In vivo evidence for an instructive role of fms-like tyrosine kinase-3 (FLT3) ligand in hematopoietic development

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ABSTRACT

Cytokines are essential regulators of hematopoiesis, acting in an instructive or permissive way. Fms-like tyrosine kinase 3 ligand (FLT3L) is an important cytokine for the development of several hematopoietic populations. Its receptor (FLT3) is expressed on both myeloid and lymphoid progenitors and deletion of either the receptor or its ligand leads to defective developmental potential of hematopoietic progenitors. *In vivo* administration of FLT3L promotes expansion of progenitors with combined myeloid and lymphoid potential. To investigate further the role of this cytokine in hematopoietic development, we generated transgenic mice expressing high levels of human FLT3L. These transgenic mice displayed a dramatic expansion of dendritic and myeloid cells, leading to splenomegaly and blood leukocytosis. Bone marrow myeloid and lymphoid progenitors were significantly increased in numbers but retained their developmental potential. Furthermore, the transgenic mice developed anemia together with a reduction in platelet numbers. FLT3L was shown to rapidly reduce the earliest erythroid progenitors when injected into wild-type mice, indicating a direct negative role of the cytokine on erythropoiesis. We conclude that FLT3L acts on multipotent progenitors in an instructive way, inducing their development into myeloid/lymphoid lineages while suppressing their megakaryocyte/erythrocyte potential.

Introduction

The development of hematopoietic cells is a highly complex and tightly regulated process that in adults is initiated in the bone marrow (BM) from hematopoietic stem cells and continues throughout life. Hematopoietic lineages are derived via intermediate multipotent progenitors, which gradually lose their multipotentiality and eventually become committed to one lineage. Several molecules are considered pivotal for the regulation of this process, including transcription factors, signaling proteins, adhesion molecules and cytokine receptors. Cytokines impart environmental signals into hematopoietic development and several of them have been identified as crucial for the generation of hematopoietic lineages.[1] The role of cytokines in hematopoiesis is considered to be either instructive, by directly promoting differentiation of multipotent progenitors into a specific lineage, or permissive, by selectively promoting the survival and/or proliferation of a particular lineage at the expense of others.[2] For most hematopoietic cytokines, their precise mode of action remains unknown.

As for other type III receptor tyrosine kinases, the Fms-like tyrosine kinase 3 (FLT3), or CD135, has an extracellular domain composed of five immunoglobulin-like domains and a tyrosine kinase motif in the cytoplasmic domain.[3-5] These features of FLT3 are shared with other hematopoietic cytokine receptors, such as stem cell factor receptor and platelet-derived growth factor receptor.[3] FLT3-ligand (FLT3L) is the only known ligand for FLT3.[6] Both the soluble and the membrane-bound form of FLT3L can bind FLT3, leading to receptor dimerization and subsequent activation of the tyrosine kinase domain.[7] Receptor activation initiates a signaling cascade involving proteins such as STAT5a, ERK1/2 and PI3K.[8]

FLT3 and its ligand have been the focus of considerable research due to their involvement in leukemias, since several mutations in FLT3 have been identified in acute myeloid leukemia.[7] Among them, the most common is an internal tandem duplication of exon 14 of the *FLT3* gene (*FLT3*-ITD) which results in constitutive activation of the kinase domain.[9] This mutation is found in ~25% of cases of acute myeloid leukemia and its presence constitutes a poor prognostic factor. *FLT3*-ITD confers growth factor-independent proliferation to leukemic cell lines and its expression in transgenic mice results in a fatal myeloproliferative syndrome.[10]

FLT3 is expressed by several hematopoietic cell populations.[11] Initially, it is expressed by non-self-renewing, short-term hematopoietic stem cells.[12,13] Several downstream progenitors with myeloid and/or lymphoid potential continue to express FLT3 whereas megakaryocyte/erythrocyte progenitors do not.[11,14-18] With the exception of dendritic cells (DC), which retain FLT3 on their surface, FLT3 expression is downregulated as cells undergo myeloid or lymphoid commitment. Deletion of either FLT3 or FLT3L results in defects in the developmental potential of myeloid/lymphoid progenitors underscoring the importance of FLT3 in their development.[19,20] In addition, FLT3L-deficient mice displayed reduced numbers of B cells, DC and natural killer cells,[20] while FLT3L has been

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shown to be crucial in sustaining adult B-cell lymphopoiesis.[21] However, ablation of the FLT3/FLT3L axis alone did not result in a complete block in the generation of any hematopoietic lineage, suggesting that FLT3L might exert its crucial role in hematopoiesis through interactions with other cytokines, such as interleukin-7 (IL-7) or stem cell factor.[19,22,23]

To elucidate the specific action of FLT3L on hematopoiesis *in vivo*, FLT3L has been administered, with the results confirming the important role of FLT3L in DC generation.[24] We have previously shown that apart from DC, FLT3L injection leads to transient expansion of a $FLT3^+$ progenitor population with lymphoid and myeloid potential.[25] In order to evaluate the role of FLT3L in the development of different hematopoietic lineages, in this study we investigated the effects of sustained over-expression of FLT3L in a transgenic mouse model. Our study confirms the positive role of FLTL in DC development and highlights the importance of this cytokine in the survival and expansion of lymphoid and myeloid progenitors. Furthermore, our data provide evidence for an instructive role of FLT3L in hematopoietic development.

Methods

Mice

All mice used in this study were bred and maintained in our animal facility under pathogen-free conditions and all animal experiments were performed in accordance with institutional guidelines (permission numbers 1887 and 1888). Immunization to induce a T-dependent antibody response and FLT3L treatment of mice were carried out as previously described.[25]

Cell cultures

ST2, OP9 and OP9 stromal cells expressing Notch ligand Delta-like 1 (OP9DL1) were maintained in IMDM supplemented with 5 x 10^{-5} M β-mercaptoethanol, 1 mM glutamine, 0.03% w/v Primatone (Quest Naarden, the Netherlands), 100 U/mL penicillin, 100 μg/mL streptomycin and 5% fetal bovine serum. Co-cultures of stromal cells with sorted progenitor cells were performed as previously described[25] (and *Online Supplementary Materials and Methods*).

Platelet counts

Blood was drawn from the tail vein of mice and incubated with 1% ammonium oxalate for 10 min at room temperature. Following incubation, live cells were counted in a Neubauer hemocytometer.

Immunofluorescence

Spleens were snap-frozen and embedded in OCT-compound (Sakura, Zoetermeer, the Netherlands), and 5 μm sections were prepared. Sections were fixed in acetone for 10 min, air-dried for 60 min and subsequently stained with fluorescein isothiocyanate-labeled anti-CD90, phycoerythrin-labeled anti-IgM and allophycocyanin-labeled anti-CD11c antibodies for 30 min.

Results

Splenomegaly and lymphadenopathy in FLT3L transgenic mice

To investigate the effect of prolonged FLT3L over-expression, we generated mice expressing the human *FLT3L* gene under the control of the *β-actin* promoter (hereafter referred to as FLT3L-Tg mice). FLT3L levels in the blood were in the range of 500-1000 ng/mL, as assesed by enzyme-linked immunosorbent assay using an anti-human FLT3L antibody developed in our laboratory (*data not shown*). FLT3L-Tg mice were viable and fertile with no apparent signs of disease until the age of 2-3 months, when many developed diarrhea and tail necrosis. Examination of internal organs revealed a striking increase in spleen size. Indeed, total spleen cellularity in 8- to 14-week old FLT3L-Tg mice was 451±127x10^6 cells compared to 71±12x10^6 cells in wild-type (WT) littermate controls, representing a 6.3-fold increase in total cell number (Figure 1A). Splenic architecture was disrupted with no clearly formed T-cell follicles and a dramatic increase in $CD11c^+$ DC (Figure 1B). Analysis of axillary, brachial and inguinal lymph node cellularity demonstrated an increase from 23±2.6x10^6 cells in WT to 69.6±3.5x10^6 cells in FLT3L-Tg mice (Figure 1A). Thymus cellularity and $CD4^+/CD8^+$ T-cell subpopulations did not differ between WT and FLT3L-Tg mice (*data not shown*).

Increased FLT3L availability leads to alterations in the numbers of dendritic cells and B cells in the bone marrow

Bone marrow cellularity (determined in 2 femora and 2 tibiae) was increased from 40±7.7x10^6 cells in WT to 73.8±14.5x10^6 cells in FLT3L-Tg mice (Figure 1A). Further analysis of BM myeloid and lymphoid populations revealed no significant change in the numbers of $GR1^+CD11b^+$ myeloid cells (*data not shown*). Staining for $CD19^+$ B-cell progenitors revealed no change in the earliest B-cell committed $CD19^+CD117^+$ preB1 population but dramatic 12-fold (from 7±2x10^6 in WT to 0.6±0.3x10^6 cells in FLT3L-Tg) and 144-fold (from 8.3±2.2x10^6 in WT to 0.06±0.09x10^6 in FLT3L-Tg mice) decreases in $CD19^+CD117^-IgM^-$ preB2 and $CD19^+IgM^+$ B cells, respectively (Figure 1C). The apparent increase in total BM cellularity in FLT3L-Tg mice was partly due to a marked increase in DC populations. As shown in Figure 1D, $CD11c^+SiglecH^+$ plasmacytoid DC showed a 41-fold increase (from 0.74±0.15x10^6 in WT to 30±8.8x10^6 in FLT3L-Tg mice), while $CD11c^+SiglecH^-$ conventional DC numbers were increased 13-fold (from 0.3±0.06x10^6 in WT to 2.5±1.2x10^6 in FLT3L-Tg mice). Overall, analysis of the FLT3L-Tg BM lymphoid and myeloid populations revealed decreased numbers of B-cell progenitors and a significant increase in DC numbers.

Diminished megakaryocyte/erythrocyte lineage differentiation, anemia and leukocytosis in FLT3L-transgenic mice

Alterations in BM erythroid progenitors in FLT3L-Tg mice were also investigated, revealing a significant 5.4-fold decrease in $TER119^+$ erythroid progenitor numbers, from 2.7±0.2x10^6 cells in WT to 0.5±0.2x10^6 cells in FLT3L-Tg mice (Figure 2A). This decrease in $TER119^+$ erythroid progenitors prompted us to analyze the development of the megakaryocyte/erythrocyte lineage in FLT3L-Tg mice. Hematocrit analysis showed that FLT3L-Tg mice manifested a significant decrease in hematocrit (from 46.4±2 in WT to 34±3 in transgenic mice) already at 8-10 weeks of age, which dropped even further to 21.6±5.9 at 19-22 weeks of age (Figure 2B). Blood platelet counts also

showed a significant drop, from 400±89x10⁸ cells/mL in WT to 207.5±61x10⁸ cells/mL in FLT3L-Tg mice (Figure 2C). Thus, megakaryocyte/erythrocyte lineage development seemed to be diminished, leading to anemia in FLT3L-Tg mice. Finally, blood smear preparations showed a marked increase in the numbers of leukocytes in FLT3L-Tg blood compared to WT blood (Figure 2D), which was confirmed by a quantitative analysis demonstrating a 27-fold increase in numbers of leukocytes in FLT3L-Tg blood (*Online Supplementary Figure S1*).

Expansion of myeloid and lymphoid populations in spleens of FLT3L-transgenic mice

Due to the observed splenomegaly we extended the analysis to mature hematopoietic populations in the spleen. Contrary to BM, the spleen of FLT3L-Tg mice displayed a significant (15-fold) increase of GR1⁺CD11b⁺ myeloid cells from 1.9±0.7x10⁶ cells in WT to 30.1±7.6x10⁶ cells in the transgenic animals (Figure 3A). NK1.1⁺ natural killer cells were also significantly increased from 2.7±0.8x10⁶ in WT to 23±6.3x10⁶ in the transgenic

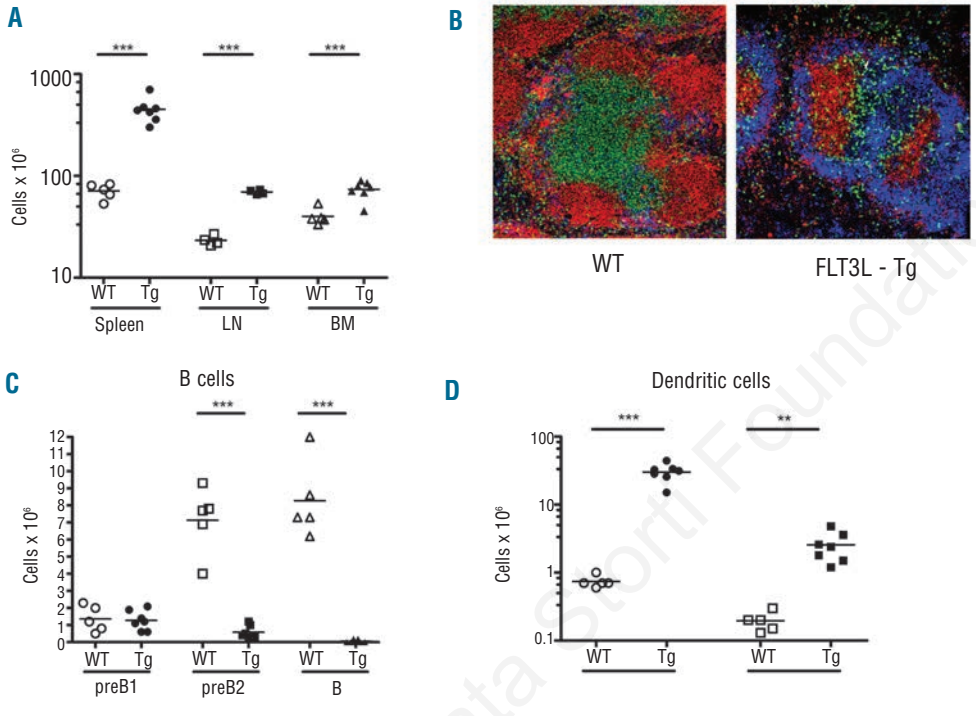

Figure 1. Splenomegaly, lymphadenopathy, disrupted spleen architecture and altered B and DC bone marrow populations in FLT3L-Tg mice. (A) Total cellularity in spleen, lymph nodes (axillary, brachial and inguinal) and bone marrow (2 femora and 2 tibiae) of 8- to 14- week old wild type (WT - white symbols) and FLT3L-Tg (Tg - black symbols) mice (5-7 mice per group). ***P<0.0001. (B) Immunofluorescence of spleen sections from 8- to 14- week week old WT and FLT3L-Tg mice stained for B cells (anti-IgM, red), T cells (anti-CD90, green) and dendritic cells (anti-CD11c, blue). (C) Numbers of CD19⁺CD117⁺ (preB1), CD19⁺CD117⁻IgM⁻ (preB2) and CD19⁺IgM⁺ B cells in WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***P<0.0001. (D) CD11c⁺SiglecH⁺ plasmacytoid DC and CD11c⁺SiglecH⁻ DC numbers in WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***P<0.0001, **P=0.0022.

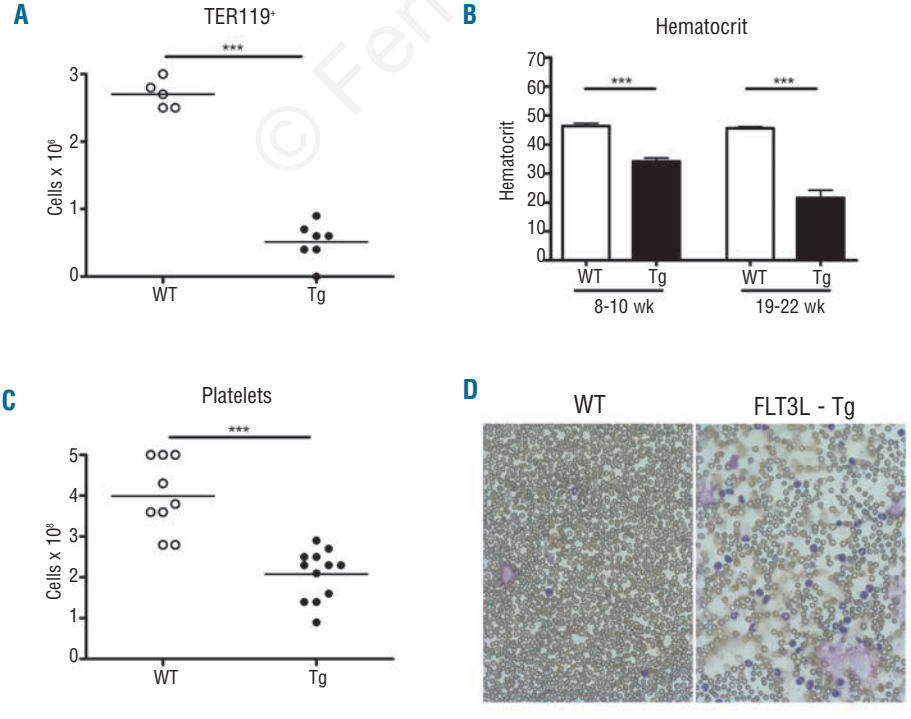

Figure 2. Diminished megakaryocyte/erythrocyte lineage development, anemia and leukocytosis in FLT3L-Tg mice. (A) Numbers of TER119⁺ erythroid progenitors in the bone marrow (2 femora and 2 tibiae) of 8- to 14-week old WT (white circles) and FLT3L-Tg (black circles) mice (5-7 mice per group). ***P<0.0001. (B) Hematocrit levels in WT (white bars) and FLT3L-Tg (black bars) mice (5-7 mice per group). ***P<0.0001. (C) Platelet numbers (per mL) in the blood of WT (white circles) and FLT3L-Tg (black circles) mice (9-12 mice per group). ***P<0.0001. (D) Hematoxylin-eosin staining of blood smears from WT and FLT3L-Tg mice (40x magnification).

mice (Figure 3A). A detailed analysis of DC subsets in the spleen of FLT3L-Tg mice showed a dramatic increase in DC numbers, reaching 368-fold for CD11c$^+$SiglecH$^+$ plasmacytoid DC (95.8±26x10^6 *versus* 0.26±0.06x10^6 in WT mice), 208-fold for CD11c$^+$CD11b$^+$ conventional DC (91.7±25x10^6 *versus* 0.44±0.29x10^6 in WT mice) and 161-fold for CD11c$^+$CD8α^+ conventional DC (21.6±7.2x10^6 *versus* 0.13±0.05x10^6 in WT mice) (Figure 3B,D). These expanded DC populations in our FLT3L-Tg mice were shown to be functional (*Online Supplementary Figure S2*). We conclude from these data that the massive expansion of myeloid, natural killer and, mainly, DC populations accounts for the splenomegaly observed in FLT3L-Tg mice.

The apparent reduction in BM B lymphopoiesis was not reflected in a reduction of CD19$^+$IgM$^+$ mature B-cell numbers in the spleens of FLT3L-Tg mice (Figure 3C). In contrast, despite the similarity in thymus cellularity, there was nevertheless a 3.2-fold increase (from 11.5±4.3x10^6 in WT to 37.4±19x10^6 cells in FLT3L-Tg mice) in splenic CD4$^+$ T cells (Figure 3C). The increase in the number of CD8$^+$ T cells was smaller (2.4-fold), being from 7.8±2.8x10^6 cells in WT to 18.9±8.4x10^6 cells in FLT3L-Tg mice. Using intracellular FACS staining, a 7.4-fold increase in Foxp3$^+$CD4$^+$ regulatory T cells (from 1.36±0.2x10^6 in WT to 10.14±1.3x10^6 in FLT3L-Tg mice), was also detected in the spleen of FLT3L-Tg mice (Figure 3C), as shown previously by *in vivo* FLT3L injection.[26]

The abnormal splenic architecture and alterations in some T-cell numbers motivated us to test the quality of the immune response in the FLT3L-Tg mice. Thus, transgenic and WT control littermates were immunized with NIP protein and the levels of serum anti-NIP IgG antibodies were quantified 13 days later by enzyme-linked immunosorbent assay. Anti-NIP IgG responses were somewhat weaker in FLT3L-Tg mice than in WT ones, even though a significant increase in IgG titers was still observed after immunization (*Online Supplementary Figure S3*).

Expansion of hematopoietic progenitors in the bone marrow of FLT3L-transgenic mice

Increased availability of FLT3L was previously shown to expand DC populations and increase numbers of an FLT3$^+$ progenitor population named early progenitor with lymphoid and myeloid potential (EPLM).[25] In addition to EPLM, other lymphoid and myeloid progenitor populations such as the lymphoid-primed multipotent progenitor population (LMPP),[27] common lymphoid progenitors

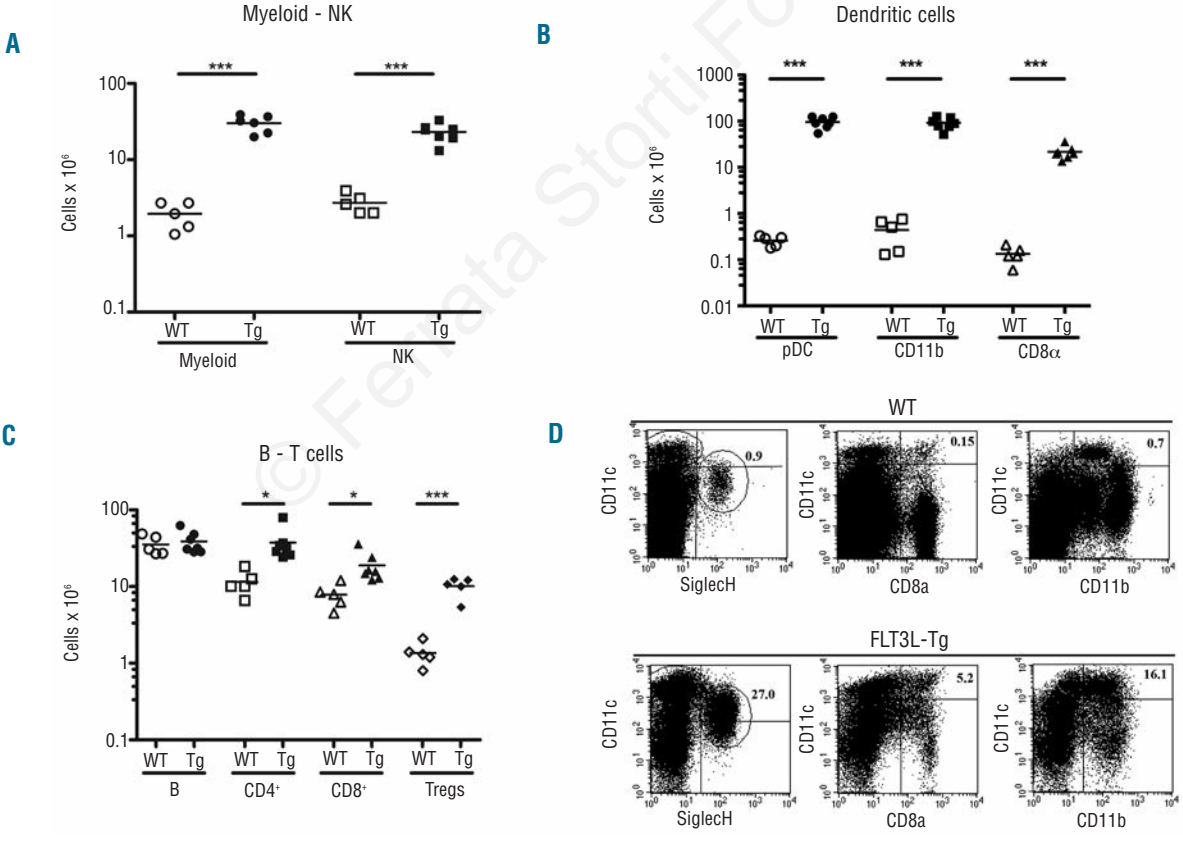

Figure 3. Alterations in myeloid, lymphoid and dendritic cell populations in the spleens of FLT3L-Tg mice. (A) Numbers of CD11b$^+$GR1$^+$ myeloid and NK1.1$^+$ natural killer (NK) cells in spleens of 8- to 14-week old WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). ***P<0.0001. (B) Numbers of CD11c$^+$SiglecH$^+$ plasmacytoid DC (pDC), CD11c$^+$CD11b$^+$ conventional DC (CD11b) and CD11c$^+$CD8α^+ conventional DC (CD8α) in spleens of WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). ***P<0.0001. (C) Numbers of CD19$^+$IgM$^+$ B cells, CD4$^+$ T cells, CD8$^+$ T cells and Foxp3$^+$CD4$^+$ regulatory T cells (Tregs) in the spleens of WT (white symbols) and FLT3L-Tg (black symbols) mice (5 or 6 mice per group). *(CD4): P=0.149, *(CD8): P=0.0192, ***P<0.0001. (D) FACS plots showing the gating strategy used for staining the DC subpopulations included in (B).

(CLP)[28,29] and common myeloid progenitors[30] are known to be FLT3$^+$. To assess the potential effects of constitutive FLT3L over-expression on these progenitor populations, we analyzed progenitors in the BM of FLT3L-Tg mice. Most of the CLP, EPLM and a fraction of myeloid-restricted CD117$^+$CD115$^+$Sca1$^-$ cells were indeed FLT3$^+$, but FLT3 expression was downregulated in FLT3L-Tg mice, presumably due to continuous engagement and internalisation of the receptor (*Online Supplementary Figure S4*). We did not, therefore, use FLT3 as a marker in our analysis. As shown previously,[25] upon increased FLT3L availability, there was a 14-fold increase in EPLM (CD117$^+$B220^{low}CD19/NK1.1$^-$) numbers from 0.2 ± 0.1 x 106 cells in WT to 2.8±0.9x10^6 cells in FLT3L-Tg mice (Figure 4A,B). Increases in CLP (CD117$^+$Sca1$^+$CD127$^+$) progenitors were even higher with a 75-fold increase from 0.04±0.01x10^6 cells in WT to 3.2±1.3x10^6 cells in FLT3L-Tg mice. While staining for CLP, we noted a CD117$^-$CD127$^+$ population that was increased in FLT3L-Tg mice (Figure 4A). This population could not represent B-cell progenitors, since they were reduced in FLT3L-Tg mice. Indeed, further analysis revealed that these cells were CD11c$^+$SiglecH$^+$ plasmacytoid DC, which had expanded 41-fold in FLT3L-Tg mice BM and were all CD127$^+$ (*Online Supplementary Figure S5*). CD117$^+$CD115$^+$Sca1$^-$ myeloid progenitors were also found to be significantly increased in FLT3L-Tg mice (from 0.4±0.1x10^6 cells in WT to 5.1 ±1.4x10^6 in FLT3L-Tg mice). The Lineage$^-$Sca1$^+$CD117$^+$ (LSK) fraction, which is to a large extent FLT3$^+$ (*Online Supplementary Figure S4*), was also significantly increased in FLT3L-Tg mice (Figure 4C,D). Further staining of LSK cells with CD150 and CD48[31] revealed that this increase is mainly due to an increase in CD150$^-$CD48$^+$ MPP (*Online Supplementary Figure S6*), which are predominantly FLT3$^+$.[32]

The anemia and reduction in TER119$^+$ erythroid progenitors and platelets prompted us to investigate the earliest identified megakaryocyte/erythroid progenitors. Staining the CD117$^+$Sca1$^-$CD127$^-$ fraction of BM with CD34 and CD16 allows the identification of common myeloid progenitors, as well as progenitors with restricted granulocyte-macrophage (GMP) and megakaryocyte-erythrocyte (MEP) potential.[30,33] We found a dramatic 9.7-fold decrease

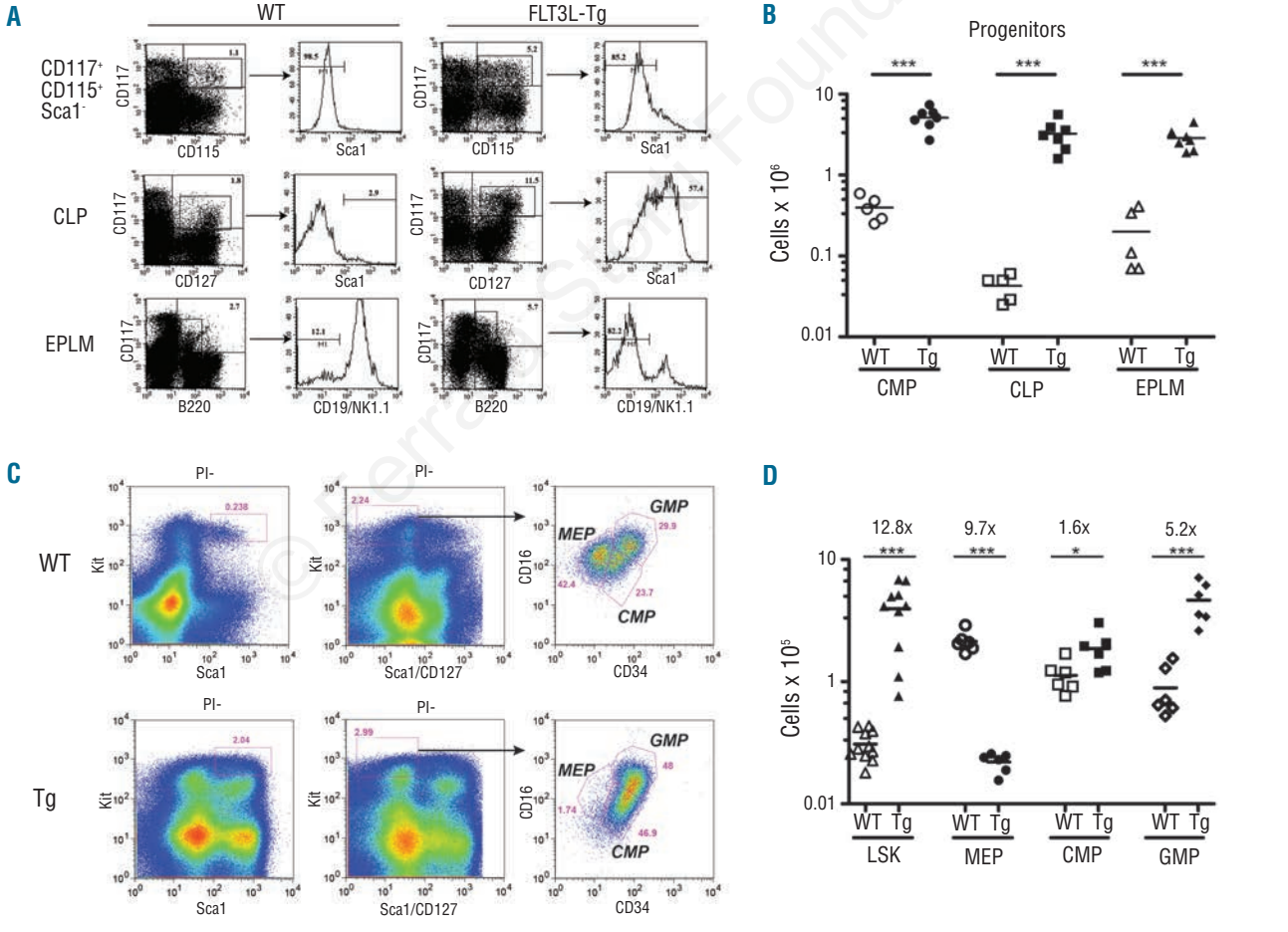

Figure 4. Hematopoietic progenitor populations in FLT3L-Tg mice. The figure shows the FACS stainings (A and C) and numbers (B and D) of hematopoietic progenitor populations in the bone marrow (2 femora and 2 tibiae) of 8- to 14-week old mice. (A) Representative FACS plots demonstrating the gating strategy used for staining CD117$^+$CD115$^+$Sca1$^-$ (upper panel), CLP (middle panel) and EPLM (lower panel) in WT and FLT3L-Tg bone marrows. (B) Total numbers of CD117$^+$CD115$^+$Sca1$^-$, CLP and EPLM in the bone marrow of WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***P<0.0001. (C) Representative FACS plots demonstrating the gating strategy used for staining LSK (left panel), MEP, CMP and GMP (center and right panel) in WT and FLT3L-Tg bone marrows. (D) Total numbers of LSK, MEP, CMP and GMP in the bone marrow of WT (white symbols) and FLT3L-Tg (black symbols) mice (MEP, CMP, GMP: 6 mice per group; LSK: 10 mice per group).

in the numbers of MEP in FLT3L-Tg mice compared to WT, as well as a significant 5.2-fold increase in GMP (Figure 4C,D). Importantly, gene expression analysis of the LSK compartment demonstrated reduced levels of the megakaryocyte/erythrocyte lineage genes *Mpl* and *Klf1*, while the expression of myeloid-specific genes *Mpo*, *Csf3r* and *Cebpa* was increased (*Online Supplementary Figure S7*). These results indicate that increased FLT3L levels skew development towards the myeloid/lymphoid pathway and away from the megakaryocyte/erythroid compartment and that this skewing occurs already at the level of FLT3$^+$ multipotent progenitors.

Next, we determined whether the expansion of lymphoid and myeloid progenitors in FLT3L-Tg mice was accompanied by a change in their developmental potential. Therefore, EPLM, CLP and CD117$^+$CD115$^+$Sca1$^-$ cells were sorted from WT or FLT3L-Tg mice and plated in 96-well plates under differentiation conditions promoting myeloid (ST2 stromal cells), B-cell (OP9 stromal cells plus IL-7) and T-cell (OP9DL1 stromal cells plus IL-7) development. As shown in Table 1, our *in vitro* differentiation analysis showed no significant change in the developmental potential of the analyzed progenitor populations. To assess the *in vivo* potential of progenitors, CLP and EPLM were sorted from CD45.2 FLT3L-Tg mice and transplanted into CD45.1 congenic mice. Analysis of the recipient mice confirmed the B-cell potential of both FLT3L-Tg CLP and EPLM, while the T-cell potential of transgenic CLP was retained but compromised by poor donor reconstitution of the thymus (*Online Supplementary Figures S8* and *S9*). Finally, transplantation of FLT3L-Tg LSK in a similar setting demonstrated their potent myeloid reconstitution potential but a reduced erythroid potential compared to WT LSK (*Online Supplementary Figure S10*). We conclude that the lymphoid and myeloid potential of FLT3L-Tg progenitors is retained, while their erythroid potential is reduced.

Kinetics of hematopoietic population changes suggest an instructive role of FLT3L in hematopoiesis

The observed alterations in hematopoietic populations in FLT3L-Tg mice could be the consequence of a direct, "instructive", action of FLT3L on multi-potent progenitors, actively guiding them to acquire a particular cell fate at the expense of other options ("instructive" model).

Table 1. ***In vitro*** **developmental potential of CD117$^+$CD115$^+$Sca1$^-$, CLP and EPLM populations from wild-type and FLT3L-Tg mice.**

		B-cell potential	T-cell potential	Myeloid potential
CD117$^+$CD115$^+$ Sca1$^-$	WT	<1 in 120	1 in 76	1 in 3
	TG	<1 in 120	1 in 42	1 in 2
CLP	WT	1 in 10	1 in 15	1 in 12
	TG	1 in 5	1 in 3	1 in 5
EPLM	WT	1 in 5	1 in 10	1 in 16
	TG	1 in 10	1 in 3	1 in 6

Frequencies of progenitors with B-cell, T-cell and myeloid cell potential as assessed by in vitro *limiting dilution analysis. One representative experiment is shown out of three to six for the different populations. CLP: common lymphoid progenitor, EPLM: early progenitors with lymphoid and myeloid potential, WT: wild-type, TG: FLT3L transgenic.*

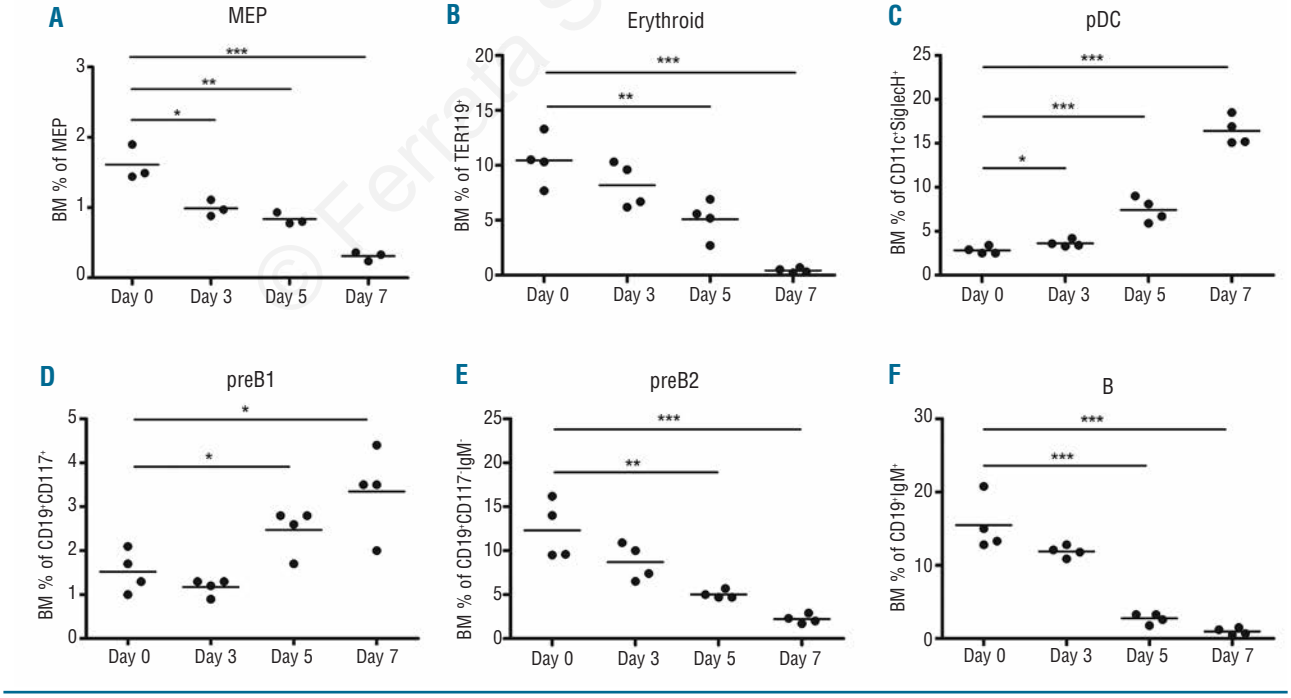

Figure 5. Kinetics of changes in percentages of hematopoietic populations following FLT3L injections into wild-type mice. The figure shows percentages of hematopoietic cells in the BM of WT mice injected daily with 10 μg of recombinant FLT3L 0, 3, 5 and 7 days after injection. (A) Percentages of MEP. *P=0.0177, **P=0.0073, ***P=0.001. (B) Percentages of nucleated TER119$^+$ bone marrow cells. **P=0.01, ***P=0.0001. (C) Percentages of CD11c$^+$SiglecH$^+$ plasmacytoid dendritic cells. *P=0.0345, **P=0.0007, ***P<0.0001. (D) Percentages of CD19$^+$CD117$^+$ preB1 cells. *(Day 5): P=0.0368, *(Day 7): P=0.0163. (E) Percentages of CD19$^+$CD117$^-$IgM$^-$ preB2 cells. **P=0.0049, ***P=0.0001. (F) Percentages of CD19$^+$IgM$^+$ B cells. *(Day 5): P=0.0005, ***(Day 7): P=0.0002.**

Alternatively, over-expression of FLT3L could result in a vast expansion of FLT3$^+$ cells which could leave little space and/or recourse for non-expanding cells, thus leading to a reduction in their numbers ("space" model). In a system with sustained elevated levels of FLT3L it would be difficult to distinguish between the two possibilities. To acquire data supporting either model, we injected WT mice with recombinant FLT3L and monitored kinetic changes in numbers of different hematopoietic lineages. As shown in Figure 5A and B, and in accordance with the anemic phenotype of FLT3L-Tg mice, the percent nucleated BM MEP and TER119$^+$ erythroid progenitors showed a reduction by 3 days after FLT3L injection, which was already significant in the case of MEP. Considering the turnover of TER119$^+$ erythroid progenitors, the speed by which increased FLT3L availability leads to MEP and TER119$^+$ progenitor reduction would argue for an instructive, negative role of FLT3L in their generation.

In addition, we quantified the percentages of other hematopoietic cells whose numbers were significantly altered in FLT3L-Tg mice, namely plasmacytoid DC and CD19$^+$ B-cell progenitors. Plasmacytoid DC seemed to increase, demonstrating a 2.6-fold increase at 5 days after FLT3L injection and reaching a 5.8-fold increase at day 7 (Figure 5C). This would be consistent with a role of FLT3L in the expansion of these FLT3$^+$ cells, shown previously.[24,25] CD19$^+$CD117$^+$ preB1 cells showed little reduction; indeed, an up to 2-fold increase in their percentage was observed at day 7 (Figure 5D). In contrast, and in accordance with our FTL3L-Tg analysis, both CD19$^+$CD117$^-$IgM$^-$ preB2 and CD19$^+$IgM$^+$ B cells were reduced after FLT3L injection, with the difference becoming significant after 5 days (Figure 5E,F). Overall, our FLT3L injection data point towards an instructive role of FLT3L in the development of certain hematopoietic lineages.

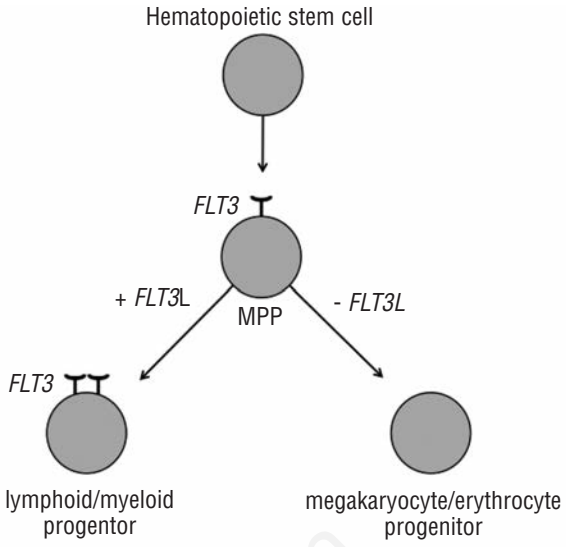

Figure 6. Proposed model of the instructive action of FLT3L in determining lymphoid/myeloid *versus* megakaryocyte/erythrocyte lineage development.

Discussion

Several lines of evidence point towards an important role of FLT3/FLT3L in hematopoiesis. The relatively mild phenotype of both FLT3 and FLT3L knock-out mice suggest that this cytokine exerts its role mainly in concert with other hematopoietic cytokines, such as stem cell factor or IL-7.[19,22] Administration of FLT3L to adult mice *in vivo* has been used as a means of elucidating the ligand's exact role in regulating hematopoiesis.[24,25] In the present study we report for the first time a detailed *in vivo* analysis of the effect of elevated and sustained transgenic expression of FLT3L in different hematopoietic lineages. Our results suggest an instructive role of FLT3L in hematopoietic development.

Analysis of FLT3L-Tg mice showed significant alterations in several hematopoietic lineages. The population with the highest increase was DC. Both in BM and spleen, all DC populations displayed a dramatic expansion ranging from 7- to 368-fold compared to numbers in WT mice. Previously, FLT3L was shown to be a crucial factor for the *in vitro* generation of DC,[34] whereas *in vivo*, absence of FLT3L resulted in a marked decrease in DC numbers.[20] Increases in DC were also noted in studies in which either FLT3L was administered *in vivo*[24,25] or FLT3L was conditionally over-expressed by transgenesis.[35] The importance of FLT3L in DC generation has been shown for conventional DC and plasmacytoid DC, both of which are FLT3$^+$ populations. Our results are in accordance with these observations. The elevated numbers of plasmacytoid DC, the population most increased in our transgenic mice, was particularly striking. Plasmacytoid DC are considered an important part of anti-viral immunity, mainly through their production of interferon-α.[36] The dramatic increase of interferon-α-producing plasmacytoid DC upon sustained over-expression of FLT3L suggests a potential therapeutic use of this cytokine to combat chronic viral infections. Furthermore, our transgenic system constitutes a source for *ex vivo* isolation of vast numbers of functional DC populations.

A significant reduction in CD19$^+$CD117$^-$IgM$^-$ preB2 and CD19$^+$IgM$^+$ B-cell populations was noted in the BM of FLT3L-Tg mice. This phenotype was somewhat surprising, considering the decreased numbers of B-cell progenitors in mice deficient in functional FLT3 or FLT3L, results that had suggested a positive role of FLT3L in B-cell development.[19,20,37] Furthermore, coincident with CD19 expression and due to repression by PAX5, FLT3 is down-regulated in B-cell progenitors,[38] thereby excluding the possibility that FLT3L is necessary for the survival of CD19$^+$ cells. Moreover, there was no evidence for reduced B-cell potential among CLP and EPLM from FLT3L-Tg mice in which there were normal numbers of the earliest committed CD19$^+$CD117$^+$ preB1 cells. We consider decreased IL-7 availability the most likely explanation for this apparent reduction in BM CD19$^+$ cells. There was a dramatic increase in IL7Rα$^+$ (CD127$^+$) lymphoid progenitors, such as CLP (76-fold) and EPLM (14-fold) in FLT3L-Tg mice. Furthermore, plasmacytoid DC, which we found to be IL7Rα$^+$, were also increased (41-fold). Hence, there is an enormous expansion of IL7Rα$^+$ cells in FLT3L-Tg mouse BM which, by absorbing IL-7, could lead to reduced levels of available IL-7 necessary for CD19$^+$ cell survival and/or proliferation. As a population, preB2 cells are particularly sensitive to IL-7 availability, while the reduced numbers of IgM$^+$ B cells might reflect the reduced input from the preB2 stage. To test this hypothesis we injected FLT3L-Tg

mice with IL-7/anti-IL-7 complexes[39] and were able to increase preB2 and B-cell percentages almost to the same levels as in WT mice (*Online Supplementary Figure S11*). Despite the reduction in BM preB2 and B cells, FLT3L-Tg BM output seemed sufficient to reconstitute the splenic B-cell compartment. Overall, we believe that the diminished numbers of BM CD19$^+$ B-cell progenitors in FLT3L-Tg mice is a secondary effect, caused by the decreased availability of IL-7, rather than a negative role of FLT3L in B-cell development and survival.

The numbers of FLT3$^+$ lymphoid and myeloid progenitors were dramatically increased in the BM of FLT3L-Tg mice. In a previous study, EPLM were also increased following administration of FLT3L *in vivo*, while their B lineage potential in limiting dilution assays was decreased.[25] Despite their expansion in our FLT3L-Tg mice, the B, T and myeloid lineage potential of EPLM was unaltered. This apparent difference from the *in vivo* administration data could be due to the sustained elevated levels of FLT3L in FLT3L-Tg mice. This dramatic expansion without any apparent alteration in the developmental potential of lympho-myeloid progenitors analyzed would indicate a role of FLT3/FLT3L signaling in their survival and/or proliferation, rather than in instructing them towards a particular downstream lineage fate. In addition, elevated FLT3L levels could enhance the generation of these FLT3$^+$ progenitors from hematopoeitic stem cells. Given the very small numbers of common myeloid progenitors, CLP and EPLM in WT BM, our FLT3L-Tg mice provide an excellent mouse model for the isolation of large numbers of these progenitors for further *in vitro*, *in vivo* or molecular biology analyses.

Strikingly, FLT3L-Tg mice became severely anemic a few months after birth. Both platelets and erythrocytes were diminished in these mice, suggesting a defect in the generation of the megakaryocyte/erythrocyte lineage. One explanation for this phenotype could be that the dramatic expansion of several other cell types in FLT3L-Tg mice leads to a reduction of FLT3$^-$ megakaryocyte/erythrocyte progenitors due to competition for space and/or resources. Nevertheless, the decreased expression of megakaryocyte/erythrocyte-specific genes already in the LSK compartment of FLT3L-Tg mice as well as the dramatic reduction in MEP and their rapid decrease following FLT3L treatment argue for a defect in their development rather than having no space to expand. This developmental defect would in turn suggest that erythrocytes originate from an FLT3L-responsive population that can be induced by FLT3 signaling to develop into lympho-myeloid lineages at the expense of the erythroid lineage. Based on our data, we propose that FLT3$^+$ MPP that receive a sufficient FLT3L signal differentiate to lympho-myeloid progenitors, while MPP that do not activate FLT3 signaling, due to either low FLT3 expression and/or low FLT3L levels in their microenvironment, develop into megakaryocyte-erythrocyte progenitors (Figure 6). Increased FLT3L availability would result in very few, if any, MPP not receiving an adequate FLT3L signal, thus leading to decreased megakaryocyte/erythrocyte developmental input and an increased lymphoid-myeloid progenitor compartment, as is the case in our FLT3L-Tg mice. Our data strengthen the suggested importance of FLT3 signaling in promoting lympho-myeloid *versus* megakaryocyte-erythroid lineage development[27] and provide evidence for an instructive role of FLT3L in this process. Furthermore, they are in accordance with recent data demonstrating that platelets and erythrocytes originate from *Flt3*-expressing progenitors.[40-42]

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