CD150high CD4 T cells and CD150high regulatory T cells regulate hematopoietic stem cell quiescence via CD73

Yuichi Hirata,[1,2,3,4] Miwako Kakiuchi,[1,2,3] Simon C Robson[5] and Joji Fujisaki[1,2,6*]

[1]Columbia Center for Translational Immunology, Columbia University College of Physicians and Surgeons, New York, NY, USA; [2]Columbia Stem Cell Initiative, Columbia University College of Physicians and Surgeons, New York, NY, USA; [3]Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY, USA; [4]MSD K.K., Tokyo, Japan; [5]Department of Medicine, Liver Center and Transplantation Institute, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA and [6]Department of Pediatrics, Division of Hematology and Oncology, Columbia University College of Physicians and Surgeons, New York, NY, USA

ABSTRACT

Various extrinsic signals tightly control hematopoietic stem cell quiescence. Our recent study showed that hematopoietic stem cells are regulated by a special FoxP3+ regulatory T-cell population with high expression of a hematopoietic stem cell marker, CD150. Extracellular adenosine generated via a cell-surface ectoenzyme CD39 on CD150high regulatory T cells maintained hematopoietic stem cell quiescence. It remains unclear how conventional T cells and the other cell-surface ectoenzyme, CD73, contribute to regulation of hematopoietic stem cells. This work shows that CD150high regulatory T cells as well as unique CD150high CD4+ conventional T cells regulate hematopoietic stem cells via CD73. Global CD73 deletion increased the numbers of hematopoietic stem cells, cycling stem cell frequencies, and levels of reactive oxygen species in hematopoietic stem cells. *In vivo* antioxidant treatment inhibited the increase of hematopoietic stem cells in CD73 knockout mice, suggesting that CD73 maintains stem cell quiescence by preventing oxidative stress. High levels of CD73 expression were frequently found on CD150high regulatory T cells and CD150high FoxP3-CD4+ T cells within the bone marrow. Transfer of these CD150high regulatory T cells and CD150high CD4+ conventional T cells abolished the increase of hematopoietic stem cells in CD73 knockout mice. In addition, the increase of stem cells in CD73 knockout mice was also inhibited by pharmacological activation of adenosine receptor 2A which is highly expressed by hematopoietic stem cells. Taken together, these results suggest that CD73 of CD150high regulatory T cells and CD150high CD4+ conventional T cells protects hematopoietic stem cells from oxidative stress, maintaining stem cell quiescence via adenosine receptor 2A.

Introduction

The bone marrow (BM) microenvironment provides various cues to regulate hematopoietic stem cell (HSC) quiescence, self-renewal, and multilineage differentiation,[1-4] and to protect HSC from various stresses, such as oxidative stress[5] and toxic substances.[6] Different mesenchymal subsets and megakaryocytes form a specialized regulatory zone for HSC residence, called the niche, within the BM.[1-4] It is thought that tight control of HSC quiescence and function helps to prevent HSC exhaustion and genetic mutation. Due to a growing demand for clinical BM transplantation, understanding how the BM microenvironment regulates HSC remains important.

Our recent study demonstrated that HSC were regulated by a unique population of regulatory T cells (Treg) with high expression of a HSC marker, CD150.[7] These CD150high Treg frequently localized adjacent to HSC.[7] Treg-mediated HSC regulation depended on a cell-surface ectoenzyme, CD39, which was highly

expressed by CD150^{high} Treg.[7] CD39 is known to convert extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophosphate (AMP) which is further hydrolyzed by the other cell-surface ectoenzyme, CD73, into extracellular adenosine, a purine nucleotide with various tissue-protective effects.[8] The results of our study using conditional knockout (KO) of CD39 in Treg suggested that extracellular adenosine generated via CD39 on Treg protected HSC from oxidative stress, maintaining HSC quiescence.[7] It remains unclear how the other cell-surface ectoenzyme, CD73, contributes to HSC regulation and which BM cell populations regulate HSC via CD73. In addition, while the BM serves as a reservoir of memory T cells,[9,10] little is known about the role of these conventional T cells in HSC regulation.

This work identified unique CD150^{high}CD4^{+}FoxP3^{-} conventional T cells (nonTreg) which highly expressed CD73 and CD39, like CD150^{high} Treg. Our observations in CD73 KO mice into which these CD150^{high} T-cell populations were transferred suggest that CD73 of CD150^{high} CD4^{+} nonTreg and CD150^{high} Treg maintain HSC quiescence and abundance.

Methods

Animals

C57BL/6J mice, SJL mice, BALB/c mice, CD73 KO mice, FoxP3-YFP mice, and Lep-cre mice (Jackson Laboratory, Bar Harbor, ME, USA) were housed in a specific pathogen-free environment. CD39-flox mice were kindly provided by Dr. Simon C. Robson (Harvard Medical School). Seven-week old CD73 KO mice were analyzed. The mice were sacrificed by CO_2 inhalation and cervical dislocation. Studies were conducted with approval from Institutional Review Boards and Animal Care and Use Committees at Columbia University.

Antibodies and reagents

We used FITC-conjugated Lineage monoclonal antibodies (B220, Mac1, GR-1, CD2, CD3a, CD8a, CD4, CD19 and Ter119), APC-780-conjugated cKit monoclonal antibodies, PECy7- or APC-conjugated CD39 monoclonal antibodies, FITC-conjugated FoxP3 monoclonal antibodies, PE-conjugated Ki67 monoclonal antibodies (all purchased from eBioscience), APC/Cy7-conjugated NK1.1 monoclonal antibodies, BV510-conjugated CD3 monoclonal antibodies, PE/Cy7-, or BV605-conjugated CD4 monoclonal antibodies, Alexa700- or Pacific blue-conjugated CD48 monoclonal antibodies, PerCP/Cy5.5-, APC-, or BV605-conjugated CD73 monoclonal antibodies, PE- or PE/Cy7-conjugated CD150 monoclonal antibodies (all from Biolegend), and BV605-conjugated Sca-1 monoclonal antibodies (from BD Pharmingen or Biolegend).

N-acetyl-L-cysteine (A9165) was purchased from Sigma-Aldrich.

Competitive reconstitution assay

SJL (CD45.1) mice were irradiated at 475 cGy twice (950 cGy in total) at least 2 h apart. Two hours after the last irradiation, donor BM cells (CD45.2), together with competitor BM cells (SJL) (3 x 10^{5}/each), were injected into the tail veins of SJL recipients. Peripheral blood samples were analyzed periodically. Red blood cells were lysed with an ammonium chloride potassium buffer. The antibodies used to analyze donor chimerism were anti-CD45.1, anti-CD45.2, anti-GR1, anti-CD11b, anti-B220, and anti-TCR-β (all from Biolegend).

Flow cytometric analysis

BM cells were isolated by crushing tibiae and femora. Following treatment with a red blood cell lysis buffer (Biolegend), the cell suspension (2x10^{6} cells) was plated onto 96-well plates and incubated with culture media containing 2 µM CellROX Deep Red (Invitrogen) for 30 min. Flow cytometry was performed using an LSRII (BD Biosciences), LSRFortessa (BD Biosciences), or FACSCanto (BD Biosciences) cytometer followed by analysis using FlowJo software (Tree Star Inc.).

Colony-forming assay

BM cells (2.0x10^{4} cells/each well) were plated in six-well plates (Corning, NY, USA) containing 1 mL MethoCult™ (M3234, Stemcell Technologies Inc.) supplemented with 1% penicillin/streptomycin (Gibco), stem cell factor (50 ng/mL), interleukin-3 (15 ng/mL), interleukin-6 (20 ng/mL), and granulocyte-macrophage colony-stimulating factor (15 ng/mL). Colonies were maintained at 37°C in humidified incubators. Colony formation was scored on day 10.

Flow cytometry following intracellular staining of FoxP3

Intracellular FoxP3 staining was performed according to the manufacturer's protocol (eBioscience).

Stromal cell analysis

For analysis of stromal cells, long bones were gently crushed using Hanks balanced saline solution to harvest the BM cells. Whole bone marrow was digested with collagenase IV (200 U/mL) and DNase I (200 U/mL) at 37°C for 30 min. Following treatment with a red blood cell lysis buffer (Biolegend), the cell suspension (2x10^{6} cells) was plated onto 96-well plates and then stained with antibodies. Anti-CD140a (APA5), anti-CD140b (APB5), anti-CD45 (30F-11), anti-CD31 (390) and anti-Ter119 antibodies (all from Biolegend) were used to stain perivascular stromal cells.

T-cell transfer assay

HSC numbers were determined in CD73 KO mice 7 days after intravenous injection of CD150^{high} BM Treg, CD150^{low} BM Treg, CD150^{high} BM nonTreg, or CD150^{low} BM nonTreg (30,000 cells/mouse). Data were pooled from three independent experiments (4-10 mice/group).

Adenosine 2A receptor agonist treatment

CD73 KO mice were given PSB0777, a potent adenosine 2A receptor (A2AR) agonist, daily for 7 consecutive days (25 µg/mouse, intraperitoneally). Total HSC numbers in one tibia and one femur were analyzed 1 day after the final injection.

Statistics

Statistical analyses were performed with GraphPad Prism software (version 6.0). Statistical significance was determined using a two-tailed t-test or one-way analysis of variance (ANOVA) with a Bonferroni post-test correction. P values less than 0.05 were considered to be statistically significant. All data are presented as mean ± standard deviation (SD).

Results

CD73 deletion increased hematopoietic stem cell pool size

The effect of global CD73 deletion on hematopoiesis was first analyzed. CD73 KO mice showed increases in

BM cellularity (Figure 1A). CD73 deletion significantly increased the frequencies of cycling cKit+Sca1+Lin- hematopoietic stem and progenitor cells (HSPC) and CD150+CD48-cKit+Sca1+Lin- HSC, as well as numbers of HSPC and HSC (Figure 1A-C, *Online Supplementary Figure S1A,B* and *Online Supplementary Table S1*). Consistently, the numbers of colonies formed following *in vitro* culture of BM cells isolated from CD73 KO mice were significantly higher than those from wild-type mice (*Online Supplementary Figure S1C*). The numbers of other BM cell populations were not significantly altered by CD73 deletion (*Online Supplementary Figure S1D*). To assess the size of the pool of functional HSC and HSPC, we performed competitive BM transplantation assays to evaluate the reconstituting potential of BM cells. BM cells of CD73 KO mice or control wildtype mice (B6 CD45.2; 3 x 10⁵ cells/mouse) were intravenously injected into lethally-irra-

diated B6 SJL mice (CD45.1), together with competitor SJL BM (CD45.1; 3 x 10⁵ cells/mouse). CD45.2 donor blood chimerism from CD73 KO BM cells remained significantly higher than that from control BM cells for 6 months after transplantation, suggesting that CD73 deletion increased functional HSC and HSPC frequencies (Figure 1D). Additionally, no myeloid skewing was observed in donor hematopoietic cells derived from CD73KO BM (*Online Supplementary Figure S1E*). Taken together, these results indicate that CD73 maintains quiescence and pool size of HSPC and HSC.

CD73 maintains hematopoietic stem cell pool size by preventing oxidative stress

CD73 KO mice showed slight but significant increases in the levels of reactive oxygen species (ROS) in HSPC and HSC but not in Lin+ cells (Figure 2A, *Online Supplementary*
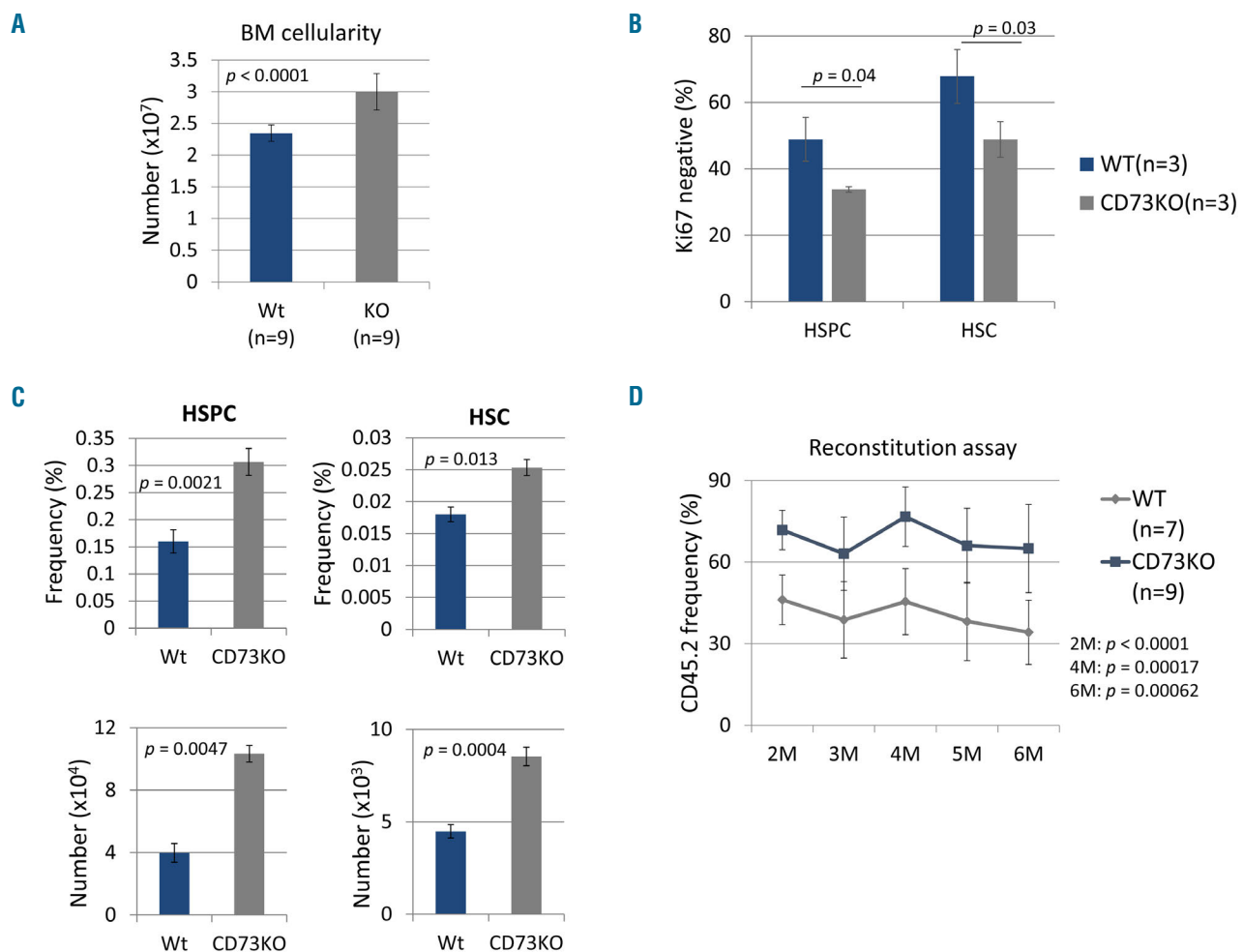

Figure 1. CD73 deletion increased hematopoietic stem cell pool size. (A) Bone marrow (BM) cellularity of CD73 knockout (KO) mice. We analyzed the numbers of total BM cells isolated from one tibia and one femur by crushing. Data from three independent experiments with nine mice/group were pooled. Data are presented as mean ± SD and analyzed by a two-tailed *t*-test. (B) Flow cytometric analysis of frequencies of Ki67- cells among hematopoietic stem and progenitor cells (HSPC: cKit+Sca1+Lin-) and hematopoietic stem cells (HSC: CD150+CD48-cKit+Sca1+Lin-) in CD73 KO mice. The results were reproducible in two independent experiments (7 mice/group total). A representative figure from one independent experiment is shown here. Data are presented as mean ± SD and analyzed by a two-tailed *t*-test. (C) Flow cytometric analysis of HSPC and HSC frequencies and numbers in CD73 KO mice. The results were reproducible in three independent experiments (3 mice/group in each experiment; refer to *Online Supplementary Table S1*). Data are presented as mean ± SD and analyzed by a two-tailed *t*-test. (D) Donor chimerism in the peripheral blood of lethally irradiated mice that received wildtype SJL bone marrow (BM) cells (CD45.1) together with BM cells of CD73 KO or control mice (CD45.2). BM cells from each donor mouse (7 control mice and 9 CD73 KO mice) were transplanted into one recipient. The results were pooled from two independent experiments. Data are presented as mean ± SD and analyzed by a two-tailed *t*-test. wt: wildtype: M: months.

Figure S2A), suggesting that CD73 prevents oxidative stress against HSC and HSPC but not against mature cells. To test whether CD73 maintains HSC quiescence in a ROS-dependent manner, we used treatment with an anti-oxidant, N-acetylcysteine (NAC), which reversed the increases in HSC numbers and reconstituting potential of BM cells in CD73 KO mice (Figure 2B,C). These results indicate that CD73 maintains HSC quiescence by preventing oxidative stress.

This ROS-mediated expansion of HSC pool size in CD73 KO mice was consistently observed in our recently reported study, using two models: (i) mice with conditional deletion of CD39 in Treg; and (ii) mice with reduction of BM Treg achieved by CXCR4 deletion in Treg.[7] In contrast, some previous studies showed that increased ROS levels in HSC led to loss of HSC quiescence, and HSC exhaustion.[11-13] HSC proliferation in our models is likely explained by moderate increases in ROS levels (1.2- to 1.5-fold) compared to greater increases in other models (3- to 5-fold).[11-13] Indeed, some studies showed that a moderate increase in ROS levels induced HSC proliferation.[14,15] Moreover, the peripheral blood of CD73 KO mice showed a non-significant trend toward myeloid skewing (*Online Supplementary Figure S2B*), which may also reflect a moderate increase in ROS levels in HSC.

CD73^{high} cells were frequently found in CD150^{high} regulatory T cells and CD150^{high}CD4$^+$ non-regulatory T cells

To identify cell populations which play important roles in CD73-mediated HSC regulation, flow cytometric analysis was performed to measure CD73 and CD39 expression levels on hematopoietic cell populations within the BM. Intermediate to high expression of CD39 was found on the following hematopoietic cell populations: HSC; HSPC; CD11b$^+$Gr1^{int} cells; CD11b$^+$Gr1^{high} cells; B220$^+$ B cells; CD4$^+$FoxP3$^-$ cells (CD4$^+$ nonTreg); CD4$^+$FoxP3$^+$ Treg; CD8$^+$ T cells; CD4$^+$CD3$^+$NK1.1$^+$ NKT cells; and NK cells (*Online Supplementary Figure S3A*). In contrast, high levels of CD73 expression were mainly observed within CD4$^+$ T cells (Treg, CD4$^+$ nonTreg) (Figure 3A,B). While CD8$^+$ T cells, CD11b$^+$Gr1^{high} cells, and CD4$^+$ NKT cells showed intermediate levels of CD73 expression, CD73 was not expressed by HSC, B cells, or CD11b$^+$Gr1^{int} myeloid cells (Figure 3A,B).

As previously reported,[7] CD73^{high} cells among Treg were predominantly CD150^{high}, showing equivalent levels of expression of CD150 as those of HSC (Figure 3B-D). These CD150^{high} Treg also highly expressed CD39 (Figure 3D, *Online Supplementary Figure S3A*). Notably, there were also CD150^{high} fractions among BM CD4$^+$ nonTreg, which
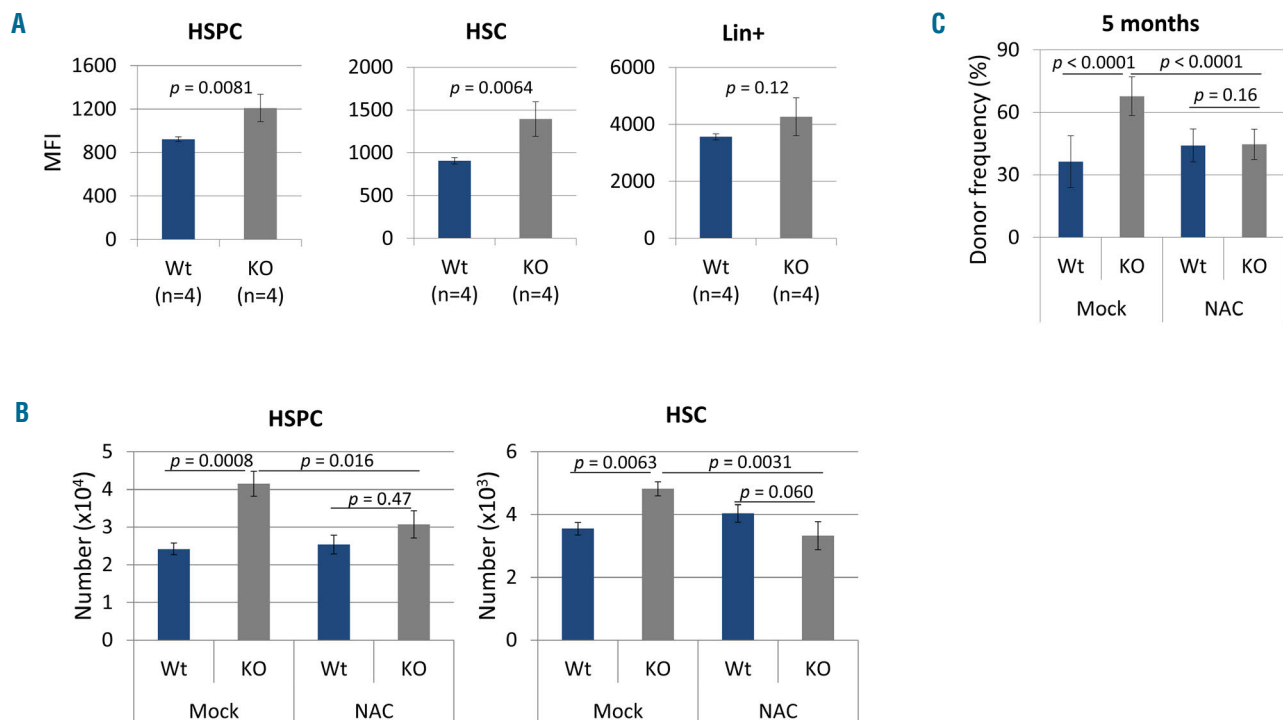
Figure 2. CD73 maintains hematopoietic stem cell quiescence and pool size by preventing oxidative stress. (A) Flow cytometric analysis of reactive oxygen species (ROS) levels in hematopoietic stem and progenitor cells (HSPC: cKit$^+$Sca1$^+$Lin$^-$) and hematopoietic stem cells (HSC: CD150$^+$CD48$^-$cKit$^+$Sca1$^+$Lin$^-$) in CD73 knockout (KO) or control B6 mice. The results were reproducible in two independent experiments (7 mice/group total). A representative figure from one independent experiment is shown here. Data are presented as mean ± SD and were analyzed by a two-tailed *t*-test. (B) Flow cytometric analysis of HSPC and HSC numbers in CD73 KO mice treated with N-acetylcysteine (NAC). CD73 KO or B6 control mice were given NAC (100 mg/kg) or mock treatment (daily; subcutaneously) for 4 weeks prior to bone marrow (BM) isolation. The results were reproducible in two independent experiments (7 mice/group total). Data are presented as mean ± SD and were analyzed by one-way analysis of variance (ANOVA) with a Bonferroni post-test. (C) Donor chimerism in the peripheral blood of lethally irradiated SJL mice that were administered wildtype SJL BM cells (CD45.1) together with BM cells of NAC-treated CD73 KO or control B6 mice (CD45.2) 5 months after transplantation. CD73 KO or control mice received NAC (100 mg/kg) or mock treatment (daily; subcutaneously) for 4 weeks prior to isolation. BM cells from each donor mouse were transplanted into one or two recipients. Experiments included three donors and 11-12 recipients/group. Data are presented as mean ± SD and were analyzed by one-way ANOVA with a Bonferroni post-test. MFI: mean fluorescence intensity; wt: wildtype.

highly expressed CD39 and CD73 as compared to the rest of the CD4^{+} nonTreg (CD150[neg-low]) (Figure 3B-D, *Online Supplementary Figure S3B*). CD150^{high} populations comprised 20% of CD4^{+} nonTreg and 40% of Treg (Figure 3E). CD150^{high} nonTreg frequently showed a CD44^{high}CD62L^{low} effector memory phenotype as compared to the rest of the CD4^{+} nonTreg (CD150[neg-low]) (Figure 3F; *Online Supplementary Figure S3C*), which is consistent with our previous observations in CD150^{high} Treg.[7] The frequencies of CD39^{high} and CD73^{high} cells among CD150^{high} nonTreg were equivalent to those among CD150^{high} Treg, and high-

er than those in BM CD150^{low} Treg, BM CD150[neg-low]CD4^{+} nonTreg, and lymph node CD4^{+} nonTreg (Figure 3B,D, *Online Supplementary Figure S3A,D*).

CD39 and CD73 expression among CD45^{-} mesenchymal cells was further analyzed following division of the CD45^{-} cells into the following three populations; CD45^{-}CD31^{+} vasculature; CD45^{-}CD31^{-}CD140a^{+}CD140b^{+} cells; and CD45^{-}CD31^{-}CD140a^{-}CD140b^{-} cells (*Online Supplementary Figure S3E*). Previous studies suggested that the former two populations were putative cellular constituents of the HSC niche.[2,3] CD45^{-}CD140a^{+}CD140b^{+} cells
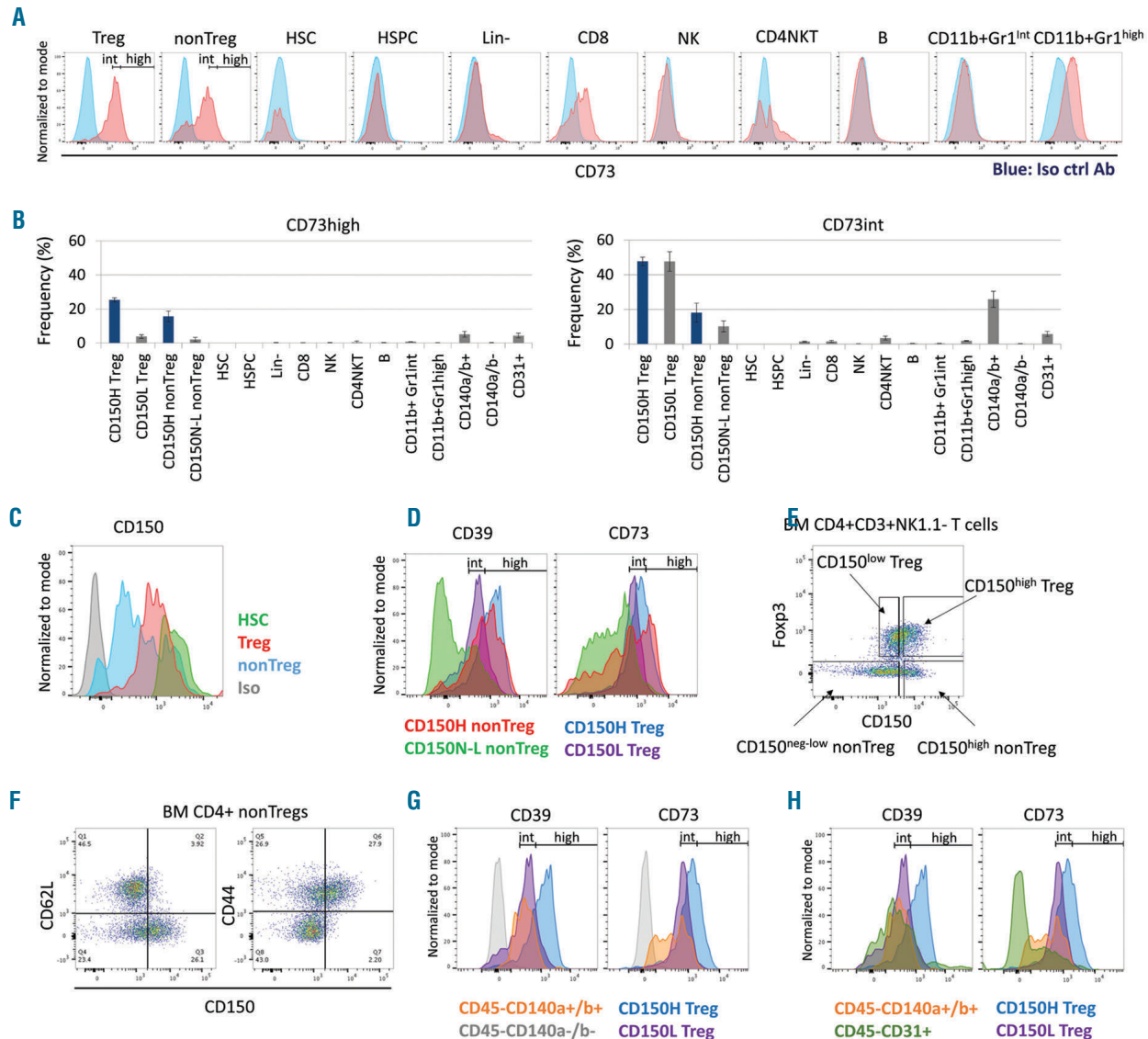

Figure 3. CD39 and CD73 expression levels in various bone marrow cell populations. (A) Flow cytometric analysis of CD73 expression levels in different bone marrow (BM) cell populations: regulatory T cells (Treg: CD4^{+}CD3^{+}NK1.1^{-}FoxP3YFP^{+}); non-regulatory T cells (nonTreg: CD4^{+}CD3^{+}NK1.1^{-}FoxP3YFP^{-}); hematopoietic stem cells (HSC: CD150^{+}CD48[-]cKit^{+}Sca1^{+}Lin^{-}); hematopoietic stem and progenitor cells (HSPC: cKit^{+}Sca1^{+}Lin^{-}); CD8 cells (CD8^{+}CD3^{+}NK1.1^{-}); natural killer cells (NK: NK1.1^{+}CD3^{-}); CD4NKT cells (NK1.1^{+}CD3^{+}CD4^{+} NKT cells); and B cells (B220^{+} cells). (B) Frequencies of CD73^{high} and CD73^{int} cells in different BM cell populations with the thresholds of CD73 expression levels determined as shown in Figure 3A, D, G and H: CD150H Treg (CD150^{high} Treg); CD150L Treg (CD150^{low} Treg); CD150H nonTreg (CD150^{high} nonTreg); and CD150N-L nonTreg (CD150[neg-low] nonTreg). Thresholds of CD150 expression levels were determined as in Figure 3E. CD140a/b^{+}: CD45^{-}CD140a^{+}CD140b^{+}CD31^{-}. CD140a/b^{-}: CD45^{-}CD140a^{-}CD140b^{-}CD31^{-}. CD31^{+}: CD45^{-}CD31^{+}CD140a^{-}CD140b^{-}. The results were reproducible in two independent experiments (n=6). A representative figure is shown here. Data are presented as mean ± SD. (C) Representative histograms showing levels of CD150 expression on HSC, Treg, and CD4^{+} nonTreg. (D) Representative histograms showing levels of CD39 or CD73 expression on CD150^{high} Treg, CD150^{low} Treg, CD150^{high} nonTreg, and CD150[neg-low] nonTreg. (E-F) Representative flow cytometric dot plots of BM CD4^{+}CD3^{+}NK1.1^{-} T cells including both Treg and nonTreg (E) and BM CD4^{+} nonTreg (F). (G-H) Representative histograms showing levels of CD39 and CD73 expression of CD150^{high} Treg, CD150^{low} Treg, CD140a^{+}CD140b^{+}CD45^{-}CD31^{-} cells, CD140a^{-}CD140b^{-}CD45^{-}CD31^{-} cells (G), and CD31^{+}CD45^{-} cells (H). Iso ctrl Ab: isotype control antibody.

were shown to overlap exclusively with leptin receptor-positive (lepr^{+}) perivascular niche cells.[2,3] CD39 and CD73 expression was observed on CD31^{+} vasculature and CD45^{-}CD31^{-}CD140a^{+}CD140b^{+} cells, but not on CD45^{-}CD31^{-}CD140a^{-}CD140b^{-} cells (Figure 3G,H, *Online Supplementary Figure S3F*). The frequencies of CD39^{high} and CD73^{high} cells within CD31^{+} vasculature and CD45^{-}CD140a^{+}CD140b^{+} cells were comparable to those in CD150^{low} Treg, and lower than those in CD150^{high} Treg and CD150^{high} nonTreg (Figure 3B,G-H, *Online Supplementary Figure S3A*). Taken together, these observations indicate that, while various BM cell populations expressed CD39 and/or CD73, CD73^{high} cells were frequently found within CD150^{high} Treg and CD150^{high} nonTreg.

The increase of hematopoietic stem cells in CD73 knockout mice was reversed by transfer of CD150^{high} regulatory T cells and CD150^{high} non-regulatory T cells and by *in vivo* adenosine receptor agonist treatment

To assess the role of CD150^{high} Treg and CD150^{high} CD4^{+} nonTreg in CD73-mediated HSC regulation, we analyzed how transfer of these T-cell populations influences HSC. Transfer of CD150^{high} Treg and of CD150^{high} CD4^{+} nonTreg significantly reversed the increase of HSC in CD73 KO mice relative to control wildtype mice. In contrast, the size of the HSC pool was not significantly altered by transfer of CD150[neg-low] nonTreg or CD150^{low} Treg (Figure 4A). These observations are consistent with our previous

observations that transfer of CD150^{high} Treg reversed the increase of HSC in mice with conditional deletion of CD39 in Treg.[7] These results suggest that CD150^{high} Treg and CD150^{high} nonTreg contribute largely to CD73-mediated HSC regulation.

The downstream signaling of CD73 in HSC regulation was further assessed. HSC showed higher levels of expression A2AR than various other BM cell populations (Figure 4B). *In vivo* A2AR agonist treatment significantly reversed the increase of HSC in CD73 KO mice (Figure 4C). These observations suggest that A2AR signaling plays an important role in CD73-mediated HSC regulation.

Discussion

This study identified CD73 and CD150^{high} nonTreg as important regulators of HSC quiescence and abundance in the adult BM. Global CD73 deletion increased ROS levels in HSC, and HSC numbers. This increase of HSC was reversed by anti-oxidant treatment, suggesting that CD73 maintains HSC quiescence by preventing oxidative stress. Because HSC did not express CD73 but CD39, CD73-mediated HSC regulation is driven by the microenvironment. While various BM cells showed intermediate levels of CD73 expression, CD73^{high} cells were frequently found within unique CD150^{high} Treg and CD150^{high} nonTreg.

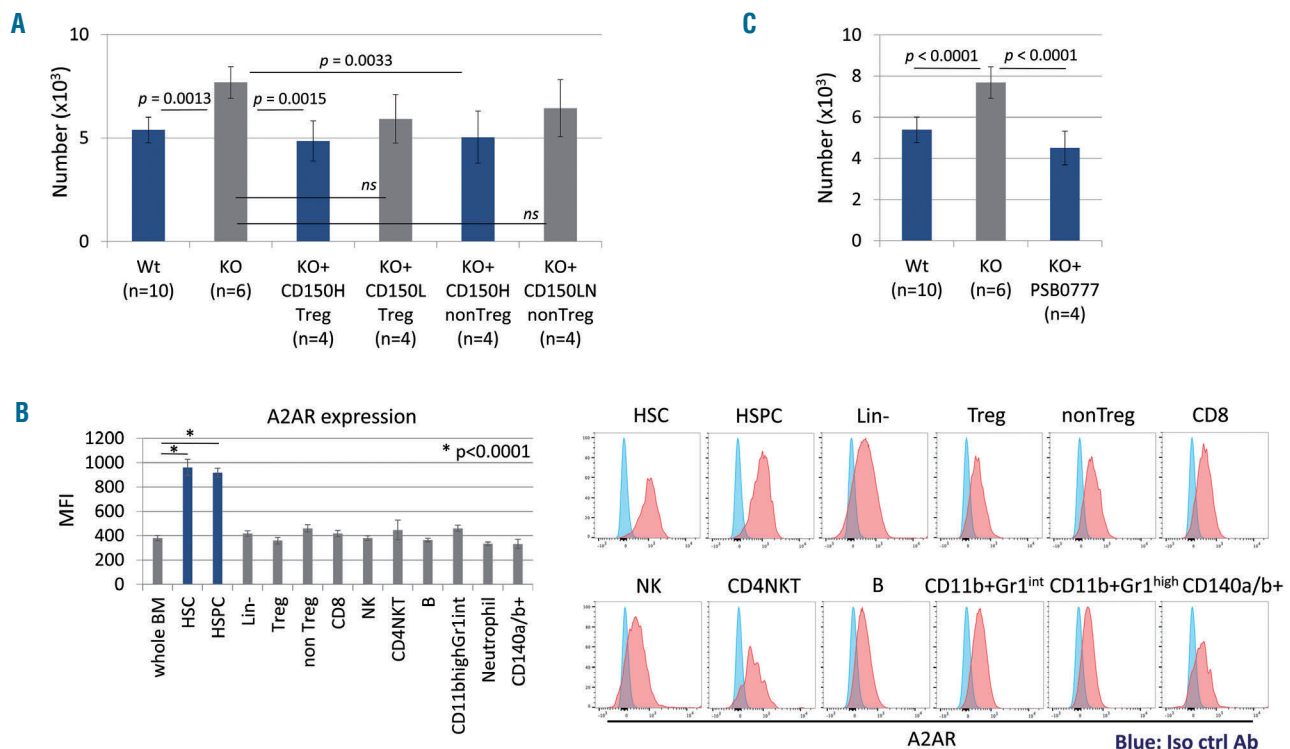**Figure 4. CD73 of CD150^{high} regulatory T cells and CD150^{high} non-regulatory T cells regulates hematopoietic stem cells via adenosine 2A receptors.** (A) Numbers of hematopoietic stem cells (HSC) in CD73 knockout (KO) mice 7 days after intravenous injection of CD150^{high} bone marrow (BM) regulatory T cells (Treg), CD150^{low} BM Treg, CD150^{high} BM nonTreg, or CD150^{low} BM nonTreg (30,000 cells/mouse). Data were pooled from three independent experiments (total 4-10 mice/group) and analyzed by one-way analysis of variance (ANOVA) with the Bonferroni multiple comparison test. (B) Levels of adenosine 2A receptor (A2AR) expression in different BM cell populations. MFI: mean fluorescence intensity. Four mice were used in the experiment. (C) HSC numbers in CD73 KO mice that received daily intraperitoneal injections of PSB0777 (25 μg/mouse) for 7 days. The total HSC numbers in one tibia and one femur were analyzed 1 day after the final injection. The results from two independent experiments were pooled (total 4-10 mice/group).

Transfer of these CD150^{high} Treg and CD150^{high} nonTreg, but not of CD150^{low} Treg or CD150[neg-low] nonTreg, reversed the increase of HSC in CD73 KO mice. Additionally, pharmacological activation of A2AR, highly expressed by HSC, reversed the increase of HSC in CD73 KO mice. Taken together, these results suggest that CD73 of CD150^{high} Treg and CD150^{high} nonTreg regulates HSC quiescence and abundance via A2AR.

To the best of our knowledge, this is the first study showing the role of conventional T cells in HSC regulation. This work is complemented by our recent study[7] showing that CD39 on CD150^{high} Treg played a critical role in maintaining HSC quiescence. As both CD150^{high} Treg and CD150^{high} nonTreg frequently displayed an effector memory T-cell phenotype,[7] the observations of our current and previous studies[7] suggest that BM CD4 memory T cells and memory Treg coordinate each other to generate extracellular adenosine via CD39 and CD73, maintaining HSC quiescence. As the BM is known to be a site to which memory T cells frequently home and in which they are maintained,[9,10] memory T cells and Treg generated following infection may play important roles in protecting BM HSC from oxidative and inflammatory stresses, controlling hematopoiesis. As CD150^{high} Treg frequently localized adjacent to HSC, a future histological analysis is warranted to identify the spatial distribution of CD150^{high} nonTreg (CD3^{+}CD4^{+}NK1.1^{-}FoxP3^{-}) with respect to HSC (CD150^{+}CD48^{-}CD41^{-}Lin^{-}), although such a study is technically challenging because of the requirement of multiple colors.

Our study does not rule out the possibility that HSC are regulated by other adenosine receptors or by P2 receptors that bind ATP metabolized by CD39. HSC expression of CD39, but not of CD73, may reflect the possibility that tight control of ATP/adenosine ratios is required for the maintenance of HSC quiescence. Indeed, a previous study showed that global P2YR deletion abrogated the radioresistance of HSC.[16] However, under normal conditions, P2YR KO mice did not show significant alteration of HSC numbers,[16] suggesting that the observed phenotypes in CD73 KO mice and FoxP3[cre] CD39[fl/wt] mice under normal conditions were not attributable to P2YR.

CD150^{high} nonTreg and CD150^{high} Treg are likely to generate adenosine in concert with various CD39^{+} or CD73^{+} BM cell populations, including HSC and the following two niche constituents: CD31^{+} vasculature and CD140a^{+}CD140b^{+}CD45^{-} mesenchymal cells which exclusively overlap with lepr^{+} perivascular cells.[2,3] Nevertheless, these two niche constituents are unlikely to be the major source of adenosine, because the frequencies of CD39^{high} and CD73^{high} cells in these mesenchymal cells were comparable to those in CD150^{low} Treg and transfer of these latter cells did not alter HSC numbers in CD73 KO mice. Indeed, our additional study using leprcre CD39[fl/wt] mice showed that conditional deletion of CD39 in lepr^{+} cells did not alter HSC number or reconstituting potential of BM cells (*Online Supplementary Figure S4A-C*). This observation further supports the important role of CD150^{high} Treg and CD150^{high} nonTreg in adenosine-mediated HSC regulation.

In summary, this work showed that CD150^{high} Treg and CD150^{high} nonTreg maintain HSC quiescence via CD73. An examination of the roles of adenosine and memory T cells in human hematopoiesis and transplantation is warranted.

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