Normal Blood and Bone Marrow Populations

It is essential to have a sound understanding of the nature and immunophenotypic characteristics of the normal cell populations encountered in bone marrow and peripheral blood. It is also important to have some understanding of lymphoid cellular interactions in the normal lymph node and thymus. This process is necessary if we are to understand the origin of the myeloid and lymphoid neoplasms encountered in routine diagnostic immunophenotyping and helps us understand why certain neoplasms carry a particular repertoire of antigens. It not only helps in the diagnosis of neoplastic disorders but equally gives support to the recognition of reactive phenomena.

The populations of cells which inhabit the bone marrow are constantly changing throughout the life of a normal individual. When this environment is challenged by infection, drugs, intercurrent medical disease and, of course, primary haematological disease, the populations change or can respond and react in a variety of ways. We need to be aware of these reactions such that we do not over or under interpret the findings. This chapter is set out as a framework or foundation of normal myeloid and lymphoid ontogeny. Our understanding of this process is still incomplete but we can only safely recognize abnormal situations once we are fully familiar with the normal resting blood and bone marrow cellular environment.

Normal stem and precursor cell populations

Haematopoietic progenitor cells
Recognition of the immunophenotypic patterns of maturation in normal bone marrow facilitates the identification of acute leukaemia (AL) blast cells, even if they occur at low frequencies. The sequential expression of antigens during normal marrow cell maturation and differentiation from a common stem cell population is well described in the literature but needs to be fully understood.

Normal haematopoietic stem cells (HSC) are CD34+ (conversely not all leukaemic blasts are CD34+, a common source of confusion) [1]. In normal steady state marrow the total CD34+ fraction comprises HSC (early progenitor cells; that is, uncommitted cells that can produce multiple lineages), myelomonocytic (also CD123+), erythroid (CD71+) and B-cell (CD19+) precursor cells [2]. Other antigens expressed by uncommitted HSC include HLA-DRdim and CD133, with the latter broadly correlating with CD34 expression. As the HSC population differentiates, they gain CD38 expression, a so-called ‘activation marker’ in conjunction with the early lineage commitment antigens detailed above.

By definition, true HSC lack lineage commitment antigens (i.e. markers of lymphoid or myeloid differentiation), a situation often abbreviated to lin-. Furthermore, many primitive haematopoietic stem cells are quiescent (out of cell cycle), with absence of both activation markers and markers of cell division, such as Ki67. These cells comprise only a very small fraction of total marrow CD34+ cells, however, as this antigen continues to be expressed into the early-committed myeloid and lymphoid progenitor compartments before it is lost during further maturation. Despite this caveat, CD34 is a very useful surrogate marker for the normal HSC compartment, and it is routinely used for assessment of stem cell harvests in the transplantation setting, for example, as well as in stem cell research. CD34lin+ cells are therefore committed progenitors and represent the first
identifiable stage through which cells mature sequentially down a particular differentiation pathway.

Normal precursor cell populations in the marrow may occasionally cause confusion in the diagnosis and monitoring of acute leukaemias. This is largely because they share many antigens of immaturity in common with leukaemic blast cells as well as morphological similarities. One of the most obvious differences is that AL blasts are usually much more numerous than normal precursors; for the most part, progenitor cells in normal steady-state marrow samples are present at low levels. CD34⁺ cells are generally no more than 1–2% of all marrow cells, while CD117⁺ cells, an antigen that is expressed beyond CD34 in myeloid progenitor cell populations, are generally no more than 2–4% [3–5]. Approximately 25% of CD34⁺ cells are CD117⁺ [6]. However, regenerating marrows often express proportionally higher levels of progenitor cell antigens than marrows in steady state, particularly in the case of normal B-cell precursors (haematogones) after chemotherapy or stem cell transplantation for example [7, 8]. In general, for practical purposes, the authors consider a CD34⁺ population of ≥ 3% or a CD117 population ≥ 5% as significant and worthy of full characterization, although this is dependent on the clinical scenario and assessing lower levels than this may be relevant in some circumstances (e.g. post induction therapy in AL or distinguishing MDS from aplastic anaemia in a hypoplastic marrow). Particularly at low levels of blast numbers, demonstration of an abnormal phenotype is of more significance than accurate quantification in most circumstances, particularly when the potential pitfalls in blast enumeration are taken into account (see below). One further complication is that aberrant or asynchronous maturation can be observed in normal situations, for example expression of CD56 on granulocyte and monocyte precursor cells in regenerating marrows both with and without G-CSF administration. Furthermore, unusually synchronous maturation may be observed in this situation (e.g. accumulation of promyelocytes as the regenerating cells expand and pass through maturational stages together) requiring meticulous attention to flow, morphology, genetics as well as the clinical history to avoid erroneous interpretation. These situations are little mentioned in the literature but should be recognized relatively easily by an experienced cytometrist.

CD45 (a pan-leucocyte antigen) is generally dimly expressed on marrow precursor cells, including myeloblasts, monoblasts, precursor B-cells and erythroblasts [9]. It is, by comparison, bright on mature cells such as monocytes, neutrophils and lymphocytes, although it is lost altogether by later erythroid precursors. CD45dim is therefore a useful feature to allow gating on blast cells of many AL, and it is commonly used in conjunction with the low SSC that is also seen in precursor cells [10], redrawn schematically in Figure 4.1.

By incorporating CD13, an early marker of myeloid differentiation, the following progenitor populations can therefore be easily defined in normal marrow:

1. A CD34⁺CD117⁺CD45dimCD13⁺ pattern, which defines the normal myeloid/monocytic progenitor population.
2. A CD34⁺CD117⁻CD45dimCD13⁻ pattern, which defines the normal precursor B-cell population. These cells would in addition express B-cell antigens.

Figure 4.1 Distribution of normal bone marrow populations according to CD45 expression and side scatter properties.
For the purposes of further discussion of normal maturation patterns we will define two major populations:

1. Myeloid (neutrophils, eosinophils, basophils, monocytes, erythroid, megakaryocytes, mast cells and dendritic cells)

2. Lymphoid (B and T-cells)

It is worth drawing attention to two excellent resources with regards precursor cells and normal marrow maturation that are freely available. First, there exist useful consensus descriptions of cytology in normal and abnormal myelopoiesis which have recently been produced by the International Working Group on Morphology of Myelodysplastic Syndrome (IWGM-MDS) [11, 12]. Second, is the most recent of several attempts to accurately document the maturation patterns of precursor cells in normal marrow. The most comprehensive study to date has been performed on behalf of the European Leukaemia Net (ELN) [13]. This study exhaustively documents scatter plot patterns of antigen expression using predefined standard antibody panels, and provides a normal framework upon which abnormal populations can be identified.

Myeloid maturation

As well as being present on pluripotent stem cells, CD34 remains brightly and consistently expressed by committed normal lymphoid and granulocyte – monocyte progenitors, but rapidly reduces in intensity as further differentiation occurs. Along with HLA-DR, it is lost as normal myeloblasts mature into promyelocytes. CD117, the c-kit receptor, is, however, expressed beyond CD34 (including promyelocytes), and is only lost as the cells mature towards the myelocyte stage [14]. As a consequence CD117 therefore describes a larger and slightly more mature myeloid progenitor population than CD34 (as well as being a hallmark of mature mast cells, although these are present at very low frequency in normal marrow). Note that this typical immunophenotype of normal promyelocytes (i.e. CD34+HLA-DR−CD117+) is partly ‘preserved’ in the leukaemic process, and is of use in identifying typical acute promyelocytic leukaemia (APL). However, as with almost all situations where AL may be seen to mirror a particular maturational stage, there are notable differences in antigen expression; malignant promyelocytes are therefore ‘similar but different’ to their normal counterparts. Knowledge of these differences is important in identifying such cells as leukaemic.

Variations in antigen intensity also occur during maturation; for example CD13 and CD33 expression on myeloblasts progressively increase in intensity up to and including the promyelocyte stage. This CD33bright population then loses intensity (but remains positive) as maturation to neutrophils takes place. This is in contrast to monocyte maturation which is characterized by a continual increase in CD13 and CD33 intensity as cells mature. Late antigens appearing during the myelocyte-neutrophil stages include CD15 (which first appears at a late promyelocyte stage) and CD11b [14]; CD16 and CD10 are expressed from the metamyelocyte/band-form stage onwards. Mature neutrophils therefore have a CD45+, CD13+, CD33+, CD11b+, CD15+, CD16+ phenotype. CD64 may be expressed on early myeloblasts, but disappears beyond this stage. It may be re-expressed by activated neutrophils, where bright CD64 expression can be indicative of sepsis [15]. A graphic summary of myeloid maturation is shown in Figure 4.2.

Monocyte maturation follows a similar course (Figure 4.3). Very early monoblasts are CD34+CD117+ (and are largely indistinguishable from normal myeloblasts), both of which diminish and disappear with evolution to a promonocyte stage [9]. HLA-DR, in contrast to the situation with granulocytic maturation where it disappears at the promyelocyte stage, remains expressed throughout monocyte maturation. Further differences include the acquisition of CD4 at the promonocyte stage (with normal monocytes remaining CD4dim), followed closely by CD64 and CD11c. CD14 becomes brightly expressed at the mature monocyte stage. CD4, CD64 and CD14 are therefore useful markers to identify populations of monocytic lineage. CD14 is specific to monocytes. However, CD4 and CD64 may be found on other normal populations (i.e. CD4 on T-cells and CD64 on myeloblasts, dendritic cells and activated neutrophils). CD15 is expressed on both neutrophils and monocytes. Overall frequency of monocytes is variable in normal steady-state marrow samples, but is in the region of 1–8% [9]. This can increase in reactive conditions and neoplastic processes involving the monocyte lineage.

Eosinophils are present in small numbers in normal bone marrow but their acknowledgement is important in that they should not be mis-identified in circumstances where they are increased, for example myeloid
Figure 4.2 Sequential bone marrow maturational stages of the myeloid lineage.

Figure 4.3 Sequential bone marrow maturational stages of monocyte lineage. Note the immunophenotypic characteristics of monocyte precursors on the maturational pathway to mature monocytes and macrophages encountered in blood and tissues, respectively. The characteristics noted here are important in understanding the immunophenotypic profile of monocytic neoplasms.
disorders, especially chronic myeloid leukaemia (CML) and reactive conditions such as allergy, inflammation and vasculitis. On the FSC/SSC plot eosinophils show high side scatter due to their intense granularity and appear slightly smaller on FSC than neutrophils. They express CD45\text{mod}, CD13, CD11b, CD66 and CD16\text{dim}.

Basophils are also present at low levels in normal blood and marrow, but are increased in CML and some subtypes of AML; they have a low side scatter compared to other myeloid cells and are difficult to distinguish from lymphocytes and monocytes based on light scatter characteristics. They express CD45\text{mod}, CD13, CD33 and CD38. Interestingly, they also express CD123, CD25\text{dim}, CD9 and CD22 \cite{16}, the latter in the absence of any other B-cell associated antigens. They can also show an aberrant phenotype in clonal disease states \cite{17}.

**Erythroid maturation**

Primitive erythroblasts have a generic early myeloid precursor phenotype, namely CD34\text{+} CD117\text{+} CD45\text{dim} CD38\text{+}. As with the other lineages these markers of immaturity are rapidly lost during maturation through the pro-erythroblast to basophilic erythroblast phases, and expression of CD71 and CD235a (glycophorin) becomes established. Later forms express CD36 as CD45 is progressively lost. Note that CD71, the transferrin receptor, is detectable on most actively proliferating cells (i.e. it is another ‘activation marker’) and is not lineage specific. Expression is, however, at its brightest on the erythroid series, presumably due to the need for large amounts of iron. CD71 is then lost as reticulocytes become mature red cells.

**Megakaryocytic maturation**

These mature from the generic normal myeloid precursor phenotype (as above) with progressive loss of precursor markers, as lineage-specific CD41, CD42 and CD61 are gained. Platelets maintain expression of these antigens (discussed in more detail in Chapter 10) but are CD45\text{−}.

**Lymphoid maturation**

Early B-cells are produced in the marrow, where they also undergo the first stages of maturation. The antigen expression patterns of maturing B-cells have been described in meticulous detail by several groups \cite{7, 18, 19}. While this process occurs as a physiological continuum, particular stages of antigen expression are seen and can be broadly correlated with the maturation arrest stages typical for B-ALL \cite{4}. An understanding of these patterns can, for example, prevent misidentification of Burkitt’s leukaemia/lymphoma as a precursor cell neoplasm.

Mature circulating B-cells express CD19, CD20, CD79a and surface immunoglobulin, but lack CD10 and precursor cell markers. They are therefore easy to separate from the earliest B-cell precursors identifiable in the marrow, which are CD34\text{+} TdT\text{+} CD10\text{bright} CD19\text{dim} CD22\text{dim} CD79b\text{+} CD20\text{+} \cite{7, 18}. No cytoplasmic or surface immunoglobulin is present in these cells. As these cells mature CD20 becomes positive and steadily more intense as does CD22 (brightly expressed on mature B-cells). Note, though, that this does not imply that CD20 is only expressed in mature B-cell malignancies; again this is a common misconception amongst trainees, perhaps reinforced by the use of anti-CD20 antibody therapy (largely) in the setting of mature B-cell disorders. This therapy is currently being tested in precursor cell ALL within clinical trials (UKALL14 trial protocol), precisely because these disorders are often found to express it (perhaps only 20\% at diagnosis, but some experimental data suggest that steroids may promote expression and hence susceptibility to anti-CD20 \cite{20}). As B-cells mature cytoplasmic immunoglobulin heavy chains (μ) are expressed and markers of immaturity (CD34, TdT and CD10) are steadily lost as surface immunoglobulin is gained (Figure 4.4).

The proportions of these maturational stages in bone marrow may differ significantly. In normal paediatric marrow specimens B-cell precursors may comprise up to 40\% of all mononuclear cells, even in steady state conditions. When marrow recovery post-chemotherapy is analysed there is a marked left shift to favour the most immature precursors (see dedicated section on haemato-gones below). Conversely, during ageing there is a steady reduction of immature B-cell progenitor activity in the marrow, with similar levels of the more mature forms preserved throughout life. The presence of these normal populations must be borne in mind when assessing for possible B-cell progenitor neoplasms. As a general rule, normal maturation is characterized by gradual gain or loss of relevant antigens, producing a smear of expression from low
to high or vice versa. This can be contrasted with a much more homogenous expression, often of abnormal intensity (i.e. too bright or too dim for the relevant normal maturational stage) in lymphoid malignancy.

T-cell development in contrast does not normally occur in the BM, but is initially confined to the thymus. In a manner similar to B-cell development several stages of T-cell development can be described, which bear some resemblance to the maturational stages of T-cell lymphoblastic leukaemia. These stages are summarized in Figure 4.5.

In parallel to this process the T-cell receptor is progressively rearranged and finally expressed on the cell surface in a complex with surface (s) CD3. The majority (>95%) of circulating T-cells express a receptor containing chains designated alpha and beta (TCR alpha/beta). The remainder express receptors for gamma and delta chains (TCR gamma/delta). Alpha-beta and gamma-delta T-cell development diverge at an early stage of development within the thymus and these subclasses differ in their tissue distribution, function and role in the immune response. Restricted surface expression of alpha-beta or gamma-delta receptors is an indicator of clonality and to some extent helps classify the neoplastic T-cell disorders. Finally, T-regulatory cells (Tregs) express a transcription factor FoxP3 which appears integral to the function of these CD4\(^{+}\), CD25\(^{+}\) cells. Tregs are important in countering inappropriate immune responses to external or internal antigens, in the form of allergy and autoimmunity, respectively. Depletion of these regulatory cells by treatment, for example purine analogues, may be one of the factors which allow autoimmune phenomena to develop.

**Haematogones**

It is important to pay particular attention to a population of normal bone marrow cells called haematogones. These are normal B-lymphocyte progenitor cells which are prominent in the bone marrow in infants and children and in adults recovering from chemotherapy. They do not cause problems in the assessment of remission status in myeloid leukaemias but they can be easily mistaken

![Figure 4.4](image-url) Sequential bone marrow maturational stages of B-cell precursors. The progressive maturation from primitive B precursor cells to mature circulating B-cells is illustrated above. Note the gradual and sequential changes in antigen expression, which contrasts with the maturational arrest picture of a B lymphoblastic leukaemia. Note that the earliest stage is typified by TdT, CD34 and CD10\(^{bright}\) expression. CD10\(^{+}\) subpopulations predominate in children, whilst in adults the CD10 \(\text{CD20}^{+}\) population is the most common [9]. This figure is adapted from data presented in work by McKenna *et al.* [21].

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**Figure 4.5** Table of antigen expression stages of B-cell development.
for low level residual disease in precursor B acute lymphoblastic leukaemia. As the latter entity is the commonest malignancy in children and as paediatric marrow often shows prominent haematogones, it is clear why this issue is important.

Haematologists have been aware of haematogones for over 70 years, but their nature has only been adequately studied with the introduction of flow cytometry. These studies have allowed the characterization of haematogones as a distinct normal bone marrow constituent and their immunophenotypic characteristics are now well characterized.

Morphologically, not surprisingly, haematogones show a spectrum of features. Very early type I haematogones are medium to large cells with a B lymphoblast type appearance showing heterogenous chromatin, sparse agranular cytoplasm and possibly nucleoli (Figure 4.6). Later type II and type III haematogones show increasing morphological maturity with condensation of nuclear chromatin, loss of nucleoli and decreasing size [22]. These later cells are difficult to differentiate on microscopy from normal mature marrow B-cells. Haematogones can be detected in small numbers in most bone marrow specimens and constitute up to 1% of nucleated cells in adults [23], or a mean of 7.15% of nucleated cells in children [18]. They are present at extremely low levels in peripheral blood except in neonates and cord blood [24].

Haematogones have a B-cell precursor type immunophenotype. It is important to recognize, however, that they show a changing surface antigen profile according to their

**Figure 4.5** Sequential thymic maturation of T-cell precursor cells.

T-cell maturation takes place largely within the thymus (over a period of three weeks or so), arising from a common lymphoid progenitor cell in the marrow. The earliest T-cell precursor is known as a triple negative (TN) due to lack of expression of CD3, CD4 and CD8. Note that they express CD34, nuclear TdT and bright CD7. Cortical thymocytes have lost CD34, gained CD1a and then mature from double negative (DN, CD4- and CD8-) to double positive cells (DP, CD4+ and CD8+). As surface CD3 (sCD3) is expressed, TdT is lost. The final stage of maturation, in the thymic medulla, is characterized by the selection of either CD4 (T-helper cell) or CD8 (T-cytotoxic/suppressor cell) phenotypes.
stage of maturity. The early forms express CD34, TdT, DR, CD19 and CD10, whilst later maturing haematogones start to lose CD34 and TdT and acquire CD20. The intensity of antigen expression also changes as these cells progress through stages of maturation [18, 21, 25]. The immunophenotypic maturation of haematogones in relation to mature B-cells is summarized in Table 4.1 above.

Each stage of haematogone maturation is normally present in different proportions. Stage II haematogones, the intermediate stage, normally constitute about two-thirds of the total, whilst the remainder is equally made up by stages I and III. Bone marrows recovering post chemotherapy often show a significant increase in haematogones, particularly in the stage I population (left shift). Similar increases can be seen in autoimmune cytopenias and in bone marrows recovering after viral infection. Reduced numbers are seen in bone marrow infiltration, bone marrow hypoplasia and myelodysplasia. In many respects they are reflective of the degree of potential bone marrow reserve. In bone marrow failure syndromes a bone marrow transplant can successfully regenerate normal haematogone populations [22].

Precursor B lymphoblasts have a variable immunophenotype according to the maturity of the cell of origin and this explains why it is not possible to arrange a straight comparison. The individual patient precursor B-ALL phenotype has to be considered in relation to haematogones in the context of assessing remission status. For example, a pro B-ALL with absent CD10 and aberrant myeloid antigen expression can easily be identified in low numbers in bone marrow (discussed in greater detail in the next chapter). Similarly, a pre B-ALL expressing uniform CD20, with absent CD34/TdT may be more easily discerned from haematogones. The common ALL immunophenotype CD34+, TdT+, CD19+, CD10+ is the most frequently seen lymphoblastic

Figure 4.6 Type I haematogones. Later haematogones are difficult to photograph as they cannot be differentiated from normal maturing B-cells.

Table 4.1 The stages of haematogone maturation in contrast to mature B-cells.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Mature B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TdT</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>CD34</td>
<td>Pos</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>CD10</td>
<td>Pos_{bright}</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>CD19</td>
<td>Pos_{dim}</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>CD38</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos</td>
<td>Pos / neg</td>
</tr>
<tr>
<td>CD20</td>
<td>Neg</td>
<td>Pos_{dim}</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>Cyt IgM</td>
<td>Neg</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
</tr>
</tbody>
</table>
When assessing remission here, it is of utmost importance to compare the diagnostic ALL immunophenotype, including fluorescence intensity for each antigen, with the immunophenotype of any coexisting haematogone population. It is low levels of common ALL which can cause most difficulty, particularly as CD19+CD10+ haematogones are normally increased in bone marrow samples taken during recovering from chemotherapy.

A schematic comparison of immunophenotypic characteristics of haematogones with mature B-cells and common ALL blasts is shown in Figures 4.7, 4.8, 4.9, 4.10, 4.11 and is derived from a

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**Figure 4.7** A schematic scatter plot showing the distribution of haematogones and normal B-cells according to CD19/CD10 expression (the inverted S pattern).

**Figure 4.8** As per Figure 4.6, with common ALL blasts superimposed.

**Figure 4.9** A schematic scatter plot showing the distribution of haematogones and normal B-cells according to CD34/CD20 expression.

**Figure 4.10** As per Figure 4.9, with common ALL blasts superimposed.

**Figure 4.11** Expected distribution of haematogones and ALL blasts according to CD38 and CD45 expression.
WORKED EXAMPLE 4.1

Illustrated below are a series of plots from a bone marrow analysis of a patient with a residual population of common ALL blasts alongside haematogones. First, note the separation of lymphoblasts (red, 25% events) from haematogones (blue, 75% events) according to the CD45^dim gate: haematogones appear less dim than blasts. The blasts have a CD34^+ TdT^+ CD79a^+ CD10^{bright} CD20^- phenotype whilst the haematogones (which are predominantly type II) are CD34^- TdT^- CD79a^+ CD10^{mod}. Second, note the particular characteristic of the haematogones in terms of the smeared acquisition of CD20 (CD10/CD20 plot) unlike the blasts which have a ‘fixed’ phenotype. Finally, note the difference in strength of CD10 expression between the two populations.
condensation of a number of important publications [18, 19, 21–23, 25, 26]. It is clear that it is not just the nature, but also the intensity of antigen expression, which is important. Haematogones demonstrate a consistent reproducible maturational pattern and should not show a discordant or aberrant immunophenotype. One exception to the rule might be following anti-CD20 monoclonal antibody therapy as this can cause diminished or blocked CD20 expression not only in neoplastic cells but also in marrow type II/III haematogones [22].

So the template is set and the standard immunophenotypic profiles of the normal blood and bone marrow populations have been described. Proceed to work through the subsequent chapters where the wide spectrum of disorders encountered in the diagnostic immunophenotyping laboratory will be considered.

References


