Leukemia (2017) **31,** 2468–2478

www.nature.com/leu

ORIGINAL ARTICLE

DPP4 truncated GM-CSF and IL-3 manifest distinct receptor-binding and regulatory functions compared with their full-length forms

HA O'Leary[1,2], M Capitano[1], S Cooper[1], C Mantel[1], HS Boswell[2], R Kapur[3], B Ramdas[3], R Chan[3,4], L Deng[4], C-K Qu[5] and HE Broxmeyer[1]

Dipeptidylpeptidase 4 (DPP4/CD26) enzymatically cleaves select penultimate amino acids of proteins, including colony-stimulating factors (CSFs), and has been implicated in cellular regulation. To better understand the role of DPP4 regulation of hematopoiesis, we analyzed the activity of DPP4 on the surface of immature blood cells and then comparatively assessed the interactions and functional effects of full-length (FL) and DPP4 truncated (T) factors (T-granulocyte–macrophage-CSF (T-GM-CSF)) and T-interleukin-3 (T-IL-3)) on both *in vitro* and *in vivo* models of normal and leukemic cells. T-GM-CSF and -IL-3 had enhanced receptor binding, but decreased CSF activity, compared with their FL forms. Importantly, T-GM-CSF and -IL-3 significantly, and reciprocally, blunted receptor binding and myeloid progenitor cell proliferation activity of both FL-GM-CSF and -IL-3 *in vitro* and *in vivo*. Similar effects were apparent *in vitro* using cluster-forming cells from patients with acute myeloid leukemia regardless of cytogenetic or molecular alterations and *in vivo* using animal models of leukemia. This suggests that DPP4 T-molecules have modified binding and functions compared with their FL counterparts and may serve regulatory roles in normal and malignant hematopoiesis.

Leukemia (2017) **31,** 2468–2478; doi:10.1038/leu.2017.98

KEY POINTS

- DPP4 truncated granulocyte–macrophage colony-stimulating factor (T-GM-CSF) and interleukin-3 (T-IL-3) reciprocally bind with higher affinity to both GM-CSF and IL-3 receptors compared with their full-length (FL) forms.
- DPP4 T-GM-CSF and -IL-3 reciprocally blunt the activities of both FL-GM-CSF and -IL-3 *in vitro* and *in vivo* in primary acute myeloid leukemia patient samples and mouse models of normal/malignant hematopoiesis.

INTRODUCTION

Dipeptidylpeptidase 4 (DPP4/CD26), a serine protease found as membrane-bound and soluble forms, enzymatically cleaves select penultimate amino acids of proteins that regulate multiple aspects of hematopoiesis.[1,2] We reported that DPP4 truncated granulocyte–macrophage colony-stimulating factor (T-GM-CSF) has diminished colony-stimulating activity, and intracellular signaling compared with full-length (FL)-GM-CSF, and blunted the *in vitro* colony-forming effects of FL-GM-CSF.[3] Although a large number of cytokines, chemokines and growth factors (including GM-CSF and IL-3) have previously unrecognized putative DPP4 truncation sites,[1–3] their potential interactive roles in hematopoiesis (including modifying the function of other molecules and acting as regulatory molecules) are poorly understood. Our objective was to clarify the regulatory effects of DPP4 T-GM-CSF and -IL-3 on *in vivo* and *in vitro* modulation of steady- and diseased-state hematopoiesis.

Hematopoietic progenitor (HPCs)/precursor cells from patients with Acute Myeloid Leukemia (AML) respond to cytokines such as GM-CSF and IL-3 for enhanced growth, survival and resistance to therapy.[4–8] GM-CSF and IL-3 have additive to synergistic proliferative effects on normal and malignant cell growth,[9-15] and patients with AML manifest increased serum GM-CSF and IL-3 before induction therapy compared with normal controls, and a decline of GM-CSF and IL-3 after successful remission.[16] GM-CSF and IL-3 share a common receptor β-chain, IL-3 competes for GM-CSF binding on AML blasts,[10] and targeting the IL-3 receptor has been suggested as a useful therapeutic in AML.[16,17] Therefore, it is important to understand how T-GM-CSF and -IL-3 may influence normal and malignant hematopoiesis. We now report that T-GM-CSF and -IL-3 reciprocally blunt receptor binding, as well as functional interactions, of both FL-GM-CSF and -IL-3 *in vitro* in primary human cord blood (CB) HPCs, primary AML HPCs/precursors, and in the human growth factor-dependent TF-1 cell line. Moreover, these blunting effects of T-GM-CSF and -IL-3 were recapitulated *in vivo* in murine models of normal and malignant hematopoiesis, thus demonstrating an additional, and perhaps crucial, layer of cell regulation for normal and leukemic hematopoiesis.

[1]Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN, USA; [2]Department of Medicine, Division of Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN, USA; [3]Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN, USA; [4]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN, USA and [5]Department of Pediatrics, Division of Hematology/Oncology, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Emory University School of Medicine, Atlanta, GA, USA. Correspondence: Dr HA O'Leary, Department of Medicine, Division of Hematology/Oncology, Department of Microbiology/Immunology, 950 West Walnut Street, R2-302, Indianapolis, IN 46202 5181, USA or Dr HE Broxmeyer, Department of Microbiology/Immunology Indiana University School of Medicine, 950 West Walnut Street, R2-302, Indianapolis, IN 46202 5181, USA.
E-mail: haoleary@iupui.edu or hbroxmey@iupui.edu
Received 23 September 2016; revised 24 February 2017; accepted 16 March 2017; accepted article preview online 27 March 2017; advance online publication, 21 April 2017

MATERIALS AND METHODS

Mice, mouse cells, human CB, TF-1 and primary patient AML cells

C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and treated with Diprotin A (DPA; Peptides International, Louisville, KY, USA) or phosphate-buffered saline control as reported.[3] *dpp4*$^{-/-}$,[18] FMS-like tyrosine kinase 3 gene-internal tandem duplication (*FLT3-ITD*)[19] and *PtpnE76K/+LysM-Cre+ (Ptpn11E76K)*[20] mice are all on a C57BL6/J mouse strain background, and have been described previously. Mouse bone marrow (mBM) and human CB (from the Eskenazi (formerly Wishard) Hospital, Indiana University School of Medicine, Indianapolis, IN, USA, and from Cord:Use Cord Blood Bank, Orlando, FL, USA) were used as described.[21] AML patient samples were obtained from peripheral blood

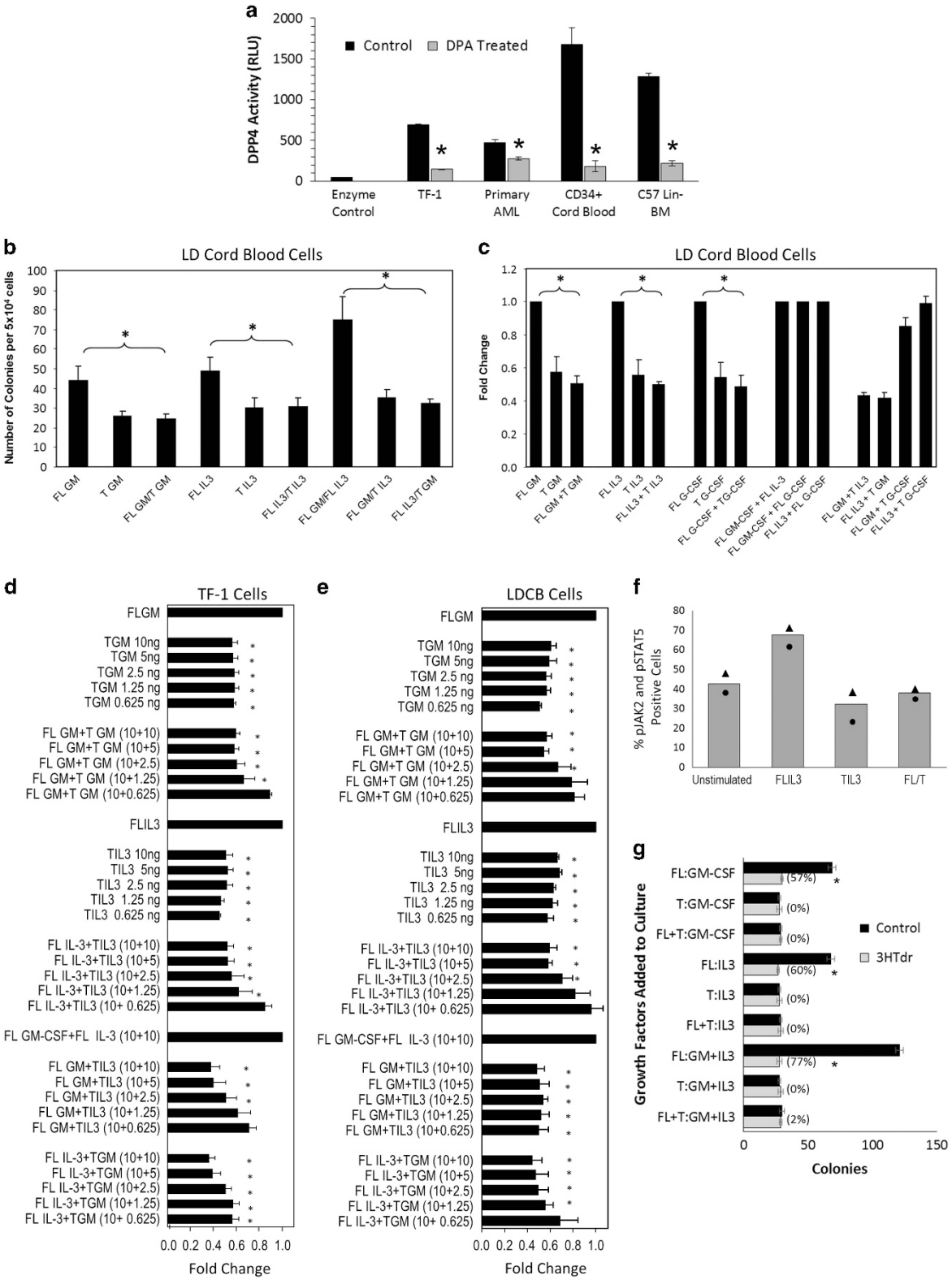

Figure 1. For caption see next page.

samples of patients under an approved institutional review board (9402-10 and 9812-11).

The Indiana University Committee on Use and Care of Animals, and the Indiana University Institutional Review Board, respectively, approved mBM, human CB and AML studies. CB received from Eskenazi (formerly Wishard) Hospital and Cord:Use was to be discarded material. TF-1 cells, originally obtained from T Kitamura, Japan, are available from American Type Culture Collection (CRL-2003) and were used as described.[3,22,23]

HPC colony assay

C57BL6/J, *dpp4*$^{-/-}$, *FLT3/ITD* and *Ptpn11E76K* mice were injected subcutaneously with combinations of 10 μg of recombinant murine (rm) FL- or T-GM-CSF and/or -IL-3. After 24 h, mice were killed, femurs flushed and mBM cells plated at $\sim 5 \times 10^4$ cells per ml in 1% methylcellulose culture medium in the presence of hemin 0.1 mM, 30% fetal bovine serum (Hyclone, Logan, UT, USA) with the following growth factors, unless otherwise noted: 1 U/ml recombinant human (rh) erythropoietin (Amgen Corporation, South San Francisco, CA, USA), 50 ng/ml rm stem cell factor (R&D Systems, Minneapolis, MN, USA) and 5% (vol/vol) pokeweed mitogen mouse spleen cell-conditioned medium.[18,21] Percent HPCs in the S phase of the cell cycle was estimated by high specific activity tritiated thymidine kill technique. Colonies were scored after a 7-day incubation, and colony-forming unit-granulocyte–macrophage, burst-forming unit-erythroid and colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte progenitors distinguished.[21] Human CB cells and primary AML patient samples were separated into a low-density fraction and plated at $\sim 5 \times 10^4$ cells per ml, and TF-1 cells were plated at 250–1000 cells per ml in 0.3% semisolid agar medium (Difco/BD, Franklin Lakes, NJ, USA) with 10 ng/ml (or concentrations listed) of rhFL- or T-GM-CSF or -IL-3. Colony formation for CB was counted on day 14, and colony formation for TF-1, or cluster formation of AML patient samples,[24–26] at days 7–10. CD34+ CB cells (>95% CD34$^+$) were purified.[27] RmGM-CSF and rhGM-CSF, IL-3 and stem cell factor were from R&D Systems. Cell cultures were incubated at lowered (5%) O_2 tension in 5% CO_2 in a humidified chamber.[3,21,27]

DPP4 activity assay

CB, AML patient samples, TF-1 or mBM were harvested and enriched (CD34$^+$) or depleted (Lineage$^-$) using Miltenyi (San Diego, CA, USA) commercially available kits.[3,28] Approximately $1–5 \times 10^5$ cells (untreated or incubated with 5 mM DPA for 30 min) were plated/well in duplicate (N = at least three individual samples or mice), and left intact in 50 μl of phosphate-buffered saline in a 96-well flat-bottom microtiter plate. The assay was carried out by combining cells with 50 μl of the DPP4 substrate Gly-Pro-aminoluciferin and assay buffer optimized for this assay (DPP4-Glo Protease Assay; Promega, Madison, WI, USA). Plates were incubated at 37 °C for 30 min, and surface DPP4 enzyme activity was measured using an LMAX luminometer (Molecular Devices, Sunnyvale, CA, USA).[3]

Flow cytometry

Human TF-1 cells were fixed with 1% formaldehyde after stimulation (with 10 ng/ml rhFL, T or FL:T IL-3), permeabilized using BD Phosflow Perm Buffer III (No. 558050, BD) and stained in BD staining buffer with primary anti-P-Stat5 (BD) and anti-P-Jak2 antibodies (Cell Signaling, Danvers, MA, USA) and secondary antibody (Cell Signaling). mBM was washed in phosphate-buffered saline and 2×10^6 cells were stained with the following phenotyping antibodies to determine hematopoietic stem cell and progenitor populations[29] before fixation (Lineage cocktail (BioLegend), CD34, SCA, KIT, FLT3, Fcγ receptor (BD). All samples were run on BD machines (LSR 4 or Fortessa) and analyzed using the FlowJo Software (FLOWJO, LLC, Ashland, OR, USA).

Generation of DPP4 T-GM-CSF and -IL-3

Soluble human or porcine DPP4, prepared from human placental tissue or porcine kidney, were purchased from MP Biomedicals, LLC or Sigma-Aldrich (No. D7052), respectively, and used at ~0.25 μg for every 1 μg of FL protein per digestion at 37°C for at least 18 h.[3]

Equilibrium receptor binding

Receptor-binding analysis of FL- and T-rhGM-CSF and -IL-3 were carried out under equilibrating conditions, and data were analyzed by Scatchard plotting[3,30,31] using TF-1 cells, CD34$^+$ CB cells and AML primary patient samples. Carrier-free rhGM-CSF and IL-3 (R&D Systems, Minneapolis, MN, USA) were radio-iodinated by chloramine-T method by Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). The [^{125}I]GM-CSF and IL-3 were repurified to a concentration of ~0.67 μg/μl and had a specific radioactivity of ~60 μCi/mmol. The chloramine-T method iodinates on tyrosine residues in proteins; therefore, truncation by DPP4 does not reduce the specific radioactivity of the protein by the enzyme's removal of the N-terminal Ala-Pro. Nonspecific binding was assessed by competing rh [^{125}I]GM-CSF or IL-3 with a 1000-fold excess of unlabeled rhGM-CSF or IL-3. Using TF-1 cells, this results in >95% reduction in non-competed counts per minute of bound ligand, which was near background radiation, thus specific binding under these conditions was considered to be 100% of measured ^{125}I, as determined by Beckman Coulter Gamma 5500B gamma counter (Brea, CA, USA). Linear-regression coefficient of correlation (r^2) was considered acceptable at a value of 0.90 or greater.

Statistical analyses

For all colony assays, three plates per experimental point were scored. Results of colony assays and animal studies were assessed by two-tailed Student's *T*-test or analysis of variance. Receptor-binding studies were assessed by Student's *T*-test. Numbers of experiments per group are noted in the figure legends, and all animal experiments analyzing effects *in vivo* contained at least three mice per group.

RESULTS

Interactions of FL- and T-GM-CSF and -IL-3 on cellular proliferation *in vitro* and *in vivo*

DPP4 is active on the cell surface of immature cells. DPP4 has been shown to be present as an active, membrane-bound form on specific cell types. We detected active DPP4 on the surface of the human growth factor-dependent TF-1 cell line, human CD34$^+$ CB cells, primary AML patient samples and Lineage$^-$ mBM, and this activity was blunted by DPA, an ILE-PRO-ILE DPP4 inhibitor (Figure 1a), on all samples evaluated.[3,18,28]

Figure 1. DPP4 is active on the cell surface of immature blood cells and DPP4 truncated molecules (T-GM-CSF and -IL-3) block colony formation across molecules *in vitro* at less than a 1:1 ratio. (**a**) A total of $1–5 \times 10^5$ cells per well from TF-1, primary AML samples, CD34$^+$ CB and Lineage$^-$ C57BL6/J mBM were analyzed for baseline DPP4 activity. DPA, a DPP4 inhibitor, was added as a control 30 min before the start of the assay (for some samples) to show assay specificity for DPP4 activity on the cell surface and ability to inhibit the DPP4 enzyme. $N = 3$ samples per group was carried out in triplicate for all except for the primary AML samples where $N = 5$ and plating for data shown was lower than other samples ($\sim 1 \times 10^5$/well) due to sample availability. AML samples were from those shown in Table 1. Low-density CB cells (**b**, **c** and **e**) or TF-1 cells (**d**) were stimulated with 10 ng/ml of FL or DPP4 T-GM-CSF, IL-3 or G-CSF, unless otherwise listed. (**f**) TF-1 cells were factor starved and treated with 10 ng/ml of FL, T or 1:1 ratio of IL-3 for 5 min, fixed and assessed for induction of JAK/STAT signaling by flow cytometry ($n = 2$ independent experiments). (**g**) Mouse BM was plated at 5×10^4 cells per ml in the presence of 10 ng/ml rmFL, T or a mixture of FL/T-GM-CSF and/or IL-3 and colony formation and cycling status of the progenitor cells were determined. All experiments contained three mice per group or were performed at least three times (unless noted otherwise) and analysis of variance (ANOVA) was used to determine statistical significance with the exception of 1A (Student's *T*-test). *$P \leqslant 0.05$ compared with control (FL-GM, IL-3 or FL-GM/IL-3, FL-G-CSF and so on). For the combined experiments shown as fold change, the average number of control colonies were: FL-GM = 45, IL-3 = 55, FL-GM/IL-3 = 95 for LDCB and FL-GM = 220, IL-3 = 170, FL-GM/IL-3 = 310 for TF-1.

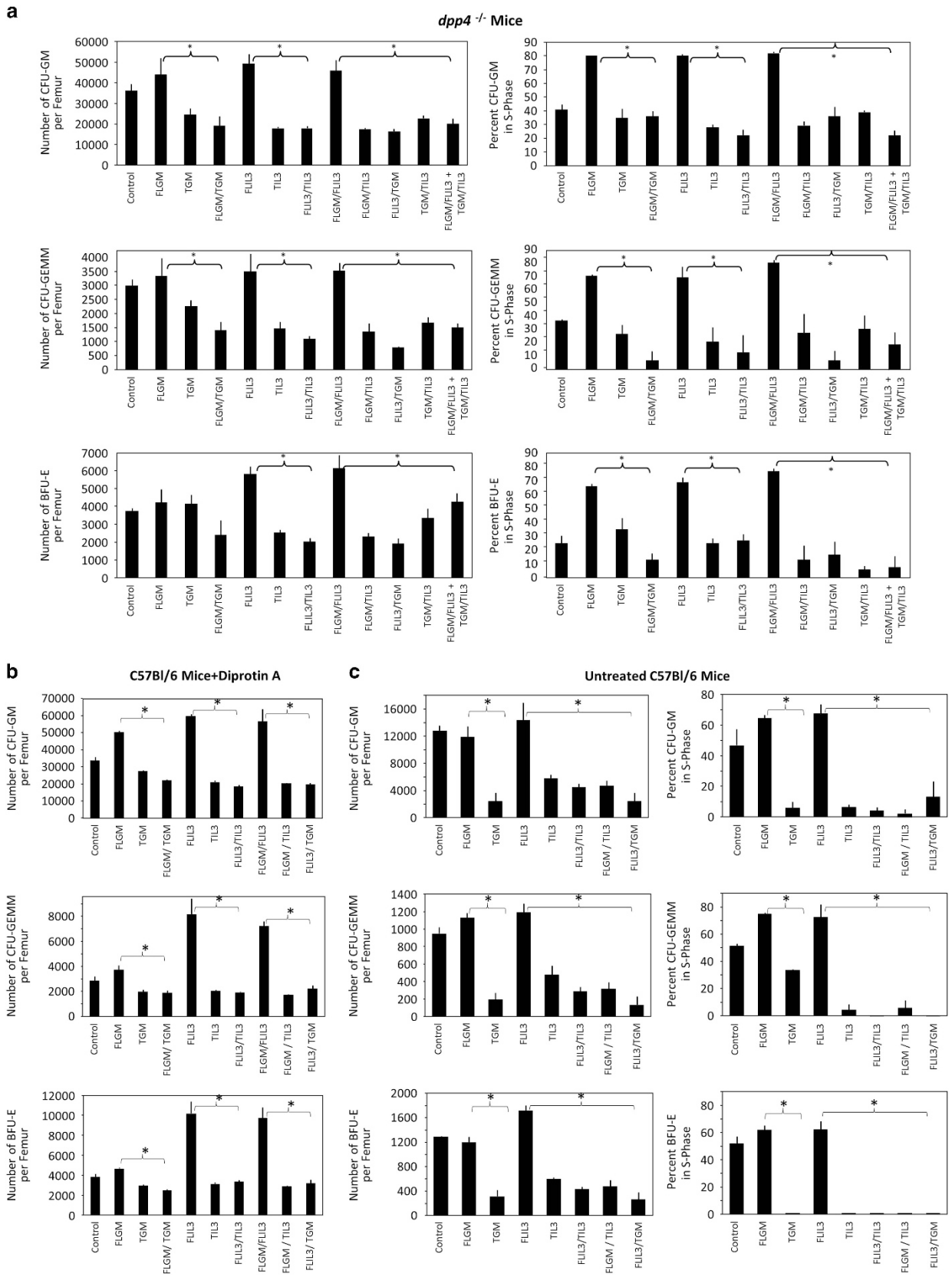

Figure 2. T-GM-CSF and -IL-3 block colony formation across molecules *in vivo*. *dpp4*$^{-/-}$ mice (**a**), C57BL6/J mice treated with DPA (**b**) or untreated C57BL6/J mice (**c**) were injected with 10 μg of either rmFL, T or a combination of FL/T-GM-CSF and/or IL-3 subcutaneously at different sites. Mice were killed 24 h later, femurs were flushed, and colony formation and cycling status of the progenitor cells were determined. *$P \leqslant 0.05$ or less by analysis of variance (ANOVA). $N = 3$ animals per group with each mouse individually accessed. One of two representative experiments shown for *dpp4*$^{-/-}$ mice.

T-GM-CSF and -IL-3 blunt colony-stimulating effects of both FL-GM-CSF or -IL-3 in vitro on human (CB and TF-1) and mouse cells. To delineate the ability of the FL- and T-GM-CSF and -IL-3 molecules to reciprocally modulate each other's function, as well as to better understand the practical relevance of their functions, we analyzed the colony-stimulating activity of these molecules using primary human low-density CB HPCs (Figures 1b and c). This reciprocal interaction was specific for GM-CSF and IL-3, and likely involved the common β-chain they share in their receptors, as DPP4 T-G-CSF (whose receptor does not share the common β-chain) was only able to blunt colony formation induced by FL-G-CSF, but not that of FL-GM-CSF or -IL-3 (Figure 1c). Importantly, the T-factors could be serially diluted to ~12% of the FL amount and still significantly blunt stimulation by either FL-GM-CSF or -IL-3 in both TF-1 (Figure 1d) and CB (Figure 1e) cells. Further, as previously demonstrated for GM-CSF,[3] T-IL-3 induced less activation of the JAK/STAT pathway than FL-IL-3 and blunted the activation induced by FL-IL-3 (Figure 1f). T-GM-CSF and -IL-3 alone or in combination blunted numbers of mBM colonies stimulated by their FL forms and manifested decreased percentages of HPCs in the S phase of the cell cycle (Figure 1g).

Effects of T- and FL-CSFs on HPCs in vivo mimic in vitro effects. To evaluate if the functional data above would be replicated in an *in vivo* situation, we studied effects of mFL- and T-GM-CSF and -IL-3 for effects on the absolute numbers and cycling status of mBM HPCs. We used *dpp4*$^{-/-}$ mice on a C57BL/6 strain background (Figure 2a), C57BL/6 mice that were pretreated with DPA (Figure 2b), or C57BL/6 mice left untreated (Figure 2c). Mice were administered a single subcutaneous dose of rmFL- or T-GM-CSF or -IL-3, or various combinations of FL-, and T-, GM-CSF or -IL-3. FL- vs T-CSFs were injected separately at different sites in the same mouse. In all three animal models, T-GM-CSF and T-IL-3 resulted in less *in vivo* stimulatory activity than their respective FL-CSFs with respect to HPC numbers (colony-forming unit-granulocyte–macrophage, colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte and burst-forming unit-erythroid)/femur and their cell cycling, as assessed by percent of HPCs in the S phase of the cell cycle (Figure 2). Moreover, T-GM-CSF and T-IL-3 blunted effects of both FL-GM-CSF and -IL-3 similar to that seen *in vitro*. T-GM-CSF or T-IL-3 each resulted in *in vivo* suppression of the effects of both FL-GM-CSF and -IL-3 (Figure 2). Most importantly, and impressively, combinations of T-CSFs were able to blunt the increased additive potency of combinations of FL-GM-CSF plus -IL-3. This functional blunting, with respect to HPC numbers/femur and cycling *in vivo,* demonstrates that both T-GM-CSF and T-IL-3 functionally display down-modulating effects on HPC over both FL factors.

Primary progenitor/precursor cells from patients with AML respond *in vitro* to DPP4 T-rhGM-CSF and -IL-3, in a manner similar to normal progenitors

GM-CSF and IL-3 are important in growth and maintenance of AML cells.[6,10,32–35] Modifications in cytogenetic and molecular status, such as ITD mutations in the FLT3/ITD, are common and can alter growth and aggressive progression of disease. Therefore, to understand if HPCs/precursors from patients with AML, with various cytogenetic backgrounds and molecular mutations (Table 1), are sensitive to T-factors in a manner similar to that of normal HPC, we assessed AML patient samples with varied cytogenetic and molecular alterations for responsiveness. Patients with newly diagnosed, or relapsed, AML have varied growth patterns compared with normal cells, and usually form clusters (3–40 cells) rather than colonies (>40 cells) or no colonies or clusters in semisolid agar culture medium.[24–26] In the cases shown (Table 1 and Figures 3a–d), cells from these patients with AML did not grow without CSFs, but formed clusters (~3–20 cells per clone) upon stimulation with either rhFL-GM-CSF or -IL-3, and T-GM-CSF and -IL-3 were each less effective stimulating factors on these cluster-forming cells than their FL forms. The FL-GM-CSF- and -IL-3-stimulated cluster formation of the AML patient samples was reciprocally diminished by either T-GM-CSF or -IL-3 in patient samples tested, regardless of cytogenetic or molecular status. Additionally, for two patient samples tested, and as seen in the CB and TF-1 *in vitro* models (Figure 1), less than a 1:1 ratio of T-CSF to FL-CSF downmodulated maximal stimulation noted with combinations of FL-GM-CSF and -IL-3 (Figures 3c and d, patients 3135 and 3131). Further, cycling analysis showed an identical trend to that seen in the *in vivo* studies evaluating normal HPCs, where T-molecules induced less stimulatory activity than the FL-CSF counterparts with respect to the number of clusters formed and the percent of cells in the S phase of the cell cycle (Figure 3e). This marked suppressive response of AML patient samples in response to the T-factors, regardless of aggressive cytogenetic or molecular alterations, prompted us to compare the extent of the inhibition by fold change in primary CB vs primary AML patient samples. Figure 3f shows that, in general in this small sampling of patient cells, AML samples were significantly more sensitive to the T-factors than the CB cells, regardless of cytogenetic/molecular status, suggesting a possibility for consideration of future use of DPP4 T-factors to downmodulate growth of AML progenitors/precursors. We then assessed effects of T-CSFs *in vivo* in two mouse models of leukemia.

Effects of T- and FL-CSFs in vivo in mouse models of leukemia. AML primary patient samples were sensitive to the FL- and T-factors *in vitro*, regardless of cytogenetic or molecular status. Therefore, to begin to assess the potential clinical utility of T-GM-CSF and -IL-3 in models of malignant hematopoiesis *in vivo*, *FLT3/ITD* mice were first used.[19] Similarly, to *in vivo* experiments with normal mice (Figure 2), *FLT3/ITD* mice were injected with rmFL- and T-GM-CSF and -IL-3, and effects on absolute numbers and cycling status of BM HPCs were analyzed. Mice showed a leukemia phenotype with significantly enhanced numbers of stem and progenitor cells in both the BM and spleen (Supplementary Figures 1A and B, respectively) compared with control. *In vivo* treatment of *FLT3-ITD* mice with rmFL-factors (GM-CSF and IL-3 in combination to maximize stimulation) resulted in functional increases in the number of progenitors (Figures 4a and c) and cycling status (Figures 4b and d), respectively, in the BM and spleen. T-factors (GM-CSF and IL-3 combination) not only reduced numbers and cycling status of HPCs compared with baseline levels of control mice but also significantly blunted the enhanced stimulation obtained with FL-CSFs in both the BM (A and B) and spleen (C and D). Another model of murine leukemia containing an E76K-activating mutation in protein tyrosine phosphatase, non-receptor type 11 (*Ptpn11, Ptpn11E76K*),[20] commonly detected in childhood acute leukemia and myeloproliferative disorders, was also evaluated for response to rmFL- and T-GM-CSF and IL-3. These mice showed a leukemia phenotype with significantly enhanced numbers of progenitors in both the BM and spleen (Supplementary Figures 2A and B, respectively) compared with control. Identical trends to those seen with the *FLT3/ITD* mice were detected with respect to progenitor cell numbers and cycling in the spleen (Figures 4e and f). The BM of the *Ptpn11E76K* mice showed marked reductions in multiple phenotypically and functionally defined cell populations, in response to both FL- and T-factors, such that there was >90% reduction in BM short-term-hematopoietic stem cells, multipotent progenitors, common myeloid progenitors and granulocyte–monocyte progenitors with few or no colony-forming unit-granulocyte–macrophage, burst-forming unit-erythroid or colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte detected in these mice after FL-, T- or FL+T-GM-CSF plus -IL-3 administration (data not shown). These decreases in HPC numbers in BM likely

reflect large mobilization effects, suggesting that these mice may be ultrasensitive to CSF-induced mobilization regardless of treatment with FL- or T-molecules. Data from these two leukemia mouse models suggest that T-CSFs are able to modify growth and cycling of leukemia cells with molecular alterations *in vivo*, which may represent a heretofore unknown mechanism of leukemia cell regulation.

T-GM-CSF and -IL-3 have high receptor binding affinity and compete with FL-GM-CSF and -IL-3 for receptor binding in primary CB and AML cells

To further identify potential mechanistic interactions of T- vs FL-GM-CSF and -IL-3 at a receptor level, receptor equilibrium binding studies were performed (Figure 5). We first used the TF-1 cell line stimulated with rhFL- and T-GM-CSF and -IL-3 that was I^{125} labeled or unlabeled. Both high- and low-affinity binding sites for FL- and T-GM-CSF and -IL-3 were detected by Scatchard analysis (Figure 5). By calculating dissociation constants (K_d), we determined that T-GM-CSF and -IL-3 bind with higher affinity than their FL counterparts, and both truncated forms are better competitors for receptor binding than FL-GM-CSF and -IL-3 (Figure 5a). We also noted, through binding competition studies using pooled $CD34^+$ CB cells (Figure 5b) and multiple primary AML samples (Figure 5c), that the T-GM-CSF and -IL-3 reciprocally competed with and blunted the receptor binding of both their own, as well as the other FL-CSFs.

DISCUSSION

Our studies have now demonstrated that DPP4 exists in an active form on the cell surface of murine as well as immature normal and malignant human cells, and that DPP4 T-GM-CSF and -IL-3 have enhanced receptor binding as well as modified HPC functional regulatory activity compared with their FL counterparts both *in vitro* and *in vivo* for both normal and leukemia progenitors/precursor cells. These results allude to the importance of DPP4, via its regulation of normal and malignant cells by acting as a scaffolding molecule and truncating physiologically and pathologically relevant proteins, thus adding another layer of interactions and complexity to growth modulatory factors and their role in the BM microenvironment. Alterations in receptor binding and signaling after DPP4 truncation is likely not only important for the effects seen on normal and leukemic cells in the settings of GM-CSF and IL-3 that we have elucidated here but also suggests the possibility for broader relevance for the many factors we have recently noted to have DPP4 putative or confirmed T-sites[1–3] and potentially for other proteins with DPP4 T-sites that have not yet been identified. This highlights the critical need to understand how DPP4 T alters receptor binding of individual proteins and how it may alter their ability to act as a negative or positive regulator on normal and malignant hematopoiesis, as well as other steady and disease states outside hematopoiesis.[1–3] Both T-GM-CSF and -IL-3 diminished receptor binding and function of both FL-GM-CSF and -IL-3, at less than a 1:1 ratio, likely through their shared common β-chain receptor. As DPP4 may have a more active role in hematopoiesis under stress conditions,[3] a role for T-proteins in the regulation of hematopoiesis may be even more apparent under stress.

DPP4-specific regulation of GM-CSF and IL-3 via its truncation, and in general as a modulator of CSF signaling and consequently alterations in hematopoietic function, may have potential clinical application. Patients with leukemia may respond to T-factors regardless of molecular alterations or may have altered sensitivity to DPP4 T-factors based on leukemia type, similar to effects seen

Table 1. Characterization of AML primary patient samples based on disease state, cytogenic/molecular phenotype and utilization

Patient number	*De novo or relapse*	*Cytogenetic/molecular alterations*	*Experimental use*
3011	*De novo*	inv 16	Yes (cycling)
3099	*De novo*	FLT3-ITD	N/A receptor binding only[a]
3127	*De novo*	t(9;22) (q34;q11.2)	Yes[a]
3130	*De novo*	Normal karyotype, FLT3-TKD	Yes[a]
3131	*De novo*	7q− FLT3-ITD	Yes[a] (cycling)
3135	*De novo*	inv 3(q21;q26) [Evl 1 activation]	Yes[a]
3136	*De novo*	Normal karyotype, FLT3-TKD, Mnpm1	No[a]
3142	Relapse	T(12;15) (Q13,Q11.2), FLT3-ITD	N/A receptor binding only[a]
3146	*De novo*	46(X;X) [13 metaphases]; t(1;13) (p32;q12) [8 metaphases] dmut CEBPA, and negative FLT3-ITD/TKD, negative NPM1	[b]
3152	Relapse	Normal karyotype (46[X,Y]), $NPM1^-$, $CEBPA^-$, $FLT3\text{-}ITD^-$, FLT3 $D835^+$	[b]
3155	*De novo*	Complex, with monosomy 7, negative for FLT3-ITD/TKD and negative for NPM1	[b]
3157	*De novo*	46(X;X)-negative FLT3-ITD, neg NPM1, neg CEBPA	[b]
3159	*De novo*	Normal karyotype; $FLT3\text{-}ITD^+$, $NPM1^-$, $CEBPA^-$	[b]
3224	Relapse	Normal karyotype, [46(X,Y)], WT 118%	Yes (cycling)[b]

Abbreviations: AML, acute myeloid leukemia; CEBPA, CCAAT/enhancer-binding protein alpha; DPP4, dipeptidylpeptidase 4; FLT, FMS-like tyrosine kinase; ITD, internal tandem duplication; N/A, not applicable; NPM1, nucleophosmin 1; WT, wild type. Yes or No refers to growth in culture, 'cycling' refers to being used in cycling assays. Peripheral blood samples were collected from patients with AML. Samples determined to contain 90% or greater blasts were used for studies and patient information based on disease state, as well as Cytogenetic and molecular alterations is included. [a]Designates use for receptor-binding studies. [b]Designates use in DPP4 activity assay.

Figure 3. DPP4 truncation of GM-CSF and IL-3 inhibit AML cluster formation stimulated by FL-CSFs. (**a**–**d**) A total of 5×10^4 cells from ficolled peripheral blood of AML patients with 90% or greater blast burden (Table 1) were plated in agar with 10 ng/ml (unless otherwise noted) of rhFL- and/or T-GM-CSF and/or IL-3. Cells were plated in triplicate and incubated at 37°C/5% O_2 for 7–10 days before enumeration of clusters formed. (**e**) Cell cycling evaluation of AML patient samples ($n = 3$, 5×10^4) treated with FL- or T-factors. (**f**) Fold-change comparison of CB response to DPP4 T-factors compared with AML response. $N = 4$ for AML samples and $N = 7$ for LDCB $^*P \leqslant 0.05$ or less by analysis of variance (ANOVA) compared with control (FL-GM, FL3 or FL-GM/FL3).

in our sampling of AML patient cells and the two *in vivo* models of leukemia assessed in our present studies. The knowledge we have gained in this research can possibly be used for investigation of the potential use of DPP4 T-proteins for future treatment of leukemia patients. This will require further mechanistic and therapeutic insight into the actions of T- vs FL-proteins in terms

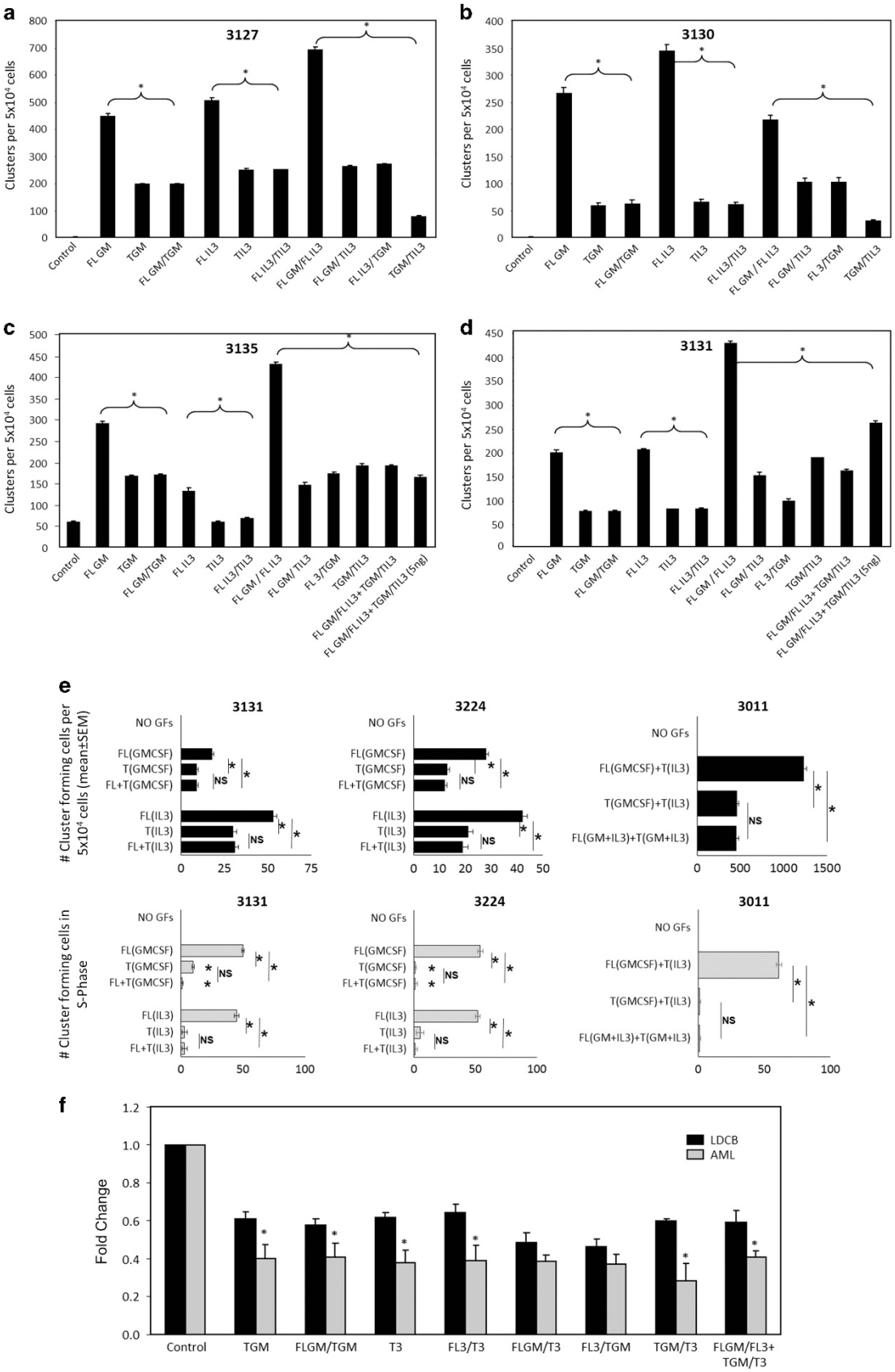

Figure 3. For caption see previous page.

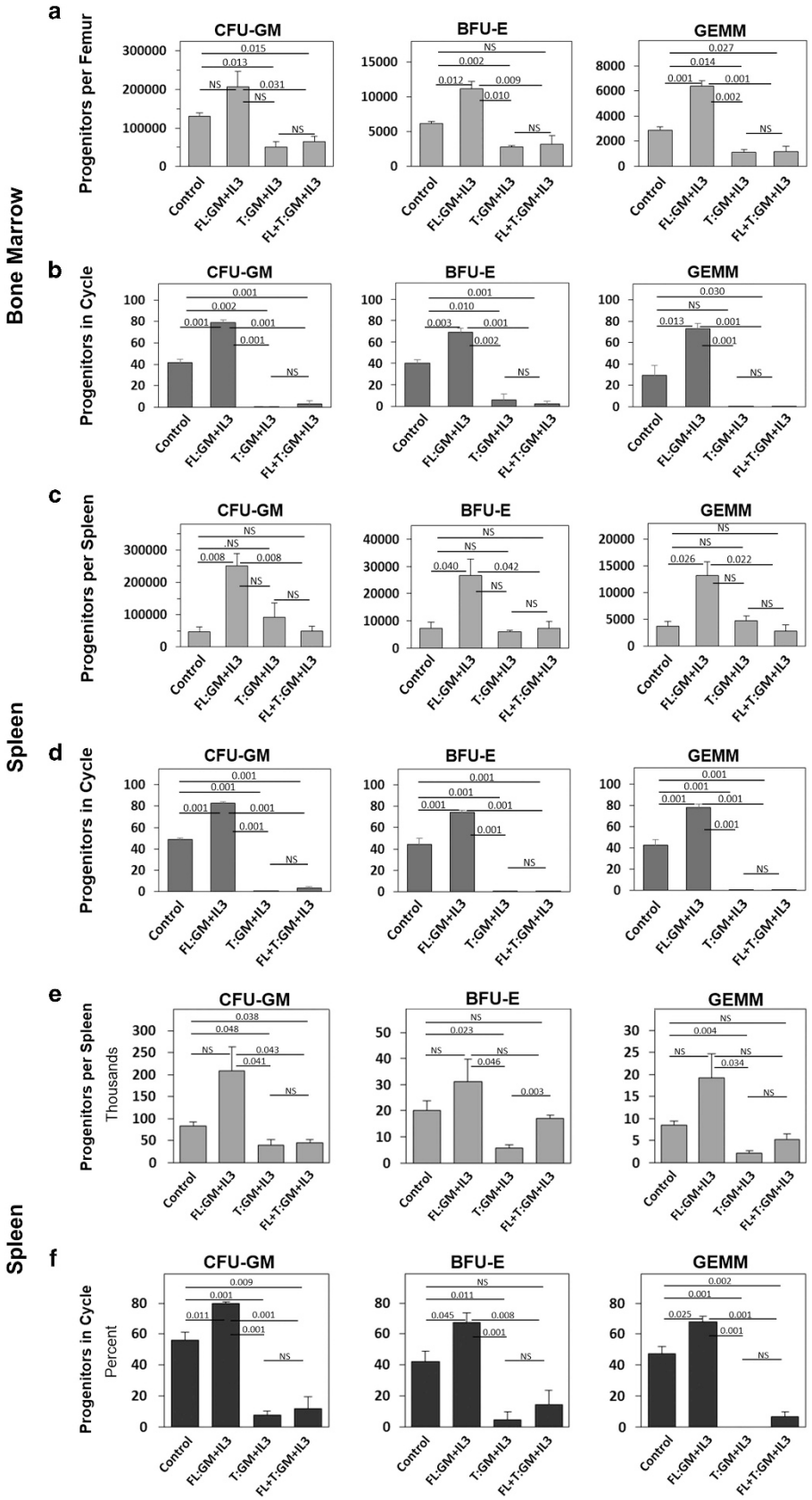

Figure 4. Effects of T- and FL-CSFs on *in vivo* model of leukemia. *FLT3-ITD* (**a**–**d**) and *Ptpn11E76K* (**e** and **f**) mice were injected with 10 μg of a combination of either rmFL-GM-CSF and FL-IL-3, T-GM-CSF and T-IL-3 or a mixture of both FL- and T- (GM-CSF and IL-3) subcutaneously at different sites. Mice were killed 24 h later, femurs flushed, spleens made into single-cell suspensions and colony formation (**a**, **c** and **e**) and cycling status (**b**, **d** and **f**) of the progenitor cells were determined.

of what overlapping and distinct intracellular signals they elicit, and more in-depth analysis of *in vivo* mouse models of leukemia to determine the effects on disease progression/relapse and animal survival.

Importantly, current assessment of protein levels, for the most part, do not distinguish between the FL- and DPP4 T-forms of these proteins and hence assessments of proteins by conventional methods such as enzyme-linked immunosorbent assay, bioplex

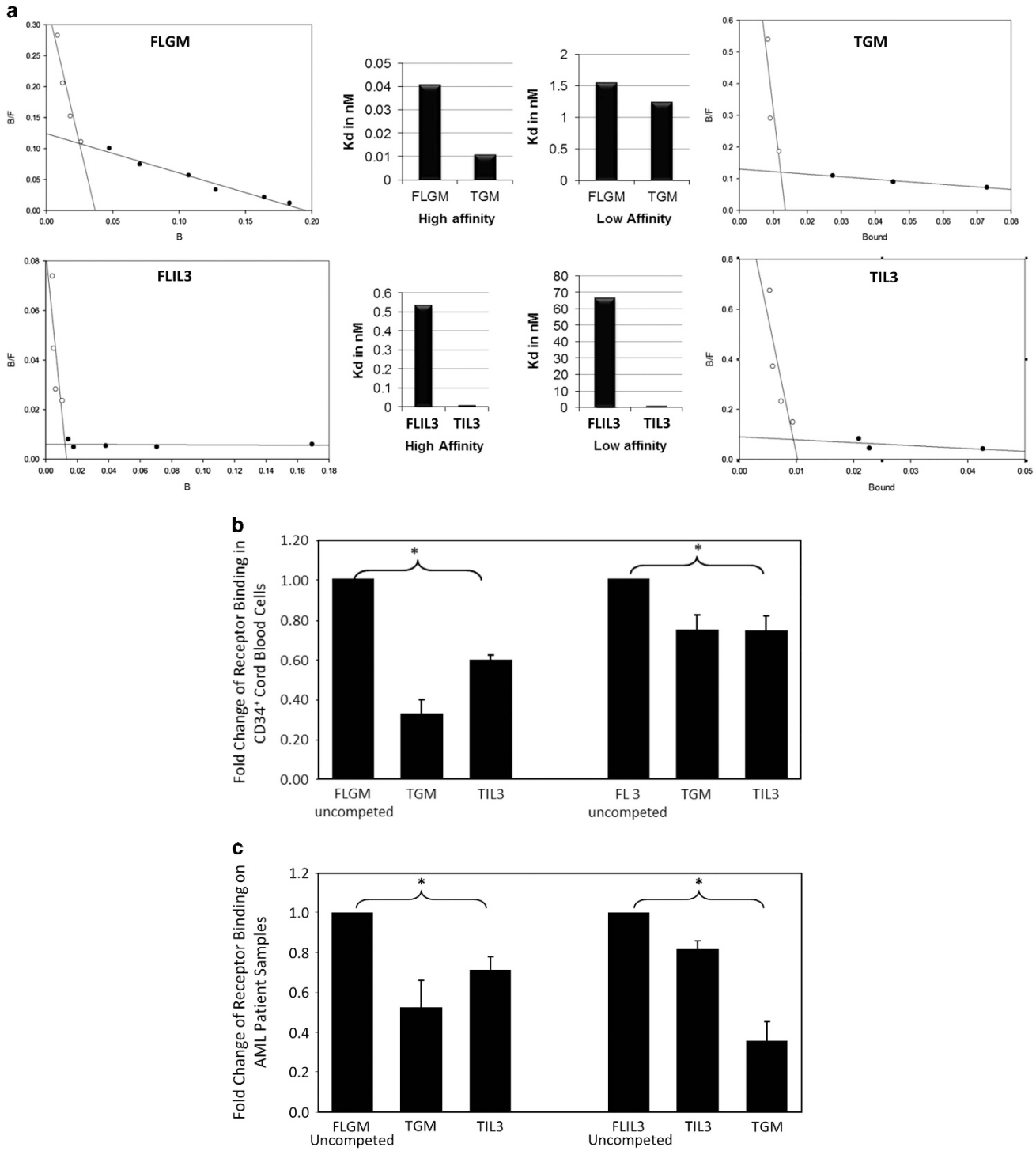

Figure 5. T-IL-3 and -GM-CSF bind with greater affinity than FL-IL-3 or -GM-CSF, and T-IL-3 and -GM-CSF blunt receptor binding of both FL-IL-3 and -GM-CSF. (**a**) Scatchard analysis and K_d determination of TF-1($n=2$) was performed with I^{125}-labeled IL-3. A total of 1×10^6 TF-1 cells were plated per well, and iodinated FL- or T-IL-3 was added for 1 h. Cells were harvested and levels of I^{125}-labeled ligand bound were detected using a Beckman Coulter Gamma 5500B and low- and high-affinity binding sites, as well as K_d were derived as published previously.[3] Pooled $CD34^+$ CB (**b**) or primary AML patient samples (**c**) were incubated with I^{125}-labeled FL-IL-3 or -GM-CSF alone or in addition to cold T-IL-3 and -GM-CSF in excess for 1 h. Cells were harvested and the counts per minute (CPM) (or levels of I^{125}-labeled ligand bound) were detected using a Beckman Coulter Gamma 5500B to analyze the ability of T-IL-3 and -GM-CSF to compete with FL-IL-3 and -GM-CSF. *$P\leqslant0.05$ AML patient samples $n=7$ distinct samples; CB $n=5$ independent experiments with $CD34^+$ cells pulled from multiple CBs for each experiment.

and other methods that use antibodies that do not distinguish the FL- from the T-forms of the proteins therefore may not be fully revealing in terms of the specific physiology or biological activities associated with these molecules. To that end, the possibility exists that functional outcomes that have been previously attributed to FL-molecules may, in fact, actually be due to T-molecules or a mixture of activities between the FL- and T-molecules. Thus, efforts to develop antibodies that can distinguish DPP4 T- from FL-proteins may be of practical, scientific and potentially clinical value.

CONFLICT OF INTEREST

HEB is a member of the Medical Scientific Advisory Board (MSAB) of Corduse, a cord blood banking company.

ACKNOWLEDGEMENTS

These studies were supported by (1) US Public Health Service Grants from the National Institutes of Health: R01HL112669, R01HL67384, R01HL56416, P01DK90948 and U54DK106846 (to HEB) and (2) Indiana University Bioinformatics Core Proposal Grant (to HAO). HAO and MC were supported by NIH T32DK07519 (HEB). We thank Giao Hangoc for help with some of the experiments and Heath D Skinner for clinical insight.

AUTHOR CONTRIBUTIONS

HAO provided concepts, designed and performed experiments and wrote the manuscript. CM, SC and MC performed experiments, HSB provided clinical samples/knowledge, RK, BR, RC, LD and CKQ provided mouse models of leukemia and insight into their use. HEB provided concepts, designed and performed experiments and helped in writing and editing of the manuscript. All authors read the manuscript and provided feedback for clarity and context.

REFERENCES

1 Ou X, O'Leary HA, Broxmeyer HE. Implications of DPP4 modification of proteins that regulate stem/progenitor and more mature cell types. *Blood* 2013; **122**: 161–169.
2 O'Leary H, Ou X, Broxmeyer HE. The role of dipeptidyl peptidase 4 in hematopoiesis and transplantation. *Curr Opin Hematol* 2013; **20**: 314–319.
3 Broxmeyer HE, Hoggatt J, O'Leary HA, Mantel C, Chitteti BR, Cooper S *et al.* Dipeptidylpeptidase 4 negatively regulates colony-stimulating factor activity and stress hematopoiesis. *Nat Med* 2012; **18**: 1786–1796.
4 Favreau AJ, Sathyanarayana P. miR-590-5p, miR-219-5p, miR-15b and miR-628-5p are commonly regulated by IL-3, GM-CSF and G-CSF in acute myeloid leukemia. *Leuk Res* 2012; **36**: 334–341.
5 Miller PH, Cheung AM, Beer PA, Knapp DJ, Dhillon K, Rabu G *et al.* Enhanced normal short-term human myelopoiesis in mice engineered to express human-specific myeloid growth factors. *Blood* 2013; **121**: e1–e4.
6 Riccioni R, Diverio D, Riti V, Buffolino S, Mariani G, Boe A *et al.* Interleukin (IL)-3/granulocyte macrophage-colony stimulating factor/IL-5 receptor alpha and beta chains are preferentially expressed in acute myeloid leukaemias with mutated FMS-related tyrosine kinase 3 receptor. *Br J Haematol* 2009; **144**: 376–387.
7 Schiller GJ. High-risk acute myelogenous leukemia: treatment today... and tomorrow. *Am Soc Hematol Educ Program* 2013; **2013**: 201–208.
8 Thomas D, Powell JA, Green BD, Barry EF, Ma Y, Woodcock J *et al.* Protein kinase activity of phosphoinositide 3-kinase regulates cytokine-dependent cell survival. *PLoS Biol* 2013; **11**: e1001515.
9 Broughton SE, Dhagat U, Hercus TR, Nero TL, Grimbaldeston MA, Bonder CS *et al.* The GM-CSF/IL-3/IL-5 cytokine receptor family: from ligand recognition to initiation of signaling. *Immunol Rev* 2012; **250**: 277–302.
10 Onetto-Pothier N, Aumont N, Haman A, Park L, Clark SC, De Lean A *et al.* IL-3 inhibits the binding of GM-CSF to AML blasts, but the two cytokines act synergistically in supporting blast proliferation. *Leukemia* 1990; **4**: 329–336.
11 Taketazu F, Chiba S, Shibuya K, Kuwaki T, Tsumura H, Miyazono K *et al.* IL-3 specifically inhibits GM-CSF binding to the higher affinity receptor. *J Cell Physiol* 1991; **146**: 251–257.
12 Broxmeyer HE, Williams DE, Cooper S, Shadduck RK, Gillis S, Waheed A *et al.* Comparative effects in vivo of recombinant murine interleukin 3, natural murine colony-stimulating factor-1, and recombinant murine granulocyte–macrophage colony-stimulating factor on myelopoiesis in mice. *J Clin Invest* 1987; **79**: 721–730.
13 Broxmeyer HE, Williams DE, Hangoc G, Cooper S, Gillis S, Shadduck RK *et al.* Synergistic myelopoietic actions in vivo after administration to mice of combinations of purified natural murine colony-stimulating factor 1, recombinant murine interleukin 3, and recombinant murine granulocyte/macrophage colony-stimulating factor. *Proc Natl Acad Sci USA* 1987; **84**: 3871–3875.
14 Williams DE, Straneva JE, Cooper S, Shadduck RK, Waheed A, Gillis S *et al.* Interactions between purified murine colony-stimulating factors (natural CSF-1, recombinant GM-CSF, and recombinant IL-3) on the in vitro proliferation of purified murine granulocyte-macrophage progenitor cells. *Exp Hematol* 1987; **15**: 1007–1012.
15 Williams DE, Straneva JE, Shen RN, Broxmeyer HE. Purification of murine bone-marrow-derived granulocyte-macrophage colony-forming cells. *Exp Hematol* 1987; **15**: 243–250.
16 Elbaz O, Shaltout A. Implication of granulocyte–macrophage colony stimulating factor (GM-CSF) and interleukin-3 (IL-3) in children with acute myeloid leukaemia (AML); malignancy. *Hematology* 2001; **5**: 383–388.
17 Jordan CT, Upchurch D, Szilvassy SJ, Guzman ML, Howard DS, Pettigrew AL *et al.* The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells. *Leukemia* 2000; **14**: 1777–1784.
18 Christopherson KW II, Hangoc G, Mantel CR, Broxmeyer HE. Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science* 2004; **305**: 1000–1003.
19 Lee BH, Tothova Z, Levine RL, Anderson K, Buza-Vidas N, Cullen DE *et al.* FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in a murine model of chronic myelomonocytic leukemia. *Cancer Cell* 2007; **12**: 367–380.
20 Xu D, Liu X, Yu WM, Meyerson HJ, Guo C, Gerson SL *et al.* Non-lineage/stage-restricted effects of a gain-of-function mutation in tyrosine phosphatase Ptpn11 (Shp2) on malignant transformation of hematopoietic cells. *J Exp Med* 2011; **208**: 1977–1988.
21 Broxmeyer HE, Orschell CM, Clapp DW, Hangoc G, Cooper S, Plett PA *et al.* Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* 2005; **201**: 1307–1318.
22 Gotoh A, Takahira H, Mantel C, Litz-Jackson S, Boswell HS, Broxmeyer HE. Steel factor induces serine phosphorylation of Stat3 in human growth factor-dependent myeloid cell lines. *Blood* 1996; **88**: 138–145.
23 Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miyagawa K *et al.* Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J Cell Physiol* 1989; **140**: 323–334.
24 Broxmeyer HE, Grossbard E, Jacobsen N, Moore MA. Evidence for a proliferative advantage of human leukemia colony-forming cells *in vitro*. *J Natl Cancer Inst* 1978; **60**: 513–521.
25 Broxmeyer HE, Grossbard E, Jacobsen N, Moore MA. Persistence of inhibitory activity against normal bone-marrow cells during remission of acute leukemia. *N Engl J Med* 1979; **301**: 346–351.
26 Moore MA, Spitzer G, Williams N, Metcalf D, Buckley J. Agar culture studies in 127 cases of untreated acute leukemia: the prognostic value of reclassification of leukemia according to *in vitro* growth characteristics. *Blood* 1974; **44**: 1–18.
27 Broxmeyer HE, Lee MR, Hangoc G, Cooper S, Prasain N, Kim YJ *et al.* Hematopoietic stem/progenitor cells, generation of induced pluripotent stem cells, and isolation of endothelial progenitors from 21- to 23.5-year cryopreserved cord blood. *Blood* 2011; **117**: 4773–4777.
28 Christopherson KW II, Hangoc G, Broxmeyer HE. Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. *J Immunol* 2002; **169**: 7000–7008.
29 Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell Stem Cell* 2012; **10**: 120–136.
30 Niu L, Golde DW, Vera JC, Heaney ML. Kinetic resolution of two mechanisms for high-affinity granulocyte–macrophage colony-stimulating factor binding to its receptor. *Blood* 1999; **94**: 3748–3753.
31 Woodcock JM, Bagley CJ, Zacharakis B, Lopez AF. A single tyrosine residue in the membrane-proximal domain of the granulocyte–macrophage colony-stimulating factor, interleukin (IL)-3, and IL-5 receptor common beta-chain is necessary and sufficient for high affinity binding and signaling by all three ligands. *J Biol Chem* 1996; **271**: 25999–26006.
32 Lemoli RM, Gulati SC, Strife A, Lambek C, Perez A, Clarkson BD. Proliferative response of human acute myeloid leukemia cells and normal marrow enriched

progenitor cells to human recombinant growth factors IL-3, GM-CSF and G-CSF alone and in combination. *Leukemia* 1991; **5**: 386–391.

33 Feuring-Buske M, Gerhard B, Cashman J, Humphries RK, Eaves CJ, Hogge DE. Improved engraftment of human acute myeloid leukemia progenitor cells in beta 2-microglobulin-deficient NOD/SCID mice and in NOD/SCID mice transgenic for human growth factors. *Leukemia* 2003; **17**: 760–763.

34 Vellenga E, Ostapovicz D, O'Rourke B, Griffin JD. Effects of recombinant IL-3, GM-CSF, and G-CSF on proliferation of leukemic clonogenic cells in short-term and long-term cultures. *Leukemia* 1987; **1**: 584–589.

35 Wunderlich M, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M *et al.* AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia* 2010; **24**: 1785–1788.

Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)