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Bone Marrow Lin-/Sca-1+/C-Kit+ and Lin-/Sca-1-/C-Kit+ cells Induce Stable Mixed Chimerism and Permanent Skin Graft Acceptance in a Mouse Model

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Abstract

Mixed chimerism is a potential induction method for alloantigen-specific tolerance. Although the phenomenon has been broadly studied over recent years, some important aspects remain to be elucidated. The aim of our study was to identify the optimal mouse stem/progenitor cell population for mixed chimerism induction purposes.

To induce stable mixed chimerism, B6.SJL-*PtprcaPep3b* (CD45.1; H-2^s; I-E-) mice were exposed to 3-Gy total body irradiation (Day -1) as well as the CD40L (Day 0, and 4) and CD8 (Day -2) blocking antibodies. The animals were transplanted with 10×10^6 Balb/c (CD45.2; H-2^d; I-E^+) unfractionated bone marrow cells (Day 0). Since mouse stem/progenitor cells are found among lin- population and they possess Sca-1 and c-kit antigens, mice were given 2×10^5 of lin-/Sca-1+/c-kit-, lin-/Sca-1+/c-kit+ or lin-/Sca-1-/c-kit+ bone marrow-derived cells. Mixed chimerism was measured in peripheral blood leukocytes several times during the 26-week experiment. In addition, the chimerism rate and the kinetics of the percentage of CD4, CD8 and NK1.1 cells in the peripheral blood were assessed. The tolerance to Balb/c mouse antigens induced by the stem/progenitor cells was tested by analyzing the proportions of the Vβ5 and Vβ11 TCR-expressing lymphocytes as well as by assessing skin graft (Day 0) acceptance.

The lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+ cells, but not the lin-/Sca-1+/c-kit- cells, induced a high degree of stable (26 weeks) multilineage mixed chimerism. In the chimeric mice, we observed an elimination of donor-reactive lymphocytes as well as permanent skin graft acceptance. We found a correlation between the initial chimerism rate, especially in the mononuclear cell populations, and graft survival.

Based on our study, we can recommend well-selected cell populations expressing c-kit receptor that possibly facilitate the induction of mixed chimerism and immune tolerance. Therefore, our findings contribute to a better understanding of mixed chimerism and may promote its use in clinical practice.

Keywords: Antigenic tolerance; Lin-/Sca-1+/c-kit-; Lin-/Sca-1+/c-kit+; Lin-/Sca-1-/c-kit+ cells; Mixed chimerism; Skin graft

Introduction

The phenomenon of mixed chimerism gives hope for the induction of specific immune tolerance. Mixed chimerism is defined as the coexistence of two types of hematopoietic cells from both a recipient and a donor. In mixed chimeric organisms, transplanted donor antigen-presenting cells migrate to the recipient's thymus and present donor antigens to developing thymocytes [1,2]. Negative selection eliminates the cells that are donor reactive. Because of an increasing number of potential transplant recipients, an inadequate number of appropriate organ donors and immunosuppression that can be ineffective and harmful, the identification of a method that is able to induce specific immunological tolerance towards alloantigens should be the main goal of transplantation research [1-3].

Although many experiments have been performed to explain mixed chimerism, this phenomenon remains only partially understood. Consequently, the induction protocol, which should be effective but non-toxic to the recipient, is of great importance. Donor cell populations that can help to create stable mixed chimerism with the lowest possible dose of transplanted cells are also under investigation [4,5]. Various cells have been suggested to create tolerance in mixed chimerism studies. Because the transplantation of hematopoietic cells has been known to induce tolerance to donor antigens for over half a century [6], bone marrow stem cells have attracted the interest of many scientists. These are cell populations enriched in hematopoietic stem/progenitor cells: CD34+ cells in humans [4,7,8] and c-kit+ cells in mice [5]. Moreover, diverse cell populations have been tested as additions to unfractionated bone marrow to support tolerance induction. These populations are CD8+/TCR- facilitating cells [9], donor spleen cells [10], CD4+/CD25+ regulatory cells [11], and immature dendritic cells [12]. Furthermore, flk+/Sca-1- mesenchymal stem cells [13] and CD45+ mouse embryonic cells have also been used in mixed chimerism experiments [14].

We have been studying various protocols to induce mixed chimerism in mice for last few years. In our search for a "protolerant" cell population in the mouse hematopoietic stem/progenitor cell compartment, we selected an induction method that consists of total body irradiation (TBI) at a dose of 3 Gy as well as treatment with blocking CD40L and CD8 antibodies. Mice hematopoietic stem cells are found among cells that possess Sca-1 (Stem cell antigen-1) surface protein and c-kit receptor both playing a role in hematopoietic stem cells self-renewal and differentiation [13,15-17]. They can be distinguished from mature blood cells by their lack of lineage-specific

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distinguished from mature blood cells by their lack of lineage-specific markers, therefore these cells do not express markers associated with the terminal maturation, characteristic of T lymphocytes (CD4, CD8), myelomonocytic cells (Mac-1α), granulocytes (Gr-1), B lymphocytes (CD45R/B220) and erythrocytes (TER-119) [15]. Many experiments have demonstrated that the lin-/Sca-1⁺/c-kit⁺ cells repopulated lethally irradiated mice [15,16], but their role in the induction of mixed chimerism has not been elucidated. To our knowledge, we were the first to consider three diverse well-defined mouse bone marrow-derived cell populations among lin- cells, i.e. $lin^-/Sca\text{-}1^+/c\text{-}kit^-$ cells, $lin^-/Sca\text{-}1^+/c\text{-}kit^+$ cells and $lin^-/Sca\text{-}1^-/c\text{-}kit^+$ cells, in a mixed chimerism study. We sought to examine if the Sca-1 and c-kit antigens may play a pivotal role and whether they both are equally potential in this phenomenon induction. We measured the mixed chimerism rate in peripheral blood leukocytes for 26 weeks. Moreover, we expanded the chimerism analysis to selected leukocyte subpopulations that participate in graft rejection, i.e., CD4, CD8, and NK1.1 cells [2,18]. In addition to chimerism proportions, we also assessed the kinetics of these cells in the peripheral blood.

The most important part of our research was the assessment of donor antigen tolerance induced by the transplantation of $lin^-/Sca\text{-}1^+/c\text{-}kit^-$, $lin\text{-}/Sca\text{-}1^+/c\text{-}kit^+$ or $lin^-/Sca\text{-}1^-/c\text{-}kit^+$ cells. For this assessment, we employed two different methods: an assessment of the elimination of peripheral blood lymphocytes with T cell Vβ5 and Vβ11 receptors that are potentially donor-reactive and an *in vivo* analysis of skin graft acceptance, which is known to be the most stringent test in tolerance studies.

Materials and Methods

Mice

Male, 12-16-week old Balb/c (MHC class I: $H\text{-}2^d$ and MHC class II: I-E+) and B6.SJL-*PtprcaPep3b* ($H\text{-}2^s$, $I\text{-}E^-$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) [19]. Different isoforms of the CD45 antigen in selected mice (CD45.1 in B6.SJL-*PtprcaPep3b* and CD45.2 in Balb/c) enabled the analysis of chimerism by flow cytometry. Various MHC class I and II antigens made these animals ideal for tolerance studies because of their histo-incompatibility and option for the analysis of alloreactive TCR expressing lymphocytes deletion. Thus, we have chosen the Balb/c mice, which possess the Ly-6E.1 form of the Sca-1 antigen as donors, because in the bone marrow of these animals the Sca-1 antigen is highly expressed on hematopoietic stem/progenitor cell population and weakly on unstimulated lymphocytes. Such antigen constellation allowed to detect strictly hematopoietic cells in the donor animals before sorting. In contrast, the mice with Ly-6A.2 reveal similar Sca-1 antigen expression on lymphocytes as compared to hematopoietic cells, what would make the sorting less reliable [20]. Moreover, different colors of mice (donor–white; recipient–black) facilitated the observation of the skin graft conditions. The animals were kept under specific pathogen-free conditions in a Bio. A.S. Blower Unit Vent II system (Ehret, Labor- und Pharmatechnik, Emmendingen, Germany). All experiments were approved by the Local Ethical Committee and were performed in accordance with the guidelines of laboratory animal care.

Induction of chimerism

Conditioning was accomplished by the total body irradiation of B6.SJL-*PtprcaPep3b* mice with gamma rays (60°C) at a dose of 3 Gy (Day -1), that is a standard dose used in “reduced toxicity” induction protocols [19,21]. Antibodies against mouse CD40L (anti-CD154 mAb, clone MR1, Becton Dickinson, Franklin Lakes, NJ, USA) and CD8a (anti-CD8a mAb, clone 53-6.7, Becton Dickinson, USA) were given intraperitoneally (i.p.) [19]. The doses and antibody injection times are indicated in Table 1. A total of 10×10^6 of unfractionated Balb/c bone marrow cells were transplanted intravenously on day 0 (in 0.2 mL PBS). Whole bone marrow cells were used to assure initial donor engraftment. The cells were obtained by flushing the femurs and tibias of the Balb/c mice with cold phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), L-glutamine (Sigma-Aldrich, USA) and antibiotics (penicillin+streptomycin, Sigma-Aldrich, USA). After the erythrocytes were depleted by a 15-minute incubation with a lysing solution (BD Pharm Lyse, Lysing Buffer, Becton Dickinson, USA) containing ammonium chloride, the cells were passed through a 40-μm nylon mesh to remove potential clots (Cell Strainer, Becton Dickinson, USA). Cell washing was performed at 4°C [19].

Additionally, the B6.SJL-*PtprcaPep3b* mice received 2×10^5 $lin^-/Sca\text{-}1^+/c\text{-}kit^-$ (G1), $lin^-/Sca\text{-}1^+/c\text{-}kit^+$ (G2) or $lin^-/Sca\text{-}1^-/c\text{-}kit^+$ (G3) cells, which were obtained by cell sorting (Table 1). The procedure was performed as follows. A total of 5×10^8 Balb/c bone marrow cells were incubated with the anti-mouse CD16/CD32 monoclonal antibody 2.4G2 (BD Fc Block, Becton Dickinson, USA) to block non-specific FcγR binding. Next, the cells were incubated on ice for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-TCRαβ (clone H57-597), anti-TCRγδ (clone GL3), anti-CD45R/B220 (clone RA3-6B2), anti-Gr-1 (clone RB6-8C5), anti-Mac-1α (CD11b, clone M1/70) and anti-TER-119 (clone TER-119) antibodies as lineage markers, phycoerythrin (PE)-conjugated anti-Sca-1 (clone D7) and allophycocyanine (APC)-conjugated anti-c-kit (clone 2B8). After washing, the cells were passed through a 40-μm nylon mesh and suspended in cold PBS. The final sample concentration was 1×10^8 cells/mL. The sorting of the $lin^-/Sca\text{-}1^+/c\text{-}kit^-$, $lin^-/Sca\text{-}1^+/c\text{-}kit^+$ and $lin^-/Sca\text{-}1^-/c\text{-}kit^+$ cells was performed using a FACSAria II Cell Sorter (Becton Dickinson, USA) with high speed sorting settings and a 70-μm nozzle [22]. The principles of gating are shown in Figure 1. Briefly,

Group	Dose of radiation	Transplanted cells	Dose of antibodies
G1	3 Gy	2×10^5 $Lin^-/Sca\text{-}1^+/c\text{-}kit^-$+$10\times10^6$ of unfractionated bone marrow	Anti-CD8 0.5 mg i.p. (Day−2) Anti-CD40L 2×0.5 mg i.p. (Day 0 and Day 4)
G2	3 Gy	2×10^5 $Lin^-/Sca\text{-}1^+/c\text{-}kit^+$+$10\times10^6$ of unfractionated bone marrow	Anti-CD8 0.5 mg i.p. (Day−2) Anti-CD40L 2×0.5 mg i.p. (Day 0 and Day 4)
G3	3 Gy	2×10^5 $Lin^-/Sca\text{-}1^-/c\text{-}kit^+$+$10\times10^6$ of unfractionated bone marrow	Anti-CD8 0.5 mg i.p. (Day −2) Anti-CD40L 2×0.5 mg i.p. (Day 0 and Day 4)
C	(−)	(−)	(−)
CP	3 Gy	(−)	Anti-CD8 0.5 mg i.p. (Day −2) Anti-CD40L 2×0.5 mg i.p. (Day 0 and Day 4)

Table 1: The mixed chimerism induction protocols.

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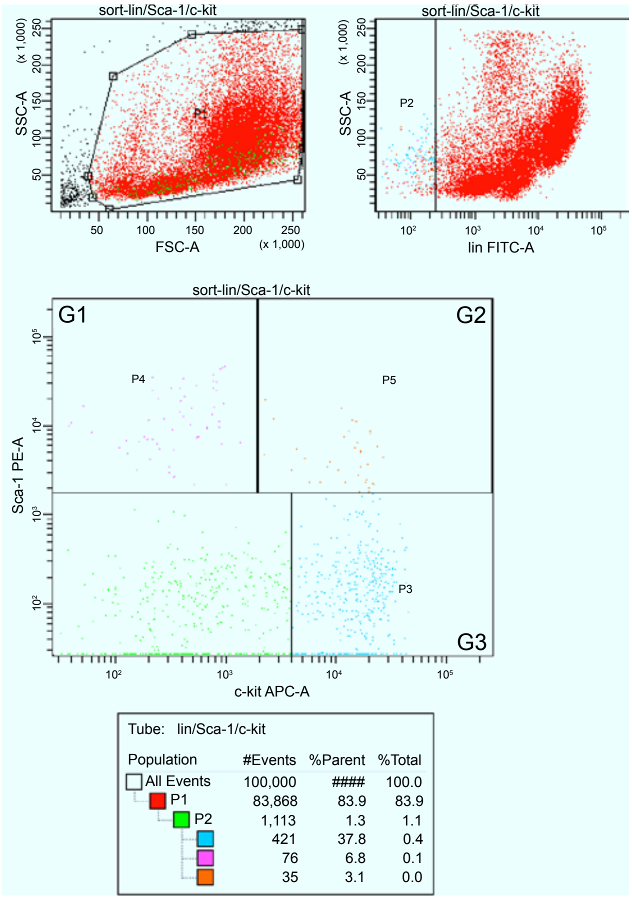

Figure 1: Principles of gating. The whole population of bone marrow cells was gated in the FSC/SSC cytogram (P1), and then the non-lineage marker-expressing cells were gated in the P1-restricted cytogram (P2). The lin- cells from the P2 gate were divided into three populations: P3-lin-/Sca-1+/c-kit- cells (the G1 group); P4-lin-/Sca-1+/c-kit+ cells (G2); and P5-lin-/Sca-1-/c-kit+ cells (G3).

the P1 gate on the FSC/SSC dot plot was set on the whole leukocyte region, and then non-lineage marker-expressing cells were gated in the P1-restricted cytogram (P2). Next, the three desired cell populations were gated in the P2-restricted PE/APC cytogram. The cells were collected into tubes coated with 10% FBS. The purity of the sorted cells was 90.8% ± 3.8, as assessed in triplicate for each sorted population. The viability was established using the trypan blue exclusion test and was determined to be approximately 94% for each population. After being sorted for two hours, the cells were administered to the mice of the G1-G3 experimental groups four hours after the injection of the unfractionated bone marrow cells.

Detection of chimerism

The presence of mixed chimerism in the peripheral blood leukocytes was measured using flow cytometry in the 1st, 2nd, 3rd, 4th, 6th, 8th, 12th, 16th and 26th weeks after bone marrow cell infusion by analyzing the CD45.1 (B6.SJL-*PtprcaPep3b*) and CD45.2 (Balb/c) antigens as previously described [19]. Chimerism was analyzed for 2×10^4 cells, which included total leukocytes, granulocytes, and mononuclear cells selected from the FSC/SSC cytogram (Figures 2A-2C). The data were collected with a BD LSR II Cytometer (Becton-Dickinson, USA) and analyzed using the FACSDiva program.

In addition, we analyzed the percentage of the CD4, CD8, and NK1.1 cell populations and the rate of mixed chimerism (percentage of donor cells) in these cells in the peripheral blood in the 2nd, 4th, 8th, 12th, 16th week of the experiment. After blocking non-specific FcγR binding, leukocytes were stained with FITC-conjugated anti-CD45.1 and peridinin-chlorophyll-protein complex-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-CD45.2. Additionally, PE-conjugated anti-CD4 (clone GK1.5), anti-CD8a (clone 53-6.7) or anti-NK1.1 antibodies (clone PK136) were used (Becton Dickinson, USA). First, the levels of the CD4, CD8, and NK1.1 cells were assessed in the lymphGate. Next, CD45 analysis was performed to measure the proportion of mixed chimerism in these cells. Appropriate isotype antibodies were used as negative controls.

Analysis of mice TCR Vβ5 and TCR Vβ11 lymphocytes

To estimate the effectiveness of eliminating potentially alloreactive lymphocytes, peripheral blood cells were collected in the 26th week of the experiment. After erythrocyte depletion and blocking of non-specific FcγR binding, the cells were stained with FITC-conjugated anti-TCR Vβ5.1/5.2 (clone MR9-4) or anti-TCR Vβ11 (clone RR3-15) antibodies and in addition with PE-conjugated anti-CD4 (clone GK1.5) or anti-CD8 antibodies (clone PC61). We measured the $CD4^+$ or $CD8^+$ cells with Vβ5 or Vβ11 receptors in the recipient mice (B6.SJL-*PtprcaPep3b*) [23]. Routinely, at least 2×10^5 cells were analyzed by flow cytometry. We added two control groups to the analysis, a group of B6.SJL-*PtprcaPep3b* mice without any treatment (C) and donor Balb/c mice (CD).

Skin transplantation

Square fragments of full-thickness tail skin with areas of 1,0 cm^2 were taken from Balb/c mice and transplanted into prepared graft beds on the tails of anaesthetized B6.SJL-*PtprcaPep3b* recipient mice (Day 0). The skin grafts were secured by 6-0 sutures (Dafilon, B. Braun Melsungen AG, Melsungen, Germany), and local antibiotic (Neomycin, Polfa Tarchomin, Warszawa, Poland) was applied. The grafts were observed by a dermatologist daily for the first two weeks and then three times a week for the remainder of the experiment. The grafts were considered to be rejected when less than 30% of the graft was visible or when it had formed a dry scar [24,25]. Skin survival is expressed using a Kaplan-Meier curve. Skin grafts were given to experimental mice (n=12), the CP group of animals that were exposed to the induction protocol (3 Gy, anti-CD40L×2, anti-CD8) but did not receive any Balb/c cells (n=4), and the following control groups, which were not subjected to any additional treatment: i) Balb/c grafts to B6.SJL-*PtprcaPep3b* mice (CW–control white, n=7), ii) B6.SJL-*PtprcaPep3b* grafts to B6.SJL-*PtprcaPep3b* mice (CB–control black, n=8). Graft survival is expressed as median survival time.

Statistical analysis

Means and standard deviations were calculated using MS Excel v. 97 and Statistica 7.1 (Statsoft) software. Non-parametric tests were used because the analyzed data were abnormally distributed (the Shapiro-Wilk test). Significant changes in the chimerism kinetics were evaluated with the Friedman ANOVA and the Wilcoxon signed-rank test. The Kruskal-Wallis ANOVA and the Mann-Whitney test were used to calculate differences between the populations of lymphocytes. Skin graft survival was analyzed using the Kaplan-Meier method, and the log rank test was used for comparisons between groups. Statistical significance was defined as $p<0.05$.

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Results

The rate and kinetics of chimerism depends on the type of transplanted mouse stem/progenitor cells

To identify the optimal stem/progenitor cell population for the induction of chimerism, we first compared the level and stability of mixed chimerism in the peripheral blood cells of three groups of mice transplanted with either lin$^-$/Sca-1$^+$/c-kit$^+$ (G1), lin$^-$/Sca-1$^+$/c-kit$^+$ (G2) or lin$^-$/Sca-1$^-$/c-kit$^+$ (G3) cells over a long-term 26-week observation period (Figure 2). All of the 12 experimental animals became chimeric. We found that the lowest chimerism rate, which did not exceed 10% at any time point, was induced in the G1 group. Only 50% of the animals had a chimerism rate that was higher than 5% by the 6th week of the experiment, which decreased by the 16th week of the study. Higher and more stable mixed chimerism rates were observed in the G2 and G3 groups (the differences between the G2 and G3 mice were not statistically significant). During the 26-week analysis, the highest mixed chimerism level was found in the 6th week, which gradually decreased.

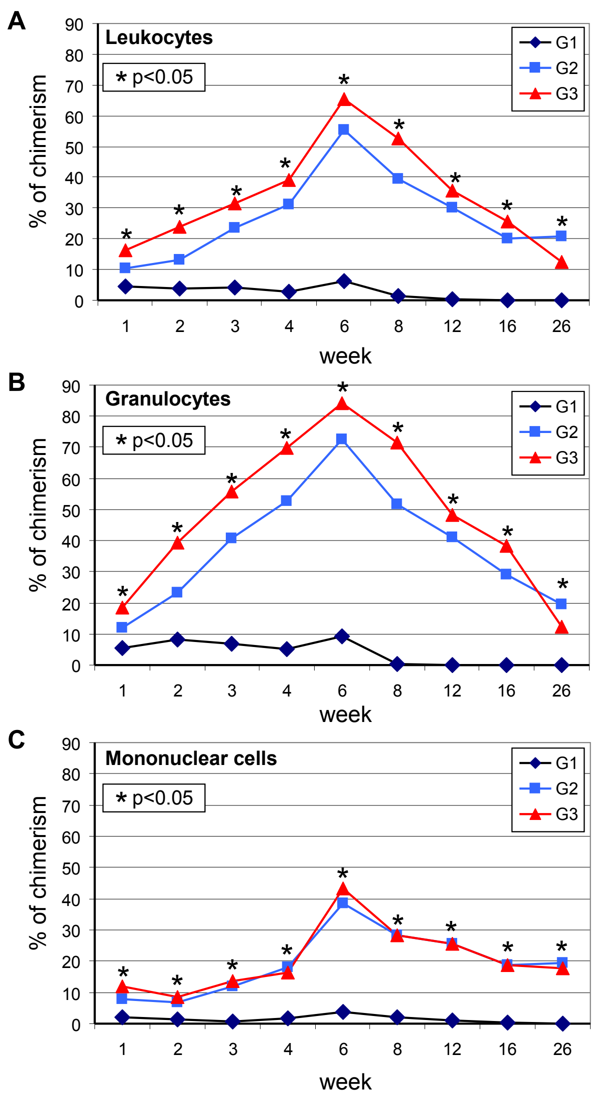

Figure 2: Kinetics of chimerism for the total leukocytes (A), granulocytes (B), and mononuclear cells (C) in the peripheral blood after various stem/progenitor cell transplantations. Data are from three independent experiments (n=12; n=4/per experimental group). *p<0.05, comparison between groups: all panels–G1 *vs.* G3 in the 1st week; G1 *vs.* G3 in the 2nd week; G1 *vs.* G2, and G1 *vs.* G3 in the 3rd, 4th, 6th, 8th, 12th, 16th and 26th weeks.

In the G2 and G3 groups, we detected a higher level of mixed chimerism in the granulocyte population than in the mononuclear cell population at many time points (Figure 2). In addition, to make the analysis of mixed chimerism more complete, we investigated the level of chimerism in cell populations that participate in graft rejection: CD4, CD8, and NK1.1 (Figure 3). We did not observe any statistically significant differences when we compared the chimerism of these cells (CD4 *vs.* CD8 *vs.* NK1.1). However, the differences were found in a few cases of chimerism assessment between experimental groups (Figures 3A-C). As stated above, the rates were higher in the G2 and G3 animals than in the G1 animals. The peak in the donor cell percentage occurred during the 8th-12th weeks of the study.

To investigate which leukocyte subpopulation best illustrates the establishment of chimerism in general, we correlated the mixed chimerism rate of all experimental animals observed in the total leukocyte population with those observed in the CD4, CD8, NK1.1 cell populations at the same time points. Associations were found in each situation, except in the 2nd week of the study. Spearman correlation coefficients did not differ significantly between the cells (Table 2A). Further analysis showed that the highest correlation coefficients were established between the total leukocyte and granulocyte populations (Table 2B).

To summarize, the stable multilineage mixed chimerism was observed in the G2 and G3 groups, but not in the G1 group (lin$^-$/Sca-1$^+$/c-kit$^-$). The highest chimerism rates were observed for granulocytes, and the strongest correlation was found for total leukocytes and granulocytes.

The rates of the CD4 lymphocytes, but not CD8 or NK1.1 lymphocytes, are higher in the mice that received the lin$^-$/Sca-1$^+$/c-kit$^-$ cells (G1)

Next, we assessed the kinetics of the CD4, CD8, and NK1.1 cell percentages over 16 weeks (Figure 4). We did not notice any statistically significant differences between consecutive weeks in the experimental mice. However, the percentages varied between the first and last analysis (2nd week *vs.* 16th week). This result reflects the fact that the CD4 and NK1.1 cell percentages gradually decreased, whereas the CD8 cell levels noticeably increased over the course of the study.

We observed an increased percentage of CD4 cells in the G1 mice compared to the other animals at many time points (Figure 4A). Taking the extremely low chimerism rate in this subpopulation in the G1 animals into consideration (Figure 3A), we assumed that the CD4 cells were mainly of recipient origin. We analyzed the CD4 chimerism and the CD4 cell percentages, but we did not find any correlation. In contrast to the enhanced percentage of CD4 recipient cells in the G1 animals, the percentages of the CD8 and NK1.1 cells remained the same in all groups.

Administration of lin$^-$/Sca-1$^+$/c-kit$^+$ (G2) and lin$^-$/Sca-1$^-$/c-kit$^+$ (G3) cells leads to the elimination of alloreactive T cell clones

The crucial part of this study was the analysis of tolerance induction by the three selected stem/progenitor cell populations. For this analysis, we assessed the percentages of Vβ5 and Vβ11 TCR lymphocytes in peripheral blood CD4 and CD8 cells in the 26th week of our study.

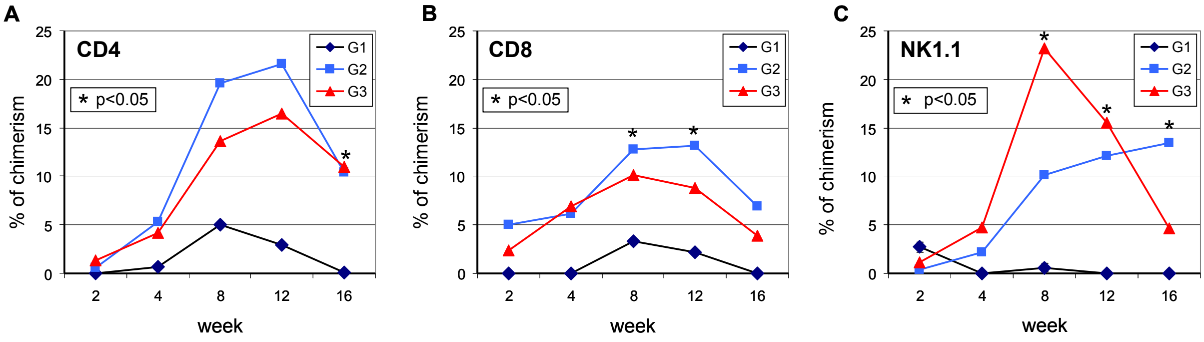

Figure 3: Kinetics of chimerism for the CD4 cells (A), CD8 cells (B), and NK1.1 cells (C) in the peripheral blood after various stem/progenitor cell transplantations. Data are from two independent experiments (n=12; n=4/per experimental group). *p<0.05, comparison between groups: panel A–G1 *vs*. G3; panel B–G1 *vs*. G2; panel C-G1 *vs*. G2 in the 8th, 12th and 16th weeks; G1 *vs*. G3 in the 8th and 12th weeks.

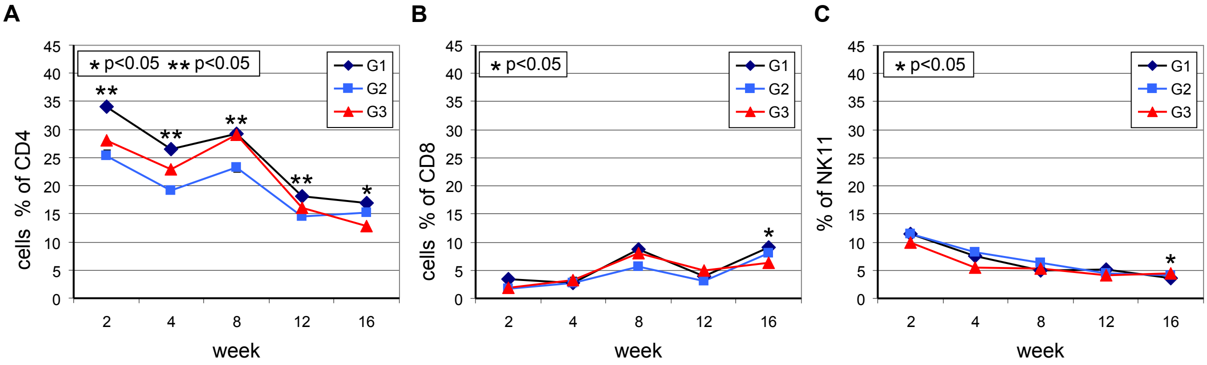

Figure 4: Kinetics of the CD4 (A), CD8 (B), and NK1.1 (C) cell percentages in the peripheral blood. Data are from two independent experiments (n=12; n=4/per experimental group). *p<0.05, comparison to 2nd week; **p<0.05, comparison between groups: panel A–G1 *vs*. G2 in the 2nd week; G1 *vs*. G2 and G3 in the 4th week; G1 *vs*. G2 in the 8th and 26th weeks.

Because of their reactivity to superantigens encoded by endogenous proviruses, the TCR Vβ5- and TCR Vβ11-expressing lymphocytes are completely or partially eliminated in the thymi of mice expressing I-E (i.e., Balb/c) as MHC class II antigens [23]. After transplantation of Balb/c hematopoietic cells to I-E-negative B6.SJL-*PtprcaPep3b* mice, the aforementioned antigens are presented in the recipient thymus, and as a consequence, TCR Vβ5 and TCR Vβ11 lymphocytes are eliminated during the double-positive stage of T cell development [23,24]. The elimination of these lymphocytes is associated with induced tolerance of donor antigens in chimeric mice [23,24]. We observed a decrease in analyzed cell percentages in the G2 and G3 chimeric mice to the level observed in the Balb/c donor animals (the CD group) (Figure 5). This effect was not observed in the G1 group, in which the level of chimerism was the lowest and disappeared by the end of experiment. These results suggest that antigen tolerance was effectively induced in the G2 and G3 animals, but not in the G1 animals.

Lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+ cell transplantations support permanent skin graft survival

In addition to analyzing the elimination of alloreactive lymphocytes, we also studied tolerance by performing skin transplantation (Figure 6A) [23]. Skin grafting is considered the most stringent *in vivo* examination of transplantation tolerance [6]. Skin transplantation was achieved by syngeneic graft in the recipient animals (the CB group). All transplants were permanently accepted and were growing hair in 40 days (Figure 6B). All control B6.SJL-*PtprcaPep3b* mice (the CW group), which received Balb/c skin grafts without any additional treatment, rejected the allografts with a median survival time of 9 days. Mice subjected to the induction protocol (the CP group) demonstrated a prolonged survival time and rejected grafts within 20 days (Figure 6C), which was statistically significant. In the G1 (lin-/Sca-1+/c-kit-) animals, the median value of skin tolerance was prolonged to 26 days, which did not differ from the CP mice (Figure 6A). Skin rejections were preceded by a decrease in the chimerism percentage. In contrast, only one skin graft was rejected in each of the G2 (lin-/Sca-1+/c-kit+) and G3 (lin-/Sca-1-/c-kit+) chimeric mouse groups at the 84th and 119th days, respectively. All other grafts in the G2 and G3 groups were permanently accepted. All mice with mixed chimerism rates of total leukocytes of at least 5% accepted the skin grafts. On the Figures 6D and 6E the skin transplanted to the experimental animals is presented after 7th and 73rd days respectively.

Next, we correlated skin graft survival with the rate of mixed chimerism in all of the measured cell populations (total leukocytes, granulocytes, mononuclear cells, and CD4, CD8, and NK1.1 cells). The Cox regression model showed a positive association between a high mixed chimerism rate and graft survival. Hazard ratios were <1 for each time point and were lowest in the 1st week of the study for leukocytes (0.64), granulocytes (0.66) and mononuclear cells (0.62), but in the 4th week for the CD4 cells (0.56). The hazard ratios were significantly

A			
Week	CD4	CD8	NK1.1
4th	0.66	0.68	0.68
8th	0.67	0.68	0.71
12th	0.85	0.86	0.94
16th	0.84	0.79	0.59
B			
Week	Granulocytes	Mononuclear cells	
1st	0.97	0.93	
2nd	0.97	0.9	
3rd	0.98	0.97	
4th	0.98	0.90	
6th	0.91	0.87	
8th	0.88	0.88	
12th	0.90	0.99	
16th	0.97	0.91	
26th	0.96	0.94	

Table 2: The coefficients of Spearman correlation between total leukocyte chimerism and (A) the CD4, CD8 and NK1.1 cell chimerism (B) granulocytes and mononuclear cell chimerism.

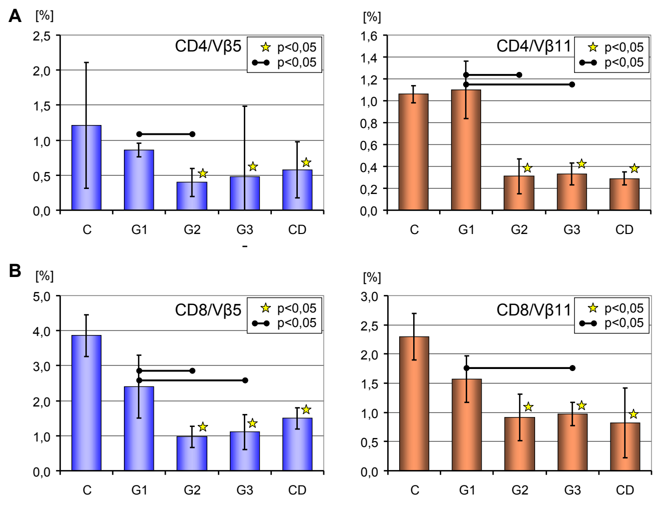

Figure 5: Analysis of the CD4 and CD8 lymphocytes with Vβ5 and Vβ11 antigens: CD4/Vβ5 and CD8/Vβ5 cells (A), and CD4/Vβ11 and CD8/Vβ11 cells (B) in the 26th week of the experiment (n=20; n=4/per experimental group). The values for the CD group are the control levels measured in the donor mice. *p<0.05, comparison to the control group; ●–● p<0.05, comparison between experimental groups.

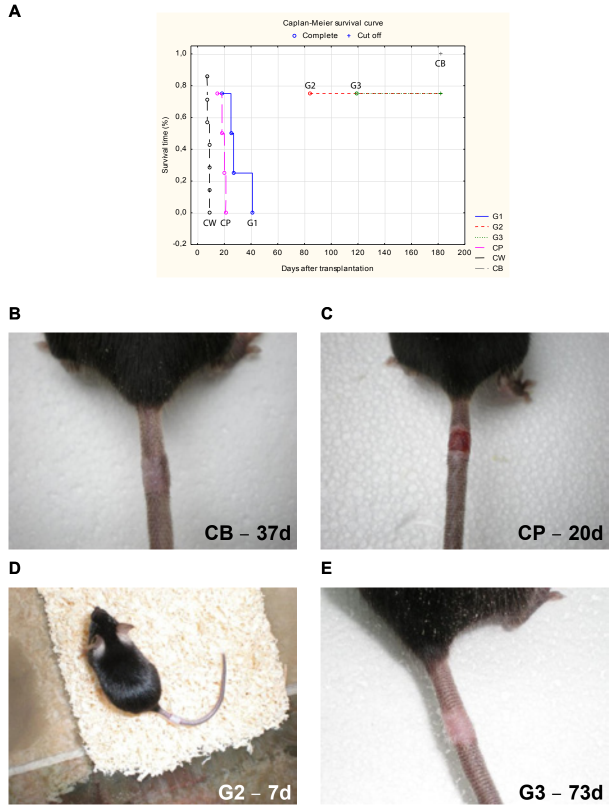

Figure 6: Kaplan-Meier curve of skin graft survival (A) (n=31; n=4/per experimental group). The experimental (G1-G3) and control (CP–mice exposed to induction protocol without bone marrow transplantation, CW–mice with skin allografts without any other treatment, CB–mice with syngeneic skin grafts without any other treatment) groups are shown. Examples of skin grafts for the CB group (control mice that received syngeneic grafts-B), the CP group (control group of mice that was subjected to the induction protocol only-C) and the G2 and G3 experimental groups on various days after transplantation (D, E).

lower for the chimerism rates of the mononuclear cells as compared to those of the granulocytes and total leukocytes (data not shown). In the case of the CD4, CD8, and NK1.1 cells, the differences were not always statistically significant, probably due to high standard deviations in the chimerism data.

Taken together, skin graft acceptance was found to be associated with the administration of the lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+ cells, and graft survival was strongly correlated with the presence of mixed chimerism among mononuclear cells in the beginning of the study.

Discussion

Mixed chimerism has been considered a valuable tool for the creation of alloantigen tolerance for many years, but this phenomenon is still not completely understood. One of the major areas under investigation is the search for a population of cells that can be transplanted in order to effectively induce mixed chimerism as well as alloantigen tolerance. To date, diverse cells have been studied. Because mixed chimerism is defined as the coexistence of donor and recipient hematopoietic cells, hematopoietic stem/progenitor cells have become the populations of major interest [4,5]. Finding the appropriate cell population could help to increase the level of mixed chimerism, lower the number of transplanted bone marrow cells required for mixed chimerism induction and promote tolerance to donor antigens. Consequently, in this study, we aimed to identify such cells among the murine stem/progenitor cell compartment in the bone marrow. CD34 antigen, the most commonly applied marker to receive population enriched in human hematopoietic stem/progenitor cells, is not broadly expressed in mice [15]. Murine hematopoietic stem cells are known to be found among lin- population and possess the c-kit receptor and Sca-1 antigens [13,15-17]. Furthermore, mouse embryonic cells, which were shown to induce mixed chimerism and tolerance to cardiac allografts, express these antigens [14]. To date, only c-kit+ and lin-/Sca-1+ cells individually were examined in mixed chimerism experiments [5,26]. Taking these facts into consideration, we assessed for the first time the efficacy of lin-/Sca-1+/c-kit+ cells in inducing mixed chimerism in a 26-week study. Moreover, we compared their effectiveness with lin-/Sca-1+/c-kit- and lin-/Sca-1-/c-kit+ cell populations.

In our experiment, two of the three cell populations used, the lin-/Sca-1+/c-kit+ (the G2 group) and lin-/Sca-1-/c-kit+ (G3) cells, were comparably effective in their ability to induce mixed chimerism, and both resulted in high rates of stable multilineage chimerism. Mixed chimerism is defined as "stable" if the leukocyte population of the recipient consists of more than 5% donor cells for at least 100 days [13]. The third population, the lin-/Sca-1+/c-kit- (G1) cells, appeared to be totally ineffective in this regard; the chimerism disappeared before the 16th week of the experiment, and only 50% of these animals had a chimerism rate that was higher than 5% by the 6th week of the study.

In our previous experiment, we assessed the mixed chimerism proportions in the mouse stem/progenitor cell compartments in a 8-week study [19]. Applying the same induction protocol as the present study (3 Gy, anti-CD40L x 2, anti-CD8) but using unfractionated bone marrow cells for transplantation, we established a lower chimerism rate among the lin-/Sca-1+/c-kit- cells compared to the lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+ cells. In the latter cells, which expressed c-kit receptor, the chimerism rate was high and did not differ between the two cell types. This effect can be explained by the effective elimination of the c-kit+ cells in the recipient mice by the induction protocol and/or their faster rate of renewal from the donor cells. The c-kit antigen is a tyrosine kinase family receptor that is only present on hematopoietic cells and binds stem cell factor (or kit ligand, KL) [15]. KL is the most important hematopoietic growth factor, and its binding to the receptor influences hematopoietic cell proliferation and differentiation [15,17]. Among c-kit+ bone marrow cells, there are lin-/Sca-1+/c-kit+ hematopoietic stem cells and multipotent progenitors (MPPs) and lin-/Sca-1-/c-kit+ cells, which are probably more mature progenitors or precursor cells [15,27]. In contrast, the cells that do not possess the c-kit receptor can be less mature, and as such, they are resistant to the induction protocol. The study of Randal et al. has at least partially confirmed this suggestion. The authors examined lin-/Sca-1+/Thy-1.1low/c-kit- cells, which appeared to be more primitive than c-kit+ cells. They are enriched in the quiescent cell population and, thus, are unsusceptible to cytotoxic drugs as well as non-sensitive to hematopoietic factors; also, they are unable to reconstitute irradiated mice [16]. Consequently, lin-/Sca-1+/c-kit- cells are probably less potent inducers of mixed chimerism, as we observed in our study and these cells transplanted with unfractionated bone marrow (G1) constitute lower number of hematopoietic cells to create mixed chimerism in comparison to others groups. To confirm this concept, we analyzed the size of the cells within the sorted populations; quiescent cells should be smaller than more mature ones [16]. The lin-/Sca-1+/c-kit- population consisted of approximately 2-fold smaller cells than the c-kit populations (data not shown).

To explore why the lin-/Sca-1+/c-kit- cells did not facilitate stable mixed chimerism, we also examined the viability of the sorted cells because apoptotic/necrotic cells may decrease donor engraftment [28], but viability did not differ between the experimental groups. It is worthy to mention that lin-/Sca-1+/c-kit- cells might also involve early non-hematopoietic stem/progenitor cells, which are present in the bone marrow or which migrate to bone marrow from other organs, to produce non-hematopoietic cells [13,27,29,30]. As non-hematopoietic cells, these cells are unable to induce or even support hematopoietic cell chimerism. We analyzed lin-/Sca-1+/c-kit- cells in Balb/c mice (donor) for the presence of the CD45 hematopoietic antigen, and 4.3% ± 1.7 of cells appeared to be CD45-. Such a low level of CD45- cells probably had an insignificant impact on chimerism development.

We have previously studied mixed chimerism in the same mice strains (and age) that were exposed to the identical induction protocol but received 2-fold higher amount (20×10^6) of unfractionated bone marrow cells only [19]. We have observed mixed chimerism analogously for eight weeks and the rates were as follows: 1st week–10.2%; 2nd week–27.2%; 3rd week–31.4%; 4th week–31.1%; 6th week–33.4%; 8th week–33.8% [19]. The chimerism proportions in those mice were significantly higher than in the animals that received additionally the lin-/Sca-1+/c-kit- cells (G1), in each week of this experiment, and were lower than in the G2 and G3 mice, even in the case of the 2-fold higher amount of transplanted bone marrow cells, in the 6th and 8th week of this study, what maintained our hypothesis that the c-kit+ cells noticeable supported mixed chimerism induction.

In a study by Bachar-Lustig et al., lin-/Sca-1+ cells were shown to induce mixed chimerism and specific tolerance towards skin grafts, but the mice had to be irradiated with larger 7-Gy dose of TBI. In addition, the number of injected cells was substantially higher than in our experiment because a smaller dose was not able to induce chimerism [26]. The number of transplanted cells and TBI dose are critical factors in the induction of mixed chimerism. Importantly, in the Bachar-Lustig study, the authors did not examine the c-kit antigen in the lin-/Sca-1+ population [26]. A proportion of the lin-/Sca-1+ cell population is c-kit+ (23.2% in our donor strain), and these cells have great potential to induce mixed chimerism according to our results.

The next part of our study focused on comparing the mixed chimerism rates among total leukocytes, granulocytes and mononuclear cells. Granulocytes demonstrated the highest rates. These results were similar to those obtained in our previous studies [19,21] and can be explained by the effective elimination of host granulocyte precursors by the induction protocol and the faster renewal of granulocytes from the transplanted donor cells. Finally, we correlated the mixed chimerism rate measured in the total leukocyte population with that of the other cells because an association between high levels of initial donor T cell reconstitution and stable chimerism has been reported [31]. In our study, a superior correlation was observed between the total leukocyte and granulocyte chimerism rates (Table 2B). This result can be explaind by the fact that granulocytes are the most numerous cells in the peripheral blood of our recipient mice (>40%) and their turn-over time is the quickest.

The next step of our experiment was to analyze the effect of each transplanted stem/progenitor cell type on the percentage of selected subpopulations of leukocytes (Figure 4). We found that the percentage of CD8 cells was low after transplantation and increased over the course of the experiment. This fact can be explained by the elimination of these cells resulting from treatment with the anti-CD8 antibody in the induction procedure two days before the cell transplantation was performed. Conversely, the percentages of the CD4 and NK1.1 cells decreased over the course of the study, which is probably related to the aforementioned increase of the CD8 cells. Moreover, we observed that the percentage of CD4 cells was higher in the group of animals with the lowest chimerism rate (G1). Based on our chimerism assessment, we proved that the CD4 cells were markedly of recipient origin (Figure 3A), especially at the beginning of the study (percentages of donor cells among the CD4 population were 0 and 0.7% in the first two weeks respectively). The animals of each group were exposed to the same induction protocol, suggesting that the increase in CD4 cells in the G1 mice was not related to the induction protocol, but to the transplanted lin-/Sca-1+/c-kit- cells or chimerism. There was no correlation found between the percentage of CD4 cells and the chimerism rate in this population. Therefore, we ruled out the possibility that chimerism is involved in the increase in the level of CD4 cells. We propose that the lin-/Sca-1+/c-kit- population had an impact on the percentage of CD4 cells. Whether these cells indeed influenced the CD4 population, but not other populations, and even more interesting question if this provoked disappearance of chimerism in the G1 group requires further study.

As the crucial part of our experiment aimed at finding the optimal stem/progenitor cell population to induce mixed chimerism, we investigated if the transplanted cells created not only mixed chimerism but tolerance for donor antigens as well. For this analysis, we assessed tolerance by measurement the elimination of Vβ5 and Vβ11 TCR lymphocytes and skin graft survival. Lymphocytes with Vβ5 and Vβ11 TCR are alloreactive T cell clones deleted in I-E- mice (recipients) after the transplantation of cells from I-E+ mice (donors) as a consequence of the appearance of the donor's antigens in the recipient's thymus [23,24]. This effect has been reported to be the main mechanism of tolerance generation through mixed chimerism [31]. As we presumed, the levels of alloreactive CD4 and CD8 lymphocytes in the G2 and G3 chimeric animals decreased to the levels observed in the control donor mice, which confirms tolerance induction, but in the G1 group, these lymphocytes were not eliminated (Figure 5).

In addition, we performed skin transplantation to determine the role of the selected stem/progenitor cell populations in prolonging skin graft survival. Because the immune system of the skin is well developed and its immunogenicity is high, skin grafts are frequently used in tolerance studies [32]. In our experiment, two populations of transplanted cells, lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+, prolonged skin graft survival (Figure 6). Only 25% of the skin grafts were rejected in these groups, but the others were permanently accepted, as was observed over our 26-week study (>100 days). The third cell population (G1), lin-/Sca-1+/c-kit-, prolonged the survival just up to the level observed in the control mice that were only subjected to the induction protocol and not cell transplantation. The costimulatory blockade used in our induction method without bone marrow transplantation has been reported to prolong skin graft survival, which we found in the CP control group as well, but it has not been shown to create tolerance [5]. As we confirmed in our experiment, each graft rejection was preceded by a decrease in chimerism rate, which is consistent with previous studies [5,12]. When we correlated skin graft survival with chimerism in different cell populations, skin graft survival was found to be related to chimerism at early time points in this study and this correlation was significantly higher for mononuclear cells (lower hazard ratios). Sykes et al. reported the relationship between high initial donor T-cell reconstitution and stable chimerism [31]. Moreover, in studies of some rodents and large animals, the animals that achieved mixed chimerism accepted allografts; however, most of them lost their mixed chimerism over time but did not reject their grafts, suggesting that mixed chimerism is crucial directly following transplantation [8,14]. As the mononuclear cell population consists of cells that are essential for graft rejection, we decided to broaden our study to include the CD4, CD8, and NK1.1 subpopulations [2,18]. It is important to note that the lowest hazard ratio was found for CD4 cell chimerism in the 4th week of the study. Because monocytes are also found in mononuclear cell population and they are precursors of antigen presenting cells [33], we intend to enlarge our correlation study on these cells in further experiments. We have previously measured mixed chimerism rates in monocytes and the rates were always higher than in lymphocytes and lower than in granulocytes [19,21], but their correlation with graft survival is particularly interesting.

In conclusion, we established, for the first time, that hematopoietic lin-/Sca-1+/c-kit+ and lin-/Sca-1-/c-kit+ cells derived from mouse bone marrow can be used as "protolerant" cell populations in mixed chimerism studies. These cells effectively induced mixed chimerism and donor antigen tolerance in a mouse model as observed both by the elimination of donor-reactive lymphocytes as well as permanent skin graft acceptance. In contrast, the more primitive lin-/Sca-1+/c-kit- population did not have this effect. This study may bring us closer to being able to effectively induce specific immune tolerance. Such tolerance could solve major problems in clinical transplantation, including chronic graft rejection, organ shortage and complications of long-term non-specific immunosuppressive therapy. Identification of a "protolerant" cell population will enhance the chance for tolerance creation. Moreover, such cells will decrease the required dose of transplanted bone marrow cells without any loss in efficacy and could even promote the development of an expansion system for this population, which may allow many recipients to use these cells from one donor.

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References

1. Delis S, Ciancio G, Burke GW 3rd, Garcia-Morales R, Miller J (2004) Donor bone marrow transplantation: chimerism and tolerance. Transpl Immunol 13: 105-115.
2. Sykes M (2007) Immune tolerance: mechanisms and application in clinical transplantation. J Intern Med 262: 288-310.
3. Golshayan D, Buhler L, Lechler RI, Pascual M (2007) From current immunosuppressive strategies to clinical tolerance of allografts. Transpl Int 20: 12-24.
4. Sykes M (2009) Hematopoietic cell transplantation for tolerance induction: animal models to clinical trials. Transplantation 87: 309-316.
5. Akpinar E, Craighead N, Smoot D, Hale DA (2004) Potent skin allograft survival prolongation using a committed progenitor fraction of bone marrow in mice. Transplantation 78: 383-391.
6. Billingham RE, Brent L, Medawar PB (1953) Actively acquired tolerance of foreign cells. Nature 172: 603-606.
7. Liesveld JL, Rothberg PG (2008) Mixed chimerism in SCT: conflict or peaceful coexistence? Bone Marrow Transplant 42: 297-310.
8. Fehr T, Sykes M (2008) Clinical experience with mixed chimerism to induce transplantation tolerance. Transpl Int 21: 1118-1135.
9. Jacquet EG, Schanie CL, Fugier-Vivier I, Willer SS, Ildstad ST (2003) Facilitating cells as a venue to establish mixed chimerism and tolerance. Pediatr Transplant 7: 348-357.
10. Takeuchi Y, Ito H, Kurtz J, Wekerle T, Ho L, et al. (2004) Earlier low-dose TBI or DST overcomes CD8+ T-cell-mediated alloresistance to allogeneic marrow in recipients of anti-CD40L. Am J Transplant 4: 31-40.
11. Gross DA, Chappert P, Leboeuf M, Monteilhet V, Van Wittenberghe L, et al. (2006) Simple conditioning with monospecific CD4+CD25+ regulatory T cells for bone marrow engraftment and tolerance to multiple gene products. Blood 108: 1841-1848.
12. Yu P, Xiong S, He Q, Chu Y, Lu Ch, et al. (2009) Induction of allogeneic mixed chimerism by immature Dendritic cells and bone marrow transplantation leads to prolonged tolerance to major histocompatibility complex disparate allografts. Immunology 127: 500-511.
13. Deng W, Han Q, Liao L, Li C, Ge W, et al. (2004) Allogeneic bone marrow-derived flk-1+Sca-1- mesenchymal stem cells leads to stable mixed chimerism and donor-specific tolerance. Exp Hematol 32: 861-867.
14. Bonde S, Chan KM, Zavazava N (2008) ES-cell derived hematopoietic cells induce transplantation tolerance. PLoS One 3: e3212.

15. Wognum AW, Eaves AC, Thomas TE (2003) Identification and isolation of hematopoietic stem cells. Arch Med Res 34: 461-475.

16. Randall TD, Weissman IL (1998) Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: resting stem cells or mystery population? Stem Cells 16: 38-48.

17. Bradfute SB, Graubert TA, Goodell MA (2005) Roles of Sca-1 in hematopoietic stem/progenitor cell function. Exp Hematol 33: 836-843.

18. Westerhuis G, Maas WG, Willemze R, Toes RE, Fibbe WE (2005) Long-term mixed chimerism after immunologic conditioning and MHC-mismatched stem-cell transplantation is dependent on NK-cell tolerance. Blood 106: 2215-2220.

19. Baśkiewicz-Hałasa M, Pius E, Hałasa M, Dziedziejko V, Grymuła K, et al. (2012) Different strategies of mixed chimerism induction may determine stem/progenitor cell populations in recipient mice. Transpl Immunol 26: 34-41.

20. Jurecic R, Van NT, Belmont JW (1993) Enrichment and functional characterization of Sca-1+WGA+, Lin-WGA+, Lin-Sca-1+, and Lin-Sca-1+WGA+ bone marrow cells from mice with an Ly-6a haplotype. Blood 82: 2673-2683.

21. Baśkiewicz-Masiuk M, Grymuła K, Hałasa M, Pius E, Boehlke M, et al. (2009) Induction of mixed chimerism in mice by employing different conditioning protocols and bone marrow cell transplantation. Transplant Proc 41: 1894-1899.

22. Halasa M, Baskiewicz-Masiuk M, Dabkowska E, Machalinski B (2008) An efficient two-step method to purify very small embryonic-like (VSEL) stem cells from umbilical cord blood (UCB). Folia Histochem Cytobiol 46: 239-243.

23. Colson YL, Lange J, Fowler K, Ildstad ST (1996) Mechanism for cotolerance in nonlethally conditioned mixed chimeras: negative selection of the Vbeta T-cell receptor repertoire by both host and donor bone marrow-derived cells. Blood 88: 4601-4610.

24. Bigenzahn S, Blaha P, Koporc Z, Pree I, Selzer E, et al. (2005) The role of non-deletional tolerance mechanisms in a murine model of mixed chimerism with costimulation blockade. Am J Transplant 5: 1237-1247.

25. Koporc Z, Pilat N, Nierlich P, Blaha P, Bigenzahn S, et al. (2008) Murine mobilized peripheral blood stem cells have a lower capacity than bone marrow to induce mixed chimerism and tolerance. Am J Transplant 8: 2025-2036.

26. Bachar-Lustig E, Li HW, Gur H, Krauthgamer R, Marcus H, et al. (1999) Induction of donor-type chimerism and transplantation tolerance across major histocompatibility barriers in sublethally irradiated mice by Sca-1(+)Lin(-) bone marrow progenitor cells: synergism with non-alloreactive (host x donor)F(1) T cells. Blood 94: 3212-3221.

27. Gupta R, Karpatkin S, Basch RS (2006) Hematopoiesis and stem cell renewal in long-term bone marrow cultures containing catalase. Blood 107: 1837-1846.

28. Li JM, Gorechlad J, Larsen CP, Waller EK (2006) Apoptotic donor leukocytes limit mixed-chimerism induced by CD40-CD154 blockade in allogeneic bone marrow transplantation. Biol Blood Marrow Transplant 12: 1239-1249.

29. Kwon SM, Lee YK, Yokoyama A, Jung SY, Masuda H, et al. (2011) Differential activity of bone marrow hematopoietic stem cell subpopulations for EPC development and ischemic neovascularization. J Mol Cell Cardiol 51: 308-317.

30. Kucia M, Reca R, Campbell FR, Zuba-Surma E, Majka M, et al. (2006) A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20: 857-869.

31. Sykes M, Szot GL, Swenson KA, Pearson DA (1997) Induction of high levels of allogeneic hematopoietic reconstitution and donor-specific tolerance without myelosuppressive conditioning. Nat Med 3: 783-787.

32. Sykes M (2009) Mechanisms of transplantation tolerance in animals and humans. Transplantation 87: 67-69.

33. Strasser EF, Eckstein R (2010) Optimization of leukocyte collection and monocyte isolation for dendritic cell culture. Transfus Med Rev 24: 130-139.

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