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M-CSF instructs myeloid lineage fate in single haematopoietic stem cells

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Abstract

Under stress conditions such as infection or inflammation the body rapidly needs to generate new blood cells that are adapted to the challenge. Haematopoietic cytokines are known to increase output of specific mature cells by affecting survival, expansion and differentiation of lineage-committed progenitors^{1,2}, but it has been debated whether long-term haematopoietic stem cells (HSCs) are susceptible to direct lineage-specifying effects of cytokines. Although genetic changes in transcription factor balance can sensitize HSCs to cytokine instruction³, the initiation of HSC commitment is generally thought to be triggered by stochastic fluctuation in cell-intrinsic

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regulators such as lineage-specific transcription factors⁴⁻⁷, leaving cytokines to ensure survival and proliferation of the progeny cells^{8,9}. Here we show that macrophage colony-stimulating factor (M-CSF, also called CSF1), a myeloid cytokine released during infection and inflammation, can directly induce the myeloid master regulator PU.1 and instruct myeloid cell-fate change in mouse HSCs, independently of selective survival or proliferation. Video imaging and single-cell gene expression analysis revealed that stimulation of highly purified HSCs with M-CSF in culture resulted in activation of the *PU.1* promoter and an increased number of PU.1⁺ cells with myeloid gene signature and differentiation potential. *In vivo*, high systemic levels of M-CSF directly stimulated M-CSF-receptor-dependent activation of endogenous PU.1 protein in single HSCs and induced a PU.1-dependent myeloid differentiation preference. Our data demonstrate that lineage-specific cytokines can act directly on HSCs *in vitro* and *in vivo* to instruct a change of cell identity. This fundamentally changes the current view of how HSCs respond to environmental challenge and implicates stress-induced cytokines as direct instructors of HSC fate.

Lineage-specific cytokines such as M-CSF can be strongly induced during physiological stress or infection^{10,11} and potentially increase the production of mature cells from lineage-committed progenitors^{1,2}. According to the prevailing model, however, they are generally not believed to influence differentiation decisions of HSCs directly^{9,12,13}. Cell fate choice of HSCs has traditionally been explained by stochastic models¹⁴. In this view transcriptional noise¹⁵ and random variations in competing lineage-determining transcription factors lead to cross-antagonistic switches that initiate lineage choice⁴⁻⁷, whereas cytokines are thought to only act on the resulting progeny cells by stimulating their survival and proliferation^{8,9}. A key example of such a master regulator is the transcription factor PU.1 that induces myelomonocytic differentiation^{16,17}. It is generally unknown whether external signals could drive the initial activation of such intrinsic master regulators. Because HSCs deficient for the transcription factor MAFB are sensitized to PU.1 activation in response to M-CSF³, we have investigated whether high systemic M-CSF levels could induce PU.1 and instruct myelomonocytic fate in wild-type HSCs without previous modification of transcription factor balance.

We observed that lipopolysaccharide (LPS), a strong mimetic of bacterial infection stimulating high systemic levels of M-CSF¹¹ (Supplementary Fig. 1a), induced an upregulation of GFP in long-term HSCs (CD117⁺Sca⁺Lin⁻CD135⁻CD34⁻CD150⁺) of *PU.1-GFP* reporter mice¹⁸ (Supplementary Fig. 1b, c). Consistent with the expression of the M-CSF receptor (*M-CSFR*; also called *Csf1r*) in HSCs (Supplementary Fig. 2)^{3,19}, direct intravenous injection of recombinant M-CSF also induced significantly increased *PU.1* activation in HSCs after 16 h (Fig. 1a, b). The treatment caused no significant change in *M-CSFR* or *Maib* expression (Supplementary Fig. 3), arguing against selection of myeloid primed HSCs with high M-CSFR or low MAFB levels. M-CSF also induced no change in the proportion of CD150^{hi} HSCs, reported to have myeloid lineage bias²⁰, in GFP-positive or GFP-negative HSCs (Supplementary Fig. 4a-c) and activated *PU.1* to a similar extent in CD150^{hi} HSCs (Fig. 1c) as in total HSCs (Fig. 1a, b). Finally, cultured CD150^{hi} HSCs revealed no proliferation or survival advantage in the presence of M-CSF (Supplementary Fig. 5a). Together, these data argued against selective amplification or survival of a pre-existing HSC sub-population and indicated that M-CSF could newly induce PU.1 expression in HSCs.

As shown in Fig. 1d, the effect of M-CSF on stem cells was direct and specific, as fluorescence-activated cell sorting (FACS)-purified HSCs showed increased *PU.1* expression after 16 h in culture with M-CSF but not with GM-CSF or G-CSF, cytokines that may also be released during infection²¹. The observed changes in gene expression cannot be explained by M-CSF-dependent selection of *PU.1*-reporter positive (*PU.1*⁺) cells, as video

microscopy of cultured HSCs showed no proliferation or survival advantage in M-CSF and *PU.1* was induced before the onset of cell division (Supplementary Figs 5 and 6). Continuous observation of individual GFP-negative sorted HSCs from *PU.1-GFP* mice by video imaging confirmed that M-CSF could induce *PU.1* expression in previously *PU.1*-negative cells (Fig. 2a–c and Supplementary Videos 1–3). We recorded the fate of HSCs between 18 h and 42 h of culture, when both the induction of *PU.1* in previously negative cells and the division of *PU.1*⁺ cells could theoretically occur. At the end of the 24-h observation period, over twofold more *PU.1*⁺ cells had developed in M-CSF than under control conditions (Fig. 2d); backtracking the origin of these cells revealed that all *PU.1*⁺ cells were derived from previously *PU.1*[−] cells, but none from divisions of *PU.1*⁺ cells. Although the absence of *PU.1*⁺ cell division may be partially due to the phototoxic effects of GFP excitement^{22,23}, we could conclude that the observed increase in *PU.1*⁺ cells was entirely due to M-CSF-induced activation of the *PU.1* reporter. These commitment events of *PU.1* activation occurred 8 h earlier and at a higher rate over the whole observation period in the presence of M-CSF (Fig. 2e). Our results indicated that M-CSF could directly increase *PU.1* promoter activation in single, previously *PU.1*-negative HSCs.

To investigate further whether M-CSF-induced *PU.1* activation changed the cell identity of individual HSCs we analysed the messenger RNA expression profile of single cells by nanofluidic real-time PCR on Fluidigm dynamic arrays. Consistent with their stem-cell identity, almost all freshly isolated HSCs expressed stem- and progenitor-cell-associated genes and about half expressed either no (*Lin*[−]) or multiple lineage-specific genes (mix). The remainder showed mainly megakaryocytic, megakaryocytic-erythroid or myeloid lineage priming (Fig. 3a and Supplementary Fig. 7). Culture for 16 h without M-CSF led to an increased number of cells with a mixed lineage profile at the expense of megakaryocytic and *Lin*[−] profiles (Fig. 3b and Supplementary Fig. 8). By contrast, culture in the presence of M-CSF induced a strong increase of cells with a myeloid gene expression signature (Fig. 3c, Supplementary Fig. 9 and Supplementary Table 1). Consistent with the video microscopy results, the increase in myeloid gene expression was associated with a doubling of the number of *PU.1*⁺ cells (Fig. 3d). Interestingly, this increase was entirely due to *PU.1*⁺ cells with a myeloid signature that did not express genes from any other lineage. By contrast, the number of *PU.1*⁺ cells that also expressed non-myeloid genes remained approximately constant (Fig. 3d). Together, this indicated that M-CSF-induced *PU.1*⁺ cells had assumed a myeloid cell identity. To evaluate whether this change in gene expression reflected a functional myeloid lineage choice *in vivo*, we compared the differentiation potential of unstimulated *PU.1*[−] HSCs to *PU.1*[−] and *PU.1*⁺ HSCs after *in vivo* priming with M-CSF (Fig. 3e). Progenitor analysis in the spleen 2 weeks after transplantation of these populations revealed a higher ratio of granulocyte/macrophage progenitors (GMP) to megakaryocytic/erythroid progenitors (MEP) developing from *PU.1*⁺ HSCs than from *PU.1*[−] HSCs (Fig. 3f, g). We observed a similar increase in myeloid differentiation potential for *PU.1*⁺ cells derived from M-CSF-stimulated *PU.1*[−] HSCs in culture (Supplementary Fig. 10a–d). Together, these data showed that M-CSF-induced *PU.1* activation led to a myeloid cell fate change in single HSCs.

To investigate further whether M-CSF could also induce a cell fate change of individual HSCs *in vivo*, we transplanted carboxy fluorescein diacetate, succinimidyl ester (CFSE)-labelled HSCs into the spleen, a site of extra-medullary haematopoiesis with adapted stem-cell niches^{3,24}, and analysed the expression of endogenous *PU.1* protein by immunofluorescence in single HSCs after 24 h (Fig. 4a). Whereas the vast majority of HSCs were *PU.1*-negative immediately after transplantation, nearly all had activated *PU.1* after transfer into spleens of LPS-challenged hosts (Fig. 4b, c). This effect was principally dependent on M-CSF signalling, as a blocking antibody against the M-CSF receptor²⁵ strongly inhibited *PU.1* activation. Furthermore, direct injection of recombinant M-CSF

resulted in a similar strong induction of PU.1 in the transplanted HSCs (Fig. 4b, c). This effect appeared to be entirely cell autonomous, as M-CSF-receptor-deficient (*M-CSFR*^{-/-})²⁶ HSCs showed no higher activation of PU.1 in M-CSF-stimulated compared with control recipients (Fig. 4d, e). Similarly, small-molecule inhibitors of the M-CSFR or phosphatidylinositol-3-OH kinase (PI(3)K), ERK and SRC kinases, which signal downstream of the receptor²⁷, also prevented induction of *PU.1* (Fig. 4f), consistent with the stimulation of transcriptional activators of the *PU.1* gene by these pathways (Supplementary Discussion). Furthermore, transplantation of *in vivo* M-CSF-primed CD45.2 HSCs into sublethally irradiated CD45.1 recipients revealed an increased ratio of GMP to MEP progenitors in the spleen after 2 weeks (Fig. 4g and Supplementary Fig. 11a, b) and an increased myeloid to lymphoid cell ratio in peripheral blood after 4 weeks (Supplementary Fig. 11c). In competitive transplantation assays, M-CSF-primed HSCs also showed a myeloid advantage compared to platelet and lymphoid contribution at 4 weeks in the blood that re-equilibrated after 6 weeks and did not compromise long-term multi-lineage contribution (Fig. 4h and Supplementary Fig. 12). Finally, this myeloid differentiation preference of M-CSF-primed HSCs could be abolished by deletion of *PU.1* (Fig. 4i and Supplementary Fig. 13). Together, these results indicated that M-CSF could directly instruct a change in cell identity of single HSCs *in vivo* that resulted in a reversible, PU.1-dependent myeloid differentiation preference.

Our results show that under haematopoietic stress conditions of infection, high systemic levels of M-CSF can directly instruct myeloid gene expression and differentiation preference of HSCs. This challenges both the current view of cytokine action and how HSCs make differentiation decisions. Whereas cytokines are commonly thought to act on lineage-committed progenitors, we show here that stem cells are direct targets of lineage instruction by cytokines. HSCs have been shown to proliferate in response to signals characteristic of bacterial²⁸ or viral infections²⁹ but without changing lineage-specific gene expression or differentiation potential. In line with the prevailing paradigm of selective cytokine action it has been proposed that distinct stem-cell subtypes could have a selective advantage in response to different stimuli³⁰. Such a mechanism is difficult to distinguish from instructive mechanisms on a population basis. We have therefore used multiple assays of single-cell analysis in culture and *in vivo* in a time window before the onset of cell division to distinguish induced changes of lineage specification from selective mechanisms. These data indicate that M-CSF can directly change stem-cell identity by activation of the myeloid master regulator PU.1 on the promoter, message and protein level, independently of selective survival or proliferation. The multi-lineage priming of gene expression in HSCs has generally been interpreted as an indication that initial cell-fate decisions are driven solely by stochastic fluctuations in the balance of lineage-specific transcription factors^{4-6,12,13}. Our data now indicate that cytokines can not only amplify random choices but also directly activate key regulators of lineage specification, such as PU.1, to instruct lineage fate of HSCs to induce an insult-tailored output of progeny. As M-CSF can transiently increase the production of myeloid progeny without compromising stem-cell activity, it may be useful to ameliorate myeloid cytopenias, particularly to protect patients from infection after stem-cell transplantation.

METHODS

Mice

CD45.1 and C57BL/6 mice were obtained from Charles River. *PU.1-GFP* (ref. 31), *M-CSFR*^{-/-} (ref. 27) and *PU.1*^{fl/fl} (ref. 32) mice have been described. Age- and sex-matched CD45.1 recipients that were reconstituted as described³ with CD45.2 fetal liver from wild-type or *M-CSFR*^{-/-} embryos²⁷ and *PU.1*^{fl/fl} or *PU.1*^{fl/fl}::*Mx-cre* bone marrow was used to isolate CD150⁺CD34⁻ KSLF HSCs not earlier than 8 weeks after reconstitution. For *in vivo*

injections, the 10 µg per mouse M-CSF, 5 mg kg⁻¹ LPS (055:B5 *Escherichia coli*) or sorted cells were injected in 100 µl of PBS into the retro-orbital sinus. For HSC transplantation, 400 CD150⁺CD34⁻ KSLF HSCs were sorted from CD45.2 mice and mixed with 100,000 Lin⁺Sca⁻ CD45.1 carrier cells before injection into sub-lethally irradiated (4.5 Gy) CD45.1 recipient mice. For competitive transplantations, 1,300 CD150⁺CD34⁻ KSLF HSCs were isolated 16 h after control or 10 µg M-CSF injection from actin-GFP CD45.2 mice³³, mixed with equal numbers of CD45.2 competitor HSCs and injected with 300,000 Lin⁺Sca⁻ RC-lysed CD45.1 carrier cells into sub-lethally irradiated (4.5 Gy) CD45.1 recipients. Contribution to platelets, CD19⁺B cells and CD11b⁺myeloid cells was analysed after 4 and 6 weeks in the blood from mice with at least 5% GFP⁺ donor cells. For *PU.1* deletion, *PU.1*^{fl/fl} or *PU.1*^{fl/fl}::*Mx-cre* reconstituted mice were intraperitoneally injected with 5 µg g⁻¹ polyinosinic:polycytidylic acid 7 and 9 days before control (PBS) or 10 µg M-CSF injection. All mouse experiments were performed under specific pathogen-free conditions in accordance with institutional guidelines.

FACS analysis

For FACS sorting and analysis we used described staining protocols³ and published stem and progenitor cell definitions³⁴, FACSCanto, LSRII and FACSARIAIII equipment and DIVA software (Becton Dickinson), analysing only populations with at least 200 events. For HSC analysis we used antibodies anti-CD34-FITC (clone RAM34, BD Biosciences), anti-CD135-PE (clone A2F10.1, BD Biosciences), anti-CD150-PE-Cy7 (clone TC15-12F12.2, Biolegend), anti-CD117-APC-H7 (clone 2B8, BD Biosciences), anti-Sca-1-PE-Cy5 (clone D7, Biolegend) and anti-CD48-APC (clone HM48-1, Biolegend). Diverging from this or in addition we used antibodies anti-CD34 Alexa 700 (clone RAM34, BD Biosciences), anti-CD16/32 PE (clone 2.4G2, BD biosciences), anti-CD11b PE-CF594 (clone M1/70, BD biosciences), anti-CD19-PE-Cy7 (clone 1D3, BD biosciences), anti-CD45.2 APC (clone 104, BD biosciences) and anti-CD45.1 Pacific blue (clone A20, BD biosciences) for progenitor and blood cell analysis. LIVE/DEAD Fixable Violet Dead cell dye (Invitrogen) was used as viability marker.

Intra-splenic injection of sorted HSCs and fluorescence microscopy

For analysis of HSCs *in vivo*, 1,500 to 7,000 FACS-sorted CD150⁺CD34⁻ KSLF HSCs were stained 10 min at 37 °C with 3 µM CFSE (Invitrogen) in PBS/0.5% BSA, washed 3× in PBS/0.5% BSA and injected in 30 µl PBS (containing or not 1 µg of isotype control or AFS98 anti-M-CSFR antibody²⁶ or 2 µM GW2580, 10 µM Ly29400, 10 µM PD98059 or 2 µM SU6656 inhibitors in 0.9% DMSO) into the spleen of anaesthetized mice. After 24 h spleens were embedded in OCT (Tissue-Tek, Sakura) and frozen at -80 °C. Cryostat sections (5 µm) were prepared from the entire organ, dried and fixed 10 min in 4% PFA/PBS at room temperature and every tenth section was further processed. After washes in PBS, slides were blocked for 1 h at room temperature in PBS/2% BSA/1% donkey serum/1% FCS/0.1% saponin, incubated for 36 h at 4 °C with anti-PU.1 polyclonal antibody (Santa Cruz) in PBS/0.05% saponin (1:50), washed and incubated with secondary Alexa 546-donkey-anti-rabbit antibody (Molecular probes) in PBS/0.05% saponin (1:500). All immunofluorescence samples were mounted with ProLong Gold DAPI antifade (Molecular probes) and analysed by multifuorescent microscopy on a Zeiss Axioplan 2. All CFSE⁺ cells were analysed for PU.1 expression up to 30 or 50 cells as indicated. Cell counts and staining were verified by a second trained microscope specialist blinded to sample identity. High-resolution photographs were obtained by confocal microscopy on a Leica SP5X.

In vitro culture of HSCs

CD150⁺CD34⁻KSLF HSCs or CD150⁺CD34⁻ CD48⁻ KSLF HSCs (single cells) were sorted into S-clone SF-03 medium (Sanko Jyunyaku) with 10% FBS supplemented with 100

U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (both Invitrogen) and cultivated in uncoated U-Shape 96-well plates (Greiner) in 100 µl SCM, 20 ng ml⁻¹ rSCF, 50 ng ml⁻¹ rTPO with or without 100 ng ml⁻¹ rM-CSF or 100 ng ml⁻¹ rGM-CSF or 100 ng ml⁻¹ rG-CSF. All cytokines were murine and from PeproTech. Cell viability was analysed by AnnexinV and Propidium iodide FACS staining³⁵.

Quantitative real-time PCR

Total RNA was isolated and reverse transcribed with µMACS One-step T7 template kit (Milenyi Biotec) and analysed by quantitative real-time PCR using TaqMan Universal PCR Master Mix and a 7500 Fast Real Time PCR System sequence detection system (both Applied Biosystem), following the manufacturers' instructions.

Single-cell gene expression profiling

Single cells were sorted using the autoclone module on an AriaIII sorter (Becton Dickinson) directly into 96-well plates in the CellsDirect Reaction Mix (Invitrogen). Individual cell lysis, cDNA synthesis and amplification were performed according to Fluidigm Advanced Development Protocol, and single-cell microfluidic real-time PCR using Dynamic Array IFCs (Biomark Fluidigm) was performed by a technical support specialist of Fluidigm Inc. Pre-amplified products (22 cycles) were diluted fivefold before analysis with Universal PCR Master Mix and inventoried TaqMan gene expression assays (ABI) in 96.96 Dynamic Arrays on a BioMark System (Fluidigm). Ct values were calculated from the system's software (BioMark Real-time PCR Analysis; Fluidigm) and filtered according to a set of quality control rules outlined below.

Gene filter: (1) for each gene, including controls, data with CtCall = FAILED and CtQuality < threshold were removed. (2) For each gene, including controls, CtValues > 32.0 were removed to filter out very low expression genes. (3) For each gene, including controls, genes with a difference of duplicate CtValues > 2.0 were considered inconsistent and removed.

Sample filter: (1) if the control gene (*Gapdh*) was not expressed or was removed according to gene filters (1–3), the whole sample was removed. (2) If the mean of the Ct values of all genes in a row was > 27.0 the whole sample row was removed.

Time-lapse imaging and analysis

Wherever possible our video microscopy protocols followed proposed guidelines²⁴. In detail, FACS-sorted CD150⁺CD34⁻ KSLF HSCs from wild-type C57BL/6 or GFP-negative CD150⁺CD34⁻ KSLF HSCs from *PU.1-GFP* reporter mouse³¹ bone marrow were suspended in SCM supplemented with 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin, 20 ng ml⁻¹ rSCF, 50 ng ml⁻¹ rTPO with or without 100 ng ml⁻¹ rM-CSF and plated in Ibidi µ-slidesVI(0.4) (Biovalley SA). Time-lapse microscopy was performed using a Cell Observer system (Carl Zeiss Microscopy) at 37 °C and 5% CO₂. Images were acquired every 10 min using 10× (A-plan 10x/0.45 Ph1) or 40× (Plan-Apochromat 40x/0.95 Korr M27) objectives in bright-field and fluorescence (GFP filters: EX BP 470/40; at 350 ms) with a CoolSNAP_{HQ2} monochrome camera (Photometrics) with a 2 × 2 binning and a metal halide 120 W source for fluorescence illumination. For image analysis a matrix of 4 × 4 images was acquired for each time point. Images were stitched with AxioVision software (Carl Zeiss Microscopy) and processed with Fiji software (<http://fiji.sc>) using a slight rolling ball subtraction of background and 1-pixel Gaussian blur. For background subtraction of bright-field images, the median of z-projection was subtracted from the time-lapse stack. Single-cell tracking was performed with basic commands of ImageJ (<http://imagej.nih.gov/ij/>) and Fiji (<http://fiji.sc>) software and with specific tracking plugin MTrackJ³⁶ in manual mode. Each cell was tracked manually frame-by-frame in the bright-field channel and cross-

controlled by two microscope specialists. Cells with non-standard morphology or size were rejected. The fluorescence signal was measured as the difference of maximum minus minimum intensity within a defined region of interest (ROI) around each cell. Cell properties and behaviour (cell division, cell death, position, fluorescence increase) were manually documented to build cumulated curves. R³⁷ and Excel (Microsoft Corporation) software was used to manage data and build graphics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. M-CSF activates the myeloid master regulator PU.1 in HSCs
a–c, Representative FACS profile (**a**) and quantification of GFP expression in HSCs (**b**) or CD150^{hi} HSCs (**c**) of *PU.1-GFP* reporter mice 16 h after control (PBS) or M-CSF injection. Horizontal bars show median. ***P* = 0.03; ****P* = 0.009, calculated by a two-tailed non-parametric Mann–Whitney *U*-test. **d**, Quantitative RT–PCR analysis of *PU.1* expression normalized to *Gapdh* expression (R.U.) in sorted HSCs after 16 h culture in the absence or presence of M-CSF, GM-CSF or G-CSF. Error bars show standard deviation of duplicates. G-CSF, granulocyte CSF; GM-CSF, granulocyte–macrophage CSF.

Figure 2. Continuous video imaging of *PU.1*⁺ cell generation from individual *PU.1*[−] HSCs
a, GFP fluorescence intensity at 10-min intervals (dots) and sliding median (lines) over 12-h observation time of three individual GFP-negative sorted HSCs from *PU.1-GFP* reporter mice after 18 h in M-CSF culture, representative of cells quantified in **e** ($n = 39$). Green, cells activating GFP; black, cell remaining GFP negative. **b**, Still photos taken at times indicated by symbols in **a** of fields with two representative HSCs (cells A, B) showing activation of *PU.1* at different time points. Cell C was outside of the shown field. **c**, Still photos taken at 40-min intervals over 8 h of three representative HSCs in M-CSF culture without (cell C) or with activation of *PU.1* (cells A, D), representative of cells quantified in **e** ($n = 39$). Complete videos are shown in Supplementary Videos 1–3. **d**, Quantification of *PU.1*⁺ cells derived from *PU.1*[−] HSCs (committed cells) with ($n = 39$) or without M-CSF ($n = 42$) as percentage of total cells after 24-h observation period. * $P < 0.1$, calculated by a two-tailed non-parametric Mann–Whitney *U*-test. **e**, Timing of *PU.1* activation in *PU.1*[−] HSCs of cells shown in **d** over 24-h observation period.

Figure 3. M-CSF activates *PU.1* and instructs myeloid identity in single HSCs

a–c, Gene expression analysis of single cells (rows) for lineage or stem-cell representative genes (columns) using duplicate nano-fluidic real-time PCR on Fluidigm array for freshly isolated HSCs (**a**) or after 16 h of culture in the absence (**b**) or the presence of M-CSF (**c**). Genes are grouped by lineage (indicated on top) and individual cells were clustered according to lineage-specific, mixed or lineage-negative gene expression profiles shown in bar and pie diagrams on the right. A full gene list and expanded view is shown in Supplementary Figs 7–9. ** $P = 0.04$ calculated by a Pearson's χ^2 test, $n = 41, 45, 45$. Blue star highlights expression of *PU.1* gene expression. Colour scale on the bottom shows correspondence between colour code and Ct values. E, erythroid; Lympho, lymphoid; Meg, megakaryocytic; MegE, megakaryocytic-erythroid; Myelo, myeloid. **d**, Individual *PU.1*⁺ cells with a myeloid gene expression profile (blue) or expressing other lineage genes (white) as a percentage of total cells. *** $P = 0.009$ (0 h) and $P = 0.005$ (–M-CSF), calculated by a two-tailed non-parametric Mann–Whitney U -test. **e**, Experimental design for transplantation of sorted *PU.1*⁻ and *PU.1*⁺ HSCs from *in vivo* M-CSF-primed CD45.2 *PU.1*-GFP mice into sub-lethally irradiated CD45.1 recipients and analysis of progeny cells after 2 weeks in the spleen (Supplementary Fig. 14). **f**, **g**, Representative FACS profiles (**f**) and quantification of the ratio (**g**) of donor GMP and MEP progenitors derived from transplanted *PU.1*⁻ or *PU.1*⁺ HSCs before or after M-CSF stimulation *in vivo*. ** $P = 0.05$; *** $P = 0.01$, calculated by a

two-tailed non-parametric Mann–Witney U -test; $n = 4, 8, 4$. Whisker plots show median (lines), upper and lower quartiles (boxes) and extreme outliers (dotted whiskers).

Figure 4. M-CSF directly induces endogenous PU.1 protein in single HSCs *in vivo* and stimulates a reversible, PU.1-dependent myeloid differentiation preference

a, Experimental design of HSC transplantation into spleens of LPS-stimulated or M-CSF-stimulated hosts and typical immunofluorescence detection of PU.1 in CFSE-labelled HSCs 24 h after transplantation for two representative PU.1⁺ and one PU.1⁻ cell. DAPI, nuclear stain. **b, c**, Representative immunofluorescence images (**b**) and percentage (**c**) of PU.1⁺ HSCs immediately (0 h) or 24 h after transplantation into LPS-stimulated host with isotype control (IC) or anti-M-CSF receptor blocking antibody (AFS98), or into M-CSF-injected hosts. (*n* = 30). **d, e**, Representative immunofluorescence images (**d**) and percentage (**e**) of PU.1⁺ cells immediately (0 h) or 24 h after transplantation of wild-type or *M-CSFR*^{-/-} HSCs into mock or M-CSF-stimulated hosts (*n* = 50). **f**, Percentage of PU.1⁺ cells 24 h after transplantation of HSCs into M-CSF-stimulated hosts in the absence or presence of kinase inhibitors for M-CSFR (GW2580), PI(3)K (LY294002), ERK/MAPK (PD98059) and SRC (SU6656) (*n* = 50). **g**, Ratio of donor GMP to MEP progenitors in the spleens of sub-lethally irradiated recipients 2 weeks after transplantation of *in vivo* M-CSF-primed or control. **h**, Ratio of donor myeloid progenitors in the blood of sub-lethally irradiated recipients 4 and 6 weeks after transplantation of *in vivo* M-CSF-primed or control. **i**, Ratio of donor myeloid progenitors in the spleen of sub-lethally irradiated recipients 4 weeks after transplantation of *in vivo* M-CSF-primed or control.

HSCs. Experimental design is shown in Supplementary Fig. 11. *** $P = 0.003$, calculated by a two-tailed non-parametric Mann–Whitney U -test; $n = 8, 9$. **h**, Donor contribution to blood of competitively reconstituted mice 4 weeks and 6 weeks after transplantation of M-CSF-primed or control HSCs, expressed as a ratio of CD11b⁺ myeloid cells to platelets or CD19⁺ lymphoid cells. Experimental design, representative FACS profiles and quantification of contribution to individual lineages are shown in Supplementary Fig. 12. * $P = 0.07$, calculated by a two-tailed non-parametric Mann–Whitney U -test, $n = 6, 4$; *** $P = 0.01$, $n = 10, 6$. **i**, Donor contribution to Mac⁺ myeloid cells in the spleen of sub-lethally irradiated recipients 2 weeks after transplantation of control or M-CSF-primed HSCs with control (fl/fl) or deleted (Δ/Δ) *PU.1* alleles. ** $P = 0.05$, calculated by a two-tailed non-parametric Mann–Whitney U -test, $n = 6, 4, 5$. NS, not significant. Whisker plots show median (lines), upper and lower quartiles (boxes) and extreme outliers (dotted whiskers).