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Myeloid cell origins, differentiation, and clinical implications

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Abstract

The hematopoietic stem cell (HSC) is a multipotent stem cell that resides in the bone marrow and has the ability to form all of the cells of the blood and immune system. Since its first purification in 1988, additional studies have refined the phenotype and functionality of HSCs and characterized all of their downstream progeny. The hematopoietic lineage is divided into two main branches: the myeloid and lymphoid arms. The myeloid arm is characterized by the Common Myeloid Progenitor and all of its resulting cell types. The stages of hematopoiesis have been defined in both mice and humans.

During embryological development, the earliest hematopoiesis takes place in yolk sac blood islands then migrates to the fetal liver and hematopoietic organs. Some adult myeloid populations develop directly from yolk sac progenitors without apparent bone marrow intermediates, such as tissue resident macrophages. Hematopoiesis also changes over time, with a bias of the dominating HSCs towards myeloid development as animals age. Defects in myelopoiesis contribute to many hematologic disorders, and some of these can be overcome with therapies that target the aberrant stage of development. Furthermore, insights into myeloid development have informed us of mechanisms of programmed cell removal. The CD47/SIRPα axis, a myeloid-specific immune checkpoint, limits macrophage removal of HSCs but can be exploited by hematologic and solid malignancies. Therapeutics targeting CD47 represent a new strategy for treating cancer. Overall,

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DISCLOSURES

K.W., P.J.S., M.P.C., A.C., and I.L.W. declare patents related to CD47-blocking therapies assigned to Stanford University and licensed to Alexo Therapeutics or Forty Seven, Inc. K.W. declares equity and/or consulting with Alexo Therapeutics and Forty Seven, Inc. M.P.C. and I.L.W. declare equity and/or consulting with Forty Seven, Inc.

an understanding of hematopoiesis and myeloid cell development has implications for regenerative medicine, hematopoietic cell transplantation, malignancy, and many other diseases.

The Hematopoietic System

The hematopoietic stem cell (HSC) is a multipotent stem cell that resides in the bone marrow and has the ability to form all of the cells of the blood and immune system. As the quintessential stem cell, it has the ability to self-replicate and differentiate into progeny of multiple lineages. Hematopoiesis describes the process of differentiating from HSCs to mature, functional cell types of the blood lineages. The existence of HSCs was first hypothesized following early experiments that demonstrated animals that received lethal doses of irradiation could be rescued by transplanting unfractionated bone marrow cells (1). The transplanted cells repopulated the bone marrow of the recipients and gave rise to all the cells of the blood. In accordance with this observation, in 1961 Till and McCulloch showed that unfractionated bone marrow cells were able to generate mixed hematopoietic (myeloid and erythroid) colonies in the spleens of lethally irradiated mice (2). They subsequently demonstrated that these colonies were formed by single cells that were capable of multilineage differentiation (3). Given the limitations in technology at the time, they were unable to purify these cells further, and the experiment that showed clonal origin of spleen colonies did not include lymphoid cells (2, 3), although a later experiment did (4). Years later, with the advent of monoclonal antibodies and fluorescence-activated cell sorting (FACS), these cells could be further characterized, purified, and evaluated in functional assays. Studies have now conclusively demonstrated that the HSC is a rare population of cells that gives rise to all of the cells comprising the two main branches of the hematopoietic lineage: the myeloid arm and the lymphoid arm. In mice, all long-term HSCs (LT-HSCs) are $Hoxb5^+$ (5) and located in the central marrow attached to the abluminal side of venous sinusoids.

In general, the hematopoietic lineage is organized such that HSCs sit atop the hierarchy and give rise to committed progenitor cells, which in turn give rise to mature, differentiated cells (Figure 1). There are two major differences between HSCs and committed progenitors: HSCs are multipotent and they have the ability to self-renew indefinitely. The transition between LT-HSC and short-term HSC (ST-HSC) is prospectively isolatable (6–8), and the poorly self-renewing ST-HSC and other multipotent progenitors (MPP) are nevertheless fully multipotent at the single cell level (9). Downstream of MPP are committed progenitors that are oligopotent and have limited ability to self-renew. As general principles, when cells progress through hematopoiesis they become more differentiated and more frequent in number. They also lose their capacity to self-renew, become more restricted in their differentiation potential, and gain expression of molecules required for functional specialization. Differentiation occurs in one direction with restriction towards a particular lineage, with no significant evidence of transdifferentiation between hematopoietic lineages under normal conditions. This chapter focuses on the characterization and isolation of the HSC in both mice and humans, as well as insight gained from the study of myelopoiesis, the development and specialization of the myeloid arm of the hematopoietic lineage.

Identifying the Hematopoietic Stem Cell in Mice

The HSC was first defined and isolated from mice. In 1988, 27 years after the work of Till and McCulloch, Spangrude et al. (1988) investigated whether distinct progenitors give rise to each cell lineage or whether a single cell could give rise to all of the cells of the hematopoietic lineage (10). Cell-sorting experiments demonstrated only a Thy-1^{lo}Lin⁻Sca-1⁺ population of bone marrow was able to form both myeloerythroid cells and lymphoid cells using colony forming assays (10) (Figure 2). As a gold standard for evaluation of HSCs, transplantation of Thy-1^{lo}Lin⁻Sca-1⁺ cells were able to produce all hematopoietic lineages in lethally irradiated mice with approximately 2,000-fold enrichment over unfractionated whole bone marrow (10). While these cells were found in very small number, representing only 0.1–0.2% of the Thy-1^{lo}Lin⁻ cells and about 0.05% of total bone marrow, even mice injected with single Thy-1^{lo}Lin⁻Sca-1⁺ cells were able to reform both lymphoid and myeloid lineages (11).

The Spangrude et al. (1988) study provided the earliest definition of the HSC. Subsequent efforts were made to refine the mouse HSC population further by defining additional surface molecules that enriched for stem cell activity. C-kit, a receptor tyrosine kinase that binds stem cell factor (SCF), was discovered as another important marker that facilitates isolation of the HSC population (12). This marker would later be incorporated to define the c-Kit⁺Thy-1^{lo}Lin⁻Sca-1⁻ population that is often used conventionally as the basis for purifying mouse HSCs. The importance of c-Kit as an HSC marker was also demonstrated by injection of monoclonal antibodies to c-Kit in vivo, which caused depletion of HSCs and bone marrow ablation (13, 14). From a translational perspective, this finding supports the use of antibody-based strategies for conditioning patients for stem cell transplant without the need for radiation or chemotherapy.

Following the successful identification of the HSC population, additional studies investigated subsequent stages of hematopoietic differentiation. Morrison and Weissman (1994) further subdivided the HSC population based on low levels of the lineage markers Mac-1 and CD4. The Thy-1.1^{lo}Sca-1^{hi}Lin⁻Mac-1⁻CD4⁻c-Kit⁺ population of bone marrow cells exhibited the greatest potency for long-term HSC (LT-HSC) activity (15). In secondary transplant experiments, in which the bone marrow of transplanted mice was used to reconstitute a second irradiated recipient, the LT-HSC population was the only population capable of sustaining long-term multilineage reconstitution (15). The LT-HSCs self-replicated and gave rise to ST-HSCs with more limited proliferative potential, establishing a hierarchical relationship between these two populations.

Subsequent investigations further refined the HSC population by introducing novel markers that distinguish it from downstream multipotent progenitors (MPP). As examples, Flk-2 was found to mark ST-HSC and MPPs (6, 7), and CD48 was identified on MPP but not HSC (8). On the contrary, CD150 was identified as a marker expressed by nearly 50% of cells within the HSC population but less than 1% of the MPP population (8). The use of these additional markers allowed for the greatest purification of LT-HSC activity, such that one out of every 2.1 CD150⁺CD48⁻Sca-1⁺lin⁻c-kit⁺ cells engrafted and gave long-term multilineage reconstitution (8). This extraordinarily enriched population of HSCs represented only

0.0058% of total bone marrow cells (8). Interestingly, new studies using transposon-based clonal markers suggest the MPP populations may make substantial contributions to hematopoiesis under normal, steady state conditions without transplantation (16). Most recently it was found that even the most refined hematopoietic stem cell population included both short-term and long-term HSC (5). Pure LT-HSC in mice have now been identified as HoxB5⁺, and represent 20–50% of the cells in the CD150⁺CD48[−]Sca-1⁺lin[−]c-kit⁺ population. Only HoxB5⁺ cells in the subset are LT-HSC, the rest are ST-HSC, as best defined by secondary transplantation (5). Over 90% of these LT-HSC are attached to the abluminal surface of the small venous endothelium mainly in marrow sinusoids, and few or none are adjacent to the endosteum or arterioles in the marrow (5). These cells represent ~ 1 in 100–200,000 bone marrow cells (5). No such marker systems have been found yet for pure long-term human HSC.

Defining Committed Progenitor Cells in the Mouse

With the establishment of the hematopoietic stem cell compartment, subsequent studies aimed to identify additional intermediate stages of development. In particular, researchers aimed to identify the earliest stages of hematopoietic development that would specify fate determination towards the two main branches of the hematopoietic system, the lymphoid or the myeloid arm. As the first demonstration that such a pure population of cells could exist, Kondo et al. (1997) identified a Lin[−]IL-7Rα⁺Thy-1[−]Sca-1^{lo}c-Kit^{lo} population within the bone marrow that exhibited limited self-renewal relative to HSCs and was restricted in its ability to differentiate: it exclusively formed cells of the lymphoid lineage, including B, T, and NK cells (17). This population was therefore defined as the Common Lymphoid Progenitor (CLP).

Investigators soon hypothesized that a counterpart to the CLP existed for the myeloid lineage. Akashi et al. (2000) identified and isolated such a cell population, deemed the Common Myeloid Progenitor (CMP). Within an IL-7Rα[−]Lin[−]c-Kit⁺Sca-1[−] fraction that contained myeloid progenitor activity, further subpopulations could be identified based on expression of Fcγ receptor-II/III (FcγR) and CD34 (18). The FcγR^{lo}CD34⁺ population gave rise to the full myeloid lineage, indicating it contained CMP activity (18). On the other hand, the FcγR^{hi}CD34⁺ fraction primarily formed macrophages or granulocytes, and the FcγR^{lo}CD34[−] cells gave rise exclusively to megakaryocytes and erythroid cells (18). Therefore, these populations represented two mutually exclusive intermediates in myelopoiesis downstream of the CMP, the granulocyte/macrophage lineage-restricted progenitor (GMP) and the megakaryocyte/erythrocyte lineage-restricted progenitor (MEP). Single-cell analyses have revealed that the CMP in mice has at least two distinct subsets (19), and recent RNA-seq data confirm that this population is still heterogeneous (20). Nevertheless, a hierarchical relationship was confirmed between these cell types, with CMP giving rise to both GMP and MEP, and the latter cells eventually giving rise to terminally differentiated progeny (18).

Identifying Murine Myeloid Progenitor Cells with Restricted Lineage Potential

The establishment of the mouse CMP, GMP, and MEP suggested that murine myelopoiesis proceeded in a stepwise, linear fashion. Therefore, efforts were aimed at identifying progenitor cells that lay downstream with more restricted lineage potential, or populations that would give rise exclusively to a single lineage of cells. As an early demonstration that such a population existed, Nakorn et al. (2003) isolated and characterized the megakaryocyte-committed progenitor (MKP) by transplanting a $CD9^{+}Fc\gamma R^{lo}c-kit^{+}Sca-1^{-}IL-7R\alpha^{-}Lin^{-}$ population into mice, which formed megakaryocytes in the spleen and platelets in the blood but no other myeloid or lymphoid cells (21). A unipotent erythrocyte-committed progenitor (EP) was also identified in a $lin^{-}c-Kit^{+}Sca-1^{-}IL-7R\alpha^{-}IL-3R\alpha^{-}TER119^{-}CD41^{-}CD71^{+}$ population. In vivo experiments using injections into irradiated mice showed EPs were able to form reticulocytes but lacked formation of other myeloid cells, providing evidence for their lineage restriction (22). Another unipotent progenitor, the eosinophil lineage-committed progenitor (EoP), was characterized as $Lin^{-}Sca^{-}CD34^{+}c-kit^{lo}IL-5R\alpha^{+}$ cells, which exclusively produced eosinophils in vitro (23).

Fogg et al. (2006) identified a bone marrow population that expressed an established mononuclear phagocyte marker, CX_3CR1 , as well as $c-Kit$ but was negative for other lineage markers (24). When transfused into the blood of recipient mice, the $CX_3CR1^{+}c-kit^{+}Lin^{-}$ cells gave rise to monocytes and populations of macrophages and dendritic cells in the spleen, identifying these cells as macrophage-dendritic cell progenitors (MDPs) (24). Specialized tissue macrophages such as peritoneal macrophages and microglia were also detected as progeny of MDPs (24). Notably, the MDP did not give rise to other cell types of the hematopoietic lineage, and the ability to form neutrophils remained within the $CX_3CR1^{-}c-kit^{+}Lin^{-}$ population (24). Traver et al. (2000) also showed that two major populations of dendritic cells, $CD8\alpha^{+}$ and $CD8\alpha^{-}$ cells, both arise downstream of the CMP (25).

Studies using $\beta 7$ integrin as a marker suggested a common origin between mast cells and basophils, cell types involved in allergic responses. Within the $Lin^{-}c-Kit^{+}Sca-1^{-}$ myeloid progenitor population, a $\beta 7^{+}T1/ST2^{+}$ subpopulation was identified that formed exclusively mast cells and was deemed the mast cell progenitor (MCP) (26). A unique $Lin^{-}c-Kit^{+}\beta 7^{hi}$ population in the spleen gave rise to mast cell and basophils, suggesting this population represented a basophil-mast cell progenitor (BMCP) (27). In the bone marrow, a $Lin^{-}CD34^{+}Fc\epsilon R1\alpha^{hi}c-Kit^{-}$ population gave rise exclusively to basophils, and thus was deemed the Basophil Progenitor (BaP) (27). A lineage hierarchy existed between these cell populations when differentiated in vitro, with BMCP capable of giving rise to either BaPs or MCPs, although some evidence also suggests the MCP could arise directly from MPPs (26, 27). Recently Chen and colleagues have found that a subset of HSC are also integrin $\beta 7^{+}$ and differ in their functional properties, and so the potential heterogeneity in the HSC (long-term and short-term) subsets and their eventual fates remain to be elucidated (28).

To refine other intermediate stages of myeloid development, Pronk et al. (2007) identified cell populations that represented distinct stages of development between CMP and downstream progenitors (29). Functional studies established new populations such as Pre-MegE and Pre-GMP, which had greater proliferative potential than their respective downstream progeny: MEP, and GMP, respectively (29). These findings helped demonstrate the multiple stepwise fashion of hematopoiesis, where as cells proceed down the hematopoietic lineage and become more limited in their differentiation potential, they also lose proliferative potential.

Defining the Hematopoietic Lineage in Humans

Based on the successful purification of the murine HSC, Baum et al. (1992) sought to prospectively isolate the human HSC. They identified a human fetal bone marrow $\text{Lin}^{-}\text{Thy-1}^{+}\text{CD34}^{+}$ population that contained multipotent hematopoietic stem cell activity (30). These candidate HSCs were highly enriched for the ability to differentiate into myeloid and lymphoid cells in vitro using long-term colony forming assays and in vivo using immunocompromised mouse models. Hematopoietic cell transplants with purified $\text{CD34}^{+}\text{Thy-1}^{-}$ HSCs have been successfully performed in patients, providing clinical evidence that the human HSC activity resides in this population (31–33).

Other studies identified markers that further enriched for human HSC activity to refine the HSC population. In particular, CD38 was validated as a marker following xenotransplantation of human hematopoietic cells into immunocompromised mice, with HSC activity residing within the $\text{Lin}^{-}\text{CD34}^{+}\text{CD38}^{-}$ fraction (34). The full subset of $\text{CD34}^{+}\text{CD90}^{+}\text{CD38}^{-}\text{Lin}^{-}$ was shown by Uchida and colleagues to contain human HSCs, and these could be genetically modified with lentiviral, but not retroviral vectors (35). Majeti et al. (2007) prospectively isolated human MPP that lie downstream of HSCs. Using CD90 (Thy-1) and CD45RA as markers, they subdivided the $\text{Lin}^{-}\text{CD34}^{+}\text{CD38}^{-/\text{lo}}$ population into $\text{CD90}^{+}\text{CD45RA}^{-}$ and $\text{CD90}^{-}\text{CD45RA}^{-}$ subpopulations. Both gave rise to myeloid and lymphoid cells, but the $\text{CD90}^{+}\text{CD45RA}^{-}$ population exhibited nine-fold greater human chimerism in the bone marrow (36). Thus, the $\text{CD90}^{+}\text{CD45RA}^{-}$ population contains the true LT-HSC in humans, while the $\text{CD90}^{-}\text{CD45RA}^{-}$ population represents an intermediate MPP.

Human Committed Progenitors and Restricted Myeloid Differentiation

The first functional demonstration of a lineage-restricted human progenitor population was achieved through studies examining lymphoid development. Isolation of $\text{Lin}^{-}\text{CD34}^{+}\text{CD45RA}^{+}\text{CD10}^{+}$ cells yielded a human bone marrow subpopulation that was devoid of myeloid potential when transplanted into immunocompromised mice (37). However, these cells were capable of forming all of the cell types of the lymphoid arm, including T and B cells, NK cells, and lymphoid dendritic cells, with bias to the B-cell lineage. Therefore, this oligopotent progenitor contains human CLP activity. Given this evidence, Manz et al. (2002) reasoned that counterparts to the mouse CMP, GMP, and MEP existed and sought to define and demonstrate their lineage potential by prospective isolation. The authors found that the $\text{lin}^{-}\text{CD34}^{+}\text{CD38}^{+}$ population of human bone marrow has limited ability to self-renew and exhibits a high proportion of myeloid-biased differentiation (38).

Expression of CD45RA and IL-3R α further subdivided this population, yielding three distinct subpopulations: IL-3R α ^{lo}CD45RA⁻, IL-3R α ^{lo}CD45RA⁺, and IL-3R α ⁻CD45RA⁻ cells. In vitro, the IL-3R α ^{lo}CD45RA⁻ population gave rise to the full range of the myeloid lineage, including mixed colonies, suggesting this population represented the human CMP (38). On the other hand, the IL-3R α ^{lo}CD45RA⁺ population only gave rise to cells of the granulocyte and macrophage lineages, and the IL-3R α ⁻CD45RA⁻ population predominantly gave rise to cells of the erythroid and megakaryocyte lineage; thereby indicating these populations represented the GMP and MEP, respectively (38). The same lineage hierarchy existed as with mouse myelopoiesis, with CMPs giving rise to GMP or MEP. Additional markers, such as the growth factor receptor TpoR, have been suggested to offer further refinement of these myeloid progenitor populations (39).

Additional studies examined unipotent committed progenitors in the human hematopoietic lineage. Some evidence suggests a unipotent human basophil/mast cell progenitor exists within a subpopulation of CD34⁺ cells that are recognized by an antibody to ectonucleotide pyrophosphatase/phosphodiesterase-3, or CD203c, although a unipotent population was not purified (40, 41). Mast cell potential was also identified within a CD34⁺c-Kit⁺CD13⁺ fraction (42). Mori et al. (2009) identified the human EoP that lay downstream of CMP and gave rise only to eosinophils. This population was defined as IL-3R α ^{lo}CD45RA⁻IL-5R α ⁺, which also helped refine the human CMP population (38, 43). Although mature basophils also express IL-5R α , their development was separate from the EoP and seemed to occur via CMP and GMP intermediates (43). Within the human MEP population, fractionation studies helped define the unipotent human erythrocyte progenitor (EP) as CD71^{intermediate(int)}/CD105⁺, and when sorted to purity, gave rise exclusively to erythrocytes in vitro with no megakaryocyte potential (44). Additionally, an erythrocyte-biased MEP (E-MEP) was identified as CD71⁺CD105⁻ that was an intermediate between the MEP and the EP (44). Downstream stages of human erythropoiesis have also been isolated to purity, including the BFU-E and CFU-E. These populations were principally distinguished as IL-3R⁻CD34⁺CD36⁻ and IL-3R⁻CD34⁻CD36⁺, respectively (45). Remaining questions surrounding intermediates in human myelopoiesis include definitive isolation and characterization of additional unipotent lineages, such as a neutrophil-lineage restricted progenitor, MDP, and MKP.

Comprehensive gene expression profiling of the hematopoietic system

To better understand the complex regulation of the hematopoietic hierarchy, a robust systems approach is needed for each molecular layer (e.g. gene expression). Microarrays and RNA sequencing (RNA-seq) have become the predominant technologies for genome-wide gene expression profiling, yet they face challenges in their analysis. First, the efficiency of detection depends on a target sequence, thus each probe set on a microarray has a different sensitivity. This limits the accuracy of profiling to relative differences in gene expression based on pair-wise comparisons. Many genes are scored as “not significantly different” regardless of the expression levels, and therefore the result is comparison-pair specific. Thus, conventional analysis depicts a profile of differences between cell types A and B instead of an overall profile of gene expression in cell type A. The second challenge pertains to the dynamic range of gene expression, which is different for every gene. Thus, even in

microarray based pair-wise comparisons or in RNA-seq, we may misinterpret the significance when comparing differences of expression between genes. For example, if gene X has a narrow dynamic range and gene Y has a wide dynamic range, a two-fold change in expression may be significant for gene X but not for gene Y.

To address these limitations, Seita et al. (2012) hypothesized that by examining expression of a particular gene across thousands of samples, a common reference for that gene could be established and applied to any new sample (46). Computer simulations demonstrated that if a random collection of over 5,000 microarray data sets could be accumulated, meta-analysis could be performed to compute robust statistical attributes for each gene, such as the dynamic range of each gene's expression levels and a threshold between active and inactive gene expression. Subsequently, by mapping new sample data against the results of the meta-analysis, a more objective genome-wide gene expression profile could be achieved for each sample individually. Based on this result, the entire microarray data collection available through the NIH Gene Expression Omnibus (GEO) public repository was subjected to meta-analysis to obtain a dynamic-range for each gene's expression level. This analysis was incorporated into a web-based platform named the Gene Expression Commons (<https://gexc.stanford.edu>). Gene Expression Commons is an open platform, thus any scientist can explore expression activity of any gene of interest and submit and analyze his or her own data. We have contributed microarray data of highly purified mouse and human hematopoietic stem and progenitor populations. The complete mouse adult hematopoiesis system containing 39 distinct populations and a partial collection of human normal and neoplastic hematopoiesis are available to the public on the Gene Expression Commons platform. This platform, designed with an intuitive interface, facilitates the identification and visualization of gene expression patterns that differ between hematopoietic populations (Figure 3).

Origins of the hematopoietic system in the embryo to fetal transition

Based on studies by Moore and Owen using avian models, Moore and Metcalf (1970) found mouse hematopoietic cells capable of forming mixed lineage myeloerythroid colonies in vitro in the embryonic day (E) 8 mouse yolk sac, the first site of appearance of blood cells (47). Weissman et al. transplanted yolk sac blood island cells from E8–9 mice into the yolk sac cavity in vivo of the same aged (E8–9) haploidentical hosts (48, 49). All such mice, when sacrificed as young adults, possessed both donor and host day 10 CFU-S, and also T cells, demonstrating HSC activity within this yolk sac population (48, 49). Dzierzak and Medvinsky challenged this finding when they cultured E10–12 yolk sac cells or aorto-gonad-mesonephros (AGM) dorsal aorta tissue in vitro, and showed higher hematopoietic activity in the AGM dorsal aorta (50). Many groups used morphological analysis to demonstrate cell clusters in the E10 ventral surface and subendothelial tissue of the dorsal aorta at those time points, consistent with an independent origin of later fetal hematopoiesis in the embryo rather than the extraembryonic yolk sacs. Keller and colleagues showed that mouse embryonic stem (ES) cells could differentiate into hematopoietic cells but they also appeared to make endothelial cells in the colonies as well; they called the common precursor the hemangioblast (51). Many groups studying birds, zebrafish, and even in vitro dorsal aortas showed images of cells 'budding' from the aortic endothelium into the blood

circulation, and dubbed the cells hemogenic endothelium. These cells have been proposed to be a common, clonal precursor to blood and blood vessel cells (for review see (52)). However, the evidence for a required common, clonal precursor for blood and blood vessel cells in the E7.5–9 mouse embryo yolk sac, which appears at least two days before the AGM dorsal aorta has blood cells, was challenged by the analyses of tetrachimeric mice. These mice were generated by the injection of 5 red, 5 green, and 5 blue fluorescently labeled ES cells into uncolored mouse blastocysts, followed by sacrifice and analyses of the E7.5–9 yolk sac blood islands (53). Each blood island was generated by multiple ES donor cells. If there was a required clonal origin for blood and blood vessel cells, the colors of endothelium surrounding and blood cells within discrete blood islands should always match; however they rarely did (53). The same result came from injecting a single colored ES cell into uncolored mouse blastocysts; the yolk sac blood islands of these mice rarely contained blood and blood vessel cells derived from the single ES cell. This was also confirmed with the use of *flk1-cre* mice crossed to our rainbow construct of three colors, with random clonal marking of each cell; again there was little or no concordance between blood and blood vessels (53). These results are consistent with the origin of blood and blood vessel cells from a common hemangioblast or hemogenic endothelium only if the cell had appeared prior to E7 (53). Lineage tracing of hematopoietic progenitors from yolk sac in *Runx1 MER-Cre-MER* mice with floxed reporters revealed that the great majority of adult blood cells of myeloerythroid and lymphoid lineages were derived from E7–8 mice (54). Later studies performed quantitative assessment of yolk sac versus AGM HSCs phenotypically separated and cultured in vitro. At all time points from E10 onward, the number of phenotypic and transplantable HSC in the yolk sac far exceeded that in the dorsal aorta, and the appearance of large numbers of HSC in the fetal liver could not be supported by the numbers of dorsal aorta HSC only (55). Thus the field has contradictory findings from these studies.

However, Merad, Geissmann and others (see elsewhere in this book) have demonstrated that the yolk sac blood islands are the origin of long-term resident macrophages, dendritic cells, and microglia, distinct from the monocyte origin of such cells in postnatal mice (56–58). With the division of resident, self-renewing macrophage/DC lineage cells and adult bone marrow HSC-derived cells of these lineages now quite clear, the clonal origin of hematopoiesis in the embryo, fetus, and postnatal needs to be re-addressed with much better experimental tools.

Changes in Myelopoiesis with Aging

A number of studies have demonstrated a decrease in the function of the innate and adaptive immune system with age. Since HSCs give rise to the entire repertoire of blood cells, aging of HSCs directly impacts downstream myelopoiesis and lymphopoiesis. Although the number of phenotypic HSCs increases with age, the regenerative capacity of these cells decreases. The balance between lymphoid and myeloid differentiation of HSCs also changes over time, resulting in a bias towards myeloid differentiation in both mice and humans with aging (59–61). Studies have suggested that the HSC population is heterogeneous in its differentiation potential, and clonal expansion of certain subsets may contribute to changes in propensity for myeloid versus lymphoid development with increasing age (62–65). Gene expression changes and altered epigenetic landscapes have been demonstrated to contribute

to these effects (60, 66, 67). Additionally, the bone marrow microenvironment can modify the degree of myeloid bias. In one study, a decrease in myeloid differentiation was observed when HSCs from aged mice were transplanted into young recipients relative to aged recipients (68). This difference was partially attributed to increases in cytokines such as Rantes within the bone marrow microenvironment of aged animals, which promotes myeloid skewing (68).

These findings have implications for HSC transplantation since the ideal selection of allogeneic donors would ensure balanced reconstitution of lymphoid and myeloid lineages in both young and aged recipients. Recent evidence indicates the small RhoGTPase Cdc42, which maintains polarity of HSCs, demonstrates an age-associated increase in expression and thereby promotes functional changes in HSCs (69). Pharmacological inhibition of Cdc42 via a novel molecule, CASIN, was sufficient to reverse the age-related changes through an increase in ACh4K16 (69). This reversal of age-related changes provides a potential clinical application for HSC transplantation of autologous or aged hematopoietic cells into aged recipients.

The existence of multiple HSC populations that differ in their lineage bias may have additional implications for health and disease. Having multiple, competing HSCs certainly allows the host to be able to produce more naïve T and B cells early in life, and more myeloid cells later in life. This enables more appropriate generation of adaptive versus innate immune cells at different ages. But why lose lymphopoiesis later in life? We propose that another class of unipotent stem cells are present in the lymphocyte lineages—T stem/memory and B stem/memory cells. In the absence of stimulation of their antigen receptors, these cells are resting and express homing receptors that allow them to patrol lymphoid organs for the appearance of antigens, just as naïve T and B cells express CD62L or integrin $\alpha_4\beta_7$ for homing to lymph nodes or Peyer's patches, respectively (for review see (70)). Upon recognizing their cognate antigens, these lymphoid stem/memory cells proliferate and self-renew to create more stem/memory cells and also differentiate to effector cells. Thousands of years ago, before trains, planes, and cars, vertebrates largely were geographically limited. Thus, by a decade or two of age, individuals had encountered most of the microbial pathogens in their environments, and by that time they had developed the groups of T and B stem/memory cells required to protect them. This likely fed back to the HSC pool, shifting gradually from lymphocyte-biased to myeloid-biased stem cells. Unfortunately, our rate of evolution could not keep pace with advances in modern transportation and exposure to pathogens from around the world. Therefore, our efficient system of shifting hematopoietic biases with age has likely enabled novel pathogens to cause pandemics in the very old and very young.

Aberrant myelopoiesis as a cause of hematologic disease

By more rigorous characterization of the hematopoietic lineage in both mice and humans, we gain an understanding of the mechanisms that promote normal development and how to intervene when these processes contribute to disease. Myelopoiesis is perturbed in many blood disorders and diseases, including chronic and acute myeloid leukemias, myelodysplastic syndrome, myeloproliferative disorders, iron or other nutrient deficiency

anemias, as well as a number of inherited bone marrow failure conditions. One potential therapeutic strategy to treat these conditions is to manipulate or reverse the abnormal myelopoiesis. Understanding normal myeloid development as well as the specific aberrant defects in myeloid development found in these diseases has been and will continue to be critical in how we treat patients.

For example, in the chronic phase of chronic myeloid leukemia (CML), there is aberrant overproduction of mature myeloid cells caused by the BCR-ABL translocation (71). When CML advances to blast crisis phase, acquisition of additional mutations, such as in GSK3beta, leads to the activation of beta-catenin and blocks myeloid maturation at the level of the GMP (72, 73). The resulting accumulation of these progenitors, or blasts, in the bone marrow and blood severely impairs normal hematopoiesis. There are currently several highly effective tyrosine kinase inhibitors that target BCR-ABL available to treat CML in chronic phase, and these medications have been very successful in controlling this phase of disease long-term as well as reducing the frequency of transformation to blast crisis CML (74, 75). However, blast crisis CML remains a therapeutic challenge today. Targeting GSK3beta or deactivating the beta-catenin pathway may cause blast crisis CML to differentiate to a milder form of disease.

In acute myeloid leukemia (AML), there is a rapid, abnormal accumulation of immature myeloid progenitors in the bone marrow and blood. In one particular subtype of AML, acute promyelocytic leukemia (APL), which is most commonly associated with a PML-RARalpha translocation, there is a differentiation block at the promyelocyte stage of myelopoiesis (76). This differentiation block can be overcome by the administration of all-trans retinoic acid (ATRA), a medication that has transformed a difficult-to-treat leukemia with extremely poor prognosis into a curable disease without the need for traditional toxic chemotherapies (76). When ATRA induces differentiation of the abnormal promyelocytes, the differentiated leukemia cells exit the bone marrow and normal hematopoiesis is able to recover. Further understanding of the mechanisms behind impaired myeloid differentiation in other subtypes of AML could be important to the development of similar therapeutic strategies for these diseases.

Myelodysplastic syndrome (MDS) is an age-associated condition characterized by ineffective production of one or more mature myeloid, erythroid, or megakaryocytic lineages. We have shown that the early mutations associated with this disease lead to HSCs that outcompete normal HSCs, presumably at the proposed HSC niches (5, 77). Thus by the time MDS is detected, over 95% of the phenotypic HSCs clonally develop from a single mutated HSC. Unfortunately, this clone cannot make blood efficiently. In MDS, there is a specific and significant reduction in the number of GMPs in the bone marrow of low-risk, or early stage, patients (77) (Figure 4). Although there is increased apoptosis of MDS GMPs compared to normal GMPs, non-apoptotic MDS GMPs also have increased cell surface calreticulin (CRT), which targets them for programmed cell removal via phagocytosis (see below) (77). Indeed, blocking CRT can prevent the phagocytosis of MDS GMPs (77). It is possible that small molecules or other therapeutics that can inhibit phagocytosis and programmed cell removal of MDS GMPs may be able to ameliorate the often debilitating cytopenia that affects MDS patients. In high risk MDS, oftentimes arising from low risk

MDS, the GMPs express cell-surface CRT and acquire cell-surface expression of CD47, which prevents phagocytosis (77). This leads to the abnormal accumulation of these progenitors in the bone marrow, which may eventually transform into AML.

In addition to BCR-ABL inhibitors and ATRA, there are other examples of our ability to successfully alter abnormal myeloid development with targeted therapies in order to treat hematopoietic disorders and diseases. Perhaps the simplest examples of this concept are iron replacement therapy for iron deficiency anemia or nutrient replacement therapy for other nutrient deficiency anemias (vitamin B12, folate, copper). In these cases, supplementation of the deficient nutrient directly alleviates the impaired proliferation of myeloid progenitors and improves the production of mature blood cells. In polycythemia vera (PV) and essential thrombocythemia (ET), which are myeloproliferative disorders characterized by overproduction of mature red blood cells and platelets, respectively, acquisition of a mutation in JAK2 causes proliferation of myeloid progenitors in their respective lineages (78, 79). JAK2 inhibitors have been successfully used to treat patients with these conditions by inhibiting the proliferation of myeloid progenitors (80, 81).

Currently, there has been development of targeted therapies for only a small number of diseases that take advantage of manipulating abnormal myelopoiesis. However, the success of these agents should only inspire us to continue exploring this therapeutic strategy for other myeloid disorders, including AML, MDS, myelofibrosis, inherited bone marrow failure syndromes, unexplained anemia of the elderly (UAE), among many other illnesses. For each of these conditions, detailed characterization of the aberrant stage in myeloid development may be crucial for the design of new targeted therapies.

CD47 and immune surveillance during normal and malignant hematopoiesis

From the study of normal hematopoiesis, we have also gained an understanding of fundamental principles that apply to other aspects of human disease. One example pertains to the CD47/SIRP α axis, which is emerging as a widespread immunotherapeutic target for many cancers. CD47 is a cell-surface molecule that is expressed on normal hematologic and non-hematologic tissues throughout the body, and it acts as a “marker of self” that limits macrophage phagocytosis. Oldenborg et al. (2000) provided an early functional demonstration of this concept when they transfused red blood cells from CD47^{-/-} mice into wild-type mice (82). The authors observed rapid elimination of the CD47^{-/-} cells from circulation by macrophages in the spleen. SIRP α , a single-pass transmembrane protein with intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), is the receptor by which CD47 transduces inhibitory signals. SIRP α is expressed on cells of the myeloid lineage, including macrophages, monocytes, granulocytes, and myeloid-derived dendritic cells (83–87).

A role for the CD47/SIRP α axis in cancer immunoevasion was further surmised from studies on HSCs. As with red blood cells, CD47 was found to function in stem cell migration by protecting circulating HSCs from host macrophage phagocytosis (88). Mobilization of mouse HSCs from the bone marrow to the periphery led to a transient

increase in CD47 expression (88). In addition, CD47 is required for in vivo HSC function, as CD47-deficient mouse HSCs failed to engraft in wild-type recipients in contrast to wild-type HSC controls (88). Preservation of the CD47/SIRP α interaction was also identified as a major factor that permits HSC engraftment across xenogeneic barriers (89, 90). From a clinical perspective, downregulation of CD47 on HSCs is a possible pathogenic mechanism of hemophagocytic lymphohistiocytosis (HLH), a life-threatening condition characterized by unrestrained macrophage phagocytosis of hematopoietic cells (91).

Given its role in protecting HSCs from host macrophages during cell migration, it was hypothesized that leukemic cells, and specifically leukemic stem cells (LSCs) could co-opt anti-phagocytic CD47 function to enable increased dissemination and immune evasion as a mechanism of tumorigenicity. In acute myeloid leukemia (AML) patients, CD47 cell surface expression was significantly increased on both leukemic blasts and LSC compared to normal cell counterparts (92). Importantly, forced overexpression of CD47 in a human leukemia cell line enabled engraftment of fulminant leukemia in vivo through specific evasion of macrophage phagocytosis (88). In addition, increased CD47 expression in AML patients predicted a worse overall survival, which was an independent prognostic factor (92). Given CD47's mechanism of immune evasion in AML, it was hypothesized that blockade of the anti-phagocytic CD47/SIRP α signaling axis could enable leukemia cells to become phagocytosed and eliminated. Indeed, a monoclonal antibody blocking CD47/SIRP α signaling led to robust phagocytosis of human AML cells but not normal hematopoietic cell counterparts (92). An anti-CD47 antibody rapidly eliminated leukemic disease in the peripheral blood and bone marrow in patient-derived xenografts (Figure 5). This therapeutic effect was dependent on macrophage effector cells as selective depletion of macrophages by liposomal clodronate completely abrogated the anti-leukemic effect of anti-CD47 antibodies. Furthermore, anti-leukemic activity was observed independent of cytogenetic and morphologic AML subtypes, suggesting that the anti-phagocytic function of CD47 is a universal mechanism across AML. Based on these preclinical data, humanized anti-CD47 antibodies have entered clinical development (93) and first-in-disease clinical trials in AML patients have begun (clinicaltrials.gov identifiers NCT02678338 and NCT02641002).

Since CD47 is normally expressed on tissues throughout the body, subsequent investigations examined whether the CD47/SIRP α axis also promoted immunoevasion in non-hematologic malignancies. Many solid tumors were found to express high levels of CD47 relative to corresponding normal tissues (94, 95). Furthermore, CD47 expression levels correlate with poor prognosis for many types of solid tumors including ovarian cancer, gliomas, and non-small cell lung cancer (94, 95), supporting the role of CD47 in immunoevasion. CD47-blocking therapies exhibited anti-tumor efficacy in a diverse range of preclinical xenograft mouse models, including ovarian, breast, colon, and bladder cancers, glioblastoma, leiomyosarcoma, pancreatic neuroendocrine tumors, small cell lung cancer, and melanoma (94, 96–99). Furthermore, therapeutic efficacy has been observed in multiple syngeneic immunocompetent models using anti-mouse CD47 antibodies, including breast cancer, lymphoma, melanoma, and neuroendocrine tumors (94, 97, 100). Based on these data, anti-CD47 antibodies are also undergoing evaluation in clinical trials for solid tumors (clinicaltrials.gov identifiers NCT02216409 and NCT02367196).

Macrophages play a critical role as effector cells in the immune response to anticancer antibodies by performing antibody-dependent phagocytosis (ADP) (101). This process is also inhibited by the CD47/SIRP α axis (102). In many cases, blockade of CD47 alone does not induce activation, but instead lowers the threshold for macrophage phagocytosis in response to an opsonizing antibody (103, 104). This principle was demonstrated using high-affinity SIRP α variants, engineered variants of the N-terminal domain of SIRP α that act as competitive antagonists to CD47 (103). When produced as high-affinity SIRP α -Fc fusion proteins, these proteins block CD47 and provide an opsonizing stimulus that induces phagocytosis, thereby yielding anticancer efficacy as a single-agent (97, 100, 103). However, when used as single domain high-affinity SIRP α 'monomers,' these proteins do not induce phagocytosis alone but synergize with anticancer antibodies that engage Fc receptors on macrophages (98, 103, 104). In this sense, the high-affinity SIRP α monomers may limit toxicity to normal cells expressing CD47 while stimulating robust anti-tumor responses by macrophages. Therapies targeting the CD47/SIRP α axis show much promise in combination with agents such as rituximab for CD20⁺ lymphoma, trastuzumab for HER2⁺ breast cancer, and cetuximab for EGFR⁺ solid tumors (103, 105). Blockade of CD47 can also stimulate macrophage antigen presentation after phagocytosis of cancer cells (106). This mechanism links the innate immune response to adaptive immunity. Moreover, CD47 may also enhance neutrophil anti-tumor responses as well as dendritic cell antigen presentation (86, 100). Thus, the CD47-SIRP α interaction acts as a myeloid-specific immune checkpoint and is a therapeutic target in many types of cancer (101).

The factors that determine the responsiveness of a tumor to CD47/SIRP α -blocking therapies are not fully understood. CD47 is expressed on both normal and malignant tissues, yet studies have found that malignant cells are more susceptible to phagocytosis. In many cases, CD47 expression levels are higher on tumors relative to normal tissues, but expression levels alone do not always predict responses (98). Some data suggests the function of CD47 may also depend on its conformation, association with integrins, and activation state (for review, see (107, 108)). It is likely that other factors such as the presence of redundant inhibitory signals or the degree of macrophage infiltration into tumors may also impact the efficacy and therapeutic window of CD47/SIRP α -blocking reagents. Further investigation of these factors in clinical studies will be valuable to identify biomarkers that predict which patients may benefit most from CD47/SIRP α blocking therapies.

Multiple CD47/SIRP α -blocking reagents have been developed, and they differ in their therapeutic and toxicity profiles. Some anti-CD47 antagonists may exert direct cytotoxicity on cells in addition to blocking the CD47/SIRP α interaction (109–111). This process seems to occur in a caspase-independent manner (109). Based on their ability to signal directly through CD47, some reagents may also have direct effects on activating other immune subsets, such as T cells (112, 113). The principal toxicity of targeting CD47 seems to pertain to red blood cell indices, and the main side effect observed in animal models has been a moderate anemia (93, 94, 103). Dosing strategies have been developed to mitigate this effect (93). Agents that block the CD47/SIRP α axis without contributing an opsonizing stimulus may also avoid this toxicity (86, 103).

Myeloid cells and programmed cell removal

Programmed Cell Removal (PrCR) of target cells by macrophages is determined by the balance between “eat me” and “don’t eat me” signaling pathways. Blockade of “don’t eat me” signals leads to the domination of “eat me” pathways in the target cells that allows them to be detected and recognized by macrophages. In 1994, we published that transgenic neutrophils that overexpressed human bcl-2 were resistant to programmed cell death, leading to their persistence in vitro for many days (114). In contrast, wild-type neutrophils died within hours due to apoptosis. In vivo, however, the number of transgenic bcl-2 neutrophils was homeostatically regulated despite their prolonged lifespan. This turned out to be due to PrCR; the wild-type neutrophils expressed cell surface “eat me” signals while undergoing cell death, but the healthy bcl-2 neutrophils expressed PrCR “eat me” signals while otherwise viable, leading to their removal by macrophages. Thus these cells, like MDS progenitors, could be removed by PrCR even if they were resistant to programmed cell death. It is of interest that the progression of MDS to AML is concurrent with the expression of CD47 by the emergent blast cells of MDS and the AML cells they generate.

We recently identified important roles of Toll-like Receptors (TLR) signaling pathways in regulating phagocytic ability of macrophages against target cancer cells (115). Agonists targeting TLR 3, 4 and 7 stimulate macrophages by triggering the phosphorylation of Bruton’s Tyrosine Kinase (Btk), which is a member of the Tec nonreceptor protein tyrosine kinase family and is expressed along the hematopoietic system except for T cells and NK cells. During PrCR, Btk in macrophages acts as a critical signal transducer by inducing the phosphorylation of calreticulin (CRT) and promoting its trafficking to the cell surface. We found that CRT, a multifunctional protein that was previously identified as a bridging molecule between phagocytes and apoptotic cells to initiate phagocytosis and also an “eat me” signal on certain types of human cancers (116), is an essential effector on the surface of macrophages mediating the recognition and phagocytosis of living tumor cells (115). PrCR of tumor cells was diminished when cell surface CRT on macrophages was blocked. Further understanding of the molecular mechanisms of “TLR-Btk-CRT” mediated signaling is critical for the development of novel anticancer immunotherapies through the activation of PrCR.

Conclusions

The hematopoietic hierarchy has been thoroughly defined in both mice and humans, and HSCs and their downstream progeny can now be isolated with extraordinary purity. The diseased counterparts to the normal stages of hematopoiesis are now being investigated, and these studies will continue to provide valuable information regarding the conversion to states of pathology. Furthermore, studies on aging will help us identify the changes that occur in the hematopoietic lineage over time. An understanding of hematopoiesis and myeloid cell development has broad implications for regenerative medicine, hematopoietic cell transplantation, malignancy, infection, among many other states of illness and disease. Through these studies, we gain insight into fundamental biological processes and we gain knowledge that can be leveraged for the benefit of patients.

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Figure 1. General organization of the hematopoietic lineage in mice and humans

The hematopoietic stem cell can give rise to all of the cells of the blood and immune system, with multiple step-wise intermediates arising before developing into fully differentiated cells. The Common Myeloid Progenitor (CMP) and the Common Lymphoid Progenitor (CLP) give rise to the two main arms of the hematopoietic hierarchy. The CMP can give rise to all myeloid cells. Conventional surface markers for purifying each population are indicated for both mice and humans. Reproduced with permission from Seita and Weissman (2010) *Wiley Interdiscip Rev Syst Biol Med* (117).

Figure 2. Purification of the first hematopoietic stem cells

A Representative examples of purified HSCs as visualized by microscopy after hematoxylin staining. **B** Myeloerythroid colonies formed in the spleen formed by the injection of purified HSCs into lethally irradiated mice. **C** A single lymphoid colony in the thymus formed by the injection of purified HSCs into lethally mice. **D** Fluorescence-activated cell sorting depicting HSCs as a $\text{Thy-1}^{\text{lo}}\text{Sca-1}^{\text{+}}$. Reproduced with permission from Spangrude et al. (1988) *Science* (10).

Figure 3. RNA expression pattern of IL-7Rα throughout the murine hematopoietic lineage
IL-7Rα is a critical surface molecule that helps distinguish the lymphoid arm of the hematopoietic system from HSCs, progenitors, and myeloid cells. Each box represents a different hematopoietic subpopulation. Blue indicates lower expression, pink indicates higher expression. Analysis performed using Gene Expression Commons (46).

Figure 4. GMP frequency is decreased in low risk MDS

Representative example of how hematopoietic progenitor cell populations can be altered in states of disease. **A** Frequency of GMPs out of total myeloid progenitors in normal, low risk MDS, and non-MDS diseased bone marrow samples. **B** Frequency of GMPs out of total lineage negative bone marrow mononuclear cells in normal and low risk MDS bone marrow samples. Reproduced with permission from Pang et al. (2013) *Proc Nat Acad Sci* (77).

Figure 5. CD47-blocking therapies are effective in preclinical models of human cancer Xenograft studies of mice engrafted with human AML samples that were then treated with anti-CD47 antibodies. **A** Anti-CD47 antibody treatment decreases leukemia burden, as assessed by the percent of human chimerism in the peripheral blood. **B** Bone marrow histology showing leukemia infiltration (images 1–2) in control mice, and eradication of disease in mice treated with anti-CD47 antibodies (images 4–5). In some mice with residual tumor burden following treatment with anti-CD47 antibodies, macrophages could be seen in the bone marrow engulfing leukemia cells (images 3–6). Reproduced with permission from Majeti et al. (2009) *Cell* (92).