into four groups: class I (HDAC1, -2, -3, and -8), IIa (HDAC4, -5, -7, and -9), IIb (HDAC6 and -10), and IV (HDAC11). Class I HDACs are ubiquitously expressed and are generally involved in cell growth and differentiation (13), whereas class II HDACs have a more restricted pattern of expression (skeletal muscle, heart, and brain) and act in association with tissue-specific transcription factors. Leukemic fusion proteins, such as PML/RARα and AML-1/ETO, form a complex with HDACs with higher affinities than their normal counterparts and aberrantly suppress the expression of genes required for cell differentiation and growth control, leading to the transformation of hematopoietic progenitor cells (14, 15). Therefore, HDACs are considered direct targets of treatment in these cases. Indeed, a variety of small compounds that inhibit HDAC activity have been developed and tested as therapeutic agents for hematologic malignancies, including AML with fusion gene products, and solid tumors (16).

HDAC inhibitors can induce differentiation, cell cycle arrest, and apoptosis in AML cells irrespective of the presence of leukemic fusion proteins, suggesting that HDACs are generally involved in leukemogenesis via multiple mechanisms (16). These effects provide a rational backbone for the clinical application of HDAC inhibitors to AML. Intriguingly, recent clinical trials have revealed that HDAC inhibitors have only moderate hematologic toxicity (17, 18), but the underlying mechanisms are to be determined. Since these observations are biologically interesting and clinically important, their molecular basis is worth investigating. In this study, we therefore examined the expression of HDACs in human hematopoietic cells and their functions during hematopoiesis and found that the expression levels of HDACs determine the fate of hematopoietic progenitor cells.

EXPERIMENTAL PROCEDURES

Cells—Human bone marrow mononuclear cells (BMMNCs) were purchased from Cambrex BioScience (Walkersville, MD). $CD34^+$ cells were purified by positive selection with CD34 MicroBeads and MACS separation columns (Miltenyi Biotec, Gladbach, Germany). More than 95% of enriched cells were shown to be positive for CD34 and negative for lineage markers (19). $CD34^+$ BMMNCs represent early hematopoietic progenitors, since most express CD38 (data not shown). The remaining cells were used as $CD34^-$ BMMNCs after depleting lineage marker-expressing cells with a lineage cell depletion kit (Miltenyi Biotec). This fraction mainly consists of committed progenitors of multiple lineages and does not contain terminally differentiated elements, such as mature myeloid cells, erythroblasts, and lymphocytes.

Human AML cell lines, HL60, U937, and K562, were differentiated in serum-free GIT medium (Nihon Pharmaceutical, Tokyo, Japan) containing appropriate chemicals, as described previously (20, 21). Primary AML cells were obtained from patients at diagnosis by sedimentation on Ficoll-Hypaque density gradients. Informed consent was obtained from all subjects in accord with the requirements of the institutional review board. Samples were selected for the study only when they contained more than 90% leukemic cells and did not carry chromosomal translocations.

Cell Culture—For clonogenic growth assays, human $CD34^+$ BMMNCs and primary AML cells were plated at $0.5–1 \times 10^3$ cells/ml in methylcellulose medium supplemented with full cytokines (H4435, Stem Cell Technologies, Vancouver, Canada) (19). AML cell lines were cultured in methylcellulose medium supplemented with 10% fetal calf serum for 7 days. To form colony-forming unit-granulocyte/macrophage (CFU-GM) and colony-forming unit-erythroid (CFU-E), murine bone marrow cells were plated at 2.5×10^3 cells/ml in methylcellulose medium supplemented with a combination of stem cell factor, interleukin-3, and interleukin-6 (M3534) and erythropoietin alone (M3334), respectively.

Semiquantitative and Real Time Quantitative RT-PCR—Total cellular RNA was isolated from 1×10^4 cells and reverse-transcribed into cDNA using SuperScript reverse transcriptase and oligo(dT) primers (Invitrogen). We performed subsequent semiquantitative PCR, as described previously (2), and real time quantitative RT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Detailed information on primers, including sequences, corresponding nucleotide positions, and PCR product sizes, is shown in supplemental Table S1.

RNA Blotting—An equal amount (15 μg) of total cellular RNA was electrophoresed in 1% agarose gels containing formaldehyde and blotted onto Hybond N synthetic nylon membranes (Amersham Biosciences). The membranes were hybridized with ^{32}P-labeled probes in Rapid-hyb buffer (Amersham Biosciences).

Immunoblotting—Immunoblotting was carried out according to the standard method using the following antibodies: anti-HDAC1 (Sigma), anti-HDAC2 (MBL International, Woburn, MA), anti-HDAC3 (BD Pharmingen, San Jose, CA), and anti-β-actin (Ab-1; Oncogene Science, Uniondale, NY). We purchased site-specific antibodies against acetylated histones (H3-Lys^9, H3-Lys^{18}, H4-Lys^{12}, and H4-Lys^{16}) from Cell Signaling Technology (Beverly, MA).

Confocal Laser Microscopy—Confocal microscopic analysis was performed using anti-HDAC1 polyclonal (Sigma) and anti-CD34 monoclonal (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. We used Alexa 488-conjugated goat antibody to mouse immunoglobulin (Molecular Probes, Inc., Eugene, OR) and Cy3-conjugated goat antibody to rabbit immunoglobulin (Amersham Biosciences) as secondary antibodies.

Flow Cytometry Analysis and Fluorescence-activated Cell Sorting—Flow cytometry analysis and cell sorting were carried out with BD FACSaria (BD Biosciences) as described previously (19).

Plasmids and Transfection—Retroviral expression vector for HDAC1 was constructed by inserting full-length cDNA (provided by Dr. Stuart Schreiber, Harvard University, Boston, MA) upstream of the internal ribosome entry site-enhanced green fluorescent protein cassette of pMYs plasmid, as described previously (22). Retrovirus production was carried out by transfecting the plasmids into Plat-E packaging cells. Expression vectors for MZF-1, C/EBPα, C/EBPβ, GATA-1, GATA-2, and Sp1 were kindly provided by Drs. Robert Hromas (University of New Mexico, Albuquerque, NM), Atsushi Iwama (Department of Cellular and Molecular Medicine, Chiba University, Chiba,

Japan), and Mitsuru Nakamura (National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan).

We used a lentiviral shRNA/siRNA expression vector pLentiLox3.7 for knockdown of HDAC1. Target sequences were designed to be homologous to wild-type cDNA sequences: *HDAC1* (forward), Tggcaaaggcaagtattatg**TTCAAGAGA**catataatacttgcctttgccTTTTTTC; *HDAC1* (reverse), TCGAGAAAAAAAggcaaaggcaagtattatgTCTCTTGAAcataatacttgcctttgcc. Scrambled sequences were used as controls. Lentiviruses were then added to cell suspensions in the presence of 8 μg/ml Polybrene and transduced for 24 h, as described previously (19).

Reporter Assays—We amplified the promoter regions of the *HDAC1* gene (−1170 to +397 and −73 to +397) by PCR and inserted them into pGL4.10 firefly luciferase vector (Promega, Madison, WI) to generate reporter plasmids. HEK293 cells were transfected with reporter plasmids along with pGL4.73 *Renilla* luciferase vector (Promega), which served as a positive control to determine transfection efficiencies, in the presence of test plasmids encoding *Sp1*, *GATA-1*, *GATA-2*, *MZF-1*, *CEBPA*, and *CEBPB* or empty vectors. After 48 h, firefly and *Renilla* luciferase activities were discriminately measured using the Dual-Luciferase reporter assay system (Promega).

Chromatin Immunoprecipitation Assays—We used the ChIP-IT chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) to perform chromatin immunoprecipitation assays. In brief, cells were fixed with 1% formaldehyde and sonicated to obtain chromatin suspensions. After centrifugation, supernatants were incubated with antibodies of interest in the presence of protein A-agarose beads. DNA fragments bound to the beads were purified with washing and subjected to PCR using primer pairs spanning −377 to −77 of the *HDAC1* gene.

Stem Cell Transplantation in Syngeneic Mice—Bone marrow mononuclear cells were isolated from C57BL/6 (Ly-5.1) donor mice (8–12 weeks of age). c-KIT-positive cells were isolated by CD117 MicroBeads in MACS separation columns (Miltenyi Biotec), and cultured overnight in Iscove's modified Dulbecco's medium supplemented with BIT 9500 and 50 ng/ml each of stem cell factor, FLT3 ligand, interleukin-3, and thrombopoietin. Prestimulated cells were infected with retroviruses harboring either pMYs-HDAC1 or an empty vector (mock) in 6-well dishes for 24 h (22, 23). Then $1-6 \times 10^5$ cells were injected through the tail vein into lethally irradiated (9.5 grays) C57BL/6 (Ly-5.2) recipient mice (8–12 weeks of age). Engraftment of transplanted cells was confirmed by measuring the percentages of GFP^+ and/or $Ly\text{-}5.1^+$ cells in the peripheral blood of recipients. All animal studies were approved by the Institutional Animal Ethics Committee and performed in accordance with the Guide for the Care and Use of Laboratory Animals formulated by the National Academy of Sciences.

RESULTS

Relative Resistance of Normal Human Hematopoietic Progenitors to HDAC Inhibitors—Given the relatively weak hematological toxicity reported in clinical trials (17, 18), we reasoned that normal human hematopoietic stem/progenitor cells are resistant to HDAC inhibitors. Indeed, normal human hematopoietic progenitors (CD34-positive/CD38-positive/lineage marker-negative bone marrow mononuclear cells ($CD34^+$ BMMNC)) generated more colonies in the presence of HDAC inhibitors than primary blasts from patients with AML and myeloid leukemia cell lines in clonogenic growth assays (Fig. 1). Interestingly, normal hematopoietic progenitors were more resistant to HDAC inhibitors than their differentiated offspring (CD34-negative and lineage marker-negative bone marrow mononuclear cells ($CD34^-$ BMMNC), which correspond to committed progenitors of multiple lineages).

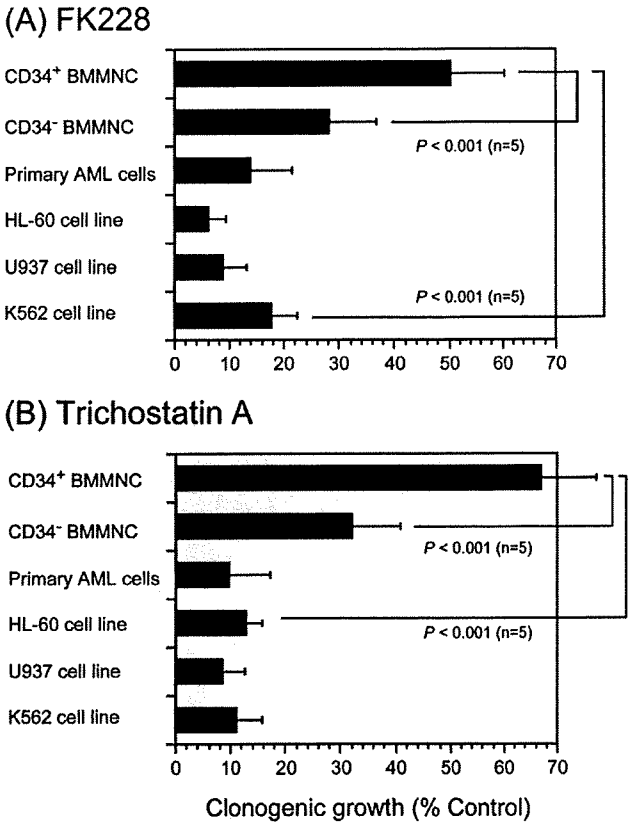

FIGURE 1. **Relative resistance of normal hematopoietic progenitor cells to HDAC inhibitors.** We seeded normal human $CD34^+$ and $CD34^-$ BMMNCs and primary AML cells at 1×10^3 cells/ml in methylcellulose medium supplemented with stem cell factor (50 ng/ml), interleukin-3 (10 ng/ml), interleukin-6 (10 ng/ml), granulocyte/macrophage colony-stimulating factor (10 ng/ml), granulocyte colony-stimulating factor (10 ng/ml), and erythropoietin (3 units/ml) and cultured in the absence or presence of either 2 nM FK228 (*A*) or 10 ng/ml trichostatin A (*B*) for 14 days. Three AML cell lines (HL-60, U937, and K562) were cultured in methylcellulose medium supplemented with 10% fetal calf serum for 7 days. Each *column* indicates the relative colony numbers setting untreated controls at 100%. The means ± S.D. (*bars*) of five independent experiments are shown. *p* values were calculated by one-way analysis of variance with the Bonferroni *post hoc* test.

Differential Expression of HDACs in Normal Hematopoietic Cells and AML Blasts—To understand the mechanisms underlying the relative resistance of normal hematopoietic progenitor cells to HDAC inhibitors, we screened for the expression of three major class I HDACs (HDAC1, -2, and -3) by immunoblotting using specific antibodies. The selection was based on the fact that these HDACs, especially HDAC1, represent the vast majority of cellular HDAC activities (13, 24) and major class II HDACs (HDAC4, -5, and -7) were scarcely expressed in hematopoietic cells in our pilot experiments (data not shown). Moreover, class I HDACs, especially HDAC1, are known to be

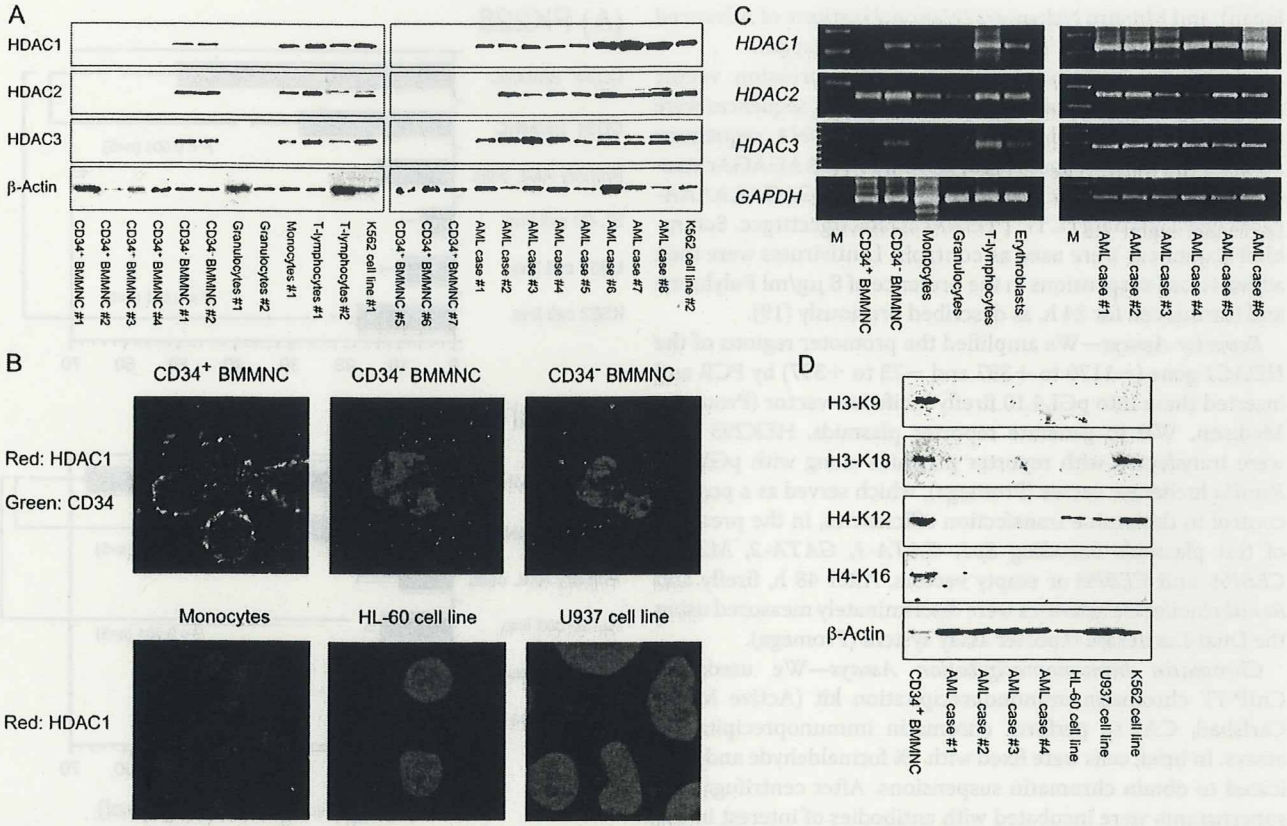

FIGURE 2. **Differential expression of HDACs in normal hematopoietic cells and AML blasts.** *A*, whole cell lysates were prepared from the indicated cell types isolated as described under "Experimental Procedures" and subjected to immunoblot analysis for the expression of HDAC1, HDAC2, HDAC3, and β-actin (loading control). Signals were obtained with the ChemiDoc XRS Molecular Imager (Bio-Rad) and used without digital manipulation except for the conversion to TIF files. See supplemental Fig. S1 for data quantification and statistical analysis. *B*, $CD34^+$ and $CD34^-$ BMNNC were stained with anti-HDAC1 (*red*) and anti-CD34 (*green*) antibodies and analyzed under confocal microscopy. Original magnification is ×600 for all *panels*. *C*, total cellular RNA was isolated from the indicated cell types, and 2.5 μg (normal hematopoietic cells) or 1.0 μg (primary AML cells) was subjected to semiquantitative RT-PCR analysis for the expression of *HDAC1*, *HDAC2*, *HDAC3*, and *GAPDH* (internal control). Five μl of the amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis. The results of suboptimal amplification cycles are shown: 35 and 30 cycles for *HDAC* genes and *GAPDH*, respectively. *M*, a molecular size marker (BioMarker Low; BioVentures, Inc., Murfreesboro, TN). See supplemental Fig. S3 for data quantification and statistical analysis. *D*, protein samples were analyzed by immunoblotting using specific antibodies against histone H3 acetylated at lysine 9 (*H3-K9*), histone H3 acetylated at lysine 18 (*H3-K18*), histone H4 acetylated at lysine 12 (*H4-K12*), histone H4 acetylated at lysine 16 (*H4-K16*), and β-actin (loading control). Data shown are the representative results of multiple independent experiments.

implicated in leukemogenesis and carcinogenesis and thus are pharmacological targets of most HDAC inhibitors (25, 26). As shown in Fig. 2*A*, the expression of HDAC proteins was below the detection limit in immature progenitor cells from normal human bone marrow ($CD34^+$ BMMNC). Immunoblotting with different antibodies yielded the same results, negating the possibility of the low sensitivity of the antibodies used for detection (data not shown). HDAC proteins were readily detectable in committed progenitors of multiple lineages ($Lin^-/CD34^-$ BMMNC) and T-lymphocytes but were weakly expressed in monocytes and nearly absent in mature granulocytes from the peripheral blood of healthy volunteers (see quantified data in supplemental Fig. S1). Primary leukemic cells from AML patients showed higher expression levels of HDACs than their normal counterparts (immature progenitor cells), although there was a minor case-to-case variation. In particular, HDAC1 was more abundantly expressed than HDAC2 and HDAC3 in approximately half of the cases with AML (Fig. 2*A*). We further confirmed the expression of HDAC1 using immunocytochemistry. As shown in Fig. 2*B*, HDAC1 was not detected in $CD34^+$ BMMNC, moderately expressed in the nuclei of $CD34^-$ BMMNC and monocytes, and apparently overexpressed in AML cells. The differential expression of HDACs does not simply reflect the proliferative states of these cells, because the abundance of HDACs was only modestly increased along with cell cycle entry of mitogen-stimulated T-lymphocytes (supplemental Fig. S2).

Next, we carried out similar analyses with semiquantitative RT-PCR. The expression of *HDAC* mRNAs was very weak in normal hematopoietic progenitors ($CD34^+$ BMMNC) as well as in mature myeloid cells (monocytes and granulocytes), except for *HDAC2* in $CD34^+$ BMMNC (Fig. 2*C*). *HDAC* transcripts were moderately expressed in committed progenitors ($Lin^-/CD34^-$ BMMNC), purified erythroblasts from bone marrow, and peripheral blood T-lymphocytes. In contrast, primary AML cells strongly expressed *HDAC* genes, especially *HDAC1*. The virtually identical pattern was obtained with real time quantitative RT-PCR (supplemental Fig. S3). Overall, the expression pattern of *HDAC* transcripts is nearly equal to that of HDAC proteins in normal and malignant hematopoietic

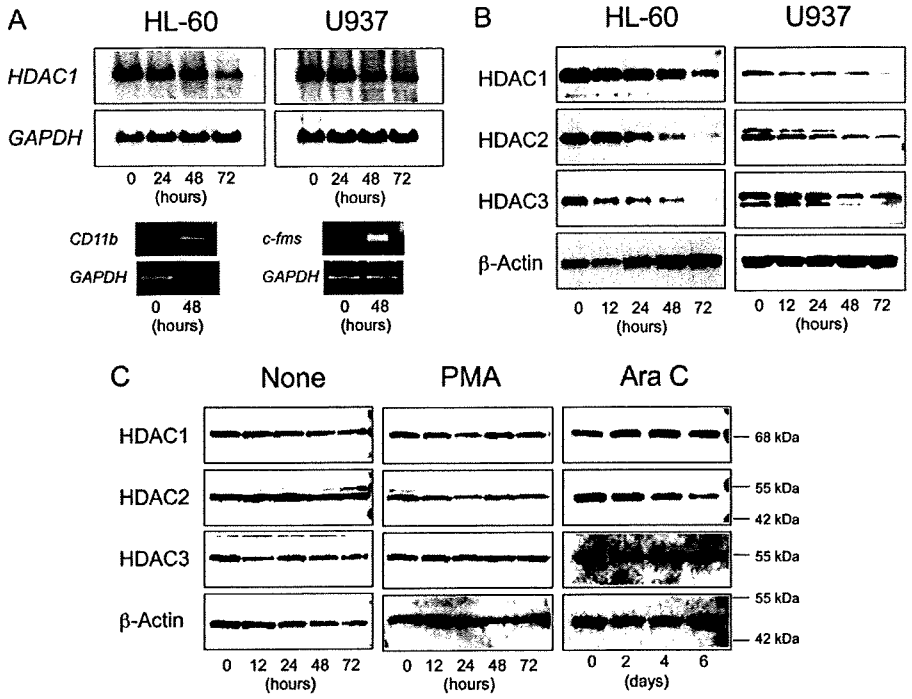

FIGURE 3. **Lineage-specific regulation of HDAC expression during hematopoietic differentiation.** HL-60 and U937 cells were cultured with all-*trans*-retinoic acid and phorbol-12-myristate-13-acetate (*PMA*) to induce granulocytic and monocytic differentiation, respectively. *A*, total cellular RNA was isolated at the indicated time points and subjected to semiquantitative RT-PCR analysis for *CD11b*, *c-fms*, and *GAPDH* (internal control) expression and Northern blot analysis for *HDAC1* and *GAPDH* expression. *B*, whole cell lysates were isolated at the indicated time points and subjected to immunoblot analysis for the expression of HDACs and β-actin (loading control). *C*, K562 cells were cultured with PMA and cytosine arabinoside (*AraC*) to induce megakaryocytic and erythroid differentiation, respectively. See supplemental Fig. S4 for the proper achievement of differentiation induction. Whole cell lysates were isolated at the indicated time points and subjected to immunoblot analysis for the expression of HDACs and β-actin (loading control). Signals were obtained with the ChemiDoc XRS Molecular Imager (Bio-Rad) and used without digital manipulation except for the conversion to TIF files. Data shown are representative results of multiple independent experiments.

cells. These results suggest that the expression of HDACs is regulated primarily at mRNA levels, but post-translational modification is also involved especially in HDAC2, as suggested by previous studies (27).

Finally, we investigated whether the acetylation status of intracellular histones reflects the distinct expression levels of HDACs in hematopoietic cells. To this end, we performed immunoblotting using site-specific antibodies against acetylated histones. As shown in Fig. 2*D*, histones H3 and H4 were heavily acetylated at Lys^9/Lys^{18} and Lys^{12}/Lys^{16}, respectively, in normal hematopoietic progenitors but not in primary AML cells. AML cell lines also lacked the acetylation of H3-Lys^9 and H4-Lys^{16}, but H4-Lys^{12} and H3-Lys^{18} were acetylated in all three cell lines examined and in K562, respectively.

Lineage-specific Regulation of HDAC Expression during Hematopoietic Differentiation—To further delineate the differential expression of HDACs in normal and malignant hematopoietic cells, we took advantage of the cell line model system. To recapitulate myeloid differentiation *in vitro*, we cultured HL-60 and U937 cells with chemical inducers of differentiation: phorbol ester, dimethyl sulfoxide, and retinoic acid (20). Successful induction of differentiation was verified by the appearance of mature myelomonocytic markers, such as *CD11b* and *c-Fms* (Fig. 3*A*, *bottom*). During myeloid differentiation, HDACs were down-regulated at both mRNA (Fig. 3*A*, *top*) and protein levels (Fig. 3*B*). We then performed similar experiments with the K562 cell line, which can be differentiated into megakaryocytic and erythroid lineages by phorbol ester and cytosine arabinoside, respectively (supplemental Fig. S4) (21). Unlike myeloid differentiation, the abundance of HDACs, especially HDAC1 and HDAC3, was unchanged during megakaryocytic and erythroid differentiation (Fig. 3*C*). Since HDAC1 seemed to be slightly increased in cytosine arabinoside-treated K562 cells, we monitored the expression of *HDAC1* during *in vitro* differentiation of primary $CD34^+$ BMMNC using real time RT-PCR. When purified $CD34^+$ BMMNCs were cultured with a combination of cytokines directing erythropoiesis (2), up-regulation of *HDAC1* expression coincided with morphological differentiation of immature progenitor cells into erythroid precursors (supplemental Fig. S5). In addition, this culture system yielded the confirmation of *HDAC1* down-regulation during myeloid differentiation of normal progenitors (data not shown).

Regulation of HDAC1 Promoter by Hematopoietic Transcription Factors—Our analyses demonstrated that HDAC expression was very low in immature hematopoietic progenitors, induced in more differentiated progenitor cells, and down-regulated during myeloid differentiation with nearly complete disappearance in mature granulocytes, whereas it was retained during erythroid and megakaryocytic differentiation. In contrast, HDACs, especially HDAC1, were overexpressed in virtually all AML cases and cell lines. To corroborate the regulatory mechanisms underlying this unique expression pattern, we isolated putative promoter regions of the *HDAC1* gene and subjected them to functional analysis. The *HDAC1* promoter contains canonical binding sites of Sp1 (GC box), MZF-1, C/EBPs (CCAAT box), and GATA transcription factors (supplemental Fig. S6). We subcloned two promoter fragments, −1179 to +397 and −73 to +397, into pGL4 luciferase vector to generate reporter plasmids as illustrated in Fig. 4*A*. The selection of analyzed regions was based on previous studies on the murine *Hdac1* promoter (28) and the results of our pilot experiments in which the segment between −1179 and +397 confers full promoter activity (data not shown). We carried out reporter assays with HEK293, because this cell line lacks the expression of transcription factors to be tested except for Sp1 (data not shown). When the −1170 construct was used, reporter assays revealed

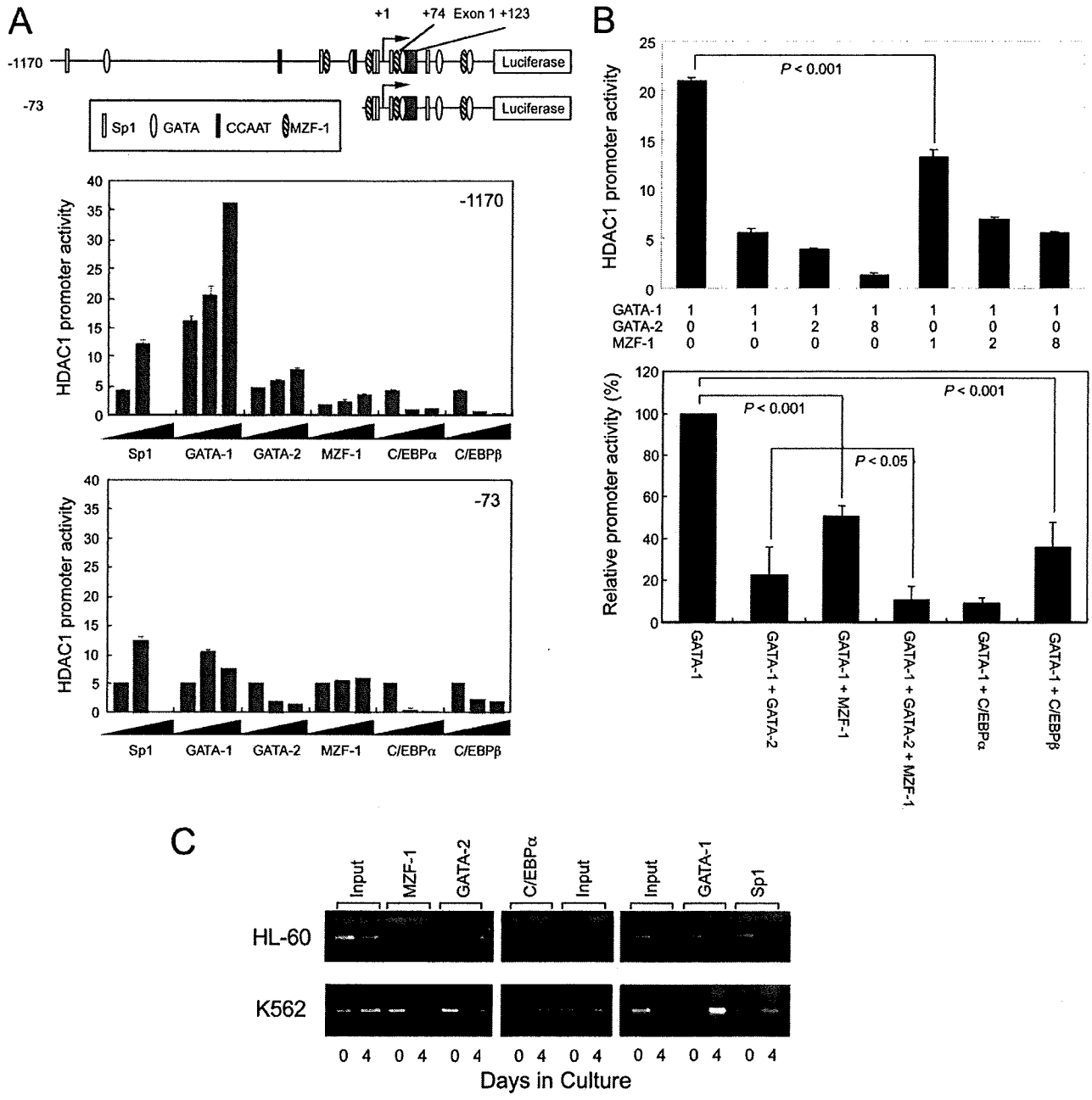

FIGURE 4. **Regulation of *HDAC1* promoter by hematopoietic transcription factors.** *A, top,* schematic representation of *HDAC1* promoter constructs used in this study. Promoter regions of the *HDAC1* gene (−1170 to +397 and −73 to +397) were linked to the luciferase gene in pGL4.10 vector as indicated. Relative locations of the putative binding sites of hematopoietic transcription factors are approximated by the *symbols* shown in the *box*. See supplemental Fig. S6 for the nucleotide sequence. *Bottom,* we transfected pGL4.10 plasmid containing *HDAC1* promoter sequences between −1170 and +397 (*−1170*) or between −73 and +397 (*−73*) into HEK293 cells along with expression vectors encoding *Sp1, GATA-1, GATA-2, MZF-1, CEBPA,* and *CEBPB* at various doses (1, 2, and 4 μg) and measured luciferase activities after 48 h. *HDAC1* promoter activity (*y* axis) was calculated as firefly luciferase activities of cells transfected with either pGL4.10−1170 or pGL4.10−73 and an empty expression vector setting at 1.0 after normalization of transfection efficiencies using *Renilla* luciferase activity. Data shown are the means ± S.D. of three independent experiments. *B, top,* we transfected pGL4.10−1170 reporter plasmid into HEK293 cells along with expression vectors encoding *GATA-1, GATA-2,* and *MZF-1* at the indicated doses (μg) and measured luciferase activity after 48 h. HDAC1 promoter activity (*y* axis) was calculated as firefly luciferase activities of cells transfected with pGL4.10−1170 and an empty expression vector set at 1.0 after normalization of transfection efficiencies using Renilla luciferase activities. *Bottom,* we transfected pGL4.10−1170 reporter plasmid and 1 μg of *GATA-1* expression vector into HEK293 cells in the absence or presence of 1 μg of expression plasmids encoding *GATA-2, MZF-1, CEBPα,* and *CEBPβ* and measured luciferase activities after 48 h. Relative promoter activity (*y* axis) was calculated as firefly luciferase activities of cells transfected with pGL4.10−1170 and *GATA-1* expression vector setting at 100% after normalization of transfection efficiencies using *Renilla* luciferase activities. Data shown are the means ± S.D. of three independent experiments. *p* values were calculated by one-way analysis of variance with the Student-Newman-Keuls multiple comparisons test. *C,* HL60 and K562 were cultured in the presence of PMA for 4 days and harvested before and after differentiation for chromatin immunoprecipitation assays. Chromatin suspensions were immunoprecipitated with the indicated antibodies and corresponding control antibodies. The resulting precipitants were subjected to PCR to amplify the promoter region (−377 to −77) of the *HDAC1* gene. The amplified products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis. Representative data of 50 cycles are shown. *Input,* PCR was performed with genomic DNA.

that *HDAC1* transactivation was driven by Sp1 and GATA-1 (Fig. 4*A*, *middle*). In contrast, members of the C/EBP family transcription factors, C/EBPα and C/EBPβ, negatively regulated transcription of the *HDAC1* gene. GATA-2 and MZF-1 showed only a modest effect on promoter activity. Sp1 was able to activate the −73 construct to a similar extent as the −1170 construct, suggesting that GC boxes surrounding the transcription start site are responsible for Sp1-mediated transactivation of *HDAC1* (Fig. 4*A*, *bottom*). However, the activity of GATA-1 was significantly diminished when the −73 construct was used, implying that consensus sequences at the positions −973 and −91 are necessary for GATA-1 to fully activate the *HDAC1* promoter. In contrast, C/EBPα and C/EBPβ were still able to repress the *HDAC1* promoter in the −73 construct. Since the −73 construct lacks CCAAT boxes, the suppressor function of C/EBPs may be mediated via interaction with activator proteins, most likely GATA-1 (7, 29). To verify this hypothesis, we examined the effects of C/EBPα and C/EBPβ on GATA-1-mediated transactivation of the *HDAC1* gene using co-transfection. As anticipated, both C/EBPα and C/EBPβ significantly suppressed *HDAC1* promoter activation by GATA-1. In particular, C/EBPα suppressed the activity of GATA-1 to about one-tenth (Fig. 4*B*, *bottom*). More importantly, GATA-2 and MZF-1 individually antagonized GATA-1-mediated activation of the *HDAC1* promoter in a dose-dependent manner (Fig. 4*B*, *top*), and the two factors synergistically suppressed it (Fig. 4*B*, *bottom*).

Next, we investigated the binding of these factors to *HDAC1* promoter *in vivo* and its changes during hematopoietic differentiation using chromatin immunoprecipitation assays. In an undifferentiated HL-60 cell line, Sp1 and GATA-1 bound to the sequence between −377 and −77, which was shown to be the active regulatory region by reporter assays (Fig. 4*C*, day 0). This suggests that these two factors confer the base-line expression of *HDAC1* in the myeloid-committed cell line HL-60. Upon differentiation, both Sp1 and GATA-1 dissociated from the *HDAC1* promoter, and GATA-2 and a small amount of C/EBPα became detectable (Fig. 4*C*, day 4). These results suggest that the exchange of positive to negative regulators on the promoter contributes to the silencing of *HDAC1* during myeloid differentiation. On the other hand, the binding of Sp1, MZF-1, and GATA-2 was demonstrated in an untreated K562 cell line, whereas GATA-1 binding was not observed (Fig. 4*C*, day 0). The difference of binding factors may be attributed to the fact that K562 is more immature than HL-60 and possesses pluripotency (21). Sp1 and GATA-2 may render the base-line expression of *HDAC1* in K562 cells, because GATA-2 appears to be a weak activator in the absence of GATA-1 in reporter assays. Upon megakaryocytic differentiation, the strongest activator GATA-1 was recruited to the promoter along with the dissociation of its inhibitors MZF-1 and GATA-2 in K562 cells. In addition, there was a slight increase in the binding of Sp1 (Fig. 4*C*, day 4). These changes may underlie the sustained expression of *HDAC1* during megakaryocytic differentiation.

Expression Levels of HDAC1 Affect the Differentiation Program of Hematopoietic Progenitor Cells—One important question regarding the expression pattern of HDACs during differentiation is whether the change is a simple consequence of differentiation or if it has a functional meaning. To address this question, we carried out loss-of-function studies using siRNA against HDAC1, which represents more than half of HDAC activities in mammalian cells and cannot be compensated by other class I HDACs (13, 24). In a myeloid-committed cell line HL-60, siRNA-mediated knockdown of HDAC1 promoted myeloid differentiation, as revealed by a decrease in the expression of CD33, a marker of immature myeloid cells (30) (Fig. 5*A*). This suggests that HDAC1 is indispensable for the maintenance of an immature state in HL-60 cells. In multipotent K562 cells, the suppression of HDAC1 caused a decrease of an erythroid marker CD235a and a reciprocal increase of a mature myeloid marker CD11b (Fig. 5*B*). This implies that overexpressed HDAC1 maintains the erythroid properties of K562 cells, and its reduction results in retrograde differentiation into a myeloid lineage. This is compatible with the expression pattern of HDACs in hematopoietic cells. In addition, we examined the effects of HDAC1 knockdown on normal progenitor cells in clonogenic assays. $CD34^+$ human BMMNCs were transduced with siRNA expression vectors and cultured in methylcellulose medium with full cytokines to induce both myeloid and erythroid differentiation. HDAC1 knockdown reduced the numbers and sizes of erythroid colonies, CFU-E and burst-forming unit-erythroid (BFU-E), with a reciprocal increase in granulocyte/macrophage colonies (Fig. 5*C*). Taken together, these results point to an instructive role of HDACs in lineage specification during hematopoiesis.

HDAC1 Overexpression Blocks Myeloid Differentiation in a Murine Stem Cell Transplantation Model—The data so far suggest that HDAC expression should be repressed in normal hematopoietic progenitors, and HDAC overexpression alters the differentiation program, leading to myeloid leukemogenesis. To investigate whether HDAC overexpression actually results in the deregulation of hematopoietic differentiation, we performed stem cell transplantation studies in syngeneic mice (22, 23). We isolated c-KIT-positive bone marrow mononuclear cells from C57BL/6 (Ly-5.1) mice and infected them with retroviruses carrying either *HDAC1* cDNA with green fluorescent protein (GFP) or GFP alone (mock). The validity of the expression vector was confirmed by checking HDAC1 overexpression and target gene silencing in transfected cells (supplemental Fig. S7). We transplanted HDAC1- or mock-transfected cells into a lethally irradiated C57BL/6 (Ly-5.2) strain and monitored engraftment by measuring the percentages of GFP-positive and/or Ly-5.1- positive cells in peripheral blood serially. As shown in Fig. 6*A*, both HDAC1- and mock-transfected progenitor cells successfully reconstructed hematopoiesis in recipient mice 4 weeks after transplantation. However, donor-derived leukocytes gradually declined in the peripheral blood of mice that received HDAC1-transduced cells after 12 weeks and became nearly undetectable after 28 weeks (Fig. 6*A*). In contrast, there were no significant changes in the numbers of red blood cells and platelets between HDAC1- and mock-transplanted mice (data not shown). This is in line with the results of our *in vitro* studies indicating that HDAC expression is required for erythro-megakaryocytic differentiation. Leukemic transformation of transplanted cells was not observed up to 60 weeks after transplantation.

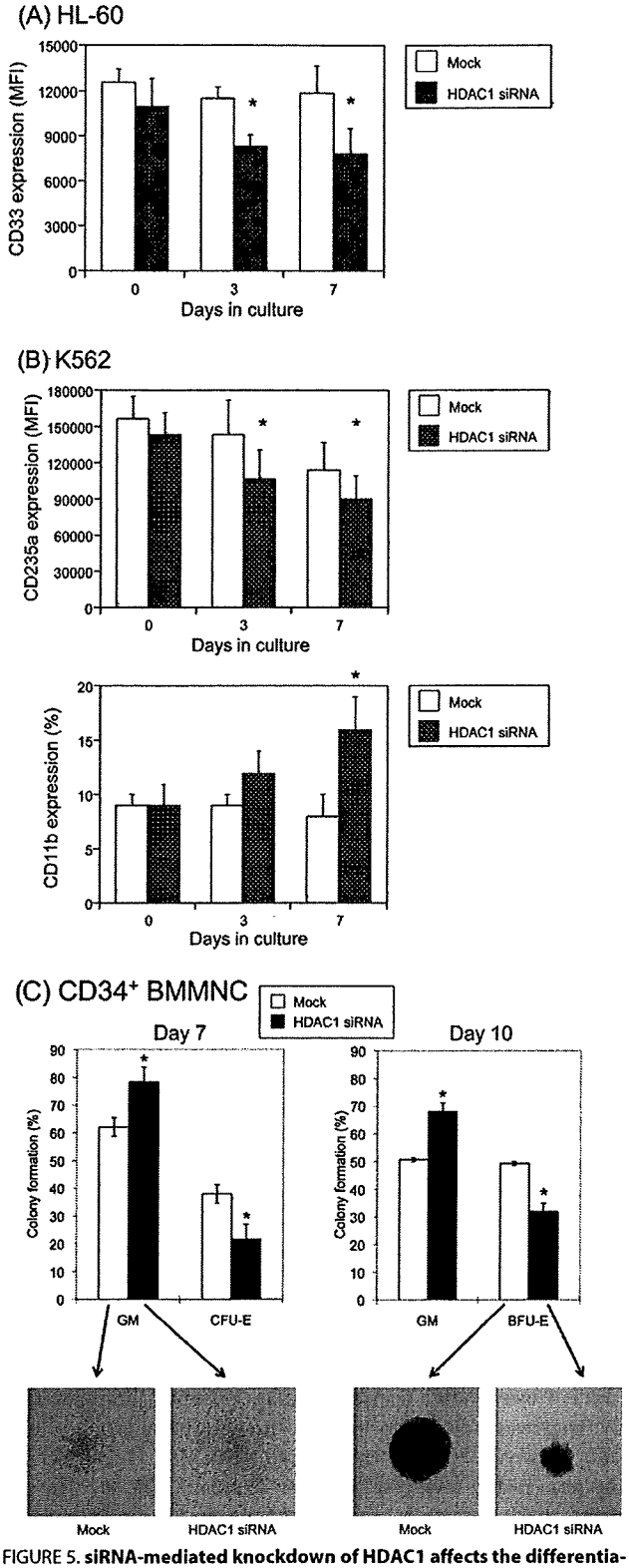

FIGURE 5. **siRNA-mediated knockdown of HDAC1 affects the differentiation program of hematopoietic progenitor cells.** *A*, HL-60 cells were transfected with lentivirus vectors carrying shRNA/siRNA against scrambled sequences (*Mock*) or *HDAC1* (*HDAC1 siRNA*) and subjected to flow cytometric analysis at the indicated time points. Data shown are the means ± S.D. (*bars*) of the mean fluorescence intensity (*MFI*) of CD33 in GFP-positive fractions ($n = 3$). p values were calculated by paired Student's t test (*, $p < 0.05$). *B*, K562 cells were transfected with lentiviral vectors carrying shRNA/siRNA against scrambled sequences or *HDAC1* and subjected to flow cytometric analysis at the indicated time points. Data shown are the means ± S.D. (*bars*) of the mean fluorescence intensity of CD235a and a percentage of CD11b-positive cells in GFP-positive fractions ($n = 3$). p values were calculated by paired Student's t test (*, $p < 0.05$). *C*, CD34$^+$ human bone marrow mononuclear cells were transfected with lentivirus vectors carrying shRNA/siRNA against scrambled sequences or *HDAC1*. GFP-positive cells were sorted and seeded at 0.5–1 × 10^3 cells/ml in methylcellulose medium supplemented with full cytokines as described in the legend to Fig. 1. The numbers of CFU-GM/CFU-E and CFU-GM/BFU-E were counted after 7 and 10 days of culture, respectively, and expressed as relative percentages. Data shown are the means ± S.D. of three independent experiments. p values were calculated by paired Student's t test (*, $p < 0.05$). Representative photographs are shown *below* (original magnification, ×100).

Next, we analyzed the components of bone marrow 10 weeks after transplantation. In HDAC1-transduced mice, there was a significant reduction of c-KIT-positive progenitors and CD11b-positive myeloid cells, whereas CD3- and B220-positive lymphoid cells were relatively increased compared with mock-transfected mice (Fig. 6*B*). Consistent with normal numbers of red blood cells and platelets in peripheral blood, immature cells of both lineages were normal or slightly increased in the bone marrow of HDAC1-transduced mice. These results indicate that HDAC1 exclusively perturbs myelopoiesis when overexpressed in hematopoietic progenitor cells. To corroborate this notion, we isolated c-KIT/GFP double-positive cells from bone marrow of mice transplanted with either HDAC1- or mock-transduced progenitors and subjected them to *in vitro* clonogenic growth assays. As shown in Fig. 6*C*, HDAC1 overexpression reduced the formation of CFU-GM, which corresponds to committed progenitors of myeloid lineage, without affecting mature erythroid progenitors CFU-E. HDAC1-transduced progenitors did not produce spontaneous colonies in this assay (Fig. 6*C*, *No CSF*). Furthermore, replating experiments yielded few or no secondary and tertiary colonies in semisolid medium even in the presence of growth factors (data not shown). These results suggest that HDAC1 overexpression alone cannot immortalize hematopoietic stem/progenitor cells, which is in line with the observation of transplantation studies.

DISCUSSION

Class I histone deacetylases are globally implicated in the growth and differentiation of mammalian cells by modifying chromatin structures and gene expression (12, 13). However, relatively little is known about their specific roles in normal hematopoiesis. In this study, we investigated the expression and function of major class I HDACs in normal human hematopoietic cells and obtained the following novel findings. First, the expression of HDACs is very low in CD34$^+$ hematopoietic progenitor cells. Second, HDAC1 plays an instructive role in lineage specification during hematopoiesis. Finally, HDAC1 is overexpressed in AML cells and contributes to leukemogenesis by perturbing myeloid differentiation. Overall, these findings indicate that HDAC is not merely an auxiliary factor of genetic elements but plays a direct role in the cell fate decision of hematopoietic progenitors.

The biological significance of the low level expression of HDACs in hematopoietic progenitor cells has yet to be determined; however, this phenomenon explains why hematological

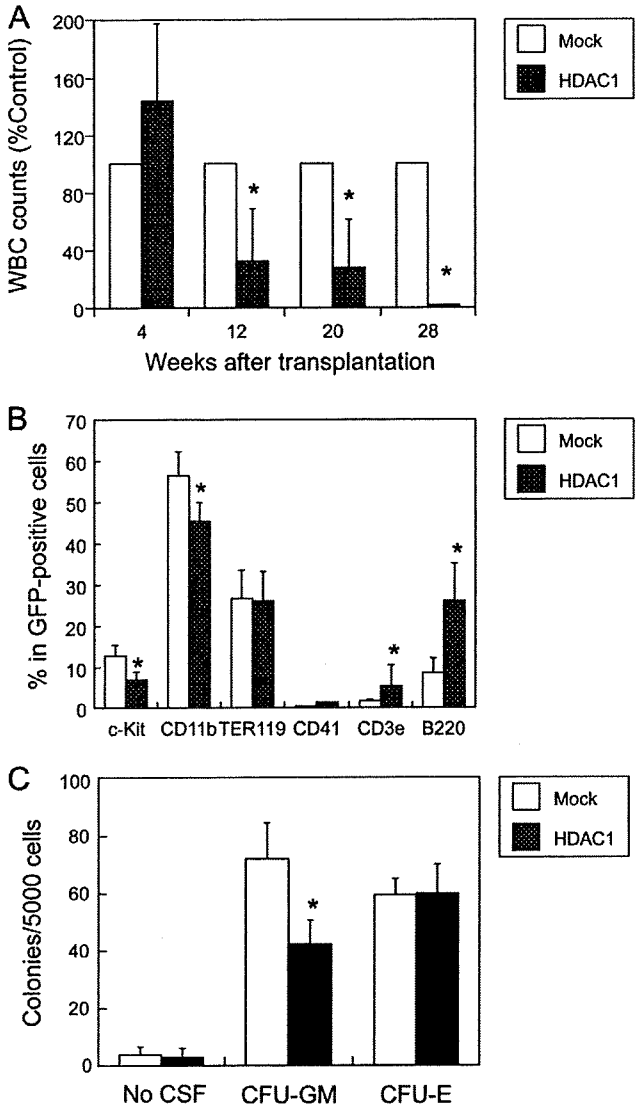

FIGURE 6. **Overexpression of HDAC1 blocks myeloid differentiation in a murine stem cell transplantation model.** Purified c-KIT-positive bone marrow cells from C57BL/6 (Ly-5.1) mice were transfected with retroviruses carrying either GFP alone (*Mock*) or HDAC1/GFP (*HDAC1*) and transplanted into lethally irradiated C57BL/6 (Ly-5.2) mice. *A*, peripheral blood was drawn at the indicated time points and subjected to blood counting. The numbers of white blood cells are shown as percentages of those of mock-transplanted mice. *B*, bone marrow mononuclear cells were subjected to flow cytometric analysis of surface marker expression. Data shown are the means ± S.D. (*bars*) of percentages of each surface marker in GFP-positive fractions ($n = 3$). *C*, GFP-positive bone marrow mononuclear cells were cultured in semisolid culture medium supplemented with appropriate cytokines as described under "Experimental Procedures." Colony numbers were counted after 14 days. Data shown are the means ± S.D. of three independent experiments. *p* values were calculated by paired Student's *t* test (*, $p < 0.05$).

toxicity is relatively weak in patients treated with HDAC inhibitors (17, 18). Inoue *et al.* (31) have also reported that the expression of *Hdac1* mRNA is extremely low in purified murine hematopoietic stem cells compared with cumulus and other somatic cells. They speculate that low HDAC activity underlies insufficient reprogramming of the genome from hematopoietic stem cells after nuclear transfer, which is consistent with the general consensus that hematopoiesis is difficult to reconstruct *in vitro* by genetic manipulation (32). Our findings fully support their view and further suggest that hematopoietic stem/progenitor cells may possess a unique genetic program, which characterizes the hematopoietic system.

Accumulating evidence indicates that hematopoietic stem/progenitor cells express a wide variety of genes required for multiple differentiation programs, most of which become repressed as lineage choices are restricted during terminal differentiation (33–35). This "priming" of multiple genes is considered to be essential for hematopoietic stem/progenitor cells to maintain multipotency. To accomplish this unique property, hematopoietic stem/progenitor cells have an open chromatin state, which is achieved by hyperacetylation of promoter histones. The low abundance of HDACs should contribute to histone hyperacetylation of "primed" genes in hematopoietic stem/progenitor cells. Indeed, multiple sites of core histones were hyperacetylated in $CD34^+$ progenitor cells compared with AML cells in our study. Moreover, up-regulation of HDACs in more differentiated/committed progenitors is consistent with this notion. If this scenario is true, exogenous interference with the up-regulation of HDACs may inhibit the differentiation of progenitor cells, leading to their expansion. Recent investigations suggest that this is the case: pharmacological down-regulation of HDACs effectively expands hematopoietic progenitor cells *ex vivo* (36, 37). Similarly, Haumaitre *et al.* (38) succeeded in amplifying endocrine progenitor cells using HDAC inhibitors.

In this study, we also found that HDAC1 has an instructive role in lineage specification of human hematopoietic cells. *HDAC1* is up-regulated in differentiating progenitor cells of multiple lineages by down-regulation of GATA-2 and MZF-1, which are abundantly expressed in early progenitor cells (39, 40). Common myeloid progenitors differentiate into erythro-megakaryocytic lineages when *HDAC1* expression is sustained by GATA-1. In contrast, they differentiate into myeloid cells, especially granulocytes, when *HDAC1* is down-regulated by C/EBPs. This view is compatible with the recent report by Yamamura *et al.* (41), in which pharmacological inhibition of HDAC activities enhances interleukin-3 and stem cell factor-mediated generation of committed progenitors from human peripheral blood-derived $CD34^+$ cells and inhibits erythropoietin-induced erythroid differentiation of committed progenitors. Furthermore, experiments using HDAC inhibitors have revealed the roles of HDACs in the differentiation of several cell types, including neurons, oligodendrocytes, osteoblasts, intestinal epithelial cells, adipocytes, and regulatory T cells (42–44). Our results provide the molecular basis of these observations and the rationale for medical application of HDAC inhibitors to tissue regeneration.

Finally, we found that HDACs are overexpressed in nearly all primary AML cells and cell lines without fusion gene products caused by chromosomal translocations. The involvement of HDACs in leukemogenesis in AML cells carrying fusion gene products has been well characterized and provides a theoretical basis for the efficacy of HDAC inhibitors for acute leukemias with chromosomal translocations (14, 15). Our findings extend this view and propose more generalized roles of HDACs in leukemogenesis and the validity of class I HDACs as therapeutic

targets in various hematological malignancies. As a result of HDAC overexpression, histone acetylation was diminished at multiple residues, including histone H4-lysine 16. Loss of acetylation at histone H4-lysine 16 was shown to be an epigenetic hallmark of human cancer, including AML, by Fraga *et al.* (45). Therefore, overexpression of HDACs may be a common feature of malignant cells, playing a fundamental role in oncogenesis. Indeed, overexpression of HDACs, mostly HDAC1, has been described in gastric, prostate, breast, cervical, and colon cancers (46, 47). As for the mechanisms of HDAC overexpression in AML, we speculate that loss-of-function mutations of C/EBP or abnormalities in the expression of MZF-1 and GATA2 cause aberrant expression of *HDAC1* in hematopoietic stem/progenitor cells. Although further investigation is required to elucidate the underlying mechanisms, HDAC overexpression justifies the application of HDAC inhibitors for cancer treatment.

According to the two-hit theory, two independent genetic abnormalities are required for the development of AML (11). Class I mutations confer a growth advantage on hematopoietic stem cells, and class II mutations block differentiation. The results of our transplantation studies suggest that *HDAC1* acts as a novel class II transforming gene upon overexpression in hematopoietic stem/progenitor cells. From a mechanistic standpoint, two questions emerge immediately. What is the identity of the downstream effectors? What are the accompanying class I mutations? To address the first question, we performed DNA microarray analysis of HDAC1-transduced c-KIT-positive bone marrow cells from recipient mice. Among 22,201 genes screened, the expressions of 153 genes (0.69%) were up-regulated more than 2-fold in HDAC1-transduced c-KIT$^+$ progenitor cells compared with mock-transfected control (supplemental Fig. S8). Similarly, down-regulation was observed in 63 genes (0.28%). Supplemental Table S2 shows part of the HDAC1-regulated genes, in which the alteration of the expression levels was confirmed by real time quantitative RT-PCR. Among them, up-regulation of c-*Mpl* is most interesting, because it is essential for megakaryocyte development (48) and for maintaining hematopoietic stem cells in the G_0 phase of the cell cycle (49). It is possible that up-regulated c-MPL directs HDAC1-expressing progenitors into quiescence and also skews their differentiation program into erythro-megakaryocytic lineage. Regarding the second question, we have found a positive correlation of the presence of internal tandem duplication of FLT3 (FLT3-ITD), the most frequent class I abnormality in *de novo* AML (50), with the expression levels of HDAC1 (supplemental Fig. S9). This strongly suggests that HDAC1 overexpression and FLT3 mutations collaborate to transform progenitor cells into leukemic clones. Extensive investigation is currently under way in our laboratory to address the functional interplay between HDAC1 overexpression and c-MPL up-regulation and/or FLT3 mutations in leukemogenesis.

In summary, our findings add new insight into the epigenetic regulation of normal and malignant hematopoiesis and ultimately contribute to the development of better treatment strategies in diseases of the hematopoietic system.

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Evidence That Integrin αIIbβ3-dependent Interaction of Mast Cells with Fibrinogen Exacerbates Chronic Inflammation*

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Integrin αIIbβ3 is expressed in mast cells as well as in megakaryocytes/platelets. A recent study has shown that surface expression levels of integrin αVβ3 are elevated in integrin αIIb-deficient bone marrow-derived mast cells (BMMCs) as compared with wild-type (WT) counterparts, but the underlying mechanism remains obscure. Here we demonstrate by transducing integrin αIIb into integrin αIIb-deficient BMMCs that surface expression levels of integrin αVβ3 are inversely related to those of integrin αIIbβ3. Thus, competitive association of integrin β3 with integrin αIIb or integrin αV determines surface expression levels of integrin αIIbβ3 or αVβ3 in mast cells. We compared WT and integrin αIIb-deficient BMMCs as well as integrin αIIb-deficient BMMCs transduced with integrin αIIb(WT) or non-functional αIIb(D163A) mutant and found that enhancement of proliferation, degranulation, cytokine production, and migration of BMMCs through interaction with fibrinogen (FB) depended on integrin αIIbβ3. In addition, elevated surface expression of integrin αVβ3 failed to compensate for loss of FB-associated functions in integrin αIIb-deficient BMMCs while enhancing adhesion to vitronectin or von Willebrand factor. Importantly, integrin αIIb deficiency strongly suppressed chronic inflammation with the remarkable increase of mast cells induced by continuous intraperitoneal administration of FB, although it did not affect acute allergic responses or mast cell numbers in tissues in steady states. Interestingly, soluble FB promoted cytokine production of BMMCs in response to *Staphylococcus aureus* with FB-binding capacity, through integrin αIIbβ3-dependent recognition of this pathogen. Collectively, integrin αIIbβ3 in mast cells plays an important part in FB-associated, chronic inflammation and innate immune responses.

Mast cells play a critical role in IgE-associated allergic disorders, but recent advances have delineated the involvement of mast cells in IgE-independent physiological and pathological processes, including certain innate immune responses. In fact, various stimuli, in addition to IgE and specific antigens, can activate mast cells to release a diverse array of preformed and newly synthesized pro-inflammatory mediators such as histamine, lipids, cytokines, and chemokines (1–4). Although mast cell numbers and activation in tissues are closely related to mast cell-mediated immunity, the underlying mechanism remains incompletely understood. As one of the key phenomena, mast cells interact with the extracellular matrix (ECM)[2] through integrins composed of two subunits (α and β), thereby regulating mast cell functions. As previously reported (5–9), integrins α4β1 and α5β1, or integrin αVβ3, expressed in mast cells mediate binding to fibronectin (FN) or vitronectin (VN), respectively. Interestingly, integrin α4β7 is involved in intestinal homing of mast cell progenitors via interaction with mucosal vascular addressin cell adhesion molecule-1 (10). In view of the implication of mast cell integrins in innate immunity, integrin α2β1 expressed in peritoneal mast cells is required for the induction of inflammatory responses to infection (11). In addition, integrin αVβ6 is essential for nematode-induced mucosal mast cell hyperplasia and for expression of the granule chymase (12).

Integrin αIIb, also known as CD41, which forms a complex with integrin β3, is a well known marker of the megakaryocyte/platelet lineage. Integrin αIIbβ3 is required for normal platelet hemostatic function (13–17). Previously, we reported that integrin αIIbβ3 is also highly expressed in mast cells (9, 18). In addition, we demonstrated that mast cell interaction with fibrinogen (FB) via integrin αIIbβ3 enhances *in vitro* mast cell functions, by using a blocking Ab specific for integrin αIIb (9). On the other hand, higher surface expression levels of integrin αV and enhanced adhesion to VN were found in integrin αIIb-deficient BMMCs as compared with wild-type (WT) counterparts (18), suggesting that integrin αIIb and integrin αV counter-regulate their surface expression levels and functions in mast cells. Therefore, we attempted to carefully analyze the regulatory mechanisms by utilizing retroviral transduction with integrin αIIb WT or non-functional mutant into integrin αIIb-deficient BMMCs.

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[2] The abbreviations used are: ECM, extracellular matrix; BMMCs, bone marrow-derived mast cells; FB, fibrinogen; FN, fibronectin; SA, fixed *S. aureus* Cowan I; PCA, passive cutaneous allergic reaction; SCF, stem cell factor; VN, vitronectin; vWF, von Willebrand factor; WT, wild type; Ab, antibody; mAb, monoclonal antibody; IL-3, interleukin-3; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; TNP, trinitrophenol; DNP, dinitrophenol; WT, wild type; KO, knockout.

FB abundant in plasma contributes to blood clotting (19). In addition, FB as well as its degradation product fibrin are also present in ECM outside blood vessels, where they play important roles in inflammation and wound healing through recruitment and activation of inflammatory cells expressing FB-binding receptors (19–22). Interestingly, surface proteins that bind to FB are also expressed by several types of bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, which modulate immune responses to bacterial infections (23–25).

In the present study, we showed that integrin αIIb expression levels regulate surface expression levels of integrin αVβ3 as well as integrin αIIbβ3 in mast cells and that mast cell functions augmented by interaction with FB are dependent on integrin αIIbβ3 and independent of integrin αVβ3. In accordance, integrin αIIb deficiency strongly suppressed FB-induced chronic inflammation. Notably, the interaction with soluble FB via integrin αIIbβ3 helps mast cells recognize and respond to *S. aureus* (Cowan I) with FB-binding capacity. Thus, integrin αIIbβ3 in mast cells modulates FB-associated, chronic inflammation and innate immune responses.

EXPERIMENTAL PROCEDURES

Mice—All experimental mice were sex- and age-matched (6–16 weeks old). Balb/c mice were purchased from Charles River Japan (Tokyo, Japan). Integrin aIIb$^{-/-}$ mice were generated as described previously (14) and backcrossed to Balb/c mice for at least six generations. Animal studies were performed according to the guidelines of the animal care committee of the Institute of Medical Science, University of Tokyo.

Antibodies and Other Materials—Source of antibodies (Abs) were as follows: anti-mouse integrin αIIbβ3 mAb (1B5) was a kind gift from Dr. B. S. Coller (Rockefeller University, New York, NY) (26). Anti-mouse integrin αV (8B3) and anti-mouse integrin β3 (8B11) mAbs were kind gifts from Drs. D. J. Gerber and S. Tonegawa (Picower Center, Massachusetts Institute of Technology, Boston, MA) (27). Anti-dinitrophenol (DNP) IgE (SPE-7) was from Sigma. Anti-trinitrophenol (TNP) IgE (C38-2), anti-mouse αIIb (MWReg30), anti-mouse αV (RMV-7 and H9.2B8), anti-mouse α4 (9C10), anti-mouse α5 (5H10–27), anti-mouse LFA1 (M17/4), anti-mouse β1 (Ha2/5), anti-mouse β2 (GAME-46), and anti-αVβ3 (2C9.G2) mAbs and other Abs were from BD Pharmingen. Cytokines such as mouse IL-3 and SCF were obtained from R&D Systems. Bovine serum VN and TNP-conjugated BSA (TNP-BSA) were from Sigma. Human plasma FB and von Willebrand factor (vWF) were from Chemicon. Formalin-fixed *S. aureus* Cowan I was purchased from Calbiochem.

Cells—To generate BMMCs with 95% purity (c-kit$^+$/FcεRI$^+$ by flow cytometry), bone marrow cells from 6-week-old male mice were cultured for 5–8 weeks in the presence of 10 ng/ml IL-3 with or without 20 ng/ml SCF as described previously (9, 28).

DNA Constructs, Transfection, and Infection—To generate mouse integrin αIIb(D163A) mutant, two-step PCR mutagenesis was performed by using mouse integrin αIIb wild-type (WT) cDNA (provided by Dr. R. B. Basani, Children's Hospital of Philadelphia, PA) as a template. Retroviral transfection was as described in a previous study (29). Briefly, integrin αIIb(WT) or αIIb(D163A) mutant cDNA was subcloned into pMXs-IRES-puro^r (pMXs-IP) to generate pMXs-IP-integrin αIIb(WT) or αIIb(D163A), respectively. To generate recombinant retroviruses, pMXs-IP plasmids were transfected into PLAT-E packaging cells (30) with FuGENE 6 (Roche Diagnostics). Cells were infected with retroviruses in the presence of 10 μg/ml Polybrene. Selection with puromycin was started 48 h after infection.

Flow Cytometric Analysis—Cells were stained as described before (9, 28). Cells stained with the indicated Abs were analyzed with a FACSCalibur equipped with CellQuest software (BD Biosciences) and Flowjo software (Tree Star).

Adhesion Assay and Migration Assay—Adhesion assay was done as described (6, 9). In brief, 96-well plates were coated with 20 μg/ml FB, FN, VN, or vWF. BMMCs resuspended at 5×10^5 cells/ml were transferred into coated wells with or without stimulant for 1 h at 37 °C. After washing, cell adhesion was quantitated using CellTiter-Glo™ (Promega, Madison, WI) and a Micro Lumat Plus luminometer (EG&G Berthold), according to the manufacturer's instructions. In assays using blocking Abs, BMMCs were preincubated with 20 μg/ml Abs for 1 h before adding the cells to the plate. Migration assays were carried out as described (7, 9), using 24-well Transwell chambers with 5-μm polycarbonate filters (Corning).

Measurement of Cytokines—The cells were transferred into FB-coated 96-well plates (1×10^4 cells/well) with or without stimulants. After incubating for 12 h at 37 °C, the supernatant of each well was collected, and the concentration of IL-6 or TNF-α was quantified by enzyme-linked immunosorbent assay with OptiEIA for IL-6 or TNF-α (BD Pharmingen) as described (9, 28).

β-Hexosaminidase Release Assay—β-Hexosaminidase release assay was as described before (31). Briefly, 5×10^4 cells of IgE-sensitized BMMCs in Tyrode buffer (10 mM HEPES buffer (pH 7.4), 130 mM NaCl, 5 mM KCl, and 5.6 mM glucose) containing 0.1% BSA, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$ were stimulated with the indicated concentration of TNP-BSA in BSA- or FB-coated 96-well plates for 1 h at 37 °C. Cell supernatants and total cell lysates solubilized with 1% Nonidet P-40 were collected, and β-hexosaminidase in the supernatants and cell lysates was quantified by spectrophotometric analysis of hydrolysis of *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (Sigma). The percentage of β-hexosaminidase release was calculated.

PCA Reactions—Passive cutaneous anaphylactic (PCA) reactions were performed as described (32–34). Briefly, anti-DNP IgE was intradermally injected into the ears of mice. After 24 h, 250 μg of DNP-BSA and 0.5% Evans blue dye was intravenously injected. The amounts of extravasated dye were measured after 30 min by extracting ears. In another type of experiment, mice received anti-DNP IgE intravenously. After 24 h, a skin reaction was elicited by applying 0.75% dinitrofluorobenzene acetone-olive oil solution to both sides of the ears. The reaction was assessed by measuring the ear thickness 1 h and 12 h after antigen challenge.

FB-induced Chronic Inflammation Model—200 μl of 0.5 mg/ml FB or PBS was intraperitoneally injected into WT or integrin αIIb$^{-/-}$ mice every 2 days. After 1 month, total peritoneal cells of the sacrificed mice were collected by using 3 ml of PBS. Total cell numbers were counted by using a hemocytometer; the percentages of mast cells (c-kit$^+$/FcεRI$^+$), granulo-

cytes (Gr-1^{high}/CD11b^{+}), and macrophages (F4/80^{+}) were calculated by fluorescence-activated cell sorting analysis.

Responses to Fixed S. aureus Cowan I in BMMCs—Fixed *S. aureus* Cowan I (SA) purchased from Calbiochem was stained with Cell Tracker Orange (Molecular Probes). Five × 10^4 BMMCs suspended in 10% BSA/Tyrode buffer were incubated with SA in the presence or absence of 0.5 mg/ml soluble FB in BSA-coated 96-well plates for 2 h. The interaction between mast cells and SA was observed by using a fluorescence microscope. After the supernatant of each well was collected, bacterial cells were removed with a 0.22-μm filter. Cytokine concentrations in the supernatants were quantified by enzyme-linked immunosorbent assay.

Quantitation of Tissue Mast Cells—Tissue mast cells in ear skin, back skin, peritoneal wall, and intestine were quantified by light microscopy at ×400 by an observer who was unaware of the identity (*i.e.* mouse genotype) of the individual specimens, in Giemsa-stained sections, as previously described (28, 35, 36). Results were expressed as mast cells (mean ± S.E.) per mm^2.

Statistical Analysis—Data are shown as the mean ± S.D. Statistical significance was determined by Student's *t* test, with $p < 0.01$ (**) and $p < 0.05$ (*) taken as being statistically significant.

RESULTS

Surface Expression Levels of Integrin αV Are Elevated in Integrin αIIb-deficient BMMCs—To investigate the role of integrin αIIb in mast cells, bone marrow cells from WT and integrin αIIb$^{-/-}$ mice were cultured in the presence of IL-3 for 5 weeks to generate comparable numbers of morphologically pure (>95%) mast cells. BMMCs from WT and integrin αIIb$^{-/-}$ Balb/c mice exhibited similar levels of FcεRI and c-kit on their cell surfaces as determined by flow cytometry (Fig. 1*A*). In addition, proliferative responses to IL-3 as well as apoptosis induced by growth factor (IL-3) deprivation were comparable between both BMMCs (Fig. 1, *C* and *D*). Thus, integrin αIIb deficiency did not affect Balb/c mice-derived mast cell development and growth in suspension culture, as previously reported in C57BL/6 mice (18). Moreover, when IgE-sensitized BMMCs were stimulated with the indicated doses of antigen, we found comparable levels of β-hexosaminidase release and cytokine (IL-6 and TNF-α) production (Fig. 1, *E* and *F*, and data not shown). This also suggested that integrin αIIb deficiency did not modulate FcεRI signaling in suspension culture of mast cells. However, in keeping with previous findings (18), we confirmed the striking differences between the two cell types: surface expression levels of integrin αV and integrin αVβ3 were 10-fold higher in integrin αIIb-deficient BMMCs as compared with WT counterparts, despite comparable expression levels of other integrins such as integrins α4, α5, and β1, and no detectable expression of integrin β2 in either BMMC (Fig. 1*E*). Collectively, these results led us to postulate that integrin αIIb deficiency influenced mast cell functions through interaction with ECM.

Surface Expression Levels of Integrin αV Are Inversely Correlated with Those of Integrin αIIb—We next investigated the mechanism by which surface expression levels of integrin αV were elevated in integrin αIIb-deficient BMMCs. As previously reported (18), mRNA levels of integrin αV and integrin β3 were comparable between WT and integrin αIIb-deficient BMMCs (data not shown), suggesting the post-translational regulation of surface expression levels of integrin αV in BMMCs. Because integrin β3 forms a complex with integrin αIIb or integrin αV, we hypothesized that integrin αV competed with integrin αIIb in the association with integrin β3. To test this, integrin αIIb-deficient BMMCs were retrovirally transduced with integrin αIIb WT or mock. Notably, flow cytometric analysis demonstrated that transduction with integrin αIIb(WT) strongly down-regulated surface expression of integrin αV in integrin αIIb-deficient BMMCs (Fig. 2*A*). In addition, integrin αIIb-(D163A) mutant (37), which lost the capacity to bind to FB, was transduced into integrin αIIb-deficient BMMCs. Consistent with a previous report (37), surface expression levels of integrin αIIb(D163A) mutant were weaker than those of integrin αIIb(WT) in the transduced cells. In proportion to less induction of integrin αIIb(D163A), surface expression levels of integrin αV in integrin αIIb(D163A) mutant-transduced cells were less down-regulated as compared with those in integrin αIIb(WT)-transduced BMMCs (Fig. 2*A*). Furthermore, similar experiments were performed using murine T cell lymphoma cell line BW5147, which originally expressed integrin αVβ3 but not integrin αIIbβ3. As shown in Fig. 2*B*, transduction with integrin αIIb(WT) into BW5147 cells down-regulated surface expression of integrin αV to a greater degree as compared with transduction with integrin αIIb(D163A) mutant. Collectively, surface expression levels of integrin αV were inversely related to those of integrin αIIb, and even non-functional integrin αIIb competed with integrin αV for integrin β3.

Reduced Adhesion to FB and Enhanced Adhesion to VN and vWF in Integrin αIIb-deficient BMMCs—Next, we examined the effects of integrin αIIb deficiency on mast-cell adhesion to ECM proteins such as FN, FB, VN, and vWF. IgE stimulation-dependent adhesion to FB was strongly suppressed in integrin αIIb-deficient BMMCs, whereas adhesion to VN or vWF was drastically enhanced in integrin αIIb-deficient BMMCs, presumably because of increased surface expression of integrin αVβ3 in integrin αIIb-deficient BMMCs when compared with WT BMMCs (Fig. 3*A*). On the other hand, integrin αIIb deficiency did not significantly affect the adhesion to FN (Fig. 3*A*). In addition, we examined the inhibitory effect of pretreatment with blocking Abs against integrin αIIbβ3 or integrin αVβ3 on mast-cell adhesion to ECM proteins, confirming that, in WT BMMCs, the binding to FB, VN, or vWF was dependent on integrin αIIbβ3, integrin αVβ3, or both (Fig. 3*B*). Similar experiments were also performed with regard to integrin αIIb-deficient BMMCs, demonstrating that pretreatment with blocking Abs against integrin αV dampened IgE stimulation-dependent strong adhesion to VN or vWF as well as weak adhesion to FB, whereas it did not affect the adhesion to FN (Fig. 3*B*). In contrast, pretreatment with blocking Ab for integrin αIIb did not reduce the adhesive property at all (Fig. 3*B*). Collectively, these results suggested that elevated surface expression levels of integrin αVβ3 in αIIb-deficient BMMCs enhanced the adhesion to VN or vWF, but it did not compensate for the defective adhesion to FB owing to integrin αIIbβ3 deficiency.

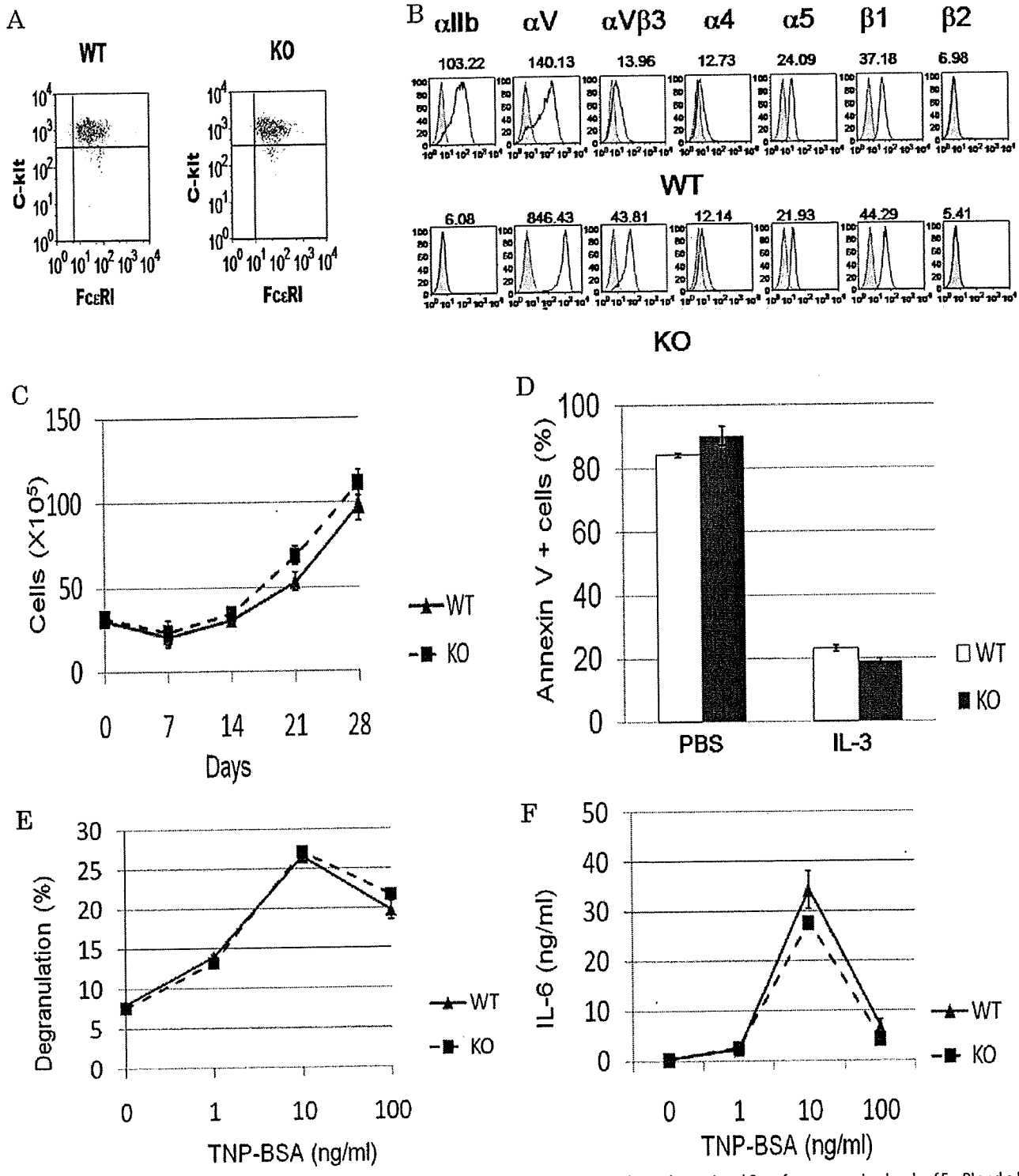

FIGURE 1. **Functional analysis of WT and integrin αIIb-deficient BMMCs in *in vitro* suspension culture.** *A* and *B*, surface expression levels of FcεRI and c-kit as well as several integrins such as integrin αIIb, αV, αVβ3, α4, α5, β1, and β2 in WT and integrin αIIb-deficient BMMCs. Mean fluorescent intensities of staining were indicated. Data are representative of three independent experiments. *C*, *in vitro* growth curves of bone marrow cells derived from WT and integrin αIIb-deficient mice. Numbers of trypan blue-excluding cells in bone marrow cell cultures in IL-3-containing medium were counted weekly. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. *D*, IL-3 deprivation-induced apoptosis of WT and integrin αIIb-deficient BMMCs. Percentage of annexin V-positive cells after 48 h was measured by flow cytometric analysis. Data represent three independent experiments. All data points correspond to the mean ± S.D. *E* and *F*, after IgE-sensitized WT and integrin αIIb-deficient BMMCs were stimulated with the indicated concentrations of antigen for 50 min or 24 h, the amounts of β-hexosaminidase (*E*) or IL-6 (*F*), respectively, released into medium were measured. Data represent three independent experiments. All data points correspond to the mean ± S.D.

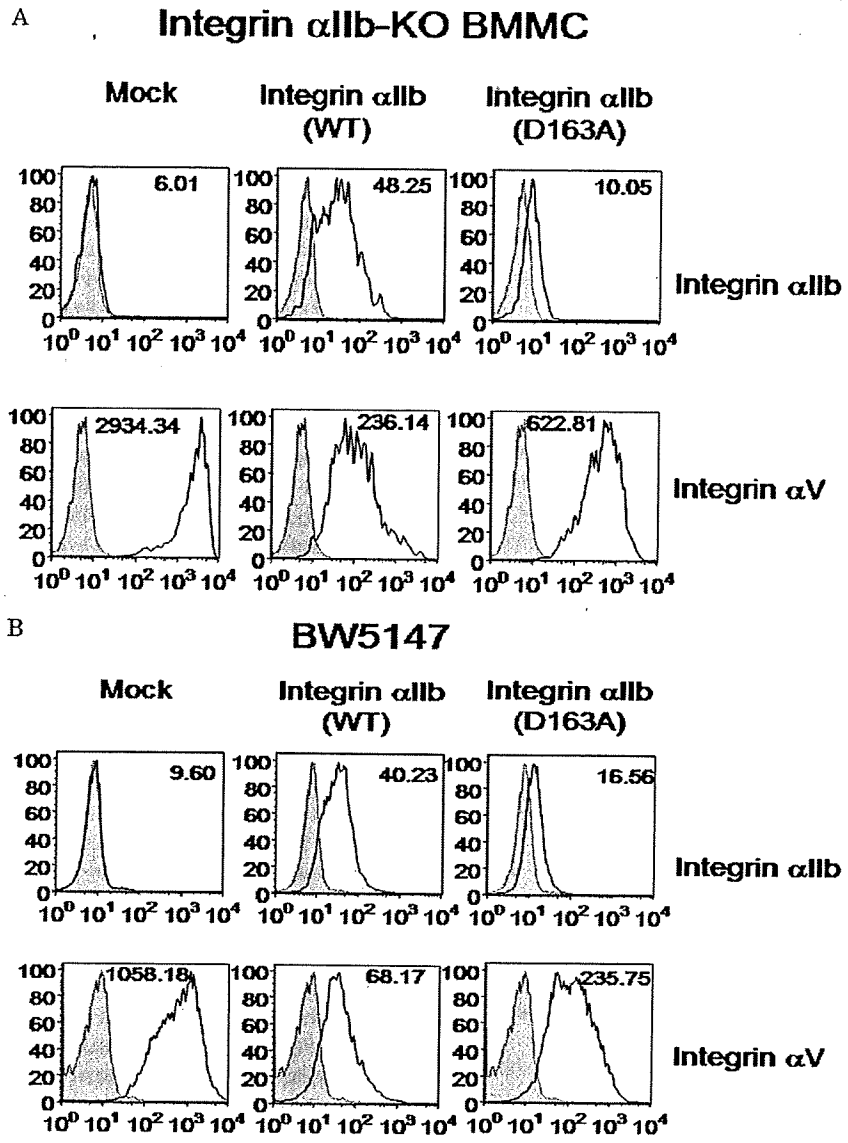

FIGURE 2. **Elevated surface expression levels of integrin αV in integrin αIIb-deficient BMMCs were reduced by transduction with integrin αIIb.** *A* and *B*, integrin αIIb-deficient BMMCs (*A*) or BW5147 cells (*B*) were transduced with integrin αIIb(WT), αIIb(D163A) mutant, or mock. Surface expression levels of integrin αIIb or integrin αV in these transfectants were analyzed by flow cytometry. Mean fluorescent intensities of staining were indicated. Data represent three independent experiments.

specific ligand for integrin αIIbβ3. Moreover, it was found in WT, but not integrin αIIb-deficient, BMMCs that SCF-stimulated mast-cell proliferation was accelerated in FB-coated plates as compared with BSA-coated plates (Fig. 4*A*). Similarly, when stimulated by IgE plus antigen, WT, but not integrin αIIb-deficient, BMMCs enhanced β-hexosaminidase release and cytokine (IL-6 and TNF-α) production through interaction with FB (Fig. 4, *B–D*). Altogether, integrin αIIbβ3 plays crucial roles in enhancing mast-cell functions through interaction with FB.

Transduction with Integrin αIIb(WT), but Not Integrin αIIb(D163A) Mutant, into Integrin αIIb-deficient BMMCs Recovered Mast Cell Functions through Interaction with FB—To further reduce the possibility that enhanced expression levels of integrin αVβ3 modulated mast-cell functions through interaction with FB, we performed similar experiments on adhesion and cytokine production in integrin αIIb-deficient BMMCs transduced with integrin αIIb(WT), integrin αIIb-(D163A) mutant, or mock. As depicted in Fig. 5*A*, transduction with integrin αIIb(WT), but not integrin αIIb(D163A) mutant, augmented the adhesion to FB as compared with transduction with mock. On the other hand, transduction with integrin αIIb(D163A) as well as integrin αIIb(WT) diminished the adhesion to VN, with the degree of the former being a little lower than that of the latter, which was consistent with surface expression levels of integrin αVβ3 in integrin αIIb-deficient BMMCs transduced with integrin αIIb(WT) and (D163A) mutant (Fig. 2*A*). Moreover, transduction with integrin αIIb(WT), but not integrin αIIb(D163A), induced the enhancement of cytokine production through interaction with FB in integrin αIIb-deficient BMMCs (Fig. 5*B*). Collectively, these results definitively confirmed that enhanced mast cell functions through interaction with FB were dependent on integrin αIIbβ3 but not integrin αVβ3.

Enhancement of Migration, Proliferation, Degranulation, and Cytokine Production of BMMCs through Interaction with FB Is Dependent on Integrin αIIb—We next examined the effect of integrin αIIb deficiency on mast-cell functions through interaction with FB. As previously reported, SCF induced migration of WT BMMCs when the lower membranes of the Transwells were pre-coated with FB, FN, or VN. Comparison of the migrating cell numbers between WT and integrin αIIb-deficient BMMCs revealed that integrin αIIb deficiency strongly diminished or enhanced the migration of BMMCs through interaction with FB or VN, respectively, whereas it did not affect mast cell migration through interaction with FN (Fig. 3*C*). These results also suggested that, in integrin αIIb-deficient BMMCs, both the adhesive and migratory ability were altered toward integrin αVβ3, whereas there was little interaction with FB, a

Integrin αIIb Deficiency Affected Neither Tissue Mast Cell Numbers in Steady States nor Mast Cell-mediated Acute Allergic Reactions—Because enhanced proliferation and migration of BMMCs through interaction with FB were suppressed by integrin αIIb deficiency, we compared the quantity of tissue mast cells in WT and in integrin $\alpha IIb^{-/-}$ mice. Microscopic

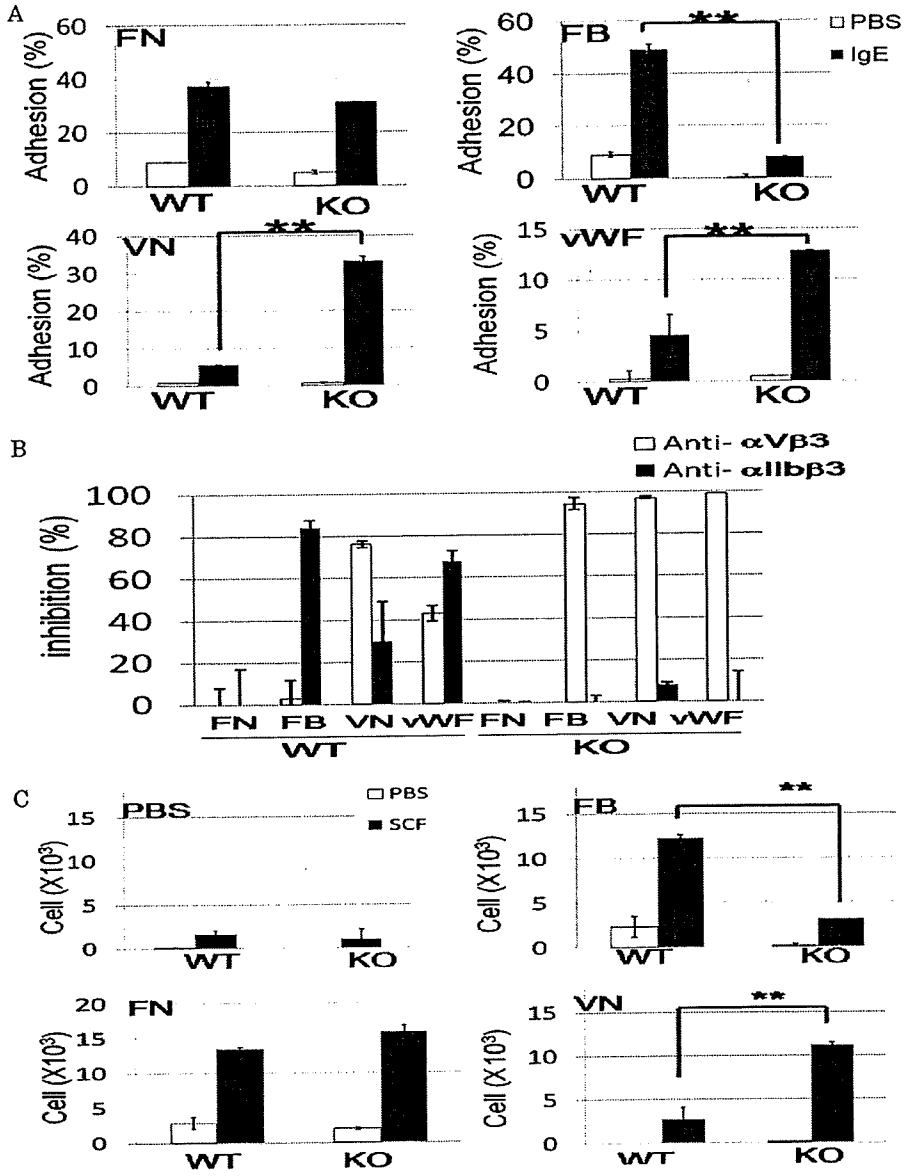

FIGURE 3. **Enhanced adhesion to VN or vWF and deteriorated adhesion to FB in integrin αIIb-deficient BMMCs.** *A*, WT and integrin αIIb-deficient BMMCs were incubated with or without 5 μg/ml SPE-7 IgE in FN-, FB-, VN-, or vWF-coated plates. Percentage of adherent cells was measured. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. ** ($p < 0.01$) and * ($p < 0.05$) indicate statistical differences. *B*, pretreatment with blocking Ab for integrin αIIb or integrin αV inhibited to various degrees the adhesion of WT- or integrin αIIb-deficient BMMCs stimulated by 5 μg/ml IgE in FN-, FB-, VN-, or vWF-coated plates. Percentage of inhibition was measured. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. *C*, WT or integrin αIIb-deficient BMMCs in the upper wells were attracted by 100 ng/ml SCF in the lower wells through BSA-, FB-, FN-, or VN-coated Transwells. Migrated cells were counted. Data represent three independent experiments. All data points correspond to the mean ± S.D. ** ($p < 0.01$) indicates statistical differences.

analysis demonstrated that mast-cell numbers in the ear skin, back skin, peritoneum wall, and small intestine were not different in these mice (Table 1). Based on this, we addressed the question of whether tissue FB extravasated by acute inflammation modulated mast cell-associated allergic reactions of WT and integrin $\alpha IIb^{-/-}$ mice. However, no significant difference of two types of PCA reaction was observed in these mice (data not shown), despite enhanced *in vitro* degranulation and cytokine production of mast cells through integrin αIIbβ3-dependent interaction with FB (Fig. 4, *B–D*). These results indicated that integrin αIIbβ3 was not involved in tissue mast-cell numbers and distributions in steady states or IgE-mediated acute allergic responses.

Integrin αIIb Deficiency Suppressed Peritoneal Chronic Inflammation with a Remarkable Increase of Mast Cells Induced by Repetitive Intraperitoneal FB Administration—We next asked whether integrin αIIb deficiency influenced chronic inflammation with extravascular FB and fibrin deposition. To explore the direct effects of FB, we adopted FB-induced chronic inflammation models where FB was administered into peritoneal cavities every other day. After 1 month, we counted total peritoneal cell numbers and estimated cell populations by flow cytometric analysis. In steady states before the stimulation, we found no significant differences in total peritoneal cell numbers or in mast cell numbers between WT and integrin $\alpha IIb^{-/-}$ mice (Fig. 6*A* and data not shown). Interestingly, repetitive intraperitoneal injection of FB, but not PBS as a control, induced severe chronic inflammation with a remarkable increase of mast cells as well as total inflammatory cells in the peritoneal cavities of WT mice (Fig. 6, *A* and *B*). Thus, an FB-induced chronic inflammation model was established. Intriguingly, integrin αIIb deficiency strongly suppressed the number of mast cells as well as the total number of inflammatory cells in the peritoneal cavities (Fig. 6, *A* and *B*), although the percentages of granulocytes and macrophages were not significantly different in the WT and integrin $\alpha IIb^{-/-}$ mice (Fig. 6*B*, *right panel*). Considering the *in vitro* roles of mast cell integrin αIIβ3-FB interaction, these results strongly suggested that *in vivo* FB-induced chronic inflammation was largely dependent on integrin αIIbβ3 in mast cells, although the effect of few, if any, platelets in the peritoneal cavities on this phenomenon was not completely ruled out. In addition, we found comparable numbers of inflammatory cells in WT and integrin αIIb-deficient mice 24 h after single dose of FB injection (data not shown). Therefore, FB-induced chronic inflammation required continuous administration of FB. Collectively, integrin αIIbβ3 in mast cells played

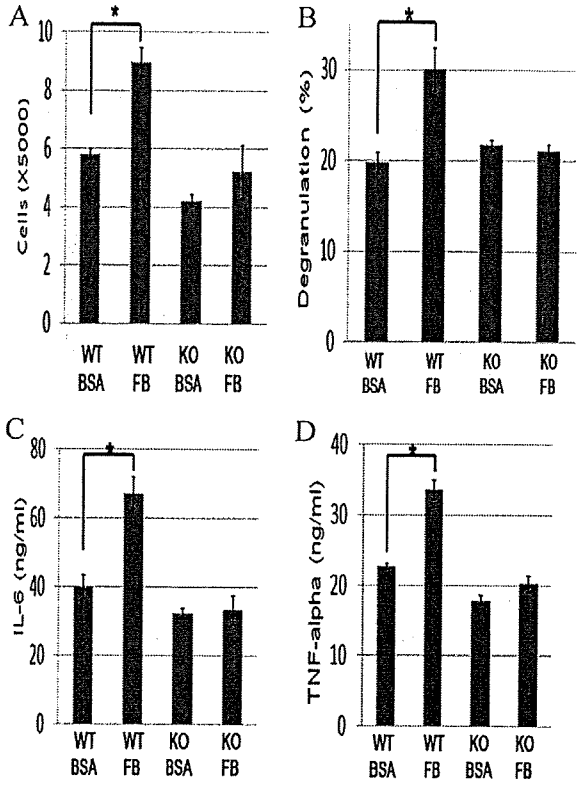

FIGURE 4. **Enhanced proliferation, degranulation, and cytokine production of WT, but not integrin αIIb-deficient, BMMCs through interaction with FB.** *A*, cell numbers of WT or integrin αIIb-deficient BMMCs stimulated by 10 ng/ml IL-3 plus 100 ng/ml SCF for 5 days in BSA- or FB-coated plates. *B*, β-hexosaminidase release of IgE-sensitized WT or integrin αIIb-deficient BMMCs stimulated by 30 ng/ml TNP-BSA for 60 min in BSA- or FB-coated plates. *C* and *D*, IL-6 (*C*) and TNF-α (*D*) production of IgE-sensitized WT or integrin αIIb-deficient BMMCs stimulated by 30 ng/ml TNP-BSA for 16 h in BSA- or FB-coated plates. All data are representative of four independent experiments. All data points correspond to the mean ± S.D. * ($p < 0.05$) indicates statistical differences.

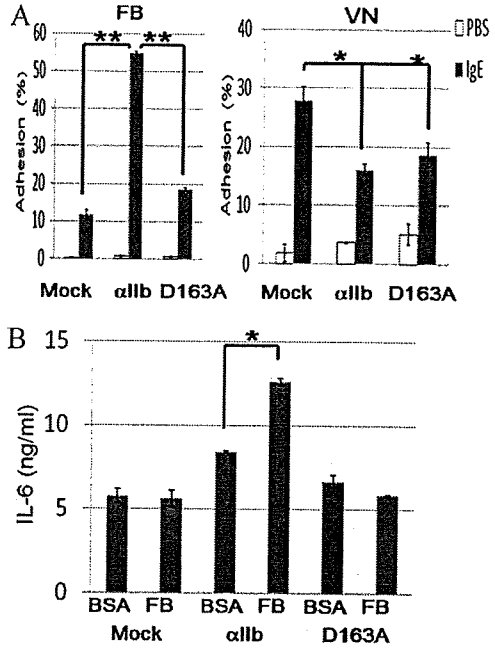

FIGURE 5. **Transduction with integrin αIIb(WT) enhanced or suppressed the adhesion to FB or VN, respectively, in integrin αIIb-deficient BMMCs.** *A*, integrin αIIb-deficient BMMCs transduced with integrin αIIb(WT), αIIb(D163A) mutant, or mock were incubated with 5 μg/ml SPE-7 IgE for 60 min in FB- or VN-coated plates. The percentage of adherent cells was measured. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. ** ($p < 0.01$) and * ($p < 0.05$) indicate statistical differences. *B*, integrin αIIb-deficient BMMCs transduced with integrin αIIb(WT), αIIb(D163A) mutant, or mock were incubated with 5 μg/ml SPE-7 IgE for 16 h in FB-coated plates. The amounts of IL-6 released into medium were measured. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. * ($p < 0.05$) indicates statistical differences.

TABLE 1

Numbers of mast cells in ear skin, back skin, peritoneum wall, and small intestine

Numbers of mast cells per ten randomly selected high power fields were determined under light microscopy. Results are the mean values ± S.E. for four mice/group. WT, wild type; KO, knockout.

Tissue	WT	KO
Ear skin	112 ± 11.7	109 ± 3.2
Back skin	29.7 ± 23	41.3 ± 13
Peritoneum wall	9.3 ± 5.0	10 ± 2
Small intestine	5.3 ± 3.3	6 ± 3.0

an important role in FB-mediated chronic, but not acute, inflammatory responses.

Soluble FB Enhanced Cytokine Production of WT, but Not Integrin αIIb-deficient BMMCs, in Response to S. aureus Cowan I with FB-binding Capacity—As previously reported, mast cells adhered to soluble FB as well as plate-coated FB via integrin αIIbβ3. Because soluble FB is bound by certain types of bacteria such as *S. aureus* (Cowan I), the immune cells expressing FB-binding receptors are thought to modulate the immune responses to these pathogens (23–25). We then investigated whether soluble FB influenced the response of mast cells to *S. aureus* (Cowan I). When WT or integrin αIIb-deficient BMMCs were incubated with *S. aureus* (Cowan I) for 2 h in the presence of soluble FB, fluorescent microscopic analysis demonstrated that WT, but not integrin αIIb-deficient, BMMCs were completely surrounded by aggregated *S. aureus* (Cowan I) probably through interaction with soluble FB (Fig. 7*A*). On the other hand, BMMCs were not apparently covered with *S. aureus* (Cowan I) in the absence of soluble FB. These results suggested that integrin αIIbβ3-dependent interaction of BMMCs with *S. aureus* (Cowan I) via soluble FB probably helped mast cells recognize this pathogen. Moreover, IL-6 released into each supernatant was quantified by enzyme-linked immunosorbent assay, demonstrating that soluble FB-induced enhancement of IL-6 production was observed only in WT, but not integrin αIIb-deficient, BMMCs in response to *S. aureus* (Cowan I) (Fig. 7*B*). To examine the specificity of this phenomenon, similar experiments were performed using *Escherichia coli* without FB-binding capacity. As shown in Fig. 7*B*, soluble FB-dependent enhancement of IL-6 production of BMMCs stimulated by *E. coli* was not observed irrespective of integrin αIIb expression, suggesting that soluble FB induced the enhancement of cytokine production of BMMCs in response to bacteria with, but not without, FB-binding capacity. Because Toll-like receptors primarily play an important part in the recognition of and response to bacteria (3), we also asked if soluble FB or immobilized FB enhanced cytokine production of both BMMCs stimulated by LPS, a Toll-like receptor 4 agonist. As

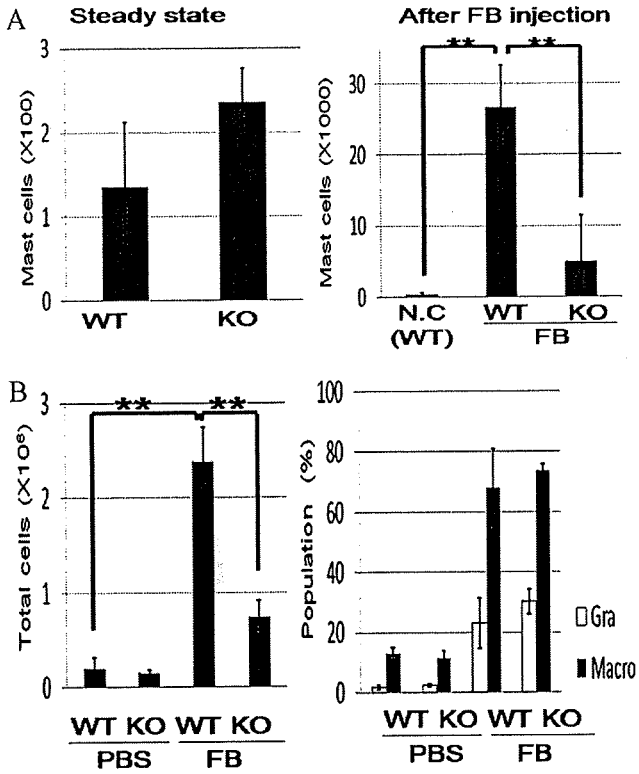

FIGURE 6. **Repetitive injection of FB into peritoneal cavities induced chronic inflammation more severely in WT mice in comparison to integrin αIIb-deficient mice.** *A*, peritoneal mast cell numbers of WT and integrin αIIb-deficient mice before (*left panel*) and after (*right panel*) FB injection. *B*, total peritoneal cell numbers (*left panel*) and cell populations (*right panel*) of WT and integrin αIIb-deficient mice after continuous intraperitoneal inoculation of FB or PBS for 1 month ($n = 5$/genotype). All data points correspond to the mean ± S.D. ** ($p < 0.01$) indicates statistical differences.

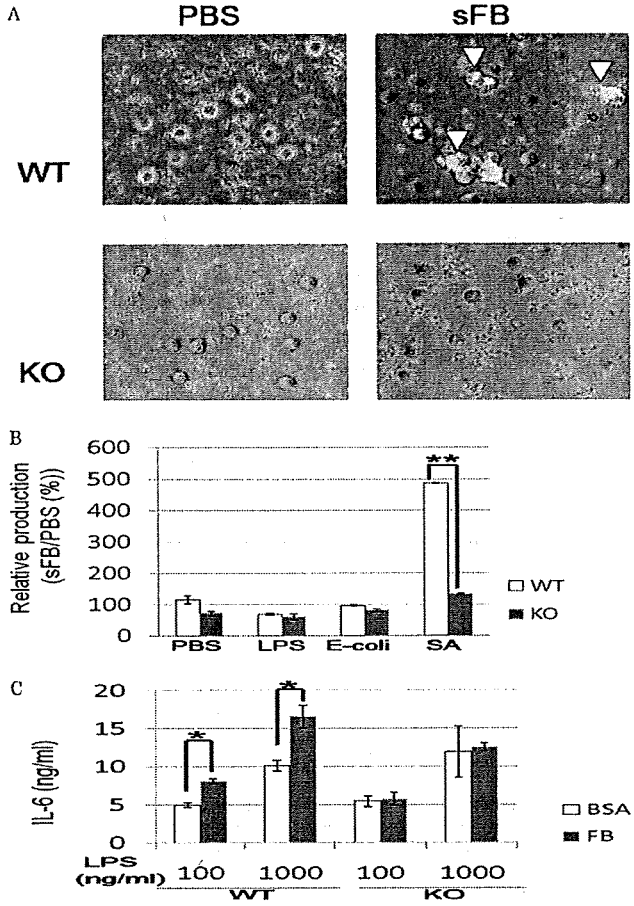

FIGURE 7. **Soluble FB enhanced cytokine production of WT, but not integrin αIIb-deficient, BMMCs in response to *S. aureus* with FB-binding capacity.** *A*, WT or integrin αIIb-deficient BMMCs were incubated with heat-killed *S. aureus* labeled by Cell Tracker Orange in the presence or absence of 500 μg/ml soluble FB for 2 h. WT, but not integrin αIIb-deficient, BMMCs were covered with SA aggregates in the presence of soluble FB (*arrowhead*). *B*, WT or integrin αIIb-deficient BMMCs were incubated with 100 ng/ml LPS, 100 μg/ml heat-killed *E. coli*, 100 μg/ml *S. aureus*, or PBS as control in the presence or absence of 500 μg/ml soluble FB for 8 h. The ratio of the amounts of IL-6 released in the presence of soluble FB to those of IL-6 in the absence of soluble FB was measured. Data are representative of three independent experiments. All data points correspond to the mean ± S.D. ** ($p < 0.01$) indicates statistical differences. *C*, WT or integrin αIIb-deficient BMMCs were incubated with the indicated concentrations of LPS in FB- or BSA-coated plates. Data represent three independent experiments. All data points correspond to the mean ± S.D. * ($p < 0.05$) indicate statistical differences.

depicted in Fig. 7 (*B* and *C*), soluble FB did not affect IL-6 production of either WT or integrin αIIb-deficient BMMCs stimulated by LPS, whereas immobilized FB enhanced IL-6 production of WT, but not integrin αIIb-deficient, BMMCs stimulated by LPS. These results suggested the synergism of Toll-like receptor 4 signaling and integrin αIIbβ3 signaling through interaction with immobilized FB, but not soluble FB. Altogether, soluble FB enhances the cytokine production of BMMCs in responses to *S. aureus* (Cowan I), probably because mast cell-soluble FB-*S. aureus* (Cowan I) complex formation promoted the quick and tight recognition of this pathogen by mast cells.

DISCUSSION

In a previous study, we found that integrin αIIbβ3 is highly expressed in mast cells, in addition to the megakaryocyte/platelet lineage and a subset of hematopoietic progenitors (9, 14, 18). Experiments using blocking Abs specific for integrins demonstrated that adhesion to FB, VN, or vWF was mediated through integrin αIIbβ3, integrin αVβ3, or both, respectively (9). In the follow-up study, we first paid attention to the interesting results shown by Berlanga O *et al.* that integrin αIIb-deficient BMMCs displayed extremely higher surface expression levels of integrin αVβ3 as compared with WT counterparts (18). Because counter-regulation of integrin αIIbβ3 and integrin αVβ3 on their surface expression levels might affect *in vivo* functions of integrin αIIbβ3 in mast cells, we attempted to delineate the underlying mechanism. Our hypothesis that integrin αIIb competed with integrin αV in heterodimerization with integrin β3 in mast cells was illustrated by experimental results as follows: retroviral transduction with integrin αIIb(WT) into integrin αIIb-deficient BMMCs reduced surface expression of integrin αVβ3 at levels comparable to those in WT BMMCs (Fig. 2*A*). In addition, transduction with integrin αIIb(D163A) mutant led to less reduction in surface expression levels of integrin αVβ3 together with less induction in those of integrin αIIbβ3 in integrin αIIb-deficient BMMCs. Thus, surface expression levels of integrin αVβ3 were conversely related to those of integrin αIIbβ3 in mast cells (Fig. 2*A*). Notably, this phenomena was true for BW5147 cells transduced with integrin αIIb(WT) or

αIIb(D163A) mutant (Fig. 2*B*). However, integrin αIIb deficiency did not affect surface expression levels of integrin αVβ3 in platelets (data not shown). These results suggested that regulatory mechanisms on surface expression levels of integrin αVβ3 differed between mast cells and platelets. One possible explanation is as follows: integrin αIIb deficiency might fail to influence surface expression levels of integrin αVβ3 if integrin β3 expression were sufficient in platelets, whereas it might promote the association of integrin αV with integrin β3 if integrin β3 expression were insufficient in mast cells. Further examination is necessary to fully understand the mechanism. Importantly, all the functional analyses (Figs. 2–4) showed that higher surface expression levels of integrin αVβ3 in integrin αIIb-deficient BMMCs enhanced adhesion to VN or vWF but did not compensate for the loss of mast-cell functions through interaction with FB. Based on this, we compared *in vivo* mast cell functions between WT and integrin αIIb-deficient mice.

First, integrin αIIb deficiency did not affect mast-cell numbers in tissues under normal conditions (Table 1). This seems reasonable, given that FB is not abundant outside blood vessels under normal conditions. Second, integrin αIIb deficiency did not affect two types of PCA estimated by ear dye extravasation or swelling (data not shown), which was reported to be mast cell-dependent (32–34). These results indicate that extravascular FB and fibrin accompanied by acute inflammation are unable to enhance mast-cell functions. On the other hand, recent advances demonstrate that FB is a central regulator of the inflammatory response as well as of hemostasis. Analysis of gene-targeted mice expressing a mutant form of FB, lacking the integrin $\alpha_M\beta2$-binding motif, demonstrated that the high affinity engagement of FB by integrin $\alpha_M\beta2$ in neutrophils and macrophages was critical for inflammatory responses (38, 39). Therefore, we speculated that FB extravasated at acute inflammatory sites activated neutrophils and macrophages via integrin $\alpha_M\beta2$ but failed to enhance mast-cell functions via integrin αIIbβ3. In contrast, we found striking differences between WT and integrin $\alpha IIb^{-/-}$ mice in FB-induced chronic inflammation: integrin αIIb deficiency strongly suppressed the increase of total inflammatory cells with mastocytosis in the peritoneal cavities. However, administration of single dose FB did not lead to any significant difference of initial inflammatory responses in these mice 24 h after inoculation (data not shown), confirming the negligible role of integrin αIIb in acute inflammation. Taking into consideration that platelets are absent in the peritoneal cavities and that integrin αVβ3 did not significantly affect *in vitro* mast-cell functions through interaction with FB, we concluded that FB-induced chronic inflammation depended on integrin αIIbβ3 in mast cells. The relevant mechanism might be as follows: FB activates macrophages and granulocytes via integrin $\alpha_M\beta2$ to produce inflammatory cytokines and chemokines in the initial phase, leading to the gradual recruitment, proliferation, and activation of mast cells in the presence of FB. Alternatively, activated mast cells also produce a diverse array of chemical mediators, accelerating chronic inflammation. Thus, FB-mediated inflammation appears to be augmented with the increase of mast cells in tissues. This scenario may explain in part why mast cell numbers increase in a variety of chronic inflammatory diseases such as atopic dermatitis and asthma that are thought to cause continuous extravasation of FB in tissues. Further analysis of WT and integrin αIIb-deficient mice using different types of chronic inflammation models will be required to delineate the role of mast cell integrin αIIbβ3 in chronic inflammatory diseases.

Another important finding in this study was that soluble FB enhanced IL-6 production of WT, but not integrin αIIb-deficient, BMMCs in response to *S. aureus* (Cowan I) with FB-binding capacity. On the other hand, soluble FB failed to enhance IL-6 production of WT BMMCs stimulated by *E. coli* harboring no FB-binding capacity, LPS, or bacterial lipopeptide (Fig. 7*B* and data not shown). Because WT, but not integrin αIIb-deficient, BMMCs apparently kept a strong contact with aggregated *S. aureus* (Cowan I) in the presence of soluble FB, integrin αIIbβ3-dependent recognition of *S. aureus* (Cowan I) in mast cells may augment the innate response to this pathogen. Considering that soluble FB facilitates the interaction of platelets with *S. aureus* by bridging clumping factor A in *S. aureus* and integrin αIIbβ3 in platelets (23–25), a similar mechanism probably occurs in mast cells: the complex formation of mast cell integrin αIIbβ3-FB-*S. aureus* (Cowan I) promotes quick and tight recognition of this pathogen by mast cells, thereby enhancing innate immune responses. Collectively, these results suggested that integrin αIIbβ3 plays an important part in the innate responses of mast cells to certain types of bacteria with FB-binding capacity.

In conclusion, the integrin αIIbβ3-dependent interaction of mast cells with FB augments FB-associated chronic inflammation or innate responses to FB-binding bacteria. Elucidation of the *in vivo* function of integrin αIIbβ3 in mast cells will lead to new approaches in the prevention of and therapy for the relevant inflammatory and infectious diseases.

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