SUPPLEMENTARY APPENDIX

CD14+ cells from peripheral blood positively regulate hematopoietic stem and progenitor cell survival resulting in increased erythroid yield

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Supplementary Methods

Mobilized peripheral blood, cord blood and bone marrow cultures

Mononuclear cells were isolated from bone marrow and cord blood. CD34+ cells from both cord blood and mobilized peripheral blood were isolated by MACS sorting. CD34+ cells from bone marrow were FACS sorted. CD34+ cells were co-cultured with PBMC-derived CD14+ cells at a ratio of 1:100. Media was replenished every two days. Informed consent from donors was given in accordance with the Declaration of Helsinki.

Quantitative PCR analysis

To assess the relative *GATA1* and *EPOR* mRNA levels during differentiation of CD34+ cells, aliquots were taken at day 0, 2, 4 and 8 of culture. To assess the relative mRNA levels of *BCL2* and *BCL2L1* after co-culturing CD34+ cells with CD14+ cells, CD34+ cells were cultured in a transwell with or without CD14+ cells for 2 days. RNA was extracted with TRIzol reagent (Invitrogen Technologies; Carlsbad, CA) and complementary DNA was synthesized with random hexamers as described in the product protocol (Invitrogen). RT-PCR was performed in duplicate with Express SYBR GreenER reagents (Invitrogen) on the StepOnePlus RT PCR system (Applied Biosystems; Foster City, CA). Values were normalized using *S18* as a reference gene and calibrated relative to expression in CD34+ cells at day 0 (for *GATA1* and *EPOR*) and CD34+ cells cultured in the absence of CD14+ cells (for *BCL2* and *BCL2L1*). The following primer sets were used: *S18* (forward: 5'-GGACAACAAGCTCCGTGAAGA-3', reverse: 5'-CAGAAGTGACGCAGCCCTCTA-3'), *GATA1* (forward: 5'-ATCACACTGAGCTTGCCACA-3', reverse: 5'-ACCAGAGCAGGATCCACAAA-3'), *EPOR* (forward: 5'-CTCATCCTCGTGGTCATCCT-3', reverse: 5'-GCTGGAAGTTACCCTTGTGG-3'), *BCL2* (forward: 5'-ATGTGTGTGGAGAGCGTCAA-3', reverse: 5'-CGTACAGTTCCACAAAGGCA-3') and *BCL2L1* (forward: 5'-GCGTGGAAAGCGTAGACAAG-3', reverse: 5'-TGCTGCATTGTTCCCATAGA-3').

Western blotting

During CD34+ cell differentiation, aliquots were taken at day 0, 2, 4 and 8 of culture. Cells were pelleted, washed once with PBS and lysed (20mM Tris-HCl pH8.0, 138mM NaCl, 10mM EDTA, 100mM NaF, 1% Nonidet P-40, 10% glycerol; Protease inhibitor cocktail V (Calbiochem; Darmstadt, Germany), 100µM PMSF (Sigma Aldrich; St. Louis, MO; 1:1000) and 1mM sodium orthovanadate (New England Biolabs, Leusden, the Netherlands)). Protein concentrations were determined by Bradford assay according to the manufacturer's protocol (Biorad; Hercules, CA, USA). Lysates were mixed with Laemmli loading dye (Invitrogen Life Technologies; Breda, the Netherlands) and incubated for 5min at 95°C. Proteins were separated on a 7.5% SDS-PAGE gel loading equal protein concentrations or equal cell numbers and subsequently transferred to nitrocellulose membranes (Invitrogen). A 250-kda precision marker was used as size standards (Invitrogen). Membranes were blocked with 5% bovine serum albumin (BSA; Sigma) and subsequently incubated for 1hr

with primary antibodies; anti-RhoGDI (Abnova; Taipei, Taiwan; 1:1000), anti-gata1 (a kind gift from Thamar van Dijk, Erasmus Medical Center, the Netherlands; 1:1000), anti-band3 (BRIC170; IBGRL; Bristol, UK; 1:1000) and anti-α/β-spectrin (Sigma; 1:1000)) in Tris-buffered saline and 0.1% Tween 20 (pH8.0) containing 5% BSA. Membranes were washed, followed by incubation with IRDye 800-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA; 1:2500-10000). Fluorescence was detected on an Odyssey Infrared Imaging system using Odyssey V3.0 software (both LI-COR Biosciences).

Flow cytometry

Antibodies used: BD Biosciences: anti-CD3 (PE 1:50; Pacific Blue 1:80), anti-CD14 (PE 1:50; Pacific Blue 1:150), anti-CD16 (Pacific Blue 1:80), anti-CD19 (PE 1:50; PE-Cy7 1:20), anti-CD34 (FITC 1:10), anti-CD38 (PE 1:50), anti-CD41 (APC 1:20), anti-CD42 (PE 1:20), anti-45RA (PE 1:10), anti-CD56 (PE 1:20), anti-CD117 (PE 1:50), anti-CD169 (APC 1:100), anti-CD206 (APC 1:100) and anti-BAH1.1 (PE 1:100; APC 1:10); Miltenyi Biotec: anti-CD14 (APC 1:50; PE 1:50), anti-CD71 (APC 1:150) and anti-CD163 (PE 1:100); Pelicluster (Amsterdam, The Netherlands): anti-CD4 (PE 1:50), anti-CD16 (FITC 1:100) and anti-CD36 (FITC 1:100); Dako (Glostrup, Denmark): anti-CD41 (PE 1:20) and anti-CD235a (PE 1:100); eBioscience (Vienna, Austria): CD184 (PE 1:100); IQ Products (Groningen, The Netherlands): anti-CD34 (APC 1:10).

Supplementary Table Legends

Online Supplementary Table S1. Statistical analysis of marker expression between the different CD14+ populations in PBMC before and after two days of culture (Figure 7C).

P values T-Test	CD16	CXCR4	CD163	CD169	CD206
Classical vs Intermediate	0.00005	0.00317	0.00416	0.17957	N/A
Classical vs Non-classical	0.00109	0.16771	0.03652	0.23951	N/A
Intermediate vs Non-classical	0.00225	0.00812	0.01267	0.07984	N/A
Classical vs Day 2	0.00152	0.03255	0.00460	0.05191	0.01646
Intermediate vs Day 2	0.03347	0.03387	0.00492	0.05456	0.01714
Non-classical vs Day 2	0.00378	0.03247	0.00315	0.03645	0.01727

Supplementary Figure Legends

Online Supplementary Figure S1. Characterization of BAH1.1 as a MEP marker. (A) Gating strategy to define CMP, GMP and MEP populations. CD34+ cells were isolated from peripheral blood mononuclear cells (PBMC) and cultured for 4 days in StemSpan supplemented with IL6 (10ng/ml), IL3 (1ng/ml), SCF (100ng/ml) and N-PLATE (50ng/ml; thrombopoietin agonist). Cells were analyzed by flow cytometry. Living cells were gated on FSC/SSC, followed by a positive gate for CD34. The CD34+ cells were further separated on the basis

of BAH1.1 and CD45RA. CD34+BAH1.1+CD45RA- cells were FACS-sorted and subjected to colony assays **(B)** or cultured in specific media **(C)**. **(B)** Colony assays indicating that CD34+BAH1.1+CD45RA- cells primarily display BFU-E colony forming ability. Note that CD34+BAH1.1-CD45RA-CMP produce both BFU-E and non BFU-E colonies. **(C)** CD34+BAH1.1+CD45RA- cells were cultured in megakaryopoiesis specific media containing StemSpan supplemented with SCF (100ng/ml), IL1β (1ng/ml), IL6 (10ng/ml) and N-PLATE (50ng/ml) for 4 days followed by 4 days with IL1β (1ng/ml) and N-PLATE (50ng/ml) only (i-ii) or erythropoiesis specific media containing StemSpan supplemented with erythropoietin (2U/ml), stem cell factor (100ng/ml) and dexamethasone (1μM) for 8 days (iii-iv). Cells were analyzed by flow cytometry. Both CD41a+CD42b- megakaryoblasts and CD41a+CD42b+ megakaryocytes were found in megakaryopoiesis specific media (ii; isotype in i) and CD71highCD235adim erythroblasts were found in erythropoiesis specific media (iv; isotype in iii). Together the colony assays and liquid cultures show that BAH1.1 can be used to delineate MEP from other hematopoietic lineages. FSC(-H): forward scatter; SSC: side scatter; MEP: megakaryocyte-erythroid progenitor; CMP: common myeloid progenitor; GMP: granulocyte-monocyte progenitor; BFU-E: erythroid burst-forming units.

Online Supplementary Figure S2. Population definitions and marker expression on erythroblasts. (A) CD34+ cells isolated from PBMC were separated using CD36/CD38 and CD36/CD117 antibodies. The dot plot (iii) shows a representative experiment indicating that CD34+ cells (i) can be further separated into 72% CD38+ (short term hematopoietic stem cells; HSC), 22% CD38- (long term HSC) and 4% CD38+CD36+ (megakaryocyte-erythroid progenitors; MEP). CD117 positivity mirrors CD38 expression patterns (iv). The isotype control is displayed in ii. **(B-C)** RT-PCR analysis of *GATA1* **(B)** and *EPOR* **(C)** mRNA levels in CD34+ cells during culture at day 0, 2, 4 or 8 normalized to *S18* and relative to CD34+ cells at day 0 (n=3). **(D)** CD34+ cells from mobilized peripheral blood were cultured for 0, 2, 4, or 8 days and western blot analysis demonstrated increasing spectrin (250kda), band3 (100kda) and gata1 (50kda) protein expression during CD34+ cell differentiation, lanes were loaded according to equal protein concentrations (left) or to equal cell numbers (right). Western blot images show a representative result (n=3). Note that the strictly erythroid marker band3 (SCLa41) is only expressed on day 8. **(E)** Gating strategy to show erythroblasts according to CD71/CD235a expression. PBMC were cultured for 8 days and erythroblasts were gated based on size (FSC/SSC), single cells (FSC-H/FSC-A) and CD71/CD235a positivity. Representative histograms showing PBMC-derived erythroblasts which do not express CD34, CD38 or CD41 but are CD117+, CD71high and CD235adim. FSC(-H): forward scatter(-height); SSC: side scatter; M: precision marker.

Online Supplementary Figure S3. Depletion and isolation efficiencies of CD3+, CD14+ and CD19+ cells. Graphs display the percentage of **(A)** CD3+, **(B)** CD14+ and **(C)** CD19+ cells before and after depletion by magnetic-activated cell sorting (n=3). Dot plots showing the MACS isolation efficiencies of **(D)** CD34+, **(E)**

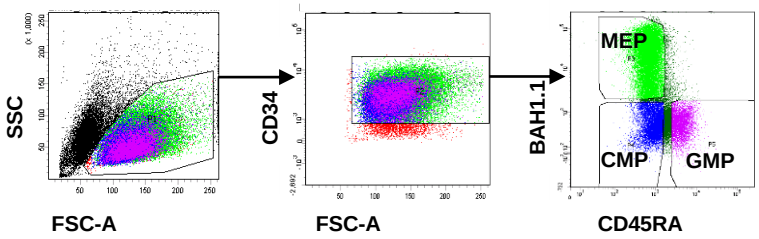
CD3+, **(F)** CD14+ and **(G)** CD19+ cells. The dot plots depict representative depletion and isolation experiments. PBMC: peripheral blood mononuclear cells; FSC: forward scatter.

Online Supplementary Figure S4. PBMC-derived CD14+ cells positively affect CD34+ cells isolated from cord blood and bone marrow. (A-B) CD34+ cells isolated from **(A)** cord blood or **(B)** bone marrow were cultured in the absence or presence of PBMC-derived CD14+ cells. After 7 (bone marrow) or 8 (cord blood) days, the erythroblasts yield was quantified by analyzing the number of CD71/CD235a positive cells by flow cytometry and depicted in a bar graph (n=3-4). **(C)** Representative histograms of CD14+ isolated cells using MACS sorting from PBMC stained with markers as indicated in the figure. Note the presence of CD16, CD163, CD4 and CD38 but absence of CD34, CD3, CD19 and CD117 indicating that MACS sorted CD14+ cells are not contaminated with HSPC, lymphocytes or B cells. **(D)** The percentage of CD14+ cells in total PBMC during time was assessed by flow cytometry (n=5). **(E-F)** CD34+ cells isolated from cord blood **(E)** or bone marrow **(F)** were cultured in the absence or presence of PBMC-derived CD14+ cells. After 2 (cord blood) or 3 (bone marrow) days, the number of CD34+ cells was analyzed by flow cytometry and depicted as bar graphs (n=3-4). The blunted response of the more heterogeneous bone marrow- and cord blood-derived CD34+ populations underscores that co-culture with CD14+ cells mainly improves the survival of early, uncommitted CD34+ cells. PB: pacific blue.

Online Supplementary Figure S5. CD14+ cells control the survival of HSPC. (A) Representative dot plots belonging to Figure 5C showing CD34/CD36 expression (populations defined in Figure 1C of sorted population A (Lin-CD34+CD36) in the presence or absence of CD14+ cells after 2 days of culture. **(B)** Ratio depicting the absolute cell numbers in population B (megakaryocyte-erythroid progenitors; MEP) divided by population A (HSPC) from the experiment in Figure 1C (n=3). **(C)** Representative CD34/CD36 dot plots from total PBMC (i), PBMC depleted for CD14+ cells (ii) and CD14+ cells (iii) belonging to the bar diagraphs of Figure 5D. RT-PCR analysis of **(D)** *BCL2* and **(E)** *BCL2L1* mRNA levels in CD34+ cells during a 2-day culture in transwell assays in the presence or absence of CD14+ cells. The bar graph depicts the relative mRNA levels normalized to the S18 reference gene with the level of mRNA in CD34+ cells without co-culture set to 1 (n=4). PBMC: peripheral blood mononuclear cells; Lin-: lineage-negative cells.

Online Supplementary Figure S1

A



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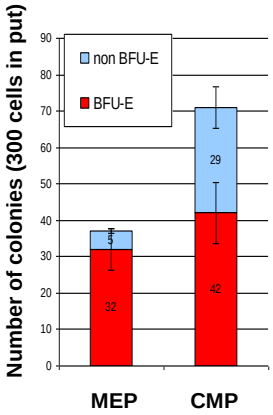

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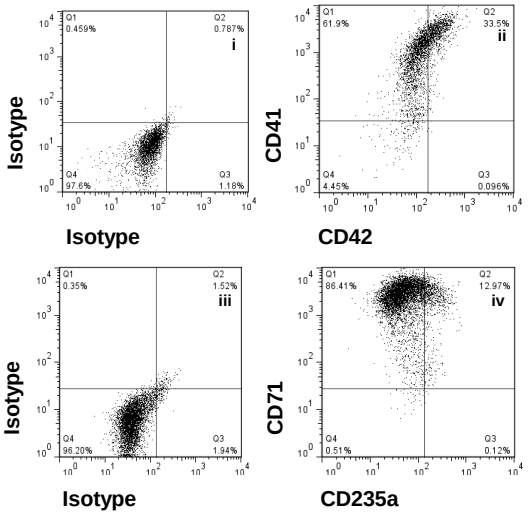

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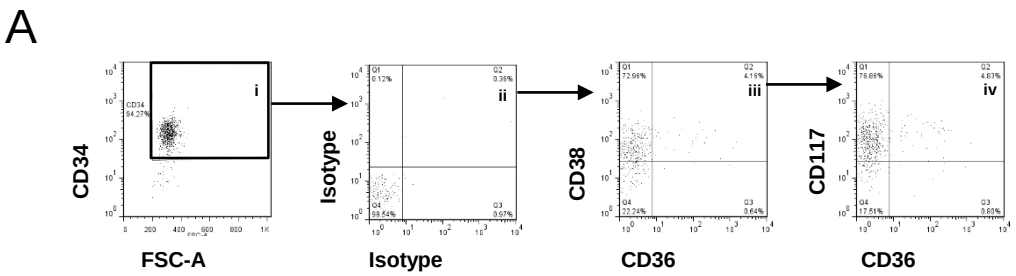

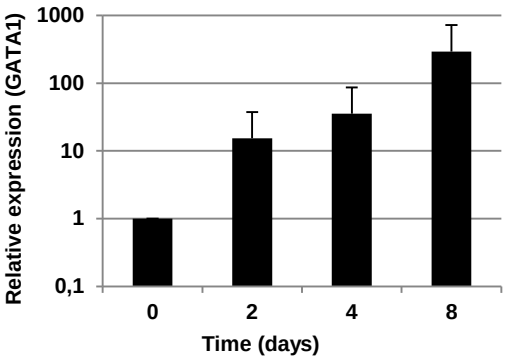

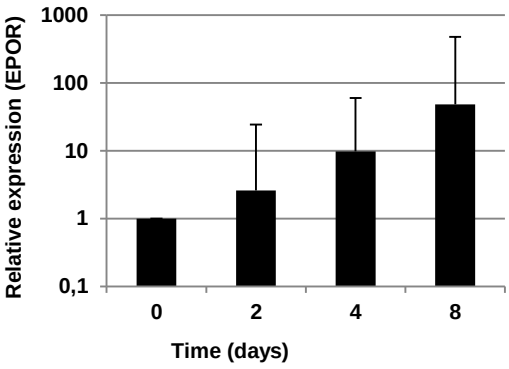

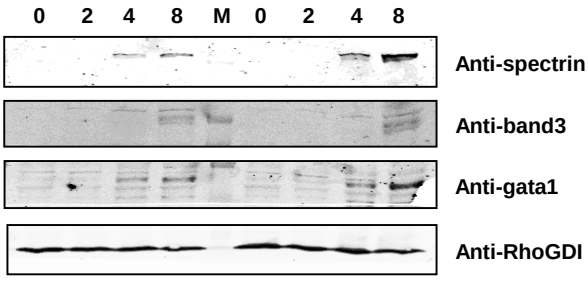

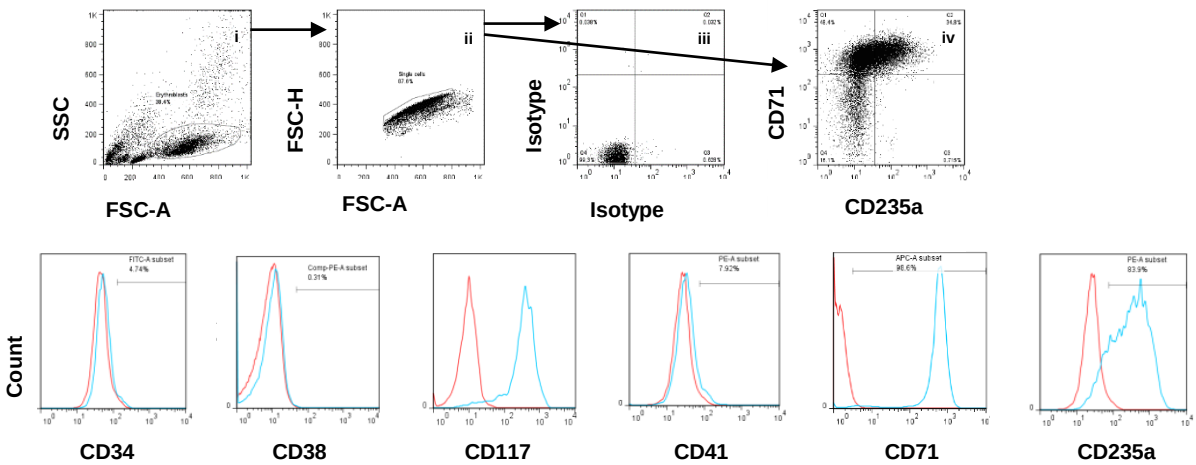

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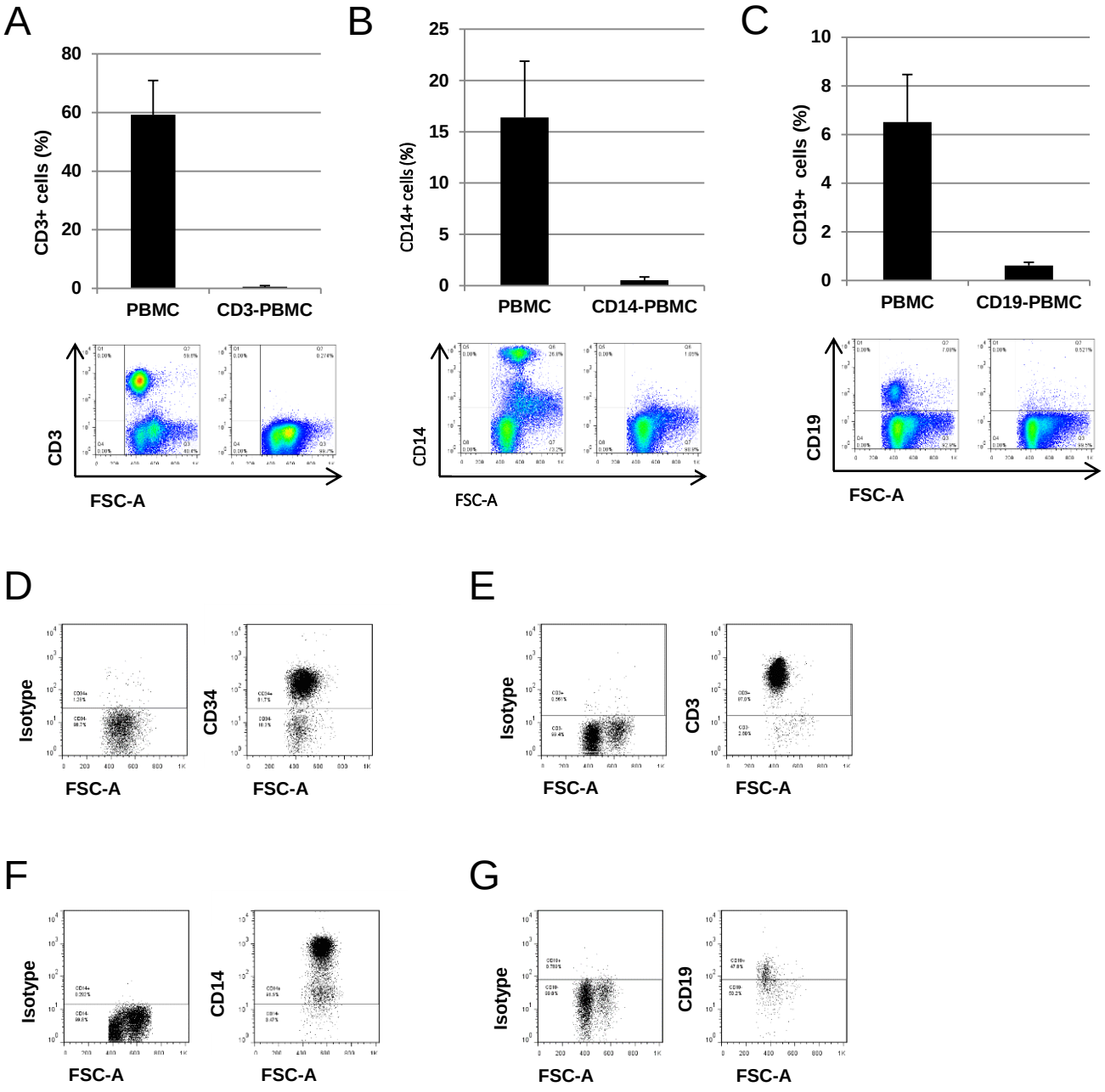

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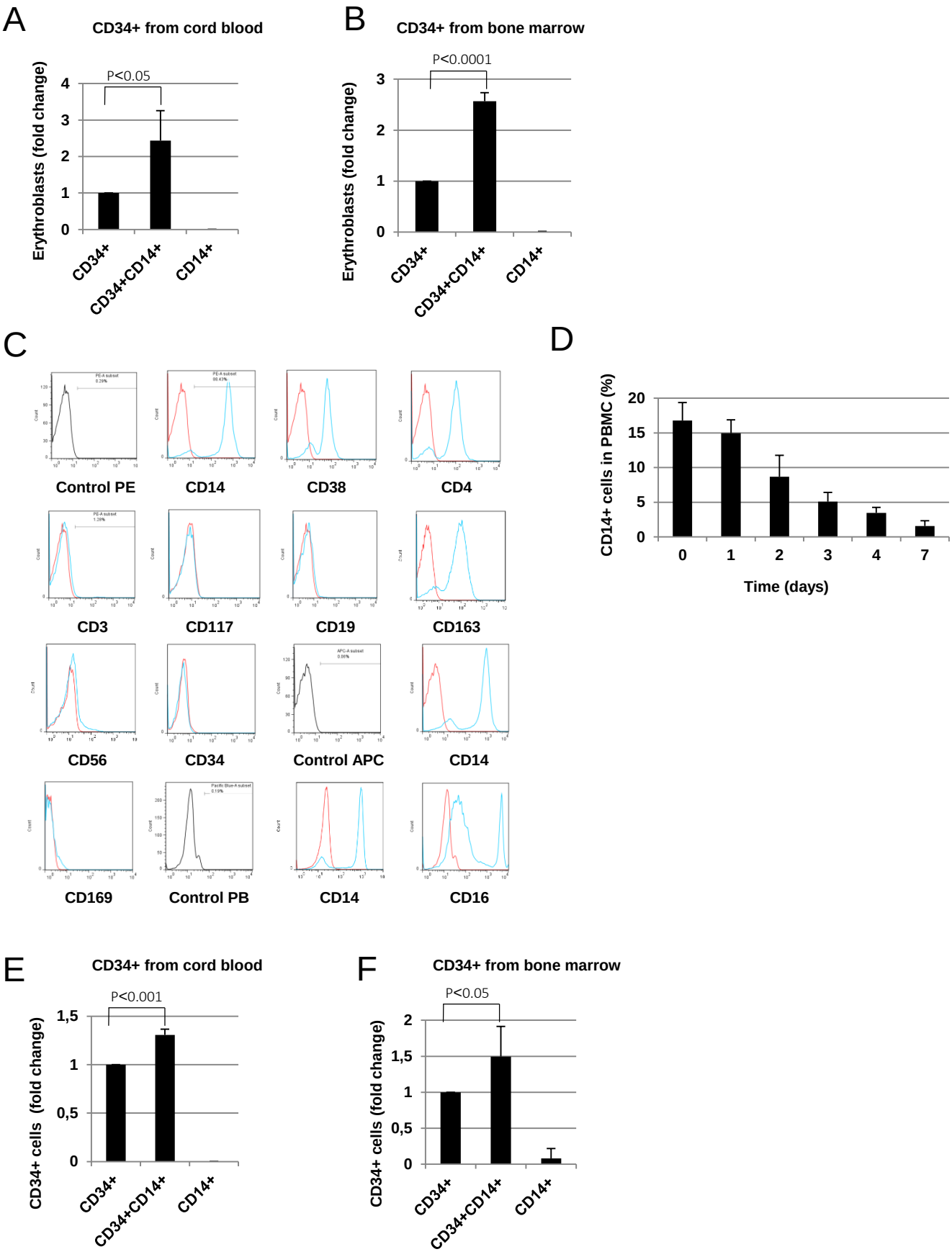

Online Supplementary Figure S5