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Supporting Online Material

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Materials and Methods
Figs. S1 to S12
References

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Hematopoietic Cytokines Can Instruct Lineage Choice

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The constant regeneration of the blood system during hematopoiesis requires tightly controlled lineage decisions of hematopoietic progenitor cells (HPCs). Because of technical limitations, differentiation of individual HPCs could not previously be analyzed continuously. It was therefore disputed whether cell-extrinsic cytokines can instruct HPC lineage choice or only allow survival of cells that are already lineage-restricted. Here, we used bioimaging approaches that allow the continuous long-term observation of individual differentiating mouse HPCs. We demonstrate that the physiological cytokines, macrophage colony-stimulating factor and granulocyte colony-stimulating factor, can instruct hematopoietic lineage choice.

All blood cells are generated from progenitor cells with more than one lineage potential (hematopoietic progenitor cells, HPCs). Hematopoiesis depends on tightly controlled lineage choice. Cytokines are necessary and sufficient for the production of specific mature blood cell types (*1*). However, despite decades of research, it is disputed whether cytokines instruct HPCs to differentiate into a specific lineage (*2*). Alternatively, cytokines may simply allow the survival or proliferation of cells that had already independently decided for this lineage. The cytokines' function would then only be to select the right cell types from a pool of already lineage-restricted cells (*3*). Cell-intrinsic transcription factors (*4*–*7*) and activation of ectopically expressed cytokine receptors (*8*–*10*) were shown to instruct lineage decisions. However, because of technical limitations, the instructive action of cytokines acting physiologically on unmanipulated HPCs could not be demonstrated (*1*, *11*). As illustrated in fig. S1 (*12*), the discontinuous analysis of HPCs does not allow conclusive answers (*13*): In order to exclude the selective model, the exact kinship, lineage commitment, and cell death of all individual cells in HPC colonies must be identified (*14*, *15*). Prior analyses had not continuously followed all individual cells in constantly mixing HPC cultures long enough and with sufficient resolution. The selective model is currently favored in the literature (*11*, *16*).

Using bioimaging approaches that allowed continuous long-term observation at the single-cell level (*17*) (fig. S2 and movies S1 to S4), we analyzed cultures of murine granulocyte-macrophage progenitors (GMPs) (*18*) (fig. S3) in the presence of only macrophage- or granulocyte colony-stimulating factor (M- or G-CSF). Single-cell tracking showed that GMPs functionally respond to both cytokines with high cloning efficiency (figs. S4 and S5). Culture in only M- or G-CSF leads almost exclusively to mature monocytic (M) or neutrophil granulocytic (G) cells, respectively (fig. S6). We utilized LysM::GFP mice (*19*), expressing enhanced green fluorescent protein (GFP) from the *lysozymeM* gene locus as an early molecular reporter for unilineage commitment. Whereas only extremely weak LysozymeM::GFP expression (LysM::GFP$^-$) is found in undifferentiated GMPs, LysM::GFP is drastically up-regulated (LysM::GFP$^+$) upon differentiation (*19*–*21*) (figs. S2B and S7 to S9 and movie S5). LysM::GFP$^+$ cells have lost their colony-forming potential (Fig. 1, A and B, and fig. S9) and are unilineage-restricted to either the M or G lineage (Fig. 1, A and C).

This approach allows detection of cell death and unilineage commitment of all cells in GMP cultures. We continuously observed hundreds of GMPs and all of their progeny throughout development into only M- or G-committed cells in the presence of only M- or G-CSF (375 pedigrees for M-CSF, 318 for G-CSF) (figs. S10 and S11). Colonies without cell death can be explained in two ways: (i) the colony-initiating cell was a bipotent GMP, and with the absence of selective cell deaths, it must have differentiated exclusively into the lineage supported by the present cytokine, or (ii) the colony-initiating cell was already unilineage-restricted to this lineage (Fig. 2A). We determined that the original GMP population contained a maximum of 23 ± 6% and 53 ± 7% potentially unilineage-restricted M and G cells, respectively (fig. S3).

Quantifying the frequency of GMP pedigrees without cell death in single-cytokine conditions allowed us to identify the lineage-instructive effect of M- and G-CSF. In 88 ± 2% (M-CSF) and

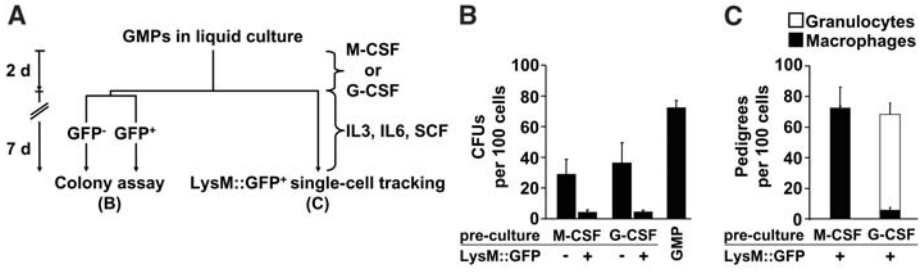

Fig. 1. LysM::GFP is a marker for unilineage-restricted G or M cells. (**A**) Experimental procedure. (**B** and **C**) LysM::GFP$^+$ cells derived from GMPs cultured with only M- or G-CSF for 48 hours have lost colony-forming potential (B) and are unilineage-restricted (C). Means ± SD of 50 pedigrees per condition and experiment (n = 3). CFU, colony-forming unit; IL, interleukin; SCF, stem cell factor.

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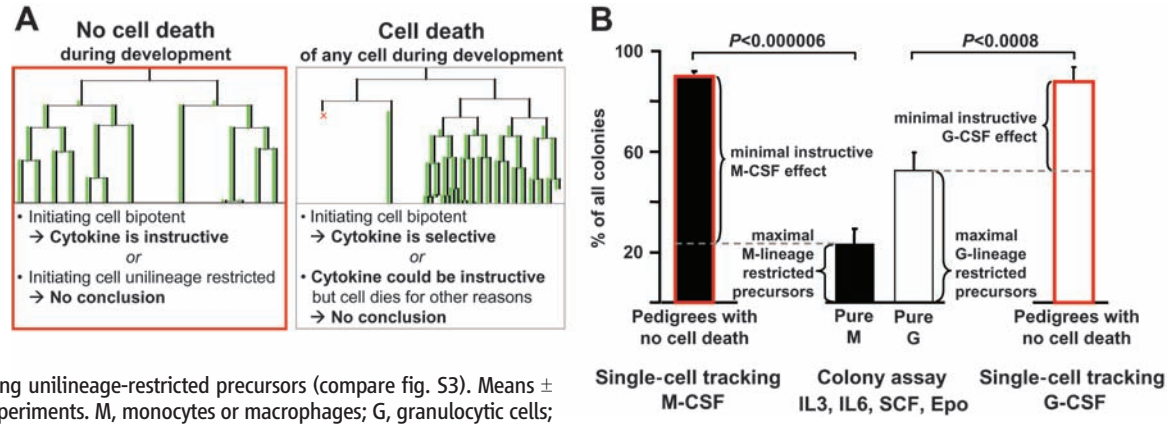

Fig. 2. M-CSF and G-CSF instruct lineage choice in GMPs. (**A**) Pedigrees were grouped according to the existence of cell death events. Green lines illustrate LysM::GFP expression. (**B**) The frequency of colonies without cell death until development into only M cells (black bars) or G cells (white bars) cannot be explained by contaminating unilineage-restricted precursors (compare fig. S3). Means ± SD, three independent experiments. M, monocytes or macrophages; G, granulocytic cells; Epo, erythropoietin.

87 ± 6% (G-CSF) of pedigrees leading exclusively to M and G cells, respectively, no cell death occurred (Fig. 2B). These percentages far exceed those of colonies that could have been generated from unilineage-restricted precursors potentially contaminating the GMP population that we used ($P < 0.000006$ and $P < 0.0008$ for M- and G-CSF, respectively). M- and G-CSF therefore instructed at least 65% and 34% of bipotent GMPs to differentiate into the M and G lineage, respectively (Fig. 2, A and B). Moreover, depending on the cytokines present, around 90% of the identical GMP population differentiates exclusively into different lineages, which demonstrates that these GMPs are bipotent. Thus, our assumption of maximally 23% (M) and 53% (G) contaminating unilineage-restricted progenitors in the starting GMP population is too conservative, and both cytokines instruct most GMPs into alternative lineages. In addition, we show that M- and G-CSF not only instruct lineage choice of differentiating cells, but also induce or accelerate differentiation: The onset of LysM::GFP expression in GMP progeny was accelerated by both M-CSF ($P < 0.00000026$) and G-CSF ($P < 0.000012$) (fig. S12).

Previous studies had postulated an exclusive selective effect of cytokines (*22*–*28*). In those studies, however, cytokine receptors were ectopically expressed in mutated progenitor cell lines or inappropriate cell types with molecular composition different from that of primary progenitors physiologically responding to those cytokines (*24*, *26*). Studies with mice either lacking cytokine receptors (*22*, *25*) or expressing chimeric cytokine receptors (*23*, *27*, *28*) demonstrated the interchangeability of cytokine receptor–derived signals for the generation (survival, proliferation, or lineage choice) of specific lineages. However, compensatory effects of other cytokines specifically for lineage choice could not be excluded. Our study shows that M- and G-CSF can instruct the lineage choice of genetically unmanipulated GMPs that physiologically respond to these cytokines (fig. S1). This demonstrates that signal transduction pathways of cell-extrinsic cytokines can influence the intracellular lineage commitment machinery (*29*). The technology described here for cell fate analysis will be useful for identifying these signaling pathways and for analyzing complex cellular systems in which a few individual cells (such as stem cells) control tissue development, homeostasis, and repair.

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Materials and Methods
Figs. S1 to S12
References
Movies S1 to S5

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