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Functional Diversity of Stem and Progenitor Cells with B-Lymphopoietic Potential

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Summary

Technical advances have made it possible to separate hematopoietic tissues such as the bone marrow into ever smaller populations, complicating our understanding of immune system replenishment. Patterns of surface marker expression and transcription profiles as well as results obtained with reporter mice suggest that lymphopoietic cells are not closely synchronized, and there is considerable cell to cell variation. Loss of differentiation options is gradual, and ultimate fate can be established at different stages of lineage progression. For example, individual hematopoietic stem cells can be biased such that some are very poor sources of lymphocytes as contrasted to ones with balanced outputs. Still other hematopoietic stem cells are effective at generating B and T cells but are defective with respect to expansion and difficult to distinguish from early lymphoid progenitors. That diversity carries forward to later events, and similar appearing cells in the immune system can arise from alternate differentiation pathways. In fact, new categories of lymphoid progenitors are still being discovered. Heterogeneity provides adaptability as hematopoiesis can be dramatically altered during infections, influencing numbers and types of cells that are produced.

Keywords

stem cells; B cells; hematopoietic progenitor cells; immunodeficiency diseases; lipopolysaccharide; Toll-like receptors/pattern recognition receptors

Introduction

Adult stem cells of several types have been described with respect to their micro-anatomical locations and relationships to tissue specific progenitors. For example, differentiation of stem cells in the skin, gonads, and intestine is well organized (1). However, it has proven surprisingly difficult to appreciate the same with hematopoietic stem cells (HSCs), and textbook differentiation schemes are being challenged. Although HSCs are extremely rare (1/10,000 marrow cells), they appear to be divisible into functionally specialized subsets. The reason for that diversity is not clear, but many are sessile within trabecular bone, others reside in perivascular zones of marrow, and still others patrol peripheral tissues via blood and lymph. While most divide infrequently and contribute to hematopoiesis, an extremely small fraction of HSCs is deeply quiescent. Some HSC categories may be biased to become lymphocytes, while others preferentially produce myeloid cells or a full spectrum of blood cells. Much remains to be learned about relationships between HSCs and progenitors that

replenish the immune system. However, it is already clear that lymphoid progenitors are as functionally diverse as HSCs and do not differentiate in synchronous fashion. We have used recombination-activating gene-1 (RAG-1)-based reporter mice to identify and track primitive lymphoid progenitors. Patterns that are emerging from those experiments suggest that multiple differentiation pathways can be alternatively utilized during health and disease to replenish the immune system.

HSC heterogeneity

As with other stem cells, all HSCs self-renew extensively and give rise to multiple differentiated cell types. Operationally, this is defined as ability to sustain multi-lineage blood formation for at least 16 weeks following transplantation (2). Despite extensive effort and extraordinary progress, procedures for isolating HSCs are not completely standardized and uniformly applicable. A lineage marker-negative Sca-1⁺ c-Kit^{Hi} (LSK) fraction of adult bone marrow has long been considered inclusive of all short- and long-term repopulating HSCs (LT-HSCs). Shortcomings of this method include the fact that c-Kit levels decrease during rebound from myeloablation, while Sca-1 is not useful for all strains of mice and is upregulated in response to inflammatory cytokines. It later became possible to obtain more enriched LT-HSCs by exclusion of CD34 and/or Flt3-bearing short-term HSCs and progenitors (3-5), but even that preparation can be subdivided in multiple ways. As one example, a recent study revealed that many HSCs in highly enriched suspensions are intermediate term HSCs lacking the ability to sustain erythropoiesis long term (6). The ability to exclude Hoechst dye has been extensively used to enrich HSCs (7). These 'side population' cells are heterogeneous and may not include all HSCs (8). Regardless, the degree of dye exclusion partially correlates with functionally restricted HSC subsets (see below).

Functional heterogeneity of HSCs has been appreciated for some time and with many experimental protocols. For example, Muller-Sieburg and colleagues (9) used limiting dilution cultures to clone HSCs before serial transplantation. Hereditary properties of individual clones suggested that a minority of HSCs are imbalanced with respect to myeloid versus lymphoid cell production. Predetermined properties of HSCs were speculated to result from epigenetic events occurring early in life. Subsequent studies confirmed that biased HSCs exist in young animals and that myeloid biased ones produce functionally defective lymphoid progenitors (10-12). Myeloid biased HSCs generate progeny slowly following transplantation, can be serially propagated extensively, and are predominate in marrow of aged mice (13,14). In contrast, lymphoid biased HSCs serially transplant less well. As discussed below, this characteristic complicates their distinction from early lymphoid progenitors (ELPs). Balanced and myeloid HSCs are labile in culture, and only the lymphoid skewed ones survive for 10 days (11).

The discovery that LT-HSCs preferentially express CD150 represented a major advance, and it is even more powerful when used for sorting CD48⁻Flt⁻CD34⁻ LSKs (15). CD150 is not reported to be altered during inflammation, and the marker is linked to stem cell activity throughout life (16). At least one out of two CD150⁺CD48⁻Flt3⁻CD34⁻ LSKs are LT-HSCs, but debate continues over whether these cells are homogeneous and inclusive. Depending on the study, less than 5% or a 'substantial number' of LT-HSCs lack CD150 (17,18). As we now consider, many of the CD150⁻ HSCs lack the full range of differentiation options.

It would be ideal if combinations of cell surface markers corresponded with HSCs differing in self-renewal potential and degree of lineage bias. Kent and colleagues (19) found that only 7% of CD150⁻ HSCs had durable transplantation potential, none were myeloid skewed, and a majority were B and/or T-lymphoid skewed. This observation contrasts with

the CD150⁺ cohort, of which 43% self-renewed efficiently and 10% were myeloid skewed. Another report concluded that CD150⁻ HSCs repopulate multiple lineages but to lower degrees than CD150⁺ HSCs (20). Interestingly, the CD150⁻ HSCs tended to replicate themselves, while CD150⁺ HSCs generated both subtypes on transplantation. Collectively, these observations indicate that CD150 marks most highly renewing LT-HSCs with balanced lineage potentials but that a fraction of CD150⁺ HSCs is myeloid biased. HSCs lacking this marker include ones that are T and/or B-lineage lymphoid skewed.

Goodell and colleagues (12,18) described two sub-categories of side population cells. Ones excluding the most Hoechst dye were referred to as 'lower SP' and may be the most primitive. While upper and lower SPs produced B cells equally well, the upper SPs were more effective in T lymphopoiesis and deficient with respect to sustained production of myeloid cells. An imbalance was also evident with lineage-restricted progenitors. CLPs were more effectively made from upper SPs, while the reverse was true of myeloid progenitors. Both of these SP categories include subsets of CD150⁺ HSCs, with approximately twice as many (49%) in the lower SP. Using these two criteria together resolved four populations of HSCs, and each was subjected to single cell transplantation analysis. Consistent with a previous study (19), absence of CD150 tended to correlate with T-lineage bias, but only among cells in the upper SP. As in other attempts to partition functionally specialized HSCs, lineage bias was not all or none. That is, even heavily skewed HSCs made some blood cells of all types. Myeloid biased HSCs have been previously shown to lack both T and B potential, so it is curious that T but not B-lineage bias was segregated in SP subsets (10,12). As noted above, myeloid biased HSCs predominate in old mice, corresponding to increased numbers in the lower SP and CD150⁺ fractions (12-14). That provides a potential explanation for the age dependence of myeloid versus lymphoid leukemias (21).

All HSCs in adult marrow divide infrequently, but 1/7 of CD150⁺CD48⁻CD34⁻ LSKs are even more quiescent (22,23). These metabolically 'dormant' HSCs can be driven into cycle in emergencies and are extremely potent in restoring all blood cell lineages. In contrast, HSCs with lymphoid bias (upper side population and absence of CD150) are more proliferative than the majority of HSCs (12,18). Dividing HSCs are known to engraft poorly on transplantation (24). These findings suggest that cell cycle status could correlate with differences in HSC functions.

These studies reveal surprising diversity among highly enriched HSCs. A degree of subset distinction can be achieved by transplanting limiting numbers, antibody staining, dye exclusion, or long-term label retention. Given the technical difficulty associated with studying these rare cells, unifying principles are yet to emerge. However, truly multipotent HSCs could be among the 600 dormant stem cells estimated to exist in a single mouse (22). That ancestral population would most likely be called on in situations of unusual demand. HSCs may oscillate between states, and expression of some genes fluctuates within individual HSCs (25,26). However, many HSCs are intrinsically biased with respect to differentiation potential, and that property can be maintained through months of serial transplantation. No doubt this involves epigenetic regulation, as first proposed by Muller-Sieburg (9). The stability is not absolute, and a degree of conversion occurs between some subsets, e.g. myeloid and balanced to lymphoid biased, CD150⁺ to CD150⁻, and lower SP to upper SP (11,12,20). Studies discussed below suggest that diversity at the level of HSCs carries forward to early- and late-stage progenitors of lymphocytes.

ELPs

The existence of primitive, lymphoid biased progenitors was first suggested by their sensitivity to steroid hormones (27). We found that cells in the LSK fraction expressed all

possible combinations of RAG-1, terminal deoxynucleotidyl transferase (TdT), and a human μ transgene. They were depleted in estrogen-treated mice in parallel with potential for generating B and T lymphocytes, and some could be isolated from RAG-1/green fluorescence protein (GFP) knockin mice as viable progenitors (28). Other procedures that can be used to enrich ELP have been reviewed (29). A consensus is that ELPs are extremely potent in restoring lymphocytes in transplantation assays but have reduced potential for generating myeloid cells. In addition, they represent a subset of Flt3^{Hi} CD27⁺ LSK [lymphoid primed multipotent progenitors (LMPPs)] that are poor sources of erythrocytes and megakaryocytes (30). It is important to stress that ELPs are not homogenous and are not synchronized with respect to lymphoid gene expression (27,28) (Fig. 1A).

As discussed above, lymphoid biased cells are included in highly enriched HSC preparations, raising the question of how different they are from ELPs. HSCs can be easily distinguished from erythroid and myeloid lineage progenitors on the basis of self-renewal potential. That is, stem cells generate large numbers of offspring for extended periods, while progenitors do not sustain blood cell production beyond an initial wave. The distinction can be more difficult for lymphoid progenitors, because mature lymphocytes can live for years and expand during immune responses. We estimate that a single ELP can generate at least 20,000 thymocytes and for at least 12 weeks. The resulting T cells eventually appear in the circulation, where many studies assess degrees of chimerism in transplantation assays. Where this was the case, it is possible that lymphoid biased HSCs were not sufficiently distinguished from lymphoid progenitors. In one study, myeloid bias of HSCs clearly correlated with production of functionally defective T and B-cell progenitors (10). Myeloid and lymphoid bias was also evident at the level of marrow progenitors in transplants prepared from upper versus lower SP HSCs (12).

The side population method gives impressive enrichment of HSCs, but small numbers of RAG-1 reporter positive ELPs can be detected in the upper SP fraction (authors' unpublished observations). It will be interesting to learn if the same is true for HSCs isolated with other staining protocols (11). Exclusion of Flt3⁺ or CD48⁺ cells while positively selecting for CD150 yields HSC preparations with no observed ELP contamination, and in one study, that strategy reduced numbers of lymphoid-biased HSCs (19). This observation would again suggest that there can be some ELP contamination in preparations isolated as CD150⁺ or upper SP cells. HSCs spend long periods between cell divisions, and approximately 70% of RAG-1/GFP⁺ ELPs are also in a quiescent G₀ state (31). Both HSCs and ELPs are in rapid cycle during fetal life.

Microarray and single cell polymerase chain reaction (PCR) experiments revealed that transcription profiles of LT-HSCs and progenitors are quite different (32,33), and indeed we identified a new HSC marker on that basis. While the endothelial cell antigen ESAM-1 is not exclusive to HSCs, it is absent on RAG-1/GFP⁺ ELPs (34). Low level expression of myeloid rather than lymphoid associated genes was said to be characteristic of HSCs and to reflect 'lineage priming' (32,33). However, that depends on the definition of 'lymphoid' genes and the method used for HSC isolation. One study found that 20% of HSCs sorted as Ikaros reporter-negative LSK cells had detectable lymphoid gene priming (35). That could result from contamination of the HSCs by ELPs. However, some of the same genes (*Dnnt*, *Igh-6*, and *Lck*) were also detectable in our microarrays of CD150⁺CD48⁺Flt3⁺ LSKs. However, HSCs defined in that way do not express TdT protein or RAG-1-based reporters, characteristics we used to define ELPs (authors' unpublished observations). Therefore, only selected lymphoid genes are expressed by subsets of highly purified HSCs. It would be interesting to know if these correlate with ones that have functional lymphoid bias.

We conclude that lymphoid biased HSCs were distinguished from ELPs in some but not all studies of HSC diversity. Similar to HSCs, ELPs are not homogeneous, many are quiescent, and individual cells express different combinations of lymphoid genes. As discussed below, some ELPs are precocious in this regard, expressing RAG-1 at a functionally immature stage, while the same locus is not activated in other progenitors until much later. That is, ELPs are not synchronized. Diversity of HSCs is matched by diversity among cells responsible for replenishing the immune system.

CLPs are divisible in multiple ways

Common lymphoid progenitors (CLPs) were first described by Kondo and colleagues (36) as $\text{Lin}^- \text{IL-7R}\alpha^+ \text{c-Kit}^{\text{Lo}} \text{Sca-1}^+$ marrow cells. They represent major differentiation intermediates between ELPs and B lymphocytes or NK cells, but much confusion surrounds them in the literature. This confusion is partly because the same name is used for cells isolated in different ways, and their definition is not standardized (29,37). Also, there is debate over how much CLPs contribute to T-cell formation and whether they retain significant potential for myelopoiesis (see discussion below). Many laboratories have evidence that CLPs do not represent a crisp branching point for T/B/NK versus myeloid options, and it is clear that CLPs are heterogeneous (Fig. 1A).

Progress in understanding stem and progenitor cells has been highly dependent on the identification of differentiation milestones. Sakaguchi and colleagues developed RAG-1/GFP knockin mice that our laboratory has used for some time (28,38). Green fluorescence in this model corresponds to RAG-1 transcripts, making it possible to isolate viable ELPs from fetal as well as adult hematopoietic tissues (39,40). Sorting $\text{Lin}^- \text{c-Kit}^{\text{Lo}} \text{Sca-1}^+ \text{RAG-1/GFP}^+$ cells from these mice resulted in a population containing most CLPs that we designated pro-lymphocytes (28). ELPs and pro-lymphocytes differ in the time required to generate lymphocytes in culture and their potency for thymus repopulation. More recently, another model was created by crossing RAG-1/Cre knockin mice with Rosa26-tdRFP mice (41). We refer to these as RAG1/Red mice. All cells that have ever expressed RAG-1 are marked by strong red fluorescence, and we found good agreement with marking of RAG-1/GFP⁺ ELP and pro-lymphocytes (Fig. 1A). Furthermore, the results are complementary to those obtained with a recombination substrate model, where cells are marked if both RAG proteins are expressed (42). As mentioned above, small numbers of RAG-1⁺ ELPs are present in the upper side population but not among HSCs defined as $\text{CD150}^+ \text{CD48}^- \text{LSKs}$. With either of these models, approximately half of the Kondo CLPs are detected as RAG-1⁺. We found that the densities of c-Kit were slightly lower on red than non-red CLPs, while the reverse was true for levels of IL-7R α . This observation suggests that RAG locus activation corresponds to some degree with lymphoid lineage progression and differentiation options (see below). However, it is remarkable that there was poor concordance with two other CLP characteristics. That is, both red and non-red CLPs could be sub-divided on the basis of AA4.1/CD93 or Flt3 staining, although both of these markers have been effectively used to enrich functional CLPs (43-45). Sigvardsson and colleagues (46) used a λ_5 based reporter system in conjunction with RAG-1/GFP knockin mice and single cell PCR assays to demonstrate additional diversity among CLPs. Thus, these progenitors do not necessarily regulate proteins in synchrony with their position on the assembly line. This is particularly the case for the presumably more primitive RAG-1⁻ CLPs. Two step culture of those progenitors revealed that while most acquire B220 before giving rise to CD19⁺ lymphocytes, a subset can become B220⁺ before RAG-1 expression (Fig. 2).

Differentiation potential of CLP subsets

A surprising number of options are available to CLPs that remain within the marrow (Fig. 1A). For example, those destined to become conventional B lymphocytes acquire B220/

CD45R before CD19 and undergo tremendous expansion in concert with Ig gene rearrangement and maturation (37). Another sequence of events includes CD19 display before B220 and leads to production of specialized B1 cells (47,48). Many rate-limiting factors associated with this process await discovery, but retinoids remarkably accelerate progression in the B lineage (49,50). Sub-division on the basis of RAG-1 locus and/or λ_5 reporter activation revealed that these events bring CLPs closer to the pro-B and subsequent stages of differentiation (41,46).

CLPs can acquire the IL-2/IL-15 (CD122) receptor coincident with commitment to the NK lineage, which includes a curious category of B220⁺Ly6C⁺DX5⁺NK1.1⁺CD11c⁺ interferon-producing killer dendritic cells (IKDCs) (51-53). Like NK cells, IKDCs are dependent on the Id2 transcriptional repressor and respond to IL-15. However, IKDCs are most efficiently generated from ELPs, whereas CLPs are the best source of NK cells in transplantation assays (54). It has been known for some time that individual CLPs can give rise to NK plus B cells or be restricted to a single fate (51). Furthermore, some NK cells undergo immunoglobulin D-J rearrangements (55). More recently, sub-division of CLPs on the basis of reporters revealed that the primitive RAG-1⁻ fraction is more prone to generating NK cells, while RAG-1⁺ CLPs progress to become B-cell restricted (41,46).

CLPs give rise to small numbers of conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) (56). A conspicuous subset (25-60%) of pDCs are labeled in RAG-1/GFP knockin and RAG-1/Red mice, a distinction that correlates with cytokine-producing potential (57). The RAG-1⁺ subset was also more closely affiliated with the B-lymphocyte lineage, expressing Ebf1, Pax5, and Bcl11a transcription factors, as well as immunoglobulin D-J rearrangements. Percentages of RAG-1⁺ pDCs in marrow, spleen, and thymus fluctuated in wave-like fashion following sublethal irradiation (Fig. 3). Conventional DCs, NK-related IKDCs, and NK cells were remarkably stable in this respect.

A much smaller percentage (approximately 7%) of the conventional B220⁻CD19⁻NK1.1⁻CD3⁻CD11c⁺ DCs found in marrow have a history of RAG-1 expression (41). Culture experiments suggested that the propensity to produce cDCs and pDCs is highest among the RAG-1⁻ CLP subset. A major pathway of DC production involves a Lin⁻c-Kit^{Lo}Flt-3⁺CD115⁺ category of progenitors (proDC/CDP) (58,59). Elegant single cell experiments revealed that pro-DCs are functionally heterogeneous, including subsets with full or restricted options for generation of DC types (59). Some very early progenitors become committed to a single DC category, while that decision is made for others at a much later stage. Several findings suggest complete independence of pro-DCs from CLPs. For example, we found no labeling of pro-DCs in RAG-1/Red mice, and they were not depleted by estrogen treatment (41). Furthermore, no pro-DCs have a history of RAG-1 expression even under emergency conditions (see below).

Contribution of marrow derived cells to the thymus

Few topics have been as complicated and contentious as the nature of cells that sustain T-lymphocyte production. Unlike the marrow, the thymus does not have a resident population of HSCs and must periodically be re-supplied with progenitors. The most primitive cells in the thymus are referred to as early T-lineage progenitors (ETPs). A consensus is emerging that several types of marrow cells, but not HSCs, can travel via the bloodstream to the thymus and acquire ETP characteristics. Here we summarize observations made with RAG-1-based reporters and conclude that the findings represent another example of progenitor heterogeneity.

Many marrow progenitors and especially CLPs have T-lineage potential in stromal cell co-cultures if Notch signaling is induced (60,61). We found that DN2/DN3 stage thymocytes

similarly accumulated in those cultures regardless of the starting population (61). In contrast to those results, ELPs sorted on the basis of RAG-1 expression were much more potent T progenitors than CLPs when transplanted to sub-lethally irradiated mice (62,63). This potency was particularly the case when total numbers of thymocytes produced rather than percentage chimerism was assessed. In recent studies, the definition of CLPs was changed to be more similar to ELPs, i.e. $\text{Flt3}^+\text{CD27}^+\text{c-Kit}^{\text{Hi}}\text{IL-7R}\alpha^{-/\text{Lo}}$ cells were included in sorted populations that had considerable T potential in sub-lethally irradiated recipients (64). Although ELPs are normally present in the circulation, it is possible that transplanted ones temporarily reside in the marrow before seeding the thymus (62,64).

It is unlikely that any experimental model precisely replicates normal conditions, and it is impressive that HSCs do not colonize the un-irradiated thymus (65). Thus, experiments involving irradiated recipients may exaggerate the range of progenitors that normally generate T lymphocytes. While levels of chimerism are very low when cells are transferred to normal mice without irradiation, Petrie and colleagues (66) found that $\text{IL-7R}\alpha^{-/-}$ mice were deficient in functional ETPs. That is, it was possible to transplant them without prior irradiation and restore the thymus to normal size. A recent study exploited that method together with selective depletion of particular marrow cells prior to transplantation (67). While Flt3^+ CLPs had transient T potential, more durable T lymphopoiesis was associated with ELP-like ($\text{c-Kit}^{\text{Hi}}\text{Flt3}^+\text{CD27}^+\text{IL-7R}\alpha^-$) progenitors. The authors concluded that multiple cell types may enter the circulation and replenish the thymus under physiological circumstances.

The definition of CLPs embraces the concept that non-lymphoid differentiation potential is absent in progenitors responsible for B, T, and NK cell formation (36). Although most DCs may be made via progenitors and pathways unrelated to CLPs, CLPs are a DC source in some circumstances (see discussion above). Granulocytes and macrophages are considered to be 'myeloid', and the definition predicts that they would never arise from CLPs. However, the fetal equivalent of CLPs has myeloid potential, and B/T/NK restricted progenitors were not found in fetal tissues with elegant single cell assays (68,69). Some laboratories have found that carefully sorted CLPs from adult marrow have myeloid potential that is detectable under certain conditions (43,70). Also, ETPs are precursors of many macrophages and granulocytes in the thymus (71,72). An exceptionally large number of DCs in the thymus have a history of RAG-1 expression (41), and we recently found the same to be true for NK cells (*Figs 1 and 2*). Indeed, the fraction of non-T cells that were labeled in RAG-1/Red mice was remarkably stable during depletion and recovery from sub-lethal irradiation.

ETPs have substantial potential to generate thymocytes and might resemble recent immigrants to that organ. Like ELPs in bone marrow, ETPs are $\text{Lin}^-\text{Sca-1}^+\text{c-Kit}^{\text{Hi}}$ and are additionally defined as $\text{CD44}^{\text{Hi}}\text{CD25}^-$. However, ELPs have high densities of Flt3, and this marker is low to negative on ETPs (73,74). Only Flt3^{Lo} ETPs have residual B-lineage potential. In an earlier study, we concluded that ETPs were unmarked in RAG-1/GFP mice, a result that accords with published reverse transcriptase-PCR results (62,75). However, we recently re-examined this issue with more stringent ETP gates (including only cells with very high levels of CD44 and cKit) as well as a specialized filter and found that 50-80% had low levels of RAG-1, as also reported by Lai and Kondo (63). A similar percentage of ETPs was marked in RAG-1/Red mice, and there was 60% agreement with RAG-1/GFP⁺ cells when we crossed the two reporter mouse strains (authors' unpublished data). In contrast to this heterogeneity of ETPs, nearly all $\text{CD4}^-\text{CD8}^-\text{CD44}^{\text{Hi}}\text{CD25}^+$ (DN2) cells and subsequent stages of T-lineage development are marked in either of the RAG-1 reporter models (*Fig. 2*). The variable RAG-1 marking of ETPs would be consistent with

colonization by multiple progenitors, and even those destined to become NK, dendritic and myeloid cells can subsequently activate the RAG-1 locus.

As discussed above, some HSCs may already be biased to T-lineage lymphocyte fates, while a spectrum of progenitors can enter the thymus and subsequently opt to become T, NK, dendritic, or non-lymphoid cells. Our own observations suggest that RAG-1 expression can occur in some potential T cells before they leave the marrow. However, this essential event is again not synchronized and occurs in some progenitors during the ETP to DN2 transition.

Complexity of a new category of lymphoid progenitors

Randall and Weissman (76) discovered a small population of Lin⁻Sca-1⁺c-Kit⁻ cells in bone marrow that appeared to be quiescent and not actively contributing to blood cell formation. Another group identified the same subset and concluded that they could give rise to c-Kit⁺ cells more similar to HSCs or multipotent progenitors (77). Conditions were subsequently developed to support this transition in culture (78). More recently, two laboratories ascribed lymphoid biased progenitor characteristics to some of the Lin⁻Sca-1⁺c-Kit⁻ cells (79,80). Allman and colleagues focused on an IL-7R α ⁻AA4.1/CD93⁺ subset of these ‘mystery’ cells, demonstrating they could rapidly generate B or T cells following intravenous transplant or intrathymic injection, respectively (80). Simultaneous stimulation with Flt3 ligand and IL-7 was needed for them to generate small numbers of B-lineage cells in culture. Even though there was low level expression of a RAG-2 transgene-based reporter, this subset showed no evidence of Ig gene recombination activity. A different approach was taken by Snoeck and colleagues (79), who also showed that Lin⁻Sca-1⁺c-Kit⁻ cells could be sub-divided with two additional markers. B and T potential was demonstrable in a Flt3⁺CD25⁻ subset that had evidence of immunoglobulin D-J rearrangements but low levels of RAG transcripts. This subset likely includes the lymphoid-biased progenitors described by Allman. A different, Flt3⁻CD25⁺ population of cells had little differentiation potential, even though they expressed high levels of IL-7R α . This possibly defective subset accumulated in marrow of aged mice.

We recently determined that Lin⁻Sca-1⁺c-Kit⁻ cells did not stain for the Ki67 proliferation antigen (authors’ unpublished observations). Also consistent with dormancy, a small fraction of these cells had side population characteristics (i.e. excluded Hoechst dye), and all of those had a history of RAG-1 expression in RAG-1/Red mice.

Further subdivision revealed that very few of the Flt3⁻CD25⁺ cells were marked, and the relevance of this population to lymphopoiesis remains unknown. They were uniformly small with stem cell/lymphocyte morphology and will not be considered further. Most cells in the lymphoid progenitor containing Flt3⁺CD25⁻ category were not actively making RAG-1 but had a history of RAG-1 expression, as assessed with reporter mice. This category was still heterogeneous and included some large cells with plasmacytoid morphology.

It is difficult to position these lymphoid progenitors in a differentiation scheme, and it has been suggested that they could be intermediates in an alternate differentiation pathway (81). The RAG-1-based fate mapping results and absence of RAG-1/GFP indicates that most are downstream of or at least distinct from CLPs. The available information suggests that Lin⁻Sca-1⁺c-Kit⁻Flt3⁺CD25⁻ cells are quiescent lymphoid progenitors, and more needs to be learned about their contribution to lymphopoiesis.

Lymphopoiesis during disease

It has long been known that the marrow responds to infections, if only indirectly via the actions of colony-stimulating factors (82). More recent studies showed that injection of adjuvant is sufficient to cause rapid elevation of TNF α and IL-1 that in turn mobilize newly

formed B cells and granulocytes, respectively, from the marrow (83,84). Another perspective was provided by the discovery that stem and progenitor cells express functional Toll-like receptors (TLRs) and can directly recognize pathogen products (85). We found that committed myeloid progenitors differentiated without the normal requirement for colony-stimulating factors when exposed to lipopolysaccharide (LPS) under defined culture conditions, while quiescent HSCs were stimulated to enter cell cycle and acquire lineage markers. Injecting mice with LPS increases numbers of HSCs in the spleen, findings consistent with mobilization from the marrow or extramedullary action of pathogen products (86,87). In addition, absence of TLR4, TLR9, or the TLR adapter protein myeloid differentiation factor 88 (MyD88) gives HSCs an advantage in transplantation assays (Fig. 4). This observation suggests that HSCs could be vulnerable to certain TLR ligands during handling or in the damaged environment of irradiated recipients. If that interpretation is correct, transient blocking of TLR signaling could improve the efficiency of transplantation. Another possibility is that normal microflora and/or endogenous TLR ligands function to limit numbers of stem and progenitor cells in bone marrow under steady state conditions. Regardless, the findings show that TLRs are functional on hematopoietic cells under physiologic conditions.

Lymphoid progenitors are also heavily influenced by pathogen products. For example, exposure of CLPs to TLR2, TLR4, or TLR9 ligands blocks their ability to generate B cells and increases production of DCs (41,85,88). There are subtle differences between proportions of cells produced in cultures containing these ligands, suggesting there is a degree of specificity in responding to complex pathogens. Depletion of newly formed lymphocytes occurs in bone marrow of mice infected with HSV-1, and we found this was primarily mediated by TNF α (88). However, CLPs were even directed to generate DCs rather than B cells in TNF $\alpha^{-/-}$, IFN α/β R $^{-/-}$ and IFN $\gamma^{-/-}$ mice. This accords with our culture results where the fate of CLPs was directly altered by CpG oligodeoxynucleotides. While herpesvirus glycoproteins can cause cytokine production via ligation of TLR2, alterations in hematopoiesis were almost completely regulated via TLR9. Overall thymus size is not immediately altered by treatment of mice with CpG ODN or LPS. However, we have found that numbers of ETPs in the thymus as well as T potential of marrow cells are reduced (authors' unpublished observations).

Several observations suggest alternate pathway utilization occurs in response to pathogen products (Fig. 1B). Myeloid progenitors are normally a better source of DCs than CLPs, but they did not have altered DC potential in herpes simplex virus-1 (HSV-1)-infected mice (88). CLPs did not acquire CD115, a defining marker of proDCs, and CD115 $^{+}$ proDCs actually declined in acutely LPS-treated mice (authors' unpublished observations). IKDCs in HSV-1-infected mice made both major classes of interferon, while freshly isolated ones are type II restricted (54,88). Furthermore, our transplantation experiments suggest IKDCs are usually derived from primitive progenitors rather than CLPs.

The CLP heterogeneity revealed in RAG-1 reporter mice made it possible to ask how subsets respond to TLR ligation (41). B lymphopoiesis was blocked in the less differentiated RAG-1 $^{-}$ CLPs by exposure to LPS, and they became efficient DC progenitors. In contrast, CLPs with a history of RAG-1 expression were resistant to re-direction, consistent with their reduced potential for generating NK cells. Therefore, lymphopoietic cells gradually become lineage restricted and progressively less influenced by pathogen products. Endogenous TLR ligands such as fatty acids, hyaluronan fragments and heat shock proteins might also be influential in health and disease (89,90).

Concluding thoughts

Although homogeneous in appearance, individual HSCs differ with respect to differentiation potential, cell cycle status, dye efflux, patterns of gene expression and surface markers, as well as homing, engraftment, and duration of blood cell formation following transplantation. Similar diversity is characteristic of progenitors with potential to replenish the immune system. Proximity to endogenous environmental cues such as Wnt family molecules, Notch ligands, adhesion molecules, chemokines, and cytokines is likely to dictate these properties.

Placement of stem and progenitor cells in a sequence is no longer simple. As discussed above, there is a low rate of conversion between HSC subsets, and this need not represent the progression suggested by clonal succession models (11-13). An alternative interpretation is that there is a degree of oscillation or refluxing among HSC types (25,91). Less information is available about ELPs, but exposure to Wnt3a in culture allowed them to acquire erythropoietic potential (91). Movement within and from the marrow might be linked to transition between states. Additionally, external factors such as pathogen products can directly and indirectly interact with hematopoietic cells to dramatically alter differentiation pathway utilization.

The question then arises as to why the process is so complex. Why can't it be depicted as a series of binary fate decisions linked to synchronous changes in surface markers and differentiation potentials? One answer is suggested by changes that occur during normal aging, i.e. HSCs tend to become less diverse and myeloid biased. Further study should reveal if that reduced complexity contributes to immune senescence and poor protective responses to vaccination. Thus, the availability of options could have survival value. Experiments with RAG-1-based reporters suggest there might be an advantage to having subsets of NK and DCs develop with some lymphoid affiliation. Ones that do may become functionally specialized through the actions of lymphoid related transcription factors. Whatever the reason, the phenomenon is particularly striking in the thymus and remarkably stable during rebound from sub-lethal irradiation (Fig. 3). The pattern is consistent with other kinds of pre-determination that can occur at the level of HSCs and ELPs, and underlying mechanisms of epigenetic regulation should be explored.

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Fig. 1. (A) A simplified scheme of hematopoiesis illustrates the diversity of stem and progenitor cells

Although drawn as discrete, separable cell types, the process is in fact a continuum and properties of progenitors can overlap. RAG-1 expressing cells (in RAG-1/GFP knockin mice), and cells with a history of RAG-1 expression (in RAG-1/red mice) are used to chart lymphopoiesis. Recent studies suggest rare subsets of HSCs may be lymphoid biased, as is the case for early lymphoid progenitors (ELPs). Several cell types may replenish the thymus, acquiring characteristics of early T-lineage progenitors (ETPs), but many myeloid, DC, and NK cells in that site are lymphoid related. Subsets of common lymphoid progenitors (CLPs) in marrow generate a variety of cell types as indicated. Lymphoid biased progenitors and B1-restricted progenitors are not shown. Differentiation patterns can change dramatically during infections (see text). (B) Culture and *in vivo* experiments suggest that pathogen products can alter hematopoiesis in multiple ways. HSCs exit quiescence and probably are exported to tissues such as the spleen. Many HSCs start to acquire lineage markers. T and B lineage differentiation potential is reduced, while formation of NK-like IKDCs, cDCs, and pDCs is augmented. Cytokine dependence of myeloid progenitors is altered. These responses were observed with purified HSCs or progenitors in culture, suggesting direct engagement of their TLR. Immunization and inflammation cause mobilization of B-lineage cells, and that response is largely TNF α dependent (see text).

Fig. 2. Alternative pathways to the B-lymphoid lineage are suggested by the results of two step cultures

Common lymphoid progenitors with no history of RAG-1 expression were sorted from RAG-1/Red reporter mice and cultured for 36 h. At that time, a majority of cells in the culture acquired B220 but still had no evidence of RAG-1-based marking. A smaller population had sufficient RAG-1 locus activation to trigger the reporter but were still B220^- . Re-culture of these newly generated subsets revealed that nearly all RAG-1^+ cells acquired B220 and some even became CD19^+ lymphocytes in these short term cultures. Thus, the order of expression of RAG-1 and B220 is not rigid.

Fig. 3. Proportions of non-T/non-B cells with a history of RAG-1 expression are tissue specific and remarkably stable

Subsets of cells that lack specific antigen receptors derive from RAG-1⁺ progenitors and fluoresce in RAG-1/Red reporter mice. The fraction that is marked depends on the tissue and is particularly high in the thymus. Some plasmacytoid dendritic cells (pDCs) express RAG-1, even after leaving the marrow, and their relative abundance fluctuates in wave-like fashion during recovery from sublethal irradiation (600R). Frequencies of other non-T/non-B cells with a history of RAG-1 expression are relatively constant. The findings would be consistent with intrinsic heterogeneity established at a very early stage.

Fig. 4. Long-term repopulating stem cells from TLR-deficient mice have an advantage when transplanted with stem cells from wildtype mice

Equal part mixtures of TLR2, TLR4, or TLR7 knockout (CD45.2 marked) and wildtype (CD45.1) bone marrow cells were transplanted intravenously to lethally irradiated recipients. The chimerism results are based on staining myeloid cells in peripheral blood. Similar experiments were done with MyD88^{-/-} marrow and a single three month time point is compared to TLR9^{-/-}. Numbers of HSCs in the donor inoculum appeared to be equivalent on the basis of staining with CD150 and CD48.