Chapter 3
Diagnostic Flow Cytometry and Immunophenotypic Classification

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3.1 Introduction

Haemopoiesis begins with a quiescent stem cell that gives rise to daughter cells capable of differentiation along multiple lineages. Differentiation progresses in a series of stages to produce functional, mature cells of all lineages and is orchestrated by sequential gene expression [1]. CD antigens are cell surface proteins which have diverse functional roles in haemopoiesis including signal transduction, enzymes, growth factor receptors and adhesion molecules and can be widely expressed or restricted to a specific stage of maturation/activation of a defined lineage. Thus, patterns of CD antigen expression can identify the lineage, maturation and functional stages of cells and are invaluable for evaluating normal haemopoiesis and the malignant state, including ALL.

While the immunophenotypic diagnosis of ALL was initially performed using fluorescence microscopy and immunocytochemistry, because of the increasing requirement for more extensive antigen expression, these methods have been supplanted by flow cytometry. This powerful methodology allows objective analysis of large numbers of cells in a short time and is multi parameter, with most routine cytometers having the capacity to measure expression levels of at least six antigens in each cell simultaneously. As such, it has become preferred method for the immunophenotyping for acute leukaemias (AL) and other haematological malignancies. Figure 3.1 shows the maturation cascade of B cells in a normal bone marrow compared to that of a patient with B-LL/Lymphoma, where the cells are arrested at an early stage of differentiation.

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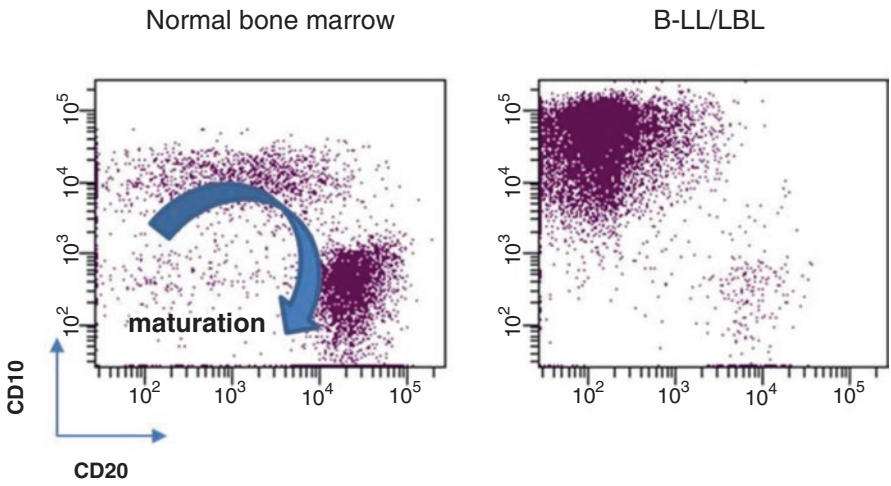

Fig. 3.1 Normal and arrested B cell maturation. Normal bone marrow and a newly diagnosed B-LL samples were labelled with antibodies to CD19, CD10 and CD20 and analysed by flow cytometry. *Dot plots* of CD10 and CD20 are shown of cells already gated for lymphoid and CD19 positivity

3.2 Immunophenotype of T-Lymphoblastic Leukemia/Lymphoma

The WHO classification defines T Lymphoblastic Leukemia/Lymphoma as a neoplasm of lymphoblasts committed to the T cell lineage involving BM, blood or presenting as a tissue-based mass involving thymus, lymph nodes or extranodal sites. By convention, a T-LBL diagnosis is made when there is no or minimal blood or BM involvement (<20% infiltrate), while a diagnosis of T-ALL is made when there is extensive blood and BM disease. T-ALL is less common that B lineage ALL, accounting for around 15% of all cases and is associated with older adolescents and a male predominance. In contrast, almost 90% of LBL are of T cell lineage.

While the earliest marker of T cell lineage is CD7, the most specific marker is CD3, thus CD7 and CD3 expression are required for the diagnosis of T-lineage leukaemia/(Table 3.1) [2]. Cytoplasmic CD3 precedes surface CD3 during normal lymphoid development and in two-thirds of T-ALL is solely cytoplasmic, with about half of these cases co-expressing cytoplasmic TCR proteins [3, 4]. In the remainder, CD3 is expressed on the cell surface along with TCR proteins. Other CD antigens usually expressed include CD2, CD5 and TdT and commonly expressed are CD1a, CD4 and/or CD8, CD10 and/or CD21. CD34, CD10 and myeloid antigens, including CD13 and/or CD33 can be expressed too [5]. CD79a is weakly expressed in about one third of cases. The antigen expression can define intrathymic

Table 3.1 Immunophenotypic subgroups of childhood ALL

Antigen expression -% of cases positive											
Subtype	CD19	CD22[a]	CD79	CD10	CD7	CD5	CD3	Cytoplasmic IgM	Surface IgM	Surface Ig κ or λ	Prevalence (%)
Early Pre-B	100	>95	>95	95	5	0	0	0	0	0	60–65
Pre-B	100	100	100	>95	0	<2	0	100[b]	0	0	20–25
B	100	100	100	50	0	0	0	>95	>95	>95	2–3
T	<5	0	30	45	100	95	100[a]	0	0	0	15–18

Modified with permission from Pui et al. [2]
[a]May be only present in the cytoplasm
[b]IgM heavy chains only

differentiation stages including early T-ALL (surface CD3−, CD4− and CD8−), mid or common (surface CD3−, CD4+, CD8+, and CD1a +) and late (surface CD3+, CD1a− and either CD4 + or CD8+). T-ALL usually have a more immature immunophenotype compared to T-LBL but there is overlap [6].

3.3 Early T-Cell Precursor ALL

In 2009, Coustan-Smith et al. used gene expression profiling to identify a distinct subtype of T ALL, known as early T-cell precursor (ETP) ALL [7]. ETP-ALL constitutes up to 16% of all T ALL and is characterised by the immunophenotype, CD1a and CD8 negative, CD5 weak positive or negative (<75% blasts positive) and positivity (>25% blasts) for at least one stem-cell or myeloid marker antigen including CD34, CD117, HLADR, CD13, CD33, CD11b and CD65. They also typically express CD2 and cytoplasmic CD3 and may express CD4, but these are not part of the definition. Although the gene expression profile of ETP-ALL is similar to that of the murine ETP, there is overlap with normal and myeloid leukaemia haematopoietic stem cell profiles and the mutational spectrum is more typical of myeloid malignancies (discussed further in Chap. 4) [8]. In early studies, ETP-ALL patients were shown to be high risk, with increased rates of remission failure, relapse and a poorer overall survival [7, 9, 10]. The high risk nature has also been observed in adolescent/adult studies, suggesting the need for development of a more effective clinical management strategy for this subgroup [11]. However, recent data from larger patient cohorts treated on more contemporary regimens show a non-significant (UKALL 2003) or no difference (COG AALL0434) in outcome for ETP versus non ETP T-ALL, thus at present there are insufficient grounds to alter risk stratification of children with an ETP-ALL immunophenotype [12, 13]. However, ETP-ALL has been added as a provisional new entity in the revised WHO classification of myeloid neoplasms and acute leukaemia [14].

3.4 Immunophenotype of B-Lymphoblastic Leukemia/ Lymphoma

B-ALL/LBL cells are characterized by the expression of the B-cell markers CD19, cCD22, and cCD79a and lack expression of cytoplasmic (or surface) CD3 and of myeloperoxidase. Most are positive for CD10, CD24, surface CD22, CD34 and Tdt [5], while CD20 is variable [15]. CD45 is often absent and is a useful for tracking disease during treatment [16] (see Chap. 6). There are three recognised stages, early pre-B ALL, pre-B ALL, and B-cell ALL. The first two are often grouped and referred to as precursor B ALL. Early pre-B ALL is characterized by absent immunoglobulin synthesis, thus surface immunoglobulins and cytoplasmic IgM heavy chains are undetectable and are the major group, representing 60–65% of the total. The next maturation step, pre-B ALL, is defined by the presence of cytoplasmic IgM heavy chains but no detectable surface immunoglobulins. The more mature, B-cell ALL, has expression of complete surface immunoglobulins and represents ~3% of childhood ALL cases. CD20 and CD10 are often expressed and CD34 is negative. Morphologically, they have a FAB L3 appearance and are associated with c-MYC gene rearrangements (see Chap. 4). B-cell ALL and the leukaemic phase of Burkitt's lymphoma are often indistinguishable. Mature B-ALL is treated as stage IV Burkitt's lymphoma.

3.5 Acute Leukaemia of Ambiguous Lineage

While most acute leukaemias (AL) can be classified as derived from the myeloid or lymphoid lineage, in up to 5%, blasts have immunophenotypic features of both or neither lineage. They may have blasts which co express myeloid and lymphoid markers (biphenotypic or BAL) or there are two different distinct populations of blasts (bilineal) (Fig. 3.2). Two diagnostic algorithms have been used to define this entity. The first of these was published in 1995 by the European Group for Immunological Characterization of Acute Leukemias (EGIL) who described a point system to score biphenotypic ALL [17, 18] (Table 3.2). The second, published in 2008 by the World Health Organization (WHO) defined acute leukaemia of ambiguous lineage as *showing no clear evidence of*

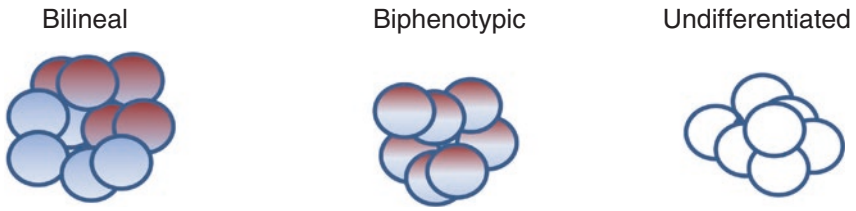

Fig. 3.2 Diagrammatic representation of bilineage, biphenotypic and undifferentiated acute leukaemia. *Red* and *blue* colouring representing myeloid and lymphoid CD antigen expression

Table 3.2 EGIL scoring matrix for lineage assignment

Points	T cell lineage	B cell lineage	Myeloid lineage
2 points	CD79	CD3	MPO
		TCR	
1 points	cCd22	CD2	cd13
	CD10	CD5	CD33
	CD19	CD8	CDw65
	CD20	CD10	CD117
0.5 points	Tdt	Tdt	CD15
	CD24	Cd17	CD15
		cd1A	CD24

Modified with permission from Bene et al. [17]
A score of 2 or more is necessary to assign a lineage

Table 3.3 2016 WHO criteria for lineage assignment for a diagnosis of mixed phenotype acute leukaemia

Myeloid lineage
[a]Myeloperoxidase (detected by flow cytometry, immunohistochemistry or cytochemistry)
or
Monocytic differentiation -at least 2 of the following: nonspecific esterase, CD11c, CD14, CD64, lysozyme
T lineage
Strong[b] cytoplasmic CD3 (detected by flow cytometry with antibodies to CD3 epsilon chain)
or
Surface CD3 (rare in mixed phenotype acute leukemia)
B lineage (multiple antigens required)
Strong[b] CD19 with at least *1* of the following strongly expressed: CD79a, cytoplasmic CD22, CD10
or
Weak CD19 with at least *2* of the following strongly expressed: CD79a, cytoplasmic CD22, CD10

Modified from Arber et al. [14]
[a]There are caveats related to weaker antigen expression, or to expression by IHC only
[b]Strong defined as equal or brighter than the normal B or T cells in the sample

differentiation along a single lineage and included acute undifferentiated leukaemia, where leukaemic cells have no lineage specific antigens and grouped bilineal and biphenotypic acute leukaemias under a new heading of Mixed Phenotype Acute Leukaemia (MPAL) [19] (Table 3.3). This new algorithm relies on fewer, more lineage-specific markers, with myeloid lineage designation requiring the presence of myeloperoxidase (detected by flow cytometry, immunohistochemistry, or cytochemistry) or at least two monocytic parameters including nonspecific esterase, CD11c, CD14, or CD64; T lineage, cytoplasmic or surface CD3 and B lineage, at least two antigens, including CD19, CD79a, CD22, and CD10. MPALs that are Ph+ or MLL rearranged are considered a distinct subgroup.

All possible combinations of MPAL are observed, with B/myeloid and T/myeloid representing ~90% of all cases, while and B/T and B/T/myeloid are more rare [20, 21]. Because of the more limited set of lineage markers and cytogenetic exclusions, several studies show fewer patients classified as WHO-MPAL, compared to the EGIL-BAL [22, 23]. However, whichever classification is used, they appear to have an inferior survival compared to standard ALL and are more in line with AML [24, 25]. While there is no consensus policy on the best treatment for MPAL, the iBFM AMBI2012 Study/Registry [26] aims to retrospectively analyse immunophenotype, molecular genetics, therapy and outcome of MPAL as a first step towards standardizing therapy and to better understand the biology of this AL subtype.

3.6 Antigen Expression: Correlation to Prognosis and Cytogenetics

While the above text describes the key antigens necessary to diagnose and classify ALL, the increasing availability of antibodies to a wide range of CD antigens has revealed substantial phenotypic heterogeneity. This is simply depicted in Fig. 3.3 showing the variability in expression levels of CD19, CD34 and CD10 in four diagnostic B lineage ALL. Clearly this heterogeneity is not fully represented by simply stating the percentage of positive cells and while mean fluorescent intensity is a gauge

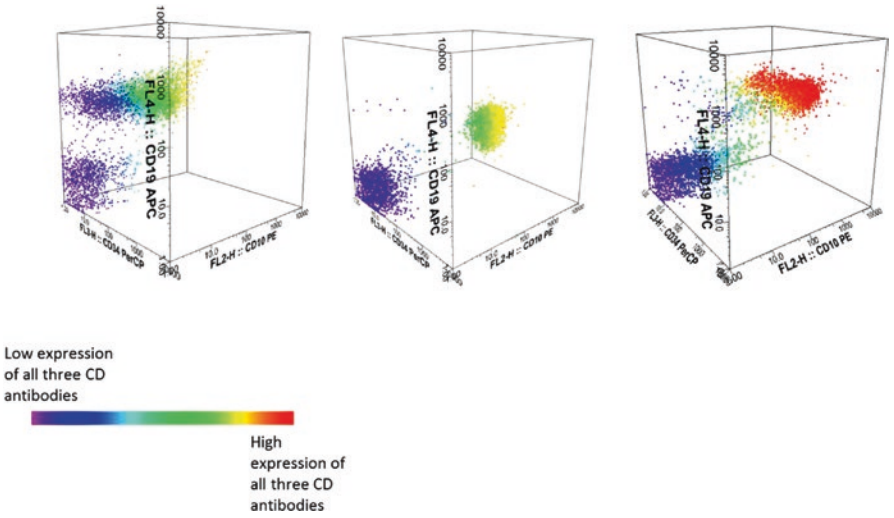

Fig. 3.3 Heterogeneity of CD antigen expression in ALL. Expression of CD34, CD10 and CD19 was assessed in three newly diagnosed precursor B ALL cells by flow cytometry and analysed using FlowJo software and depicted in three dimensions. Non leukaemic cells, negative for all three antigens, are *purple*, while leukaemic cells show a spectrum of colour depending on the intensity of all three antigens

of antigen levels, it is relative and can vary over time due to cytometer age, antibody batches and with instrument service and/or laser replacement. Absolute fluorescence can be measured using beads with varying amounts of known levels of fluorochromes which are used to create a standard curve from which fluorescence of an antigen in ALL cells can be expressed in units termed mean equivalents of soluble fluorescein (MESF). Using these approaches, several studies have investigated whether more extensive immunophenotyping can offer further prognostic relevance.

In a large American study (POG 1991) of more than 1200 children with B lineage ALL, fluorescent intensity as measured in absolute terms using the MESF approach found that two CD antigens, CD45 and CD20 were highly prognostic [27]. Patients with the brightest expression of CD45 (>75th percentile) or relatively bright CD20 (>25th percentile) on their ALL blasts had an increased risk of treatment failure which was independent of traditional risk factors including age, white blood cell count, DNA ploidy or poor risk chromosomal translocations. More recent data from a European clinical trial (IBFM 2000) confirmed these observations in both Pre B and T lineage ALL [28]. In this case, CD45 levels in ALL cells were expressed relative to that of normal mature lymphocytes in the same sample. Similar to the American trial, children with high CD45 expression were associated with a lower event-free survival (EFS) rate; for PreB this was 72% compared to 86% and for T ALL, 60% compared to 78%. The difference in EFS was mainly attributable to a higher cumulative relapse rate and again, CD45 expression maintained its significance in multivariate analyses. These findings may be explained by the functional role of CD45 as an integral membrane protein tyrosine phosphatase which regulates antigen receptor and cytokine signalling by dephosphorylating SRC and JAK family kinases, key pathways which regulate cell growth and survival and are known to be aberrant in ALL. Interestingly, the gene encoding CD45 (*PTPRC*) has recently found to be inactivated by gene deletion in T ALL [29], thus appropriate expression levels of CD45 appear critical for normal lymphocyte cell function.

While the CD45 correlative data are mirrored in two large independent clinical trials, for CD20, two separate reports (three consecutive St Jude Total Therapy trials and NILG-ALL 09/00) concluded that CD20 expression was not associated with inferior outcome [15, 30]. Whether this is due to differences in relative rather than absolute quantitation methodologies or loss of prognostic relevance in more contemporary regimens, is not clear. Other antigens of interest are those associated with the myeloid lineage but data are not consistent and their independence as prognostic markers is less clear. Another study showed that expression of CD38 was highly variable and low expression on leukaemic blasts relative to that of normal B cell progenitors was significantly associated with MRD positivity [31]. Since MRD positivity is a surrogate marker of outcome, it suggests that levels of this antigen too may have prognostic relevance.

One difficulty with correlating antigen levels with prognosis is the interdependence of immunophenotype with cytogenetic abnormalities which have well defined prognostic relevance and thus has prompted investigations into whether immunophenotyping can accurately classify key cytogenetics subtypes [32]. For example, those with the good prognostic *ETV6-RUNX1* ALL gene fusion, de Zen et al. showed

a higher intensity of CD10 and HLADR and lower levels of CD20, CD45, CD135 and CD34, compared to *ETV6-RUNX1* negative cases [26]. This 'characteristic' immunophenotype classified *ETV6-RUNX1* ALL with a sensitivity of 86% (i.e. the true positive rate) and a specificity of 100% (i.e. true negative rate). Another study prospectively evaluated an immunophenotypic signature for *ETV6-RUNX1* ALL which consisted of dual CD9 and CD20 negative/weak positivity in more than 200 children and found it to have an 88% sensitivity and 71% specificity for the presence of the gene fusion [33]. While these data are good, the general consensus is that they are not sufficiently robust to replace standard cytogenetic analyses to classify *ETV6-RUNX1* ALL, particularly since the fluorescence in situ hybridisation methodology used as standard to detect this gene fusion, will also identify high risk cases with iAMP21 (see Chap. 4) [34].

3.7 Extended Leukaemia Immunophenotyping

The EuroFlow Consortium [35] are international experts in the fields of flow cytometry and molecular diagnostics and aim to develop and standardize fast, accurate and highly sensitive flow cytometric tests for the diagnosis, prognosis and treatment effectiveness in haematological malignancies. The ALL immunophenotyping panel designed by the Euroflow consortium enables the diagnosis and subclassification of ALL according to the WHO classification but also goes on to provide more extensive immunophenotypic characterization [23]. This additional information allows discrimination of the leukaemia from normal and regenerating precursor B-cells, termed a leukaemia-associated immunophenotype, that can subsequently be used to track minimal residual disease during ALL therapy [36] (see Chap. 6) and also includes markers associated with genetic aberrations, such as the chondroitin sulphate proteoglycan, NG2, associated with 11q2 3 rearrangements [33]. In addition, they have developed a simple flow cytometric immunobead assay to detect ALL related fusion proteins in cell lysates which utilises a bead-bound catching antibody to detect one half of the fusion protein and a fluorochrome-conjugated antibody to bind the other half [37, 38]. Such assays have been developed for a number of fusion proteins relevant to ALL and have high specificity and sensitivity. For example, fully concordant results were obtained between the immunobead assay and reverse transcriptase PCR of fusion gene transcripts for BCR-ABL [38].

3.8 Conclusions and Perspective

Immunophenotyping has played an important role in the impressive improvement in survival rates for ALL over the last few decades and will continue to be essential in diagnosis and patient stratification. The recent impressive activity of novel antibody therapies such as the bi-specific T-cell engaging antiCD19/CD3 antibody,

Blinatumomab [39] and targeted immunotherapy using patient-specific chimeric antigen receptor T cells [40, 41], further emphasises the importance of immunophenotyping, since expression levels of the target antigen predict response and emerging resistance to these new agents. In one of the largest trials for relapsed ALL (IntReALL) [42], standard risk patients are being randomized to receive the targeted anti-CD22 drug, Epratuzumab during consolidation. CD22 levels have been shown to vary 100-fold [43], thus IntReALL flow laboratories have developed a standardised methodology to quantify both the levels of CD22 and amount of Epratuzumab binding in ALL cells relative to that of mature B cells in a fixed preparation of peripheral blood (Mejstrikova et al. unpublished observations). Subsequent correlation of these parameters with response will determine their value as predictive biomarkers of Epratuzumab response and optimise the use of this drug in future trials. Following the impressive clinical benefit of the tyrosine kinase inhibitor (TKI), imatinib, for children with Ph+ ALL [44], this other class of targeted drug is also likely to be increasingly used in ALL therapy. In this respect too, immunophenotyping may have an emerging role. The development of robust antibodies that are highly specific to phosphorylated antigens and suitable for flow cytometry allows key signalling pathways to be monitored for hyper-activation in ALL subgroups such as Ph-like (e.g. phosphorylated CRKL) or Ras pathway mutated ALL (e.g. phosphorylated ERK) [45, 46]. Patients with these high risk ALL types may be candidates for TKI therapies, such as dasatinib and MEK inhibitors, respectively.

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