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Hematopoietic Cells from Ube1L-Deficient Mice Exhibit an Impaired Proliferation Defect under the Stress of Bone Marrow Transplantation

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Abstract

Following bone marrow transplantation, donor stem cells are recruited from their quiescent status to promote the rapid reconstitution in recipients. This dynamic process is tightly regulated by a complex of internal and external signals. Protein modification by the ubiquitin like modifier ISG15 (ISGylation) is strongly induced by Type I Interferons (IFNs). There are higher levels of Type I IFNs and protein ISGylation in the bone marrow of recipients shortly after transplantation. In order to clarify the physiological function of protein ISGylation, we generated a mouse model that lacks protein ISGylation due to deficiency of ISG15 conjugating enzyme Ube1L (Ube1L$^{-/-}$). In this report, we focused on the analysis of the hematopoietic system in Ube1L$^{-/-}$ mice in steady-state hematopoiesis and its potential protective role during bone marrow reconstitution. Here we demonstrated that In Ube1L$^{-/-}$ mice, steady-state hematopoiesis was unperturbed. However, transplantation experiment revealed a 50% reduction in repopulation potential of Ube1L-deficient cells at 3 weeks posttransplantation, but no differences at 6 and 12 weeks. A competitive transplantation experiment magnified and extended this phenotype. Cell cycle analysis revealed that under the condition with high levels of IFNs and protein ISGylation, the Ube1L deficiency can cause G2/M phase block of cell cycle in hematopoietic multipotential progenitors. These observations indicate that although protein ISGylation is dispensable for steady-state hematopoiesis, it plays a significant role during interferon related stress response, such as bone marrow transplantation.

Keywords

Hematopoiesis; Bone marrow transplantation; Protein ISGylation; Ube1L

Introduction

Hematopoiesis relies on the unique abilities of relative few hematopoietic stem cells (HSCs) to self-renew and generate progenitors that will differentiate into the mature cells forming the blood system. At steady state, cytokines and chemokines drive a small percentage of HSCs to self-renew in order to maintain a constant number of stem cells. Following bone marrow ablation, by cytotoxic agents or radiation, stem cells are recruited from their

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quiescent status to promote the rapid reconstitution of a depleted hematopoiesis system. This dynamic process is tightly regulated by a complex of internal and external signals, such as transcription factors, growth factors, and cell cycle regulators[1;2]. Therefore, the proper proliferation and differentiation of HSCs are crucial for reconstitution of hematopoiesis on transplantation into recipients with bone marrow ablation.

Interferons (IFNs) include the type I interferon family (mainly α and β-interferon), and type II or γ-interferon. Type I IFNs are induced in various types of cells by different stimuli [3]. IFNs activate the expression of hundreds of genes via JAK-STAT pathways and effect gene expression, protein processing, cell proliferation and survival to regulate cellular immune responses [4;5]. ISG15 is a 17 KDa protein encoded by an IFN stimulated gene (ISG) [6–8]. Its expression is highly induced upon IFN treatment. In most cell types and tissues, ISG15 expression and protein ISGylation is very low under normal conditions. Upon IFN stimulation, ISGylation is strongly induced [9]. Ubiquitin-activating enzyme E1 like (Ube1L) is a critical enzyme for protein ISG15 modification. Ube1L deficient cells are unable to undergo protein ISGylation [10]. Although numerous proteins are modified by ISG15 upon IFN stimulation, the biological function of protein ISGylation is still largely unknown. Therefore, we used Ube1L knockout mice as a model to analyze protein ISGylation in hematopoiesis, especially hematopoietic stem cells and progenitors under the stress induced by the bone marrow transplantation (BMT).

Here, for the first time, we report the increased IFN production and protein ISGylation in hematopoietic cells shortly after BMT. Furthermore, our findings revealed that Ube1L deficiency has a limited effect on stem cell behavior under steady-state conditions with the result that the Ube1L-deficient mice have unperturbed cell numbers and lineage distribution in peripheral blood (PB), BM, and the spleen, as well as normal numbers of primitive Lin^{-} $Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells and committed progenitors in the bone marrow. However, we observed a substantially delayed hematopoietic reconstitution in recipients after 3 weeks following BMT but not 6 and 12 weeks. This decreased reconstitution capacity is accompanied by a G2/M phase block of cell cycle in multipotent progenitors of hematopoietic cells. These results suggest a crucial role of protein ISGylation in facilitating the generation and proliferation of multipotent progenitor compartment under IFN related stress conditions.

Materials and Methods

Experimental animals

Mice deficient in Ube1L ($Ube1L^{-/-}$) were described previously [10] and maintained on a C57BL/6 inbred genetic background. Six- to 10-week-old (age-matched) mice from mouse strain $Ube1L^{-/-}$ (CD45.2), C57BL/6 (CD45.2, WT control), and congenetic B6.SJL-$Ptprc^{a}Pep^{b}$/BoyJ (Pep3 CD45.1, WT) and F1 C57/Pep3 (CD45.1/CD45.2) were used in these experiments. All mice were maintained under specific pathogen-free conditions. All procedures were approved by institutional IACUC.

In vitro colony-forming unit (CFU) assays

Clonogenic progenitors were determined in methylcellulose colony assay medium (MethoCult GF M3434, StemCell Technologies) following the instructions of the manufacturer. In brief, cells were plated in methylcellulose media at a density 3×10^4 BM cells/1.1 ml. 250 ul were plated in triplicate wells of a 24-well plate. The plate was incubated at 37°C in 5% CO2 incubator for 10–12 days. The total numbers of colony were scored and expressed as number of CFUs per 3×10^4 BM cells.

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In vivo assay for colony-forming units-spleen (CFU-S)

The content of multipotent myeloid progenitors was determined by the day 12 CFU-S assay as original described [11]. Briefly, recipient mice were lethally irradiated with 10Gy of whole-body irradiation, and injected with 8×10^4 fresh bone marrow cells intravenously. Spleens were dissected 12 days posttransplant and fixed in Bouin's solution. Colonies per spleen were counted by visual inspection and expresses as number of CFU-S12 colonies per 8×10^4 BM cells.

Bone marrow transplantation experiments

Recipient mice were lethally irradiated (10Gy) and one million BM cells were injected intravenously into recipient mice. Animals were sacrificed at 3, 6, and 12 weeks post-transplantation. Hematopoietic reconstitution was monitored in PB and BM. For standard competitive transplantation experiments, 4×10^5 fresh BM cells from Ube1L$^{-/-}$ or control littermates (CD45.2) were mixed together with 4×10^5 competitor BM cells from pep3 (CD45.1). The mixture was injected into lethally irradiated F1 pep3/C57BL/6 recipient mice (CD45.1/ CD45.2). To measure reconstitution of CD45.2-derived cells, PB and BM cells were taken at weeks 3, 6, and 12 when the mice were sacrificed, and the cells were stained with anti-CD45.1 and CD45.2 antibodies and were analyzed for populations of CD45.1$^+$ cells and CD45.2$^+$ cells.

Homing assays

The homing efficiency of hematopoietic cells was assayed by labeling BM cells with FACS antibody. One million whole BM cells from CD45.2$^+$ Ube1L$^{-/-}$ or wild-type control donor were injected into lethally irradiated CD45.1$^+$ recipients. Recipients were sacrificed at 3 or 16 h later for FACS analysis of donor-derived cells in BM by using PE-conjugated CD45.2 and FITC-conjugated CD45.1. The percent of transplanted CD45.2$^+$ donor cells that had homed to bone marrow was calculated as follows:

$$\%\,\text{Homing} = [(A \times B)/C] \times 100\%$$

Where:

A = % CD45.2$^+$ cells determined by flow cytometry

B = Total bone marrow cellularity

C = Number of cells transplanted

In measuring homing to the BM, cells from both femora and tibiae were assumed to represent 25% of the total marrow compartment.

Cell cycle analysis

At 7 days posttransplantation, recipient mice were injected with 2 mg BrdU. After 90 minutes, bone marrow cells were isolated and lineage negative purified using MACS magnetic cell sorting (Miltenyi Biotec) and then stained with c-kit PE-Cy7, Sca-1 PE, and flt3 (biotin)-APC-Cy7 antibodies (BD Biosciences). Stained cells were then fixed, permeabilized, and incubated with APC-labeled anti-BrdU and 7AAD according to the manufacturer's suggestions (BD Biosciences). Per sample, 1×10^6 events were analyzed and the LSK and different stem cell populations were defined according the surface antibodies staining. The analysis of cellular DNA levels in selected subpopulations was done.

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Flow cytometry analysis

Flow cytometry was used to quantify the hematopoietic cells in the PB and BM. For analysis of reconstitution in transplanted mice, PE-conjugated CD45.2 and FITC-conjugated CD45.1 antibodies were used. For estimation of stem cell populations, the cells were incubated in the lineage cocktail containing Percp-5.5-conjugated CD3, CD4, CD8, CD19, B220, CD11b, and Gr-1 antibodies (BD Biosciences) and then labeled with Sca1-APC, c-kit-PE-Cy7, CD34-FITC, and flt3-(biotin)-APC-Cy7.

Real-time PCR analysis of IFNs expression

The expression of IFNs mRNA level was analyzed by reverse transcription (RT) followed by quantitative PCR. The total RNA from the bone marrow cells was extractive using TriZol (Gibco BRL, Grand Island, NY). RT reaction was performed using total RNA with random hexamers as primers. Quantitative PCR amplification was performed using the following previously described oligonucleotide primers including GAPDH, IFN-α_{1-2} and IFN-β primers: IFN-α_{1-2} sense, TGTCTGATGCAGCAGGTGG; IFN-α_{1-2} antisense, AAGACAGGGCTCTCCAGAC [12]; IFN-β sense, CACTTGAAGAGCTATTACTGGAGGG; IFN-β antisense, CTCGGACCACCATCCAGG [13]. Values for each PCR product were normalized against GAPDH giving a relative intensity for comparison of knockout and normal mice.

Western blot analyses

At different time points of posttransplantation, BM cells were separately collected from recipient mice and protein extract was prepared. Rabbit anti-mouse ISG15 polyclonal antibodies used in this study were described previously [14]. Western blotting was performed as described previously [15].

Statistical analysis

Each value represents at least three independent experiments. Statistical significance was evaluated with the two-tailed Student's t-test. $p < 0.05$ was considered statistically significant.

Results

Steady-state hematopoiesis is unperturbed in Ube1L$^{-/-}$ mice

All live-born Ube1L$^{-/-}$ and wild-type mice appeared to develop normally into adults with equivalent average size and weight. The absolute spleen and thymus weights and overall cellularities of the BM and PB analysis from knockout and wild-type controls were approximately equal. Furthermore, FACS analysis on cells derived from PB, BM, spleen, and thymus showed no significant difference in the ratios of myeloid cells (Gr-1, CD11b), B-cells (B220), and T cells (CD3, CD4, CD8) between knockout and normal littermates (Table. 1 and Fig. 1).

Next, we analyzed the ability of Ube1L-deficient BM to form committed progenitors by plating fresh BM cells in cytokine-containing methylcellulose medium. The results revealed no difference in size or number of myeloid colonies formed (Fig. 2A), suggesting that neither the number of hematopoietic myeloid progenitors nor their capacity to proliferate or differentiate in vitro was affected by the loss of Ube1L.

In order to analyze hematopoietic stem cells directly, we further performed FACS analysis of stem cells. Several studies have shown that antibodies against different combinations of cell surface markers can be used to isolate and enumerate immunophenotypic HSCs [16;17].

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Based on anti-CD34 and anti-flt3 antibodies, three distinct LSK populations could be observed in adult BM: LSKCD34^{-}flt3^{-} (Long-term HSCs), LSKCD34^{+}flt3^{-} (Short-term HSCs), and LSKCD34^{+}flt3^{+} cells (multipotent progenitors, [MPPs]) [18]. Using this approach, we further analyzed the stem cell populations under normal conditions in adult Ube1L$^{-/-}$ and wild-type mice. In three different experiments, the fractions of LT-HSCs, ST-HSCs, and MPPs cells within the LSK compartment were almost identical in the BM of Ube1L$^{-/-}$ and wild-type mice (Fig. 2B). The results showed that the generation or maintenance of HSCs was normal in adult Ube1L$^{-/-}$ mice bone marrow. Taken together, these findings indicate that Ube1L is dispensable for the maintenance of the hematopoietic system under steady-state conditions.

Higher levels of IFNs and protein ISGylation in mice receiving total bone marrow transplantation

In most cell types and tissues, ISG15 expression and protein ISGylation are very low under normal conditions. However, ISG15 expression and ISGylation are highly induced upon IFNs treatment [9]. We performed total BMT using wild-type mice as both donors and recipients, and then collected the BM cells from recipients at different time points in order to check the expressions of protein ISGylation and IFNs level under transplantation condition in order to check Type I IFN expression and protein ISGylation. We used real-time PCR to check the Type I IFNs mRNA levels in the BM cells from the lethally irradiated recipient mice. Setting the control as one, the relative fold change at different time-points was compared to the control. The results (Fig. 3A–B) showed that there were increased levels of IFN-alpha and beta in the BM cells of recipient mice at 4, 6, 8, and 10 days after transplantation. Compared with the mice simply receiving lethal-dosage irradiation, the BM cells from the transplanted mice showed much higher and earlier expression of both (Supplementary. Figure S1). We also used western blotting method to investigate the protein ISGylation levels in the BM, Increased levels of protein ISGylation were observed at 2, 4, 6, and 8 days after transplantation, which then began to return to normal levels (Fig. 3C). Taken together, bone marrow transplantation triggers the production of Type I IFNs and enhances ISG15 expression and protein ISGylation.

Delayed proliferative response of primitive hematopoietic cells of Ube1L$^{-/-}$ mice

To compare the hematopoietic reconstitution potential of BM cells, we performed the total BMT using fresh BM cells from Ube1L$^{-/-}$ and wild-type mice. Three weeks after transplantation, the majority of circulating cells in all recipient mice were derived from donor cells. The delayed engraftment of Ube1L$^{-/-}$ donor cells compared to wild-type cells was illustrated by the total bone marrow cell numbers in recipients. The result showed that the repopulating cell number in recipient mice receiving Ube1L-deficient BM cells was less than 50% of the number in the mice that received wild type cells at 3 weeks after transplantation ($p = 0.0015$) (Fig. 4A). The lineage commitment of engrafted Ube1L-deficient cells was not affected (Supplementary. Figure S2). However, at 6 weeks and 12 weeks later, there was no significant difference in the repopulating donor cell number in the recipients receiving Ube1L$^{-/-}$ or wild-type donor cells. These results indicate that the Ube1L-deficient cells have a reduced regenerative capacity under the proliferative stress condition after bone marrow transplantation.

The in vivo CFU-S assay detects primitive multipotent progenitor/stem cells capable of rapidly producing myeloerythroid cells in vivo [11]. Next, we used this assay as a measure of the multipotent myeloid progenitor cells that were transplanted into recipients in spleen. In four individual experiments, 80,000 fresh knockout or wild-type BM cells were injected into lethally irradiated normal recipients; 12 days later, the spleens were harvested and the macroscopic colonies were counted. The frequency of CFU-S12 was normal in mice

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receiving Ube1L-deficient cells compared to wild-type cells (Fig. 4B), however, the size of the colonies derived from Ube1L deficient cells was clearly reduced compared to wild-type cells ($Ube1L^{-/-}$ is about 50% of wild-type) (Fig. 4C), indicating that $Ube1L^{-/-}$ mice have normal number of multipotential myeloid progenitors, but have a reduced proliferative ability in the condition of transplantation.

Normal homing of Ube1L-deficient BM cells

Before evaluating the repopulating capacity of Ube1L-deficient hematopoietic cells, we sought to determine whether the defective engraftment potential of hematopoietic cells from $Ube1L^{-/-}$ mice is due to their homing efficiency in irradiated adult recipients. For testing this possibility, one million BM cells from Ube1L-deficient and wild-type mice (CD45.2) were transplanted into irradiated recipients (CD45.1), and homing into BM was evaluated by FACS analysis of donor-derived cells in BM at 3 and 16 hours after transplantation. The results showed that the homing capacity of Ube1L-deficient cells was not affected by the lack of protein ISGylation when compared to control mice (Fig. 4D), suggesting that the reduced hematopoietic cell numbers in the mice receiving Ube1L-deficient BM cells was not simply due to an impaired ability to migrate to hematopoietic sites. There was also no difference in the ability of normal donor cells to home into the BM stroma of $Ube1L^{-/-}$ mice compared to control mice, suggesting that the defect of $Ube1L^{-/-}$ hematopoietic cells was not derived from hematopoietic microenvironment (data not shown).

Reduced competitive repopulating ability of Ube1L deficient hematopoietic cells

Iin order to further address the physiologic role of Ube1L in the proliferation of primitive hematopoietic progenitor and stem cells, a competitive repopulation experimental design was chosen [19]. Our experiments were designed to reveal deficiencies of Ube1L-deficient hematopoietic cells when they were in competition with wild-type cells. Fresh 4×10^5 BM cells from $Ube1L^{-/-}$ or wild-type mice (CD45.2) were mixed with competitor BM cells from wild-type pep3 mice (CD45.1) at a ratio of 1:1 and engrafted into lethally irradiated recipient mice (CD45.1/CD45.2) (The recipient mice are F1s of CD45.1 × CD45.2 mice) (Fig. 5A). We selected three time-points following transplantation (3, 6, and 12 weeks) to measure the reconstitution process, and the BM and PB cells of recipient mice were monitored by FACS analysis of the presentation of $CD45.2^+$ donor cell. We set the wild type competitor (CD45.1) donor cells at a value of one, and then calculated the relative value of $CD45.2^+/CD45.1^+$ donor cells for the two groups. Higher value represents higher cell proliferation ability. At 3 weeks posttransplantation, this analysis revealed a significant reduction in repopulation potential of Ube1L-deficient cells compared with wild-type cells ($p = 0.0141$) (Fig. 5B–C). Reconstitution analysis was also performed at 6 and 12 weeks after transplantation, and no difference was observed between wild-type and Ube1L-deficient cells contribution to total BM reconstitution and the two transplanted cell populations contributed approximately equally (Fig. 5D–E). Furthermore, the contribution of $Ube1L^{-/-}$ derived cells to overall PB reconstitution was not significantly different from that of mice transplanted with wild-type control cells at three time-points, although showed the same trend as seen in BM (Supplementary. Figure S3). Conclusively, these results strongly suggest that although Ube1L-deficient BM contains normal primitive hematopoietic cells, the repopulating capacity of MPPs population, not LT- or ST-HSCs, is reduced in $Ube1L^{-/-}$ cells compared to wild-type cells in a competitive situation by responding to a stress of transplantation.

MPPs population of Ube1L-deficient cells showed a G2/M phase block in cell cycle

To understand the less efficient repopulation of primitive $Ube1L^{-/-}$ cells in the stress condition, we performed BrdU incorporation assay to analyze the cell cycle status of hematopoietic cells under the condition with high levels of IFNs and protein ISGylation

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(Fig. 3). Ube1L-deficient and wild-type BM cells were transplanted into lethally irradiated recipients; 7 days posttransplantation, BrdU was injected and the LSK population was stained with BrdU antibody and 7AAD for an analysis of cell cycle distribution. A significantly higher proportion of Ube1L$^{-/-}$ MPPs were found in the active G2/M phase ($p = 0.012$) compared to control cells (Fig. 6A–B). In the LT- and ST-HSC populations, we did not observe this phenomenon (data not shown). Collectively, these findings suggest that the G2/M phase block in Ube1L-deficient MPP population causes the delayed short-term reconstitution in recipients within a short period after hematopoietic stress that is induced by transplantation.

Discussion

To date, allogeneic hematopoietic stem cell transplantation (HSCT) is the only therapy with the potential to cure patients with leukemia. Most strategies for experimental or clinical BMT still have involved treatments of recipients with irradiation with the result that the intramedullary environment in hosts and elevations of a variety of cytokines differ dramatically from the normal intramedullary environment. However, little is known about these effects on bone marrow engraftment or long-term renewal and whether their influence may be positive or negative. Furthermore, rapid and efficient replacement of short-lived myeloerythroid cell lineages is critical to overcome life-threatening cytopenia following severe insults to the hematopoietic system, as well as in the first weeks after transplantation [20].

Type I IFN signaling plays a critical role in ISG15 modification and ISG15 modifies many cellular proteins following IFN treatment [21;22]. In this study we demonstrated for the first time the increased high levels of IFNs and protein ISGylation in recipient BM shortly after transplantation. This fact may indicate that the IFNs signaling and IFNs stimulated protein ISGylation are involved in the regulation of hematopoietic stem and progenitor cell behavior. Here, we have for the first time evaluated the effects of the protein ISGylation in adult murine hematopoiesis.

Our initial study shows that Ube1L deficiency is dispensable in normal homeostasis condition. In the further transplantation experiment, in the case of Ube1L$^{-/-}$ mice, the repopulation of BM cells in lethally irradiated recipients after total BMT was clearly delayed, and the deficiency led to a 50% reduction in cellularity of BM at 3 weeks after transplantation and caused a mild reduction in peripheral blood cellularity without affecting differentiation or lineage choice, however, no differences were observed at 6 and 12 weeks posttransplantation. The reduction is not caused by alterations in lineage commitment of hematopoietic cells, but the defect seems to be at a more primitive level, which suggested a beneficial role of protein ISGylation in the accelerating repopulation of hematopoietic cells. The colony-forming ability of clonogenic progenitors seems not to be significantly affected by the deficiency of Ube1L; however, in CFU-S12 assay, the colony size derived from the Ube1L-deficient cells is significantly smaller presumably owing to the delayed repopulation rate, indicating that protein ISGylation can influence the proliferation of multipotent progenitor cell population under stress condition of transplantation. Furthermore, competitive repopulation assay with BM-derived cells demonstrates an impaired repopulation rate of Ube1L-deficient cells in recipients in the first three weeks. Interestingly, when we analyze the cell cycle status of hematopoietic cells under the condition with high levels of IFNs and protein ISGylation (shortly after transplantation), the Ube1L deficiency can cause G2/M phase block in multipotent progenitor cell population at day 7 posttransplantation, which may be responsible for the delayed reconstitution at three weeks after transplantation. However, other important factors such as survival, differentiation, and homing of these primitive cells seem not to be affected.

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The key question emerging from this study is why the deficiency of protein ISGylation results in such an obvious reduction in repopulating capacity of MPPs. One potential explanation for this defect is that under transplantation condition, the high levels of IFNs and protein ISGylation in recipients bone marrow are showed shortly (from the second day to 8th days) after transplantation, and that period is very important for MPP population to proliferate and differentiate into mature cells. It is reported that in the bone marrow reconstitution process, at three weeks after transplantation, both ST-HSCs and MPPs have a combined myeloid and lymphoid differentiation potential and are equally efficient at rapidly reconstituting total blood cell levels in lethally irradiated recipients. However, at five weeks after transplantation the contribution of ST-HSCs to total PB reconstitution was further enhanced, whereas, MPP reconstitution was reduced almost 10-fold [18]. It indicates that MPP cells population, either from the infused donor cells or derived from ST-stem cells population, is the major cells responsible for the bone marrow early-stage reconstitution. Therefore, the protein ISGylation could function within this subpopulation of stem cells for active hematopoiesis, suggesting a protein ISGylation-dependent development program that regulates MPP population in commitment and proliferation processes rather than self-renewal events during transplantation.

It is well known that to meet the need of a variety of situations, ranging from normal homeostasis to acute blood loss, hematopoiesis must be rapidly and tightly regulated, particularly during the transplantation of HSCs. Positive and negative regulators ultimately balance the transition from quiescence to proliferation of HSCs. Furthermore, effective engraftment of HSCs during transplantation may depend on their cell cycle status [23]. In this study, as a result of protein ISGylation loss, MPPs tend to proliferate slowly due to G2/M phase block. Given the potential importance of protein ISGylation in accelerating repopulation, we propose a model that protein ISGylation facilitates primitive progenitor proliferation through hastening the G2/M phase transition in cell cycle shortly post transplantation.

Till now, we have found that two proteins, 14-3-3σ and UbcH10, related to G2/M phase regulation have been modified by ISG15. 14-3-3σ is involved in the G2 checkpoint, which guards entry in mitosis by regulating the Cdc25 activation [24;25]. Considering our previous data that the E3 ubiquitin ligase Efp and the E2 ubiquitin conjugating enzyme UbcH8 mediate ISG15 modification of 14-3-3σ, which can be activated by type I interferon stimulation [26], it is a possibility that 14-3-3σ ISGylation can regulate its function and then to hasten cells into mitosis and proliferation responsive to the stress condition. Besides protein 14-3-3σ, UbcH10, the mitotic specific E2 protein, is also modified by ISG15, based on both our unpublished data and other report [27]. UbcH10 can be used by the anaphase-promoting complex/cyclosome (APC/C) to target mitotic regulators - such as Cyclin A, Cyclin B and securin - to the proteasome for degradation [28]. This UbcH10-dependent destruction is required for anaphase onset and forces cells to exit mitosis around the time of the metaphase – anaphase transition. Considering the fact that MPPs population shows G2/M phase block, combined with the delay reconstitution on the early-stage, it is a possibility that ISG15 modification of UbcH10 enhances its proteolysis function to increase cell cycle progress (exit from mitosis) during the hematopoiesis stress. Taken together, these observations indicate that the protein ISGylation modification may regulate the function of 14-3-3σ or UbcH10 during cell cycle transition; however, it is difficult to clearly demonstrate the protein expression changes due to the specific cell population and complicated cytokines regulation network during the hematopoiesis reconstitution.

In conclusion, we have for the first time evaluated the physiologic role of the protein ISGylation in steady-state hematopoiesis and its potential protective role during repopulation. We conclude that the physiologic role of protein ISGylation has a limited, if

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any, effect on the hematopoietic cells during steady-state hematopoiesis, however, in conditions that call for rapid proliferation, such as BMT, protein ISGylation appears to be critically involved in promoting and enhancing the proliferative response of accelerating repopulation from MPP population by hastening G2/M phase transition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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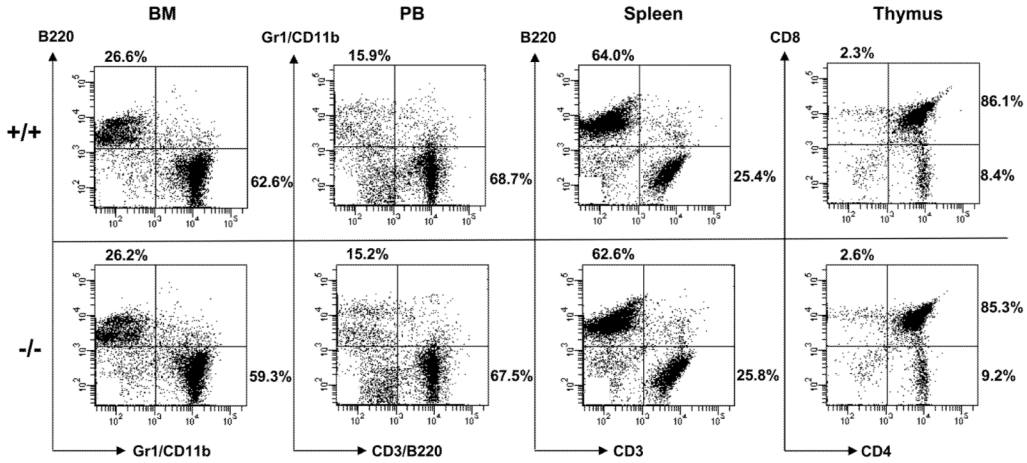

Figure 1. Normal hematopoietic lineage distribution in Ube1L$^{-/-}$ mice on steady state

Representative FACS analysis on cells derived from PB, BM, spleen, and thymus showed no significant difference in the ratios of myeloid cells (Gr-1, CD11b), B-cells (B220), and T cells (CD3, CD4, CD8) between knockout and wild-type littermates (n = 3 for both Ube1L$^{+/+}$ and Ube1L $^{-/-}$ mice).

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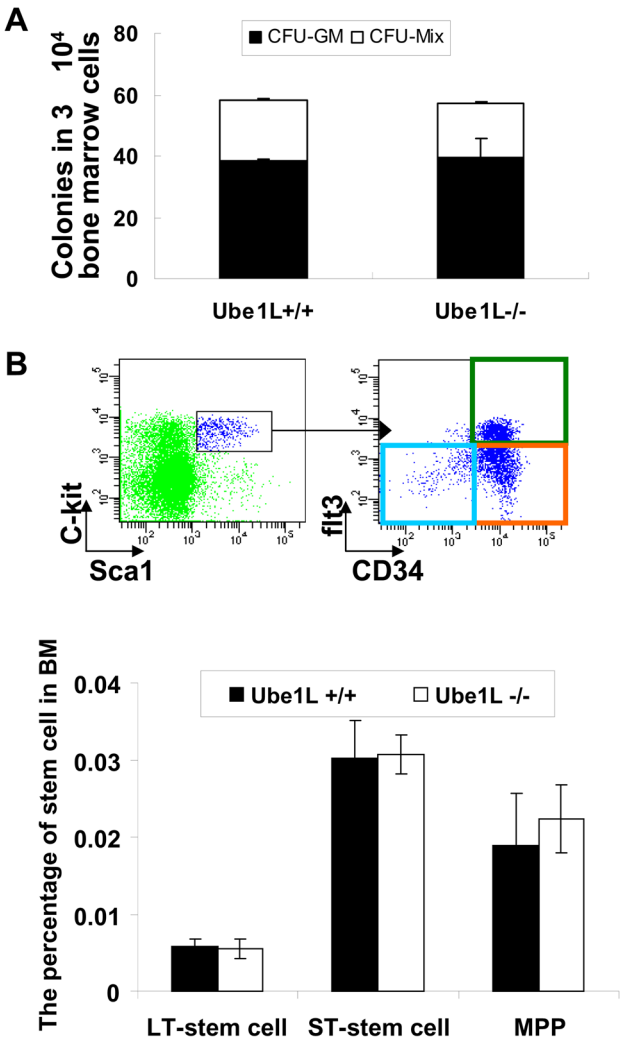

Figure 2. Normal numbers of committed myeloid progenitor and stem cell in Ube1L$^{-/-}$ mice

(A) Methylcellulose assay indicating the number of myeloid colony-forming units (CFUs) per 30,000 cells plated. Platings from each animal were performed in triplicate using bone marrow from a total of 3 animals per genotype. (B) The steady-state level of stem cells was evaluated using FACS staining analysis for LT-, ST-stem cells and MPPs. There was no difference in the distribution of stem cells between wild-type and knockout mice. Data from 4 mice per each group (mean ± SD).

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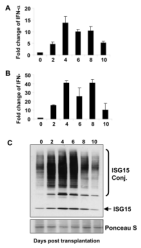

Figure 3. Increased IFNs and protein ISGylation levels in recipient mice after BMT

Using real-time PCR method to test the IFN-α (A) and IFN-β (B) mRNA levels in BM cells from lethally-irradiated recipient mice receiving total bone marrow cells at 2, 4, 6, 8 and 10 days after transplantation. Set day 0 control as the one, the relative fold change in different time points is compared to control. Each bar represents the average expression level from at least six animals in two independent experiments. Error bars represent the standard deviation of the average relative expression among animals. (C) Using Western-blotting method to test the protein ISGylation levels in the bone marrow cells from lethally-irradiated recipient mice receiving total bone marrow cell transplantation. Examples shown are representative of two independent experiments.

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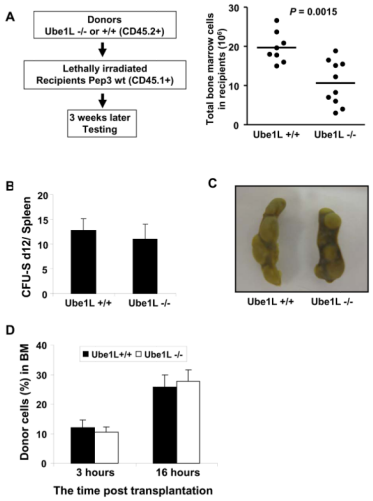

Figure 4. Transplantation studies to assess hematopoietic cell proliferation

(A) Reduced repopulating ability of Ube1L deficient HSCs in lethally irradiated recipients. 1 × 10^6 bone marrow cells taken from either Ube1L$^{+/+}$ or Ube1L$^{-/-}$ mice were directly injected to lethally irradiated recipient mice. 21 days after BMT, the recipient mice were sacrificed and the total bone marrow cells were counted. Data were collected from four batches of individual transplantation experiments. (B) Normal multipotent myeloid progenitors but less proliferation capacity in Ube1L$^{-/-}$ mice under transplantation. 80,000 fresh knockout or wild-type BM cells were injected into lethally irradiated normal recipients for CFU-S12. The findings show that there is no difference in frequency of hematopoietic progenitors. (C) Representative CFU-S12 from recipients. Although frequency of CFU-S was similar (four experiments, three to four donors of each genotype per experiment), the colonies generated by Ube1L$^{-/-}$ BM were estimated to be 50% smaller than wild type colonies. Data represent mean ± SD. (D) Normal homing ability of Ube1L$^{-/-}$ bone marrow cells. 1 × 10^6 Ube1L$^{+/+}$ or Ube1L$^{-/-}$ (CD45.2) BM cells were transplanted into lethally irradiated pep3 wt (CD45.1) recipients. Homing of donor cells in BM of recipients was measured as a percentage of engrafted CD45.2$^+$ donor cells by FACS analysis at 3 and 16 hours after transplantation. Shown are the mean ± SD. Pooled data from two independent experiments per time point.

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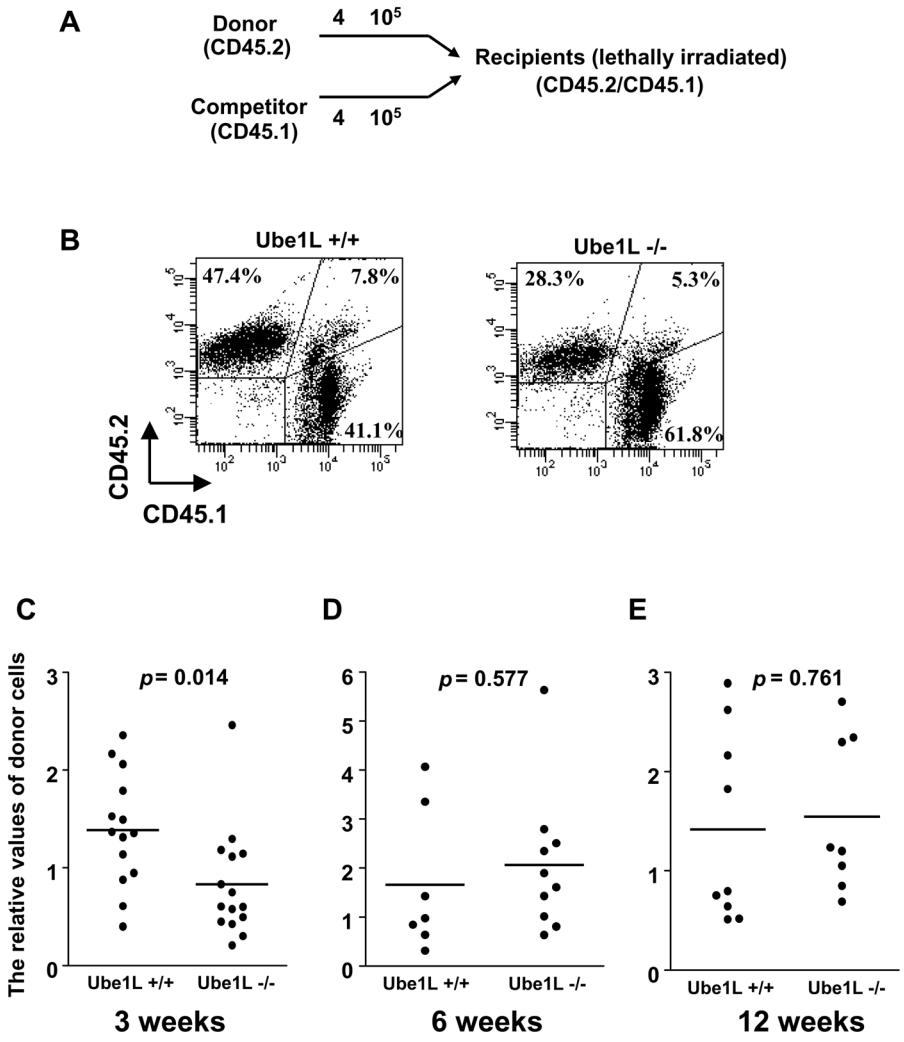

Figure 5. Reduced competitive repopulation capacity of Ube1L$^{-/-}$ hematopoietic cells under transplantation

(A) Total bone marrow cells (4×10^5) from adult Ube1L$^{+/+}$ and Ube1L$^{-/-}$ (CD45.2$^+$) mice were injected into lethally irradiated normal recipient mice (CD45.1$^+$/CD45.2$^+$) together with an equal number of competitor bone marrow cells from wild-type pep3 (CD45.1$^+$) mice. (B) Representative CD45.1 and CD45.2 staining of recipient bone marrow cells at 3 weeks post-transplantation. (C–E) Contribution of Ube1L$^{+/+}$ and Ube1L$^{-/-}$ cells in bone marrow of recipients was analyzed at 3, 6, and 12 weeks after transplantation. Set the value for Pep3 wt (CD45.1$^+$) donor cells at one, and then compared the relative value of CD45.2$^+$ and CD45.1$^+$ donor cells for the two groups. Higher value represents higher cell proliferation ability. Results represent the average values.

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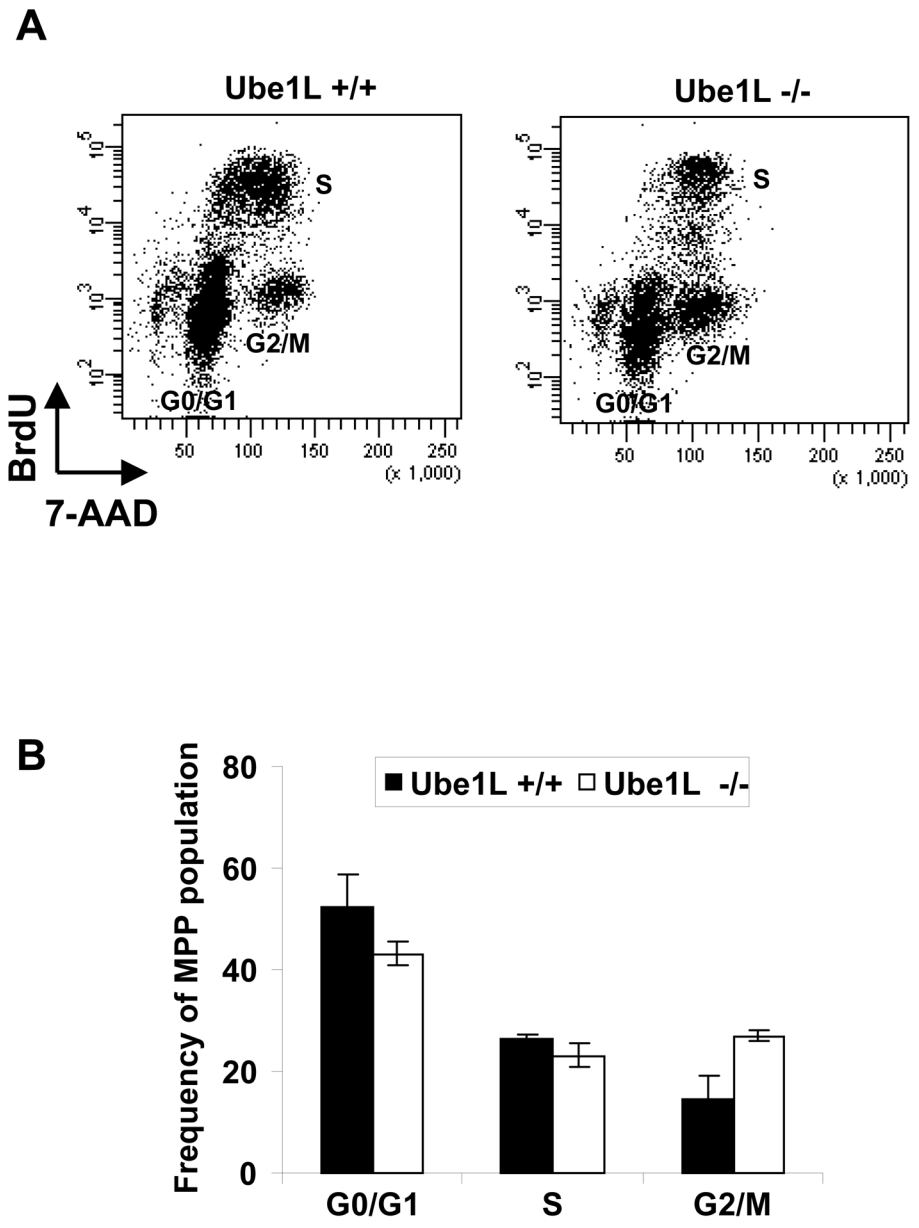

Figure 6. MPP population of Ube1L$^{-/-}$ hematopoietic cells showed a G2/M phase stock in cell cycle at the initial stage of transplantation

(A) Representative FACS analysis of BrdU and 7-AAD incorporation in MPP subsets at 90 min post-IP injection of a single dose (2 mg) of BrdU. (B) Numbers indicate the frequency of cells in G0/G1, S, and G2/M phases of the cell cycle (three separate experiments, three donors of each genotype per experiment). Data represent mean ± SD.

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Table I

Cellularities in hematopoietic organs are similar between Ube1L$^{+/+}$ and Ube1L$^{-/-}$ mice.

Mouse genotype	Spleen wt (mg)	Thymus wt (mg)	RBC count (10^{12}/liter)	Hemoglobin (g/liter)	WBC count (10^{9}/liter)	BM cellularity (two femurs/tibeas[10^{6}])
Ube1L$^{+/+}$	80.3 ± 13.4	48.0 ± 4.0	8.8 ± 0.2	12.3 ± 0.3	10.1 ± 0.9	63.1 ± 12.5
Ube1L$^{-/-}$	77.7 ± 9.5	47.7 ± 4.7	8.8 ± 0.5	12.7 ± 0.9	9.5 ± 1.1	61.2 ± 14.8

Data presented as means ± SD; n=4. wt: weight; RBC: red blood cell; WBC: white blood cell; BM: bone marrow.