CD97 is differentially expressed on murine hematopoietic stem- and progenitor-cells

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ABSTRACT

Background

CD97 is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) family of adhesion receptors and is broadly expressed on hematopoietic cells. The aim of this study was to investigate the expression of CD97 on hematopoietic stem- and progenitor cells (HSC/HPC).

Design and Methods

CD97 expression on hematopoietic stem- and progenitor cells was studied in BALB/c, C57BL/6 and DBA/1 mice using flow cytometry. Functional hematopoietic stem- and progenitor cell characteristics were investigated *in vitro* and *in vivo* by progenitor cell assays, cobblestone area forming cell assays and bone marrow cell transplantation.

Results

Analysis of CD97 expression on murine bone marrow cells showed three major populations i.e. $CD97^{HI}$, $CD97^{INT}$ and $CD97^{NEG}$ cells. Functional studies revealed that radioprotective capacity and cobblestone area forming cell day 28-35 activity resides in the $CD97^{INT}$ bone marrow cell fraction while CFU-GM colony-forming capacity mainly resides in the $CD97^{NEG}$ population in all strains. In C57BL/6 and DBA/1 mice $CD97^{NEG}$ and $CD97^{HI}$ bone marrow cells show hematopoietic stem cell characteristics as well. Further functional analysis of BALB/c $CD97^{INT}$ bone marrow cells revealed that c-$Kit^{HI}CD97^{INT}$ bone marrow cells exhibit HSC activity and are 1.5-fold enriched for cobblestone area forming cell-day 35 activity compared to c-Kit^{HI} bone marrow cells. Moreover, phenotypical analysis showed that BALB/c and C57BL/6 HSC are $CD97^{INT}$, while DBA/1 HSC are $CD97^{HI}$.

Conclusions

CD97 is differentially expressed on hematopoietic stem cells and hematopoietic progenitor cells. Committed progenitor cell activity is largely comprised in the $CD97^{NEG}$ fraction, while the $CD97^{INT}$ population contains hematopoietic stem cell activity. In BALB/c mice, CD97 expression can be applied to almost completely separate colony-forming cells and cells exhibiting radioprotective capacity. In addition we propose that the $CD97^{INT}$c-Kit^{HI} phenotype allows simple and rapid purification of murine hematopoietic stem cells.

Key words: hematopoietic stem cells, CD97, mouse model, hematopoietic progenitor cells.

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Introduction

Hematopoietic stem cells (HSC) are capable of extensive self-renewal and confer long-term multilineage repopulating ability upon infusion into a myeloablated recipient.[1] Although human HSC are characterized by only a few cell surface markers,[1-3] the phenotype of murine HSC is defined by a variety of cell surface molecules. Murine HSC have been identified as c-Kit^{POS}Thy-1^{LO}Lin$^{NEG/LO}$Sca-1^{POS}.[4-6] However, this population contains a mixture of HSC and hematopoietic progenitor cells (HPC) that exhibit long-and short-term repopulating ability, respectively.[7] To increase the frequency of HSC with long-term repopulating capacity within this Kit^{POS}Thy-1^{LO}Lin$^{NEG/LO}$Sca-1^{POS} population, further selection using rhodamine-123, wheat germ agglutinin or a combination of anti-CD11b and anti-CD4 antibodies has been used.[8-10]

CD97 is a member of the epidermal growth factor-seven transmembrane (EGF-TM7) receptor family.[11,12] CD97 is characterized by an extended extracellular region comprising several tandemly arranged EGF-like domains coupled to the TM7 region by a spacer. As a result of alternative RNA splicing three isoforms, comprising different numbers of EGF-like domains, are expressed in humans and mice.[12-14] Except for smooth muscle cells and a restricted set of epithelial tumors, CD97 is only expressed on a broad array of hematopoietic cells including (activated) lymphocytes, granulocytes, monocytes, macrophages and dendritic cells.[15,16]

CD55 (decay accelerating factor), chondroitin sulfate and the integrin α5β1 (also known as VLA-5) have been identified as cellular ligands for CD97.[12,13,17,18,19] The composition of the EGF domain region defines the ligand specificity of the different CD97 isoforms. The first and second EGF domains interact with CD55, whereas the fourth EGF domain binds chondroitin sulphate.[17,18,20] The ligand affinity of the CD97 isoforms differs. While affinity for CD55 is significantly higher for the smaller isoforms,[21,22] chondroitin sulphate interacts exclusively with the largest isoforms.[18]

In this study we investigated the expression of CD97 on HSC and HPC obtained from different mouse strains.

Design and Methods

Animals

Eight to 12-week old male BALB/c and DBA/1 mice were purchased from Charles River (Maastricht, The Netherlands), and male C57BL/6-Ly5.2 mice (8-12 weeks old) were obtained from Bioservices (Uden, The Netherlands). C57BL/6-Ly5.1 congenic recipients were bred in our own facilities and were used at 8-12 weeks of age. The animals were fed commercial rodent chow and allowed acidified water *ad libitum* and were maintained in the animal facility of the Leiden University Medical Center under conventional conditions. All experimental protocols were approved by the institutional ethics committee on animal experiments.

Antibodies for cell sorting and analysis

CD97 expression was assessed by flow cytometry using a biotinylated hamster anti-murine CD97 monoclonal antibody (clone 1B2)[23] directed against the first EGF-like domain of murine CD97 and was stained with streptavidin-APC or streptavidin-PE-Cy7 (both obtained from Caltag Laboratories, Burlingame, CA, USA). To analyze the phenotype of CD97 subpopulations, cells were stained with FITC-labeled anti-CD8 (clone 53-6.7), anti-CD4 (clone GK1.5), anti-B220 (clone RA3-6B2), anti-CD11b (clone M1/70), anti-GR-1 (clone RB6-8C5), anti-CD3 (clone 145-2C11; all obtained from Pharmingen, San Diego, CA, USA) and anti-TER119 (clone Ter-119; obtained from Caltag Laboratories). These markers are further designated as *Lin-markers*. Phycoerythrin-labeled anti-CD117 (c-Kit, clone 2B8) and biotinylated anti-CD90.2 (Thy1.2, clone 53-2.1) were obtained from Pharmingen. CD90.2 was stained with streptavidin PE-Cy5. Biotinylated Sca-1 (clone D7) was purchased from Pharmingen and was stained with streptavidin APC-Cy7 (Pharmingen).

Hoechst 33342 staining was performed as previously described.[24] Briefly, bone marrow cells were resuspended in RPMI (Life Technologies, Paisley, Scotland), containing 2% heat inactivated fetal calf serum, 25 mM HEPES, penicillin, streptomycin and Hoechst 33342 (5 μg/mL, Molecular Probes Europe, Leiden, The Netherlands) and incubated for 90 minutes at 37°C. Next the cells were washed in cold medium and maintained at 4°C until analysis on a LSRII flowcytometer (Beckton Dickinson, Mountain View, CA, USA).

Preparation of cell suspensions

Using sterile procedures, bone marrow cells were obtained by flushing femoral, tibial and humeral bones from donor mice with RPMI medium (Life Technologies, Paisley, Scotland) supplemented with 2% heat inactivated fetal calf serum, 10% heparin, penicillin and streptomycin. After incubation with DNAse (1.33 μg/mL), erythrocytes were lysed. Subsequently, the cells were sorted on a FACS Aria (Beckton Dickinson). As a control, total bone marrow cells were sorted for the life-gate, based on forward scatter/side scatter characteristics. All populations used in experiments were of ≥95% purity.

Progenitor cell assays

Colony forming units-granulocyte monocyte (CFU-GM) were cultured as described previously.[25] Briefly, peripheral blood mononuclear cells were cultured in 3.5-cm dishes containing 5×10^5 cells/mL in semisolid medium in the presence of recombinant murine granulocyte-monocyte colony-stimulating factor (1.25 ng/mL, Pharmingen). Bone marrow cells were cultured at a concentration of 5×10^4 cells/mL. After 6 days of culture in a fully humidified atmosphere of 37°C containing 5% CO_2, the number of colonies (defined as an aggregate of ≥20 cells) was scored using an inverted light microscope.

Cobblestone area forming cell (CAFC) assay

Confluent stromal layers of FBMD-1 cells in flat-bot-

tomed 96-well plates (Falcon, Etten-Leur, The Netherlands) were overlaid with various dilutions of freshly sorted bone marrow cells to allow limiting dilution analysis of the precursor cells forming hematopoietic clones under the stromal layers. To assay a particular cell suspension, we used eight dilution steps differing by a factor of 2.5, with 15 wells per dilution. The cells were cultured at 33°C in 7% CO_2 and were fed weekly by changing half of the medium. Between 7 and 42 days after overlay, all wells were inspected at weekly intervals and scored positive if at least one phase-dark hematopoietic clone (cobblestone area, at least 5 cells) was observed. The CAFC frequencies were calculated using Poisson statistics.

Bone marrow cell transplantation

Recipient mice were irradiated in perspex chambers with a linear accelerator (Philips SL 75-5/6 mV, Philips Medical Systems, Best, The Netherlands) at a dose rate of 98 cGy/min. Total doses of 9.5 Gy (BALB/c and C57BL/6) and 9.0 Gy (DBA/1; lethal irradiation) were administered. Four to 8 hours following total body irradiation, bone marrow cells were injected into the caudal vein in 0.2 mL of saline, containing 0.2% bovine serum albumin.

Statistical analysis

Statistical analyses were performed with the Student's *t*-test and the log-rank test, using GraphPadPRISM (GraphPad Software, San Diego, CA, USA). p values <0.05 were considered statistically significant.

Results

The CD97^{NEG} bone marrow cell fraction is enriched in colony-forming cells

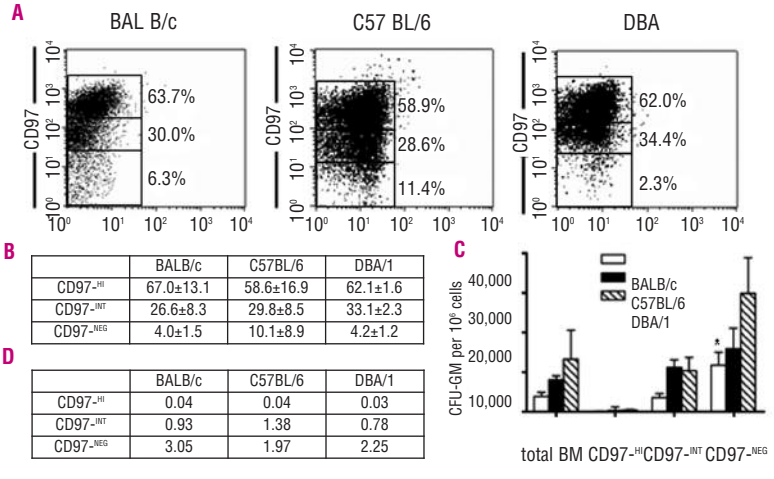
To evaluate the expression of CD97 on murine bone marrow cells, primary BALB/c, C57BL/6 and DBA/1 bone marrow cells were stained with anti-CD97 monoclonal antibodies. FACS analysis showed three major populations i.e. CD97^{HI}, CD97^{INT} and CD97^{NEG} cells (Figure 1A). The majority of bone marrow cells stained highly positive for CD97 (range 58-67% of total bone marrow cells), while 27-33% showed an intermediate expression of CD97. Only a small proportion of bone marrow cells did not express CD97 (range 4-10%; Figure 1B). No significant differences were detected between the different strains of mice.

Based on CD97 expression, bone marrow cells were sorted into highly pure CD97^{HI}, CD97^{INT} and CD97^{NEG} populations. Subsequently, CFU-GM colony-forming capacity was analyzed in the different CD97 subsets. Although the frequency of progenitor cells in total bone marrow cells varied between the different mouse strains (3,840±1,086, 8,070±970.6 and 13,251±7,342 CFU-GM per 10^6 cells for BALB/c, C57BL/6 and DBA/1, respectively) the lowest frequency of CFU-GM colonies was detected in the CD97^{HI} group in all strains. Furthermore, the highest frequency of progenitors was detected in the CD97^{NEG} population, while the CD97^{INT} fraction contained colony-forming cells at levels comparable to those in total bone marrow cells (Figure 1C and 1D). These results indicate that the majority of HPC with colony-forming capacity are present in the CD97^{NEG} bone marrow cell fraction.

Enrichment of repopulating hematopoietic stem cell in the CD97^{INT} bone marrow cells subset

To enumerate the frequency of primitive stem cells with long-term repopulating ability in the CD97^{HI}, CD97^{INT} and CD97^{NEG} populations, sorted BALB/c, C57BL/6 and DBA/1 bone marrow cells were tested in CAFC assays.

In bone marrow cells obtained from BALB/c mice, the highest proportion of CAFC-day 7, representing cells with progenitor cell activity, was found in the CD97^{NEG} fraction compared to in CD97^{HI}, CD97^{INT} and total bone marrow cells (Figure 2A). However, the frequency of CAFC in the CD97^{NEG} population decreased during the weeks of culture and no CAFC-day 28/35 were present in the CD97^{NEG} fraction. The CD97^{HI} bone marrow cells fraction contained very few CAFC throughout the whole culture period and did not contain CAFC-day 28/35. In contrast, the CD97^{INT} bone marrow cells showed a high frequency of CAFC-day 7



	BALB/c	C57BL/6	DBA/1
CD97-^{HI}	67.0±13.1	58.6±16.9	62.1±1.6
CD97-^{INT}	26.6±8.3	29.8±8.5	33.1±2.3
CD97-^{NEG}	4.0±1.5	10.1±8.9	4.2±1.2

	BALB/c	C57BL/6	DBA/1
CD97-^{HI}	0.04	0.04	0.03
CD97-^{INT}	0.93	1.38	0.78
CD97-^{NEG}	3.05	1.97	2.25

Figure 1. Hematopoietic progenitor cells are CD97^{NEG}. (A) Murine bone marrow cells can be divided into three major populations according to CD97 expression. Bone marrow cells were stained with anti-CD97 monoclonal antibody and CD97 expression was evaluated by flow cytometric analysis. The percentages indicate the cell fractions defined by the gates shown. (B) Mean percentage ± SD of bone marrow cells in each CD97 fraction. (C) Based on CD97 expression, bone marrow was sorted into CD97^{HI}, CD97^{INT} and CD97^{NEG} populations. The colony-forming capacity of each CD97-sorted bone marrow cell population was analyzed. Results of six independent experiments are shown as mean±SD. *$p\leq$ 0.01 *vs.* CD97^{INT} and total bone marrow. (D) Relative increase in CFU-GM colony formation compared to total bone

compared to that in total bone marrow cells, and the frequency of CAFC remained high throughout the entire culture period. In bone marrow cells obtained from BALB/c mice, the CD97^{INT} population was the only population containing CAFC-day 28/35. This indicates that HSC are contained in the CD97^{INT} population.

Analysis of bone marrow cells obtained from C57BL/6 and DBA/1 mice indicated that the majority of CAFC-day 7 were found in the CD97^{INT} population and the frequency of CAFC remained highest in the CD97^{INT} population throughout the entire culture period (Figure 2A). In bone marrow cells obtained from C57BL/6 mice, CAFC-day 35 were also found in the CD97^{NEG} fraction at very low frequencies (0.1±0.1 CAFC-day 35 per 10^5 CD97^{NEG} bone marrow cells). In DBA/1 bone marrow cells, CAFC-day 35 cells were also detected in the CD97^{HI} fraction (0.3±0.3 CAFC-day 35 per 10^5 CD97^{HI} bone marrow cells; Figure 2B).

CD97^{INT} bone marrow cells are radioprotective in vivo

To investigate the repopulating ability of the different CD97-sorted bone marrow cells fractions, 1×10^5 CD97-sorted bone marrow cells were transplanted into lethally irradiated syngeneic (BALB/c and DBA/1) or congenic (C57BL/6) recipient mice. Transplantation of 1×10^5 CD97^{INT} bone marrow cells showed a radioprotection rate of ≥70% at 150 days following transplantation in all strains (Figure 3A). Furthermore, CD97^{HI} bone marrow cells obtained from C57BL/6 donors gave 40% radioprotection in lethally irradiated congenic recipients. In DBA/1 recipients, CD97^{HI} and CD97^{NEG} bone marrow cells gave radioprotection rates of 90% and 67%, respectively (n ≥10; two independent experiments). None of the control mice that received total body irradiation alone (n=6 per strain; two independent experiments) was radioprotected at 8 weeks following transplantation. To confirm that CD97^{INT} bone marrow cells exhibited long-term repopulating ability, lethally irradiated (9.5 Gy) secondary recipient mice were transplanted with 1×10^5 total bone marrow cells obtained either from mice that had previously received 1×10^5 CD97^{INT} bone marrow cells or from recipients of 1×10^5 total bone marrow cells. At 150 days following transplantation, four of the five mice that were secondary recipients of CD97^{INT} bone marrow cells and four of the five secondary recipients of 1×10^5 total bone marrow cells were radioprotected ($p<0.01$ compared to irradiated controls; Figure 3B). FACS analysis revealed that all hematopoietic lineages were reconstituted in all of the radioprotected recipient mice (*data not shown*).

Together, these results indicate that the CD97^{INT} bone marrow cell fraction contains HSC with long-term repopulating ability *in vitro* and *in vivo*.

CD97 is differentially expressed on BALB/c, C57BL/6 and DBA/1 hematopoietic stem cell

To evaluate the expression of CD97 on cells that are phenotypically characterized as HSC, total unseparated bone marrow cells were stained for CD97, c-Kit, Thy-

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	Population	Weeks of culture 1	2	3	4	5
BALB/c	Totale BM	108.8±18.0	77.9±20.9	58.0±21.2	10.4±3.8	1.2±0.2
	CD97-HI	0.6±0.7	0.5±0.6	0.6±0.8	0.0±0.0	0.0±0.0
	CD97-INT	211.6±184.7	262.2±131.8	162.0±76.2	33.7±9.9	3.1±0.2
	CD97-NEG	649.0±355.1	33.2±21.9	3.7±5.0	0.0±0.0	0.0±0.0
C57BL/6	Totale BM	55.2±23.8	30.7±18.9	8.2±5.3	2.0±0.8	0.8±0.6
	CD97-HI	12.3±9.7	11.5±10.6	1.9±1.1	0.4±0.4	0.0±0.0
	CD97-INT	124.2±70.3	59.6±53.6	13.3±10.9	5.1±4.8	1.9±2.4
	CD97-NEG	18.0±7.8	0.8±0.5	0.2±0.2	0.1±0.1	0.1±0.1
DBA	Totale BM	9.1±4.4	25.3±10.8	7.5±1.8	1.5±0.3	0.8±0.3
	CD97-HI	0.2±0.2	1.4±0.3	1.3±0.4	0.7±0.7	0.3±0.3
	CD97-INT	26.9±20.9	88.1±69.1	2.3±1.9	0.4±0.0	0.4±0.5
	CD97-NEG	6.8±2.9	0.5±0.7	0.0±0.0	0.0±0.0	0.0±0.0

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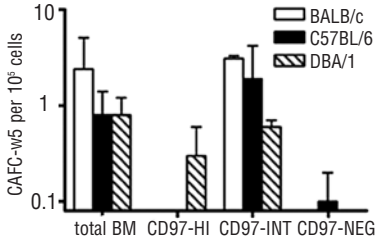

Figure 2. CAFC-day 35 cells are CD97^{INT}. (A) CD97^{HI}, CD97^{INT} and CD97^{NEG} bone marrow (BM) populations were analyzed for CAFC-forming capacity over 5 weeks. Results of three independent experiments are shown as mean ± SD. (B) CAFC-day 35 forming capacity of bone marrow cells that were sorted based on CD97 expression. Results are shown as mean ± SD.

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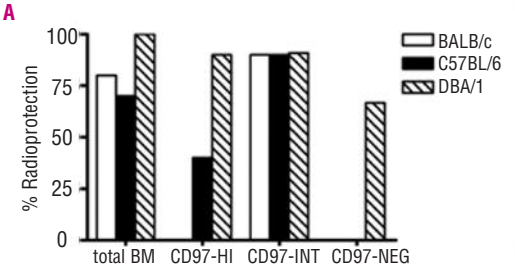

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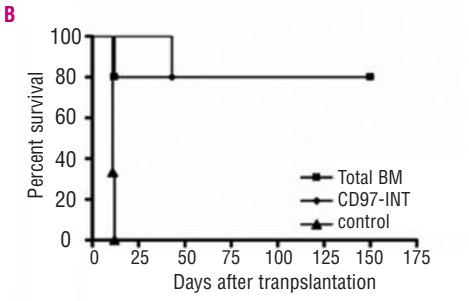

Figure 3. CD97^{INT} bone marrow cells are radioprotective *in vivo*. (A) Radioprotection by 1×10^5 CD97-sorted bone marrow cells. Lethally irradiated recipients (9.5 Gy; n=10 per group, two independent experiments) were transplanted with 1×10^5 total bone marrow cells or 1×10^5 purified CD97^{HI}, CD97^{HI} and CD97^{NEG} bone marrow cells. (B) Radioprotection by 1×10^5 sorted bone marrow cells in second recipients. Lethally irradiated recipients of 1×10^5 CD97^{INT} transplanted bone marrow cells were sacrificed on day 126 after bone marrow transplantation and bone marrow cells were harvested. Subsequently, 1×10^5 total bone marrow cells were administered to lethally irradiated (9.5 Gy) second recipients (n=5 per group).

1, Lin markers, Sca-1 and Hoechst 33342. Subsequently, the expression of CD97 was analyzed within the c-Kit^{HI}Thy-1^{LO}Lin^{NEG} population (BALB/c) or within the c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS} (C57BL/6 and DBA/1) population. In both BALB/c and C57BL/6 mice, the majority of c-Kit^{HI}Thy-1^{LO}Lin^{NEG} (BALB/c) and c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS} (C57BL/6) bone marrow cells are CD97^{INT}. In contrast, in DBA/1 mice, c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS} bone marrow cells are CD97^{HI} (Figure 4). Next, Hoechst 33342 dye efflux was used as a marker to identify the hematopoietically active bone marrow cell subset known as the side population.[24] We used the side population as an additional selection criterion for HSC, in combination with c-Kit^{HI}Thy-1^{LO}Lin^{NEG}(Sca-1^{POS}). In bone marrow cells obtained from BALB/c mice, 96.7% of the c-Kit^{HI}Thy-1^{LO}Lin^{NEG}-side population cells were CD97^{INT} and 95.0% of the c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS}-side population C57BL/6 BMC were CD97^{INT} (Figure 4). In contrast, in bone marrow cells that were obtained from DBA/1 mice, 100% of the c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1POS-side population were CD97^{HI} (Figure 4).

The CD97^{INT}c-Kit^{HI} phenotype confers long-term repopulating ability and is highly enriched for HSC

The isolation of murine HSC is a multistep process, consisting of both positive and negative selection.[4] To investigate whether long-term repopulating HSC in BALB/c mice can be isolated based on CD97 and c-Kit expression levels, we investigated the HSC and HPC content of CD97^{INT}c-Kit^{HI} *in vitro* as well as their repopulating ability *in vivo*.

The usage of intermediate CD97 expression as an isolation marker for HSC in addition to high c-Kit expression, led to a further 2.2-fold increase in CAFC-day 28 and a 1.5-fold increase in CAFC-day 35 cells as compared to the use of c-Kit^{HI} alone (Figure 5A). Moreover, intermediate expression of CD97 is a useful marker to further enrich c-Kit^{HI}Thy-1^{LO}Lin^{NEG} bone marrow cells for HSC activity, since the frequency of CAFC-day28/35 in the c-Kit^{HI}Thy-1^{LO}Lin^{NEG} population was increased 1.5-fold when c-Kit^{HI}Thy-1^{LO}Lin^{NEG} cells were also selected for CD97^{INT} expression (Figure 5A, 5B).

To investigate the repopulating ability of bone marrow cell subsets that are enriched for HSC activity, lethally irradiated recipient mice were transplanted with either 1×10^5 c-Kit^{HI} or 1×10^5 c-Kit^{HI}CD97^{INT} bone marrow cells (n=5 per group). Sixty percent of the mice that were transplanted with c-Kit^{HI} bone marrow cells survived long-term (i.e. >150 days), whereas the radioprotection rate of c-Kit^{HI}CD97^{INT} bone marrow cells was 100% (Figure 5C).

In conclusion, these *in vitro* and *in vivo* data indicate that addition of CD97^{INT} as a selection marker for primitive HSC leads to a significant further enrichment of primitive stem cells.

Discussion

CD97 is a cell-surface molecule that is expressed on cells of the immune system.[15,16] It is a member of the EGF-TM7 family of adhesion class heptahelical receptors. In the present study, we investigated the expression of CD97 on hematopoietic stem- and progenitor cells obtained from different mouse strains. We show that committed progenitor cell activity is largely present in the CD97^{NEG} fraction, while the CD97^{INT} popula-

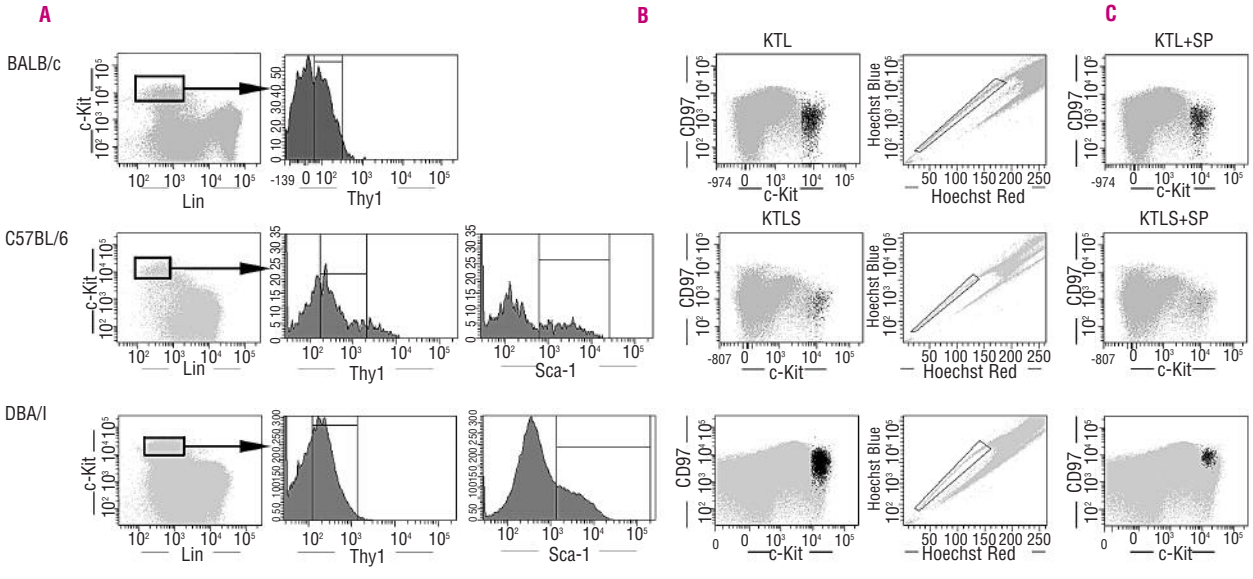

Figure 4. CD97 is differentially expressed on BALB/c, C57BL/6 and DBA/1 HSC. (A) Bone marrow cells obtained from BALB/c, C57BL/6 and DBA/1 mice were stained for CD97, c-Kit, Thy-1, Lin markers, Sca-1 and Hoechst 33342. (B) CD97 expression was analyzed on c-Kit^{HI}Thy-1^{LO}Lin^{NEG} (KTL; BALB/c) or within the c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS} (KTLS; C57BL/6 and DBA/1) cells. (C) Hoechst 33342 dye efflux was used to identify the side population (SP) and CD97 expression was analyzed within the KTL(S)-SP population.

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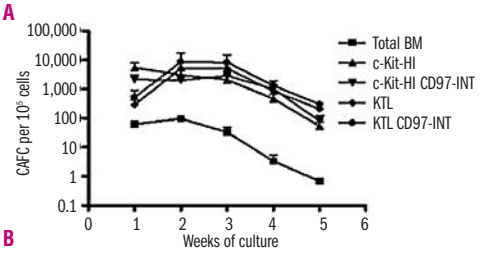

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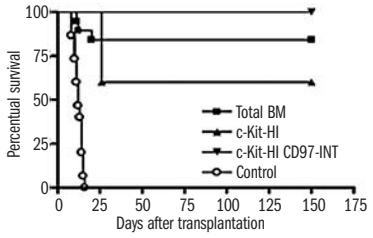

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	Weeks of culture				
Population	1	2	3	4	5
Totale BM	62.0±16.5	95.5±27.9	33.1±17.4	3.3±2.2	0.7±0.2
c-Kit-HI	5477.8±2428.0	3072.2±1252.2	2112.4±901.7	464.8±190.4	53.0±22.7
c-Kit-HI CD97-INT	2137.4±953.1	2011.0±814.7	2910.9±1211.6	1012.9±441.1	79.9±44.3
KTL	292.5±124.5	5122.9±1118.6	5143.1±2704.8	859.4±472.3	208.8±31.8
KTL-CD97-INT	521.8±375.2	8536.4±8969.2	8271.1±6547.0	1317.1±604.8	297.5±30.5

Figure 5. Stem cells with long-term repopulating ability are contained in the c-Kit^{HI}CD97^{INT} bone marrow cell fraction. (A) c-Kit^{HI}, c-Kit^{HI}CD97^{INT}, c-Kit^{HI}Thy-1^{LO}Lin^{NEG} (KTL) and KTL-CD97^{INT} bone marrow cell populations were analyzed for CAFC-forming capacity over 5 weeks. Results of three independent experiments are shown as mean ± SD. (B) c-Kit^{HI}, c-Kit^{HI}CD97^{INT}, KTL and KTL-CD97^{INT} bone marrow cell populations were analyzed for CAFC-forming capacity over 5 weeks. Results are shown as mean ± SD. (C) Radioprotection by 1x10^5 bone marrow cells. Lethally irradiated recipients (9.5 Gy; n=5-10 per group) were transplanted with 1x10^5 total bone marrow cells or 1x10^5 purified c-Kit^{HI}, c-Kit^{HI}CD97^{INT} bone marrow cells.

tion of all mouse strains contains HSC activity, although there are differences in levels of CD97 expression between the individual strains. To investigate HSC characteristics, *in vivo* repopulation assays are used in which the radioprotective capacity of transplanted cell populations are examined. Furthermore, surrogate markers for stem cell activity are measured *in vitro,* including colony-forming capacity to determine progenitor cell content and the CAFC assay. The latter assay measures a spectrum of hematopoietic cells and allows early progenitor cells (arising at day 7-14 of culture) to be separated from more primitive HSC with long-term repopulating ability (day 28-35 CAFC).

In BALB/c mice, CD97^{INT} cells are the only bone marrow subpopulation with HSC characteristics, including repopulating capacity *in vivo* and CAFC-day 35 activity *in vitro*. The majority of colony-forming progenitor cells and CAFC-day 7 cells, representing HPC with short-term repopulating ability, reside in the CD97^{NEG} subpopulation and transplantation of 1×10^5 CD97^{NEG} bone marrow cells was not radioprotective *in vivo*, indicating that this population is depleted of cells with repopulating ability. In addition, CD97^{NEG} bone marrow cells do not exhibit CAFC-day 28-35 activity *in vitro* and side population cells are not contained in the CD97^{NEG} population. CD97^{HI} bone marrow cells do not show any HSC or HPC activity *in vivo* or *in vitro*. In addition, phenotypic analysis revealed that these cells are mainly Lin^{POS}c-Kit^{NEG} cells and are not side population cells. This indicates that the CD97^{HI} bone marrow subset contains lineage committed cells that do not have any repopulating ability *in vivo*. These data suggest that, upon differentiation to colony-forming cells, HSC lose their CD97 expression and on further lineage commitment CD97 is re-expressed. In addition, transwell migration studies showed that CD97^{INT} bone marrow cells are the only CD97 population that actively migrates towards SDF-1 (*data not shown*). Although we did not evaluate CXCR4 expression on CD97^{INT} bone marrow cells directly, these data strongly suggest that CD97^{INT} bone marrow cells express CXCR4.

In C57BL/6 and DBA/1 mice, CD97 expression on HSC is less unambiguous. As for BALB/c, CD97^{INT} bone marrow cells exhibit functional characteristics of HSC, including repopulating capacity *in vivo* and CAFC-day 35 activity *in vitro*. However, CD97^{NEG} bone marrow cells obtained from C57BL/6 mice and CD97^{HI} bone marrow cells obtained from DBA/1 mice exhibit CAFC-day 35 activity. Furthermore, CD97^{HI} bone marrow cells obtained from both C57BL/6 and DBA/1 mice and DBA/1 CD97^{NEG} cells are radioprotective *in vivo*. Phenotypically, c-Kit^{HI}Thy-1^{LO}Lin^{NEG}Sca-1^{POS}-side population cells obtained from C57BL/6 bone marrow cells are CD97^{INT}, while c-Kit^{HO}Thy-1^{LO}Lin^{NEG}Sca-1^{POS}-side population cells originating from DBA/1 donors are CD97^{HI}.

Murine HSC are phenotypically defined by a complex expression of different surface molecules.[4,5,6] As a consequence, the isolation of primitive HSC involves a procedure that requires both magnetic and fluorescent cell sorting steps. Here, we show that primitive HSC can be easily isolated on the basis of CD97 and c-Kit expression. Furthermore, we show that using CD97 as a marker, in combination with the conventional HSC isolation methods, leads to enrichment of a bone marrow cell population that contains a higher frequency of HSC with radioprotective and long-term repopulating abilities. We show that CD97 can be successfully applied to enrich for primitive HSC. Addition of CD97^{INT} as a selection marker to c-Kit^{HI} leads to a more than 2-fold increase in CAFC-day 28 activity. Furthermore, addition of selection of CD97^{INT} bone marrow cells in combination with c-Kit^{HI}Thy-1^{LO}Lin^{NEG}, leads to a further increase in CAFC-day 28 activity compared to that obtained by c-Kit^{HI}Thy-1^{LO}Lin^{NEG} selection alone. These data indicate that the addition of CD97^{INT} as a selection marker for the isolation of primitive cells with long-term repopulating ability can strongly increase the frequency of primitive HSC.

Preliminary data on CD97 expression on human bone marrow cells showed that, as for murine bone marrow cells, human bone marrow cells can be divided

according to CD97 expression into $CD97^{HI}$, $CD97^{INT}$ and $CD97^{NEG}$ subpopulations. Co-staining with CD45, CD34 and CD38 revealed a small subpopulation that is $CD45^{POS}CD34^{POS}CD38^{NEG}CD97^{INT}$.[26] Although further studies are required, these data suggest that CD97 may also be differentially expressed on human HSC and HPC.

Despite accumulating data on the molecular structure and expression of CD97, we are only beginning to understand the physiological role of this molecule. Increased expression of CD97 has been implicated in several diseases linked with autoimmunity, such as multiple sclerosis[27] and rheumatoid arthritis,[28,29] and with tumor migration and invasiveness.[30,31] Recently, it has been shown that CD97 is involved in neutrophil migration and angiogenesis.[19,23] The role of CD97 in neutrophil migration is further strengthened by our finding that neutralizing antibodies directed against CD97 inhibit interleukin-8-induced HSC mobilization,[32] since neutrophils are indispensable for interleukin-8-induced HSC/HPC mobilization.[33] However, the molecular function of CD97 on HSC and HPC is currently unclear and requires further investigation.

In conclusion, we show that CD97 is intermediately expressed on HSC and that $CD97^{NEG}$ bone marrow cells are colony-forming progenitor cells that are not radioprotective *in vivo*. In addition, in DBA/1 and C57BL/6 mice, $CD97^{INT}$ is not the only HSC population, since $CD97^{HI}$ and $CD97^{NEG}$ bone marrow cells also show HSC characteristics. Moreover, we found that addition of $CD97^{INT}$ as a marker for HSC leads to a further enrichment in primitive HSC, compared to the $c\text{-}Kit^{HI}Thy\text{-}1^{LO}Lin^{NEG}$ phenotype. We conclude that the $CD97^{INT}c\text{-}Kit^{HI}$ phenotype separates colony-forming cells from cells with repopulating capacity and allows simple and rapid purification of murine HSC.

Authorship and Disclosures

MVP: conception and design of the study, collection, analysis and interpretation of the data and writing of the paper; HH: collection, analysis and interpretation of the data and writing of the paper; JH: conception and design of the study and critical revision of the manuscript; WEF: conception and design of the study, interpretation of the data and critical revision of the manuscript. All authors approved the final version of the manuscript. The authors reported no potential conflicts of interest.

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