Original Article

T-lymphopoiesis is Severely Compromised in Ubiquitin-Green Fluorescent Protein Transgenic Mice

(UBC-GFP mice / C57Bl/J mice / lymphopoiesis / myelopoiesis / haematopoiesis / stem cell / transplantation / green fluorescent protein / T cell / B cell)

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Abstract. Tagging cells of experimental organisms with genetic markers is commonly used in biomedical research. Insertion of artificial gene constructs can be highly beneficial for research as long as this tagging is functionally neutral and does not alter the tissue function. The transgenic UBC-GFP mouse has been recently found to be questionable in this respect, due to a latent stem cell defect compromising its lymphopoiesis and significantly influencing the results of competitive transplantation assays. In this study, we show that the stem cell defect present in UBC-GFP mice negatively affects T-lymphopoiesis significantly more than B-lymphopoiesis. The production of granulocytes is not negatively affected. The defect in T-lymphopoiesis causes a low total number of white blood cells in the peripheral blood of UBC-GFP mice which, together with the lower lymphoid/myeloid ratio in nucleated blood cells, is the only abnormal phenotype in untreated UBC-GFP mice to have been found to date. The defective lymphopoiesis in UBC-GFP mice can be repaired by transplantation of congenic wild-type bone marrow cells, which then compensate for the insufficient production of T cells. Interestingly, the wild-type branch of haematopoiesis in chimaeric UBC-GFP/wild-type mice was more active in lymphopoiesis, and particularly towards production of T cells, compared to the lymphopoiesis in normal wild-type donors.

Introduction

We have recently reported that transgenic mice carrying the gene encoding the enhanced green fluorescent protein and expressed strongly in haematopoietic tissue (UBC-GFP mice; Schaefer et al., 2001) engraft transplanted bone marrow of wild-type mice without previous conditioning (Faltusová et al., 2018). Analysis of this unexpected finding revealed a latent stem cell defect negatively affecting the lymphopoiesis in UBC-GFP mice (Faltusová et al., 2018). This is a very serious finding, because UBC-GFP mice are widely used in experimental haematology research (Huang et al., 2010; Dholakia et al., 2015; Zhou et al., 2017; Ayoub et al., 2018; Grinenko et al., 2018) and are generally assumed to have no abnormal phenotype except for the fluorescent cellular label (Okabe et al., 1997; Li et al., 2018).

This defect in the haematopoietic stem cells of UBC-GFP mice manifested as a weak potential of the UBC-GFP bone marrow cells to compete with the bone marrow cells of wild-type mice in competitive transplantation assays. Interestingly, this stem cell defect negatively affecting the lymphopoiesis in UBC-GFP mice is not associated with reduced fitness of these transgenic mice (Faltusová et al., 2018).

In this study we focused on analysis of the B- and T-lymphopoiesis in untreated or submyeloablatively irradiated UBC-GFP mice and also in mice with post-transplantation UBC-GFP/wild-type chimaeric haematopoiesis. We show that the low number of white blood cells (WBC) in the peripheral blood of untreated UBC-GFP mice is mainly due to the low number of T cells and that production of T cells is significantly more negatively affected in UBC-GFP mice than the production of

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Abbreviations: B cell – B220-positive cell, B/T – ratio between percentage of B220 and T cells, CD45.1 and CD45.2 – two isoforms of CD45 marker expressed in nucleated blood cells in C57Bl/6 mice, CTRL – control mice, FC – flow cytometry, GFP – green fluorescent protein, GM – Gr1/Mac-1-positive cells, HA – haematology analyser, LSK – lineage-negative, Sca-1 and c-Kit-positive cell, $LS^{neg}K$ – lineage-negative Sca-1-negative and c-Kit-positive cell, Ly – lymphocytes ($B220^+$ plus $CD4^+$ plus $CD8^+$ cells; My – myeloid cells (neutrophils + eosinophils + basophils + monocytes) or GM cells, PBS – phosphate-buffered saline containing 0.5% bovine serum albumin, UBC – ubiquitin, T cells – $CD4^+$ plus $CD8^+$ cells, WBC – white blood cell(s).

B cells. Transplanted wild-type bone marrow cells substitute the T cell defect by enhanced production of T cells.

Material and Methods

Mice

C57BL/6J (CD45.2 mice), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1 mice), their F1 hybrids, and C57Bl/6-Tg(UBC-GFP)30Scha/J (UBC-GFP mice) mice of both sexes were used. The breeding pairs of CD45.2 and CD45.1 were from Charles River (Velaz, Prague, Czech Republic). The breeding pairs of UBC-GFP mice were obtained from Dr. Radek Skoda (University of Basel, Switzerland) in 2009 and since then have been bred in the specific pathogen-free barrier area of the institutional animal facility. During the experiments, mice were maintained in a clean conventional part of the facility. Two to four-month-old mice of both sexes were used in the experiments. The experiments were performed in accordance with the national and international guidelines for laboratory animal care and were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic (MSMT-4502/2017-2 and MSMT-32441/2018-8).

Blood cell analysis

Blood samples obtained from retro-orbital venous plexus of mice were analysed by Auto Haematology Analyser BC-5300 Vet (Mindray, Nanshan, China).

Bone marrow, spleen and thymus cell collection

Bone marrow, spleen and thymus cells were collected from mice killed by cervical dislocation. Femurs were flushed with 1 ml PBS (phosphate-buffered saline) containing 0.5% bovine serum albumin (hereafter referred to as PBS). Spleen and thymus tissues were disrupted by a loosely fitting glass homogenizer. The cells were counted by either Cellometer (Nexcelom Bioscience, Lawrence, MA) or by BC-5300Vet haematology analyser.

Irradiation

Total body irradiation with γ rays from a ^{60}Co source from a distance of 123.5 cm or 102.0 cm (≈ 0.58 Gy/min) was used.

Bone marrow or spleen cell transplantation

The single-cell suspension of bone marrow cells was transplanted intravenously via the retro-orbital route. Recipient mice were groups of three to eight mice either untreated or conditioned with irradiation. Irradiated recipients were transplanted with donor cells within four hours after irradiation.

Analysis of chimaeric haematopoiesis in transplanted mice

The donor and host cells were determined in the peripheral blood of transplanted mice and in the bone marrow, spleen and thymus after the mice were sacrificed. Blood samples were withdrawn from the retro-orbital venous plexus into heparinized capillaries. To exclude adhering of GFP-containing particles from lysed red blood cells to nucleated cells and thus to eliminate false-positive GFP signals on non-GFP nucleated cells, we skipped the red blood cell lysis and excluded red blood cells by the gating strategy, previously described by us (Faltusová et al., 2018).

Analysis of bone marrow and spleen cells

The cell suspension of bone marrow or spleen cells was filtered through a 70 μm nylon cell filter (BD Biosciences, Tenecula, CA) and stained with fluorochrome-labelled antibodies. The antibodies used are listed in Table 1. The staining was performed on ice for 30 min in the dark with optimal dilutions of antibodies. Digital FACS Canto II and FACSAria IIu flow cytometers (BD Biosciences) and the BD FACSDiva software, version 6.1.3, were used. Data were analysed using FlowJo vX software (FlowJo, Becton, Dickinson & Company, Franklin Lakes, NJ).

Statistics

Statistical analyses were performed with the GraphPad Prism software (GraphPad Software, San Diego, CA, www.graphpad.com). Data are presented in figures as the mean ± standard error of the mean (SEM). For multi-group comparisons, one-way ANOVA was used. For comparison between two experimental groups, the Student's *t*-test was used, with two-tailed distribution assuming equal sample variance. The following asterisk marks were used to indicate the level of significance: *P < 0.05, **P < 0.01, ***P < 0.001.

Results

1. The defect in lymphoid-primed stem cells in UBC-GFP mice is more intensively expressed in T-lymphopoiesis than in B-lymphopoiesis and causes lymphopoenia

To investigate the previously reported stem cell defect in the haematopoiesis of UBC-GFP transgenic mice in more depth, we analysed the peripheral blood in untreated UBC-GFP mice and wild-type CD45.1 mice by flow cytometry and also determined the total number of WBC with a haematology analyser. The flow cytometry analysis of nucleated blood cells revealed a significantly decreased ratio between lymphocytes and myeloid blood cells (Ly/My_{FC}) in the blood of female UBC-GFP mice compared to the ratio in CD45.1 female wild-type mice (Fig. 1b). In male mice, the difference did not

Table 1. Antibodies used in phenotypic analysis of immature haematopoietic cells

Antibody	Clone	Producer	Fluorochrome
anti-Lineage cocktail of antibodies CD45R(B220)/CD3/ Ly-6G(Ly-6C)/CD11b/Ter-119)		http://www.biolegend.com/	Alexa Fluor® 700
CD117 (c-kit)	2b8	http://www.biolegend.com/	Brilliant Violet 421™
Ly-6A/E (Sca-1)	D7	http://www.biolegend.com/	PerCP
Ly-6A/E (Sca-1)	E13-161.7	http://www.biolegend.com/	PE/Cy7
CD48	HM48-1	http://www.biolegend.com/	PE
CD150 (SLAM)	TC15-12F12.2	http://www.biolegend.com/	APC
CD150 (SLAM)	TC15-12F12.2	http://www.biolegend.com/	Brilliant Violet 605™
CD71	RI7217	http://www.biolegend.com/	PE
CD45.2	104	http://www.biolegend.com/	PE/Cy7
CD45.1	A20	http://www.biolegend.com/	APC
CD45R/B220	RA3-6B2	http://www.biolegend.com/	A700
CD4	53-6,7	http://www.biolegend.com/	PerCP
CD8	GK1.5	http://www.biolegend.com/	PerCP
CD8	GK1.5	http://www.biolegend.com/	APC
Ly-6G(Gr-1)	RB6-8C5	http://www.biolegend.com/	Brilliant Violet 421™
CD11b	M1/70	http://www.biolegend.com/	Brilliant Violet 421™
TER-119/Erythroid cells	TER119	http://www.biolegend.com/	PerCP
CD 127 IL7	93	http://www.biolegend.com/	Brilliant Violet 785™
CD 16/32	A7R34	http://www.biolegend.com/	Brilliant Violet 510™
CD 34	RAM34	http://www.ebioscience.com/	Biotin
CD 135	A2F10	http://www.biolegend.com/	APC
Streptavidin		http://www.biolegend.com/	PE/Cy7

reach statistical significance (Fig. 1a). The percentage of T cells among all lymphocytes was significantly lower in UBC-GFP mice of both sexes compared to CD45.1 mice, and this led to a significantly increased ratio between B cells (B220) and T cells (CD4 + CD8) in UBC-GFP mice (Fig. 1 a,b).

The absolute number of WBCs in the peripheral blood of UBC-GFP and CD45.1 mice was then used, together with the relative percentage of B cells, T cells and myeloid GM cells determined by flow cytometry, to calculate the total number of B cells, T cells and GM cells in one microliter of the peripheral blood. The number of WBCs was lower in UBC-GFP mice of both sexes than the WBC number in CD45.1 mice (Fig. 1 a,b). The absolute number of B cells was significantly lower only in female UBC-GFP mice (Fig. 1b). The absolute number of T cells was significantly lower in UBC-GFP mice of both sexes (Fig. 1a,b). The absolute number of GM cells did not differ between UBC-GFP mice and CD45.1 mice (Fig. 1a,b). The lower number of WBCs, and particularly the lower number of T cells, in the peripheral blood of untreated UBC-GFP mice is thus the only known phenotype by which these transgenic mice differ from CD45.1 wild-type mice.

2. Defective lymphopoiesis of UBC-GFP mice can be repaired by transplantation of bone marrow from wild-type mice

We have previously shown that the defect in lymphoid-primed HSCs in UBC-GFP revealed by Faltusová et al. (2018) makes their haematopoiesis permissive for the engraftment of transplanted bone marrow cells of wild-type mice without prior conditioning (Faltusová et al., 2018). However, the engraftment level of transplanted bone marrow is then highly enhanced in the UBC-GFP mice that have fully recovered from previous sub-myeloablative irradiation (Faltusová et al., 2018). We have now tested whether the engraftment of wild-type bone marrow cells would correct the low number of T cells in the peripheral blood of UBC-GFP mice.

Two groups of male and two groups of female UBC-GFP mice were either irradiated with a dose of 6 Gy (groups 2 in Fig. 2a,c) or left untreated as age-matched controls (groups 1 in Fig. 2a,c). After 84 days, the peripheral blood of irradiated mice recovered almost fully to the values in unconditioned mice (Fig. 2b). Importantly, the number of lymphocytes also recovered, and the ratio between the lymphoid and the myeloid cells (Ly/My_{HA}) was close to that in untreated UBC-GFP control mice (Fig. 2b).

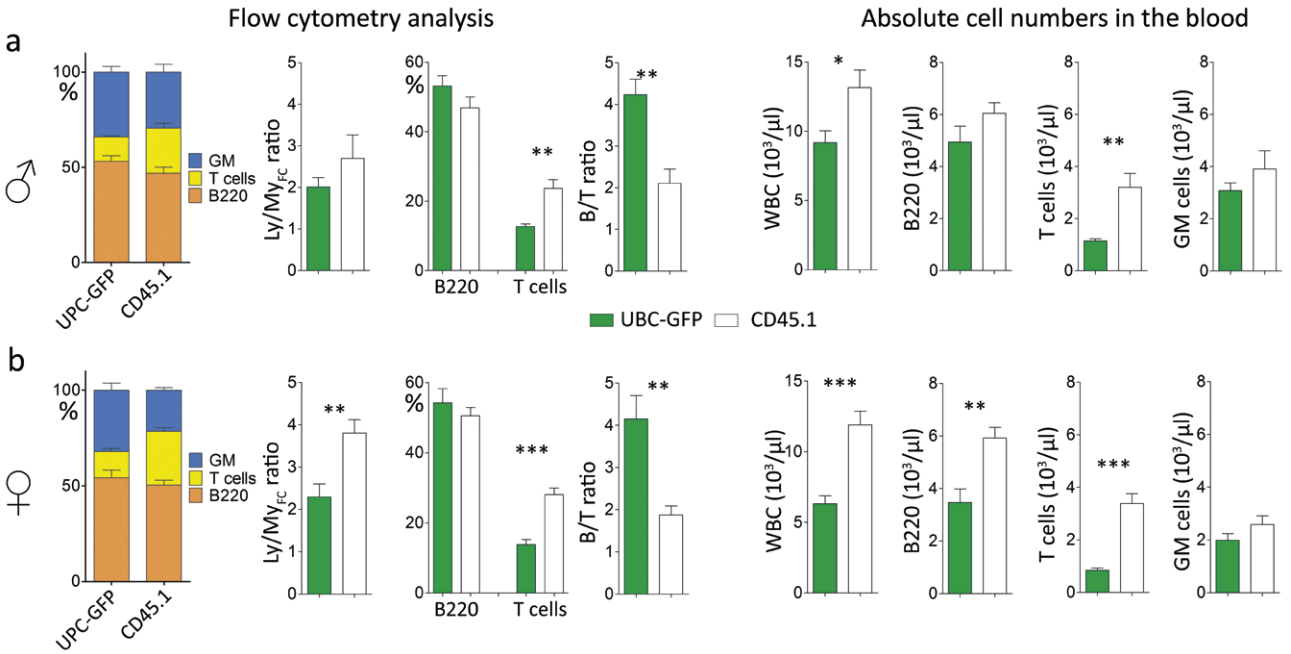

Fig. 1. Peripheral blood in UBC-GFP mice differs from the blood of CD45.1 wild-type mice and has a low number of T cells. A sample of blood was collected from the retro-orbital sinus of 5 male and 6 female UBC-GFP mice and from 7 male and 7 female CD45.1 mice, age-matched to UBC-GFP mice. (**a**) male mice, (**b**) female mice.

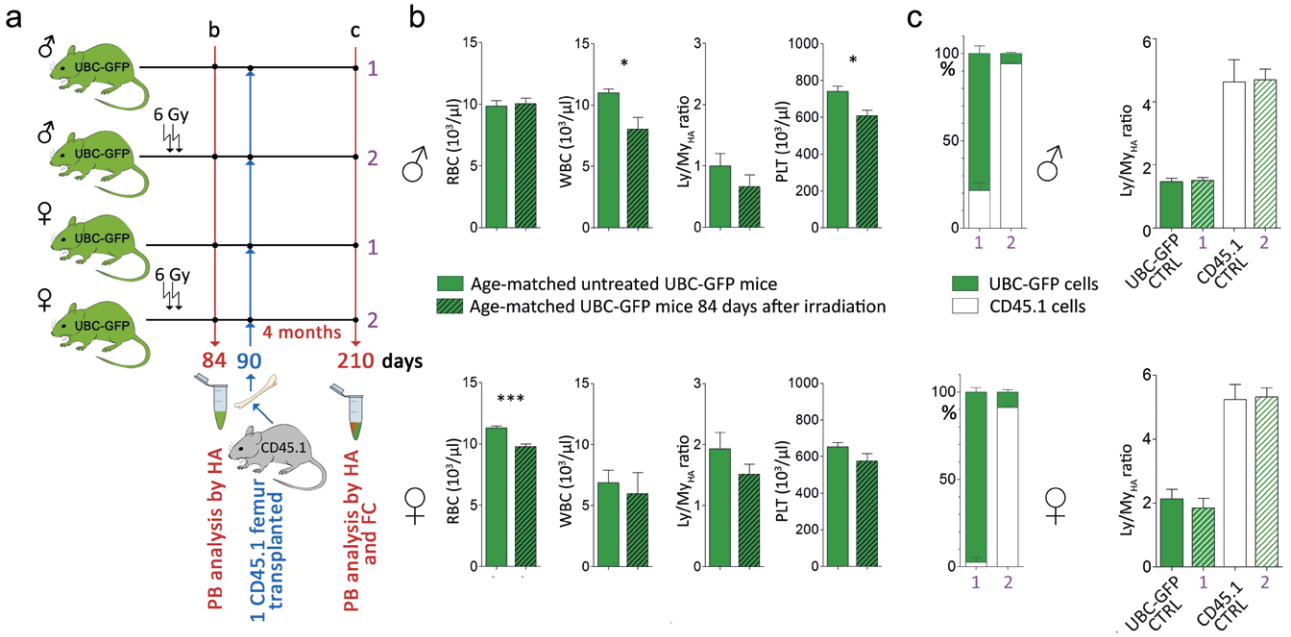

Fig. 2. Unconditioned UBC-GFP mice (groups 1) engraft transplanted bone marrow of wild-type mice and reconstituted haematopoiesis in submyeloablatively irradiated UBC-GFP mice (groups 2) remains highly permissive for engraftment of transplanted bone marrow cells.
(**a**) Experimental set-up: five UBC-GFP male and five female mice were pre-conditioned by irradiation with a dose of 6 Gy (groups 2) and five male and five female UBC-GFP mice were kept as controls (groups 1). (**b**) Samples of peripheral blood were analysed 84 days (84) after irradiation with a haematology analyser. Ninety days after starting the experiment, all 20 mice were transplanted with bone marrow obtained from 20 femurs of 10 CD45.1 male donor mice. (**c**) Four months after the transplantation, the UBC-GFP and CD45.1 nucleated cells were determined in samples of peripheral blood by flow cytometry. The blood samples were also examined with the haematology analyser together with blood samples from untreated age-matched UBC-GFP (8 males and 5 females) and CD45.1 (5 males, 4 females) mice. The Ly/My$_{HA}$ ratio from the haematology analyser data were compared in the blood of mice with chimaeric haematopoiesis (1 and 2) with those in untreated either UBC-GFP or CD45.1 mice (CTRL).

All these mice were transplanted with bone marrow cells of CD45.1 wild-type mice in an amount equivalent to one femur to each mouse (see Fig. 2a). This transplantation established chimaeric UBC-GFP/CD45.1 haematopoiesis, which was then analysed in the peripheral blood after four months (210 days from the very beginning of the experiment). The proportion of CD45.1 nucleated blood cells was 21.6 % and 2.6 % in previously unconditioned male and female UBC-GFP mice (Fig. 2c), respectively. However, there were 94.0 % of CD45.1 nucleated blood cells in male and 91.1 % in female mice with the reconstituted haematopoiesis after previous irradiation (Fig. 2c). In mice with the high CD45.1 chimaerism level of 94.0 % in males and 91.4 % in females, the Ly/My_{HA} ratio was significantly increased and corresponded to the values found in normal CD45.1 mice (Fig. 2c). These results show that the transplantation of bone marrow cells from wild-type CD45.1 mice to UBC-GFP mice efficiently corrects the defect in lymphopoiesis in UBC-GFP transgenic mice and confirm that the defect is stem cell-based and not caused by a haematopoietic stroma defect.

3. Transplantation of wild-type bone marrow to UBC-GFP mice replenishes T cells and B cells in UBC-GFP mice

Next we tested whether the transplantation of wild-type CD45.1 bone marrow to either unconditioned UBC-GFP mice or UBC-GFP mice pre-conditioned by irradiation 90 days before the transplantation would correct both the T-cell and B-cell lymphopoiesis. Blood samples from the twenty mice with the chimaeric UBC-GFP/CD45.1 haematopoiesis, established as described in Fig. 2a by transplantation of bone marrow cells of CD45.1 mice to either unconditioned or pre-conditioned (by 6 Gy-irradiation before 90 days) UBC-GFP mice, were collected and analysed by flow cytometry for the frequency of B cells (B220), T cells (CD4 + CD8) and GM cells (Gr1/Mac-1) four months after the transplantation. The analysis was done separately in the GFP-positive (UBC-GFP) and GFP-negative (CD45.1) nucleated blood cells. The results of this examination of the chimaeric UBC-GFP/CD45.1 peripheral blood can also be compared to the flow cytometry data from the peripheral blood in normal UBC-GFP and CD45.1 mice shown in Fig. 1.

The CD45.1 haematopoiesis in unconditioned male UBC-GFP/CD45.1 chimaeric mice (21.6 % of total nucleated blood cells) was significantly biased towards lymphoid cells, as indicated by the Ly/My_{FC} ratio of 30.3 (Fig. 3a; males). Transplanted CD45.1 cells produced a few GM cells and approximately equal numbers of B cells and T cells, as shown by the B/T ratio ≈ 1 (Fig. 3a males; compare to the B/T ratio ≈ 2 in untreated CD45.1 mice shown in Fig. 1a). This demonstrates that the CD45.1 haematopoiesis derived from bone marrow cells transplanted to unconditioned male UBC-GFP mice was strongly biased towards production of T cells. There were only 2.6 % of CD45.1 nucleated blood cells in the peripheral blood in unconditioned UBC-GFP female recipients of CD45.1 bone marrow transplants. The CD45.1 haematopoiesis in these mice was also biased towards lymphopoiesis and strongly towards production of T cells (Fig. 3a; females). The UBC-GFP branch of haematopoiesis in these UBC-GFP/CD45.1 chimaeric male and female mice did not differ from that in normal UBC-GFP mice (compare the results for UBC-GFP cells in Fig. 3a and Fig. 1a).

In the pre-conditioned UBC-GFP recipient mice with the haematopoiesis spontaneously reconstituted 90 days after submyeloablative irradiation and then transplanted with CD45.1 bone marrow cells, the CD45.1 cells represented 94.0 % of nucleated blood cells in male and 91.6 % in female mice in the chimaeric UBC-GFP/CD45.1 blood (Fig. 3b). The major CD45.1 branch of the chimaeric haematopoiesis was also biased towards lymphoid cells, as shown by the Ly/My_{FC} ratio of 7.3 in males and 11.1 in females (Fig. 3b), significantly different from the values of 2.5 and 3.9 in the blood of untreated CD45.1 mice (Fig. 1a,b). Note that in these mice pre-conditioned by irradiation before 90 days, the B/T ratio was significantly increased in CD45.1 cells, showing that the CD45.1 lymphopoiesis was biased towards B cells (Fig. 3b). This is in strong contrast to the CD.45.1 lymphopoiesis in the chimaeric mice generated in unconditioned UBC-GFP mice, which was biased towards T-cell production (see Fig. 3a). Further, in contrast to the results from transplanted unconditioned UBC-GFP mice, the residual UBC-GFP lymphopoiesis in the UBC-GFP/CD45.1 chimaeric haematopoiesis of previously irradiated and spontaneously recovered UBC-GFP recipients (6.0 % of total WBCs in males and 8.9 % in females) proved to be biased towards T cells. Despite these partial controversies in results from unconditioned and pre-conditioned UBC-GFP recipients of transplanted CD45.1 bone marrow cells discussed later on, the transplanted CD45.1 bone marrow significantly contributed to both T-cell and B-cell production in UBC-GFP recipient mice.

4. CD45.1 haematopoiesis is more strongly expressed in the spleen than in the bone marrow in UBC-GFP/CD45.1 chimaeric mice

Previously, we have reported that transplantation of CD45.1 bone marrow to UBC-GFP recipient mice results in higher engraftment of CD45.1 cells in the spleen compared to the bone marrow (Faltusová et al., 2018). We tested this finding in the present new experiments. The peripheral blood, bone marrow, spleen and thymus of five mice with established chimaeric UBC-GFP/CD45.1 haematopoiesis, generated by transplantation of CD45.1 bone marrow cells from one femur to unconditioned UBC-GFP male mice, was examined for the frequency of CD45.1 cells (Fig. 3c). In the bone marrow and spleen, the analysis also included immature $Lin^{-}Sca^{+}c\text{-}Kit^{+}$ (LSK) and $Lin^{-}Sca\text{-}1^{-}c\text{-}Kit^{+}$ ($LS^{neg}K$) cells (Fig. 3d).

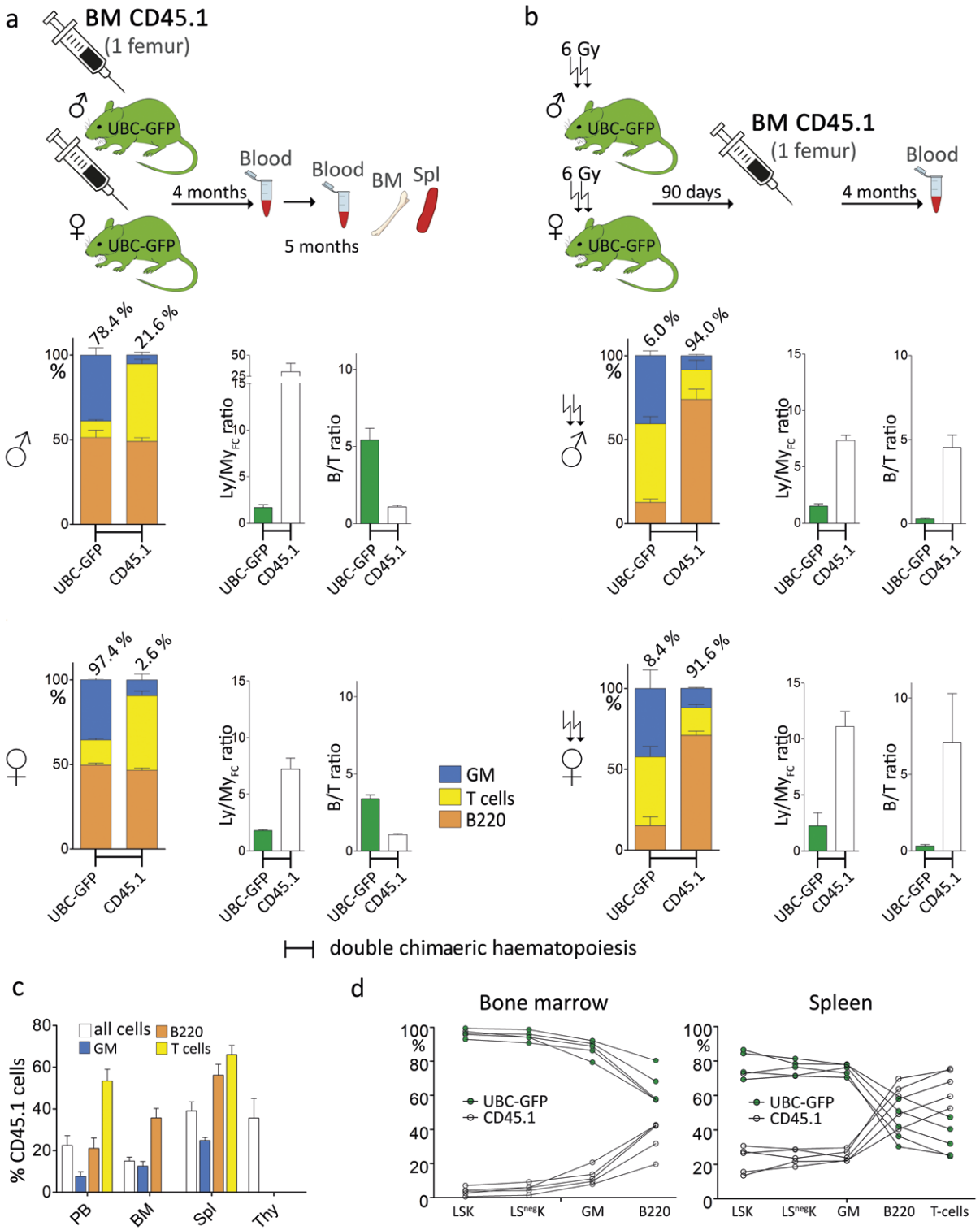

Fig. 3. CD45.1 bone marrow transplanted to either unconditioned UBC-GFP mice (**a**) or UBC-GFP mice with spontaneously reconstituted haematopoiesis after irradiation (**b**) contribute to production of T cells and B cells.
(**a,b**) Transplanted UBC-GFP mice, also analysed in Fig. 2, were subjected to further analysis of their blood and haematopoietic tissues 4 and 5 months after transplantation with bone marrow of CD45.1 mice. Blood samples were analysed for the proportions of Gr1/Mac-1$^+$, B220$^+$ and CD4$^+$ + CD8$^+$ cells in the UBC-GFP and CD45.1 branches of chimaeric haematopoiesis. Each group consisted of five mice. The numbers above the columns indicate the percentage of cells with the particular UBC-GFP or CD45.1 phenotype in all WBCs. (**c, d**) Five months after transplantation, the peripheral blood, femoral bone marrow, splenic and thymus cells were analysed by flow cytometry. (**c**) The percentage of CD45.1-positive cells in peripheral blood (PB), bone marrow (BM), spleen (Spl) and thymus (Thy). (**d**) The percentage of GFP-positive and CD45.1-positive cells in Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK), Lin$^-$Sca-1$^-$, c-Kit$^+$ (LS^{neg}K), Gr-1/Mac-1-positive, B220-positive and CD4- plus CD8-positive (T cells) cells in bone marrow and in the spleen.

The CD45.1 cells were more frequent in nucleated cells in the spleen and thymus (the thymus cells were not stained for the CD4 and CD8 markers; therefore, the result shows only the percentage of CD45.1 cells in all thymus cells; Fig. 3c) than in the bone marrow and peripheral blood (Fig. 3c; all cells). In nucleated blood cells, the fraction of CD45.1 cells was higher in the lymphoid cells (B220 and T cells) than in the myeloid GM cells (Fig. 3c). The CD45.1 cells were also more frequent in the spleen than in the bone marrow in LSK and $LS^{neg}K$ cells (Fig. 3d). These results demonstrate that transplanted wild-type CD45.1 cells engraft more in the spleen than in the bone marrow (Fig. 3d). CD45.1 cells then become further enhanced, compared to $LS^{neg}K$ and LSK cells, in the lymphoid cells (B220; T cells) both in the bone marrow and spleen (Fig. 3d).

5. Production of wild-type T cells is enhanced in chimaeric UBC-GFP/CD45.1 mice

The UBC-GFP bone marrow transplanted to unconditioned CD45.1 mice does not engraft (Faltusová et al., 2018). However, conditioning of CD45.1 mice by submyeloablative irradiation allows for the engraftment of the bone marrow of UBC-GFP mice that results in stable UBC-GFP/CD45.1 chimaeric haematopoiesis (Faltusová et al., 2018). We generated mice with UBC-GFP/CD45.1 chimaeric haematopoiesis by transplanting bone marrow from UBC-GFP mice to CD45.1 mice irradiated with submyeloablative doses of 3 Gy, 4 Gy, 5 Gy and 6 Gy. The peripheral blood of the mice was examined after six months. Figure 4 shows the percentage of all GFP-positive nucleated blood cells and also separately in granulocyte-monocytes (GM cells), B cells (B220) and T cells (CD4 + CD8). The proportion of GFP-positive cells increased from 15.7 ± 5.2 % in the CD45.1 mice irradiated with 3 Gy to 56.8 ± 8.5 % in the mice irradiated with 6 Gy. The proportion of GFP cells was the highest in GM cells, medium in B220 cells and the lowest in T cells (Fig. 4b). Figure 4c shows the total number of WBC in the mice with chimaeric haematopoiesis.

Nucleated blood cells in UBC-GFP/CD45.1 chimaeric haematopoiesis were analysed in their UBC-GFP and CD45.1 branches for the proportions of GM cells, B220 cells and T cells. There were very few T cells in GFP-positive nucleated blood cells (Fig. 4d). Interestingly, the proportion of CD45.1 T cells was significantly increased in the mice pre-conditioned with either 5 Gy or 6 Gy in comparison with the proportion of T cells in the blood of normal CD45.1 mice (Fig. 4d). These results confirm that the defective haematopoiesis in UBC-GFP mice can be corrected by transplantation of the bone marrow of wild-type mice. The results also suggest that the wild-type haematopoiesis responds to the defective production of T cells in UBC-GFP mice by enhanced T-cell lymphopoiesis.

6. Wild-type haematopoiesis in chimaeric UBC-GFP/wild-type mice compensates for insufficient production of GFP T cells

The previous findings (Fig. 4; 5 Gy and 6 Gy groups) showed enhanced production of T cells by CD45.1 bone marrow transplanted to submyeloablatively irradiated UBC-GFP mice. To test this finding, we set up experiments in which various mixtures of bone marrow cells from UBC-GFP and CD45.1 mice were transplanted to 7.5 Gy-irradiated CD45.2 recipient mice (Fig. 5a). Two groups of CD45.2 mice were transplanted with exclusively either UBC-GFP or CD45.1 bone marrow cells. Six months after the transplantation, nucleated cells in the peripheral blood were analysed. The resulting triple or double chimaeric haematopoiesis consisted of UBC-GFP/CD45.1/CD45.2 cells, or of only UBC-GFP/CD45.2 and CD45.1/CD45.2 cells in the two groups transplanted exclusively with either UBC-GFP or CD45.1 bone marrow cells. The CD45.2 non-GFP cells reflected the background haematopoiesis persisting after irradiation of recipient mice with a dose of 7.5 Gy. Figure 5b shows the proportion of GFP, CD45.1 and CD45.2 cells in nucleated blood cells in the triple or double chimaeric haematopoiesis.

Each of the three or two branches of phenotypically distinct nucleated cells in the blood of mice with chimaeric haematopoiesis was also analysed for the proportions of GM, B220 and T cells. T cells were predominantly of wild-type mouse origin, either CD45.1 or CD45.2 (Fig. 5c). The percentage of T cells was consistently higher in CD45.1 cells transplanted together with UBC-GFP cells compared to their percentage in CD45.1 cells transplanted without UBC-GFP cells (CD45.1 part in Fig. 5c), but the difference was not statistically significant in any of the three experimental groups. The proportion of T cells was consistently low in UBC-GFP cells compared to the CD45.1 cells. There was a very high proportion of T cells in the CD45.2 nucleated blood cells in 7.5 Gy-irradiated recipient mice (Fig. 5c). We hypothesize that long-lived radio-resistant T cells persisted in the peripheral blood of irradiated CD45.2 mice, while GM and B cells were more severely reduced.

7. UBC-GFP mice have fully potent haematopoietic stem cells for myelopoiesis and partly also for B-lymphopoiesis

To test how the triple chimaeric haematopoiesis from the previous experiment reflected the activity of transplantable stem and progenitor cells, we collected the UBC-GFP/CD45.1/CD45.2 chimaeric bone marrow from six mice 10 months after transplantation with a mixture of UBC-GFP and CD45.1 bone marrow in the ratio 3 : 1, and re-transplanted it to secondary recipient dual CD45.2/CD45.1 F1 hybrid mice that were 6.5 Gy-irradiated. The transplanted triple chimaeric bone marrow contained 59 % of UBC-GFP cells, 36 % of CD45.1 cells and 5 % of CD45.2-non-GFP cells. Three

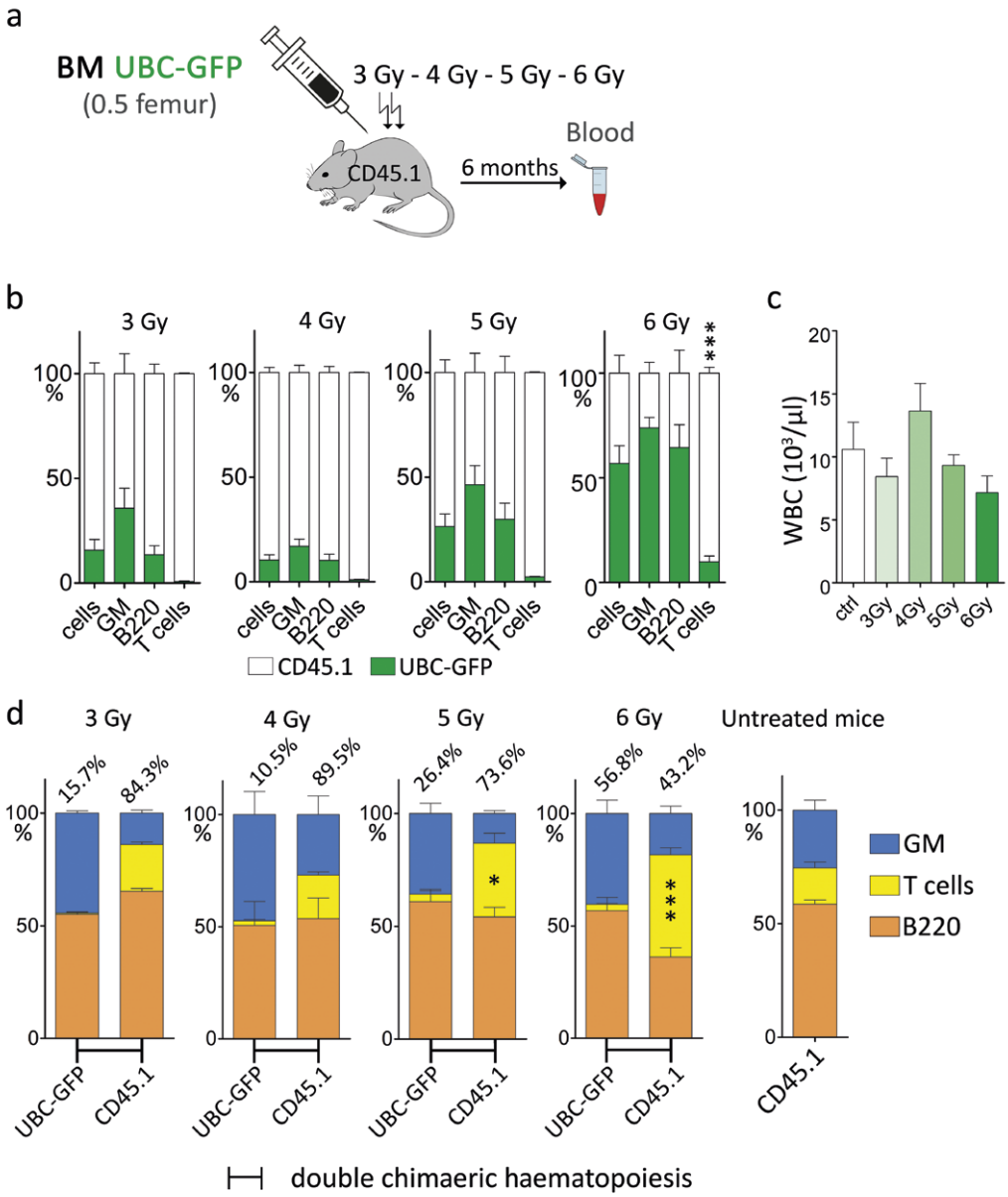

Fig. 4. Chimaeric UBC-GFP/CD45.1 haematopoiesis in submyeloablatively irradiated CD45.1 mice transplanted with bone marrow of UBC-GFP mice

(**a**) Experimental set-up: groups of 5–6 CD45.1 male mice were irradiated with a dose of 3 Gy, 4 Gy, 5 Gy or 6 Gy and within 4 hours transplanted with bone marrow cells of female UBC-GFP mice in the amount equivalent to half of the femur. Three CD45.1 male untreated mice were kept as age-matched controls (CTRL). (**b**) Six months after the transplantation, samples of peripheral blood were analysed for the frequency of GFP-positive nucleated blood cells and also of their GM, B220 and T-cell subtypes. (**c**) The total number of WBCs is shown. (**d**) The UBC-GFP and CD45.1 blood cells in chimaeric haematopoiesis and in the blood of three CD45.1 control mice were analysed for the proportion of GM, B220 and T cells. The proportion of T cells is significantly increased in the CD45.1 cells in chimaeric blood of 5 Gy and 6 Gy irradiated mice compared to the proportion of T cells in the blood of control CD45.1 mice (**d**) (*P < 0.5; ***P < 0.001, Dunnett's multiple comparison test). The numbers above the columns in (d) indicate the percentage of cells with the particular phenotype in all WBCs.

and a half months later, the nucleated blood cells, which consisted of cells with four different phenotypes in the quadruple chimaeric haematopoiesis, were analysed by flow cytometry (Fig. 6). The F1 cells, ≈ 50 % of all nucleated blood cells, represented the background haematopoiesis in the secondary transplant recipients (Fig. 6b). UBC-GFP haematopoiesis produced very little CD4 and CD8 T cells (Fig. 6b). However, UBC-GFP cells produced both GM and B220 cells, but the production was biased towards GM cells (compare with Fig. 1a,b).

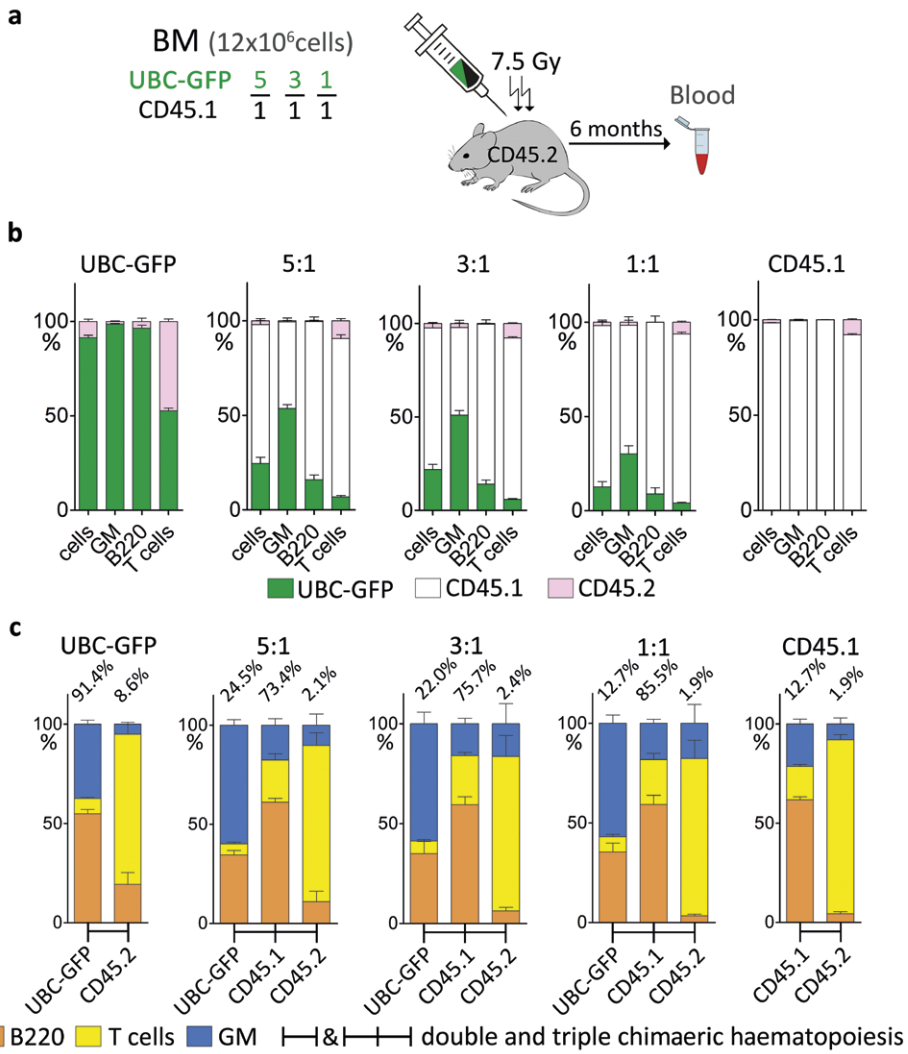

Fig. 5. T cells are mostly of wild-type mouse origin in triple chimaeric UBC-GFP/CD45.1/CD45.2 mice (**a**) Experimental set-up: 28 CD45.2 male mice were irradiated with 7.5 Gy and transplanted with only UBC-GFP, only CD45.1 bone marrow cells, or with a mixture of UBC-GFP/CD45.1 cells in the ratio 5 : 1, 3 : 1 or 1 : 1. Six months after transplantation, samples of peripheral blood were stained with antibodies against CD45.1, CD45.2, Gr-1/Mac-1, B220, CD4, and CD8 markers and analysed by flow cytometry. (**b**) The proportion of UBC-GFP, CD45.1 and CD45.2 cells in all nucleated blood cells and their GM, B220 and T-cell (CD4 + CD8) subtypes. (**c**) The proportion of B220 T cells and GM cells in the three or two branches of the double or triple chimaeric haematopoiesis.

These results demonstrate that haematopoietic stem cells in UBC-GFP mice are fully potent in the production of granulocytes and macrophages (GM cells) and partially also in the production of B cells.

8. CD4 T cells are more negatively affected in UBC-GFP mice than CD8 T cells

To test whether the stem cell defect in UBC-GFP mice negatively affecting T-lymphopoiesis is equally expressed in CD4 and CD8 T cells, we analysed the ratio between CD8 and CD4 T cells in the peripheral blood of normal UBC-GFP, CD45.1 or CD45.2 mice (Table 2) and also in the UBC-GFP and CD45.1 branches of UBC-GFP/CD45.1 chimaeric haematopoiesis (Table 3). The ratio between CD8 and CD4 T cells in untreated UBC-GFP mice, both males and females, was consistently significantly higher than the corresponding values in CD45.1 or CD45.2 wild-type mice (Table 2). It was also higher in the UBC-GFP branch compared to the CD45.1 branch in UBC-GFP/CD45.1 chimaeric haematopoiesis (Table 3). These results suggest that the production of CD4 T cells is more negatively affected than the production of CD8 cells in UBC-GFP mice.

Discussion

We discovered a defect in lymphoid-primed haematopoietic stem and progenitor cells in the transgenic mice carrying a gene construct with the enhanced green fluo-

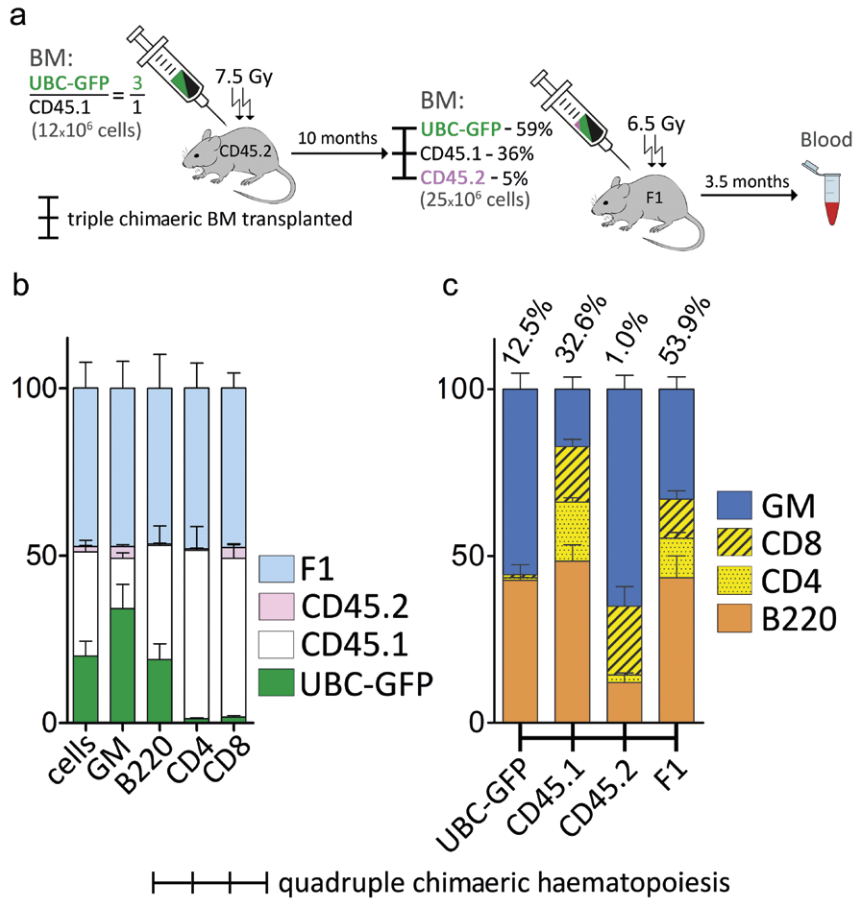

Fig. 6. Longevity of performance of haematopoietic stem cells of UBC-GFP mice after their transplantation
(**a**) Experimental set-up: five CD45.2 male mice were irradiated with 7.5 Gy and transplanted with a mixture of UBC-GFP/CD45.1 cells in a 3 : 1 ratio. Ten months later, the resulting triple chimaeric (UBC-GFP/CD45.1/CD45.2) bone marrow was collected from these mice, analysed by flow cytometry, and 25 × 10^6 cells were transplanted to a group of 6.5-Gy-irradiated dual CD45.1/CD45.2 F1 hybrid mice. Samples of peripheral blood were withdrawn after three and a half months, stained with antibodies against CD45.1, CD45.2, Gr-1/Mac-1, B220, CD4, and CD8 markers and analysed by flow cytometry. (**b**) The proportion of UBC-GFP, CD45.1, CD45.2 and F1 cells in all nucleated peripheral blood cells and their GM, B220 and T cell (CD4 + CD8) subtypes. (**c**) The proportion of GM, CD4, CD8 and B220 in the four branches of the quadruple chimaeric haematopoiesis. The numbers above the columns in "c" indicate the percentage of cells with the particular phenotype in all nucleated peripheral blood cells

Table 2. Ratio between CD8 and CD4 T cells in the blood of untreated UBC-GFP and CD45.1 or CD45.2 mice

	sex	N	CD8/CD4	P value
UBC-GFP	♂	5	1.15 ± 0.09	0.0018
CD45.1	♂	5	0.70 ± 0.03	
UBC-GFP	♂	8	1.15 ± 0.06	0.0004
CD45.1	♂	5	0.71 ± 0.06	
UBC-GFP	♂	5	1.76 ± 0.20	0.0002
CD45.1	♂	6	0.63 ± 0.03	
UBC-GFP	♀	6	0.82 ± 0.05	0.0006
CD45.1	♀	5	0.55 ± 0.01	
UBC-GFP	♀	5	1.07 ± 0.06	0.0002
CD45.2	♀	5	0.70 ± 0.01	

Results from five independent measurements in the groups of normal mice are presented.

Table 3. CD8/CD4 T-cell ratio in UBC-GFP/CD45.1 chimaeric mice

sex	N	Chimaeric blood		CD8/CD4	P value
♂	5	UBC-GFP	77.5 %	2.14 ± 0.15	
		CD45.1	22.5 %	0.36 ± 0.04	0.0001

Chimaeric haematopoiesis was established by transplanting bone marrow from one femur to each of five unconditioned UBC-GFP male mice five months before blood analysis.

rescent protein expressed under the promoter of the human ubiquitin gene (Schaefer et al., 2001; Faltusová et al., 2018). This defect became strongly manifested when the haematopoiesis of UBC-GFP mice competed with the haematopoiesis of wild-type mice in chimaeric haematopoiesis established by transplantations (Faltusová et al., 2018). Though mostly latent in normal untreated mice, the defect can cause quite unexpected results in some experimental settings (Faltusová et al., 2018). This was thus another report on a nonstandard response of mice carrying the GFP transgene after those of Spangrude et al. (2006) and Challen and Goodell (2008), who reported limitations in using mice with the GFP transgene in haematopoietic stem cell research.

In this study, we analysed the altered phenotype of UBC-GFP mice in more depth, because it has remained unrecognized for a long time. The whole blood count and the flow cytometry analysis of peripheral blood of untreated UBC-GFP mice showed that the defect manifests by the low total WBC number and particularly by the low number of T cells in peripheral blood. Surprisingly, the low number of T cells does not seem to negatively affect the general fitness of these mice, which breed well and survive well under conventional clean conditions, and also when irradiated with γ rays (Faltusová et al., 2018; the present experiments).

After analysis of the peripheral blood in UBC-GFP and wild-type CD45.1 untreated mice, we focused on the analysis of double, triple and quadruple chimaeric haematopoiesis consisting of the haematopoiesis of UBC-GFP mice and haematopoiesis of CD45.1, CD45.2, and dual CD45.1/CD45.2 mice. The chimaeric haematopoiesis was established by five different approaches: first, unconditioned UBC-GFP mice were transplanted with bone marrow cells from CD45.1 donors; second, UBC-GFP mice were irradiated with a submyeloablative dose of 6 Gy, and when their haematopoiesis had spontaneously recovered, they were transplanted with CD45.1 bone marrow cells; third, CD45.1 mice irradiated with submyeloablative doses were transplanted with bone marrow cells from UBC-GFP donors; fourth, lethally irradiated CD45.2 mice were transplanted with a mixture of UBC-GFP and CD45.1 bone marrow cells; fifth, UBC-GFP/CD45.1/CD45.2 chimaeric bone marrow was transplanted into submyeloablatively irradiated CD45.1/CD45.2 F1 hybrid mice.

The transplantation of unconditioned UBC-GFP mice (Fig. 2; groups 1) with the bone marrow of CD45.1 mice confirmed previous results (Faltusová et al., 2018) consisting in higher engraftment of CD45.1 cells in male UBC-GFP recipient mice compared to UBC-GFP female mice. This finding likely reflects the suppressive effect of oestrogens on lymphopoiesis derived from transplanted wild-type bone marrow in female mice. The suppressive action of oestrogens was reported by Pelichovská et al. (2008) and Hlobeňová et al. (2012). These results confirmed the previous demonstration that transplanted CD45.1 cells contributed more to lymphoid cells than to the granulocytes and monocytes, as reported in Faltusová et al. (2018). However, we have newly observed that the CD45.1 cells were more frequent in LSK and $LS^{neg}K$ haematopoietic progenitor cells in the spleen than in the bone marrow (Fig. 3d). The T cells derived from transplanted CD45.1 bone marrow amounted to about 50 % of all CD45.1 nucleated blood cells, which is approximately twice their occurrence in CD45.1 mice (see Fig. 1). Hence, the haematopoiesis derived from the haematopoietic stem cells of wild-type mice responded to the low T-cell number by enhanced production of T cells.

A robust engraftment of wild-type eGFP-labelled HSCs in unconditioned severe combined immunodeficient (SCID) mice, leading to the rescue of lymphoid deficiencies through expansion of donor lymphoid progenitors, was reported by Bhattacharya et al. (2006).

The transplantation of CD45.1 bone marrow to UBC-GFP mice with haematopoiesis spontaneously reconstituted after previous damage has confirmed that such haematopoiesis remains highly permissive to the engraftment of bone marrow of wild-type mice (Faltusová et al., 2018) both in male and female mice. There were more than 90 % CD45.1 nucleated blood cells in the peripheral blood of these mice, and the ratio between lymphoid and myeloid cells was normalized to that of CD45.1 mice (Fig. 2). Surprisingly, the lymphopoiesis derived from transplanted CD45.1 cells mainly produced B lymphocytes in this case (Fig. 3b), which was in contrast to all other results. The result was checked for a possible error, but remained unexplained. Also unexpectedly, the nucleated blood cells of UBC-GFP origin contained a high number of T cells (Fig. 3b). We hypothesize that long-lived T cells resistant to irradiation could have survived for seven months after irradiation in the blood of UBC-GFP recipient mice, while other types of WBCs were more severely reduced after irradiation.

The transplantation of UBC-GFP bone marrow to CD45.1 mice pre-conditioned by progressive doses of irradiation resulted in chimaeric haematopoiesis with a proportion of GFP-positive cells exceeding 50 % of nucleated blood cells. T cells were almost exclusively of CD45.1 type in these mice. In mice that had been irradiated with radiation doses of 5 Gy or 6 Gy, the proportion of CD45.1 T cells in CD45.1 nucleated blood cells were significantly increased compared to normal mice.

The co-transplantation of UBC-GFP and CD45.1 bone marrow cells to lethally irradiated CD45.2 recipients (Fig. 5) further demonstrated the relative competitive weakness of stem cells of the UBC-GFP mice. The transplanted bone marrow of UBC-GFP mice produced mainly myeloid (GM) cells in this experimental setting. The high percentage of CD45.2 T cells is likely due to long-living T cells resistant to irradiation. When the triple chimaeric haematopoiesis consisting of UBC-GFP/CD45.1/CD45.2 cells was transplanted to secondary recipient (dual CD45.1/CD45.2 cells F1 hybrids) mice (Fig. 6), all three types of cells were produced in parallel with the background dual CD45.1/CD45.2 cells.

The wild-type CD45.1 cells mainly produced lymphoid cells (Fig. 6) and the UBC-GFP cells produced both myeloid (GM) and B cells (Fig. 6). The implication of this finding might be that the total number of T cells is regulated in peripheral blood and that the haematopoiesis in UBC-GFP transgenic mice is incapable to meet this regulation. Hence, the stem cell deficiency in UBC-GFP mice negatively affecting T-lymphopoiesis is a certain analogy to the defect in the myelopoiesis of W/W^v mice, which mainly suffer from anaemia. The W/W^v mice also accept the transplanted bone marrow of wild-type mice without conditioning, and this corrects the anaemia (Boggs et al., 1982; Maloney et al., 1982). However, compared to the regulation of red blood cell numbers by systemic factor erythropoietin, little is known about the systemic control of the number of circulating T lymphocytes and their subsets (Almeida et al., 2005; Arias et al., 2017).

The low number of T cells in the peripheral blood of UBC-GFP mice could be caused either by inadequate production of T cells or by their increased death rate. The results we have at disposal are not sufficient to distinguish between these two possibilities. In our previous study (Faltusová et al., 2018), we studied the survival of transfused nucleated blood cells in the circulation system and did not observe a significant difference in the survival of the cells collected from UBC-GFP mice and those collected from wild-type mice. However, the assay might not have been sensitive enough to detect a mild difference in the survival of the transfused cells. The T cells of UBC-GFP mice could also be subject to an increased death rate during their differentiation and maturation in the thymus.

Because of the readiness with which unconditioned UBC-GFP male mice engraft the transplanted haematopoietic progenitors and stem cells of wild-type mice, the defect in the haematopoiesis of UBC-GFP mice, which mainly negatively affects T-lymphopoiesis, targets stem cells. This is also supported by the possibility of transferring the defect to wild-type mice by bone marrow transplantation, and its persistence after retransplantation of such bone marrow to secondary recipients (Fig. 6; Faltusová et al., 2018). However, it is still unclear at what stage of the development of haematopoietic cells this stem cell defect impacts the production of T cells and their precursors. This enigma includes the interesting possibility of the existence of T cell-primed haematopoietic stem cells with a long-term repopulating capacity.

Conclusions

The haematopoiesis of UBC-GFP mice fails when it competes with the haematopoiesis of wild-mice because of a defect in the lymphoid-primed stem cells. This defect manifests itself mostly as a low number of T cells, which causes general lymphopoenia and a decreased lymphoid/myeloid cell ratio in nucleated cells in the peripheral blood. The defect in T-lymphopoiesis in UBC-GFP mice can be compensated for by transplantation of the wild-type congenic bone marrow. The transplantation of wild-type bone marrow to UBC-GFP mice is also effective without conditioning, but it is highly enhanced by submyeloablative irradiation of recipient UBC-GFP mice. The results of the transplantation experiments giving rise to chimaeric UBC-GFP/wild-type haematopoiesis suggest a tight systemic control of the T-cell number in the peripheral blood.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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