Designing Multicolor Flow Cytometry Panels in Hematolymphoid Neoplasms

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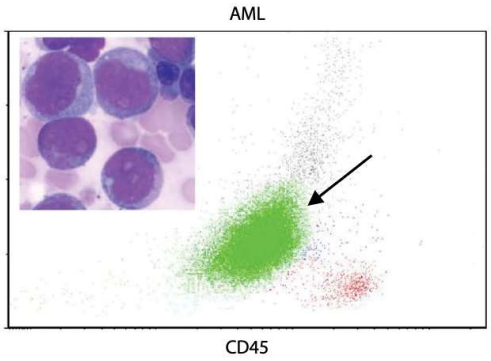
Acute Myeloid Leukemia

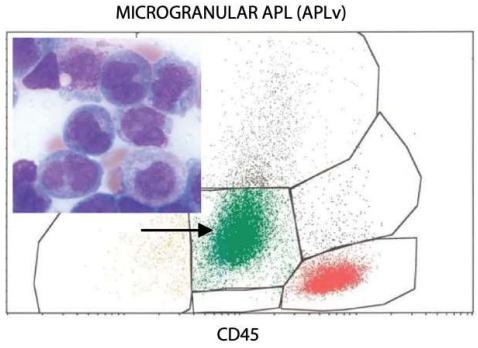
WHO classification of acute myeloid leukemia

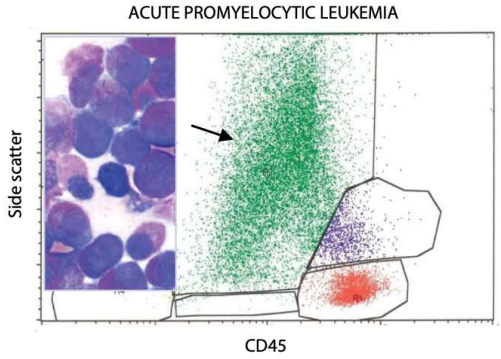
- AML with defining genetic abnormalities
- AML, myelodysplasia-related
- Therapy-related myeloid neoplasm
- AML, defined by differentiation

Acute Myeloid Leukemia

- Myeloid blasts in AML are usually CD45 intermediate, of about the same intensity as neutrophils, but with SSC low/intermediate, except for acute promyelocytic leukaemia (APL) that may show similar SSC to normal neutrophils.



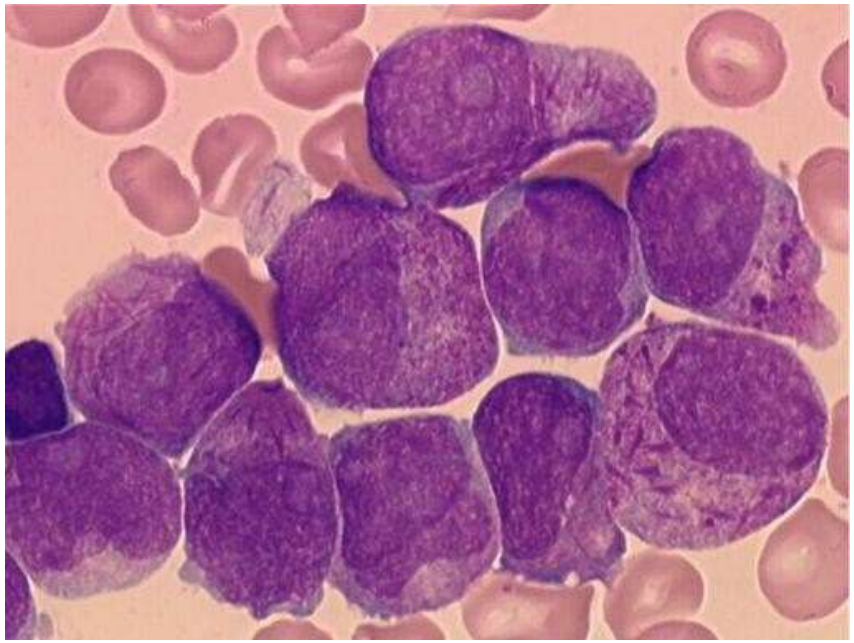




Current AML Classification Approach

Acute myeloid leukaemia with defining genetic abnormalities
Acute promyelocytic leukaemia with PML::RARA fusion
Acute myeloid leukaemia with RUNX1::RUNX1T1 fusion
Acute myeloid leukaemia with CBFB::MYH11 fusion
Acute myeloid leukaemia with DEK::NUP214 fusion
Acute myeloid leukaemia with BCR::ABL1 fusion
Acute myeloid leukaemia with KMT2A rearrangement
Acute myeloid leukaemia with MECOM rearrangement
Acute myeloid leukaemia with NUP98 rearrangement
Acute myeloid leukaemia with NPM1 mutation
Acute myeloid leukaemia with CEBPA mutation
Acute myeloid leukemia with myelodysplastic related
Acute myeloid leukaemia, defined by differentiation
Acute myeloid leukaemia with minimal differentiation Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute basophilic leukaemia
Acute myelomonocytic leukaemia Acute monocytic leukaemia
Acute erythroid leukaemia
Acute megakaryoblastic leukaemia

Acute Promyelocytic Leukemia (APL)



- The scattergram of classic APL is very characteristic, almost mimicking normal BM with slightly degranulated neutrophils on the CD45/SSC histogram

- this is easily ruled out by demonstrating the absence of expression of CD16 on these cells

Immunophenotypic Profile of Hypergranular APL

	Frequency (%)	Comments
Side scatter		High ("granulocytic" region on CD45 vs SSC)
$CD2^+$	17	
$CD4^+$	16	Dim expression
$CD7^+$	1.2	
$CD11b^+$	0	
$CD11c^+$	1.2	1 case (1.2%) showed dim expression; additional 5 cases (6%) showed dim expression on minor subset)
$CD13^+$	94	
$CD14^+$	0	
$CD16^+$	0	
$CD19^+$	0	
$CD33^+$	100	Moderate, 15%; bright 85%
$CD34^+$	14	Mostly dim or partial expression
$CD45^+$	100	Moderate expression
$CD56^+$	14	
$CD64^+$	61	Mostly dim expression
$CD117^+$	100	Moderate expression
$HLA\text{-}DR^+$	0	

Hypergranular APL

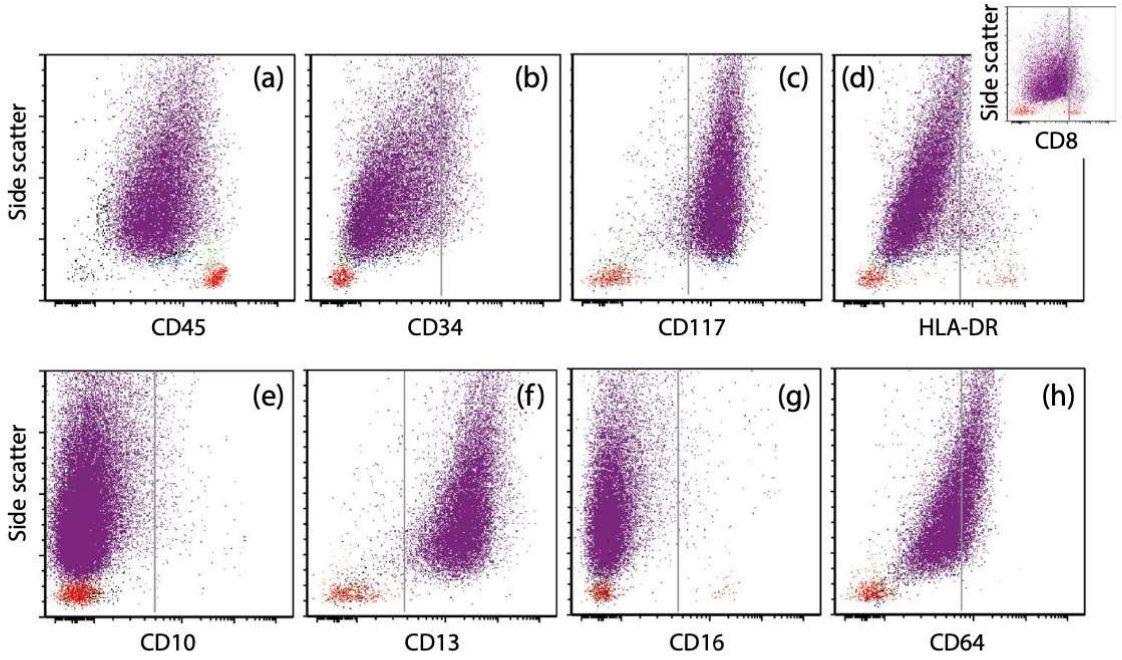


FIGURE 23.10 APL – flow cytometry of hypergranular variant (pattern 1). Neoplastic promyelocytes are characterized by very high SSC and moderate CD45 (a), negative CD34 (b), positive CD117 (c), negative HLA-DR (d; compare with negative staining with the same fluorochrome for CD8, inset), negative CD10 (e), positive CD13 (f), negative CD16 (g), and dim CD64 (h).

Hypergranular APL

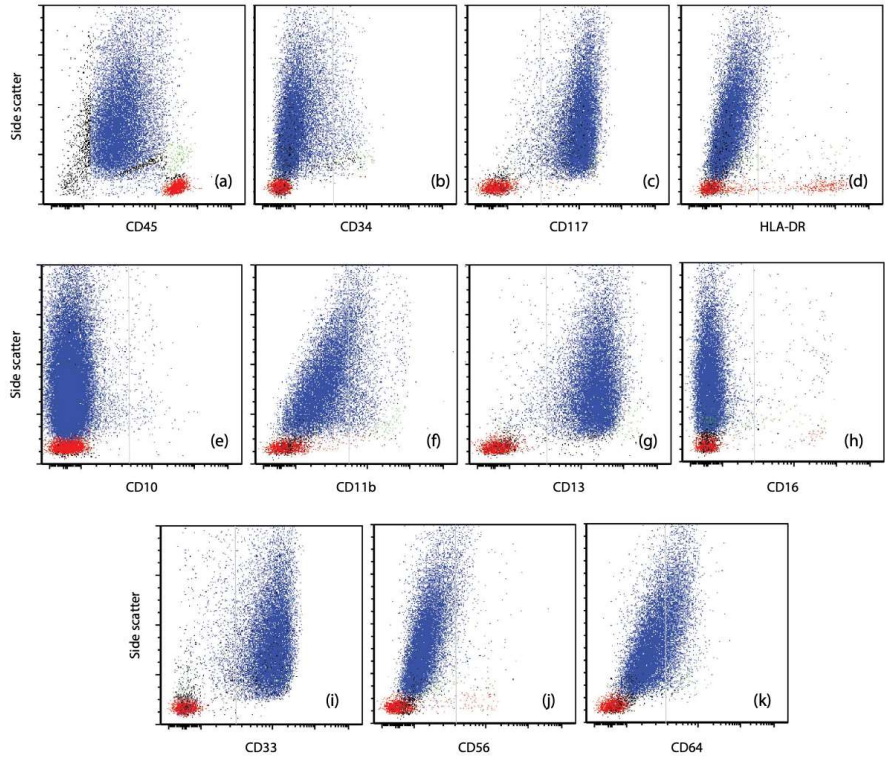
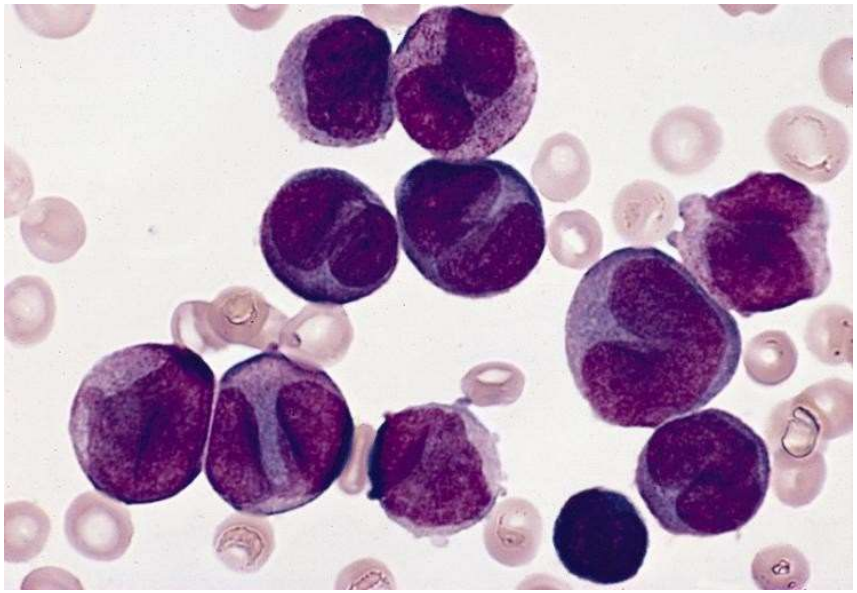


FIGURE 5.11 Acute promyelocytic leukemia (APL). Neoplastic promyelocytes (blue dots) are characterized by high side scatter (a–k; y axis), positive CD45 (a) negative CD34 (b), positive CD117 (c), negative HLA-DR (d), negative CD10 (e), negative CD11b (f), positive CD13 (g), negative CD16 (h), positive CD33 (i), negative CD56 (j), and negative to dimly positive CD64 (k). APL often show high non-specific "background" fluorescence and therefore the expression of specific markers need to be correlated with negative or "build-in" controls (e.g., with markers known to be negative such as CD3, CD8, CD19, CD20, etc.).

APL Variant

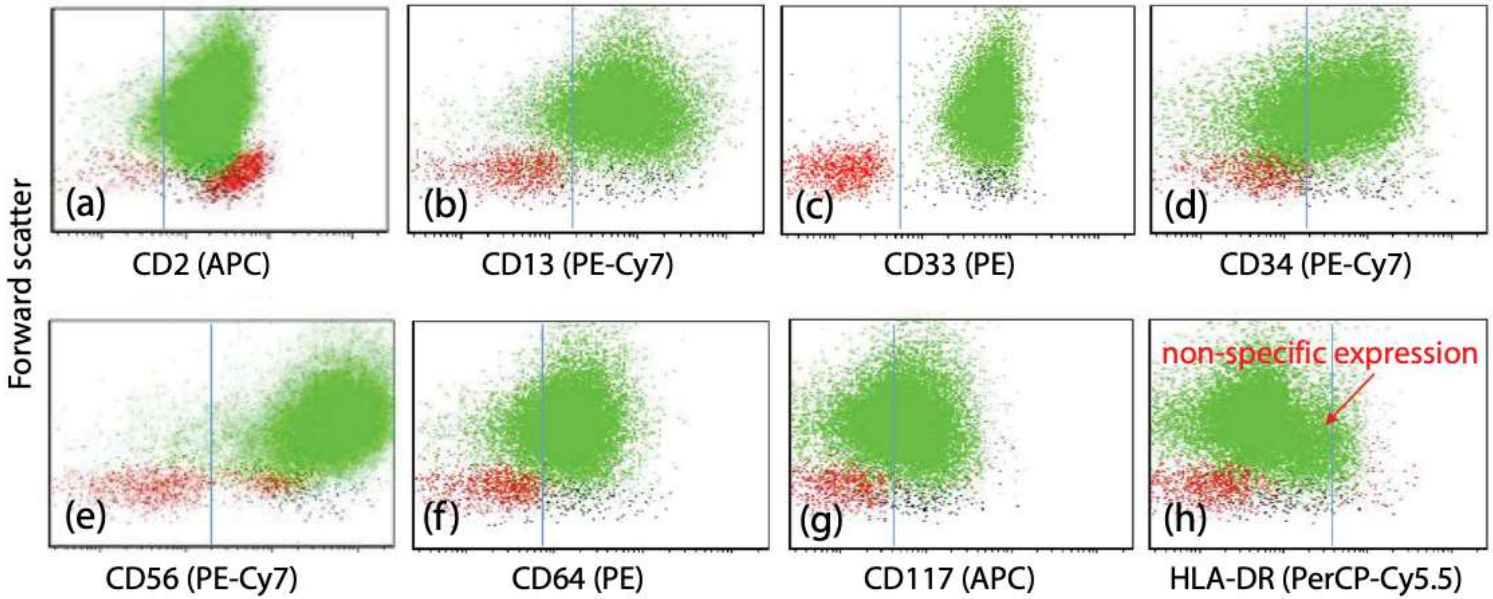


- The blasts display less granularity but could still be mistaken for granulocytes or monocytes.
- HLA-DR is absent on the blasts and CD34 is usually negative/weak. However, cases with intermediate or even strong CD34 and/or HLA-DR expression have been described.
- CD33, CD13 and MPO are strongly expressed.
- **Partial or more frank expression of CD2 can be observed in APL variant (APLv).**

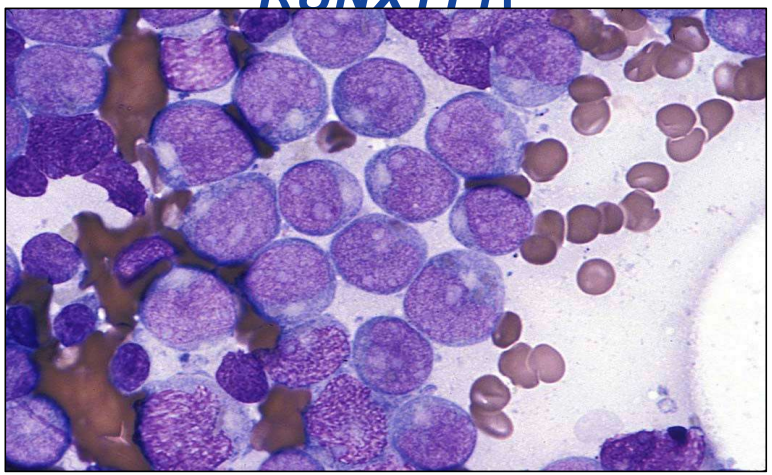
Immunophenotypic F
Hyporgranular APL

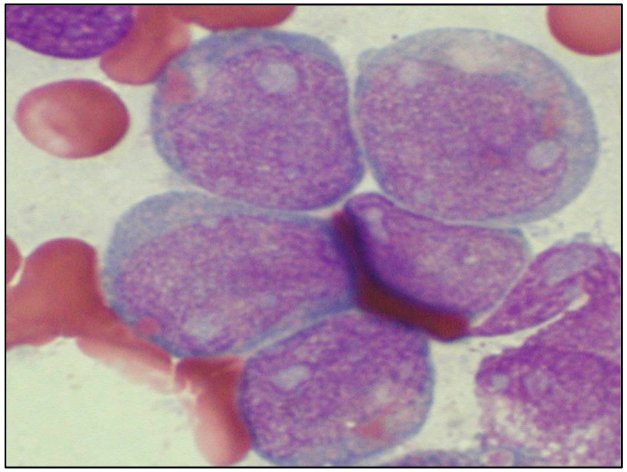
	Frequency (%)	Comments
Side scatter		Low ("blastic" region)
CD2+	79	Dim expression
CD4+	18	Dim expression
CD7+	0	
CD11b+	0	
CD11c+	0	
CD13+	94	Dim, 30%; moderate 65%; negative 6%
CD14+	0	
CD16+	0	
CD19+	0	
CD33+	100	Moderate expression, 10%; bright expression,90%
CD34+	76.5	8 cases (23.5%) were completely negative 3 cases showed dim expression on minor subset
CD45+	100	Moderate expression
CD56+	26	
CD64+	79	Mostly dim expression
CD117+	100	
HLA-DR+	0	

Hypogranular APL



Acute myeloid leukemia with t(8;21) [*RUNX1-RUNX1T1*]





- an example of a **core-binding factor (CBF)-**associated leukemia.

- The blasts express CD34 strongly and there is a maturation pathway towards CD34−/CD33++ monocytes
- **Very strong CD34 expression and CD19 co-expression are characteristic features of this leukemia**
- As in APL, **CD56** expression was reported as a negative prognostic feature in t(8;21) AML cases

- These morphologic and immunophenotypic features have a high correlation with t(8;21)(q22;q22.1)

Acute myeloid leukemia with t(8;21) [*RUNX1-RUNX1T1*]

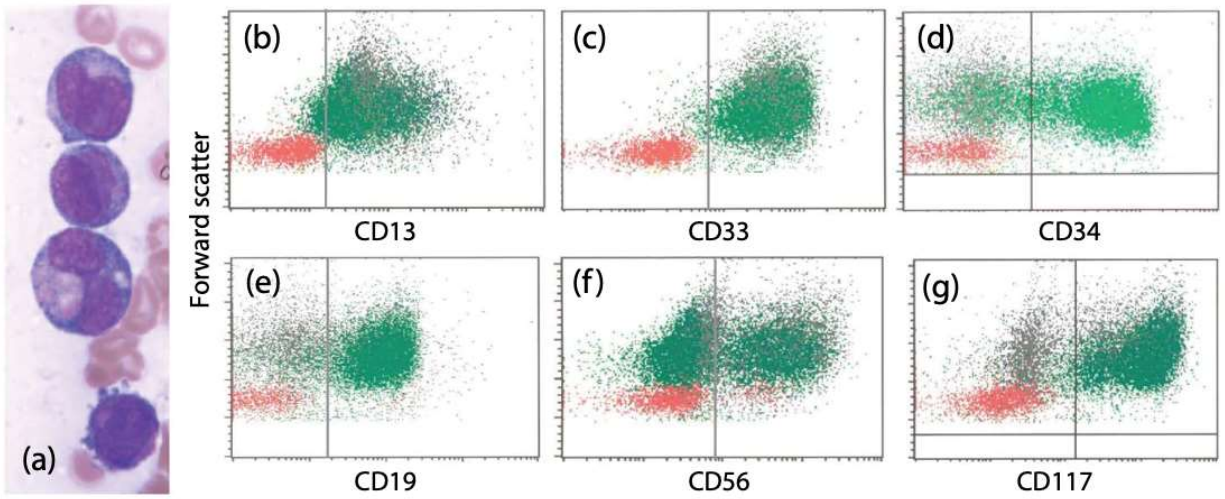


FIGURE 23.29 AML with t(8;21) – flow cytometry. Myeloblasts have granular cytoplasm (a). They are positive for CD13 (b; green dots), CD33 (c), CD34 (d), CD19 (e), CD56 (f), and CD117 (g).

Acute myeloid leukemia with t(8;21) [*RUNX1-RUNX1T1*]

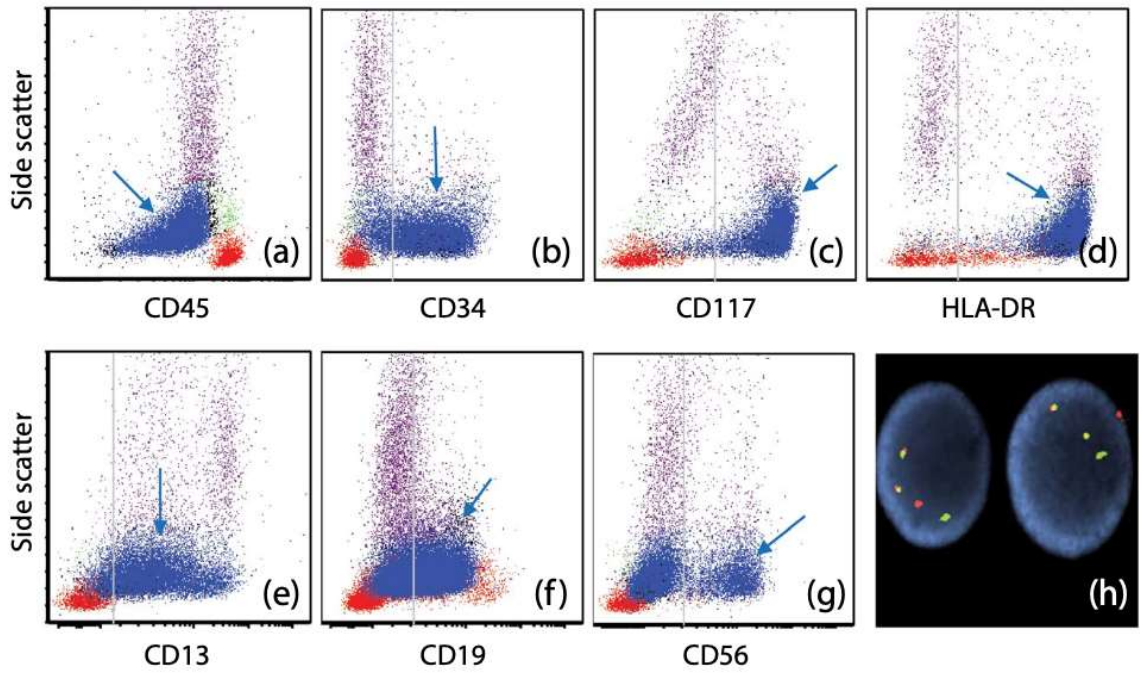
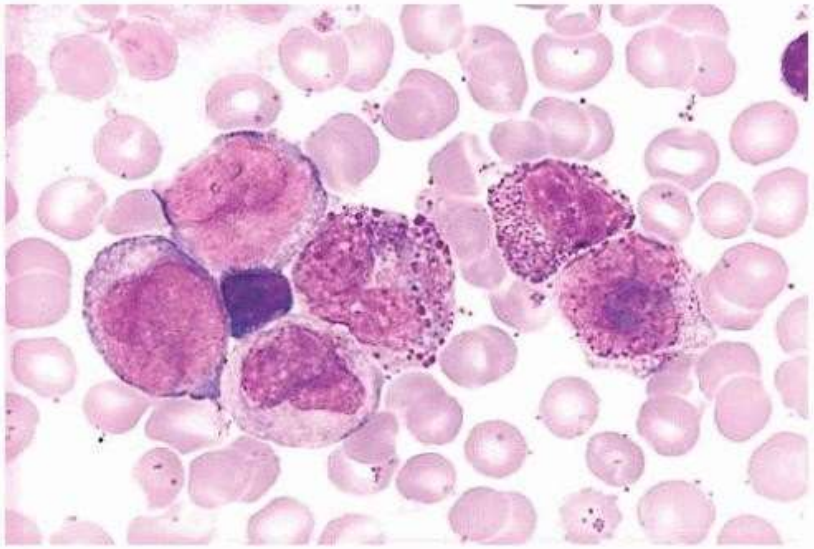


FIGURE 23.30 AML with t(8;21)/*RUNX1-RUNX1T1*. The flow cytometry pattern is often similar to AML with maturation. Blasts (blue dots; arrow) are positive for CD45 (a), CD34 (b), CD117 (c), HLA-DR (d), and CD13 (e). Blasts in AML with t(8;21)/*RUNX1-RUNX1T1* often show aberrant expression of CD19 (f) and CD56 (g; partial positivity). FISH studies (h) revealed fusion between *RUNX1-RUNX1T1* (yellow signal).

AML with *CBFB-MYH11*



- CBF-associated leukemia usually presenting with typical morphologic features of **myelomonocytic AML with eosinophilia.**
- Blasts co-express the immature markers CD34 and CD117
- There is also a monocytic compartment identified by the expression of CD14, absent from the majority of blasts.
- there is no specific immunophenotype for this disease

AML with *CBFB-MYH11*

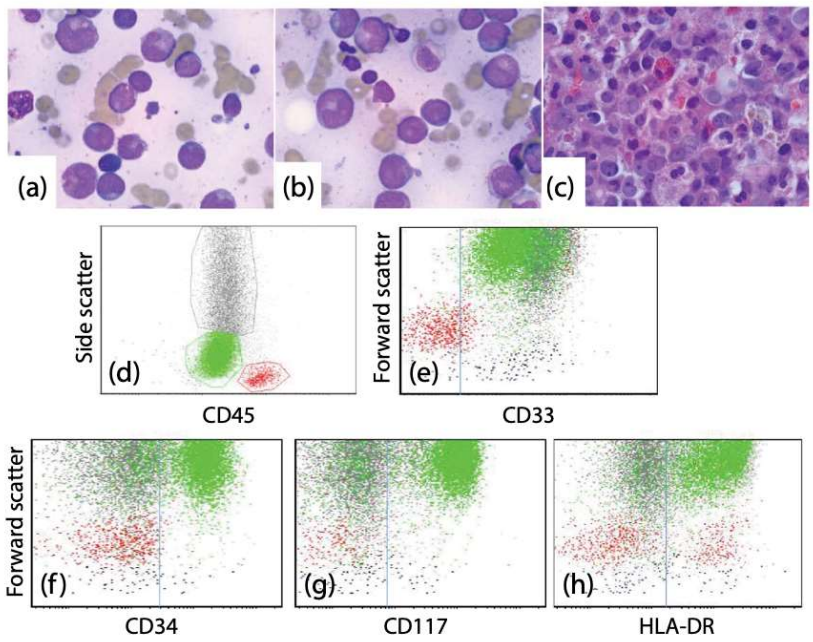


FIGURE 23.33 AML with *CBFB-MYH11.* Aspirate smear (a–b) shows large blasts with irregular nuclei and prominent cytoplasmic granules and occasional Auer rods (mimicking APL). BM core biopsy (c) shows hypercellular marrow with sheets of blasts and increased eosinophils. Flow cytometry analysis (d–h) shows blasts (green dots) with low SSC (d), dim CD33 (e), positive CD34 (f), CD117 (g), and HLA-DR (h).

AML with *CBFB-MYH11*

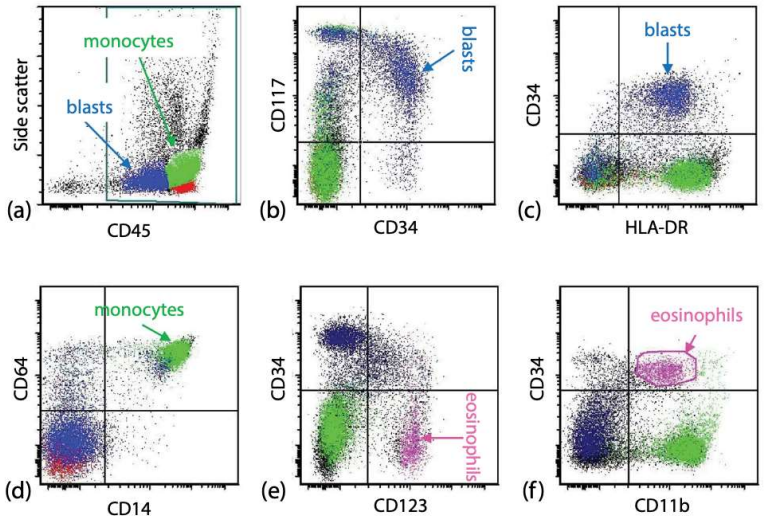
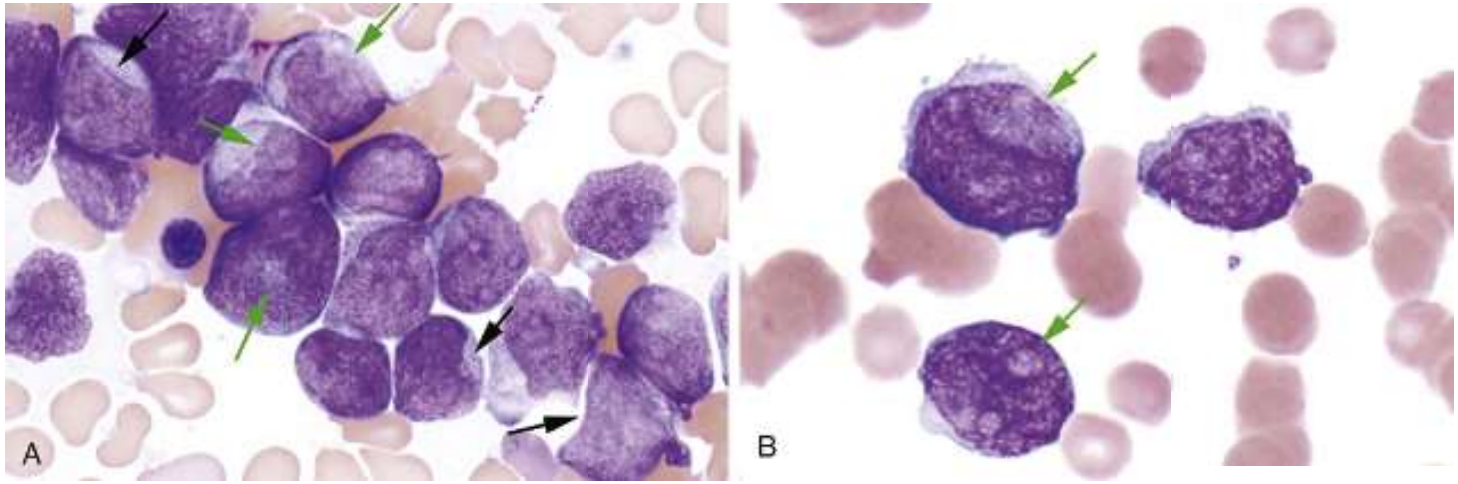


FIGURE 23.35 AML with *CBFB-MYH11* – flow cytometry. Flow cytometry analysis shows increased blasts (blue dots) and monocytes (green dots). In addition, maturing myeloid cells (gray dots) and atypical eosinophils (pink dots) are present. Blasts show dimmer CD45 when compared to monocytes (a); they are positive for CD34 (b), CD117 (b), and HLA-DR (c). Monocytes are negative for CD34 and CD117 (b) and are positive for HLA-DR (c; brighter expression than in blasts), CD14 (d), CD64 (d), and CD11b (f). Atypical (immature) eosinophils are positive for CD123 (e) and CD11b (f; dimmer expression than in monocytes).

AML with mutated *NPM1*

- the characteristic feature associated with NPM1 mutation, is that most of these cells **lack CD34 expression.**

- **This immunophenotype can raise the suspicion of APL, but APL cases do not show signs of monocytic differentiation**.
- CD110 was suggested as an antigen usually positive in NMP1 but negative in APL



AML with mutated *NPM1*

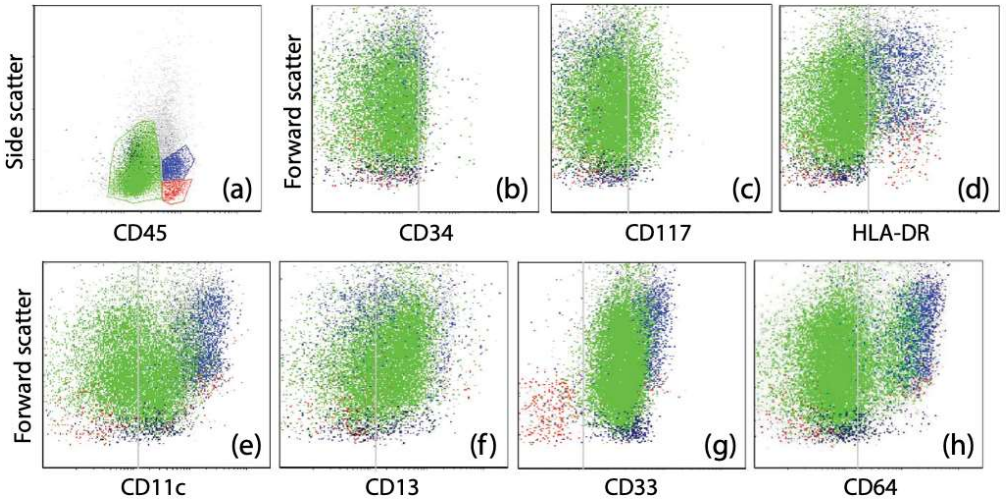


FIGURE 23.37 AML with *NPM1* mutation (hypogranular APL-like flow cytometry pattern). Blasts (green dots) show low SSC (a), negative CD34 (b), dim (partial) CD117 (c), negative HLA-DR (d), dim CD11c (e), dim CD33 (f), positive CD33 (g), and negative CD64 (h).

AML with mutated *NPM1*

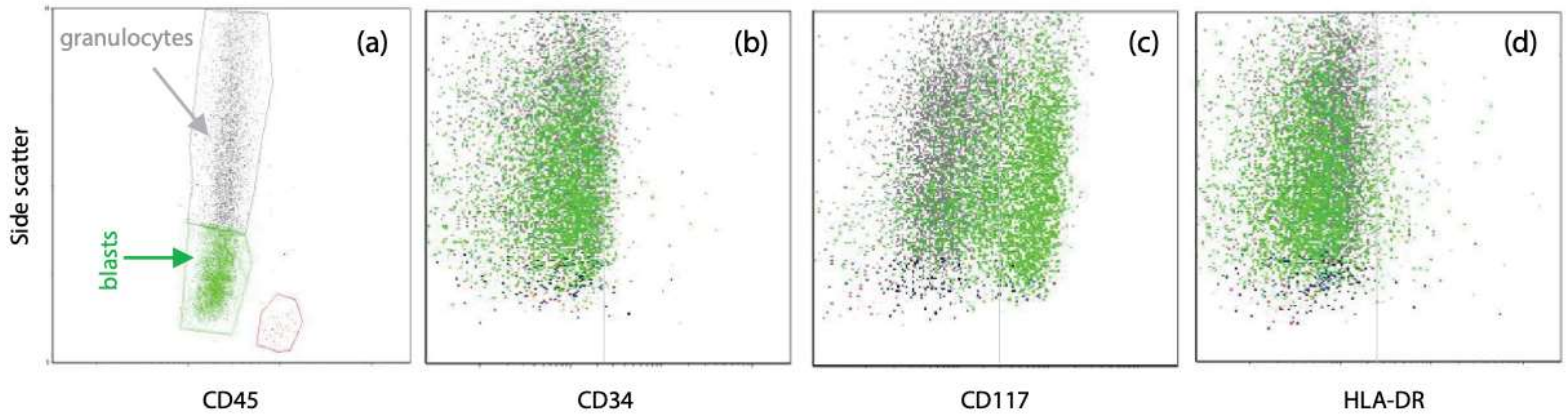


FIGURE 5.12 AML with *NPM1* mutations. Blasts (a–d; green dots) have low side scatter (a), moderate CD45 (a), negative CD34 (b), positive CD117 (c), and negative HLA-DR (d).

ACUTE MYELOID LEUKEMIA, DEFINED BY DIFFERENTIATION

- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelomonocytic leukemia (AMML)
- Acute monoblastic (monocytic) leukemia
- Acute erythroid leukemia (AEL)
- Acute megakaryoblastic leukemia

AML with Minimal Differentiation

- AML with minimal differentiation corresponds to previous AML M0, according to the FAB2 classification
- Blasts in minimally differentiated AML lack MPO and also lack markers that would classify the case as ALL or MPAL (no CD19 and no cyCD3).
- The blasts lack MPO expression .
- However, the presence of CD117 and CD13 on this population confirms its myeloid lineage.

AML with Minimal Differentiation

Phenotype of AML with minimal differentiation: s.CD3−, c.CD3−, CD4−, CD7+/−, CD11b−, CD11c−, CD13+, CD14−, CD15−, CD19−, c.CD22−, CD33+/−, CD34+, CD38+, CD36−, CD56−, CD64−, CD65−, c.CD79a−, CD117+, HLA-DR+, MPO−/few blasts+, TdT+/−

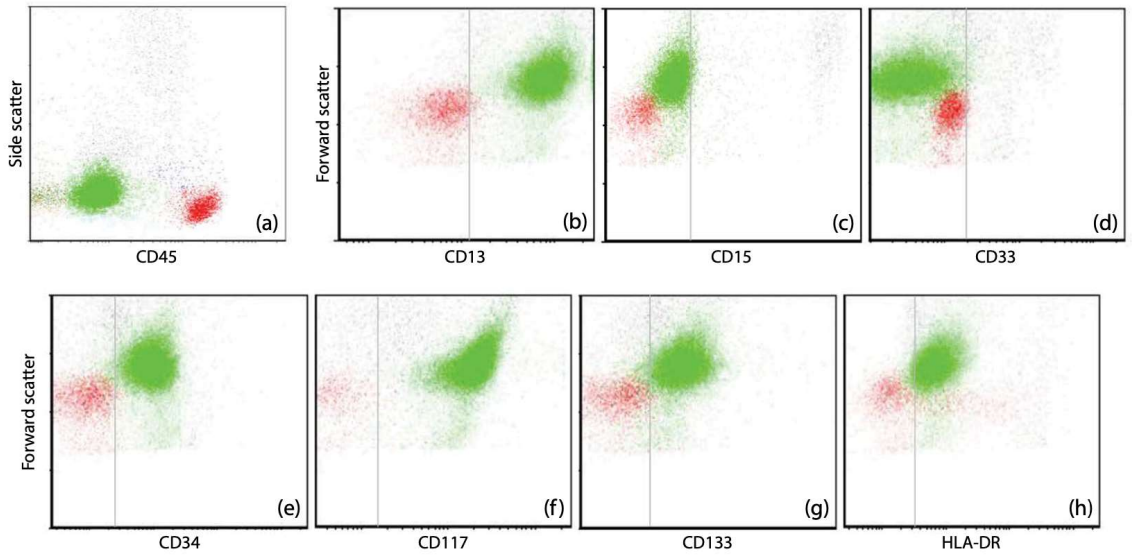
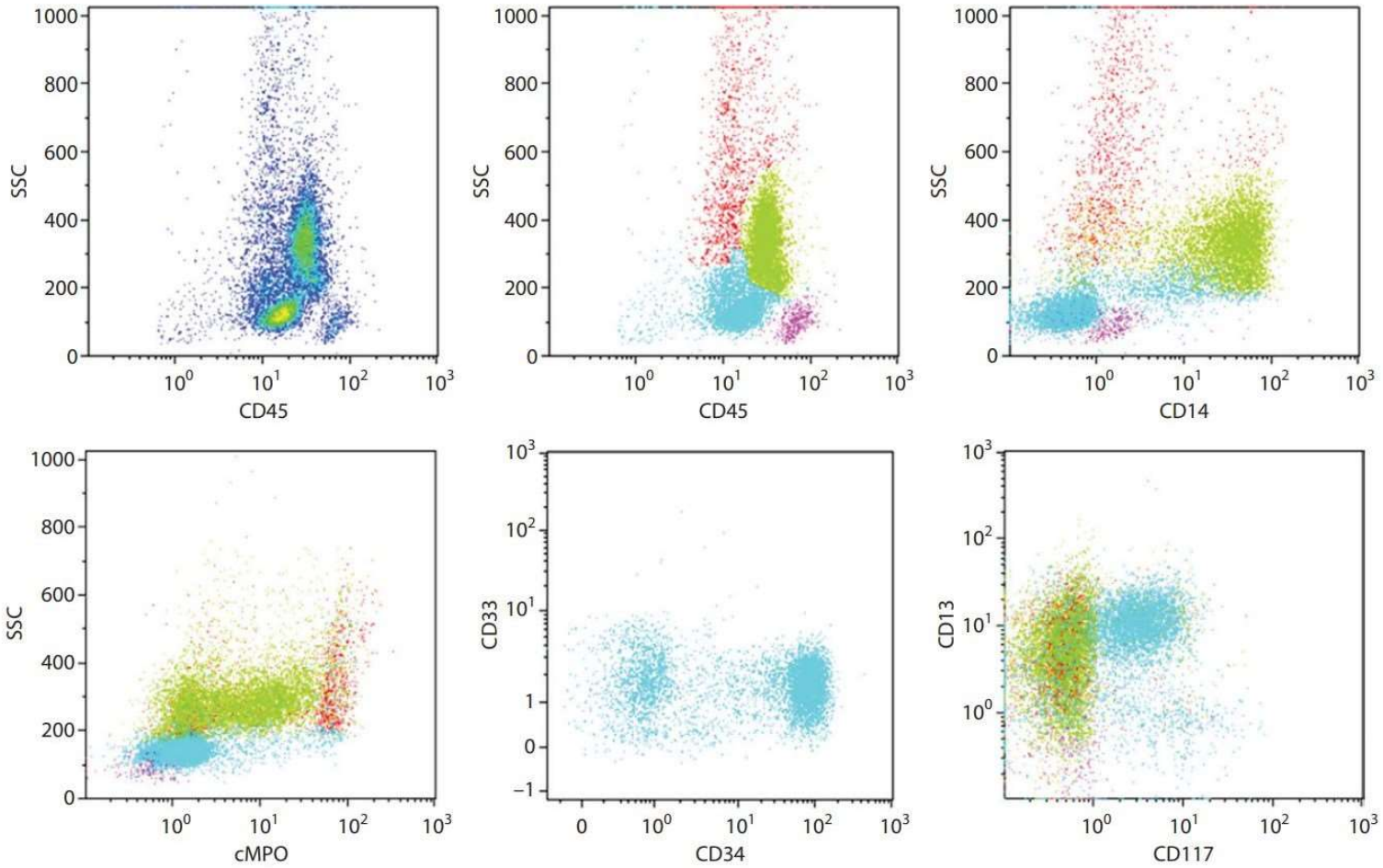


FIGURE 22.2 Minimally differentiated AML. Blasts (green dots) show the following phenotype: dim $CD45^{+}$ (a), $CD13^{+}$ (b), $CD15^{-}$ (c), $CD33^{-}$ (d), $CD34^{+}$ (e), $CD117^{+}$ (f), $CD133^{+}$ (g) and $HLA\text{-}DR^{+}$ (h).

AML with Minimal Differentiation



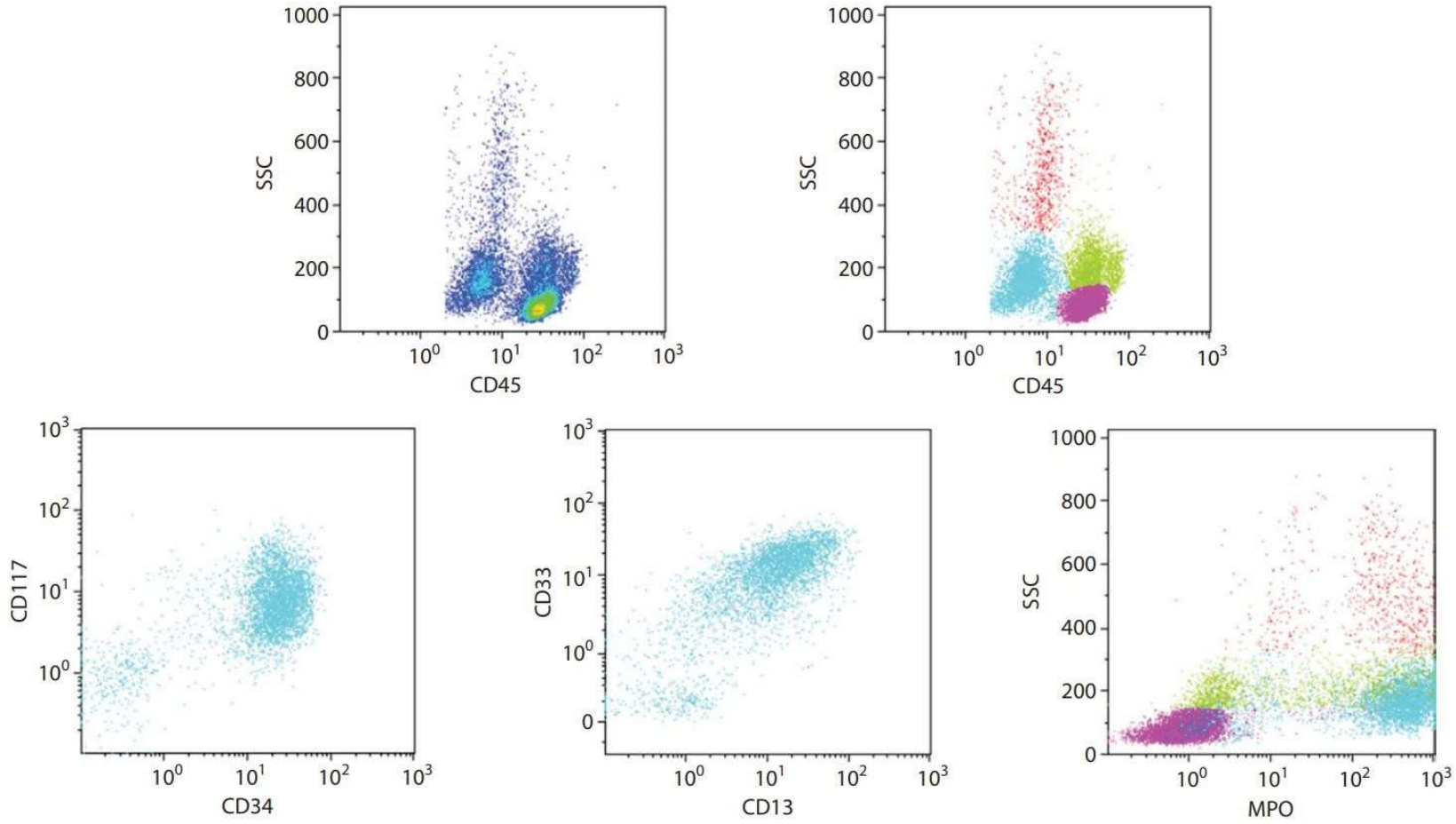
AML without Maturation

- AML without maturation corresponds to previous AML M1 in the FAB classification.
- In this example the blasts co-express the immature markers CD34 and CD117 as well as CD13 and CD33.
- They are also characterized by a strong expression of MPO.
- Other markers showed no evidence of differentiating granulocytic or monocytic populations.

AML without Maturation

Phenotype of AML without maturation: CD2−/rarely+, s.CD3−, c.CD3−, CD4−/+, CD7+/−, CD11b−/rarely+, CD13+, CD14−, CD15−, CD19−/rarely+, c.CD22−, CD33+/−, CD34+/rarely−, CD38+/rarely−, CD36−, CD64−, CD65−, c.CD79a−, CD117+, CD133+/−, HLA-DR+/rarely−, MPO blasts+, TdT+/−

AML without Maturation



AML without Maturation

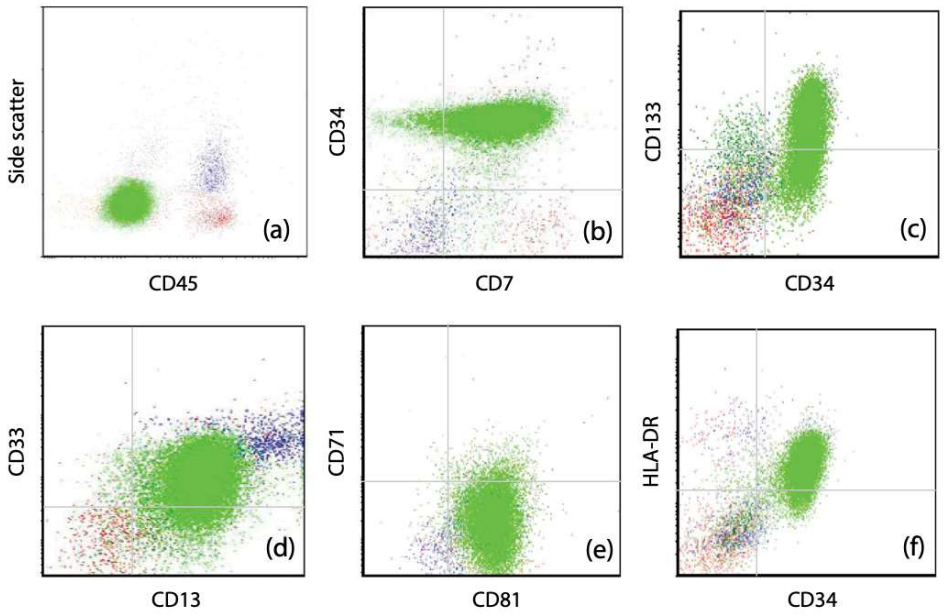


FIGURE 5.13 AML without maturation. Blasts (green dots) are characterized by low side scatter (a), dim CD45 expression (a), positive CD34 (b–c), aberrant expression of CD7 (b), positive CD133 (c), positive CD13 and CD33 (d), negative CD71 (e), dim expression of CD81 (e), and positive HLA-DR (f).

AML with Maturation

- AML with maturation corresponds to the previous AML M2 category in the FAB classification.
- Blasts occupy the whole 'bermudes' area, here, with a CD45 intensity slightly lower than that of neutrophils and SSC low.
- They co-express the immature markers CD34 and CD117 as well as CD13 and CD33.
- Note the CD13 bright/CD33 dim expression of remaining mature neutrophils.
- Here, the four major markers CD34, CD117, CD33 and CD13 are co-expressed.
- However, a lack of CD34 and/or any other combination of these four markers can be observed in almost 50% of AML cases.
- CD64 and CD36 showed no evidence for any population with monocytic differentiation

AML with Maturation

Phenotype of AML with maturation: CD2−/rarely+, s.CD3−, c.CD3−, CD4−/+, CD7+/−, CD11b−/rarely+, CD11c−/+, CD13+, CD14−, CD15−/rarely+, CD19−/rarely+, c.CD22−, CD33+/−, CD34+/rarely−, CD38+/rarely−, CD36−, CD56−/rarely+, CD64−, CD65−/rarely+, c.CD79a−, CD117+/rarely−, CD133+/−, HLA-DR+/rarely−, MPO+, TdT−/+

AML with Maturation

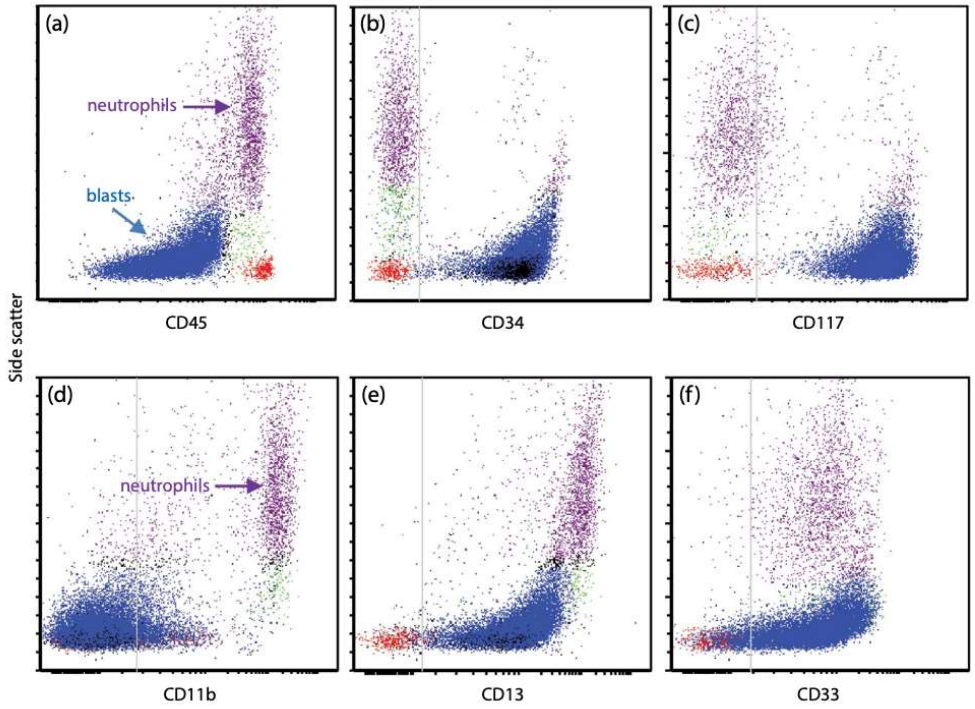


FIGURE 22.7 AML with maturation. Blasts (blue dots) show dim CD45 (a), positive CD34 (b), positive CD117 (c), negative CD11b (d), and positive CD13 (e) and CD33 (f). Granulocytes (purple dots) are strongly positive for CD11b (d) and do not express CD34 or CD117.

AML with Maturation

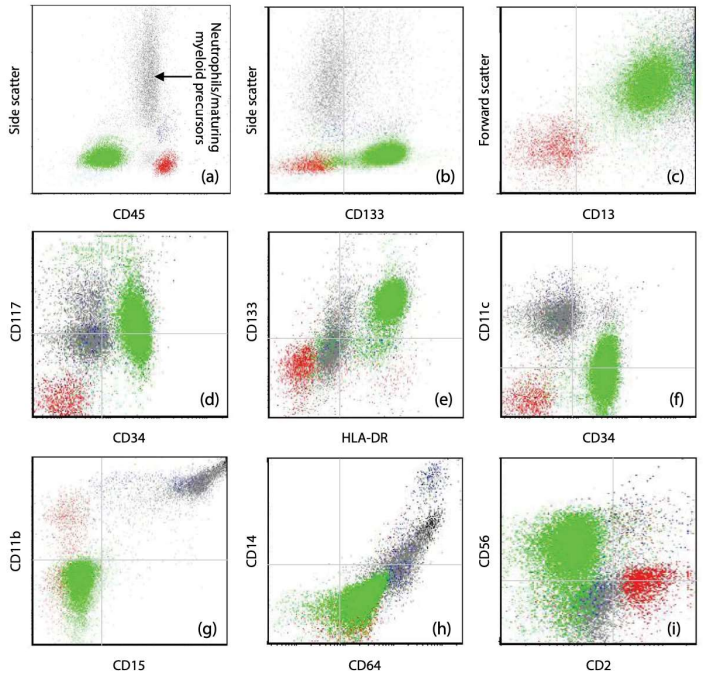


FIGURE 5.14 AML with maturation. Blasts (green dots) show dim expression of CD45 (a), low side scatter (a–b) and high forward scatter (c). Note the presence of maturing myeloid precursors and neutrophils (a, grey dots, arrow). Blasts display the following phenotype: CD133^{+} (b), CD13^{+} (c), CD34^{+} (d), CD117^{dim+} (d), HLA-DR^{+} (e), partially CD11c^{+} (f), CD11b^{-} (g), CD15^{-} (g), CD14^{-} (h), CD64^{-} (h), and CD56^{+} (i).

Acute myelomonocytic leukemia (AMML)

Phenotype of AMML

Myeloblastic component: CD2−/rarely+, s.CD3−, c.CD3−, CD4−/+, CD7+/−, CD11b−/rarely+, CD11c−/+, CD13+, CD14−, CD15−/+, CD19−/rarely+, c.CD22−, CD33+/−, CD34+/rarely−, CD38+/rarely−, CD56−/rarely+, CD64−, CD65−/+, c.CD79a−, CD117+/rarely−, CD133+/−, HLA-DR+/rarely−, MPO+, TdT−/+

Monocytic component: CD2+/−, CD4+, CD11b+, CD11c+, CD14+, CD15+, CD34−, CD36+, CD56−/+, CD64+, CD117−, HLA-DR+

Acute myelomonocytic leukemia (AMML)

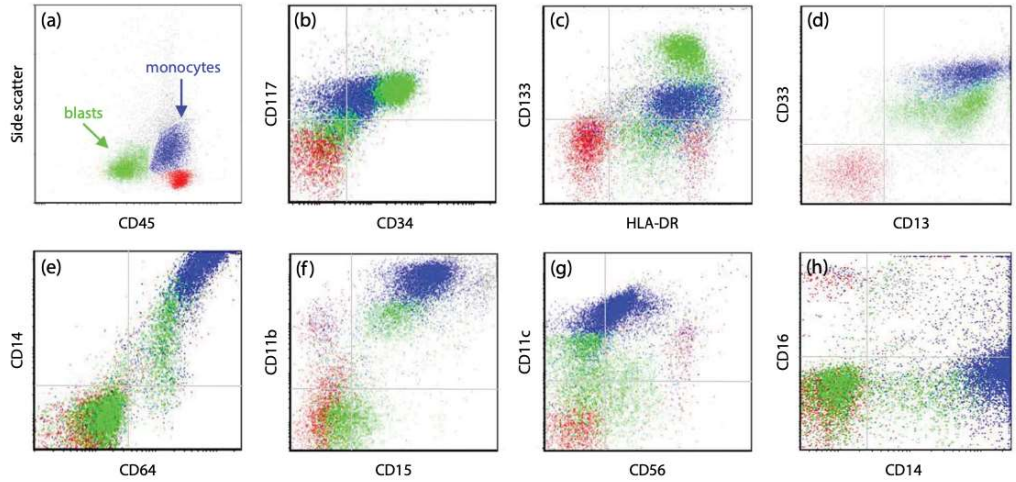


FIGURE 22.12 AMML is characterized by presence of blasts (≥20%) and monocytes (≥20%), Blasts (green dots) have moderate CD45 (a) and low side scatter (a), and monocytes (blue dots) have bright CD45 and minimally increased side scatter (a). Blasts show the following phenotype: $CD34^+$ (b), $CD117^+$ (b), $CD133^+$ (c), $HLA\text{-}DR^+$ (c), $CD13^+/CD33^+$ (d), $CD14^-/CD64^-$ (e), $CD11b^-/CD15^-$ (f), CD11c $dimly^+$ (g), and $CD14^-/CD16^-$ (h). Monocytes show the following phenotype: CD34-/CD117+/$CD133^+$ (b-c), $HLA\text{-}DR^+$ (c), $CD13^+/CD33^+$ (d), $CD14^+/CD64^+$ (e), $CD11b^+/CD15^+$ (f), CD11c $bright^+$ (g), and $CD14^+/CD16^-$ (h).

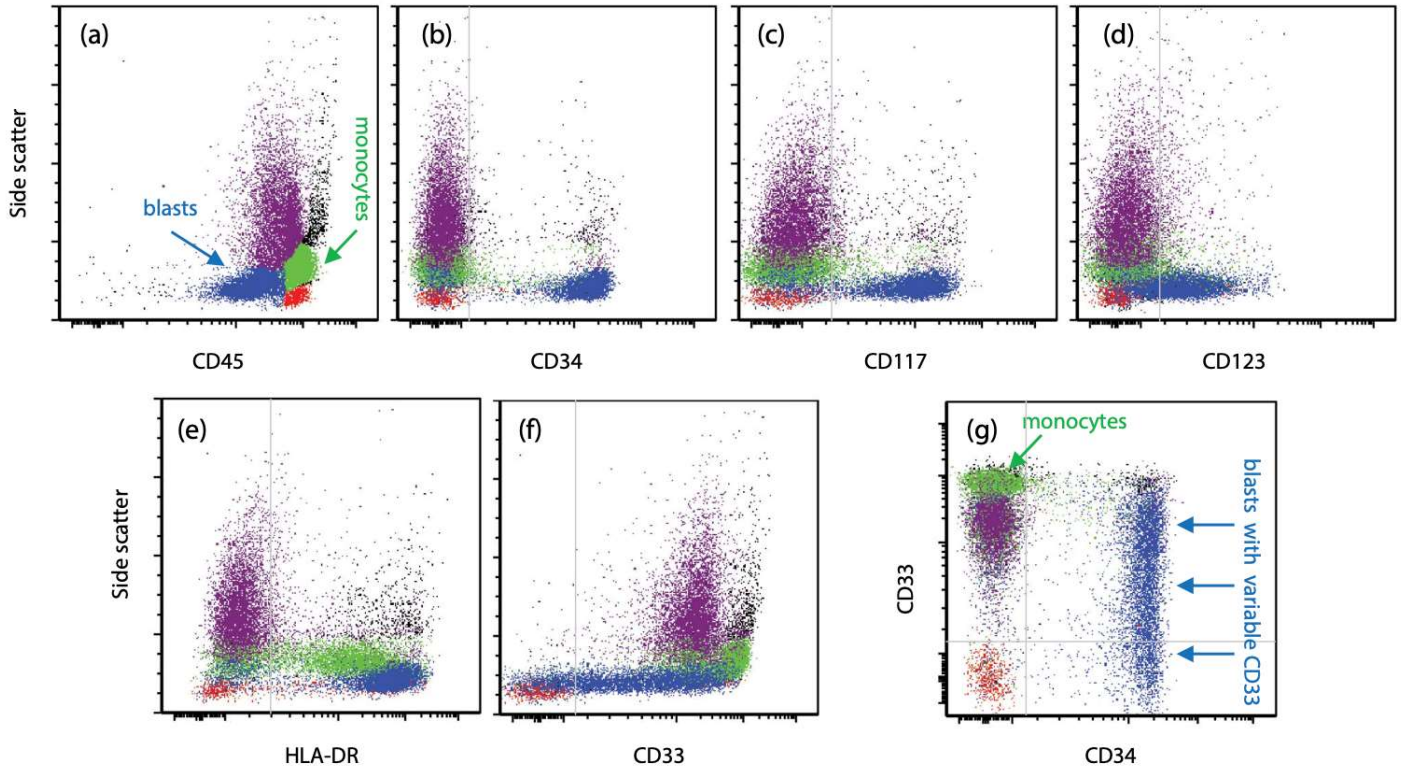
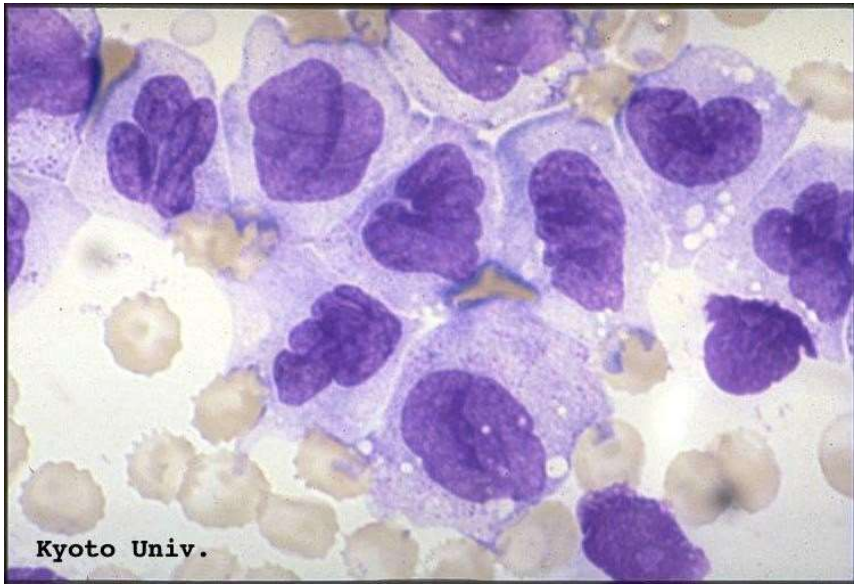


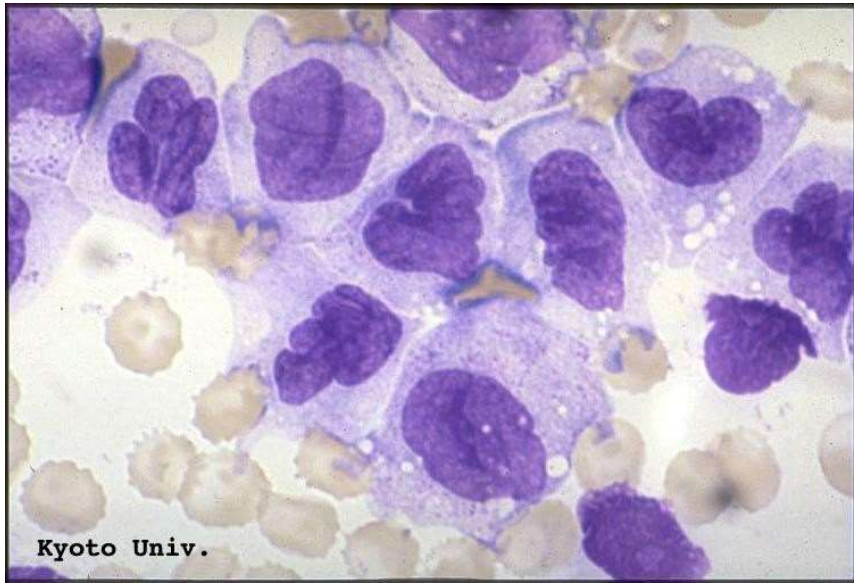
FIGURE 5.17 Acute myelomonocytic leukemia. Flow cytometry analysis shows blasts (≥20%; blue dots), monocytes (green dots), and granulocytes (purple dots). Blasts have low side scatter and dim to moderate CD45 (a), positive CD34 (b), CD117, (c), HLA-DR (d), and CD33 (e–f; aberrant variable expression). Monocytes have bright CD45 (a), positive HLA-DR (e), and bright CD33 (e–f).

Acute monoblastic (monocytic) leukemia

Phenotype of acute monoblastic (monocytic) leukemia: CD2−/+, s.CD3−, c.CD3−, CD4+, CD7+/−, CD11b+(variable)/rarely−, CD11c+, CD13+, CD14−/+(variable), CD15+, CD19−, c.CD22−, CD33+, CD34−/rarely+, CD38+/rarely−, CD36+, CD56+/rarely−, CD64+, CD56+/-, c.CD79a−, CD117−/rarely+, CD133−, HLA-DR+/rarely−, MPO−/rarely+



AML with Monocytic Differentiation



- AML with monocytic differentiation corresponds to the previous FAB AML M5a and M5b categories.
- The blasts in the illustrated case show a strong CD45 positivity and higher granularity than seen in undifferentiated cases.
- They **express CD14 with intermediate** intensity but are **strongly positive for CD33**.
- In general, monoblastic leukemia is characterized by variable CD34 and CD117 expression together with CD64, CD36, HLA-DR and CD33bright, while more differentiated monocytic leukemias express CD11c, CD14, CD35 and/or CD303 but may be negative for CD34 and CD117
- **MPO is negative or weakly** expressed in a fraction of cells.
- **CD56 is often aberrantly expressed in leukemias with monocytic differentiation**

Acute monoblastic leukemia

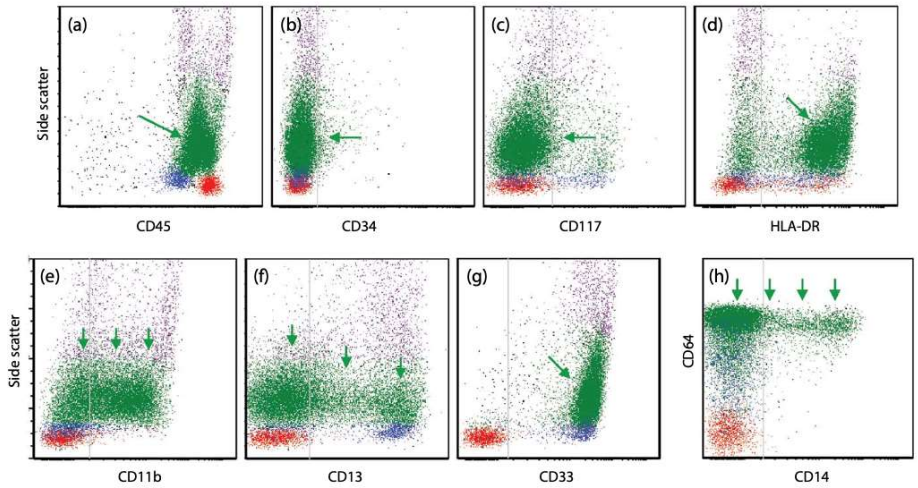


FIGURE 22.20 Acute monoblastic leukemia – flow cytometry. Monoblasts (green dots, arrow) show bright expression of CD45 and slightly increased side scatter (a). CD34 and CD117 are not expressed (b–c). HLA-DR is positive (d) and both CD33 and CD64 are brightly expressed (g–h). There is aberrant expression of CD11b (e: variable with subset negative and dimly positive), CD13 (f: mostly negative, only minor population is positive) and CD14 (h: mostly negative with only minute population showing variable expression).

Acute monoblastic leukemia

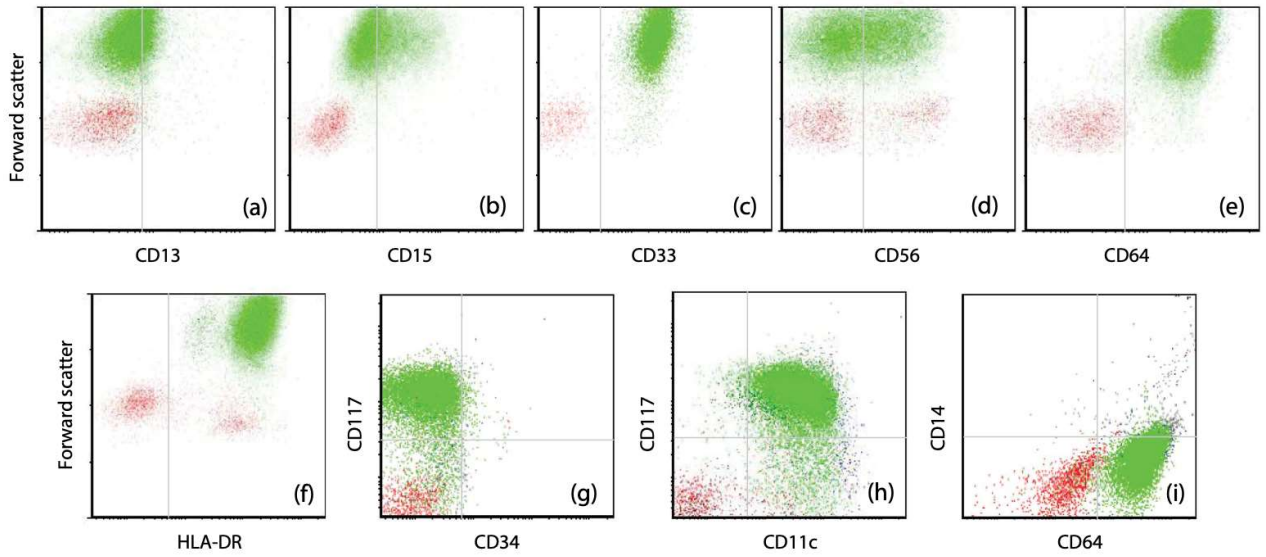


FIGURE 5.18 Acute monoblastic leukemia. Monoblasts (green dots) are characterized by high forward scatter (a–f), aberrant loss of CD13 (a), partially positive CD15 (b), bright expression of CD33 (c), partially positive CD56 (d), bright expression of CD64 (e), positive HLA-DR (f), negative CD34 (g), positive CD117 (g–h), positive CD11c (h), and aberrant loss of CD14 (i).

Acute monoblastic leukemia

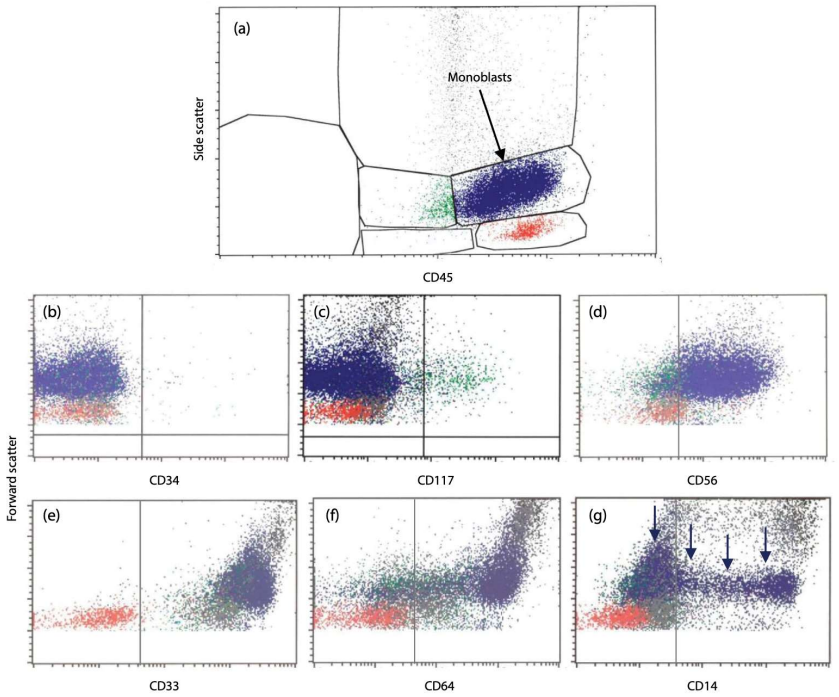
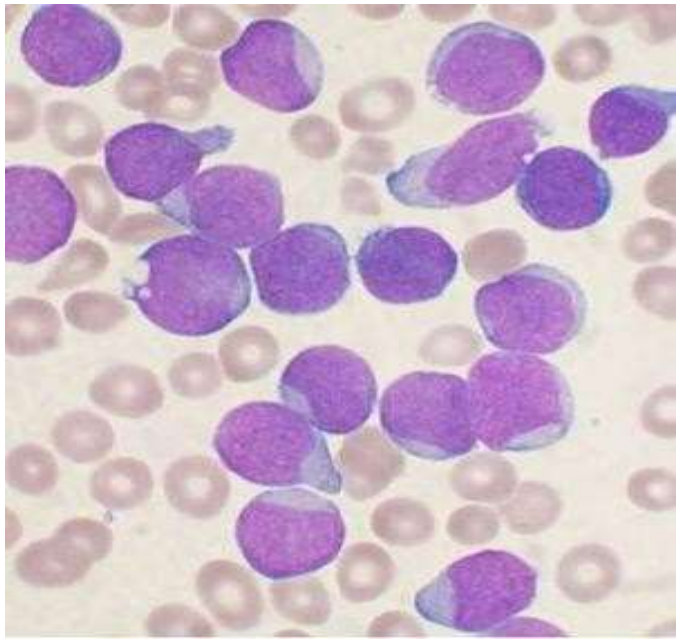


FIGURE 5.19 Acute monoblastic leukemia: monoblasts are CD45^{+} (a), CD34^{-} (b), CD117^{-} (c), CD56^{+} (d), CD33^{+} (e), and CD64^{+} (f). CD14 is variably expressed (g; arrows) with many blasts being negative.

TABLE 22.1: Immunophenotypic Profile of Acute Monocytic (Monoblastic) Leukemia ($n = 94$)

Marker	Frequency	Comments
$CD2^+$	34%	Usually dim or partial expression
$CD4^+$	93%	Usually dim expression
$CD7^+$	2%	
$CD10^+$	3%	
$CD11b^+$	92%	Including bright expression in 29.9%; moderate in 29.9%; dim in 8%, variable in 17.2%, and partial in 6.9%
$CD11c^+$	99%	Including bright expression in 64.4%; moderate in 26.4%; dim in 3.4%, variable in 2.3%, and partial in 2.3%,
$CD13^+$	78%	Including bright expression in 4.6%; moderate in 24.1%; dim in 14.9%, variable in 16.1%, and partial in 17.2%,
$CD14^+$	55%	Including bright expression in 14.9%; moderate in 3.5%; dim in 3.5%, variable in 19.5%, and partial in 12.6%,
$CD16^+$	8%	
$CD19^+$	0	
$CD23^+$	15%	
$CD33^+$	100%	Including bright expression in 73.6%, moderate in 24.1%, and variable in 2.3%,
$CD34^+$	13%	May be partial expression
$CD45^+$	99%	Predominantly bright or moderate expression
$CD56^+$	68%	
$CD64^+$	99%	Mostly bright expression, rarely moderate or dim
$CD117^+$	23%	May be partial expression
$CD123^+$	28%	
HLA-DR^+	89%	

Acute Lymphoblastic Leukemia



- It is important to be aware that lymphoid blasts in ALL can express CD45 at variable levels and may also be CD45−.

- In case of ALL suspicion, the blasts must therefore be looked for using CD19 versus SSC or CD7 versus SSC plots since they can be seen all along the CD45/SSClow region when gated on viable cells after excluding debris.

- The diagnosis of ALL by FCM should follow three specific steps:

1. identification of the B- or T- origin
2. characterization of specific subtypes
3. evaluation of immunophenotypic profiles associated with recurrent genetic abnormalities

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

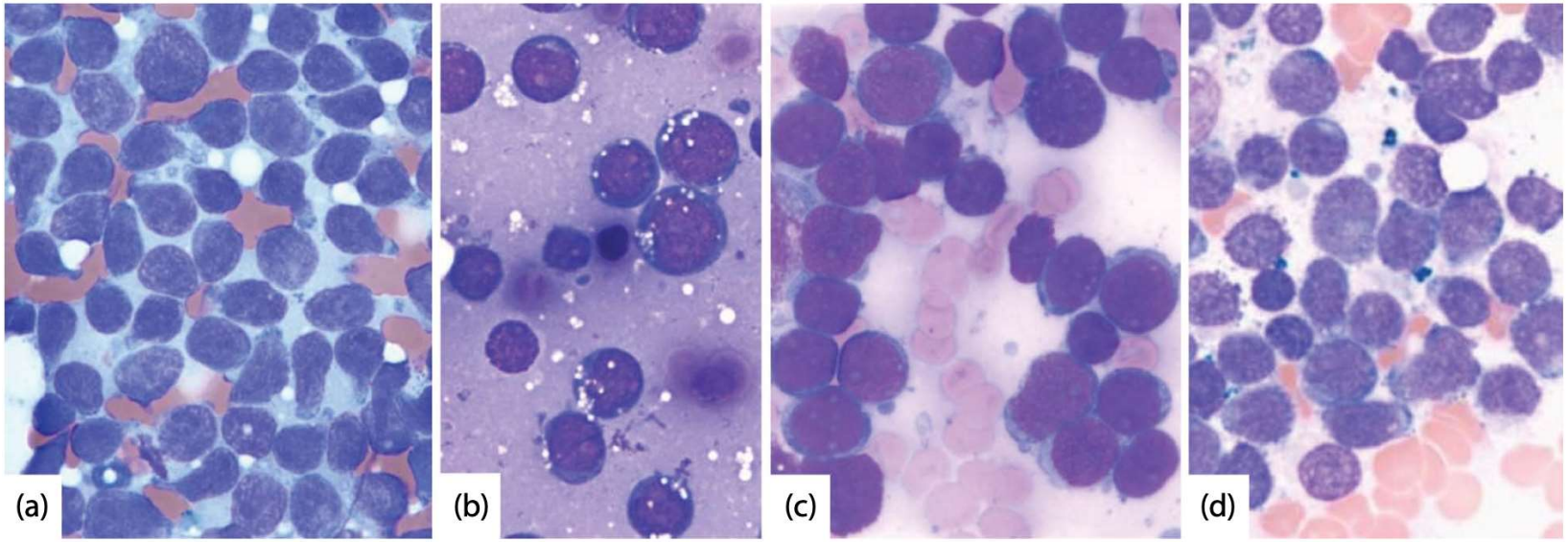


FIGURE 17.1 B-lymphoblastic leukemia – cytology. BM aspirate smears from four B-ALL cases (a–d) show lymphoblasts with increased nuclear-cytoplasmic ratio, scanty cytoplasm, nucleoli, and occasional cytoplasmic vacuoles (Wright-Giemsa, ×1000).

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

B-ALL can be subdivided into following categories (stages of maturation):

•**Pro-B-ALL**: CD10−, CD19+, TdT+, CD45+dim
•**Common ALL**: CD10+ , CD19+, CD34+, TdT+
•**Pre-B-ALL**: CD10+(dim)/−; cytoplasmic (c) IgM+; CD34+/−, TdT+/−, CD45+moderate /CD20+
•**Mature B-ALL**: surface IgM+, CD34− (maybe positive), TdT− (maybe positive)
• **Philadelphia chromosome+ B-ALL**: bright CD10+, CD11b+/−, CD13+ (dim)/−, CD15+/−, CD19+, CD20− (rarely dimly+), CD22+, CD25+, CD33+dim (maybe negative), CD34+/− (maybe partial), CD38+/−, CD45+(dim)/−, CD66c+, and TdT+/−

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

- The expression of CD10 is most often bright, which is helpful in differential diagnosis from mature CD10+ B-cell neoplasms (which usually show dim or moderate expression).
- The expression of CD38 is either dim or moderate.

- The expression of CD81 is usually dim and CD200 may be either positive or negative. Subset of B-ALL may display expression of CD123. Minor subset of B-ALL cases is negative for CD34 (~29%) or TdT (~15%), and very rare cases (~5%) lack both TdT and CD34.
- CD20 expression has adverse prognostic significance in ALL, in both Philadelphia− and Philadelphia+ cases, but its significance is limited mostly to younger patients

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

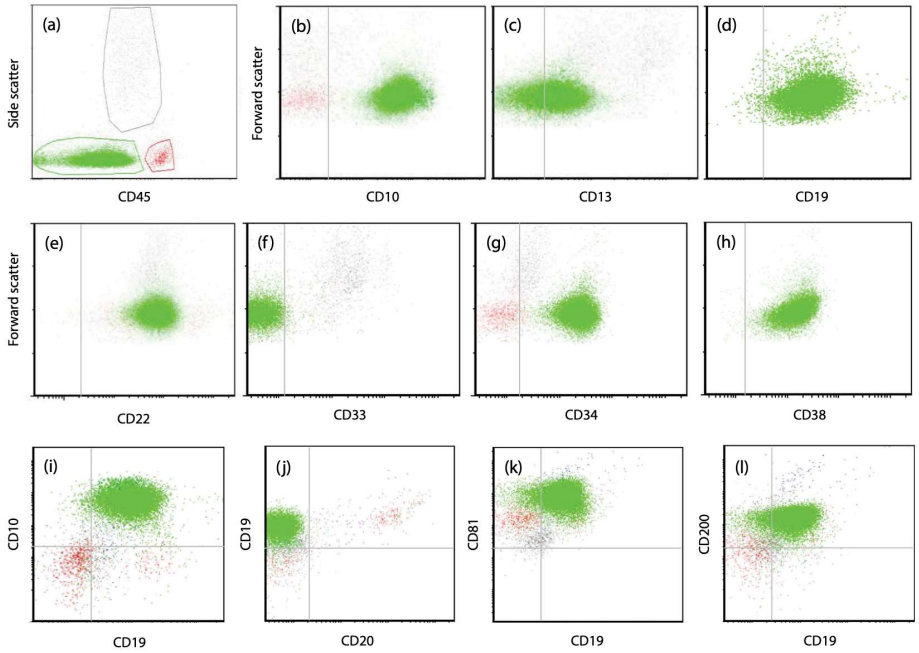


FIGURE 17.4 B-ALL – flow cytometry (bone marrow from 5-year-old patient). Blasts (green dots) have the following phenotype: CD45-/dim^{+} (a), CD10^{+} (bright; b), CD13^{+} (dim/partial; c), CD19^{+} (d), CD22^{+} (e), CD33^{-} (f), CD34^{+} (g), and CD38^{+} (h). Note typical for B-ALL bright expression of CD10 (i) and negative CD20 (j). Majority of B-ALL are positive for CD81 (k). The expression of CD200 varies, ranging from positive (l) to negative.

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

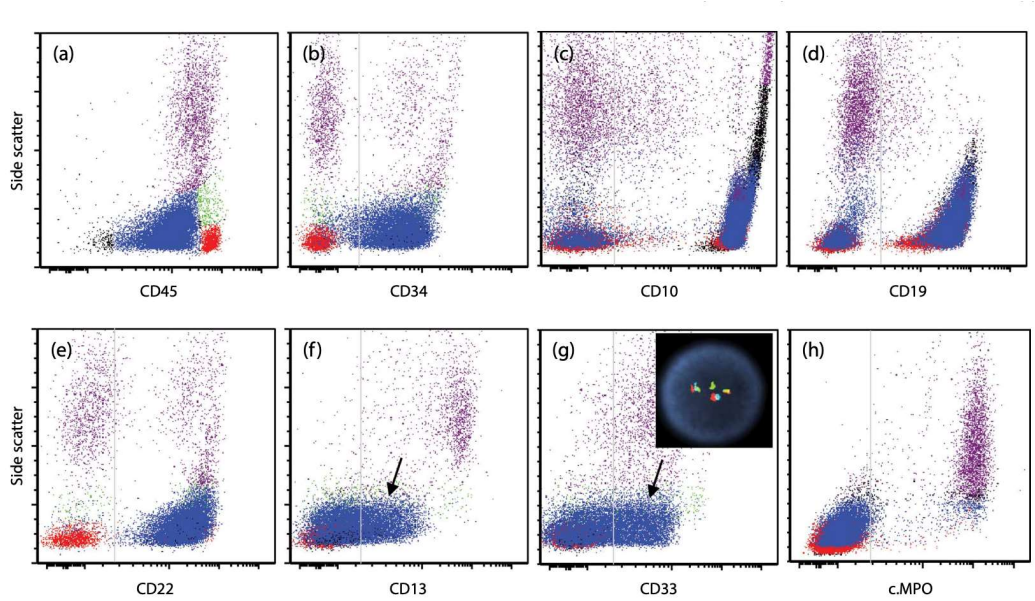


FIGURE 17.8 B-ALL with aberrant expression of pan-myeloid antigens – flow cytometry. B-lymphoblasts (blue dots) are positive for CD45 (a), CD34 (b), CD10 (c; bright expression), CD19 (d; bright expression), CD22 (e), and partially for both CD13 (f; arrow) and CD33 (g; arrow). Negative cytoplasmic MPO excludes mixed phenotype acute leukemia (MPAL). FISH (g; inset) shows several copies of BCR-ABL fusion; (*ASS1*-aqua, *ABL1*-orange, *BCR*-green).

Pro-B-ALL

- **shows the characteristic expression of the pan-B marker CD19, but the absence of CD10**.
- B-lineage is confirmed showing the co-expression of surface CD19 and intracytoplasmic CD79a (gating on ‘bermudes’).
- classification must be confirmed by demonstrating the **absence of intracytoplasmic μ chains**.
- the blasts retain CD34 expression.

Common B-Cell ALL

- Blasts express CD45 weakly
- The blasts **co-express CD19 and CD10 but display no intracytoplasmic μ chains**
- expression of the myeloid marker **CD33** is clearly present.
- *This is not a rare feature in B-cell ALL (B-ALL) and has been associated with **t(9;22)/BCR-ABL1 or t(11;12).***
- Blasts brightly express CD34
- Among other markers, a combination of bright CD10 and CD123 expression has been associated with hyperdiploidy in common ALL.

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

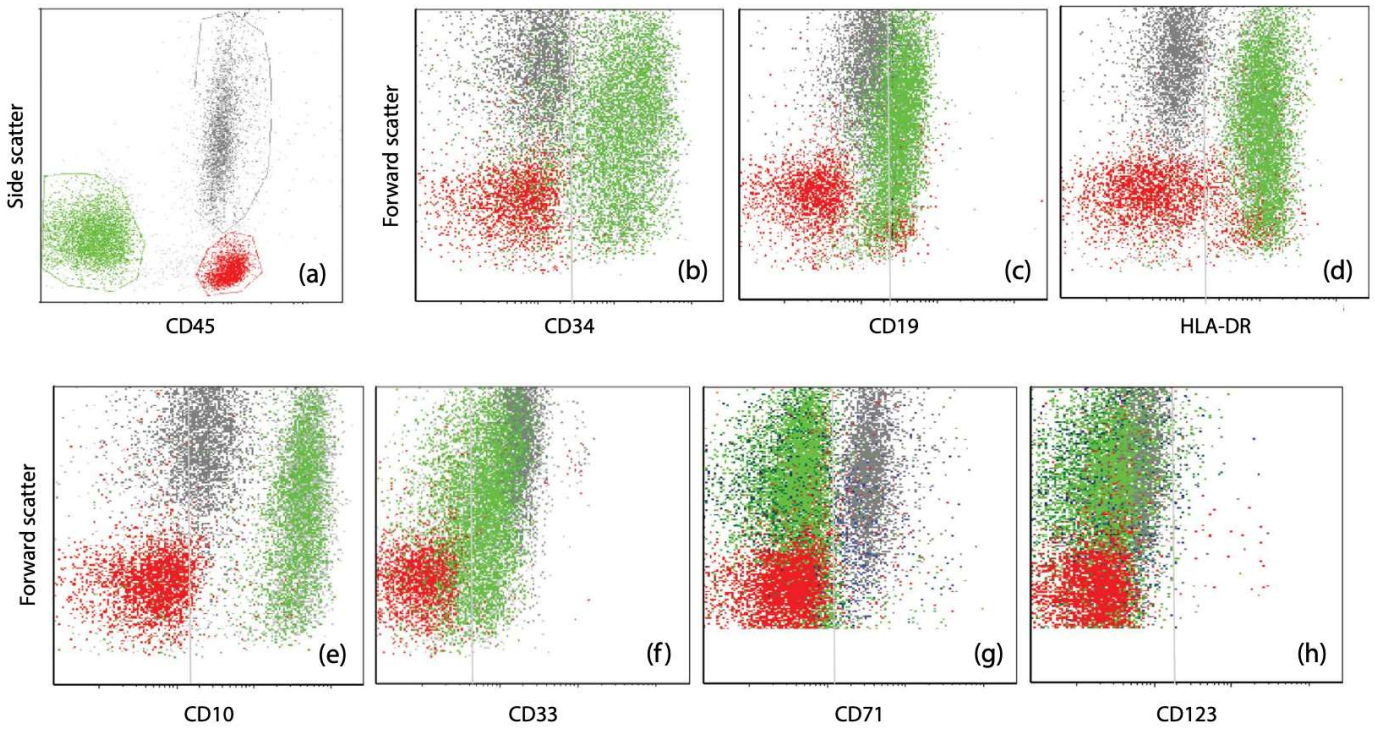


FIGURE 17.6 B-ALL – flow cytometry. Blasts (green dots) show low side scatter (a), negative CD45 expression (a) and increased forward scatter (b–h). They are positive for CD34 (b), CD19 (c), HLA-DR (d), CD10 (e), and CD33 (f), whereas CD71 (g) and CD123 (h) are negative. Note very bright CD10 expression (e; much brighter when compared to neutrophils represented by gray dots).

Pre-B-ALL

- blasts express CD45 at an intermediate level.
- Co-expression of **CD19 and CD10 remains, although the latter is weaker than in the previous case and sometimes may be found only on a fraction of cells**.
- The blasts now **lack CD34**, showing their more mature stage and clearly contain **intracytoplasmic μ chains**.
- In some cases, CD34 may still be found on a fraction of cells, CD20+
- Of note neither **κ nor λ intracytoplasmic light chains** are expressed

B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

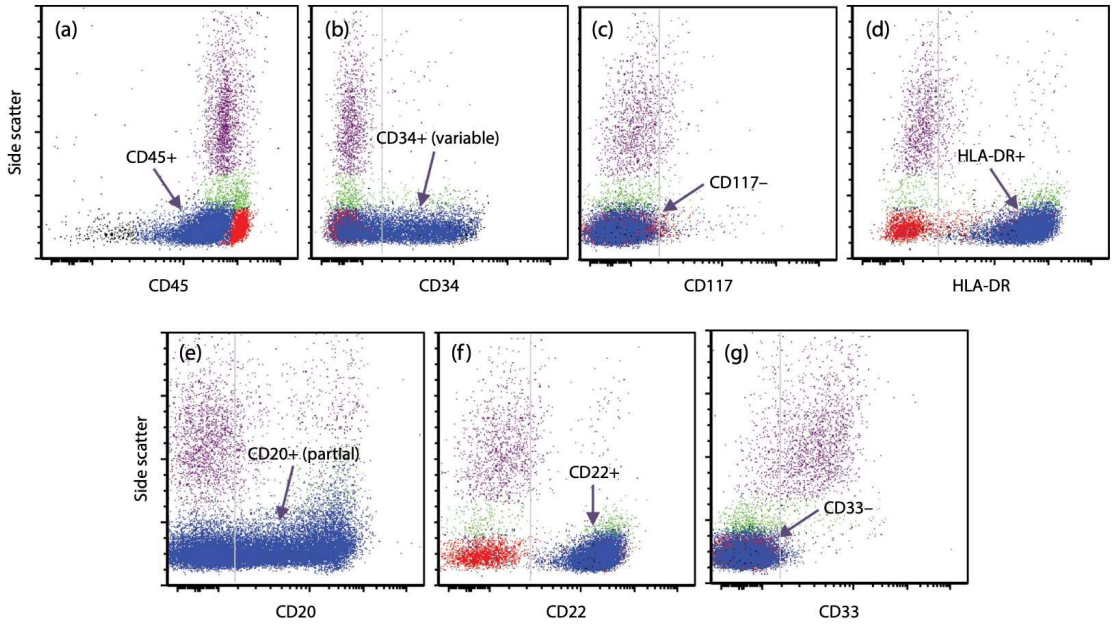
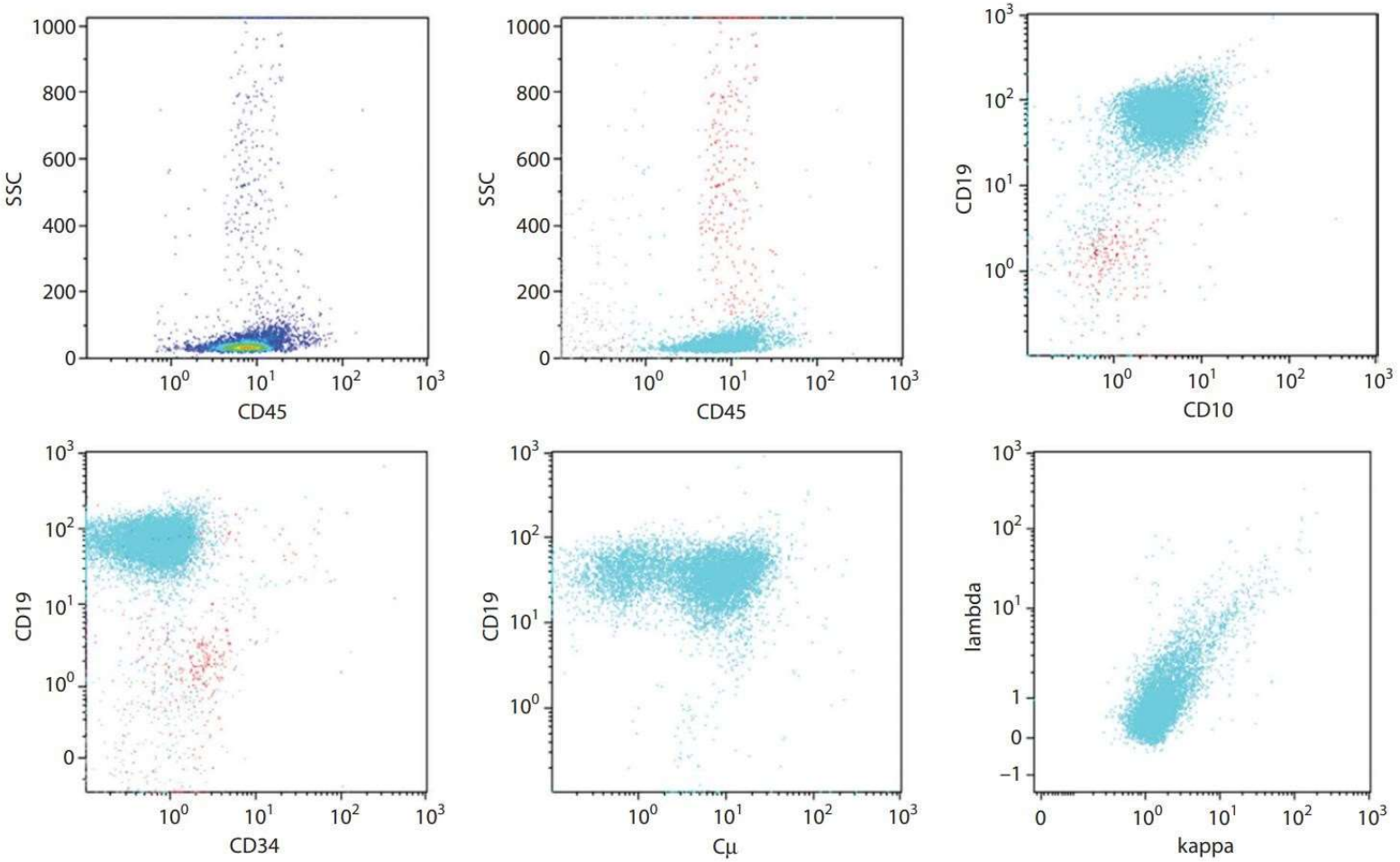


FIGURE 17.11 B-ALL – flow cytometry. B-lymphoblasts (blue dots) are positive for CD45 (a), CD34 (b; variable expression), HLA-DR (d), CD20 (e; variable and partial expression), and CD22 (f). CD117 (c) and CD33 (g) are not expressed.

Pre-B-ALL



B-lymphoblasts versus maturing B-cell precursors (hematogones)

- hematogones can be identified by FC in majority of samples from BM (~1% of marrow elements).
- Their number decreases with age but may be increased in regenerating marrow from patients after chemotherapy or stem cell transplantation, in some neoplasms, autoimmune disorders (ITP), iron deficiency or infections.
- Majority of patients with MDS and MPN show lack of hematogones or significant decrease in their number.
- The identification of hematogones is important in FC monitoring B-ALL patients after treatment to exclude MRD

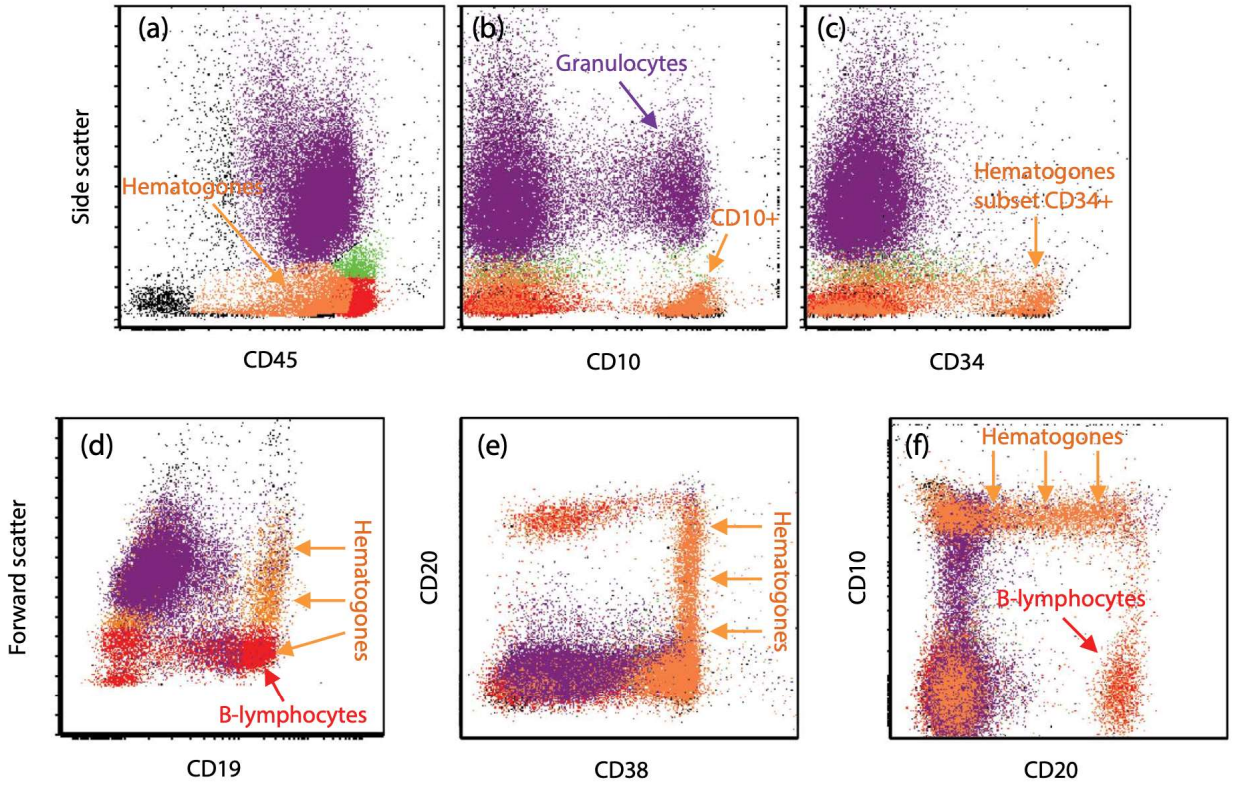


FIGURE 6.10 Hematogones (benign B-cell precursors). Hematogones (orange dots) are positive for CD45 and display very low side scatter (a). They are positive for CD10 (b), CD34 (c; partial), CD19 (d), and CD38 (e). One of the characteristic features of hematogones is increased, but variable forward scatter (d; arrows) and variable expression of CD20 (e–f; arrows).

Hematogones VS B-ALL

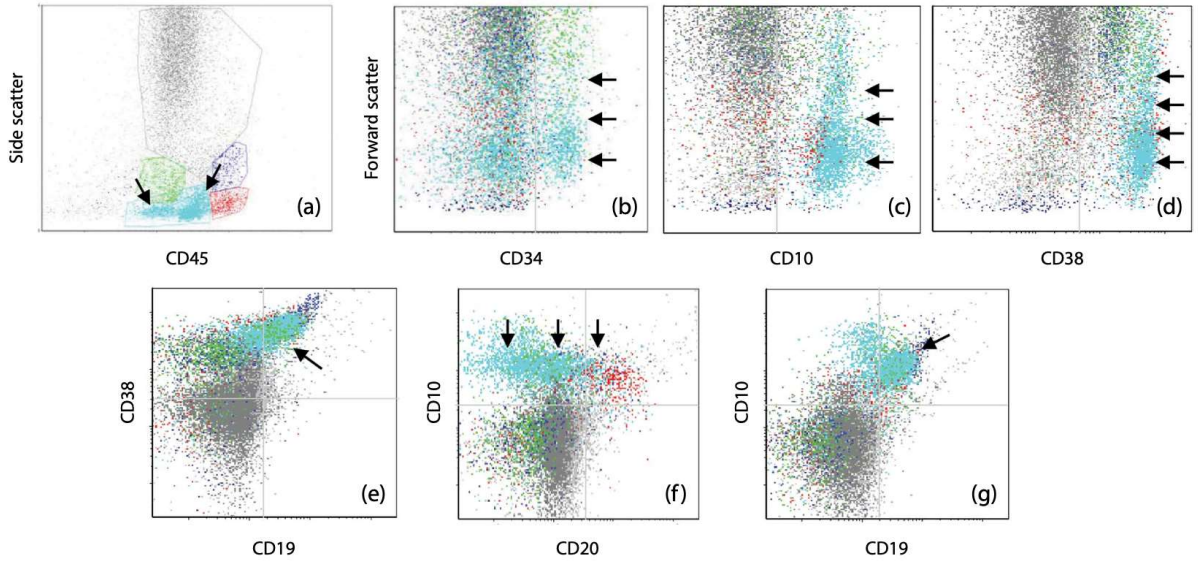


FIGURE 17.13 Flow cytometry features of hematogones. On the CD45 versus side scatter (SCC; a) hematogones show two distinct populations, one with dim CD45 and one with moderate CD45 expression (arrows). Subset of hematogones is positive for CD34 (b). Forward scatter (FSC) is characteristically variable (b–d; arrows) ranging from low to high, with majority of hematogones display low FSC. Hematogones are positive for CD10 (c, f–g) and CD38 (d–e), with CD10 being dimmer than in B-ALL and CD38 being brighter than in B-ALL. The expression of CD20 (f) is variable, ranging from negative (majority of hematogones) to dimly positive (minor subset).

Hematogones VS B-ALL

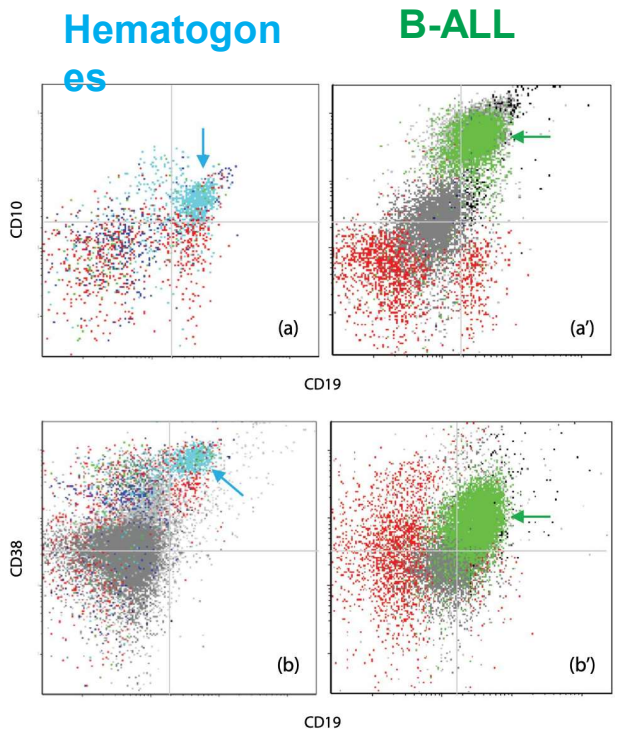
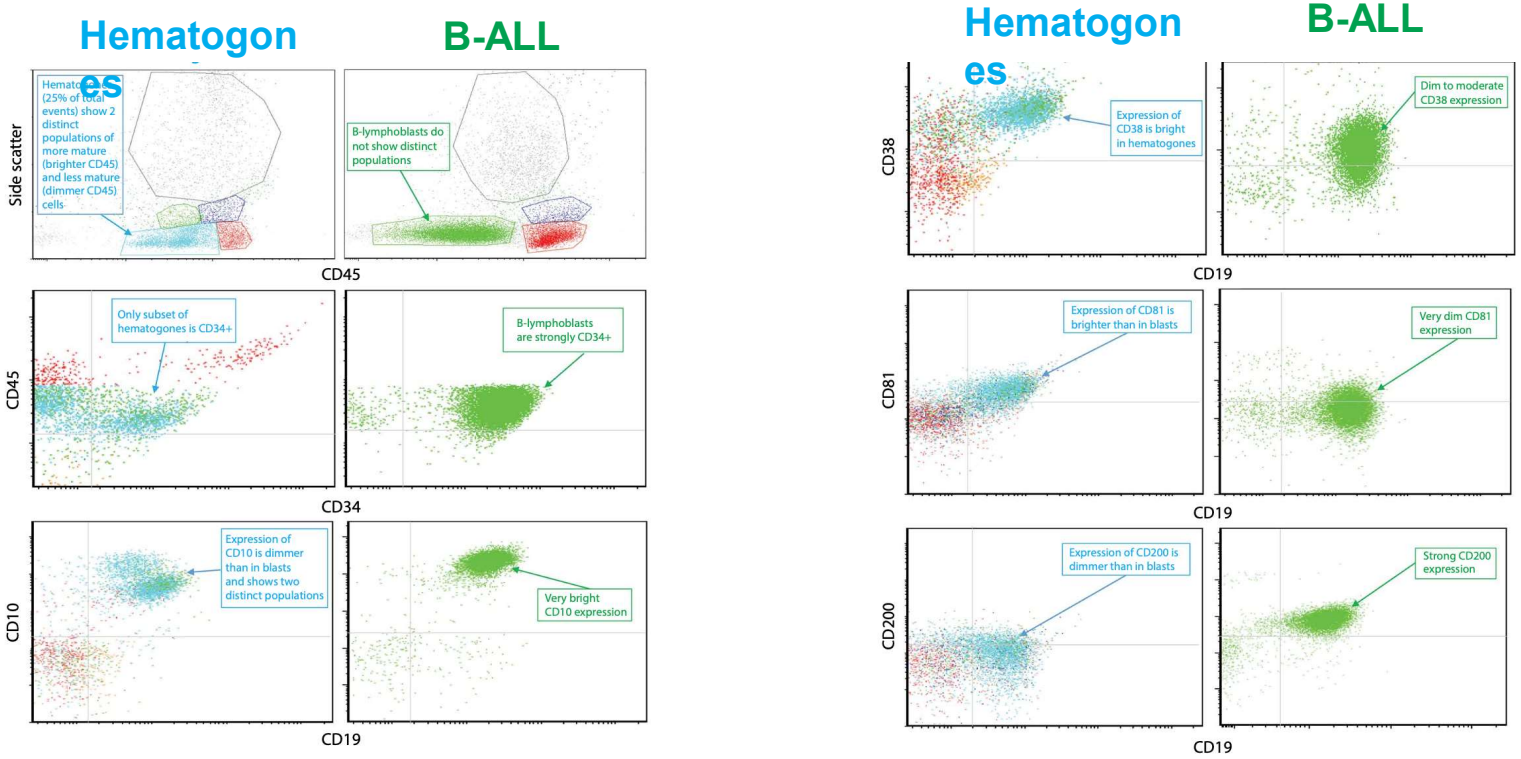


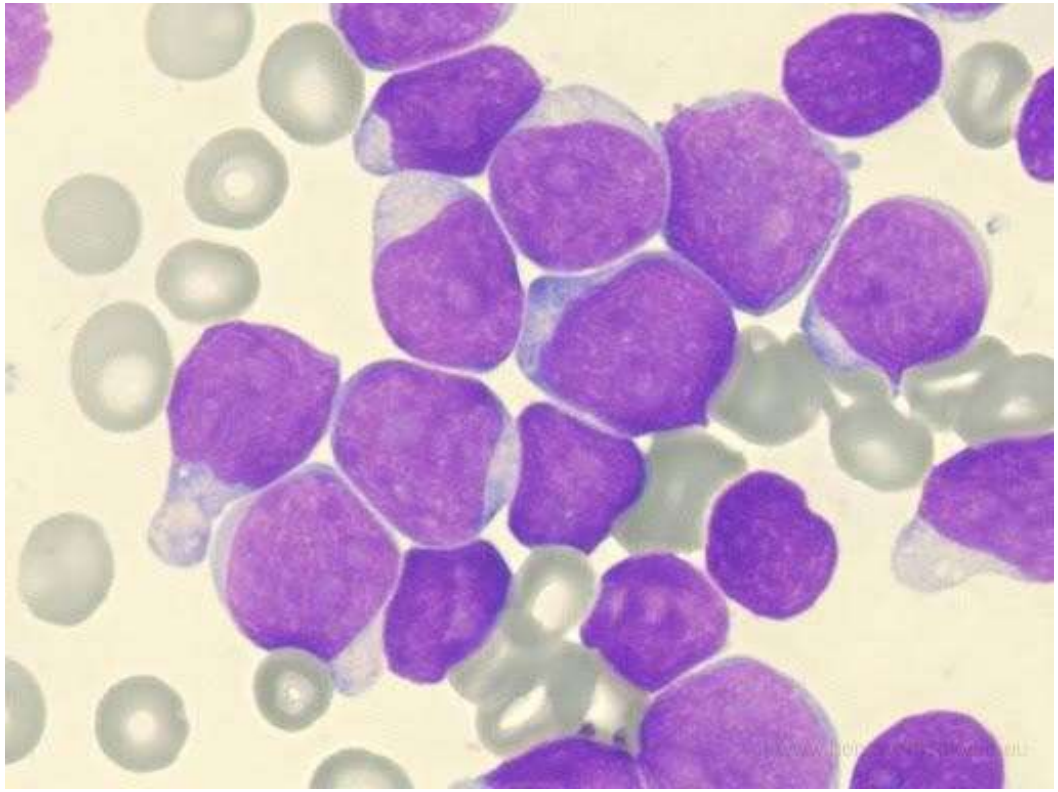
FIGURE 17.14 Comparison of the expression of CD10 and CD38 versus CD19 in hematogones (a, b) and B-ALL (a′, b′). Hematogones (a–b; blue dots) and B-ALL (a′–b′; green dots) are $CD19^+$, $CD10^+$, and $CD38^+$, but deferrer in the intensity of staining: CD10 is much brighter in B-ALL and CD38 is dimmer in B-ALL, when compared to hematogones.

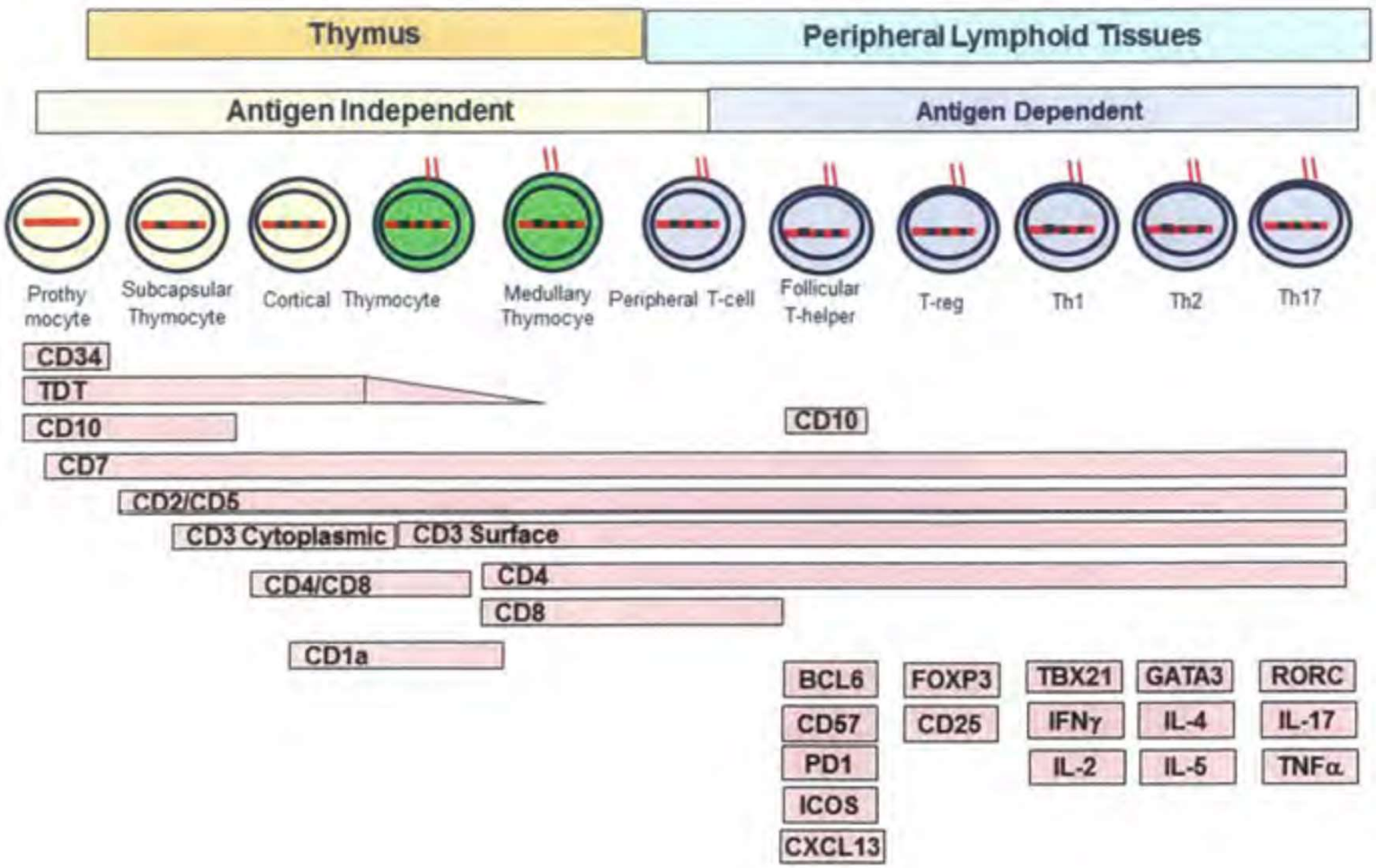
Hematogones VS B-ALL

Comparison of FC pattern of hematogones (blue dots, left column) and B-ALL (green dots, right column).



T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA




Thymus
Peripheral Lymphoid Tissues
Antigen Independent
Antigen Dependent
Prothy mocyte
Subcapsular Thymocyte
Cortical Thymocyte
Medullary Thymocye
Peripheral T-cell
Follicular T-helper
T-reg
Th1
Th2
Th17
CD34
TDT
CD10
CD10
CD7
CD2/CD5
CD3 Cytoplasmic
CD3 Surface
CD4/CD8
CD4
CD8
CD1a
BCL6
CD57
PD1
ICOS
CXCL13
FOXP3
CD25
TBX21
IFNγ
IL-2
GATA3
IL-4
IL-5
RORC
IL-17
TNFα

T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

The phenotypic stages of T-cell development can be divided into :

- **Pro-T (T-I):** CD7+, s.CD3−, c.CD3+, CD1a−, CD34+/−, CD2−, CD5−, CD4−/CD8−;
- **Pre-T (T-II):** CD2+ and/or CD5+, s.CD3−, c.CD3+, CD7+, CD1a−, CD34+/−, CD4−/CD8− or CD4+/CD8+), TdT+
- **Cortical T (T-III):** CD1a+, s.CD3+/−, c.CD3+, CD2+, CD5+, CD1a+, CD34−, CD4+/CD8+, TdT+;
- **Mature (medullary) T cells (T-IV):** CD4+ or CD8+, CD2+, CD5+, CD7+, s.CD3+, c.CD3+, CD1a−, CD34−
- **ETP-ALL phenotype :** CD1a−, CD2+, surface CD3−, cytoplasmic CD3+, CD4−/CD8−, CD5−/dim+(<75%), CD7+,

CD10−/+, CD13+, CD15−/rarely+, CD33+, CD34+/−, CD38+, CD56−/rare cases+, CD117−/+, , HLA-DR−, MPO−, TdT+/rarely−

WHO recognizes the following categories of T-ALL:

- *T-lymphoblastic leukaemia / lymphoma, NOS*

T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

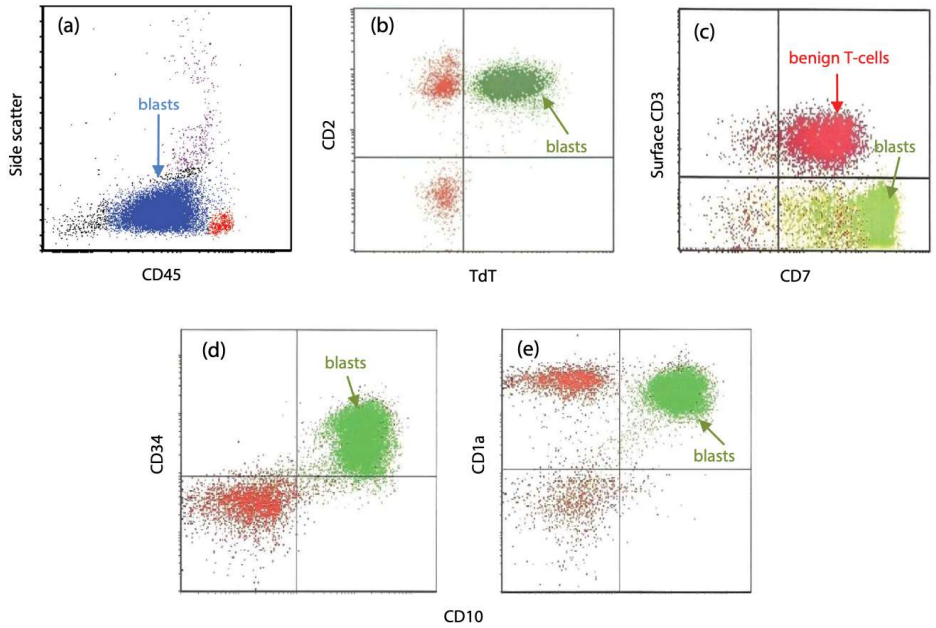


FIGURE 6.19 Identification of T-lymphoblasts by flow cytometry: T-lymphoblasts can be identified by moderate CD45 expression and low side scatter (a), positive TdT (b), lack of surface CD3 expression (c), positive CD34 (d), positive CD10 (d–e), and positive CD1a (e).

T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA/LYMPHOMA

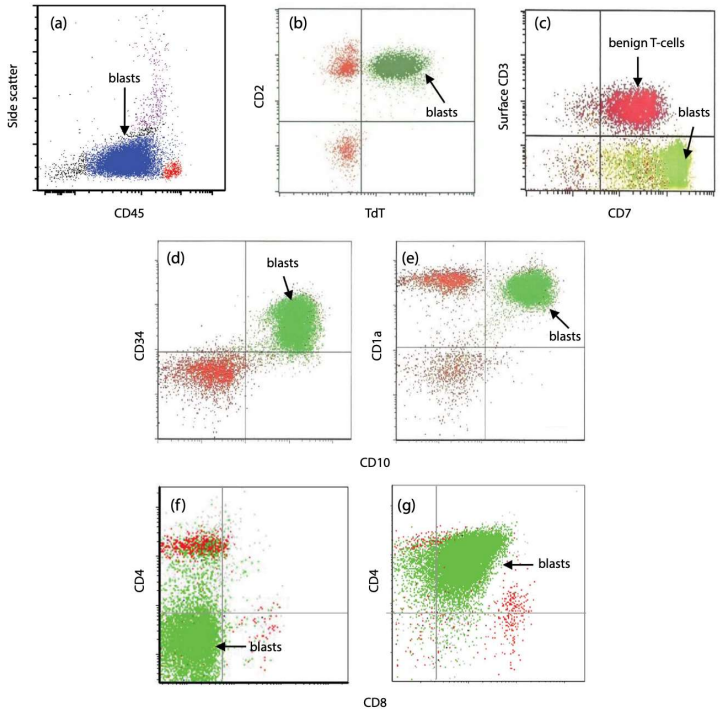


FIGURE 18.5 Identification of T-lymphoblasts by flow cytometry: T-lymphoblasts can be identified by moderate CD45 expression (a), positive TdT (b), lack of surface CD3 expression (c), positive CD34 (d), positive CD10 (d–e), positive CD1a (e), and by either dual CD4/CD8 negativity (f) or dual CD4/CD8 positivity (g).

Early T-precursor lymphoblastic leukaemia / lymphoma

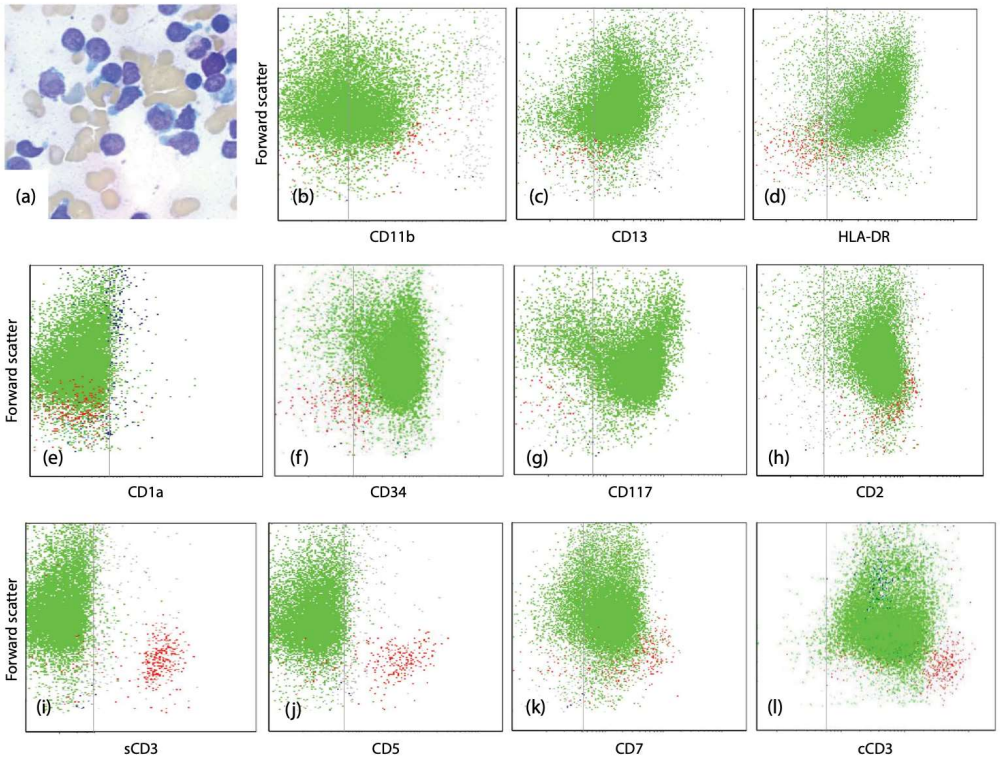


FIGURE 18.18 ETP-ALL (flow cytometry from BM). (a) Aspirate smear shows lymphoblasts with hand-mirror appearance. Blasts display typical phenotype for ETP-ALL: CD11b^{+}, CD13^{+}, HLA-DR^{+}, CD1a^{-}, CD34^{+}, CD117^{+}, CD2^{+}, surface CD3^{-}, CD5^{-}, CD7^{+}, and cytoplasmic CD3^{+}.

Immunophenotyping of Mature B/T-Cell Lymphomas

Limitation of flow cytometry in diagnosis of lymphoma

- Small number of neoplastic cells are present (Hodgkin ,T cell rich B cell lymphoma)
- Focal marrow involvement

B lymphoproliferative disorders

Among B cell lymphoma, there are two entities that can be both diagnosed and classified with a high degree of confidence based on immunophenotypic data alone: chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and hairy cell leukemia

Tools for Evaluating T-cell Lymphoproliferative Disorders

- Clinical and Morphologic evaluation (most important)
- TCR to determine clonality (watch-out for false positives)
- Antigen drop-out:
  Limited due to reactive T-cells which can show loss of pan T-cell antigen expression:
    CD7 loss in reactive T-cells
    CD2 loss in reactive NK-cells
- Aberrant antigen expression:
  Abnormally bright expression of antigens (CD25)
  T-ALL (expression of myeloid antigens)
  Significantly increased or decreased CD4:CD8 ratio (watch-out as reactive conditions can show this as well as immunodeficiency states (HIV))

  So the role of immunophenotyping in the classification of T cell lymphoma is less well described

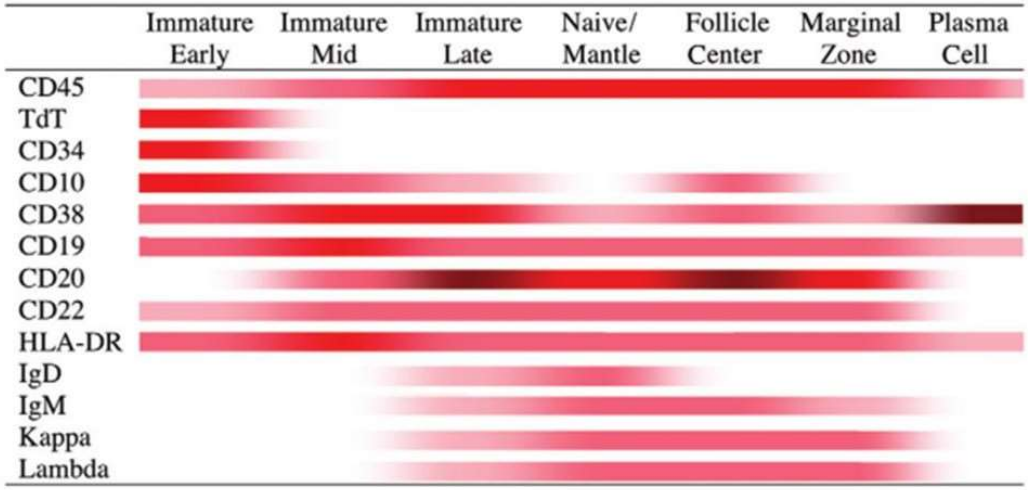
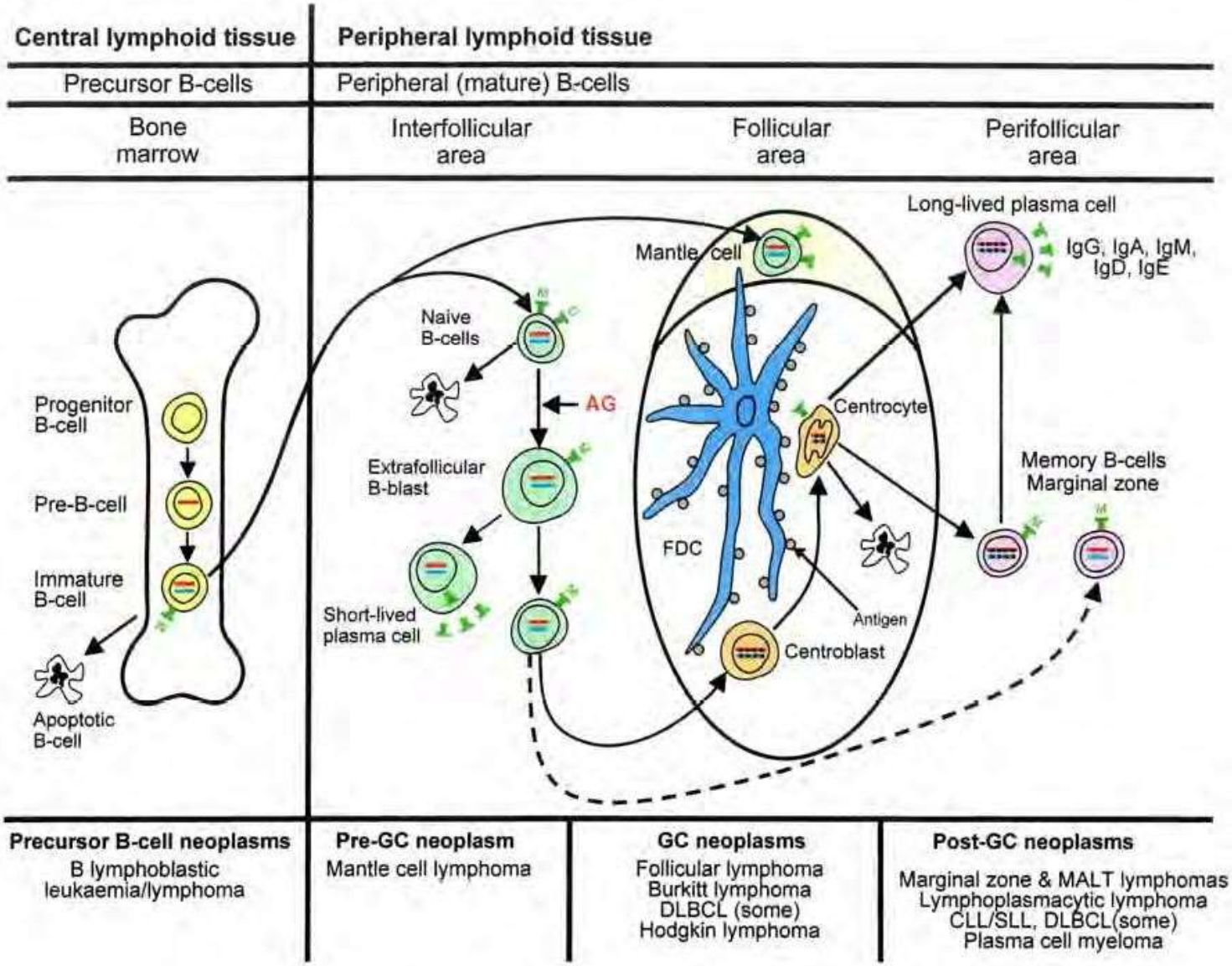


Figure 34-6 B cell differentiation. The intensity of antigen expression throughout B cell maturation is depicted for antigens commonly used in the clinical laboratory. Normal mature B cells express κ or λ light chains, never both. Darker colors represent higher levels of antigen expression.


Central lymphoid tissue
Peripheral lymphoid tissue
Precursor B-cells
Peripheral (mature) B-cells
Bone marrow
Interfollicular area
Follicular area
Perifollicular area
Progenitor B-cell
Pre-B-cell
Immature B-cell
Apoptotic B-cell
Naive B-cells
AG
Extrafollicular B-blast
Short-lived plasma cell
Mantle cell
FDC
Centrocyte
Centroblast
Antigen
Long-lived plasma cell
IgG, IgA, IgM, IgD, IgE
Memory B-cells Marginal zone
Precursor B-cell neoplasms
B lymphoblastic leukaemia/lymphoma
Pre-GC neoplasm
Mantle cell lymphoma
GC neoplasms
Follicular lymphoma
Burkitt lymphoma
DLBCL (some)
Hodgkin lymphoma
Post-GC neoplasms
Marginal zone & MALT lymphomas
Lymphoplasmacytic lymphoma
CLL/SLL, DLBCL(some)
Plasma cell myeloma

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

- Immunophenotyping plays a central role in the diagnosis of CLL/SLL, which is the most common chronic LPD in the Western world.
- Current criteria for the diagnosis of CLL are based on morphology and detection of **CD19+, CD5+, CD23+** B-cell population with dim expression of **CD20 and dim or absent light-chain restricted sIg.**

- **This population must be present at a level of ≥5 × 10^9 /L in blood.**

- Patients who do not have extranodal disease fulfilling the criteria for SLL and present with lower levels of B-cells with a CLL phenotype are diagnosed as **monoclonal B-cell lymphocytosis (MBL)**

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

- The typical CLL immunophenotype is characterized by both **CD19 and CD20 expression lower than in normal B-cells**, which allows separation of malignant from normal B-cells whenever those are present in the sample.

- The lowest CD20 expression is seen in CLL with **del(11q)**, while CLL with **trisomy 12 shows relatively higher CD20 expression** levels In most cases, light-chain restriction is detected with low intensity
- **In some cases, surface light chains are undetectable but light-chain restriction can be confirmed by analysis of cytoplasmic Ig expression** .
-
- **CD5** expression in CLL is typically weaker than in normal T-cells but higher than the levels seen in the minute normal CD5+ B-cell subsets
- **CD23** expression in CLL is variable and a heterogeneous expression within the malignant B-cell population is often seen.

- CD5+ T-cells in the sample can be used as a negative reference population to determine the threshold for CD23 expression in B-cells.
- Low CD23 appears to be most often related to trisomy 12.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

- **Other markers such as CD10, CD43, CD79b, CD81, CD200** are useful in differential diagnosis and are recommended to deal with borderline cases.
- A search for surrogate markers for CLL mutational status revealed at first a strong correlation between CD38 expression and unmutated CLL with a better prognosis for **CD38–** cases

- CD38 seems to be an independent prognostic factor in CLL.

- Another antigen overexpressed in unmutated CLL cases is zeta chain of T-cell receptor-associated protein kinase 70 (**ZAP70).**

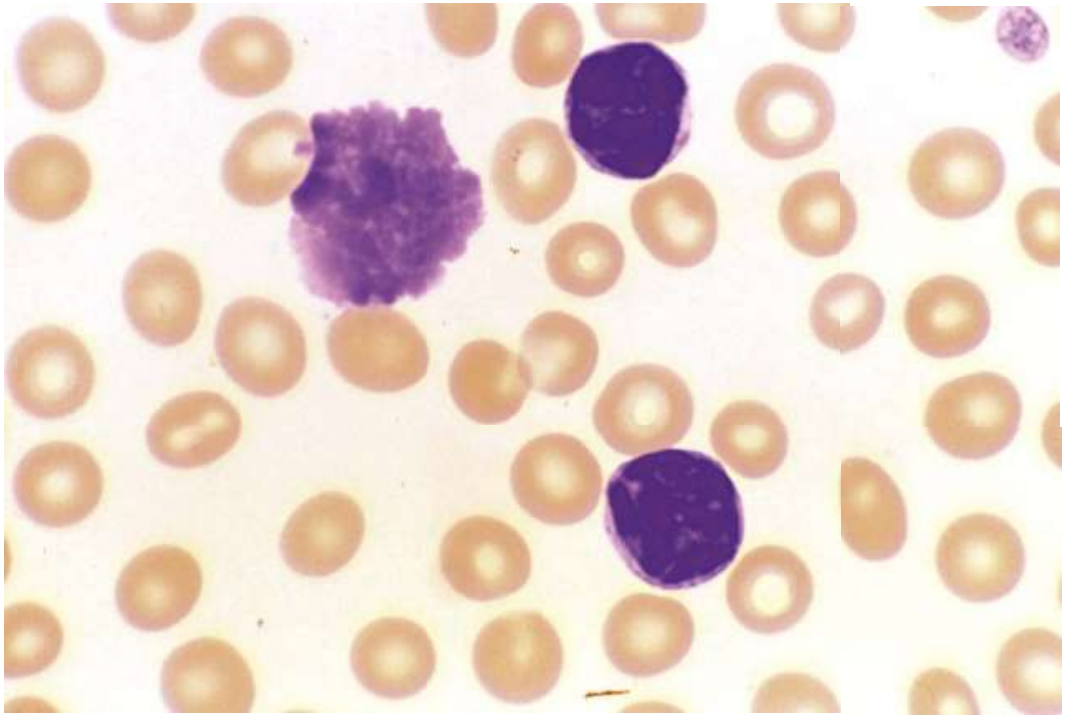
- FCM detection of ZAP70 has not been widely applied in clinical practice due to problems with standardisation of the determination of expression level

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

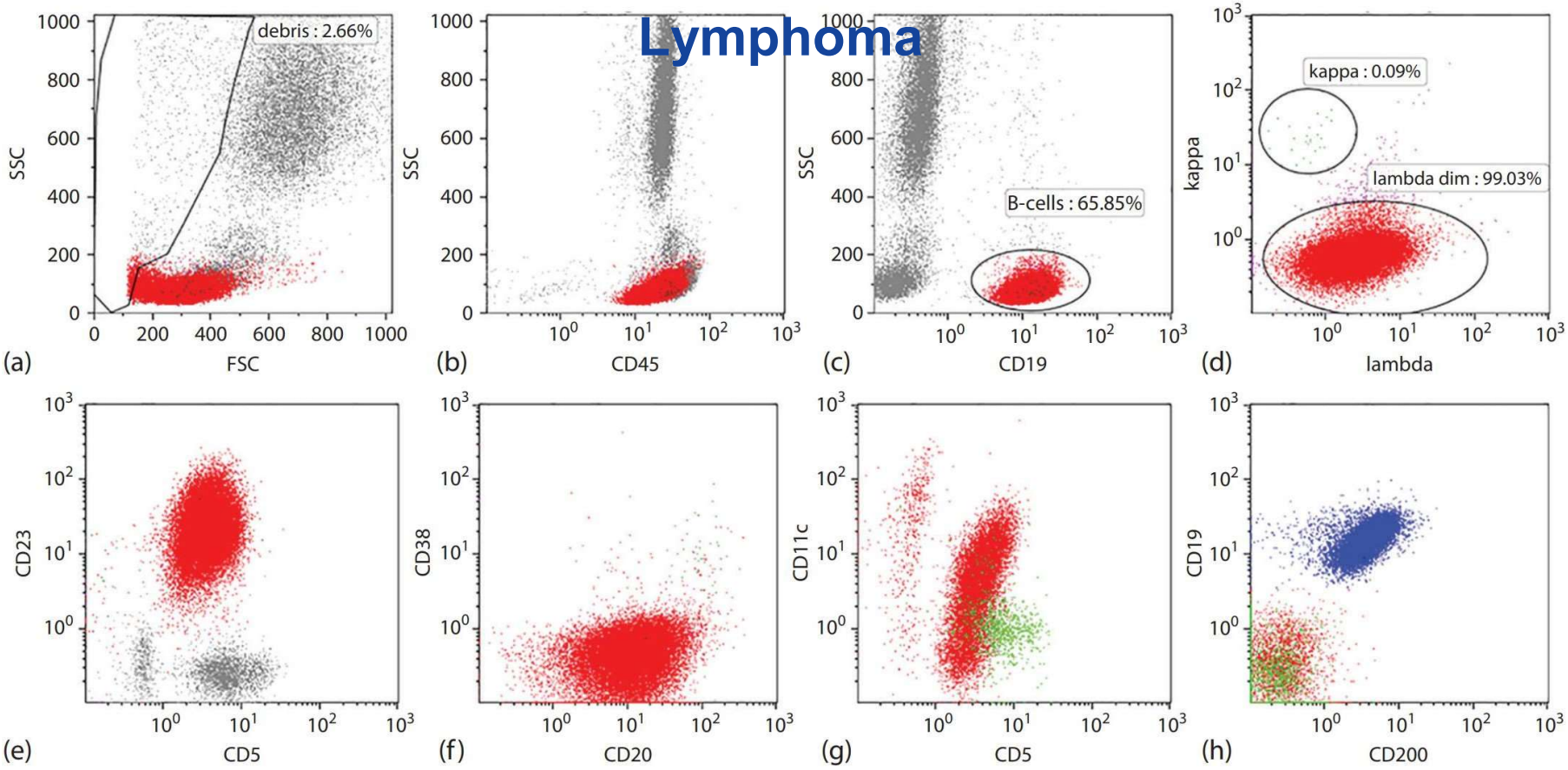
- Patients with CLL cells negative for both ZAP70 and CD38 are mostly those with mutated CLL and indolent disease.

- Another FCM marker strongly related to prognosis in patients with CLL is **CD49d** with high expression (cut-off 30%) being a negative prognostic factor CD49d is more often highly expressed in unmutated CLL and in CD38+ cases but its prognostic significance is independent of the mutational status.

Broken CLL cells (so-called smudge cells) are evident on this peripheral blood smear from a patient with CLL. Smudge cells consist largely of nuclear material.



Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma



Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

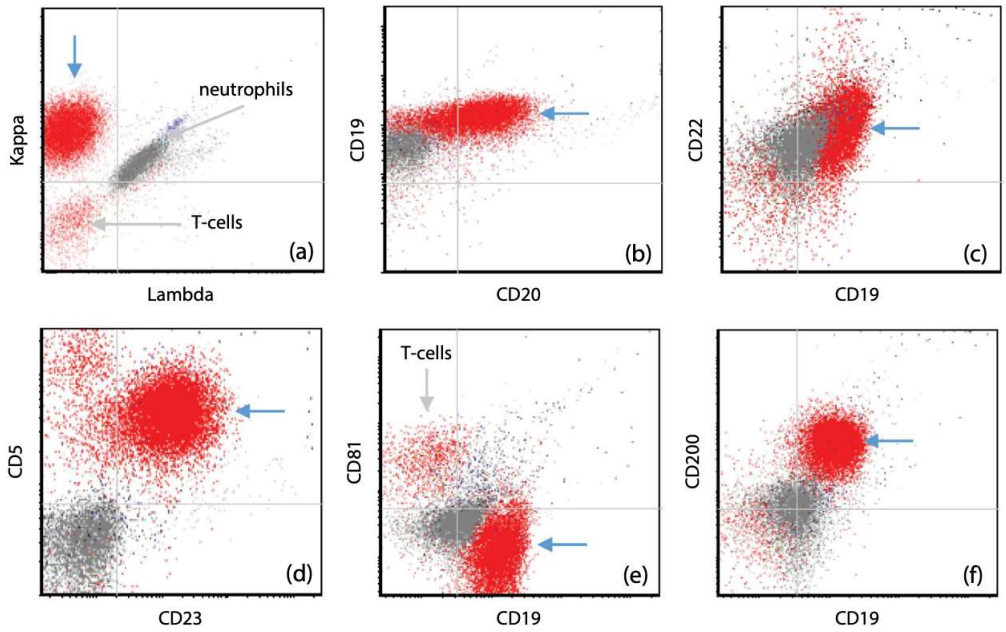


FIGURE 13.3 Typical immunophenotypic profile of CLL. Leukemic B-cells (red dots, arrow) show the following phenotype: clonal expression of surface immunoglobulins (kappa in this case, a), positive CD19 and dimly positive CD20 (b), dimly positive CD22 (c), co-expression of CD5 and CD23 (d), negative CD81 (e), and moderate expression of CD200 (f).

Hairy cell leukemia

- HCL accounts for approximately 2% of LPDs.
- Typically, only low numbers of circulating cells are found in the PB.
- The tumor cells mainly reside in the BM and spleen, and symptoms are chiefly related to cytopenia.
- **HCL tends to present as CD19+/SSC high cells.**
- BM involvement is typically extensive but fibrosis makes dry tap a characteristic feature in the majority of patients, and the diagnosis is typically made on PB or a BM biopsy .
- **A characteristic laboratory finding is an abnormally low monocyte count.**
- However, because **HCL cells display a high SSC, they can be mistaken for monocytes by automated cell counters.**
- HCL cells have a highly characteristic immunophenotype and express **CD11c, CD103, CD25 and CD123 with a bright expression of CD20 and CD22.**
- They lack **CD27, and CD23** expression is typically absent or dim.
- Whereas expression of CD5 is rare (2%), CD10 expression is seen in approximately 15% of the cases.

Hairy cell leukemia

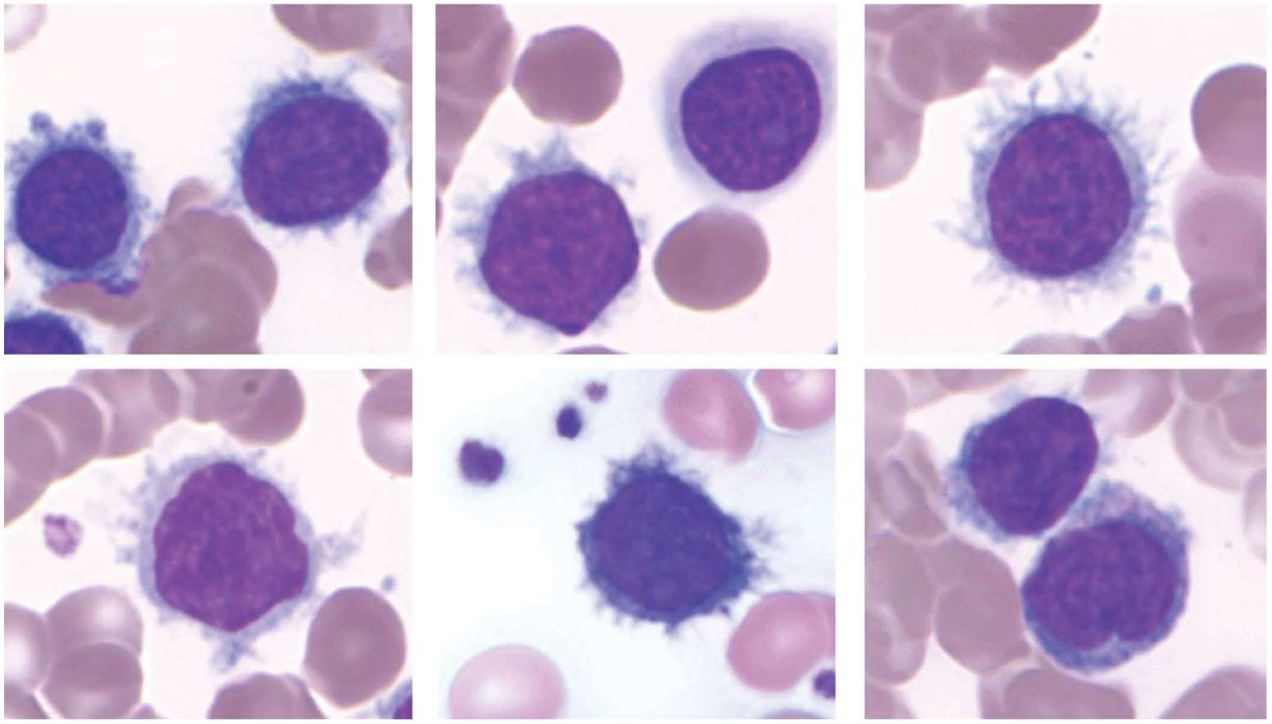
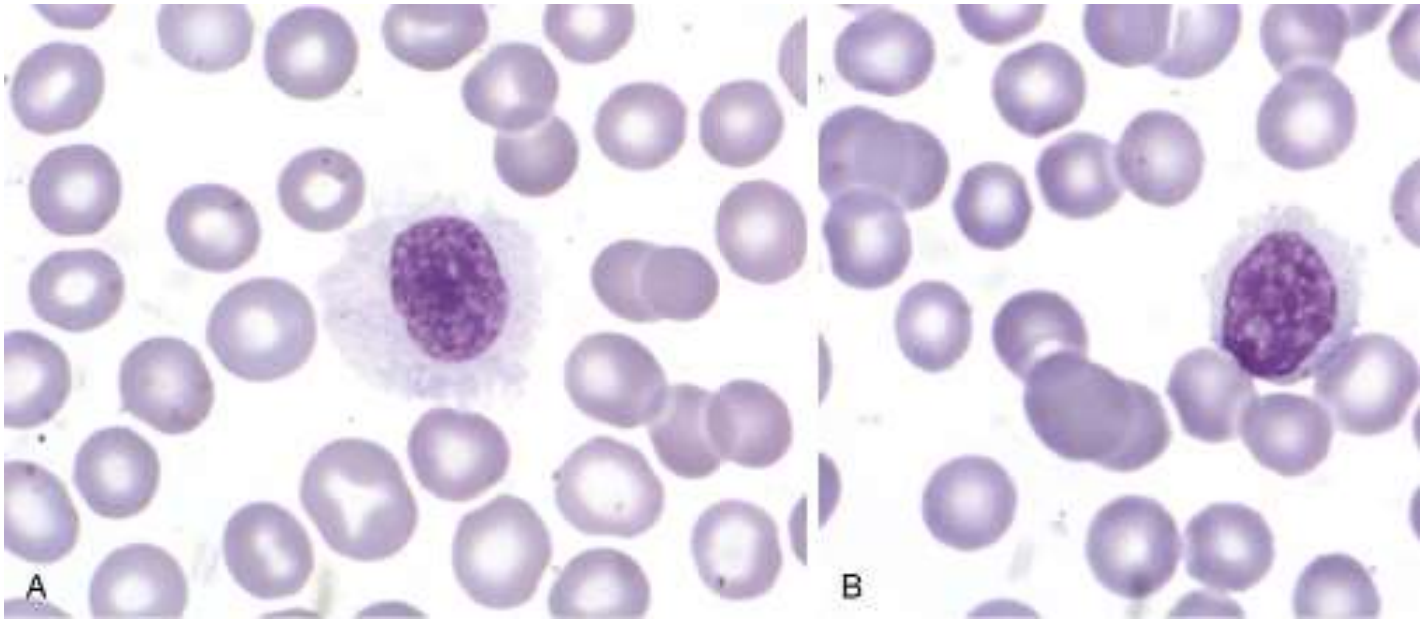
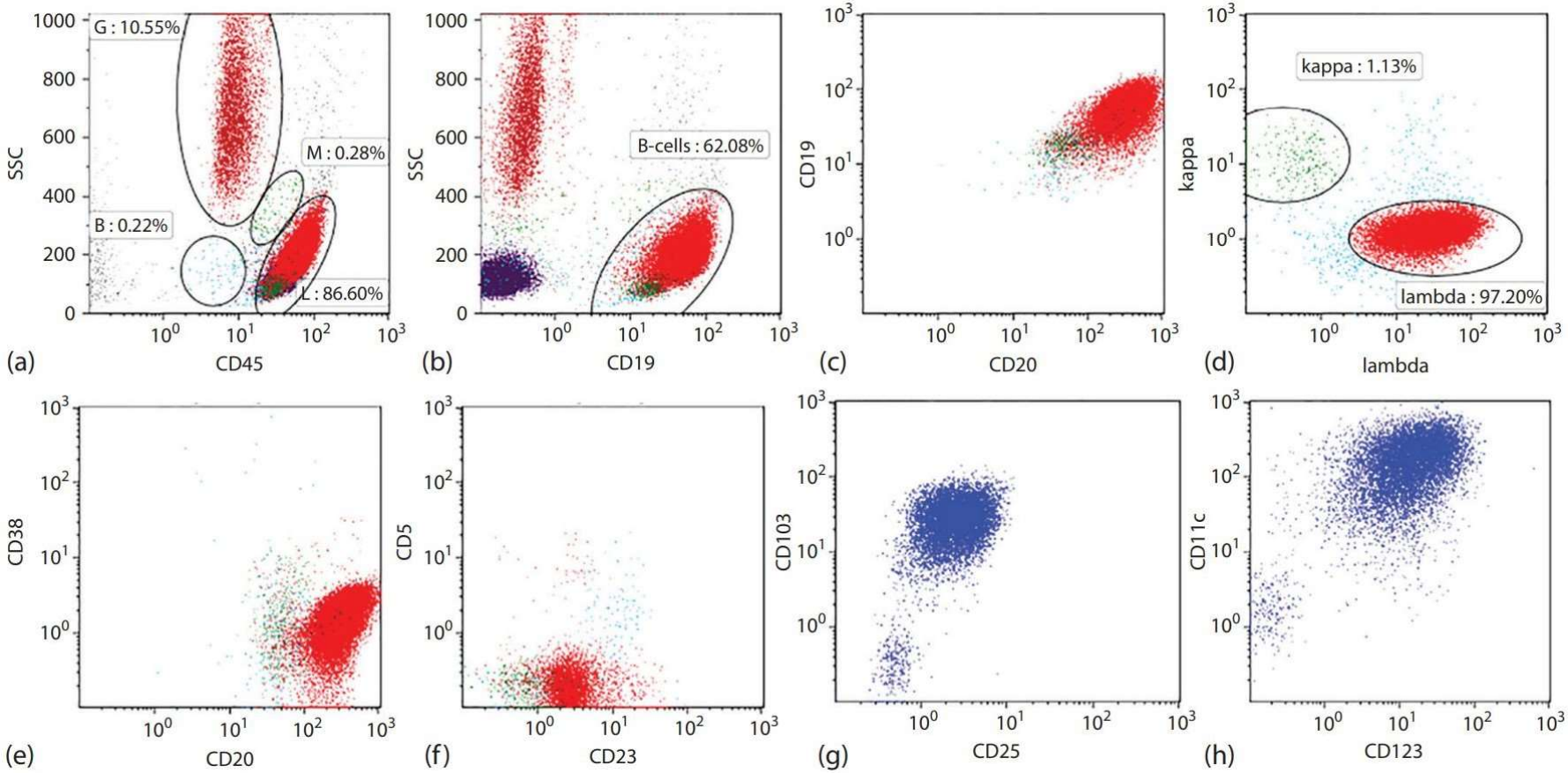


FIGURE 13.28 Hairy cell leukemia – cytology (blood smear of several cases of HCL).

A, Hairy cell on peripheral blood smear. The nucleus is oval, with slightly dispersed chromatin, and the cytoplasm is pale blue with an ill-defined, ruffled border. B, Hairy cell on peripheral blood smear. In thicker areas of the smear or in bone marrow aspirates, the cytoplasm may appear more scant. Small nucleoli may be present in some hairy cells.



Hairy cell leukemia



Hairy cell leukemia

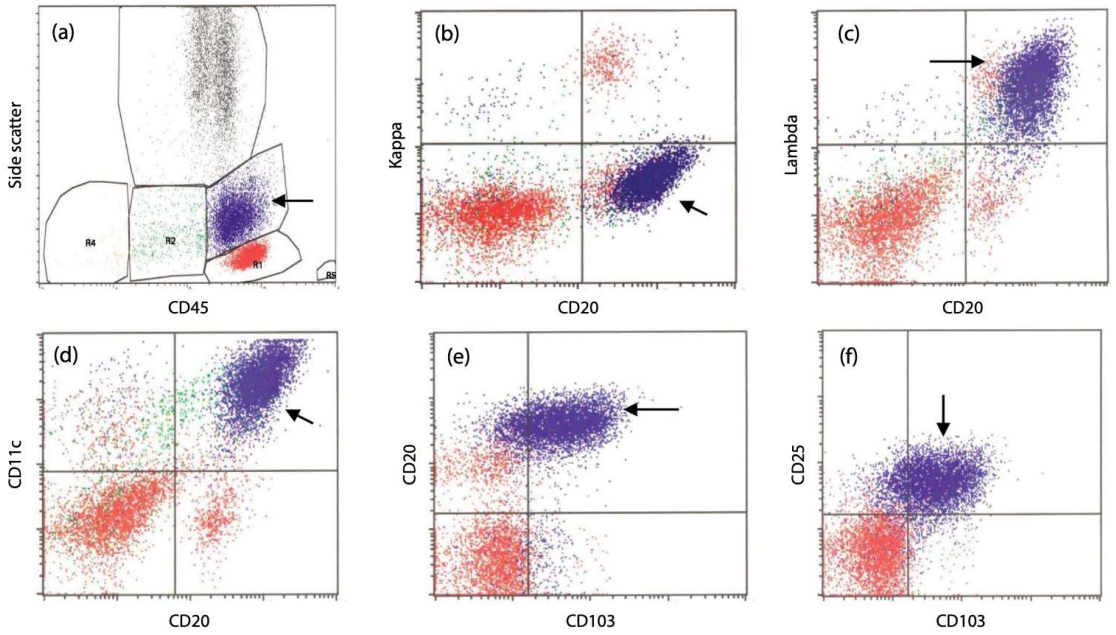


FIGURE 13.31 Hairy cell leukemia – flow cytometry (FC). delete this part FC analysis shows increased orthogonal side scatter (a; SSC, arrow), which places the cells in the "monocytic region", above normal lymphocytes (red dots). HCL cells show moderate to bright expression of CD20 (b–e), surface immunoglobulin (lambda, c), bright expression of CD11c (d), and co-expression of CD25 and CD103 (e–f).

Hairy cell leukemia

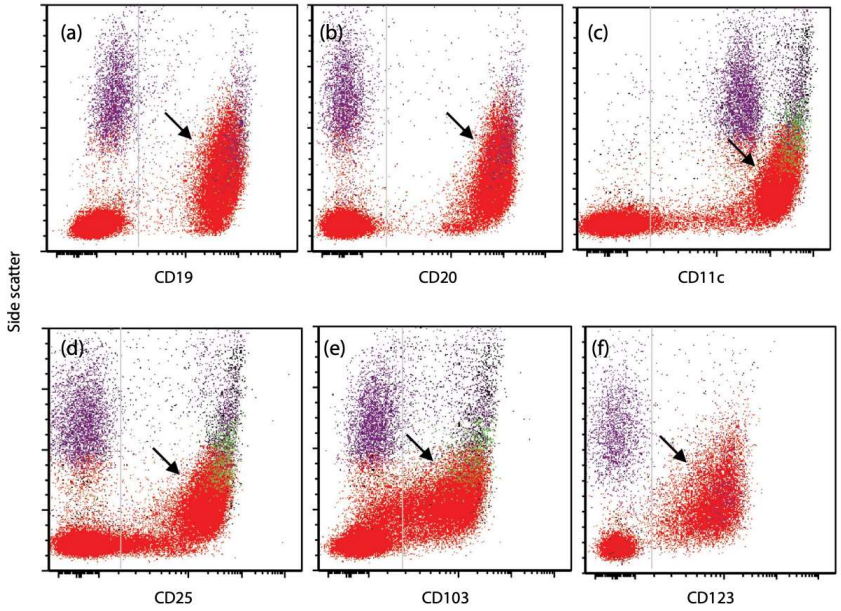


FIGURE 13.32 HCL – flow cytometry analysis (blood). HCL cells (arrow) are strongly positive for CD19 (a), CD20 (b), CD11c (c), CD25 (d), CD103 (e), and CD123 (f).

Mantle Cell Lymphoma

- MCL constitutes approximately 5% of lymphomas.
- In most cases, MCL is an aggressive, rapidly progressing adenopathic disease, although in 10%–15% of cases it follows a clinically more indolent course with mainly a leukemic, non-nodal manifestation.
- At diagnosis, the disease is usually widespread and the BM is involved in 80%–90% of the patients.
- More indolent cases arise from cells with different gene expression profiles that appear to have entered the GC and undergone IGHV somatic hypermutation, whereas aggressive MCL seem to evolve from cells of the mantle or marginal zones.
- **Due to the typical presence of the t(11;14) translocation, MCL cell are characterized by overexpression of nuclear cyclin D1.**
- FCM for the detection of cyclin D1 expression has been demonstrated to be feasible but has not reached widespread application, most likely due to the broad availability of immunohistochemical methods.
- FCM detection and quantification of the transcription **(SOX11**), which is usually highly upregulated in MCL, has also been described . However, more MCL indolent cases with leukemic presentation tend to be SOX11− .

Mantle Cell Lymphoma

- By FCM, MCL cells are typically positive for **CD5 together with the pan-B-cell antigens CD19, CD20 and CD22, and negative for CD10 and CD23**. However, CD5− or CD10+ cases have been described with a frequency of approximately 10%.
- CD23 is expressed in about 25% of the cases and may be associated with the more indolent, leukemic form.
- **CD11c is most often negative, while CD38** is positive but not overexpressed.
- The expression of **CD200** is very rare (0%–5% in various studies) and appears to be particularly useful in distinguishing MCL from CLL, where it is consistently expressed.
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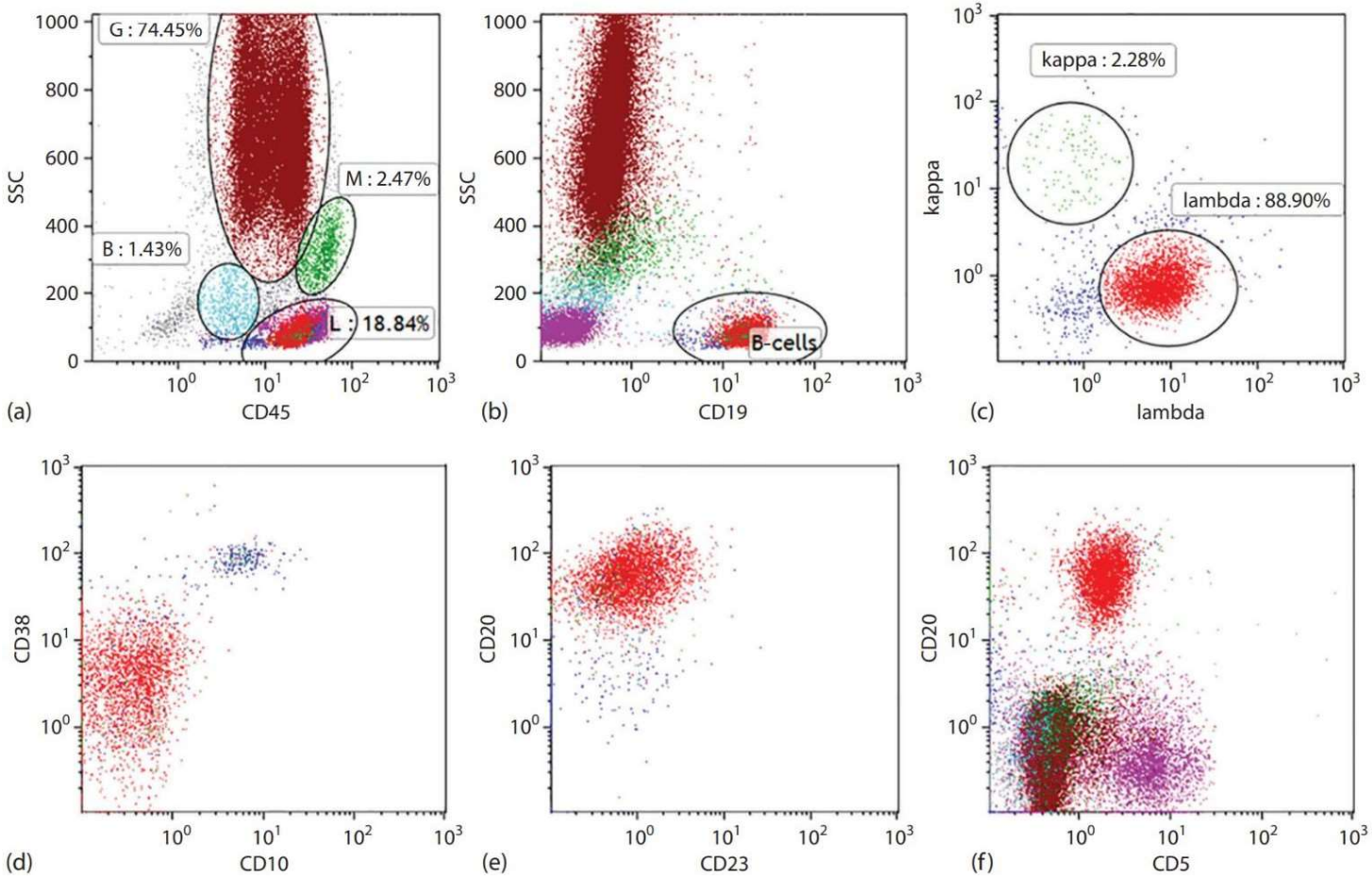
Mantle Cell Lymphoma



Mantle Cell Lymphoma

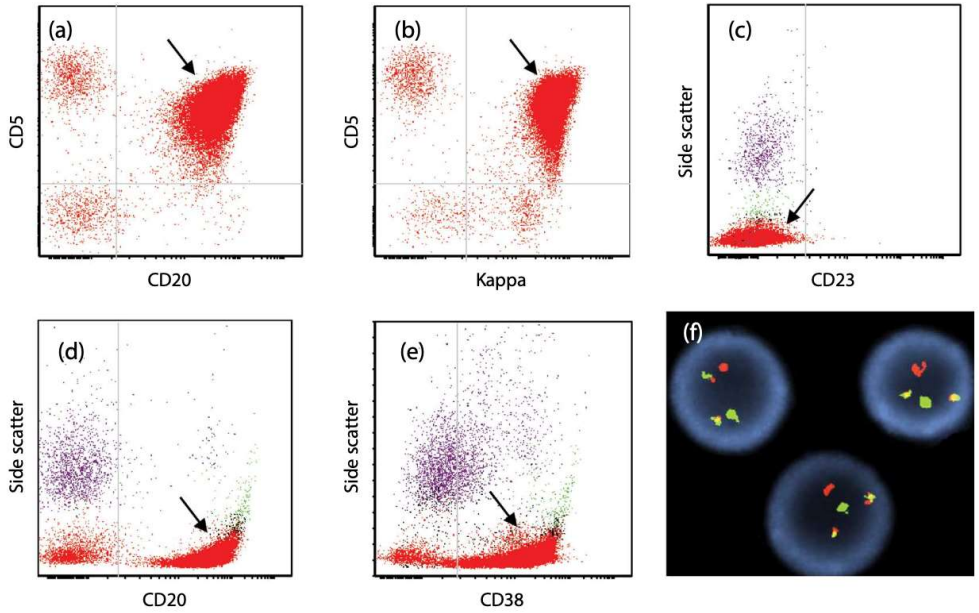


FIGURE 13.76 Mantle cell lymphoma – flow cytometry (blood). Lymphomatous cells display moderate expression of CD20 (a, d) and kappa (b), positive CD5 (a–b), negative CD23 (c), and positive CD38 (e). FISH studies performed on blood sample confirms the diagnosis of MCL by positive rearrangement of *CCND1* (f; yellow fusion signal).

Mantle Cell Lymphoma

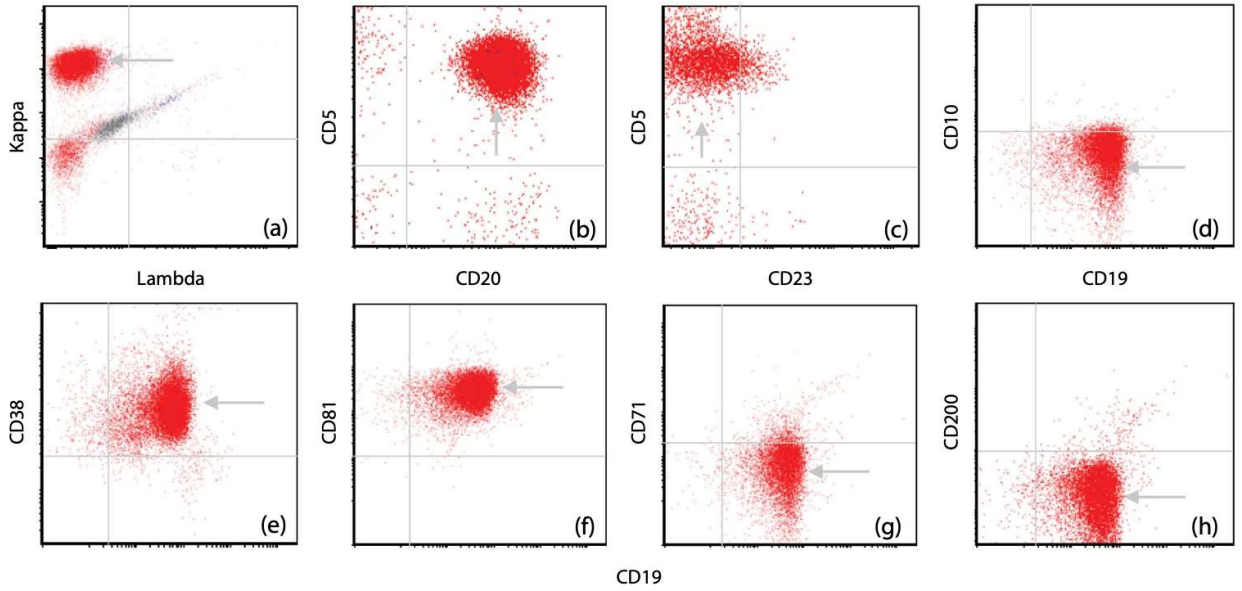


FIGURE 13.77 MCL – typical flow cytometry pattern. MCL is characterized by moderate expression of surface immunoglobulins (a) and CD20 (b), positive CD5 (b–c), negative CD23 (c), negative CD10 (d), positive CD38 (e), positive CD81 (f), negative CD71 (g), and negative CD200 (h).

The End