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Dnmt3a-null hematopoietic progenitor stem cells expand after busulfan treatment

Jichun Chen¹, Katie A. Matatall², Xingmin Feng¹, Daniel Hormaechea-Agulla², Mukesh Maharjan², Neal Young¹, Katherine Y. King²

¹Hematology Branch, National Heart Lung and Blood Institute, National Institutes of Health, Baylor College of Medicine, Houston, Texas

²Section of Infectious Diseases, Department of Pediatrics, Stem Cells and Regenerative Medicine Center, Baylor College of Medicine, Houston, Texas

Abstract

Mutations in the gene encoding DNA Methyltransferase 3A (DNMT3A) are the most common in clonal hematopoiesis (CH), an age-related condition that was recently demonstrated to affect outcomes in patients undergoing hematopoietic stem cell transplant (HSCT). Recent studies showed patients with CH have worse prognoses after HSCT, suggesting stress imposed by HSCT preconditioning agents may impact hematopoietic stem cell (HSC) dynamics in transplant recipients. In this study, we used a competitive transplantation mouse model to investigate how treatment with common preconditioning agents 5-fluorouracil (5-FU) or busulfan (BU) affects the prevalence of Dnmt3a^{-/-} HSCs and progenitor cells in competition with WT cells. We found that, though sufficient to deplete peripheral blood counts, 5-FU preconditioning did not significantly alter the frequency of Dnmt3a-null hematopoietic stem and progenitor cells (HSPCs) in mosaic mice. In contrast, mice treated with BU had a 7-fold decline in total bone marrow cells and an increase in Dnmt3a-null HSPCs that was detectable in PB. Indeed, even though all mosaic mice had starting engraftment of ~10–40%, 85–100% of HSPCs were Dnmt3a-null in four of seven mice following BU treatment, indicating these cells expand dramatically during recovery. Overall, these results suggest that different preconditioning regimens have different effects on the expansion of Dnmt3a mutant cells in patients with pre-existing CH. Thus, the presence of CH-associated mutants should be evaluated prior to selecting pre-conditioning regimens for HSCT.

Graphical Abstract

Corresponding Author: Katherine Y. King, Associate Professor, Pediatric Infectious Diseases, Baylor College of Medicine, 1102 Bates Street, Suite 1150, Houston, TX 77030 kyk@bcm.edu, 832-824-4330.

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Keywords

Clonal hematopoiesis; busulfan; 5-fluorouracil; clonal competition; pre-conditioning regime; bone marrow transplant

Introduction

Preconditioning regimens are necessary before hematopoietic stem cell transplantation (HSCT) to ablate the host immune system, reduce tumor burden, promote engraftment, and prevent graft rejection (1). High-dose chemotherapy-based preconditioning regimens have been developed to replace standard high-dose total body irradiation (TBI), which is associated with short- and long-term toxicities (1). Multiple drugs such as 5-fluorouracil (5-FU), busulfan (BU), fludarabine, cyclophosphamide, and melphalan have been explored as alternative conditioning protocols (1, 2). Indeed, the alkylating drug BU often is a major component of chemotherapy-based preconditioning (3). Historically, oral BU in combination with cyclophosphamide was reported to produce equivalent immune-ablation to high-dose TBI and cyclophosphamide (4). More recent reports clearly show that intravenous (IV) BU significantly reduces non-relapse mortality and improves overall and leukemia-free survival compared to TBI (5, 6).

Clonal hematopoiesis (CH), a common age-related condition that is associated with an increased risk of leukemia, cardiovascular diseases and all-cause mortality (7) was recently demonstrated to affect HSCT outcomes (8). CH develops when hematopoietic stem cells (HSCs) with certain genetic mutations produce a disproportionate number of leukocytes in the peripheral blood (PB) of otherwise healthy people (9). The prevalence of CH reaches 15–20% by 70 years of age, but is expected to occur at some level in all people over time (7, 10). Among mutations recurrently found in CH, the most common by far are truncating loss-of-function mutations in the gene encoding DNA Methyltransferase 3A (*DNMT3A*) (9).

While aging is the dominant factor associated with CH development, some recent studies have highlighted environmental and external contexts that predispose to CH (11, 12). For instance, conditions that generate cellular stress may provide a fitness advantage to certain HSC-mutant populations harboring mutations in genes associated with CH (i.e., *DNMT3A*, *TET2*). HSCs with mutations in *DNMT3A* survive better due to a bias toward self-renewal instead of cell differentiation in murine models of serial bone marrow transplantation, increasing their frequency in the bone marrow and eventually in the PB (13, 14). Recent studies have shown that mutations in *TP53* (15) and *PPM1D* (16) confer HSCs with tolerance to DNA damage and cellular stress, allowing these mutants to expand after treatment with certain chemotherapies (11). One report showed that *Dnmt3a*-mutant cells were more resistant to anthracycline treatment due to impaired nucleosome remodeling (17). In summary, external conditions of stress may allow mutant HSCs to increase in number at the expense of wildtype HSC populations.

These observations suggest that the stress imposed by preconditioning agents prior to HSCT may impact the dynamics of HSC clones in transplant recipients. Indeed, patients with CH or pre-CH have a worse prognosis after HSCT (18). Given that almost all people harbor some degree of *DNMT3A* mutation by the age of 50, CH is gaining attention as an important screening criterion prior to transplant (19). A recent study of patients undergoing autologous HSCT found that CH mutations present before HSCT were more likely to dominate blood reconstitution over wild-type HSCs after transplant (20). Therefore, evaluating whether different preconditioning regimens used in HSCT alter risk for selection and propagation of these premalignant clones is critical to improving patient treatment and outcomes.

In this study, we use a competitive transplantation mouse model to investigate how treatment with 5-FU or BU affects prevalence of *Dnmt3a*^{-/-} (*Dnmt3a*-null) HSCs and progenitor cells (LSK) while in competition with WT cells. We found that, whereas 5-FU treatment resulted in little change in the prevalence of *Dnmt3a*-null cells in mixed mosaic mice, *Dnmt3a*-null HSCs and LSK increased after treatment with BU, and these increases were detectable in PB. Our results suggest that the choice of preconditioning regimen affects the expansion of *Dnmt3a*-mutant cells in patients with pre-existing CH before HSCT.

Materials and methods

Animals and treatment with 5-fluorouracil and busulfan

For 5-FU, congenic B6-CD45.1 mice were lethally irradiated (11 Gy of TBI using a ¹³⁷cesium gamma source from J. L. Shepherd & Associates, Glendale, CA, USA) and reconstituted with *Dnmt3a*-null donor (CD45.2) and WT competitor (CD45.1) whole bone marrow (WBM) cells, or with or Mx1-Cre- or *Dnmt3*^{fl/fl} donor (CD45.2) and competitor (CD45.1) cells at Baylor College of Medicine. B6 and congenic B6-CD45.1 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), while Dr. Margaret Goodell generously provided *Dnmt3a*-null mice. At 10 weeks after reconstitution, mice were divided into control and treatment groups. Animals in the treatment groups received 5-FU at 150 µg/gBW through intraperitoneal (IP) injection while animals in the control groups received

the same volume of solvent. 5-FU was injected 2 times on days-1 and day-14 and animals were bled and euthanized on day 28 for analyses.

For BU experiments, lethally irradiated B6-CD45.1 mice were reconstituted with Dnmt3a-null donor (CD45.2) and WT competitor (CD45.1) WBM cells, or with WT B6 donor (CD45.2) and WT competitor (CD45.1) cells at Baylor College of Medicine. Reconstituted mice were transferred to the NIH for further experimentation. At 10 weeks after reconstitution, mice were divided into control and treatment groups. Animals in the treatment groups received IP injection of BU (Sigma-Aldrich, St. Louis, MO, USA) at 9.5 µg/gBW and animals in the control groups received the same volume of solvent. In the short-term study, BU was injected 10 times on days 1, 3, 5, 8, 10, 12, 15, 17, 19 and 21 and animals were bled and euthanized for analyses at 15 days after the last injection. In the long-term study, BU was injected 7 or 9 times for 15 or 19 days, respectively, following the same time schedule, and animals were bled and euthanized after 15 weeks for analyses. BU was initially dissolved in acetone (at 6 mg/mL) for storage, and right before injection was diluted 1:5 with deionized water to 1 mg/mL and filtered through 0.2 µm Millex-Gs sterile filter (Merck Millipore Ltd, Cork Ireland).

The selection of 5-FU and busulfan doses and administration schedules was based on to previously published studies in combination with our prior experience using these agents in mouse models that show effective hematopoietic suppression(21–23). All animal studies were approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine and the National Heart, Lung, and Blood Institute.

Sample preparation, blood counts and flow cytometry

Blood was collected from the retro-orbital sinus into EDTA-added Eppendorf tubes. Complete blood counts (CBC) were performed in a HemaVet 950 analyzer (Drew Scientific, Inc. Waterbury, CT, USA). After mouse euthanasia by CO₂ inhalation, BM cells were extracted from bilateral tibiae and femurs in Iscove's Modified Dullbecco's Medium (IMDM, Life Technologies Corporation, Grand Island, NY, USA), filtered through 90µM nylon mesh (Small Parts, Miami Lake, FL, USA), and counted by a Vi-Cell counter (Counter Cooperation, Hialeah, FL, USA). Peripheral blood leukocytes and BM cells were first incubated with ACK buffer twice for 10 minutes to lyse red blood cells (RBCs). Residual leukocytes were stained with the antibodies listed below and analyzed on a Canto II flow cytometer using the FACSDiva software (Becton Dickson, San Diego CA).

We used the following monoclonal antibodies for murine CD3 (clone 145–2C11), CD4 (clone GK 1.5), CD8 (clone 53–6.72), CD11b (clone M1/70), erythroid cells (clone Ter119), and granulocytes (Gr1/Ly6-G, clone RB6–8C5) from BD-Biosciences (San Diego, CA). We used anti-mouse CD45.1 (clone A20), CD45.2 (clone 104), CD45R (B220, clone RA3–6B2), CD117 (c-Kit, clone 2B8), CD150 (clone TC15–12F12.2), and stem cell antigen 1 (Sca-1, clone D7) from Biolegend (San Diego, CA). Antibodies were conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanin 5 (PE-Cy5), phycoerythrin-cyanin 7 (PE-Cy7), allophycocyanin (APC), allophycocyanin 7 (APC-Cy7), or brilliant violet 421 (BV421).

Pathology and histology

Sternum were collected, fixed in 10% neutral buffered formalin, sectioned at 5 μ thickness, and stained with H&E (GeneCopoeia Inc., Rockville, MD, USA). We examined slides under a Zeiss Axioskop2 plus microscope and captured images at 20x magnification using a Zeiss AxioCam HRC camera (Carl Zeiss MicroImaging GmbH, Jena, Germany).

Statistical analysis

We used GraphPad Prism 8 statistical software to analyze CBC and BM cellular composition data through variance analysis and t-tests and statistical significance was considered $p < 0.05$ and $p < 0.01$, respectively.

Results

5-FU preconditioning causes bone marrow suppression but no expansion of Dnmt3a-null HSCs in transplant recipients

In order to model CH, we created mosaic mice by lethally irradiating 6-week-old B6-CD45.1 mice and reconstituting them with a 1:4 ratio of Dnmt3a-null (CD45.2) or WT (CD45.2) control and WT (CD45.1) competitor whole bone marrow cells. To study the effect of HSCT preconditioning agent 5-FU, an antimetabolite chemotherapy drug, on Dnmt3a-null clonal expansion, we waited 10 weeks for engraftment and then treated mice with 5-FU or PBS control. We administered 5-FU as two IP doses of 150 μ g/gBW two weeks apart. Mice recovered for two weeks before we assessed their peripheral blood (PB) count and bone marrow (BM) composition (Figure 1A). 5-FU treatment decreased overall white blood cells (WBC), red blood cells (RBC), and whole bone marrow (WBM) cells, but increased platelets (Figure 1B–E). To ascertain if Dnmt3a-null cells displayed a competitive advantage compared to WT in mosaic mice during recovery from 5-FU, we compared the frequency of CD45.2 donor cells in the PB of mosaic mice. As shown in Figure 1F, there was no significant difference in the chimerism of Dnmt3a-null cells in mice treated with 5-FU or control. Similarly, there was no significant difference in the frequency of Dnmt3a-null cells in WBM, BM progenitors (lineage-negative, Sca+, cKit+; LSK), or phenotypically-defined HSCs (LSK CD150+ CD48-) of the BM in the presence or absence of 5-FU (Figure 1G, Supplementary Figure 1). These data suggest that, though sufficient to deplete peripheral blood counts, 5-FU preconditioning does not significantly alter the frequency of Dnmt3a-null HSPCs in mosaic mice.

Dnmt3a-null cells expand after intensive BU treatment in mosaic mice

To assess the impact of the common preconditioning agent BU on the frequency of Dnmt3a-null cells in the mosaic mice described above, we injected mice 10 weeks post-transplant with BU 10 times at a dose level of 9.5 μ g/gBW over 23 days (Figure 2A). As previously described (22), 15 days after the last BU injection this treatment regimen produced severe BM failure in recipient mice with large reductions in red blood cells, neutrophils, and platelets (Figure 2B–D). Relative to mosaic mice treated with PBS only, mice treated with BU showed a 7-fold decline in total bone marrow cells (Figure 2E), as well as hematopoietic progenitor cells (LSK) per mouse (Figure 2F). Consistent with pancytopenia and marrow

hypoplasia, sterna from BU-treated animals appeared empty relative to PBS-treated controls, in which sterna were full of hematopoietic cells (Figure 2G). We detected a significant enrichment in T and B cells in BU-treated animals (Supplemental Figure 2A), with 10-fold increase in the proportion of CD3 T cells, especially CD8 T cells (Supplemental Figure 2B), in the surviving BM cells relative to control animals treated with solvent. These results indicate a slight chemoresistance in the BM T cell subset, though, as expected, BU treatment was fully myeloablative in recipient mice.

Next, we analyzed the effect of BU on donor contributions to LSK, total BM cells and PB leukocytes 15 days after the final treatment (Figure 2H–I). Despite large inter-individual variability among all the mice, CD45.2 Dnmt3a-null cells expanded significantly in the WBM after BU treatment compared to treatment with solvent. Furthermore, after 15-day recovery from BU treatment, there was a clear trend toward expansion in donor Dnmt3a-KO CD45.2-derived LSK progenitor cells and PB leukocytes. Indeed, even though all mosaic mice had starting engraftment of ~10–40% (Supplementary Figure 2C), following BU treatment four of seven mice had CD45.2 LSK frequencies between 85–100%, indicating dramatic expansion of Dnmt3a-null LSK BM during recovery.

We also looked at the percentage of CD45.2 cells in the PB relative to starting engraftment. As shown in Figure 2J, CD45.2 engraftment did not change before or after treatment in vehicle-treated mice (Fold change=1.15). In contrast, the frequency of CD45.2 in the PB of BU-treated mice tended to increase (average fold change=6.47) (Figure 2J). Indeed, we found that CD45.2 PB leukocytes increased in 5 of 7 mice after recovery from BU, with one mouse showing an increase of 29.9% from starting engraftment (Figure 2L). We did not see similar changes in PBS-treated mice (Figure 2K). These results indicate that Dnmt3a-null hematological populations expand preferentially after BU treatment.

To assess the long-term effects of BU treatment on mosaic mice, we performed a similar treatment of mosaic mice with BU, but this time we waited 15 weeks after treatment before assessing of the blood and bone marrow (Figure S3A). In the peripheral blood, RBCs and platelets were fully recovered in all mice at 15 weeks after myeloablative treatment (Figure S3B and S3C). However the WBC remained significantly depleted in BU-treated mosaic mice, even 15 weeks after treatment. The WBC of Dnmt3a-null mosaics was low even in the absence of BU treatment and tended to be even lower in the BU-treated recipients (Figure S3D). These findings may be consistent with the known differentiation defect of Dnmt3a-null mice, hindering peripheral blood production in these mice. In the bone marrow, the total cell count was relatively suppressed in the BU treated mice, and this difference was more dramatic in mice transplanted with Dnmt3a-null marrow (Figure S3E). When looking at the proportion of Dnmt3a-null cells in the peripheral blood and the bone marrow at 15 weeks post BU treatment, we saw significant expansion, with 80–100% chimerism in many of the mice, but this expansion was also evident in many of the PBS-treated Dnmt3a-null mosaic mice, indicative of the natural tendency of Dnmt3a-null hematopoietic cells to expand in the marrow over time (Figure S3F).

Discussion

We studied how BU and 5-FU, two agents used in cytotoxic preconditioning therapy for HSCT or bone marrow transplant (BMT), affect the selection of *Dnmt3a*-null clones following HSCT-induced stress. We saw an increase in the *Dnmt3a*-null population following treatment with BU but not 5-FU compared to PBS-treated controls. The most significant increase occurred in HSC and progenitor populations, but also was reflected in the PB, where the mutant population increased 5.6 times more in the BU treatment group compared to controls. Thus, DNMT3A mutations provide HSCs with a selection advantage in the setting of chemotherapy stress.

5FU drives HSCs into cell cycle and has been used in many animal studies to disrupt HSC quiescence(24–27). We speculated that increased cell cycle activity induced by 5-FU would amplify selection advantages of *Dnmt3a*-null versus WT HSCs. However, our data indicate that 5-FU did not exert differential effects on WT versus *Dnmt3a*-null HSCs, and 5-FU treatment did not significantly affect the proportions of *Dnmt3a*-null and WT HSCs in mosaic mice. These findings suggest that disruption of quiescence alone is not sufficient to provide a selection advantage for *Dnmt3a*-null HSCs.

In contrast, our studies demonstrate that BU treatment results in selection and expansion of *Dnmt3a*-null HSCs. A previous study found similar results in AML cells and patients treated with anthracycline chemotherapy, where DNMT3A-mutant cells were more viable and resistant to chemotherapy (17). These findings are also reminiscent of a study in which HSCs with PPM1D mutations increased following treatment with certain chemotherapy drugs (16). Collectively, these studies highlight the importance of evaluating for the presence of CH-associated mutations prior to selecting pre-conditioning regimens for BMT or HSCT (20).

Previous clinical studies have shown CH mutations present at the time of autologous stem cell transplant (ASCT) are strongly associated with later dominance of those mutant HSCs and, hence, risk of therapy-related myeloid neoplasms (tMN) and non-relapse mortality (NRM) (19, 20, 28, 29). A study of 81 patients with solid tumors or myeloid or lymphoid disease who underwent ASCT after cytotoxic therapy clearly showed CH-mutated HSCs dominated non-mutant clones (20). In CH after transplant, mutations in *DNMT3A*, *TET2*, *PPM1D*, *TP53* and *RAD21* were the most common. The study also showed that these mutations did not result from cytotoxic therapy but were present prior to treatment and gained a reconstitution advantage after hematopoietic stress. Furthermore, post-transplant patients with CH mutations required significantly longer hospitalization and time for neutrophil reconstitution. This association between CH before ASCT and NRM after transplant was independent of patient age (19, 20, 28, 29). The impact of CH on allogeneic transplant is also beginning to be recognized. A recent study indicated that CH was more likely to show increased clone size and have increased telomere shortening in transplant recipients compared to donors, indicating that the stress of transplant contributes to clonal expansion(30). While our studies were designed to test the relative responses of *Dnmt3a*-null versus WT HSCs to BU treatment (relevant to the recipient of an autologous transplant),

these data do not exclude the possibility that BU leads to lasting effects on the bone marrow niche that may contribute to clonal selection, including in HSCs from an allogeneic donor.

The link we identified between CH mutations and clonal dominance in HSCT recipients after treatment with the cytotoxic pre-conditioning agent busulfan, but not 5-FU, strengthens the argument for prospective evaluation of CH in human patients diagnosed with hematopoietic malignancy. To take CH mutation into account prior to ASCT pre-conditioning regimen, Soerensen et al. (28) combined next generation sequencing and flow cytometry to predict which patients were at risk for developing tMN after ASCT. Aberrant expression of CD7 on HSCs at the time of ASCT was found to identify patients at the higher risk of developing tMN with positive predictive value of 75%. The fact that not all preconditioning agents affect preexisting CH clones in the same way highlights the importance of screening for CH in order to choose the safest and most effective HSCT preconditioning agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Busulfan and a variety of chemotherapy agents such as 5-fluorouracil are commonly used as preconditioning agents before hematopoietic stem cell transplant.
- Busulfan treatment, but not 5-fluorouracil, leads to a relative expansion of Dnmt3a-null HSCs compared to WT HSCs in the bone marrow of mice with mixed bone marrow.
- The mutational profile of HSCT recipients and donors, especially older individuals who have a high likelihood of clonal hematopoiesis, should be taken into consideration when choosing a preconditioning agent.

Figure 1: Effect of 5-FU treatment on Dnmt3a clonal competition.

(A). B6-CD45.1 mice were irradiated and reconstituted with CD45.1 BM cells (competitor) and WT (n=10 mice) or Dnmt3a-null CD45.2 BM (n=23 mice) donor cells. At 10 weeks after reconstitution, mice were divided into control and treatment groups. 5-FU treatment consisted in two injections of 150 mg/Kg with two weeks of separation and then mice were allowed to recover for another 2 weeks. (B). White blood cells (WBC) counts. (C). Red blood cells (RBC) counts (D). Total WBM cells. (E). Platelet counts. (F). Percentage of CD45.2 donor cells in peripheral blood (PB) from 5-FU treated and PBS treated mice. (H). Percentage of CD45.2 cells (WT or Dnmt3a-null) in WBM, LSK and HSCs (LSK CD150+ CD48-) from 5-FU treated and PBS treated mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 2: Recovery after intense BU treatment shows Dnmt3a-null expansion.

(A). Lethally irradiated B6-CD45.1 mice were reconstituted with Dnmt3a-null donor (CD45.2) and WT competitor (CD45.1) WBM cells. BU-treated mice (n=7) received IP injection of BU at 9.5 μ g/gBW and animals in the control group (n=4) received the same volume of PBS. BU was injected 10 times on days 1, 3, 5, 8, 10, 12, 15, 17, 19 and 21 and animals were bled and euthanized for analyses at 15 days after the last injection. (B). RBC counts. (C). Neutrophils counts. (D). Platelets counts. (E). WBM cells. (F). LSK cells. (G). Consistent with pancytopenia and marrow hypoplasia, sternums from BU-treated animals

appeared empty when sectioned and H&E stained for observation under microscope relative to recipients without BU treatment in which sternums were full of hematopoietic cells (**H**). Gating strategy showing CD45.2 (Dnmt3a-null) ckit+ cells accumulation in BU-treated mice. (**I**). Percentage of CD45.2 cells (Dnmt3a-null) in LSK, WBM and PB from BU-treated and PBS-treated mice. (**J**). Fold change variation in PB CD45.2 cells (Dnmt3a-null) after PBS or BU treatment from starting engraftment. (**K-L**). Paired analysis performed in each mouse shows percentage of CD45.2 cells (Dnmt3a-null) pre (starting engraftment) and post PBS treatment (**K**) or BU treatment (**L**). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.