Table 2. Prognostic factors following CBT in multivariate analysis

	Factors		Hazard ratio	P-value	95% Confidence interval
Neutrophil engraftment	Number of CD34-positive cells	$<1 \times 10^5$/kg vs $\geqslant 1 \times 10^5$/kg	1.58	<0.0001	1.27-1.95
Platelet engraftment ($\geqslant 20\,000$/mm^3)	Number of CD34-positive cells	$<1 \times 10^5$/kg vs $\geqslant 1 \times 10^5$/kg	1.92	<0.0001	1.33-2.26
	Disease status	non-remission vs remission	1.88	0.004	1.23-2.88
	ABO misatch	Major/minor mismatch vs match	1.34	0.003	1.10-1.64
	Number of nucreated cells	per 1×10^7/kg	0.63	0.005	0.46-0.87
	GVHD prophylaxis	With MTX vs no MTX	0.62	0.02	0.42-0.92
Relapse	Disease status	non-remission vs remission	2.28	0.0012	1.38-3.76
	Number of nucreated cells	per 1×10^7/kg	1.84	0.002	1.25-2.71
Acute GVHD	*	*			
Chronic GVHD	Acute GVHD	3-4 vs 0-2	1.92	0.02	1.12-3.28
	Number of CD34-positive cells	$<1 \times 10^5$/kg vs $\geqslant 1 \times 10^5$/kg	1.51	0.04	1.02-2.23
Overall survival	Disease status	non-remission vs remission	2.2	< 0.0001	1.53-3.15
	Age	51 or older vs younger than 50	1.89	0.0009	1.30-2.75
	Acute GVHD	3-4 vs 0-2	1.98	0.006	1.21-3.24
	Chronic GVHD	Absent vs present	2.39	0.02	1.13-5.08
Disease-free survival	Disease status	non-remission vs remission	2.51	< 0.0001	1.78-3.56
	Acute GVHD	3-4 vs 0-2	1.65	0.04	1.02-2.66

Abbreviations: CBT, cord blood transplantation; GVHD, graft-versus-host disease. *Indicates no prognostic factors were identified in multivariate analysis.

($n = 13$), thrombotic microangiopathy ($n = 20$) and hemorrhage from any sites ($n = 18$).

A total of 89 patients (35%) died of TRM at a median day of 46 days (range, 4-466). The cumulative incidence of TRM at 2 years after CBT was 32% (95% CI, 26-38%). Causes of death included infection ($n = 36$), acute GVHD ($n = 11$), veno-occlusive disease ($n = 10$), graft failure ($n = 6$), idiopathic pneumonia syndrome ($n = 7$), thrombotic microangiopathy ($n = 6$), multiple organ failure ($n = 6$), hemorrhage ($n = 5$) and infarction ($n = 2$).

Response to CBT

Out of 78 patients, 24 who were transplanted in non-remission achieved durable remission lasting 60 days or longer and 19 survived without relapse longer than 1 year.

Relapse

The cumulative incidence of relapse at 2 years was 43% (95% CI, 38-47%). Disease status and the number of nucleated cells at cryopreservation were the prognostic factors in multivariate analysis (Table 2).

DFS and OS

Probabilities of 2-year DFS and OS were 36% (95% CI, 33-39%) and 42% (95% CI, 39-45%), respectively. In the 177 patients transplanted in remission, DFS and OS were 45% (95% CI, 41-49%) and 51% (95% CI, 46-55%), respectively. In those who were transplanted in non-remission, DFS and OS were 16% (95% CI, 12-21%) and 23% (95% CI, 17-28%), respectively (Figure 1). In patients with Ph-positive ALL, probabilities of 2-year DFS and OS were 32% (95% CI, 27-37%) and 43% (95% CI, 37-48%), respectively.

Multivariate analysis showed that age (51 or older vs younger than 50), disease status (non-remission vs remission), absence of chronic GVHD and grade III-IV acute GVHD were negatively associated with OS (Table 2). Disease status and grade III-IV acute GVHD were negatively associated with DFS (Table 2).

OS was shown according to the presence of acute and chronic GVHD among the 180 patients who survived longer than 100 days (Figure 2). Patients with grade III-IV acute GVHD had poor prognosis compared with those with grade 0-I and grade II acute GVHD. The patients who developed chronic GVHD was expected higher survival rates than patients of absence chronic GVHD.
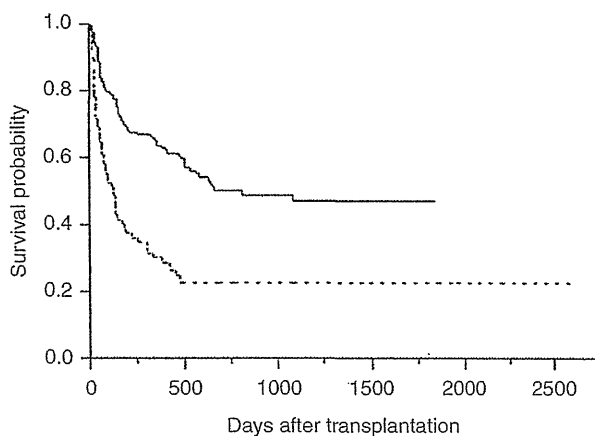

Figure 1. OS according of disease status at CBT. Probability of 2-year OS rates of patients in remission and non-remission were 51% (95% CI, 46-55) and 23% (95% CI, 17-28), respectively ($P<0.001$). Solid line indicates patients in remission ($n = 177$). Broken line indicates patients in non-remission ($n = 78$).

DISCUSSION

The present study showed that 24 of the 78 patients transplanted in primary refractory or relapse achieved durable remission. Considering that combination chemotherapy is usually ineffective at prolong survival in adult patients with advanced or Ph-positive ALL,[23,24] the long-term DFS after CBT suggests that durable allogeneic immune reactions continue to suppress leukemic progression after CBT. These findings support the hypothesis that graft-versus-leukemia effects exist after CBT for adult ALL.

Cumulative incidence of grade II-IV acute GVHD at day 100 is 37%. These findings were comparable with previous studies on CBT (26-60%).[6,25] However, it should be noted that grade III-IV acute GVHD negatively impacted OS, and that there was no significant differences between patients with grade 0-I acute GVHD and those with grade II acute GVHD. These findings suggest that control of acute GVHD is important in CBT for ALL.

Previous studies showed that presence of chronic GVHD protected relapse following CBT for adult ALL,[9,14] whereas it had
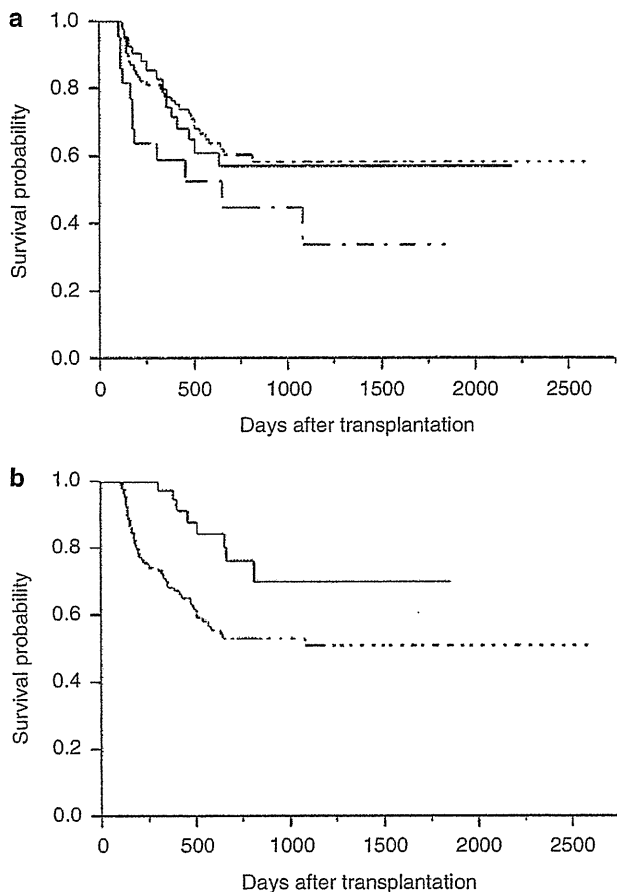
Figure 2. (a) Influence of acute GVHD on OS. Solid line indicates patients with acute GVHD less than grade II ($n = 99$). Broken line indicates patients with grade II acute GVHD ($n = 48$). Wavy line indicates patients with grades III–IV acute GVHD ($n = 33$).
(b) Influence of chronic GVHD on OS. Solid line indicates patients with chronic GVHD ($n = 41$). Broken line indicates patients without chronic GVHD ($n = 139$).

TRM is a significant concern in CBT. The present study demonstrated that most TRM occurred early after transplantation, as consistent with the previous studies.[9,17] Early infection before engraftment is a significant complication in CBT, and the long duration of neutropenia might have a major role in its pathogenesis. As the higher number of infused CD34-positive cells was a significant predictive factor for engraftment in this study, selection of cord blood units with a higher number of CD34-positive cells may be reasonably expected to reduce the risk of graft failure. Use of double CBT was another option. Some researchers reported that infused cell doses and increased alloreactivity induced by interaction between the two CB units may be responsible for reduced risk of relapse.[11]

Our study indicates that CBT is worth considering options for adult ALL further intense evaluation. As a result of the advances in supportive care with better management of infectious complications, and the accumulation of knowledge of better cord blood selection, the outcomes of CBT were significantly improved over time.[8] In addition, reduced-intensity regimens are used widely for CBT, and some small-sized clinical trials suggested that reduced-intensity transplantation potentially results in modest TRM, limited risk of relapse and promising survival in adult patients with ALL.[26] Use of reduced-intensity regimen might offer chance of cure to elderly patients with ALL. Further investigations are warranted to investigate its role in the therapeutic options for adult patients with ALL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Author contribution

TM designed the research and wrote the manuscript. TY analyzed the data. MK, KY, EK, STaniguchi, STakahashi, MO and HS performed the research. HA, MT, HK, SKai and TI-N managed the data. KK and SKato took the leadership of authors.

no significant impact on OS and DFS. Chronic GVHD was not identified as a prognostic factor for relapse in either analysis for patients transplanted in remission or that for patients with active disease at time of transplant. These findings were in contrast to our study, in which chronic GVHD was associated with better OS. Notably, chronic GVHD was limited-type in two-thirds of the patients in this study, and extensive-type chronic GVHD was dominant in the previous study.[14] The mild features of chronic GVHD among Japanese patients following CBT probably lead to the favorable impact of chronic GVHD on OS.[15]

This study provided detailed information on the prognostic factors of CBT for adult ALL patients. The number of infused CD34-positive cells was associated with engraftment. Concerning OS, patients' age, disease status and development of acute and chronic GVHD were significant risk factors. These findings were comparable to previous reports.[7,25]

Clinical decision making as to when to proceed with CBT in patients with ALL is difficult. Disease status is an important prognostic factor in CBT. As some patients with second or further remission and those with induction failure achieved durable remission after CBT, they are candidates for CBT. However, it is controversial whether CBT should be offered to patients in first remission, as CBT is associated with a high mortality.

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Unrelated cord blood transplantation after myeloablative conditioning regimen in adolescent and young adult patients with hematologic malignancies: A single institute analysis

Yasuhiro Ebihara [a,*], Satoshi Takahashi [b,e,f], Shinji Mochizuki [a,d], Seiko Kato [b], Toshiro Kawakita [b,e], Jun Ooi [b], Kazuaki Yokoyama [f], Fumitaka Nagamura [c], Arinobu Tojo [b,e,f], Shigetaka Asano [g], Kohichiro Tsuji [a,d]

[a] Department of Pediatric Hematology/Oncology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[b] Department of Hematology/Oncology, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[c] Division of Clinical Trial Safety Management, Research Hospital, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[d] Division of Stem Cell Processing, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[e] Division of Stem Cell Transplantation, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[f] Division of Molecular Therapy, Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[g] Department of Integrative Bioscience and Biomedical Engineering, Waseda University, Tokyo, Japan

ARTICLE INFO

ABSTRACT

We report the results of unrelated cord blood transplantation (CBT) after myeloablative conditioning regimen in 16 patients with hematologic malignancies from 15 to 20 years old. The median times of myeloid and platelet engraftment were 21 and 38 days, respectively. The cumulative incidences of acute graft-vs-host disease (GVHD) was 62.0%, all of which were grade I or II, and that of extensive-type chronic GVHD was 12.5%. The probabilities of overall and disease-free survival at 3 years were 68.2% and 48.6%, respectively, comparable to adult or childhood cases. Adolescents and young adult patients with hematologic malignancies who have no HLA-matched adult donors could be considered as candidates for CBT.

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1. Introduction

Recently the medical needs of adolescents and young adults with hematologic malignancies have become better defined. In comparison of outcome of patients with 16–21 years of age with acute lymphoblastic leukemia (ALL) treated with pediatric vs adult clinical trials, pediatric trials yielded better outcome than adult trials [1]. In patients with acute myelocytic leukemia (AML), outcome of children younger than age 15 years has significantly improved for the last several decades, but that of patients with 15–19 years remains poor [2]. Thus, adolescents and young adults with hematologic malignancies are distinct in terms of their therapeutic requirements compared to adults or children. However,

there have been no data defined adolescent and young adult patients for cord blood transplantation (CBT) after conventional myeloablative conditioning regimen. We here first report the clinical results for a group of 16 adolescent and young adult patients with hematologic malignancies treated with CBT in our institute, showing the safety and efficacy comparable to those for adults and children.

2. Patients and methods

This is a retrospective single-center analysis. Between September 1999 and July 2009, 16 patients at adolescent and young adult ages from 15 to 20 years old were treated with CBT as the first allogeneic stem cell transplantation at The Research Hospital, Institute of Medical Science, University of Tokyo. One patient received an autologous bone marrow transplantation before he had come to our hospital. Written informed consent for treatment was obtained from all patients with the Declaration of Helsinki. Patients were qualified as being standard risk and high risk according to the criteria in the previous reports [3,4].

* Corresponding author at: Department of Pediatric Hematology/Oncology, Research Hospital, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Tel.: +81 354 495 694; fax: +81 354 495 428.
E-mail address: ebihara@ims.u-tokyo.ac.jp (Y. Ebihara).

2.1. HLA typing and donor selection

HLA-A and HLA-B antigens were identified by serological typing. HLA-DRB1 alleles were determined by high-resolution molecular typing using polymerase chain reaction sequence-specific primers. Patients who did not have HLA-matched family or unrelated adult donors were considered to be eligible for CBT. In the selection of cord blood unit for transplantation, all cord blood grafts were evaluated by HLA-A, HLA-B and HLA-DRB1 typing, and nucleated cell counts. Preferred cord blood units matched 4 of 6 to 6 of 6 HLA loci and contained a minimal cell count of 1.5×10^7 nucleated cells/kg body weight before freezing according to the criteria of our institution as shown in the previous reports [3,4]. All cord blood units were obtained from cord blood banks belonging to the Japan Cord Blood Bank Network.

2.2. Conditioning regimen, GVHD prophylaxis, and supportive care

All patients received fractionated 12 Gy total body irradiation and chemotherapy as a myeloablative conditioning regimen (Table 1). Fifteen patients received standard cyclosporin (CsA) and methotrexate (MTX), and one patient received CsA alone for a graft-vs-host disease (GVHD) prophylaxis [3,4]. Fifteen mg/m^2 of MTX was given intravenously on day 1, and 10 mg/m^2 on days 3 and 6 as previously reported [3,4]. Both acute and chronic GVHD (aGVHD and cGVHD, respectively) were graded according to the previously published criteria [5,6]. The criterion to stop immunosuppression depended on patients' disease status. All patients received recombinant human granulocyte colony-stimulating factor starting on day 1 until durable granulocyte recovery was achieved.

2.3. Endpoints and statistical analysis

The chimerism status after CBT, engraftment, graft failure, treatment-related mortality (TRM), and disease-free survival (DFS) were defined as described in the previous reports [3,4].

Data analysis was performed on 1 December 2010. The probability of overall survival (OS) and DFS were estimated using the Kaplan–Meier method.

3. Results and discussion

The characteristics of the 16 patients and the cord blood units are shown in Table 1. Six patients were classified as standard risk while 10 patients as high risk. Six patients (2 ALL, 3 myelodysplastic syndrome (MDS) and 1 chronic myelocytic leukemia (CML)) were initially treated by Pediatric units. All patients received a single and HLA-mismatched cord blood unit. The median numbers of cryopreserved nucleated cells and CD34$^+$ cells were 2.50×10^7/kg (range 2.05 to 3.73×10^7/kg) and 0.94×10^5/kg (range 0.46 to 1.33×10^5/kg), respectively. The median numbers of infused nucleated and CD34$^+$ cells were 2.11×10^7/kg ($n = 11$; range 1.36 to 2.38×10^7/kg) and 0.76×10^5/kg ($n = 11$; range 0.25 to 2.55×10^5/kg), respectively.

Fourteen patients (87.5%) successfully achieved myeloid reconstitution and 2 patients went into graft failure regardless of above of median number of total nucleated cells (2.71 and 2.45×10^7/kg, respectively) and CD34$^+$ cells (1.09 and 1.13×10^5/kg, respectively) transplanted. One had full recovery with 100% of host chimerism by day 52, and the other took a second cord blood graft on day 30. All patients with myeloid reconstitution showed full donor chimerism at the first bone marrow examination after CBT. The median time to an absolute neutrophil count $>0.5 \times 10^9$/L among the patients with engraftment was 21 days (range 19–32 days). The cumulative

Table 1
Characteristics of patients, cord blood units, and outcomes.

Characteristics	
Patients, n	16
Male/female, n	9/7
Median age, years (range)	17 (15–20)
Median weight (kg) (range)	52 (45–71)
Median number of cryopreserved nucleated cells $\times 10^7$/kg (range)	2.50 (2.05–3.73)
Median number of cryopreserved CD34$^+$ cells $\times 10^5$/kg (range)	0.94 (0.46–1.33)
Median number of infused nucleated cells, n $\times 10^7$/kg (range)	11 2.11 (1.36–2.38)
Median number of infused CD34$^+$ cells, n $\times 10^5$/kg (range)	11 0.76 (0.25–2.55)
Median time from diagnosis to transplantation Days (range)	429 (65–1898)
Recipient CMV status, positive/negative, n	13/3
Diagnosis	
De novo AML [n (%)]	3 (19)
CR1, n	1
CR2, n	1
Not in remission, n	1
ALL [n (%)]	7 (44)
CR1, n	2
CR2, n	4
CR3, n	1
CML BC [n (%)]	1 (6)
MDS [n (%)]	4 (25)
RA, n	1
RCMD, n	1
Advanced (n)	2
MDS/MPD [n (%)]	1 (6)
Disease status at transplant*	
Standard risk [n (%)]	6 (37)
High risk [n (%)]	10 (63)
Conditioning regimen	
TBI + CY + AraC, n	1
TBI + CY + AraC/G-CSF, n	9
TBI + CY, n	4
TBI + CY + Tepa, n	1
TBI + Flu + Mel, n	1
GVHD prophylaxis	
CSP + sMTX, n	15
CSP, n	1
Number of HLA-A, B, DRB1 mismatches	
1, n	4
2, n	5
3, n	6
4, n	1
Engraftment [day (range)]	
Median time to neutrophil count $>0.5 \times 10^9$/L	21 (19–32)
Median time to platelet count $>50 \times 10^9$/L	38 (33–98)
Acute GVHD [n (%)]	
0	1 (7)
Grade I	6 (43)
Grade II	7 (50)
Grade III	0
Grade IV	0
Chronic GVHD [n (%)]	
None	0
Limited	10 (83)
Extensive	2 (17)
Immunosuppressant termination (n=7)	
Median time [day (range)]	267 (83–952)
Cause of death [n (%)]	
Relapse	4 (80)
MOF	1 (20)

CMV, cytomegalovirus; AML, acute myelogenous leukemia; CR1, CR2, CR3: first, second, third complete remission, respectively; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; BC, blast crisis; MDS, myelodysplastic syndrome; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; Advanced, patients with MDS-related secondary AML; MPD, myeloproliferative disease; TBI, total body irradiation; Ara-C, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; CY, cyclophosphamide; Tepa, thiotepa; Flu, fludarabine; Mel, melphalan; CsA, cyclosporine; sMTX, short-term methotrexate; MOF, multiple organ failure.

* Patients qualified as being standard risk or high risk according to the criteria described in previous reports [3,4].
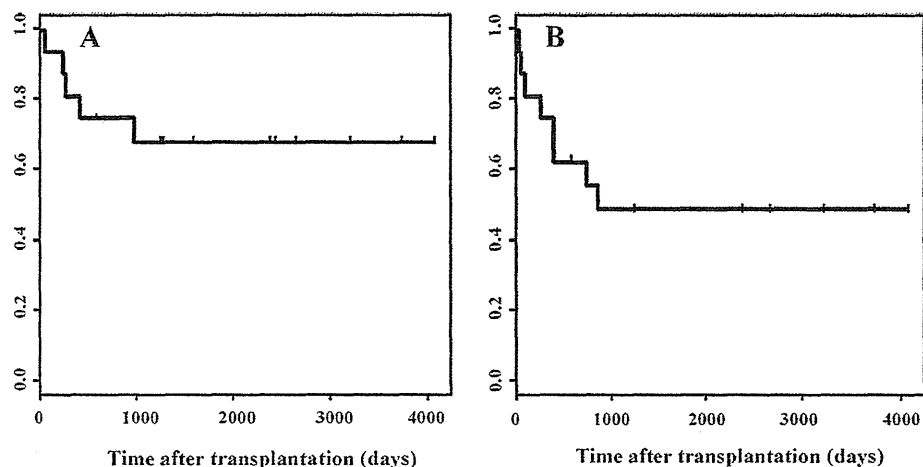
Fig. 1. Kaplan–Meier estimates of overall survival (A) and disease-free survival (B) after cord blood transplantation in adolescent patients with hematologic malignancies. The probability of OS (A) and DFS (B) at 3 years was 67.5% (95% CI, 47.6–95.8%) and 48.6% (95% CI, 29.0–81.4%), respectively.

incidence of neutrophil recovery at day 42 was 87.5% (95% confidence interval (CI), 68.9–100%). A self-sustained platelet count >50 × 10⁹/L was achieved in 13 patients at a median time of 38 days (range 33–98 days). The cumulative incidence of platelet recovery at day 100 was 81.3% (95% CI, 57.3–100%). These results using single units showed that the hematopoietic reconstituting ability of unrelated CB in adolescent patients was similar to those in pediatric and adult ones [7–10].

aGVHD occurred in 13 of 14 evaluable patients who survived for more than 100 days, but there were no patients with grades III and IV aGVHD (Table 1). The cumulative incidence of grade I or II aGVHD at day 100 was 62.0% (95% CI, 27.8–96.2%). cGVHD occurred in all of 12 evaluable patients, and 2 among them displayed the extensive type. The cumulative incidence of cGVHD and extensive-type cGVHD at 1 year was 68.8% (95% CI, 43.6–94.0%) and 12.5% (95% CI, 0–29.4%), respectively. There was no relationship between the immunosuppressive therapy and the occurrence of aGVHD and cGVHD. TRM only occurred in one patient. This patient suddenly suffered an infarction in the pons on day 22 after CBT, and died of multiple organ failure (MOF) on day 43. The cumulative incidence of TRM at 1 year was very low (6.3%). Consequently GVHD was not related to TRM in our study. The relatively higher incidence of GVHD in our study may come from earlier discontinuation of immunosuppressant as reported previously [3,4].

However, 2 of 6 standard risk patients and 5 of 10 high risk patients relapsed. The cumulative incidence of relapse at 3 years was 45.1% (95% CI, 18.8–71.4%). All of the relapsed patients received chemotherapy to obtain remission, and five patients received a second CBT. To find the risk factors to relapse after CBT in adolescent and young adult patients, we analyzed the relationships between the relapse and the numbers of infused nucleated and CD34⁺ cells, GVHD prophylaxis and the number of HLA-mismatches, but there were no significant relationships. In the patients with ALL, 1 out of 2 patients, who were treated by pediatric-type regimen before CBT, and 3 out of 5 patients, who were treated by non-pediatric-type regimen, relapsed. Although it was shown that pediatric-type regimen is favorable for the treatment of adolescent and young adult patients in ALL [1], there was no significant relationship between the relapse after CBT and the treatment with pediatric-type regimen in this study. However, since the number of patients was small in our single institute analysis, further study with a larger number of patients may be needed to find the risk factor to relapse after CBT.

Eight patients are alive and disease-free at the median 97 months (range 20–135 months) after transplantation. The

disease-free patients included the patient who underwent autologous bone marrow recovery without relapse. All of the alive patients had a good performance status with 90–100% Karnofsky score at the time of analysis. The probabilities of OS and DFS at 3 years were 67.5% (95% CI, 47.6–95.8%) and 48.6% (95% CI, 29.0–81.4%), respectively (Fig. 1A and B). The probability of DFS at 3 years in the standard risk patients was 66.7% (95% CI, 37.9–100%) while that in the high risk group was 40% (95% CI, 18.7–85.5%). Atsuta et al. reported that OS and DFS at 2 years in adult cases who received CBT were 48% and 42% in AML, and 52% and 46% in ALL, respectively [11]. Eapen et al. also showed that DFS at 2 years was 44% in remission cases and 15% in non-remission cases at CBT in adults [12]. Pediatric studies reported that OS at 2 years was 45.5%[9] and DFS at 5 years was 33–60% [10]. Thus, both DFS and OS in the present study were comparable to those in adult and pediatric patients.

However, in the previous report by our institute, the 3-year probability of DFS after unrelated CBT for hematological malignancies was higher (70%) than that in the present report, especially in the standard risk patients (93% vs 67%) [4]. In comparison with the previous report, the overall rate of high risk patients (62% vs 57%), the 1-year incidence of TRM (6% vs 9%), the 100-day incidence of aGVHD (62% vs 52%) and the cumulative incidence of cGVHD in patients surviving more than 100 days (69% vs 71%) were almost similar. In contrast, the 3-year cumulative incidence of relapse was significantly higher in the present study (45% vs 17%). Therefore, the difference in DFS between the present and previous reports might have been caused by the biological characteristics of hematological malignancies in adolescence, such as the resistance of malignant cells to anti-cancer drugs or immunological immaturity reducing the graft-vs-malignant cell effect. Accordingly, further improvement in the pre-transplantation chemotherapy, conditioning regimen and post-transplantation immunomodulation may be needed to achieve better outcomes during the treatment of adolescent hematologic malignancies with unrelated CBT.

In summary, although our patient cohort was small, our results suggested that CBT after myeloablative conditioning regimen could be safe for adolescents and young adult patients with hematologic malignancies as well as pediatric and adult patients. However, since the adolescent hematologic malignancies are thought to be relatively chemoresistant, a therapeutic regimen that takes the biological characteristics of these malignancies into account would contribute to achieve better outcomes.

Conflicts of interest statement

Acknowledgments

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MT1-MMP plays a critical role in hematopoiesis by regulating HIF-mediated chemokine/cytokine gene transcription within niche cells

Chiemi Nishida,[1] Kaori Kusubata,[2] Yoshihiko Tashiro,[1] Ismael Gritli,[1] Aki Sato,[1] Makiko Ohki-Koizumi,[1] Yohei Morita,[1] Makoto Nagano,[3] Takeharu Sakamoto,[3] Naohiko Koshikawa,[3] Takahiro Kuchimaru,[4] Shinae Kizaka-Kondoh,[4] Motoharu Seiki,[3] Hiromitsu Nakauchi,[1] *Beate Heissig,[1,2,5] and *Koichi Hattori[1,5]

[1]Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [2]Frontier Research Initiative, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [3]Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo, Japan; [4]Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama, Japan; and [5]Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo, Japan

HSC fate decisions are regulated by cell-intrinsic and cell-extrinsic cues. The latter cues are derived from the BM niche. Membrane-type 1 matrix metalloproteinase (MT1-MMP), which is best known for its proteolytic role in pericellular matrix remodeling, is highly expressed in HSCs and stromal/niche cells. We found that, in MT1-MMP$^{-/-}$ mice, in addition to a stem cell defect, the transcription and release of kit ligand (KitL), stromal cell–derived factor-1 (SDF-1/CXCL12), erythropoietin (Epo), and IL-7 was impaired, resulting in a trilineage hematopoietic differentiation block, while addition of exogenous KitL and SDF-1 restored hematopoiesis. Further mechanistic studies revealed that MT1-MMP activates the hypoxia-inducible factor-1 (HIF-1) pathway via factor inhibiting HIF-1 (FIH-1) within niche cells, thereby inducing the transcription of HIF-responsive genes, which induce terminal hematopoietic differentiation. Thus, MT1-MMP in niche cells regulates postnatal hematopoiesis, by modulating hematopoietic HIF-dependent niche factors that are critical for terminal differentiation and migration. (*Blood*. 2012;119(23):5405-5416)

Introduction

The adult hematopoietic system is maintained by a small number of HSCs that reside in the BM in a specialized microenvironment (the niche).[1,2] Here, HSCs undertake fate decisions including differentiation to progenitor cells and self-renewal, which ensures a lifelong supply of terminally differentiated blood cells. Intrinsic cellular programming and external stimuli such as adhesive interactions with the microenvironmental stroma and cytokine activities regulate HSC fate. However, it is unclear how niche factor production is controlled to adjust to external demand with a fine-tuned response.

Hypoxia-inducible factors (HIFs) consist of an α (HIF-α) and a β (HIF-β, or ARNT) subunit and activate the expression of genes encoding proteins that regulate cell metabolism, motility, angiogenesis, hematopoiesis, and other functions. HSCs maintain cell-cycle quiescence by regulating HIF-1α levels.[3,4] Mice with mutations in the heterodimeric transcription factor HIF develop extensive hematopoietic pathologies: embryos lacking *Arnt* have defects in primitive hematopoiesis.[5] Mice lacking endothelial PAS domain protein 1 (EPAS1, also known as HIF-2alpha/HRF/HLF/MOP3), a second HIF family member, exhibited pancytopenia, and it was shown that EPAS1 is necessary to maintain a functional microenvironment in the BM for effective hematopoiesis.[6] HIFs bind to canonical DNA sequences in the promoters or enhancers of target genes such as erythropoietin (*Epo*), vascular endothelial growth factor-A, *SDF-1α*/CXCL12, angiopoietin-2, platelet-derived growth factor-B and Kit Ligand (*KitL*)/stem cell factor, which are involved in HSC maintenance within the BM niche.[7-10] The chemokine

SDF-1α/CXCL12 (SDF-1α) is expressed by perivascular, endosteal, mesenchymal stem and progenitor cells as well as by osteoblasts.[11,12] SDF-1α deficiency leads to a reduction in HSCs and impaired B-cell development in mice.[13,14] IL-7 is another stromal cell–derived niche factor, which, in cooperation with CXCL12, functions at sequential stages of B-cell development.[15,16] IL-7 or IL-7R deficiency results in impaired B-cell development.[17,18]

Proteases such as matrix metalloproteinase-9 (MMP-9) and the serine proteinase plasmin(ogen) regulate HSC fate through KitL release in the BM.[10,19] Membrane type-1 MMP (MT1-MMP, also known as MMP-14) can proteolytically degrade extracellular matrix (ECM) components and cleave membrane receptors and ligands.[20] MT1-MMP is expressed within mesenchymal stem and immature hematopoietic cells.[21,22] MT1-MMP can control the migration of hematopoietic stem/progenitor cells and monocytes.[21,23,24] MT1-MMP function is essential for angiogenesis, wound healing, connective tissue remodeling, arthritis, tumor growth, and metastasis.[25-27] MT1-MMP–deficient (MT1-MMP$^{-/-}$) mice showed skeletal dysplasia, arthritis, and osteopenia.[28] However, the role of MT1-MMP in hematopoiesis is unclear.

We found that MT1-MMP inactivation in mice resulted in severe pancytopenia, characterized by an impaired stem cell pool and a block in hematopoietic differentiation. MT1-MMP deletion from hematopoietic cells generated normal hematopoiesis in recipient mice, thereby demonstrating that MT1-MMP is an essential regulator of the BM microenvironment. Mechanistically, we demonstrate that MT1-MMP deficiency blocked the transcription of

typical HIF-1–dependent niche factors including Epo, SDF-1, KitL, and IL-7 by modulating the HIF-1 pathway through factor inhibiting HIF-1 (FIH1), resulting in a 3-lineage terminal differentiation block. Thus, MT1-MMP controls HSC fate by regulating the BM niche.

Methods

Animals

Age-matched (14-day) MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice were obtained by heterozygous breeding.[24] Animal procedures were approved by the Animal Care Committee of The Institute of Medical Science (University of Tokyo). C57BL/6 and C57BL/6-Tg (CAG-EGFP) mice were purchased from Japan SLC Inc, and Ly5.1 mice were purchased from Sankyo Lab Service.

In vivo assays

Competitive transplantation experiments. Lethally irradiated Ly-5.1 mice were injected with BM cells (10 recipient mice per cell concentration) from MT1-MMP$^{+/+}$ or MT1-MMP$^{-/-}$ mice together with 2×10^5 CD45.1 BM competitive cells. Peripheral blood (PB) cells of the recipient mice were analyzed 4 months after transplantation. Cells were stained with PE-conjugated anti-CD4 and anti-CD8, FITC-conjugated anti-CD45.2, allophycocyanin-conjugated anti-CD11b and anti-Gr-1, PE-cy7–conjugated anti-B220, and biotinylated anti-CD45.1 Abs. The biotinylated Ab was developed using streptavidin-PE–Cy5. The percentage of donor-derived lineage contributions in PBMCs was assessed using Abs against CD45.2, Gr-1/CD11b, or B220. Total chimerism of > 1% for all Abs tested using PBMCs was considered as long-term reconstitution. The frequencies were determined using L-Calc software (StemCell Technologies).

Growth factor rescue experiments. Recombinant mouse KitL (Pepro-Tech) was administered IP into MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice at a concentration of 150 μg/kg body weight, daily from postnatal day 7 to day 10. Recombinant mouse SDF-1α (PeproTech; 100 ng/mice) was injected twice intraperitoneally on postnatal day 10. Blood was collected and blood cells counted on day 12.

CFU-S assay. Mobilized PBMCs were obtained and subjected to a CFU-S assay as previously described.[29] Mice were killed on day 12. The number of visible splenic colonies was counted.

In vitro assays

Peripheral blood analysis. Blood was collected from mice by retro-orbital bleeding using heparinized capillaries. White blood cell (WBC), RBC, and platelet (PLT) counts were determined. Plasma samples were stored at −80°C until further analysis.

Hematopoietic progenitor assay. BM mononuclear cells (BMMCs; 10^4 cells/plate) were plated in triplicate in 1 mL of a commercially available methylcellulose-based assay solution (Methocult; StemCell Technologies).

Lineage-negative cell separation. Murine BM cells were obtained after flushing mouse femur and tibiae. Cells were stained using a lineage cell separation kit (StemCell Technologies). After MACS cell separation (Miltenyi Biotec), cells were stained with c-Kit, Sca-1, and lineage Abs (BD Pharmingen), and were then analyzed by FACS.

B-cell colony-forming assay (CFU–IL-7). The CFU–IL-7 assay was carried out in medium (Invitrogen) containing 1.2% methylcellulose (StemCell Technologies), 30% FCS (HyClone), 1% BSA, 0.1mM 2-ME, and mouse IL-7 (PeproTech). On day 7 of culture, aggregates consisting of > 50 cells were scored as a colony.

Cell culture. Mouse stromal cells (MS-5) were maintained in IMDM supplemented with 10% FBS. Human BM endothelial cells (BMEC-1) were maintained in Medium 199 supplemented with 10% FBS, 0.146 mg/mL L-glutamine, and 2.2 mg/mL sodium bicarbonate. Mouse embryonic fibroblast cells (NIH3T3) were maintained in DMEM supplemented with 10% FBS. These cells were cultured at 37°C, in a 5% CO_2 incubator. Human osteoblastic cells (FOB) were maintained in a 1:1 mixture of Ham F12

medium DMEM supplemented with 2.5mM L-glutamine and 10% FBS, and the cells were cultured at 34°C, in a 5% CO_2 incubator. MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mouse embryonic fibroblasts (MEF) cells (kindly provided by M.S.) were maintained in DMEM (Invitrogen) supplemented with 10% FBS at 37°C, in a 5% CO_2 incubator.[30]

Knockdown experiment using shRNA. The shRNA sequences used for knockdown of mouse MT1-MMP and FIH-1 were: 5′-caccgctgtggtgttc-cggataagtcgaaacttatccggaacaccacagc-3′ and 5′-caccggacctcgaatacctgcaagac-gaatcttgcaggtattcgaggtcctttt-3′, respectively. These sequences were sub-cloned into pENTR/U6 TOPO (Invitrogen) and then transferred via recombination into the lentivirus vector pLenti6 BLOCKiT (Invitrogen). shRNA-expressing lentiviral vectors were generated and used according to the manufacturer's instructions.

FACS analysis. Cells were flushed out from mouse BM. For cell-surface analysis, cells were stained with the following Abs: PE-conjugated anti-CD19, -Sca-1, -B220, CD44, c-kit, and -Gr1, FITC-conjugated anti-CD43, -CD34, -NK1.1, and -CD8, allophycocyanin-conjugated anti-B220, -CD4, CD11b, and a lineage cocktail, PerCP-Cy5.1–conjugated anti-c-kit. Cells were analyzed using FACS Aria (BD Biosciences). Common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), and mega-karyocyte/erythroid progenitor (MEP) were determined in Lin$^-$ BM cells of 14-day-old MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice. The following Abs were used: c-kit–allophycocyanin, Sca1-PE/Cy7, CD34-FITC, FcgRIII-PE all from BD Pharmingen. For the detection of Lin$^-$ cells, biotinylated Abs (B220-bio, Gr-1-bio, CD11b-bio, CD5-bio, Ter119-bio, 7-4-bio, from Miltenyi Biotec) were costained with allophycocyanin/Cy7-conjugated (BD Biosciences/BD Pharmingen) streptavidin. For mesenchymal stem cells (MSCs) detection, cells were stained with the following Abs: CD45-PE, Sca1-FITC, PDGFRα-allophycocyanin (eBioscience), TER119-PE (BD Bioscience), CD45–Pacific Blue (BioLegend), and Alexa 488–conjugated nestin Ab (Abcam).

RNA extraction, RT-PCR, and quantitative real-time PCR analysis. Total RNA was extracted using RNA TRIzol (Invitrogen), and cDNA was generated according to the manufacturer's protocols. This cDNA (10 ng) was used as a template for each PCR amplification using the following specific forward and reverse primers, respectively: mouse β-actin (5′-tggaatcctgtggcatccatgaaac-3′) and (5′-taaaacgcagctcagtaacagtccg-3′); mouse *MT1-MMP* (5′-tccggataagtttgggactg-3′) and (5′-ccttcaccatcaaagggtgt-3′); human *GAPDH* (5′-gagtcaacggatttggtcgt-3′) and (5′-ttgattttggagggatctcg-3′); human *MT1-MMP* (5′-caagcattgggtgtttgatgatg-3′) and (5′-cttgggg-tactcgctatcca-3′). For quantitative real-time PCR, PCR mixtures were prepared using SYBR Premix Ex TaqII (Takara) containing 0.2mM of each primer, and amplification reactions were performed. Specific forward and reverse primers, respectively, were designed as follows: β-actin (5′-gctggaaggtggacagtgag-3′) and (5′-tgacaggatgcagaaggaga-3′); *KitL* (5′-gcctagtcattgttggctac-3′) and (5′-cccaagtttgtctatgatgg-3′); *SDF-1* (5′-agaaaccttccaccagagca-3′) and (5′-aacggctaggaaagggtctc-3′); *IL-7* (5′-tgcaatcatgtcaactgcaa-3′) and (5′-tgcaatcatgtcaactgcaa-3′); *EPO* (5′-catctgcgacagtcgagttctg-3′) and (5′-cacaacccatcgtgacattttc-3′); *G-CSF* (5′-cctgcttagagcagagagag-3′) and (5′-cagcagcaggaatcaatact-3′). Gene expression levels were measured using the ABI Prism 7500 sequence detection system (Applied Biosystems). PCR product levels were estimated by measurement of the intensity of SYBR Green fluorescence. Gene expression levels were normalized to β-actin mRNA.

Knockdown experiment using siRNA. Target sequences for siRNA were commercially designed and synthesized (B-bridge). siRNAs were provided as a mixture containing 3 different siRNA target sequences. The RNAi transfection solution was prepared by preincubating a mixture of 5nM siRNAs dissolved in 1 mL of serum-free and antibiotic-free medium (OptiMEM; Invitrogen) and 10 μL of RNAiMAX for 20 minutes at room temperature. Cells (3-4.5 × 10^5 cell/4 mL growth medium without antibiotics) suspended by trypsinization were added to the mixture and cultured overnight, and the medium was replaced with fresh growth medium.

Overexpression of MT1-MMP. The transfection solution was prepared by preincubating a mixture of 1 μg of plasmid DNA dissolved in 250 μL of OptiMEM and 3 μL of Lipofectamine 2000 (Invitrogen) dissolved in 250 μL of OptiMEM for 20 minutes at room temperature. The transfection mixture (500 μL in total) was then added to the cells. After

48 hours of culture, the culture media were replaced with fresh growth medium.

Immunoprecipitation. Cells were lysed with cell lysis buffer (Cell Signaling Technology) and the supernatants were collected. Total protein content was measured using the Bradford assay (Bio-Rad). Lysates were incubated with protein-G–Sepharose beads (Santa Cruz Biotechnology) and anti–HIF-1α Ab (BD Biosciences) overnight and were then spun down for 1 minute at 5800g. The supernatant was removed and the pellets were solubilized with 6 × SDS sample buffer (0.35M Tris-HCl, pH 6.8, 10% SDS, 30% glycerol, 9.3% DTT) and analyzed by immunoblot analysis.

Immunoblotting. Cells were lysed with lysis buffer (Cell Signaling Technology) according to the manufacturer's instructions. The supernatants were collected, and total protein content was measured using the Bradford assay (Bio-Rad). The subcellular proteome extraction kit (Merck) was used to fractionate subcellular proteins. Lysates were separated by SDS-PAGE, transferred to a PVDF membrane, subjected to immunoblotting using anti–FIH-1 (1:200; Santa Cruz Biotechnology), anti-integrinβ1 (1/1000; Chemicon), and anti–α/β tubulin (1:1000; Cell Signaling Technology), washed with TBS-T, and immunoprobed for 1 hour at room temperature with HRP-conjugated or alkaline phosphatase–conjugated Ab, then washed with PBS-T. Finally, membranes were incubated with ECL-Plus (Amersham), and the chemiluminescent signal was detected using LAS4000 (Fujifilm) according to the manufacturer's instructions. The alkaline phosphatase signal was then detected using the Histofine Kit (Nichirei).

Immunoassay. After cell transfection with siRNA, the growth medium was replaced and the cells were cultured for 48 hours. Supernatants were then collected and analyzed for murine KitL, SDF-1α, and IL-7 using commercially available ELISAs (R&D Systems).

Stromal-based expansion cultures. A total of 1 × 10⁴ Lin⁻ cells from the BM of GFP mice were cocultured with a confluent layer of stromal cells (MS-5). Recombinant mouse KitL (20 ng/mL, every other day) or recombinant mouse SDF-1α (100 ng/mL, every other day) was added to MT1-MMP KD MS-5 and control MS-5 cell cultures. Twelve days later, adherent cells were retrieved using trypsin (Sigma-Aldrich) and pooled with the nonadherent cells. Hematopoietic cells (GFP positive) were counted.

Migration assay. Migration assays were performed in 24-well plates using 5-μm polycarbonate Transwell inserts (Costar). BM Lin⁻ cells were collected and isolated from normal adult mice. The cells were resuspended in X-vivo 15 (Lonza). MT1-MMP⁺/⁺ and MT1-MMP⁻/⁻ MEF culture supernatant was aliquoted (600-μL aliquots) into 24-well plates, which formed the bottom chamber, in the presence or absence of neutralizing Abs against mouse SDF-1 (R&D Systems). Lin⁻ cells derived from the BM cells of GFP mice (2 × 10⁵ cells in 100 μL) were added into the Transwell insert (top chamber), and the cells were allowed to migrate through the porous bottom for 4 hours at 37°C. The number of cells that migrated into the lower chamber was determined using flow cytometry. Cells had been stained with Abs against CD11b and Gr1 (BD Biosciences). The medium from the lower chamber was passed through a FACSCalibur for 60 seconds, gating on forward (FSC) and side scatter to exclude cell debris. The number of live cells was compared with a 100% migration control in which 2 × 10⁵ cells had been added directly into the lower chamber and then counted on the FACScalibur for 60 seconds.

Immunohistochemistry. BM sections were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100 for 20 minutes. After blocking in PBS containing 5% goat serum and 0.2% BSA, the sections were incubated with anti–FIH-1 Ab (Novus Biologicals) for 16 hours, washed 3 times with PBS and incubated for 1 hour with anti–rabbit Alexa 568 (Invitrogen). After washing, the sections were incubated with Alexa 488–conjugated nestin Ab (Abcam) for 2 hours. For HIF-1 staining, fixed and blocked sections were incubated with Alexa 488–conjugated HIF-1 Ab (BD Pharmingen) for 2 hours. The sections were mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were acquired with an Olympus DP71 camera.

Micro-CT analysis. We evaluated the BM shaft surface area by using micro-CT (Rigaku). We measured the shaft area at the center of the femur by NIH ImageJ software.

Proliferation assay. A total of 2 × 10⁴ Lin⁻ cells isolated by MACS (Miltenyi Biotec) from BM cells of GFP mice were cocultured with a confluent layer of MEF cells. The cells were maintained in X-vivo 15 supplemented recombinant mouse IL-3 (20 ng/mL, every other day) with or without neutralizing Abs against mouse KitL (R&D Systems). One week later, all cells were retrieved using trypsin (Sigma-Aldrich). GFP-positive were counted using FACS.

Statistical analysis

Data were analyzed using the unpaired 2-tailed Student *t* test and are expressed as means ± SEM. *P* values of < .05 were considered significant.

Results

MT1-MMP deletion leads to severe pancytopenia

We used 14-day-old MT1-MMP⁻/⁻ mice to explore the role of MT1-MMP in the regulation of postnatal hematopoiesis, as mortality of older MT1-MMP⁻/⁻ mice is very high.[24,28] Compared with MT1-MMP⁺/⁺ mice, the body weight of MT1-MMP⁻/⁻ mice was decreased (7.22 ± 0.48 and 2.99 ± 0.05 g/mouse, respectively; n = 5, *P* < .001) and there was a decline in all hematopoietic lineages examined in MT1-MMP⁻/⁻ animals, including WBCs (Figure 1A), PLTs (Figure 1B), and RBCs (Figure 1C) in the PB. MT1-MMP⁻/⁻ hematopoietic femurs were smaller than MT1-MMP⁺/⁺ femurs (Figure 1D). Under steady-state conditions, BM cellularity in MT1-MMP mice was decreased compared with MT1-MMP⁺/⁺ mice (Figure 1E). We next analyzed the number of MSCs by flow cytometry. The frequency of PDGFRα⁺/Sca1⁺/CD45⁻/Ter119⁻ BM-derived MSCs and nestin⁺ niche cells was lower in MT1-MMP⁻/⁻ BM cells than in MT1-MMP⁺/⁺ BM cells (Figure 1F).[31] Because the skeletal malformation described in MT1-MMP⁻/⁻ mice could affect the intrafemural space where hematopoiesis occurs, the BM shaft surface area was determined by microcomputer tomography (microCT). The BM shaft surface area was reduced in MT1-MMP⁻/⁻ femurs compared with MT1-MMP⁺/⁺ mice (Figure 1G).

Histologic analysis of BM sections showed that MT1-MMP⁻/⁻ mice exhibited a striking paucity of hematopoietic cells within the BM shaft and a reduction in the number of megakaryocytes, which are responsible for platelet production (Figure 1H-I).

We next examined HSCs and hematopoietic progenitor populations. In wild-type mice, MT1-MMP is expressed in primary BMMCs within the lineage-negative (Lin⁻) cell fraction, which contains mostly hematopoietic progenitor cells and a small fraction of HSCs, and in BM stromal cells, including BM endothelial cells (BMEC-1), fetal osteoblasts (FOB), and fibroblast-like stromal cells (MS-5; Figure 1J-K). The absolute number of CFU cells (CFU-C; Figure 1L) and of more primitive progenitor populations within BM cells, including day-8 CFU-spleen (CFU-S; Figure 1M), CMPs, GMPs, and MEPs and HSC-enriched CD34⁻c-Kit⁺, Sca-1⁺, Lin⁻ (KSL) cells were diminished in MT1-MMP⁻/⁻ BMMCs. To determine the HSC content of the BM, we performed limiting-dilution competitive repopulation analysis using MT1-MMP⁺/⁺ or MT1-MMP⁻/⁻ BMMCs. There was a lower frequency, and a significantly reduced absolute number of competitive repopulation units (CRUs) per femur in MT1-MMP⁻/⁻ BMMCs than in MT1-MMP⁺/⁺ BMMCs. These data indicate that MT1-MMP is required for HSC maintenance and for normal hematopoietic differentiation.
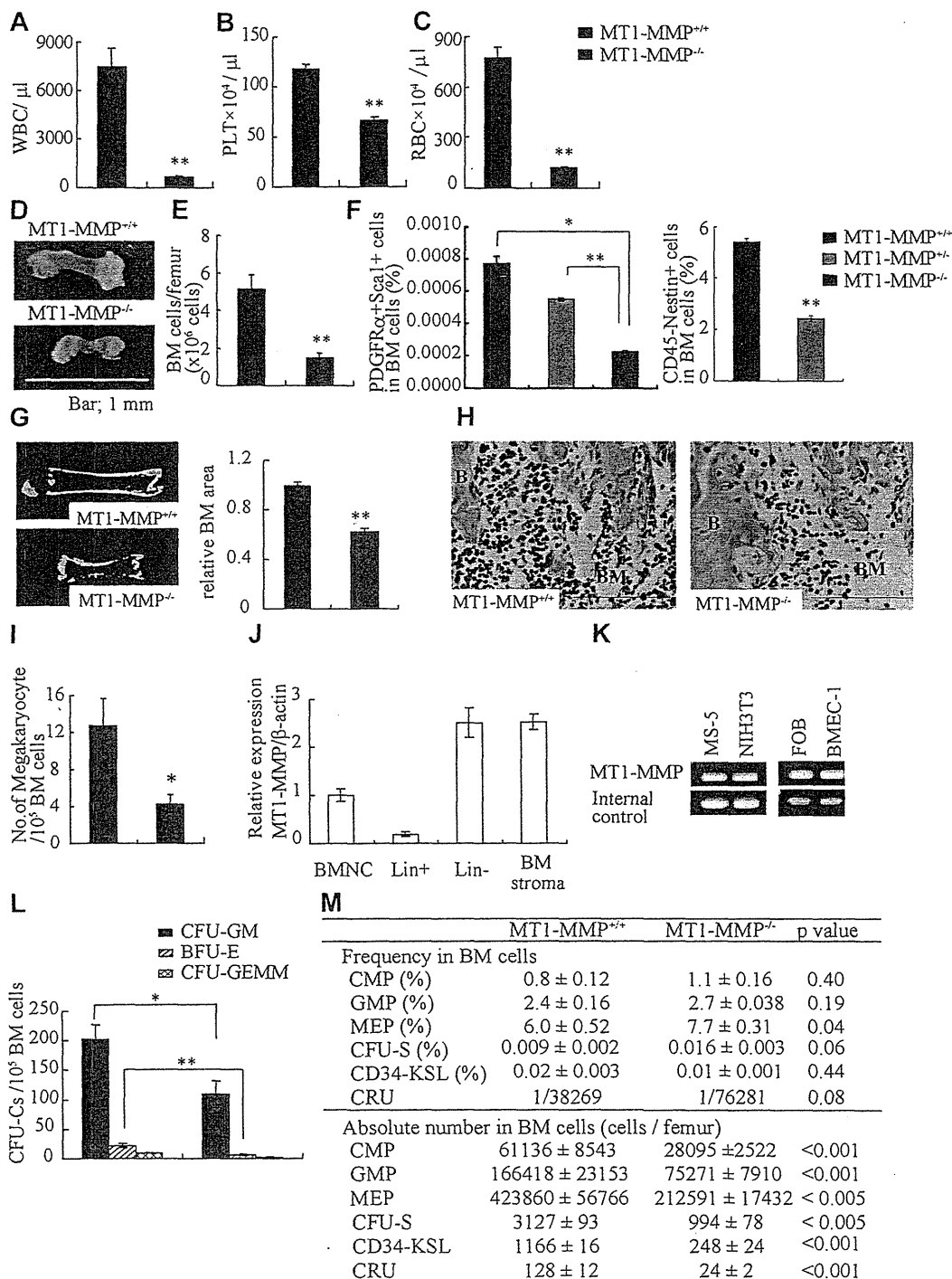
Figure 1. MT1-MMP gene deletion causes myelosuppression. (A-C) The number of (A) WBCs, (B) platelets (PLT), and (C) RBCs in the PB of 14-day-old mice was counted (n = 8). (D) Images of mouse femurs (bars, 1 mm). (E) Total BM cell number per femur. (F) The percentage of PDGFRα+Sca1+ cells (MSCs) was determined by flow cytometry (n = 2). (G) (left panel) Representative MicroCT scan images, and (right panel) quantification of the relative BM area within the BM femur shaft are presented (n = 3). (H) H&E-stained femur sections. Right panel shows the quantification of the BM area per femur. B indicates bone, (bars, 200 μm). (I) Megakaryocyte number (n = 5). (J-K) Real-time PCR analysis of MT1-MMP expression in (J) BM nuclear cells (BMNCs), Lin+, Lin−, and BM stroma cells, and (K) in MS-5, NIH3T3, FOB, and BMEC-1 cells. (L-M) The (L) absolute number of CFU-C per 10^5 BM cells and (M) frequency and absolute number/femur of CFU-S, CMP, GMP, MEP, CD34-KSL cells, and competitive repopulating units (CRU) in wild-type and knockout BM cells (n = 3). For CRU determination, freshly isolated BM cells of different cell concentration were transplanted into recipients (n = 10). Four months posttransplantation, the repopulating unit (RU) with trilineage engraftment (> 1% of donor-derived cells) was calculated. The absolute number of CRU was calculated based on the observed number of BM cells per femur. Errors in bar graphs are SEM; *P < .05, **P < .01. CFU-C indicates CFU cells; CFU-S, CFU-spleen; CMP, common myeloid progenitor; GMP, granulocyte/macrophage lineage-restricted progenitor; and MEP, megakaryocyte/erythrocyte lineage-restricted progenitor.

MT1-MMP$^{-/-}$ mice show a T- and B-cell differentiation defect

Compared with MT1-MMP$^{+/+}$ mice, the absolute numbers of PB CD3$^+$ T and B220$^+$ B lymphocytes were reduced (Figure 2A) and the spleen was much smaller (Figure 2B) in MT1-MMP$^{-/-}$ mice.

Similar to the BM, histologic examination revealed less megakaryocytes in the MT1-MMP$^{-/-}$ spleen (Figure 2C). The white pulp appeared smaller in MT1-MMP$^{-/-}$ spleens (Figure 2C), which correlated with a reduction in the absolute numbers of lymphoid
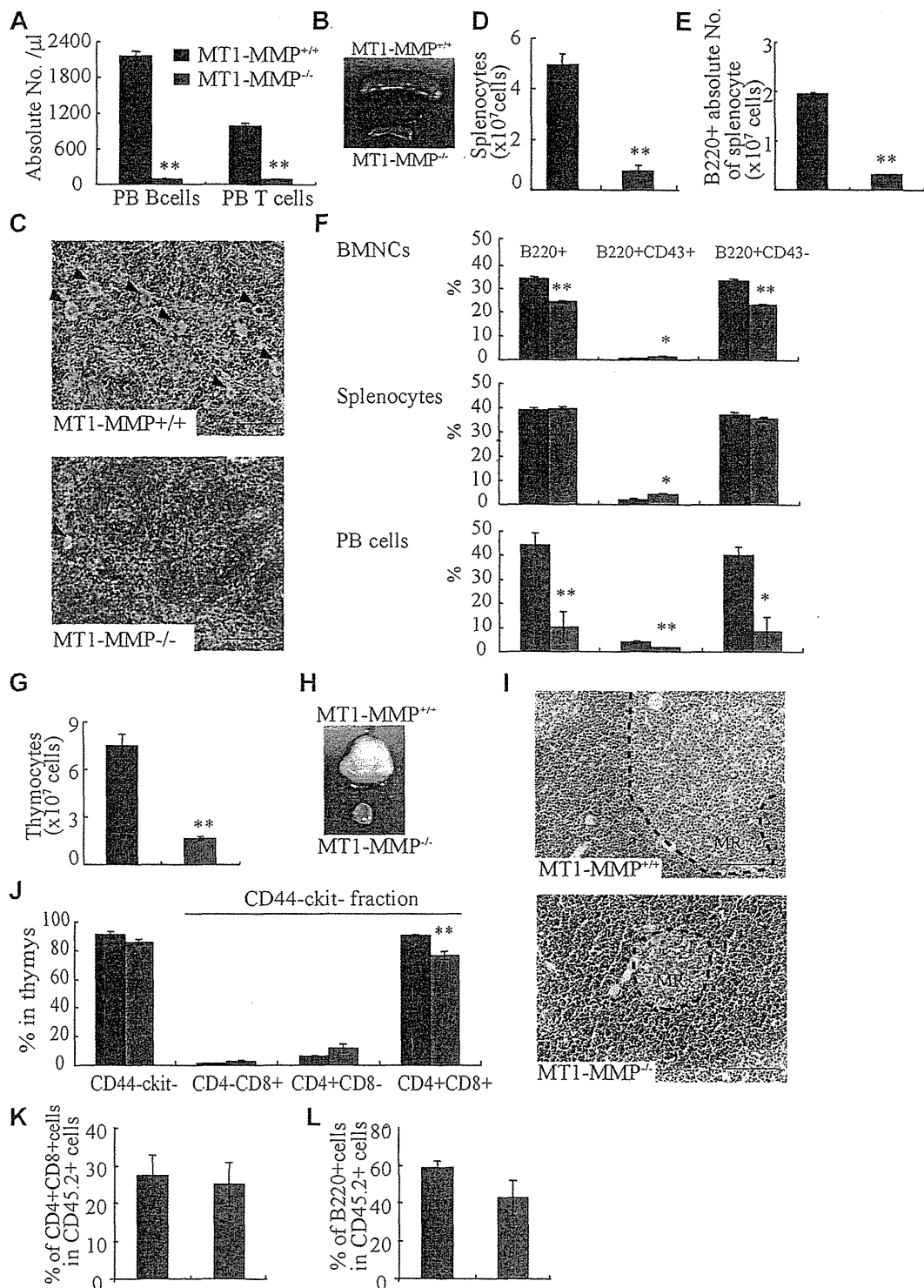
Figure 2. T and B lymphopoiesis are impaired in MT1-MMP$^{-/-}$ mice. (A) PB B- and T-cell numbers (FACS analysis). (B) Images of mouse spleens. (C) H&E-stained spleen sections. Arrowheads indicate megakaryocytes (bars, 100 μm). (D-E) The (D) total number of splenocytes and (E) B220$^+$ cells in splenocytes (n ≥ 6). (F) The percentage of B220$^+$ populations in BMNCs (n = 6), splenocytes (n = 3), and PB cells (n = 2). (G) Thymocyte number (n = 4). (H) Images of thymi. (I) H&E-stained thymus sections. MR indicates medullar region (bars, 100 μm). (J) The percentage of thymic T-lineage subpopulations. (K-L) BM cells from MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice were transplanted into wild-type animals (CD45.2). (K) The percentage of donor CD4$^+$/CD8$^+$ T and (L) B220$^+$B cell lineage contribution of donor-derived cells in the PB 4 months after transplantation (n = 10/group). Errors in bar graphs are SEM; *P < .05, **P < .01.

and total B (B220$^+$) spleen cells (Figure 2D-E). The percentage of B220$^+$, early- (B220$^+$CD43$^+$), and late-stage (B220$^+$CD43$^-$) B cells was reduced in MT1-MMP$^{-/-}$ BM, spleen, and PBMCs (Figure 2F). Although the absolute number of early- and late-stage B cells in splenocytes was reduced in MT1-MMP$^{-/-}$ mice (data not

shown), the relative percentage of early (B220$^+$CD43$^+$) B cells was augmented in MT1-MMP$^{-/-}$ splenocytes. These data demonstrate that MT1-MMP is required for normal terminal B-cell differentiation within the BM with impaired release of B cells into the circulation.
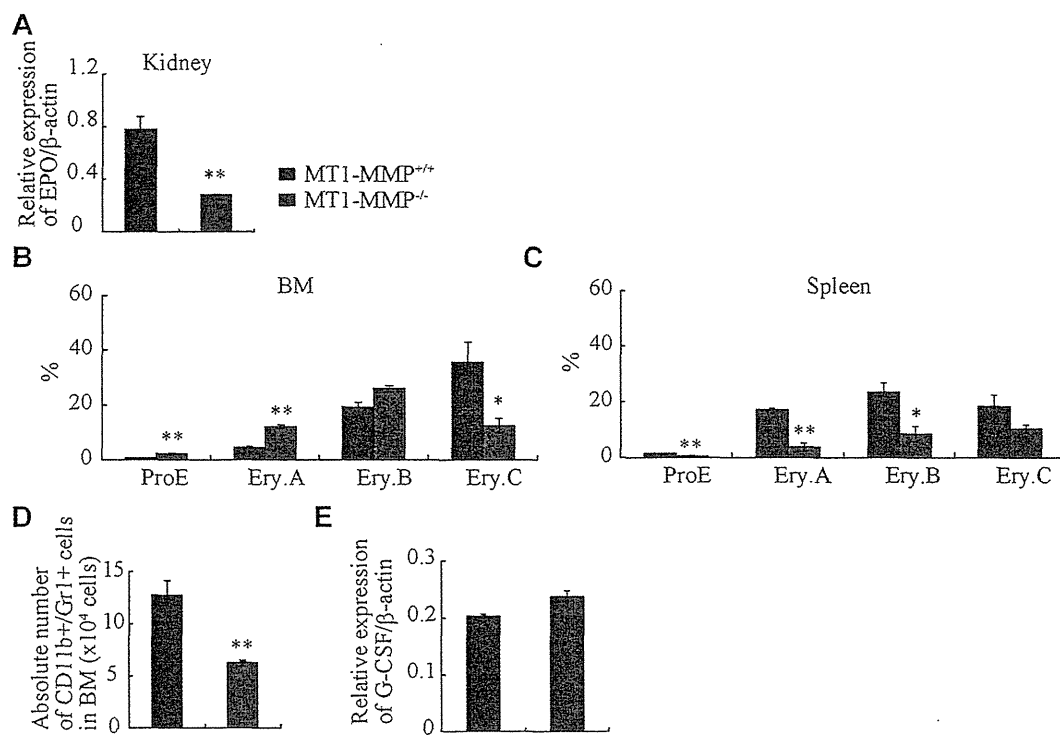
Figure 3. Erythroid and myeloid differentiation block in MT1-MMP$^{-/-}$ mice. (A) Real-time PCR of *Epo* in kidney (n = 3). (B-C) Percentage of different erythroblast populations during erythroid development (proE-EryA-EryB-EryC) of (B) BM cells or of (C) dissociated mouse spleen cells from MT1-MMP$^{+/+}$ (n = 5) and MT1-MMP$^{-/-}$ mice (n = 3). (D) Percentage of CD11b$^+$/Gr-1$^+$ cells per femur as determined by FACS (n = 5). Errors in bar graphs are SEM; **P < .01. (E) Real-time PCR of *G-CSF* in BM cells (n = 3).

The earliest T-cell progenitors are produced in the BM. We found an increase in the percentage of immature CD4$^-$CD8$^-$c-Kit$^-$CD44$^-$ cells T cells in MT1-MMP$^{-/-}$ BM cells (4.1 × 10^5 ± 0.2 × 10^5 cells in MT1-MMP$^{-/-}$ and 2.6 × 10^5 ± 0.3 × 10^5 cells in MT1-MMP$^{+/+}$ BM, respectively; n = 4, P < .05). The MT1-MMP$^{-/-}$ thymus (Figure 2G) was small with a low number of thymocytes (Figure 2H). Thymic lobes, and specifically the medullar region, appeared smaller than in MT1-MMP$^{+/+}$ mice (Figure 2I). Flow cytometric analysis of thymocytes revealed no difference in the percentage of immature CD4$^-$ or CD8$^-$ double-negative (DN) thymocytes or of CD4$^-$CD8$^-$c-Kit$^-$CD44$^-$ thymocytes between the MT1-MMP$^{+/+}$ and the MT1-MMP$^{-/-}$ mice. However, a relative decrease in the percentage of the more mature CD4$^+$CD8$^+$ double-positive (DP) thymocyte population and in the CD4$^+$ single-positive (SP) cell population was revealed in thymocytes from the MT1-MMP$^{-/-}$ mice (Figure 2J). In absolute terms, CD4 SP numbers were reduced 2.5-fold, CD8 SP 2:1-fold, and DP thymocytes 5.3-fold, whereas the DN population remained unaffected. These data indicate that T-cell progenitors developed within the BM even in the absence of MT1-MMP, but that their further differentiation in the thymus was blocked at or before development of the immature DN thymocyte population, causing default differentiation into CD4 cell SP and NK-cell lineages.

To assess whether the abnormal thymic colonization observed in MT1-MMP$^{-/-}$ mice was because of the loss of MT1-MMP in hematopoietic cells or in stromal cells, MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ BM donor cells were transplanted into lethally irradiated wild-type recipients. Chimeras were analyzed 4 months after transplantation. T- and B-cell differentiation of transplanted MT1-MMP$^{-/-}$ cells (expressing the Ag Ly5.2 (CD45.2) on leukocytes) within the BM were normal (Figure 2K-L). These data indicated that MT1-MMP$^{-/-}$ mice showed impaired T- and B-cell

development. Even though MT1-MMP$^{-/-}$ mice showed a hematopoietic cell defect (see Figure 1), MT1-MMP$^{-/-}$ BM cells retained the potential to differentiate into T- and B-cell lineages in an MT1-MMP$^{+/+}$ environment. These data indicate that, aside from its influence on stem cells, MT1-MMP also plays an additional role in regulating the BM niche.

MT1-MMP ablation impairs erythroid and myeloid differentiation

Anemia, as seen in 14-day-old MT1-MMP mice (see Figure 1C), is expected to trigger a compensatory response that is mediated principally through increased serum levels of erythropoietin (*Epo*), a master cytokine of erythropoiesis. Epo controls growth, survival, and differentiation of erythroid progenitors, either cooperatively with, or independently of KitL.[32-34] Despite severe anemia, *Epo* mRNA expression in kidney tissues of MT1-MMP$^{-/-}$ mice was low (Figure 3A) and the expected compensatory increase in spleen size did not occur (see Figure 2B).

To assess erythroid differentiation, we examined CD71 and TER119 expression in the BM and splenic erythroblasts, and classified erythroid cells into 4 populations at progressive levels of differentiation: proerythroblasts TER119^{med}CD71^{high}FSC^{high} (ProE), TER119^{high}CD71^{high}FSC^{high} (Ery.A), TER119^{high}CD71^{high}FSC^{low} (Ery.B), and TER119^{high}CD71^{low}FSC^{low} (Ery.C).[35]

MT1-MMP$^{-/-}$ BM erythroid lineage cells accumulated at the ProE, Ery.A, and Ery.B stage, and cells at the late erythroblast differentiation stages (Ery.C) were reduced (Figure 3B). In spleen, all stages of erythroid lineage cells were reduced in MT1-MMP$^{-/-}$ mice (Figure 3C). These data suggest that MT1-MMP is involved in both BM and splenic erythropoiesis, and that the defect in erythropoiesis in MT1-MMP$^{-/-}$ mice might be due, in part, to impaired Epo production.

Granulocytic cells of adult mouse BM express CD11b and high amounts of Gr-1. The absolute number of CD11b$^+$/Gr-1$^+$ granulocytes was lower in MT1-MMP$^{-/-}$ than in MT1-MMP$^{+/+}$ BM cells (Figure 3D). G-CSF is a growth, differentiation, and activating factor for neutrophils and their precursors. Regardless of the observed neutropenia, no difference in *G-CSF* (Figure 3E) or thrombopoietin (*Tpo*; data not shown) expression was observed in the BM cells of these mice, indicating that other factors might be responsible for the impaired myelopoiesis.

MT1-MMP deficiency reduces stromal cell–derived cytokine production

Chimera experiments indicated that the observed hematopoietic defect in MT1-MMP–deficient mice was in part because of a niche defect, suggesting that dysregulated production of stromal cell–derived cytokine(s) may be responsible for the hematopoietic differentiation block observed in MT1-MMP$^{-/-}$ mice. BM and thymic stromal cells produce IL-7.[36] Plasma levels of the stromal cell–derived factors KitL, SDF-1α, and IL-7 were lower in MT1-MMP$^{-/-}$ mice than in MT1-MMP$^{+/+}$ mice (Figure 4A-C). We observed a reduced number of nestin$^+$ niche cells in MT1-MMP$^{-/-}$ mice and, interestingly, nestin$^+$ niche cells highly express KitL and SDF-1.[31] In accordance with these data, MT1-MMP$^{-/-}$ BM cells and MT1-MMP$^{-/-}$ primary stromal cells showed lower mRNA expression of *IL-7, KitL*, and *SDF-1α/CXCL12* compared with MT1-MMP$^{+/+}$ cells (Figure 4D, supplemental Figure 1). In addition, very few primary stromal cells grew in cultures established using MT1-MMP$^{-/-}$ BM cells (supplemental Figure 1).

To exclude the possibility that the growth/chemokine factor decrease in MT1-MMP$^{-/-}$ mice was because of a lower number of the growth factor–producing stromal cells, we modulated MT1-MMP expression on a murine stromal cell line (MS-5). Overexpression of MT1-MMP in MS-5 stromal cells (MT1-MMP TF) increased *KitL, SDF-1α*, and *IL-7* gene expression (Figure 4E). In contrast, MT1-MMP knock down in MS-5 murine stromal cells using shRNA (*MT1-MMP KD*) reduced the expression of *KitL, SDF-1α*, and *IL-7* mRNA than control MS-5 cells (Figure 4F). Consistent with this result, less KitL, SDF-1α, and IL-7 protein was detected in supernatants of MT1-MMP KD cultures compared with control cultures as determined by ELISA (Figure 4G). The frequency of CXCR4 (the SDF-1 receptor) and c-Kit (the KitL receptor) expressing Sca-1$^+$ MT1-MMP$^{-/-}$ BMMCs was not significantly changed (data not shown). These data indicate that loss of MT1-MMP activity in stromal cells reduced signaling by IL-7, KitL, and SDF-1α, factors known to regulate B and T lymphopoiesis and erythropoiesis.

We next examined the release and expression of these factors by primary *MEFs*. Confirming our BM cell and primary stromal cell cytokine/chemokine data, low expression of *KitL, SDF-1α*, and *IL-7* was found in MT1-MMP$^{-/-}$ MEFs (Figure 4H-I).

To investigate whether the observed impaired production of cytokines/chemokines would also result in impaired function, we set up an MEF feeder–based culture supplemented with IL-3. Whereas Lin$^-$ cells differentiated in MT1-MMP$^{+/+}$ MEF-supported cultures, the number of CD11b$^+$/Gr-1$^+$ cells in MT1-MMP$^{-/-}$ cultures did not change significantly (Figure 4J). Addition of neutralizing Abs against KitL confirmed that the observed myeloid cell differentiation was mainly because of the KitL that was released by the cultures.

We set up a migration assay to investigate SDF-1α function. Lin$^-$ cells derived from BM cells migrated toward an MT1-MMP$^{+/+}$ MEF supernatant, a process that is mediated by SDF-1, as shown by using neutralizing Abs against SDF-1. In contrast, no migration of Lin$^-$ cells was observed toward an MT1-MMP$^{-/-}$ supernatant (Figure 4K).

MT1-MMP increases cytokine/chemokine production in niche cells by suppressing HIF-1α

The *HIF* gene is known to regulate VEGF, placental growth factor, angiopoietin 2, platelet-derived growth factor-B, SDF-1α, Epo, and KitL/SCF expression.[7] HIF-1α activity under normoxia depends on the FIH-1. FIH1-mediated hydroxylation disrupts a critical interaction between HIFα and the coactivators p300/CBP, impairing HIF transcriptional activity.[37,38] To determine whether HIF signaling is involved in MT1-MMP modulation of hematopoietic differentiation, we assessed HIF-1α, HIF-2α, and FIH-1 protein levels in MT1-MMP knockdown and control MS-5 cells. MT1-MMP knockdown did not affect HIF-1α or HIF-2α protein expression (supplemental Figure 2), but did up-regulate FIH-1 protein expression within the cytosol of MS-5 cells (Figure 5A), where FIH-1 can prevent HIF-1α binding to the transcriptional coactivator p300/CBP thereby blocking HIF-1-induced transcription of genes such as SDF-1 and KitL.[39] Immunohistochemical analysis showed that less nestin$^+$ stromal cells coexpressing FIH-1 were found in MT1-MMP$^{-/-}$ BM cells than in MT1-MMP$^{+/+}$ BM cells (Figure 5B). These findings are consistent with previous data that, in monocytes, the cytoplasmic tail of MT1-MMP binds to FIH-1, leading to inhibition of FIH-1 activity by its inhibitor, Mint3/APBA3.[23]

HIF-1 immunostaining showed that HIF-1, in contrast to its expression in MT1-MMP$^{+/+}$ BM cells, was preferentially expressed in the cytoplasm of MT1-MMP$^{-/-}$ BM cells (Figure 5C).

Our data suggested that MT1-MMP can activate the HIF1 pathway in stromal cells as it does in monocytes. We therefore hypothesized that FIH-1 overexpression in stromal cells would cause a reduction in cytokine/chemokine production, which was indeed the case (Figure 5D-F). These data suggested that FIH-1 overexpression reduced SDF-1, KitL, and IL-7 gene transcription in stromal cells. Furthermore, although the knockdown efficiency of FIH-1 by shRNA was only 30%, FIH-1 knockdown rescued *KitL, SDF-1α*, and *IL-7* gene expression in MT1-MMP knockdown (70% reduction by siRNA) MS-5 cells (Figure 5G-I) indicating that the decreased HIF-mediated cytokine gene transcription in MT1-MMP knockdown stromal cells can be partially rescued by blocking FIH-1 activity. These data highlight the importance of MT1-MMP–mediated HIF-1 activation for the transcriptional regulation of critical hematopoietic niche factors.

Exogenous SDF-1α and KitL addition restores leukopenia and thrombopenia in MT1-MMP$^{-/-}$ mice

Finally, we asked whether growth factor addition would rescue the observed block in leukopenia and thrombopenia because of MT1-MMP deficiency. Indeed, addition of recombinant IL-7 to MT1-MMP$^{-/-}$ or wild-type BMMC cultures induced a similar number of B-cell colonies (CFU–IL-7) in the MT1-MMP$^{-/-}$ as in the wild-type cells (Figure 6A).

MS-5 cells support myeloid cell differentiation of Lin$^-$ wild-type cells in vitro. However, fewer GFP$^+$ hematopoietic cells were generated from Lin$^-$ wild-type BM cells on MT1-MMP KD MS-5 stromal cells than on control MS-5 cells. Addition of KitL restored hematopoietic cell growth on the MT1-MMP KD MS-5 feeder cultures (Figure 6B).
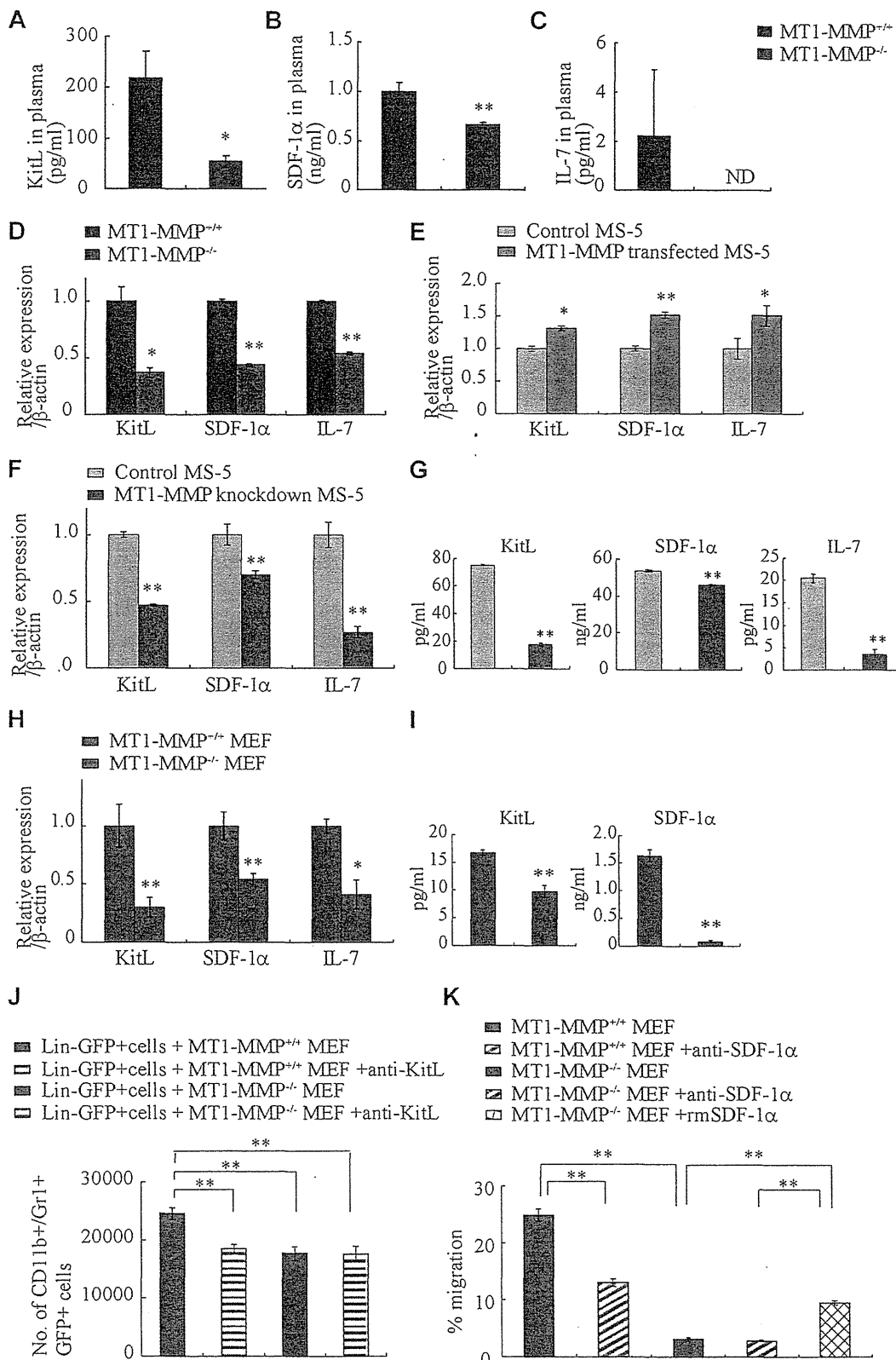
Figure 4. MT1-MMP deficiency prevents transcription of niche chemokines/cytokines. (A-C) KitL, SDF-1α, and IL-7 plasma levels in MT1-MMP^{+/+} and MT1-MMP^{−/−} plasma were measured by ELISA (n > 6). *KitL, SDF-1α,* and *IL-7* gene expression in (D) total BM cells, (E) MS-5 control and MT1-MMP–overexpressing cells, and (F) MT1-MMP knockdown (KD) and control MS-5 cells were analyzed using real-time PCR. The results are expressed relative to expression of a β-actin. (G) KitL, SDF-1α, and IL-7 protein levels in MT1-MMP knockdown (KD) and control MS-5 cell-culture supernatants were determined by ELISA. (H) *KitL, SDF-1α,* and *IL-7* gene expression in MT1-MMP^{+/+} and MT1-MMP^{−/−} MEF cells was determined using real-time PCR. The results are expressed relative to expression of β-actin. (I) KitL and SDF-1α protein levels in the indicated MEF cell-culture supernatants were evaluated by ELISA. (J) Lin⁻GFP⁺ cells were cultured on MT1-MMP^{+/+} and MT1-MMP^{−/−} MEF cells in the presence or absence of neutralizing Abs against KitL. The number of CD11b⁺Gr1⁺ cells was assessed after 7 days by FACS (n = 5). (K) Lin⁻ cells were plated in transwells. MT1-MMP^{+/+} and MT1-MMP^{−/−} MEF cell-culture supernatants supplemented with recombinant SDF-1α were added to the lower chamber. Neutralizing Abs against SDF-1α were added to both chambers. The percentage of migrated cells was determined (n = 9 from 2 independent experiments). Errors in bar graphs are SEM; *P < .05, **P < .01. Data shown are representative of 3 to 4 independent experiments.
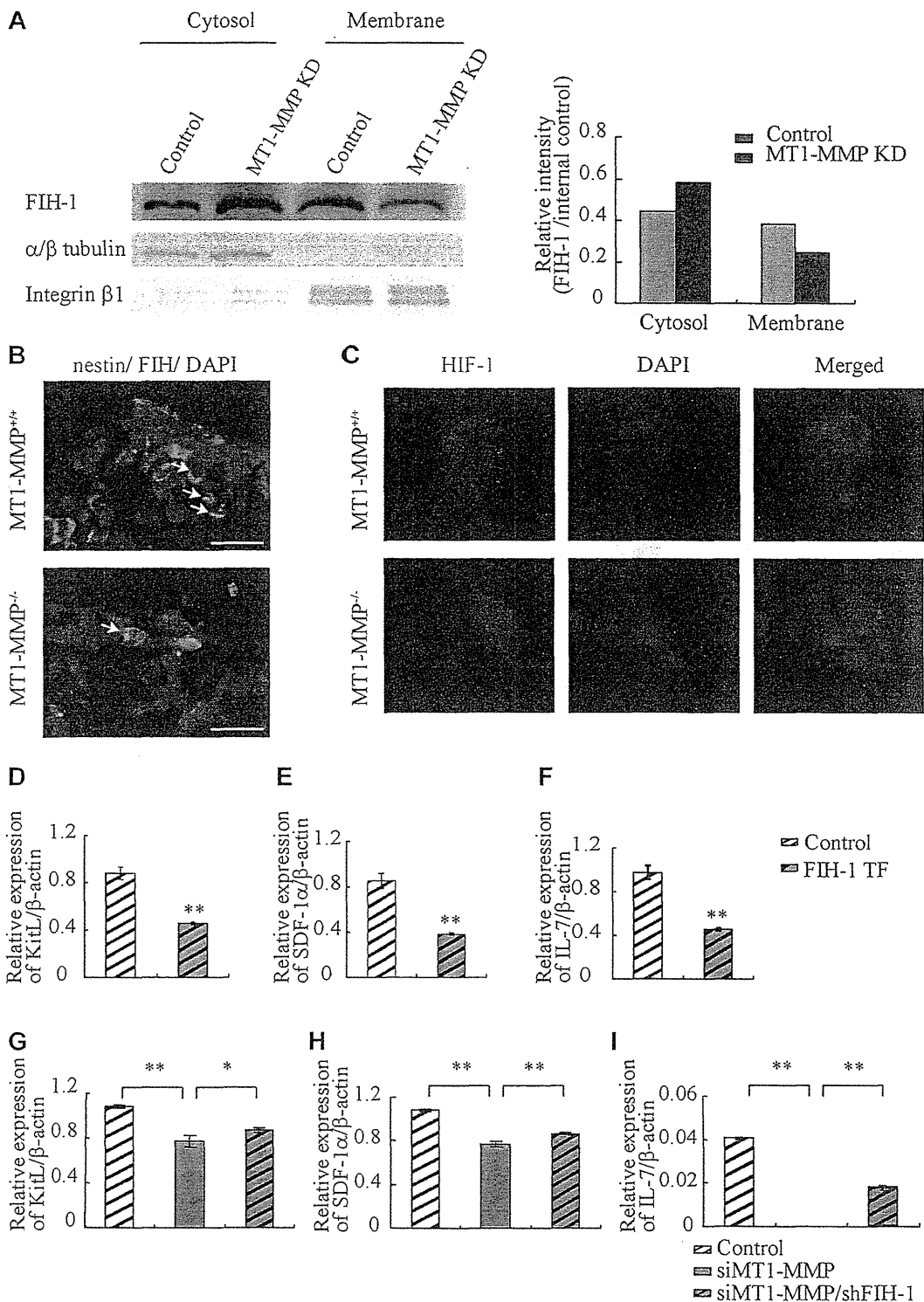
Figure 5. MT1-MMP deficiency prevents HIF-mediated transcription of niche factors. (A) FIH-1 expression in subcellular fractions was analyzed by Western blotting. α/β tubulin and integrin-β1 are representative cytosolic and membrane proteins, respectively. (B) Representative images of immunofluorescent staining of nestin (green fluorescence) and FIH-1 (red fluorescence) in BM sections derived from MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice. The arrows indicate nestin$^+$/FIH-1 cells. Nuclei were counterstained with DAPI (blue; bars, 100 μm). (C) Representative images of immunofluorescent staining of HIF-1α (green fluorescence) in BM cells derived from MT1-MMP$^{+/+}$ and MT1-MMP$^{-/-}$ mice. Nuclei were counterstained with DAPI (blue). (D-I) KitL, SDF-1α and IL-7 gene expression in (D-F) MS-5 cells overexpressing FIH-1, and (G-I) in MT1-MMP–deficient MS-5 cells with or without FIH-1 knockdown as analyzed using real-time PCR. The results are expressed relative to expression of a β-actin, which was set at 1.0. Data shown are representative of 3 to 4 independent experiments.

Similarly, KitL treatment restored WBC and PLT numbers in the PB in MT1-MMP$^{-/-}$ mice to the values of wild-type controls (Figure 6C-D) and a single injection of SDF-1α increased both WBC and PLT counts in the PB of MT1-MMP$^{-/-}$ mice 2 days after injection (Figure 6E-F). Although hematopoietic cell growth was

restored in MT1-MMP$^{-/-}$ mice by injection of KitL or SDF-1, survival was not improved (data not shown).

Collectively, these results suggest that MT1-MMP alters the HSC niche by modulating HIF signaling, which promotes cytokine production and enhances cell differentiation and migration.
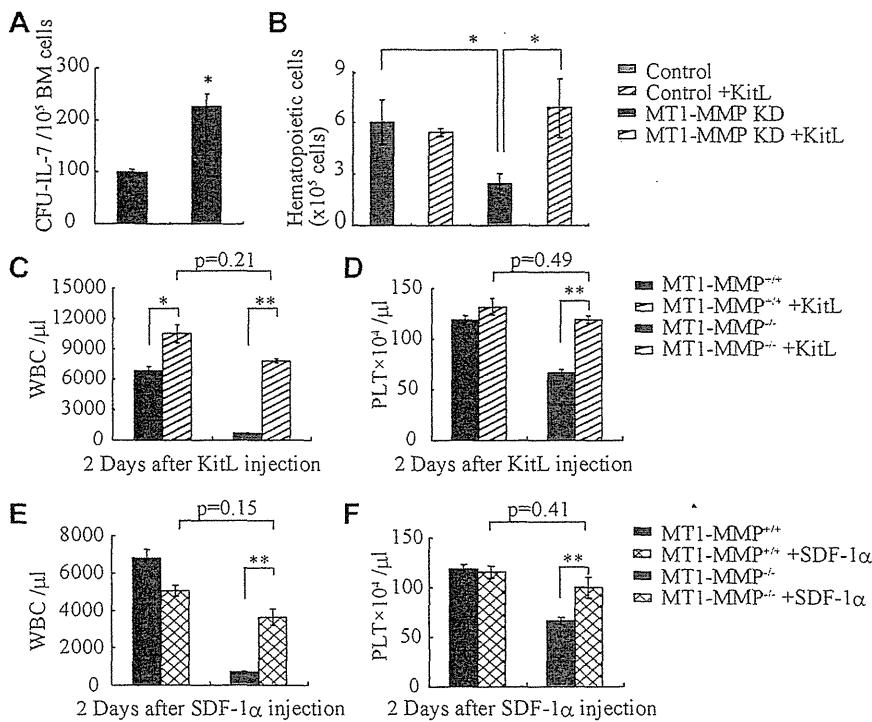
A

B

Legend:
- ▨ Control
- ◫ Control +KitL
- ▬ MT1-MMP KD
- ▱ MT1-MMP KD +KitL

C   p=0.21

D   p=0.49

Legend:
- ▬ MT1-MMP−/+
- ◫ MT1-MMP−/+ +KitL
- ▨ MT1-MMP−/−
- ▱ MT1-MMP−/− +KitL

E   p=0.15

F   p=0.41

Legend:
- ▬ MT1-MMP+/+
- ▨ MT1-MMP+/+ +SDF-1α
- ▨ MT1-MMP−/−
- ▨ MT1-MMP−/− +SDF-1α

Figure 6. MT1-MMP–deficient mice have a defective BM stromal niche with impaired terminal differentiation because of impaired release of HIF-1α–associated factors. (A) Number of colonies in IL-7–containing cultures of MT1-MMP+/+ and MT1-MMP−/− BM cells (n = 3). (B) Coculture of wild-type Lin− BM GFP+ cells on confluent MT1-MMP knockdown or control MS-5 cells with/without KitL. (C-D) PB WBCs (C) and PLT (D) after KitL injections into MT1-MMP+/+ (n ≥ 10) and MT1-MMP−/− mice (n = 3). (E-F) PB WBCs (E) and PLT (F) counts 2 days after initiation of SDF-1 treatment of MT1-MMP+/+ (n ≥ 10) and MT1-MMP−/− mice (n = 2). Errors in bar graphs are SEM; *$P < .05$, **$P < .01$.

Discussion

In this report, we identified MT1-MMP as a key player of postnatal hematopoiesis. We demonstrate that MT1-MMP–expressing cells serve as key links between the HIF-1 regulatory system and transcriptional regulation of vital niche chemokines/cytokines necessary for HSC maintenance and cell differentiation (Figure 7). Specifically, we show that MT1-MMP deficiency leads to impairment of steady-state hematopoiesis because of a reduced HSC pool and a trilineage differentiation block. Mechanistically, we provide

evidence that MT1-MMP deficiency in niche/stromal cells increases cytosolic FIH1 on expense of the membrane-associated FIH1 expression, thereby preventing transcription of the HIF-responsive genes EPO, KitL, IL-7, and SDF-1α. Thus, this study identifies MT1-MMP as a key molecular link between hypoxia and the regulation of vital HSC niche factors.

We reported that MMP-9 and plasminogen activation is important for the ectodomain shedding of the hematopoietic growth factor like KitL.[10,19] MT1-MMP can activate various proteases, including plasminogen or MMP-2, which in turn can activate MMP-9. It has been reported that MMP-2 activation can process

Figure 7. Proposed model of the role of MT1-MMP–expressing niche cells, which serve as key links between the HIF-1 regulatory system and transcriptional regulation of vital niche chemokines/cytokines necessary for HSC maintenance and cell differentiation. MT1-MMP deficiency in niche cells up-regulates FIH1 expression, thereby preventing transcription of the HIF-responsive genes EPO, KitL, IL-7, and SDF-1α. MT1-MMP deficiency leads to impaired steady-state hematopoiesis because of a trilineage differentiation block.
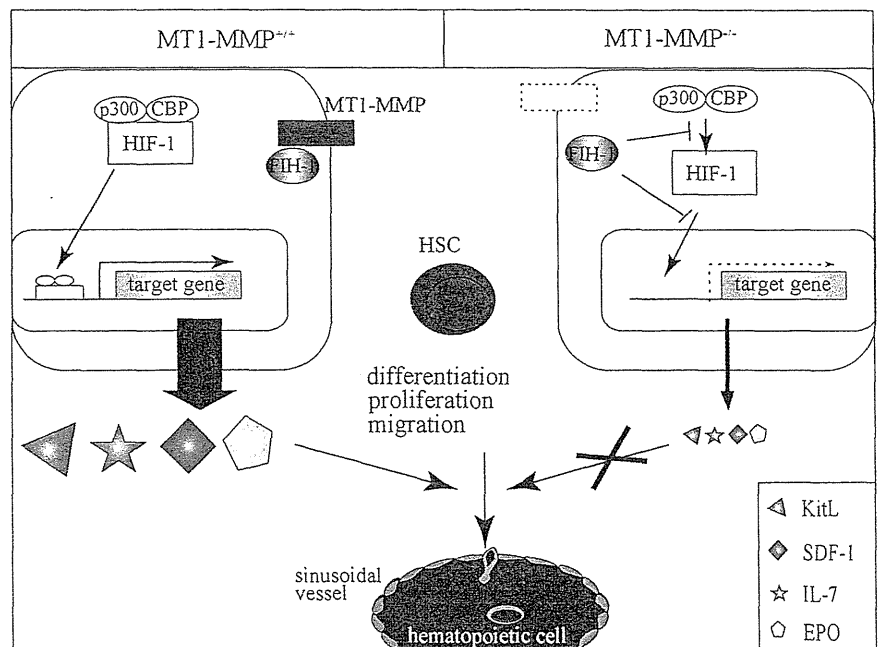
SDF-1/CXCL12.[40] We are currently examining whether ectodomain shedding by MT1-MMP–activated MMPs could be another reason for the low cytokine levels observed in MT1-MMP$^{-/-}$ mice.

Other known modulators of the BM and of niches such as the cancer stem cell niche, including vascular endothelial growth factor-A, angiopoietin 2, placental growth factor, and platelet-derived growth factor B, are also HIF-regulated target genes and therefore might also depend on MT1-MMP function.[7,8,41-43] But further studies will be needed to proof this concept.

Using BM chimeras generated using MT1-MMP$^{-/-}$ and MT1-MMP$^{+/+}$ donor cells, we showed that the developmental T-cell differentiation arrest observed in MT1-MMP$^{-/-}$ mice was mainly because of a niche defect, and not because of the impaired stem cell pool observed in these mice. In MT1-MMP$^{-/-}$ mice, we found preferential differentiation into CD4 SP thymocytes, which is most likely because of impaired Notch signaling.[44] Indeed, a recent study demonstrated that MT1-MMP directly cleaves Dll-1, a Notch ligand on BM stromal cells.[45] The continued presence of Dll-1 is required for T-cell commitment and maintenance at the DN1 and DN2 stages of thymocyte development. In the absence of Notch signaling, the developing DN1 and DN2 thymocytes adopt a NK-cell fate by default, a phenomena that we indeed observed in the MT1-MMP$^{-/-}$ mice.[46]

IL-7 signaling and Notch signaling are implicated in B-cell lymphopoiesis.[47] We found that HSC differentiation toward B lymphocytes was compromised in MT1-MMP–deficient BM cells. The long-term proliferation capacity of BM pre-B1 cells is known to be critically dependent on KitL and IL-7 expression and signaling, factors that we have shown require the presence of MT1-MMP for their expression.[48,49] Furthermore, we provide evidence that MT1-MMP deficiency in stromal cells also impairs the expression of SDF-1α, which is a key regulator of B-cell lymphopoiesis and BM myelopoiesis.[50] Our data are consistent with those of a previous report, which observed that defects in B lymphopoiesis are modulated by MT1-MMP–mediated cleavage of Dll-1 on BM stromal cells.[45] Impaired BM myelopoiesis was found in MT1-MMP–deficient mice, regardless of the fact that G-CSF expression was normal. Factors such as KitL and SDF-1α have also been implicated in myelopoiesis, and therefore might be at least partially responsible for the observed phenotype.

In addition, in vivo administration of KitL and SDF-1α rescued the pancytopenia in MT1-MMP–deficient mice. As both growth factors can increase the egress and mobilization of mature hematopoietic cells, but also can promote hematopoietic cell differentiation, the restoration of PB counts in MT1-MMP–deficient mice could be because of improved hematopoietic cell migration and/or differentiation. We confirmed data by Vagima et al demonstrating that MT1-MMP is expressed on hematopoietic progenitor cells.[21] This group showed that MT1-MMP is required for G-CSF–mediated hematopoietic progenitor cell mobilization.

SDF-1α is required for the maintenance of BM HSCs and is expressed by both perivascular and endosteal cells.[11,13] Deficits in the maintenance of the HSC pool in the absence of CXCR4 are HSC autonomous. Indeed, we could show that MT1-MMP deficiency altered SDF-1α/CXCL12-CXCR4 signaling and impaired the stem cell pool.

Our studies on the role of MT1-MMP in hematopoietic niche/stromal cells provide the rationale for further exploration of MT1-MMP in other hypoxic niches, for example, the cancer niche or the "ischemia-associated niche," as MT1-MMP seems to control the hematopoietic cell response in those diseases by controlling cytokine production.

Authorship

Contribution: C.N., B.H., and K.H. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; K.K., Y.T., I.G., A.S., M.O.-K., Y.M., M.N., T.S., N.K., T.K., and S.K.-K. participated in performing experiments, data analysis, and discussion; M.S. and H.N. interpreted data; and H.N., B.H., and K.H. assisted with experimental design and manuscript writing.

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Correspondence: Koichi Hattori, MD, PhD, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; e-mail: khattori@ims.u-tokyo.ac.jp.

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Inhibition of PAI-1 induces neutrophil-driven neoangiogenesis and promotes tissue regeneration via production of angiocrine factors in mice

Yoshihiko Tashiro,[1,2] Chiemi Nishida,[1] Kaori Sato-Kusubata,[3] Makiko Ohki-Koizumi,[1] Makoto Ishihara,[1] Aki Sato,[1] Ismael Gritli,[1] Hiromitsu Komiyama,[1,2] Yayoi Sato,[1] Takashi Dan,[4] Toshio Miyata,[4] Ko Okumura,[5] Yuichi Tomiki,[2] Kazuhiro Sakamoto,[2] Hiromitsu Nakauchi,[1] *Beate Heissig,[1,3,5] and *Koichi Hattori[1,5]

[1]Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, Tokyo, Japan; [2]Department of Coloproctological Surgery, Juntendo University Faculty of Medicine, Tokyo, Japan; [3]Department of Stem Cell Dynamics, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science at the University of Tokyo, Tokyo, Japan; [4]United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan; and [5]Atopy (Allergy) Center, Juntendo University School of Medicine, Tokyo, Japan

Plasminogen activator inhibitor-1 (PAI-1), an endogenous inhibitor of a major fibrinolytic factor, tissue-type plasminogen activator, can both promote and inhibit angiogenesis. However, the physiologic role and the precise mechanisms underlying the angiogenic effects of PAI-1 remain unclear. In the present study, we report that pharmacologic inhibition of PAI-1 promoted angiogenesis and prevented tissue necrosis in a mouse model of hind-limb ischemia. Improved tissue regeneration was due to an expansion of circulating and tissue-resident granulocyte-1 marker (Gr-1$^+$) neutrophils and to increased release of the angiogenic factor VEGF-A, the hematopoietic growth factor kit ligand, and G-CSF. Immunohistochemical analysis indicated increased amounts of fibroblast growth factor-2 (FGF-2) in ischemic gastrocnemius muscle tissues of PAI-1 inhibitor-treated animals. Ab neutralization and genetic knockout studies indicated that both the improved tissue regeneration and the increase in circulating and ischemic tissue-resident Gr-1$^+$ neutrophils depended on the activation of tissue-type plasminogen activator and matrix metalloproteinase-9 and on VEGF-A and FGF-2. These results suggest that pharmacologic PAI-1 inhibition activates the proangiogenic FGF-2 and VEGF-A pathways, which orchestrates neutrophil-driven angiogenesis and induces cell-driven revascularization and is therefore a potential therapy for ischemic diseases. (*Blood.* 2012;119(26): 6382-6393)

Introduction

Approximately 500 to 1000 people per million per year are diagnosed with critical ischemia of the limb, which in most cases results in serious morbidity and mortality. Therapeutic restoration of blood flow by, for example, the induction of the formation of new capillaries (angiogenesis) is the ultimate goal for critical limb ischemia patients. Growth of new blood vessels in the adult occurs through angiogenesis or arteriogenesis (vessel maturation via recruitment of smooth muscle cells) and vasculogenesis (mobilization of BM-derived cells).[1,2] In contrast to promising results from animal studies, administration of proangiogenic factors such as fibroblast growth factor 2 (FGF-2, also known as basic FGF) or VEGF-A failed to induce significant improvement in ischemia in several phase 1 clinical trials.[3]

The plasminogen activation system and matrix metalloproteinases (MMPs), which can cleave growth factors, growth factor receptors, and adhesion molecules and mediate the extracellular matrix degradation that is necessary for cell migration, are widely recognized as being involved in the process of angiogenesis.[2,4] Although plasminogen activator inhibitor-1 (PAI-1) is one of the primary regulators of the fibrinolytic system, it also has dramatic effects on cell adhesion, detachment, and migration[5] and can inhibit cellular migration by affecting cell adhesion.[6,7] PAI-1–deficient (PAI-1$^{-/-}$) mice showed improved vascular wound healing in models of perivascular electric or transluminal mechanical injury[8] due to improved migration of PAI-1$^{-/-}$ smooth muscle cells. The

52-kDa serine protease inhibitor PAI-1 is the major plasma inhibitor of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) and inhibits plasmin-mediated fibrinolysis.[9] Studies in mice have indicated that the PAI-1 mRNA concentration is high in the heart, lung, aorta, and adipose and muscle tissue.[10] Plasma and tissue concentrations of PAI-1 increase under pathologic conditions. This increase is mediated by many factors, including reactive oxygen species. PAI-1 is secreted from endothelial cells after ischemia, such as that which occurs in acute myocardial infarction, atherosclerosis, and restenosis.[11,12] However, the role of PAI-1 in both promoting and inhibiting vascular remodeling or tissue regeneration and tumor growth or neoangiogenesis is controversial.[13-15]

The proteases plasmin and MMP-3 cleave and inactivate PAI-1.[16,17] The balance between PAI-1 inhibition of plasmin and other proteases and the cleavage of PAI-1 by these proteases, my play a critical role in the modulation of vascular proliferative responses. However, the exact mechanisms by which PAI-1 affects ischemic tissue regeneration and cell migration are not completely understood. In the present study, we demonstrate that, under ischemic conditions, drug-induced PAI-1 inhibition accelerates neoangiogenesis in a model of hind-limb (HL) ischemia. Moreover, PAI-1 inhibition also increases the proangiogenic factors FGF-2 and VEGF-A, the hematopoietic growth factor kit ligand (KitL), and G-CSF. tPA and MMP-9 deficiency and VEGF-A and FGF-2