

1 **Identification of the Minimum Requirements for Successful Haematopoietic**
2 **Stem Cell Transplantation**

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4 Running short title; Identification of the Minimum Subset for HSCT

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25 **SUMMARY**

26 Historically, defining haematopoietic subsets, including self-renewal, differentiation, and
27 lineage restriction, has been elucidated by transplanting a small number of candidate cells
28 with many supporting bone marrow (BM) cells. While this approach has been invaluable
29 in characterising numerous distinct subsets in haematopoiesis, this approach is arguably
30 flawed. The haematopoietic stem cell (HSC) has been proposed as the critical
31 haematopoietic subset necessary for transplantation. However, due to the presence of
32 supporting cells, the HSC has never demonstrated sufficiency.

33 Utilising the Hoxb5-reporter system, we found that neither long-term HSCs nor short-
34 term HSCs alone were sufficient for long-term haematopoietic reconstitution. Critically,
35 reconstitution can be rescued by transplanting a combined LT-HSCs and ST-HSCs,
36 without supporting cells– a fraction we term the Minimum Subset for Transplantation
37 (MST). MST accounts for only 0.005% of nucleated cells within mouse bone marrow,
38 and this MST can be cultured, expanded, and genetically modified while preserving its
39 rapid haematopoietic engraftment potential. These results support the consideration of an
40 MST approach for clinical translation, especially for gene therapy which require the HSC
41 compartment modification.

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43 **Keywords;** Long term haematopoietic stem cell, Short term haematopoietic stem cell,
44 Hoxb5, Purified haematopoietic stem cell transplantation, Gene therapy

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49 **Introduction**

50 Haematopoietic stem cell transplantation (HSCT) has been primarily developed as a
51 treatment for haematopoietic malignancies and has been performed in more than one
52 million patients.¹ In recent years, gene therapy approaches, wherein a patient's
53 haematopoietic cells are genetically engineered and transplanted back to normalise the
54 malfunctioning haematopoietic system, have been performed for monogenic diseases,
55 including β -thalassemia^{2,3} and severe combined immunodeficiency.^{4,5} Given that
56 autologous HSCT can be applied to many more diseases, including other primary
57 immunodeficiency disorders,⁶ inherited metabolic disorders,⁷ and autoimmune
58 diseases,^{8,9} further development of the technology is desirable.

59 Currently, autologous HSCT with genetic modification has only been performed in a
60 few hundred cases worldwide limited in part to the technical hurdles of haematopoietic
61 stem cell (HSC) genetic modification,¹⁰ lack of persistent gene-modified cell
62 engraftment,^{11,12} and genotoxicity by random insertion of the vectors throughout the
63 genome of targeted haematopoietic cells.¹³ Although genetic engineering challenges of
64 HSCs to solve the genotoxicity have been previously described,¹⁴ efforts aimed at
65 optimising genetic modification and improving insufficient therapeutic efficacy should
66 logically utilise a purified cell fraction containing only the necessary and minimally
67 sufficient cells for HSCT.

68 While the HSC field has been able to ascribe the fundamental stem cell and
69 haematopoiesis properties of self-renewal,^{15,16} multi-potency,¹⁷ and lineage restriction¹⁸
70 to over 23 distinct subsets of the haematopoietic hierarchy, these studies have all
71 depended on a transplantation assay in which a small number of candidate cells are co-
72 transplanted with a large number of unfractionated BM cells, termed supporting cells (aka

competitor cells).¹⁹ From these studies the field has concluded that the long-term (LT) HSC, even as a single cell transplant,^{20,21} is both necessary and sufficient for successful HSCT. However, this conclusion relies on the assumption that there is a lack of interaction and co-dependence of the LT-HSC on the unfractionated marrow.

Therefore, we sought to identify the cell fractions that are minimally necessary and critically sufficient for safe and long-term haematopoiesis after transplantation, a subset we term the “Minimum Subset for Transplantation” (MST). We systematically re-evaluated the distinct cell types in the haematopoietic hierarchy for MST, but were unable to identify a single fraction meeting these criteria. Only by utilising the *Hoxb5* mouse model which enabled us to isolate the LT-HSCs and short-term (ST) HSCs with high purity,²² were we able to identify the MST required for long-term and rapid haematopoietic recovery in transplantation.

Methods

Mice

Hoxb5-tri-mCherry (C57BL/6J background) mice derived from our previous work²² were used as donors for transplantation assays. CAG-EGFP mice (Japan SLC, Shizuoka, Japan) were bred with *Hoxb5*-tri-mCherry mice for transplantation experiments. C57BL/6-Ly5.1 mice (Sankyo Labo Service, Tokyo, Japan) were used as recipients for transplantation assays. The 10-18-week-old mice were used as donors and 8-11-week-old mice were used as recipients. As an exception to the above, in Fig 4F, C57BL/6-Ly5.1 mice were used as donor cells, and C57BL/6J mice (CLEA Japan, Tokyo, Japan) were used as recipient mice. All mice were housed in pathogen-free conditions. Mice were

bred accordingly with RIKEN guidelines. All animal protocols were approved by RIKEN Centre for Biosystems Dynamics Research.

More detailed information of bone marrow analyses, sorting and transplantation without supporting cells, peripheral blood analyses, Sca-1⁺ depletion, lentiviral gene transduction, Ex vivo expansion of haematopoietic cells, 5-FU conditioning, quantification and statistical analyses are described in the supplementary materials and methods.

Result

Non-LKS fraction alone does not rescue lethally irradiated mice

Several reports have demonstrated that the Lineage⁻c-Kit⁺Sca-1⁺ (LKS) fraction (**Fig S1A**), which accounts for 0.1% of the mouse BM, enables rapid haematopoietic recovery after myeloablative conditioning.^{23,24} On the other hand, it has been reported that the non-LKS fraction, which is believed to be free of undifferentiated cells, may also contribute to continuous haematopoiesis, albeit to a dramatically lesser extent.²⁵ Therefore, we first tried to confirm whether the non-LKS fraction, which accounts for 99% of the BM cells, could rescue lethally irradiated recipient mice. Our previous report²² showed that 2×10^5 whole bone marrow (WBM) cells are enough to rescue lethally irradiated mice. Therefore, we compared the haematopoietic reconstitution between the group transplanted with 2×10^5 WBM cells and the group transplanted with 2×10^5 non-LKS cells (**Fig 1A**).

Non-LKS BM cells were prepared by depleting Sca-1-positive cells from the WBM by magnetic separation (**Figs 1B, C**). The survival rate of the WBM-transplanted group was 83%. In contrast, none of the mice transplanted with the same number of the non-LKS cells survived (**Fig 1D**). No donor cells were detected in the peripheral blood (PB), even

in the survived recipient mouse receiving three doses of non-LKS cells (**Fig 1E**). These results indicated that non-LKS fraction alone were insufficient for rescuing recipient mice.

Transplantation with different doses of LKS cells revealed a threshold for rapid haematopoietic recovery after HSCT

Although LKS cells were considered necessary for rapid haematopoietic recovery based on the above results, it is desirable to establish a sufficiently safe transplantation dose using fewer donor cells to improve the efficiency of genetic modification.^{26,27} Therefore, we transplanted different numbers of LKS cells into lethally irradiated mice to determine the minimum cell dose (**Fig 2A**). The groups that received more than 1×10^3 cells showed an average survival rate of 95.8%. In contrast, the groups that received less than 3×10^2 cells showed a 50% survival rate (**Fig 2B**). PB analysis revealed that the groups receiving less than 1×10^3 cells showed significantly reduced donor chimaerism and delayed lymphocyte recovery (**Figs 2C-E**). On the other hand, the groups receiving more than 3×10^3 cells showed stable donor chimaerism and rapid lymphocyte recovery 8 weeks after transplantation (**Figs 2C-E**). These results suggested that the cell dose should be determined based not only on the survival rate but also on the rate of haematopoietic reconstitution to ensure safe transplantation.

Non-HSCs in the LKS fraction do not play a pivotal role in post-transplant haematopoietic reconstitution

Although we demonstrated that the LKS fraction alone was necessary for successful transplantation, we have not determined what is minimally necessary and sufficient as this fraction consists of multiple cell types, including HSCs and multipotent progenitors

(MPPs), the latter which have lost their self-renewal capability, and other differentiated cells. The next question is whether all of the constituents are necessary for haematopoiesis after transplantation or not. Then, we sought to identify the minimum subsets that were truly responsible for post-transplant haematopoiesis. For this, we analysed the numerical contribution of the LT-HSC ($\text{Hoxb5}^+\text{Lineage}^-\text{c-Kit}^+\text{Sca-1}^+\text{CD150}^+\text{CD34}^{-/\text{lo}}\text{Flt3/Flk2}^-$), ST-HSC ($\text{Hoxb5}^-\text{Lineage}^-\text{c-Kit}^+\text{Sca-1}^+\text{CD150}^+\text{CD34}^{-/\text{lo}}\text{Flt3/Flk2}^-$), MPP subset A (MPPa; $\text{Lineage}^-\text{c-Kit}^+\text{Sca-1}^+\text{CD150}^+\text{CD34}^+\text{Flt3Flk2}^-$), MPP subset B (MPPb; $\text{Lineage}^-\text{c-Kit}^+\text{Sca-1}^+\text{CD150}^-\text{CD34}^+\text{Flt3/Flk2}^-$) and Flt3/Flk2-positive cells (Flt3/Flk2^+ ; $\text{Lineage}^-\text{c-Kit}^+\text{Sca-1}^+\text{Flt3/Flk2}^+$) from the LKS population along the haematopoietic hierarchy (**Fig S1A**). In our analyses, 1000 LKS cells consists of 500 Flt3/Flk2^+ cells, 300 MPPb cells, 75 MPPa cells, 38 ST-HSCs, and 12 LT-HSCs, excluding outlier cells (**Fig S1A**).

To determine the sufficiency of each of these fractions to the Minimum Subset for Transplantation (MST), we first prepared four different cell mixtures starting from the most differentiated population (Flt3/Flk2^+) and adding back the upstream population one by one according to the haematopoietic hierarchy (**Fig 3A**). We used 1000 LKSs as a positive control, which is the minimum number of cells to ensure recipient survival (**Fig 2B**). All mice receiving non-HSC containing fractions ($\blacktriangledown \text{Flt3/Flk2}^+$, $\blacktriangledown \text{MPPb}$, and $\blacktriangledown \text{MPPa}$) died within a short period of time except for one case in the $\blacktriangledown \text{MPPb}$ group (**Fig 3B**). Even in this case, no donor myeloid cells were detected in the PB, representing typical graft failure (**Fig 3C**). On the other hand, adding 38 ST-HSCs to the mixture of Flt3/Flk2^+ , MPPb, and MPPa ($\blacktriangledown \text{ST-HSC}$) dramatically improved the survival rate and myeloid cells output 4 weeks after transplantation (**Figs 3B, C**). However, compared to the LKS group which critically contains LT-HSCs, the survival outcomes were inferior, suggesting that the LT-HSCs, while only contributing a miniscule cell number to the LKS,

were crucial for safer outcomes. These results suggested that more than 90% of the LKS fraction, which consists of MPPa, MPPb, and Flt3/Flk2⁺, did not play a critical role in haematopoietic reconstitution after transplantation.

A combined fraction is the minimum necessary and sufficient subset for haematopoietic recovery after transplantation

As the field has concluded that the LT-HSC is both necessary and sufficient for successful transplantation, the results above strongly suggest that the LT-HSC is the key fraction. However, the recipient mice showed significant improvement of survival by adding ST-HSCs. Therefore, we sought to clarify whether either or both fractions were required to achieve successful transplantation. We transplanted isolated 12 LT-HSCs, 38 ST-HSCs, 50 combined HSCs, and 1000 LKSs, respectively (**Fig 4A**). Surprisingly, neither 12 LT-HSCs alone nor 38 ST-HSCs alone rescued recipient mice (**Fig 4B**). In contrast, 50 combined HSCs had nearly equivalent rescue potential to 1000 LKSs (**Fig 4B**).

Next, we tried to determine the sufficient dose of the combined HSC for rapid haematopoietic recovery and long-term survival after transplantation by titrating the combined HSC cell dose (**Fig 4C**). The mice receiving more than 300 combined HSCs showed rapid haematopoietic recovery 4 weeks after transplantation (**Fig 4D**), resulting in a high survival rate (**Fig 4E**). Another group receiving 300 combined HSCs confirmed 100% survival and a year-long stable haematopoietic reconstitution deriving from the donor cells with very high chimaerism (**Figs 4F, G**). In contrast, more than 90% of the LKS fraction, which consists of MPPa, MPPb, and Flt3/Flk2⁺, contributed to haematopoiesis only in the very short term and did not play a critical role in the survival of the recipient mice after transplantation. In summary, the combined fraction, LT-HSC

and ST-HSC, was the minimal necessary and sufficient subset to ensure both rapid haematopoietic recovery and long-term haematopoietic reconstitution, the Minimum Subset for Transplantation (MST).

LT-HSCs and ST-HSCs work complimentary in rapid haematopoietic recovery and long-term haematopoiesis

Although we found that LT-HSCs or ST-HSCs alone were insufficient for rescuing irradiated mice, this may be due to the scarcity of transplanted cells. To confirm this, we transplanted approximately ten times as many LT-HSCs or ST-HSCs compared to the previous experiments (**Figs S2A, C**). While the group receiving 100 LT-HSCs showed little improvement in survival, the group receiving 380 ST-HSCs showed a significant improvement in survival and haematopoiesis not only in the short term but also in the long term (**Figs S2B, D, and E**). These results indicated that LT-HSCs and ST-HSCs played different roles in transplantation.

To properly understand the differences, we performed a co-transplantation assay in which we sorted LT-HSCs from *Hoxb5*-tri-mCherry mice and ST-HSCs from CAG-EGFP; *Hoxb5*-tri-mCherry mice, or vice versa. These cells were then co-transplanted into the same recipients in a 25 LT-HSC: 75 ST-HSC ratio, as seen in healthy young mouse BM (**Figs 5A, and S1A**). Except for one case, all mice survived the transplantation with 300 MST cells which included 75 LT-HSCs and 225 ST-HSCs. PB analysis revealed that both LT-HSCs and ST-HSCs in the donor fraction produced myeloid lineage cells predominantly in the first 4 weeks after transplantation. However, ST-HSCs started losing the ability to produce myeloid lineage cells 8 weeks after transplantation (**Figs 5B, C**). On the other hand, LT-HSCs maintained multilineage haematopoietic reconstitution

throughout the observation period (**Figs 5B, C**). A direct comparison of the haematopoietic reconstitution ability between LT-HSCs and ST-HSCs within the donor fraction revealed that haematopoiesis by ST-HSCs is predominant in the early post-transplant period, but LT-HSCs are primary source for all the blood lineage over time (**Figs 5D, E**). Taken together with the results showing insufficiency of the LT-HSC to rescue, these findings suggested that rapid haematopoietic recovery by ST-HSCs was essential in the early post-transplant period, while LT-HSCs were essential to ensuring robust long-term multilineage haematopoiesis and survival.

Gene modified MST represent persistent reporter expression in recipient mice.

Next, we examined whether MST was applicable for gene modified HSCT. For this, we used lentivirus to genetically modify the minimum subset and confirmed their transduction efficiency and persistence by monitoring the ZsGreen reporter expression (**Figs 6A, B**). We achieved $59.6 \pm 15.2\%$ of infection efficiency with 4×10^3 multiplicity of infection (MOI) (**Fig 6C**). Under these conditions, transplanting 300 genetically modified MST cells, we detected stable donor cell contribution and persistent reporter expression for up to 16 weeks without decline (**Fig 6D**).

Ex vivo expansion of MST allows rapid and robust multilineage reconstitution

Although we have shown that transplantation using MST is feasible, as several previous studies have noted, graft failure and delayed haematopoietic recovery are the major impediments to expand the applicability of purified HSCT to various diseases.²⁸ Recent studies have demonstrated that *ex vivo* expansion of umbilical cord blood stem cells promoted rapid haematopoietic recovery,²⁹ while maintaining the capacity for long-term

haematopoiesis. Taking this into account, we sought to evaluate the effect of *ex vivo* expansion of MST on haematopoietic recovery.

We cultured 300 MST cells for 7 days in the F12-based culture medium with TPO³⁰ and SCF.^{31,32} After the incubation, we collected the cultured cells and transplanted all expanded cells into lethally irradiated mice. For comparison, we transplanted freshly isolated 300 MST cells into lethally irradiated mice (**Fig 7A**). PB analysis showed that donor chimaerism at 4 weeks was significantly higher in the group receiving cultured cells than in the group receiving freshly isolated cells (**Fig 7B**). We also observed faster recovery of lymphocytes in the PB in the group receiving cultured cells than in the group receiving freshly isolated cells (**Figs 7C, D**). To directly compare haematopoietic reconstitution potential between freshly isolated MST and expanded MST, we co-transplanted freshly isolated 300 GFP⁺ MST cells and 7-day cultured cells derived from 300 MST cells (**Fig 7E**). We found that cultured cells outcompeted freshly isolated cells in haematopoietic reconstitution throughout the observation period (**Figs 7F, G**). In conclusion, these results indicated that transplantation using *ex vivo* expansion of the MST (*ex vivo* MST) improved short-term haematopoietic recovery while maintaining long-term haematopoietic capacity.

5-FU conditioning followed by MST transplantation enables long-term donor haematopoiesis

Based on the aforementioned results, we have concluded that the combined HSC fraction consisting of LT-HSCs and ST-HSCs is the minimum subset sufficient for successful transplantation. MST can contribute to expand the applicability of purified HSCT by improving gene modification efficiency. However, due to life-threatening events caused

by myeloablative conditioning, HSCT has not been commonly practised. To potentially address this issue, we sought to explore the utility of MST to non-myeloablative conditioning.

5-FU has been widely used in various cancer treatments,³³ partially due to 5-FU's low-cost and less toxicity,³⁴ contrary to other reagents such as Busulfan, Cyclophosphamide, and Fludarabine.³⁵⁻³⁹ In addition, a previous report⁴⁰ demonstrated that 5-FU conditioning followed by transplantation of 5×10^6 WBM cells achieved 10% donor chimaerism. Therefore, we attempted to evaluate the same 5-FU regimen followed by transplanting 3×10^3 MST cells instead of WBM cells, which represent less than 0.1% of 5×10^6 WBM cell dose (**Fig 8A**). We observed approximately the same donor chimaerism as in the previous report (**Fig 8B**).⁴⁰ As expected, no donor cell engraftment was observed in the non-conditioned group throughout the observation period (**Fig 8B**). Donor chimaerism analysis in each lineage showed that MST demonstrated persistent contribution to all cell lineages in the PB (**Fig 8C**). We also critically detected persistent MST contribution to all haematopoietic stem and progenitor cell fractions in the BM (**Figs 8D, E and S1B**), suggesting that MST maintained long-term self-renewal capacity and multipotency. These data demonstrated that MST transplantation following 5-FU conditioning could provide an efficient therapeutic platform including gene therapy without fatal complications while maintaining long-term haematopoiesis.

Discussion

In this study, using the Hoxb5-reporter system, we have shown that the minimum subset consisting of LT-HSCs and ST-HSCs, which account for only 0.005% of nucleated cells within mouse BM, is critically sufficient for post-transplant haematopoiesis and that MST

transplantation is practically feasible without graft failure and delayed haematopoietic recovery by transplanting a sufficient amount of MST cells.

In conventional HSCT, unpurified WBM cells, which are comprised of mostly differentiated cells, have been intentionally used as donor sources to ensure high engraftment by preventing loss of effective cells and anti-tumour effect (called GVT effect) by transfusing donor mature T cells.⁴¹ On the other hand, recent innovations including sequencing technology, gene modification, and big data analytics significantly impact on selecting treatment strategies.⁴² For instance, molecular targeted drugs,⁴³ CAR-T,⁴⁴ and immune checkpoint inhibitors⁴⁵ have the potential to replace HSCT as the standard curative therapy for haematopoietic malignancies in the near future. These changes in clinical practise indicate the need to reconsider the use of HSCT in various situations.

Recently, clinical trials have shown that autologous HSCT is very effective in treating autoimmune diseases.⁴⁶ Furthermore, genetic engineering technology is highly expected to expand the applicability of autologous HSCT to many diseases, including primary immunodeficiencies⁴ and inborn errors of metabolism.⁷ On the other hand, *ex vivo* manipulations of HSCs, such as cell sorting, genetic engineering, and cell culture, are likely to lose or abnormalise significantly HSC functions, resulting in the lack of persistent therapeutic effect^{11,12} or the increased risk of leukemogenesis.¹³ To solve these issues, starting from purified cell populations is a practical solution to minimise risks around *ex vivo* modifications.

Furthermore, to explore the applicability of MST to humans, current myeloablative conditioning regimens need to be revised due to acute and chronic fatal effects. Although several groups have developed non-intensive conditioning regimens that could be

implemented into clinical practice in the future, such as anti-c-Kit antibody treatment,⁴⁷ dietary valine restriction,⁴⁸ and nonconditioned transplantation,⁴⁹ we hope that 5-FU conditioning will be an optimal option because of not only low myeloablative toxicity but also low-cost and very mild gonadotoxic for young patients. Using a single MST (3000 cells) transplantation following 5-FU conditioning, we achieved approximately 10% donor chimaerism comparable to that of the nonconditioned transplantation using the massive HSC expansion *ex vivo* previously reported.⁵⁰ For severe combined immunodeficiency and chronic granulomatous disease, 5-10% myeloid chimaerism is high enough to improve their clinical symptom.^{51,52} However, given that donor chimaerism depends on the dose of transplanted cells,⁴⁹ increased cell number of the MST or combination with *ex vivo* expanded MST will be expected to achieve even higher chimaerism which can further expand the application of the MST for gene therapy⁵³.

MST transplantation has also brought new insights into basic research. When assessing the function of HSCs, WBM cells, in addition to donor cells, were commonly co-transplanted as a supporting fraction to ensure the survival of recipient mice. However, the supporting cell fraction also contains functional HSCs. As we reported recently that the cell fate of transplanted HSCs could be flexibly changed depending on the intensity of haematopoietic stress,⁵⁴ the outcome of donor cells after transplantation are potentially skewed by the supporting cells, especially when low dose of donor cells is used. Using MST transplantation, which does not require any supporting cells, we can evaluate the function of the transplanted cell fraction specifically. Based on this approach, we found that LT-HSCs did not improve early haematopoietic recovery even with increasing numbers of transplanted cells, indicating that LT-HSCs are specialized for late and long-term haematopoiesis after transplantation. On the other hand, we also demonstrated that

ST-HSCs could maintain long-term haematopoiesis by transplanting a large number of ST-HSCs, although their efficiency was lower than that of the minimum subset. This result strongly supports our recent finding that ST-HSCs can behave like LT-HSCs after transplantation by reducing the haematopoietic stress per transplanted cell.⁵⁴ This may also explain why transplanting large numbers of HSCs grown *ex vivo*, including our result, show improved haematopoietic recovery despite a dramatic decrease in the frequency of HSC fractions. Furthermore, we showed that the modified MST, in which the minimum subset was the target for genetic modification, could largely reduce development costs and sustain therapeutic effects.

Since we have utilised only a mouse model to identify the MST, further research is necessary to transfer this knowledge into a human context. *In vivo* tracking of human haematopoietic reconstitution after transplantation revealed bimodal peaks in the early and late stages,⁵⁵ strongly suggesting the existence of LT-HSCs and ST-HSCs in humans as well. The establishment of a purification method of these fractions will solve this in the future.

Finally, through detailed analysis of purified cell fractions within BM, we identified the minimum subset which was minimally necessary and critically sufficient for post-transplant haematopoiesis over time. Based on these findings, we have shown that MST transplantation can be a feasible method, which can be developed as a safer transplantation method by combining minimally invasive pre-treatment and *ex vivo* cell expansion, further applying to genetic disease by high-efficiency gene modification in the minimum subset.

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Authorship Contributions

K.N. conceived, performed, analysed the experiments. T.S. and K.S. performed transplantation experiments. K.S. and M.F. provided technical supports for the experiments. M.M. conceived, performed, analysed, and oversaw the experiments. K.N., M.M., J.Y.C. wrote the manuscript. K.S., T.S., M.F., A.T., J.Y.C. provided comments on the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest in association with the present study.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Antibodies used for flow cytometry

Fig S1. Gating scheme for haematopoietic cells.

Fig S2. Either LT-HSCs or ST-HSCs are not sufficient for the rapid haematopoietic recovery and long-term haematopoiesis.

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Figure.1

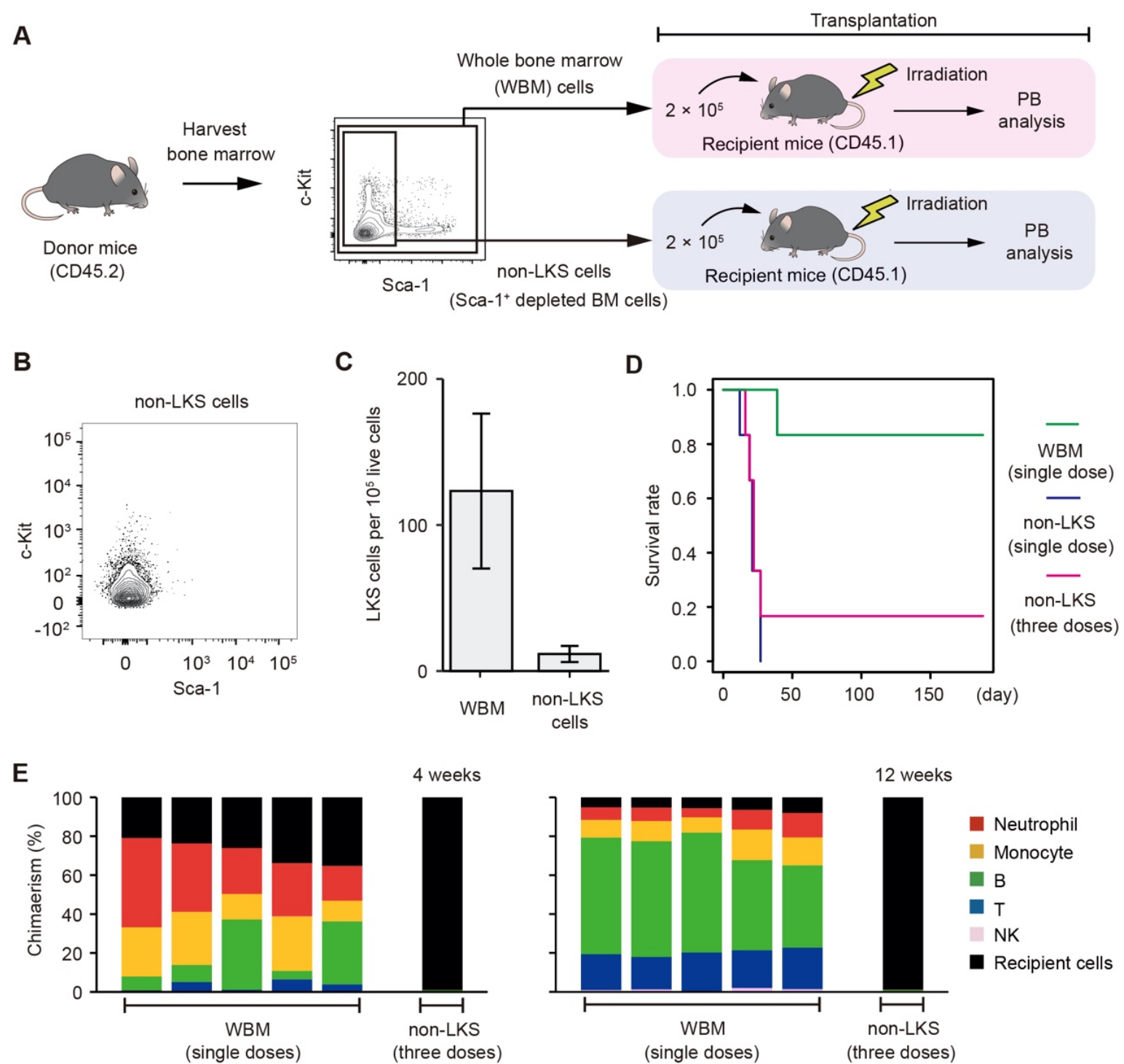


Fig 1. Transplantation of non-LKS cells does not rescue lethally irradiated mice. (A)

Experimental design for assessing the haematopoietic reconstitution capacity. Two hundred thousand WBM cells or non-LKS cells (Sca-1⁺ depleted BM cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice. (B) Representative flow cytometry plots of non-LKS cells after exclusion of doublets and dead cells. (C) Number of LKS cells per 1×10^5 live cells in WBM or non-LKS cells ($n = 3$). (D) Survival rate of recipient mice receiving single dose of 2×10^5 WBM cells or single dose of 2×10^5 non-LKS cells at day 0 or three doses of 2×10^5 non-LKS cells at day 0, 3, and 7 after

593 irradiation ($n = 6$). (E) PB chimaerism in each group 4 and 12 weeks after transplantation.

594 Each column represents an individual mouse.

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Figure.2

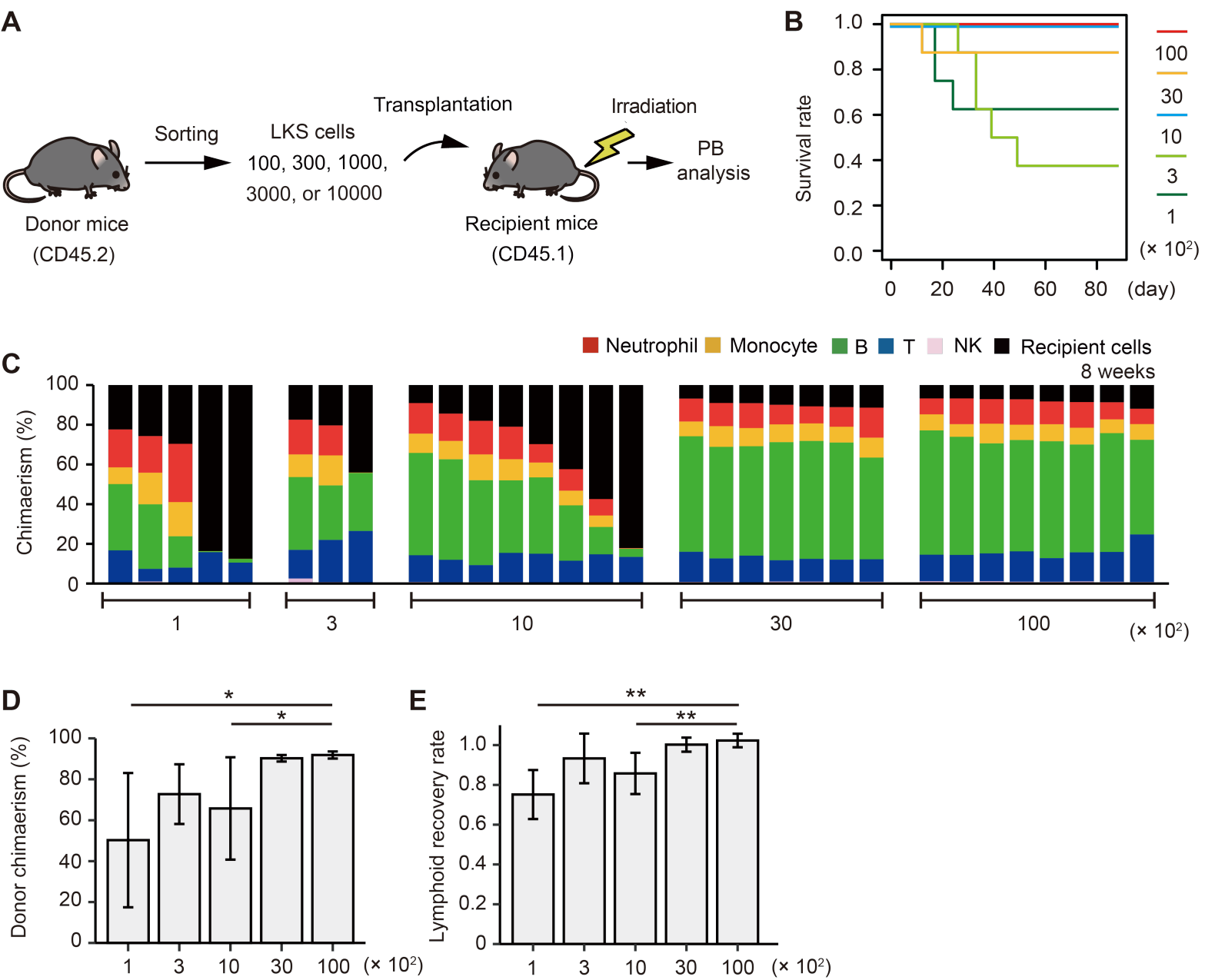


Fig 2. Transplantation with different numbers of LKS cells revealed a threshold for rapid haematopoietic recovery after LKS transplantation. (A) Experimental design for determining the cell number required for successful transplantation. Different number of LKS cells (100, 300, 1000, 3000, or 10000 cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice ($n = 8$). (B) Survival rate of each group after LKS transplantation. (C) PB chimaerism in each group 8 weeks after LKS transplantation. Each bar represents an individual mouse. (D) Average donor chimaerism in each group 8 weeks after LKS transplantation. (E) Average of lymphoid recovery rate in each group 8 weeks after LKS transplantation. The lymphoid recovery rate was defined

as the ratio of the lymphocyte percentage in the peripheral blood of recipient mice to the average percentage of untreated 3-month-old *Hoxb5*-tri-mCherry mice ($n = 5$). All error bars denote *s.d.* * $p < 0.05$. ** $p < 0.01$.

Figure.3

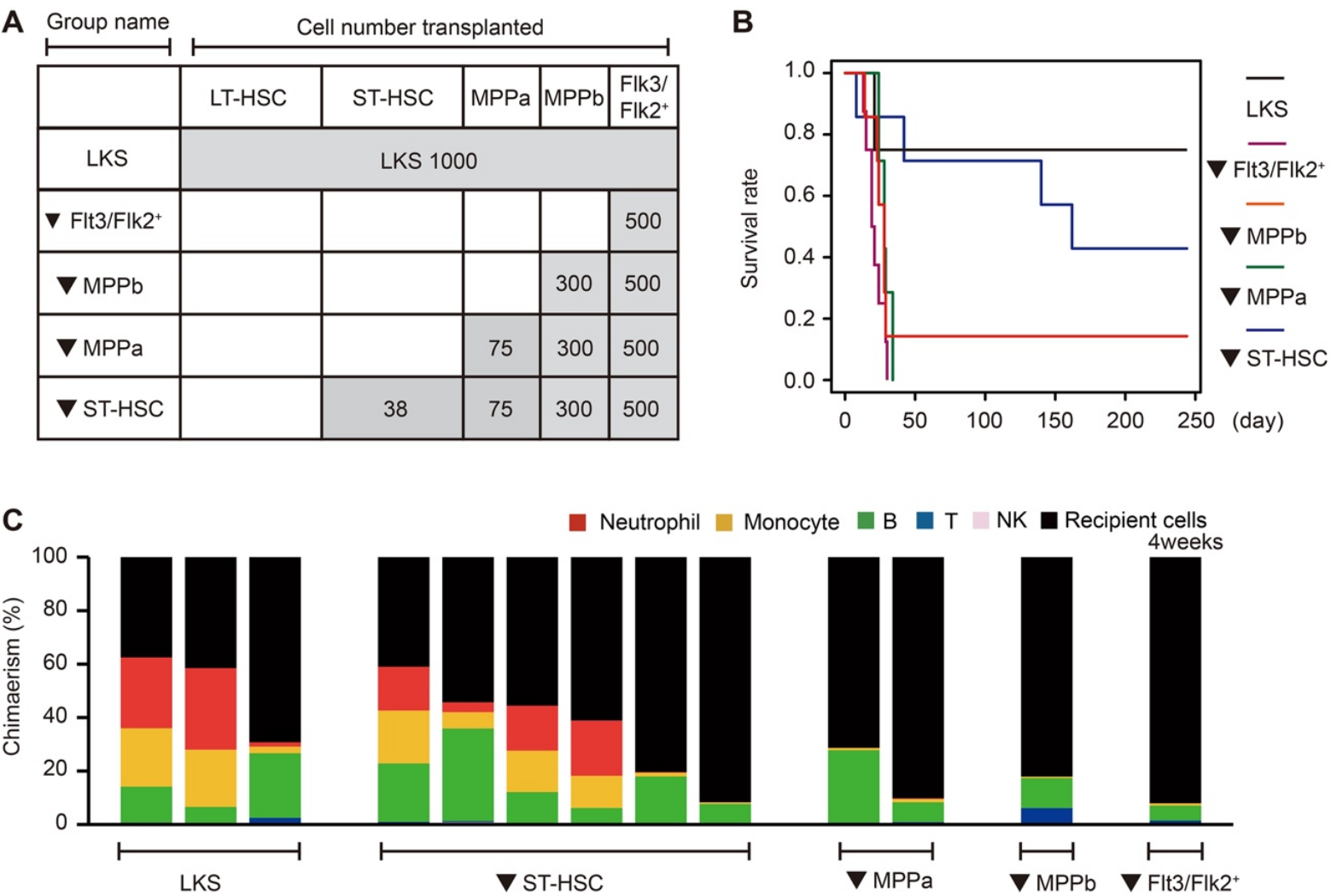


Fig 3. Non-HSC fractions do not play a critical role in haematopoietic reconstitution after transplantation (A) Summary of cell types and numbers transplanted in each group. The first column represents group names and the first row represents cell types. The total number of cells shown in each row were transplanted (LKS, $n = 4$; ▼Flt3/Flk2⁺, $n = 8$; ▼MPPb, $n = 7$; ▼MPPa, $n = 7$; ▼ST-HSC, $n = 7$). (B) Survival rate of each group. (C) PB chimaerism 4 weeks after transplanting each fraction. Each bar represents an individual mouse.

Figure.4

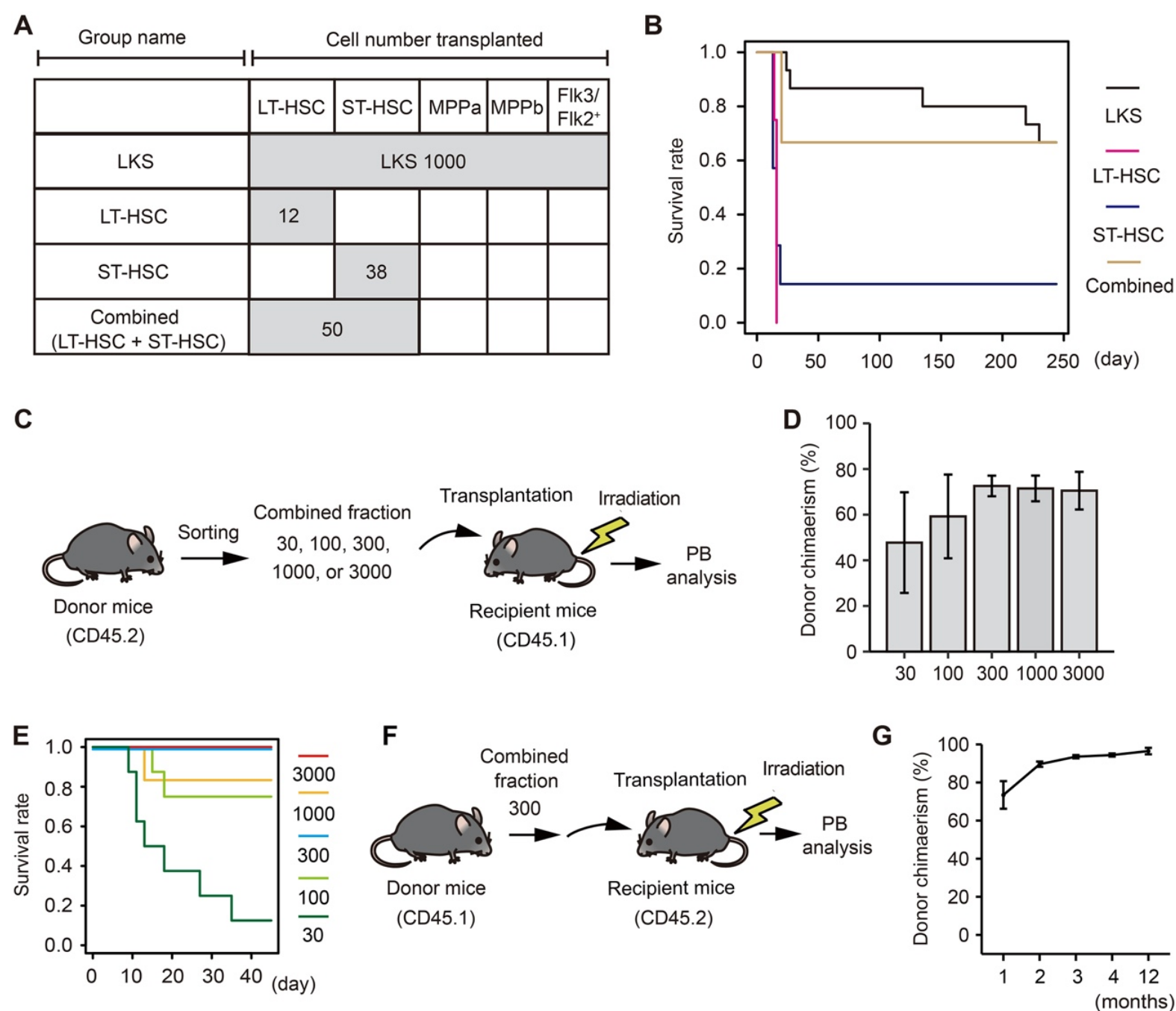
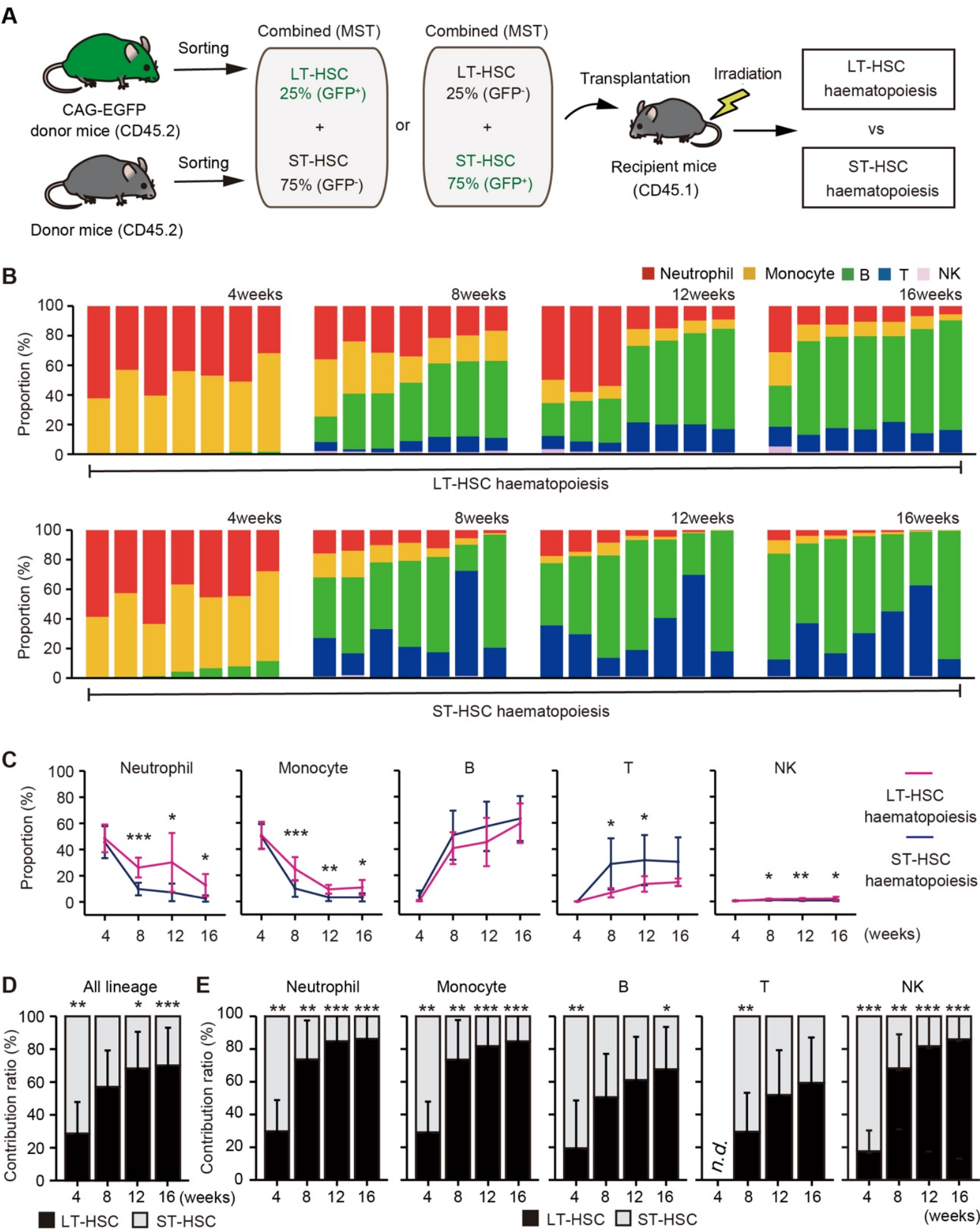


Fig 4. A combined HSC fraction is sufficient for haematopoietic recovery after transplantation. (A) Summary of cell types and numbers transplanted in each group (LKS, $n = 15$; LT-HSC, $n = 4$; ST-HSC, $n = 7$; combined, $n = 6$). (B) Survival rate of each group. (C) Experimental design for determining the cell number required for successful transplantation. Different cell number of combined HSCs (30, 100, 300, 1000, or 3000 cells) from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient mice. ($n = 6-8$ except for $n = 4$ in the 3×10^3 group). (D) Average donor chimaerism in each group 4 weeks after combined HSC transplantation. (E) Survival rate of each group. (F) Experimental design for assessing the long-term haematopoiesis after

combined HSC transplantation. Three hundred combined HSCs from CD45.1 mice were transplanted into lethally irradiated CD45.2 recipient mice ($n = 7$). (G) Kinetics of average donor chimaerism 1, 2, 3, 4, and 12 months after combined HSC transplantation. All error bars denote *s.d.*

Figure.5



GFP mice or *Hoxb5*-tri-mCherry mice and 225 ST-HSCs from *Hoxb5*-tri-mCherry mice or *Hoxb5*-tri-mCherry GFP mice were co-transplanted into lethally irradiated CD45.1 recipient mice ($n = 8$). (B) Individual lineage proportion from LT-HSCs and ST-HSCs in the donor PB 4, 8, 12, and 16 weeks after MST transplantation. Each bar represents an individual mouse. (C) Kinetics of average individual lineage proportion from LT-HSCs and ST-HSCs 4, 8, 12, and 16 weeks after MST transplantation. (D) Average contribution ratio from LT-HSCs and ST-HSCs in donor fraction 4, 8, 12, and 16 weeks after MST transplantation. The contribution ratio was calculated as the proportion of LT-HSC derived cells vs. ST-HSC derived cells in donor fraction. Statistical significance was calculated using Welch's t test between the proportion of LT-HSC derived cells vs. ST-HSC derived cells in donor fraction. (E) Average contribution ratio from LT-HSCs and ST-HSCs in each lineage 4, 8, 12, and 16 weeks after MST transplantation. All error bars denote *s.d.* $*p < 0.05$. $**p < 0.01$. $***p < 0.001$. "n.d." stands for "not detected."

Figure.6

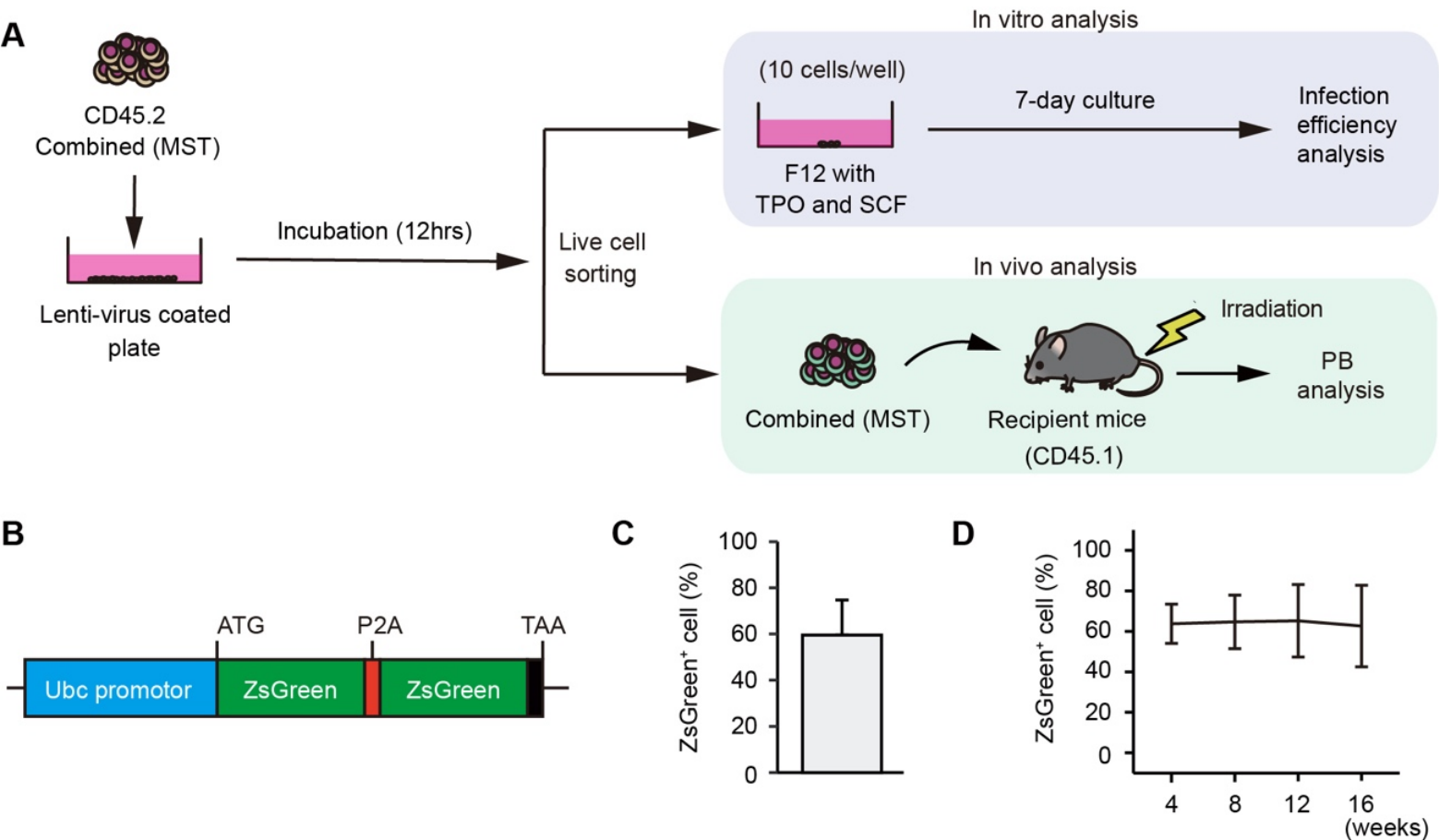


Fig 6. Gene transduction into the minimum subset by the lentiviral system. (A)

Experimental design for assessing the gene transduction efficiency into the minimum subset. (*In vitro* analysis, $n = 5$; *In vivo* analysis, $n = 8$). (B) Schematic of viral construct. Ubc: Ubiquitin C promoter. (C) Average percentage of ZsGreen positive cells after 7-day culture. (D) Kinetics of average percentage of ZsGreen positive cells in donor fraction 4, 8, 12, and 16 weeks after MST transplantation. All error bars denote *s.d.*

Figure.7

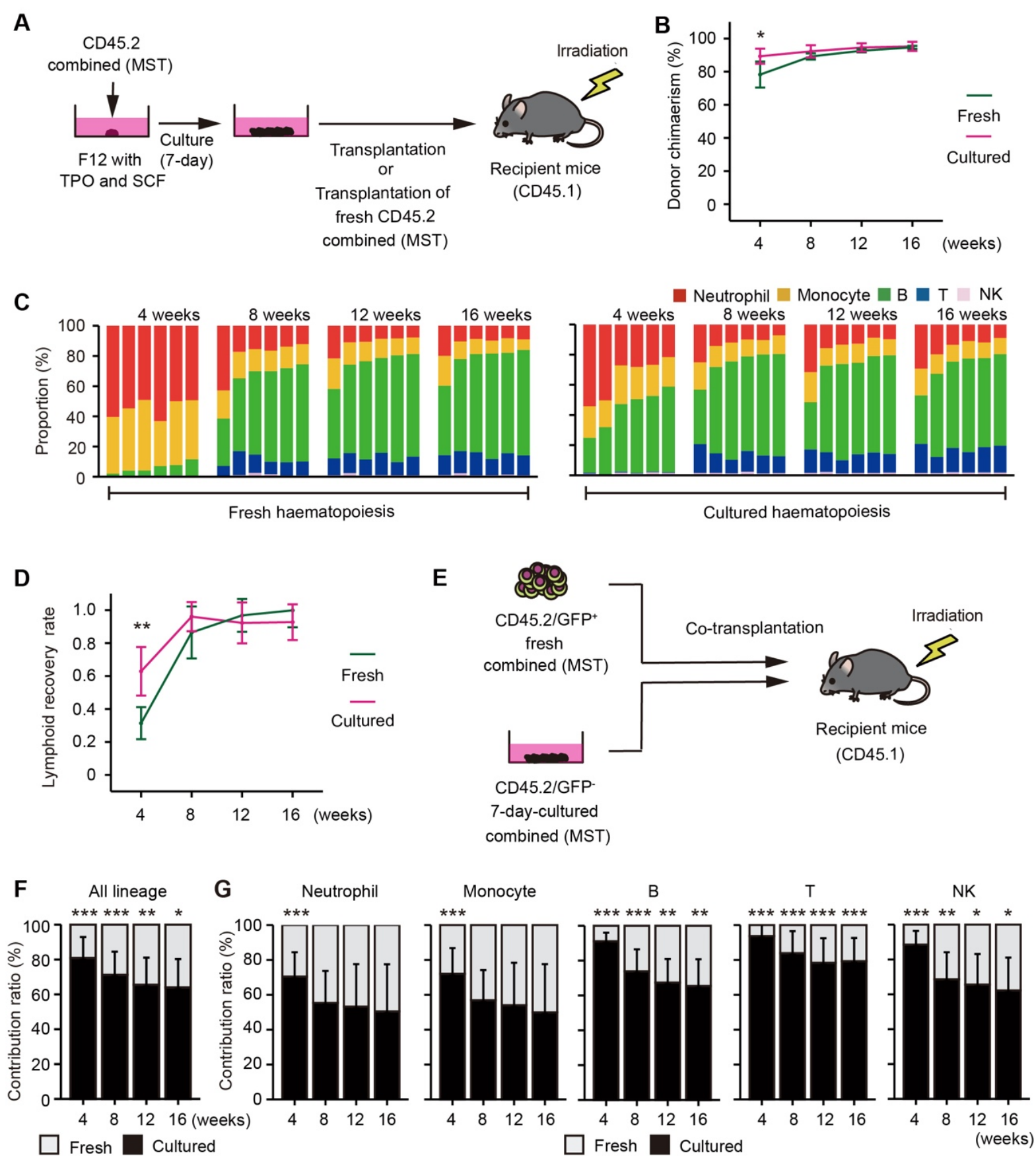


Fig 7. *Ex vivo* expansion of MST accelerates rapid and robust multilineage reconstitution. (A) Experimental design for assessing the haematopoietic reconstitution ability of fresh or cultured MST. Fresh MST cells from CD45.2 mice or 7-day-cultured MST cells from CD45.2 mice were transplanted into lethally irradiated CD45.1 recipient

mice ($n = 6$). (B) Kinetics of average donor chimaerism in each group 4, 8, 12, and 16 weeks after MST transplantation. (C) Lineage proportion in the donor PB from fresh or cultured MST cells 4, 8, 12, and 16 weeks after MST transplantation. (D) Kinetics of average lymphoid recovery rate in each group 4, 8, 12, and 16 weeks after MST transplantation. (E) Experimental design for co-transplantation assay. Fresh MST (CD45.2/GFP⁺) and cultured MST (CD45.2/GFP⁻) cells were co-transplanted into lethally irradiated CD45.1 recipient mice ($n = 6$). (F) Average contribution ratio from fresh and cultured MST cells 4, 8, 12, and 16 weeks after MST transplantation. The contribution ratio was calculated as the proportion of cultured MST derived cells vs. fresh MST derived cells in donor fraction. Statistical significance was calculated using Welch's t test between the proportion of cultured MST derived cells vs. fresh MST derived cells in donor fraction. (G) Average contribution ratio from cultured MST derived cells vs. fresh MST derived cells in each lineage 4, 8, 12, and 16 weeks after MST transplantation. All error bars denote *s.d.* * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Figure.8

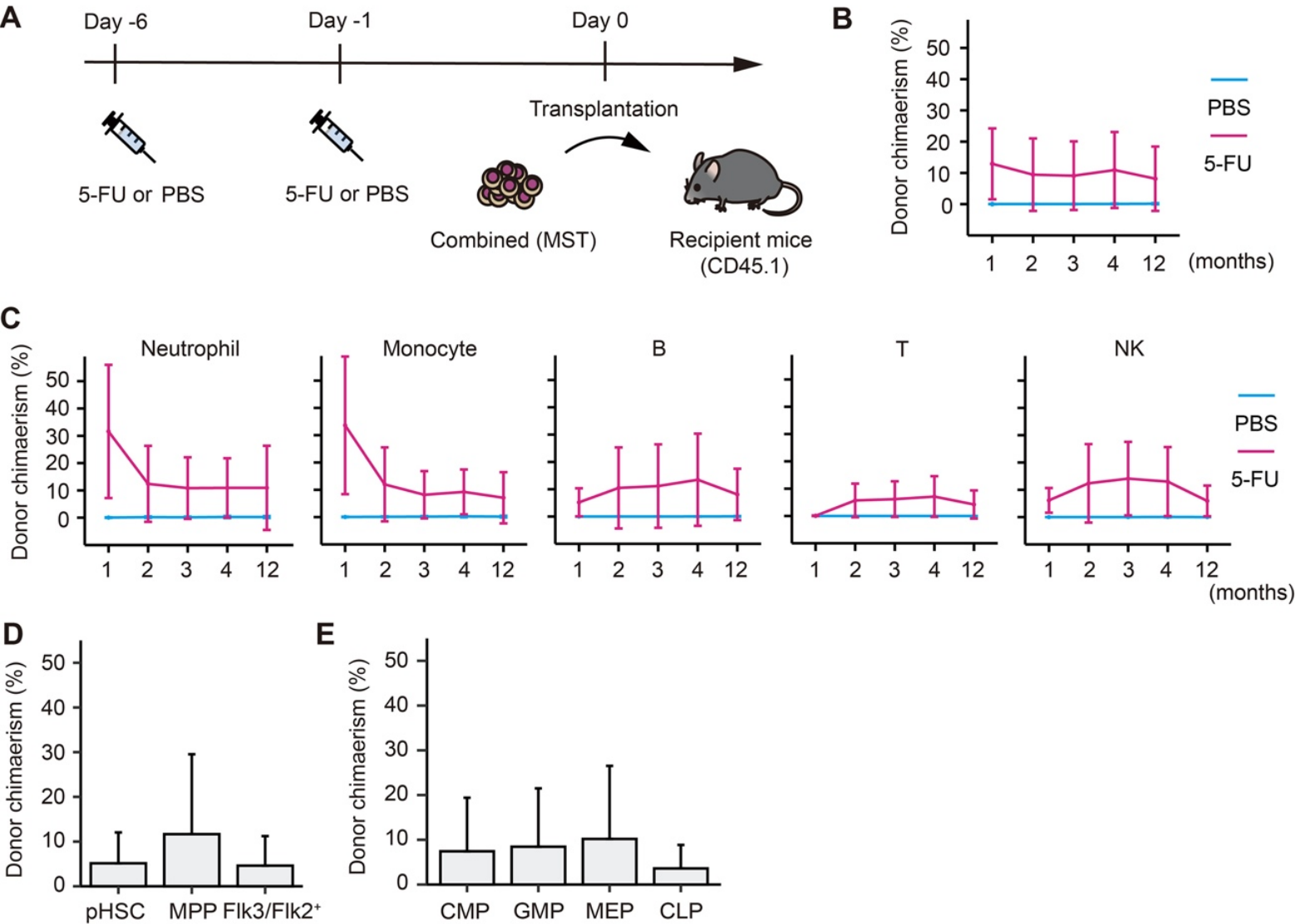


Fig 8. 5-FU conditioning followed by MST transplantation enables long-term donor haematopoiesis. (A) Experimental design for the 5-FU conditioning regimen. Three-thousand MST cells from CD45.2 mice were transplanted into 5-FU-treated or PBS-treated CD45.1 recipient mice (5-FU-treated, $n = 6$; PBS-treated, $n = 5$). (B) Kinetics of average donor chimaerism in the PB 1, 2, 3, 4, and 12 months after MST transplantation. (C) Kinetics of average donor chimaerism in each lineage fractions 1, 2, 3, 4, and 12 months after MST transplantation. (D) Average donor chimaerism in the haematopoietic stem and progenitor fractions 12 months after MST transplantation. (E) Average donor chimaerism in the oligopotent progenitor fractions 12 months after MST transplantation. All error bars denote *s.d.*

1 **Supplemental materials and methods**

2 ***Bone marrow cell analysis and sorting***

3 Flow cytometry and cell sorting were performed on a FACS Aria II cell sorter (BD
4 Biosciences, CA) and analysed using FlowJo™ software (BD Biosciences). Bone
5 marrow cells were collected from bilateral tibias, femurs, humeri, and pelvises in Ca²⁺
6 and Mg²⁺ free PBS supplemented with 2% heat-inactivated bovine serum (Thermo Fisher
7 Scientific, MA) and 2 mM EDTA. Cells were passed through 100 µm and 40 µm strainers
8 before analysis and sorting. Prior to staining, samples were blocked with 50 µg/ml rat IgG
9 (Sigma-Aldrich, MO) for 15 minutes. To enrich HSCs and progenitor populations, cells
10 were stained with APC-eFluor780-conjugated anti-c-Kit antibody (Thermo Fisher
11 Scientific) and fractionated using anti-APC magnetic beads and LS columns (both
12 Miltenyi Biotec, Bergisch Gladbach, Germany). c-Kit⁺ cells were then stained with a
13 combination of antibodies as described in supplemental table S1. All stainings were
14 performed at 4 °C for 30 minutes, with the exception of CD34 staining which was
15 incubated for 90 minutes. Samples were washed twice after staining. Prior to flow
16 cytometry or cell sorting, samples were stained with SYTOX Red Dead Cell Stain
17 (Thermo Fisher Scientific) or 7-aminoactinomycin D (BioLegend, CA). The donor and
18 recipient fraction were determined by the expression of CD45.1 and CD45.2 antigens.

19

20 ***Supporting cell-free Transplantation***

21 Twelve-to-twenty hours prior to transplantation, recipient mice were lethally irradiated
22 with a single dose of 8.7 gray. Donor cells in 200 µl of PBS with 2% heat-inactivated
23 bovine serum (Thermo Fisher Scientific), were then injected into the retro-orbital venous
24 plexus.

25

26 ***Peripheral blood analyses***

27 For evaluation of haematopoietic recovery after transplantation, peripheral blood was
28 collected. At each time point, 50 µl of blood was collected from the tail vein and
29 resuspended in Ca²⁺ and Mg²⁺ free PBS supplemented with 2 mM EDTA. Red blood cells
30 were lysed twice on ice for 3 minutes with BD Pharm Lyse Buffer (BD Biosciences).
31 Identification of leukocyte subsets was performed by staining with antibodies. Antibody
32 information was described in Supplemental table S1. The gating strategy of each lineage
33 (NK cell, B cell, T cell, granulocyte, and monocyte) was described in Figure S1C. The
34 donor and recipient fractions were determined by the expression of CD45.1 and CD45.2
35 antigens. The donor lineage proportion was calculated as the frequency of each lineage
36 among all donor cells. The donor chimaerism in the peripheral blood was calculated as
37 the frequency of the donor fraction among all live cells. Lymphoid recovery rate was
38 calculated as the ratio of lymphocyte percentage in the peripheral blood in recipient mice
39 to the average percentage of untreated 3-month-old *Hoxb5*-tri-mCherry mice ($n = 5$).
40 Peripheral blood data represent mice which survived until the last peripheral blood
41 analysis.

42

43 ***Sca-1⁺ depletion***

44 Bone marrow cells were collected as mentioned above. To deplete Sca-1⁺ fraction, cells
45 were stained with APC-conjugated anti-Sca-1 antibody (BioLegend) and fractionated
46 using anti-APC magnetic beads and LD columns (both Miltenyi Biotec). Sca-1⁺ depleted
47 cells were also stained for analysis with a combination of antibodies as described in
48 Supplemental table S1. All stainings were incubated at 4 °C for 30 minutes. Prior to flow

49 cytometry, samples were stained with SYTOX Blue Dead Cell Stain (Thermo Fisher
50 Scientific).

51

52 *Lentiviral gene transduction*

53 Lentiviruses were produced in the Lenti-X 293T cell line (Takara Bio, Shiga, Japan) by
54 transient co-transfection of pCAG-HIVgp (provided from RBRC, RDB04394), pCMV-
55 VSV-G RSV-Rev (provided from RBRC, RDB04393), and a lentiviral vector to express
56 ZsGreen. Transfections were performed with X-tremeGENE HP DNA Transfection
57 Reagent (Sigma-Aldrich), as per manufacturer instructions, at a ratio of 1.7:1:1, lentiviral
58 vector: pCAG-HIVgp: pCMV-VSV-G RSV-Rev. After 48 hours, supernatant was
59 collected and filtered through a 0.45 µm filter. Lentiviral particles were concentrated by
60 ultracentrifugation for 1.5 hours at 22,000 rpm at 4 °C.¹ Next, lentiviral transduction was
61 performed on 96 well plates (Corning, NY) coated with RetroNectin (Takara Bio)
62 following manufacturer instructions. Cells were collected the following day and sorted
63 by a FACS Aria II (BD Biosciences) to adjust cell numbers transplanted per mouse. For
64 *in vitro* assays, 10 cells per well on 96 well plate were cultured in F12 medium (Thermo
65 Fisher Scientific), 1% insulin–transferrin–selenium–ethanolamine (ITSX; Thermo Fisher
66 Scientific), 1% penicillin/streptomycin/glutamine (P/S/G; Thermo Fisher Scientific), 10
67 mM HEPES (Thermo Fisher Scientific), at 37 °C with 5% CO₂. Cultures were
68 supplemented with 12.5 ng/ml recombinant mouse SCF (Peprotech) and 12.5 ng/ml
69 recombinant mouse TPO (Peprotech).

70

71 *Ex vivo expansion of haematopoietic cells*

72 The culture medium was composed of F12 medium (Thermo Fisher Scientific), 1% ITSX
73 (Thermo Fisher Scientific), 10 mM HEPES (Thermo Fisher Scientific), 1% P/S/G
74 (Thermo Fisher Scientific), 12.5 ng/ml mouse TPO (Peprotech, NJ), 12.5 ng/ml mouse
75 SCF (Peprotech). Fifty of the minimum subset cells were sorted and cultured in a well in
76 96 well plate for 7 days at 37 °C with 5% CO₂.

77

78 ***5-FU conditioning***

79 5-Fluorouracil (Sigma-Aldrich) was dissolved in DMSO and prepared as 5-FU solution
80 in PBS. Recipient mice were injected intraperitoneally twice with 150 mg of 5-FU per kg
81 body weight from the 5-FU solution.²

82

83 ***Quantification and statistical analysis***

84 The sample size for each experiment is included in the figure legends. Statistical analyses
85 were performed using ggplot2 in the R statistical programming environment or Microsoft
86 Excel. Statistical significance was determined by Welch's t test. *P* values < 0.05 were
87 considered significant. Error bar denotes s.d. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001, n.s.
88 not significant.

89

90 **References for Supplementary Methods**

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Table S1 Antibodies used for flow cytometry

	Antigen	Clone	Fluorochrome	Vendor
Mouse bone marrow analysis	Flk2	A2F10	PerCP-eFluor710	Thermo Fisher Scientific
	CD34	RAM34	FITC	Thermo Fisher Scientific
		RAM34	A647	BD Bioscience
	CD11b	M1/70	BV711	BD Bioscience
		M1/70	BV650	BD Bioscience
	CD150	TC15-12F12.2	BV421	Biolegend
		TC15-12F12.2	A647	Biolegend
	CD3ε	145-2C11	Biotin	Biolegend
	CD3	17A2	A700	Biolegend
	CD4	GK 1.5	Biotin	Biolegend
		GK 1.5	A700	Biolegend
	CD8a	53-6.7	Biotin	Biolegend
		53-6.7	A700	Biolegend
	Ter-119	TER-119	Biotin	Biolegend
		TER-119	A700	Biolegend
	Gr-1	RB6-8C5	Biotin	Biolegend
		RB6-8C5	A700	Biolegend
	B220	RA3-6B2	Biotin	Biolegend
		RA3-6B2	A700	Biolegend
	CD127	A7R34	A-700	Thermo Fisher Scientific
		A7R34	PE	Biolegend
	CD16/32	93	A-700	Thermo Fisher Scientific
		93	FITC	Biolegend
	Sca-1	D7	BUV395	BD Bioscience
	c-Kit	2B8	APC-eFluor 780	Thermo Fisher Scientific
	Streptavidin		BUV737	BD Bioscience
			BV421	Biolegend
	CD45.1	A20	Biotin	Biolegend
	CD45.2	104	BV785	Biolegend
Mouse peripheral blood analysis	CD45.1	A20	FITC	BD Bioscience
		A20	Biotin	Biolegend
	CD45.2	104	PE	BD Bioscience
	Gr-1	RB6-8C5	A700	Biolegend
	CD11b	M1/70	BUV395	BD Bioscience
		M1/70	BV711	BD Bioscience
	NK1.1	PK136	PerCP-Cy5.5	Biolegend
	CD3ε	145-2C11	BV421	Biolegend
		145-2C11	APC-Cy7	Biolegend
	TCRβ	H57-597	BV421	Biolegend
		H57-597	APC-Cy7	Biolegend
	B220	RA3-6B2	BV786	BD Bioscience
Sca-1+ depletion	c-Kit	2B8	FITC	Biolegend
	CD11b	M1/70	BV711	BD Bioscience
	CD3ε	145-2C11	A700	Biolegend
	CD4	GK 1.5	A700	Biolegend
	CD8a	53-6.7	A700	Biolegend
	Ter-119	TER-119	A700	Biolegend
	Gr-1	RB6-8C5	A700	Biolegend
	B220	RA3-6B2	A700	Biolegend
	Sca-1	D7	APC	Biolegend

Supplemental Figure 1

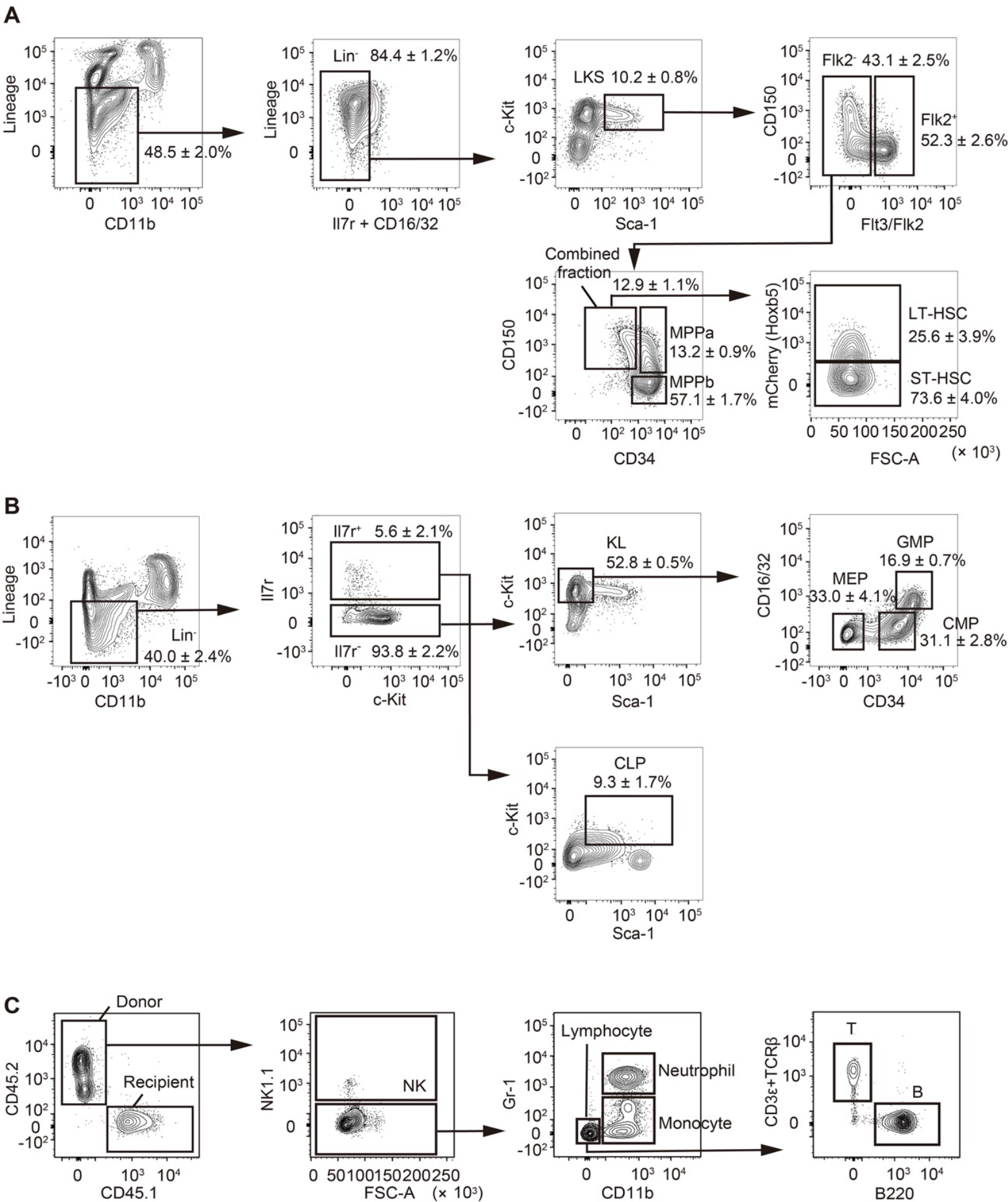


Fig S1. Gating scheme for haematopoietic cells. (A) Representative flow cytometry gating to isolate LKS, Flk2⁺, MPPa, MPPb, Combined fraction, ST-HSC, and LT-HSC after exclusion of doublets and dead cells. Values indicate the percentage of each fraction

± *s.d.* (*n* = 3). (B) Representative flow cytometry gating to isolate common lymphoid progenitor (CLP), granulocyte-macrophage progenitor (GMP), common myeloid progenitor (CLP), and megakaryocyte-erythrocyte progenitor (MEP) after exclusion of doublets and dead cells. Values indicate the percentage of each fraction ± *s.d.* (*n* = 3). (C) Gating scheme to identify peripheral blood cells (NK cell, Neutrophil, Monocyte, T cell, and B cell) in recipient mice after exclusion of doublets and dead cells.

Supplemental Figure 2

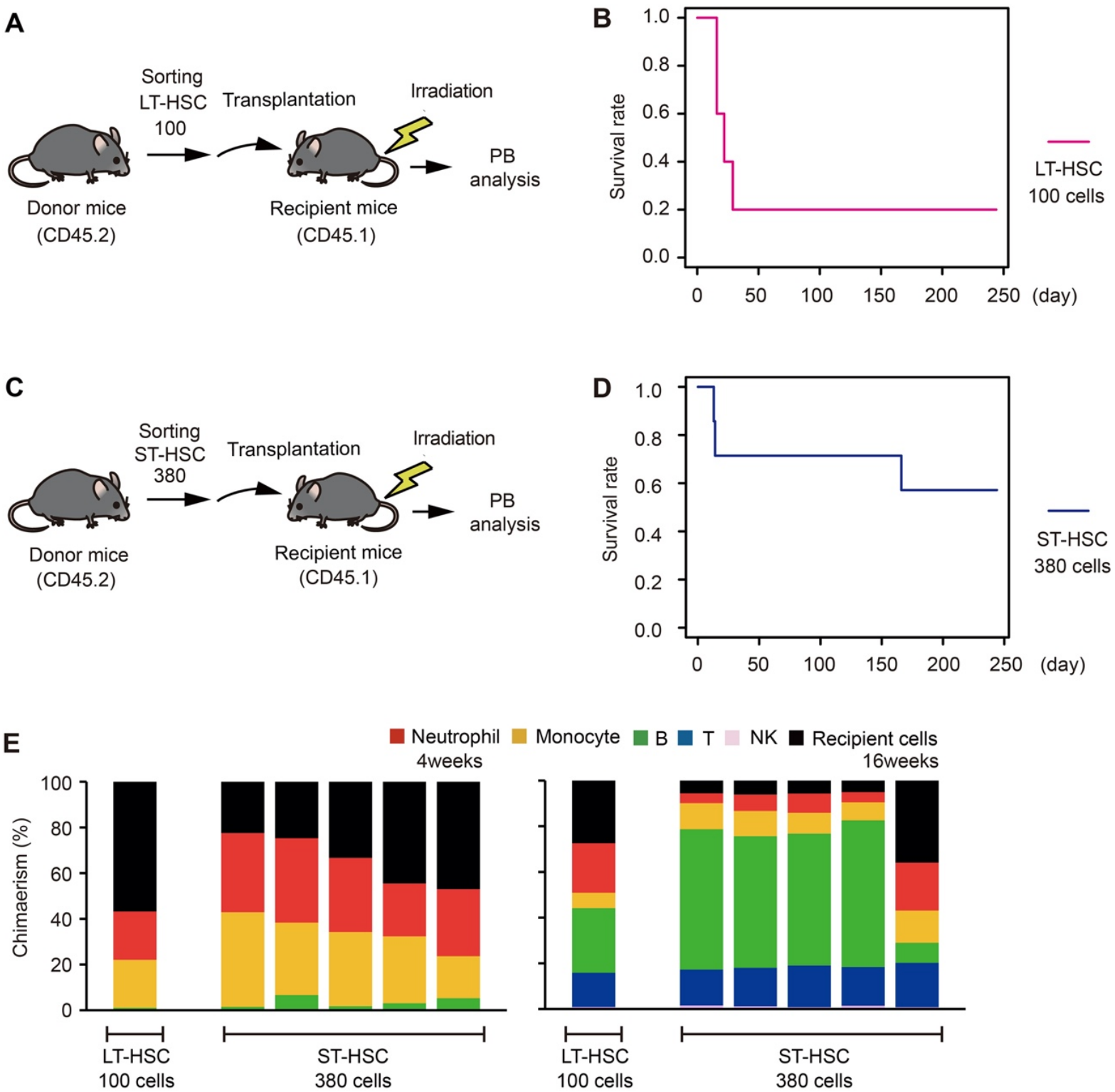


Fig S2. Either LT-HSCs or ST-HSCs are not sufficient for the rapid haematopoietic recovery and long-term haematopoiesis. (A) Experimental design for assessing the effect of a high dose of LT-HSC transplantation ($n = 5$). (B) Survival rate after LT-HSC transplantation. (C) Experimental design for assessing the effect of a high dose of ST-HSC transplantation ($n = 7$). (D) Survival rate after ST-HSC transplantation. (E) Peripheral blood chimaerism 4 and 16 weeks after LT-HSC or ST-HSC transplantation. Each bar represents an individual mouse.