Cathepsin K is required for maintenance and regeneration of lymphopoiesis in vivo

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Abstract

Cathepsin K (CTSK) is a major collagen-degrading protease in osteoclasts. Defects in CTSK activity are directly linked to skeletal abnormalities such as osteoporosis and osteopetrosis. The role of CTSK in cell types other than osteoclasts is poorly understood. Stromal cells, which maintain hematopoietic stem cells (HSCs) during *in vitro* culture, show high transcription of the *Ctsk* gene. Here, we investigated the influence of the absence of *Ctsk* on the hematopoietic system. We found that *in vitro* cultures with Lineage^{-} SCA1^{+} KIT^{+} (LSK) cells on *Ctsk* deficient stromal cells display reduced colony formation and proliferation, with increased differentiation, whilst subsequent *in vivo* experiments show impaired HSC maintenance of lymphopoiesis of *in vitro* cultured cells. *In vivo*, deletion of *Ctsk* does not show gross effects on early hematopoiesis or myelopoiesis. But, in the bone marrow (BM), T cell numbers are significantly reduced. Lymphocyte deregulation is also found in transplantation experiments, which revealed that *Ctsk* is required for optimal regeneration not only of T cells, but also of B cells. Interestingly, environmental *Ctsk* is involved in both B- and T-lymphopoiesis, but T cell development in the bone marrow additionally requires an intrinsic *Ctsk*-dependent process. Thus, our study shows that *Ctsk* is required for the maintenance of HSCs and proper lymphopoiesis *in vitro* and *in vivo*.

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Introduction

Skeletal tissues are constantly remodeling due to the activity of bone-remodeling osteoblasts and osteocytes, and bone-resorbing osteoclasts. The bone marrow (BM) is further harboring hematopoietic stem cells (HSCs), which are maintained in endosteal arteriolar and sinusoidal niches (1). Mobilization of HSCs to the periphery in hematopoietic stress or when induced by therapeutic granulocyte colony-stimulating factor is mediated by the release of proteases, such as dipeptidyl peptidases (including CD26), metalloproteases (such as MMP9), and cathepsins secreted from osteoclasts (2). The lysosomal cysteine protease cathepsin K (gene *Ctsk*, protein: CTSK) cleaves collagens, osteonectin and fibrinogen and as such, plays a crucial role in bone resorption (3). Deregulation of CTSK may result on osteoporosis by overactivation such as in osteoclast-mediated bone destruction in bone marrow tumors as well as in postmenopausal osteoporosis. Inactivation of CTSK by mutations in the *Ctsk* gene are further associated with increased bone density (osteopetrosis) in pycnodysostosis, an inheritable condition marked by skeletal abnormalities (4). *Ctsk* is not only expressed by osteoclasts, but it is also found in other cell types such as BM stromal cells.

The role of *Ctsk* expression in BM stromal cells is poorly investigated. We have previously described that expression of genes coding for several niche factors such as *Ctsk*, *Ptn*, *Sfrp1*, *Sfrp2*, and *Wnt5a* are preferentially expressed by HSC-supportive stromal cells (5, 6). Deletion of *Ptn (7)*, *Sfrp1 (8)*, *Sfrp2 (9),* or *Wnt5a (10)* in stromal cells results in secondary stimulation of cell cycle progression of HSCs with reduced or complete loss of HSC self-renewal (11). However, the physiologic role of CTSK in stromal cells of the hematopoietic niche has, so far, not been explored. We here show that knockdown of *Ctsk* in stromal cells impairs maintenance and engraftment potential of HSCs in co-cultures. *In vivo*, we find that *Ctsk* is particularly important for optimal regeneration of B- and T-lymphocyte compartments in different hematopoietic tissues.

Materials and Methods

Mice

Ctsk knockout mice (12) were bred for at least six generations on C57BL/6.J (B6) background. Age- and sex-matched B6 (CD45.2) control mice were obtained from Harlan Laboratories. In extrinsic transplantations, C57BL/6.Pep3b.Ptpcr (CD45.1) mice obtained from Taconic served as donors and recipients in intrinsic transplantations. 129S2/SvPasCrl (129; CD45.2) were obtained from Charles River Laboratories. *In vitro* experiments were conducted with 129xLy5.1(F1) (129xLy5.1) mice.

Animals were kept according to the Federation of Laboratory Animal Science Associations and institutional guidelines. The animal experiments were approved by the Government of Upper Bavaria.

Transplantation assay

Transplantation experiments for repopulating capacity were performed as previously described (7, 8, 10). All co-cultures were set-up with Lineage$^-$ (Lin$^-$) cells from 129xLy5.1 mice and transplanted into 129B6 (Ly5.2) recipients. For transplantation of primary cells, 2.5×10^5 freshly isolated BM cells were injected into lethally irradiated recipients. In extrinsic transplantations, Ly5.1 WT cells were transplanted into either Ly5.2 WT or *Ctsk*$^{-/-}$ recipients. In intrinsic transplantation settings, Ly5.2 WT or *Ctsk*$^{-/-}$ BM cells were transplanted into WT Ly5.1 recipients.

During the first five weeks after transplantation, recipient mice received antibiotic-treatment. Sixteen weeks after transplantation, mice were sacrificed and PBs, BM, spleens, thymi and lymph nodes were analyzed for engraftment of donor cells using the congenic system (Ly5.1/Ly5.2). Positive engraftment was defined as >1% positive myeloid and >1% lymphoid donor engraftment.

Flow cytometry analysis and cell sorting

Staining of cell surface antigens was performed with antibodies from Invitrogen except for PE-Cy5.5-streptavidin conjugate, which was purchased from eBioscience-ThermoFisher. The cell suspension was incubated in HF2+ buffer (Hank's balanced salt solution with 2% FCS and 10 µM HEPES buffer) for 15 min on ice in the dark. Hematopoietic subpopulations were separated as described previously (7, 8, 10). EPICS XL (Beckman Coulter) or CyAn ADP Lx P8 (Coulter- Cytomation) were used for FACS analysis; data were analyzed using FlowJo

software (Tree Star). Cell sorting was done with a MoFlo High Speed cell sorter (Beckmann Coulter).

Knock down of *Ctsk* UG26-1B6 and co-cultures with HSCs

UG26-1B6 stromal cells were cultured on 0,1% gelatin coated wells as described (13, 14). As previously described (7, 8), we used established stable *Ctsk* gene knockdowns in stromal cells. In brief, sh*RNA*^{mir} contructs for *Ctsk* gene (Open Biosystems - ThermoFisher) were transfected into Phoenix E packaging cell line. Collected virus particle were used for the infection of UG26-1B6 cells followed by selection with 5 µg/ml puromycin. As a control, empty vector pLKO.1 was used.

For cocultures, UG26-1B6 derivatives sh*Ctsk* and pLKO.1 were grown in 6 well plates to 90% confluence and irradiated with 30 Gy. $1.0x10^4$ Lin^- BM cells were plated on stromal cells and cultured in long–term culture medium (M5300; Stem Cell Technologies) supplemented with Hydrocortisone in a 1:1000 ratio, Penicillin/Streptomycin and GlutaMax (200mM, GIBCO-ThermoFisher) in a 1:100 ratio for two to four weeks. Each week half of the supernatant was replaced with fresh medium. After co-culture, cells were either used for colony forming unit and FACS analysis or for *in vivo* repopulation assay.

Hematopoietic Colony Assays

Colony forming assay was performed with growth factor-supplemented methylcellulose (Metho Cult GF 3434, STEMCELL Technologies). $2.5x10^4$ total BM cells, $1x10^4$ Lin^- BM cells or fractions of cell cultures were seeded into methylcellulose on two 3.5 cm dishes. After ten days of culture at 37°C, 5 % CO_2 and ≤95% humidity, the colonies formed were counted under the microscope and analyzed using flow cytometry.

Single cell culture

Serum-free Conditioned Medium (CM) was essentially generated as described (10, 15, 16) by incubating serum-free medium (BIT 9500, STEMCELL Technologies) for three days on irradiated (30 Gy) and confluent pLKO.1 and sh*Ctsk* stromal cells.

Single cell cultures were performed as previously described (10, 15, 16). Briefly, HSC-enriched $CD34^-$ $CD48^-$ $CD150^+$ Lin^- $SCA1^+$ KIT^+ ($CD34^-$ SLAM) cells were sorted from the lineage-depleted BM cells of 8 weeks old B6 control mice and deposited as single cells in round-bottomed 96-well plates. Each well was filled with 100µl of 0,22µm filtered CM, supplemented with 100ng/ml mSCF and 20ng/ml IL-11 (both from R&D Systems). The clone

size was counted each day under the light microscope. After five days, cells were harvested and analyzed with flow cytometry.

Statistical methods

Significance levels were determined with unpaired Students t-test when data followed a Gaussian distribution. When data were not normally distributed, we used the Mann-Whitney test, using GraphPad Prism 7.

Results

Reduction of stromal Cathepsin K impairs proliferation and clonogenic capacity of HSCs *in vitro*

To study how the absence of stromal *Ctsk* affects hematopoiesis, we generated *Ctsk*-knockdowns in the HSC supportive stromal cell line UG26-1B6 (5, 13) with sh*RNA*^{mir} (sh*Ctsk*). As control cells, we transduced stromal cells with pLKO.1 empty vector (control) (Figure 1A, 1B). To determine whether reduction of *Ctsk* mRNA would affect production of colony-forming cells, we set up co-cultures of WT Lin^{-} BM-cells with control and sh*Ctsk* UG26-1B6 cells. These experiments showed, that four weeks after co-culture the number of granulocytic colony-forming cells was significantly reduced when stromal *Ctsk* was down-regulated (Figure 1C).

To assess whether *Ctsk* deficiency in UG26-1B6 affects the ability of its secreted factors to sustain survival, proliferation and differentiation of HSCs we set up single cell cultures with conditioned medium (CM) from control and sh*Ctsk* cells supplemented with stem cell factor (SCF, Kitl) and IL-11 (Figure 1D). We observed that HSCs cultured in CM from sh*Ctsk*-stromal cells display significantly reduced proliferation rate and clone size (Figure 1E, 1F). Importantly, the frequency of HSC-enriched LSK cells was strongly reduced, while myeloid progenitor (MP) frequency was increased in WT HSC cultured in CM from sh*Ctsk* (Figure 1G), suggesting that HSC maintenance is impaired when *Ctsk* is reduced in stromal niche cells.

To determine whether reduction of *Ctsk* expression in stromal cells also impairs the repopulating ability of HSCs isolated from co-cultures, we transplanted WT Lin^{-} cells after three weeks of co-culture with either pLKO.1 or sh*Ctsk* stromal cells. Cells from both types of culture showed similar total engraftment in the BM of recipient mice. However, donor regeneration of the HSC-enriched LSK compartment and mature T-lymphocytes in the BM were significantly reduced (Figure 1H-J).

Loss of Cathepsin K impairs *in vivo* lymphopoiesis

Since reduction of stromal *Ctsk* reduces both number and function of early hematopoietic cells *in vitro*, we wondered whether *Ctsk* deletion similarly affects hematopoiesis *in vivo* in *Ctsk* knockout (*Ctsk*$^{-/-}$) mice (12). Under steady-state conditions, 8-12-week old *Ctsk*$^{-/-}$ mice showed unchanged early hematopoiesis and myelopoiesis compared to WT controls (Figures 2A, B). Interestingly though, the clonogenic activity of granulocytic progenitors (CFU-GM) from the BMs of *Ctsk*$^{-/-}$ mice was increased as compared with their WT littermates (Figure 2C). In the periphery, we observed an increase in circulating B cells (Figure 2D). Furthermore, the

absolute numbers of both mature B and T cells in the thymus (THY) of *Ctsk*$^{-/-}$ mice was increased, whereas T cells showed a severe reduction in numbers in the BM of *Ctsk*$^{-/-}$ mice (Figures 2D-G) compared with WTs, suggesting a deregulation of lymphopoiesis in the absence of *Ctsk*.

Both extrinsic and intrinsic *Ctsk* are required for regeneration of donor lymphopoiesis

In order to separate effects of extrinsic *Ctsk* deficiency on T lymphopoiesis, we transplanted WT (Ly5.1) BM cells into lethally irradiated *Ctsk*$^{-/-}$ (Ly5.2) mice and their WT littermates (Figure 3A). Sixteen weeks after transplantation, we observed unchanged engraftment of donor HSC-enriched CD34^{-} LSKs and myeloid progenitors (MPs) in the BM of *Ctsk*$^{-/-}$ mice (Figures 3C, D). Similarly, no changes were observed in engrafted mature myeloid cells in PBs of *Ctsk*$^{-/-}$ animals (Figure 3D). However, similar to the engrafted cells from co-cultures, we found deregulation of both B-and T-lymphopoiesis in several hematopoietic tissues. For instance, we noted a significant reduction of B cells in BM and lymph nodes (LNs) of recipient mice (Figure 3F, I). Interestingly, although lymphocytes in general seemed to be reduced, the number of B cells in the spleen was significantly increased (Figure 3G). In addition to changes in B lymphopoiesis, donor T cell repopulation was dramatically decreased in THY and LNs of *Ctsk*$^{-/-}$ recipient mice as compared with their WT littermates (Figure 3H, I). These experiments show that host (extrinsic) expression of *Ctsk* is required for optimal donor lymphopoiesis.

Since *Ctsk* may also be expressed by hematopoietic cell populations, we studied whether intrinsic donor *Ctsk* is important for regeneration of donor hematopoiesis. For this purpose, we transplanted BM cells from either WT or *Ctsk*$^{-/-}$ mice into lethally irradiated WT recipient mice (Figure 4A). Sixteen weeks after transplantation, we found that *Ctsk*-deficient BM cells showed a significantly reduced engraftment in PB (Figure 4B). As in the experiments in *Ctsk*$^{-/-}$ recipients, we found unaltered early donor hematopoiesis in the BM (Figure 4C). Similarly, with steady-state *Ctsk*$^{-/-}$ mice and transplantations of cells co-cultured on sh*Ctsk* stroma or WT donor cells into *Ctsk*$^{-/-}$ mice, we observed decreased repopulation of donor T cells in the BM of recipient mice. This finding suggests that HSCs from *Ctsk*$^{-/-}$ mice were primed in the *Ctsk*-deficient microenvironment prior to transplantation and therefore not rescued by the presence of recipient *Ctsk* (Figure 4E).

Thus, our experiments strongly suggest that both intrinsic and extrinsic *Ctsk* are required for B- and T-lymphopoiesis, particularly for regeneration of the T-lymphocyte compartment in the BM.

Discussion

We describe a role of stromal *Ctsk* in the maintenance of HSCs *in vitro* and its importance for the regulation of steady state lymphopoiesis and regeneration of lymphopoiesis *in vivo*. Our experiments show that CTSK shapes the secretion of stromal factors required for maintaining HSC proliferation and preventing HSC exhaustion *in vitro*. Despite these clear indications of the importance of CTSK for maintaining HSCs *in vitro*, we did not find direct effects of *Ctsk* loss on HSC maintenance or myelopoiesis *in vivo*. However, we observed that *Ctsk* is required for T-lymphopoiesis in the BM under steady-state conditions and donor regeneration of both B and T cells after transplantation. Furthermore, HSCs generated in a *Ctsk*-deficient microenvironment reproduce this phenotype during the regeneration in WT recipient mice.

An interesting finding from our study is that the CM from sh*Ctsk* stromal cells reduces HSC proliferation and the maintenance of their repopulating ability. Thus, our results strongly suggest that CTSK shapes the HSC-regulating secretome of stromal cells. Considering that CTSK is a cysteine protease, this result suggests that degradation of CTSK substrates is required for maintaining HSC. It remains to be resolved which of these stromal CTSK substrates are important for HSC maintenance. A prime candidate is collagen, which we previously found to be required for optimal survival of HSCs (15). However, we found no effect of *Ctsk* depletion on the survival-promoting activity of stromal CM *in vitro*, suggesting that collagen may also not be the dominant stromal factor in HSC maintenance *in vitro*. Other CTSK substrates are SCF, osteopontin, and the chemokine CXCL12 (2), which all have documented activity on HSC and which are required for HSC maintenance. However, in previous studies of the transcriptome of UG26-1B6 cells, we did not detect expression of these three factors (6, 16), suggesting that these factors may also not be the CTSK targets in these stromal cells Thus, the relevant CTSK substrates for HSC maintenance remain to be established.

In our study, we found a loss of LSK cells in single HSC cultures and reduced regenerative capacity of cultured cells on sh*Ctsk* stromal cells. In addition, *Ctsk*$^{-/-}$ BM cells engrafted the WT periphery inefficiently. Considering the similarity to the in vitro results, the latter finding suggests that HSCs are primed for loss of HSC activity by the *Ctsk*-deficient environment. These findings are reminiscent of a study, in which the loss of *Ctsk* resulted in reduced numbers of immature LSK cells and mature Ly6C^{lo} and CD11c^{+} cells in the BM (17). However, under steady-state conditions, or in both intrinsic and extrinsic transplantation settings, immature donor LSK cells, myeloid progenitors and mature myeloid cells engrafted to a similar level as their WT counterparts. Although both the published report (17) and our present study represent HSC stress-models, there is an apparent disparity of the findings in myelopoiesis. The causes of the disparity between the two studies could lie in differences in stress levels

during housing, the strain of mice used (B6 in the present study vs. B6/L129 (17)), or in the subpopulations of non-lymphoid ($B220^-$ $CD3^-$) myeloid cells studied ($CD11b^+$ $Gr1^+$ in the present study vs. $CD11b^+$ Ly6-C^{lo}, Ly6-G^+, F4/80^+, or $Cd11c^+$ (17)).

We here uncovered an interesting disbalance in lymphopoiesis, both in the numbers and distribution of both B- and T-cells in four different experimental settings. Importantly, this disbalance is observed under both steady-state and transplantation conditions, suggesting a stress-independent role of *Ctsk* expression in lymphopoiesis, particularly in the BM. CTSK is a member of the papain family of cysteine proteases most closely related to cathepsin S (CTSS) and L (CTSL). Interestingly, both these cathepsins have previously been described to regulate lymphopoiesis. For instance, CTSL has been shown to be a negative regulator of B lymphopoiesis (18), as well as $CD4^+$ T lymphopoiesis (19) and CTSS is involved in Th17 differentiation (20). Furthermore, both CTSL and CTSS and other cathepsins like cathepsin B (CTSB) have been implicated in different stages of endosomal processing during antigen presentation (21), and failure to correctly present antigens in the context of MHC Class II or CD1d molecules may lead to defective Th2 lymphocyte responses (21). Our experiments thus suggest that CTSK may play a hitherto unrecognized role in regulating antigen-presenting cell-dependent T cell responses. Thus, although the effects of *Ctsk* deletion on lymphopoiesis are not entirely unexpected, it remains to be resolved which specific lymphocyte subsets are affected and through which cathepsin K-dependent mechanisms.

Such questions may be of importance when considering the use of cathepsin K inhibitors, as in reducing bone resorption in secondary osteoporosis, such as in metastatic bone disease (22), or in postmenopausal osteoporosis (23-25). In addition, CTSK inhibitors are being considered for treatment of periodontal diseases (26). Our results in *Ctsk* knockout mice would urge caution in patients, not only because of the increased risk of cardiovascular disease noted upon treatment of postmenopausal women with the CTSK inhibitor odanacatib (25), but also because deleterious effects on lymphocyte responses can be expected, for instance in infectious disease. Indeed, the inhibitor odanacatib was successful as anti-inflammatory agent in periodontal disease (26), but its effects on de novo infections need to be established.

In conclusion, *Ctsk* is expressed by stromal cells of the hematopoietic niche. *In vitro*, *Ctsk* deficiency in stroma cells resulted into decreased percentage of HSC-enriched LSK cells due to decreased proliferation rates and increased differentiation to myeloid progenitors. Although these cells engrafted normally in WT recipient mice, we here also observed a decrease in donor LSK repopulating activity. Interestingly, in all of our experiments (culture, steady state, transplantations), we noted recurrence of dramatic reductions in T lymphocytes. Indeed, *in vivo Ctsk* deletion affects lymphopoiesis in different organs at steady state and in transplantation experiments. Thus, our study shows that intact activity of CTSK is required for

in vitro HSC maintenance in stromal co-cultures, and, *in vivo*, CTSK is non-redundantly required for regulating T lymphopoiesis, particularly in transplantation settings.

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Author contributions

RH, MH, AJA, MK, SRM, and FH performed experiments. RI, and RAJO designed experiments. RH, RI and RAJO analyzed data and wrote the manuscript

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Legends to the Figures

Figure 1. *In vitro* experiments with UG26-1B6 -pLKO.1 and -sh*Ctsk* knockdown stromal cells. **(A)** Relative mRNA level, measured by real- time PCR using *Ythdf1* as housekeeping gene, comparing the relative expression of *Ctsk* in the knockdown clone of the UG26-1B6 (sh*Ctsk1-6, 8, closed columns*) cells and in the pLKO.1 empty vector control (open column). *ShCtsk2* was used in the followed-up experiments, unless otherwise indicated **(B)** Western Blot showing the relative CTSK protein content to ß- Actin in UG26-1B6- pLKO.1 and sh*Ctsk2* cells, quantified by ImageJ. **(C)** Co-culture of 10.000 Lin^- cells on irradiated *shCtsk* and pLKO.1 stromal cells in 10 cm^2 round dishes for 4 weeks (n=6). After culture, cells were seeded in methylcellulose, after ten days colonies were counted under the microscope. **(D)** Experimental design of single cell experiments: Conditioned medium was generated by radiation of stromal cells and incubation with serum free medium for three days. Single $CD34^-$ SLAM cells were sorted in 96-well plates with pLKO.1 and *shCtsk2* and *shCtsk4* conditioned medium supplemented with SCF and IL11 and every 24h microscopically evaluated for cell number (n=4). After five days (d1-d5), the clones were harvested and analyzed using flow cytometry (n=5). **(E)** Mean clone size of $CD34^-$ SLAM cells cultured with pLKO.1 and *shCtsk* conditioned medium. Shown are the results of four independent experiments. **(F)** Heat map showing proliferation frequency of single clones. **(G)** Content of LSK and MP cells per cultured plate. Cells from all wells were pooled and stained for lineage markers (B220, CD4/CD8a, CD11b, and Gr1), SCA1 and KIT. Shown are the results of two independent experiments, each with three plates per condition **(H)** Experimental design of co-culture transplant experiments: 10.000 Lin- cells were co-cultured on irradiated stromal cells for three weeks and then transplanted into lethally irradiated mice. **(I)** FACS analysis of BM, 16-weeks post transplantation, showing mature and **(J)** early stage hematopoiesis. Results shown, are from two to four independent experiments. *: p<0,05 using Mann-Whitney U test.

Figure 2. Effects of *Ctsk* loss on steady state hematopoiesis. (**A**) Representative FACS plots of the BM stem and progenitor cells. (**B**) Absolute numbers of LSK, $CD34^-$ LSK, MPs, granulocytes and monocytes in the BM of WT and $Ctsk^{-/-}$ mice. (**C**) Clonogenic activity of progenitor cells; n=8 (WT), n=5 ($Ctsk^{-/-}$). (**D**) Percentages of B- and T- lymphocytes in the peripheral blood (PB), and (**E**) absolute numbers of B and T cells in the BM, (**F**) Spleen (SPL) and (**G**) Thymus (THY). Data represents results of two to three independent experiments. In B, D-G each dot represents one animal. Open symbols represent results from WT control animals, and closed symbols represent $Ctsk^{-/-}$ animals. *: p<0,05 using the Mann-Whitney U test.

Figure 3. Role of extrinsic *Ctsk* for regeneration of WT HSCs. (**A**) Experimental design. WT Lin$^-$ cells from congenic Ly5.1 mice were transplanted into Ly5.2 *Ctsk*$^{-/-}$ mice and their WT littermates. Donor cells were analyzed 16 weeks post transplantation. (**B**) Percentage of donor engraftment in the PB, BM, and SPL. (**C**) Representative FACS plots of the BM stem and progenitor cells. (**D**) Absolute numbers of LSKs, CD34$^-$ LSKs, MPs, granulocytes (non-lymphocyte CD11b$^+$ Ly6G^{hi}) and monocytes (non-lymphocyte CD11b$^+$ Ly6G^{med}) in the BM from the extrinsic transplant recipients. (**E**) Percentage of B- and T- lymphocytes in the PB. (**F**) Absolute numbers of B- and T- lymphocytes in the BM, (**G**) SPL, (**H**) THY, (**J**) and lymph nodes. Results of two or three independent experiments are shown. In B, D-I, each dot plot represents one animal. Open symbols represent results from WT control animals, and closed symbols represent *Ctsk*$^{-/-}$ recipients. *: $p<0.05$ statistically significant using Mann-Whitney U-test.

Figure 4. Repopulation capacity of *Ctsk*$^{-/-}$ HSCs. (**A**) Experimental design. Ly5.2 BM cells from *Ctsk*$^{-/-}$ and WT control littermates were transplanted into Ly5.1 WT recipient mice and analyzed 16 weeks post transplantation. (**B**) Percentage of donor engraftment in PB, BM, and SPL. (**C**) Absolute numbers of LSKs, CD34$^-$ LSKs, MPs, granulocytes and monocytes in the BM of recipient mice. (**D**) Percentage of the B- and T- lymphocytes in the PBs. Absolute numbers of donor B and T cells in the (**E**) BM and (**F**) SPL. The results of two independent experiments are shown. In B-F, each dot represents one animal. Open symbols represent results from WT donor animals, and closed symbols represent *Ctsk*$^{-/-}$ donors. *: $p<0.05$ statistically significant difference between control and *Ctsk*$^{-/-}$ donor cells in WT recipients using Mann-Whitney U-test.

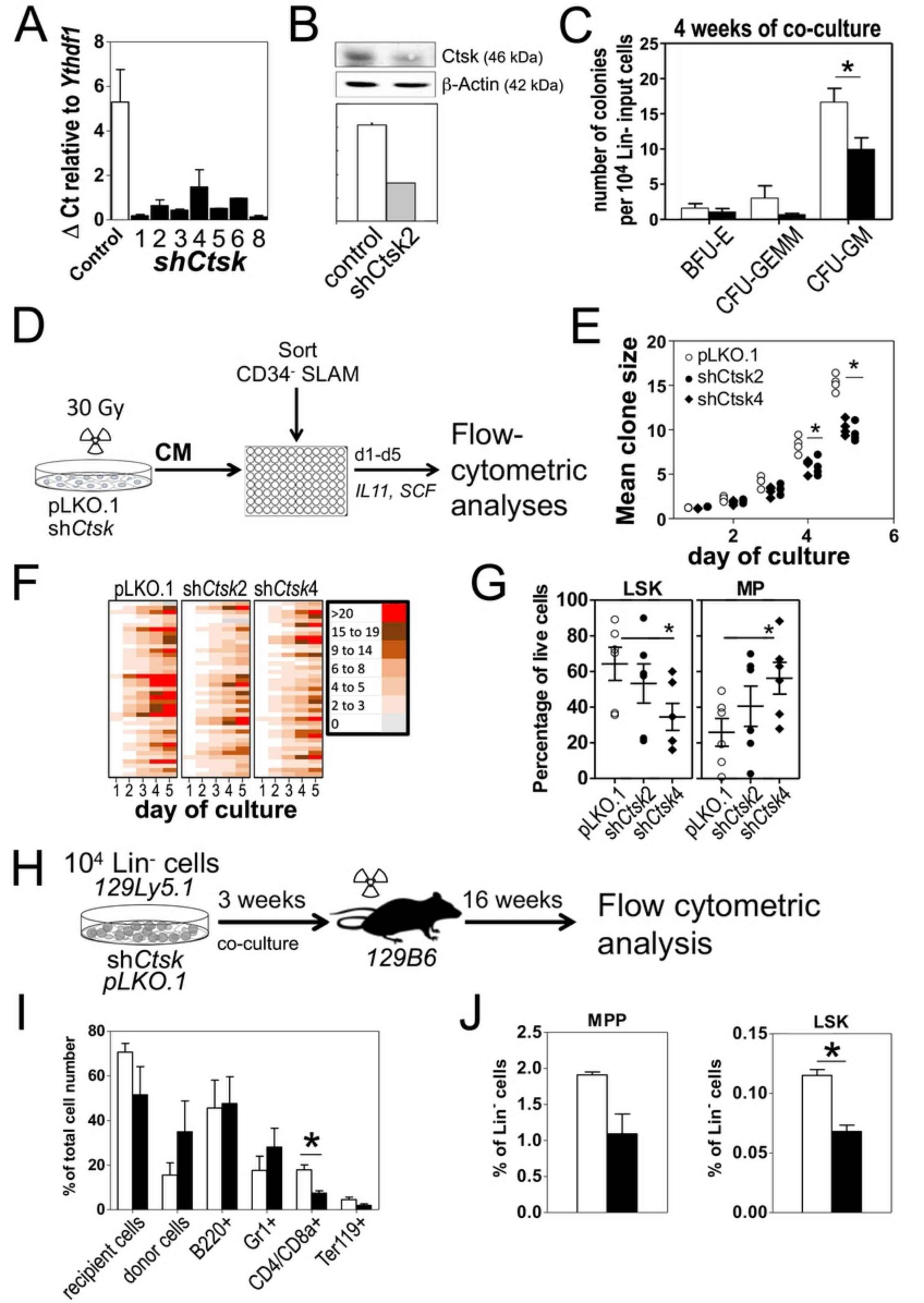
A
Δ Ct relative to Ythdf1
Control
1 2 3 4 5 6 8
shCtsk
B
Ctsk (46 kDa)
β-Actin (42 kDa)
control
shCtsk2
C
4 weeks of co-culture
number of colonies per 10⁴ Lin- input cells
BFU-E
CFU-GEMM
CFU-GM
D
30 Gy
pLKO.1
shCtsk
CM
Sort
CD34- SLAM
d1-d5
IL11, SCF
Flow-cytometric analyses
E
pLKO.1
shCtsk2
shCtsk4
Mean clone size
day of culture
F
pLKO.1
shCtsk2
shCtsk4
>20
15 to 19
9 to 14
6 to 8
4 to 5
2 to 3
0
day of culture
G
LSK
MP
Percentage of live cells
pLKO.1
shCtsk2
shCtsk4
H
10⁴ Lin- cells
129Ly5.1
shCtsk
pLKO.1
3 weeks
co-culture
129B6
16 weeks
Flow cytometric analysis
I
% of total cell number
recipient cells
donor cells
B220+
Gr1+
CD4/CD8a+
Ter119+
J
MPP
LSK
% of Lin- cells

Figure 1

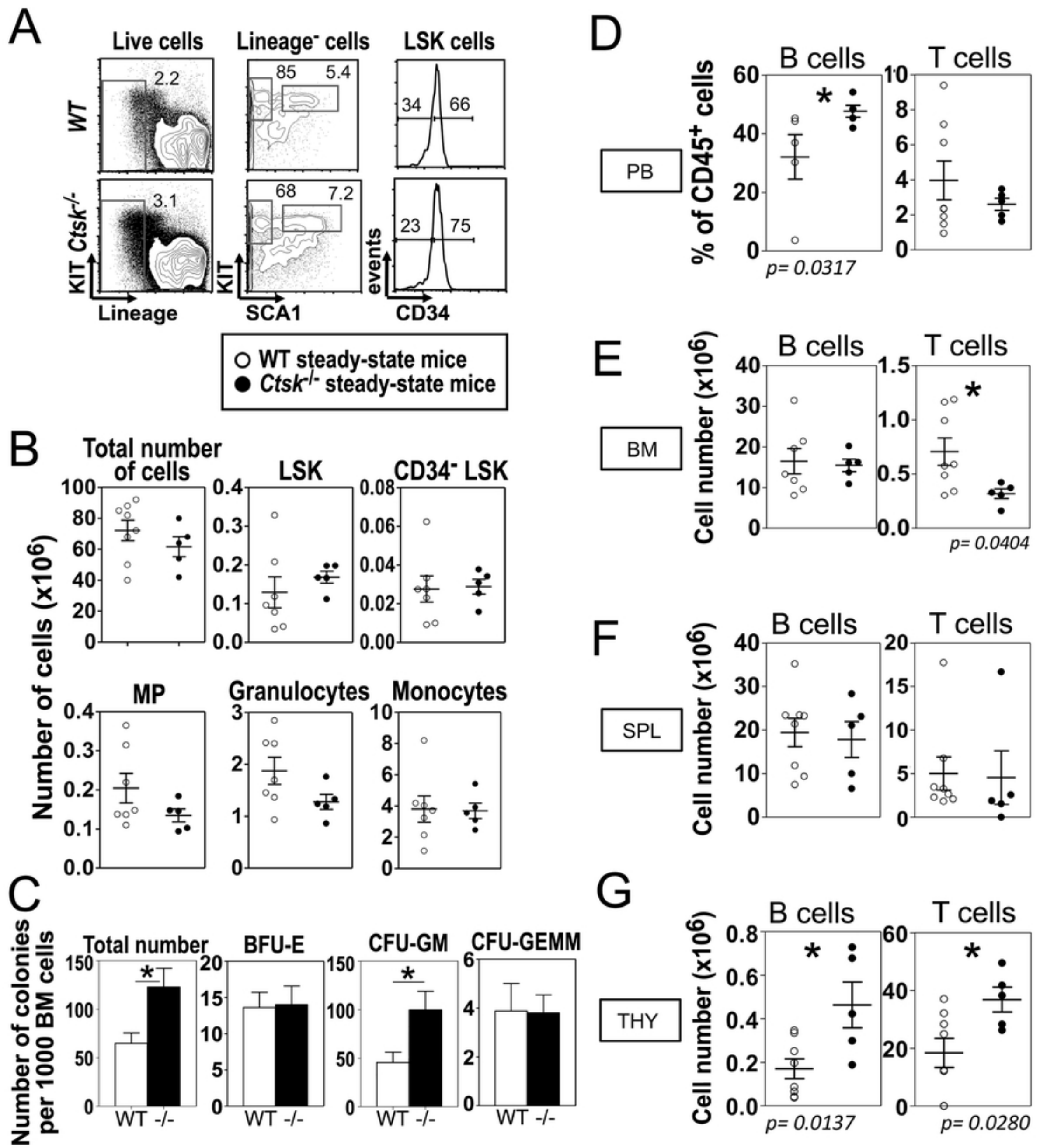
A
Live cells
Lineage⁻ cells
LSK cells
WT
Ctsk-/-
2.2
85
5.4
34
66
3.1
68
7.2
23
75
KIT
Lineage
SCA1
events
CD34
WT steady-state mice
Ctsk-/- steady-state mice
B
Total number of cells
LSK
CD34⁻ LSK
MP
Granulocytes
Monocytes
Number of cells (x10^6)
C
Total number
BFU-E
CFU-GM
CFU-GEMM
Number of colonies per 1000 BM cells
WT
-/-
D
PB
B cells
T cells
% of CD45+ cells
p= 0.0317
E
BM
Cell number (x10^6)
p= 0.0404
F
SPL
G
THY
p= 0.0137
p= 0.0280

Figure 2

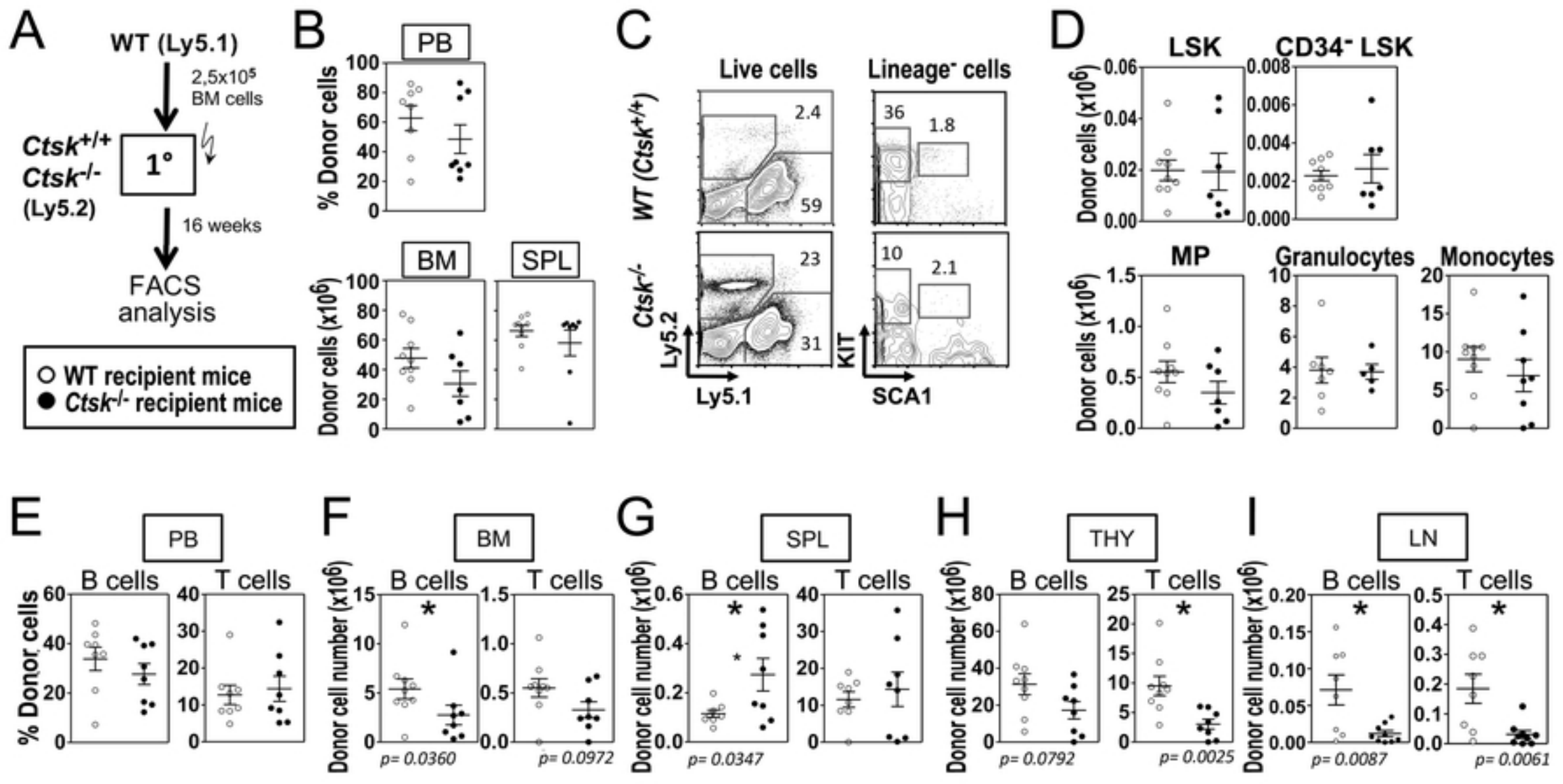
A
WT (Ly5.1)
2,5x10⁵ BM cells
Ctsk+/+
Ctsk-/-
(Ly5.2)
1°
16 weeks
FACS analysis
WT recipient mice
Ctsk-/- recipient mice
B
PB
% Donor cells
BM
SPL
Donor cells (x10⁶)
C
Live cells
Lineage⁻ cells
WT (Ctsk+/+)
Ctsk-/-
2.4
59
23
31
36
1.8
10
2.1
Ly5.2
Ly5.1
KIT
SCA1
D
LSK
CD34⁻ LSK
MP
Granulocytes
Monocytes
Donor cells (x10⁶)
E
PB
B cells
T cells
% Donor cells
F
BM
B cells
T cells
Donor cell number (x10⁶)
p= 0.0360
p= 0.0972
G
SPL
B cells
T cells
Donor cell number (x10⁶)
p= 0.0347
H
THY
B cells
T cells
Donor cell number (x10⁶)
p= 0.0792
p= 0.0025
I
LN
B cells
T cells
Donor cell number (x10⁶)
p= 0.0087
p= 0.0061

Figure 3

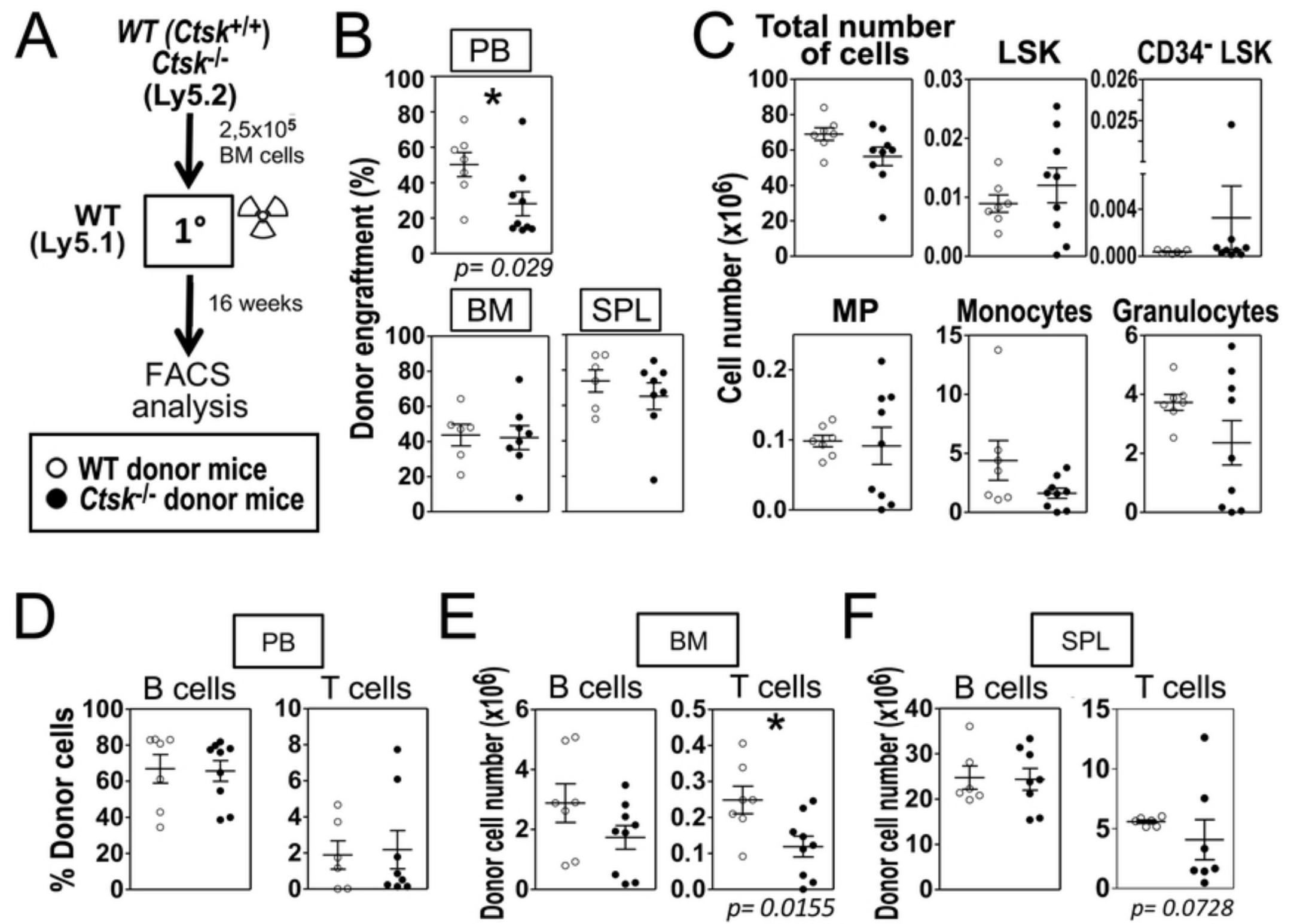
A
WT (Ctsk+/+)
Ctsk-/-
(Ly5.2)
2,5x10^5
BM cells
WT
(Ly5.1)
1°
16 weeks
FACS
analysis
WT donor mice
Ctsk-/- donor mice
B
PB
Donor engraftment (%)
p= 0.029
BM
SPL
C
Total number
of cells
LSK
CD34- LSK
Cell number (x10^6)
MP
Monocytes
Granulocytes
D
PB
B cells
T cells
% Donor cells
E
BM
B cells
T cells
Donor cell number (x10^6)
p= 0.0155
F
SPL
B cells
T cells
Donor cell number (x10^6)
p= 0.0728

Figure 4