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

Esther Heideveld,[1] Francesca Masiello,[2] Manuela Marra,[2] Fatemehsadat Esteghamat,[1] Nurcan Yağcı,[1] Marieke von Lindern,[1] Anna Rita F. Migliaccio,[2,3] and Emile van den Akker[1]

[1]Sanquin Research, Dept. of Hematopoiesis, and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, The Netherlands; [2]Department of Hematology, Oncology and Molecular Medicine, Istituto Superiore di Sanita, Rome, Italy; and [3]Division of Hematology and Medical Oncology, Mount Sinai School of Medicine and the Myeloproliferative Disorders Research Consortium, New York, NY, USA

ABSTRACT

Expansion of erythroblasts from human peripheral blood mononuclear cells is 4- to 15-fold more efficient than that of CD34+ cells purified from peripheral blood mononuclear cells. In addition, purified CD34+ and CD34- populations from blood do not reconstitute this erythroid yield, suggesting a role for feeder cells present in blood mononuclear cells that increase hematopoietic output. Immunodepleting peripheral blood mononuclear cells for CD14+ cells reduced hematopoietic stem and progenitor cell expansion. Conversely, the yield was increased upon co-culture of CD34+ cells with CD14+ cells (full contact or transwell assays) or CD34+ cells re-constituted in conditioned medium from CD14+ cells. In particular, CD14++CD16+ intermediate monocytes/macrophages enhanced erythroblast outgrowth from CD34+ cells. No effect of CD14+ cells on erythroblasts themselves was observed. However, 2 days of co-culturing CD34+ and CD14+ cells increased CD34+ cell numbers and colony-forming units 5-fold. Proliferation assays suggested that CD14+ cells sustain CD34+ cell survival but not proliferation. These data identify previously unrecognized erythroid and non-erythroid CD34- and CD34+ populations in blood that contribute to the erythroid yield. A flow cytometry panel containing CD34/CD36 can be used to follow specific stages during CD34+ differentiation to erythroblasts. We have shown modulation of hematopoietic stem and progenitor cell survival by CD14+ cells present in peripheral blood mononuclear cells which can also be found near specific hematopoietic niches in the bone marrow.

Introduction

Hematopoiesis occurs in niches that ensure specific interactions and cross-talk of hematopoietic cells with the surrounding stromal cells and among different hematopoietic cells themselves. These niches dictate processes such as lineage specification, cell survival and mobilization. Hematopoietic stem and progenitor cells (HSPC) reside in perivascular niches and within the non-endosteal parenchyma.[1-4] This hematopoietic niche consists of mesenchymal stem cells, osteoblasts, and hematopoietic effector cells, such as T regulatory cells and tissue-resident macrophages. The niche is important for hematopoietic stem cell (HSC) homeostasis as well as hematopoietic lineage development including erythropoiesis.[5] In mice, tissue-resident macrophages are important regulators of HSC retention within the bone marrow,[6,7] and ablation of CD163+CD169+ macrophages leads to mobilization of HSPC, committed progenitors[8] and erythrocyte precursors.[8] These myelodepleted mice experience compensated anemia with increased splenic erythroblasts. Increased erythrocyte survival in these mice is likely due to reduced phagocytosis of aging red cells by red pulp macrophages.

Central tissue-resident macrophages also contribute to the erythroid islands in the bone marrow (the erythron) which regulate erythroblast differentiation, the final stages of enucleation, and reticulocyte maturation.[9-12] However, macrophage colony-stimulating factor (M-CSF)-deficient *op/op* mice and *CSFR*$^{-/-}$ mice, which display reduced F4/80 tissue-resident macrophages, are characterized by hypocellularity of the bone marrow and reduced erythroid burst-forming units (BFU-E),[13] which indicates a major effect of macrophages on HSPC. In addition, erythroblast proliferation in bone marrow was unchanged in myeloablated mice and the ratios between the different stages during erythroblast maturation were comparable.[8] Together, these observations suggest a major role for (tissue-resident) macrophages in the control of proliferation and/or differentiation of HSPC, which needs to be investigated.

We have previously shown that erythroblast expansion from unsorted peripheral blood mononuclear cells (PBMC) is 4- to 15-fold increased compared to CD34+ cells purified from a similar number of PBMC.[14] This increased erythroid yield may partly be explained by the presence and differentiation of CD34- cells in peripheral blood.[14] However, erythroid differentiation from CD34- PBMC does not fully account for the increased erythroid yield from unselected PBMC. This suggests that additional processes underlie the increased erythroid yield from total PBMC compared to CD34+ cells isolated from PBMC. We hypothesize that PBMC with feeder/stromal cell-like properties can positively influence HSPC proliferation, differentiation, and/or survival to yield more erythroblasts. A role for these effector cells during *ex vivo* culture may also reveal clues to their function in the bone marrow niche.

 doi:10.3324/haematol.2015.125492
The online version of this article has a Supplementary Appendix.
Manuscript received on February 13, 2015. Manuscript accepted on August 12, 2015.
Correspondence: e.vandenakker@sanquin.nl

In this study we showed that human PBMC-derived CD14+ cells, in particular CD14++CD16+ intermediate monocytes/macrophages, increased the erythroid yield from CD34+ HSPC in co-culture experiments. Macrophages sustained HSPC that precede the erythroblast stage, which resulted in increased erythroid expansion from CD34+ cells in *ex vivo* cultures.

Methods

Cell sorting

CD3, CD19, CD14 and CD34 MicroBeads (Miltenyi Biotec; Bergisch Gladbach, Germany) were used for magnetic-activated cell sorting (MACS) from PBMC (manufacturer's protocol). Prior to sorting, monocytes/macrophages were purified from PBMC by counterflow centrifugal elutriation (JE-6B Beckman-Coulter centrifuge, Beckman Instruments Inc.; Palo Alto, CA, USA). Monocyte/macrophage subsets and hematopoietic precursors were sorted on a FACS-Aria II/III (BD Biosciences; Oxford, UK).

Cell culture

Human cells were cultured in StemSpan (Stem Cell Technologies; Grenoble, France) supplemented with stem cell factor (SCF; supernatant equivalent to 100 ng/mL), erythropoietin (2 U/mL, ProSpec; East Brunswick, NJ, USA), dexamethasone (1 μM, Sigma; St. Louis, MO, USA) and cholesterol-rich lipids (40 μg/mL, Sigma) as described elsewhere.[14,15] Informed consent was given in accordance with the Declaration of Helsinki and Dutch national and Sanquin internal ethic boards. Conditioned media were collected from CD14+ cells cultured for 2 days at 5-10x10^6 cells/8 mL, filtered (0.22 μm) and stored at 4°C. Isolated CD34+ cells were cultured with conditioned media diluted 1:2 with fresh culture medium. The media were replenished every 2 days.

Co-culture experiments

CD34+ cells were co-cultured with purified hematopoietic effector cells using ratios found in PBMC (1:100 CD14+ cells; 1:430 CD3+ cells and 1:25 CD19+ cells). CD34+ cells were co-cultured with CD14++CD16-, CD14++CD16+ or CD14+CD16+ cells (at a ratio of 1:100).

Transwell assays

CD14+ and CD34+ cells were seeded into transwells (0.4 μm polyester membrane, Corning; NY, USA) with CD34+ cells inside the transwell and CD14+ cells in the well (at a ratio of 1:100). Cells were analyzed after 2-8 days on the flow cytometer.

Colony assays

Colony assays were started with freshly purified, sorted, or cultured cells mixed with methacult (Medium ColonyGel™ Cell Systems; Troisdorf, Germany). After 14 days, total colony numbers and colony-forming units were manually scored twice using a wide-field microscope (Axiovert 200M, Carl Zeiss Inc.; Thornwood, NY, USA).

Proliferation assays

CD34+ cells were washed in phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA, Sigma; PSA). Cells were labeled with 0.5 μM carboxyfluoroscein succinimidyl ester (CFSE, Molecular Probes; Leiden, The Netherlands) for 8 min with gentle shaking at 37°C. Cells were washed in 1% PSA and cultured with or without CD14+ cells (at a ratio of 1:100) for 2 days. CFSE dilution was measured by flow cytometry.

Flow cytometry

Cells were washed in PBS and re-suspended in 1% PSA. Cells were incubated with primary antibodies for 30 min at 4°C, washed in PBS and measured on a FACS Canto II or LSR Fortessa (both BD Biosciences) and analyzed using FlowJo software (FlowJo v7.6.4/v10; Ashland, OR, USA). The antibodies are detailed in the *Online Supplementary Methods*. For apoptosis experiments, cells were stained in 100 μL binding buffer with annexin V-APC (BD Biosciences; 1:200) at 4°C. After 30 min, 100 μL binding buffer and propidium iodide (50 mg/mL; Sigma) were added.

Cytospins

Cells (5×10^5) were cytospun onto glass slides, fixed in methanol, and stained with May-Grünwald-Giemsa stain (manufacturer's protocol). Images were processed using Adobe Photoshop 9.0 (Adobe Systems Inc.; CA, USA).

Statistical analysis

The Student t-test was used for the statistical analyses. *P* values ≤0.05 are considered statistically significant.

Results

Hematopoietic cell fractions in peripheral blood mononuclear cells

The erythroid yield is significantly higher from total PBMC than from CD34+ cells purified from PBMC.[14] To investigate which cells contribute to this phenomenon we first examined the presence, frequency and erythroid potential of CD34+ and CD34- HSPC and their downstream precursor cells in PBMC. PBMC contained low numbers of CD34+ cells (0.16±0.08%; Figure 1A) which could be subdivided into 69% HSC/common myeloid progenitors (CMP), 29% granulocyte-monocyte progenitors (GMP) and 1% megakaryocyte-erythroid progenitors (MEP; Figure 1B and *Online Supplementary Figure S1*). Around 22% of the CD34+ cells consisted of CD34+CD38-CD36- long-term HSC (*Online Supplementary Figure S2A*). The erythroblasts expanded and differentiated from PBMC may potentially also originate from erythroblasts present in PBMC. We, therefore, evaluated the frequency of erythroblasts in PBMC. T cells (CD3) and monocytes/macrophages (CD14), further denoted as Lineage (Lin), were excluded from the analysis. Lin-CD34+CD36- (A; HSPC morphology), Lin-CD34+CD36+ (B; blast morphology), Lin-CD34-CD36+ and Lin-CD34-CD36^{dim} (C,D; blast, basophilic, orthochromatic, polychromatic, reticulocytes) cells were identified in PBMC (Figure 1C,D and Table 1). The Lin-CD34-CD36- population E consisted primarily of the remaining assorted cells (e.g. B cells, natural killer cells, CD34- HSC and other CD3-CD14-CD34-CD36- cells).

Supplementing lineage depletion with CD117 (c-kit) antibodies resulted in severe reductions of populations A and B indicating that the majority of these cells are CD117+ (Figure 1E). Supplementing lineage depletion with CD38 resulted in a loss of population D and reductions in populations A, B, C and E. Combining CD117 and CD38 in the lineage depletion resulted in complete loss of populations A, B and D and reductions in C and E (Figure 1E). These results suggest that the CD34+CD36+CD38+CD117+ population B is enriched for MEP. This was further confirmed through supplementing lineage depletion with

BAH1.1 which resulted in complete loss of population B and only a modest loss in population A (Figure 1E). Populations A-E were seeded in colony assays. Populations A and B contained the majority of the colony formation capacity, whereas populations C, D and E displayed low to no colony-forming capability (Table 1). This is in line with our previously published data showing that the CD34$^-$ population has severely limited colony-stimulating capacity.[14] Population A can be discriminated from B by its greater ability to generate granulocyte-monocyte colony-forming units (CFU-GM), indicating that population A is composed of more immature hematopoietic pro-

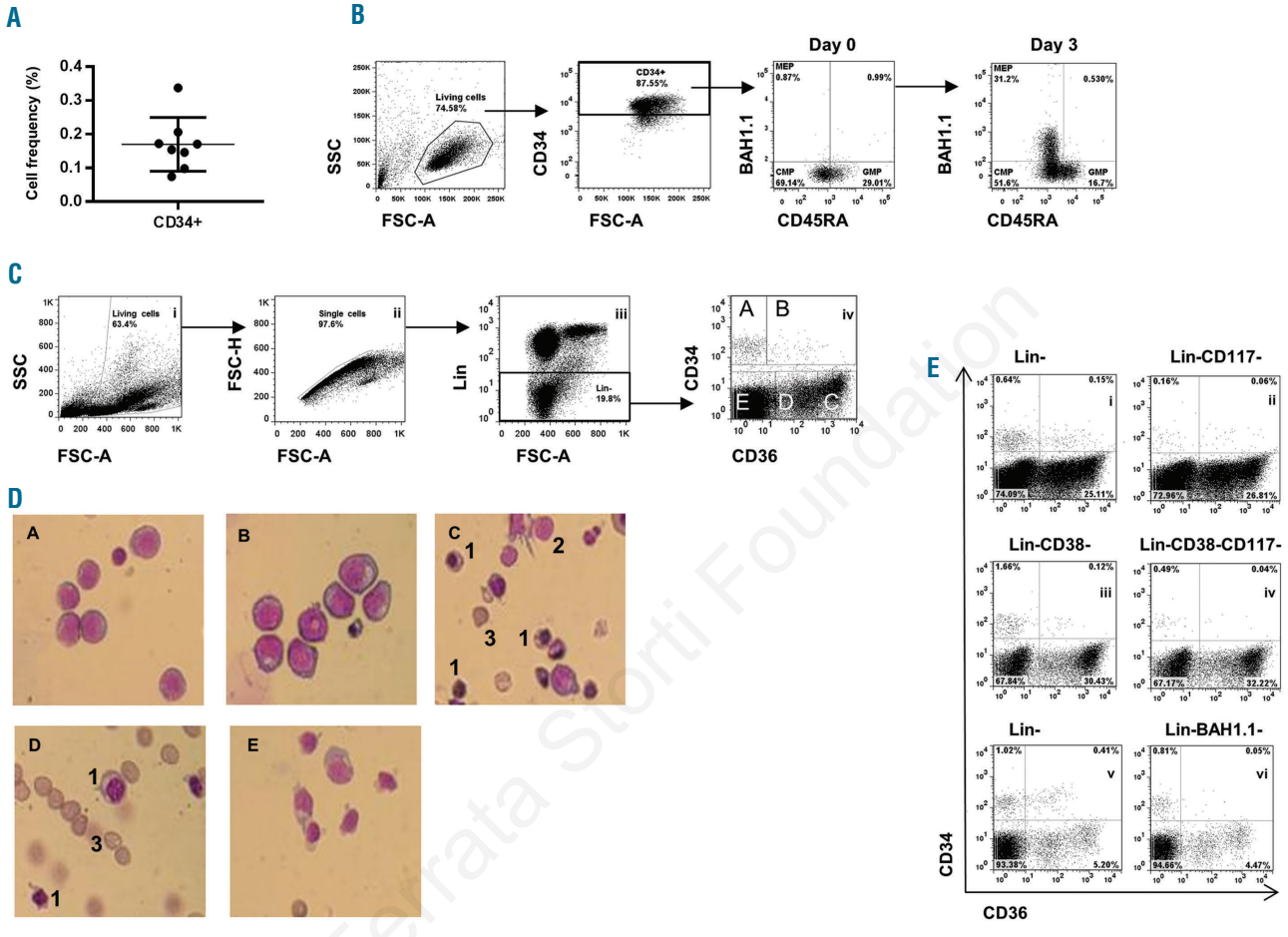

Figure 1. Defining hematopoietic progenitor cells and frequencies in peripheral blood mononuclear cells (PBMC). (A) The percentage of CD34$^+$ cells in PBMC measured using flow cytometry (n=8). (B) Representative dot plots of CD34$^+$ cells from panel A stained for CD45RA and BAH1.1 to discriminate between MEP (CD34$^+$BAH1.1$^+$CD45RA$^-$), GMP (CD34$^+$BAH1.1$^-$CD45RA$^+$) and CMP (CD34$^+$BAH1.1$^-$CD45RA$^-$) at day 0 and at day 3 of *ex vivo* culture. The low abundant MEP population was quantified using a specific MEP marker BAH1.1 (characterization in *Online Supplementary Figure S1*). (C) Gating strategy to show hematopoietic precursors according to CD34/CD36 expression. Live cells were gated (FSC/SSC; i), followed by gating of single cells (FSC-A/FSC-H; ii). A dump-channel containing CD14$^+$ and CD3$^+$ cells, henceforth denoted as Lin, was set up to exclude specific populations (iii). Lin-negative cells are depicted in a CD34/CD36 dot plot (iv) and five distinct populations are indicated (A-E; frequencies in Table 1). (D) May-Grünwald-Giemsa stained cytospins of FACS-sorted populations as indicated in (C) (iv). (E) Representative CD34/CD36 dot plot as indicated in (C) (i), supplementing the Lin-negative cells with anti-CD117 (ii), anti-CD38 (iii) or both anti-CD38/anti-CD117 (iv) or BAH1.1 (vi; control in v). FSC(-A/H): forward-scatter(-area/height); SSC: side-scatter; MEP: megakaryocyte-erythroid progenitor; CMP: common myeloid progenitor; GMP: granulocyte-monocyte progenitor; Lin: lineage.

Table 1. Frequency of the five populations identified in Figure 1C on the basis of CD34/CD36 staining in PBMC and respective colony formation capacity. Colonies shown per 500 cells. Results are presented as mean (±SD) of three to five separate experiments.

Population	Phenotype	Frequency	CFU-E	BFU-E	CFU-GM	CFU-GEMM	Total CFU
PBMC	N/A	N/A	0.75±0.03	0.5±0.3	0.2±0.1	0.006±0.005	0.8±0.7
A	CD34$^+$CD36$^-$	0.14±0.05	320±32	112±91	67±46	4±6	263±281
B	CD34$^+$CD36$^+$	0.04±0.05	272±16	152±130	11±14	4±5	303±306
C	CD34$^-$CD36$^+$	0.96±0.52	0.1±0.01	0.1±0.01	0.1±0.002	0.1±0.05	0.35±0.05
D	CD34$^-$CD36^{dim}	2.04±0.76	0	0	0	0	0
E	CD34$^-$CD36$^-$	83.5±20	0	0.1±0.1	0.02±0.03	0.001±0.002	0.1±0.1

PBMC: peripheral blood mononuclear cells; CFU: colony-forming units; BFU-E: erythroid burst-forming units; E: erythroid; GM: granulocyte-monocyte; GEMM: granulocyte-erythrocyte-monocyte-megakaryocyte.

genitors (e.g. HSC, CMP, GMP), whereas population B contains MEP committed to the megakaryocyte-erythroid lineage. In conclusion, the cells in population A are early HSPC, population B is composed of MEP and cells in transition to become erythroblasts. Populations C and D are mixed pools of cells that also contain erythroid cells at different stages.

Tracking hematopoietic stem and progenitor cell differentiation to erythroblasts

The Lin$^-$CD34/CD36 flow cytometer readout provides a novel tool to follow the differentiation of HSPC through the MEP stage to erythroblasts. CD34$^+$ cells were isolated from peripheral blood and cultured *ex vivo* for 8 days. Differentiation of CD34$^+$ cells was monitored daily by assessing Lin$^-$/CD34/CD36/CD71/CD235a membrane expression and *GATA1* and erythropoietin receptor (*EPOR*) mRNA expression during culture (Figure 2 and *Online Supplementary Figure S2B,C*). CD71 (transferrin receptor) and CD235a (glycophorin A; GPA) are more classical markers for erythroblasts and subsequent stages of differentiation towards erythrocytes,[16] but fail to identify the upstream progenitor progression of HSC towards erythroblasts. At day 0, cells are primarily CD34$^+$CD36$^-$ HSPC (P1) but progress towards CD34$^+$CD36$^+$ MEP (P2,3) during days 1-5, mirrored by an increase in *GATA1* mRNA levels. From day 5 to day 7, the cells finally become CD34$^-$CD36$^+$ erythroid cells (P4), accompanied by a 100-fold increase in *GATA1* and *EPOR* mRNA levels compared to levels in CD34$^+$ cells at day 0. CD71^{high}CD235a^{dim} erythroid cells

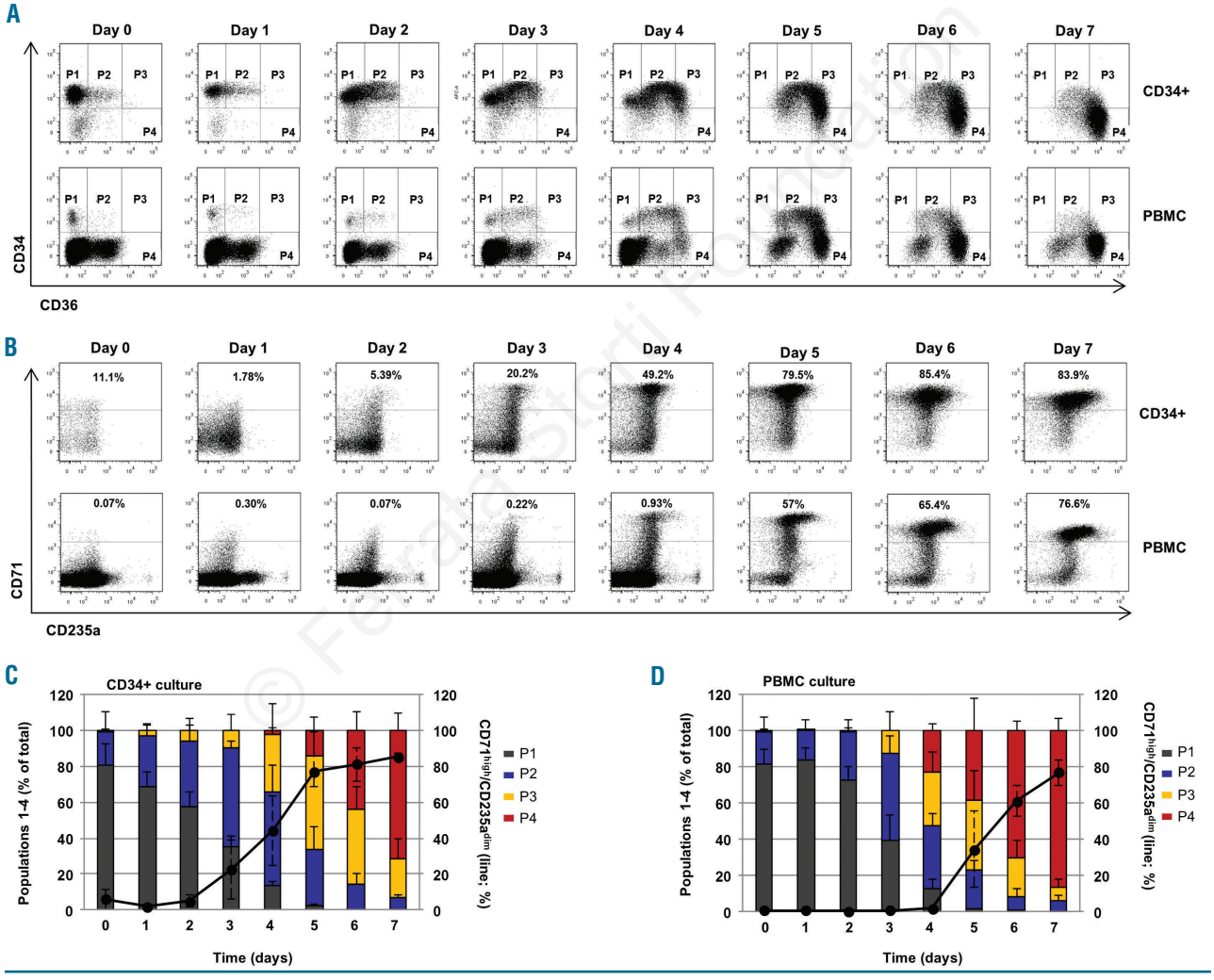

Figure 2. Tracking hematopoietic stem and progenitor cell (HSPC) differentiation to erythroblasts. CD34$^+$ cells purified from PBMC and total PBMC (n=3) were cultured for 7 days. (A) Dot plots depicting delineated CD34/CD36 populations from a representative donor over time (CD34$^+$ culture: top; total PBMC culture: bottom). The specific stages, hematopoietic stem and progenitor cells (HSPC; P1), megakaryocyte-erythroid progenitors (MEP; P2-P3) and erythroblasts (P4) are indicated within the dot plots. Note the progression from stage 1 to 4 during culture. (B) Dot plots depicting CD71/CD235a expression from CD34$^+$ cell (top panels) and total PBMC (bottom panels) cultures over 7 days. Note that CD71^{high}CD235a^{dim} cells appear gradually during culture. Histograms showing the percentage of cells in stage P1-4 of panel A for (C) CD34$^+$ cell cultures and (D) total PBMC cultures. The sum of the percentages within P1-4 is set to 100% to better visualize the relative changes during culture. The line depicts the percentage of CD71^{high}CD235a^{dim} erythroid cells in the total culture. Note that erythroid cells appear faster in the CD34$^+$ cell culture than in total PBMC cultures. This is because CD34$^+$ cell cultures do not contain any other cells then CD34$^+$ cells at the onset of culture in contrast to total PBMC, which contain large numbers of T cells, B cells and CD14$^+$ monocytes/macrophages during the first days of culture. However, these cells are outcompeted by the proliferating erythroid cells after 6-7 days (see also Figure 2B). PBMC: peripheral blood mononuclear cells.

start to appear after 4 days (Figure 2B,C) and are the predominant population by day 7. This coincides with the up-regulation of *GATA1* and *EPOR* mRNA and markedly increased protein levels of gata1 and the erythroid specific markers α/β-spectrin and band3 (SLC4A1; *Online Supplementary Figure S2B-D*). This pattern of marker expression is similar in cultures initiated from total PBMC, although the erythroid compartment is enhanced compared to that from CD34$^+$ cultures (Figure 2C,D). In conclusion, the Lin$^-$CD34/CD36 scheme allows sorting for specific populations of HSPC, MEP and erythroblasts but also for cells between these specific stages.

Expansion of erythroblasts from sorted peripheral blood mononuclear cell populations

Next, we investigated the contribution of each specific population in Figure 1C to the outgrowth of erythroid cells from total PBMC (Figure 3A,B). Populations A and B have the majority of the proliferative potential. Eight days after initiation of the cultures, the cells stained negative for CD34, CD38 and CD41, and positive for CD117, CD71 and CD235a (*Online Supplementary Figure S2E*), indicating a pure erythroblast population. To assess the relative contribution of each population to the erythroid expansion capacity of total PBMC, the absolute number of cells in each fraction was corrected for the frequency with which the initiating cells were present in PBMC (Table 1). The number of CD71^{high}CD235a^{dim} erythroblasts produced by each population at day 17 of culture was compared to the number of erythroblasts obtained from total PBMC (Figure 3B). The CD34$^+$ populations A and B accounted maximally for 43.6% of the total erythroid yield from PBMC, and the CD34$^-$ fraction accounted for 5.6% of the erythroid yield. A relatively small proportion (<3%) of this yield could be attributed to the Lin$^-$CD34$^-$CD36^{dim} population D in PBMC. Importantly, the total number of erythroblasts from populations A-E did not recapitulate the erythroid yield from total PBMC.

CD14$^+$ monocytes/macrophages enhance erythroblast expansion from CD34$^+$ cells

As we observed an increased yield of erythroblasts from total PBMC compared to CD34$^+$ cells, we hypothesized that PBMC contain a cell fraction that functions as ´helper/feeder cells´. PBMC mainly contained CD3$^+$ T cells, CD19$^+$ B cells and CD14$^+$ monocytes (Figure 4A). Depletion of CD14$^+$ cells, but not CD3$^+$ or CD19$^+$ cells, from PBMC consistently reduced expansion of CD71^{high}CD235a^{dim} erythroid cells by 35-40% (Figure 4B and *Online Supplementary Figure S3* for isolation/depletion efficiencies). The CD14$^+$ fraction did not contain cells with expansion capacity, which indicates that hematopoietic progenitors are not co-depleted with CD14$^+$ cells. Conversely, the addition of CD14$^+$ cells to CD34$^+$ cells increased the number of erythroid cells (Figure 4C). In addition, co-culture of PBMC-derived CD14$^+$ cells with CD34$^+$ cells isolated from cord blood or bone marrow also increased the erythroblast yield (*Online Supplementary Figure S4A,B*). PBMC-derived CD14$^+$ cells expressed CD16, CD4, CD38 and CD163, but not CD117, CD34, CD3 or CD19 (*Online Supplementary Figure S4C*). The addition of CD3$^+$ and CD19$^+$ cells only marginally affected outgrowth of erythroblasts from CD34$^+$ cells. Together, the data indicate that CD14$^+$ cells isolated from PBMC positively influence hematopoiesis/erythropoiesis.

CD14$^+$ monocytes/macrophages do not exert their effect on erythroblasts but increase the numbers of CD34$^+$ hematopoietic stem and progenitor cells during co-culture

CD14$^+$ cells persisted in culture until day 7 (*Online Supplementary Figure S4D*), at which time erythroblasts are emerging. CD14$^+$ cells could, therefore, elicit their effect on all stages from HSC to erythroblasts. To test this, CD14$^+$ cells were added to day 8 cultures of erythroblasts only; the expansion of erythroblasts was similar in the presence or absence of CD14$^+$ cells (Figure 5A).

To examine whether CD14$^+$ cells exert their effect on HSPC prior to the erythroblast stage, PBMC-derived CD14$^+$ and CD34$^+$ cells were co-cultured for 2 days. This resulted in a 5-fold increase in CD34$^+$ cell numbers compared to when CD34$^+$ cells were cultured alone (Figure 5B). In addition, CD34$^+$ cell numbers from cord blood or bone marrow were 1.5-fold increased in the presence of PBMC-derived CD14$^+$ cells (*Online Supplementary Figure S4E,F*). The CD14$^+$ cell fraction did not contribute to ery-

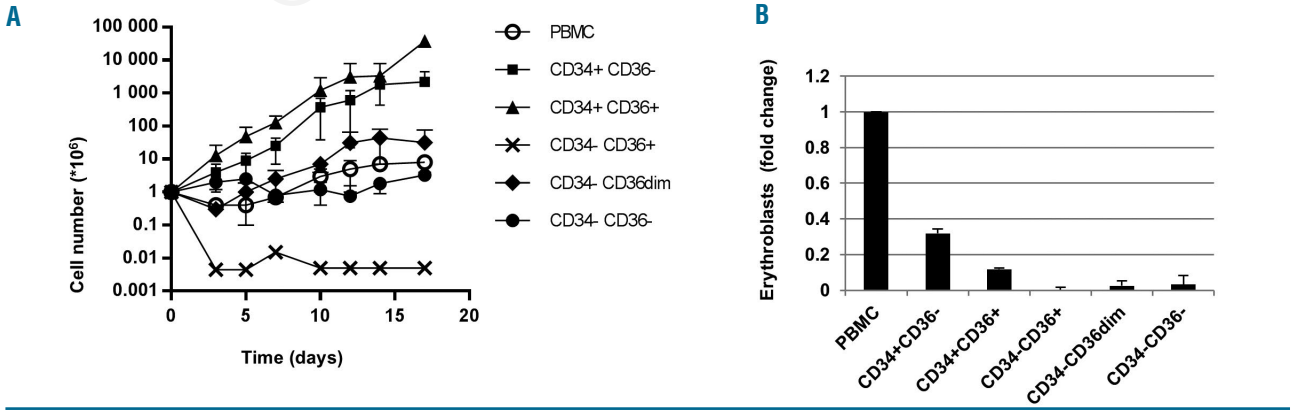

Figure 3. Cumulative yield of erythroblasts from sorted precursor populations present in PBMC is inferior to total PBMC erythroid yield. (A) One million cells of each population as defined in Figure 1C were cultured for 17 days. The growth curve depicts the cell number in millions as a function of time in days (n=5). (B) To normalize the yield observed in (A) to the frequencies of the individual populations observed in PBMC, the cell number at day 17 was multiplied by the frequencies presented in Table 1. The data are shown as fold change with respect to the erythroblast yield of PBMC (n=5). PBMC: peripheral blood mononuclear cells.

throid outgrowth nor did these cells become CD34+ (Figure 5B,E). Population A (Lin-CD34+CD36-) was sorted and cultured in the presence or absence of CD14+ cells for 2 days. Cell numbers increased 3-fold in population A and 5-fold in population B (Figure 5C). However, the ratio between A and B did not increase upon co-culture (*Online Supplementary Figure S5A,B*). These data suggest that proliferation and/or survival of the HSPC and MEP populations is affected by CD14+ cells. They predict that depletion of CD14+ cells from PBMC should decrease the total number of CD34+ cells during culture. Indeed, PBMC depleted of CD14+ cells resulted in decreases in populations A and B within 2 days (Figure 5D and *Online Supplementary Figure S5C*).

To determine whether co-culture is required or whether the effect is exerted immediately, we seeded CD34+ cells in colony assays in the presence or absence of CD14+ cells, and with or without 2 days of co-culture. Without co-culture, the presence or absence of CD14+ cells did not increase colony numbers. However, co-culture of CD34+ with CD14+ cells resulted in a 5-fold increase in hematopoietic colony formation compared to CD34+ cell cultures or cells seeded in colony assays at the initiation of culture (Figure 5E). Note that CD14+ cells do not form any colonies and that co-cultures of CD34+ with CD14+ cells yielded the same number of colonies as 2-day cultures of total PBMC. CD14+ cells may increase CD34+ cell numbers in the erythroid biased culture conditions through: (i) enhanced lineage specification; (ii) induction of cell survival; or (iii) promotion of proliferation. To examine an effect on lineage specification the number of CFU-GM (myeloid), granulocyte-erythrocyte-monocyte-megakaryocyte colony-forming units (CFU-GEMM; mixed) and BFU-E (erythroid) colony-forming cells was determined. The overall distribution of CFU-GM, CFU-GEMM and BFU-E after 2 days of co-culture with CD14+ cells was not biased towards erythropoiesis (Figure 5F). This suggests that CD14+ cells do not push CD34+ cells towards a lineage, but affect the overall yield of HSPC.

The effect of CD14+ cells is independent of cell contact, in particular CD14++CD16+ intermediate monocytes/macrophages enhance erythroblast expansion

To investigate whether CD14+ cells increase proliferation of CD34+ cells, the CD34+ cells were loaded with CFSE and CFSE dilution was measured during 2 days of culture. Both in the presence and absence of CD14+ cells, the CFSE signal in CD34+ cells was reduced to similar levels, indicating comparable numbers of cell divisions (Figure 6A). When lineage specification and proliferation are similar, CD14+ cells most likely enhance survival of CD34+ cells. Indeed, the presence of CD14+ cells decreased apoptosis of CD34+ cells (Figure 6B). However, this was not regulated via B-cell CLL/lymphoma2 (*BCL2*) or BCL2-like 1 (*BCL2L1*) anti-apoptotic signaling pathways as mRNA levels of these proteins were unchanged in co-culture conditions compared to the levels in CD34+ cultures alone (*Online Supplementary Figure S5D,E*).

Monocytes/macrophages express various integrins, membrane-bound ligands of integrins, and other sequestering membrane proteins. These molecules facilitate macrophage functions in innate immune responses, and regulate cell-cell contact-mediated signal transduction in CD14+ and associated cells. We hypothesized that cell contact may be important for CD14+ cells to exert their effect on CD34+ cells. However, a dose-dependent effect of the number of CD14+ cells in a transwell assay supported the involvement of secreted factors rather than cell-cell contact (Figure 6C). These experiments did not discriminate between an exclusive effect of CD14+ cells or an interplay between secreted factors by CD34+ cells that regulate the

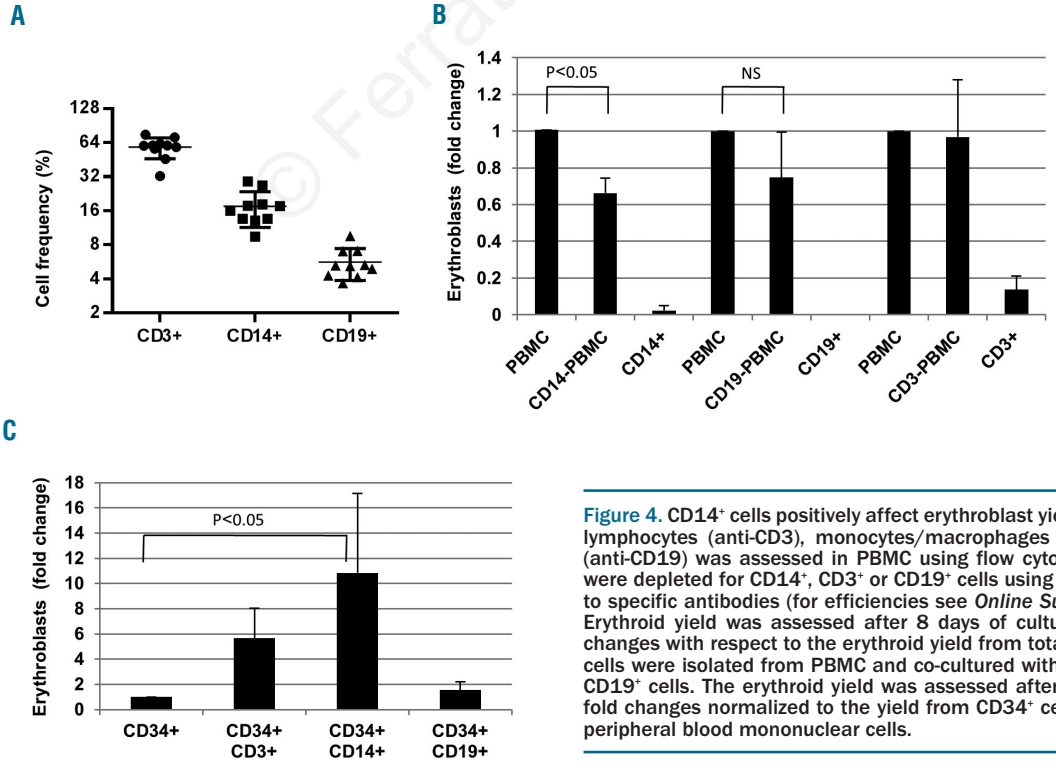

Figure 4. CD14+ cells positively affect erythroblast yield. (A) The percentage of lymphocytes (anti-CD3), monocytes/macrophages (anti-CD14) and B cells (anti-CD19) was assessed in PBMC using flow cytometry (n=10). (B) PBMC were depleted for CD14+, CD3+ or CD19+ cells using magnetic beads coupled to specific antibodies (for efficiencies see *Online Supplementary Figure S3*). Erythroid yield was assessed after 8 days of culture and depicted as fold changes with respect to the erythroid yield from total PBMC (n=3). (C) CD34+ cells were isolated from PBMC and co-cultured with isolated CD3+, CD14+ or CD19+ cells. The erythroid yield was assessed after 8 days and depicted as fold changes normalized to the yield from CD34+ cells alone (n=3-6). PBMC: peripheral blood mononuclear cells.

factor secretion profile of CD14+ cells. To investigate this, conditioned media from cultured CD14+ cells were added to cultures of isolated CD34+ cells. Similar to CD14+ cells, supernatant increased expansion of erythroid cells, suggesting that the spectrum of factors secreted by CD14+ cells is not influenced by CD34+ cell-induced signaling. Of note, the reduced erythroid yield of the conditioned media (Figure 6D) compared to the transwell assays (Figure 6C) is probably explained by: (i) the half-life of the secreted factors in the conditioned media, rather than continuously replenished factors in the transwell setting; and (ii) the dilution of the supernatant 1:2 with fresh culture medium. Together, these results show that CD14+ cells exert their effect on HSPC primarily through secreted factors.

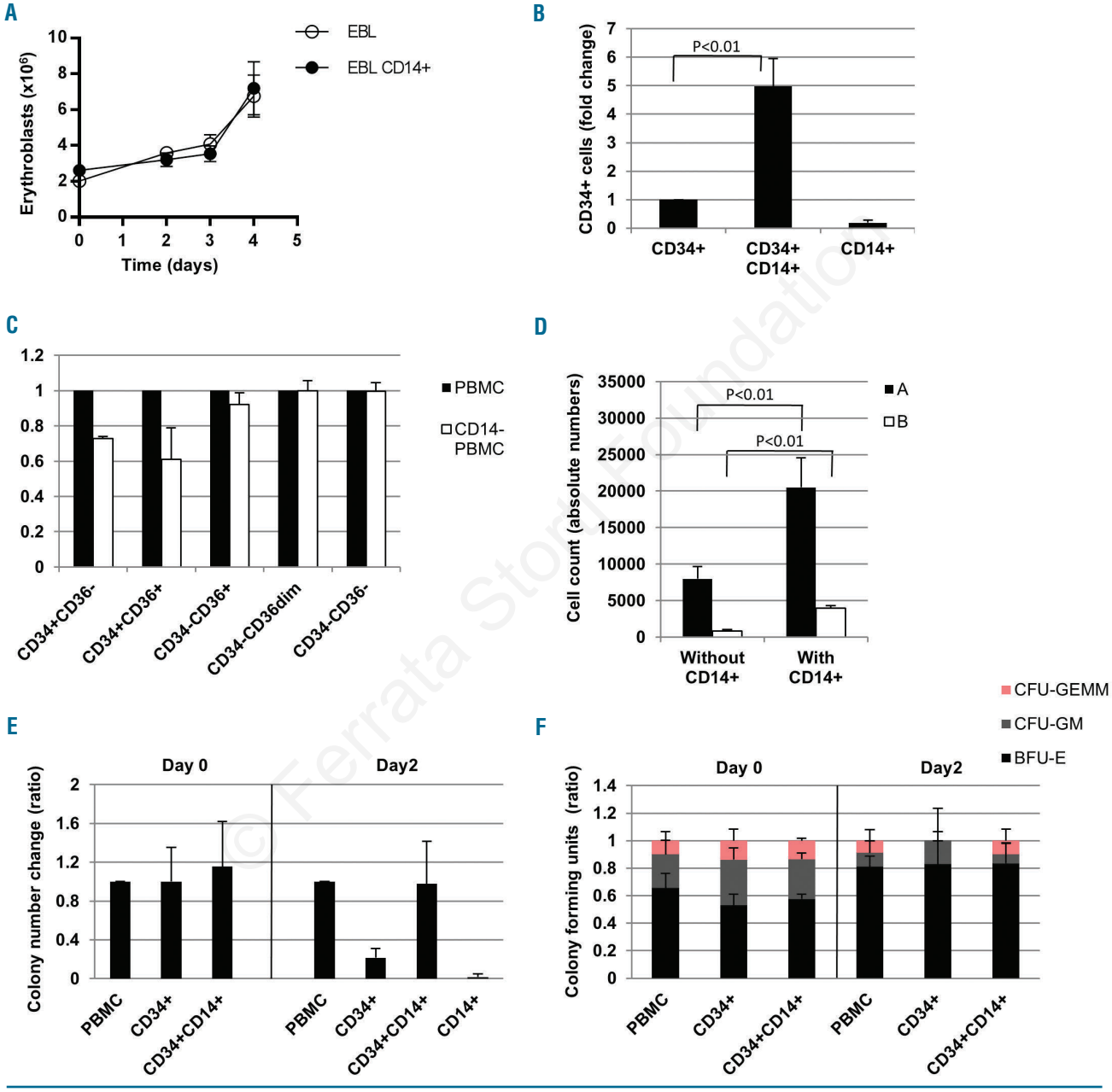

Figure 5. CD14+ cells do not affect erythroblasts, however they positively influence hematopoietic stem and progenitor cells (HSPC). (A) Erythroblasts were expanded from PBMC and were cultured with or without CD14+ cells for 4 additional days (n=6). (B) CD34+ cells were cultured in the presence or absence of CD14+ cells for 2 days. CD34+ cell numbers were assessed by multiplying the percentage of CD34+ cells by total cell counts. The data are depicted as fold changes normalized to CD34+ cell numbers of CD34+ cell cultures (n=4). Note the absence of CD34+ cells in the CD14+-only condition. (C) Population A, as defined in Figure 1C, was FACS-sorted from CD34+ cells and cultured for 2 days with or without CD14+ cells. Populations A and B were quantified and absolute counts are displayed (n=3). (D) PBMC depleted for CD14+ cells were cultured for 2 days. CD34/CD36 populations were delineated and normalized to total PBMC. Note the reduction in population A and B when CD14+ cells were absent (n=3). (E-F) Total PBMC, CD34+ cells and CD34+ with CD14+ cells were subjected to colony assays at initiation (day 0) and after 2 days of culture (day 2). Total colony numbers were normalized to colony numbers from total PBMC (n=4) (E). Quantification of colony-forming units. Total colony number is set to 1 to visualize any bias towards a specific hematopoietic lineage (n=4) (F). EBL: erythroblasts; PBMC: peripheral blood mononuclear cells; CFU: colony-forming units; GEMM: granulocyte-erythrocyte-monocyte-megakaryocyte; GM: granulocyte-monocyte; BFU-E: erythroid burst-forming units.

CD14+ monocytes/macrophages from PBMC can be subdivided into three populations based on CD14 and CD16 expression: classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16+) monocytes/macrophages (Figure 7A).[17] Each subpopulation has its own functional and molecular characteristics.[18-20] To investigate whether a specific subset is responsible for the increased erythroid yield, CD14++CD16- (P1), CD14++CD16+ (P2) or CD14+CD16+ (P3) subpopulations were sorted and co-cultured with CD34+ cells (Figure 7B). Of note, the percentages of the specific subsets in PBMC reflect literature values (Figure 7A). Co-culture of CD14++CD16+ cells (P2) or CD14++CD16- cells (P1) with CD34+ cells resulted in 5-fold and 3-fold increases in erythroblast yield, respectively, compared to culture of CD34+ cells alone (Figure 7B). Co-culturing CD34+ cells with CD14+CD16+ cells (P3) led to only a modest increase in erythroid yield, indicating that this population is not involved. Additional analysis showed that CD14++CD16+ intermediate cells (P2) can be further discriminated from classical and non-classical cells by the combination of CXCR4, CD163, CD169 (Figure 7C) and HLA-DR (*data not shown*). All peripheral CD14+ cells are negative for the M2 macrophage marker CD206 (mannose receptor). CD14+ cells cultured for 2 days showed differentiation towards a CD14++CD16+CD206+ macrophage population that also

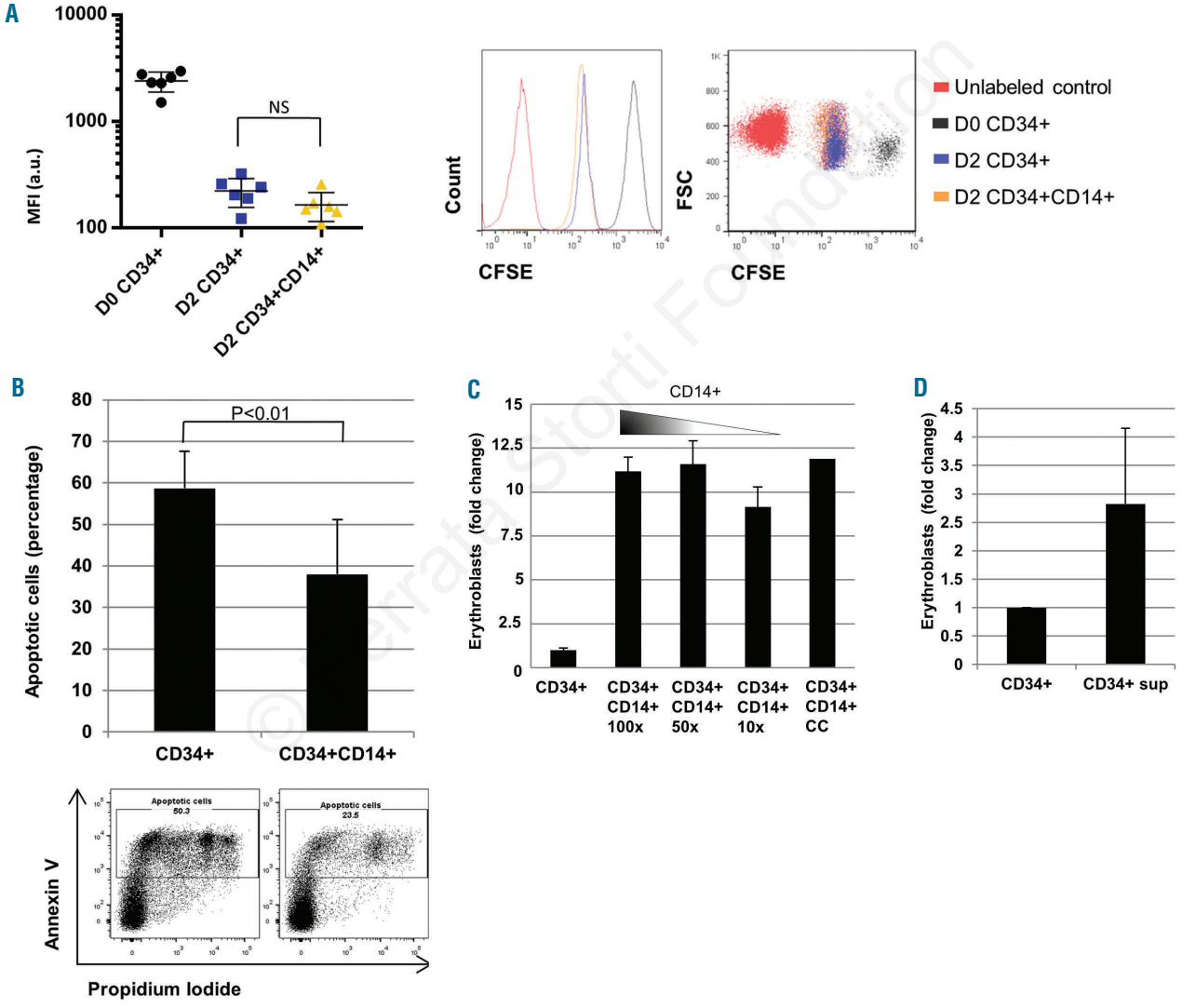

Figure 6. CD14+ cells affect CD34+ cell survival by secreted factors. (A) PBMC-derived CD34+ cells were loaded with CFSE and cultured for 2 days with or without CD14+ cells. The CFSE dilution is displayed as arbitrary units (a.u.) of mean fluorescence intensity (MFI) (n=6). The histogram and dot plot show a representative experiment. (B) CD34+ cells were isolated from PBMC and cultured in a transwell assay with or without CD14+ cells. After 2 days, apoptosis was measured by assaying the cumulative number of annexinV/propidium iodide-positive cells, which is displayed as the percentage of apoptotic cells in the bar graph (n=4). Note the high baseline rate of apoptosis (60%) of CD34+ short-term cultures on erythropoietin, stem cell factor and dexamethasone as previously described.[14] The dot plots depict a representative experiment. (C) PBMC-derived CD34+ cells were cultured with or without CD14+ cells in a transwell assay preventing cell-cell contact (using various concentrations of CD14+ cells) or in full contact co-culture (CC). The bar diagram displays the erythroid yield after 8 days of culture normalized to the erythroid yield ($CD71^{high}CD235a^{dim}$) in CD34+ conditions without any CD14+ cells (n=4). (D) CD34+ cells isolated from PBMC were cultured for 8 days in conditioned medium as described in the Methods. The erythroid yield was normalized to the erythroid yield observed in CD34+ cells (n=3). MFI: mean fluorescence intensity; CFSE: carboxyfluorescein succinimidyl ester; FSC: forward-scatter; CC: co-culture.

upregulate tissue residency markers like CXCR4, CD163 and CD169 (Figure 7C and *Online Supplementary Table 1*). Note that CD16 expression is similar to intermediate monocytes freshly isolated from peripheral blood. Low to no expression of CD209 was observed thereby excluding the presence of dendritic cells (*data not shown*).

Discussion

Survival and proliferation of HSPC is crucial to prevent bone marrow failure *in vivo*, and to enable expansion of HSPC for cellular therapy *in vitro*. For future transfusion medicine, the *in vitro* production of erythrocytes is the

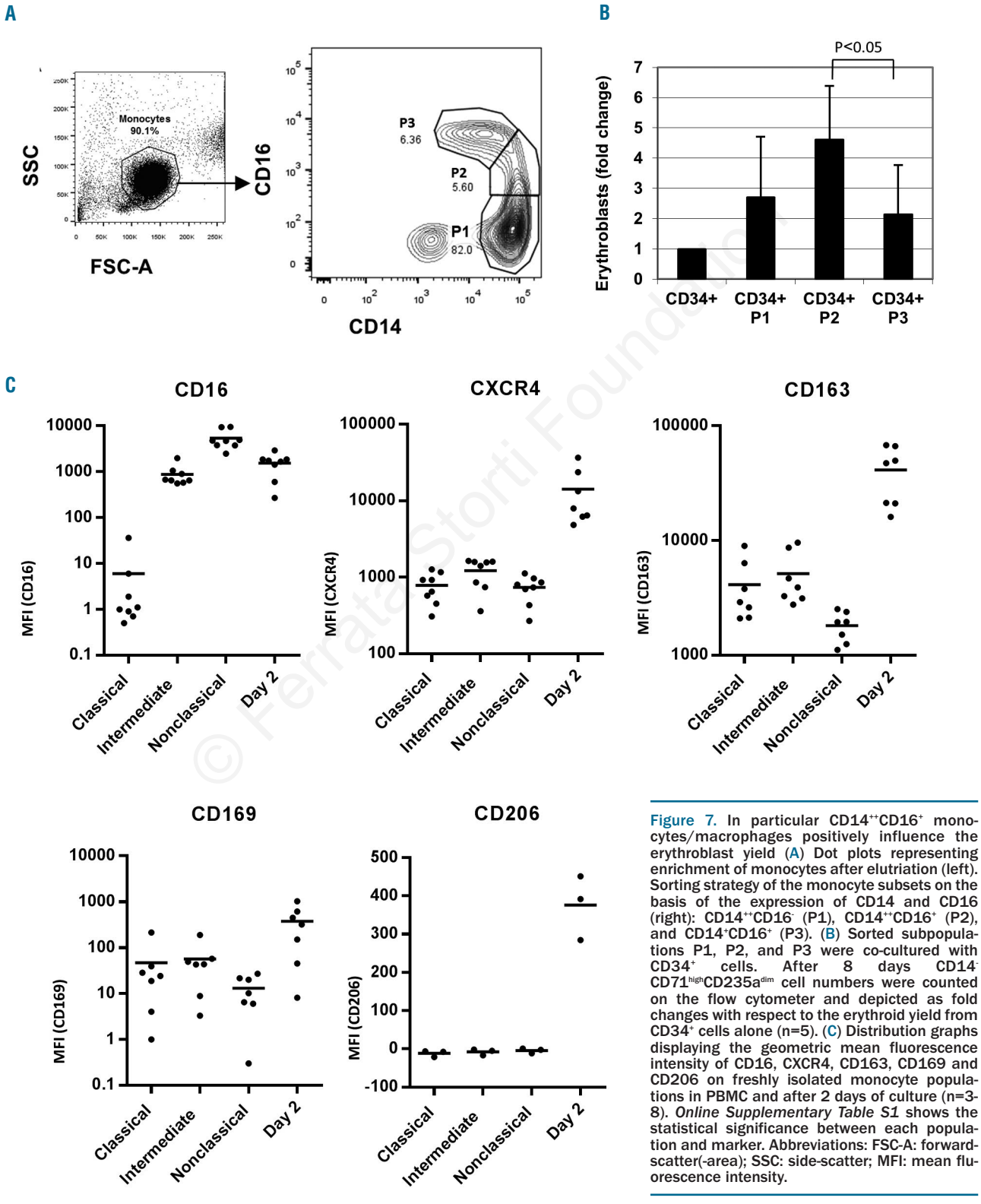

Figure 7. In particular $CD14^{++}CD16^{+}$ monocytes/macrophages positively influence the erythroblast yield (A) Dot plots representing enrichment of monocytes after elutriation (left). Sorting strategy of the monocyte subsets on the basis of the expression of CD14 and CD16 (right): $CD14^{++}CD16^{-}$ (P1), $CD14^{++}CD16^{+}$ (P2), and $CD14^{+}CD16^{+}$ (P3). (B) Sorted subpopulations P1, P2, and P3 were co-cultured with $CD34^{+}$ cells. After 8 days $CD14^{-}$ $CD71^{high}CD235a^{dim}$ cell numbers were counted on the flow cytometer and depicted as fold changes with respect to the erythroid yield from $CD34^{+}$ cells alone (n=5). (C) Distribution graphs displaying the geometric mean fluorescence intensity of CD16, CXCR4, CD163, CD169 and CD206 on freshly isolated monocyte populations in PBMC and after 2 days of culture (n=3-8). *Online Supplementary Table S1* shows the statistical significance between each population and marker. Abbreviations: FSC-A: forward-scatter(-area); SSC: side-scatter; MFI: mean fluorescence intensity.

Holy Grail to provide anemic patients with fully matched erythrocytes, which requires a maximum expansion capacity during all stages of erythropoiesis. We previously found that total PBMC cultures display a better expansion of erythroblasts compared to CD34+ cells isolated from a similar amount of PBMC.[14] Unraveling the mechanisms that drive this process is important to optimize *in vitro* erythroid expansion protocols and potentially unlocks clues of CD34+ cell homeostasis. Here, we show that CD14+ monocytes/macrophages in PBMC secrete factors that enhance the expansion of CD34+ HSPC. In particular, it is the CD14++CD16+ subset of intermediate monocytes/macrophages[17] that is involved. Interestingly, they affect early CD34+CD36- HSPC and CD34+CD36+ MEP but not erythroblasts. Besides erythroid expansion from CD34+ cells, also myeloid colony forming potential increased following co-culture of CD34+ cells with CD14+ monocytes/macrophages. Analysis of cell divisions using CFSE labeling showed similar proliferation capacity of CD34+ cells in culture with or without CD14+ cells, indicating that the increased erythroid yield is through activation of survival pathways.

Tissue-resident macrophages have been found in hematopoietic niches near the HSC where they provide retention signals to HSPC.[6] Depletion of macrophages in mice reduces erythropoiesis not because of impaired erythroblast proliferation *per se*, but rather as a result of HSPC and erythroblast mobilization.[8] This is in agreement with our data showing that CD14+ cells enhance the number of CD34+ HSPC but not the proliferation rate of human erythroblasts. We showed that CD14+ cells do not alter lineage commitment or proliferation. Instead, we show that CD14+ cells support CD34+ cell survival, although this remains to be proven *in vivo*. Reduced BFU-E formation and hypocellularity of the bone marrow was observed in M-CSF deficient *op/op* mice and *CSFR*-/- mice which are characterized by reduced F4/80 tissue-resident macrophages.[13] The combination of retention signals and survival signals could explain the hematopoietic phenotypes observed in *op/op* mice and underscores that macrophages play a role in hematopoietic/erythropoietic homeostasis. Thus, besides being involved in the retention of HSPC in the bone marrow, we discovered a potential additional role for macrophages in the survival of CD34+ HSPC during differentiation. An important remaining question is whether survival and retention of CD34+ cells by the CD14+ monocytes/macrophages are the same, or originate from independent signal transduction pathways. We observed that the increased survival of CD34+ cells and subsequent yield of erythroblasts is caused by a secreted factor, whilst the retention of CD34+ HSPC in the bone marrow was dependent on cell contact through VCAM-1 (CD106).[6] This favors a model in which these two macrophage functions can be separated.

CD14+ cells in peripheral blood are a mixed population of cells that includes phenotypes resembling bone marrow tissue-resident macrophages (CD14+CD163+CD169+).[6,8] We find that these cells are present in the intermediate monocyte population in PBMC. This population also displays the best HSPC support during co-culture experiments. During (co-)culture these monocytes differentiate towards CD206+ macrophages that further upregulate tissue resident markers like CXCR4, CD163, CD169 resembling tissue resident M2 macrophages.[21,22] It is important to compare this specific subset and their similarity to bone marrow isolated resident macrophages. The observed effects are caused by secreted factors and hence the identity of these growth factors is of paramount importance to optimize *in vitro* erythropoiesis and to better understand HSPC survival in the hematopoietic niches. At present, hematopoietic stem cell transplantation is performed with total mobilized PBMC rather than purified CD34+ HSPC from PBMC or bone marrow. However, the effect of different hematopoietic effector cells on HSPC engraftment is unclear. As we found that CD14+ cells promote survival of CD34+ HSPC, a putative beneficial effect of CD14+ cells in HSPC survival after engraftment is possible.

Analysis of total peripheral blood and PBMC-derived CD34+ cell cultures on the basis of lineage depletion (CD3/CD14) and CD34/CD36 during time, revealed a gradual disappearance of CD34 and appearance of CD36 during HSPC differentiation as recently observed for cord blood CD34+ cells.[23] Colony assays confirmed that CD34+CD36- cells were HSPC, CD34+CD36+ cells were highly enriched for MEP and CD34-CD36+ cells were erythroblasts. This Lin-CD34/CD36 staining provides a novel tool to follow HSPC differentiation towards erythroblasts and enables sorting of specific differentiation intermediates. Subsequently, the erythroblasts can be further analyzed during differentiation to enucleated reticulocytes using previously described protocols.[16,24] Here, we found that CD14+ cells provide support to HSPC cells leading to increased survival of CD34+ cells. The observations explain the increased erythroid expansion from total PBMC compared to CD34+ cells isolated from a similar amount of PBMC and unravel a novel role for CD14+ cells in survival of hematopoietic progenitors. Furthermore, we designed a novel tool to follow specific stages during CD34+ differentiation to erythroblasts in adult erythropoiesis using a Lin- CD34/CD36 flow cytometry panel.

Acknowledgments

This study was supported by funding from the Landsteiner Foundation for Blood Transfusion Research Landsteiner (EvdA, EH, FE; LSBR: 1141), Sanquin (NY, MvL, EvdA; PPOC: 12-025), NHLBI (AM; RFA on terminal erythroid maturation HL116329-01) and the National Cancer Institute (AM; P01-CA108671), Centro Nazionale Sangue and Associazione Italiana Ricerca sul Cancro (FM, MM; AIRC). We would like to thank Erik Mul (Sanquin Central Facility) and Gijs van Schijndel (Immunopathology; Sanquin Research) for technical assistance regarding the isolation of monocytes/macrophages from PBMC by elutriation and FACS sorting and Thamar van Dijk (Erasmus Medical Center, Rotterdam, The Netherlands) for providing gata1 antibodies.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Kiel MJ, Iwashita T, Yilmaz OH, Morrison SJ. Spatial differences in hematopoiesis but not in stem cells indicate a lack of regional patterning in definitive hematopoietic stem cells. Dev Biol. 2005;283(1):29-39.
2. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity. 2006;25(6):977-988.
3. Lo Celso C, Wu JW, Lin CP. In vivo imaging of hematopoietic stem cells and their microenvironment. J Biophotonics. 2009;2 (11):619-631.
4. Mendez-Ferrer S, Michurina TV, Ferraro F, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature. 2010;466(7308):829-834.
5. Nwajei F, Konopleva M. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. Adv Hematol. 2013;2013:953982.
6. Chow A, Lucas D, Hidalgo A, et al. Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. J Exp Med. 2011;208(2):261-271.
7. Winkler IG, Sims NA, Pettit AR, et al. Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. Blood. 2010;116(23):4815-4828.
8. Chow A, Huggins M, Ahmed J, et al. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. Nat Med. 2013;19(4):429-436.
9. Mohandas N, Prenant M. Three-dimensional model of bone marrow. Blood. 1978; 51(4):633-643.
10. Lee G, Lo A, Short SA, et al. Targeted gene deletion demonstrates that the cell adhesion molecule ICAM-4 is critical for erythroblastic island formation. Blood. 2006;108(6): 2064-2071.
11. Rhodes MM, Kopsombut P, Bondurant MC, Price JO, Koury MJ. Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. Blood. 2008;111(3):1700-1708.
12. Hanspal M, Golan DE, Smockova Y, et al. Temporal synthesis of band 3 oligomers during terminal maturation of mouse erythroblasts. Dimers and tetramers exist in the membrane as preformed stable species. Blood. 1998;92(1):329-338.
13. Dai XM, Ryan GR, Hapel AJ, et al. Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects. Blood. 2002;99(1):111-120.
14. van den Akker E, Satchwell TJ, Pellegrin S, Daniels G, Toye AM. The majority of the in vitro erythroid expansion potential resides in CD34- cells, outweighing the contribution of CD34+ cells and significantly increasing the erythroblast yield from peripheral blood samples. Haematologica. 2010;95(9):1594-1598.
15. Migliaccio G, Sanchez M, Masiello F, et al. Humanized culture medium for clinical expansion of human erythroblasts. Cell Transplant. 2010;19(4):453-469.
16. Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. Blood. 2001;98(12):3261-3273.
17. Ziegler-Heitbrock L, Ancuta P, Crowe S, et al. Nomenclature of monocytes and dendritic cells in blood. Blood. 2010;116(16):e74-80.
18. Zawada AM, Rogacev KS, Rotter B, et al. SuperSAGE evidence for CD14++CD16+ monocytes as a third monocyte subset. Blood. 2011;118(12):e50-61.
19. Schmidl C, Renner K, Peter K, et al. Transcription and enhancer profiling in human monocyte subsets. Blood. 2014;123 (17):e90-99.
20. Wong KL, Tai JJ, Wong WC, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood. 2011;118(5):e16-31.
21. Moestrup SK, Moller HJ. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. Ann Med. 2004;36(5):347-354.
22. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. J Immunol. 2006;177(10):7303-7311.
23. Li J, Hale J, Bhagia P, et al. Isolation and transcriptome analyses of human erythroid progenitors: BFU-E and CFU-E. Blood. 2014;124(24):3636-3645.
24. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci USA. 2009;106(41):17413-17418.