이학석사 학위논문

DARC on macrophage maintains the dormancy of long-term hematopoietic stem cells

대식세포의 DARC가 최상위 조혈모세포의 휴면을 조절하는 기전에 관한 연구

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채 정 환

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이 논문을 이학석사학위논문으로 제출함

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Abstract

DARC on macrophage maintains the dormancy of long-term hematopoietic stem cells

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Among many types of mouse hematopoietic stem-progenitor cell s (HSPCs), CD82/KAI1 is expressed exclusively by long-term he mato-poietic stem cells (LT-HSCs), which is at the top of the hema top-oietic hierarchy. In this study, we searched for binding partner of

CD82/KAI1 and investigated its influence on regulating HSC quiescence. Duffy antigen receptor for chemokines (DARC/CD234), the counter molecule of CD82/KAI1, was found to be expressed by bone marrow (BM) macrophages located in the endosteal and arteriolar niches. Upon BM macrophage ablation, LT-HSCs lost direct contact with DARC, resulting in ubiquitination and endocytosis of surface CD82/KAI1 molecules. Decreased surface CD82 level of LT-HSCs led to cell cycle entry and proliferation, which could be reversed by macrophage co-culture. Taken together, our findings highlight the importance of BM niche components – DARC+ macrophages – in maintenance of LT-HSC dormancy. Furthermore, human primitive hematopoietic stem cells derived from umbilical cord blood exhibited significant surface KAI1 levels. Particularly KAI1 was predominantly expressed on dormant HSCs while much less was expressed on actively cycling HSCs and precursors. As in mouse, DARC was mainly expressed by human monocytes/macrophages and maintained KAI1 expression on human HSCs, thereby down-regulating cell cycle progression of HSCs. Recombinant human DARC protein also preserved surface CD82 expression and dormancy of HSCs. The KAI1/DARC axis represents a new strategy to make HSC therapies available for broader spectrum of patients (e.g. ex vivo expansion of umbilical cord blood).

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Keywords : Hematopoietic stem cell, KAI1/CD82, DARC, Quiescence, Macrophage, Umbilical cord blood, Bone marrow
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Contents

I . INTRODUCTION

CD82/KAI1(CD82 hereafter) which belongs to the tetraspanin super-family, initially identified in studies of T-cell activation (Liu and Zhang, 2006; Miranti, 2009). These days, CD82 has attracted attention because CD82 expression has been shown to suppress metastasis (Kim et al., 2005; Miranti, 2009). Furthermore, CD82 is ubiquitously expressed and evolutionally conserved (Liu and Zhang, 2006). It also has its significant function in non-tumor cells as well.

Duffy antigen receptor for chemokines (DARC)/CD234(DARC hereafter) is a seven-transmembrane domain protein which is mainly expressed on erythrocytes, vascular endothelium, and a subset of epithelial cells (Peiper et al., 1995). Previous research has reported that endothelial DARC induces CD82+ tumor cells senescence through directly binding to CD82 for CD82-mediated suppression of cancer metastasis (Bandyopadhyay et al., 2006; Khanna et al., 2014).

Hematopoietic stem cells (HSCs) reside in bone marrow and supply all blood cells by their self-renewal and differentiation capabilities. While most HSCs remain dormant in the BM stem cell niche, depending on blood cell requirements they undergo cycles of quiescence and self-renewal (Tesio and Trumpp, 2011; Zon, 2008). LT-HSCs which are at the top of the hematopoietic hierarchy, have long-lasting self-renewal and differentiation capacity. LT-HSCs also enables life-long production of all hematopoietic lineages, while multipotent progenitor cells (MPPs) only possess transient differentiation capacity (Doulatov et al., 2012).

Previous reports have shown that CD82 is expressed predominantly

on LT-HSCs, but rarely expressed on ST-HSCs or MPPs in the BM niche, and CD82 molecule is key functional marker to maintain quiescence of LT-HSCs (Hur et al,. 2016). However, there is no published data in terms of binding partner in BM. In addition to significant molecules on LT-HSCs, other important factors that may regulate patterns of expressing protein on HSCs, particularly the quiescence-proliferation decision, include diverse environmental factors, such as angiopoietin-1, osteopontin, stromal derived factor-1 (SDF-1), thrombopoietin, and hypoxia inducible factor-1 alpha (HIF-1a) (Kiel and Morrison, 2008; Takubo et al., 2010). Here, we found duffy antigen receptor for chemokines (DARC/CD234), the counter molecule of CD82, expressed by bone marrow (BM) macrophages was located in the endosteal and arteriolar niches. Furthermore, DARC on macrophage which was a binding partner of CD82 on LT-HSCs and investigated its influence on regulating HSC quiescence in mouse and human as well.

Ⅱ. Materials and Methods

Immunofluorescence analysis

Immunocytochemistry was performed as previously described with minor modifications. LT-HSCs, ST-HSCs and MPPs were fixed with 2% paraformaldehyde (PFA, Wako). After blocking them for 1 hr at room temperature, cells were incubated with a primary antibody for 12 hrs at 4°C. Then, the cells were washed and incubated with a secondary antibody for 1 hr at room temperature. Whole and sectioned BM samples were stained with following many antibodies: CD82/KAI1 (sc-1087), all from Santa Cruz Biotechnology; CD38, CD93, and CD34, phosphorylation-PKCα, DARC from Santa Cruz Biotechnology (sc-27817, sc-27813)/Miltenyi Biotec (130-105-683, 130-105-684)/R&D (FAB6695A, FAB6695P);. Confocal images were acquired using Zeiss LSM-710 META confocal microscope and ZEN 2008 analysis software.

EML cell culture

The EML cell line was maintained in medium containing stem cell factor (SCF) as previously described (Arai et al., 2004). EML cells were grown in IMDM (Gibco) containing 20% FBS (Gibco) and SCF (200 ng/ml, PeproTech), which is referred to as 'proliferation conditions' in the manuscript.

Flow cytometry

Under sterile conditions, the femur and tibia were excised and all

connective tissue was removed from the bones. BM cells were harvested from the femur and tibia by flushing the BM cavity with DPBS. BM cells were dispersed by passing through a 40μm strainer (Falcon). After suspended BM cells were obtained, cells were separated into mononuclear cells by Histopaque-1083 (Sigma-Aldrich). Collected mononuclear cells were washed once at 1800 rpm at 4°C, and suspended in FACS buffer. Flow cytometry was performed as previously described with slight modifications (Passegue and Wagers, 2006; Wang et al., 2011). Flow cytometry analysis and sorting (BD Canto II, LSR II and FACS Aria III) were performed using several antibodies specific for the following: CD45.2, CD45.1, Sca-1, CD34, CD48, CD3, CD4, CD8a, CD31, CD45, CD11b, TER-119, Ly-6G and Ly-6C, lineage antibody-cocktail (M1/70 RA3-6B2, 145-2C11, TER-119, RB6-8C5), lineage cocktail 1 (SK7, 3G8, SJ25C1, L27, MΦP9, NCAM16.2 and GA-R2), CD34, CD38, Ki-67, CD244, and CD229.1, all from BD Pharmingen; CD117, F4/80, CD135, CD45-RA, TGB-β1, all from e-Bioscience; CD16/32 and CD150 from Biolegend; TGF-βR2, TGF-βR1 from R&D systems; α-SMA (Cat no. 509760-82; Clone no. 1A4) from eBioscience; Hoechest 33342 from Invitrogen. A Miltenyi Biotec antibody (fluorochrome-conjugated) was used for detection of human CD82, and R&D Systems (FAB4616A) and Abcam (ab135779) antibodies for mouse CD82. R&D Systems antibodies were used to detect DARC of human (FAB4139P, FAB4139A) and mouse (FAB6695P) origin. Fc receptor blocker (Miltenyi Biotec), Fluorescence Minus One (FMO) controls, appropriate isotype controls and secondary antibodies were used where necessary.

Preparation of human umbilical cord blood cells

Human umbilical cord blood was collected as follows. After delivery, the cord was clamped and cord blood was collected in a closed system from the umbilical vein using a heparin coated syringe. Donors were informed with consent guidelines provided by the Institutional Review Board (IRB) of Seoul National University Hospital (IRB number: H-1210-032-430). After collection, mononuclear cells were obtained using Ficoll-Paque Plus (GE Healthcare Life Sciences) as previously described with slight modifications (Hur et al., 2004). Isolated cells were maintained in 'proliferation conditions' (i.e. StemSpan^{TM}H3000(Stemcell Technologies) media with a cocktail of growth-stimulating cytokines comprising flt3l, Scf, Il3, and Tpo (all from Peprotech))

Immunoprecipitation

Immunoprecipitation was performed as previously described (Lee et al., 2014) with slight modifications. Light-chain specific secondary antibodies (Jackson ImmunoResearch) were used to avoid detection of heavy chain at 40kDa, the predicted size for CD82 and DARC.

Endocytosis assay

The quiescent EML population (qEML, c-Kit^{+}Lin^{-}CD34^{-}) was MACS-sorted from EML cells and incubated with biotin-conjugated monoclonal CD82 antibody (Miltenyi Biotec) for 1 hour. After 3-5 times of wash, cells underwent 3-hr incubation in normal culture medium with or without SCF and rhDARC protein (Abnova, H00002532-G01). rhDARC, a full-length transmembrane protein with seven membrane-spanning domains, was synthesized by *in vitro* translation and then trapped by liposomes, folding into native

three-dimensional structure. Mock or *Darc* K/D Raw 264.7 cells were co-cultured where appropriate. Then, membrane-bound antibodies were removed using a buffer (50 mM glycine, 100 mM NaCl (pH 2.5)). Streptavidin-conjugated secondary antibodies were used in resultant cells and observed with confocal microscopy.

Ⅲ. Results

1. DARC is highly expressed on Mϕ in the BM niche

Previous reports have suggested that CD82 binds to DARC (Bandyopadhyay et al., 2006). Cells such as ECs, MSCs, OBs and Mϕ have all been reported to support the BM stem cell niche(Kiel and Morrison, 2008; Morrison and Scadden, 2014). Therefore, we examined whether, and if so, which of these cells express DARC. Interestingly, 38.4±1.2% of BM Mϕ ($CD11b^{+}Gr1^{low}F4/80^{+}SSC^{low}$)and as many as 64±5% of $F4/80^{+}$ BM Mϕ were $DARC^{high}$ (Figures 1A and 1B). Other niche-supporting cells (OBs, ECs and MSCs) rarely expressed DARC (Figures 1A). $F4/80^{+}DARC^{+}$ Mϕ was detected both in the arteriolar and endosteal niches(Figures 1C,and 1D). Therefore, we focused on investigating how DARC-expressing Mϕ function through the molecule.

2. DARC on macrophages and CD82 on LT-HSCs directly interact with each other

We examined whether DARC on Mϕ directly interacts with CD82 on LT-HSCs. In in vitro co-culture experiments, DARC on Mϕ was adjacent to CD82 on HSPCs (Figures 2A). Direct interaction of CD82/DARC was also confirmed by co-immunoprecipitation of EML cell lysates(Figures 2B). Thus, DARC, which is highly expressed on Mϕ but rarely on ECs, regulates LT-HSC quiescence through direct contact with CD82 on LT-HSCs in the BM niche.

3. In response to ablation of DARC-expressing macrophages, LT-HSCs lose surface expression of CD82 by ubiquitination and endocytosis

To examine the role of CD82 and DARC in maintaining LT-HSC quiescence and reconstituting the BM after ablative intervention, we investigated the effect of Mϕ depletion by clodronate (Petersen et al., 2014) on $CD82^{+}$LT-HSCs. Clodronate effectively removed total and $DARC^{+}$Mϕ(Figures 2C) leading to a marked reduction in the number of total and $CD82^{+}$LT-HSCs(Figures 2D).

Next, we mimicked in vivo BM ablation by co-culturing qEML cells with various numbers of Raw 264.7 cells (mouse macrophage cell line). Of interest, CD82 expression was maintained on qEML co-cultured with Raw 264.7 cells, and CD82 levels on qEML were pos-

itively correlated with the number of co-cultured Raw 264.7 cells (Figures 2E).

To explore the direct influence of macrophagic DARC to regulate CD82 expression on HSC, we established a Darc K/D Raw 264.7 cell line(Figures 3B). We further investigated the mechanism through which CD82 is decreased. It has been reported that CD82 expression level is modulated by ubiquitination (Tsai et al., 2007) and endocytosis (Xu et al., 2009). Thus, we speculated that after losing a direct contact with DARC on macrophage, CD82 on LT-HSCs might first be ubiquitinated and then endocytosed. We detected CD82 ubiquitination when qEML cells were cultured in proliferation conditions(Figures 3A). Also, $CD82^{+}$ qEML cells maintained under proliferation conditions for 2 days exhibited markedly reduced CD82 expression. Notably, CD82 expression on qEML cells was preserved by treatment with the proteasome inhibitor MG-132, similar to $DARC^{+}$ Mϕ co-culture(Figures 3B).

Next, we performed an endocytosis assay on co-cultured qEML and Raw 264.7 cells. When co-cultured with DARC-positive 'mock' Mϕ, qEML showed no sign of CD82 endocytosis. However, when qEML cells were cultured alone or with Darc K/D Mϕ, CD82 endocytosis was significantly increased, which was reversed by treatment with recombinant human DARC (rhDARC) or MG-132 (Figures 3C and 3D).

For further validation of our hypothesis, we measured surface CD82 expression of qEML by FACS without removing surface-bound antibodies. As expected, surface level of CD82 decreased when mono-cultured in proliferation conditions, but was maintained when co-cultured with Mϕ(Figures 3E). Taken together, our IF and FACS

data indicate that qEML mono-culture in proliferation conditions induced CD82 endocytosis and concomitant reduction of surface CD82 expression, which can be blocked by either Mϕ DARC or rhDARC. Importantly, while CD82 protein levels fluctuated in reaction to various experimental conditions, CD82 displayed no significant changes at the mRNA level(Figures 3F).

To our excitement, although surface CD82 levels had been lowered by ubiquitination-endocytosis in qEML mono-culture, subsequent Mϕ co-culture or rhDARC treatment rapidly restored CD82 expression. In contrast, sustained mono-culture or Darc K/D Mϕ co-culture failed to restore surface CD82 expression(Figures 3G).

4. In humans as well, HSC-specific CD82 expression maintains HSC quiescence through interactions with DARC on macrophages

To determine whether CD82 is selectively expressed by and maintains quiescence of Primitive HSCs (hereafter HSCs) in humans as well as mice, we examined HSCs from human umbilical cord blood (hUCB). Previous reports have used the marker for CD82 expression on human HSCs; $Lin^-CD34^+CD38^-$ and $Lin^-CD34^-CD38^-CD93^{high}CD45RA^-$. (Anjos-Afonso et al., 2013; Laurenti et al., 2015). CD82 was expressed on approximately 98±0.5% of $Lin^-CD34^+CD38^-$ cells, a human HSC population with high reconstituting potential (Figure 4A), and on one-quarter of $Lin^-CD34^-CD38^-CD93^{high}CD45RA^-$ HSCs, another quiescent HSC population with severe combined immunodeficiency (SCID)-repopulating capacity at the top of the human hematopoietic hierarchy (Anjos-Afonso et al., 2013). As in mice, human monocytes/Mϕ exhibited the highest DARC expression level among hUCB component cells. T cells, B cells, Lin^- cells,and ECs exhibited very weak DARC expression (Figure 4B). The majority of $CD82^-$ HSCs were positive for Ki-67, a proliferation marker, whereas $CD82^+$ HSCs were primarily Ki-67 negative (Figure 4C). Also, the $CD82^+$ HSC fraction exhibited upregulation of CDK inhibitors (Figure 4C).

To confirm that PKCα phosphorylation is upstream of the CDK inhibitors, $CD82^+$ and $CD82^-$ HSC fractions were treated with rhDARC. PKCα signaling was activated in rhDARC-treated $CD82^+$ HSCs, but not in $CD82^-$HSCs (Figure 4D). Furthermore, rhDARC blocked G_0 exit

of $CD82^{+}$HSCs (Figure 4E and 4F).

To demonstrate DARC functionality in humans, HSPCs ($Lin^{-}DARC^{-}$) were subjected to proliferation conditions, and cultured with or without $DARC^{+}$ monocyte/Mϕ or rhDARC. HSCs co-cultured with $DARC^{+}$ monocyte/Mϕ or treated with rhDARC displayed marked upregulation of CD82 (Figure 4G).

In conclusion, CD82 is selectively expressed on primitive HSCs in humans as well; in addition, the majority of $CD82^{+}$HSCs are quiescent, which is maintained by DARC-expressing monocytes/Mϕ.

Ⅳ. DISCUSSION

In summary, we found that 1) DARC on Macrophage is prodominently especially in the arteriolar and endosteal niches, and directly interacts CD82 on LT-HSCs surface. 2) Upon ablation of macrophage by treating clodronate, a number of DARC expressing macrophages are reducing significantly, but also CD82 expressing LT-HSCs are decreased largely. 3) DARC on macrophage regulates surface CD82 level of LT-HSCs by ubiquitination and endocytosis. 4) Newly generated DARC-expressing macrophage promote reduced CD82 expression on LT-HSCs to normal levels in qEML. 5) DARC on macrophage maintains quiescence of CD82 expressing HSCs in human as well.

These findings demonstrate that CD82 is a functional surface marker of LT-HSCs and that the molecule maintains LT-HSC quiescence by interactions with DARC-expressing Mϕ. Decreased CD82 level of LT-HSCs led to cell cycle entry and proliferation, which could be revesed by macrophage co-culture or rhDARC. Furthermore, we isolated human primitive HSCs from human umbilical cord blood, and found that selective expression of KAI1 on human primitive hematopoietic stem cells regulates their quiescence by interaction with DARC. Taken together, our findings highlight the importance of BM niche components - DARC+ macrophages - in terms of maintaining of LT-HSC dormancy in mouse and human as well.

The possible therapeutic scheme would be treatment for leukemia.

Acute myeloid leukemia (also called AML) is a cancer of bone marrow cells. Acute means that a cancer of bone marrow cells produces and develops quickly, and requires immediate treatment. One of AML treatments is bone marrow transplantation or hematopoietic cell transplantation (HCT) after very high doses of chemotherapy. Further research is also needed regarding effective soluble DARC to eradicate cancer. HSCs with soluble DARC at a specific time point might be more effective therapeutic source for BMT through ex vivo culture. In order to determine the effect of soluble DARC protein on the repopulation capacity of HSCs, further study must be needed. Identifying other factors regulating CD82 expression in HSCs and elucidating DARC downstream will facilitate the development and optimization of a treatment protocol capable of exploiting the CD82/DARC axis.

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Zon, L.I. (2008). Intrinsic and extrinsic control of haematopoietic stem-cell selfrenewal. Nature 453, 306 – 313.

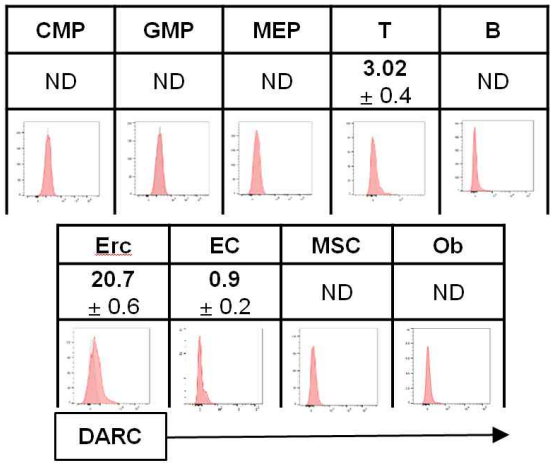
Zou, P., Yoshihara, H., Hosokawa, K., Tai, I., Shinmyozu, K.,

Tsukahara, F., Maru, Y., Nakayama, K., Nakayama, K.I., and Suda, T. (2011). p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell Stem Cell 9, 247 – 261. cell

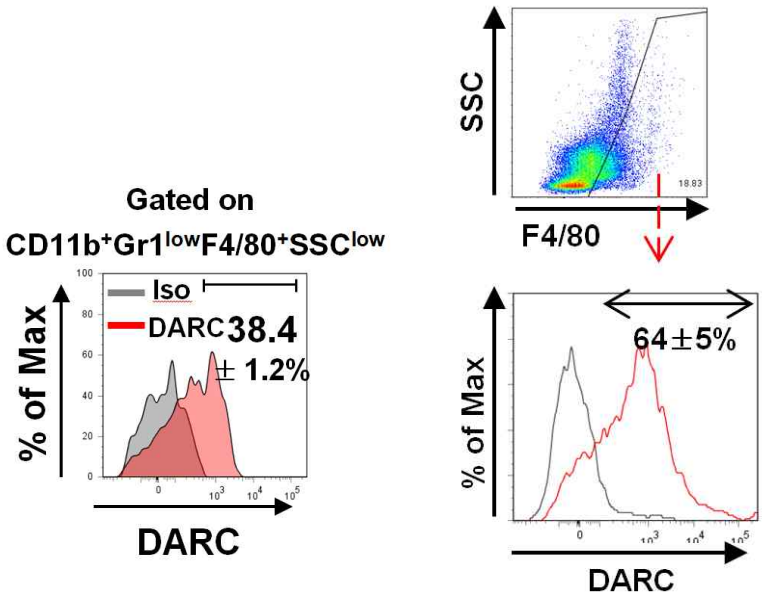
Ⅵ. Figures

[Figure 1]

A

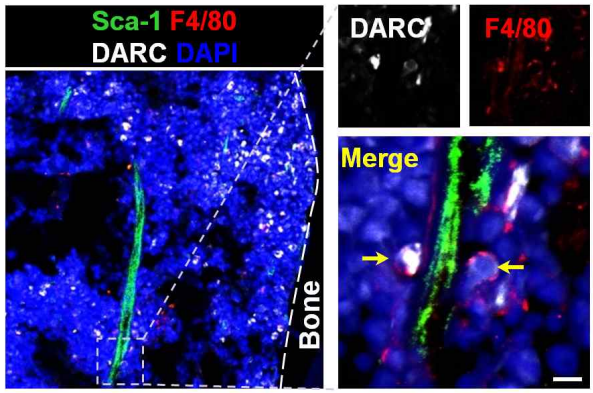


B

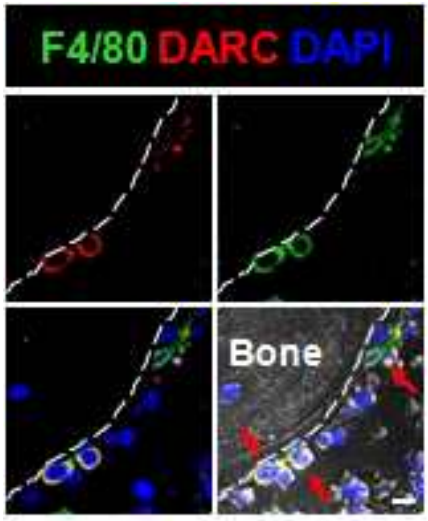


[Figure 1](Continued)

C

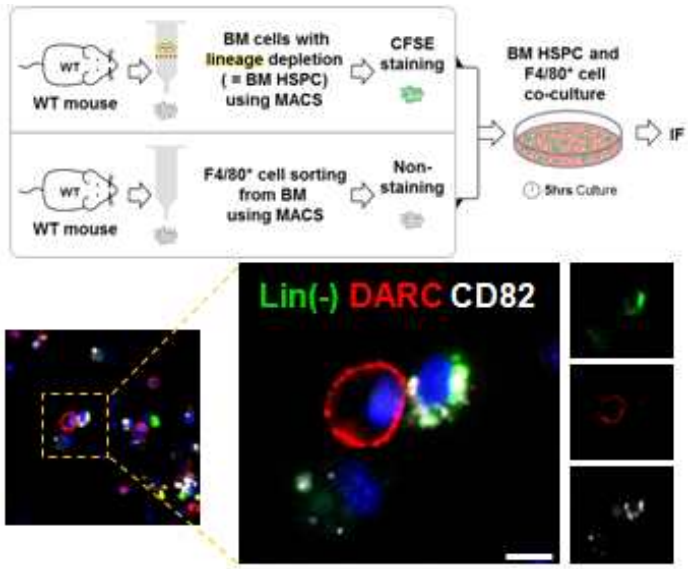


D

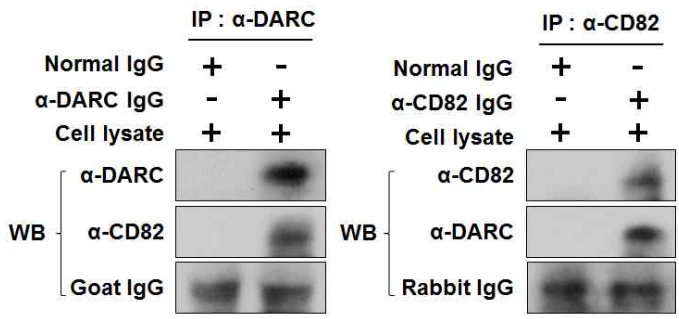


[Figure 2]

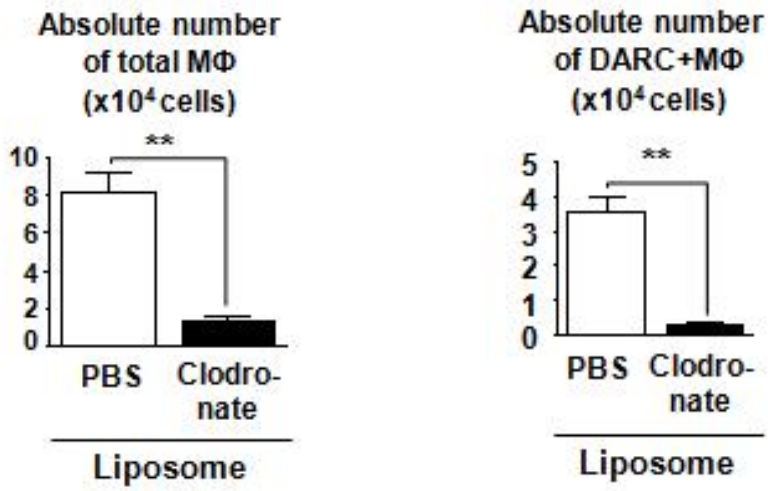
A

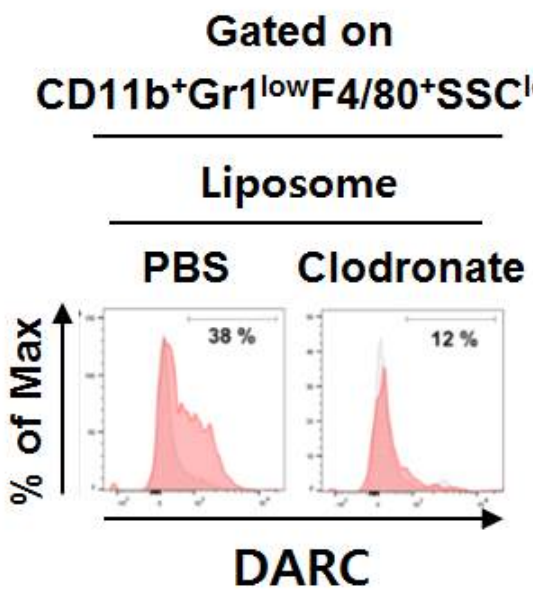

WT mouse
BM cells with lineage depletion (= BM HSPC) using MACS
CFSE staining
WT mouse
F4/80+ cell sorting from BM using MACS
Non-staining
BM HSPC and F4/80+ cell co-culture
IF
5hrs Culture
Lin(-) DARC CD82

B


IP : α-DARC
Normal IgG + -
α-DARC IgG - +
Cell lysate + +
WB
α-DARC
α-CD82
Goat IgG
IP : α-CD82
Normal IgG + -
α-CD82 IgG - +
Cell lysate + +
WB
α-CD82
α-DARC
Rabbit IgG

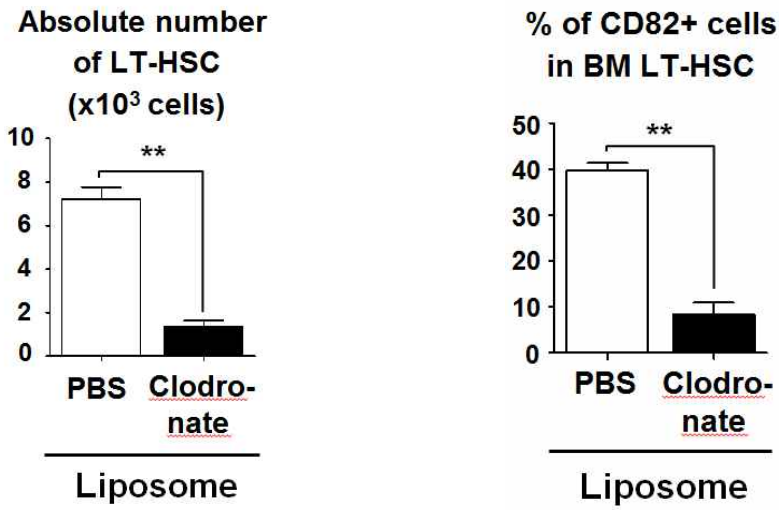
C


Absolute number
of total MΦ
(x10⁴cells)
**
10
8
6
4
2
0
PBS
Clodro-
nate
Liposome
Absolute number
of DARC+MΦ
(x10⁴cells)
**
5
4
3
2
1
0
PBS
Clodro-
nate
Liposome

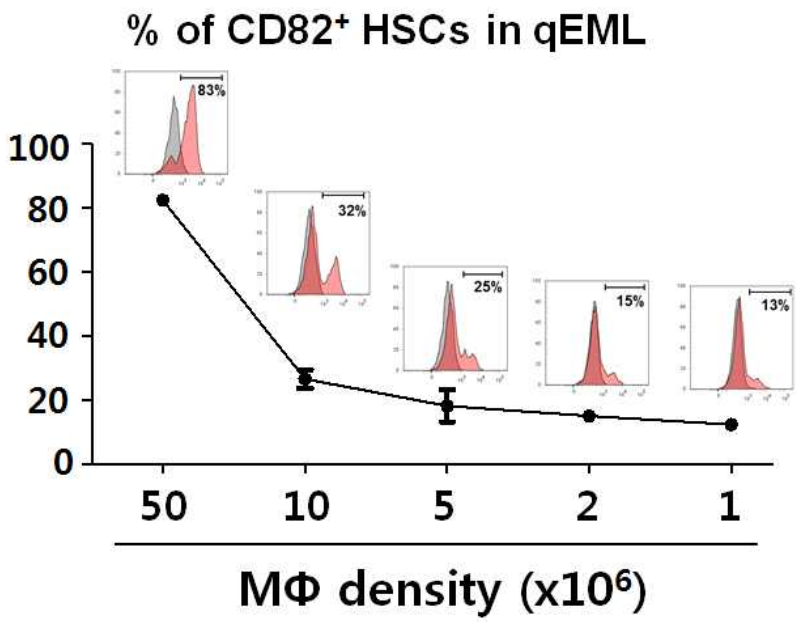

Gated on
CD11b⁺Gr1ˡᵒʷF4/80⁺SSCˡᵒʷ
Liposome
PBS
Clodronate
% of Max
38 %
12 %
DARC

[Figure 2](Continued)

D

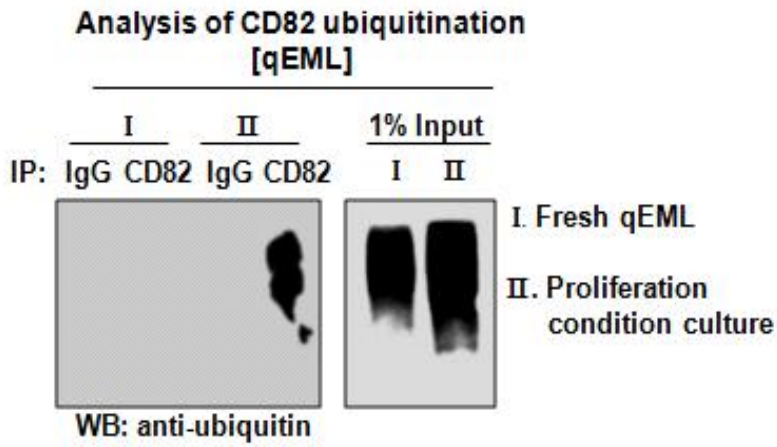


E

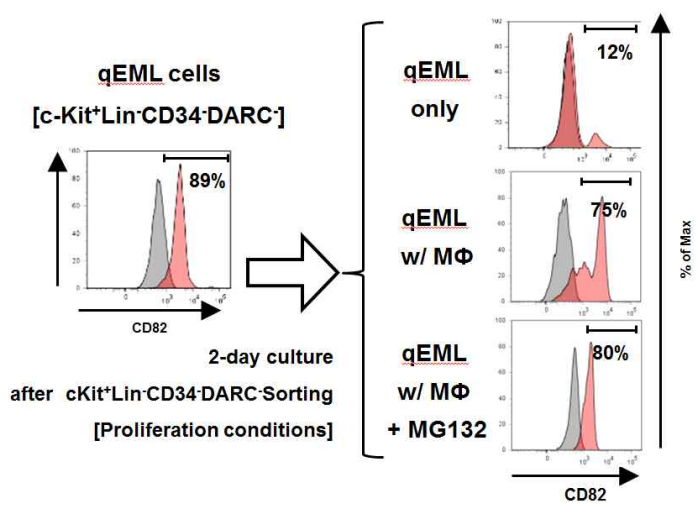


[Figure 3]

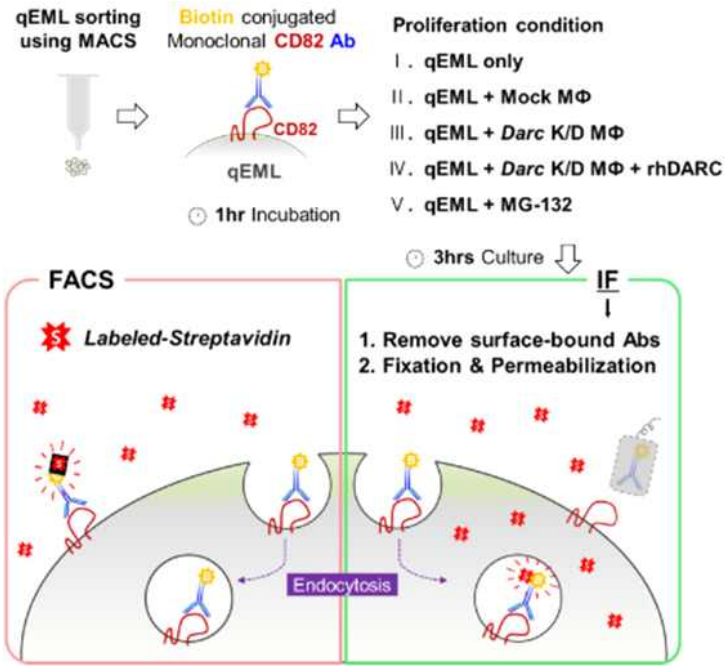
A


Analysis of CD82 ubiquitination
[qEML]
I
II
1% Input
IP: IgG CD82 IgG CD82
I II
I. Fresh qEML
II. Proliferation condition culture
WB: anti-ubiquitin

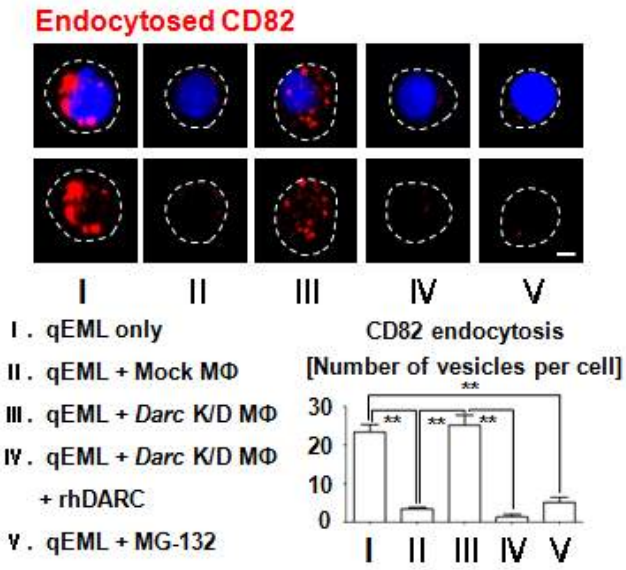
B


qEML cells
[c-Kit+Lin-CD34-DARC-]
89%
CD82
2-day culture
after cKit+Lin-CD34-DARC-Sorting
[Proliferation conditions]
qEML only
qEML w/ MΦ
qEML w/ MΦ + MG132
12%
75%
80%
% of Max
CD82

C

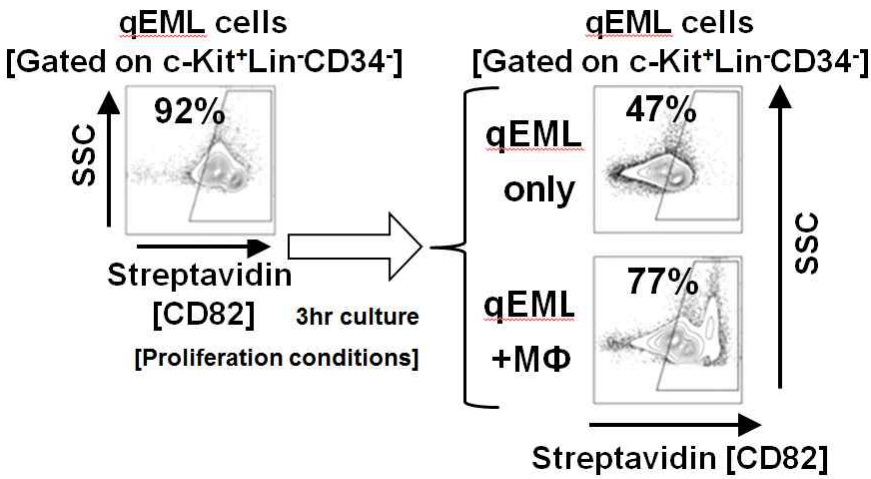

qEML sorting using MACS
Biotin conjugated Monoclonal CD82 Ab
CD82
qEML
1hr Incubation
Proliferation condition
Ⅰ. qEML only
Ⅱ. qEML + Mock MΦ
Ⅲ. qEML + Darc K/D MΦ
Ⅳ. qEML + Darc K/D MΦ + rhDARC
Ⅴ. qEML + MG-132
3hrs Culture
FACS
Labeled-Streptavidin
IF
1. Remove surface-bound Abs
2. Fixation & Permeabilization
Endocytosis

D

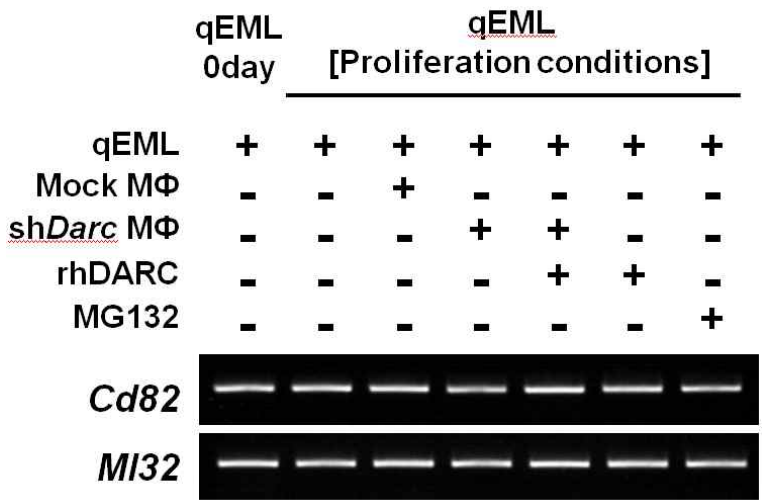

Endocytosed CD82
Ⅰ
Ⅱ
Ⅲ
Ⅳ
Ⅴ
Ⅰ. qEML only
Ⅱ. qEML + Mock MΦ
Ⅲ. qEML + Darc K/D MΦ
Ⅳ. qEML + Darc K/D MΦ + rhDARC
Ⅴ. qEML + MG-132
CD82 endocytosis
[Number of vesicles per cell]
30
20
10
0
**

[Figure 3](Continued)

E

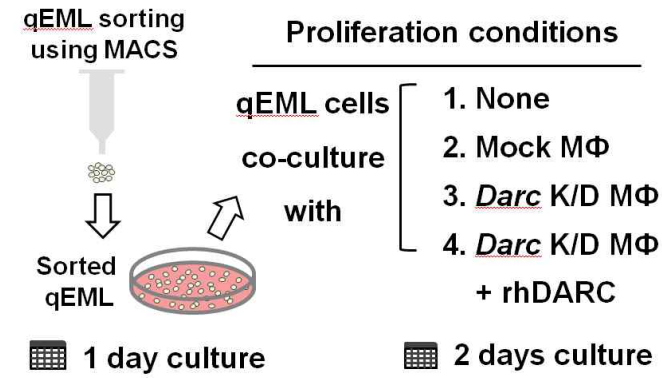


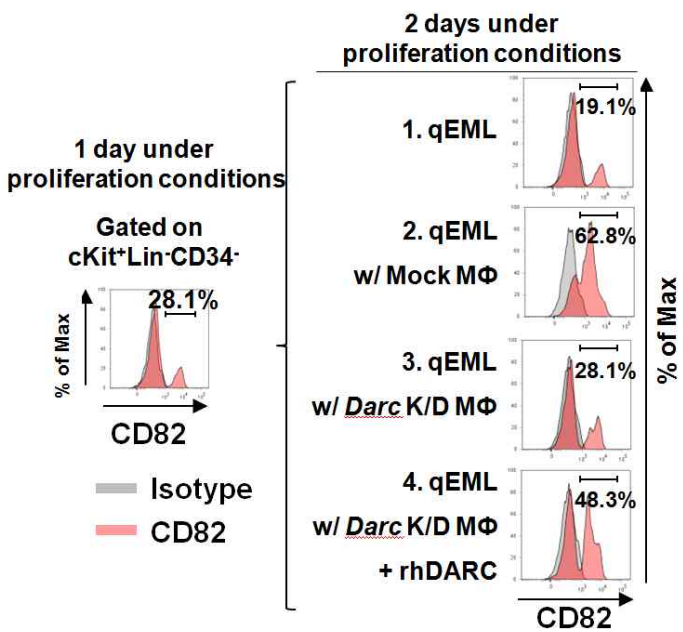
F



[Figure 3](Continued)

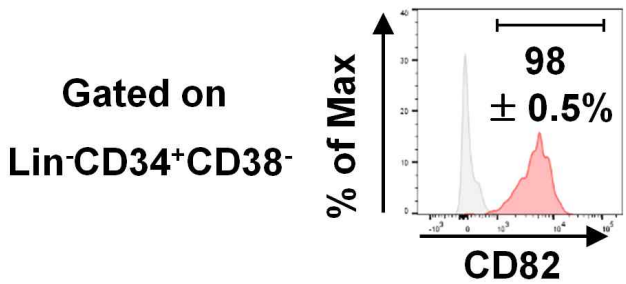
G



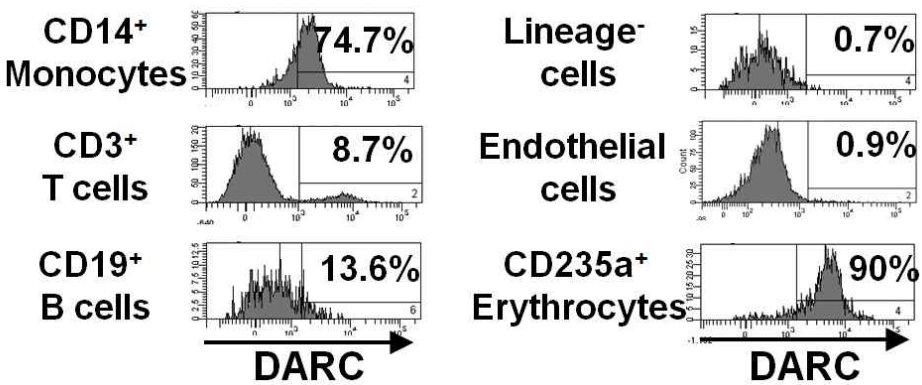


[Figure 4]

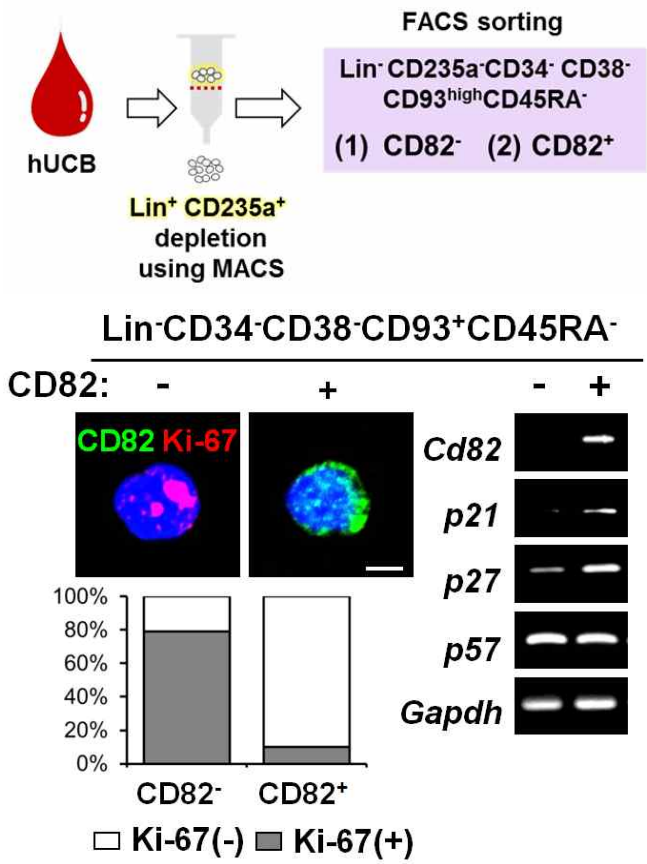
A


Gated on
Lin-CD34+CD38-
% of Max
98
± 0.5%
CD82

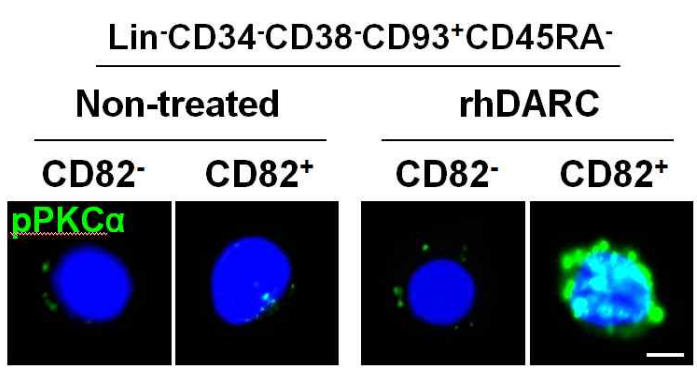
B


CD14+
Monocytes
74.7%
Lineage-
cells
0.7%
CD3+
T cells
8.7%
Endothelial
cells
0.9%
CD19+
B cells
13.6%
CD235a+
Erythrocytes
90%
DARC
DARC

C

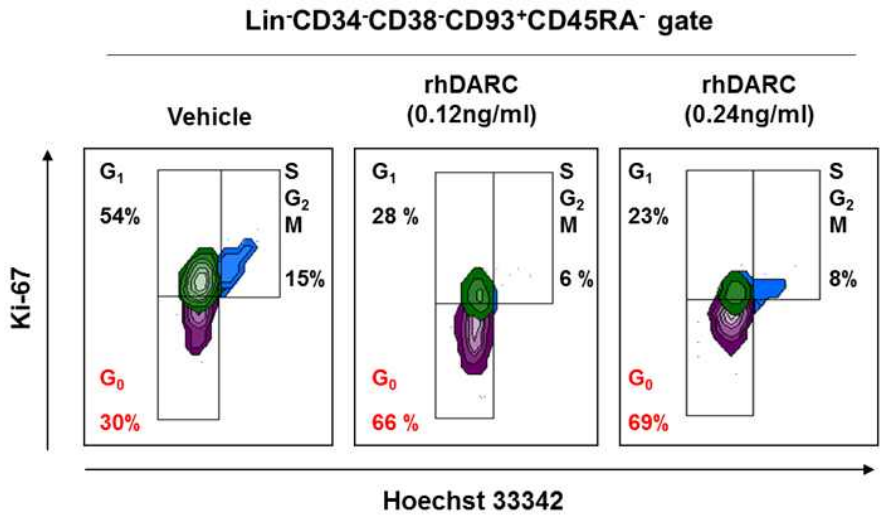

FACS sorting
Lin- CD235a- CD34- CD38-
CD93highCD45RA-
(1) CD82- (2) CD82+
hUCB
Lin+ CD235a+
depletion
using MACS
Lin-CD34-CD38-CD93+CD45RA-
CD82: - + - +
CD82 Ki-67
Cd82
p21
p27
p57
Gapdh
100%
80%
60%
40%
20%
0%
CD82- CD82+
Ki-67(-) Ki-67(+)

D

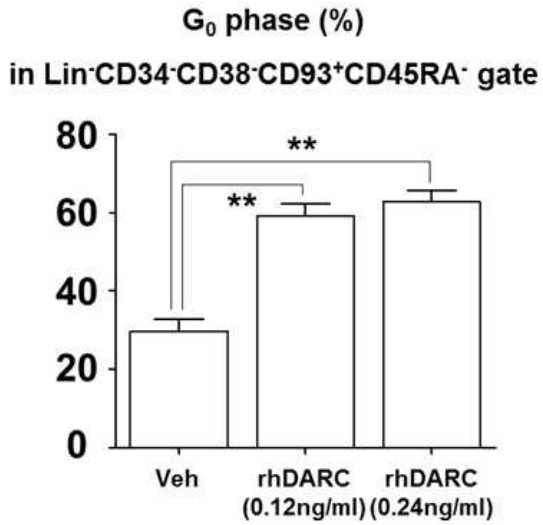

Lin-CD34-CD38-CD93+CD45RA-
Non-treated
rhDARC
CD82- CD82+
CD82- CD82+
pPKCα

[Figure 4](Continued)

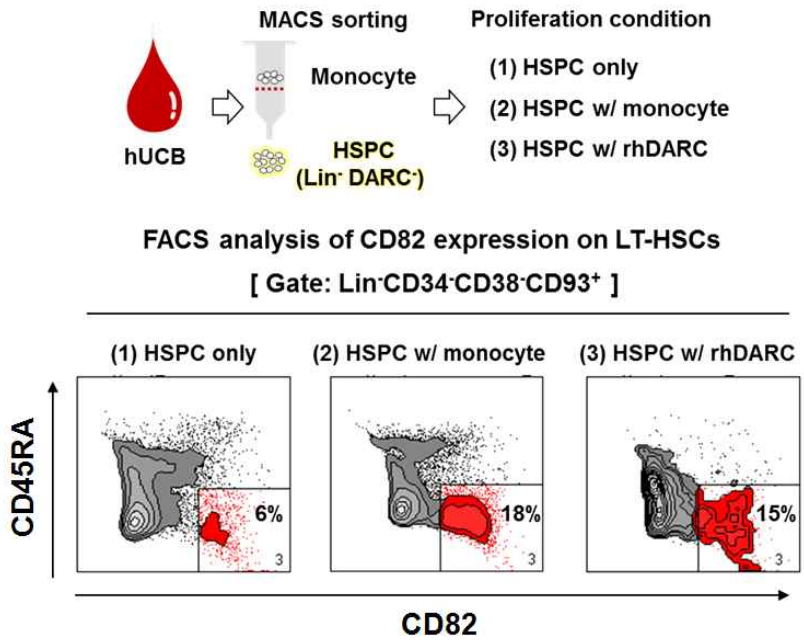
E



F



G


MACS sorting
Proliferation condition
Monocyte
(1) HSPC only
(2) HSPC w/ monocyte
(3) HSPC w/ rhDARC
hUCB
HSPC
(Lin- DARC-)
FACS analysis of CD82 expression on LT-HSCs
[Gate: Lin-CD34-CD38-CD93+]
(1) HSPC only
(2) HSPC w/ monocyte
(3) HSPC w/ rhDARC
CD45RA
CD82
6%
18%
15%

Ⅶ. Figure legends

Figure 1. Macrophages are the major source of DARC in the murine BM endosteal and arteriolar niches.

(A) DARC expression on various types of BM cells defined by following marker sets (n=3): Common myeloid progenitor (CMP, $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34^{+}Fc\gamma RⅡ/Ⅲ^{-}$); Granulocyte-monocyte progenitor (GMP, $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34^{+}Fc\gamma RⅡ/Ⅲ^{+}$); Megakaryocyte/erythrocyte progenitor (MEP, $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}CD34^{-}Fc\gamma RⅡ/Ⅲ^{-}$); T cell (T, $CD3^{+}$); B cell(B,CD45R/$B220^{+}$); Erythrocyte(Erc,$CD45^{-}Ter119^{+}$); Endothelial cell(EC,$CD45^{-}Ter119^{-}CD31^{+}$); Mesenchymal stromal cell(MSC,$CD45^{-}Ter\text{-}119^{-}CD31^{-}LepR^{+}$); Osteoblast(Ob,$CD45^{-}Ter119^{-}CD31^{-}Sca1^{-}CD51^{+}$). ND stands for 'Not Detectable'.

(B) Representative FACS plot showing surface expression of DARC on BM Mϕ ($CD11b^{+}Gr1^{low}F4/80^{+}SSC^{low}$) and DARC expression among $F4/80^{+}$population(n=3).

(C) (Left) $DARC^{+}F4/80^{+}$ Mϕ in the BM arteriolar niche($Sca\text{-}1^{+}$). (Right) High magnification images of the boxed area in the left figure. Arrows indicate $F4/80^{+}DARC^{+}$ Mϕ in the arteriolar niche.

(D) Arrows indicate $DARC^{+}$ Mϕ in the BM endosteal niche. Scale bar,5μm.

Figure 2. DARC on macrophages and CD82 on LT-HSCs directly contact and regulate CD82 level on LT-HSCs.

(A) (Top) Schematic representation of the experiment. Lin^-cells,MACS-sorted from the mouse BM, were CFSE-stained and co-cultured with $F4/80^+$Mϕ which were also MACS-sorted from the BM. (Bottom) IF analysis was performed to confirm CD82/DARC interaction. Green and white-labeled cells are CFSE-stained $CD82^+Lin^-$cells, the red cell is an$F4/80^+DARC^+$macrophage. Scale bar,10μm.

(B) CD82/DARC interaction in EML cells was confirmed by co-immunoprecipitation (co-IP).

(C) (Left) The number of BM Mϕ population from WT mice that were injected either with control or clodronate liposome. BM Mϕ were isolated from the femur and tibia of a single hindlimb of WT mice (**p-value<0.05, n=3). (Right) DARC positivity in BM Mϕ from WT mice that were injected either with control or clodronate liposome. BM Mϕ were isolated from the femur and tibia of a single hindlimb of WT mice. (Bottom) Quantification of the left plot (**p-value<0.05, n=3).

(D) (Left) Cell count of primary LT-HSCs from WT mouse BM after control or clodronate liposome injection. (Right) Percentage of $CD82^+$cells in LT-HSCs from WT BM after control or clodronate lip-

osome injection. BM LT-HSCs were isolated from the femur and tibia of a single hindlimb of WT mice (**p-value<0.05, n=3).

(E) Changes in $CD82^{+}$ positivity of qEML cells depending on the density of co-cultured MΦ(n=3).

Figure 3. In the absence of DARC-expressing macrophages, LT-HSCs lose surface expression of CD82

(A) MACS-sorted qEML cells were subjected to proliferation conditions and their protein expression was analyzed by co-IP (CD82) and immunoblot (ubiquitin).

(B) Percentage of CD82-expressing qEML cells before and after 2-day mono-culture and MΦ co-culture with or without MG132.
(C)Schematic illustration of the experimental procedure to detect the endocytosed CD82 in various conditions.

(D) (Top) To analyze CD82 internalization, qEML cells were incubated with biotin-conjugated CD82 antibody and then either mono-cultured (I), cultured with mock Raw 264.7 cells (II), cultured with Darc K/D Raw 264.7 cells (III) or cultured with Darc K/D Raw 264.7 cells in the presence of rhDARC (IV) or MG-132 (V). After removal of un-internalized antibodies with acid wash, cells were fixed, permeabilized, stained with a fluorescent streptavidin conjugate, and observed using confocal microscopy. (Bottom) CD82 endocytosis was quantified in terms of the number of vesicles per cell (**$p<0.05$, n=5).

(E) FACS plots showing that endocytosis-induced decrease of surface CD82 on qEML is prevented by MΦ co-culture.

(F) mRNA expression of Cd82 in qEML cells which were co-cultured

with Raw 264.7 cells (mock or Darc K/D) or treated with rhDARC or MG132. Ml32 served as an endogenous control.

(G) (Top) Schematic illustration of the experimental procedure. After CD82 down-regulation in proliferation conditions, qEML cells were mono-cultured (1) or co-cultured with mock Raw 264.7 cells (2), Darc K/D Raw 264.7 cells (3) or one-hour pre-treatment of rhDARC followed by co-culture with Darc K/D Raw 264.7 (4). CD82 expression on qEML population was examined by FACS analysis. (Bottom) Percentage of $CD82^{+}$cells(in the c-$Kit^{+}Lin^{-}CD34^{-}$gate)was measured(Left) after one-day mono-culture of qEML cells. (Right) Later, the cells were further subjected to either mono-culture (1), co-culture with mock Mϕ (2), co-culture with Darc K/D Mϕ (3), or co-culture with Darc K/D Mϕ after 1 hr pre-treatment with rhDARC. Two days later, surface CD82 levels were estimated by FACS.

Figure 4. Quiescence of human $CD82^+$primitive HSCs is maintained by rhDARC and DARC-expressing Mϕ.

(A) expression of $Lin^-CD34^+CD38^-$ hUCB cells

(B) Flow cytometry analysis of human DARC expression on $CD14^+$monocytes, $CD3^+$Tcells, $CD19^+$Bcells, lineage-negative cells, human umbilical vein endothelial cells(HUVECs) and $CD235a^+$ erythrocytes (all derived from hUCB)

(C) (Top) Schematic figure showing purification of HSCs. MACS-sorted $Lin^-CD235a^-$ hUCB cells were further sorted into two groups ($CD34^-CD38^-CD93^{high}CD45RA^-CD82^-$ and $CD34^-CD38^-CD93^{high}$ $CD45RA^-CD82^+$ populations) by FACS. (Left) Representative IF images showing Ki-67 expression in $CD82^-$ and $CD82^+$ HSCs. Quantification of the IF analysis. Scale bar, 2 μm. (Right) mRNA expression of CD82 and CDK inhibitors (p21, p27 and p57) in $CD82^-$and $CD82^+$ HSCs.

(D) $CD82^+$ and $CD82^-$ HSCs were separated by FACS, and rhDARC-induced PKCα phosphorylation (pPKCα) was observed by confocal imaging. Scale bar, 2 μm.

(E) FACS analysis revealed rhDARC blocked G_0 exit of HSCs.

(F) Quantification of the (E) plot (**$p<0.05$, n=4).

(G) (Top) Schematic figure of the experiment. Lin^-DARC^- HSPCs and monocytes were isolated from hUCB. Lin^-DARC^-cells were then cultured alone, co-cultured with monocytes, or treated withrh DARC. (Bottom) CD82 surface expression of the three groups of HSCs (gated on $Lin^-CD34^-CD38^-CD93^{high}$) was estimated with FACS.

국문 초록

대식세포의 DARC가 최상위 조혈모세포의 휴면을 조절하는 기전에 관한 연구

서울대학교 융합과학기술대학원
분자의학 및 바이오제약학과
분자의학 및 바이오제약전공
채 정 환

마우스 HSPC의 많은 종류 중에, 카이원(CD82/KAI1)은 조혈모세포 계층 중에 가장 최상위인 최상위 조혈모세포에 특이적으로 많이 발현한다. 이 연구에서는 카이원의 결합 물질을 밝혔으며, CD82의 상대 결합 물질이 조혈모세포 휴면을 조절하는데 어떠한 영향을 미치는지에 대해 밝혔습니다. DARC는 CD82의 상대결합 물질로써, 골내막 또는 소동맥 부근의 골수에 존재하는 대식세포에서 많이 보여집니다. 골수 내 대식세포가 제거되면, 최상위 조혈모세포는 DARC와의 직접적 결합이 끊어지게 되

고, 그 결과 표면에 존재하는 CD82 분자들은 유비퀴틴화 그리고 내포작용이 일어나게 됩니다. 표면에 존재하는 막 CD82 단백질이 줄어들면, 최상위 조혈모세포는 세포주기가 활성화되며 분화, 증식하게 되는데, 이 현상은 대식세포 공배양에 의해 다시 돌아옵니다. 종합해보면, 우리의 연구는 골수 주변 구성물질인, DARC 양성 대식세포가 최상위 조혈모세포의 휴면에 중요하다 라는 것을 밝혔습니다. 더하여, 제대혈 내 인간 유래 조혈모세포 또한 상당한 양의 CD82/KAI1분자를 표면에 발현하고 있습니다. 특히, 카이원은 휴면상태의 조혈모세포에서 주로 발현하고 있는 반면에, 활발하게 세포주기가 돌아가고 있는 조혈모세포 또는 전구체에서는 적은 양의 발현을 보입니다. 마우스와 마찬가지로, DARC는 주로 인간유래 대식세포 또는 단핵구에서 발현하며, 인간 조혈모세포의 CD82/KAI1 발현을 유지시킵니다. DARC 재조합단백질 또한 조혈모세포의 표면 CD82/KAI1 막 단백질 발현을 유지시키며, 그 결과 조혈모세포의 세포주기 진행을 더디게 만듭니다. CD82/KAI1 결합은 다양한 환자군에서 사용가능한 줄기세포 체외증폭배양방법과 같은 새로운 조혈모세포 치료법을 제시할 수 있습니다.

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주요어 : 조혈모세포, KAI1/CD82, DARC, 휴면, 대식세포, 제대혈, 골수

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