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DOI:
10.1111/imcb.12055

Document Version
Peer reviewed version

Citation for published version (Harvard):
Brown, G, Tsapogas, P & Ceredig, R 2018, 'The changing face of haematopoiesis: A spectrum of options is available to stem cells', *Immunology and Cell Biology*. https://doi.org/10.1111/imcb.12055

The changing face of haematopoiesis: A spectrum of options is available to stem cells

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Keywords

Haematopoiesis, haematopoietic stem cells, fate determination, blood and immune cells

ABSTRACT

For more than 30 years the scheme whereby bone marrow haematopoietic stem cells give rise to the many different types of blood and immune cells has been represented as a lineage tree diagram. In this model, haematopoietic stem cells follow a preferred route to each of the end cell types and gradually restrict their other lineage options *via* a series of intermediate oligo-potent progenitors. Recent findings of lineage biases or affiliations within haematopoietic stem and progenitor cells that are either pluripotent or uni-potent show that a continuum of fate options is open to haematopoietic stem cells. These results support the view that in order to close down developmental options, haematopoietic stem cells can make an immediate lineage choice rather than become gradually committed as they progress step-wise through a series of intermediate progenitors. In this scenario, there is inherent versatility in that developing cells are still able to move sideways to adopt an alternative lineage fate. Here we examine the information that is leading towards this very different viewpoint of blood cell development.

INTRODUCTION

A textbook account of haematopoiesis has been in existence since the early 1980s. The model, often referred to as the 'classic model', is hierarchical, with self-renewing pluripotent haematopoietic stem cells (HSC) at the apex. These cells give rise to haematopoietic progenitor cells (HPC) and a tree lineage map depicts the progressive restriction of their developmental options *via* a series of bifurcations.[1] The first such bifurcation is when the progeny of HSC become either common lymphoid progenitors (CLP), that generate lymphoid cells, including T and B lymphocytes and natural killer cells, or common myeloid progenitors (CMP), that become myeloid cells including all other cell lineages. A plethora of variants of the 'classic model' have been proposed,[2] some of which challenge its cardinal properties. In 2008, Graf emphasised the need to re-wire our ideas of haematopoiesis.[3]

In 2009, we argued that a continuum of fates is available to HSC[2,4] and our view of the architecture of haematopoiesis was the pair-wise model, shown in Figure 1. In contrast to tree-like models, this model envisages HSC as being able to commit directly to a lineage option without the need to sequentially restrict alternative fates (see later). Another aspect of this model is its emphasis on close relationships between cell lineages. The lineage options available to bi-potent cell populations, as revealed by colony-forming assays of bone marrow cells using semi-solid medium, and the incomplete sets of options available to other progenitor populations were assumed to indicate close relationships between pathways. Sets of potencies that overlapped were combined and shown in our model.[2,5] The shared cell usage of transcription factors and responsiveness to promiscuous cytokines[6] accord with the particular close relationships between cell lineages. Transcription factors can promote the development of a group of adjacent cell types, can suppress the development of cells lying on either side of a fate or set of fates and can divert the development of a progenitor that has provisionally adopted a fate to an adjacent fate.[2] As a result, the concerted action of multiple

transcriptions factors might narrow fate trajectories in a centrifugal manner until only one option remains.[2]

Haematopoietic stem cells and various progenitors are heterogeneous population of cells

Conventionally, HSC and populations of HPC are shown to be phenotypically homogeneous, or 'ring-fenced', by the use of a panel of antibodies to cell surface markers [Figure 2]. Mouse HSC lie within a very small fraction of bone marrow cells that lack expression of cell lineage markers (Lin^-) but express the Sca-1 antigen and the receptor for the stem cell factor CD117 or c-kit; they are thus called LSK. Further analysis with additional markers shows that LSK include the following: $CD150^+$ $CD48^-$ HSC; $CD150^-$ $CD48^-$ multipotent progenitors (MPP); $CD150^-$ $CD48^+$ HPC1; $CD150^+$ $CD48^+$ HPC2 and $CD48^+$ $CD135^+$ ($Flt3^+$) lymphoid-primed multipotent progenitors (LMPP). Furthermore, on the basis of CD34 expression, HSC are subdivided into a) $CD150^+$ $CD48^-$ $CD34^-$ cells capable of reconstituting the blood cell system of a lethally irradiated mouse long-term (LT-HSC); and b) $CD150^+$ $CD48^-$ $CD34^+$ short term (ST-HSC) (reviewed in [7]). One difficulty with compartmentalising cells in this manner is a lack of knowledge about the functions of some of these surface molecules during developmental progression. For example, the cellular functions of CD34, CD48 and CD150, and of markers such as Ly6D and SiglecH that are used to subdivide HPC in the case of early progenitors with lymphoid and myeloid potential (EPLM), are not known.[8,9]

Can HSC be described simply as either LT-HSC or ST-HSC? The first indication that this is not the case was the identification of HSC with lineage biases. Myeloid- and lymphoid-biased mouse HSC have been identified by transferring single cells into irradiated mice. Myeloid-biased HSC express a higher level of CD150 than lymphoid-biased HSC and exclude the DNA-binding dye Hoechst 33342 more effectively. The surface markers CD41 and CD86, respectively, have also been used to denote myeloid- *versus* lymphoid-biased HSC.[10,11] Myeloid-biased HSC predominate in aged mice, in which the supply of lymphoid-biased

HSC appear to have been exhausted as a result of more extensive proliferation.[12] Using reconstitution experiments, the Jacobsen group described a mouse HSC that was biased towards platelets and myeloid cells, expressing von Willebrand factor and requiring thrombopoietin for its maintenance. Cells that did not express von-Willebrand factor were lymphoid-biased.[13]

Mouse HSC express the receptors for macrophage colony-stimulating factor (M-CSF) and erythropoietin (Epo) and the fms-like tyrosine kinase 3 (Flt3), which binds the lympho/myeloid-affiliated Flt3 ligand (Flt3L).[14-17] The receptors for M-CSF (CD115) and Epo (Epo-R) are expressed by HSC; some HSC co-express the M-CSF receptor and Flt3 and co-expression of the Epo-R and Flt3 mRNAs was rarely seen.[15] Figure 3 therefore shows HSC as a mixed population of cells. As discussed later, M-CSF, Epo and Flt3L have been shown to instruct lineage choice and therefore expression of the receptors by HSC is indicative of lineage affiliation and/or commitment.

Using *in vitro* culture experiments, Notta and colleagues examined the extent to which human HSC are already lineage-affiliated by mapping the fates of single $CD34^+$ cells. For adult bone marrow, they observed that cells with uni-potent myeloid or erythroid potential predominated alongside some multipotent cells. There were few oligopotent progenitor intermediates in adult bone marrow, but foetal liver contained large numbers of HPC with megakaryocyte/erythroid/myeloid and megakaryocyte/erythroid fates. These findings led to the proposition of a ‘two-tier’ hierarchy with different routes for cell lineage development during foetal and adult haematopoiesis.[18] In contrast, Kauts and colleagues have argued that HSC in the mouse embryo produce blood cells without the need for intermediate progenitors, and that adult blood cells are produced *via* intermediate progenitor populations.[19]

If we accept that lineage-affiliation occurs as early as the HSC stage of haematopoiesis, then the HPC down-stream of HSC that have been ring-fenced as multipotent are most likely a mixture of cells with different lineage potentials. This has indeed been shown to be the case for some mouse populations. LMPP were described as primitive cells with little potential for megakaryocyte and erythroid development.[20] LMPP have now been divided into lymphoid-, myeloid- and dendritic cell lineage-biased progenitors[21] and their fate is determined with the siblings of a single cell often sharing the same fate. EPLM were described as cells lacking megakaryocyte and erythroid potentials and able to generate T and B lymphocytes, NK cells, dendritic cells and macrophages.[22] Phenotypic analysis using Ly6D, SiglecH and CD11c has revealed that EPLM comprise four subpopulations with distinct lineage developmental biases; the population of cells retaining the most lineage options as a whole expresses none of the above markers and were called “triple negative” EPLM. RNA sequencing of single triple negative EPLM subpopulation revealed that genotypically they already have either myeloid, dendritic cell or lymphoid signatures, with extremely few having a combined lymphoid and myeloid potential.[8]

The CMP population is viewed as giving rise to all myeloid cells. However, the use of an optimised single cell *in vitro* assay has shown that human CMP are a mixture of cells with either myeloid, erythroid or megakaryocyte developmental potential.[18] Hoppe and colleagues have analysed RNA expression data from individual murine HSC throughout their development towards megakaryocytic, erythroid and myeloid cells and concluded that CMP are a mixture of bipotent granulocyte/monocyte (GMP) and megakaryocyte/erythroid (MEP) progenitors.[23] Paul and colleagues have examined, at the single cell level, the transcriptional heterogeneity and lineage commitment of myeloid progenitors from normal as well as mice in which myelopoiesis had been perturbed by knocking out the myeloid regulator Cebpα. Single bone marrow progenitors were sorted into 7 clusters with the transcriptional properties

of neutrophils, basophils, eosinophils, monocytes, dendritic cells, erythrocytes and megakaryocytes. In total, 18 different populations were identified with variable degrees of specification towards these pathways. Normally, cells with mixed gene expression profiles were not observed but were present when Cebpα had been knocked out, revealing that mixed states are possible.[24] These studies using recently-developed single cell genomic analysis highlight the fact that phenotypically ring-fenced populations can nevertheless be genotypically and developmentally heterogeneous.

From all of the above there is the need to move away from a tree-like depiction of haematopoiesis which does not envisage the direct commitment of HSC to a cell type. Another conclusion from all of the the above is that the distinction between HSC and HPC has become somewhat blurred, and perhaps redundant. HSC can no longer be defined strictly by their lack of a lineage affiliation coupled invariably to a capacity to self-renew. Additionally, Hofer and colleagues have highlighted that the hallmarks used to delineate HSC and HPC, namely self-renewal and the extent of multi-potency as revealed by cell transplantation experiments and *in vitro* assays, may not be features that occur naturally in these cells.[25] The nomenclature used to describe LMPP, EPLM and CMP is misleading, particularly that each of these populations is a mixture of cells. Perhaps we should describe all cells that give rise to the haematopoietic system as progenitors that are either multipotent, termed HPC-MP or with a designation regarding their lineage affiliation, for example HPC-E as to erythroid.

The pair-wise model has been annotated in Figure 3 to show that HPC have been sub-divided into cells with more restricted, including uni-potent, lineage options. The new sub-divisions combine data from studies of human and mouse cells and also the various means of identifying individual cell populations, including global transcriptome analyses,

transplantation experiments to measure lineage biases and single cell culture experiments. Uni-lineage biases or affiliations that are evident at the HSC stage lead to threads of development pathways that are commensurate with cells not having to progress to an end-cell type via a series of intermediate and oligopotent progenitor states. In essence, cell lineage commitment can occur at the level of HSC which is in keeping with the spectrum of options that is available in the pair-wise model. Both these matters have important implications for how HSC determine their fate. There are two possibilities. First, that lineage fate is determined in a cell-autonomous manner and therefore set by the genetic/epigenetic nature of the cell. The second is that fate is influenced by environmental factors such as cytokines or the cell's interactions with stromal cells. In favour of the second argument, discussed later, is the fact that cells do not have a 'mind' of their own and are not capable of "making decisions". They are essentially 'social', responsive to nurturing and functioning for the benefit to the whole organism.

Why might experimental approaches have misled mapping haematopoiesis?

Commitment at the level of HSC questions the validity of information from assays in which HPC were dispersed in semi-solid agar or methycellulose medium and colonies observed containing many different cell types. The founding colony-forming cell is clearly oligo-potent and the nature of mature cells within the various types of colonies has underpinned tree lineage maps for haematopoiesis. Whilst the colony assay was the best available at the time, the mixtures of mature cells seen *in vitro* appear now to have misled us as to how progenitors behave *in vivo*. Of course, what happens *in vitro* might be very different to what happens *in vivo*, particularly as the *in vitro* experiments are performed in 5-10% CO_2-in-air incubators with 20% oxygen; conditions very different from the niches that cells occupy *in vivo*. Moreover, single cells dispersed in agar are clearly out of their normal social environment with agar not a substitute for extracellular matrix. In particular, the heterogeneity within the

in vivo microenvironment that may influence the restriction of lineage options is impossible to replicate *in vitro*.

The transfer of bulk and purified sub-populations of HSC into an irradiated mouse and the variable capacity of these cells to reconsitutute blood cell lineages has also been used as an assay to map haematopoiesis. There are also numerous concerns regarding the use of this assay. The engraftment efficiency, that is the proportion of transferred cells finding a suitable microenvironment and repopulating hosts, is frequently low. These concerns can be overcome by transfers of single cells or by injecting HSC together with bone marrow stromal cells directly into the bone marrow cavity. Perhaps of greater concern is the fact that recipient mice need to be depleted of endogenous haematopoiesis, frequently by irradiation. This is a very stressful procedure that kills most hematopoietic cells and results in the release of cytokines and extracellular material and damage to the tissue niches that support hematopoiesis. The niches that HSC and progenitors riside in are known to be heterogenous and, as alluded to above, the availability of haematopoietic cytokines within niches might well determine the lineage bias/affiliation of HSC and HPC (see also later). Recent studies have used *in vivo* genetic marking of HSC/HPC, thus enabling the investigation of the dynamics of steady-state haematopoiesis, in some cases at the single-cell level without the need for irradiation.[26-28] Interestingly, the results obtained from these investigations suggest significant differences between steady-state physiological haematopoiesis and that observed following transplantation into irradiated animals. Thus, while reconstitution of the depleted haematopoietic system after transplantation occurs through the expansion and differentiation of a small number of donor HSC, the normal replenishment of blood cells seems to involve a larger number of HSC clones, which are successively recruited to regenerate mature haematopoietic cells.[28] In addition, steady-state haematopoiesis seems to be sustained to a large extent through the contribution of multi- and oligo-potent HPC, rather than HSC.[28,29]

These differences between physiological and transplantation-based haematopoiesis could be the result of the different engraftment capabilities of various HSC clones and could be consequences of the changes that occur in the haematopoietic niches after irradiation.

Steady state haematopoiesis in adult mice is most often the model that is studied to provide a map of haematopoiesis. But, how blood cell types are produced in early life, during established steady state and during infection may well differ. For example, there are very few cells with dual lineage potential in the bone marrow of humans and adult mice whereas oligo-potent cells are frequent in the human foetal liver.[18,30] Two studies in zebrafish and mice have used multicolour approaches to track the clonality of HSC. The zebrafish provides an insight to the early life of HSC and the analysis of colour 'bar codes' has shown that a relatively small number (20-26) of HSC clones is generated. A reduced clonal diversity was observed in the blood pool of zebrafish during stress, indicating that further clonal selection of a small number of selected HSC repopulate the marrow after injury. A small number of HSC clones has been proposed for mammalian models. As HSC arise, perhaps the clones generated acquire, or evolve, the uni-potent propensities necessary to generate all the different types of blood and immune cells, to 'set' an established and complete pattern of diversity. Even so and as mentioned above, mixed lineage states were observed when haematopoiesis was perturbed in the adult mouse, by Cebpα knock-out,[24] which may mean that propensities can be re-set in a particular circumstance.

The nature of the trajectories available to HSC

Affiliation of HSC/HPC to a single fate does not preclude there being particular relationships between lineage trajectories. As previously mentioned, the identification of cells that share just two particular lineage options has been a long standing feature of the outcomes from many experiment studies. A prime example of this is that primitive haematopoiesis is bi-

lineage in nature with megakaryocyte/erythroid progenitors emerging in the mouse yolk sac at embryonic day 7.25, along with megakaryocytes and primitive erythroid progenitors.[31] Holtzer argued in favour of bipotent lineage relationships from the point of view that cells can only make either/or type decisions[32] and support for the notion of bi-potency at a particular developmental moment has been provided by single-cell RNA-Seq analysis of the lineage status of cells. Olsson and colleagues analysed the lineage states of MPP and observed bi-potential patterns of gene expression during myelopoiesis. In particular, GMP have low-level expression of the mRNAs for Gfi1 and Irf8 and increased expression of these genes coincides with, and appears to be necessary for, neutrophil and monocyte specification, respectively. Accordingly, mixed lineage cells appear to be poised for binary fate choices. The above investigators have proposed that bi-lineage expression states are meta-stable, due to bursts of alternative lineage gene expression, and are obligatory for cell-fate specification.[33]

Velten and colleagues have constructed developmental trajectories by integrating transcriptomic data obtained from single-cell RNA-seq with data from single cell cultures to provide compelling evidence that lineage commitment of human HSC is a continuous process.[30] Genes relating to the degree of transcriptome priming were clustered into modules which were then plotted against the extent of lineage-specific priming. The data fitted the pair-wise/continuum model of haematopoiesis[2] whereby cell lineages that are closely related were placed in the order B cell, monocyte/dendritic cell, neutrophil, eosinophil/basophil/mast cell, megakaryocyte and erythrocyte. In contrast to Olsson and colleagues, Velten and coleagues observed very few cells with a dual-lineage transcriptomic state and concluded that primed MPP make a direct transition towards a uni-lineage transcriptome state. They likened their module to Waddington's epigenetic landscape model[34] with barriers between lineages that are near neighbours arising early, and the cells following valleys with the barriers

becoming more pronounced as cells progressed towards an end-cell type. Accordingly, cells at early stages of development are viewed as able to cross into the adjacent valley rather than having to follow their primary trajectory. In other words, the transition from one stable cell state to another does not need to be sequential and cells can inter-convert to an adjacent fate, particularly at early stages of development.

The availability of lineage options to either the left or right of a developmental trajectory is supported by the studies of Nestorowa and colleagues.[35] By single-cell RNA sequencing, they profiled more than 1600 single haematopoietic stem and progenitor cells (HSPC), and then built expression maps to reveal the changes relating to the early stages of differentiation along the erythroid, granulocyte/macrophage and lymphoid pathways. Nestorowa opted to construct broad trajectories for the progression of HSC to lineage restricted progenitors with immature cells having the option of moving sideways. This sideways plasticity adds further to the debate about whether cells do just follow a single, preferred and shortest trajectory to an end cell type.

An interesting question is whether there is a hierarchy to the availability of options of HSC. As early as 1981, Nicola and Johnson argued that commitment to megakaryocyte and erythroid differentiation was an obligatory step during the development of HSC and that erythroid cells were invariably present in mixed colonies of bone marrow-derived cells plated in semi-solid medium.[36] Sanjuan-Pla and colleagues have argued that a platelet-biased stem cell resides at the apex of the haematopoietic hierarchy[13] and in particular that megakaryocytes and HSC share key transcription factors.[37] The myeloid-based model of haematopoiesis proposed by Kawamoto and Katsura postulates that myeloid cells are prototypic and the myeloid activities are modified or switched-off to give rise to the more specialised B and T lymphocytes and erythroid cells.[38] That ontogeny might recapitulate phylogeny supports this viewpoint whereby the hemostatic function of platelets may be part

of the angiogenesis program in development and macrophages evolved as a primordial mechanism for the elimination of apoptotic cells generated as part of development. This function of macrophages would precede their phagocytic function as part of natural immunity. Specific immunity associated with lymphocytes clearly appeared later in ontogeny and phylogeny than the phagocytic capacity of macrophages. However, evolution might merely have added to the spectrum of options open to HSC as opposed to instilling a genomic hierarchy.

To add to the debate about trajectories, some investigators have argued that the ability of HSC/HPC to choose a particular cell fate varies during the cell cycle.[39] In this case, noise relating to random variations in the levels of transcription factors has been postulated to control lineage choice. Additionally, cell fate decisions in model organisms such as Drosophila and *C. elegans* are controlled by asymmetric cell divisions; and these may also play a role in T cell fate and haematopoiesis.[40] In a more general sense, cells are inherently dynamic, as double negative (DN) 1 and DN2 cells that have developed some way towards becoming mature T cells can still give rise to natural killer and myeloid cells.[22,41]

The role of the epigenome

One piece of evidence that a continuum of options is open to HSC is that just after cell division the chromatin of human $CD34^+$ HPC is completely devoid of the repressive histone mark H3K27me3. In terms of the timing of the recruitment of the transcription factors that play a key role in regulating myeloid (C/EBPα, PU.1) *versus* erythroid (GATA-1) differentiation[42] [Figure 4] this aspect of chromatin structure is important for lineage specification. Arinobu and colleagues have argued that activation of PU.1 and GATA-1 within HSC specifies myeloid/lymphoid *versus* myeloid/erythroid fates, respectively.[43] The use of PU.1-GFP mice has shown that M-CSF stimulates expression of PU.1 in some LT-

HSC thereby eliciting myeloid lineage specification.[16] As considered later, G-CSF/M-CSF and Epo are reported to be instructive to the myeloid and erythroid pathways, respectively ([14,16,44] and see below). Upon treatment of $CD34^+$ HPC with G-CSF/M-CSF and Epo, the appropriate lineage-affiliated transcription factors (see above) are recruited to DNA just after DNA replication. Cytokine-driven differentiation was suppressed by increasing H3K27me3 levels or blocking DNA replication.[42] A caveat to the above is that by demonstrating that random PU.1 to GATA-1 protein ratios do not initiate lineage choice, Hoppe and colleagues have argued that PU.1 and GATA-1 merely reinforce a choice that has already been made.[23] Even so, there is a "pecking-order" to events, and the accessibility of appropriate transcription factors to DNA is clearly a pre-requisite of whether transcription factors instruct or reinforce lineage choices (Figure 4).

The findings of Roy and Sridharan[45] support the importance of chromatin marks. They examined the epigenome to model the relationship between haematopoietic cell types and chromatin-level decision points for diversification. They captured the dynamics of the state of chromatin by using a clustering approach, identifying chromatin modules as a set of gene loci with the same chromatin activating and repressive histone modifications.[45] Four chromatin marks, including enhancer-enriched (H3K27ac, H3K4me1 and H3K4me2) and promotor-enriched (H3K4me3) marks, were examined for 15 types of haematopoietic cells. Closely related cell types had modules that were more similar than those for distantly related cell types. For example, each of the following groups of cell types - immature erythroid cells/mature erythroid cells, GMP/macrophages/monocytes and CD4/CD8 T lymphocytes - have marks that are more similar to one another that to any other cell types. For the future, more details of the dynamic interaction between chromatin marks may well reveal that the erasure of repressive marks and addition of activating marks leads either to the expression of

genes encoding transcription factors or to recruitment of these factors to DNA which, in turn, affect lineage specification.

Interest in the importance of the epigenome for lineage specification extends to microRNAs (miRNAs). There are many new classes of noncoding RNAs, including both enhancer and antisense, which are important for the regulation of haematopoiesis and disease. A single type of miRNA can interact simultaneously with many targets thereby influencing the transcriptional heterogeneity of cells and alterating various signalling pathways and cell functions. MiRNAs are therefore viewed as possible key triggers of cell fate. In keeping with miRNAs orchestrating haematopoiesis is that comprehensive profiling of miRNAs with regard to developmental hierarchy has been used to infer the relationships between cell lineages and the functional similarities of cells.[46] Additionally, long non-protein-coding RNAs have been shown to play a role in cell plasticity in the trans-differentiation of osteoblasts to adipocytes (reviewed in [47]).

Do haematopoietic cytokines instruct cell lineage choice?

The first evidence for a humoral factor that influences haematopoiesis, one that promotes erythropoiesis, was published more than 60 years ago. Since then, a plethora of haematopoietic cytokines has been characterized. Other extra-cellular factors, such as cell-adhesion or extra-cellular matrix molecules, might also influence haematopoietic lineage decisions. Here we will focus on cytokines as prototypic examples of environmental signals that regulate the lineage outcomes of HSC/HPC. Haematopoietic cytokines have been shown both to regulate the survival and proliferation of HSC/HPC, and to influence their lineage choices. However, their roles as lineage-determining factors remain controversial. When it comes to regulating fate choice at the single cell level, cytokines can be either instructive or permissive. An instructive cytokine acts on an un-committed and oligopotent HPC to induce

a signaling cascade and activate a genetic program that leads to the commitment of the cell to a particular haematopoietic lineage (Figure 5). In contrast, a permissive cytokine acts as a selection factor, promoting the survival and/or proliferation of some of its progeny. In this latter model, lineage commitment occurs in a cell-autonomous manner and independently of a particular cytokine. Proponents of the permissive model often refer to the commitment of progenitors as “stochastic”, a term that has caused some misunderstanding when used in developmental biology.

The discovery of low level and promiscuous expression of lineage-specific genes in early HPC (lineage priming)[48] led to the hypothesis that random initiation of a self-reinforcing lineage-specific genetic program leads to the commitment of HPC to that lineage and to the subsequent expression of cytokine receptors that then facilitate the permissive function of the corresponding cytokines (Figure 5, right panel). However, this initial lineage specification could be the result of other extra-cellular cues or epigenetic assymetries originating from the progenitor’s developmental history; factors that cannot be considered as truly random, but rather as deterministic. Therefore, when discussing the permissive model, we herein refer to the lineage commitment of HPC merely as independent of the cytokine in question.

Whether a cytokine is instructive or permissive for a lineage, its presence or absence will influence the production of lineage-committed HPC *in vivo* or *in vitro*, but its exact mode of action is unknown in most cases. Identifying the precise role of cytokines in haematopoietic cell fate decisions is clinically significant, as this might lead to interventions to increase haematopoietic cell production, but is technically challenging. This has led to apparently contradictory results and has fueled a long-standing and lively debate. Genetic deletion of cytokines or their receptors reduces the developmental output of haematopoietic lineages regulated by the corresponding cytokines, with the reductions ranging from modest to severe. However, deletion of a single cytokine or cytokine receptor never results in a complete

absence of a haematopoietic lineage. This has been considered as evidence for the permissive action of cytokines but could also be the result of compensatory mechanisms and of some level of redundancy *in vivo*. Over-expression of anti-apoptotic genes, such as *Bcl2*, has sometimes been sufficient to rescue the differentiation of HPC to the affected lineage, as seen with T cell development in the absence of IL-7.[49] These results identify a role for these cytokines as survival factors for the corresponding HPC and provide evidence for their permissive role. With regards to B cell development, *Bcl2* over-expression is sufficient to rescue B cell generation in mice deficient in Stat5 expression (a crucial signaling mediator of IL-7 action),[50] while *in vivo* over-expression of the cytokine Flt3L renders IL-7 signaling dispensable for commitment of $Ly6D^+$ CLP and EPLM progenitors to the B cell fate.[9] These studies have seriously challenged the hypothesis that IL-7 is the extracellular signal responsible for Ebf1/Pax5 transcription factor up-regulation and subsequent commitment of HPC to the B cell lineage, and suggest an exclusively permissive role for IL-7 in lymphoid development, but *in vivo* compensatory mechanisms cannot be excluded.

Further transgenic approaches - by ectopically expressing cytokine receptors or through the generation of chimeric receptors - have attempted to investigate the role of haematopoietic cytokines in lineage decision of HPCs. For example, enforced expression of Epo-R in multi-potent HPC did not result in increased erythroid production.[51] And mice carrying a chimeric receptor comprised of the extra-cellular part of thrombopoietin receptor (mpl) and the intra-cellular part of G-CSF receptor had normal numbers of both platelets and granulocytes, arguing against an instructive role of both cytokines.[52] Similarly, replacement of the signalling domain of the G-CSF receptor with the corresponding domain of the Epo-R did not cause lineage skewing.[53] Such experimental approaches have though provided some evidence for cytokine-mediated lineage instruction. Ectopic expression of M-CSF receptor in multi-potent haematopoietic cell lines significantly changed their lineage bias.[54] And, ectopic

expression of the GM-CSF receptor in primary CLP and pro-T cells significantly increased their myeloid differentiation potential, although this was not the case for pro-B and MEP progenitors.[55,56] Similarly, Flt3 over-expression in MEP led to up-regulation of the myeloid-affiliated transcription factors Stat3 and PU.1 and differentiation of the cells towards granulocyte/macrophage lineages.[57] Again, ubiquitous *in vivo* Flt3-ligand expression developmentally skewed LSK progenitors towards myeloid-lymphoid lineages and suppressed the generation of erythroid and megakaryocyte progenitors,[58] indicating the possible instructive role of Flt3 signalling in early stages of haematopoiesis. Similarly, an instructive role of Epo has been shown by increasing the level of this cytokine *in vivo*.[14] These studies have illustrated that not only knock-out, but also over-expression of cytokines *in vivo* can provide valuable insight into their function as differentiation factors in haematopoiesis.[17,58] The inconclusive, and in some cases conflicting results discussed, highlight the cell-context dependency of the response of progenitors to cytokine signalling, as well as the importance of investigating the role of cytokines at the single cell level in order to avoid misinterpretation of results due to the heterogeneity of progenitor populations.

By separating daughter cells of bi-potent granulocyte-macrophage colony forming cells (GM-CFC) after one division and assessing their developmental output after stimulation with G- or GM-CSF, Metcalf and Burgess in 1982 provided evidence for an instructive role of these two cytokines in the generation of granulocytes and macrophages, respectively.[59] Since then, significant improvements in imaging technology and software have allowed the continuous *in vitro* tracing of cells and their progeny, enabling Rieger and colleagues to confirm these findings by unequivocally demonstrating the instructive role of G- and M-CSF cytokines on GMP progenitors.[44] Even though these were *in vitro* experiments and focused exclusively on a bi-potent progenitor stage, they have provided proof that cytokines can instruct lineage choice and further highlight the importance of single-cell technology in shedding new light

on old questions. Analyses at the single-cell level have also demonstrated M-CSF action on HSC can induce PU.1 and instruct their myeloid differentiation, rather than promoting survival or proliferation of daughter cells.[16] Similarly, Epo can induce the initiation of a genetic program, including GATA1 expression, that commits multipotent HPC to the erythroid fate.[14]

Identifying the instructive or permissive function of cytokines in haematopoietic lineage commitment is technically challenging and still remains less than conclusive for most of the cytokines and lineages studied. For a long time this debate has been of an "either/or" nature. However, experimental results indicate that the effect of cytokines on haematopoietic progenitors can be highly cell-context dependent. Flt3-ligand, for example, can act instructively on early progenitors, such as MEP or LSK,[57,58] but permissively on downstream progenitors, such as CLP and EPLM, at least as regards their commitment to the B cell lineage.[9] Such versatility in the activity of haematopoietic cytokines can be attributed to the particular genetic, epigenetic and signalling landscape of the progenitor cell on which the cytokine acts. This may partly explain some conflicting data on the instructive action of cytokines and it emphasizes the need to focus on how cytokine signals are integrated to the already existing transcription factor networks in haematopoietic progenitors.

Furthermore, a particular cytokine might act in both instructive and permissive manners on the same HPC type, depending on its signal strength. It should be noted that most of the experimental results mentioned above showing a clear instructive function of cytokines are derived from *in vitro* experiments where the amount of cytokines used might have been above the level which an HPC experiences *in vivo* and in steady-state conditions. As previously, there is evidence that steady-state haematopoiesis differs significantly from haematopoiesis under stress conditions (e.g. irradiation, inflammation). Cytokine levels can increase significantly under stress conditions, not only locally but also systemically, which

may expose HSC/HPC to stronger cytokine signals than they normally encounter *in vivo*. Strong signals could in turn affect the differentiation rate and/or lineage output of HPCs in a manner different from that occurring under normal conditions.

Hence, it could be envisaged that during steady-state haematopoiesis, cytokines facilitate the continuous production of blood cells in normal ratios, mainly through their action as permissive factors. However, when the haematopoietic system needs to respond to an emergency, such as injury, bleeding, inflammation or infection, situations that require a prompt increase in the production of particular lineage(s), increased cytokine concentrations might trigger instructive actions of cytokines on the relevant HPC, inducing their differentiation towards the required lineage(s). This ability to alternate between instructive and permissive cytokine action might be one of the mechanisms that contribute to the immense adaptability and dynamic nature of haematopoiesis. This might help to explain some of the apparent discrepancies in the experimental data and their interpretations in which disruptions of cytokine signalling point mainly to a permissive function, whereas over-expression of cytokines or cytokine receptors and *in vitro* cytokine treatments often suggest instructive roles.

An interesting question arising from this interpretation is whether all haematopoietic lineages would require such an “emergency” instruction by cytokines. Due to the clonal nature of the adaptive immune response, only a very small proportion of lymphoid cells participate in the response to a particular immunological challenge. Therefore, cytokine-mediated instruction of bone marrow HSC/HPC to increase B cell output upon infection would not be advantageous (as is the case for erythrocytes, platelets and cells of the innate immune system in other stress conditions), since the vast majority of produced B cells would have an antigenic specificity irrelevant to the particular pathogen. A steady production of naïve B and T cells (which mostly occurs quite early in life) is sufficient to generate a pool of naïve

lymphocytes able to respond to pathogens through clonal activation and subsequent immunologic memory. Thus, the hypothesis of stress-triggered instruction of haematopoiesis by cytokines would rather seem to apply to cells of the erythroid, megakaryocyte and myeloid lineages, and not to lymphocyte progenitors.

Implications to the nature and origin of leukaemia

A precise understanding of the events that establish and stabilise a cell's differentiated status is essential to resolving how these become aberrantly wired to set a malignant phenotype. Often and to variable extents, the majority of leukaemia cells are undifferentiated. However, it has been clear for many years that the bulk of cells of each leukaemia belong to just one cell lineage, and leukaemias are categorised accordingly. Similarly, molecular and genetic analyses have shown associations between a certain genetic lesion and a particular sub-type of leukaemia, and of other cancers (reviewed in [60]). Hence, there is an intimate association between a genotypic lesion and phenotype, and, as above, the cell lineage nature of the leukaemia cells. The cell targeted by an event(s) that initiates the leukaemia might well be a differentiated cell type, and this explains the association between a lesion and the leukaemia cells belonging to just one lineage. However, it was clear 40 years ago that chronic myeloid leukaemia, a disease that presents as an expansion of relatively mature myeloid cells, arises from transformation of an HSC that spawns an abundance only of myeloid cells. In short, the offspring of the transformed HSC are dumped down the neutrophil pathway. Other examples include common acute lymphoblastic leukaemia and acute promyelocytic leukaemia, in which the bulk of the leukaemia cells belong respectively to the B lymphocyte and neutrophil lineage and there is evidence of transformation of a HSC (reviewed in [7]). In keeping with this, the "cancer stem cell" theory has recently been discussed as the probable root cause of cancer. In this view, the genotypic alteration occurs in the stem cell compartment and the aberrant cancer stem cell produces abundant malignant cells of a specific phenotype.

As argued previously, a landscape exists for normal haematopoiesis whereby HSC are able to commit directly to a particular cell lineage. We might presume that these cells are equally competent to commit to any one of the array of pathway options, by virtue of having a high degree of transcriptional plasticity. Hence, HSC are versatile in their capacity to populate the mature cell compartments. By contrast, the leukaemia stem cell (LSC) appears to be biased towards or more competent to commit to just one lineage. Estimates of the proportion of LSC within a leukaemia, and CSC within other cancers, vary from very few up to 25%. However, the important point is that, whether few or a high percentage, they all give rise to one cell type, that is possibly related to their characteristic genetic lesion(s). A loss of versatility regarding the capacity of LSC to commit to any one of a number of lineage options might well be a cardinal feature of leukaemia, and other cancers (see Figure 6). Another possiblilty is that some of the progeny of a LSC can drive the leukaemia whereas others are non-tumorigenic and cannot: a clone that is biased or committed directly to one pathway is generating the bulk of the leukaemia cells. The epigenome is of interest regarding both possibilities as, in part, cancers are epigenetically-driven, whereby dysregulation of histone modifications, DNA methylation and miRNAs act in concert with genetic abnormalities.

Concluding remarks

New findings support a movement away from describing the haematopoietic system as a strictly hierarchical tree lineage with intermediate and oligo-potent progenitors for each mature cell type. A continuum of lineage options appears to be directly available to HSC. Whilst HSC can affiliate to a lineage pathway immediately, their progeny remain versatile. HPC are able to step sideways developmentally, to follow a route to a different end cell. Perhaps we have come full circle to Waddington's postulated viewpoint of an epigenetic landscape and, as considered above, he may well be right (Figure 4). Much water must pass under the bridge before a consensus can be reached as to how to best represent

haematopoiesis; use of single cell technologies will be essential to provide an accurate map. But, the techniques that are generally used to interrogate the lineage options of a single cell themselves destroy the cell and, therefore, can only provide information about a cell at a single moment in time. It is important that this is borne in mind when unravelling trajectories. Also, a complex network of events including gene regulation, by more than 50 transcription factors, over 2000 cis-regulatory enhancers and cellular signalling are clearly important for the establishment and stabilisation of a cell phenotype. As a result of rapid advances in global profiling of cellular events combined with bioinformatics, understanding how this dynamic is altered to give rise to diverse cell types now seems more accessible. A bifurcating lineage tree model has for years led us to examine how a developing cell is committed to either one pathway or another. This has dramatically influenced our thinking, particularly as regards the lymphoid versus myeloid lineage choice. By contrast, lineage commitment might now be best viewed as one of understanding how HSC reduce their effective potentiality. Presumably, pleiotropic cues elicit cascade responses that increasingly drive cells to acquire the functional and morphological features of a cell type. In this case, and as cells can step sideways, differentiation might proceed determination of a stable fate.

CONFLICT OF INTEREST

There is no conflict of interest to disclose.

ACKNOWLEDGEMENTS

This project received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 315902. GB and RhC were Partners within the Marie Curie Initial Training Network DECIDE (Decision-making within cells and differentiation entity therapies). We dedicate this article to our friend, colleague and mentor, Prof. Antonius G Rolink whose incisive

comments greatly influenced our way of viewing hematopoiesis. We thank Prof. Bob Michell for his comments on the manuscript.

FIGURE LEGENDS

Figure 1 A continuum or pair-wise model for haematopoiesis

In 2009, we proposed that a continuum of fates is available to HSC.[2] The model also envisages simple pair wise relationships between cell fates. We inferred close relationships between cell lineages from the different groups of fates that are available to a number of known intermediary progenitor cells, represented by a partial arc for each progenitor. The model is in keeping with decision-making at the level of the HSC. In this case and unlike the 'classic model' the partial arcs would therefore represent options that are clandestine or latent such that cells that have made a lineage choice still have the capacity to move sideways to adopt an alternative, though closely related, fate. The figure is, with permission, from [2] © Macmillan Magazines Ltd. HSC, haematopoietic stem cell; LMPP, lymphoid-primed multipotent progenitor; EPLM, early progenitor with lymphoid and myeloid potential; DC, dendritic cell; NK cell, natural killer cell.

Figure 2 Markers used to describe haematopoietic stem and progenitor cells

Expression of CD135 (Flt3) is used to subdivide the Lineage markers$^-$, Sca-1$^+$, c-Kit$^+$ (LSK) population of bone marrow cells into LT-HSC (Flt3$^-$) and ST-HSC/MPP (Flt3$^+$). Use of CD34 and Flt3 expression subdivides LSK cells into LT-HSC (CD34$^-$ Flt3$^-$), ST-HSC (CD34$^+$ Flt3$^-$) and MPP (CD34$^+$ Flt3$^+$). HPC1 and HPC2 express the signalling lymphocyte activation molecules CD48 and CD150 as above. HSC, haematopoietic stem cell; LT, long term reconstituting HSC; ST, short term reconstituting HSC; HPC, haematopoietic progenitor cell; LMPP, lymphoid-primed multipotent progenitor; EPLM, early progenitor with lymphoid and myeloid potential; CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor; MEP, megakaryocyte and erythrocyte progenitor; CLP, common lymphoid progenitor. The following lineage potentials are shown: Meg, megakaryocyte; Ery,

erythrocyte; Eos, eosinophil; Bas, basophil; Neut, neutrophil; Mono, monocyte; DC, dendritic cell; NK cell, natural killer cell.

Figure 3 Haematopoietic stem and various progenitors are a heterogeneous population of cells

The known sets of fates of lymphoid-primed multipotent progenitors (LMPP), early progenitors with lymphoid and myeloid potential (EPLM), common lymphoid progenitors (CLP), common myeloid progenitors (CMP) and other downstream progenitors, shown by the partial arcs, were used to construct the relationships between cell lineages in the pair wise model (see Figure 1). Cells that are marked with a red asterisk were each thought to be a homogeneous population of cells. They are now known to be a mixture of cells with the lineage affiliations B lymphocyte (B), dendritic cell (DC), monocyte (Mo), eosinophil (Eo), basophil (Ba), erythroid (E) and megakaryocyte (M), as shown by cells added to the arcs for LMPP,[21] CMP[18,23,24] and CLP/EPLM.[8] The lineage affiliations seen regarding LMPP, EPLM and CMP have occurred even earlier in development, as early as at the level of the haematopoietic stem cell (HSC) as these cells are a heterogenous population of cells. There are HSCs that are lymphoid biased (Ly) and expressing the fms-like tyrosine kinase 3 receptor ($Flt3^+$)[15], myeloid biased or committed (My)[10] and expressing Flt3[15] and/or the receptor for thrombopoietin ($TpoR^+$)[13], committed to the erythroid pathway (E)[18] and affiliated as to expression of the receptor for erythropoietin ($EpoR^+$)[14,15] and expressing the receptor for macrophage colony stimulating factor ($M\text{-}CSFR^+$) and monocyte-affiliated (Mo)[15,16]. These lineage HSC biases or affiliations that are seen also for various downstream progenitors, from their heterogenous nature, are commensurate with HSCs not having to progress to an end-cell type via a series of intermediate and oligopotent progenitor states. Decision-making at the level of the HSC is very different from these cells and their progeny making a series of binary choices. A possibility is HSCs that are affiliated to/biased towards a

particular lineage narrow fate trajectories in a progressive and centrifugal manner regarding adjacent options. DC/Pro-B, dendritic cell and B lymphocyte progenitor; Eo/B-CFU, eosinophil and basophil progenitor; GMP, granulocyte and macrophage progenitor; MEP, megakaryocyte and erythrocyte progenitor; Mon/B/DC?, monocyte, B lymphocyte and dendritic cell? progenitor; Mon/DC, monocyte and dendritic cell progenitor, NK/T, natural killer cell and T lymphocyte progenitor and NK/ILC, natural killer cell and inate lymphoid cell progenitor.

Figure 4 Schema of some of the controls on myeloid specification

According to the cell's developmental stage, myeloid-specific clustering of the locus control region (blue circles) leads to clustering of the binding sites for transcription factors (TF) to allow efficient transcription of a gene(s) (shown by the arrow) that is/are associated with myeloid specification/development. Macrophage colony-stimulating factor (M-CSF) binding to its receptor (M-CSFR) stimulates expression of the master myeloid regulator PU.1 in some LT-HSC to effect myeloid gene expression.[16] Chromatin that is globally devoid of the repressive histone marks (red triangle), such as H3K27me3, is essential to the recruitment of PU.1 as increasing H3K27me3 levels block M-CSF-induced differentiation.[42] Having permissive histone marks (green triangle) is also important.

Figure 5 Schematic description of the instructive and permissive models for cytokine function in haematopoietic lineage commitment.

The upper part shows an un-committed haematopoietic progenitor (in grey) which has the potential to differentiate towards three different lineages (in colours). In the instructive model (lower left), the cytokine in question "instructs" the progenitor to differentiate exclusively towards one cell fate by inducing the initiation of the corresponding lineage-specific genetic program. In the permissive model (lower right), the progenitor retains its potential to generate

all three downstream lineages and does so, either stochastically or under the influence of other extra-cellular cues. The cytokine acts as a selection factor, promoting the survival and/or proliferation of one lineage at the expense of the other lineages, which are nevertheless still generated from the progenitor.

Figure 6. Schematic representation of the versatility of normal and leukaemia stem cells

In **A**, a spectrum of options is available to normal HSC and they can commit directly to any one of the lineage options. In **B**, a leukaemia stem cell, by virtue of a genetic insult, is shown to be restricted as to versatility and only able to commit directly, or is biased towards, a single or limited range of options, as shown by the green section of the arc. HSC, haematopoetic stem cell; LSC leukaemia stem cell; T cell, T lymphocyte; NK cell, natural killer cell; B cell, B lymphocyte, DC, dendritic cell, Mon, monocyte; Neut, neutrophil; Eos, eosinophil, Bas/MC, basophil/mast cell; Ery, erythroid and Pl, platelets

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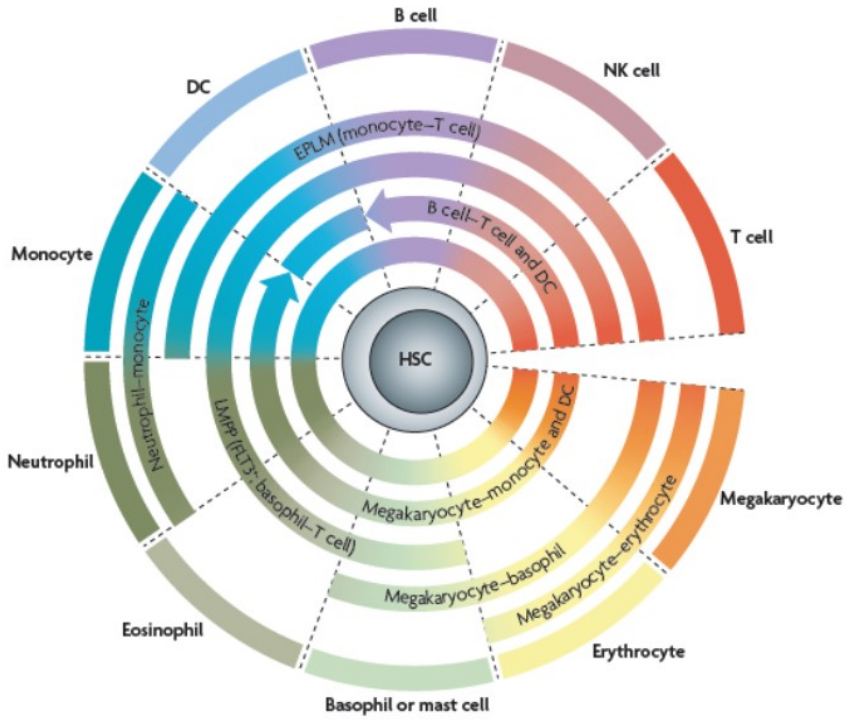
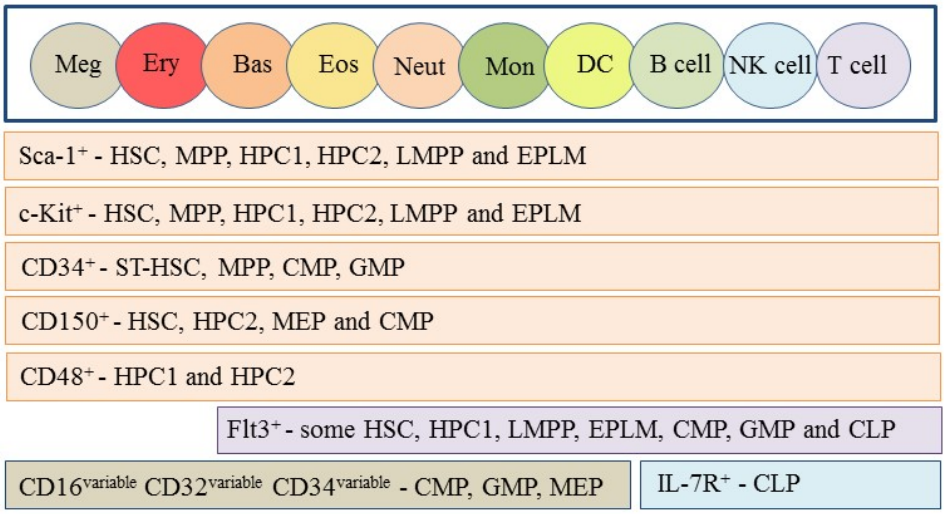

B cell
NK cell
DC
EPLM (monocyte–T cell)
B cell–T cell and DC
T cell
Monocyte
HSC
Neutrophil–monocyte
LMPP (FLT3; basophil–T cell)
Megakaryocyte–monocyte and DC
Neutrophil
Megakaryocyte
Megakaryocyte–basophil
Megakaryocyte–erythrocyte
Eosinophil
Erythrocyte
Basophil or mast cell

Figure 1


Meg
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c-Kit+ - HSC, MPP, HPC1, HPC2, LMPP and EPLM
CD34+ - ST-HSC, MPP, CMP, GMP
CD150+ - HSC, HPC2, MEP and CMP
CD48+ - HPC1 and HPC2
Flt3+ - some HSC, HPC1, LMPP, EPLM, CMP, GMP and CLP
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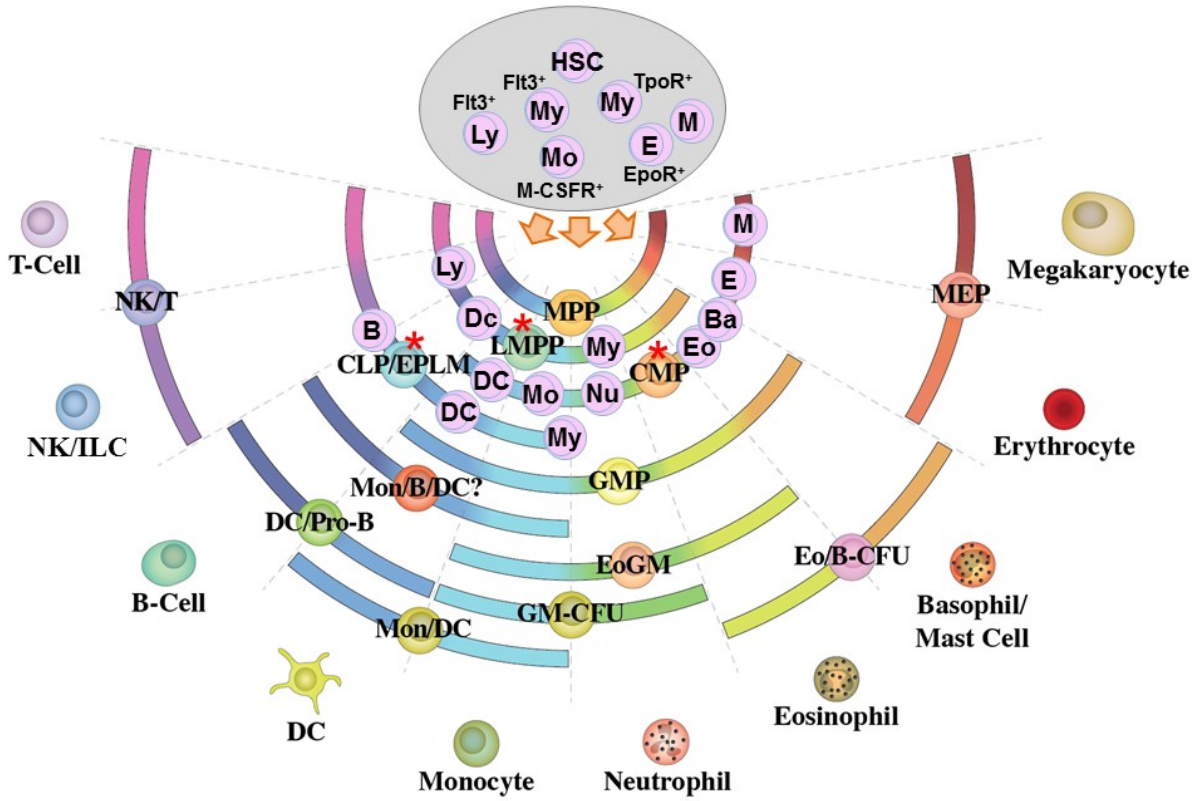
IL-7R+ - CLP

Figure 2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NK/ILC
B-Cell
DC
Monocyte
Neutrophil
Eosinophil
Basophil/
Mast Cell
Erythrocyte
Megakaryocyte
MEP
Eo/B-CFU
Ly
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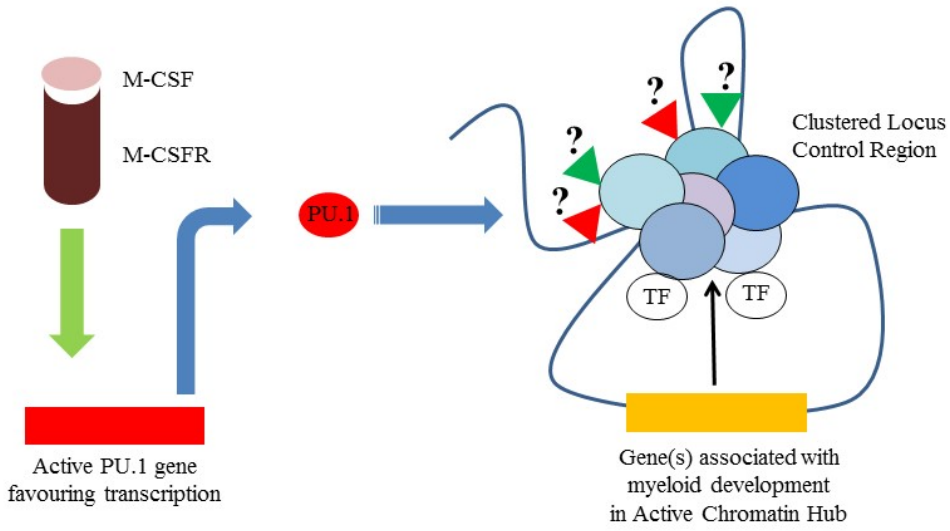
Figure 3


M-CSF
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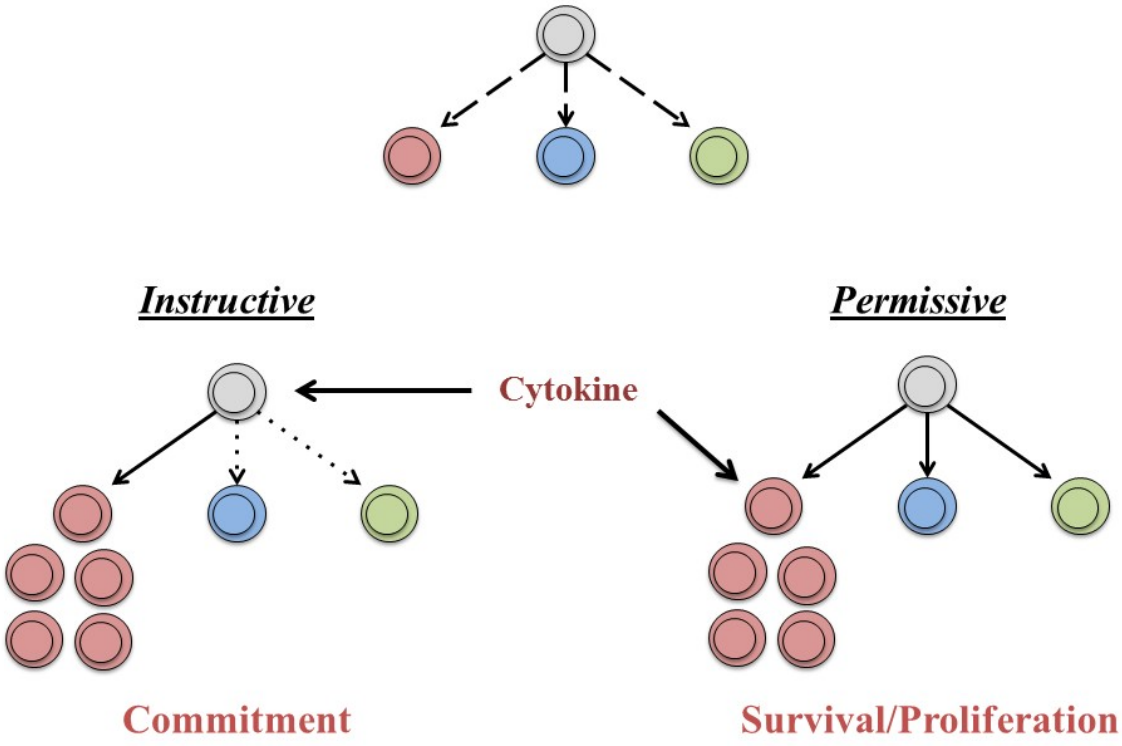
in Active Chromatin Hub

Figure 4


Cytokines in Haematopoiesis
Instructive
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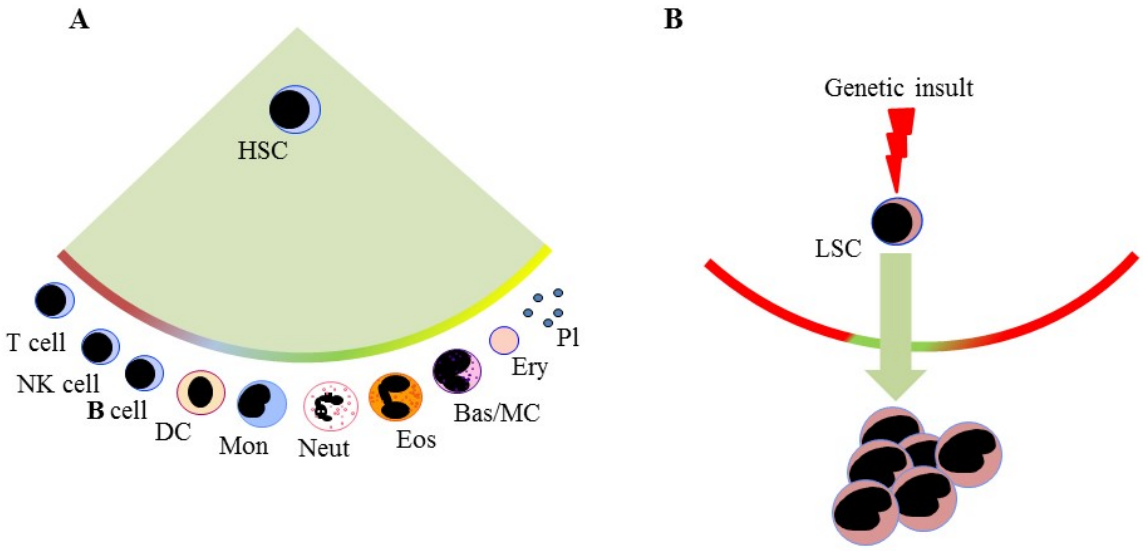
Survival/Proliferation

Figure 5


A
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Neut
Eos
Bas/MC
Ery
Pl
B
Genetic insult
LSC

Figure 6