Interpreting Flow Cytometry Minimal Residual Disease in Acute Leukemia

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Contents

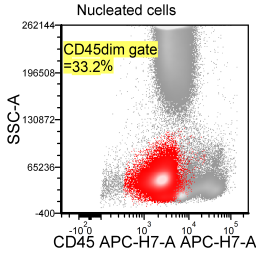
Basic principle in MRD testing

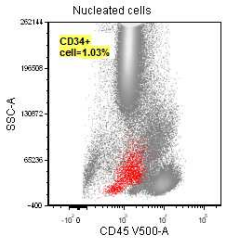
How to detect MRD in

- B-lymphoblastic leukemia
- T-lymphoblastic leukemia
- Acute myeloid leukemia

The Basic Principle of MRD in Acute Leukemia Panel Set Up

1) Target a specific population, reduce the noise
   - A marker to pull out the population of interest for analysis;

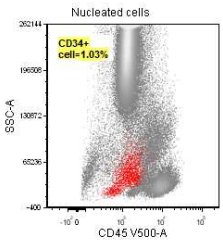


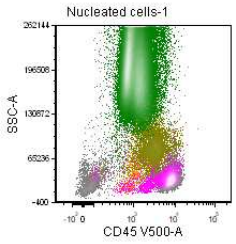


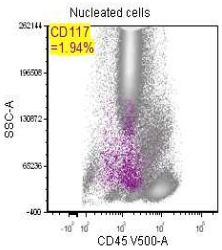
CD45dim "blasts" gate

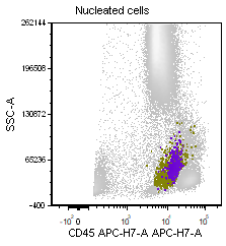
CD45dim gate is where blasts reside, but it also contains many different cells:

1. Myeloid precursors/myeloblasts
2. Hematogones (immature B cells)
3. Basophils
4. Plasmacytoid dendritic cells (PCD)
5. Plasma cells
6. Hypogranulated granulocytes
7. Some promyelocytes
8. Immature erythroid precursors
9. Immature monocytes
10. Early NK cells









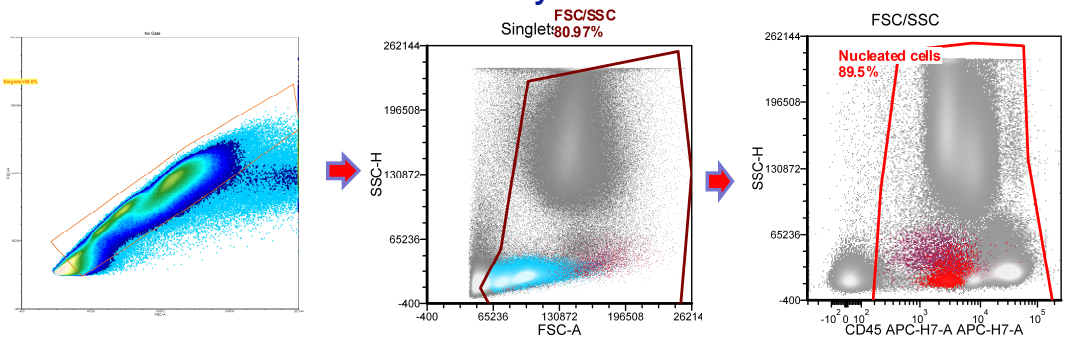
The Basic Principle of MRD in Acute Leukemia Sensitivity

2). Acquire sufficient events (nucleated events)

- Sensitivity is directly related the events you acquired for analysis;
  - 20 cells in a clusters are required to define an abnormal population; if to reach a 0.01% sensitivity, 200,000 nucleated events are minimal (20/200,000=0.01%)
  - 50 cells in a cluster are required for precise quantitation, 500,000 nucleated events are minimal

Acquire sufficient events

Only nucleated events should be included in the final analysis



- Average total events: 1493064
- Nucleated cells=42.5%
- Nucleated events=574477

The Basic Principle of MRD in Acute Leukemia Panel design

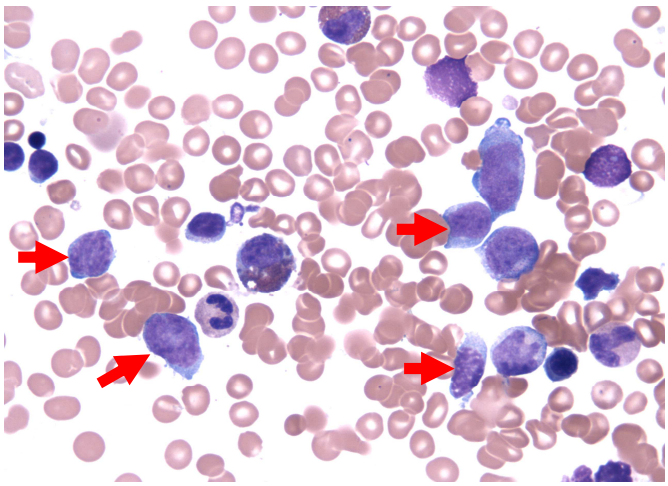
3). Panel includes sufficient markers to cover the heterogeneity of blast phenotype, and differentiate blasts from normal

Look for cells showing phenotypic deviation from normal (DfN); and leukemia associate immunophenotype (LAIP)

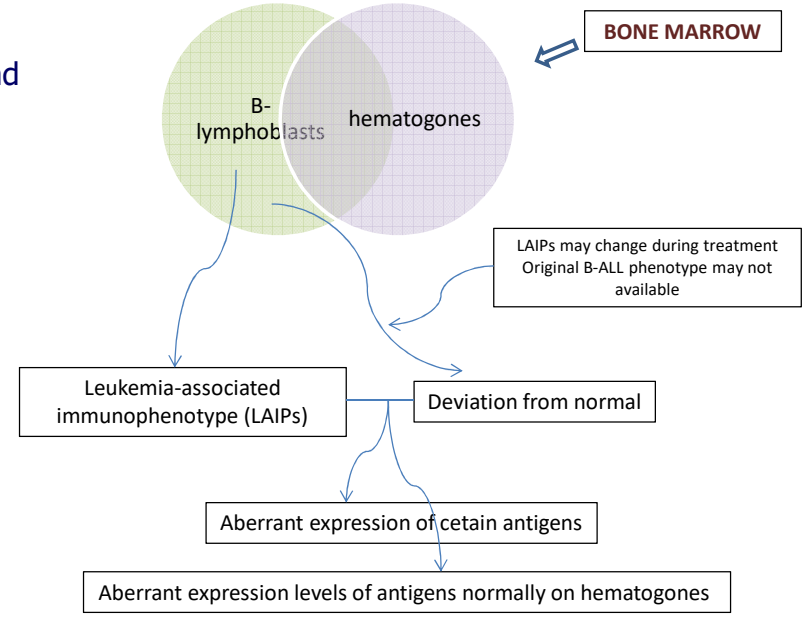
- the normal counterparts/Mimics

MRD flow cytometry detection for Precursor B-lymphoblastic leukemia (B-ALL)

- Gating on CD19
- BM CD19+ cells include
  - Immature B cells (Hematogones)
  - Mature B- cells
  - Plasma cells



Flow cytometry panel design and analytical approaches



MRD flow cytometry panels/markers

ECOG panel

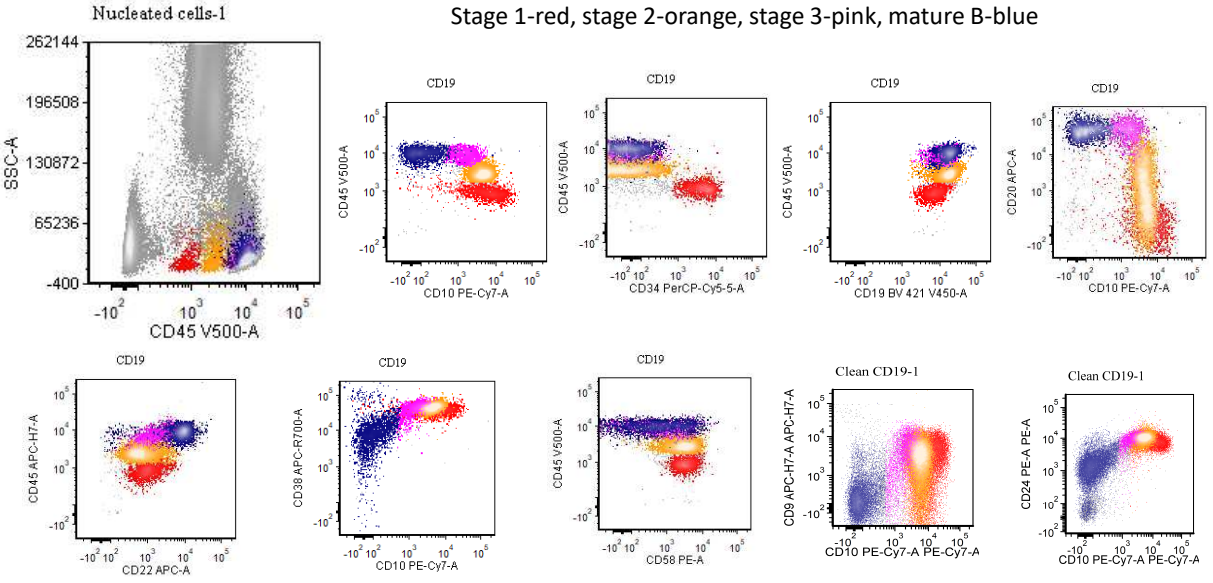
FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-H7	V450	V500	BV605
CD20	CD10	CD38	CD19	CD58		CD45			
CD9	CD13/33	CD34	CD19	CD10		CD45			

10-color panel (MDACC)

FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R718	APC-H7	V450/BV421	V500	BV605
CD13/33	CD58	CD34	CD10	CD20	CD38	CD9	CD19	CD45	
CD66c	CD24	CD34	CD10	CD22		CD45	CD19	CD15	CD25

CD19+ B cells in BM

Stage 1-red, stage 2-orange, stage 3-pink, mature B-blue



CD19+ cells in a normal bone marrow

Hematogones: (Normal precursor B cells in bone marrow)

- **Stage I:** CD19dimmer+, CD10brighter+, CD34+, TdT+, CD20-/weaker+, may have dim partial CD13
- **Stage II:** CD19+, CD10moderate+, CD34-, TDT-, CD20hterogeneously+,
- **Stage III:** CD19+, CD10dim+, CD34-, CD20 bright+,

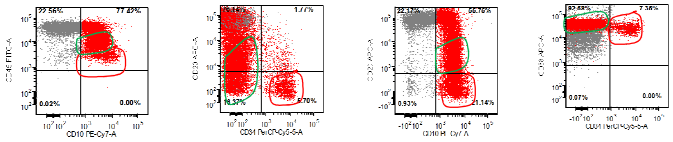
All three stages are CD38bright+, CD22dimmer+, CD24+, variable CD9, negative for **CD15, CD25, CD66c, CD33**

Mature B cells: CD38moderate+, CD22bright+

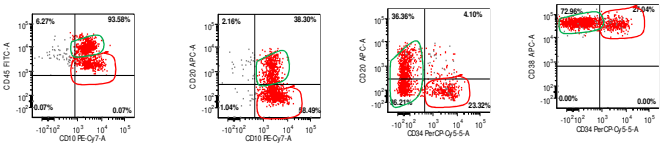
Plasma cells: CD19+, CD38 bright+, CD10-, CD34-, CD22-, CD20-

Post treatment, BM may not have all three stages of hematogones

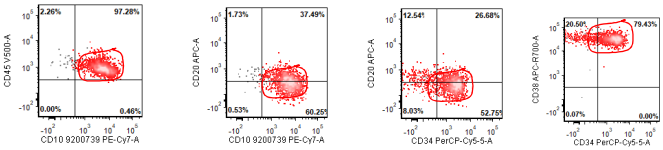
All three stages of hematogones present



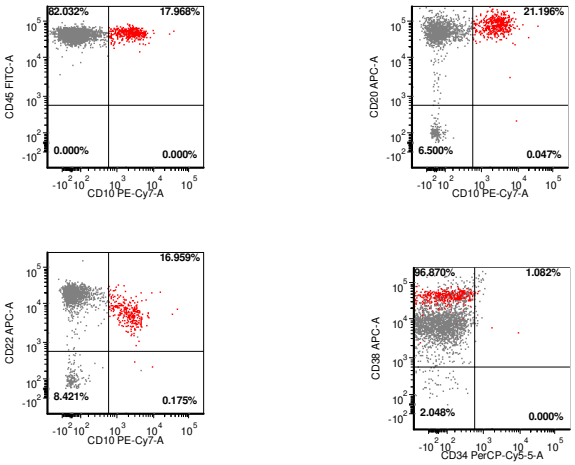
Predominantly stage I and II hematogones



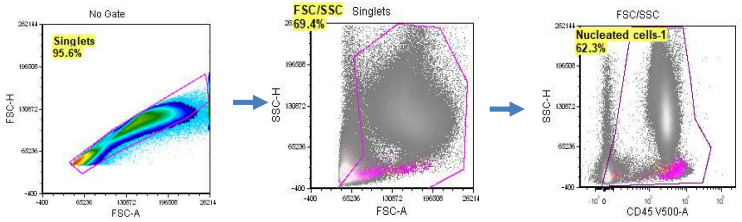
Predominantly stage I hematogones



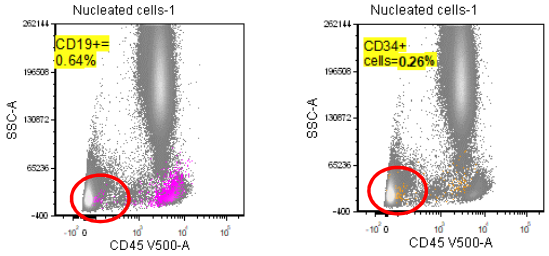
Predominantly stage III Hematogones, (transition cells) in peripheral blood



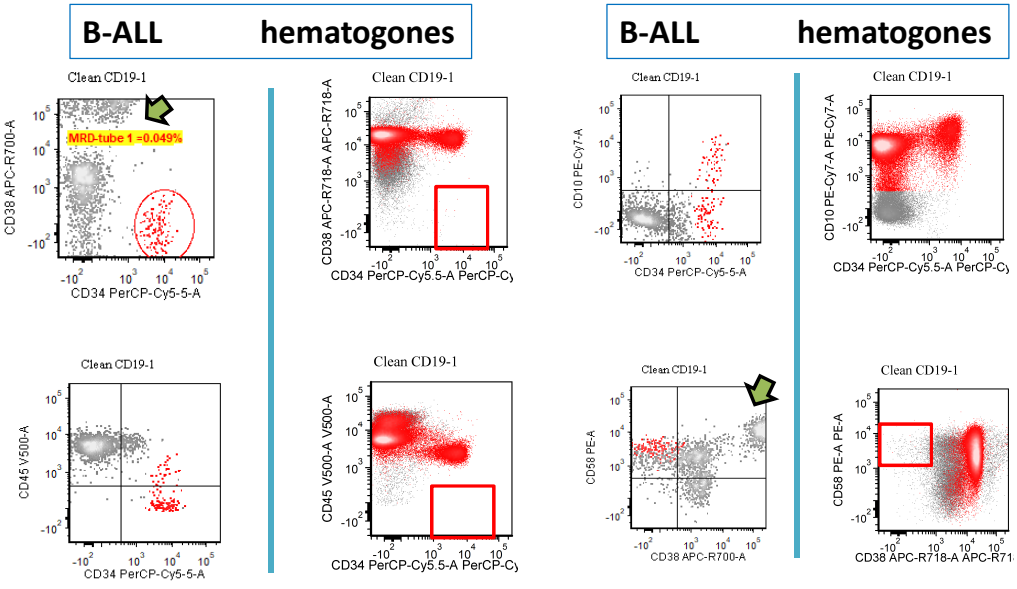
Gating strategy and analysis



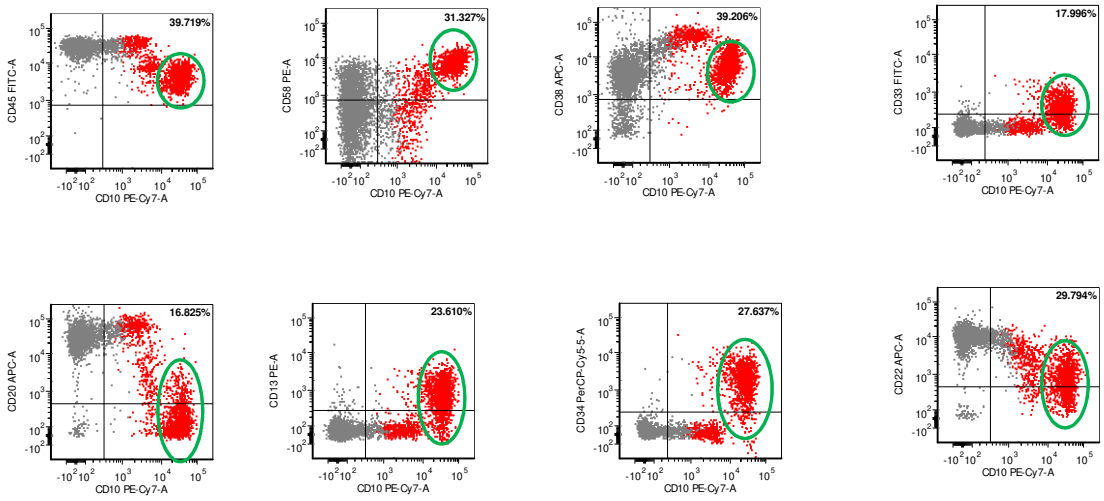
Nucleated events 328849



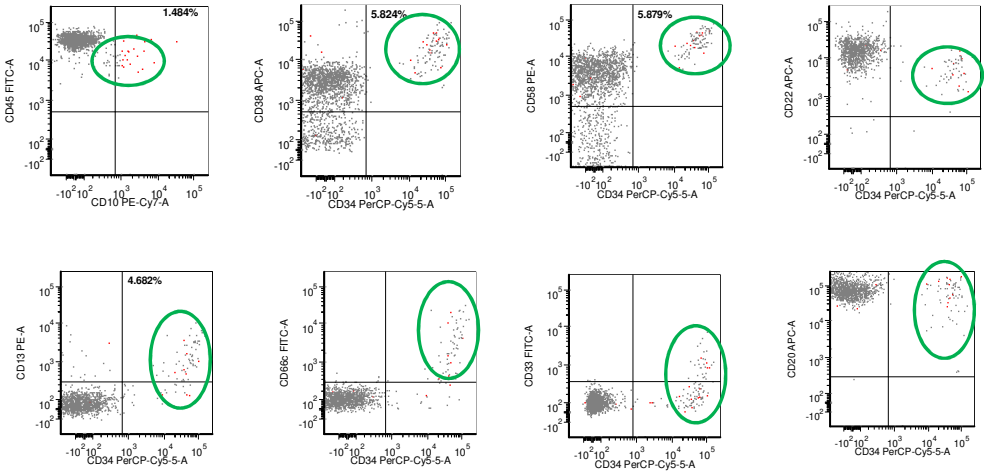
B-ALL MRD identification-Case1



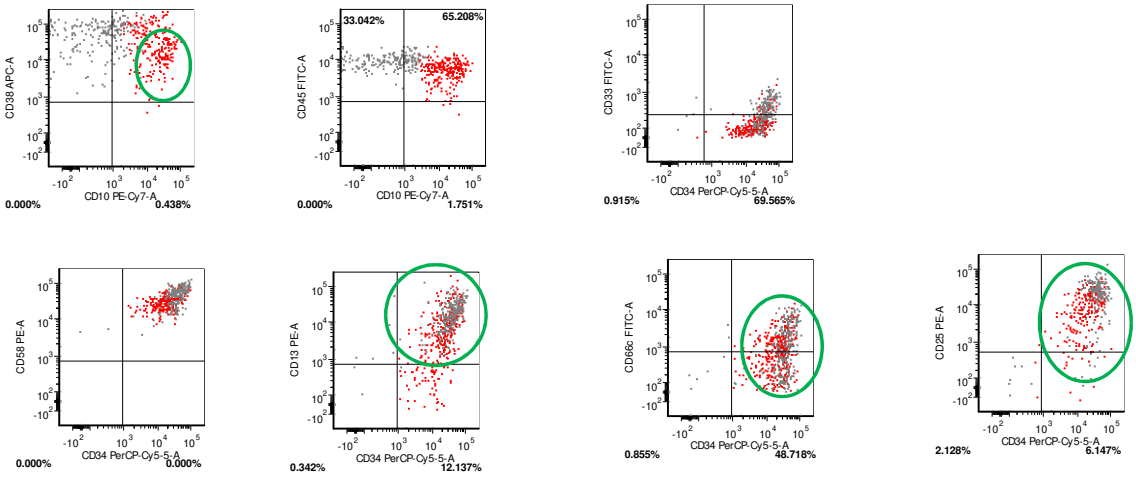
Case 2, B-ALL mixed with hematogones (0.71%)



Case 3, CD10 negative/dim+ B-lymphoblasts (0.06%)

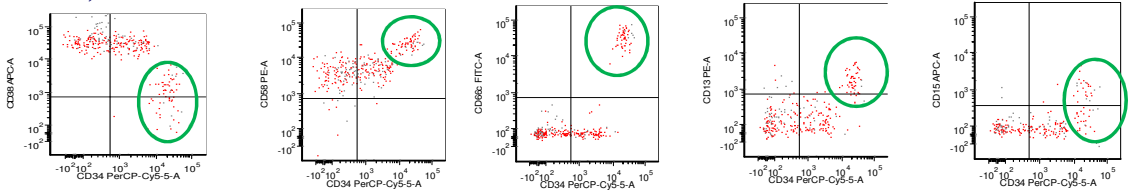


Case 4: B-ALL cells with heterogeneous CD10, CD38 and CD45 expression

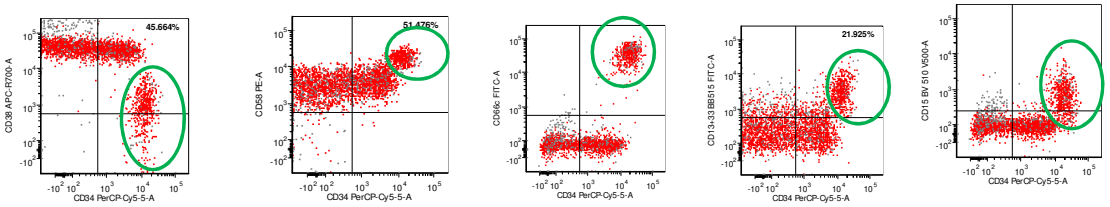


Compare events acquired 200,000 vs 1,000,000

200,000



1,000,000



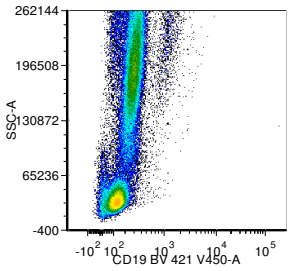
Targeted therapies

- Immunotherapy targets disease independent of chemo-radiotherapy, with non-overlapping toxicities
  - HyperCVAD-rituximab (anti-CD20);
  - HyperCVAD-inotuzumab ozogamicin (anti-CD22 immunotoxin)[2]
  - Blinatumomab (anti-CD19/anti-CD3)[3]
  - Chimeric Antigen Receptor T-cell (CART)-19 Therapy

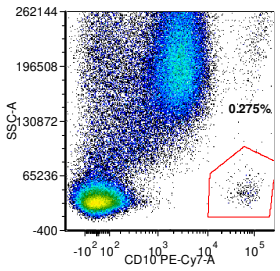
1. **Thomas et al. *JCO* 2010; 28:3880**
2. **Kantarjian et al. Cancer 2013; 119:2728**
3. **Topp et al. *JCO* 2011; 29:2493**

A patient treated with CAR-T

Original B-ALL was CD19+, CD10bright+, CD34 partial+, CD20+, CD22+, CD58bright+, CD38dimmer+

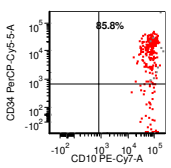


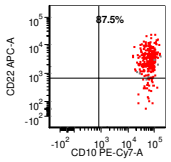
There are no CD19 positive cells

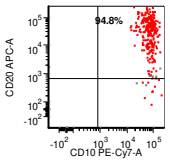


Change the CD19 gating to CD10/low SSC gating. A total of 0.275% CD10/low SSC+ cells seen

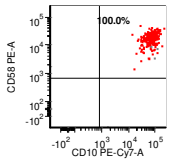
CD10/low SSC cells

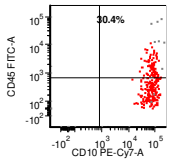


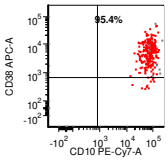




These cells expressing CD34, CD22, and bright CD20, consistent with B-lymphoblasts



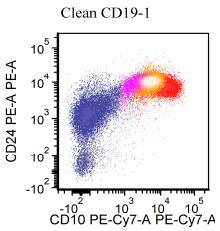


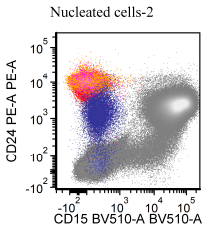


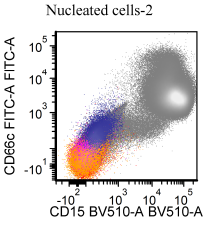
These lymphoblasts show increased CD58expression, and decreased CD45 and CD38 expression

Introducing CD24 as another B cell marker

- Positive in mature B cells, hematogones, granulocytes and granulocytic precursors
- Need to have a myeloid marker



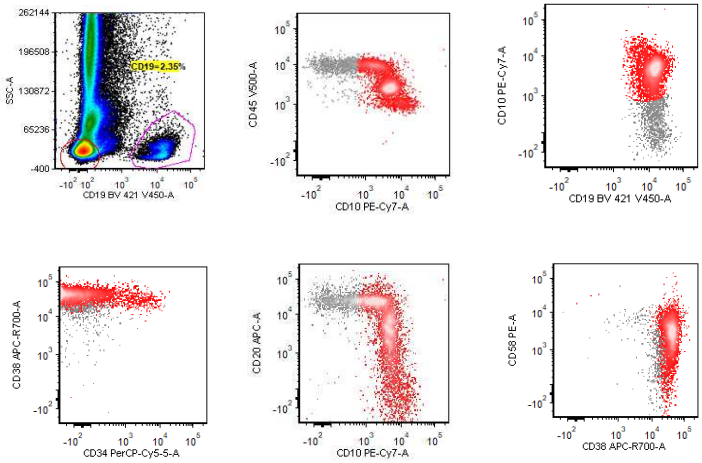




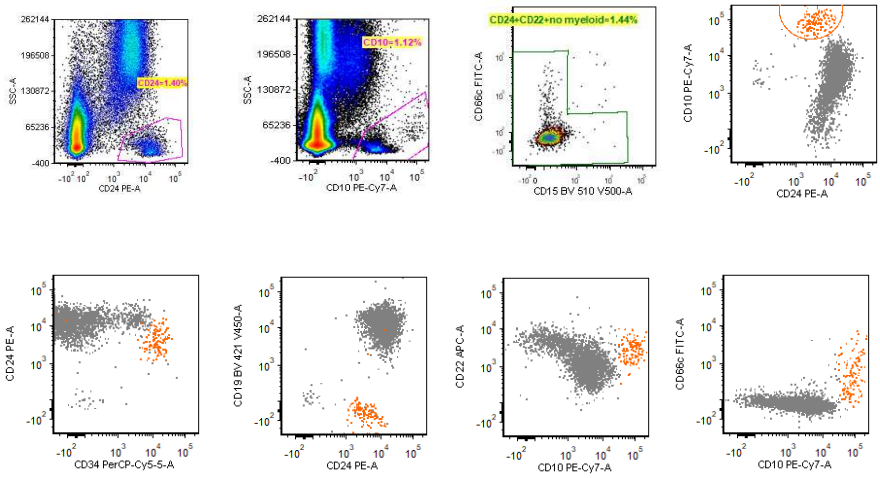
Case example

62 yo male, initially diagnosed with B-ALL in 2016 and is s/p blinatumomab x 2 cycles at OSH and he presented to MD Anderson.

Regular CD19 gating



CD24 or CD10+ cells



Summary of B-ALL MRD by flow cytometry

- Design/Use a good panel, acquire as many cells as your lab allows
  - half million, and recommend 2 millions
- Use a B cell marker (CD19) for gating,
  - Alternative markers needed in patients received anti-CD19 therapy
- B-ALL MRD can be reliably differentiated from benign counterparts
  - hematogones, mature B cells and plasma cells
- The most common abnormalities observed in B-ALL:
  - ↓CD38,
  - ↑CD10,
  - ↑ CD58,
  - ↓/negative CD45,
  - abnormal loss or ↑ CD9,
  - aberrant CD13/CD33, CD66c, CD25, CD15 (especially useful in *KMT2A*-rearranged cases)
- Be careful with CD10-negative, CD34-negative cases, or CD10/CD34 double negative cases
- Be aware of immunophenotypic heterogeneity of blasts

Precursor T-lymphoblastic leukemia MRD

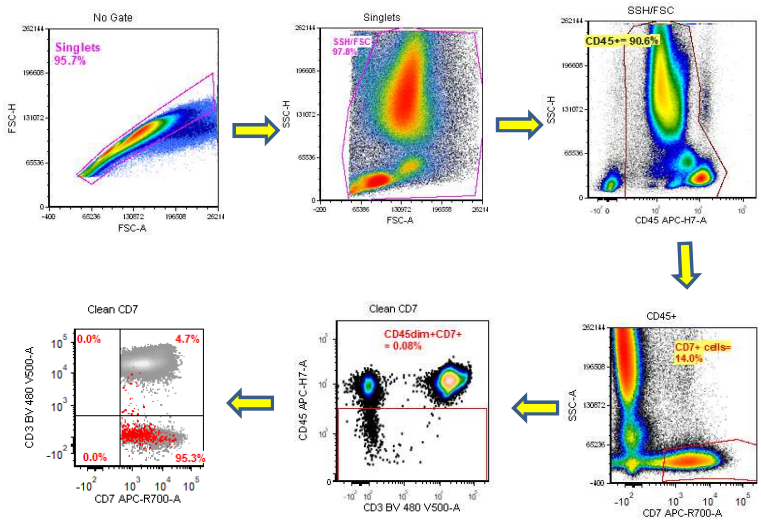
Something unique about T-ALL MRD by flow cytometry

- T- lymphocyte maturation occurs in thymus; Immature T cells are not supposed to be seen in blood or bone marrow;
- Immunophenotypic drift occurs significantly more frequently than B-ALL, tend to lose immature markers following therapy

  >40% of cases of T-ALL do not express immature markers following induction therapy
- Some T-ALL, especially ETP, cCD3 may be partial and or dim to begin with, which may further got lost after therapy;
- ***CD7:*** almost always positive in T-lymphoblastic leukemia/lymphoma

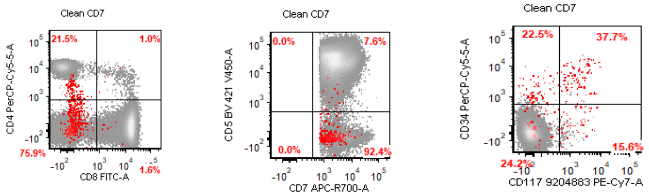
Roshal, M. et al. Cytometry B Clin Cytom 78, 139-146 (2010).
Mori N. Am J Clin Pathol. 1988; 90(3):244-9
Ginaldi L et al. Br J Haematol. 1996 Jun;93(4):921-7

T-MRD analysis, normal BM



Normal CD7+ cells in the dimCD45 region, not mistaken as T-ALL cells, including

- Normal myeloid precursors/erythroid precursors (CD34+CD117+CD13+CD33+CD4dim+HLADR+);

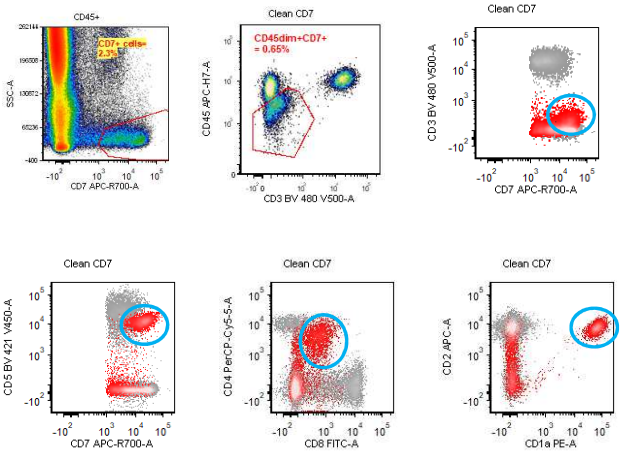


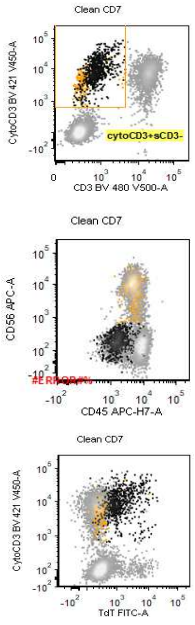
- Plasmacytoid dendritic cell precursors (CD2+, CD4+, HLADR+);
- NK cells, especially immature NK cells (CD7+, CD2+, CD56+, CD16+, CD117 partial+, CD33 partial+, CD45dim+, CD48-)

T-ALL MRD flow cytometry panel

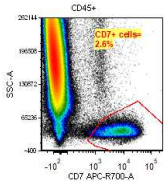
FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-R700	APC-H7	V450	V500	BV605	BV711	BV786
CD8	CD1a	CD4	CD2	CD10	CD7	sCD3	CD5-BV421	CD45	CD56		CD19
CD13/CD33-BB515	CD48	CD34	CD117	CD7	CD38	sCD3	HLA-DR-V450	CD45	CD56		
TDT	CD7	CD34		cytoCD3			sCD3-BV421	CD45	CD56		

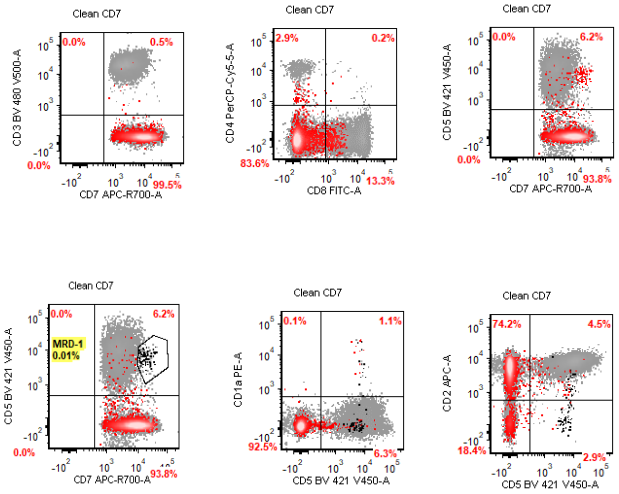
Case 1, TMRD+





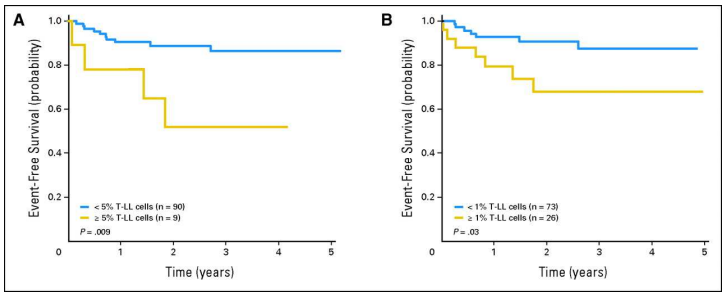
Case #2 T-MRD+





Minimal disseminated disease of T-lymphoblastic lymphoma

- By flow cytometry, >2/3 of children with T-lymphoblastic lymphoma have disseminated disease at diagnosis (positive blood and bone marrow)



Coustan-Smith E, et al. J Clin Oncol. 2009 Jul 20;27(21):3533-9.

B and T-lymphoblastic leukemia MRD PB vs BM

- There is high concordance, T-ALL (91%) better than B-ALL (83%)
- In positive cases, BM shows higher number of blasts than PB
- Bone marrow is still preferred samples for MRD detection

Cytometry Part B: Clinical Cytometry
Volume 88, Issue 1, pages 21-29, 1 NOV 2014 DOI: 10.1002/cyto.b.21195
http://onlinelibrary.wiley.com/doi/10.1002/cyto.b.21195/full#cytob21195-fig-0004

AML, Assessment of Remission

- Conventional remission criteria:
  - <5% BM blasts
  - absolute neutrophil count >1K and platelets >100K
- Minimal residual/measurable disease:
  - Examine residual AML in a morphologically remission bone marrow

Reality check
AML MRD by multicolor flow cytometry

- AML MRD, considered to be the most challenging test:
  - AML pathogenesis (especially in elderly) is complex, multi-step, accumulations of molecular genetic events;
    - Subclones
    - Preleukemic cells, MDS cells
    - Immunophenotypic switch

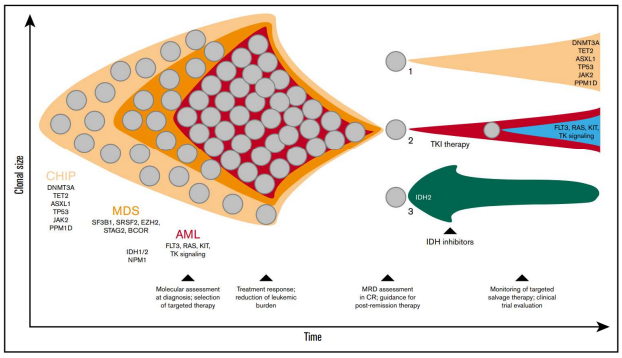
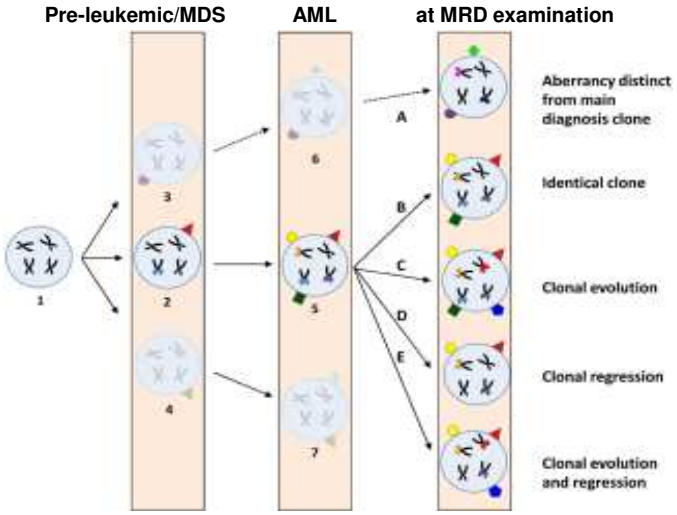


Figure 1. The role of molecular profiling in AML diagnosis, therapeutic selection, and disease monitoring. In this schematic, AML development is depicted as the terminal stage in a phenotypic continuum from prior clonal hematopoiesis and myelodysplasia. A sample of acquired mutations (not all encompassing) driving disease progression is listed with respect to time (*x*-axis), in addition to a model for longitudinal molecular assessment. Relative clonal size is depicted on the *y*-axis. In this sample case, mutational profiling at diagnosis aids in selection of a therapy that leads to a significant reduction of leukemic burden and establishment of a first complete remission (CR). From here, 3 possible scenarios are depicted: sustained remission with expansion of preexisting nonmalignant clones (clonal hematopoiesis of indeterminate potential [CHIP],[13-17] disease relapse with development and outgrowth of a leukemic subclone (blue),[61] and control of persistent disease with targeted agents leading to cellular differentiation without killing (eg, IDH1/2 inhibitors, hypomethylating agents).[4] IDH, isocitrate dehydrogenase; MRD, minimal residual disease.

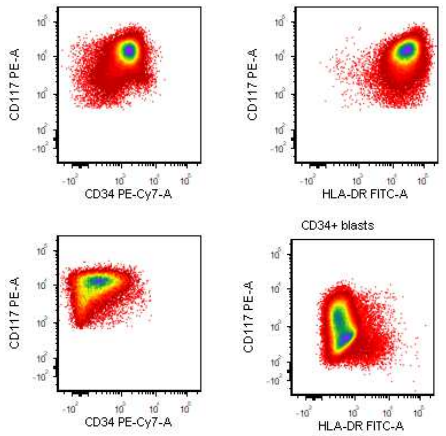
Tumor heterogeneity makes AML a "moving target" for detection of residual disease



Cytometry Part B: Clinical Cytometry
Volume 86, Issue 1, pages 3-14, 21 OCT 2013 DOI: 10.1002/cyto.b.21134

Reality check
AML MRD by multicolor flow cytometry

- AML blasts are immunophenotypically diverse
  - CD34+, CD34-, APL-like, monocytic, megakaryoblastic, erythroblastic…
- AML treatment varies greatly, there is great deal of reactive changes;
- Bone marrow ablation/suppression is common, and often specimens are suboptimal or insufficient for analysis



Markers of recommendation by European Leukemia Network (ELN):

Minimal: CD7, CD11b, CD13, CD15, CD19, CD33, CD34, CD45, CD56, CD117, HLA-DR;

May be useful: **CD38, CD123,** CD133;

If necessary, add a "monocytic tube" containing: CD64/CD11b/CD14/CD4/CD34/HLA-DR/CD33/CD45.

blood® 22 MARCH 2018 | VOLUME 131, NUMBER 12 1275

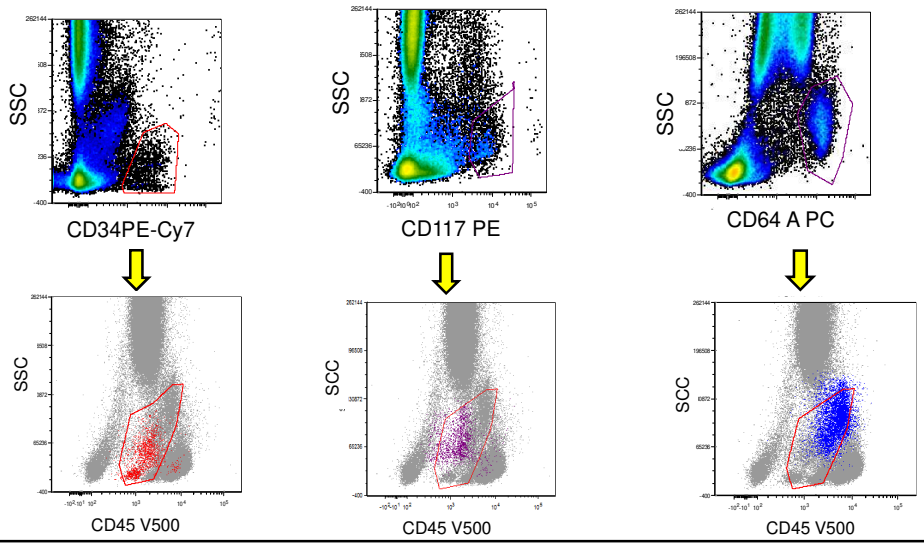
Heuser M, et al. 2021 Update on MRD in acute myeloid leukemia: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 2021 Dec 30;138(26):2753-2767.

Flow Cytometry Panel for AML MRD at MDACC
12-color, two tubes, FDA cleared for IDE

CD7	CD117	CD34	CD33	CD13	CD38	CD45	CD133	CD5	CD4	CD54	CD19
CD36	CD117	CD34	CD64	CD123	CD38	CD45	DR-V450	CD15	CD56	CD25	CD14

CD133: myeloid committed precursors, can be aberrantly lost in AML blasts
CD54: stem cell marker, also stain some monocytes, can be aberrantly increased in AML blasts
CD25: also helps identifying aberrant mast cells, can be aberrantly increased in AML blasts
CD36: positive in erythroids, monocytes, megakaryocytes, PDC, basophils

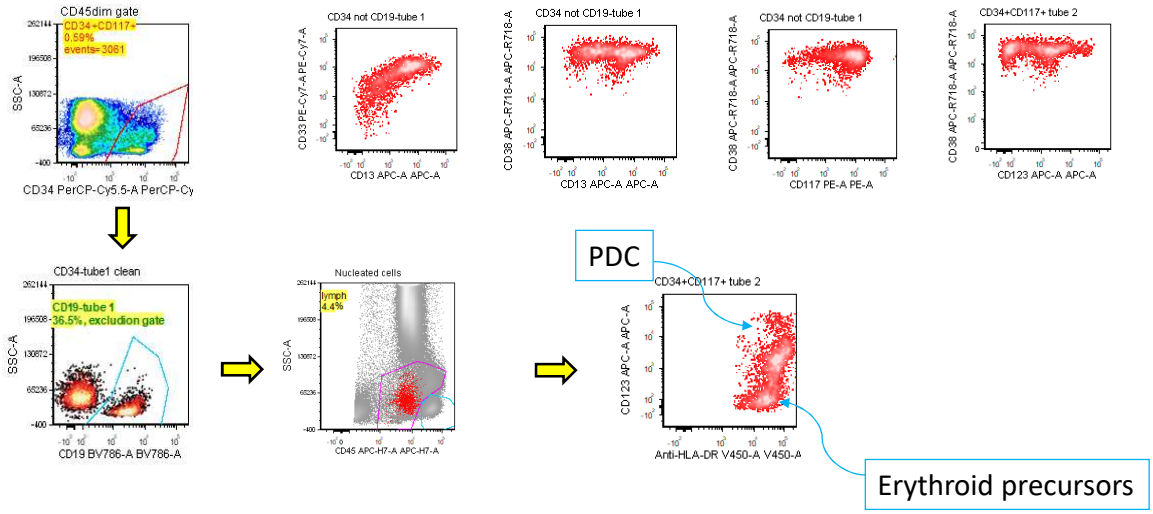
Analysis strategy (CD45dim gate, CD34 gate and CD117 gate)



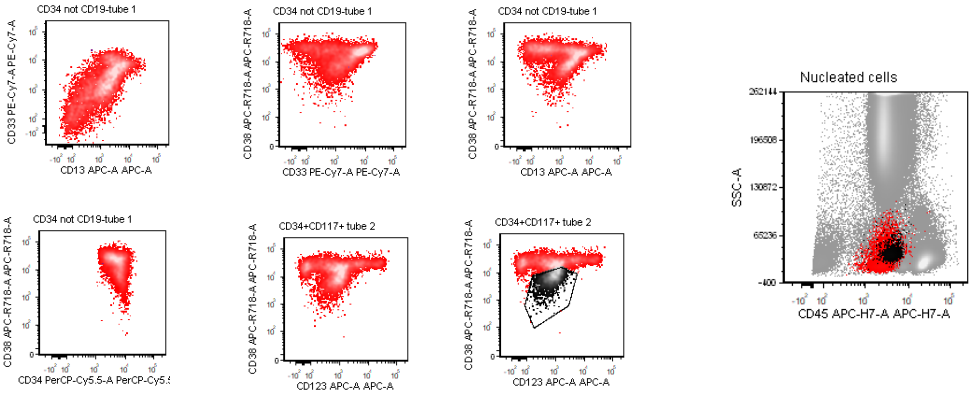
Why CD34+ myeloid precursors?

- Rationale to put great emphasis on CD34+ myeloid precursors
  - The more immature cells/leukemic stem cells tends to be treatment resistant, these cells are CD34+;
  - For monocytic leukemia or CD34- AML, recovery of normal CD34+ cells is a reliable sign of remission. Lacking of normal CD34+ myeloid precursors will be either insufficient sample or with overt residual AML

Normal pattern of CD34+ myeloid precursors

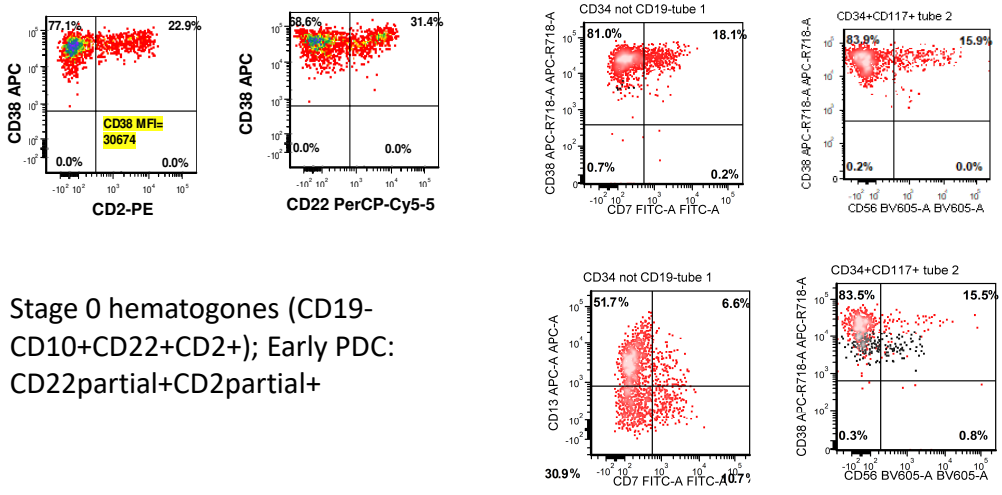


Exuberant regenerating BM



Early normal/precursors stem cells: CD38dimmer+, brighter for CD34, CD45, CD4, dimmer for CD13, CD33, CD117, CD123, HLA-DR

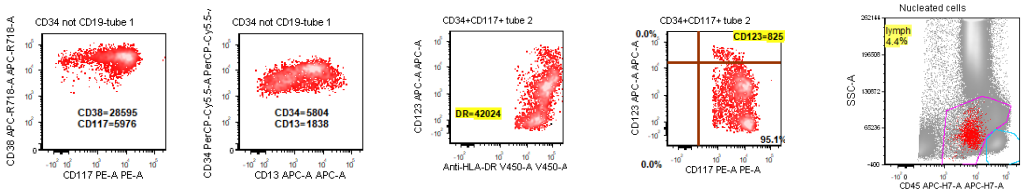
Lymphoid antigens and mature myeloid antigens can be normally expressed on CD34+CD19- cells



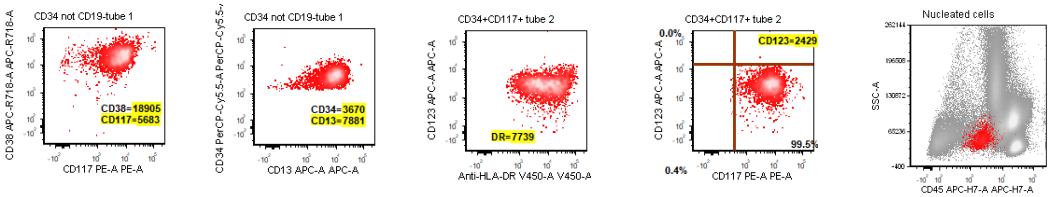
Stage 0 hematogones (CD19-CD10+CD22+CD2+); Early PDC: CD22partial+CD2partial+

MFI can provide a number helping pattern recognition

Normal



Abnormal

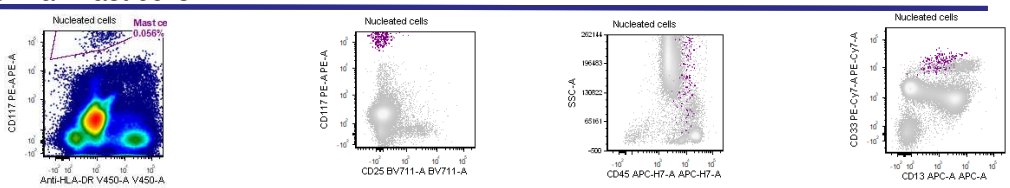


Alterations often observed in AML leukemic cells (CD34+ cells)

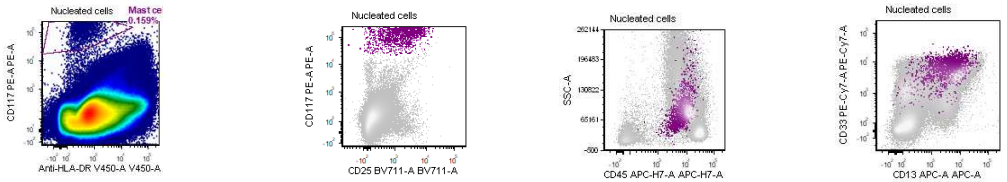
1) Altered expression of levels	
• Increased/over-expressed/abnormal gain	CD34, CD117, CD123, CD13, CD4
• Decreased/Negative (lost)	**CD38,** HLA-DR (CD4, CD34, CD13, CD33, CD117, CD133)
• Increased or decreased	CD45/SSC
2). Aberrant lymphoid antigens or other antigens	
• CD2, CD5, CD7, CD19, CD56, CD54, CD25	
3). Expressing mature myelomonocytic antigens	
• CD11b, CD15, CD64 (if a lot)	

CD25, identifies abnormal mast cells

Normal mast cells

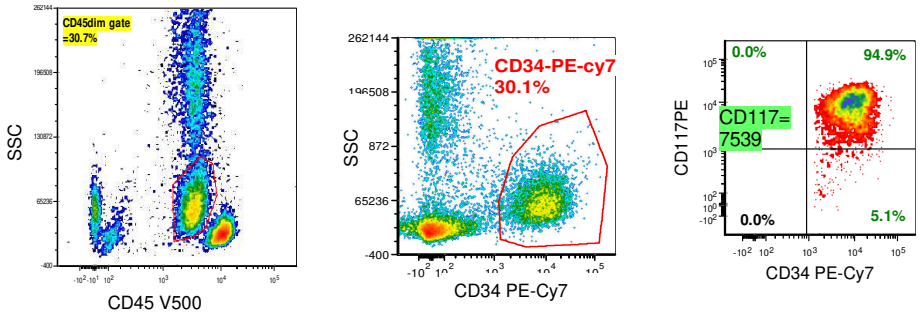


Abnormal

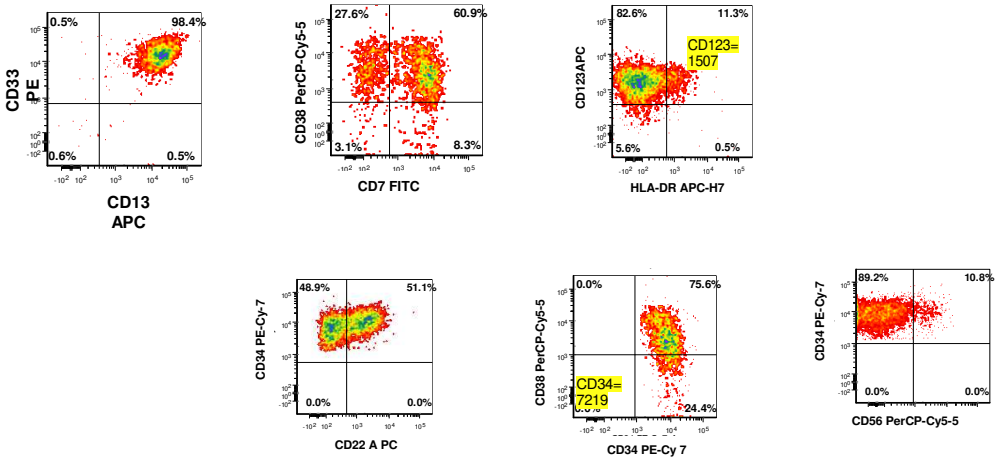


CASE ILLUSTRATION:
CASE 1:

79 yo with a newly diagnosed AML, here for treatment options



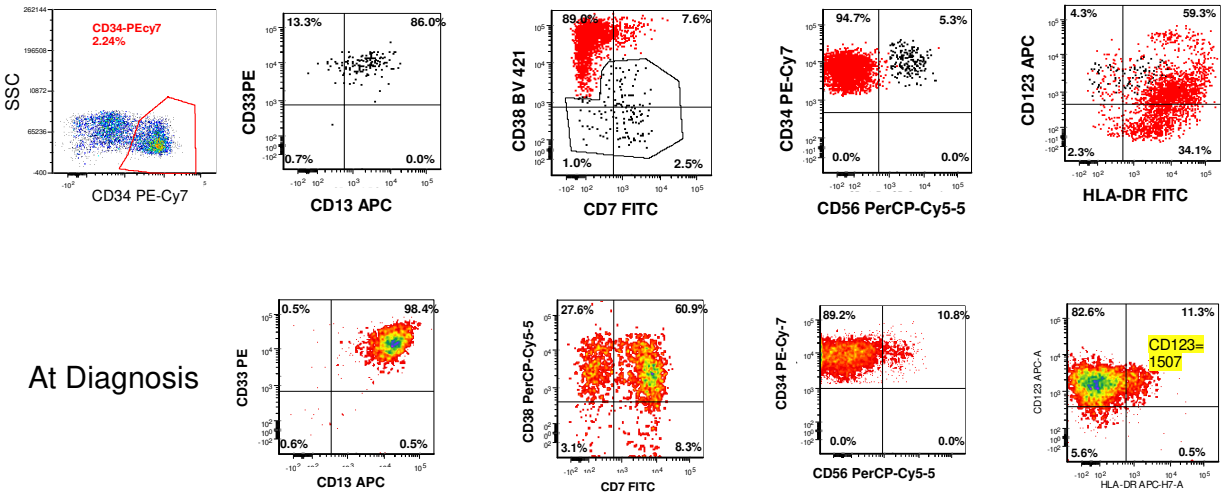
Myeloblasts exhibit multiple aberrancies



- Karyotype: 49,XY,-3,del(5)(q15q33),+8,+10,+13, -17,+21,+22[8]/46,XY[12]
- *TP53* gene mutation

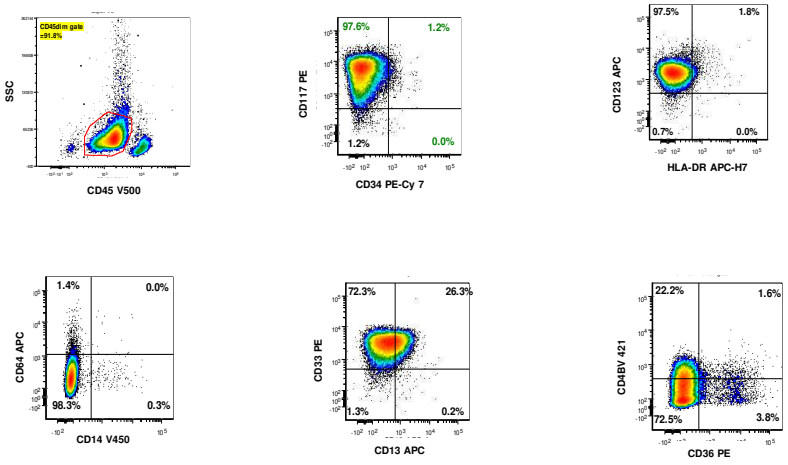
The patient continued treatment with SGI-110 (DNMT inhibitor), 3 months later

Normal Karyotype:
TP53 gene mutation negative



CASE ILLUSTRATION:
CASE 2: AML, Blasts CD34-negative, not monocytic

- 49 year old with newly diagnosed AML



AML, Blasts CD34-negative, not monocytic

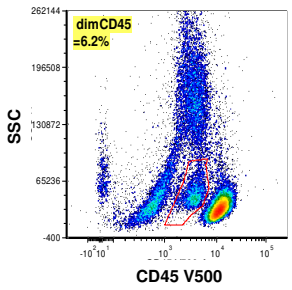
Karyotype: 46,XX[20]

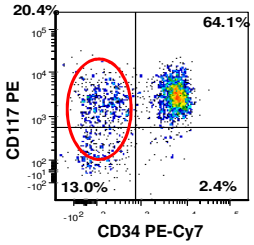
NGS: positive for *NPM1, DNMT3A and IDH2* mutations

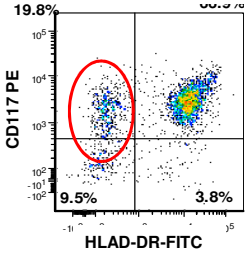
Treated with Cladribine plus Idarubicin plus Cytarabine (ARAC) induction

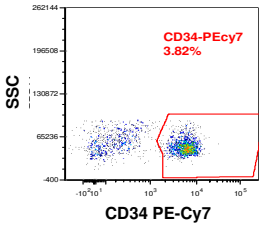
- WBC 2.7, Hb 9.8, plt 594
- BM 3% blasts

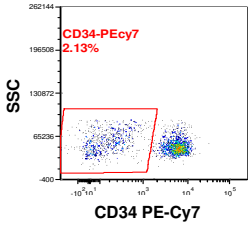
AML, Blasts CD34-negative, not monocytic, post treatment





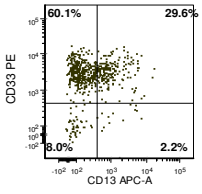


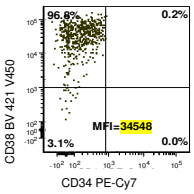


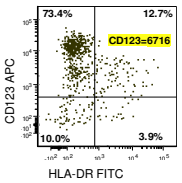


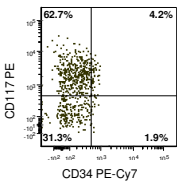
AML, Blasts CD34-negative, not monocytic, post treatment

CD34-negative blasts

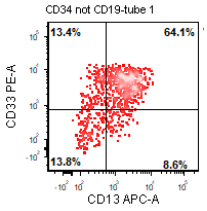


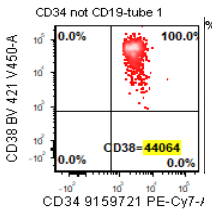


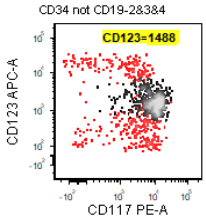


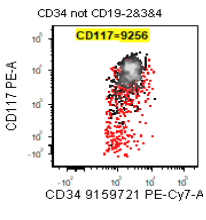


CD34-positive blasts









Case 2. Molecular correlations

At diagnosis → ***NPM1+, DNMT3A+, IDH2+***

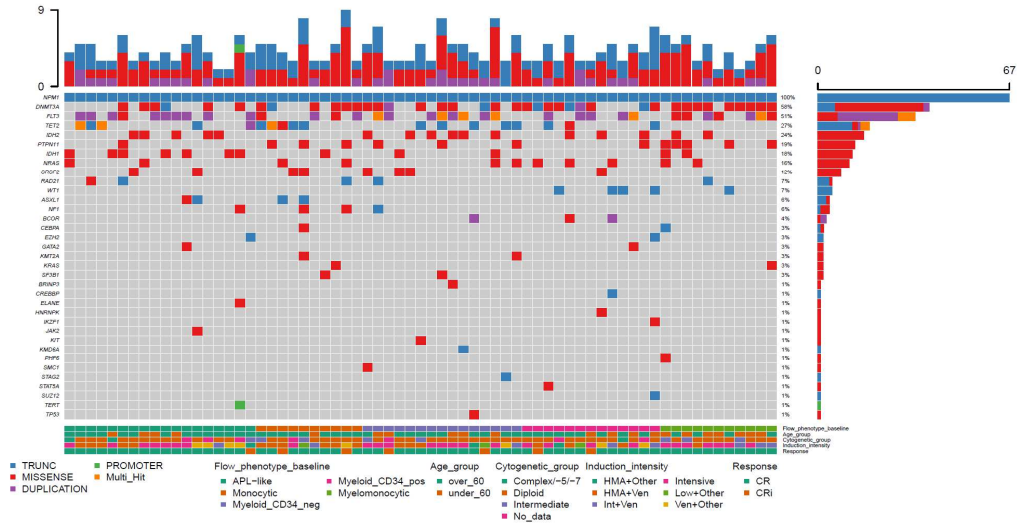
↓

Post Induction → ***NPM1+(<5%), DNMT3A-, IDH2+***

↓

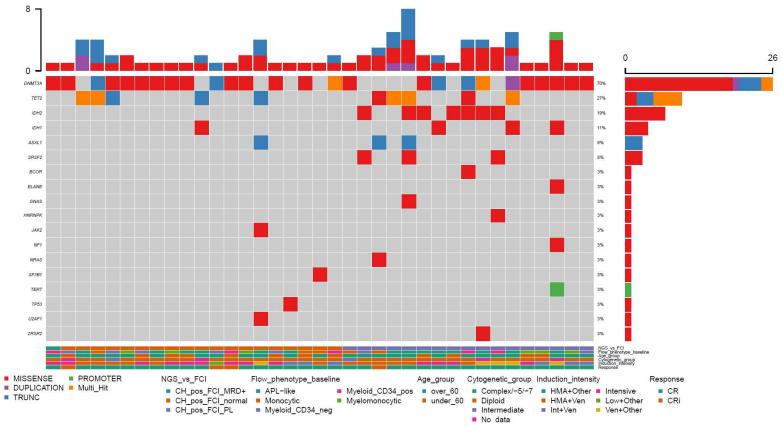
Consolidation → ***NPM1-, DNMT3A-, IDH2+***

Co-mutations are extremely frequent in *NPM1*-mut AML (n=67, 100% with comutation)



Loghavi S et al and Wang SA, Br J Haematol. 2021 Mar;192(6):1054-1063

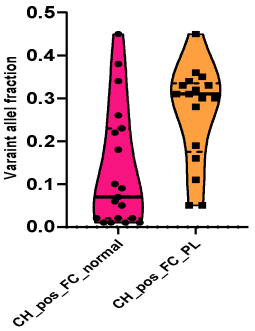
Persistent clonal hematopoiesis/mutations in *NPM1*-AML MRD negative BM (37/50, 74%)



Preleukemic immunophenotypical alterations not seen in cases with no persistent CH, and in 46% of cases with persistent CH

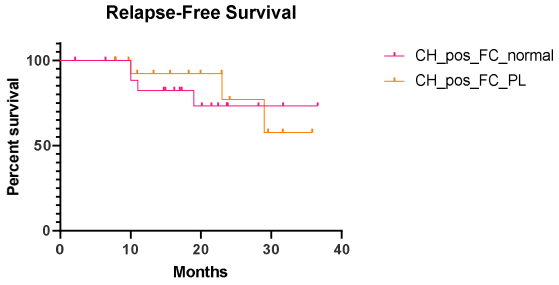
Loghavi S et al and Wang SA, Br J Haematol. 2021 Mar;192(6):1054-1063

Persistent clonal hematopoiesis/mutations with (n=17) or without (n=20) immunophenotypical alterations



	ASXL1	IDH1	SRSF2	IDH2	TET2	DNMT 3A
CH_pos_FC_normal	-1	-1	0	0	-5	-14
CH_pos_FC_PL	2	3	3	7	5	11

CH_pos_FC_normal CH_pos_FC_PL



Pre-leukemic immunophenotype

- Pre-leukemic phenotype
  - Decreased CD38, HLA-DR, increased CD117 and increased CD123; <1%, very similar to low grade MDS;
- Correlates with persistent clonal hematopoiesis/mutation, or an underlying MDS;
  - High VAF and certain specific mutations
  - More cases with dyspoiesis
- Please don't to call AML MRD;
  - DfN approach needs to be limited to:
    - Share a few resemblance to original AML even though other markers are different
    - Very aberrant immunophenotype

CASE ILLUSTRATION:
CASE 3: AML with myelomonocytic differentiation (M4)

46,XY,inv(16)(p13.1q22)[20]

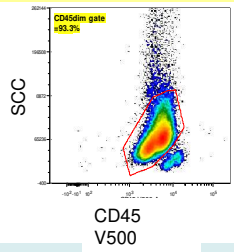
AML with inv(16) [*CBFB-MYH11*]

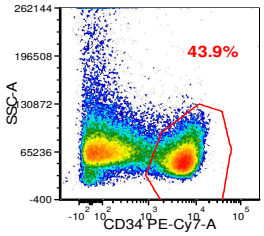
Patient was treated with induction chemotherapy (FLAG-IDA)

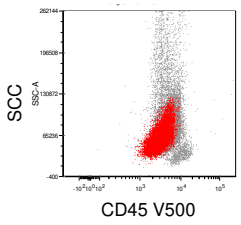
- BM 4% blasts and 6% monocytes

CASE ILLUSTRATION:
CASE 4: AML with myelomonocytic differentiation (M4)

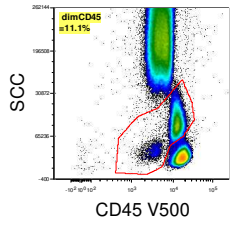
At the time of diagnosis

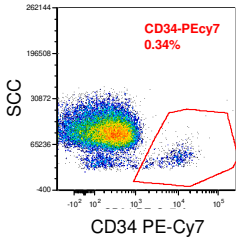


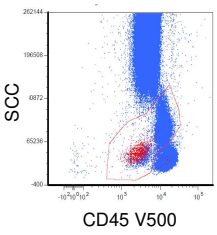




Post induction

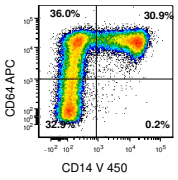


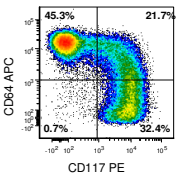


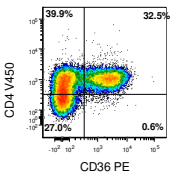


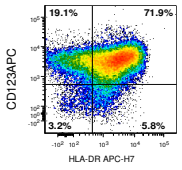
CASE ILLUSTRATION:
CASE 3: AML with myelomonocytic differentiation (M4)

At the time of diagnosis

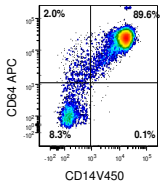


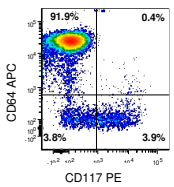


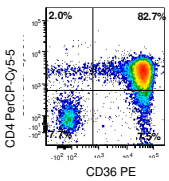


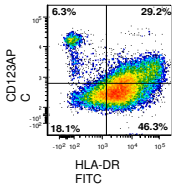


Post induction



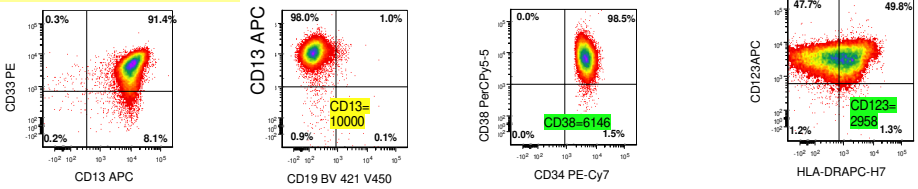




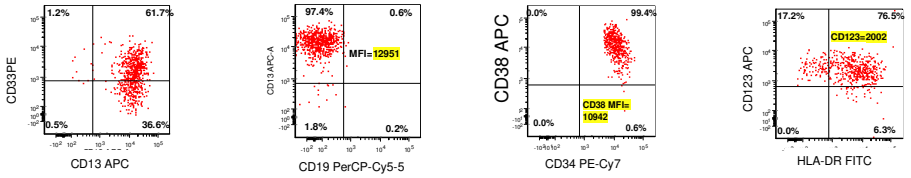


AML with myelomonocytic differentiation (M4)

At the time of diagnosis



Post induction



CASE ILLUSTRATION:
CASE 3: AML with myelomonocytic differentiation (M4)

Molecular cytogenetic correlation, supporting AML MRD

The percentage of *CBFB-MYH11* to *ABL1* determined by quantitative real-time RT-PCR is 1.28

FISH was positive for 5.5%

Questions?