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Non-Myelotoxic Agents as a Preparatory Regimen for Hematopoietic Stem Cell Gene Therapy

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Abstract

RAG2 deficiency is characterized by a lack of B and T lymphocytes, causing severe lethal infections. Currently, RAG2 deficiency is treated with a Hematopoietic Stem Cell transplantation (HSCT). Most conditioning regimens used before HSCT consist of alkylating myelotoxic agents with or without irradiation and affect growth and development of pediatric patients. Here, we developed a non-myelotoxic regimen using G-CSF, VLA-4I or AMD3100. These agents are known HSC mobilizers or affect bone marrow (BM) permeability and may support the homing of HSCs to the BM, without inducing major side effects. Female *Rag2*$^{-/-}$ mice were pre-treated with Busulfan (BU), G-CSF, VLA-4I or AMD3100 and transplanted with male BM cells transduced with a lentiviral vector carrying codon optimized human RAG2 (RAG2co). Peripheral blood cell counts increased significantly after G-CSF, VLA-4I and AMD3100 treatment, but not after BU. Reconstitution of PB lymphocytes was comparable for all groups with full immune reconstitution at 6 months post transplantation, despite different methods of conditioning. Survival of mice pre-treated with non-myelotoxic agents was significantly higher than after BU treatment. Here we show that the non-myelotoxic agents G-CSF, VLA-4I, and AMD3100 are highly effective as conditioning regimen before HSC gene therapy and can be used instead of BU.

Keywords

RAG2; Hematopoietic Stem Cell Transplantation; Conditioning, Gene Therapy; G-CSF; VLA-4I; AMD3100

Introduction

Severe Combined Immune Deficiency (SCID) is an inherited primary immunodeficiency (PID), characterized by the absence or dysfunction of lymphocytes and affects both cellular and humoral adaptive immunity (1, 2). The main symptoms of SCID are persistent or recurrent infections with or without growth-development retardation. If left untreated, patients die in the first year of life due to severe, uncontrollable infections (3, 4). RAG2 (Recombination activating gene 2) SCID is characterized by a lack of both mature B and T lymphocytes, causing severe viral and bacterial infections (1, 3-7). Currently, the only curative treatment for RAG2 SCID is Hematopoietic Stem Cell (HSC) transplantation (HSCT) (8, 9). However, in the absence of a suitable donor, there is no other treatment option, except for supporting treatments for RAG2 SCID. Recently, successful retroviral and/or lentiviral (LV) hematopoietic stem cell gene therapies (HSC-GTx) have been developed or are under development for other SCIDs, such as adenosine deaminase (ADA) SCID and X-linked SCID (X-SCID) (8-12). Thus far several lentiviral constructs that have been developed using codon optimized RAG2 sequences to treat RAG2 SCID show potential for clinical development (13, 14).
Usually, the conditioning regimen applied to patients before HSCT for treatment of malignancies or inherited genetic diseases consists of a combination of alkylating myelotoxic agents or Fludarabine with or without radiation in order to eradicate malignant cells and/or knockout the remaining immune system (15). However, use of myeloablative conditioning (MAC) regimens in children has been shown to affect growth and may result in developmental retardation, infertility, organ damage and secondary malignancy (16-18). Since SCID patients are mostly young children with an already severely weakened immune system, most preparatory regimens for HSCT or HSC-GTx for SCIDs consist of reduced intensity conditioning (RIC) protocols (19, 20). The RIC-based regimens are largely used to open up space in the bone marrow and thymic niches to allow proper engraftment of healthy BM cells and has been associated with a better immune reconstitution 1 year after transplant (21) and with an overall better survival and prognosis than MAC (22). Furthermore, complete absence of any form of preparatory conditioning has been shown to result in inadequate or unbalanced engraftment (22, 23), worse immune reconstitution, but less acute Graft versus host disease (aGvHD) (24). In addition, for SCID patients with a RAG deficiency, who may still have some residual NK cell function, it may be impossible to completely abolish a pre-transplant preparatory regimen (25). Therefore, there is a need to develop new preparatory regimens that support adequate engraftment and immune reconstitution, but that induce minimal toxicity.
Here, we aimed to develop a non-myelotoxic regimen that can be used as preparatory conditioning before HSCT or HSC-GTx, using different agents that are known HSC mobilizers or have otherwise been shown to affect BM permeability. We hypothesized that increased BM permeability may not

only result in mobilization of stem cells to the peripheral blood, but that this may also support the homing of intravenously infused (transduced) HSCs back to the BM niche. In a previous study, we showed that transplantation of Il2Rγ$^{-/-}$ knockout mice pre-treated with G-CSF or a low dose of total body irradiation (2.0 Gy) was sufficient for engraftment of healthy Balb/c Lin- BM cells or Il2Rγ$^{-/-}$ Lin- cells transduced with therapeutic IL2Rγ lentiviral vectors without inducing any treatment related harm (26). In addition, we found low chimerism in the absence of any conditioning, but similar levels of chimerism after G-CSF or low dose TBI. Furthermore, whereas T cell reconstitution was observed in all treatment groups, B-cell reconstitution was 3-fold increased after G-CSF conditioning in comparison to saline and almost as high as in irradiated mice.

In this study, we used granulocyte colony stimulating factor (G-CSF), which has been routinely used for therapeutic mobilization of HSCs (27, 28); AMD3100 (Plerixafor), which has been shown to mobilize HSCs by antagonizing CXCR4 at the CXCR4/CXCL12 junction (29, 30); and a VLA4 inhibitor (Very late antigen 4 inhibitor, BIO5192), as a preparatory conditioning regimen in a mouse model of RAG2 deficiency (13). VLA4, together with vascular cell adhesion molecule-1 (VCAM-1) plays an important role in anchoring HSC progenitors to BM stromal cells and regulates HSC traffic between the BM and peripheral regions (31, 32). Inhibition of VLA4 has been shown to mobilize HSCs by interruption of the VCAM-1/VLA-4 axis (33). After conditioning, we transplanted the *Rag2*$^{-/-}$ mice with either healthy Balb/c Lin-/- BM cells or RAG2 expressing, lentivirally corrected c-Kit+ HSCs and assessed engraftment, immune reconstitution, and survival.

Methods

Animals

Healthy Balb/c mice were purchased from the Ankara University Experimental Animals Laboratory. *Rag2*$^{-/-}$ mice were kindly provided by Prof. Dr. Gerard Wagemaker (Erasmus University Medical Center, Rotterdam, The Netherlands) (13). All mouse studies were carried out at the Hacettepe University Experimental Animals Application and Research Center after approval by the Hacettepe University Animal Experiments Ethical Committee (2020/06-02).

Hematopoietic Stem Cell isolation and characterization

BM from male *Rag2*$^{-/-}$ mice was collected and lineage depletion was performed using a Lineage Depletion kit (Miltenyi Biotech, #130-090-858), followed by positive selection for c-Kit (Miltenyi Biotec, #130-091-224). The c-Kit+ positive fraction was labeled using anti-c-Kit antibody

(BioLegend, #105812) and anti-Sca-1 antibody (BioLegend, #108116), measured using a BD Accuri™ C6 Plus (Becton Dickinson) and analyzed using the BD CSampler software.

Construction of lentiviral vector plasmids and lentiviral transduction

Human codon-optimized RAG2 was expressed in the pRRL.PPT.UCOE.hRAG2co.bPRE4*.SIN lentiviral vector (briefly, LV-hRAG2co), as previously described (13, 34) and contained the silencing resistant UCOE promoter (35, 36). LV production was done according to standard procedures (13, 34). For LV transductions, 10^5 c-Kit+ $Rag2^{-/-}$ BM cells were incubated overnight in StemSPAN HSC expansion medium (Stem Cell Technologies, #09650), with 30 ng/mL recombinant mTPO (Peprotech, #315-14) at an MOI of 30. Healthy Balb/c c-Kit+ BM cells subjected to the same culture conditions in the absence of virus were used as a positive control in the transplantation assays.

Measurement of Rag2 protein levels and confirmation of genomic integration

Flow cytometry: Cells were fixed with 90% MeOH and incubated with blocking buffer (3% BSA + PBS). Cells were incubated with rabbit-anti-RAG2 primary (Proteintech: #11825-1-AP) antibody for 2 hours and with a secondary AF488-conjugated anti-rabbit-antibody (Abcam: #ab150077) for 1 hour. Protein expression was analyzed using a BD Accuri™ C6 Plus.

Immunofluorescent staining: Control and transduced c-Kit+ cell smears were fixed with 4% paraformaldehyde for 10 minutes, followed by permeabilization with 0,2% Triton X-100 (BioRad #1610407). Cells were incubated with blocking buffer and stained with 1:25 rabbit-anti-RAG2 primary antibody for 2 hours. After washing, cells were incubated with 1:1000 diluted secondary goat-anti-rabbit IgG-AF568 (Abcam #ab175471) for 45 minutes and counterstained with 5 µg/mL DAPI (Sigma #D8417). Photographs were taken using an Olympus Inverted Fluorescent Microscope.

qRT-PCR: RNA isolation was performed using the Direct-zol RNA Miniprep kit (Zymo research, #R2050) with an additional DNAse step. cDNAs were prepared using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) and followed by RT-qPCR for hRAG2co using a LightCycler 480 II (Roche). Measurement of Gadph expression was used as internal control. Changes in expression were calculated using the ($2^{-\Delta\Delta Ct}$) method.

qPCR: Genomic DNA was isolated from the spleens of transplanted female mice using the FastPure® Cell/Tissue DNA Isolation Mini Kit (Vazyme Biotech #DC102) and assessed for integration sequences (wPRE, HIV) of the transfer vector, as well as for hRAG2co to assess engraftment of transduced hematopoietic stem and progenitor cells. Sry sequences were used to assess total engraftment of male cells in the female recipients using qPCR (Roche #50-720-3180). All primer sequences used, are shown in Table S1.

Conditioning regimens

Female *Rag2*$^{-/-}$ mice aged 3-6 months received different conditioning regimens prior to transplantation (Figure S1) and were divided into 4 groups: 1) 25 mg/kg BU intraperitoneal (i.p.), day -1 (Sigma, #BCBS8240V); 2) 6 µg/kg/d G-CSF subcutaneous (s.c.) from day -4 to -1 (Dong-a St Co. Ltd, Leucostim 30); 3) 1 mg/kg VLA-4I intravenous (i.v.) (BIO5192, Selleck Chemicals, #S7198) 1 hour before transplant and; 4) 1 mg/kg AMD3100 s.c. (Cayman, #10011332) 1 hour before transplant. PB white blood cell numbers (n=4-6) were manually counted before and after treatment.

To assess the direct effect of the non-myelotoxic conditioning regimens on engraftment with healthy Balb/c BM cells, the female *Rag2*$^{-/-}$ mice were transplanted with 1x10^6 lineage depleted (Lin-) male BM cells. To assess the effect of the non-myelotoxic conditioning on engraftment of genetically corrected cells, female *Rag2*$^{-/-}$ mice were transplanted with 1x10^5 LV-hRAG2co transduced male *Rag2*$^{-/-}$ c-Kit+ BM cells or healthy non-transduced Balb/c c-Kit+ BM cells, maintained under the same culture conditions. Expression of CD45 (BioLegend, #147708), CD3 (BioLegend, #100204), CD19 (BioLegend, #152408), CD11b (BioLegend, #108706), CD45R (BioLegend, #103212) and NK1.1 (BioLegend, #108706) in PB was measured at 1 and 3 months after transplantation. Spleen and BM cellularity and cellular content (CD45, CD3, CD19, CD11b, CD45R, NK1.1, c-Kit, and Sca-1) were measured 6 months post-transplant. Total BM CFU-c counts (including CFU-GEMM, CFU-GM and BFU-E) were assessed before and after transplantation using Methocult Classic (Stem Cell Technologies, #M3434).

In vitro spleen cell proliferation assay

Spleen cells of mice sacrificed at 6 months were stained with Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE, ThermoFisher Scientific #C34554). Briefly, cells were incubated with 10 µm CFSE for 15 min at 37°C. Cells were seeded at 1x10^6 cells/well in 24 well plates in presence of 5 µg/mL Phytohemagglutinin (PHA-P, Sigma Aldrich #L1668) and 5 ng/mL human interleukin-2 (hIL-2, Immunotools #11340025) and analyzed by FACS after 4 days.

Statistical Analysis

Significant differences between 2 groups were calculated with Student's T-test and ANOVA for comparison of >2 groups using Microsoft Excel 365 (Microsoft, Redmond, WA). A p-value <0.05 was considered statistically significant. Survival rates of mice treated with different conditioning regimens were evaluated 6 months after transplantation and depicted as Kaplan Meier survival plots. Differences were calculated using the Cox-Mantel version of the log-rank test to compare survival curves (37, 38). PCR gel band intensities were analyzed using the ImageJ program.

Results

PB white blood cell numbers increase after treatment with non-myelotoxic conditioning

Total blood cell counts were assessed before and after conditioning with BU, G-CSF, VLA-4I and AMD3100. Peripheral blood cell counts increased significantly in the groups treated with G-CSF ($p<0.05$), VLA-4I ($p<0.01$) and AMD3100 ($p<0.05$) in comparison to baseline levels, but not in the BU-treated group. There were no significant differences in the number of mobilized cells between the G-CSF, VLA-4I and AMD3100 groups, indicating that the mobilizing potential of the tested non-myelotoxic conditioning regimens was comparable despite different mobilization mechanisms (Figure 1).

Non-myelotoxic conditioning supports engraftment and immune reconstitution after transplantation

c-Kit+ BM cells were isolated from $Rag2^{-/-}$ mice and transduced overnight with LV-hRAG2co at an MOI of 30. After confirmation of RAG2 protein and gene expression (Figure 2), the transduced cells were transplanted at a dose of 10^5 i.v. in mice pretreated with different conditioning regimens. Reconstitution of CD3+ (Figure 3A) and CD19+ (Figure 3B) PB cells after transplantation of mice with Lin- cells or c-Kit+ BM cells was similar in all groups, although mice subjected to BU treatment showed a slight advantage in terms of absolute CD19+ numbers, starting from 1 month after transplantation ($p<0.05$). However, after 6 months of transplantation, mice from all groups displayed full immune reconstitution, despite different methods of conditioning. Likewise, mice treated with LV-hRAG2co transduced c-Kit+ BM cells showed a similar reconstitution at 6 months after transplantation to untransduced healthy Balb/c c-Kit+ BM cells, independent of the conditioning regimen used. However, subtle differences changes were observed in reconstitution kinetics (speed and absolute numbers) between groups, with mice conditioned with G-CSF showing a relatively rapid reconstitution, and AMD3100 treatment generally resulted in a delayed and less robust reconstitution of especially CD3+ cells at 6 months post-transplantation. In addition, whereas PB-CD3 numbers showed a steady increase in all groups, absolute PB-CD19 numbers varied both widely in time and between mice.

At sacrifice 6 months post-transplantation, BM and spleen cellularity were increased in mice transplanted with healthy Lin- BM cells (orange bars: $p<0.01$, except for the BU-treated group) and healthy c-Kit+ BM cells (green bars: $p<0.05$, except for the AMD3100 group). However, cellularity remained largely unchanged in mice transplanted with lentivirally transduced RAG2 c-Kit+ BM cells (blue bars, $p>0.05$ for all groups) in comparison to untreated $Rag2^{-/-}$ mice. No significant differences were found between the different methods of conditioning. All transplanted mice showed an increase in spleen weight in comparison to pre-transplant weights (Figure S2).

BM c-Kit+ cell numbers increased in all groups after transplantation (Figure 4A), but the increase was most prominent in mice transplanted with healthy Balb/c Lin- BM and LV-hRAG2co transduced c-Kit+ BM cells. This observation was independent of the method of conditioning and showed that G-CSF, VLA-4I and AMD3100 conditioning can all be used as an alternative to BU conditioning in terms of engraftment. In contrast, although BM Sca-1+ cell numbers did not notably alter after transplantation with healthy Balb/c c-Kit+ BM cells, BM Sca-1+ cell numbers did increase 2.41- and 2.86-fold in mice pre-treated with AMD3100 and VLA-4I and transplanted with hRAG2co transduced cells, respectively, in comparison to pre-transplant levels (Figure 4B). Furthermore, this increase was particularly striking for the VLA-4I treated group in comparison to BU-conditioned mice, which showed no increase in BM Sca-1+ cells ($p<0.05$). Colony-forming unit (CFU-c) capacities of $Rag2^{-/-}$ mice before transplantation, healthy Balb/c mice and BM cells obtained from transplanted mice at sacrifice (6 months post-transplant) were evaluated. No differences in total colony numbers were found between healthy Balb/c and untreated $Rag2^{-/-}$ mice before transplantation. After transplantation significant differences were found between mice transplanted with healthy Balb/c Lin- BM cells and untreated $Rag2^{-/-}$ mice ($p<0.01$) and mice treated with healthy Balb/c c-Kit+ BM cells and untreated $Rag2^{-/-}$ mice ($p<0.01$). In addition, no significant differences were observed between conditioning regimens, although CFU-c numbers were modestly lower in the VLA-4I treated group, in comparison to untreated $Rag2^{-/-}$ mice ($p<0.05$, Figure S3).

Whereas spleen CD3 counts only moderately increased after transplantation with LV-hRAG2co transduced cells, CD3 cell numbers displayed a 30- to 40-fold increase after transplantation with healthy Balb/c c-Kit+ or Lin- BM cells (Figure 4C). This increase was significant for all groups pre-treated with G-CSF ($p<0.05$), for the AMD3100 group ($p<0.01$) transplanted with healthy Balb/c c-Kit+ BM cells and for mice, that were pretreated with VLA-4I and transplanted with LV-hRAG2co transduced c-kit+ BM cells ($p<0.05$). Although CD19 cell numbers showed a wide variability between treatment groups, the best results were obtained with BU and VLA-4I conditioning (Figure 4D). However, significance in comparison to the BU control group could not be determined because of low survival in the BU group at 6 months post-transplantation.

Expression of *hRAG2co* in BM cells transduced with the LV-UCOE-hRAG2co vector was measured directly after transduction and 6 months after transplantation of animals pre-treated with BU, G-CSF, AMD3100 and VLA-4I. Although the level of *hRAG2co* expression decreased after transplantation, this may be due to dilution of the donor cells with cells from the recipient and also because not all transduced cells may have resulted in engraftment (Figure S4). No significant differences were observed between groups ($p>0,05$). Therefore, these data confirm the presence of gene

corrected cells in the BM of all mice, independent of the conditioning regimen, supporting the use of non-myelotoxic agents in transplantation of immune compromised mice.

Spleen lymphocytes obtained from mice transplanted with healthy Balb/c c-Kit+ BM cells showed an overall better proliferative response to stimulation with PHA, but no differences were observed between the different conditioning groups ($p>0.05$). Spleen lymphocytes obtained from mice transplanted with LV-hRAG2co transduced cells showed the highest proliferative potential (i.e. at least 3 divisions in response to stimulation) after conditioning with BU in comparison to any of the other pre-conditioning methods ($p<0.05$ for all groups, Figure S5).

To determine the effects of different conditioning regimens on total cell engraftment, sequences of Sry, which encodes the sex-determining region Y protein and is only found in the male donor-derived cells, were assessed in gDNA obtained from the spleen total nuclear cell fraction at 6 months after transplantation. Although Sry was present in all transplanted animals, levels were lowest in the group pre-treated with AMD3100 and highest in the group treated with VLA-4I, whereas G-CSF treatment resulted in comparable engraftment to the BU-treatment (Figure 5). The presence of hRAG2co and HIV sequences further confirmed permanent integration into the host genome (Figure S6). Interestingly, although present in all groups, Sry levels were found to be lower in the AMD3100 treated animals, indicating overall poorer engraftment in comparison to the other groups and this further correlated with a weaker signal for hRAG2co and HIV. These data further confirm the above data, where we show stable long-term engraftment and immune reconstitution after treatment with both VLA-4I and G-CSF, but lower levels of bone marrow engraftment and immune reconstitution in spleen and peripheral blood after treatment with AMD3100.

Use of non-myelotoxic conditioning promotes increased survival after transplantation

Survival was monitored until termination at 6 months after transplant (Figure 6). Survival of mice treated with the myelotoxic BU was significantly lower than survival of mice in any of the other groups ($p<0.0007$), with survival at 6 months post-transplant being approximately 32%. Interestingly, survival was the highest in the AMD3100-treated group. However, this increased survival did not correlate with increased engraftment or improved immune reconstitution. In contrast, the survival of mice pre-treated with G-CSF or VLA-4I was similar, i.e. 71.4 and 70.4%, respectively, and these mice showed an overall better immune reconstitution than the mice treated with AMD3100.

Discussion

Children that receive conventional myeloablative conditioning (MAC) regimens have been shown to display an increased risk for developmental delay, infertility, organ damage and secondary malignancy (16-18). Since SCID patients are often young children with a severely compromised

immune system, who do not require eradication of malignant cells, currently preferred preparatory regimens consist of more benign immune suppression (IS), a variety of reduced intensity conditioning (RIC) protocols, or absence of any form of conditioning (19-21). The latter, however, is correlated with decreased chimerism, low PB lymphocytes and increased graft failure/rejection (21), whereas the use of RIC/MAC is associated with lower graft failure/rejection and increased independence from immunoglobulin replacement (39), indicating that some form of conditioning is required for successful engraftment.

CXCR4/CXCL12 and VLA-4/VCAM-1 interactions are important for homing and retention of hematopoietic stem and progenitor cells (HSPCs) in the BM niche. Disruption of interactions between HSPCs and its environment by serine proteases and metalloproteinase-9 (MMP-9) induced by G-CSF treatment (28, 40), the use of CXCR4 antagonists, such as AMD3100 (41), or the use of Integrin antagonists, such as BIO5192, results in release of the HSPCs and mobilization to the PB (42, 43). Here, we assessed whether G-CSF, a VLA-4I specific inhibitor BIO5192 and AMD3100, three chemicals that display no known myelotoxicity, can be used as a HSCT preparatory regimen in RAG2 SCID by creating space in the BM and/or increasing BM permeability.

G-CSF, VLA-4I and AMD3100 have previously been shown to be potent HSC mobilizers, when used alone or in combination (33, 44, 45) and similar to these studies we found a significant increase in PB white blood cell counts in response to any of these three mobilizing agents, but not after treatment with BU. We then proceeded by transplanting the *Rag2*$^{-/-}$ mice with c-Kit+ BM cells from healthy Balb/c mice or *Rag2*$^{-/-}$ mice after lentiviral transduction with hRAG2co. Overall engraftment and T cell immune reconstitution was similar in all groups despite different methods of conditioning. However, subtle differences could be observed in reconstitution kinetics, i.e. onset and robustness of increase in absolute numbers between groups, with the most rapid reconstitution observed in G-CSF treated mice and the slowest reconstitution in mice treated with AMD3100. Whether these differences in reconstitution patterns are related to the intrinsic mechanistic effects of G-CSF, AMD3100 and VLA-4I on the bone marrow niche are currently not known. The three mobilizing agents work through very different actions, with G-CSF treatment resulting in slow mobilization of stem cells and delayed recovery of neutrophils, AMD3100 is known for its rapid mobilization and rapid normalization of PB-WBC counts, in contrast to VLA-4I which similar to AMD3100 is a rapid mobilizer, but displays delayed recovery of PB-WBC numbers (43, 46-50). These mechanisms may affect the BM consistently and therefore it is possible that the effects on PB-WBC (and CFU-c) are a mirror for the function of the BM. The kinetics of normalization may perhaps predict the effects of the recovery after transplantation in this study, with the effects of G-CSF lasting longer than those of AMD3100 and VLA-4I, thus providing a longer period for the circulating stem cells to return to

the BM. Therefore, it may be necessary to adjust the transplantation protocol according to the mobilizing agents, with infusion of cells at different time points based on the preferred treatment.

At 6 months post-transplant all mice showed the presence of PB B-cells, but absolute B-cell numbers varied widely between mice and in time. Garcia-Perez et al. tested the effects of combined pretreatment of NSG mice with G-CSF or AMD3100 in combination with a low dose of BU followed by transplantation of human CD34+ cord blood cells and showed that use of mobilizing agents alone was not sufficient for immune reconstitution in NSG mice in the absence of BU (51). However, these researchers used a xeno-transplant model, which requires a higher level of conditioning to sustain long-term engraftment. In our syngeneic transplant setting, we found low chimerism in the absence of any conditioning, but similar levels of chimerism after G-CSF or low dose TBI conditioning in *Il2rγ*$^{-/-}$ knockout mice (26) and improved B-cell reconstitution. Omer-Javed et al. tested a combination of G-CSF/AMD3100 with or without VLA-4I (BIO5192) as pre-conditioning before transplantation of LV transduced GFP+ Lin- BM cells into C57Bl/6 mice (52). They found that after addition of VLA-4I to the G-CSF/AMD3100 conditioning protocol donor chimerism increased and remained stable for up to 6 months. In contrast to some of the other studies described above [46,48], we did not aim to prove the efficacy of the mobilized HSPCs in competitive transplantation studies or confirm the stemness of the mobilized cells. Our study confirms here that a basic mobilization protocol might be sufficient to support sustained engraftment of autologous genetically modified HSPCs in immune deficient mice. In addition to the encouraging results obtained in terms of engraftment and immune reconstitution, we also found a significantly increased survival in mice subjected to any of the non-myelotoxic conditioning protocols in comparison to BU pre-treated mice, with the highest survival and best immune reconstitution in mice treated with either G-CSF or VLA-4I.

In conclusion, here we show in the immune deficient *Rag2*$^{-/-}$ mouse model that the use of non-toxic, mobilizing agents may be developed as a viable alternative to the standard (RIC) regimens. Other studies using different immune deficient mouse models, such as the NSG, *Il2rγ*$^{-/-}$ and *Il2rγ*$^{-/-}$ / *Rag2*$^{-/-}$ double knockout models, further underline that treatment of SCID using different methods/combinations of non-myelotoxic conditioning followed by transplantation of healthy donor HSPCs or lentiviral-vector corrected autologous HSPCs may be feasible. However, to determine which type of conditioning protocol would be optimal for the different types of immune deficiencies, more research will be required focusing on the testing of combinations of G-CSF and VLA-4I, with or without AMD3100 and may also include the testing of newly developed alternatives to the small molecule VLA-4 inhibitors.

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Disclosure of Conflicts of Interest

The authors declare no conflicts of interest.

Availability of data and material

The data presented in this study are available upon request from the corresponding author.

Author's Contributions

Conceptualization, M.E. Şeker, F. Aerts-Kaya; Methodology, Ö.D. Erol, M.E. Şeker, N.P. van Til, F. Aerts-Kaya; Formal analysis, M.E. Şeker, N.P. van Til, F. Aerts-Kaya; Investigation, M.E. Şeker, Ö.D. Erol, B. Pervin; Resources, G. Wagemaker; Data curation, M.E. Şeker, F. Aerts-Kaya; Writing – Original draft preparations, M.E. Şeker; Writing – Review & Editing, F. Aerts-Kaya; Funding aquisition, M.E. Şeker, Ö.D. Erol, G. Wagemaker, F. Aerts-Kaya.

Ethics approval

All mouse experiments performed in this study were done after approval by the Hacettepe University Animal Experiments Ethical Committee (2020/06-02).

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figure Legends

Figure 1. Peripheral blood white blood cell counts before and after conditioning Peripheral blood (PB) white blood cell (WBC) counts were assessed before (blue bars) and after (green bars) conditioning with Busulfan (BU), Granulocyte-Colony Stimulating Factor (G-CSF), Very Late Antigen-4 Inhibitor (VLA-4I) or AMD3100 in female $Rag2^{-/-}$ mice (n=3 per group), directly before transplantation. Data are given as mean ± standard deviation. * $p<0.05$; ** $p<0.01$.

Figure 2. RAG expression in transduced c-Kit+ BM cells c-Kit+ BM cells from $Rag2^{-/-}$ mice were transduced with LV-hRAG2co at an MOI of 30. A: Bright expression of RAG2 was detected using immunofluorescence 1 day after transduction (20X magnification 50 μm); B: Flow cytometry dot plots showing high RAG2 protein expression in the FITC channel). C: hRAG2co gene expression as confirmed by RT-PCR using specific primers designed to detect hRAG2co (left: non-transduced, control RAG2- c-Kit+ BM cells; right: hRAG2co expression after lentiviral transduction).

Figure 3. Peripheral blood immune cell reconstitution after transplantation $Rag2^{-/-}$ mice were subjected to conditioning with Busulfan (BU, blue lines), Granulocyte-Colony Stimulating Factor (G-CSF, orange lines), Very Late Antigen-4 Inhibitor (VLA-4I, grey lines) or AMD3100 (green lines) and transplanted with healthy Balb/c lineage depleted (Lin-) bone marrow (BM) cells (left); healthy Balb/c c-Kit+ BM cells (middle) or lentivirally (LV) transduced RAG2 c-Kit+ BM cells (right). To assess immune reconstitution, peripheral blood (PB) absolute CD3+ (A) and CD19+ (B) cell counts were measured at 1, 3 and 6 months after transplantation (n=6 per group).

Figure 4. Bone marrow stem cell and spleen immune reconstitution $Rag2^{-/-}$ mice were subjected to conditioning with Busulfan (BU), Granulocyte-Colony Stimulating Factor (G-CSF), Very Late Antigen-4 Inhibitor (VLA-4I) or AMD3100 and transplanted with healthy Balb/c lineage depleted (Lin-) bone marrow (BM) cells (orange bars); healthy Balb/c c-Kit+ BM cells (green bars) or lentivirally (LV) transduced hRAG2co c-Kit+ BM cells (blue bars). To assess BM engraftment, c-Kit (A) and Sca-1+ (B) cells were measured; to assess spleen immune reconstitution, CD3+ (C) and CD19+ (D) cell counts were measured at 6 months after transplantation. Data are given as mean + standard deviation. * $p<0.05$; ** $p<0.01$ in comparison to untreated control $Rag2^{-/-}$ mice (n=6 per group).

Figure 5. Assessment of engraftment of donor-derived cells by PCR Genomic PCR showing the presence of the male Sry gene, indicating robust overall engraftment in all transplantation groups.

Figure 6. Non-myelotoxic conditioning supports survival after transplantation Animals were subjected to Busulfan (BU, n=28), Granulocyte-Colony Stimulating Factor (G-CSF, n=28), Very Late Antigen-4 inhibitor (VLA-4I, n=27) or AMD3100 (n=8) conditioning and transplanted with healthy donor Balb/c Lin- BM cells, c-Kit+ BM cells or LV-hRAG2co transduced $Rag2^{-/-}$ c-Kit+ BM cells. Survival data from mice from all groups were pooled and monitored for 6 months after transplantation.

Figures

Figure 1. Peripheral blood white blood cell counts before and after conditioning

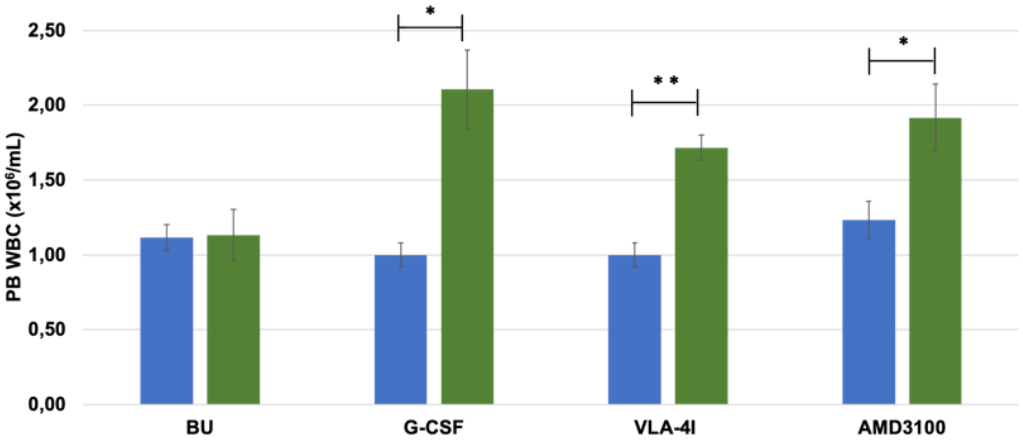


Figure 2. RAG expression in transduced c-Kit+ BM cells

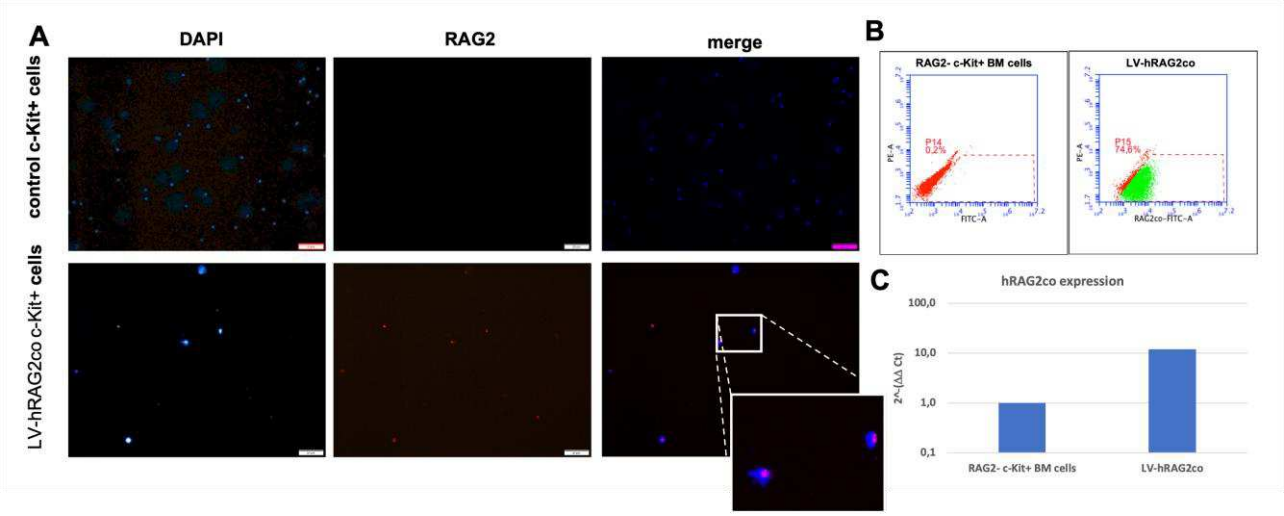


Figure 3. Peripheral blood immune cell reconstitution after transplantation

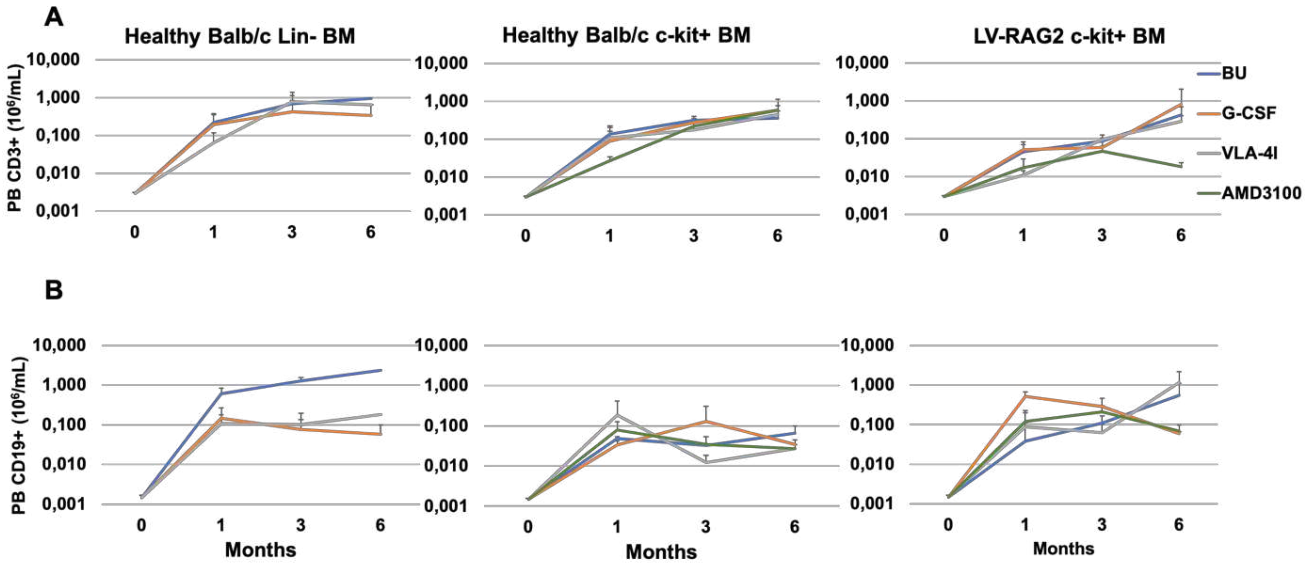


Figure 4. Bone marrow stem cell and spleen immune reconstitution

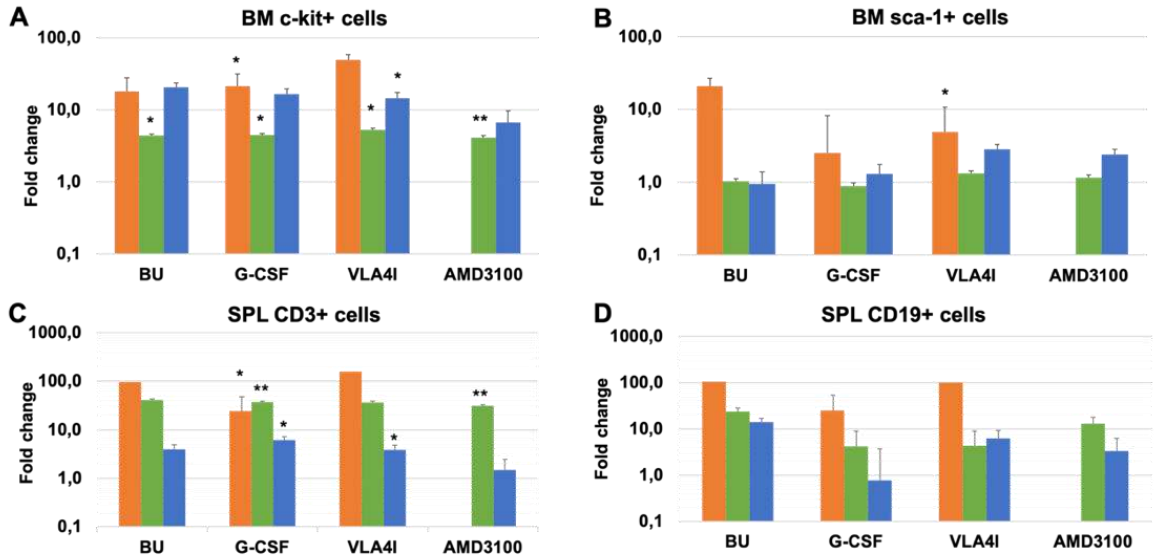


Figure 5. Assessment of engraftment of donor-derived cells by PCR

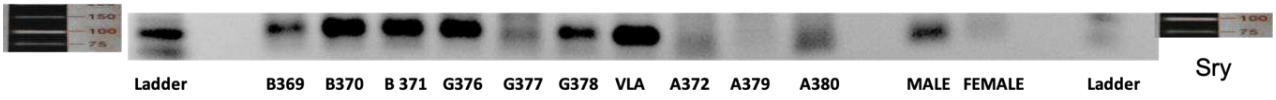
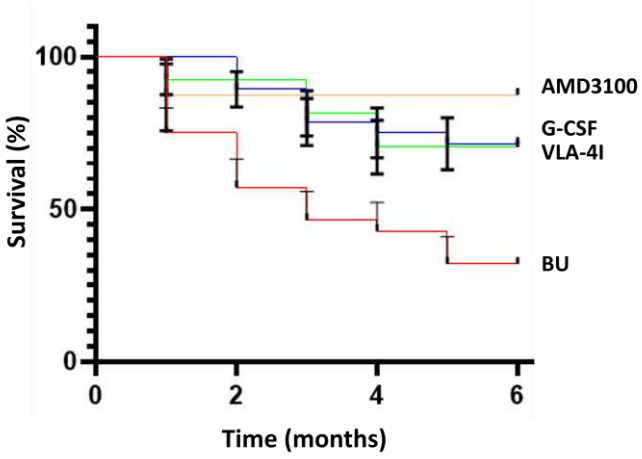


Figure 6. Non-myelotoxic conditioning supports survival after transplantation



Supplementary Files

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