Transcription intermediary factor 1γ (TIF1γ) was suggested to play a role in erythropoiesis. However, how TIF1γ regulates the development of different blood cell lineages and whether TIF1γ is involved in human hematological malignancies remain to be determined. Here we have shown that TIF1γ was a tumor suppressor in mouse and human chronic myelomonocytic leukemia (CMML). Loss of TIF1g in mouse HSCs favored the expansion of the granulo-monocytic progenitor compartment. Furthermore, Tif1g deletion induced the age-dependent appearance of a cell-autonomous myeloproliferative disorder in mice that recapitulated essential characteristics of human CMML. TIF1γ was almost undetectable in leukemic cells of 35% of CMML patients. This downregulation was related to the hypermethylation of CpG sequences and specific histone modifications in the gene promoter. A demethylation agent restored the normal epigenetic status of the TIF1G promoter in human cells, which correlated with a reestablishment of TIF1γ expression. Together, these results demonstrate that TIF1G is an epigenetically regulated tumor suppressor gene in hematopoietic cells and suggest that changes in TIF1γ expression may be a biomarker of response to demethylating agents in CMML.

Introduction

TIF1γ (also known as tripartite motif protein TRIM33) is an ubiquitous nuclear protein that belongs to the transcriptional intermediary factor 1 family (1). Four TIF1 family members (α to δ) have been identified in mammals, and orthologs are present in organisms such as Drosophila (1–6). TIF1α (also known as TRIM24) interacts with nuclear receptors and modulates their transcriptional activity either positively or negatively in a ligand-dependent fashion (5, 7). In mice, TIF1α functions as a liver-specific tumor suppressor whose deletion reveals the deleterious effect of retinoic acid receptor α aberrant activation to liver homeostasis (8). TIF1β, a component of the histone deacetylase N-CoR1/HDAC3 complex (9), functions as a corepressor for the large family of Krüppel-associated box (KRAB) zinc finger transcription factors (3, 10) and is required for post-implantation embryogenesis and mesoderm induction (11). TIF1δ is involved in heterochromatin-mediated gene silencing (4). Human and mouse TIF1G is closely related to zebrafish moonshine (mon), a gene whose mutations disrupt embryonic and adult hematopoiesis with severe red blood cell aplasia (12). Targeted deletion of Tif1g is embryonic lethal in mice (13, 14). In zebrafish and human CD34+ cells, TIF1γ functionally links positive elongation factors such as p-TEFb and FACT to blood-specific transcription complexes (e.g., the SCL/TAL1 complex) to regulate elongation of genes by antagonizing RNA polymerase II (RNA Pol II) pausing (15). TIF1γ also affects the human hematopoietic progenitor cell response to the cytokines of the TGF-β superfamily through various mechanisms (14, 16–18).

To further explore the role of TIF1γ in hematopoiesis, we examined the effects of hematopoietic tissue–targeted deletion of Tif1g in mice. Tif1g deletion affects the transition from very primitive progenitors (i.e., LT-HSC population) to common myeloid progenitors and leads to a selective expansion of granulo-monocytic progenitors. This effect correlates with an inhibition of the hematopoietic progenitor cell response to TGF-β and provokes the age-dependent appearance of a cell-autonomous phenotype that recapitulates important features of human chronic myelomonocytic leukemia (CMML). Interestingly, a downregulation of TIF1G gene expression is observed in hematopoietic cells of approximately 35% of patients with CMML. While no inactivating mutations were identified, a low level of TIF1γ expression in CMML cells was related to the hypermethylation of the gene promoter, and the expression of TIF1γ was reestablished after treatment with the demethylating agent decitabine, suggesting that changes in TIF1γ expression may be a biomarker of HSC fate that behaves as a tumor suppressor gene.

Results

The Tif1g deletion affects hematopoietic progenitor populations in mice. To obtain further insights into the contribution of TIF1γ to adult hematopoiesis, and in particular HSCs, we generated mice selec-
tively deficient for Tif1γ by breeding floxed Tif1g mice (Tif1g*Δ/Δ) (19) with cFES-Cre transgenic animals (ref. 20 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45213DS1). In litters from crosses between Tif1g*Δ/Δ mice and cFES-Cre transgenic animals, cFES-Cre;Tif1g*Δ/Δ (Tif1g*Δ/Δ) mice correspond to the hematopoietic tissue–restricted knockout mice, whereas Tif1g*Δ/Δ mice represent controls (Supplemental Figure 1A). Although Tif1g-null mice invariably died perinatally (13, 14), Tif1g*Δ/Δ mice reached adulthood and were fertile. As expected, the deletion of Tif1g floxed alleles was observed in hematopoietic organs and cells, including long-term HSCs (LT-HSCs) (Supplemental Figure 1, B and C, and ref. 20), and was associated with a low expression of Tif1g at both the RNA and protein levels in the hematopoietic organs (Supplemental Figure 1, D and E).

Mice younger than 6 months old did not display any macroscopic and blood peripheral abnormalities (data not shown). Nevertheless, the proportion of granulocyte/monocyte progenitors (GMPs; Lin−Sca1−c-Kit−CD34−CD16/32+) was increased (~40%) at the expense of common myeloid progenitors (CMPs; Lin−Sca1−c-Kit−CD34+CD16/32−) and megakaryocyte-erythroid progenitors (MEPs; Lin−Sca1−c-Kit−CD34+CD16/32−; ~50%) and megakaryocyte-erythroid progenitors (MEPs; Lin−Sca1−c-Kit−CD34+CD16/32−; ~60%) (Figure 1). A significant increase in the Lin−Sca1−c-Kit+ (LSK) fraction was also observed (Figure 2, A and B), including an increase in the proportion of short-term HSCs/multipotent progenitors (ST-HSCs/MPPs; LSK CD34+) (Figure 2, C and D) and a decrease in the fraction of primitive LSK (long-term reconstituting HSCs [LT-HSCs]) identified on the “SLAM code” (signaling lymphocyte activation molecule: CD150+CD48−) (ref. 21 and Figure 2, E and F). These data demonstrate that Tif1γ is a key regulator of HSC fate.

Tif1g*Δ/Δ mice develop a CML-like myeloproliferative disease with monocytic features. Mice older than 6 months developed a progressive hyperleukocytosis (data not shown). Although the number of MEPs was decreased, there were no obvious signs of anemia before or after the onset of the disease (Supplemental Figure 2A). The number of Ter119+CD71+ erythroid progenitors was slightly decreased in the bone marrow, while it is increased in the spleen (Supplemental Figure 2B). Morphologic analysis of peripheral blood identified Howell-Jolly bodies and stomatocytes in mice older than 6 months (Supplemental Figure 2C). The progressive hyperleukocytosis observed was due to the accumulation of monocytes (Figure 3A), which was confirmed by FACS analysis (Gr1+Mac1+ cells) (Figure 3B). Bone marrow examination showed an accumulation of monocytes (Figure 3, C and D), which was also observed in the spleen and in the liver (Figure 3, D–H), leading to a severe hepatosplenomegaly (Supplemental Figure 3A). The splenic organization was destroyed (Figure 3E) by mature highly proliferative Mac1+ cells (Figure 3, F and G) that invaded the red pulp (Supplemental Figure 3B). Immature hematopoietic cells including erythroblast cells were observed in the spleen (not shown), and the liver was also infiltrated by highly proliferative cells (Figure 3H). Kinetics of hematopoietic recovery after sublethal irradiation of control or Tif1g*Δ/Δ mice did not reveal another lineage specificity of Tif1g deletion, i.e., these mice recovered normally and accumulated monocytes in their blood and spleen when progressing in age (data not shown).

Tif1g*Δ/Δ myeloproliferative disease is transplantable into secondary recipients. Mice transplanted with Tif1g*Δ/Δ bone marrow cells from 4-month-old mice (phenotypically normal) survived lethal irradiation, indicating the reconstitution of hematopoiesis. Two months after transplantation, Tif1g-deleted cells were detected by quantitative PCR (Q-PCR) in the peripheral blood of the transplanted mice (Supplemental Figure 4A), this deletion being correlated to the decreased expression of Tif1g mRNA (data not shown). These observations suggested that the deletion occurred in HSCs. Two months after transplantation, the mice developed the same myeloproliferative disease (i.e., monocytosis, hepatosplenomegaly) (data not shown), supporting the notion that the phenotypic effects of the Tif1g deletion were cell autonomous. We observed an increased number of Gr1+Mac1+ cells in the bone marrow and in the spleen (Supplemental Figure 4C) and an augmentation of the LSK fraction in Tif1g*Δ/Δ mice compared with control littermates (Supplemental Figure 4D). The abnormal distribution and frequency of progenitors were also reproducible (Supplemental Figure 4E). A second transplantation into lethally irradiated recipients reproduced the same disorder after 2 months (data not shown). We performed reciprocal transplant experiments in which wild-type donor cells were transplanted into either control or Tif1g*Δ/Δ mutant recipients. We did not observe any alteration in the distribution of LSK (Supplemental Figure 4F) and progenitors (CMPs, GMPs, MEPs) (Supplemental Figure 4G) in the Tif1g*Δ/Δ mutant recipients. These observations indicate that the disease generated by the deletion of Tif1g is initiated from the HSC compartment and is cell autonomous.

The hematopoietic cell response to TGF-β is inhibited in Tif1g*Δ/Δ mice. Tif1γ was identified as part of the TGF-β signaling pathway, including its E3 ubiquitin ligase activity on Smad4 (16–18, 22).
Tif1g-deficient LSK CD34+ cells (ST-HSCs/MPPs) were cultured in the presence or absence of TGF-β and TGF-β inhibitor (Figure 4, A and B). Stimulation with TGF-β dramatically (~80%) reduced the formation of myeloid cells from control LSK CD34+ cells as compared with untreated cultures, whereas it only slightly decreased (~25%) myelopoiesis of Tif1g-deficient cells (Figure 4, A and B). We also treated i.p. 6-month-old Tif1gΔ/Δ or control mice with either PBS or 15 mg/kg of a neutralizing pan–TGF-β monoclonal antibody that blocks all 3 TGF-β mammalian isoforms (TGF-β1, -β2, and -β3) (23, 24). Treatment was administered once a week for 5 weeks, when the mice were sacrificed and evaluated for hematopoietic progenitor populations. In control mice, treatment with body that blocks all 3 TGF-β inhibited all 3 TGF-β activity, whereas it only slightly decreased the expression of Csf1r (Supplemental Figure 5, A and B). Stimulation with TGF-β and TGF-β inhibitor (Figure 4D), whereas the antibody did not affect the distribution of these cells in Tif1gΔ/Δ mice (Figure 4D). Thus, low expression of TIF1G in CMML cells correlates with a low level of TIF1G (r = 0.915) (Figure 5C).

TIF1G is an epigenetically regulated tumor suppressor gene in chronic myelomonocytic leukemia. Sequencing of all the TIF1G exons in the monocytes of a cohort of 66 CMML patients (Supplemental Table 2) with various TIF1G expression correlated with a specific pattern of histone modifications, which are highly related to the epigenetic status of DNA, may be correlated with gene expression levels (25). The decrease in TIF1G gene expression observed in normal human monocytes upon M-CSF exposure for 1 day was correlated with a specific pattern of histone modification (acetylations and methylations) on the TIF1G promoter. Histone modifications, which are highly related to the epigenetic status of DNA, may be correlated with gene expression levels (25). The decrease in TIF1G gene expression observed in normal human monocytes upon M-CSF exposure for 1 day correlated with a specific pattern of histone modification (acetylations and methylations) on the TIF1G promoter. Histone modifications, which are highly related to the epigenetic status of DNA, may be correlated with gene expression levels (25).

To determine whether Tif1g affects expression of Csf1r and Csf2ra (G-CSFR), Csf2ra (GM-CSFR), and Csf3r, 3 genes known to participate in monocyte or granulocyte differentiation. Unlike Csf3r and Csf2ra, Csf1r expression was significantly decreased by the lack of Tif1g (Supplemental Figure 5, A and B), which could account for the altered production of peritoneal macrophages observed in Tif1gΔ/Δ mice (Supplemental Figure 5C). These Tif1gΔ/Δ macrophages did not adhere to the plastic and were morphologically abnormal in vitro (Supplemental Figure 5D). The plasma levels of M-CSF (CSF-1) and G-CSF remained unchanged in Tif1gΔ/Δ compared with control mice (Supplemental Figure 5E).

**Figure 2**

The Tif1g deletion affects HSCs in mice younger than 6 months. (A and B) Analysis of LSK cells from representative control and healthy Tif1gΔ/Δ mice demonstrated an increase in the LSK population in Tif1gΔ/Δ mice. (C and D) Analysis of ST-HSCs/MPPs from representative control and Tif1gΔ/Δ mice demonstrated an increase in ST-HSCs/MPPs in Tif1gΔ/Δ mice. (E and F) Analysis of LT-HSCs from representative control and Tif1gΔ/Δ mice demonstrated a decrease in the LT-HSC population in Tif1gΔ/Δ mice. **P < 0.01, ***P < 0.001.
patients before decitabine treatment (Figure 7D) was no longer detected after 7 cycles of decitabine (Figure 7D), which correlated with Tif1g mRNA reexpression (Figure 7B).

**Discussion**

We demonstrate that hematopoietic tissue–targeted deletion of TIF1G gene, which encodes an E3 ubiquitin ligase that is also a transcription and elongation coregulator, deregulates the HSC compartment and leads in aging mice to a myeloproliferative disease with myelodysplastic features. Its epigenetically regulated down-regulation in leukemic cells of a subset of patients with CMML also suggests a tumor suppressor function in human hematopoiesis.

cFES-Cre–mediated recombination of the floxed phosphatidylinositol glycan class A (Piga) allele induced the deletion of Piga in adult HSCs and all blood cell lineages (20), which prompted us to investigate the role of TIF1γ in adult hematopoiesis by crossing cFES-Cre with Tif1gΔ/Δ mice (19). Mutations in the zebrafish mon (tif1g) gene cause a disruption in both primitive embryonic and definitive adult hematopoiesis, resulting in a severe loss of erythroid cells (12). In zebrafish and human stem/progenitor CD34+ cells, TIF1γ functionally links positive elongation factors to blood-specific transcription complexes to regulate the erythroid commitment (15). Here, we show that targeted inactivation of Tif1g in stem cells does not affect the number of peripheral blood erythrocytes. The discrepancy with previous models may be related to compensatory mechanisms operating in the mouse model, i.e., the decrease in bone marrow erythroblast is compensated by splenic erythropoiesis, by a function of TIF1γ in zebrafish embryonic primitive erythropoiesis, or by the requirement of TIF1γ for in vitro erythroid commitment of human CD34+ cells while being dispensable for mammalian adult erythropoiesis in vivo. Based on the expression of c-myb, mon mutants were suggested also to have
normal myeloid development (12). We show here that inactivation of Tif1g in mouse stem cells expanded the LSK population, in which LT-HSC number was decreased while ST-HSC/MPP number was dramatically increased. The Tif1γ defect also promotes the expansion of the GMP compartment at the expense of the CMPs and the MEPs. At an older age (>6 months), all Tif1g−/− mice exhibit an accumulation of monocytes in peripheral blood, bone marrow, liver, and spleen, suggesting that aging favors the dysregulation of myelopoiesis. This phenotype recapitulates the human CMML that is observed in patients older than 50 (mean age, 70 years old) and associates proliferative and dysplastic features (27).

Transplantation experiments demonstrate that hematopoietic cells are capable of propagating the Tif1g−/− CMML-like phenotype in recipient mice, indicating a cell-autonomous process. In addition, similarly to mice in which Junb has been deleted (28), and in contrast to those deficient in Rarg (27) and Rb (29, 30), Tif1g−/− mice receiving control bone marrow transplants did not develop the phenotype.

TIF1γ was identified as a modulator of the TGF-β signaling pathway (22). Cytokines of the TGF-β family bind membrane heterodimeric serine/threonine kinases that in turn phosphorylate intracellular mediators known as Smads (31). Phosphorylated Smad2 and Smad3 accumulate into the nucleus and bind Smad4 to regulate target gene transcription. In hematopoietic, mesenchymal, and epithelial cells exposed to TGF-β, TIF1γ could selectively bind phosphorylated Smad2/3 in competition with Smad4 (17). TIF1γ could also antagonize Smad4 through its ubiquitin ligase properties (16). Here, we show that decreased expression of TIF1γ impairs the HSC response to TGF-β (32–34). Recent results indicate that the gene deletion also affects transcriptional elongation of blood genes such as SCL/TAL1 by antagonizing Pol II pausing (15). Hence, TIF1γ may act on cell fate and lineage commitment through transcription regulation, elongation, and protein degradation. These effects may account for the decrease in Csf1r gene expression observed in Lin− and total bone marrow cells of Tif1g−/− mice, which correlates with altered monocyte differentiation into macrophages.
TIF1G gene expression is almost undetectable in sorted leukemic cells of approximately 35% of patients with CMML, which, as in the mouse model, correlates with decreased expression of the CSF1R gene. In this disease, the most frequently identified somatic mutations involve TET2 (35), RUNX1 (36, 37), and ASXL1 (38); RAS and CBL mutations may be less frequent and mutually exclusive (36, 39); while mutations in several other genes encoding epigenetic regulators or signaling molecules (UTX, EZH2, IDH1, IDH2) were identified with a low frequency (40–47). It remains difficult to distinguish in these somatic events those that drive the disease pathogenesis from those acquired as a consequence of clonal evolution. The copy-neutral uniparental disomy that is frequently observed in CMML cells can affect the 1p chromosomal region containing the TIF1G gene (48), but we failed to detect any mutation in the coding sequence of the gene that could account for its decreased expression in patient cells.

TIF1G downregulation correlates with the hypermethylation of CpG islands and a specific pattern of histone modifications on the gene promoter in CMML samples (26, 49). The link between mutations in epigenetic genes such as TET2, ASXL1, EZH2, UTX, IDH1, and IDH2 and the downregulation of TIF1G has now to be explored (50–53). TIF1γ downregulation could also cooperate with mutated signaling molecules such as K-Ras, as recently demonstrated in a mouse model of pancreatic cancer (54). Epigenetic drugs including DNA methyltransferase inhibitors, histone deacetylase inhibitors, and their combination are currently tested as therapeutic approaches in CMML (55). Interestingly, the expression of TIF1γ was restored in monocytes of patients who responded to the demethylating agent decitabine, which could indicate either a demethylating effect of the treatment on the gene promoter or the selection of cells in which the gene was not repressed. Whatever the explanation, changes in TIF1γ expression may be a biomarker of response to demethylating agents in CMML (56).

Together, our results suggest that TIF1G is an epigenetically regulated tumor suppressor gene in hematopoietic cells. The decreased expression of this gene favors the proliferation of MPPs and leads to the age-dependent expansion of the monocyte population. Changes in TIF1G expression may be tested as a biomarker of response to demethylating agents and other chromatin structure modifiers such as HDAC inhibitors (57) that are currently being developed for the treatment of this disease.

**Methods**

Mice. Mice were housed in a temperature-controlled environment under a 12-hour light/12-hour dark cycle with free access to water and a standard rodent chow diet; all mice used were maintained under specific patho-
gen-free conditions according to animal study protocols reviewed and approved by the Animal Experiment Ethics Committee of the University of Burgundy. cFES-Cre mice were provided by P.P. Pandolfi (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA). For genotyping, genomic DNA was prepared from blood, bone marrow, or tail biopsies using the Gentra Puregene Kit (QIAGEN). Floxed (531 bp), deleted (360 bp), and wild-type (498 bp) Tif1g alleles were identified by PCR with primers AFI 124 (5′-AGCTCGGAAGCTGTCAGATCCTCTAGAACT-3′), AFI 125 (5′-GGTAAGTCAGCAAGACCTCA-3′), and AFM 257 (5′-GGTAGTACCTGTATGAGGT-3′). For genomic Q-PCR, a specific sequence located between exons 3 and 4 was amplified on a 7500 FAST thermocycler (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer (Applied Biosystems). Mouse specific forward (5′-TACTTGTATGGAGGT-3′) and reverse primers were: Moz (5′-GGTAGTACCTGTATGAGGT-3′), and AFM 257 (5′-GGTAGTACCTGTATGAGGT-3′), used as a standardizing control, and Tif1g, 5′-GTGCCGCAGTGCCCTATTTG-3′ and 5′-AATGCAGAGAGCAGCCAGTTCC-3′.

Bone marrow transplantation. To generate bone marrow–reconstituted mice, we injected a total of 2 × 10⁶ bone marrow cells from control or Tif1gΔ/Δ donor mice into the retro-orbital sinus of lethally irradiated recipient (11 Gy) BALB/c mice. For the second transplantation, the same protocol was followed. For reciprocal transplantations, we injected a total of 2 × 10⁶ BM cells from control donor mice into the retro-orbital sinus of Tif1gΔ/Δ or control lethally irradiated recipient (16 Gy) C57BL/6 mice.

Blood cell analysis. Adult control and Tif1gΔ/Δ mice were anesthetized with 1%-2% isoflurane. Blood was collected from the conjunctival vein in the eye using an EDTA-coated tube. Complete blood counts were performed using an automated hematology analyzer (MS Laboratories). Collected blood was also used to prepare blood smears, which were stained with May-Grünwald-Giemsa (MGG) stain.

Cell preparation. Following sacrifice, mice were examined for the presence of abnormalities, and organs were collected for further cell and histopathology analyses. For the bone marrow cell preparations, femurs and tibias were removed aseptically and cells were flushed. For splenocytes, spleens were dilacerated, red blood cells were lysed, and cells were washed in 1× PBS (Lonza).

Responsiveness to TGF-β. For myeloid progenitor assays studying the responsiveness to TGF-β ligand, we purified Lin- cells from bone marrow cells using the Lineage Cell Depletion magnetic isolation kit and AutoMACS separator according to the manufacturer’s instructions (Miltenyi Biotec). ST-HSCs/MPPs (LSK CD34⁺) were sorted from these cells.

Histopathology and immunohistochemistry analyses. Sectioned femoral bone marrow, spleens, and livers were fixed at least 72 hours in 10% neutral buffered formalin, dehydrated in alcohol, cleared in xylene, and infiltrated with paraffin before dehydration in alcohol and rehydration in water and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on paraffin-embedded tissue sections using primary antibodies directed against Mac1 (BD Biosciences – Pharmingen) or Ki67 (Abcam).

Flow cytometry and cell sorting analysis. FACS analysis was performed on blood cells, bone marrow cells, or splenocytes from mice. Briefly, single-cell suspension was prepared and stained with primary and secondary antibodies if necessary. The cells were washed twice in 1× PBS and resuspended in 1× PBS/30% FBS. Lin- cells were identified by absence of signal after staining with a biotinylated antibody cocktail containing B220, CD3, Ter119, Mac1, and Gr1 (BD Biosciences – Pharmingen) and developed by streptavidin–Aluor Fluor 405 (Invitrogen). Antibodies Gr1-FITC, Mac1–Alexa Fluor 780, T-Cell, and NKP46-PE were from eBioscience (San Diego, CA).

**Figure 6** TIF1G is an epigenetically regulated tumor suppressor gene in CMML. (A) Sequencing of the bisulfite modified TIF1G promoter sequence from normal monocytes (Control) or CMML monocytes. (B) ChIP analysis of the status of histone modifications on the TIF1G promoter. Chromatin was immunoprecipitated using specific antibodies directed against histone modifications. Ac, acetylated; Me, methylated; Me3, tri-methylated.
Fluor 647, Ter119–Alexa Fluor 405, CD71-PE, Sca1-PE, c-Kit–APCH7, CD150-APC, CD48-FITC, CD34-FITC, and CD16/32-PECya7 were purified from bone marrow cells using the Lineage Cell Depletion magnet Minus One (FMO) method, which was performed by sequentially adding the manufacturer’s instructions. cDNA was obtained from 150 ng total RNA using the TaqGold polymerase in a 7500 FAST thermocycler (Applied Biosystems) and subjected to RQ-PCR analysis. One cycle of decitabine corresponds to 72 μg/m2 i.v. for 5 days every 22 days. For in vitro culture, monocytes isolated by Ficoll Hypaque (Eurobio), and monocytes were sorted using the Fluorochrome or with a combination of cells stained by the Fluorescence minus One (FMO) method, which was performed by sequentially adding fluorescence-labeled antibodies to the staining cocktail.

Real-time Q-PCR in mice. Total RNA was isolated from bone marrow cells and splenocytes using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was obtained from 150 ng total RNA using M-MLV Reverse Transcriptase (Promega). Real-time Q-PCR (RQ-PCR) was performed in triplicate using TaqMan probes (Applied Biosystems) and analyzed in an Applied Biosystems 7500 Real-Time PCR System. The Tif1g TaqMan assay was Mm01308706_m1. Values for each PCR were normalized to Hprt levels (Mm00479084_m1). For the study of Csflr, Csfr3, and Csfr2a expression, real-time PCR was performed in a 7500 FAST thermocycler (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer. Mouse specific forward and reverse primers were: Csflr, 5′-CATGGCCTCTCTCTCTTCAAA-3′ and 5′-CAGCAGCTTTTGAGCT-GCTA-3′; Csfr3, 5′-GGCGCCGACTGTAGTACCA-3′ and 5′-GGAGCAGTT-GTCTGCCTCTTC-3′; and Csfr2a, 5′-ACGTTGGCCGATGCA-3′ and 5′-TCAGACCAAGTGGCCCTC-3′. Hprt was used as internal control.

CMML sample collection and analysis. Blood samples from patients with CMML were collected, and informed consent was obtained. CMML tissue studies were reviewed and approved by the institutional review board of Hospital Cochin (Paris, France). Peripheral blood mononucleated cells were isolated by Ficoll Hypaque (Eurobio), and monocytes were sorted using the CD14+ magnetic isolation kit and AutoMACS separator according to the manufacturer’s instructions (Miltenyi Biotec). Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was reverse transcribed by M-MLV reverse transcriptase with random hexamer primers (Promega). Real-time PCR was performed with AmpliTaq Gold polymerase in a 7500 FAST thermocycler (Applied Biosystems) using the SYBR Green detection protocol as outlined by the manufacturer. Briefly, 15 ng of total complementary DNA, 50 nM of each primer, and 1× SYBR Green mix were used in a total volume of 20 μl. Human specific forward and reverse primers were L32, 5′-TGTCCTGAATGGTGCTACCTGA-3′ and 5′-CTGACTCTCCTGACACCT-3′ used as a standardizing control, TIF1G, 5′-AGCAGCCGACATCAA-3′ and 5′-TGCATTTCTGCGGGCA-TA-3′, and CSF1R, 5′-GCCCCCATCACCTCCTA-3′ and 5′-GTTTTTGTG-GAAGTAGCCTTT-3′. L32 was used as internal control.

Treatment of CMML with decitabine. Adults with a diagnosis of CMML were enrolled in “A Phase II Study of Decitabine in Patients with Chronic Myelomonocytic Leukemia” (GFM-DEC-LMMC-2007-02), and informed consent was obtained. CMML tissue studies were reviewed and approved by the institutional review board of Hospital Cochin. Monocytes were isolated, as described in CMML sample collection and analysis, after decitabine treatment and subjected to RQ-PCR analysis. One cycle of decitabine corresponds to 20 mg/m2/d i.v. for 5 days every 22 days. For in vitro culture, monocytes isolated from CMML patients were cultured in RPMI 1640 Glutamax medium (BioWhittaker) supplemented with 10% fetal calf serum (BioWhittaker),
penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (BioWhittaker) at 37°C in a 5% CO₂ humidified atmosphere. Cells were plated at 0.5 x 10⁶ cells/ml and incubated for 3 days with M-CSF (100 ng/ml) in the presence or absence of decitabine (3 μM).

Bisulphite DNA treatment and sequencing. Genomic DNA was isolated from monocytes of CMML patients before and after 7 cycles of decitabine using QIAGEN's standard procedures. Two hundred nanograms of total genomic DNA were subsequently converted and used for bisulphite treatment and sequencing (MethylDetector, Active Motif). Converted DNA was identified by PCR with converted primers forward (5′-GGCTTAAAAA-AAAAATCTCCCTTT-3′) and reverse (5′-CCACCATTTTCCCTTAAACCCCG-3′), and direct sequencing reaction was performed using standard conditions according to the manufacturer's instructions (Applied Biosystems).

ChIP procedure. One million cells were fixed with 1% formaldehyde to crosslink DNA with proteins, then lysed and sonicated. The ChIP procedure was carried out according to the manufacturer’s instructions with modifications (Upstate Biotechnology) and as previously described (58). After pre-clearing with salmon sperm DNA/protein A/G agarose beads, the samples underwent immunoprecipitation with 3′-CCCTCAGGCCCTTTGCA-3′ and 5′-GGCTTAAAAA-AAAAATCTCCCTTT-3′, and reverse (5′-CCACCATTTTCCCTTAAACCCCG-3′), and direct sequencing reaction was performed using standard conditions according to the manufacturer's instructions (Applied Biosystems).

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