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

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Running title: “Instructive role of FLT3-ligand in hematopoiesis”

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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. 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, 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 (HSC) and continues throughout life. Hematopoietic lineages are derived via intermediate multi-potent progenitors, which gradually lose their multi-potentiality 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 multi-potent 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 5 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 (SCF) receptor and platelet-derived growth factor (PDGF) 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 implication in leukemias, since several mutations in FLT3 have been identified in Acute Myeloid Leukemia (AML)(7). Amongst them, the most common is an internal tandem duplication of exon 14 of the FLT3 gene (FLT3-ITD) that results in constitutive activation of the kinase domain(9). This mutation is found in ~25% of AML 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 HSC(12, 13). Several downstream progenitors with myeloid and/or lymphoid potential continue to express FLT3 whereas megakaryocyte/erythocyte progenitors do not(11, 14-18). With the exception of dendritic cells (DC), which retain FLT3 on their surface, FLT3 expression is down-regulated as cells undergo myeloid or lymphoid commitment. Deletion of either FLT3 or FLT3L resulted 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 (NK) cells(20), while FLT3L has been shown crucial in sustaining adult B 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 Inteuleukin-7 (IL-7) or SCF(19, 22, 23).
To elucidate the specific action of FLT3L on hematopoiesis in vivo, administration of FLT3L has been carried out. Results obtained confirmed 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 on the development of different hematopoietic lineages, we describe herein 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 herein were bred and maintained in our animal facility under pathogen free conditions and all animal experiments were performed within institutional guidelines (permission numbers 1887 and 1888). Immunizations 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 the Notch ligand Delta-like 1 (OP9DL1) were maintained in IMDM supplemented with 5 x 10⁻⁵M β-mercaptoethanol, 1mM glutamine, 0.03% w/v Primatone (Quest Naarden, The Netherlands), 100U/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 Suppl. Materials and Methods].
Platelet counts

Blood was drawn from the tail vein of mice and incubated with 1% ammonium oxalate for 10 minutes 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, NL), and 5 µm sections were prepared. Sections were fixed in acetone for 10 min, air dried for 60 min and subsequently stained with FITC-labeled anti-CD90, PE-labeled anti-IgM and APC-labeled anti-CD11c antibodies for 30 minutes.

Results

Splenomegaly and lymphadenopathy in FLT3L transgenic mice.

To investigate the effect of prolonged FLT3L over-expression, we generated mice expressing the human \( \text{FLT3L} \) gene under the control of the \( \beta\text{-actin} \) promoter (hereafter FLT3L-Tg mice). FLT3L levels in the blood were at the range of 500-1000 ng/ml, as assessed by ELISA using an anti-hFLT3L antibody developed in our laboratory (data not shown). FLT3L-Tg mice were viable, 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 8-14 week old FLT3L-Tg mice was \( 451 \pm 127 \times 10^6 \) cells compared to \( 71 \pm 12 \times 10^6 \) 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\(^+\) dendritic cells (DC) (Figure 1B). Analysis of axillary, brachial and inguinal lymph node cellularity demonstrated an increase from \( 23 \pm 2.6 \times 10^6 \) cells in WT to \( 69.6 \pm 3.5 \times 10^6 \) cells in FLT3L-Tg mice (Figure 1A). Thymus cellularity and CD4\(^+\)/CD8\(^+\) T cell
subpopulations did not show any difference 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 (2 femurs and 2 tibias) was increased from $40 \pm 7.7 \times 10^6$ cells in WT to $73.8 \pm 14.5 \times 10^6$ 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$^+\text{CD117}^+$ preB1 population but a dramatic 12-fold (from $7 \pm 2 \times 10^6$ in WT to $0.6 \pm 0.3 \times 10^6$ cells in FLT3L-Tg) and 144-fold (from $8.3 \pm 2.2 \times 10^6$ in WT to $0.06 \pm 0.09 \times 10^6$ in FLT3L-Tg) decrease in CD19$^+\text{CD117}^+\text{IgM}^-$ preB2 and CD19$^+\text{IgM}^+$ 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$^+\text{SiglecH}^+$ plasmacytoid DC (pDC) showed a 41-fold increase (from $0.74 \pm 0.15 \times 10^6$ in WT to $30 \pm 8.8 \times 10^6$ in FLT3L-Tg), while CD11c$^+\text{SiglecH}^-$ conventional DC (cDC) numbers were increased 13-fold (from $0.3 \pm 0.06 \times 10^6$ in WT to $2.5 \pm 1.2 \times 10^6$ in FLT3L-Tg). 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-Tg 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 $2.7 \pm 0.2 \times 10^6$ cells in WT to $0.5 \pm 0.2 \times 10^6$ cells in FLT3L-Tg mice (Figure 2A). This decrease in TER119$^+$ erythroid progenitors prompted us to analyze the development of megakaryocyte/erythrocyte lineage in FLT3L-Tg mice. Hematocrit analysis showed that FLT3L-Tg mice manifested a significant decrease in hematocrit (from $46.4 \pm 2$ in WT to $34 \pm 3$ in transgenics) already at 8-10 weeks of age, which dropped even further to $21.6 \pm 5.9$ at 19-22 weeks of age (Figure 2B). Blood platelet counts also showed a significant drop, from
400 ± 89 x 10^8 cells/ml in WT to 207.5 ± 61 x 10^8 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 (Figure 2D), which was confirmed by a quantitative analysis demonstrating a 27-fold increase in numbers of leukocytes in FLT3L-Tg blood (Suppl. Figure 1).

Expansion of myeloid and lymphoid populations in spleens of FLT3L-Tg 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 ± 0.7 x 10^6 cells in WT to 30.1 ± 7.6 x 10^6 cells in transgenics (Figure 3A). NK1.1⁺ natural killer (NK) cells were also significantly increased from 2.7 ± 0.8 x 10^6 in WT to 23 ± 6.3 x 10^6 (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⁺ pDC (95.8 ± 26 x 10^6 versus 0.26 ± 0.06 x 10^6 in WT), 208-fold for CD11c⁺CD11b⁺ cDC (91.7 ± 25 x 10^6 versus 0.44 ± 0.29 x 10^6 in WT) and 161-fold for CD11c⁺CD8α⁺ cDC (21.6 ± 7.2 x 10^6 versus 0.13 ± 0.05 x 10^6 in WT) (Figures 3 B, D). These expanded DC populations in our FLT3L-Tg mice were shown to be functional (Suppl. Figure 2). We conclude from this data that the massive expansion of myeloid, NK 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 ± 4.3 x 10^6 in WT to 37.4 ± 19 x 10^6 cells in FLT3L-Tg) in splenic CD4⁺ T cells (Figure 3C). The increase in CD8⁺ T cell number was smaller (2.4-fold); from 7.8 ± 2.8 x 10^6 cells in WT to 18.9 ± 8.4 x 10^6 cells in FLT3L-Tg mice. Using intracellular FACS staining, a 7.4-fold increase in Foxp3⁺CD4⁺ regulatory T cells (Tregs) (from 1.36 ± 0.2 x 10^6 in WT to 10.14 ± 1.3 x 10^6 in FLT3L-Tg), 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 immune response in FLT3L-Tg. 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 ELISA. Anti-NIP IgG responses were somewhat weaker in FLT3L-Tg mice compared to WT, even though a significant increase in IgG titers was still observed after immunization (Suppl. Figure 3).

**Expansion of hematopoietic progenitors in the BM of FLT3L-Tg mice.**

Increased availability of FLT3-ligand 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 LMPP (Lymphoid-primed Multipotent Progenitor Population)(27), CLP (Common Lymphoid Progenitors)(28, 29) and CMP (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 (Suppl. Figure 4). Therefore, we have not used 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^lowCD19/NK1.1^-) numbers from 0.2 ± 0.1 x 10^6 cells in WT to 2.8 ± 0.9 x 10^6 cells in FLT3L-Tg mice (Figures 4 A, B). Increases in CLP (CD117^Sca1^-CD127^-) progenitors were even higher with a 75-fold increase from 0.04 ± 0.01 x 10^6 cells in WT to 3.2 ± 1.3 x 10^6 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 are reduced in FLT3L-Tg mice. Indeed, further analysis revealed these cells were CD11c^-SiglecH^ pDC, which had expanded 41-fold in FLT3L-Tg mice BM and were all CD127^ (Suppl. Figure 5). CD117^CD115^-Sca1^- myeloid progenitors were also found to be significantly increased in FLT3L-Tg mice (from 0.4 ± 0.1 x 10^6 cells in WT to 5.1 ± 1.4 x 10^6 in FLT3L-Tg). The Lineage^-Sca1^-CD117^ (LSK) fraction, which is to a large extent FLT3^+ (Suppl. Figure 4), was also significantly increased in FLT3L-Tg mice (Figures 4 C, D). Further staining of LSK cells with CD150 and
CD48 (31) revealed that this increase is mainly due to an increase in CD150
CD48 MPPs (Suppl. Figure 6), 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 (CMP), as well as progenitors with restricted
Granulocyte-Macrophage (GMP) and Megakaryocyte-Erythrocyte (MEP) potential(30, 33).
We found a dramatic 9.7-fold decrease in numbers of MEPs in FLT3L-Tg mice compared to WT,
as well as a significant 5.2-fold increase in GMPs (Figures 4 C, D). Importantly, gene expression
analysis of the LSK compartment demonstrated reduced levels of the
megakaryocyte/erythroid lineage genes Mpl and Klf1, while the expression of myeloid-
specific genes Mpo, Csf3r and Cebpa was increased (Suppl. Figure 7). These results indicate
that increased FLT3L levels skew development towards the myeloid/lymphoid and away
from the megakaryocyte/erythroid pathway 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, CLPs and EPLMs were sorted from CD45.2 FLT3L-Tg mice
and transplanted into CD45.1 congenic mice. Analysis of recipient mice confirmed the B-cell
potential of both FLT3L-Tg CLPs and EPLMs, while the T-cell potential of transgenic CLPs
was retained but compromised by poor donor reconstitution of the thymus (Suppl. Figures 8
and 9). 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 (Suppl. Figure 10). 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). Alternatively, over-expression of FLT3L could result in a vast expansion of FLT3+ cells which could leave little space and/or recourses 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 supportive of either model, we injected WT mice with recombinant FLT3L and monitored kinetic changes in numbers of different hematopoietic lineages. As shown in Figures 5 A 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 post 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 pDC 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 expansion of these FLT3+ cells, shown previously(24, 25). CD19+CD117+ preB1 cells showed little reduction; rather 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+CD117IgM- preB2 and CD19+IgM+ B cells were reduced after FLT3L injection, becoming significant after 5 days (Figure 5 E, F). Overall, our FLT3L injection data would point towards an instructive role of FLT3L in development of certain hematopoietic lineages.
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 would suggest that this cytokine exerts its role mainly in concert with other hematopoietic cytokines, such as Stem Cell Factor (SCF) or Interleukin-7 (IL-7)(19, 22). Administration of FLT3L to adult mice in vivo has been used as a means of elucidating its 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 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 where either FLT3L was administered in vivo(24, 25) or when FLT3L was conditionally over-expressed by transgenesis(35). The importance of FLT3L in DC generation has been shown for cDC and pDC, both of which are FLT3+ populations. Our results are in accordance with these observations. Particularly striking was the elevated numbers of pDC, the population most increased in our transgenic mice. Plasmacytoid DC are considered an important part of anti-viral immunity, mainly through their production of IFN-α(36). The dramatic increase of IFN-α producing pDC 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 the ex vivo isolation of vast numbers of functional DC populations.

A significant reduction in CD19+CD117IgM- 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, pDC, 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 WT levels (Suppl. Figure 11). 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.

In the BM of FLT3L-Tg, numbers of FLT3+ lymphoid and myeloid progenitors were dramatically increased. 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 to 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 HSC. Given the very small numbers of CMP, CLP and EPLM in a WT BM, our FLT3L-Tg mice provide an excellent mouse model for the isolation of these progenitors in large numbers 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 would lead 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, the dramatic reduction in MEP and their rapid decrease following FLT3L treatment argues 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 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 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).

**Authorship and disclosures**

P.T., R.C. and A.R. designed the overall research, analyzed the data and wrote the manuscript; P.T. performed cell culture experiments, FACS analysis and PCR; L.K.S. generated the transgenic mice and performed initial FACS analysis; A.N. and G.C. performed FACS analysis; N.N. and M.K. performed the *in vivo* FLT3L and IL-7 treatment; H.R. performed histology; A.R. performed cell sorting and the *in vivo* transplantations.

The authors declare no financial or commercial conflict of interest.
References

Tables

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

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<td>WT</td>
<td>1 in 5</td>
<td>1 in 10</td>
</tr>
<tr>
<td>TG</td>
<td>1 in 10</td>
<td>1 in 3</td>
<td>1 in 6</td>
</tr>
</tbody>
</table>

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 3-6 for the different populations. CLP: Common Lymphoid Progenitor, EPLM: Early Progenitors with Lymphoid and Myeloid potential, WT: wild-type, TG: FLT3L transgenics.
Figure legends

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 femurs and 2 tibias) of 8-14 week old wild type (WT - white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001. B. Immunofluorescence of spleen sections from 8-14 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) B cells in WT (white symbols) and FLT3L-Tg (black symbols) mice (5-7 mice per group). ***: P<0.0001. D. CD11c-SiglecH+ plasmacytoid (pDC) and CD11c~SiglecH~ conventional (cDC) dendritic cell 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 femurs and 2 tibias) of 8-14 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).
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⁺ NK cells in spleens of 8-14 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.

Figure 4. Hematopoietic progenitor populations in FLT3L-Tg mice.

Figure depicts numbers (B and D) and FACS stainings (A and C) of hematopoietic progenitor populations in the bone marrow (2 femurs and 2 tibias) of 8-14 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 (centre 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).
Figure 5. Kinetics of changes in percentages of hematopoietic populations following FLT3L injections into wild type mice.

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+CD117IgM- 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.

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

\[ FLT3 \]

MPP

+ FLT3L

- FLT3L

\[ FLT3 \]

lymphoid/myeloid progenitor

megakaryocyte/erythrocyte progenitor
Supplementary Figure 1

Leukocytosis in FLT3L-Tg blood.

Blood was drawn from the tails of 8-14 week old WT (white circles) and FLT3L-Tg (black circles) mice. Following 5 minute incubation with NH₄Cl to lyse erythrocytes, live white blood cells were counted in a Neubauer hemocytometer. ***: P<0.0001
Supplementary Figure 2

Functional analysis of FLT3L-Tg DC subsets

A. CD11c+SiglecH+ pDC were sorted from FLT3L-Tg spleens and stimulated with increasing amounts of CpG for 24 hours. Following stimulation, supernatants were collected and the amount of IFN-α produced was quantified using the Mouse IFN-alpha Platinum ELISA (eBioscience) according to manufacturer’s instructions. B. CD11c+CD11b+ cDC were sorted from spleens of FLT3L-Tg mice [(C57BL/6 x DBA/2)F1, herein named BDF1] and incubated with lymph node (LN) cells from WT C57BL/6 mice, as a source of T cells, for 5 days. Following incubation, proliferation of LN cells was quantified by measuring 3[H]-Thymidine incorporation. As a negative control, spleen cells from WT C57BL/6 were incubated with WT C57BL/6 LN cells (B6), while incubation of WT BDF1 spleen cells with WT C57BL/6 LN cells was used as a positive control for T cell proliferation (BDF1).
Supplementary Figure 3

IgG anti-NIP titers of FLT3L-Tg sera after immunization.

Levels of anti-NIP IgG as assessed by ELISA analysis in the blood of WT (white symbols) and FLT3L-Tg (black symbols) mice before (circles) and 13 days after (squares) immunization with NIP
Supplementary Figure 4

FLT3 expression in WT and FLT3-Tg hematopoietic progenitors.

FACS stainings of hematopoietic progenitor populations in WT (red line) and FLT3L-Tg (blue line) mice. Cells were identified by FACS following the staining strategy showed in Figure 4A and additionally stained with an anti-CD135 (FLT3) biotinylated antibody, followed by Streptavidin-PECy7 staining. Grey filled histogram: no anti-FLT3 antibody.
Supplementary Figure 5

CD127 expression on FLT3L-Tg pDC

FACS analysis of CD11c^+SiglecH^+ pDC from FLT3L-Tg BM incubated with a biotinylated anti-CD127 antibody, followed by incubation with PE-labeled streptavidin. Thin line histogram: no anti-CD127 antibody, thick line histogram: staining with anti-CD127 antibody.
Supplementary Figure 6

CD150/CD48 staining of LSK cells in WT and FLT3L-Tg mice.

Upper panels: representative FACS plots of LSK cells in WT and FLT3L-Tg mice. LSK cells were stained as shown in Figure 4C and additionally stained with an anti-CD150-PE and anti-CD48-APC-Cy7 antibodies. Percentages in gates indicate the percentage of cells within the corresponding LSK population. Lower panel: total numbers of CD150<sup>+</sup>CD48<sup>+</sup> (circles), CD150<sup>+</sup>CD48<sup>-</sup> (squares) and CD150<sup>-</sup>CD48<sup>-</sup> (triangles) LSK cells in WT (white) and FLT3L-Tg (black) mice (7 mice per group).
Supplementary Figure 7

Quantitative PCR gene expression analysis of LSK cells in WT and FLT3L-Tg mice.

LSK cells from WT and FLT3L-Tg mice were sorted after staining as in Figure 4C. RNA was extracted, cDNA synthesized and quantitative real-time PCR performed for detection of the indicated genes expression levels relative to the $Hprt$ gene. Bars indicate fold difference in expression levels between FLT3L-Tg and WT (set as 1). Figure shows the results from one of two independent experiments.
Supplementary Figure 8

T and B cell potential of FLT3L-Tg CLP.

CD117\textsuperscript{+}Sca1\textsuperscript{+}CD127\textsuperscript{+} CLP were sorted from CD45.2 WT or FLT3L-Tg mice and intravenously transplanted into lethally irradiated congenic CD45.1 recipients (2.000 WT and 4.000 FLT3L-Tg CLP per mouse) together with 5 x 10\textsuperscript{5} unfractionated CD45.1 BM cells. Analysis of T and B cells was performed 3 weeks after transplantation. Figure shows analysis of one representative out of 3 recipient mice, for each genotype. A. CD4 and CD8 staining of thymus. B. CD19 and IgM staining of spleen. All donor and recipient mice used were 8-12 weeks old.
Supplementary Figure 9

*In vivo* B cell potential of FLT3L-Tg EPLM

Four thousand (WT) or 8,000 (FLT3L-Tg) EPLM were sorted from CD45.2 mice and intravenously injected into lethally irradiated congenic CD45.1 recipients together with 5 x 10^5 unfractionated CD45.1 BM cells. Spleens were analyzed 3 weeks following transplantation. Figure shows CD19/IgM FACS analysis of one representative out of 4 recipient mice, for each genotype. All donor and recipient mice used were 8-12 weeks old.
Supplementary Figure 10

Myeloid and erythroid in vivo potential of WT and FLT3L-Tg LSK cells.

Ten thousand WT and 15,000 FLT3L-Tg LSK cells (CD45.2) were sorted and intravenously injected into congenic CD45.1 recipients together with 3 x 10^5 unfractionated CD45.1 BM cells, after lethal irradiation of the hosts. BM were analyzed 7-10 days after transplantation and the percentage of the indicated hematopoietic cells within the CD45.2 compartment was calculated. For WT LSK 6 mice and for FLT3L-Tg 2 mice were transplanted. Upper-right panel: total percentage of CD45.2^+ cells at the time of the analysis. *:P=0.0449. Error bars indicate Standard Error of the Mean (SEM). All donor and recipient mice used were 8-12 weeks old.
Supplementary Figure 11

CD19+ cell percentages following IL-7/anti-IL-7 complexes injections in mice

WT (squares) and FLT3L-Tg mice (circles) were injected with PBS (white symbols) or 15 µg/mouse IL-7/anti-IL-7 complexes (black symbols) three times with 3-day intervals. BM (2 femurs and 2 tibias) were analyzed 2 days after the last injection for the percentages of CD19+CD117+ (preB1), CD19+CD117IgM− (preB2) and CD19−IgM+ (B) cells. ***(preB1): P=0.0006, ***(preB2): P=0.0001, **: P=0.0014.
Supplementary materials and methods

Generation of FLT3L-Tg mice.

Transgenic mice were initially generated in a C57BL/6 background. We have previously shown that in a model of C57BL/6 lymphocyte transplantation into (C57BL/6 x DBA/2)F1 hosts, treatment of the hosts with FLT3L provides protection against acute Graft Versus Host Disease(1). This prompted us to cross our C57BL/6 FLT3L-Tg mice to DBA/2 mice in order to obtain (C57BL/6 x DBA/2)F1 mice with sustained elevated levels of FLT3L. Subsequent analysis of both the C57BL/6 and (C57BL/6 x DBA/2)F1 genotypes revealed similar levels of FLT3L in their blood and an identical effect of FLT3L over-expression in hematopoietic populations (data not shown), with the exception of more profound splenomegaly in (C57BL/6 x DBA/2)F1 mice. All the data presented herein are from (C57BL/6 x DBA/2)F1 FLT3L-Tg mice, with the exception of certain transplantation experiments (Suppl. Figures 8, 9 and 10) where CD45.2 C57BL/6 FLT3L-Tg mice were used.

Flow cytometry and cell sorting.

FITC-, PE-, APC-, PE-Cy7- and APC-Cy7- and biotin-conjugated monoclonal antibodies specific for CD11c (HL3), CD11b (M1/70), CD117 (2B8), CD19 (1D3), CD127 (A7R34), CD115 (AFS98), CD8α (53-6.7), CD4 (GK1.5), SiglecH, GR1 (RB6-8C5), IgM (M41), B220 (RA3-6B2), Sca1 (D7), TER119, F4/80, CD34 (RAM34), CD16 (2.4G2), CD150 (TC15-12F12.2), CD48 (HM48-1), FLT3 (A2F10), NK1.1 (PK136), CD45.1 (A20) and CD45.2 (1D4-2.1) were purchased from BD Biosciences or e-Biosciences or were made in our laboratory. Staining, flow cytometry and cell sorting were performed as previously described(2, 3).
**In vitro** limiting dilution assays.

OP9, OP9DL1 or ST2 stromal cells were plated in 96-well plates one day before the initiation of the experiment at 4000 cells per well. At the day of the experiment stromal cells were irradiated and subsequently co-cultured with hematopoietic progenitors at different concentrations. Cultures have been monitored with inverted microscope for generation of lymphoid or myeloid cell colonies and after 2 (for OP9 cell cultures) or 3 weeks (for OP9DL1 and ST2 cell cultures) the total number of wells with no colonies was scored. For each experiment the number of wells with no colonies was plotted against the number of hematopoietic progenitors plated and the fraction of progenitor cells developing lymphoid or myeloid colonies was estimated.

**Quantitative real-time PCR.**

RNA extraction was performed using TRI Reagent® (Life Technologies) followed by cDNA synthesis using GoScript™ Reverse Transcriptase (Promega). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). Primers used were: \textit{Mpl}: Mpl-F: CATCCCAACCGCATGGACC; Mpl-R: TGGAGCCAGTAGGATTTGCC; \textit{Klf1}: Klf1-F: TACACCAAGAGCTCGCACCT; Klf1-R: GACGATGTCCAGTGTGCTTC; \textit{Mpo}: Mpo-F: GCTGGAGAGTCGTGTTGGAA; Mpo-R: GAGCAGGCAAATCCAGTCCT; \textit{Csf3r}: Csf3r-F: GATGTTGCCCCCACCATCAG; Csf3r-R: ATCTGGGGAACTCCAGGACA; \textit{Cebpa}: Cebpa-F: CCATGCCGGGAGAAGTCTCAA; Cebpa-R: CTCTGGGAGGTGACTGCTCATC.

**Supplementary References**