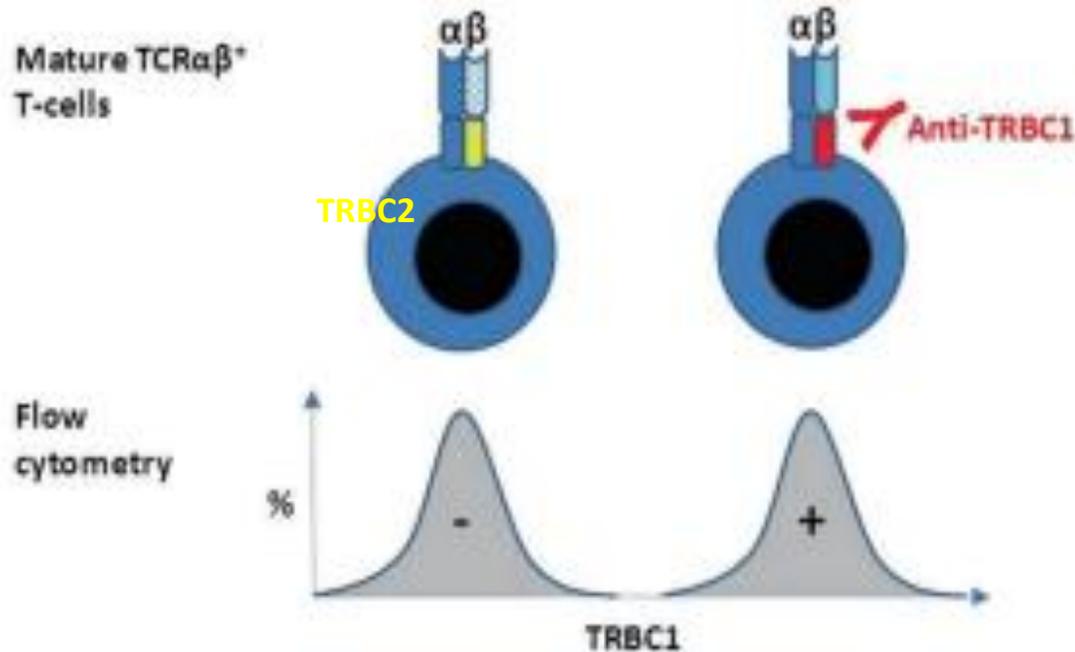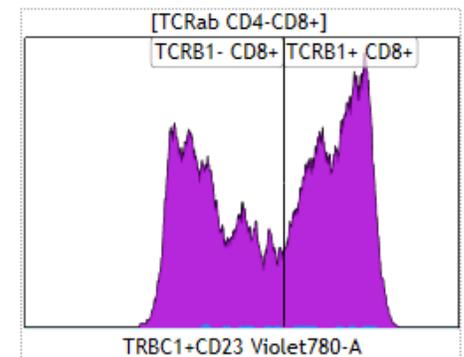
- ## How to differentiate between normal and malignant T- cells
  1. CD4/CD8 ratio
  2. JOVI-1 (TRBC1)

Malignant cell populations

- How to **differentiate** between normal and **malignant** T- cells
  1. CD4/CD8 ratio
  2. JOVI-1/TRBC1
  3. Aberrant antigen expression
     - CD7
     - CD2
     - CD5
     - HLA-DR
     - CD26
     - CD16

     - CD56
     - CD57
     - CD52
     - CD10
     - CD1a
     - CD99

     - cyTCL1
     - CD94
     - TRBC2
     - PD-1
     - …

Minimale Panel MTCN 2024

Verplicht
- CD45
- CD2
- CD3
- CD4
- CD5
- CD7
- CD8
- CD56
- TCR-GD
- TRBC1 of Vβ-kit

Additioneel verplicht bij subtypering
- CD57 (T-LGL)
- cTCL1 (T-PLL)
- CD25 (ATLL)
- CD26 (MF/SS)

Optioneel
- CD16 (NK-LGL)
- CD94 (NK-LGL)
- CD158a/b/e (NK-LGL)
- CD279 (AITL)
- CD30 (ALCL)
- CD52 (target alemtuzumab)

RIJPE T-CEL MALIGNITEITEN (MTCN)

- TRBC1 geaccepteerde klonaliteitsmarker naast moleculair TCR-onderzoek
- TRBC2 inmiddels op de markt, nog niet in minimale panel door gebrek aan ervaring, volgt!
- Bij afwezigheid van sCD3, wees beducht op T-ALL en overweeg onrijpe T-cel markers
- T-LGL ≠ T-LGLL; niet elke kloon is maligne!
- T-celmaligniteiten zeldzaam en moeilijk, volledig subtyperen.

Normal cell populations

- Whole blood: monocyte subset analysis

  - Selection based on CD45/CD4+/CD3-
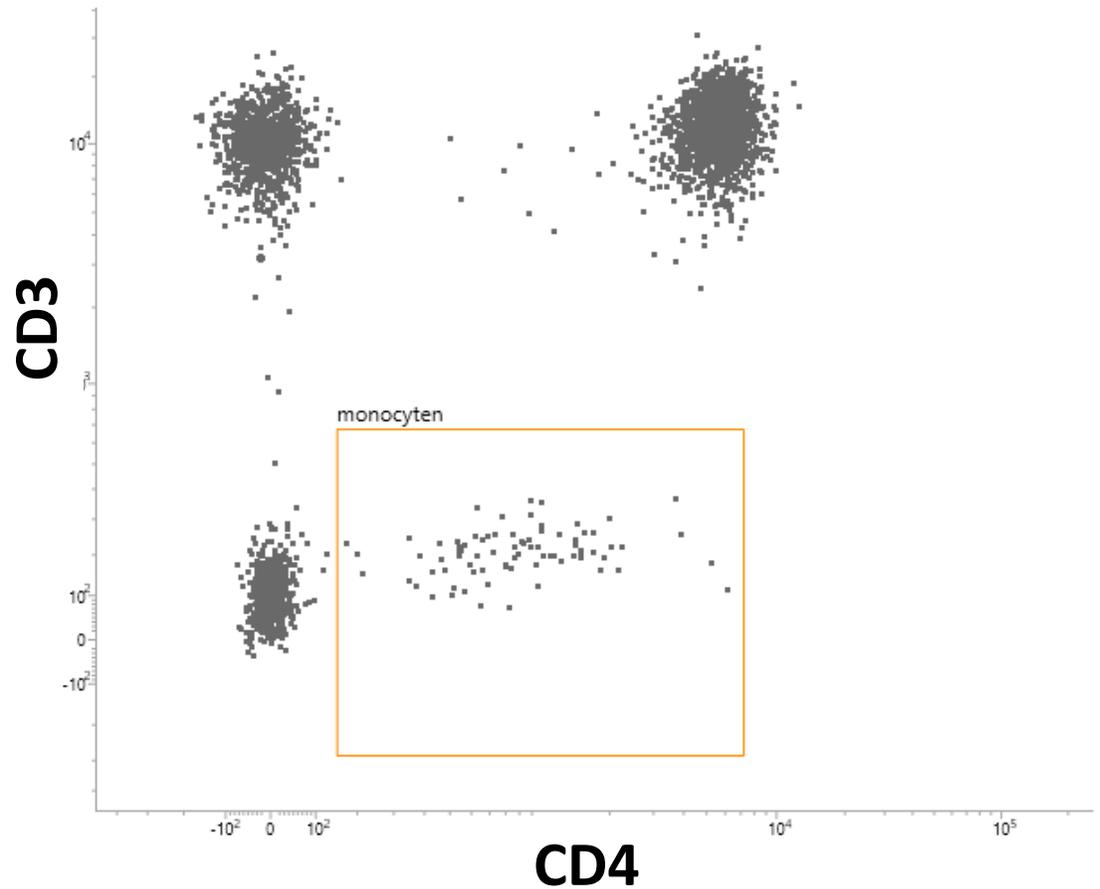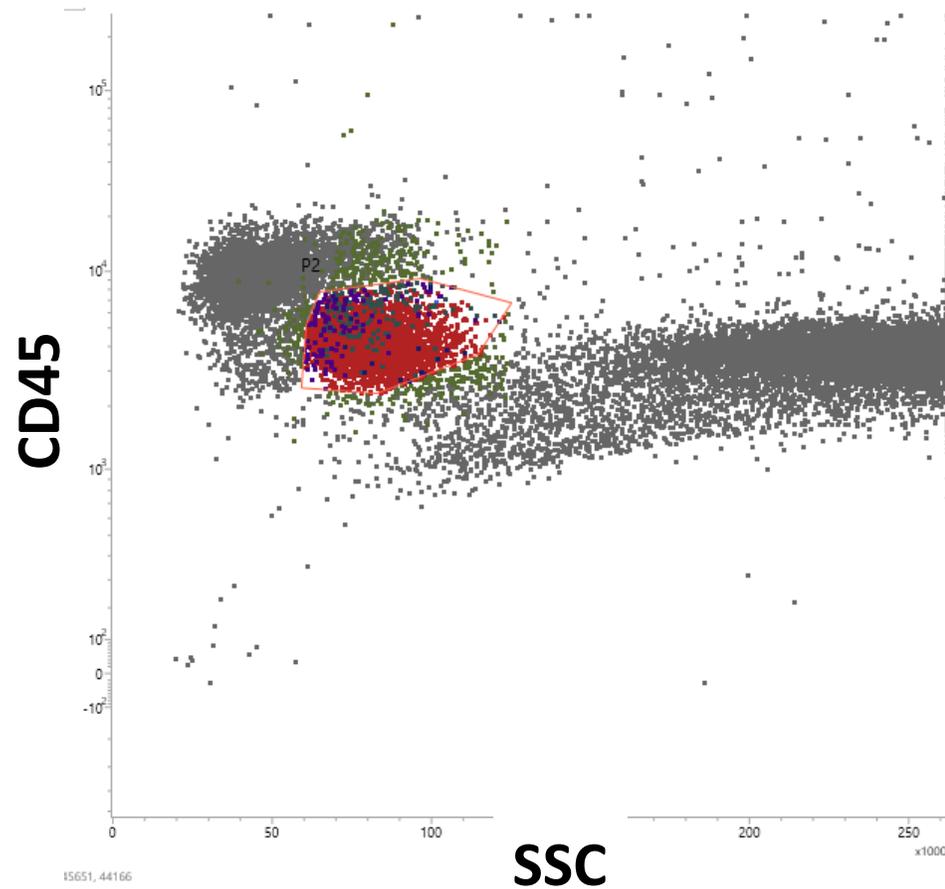
Normal cell populations

- Whole blood: monocyte subset analysis

    - Selection based on CD45/CD4+/CD3-
    - **3 groups** circulating mature monocytic cells (Mukerjee et al 2015)
        1. CD14+/CD16- (classical, 80-85%): cMo
        2. CD14+/CD16+ (intermediate, <15%): iMo
        3. CD14lo/CD16+ (non-classical, <15%): ncMo
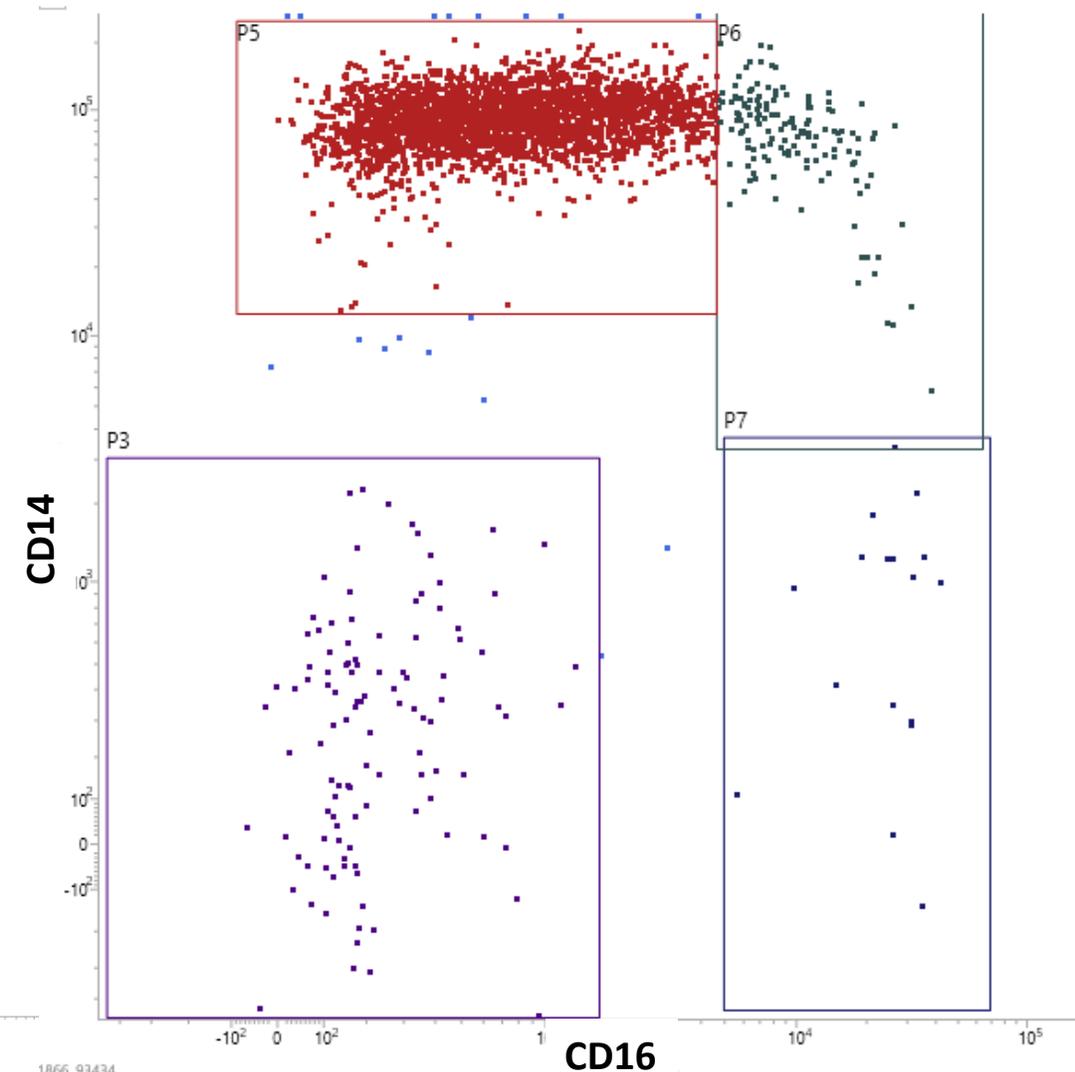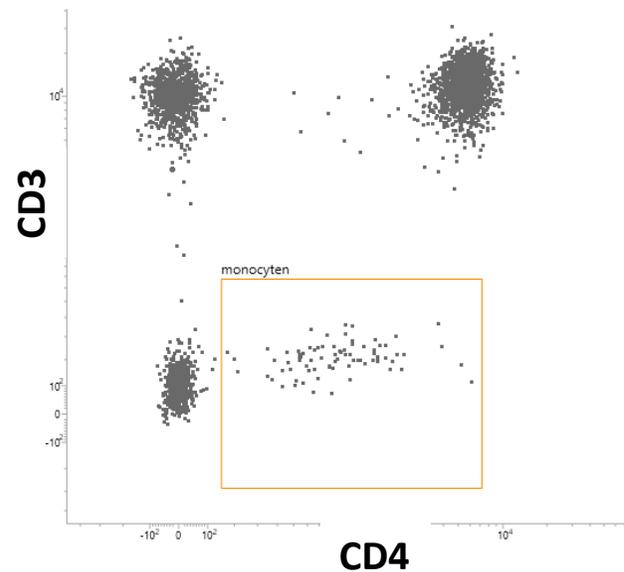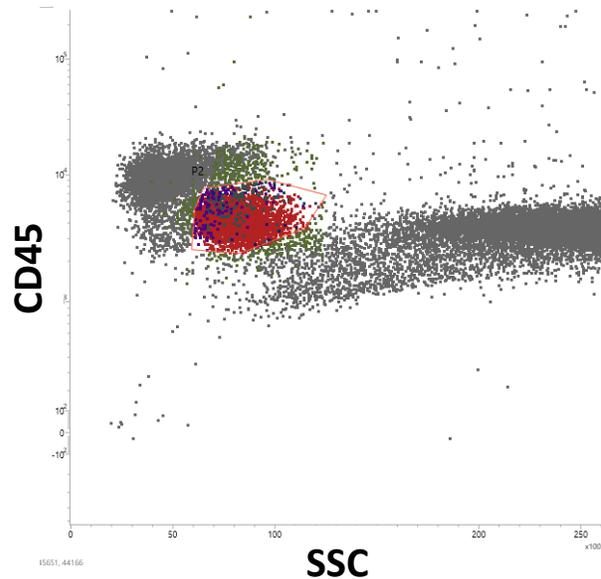
Malignant cell populations

- FCM helpful to distinguish **neoplastic** from **reactive** monocytosis
    - **cMo** (CD14+/CD16-) are **increased** in CMML
    - **iMo** (CD14+/CD16+) and **ncMo** (CD14-/CD16+) are **decreased** in CMML

CMML

Reactive monocytosis
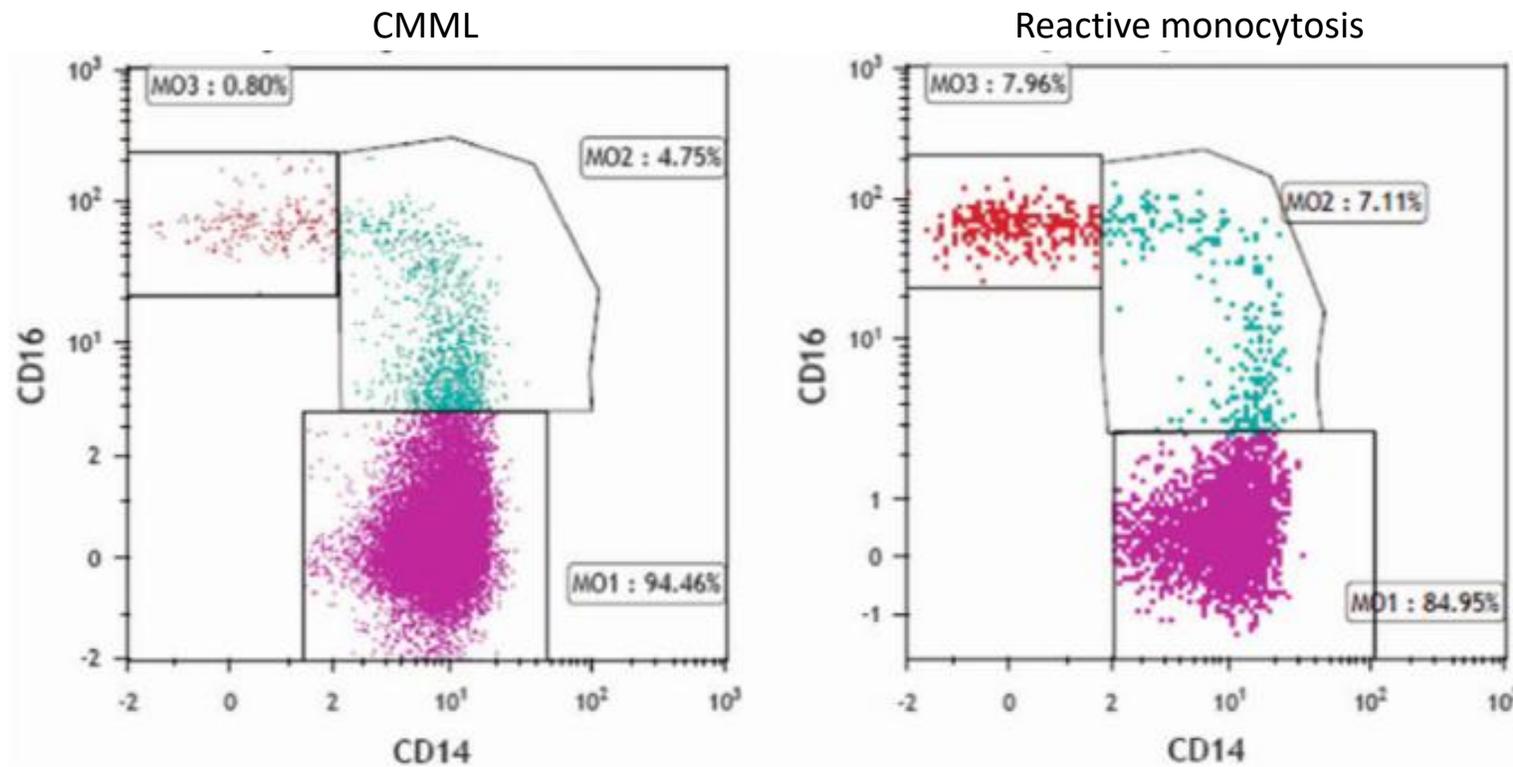
cMO⩾94%: CMML
(96% (14/15) and 92% (98/107) of patients)

cMO<92%: other monocytosis

Co-occurence inflammatory disease (increased iMO)

(1) Selimoglu-Buet D et al (2015, (2) Talati et al (2017), Jestin et al (2020)

Malignant cell populations

- <mark>ncMo</mark> are CD45++/CD33+weaker/CD14-
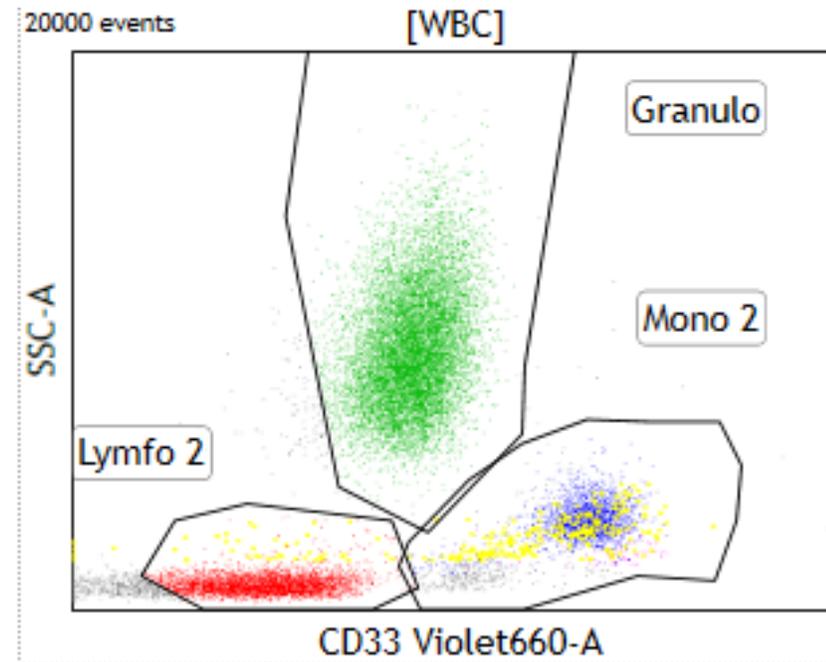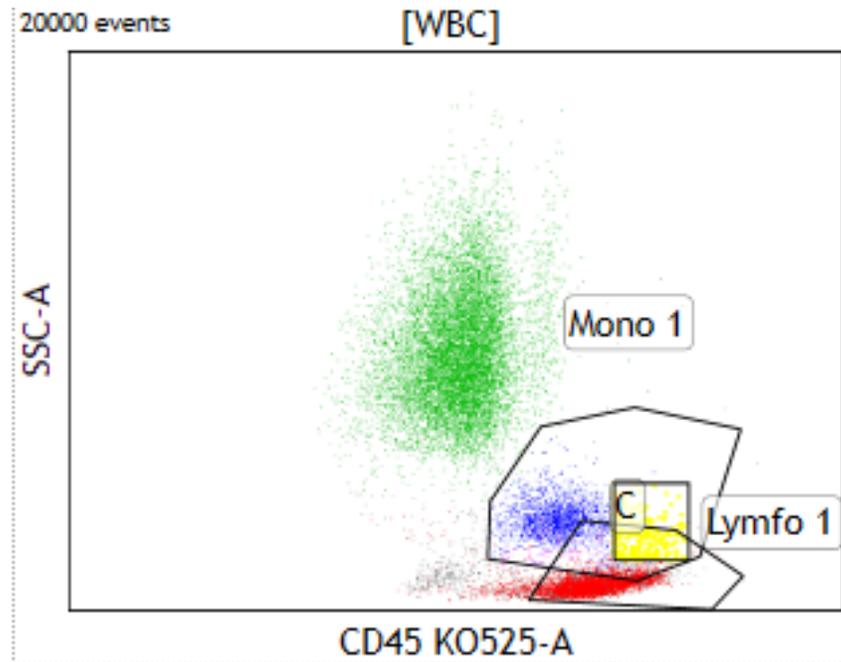
Normal cell populations

- Bone marrow: monocytic compartment
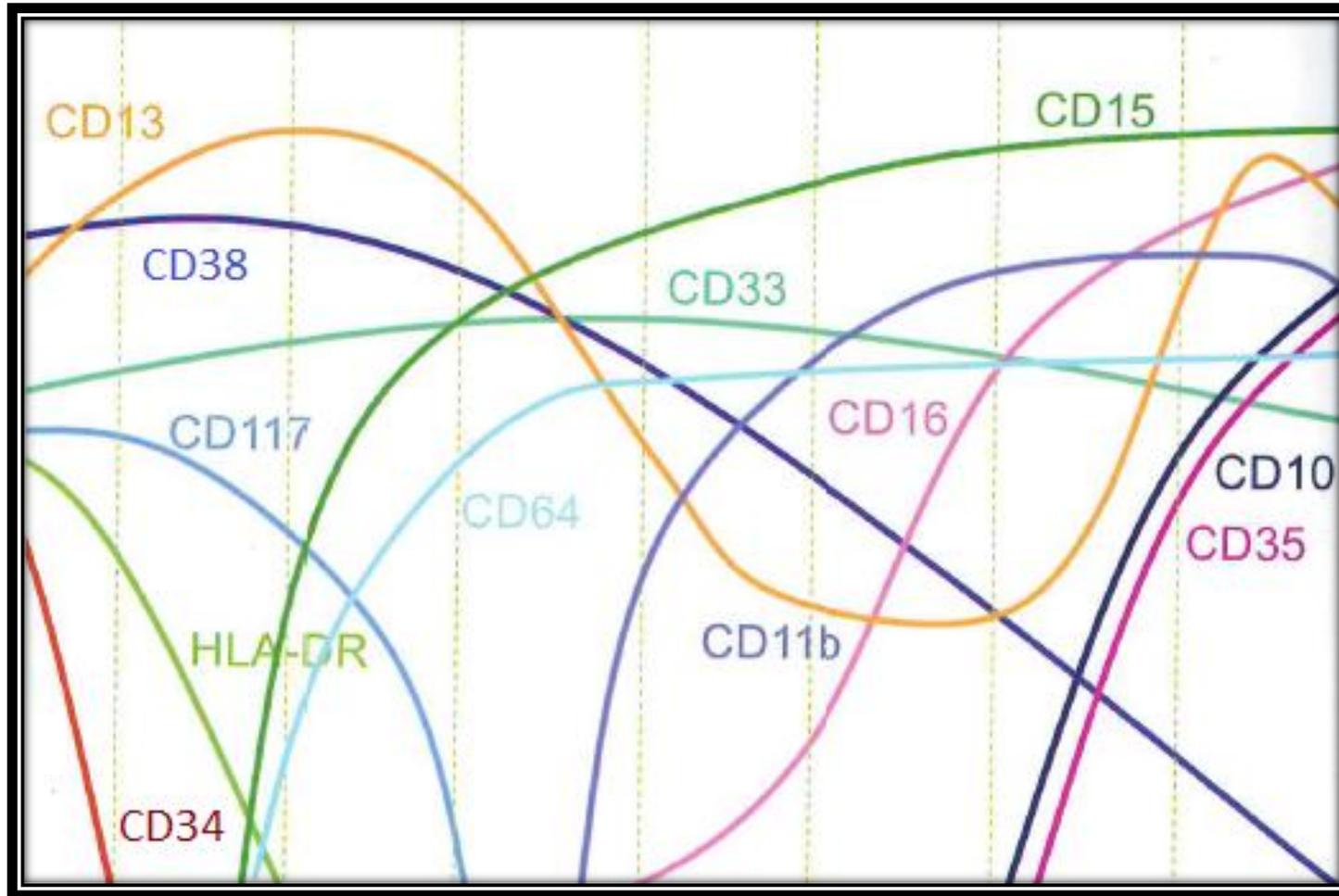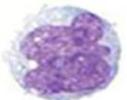
- Monoblasts
- Promonocytes
- Monocytes
  - →Macrophages
    - →Dendritic cells

Monocytic development
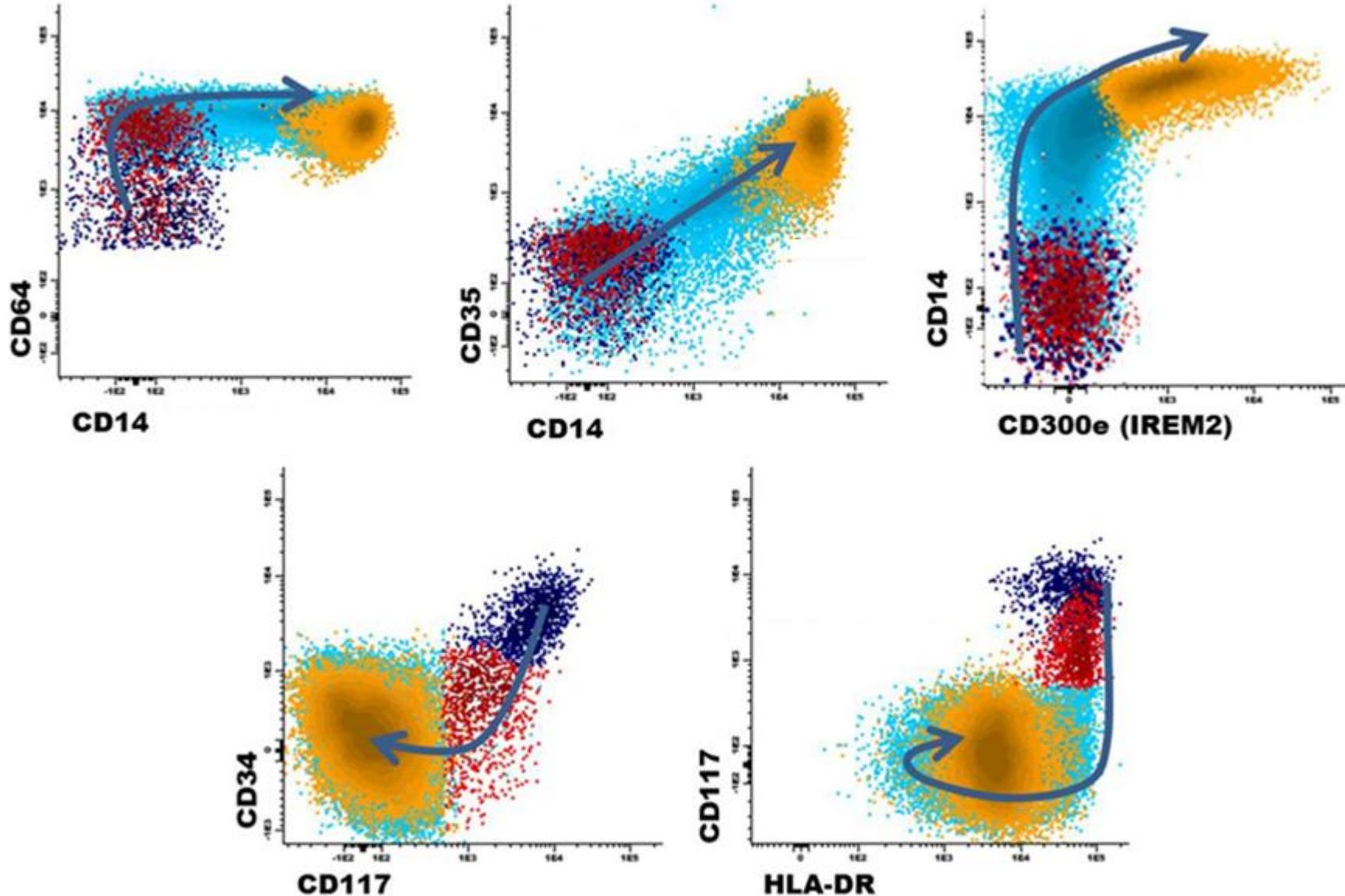
Monocytic precursors incl. monoblasts

CD34±/CD117+/CD64zw+/ CD14-/DR+/CD33+

Promonocytes

CD34-/CD117-/CD64++/ CD14±/CD300e-/DR+/CD33++

Mature monocytes

CD34-/CD117-/CD64++/CD14++/ CD300e+/DR+/CD33++

Matarraz, S et al. (2017); van Dongen J et al. (2012)

Monocytic development

CD marker	Monoblast	Promonocyte	Monocyte
CD4	++	++	++
CD11b	-	++	+++
CD11c			
CD13	+	+/++	++/+++
CD14	-	+/-	+++
CD15	-	++	+/++
CD16	-	-	-/+
CD33	+++	+++	+++
CD34	+/-	-	-
CD35	-	+/-	+
CD36	-	++	+++
CD45	+	++	+++
CD64	+(++)	+++	+++
CD117	+	+/-	-
CD300e	-	-	+
HLA-DR	+++	+++	++/+++

Immunohenotypic changes

~ maturation

~ activation

Monocytic development

CD marker	Monoblast	Promonocyte	Monocyte
CD4	++	++	++
CD11b	-	++	+++
CD11c			
CD13	+	+/++	++/+++
CD14	-	+/-	+++
CD15	-	++	+/++
CD16	-	-	-/+
CD33	+++	+++	+++
CD34	+/-	-	-
CD35	-	+/-	+
CD36	-	++	+++
CD45	+	++	+++
CD64	+++	+++	+++
CD117	+	+/-	-
CD300e	-	-	+
HLA-DR	+++	+++	++/+++

Some anti-CD14 clones only recognize mature monocytic cells, while others recognize promonocytes and mature monocytes.

Neoplastic monoblasts or promonocytes may not always be CD14 negative
!correlation with morphology

ncMo have decreased expression of CD14 should not be confused with immature monocytic cells.

Yang et al (2005)

Monocytic development

CD marker	Monoblast	Promonocyte	Monocyte
CD4	++	++	++
CD11b	-	++	+++
CD11c			
CD13	+	+/++	++/+++
CD14	-	+/-	+++
CD15	-	++	+/++
CD16	-	-	-/+
CD33	+++	+++	+++
CD34	+/-	-	-
CD35	-	+/-	+
CD36	-	++	+++
CD45	+	++	+++
CD64	+++	+++	+++
CD117	+	+/-	-
CD300e	-	-	+
HLA-DR	+++	+++	++/+++

Activation influences the level of CD15 and HLA-DR expression

Normal cell populations

- Peripheral blood: granulocytic/myeloid compartment
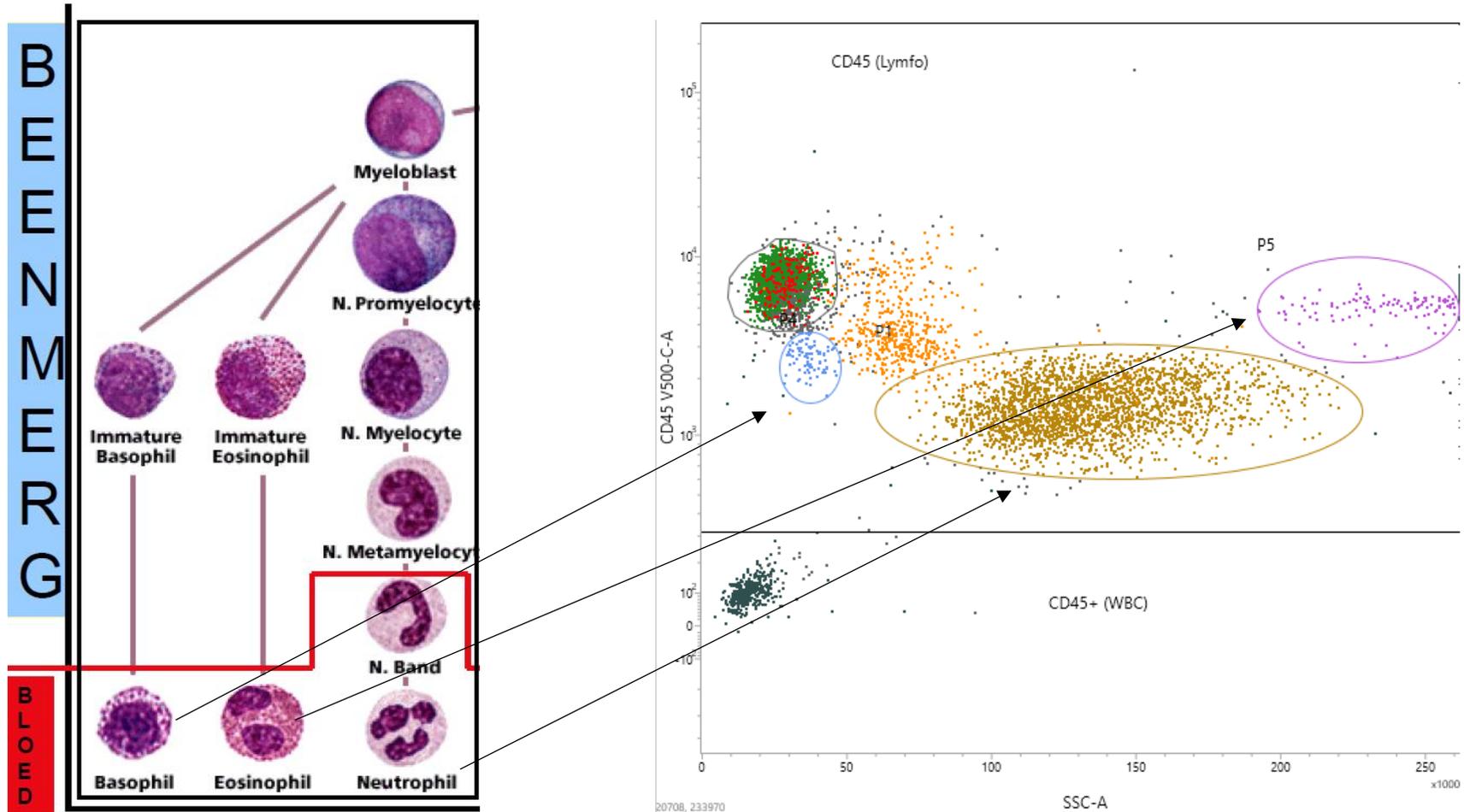
Normal cell populations

- Peripheral blood: granulocytic/myeloid compartment

Basophils: CD45^{int}/**CD123++/HLA-DR-/CD9+/**CD22+/CD25+/CD13+/CD33+/CD36+/**CD38++** (subset ook CD11b+)

Eosinophils: **SSC^{high}**/FSC^{low}/CD45++/CD14-/**CD16-**/HLA-DR-/CD11b+/CD11c+/CD13+/**CD15+**/CD33+

Granulocytes: CD11b+/CD11c+/CD13++/CD15+/CD33+/**CD16+/CD10+**

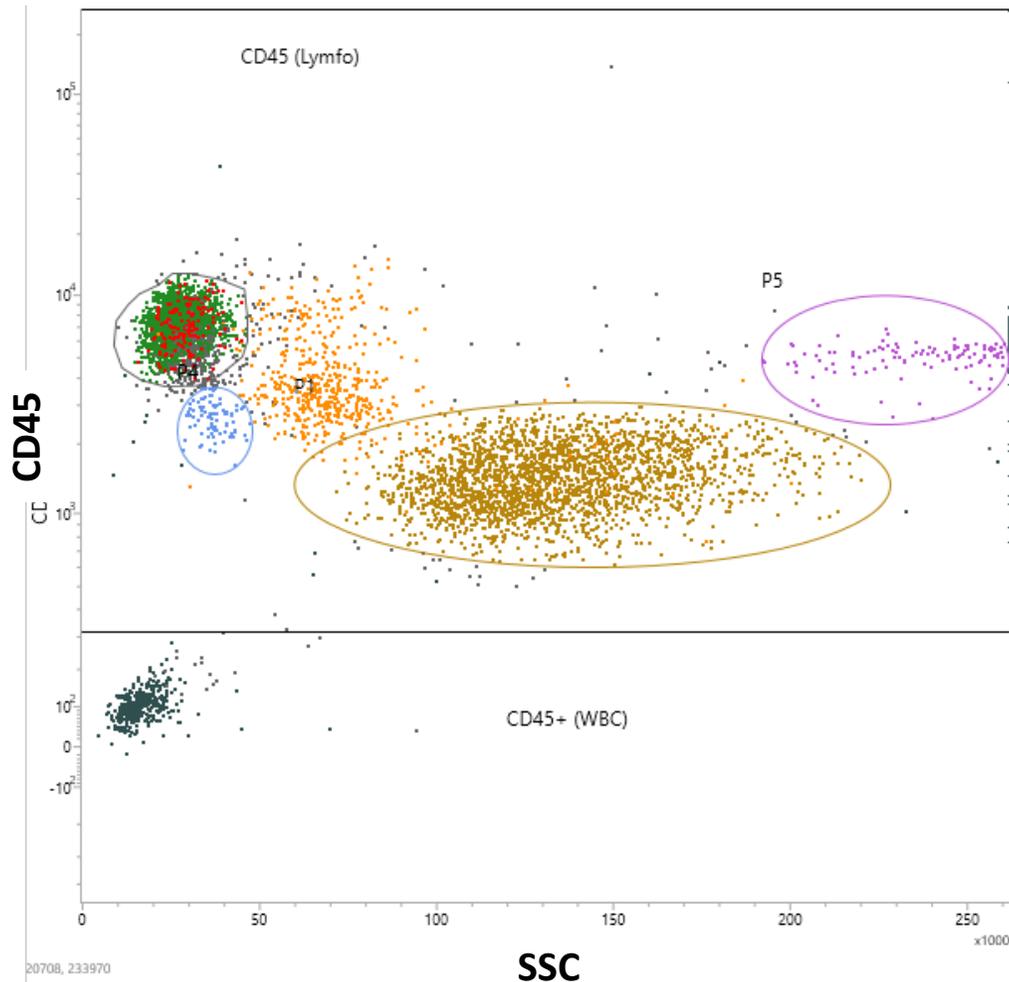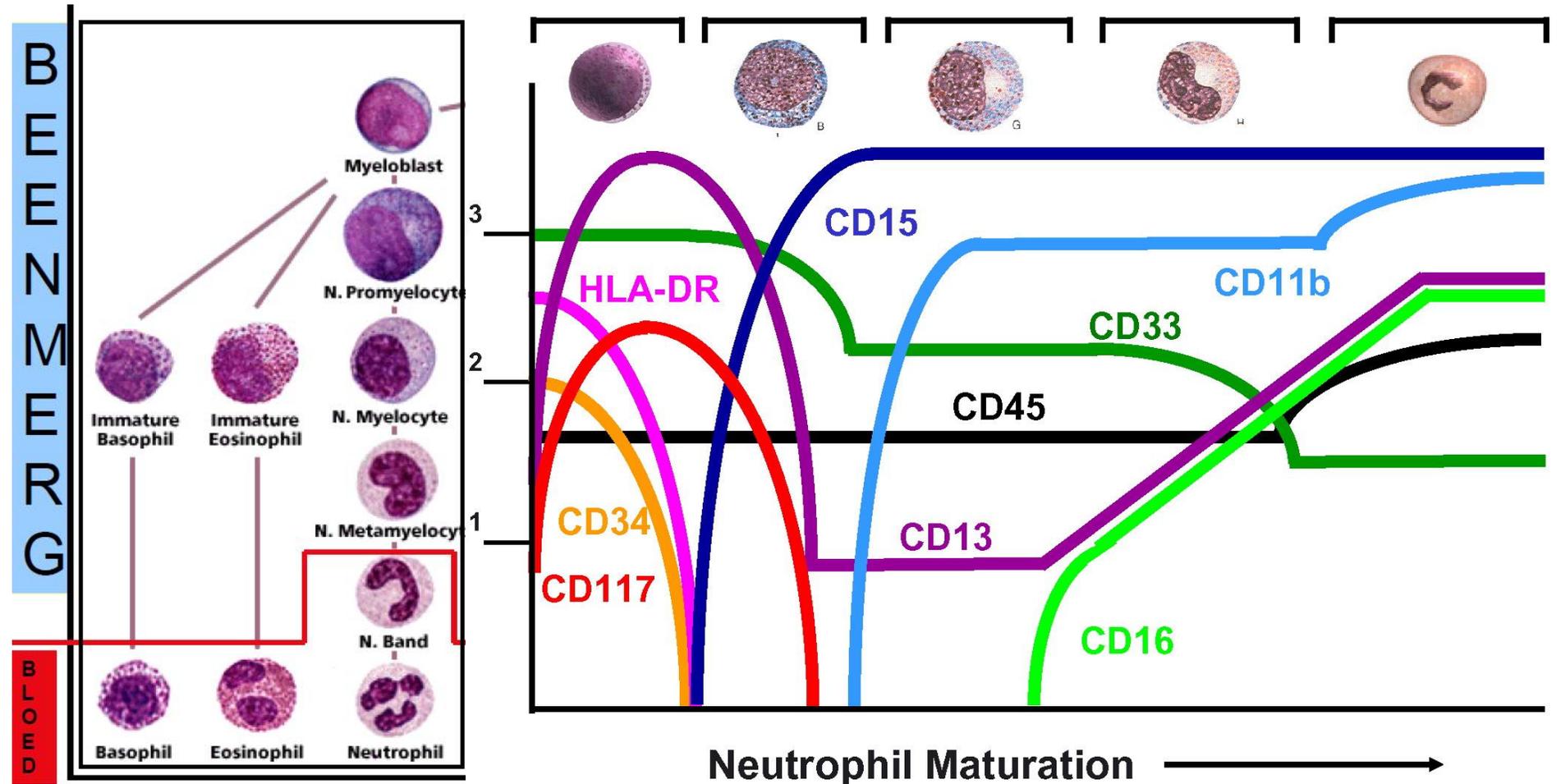# Normal cell populations

- Bone marrow: granulocytic/myeloid compartment

Normal cell populations

- Bone marrow: granulocytic/myeloid compartment

Recommendations 2023 of the ELN /MDS Flow WG
Recommended antibodies

TABLE 1   Recommended antibodies for FCM analysis of various cell types

Cell subset	Backbone markers	Recommended markers	Optional
Myeloid progenitor	CD45, CD34, CD117, HLA-DR	CD13, CD33, CD10, CD11b, CD15, CD38, CD7, CD56	TdT, CD5, CD19, CD25, CD133
Lymphoid progenitor	CD45, CD34	HLA-DR, CD10, CD19	CD22
Granulocyte	CD45, CD117	HLA-DR, CD13, CD33, CD11b, CD16, CD10, CD15, CD14, CD64, CD56	CD34, CD5, CD7
Monocyte	CD45	HLA-DR, CD13, CD33, CD11b, CD14, CD34, CD36, CD64, CD16, CD56, CD117	CD2, MDC8 (Slan), CD300e
Erythroid	CD45, CD34, CD117	HLA-DR, CD36, CD71, CD105, CD13, CD33	CD235a
Optional cell subsets for analysis:			
Basophil	CD45	CD123, HLA-DR	CD203c
Mast cell	CD117	CD45, HLA-DR	CD2, CD25
Dendritic cell	CD45, CD34, CD117	HLA-DR, CD123	CD11c CD1c, CD141, CD303

Normal cell populations

- Bone marrow : granulocytic/myeloid compartment
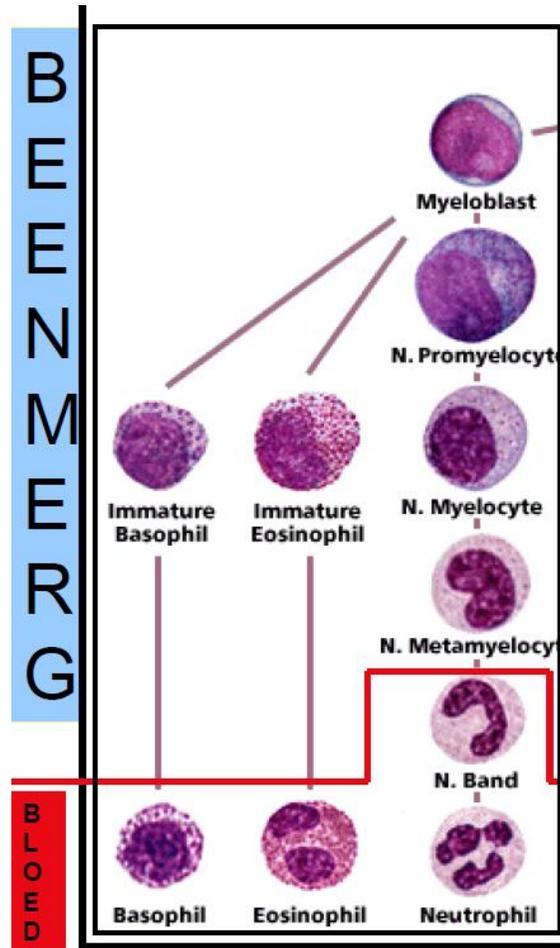
- HSC/LSC:
  SSC^{low}/CD45moderate/CD34+/CD38-

- **Blast window = intermediate CD45 and low SSC**
  o CD34+ (myeloblasts)
  o CD34- (monoblasts and megakaryoblasts)
- Myeloblasts:
  SSC^{low}/CD45moderate/CD34+/CD38+/CD117+/CD13+/HLA-DR+/CD33+/TdT±
  o Monocytoid: CD36/CD64/CD14/CD33
  o Erytroid: CD36/CD71
  o Megakaryocytic: CD36/CD9/CD41/CD64

Normal cell populations

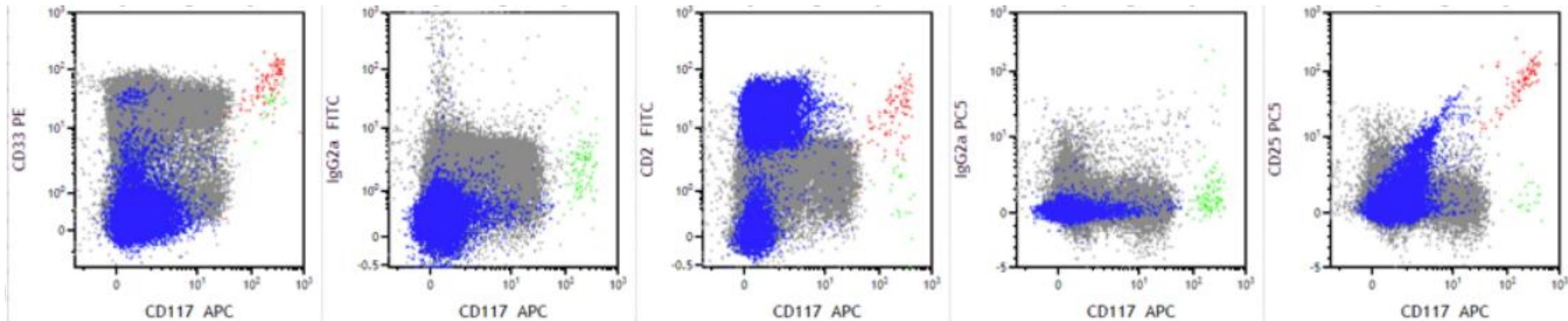- Bone marrow : granulocytic/myeloid compartment
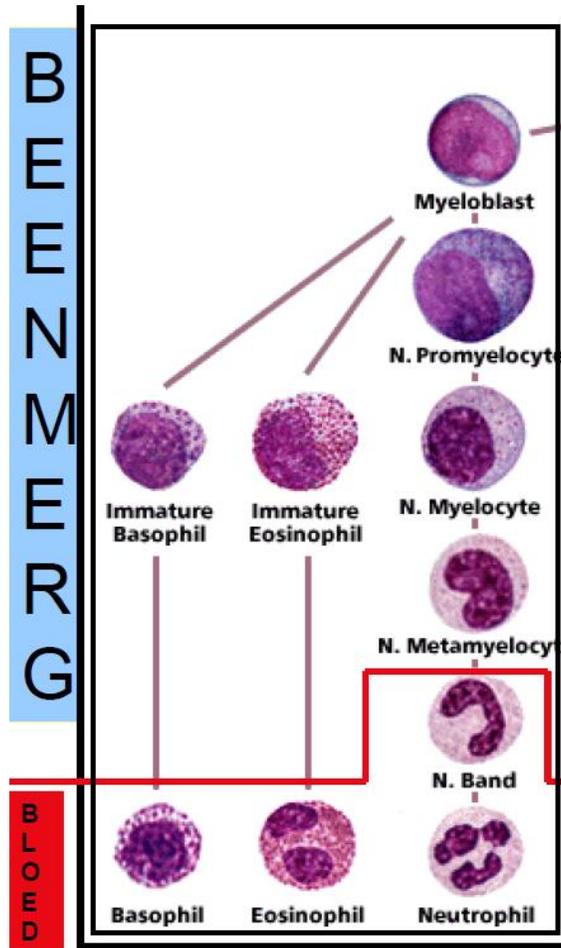
- Promyelocytes:
  SSC^{high}/**CD34-**/**CD117+/**CD13++/CD33+/**HLA-DR-/CD15-/CD16-/CD11b-/CD11c-**

- Myelocytes:
  SSC^{high}/CD34-/**CD117-/CD13+-/**CD33+/HLA-DR-/**CD16-/CD15+/**CD11b-/**CD11c±**

- Metamyelocytes:
  SSC^{high}/CD34-/CD117-/CD13+/CD33+/HLA-DR-/CD16+/CD15+/CD11b±/**CD11c±**
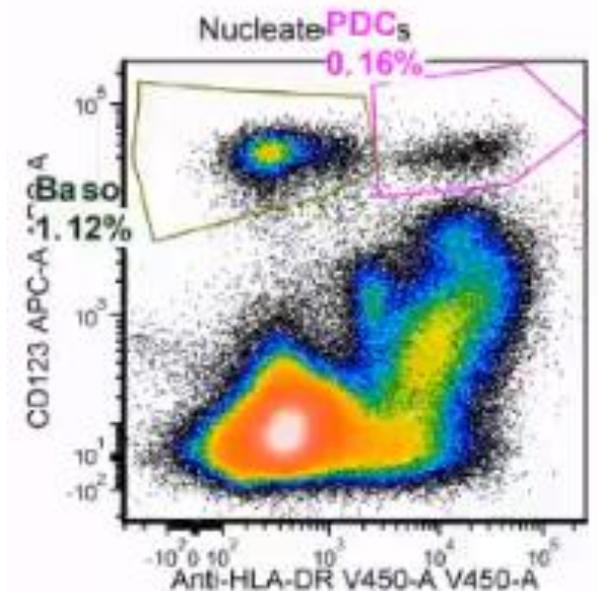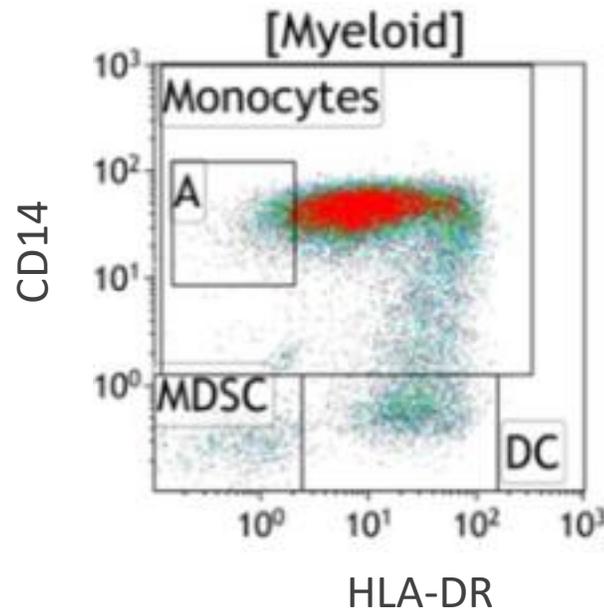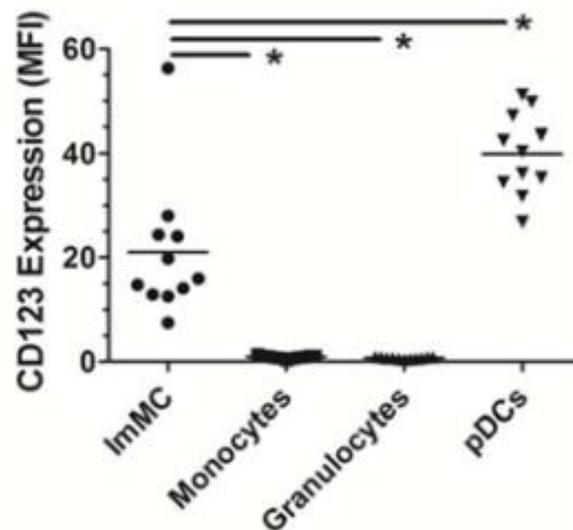
Normal cell populations

- Bone marrow : granulocytic/myeloid compartment

- **Mast cells**
  - <1% BM
  - SSC^{high}/FSC^{low}/CD45++ (~eosinophils)
  - **CD117++/**CD33+/CD9+/CD71+/CD11b^{low}/HLA-DR^{low}
  - Systemic mastocytosis: aberrant expression of **CD25**, with or without **CD2**

🟩 Normal mast cells
🟥 Abnormal mast cells

Normal cell populations

- Bone marrow : granulocytic/myeloid compartment

  - **Dendritic cells (DC):** CD45+/CD14-/CD33+/CD11c+/**HLA-DR++/CD4+/CD45RA+/CD36+**
    - BM: 0.082% ± 0.025%; PB ± 0,65%
      - CD16-/**CD123- "myeloid"** DCs (MDSC, zijn ook HLA-DR-)
      - CD16+/**CD123dim** DCs
      - **CD16-/CD123++ "plasmacytoid" DCs**

Gustafson et al (2015), Zhang et al (2019), Matarraz, S et al. (2017)

Normal cell populations

- Bone marrow : granulocytic/myeloid compartment

    - **(advanced)** Myeloid-derived suppressor cells (MDSCs): CD33+/HLA-DR-
        - PMN-MDSC: CD3-/CD19-/CD56-/**CD123-/CD11b+/**CD14−/CD33+/**CD15+/HLA-DRlow/-**
        - M-MDSC: CD3-/CD19-/CD56-/**CD123-/CD11b+/**CD14+/CD33+/**CD15−/HLA-DRlow/-**

(Bronte et al., 2016; Gabrilovich et al., 2007; Li et al., 2021; Llitjos et al., 2016; Tumino, Fiore, Pelosi, Moretta, & Vacca, 2022; Veglia, Perego, & Gabrilovich, 2018; Gustafson et al. 2015).

Thank you for your attention!

Barbara.depreter@azdelta.be