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STEM CELLS / Volume 35, Issue 4

Tissue-Specific Stem Cells |

Pre-Transplantation Blockade of TNF- α -Mediated Oxygen Species Accumulation Protects Hematopoietic Stem Cells

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First published: 18 October 2016

<https://doi.org/10.1002/stem.2524>

Cited by:2

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ABSTRACT

Hematopoietic stem cell (HSC) transplantation (HSCT) for malignancy requires toxic pre-conditioning to maximize anti-tumor effects and donor-HSC engraftment. While this induces bone marrow (BM)-localized inflammation, how this BM environmental change affects transplanted HSCs in vivo remains largely unknown. We here report that, depending on interval between irradiation and HSCT, residence within lethally irradiated recipient BM compromises donor-HSC reconstitution ability. Both in vivo and in vitro we demonstrate that, among inflammatory cytokines, TNF- α plays a role in HSC damage: TNF- α stimulation leads to accumulation of reactive oxygen species (ROS) in highly purified hematopoietic stem/progenitor cells (HSCs/HSPCs). Transplantation of flow-cytometry—sorted murine HSCs reveals damaging effects of accumulated ROS on HSCs. Short-term incubation either with a specific inhibitor of tumor necrosis factor receptor 1 signaling or an antioxidant *N*-acetyl-L-cysteine (NAC) prevents TNF- α -mediated ROS accumulation in HSCs. Important pre-transplantation exposure to NAC successfully demonstrates protective effects in inflammatory BM on graft-HSCs, exhibiting better reconstitution capability than that of



Hematopoietic reconstitution kinetics in transplantation with myeloablative conditioning that inevitably causes inflammation in recipient BM. *STEM CELLS* 2017;35:989-1002

SIGNIFICANCE STATEMENT

This study shows the following: (a) depending on interval between TBI and HSCT, residence within lethally irradiated recipient BM compromises donor-HSC reconstitution ability; (b) elevated levels of TNF- α in inflamed BM is responsible for the effect; (c) TNF- α induces formation of ROS through an NADPH oxidase in donor HSCs, leading to impaired reconstitution ability; (d) Pre-incubation with an antioxidant NAC that suppresses TNF- α -stimulated ROS production successfully protects transplanted HSCs from BM inflammation. We eventually came up with the proposal of “in vivo stem cell protection” as a novel therapeutic concept in HSCT. These findings have implications for the basic HSC biology and for the improvement of an HSCT outcome for patients.

Introduction

Hematopoietic stem cell (HSC) transplantation (HSCT) for hematological malignancy general includes aggressive chemotherapy and irradiation, with effects that exceed dose-limiting toxicity. Although intense pre-conditioning is considered necessary to eradicate tumor cells and to create a niche for HSCs, it may also induce inflammation in recipient BM. While inflammation within the devastated BM is hypothesized to affect transplanted HSCs, such effects have not yet been experimentally illustrated [1](#). We therefore sought to clarify how the pre-conditioned BM environment might affect donor HSCs, focusing especially on inflammatory effects and on developing protective measures against them.

Stimulation of HSCs with inflammatory cytokines such as IFN- γ , TNF- α , or IL-1 generally appears to drive differentiation, leading to stem cell exhaustion and sometimes to malignancy [2-10](#). In some cases, however, inflammatory cytokines can be essential for HSC function [3, 11](#). These findings rely on mostly in vitro studies; in vivo effects of cytokines on transplanted wild type HSCs have been difficult to model. Among the above inflammatory cytokines, TNF- α was demonstrated to be a relevant in vivo suppressor of HSCs in the context of transplantation [12](#).



irradiated recipients. These results clearly indicate that negative effects are mediated by TNF- α on post-transplant hematopoiesis in irradiated hosts. This may translate into the idea that blockade of such negative effects will lead to improved HSC transplantation outcomes [8](#), [12](#).

To develop a clinically relevant measure to protect HSCs without genetic modification from deleterious effects inherent to post-transplantation environment, we thought it essential to know if the impairment of HSC function could occur even within a short-term period after transplantation. This is critical because if that is the case, a protective measure will be still effective for HSCs with only short-acting treatment. We therefore have developed a series of novel in vivo systems that allow testing the effects of BM inflammation on HSCs at transplantation and during engraftment ("graft-HSCs"). Of note is a short-term HSC exposure assay in which graft-HSCs are exposed only for a limited period to the inflammatory BM environment generated by preconditioning treatments. Using these assays, we indeed identified elevated levels of TNF- α in inflamed BM and demonstrated that these, by inducing formation of reactive oxygen species (ROS), impaired reconstitution ability of donor HSCs in secondary transplantation. We further demonstrated that protection was feasible by pre-incubation of HSCs with an anti-oxidant *N*-acetyl-L-cysteine (NAC) against ROS accumulation mediated by TNF- α in inflamed BM, leading to improved graft-HSC reconstitution capability. We propose "stem cell protection" as a novel therapeutic concept in HSCT.

Materials and Methods

Mice

C57BL/6 (Ly5.2⁺, B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan). Ly5.1⁺ congenic B6 (B6-Ly5.1) and Ly5.1⁺/5.2⁺ B6 F1 (B6-Ly5.1/5.2) mice were purchased from Sankyo Laboratory Service (Tsukuba, Japan). TNF- α knockout B6 (Ly5.2⁺) mice were provided by the Laboratory of Molecular Pathogenesis, Center for Experimental Medicine, Institute of Medicine, University of Tokyo.

Purification of Mouse Hematopoietic Stem/Progenitor Cells

Purification of mouse hematopoietic stem/progenitor cells (HSPCs), phenotypically defined as KSL cells (cKit⁺, Sca-1⁺, Lineage-marker⁻), is described [13](#), [14](#). Where indicated, highly purified HSCs were used (CD34^{-/low} cells among the KSL fraction: CD34⁻KSL cells), with the purity being comparable to that of SLAM-HSCs [15](#) due to our strict gating strategy [16](#). Briefly, BM cells isolated from femora, tibiae, and pelvic bones of adult mice (8-12 weeks) were incubated with allophycocyanin (APC)-conjugated anti-c-Kit monoclonal Abs (BD Biosciences, San Jose, CA).



using a cocktail of biotinylated mAbs specific for the antigens CD4, CD8, B220, IL-7 α , Gr-1, Mac-1, and Ter-119 (BD Biosciences), with phycoerythrin (PE)-conjugated anti-Sca-1 (eBioscience, San Diego, CA) and APC-conjugated anti-c-Kit (BD Biosciences) mAbs and APC/cyanin7 (Cy7)-streptavidin (eBioscience). FITC-anti-CD34 mAb (eBioscience) was added to obtain CD34⁻KSL cells. Cells were sorted on a FACS Aria II cell sorter (BD Biosciences).

Transplantation

Recipient mice fed with acidified water (~pH 2.5) for over 1 week before BMT were lethally irradiated with a total dose of 9.8 Gy (2 split doses 3 hours apart) or a single dose of 9.5 Gy at stated times and were intravenously transplanted with cells as specified. After transplantation they were fed with acidified water containing enrofloxacin (Baytril; Bayer Animal Health, Leverkusen, Germany) for at least 2 wks. Serial transplantation was performed either with unfractionated BM cells from primary recipients in a noncompetitive manner or with sorted cell populations in a competitive manner.

Treatment of Mouse Hematopoietic Stem/Progenitor Cells

For in vitro ROS detection, 3,000 KSL cells were sorted into individual wells of a 96-well plate containing 200 μ l of S-clone (Eidia, Tokyo, Japan) supplemented with 1% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO), 1% penicillin-streptomycin-glutamine mix (PSG; Life Technologies, Carlsbad, CA), 100 ng/ml murine stem cell factor (SCF), and 100 ng/ml murine thrombopoietin (TPO) with or without murine TNF- α (all Peprotech, Rocky Hill, NJ).

Competitive Repopulation Assays

Competitive repopulation assays were performed using a Ly5-congenic mouse system as described [17](#). Freshly isolated CD34⁻KSL cells from B6-Ly5.1 mice or cultured CD34⁻KSL cells, the numbers shown, were transplanted into lethally irradiated B6-Ly5.2 mice together with 10^5 or 5×10^5 whole BM competitor cells from B6-Ly5.1/5.2 F1 mice. Intervals between TBI and transplantation were as stated. Peripheral blood cells of the recipient mice were analyzed at the indicated times. After treatment with ACK lysis buffer (NH₄Cl 8,024 mg/l, KHCO₃ 1,001 mg/l, EDTA.Na₂.2H₂O 3.722 mg/l), the nucleated cells were stained with the following mAbs: FITC-conjugated anti-CD45.2 (Ly5.2, BioLegend), PE/Cy7-conjugated anti-CD45.1 (Ly5.1, eBioscience), PE-conjugated anti-Mac1 (eBioscience), PE-conjugated anti-Gr-1 (eBioscience), APC/Cy7-conjugated anti-CD45R/B220 (eBioscience), APC-conjugated anti-CD4 (BioLegend), and APC-conjugated anti-CD8a (BioLegend). Donor chimerism was determined as the percentage of Ly5.1⁺ cells within each cell population (Ly5.1⁺ + Ly5.1⁺/Ly5.2⁺) using a FACS Canto flow



Test KSL cells (B6-Ly5.1, male, 8 weeks) were directly sorted into 96-well round-bottom plate at 1,000 cells per well. Each well contained S-clone + 1% BSA with SCF and TPO (100 ng/ml) as basal medium. Plates were incubated at 37°C, 5% CO₂ for 4 hours, with control wells containing vehicle alone (nonprotected) and other wells containing NAC (Sigma-Aldrich) at either 0.1 mM or 4 mM (protected). These cells were combined with fresh (nonprotected) 1 × 10⁶ unfractionated BM cells (B6-Ly5.1/5.2, male, 8 weeks), and transplanted into recipient mice ($n = 8$, B6-Ly5.2, female, 8 weeks). In these particular assays, recipient mice were lethally irradiated (4.75Gy × 2) 2 days before transplantation. For secondary transplantation, BM cells were collected at 20 weeks from all available primary recipients in each group. These cells were pooled, and transplanted into lethally irradiated mice (each received cells corresponding to ~70% BM cells in a single femur of primary recipient mice; $n = 8$, B6-Ly5.2).

Isolation of BM Stromal Cells

To assess induction of gene expression after irradiation, stromal cells were isolated from BM samples as described [18](#). Briefly, femora and tibiae obtained from B6 mice ($n = 6$) either nonirradiated or after 9.5 Gy irradiation, at indicated times were cut into small fragments and digested for 30 minutes with 0.2% collagenase type 1 (Wako, Tokyo, Japan). After removal of undigested materials, the cell suspension was collected and treated with a hemolysis buffer. Hematopoietic cells containing residual erythrocytes were removed by a magnetic separator after incubating cells with biotin-anti-CD45.2 and -Ter-119 mAbs (e-Bioscience) followed by streptavidin beads (Dynabeads, Life Technologies). Finally, highly purified nonhematopoietic cells were obtained through flow cytometry and sorting (FACS Aria II, BD Biosciences) as a population lacking CD45 and Ter-119 expression.

Quantitative PCR Analysis

For IL-1 β , TNF- α , and IFN- γ RT-PCR, 1.2 × 10⁵ stromal cells were collected; for Tumor Necrosis Factor Receptor 1 (TNFR1), riboflavin kinase, and p22phox RT-PCR, 5.4 × 10⁵ KSL cells were collected, by flow cytometry and subjected to RNA isolation (RNeasy MicroKit; QIAGEN, Venlo, Netherlands). cDNA samples were synthesized using High Capacity cDNA Reverse Transcription Kits (Life Technologies). For quantitative PCR (qPCR) reactions, samples were prepared in triplicate and reactions were run for each sample in 20 μ l of mixed reagents including 2 μ l of cDNA, Eagle Taq Master Mix with ROX (Roche Life Science, Basel, Switzerland), 10 μ M probe (from a Roche-Universal Probe Library, Basel, Switzerland), and 10 μ M of the following primers (5' → 3'), with IL-1 β : forward, agttgacggaccccaaaaag/reverse,



ggaaagatgcccataagaaaca/ reverse, agtcaaccacccaaagtaggctctt, riboflavin kinase: forward, tccaagcaactgggcatc/reverse, gctggaagattgtccaccac; and p22phox: forward, tgccctccacttctgtt/reverse, gcagatagatcacactggcaat. Annealing temperature was 60°C. Results were analyzed using the QuantStudio TM 7 Flex Real-Time PCR System and ViiA TM 7 Software (Life Technologies). Transcript levels were normalized against Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression (Taqman Rodent Gapdh Control Reagents, Life Technologies). The data were analyzed by a comparative cycle threshold method (IL-1 β , TNF and IFN- γ) or absolute quantification using standard curves (TNFR1, riboflavin kinase and p22phox)

Microarray Analysis

Preparation of cDNA samples from BM-resident stromal cells is described above. The data were obtained and analyzed using a high-sensitivity DNA Chip 3D-Gene system at TORAY (Tokyo, Japan). The microarray data have been deposited in the Gene Expression Omnibus at the National Center for Biotechnology Information (accession number: GSE72235; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qtohowsejrapfad&acc=GSE72235>)

ROS Detection Assay

After culture of CD34⁺KSL or KSL cells for indicated periods, cells were collected, stained with 2',7'-dichlorofluorescein (DCF, excitation: 495 nm, emission: 529 nm, Sigma-Aldrich) or HySC (555 nm, 575 nm, GORYO Chemical, Sapporo, Japan) [19](#) for 30 minutes at 37°C, and analyzed by flow cytometry (FACS Calibur, BD Biosciences). ROS production was quantitated in terms of dye fluorescence intensities.

TNFR1-Derived Peptide (PepTNFR1)

A TNFR1-derived peptide, PepTNFR1 (amino-acid sequence: YARAAARQARAP-YAVVENVPPLRWKEFVRRRLGLSDHEIDRLELQN; molecular mass: 5,275.97 Da), was synthesized by Life Technologies as described [20](#). The scramble peptide, PepScr (amino-acid sequence: YARAAARQARAP-AEPDEPVVDRLNVVLYRLGQFSKRRLNIWLH; molecular mass: 5,275.97 Da), was used as control. Both PepTNFR1 and PepScr were applied (60 μ M) for 4 hours to test cells before stimulation by TNF- α . ROS production was measured 48 hours after TNF- α addition.

Statistics

Two groups were compared with a Mann-Whitney test, whereas multiple groups were compared using one-way ANOVA followed by a Tukey's post hoc test. A value of $p < .05$ was



All animal experiments were performed under the approval of the Institutional Animal Care and Use Committee of the Institute of Medical Science, University of Tokyo.

Results

Initial In Vivo Exposure to Ongoing Inflammation in an Irradiated BM Environment Affects Graft-HSCs' Reconstitution Ability

We first tested if the recipient BM environment would affect graft-HSCs differently depending on interval between total body irradiation (TBI) and transplantation. To this aim, test HSCs (purified CD34⁻/low cKit⁺ Sca-1⁺ Lineage-marker⁻ cells, CD34⁻KSL cells hereafter) obtained from Ly5.1⁺ C57BL/6 (B6) mice were transplanted at a dose of 50 cells together with 1×10^6 competitor BM cells (from Ly5.1⁺/5.2⁺ B6 mice) into primary recipient mice (Ly5.2⁺ B6) irradiated at different times (Fig. 1A, Primary transplantation). All mice transplanted 5 days after TBI ("day 5" group) soon died, precluding subsequent experiments. Some mice also die in a group transplanted 3 days after TBI ("day 3" group); however, serial analysis of donor cell chimerism was feasible in the survivors for up to 24 wks, allowing comparison with another group transplanted on the day of TBI ("day 0" group). As shown in Fig. 1B, left, no significant difference in donor cell chimerism existed between groups. We interpreted this as reflecting equality of recipient BM environmental influence (or lack of influence) on both test HSCs and competitor graft cells. We thus purified Ly5.1⁺ hematopoietic stem/progenitor cells (HSPCs) from these primary recipient mice and competitively transplanted 1,000-cell aliquots into secondary recipient mice to compare reconstitution ability among HSCs that had experienced the TBI-BM environment under different conditions (Fig. 1A, Secondary transplantation). Remarkably, HSPCs initially transplanted 3 days after TBI ("day 3") contributed significantly less to hematopoietic reconstitution in the secondary recipients than did "day 0" HSPCs (Fig. 1B, Secondary), indicating impaired stem cell functions in the former.

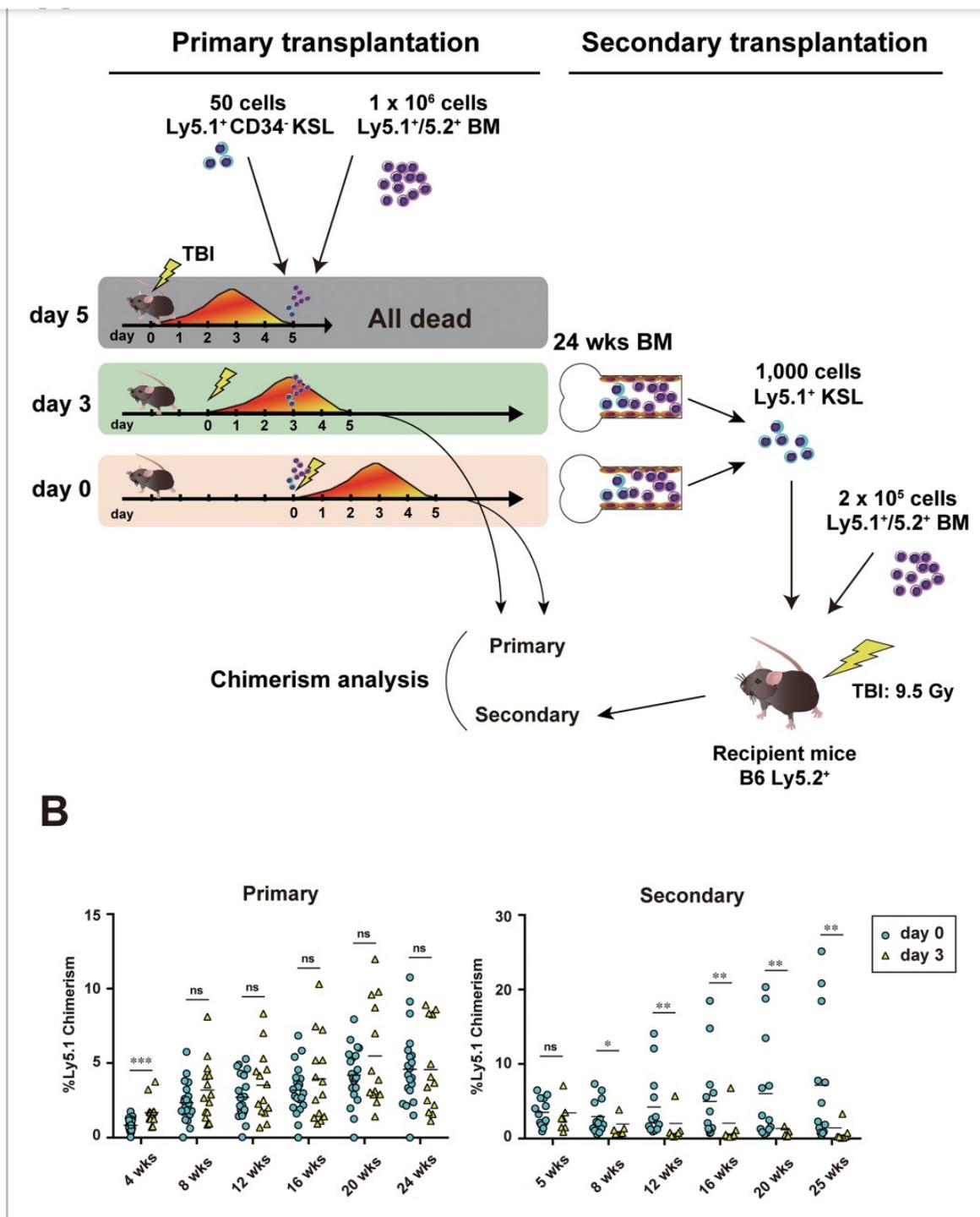


Figure 1

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The influence of an irradiated BM environment on reconstituting HSCs long-term after transplantation. **(A)**: Primary transplantation. Test HSCs (50 cells per mouse, Ly5.1⁺ CD34⁻KSL cells) were competitively transplanted with 1 × 10⁶ cells of BM (Ly5.1⁺/5.2⁺ BM cells) into recipient mice (B6 Ly5.2⁺) after TBI. All mice died by day 5. **(B)**: Secondary transplantation. Test HSCs (1,000 cells per mouse, Ly5.1⁺ KSL cells) were competitively transplanted with 2 × 10⁵ cells of BM (Ly5.1⁺/5.2⁺ BM cells) into recipient mice (B6 Ly5.2⁺) after TBI (9.5 Gy). Chimerism analysis was performed at 4, 8, 12, 16, 20, and 24 weeks for primary and 5, 8, 12, 16, 20, and 25 weeks for secondary transplantation. The graphs show that chimerism levels were significantly higher in the secondary group, especially at later time points.



We next sought to test how “short-term” (~24 hours) exposure to an irradiated BM environment could affect graft-HSCs. To this aim, we developed new transplantation experiments (Fig. 2). In brief, purified HSCs alone (400 cells) were first transplanted into recipient mice at different times after lethal-dose TBI (Fig. 2A, 2B, In vivo HSC exposure transplantation). After 24 hours, when a substantial proportion of transplanted cells was thought to reside within BM, whole-BM cells (equivalent doses were ensured on a bone-volume basis, thereby taking into account variable cell yields after TBI. See Materials and Methods) were competitively transplanted into lethally irradiated recipient mice. In this experiment, transplanting whole BM cells ~24 hours after HSC infusion with competitor cells into another cohort of irradiated mice permits assessment of reconstitution ability in test HSCs residing in BM of the initial recipient (Fig. 2A, 2B, Primary transplantation). As shown in Fig. 2, achieved chimerism tended to decrease with interval between TBI and exposure to the irradiated BM environment; thus chimerism was least with HSCs exposed to irradiated BM between 48 and 72 hours after TBI (d2-3), while with those exposed to irradiated BM in the first 24 hours after TBI (d0-1), chimerism was greatest (Primary). When HSCs were serially transplanted, loss of contribution to hematopoiesis became evident with exposure late after TBI (Fig. 2C, Secondary, d2-3 and d3-4). Of importance is that the first two groups also clearly differed; in secondary transplantation, HSCs exposed to BM for the first 24 hours after TBI (d0-1) outperformed those exposed for the second 24 hours after TBI (d1-2). These results indicate collectively that HSCs’ reconstitution ability can be impaired within an “inflamed” BM environment even during a short-term (~24 hours) time window.

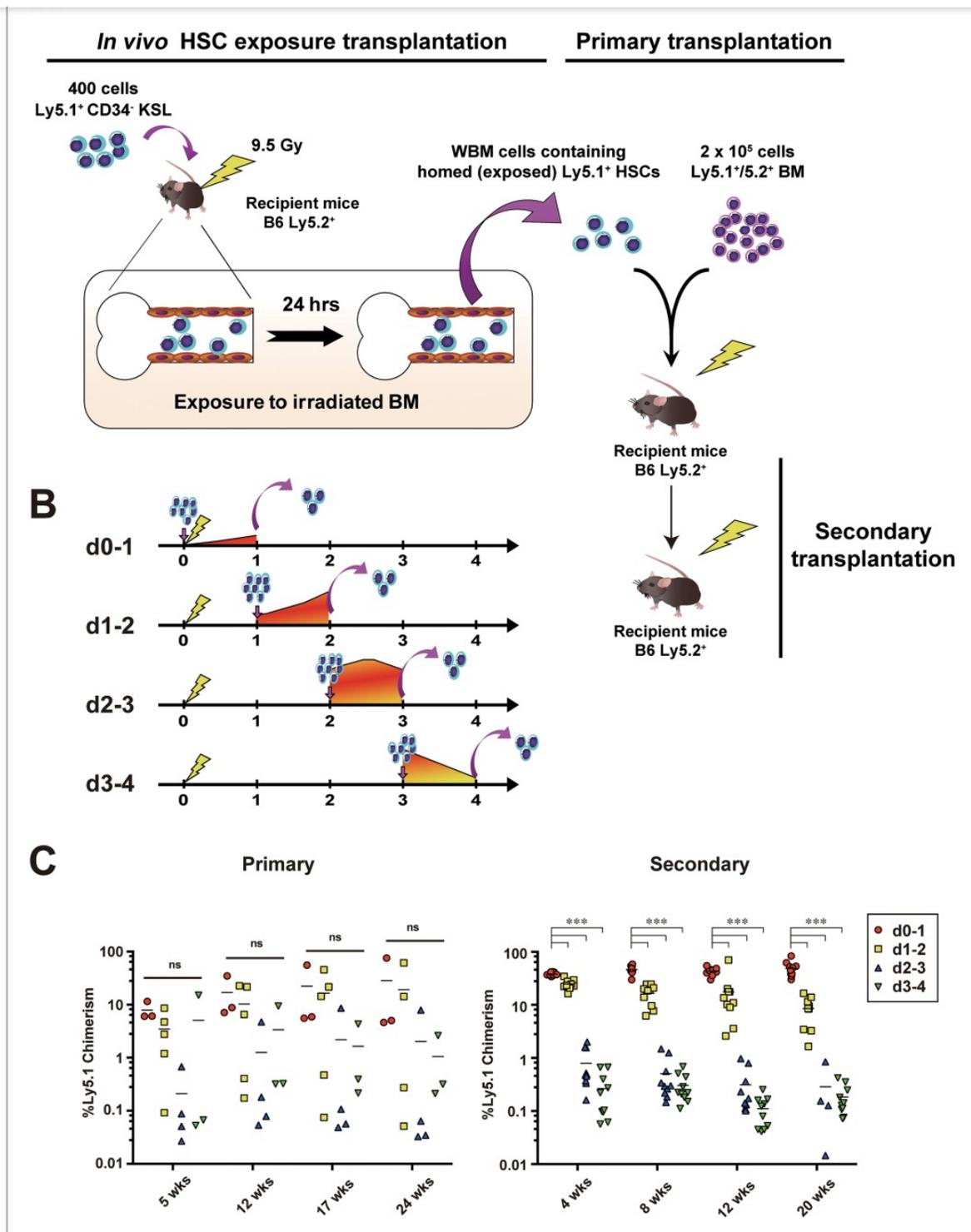


Figure 2

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The influence of short-term exposure to an irradiated BM environment on reconstitution ability of HSCs. **(A)**: Schematic representation of a newly designed experiment to test the influence of short-term (24 hrs) exposure to



For an overview of BM environmental change caused by TBI, we performed comprehensive gene expression analysis in BM-resident stromal cells considered to constitute the HSC niche. Expression of genes encoding certain inflammatory cytokines and chemokines rose one day after TBI, quickly returning to basal levels (Fig. 3A). We focused on 3 major inflammatory cytokines, IFN- γ , IL-1 β , and TNF- α (Fig. 3B); induction of gene expression for the latter two was confirmed by re-analysis (Fig. 3C). To compare the effects of these cytokines on HSCs, we conducted in vitro HSC colony forming assays as previously described 13, 21. Only TNF- α inhibited colony formation of HSCs in a dose dependent manner. The other 2 cytokines' effects were marginal (Fig. 3D). To examine in vivo relevance of TNF- α 's role, we next used in vivo short-term HSC exposure assays to test if deletion of host-derived TNF- α could preserve HSC functions, comparing TNF- α KO BM with wild type BM (Fig. 4A). As shown in Fig. 4B, left, graft-HSCs exposed to TNF- α KO BM tended to achieve greater chimerism, although no statistical difference existed between the groups' results (WT vs. TNF- α KO, Primary). Interestingly, however, this difference was clearly magnified upon secondary transplantation with better preservation of reconstitution ability in graft-HSCs initially exposed to inflammatory BM lacking environment-derived TNF- α (Secondary). Detailed chimerism analysis revealed that the absence of TNF- α in inflammatory BM favored lymphoid potential more than myeloid potential in the test HSCs (Supporting Information Fig. 3). We concluded, in summary, that TBI provoked inflammation within BM and that TNF- α could impair graft-HSC function even in a short-term time window in a time-dependent fashion.

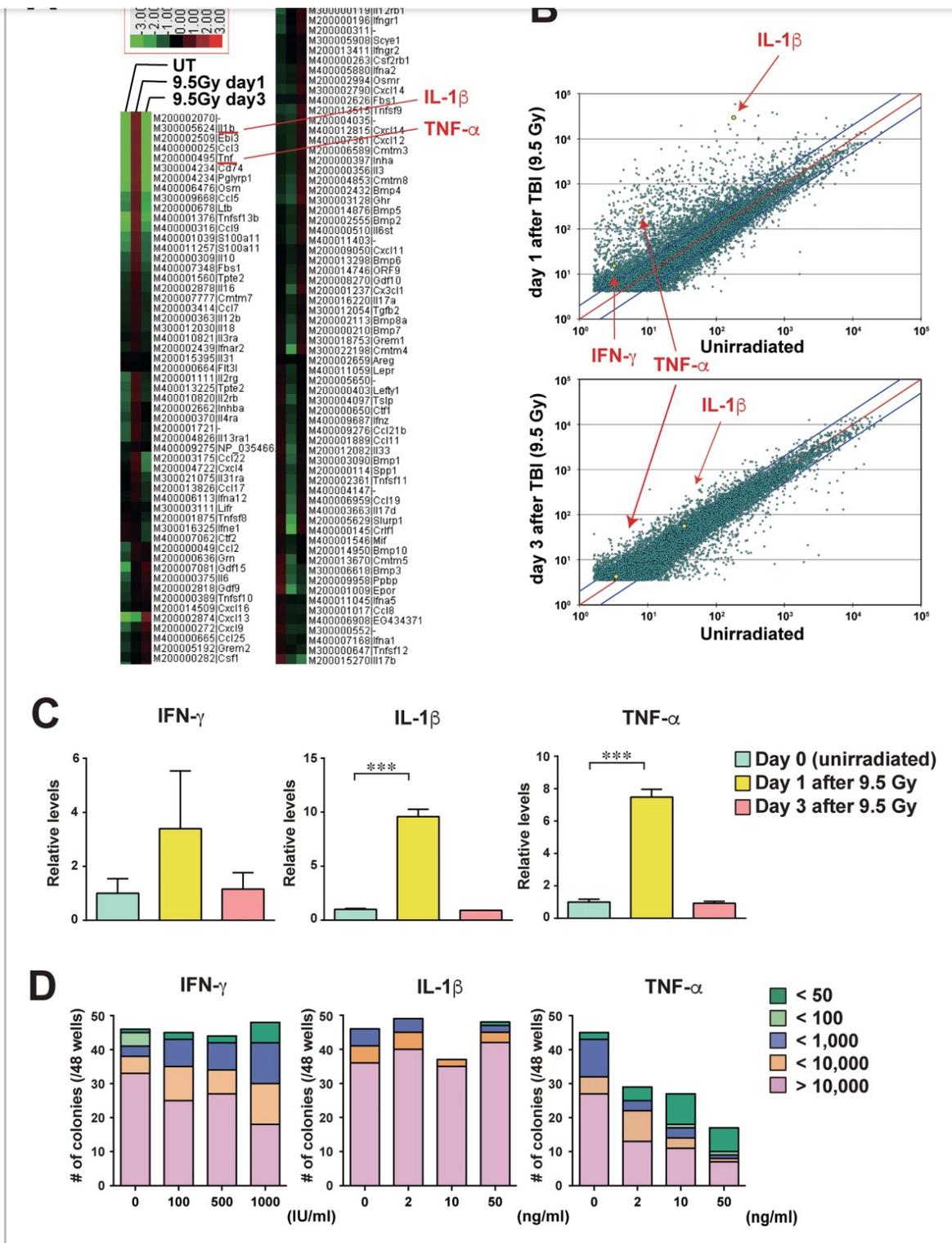


Figure 3

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Induction of inflammatory cytokine synthesis in BM-resident stromal cells by total body irradiation and a causal role for TNF- α in impairing reconstitution

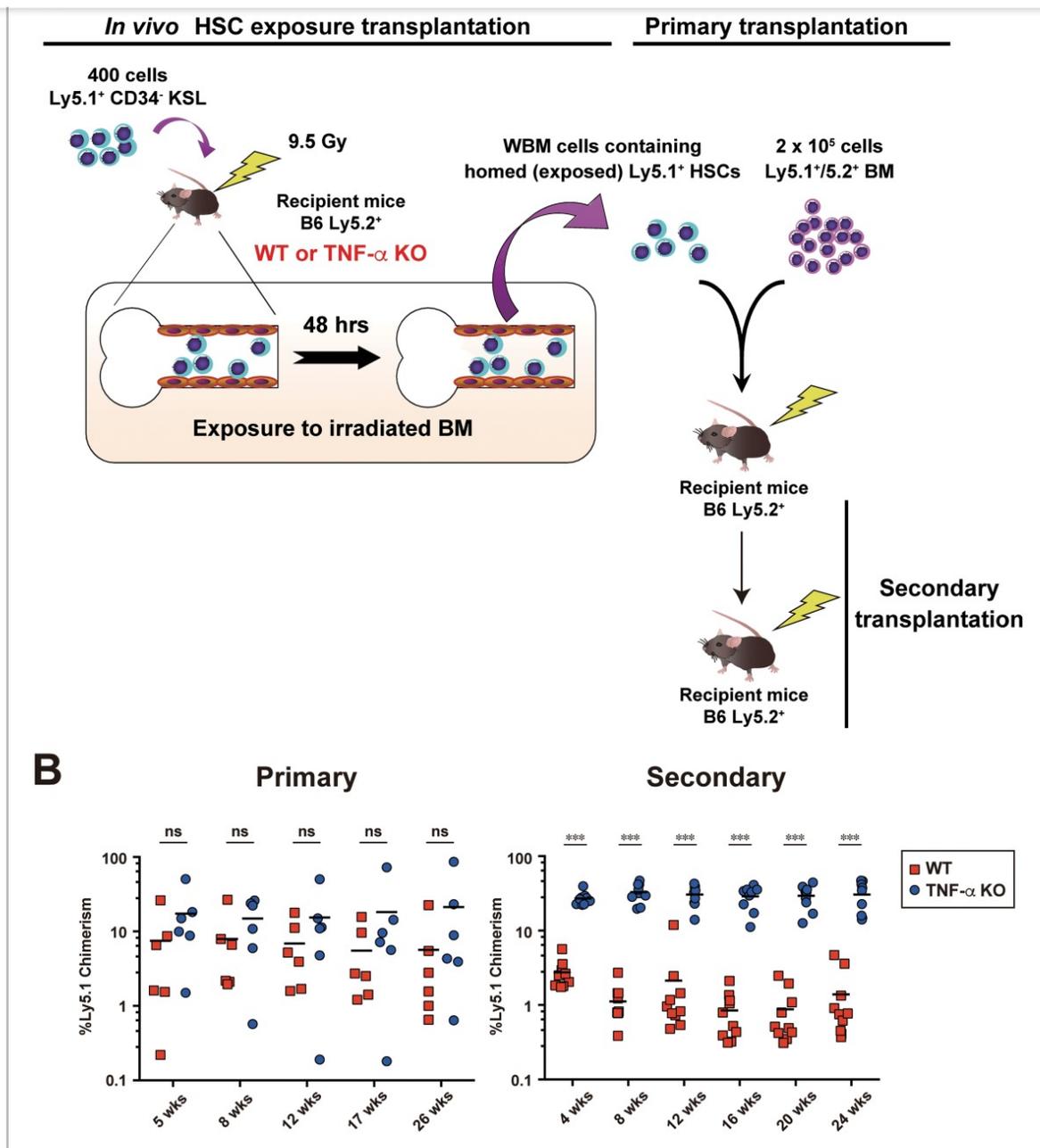


Figure 4

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Testing a causal role for TNF- α in impairment of HSCs' reconstitution ability within an irradiated BM environment. **(A)**: Schematic representation of an experiment to test whether the absence of TNF- α from an irradiated BM environment improves graft-HSCs' reconstitution Ly5.1 ability. An in vivo short-term HSC exposure assay was carried out as shown in Fig. 2 with some modifications. In vivo HSC exposure transplantation. As inflammation kinetics might differ between WT and TNF- α KO backgrounds, exposure time was set



ability. TNF- α stimuli activate Nicotinamide Adenine Dinucleotide mono Phosphate Hydride (NADPH) oxidase, with ROS produced through coupling riboflavin kinase to NADPH oxidase. While accumulation of ROS results in DNA damage and functional impairment in HSCs [22](#), a relationship between TNF- α and ROS in these cells remains to be investigated. We first confirmed expression of key components in the putative complex of TNF- α -ROS signaling in HSPCs (Fig. [5A](#)) and accordingly determined whether TNF- α stimulation led to excessive production of ROS in highly purified murine HSPCs by culturing them with or without TNF- α stimulation for up to 48 hours and staining them with dichlorodihydrofluorescein (DCF) to quantify the accumulation of ROS (Fig. [5B](#)). As shown, TNF- α induced ROS production in HSP in a dose- and time-dependent manner (Fig. [5C](#), [5D](#)). That TNF- α stimulation induced HSPCs produce ROS was confirmed more specifically using HySOx [19](#), a stable, auto-oxidation—resistant novel fluorescent probe uniquely specific for hypochlorous acid (Supporting Information Table).

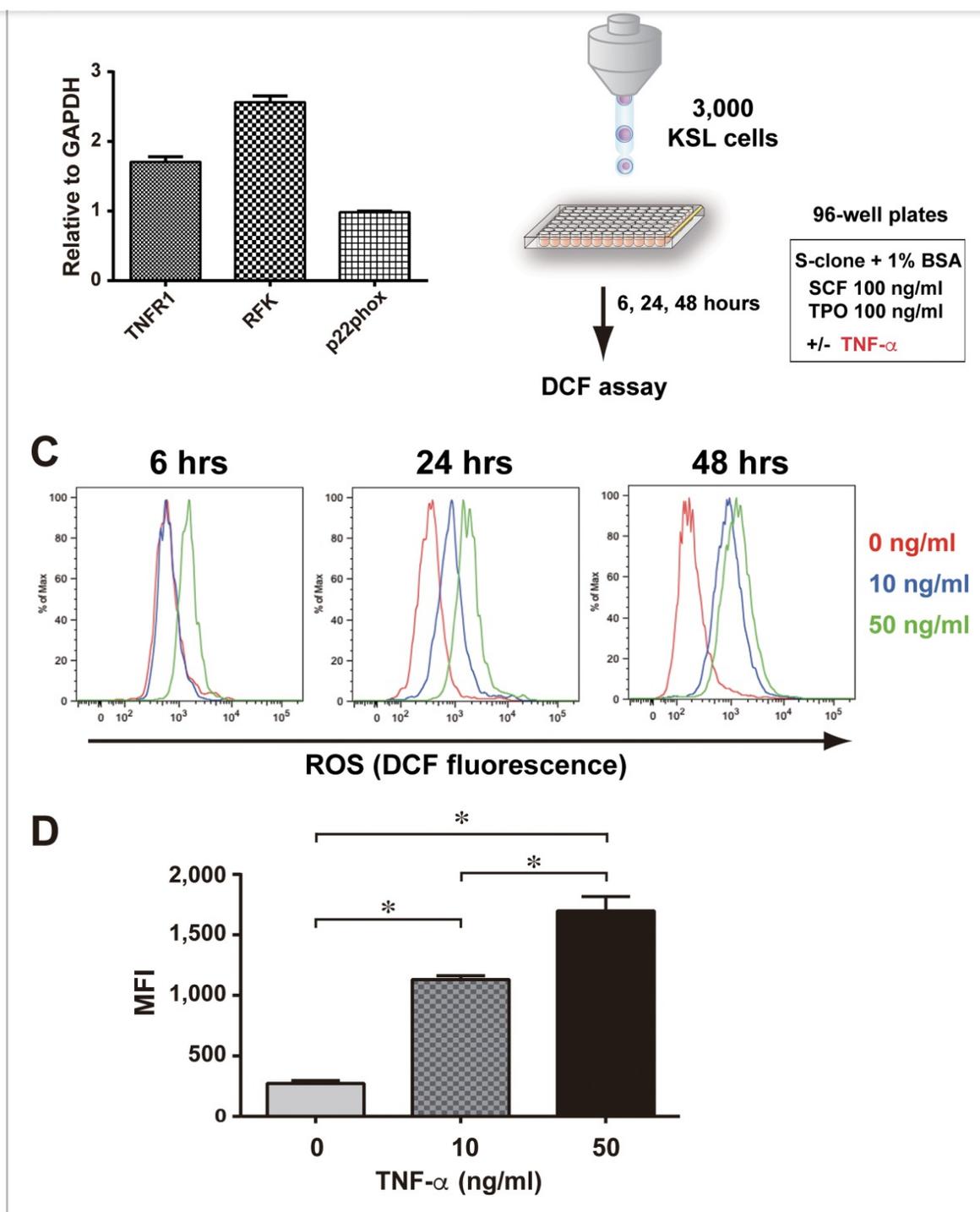


Figure 5

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TNF- α induces ROS production in HSPCs. **(A)**: qPCR analysis of gene expression in murine HSPCs (KSL cells) in relation to TNF- α -ROS signaling pathway. KSL cells isolated from B6 mice were used. Shown are mean expression levels of each indicated molecules to *Candb* expression. **(B)**: KSL cells were sorted at



We next sought to assess if a causal relationship existed between TNF- α -mediated ROS accumulation and impaired reconstitution ability of HSCs. We first confirmed that cultivation of HSCs with TNF- α for 48 hours lowered their reconstitution ability in competitive repopulation assays (Supporting Information Fig. 1A, 1B). We then compared reconstitution abilities of HSCs between groups exhibiting different levels of ROS using flow-cytometry sorting (Supporting Information Fig. 2A). As shown in Supporting Information Fig. 2B, competitive repopulation assays revealed that reconstitution ability was less in HSCs with high levels of ROS (DCF-high) than in HSCs with low/medium levels of ROS (DCF-low/med). Interestingly, the difference was highly significant with respect to lymphoid reconstitution, but not myeloid reconstitution. These results suggest that short term-exposure to TNF- α affects HSCs' reconstitution ability through induction of ROS.

Inhibition of TNF- α Mediated ROS Accumulation is Feasible by Simple Pre-Incubation of HSPCs with Reagents

As ROS accumulation in response to TNF- α within an inflammatory BM environment quickly reduces graft-HSCs' reconstitution ability, we hypothesized that to inhibit ROS production even for a short-term might improve transplantation outcomes. It was reported that the TNF receptor 1 (TNFR1) blocking peptide (PepTNFR1) specifically blocked NADPH oxidase-mediated ROS production in cells after stimulation with TNF- α [20](#). We thus tested if PepTNFR1 could block ROS accumulation induced by TNF- α also in our experimental settings. As shown, pre-incubation (~4 hours) of murine HSPCs with PepTNFR1, but not with a scrambled control peptide (Scr), inhibited TNF- α -mediated ROS accumulation; this was confirmed using 2 independent dye-indicators, DCF and HySOx (Fig. [6A](#), [6B](#)).

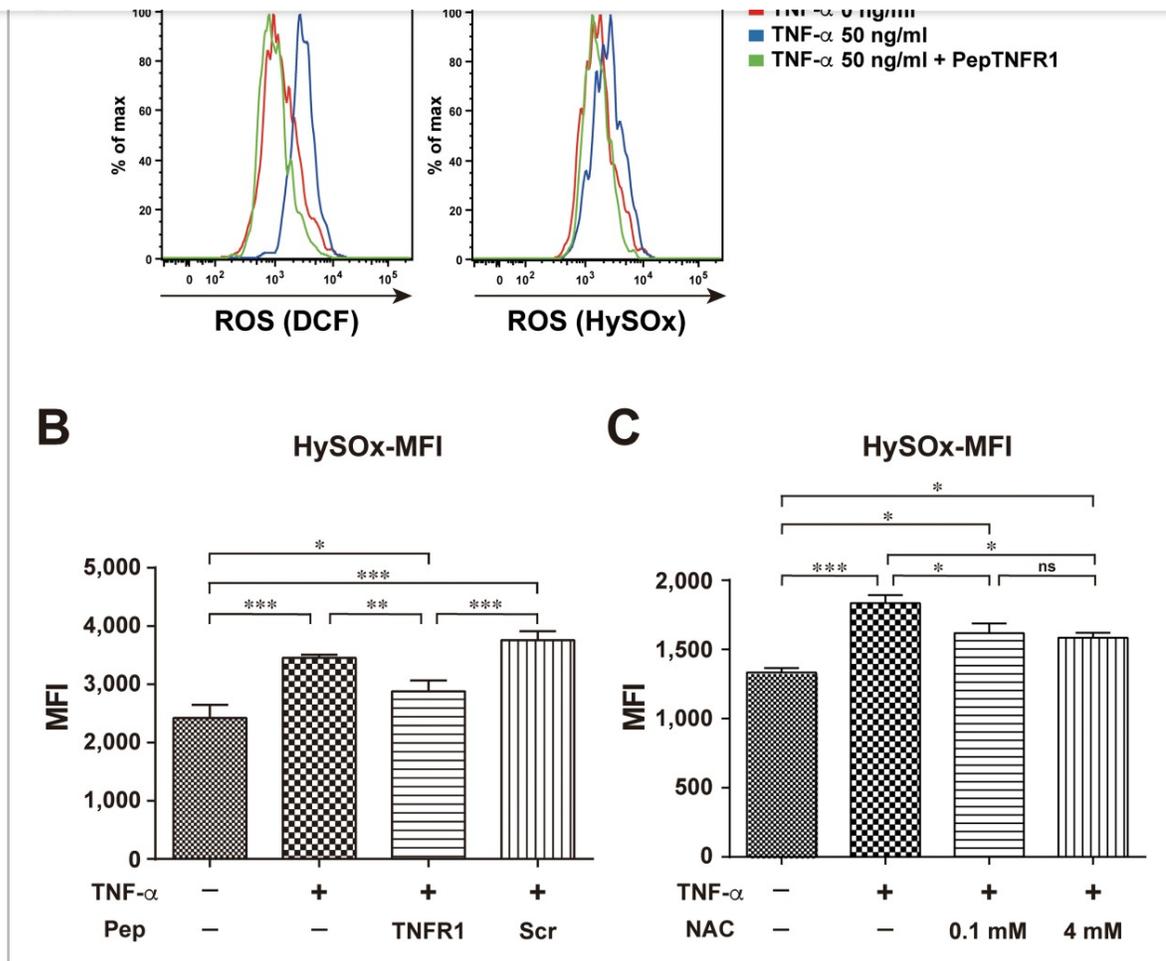


Figure 6

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Inhibition of TNF- α -mediated ROS production in HSC/HSPCs. **(A)**: KSL cells were pre-incubated with 60 μ M PepTNFR1 for 4 hours, washed, and cultured for 48 hours. Shown are histogram-overlay images obtained by flow cytometry analysis of ROS levels using DCF and HySOx. **(B)**: Graphic representation of results (HySOx) shown in **A**. Mean MFI values ($n=3$) \pm SD are shown. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test. * $p < .05$, ** $p < .01$, *** $p < .001$. For simplicity, a histogram for Scr is not shown in **A**. **(C)**: Inhibition of TNF- α -mediated ROS production in KSL cells by NAC. NAC was included for 48 hours at 0.1 mM. With 4 mM NAC, pre-incubation, wash, and cultivation was carried out similarly as peptide treatment. Shown is graphic representation of ROS levels using HySOx. Mean MFI values ($n=4$) \pm SD are shown. Data were analyzed by one-way ANOVA with Tukey's multiple comparison test. * $p < .05$, *** $p < .001$. Abbreviations: ANOVA, analysis of variance; DCF, dichlorofluorescein diacetate; HSC, hematopoietic stem cell; HSPC, hematopoietic progenitor cell; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; PepTNFR1, peptide TNF receptor 1; ROS, reactive oxygen species; Scr, scrambled peptide; TNF- α , tumor necrosis factor- α .



representative antioxidant NAC, by which sort of protective effects were demonstrated on HSCs in serial transplantation experiments when given systemically to recipients for long-term. As expected, continuous incubation of HSPCs with low concentration (0.1 mM) of NAC significantly inhibited TNF- α -mediated ROS accumulation at 48 hours (Fig. 6C, 0.1 mM). Of note is that a pre-incubation method (4 hours) was shown also applicable to NAC (4 mM), with lowered ROS levels evident even 48 hours after TNF- α stimulation.

Pre-Transplantation Incubation with NAC Protects Graft-HSCs from Loss of Long-Term Reconstitution Ability by Inflammatory BM Environment

We finally tested if reconstitution ability in HSPCs was protected in inflammatory BM by pre-transplantation incubation with NAC. In these experiments, test cells were left unprotected or protected by NAC pre-treatment (0.1 and 4 mM, 4 hours), and transplanted into recipient mice lethally irradiated at day-2, competitively with "non-protected" BM cells (Fig. 7A). As shown, donor cell chimerism in primary recipients showed tendency to decline over time in a control group (Fig. 7B, Primary transplantation, Control). In contrast, some recipient mice seemed to be showing some protective effects on graft-HSCs by NAC pre-treatment in both 0.1 and 4 mM groups, although only one occasion reached statistically significant difference (%Ly5.1 chimerism in T cells at 16 weeks, Control vs. 4 mM NAC). Remarkably, protective effects became apparent upon secondary transplantation as shown by higher donor-cell chimerism in recipients of "protected" HSCs (NAC) than in those of nonprotected HSCs (Control); the results were highly reproducible in independent experiments (Secondary transplantation, Fig. 7B and Supporting Information Fig. 4). Interestingly, NAC effects by pre-incubation were visible even with 0.1 mM concentration. Moreover, two NAC concentrations affected HSCs in a distinct manner: low concentration favored lymphocyte reconstitution better, whereas high concentration helped myeloid cell reconstitution more. This observation was also highly reproducible (Fig. 7B and Supporting Information Fig. 4).



BM microenvironment [24](#); TBI stimulates release of inflammatory cytokines from macrophages [24](#) and of TLR ligands from bacterial flora [25](#). Moreover, anti-tumor chemotherapy may sensitize tissues to the toxic effects of conditioning [26](#). Such inflammation is of particular significance in relation to graft-versus-host disease (GvHD) [24-26](#). However, the possible effects of such inflammation on transplanted HSCs/HSPCs have not been formally examined. We thus explored in a BMT setting the new strategy of stem cell protection from preconditioning-related inflammatory changes in the BM environment.

The effects of inflammatory cytokines on HSCs are controversial [9, 10, 27-31](#). TNF- α primarily inhibits growth and induces apoptosis of HSCs in both human [10, 27, 28, 31](#) and mouse [29, 32](#) systems; our results in mouse HSCs/HSPCs were overall consistent with those reports. Other researchers report different effects; in a TNF- α pathway, TNF Receptor Superfamily1a might be crucial for maintenance of HSCs [9, 33, 34](#). That stimulation of progenitors via TNFR signaling, with activation of the caspase cascade, supports better engraftment [35](#) indicates that TNF- α effects must be evaluated cautiously in light of specific experimental and clinical conditions. Furthermore, other inflammatory cytokines are also necessary to support HSC function [3, 4](#).

Our study focuses on adverse effects on transplanted HSCs of an inflammatory BM environment, an inevitable consequence of TBI pretreatment in HSCT. Although the influence of serum TNF- α levels on graft cells is clinically appreciated [1, 26](#), detailed analysis of BM levels of inflammatory cytokines have not yet been done; nor have effects of preconditioning on purified HSCs been tested, and measures to improve transplantation outcomes based on "stem cell protection" have not been sought. To our knowledge, ours is the first demonstration of a link between TNF- α and ROS accumulation that affects the reconstitution ability of purified HSCs/HSPCs.

The relationship between HSCs and ROS is of great interest. Hypoxia in the BM niche maintains HSCs' self-renewal activity [36](#), and a ROS^{low} HSC population exhibited higher self-renewal activity than a ROS^{high} population [37](#). Accumulation of ROS resulted in DNA damage and functional impairment of human HSPCs [22](#). In addition, ROS levels in the BM environment and not in graft-HSCs may affect transplanted human HSPCs [38](#). These observations generally highlight adverse effects of ROS on transplanted HSCs/HSPCs, but other data suggest that ROS enhance early hematopoietic reconstitution after transplantation [37, 39](#). We have recently demonstrated in a comprehensive in vitro study that inflammatory cytokines alter ROS levels variously in human HSCs/HSPCs, which in turn seem to affect cellular fates [40](#). It might be intriguing that in our HSC protection experiments, different NAC concentrations did exhibit



exposure assay also showed bias in myeloid lymphoid lineages in secondary recipients where reconstitution ability of graft-HSCs was preserved by the absence of TNF- α . We thus believe crucial to regulate ROS levels within appropriate windows at varying times to regulate outcomes in a favorable way in transplantation medicine.

Several issues still remain to be addressed. For example, although we have demonstrated that stem cell protection applies to murine HSPCs, transplantation experiments with human HSPC with or without protection have not been conducted. This requires xenotransplantation with immunodeficient mice and allows only very low-dose TBI, most likely not enough to induce meaningful levels of TNF- α synthesis. Other approaches will be needed, such as using mice that permit timely BM induction of TNF- α in the absence of lethal TBI while still accepting human hematopoiesis. Similarly, to test stem cell protection in allogeneic BMT will be interesting. We here used a congenic transplantation system to allow a proof-of-concept study while minimizing variables such as GvHD, which might affect hematopoietic reconstitution by several unidentifiable factors. Inflammation may be more severe in allogeneic than in autologous transplantation, due to disparity between donor and recipient MHC types, with systemic and sustained elevation of TNF- α ; it thus remains to be determined whether the current stem cell protection method still exhibits visible benefits on graft-HSCs.

We believe that in combination with other strategies such as amplification of HSCs, some of which are already being tested in clinical trials [41-43](#), establishment of stem cell protection measures will help improving transplantation medicine. Notably, Mantel et al. reported that the mitochondrial respiratory dysregulation in murine HSCs/HSPCs would cause ROS overproduction leading to their functional defects [44](#). Furthermore, they also demonstrated that the measures to prevent such unfavorable events during collection and processing could benefit HSC recovery [45](#). We thus expect that the two measures will work cooperatively to protect HSCs, at the stage *ex vivo* by the latter, whereas *in vivo* by ours. Overall, the cumulative effort to optimize the appropriate use of all these HSC manipulation techniques in combination will eventually lead to better engraftment and reconstitution kinetics in transplantation, rescuing many patients who require HSCT.

Conclusions

We here provide a proof of benefit that stem cell protection measures against TNF- α -mediated ROS accumulation in the context of transplantation will eventually help in combination with other available approaches better engraftment and improved reconstitution kinetics, thereby ameliorating outcomes of HSCT.



S. Knisely for critical reading of the manuscript. We also thank Dr. H.-T. Lin for his advice on graphical abstract. This work was supported in part by JSPS KAKENHI 21249059, 24659127, 15K15109.

Author Contributions

T.I.: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.S.: Conception and design, collection and/or assembly of data, data analysis and interpretation; C.-Y.L.: Collection and/or assembly of data; S.Y.: Conception and design, data analysis and interpretation; S.K.: Provision of study material or patients; Y.I.: Provision of study material or patients; M.N.: Data analysis and interpretation; Y.T.: Conception and design; M.H.: Administrative support, final approval of manuscript; H.N.: Administrative support, final approval of manuscript; M.O.: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

Supporting Information



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