CD74 is a regulator of Hematopoietic stem cell maintenance

Shirly Becker-Herman[1], Milena Rozenberg[1], Naama Gil-Yarom[1], Mattias Karmer[1], Avital Barak[1], Lital Sever[1], Keren Gavish[1], Lihi Radomir[1], Hadas Lewinsky[1], Richard Bucala[2], Amnon Peled[3] and Idit Shachar[1*]

[1]Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel; Internal Medicine, Yale School of Medicine, New Haven, CT, USA 06510; [3] Hadassah Hebrew University Hospital, Goldyne Savad Institute of Gene Therapy, P.O. Box 12000, Jerusalem, 91120 Israel

* Corresponding author: Idit Shachar, Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel; Tel: 972-8-9344257, Fax: 972-8-9344141, Email: idit.shachar@weizmann.ac.il

Key points

CD74 regulates HSPC maintenance, and its absence can result in expansion of this highly potent population.

Our study could lead to improved clinical insight into the pathophysiology of bone marrow transplantation protocols, as well as into diseases associated with hematopoietic failure. Since CD74 deficiency results in increased numbers of efficient stem cells, we suggest that blocking CD74 may lead to expansion of the stem cell pool and enhanced engraftment.

Abstract

The Hematopoietic stem cells (HSCs) are a small population of undifferentiated cells that have the capacity for self-renewal and differentiate into all blood cell lineages. These cells are the most useful cells for clinical transplantations and for regenerative medicine. So far, it has not been possible to expand adult HSCs without losing their self-renewal properties. CD74 is a cell surface receptor for the cytokine macrophage migration inhibitory factor (MIF), and its mRNA is known to be expressed in HSCs. Here, we demonstrate that mice lacking CD74 exhibit an accumulation of Hematopoietic stem cells in the BM due to their increased potential to repopulate and compete for BM niches. Our results suggest that CD74 regulates the maintenance of the HSCs. Its absence leads to induced survival of these cells and accumulation of quiescent and proliferating cells. Thus, we suggest that blocking CD74 could lead to improved clinical insight into bone marrow transplant protocols enabling improved engraftment.

Introduction

Host immunity requires a constant renewal of red blood cells and leukocytes throughout life, as these cells have a restricted lifespan. This process is enhanced following acute stress situations, such as infections or irradiation, by the proliferation of hematopoietic stem cells (HSCs) and progenitor cells (HPCs), which respond to these conditions. The hematopoietic stem and progenitor cells (HSPCs) are a small population of undifferentiated cells that reside in the bone marrow (BM). HSCs are defined by their capacity for self–renewal, and ability to differentiate into all blood cell lineages. Another distinct feature of these cells is their ability to migrate out of the BM to the peripheral blood. This process is enhanced under stress as a part of the host mechanisms of defense and repair. In addition, HSCs injected to the blood stream, as performed in BM transplantation, can home to the BM and re-establish the HSC pool as a lifelong reservoir of new blood and immune cells (Ludin et al., 2014).

The BM is the main site of adult hematopoiesis, and the majority of HSPCs remain confined to the BM microenvironment in a quiescent non-motile state maintained via adhesive interactions (Boulais and Frenette, 2015; Mendelson and Frenette, 2014; Morrison and Scadden, 2014). Under stress conditions, undifferentiated progenitor cells can be triggered by their microenvironment to undergo enhanced proliferation and differentiation, to address the demand of the immune and hematopoietic systems for new leukocytes and blood cells.

Cells in the BM microenvironment maintain a functioning pool of precursor cells regulated by cytokines, chemokines, and by additional lipid effectors. The chemokine CXCL12 and its primary receptor CXCR4 are essential for adhesion and retention of HSPCs in the mouse BM (Peled et al., 1999; Sugiyama et al., 2006). During homeostasis in the steady-state, CXCR4 is expressed by hematopoietic cells in addition to stromal cells, which are the main source for CXCL12 in the BM. $CXCR4^{+}$ HSPCs tightly adhere to bone marrow stromal cells, which express functional, membrane-bound CXCL12 (Sugiyama et al., 2006). The CXCL12/CXCR4 pathway is involved in regulation of migration, survival, and development of human hematopoietic cells. Increased expression levels of CXCL12 and CXCR4 induce proliferation of hematopoietic progenitors, and recruitment of bone-resorbing osteoclasts, osteoblasts, neutrophils, and other myeloid cells, leading to leukocyte

mobilization (Dar et al., 2006). In addition, reduced CXCR4 expression levels might result in an amplified mobilization response and cell proliferation in the BM (Karpova et al., 2017).

CD74 mRNA is expressed in HSPCs (Kiel et al., 2005; Klimmeck et al., 2014; Luckey et al., 2006); however, the role of this receptor in HSPCs was never analyzed. CD74 is a type II integral membrane protein that is expressed many cell types. The CD74 chain was initially described to function mainly intracellularly as an MHC class II chaperone (Stumptner-Cuvelette and Benaroch, 2002). A small proportion of CD74 is modified by the addition of chondroitin sulfate (CD74-CS), and this form of CD74 is expressed on the cell surface. It was previously shown that macrophage migration inhibitory factor (MIF) binds to the CD74 extracellular domain, a process that results in the initiation of a signaling pathway (Leng et al., 2003).

MIF is also a noncognate ligand of the CXCRs CXCR2 and CXCR4, and biochemical evidence suggests that these chemokine receptors could act as additional signal-transducing CD74 co-receptors upon MIF stimulation (Bernhagen et al., 2007; Schwartz et al., 2009). Importantly, structural and functional interactions between CD74 and the MIF chemokine receptor, CXCR4, have been proposed (Bernhagen et al., 2007; Klasen et al., 2014).

Our previous studies have shown that CD74 expressed on healthy and malignant B cells is directly involved in regulating murine mature B cell survival (Binsky et al., 2007; Matza et al., 2003; Matza et al., 2002b; Shachar and Flavell, 1996) through a pathway leading to the activation of transcription mediated by the NF-κB p65/RelA homodimer and its co-activator, TAFII105 (Matza et al., 2001). NF-κB activation is mediated by the cytosolic region of CD74 (CD74-ICD), which is liberated from the membrane and translocates to the nucleus (Matza et al., 2002a). Moreover, we demonstrated that CD74 stimulation by MIF enables augmented expression of anti-apoptotic proteins in a CD44-dependent manner. In addition, we recently characterized the transcriptional activity of CD74-ICD. We showed that following CD74 activation, CD74-ICD interacts with the transcription factors RUNX and NF-κB and binds to proximal and distal regulatory sites enriched for genes involved in apoptosis, immune response and cell migration. This leads to regulation of expression of these genes.

In the current study, the role of MIF/CD74 axis in HSPCs was followed. We show that CD74 plays a crucial role in HSCs maintenance. Deficiency of CD74 and MIF leads to enhanced survival and accumulation of HSPCs in

the BM. The enlarged pool of HSCs give rise to higher numbers of HSPCs and the various immune cell lineages. Cells lacking CD74 demonstrated an advantage in repopulating the host environment, as seen by the significantly higher levels of those cells when compared to the WT cells in mixed chimera. Thus, our study could lead to improved clinical insight into factors governing the efficacy of bone marrow transplantation protocols, as well as diseases associated with hematopoietic failure.

Results

Expansion of hematopoietic stem and progenitor cell populations in CD74$^{-/-}$ mice

CD74 mRNA levels were analyzed in HSPCs (Lin-Sca-1+c-Kit+; LSK) (Kiel et al., 2005; Klimmeck et al., 2014; Luckey et al., 2006), which are enriched for hematopoietic stem (CD34-Lin-Sca-1+c-Kit+; HSCs) and progenitor (CD34+ Lin-Sca-1+c-Kit+; HPCs) cells (gating is shown in Supplementary Fig 1 A-D) (Kiel et al., 2005; Osawa et al., 1996; Yang et al., 2005). To determine the role of CD74 in these cells, we first confirmed by FACS that CD74 protein is expressed on HSPCs, in low levels as expressed on mature B cells (Stumptner-Cuvelette and Benaroch, 2002)

.As shown in Fig 1A, CD74 was expressed on the surface of these populations. To determine CD74 function in these cells, cell numbers of the various stem and progenitor populations in WT and CD74$^{-/-}$ mice were compared. While similar numbers of BM cells were detected in WT and CD74$^{-/-}$ mice (Fig 1B), a significant increase in the Lineage marker-negative (CD11b-,Gr-1-,CD3-,B220-and Ter117-; Fig 1C) and LSK (Fig 1 D-E) populations was observed in the CD74$^{-/-}$ animals. Next, the numbers of HSCs and HPCs were compared. As shown in Fig 1 F-G, a significant increase in both the CD34^{+} and CD34^{-} populations was detected in mice lacking CD74, with a more significant elevation of the HSC CD34^{-} population. These populations are also characterized by expression of the SLAM family members, CD150 and CD48 (Kiel et al., 2005; Osawa et al., 1996; Yang et al., 2005). Similarly, we observed an increase in the number of HSCs and progenitors by FACS analysis for CD150, CD48, Lin^{-}, ckit^{+} and Sca-1^{+} (Fig 1 H-J). These results suggest elevated stem and progenitor cell populations in mice lacking CD74. Since CD74 regulates the expression of SLAM receptors (Binsky-Ehrenreich et al., 2014), we decided to focus on the CD34 marker for stem cell analysis in this study.

To examine the *in vitro* repopulation potential of CD74-deficient stem cells, the ability of HSPCs to proliferate and differentiate into colonies *in vitro* was analyzed by a colony-forming unit cells assay (CFU-C assay). As seen in Fig 1K, higher numbers of colonies were generated from CD74$^{-/-}$ BM when compared to WT cells.

Since MIF is the natural ligand for CD74 (Leng et al., 2003), we next analyzed the HSPCs populations in MIF deficient mice (*Mif*$^{-/-}$ mice). Total BM cells were extracted from WT and MIF$^{-/-}$ mice and progenitor populations were analyzed. Higher numbers of Lin neg and HSC populations were observed in the MIF$^{-/-}$ mice compared to

WT animals (Supplementary Figs 1 E-H). However, the differences were not as significant as in the CD74 deficient mice. This could be explained by partial compensation by the MIF homologue, MIF2 (Merk et al., 2011). These results suggest that the role of MIF/CD74 in HSPCs is opposite to its survival effect in B cells.

CD74$^{-/-}$ HSPCs demonstrate enhanced long-term self-renewal capacity

To determine whether the expansion of HSPCs in CD74$^{-/-}$ results from an effect intrinsic to the cells themselves, or whether the differences are due to extrinsic environmental factors, chimeric mice were generated. Total BM cells from WT or CD74$^{-/-}$ mice were transplanted into lethally irradiated WT or CD74$^{-/-}$ recipients. The animals were sacrificed after 16 weeks, and their HSPCs were analyzed. As seen in Fig 2 A-C, elevation in Lin, LSK and CD34^{-} populations was detected in mice transplanted with CD74$^{-/-}$ BM compared to WT donors. Thus, the lack of CD74 in the cells rather than in the microenvironment contributed to HSPC accumulation. Taken together, these results indicate that the lack of MIF/CD74 signaling results in an intrinsic increase in the HSPC population in the bone marrow.

Next, we followed the *in vivo* potential of CD74 negative HSPCs to repopulate and compete with WT cells, WT (CD45.1) BM cells were transplanted at a 1:1 ratio with either CD74$^{-/-}$ (CD45.2) or WT (CD45.2) cells into lethally irradiated recipient mice (CD45.1). BM populations of the mixed chimeras were analyzed at 6, 16 and 24 weeks after transplantation. While WT CD45.1 and CD45.2 chimera maintained a 1:1 ratio (Supplementary Fig 2), CD74$^{-/-}$ derived BM cells exhibited a growth advantage over the WT populations (Fig 3). The dramatic takeover of CD74$^{-/-}$ cells was observed as early as 6 weeks post-transplant and was maintained throughout the experiment. A significant advantage of CD74$^{-/-}$ total BM cells (Fig 3 A-B), myeloid (Fig 3C), total B cells (Fig 3D), and HSPC populations (Fig 3 E-F) was observed at various time points tested. In the B cell lineage, the growth advantage of the CD74$^{-/-}$ population was detected from early stages of B cell differentiation through the formation of immature B cells in the BM (Fig 3G). This advantage disappeared at the mature stage (Fig 3H), due to the role of CD74 as a survival receptor on these cells (Cohen and Shachar, 2012). These results show that the CD74 deficient BM cells have an advantage in repopulation.

Next, to directly test the capacity of CD74$^{-/-}$ HSPCs to repopulate the immune system, sorted LSK populations from WT (CD45.1) and CD74$^{-/-}$ (CD45.2) mice were transplanted at a 1:1 ratio to irradiated recipient mice (CD45.1) to generate competitive BM chimera. Bone marrow populations of the mixed chimeras were

analyzed 6 and 18 weeks following the transplantation. As shown in Figs 4 A-E, a significant advantage of $CD74^{-/-}$ BM cells, was observed at both 6 and 18 weeks post engraftment. These results indicated that LSK lacking CD74 are more efficient in repopulating the host environment, as seen by the significantly higher levels of those cells when compared to the WT (CD45.1).

$CD74^{-/-}$ derived BM cells show an advantage over the WT populations in BM transplantation. The dramatic overgrowth of $CD74^{-/-}$ cells might result from their higher numbers of progenitor cells, from their higher potential to repopulate, or both. To determine the basis for the enhanced ability of $CD74^{-/-}$ HSCs to repopulate the BM, we wished to follow the competition when the HSC WT and $CD74^{-/-}$ numbers are similar. Therefore, chimeric mice were generated with a ratio of about 3:1 in favor of the WT, which results in injection of similar numbers of HSCs. As shown in Fig 5, $CD74^{-/-}$ derived BM cells retained an advantage over the WT populations. While the WT (CD45.1):WT (CD45.2) chimeric mice preserved a ratio of 3:1, there was a dramatic takeover of $CD74^{-/-}$ cells as early as 6 weeks post-transplant that was maintained throughout the experiment (16 weeks) (Supplementary Fig 2F). A significant numerical advantage of $CD74^{-/-}$ total BM cells (Fig 5A), myeloid cells (Fig 5B), immature B cells (Fig 5C), LSK (Fig 5D), and HSC populations (Fig 5E) was observed at this time point. The only population that could not compete was the mature B population (Fig 5F), whose survival is dependent on CD74 expression (Bucala and Shachar, 2014). These results show that CD74-deficient HSCs have a stronger potential to repopulate the BM and compete for the niches than WT HSPCs.

To evaluate the long-term self-renewal and functional properties of $CD74^{-/-}$ HSCs, a serial BM transplantation assay was performed. BM cells from six WT and six $CD74^{-/-}$ mice were isolated, and the cells were serially transplanted into lethally irradiated WT mice. Each host transplanted with $2X10^6$ BM cells. During the first three first cycles, no significant differences were observed between the WT and $CD74^{-/-}$ groups, with high survival rates of all mice (Fig 5 G-I). However, in the fourth transplantation cycle, $CD74^{-/-}$ transplanted mice showed a better survival rate compared to the WT mice (57% compared to 33%) (Fig 5J). Thus, the absence of CD74 in HSCs results in accumulation of cells with a higher potential to repopulate the BM.

CD74 regulates stem cell survival

Next, we wished to identify the molecular mechanism regulating the HSPCs accumulation in CD74 deficient

mice. The accumulation of stem cells might result from their elevated retention in the BM niche or upregulation in their proliferation or survival. Since CXCR4 plays a major role in retention of HSCs and HSPCs (Karpova and Bonig, 2015; Karpova et al., 2017; Lapidot, 2001), we next wished to follow the role of CD74 in CXCR4 expression and function in HSPCs. We previously showed that following activation of CD74 expressed on CLL cells, CD74-ICD binds the chromatin of the CXCR4 promoter ((Gil-Yarom et al., 2017); Supplementary Fig 3A). Expression of cell surface CXCR4 HSPCs was therefore analyzed on WT and CD74 deficient mice. As shown in Fig 6 A-B, a reduction in the expression of CXCR4 on the cell surface was observed on $CD74^{-/-}$ cells. These results show that CD74 regulates CXCR4 expression. To determine whether the reduced levels of CD74 affect HSPCs retention, the total counts of HSPCs in WT and $CD74^{-/-}$ PB were compared. As shown in Fig 6 C-E, a dramatic elevation in the number of HSPCs in the circulation of CD74 deficient mice was detected. Thus, in the absence of CD74, lower CXCR4 levels result in reduced retention of HSPCs in the BM.

All studies performed with CXCR4 deficient HSPCs, demonstrated a requirement of this axis for efficient engraftment. However, transiently inhibition of CXCR4, indicated no adverse effects of the treatment on the engraftment capacity of the HSPCs (Karpova et al., 2017). Since CD74 deficiency partially reduce CXCR4 expression, we suggest that homing of injected cells to the BM is not affected, as shown in the chimeric mice experiments.

It was recently shown that blocking CXCR4 induces an increase in the cycling activity of the HSPCs (Karpova et al., 2017). We therefore analyzed the role of CD74 in HSPCs proliferation. To determine whether CD74 controls cell proliferation and cycle, Ki67 levels were followed. Higher numbers of both quiescent ($Ki67^{-}$) and cycling ($Ki67^{+}$) stem cells (CD34-LSK) (Fig 6F) and progenitors (CD34+LSK) (Fig 6G) were detected in $CD74^{-/-}$ mice. However, the ratio between $Ki67^{-}$ and $Ki67^{+}$ did not change (Fig 6H). To further follow cell proliferation in mice lacking CD74, a 5-bromodeoxyuridine (BrdU) labeling experiment was performed. Mice were fed with 0.8 mg/ml BrdU in the drinking water for 3 days and BrdU incorporation was followed. As shown in Fig 6 I-J, although higher numbers of both quiescent and cycling cells were detected $CD74^{-/-}$ mice, no significant change in the ratio of these populations was observed. These results imply that although the HSC and HSPC compartments are larger in mice lacking CD74, there is no over proliferation of a specific population and the proportion of proliferating cells is similar in the WT and $CD74^{-/-}$ mice.

Electron transfer along the mitochondrial respiration chain induces the formation of reactive oxygen species (ROS) (Kobayashi and Suda, 2012). Emerging evidence shows that oxidative stress and in particular, ROS content, influences stem cell migration, development, and self-renewal as well as their progression through the cell cycle (Ludin et al., 2014). To determine whether oxidative phosphorylation is elevated in the absence of CD74 in stem cells, ROS levels were compared in HSPCs of $CD74^{-/-}$ and WT cells. As can be seen in Fig 7A, higher number of ROS^{high} cells were detected in the $CD74^{-/-}$ HSPCs compared to the WT. However, no difference was observed in the ratio between ROS^{high} to ROS^{low} expressing cells (Fig 7B), suggesting that level of ROS expression in each subpopulation remains constant. To determine whether excess of ROS contributes to the expansion of $CD74^{-/-}$ stem cells, ROS levels were reduced using the antioxidant, N-acetyl-L-cysteine (NAC) (Ito et al., 2006). As shown in Fig 7 C-D, treatment with NAC for 6 days partially reduced the levels of HSPCs in $CD74^{-/-}$ mice. This suggests that ROS levels play a partial role in HSPC accumulation in mice lacking CD74.

To further probe the mechanism of action of CD74 in HSPCs, we wished to determine whether the higher number of $CD74^{-/-}$ HSPCs results from enhanced cell survival. Therefore, HSPCs cells were analyzed for cell survival using an Annexin V staining assay. As shown in Fig 7E**,** reduced apoptosis was observed in the $CD74^{-/-}$ $CD34^{-}$ LSK cells compared to the WT population. Thus, the higher number of $CD74^{-/-}$ stem cells can result from an increase in their survival.

Since CD74 regulates mature B cell survival, and its absence leads to cell death (Gore et al., 2008; Starlets et al., 2006), we wished to understand the different roles of CD74 in stem cells. Our hypothesis was that hypoxic conditions that exist in the BM environment and especially the perivascular niches where the non-dividing HSCs reside, control the enhanced survival of CD74-/- cells (Mohyeldin et al., 2010). WT and $CD74^{-/-}$ BM cells were incubated under hypoxia or with normal oxygen levels for 24 hours and cell survival was analyzed by Annexin V staining. As can be seen in Fig 7F, CD74 deficient stem cells survived better under hypoxia. Transfer of the $CD74^{-/-}$ cells to normoxic conditions reduced the advantage of these cells compared to WT stem cells (Fig 7G). These results support our suggestion that the BM hypoxic conditions play a role in the control of CD74 function.

Finally, to directly demonstrate the advantage of CD74 deficient cells in chemotherapy, WT and $CD74^{-/-}$ mice

were injected weekly with the cell-cycle dependent myelotoxic agent 5-fluorouracil (5-FU), which kills proliferating cells and thereby stimulates HSCs to proliferate and replenish the hematopoietic system (Cheng et al., 2000). After the second injection of this drug, at day 14, only 30% of the WT mice were alive, in contrast to 80% of the $CD74^{-/-}$ mice (Fig 7H). These results further suggest that elevated survival of CD74-/- HSCs allows a better replenishment of the immune system and increased survival of these mice under hemodepleting conditions.

Discussion

Despite the enormous experience in the manipulation and therapeutic use of HSCs, the biology of these cells is still not fully understood. HSCs are useful cells for transplantation and for regenerative medicine. However, it has been not possible to date to expand adult HSCs without losing their self-renewal properties. We suggest that CD74 regulates HSPC maintenance, and its absence or inhibition can result in expansion of this highly potent population.

CD74 is a membrane-associated protein that serves as a cell surface receptor for the cytokine MIF (Leng et al., 2003). Here we show that potent stem and progenitor cells are accumulated in the BM of CD74 or MIF-deficient mice due to intrinsic changes in these cells. The increased quiescent stem cell population in mice lacking CD74 allows enhanced replenishment of the immune system and increased survival following chemotherapy. In the absence of CD74, there is an accumulation of higher numbers of HSPCs, which show a stronger potential to repopulate and compete for the BM niches compared to WT HSPCs. The accumulation does not result from induced stem cell retention. Our results show that the absence of CD74 leads to elevated survival of the HSCs. This results in elevated numbers of HSCs that express similar levels of ROS as wt HSPCs and proliferate in a similar rate. However, due to the elevated numbers of HSCs, the total production of ROS and the number of proliferating cells is increased. This allows the production of elevated numbers of HSPCs that results in generation of hematopoietic cells of the different lineages.

CD74 regulates CXCR4 expression (Gil-Yarom et al., 2017). It was recently shown that inhibition of the CXCR4/CXCL12 axis on the HSPC compartment results in an increase in mobilization efficiency. In addition, a

concurrent increase in the cycling activity, self-renewing proliferation of the HSPC pool and expansion of this population in the BM was observed (Karpova et al., 2017). Our results show that CD74 deficiency downregulates CXCR4 cell surface expression. However, this downregulation does not affect cell proliferation, but rather induces survival of the cells.

CD74 functions as a survival receptor in mature B cells (Gore et al., 2008; Starlets et al., 2006), while results presented in this study show that the lack of CD74 leads to accumulation of functional HSPCs in the BM due to their induced survival. The CD74-dependent cell type-specific regulation of survival might result from its association with different cell surface receptors resulting in induction of cell type specific signals. Alternatively, it is possible that the environment of these two cell types controls the different outcomes. While the blood and spleen environment is normoxic, the BM environment is hypoxic. Niches of various stem cells, including hematopoietic cells, are microenvironments with low oxygen tension, ranging from 1% to 8% O_2 (Mohyeldin et al., 2010). The hypoxic environment plays a critical role in the regulation of stem cell self-renewal and differentiation (Mohyeldin et al., 2010). Our results show that the hypoxic microenvironment plays a role in the CD74-induced outcome, showing an advantage for the CD74 deficient HSPCs in the hypoxic microenvironment. Thus, we suggest that in normal conditions, CD74 fine tune the maintenance of the BM stem cells inducing a cascade that leads to cell death. It was previously shown that mesenchymal stem cells produce MIF to delay hypoxia-induced senescence (Palumbo et al., 2014). In addition, MIF protects against hypoxia/serum deprivation-induced apoptosis of mesenchymal stem cells by interacting with CD74 to stimulate c-Met, leading to downstream PI3K/Akt-FOXO3a signaling and decreased oxidative stress (Xia et al., 2015). Thus, CD74 induces a cell type specific cascade that leads to different cell death outcomes.

Several novel HSC transplantation protocols are based on the administration of high numbers of donor cells. The need for large numbers of engraftable cells becomes particularly challenging in case of cord blood (CB)-transplantation and adult HSC-gene therapy protocols, because of suboptimal HSC doses available for infusion, and impaired engraftment of the transplanted cells (Psatha et al., 2016). Methods to improve maintenance and expansion of HSPCs resulting in increased numbers of cord blood stem cells may shorten time to engraftment and improve survival in adult recipients. Since CD74 deficiency results in a higher numbers of efficient stem

cells, we suggest that blocking CD74 in HSCs may lead to expansion of the stem cells and an improved transplantation protocol.

Materials and Methods

Mice

C57BL/6, CD74-/- (Shachar and Flavell, 1996), MIF-/- and CD45.1 mice were used in this study. All mice were used at 6-8 weeks of age.

N-acetyl-L-cysteine (NAC) was administered by I.P. injection (50mg kg $^{-1}$; Sigma) for 6 consecutive days before the experiments. 5FU was administered by I.P. injection (150 mg kg $^{-1}$; ABIC, Teva group) once a week. The Weizmann Institute Animal Care and Use Committee approved all animal experiments.

Cells- Bone marrow cells were obtained by flushing long bones with PBS, and peripheral blood was collected from the eye or heart using heparinized syringes.

Flow Cytometry

For flow cytometry analysis we used the following monoclonal antibodies: anti mouse lineage cocktail (cat: 13302), CD11b (clone: M1/70), Gr-1 (clone: RB6-8C5), Ter119 (clone: TER-119), CD3 (clone: 145-2C11), B220 (clone: RA3-6B2), c-Kit (clone: 2B8), , IgM (clone: RMM-1), IgD (clone: 11-26C), CD115 (clone: AFS98), CD150 (clone: TC15-12F12.2), CD48 (clone: HM48-1), CD45.1 (clone: A20), CD45.2 (clone: 104), anti Rabbit (clone: poly4064) all from Biolegend USA. Anti-Sca-1 (clone: D7), CD34 (clone: RAM34) were purchased from eBioscience, USA. Anti-CD74 (cat: FAB7478A) was purchased from R & D Systems, USA, and anti-CXCR4 (cat:TP-503) from Torrey Pines Biolabs. All analyses were done using a FACS Canto II flow cytometer (BD Bioscience, USA).

Sorting of the LSK CD45.1 WT and CD45.2 CD74-/- cells was performed using a FACS Aria II system (BD Bioscience, USA), following enrichment using CD117 (c-Kit) MicroBeads (cat: 130-091-224) on LS MACS Separation Columns (cat: 130-042-401), both obtained from Miltenyi Biotec, UK.

Generation of chimeric mice

Lethally irradiated (950 Rad) C57BL/6 (WT) recipient mice were reconstituted with $5*10^6$ either WT or CD74-/- BM cells. Additionally, lethally irradiated (950 rad) CD74-/- recipient mice on a C57BL/6 background were reconstituted with $5 *10^6$ WT or CD74-/- total BM cells. Long-term reconstitution of the peripheral blood and bone marrow was evaluated at 16 weeks post-transplant.

Competitive total BM transplant- Lethally irradiated (950 Rad) WT recipient mice (CD45.1 on a C57BL/6 background), were reconstituted with 2.5 $x10^6$ WT CD45.1 total BM cells together with either 2.5 $x10^6$ CD45.2 WT or 2.5 x 10^6 CD45.2 CD74-/- total BM cells (1:1 ratio), or 1.5$x10^6$ WT CD45.1 together with 0.5$x10^6$ CD45.2 WT or 0.5 x 10^6 CD45.2 CD74-/- (3:1 ratio). Short and long-term donor reconstitution (CD45.1 and CD45.2) was monitored 6, 16 and 24 weeks post transplantation.

Competitive LSK cell transplantation- Lethally irradiated (950 Rad) WT recipient mice (CD45.1 on a C57BL/6 background) were reconstituted with 7.5 * 10^4 WT CD45.1 sorted LSK (Lin-/Sca-1+/c-Kit+) cells together with 7.5 * 10^4 CD45.2 CD74-/- sorted LSK (Lin-/Sca-1+/c-Kit+) cells.

Colony-forming assay (CFU-C)

BM mononuclear (BM-MNC) cells were isolated by Ficoll separation, and were seeded (15$x10^3$ cells/ml) in CFU-C semisolid medium supplemented with EPO, IL-3, GM-CSF and SCF, as described (Kollet et al., 2006). CFU-C were scored 7 days after plating, and presented as CFU-C per number of seeded cells.

Serial Transplantation

For serial transplantation assay, 2$x10^6$ BM cells were obtained from 6 WT donors and 6 CD74-/-donor mice and transplanted to lethally irradiated WT CD45.1 animals. Each donor population was transplanted to 4-5 recipient mice. After 10-12 weeks post transplantation, one mouse from each donor served as a donor for the subsequent transplant.

Cell-cycle analysis

To analyze quiescent cells, total bone-marrow cells were stained for the designated markers (lineage/Sca-1/c-Kit/CD34), fixed and permeabilized using BD Cytofix/Cytoperm Plus kit (BD Bioscience, USA) and stained with the Ki-67 antibody (cat: 556026; BD Pharmingen, USA). To determine proliferation, C57BL/6 and CD74-/- mice were fed with 0.8 mg/ml BrdU in their drinking water for 3 days. BrdU incorporation was followed in LSK cells from bone-marrow using the BrdU flow kit (BD Pharmingen)

ROS analysis

Cellular ROS levels were analyzed by incubation of BM cells with 2 μM hydroethidine (Molecular Probes, USA) for 10 minutes at 37°C. Cells were then washed with PBS and stained for lineage/Sca-1/c-Kit/CD34 markers, and analyzed by FACS.

Apoptosis analysis

Total bone marrow cells were stained with the appropriate antibodies, using the Annexin V binding buffer (1:10 in ddW; cat: 556454, BD Pharmingen, USA) and mixed with Annexin V (FITC Annexin V, cat: 556419; BD Pharmingen, USA). All samples were incubated for 15 minutes at room temperature. For measuring Annexin levels under hypoxia, the BM cells were incubated in a hypoxia chamber at 1% O_2 for 24 hours before staining.

Statistical analysis

All statistical analyses were conducted using Prism 7 version. All data are expressed as mean ± SEM, unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001. Unless indicated otherwise in the figure legends.

n values represent biological replicates.

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The authors declare no competing interests

Author contributions

S. B.-H.- designed research, performed research, analyzed data and wrote the paper.

M. R.- designed research, performed research and analyzed.

N. G.-Y.- designed research, performed research and analyzed data.

M. K.- designed research and analyzed data.

A. B- designed research, and analyzed data.

L. S.- designed research and analyzed data.

K. G.- designed research and analyzed data.

L. R.- designed research and analyzed data.

H. L.- designed research and analyzed data.

R. B.- designed research, analyzed data and wrote the paper.

A. P.- designed research, analyzed data and wrote the paper.

I. S.- designed research, analyzed data and wrote the paper.

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Legends

Figure 1- Expansion of HSPCs in the BM of CD74-/- mice. (A) BM cells derived from WT or CD74-/- were purified. Histograms show representative analysis of CD74 expression on HSPCs in WT and CD74-/- mice. n=3. **(B)** Total number of BM cellularity per femur and tibia in WT and CD74-/-mice. **(C-J)** The percent of the different populations in WT and CD74-/-derived BM cells. (C) Lin-; (D) Representative FACS analysis for WT and CD74-/- HSPCs analysis; (E) LSK; (F) CD34-/LSK and (G) CD34+; (H) CD150+CD48-LSK; (I) CD150-CD48-/LSK; and (J) CD150-CD48+/LSK. n=14-18. **(K)** CFUC assay: Total BM cells from WT and CD74-/- mice were seeded at 15,000 cells/mL in semisolid cultures supplemented with cytokines and nutrients. Colony-forming unit cells (CFU-C) were counted 7 days later, n=7. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001.

Figure 2- CD74-/- HSPCs expansion is cell-intrinsic. Lethally irradiated WT or CD74-/- mice were transplanted with either WT or CD74-/- total BM cells. Long-term reconstitution was evaluated 16 weeks post transplantation. Percent of total BM cells was calculated for **(A)** LIN**-, (B)** LSK, and **(C)** CD34-/LSK; n=5-12. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001.

Figure 3- CD74-/- HSPCs have an advantage in BM repopulation. Lethally irradiated WT (CD45.1) mice were transplanted with bone marrow derived from WT (CD45.1) and WT (CD45.2) at a 1:1 ratio or bone marrow derived from WT (CD45.1) and CD74-/- (CD45.2) mice at a 1:1 ratio. **(A)** Representative BM FACS staining. Percent of donor-derived cells was analyzed in the BM after 6, 16 and 24 weeks in **(B)** Total BM cells; **(C)** Myeloid cells; **(D)** B cells; **(E)** LSK; **(F)** CD34-/LSK; **(G)** immature BM B cells; **(H)** mature BM B cells. n=8-18. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001.

Figure 4- CD74-/- HSPCs show a higher potential to repopulate the BM. Lethally irradiated WT CD45.1 recipient mice were reconstituted with $7.5*10^4$ sorted LSK cells from WT (CD45.1) and $7.5x10^4$ sorted LSK from CD74-/- (CD45.2) at a 1:1 ratio. Percent of donor-derived cells were analyzed in the BM after 6 and 18 weeks. **(A)** Total BM cells; **(B)** Myeloid cells; **(C)** immature BM B cells; and **(D)** mature BM B cells. **(E)** Percent of donor-derived cells was analyzed in LSK and CD34-LSK cells 18 weeks post-transplant. n=6-8. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001.

Figure 5- CD74-/- HSPCs demonstrate enhanced long-term self-renewal capacity. Lethally irradiated WT(CD45.1) mice were transplanted with bone marrow derived from WT (CD45.1) and WT(CD45.2) at a 3:1 ratio or bone marrow derived from WT (CD45.1) and $CD74^{-/-}$ (CD45.2) mice at a 3:1 ratio. Mice were analyzed 16 weeks after transplantation. Graphs show percent of donor derived cells from both WTCD45.1/WTCD45.2 and WTCD45.1/CD74-/-CD45.2 chimera. **(A)** Total BM cells; **(B)** BM myeloid cells; **(C)** BM immature B cells; **(D)** LSK; **(E)** CD34-LSK; **(F)** mature BM B cells; n=13. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001. **(G-J)** Survival curves for serial transplantation assay. Six donors from each genotype were transplanted to 4-5 lethally irradiated hosts. After 10-12 weeks, one mouse from each donor served as a donor for the following transplant. $2x10^6$ BM cells were transplanted to each host in all the transplantations. Log-rank test *<0.05, n=20-30 mice in each transplant per genotype.

Figure 6- Accumulation of HSPCs does not result from their elevated retention. (A-B) FACS analysis of CXCR4 expression on BM LSK and BM CD34-/LSK of WT and CD74-/- mice, n=7. Representative histograms are shown. **(C-E)** FACS analysis for HSPCs in the peripheral blood (PB) of WT and CD74-/-. (C) Dot plot analysis of LSK in WT and CD74-/- mice. **(D-E)** Cell number of (D) LSK, and (E) CD34-LSK in 600 μl blood. WT n=6 CD74-/- n=7. **(F-H)** FACS staining of WT and CD74-/- HSPCs for Ki-67. Results are presented as: (F) percent of CD34-/LSK Ki-67- and CD34-/LSK Ki-67+ from total BM cells, (G) percent of CD34+/LSK Ki-67- and CD34+/LSK Ki-67+ from total BM cells, and (H) percent of Ki-67+ from CD34-/LSK and percent of Ki-67+ from CD34+LSK, n=15. **(I-J)** Mice were fed with 0.8 mg/ml BrdU in the drinking water for 3 days, and BrdU

incorporation was analyzed by FACS. Results are represented as: (I) percent of LSK BrdU- and LSK BrdU+ from total BM cells (J), percent of BrdU+ in LSK n=12-14. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01***< 0.001****< 0.0001.

Figure 7- Elevated survival in CD74-/- HSPCs. (A-B) FACS staining of WT and CD74 deficient HSPCs for ROS. (A) Results are presented as number of ROS high cells per 10^6 cells, n=9 (B) Percentage of ROS^{high} in LSK. **(C-D)** Percent of LSK (C) and CD34- (D) after 6 days of NAC injections (50mg kg^{-1}), n=5. **(E-F)** FACS analysis of HSPCs from WT and CD74-/- mice for Annexin V (E), n=10-12, and after 24h in hypoxia conditions (F), n=3 (each dot represents a duplicate determination). **(G)** Ratio of Annexin V+ $CD74^{-/-}$ to WT of HSPCs in hypoxia and normoxic conditions. Bars show SEM. Unpaired t-test two tailed *<0.05 **<0.01. **(H)** Survival curve: 5FU (150mg/kg) was injected to WT and CD74-/- mice once a week for 2 weeks. Log-rank test *<0.05, n=10 in each group.

Figure 1

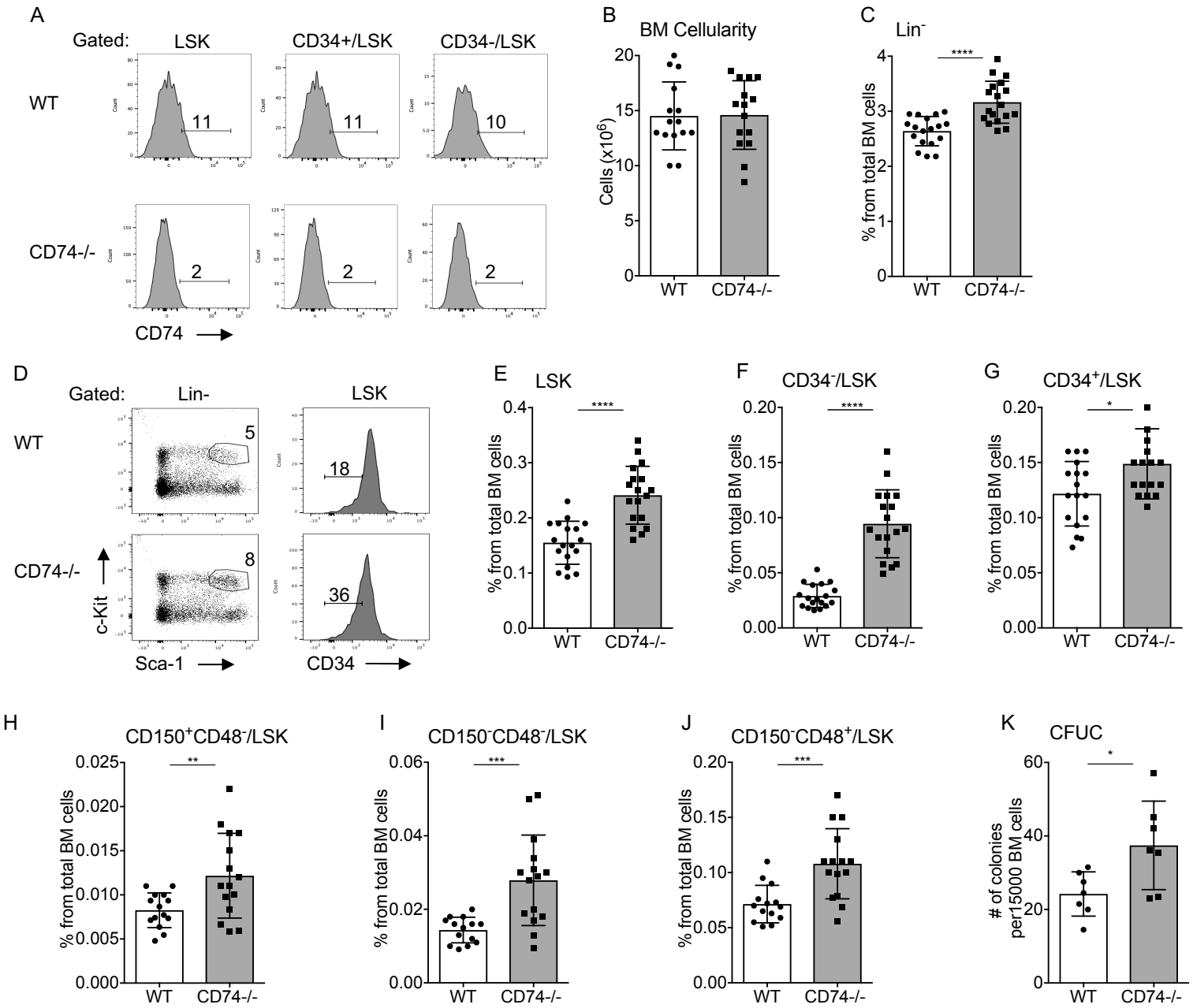

A
Gated:
LSK
CD34+/LSK
CD34-/LSK
WT
CD74-/-
11
11
10
2
2
2
CD74
B
BM Cellularity
Cells (x10^6)
WT
CD74-/-
C
Lin-
% from total BM cells
****
D
Gated:
Lin-
LSK
5
18
8
36
c-Kit
Sca-1
CD34
E
LSK
F
CD34-/LSK
G
CD34+/LSK
*
H
CD150+CD48-/LSK
**
I
CD150-CD48-/LSK
***
J
CD150-CD48+/LSK
K
CFUC
# of colonies per15000 BM cells

Figure 2

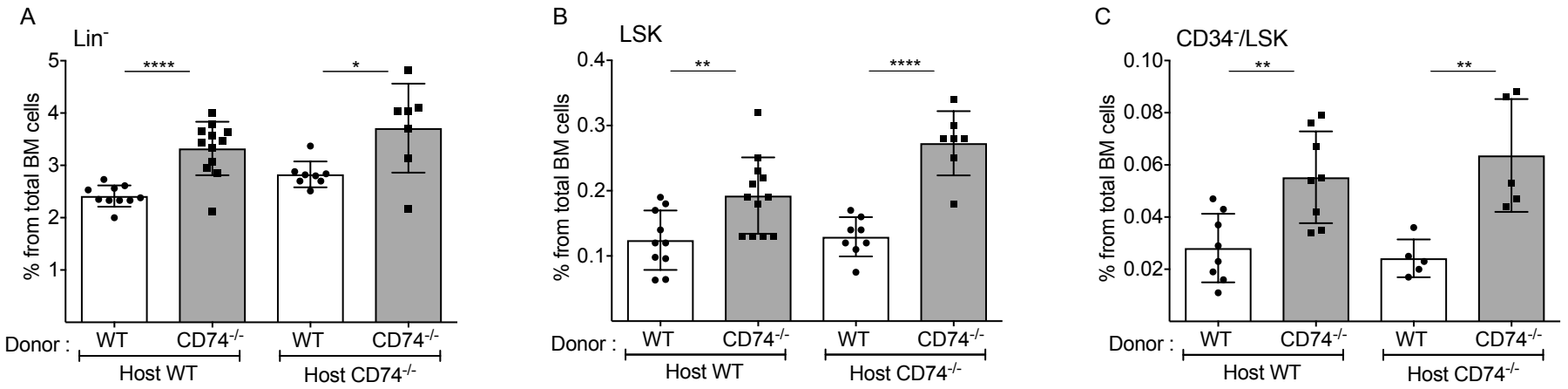

A
Lin-
% from total BM cells
5
4
3
2
1
****
*
Donor :
WT
CD74-/-
WT
CD74-/-
Host WT
Host CD74-/-
B
LSK
% from total BM cells
0.4
0.3
0.2
0.1
**
****
Donor :
WT
CD74-/-
WT
CD74-/-
Host WT
Host CD74-/-
C
CD34-/LSK
% from total BM cells
0.10
0.08
0.06
0.04
0.02
**
**
Donor :
WT
CD74-/-
WT
CD74-/-
Host WT
Host CD74-/-

Figure 3

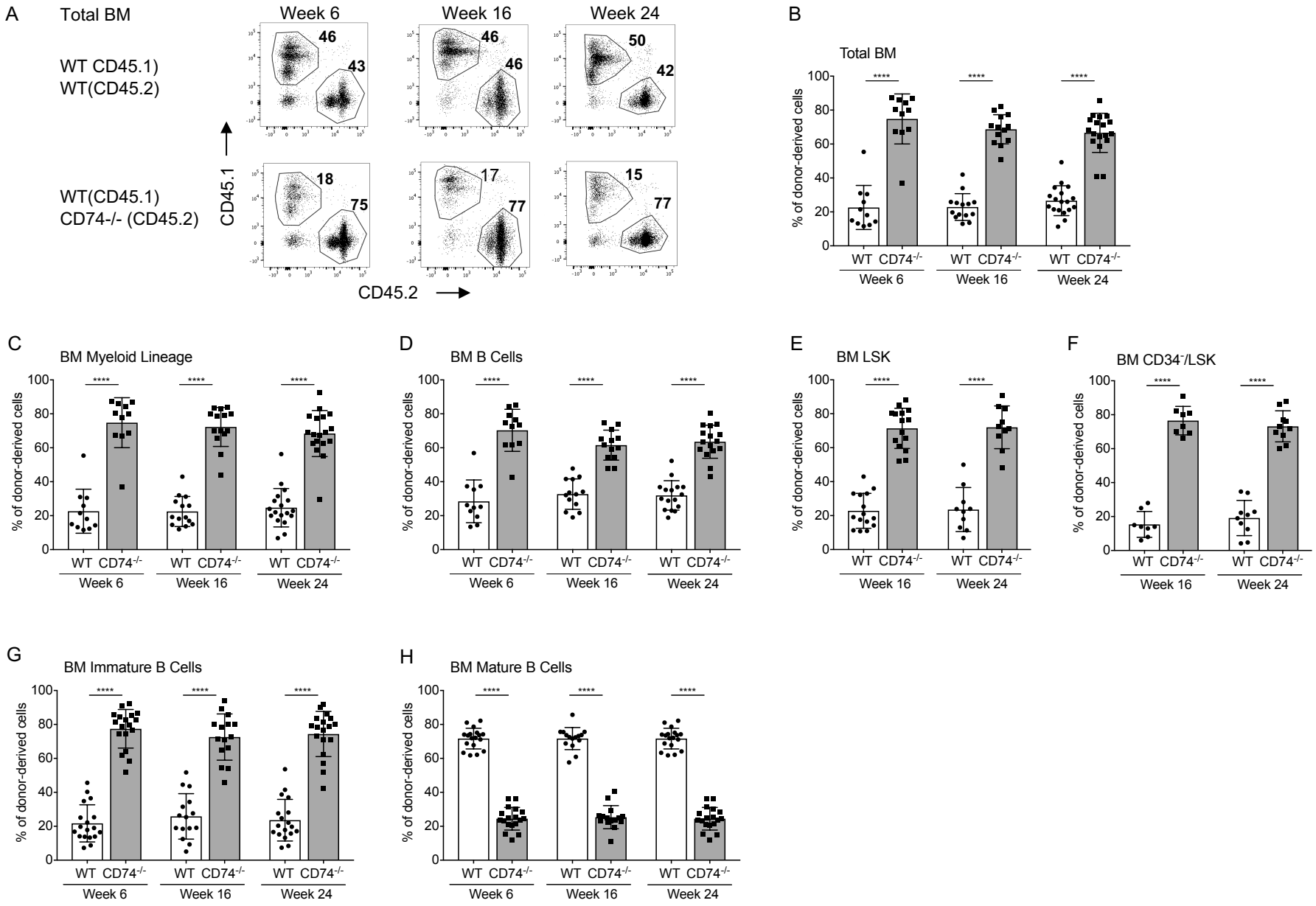

A
Total BM
WT CD45.1)
WT(CD45.2)
WT(CD45.1)
CD74-/- (CD45.2)
Week 6
Week 16
Week 24
46
43
46
46
50
42
18
75
17
77
15
77
CD45.1
CD45.2
B
Total BM
% of donor-derived cells
WT CD74-/-
Week 6
Week 16
Week 24
C
BM Myeloid Lineage
D
BM B Cells
E
BM LSK
F
BM CD34-/LSK
G
BM Immature B Cells
H
BM Mature B Cells

Figure 4

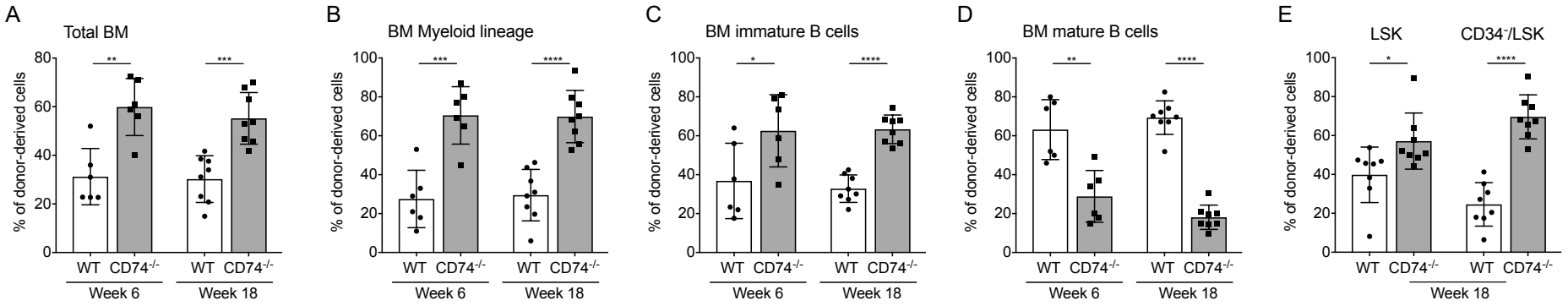

A
Total BM
B
BM Myeloid lineage
C
BM immature B cells
D
BM mature B cells
E
LSK
CD34-/LSK
% of donor-derived cells
WT
CD74-/-
Week 6
Week 18

Figure 5

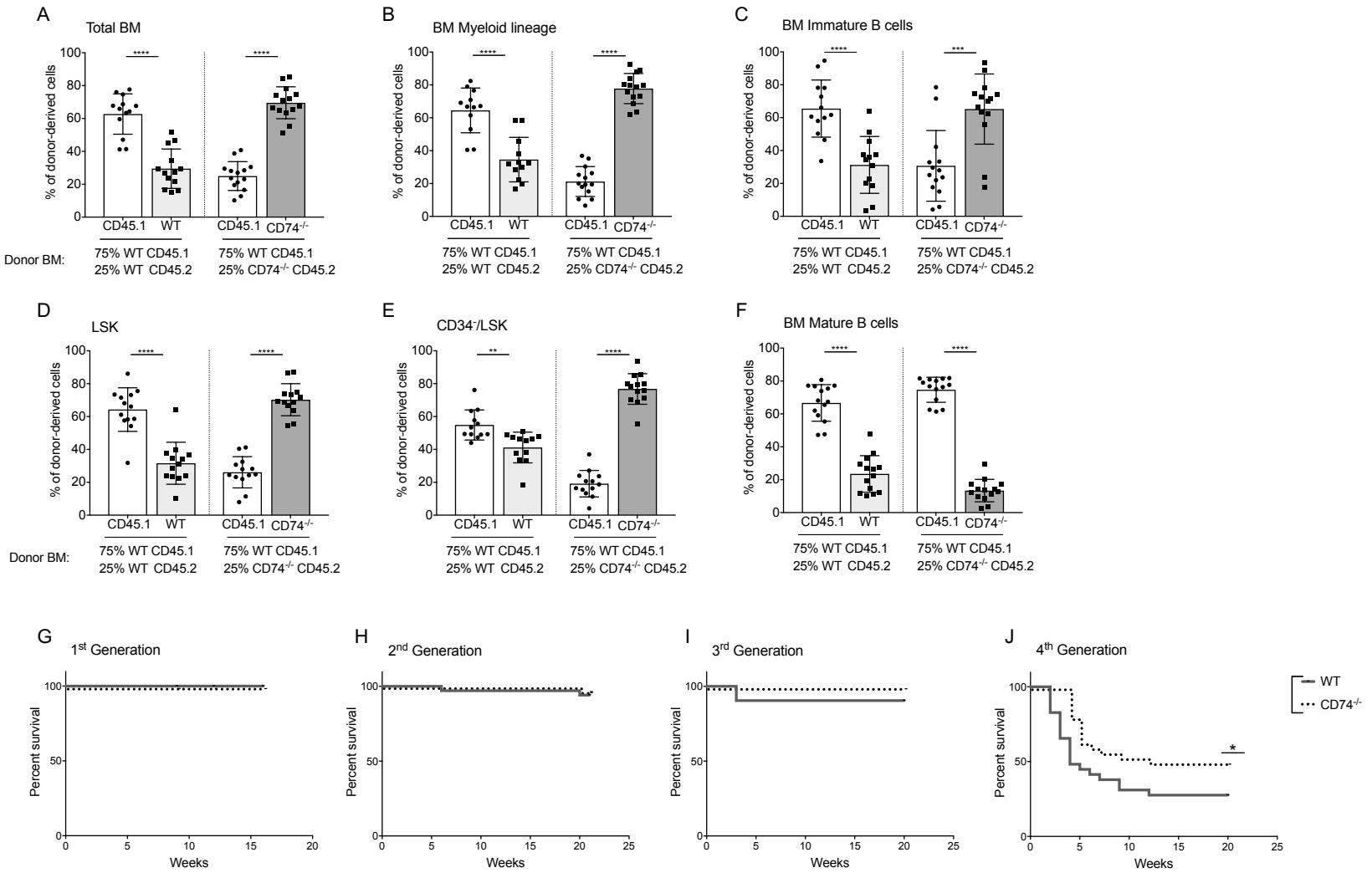

A
Total BM
B
BM Myeloid lineage
C
BM Immature B cells
D
LSK
E
CD34-/LSK
F
BM Mature B cells
% of donor-derived cells
CD45.1
WT
CD74-/-
Donor BM:
75% WT CD45.1
25% WT CD45.2
75% WT CD45.1
25% CD74-/- CD45.2
G
1st Generation
H
2nd Generation
I
3rd Generation
J
4th Generation
Percent survival
Weeks
WT
CD74-/-

Figure 6

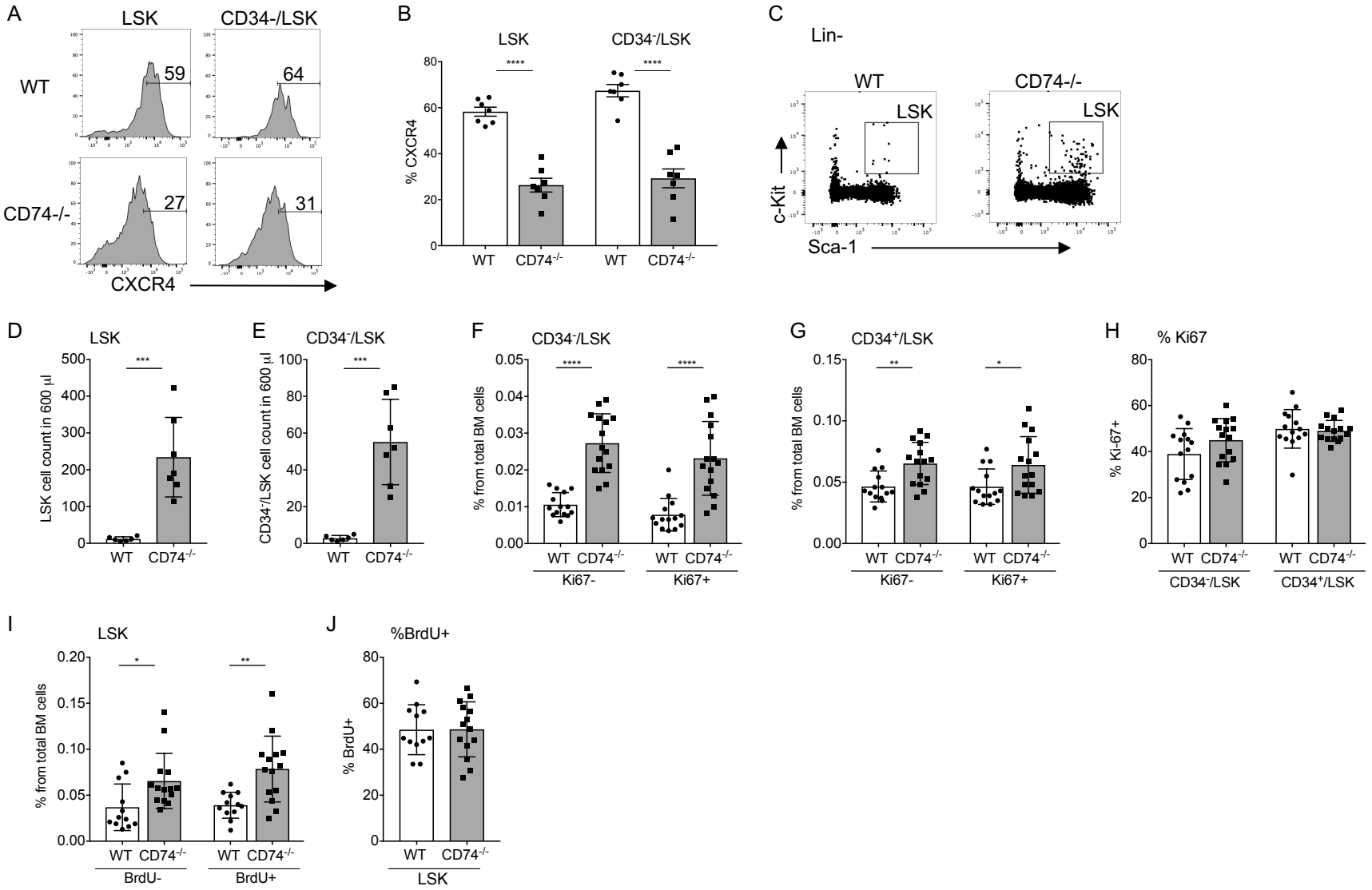
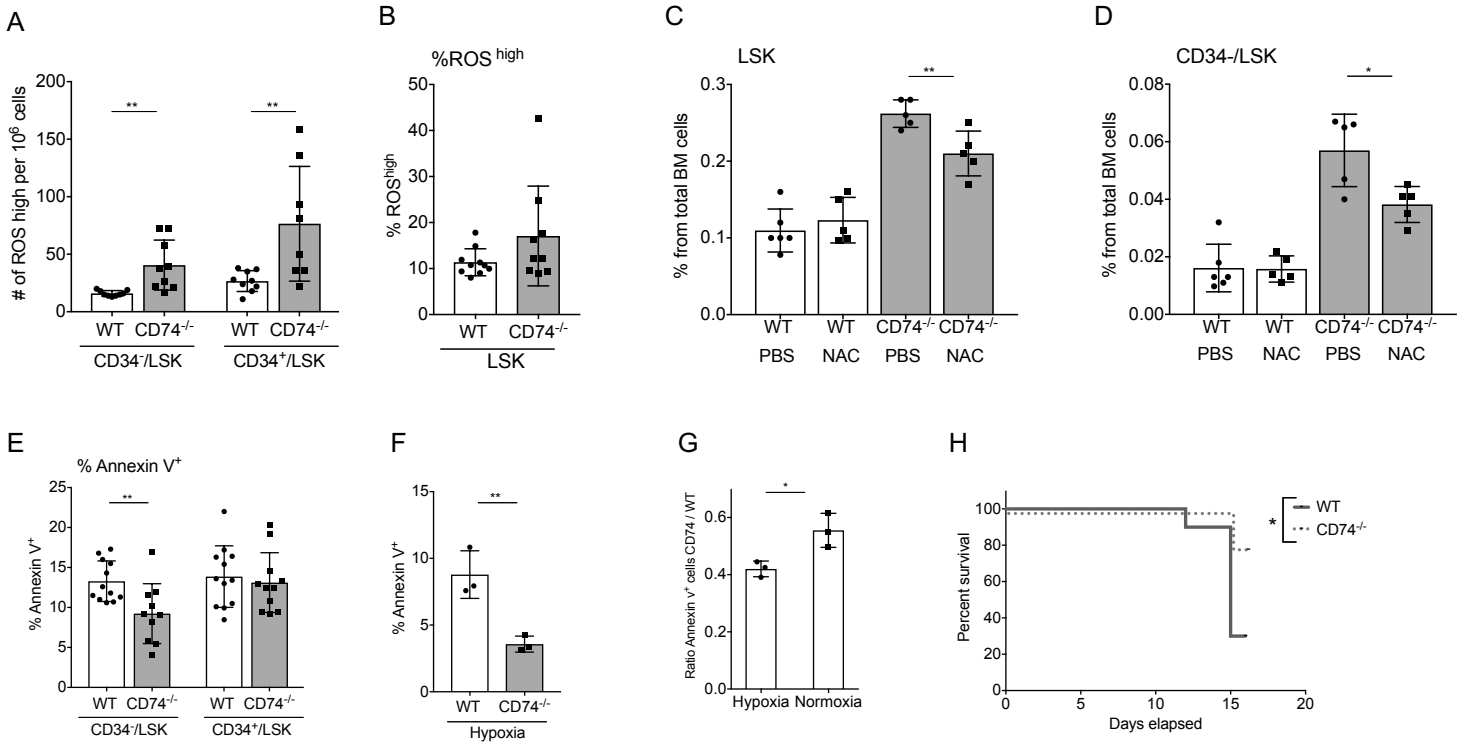

A
LSK
CD34-/LSK
WT
CD74-/-
59
64
27
31
CXCR4
B
LSK
CD34-/LSK
% CXCR4
WT
CD74-/-
C
Lin-
WT
CD74-/-
LSK
c-Kit
Sca-1
D
LSK
LSK cell count in 600 µl
E
CD34-/LSK
CD34-/LSK cell count in 600 µl
F
CD34-/LSK
% from total BM cells
Ki67-
Ki67+
G
CD34+/LSK
% from total BM cells
Ki67-
Ki67+
H
% Ki67
% Ki-67+
CD34-/LSK
CD34+/LSK
I
LSK
% from total BM cells
BrdU-
BrdU+
J
%BrdU+
% BrdU+
LSK

Figure 7


A
# of ROS high per 10^6 cells
WT CD74-/- WT CD74-/-
CD34-/LSK
CD34+/LSK
B
%ROS high
% ROShigh
WT CD74-/-
LSK
C
LSK
% from total BM cells
WT WT CD74-/- CD74-/-
PBS NAC PBS NAC
D
CD34-/LSK
% from total BM cells
WT WT CD74-/- CD74-/-
PBS NAC PBS NAC
E
% Annexin V+
% Annexin V+
WT CD74-/- WT CD74-/-
CD34-/LSK
CD34+/LSK
F
% Annexin V+
WT CD74-/-
Hypoxia
G
Ratio Annexin V+ cells CD74 / WT
Hypoxia Normoxia
H
Percent survival
Days elapsed
WT
CD74-/-