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Versatility of stem and progenitor cells and the instructive actions of cytokines shape haematopoiesis

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Abstract

For many years developing haematopoietic cells have been strictly compartmentalised into a rare population of multi-potent self-renewing haematopoietic stem cells (HSC), multi-potent haematopoietic progenitor cells (MPP) which are undergoing commitment to particular lineage fates, and recognisable precursor cells which mature towards functional blood and immune cells. A single route to each end-cell type is prescribed in the 'classical' model for the architecture of haematopoiesis. Recent findings have led to the viewpoint that HSCs and MPPs are more versatile than previously thought. Underlying this are multiple routes to a particular fate and cells having clandestine fate options even when they have progressed some way along a pathway. The primary role of cytokines during haematopoiesis has long been seen to be regulation of the survival and proliferation of developing haematopoietic cells. Some cytokines now clearly have instructive actions on cell-fate decisions. All of this leads to a new way of viewing haematopoiesis whereby versatile HSC and MPP are directed towards lineage outcomes *via* cytokine regulated cell-fate decisions. This means greater flexibility to the shaping of haematopoiesis.

Keywords: Haematopoiesis, cell-fate decisions, cytokines, cell differentiation, leukaemia

Abbreviations: **ALL**, acute lymphoblastic leukaemia; **AML,** acute myeloid leukaemia; **Bas**, basophil; **CLP**, common lymphoid progenitor; **CMP**, common myeloid progenitor; **CSC**, cancer stem cell; **DC**, dendritic cell; **DN**, double-negative; **DP**, double-positive; **Eos**, eosinophil; **Eo/B-CFU**, eosinophil and basophil progenitor; **Epo**; erythropoietin; **EPLM**, Early Progenitors with Lymphoid and Myeloid potential; **Ery**, erythroid; **FLT3**, Fms-like tyrosine kinase 3 receptor; **G-CSF**, granulocyte colony-stimulating factor; **GM-CFU**, granulocyte/macrophage colony-forming unit; **GM-CSF**, granulocyte/macrophage colony-stimulating factor; **GMP**, granulocyte and monocyte progenitor; **HSC**, haematopoietic stem cell; **IL**, interleukin; **IL-7R**, interleukin-7 receptor; **LIC**, leukaemia initiating cell; **LMPP**,

Lymphoid-primed Multipotent Progenitors; **LSK**, lineage markers$^-$, Sca-1$^+$, c-Kit$^+$ population of bone marrow cells; **LT-HSC**, long-term reconstituting haematopoietic stem cell; **Ly**, lymphoid; **MC**, mast cell; **M-CSF**, macrophage colony-stimulating factor; **Meg**, megakaryocyte; **MegE**; megakaryocyte/erythroid; **MEP**, megakaryocyte and erythroid progenitor; **Mon**, monocyte; **MPP**, multi-potent haematopoietic progenitor cells; **My**, myeloid; **Neut**, neutrophil; **NK**, natural killer cell; **SCF**, stem cell factor; **SLAM**, signalling lymphocyte activation molecule; **ST-HSC**, short-term reconstituting haematopoietic stem cell; **TCR**, T-cell receptor; **TGF-β1**, transforming growth factor-β1; **Tpo**, thrombopoietin; **TSP**, thymus-settling progenitor; **vWF**, von-Willebrand factor;

Introduction

The haematopoietic stem cell (HSC) gives rise to a wide range of blood and immune cell types. The generation of large numbers of each cell type is a complex and tightly regulated process that is ultimately governed by commitment of rare, and generally quiescent, HSCs to pathways of cell differentiation. These cells, which reside within the bone marrow in adult mammals, are the apex of the haematopoietic hierarchy. HSCs, which can self-renew, give rise to multi-potent progenitor cells (MPPs) which undergo decision-making, expansion and differentiation, *via* recognisable lineage precursors, to give rise to the final compartment of functional cells. Since the early 1980s the end cell types have been viewed as two families; lymphoid that includes B and T lymphocytes and natural killer (NK) cells and myeloid consisting of the rest of the blood and immune cells. Commensurate with this has been the identification of progenitors of each family; namely the common lymphoid progenitor (CLP) (1) and common myeloid progenitor (CMP) (2). This lymphoid/myeloid dichotomy is the basis of a long standing model for the architecture of haematopoiesis which also encompasses single routes of differentiation towards individual fates (3). Over the past 15 years, the above strict architecture has become less black and white. HSCs and haematopoietic progenitor cells (HPCs) are now viewed as more versatile and developing HSCs/HPCs less rigorously compartmentalised.

Cell heterogeneity and versatility during haematopoiesis

Heterogeneity of haematopoietic stem cells

HSCs are better described in mouse than in human and mouse HSCs can be purified to homogeneity to a greater extent (4). There are differences between mouse and human HSCs, and between foetal and adult HSCs. Human HSCs are isolated for transplantation on the basis of expression of the cell surface molecule CD34. By contrast, a single mouse $CD34^{low/-}$ HSC

reconstitutes haematopoiesis long-term in a lethally irradiated mouse (5, 6). Matsuoka and co-workers observed that HSCs of bone marrow, liver, and spleen from foetal and neonatal mice express CD34, whereas HSCs are enriched in the $CD34^-$ fraction and MPPs are $CD34^+$ in mice older than 10 weeks (7). Albeit, the principles of haematopoiesis derived from human and mouse studies, including of foetal and adult cells, are substantially similar.

The description of HSCs, and isolation by fluorescence activated cell sorting, relies on the presence and absence of a range of cell surface molecules. Studies of HSCs in the mouse have the advantage that HSCs can be rigorously defined during purification as cells that repopulate long-term (LT-HSC) and short term (ST-HSC) the entire haematopoietic system. A cardinal aspect of HSCs is they do not express markers that are associated with the various haematopoietic cell lineages (Lin^-), including, for example, CD3 (T lymphocytes), B220 (B lymphocytes), CD11b (monocytes/macrophages), Ly-6G (neutrophils), and TER-119 (erythroid cells). HSCs and MPPs express the two molecules c-Kit, a mast/stem cell growth factor receptor and tyrosine kinase, and Sca-1, a phosphatidylinositol-anchored membrane protein. LT-HSC and ST-HSC reside in the Lin^-, Sca-1^+, c-Kit^+ population of bone marrow cells, termed LSK.

Originally the Weissman group sub-divided the LSK compartment on the basis of expression of Thy-1.1 and the Flt3 (fms-like) tyrosine kinase, a type 3 receptor kinase (3, 8). The loss of a low level of expression of Thy-1.1 and gain of expression of Flt3 was observed to correlate with a loss of the capacity of HSCs to self-renew (Figure 1). LT-HSCs were isolated from adult bone marrow as LSK $Flt3^-$, and transplantation of LSK $Flt3^+$ cells resulted in short-term multi-lineage reconstitution. However, foetal liver HSCs are contained within the $Flt3^+$ and $Flt3^-$ LSK cells. MPPs are $Thy1^-Flt3^+$. In 2005, Yang and co-workers combined LSK markers with CD34 and Flt3. The definitions provide by the Jacobsen group, which are commonly

used, are: LT-HSC as LSK $CD34^{-}Flt3^{-}$, ST-HSC as LSK $CD34^{+}$ $Flt3^{-}$, and MPPs as LSK $CD34^{+}$ $Flt3^{+}$ (Figure 1) (9).

Recently, the signalling lymphocyte activation molecule (SLAM) family of membrane receptors (CD150, CD48, CD229 and CD244) has been used to describe subpopulations of HSCs and MPPs (Figure 1). Kiel and co-workers purified HSCs and MPPs as $CD150^{+}CD48^{-}CD244^{-}$ and $CD150^{-}CD48^{-}CD244^{+}$, respectively. Lineage restricted progenitors can be identified by acquired expression of CD48, and are $CD150^{-}CD48^{+}CD244$ (10). Adding CD229 to the panel of SLAM, CD34 and LSK markers led Oguro and co-workers to propose HSC-1 and HSC-2 populations, which have different lineage biases, and three populations of MPPs (Figure 1). $CD229^{-}$ HSC-1 are cells that rarely divide and are myeloid-biased as revealed by transplantation studies. $CD229^{+}$ HSC-2 cells divide more frequently and are lymphoid-biased (10). Contrary to the findings of the Morrison group, the level of expression of CD150 has also been used to distinguish myeloid- and lymphoid-biased HSCs. Ema and co-workers have observed that myeloid-biased HSCs are enriched in the $CD150^{high/med}CD34^{-}$ LSK population and lymphoid-biased HSCs are enriched in $CD150^{low/-}CD34^{-}$LSK population. These workers have proposed that these two populations of cells overlap with LT-HSCs and ST-HSCs, respectively (11).

As mentioned above and for many years, the two compartmentalising properties of HSCs are their capacities to reconstitute the entire haematopoietic system and to self-renew. Oguro in summarising the sub-populations of hematopoietic stem and progenitor cells distinguished using SLAM markers provides a scheme whereby the self-renewal potential of HSC-1 is long-term, of HSC-2 is long to intermediate, of MPP-1 is intermediate to transient, and of MPP2 and 3 is transient (10). Oguro also described haematopoietic progenitor cells (HPC-1 and 2) which do not self-renew. In essence, the boundary between HSCs and MPPs as to the property of self-renewal is blurred, and a distinction between HSCs and MPPs is perhaps somewhat

redundant. Both these cell types might best be viewed, and classified, as a continuum of haematopoietic progenitor cells that reduce their capacity to self-renew as they mature.

Strict multi-potency does not always go hand in hand with self-renewal as to the identification of HSCs with lineage biases. As mentioned above, the $CD34^{-}$LSK population of bone marrow cells, which engraft mice including secondary hosts, has been divided into myeloid-biased HSCs and lymphoid-biased HSCs. In addition to expressing a higher level of CD150, myeloid-biased HSCs exclude Hoechst 33342 more effectively than lymphoid-biased HSC, and these two HSC sub-types are differentially regulated by transforming growth factor-β1 (TGF-β1) (12). There is additional heterogeneity within the $CD150^{high}CD34^{-}$LSK population of cells, as revealed by engraftment of single cells in irradiated mice. In primary hosts some single cells readily gave rise to myeloid cells and cells that were able to engraft a secondary host. Some single cells produced few myeloid cells in primary hosts and cells which when transferred to secondary hosts give rise progressively to multiple lineages (13). Furthermore, CD41 and CD86 expression on HSCs has been reported to distinguish myeloid-biased and lymphoid-biased cells, respectively (14, 15). As mice age there are quantitative differences in lineage biases in the HSC population. The data support a model whereby myeloid-biased HSCs have clonally expanded, while lymphoid-biased HSCs exhaust themselves due to their more extensive proliferative nature (16).

Recently Jacobsen's group have described a platelet-biased LT-HSC that expresses von-Willebrand factor (vWF) (17). Transplantation of single vWF^{+} HSCs into irradiated hosts resulted in reconstitution biased towards platelets and myeloid cells. vWF^{-} HSCs gave rise to a lymphoid-biased reconstitution. vWF^{+} HSCs require thrombopoietin (Tpo) for their maintenance as these cells were significantly reduced in number in $Tpo^{-/-}$ mice. vWF^{+} HSCs give rise to vWF^{-} HSCs, and vWF^{-} HSCs were not able to give rise to vWF^{+} HSCs. This led

the Jacobsen group to propose that platelet-biased HSCs are the apex of the haematopoietic hierarchy.

Heterogeneity of haematopoietic progenitor cells

In the 'classical' lymphoid/myeloid dichotomy model of haematopoiesis the sets of potentials observed for different types of progenitors align themselves to developmental progression along each arm of the dichotomy. However, this is not the case for all of the progenitors that have been described to date. At odds with an irrevocable commitment of HSCs to either a lymphoid or myeloid pathway is the early description of a progenitor in mouse foetal liver with just the potentials for B lymphoid and macrophage differentiation (18). This cell was later shown to be present in adult bone marrow (19). A further finding that contradicts the notion that HSCs make an immediate and irrevocable decision to commit to either the lymphoid or myeloid pathways of differentiation is the identification of cells with lymphoid potentials and an incomplete set of myeloid potentials. These cells are Early Progenitors with Lymphoid and Myeloid potential (EPLM), that can give rise to T and B lymphocytes, NK cells, dendritic cells (DCs), and macrophages,(20) and Lymphoid-primed Multipotent Progenitors (LMPP), that have little potential for megakaryocyte or erythroid development whilst retaining other potentials.(21)

Progenitor cells that contradict a lymphoid/myeloid dichotomy led us to propose the pair-wise model of haematopoiesis (22-24). This model does not assume lineage branching patterns nor prescribe a single preferred route to a particular end-cell fate. Instead mature cell fates are shown to be near-neighbours within a continuum of lineage fates (Figure 2). As HSCs mature towards a specific dominant cell fate, fates that are distantly related to this fate are lost first and more closely related fates remain possible as latent fates. The model envisages versatility of HSCs and MPPs, as to allowing an end-cell type to be reached by more than one route (see

also below). Mapping of transcription factor usage and the responsiveness of progenitors to growth factors supports the proposed close relationships between cell lineages (22-24).

A number of progenitor cells have been described that have different sets of potentials. The various combinations of lineage potentials that exist within progenitors are also reflected in a number of different cell lines [reviewed in (27)]. Figure 3 shows that the different combinations of differentiation capabilities described for normal progenitors can be mapped to the pair-wise model. Of particular importance to placing cell lineages close to one another in the continuum are progenitor cells that have just two differentiation capacities. In other words, the continuum infers that only certain bi-potentialities are permissible. For example, a cell with the potential for megakaryocyte and T cell differentiation should not exist, and has not been described to date. Bi-potent progenitor cells can be placed within the model with the exception of a bi-potent B lymphocyte/macrophage cell (18, 19). Whether this cell can give rise to DCs has not been studied.

Already there is a considerable variety of stem cells, with differing biases, and progenitor cells, with differing sets of potentials. The latter in turn give rise to end cell types which can be divided into numerous sub-types, for example, as is the case for T helper cells and DCs. The full extent of the heterogeneity of progenitor cell populations, and their mature progeny, is very much contingent on the extent to which new and existing cell surface markers can be used to define new sub-populations. It is highly likely that progenitor cells with multiple lineage options and which we presently view as a homogeneous population of cells will be divided into cells with lineage biases in various directions.

Versatility of haematopoietic progenitor cells

The pair-wise model allows developmental pathways to be flexible. One aspect of this is HSCs and their progeny using more than one route to a particular end-cell type. This principle

was demonstrated by deriving DCs *ex vivo* from cells purified as CLPs and CMPs. When the transcription profiles of the two DC populations were compared they were found to be the same (26). Ishikawa and co-workers concluded that the developmental program of human DCs operates independently of the pathways for myeloid and lymphoid cells.

Alongside new findings there has been a plethora of new models for the architecture of haematopoiesis. Some of the models depict multiple routes towards granulocytes and monocytes. For example, a model provided by Jacobsen considers the possibilities of these myeloid cells arising from ST-HSC/MPP via: *(i)* CMP and GMP; (ii) LMPP giving rise to GMP; and *(iii)* LMPP giving rise to a granulocyte/monocyte/T lymphocyte progenitor (32) which in turn gives rise to GMP. In a model proposed by Katsura the two routes towards myeloid cells are HSCs veering towards: *(i)* a cell with the potentials for myeloid, erythroid and megakaryocyte development; and *(ii)* a cell with the potentials for myeloid and lymphoid development (33). Ye and Graf compared the production of mature cell types to flows along branches of a tree with a major branch giving rise to platelets, erythroid cells, granulocytes and monocytes in equal measure and a separate branch giving rise largely to granulocytes and monocytes (34). Of course, it is difficult to exclude the possibility that all the above routes to myeloid cells occur to some degree.

Precise tracking of a progenitor, as defined by a set of markers, giving rise to the next progenitor, also verified by markers, and so on to an end cell type(s) is an impossible task. However, examination of the sets of options available to various progenitors, as defined by appropriate markers, and which progenitors are or are not able *via* loss of a fate option(s) to give rise to one another allows configuration of multiple routes to certain end-cell types. As described by Jacobsen and c-workers, HSCs can give rise to neutrophils and monocytes through LMPP and CMP intermediates. Figure 4 shows different possible routes downstream of LMPP and CML towards neutrophils. These include potential pathways through an

eosinophil/granulocyte/monocyte progenitor (35) and/or GM-CFUs. The routes are in keeping with the pair-wise model as to their close proximity. Similarly, there are multiple potential pathways towards monocytes *via* CMP, an eosinophil/granulocyte/monocyte progenitor and GM-CFU, and *via* LMPP, EPLM and a monocyte/dendritic cell progenitor (30). In foetal liver, a myeloid/B biased progenitor is a further intermediary to myeloid cells (36). A caveat to all of this is the extent to which routes are used *in vivo*. However, the point of interest is the inherent flexibility of pathways available to progenitors. It is noteworthy that the ability of precursor cells to follow alternative developmental pathways to give rise to the same cell phenotype was described for cell lineages in the embryo of the leech as early as 1987 (37).

One aspect underpinning versatility is that progenitor cells retain fate options even after they have progressed some way along a pathway. Thymus-settling progenitors (TSP) have the potential to give rise to myeloid cells, dendritic cells, NK cells, and B lymphocytes in addition to T lymphocytes. TSPs give rise to Double-negative (DN) 1 early thymocyte progenitors (ETP) which give rise to DN2 cells. DN2 cells have lost the potential for differentiation towards B lymphocytes, but when cultured in the right environment generate myeloid, dendritic cells, and NK cells (38-40). These clandestine potentials are lost as DN2 cells progress to the DN3 stage of development.

Some cytokines have instructive actions on cell-fate decisions

Early glimpses to the instructive action of cytokines

A long-standing debate is whether the commitment of HSCs to fate options occurs in a cell-autonomous and stochastic, or is driven (in an ordered way) by instructive signals from the local environment (41-44). Cytokines are the pivotal external factors that impart environmental signals to control haematopoietic cell development. They have multiple actions that can be viewed as either instructive, by directing HSCs/MPPs towards a specific lineage,

or permissive, by selectively allowing cells committed to a particular lineage to survive and/or proliferate (45, 46). For many years, a permissive role of cytokines has been favoured. A very recent and complete turn about in our understanding of the control of haematopoiesis is the notion that cytokines instruct decision-making (47, 48).

Information to support an instructive role for cytokines has been available for quite some time. In 1982 Metcalf and Burgess concluded that granulocye/macrophage colony-stimulating (GM-CSF) factor and macrophage colony-stimulating factor (M-CSF) can "*irreversibly commit the progeny of GM-CFC respectively to granulocyte and macrophage production*" (49). When paired daughter cells of GM-CFU were split and one cultured in GM-CSF and the other in M-CSF some of the cells underwent irreversible commitment to the granulocyte and macrophage pathways, respectively. This occurred during completion of the first cell division and within 24 hours. Later in 1991 Metcalf again concluded that colony-stimulating factors have the ability to influence lineage commitment (50). Metcalf examined the relative frequencies of lineage committed progenitors when blast cell colonies were established from normal bone marrow cells in combinations of G-CSF, GM-CSF, and multi-CSF. The relative frequency of granulocyte progenitors was increased when cultures were established in the combination of GM-CSF or multi-CSF with stem cell factor (SCF).

In 2000, Kondo and co-workers provided more evidence to support the notion that cytokines can convert the fate of lymphoid-committed progenitors (47). The interleukin (IL)-2 and GM-CSF receptors were exogenously expressed in CLPs, which normally gives rise exclusively to T lymphocytes, B lymphocytes and NK cells. This resulted in cell-fate conversion to the myeloid lineage. The use of mutants of the beta-chain of the IL-2 receptor revealed that signals for the granulocyte and monocyte differentiation pathways are provoked by different cytoplasmic domains of the IL-2 receptor. Kondo and co-workers also showed that primitive HSCs express low to moderate levels of the receptors for GM-CSF and M-CSF. Hence, there

is the possibility that HSCs are receptive to the instructive actions of these growth factors. Kondo and co-workers concluded from all of the above that down-regulation of cytokine receptors that drive myeloid cell development is a critical step in commitment of cells to lymphoid development.

Recent studies confirm an instructive action of cytokines

A number of studies have now shown that cytokines have an instructive action on cell-fate decisions. M-CSF, G-CSF and erythropoietin (Epo) instruct monocytic, neutrophilic and erythroid fates, respectively. In 2009, Rieger and co-workers confirmed that M-CSF and G-CSF provided instructive cues by monitoring individual hematopoietic progenitors in culture. Using bioimaging techniques and a LysM-GFP reporter system to detect differentiation and cell death, the group observed that individual GMPs adopted monocytic or neutrophilic fates in the presence of M-CSF or G-CSF, respectively (48). More recently, Mossadegh-Keller and co-workers made us of PU.1-GFP reporter mice to show that M-CSF drives expression of myeloid-associated genes in some LT-HSCs. *In vivo* M-CSF stimulated expression of PU.1^{+}. HSCs generated increased numbers of GMPs in the spleen and peripheral myeloid cells at the expense of cells undergoing megakaryocyte, erythroid and lymphoid development when compared to non-primed PU.1^{-} HSCs (51). Epo induces priming of erythroid lineage-associated genes in LT-HSCs and *in vivo* skews the potential of these cells towards an erythroid fate (52). An increase in serum Epo levels in mice led to the expansion of committed erythroid and megakayocyte and erythroid progenitors (MEP) in the bone marrow, whereas megakaryocyte progenitors, pre-GMP, and LMPP populations were decreased.

An instructive action of Flt3 ligand in determining cell fate

One of the cytokines that is essential to cell survival and proliferation during early haematopoiesis is the ligand for Flt3 (53, 54). Flt3 ligand (Flt3L) was described two decades

ago (53), and is the only known ligand for Flt3. Upon ligand binding, the Flt3 receptor dimerizes and initiates signalling that involve STAT5a, ERK1/2 and PI3K (55). Flt3 is an important area of research since mutations in Flt3 were among the first ones discovered in acute myeloid leukaemia (AML) [reviewed in (56)]. Presently, there is substantial interest in elucidating the instructive role of Flt3L.

Flt3 expression occurs during haematopoiesis at the non-self-renewing ST-HSC stage of development (57). In fact, Flt3 up-regulation relates to the loss of self-renewal capacity (58). This may just be a coincidence or Flt3/Flt3L provokes the loss of self-renewal capacity by an as yet unknown mechanism. Thereafter, MPP express Flt3 as do several downstream progenitors with myeloid and/or lymphoid potential, while the MEP is Flt3 (57). As lineage options become more restricted Flt3 expression is down-regulated with the exception of DCs (59).

Importantly, Flt3L exerts a role by interacting with other cytokines such as IL-7 or SCF (60, 61). For example, IL-7 and Flt3L stimulate lymphoid development in a coordinated manner which occurs in a narrow window during which cells express both receptors. In the case of *in vitro* cultures of haematopoietic progenitor cells, such as ETP, CLP or EPLMs, and in the presence of IL-7, Flt3L provides an additive anti-apoptotic effect while stimulating proliferation (62, 63) [and our observations]. This additive effect is reported to result from parallel activation of the IL-7 receptor (IL-7R) and Flt3 *via* separate signalling pathways converging to activate Stat5 (61).

Mice with targeted gene disruption of Flt3 (64) or its ligand (65) have provided precise information about the actions of Flt3/Flt3L. These mice present defects in the developmental potential of myeloid/lymphoid progenitors as well as reduced numbers of B cells, DCs and NK cells(64, 65). Tsapogas and co-workers have provided evidence to support the notion that Flt3L is instructive to cell decision-making. These workers generated a Flt3L transgenic

(Flt3L-Tg) mouse that expresses human Flt3L (66). Flt3L-Tg mice have a tremendous expansion of haematopoietic progenitors in the bone marrow. Of all the progenitor populations analysed, the only progenitor that was decreased was the MEP. As such, the Flt3-Tg mice have decreased platelet counts and developed anaemia. Previous studies had reported that expression of Flt3 after the ST-HSC stage leads to a reduction of megakaryocyte and erythrocyte potentials (21). Also, a significant reduction in MEP progenitors was observed by day 3 when wild type mice were injected with recombinant Flt3L (67). Considering this rapid response, it is likely that the reduction in MEP numbers was a consequence of a Flt3L threshold response in upstream $Flt3^{+}$ progenitors, rather than a space restriction within the bone marrow caused by the over-proliferation of other progenitors. It was proposed that upstream $Flt3^{+}$ progenitors develop towards lymphoid/myeloid lineages upon receiving Flt3L stimulation above a certain threshold level and develop towards the Meg/E lineage if this threshold level was not reached. In other words, an increased level of Flt3L guides the development of cells towards the lymphoid/myeloid fates at the expense of the Meg/E fates (Figure 5). This provides an explanation of the megakaryocyte/erythrocyte developmental defect in the Flt3L-Tg mice**.** The exact mechanism by which Flt3L exerts an instructive action remains to be elucidated and whether this occurs at the CLP/EPLM level is of considerable interest.

Cytokines play different roles at various developmental stages

This is best illustrated by consideration of the actions of IL-7 at various stages of B and T lymphocyte development [reviewed in (68-70)]. These include promoting cell survival and proliferation and facilitating decision-making during differentiation (Figure 6). IL-7 was described in 1988 (71). In mice, deficiencies in IL-7 or the receptor lead to impairment of B and T lymphopoiesis (72-74). IL-7 is not required for B lymphopoiesis in humans, as deficiencies in IL-7 result in an apparent normal B cell development (70). However, the B

cells could be the result of foetal development since they can be cultured *in vitro* in the absence of IL-7 while postnatal B cells seem to be IL-7 dependent (75).

CLPs express IL-7R, which provides a criterion used to isolate these cells (1). CLP are dramatically reduced in IL-7 deficient mice (76), indicating this cell is critically dependant on IL-7. EPLM express the IL-7R (20). A strong proliferative action of IL-7 is seen when CLPs and EPLMs, sorted from mouse bone marrow, are cultured on OP9 stromal cells and treated with IL-7 (76) [and our own observations]. Furthermore, these cells are able to differentiate to the next B cell developmental stage, the pro-B (pre-B1), indicating that IL-7 acts as a differentiation factor at the CLP/EPLM level. In keeping with this notion is that CLP cells are unable to reach the pro-B (pre-B1) stage in $\gamma c^{-/-}$, IL-7R$\alpha^{-/-}$ or IL-$7^{-/-}$ mice, and instead arrest at an uncommitted level (77). These data suggest an instructive role for IL-7. This appears to occur *via* IL-7-induced STAT5 signalling which regulates the expression levels of EBF (76, 78). In turn, EBF activates transcription of Pax5 (79, 80) leading to expression of CD19. This cell surface marker indicates that cells have gained the pro-B phenotype and commitment to the B cell lineage (81, 82).

The pro-B (pre-B1) stage is also sensitive to the action of IL-7. Foetal and bone marrow pro-B (pre-B1) cells can be cultured *in vitro* in the presence of IL-7 and stromal cells for long periods of time (more than 4 months) (83). The survival action of IL-7 appears to be exerted *via* expression of the gene encoding the anti-apoptotic protein Mcl-1 (84). Moreover, there is evidence to suggest that IL-7 facilitates differentiation of pro-B (pre-B1) cells into cytoplasmic μ immunoglobulin (Ig) heavy chain expressing large pre-B2 cells (85, 86) [and our own unpublished observation]. IL-7 is not instructive, since cells differentiate irrespective of the presence of IL-7 (85, 86). Instead, the data argue for a permissive role of IL-7, in promoting survival and proliferation, during the pro-B (pre-B1)/large pre-B2 transition. However, some groups have reported that STAT5-mediated IL-7 signalling controls

chromatin accessibility and the rearrangement of distal V_H genes at the *Igh* locus (87-89). This is exemplified by a significant decrease in distal V_H rearrangements in $B220^+$ IgM^- bone marrow B lymphocytes of IL7R-/- and *Stat5-/-* deficient mice (87, 89). By contrast, Malin has argued there is no substantial difference in the distal V_H rearrangement genes seen for STAT5 deficient, IL-7R mutant and control pro-B (pre-B1) cells (84). Finally, as to the pre-B1/pre-B2 transition, it has been reported that IL-7 acts to prevent premature rearrangements of the Igκ *via* binding of IL7-mediated STAT5 to the Igκ intronic enhancer (iEκ) (84, 90).

The large pre-B2 is the last stage in B cell development that is sensitive to the action of IL-7. The proliferation of these cells is improved by the presence of IL-7 (91). However, IL-7 acts as an anti-differentiation factor towards immature B cells by blocking the rearrangement of the light chain loci (84, 90, 91). Upon withdrawal of IL-7 from the culture, cells undergo Igκ recombination and differentiation into IgM positive cells (21, 84, 92, 93). At this stage, as a consequence of the pre-B cell receptor signalling, IL-7R is down-regulated and the later stages of the B cell development are unresponsive to IL-7 (91, 94) [reviewed in (95)].

During T lymphocyte development, the thymus is seeded by ETP (96) and there is a general agreement that these cells can give rise to multiple lineages. In humans, the ETP retain lympho-myeloid potentials and can generate separate lymphoid- and myeloid-primed progenitors. Lymphoid–restricted progenitors seem to be the main thymus colonizers in the mouse (97), though several *in vitro* studies suggest these cells have myeloid potentials (62, 98). IL-7Rα is a direct target of Notch1 (99), the master signalling pathway that regulates thymopoiesis (100-102). Up-regulation of IL-7R at the ETP stage (103) is a hallmark for lymphoid commitment, and IL-7-mediated signalling triggers the first wave of expansion of lymphoid-primed progenitors (104, 105). IL-7 is essential to survival and proliferation of ETP, and appears to transmit signals for the survival and proliferation of lymphoid-committed cells at the expense of the myeloid branch (70). Sustained Notch signalling favours cell

development along the T-cell lineage by inducing the transcription of important T lymphocyte differentiation factors and blocking the development of cells towards other lineages (103, 106). IL-7 is also required for the transition of ETP cells to the DN2 stage *in vitro* (62). Moreover, an early block is observed in T lymphocyte development in IL-7 or IL-7R deficient mice (107). However, Bcl-2 is sufficient to rescue T lymphocyte development (at least the α/β branch of T lymphocytes), thus confirming a permissive role for IL-7 during the early thymopoiesis (108).

IL-7Rα chain expression increases progressively until the DN2 stage, coinciding with the first massive cellular expansion, and then steadily decreases. DN2 cells with high IL-7R expression levels are diverted to the γδ T cell lineage, at least in mice (109), and IL-7R signalling controls accessibility to the T cell receptor (TCR)γ locus and its rearrangement (110). Thus, IL-7 plays an instructive role in this developmental process and Bcl-2 is unable to rescue the development of γδ T cells (108, 111). The cells with reduced IL-7R expression and/or limited IL-7 availability progress to the DN3 stage. The proliferation of these cells is still IL-7 dependent (108, 111). However, a diminished IL-7R signalling seems necessary for DN2 mouse thymocytes to up-regulate Bcl11b, a transcription factor that is essential to the T cell lineage (112). Thereafter, upon successful rearrangement of the TCRβ chain and subsequent pre-TCR expression, IL7-R is down-regulated, and cells move to the DN4 stage (113, 114). Here, at the β selection checkpoint, there is a second wave of expansion that is controlled by Notch1 signalling and pre-TCR expression (62). As such, the DN3 to DP transition is IL-7 independent. However, the continued presence of IL-7 in *in vitro* cultures blocks differentiation and DP cells are generated only upon IL-7 removal. In this case, IL-7 is acting as an anti-differentiation factor (62, 115). This highlights the importance of the IL-7R signalling suppression during the DP transition, which is guaranteed by SOCS-1 (113). After the DP selection stage, IL-7R surface expression levels are restored and IL-7 signalling seems to be required for the DP to single positive CD8 transition [reviewed in (116)]. In keeping

with this, Park and co-workers reported differentiation of single positive CD8 cells ($CD8^+$) and RUNX-3 transcription factor up-regulation in transgenic TCR mice expressing a transgene derived IL-7Rα chain in an IL-7 over-expressed environment (117). Finally, IL-7 is required for homeostatic expansion of naïve $CD8^+$ and $CD4^+$ T cells (118, 119).

In the context of the role of IL-7 in lymphopoiesis and in particular the flexibility of the hematopoietic process, the effect of pregnancy on hematopoiesis and lymphopoiesis is worthy of a mention. Pregnancy is certainly a physiological process in which there is an increase in blood volume and the bone marrow responds by increasing erythropoiesis (119). Lymphopoiesis is also affected and two mechanisms account for the reduced B lymphopoiesis observed in pregnancy. Firstly, early B progenitor cells are sensitive to the increased levels of sex steroids in pregnancy (120) and secondly, in mice, IL-7 production and availability dramatically decrease (121). Not only is the bone marrow affected during pregnancy, but the maternal thymus also undergoes dramatic involution (122). Both B and T lymphopoiesis return to pre-pregnancy levels following parturition and weaning. A full explanation of these associated phenomena is not currently available and is certainly worthy of further investigation.

Implications of heterogeneity and versatility to leukaemia

A Darwinian viewpoint on leukaemia

A widely held viewpoint is that many leukaemias and cancers arise in a stem cell, termed leukaemia initiating cells (LIC) and cancer stem cells (CSC), respectively (123-127). These cells, which have an inherent capacity to self-renew, sustain the tumour cell population. A LIC/CSC origin of leukaemias/cancers is important to putting into practice a cancer stem cell-based therapeutic to cure patients. To add to the difficultly of designing therapies to eliminate LIC/CSC, Greaves has proposed a Darwinian viewpoint on the nature of CSC which pays

attention to the dynamics of the cancer (128, 129). Studies of single acute lymphoblastic leukaemia (ALL) cells have revealed that the leukaemia 'stem' cells are genetically diverse. Greaves has likened the complex and branching clonal architecture of ALL 'stem' cells to Darwin's evolutionary tree-like divergence diagram which was drawn in 1837. To add to the Darwinian analogy, as the leukaemia evolves, in an almost entirely clinically silent manner, cells acquire gene copy number alterations. The cytokine TGF-β has been proposed to exert a selective Darwinian advantage to expand particular clones from the diverse population of LICs. In essence, this is similar to 'natural selection' as described by Darwin.

Often what we discover from studies of cancer cells, and ascribe to these cells, turns out to be a feature of normal cells. A simple example is that the common ALL-associated antigen (CD10) was first described as a candidate leukaemia-specific antigen (130). In fact, CD10 is expressed by rare B cell progenitor cells and the presence on ALL cells was telling us something about the origin of common ALL (131). So, might we view normal haematopoiesis as a Darwinian process that is driven by selective pressures, namely cytokines, acting on an inherent diversity that is sufficient to generate the various types of blood and immune cells. In essence, inherent cell heterogeneity must have been the template to the evolution of the wide variety of immune cells that exist in higher mammals.

Are leukaemia stem cells as versatile as their normal counterpart?

An important question that arises from the above considerations is: Are LICs as versatile as normal HSCs in terms of their capacity to access routes to end-cell types? There is evidence to suggest that LICs are less versatile in this regard [reviewed in (132, 133)], as exemplified by erythroleukaemia (acute myeloid leukaemia (AML) FAB-M6) and pre-B/pro-B/common acute lymphoblastic leukaemia (ALL). The cells that sustain these leukemias appear to have become directed to generate cells of a particular cell type. An accumulation of erythroid precursors and myeloblasts in AML FAB-M6 reveals a disease origin in a cell with multi-

lineage potential (134, 135). However, the partial differentiation of the leukaemic blast cells is restricted to certain pathways as to characterisation of disease sub-sets as myeloblast-rich (FAB-M6A), proerythroblast-rich (FAB-M6B) and myeloblast- and proerythroblast-rich (FAB-M6C) (135).

In the case of childhood ALL, including pre-B ALL, pro-B ALL, and common ALL (c-ALL), there are arguments to support a disease origin in either a cell that is committed to B lymphocyte development (136, 137) or a cell that is more stem cell-like (138). The former notion is a long held viewpoint. In favour of the latter cellular origin is that c-ALL-derived cells lacking the B-lineage markers CD10 and CD19 and expressing the stem cell marker CD34 can give rise to c-ALL and pre-B ALL when transplanted into mice (138). The argument about the precise 'target' cell that is transformed in childhood ALL could be set aside as versatility of lineage options extends to progenitor cells, as illustrated by the cytokine-mediate redirection of CLPs to the myeloid lineage (47). Strikingly, the blast cells that accumulate in the blood and bone marrow in childhood ALL are restricted to B-lineage development.

Genomic stability and pluripotency of haematopoietic stem cells.

There is now good evidence to support the notion that growth factors can instruct the lineage potentials of stem and progenitor cells. This leads to an interesting question which is how do HSCs establish and, as so required for steady state haematopoiesis, maintain pluripotency. How a network of factors might participate to control HSC identity and commitment ability to drive HSC contributions to homeostasis and adaption to inflammatory conditions is as yet unclear.

Presumably to maintain HSC pluripotency there is the requirement to control genomic stability. In part, this means controlling intrinsic DNA repair machinery. As mentioned above

vWF^{+} HSCs require Tpo for their maintenance. This growth factor also plays a role in regulating the genomic stability of HSCs. De Lavel and colleagues have shown that the efficiency of DNA-Protein Kinase-dependent DNA repair, in response to DNA damage, is increased by Tpo (139). Tpo-induced activation of ERK and NF-κB in HSCs is important to damage repair (140). As to a role of Tpo in ensuring chromosomal integrity, the plot thickens regarding the roles growth factors play.

As eluded above, why might LIC/CSC be less versatile in regard to lineage options than their normal counterparts? One possibility is global genomic instability that might underlie clonal evolution curtails the availability of lineage options. However this doesn't seem to be the case. Global instability is unusual for AML (141). For both AML and pro-B ALL a very small number of mutations are required to generate the leukaemia (142). Genomic instability is a feature of the chronic phase of chronic myeloid leukaemia, resulting in BCR-ABL-1 mutations that encode resistance to the tyrosine kinase inhibitors (e.g. imatinib) used to treat the disease. Importantly, instability has been postulated to occur in primitive leukaemia progenitor cells in patients who haven't been treated with tyrosine kinase inhibitors. And, it has been suggested that the instability is due to high levels of DNA damage, by reactive oxygen species, and inefficient/unfaithful repair of DNA double-strand breaks leading to chromosomal aberrations (143).

As to all of the above, genomic stability and instability, as linked to effective DNA repair, are important to the behaviour of normal stem/progenitor cells and LIC, respectively. As yet we do not know whether LICs/CICs are as or less versatile in terms of lineage options than their normal counterparts. Selective instability in LICs/CICs in genomic elements encoding controls (e.g. transcription factors and signalling molecules) that play roles in lineage decision-making might restrict the versatility of LIC/CIC. For normal stem and progenitor

cells the versatility nature of the pair-wise model provides an appropriate template for the persuasive action of growth factors to shape haematopoiesis.

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Figure legends

Figure 1. Identification of haematopoietic stem and progenitor cells in adult bone marrow

The loss and gain of cell surface molecules is used to delineate sub-populations of haematopoietic stem cells (HSCs) and multipotent progenitors (MPPs). Weissman subdivides the Lineage markers^{-}, Sca-1^{+}, c-Kit^{+} (LSK) population of bone marrow cells on the basis of expression of Flt3 and Thy1.1. Jacobsen makes use of CD34 and Flt3 expression. Signalling lymphocyte activation molecule (CD150, CD48, CD229 and CD244) markers are used to subdivide LSK cells in the model proposed by Morrison. Flt3, fms-like tyrosine kinase; LT-HSC, long-term reconstituting haematopoietic stem cells; ST-HSC, short-term reconstituting haematopoietic stem cells.

Figure 2. A pair-wise model of haematopoiesis

A fate choice continuum with an invariant series of pair-wise developmental relationships between haematopoietic cells fates is derived from the nature of the sets of potentials of various haematopoietic progenitor cells (22-25). These are show as segments of the continuum. Dendritic cells (DC) can be derived from common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), as shown by arrowheads on two of the arcs (26). The figure, modified and with permission is from [21] © Macmillan Magazines Ltd. Bas, basophil; DC, dendritic cell; Eos, eosinophil; EPLM, early progenitor with lymphoid and myeloid potential; Ery, erythroid; HSC, haematopoietic stem cells; LMPP, lymphoid-primed multipotent progenitor; MC, mast cell; Meg, megakaryocyte; Mon, monocyte; Neut, neutrophil; NK, natural killer cell.

Figure 3 Mapping of haematopoietic progenitor cells to the pair-wise model

The set of fates available to various progenitors provide underlie near-neighbour placement of lineages in the pair-wise model. Progenitors with commensurate and contiguous lineage

potentials are CLP, common lymphoid progenitor (1); CMP, common myeloid progenitor (2); DC/Pro-B, dendritic cell and B lymphocyte progenitor (26); Eo/B-CFU, eosinophil and basophil progenitor (28); EPLM, early progenitor with lymphoid and myeloid potential (20); GMP, granulocyte and macrophage progenitor (27); LMPP, lymphoid-primed multipotent progenitor (21); MEP, megakaryocyte and erythrocyte progenitor (29); Mon/B/DC?, monocyte, B lymphocyte and dendritic cell? progenitor(18, 19); Mon/DC, monocyte and dendritic cell progenitor (30) and NK/T, natural killer cell and T lymphocyte progenitor (31); HSC, haematopoietic stem cell; Ly, lymphoid bias; Meg, megakaryocyte bias; My, myeloid bias.

Figure 4 Alternative developmental routes towards neutrophils

The sets of potentials available to known oligopotent progenitors are used to construct possible routes. The red arrows are routes delineated from studies of the progeny of progenitors and the blue dash arrows are putative routes. For abbreviations see the legend to Figure 3.

Figure 5 Instructive action of Flt3 ligand in determining lymphoid/myeloid *versus* megakaryocyte/erythrocyte lineage development

If the Flt3 ligand signal strength exceeds a certain threshold level cells enter the lymphoid/myeloid branch at the expense of the megakaryocyte/erythrocyte lineage. HSC, haematopoietic stem cell; MPP, multipotent progenitor.

Figure 6. IL-7 action during various stages of murine B and T lymphocyte development

The progressive stages of B lymphocyte development (A) and T lymphocyte development (B) are shown. The green cells represent stages responsive to the survival and proliferative action of IL-7. Pro-differentiation and anti-differentiation actions are also shown.* Indicates that the IL-7 pro-differentiation action could be instructive. For other stage transitions more evidence

is needed to conclude an instructive action. DN, double-negative; DP, double-positive; EPLM, early progenitor with lymphoid and myeloid potentials; ETP, early thymocyte progenitor.

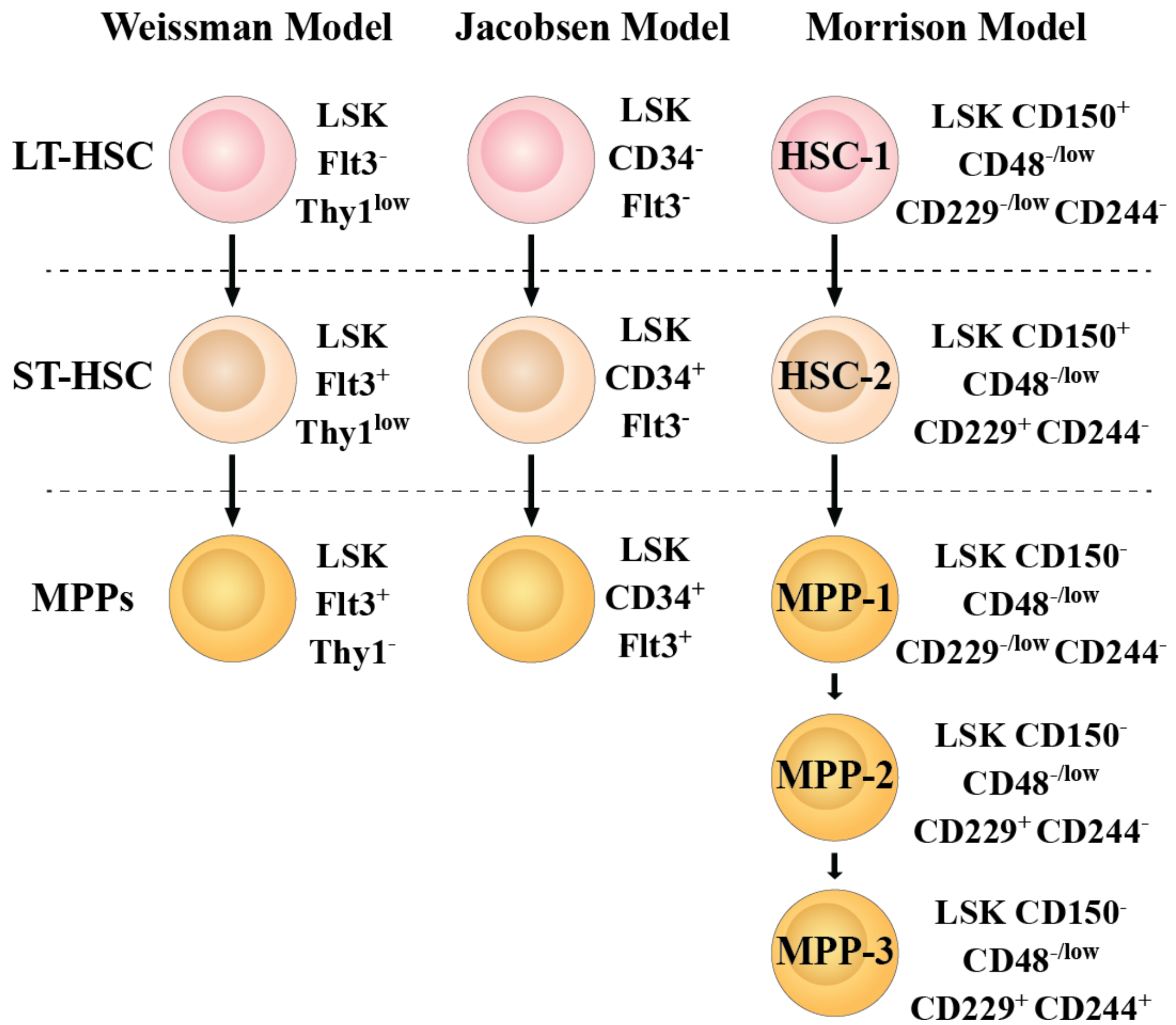

Fig. 1

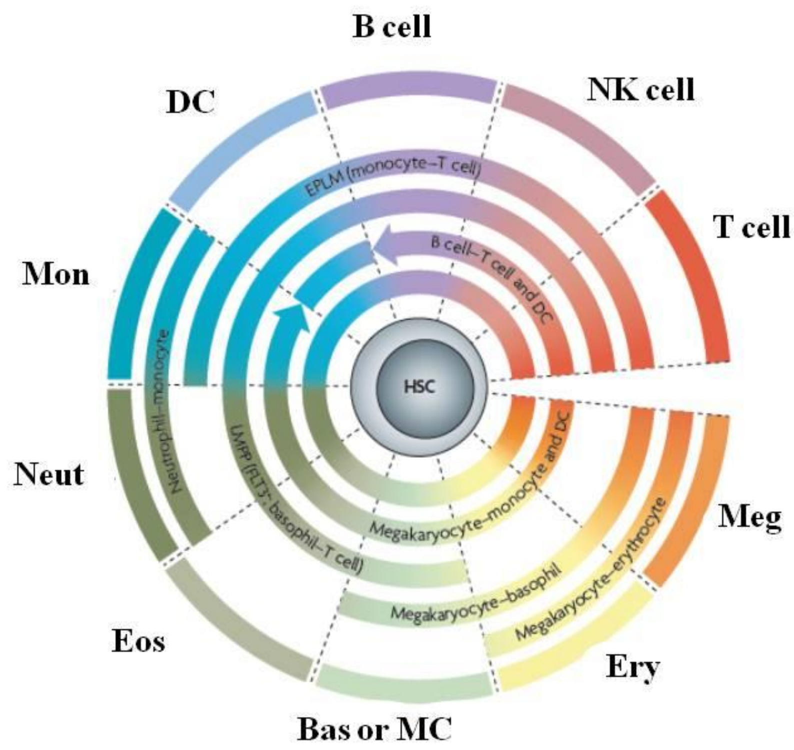

Fig. 2

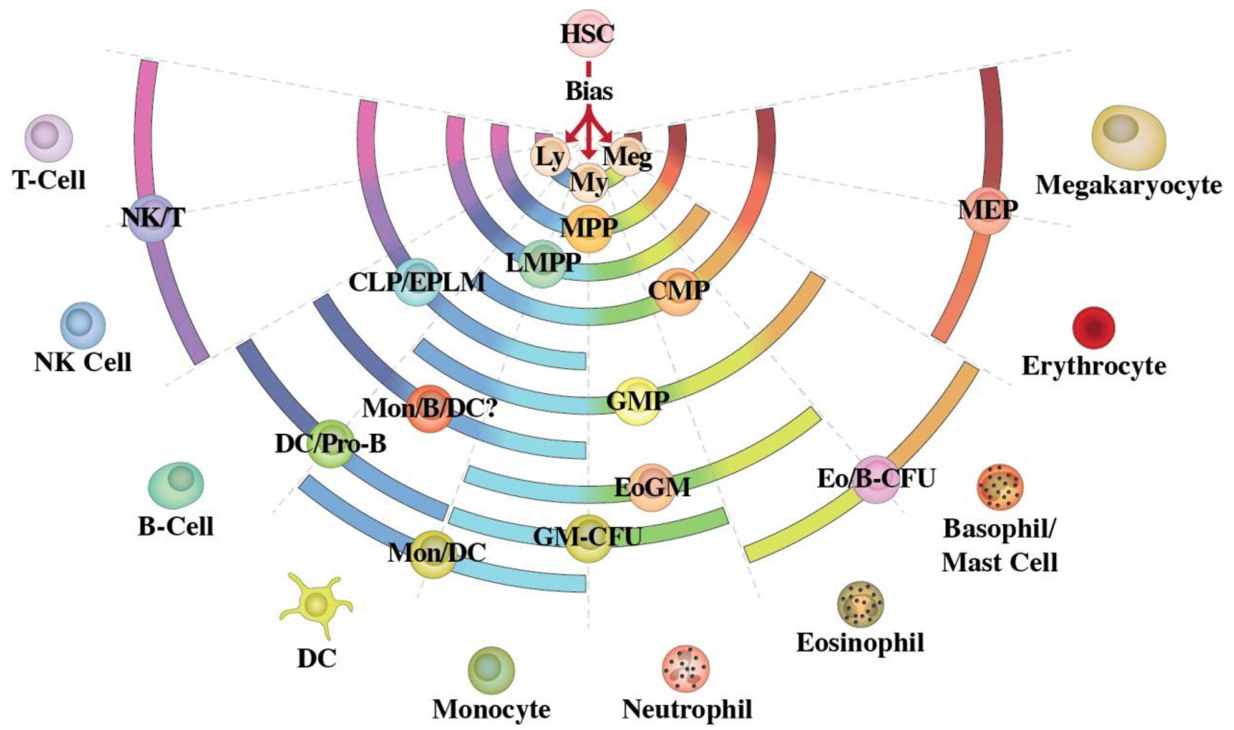

Fig. 3

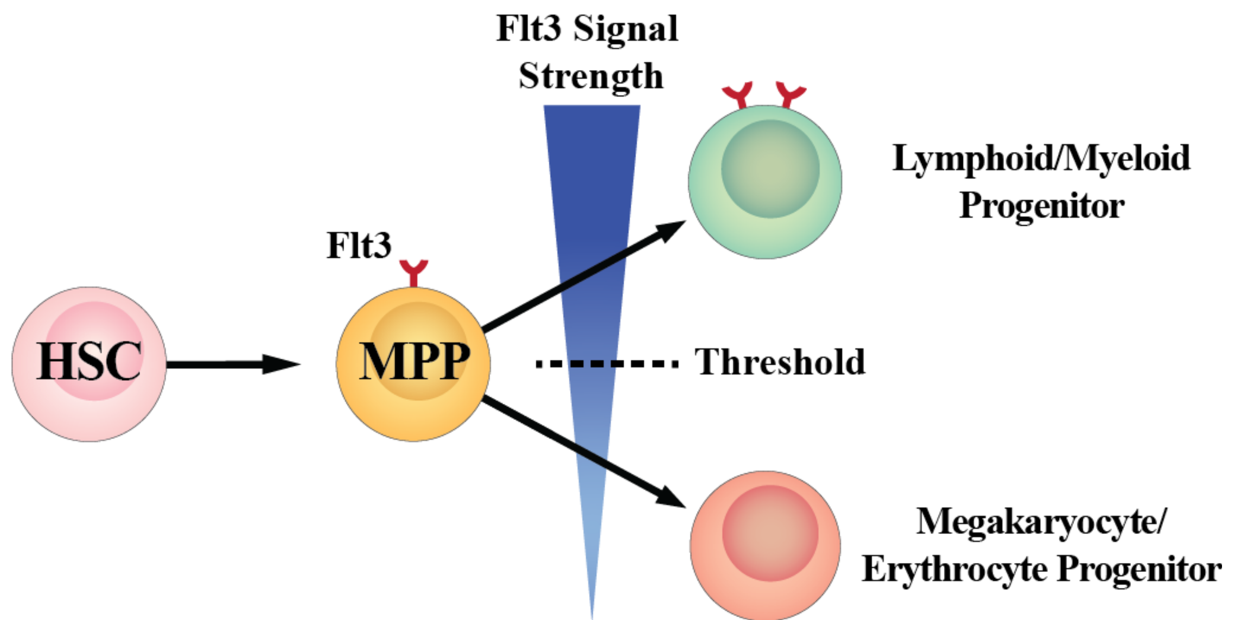

Fig. 4

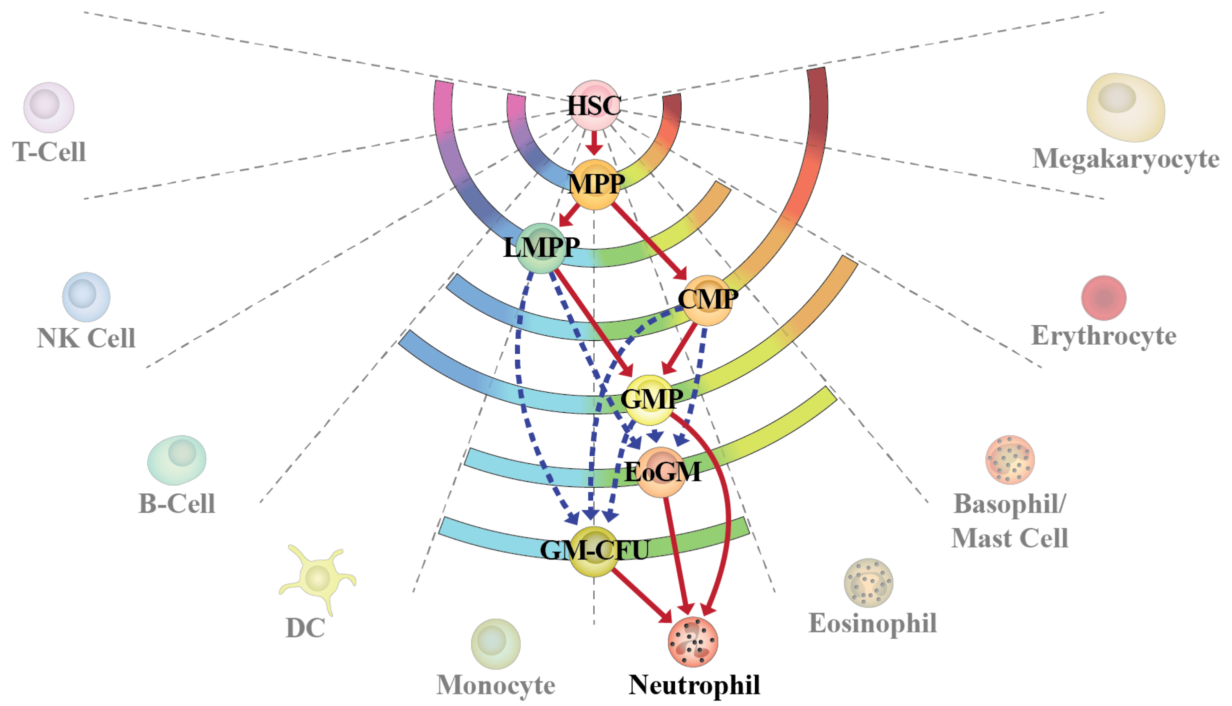

Fig. 5

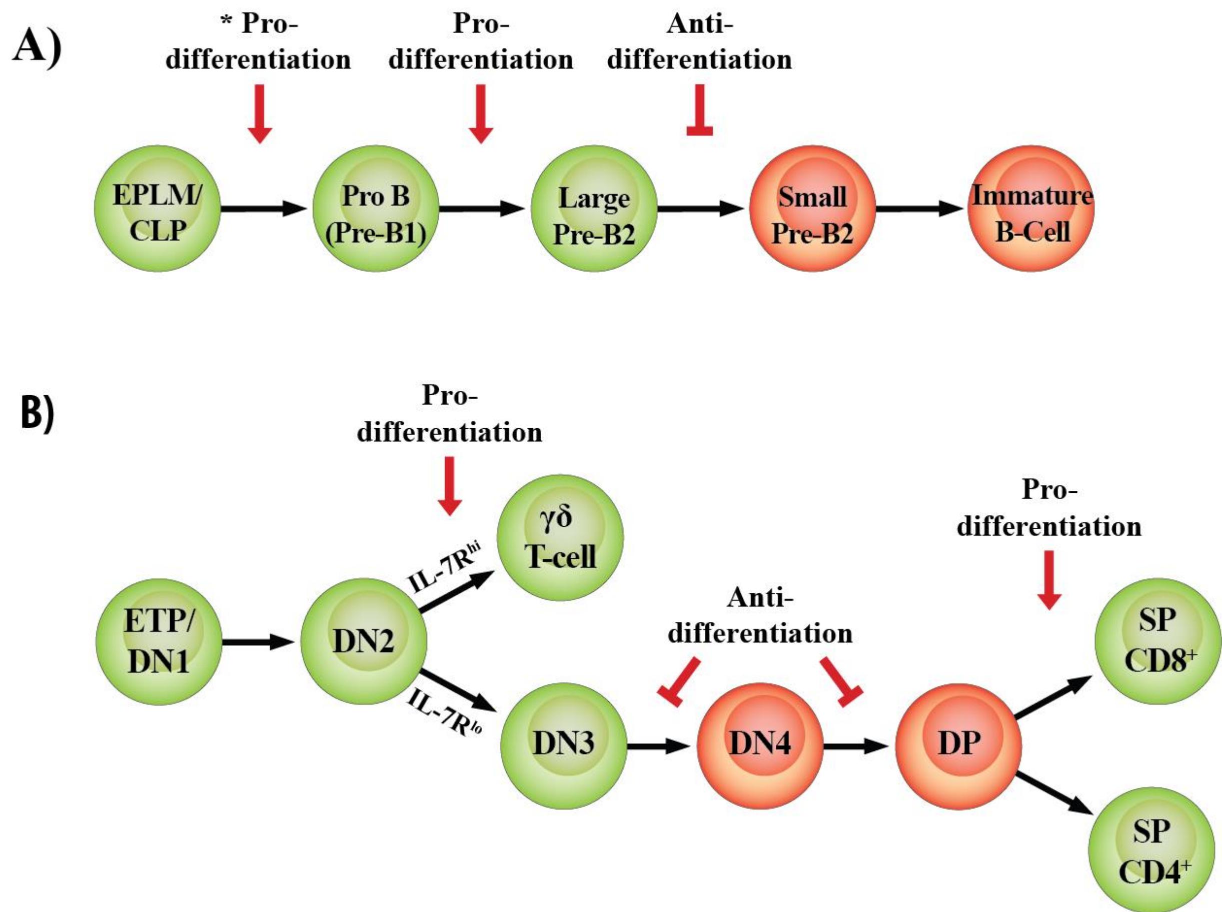

Fig. 6