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

Mossadegh-Keller, N., Sarrazin, S., Kandalla, P.K., Espinosa, L., Stanley, E.R., Nutt, S.L., Moore, J., Sieweke, M.H.

Published in final edited form as:
Nature. 2013 May 13 ; 497(7448): 239-243 | doi: 10.1038/nature12026
Nature Publishing Group ►

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

Noushine Mossadegh-Keller[1,2,3,], Sandrine Sarrazin[1,2,3,*,#], Prashanth K. Kandalla[1,2,3], Leon Espinosa[4], E.Richard Stanley[5], Stephen L. Nutt[6], Jordan Moore[7], Michael H.Sieweke[1,2,3,8,#]*

1 Centre d'Immunologie de Marseille-Luminy (CIML), Aix-Marseille Université, UM2, Campus de Luminy, Case 906, 13288 Marseille Cedex 09, France

2 Institut National de la Santé et de la Recherche Médicale (INSERM), U1104, Marseille, France

3 Centre National de la Recherche Scientifique (CNRS), UMR7280, Marseille, France

4 Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique (CNRS), UMR 7283, 31 Chemin Joseph Aiguier, 13009 Marseille, France

5 Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA

6 Walter and Eliza Hall Institute, 1G Royal Parade, Parkville, Victoria 3052, Australia

7 Fluidigm Inc., San Francisco, CA, USA

8 Max-Delbrück-Centrum für Molekulare Medizin (MDC), Robert-Rössle-Str. 10, 13125 Berlin, Germany

***** *equal contribution* *#* *Corresponding author*

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 (HSC) are susceptible to direct lineage-specifying effects of cytokines. Although genetic changes in transcription factor balance can sensitize HSC to cytokine instruction[3], the initiation of HSC commitment is generally thought to be triggered by stochastic fluctuation in cell intrinsic regulators such as lineage specific transcription factors[4,5,6,7], leaving cytokines to ensure survival and proliferation of the progeny cells[8,9]. Here we show that M-CSF, a myeloid cytokine released during infection and inflammation, can directly induce the myeloid master regulator PU.1 and instruct myeloid cell fate change in HSC, independently of selective survival or proliferation. Video imaging and single cell gene expression analysis revealed that stimulation of highly purified HSC 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 HSC and induced a PU.1 dependent myeloid differentiation preference. Our data demonstrate that lineage specific cytokines can act directly on HSC *in vitro* and *in vivo* to instruct a change of cell identity. This fundamentally changes the current view of how HSC respond to environmental challenge and implicates stress-induced cytokines as direct instructors of HSC fate.

Lineage specific cytokines such as macrophage colony stimulating factor (M-CSF/CSF-1), can be strongly induced during physiological stress or infection[10,11]. and potently increase the production of mature cells from lineage-committed progenitors[1,2]. According to the prevailing model, however, they are generally not believed to directly influence differentiation decisions of haematopoietic stem cells (HSC)[9,12,13]. Cell fate choice of HSC has traditionally been explained by stochastic models[14]. In this view transcriptional noise[15] and random variations in competing lineage determining transcription factors lead to cross-antagonistic switches that initiate lineage choice[4,5,6,7], 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 myelo-monocytic differentiation[16,17]. It is generally unknown whether external signals could drive the initial activation of such intrinsic master regulators. Since HSC deficient for the transcription factor MafB are sensitized to PU.1 activation in response to M-CSF[3], we have investigated whether high systemic M-CSF levels could induce PU.1 and instruct myelo-monocytic fate in *wt* HSC without prior modification of transcription factor balance.

We observed that lipopolysacharide (LPS), a strong mimetic of bacterial infection stimulating high systemic levels of M-CSF[11] (sup.fig.1a), induced an up-regulation of GFP in long term HSC (CD117+sca+Lin-CD135-CD34-CD150+) of PU.1-GFP reporter mice[18] (sup.fig.1b,c). Consistent with the expression of the M-CSF receptor (M-CSFR) in HSC (sup.fig.2)[3,19], direct intravenous injection of recombinant M-CSF also induced significantly increased PU.1 activation in HSC after 16h (fig.1a,b)**.** The treatment caused no significant change in M-CSFR or MafB expression (sup. fig.3), arguing against selection of myeloid primed HSC with high M-CSFR or low MafB levels. M-CSF also induced no change in the proportion of $CD150^{hi}$ HSC, reported to have myeloid lineage bias[20], in GFP-positive or –negative HSC (sup.fig.4a-c) and activated PU.1 to a similar extent in $CD150^{hi}$ HSC (fig.1c) as in total HSC (fig.1a,b). Finally cultured $CD150^{hi}$ HSC revealed no proliferation or survival advantage in the presence of M-CSF (sup.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 HSC.

As shown in fig.1d, the M-CSF effect on stem cells was direct and specific, since FACS purified HSC showed increased PU.1 expression after 16h in culture with M-CSF but not with GM-CSF or G-CSF, cytokines that may also be released during infection[21]. The observed changes in gene expression cannot be explained by M-CSF dependent selection of PU.1+ cells, as video-microscopy of cultured HSC showed no proliferation or survival advantage in M-CSF and PU.1 was induced before onset of cell division (sup.fig.5,6). Continuous observation of individual GFP-negative sorted HSC from PU.1-GFP

mice by video imaging confirmed that M-CSF could induce PU.1 expression in previously PU.1 negative cells (fig.2.a-c, sup.Video1-3). We recorded the fate of HSC between 18 hours and 42 hours 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 24h observation period over two-fold more PU.1+ cells had developed in M-CSF than under control conditions (fig.2d) and backtracking the origin of these cells revealed that all PU.1+ cells were derived from previously PU.1 negative 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 hours 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 HSC.

To further investigate whether M-CSF induced PU.1 activation changed the cell identity of individual HSC we analyzed the mRNA expression profile of single cells by nanofluidic real time PCR on Fluidigm™ dynamic arrays. Consistent with their stem cell identity almost all freshly isolated HSC 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 (Meg), megakaryocytic-erythroid (MegE) or myeloid lineage priming (fig.3a, sup.fig.7). Culture for 16h without M-CSF led to an increased number of cells with a mixed lineage profile at the expense of Meg and lin- profiles (fig.3b, sup.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, sup.fig.9, sup.table1). 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- HSC to PU.1- and PU.1+ HSC 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+ HSC than from PU.1- HSC (fig.3f,g). We observed a similar increase in myeloid differentiation potential for PU.1+ cells derived from M-CSF stimulated PU.1- HSC

in culture (sup.fig.10a-d). Together these data showed that M-CSF induced PU.1 led to a myeloid cell fate change in single HSC.

To further investigate, whether M-CSF could also induce a cell fate change of individual HSC *in vivo*, we transplanted CFSE-labelled HSC into the spleen, a site of extra-medullary haematopoiesis with adapted stem cell niches[3,24], and analyzed expression of endogenous PU.1 protein by immuno-fluorescence in single HSC after 24h (fig.4a). Whereas the vast majority of HSC 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 receptor[25] strongly inhibited PU.1 activation. Furthermore, direct injection of recombinant M-CSF resulted in a similar strong induction of PU.1 in the transplanted HSC (fig.4b,c). This effect appeared to be entirely cell autonomous, as M-CSF receptor deficient (M-CSFR$^{-/-}$)[26] HSC showed no higher activation of PU.1 in M-CSF stimulated than control recipients (fig.4d,e). Similarly, small molecule inhibitors of the M-CSFR or PI3K, ERK and SRC kinases that signal downstream of the receptor[27] also prevented induction of PU.1 (fig.4f), consistent with the stimulation of transcriptional activators of the *pu.1* gene by these pathways (sup. discussion). Furthermore, transplantation of *in vivo* M-CSF primed CD45.2 HSC into sub-lethally irradiated CD45.1 recipients revealed an increased ratio of GMP to MEP progenitors in the spleen after 2 weeks (fig.4g, sup.fig.11a,b) and an increased myeloid to lymphoid cell ratio in peripheral blood after 4 weeks (sup.fig.11c). In competitive transplantation assays M-CSF primed HSC 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, sup.fig.12). Finally, this myeloid differentiation preference of M-CSF primed HSC could be abolished by deletion of PU.1 (fig.4i, sup.fig.13). Together these results indicated that M-CSF could directly instruct a change in cell identity of single HSC *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 HSC. This challenges both the current view of cytokine action and how HSC make differentiation decision. Whereas cytokines are commonly thought to act on lineage-committed progenitors, we here show that stem cells are direct targets of lineage instruction by cytokines. HSC have been shown to proliferate in response to signals characteristic of bacterial[28] or viral infections[29] 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 stimuli[30]. Such a mechanism is difficult to distinguish from instructive mechanisms on a population basis. We have therefore employed 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 haematopoietic stem cells has generally been interpreted as indication that initial cell fate decisions are driven solely by stochastic fluctuations in the balance of lineage specific transcription factors[4,5,6,12,13]. 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 output of haematopoietic stem cells. Cytokines released during specific challenges may thus directly shunt the differentiation choice of HSC to an insult tailored output of progeny. This discovery may also provide new opportunities for the beneficial manipulation of stem cell fate under pathological or transplantation conditions.

Acknowledgements

We gratefully acknowledge grants from the 'Association pour la recherche sur le Cancer' (3422) and the 'Agence nationale de la Recherche' (BLAN07-1_205752). We thank Drs. P.Kastner and S.Chan for PU.1-GFP reporter mice, T.P.VuManh and J.Maurizio for bioinformatics and M.Barad, A.Zouine and M.-L.Thibult for flow cytometry support, L.Razafindramanana for animal handling and J.Favret, P.Perrin and L.Chasson for tissue sectioning. RS is supported by NIH grant CA 32551. SN is an Australian Research Council Future Fellow and received Victorian State Government Operational and Australian Government NHMRC Independent Research Institute Infrastructure Support. MS is a 'Fondation pour la Recherche Médicale' (DEQ20071210559; DEQ20110421320) and INSERM-Helmholtz group leader.

Author Contributions

MS conceived the study, analyzed and interpreted data and wrote the paper; SS performed experiments, analyzed and interpreted data and contributed to the preparation of the manuscript; NMK performed most experiments and analysed data; PK Performed and analysed video-microscopy and contributed to other experiments; LE analysed and interpreted video microscopy data; JM provided expertise and service on Fluidigm™ experiments; ERS and SN provided essential MCSFR/CSF-1R and PU.1 deficient hematopoietic cells. NMK and SS contributed equally to the study. NMK, SS, PK, LE and MS jointly designed experiments and SS and MS coordinated the project.

Author Information

Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests.

Corresponding Author

Correspondence to : MS (sieweke@ciml.univ-mrs.fr) or SS (sarrazin@ciml.univ-mrs.fr)

Methods summary

Flowcytometry, bone marrow transplantation and *in vivo* immunofluorescence of HSC were performed essentially as described[3]. Single cell nano-fluidics-based real-time PCR was performed using a BioMark™ HD system and 96.96 dynamic arrays (Fluidigm, CA, USA) and videomicroscopy analysis followed proposed standards[23]. Details of procedures and reagents are described in Supplementary Methods.

References

1 Metcalf, D. Hematopoietic cytokines. *Blood* **111**, 485-491 (2008).

2 Rieger, M. A., Hoppe, P. S., Smejkal, B. M., Eitelhuber, A. C. & Schroeder, T. Hematopoietic cytokines can instruct lineage choice. *Science* **325**, 217-218 (2009).

3 Sarrazin, S. *et al.* MafB restricts M-CSF dependent myeloid commitment divisions of hematopoietic stem cells *Cell* **138**, 1-14 (2009).

4 Orkin, S. H. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* **1**, 57-64 (2000).

5 Cantor, A. B. & Orkin, S. H. Hematopoietic development: a balancing act. *Curr Opin Genet Dev* **11**, 513-519 (2001).

6 Enver, T., Pera, M., Peterson, C. & Andrews, P. W. Stem cell states, fates, and the rules of attraction. *Cell Stem Cell* **4**, 387-397(2009).

7 Graf, T. & Enver, T. Forcing cells to change lineages. *Nature* **462**, 587-594 (2009).

8 Socolovsky, M., Lodish, H. F. & Daley, G. Q. Control of hematopoietic differentiation: lack of specificity in signaling by cytokine receptors. *Proc Natl Acad Sci U S A* **95**, 6573-6575 (1998).

9 Metcalf, D. On hematopoietic stem cell fate. *Immunity* **26**, 669-673 (2007).

10 Cheers, C. & Stanley, E. R. Macrophage production during murine listeriosis: colony-stimulating factor 1 (CSF-1) and CSF-1-binding cells in genetically resistant and susceptible mice. *Infect Immun* **56**, 2972-2978 (1988).

11 Roth, P., Bartocci, A. & Stanley, E. R. Lipopolysaccharide induces synthesis of mouse colony-stimulating factor-1 in vivo. *J Immunol* **158**, 3874-3880 (1997).

12 Cross, M. A. & Enver, T. The lineage commitment of haemopoietic progenitor cells. *Curr Opin Genet Dev* **7**, 609-613(1997).

13 Enver, T., Heyworth, C. M. & Dexter, T. M. Do stem cells play dice? *Blood* **92**, 348-351(1998).

14 Till, J. E., McCulloch, E. A. & Siminovitch, L. A Stochastic Model of Stem Cell Proliferation, Based on the Growth of Spleen Colony-Forming Cells. *Proc Natl Acad Sci U S A* **51**, 29-36 (1964).

15 Chang, H. H., Hemberg, M., Barahona, M., Ingber, D. E. & Huang, S. Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. *Nature* **453**, 544-547 (2008).

16 Laiosa, C. V., Stadtfeld, M. & Graf, T. Determinants of lymphoid-myeloid lineage diversification. *Annu Rev Immunol* **24**, 705-738 (2006).

17 Orkin, S. H. & Zon, L. I. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* **132**, 631-644 (2008).

18 Back, J., Allman, D., Chan, S. & Kastner, P. Visualizing PU.1 activity during hematopoiesis. *Exp Hematol* **33**, 395-402 (2005).

19 Miyamoto, T. *et al.* Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* **3**, 137-147 (2002).

20 Morita, Y., Ema, H. & Nakauchi, H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J Exp Med* **207**, 1173-1182 (2010).

21 Cheers, C. *et al.* Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte-macrophage-, and multi-CSFs. *Infect Immun* **56**, 247-251 (1988).

22 Hoebe, R. A. *et al.* Controlled light-exposure microscopy reduces photobleaching and phototoxicity in fluorescence live-cell imaging. *Nat Biotechnol* **25**, 249-253 (2007).

23 Schroeder, T. Long-term single-cell imaging of mammalian stem cells. *Nat Methods* **8**, S30-35 (2011).

24 Kiel, M. J. & Morrison, S. J. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol* **8**, 290-301 (2008).

25 Sudo, T. *et al.* Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. *Oncogene* **11**, 2469-2476 (1995).

[26] Dai, X. M. *et al.* Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. *Blood* **99**, 111-120 (2002).

[27] Pixley, F. J. & Stanley, E. R. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol* **14**, 628-638 (2004).

[28] Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. & Goodell, M. A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465**, 793-797(2010).

[29] Essers, M. A. *et al.* IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).

[30] King, K. Y. & Goodell, M. A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nat Rev Immunol* **11**, 685-692 (2011).

Figure legends

Fig.1 M-CSF activates the myeloid master regulator PU.1 in HSC

a-c)Representative FACS profile (a) and quantification of GFP expression in HSC (b) or $CD150^{hi}$ HSC (c) of PU.1-GFP reporter mice 16h after control (PBS) or M-CSF injection. ***p=0,009; **p=0,03.

d) Quantitative RT-PCR analysis of PU.1 expression normalized to GAPDH expression (R.U.) in sorted HSC after 16h culture in the absence or presence of M,CSF, GM-CSF or G-CSF. Error bars show standard deviation of duplicates.

Fig.2 Continuous video-imaging of PU.1+ cell generation from individual PU.1 negative HSC.

a) GFP-fluorescence intensity at 10 minute intervals (dots) and sliding median (lines) over 12h observation time of 3 individual GFP negative sorted HSC from PU.1-GFP reporter mice after 18h in M-CSF culture, representative of cells quantified in fig.2e (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 2 representative HSC (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 8h of 3 representative HSC in M-CSF culture without (cell C) or with activation of PU.1 (cells A,D), representative of cells quantified in fig.2e (n=39). Complete videos are shown in Sup.Video1-3.

d) Quantification of PU.1+ cells derived from PU.1 negative HSC (committed cells) with (n=39) or without M-CSF (n=42) as percentage of total cells after 24h observation period. *$p \leq 0.1$.

e) Timing of PU.1 activation in PU.1 negative HSC of cells shown in d) over 24h observation period.

Fig.3 M-CSF activates PU.1 and instructs myeloid identity in single HSC

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 HSC (a) or after 16 hours 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. Full gene list and blow up in sup.fig. 7-9. **p= 0.04, n=41,45,45.

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 (0h), and 0,005 (-M-CSF).

e) Experimental design for transplantation of sorted PU.1- and PU.1+ HSC 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 (sup.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+ HSC before or after M-CSF stimulation *in vivo*. **p=0.05, ***p=0.01, n=4,8,4.

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

a) Experimental design of HSC transplantation into spleens of LPS or M-CSF stimulated hosts and typical immuno-fluorescence detection of PU.1 in CFSE-labelled HSC 24h after transplantation for two representative PU.1+ and one PU.1- cell. DAPI, nuclear stain.

b,c) Representative immuno-fluorescence images (b) and percentage (c) of PU.1+ HSC immediately (0h) or 24h after transplantation into LPS stimulated host with isotype control (IC) or anti-M-CSF receptor blocking antibody (AFS98), or into M-CSF injected hosts. ($n \geq 30$).

d,e) Representative immuno-fluorescence images (d) and percentage (e) of PU.1+ cells immediately (0h) or 24h after transplantation of wt or M-CSFR$^{-/-}$ HSC into mock or M-CSF stimulated hosts. ($n \geq 50$).

f) Percentage of PU.1+ cells 24h after transplantation of HSC into M-CSF stimulated hosts in the absence or presence of kinase inhibitors for M-CSFR (GW2580), PI3K (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 HSC. Experimental design is shown in sup.fig.11.

***p=0.003, 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 HSC, expressed as ratio of CD11b+ myeloid cells to platelets or CD19+ lymphoid cells. Experimental design, representative FACS profiles and quantification of contribution to individual lineages in sup.fig.12.

***p=0.01, n=10,6, *p=0.07, n=6,4.

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 HSC with control (fl/fl) or deleted (Δ/Δ) PU.1 alleles. **p=0.05, n=6,4,5.

Methods

Mice

CD45.1 and C57Bl/6 mice were obtained from Charles River. *PU.1-GFP* [31] M-CSFR$^{-/-}$[27] and PU.1$^{fl/fl}$ [32] mice have been described. Age- and sex-matched CD45.1 recipients that were reconstituted as described[3] with CD45.2 foetal liver from wt or M-CSFR$^{-/-}$ embryos[27] and PU.1$^{fl/fl}$ or PU.1$^{fl/fl}$::MxCre bone marrow, were used to isolate CD150+ CD34- KSLF HSC not earlier than 8 weeks after reconstitution. For *in vivo* injections the 10µg/mouse M-CSF, 5mg/kg LPS (055:B5 *E. coli*) or sorted cells were injected in 100µl of PBS into the retro-orbital sinus. For HSC transplantation 400 CD150+ CD34- KSLF HSC were sorted from CD45.2 mice and mixed with 100.000 Lin$^+$ Sca$^-$ CD45.1 carrier cells prior to injection into sub-lethally irradiated (4,5 Gy) CD45.1 recipient mice. For competitive transplantations, 1300 CD150+,CD34-KSLF HSC were isolated 16h after control or 10µg M-CSF injection from actin-GFP CD45.2 mice[33], mixed with equal numbers of CD45.2 competitor HSC 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$^{fl/fl}$::MxCre reconstituted mice were intra-peritoneallay injected with 5µg/g Polyinosinic :polycytidylic acid 7 and 9 days prior to control (PBS) or 10µg 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 protocols[3] and published stem and progenitor cell definitions[34], 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), 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-CD19PE-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-spleenic injection of sorted HSC and fluorescence microscopy

For analysis of HSC *in vivo*, 1500 to 7000 FACS sorted $CD150^{+}$ $CD34^{-}$ KSLF HSC were stained 10 min at 37°C with 3µM CFSE (Invitrogen) in PBS / 0,5% BSA, washed 3x in PBS / 0,5% BSA and injected in 30µl PBS (containing or not 1µg of isotype control or AFS98 α-M-CSFR antibody[26] or 2µM GW2580, 10µM Ly29400, 10µM PD98059 or 2µM SU6656 inhibitors in 0.9% DMSO) into the spleen of anesthetized mice. After 24h spleens were embedded in OCT (Tissue-Tek, Sakura) and frozen at -80°C. Cryostat sections (5µm) were prepared from the entire organ, dried and fixed 10 min in 4% PFA/PBS at room temperature (RT) and every 10^{th} section was further processed. After washes in PBS, slides were blocked for 1 hour at RT in PBS / 2%BSA/ 1% Donkey serum / 1% FCS / 0.1% saponin, incubated for 36h 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 analyzed by multifluorescent microscopy on a Zeiss Axioplan 2. All CFSE+ cells were analysed for PU.1 expression up to $\geq$30 or $\geq$ 50 cells as indicated. Cell counts and staining were verified by a second trained microscopist blinded to sample identity. High-resolution photographes were obtained by confocal microscopy on a Leica SP5X.

In vitro culture of HSC

CD150+ CD34- KSLF HSC or CD150+ CD34- CD48- KSLF HSC (single cells) were sorted into S-clone SF-03 medium (Sanko Jyunyaku) with 10% FBS supplemented with 100 U/ml penicillin and 100mg/ml streptomycin (both Invitrogen) and cultivated in uncoated U-Shape 96 well plates (Greiner) in 100 µl SCM, 20 ng/ml rSCF, 50 ng/ml rTPO +/- 100 ng/ml rM-CSF or 100 ng/ml rGM-CSF or 100 ng/ml rG-CSF. All cytokines were murine and from PeproTech. Cell viability was analyzed by AnnexinV and Propidium iodide FACS staining[35].

Quantitative real time PCR

Total RNA was isolated and reverse transcribed with µMACS One-step T7 template kit (Miltenyi 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 wells plate in the CellsDirect Reaction Mix (Invitrogen). Individual cell lysis, cDNA synthetis

and amplification was 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. Preamplified products (22 cycles) were diluted 5-fold prior to 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:

(a) For each gene, including controls, data with CtCall = FAILED and CtQuality < threshold were removed.

(b) For each gene, including controls, CtValues > = 32.0 were removed to filter out very low expression genes.

(c) For each gene, including controls, genes with a difference of duplicate CtValues > = 2.0 were considered inconsistent and removed.

Sample filter:

(a) If the control gene (Gapdh) was not expressed or was removed according to gene filters (a-c), the whole sample was removed.

(b) 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 guidelines [24]. In detail, FACS sorted CD150+ CD34- KSLF HCS from wt C57/Bl6 or GFP-negative CD150+ CD34- KSLF HSC from PU.1-GFP reporter mouse [31] bone marrow were suspended in SCM supplemented with 100 U/ml penicillin and 100mg/ml streptomycin, 20 ng/ml rSCF, 50 ng/ml rTPO +/- 100 ng/ml rM-CSF and plated in Ibidi μ-slidesVI(0.4) (Biovalley SA, France). Time-lapse microscopy was performed using a Cell Observer system (Carl Zeiss Microscopy GmbH, Germany) at 37°C and 5% CO2. Images were acquired every 10 minutes using 10x (A-plan 10x/0.45 Ph1) or 40x (Plan-Apochromat 40x/0.95 Korr M27) objectives in brightfield and fluorescence (GFP filters: EX BP 470/40; at 350 ms) with a $CoolSNAP_{HQ}2$ monochrome camera (Photometrics) with a 2x2 binning and a metal halide 120W source for fluorescence illumination. For image analysis a matrix of 4x4 images was acquired for each time point. Images were stitched with AxioVision software (Carl Zeiss Microscopy GmbH, Germany) and processed with Fiji software[36] using a slight rolling ball subtraction of background and 1 pixel Gaussian blur. For background subtraction of brightfield images, the median of z-projection was subtracted from the time-lapse stack. Single cell tracking was performed with basic commands of

ImageJ[37] and Fiji[36] software and with specific tracking plugin MTrackJ[38] 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. R[39] and Excel (Microsoft Corporation) software was used to manage data and build graphics.

Statistical analysis

P values were calculated by two-tailed non parametric Mann-Witney test for direct sample comparisons or Pearson's chi[2] test for proportions (alpha=0,05). Whisker plots show median (lines), upper and lower quartiles (boxes) and extreme outliers (dotted whiskers).

Methodes References

31 Back, J., Dierich, A., Bronn, C., Kastner, P. & Chan, S. PU.1 determines the self-renewal capacity of erythroid progenitor cells. *Blood* **103**, 3615-3623 (2004).

32 Dakic, A. *et al.* PU.1 regulates the commitment of adult hematopoietic progenitors and restricts granulopoiesis. *J Exp Med* **201**, 1487-1502 (2005).

33 Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. & Nishimune, Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* **407**, 313-319 (1997).

34 Bryder, D., Rossi, D. J. & Weissman, I. L. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol* **169**, 338-346 (2006).

35 Niu, C. *et al.* c-Myc is a target of RNA-binding motif protein 15 in the regulation of adult hematopoietic stem cell and megakaryocyte development. *Blood* **114**, 2087-2096(2009).

36 Schindelin, J., Proceedings of the ImageJ conference(2008).

37 Rasband, W.S.,ImageJ, http://imagej.nih.gov/ij/ (Bethesda, ML, USA, 1997-2012).

38 Meijering, E., Dzyubachyk, O. & Smal, I. Methods for cell and particle tracking. *Methods Enzymol* **504**, 183-200 (2012).

39 R: A language and environment for statistical computing (Vienna, Austria, 2012).

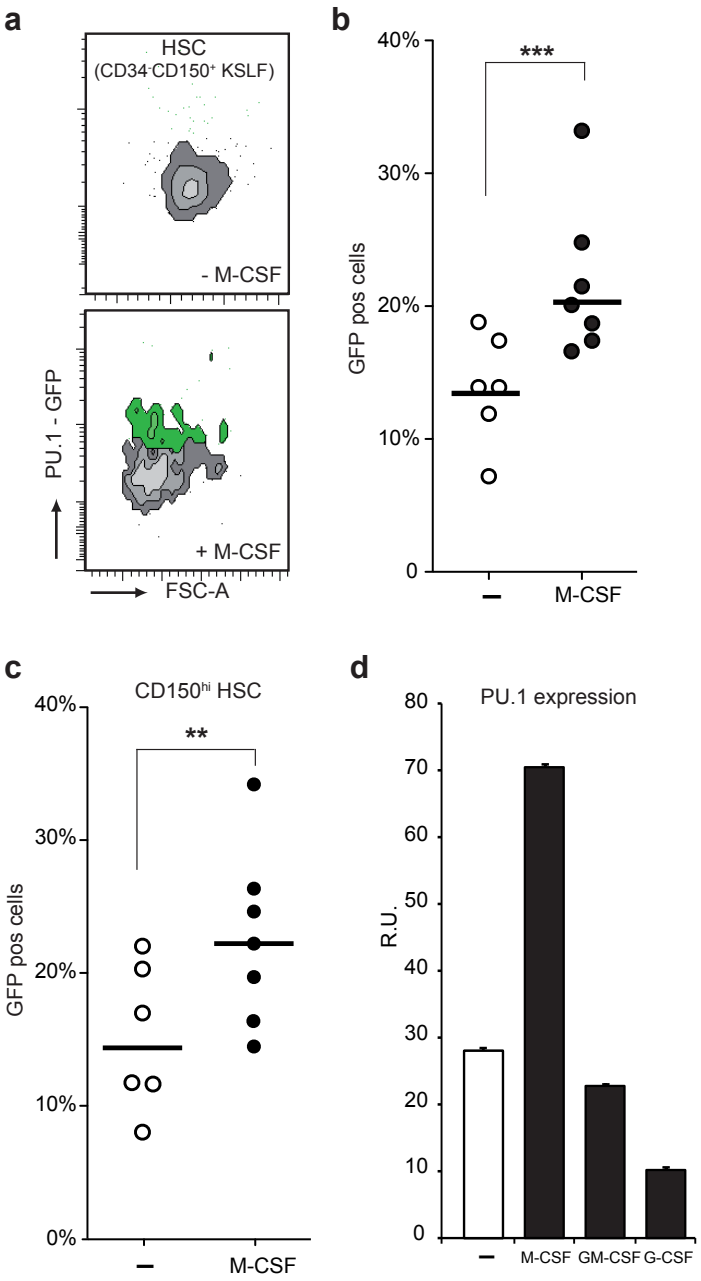

a
HSC
(CD34-CD150+ KSLF)
- M-CSF
+ M-CSF
PU.1 - GFP
FSC-A
b
***
40%
30%
20%
10%
0
GFP pos cells
–
M-CSF
c
CD150hi HSC
**
40%
30%
20%
10%
0%
GFP pos cells
–
M-CSF
d
PU.1 expression
80
70
60
50
40
30
20
10
0
R.U.
–
M-CSF
GM-CSF
G-CSF

fig.1

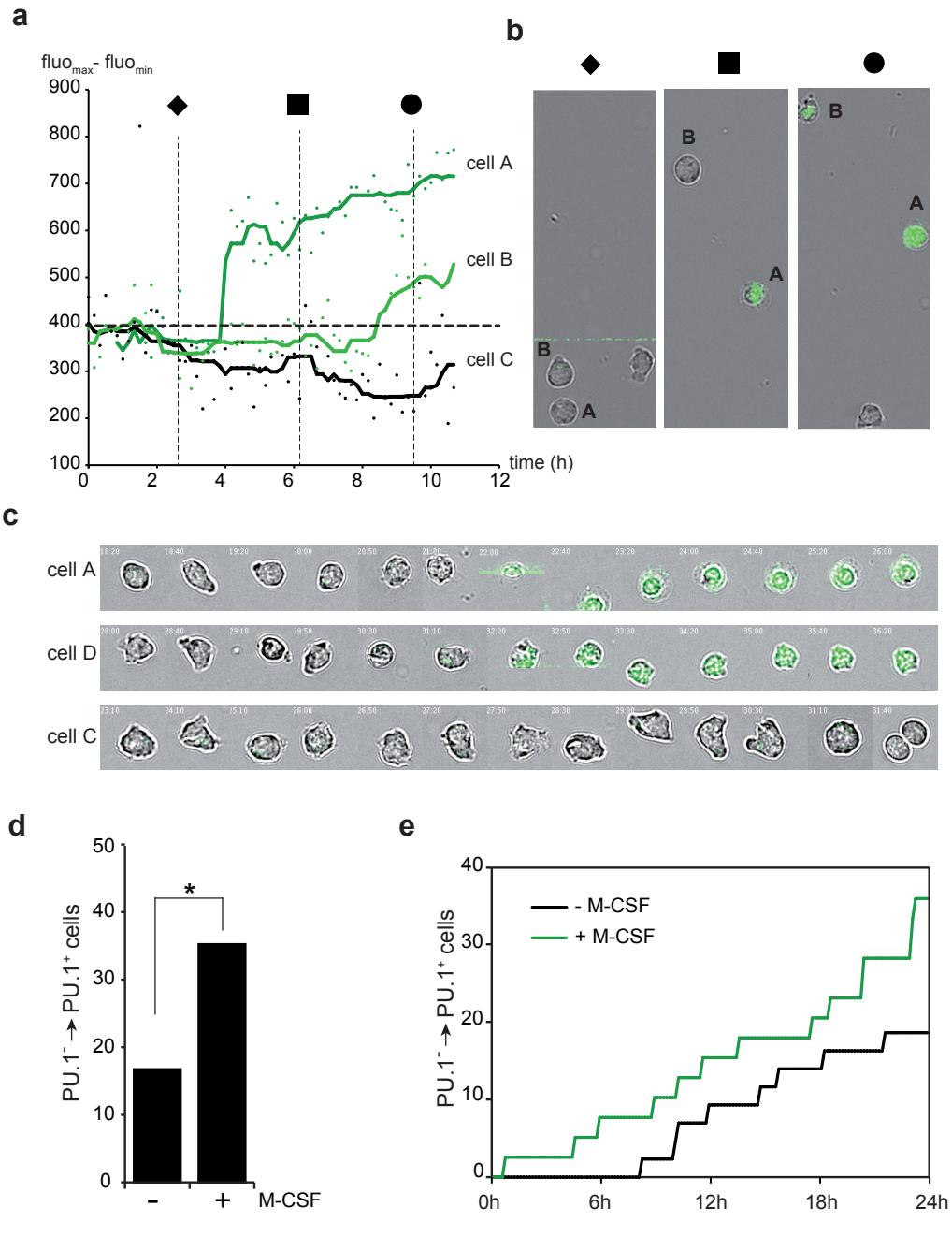

a
b
c
d
e
fluo$_{max}$- fluo$_{min}$
900
800
700
600
500
400
300
200
100
0
2
4
6
8
10
12
time (h)
cell A
cell B
cell C
B
A
cell A
cell D
cell C
PU.1$^-$→PU.1$^+$ cells
50
40
30
20
10
0
*
–
+
M-CSF
- M-CSF
+ M-CSF
0h
6h
12h
18h
24h

fig.2

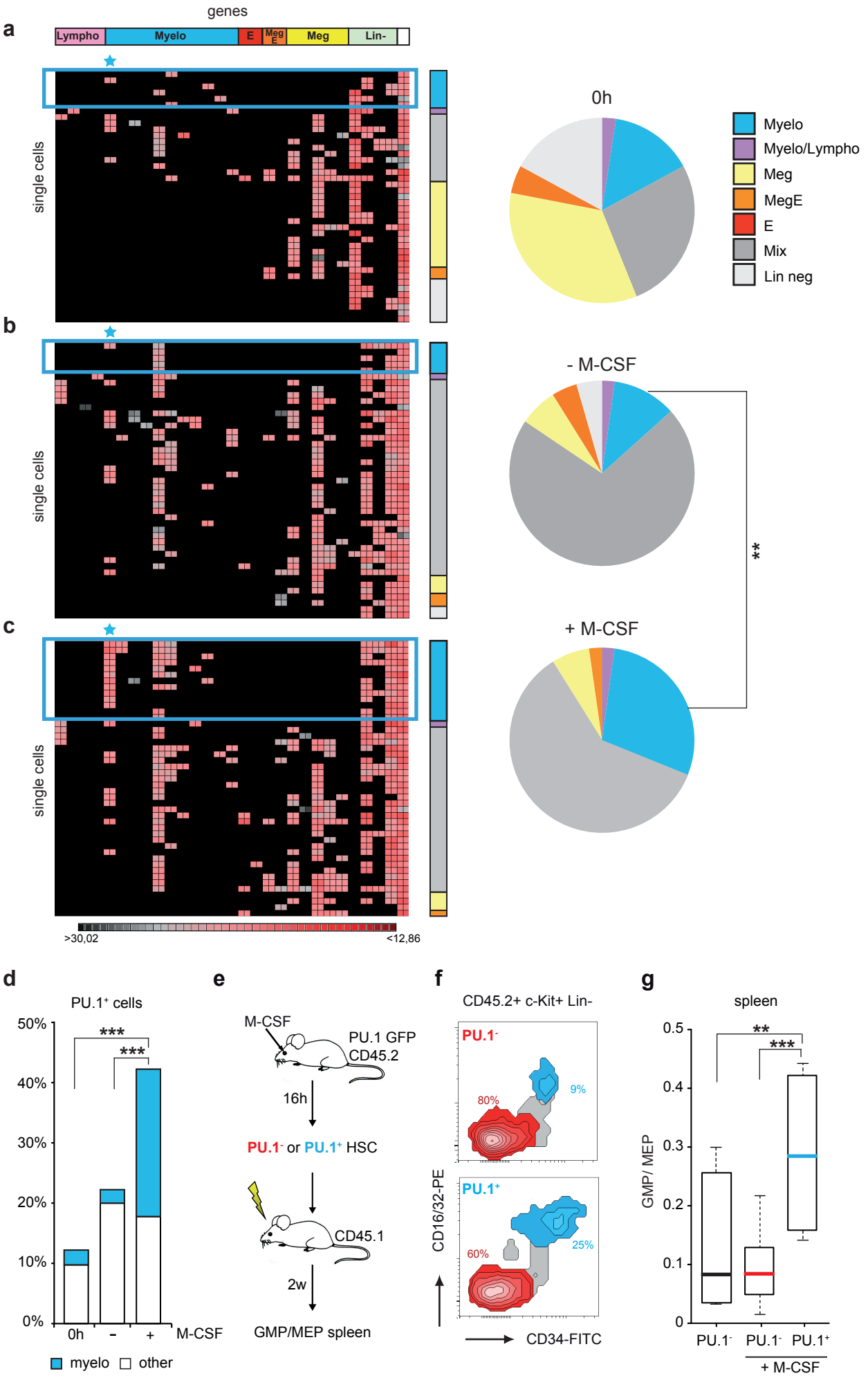

a
genes
Lympho
Myelo
E
Meg E
Meg
Lin-
single cells
0h
Myelo
Myelo/Lympho
Meg
MegE
E
Mix
Lin neg
b
single cells
- M-CSF
**
c
single cells
+ M-CSF
>30,02
<12,86
d
PU.1⁺ cells
50%
40%
30%
20%
10%
0%
***
***
0h
-
+
M-CSF
myelo
other
e
M-CSF
PU.1 GFP
CD45.2
16h
PU.1⁻ or PU.1⁺ HSC
CD45.1
2w
GMP/MEP spleen
f
CD45.2+ c-Kit+ Lin-
PU.1⁻
80%
9%
PU.1⁺
60%
25%
CD16/32-PE
CD34-FITC
g
spleen
0.5
0.4
0.3
0.2
0.1
0
**
***
GMP/ MEP
PU.1⁻
PU.1⁻
PU.1⁺
+ M-CSF

fig.3

	0h		- M-CSF		+ M-CSF	
	Myeloid	total	Myeloid	total	Myeloid	total
Experiment 1	5	23	5	22	11	24
Experiment 2	3	47	3	40	15	39
Experiment 3	6	41	5	45	13	45
total	14	109	13	107	39	108

p=0,0001

p=0,0001

Sup.table 1, related to Fig 3a,b,c
Summary of cell numbers with myeloid identity in three independent single cell nano-fluidic real time PCR experiments on FluidigmTM array for freshly isolated HSC (0h) or after 16 hour of culture in the absence or the presence of M-CSF. HSC were FACS sorted as CD150+CD34-CD48-KSLF (experiment 1,3) or CD150+CD34-KSLF (experiment 2). Experiment 3 is shown in Fig.3a and sup.Fig.7-9.

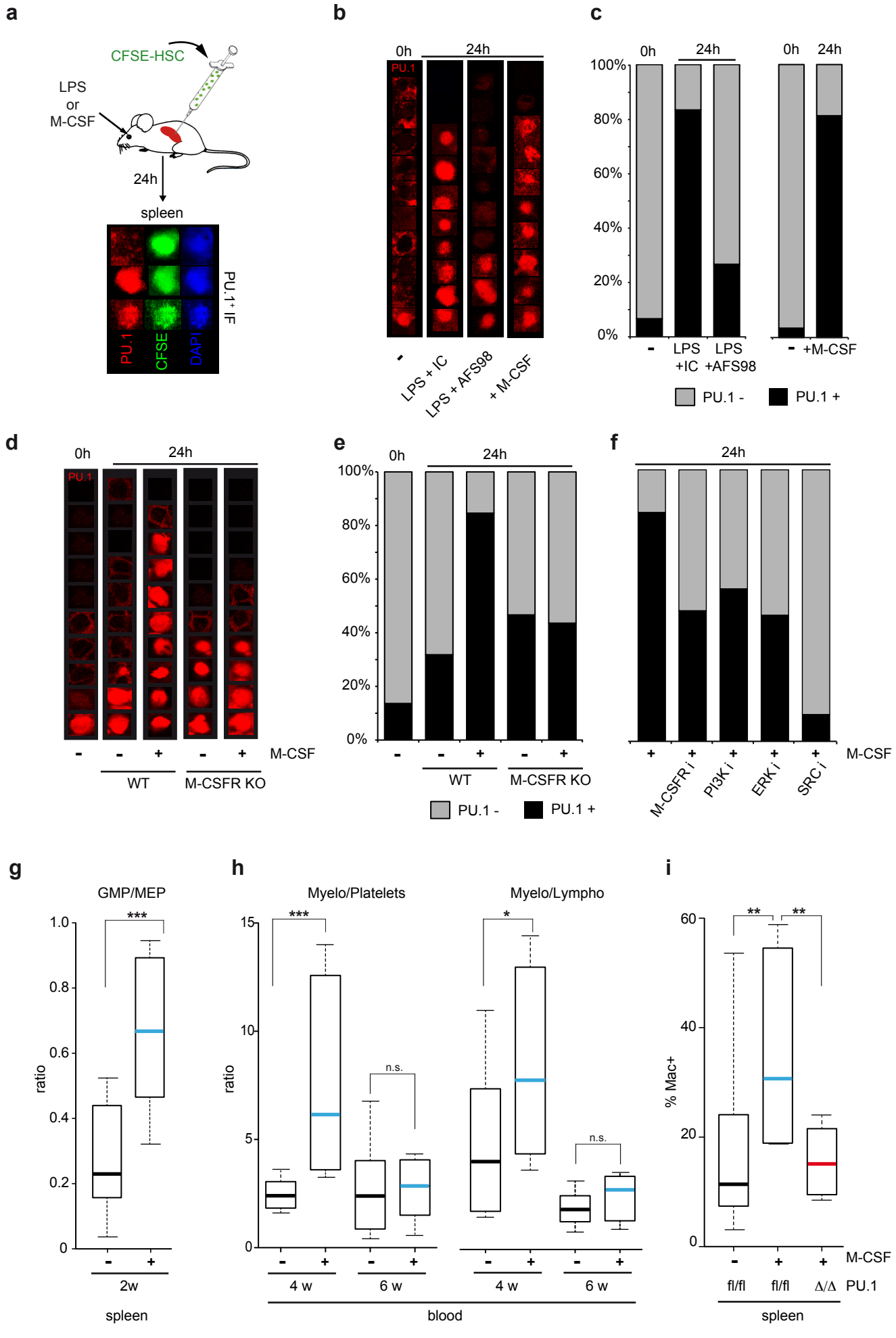
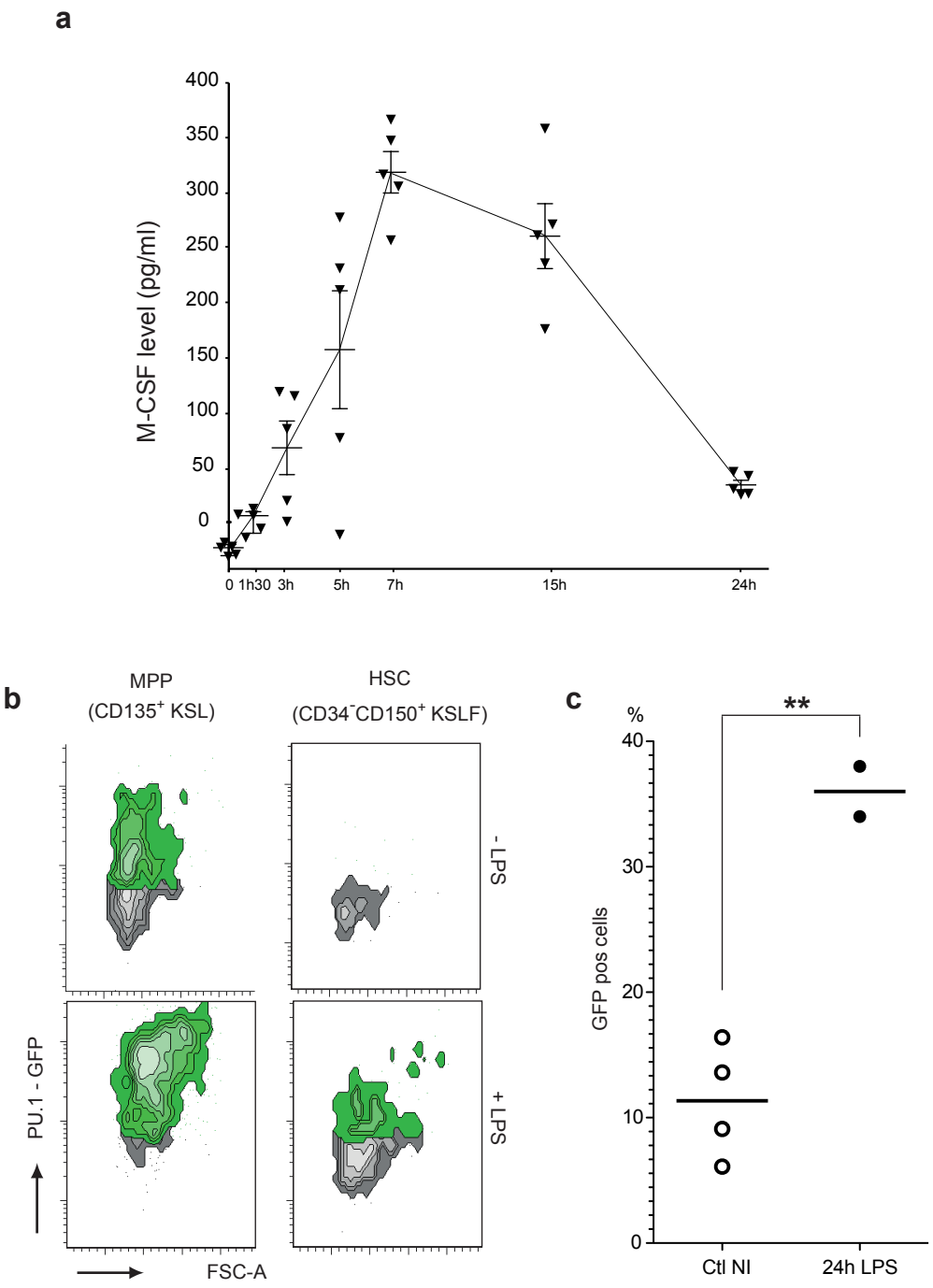
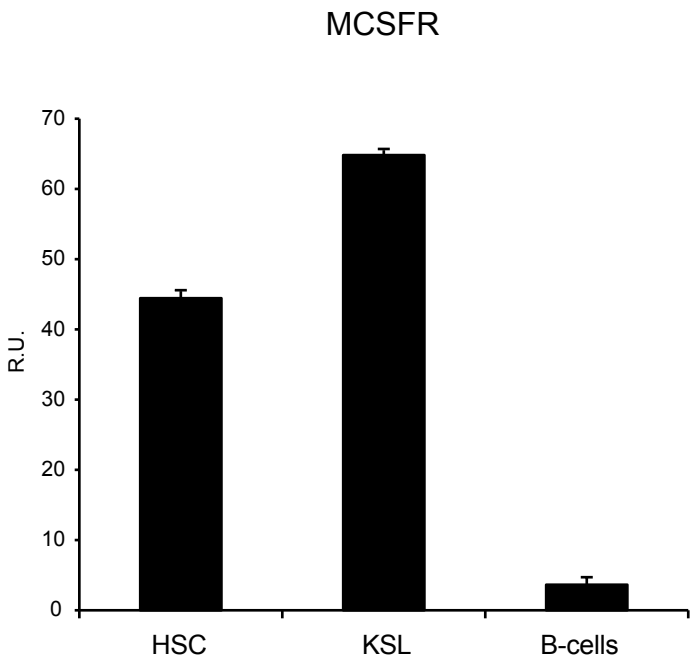

a
CFSE-HSC
LPS
or
M-CSF
24h
spleen
PU.1
CFSE
DAPI
PU.1+ IF
b
0h
24h
PU.1
-
LPS + IC
LPS + AFS98
+ M-CSF
c
0h
24h
100%
80%
60%
40%
20%
0%
-
LPS +IC
LPS +AFS98
+M-CSF
PU.1 -
PU.1 +
d
0h
24h
PU.1
M-CSF
WT
M-CSFR KO
e
0h
24h
100%
80%
60%
40%
20%
0%
WT
M-CSFR KO
PU.1 -
PU.1 +
f
24h
M-CSF
M-CSFR i
PI3K i
ERK i
SRC i
g
GMP/MEP
***
ratio
1.0
0.8
0.6
0.4
0.2
0
2w
spleen
h
Myelo/Platelets
Myelo/Lympho
***
*
n.s.
n.s.
ratio
15
10
5
0
4 w
6 w
4 w
6 w
blood
i
**
**
% Mac+
60
40
20
0
M-CSF
fl/fl
fl/fl
Δ/Δ
PU.1
spleen

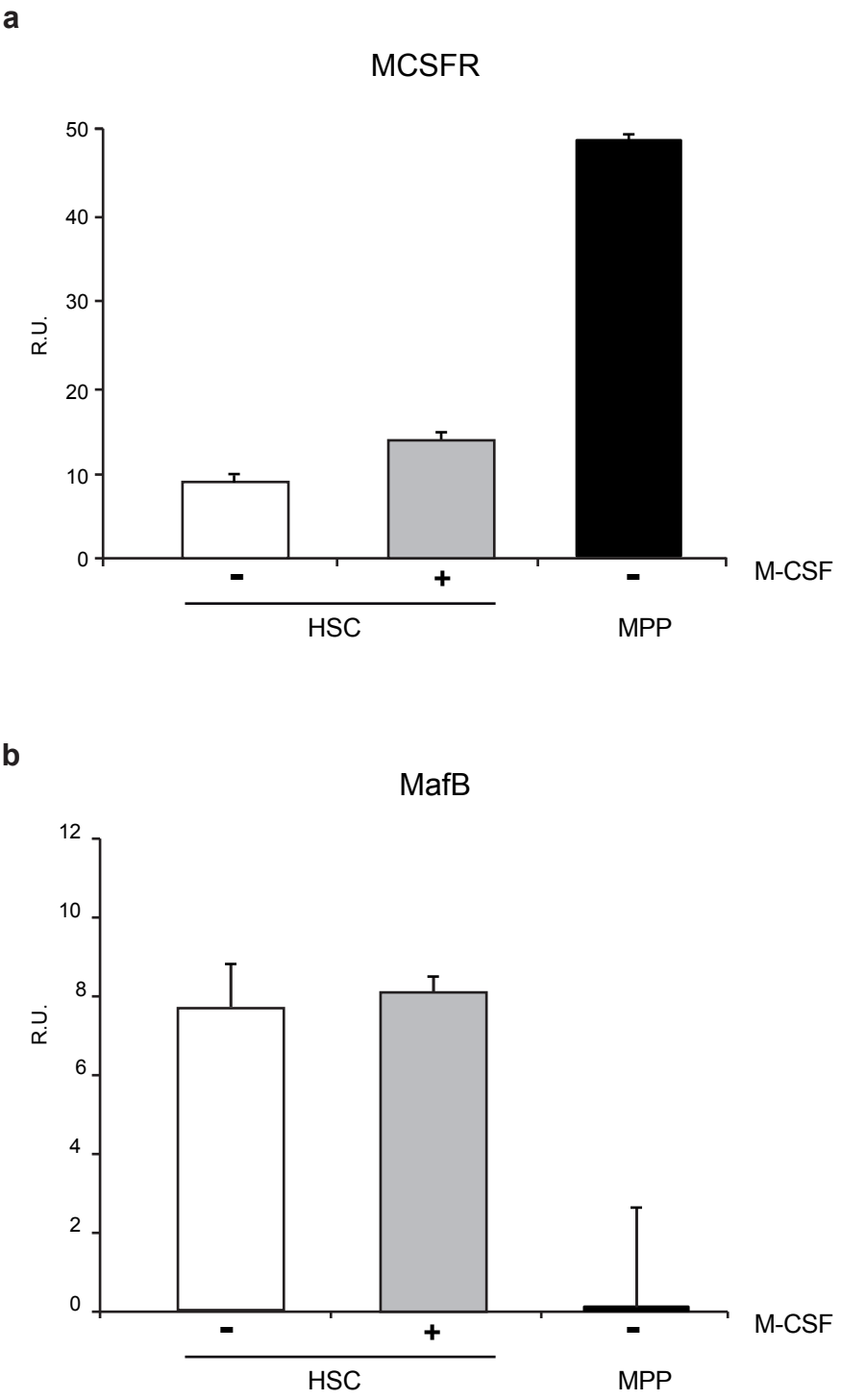
fig.4



Sup.Fig.1 Effect of LPS on M-CSF release and PU.1 induction in HSC
a) Serum levels of M-CSF after LPS stimulation
Median and individual concentrations of M-CSF in blood serum from five mice at the indicated times after 5mg/kg intra-peritoneal LPS injection.
b,c)Representative FACS profiles (b) and quantification (c) of GFP expression in MPP and HSC of PU.1-GFP reporter mice before or after 24h of 5mg/kg LPS injection.
** p = 0.03, n = 4,2

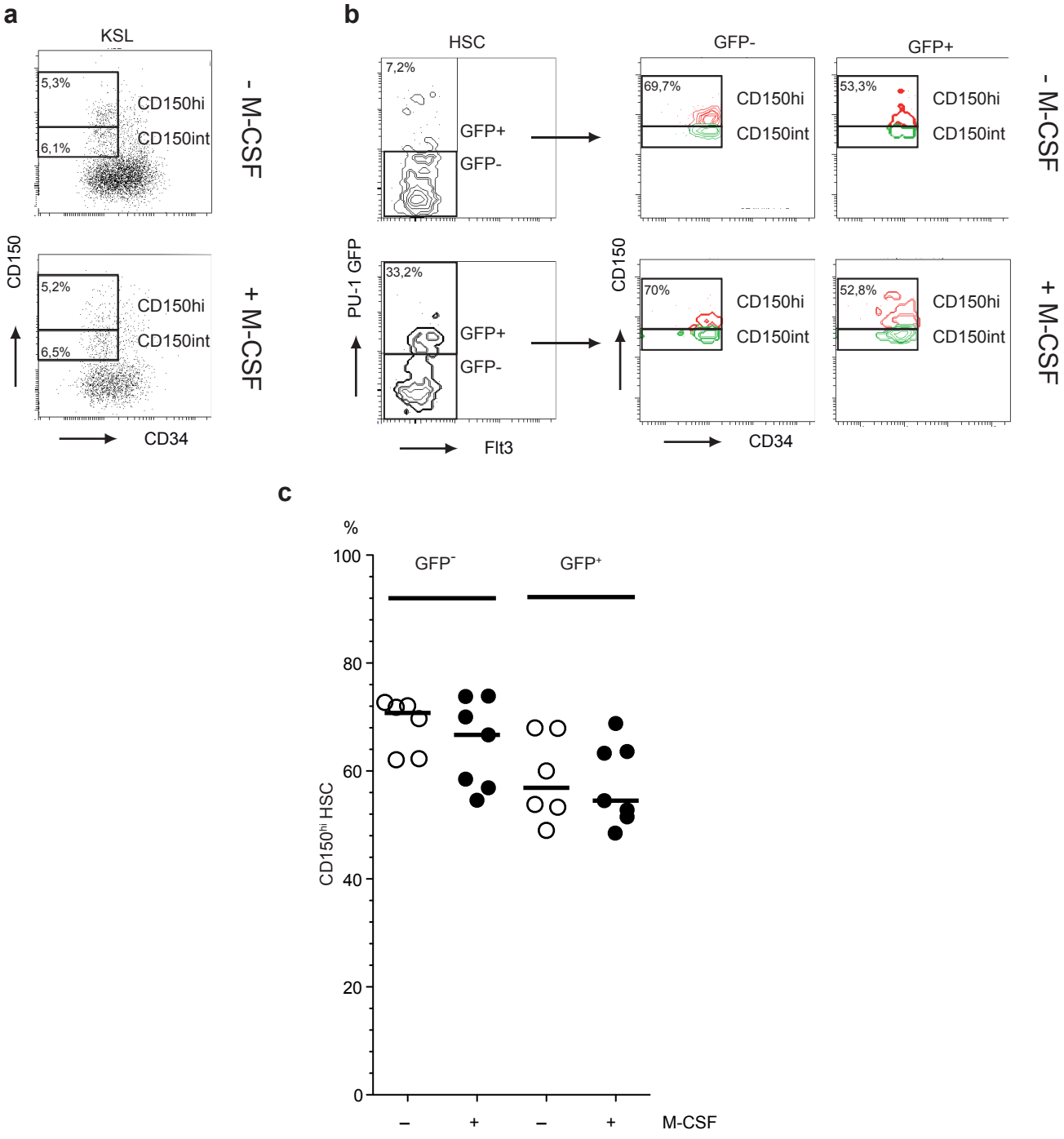


Sup.Fig.2 M-CSFR expression in HSC
Relative expression of M-CSFR normalized to GAPDH (R.U.) by qRT-PCR analysis in sorted HSC, KSL (c-kit+, sca-1+, lin–) hematopoietic stem and progenitor cells (HS/PC) and CD19+ B-cells from the bone marrow, as positive and negative control respectively. Error bars show standard error of the mean from duplicates. Error Bars show standard deviation from duplicates.



Sup.Fig.3 M-CSFR and MafB expression in HSC after M-CSF stimulation

Relative expression of M-CSFR (a) and MafB (b) normalized to GAPDH (R.U.) by qRT-PCR analysis in sorted HSC, 16h after control (PBS) or M-CSF injection, compared to untreated MPP (CD135+ KSL) as control for a population containing myeloid committed cells[3], with high M-CSFR and low MafB expression, respectively. Error bars show standard deviation from duplicates.

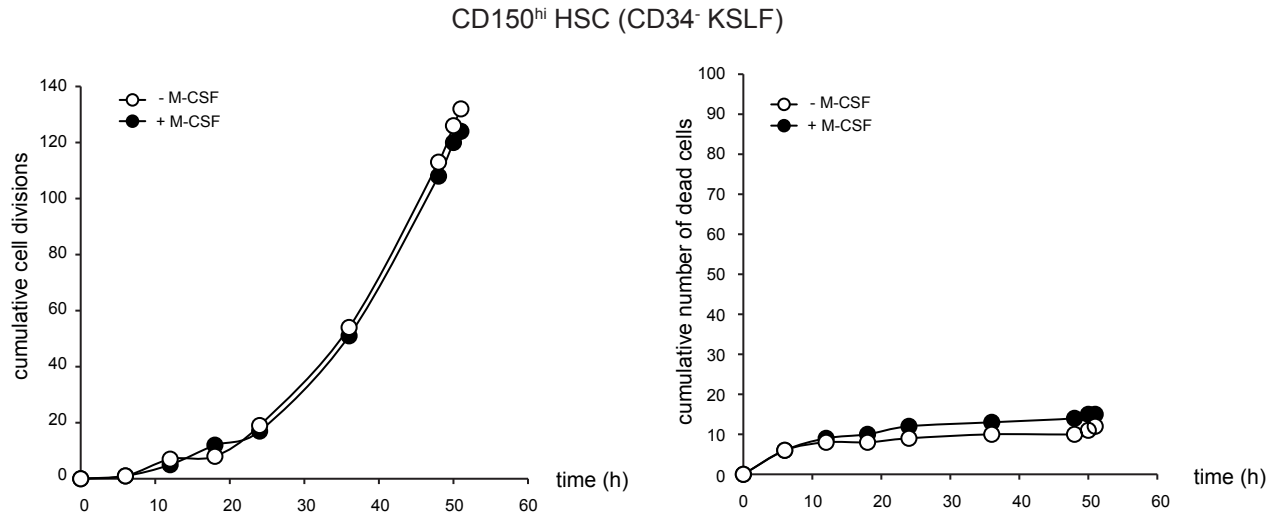


Sup.Fig.4 Distribution of CD150hi HSC in GFP+ HSC.

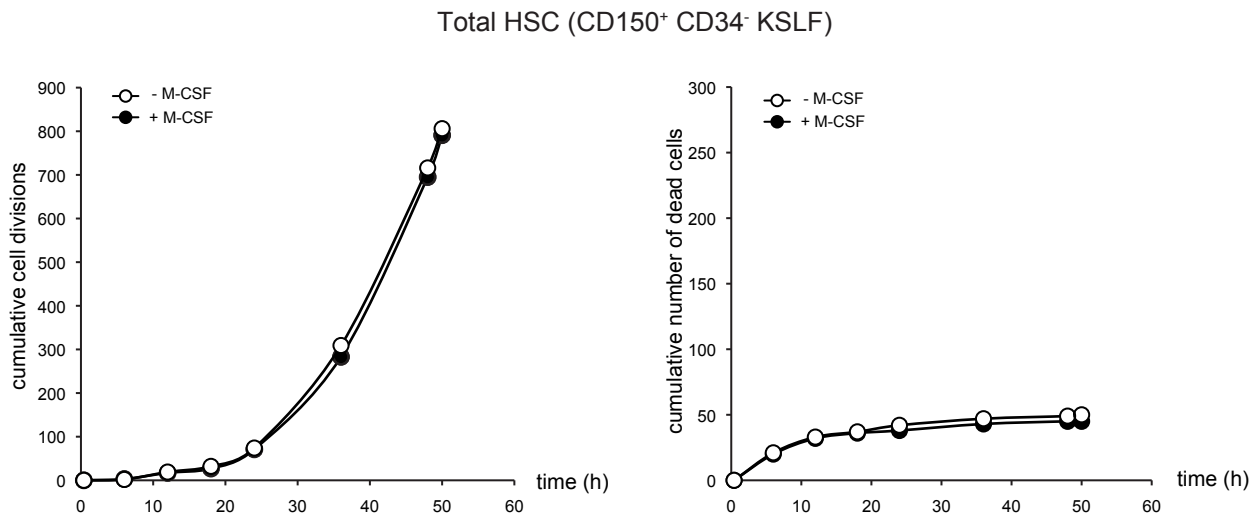
a) Gating strategy for CD150hi HSC in the KSL compartment following published definitions[20].

b,c) Representative FACS profiles (b) and quantification (c) of CD150hi HSC subpopulations in GFP+ and GFP- HSC from PU.1-GFP mice 16h after control (PBS) or M-CSF injection.

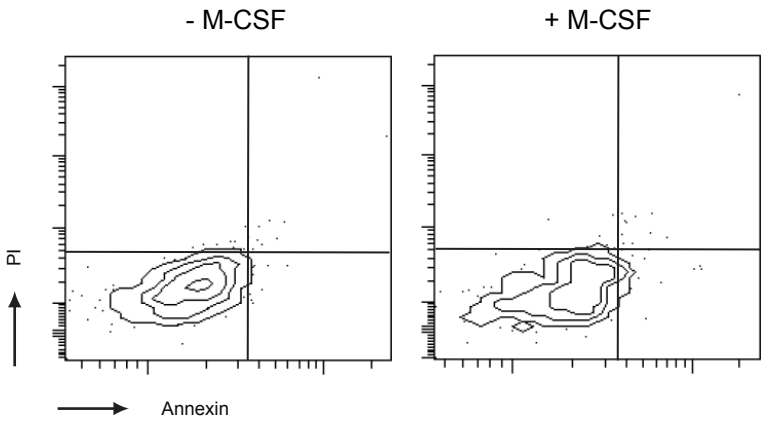
a



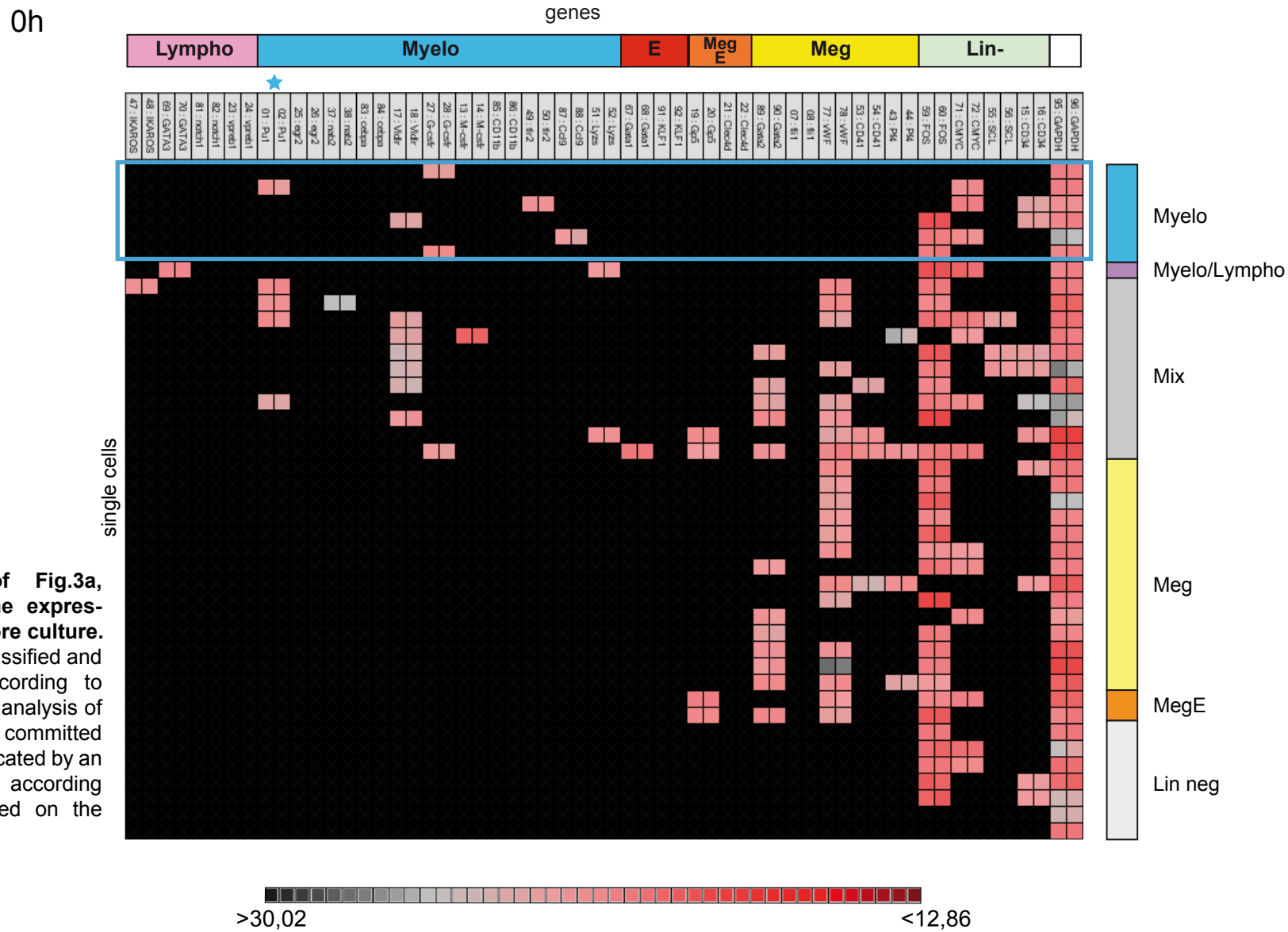
b



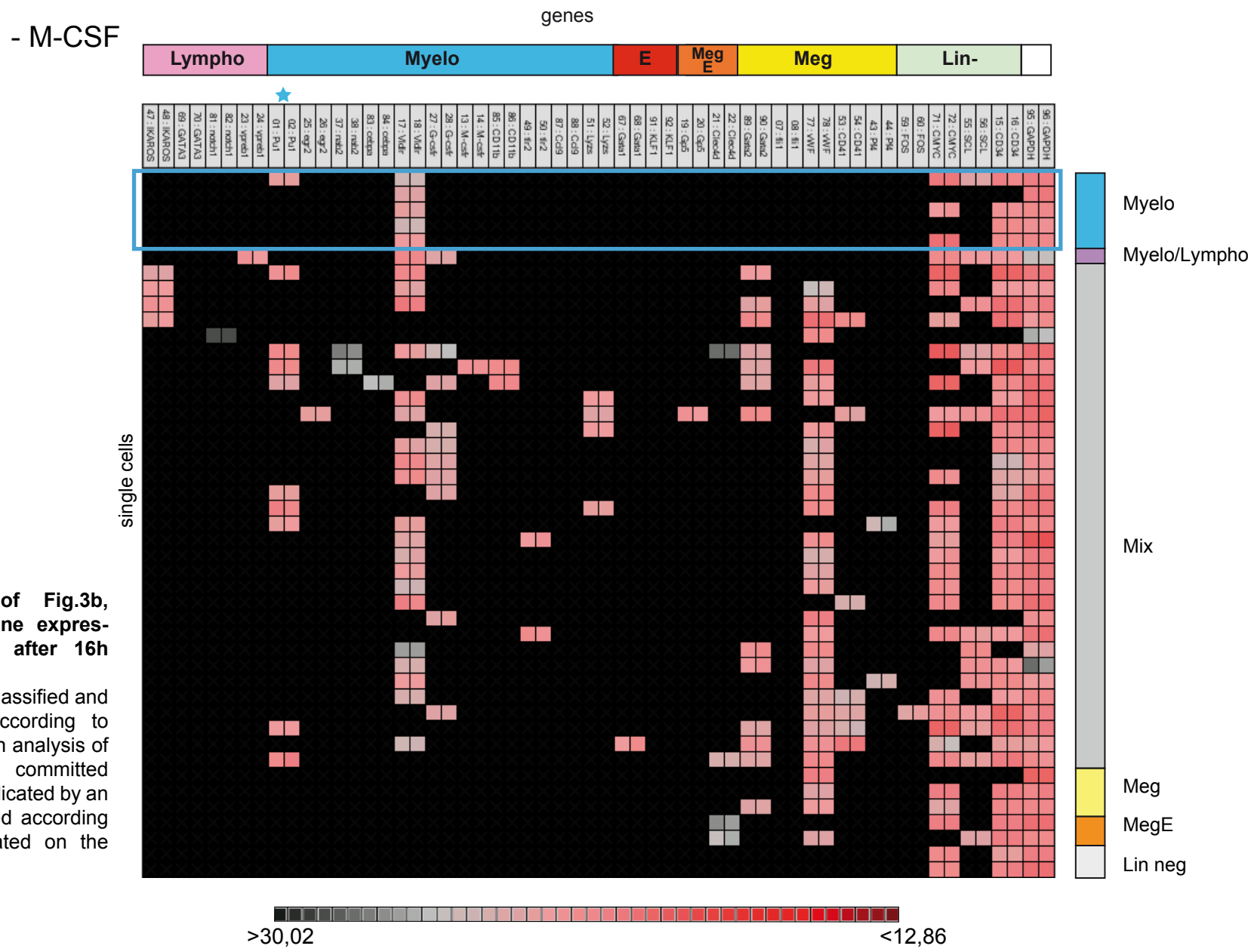
Sup.Fig.5 Recording of cell division and cell death of HSC in culture by video imaging. Cumulative cell divisions (left) and cumulative number of cells that died (right) of CD150hi (a) and total (b) HSC for 100 and 360 input cells respectively, over the indicated times in culture with or without M-CSF. The vast majority of first cell divisions occurred after 24h and nearly no additional cell death occurred after minimal initial sorting and culture stress. No significant differences in proliferation or cell death were detected between culture with or without M-CSF.



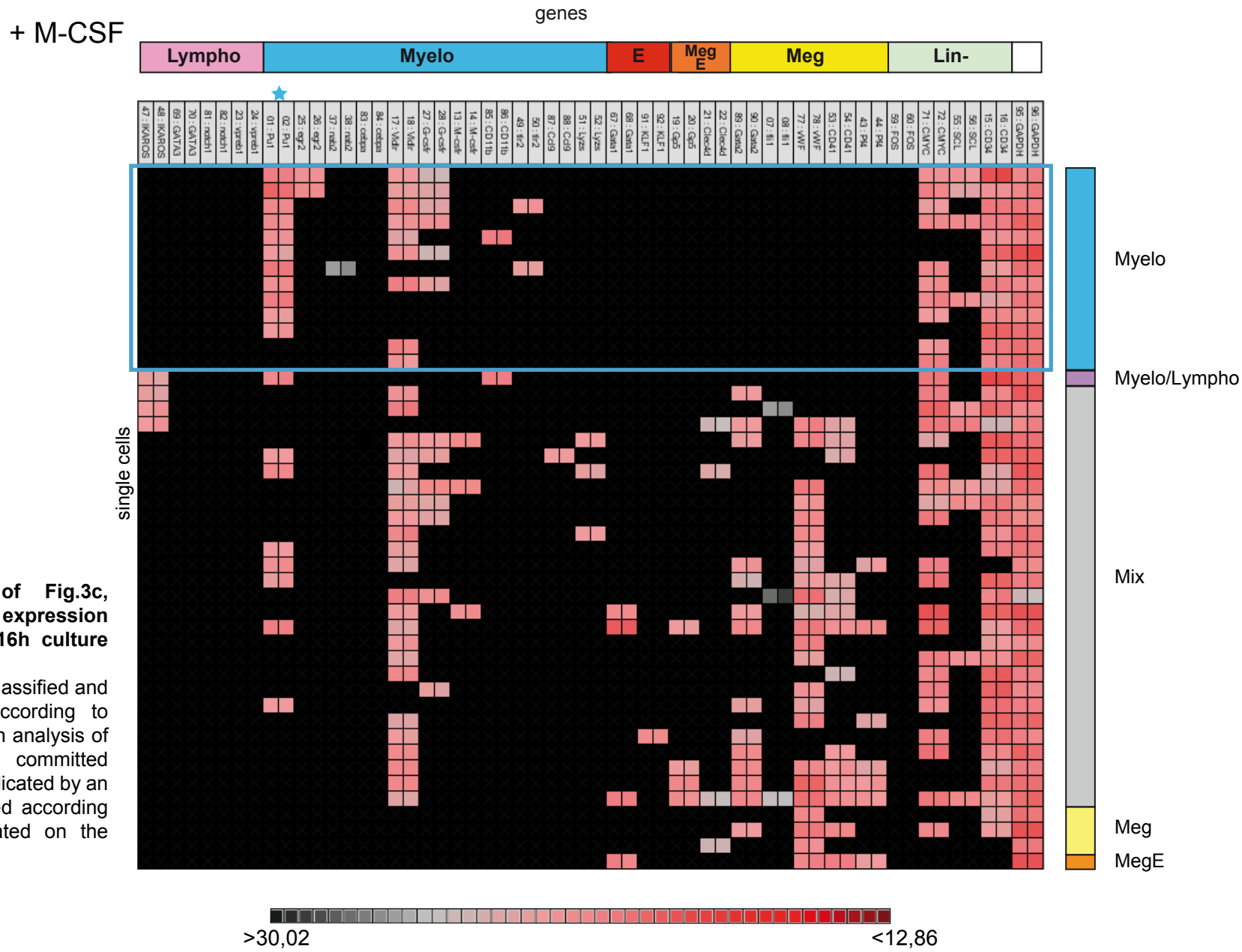
Sup.Fig.6 Analysis of viability of HSC after M-CSF stimulation
Propidium iodide / Annexin V staining of HSC after 16h in culture with or without M-CSF.



Sup.Fig.7 Blow up of Fig.3a, showing single cell gene expression analysis of HSC before culture. The genes analyzed are classified and colour coded on top according to published gene expression analysis of early lineage committed progenitors[40,41]. PU.1 is indicated by an asterix. Cells are clustered according to lineage identity indicated on the right.

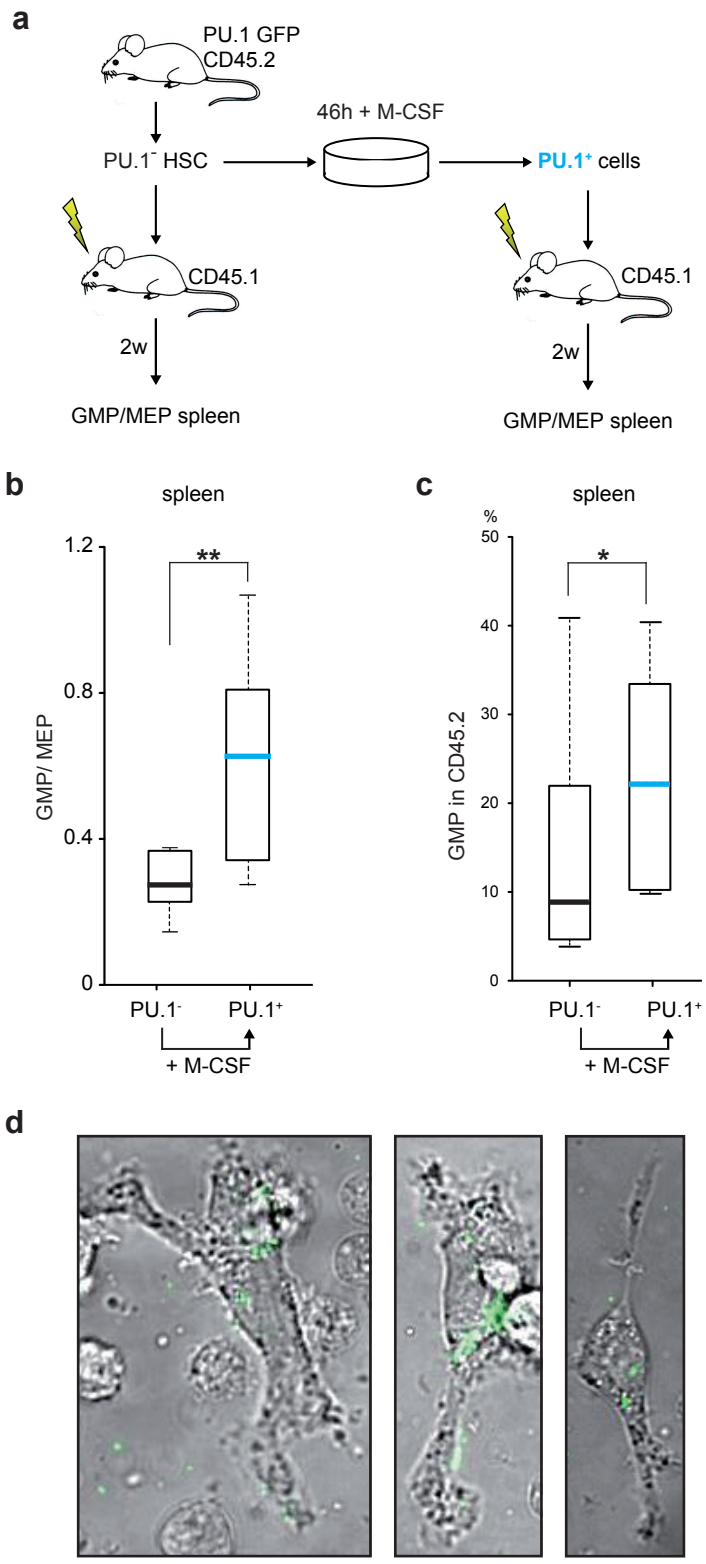


Sup.Fig.8 Blow up of Fig.3b, showing single cell gene expression analysis of HSC after 16h culture without M-CSF.
The genes analyzed are classified and colour coded on top according to published gene expression analysis of early lineage committed progenitors[40,41]. PU.1 is indicated by an asterix. Cells are clustered according to lineage identity indicated on the right.



Sup.Fig.9 Blow up of Fig.3c, showing single cell gene expression analysis of HSC after 16h culture with M-CSF.

The genes analyzed are classified and colour coded on top according to published gene expression analysis of early lineage committed progenitors[40,41]. PU.1 is indicated by an asterix. Cells are clustered according to lineage identity indicated on the right.

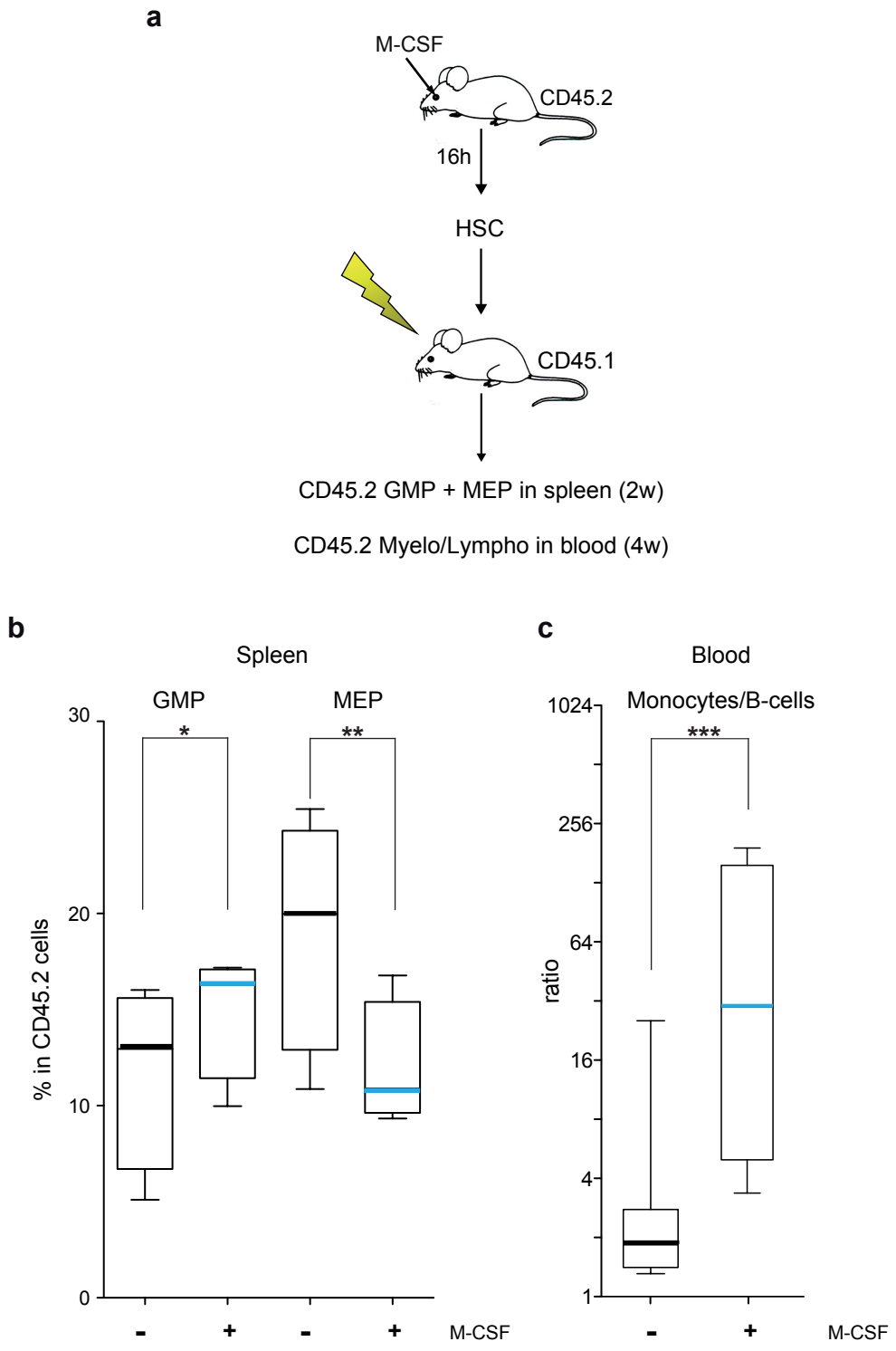


Sup.Fig.10 Differentiation potential of M-CSF induced PU.1+ cells

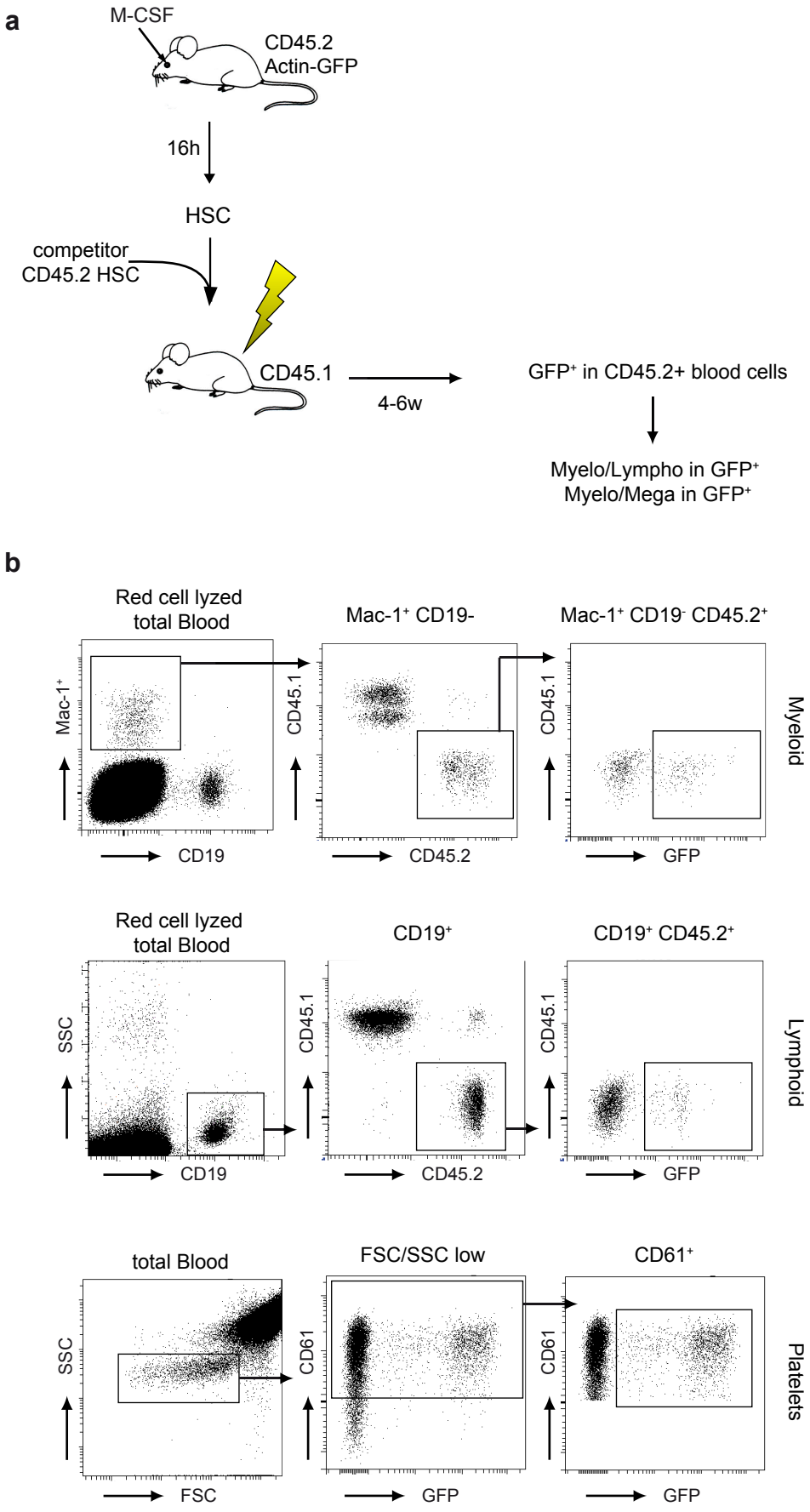
a) Experimental design for transplantation of sorted CD45.2 PU.1- HSC before and after induction of PU.1+ cells in M-CSF culture into sub-lethally irradiated CD45.1 recipients and analysis of progeny cells after 2 weeks in the spleen.

b,c) Quantification of the ratio of donor GMP to MEP progenitors (b) and total GMP (c) derived from transplanted PU.1- HSC before or PU.1+ cells after M-CSF culture. **p = 0.02, *p = 0.07, n = 6,7.

d) Cells with macrophage morphology phagocytosing fluorescent latex beads after continued culture of PU.1+ cells in M-CSF for 10 days.

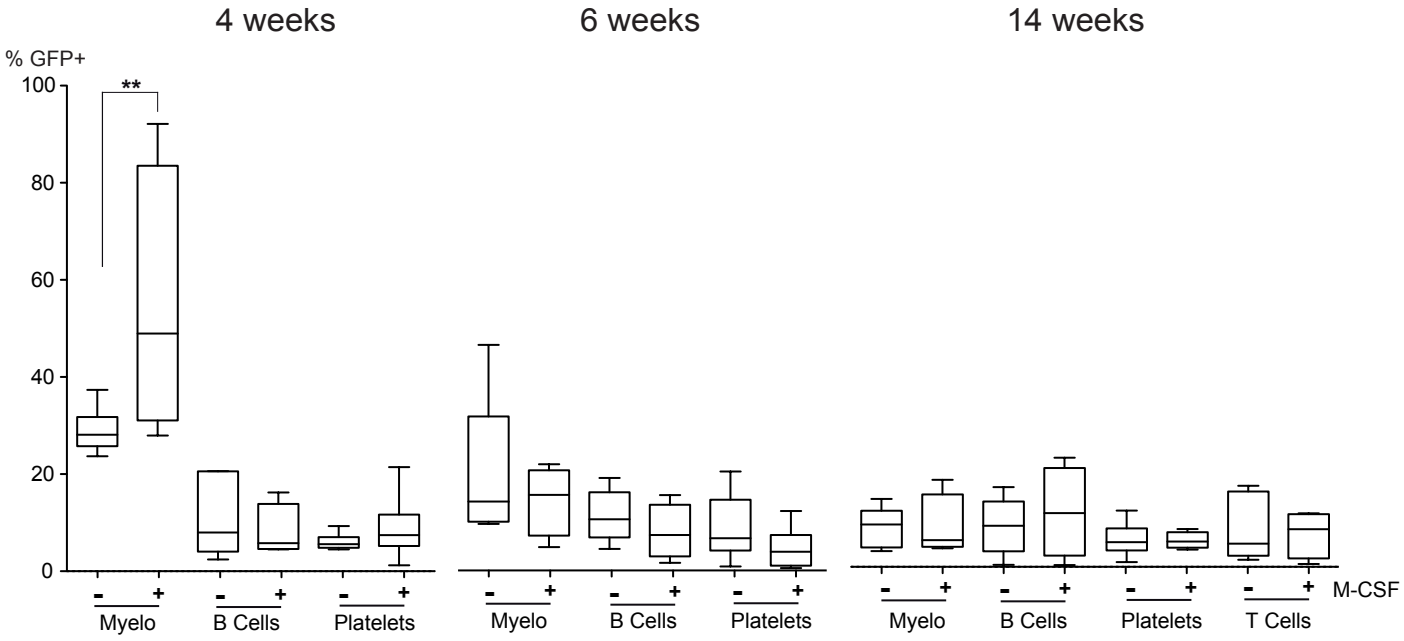


Sup.Fig.11 Differentiation potential of M-CSF primed HSC
a) Experimental design for transplantation of in vivo M-CSF primed CD45.2 HSC into sub-lethally irradiated CD45.1 recipients and analysis of progeny cells after 2 weeks in the spleen or 4 weeks in the blood.
b) Percentage of GMP and MEP progenitors in total donor cells derived from control (PBS) or M-CSF primed HSC in the spleen 2 weeks after transplantation.
*p = 0.1, **p = 0.04, n = 4,4
c) Quantification of the ratio of donor CD11b^{+} SSC^{lo} monocytes to CD19^{+} B-cells in the blood 4 weeks after transplantation. ***p = 0.009, n = 8,4.



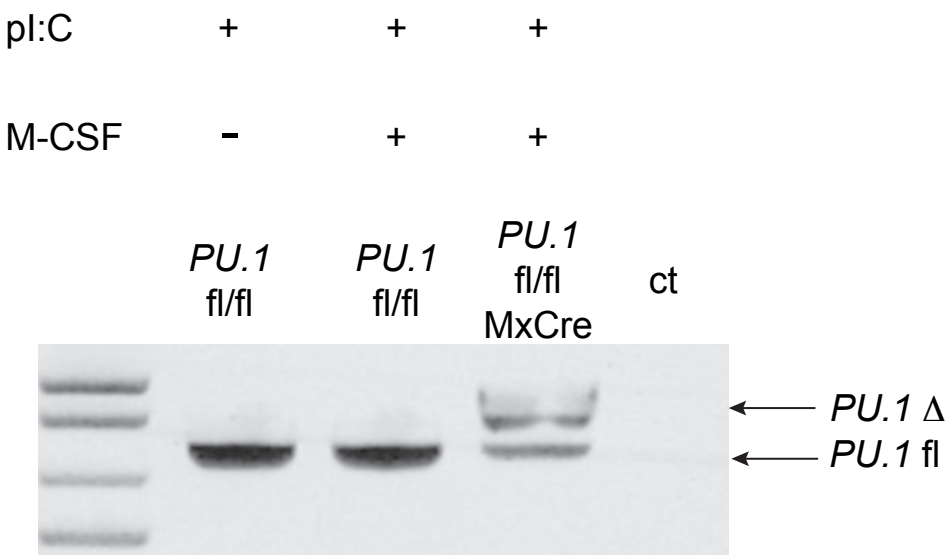
Sup.Fig.12 Competitive transplantation of M-CSF primed HSC
a) Experimental design for competitive transplantation of FACS sorted in vivo M-CSF primed HSC (CD150+CD34-CD135-KSL) from actin-GFP CD45.2 mice together with CD45.2 competitor HSC into lethally irradiated CD45.1 recipients and analysis of blood cell contribution.
b) Gating strategy for quantification of actin-GFP+ HSC derived myeloid , lymphoid blood cells and platelets.

c

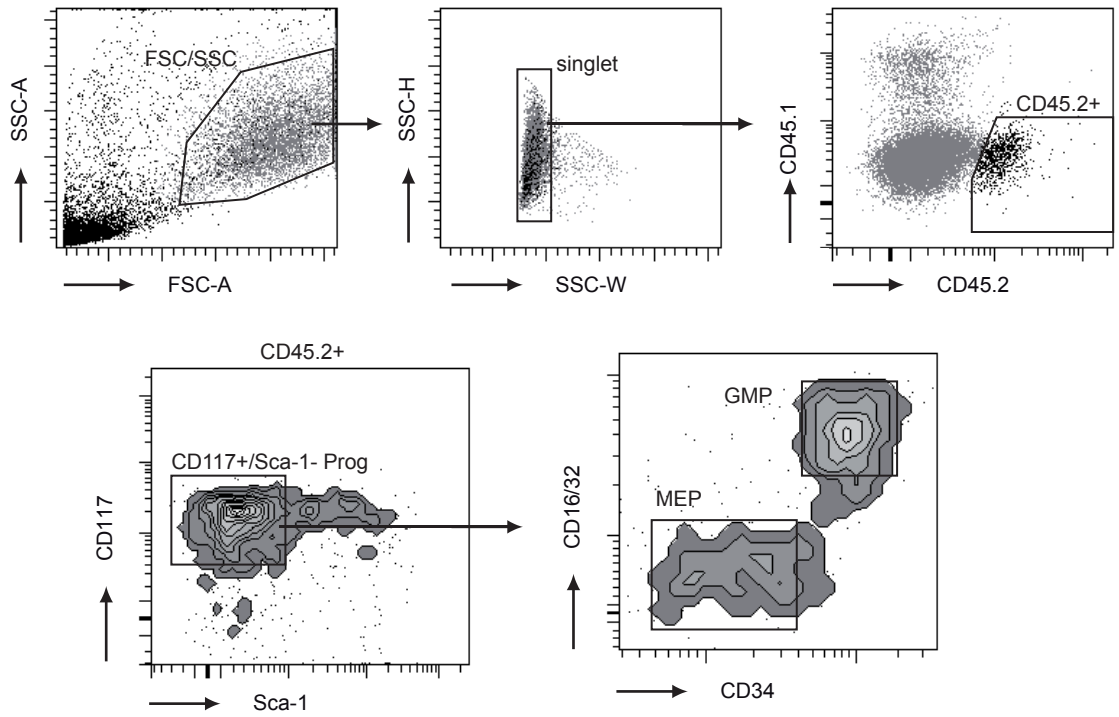


Sup.Fig.12 Competitive transplantation of M-CSF primed HSC

c) Donor contribution to blood of competitively reconstituted mice 4, 6 and 14 weeks after transplantation of M-CSF primed or control HSC, expressed as percentage of GFP+ donor cells in Mac+ myeloid, CD19+ B Cells, CD61+ Platelets (4, 6 and 14 weeks) and CD3e T Cells (14 weeks) and normalized to total GFP contribution in CD45.2 donor compartment. ** p = 0.03, n = 6,4.



Sup.Fig.13 Analysis of PU-1 deletion
PCR analysis to detect deleted (PU.1Δ) and loxP flanked (PU.1fl) PU.1 alleles in samples transplanted in fig.4i from control or M-CSF primed HSC isolated from PU.1fl/fl::MxCre or PU.1fl/fl bone marrow 7 days after last treatment with pI:C.



Sup.Fig.14 Gating strategy for detection of HSC derived donor CD45.2 GMP and MEP populations in the spleens of CD45.1 recipients.