SUPPLEMENTARY APPENDIX

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

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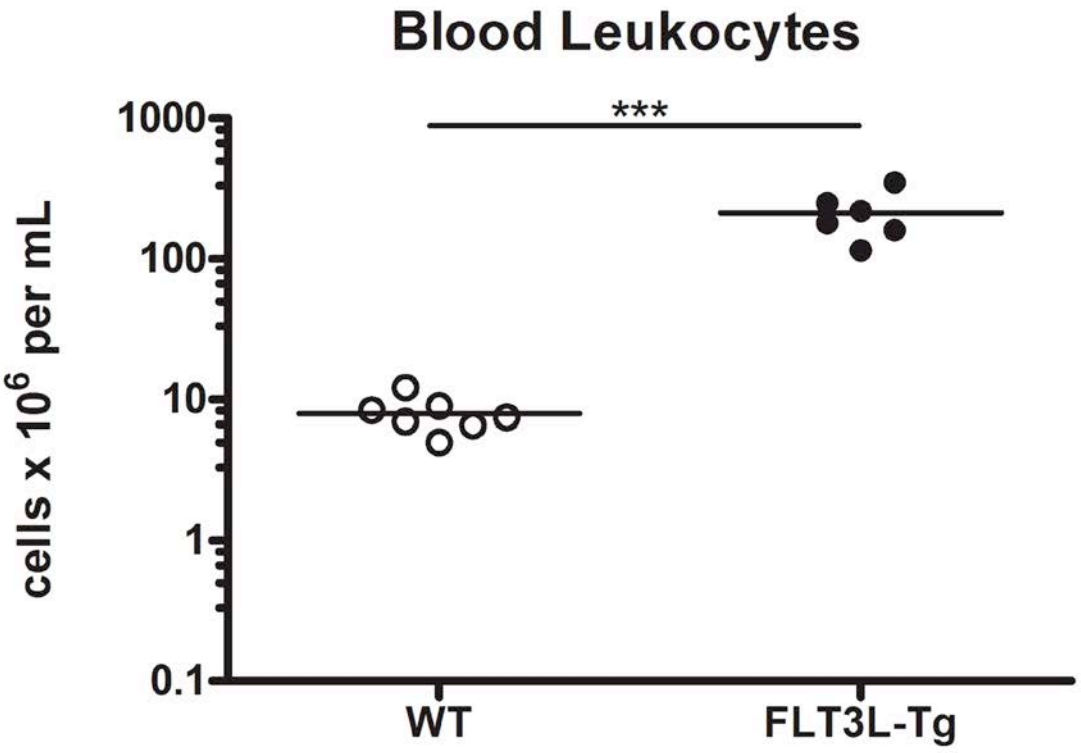
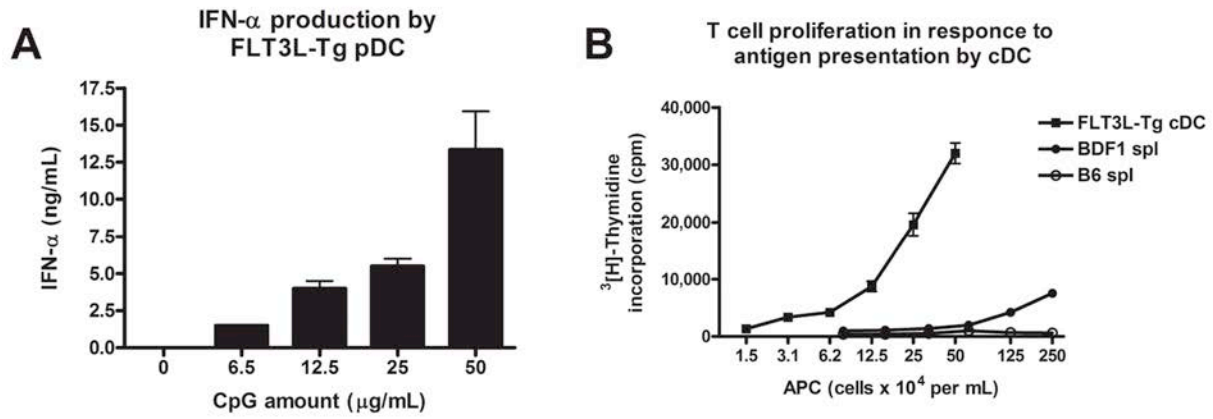


Supplementary Figure 1

Leukocytosis in FLT3L-Tg blood.

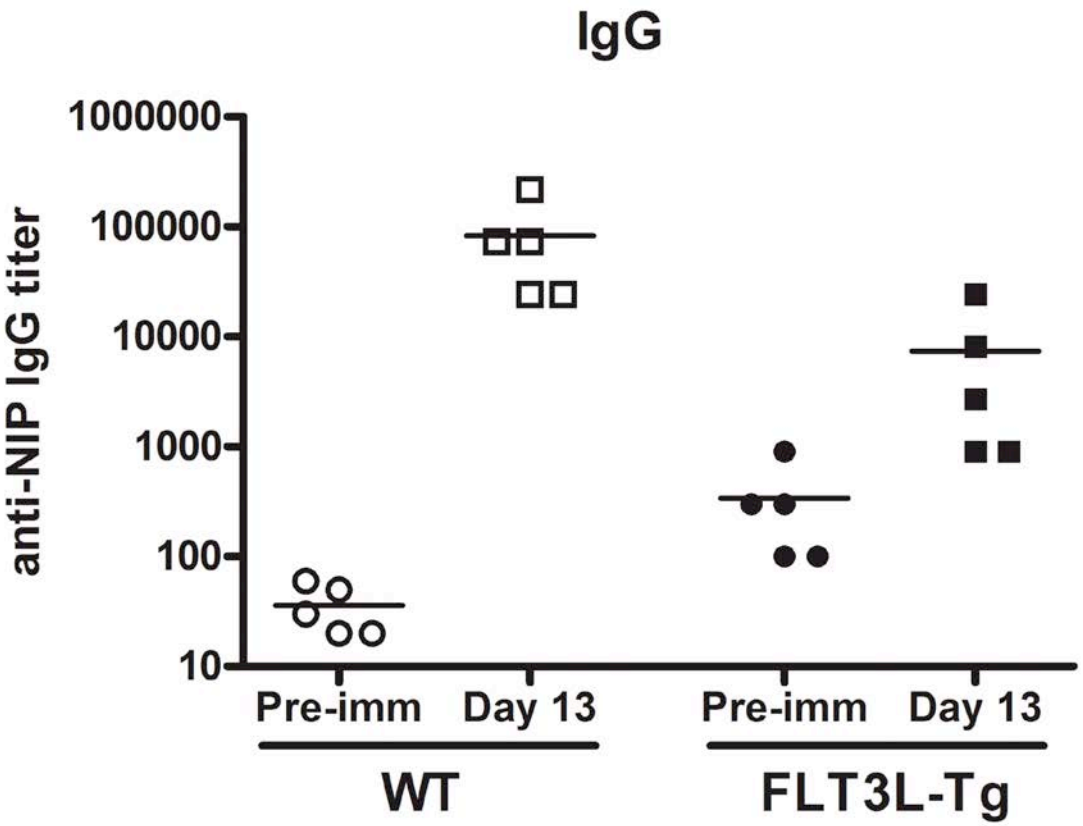
Blood was drawn from the tails of 8-14 week old WT (white circles) and FLT3L-Tg (black circles) mice. Following 5 minute incubation with NH_4Cl to lyse erythrocytes, live white blood cells were counted in a Neubauer hemocytometer. ***: P<0.0001



Supplementary Figure 2

Functional analysis of FLT3L-Tg DC subsets

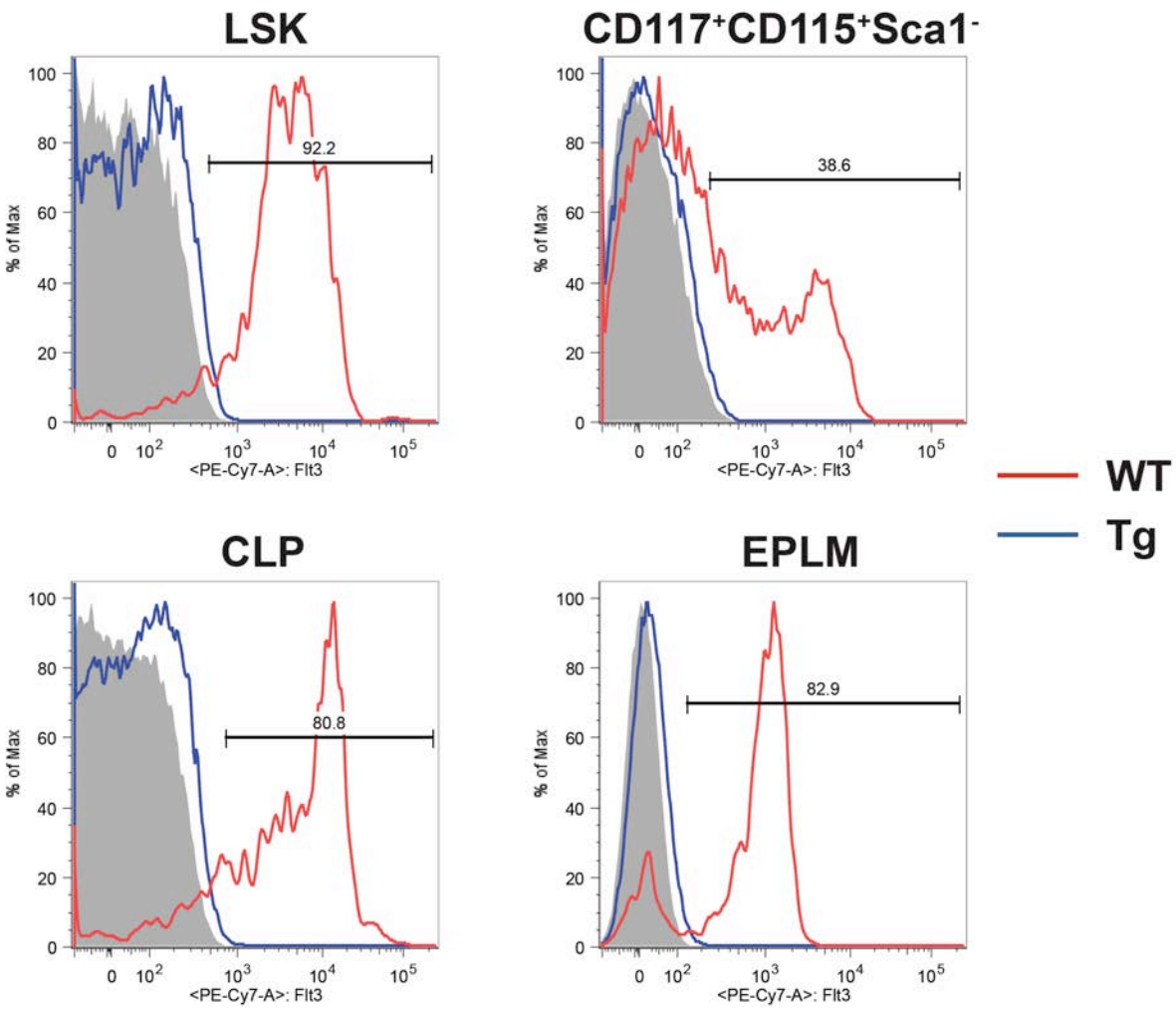
A. $CD11c^{+}SiglecH^{+}$ pDC were sorted from FLT3L-Tg spleens and stimulated with increasing amounts of CpG for 24 hours. Following stimulation, supernatants were collected and the amount of IFN-α produced was quantified using the Mouse IFN-alpha Platinum ELISA (eBioscience) according to manufacturer's 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.

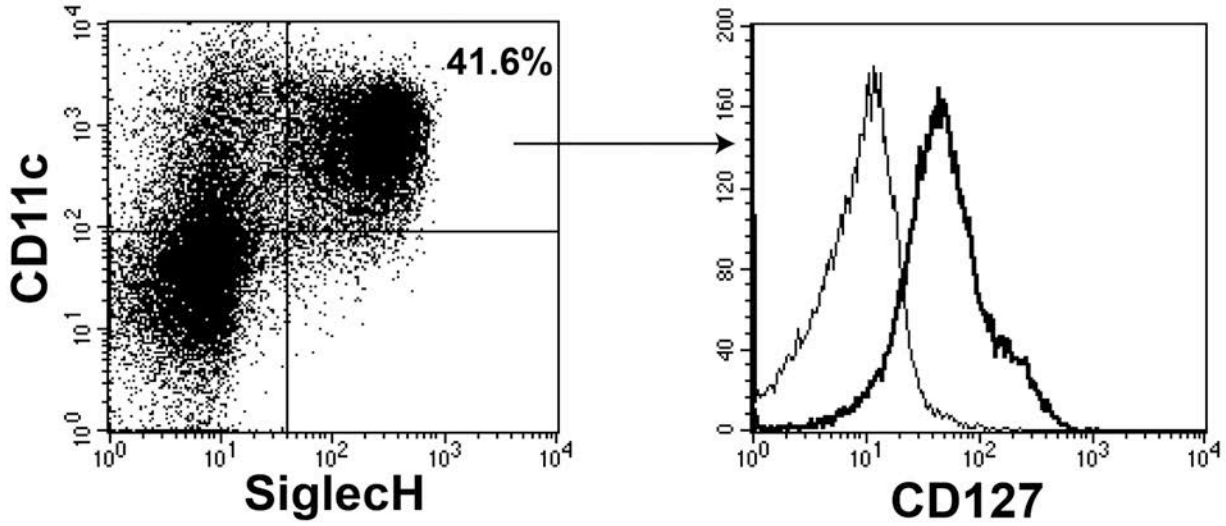
Levels of anti-NIP IgG as assessed by ELISA analysis in the blood of WT (white symbols) and FLT3L-Tg (black symbols) mice before (circles) and 13 days after (squares) immunization with NIP



Supplementary Figure 4

FLT3 expression in WT and FLT3-Tg hematopoietic progenitors.

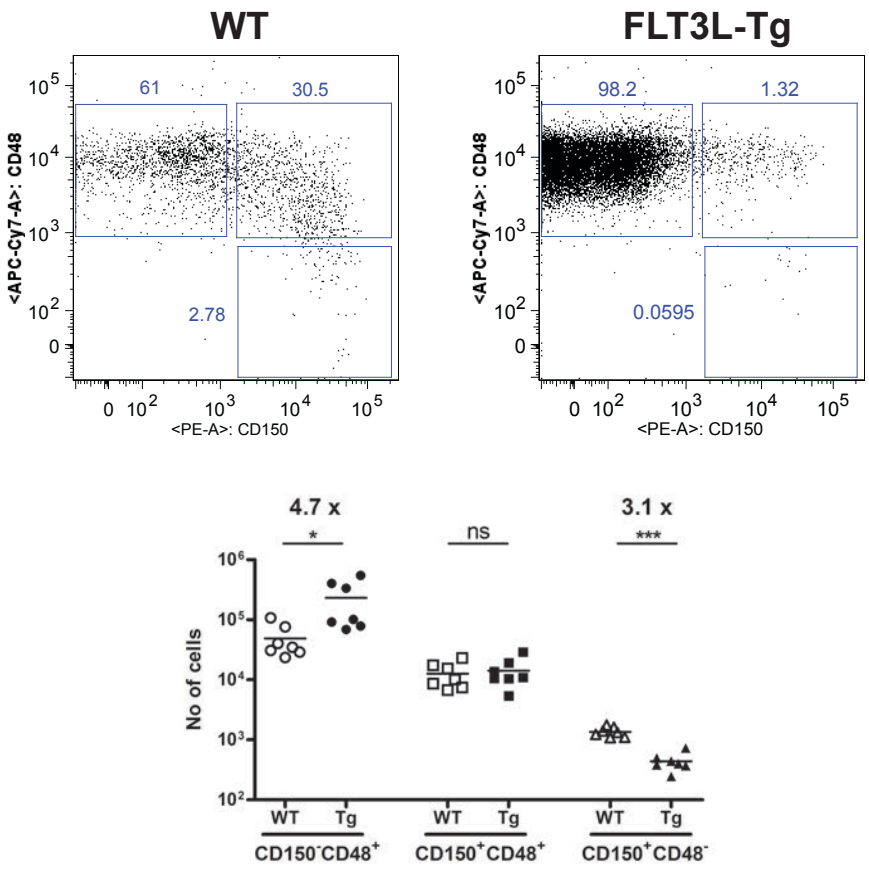
FACS stainings of hematopoietic progenitor populations in WT (red line) and FLT3L-Tg (blue line) mice. Cells were identified by FACS following the staining strategy showed in Figure 4A and additionally stained with an anti-CD135 (FLT3) biotinylated antibody, followed by Streptavidin-PECy7 staining. Grey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



Supplementary Figure 5

CD127 expression on FLT3L-Tg pDC

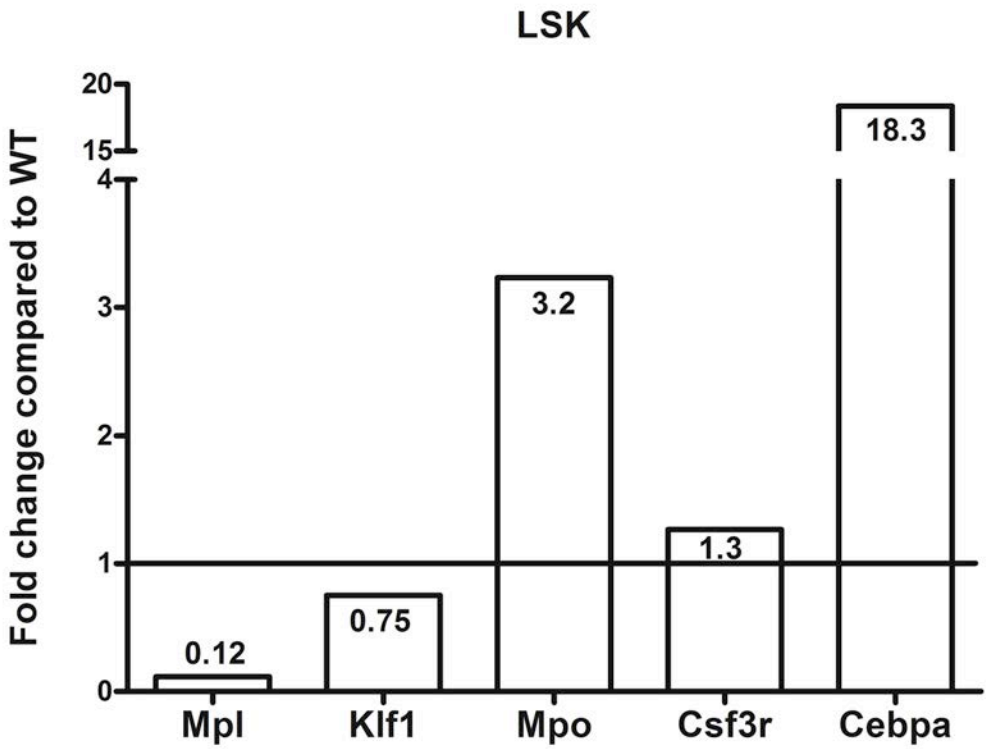
FACS analysis of $CD11c^{+}SiglecH^{+}$ pDC from FLT3L-Tg BM incubated with a biotinylated anti-CD127 antibody, followed by incubation with PE-labeled streptavidin. Thin line histogram: no anti-CD127 antibody, thick line histogram: staining with anti-CD127 antibody.



Supplementary Figure 6

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

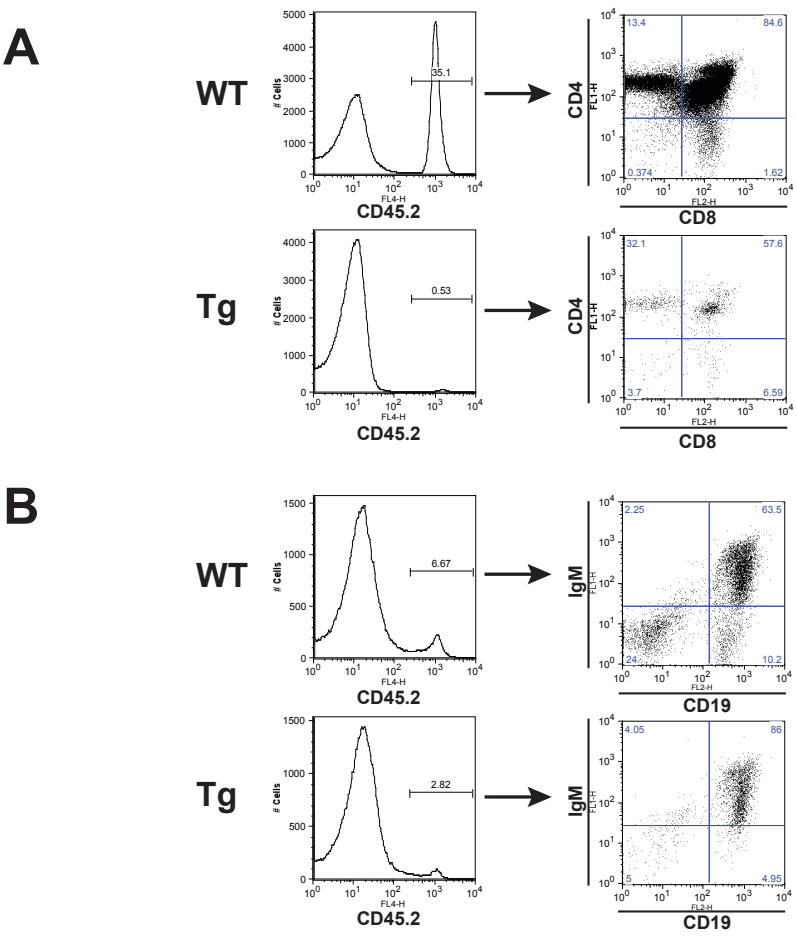
Upper panels: representative FACS plots of LSK cells in WT and FLT3L-Tg mice. LSK cells were stained as shown in Figure 4C and additionally stained with an anti-CD150-PE and anti-CD48-APC-Cy7 antibodies. Percentages in gates indicate the percentage of cells within the corresponding LSK population. Lower panel: total numbers of $CD150^{-}CD48^{+}$ (circles), $CD150^{+}CD48^{+}$ (squares) and $CD150^{+}CD48^{-}$ (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.

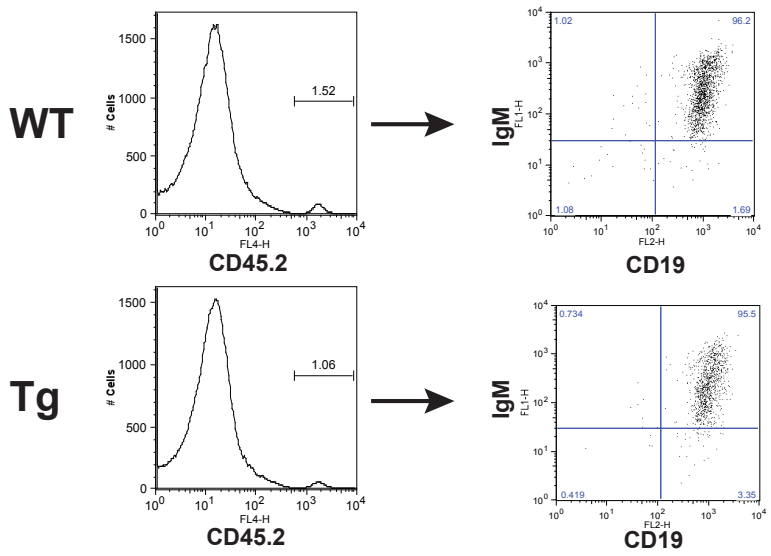
LSK cells from WT and FLT3L-Tg mice were sorted after staining as in Figure 4C. RNA was extracted, cDNA synthesized and quantitative real-time PCR performed for detection of the indicated genes expression levels relative to the *Hprt* gene. Bars indicate fold difference in expression levels between FLT3L-Tg 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.

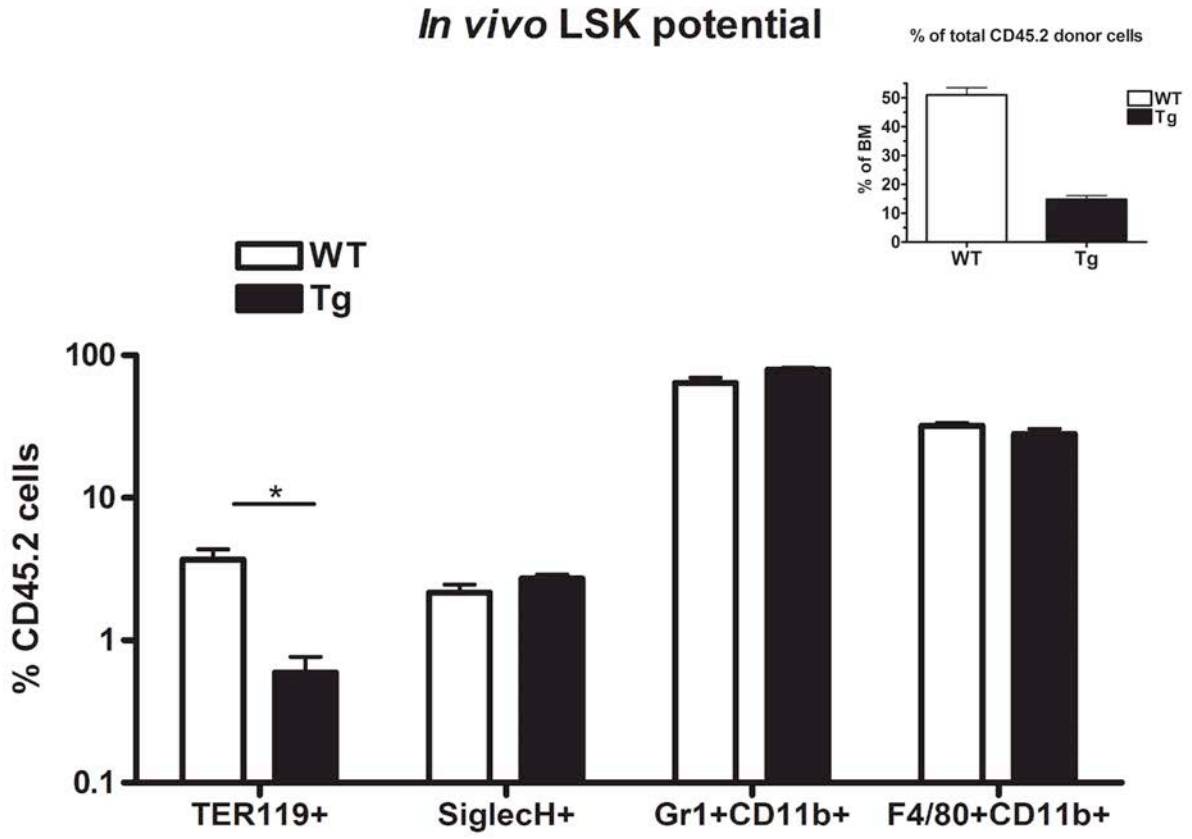
$CD117^{+}Sca1^{+}CD127^{+}$ CLP were sorted from CD45.2 WT or FLT3L-Tg mice and intravenously transplanted into lethally irradiated congenic CD45.1 recipients (2.000 WT and 4.000 FLT3L-Tg CLP per mouse) together with 5 x 10^{5} unfractionated CD45.1 BM cells. Analysis of T and B cells was performed 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**

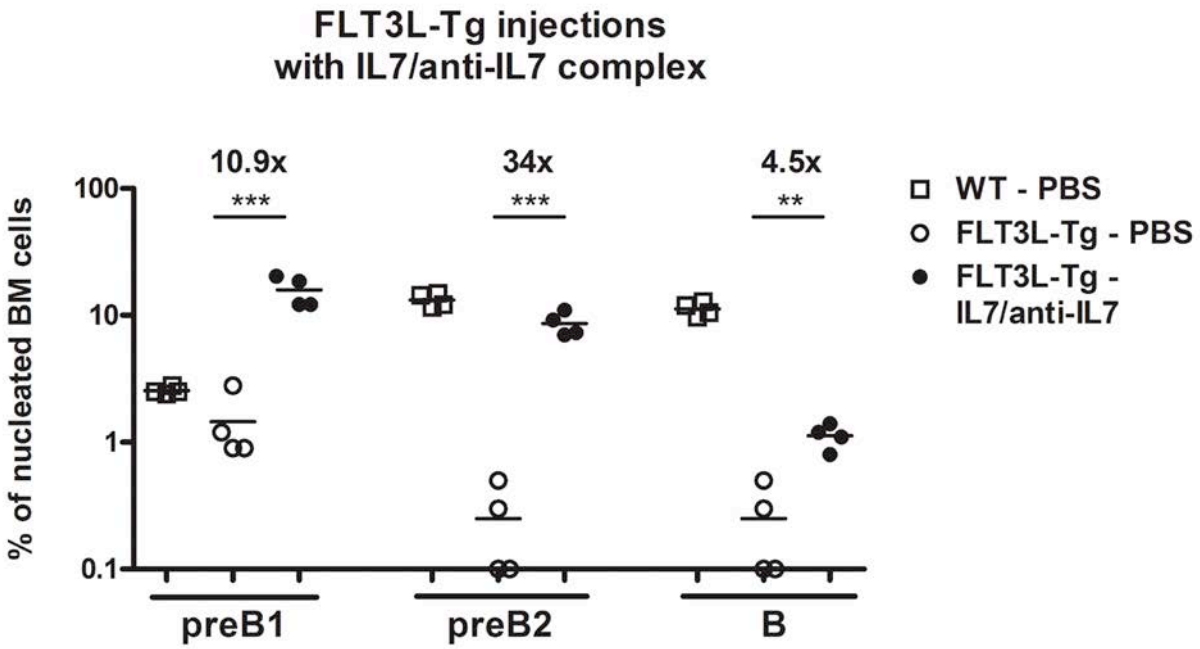
Four thousand (WT) or 8.000 (FLT3L-Tg) EPLM were sorted from CD45.2 mice and intravenously injected into lethally irradiated congenic CD45.1 recipients together with 5 x 10^5 unfractionated CD45.1 BM cells. Spleens were analyzed 3 weeks following transplantation. Figure shows CD19/IgM FACS analysis of 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$^+$CD117$^-$IgM$^-$ (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: *Mpl*: Mpl-F: CATCCCAACCGCATGGACC; Mpl-R: TGGAGCCAGTAGGATTTGCC; *Klf1*: Klf1-F: TACACCAAGAGCTCGCACCT; Klf1-R: GACGATGTCCAGTGTGCTTC; *Mpo*: Mpo-F: GCTGGAGAGTCGTGTTGGAA; Mpo-R: GAGCAGGCAAATCCAGTCCT; *Csf3r*: Csf3r-F: GATGTTGCCCCCACCATCAG; Csf3r-R: ATCTGGGGAACTCCAGGACA; *Cebpa*: Cebpa-F: CCATGCCGGGAGAACTCTAA; Cebpa-R: CTCTGGAGGTGACTGCTCATC.

Supplementary References

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3. Balciunaite G, Ceredig R, Massa S, Rolink AG. A B220+ CD117+ CD19- hematopoietic progenitor with potent lymphoid and myeloid developmental potential. European Journal of Immunology. 2005;35(7):2019-30.