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PERIPHERAL BLOOD CD34$^+$ CELLS EFFICIENTLY ENGRAFT HUMAN CYTOKINE KNOCK-IN MICE

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Key points:

- Human cytokine knock-in mice are improved in vivo models for multi-lineage engraftment of mobilized peripheral blood CD34+ cells
- Humanized mouse models might open new avenues for personalized studies of human (patho)physiology of the hematopoietic and immune system

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ABSTRACT

Human CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) can reconstitute a human hemato-lymphoid system when transplanted into immunocompromised mice. While fetal liver- and cord blood-derived CD34$^+$ cells lead to high engraftment levels, engraftment of mobilized, adult donor-derived CD34$^+$ cells has remained poor. We generated so-called MSTRG and MISTRG humanized mice on a *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ background carrying a transgene for human SIRPα and human homologues of the cytokines macrophage-colony stimulating factor, thrombopoietin, with or without interleukin-3 and granulocyte-macrophage colony stimulating factor under murine promotors. Here we transplanted mobilized peripheral blood CD34$^+$ cells in sub-lethally irradiated newborn and adult recipients. Human hematopoietic engraftment levels were significantly higher in bone marrow, spleen and peripheral blood in newborn transplanted MSTRG/MISTRG as compared to non-obese diabetic/severe combined immunodeficient *Il2rg*$^{-/-}$ or human SIRPα-transgenic *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ recipients. Furthermore newborn transplanted MSTRG/MISTRG mice supported higher engraftment levels of human phenotypically defined HSPCs in bone marrow, T-cells in the thymus, and myeloid cells in non-hematopoietic organs such as liver, lung, colon and skin, approximating the levels in the human system. Similar results were obtained in adult recipient mice. Thus, human cytokine knock-in mice might open new avenues for personalized studies of human (patho)physiology of the hematopoietic and immune system *in vivo*.

INTRODUCTION

Modeling the human hemato-lymphoid-system (HHLS) in human hematopoietic stem and progenitor cell (HSPC) xeno-grafted mice has advanced our understanding of hematopoiesis and leukemogenesis and allowed testing novel therapeutic approaches[1-4]. Although G-CSF mobilized CD34$^+$ peripheral blood cells (PBCs) are clinically widely used for autologous and allogenic hematopietic stem cell transplantation (HSCT)[5], these cells - in contrast to fetal liver (FL) and cord blood (CB) CD34$^+$ cells - have shown relatively poor engraftment at similar numbers in mice and are thus rarely utilized[6,7]. However, some cell-intrinsic pathways might differ in FL and CB compared to adult HSPCs, and fetal and neonatal cells are inappropriate to study individual patients` acquired conditions.

We recently generated immunodeficient *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice with genes encoding human M-CSF[8], combined interleukin 3 (IL-3) and GM-CSF[9], and thrombopoietin (TPO)[3,10], knocked into the respective mouse loci, either individually or in combination (MISTRG)[11]. Additionally, MISTRG mice carry a transgene encoding human SIRPα, known to bind human CD47 and thereby inhibiting mouse phagocytes of human cells[12-14]. We demonstrated that transplantation of human FLCD34$^+$ cells into newborn MISTRG mice leads to high-level engraftment, multi-lineage differentiation and functionality of innate immune cells[11]. Also, proof of principle engraftment of CB and PB CD34$^+$ cells into newborn mice has been shown[11].

To test utility and value of these novel mouse strains for PB-derived human HSPC research, we compared engraftment, lineage differentiation and tissue seeding of healthy adult mobilized PB human CD34$^+$ cells and their progeny in various immune-deficient strains, including newborn and adult recipient MISTRG mice.

METHODS

Humanized cytokine KI mice were generated as reported previously[11,16]. G-CSF–mobilized adult PBCs and cord blood cells were collected from healthy donors after obtaining informed consent. The study was approved by the ethics boards of the canton Zurich and Yale University Human Investigation Committee respectively. Human (h) $CD34^+$ cells were purified by density gradient centrifugation followed by positive immunomagnetic selection. Newborn and adult mice were sublethally irradiated. Newborn mice were intra-hepatically (*i.h.*) injected with 300,000 to 500,000 (Zurich cohort) or 100,000 to 140,000 (Yale cohort) $hCD34^+$ cells per mouse[15]. Adult mice were injected intravenously (*i.v.*) with 800,000 to 1,000,000 $hCD34^+$ cells per mouse. Mice were bled at several time points and terminally analyzed 10 to 16 weeks post transplantation. Experiments were approved by the *Veterinäramt des Kantons* Zurich and Yale University Institutional Animal Care and Use Committee. For further details see supplemental methods, available on the *Blood* web site.

RESULTS AND DISCUSSION

We first examined the engraftment of $hCD34^+$ cells isolated from G-CSF mobilized PB upon *i.h.* injection into sub-lethally irradiated newborn NSG, hSIRPα-transgenic *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (SRG)[12], and mouse strains with human cytokine KI genes for hM-CSF combined with hTPO (MSTRG) or the addition of hIL-3/hGM-CSF (MISTRG)[11]. PB analysis to determine $hCD45^+$ cells at 4, 8, and 14 to 16 weeks revealed an increasing frequency of $hCD45^+$ cells over time among total $CD45^+$ cells in cytokine KI but not in control mice (**Figure S1**). When we compared percentage and absolute numbers of engrafted human cells in the bone marrow (BM) of recipient mice, the number was greatly increased in MSTRG and MISTRG in comparison to NSG and SRG mice (**Figure 1A and 1B**). Superior engraftment efficiency of cytokine KI mice was reproducible in an independent experimental cohort at Yale, where even lower numbers of PB $CD34^+$ were transplanted (**Figure S2A**). Importantly, split donor sample transplantation into newborn cytokine KI versus NSG and SRG mice resulted in significantly higher engraftment in the KI group (**Figure 1C**). While engraftment was highest in BM, $\%hCD45^+$ cells in PB and spleen of cytokine KI mice also exceeded engraftment of control groups (**Figure 1D**). Following transplantation of adult PB $CD34^+$ cells, similar as in previous studies using FL or CB $CD34^+$ cells, we observed a lower frequency of $hCD45^+$ cells in PB and spleen in comparison to BM in all recipient strains used. Interestingly, this difference was less prominent when transplanting CB $CD34^+$ cells in newborn KI recipients (**Figure S4**). The reason for these differences needs to be further explored but might be in part attributable to extramedullary mouse hematopoiesis and/or differences in migration potential of human cells.[11] Irrespective of this observation and more importantly, adult recipient MISTRG mice transplanted with mobilized PB $CD34^+$ cells also supported substantial overall $hCD45^+$ and myeloid cell engraftment in BM, PB, spleen and liver with a trend towards superiority compared to NSG mice (**Figure 1E, Figure S5**).

We next compared immature human HSPC engraftment and maintenance in different newborn recipients. Cytokine KI mice showed significantly increased relative and absolute numbers of human hematopoietic progenitor cells ($CD34^+CD38^+$) and HSC containing $CD34^+CD38^-$ cells in BM (**Figure 1F and 1G**). Thus, we conclude that - in line with prior *in vitro* and *in vivo* observations[17-19] - human cytokines such as M-CSF, Il-3/GM-CSF and TPO support maintenance and differentiation of human HSPCs.

The percentage of human $CD33^+$ myeloid cells in the BM was not significantly different between newborn recipients, while absolute numbers were higher in KI than in conventional mice (**Figure 2A, 2B, Figure S2B),** regardless of a comparable bone marrow cellularity (Figure S6). Importantly, $hCD33^+$ cells were significantly increased in PB as well as non-lymphoid tissues such as liver and lung in the KI group (**Figure 2B, 2D, 2E and Figure** S7) and human $CD68^+$ myeloid cells were detectable in non-lymphoid tissues such as lung, liver, colon and skin of KI but not of control mice (**Figure 2F**). In adult recipients we observed a trend towards superior myeloid development in different organs (**Figure S5).**

$CD14^+CD16^-$ cells are classical monocytes, while $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ cells are categorized as non-classical "patrolling" monocytes[20]. The amount of both, $CD14^+CD16^+$ and $CD14^{dim}CD16^+$ non-classical monocytes in BM of the KI group was higher than in the control group (**Figure 2C**). In addition, $CD141^+$ ($BDCA\text{-}3^+$) DCs with cross-presentation activity were significantly increased in the BM of KI mice (**Figure S8**).

As previously reported, we observed splenomegaly due to extra-medullary mouse hematopoiesis in highly engrafted animals[11]. Immuno-histochemical staining of BM of an engrafted MISTRG mouse (32% $hCD45^+$ cells) confirmed the presence of human macrophages and showed evidence of hemophagocytosis, underlining human myeloid cell functionality (**Figure S9)**. Interestingly, and in contrast to MISTRG mice engrafted with $1x10^5$ $hCD34^+$ FL cells[11], mice engrafted with up to $5x10^5$ mobilized PB $CD34^+$ cells survived clinically healthy until terminal analysis up to 16 weeks. This might be a result of lower human BM engraftment levels, leading to relatively less reduction of mouse hematopoiesis and less phagocytosis of mouse cells by human phagocytes[11].

Analysis of human lymphocyte subsets revealed that the frequency of NK cells in BM and PB was significantly higher in KI mice (**Figure S10A**). Also, the CD4/8 ratio of T cells in the BM of MSTRG and MISTRG mice was higher, while naïve and memory $CD4^+$ T cell subsets as well as B cell subsets were similar between KI and control groups (**Figure S10B and C**). Of interest, thymus $hCD45^+$ cells and thymocyte development in KI mice were significantly enhanced as compared to control mice (**Figure 2G**).

Taken together, these data indicate that human cytokine KI mice support the development of mature lineage cells - both myeloid and lymphoid - from adult mobilized PB $CD34^+$ cells in hematopoietic, lymphoid and non-lymphoid tissues. Even though this study focused on newborn recipients, we are also showing confirmatory proof of principle data from adult recipient cytokine KI mice, a setting that might - due to easier experimental handling - facilitate clinical translational re-

search. Indeed, it will now become feasible to use these and other humanized mice for individualized patient-sample derived *in vivo* research that allows preclinical, likely predictive testing of (patho)-physiology and novel therapeutic strategies in a personalized manner in infectious and neoplastic diseases [11,21]

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AUTHORSHIP CONTRIBUTION

Y.Saito, J.M.E., Y.Song and S.H. designed research, performed experiments, analyzed data and wrote the manuscript; A.Rafiei, A.Rongvaux, D.K and M.H. performed experiments; R.A.F and M.G.M. directed the study and wrote the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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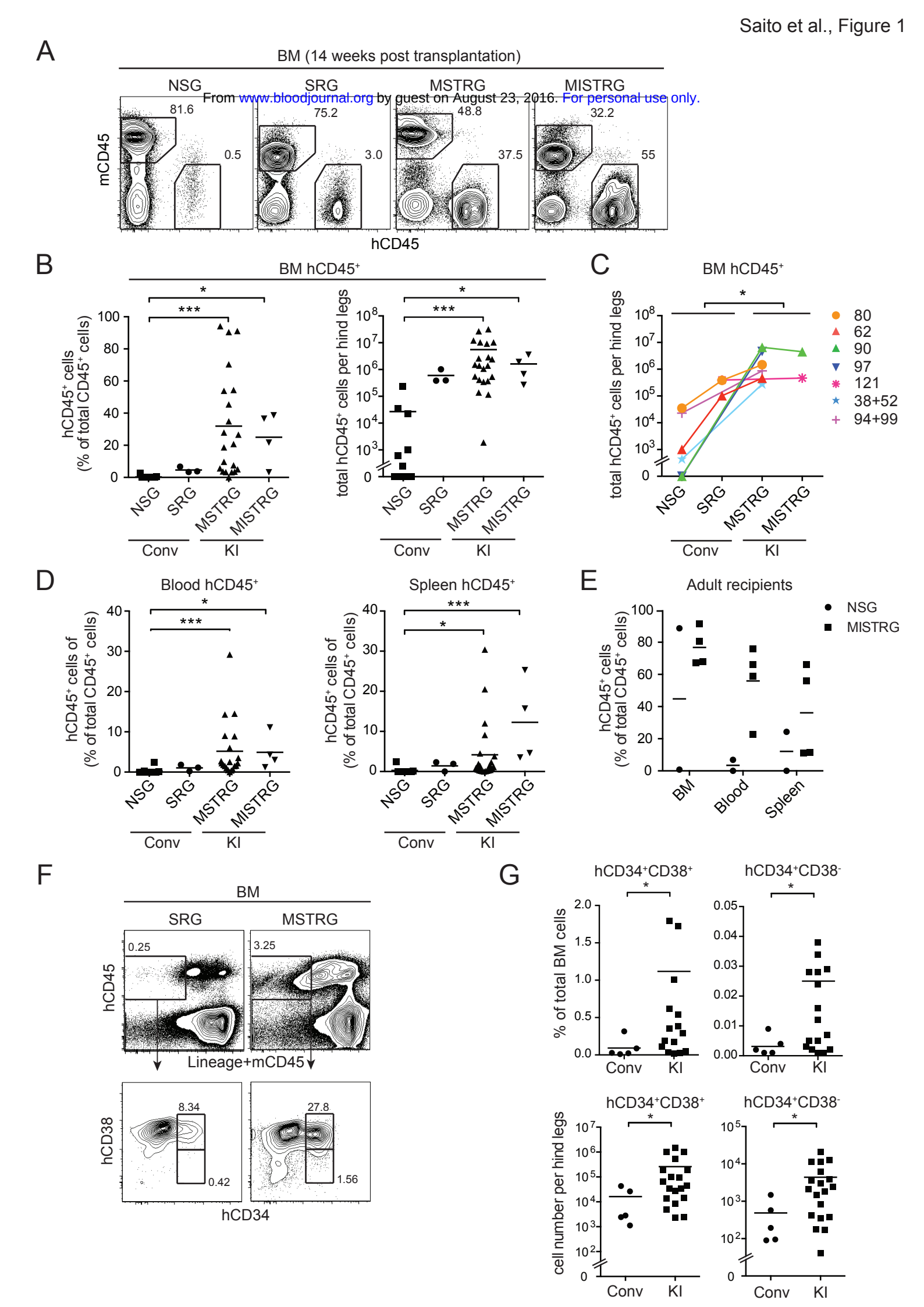
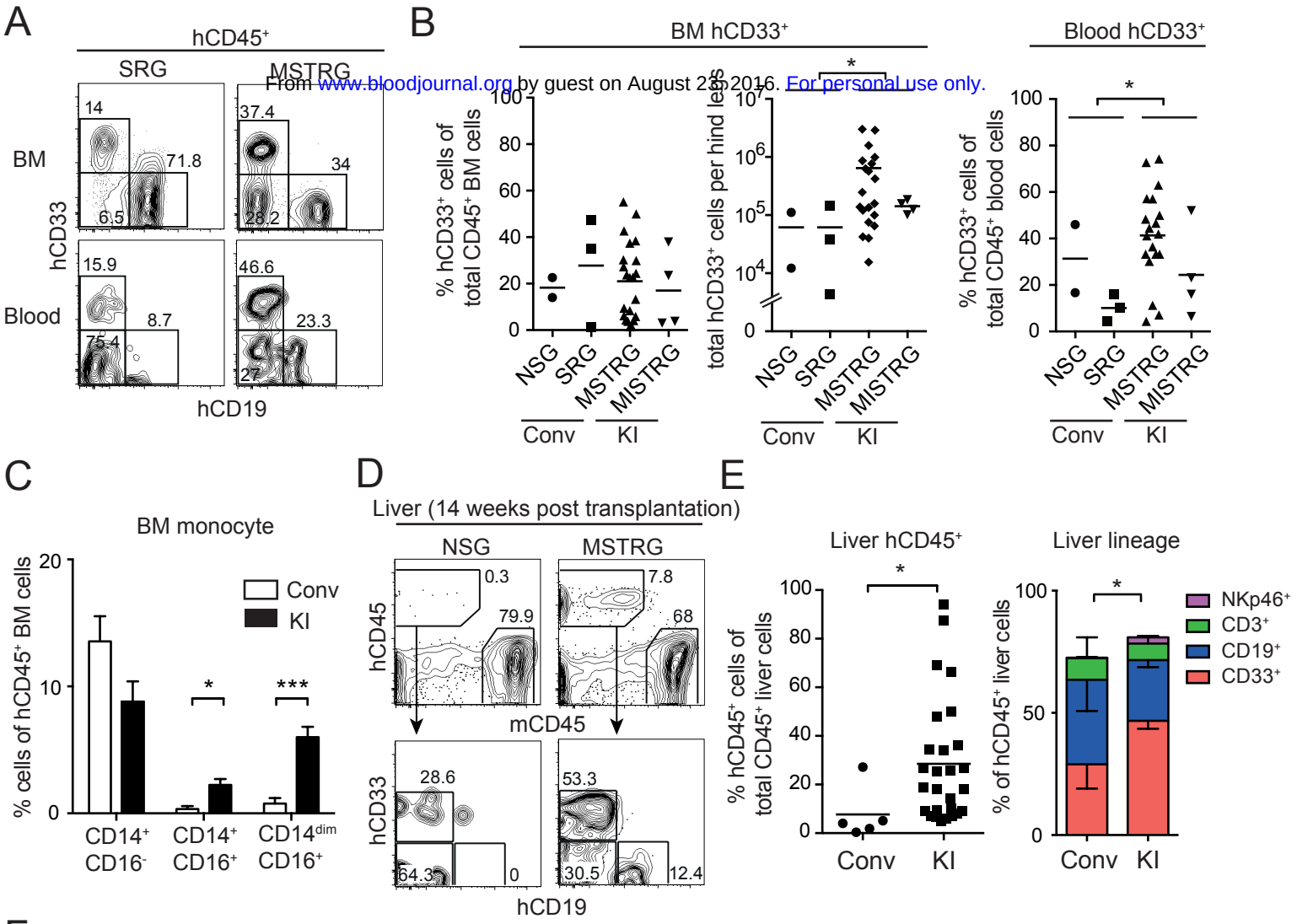
FIGURE LEGENDS

Figure 1: Human cytokine knock-in mice support engraftment of human mobilized peripheral blood CD34$^+$ cells. (A) Representative FACS analysis of the frequency of mouse and human CD45$^+$ cells in BM in newborn mice 14 weeks post transplantation. Numbers besides gated areas indicate percentages of cells. **(B)** Engraftment of hCD45$^+$ cells in BM of newborn mice 10 to 16 weeks post transplantation. Left panel indicates percentage of hCD45$^+$ cells among total CD45$^+$ cells. Right panel indicates absolute number of hCD45$^+$ cells per hind legs (2 femurs and tibias). Each symbol represents an individual mouse, bars indicate mean values; n=11 (NSG), n=3 (SRG), n=22 (MSTRG), n=4 (MISTRG); Conv=Conventional; KI=human cytokine knock-in; *P<0.05, ***P<0.001 (One-way ANOVA Kruskal–Wallis test with Dunn's Multiple Comparison Test). **(C)** hCD45$^+$ cell engraftment in BM of newborn mice from splitted, matched individual donors. Each symbol represents mean frequency of hCD45% cells among total CD45$^+$ cells. Mean frequency of human CD45+ cells in Conv versus KI mice was significantly different. *P<0.05 (Conv vs. KI; Paired t test). **(D)** Engraftment of hCD45$^+$ cells in blood (left panel) and spleen (right panel) 10 to 16 weeks post transplantation. Each symbol represents an individual mouse, bars indicate mean values; n=11 (NSG), n=3 (SRG), n=22 (MSTRG), n=4 (MISTRG); *P<0.05, ***P<0.001 (One-way ANOVA Kruskal–Wallis test with Dunn's Multiple Comparison Test). **(E)** Engraftment of hCD45$^+$ cells in BM, blood and spleen in adult mice 12 to 16 weeks after transplantation. Panel shows percentage of hCD45$^+$ cells among total CD45$^+$ cells in each organ. Each symbol represents an individual recipient engrafted with hCD45$^+$ cells, bars indicate mean values; n=2 (NSG), n=4 (MISTRG) **(F)** Representative FACS plots for hCD34$^+$CD38$^+$ and hCD34$^+$CD38$^-$ cells in newborn recipients gated on mCD45$^-$ Lineage (Lin)$^-$ hCD45$^+$ cells. Numbers beside gate indicate percentages of cells. **(G)** Frequency (upper graphs) as well as absolute number (bottom graphs) of hCD34$^+$CD38$^+$, hCD34$^+$CD38$^-$ cells per hind legs. Each symbol represents an individual newborn recipient engrafted with hCD45$^+$ cells, bars indicate mean values; n=5 (Conv; 2 NSG and 3 SRG), n=19 (KI; 15 MSTRG and 4 MISTRG); *P <0.05 (Mann-Whitney U test).

Figure 2: Human cell engraftment in lymphoid and non-lymphoid tissues in newborn recipients. (A) Representative FACS plots for hCD19$^+$ and hCD33$^+$ cells gated on hCD45$^+$ cells in BM (top panels) and PB (bottom panels). **(B)** Frequency and absolute number of hCD33$^+$ cells in BM and frequency of hCD33$^+$ cells in PB. Each plot shows data from an individual recipient engrafted with hCD45$^+$ cells. Numbers beside gates indicate percentages of cells; n=5 (Conv; 2 NSG and 3 SRG), n=20 (KI; 16 MSTRG and 4 MISTRG); *P <0.05 (Mann-Whitney U test). **(C)** Frequency of classical (CD14$^+$CD16$^-$) and non-classical monocytes (CD14$^+$CD16$^+$ or CD14^{dim}CD16$^+$) among lineage (Lin; CD3, CD19, CD20)$^-$ hCD45$^+$ cells in BM of engrafted recipient mice; data shows mean +SEM; n=5 (Conv; 2 NSG and 3 SRG), n=22 (KI; 18 MSTRG and 4 MISTRG); *P <0.05, ***P<0.001 (Mann-Whitney U test). **(D)** Representative FACS plots of recipient mice for hCD45$^+$ cells (top panels), CD33$^+$ or CD19$^+$ cells gated on hCD45$^+$ cells (bottom panels). Numbers beside

gates indicate percentages of cells shown. **(E)** Percentage of hCD45$^+$ cells among total CD45$^+$ cells (left graph); each symbol represents an individual recipient engrafted with hCD45$^+$ cells and bars indicate mean values. CD33$^+$, CD19$^+$, CD3$^+$, and NKp46$^+$ population among hCD45$^+$ cells in the liver of engrafted mice (right graph); data shows mean + SEM; n=5 (Conv; 2 NSG and 3 SRG), n=25 (KI; 21 MSTRG and 4 MISTRG), *P <0.05 (Mann-Whitney U test; left graph) and *P <0.05 (for CD33$^+$ cells, one-way ANOVA Kruskal–Wallis test with Dunn's Multiple Comparison Test; right graph). **(F)** Immuno-histological staining of human myeloid cells (hCD68$^+$) in non-lymphoid tissues (liver, lung, colon, and skin) of recipient mice. Scale bars: 20 µm. Images are representative of at least three mice analyzed per group. **(G)** Representative FACS plots for hCD45$^+$ cells (top panels) and double-negative, double-positive, CD4$^+$ single-positive, and CD8$^+$ single-positive thymocytes among hCD45$^+$ cells (bottom panels) in the thymus of indicated recipient mice. Number of hCD45$^+$ cells in the thymus is shown (right panel); each symbol represents an individual recipient engrafted with hCD45$^+$ cells, bars indicate mean values; n=4 (Conv; 2 NSG and 2 SRG), n=9 (KI; 9 MSTRG); *P <0.05, ***P<0.001 (Mann-Whitney U test).





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