

Demonstration of human mast cell progenitors in the bone marrow

To the editor

Mast cells are tissue-resident cells widely recognized for their role in asthma and allergy. In inflammatory diseases, mast cells accumulate and become activated in particular sites of affected tissues such as, for example in the bronchial smooth muscles of allergic asthmatics.¹ Using in vivo models, we and others have shown that these inflammation-induced mast cells originate from mast cell progenitors (MCp).² For decades, human mast cells have been differentiated in vitro from uncommitted progenitor cells isolated from, for example peripheral blood and bone marrow (BM). In the current study, a population of MCp in the BM of healthy adults was identified, which was more frequent, and had lower surface expression of integrin $\beta 7$ than the recently described MCp population in the blood.³ Integrin $\beta 7$ is required for transmigration of mouse MCp into the lung.⁴ Thus, we speculate that integrin $\beta 7$ is upregulated on the human MCp upon release from the BM in preparation for their transmigration from the blood to the peripheral tissues.

Using flow cytometry, BM samples from healthy donors were investigated for the possible presence of a MCp population. In comparison with peripheral blood samples from the same individuals ($n = 11$), the BM was enriched in lineage (Lin^-) ($\text{CD4}^- \text{CD8}^- \text{CD19}^- \text{CD14}^-$) cells, $\text{Lin}^- \text{CD34}^{\text{hi}}$ cells and $\text{Lin}^- \text{CD34}^{\text{hi}} \text{CD117}^+$ cells (Figure 1A). A population of $\text{Lin}^- \text{CD34}^{\text{hi}} \text{CD117}^+ \text{Fc}\epsilon\text{RI}^+$ cells was found in healthy BM (Figure 1D), which was similar but 14-fold more frequent than the known MCp population in peripheral blood (Figure 1B and I). In one set of experiments, $\text{Lin}^- \text{CD34}^{\text{hi}} \text{CD117}^+ \text{Fc}\epsilon\text{RI}^+$ cells from the BM and blood from seven donors were isolated by fluorescence-activated cell sorting (FACS) and either cultured in a myeloid-erythroid cytokine cocktail for 7 days or analysed directly. The primary BM $\text{Lin}^- \text{CD34}^{\text{hi}} \text{CD117}^+ \text{Fc}\epsilon\text{RI}^+$ cells demonstrated immunofluorescence for mast cell tryptase (Figure 1E). Re-analysis of the respective progeny cells by flow cytometry revealed that all cells had an intermediate CD117 expression after culture and that a proportion of them had lost Fc ϵ RI surface expression (Figure 1F and J). The loss of Fc ϵ RI expression upon in vitro culture of the human peripheral blood MCp during the same culture conditions was reported previously,³ and Fc ϵ RI $^-$ mast cells have been demonstrated in vitro⁵ and shown residing in the alveolar parenchyma in vivo.⁶ Regardless, there was no difference in the expression of Fc ϵ RI between the BM and blood progenies ($39.8\% \pm 10.2\%$ and $52.7\% \pm 12.0\%$, respectively). The BM

progeny cells had a similar mast cell-like morphology, determined by May-Grünwald-Giemsa staining (Figure 1G), as the blood progeny cells (Figure 1K). Further, the progeny cells from both sources expressed various levels of tryptase (Figure 1H and L). Altogether, the progenies of the $\text{Lin}^- \text{CD34}^{\text{hi}} \text{CD117}^+ \text{Fc}\epsilon\text{RI}^+$ cells from the BM and blood were similar in terms of morphology and surface expression of CD117 and Fc ϵ RI, indicating that both populations consisted of MCp. To verify this, mRNA expression of mast cell-related genes was assessed in MCp from three donors. Both MCp populations expressed similar levels of mRNA of CD117 (*KIT*), the Fc ϵ RI alpha chain (*FCER1A*), carboxypeptidase A3 (*CPA3*), hematopoietic prostaglandin D synthase (*HPGDS*), serglycin (*SRGN*) and tryptase alpha/beta I/II (*TPSAB1/TPSB2*) (Figure 1C).

As both the CD34^+ cells and the MCp were more frequent in the BM than in the blood, we investigated whether the MCp frequency was related to the frequency of CD34^+ cells. Indeed, the levels of MCp correlated with the frequency of CD34^{hi} cells in BM and blood from 11 donors (Figure 2A and B). To assess the proliferation capacity, 50 BM and blood MCp from 7 donors were cultured in a myeloid-erythroid cytokine cocktail for 7 days, before quantification by flow cytometry. The MCp proliferation capacity was variable between donors and the number of progenies after culture did not differ between the MCp from blood (866 ± 375) and BM (1028 ± 433) (Figure 2C). On average, the MCp divided approximately four times. The proliferation capacity of the MCp populations in BM ($n = 4$) and blood ($n = 3$) was also compared by determining the protein expression of the intracellular proliferation marker Ki-67. Most MCp from both sources were positive for Ki-67 (Figure 2D). As the MCp were in a highly proliferative state but divided poorly in vitro, our culture system was insufficient in supporting mast cell proliferation. Next, the surface expression of the IL-33 binding receptor ST2, integrin $\beta 7$ and the IL-3 receptor (IL-3R) on primary blood and BM MCp from the same three donors was determined by flow cytometry. The membrane-bound ST2 receptor is expressed on mouse MCp⁷ and human in vitro-derived mast cells.⁸ However, the human MCp from BM and blood lacked or had minimal ST2 surface expression (Figure 2E). Mouse MCp express integrin $\beta 7$,⁷ and the $\alpha 4\beta 7$ integrin is functionally important for the transmigration of MCp into the lung in a mouse model of allergic airway inflammation.⁴ In humans, $\alpha 4\beta 7$ is required for

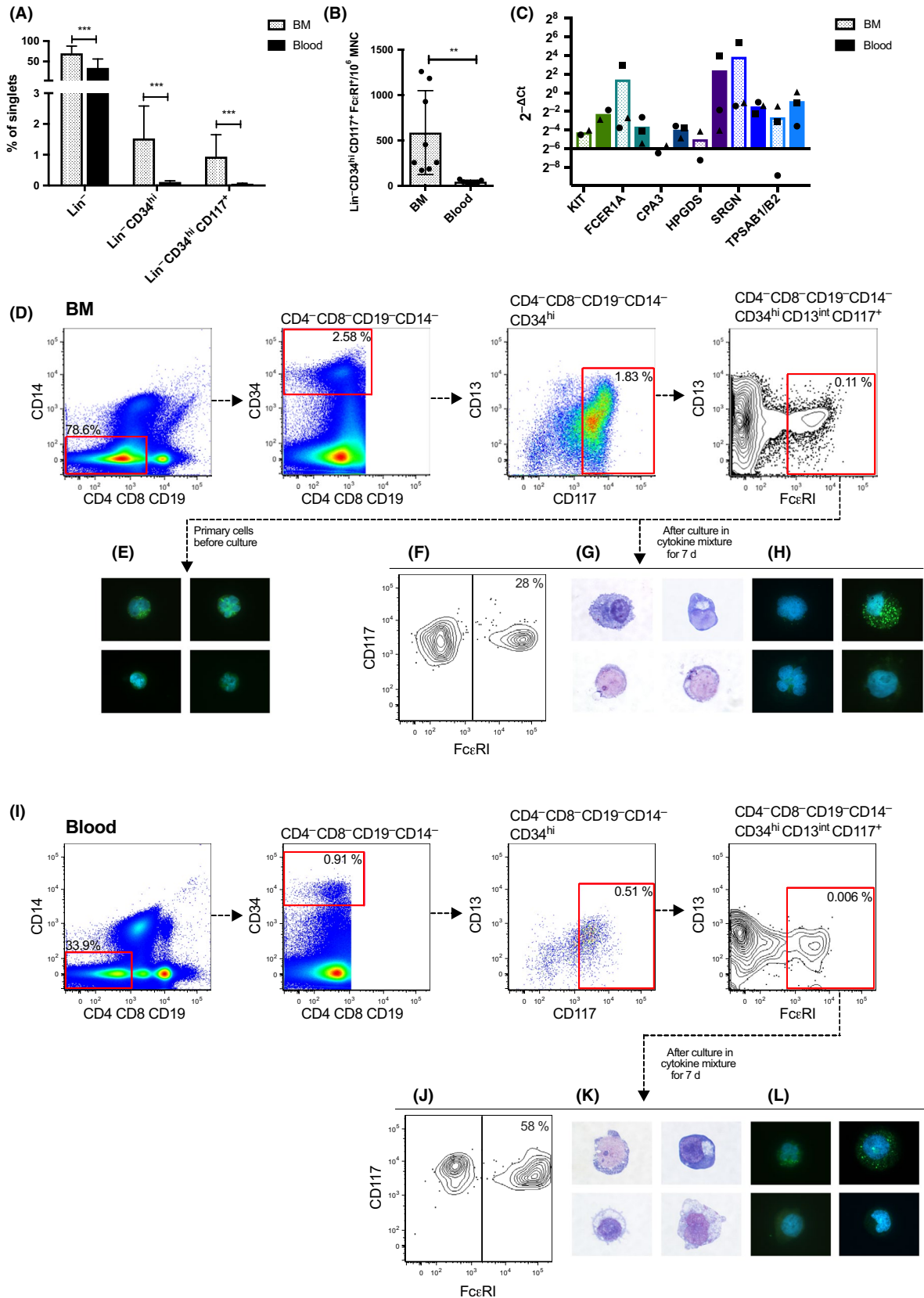


FIGURE 1 MCP in the BM are similar but more frequent than their counterparts in the blood. A and B, The frequency of BM and blood populations. C, Analysis of Lin⁻CD34^{hi}CD117⁺ FcεRI⁺ cells by qPCR using beta-actin (ACTB) as endogenous control. Each donor is labelled by a unique symbol. KIT, CPA3 and HPGDS were undetectable in one sample. D and I, Gating strategy for the Lin⁻CD14⁻CD34^{hi}CD117⁺ FcεRI⁺ BM (D) and blood cells (I). The primary cells (E) and progenies were visualized with an anti-tryptase mAb (H, L). The progenies were analysed by flow cytometry (F, J) or May-Grünwald-Giemsa (G, K). Means ± SD (**P < .001, ***P < .0001)

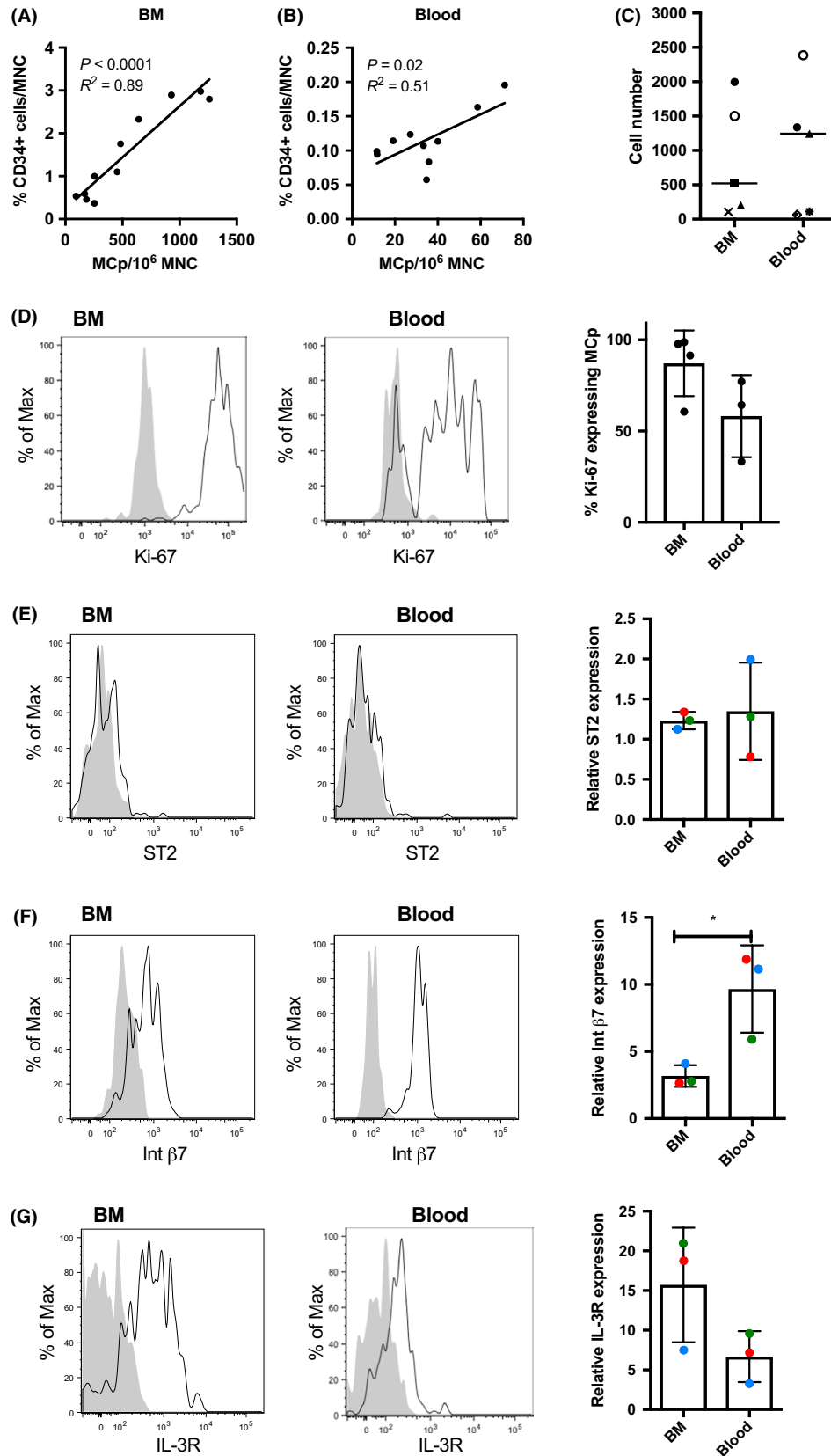


FIGURE 2 Blood MCP have higher surface expression of integrin $\beta 7$ than those in the BM. A and B, The frequency of MCP correlates with the frequency of CD34⁺ cells in the BM and blood. C, The number of MCP progenies from five blood and BM donors after 7 d in culture was quantified by flow cytometry. Each donor is depicted with a unique symbol. D–G, The expression of Ki-67 (D), ST2 (E), integrin $\beta 7$ (Int $\beta 7$) (F), and IL-3R (G) relative to the appropriate isotype control. The histograms are representative of three-four experiments. Means $\pm$ SD (* $P < .05$)

transmigration of lymphocytes into the gut and a therapeutic target in inflammatory bowel diseases. Interestingly, the surface expression of integrin $\beta 7$ was three times higher in the blood MCp than in the BM MCp (Figure 2F). We speculate that the MCp up-regulates integrin $\beta 7$ expression as they leave the BM and that integrin $\alpha 4 \beta 7$ is involved in the transmigration of human circulating MCp into tissues. As surface expression of the IL-3R was reported on SSC^{lo} CD14⁺ CD34⁺ CD117^{int/hi} Fc ϵ RI⁺ blood MCp,⁹ the surface expression of IL-3R was compared between BM and blood MCp. In all donors, the relative IL-3R expression was higher in the BM MCp compared to the blood counterparts (Figure 2G).

In conclusion, we establish the presence of a MCp population in the BM of healthy subjects, which is more frequent in numbers compared to peripheral blood MCp from the same donors. Blood and BM MCp have a similar expression of mast cell-related genes and demonstrate a mast cell-like phenotype after in vitro culture. However, the blood MCp have three times higher surface expression of integrin $\beta 7$ than the BM MCp, indicating that the circulating MCp are preparing to transmigrate into tissues.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

MS and JH conceived the idea and designed the study; JU recruited the healthy donors and arranged the sampling; MS performed the experiments; PAA-V. performed one set of experiments; MS, PAA-V., and JH analysed the data; MS and J.H wrote the manuscript; JU and PAA-V critically reviewed the manuscript and approved of submission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Online materials and methods

Demonstration of human mast cell progenitors in the bone marrow

Short title: Human bone marrow mast cell progenitors

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Online Supplementary Material and Methods

Subjects

Bone marrow and blood samples were collected from in total 11 healthy donors (two of which donated several times) recruited via Karolinska Hospital, Huddinge, Sweden. In addition, buffy coats from healthy donors at Uppsala University Hospital, Sweden were used.

Ethical statement

All subjects gave their written, informed consent to participate in the study. The study protocol was approved by the Stockholm Regional Ethics committee, Dnr 2015/1914-31/1 and conducted according to the Declaration of Helsinki.

Mononuclear cell preparation

Blood samples were collected in EDTA-treated tubes (10 ml; BD Vacutainer, BD Bioscience, Franklin Lakes, NJ, USA) or from buffy coats. Bone marrow was collected into 15 ml falcon tubes with RPMI media containing heparin. Peripheral blood mononuclear cells (PBMC) were enriched using Ficoll-Paque Premium ($\rho=1.076$ g/ml) (GE Healthcare, Little Chalfont, UK) in SepMate™-50 tubes (Stemcell Technologies, Vancouver, Canada). Platelets were removed by centrifugation (2 x 200g, 10 min).

Flow cytometry

To sort or analyze human MCp, enriched mononuclear cells from blood or bone marrow were incubated in PBS, pH 7.4 with 2% heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA) with the following fluorescence-labeled antibodies; CD4 (RPA-T4), CD8 (RPA-T8), CD13 (WM15), CD14 (M5E2), CD19 (HIB19), CD34 (581), CD117 (104D2) and FcεRI (AER-37). For analysis of surface expression, the following antibodies were added per

experiment to the above-mentioned panel: CD123/ IL-3 receptor (7G3), integrin β 7 (FIB504) or ST2-biotin (B4E6) followed by fluorescently conjugated streptavidin. For intracellular detection of Ki-67 the cells were treated with eBioscience™ Foxp3/Transcriptn Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) and incubated with Ki-67 or isotype control from the Ki-67 analysis kit (catalog number 556027, BD Biosciences). For re-analysis of the MCp-progenies after seven days in culture, the cells were incubated with CD117 (104D2) and Fc ϵ RI (AER-37) antibodies. All antibodies were obtained from BD Bioscience (Franklin Lakes, NJ, USA), MD Bioproducts (Zürich, Switzerland) and eBioscience (San Diego, CA, USA). After washing, the flow cytometry analysis and sorting were performed on a LSRII, LSRFortessa or a FACSAria III (BD Biosciences). Data analysis was preformed using FlowJo software version 9.8.

Culture conditions

The cells were cultured in serum free StemPro-34 medium supplemented with the following cytokines; 30 ng/mL interleukin (IL) -3, 100 ng/mL IL-6, 20 ng/mL IL-9, and 100 ng/mL stem cell factor, 20 ng/mL IL-5, 50 ng/mL granulocyte macrophage–colony-stimulating factor, 20 ng/mL thrombopoietin, 10 ng/mL IL-11, 10 ng/mL FMS-like tyrosine kinase 3 ligand, and 4 U/mL erythropoietin. Cytokines were purchased from PeproTech (Rocky Hill, NJ, USA) and R&D Systems (Minneapolis, MN, USA). The number of cell divisions after culture was estimated using the following formula: The number of cell divisions = $\log_2$ (the number of progeny cells/number of sorted cells).

May-Grünwald Giemsa and tryptase immunostaining

The cultured cells were cytocentrifuged on to glass slides, stained with May-Grünwald (Sigma-Aldrich) for five min, and rinsed once in phosphate buffered saline (PBS, pH 7.2) for

1.5 min. The slides were counterstained with a 5% Giemsa solution (Sigma-Aldrich). After 20 min, the slides were rinsed with distilled water and observed under a bright field microscope (Nikon Eclipse 90i). For tryptase immunostaining, the cell culture progenies were cytocentrifuged on to glass slides and fixed with 4 % formaldehyde. After 15 min, the slides were incubated in a 0.25 % Triton X-100 permeabilization solution for 5 min. After two washes with PBS, the slides were incubated with a monoclonal mouse antibody recognizing human mast cell tryptase (MA511711, 1:1500, Thermo Scientific, Waltham, MA, USA) for one hour at room temperature. The slides were washed twice using PBS and incubated for one hour at room temperature with an anti-mouse secondary antibody raised in rabbit and conjugated to Alexa 488 (1:100, Thermo Scientific). The slides were mounted using ProLong® Gold Antifade Reagent with DAPI and the images were obtained using a fluorescence microscope (Zeiss Axio Imager).

qPCR

The cells were sorted directly into RLT lysis buffer (Qiagen, Hilden, Germany) placed into cryotubes and snap frozen in liquid nitrogen and stored in liquid nitrogen until RNA extraction. The RNeasy Mini Kit (Qiagen) was used for extraction of RNA and the Whole Transcriptome Amplification Kit 1 (Sigma-Aldrich) in combination with JumpStart AccuTaq LA DNA Polymerase (Sigma-Aldrich) were used for cDNA synthesis and pre-amplification. Primers for *HPGDS*, *KIT*, *FCER1A*, *CPA3* were designed using Primer 3 software and primers for *SRGN*, *TPSAB1/TPSB2* and *ACTB* were designed according to Dahlin et al¹. The primers were produced by LGC Biosearch Technologies (Risskov, Denmark) and the sequences are listed in **Table 1**. For detection PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) was used and the samples were analysed with CFX96 Touch Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using the CFX Maestro software (Bio-Rad).

Blood and bone marrow were analysed from the same donors (n=3). In one out of three blood samples the signal was too weak to detect mRNA for *KIT*, *CPA3* and *HPGDS*. To quantify the relative gene expression, $2^{-\Delta C_t}$ was calculated for each gene with the ACTB gene as the endogenous control.

Table 1. Primers used for detection of mast cell-related genes.

Target gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>HPGDS</i>	ATG CGC CTC ATC TTA TGC AAG	GGT TGT CTA ACA GGT CAG GCT
<i>KIT</i>	CGT TCT GCT CCT ACT GCT TCG	CCC ACG CGG ACT ATT AAG TCT
<i>FCER1A</i>	CTC CAT TAC AAA TGC CAC AGT TG	TCA CGC GGA GCT TTT ATT ACA G
<i>CPA3</i>	AGG CAC AGC TAC GCA AAA TAC	TGC CAC AAT CCG TAA AAA TAG CC
<i>SRGN</i>	TGG GAG TGG CTT CCT AAC GGA	TGT CTG AGG GCA GAT TCC TGT
<i>TPSAB1/TPSB2</i>	CGA TGT GGA CAA TGA TGA GC	CGC CAA GGT GGT ATT TTG C
<i>ACTB</i>	CGC GAG AAG ATG ACC CAG AT	GAT GGG CAC AGT GTG GGT G

Statistical analysis

Statistical differences between groups were assessed using unpaired, two-tailed Student's t-test. Correlations and linear regression were analyzed with Pearsons correlation. All graphs were prepared and statistics calculated using GraphPad Prism 7.0c (GraphPad software Inc., San Diego, CA). A p-value of less than 0.05 was considered significant.

Reference

1. Dahlin JS, Malinowski A, Ohrvik H, et al. Lin- CD34hi CD117int/hi FcεRI+ cells in human blood constitute a rare population of mast cell progenitors. *Blood*. 2016;127(4):383-391.