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PU.1-mediated upregulation of *M-CSFR* is critical for MOZ-leukemia stem cell potential

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Abstract

Leukemias and other cancers possess self-renewing stem cells that help to maintain the cancer[1,2]. Cancer stem cell eradication is thought to be critical for successful anti-cancer therapy. Using an acute myeloid leukemia (AML) model induced by introducing the leukemia-associated monocytic leukemia zinc finger (MOZ)-TIF2 fusion protein, we show here that AML can be cured by the ablation of leukemia stem cells. The MOZ-fusion proteins interacted with PU.1 to stimulate the expression of macrophage-colony stimulating factor receptor (M-CSFR, also called CSF1R/c-FMS/CD115). Analysis using PU.1-deficient mice demonstrated that PU.1 was essential for MOZ-TIF2 to establish and maintain AML stem cells. Cells expressing high levels of CSF1R (CSF1R^{high} cells), but not those expressing low levels of CSF1R (CSF1R$^{low/-}$ cells), showed potent leukemia-initiating activity. Using transgenic mice expressing a drug-inducible suicide gene controlled by the *CSF1R* promoter, AML was cured by ablation of the CSF1R^{high} cells. Induction of AML was suppressed in CSF1R-deficient mice. CSF1R inhibitors slowed the progress of MOZ-TIF2–induced leukemia. Thus, CSF1R^{high} cells contain leukemia stem cells, and the PU.1-mediated upregulation of CSF1R may be a useful therapeutic target for *MOZ* leukemia.

Chromosomal translocations that involve the monocytic leukemia zinc finger (*MOZ*) gene[3] are typically associated with the FAB-M4 or -M5 subtype of human acute myeloid leukemia (AML) and often predict a poor prognosis[4]. While MOZ is essential for the self-renewal of hematopoietic stem cells[5,6], MOZ-fusion proteins enable the transformation of non–self-renewing myeloid progenitors into leukemia stem cells[7]. We generated a mouse model for AML by introducing c-KIT^{+} bone marrow (BM) cells infected with MSCV-MOZ-TIF2-ires-EGFP retrovirus into lethally irradiated mice, as previously reported[8].

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To identify leukemia-initiating cells (LIC), we investigated the BM cells of AML mice for various cell surface markers by fluorescence-activated cell sorting (FACS) analysis. CSF1R^{high} and CSF1R$^{low/-}$ cells were present in the AML mouse BM (Fig. 1a) and expressed equivalent levels of MOZ-TIF2 proteins (Fig. 1b). To determine their LIC activity, CSF1R^{high} and CSF1R$^{low/-}$ cells were isolated by cell sorting, and limited numbers (10–10^4 cells) were transplanted into irradiated mice. One-hundred CSF1R^{high} cells were sufficient to induce AML in all mice transplanted (Fig. 1c). Conversely, no mice developed AML after 10^3 CSF1R$^{low/-}$ cells were transplanted per mouse, and only half of the mice developed AML with delayed onset when 10^4 CSF1R$^{low/-}$ cells were transplanted (Fig. 1d). Thus, the CSF1R^{high} cells displayed a > 100-fold stronger LIC activity than CSF1R$^{low/-}$ cells.

FACS analysis indicated that the CSF1R^{high}-LICs were c-Kit$^+$ Sca-1$^-$ CD16/32$^+$ Mac-1^{low} Gr-1$^+$ (Fig. S1A). Comparison of the CSF1R^{high} and CSF1R$^{low/-}$ cells indicated that Mac-1 expression was lower in CSF1R^{high}-LICs than in CSF1R$^{low/-}$ cells (Fig. 1e). However, significant differences were not observed between the CSF1R^{high} and CSF1R$^{low/-}$ cells in their cell morphology (Fig. 1f), colony-forming ability in methylcellulose medium (Fig. 1g), cell cycle distribution (Fig. S1B), or HoxA9 expression levels (Fig. S1C). To investigate if downstream pathways of CSF1R were activated, we measured the phosphorylation levels of STAT5 and ERK in CSF1R^{high} and CSF1R$^{low/-}$ cells. STAT5 was highly phosphorylated in CSF1R^{high} cells but not in CSF1R$^{low/-}$ cells (Fig. 1h), while ERK was phosphorylated in both CSF1R^{high} and CSF1R$^{low/-}$ cells.

Side population (SP) cells, a feature of some normal and malignant stem cells, were present in the BM of MOZ-TIF2-induced AML mice (Fig. S2A). While most SP cells were CSF1R^{high} cells, non-SP cells contained both M-CSFR^{high} and CSF1R$^{low/-}$ cells (Fig. S2B). The LICs were ~ 10-fold more enriched in the SP fraction than in the non-SP fraction (Fig. S2, C and D). Since the SP population was very small (~ 0.12%), the population of LICs in the SP fraction was also small (~ 1% of all LICs) and most of the LICs were present in the non-SP fraction (~ 99%).

To determine if a high level of CSF1R expression also occurs in human AML cells with MOZ translocations, we investigated CSF1R expression in BM cells from an AML patient with a t(8;16) translocation expressing *MOZ-CBP*[9]. FACS analysis indicated that CSF1R^{high} and CSF1R$^{low/-}$ cells were also observed in the AML cells with t(8;16) (Fig. 1i). MOZ-CBP fusion transcripts were detected in both CSF1R^{high} and CSF1R$^{low/-}$ cells (Fig. 1j).

The above results suggest that leukemia stem cells express a high level of CSF1R, indicating that leukemia might be cured by inducing apoptosis of CSF1R^{high} cells. To test this, we used transgenic mice expressing a drug-inducible FKBP-Fas suicide gene and EGFP under control of the CSF1R promoter[10] (Fig. 2a). The suicide gene products are inactive monomers under normal conditions, but can be activated by injection of the AP20187 dimerizer that induces the apoptosis of cells expressing high levels of CSF1R[10]. The c-KIT$^+$ BM cells of transgenic mice were infected with the MOZ-TIF2 retrovirus, and transplanted into lethally irradiated wild-type mice. These mice developed AML ~ 2 months after transplantation, in which morphologically indistinguishable CSF1R^{high} and CSF1R^{low} cells were observed and endogenous CSF1R expression was proportional to EGFP and FKBP-Fas expression levels (Figs. 2b and S3A).

Next, we transplanted the BM cells of these AML mice (10^5 cells/ mouse) into secondary recipient mice. Seven days after transplantation, the mice were injected with AP20187 or a control solvent, as described[10]. An increase in the number of CSF1R^{high} cells (Fig. 2c) and splenomegaly (Fig. 2d) were observed in the control-treated mice three weeks after

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transplantation. However, neither CSF1R^{high} cells nor splenomegaly was detected in the AP20187- treated mice after a one-week course of treatment with AP20187 (Figs. 2c and 2d). Although CSF1R^{low} cells were observed in the BM and peripheral blood after the one-week treatment course, these cells were not detected after three months (Figs 2c and S3B). All control mice developed AML 4–6 weeks after transplantation, but none of the AP20187-treated mice died of AML within 6 months of transplantation (Fig. 2e). These results indicate that ablation of the CSF1R^{high} cells was sufficient to cure MOZ-TIF2–induced AML, and that a high expression level of CSF1R is a key element for leukemia stem cell potential. Since it has been reported that N-Myc overexpression rapidly causes AML in mice[11], we also generated AML mice with MSCV-N-Myc-ires-EGFP using the BM cells of suicide gene–expressing transgenic mice as control animals. In AML mice with N-Myc, the GFP^{+} leukemia cells were Mac1^{+} Gr1^{+} CSF1R^{-} blast cells (Fig. S4, A and B). Treatment of mice with AP20187 did not affect AML induction (Fig. S4C).

To investigate the role of CSFR in the development of MOZ-TIF2–induced AML, wild-type and *Csf1r*$^{-/-}$[12] mouse fetal liver cells of E16.5 littermate embryos were infected with the MOZ-TIF2 virus and transplanted into lethally irradiated mice. All mice transplanted with wild-type cells developed AML within three months. In contrast, AML induction was initially suppressed in mice transplanted with *Csf1r*$^{-/-}$ cells, but half of the mice developed AML after a longer latency period (Fig. 3a). The suppression of AML was rescued by coinfection with the MSCV-CSF1R retrovirus (Fig. 3b). STAT5, which was highly phosphorylated in CSF1R^{high} cells but not in CSF1R$^{low/-}$ cells, was phosphorylated in *Csf1r*$^{+/+}$ cells but not in Csf1r−/− cells (Fig. S5). We also generated AML mice with MSCV-N-Myc-ires-EGFP, using *Csf1r*$^{+/+}$ and *Csf1r*$^{-/-}$ fetal liver cells as controls. All of the mice transplanted with either *Csf1r*$^{+/+}$ or *Csf1r*$^{-/-}$ cells expressing N-Myc developed AML (Fig. S4D). These results indicate that CSF1R is important for AML induction by MOZ-TIF2.

The above results suggest that signaling through CSF1R may be a therapeutic target for kinase inhibitors in *MOZ* leukemogenesis. To test this, we used the CSF1R-specific inhibitor Ki20227[13] and the tyrosine kinase (including CSF1R1) inhibitor Imatinib mesylate (STI571; Glivec^{R})[14–16]. Oral administration of Ki20227 and Imatinib inhibited MOZ-TIF2-induced splenomegaly (Fig. 3C) and slowed MOZ-TIF2–induced AML onset (Fig. 3d). However, they did not affect the progress of N-MYC–induced AML (Fig. 3e).

The monocyte-specific expression of CSF1R is reportedly regulated by transcription factors such as AML1, PU.1, and C/EBP[17]. We previously found that MOZ interacts with AML1 and PU.1, but not with C/EBPα or C/EBPε, to stimulate transcription of their target genes[5,18]. Deletion analysis indicated that PU.1 interacts with the N-terminal and central regions of MOZ (Figs. 4a and S6), and that the acidic amino acid–rich region (DE region) of PU.1 was required for its high-affinity interaction with MOZ (Figs. 4a and S7A–D). While several deletions in the PU.1 protein prevent binding to N-terminal MOZ (1–513) (Fig S7C), considerable binding is retained with the full length protein (Fig S7B), suggesting there may be other PU.1-binding sites in MOZ and/or associated proteins. A pull-down assay using *E. coli*-produced GST-PU.1 or GST-AML1 and *in vitro*-produced N-terminal MOZ indicated a direct interaction between PU.1/AML1 and MOZ (Fig. S8). However, these interactions are weak in comparison to those observed in coimmunoprecipitates, suggesting that other factors may facilitate these interactions *in vivo*. Reporter analysis using a *CSF1R* promoter-luciferase construct showed that MOZ, MOZ-CBP, and MOZ-TIF2 activated the *CSF1R* promoter in the presence of PU.1, but not in the presence of AML1 (Fig. 4b). MOZ, MOZ-TIF2, and MOZ-CBP did not activate a *CSF1R* promoter mutant lacking PU.1-binding sites (Fig. 4c). These results suggest that MOZ and MOZ-fusion proteins activate *CSF1R* transcription in a PU.1-dependent manner. Deletion analysis indicated that the DE, Q, and

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ETS domains of PU.1, as well as the H15 and the central PU.1-binding domains of MOZ/ MOZ-fusions, are required for the activation of *CSF1R* transcription (Figs. S7E and S9). The MOZ mutant lacking the C-terminal region (1–1518) failed to activate the transcription, indicating that the transcriptional activity of MOZ-TIF2/MOZ-CBP requires the TIF2 or CBP sequence Hoogenkamp et al.[19] recently reported that, although chromatin reorganization of *Csf1r* requires prior PU.1 expression together with AML1 binding, once the full hematopoietic program is established, stable transcription factor complexes and active chromatin can be maintained without AML1 at the *Csf1r* locus. This might explain why AML1 was not required for the MOZ-TIF2-mediated activation of *Csf1r*.

To test for the expression of endogenous *CSF1R*, we used PU.$1^{-/-}$ myeloid progenitors expressing the PU.1-estrogen receptor fusion protein (PUER). Upon restoration of PU.1 activity by exposure to 4-hydroxytamoxifen (4-HT), PUER cells can differentiate into macrophages[20]. We infected PUER cells with MSCV-MOZ-TIF2-ires-GFP or control retroviruses, and sorted and cultured GFP^+ cells in the presence of 4-HT. The results of FACS (Fig. 4d) and quantitative RT-PCR (Fig. S10) analyses indicated that CSF1R expression was induced after exposure to 4-HT, and that MOZ-TIF2 enhanced the PU.1-induced upregulation of CSF1R. Importantly, five days after exposure to 4-HT, $CSF1R^{high}$ and $CSF1R^{low}$ cells were detected in PUER cells expressing MOZ-TIF2 but not in control cells. CSF1R expression was not induced before addition of 4-HT, even in PUER cells expressing MOZ-TIF2, indicating that functional PU.1 is required for MOZ-TIF2–induced CSF1R expression. Chromatin-immunoprecipitation (ChIP) analysis indicated that PU.1 and MOZ/MOZ-TIF2 were recruited to *Csf1r* in the BM cells of MOZ-TIF2-induced AML mice (Fig. S11A). In PU-ER cells expressing MOZ-TIF2, recruitment of MOZ/MOZ-TIF2 was detected after 4-HT treatment, but not before the treatment (Fig. S11B), suggesting that recruitment of MOZ/MOZ-TIF2 is dependent upon functional PU.1.

To determine if PU.1 is essential for the development of MOZ-TIF2–induced AML, PU.$1^{+/+}$ and PU.$1^{-/-}$ fetal liver cells of E12.5 litter mates were infected with retroviruses for MOZ-TIF2 or N-Myc as a control, and were transplanted into irradiated mice. Although mice with PU.$1^{+/+}$ cells expressing MOZ-TIF2 developed AML 8–14 weeks after transplantation, mice with PU.$1^{-/-}$ cells were quite healthy for at least 6 months (Fig. 4e). In contrast, all mice transplanted with either PU.$1^{-/-}$ or PU.$1^{+/+}$ cells expressing N-Myc developed AML 6–10 weeks after transplantation (Figure 4f). When both PU.1 and MOZ-TIF2 were introduced into PU.1-deficient fetal liver cells, the mice developed leukemia (Fig. 4g). However, introduction of either PU.1 or MOZ-TIF2 alone was not sufficient for AML induction in mice. Thus, we conclude that PU.1 is required for the initiation of MOZ-TIF2–induced AML.

To determine if PU.1 is required for the maintenance of MOZ-TIF2-induced AML, fetal liver cells of PU.1 conditional knock-out mice (PU.$1^{flox/flox}$ expressing ER-Cre) were infected with MOZ-TIF2 to induce AML. The BM cells of AML mice were again transplanted into irradiated mice, and half of the mice were then treated with tamoxifene to induce PU.1 deletion (Fig. 4h). All of the control mice died of AML within 6 weeks, but none of the tamoxifene-treated mice developed AML for at least for 6 months. These results indicate that PU.1 is also required for the maintenance of MOZ-TIF2–induced AML stem cells.

Taken together, our results indicate that MOZ and its leukemia-associated fusion proteins activate the PU.1–mediated transcription of monocyte-specific *Csf1r*. MOZ-fusions might constitutively stimulate high *Csf1r* expression levels to induce AML (Fig. 4i). In contrast, we previously found that MOZ-fusions inhibited AML1-mediated activation of granulocyte-specific *Mpo* gene transcription[18]. Since MOZ-fusions are associated with monocytic

leukemia, the lineage commitment may be determined by differential regulation of the target genes by MOZ fusions (i.e., upregulation of monocyte specific genes such as *Csf1r* and downregulation of granulocyte-specific genes such as MPO). It is also likely that normal MOZ modulates *Csf1r* expression to an appropriate level to regulate normal hematopoiesis (Fig. 4i), since *Csf1r* expression was impaired in $MOZ^{-/-}$ fetal liver cells (Fig. S12).

We observed that AML induction was suppressed in mice transplanted with $Csf1r^{-/-}$ cells, but half of these mice developed AML, albeit at a longer latency. Since MOZ-TIF2 can induce AML in the absence of *Csf1r*, MOZ-TIF2 can provoke rapid induction of AML not alone in a CSF1R-dependent manner, but also in a CSF1R-independent AML at longer latency. There are several possibilities for why CSF1R-independent AML cells escape CSF1R-dependency. Increased HoxA9 expression was observed in both $CSF1R^{high}$ and $CSF1R^{low/-}$ cells (Fig. 1h). HoxA9 overexpression is reportedly not sufficient to induce AML, but requires additional mutations or oncogene activations[21,22]. Thus, it is possible that CSF1R-independent cells require additional mutations to escape CSF1R-dependency. Since we used a retrovirus vector to introduce MOZ-TIF2, it is also possible that oncogene activation by retroviral integration may mediate AML pathogenesis. To investigate whether downstream pathways of CSF1R were activated, we checked the STAT5 phosphorylation levels in $CSF1R^{+/+}$ AML cells and $CSF1R^{-/-}$ AML cells, and found that STAT5 was highly phosphorylated in $CSF1R^{+/+}$ AML cells but not in $CSF1R^{-/-}$ AML cells (Fig. S5).

In conclusion, our results indicate that PU.1-mediated upregulation of *Csf1r* is critical for MOZ-leukemia stem cell potential. Association of CSF1R with AML has been suggested by several previous findings. CSF1R upregulation has been reported in human[23–25] and mouse[26] AML, and CSF1R is also known as the protooncogene c-Fms. Multilineage hematopoietic disorders are induced by the transplantation of BM cells expressing the v-fms oncogene[27]. It was recently reported that CSF1R is involved in a chromosomal translocation associated with AML, which results in expression of a novel fusion of RBM6 to CSF1R[28]. These results suggest that CSF1R is important for not only MOZ-leukemia, but also for a subset of AMLs.

METHODS

Mice

C57BL/6 mice were purchased from CREA Japan (Tokyo). NGF-FKBP-Fas transgenic mice[10] (Jackson Lab.), *CSF1R*-deficient mice[12], *PU.1*-null/conditional deficient mice[29], and CreERT2 mice (TaconicArtemis GmbH)[30] were maintained on a C57BL/6 genetic background. Mouse experiments were performed in a specific pathogen-free environment at the National Cancer Center animal facility according to institutional guidelines and with approval of the National Cancer Center Animal Ethics Committee.

Generation of AML mouse models

MSCV-MOZ-TIF2-ires-GFP, MSCV-N-MYC-ires-GFP, MSCV-CSF1R-pgk-pac, and MSCV-PU.1-pgk-pac were generated by inserting their cDNAs into the respective vectors. The constructs were transfected with PLAT-E[31] cells using the FuGENE 6 reagent (Roche Diagnostics), and supernatants containing retrovirus were collected 48 h after transfection. The c-Kit+ cells (1×10^5 cells), which were selected from BM or fetal liver cells using CD117 MicroBeads (Militenyi Biotec), were incubated with the retrovirus using RetroNectin (Takara Bio) for 24 h in StemPro-34 SFM medium (Invitrogen) containing cytokines (20 ng/mL SCF, 10 ng/mL IL-6, 10 ng/mL IL-3). The infectants were then transplanted together with BM cells (2×10^5) into lethally irradiated (9 Gy) 6- to 8-week-old C57BL/6 mice by intravenous (IV) injection. Secondary transplants were performed by

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intravenous injection of BM cells from the primary AML mice into sublethally irradiated (6 Gy) C57BL/6 mice.

Administration of AP20187, Imatinib, or Ki20227

AP20187 (gift from Ariad Pharmaceuticals; 10 mg/kg) was administered daily by IV injection for 5 d, and then 1 mg/kg AP20187 was administered every 3 d thereafter as described previously10. Mice were orally administrated Imatinib mesilate (Novoltis Pharma; 100 mg/kg), Ki2022713 (gift from KIRIN Pharma; 20 mg/kg), or solvent twice daily from 7 d after transplantation.

Immunofluorescent staining, flow cytometric analysis, and cell sorting

Bone marrow cells from AML mice were preincubated with rat IgG, and then incubated on ice with the following staining reagents: anti-CD115(CSF1R)-PE (eBioscience), anti–Mac-1 (M1/70)-PE-Cy7 (eBioscience), anti–Gr-1(RB6-8C5)-APC (BD Pharmingen), and anti-c-Kit-APC (2B8)-APC (BD Pharmingen). Flow cytometric analysis and cell sorting were performed using the cell sorter JSAN (Baybioscience), and the results were analyzed using FlowJo software (Tree Star).

Reporter analysis

CSF1R-luciferase constructs were generated by ligation of wild-type and PU.1-lacking CSF1R promoter[32] with pGL4. For reporter analysis, SaOS2 cells were transfected with CSF1R-luc and phRL-CMV together with various expression constructs in 24-well plates, and luciferase activity was assayed 24 h after transfection using the microplate luminometer GLOMAX (Promega). Results of reporter assays represent the average values for relative luciferase activity generated from at least three independent experiments that were normalized using the activity of the enzyme from phRL-CMV as an internal control.

Immunoprecipitation and immunoblotting

For immunoprecipitation experiments, cells were lysed in a lysis buffer containing 250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1? NP-40, 5 mM DTT, 1 mM PMSF, and protease inhibitor. Cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads (Sigma) and slightly rotated at 4°C overnight. The absorbed beads were washed 3 times with lysis buffer. Precipitated proteins were eluted from the beads by FLAG peptide and dissolved with the same volume of 2X SDS sample buffer. When immunoprecipitation was not performed, total protein lysates were prepared in 2X SDS sample buffer. Antibodies were detected by chemiluminescence using ECL plus Detection Reagents (Amersham Biosciences, Buckinghamshire, United Kingdom). The primary antibodies used in this study were anti-FLAG (M2) (Sigma), anti-HA (3F10) (Roche), and anti-MOZ[18] antibodies.

Statistical analyses

We performed unpaired two-tailed Student's *t*-tests for comparisons and a log-rank test for survival data using JMP8 software (SAS Institute).

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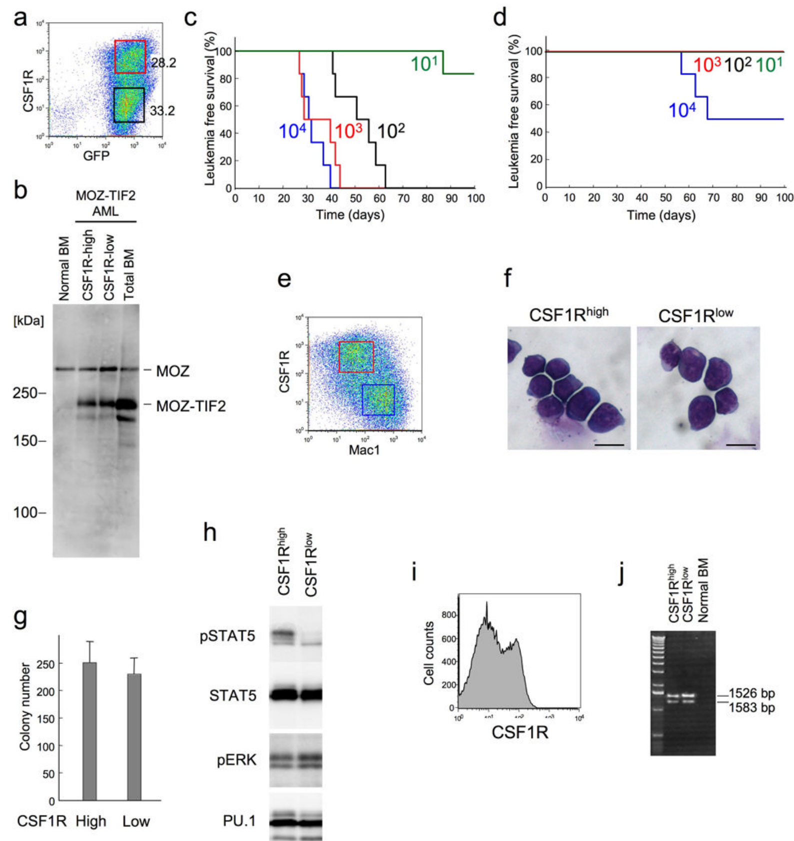

Fig. 1. CSF1R^{high} cells show potent leukemia-initiating activity

(**a**) The BM cells from MOZ-TIF2–induced AML mice were analyzed by FACS for expression of GFP and CSF1R. (**b**) CSF1R^{high} and CSF1R$^{low/-}$ cells were sorted by flow cytometry, and MOZ-TIF2 expression was investigated by immunoblot analysis using an anti-MOZ antibody. (**c, d**) The indicated numbers of flow-sorted CSF1R^{high} (c) and CSF1R$^{low/-}$ (c) cells were transplanted into sub-lethally irradiated mice, and leukemia-free survival was investigated. $n = 6$, $P = 0.0001$ (10^4, 10^3 and 10^2) and 0.3173 (10^1). Three independent experiments were performed and essentially the same results were obtained. (**e**) The BM cells from MOZ-TIF2–induced AML mice were analyzed by FACS for expression of Mac1 and CSF1R. (**f–h**) CSF1R^{high} and CSF1R$^{low/-}$ cells were sorted and analyzed for morphology by staining with May-Giemsa (f), colony-forming activity in methylcellulose medium (g), and levels of total and phosphorylated STAT5, phosphorylated ERK, and PU.1 (h). The scale bars represent 10 ?m in (f). (**i**) The BM cells from an AML patient with t(8;16) were cultured for 3 d in 10 ng/ml hM-CSF and tested by FACS for CSF1R expression. (**j**) CSF1R^{high} and CSF1R$^{low/-}$ cells of the AML patient with t(8;16) were sorted and analyzed for MOZ-CBP transcripts by RT-PCR.

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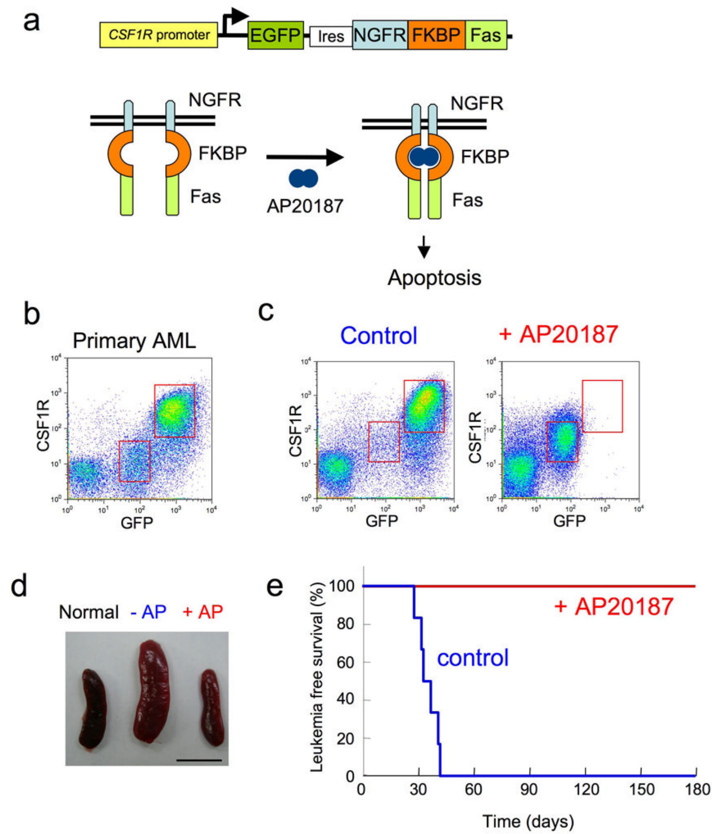

Fig. 2. Cure of AML by ablation of CSF1R^{high} cells

(**a**) Structure of genes for the *CSF1R* promoter, EGFP, the NGFR–FKBP–Fas suicide construct, and activation of NGFR–FKBP–Fas. In transgenic (Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6) mice, conditional ablation of cells expressing high levels of CSF1R can be induced by exposure to the AP20187 dimerizer. (**b**) The BM cells from transgenic mice were infected with MSCV-MOZ-TIF2-ires-GFP and were transplanted into lethally irradiated C57BL/6 mice to induce AML. Expression levels of GFP and CSF1R in BM cells were analyzed by flow cytometry two months after transplantation. (**c–e**) Bone marrow cells (1×10^5) of primary AML mice were transplanted into sub-lethally irradiated C57BL/6 mice. Administration of AP20187 or solvent (control) to the secondary AML mice was started by IV injection three weeks after transplantation. Expression of GFP and CSF1R in BM cells (c) and spleen sizes (d) were analyzed four weeks after transplantation. Bars represent 1 cm. Leukemia-free survivals of the untreated (n = 6) and AP20187-treated (n = 6) secondary transplanted mice were also investigated (e). $P < 0.0001$

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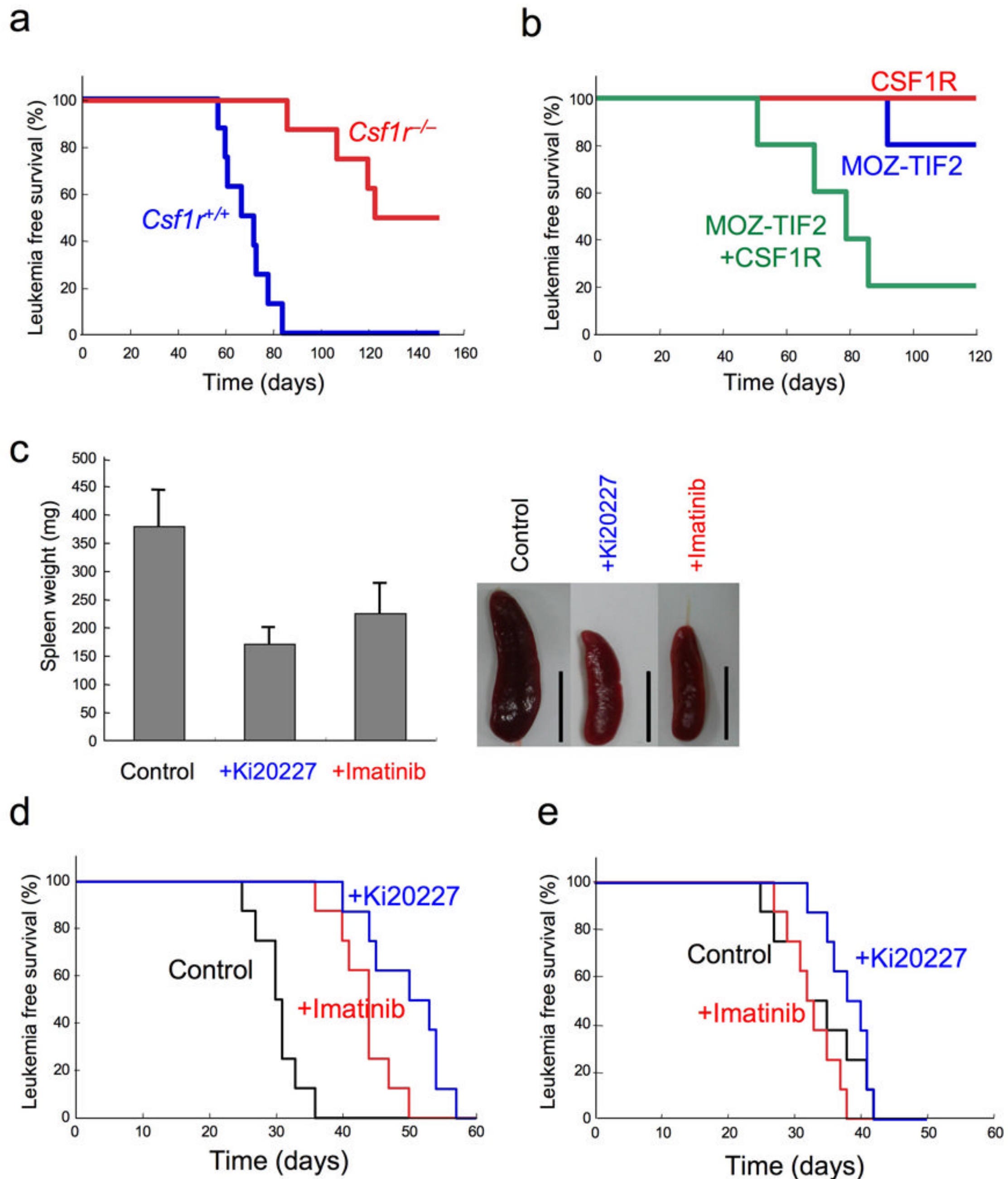

Fig. 3. Roles of CSF1R in MOZ-TIF2–induced AML

(**a**) Fetal liver cells of E16.5 CSF1R$^{+/+}$ and CSF1R$^{-/-}$ mice embryo littermates were infected with either MOZ-TIF2-ires-GFP, and transplanted into irradiated mice. The leukemia-free survivals of the mice were analyzed. $n = 8$, $P < 0.0001$ (**b**) Fetal liver cells of CSF1R$^{+/+}$ mice were infected with CSF1R, MOZ-TIF2, or both, and transplanted into irradiated mice. $n = 5$ (**c–e**) The BM cells (10^5) from AML mice with MOZ-TIF2 (c, d) or N-MYC (e) were transplanted into irradiated mice. Imatinib mesylate was administrated twice daily. Spleen sizes of the MOZ-TIF2–introduced mice were analyzed three weeks after transplantation (C). Bars represent 1 cm. Leukemia-free survivals of the mice were

analyzed (d, e). (d) $n = 8$, $P < 0.0001$ (+Ki20227 and +Imatinib) (e) $n = 8$, $P = 0.3825$ (+Ki20227) and 0.4051 (+Imatinib)

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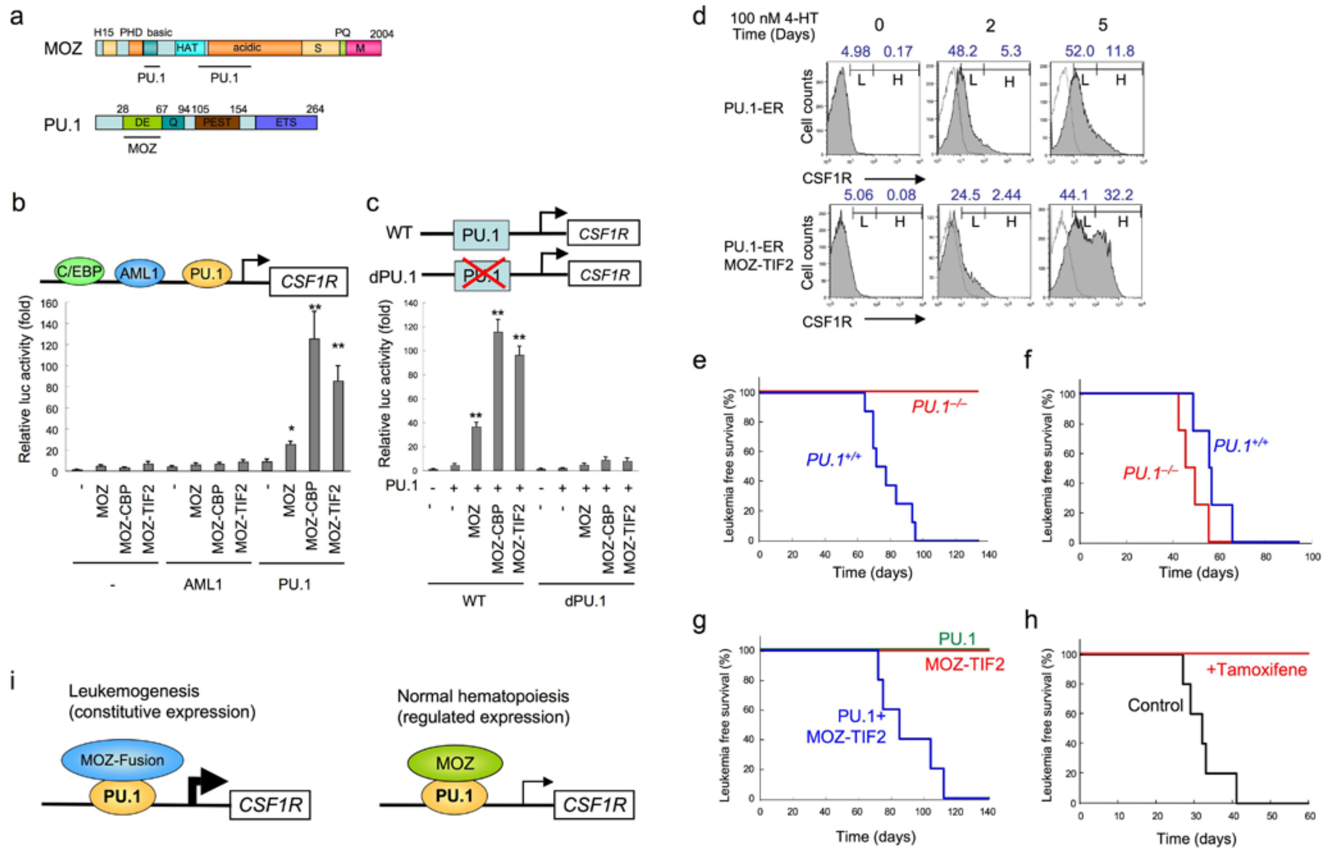

Fig. 4. PU.1-dependent upregulation of CSF1R by MOZ-fusions

(**a**) Protein-interacting domains on MOZ and PU.1. (**b**) Effects of MOZ, MOZ-CBP, and MOZ-TIF2 on AML1- and PU.1-mediated transcription of the CSF1R promoter. SaOS2 cells were transfected with the CSF1R–luciferase construct and the effectors indicated. Luciferase activity was analyzed 24 h after transfection. Error bars represent SD ($n = 3$). * $P < 0.01$ and ** $P < 0.005$. Six independent experiments were performed and essentially the same results were obtained. (**c**) SaOS2 cells were transfected with the wild-type *CSF1R*–luciferase construct or its mutant lacking the PU.1-binding site, together with effectors indicated. (**d**) PUER cells infected with MSCV-GFP or MSCV-MOZ-TIF2-ires-GFP retroviruses were exposed to 100 nM 4-hydroxytamoxifen (4-HT) for 0, 2, or 5 d and analyzed by FACS for CSF1R expression. Three independent experiments showed the same results. (**e–g**) Fetal liver cells of E12.5 PU.1$^{+/+}$ and PU.1$^{-/-}$ mouse embryo littermates were infected with either MOZ-TIF2 (e) or N-MYC (f), and transplanted into irradiated mice. Fetal liver cells of PU.1$^{-/-}$ mice were infected with PU.1, MOZ-TIF2, or both, and transplanted into irradiated mice (g). Leukemia-free survivals of the mice were analyzed. (e) $n = 8$, $P < 0.0001$ (F) $n = 4$, $P = 0.0943$ (g) $n = 5$, $P = 0.0001$ (PU.1 + MOZ-TIF2 vs. others) (**h**) The fetal liver cells of E14.5 PU.1$^{flox/flox}$ with ER-Cre were infected with MOZ-TIF2, and transplanted into irradiated mice. The BM cells of the primary AML mice were transplanted into sub-lethally irradiated wild-type mice. Tamoxifene or solvent (control) was administered to the secondary AML mice every 2 d by intravenous injection 17 d after transplantation, when GFP+ cells were detected in peripheral blood. Leukemia-free survivals of the secondary mice were investigated. $n = 5$, $P = 0.0018$ (**i**) Model for transcriptional regulation by normal and fusion MOZ proteins. MOZ-fusions stimulated constitutive CSF1R expression to induce leukemia (left panel). Normal MOZ controlled CSF1R expression by binding to PU.1 to regulate normal hematopoiesis (right panel).