PART 1
Hematopoiesis

CHAPTER 1
Normal and Malignant Hematopoiesis

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Introduction

Hematopoiesis, simply stated, describes the regulated process of hematopoietic stem cell (HSC) self-renewal and differentiation into lineage committed progeny. Pluripotent HSC are rare cells (<1 of 10 000 bone marrow cells) specifically characterized by their proliferative capacity (though under steady state conditions >95% of HSC are quiescent, non-dividing cells at any one time), pluripotency (they can regenerate the entire spectrum of mature blood derived cells), and self-renewal. The hierarchy of hematopoietic cell differentiation is depicted in Figure 1.1. HSC reside in close association with hematopoietic stromal cells within specific microenvironmental niches that function in concert with a variety of both multilineage and single lineage-specific hematopoietic growth factors, stromal cells, and extracellular matrix molecules to regulate their survival, cell cycle progression, proliferation, and differentiation. These processes of self-renewal, proliferation, differentiation, and cell death are tightly regulated under normal conditions throughout life. A normal individual maintains steady state numbers of blood cells within a very tight range with no more than a few percent variation from day-to-day, with constant production of the number of new cells required to replace the number of senescent cells that die. On average erythrocytes survive in the circulation for about 120 days, platelets for about 10 days, and neutrophils for about 6–12 hours. In order to replace senescent blood cells, the bone marrow of normal adult humans must produce about 180–250 billion erythrocytes, 60–100 billion neutrophils, and 80–150 billion platelets every day, or about 10^{16} (10 quadrillion) blood cells in a lifetime, with only minimal reduction in the bone marrow cell production capacity as a result of aging. The bone marrow can respond rapidly, in lineage-specific manner, to increase production of new blood cells by 6- to 8-fold over baseline under conditions of demand for each specific type of blood cells, such as *in vivo* destruction of erythrocytes, platelets, or neutrophils, infections requiring increased neutrophil production, and hemorrhage requiring increased erythrocyte production. Regulation of lymphocyte numbers is much less clearly understood, although it is known that some types of T and B lymphocytes may survive for many years. An understanding of these normal regulatory components in normal hematopoiesis is essential to unraveling the mechanisms that drive malignancy.

Isolation of hematopoietic progenitors

In 1961, Till and McCulloch isolated single cell-derived colonies of myeloid, erythroid, and megakaryocytic cells (CFU-S) from the spleens of lethally irradiated mice 1–2 weeks after rescue by bone marrow transplantation [1]. These colonies were

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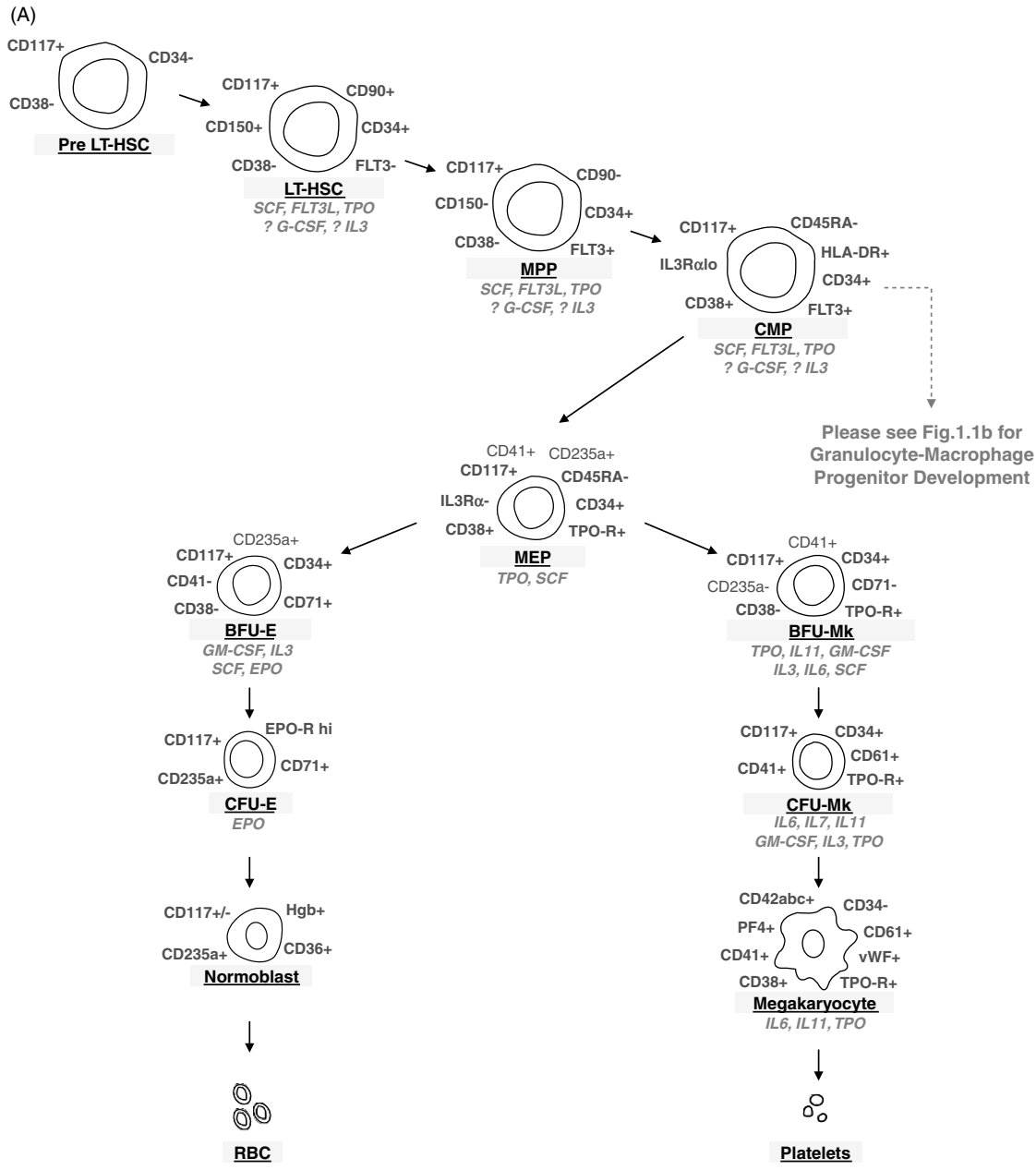
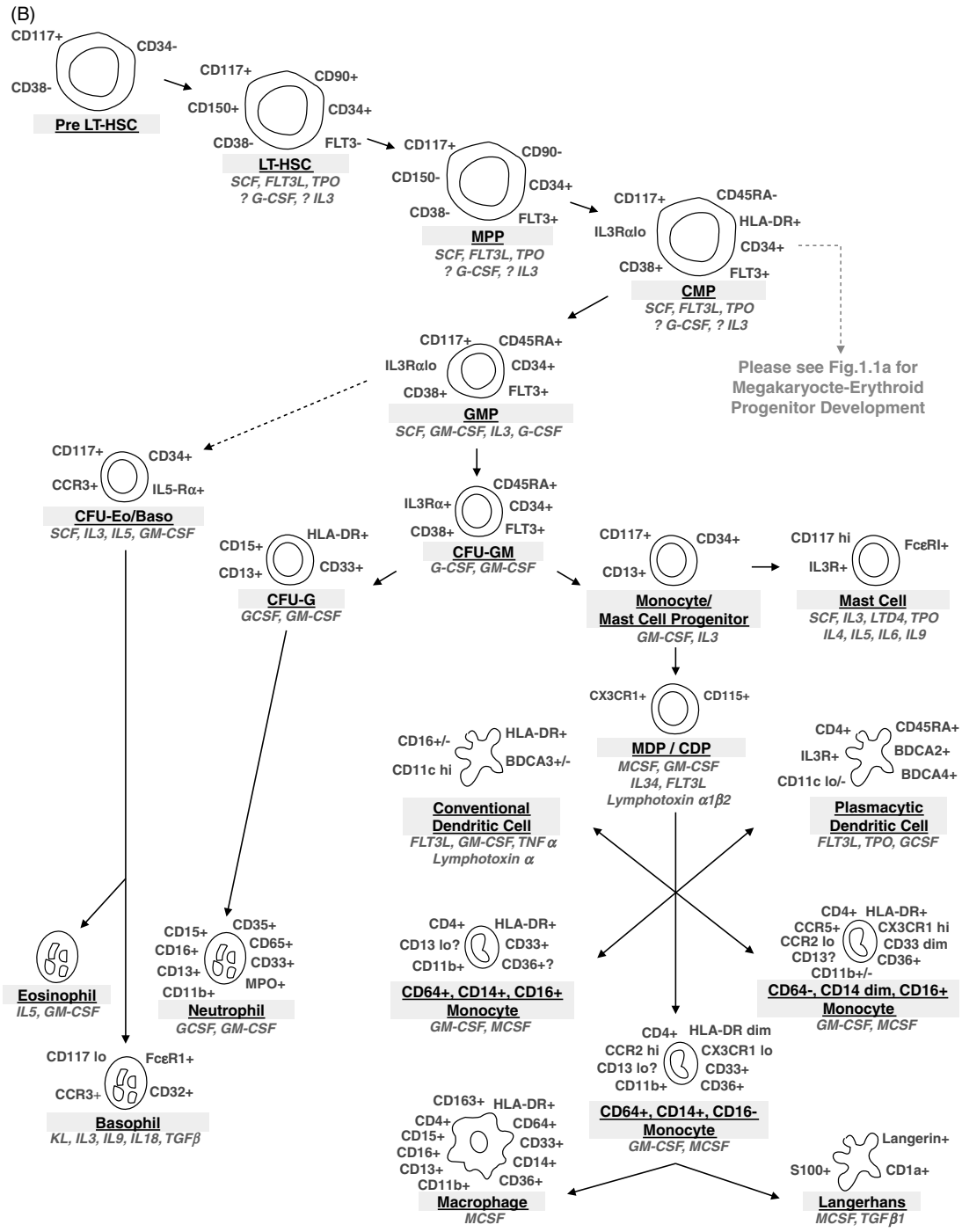


Figure 1.1 Schematic diagram of hematopoiesis highlighting identifying cell surface markers (in gray) and cytokines affecting each stage of hematopoietic differentiation (in italic). (A) Differentiation from hematopoietic stem cells through erythrocytes and megakaryocytes/platelets. (B) Differentiation from hematopoietic stem cells through granulocytes and monocytes/macrophages. (C) Differentiation from hematopoietic stem cells through lymphocytes.



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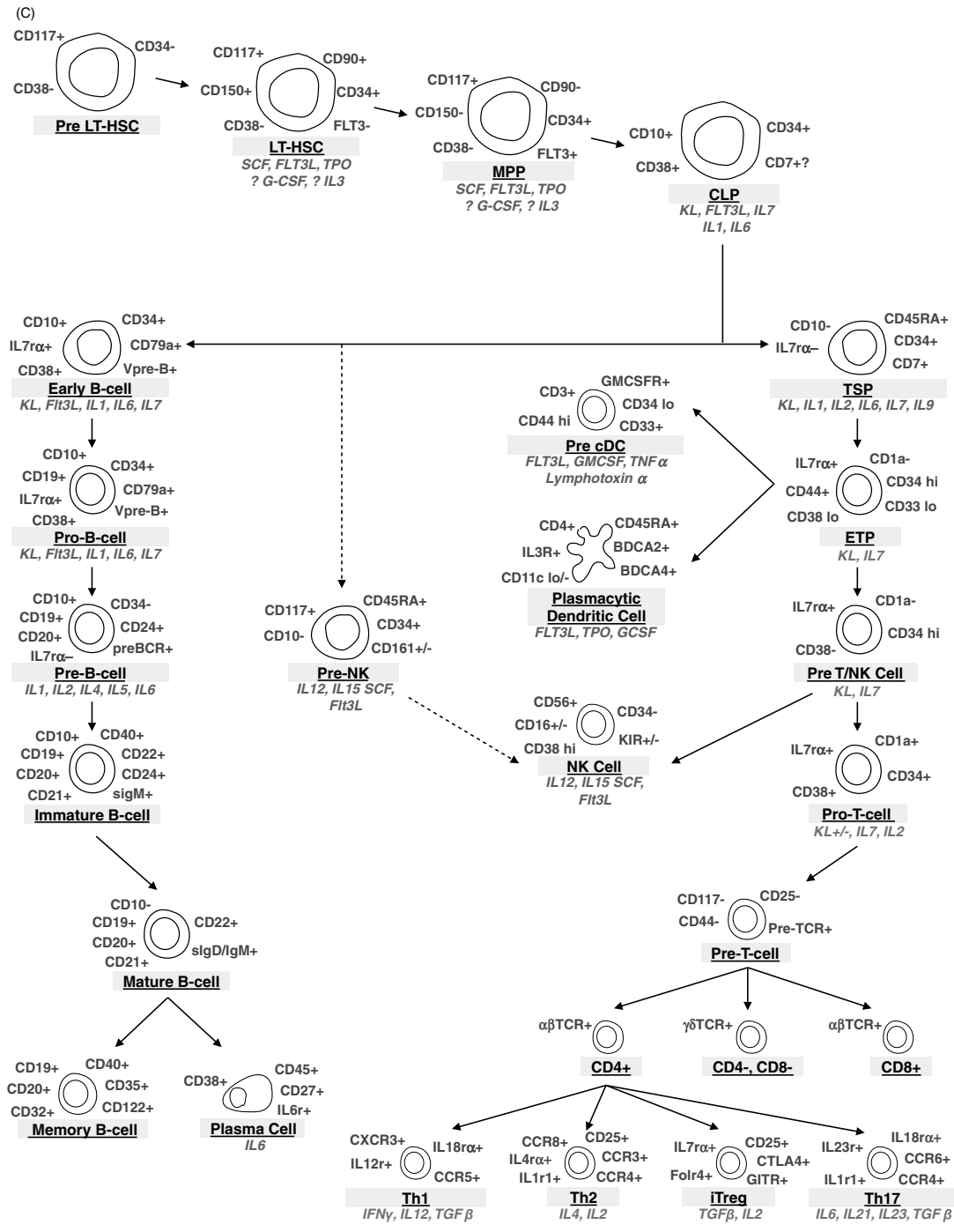

Figure 1.1 (*Continued*)

capable of extensive proliferation *in vivo*, exhibited some potential for self-renewal and, for the first time, conclusively demonstrated the presence of a multipotent hematopoietic progenitor cell. However, the lack of lymphoid colony development, as well as experiments in which 5-fluoruracil killed CFU-S without killing cells capable of replenishing CFU-S suggested that a more primitive "pre-CFU-S" must exist [2].

These data were further refined with the advent of flow cytometry, fluorescence activated cell sorting (FACS), *in vitro* hematopoietic progenitor cell systems, and xenotransplantation models, which revealed that long-term bone marrow repopulating HSCs were distinct from CFU cells, or multipotent progenitors (MPPs), and could be further subdivided into cells with short-term (ST-HSC) and long-term (LT-HSC) hematopoietic stem cell repopulation capacity. Specifically, LT-HSCs are defined by their extensive self-renewal capacity, allowing for full reconstitution of an irradiated host following transplantation of these cells. ST-HSCs, alternatively, have less capacity for self-renewal and instead more avidly differentiate into more committed MPPs. As such, ST-HSCs provide short-term hematopoietic cell reconstitution, but are incapable of permanently rescuing humans or other mammals with an aplastic bone marrow after lethal ionizing radiation.

Although some controversy exists, the most widely accepted model suggests that hematopoietic lineage commitment is both a stochastic and instructive process that occurs at specific branchpoints, manifested at the time of cell division. During cell division, HSCs can either divide asymmetrically (a maintenance event with the production of one identical immature daughter cell and one differentiating daughter cell), symmetrically (an expansion/self-renewal event which serves to generate two identically immature daughter cells (self-renewal)), or terminally differentiate (an extinction event, in which both daughter cells are committed to terminal differentiation). The hierarchy of differentiation from HSC to mature end-stage hematopoietic cells is shown in Figure 1.1. As cells progressively differentiate into functional components of the hematopoietic system, they lose proliferative and multilineage differentiation capacity. Regulation of self-renewal, cell cycling, terminal differentiation, and apoptosis is therefore critically important to maintaining the production of hematopoietic elements over a lifetime. It is now clear that extrinsic and intrinsic systems act in concert to generate a network of events that govern HSC fate.

Cytokine regulation

Cytokines/growth factors include interleukins, lymphokines, monokines, interferons, chemokines, colony-stimulating factors (CSFs), and other hematopoietic hormones. These secreted factors interact with receptors on both pluripotent stem cells and committed hematopoietic progenitor cells to affect their survival, proliferation, and differentiation. The stages of differentiation from pluripotent HSC to fully mature hematopoietic cells of all lineages and the growth factors that play roles in these differentiation events are shown in Figure 1.1. Kit-ligand (also known as stem cell factor (SCF), and Steel factor (SF)) and Flt3 ligand, which function to drive proliferation by binding to the Kit and Flt3 tyrosine kinase receptors, respectively, on $CD34^+CD38^-$ progenitors are important regulators of the early stages of hematopoietic differentiation from HSC. SCF, in particular, cooperates with multiple cytokines and cytokine receptors to influence differentiation, as well as upregulating BCL-2, BCL-X_L, and perhaps other antiapoptotic molecules to promote target cell survival. These receptors are downregulated during normal differentiation. Colony-stimulating factors, including erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and macrophage-CSF (M-CSF; CSF-1), induce the differentiation and function of specific hematopoietic cell lineages. These factors accordingly are named for the lineages that they predominantly stimulate, although several also have effects on multipotent hematopoietic progenitors and perhaps even on pluripotent HSC. Alternatively, TGFβ (tumor growth factor-β), TNFα (tumor necrosis factor-α), and IFNs (interferons) all tend to negatively influence hematopoiesis.

Table 1.1 Cytokine receptor families

Type I Cytokine receptors	
Homodimerizing receptors	G-CSF-R, EPO-R, TPO-R
Heterodimerizing receptors	
gp130 receptor family	IL6-Rα, LIF-Rβ, IL11-Rα, Oncostatin M-Rα, CNTF-Rα, CLCF-R
β_C (Common β receptor) receptor family	GM-CSFRα, IL3-Rα, IL5-Rα
IL2-R family (γ chain) receptor family	IL4-Rα, IL7-Rα, IL9-Rα, IL13-Rα, IL15-Rα, IL21-Rα
Type II Cytokine receptors (interferon family)	IFNα-R, IFNγ-R1/2, IL10-R1/2
Receptors with intrinsic tyrosine kinase activity	Flt3, c-Kit

G: granulocyte; CSF: colony-stimulating factor; R: receptor; EPO: erythropoietin; TPO: thrombopoietin; IL: interleukin; LIF: leukemia inhibitory factor; CNTF: ciliary neurotrophic factor; CLCF: cardiotrophin-like cytokine factor; GM: granulocyte-macrophage; IFN: interferon.

Although cytokine-receptor interactions would appear to generate a level of specificity with regards to transcriptional and genomic regulation and, hence, lineage-specific cell differentiation, the convergence of similar molecular pathways upon genomic targets makes it difficult to delineate this. What can be said, however, is that cytokine receptors appear to fall into specific families based upon their signal transducing subunits (see Table 1.1), and that these signaling subunits rely on three major pathways to ultimately influence transcription. These pathways include the JAK-STAT pathway, the MAPK pathway, and the PI3/AKT pathways, although other pathways involving NF-κB, TGF/SMAD, and protein kinase C pathways also play roles in the regulation of hematopoiesis. Importantly, mutations that affect these pathways are well described in lymphomas, myeloproliferative neoplasms, and leukemias [3–6].

Mechanistically, growth factors and cytokines act as ligands for transmembrane receptors that are located on the surface of hematopoietic cells, with differing receptor expression on HSC, multipotent progenitors, single lineage precursors and mature hematopoietic cells of different lineages. Dimerization (or conformational change) of receptors occurs following ligand binding. This receptor dimerization and conformational change leads to autophosphorylation of the intracellular portion of the receptors and recruitment of signaling molecules to docking sites on the activated receptors. This leads, in turn, to recruitment, phosphorylation, and activation of a broad range of cytoplasmic effector signaling molecules, such as STATs, Src-kinases, protein phosphatases, Shc, Grb2, IRS1/2 and PI3K via binding at the conserved SH2 domains and phosphorylation sites on the receptors themselves. For example, phosphorylation of STATs leads to the generation of STAT homo- and heterodimers, which are then translocated to the nucleus, where they can bind specific nucleotide sequences in the regulatory regions of specific genes to influence transcription of those genes, which determines the proliferation, survival, differentiation, and function of those cells. Similarly, phosphorylation of Grb2 facilitates the activation of SOS, which in turn, influences transcription via activation of the Ras/Raf/Mek/Erk, and the Rho/Mlk-Mekk/Mek/p38-JNK pathways. Activation of phosphatidylinositol-3 kinase (PI3K), either directly or indirectly via RAS or IRS 1 and 2, generates PIP3, which in turn activates PKC, SGK, RAC1/CDC42 and AKT. Activation of AKT is particularly relevant to both normal and malignant hematopoiesis, as it can phosphorylate multiple transcription factors, leading to activation of mTOR, MDM2, and NFκB and inhibition GSK3β, FKHR, and BAD. Notably, multiple related proteins and isoforms of many of the signal transduction molecules exist (including JAK, STAT, Mek, Mlk, Mekk, Erk, p38, JNK, PI3K, PIP3, and AKT), and appear to have different nuclear

targets depending on the cell type in which activation occurs [3–5].

Transcriptional regulation

Transcription factors are proteins that interact with the regulatory region of genes, either alone or in protein complexes, to increase or decrease expression of genes that contain specific sequences of nucleotides in these regulatory regions, which are recognized by the specific transcription factors. Transcriptional networks play a central role in the intrinsic regulation of HSC and lineage-committed progenitor cell survival, proliferation, and differentiation. Accordingly, these pathways are commonly perturbed in hematopoietic malignancies. Unfortunately, our knowledge in many cases is limited to non-human and *in vitro* models, which may not accurately reflect human hematopoiesis. Nonetheless, these experimental approaches have helped to define several important concepts in transcriptional regulation, including timing, autonomous and antagonistic pathways, cofactor regulation, and cellular signaling-related changes to transcription factor activity/function. A summary of relevant transcription factors thought to be involved in varying steps in the hematopoietic differentiation pathways is provided in Figure 1.2 and the transcriptional regulatory factors involved in each of the specific lineages of hematopoietic differentiation are described in more detail in the sections below on each of those lineages.

MicroRNA regulation

MicroRNAs (miRNA) have been recently implicated in the control of gene expression in hematopoiesis (Figure 1.2). miRNAs are small non-coding RNAs that bind to the 3′-untranslated regions and destabilize messenger RNAs (mRNAs) leading to their rapid degradation or, less commonly, may bind to the coding region of targeted genes and inhibit transcription of those genes. To date over 700 miRNAs have been identified in humans, with over 33% of human genes identified as potential targets of these miRNAs, based on identification of sequences in those genes that are reverse complements of specific miRNAs. A thorough review of the involvement of miRNAs in hematopoiesis is beyond the scope of this review; however, interested readers are referred to several recent reviews highlighting the importance of miRNA in both normal and malignant hematopoiesis [7–11].

Hematopoietic microenvironment

HSCs are most likely generated independently in the yolk sac and aorta-gonad-mesonephros (AGM) region in the developing embryo, after which they migrate to the placenta, attaching via VE-cadherin, and subsequently to the liver and spleen via β1 integrin-dependent interactions with the extracellular matrix (ECM). Mesenchymal cell development in the liver and spleen creates a unique microenvironment that fosters HSC survival and expansion. During most of human fetal development the liver is the primary source of hematopoietic cell production, with erythrocyte production predominating, and the spleen contributes a small proportion of fetal hematopoiesis. Shortly before birth, HSCs migrate to the bone marrow, presumably under the influence of CXCL12/CXCR4, c-Kit/SCF, CD44/hyaluronic acid, and α4β1 integrin (VLA-4)/ECM and stromal cell interactions. At that point, hepatic and splenic hematopoiesis virtually ceases, and essentially all subsequent human hematopoietic cell production is restricted to the bone marrow. It is now well accepted that stem cells routinely circulate into and out of the bone marrow niche throughout life, although the purpose of circulating hematopoietic stem and progenitor cells is not known. The same molecules that are involved in movement of HSCs to the bone marrow during development appear to play similar roles in HSC homing and marrow engraftment throughout adulthood. Curiously, in adults, CD44 is fucosylated, converting it to an E-selectin ligand, and accordingly facilitates binding and retention by bone marrow endothelial cells [12, 13]. CD44/hyaluronic acid and CD44/E-selectin interactions, which serve redundant roles in normal stem cell homing and engraftment, also have been found to be required for both human CML and

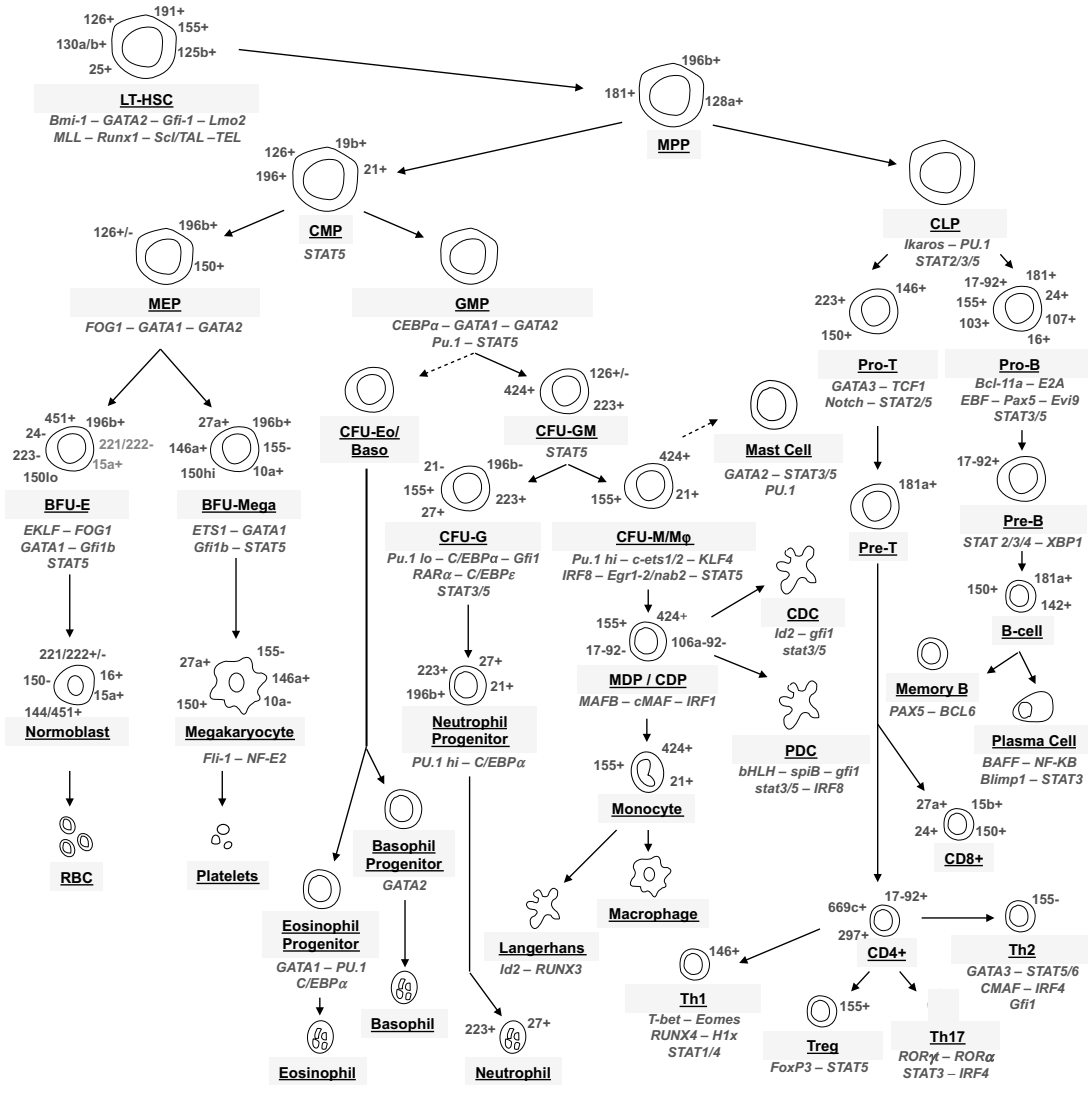

Figure 1.2 Schematic diagram of hematopoiesis highlighting transcription factors (in italic) and microRNAs (in gray) that are active at each of the stages of hematopoietic differentiation.

AML leukemia cell growth in mouse xenograft models [14, 15].

HSC and early hematopoietic progenitors tend to predominantly lodge into endosteal niches near N-cadherin-expressing osteoblasts, where they tend to remain quiescent, perhaps under the influence of osteoblast-secreted Angiopoietin-1, active at HSC TIE2 receptors. Increasing the osteoblast population via conditional inactivation of bone morphogenic protein receptor type 1A (BMPR1A) or administration of PTH leads to an increase in the number of HSCs in the marrow. PTH also increases CXCL12 expression by osteoblasts, and indeed CXCR4 appears to retain its importance in HSC repopulation even after homing. SCF and extracellular calcium-ion concentration (sensed via the calcium receptor, CaR) also may play a role in localization to the endosteum (reviewed in [12]).

Interestingly, a separate population of HSCs is also found adjacent to endothelial cells, where

N-cadherin expression is lower. Endothelial interactions likely play a role in HSC retention and egress, and may also facilitate HSC expansion and differentiation. For example, Tie2 is also expressed on endothelial cells, and blocking of this receptor impairs neoangiogenesis and delays hematopoiesis following myelosuppression. Angiopoietin-1, conversely, can rescue hematopoiesis in TPO-deficient mice [16]. Together these data suggest that two pools of HSCs may exist, a quiescent fraction adjacent to osteoblasts in the endosteal niche, and a more rapidly proliferating and differentiating fraction adjacent to blood vessels.

Adhesive interactions via osteopontin/CD44 and β1 integrins, N-cadherin, c-Kit/SCF, CXCL12/CXCR4, Jagged1/Notch and TIE2/Angiopoietin-1 all play roles in maintenance of the bone marrow niche and in HSC quiescence. These adhesive interactions are commonly altered in hematologic malignancies. Increased expression appears to confer a more aggressive and more drug-resistant "stem cell" phenotype, while decreased expression, as seen with AML1/ETO translocations, appears to confer a more migratory phenotype (reviewed in [17]). CXCL12 is particularly important in HSC retention, and interestingly has been found to be expressed at a higher level among a subset of stromal reticular cells. These CXCL12-abundant reticular cells, or CAR cells, are found throughout the marrow, generally surrounding sinusoidal endothelial cells. Rhythmic noradrenaline secretion via local sympathetic nerves modulates CXCL12 expression via β3 adrenoreceptor-mediated regulation of Sp1 levels. HSC egress is commonly provoked using high doses of G-CSF, which acts on neutrophils to facilitate proteolytic cleavage of these adhesive interactions, and may also regulate CXCL12 expression via CSF receptors found on sympathetic nerves [12]. Importantly, the marrow niche also critically regulates more mature cells as well. Osteoblast and endothelial cell niches play a role in both myelopoiesis (via G-CSF secretion) and B-cell lymphopoiesis (via IL-7 secretion and VCAM-1/cannabinoid receptor 2 expression). On the other hand, erythroid maturation is critically dependent on specialized bone marrow macrophage interactions [18].

Hematopoietic developmental pathways

Human HSCs with long-term repopulation potential were initially found in the $CD34^{+}CD38^{-}CD90^{+}$ bone marrow compartment. Later flow cytometry-based studies have disclosed a rare "side population" with a $CD34^{-}$ or $CD34^{lo}$ phenotype with 1000-fold greater repopulating potential [19]. It remains unclear whether $CD34^{-}$ cells serve as progenitors to $CD34^{+}$ cells, as expression of this protein does not appear to be a terminal event. In fact, HSCs likely cycle expression of CD34 depending on specific microenvironmental niches, wherein CD34 expression may facilitate adhesion and decreased proliferation [20].

Pluripotent, self-renewing, long-term repopulating HSCs appear to progress through several stages of MPPs, which probably have reduced self-renewal capacity, before beginning the process of what is recognizable as differentiation by proceeding down either a lymphoid (CLP, common lymphoid progenitor) or myeloid (CMP, common myeloid progenitor) developmental pathway, after which they are incapable of self-renewal in xenotransplant models. The lymphoid pathway ultimately generates T-cells, B-cells, natural killer (NK) cells, and dendritic cells. The myeloid pathway generates all the remaining mature hematopoietic phenotypes, including red blood cells (RBCs), granulocytes (neutrophils, eosinophils, basophils), mast cells, monocyte-macrophages, and megakaryocytes-platelets, and provides an additional mechanism for generating dendritic cells. This hierarchy of differentiation from HSC to the broad spectrum of mature hematopoietic cells, cell surface molecules that serve as markers of the various stages of differentiation, and the growth factors that impact the differentiation processes, are depicted in Figure 1.1.

The initial decision to pursue either a lymphoid or myeloid fate is understandably important and the product of extensive investigation. Although much remains unknown about the mechanisms of these cell fate determinations, changes in regulation of gene expression through transcription factors, miRNA expression, epigenetic changes such as histone methylation or acetylation, among others, are thought to be critical to such cell-fate decisions. An

analysis of SCL and E2A expression suggests that SCL encourages myeloid differentiation, while high levels of E2A (a helix-loop-helix protein) may be required for lymphoid development [21, 22]. Graded expression of the Ets family member, PU.1, likewise impacts myeloid/lymphoid lineage decisions, with low and high levels specifying lymphoid and myeloid commitment, respectively [23]. RUNX1 may play an early role in CMP lineage commitment by increasing PU.1 expression [24].

Common lymphoid progenitors (CLP)

Galy et al. were the first to characterize human lymphoid committed progenitors ($CD34^+CD38^+CD45RA^+CD10^+$) from the bone marrow using both xenotransplant and *in vitro* culture systems. Using limiting dilution assays, this population was found to contain B, NK, and DC progenitors. Additionally, injection of $CD34^+CD10^+$ cells into fetal thymic organs provided evidence that these cells could also develop into T-cells [25]. IL-7Rα, a critical marker for murine CLPs, has since been found among $CD34^+CD45RA^+CD10^+$ adult human marrow CLPs. In fact, these cells were found to express transcripts for both B-cells (including Pax-5 and Igβ) and T-cells (including GATA3 and pTα). Interestingly, however, *in vitro* studies suggest a bias towards B-cell development among this subset (though limited NK cell development was observed) [26]. Indeed, a bias towards T- and NK cell lineage commitment appears to be found among $CD34^+CD45RA^+CD7^+CD10^-IL\text{-}7R\alpha^-$ cells. Whether both populations derive from a $CD34^+CD45RA^+CD10^+CD7^+$ cell population remains unknown, largely due to the scarcity of this phenotype in adult marrow (0.3% of cells) [27, 28].

Low levels of PU.1, likely act in parallel with Ikaros to provide transcriptional control of the maturation of HSCs into lymphoid precursors. In this context, PU.1 promotes IL-7Rα and EBF1 expression, while Ikaros promotes Flt3 receptor expression, all important in B- and T-cell development (reviewed in [29]). Additional regulation via the Notch1 receptor appears to be critical in T lineage commitment from the CLP. Deletion or inhibition of Notch receptor signaling in CLPs prevents T-cell formation and promotes development of B-cells [30].

In the absence of Notch, the transcription factors E2A and EBF1 (early B-cell factor) appear to work together to induce expression of Pax-5. Indirect data implicating EBF in B-cell lineage commitment comes from an analysis of the EBF inhibitor, EHZF (early hematopoietic zinc finger). EHZF is highly expressed in CD34+ cells, but absent following differentiation to CD19+ B-cells [31]. Furthermore, *in vitro* inhibition of E2A with Id3 inhibits B-cell formation, possibly by inhibiting development/survival of CD10+IL-7Rα+ expressing B-cell biased lymphoid progenitors [32]. Pax-5 also plays an important role in the activation of B-cell lineage-specific genes, and repression of lineage-inappropriate genes, such as Notch1, c-Fms (which encodes the macrophage colony-stimulating factor receptor, and accordingly supports myeloid development), and CCL3 (which promotes osteoclast formation) [33–35]. Finally, Bcl11a (a zinc finger transcription factor) is also critical to B-cell lineage commitment, as its absence blocks B lymphopoiesis from the CLP [36]. Translocations involving Bcl11a are particularly relevant in malignant transformation [37].

Common myeloid progenitors (CMP)

CMPs (also known as CFU-GEMM; colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte) give rise to all myeloid lineages. CMPs are thought to give rise to two or more intermediate differentiated multipotent progenitor cell types. Granulocyte-monocyte progenitors (GMP), give rise to neutrophils, eosinophils, basophils, and monocyte/macrophages. The other, called megakaryocyte-erythroid progenitors (MEP) subsequently give rise to two separate lineages of hematopoietic cells, erythroid and megakaryocytic. The CMP, GMP, and MEP have all been isolated within the $CD34^+CD38^+$ compartment in both human marrow and cord blood. These cells lack the lymphoid markers CD10, CD7, and IL-7Rα, and can be isolated according to CD45RA and IL-3Rα expression: CMPs are $CD45RA^-IL\text{-}3R\alpha^{lo}$; GMPs are $CD45RA^+IL\text{-}3R\alpha^{lo}$; and MEPs are $CD45RA^-IL\text{-}3R\alpha^-$. Importantly

CD33 is also expressed by CMPs but lost beyond the myelocyte stage and accordingly is a recognized target for the treatment of certain types of acute myeloid leukemia (AML) [38].

Another important marker in hematopoiesis is the FMS-like tyrosine kinase 3 receptor (Flt3). Interestingly, expression of Flt3 in human progenitor populations differs considerably from that of mice. Around 40–80% of human $CD34^+$ bone marrow and cord blood cells are $Flt3^+$, and its presence appears to correlate with a capacity for long-term repopulation. Specifically, a fraction of both $Flt3^+$ and $Flt3^-$ populations generate multilineage colonies containing all the myelo-erythroid components, with $Flt3^+$ populations forming more GM colonies, and $Flt3^-$ populations more erythroid colonies [39]. Further exploration using xenotransplant models has characterized the $Flt3^+CD34^+CD38^-$ as LT-HSCs, and has identified Flt3 on both GMPs and CLPs [40, 41]. In contrast, murine Flt3 expression is limited to MPPs with both granulocytic and lymphocytic (but not megakaryocytic-erythroid) potential. Cells with similar potential have not been isolated in humans. The specific role of Flt3 is still being delineated; however, persistent activation as a consequence of activating mutations is commonly seen in AML, and is associated with a worse prognosis.

Transcriptional control of lineage bifurcation between the MEP and GMP populations is at least driven by antagonism between PU.1 and GATA1, with the former driving GMP formation, and the latter encouraging MEP development. PU.1 in association with Rb, binds to the promoters of GATA1 target genes, inhibiting their transcription, and has specifically been shown to inhibit α-globin expression and erythroid differentiation. GATA1 also suppresses myeloid differentiation via binding to the Ets domain of PU.1, blocking binding of the coactivator c-Jun and, accordingly, inhibiting PU.1 DNA-binding [42, 43].

Megakaryocytopoiesis and erythropoiesis

Megakaryocytes and erythroid cells originate from the CMP/CFU-GEMM. The process begins with differentiation of the CMP into the MEP intermediate. Progression beyond the MEP stage is associated with lineage commitment to either the erythroid or megakaryocyte lineages. Specifically, the MEP initially differentiates into a highly proliferative burst forming unit-megakaryocytic or burst forming unit-erythroid (BFU-Mk or BFU-E), which is followed by further maturation to colony forming units (CFU-Mk or CFU-E, respectively), and ultimately either megakaryocyte/platelet formation or erythroid cell production. In fact, the existence of MEP cells was postulated prior to their isolation, given the numerous similarities in transcriptional regulation (SCL, GATA1, GATA2, NF-E2), cell surface molecules (TER119, CD235a/glycophorin A), and cytokine receptors (IL-3, SCF, EPO, and TPO). Additionally, several erythroid and megakaryocytic leukemia cell lines can be induced to display features of both lineages. Furthering this concept are the structural and downstream signaling similarities after binding of EPO and TPO to their respective cell surface receptors, which display a modest degree of synergy in stimulating the growth of progenitors of both lineages.

Although the mechanisms by which these differentiation decisions are made are not fully elucidated, it is known that specific transcription factors play roles in determining whether MEPs proceed down the differentiation pathway towards erythropoiesis or megakaryocytopoiesis. Here, Fli-1 and EKLF appear to play similarly antagonistic roles, with Fli-1 supporting the development of BFU-Mk, and EKLF the formation of BFU-E. EKLF expression relies on GATA1 and CP1. Cells committed to the megakaryocytic lineage express CD41 and CD61 (integrin αIIβ3), CD42 (glycoprotein I) and glycoprotein V, von Willebrand factor, platelet factor 4 and other platelet proteins. As MEP maturation along the erythroid pathway occurs, they lose CD41 expression, and express the transferrin receptor (CD71) at the BFU-E stage, and subsequently erythroid membrane proteins, erythroid enzymes, and hemoglobins.

Megakaryocytopoiesis

The mature megakaryocyte progenitor proceeds down a regimented pathway, forming promegakaryoblasts, which generate megakaryoblasts, and in turn produce megakaryocytes. Megakaryocytes are

unique among hematopoietic cells, in that after the CFU-MK stage, DNA replication is not accompanied by cell division, resulting in production of progressively larger cells with complex nuclei containing 4N to as high as 128N chromosomes. Platelets are generated by fragmentation of the mature megakaryocyte cytoplasmic pseudopodial projections, called proplatelets. The sliding of microtubules over one another drives the elongation of proplatelet processes and organelle transportation (into the proplatelets) in a process that consumes the megakaryocyte and results in production of 2000–3000 platelets from each mature megakaryocyte (reviewed in [44]).

Although influenced by multiple cytokines (SCF, GM-CSF, IL-3, IL-6, IL-7, IL-11, EPO), TPO and IL-3 are particularly important in the generation and release of mature platelets [45]. Studies conducted following the purification of TPO have found that it is capable of stimulating the growth of 75% of all CFU-MKs, with the remainder proliferating with the addition of IL-3. Additionally, TPO and either IL-3 or SCF are required for the generation of more complex, larger hematopoietic colonies from earlier progenitor populations [46]. Consistent with these experimental data is the observation of amegakaryocytic thrombocytopenia among those with inactivating TPO receptor mutations. The relative contributions of elevated levels of TPO or increased TPO receptor expression to enhancement of megakaryocyte and platelet production remain unclear, though it is likely that both play roles *in vivo*.

Several megakaryocyte DNA promoter binding domains have been identified in mice, with some clinical homology demonstrated in humans with mutations involving associated proteins. It should be stressed that these proteins likely interact with one another, as well as with other transcriptional proteins to ultimately affect the generation of mature progeny.

GATA1 and GATA2 are the major GATA zinc finger DNA binding-proteins influencing differentiation in both the erythroid and megakaryocytic lineages. In both series, GATA1 levels increase while GATA2 levels decrease with progressive differentiation. Additionally, GATA proteins are co-regulated by FOG1 (friend of GATA), a large multifinger protein that influences transcription independent of DNA-binding. GATA1 and FOG1 knockout mice both demonstrate abnormalities in erythropoiesis and megakaryocytopoiesis. Interestingly, human mutations affecting the binding of GATA1 to FOG1 appear to have greater impact on megakaryocytopoiesis than erythropoiesis (reviewed in [47]). Indeed, GATA1-mediated expression of Gfi-1b and repression via interactions with Eto-2 are required for terminal differentiation of megakaryocytes [48, 49]. In fact, mutations involving both Gfi-1b and Eto-2 have been observed in leukemias [50, 51]. GATA2 instead contributes to proliferation of progenitor cells [52].

Another transcriptional regulator is the family of core binding factors, consisting of the DNA binding proteins RUNX1–3 and the non-DNA binding element, CBFβ. The complex of RUNX1 and CBFβ is particularly important in hematopoietic ontogeny. In fact mutations involving these proteins are commonly observed in human acute leukemias. Inactivation of either RUNX1 or CBFβ in murine models leads to a profound defect in megakaryocytic differentiation (with little impact on erythropoiesis). Clinically, RUNX1 mutations are associated with the autosomal dominant familial platelet disorder with predisposition to AML (FPD/AML), with these leukemias likely occurring as a direct consequence of perturbed HSC homeostasis [53].

Ets factor binding sites are also commonly observed among megakaryocytic promoters. The Ets family of transcription factors includes approximately 30 members, with both stimulatory and inhibitory consequences on gene expression. Four Ets factors are of particular consequence to murine megakaryocytopoiesis: Fli-1, GABPα, TEL1, and Ets-1. Selective deletion of TEL1 has been shown to increase CFU-MKs, yet produce a dramatic decrease in platelet production (with little impact on erythrocytes). Decreasing GABPα expression leads to decreased megakaryocyte formation, accompanied by decreased expression of GPIIb and the TPO receptor. Fli-1 deletion similarly decreases megakaryocyte formation with an accompanying decrease in gpIX expression. Ets-1 is normally upregulated in megakaryocytic differentiation, and, alternatively downregulated in erythroid development. Enforced

expression of Ets-1 appears to enhance megakaryocyte development, and, likewise inhibit erythroid formation. Interestingly, knockout models of Ets-1 do not significantly impact murine megakaryocyte development (reviewed in [47]).

Erythropoiesis

Akin to megakaryocytopoiesis, the initial steps in erythropoiesis are driven by SCF, GM-CSF, IL-3, and TPO. Erythropoietin (EPO) receptors are not highly expressed until the CFU-E stage, where EPO functions to prevent apoptosis, induce hemoglobin synthesis, and drive maturation to proerythroblasts, from which point erythroid maturation proceeds through nucleated normoblasts to enucleated red blood cells without further contribution by erythropoietin. Interestingly, EPO does not contribute significantly to lineage commitment among HSCs or MPPs [54].

The transition to lineage-committed erythroid cells requires both the downregulation of proliferation associated genes, such as GATA2, c-Myb, c-Myc, and c-Kit, and the upregulation of terminal differentiation genes, including P4.2, glycophorin A, α and β globins. The transcriptional regulatory process is governed by the SCL complex. Proliferation in this setting is driven by GATA1, which binds to the c-Myb promoter to enhance its expression. However, FOG1 expression, which is also induced by GATA1, subsequently binds GATA1, generating a complex that inactivates c-Myb and similarly represses GATA2 activity. GATA1 also acts to transiently increase Gfi-1b, which likely acts in concert with the EPO/GM-CSF-driven JAK/STAT pathway to increase the proliferation of erythroid progenitors [55]. *In vitro* models suggest that Gfi-1b must be downregulated beyond the proerythroblast stage to facilitate survival, though, interestingly, increased expression has been observed in erythroleukemia [50, 56]. GATA1 additionally downregulates c-Myc and indirectly maintains Rb expression, which downregulates c-Kit expression via binding to the SCL complex. Finally, GATA1 also regulates EKLF expression in conjunction with CP1; and EPO receptor genes in conjunction with Sp1 (reviewed in [57]).

Granulocytopoiesis

Progression beyond the GMP stage along the granulocytic pathway facilitates the production of neutrophils, eosinophils, and basophils. This progression occurs via similarly discrete intermediate steps in all three series, beginning with myeloblasts and progressing through promyelocytic, myelocytic, metamyelocytic, and band stages before culminating in a mature granulocyte. Primary, secondary, and tertiary granules important to granulocyte function are acquired at the promyelocyte, myelocyte, and band stages, respectively. CD11b, CD13, CD14, CD15, and CD16 are common markers of maturing and mature neutrophils and monocytes. Neutrophil elastase, myeloperoxidase, lactoferrin, and leukocyte alkaline phosphatase are markers of maturing and mature neutrophilic granulocytes. Muramidase and lysozyme are common markers of mature monocytes/macrophages. Not surprisingly, G-CSF, GM-CSF, and IL-3 are important cytokines in the generation of functional granulocytes.

Expression of the CCAAT/enhancer binding protein (C/EBP)α and interaction with c-Jun increase the activity of PU.1, while also inhibiting Pax-5 (paired box gene 5), and likely other lymphoid transcriptional elements, to further commit cells to the GMP stage. Graded expression of PU.1 beyond this stage facilitates lineage bifurcation, with low and high levels specifying granulocytic and monocytic commitment, respectively. Downstream targets of PU.1 include the EGR/Nab transcription factors. Antagonism between Egr-1/2 acting with its co-repressor Nab-2, and Gfi-1 function to drive lineage commitment down either a macrophage or neutrophil pathway. Specifically, the Egr-1/2/Nab2 complex drives macrophage-specific gene expression, while repressing neutrophil-specific genes, including Gfi-1. Gfi-1, which is downstream of C/EBPα, does precisely the opposite, and similarly represses the Egr-1/2/Nab-2 complex (reviewed in [58]).

The paradoxical role of C/EBPα may be explained by M-CSF/PLCγ/ERK-mediated phosphorylation at serine 21, potentially weakening C/EBPα granulocytic gene interactions by limiting C/EBPα homodimerization, while simultaneously fostering

monocytic gene interactions via stabilization of c-Fos and subsequent phospho-C/EBPα(S21):c-Fos heterodimers. G-CSF, alternatively, acts via STAT3-mediated phosphorylation of SHP2, which may limit PU.1:IRF8 interactions, influence HoxA9/10 genomic interactions, and alter ERK activity to favor granulocytic development [59–62]. Interestingly, activating mutations in SHP2 have been found in MDS, AML, and JMML, wherein constitutive expression of SHP2 more potently activates ERK (akin to M-CSF) [63, 64].

In addition to Gfi-1, C/EBPα and RARα (retinoic acid receptor alpha) induce C/EBPε in the context of low PU.1 to ultimately influence granulocytic maturation. C/EBPα and C/EBPβ may serve somewhat redundant roles in myelopoiesis; however, only C/EBPα is capable of inhibiting cell cycle progression to facilitate terminal differentiation. C/EBPα interacts with E2F1 to bind to the c-myc promoter, and ultimately to repress its transcription (reviewed in [58]). Indeed, C/EBPα mutations reflect a recurring theme in both adult and pediatric AML [65, 66]. Terminal differentiation of granulocytes is also afforded by repression of CAAT displacement protein (CDP) and downmodulation of the retinoid x receptor, RXRα [67–69]. Alterations in RAR:RXR signaling are commonly seen in acute promyelocytic leukemia, in which treatment with all-trans retinoic acid is instead used to facilitate terminal granulocytic differentiation. Likewise, failure to downmodulate CDP in experimental models has been shown to generate a myeloproliferative phenotype with an excess of neutrophils in the bone marrow and spleen [70].

Eosinophil formation

Eosinophils are thought to be generated from an eosinophil-basophil progenitor derived from the GMP [71]. The cytokines IL-3, IL-5, and GM-CSF are important in the regulation of eosinophils, most likely by providing permissive proliferation/differentiation signals in concert with transcriptional signals (mediated by GATA1, PU.1, and C/EBPs, see below). Of these growth factors, IL-5 is most specific to the eosinophils, promoting selective differentiation of eosinophils as well as their release from the marrow. Overproduction of these cytokines (particularly IL-5) is seen in a variety of malignancies, and has a known association with eosinophilia (reviewed in [72]).

The timing of expression, as well as interactions between GATA, PU.1, and C/EBP members influences eosinophil lineage commitment. Of these GATA1 appears to be most important, as deletion of the high affinity palindromic GATA binding site in the GATA1 promoter prevents eosinophil formation. This binding site appears to be specific to eosinophil development, as deletion does not appear to influence the development of other $GATA1^{+}$ lineages, including megakaryocyte, erythroid, and mast cell lineages. Similar binding sites exist outside of the promoter region in the regulatory regions of eosinophil specific genes, such as the eotaxin receptor, CCR3, MBP, and IL-5Rα. These palindromic binding sites may also facilitate synergy between the normally antagonistic functions of GATA1 and PU.1 (reviewed in [72]).

Basophil and mast cell formation

As indicated above, basophils likely derive from a bipotent precursor with both eosinophil and basophil differentiation capacity (although other differentiation pathways have been proposed, see [73]). The existence of this bipotent precursor has not been proven, but it is supported by *in vitro* clonogenic assays, as well as the presence of cells with a hybrid eosinophil/basophil phenotype in some patients with CML and AML [74]. Although murine mast cells appear to derive from a common basophil/mast cell progenitor, human mast cell development does not appear to conform to this pathway [75, 76]. Human mast cell progenitors, which are distinct from basophil progenitors, are marked by expression of CD34, c-Kit, and CD13, and they appear to have both monocytic and mast cell potential, which may explain why monocytosis (but not basophilia) is observed in patients with mast cell neoplasia [77, 78].

IL-3 plays a major role in basophil growth and differentiation, and basophilia (and eosinophilia) is seen shortly after exogenous administration [79].

Interestingly, IL-3-deficient mice have normal basophil counts, suggesting that the final steps in basophil maturation may occur without a growth factor requirement. In support of this theory, a 3–4 hour exposure of cord blood progenitors to IL-3 was sufficient to drive basophil differentiation for three weeks [80]. TGF-β and IL-18 work synergistically with IL-3 to inhibit eosinophil differentiation and increase IL-4/histamine production by basophils, respectively. Finally, GM-CSF and IL-5 also drive basophil (and eosinophil) production (reviewed in [73]).

Mast cell differentiation is primarily dependent on SCF and IL-3, although it is likely that other factors (TPO, leukotriene D4) and T helper type II (Th2) associated cytokines, such as IL-4, IL-5, IL-6, and IL-9, also play a role [76, 81–83]. Emphasizing the importance of SCF in mast cell development are the findings that exogenous SCF administration in humans produces mast cell proliferation and degranulation, and that there are activating mutations (D816V) of c-Kit in the majority of cases of mastocytosis [84].

Transcriptional regulation of human basophil differentiation is still being elucidated. Murine models suggest that increased GATA2 in conjunction with low levels of C/EBPα appear to be important in early basophil and mast cell lineage commitment. Failure to decrease C/EBPα at this early stage instead leads to eosinophil production. Following basophil lineage commitment, C/EBPα must again be upregulated to facilitate basophil development. Signaling via Notch2-Delta1-Hes1 may be important in repressing C/EBPα at this stage, which, alternatively, drives mast cell production [85–87]. Importantly, PU.1 and GATA2 do not appear to antagonize one another, and, indeed, elevated PU.1 is important in mast cell differentiation [88].

Monocyte/macrophage development

Monocytes derive from the GMP, proceeding through an intermediate, the macrophage dendritic cell progenitor (MDP). The MDP reflects the first committed stage of monocytic development, and is characterized by the expression of CSF-1 receptor (CD115, M-CSFR) and the chemokine receptor, CX3CR1. As the name suggests, MDPs give rise to monocytes, macrophages, and both lymphoid/non-lymphoid and plasmacytic types of dendritic cells (see below) [89].

Human monocytes are categorized into three distinct populations on the basis of CD64, CD14 and CD16 expression. Large $CD64^+CD14^+CD16^-$ monocytes comprise 80–90% of the circulating monocytes, and are characterized by high levels of the chemokine receptor CCR2, and low levels of CX3CR1. These cells possess higher phagocytic/myeloperoxidase activity, higher superoxide release, and secrete IL-10 on stimulation with lipopolysaccharide (LPS). The smaller $CD16^+$ monocytes, alternatively, express high levels of CX3CR1 and low levels of CCR2. This population is comprised of two subsets: a $CD64^+CD14^+CD16^+$ "pro-inflammatory" subtype and $CD64^-CD14^{dim}CD16^+$ subtype whose function remains largely unknown. $CD14^+CD16^+$ monocytes also express the Fc receptor CD32, produce TNF-α and IL-1 in response to LPS, and likely mediate antibody-dependent cytotoxicity (reviewed in [90]).

M-CSF and IL-34 are the two known ligands for CSF-1R (M-CSFR), and both are important in monocytic development. Other cytokines, including GM-CSF, Flt3 ligand, and lymphotoxin α1β2, play similar, but likely redundant roles.

PU.1 plays a dominant role in early monocytic lineage commitment, antagonizing GATA1 (to inhibit MEP formation), GATA2 (to inhibit mast cell formation), and C/EBPα (to inhibit granulocytic development) [88, 91]. Targets of PU.1 activity include the Egr transcription factors (and their cofactor Nab), KLF4 (Kruppel-like family 4), and, likely, ICSBP/IRF-8 (IFN consensus sequence binding protein/IFN regulatory factor 8) [58, 92, 93]. PU.1, c-Ets-1, and c-Ets-2 transactivate the M-CSFR promoter, although, M-CSFR expression cannot rescue monocytic differentiation in PU.1 knockout models [94, 95].

The MafB and c-Maf transcription factors also induce monocyte development. MafB and c-Maf are also thought to cooperate to decrease proliferation in the context of terminal differentiation and, in fact,

MafB must be downregulated to allow dendritic cell maturation [96]. These factors likely act by repressing the transcription factor Ets-1, which is important in transducing CSF-1 receptor associated proliferative signals (via c-Myc and c-Myb), as well as directly inhibiting c-Myb transactivation [97].

Dendritic cell formation

Dendritic cells (DC) are unique among human hematopoietic cells in that they are able to develop from both myeloid and lymphoid progenitors. Many DC progenitor candidates have been proposed, and these can be divided into highly proliferative early DC progenitors (EDCP), late DC progenitors (LDCP) with limited proliferative capacity, and non-proliferative $Gr1^{hi}$ monocytic precursors with immediate DC precursor potential. Multiple subtypes exist within the EDCP and LDCP categories, and are beyond the scope of this review (interested readers are referred to [98]). EDCP are lineage-negative cells characterized by c-Kit, while LDCP are negative for c-Kit, but express CD11c.

Mature DCs can be divided into two major populations: (1) migratory (non-lymphoid) and lymphoid tissue resident DCs, and (2) plasmacytic DCs (pDCs, also called interferon-producing cells). DCs can also be classified as "conventional DCs" (cDCs), which has been used to oppose lymphoid organ resident DCs with pDCs. In this schema, non-lymphoid organ DCs are referred to as "tissue DCs." Nonetheless, it is important to note that non-lymphoid tissue DCs are also different from pDCs, and that primary non-lymphoid tissue DCs can be found in LNs while migrating (but are not cDCs). Specifically, cDCs are characterized as HLA-DR^+, $CD11c^{hi}$, with a major $BDCA3^-$ (also known as thrombomodulin) and minor $BDCA3^+$ population. $BDCA3^-$ CDC can be further subdivided into $CD16^+$ and $CD16^-$ populations. PDC express very low to no CD11c, and instead demonstrate CD4, CD45RA, BDCA2 (a c-type lectin receptor), BDCA4 (a neuronal receptor often used to isolate pDCs), and high levels of IL-3R (CD123).

Flt3L appears to be of primary importance in dendritic cell development and, in fact, Flt3L levels are actively adjusted to maintain DC homeostasis in the BM and peripheral lymphoid organs. Cytokines important in differentiation of circulating precursors include GM-CSF and TNF-α, which prime monocyte-to-cDC commitment in the context of inflammation; M-CSF and TGF-β1, which are both non-redundant cytokines important in facilitating Langerhans cell development; and TPO (in cooperation with Flt3L) and G-CSF, which enhance pDC development and mobilization, respectively (reviewed in [98]).

Flt3L appears to act via STAT3, and deletion of STAT3 has been shown to decrease DC numbers [99]. Gfi-1 regulates STAT3 activation, and deletion reduces lymphoid-derived DCs while increasing LC production [100]. GM-CSF instead promotes cDC development via STAT5, which in turn suppresses IRF-8 (ICSBP) and inhibits pDC development. In fact, in the absence of STAT5, GM-CSF stimulated cultures generate pDCs. GM-CSF also increases STAT3 activation and IRF-4 expression, a transcription factor important in DC development [101]. Among its many effects, STAT3 signaling has been shown to increase PU.1 expression, which is important in both cDC and pDC development [102]. Interestingly, constitutive signaling of Flt3 via internal tandem repeat mutations appear to repress PU.1 and c/EBPα and activate STAT5, which may explain why these mutations have not been associated with DC neoplasias [103].

The basic helix-loop-helix (bHLH) transcription factors (E2-2, E2A encoded E12/E47, and HEB) also play an important role in DC homeostasis, with ectopic expression favoring pDC development. In fact, Spi-B, an Ets family member important in pDC development, may act in part by increasing E2-2 activity [104]. Id-2, an inhibitor of the bHLH factors, is increased following GM-CSF, and appears to favor production of cDCs. Likewise, TGF-β1 appears to signal increases in Id-2 (via RUNX3), which has been shown to inhibit E2A to favor LC development in the context of low C/EBPα. Haplo-insufficiency of E2-2 in humans leads to a functional pDC defect (Pitt-Hopkins syndrome), further confirming the importance of bHLH in pDC development [105].

The role of Notch1 is gaining increasing attention in the differentiation and function of DCs, with both

pro- and antagonistic effects occurring depending on the ligand interaction (Delta-1 vs. Jagged-1), and on the immature cell type studied (HSC vs. immature thymic progenitors). Accordingly, Notch1 has been shown to influence multiple downstream signaling factors, including Wnt, NFκB, GATA3, and Spi-B [106, 107].

B-cell lymphopoiesis

B-cells are derived from $CD34^+CD19^-CD10^+$ cells. The earliest lineage-committed B-cell is the pro-B-cell, which is characterized by a $CD19^+$ phenotype. The vast majority of these cells also express terminal deoxynucleotidyl transferase (TdT), and potentially VpreB (a component of the pre-B-cell receptor). It is interesting to note that commitment to B-cell differentiation may occur prior to this based on the presence of cytoplasmic Vpre-B in CD19 negative cells – though, the lineage commitment of this cell remains controversial [108]. The pro-B-cell stage is defined by immunoglobulin (Ig) heavy chain gene rearrangement, initiated via the recombinase activating genes RAG1 and RAG2. Importantly, oncogenes such as Bcl-2 lie in close proximity to immunoglobulin genes, which may partially underlie the mechanism for translocation seen in B-cell neoplasias (reviewed in [29] and [109]).

If heavy chain gene rearrangement is successful, an Ig heavy chain of μ class is expressed on the cell surface in association with ΨL (VpreB and λ5) and CD79a/b to form the pre-B-cell receptor, which signals several rounds of division and termination of rearrangement. This is accompanied by loss of CD34 and TdT expression, and marks the transition to the pre-B-cell stage of development, while a persistence of TdT positivity is commonly seen in pre-B-cell ALL. Internalization of the pre-B-cell receptor and rearrangement of the light chains occur next, defaulting to the kappa gene. Lambda gene rearrangement and expression generally occurs only if kappa gene rearrangement is unsuccessful. Successful light chain rearrangement is heralded by cell surface expression of IgM, composed of the μIg and either κ or λ light chain. Failure to rearrange either the heavy or light chains induces apoptosis. Similarly, reaction to self antigen at IgM can induce either apoptosis or anergy as a mechanism of negative selection (reviewed in [110]).

Secondary B-cell development is characterized by the migration of immature B-cells to the spleen where they differentiate into mature antigenically naïve B-cells, now characterized by surface IgD (in addition to IgM), CD21, and CD22, as well as a loss of CD10. Positive selection subsequently commences, with cells failing to react succumbing to cell death. Following this process, these cells migrate to secondary lymphoid tissue in anticipation of antigenic challenge, whereupon they can further differentiate into short-lived antibody secreting plasma cells or establish a germinal center. Within germinal centers, these antigen primed B-cells undergo rapid proliferation and a process of somatic hypermutation to increase antigen affinity. Somatic hypermutation is mediated by the enzyme activation-induced cytidine deaminase (AID). Akin to RAG1/2, AID can also generate neoplastic chromosomal translocations involving c-Myc and the IgH locus, as seen in Burkitt's lymphoma. Centrally proliferating B-cells are referred to as centroblasts, which divide to form smaller centrocytes that migrate to the periphery of the germinal center. Centrocytes with differing antigen affinity compete for antigen-binding sites on dendritic cells. Successful interaction is marked by expression of the antiapoptotic protein Bcl-XL, while unsuccessful interaction leads to apoptosis. Those surviving centrocytes subsequently depend on CD40-based interaction with T-cells to facilitate differentiation into long-lived plasma and memory-type B-cells. Secondary immune responses ultimately produce long-lived plasma cells in the spleen, which migrate back to the bone marrow where they can persist for a lifetime without need for self-renewal. However, if depleted, memory B-cells are capable of regenerating new marrow plasma cells [111].

We still have much to learn about cytokine regulation in B-cell development. Certainly, c-Kit and Flt3 are likely to play a role in the proliferation and survival of early pro-B-cells. However, the role of IL-7 in the survival and proliferation remains controversial. *In vitro* data supports a role for IL-7 in promoting Pax-5 and CD19 expression [112]. Yet,

mutations of the IL-7 receptor complex in humans do not appear to significantly affect B-cell numbers (though their function is impaired) [113].

Pax-5 is perhaps the most important transcriptional regulator in B-cell development. As described above, Pax-5 is critical to early B-cell lineage commitment, where it likely represses Flt3 expression to enable the differentiation program that results in production of the mature phenotype. Pax-5 continues to retain its importance throughout the maturation of the B-cell by facilitating VH-DJH recombination and enhancing numerous B-cell specific genes, including Ebf1, ΨL, CD79a, CD19, CD21, CD23, adhesion/migration proteins, N-myc, LEF-1, and the central adaptor protein, BLNK. Together, these suggest Pax-5-mediated maintenance of the B-cell phenotype prior to plasma cell differentiation (which requires BLIMP1 associated Pax-5 inhibition) (reviewed in [114]). Indeed, conditional inactivation of Pax-5 in mature B-cells leads to de-differentiation to a pro-B-cell stage, wherein T-cell developmental potential can be induced [115]. Additionally, inactivation/mutation of Pax-5 in committed B-cells is also associated with transformation to B-cell ALL, as well as Ki+ B-cell lymphomas [116, 117].

The transcription factors FoxP1, E2A/E47, and EBF also play roles in B-cell development, acting to control DH-JH rearrangement by activating expression of RAG1/2 and promoting accessibility of the DH-JH region. Aberrant expression of B-cell transcription factors have known associations with malignant phenotypes, including E2A in pre- and pro-B cell ALL [118, 119].

The proto-oncogene Bcl6 encodes a nuclear transcriptional repressor, which appears to be necessary in germinal center formation. Bcl6 likely plays a role in suppression of apoptosis during the process of low-level physiologic DNA breaks that occur during somatic hypermutation, and mutations of Bcl6 are commonly observed in diffuse large B-cell lymphomas and follicular lymphomas.

T-cell lymphopoiesis

Immature T-cell progenitors are likely guided to the thymus via chemokine receptor/ligand CCR9/CCL25 interactions [120]. Subsequent T-cell formation is analogous to B-cell formation, with maturation progressing through pro-T- and pre-T-cell stages marked by TCR gene rearrangement. T-cells begin their developmental paradigm by migrating to the thymus, initially as $CD34^+CD1a^-$early thymic progenitors (ETP). The transition to CD1a positivity marks an irreversible commitment to the T-cell lineage, at which time they are referred to as pro-T-cells. Importantly, these cells are negative for both CD4 and CD8, and as such, are heralded as "double-negative" T-cells. These double-negative T-cells proceed through four discrete stages of maturation, DN1–4. The DN1 stage is marked by expression of c-Kit and CD44 (an adhesion molecule), but an absence of CD25 (the α-chain of the IL-2 receptor). Within approximately one day, these thymic lymphoid progenitors begin to express CD25, which marks the transition to the DN2 stage. Progression through the DN2 and DN3 stages, is accompanied by Sox13-mediated γδ TCR expression or TCR β chain rearrangement, and is accompanied by a loss of c-Kit and CD44. Akin to B-cell gene rearrangement, this process is prone to anomalous chromosomal translocations that can drive neoplastic transformation. Successful rearrangement of the TCR β chain is followed by its expression on the cell membrane in conjunction with CD3 and pTα, to form the pre-T-cell receptor (pre-TCR), which, as in B-cells, is thought to inhibit further rearrangement and stimulate proliferation. The expression of the pre-T-cell receptor also demarcates the transition from pro-T-cells to pre-T-cells. Finally, CD25 expression is lost at the DN4 stage (reviewed in [121]).

T-cells subsequently mature by first proceeding through an immature single positive CD4 phase. Early double-positive T-cells then emerge ($CD4^+CD8\alpha^+\beta^-$), followed soon thereafter by precursor double-positive T-cells ($CD4^+CD8\alpha^+\beta^+$), which ultimately form the double-positive $TCR\alpha\beta^+$ cells. Pre-T-cells are subsequently induced to express either $CD4^+$ or $CD8^+$ cells via interaction of their TCR with MHC/peptide complexes expressed by thymic epithelial cells in a process referred to as positive selection. Successful interaction with MHC Class I molecules is associated with differentiation

towards a $CD8^+$ cytotoxic T-cell phenotype, while successful interactions with MHC Class II molecules drive formation of $CD4^+$ helper T-cells. Following the process of positive selection, T-cells migrate to the thymic medulla under the influence of CCR7 (and likely other chemokines), where they are tested against self antigens on thymic dendritic cells – with those reacting strongly being induced to undergo apoptosis [122]. This latter process is commonly referred to as "negative selection" (reviewed in [121]).

Interestingly, while IL-7Rα^+ immature CLPs more avidly develop into B-cells, the absence of critical components of the IL-7R receptor appears to influence T-cell maturation more significantly [123]. The IL-7 receptor consists of two components, an IL-7Rα and gamma common (γc) chain (which also forms the IL-2, IL-4, IL-9, IL-15, and IL-21 receptors), and signals via the JAK1, JAK3/STAT5, and the PI3K (phosphatidylinositol-3 kinase)/Akt transduction pathways. Inactivating mutations in the γc, IL-7Rα, or JAK3 genes all produce severe combined immunodeficiency (SCID), with a marked reduction in T-cells. Specifically, in patients with γc mutations, there is an absence of T- and NK cells, while B-cell development appears to be preserved. Additionally, those with IL-7Rα mutations have significantly reduced T-cell numbers, with near normal B-cell and normal NK cell numbers [123–125].

However, given the role of IL-7 in B-cell development and the absence of IL-7Rα on ETPs, it is difficult to conclude that this interleukin drives $\alpha\beta$ T-cell lineage commitment – rather, its effect is likely mediated at a later stage where it promotes T-cell maturation and/or survival. In fact, additional data demonstrate dependence of $CD34^+CD1a^+$ cells on IL-7 for transition to the $CD4^+$ immature single-positive phase, perhaps via expression of the anti-apoptotic Bcl-2-related protein Mcl-1 [126]. Interestingly, IL-7 is a prerequisite for TCR $\gamma\delta$ formation via direct regulation of TCRγ locus accessibility and rearrangement [113].

SCF, Flt3, and BMP are also important, though they appear to be active during earlier stages of T-cell development. Neutralization of SCF both inhibits T-cell proliferation and accelerates T lineage maturation from lymphoid progenitors [127]. Likewise, knockout of c-Kit leads to a dramatic reduction in DN1 cellularity, suggesting SCF may play a role in maintenance of the undifferentiated lymphoid precursors [128]. BMP signaling appears to play a similar role, with increased levels corresponding to enhanced survival of immature thymocyte progenitors, and inhibition of differentiation through the DN stages [129, 130]. Flt3 ligand likely plays many roles in T-cell development, and has recently been shown in mice to enhance expression of CCR9, allowing for enhanced thymic migration [131].

Notch1 signaling retains its importance following T-cell lineage commitment, in which it regulates the survival and maturation of CD4-/CD8- (double-negative) T-cells. Notch1 activity at the DN1 stage likely functions to maintain T-cell lineage specification, as inhibition of Notch at this stage drives production of NK cells, monocytic/DC cells and plasmacytoid dendritic cells. Notch activity at the DN2 and DN3 stages of T-cell development facilitates expression of pTα, as well as β-chain rearrangement and β selection. During β selection, Notch functions in a trophic manner, supporting Akt activation and c-Myc expression. T-cell development beyond DN4 is accompanied by decreased Notch activity, and an increase in antagonistic proteins such as Ikaros (reviewed in [121]. This transition is tightly regulated, as persistent Notch activity in double-positive T-cells is highly oncogenic. In fact, activating mutations of Notch1 have been found in over 50% of T-cell acute lymphoblastic lymphomas (T-ALL) [132].

A second regulatory system is the Wnt-β-catenin pathway. Wnt drives the release of active β-catenin, which, in double-negative T-cells, forms a bipartite transcription factor complex with the HMG-box T-cell factor (TCF)/lymphocyte enhancer binding factor (LEF) family of proteins, ultimately promoting expression of TCF1, as well as c-Fos, c-Jun, and integrins. Inhibition of Wnt signaling leads to a maturation arrest at the DN2 stage. Similarly, unsuccessful TCRβ rearrangement at the DN3 stage leads to expression of β-catenin degrading proteins, including adenomatous polyposis coli (APC) and the E3 ubiquitin ligase, SIP [133, 134]. Wnt/TCF retains importance in double-positive T-cells as well, in which it functions to promote expression of CD4 and the antiapoptotic protein BCL-XL reviewed in [121]).

The bHLH subfamily of E-box binding (E) proteins (E12, E47, HEB, and E2-2) also play a critical role in T-cell development. These proteins are naturally antagonized by Id (inhibitors of DNA binding) proteins, specifically Id2 and Id3. Ectopic expression of these Id proteins in $CD34^+$ cells drives NK formation at the expense of T-cell formation [135]. Later introduction of Id3 into immature single-positive CD4 T-cells inhibited TCRαβ development, but allowed TCRγδ development – which could be reversed by the E-protein HEB, likely via an influence on pTα expression [29, 136].

Thymocyte progenitors are dependent on hedgehog (Hh) signals for survival, expansion, and differentiation prior to pre-TCR signaling. Knockout of sonic hedgehog (Shh) in mouse embryos leads to diminished thymic cellularity, likely due to defects in DN1 and DN2 progenitor expansion [137]. Additionally, the transition from double-negative to double-positive T-cells may also be regulated by concentration-dependent effects of Shh, as high concentrations of this protein appear to antagonize this progression [138]. New data are emerging on the role of dysregulation of the Shh pathway in leukemias and lymphomas. In particular, abnormally increased Shh and cyclopamine (a Shh inhibitor) mediated apoptosis have been observed in Alk + anaplastic large cell lymphoma, mantle cell lymphoma, and cytarabine-resistant leukemic cell lines [139, 140].

The role of GATA3 in T-cell development is still being delineated. Early experiments suggested that GATA3 overexpression in $CD34^+$ thymic progenitors stimulated development of $CD4^+CD8^+$ cells. However, the absolute numbers of TCRαβ cells were markedly reduced at later time-points, suggesting an inhibition of further development [141]. Murine GATA3-deficient models add further confusion, since GATA3 appears to be necessary for TCRβ expression and pre-TCR signaling [142]. Together, this information may suggest as yet unknown cooperative factors that mediate the activation/inhibition of GATA3.

Natural killer (NK) cell development

The formation and characterization of NK cells are still being elucidated. A bipotent T/NK progenitor (TKNP) has been found in the human fetal thymus, and is characterized by being $CD34^+CD7^+CD1a^-$. Within the thymus, these TNKP are found in close relation to NK progenitors (NKP) as well as T-cells, suggesting that the TNKP are their immediate precursor. However, inducible deletion of murine notch, a membrane receptor critical for T-cell formation, does not appear to influence NK cell numbers, suggesting that NK cell formation may be a default program for cells failing to develop into T-cells.

NKP in humans have been difficult to characterize phenotypically. NK formation following IL15 signaling via CD122 is an essential feature of the NKP in mice; however, CD122 has been difficult to demonstrate in humans (despite IL-15 sensitivity). A more recent study isolated mature/functional $CD56^{hi}$ NK cells from a novel hematopoietic precursor expressing $CD34^{dim}CD45RA^+$ $\alpha4\beta7^{hi}$ in a stromal cell culture system following the addition of IL-2 or IL-15. These precursors were found in the bone marrow and peripheral blood, but appeared to be highly enriched in lymph nodes, suggesting migration to these sites. In fact, further characterization of these cells revealed expression of the lymph node-homing ligand CCR7 and CD62L.

Following activation by APC surface-bound IL-15, circulating $CD34^+$/$CD45RA^+$ pro-NK mature through pre-NK and immature-NK intermediates before forming the $CD56^{bright}$NK (characterized as $NKp46^+$, CD94/$NKG2A^+$, $cd117^{+/-}$, $KIR^{+/-}$). The LN resident $CD56^{bright}$NK cell can produce significant amount of cytokines/chemokines on activation, but only poorly eliminate tumor cells. $CD56^{dim}$NK cells, alternatively, are more adept at cancer cell killing and less so at cytokine/chemokine release. These cells are characterized as $NKp46^+$ CD94/$NKG2A^{+/-}$ $CD16^+$ (the low-affinity F_C receptor IIIA), and KIR^+, and comprise the majority of circulating NK cells. F_C binding to CD16 leads to NK cell degranulation and perforin-dependent cellular killing. In fact, NK cells harboring a CD16 polymorphism that increased affinity for Rituximab demonstrated an improved response to the drug. It is not yet clear whether $CD56^{dim}$NK cells mature from the $CD56^{bright}$NK cells, or whether these reflect two different maturation pathways.

The cytokines and transcriptional events involved in NK maturation are still being elucidated. NKP numbers are not clearly affected by mutations of c-Kit, and are only slightly reduced in the absence of Flt3, again suggesting that NK cell formation may be a default pathway. Alternatively, NK cell formation and function appear to be critically dependent on the transcription factors Ets-1 and Ikaros.

Leukemia stem cells

Leukemias appear to depend on a small population of leukemia stem cells (LSC), which also are called leukemia initiating cells (LIC), for their continued growth and propagation [143, 144]. The LSC/LIC is likely to be a crucial cellular target in the treatment of leukemias and, therefore, it is critical to understand the difference of cellular properties between LSCs and normal HSCs, and between LSCs and other proliferating leukemia cells.

Evidence for LSC/LIC was initially suggested by Buick and McCulloch, who found that AML-CFUs had a heterogeneous capacity for serial replating efficiency [145]. Dick et al. ultimately demonstrated this concept *in vivo* in 1994. In this now classic experiment, the transplantation of $CD34^+CD38^-$, but not $CD34^+CD38^+$ leukemia cells into lethally irradiated, immunodeficient NOD-SCID mice successfully repopulated the bone marrow and transmitted AML. These data suggest that, akin to normal hematopoiesis, leukemias may be maintained by a small minority of stem-like leukemia cells [146]. Furthering this concept has been the identification of LT-LSC and ST-LSC by Morrison and Weissman, and by Dick et al. [147, 148]. As was the case for LT-HSC, LT-LSC proved capable of long-term persistence in xenotransplanted mice, while ST-LSC demonstrated only an abbreviated capacity for repopulation.

It is not clear that mutational events must occur in HSCs. Transfection of MLL-GAS7, a chromosomal translocation known to produce mixed-lineage leukemias, was only able to do so in HSCs and MPPs, but not in lineage-restricted progenitors [149]. However, transfection with other leukemia encoding translocations (MOZ-TIF2, MLL-AF9, MLL-ENL) produced a leukemic phenotype regardless of the stage of differentiation of the transduced cells [150–152]. This concept is further refined by studies involving humans with leukemia arising out of the AML1-ETO translocation. In this setting, more primitive MPP cells ($CD34^+CD90^-CD38^-$) chimeric for this mutation produced normally differentiating multilineage clonogenic precursors, while more mature cells ($CD34^+CD90^-CD38^+$) produced leukemic blast colonies, suggesting that while the initial translocation may occur in a primitive stem cell, subsequent events must occur in the committed progenitor pool to enable development of LSC/LIC [153]. This notion of a "pre-leukemic" stem cell is supported by twin studies in which both share a compromising genotype (e.g., MLL, Tel-AML1), yet develop leukemia at different times [154]. In fact, Hong et al. have identified and xenotransplanted these unique preleukemic cells from the non-affected twin, and found them to be clonally related to LSC/LIC from the affected twin [155].

Alternatively, while normal hematopoiesis is thought to proceed in unidirectional manner, the genetic and epigenetic instability inherent to "committed" leukemic cells may allow for dedifferentiation to a LSC/LIC phenotype, as proposed in breast cancer [156]. Phenotypic analysis of LSC/LIC in AML suggests that they are significantly different from normal HSCs. For example, $CD34^+CD38^-$ LSCs in AML lack CD90 expression, and instead express IL-3Rα, similar to what is seen in GMPs. This phenotype could suggest evolution to an LSC/LIC from a mutated GMP via a loss of CD38. More importantly, its aberrant expression may allow targeted therapy without affecting non-leukemic resident HSCs [157]. Indeed, a clinical trial testing this hypothesis has recently completed accrual [158]. Similarly in ALL, LSC/LIC express a $CD34^+CD38^-$ phenotype while retaining CD19 expression [52].

Although stem cells have a high proliferative capacity, they spend most of their time in the G_0 phase of the cell cycle, limiting the efficacy of cell-cycle-specific chemotherapeutic agents or any treatment that requires cell replication in order to be cytotoxic. This may explain the high rate of relapse seen in acute myelogenous leukemias, even after

seemingly near-complete eradication of detectable leukemia cells with conventional regimens. Additionally, the commonly observed decreases in progression-free survival and increases in chemotherapy resistance with successive chemotherapy regimens also may be a consequence of expansion of the LSCs, which are resistant to therapy.

Little is known about the LSC/LIC niche or microenvironment in the bone marrow, although in certain situations, the niche may even promote a malignant phenotype, as seen in Rb inactivation studies [159]. Presence of the α4β1 integrin, VLA-4, on AML blast cells has been found to be associated with resistance to chemotherapy. It has been postulated that the VLA-4 induces resistance of the AML blasts to chemotherapy by mediating binding of the VLA-4^+ blasts (which may be LSC/LIC) to stromal fibronectin [160]. Emerging preclinical data are beginning to accumulate suggesting that targeted inhibition of specific chemokines (CXCR4) and cell surface molecules (e.g., CD44, CD123) may impair the ability of LSCs to interact with their niche, and allow for more successful therapeutic outcomes [15, 161, 162]. What is still unknown is whether LSC/LIC inhabit specific areas of the bone marrow microenvironment, particularly the endosteal region, which appears to protect normal LT-HSC that remain in the non-dividing G_0 state, and whether the normal niche functions to protect LSC/LIC by mechanisms similar to the effects of the niche on normal LT-HSC, or whether LSC/LIC are capable of interacting with distinct protective microenvironments of their own.

Summary

Malignancy in the hematopoietic system can be traced to multiple steps along respective developmental paradigms, reflecting distinct changes in factors that govern differentiation, survival, and proliferation. As described herein and in subsequent chapters, such stimuli are diverse, and often require perturbations in both intrinsic and extrinsic mechanisms before ultimately culminating in a neoplastic phenotype.

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