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DPP4 Truncated GM-CSF & IL-3 Manifest Distinct Receptor Binding & Regulatory Functions Compared to their Full Length Forms

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Abstract

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 factors [(T)-GM-CSF and- IL-3] on both *in vitro* and *in vivo* models of normal and leukemic cells. T-GM-CSF and T-IL-3 had enhanced receptor binding, but decreased CSF activity, compared to their FL forms. Importantly, T-GM-CSF and T-IL-3 significantly, and reciprocally, blunted receptor binding and myeloid progenitor cell proliferation activity of both FL-GM-CSF and FL-IL-3 *in vitro* and *in vivo*. Similar effects were apparent *in vitro* using cluster forming cells from patients with Acute

Myeloid Leukemia (AML) regardless of cytogenetic or molecular alterations and *in vivo* utilizing animal models of leukemia. This suggests that DPP4 T-molecules have modified binding and functions compared to their FL counterparts and may serve regulatory roles in normal and 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 (T)-GM-CSF has diminished colony stimulating activity, and intracellular signaling compared to 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 truncated GM-CSF and IL-3 on *in vivo* and *in vitro* modulation of steady and diseased state hematopoiesis.

Hematopoietic progenitor (HPC)/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 to normal controls, and a decline of GM-CSF and IL-3 after successful remission.[16] GM-CSF and IL-3 share a common receptor beta-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 T-IL-3 may influence normal and malignant hematopoiesis. We now report that T-GM-CSF and T-IL-3 reciprocally blunt receptor binding, as well as functional interactions, of both FL-GM-CSF and FL-IL-3 *in vitro* in primary human cord blood (CB) HPC, primary AML HPC/precursors, and in the human growth factor dependent TF-1 cell line. Moreover, these blunting effects of T-GM-CSF and T-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.

Materials and Methods

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

C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and treated with Diprotin A (Peptides International; Louisville, KY) or PBS control as reported.[3] *dpp4*[−/−18], *FLT3-ITD*[19], and *PtpnE76K/+LysM-Cre+ (Ptpn11E76K)*[20] mice are all on a C57BL6/J mouse strain background, and have been previously described. Mouse bone marrow (mBM) and human cord blood [(CB) from the Eskenazi (formerly Wishard) Hospital, Indiana University School of Medicine, Indianapolis, Indiana, USA, and from

Cord:Use Cord Blood Bank, Orlando, Florida, USA] were used as described.[21] AML patient samples were obtained from peripheral blood samples of patients under an approved IRB (9402–10 & 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 was from 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*^{−/−}, *FLT-3/ITD*, and *Ptpn11E76K* mice were injected s.c. with combinations of 10ug of recombinant murine (rm) FL- or T- GM-CSF and/or -IL-3. After 24 hours mice were sacrificed, femurs flushed, and mBM cells plated at ~5×10^4 cells/ml in 1% methylcellulose culture medium in the presence of hemin 0.1 mM, 30% FBS (Hyclone, Utah) with the following growth factors, unless otherwise noted: 1 U/ ml recombinant human (rh) erythropoietin (Amgen Corporation, South San Francisco, CA), 50 ng/ml rmSCF (R&D Systems, Minneapolis, MN), and 5% vol/vol pokeweed mitogen mouse spleen cell–conditioned medium (PWMSCM).[18, 21] Percent HPCs in 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 CFU-GM, BFU-E and CFU-GEMM progenitors distinguished.[21] Human CB cells and primary AML patient samples were separated into a low-density fraction and plated at ~5×10^4 cells/ml, and TF-1 cells were plated at 250–1,000 cells/ml in 0.3% semisolid agar medium (Difco) with 10ng/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 day 7–10. CD34+ CB cells (>95% CD34^{+}) were purified.(27) Rm and rhGM-CSF, IL-3, and SCF 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) commercially available kits.[3, 28] Approximately 1-5×10^5 cells (untreated or incubated with 5mM DiprotinA for 30 minutes) were plated/ well in duplicate (N= at least 3 individual samples or mice), and left intact in 50ul of PBS 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 10ng/ml rhFL, T or FL:T IL-3), permeabilized using BD Phosflow Perm Buffer III (#558050) and stained in BD staining buffer with primary anti-P-Stat5 (BD) and anti-P-Jak2 antibodies (Cell

Signaling) and secondary antibody (Cell signaling). Mouse bone marrow was washed in PBS and 2×10^6 cells were stained with the following phenotyping antibodies to determine HSC and progenitor populations[29] prior to fixation [Lineage cocktail (Biolegend,), CD34, SCA, KIT, FLT3, Fcgamma receptor (BD)]. All samples were run on BD machines (LSR 4 or Fortessa) and analyzed using FlowJo Software.

Generation of DPP4 truncated (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 (#D7052), respectively, and used at approximately 0.25ug for every 1ug of full-length protein per digestion at 37°C for at least 18 hours.[3]

Equilibrium Receptor Binding

Receptor binding analysis of FL- and T- rh GM-CSF and IL-3 were done under equilibrating conditions, and data 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) were radio-iodinated by chloramine-T method by Phoenix Pharmaceuticals, Inc. (Burlingame, CA). The [125I]-GM-CSF and IL-3 were re-purified to a concentration of approximately 0.67ug/ul and had a specific radioactivity of ~60 uCi/mmole. 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[125I]-GM-CSF or IL-3 with a 1,000-fold excess of unlabeled rhGM-CSF or IL-3. Using TF-1 cells, this results in greater than 95% reduction in non-competed c.p.m. of bound ligand, which was near background radiation, so specific binding under these conditions was considered to be 100% of measured 125I, as determined by Beckman Coulter Gamma 5500B gamma counter (Brea, CA). Linear-regression coefficient of correlation (r2) 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 students T test or Analysis of Variance (ANOVA). 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 3 mice per group.

Results

A.) Interactions of FL- and T-GM-CSF and –IL-3 on Cellular Proliferation *in vitro* and *in vivo*

Ai.) 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 negative mBM and this activity was blunted by Diprotin A (DPA), an ILE-PRO-ILE DPP4 inhibitor (Figure 1A) on all samples evaluated.[3, 18, 28]

Aii.) T-GM-CSF and T-IL-3 blunt colony stimulating effects of both FL-GM-CSF or FL-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 (Figure 1B and 1C). This reciprocal interaction was specific for GM-CSF and IL-3, and likely involved the common beta chain they share in their receptors, as DPP4 T-G-CSF (whose receptor does not share the common beta-chain) was only able to blunt colony formation induced by FL-G-CSF, but not that of FL-GM-CSF or FL-IL-3 (Figure 1C). Importantly, the T-factors could be serially diluted to approximately 12% of the FL amount and still significantly blunt stimulation by either FL-GM-CSF or FL-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 T-IL-3 alone or in combination blunted numbers of mBM colonies stimulated by their FL forms and manifested decreased percentages of HPCs in S-phase of the cell cycle (Figure 1G).

Aiii) Effects of T- and FL- CSFs on HPC *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 m FL- 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 pre-treated with DPA (Figure 2B), or C57BL/6 mice left untreated (Figure 2C). Mice were administered a single s.c. 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 3 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 (CFU-GM, CFU-GEMM, and BFU-E)/femur and their cell cycling, as assessed by percent of HPC in S-Phase of the cell cycle (Figure 2). Moreover, T-GM-CSF and T-IL-3 blunted effects of both FL-GM-CSF and FL-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 FL-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 FL-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.

B) Primary progenitor/precursor cells from patients with AML respond *in vitro* to DPP4 T-rhGM-CSF and T-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 internal tandem duplication (ITD) mutations in the FMS-like tyrosine kinase 3 gene (FLT3/ITD), are common and can alter growth and aggressive progression of disease. Therefore, to understand if HPC/precursors from patients with AML, with various cytogenetic backgrounds and molecular mutations (Table 1), are sensitive to T- factors in a fashion 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 to normal cells, and usually form clusters (3-40 cells) rather than colonies (>40 cells) or no colonies or clusters in semi-solid agar culture medium.[24–26] In the cases shown (Table 1, Figure 3 A–D), cells from these patients with AML did not grow without CSFs, but formed clusters (~3-20 cells/clone) upon stimulation with either rhFL-GM-CSF or FL-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 FL-IL-3 stimulated cluster formation of the AML patient samples was reciprocally diminished by either T-GM-CSF or T-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 down-modulated maximal stimulation noted with combinations of FL-GM-CSF and FL-IL-3 (Figure 3 C and D, patients 3135 and 3131). Further, cycling analysis showed an identical trend to that seen in the *in vivo* studies evaluating normal hematopoietic progenitors, where T- molecules induced less stimulatory activity than the FL-CSFs counterparts with respect to numbers of clusters formed and percent of cells in S-Phase of the cell cycle (Figure 3E). This dramatic 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 down-modulate growth of AML progenitors/precursors. We then assessed effects of T-CSFs *in vivo* in two mouse models of leukemia.

Bi) 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*, *FLT-3/ITD* mice were first utilized.[19] Similarly, to *in vivo* experiments with normal mice (Figure 2), *FLT-3/ITD* mice were injected with rm FL- 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 bone marrow and spleen (Supplemental Figure 1A and B, respectively) compared to 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 numbers of progenitors (Figure 4A and C), and cycling status (Figure 4B and D), respectively in BM and spleen. T-factors (GM-CSF and IL-3 combination) not only reduced numbers and cycling status of HPCs compared to baseline levels of control mice but also significantly blunted the enhanced stimulation obtained with FL-CSFs in both the bone marrow (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 bone marrow and spleen

(Supplemental Figure 2A and B, respectively) compared to control. Identical trends to those seen with the *FLT-3/ITD* mice were detected with respect to progenitor cell numbers and cycling in the spleen (Figure 4E and F). The BM of the *Ptpn11E76K* mice showed dramatic 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 ST-HSCs, MPPs, CMPs and GMPs with few or no CFU-GM, BFU-E or CFU-GEMM 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 ultra-sensitive 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.

C) 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 utilized 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 (Kd), we determined that T-GM-CSF and T-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 T-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 to 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

of 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. Since DPP4 may play a more active role in hematopoiesis under stress conditions,[3] a role for T- proteins in 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 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 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 ELISA, bioplex, and other methods that use antibodies that do not distinguish the FL- from the T- forms of the proteins therefore may not be fully telling 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

- DPP4 truncated (T) GM-CSF & IL-3 reciprocally bind with higher affinity to both GM-CSF & IL-3 receptors compared to their full length (FL) forms.

- DPP4 truncated (T) GM-CSF & IL-3 reciprocally blunt the activities of both full length (FL) GM-CSF & IL-3 *in vitro* and *in vivo* in primary AML patient samples and mouse models of normal/malignant hematopoiesis.
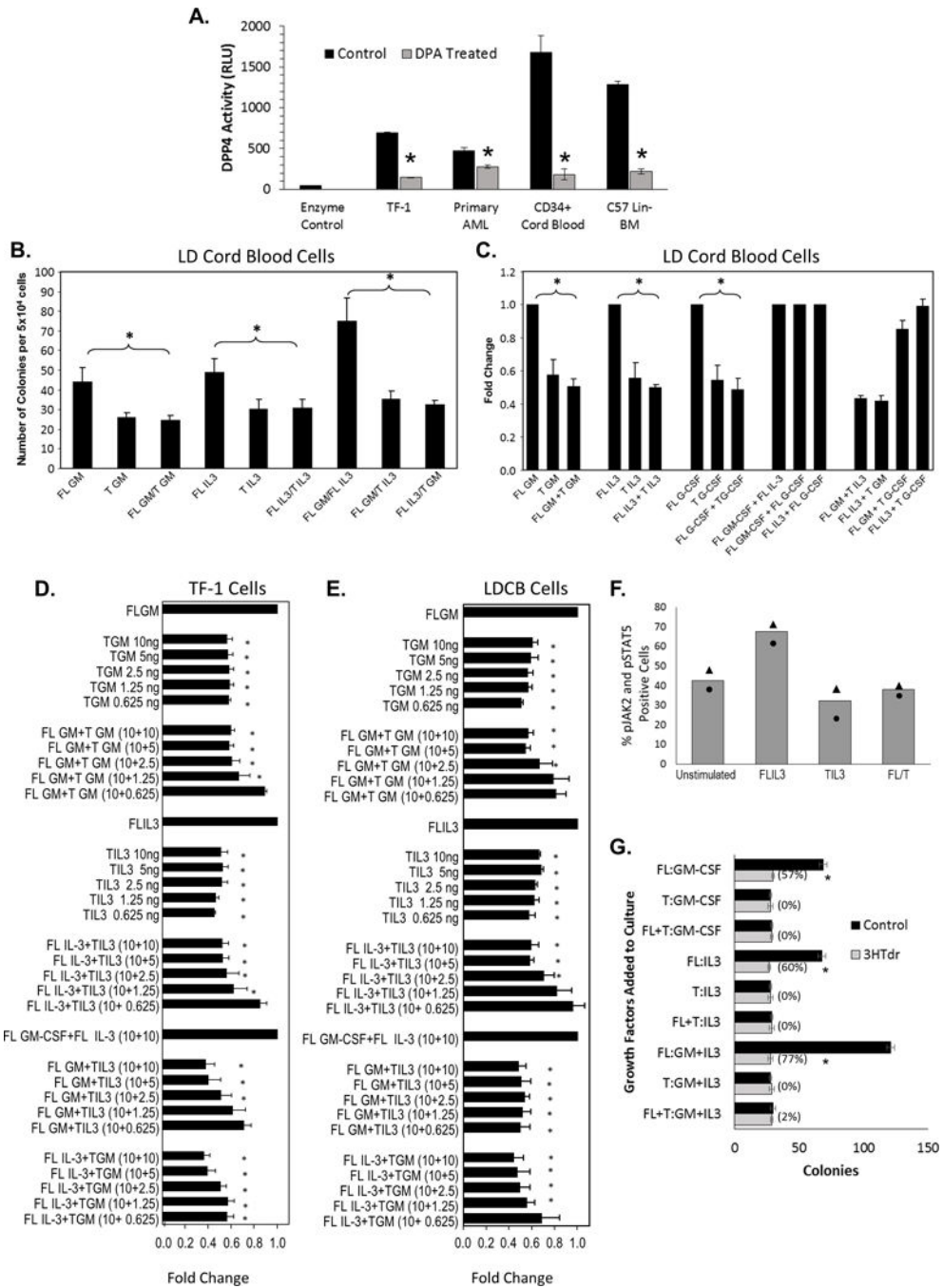
Figure 1. DPP4 is active on the cell surface of immature blood cells and DPP4 truncated molecules (T-GM CSF and T- IL3) block colony formation across molecules *in vitro* at less than a 1:1 ratio

(**A**) 1–5×10⁵ cells/well from TF-1, primary AML samples, CD34⁺ CB and lineage negative C57 BL6/J mBM were analyzed for baseline DPP4 activity. Diprotin A (DPA, a DPP4 inhibitor), was added as a control 30 minutes prior to 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 done in triplicate for all except for the primary AML samples where N=5 and plating for data shown was lower than other samples

(~1×10^5/well) due to sample availability. AML samples were from those shown in Table 1. Low density cord blood cells **(B,C, and E)** or TF-1 cells **(D)** were stimulated with 10ng/ml of full length (FL) or DPP4 truncated (T) GM-CSF, IL-3 or G-CSF unless otherwise listed. **(F)** TF-1 cells were factor starved and treated with 10ng/ml of FL, T or 1:1 ration of IL-3 for 5 minutes, 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/ml in the presence of 10ng/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 3 mice per group or were performed at least 3 times (unless noted otherwise) and ANOVA was used to determine statistical significance with the exception of 1A (students-T Test). * = p   0.05 compared to control (FL GM, IL-3 or FL GM/IL-3, FL G-CSF etc.) 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.
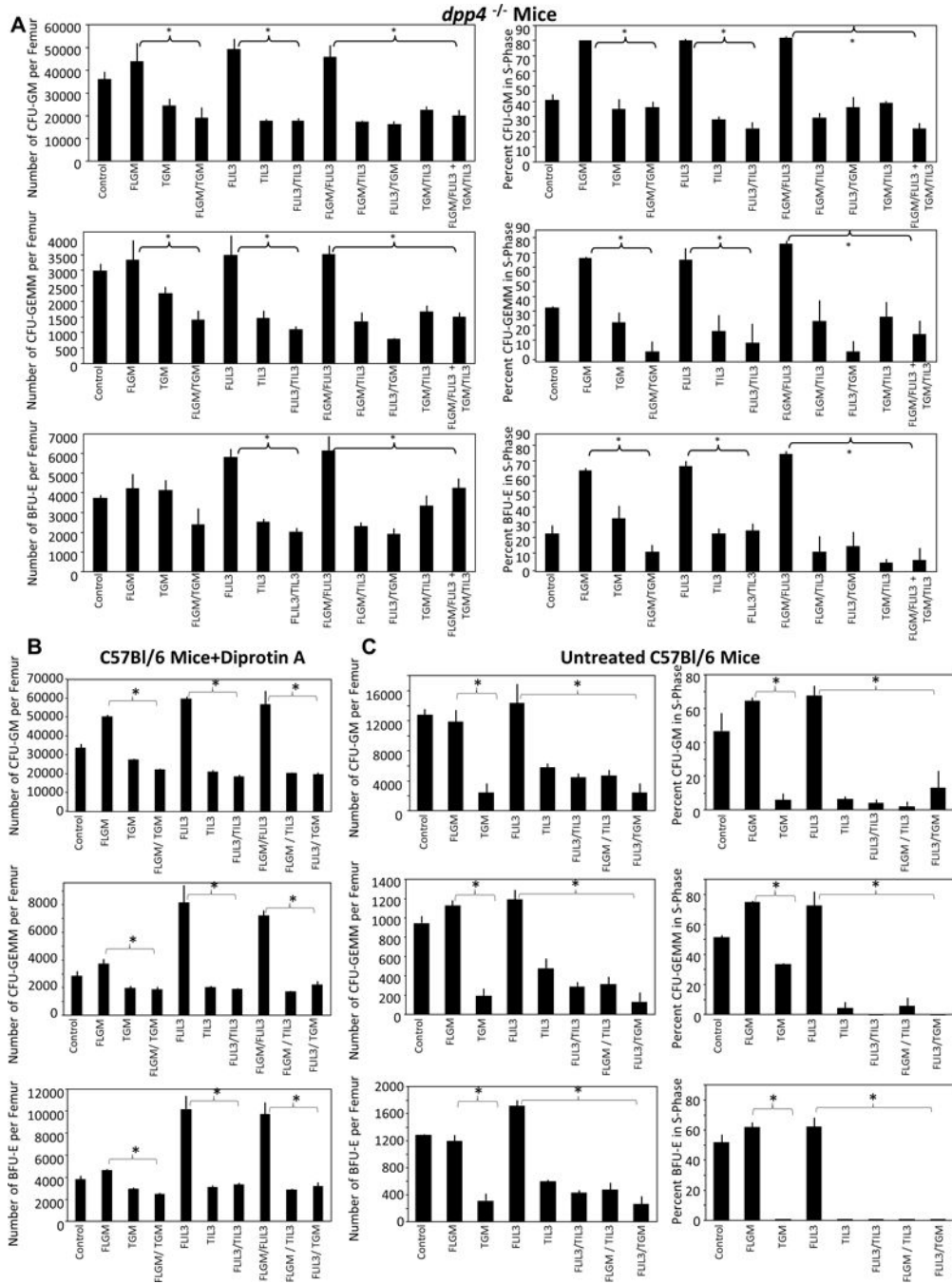
Figure 2. Truncated GM-CSF (T-GM-CSF) and IL-3 (T-IL-3) block colony formation across molecules *in vivo*

dpp4$^{-/-}$ mice (**A**), C57BL6/J mice treated with Diprotin A (**B**), or untreated C57BL6/J mice (**C**) were injected with 10ug of either rmFL, T or a combination of FL/T GM-CSF and/or IL-3 s.c. at different sites. Mice were sacrificed 24 hours later, femurs were flushed, and colony formation and cycling status of the progenitor cells were determined. *= p   .05 or less by ANOVA. N=3 animals/group with each mouse individually accessed. 1 of 2 representative experiments shown for *dpp4*$^{-/-}$ mice.
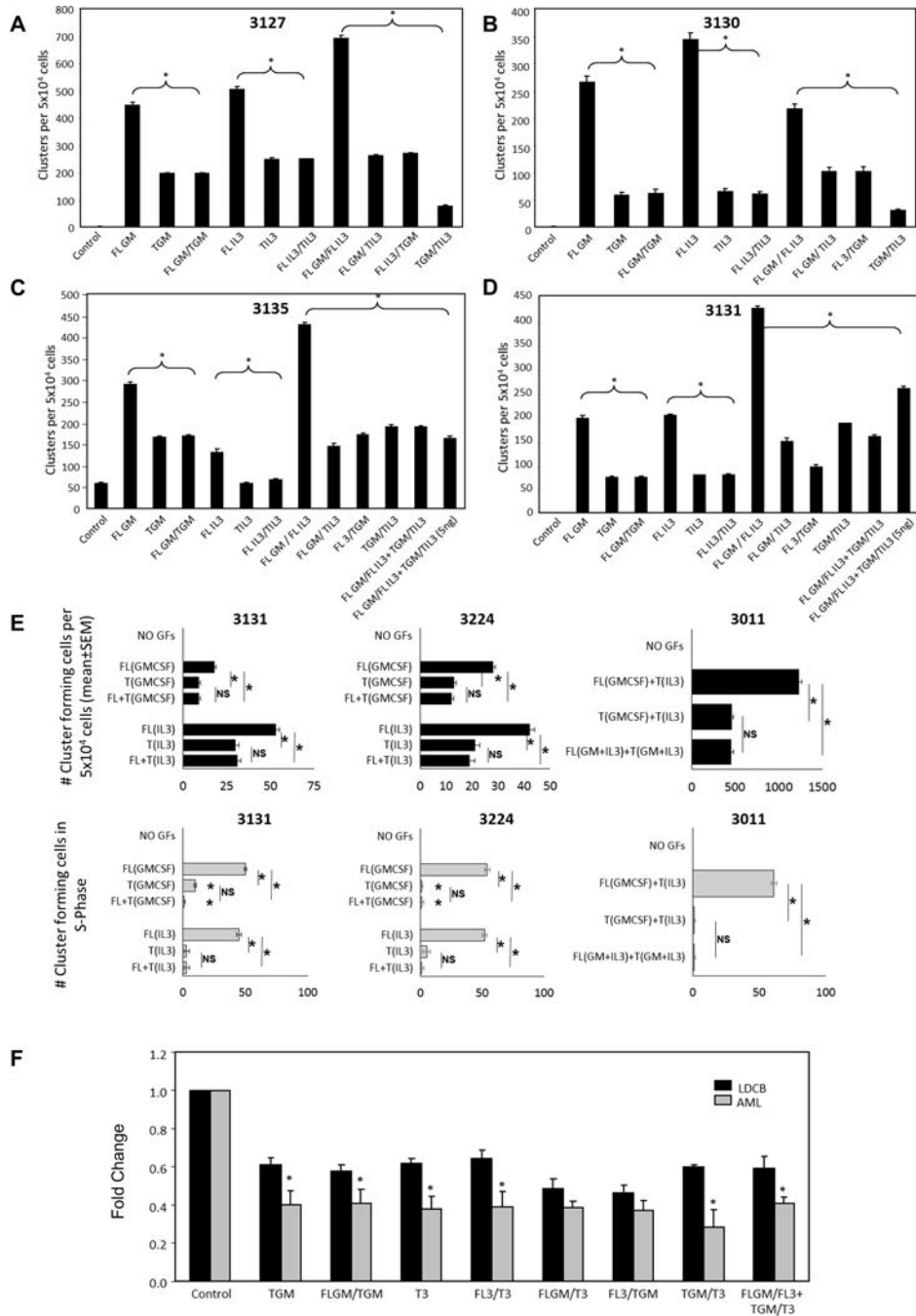
Figure 3. DPP4 Truncation of GM-CSF and IL-3 inhibit AML cluster formation stimulated by FL-CSFs

A-D) 5×10^4 cells from ficolled peripheral blood of AML patients with 90% or greater blast burden (Table 1) were plated in agar with 10ng/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% O2 for 7–10 days prior to 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 truncated factors compared to AML response. N=4 for AML samples and

N=7 for LDCB * = p   .05 or less by ANOVA compared to control (FLGM, FL3 or FLGM/ FL3)
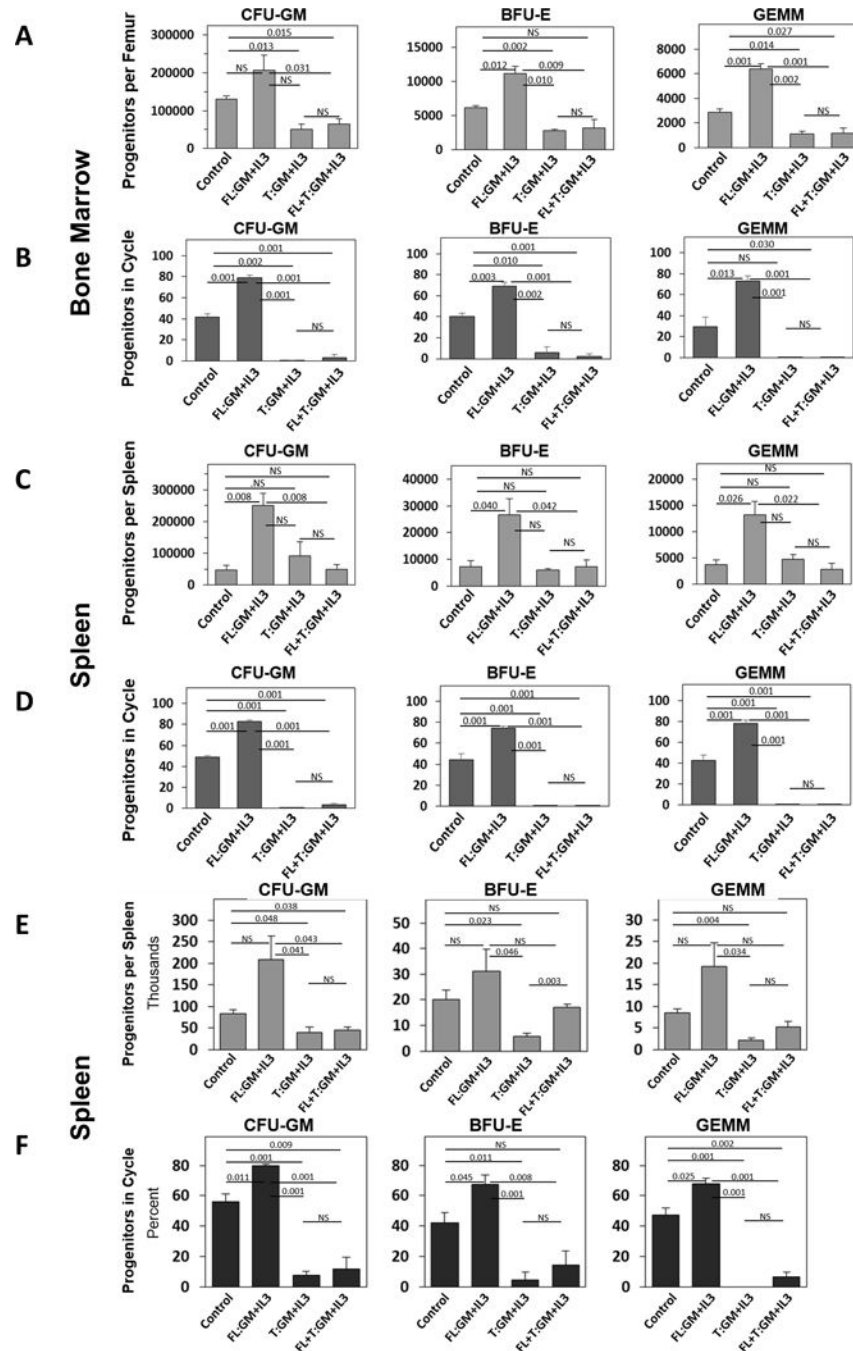
Figure 4. Effects of T- and FL- CSFs on *in vivo* model of leukemia

FLT3-ITD (**A-D**) and *Ptpn11E76K* ((**F, G**) mice were injected with 10ug 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) s.c. at different sites. Mice were sacrificed 24 hours later, femurs flushed, spleens made into single cell suspensions and colony formation (**A,C,E**) and cycling status (**B,D,F**) of the progenitor cells were determined.
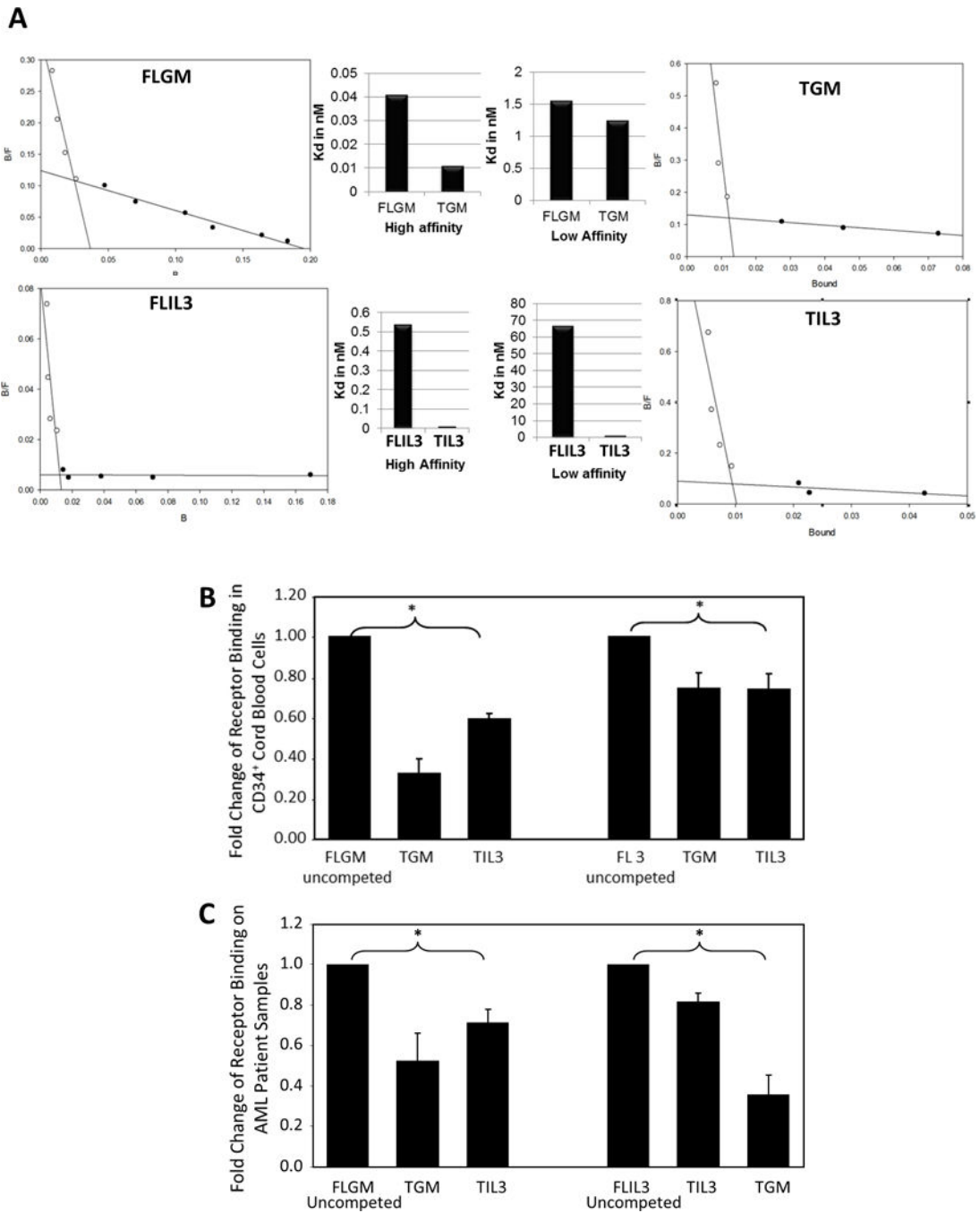
Figure 5. T-IL-3 and T-GM-CSF bind with greater affinity than FL-IL-3 or FL-GM-CSF, and T-IL-3 and T-GM-CSF blunt receptor binding of both FL-IL-3 and FL-GM-CSF

(**A**) Scatchard analysis and Kd determination of TF-1(n=2) was performed with I^{125} labeled IL-3. 1×10^6 TF-1 cells were plated per well, and iodinated full length or truncated IL-3 was added for one hour. 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 the dissociation constant (K_d) were derived as previously published.(3) Pooled $CD34^+$ CB (**B**) or primary AML patient samples (**C**) were incubated with I^{125} labeled FL-

IL-3 or FL-GM-CSF alone or in addition to cold T-IL-3 and T-GM-CSF in excess for 1 hour. Cells were harvested and the 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 T-GM-CSF to compete with FL-IL-3 and FL-GM-CSF. * = p   0.05 AML patient samples n= 7 distinct samples; CB n= 5 independent experiments with CD34^{+} cells pulled from multiple cord bloods for each experiment.

Table 1

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

AML patient samples were obtained and samples utilized were determined to be at least 90% tumor blasts. Yes or No (refers to growth in culture), "cycling" refers to being used in cycling assays

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 [*]
3127	De Novo	t(9;22)(q34;q11.2)	Yes [*]
3130	De Novo	normal karyotype, F1t3 TKD	Yes [*]
3131	De Novo	7q- Flt3 ITD	Yes [*] (cycling)
3135	De Novo	inv 3(q21;q26) [EVI 1 activiation]	Yes [*]
3136	De Novo	normal karyotype, Flt3 TKD, Mnpml	No [*]
3142	Relapse	T(12;15)(Q13,Q11.2),Flt3ITD	**N/A** receptor binding only [*]
3146	De Novo	46(X;X) [13 metaphases]; t(l;13) (p32;ql2) [8 metaphases] dmut CEBPA, and negative Flt3ITD/TKD, negative NPM1	[#]
3152	Relapse	normal karyotype (46[X,Y]), NPM1 negative, CEBPA negative, FltlTD negative, Flt3 D835+	[#]
3155	De Novo	complex, with monosomy 7, negative for Flt3ITD/TKD and negative for NPM1	[#]
3157	De Novo	46(X;X) negative Flt3ITD, neg NPM1, neg CEBPA.	[#]
3159	De Novo	normal karyotype; FLT3ITD+, NPM1−,CEBPA−	[#]
3224	Relapse	normal karyotype, [46(X,Y)], WT1 18%	Yes (cycling) [#]

[*] designates use for receptor binding studies

[#] designates use in DPP4 activity assay.