## Transfer of stem cell niche-residential regulatory T cells prevents post-irradiation bone marrow injury

Post-irradiation bone marrow (BM) injury is a major dose-limiting side effect of radiation therapy for cancers.[1] Irradiation generates free radicals which cause DNA damage and death of hematopoietic stem cells (HSC) and progenitor cells (HSPC), leading to depletion of BM and blood cells.[1] There is a substantial unmet clinical need for new treatment strategies for post-irradiation BM injury. One of the potential therapeutic targets is the HSC niche which provides various cues to regulate HSC fate and function, and protect HSC from stress. Our recent studies have demonstrated that unique BM FoxP3+ regulatory T cells (Tregs) with high expression of a HSC marker, CD150, frequently localized adjacent to HSC, rendering the HSC niche an immunological sanctuary for stem cells, termed an immune privileged site.[2-4] Little is known about roles of immune privilege in tissue injury. Here, we investigate roles and therapeutic utility of HSC niche-residential CD150high Tregs in post-irradiation BM injury. This work demonstrates that niche Treg-derived extracellular adenosine mitigates post-irradiation BM injury. Our

work further identifies niche Treg transfer and adenosine 2A receptor agonist treatment as promising treatment strategies to prevent BM injury.

Immune privilege was originally demonstrated decades ago within testis, placenta and hair follicle. In these tissues, multiple mechanisms conspire to prevent or suppress the immune reaction, even enabling persistence of transplanted allogeneic (allo-) or xenogeneic grafts without immune suppressive therapy.[5,6] Although more recent stem cell research has identified various tissue-committed stem cells and their niches, little is known about whether these niches are broadly immune privileged. Our recent study demonstrated that the HSC niche within the BM accommodates unique potent CD150high Tregs, rendering HSC immune privileged.[2-4] These CD150high Tregs comprise approximately 30% of BM Tregs and from 0.04% to approximately 0.07% of whole BM mononuclear cells. As compared to CD150low BM Tregs and lymph node Tregs, HSC niche-residential CD150high Tregs highly expressed cell-surface ectoenzymes CD39 and CD73 which generate extracellular adenosine, a nucleotide with potent immunosuppressive and tissue-protective effects.[2,7] Niche Treg-derived adenosine pro-
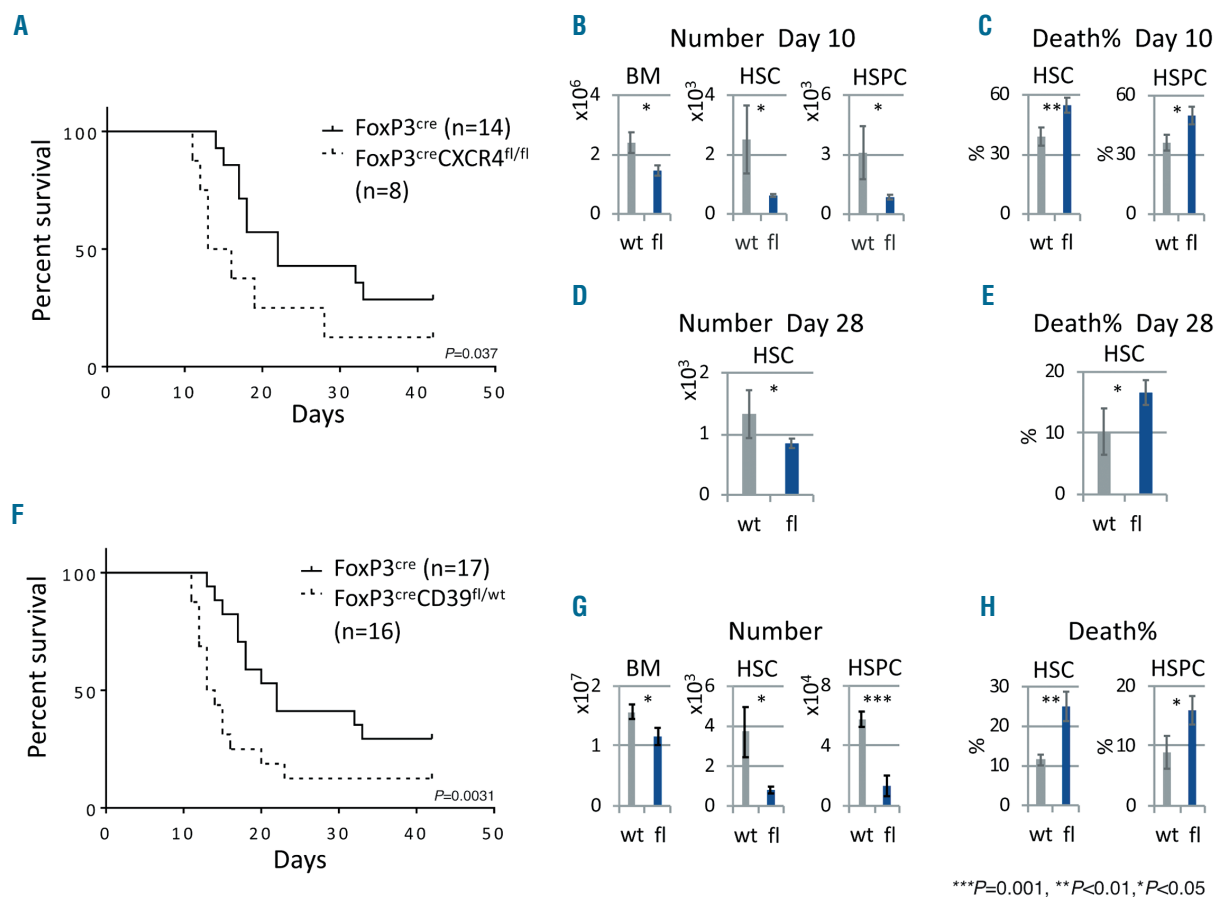
Figure 1. Reduction of bone marrow (BM) regulatory T cells (Tregs) and CD39 deletion in Tregs exacerbated post-irradiation BM failure. (A-E) Analysis of FoxP3cre-CXCR4fl/fl or FoxP3cre mice. fl: FoxP3cre-CXCR4fl/fl mice. wt: FoxP3cre. (A) Survival after 9 Gy total body irradiation (TBI). Pooled from three independent experiments. (B) Numbers of BM cells, hematopoietic stem progenitor cells (HSPC) and hematopoietic stem cells (HSC) on day 10 after 5 Gy TBI. N=3/group. (C) Death (7AAD+) frequencies in HSPC and HSC on day 10. N=3/group. (D and E) HSC numbers (D) and death frequencies (E) on day 28 after 8.5 Gy TBI. N=4/group. (F-H) Analysis of FoxP3cre-CD39fl/wt mice or FoxP3cre mice. fl: FoxP3cre-CD39fl/wt mice. wt: FoxP3cre mice. (F) Survival after 9 Gy TBI. Pooled from three independent experiments. (G) Numbers of BM cells, HSPC and HSC on day 28 after 8.5 Gy TBI in FoxP3cre-CD39fl/wt mice. N=3/group. (H) Death frequencies in HSPC and HSC on day 28 after 8.5 Gy TBI. N=3/group. Statistical analyses were performed with GraphPad Prism and EZR. Statistical significance was determined using two-tailed t-test. For survival analysis, generalized Wilcoxon test was used. All data are presented as mean±standard deviation. 7-week-old mice were used in all studies. n: number. *P<0.05; **P<0.01; ***P=0.001.
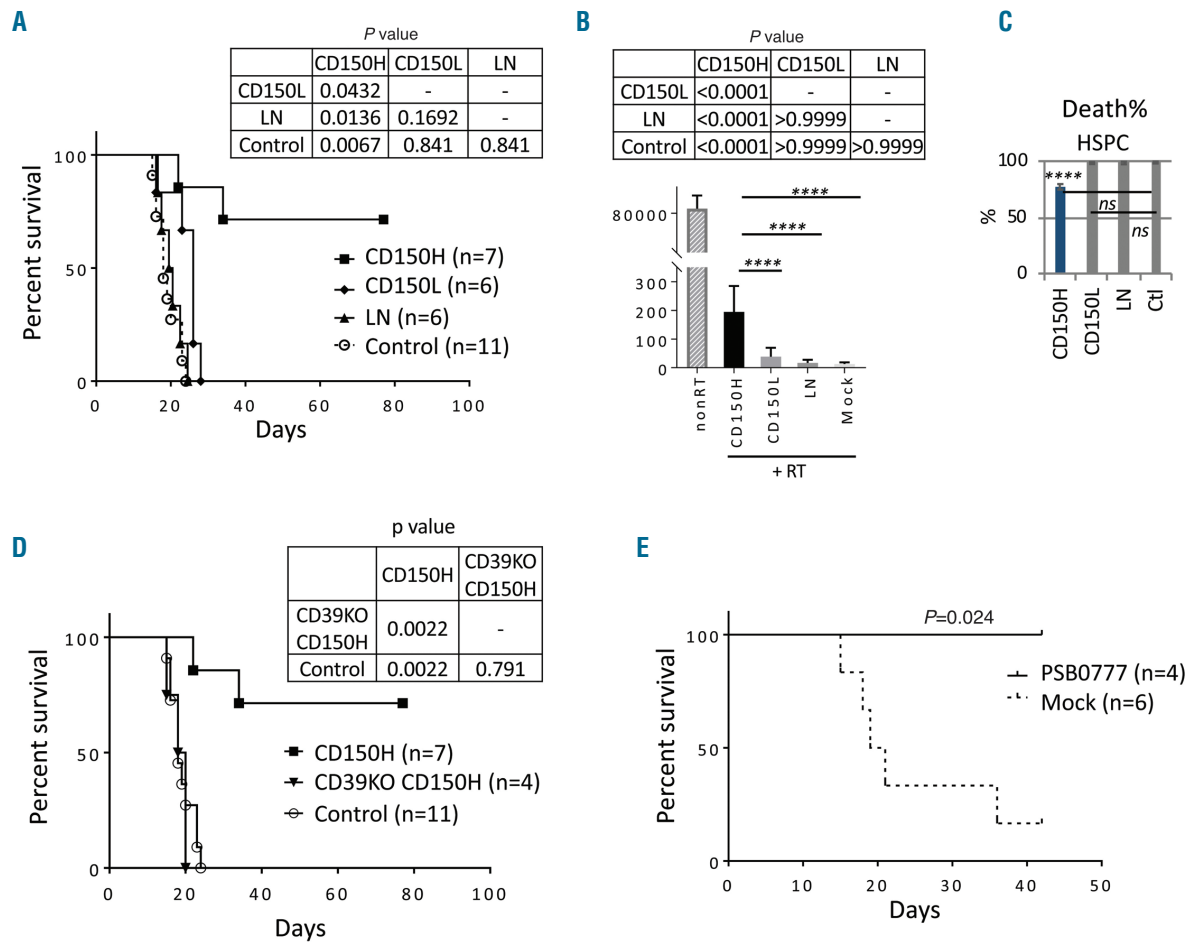
Figure 2. Niche regulatory T-cell (Tregs) transfer and A2AR agonist treatment rescued lethally irradiated mice from critical bone marrow (BM) failure. (A-C) Analysis of 9.5 Gy-irradiated B6 mice receiving tail vein injection of CD150high BM Tregs (CD150H), CD150low BM Tregs (CD150L), LN Tregs (LN) or vehicle (Control, Ctl) (30,000 cells/mouse; day -2; i.v.). CD150high Tregs were defined as Tregs with CD150 expression levels in the top 30% of BM Tregs, and CD150low Tregs were those in the bottom 30%. (A) Post-irradiation survival. Pooled from three independent experiments. (B) Hematopoietic stem progenitor cell (HSPC) numbers on day 10 after 7 Gy total body irradiation (TBI). nonRT: non-irradiated wild-type mice. N=6/group. (C) HSPC death frequencies on day 10 after 7 Gy g TBI. (D) Survival of 9.5 Gy-irradiated B6 mice receiving tail vein injection of CD150high BM Treg from Foxp3creCD39fl/wt mice (CD39KO CD150H), wild-type CD150high BM Tregs (CD150H) or vehicle (Control, Ctl) (30,000 cells/mouse; day -2; i.v.). Pooled from three independent experiments. (E) Survival of 9.5 Gy-irradiated B6 mice treated with PSB0777 (daily from day -2 till day 7; i.p.; 25 μg/dose; Tocris). Pooled from two independent experiments. Statistical analyses were performed with GraphPad Prism and Easy-R (EZR) software. Statistical significance was determined using one-way ANOVA with Bonferroni post test. For survival analysis, generalized Wilcoxon test was used, followed by Holm post-hoc multiple comparison test when needed. All data are presented as mean±standard deviation. 7-week-old mice were used in all studies. *P<0.05; ****P<0.0001.

tected donor allo-HSC from recipient immunity, enabling allo-HSC persistence without immune suppression and promoting allo-BM engraftment following non-myeloablative conditioning.[2,3] Under non-transplantation settings, niche Treg-derived adenosine protected endogenous HSC and progenitors from oxidative stress, maintaining stem cell quiescence via adenosine 2A receptors (A2AR).[2] These observations prompted us to investigate protective roles of CD150high niche Tregs and adenosine in post-irradiation BM injury.

BM Tregs (~0.1% of BM mononuclear cells) was depleted by using previously established mice with conditional deletion of CXCR4 in Tregs,[2] which is a chemokine receptor required for Treg homing to the BM, but not to the spleen or lymph nodes. FoxP3cre-CXCR4fl/fl mice showed from 70% to approximately 80% reductions in frequencies and numbers of BM Tregs and CD150high BM Tregs, while pool sizes of spleen and lymph node Tregs were not altered.[2] We assessed how this reduction of BM Tregs influences BM failure follow-

ing total body irradiation (TBI). Mortality of 9-Gy irradiated FoxP3cre-CXCR4fl/fl mice was significantly higher than that of control FoxP3cre mice (Figure 1A). Reduction of BM Tregs slowed down post-irradiation recovery of numbers of BM cells, cKit+Sca1+Lin- hematopoietic stem and HSPC, and CD150+CD48- cKit+Sca1+Lin- HSC, further increasing HSPC and HSC death on days 10 and 28 (Figure 1B-E and Online Supplementary Figure 1A and B). Irradiated FoxP3cre-CXCR4fl/fl mice and FoxP3cre mice were rescued by intravenous injection of HSPC (Online Supplementary Figure 1C), suggesting that the post-irradiation death of these models were largely attributed to hematopoiesis failure. These observations suggest that the reduction of BM Tregs exacerbated post-irradiation hematopoiesis failure which increased mouse mortality.

Next, protective roles of Treg-derived adenosine were investigated by using conditional deletion of a cell-surface ectoenzyme CD39 in Tregs, which is required for the generation of extracellular adenosine. 8.5 Gy-irradiated FoxP3cre-CD39fl/wt mice recapitulated all the phenotypes

in irradiated FoxP3^{cre}-CXCR4$^{fl/fl}$ mice (Figure 1F-H and *Online Supplementary Figure 1D-F*), suggesting Treg-derived adenosine as a critical mediator of Tregs to mitigate post-irradiation BM injury. The therapeutic utility of niche Treg transfer was further investigated. Lethally-irradiated (9.5 Gy) B6 mice received tail vein injection of CD150^{high} BM Tregs, CD150^{low} BM Tregs or LN Tregs (CD4$^+$CD3$^+$NK1.1-FoxP3-YFP cells isolated from B6 FoxP3-YFP mice) on day -2. Transfer of as few as 30,000 CD150^{high} BM Tregs per mouse rescued 9.5 Gy-irradiated mice, while transfer of CD150^{low} BM Tregs or LN Tregs did not influence post-irradiation mouse mortality (Figure 2A). Consistently, transfer of CD150^{high} BM Tregs, but not of CD150^{low} BM or LN Tregs, improved recovery of the HSPC pool, decreasing post-irradiation HSPC death (Figure 2B and C, and *Online Supplementary Figure S2*). Next, the mechanism which confers niche Treg transfer with higher therapeutic efficacy was investigated. Our previous study has demonstrated that BM homing efficacy of CD150^{high} BM Tregs was equivalent to that of other Tregs.[2] We examined whether niche Tregs' therapeutic effect is attributed to CD39 which is highly expressed on CD150^{high} BM Tregs as compared to other Tregs.[2] Transfer of CD150^{high} BM Tregs isolated from FoxP3^{cre}-CD39$^{fl/fl}$ mice did not influence post-irradiation mouse mortality (Figure 2D), suggesting that therapeutic effect of niche Treg transfer depends on Treg-derived adenosine which was previously shown to protect HSPC from oxidative stress via A2ARs on HSPC.[2] Consistently, A2AR agonist treatment (PSB0777;[7] Tocris; 25 μg/dose; i.p. daily from day -2 till day 7) rescued 9.5 Gy-irradiated mice (Figure 2E).

Taken together, these observations demonstrate protective roles of HSC niche-residential Tregs and their product, adenosine, in post-irradiation BM injury, further identifying niche Treg transfer and A2AR agonist treatment as new strategies to prevent BM injury. This report for the first time shows treatment utility of transferring niche Tregs for tissue injury, opening up new strategies to promote tissue regeneration or to counteract radiotoxicity following radiation therapy or nuclear terrorism. Our work is in line with a recent growing interest in non-canonical, tissue-protective effects of Tregs within muscle, lung, and hair follicle,[8-12] while numbers of isolatable Tregs from these tissues are too low for transfer. It would be interesting to test whether transfer of HSC niche-residential Tregs is effective in improving the outcome of injury of other tissues, as well as other BM failures, including acquired aplastic anemia which is hematopoiesis failure HSPC. It remains unknown how frequently muscle and lung Tregs reside in the stem cell niche and whether there is any immune privilege in other tissue-committed stem cell niches besides hair follicle and HSC niche.[2-4,11,12] Testing whether niches broadly possess immune privilege or harbor Tregs is warranted. Human BM Tregs were previously shown to exhibit higher suppressive potential *in vitro* than other Tregs,[13] suggesting the possibility that human Tregs confer immune privilege to HSC. In summary, this work indicates that immune privilege, niche Tregs, and adenosine represent promising therapeutic targets for tissue injury.

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