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From stem cells to functional lymphocytes: cell differentiation and gene therapy implementation for RAG-SCID

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Chapter 7

Combining mobilizing agents with busulfan to reduce chemotherapy-based conditioning for hematopoietic stem cell transplantation

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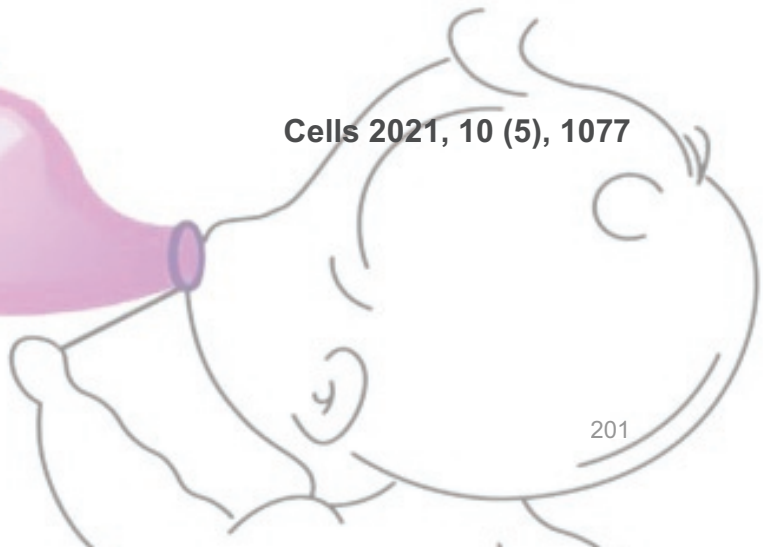

ABSTRACT

In the context of hematopoietic stem cell (HSC) transplantation, conditioning with myelo- and immune-ablative agents is used to eradicate the patient's diseased cells, generate space in the marrow and suppress immune reactions prior to the infusion of donor HSCs. While conditioning is required for effective and long lasting HSC engraftment, currently used regimens are also associated with short and long-term side effects on extramedullary tissues and even mortality. Particularly in patients with Severe Combined Immunodeficiency (SCID), which are generally less than 1-year-old at the time of transplantation and often suffer from existing co-morbidities. There is a pressing need for developing alternative, less toxic conditioning regimens. Hence, we here aimed to improve efficacy of currently used myeloablative protocols by combining busulfan with stem-cell niche-directed therapeutic agents (G-CSF or Plerixafor) that are approved for clinical use in stem cell mobilisation. T, B and myeloid cell recovery was analysed in humanized NSG mice after different conditioning regimens. Increasing levels of human leukocyte chimerism were observed in a busulfan dose-dependent manner, showing comparable immune recovery as with total body irradiation in CD34-transplanted NSG mice. Notably, a better T cell reconstitution compared to TBI was observed after busulfan conditioning not only in NSG mice but also in SCID mouse models. Direct effects reducing the stem cell compartment in the bone marrow were observed after G-CSF and Plerixafor administration, as well as in combination with low doses of busulfan. Unfortunately, these direct effects on the stem population in the bone marrow were not reflected by increased human chimerism nor immune recovery after CD34 transplantation in NSG mice. These results indicate moderate potential of reduced conditioning regimens for clinical use relevant for all allogeneic transplants.

INTRODUCTION

Allogeneic and gene-corrected autologous hematopoietic stem cell (HSC) transplantation may result in limited engraftment of progenitors without preceding conditioning regimen due to the occupation of bone marrow (BM) and thymic niches by host cells, which results in incomplete graft function, immune reconstitution and cure [1]. Conditioning agents can be employed to create space in the BM niches thus allowing transplanted HSCs to engraft efficiently. Although conditioning contributes to an improved HSCT outcome by increasing HSC engraftment, immune chimerism, immune function and by reducing the risk of graft rejection, it may also have negative impact on patient well-being due to short-term and long-term treatment-related morbidity and mortality [2, 3]. The use of irradiation-based regimens and alkylating chemotherapy in infants has an unfavorable impact on growth and fertility, and is associated with an increased risk for secondary malignancies [4-6]. Therefore, particularly in pediatric patients, total body irradiation regimens have been gradually replaced by chemotherapy-based conditioning [7]. Busulfan is a myeloablative alkylating agent that prevents DNA replication through DNA crosslinking and therefore triggering cell apoptosis [8]. Busulfan is used as conditioning agent prior HSCT as it is known to be cytotoxic to host hematopoietic stem and progenitor cells (HSPCs) [9].

In the first stem cell gene therapy protocols for Severe Combined immunodeficiency (SCID) conditioning was omitted. The absence of conditioning prior to both allogeneic and gene-corrected autologous HSCs transplantation led to limited engraftment of transplanted HSC and thus only partial correction of the immune deficiency, especially B cell function, resulting in suboptimal clinical benefit. [3, 10]. Subsequent clinical trials on gene therapy for SCID included the use of a non-myeloablative reduced intensity conditioning (RIC) regimen consisting of a low dose busulfan-based conditioning (4mg/kg), approximately 25% of the total dose usually used in myeloablative protocols. The use of RIC regimens enables the engraftment of early progenitor cells and therefore allow both T and B cell long-term correction, while limiting potential short- and long-term toxicities [11-13]. However, insufficient conditioning is associated with the risk of mixed chimerism in the HSC compartment [14] and therefore reduce the chance for a favorable outcome. Current gene therapy protocols for SCID, especially ADA-SCID and X-linked SCID, rely on the use of HSC corrected cells and a reduced-intensity busulfan-based conditioning regimen which have been shown to be successful in achieving a lasting effective engraftment with limited toxicity [11, 15, 16].

However, this reduced-intensity busulfan-based conditioning may be insufficient in other forms of SCID like the Recombinase-Activating gene 1 and 2 (RAG1/2) SCID where there is a more prominent occupancy of BM niches by precursors B cells blocked in development. For this patient group, insufficient HSC engraftment resulting in poorer T- and B-cell reconstitution have been reported in the absence of conditioning. [17-19]. In RAG1/2 SCID, precursor B cells completely occupy bone marrow niches and strongly compete with transplanted cells leading to poor immune reconstitution [20, 21]. Therefore, to achieve proper engraftment of transplanted cells, a myeloablative regimen is required to empty precursor niches. Conditioning benefits should also be weighed against its short and long-term toxicity, especially in for instance Artemis deficiency with inherent radio-

sensitivity due to impaired DNA repair and in new-born patients [3, 6]. Accordingly, a critical balance for successful engraftment together with the risk of dose-limiting toxicities must be carefully considered and highlight the need to develop alternative non-/less genotoxic conditioning regimens.

Thus, although current conditioning agents are often successfully employed, there is a pressing need for alternative, less toxic conditioning regimens to create space in the BM niches for a durable engraftment of stem cells/HSC without adverse effects on extramedullary tissues. Development of effective, non-toxic, non-alkylating-based conditioning regimens are essential to ensure a successful transplantation and good quality of life in patients with SCID or related inborn errors. In SCID, where patients are predominantly less than 1 year-old at the time of treatment and where co-morbidities including viral infections, are frequently present, reducing conditioning-related toxicity and improving the rate of immune recovery are of great importance.

Hence, we here explored alternative approaches including the added value of clinically approved mobilizing agents like G-CSF (Granulocyte-Colony Stimulating Factor) or Plerixafor. G-CSF together with Plerixafor are used to mobilize HSCs from the BM niche to the bloodstream for HSC collection in autologous transplants. G-CSF mobilizes by impairing HSC niche function in the BM by suppressing niche-supportive cells and cytokines whereas Plerixafor (also known as AMD3100) directly targets HSC without altering HSC niche function by directly antagonizing the CXCR4-mediated sensing that retains HSCs within the BM [22]. Therefore, we studied whether combining chemotherapy regimens similar to those used in clinical setting with stem cell niche directed therapeutic agents (HSC mobilizing agents) would result in engraftment of transplanted progenitor cells with equivalent efficacy at lower chemotherapy exposure in comparison with current standard chemotherapy-based conditioning. With this aim we first assessed the efficacy and tolerability of busulfan conditioning in mice. Secondly, we examined the direct effect of the chemotherapy and the HSC mobilizing agents in the BM and the HSC niches. Finally, we analysed whether alternative low toxicity conditioning regimens allowed successful and equivalent immune reconstitution in NSG mice compared to high standard chemotherapy dose.

MATERIALS AND METHODS

Human CD34+ cell enrichment

Human cord blood was obtained according to the Medical Ethical Committee and IRB guidelines at Leiden University Medical Center. Cord blood mononuclear cells were separated by Ficoll (Pharmacy Leiden Academic Hospital) gradient centrifugation, frozen in fetal bovine serum (Hyclone)/10% DMSO (Sigma-Aldrich) and stored in liquid nitrogen. After thawing, human $CD34^+$ cells were isolated using CD34 MicroBead UltraPure Kit (Miltenyi Biotec). In short, cells were incubated with FcR blocking reagent and CD34 Microbeads Ultrapure following manufacturer protocol for 30 min at 4ºC. Subsequently CD34+ cells were positively selected using the appropriate ferromagnetic columns and the MACS separator (Miltenyi Biotec). Hematopoietic progenitor Stem Cells (HSPC) count

and purity after isolation was evaluated using a customized Flexicyte Program on NucleoCounter3000 (Chemometec). Directly isolated CD34+ cells were stimulated for 2 days in StemSpan serum-free expansion medium (StemSpan-SFEM; STEMCELL Technologies) supplemented with 10 ng/ml human Stem Cell Factor (huSCF; Miltenyi Biotec), 20 ng/ml human Thrombopoietin (huTPO; R&D Systems), 20 ng/ml recombinant mouse insulin-like growth factor 2 (IGF2; R&D Systems) and 10 ng/ml recombinant human fibroblast growth factor-acidic (hIFG1; PeproTech).

Murine HSPC isolation

Lineage negative depletion was performed using the Lineage Cell Depletion kit from Miltenyi Biotec, to isolate hematopoietic stem cell from frozen murine bone marrow. In short, cells were magnetically labelled with the Biotin-Antibody Cocktail and incubated for 10min at 4ºC and subsequently incubated for 15 minutes at 4ºC with the Anti-Biotin Microbeads. Lineage negative cells were subsequently depleted using the appropriate magnetic columns and the MACS separator (Miltenyi Biotec). Directly enriched HSPC were cultured in StemSpan (SFEM) medium supplemented with Pen/Strep (Gibco), 50ng/mL recombinant mouse (rm) Flt3L, 100ng/mL rmSCF and 10ng/mL rmTPO (all from R&D Systems) at 37˚C with 5%CO_2. Depletion efficiency and purity of lineage negative population was analyzed by flow cytometry with FACSCanto (BD).

Mice

BALB/c Rag2/Il2rg double-knockout mice were a kind gift from Dr. E.J. Rombouts from the Department of Hematology at Erasmus MC (University Medical Center Rotterdam, The Netherlands). C57BL/6 wild-type, BALB/c wild-type and NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were purchased from Charles River (Netherlands & France). Mice were bred and maintained in the animal facility of Leiden University Medical Center (LUMC). All animal experiments were approved by the Dutch Central Commission for Animal experimentation (Centrale Commissie Dierproeven, CCD).

Pre-conditioning of mice

Rag2$^{-/-}$ mice were conditioned with a total body single dose irradiation 24h prior the transplantation using orthovoltage X-rays (8.08Gy) or with two consecutive doses of 25 mg/kg Busulfan (1mg/ml; Sigma-Aldrich) (48h and 24h prior transplantation). NSG mice were conditioned with injected busulfan intraperitoneally, single dose (5mg/kg, 12,5mg/kg and 25mg/kg) 24h prior to cell transplantation or with 2 consecutive doses of 25 mg/kg Busulfan (48h and 24h prior transplantation) for the highest dose (50mh/kg).

HSPC mobilization was performed with G-CSF (Neulasta®, Amgen) up to a total dose of 125µg/kg. Mice were injected subcutaneously 2 consecutive days, 24h apart with the last injection 24h before the transplantation or analysis. Plerixafor (AMD3100, Sigma) was also used as HSC mobilization agent. A single dose of 10mg/kg was injected subcutaneously 1h prior transplantation or analysis. Pre-conditioning of NSG mice with the different regimens described in the paper (Busufan, G-CSF, Plerixafor and combinations) were weight-adjusted per mice.

HSPC transplantation

Cells were harvested and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Gibco) for intravenous injection into the tail veins of pre-conditioned mice. Human CD34+ enriched cells (100.000 cells per mice) were transplanted into 5-6 week old female NSG mice, while murine HSPCs (mixed with supportive $Rag2^{-/-}$ spleen cells ($3x10^6$ cells/mouse) and transplanted into pre-conditioned $Rag2^{-/-}$ recipient mice (8-12 week old mice).

Mice used for transplantation were kept in a specified pathogen-free section. The first four weeks after transplantation mice were fed with additional DietGel recovery food (Clear H2O) and antibiotic water containing 0.07 mg/mL Polymixin B (Bupha Uitgeest), 0.0875 mg/mL Ciprofloxacin (Bayer b.v.) and 0.1 mg/mL Amfotericine B (Bristol-Myers Squibb) and their welfare was monitored daily. Peripheral blood (PB) from transplanted mice was drawn by tail vein incision and analysed every 4 weeks until the end of the experiment (20 to 24 weeks after transplantation). PB, thymus, spleen and bone marrow were obtained from CO_2 euthanized mice.

Flow cytometry analysis

Single cell suspensions from thymus and spleen were prepared by squeezing the organs through a 70 µM cell strainer (BD Falcon). Bone marrow single cell suspension was obtained from flushed or crushed bones (femur and tibias) and cells were also passed through a 0,7 µm cell strainer (BD Falcon). Erythrocytes from PB and spleen were lysed using NH_4Cl (8,4 g/L)/$KHCO_3$ (1 g/L) solution (LUMC Apotheek). Mononuclear cells were counted and stained with the antibodies listed in Table S1. Briefly, cells were incubated for 30 min at 4°C in the dark with the antibody-mix solution including directly conjugated antibodies at the optimal working solution in FACS buffer (PBS pH 7.4, 0.1% azide, 0.2% BSA). After washing with FACS buffer, a second 30 min incubation step at 4°C was performed with the streptavidin-conjugated antibody solution. When necessary, 7AAD (BD Biosciences) was used as viability dye. Data was acquired on a FACS-CantoII and a LSR Fortessa X-20 (BD Biosciences) and analysed using FlowJO software (Tree Star).

Statistics

Statistics were calculated and graphs were generated using GraphPad Prism6 (GraphPad Software). Statistical significance was determined by one-way or two-way ANOVA test (*p<0.05, **p < 0.01, ***p < 0.001 and ****p<0.0001).

RESULTS

Busulfan conditioning as an alternative to TBI in mice

The standard pre-conditioning method in mice for hematopoietic stem cell (HSC) transplantation is total body irradiation (TBI), varying the irradiation dose depending on the mouse strain. $Rag2^{-/-}$ mice were transplanted with wild-type BALB/c hematopoietic and progenitor stem cells (HSPC) after conditioning with TBI (8,09Gy) or busulfan (50mg/kg) as previously published for immunodeficient mice [23, 24]. An improved welfare and well-being of the animals was observed for mice pre-conditioned with busulfan compared to TBI, with a lower loss of weight and a faster recovery after transplantation (**Figure 1A**).

The survival rate of busulfan-conditioned mice was higher than TBI treated mice (**Figure 1B**), where mice died from irradiation side effects which requires strict and careful animal support and can lead to high mortality rates [24]. In addition, busulfan-conditioned mice showed increased T-cell reconstitution from week 12 after transplantation, represented by a more significant population in the peripheral blood (PB) (**Figure 1C**). Although T-cell development in the thymus including all development stages was comparable between busulfan and TBI conditioned mice (**Figure 1D**), the T-cell output in PB at 20 weeks after transplantation was higher for busulfan-conditioned mice (**Figure 1E**). The immune outcome of busulfan-conditioned (50mg/kg dose) NSG transplanted mice was also comparable to TBI-treated NSG mice previously published [25].

Overall human engraftment, HSC engraftment in the bone marrow (BM) and immune cell distribution of mice pre-conditioned with 50mg/kg dose busulfan (**Figure 2A, 2B** and **2C**) matched TBI-conditioned reference values (horizontal black dot line). Busulfan conditioning may lead to better conservation of tissue integrity than TBI, allowing for a higher immune output after transplantation, mainly seen in the T-cell compartments. Importantly, the welfare and well-being of the animals were improved, without compromising the overall immune recovery. Therefore, busulfan conditioning represents a favourable regimen to use in pre-clinical studies in mice, bringing the model a step closer towards mirroring clinical protocols.

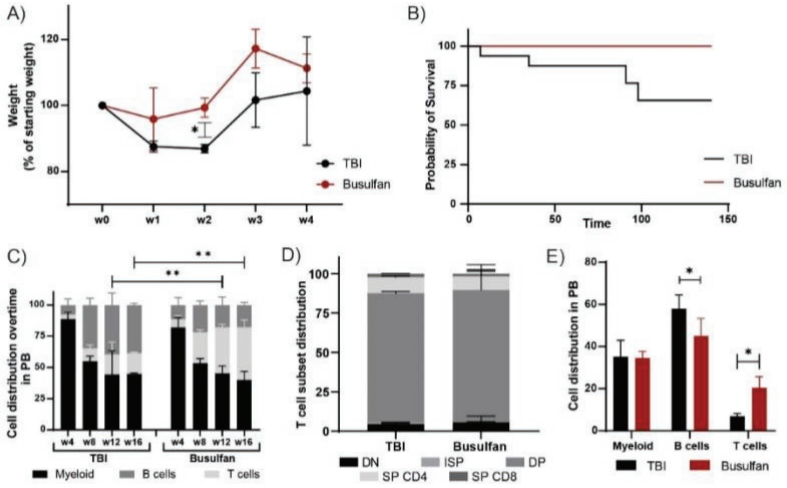

Figure 1: Busulfan conditioning as an alternative of TBI in immunodeficient mice. *Rag2-/- mice transplanted with wild-type BALB/c HSPCs were pre-conditioned by total body irradiation (TBI, 8,09 Gy) or busulfan (50mg/kg). Immune reconstitution was analysed up to 20 weeks after transplantation. A) Mice were weighted weekly during the first month after transplantation. Change of weight normalized to the starting weight before conditioning is depicted in the graph for TBI (2 mice) and busulfan (3 mice) treated mice. (Unpaired t-test; *p<0,05). B) Survival analysis of the TBI-conditioned (16 mice from historical data) and busulfan-conditioned mice after transplantation (4 mice). C) Cell distribution (myeloid, B and T cell) in peripheral blood (PB) over time of mice pre-conditioned with TBI (2 mice) or busulfan (4 mice). (two-way ANOVA; **p<0,01). D) Proportion of the different T-cell*

*developmental subsets in the thymus after TBI or busulfan conditioning. E) Cell distribution (myeloid, B and T cells) in PB 20 weeks after transplantation. (two-way ANOVA; *p<0,05).*

Modelling busulfan conditioning in NSG mice; determining a suitable dose

We first focused on setting the optimal busulfan dose in NSG mice to investigate the possibility of reducing busulfan conditioning before HSCT to reduce associated side effects. A dose of 50mg/kg busulfan was used as starting dose [23, 26-28] reducing it gradually until 5mg/kg. Mice were pre-conditioned with different doses of busulfan (control without busulfan, 5mg/kg busulfan, 12,5mg/kg busulfan, 25mg/kg busulfan and 50mg/kg busulfan as described in Material & Methods) and transplanted intravenously with $1x10^5$CD34+ cells/kg isolated from cord blood (5mice/group). Human chimerism and human immune cell reconstitution were followed up to 20 weeks after transplantation (**Suppl. Figure 1**). Mice were sacrificed and immune organs were thoroughly analysed for human HSC engraftment and human B- and T-cell development. Increasing levels of human chimerism were observed in PB, spleen and BM with increasing busulfan doses, with a significant increase in the group receiving the maximum dose (50mg/kg) compared to the control group and the lower 5mg/kg and 12,5mg/kg doses (**Figure 2A**). As NSG thymi are devoid of murine cells, human engrafted cells completely repopulated the thymus in all dosing groups, showing close to 100% human chimerism in this organ. Although comparable number of human HSC engrafted in BM across the groups (**Figure 2B**), the distribution of immune cell lineages in PB, mainly B and T cells, significantly differed for the highest dose compared to other groups, leading to a higher T-cell contribution (**Figure 2C**). All busulfan doses contributed to an overall normal B cell development in BM (**Figure 2D**) and T cell development in the thymus (**Figure 1F**) with a normal population distribution over the developmental stages. However, significantly higher B-cell (**Figure 1E**) and T-cell (**Figure 1G**) numbers were detected in the periphery (spleen and PB) with the highest dose, while following more moderate doses, immune output was comparable to that of control transplanted mice.

High dose busulfan (50mg/kg) gave reliably higher immune reconstitution and was set as high dose group for the following experiments. Consistent immune development and chimerism were detected for lower busulfan doses, and therefore we set the 12,5mg/kg dose as our low dose busulfan for following experiments where we aimed to improve our low dose busulfan immune outcome by combining with stem cell niche directed non-chemotherapeutic agents.

Short-term effect of busulfan and mobilizing agents on BM HSCs

The principal purpose of conditioning is to make space in BM before transplantation to improve HSC engraftment and immune recovery. Our aim was to reduce the dose of busulfan used, without compromising immune recovery, by combining a low dose busulfan with mobilizing agents. G-CSF (Granulocyte Colony-Stimulating Factor) and Plerixafor are clinically used mobilising agents to collect HSC cells directly from PB instead of BM. We therefore investigated the effect of busulfan, G-CSF and plerixafor as single agents, and G-CSF or plerixafor in combination with low dose busulfan on the HSC compartment of NSG mice (3 mice/group) 24h after the last injection of G-CSF and Busulfan and 1h after Plerixafor. High dose busulfan resulted in a significant reduction of total BM cells (**Figure**

3A). Spleen cell numbers and viability were also significantly compromised with the highest dose of busulfan (**Figure 3B**). In addition, only the high dose busulfan showed a reduction of the HSPC population (named LSK in mouse; lineage-Sca1+ckit-) in NSG mice (**Figure 3C**), mostly explained by the decrease of hematopoietic progenitor cells (HPC; **Lin-**Sca1+cKit+ CD48+**)** and to a lesser extent multipotent progenitor cells (MPP; Lin-Sca1+cKit+CD150-CD48-**)** in BM but no long-term HSCs (Lin-Sca1+cKit+CD150+CD48-) (**Figure 3D**).

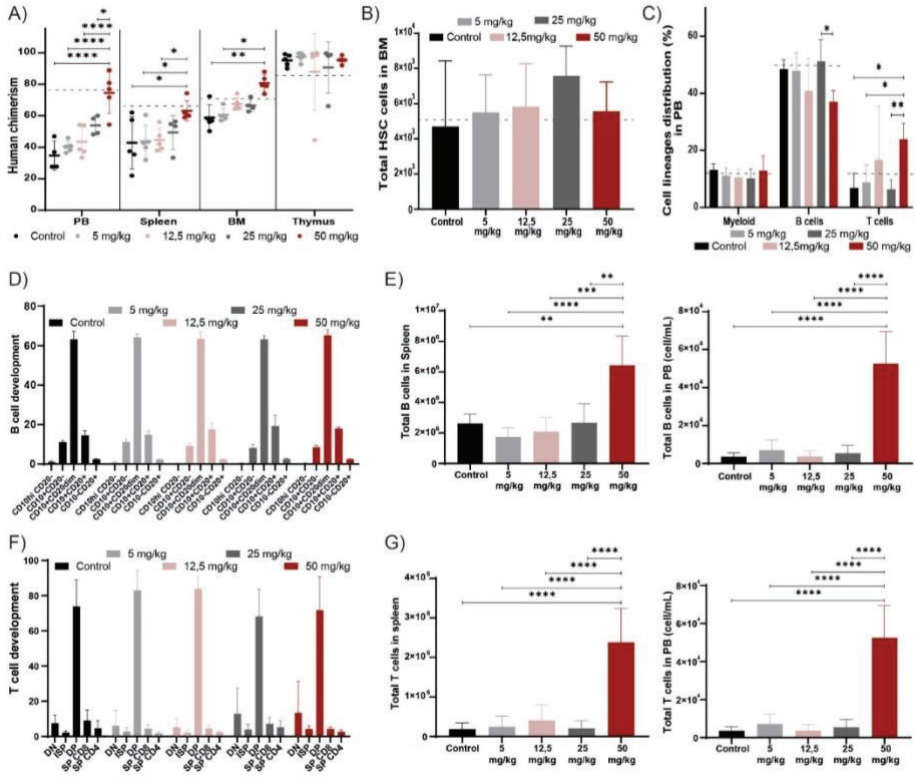

*Figure 2: Modelling busulfan conditioning in NSG mice, determining a suitable dose. NSG mice were pre-conditioned with increasing doses of busulfan (control, 5mg/kg, 12,5mg/kg, 25mg/kg and 50mg/kg) and transplanted with 100.000 human CD34 cells (5 mice/group). A) Human chimerism (% hCD45 cells) achieved in PB, spleen, bone marrow (BM) and thymus 20 weeks after transplantation. Human chimerism achieved by TBI represented by dashed line. (2-way-ANOVA; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001). B) Total number of human hematopoietic stem cells (HCS) in NSG BM 20 weeks after transplantation. C) Cell lineage distribution (myeloid, B and T cells) in PB 20 weeks after transplantation of the different conditioned groups. (2-way-ANOVA; *p<0,05, **p<0,01). D) Proportion of B cell developmental stages in BM in the different busulfan treated mice. E) Total B cell counts in spleen and in PB 20 weeks after transplantation. (One-way ANOVA; **p<0,01, ***p<0,001, ****p<0,0001). F) Proportion T cell developmental stages in the thymus in the different busulfan treated mice. G) Total T cell counts in spleen and in PB 20 weeks after transplantation. (One-way ANOVA; ****p<0,0001).*

The mobilizing efficiency to peripheral blood of G-CSF and Plerixafor was tested on NSG mice (3 mice/group) as previously published for different mouse strains and with doses adjusted to the NSG mouse strain (**Suppl. Figure 2**) [22, 29-31]. An increased HSPC (LSK) population was detected in PB of NSG mice treated with G-CSF (total 250µg/kg) or Plerixafor (10mg/kg) 24h or 1h after the last injection respectively (**Figure 3E**). In addition, in accordance with Winkler et al (2012) [22] the counts in PB highly increases after G-CSF administration due to the increased release of myeloid cells to the periphery (**Suppl. Figure 2A**). Knowing that G-CSF and Plerixafor are able to mobilise HSPCs in NSG mice, we analysed their effect directly in the BM. G-CSF alone or in combination with the low dose busulfan had no impact on BM cellularity (**Figure 3F, upper graph**). However, significant decrease of the HSPC (LSK) compartment was observed after G-CSF treatment, even more prominent than the decreased induced by the high dose busulfan (**Figure 3F, middle graph**). As for high dose busulfan, this decreased was mainly explained by a reduction of the progenitor compartment, but not of long-term HSCs (**Figure 3F, lower graph**). In contrast, total BM cells were reduced by Plerixafor comparable to high dose busulfan dose (**Figure 3G, upper graph**). Although no significant decrease of the total HSPC (LSK) population was detected, an interesting but nor significant reduction of the long-term HSCs as well as MPPs was observed in mice treated with the combination of plerixafor and low dose busulfan (**Figure 3G, lower graph**).

In summary, high dose busulfan and G-CSF administration alone showed consistent reduction in the number of progenitor cells in BM. However, while the low dose busulfan did not impact the HSPC population in BM, interesting effects were observed when combined with plerixafor, the only condition agent leading to a potential reduction of long-term HSCs**.**

Long-term immune recovery after reduced busulfan conditioning

Finally, we aimed to study if the direct effects of the different conditioning regimens on the cellular composition of the BM would also lead to better engraftment *in vivo* after CD34 transplantation. NSG mice (5 mice/group) were pre-conditioned with different conditioning regimens (low dose busulfan, high dose busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan) and transplanted with $1x10^5$ CD34/kg enriched cells from cord blood. As previously described, human chimerism increased with increasing busulfan dose. Combining low dose busulfan with either of the mobilizing agents did not increase human chimerism, achieving similar engraftment as with low dose busulfan only. In addition, G-CSF or Plerixafor alone yielded lower human chimerism in BM (**Figure 4A** & **Suppl. Figure 3**). However, no significant differences in the number of human HSC engrafted cells in BM was detected across the conditions (**Figure 4B**). B-cell development in BM was consistent across all conditions (**Figure 4C**), however a lower number B cells was observed in spleen of mice conditioned with the single mobilizing agents. In addition, no difference was observed between the combinations and the low dose busulfan group (**Figure 4D**). In parallel, T-cell development in the thymus was uniform across all conditions, both in early (**Figure 4E**) and late developmental stages (**Figure 3F**). The T-cell output, both CD4+ and CD8+ T cells, was significantly lower after

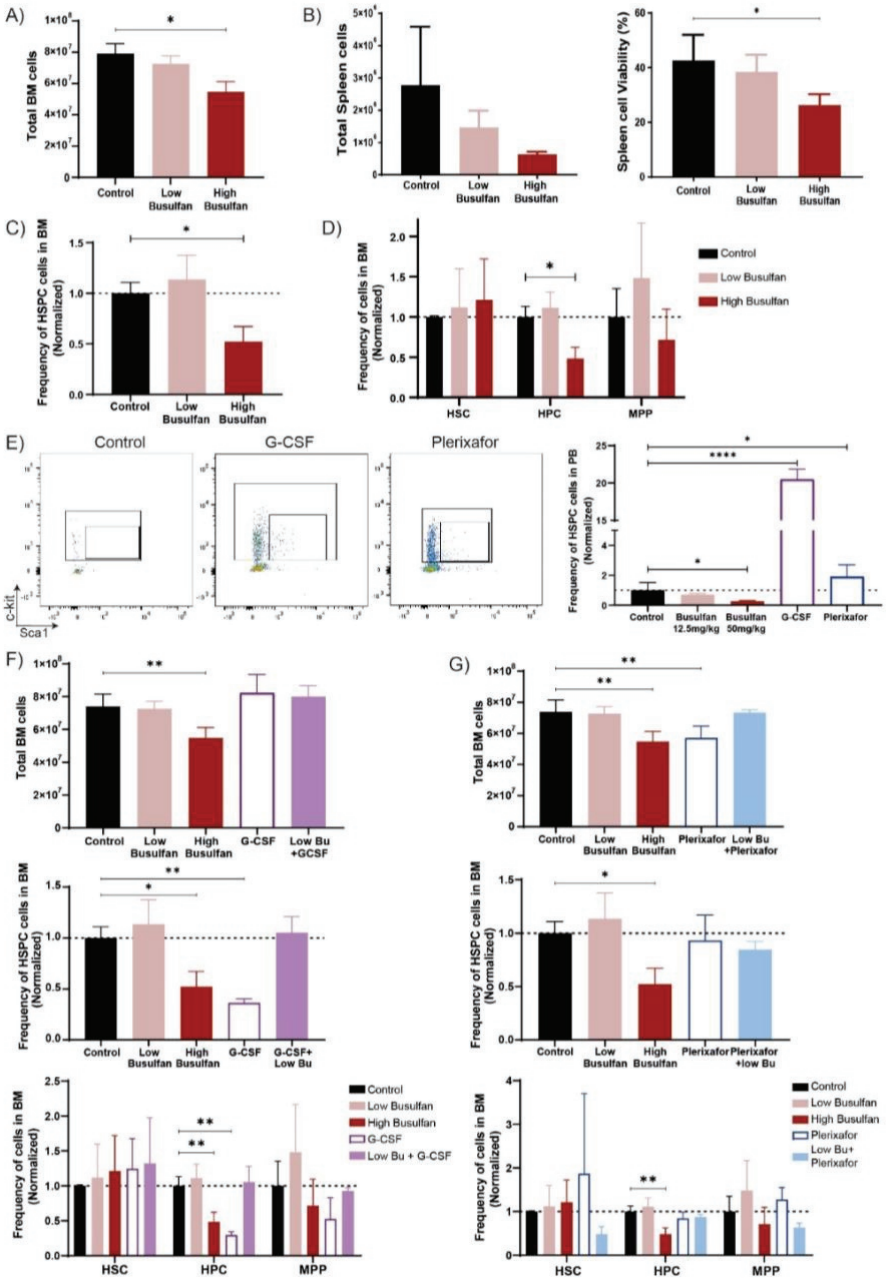

Figure 3: Effect of busulfan and mobilizing agents on BM HSCs. *A) Total BM cell numbers, 24h after busulfan conditioning (low and high dose) compared to the control group (without busulfan). (One-way ANOVA, *p<0,05). B) Total spleen cell numbers and cell viability after busulfan conditioning (24h after). (One-way ANOVA; *p<0,05). C) Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after busulfan conditioning, normalized to control mice. (One-way ANOVA; *p<0,05). D) Frequency of long-term HSCs (Lin-Sca1+cKit+CD150+CD48-), hematopoietic progenitor cells (HPC;*

*Lin-Sca1+cKit+CD48+) and multipotent progenitor cells (MPP; Lin-Sca1+cKit+CD150-CD48-). (2-way-ANOVA; *p<0,05). E) Representative FACS plots of PB HSPCs after mobilizing agents injection. G-CSF was measured 1 day after the last injection and Plerixafor 1h after injection. Quantification of HSPCs in PB is depicted in the graph. (One-way ANOVA; **p<0,01, ***p<0,001). F) Mice were conditioned with busulfan (low and high dose), G-CSF or the combination G-CSF+low dose busulfan and analysed 24h after the last injection (3 mice/group). Upper graph: Total BM cells count after conditioning. (One-way ANOVA; **p<0,01). Middle graph: Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after conditioning, normalized to control mice. (One-way ANOVA; *p<0,05, **p<0,01). Lower graph: Frequency of long-term HSCs, HPC and MPP cells. (2-way-ANOVA; **p<0,01). F) Mice were conditioned with busulfan (low and high dose), Plerixafor or the combination Plerixafor+low dose busulfan and analysed after the last Busulfan injection or 1h after Plerixafor administration (3 mice/group). Upper graph: Total BM cells count after conditioning. (One-way ANOVA; **p<0,01). Middle graph: Frequency of HSPCs (LSK; Lin-Sca1+cKit+) cells in BM 24h after conditioning, normalized to control mice. (One-way ANOVA; *p<0,05). Lower graph: Frequency of long-term HSCs, HPC and MPP cells. (2-way-ANOVA; **p<0,01).*

single G-CSF or Plerixafor conditioning and no improvement was observed with the combinations compared to using only low dose busulfan (**Figure 4G**). Only an enhanced naive T-cell compartment, most prominent for CD8+ naive cells, was detected by combining plerixafor with a low dose busulfan (**Figure 4H**).

Hence, single mobilizing agents did not yield sufficient immune reconstitution in NSG mice by themselves. In addition, the combination of a low dose busulfan with mobilizing agents did not reveal additive effects, and reconstitution efficiency was primarily driven by busulfan. Only the naïve T-cell compartment seemed to be boosted by Plerixafor. None of the novel combinations reached high dose busulfan reconstitution levels. However, Plerixafor apparently could have more impact on the lymphoid progenitors than on the myeloid which could be interesting to further investigate from a clinical perspective.

DISCUSSION

The NSG mouse model is suitable to study *in vivo* detection and quantification of human HSCs and human immune cells, and can therefore be used to evaluate the effects of stem cell based therapies. Pre-conditioning of mice prior to human HSCT is important to ensure successful homing and HSC development. In murine pre-clinical experiments, the most commonly used conditioning regimen is based on total body irradiation (TBI; x-rays or γ-rays). However, the irradiation procedure induces high stress levels and intestinal damage in the mice, and leads to weight loss and potentially death of the animal in some occasions. Therefore, it is critical to maintain irradiated mice under strict aseptic conditions and continuous health control. In addition, mice can absorb different doses of irradiation depending on their weight and position during the procedure resulting in a heterogenous group of conditioned mice. Alternative conditioning with chemotherapy like busulfan which is commonly used in human HSCT, represents a suitable alternative offering simple, convenient, individual, weight-adjusted and less-toxic conditioning regimen. Busulfan is indeed an attractive and effective alternative conditioning model that allows an improved human immune reconstitution and better well-being and survival of the mice, which is highly important when working with precious patient material.

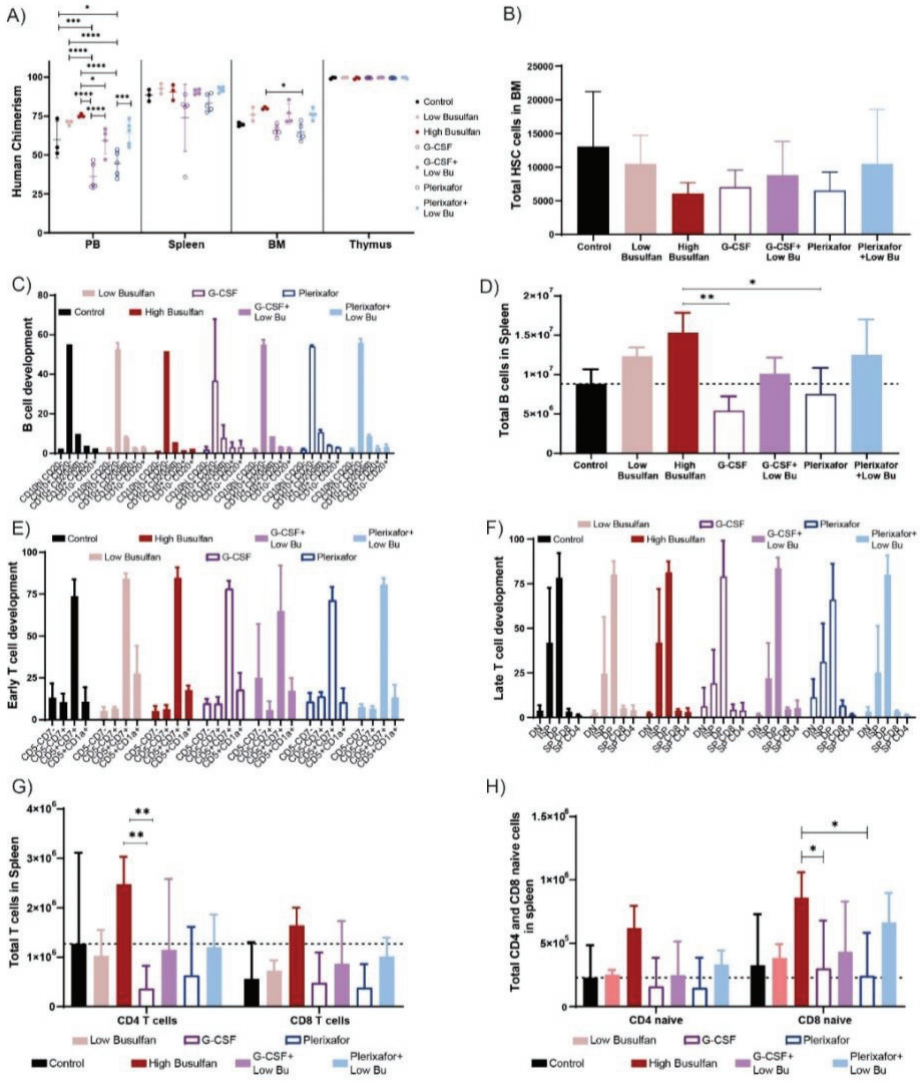

Figure 4: Long-term immune recovery after reduced busulfan conditioning*. NSG mice (5 mice/group) were pre-conditioned with different conditioning regimens (low dose busulfan, high busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan) and transplanted with 100.000 CD34 enriched cells from cord blood. A) Achieved human chimerism (% hCD45 cells) in PB, spleen, bone marrow (BM) and thymus 20 weeks after transplantation. (2-way-ANOVA; *p<0,05, **p<0,01, ***p<0,001, ****p<0,0001). B) Total number of human hematopoietic stem cells (HCS) in NSG BM 20 weeks after transplantation. C) Proportion of B cell developmental stages in BM in the different conditioned regimen groups. D) Total B cell counts in spleen 20 weeks after transplantation. (One-way ANOVA; *p<0,05; **p<0,01). E and F) Proportion T-cell developmental stages (early and late) in the thymus in the different conditioning regimen groups. G) Total T-cell numbers (CD4+ and CD8+ cells) in spleen 20 weeks after transplantation. (2-way*

*ANOVA; **p<0,01). H) Total naïve T-cell numbers (CD4+ and CD8+ cells) in spleen 20 weeks after transplantation. (2-way ANOVA; *p<0,05).*

Although previous groups already set the most suitable dose of busulfan to condition NSG mice [23, 24, 27, 28], we present here a more extensive analysis of the thymus and T-cell development, leading to higher T cells in the periphery after busulfan compared to TBI as identified also by Choi et al [24]. A more preserved and less damaged thymic tissue after busulfan conditioning compared to TBI may explain the higher T-cell outcome observed. While busulfan may have a more targeted effect on BM, TBI is a general therapy causing damage in thymic and lymphoid tissue that will impact T-cell output. A dose of 50mg/kg busulfan (split in 2 doses 24h apart) provides optimal human cell engraftment not only in NSG mice, but also for other immunodeficient mice like Rag2-/- or Rag1-/- [32]. Normal human B-and T-cell development was obtained also with lower doses of busulfan, but the output of B- and T-cells in the periphery was dose dependent. Chevaleyre et al (2013) [23] described that although increasing human CD45 chimerism was observed with increasing doses of busulfan (as we also described), no impact on the number of colony-forming cells was detected, which would explain that B- and T- cell developmental pattern we observed across the conditions. The direct effect of busulfan and mobilizing agents used in this study (G-CSF and Plerixafor) on BM and HSPC population was analysed in NSG mice. To the best of our knowledge, G-CSF and Plerixafor have not been used previously in the NSG mouse model. Therefore, G-CSF and Plerixafor doses were derived from published literature on other mouse strains [22, 29, 31] and the HSC mobilizing capacity was analysed on PB of the NSG mice. NSG mice show a significant capacity to mobilize HSC to the periphery after G-CSF or Plerixafor administration. While busulfan and G-CSF affect more mature progenitor populations such as HPC and MPP in BM, Plerixafor boosts the reduction in BM and increases mobilization of long-term HSCs.

Although interesting effects on different HSPC populations were observed in BM shortly after administration, the longer term human cell engraftment and immune development after CD34 transplantation did not reflect that direct effect. G-CSF and Plerixafor alone allowed appropriate immune development as described previously by Huston et al [29]. However, when combined with low dose busulfan, no additive effect was observed between the mobilizing agents and the chemotherapy. The main parameters of chimerism and immune development observed in the combination groups were comparable to the low dose busulfan group, meaning that immune reconstitution was triggered by the chemotherapy conditioning rather than the non-chemotherapy agents. Only the naive T-cell compartment tended to be improved by the addition of plerixafor to low dose busulfan, which can be caused by the effect of plerixafor on the long-term HSC cells in BM. More extensive pharmacokinetics and pharmacodynamics studies of busulfan, G-CSF and Plerixafor in NSG mice will help to select the most suitable doses and timings to ensure a proper model for humanized mice. As G-CSF and Plerixafor are clinically approved as mobilizing agents, a small trial with patients has been already performed where patients were pre-conditioned with myeloablative regimen together with G-CSF and Plerixafor prior transplantation. No suitable engraftment was achieved with minimal myeloablative regimen [33], however the addition of G-CSF and Plerixafor to TCRαβ+/CD19+-depletion

regimen appears to solve the problem of graft failure after HSCT, with no additional risks of toxic complications and associated morbidity [34].

To note, the conditioning field is moving towards antibody-based conditioning that will target and potentially deplete stem cells without causing off-target toxicity. Antibody-based conditioning regimens are being developed, which may ultimately achieve long-term myeloid engraftment without the associated toxicities of current chemotherapy-based regimens. Different variations of antibody-based conditioning are being tested both pre-clinically and clinically, such as antibody-drug conjugates specifically targeting HSPCs. Antibody-drug conjugates (ADC) like CD177-ADC [35-37] or CD45-ADC [38-42] have proven to be a safer conditioning regimen than conventional chemotherapy in pre-clinical models. In addition, monoclonal antibodies targeting CD117 [43-46] have been successfully developed and paved the way for the use of anti-CD117 antibody in a currently ongoing clinical trial (NCT02963064). Less toxic and more directed conditioning regimens are needed to improve outcome of all allogeneic and autologous gene therapy stem cell transplantations. The possible implications of these improvements are substantial and could potentially impact allogeneic and autologous transplants worldwide.

SUPPLEMENTARY MATERIAL

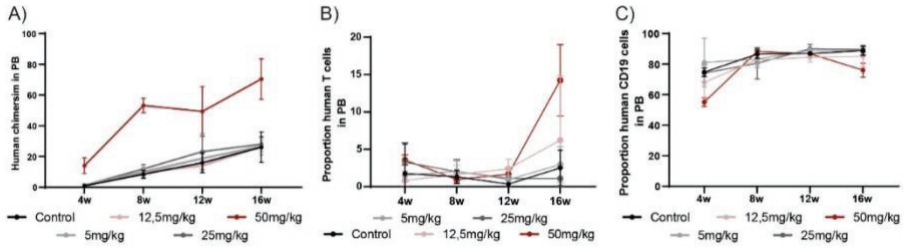

***Figure S1: Human immune reconstitution kinetics after different busulfan dose conditioning in NSG mice**. A) Human chimerism kinetics, B) human T-cell development kinetics and C) Human B-cell development kinetics over time in PB of CD34 transplanted NSG mice after pre-conditioning with different doses of busulfan (Control, 5mg/kg, 12,5mg/kg, 25mg/kg and 50mg/kg).*

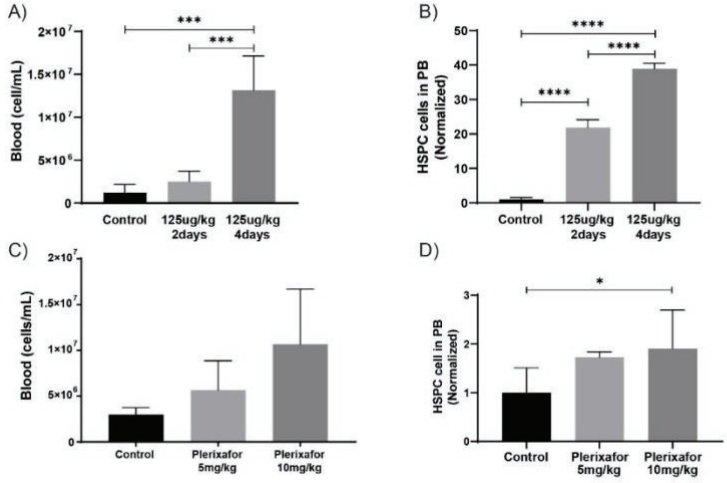

***Figure S2: Mobilizing agents (G-CSF and Plerixafor) dosage in NSG mice.** Effect of different doses of G-CSF in mice A) PB cell numbers and B) HSPC mobilization capacity. Effect of different doses of G-CSF in mice C) PB cell numbers and D) HSPC mobilization capacity. (One-way ANOVA; *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001).*

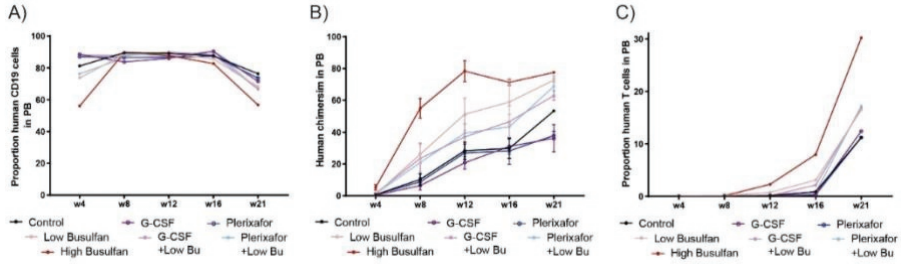

***Figure S3: Human immune reconstitution kinetics after conditioning regimens in NSG mice.** A) Human chimerism kinetics, B) human T-cell development kinetics and C) human B-cell development kinetics over time in PB of CD34 transplanted NSG mice after pre-conditioning with*

different regimens (Control, low dose busulfan, high busulfan, G-CSF, G-CSF+low dose busulfan, Plerixafor and Plerixafor+low dose busulfan).

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