Glucocorticoids induce differentiation of monocytes towards macrophages that share functional and phenotypical aspects with erythroblastic island macrophages

Esther Heideveld,[1] Lea A. Hampton-O'Neil,[2] Stephen J. Cross,[3] Floris P.J. van Alphen,[4] Maartje van den Biggelaar,[4,5] Ashley M. Toye[2,6,7] and Emile van den Akker[1]

[1]Sanquin Research, Department of Hematopoiesis, Amsterdam and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, the Netherlands; [2]Department of Biochemistry, School of Medical Sciences, Bristol, UK; [3]Wolfson Bioimaging Facility, School of Medical Sciences, Bristol, UK; [4]Sanquin Research, Department of Research Facilities, Amsterdam, the Netherlands; [5]Sanquin Research, Department of Plasma Proteins, Amsterdam, the Netherlands [6]Bristol Institute for Transfusion Sciences, NHS Blood and Transplant, Filton, Bristol, UK and [7]National Institute for Health Research (NIHR) Blood and Transplant Research Unit in Red Blood Cell Products, University of Bristol, UK

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ABSTRACT

The classical central macrophage found in erythroblastic islands plays an important role in erythroblast differentiation, proliferation and enucleation in the bone marrow. Convenient human *in vitro* models to facilitate the study of erythroid-macrophage interactions are desired. Recently, we demonstrated that cultured monocytes/macrophages enhance *in vitro* erythropoiesis by supporting hematopoietic stem and progenitor cell survival. Herein, we describe that these specific macrophages also support erythropoiesis. Human monocytes cultured in serum-free media supplemented with stem cell factor, erythropoietin, lipids and dexamethasone differentiate towards macrophages expressing CD16, CD163, CD169, CD206, CXCR4 and the phagocytic TAM-receptor family. Phenotypically, they resemble both human bone marrow and fetal liver resident macrophages. This differentiation is dependent on glucocorticoid receptor activation. Proteomic studies confirm that glucocorticoid receptor activation differentiates monocytes to anti-inflammatory tissue macrophages with a M2 phenotype, termed GC-macrophages. Proteins involved in migration, tissue residence and signal transduction/receptor activity are upregulated whilst lysosome and hydrolase activity GO-categories are downregulated. Functionally, we demonstrate that GC-macrophages are highly mobile and can interact to form clusters with erythroid cells of all differentiation stages and phagocytose the expelled nuclei, recapitulating aspects of erythroblastic islands. In conclusion, glucocorticoid-directed monocyte differentiation to macrophages represents a convenient model system to study erythroid-macrophage interactions.

Correspondence:

e.vandenakker@sanquin.nl

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Introduction

In human bone marrow (BM) and fetal liver (FL), the production of erythrocytes through erythropoiesis occurs on erythroblastic islands.[1,2] These erythroblastic islands consist of a central macrophage surrounded by erythroid cells at different stages of terminal differentiation and support proliferation, differentiation and phagocytose the extruded nuclei (or pyrenocytes) of erythroid cells.[2-6] Chow *et al.* described that mouse CD169+ (SIGLEC1) BM resident macrophages display a dual role promoting erythropoiesis and retention of hematopoietic stem and progenitor cells (HSPC).[7,8] Their absence leads to the mobilization of HSPC, reduced BM erythropoiesis and the inability to properly respond to anemia[7-10] It is, however, unclear whether CD169 identifies different macrophage populations or indicates an intrinsic dual role for the same tissue macrophage. FL macrophages that are unable to interact with erythroblasts due to disruption of the retinoblastoma tumor suppressor gene in mice lead to embryonic death as erythroblasts fail to enucleate.[11] These data show that *in vivo*, macrophages are important in regulating erythropoiesis in adults and during development.

Previously, we found that blood-derived monocytes induced to differentiate using stem cell factor (SCF), erythropoietin (EPO) and glucocorticoids enhance *in vitro* erythropoiesis by supporting HSPC survival.[12] These macrophages display a tissue-resident profile expressing CD14 (lipopolysaccharide [LPS]-receptor), CD16 (FcγRIII), scavenger receptor CD163, CD169, CD206 (mannose receptor), CXCR4 and minimal expression of dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN).[12] We hypothesized that these cultured monocyte-derived macrophages may have a similar role as mouse CD169$^+$ macrophages in both hematopoiesis and erythropoiesis. This would provide an easy-to-use *in vitro* human model system to mimic erythroblastic islands allowing for the study of functional interactions between macrophages and erythroid cells, which is currently limited to harvesting BM or involves genetic modification.[13] A better understanding of the mechanism(s) through which human macrophages interact and regulate erythroblast maturation and enucleation is important in order to understand the pathology of erythropoietic disorders, such as erythrocytosis in polycythemia vera or erythrophagocytosis in several types of hemolytic anemia, as well as to improve *in vitro* erythroid differentiation protocols for erythrocyte production.[14,15]

In mice BM, erythroblasts are bound to macrophages *via* interactions between integrin-α4β1 on erythroblasts and VCAM1 on macrophages, and blocking these molecules disrupts erythroblastic islands.[16] Chow *et al*. described human BM macrophages as also expressing VCAM1. However, Ulyanova *et al*. have shown that *Vcam*$^{-/-}$ mice do not display an erythroid phenotype during homeostasis or phenylhydrazide-induced stress.[17] During terminal differentiation erythroblasts enucleate, resulting in reticulocytes and pyrenocytes. The latter are also still encapsulated by plasma membrane. In mice, clearance of pyrenocytes occurs *via* TAM-receptors on the central macrophages that recognize and bind phosphatidylserine (PS) exposed on pyrenocytes resulting in phagocytosis in a protein S-dependent manner.[18,19] The TAM-receptor family of tyrosine kinases (TYRO3, AXL, and MERTK) play an important role in the phagocytic ability of macrophages as triple knock-out mice fail to clear apoptotic cells in multiple tissues. These mice develop normally, but eventually develop autoimmunity, such as systemic lupus erythematosus (SLE).[20] This is in line with studies showing that SLE has been associated with failure of macrophages to phagocytose apoptotic cells and pyrenocytes in both humans and mice.[21-24] In addition, anemia is found in about 50% of SLE patients; Toda *et al*. showed that embryos suffer from severe anemia caused by failure of macrophages to phagocytose pyrenocytes.[25] These data indicate that macrophages are essential during all stages of erythropoiesis, including enucleation, and display inherent features that are indispensable to the functionality of these macrophages.

Herein, we show that peripheral blood monocytes can be differentiated to erythropoiesis-supporting macrophages that interact with erythroid cells, phagocytose pyrenocytes and phenotypically resemble human CD169$^+$ BM and FL macrophages.

Methods

Human materials

Human blood, BM and FL mononuclear cells were purified by density separation, following manufacturer's protocol. Regarding blood, informed consent was given in accordance with the Declaration of Helsinki, the Dutch National and Sanquin Internal Ethic Boards, and by the Bristol Research Ethics Committee (REC; 12/SW/0199). Following informed consent, adult BM aspirates were obtained from the sternum of patients undergoing cardiac surgery, and approved by the Medical Ethical Review Board of the AMC (MEC:04/042#04.17.370). Fetal tissues (week 15-22) were obtained from elective abortions contingent on informed consent and approval by the Medical Ethical Commission of the Erasmus University Medical Center Rotterdam (MEC-2006-202).

Cell culture

CD14 and CD34 MicroBeads (Miltenyi Biotec, Gladbach, Germany) were used for cell isolation from peripheral blood. CD14$^+$ monocytes were cultured at 1.5-3x10^6 cells/well (CASY® Model TTC, Schärfe System GmbH, Reutlingen, Germany) in a 12-well plate as described.[12] Cells were treated with 1-20μM mifepristone (Sigma-Aldrich, Munich, Germany) directly after isolation or 4-24 hours after three days of culture. CD34$^+$ cells were differentiated towards erythroblasts,[12] with the addition of 1ng/ml IL-3 (R&D systems, Abingdon, UK) at the start of culture. Media was replenished every two days. After 8-10 days, cells were differentiated towards reticulocytes by removing dexamethasone, increasing EPO (10U/ml, ProSpec; East Brunswick, NJ, USA) and adding heparin (5U/ml, LEO Pharma B.V., Breda, The Netherlands), 5% pooled AB+ plasma and holotransferrin (700μg/ml, Sanquin, Amsterdam, The Netherlands). Every other day, half the media was replenished. For co-culture experiments, CD14$^+$ cells were differentiated with (GC-macrophages) or without dexamethasone for three days and co-cultured with erythroblasts (day 8-10 of culture; ratio 1:1.5) or more differentiated erythroid cells (day 6 of differentiation; ratio 1:4) for 24 hours.

Flow cytometry

Cells were washed in phosphate-buffered saline (PBS) and resuspended in 1% bovine serum albumin (BSA)/PBS. Cells were incubated with primary antibodies for 30min at 4C, measured on LSRII or LSRFortessa (both BD Biosciences, Oxford, UK) and analyzed using FlowJo software (FlowJo v10; Tree Star, Inc., Ashland, OR, USA) (antibodies listed in *Online Supplementary Methods*).

Mass spectrometry

See *Online Supplementary Methods.*

ImageStreamX and IncuCyte

GC-macrophages or unstimulated cells were incubated with 100μg/ml fluorescein isothiocyanate (FITC)-labeled zymosan (*S. cerevisiae*; MP Biomedicals, Solon, OH, USA) for 40min at 37C. Zymosan was removed and cells were fixed in 4% paraformaldehyde (PFA) for 20min at 4C. Cells were transferred to 1% BSA/PBS and stained with human leukocyte antigen-antigen D-related R-phycoerythrin (HLA-DR PE; BD Biosciences). Furthermore, erythroid cells at day seven of differentiation were stained with Deep Red Anthraquinone 5 (DRAQ5; Abcam, Cambridge, UK). Imaging was performed on the ImageStreamX (Amnis Corporation, Seattle, WA, USA) and images were analyzed using IDEAS Application v6.1 software (Amnis Corporation). For IncuCyte experiments see *Online Supplementary Methods.*

Cytospins

Cells were cytospun using Shandon Cytospin II (Thermo Scientific), dried and fixed in methanol. Cells were stained with benzidine and Differential Quik Stain Kit (PolySciences, Warrington, PA, USA) following manufacturer's instructions. Slides were dried, embedded in Entellan (Merck, Darmstadt, Germany) and images were taken (Leica DM-2500, Germany).

Reverse transcription polymerase chain reaction analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described.[12] Values were normalized using S18 and HPRT as a reference gene and calibrated relative to expression of CD14+ monocytes at day 0 (primers listed in *Online Supplementary Methods*).

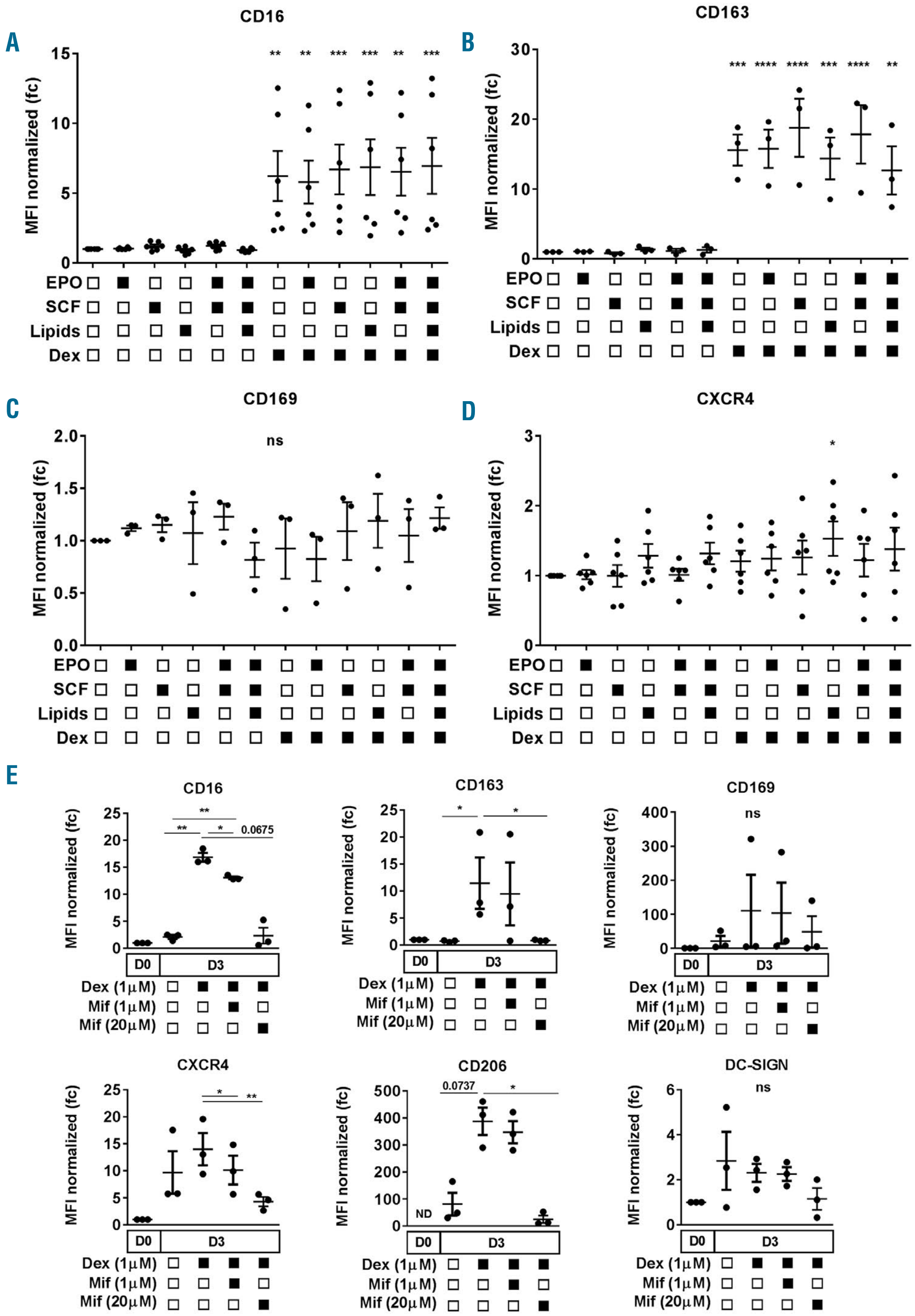

Figure 1. Glucocorticoid receptor activation directs CD14+ monocytes towards a tissue resident macrophage phenotype. (A-D) Distribution graphs displaying the relative geometric mean fluorescence intensity (MFI) of CD16, CD163, CD169 and CXCR4 on human monocytes (n=3-6) cultured for three days under various conditions (EPO, SCF, lipids or dexamethasone). MFI was normalized to change to isotype control and presented as fold change (fc). Mean ± SEM (two-way ANOVA, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). (E) Relative expression of CD16, CD163, CD169, CXCR4, CD206 and DC-SIGN on CD14+ monocytes (n=3) directly after isolation from mononuclear cells (D0) and after culture in the presence or absence of dexamethasone (Dex) and/or mifepristone (Mif). MFI was normalized to isotype control and displayed as a fold change to day 0. Mean ± SEM (ratio paired t-test, *P<0.05, **P<0.01). EPO: erythropoietin; SCF: stem cell factor; ns: not significant; ND: not detected.

Results

Glucocorticoid stimulation directs monocyte differentiation to $CD16^+CD163^+CD169^+CXCR4^+CD206^+$ macrophages

We previously found that purified peripheral blood $CD14^+$ monocytes cultured in EPO, SCF, lipids and dexamethasone differentiate within three days into CD163, CD169, CXCR4 and CD16-positive macrophages that, upon co-culture with $CD34^+$ cells, significantly increase the erythroid yield.[12] However, it remained unclear as to which growth factors were crucial to differentiate monocytes to macrophages supporting erythropoiesis. Therefore, we examined which growth factors or supplements determined this differentiation cue. Flow cytometry analysis showed that dexamethasone, exclusively, induces high expression of CD16 and CD163 in macrophages. The addition of EPO, SCF or lipids does not contribute to this high expression (Figure 1A,B). CXCR4 expression was already upregulated in the absence of dexamethasone but was further increased upon stimulation with dexamethasone and lipids, whilst the expression of tissue residency marker CD169 was also upregulated but occurred in a dexamethasone-independent manner (Figure 1C,D). *Online*

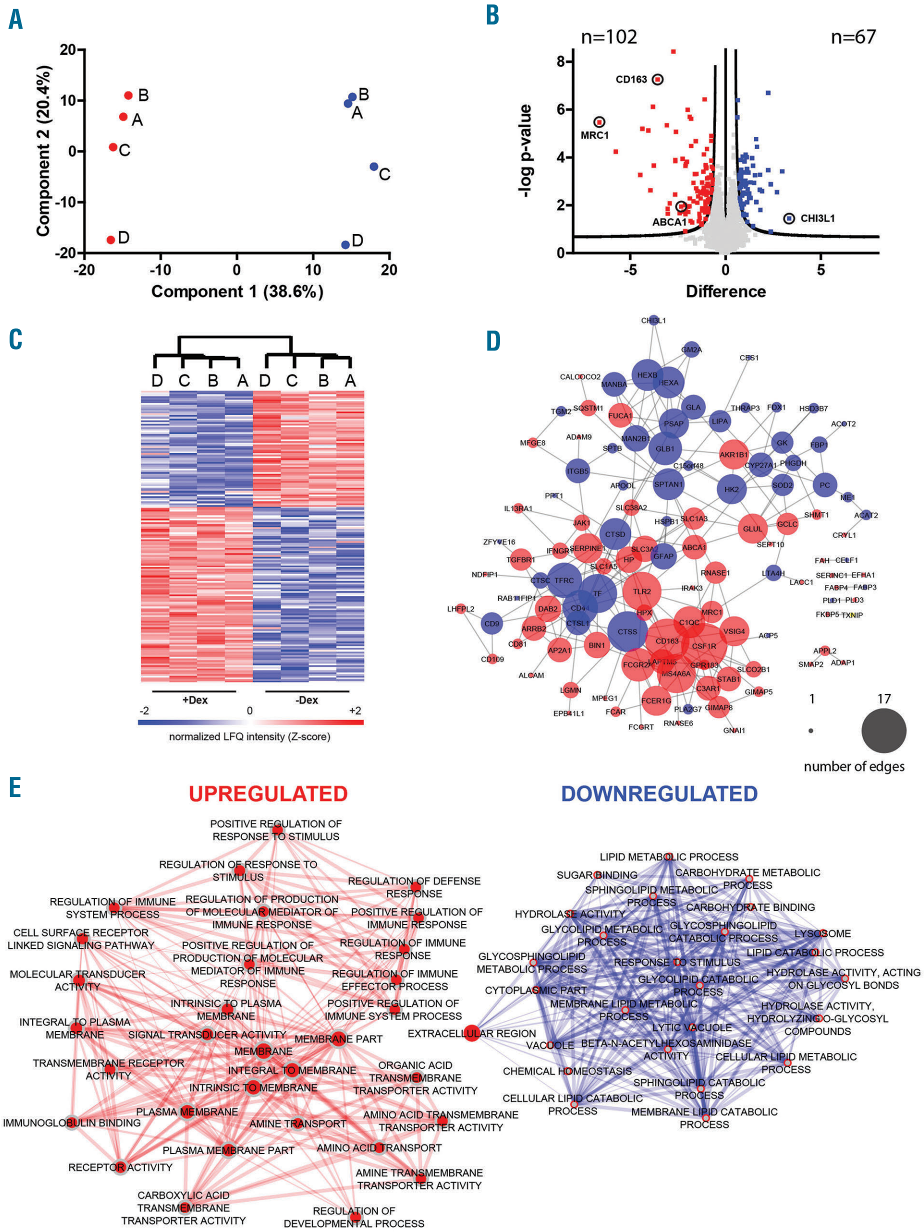

Figure 2. Proteome analysis of $CD14^+$ monocytes cultured in the presence or absence of dexamethasone revealed two distinct macrophage populations. (A) Principal component analysis of GC-macrophages (red) *versus* non-glucocorticoid stimulated cells (blue) of four donors (indicated A-D). (B) Volcano plot (false discovery rate 0.05 S0 0.4) showing *P*-values (-log) *versus* difference of cells cultured for three days in the presence or absence of dexamethasone. (C) Heatmap of differentially expressed proteins based on Z-scored label-free quantification values. (D) Interaction analysis based on STRING (all interactions) of upregulated (red) and downregulated (blue) proteins. (E) Enrichment analysis using BiNGO and enrichment mapper in GC-macrophages with upregulated (red) and downregulated (blue) processes.

Supplementary Figure S1A depicts distinct morphological changes upon dexamethasone-induced differentiation between freshly isolated CD14⁺ monocytes and cultured CD14⁺ cells. Monocytes were incubated with mifepristone, which blocks glucocorticoid receptor activation. Membrane and messenger ribonucleic acid (mRNA) expression of CD16, CD163, and CD206 was significantly reduced by mifepristone treatment, and thus dependent on glucocorticoid receptor transcriptional control (Figure 1E and *Online Supplementary Figure S1B,C*). Although neither Figure 1C nor Figure 1E show an effect of dexamethasone on the fluorescence intensity of CD169, mRNA levels of CD169 were clearly increased upon stimulation of the glucocorticoid receptor and reduced when cells were treated with mifepristone. In contrast, CXCR4 mRNA levels did not change upon mifepristone treatment, but membrane expression was increased (*Online Supplementary Figure S1B*). Monocyte differentiation increases expression of DC-SIGN independently of dexamethasone, albeit to expression levels that are significantly lower compared to dendritic cells (Figure 1E and *Online Supplementary Figure S1C*).[26] Note that cultured monocytes in all conditions are a homogeneous population, as single peaks observed in histograms and multi-color flow cytometry data revealed that monocytes stimulated with glucocorticoids are $CD16^+CD163^+CD169^+CXCR4^+CD206^+$ cells (*Online Supplementary Figure S1C,D*). Interestingly, flow cytometry data revealed that monocytes that have been differentiated for three days in the presence of dexamethasone were unable to change their phenotype after 4 or 24 hours of mifepristone treatment. Only CD163 expression was slightly reduced after 24 hours mifepristone treatment (*Online Supplementary Figure S1E*). The data indicates that glucocorticoid stimulation initiates an irreversible differentiation program of monocytes towards $CD16^+CD163^+CD169^+CXCR4^+CD206^+$ macrophages which is maintained for at least 17 days of culture (*Online Supplementary Figure S2A,B*).

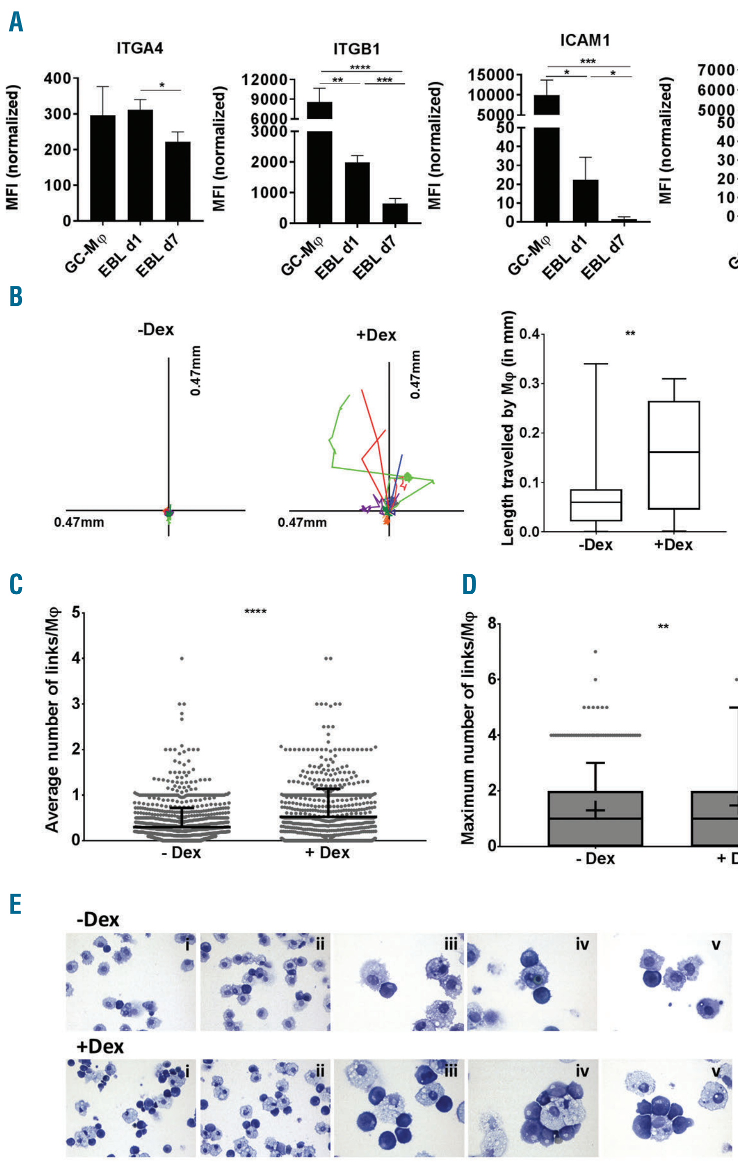

Figure 3. GC-macrophages form erythroblast clusters with increased frequency and erythroblast composition. (A) Expression of integrins ITGA4 and ITGB1 and adhesion molecules ICAM1, PECAM, and VCAM1 on GC-macrophages (Mϕ) (n=6) and erythroblasts (EBL) at day 1 and 7 of differentiation (n=3-4). Mean fluorescence intensity (MFI) has been normalized to the isotype control. Mean ± SEM (unpaired *t*-test, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001). (B) Scaled cell-displacement vector diagram (left; 20 representative macrophages in both conditions) and box-and-whisker plot (right; 68 representative macrophages in -Dex and 21 in +Dex) after three days of culture in the absence or presence of dexamethasone. (Welch's unpaired *t*-test, ***P*<0.01, n=5). (C-D) Co-culture of GC-macrophages or unstimulated cells with erythroblasts (unpaired *t*-test of 1153 (-Dex) and 749 (+Dex) macrophages, ***P*<0.01, *****P*<0.0001, n=5). Images were taken every hour during 64 hours of analysis. (C) Plot showing the average erythroblast-macrophage links for each macrophage. Mean ± SD. (D) 5-95% box plot showing the maximum number of links per macrophage. Mean is indicated by crosses. (E) Representative images of cytospins of GC-macrophages (+Dex) or unstimulated cells (-Dex) co-cultured with erythroblasts for 24 hours (in 50x magnification, panels i-ii or 100x magnification, panels iii-v; n=4). Dex: dexamethasone; ND: not detected.

Proteomics data revealed GC-macrophages display a distinct anti-inflammatory profile

To gain further insights into the dexamethasone-induced monocyte differentiation process, we performed mass spectrometry-based quantitative proteomics on these cells after three days of differentiation and compared this to non-glucocorticoid stimulated monocytes. A total of 3,210 proteins were quantified, and principal component analysis clearly separated glucocorticoid-stimulated from non-stimulated cells (Figure 2A and *Online Supplementary Table S1*). Glucocorticoid stimulation induced a distinct expression pattern compared to non-glucocorticoid stimulated monocytes, as visualized in the volcano plot and corresponding heatmap of the 169 differentially expressed proteins for individual donors (Figure 2B,C). Note that the expression of CD163 and CD206

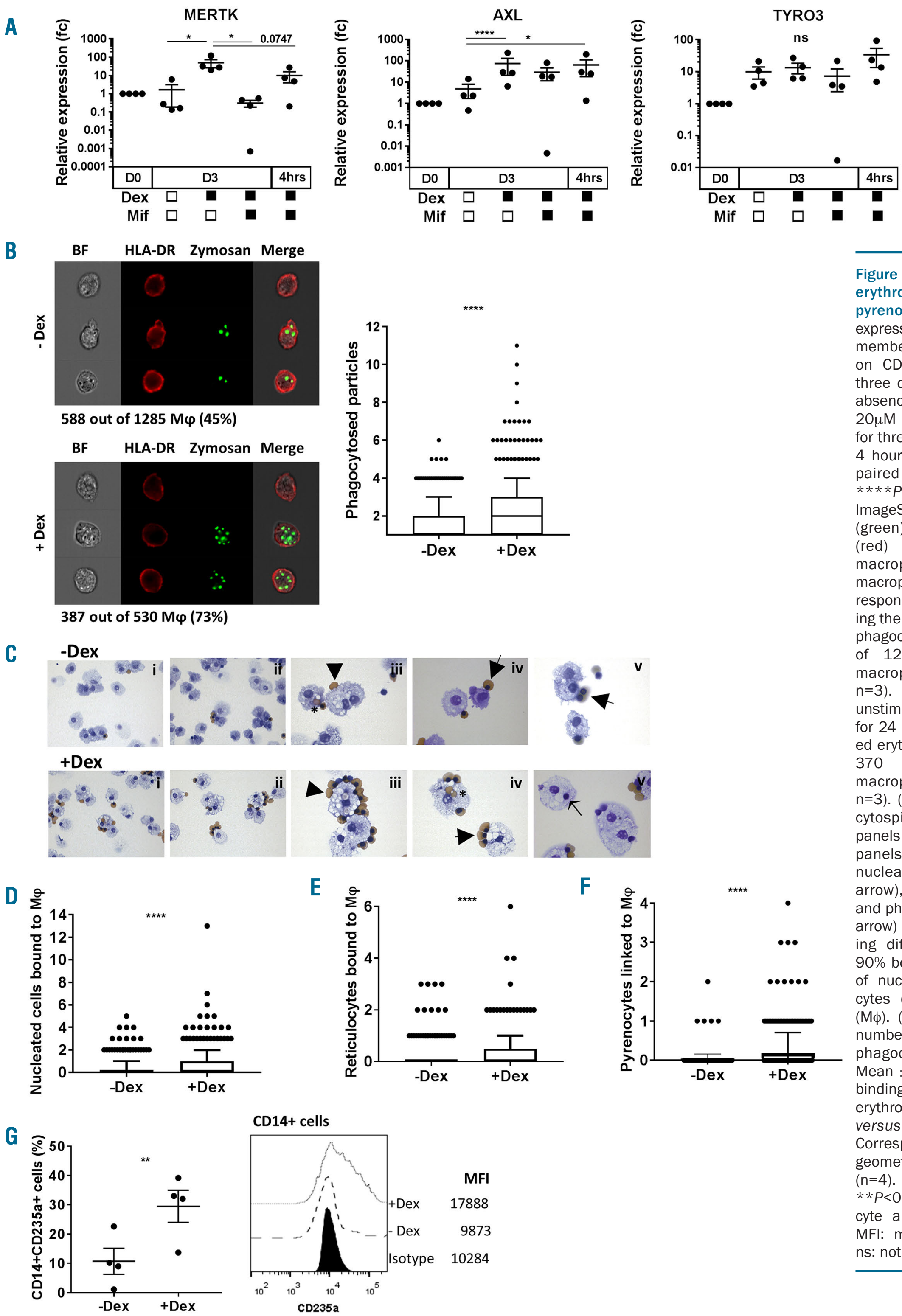

Figure 4. GC-macrophages can bind erythroid cells and phagocytose pyrenocytes. (A) Relative mRNA expression of TAM-receptor family members MERTK, AXL and TYRO3 on CD14+ cells (D0) cultured for three days (D3) in the presence or absence of dexamethasone (Dex). 20μM mifepristone (Mif) was added for three days or after three days for 4 hours (n=4). Mean ± SEM (ratio paired *t*-test, **P*<0.05, *****P*<0.0001). (B) Representative ImageStreamX images of zymosan (green) phagocytosed by HLA-DR (red) positive unstimulated macrophages (-Dex) and GC-macrophages (+Dex) (left), and corresponding 10-90% box plot showing the number of zymosan particles phagocytosed (right) (unpaired *t*-test of 1285 -Dex and 530 +Dex macrophages, *****P*<0.0001, n=3). (C-F) GC-macrophages and unstimulated cells were co-cultured for 24 hours with day 6 differentiated erythroid cells (unpaired *t*-test of 370 –Dex and 313 +Dex macrophages, *****P*<0.0001, n=3). (C) Representative images of cytospins (in 50x magnification, panels i-ii or 100x magnification, panels iii-v). Macrophages bind nucleated erythroid cells (large arrow), reticulocytes (arrowhead) and phagocytose pyrenocytes (small arrow) and some erythroid cells during differentiation (asterisk). 10-90% box plots showing the number of nucleated cells (D) or reticulocytes (E) bound to macrophages (Mϕ). (F) Scatter plot showing the number of pyrenocytes bound to or phagocytosed by macrophages. Mean ± SD. (G) Graph showing the binding of CD235a+ differentiated erythroid cells to GC-macrophages *versus* unstimulated cells. Corresponding histogram showing geometric mean of CD235a in FITC (n=4). Mean ± SEM (paired *t*-test, ***P*<0.01). HLA-DR: human leukocyte antigen – antigen D-related; MFI: mean fluorescence intensity; ns: not significant; BF: bright-field.

(MRC1) was highly induced after glucocorticoid receptor activation, corroborating the flow cytometry experiments. The most differentially expressed proteins (n=169) were mapped to evaluate specific upregulation or downregulation of functionality-linked protein networks, based on the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) analysis (Figure 2D). CD163 and CD206 are part of an interactome protein node that is specifically upregulated in dexamethasone-induced macrophages, and includes M2 macrophage markers CSF1R, stabilin-1 (STAB1) and complement proteins C3AR1, C1QC, and FcγRIIa (CD32) which has been associated with high phagocytic capacity of the cells. Moreover, VSIG4 was upregulated in dexamethasone-induced macrophages, which is restricted to resting tissue macrophages,[27] while ABCA1 was also upregulated, which has been highly associated with hemoglobin-associated macrophages.[28] In addition, proteins with a positive regulation of cell migration and motility, including DAB2, ADAM9, Serpine1 (PAI1) and CD81, are upregulated in dexamethasone-induced macrophages. Furthermore, a whole range of signaling receptors were upregulated, amongst which are TGF and IFNγ receptors (TGFBR1 and IFNGR1) and IL13RA1. These proteins belong to processes that are enriched as GO-term, e.g., membrane part, signal transducer activity, transmembrane receptor activity and molecular transduced activity. In addition, many immune regulatory processes are also enriched (Figure 2E and *Online Supplementary Table S2*). Interestingly, members of the cathepsin family involved in antigen presentation (e.g., CTSC, CTSL1, CTSD and CTSS) were downregulated. A range of pro-inflammatory proteins, clustered within an interactome node, were downregulated; these include lysosomal enzymes HEXA and HEXB, MANBA,

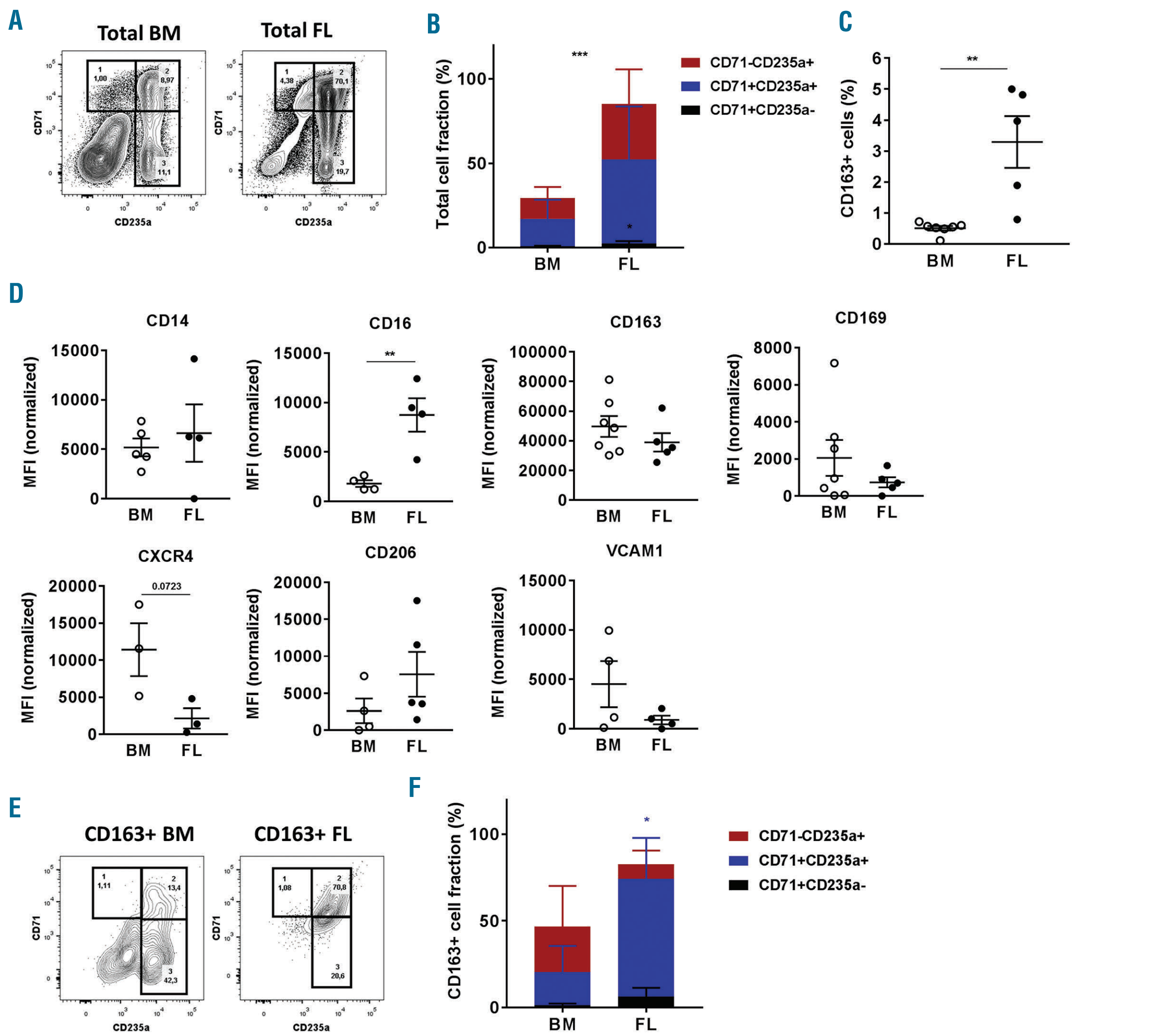

Figure 5. CD163$^+$ macrophage populations in human BM and FL. (A) Representative dot plots of erythroid cells characterized by the expression of CD71^{high} and CD235a in total human BM (n=4) and FL mononuclear cells (n=4). (B) Graph belonging to panel A. Mean ± SD (unpaired *t*-test, ****P*<0.001). (C) Graph showing the percentage of CD163$^+$ cells present in the total cell population in BM (n=7) and FL (n=5). Mean ± SEM (unpaired *t*-test, ***P*<0.01). (D) Characterization of CD163$^+$ macrophages in human BM (n=3-7) and FL (n=3-5) based on the expression of CD14, CD16, CD163, CD169, CXCR4, CD206 and VCAM1. Mean fluorescence intensity (MFI) has been normalized to the isotype control. Mean ± SEM (unpaired *t*-test, ***P*<0.01). (E) Representative dot plots of erythroid-macrophage clusters formation of erythroid cells (CD71^{high}CD235a$^+$) with CD163$^+$ BM (n=4) and FL (n=4) macrophages. (F) Graph belonging to panel E. Mean ± SD (unpaired *t*-test, **P*<0.05). BM: bone marrow; FL: fetal liver.

saponin PSAP and GLB1, in addition to other lysosome/hydrolase activity-related GO-categories (Figure 2D,E). In addition, GO-categories associated with lipid metabolic processes were also downregulated in GC-macrophages. Furthermore, CHI3L1 and CD44 are highly upregulated in non-glucocorticoid stimulated cells (Figure 2B). CHI3L1 is described as a pro-inflammatory factor,[29,30] while CD44 has been expressed on pro-inflammatory tissue macrophages.[31] In conclusion, $CD14^+$ monocytes that have been differentiated in the presence of dexamethasone display a distinct anti-inflammatory proteomic profile and are further denoted as GC-macrophages, while unstimulated cells have a more inflammatory profile.

GC-macrophages are motile and bind erythroblasts

GC-macrophages may, besides supporting the erythroid yield, also regulate terminal differentiation of erythroblasts, recapitulating aspects of erythroblastic islands. In mice, it has been shown that BM central macrophages can bind erythroblasts through various interactions: VCAM1-integrin-α4β1,[16,32] integrin-α5β1-ICAM4,[33,34] erythroblast macrophage protein (EMP)-EMP,[4,35] or EphrinB2-EphrinB4.[36] Flow cytometry data revealed that GC-macrophages express common cell adhesion molecules (CAM), such as integrins (α4 [ITGA4], β1,2 [ITGB1, ITGB2/CD18] and αL,M,X [ITGAL/CD11a, ITGAM/CD11b, ITGAX/CD11c]), the immunoglobulin (Ig) superfamily (ICAM1, PECAM, VCAM1) and E- and L-selectin (Figure 3A and *Online Supplementary Figure S3A*). Most of these CAM could be identified in the proteomics data, including ICAM3, integrin-β5 and α5, however, VCAM1, selectins and EMP were not detected (*Online Supplementary Table S1*). With the exception of integrin-β5, these CAM were not differentially expressed between GC-macrophages and non-glucocorticoid stimulated cells. Erythroblasts expressed similar ITGA4 levels compared to GC-macrophages, but exhibited a 10-fold reduction in ITGB1 expression and low expression of ICAM1 and PECAM, whereas VCAM1 was not detected (Figure 3A). When differentiating erythroblasts towards reticulocytes (*Online Supplementary Figure S3B,C*), the expression of CAM was reduced, as expected, which potentially indicates a lower binding affinity of erythroid cells to macrophages during erythroid differentiation. Next, we investigated whether GC-macrophages interact *in vitro* with erythroid cells compared to non-glucocorticoid stimulated monocytes. Indeed, live imaging cells for 2.5 days showed that GC-macrophages are highly motile and non-stimulated macrophages are non-motile (Figure 3B), a finding which corroborates the increased expression of cell migration and motility proteins (Figure 2D) whilst engaging twice as many erythroblasts (0.5 *vs.* 0.3, $P<0.0001$) at every time point measured (Figure 3C,D). In addition, cytospins of macrophages co-cultured for 24 hours with erythroblasts showed that the number of macrophages binding erythroblasts as well as the number of erythroblasts bound was increased on GC-macrophages compared to non-GC macrophages (Figure 3E and *Online Supplementary Figure S3D*). Nonetheless, no difference in interaction duration between erythroblasts and macrophages from both conditions was observed (*Online Supplementary Figure S3E*), suggesting that the unstimulated cells possess some machinery to interact with erythroblasts. In conclusion, GC-macrophages are motile, express a variety of CAM and form erythroblast interactions with increased frequency and numbers per macrophage compared to cells cultured in the absence of dexamethasone.

GC-macrophages express TAM-receptor family members and phagocytose pyrenocytes

As $CD169^+CD163^+$ macrophages promote erythropoiesis,[8] we decided to examine whether GC-macrophages can provide a similar functional role *in vitro*. In mice, pyrenocytes are phagocytosed by central macrophages in a Mer tyrosine kinase (MERTK)-dependent manner.[18] RT-PCR showed that GC-macrophages upregulate both MERTK and AXL mRNA compared to freshly isolated and non-glucocorticoid stimulated monocytes (Figure 4A). MERTK expression was inhibited by mifepristone treatment during the first three days of culture, whereas AXL was not, suggesting that AXL expression is induced *via* a trans-regulated process while MERTK needs the transcriptional activity of the glucocorticoid receptor. Note that TYRO3 levels are dexamethasone-independently increased. Besides TAM-receptors, other PS-receptors on macrophages have been reported to be involved in clearing apoptotic bodies, such as TIM3[37] (T-cell Ig and mucin-domain containing-3), STAB[38] and CD300A[39] (CMRF35-like molecule 8). TIM3 mRNA levels were increased, albeit independently of dexamethasone (*Online Supplementary Figure S4A*). This was confirmed by mass spectrometry, as peptides corresponding to TIM3 were identified in GC-macrophages (HAVCR2 in *Online Supplementary Table S1*). CD300A and STAB1 were also identified, of which STAB1 was significantly increased in GC-macrophages compared to unstimulated cells. Interestingly, proteomics data showed that lactadherin, a PS-binding glycoprotein which stimulates phagocytosis of red blood cells by macrophages,[40] was significantly induced in GC-macrophages compared to unstimulated cells. RT-PCR confirmed increased lactadherin mRNA levels, but this was dexamethasone-independent (*Online Supplementary Figure S4B*). Moreover, both GC-macrophages and unstimulated cells express DNASE2, a crucial protein required to degrade DNA within phagocytosed apoptotic bodies or pyrenocytes in macrophages.[41]

Expression of TAM-receptors and other PS-receptors on GC-macrophages may be a prerequisite to phagocytose particles, cells or pyrenocytes in case of erythropoiesis. Figure 4B shows that the number of GC-macrophages that phagocytose particles, in addition to the amount of zymosan particles per macrophage, is higher (73% *vs.* 45%, 2.3 *vs.* 1.7, respectively) compared to unstimulated cells. Subsequently, both unstimulated cells and GC-macrophages were co-cultured with a mixture of differentiating erythroblasts, reticulocytes and pyrenocytes (*Online Supplementary Figure S3B,C*) for 24 hours. Cytospin analysis showed that both GC-macrophages and unstimulated cells bind erythroid cells (Figure 4C), however, increased numbers of nucleated cells, reticulocytes and pyrenocytes bind to GC-macrophages compared to unstimulated cells (Figure 4D-F and *Online Supplementary Figure S4C*). Note that all nucleated erythroid cells are specifically aligned with their nucleus towards the macrophage as observed *in vivo* (Figure 4C). Pyrenocytes, however, were almost solely phagocytosed by GC-macrophages (Figure 4F and *Online Supplementary Figure S4D*). Importantly, GC-macrophages and unstimulated cells did not overtly phagocytose nucleated cells or reticulocytes (*Online Supplementary Figure S4E,F*). Flow cytometry data showed that indeed both GC-macrophages and unstimulated cells can bind erythroid cells, however, increased cluster formation was found for GC-macrophages compared to unstimulated cells (Figure 4G). These results demonstrate that GC-macrophages functionally resemble specific aspects of macrophages within the erythroblastic island by binding erythroblasts and reticulocytes and phagocytosing pyrenocytes.

GC-macrophages share characteristics with $CD163^+$ macrophages found in human BM and FL

To investigate whether GC-macrophages share phenotypical characteristics with macrophages found in the two major erythropoietic organs during human development and adulthood (FL and BM, respectively), mononuclear cells of both organs were analyzed. Between week 15 and 22 of human development, the FL is primarily undertaking erythropoiesis, representing a median of 85% of the total number of mononuclear cells compared to 29% in BM, with increased frequencies of $CD71^+CD235^-$ pro-erythroblasts in FL (Figure 5A,B). To prevent the presence of free immunogenic pyrenocytes and to support erythroid cell requirements in the developing embryo, it is anticipated that the FL contains significant amounts of erythroblastic islands and, thus, supporting macrophages. Indeed, Figure 5C shows a 6.5-fold increase in $CD163^+$ FL macrophages compared to BM (3.3% *vs.* 0.5%). Further characterization shows only subtle differences in expression of macrophage markers (Figure 5D and *Online Supplementary Figure S5A,B*), as both macrophage populations express high levels of CD163 and CD14 and have intermediate levels of CD169, CD206 and VCAM1. $CD163^+$ BM macrophages tend to express more CXCR4, whereas $CD163^+$ FL macrophages have higher expression of CD16. *Online Supplementary Table S3* displays the comparison between the mean fluorescence intensity (MFI) of BM, FL, non-stimulated and GC-macrophages and reveals that GC-macrophages phenotypically recapitulate macrophages found in the FL and BM. GC-macrophages are more similar to BM macrophages (CD16 and CXCR4 expression), however, they also share features of FL macrophages (CD206 expression). Unstimulated cells do not express VCAM1, and have low expression of CD206, CD163, CD14 and CD16. Figure 5E,F shows that both BM and FL $CD163^+$ macrophages bind erythroid cells (46% in BM *vs.* 83% in FL), indicating that CD163 purifies erythroid-supporting macrophages. Interestingly, FL macrophages have increased interactions with $CD71^+CD235a^+$ cells compared to BM. The similarity of marker expression levels of BM, FL and GC-macrophages and the fact that all three populations form erythroid clusters suggest that GC-macrophages share phenotypic and functional characteristics with *in vivo* erythroid-supporting macrophages. GC-macrophages could thus be used as a substitute *in vitro* model to study the supportive effects of macrophages on erythropoiesis.

Discussion

We have previously shown that monocyte-derived macrophages can support erythropoiesis by increased survival of HSPC.[12] Herein, we show that these macrophages, derived from $CD14^+$ monocytes, are differentiated in a glucocorticoid-dependent manner (termed GC-macrophages), interact with erythroid cells of all stages and phagocytose the extruded pyrenocytes. Besides these functional aspects, GC-macrophages also share phenotypic characteristics with resident macrophages from both human BM and FL, among which there is high expression of CD163 and CD206. Interestingly, $CD163^+$ BM cells appear to be more heterogeneous compared to FL cells. GC-macrophages also phenotypically resemble macrophages described recently by Belay *et al*., who employed a lentivirally introduced small molecule responsive Mpl-based cell growth switch that enabled cord blood or BM $CD34^+$ cells to be differentiated to erythroid-supporting macrophages.[13] Similar to GC-macrophages, these cells express CD14, CD163, CD169, CD206, VCAM1, ITGAM and ITGAX. Herein, we show that these macrophages can also be differentiated from peripheral blood monocytes using dexamethasone, without the need for genetic manipulation. Falchi *et al*. showed that in erythroid culture conditions, $CD34^+$ cells can also differentiate to macrophages that interact with erythroid cells, however, we can exclude this differentiation pathway as the purified $CD14^+$ monocytes we used to differentiate macrophages from peripheral blood did not show hematopoietic colony potential or $CD34^+$ contamination.[12]

The erythroid system is renowned for its rapid response to systemic decreases in oxygen pressure. Together with elevated EPO levels, glucocorticoid levels also increase upon exposure to high altitude.[42] EPO, SCF and glucocorticoids induce erythroblasts to proliferate whilst inhibiting differentiation.[43-46] Elevated systemic EPO and glucocorticoids as a response to low-oxygen stress leads to increased erythroid output due to augmented survival and proliferation of BM erythroblasts. To accommodate this increased erythropoiesis, we hypothesize that the number of central macrophages must also be increased or alternatively these cells would have to engage with more erythroblasts.

Our flow cytometry and cytospin data confirmed that GC-macrophages interact with erythroid cells of all stages, be that as it may, this does not provide information on the longevity of the interactions, as these could be transient, as previously implied.[47] *Via* live cell imaging we analyzed the interaction between GC-macrophages and erythroblasts, which revealed that GC-macrophages are more mobile compared to cells that were cultured in the absence of dexamethasone, and that this mobility, or "macrophage ranging", results in more interactions with erythroblasts. Higher mobility was accompanied by an increased expression of proteins involved in migration and motility. High motility has previously been observed in $CD34^+$ differentiated macrophages stimulated with dexamethasone.[47] Motility is an important functional aspect, as erythroblastic islands *in vivo* form away from sinusoids and migrate to the sinusoidal endothelium to release reticulocytes into the circulation.[48,49] Interestingly, this work also demonstrates that non-glucocorticoid-stimulated monocytes can interact with erythroblasts, as they form interactions for the same length of time (1.8 hours on average) when they encounter erythroblasts. This suggests that both populations express receptors that allow engagement and interaction with erythroblasts, however, GC-macrophages have significantly more interactions with erythroblasts per macrophage and bind a higher number of erythroblasts. Surprisingly, GC-macrophages display low expression of VCAM1, suggesting that erythroblast interactions may also occur in a VCAM1-independent manner. Indeed, Ulyanova *et al*. reported that *Vcam1*$^{-/-}$ mice do not display a compromised erythroid stress response in spleen and BM.[17] Whether another interaction substitutes for VCAM1 would need to be determined. The presented monocyte differentiation methodology has potential to be exploited as an imaging platform to delineate the hierarchy of contributions of various receptors within the macrophage-erythroblasts in BM and GC-macrophages in future studies.

We have also demonstrated, using proteomics and imaging, that GC-macrophages actively phagocytose pyrenocytes and express the correct putative machinery to recognize pyrenocytes. The mechanism(s) through which macrophages recognize reticulocytes but phagocytose pyrenocytes are ill-defined in human erythropoiesis. Our proteomic study and RT-PCR data demonstrate that GC-macrophages express all TAM-receptors, including MERTK and other PS-receptors, which may be used by GC-macrophages to take up pyrenocytes. This work, alongside our ability to manipulate erythroblast protein expression, now provides an excellent accessible model system to mechanistically understand how macrophages promote erythropoiesis and eventually target pyrenocytes for phagocytosis and destruction. Furthermore, it is interesting to note that GC-macrophages interact preferably to the polarized nuclear side of erythroid cells as observed in BM erythroblastic islands. In general, proteomic analysis revealed an array of processes and proteins that are differentially regulated between GC-macrophages and unstimulated cells. The data will allow further studies to delineate essential pathways that are key to glucocorticoid-stimulated differentiation of monocytes towards erythroid-supporting GC-macrophages. This is probably the concerted action of multiple pathways.

Finally, our observations have important implications for our understanding of the dynamics of the macrophage populations in human BM. We characterized both human BM and FL macrophages and found that CD163^{+} FL macrophages define a homogeneous population. In contrast, CD163^{+} BM macrophages show a more heterogeneous population, reflecting that CD163^{+} cells represent a mixed population of myeloid cells. Both human BM and FL CD163^{+} macrophages are capable of binding erythroid cells, however, this percentage is lower in BM (46%) compared to FL (83%). The FL is primarily performing erythropoiesis at week 15-22 of embryonic development, which suggests that CD163 purifies mainly central macrophages. The reduced erythroid-macrophage clusters in BM may reflect a more heterogeneous CD163^{+} population with possibly different functions. Changes observed in marker expression of macrophages in both organs could thus be due to this heterogeneity in the BM population. CD163 isolation in combination with single cell RNA-sequencing may discriminate these different populations and identify specific discriminatory cell surface markers to allow for functional experiments.

Albeit for decades it was believed that all macrophages originate from monocytes,[50] recent parabiosis and fate-mapping studies showed that most resident macrophages are maintained independently of monocytes.[51] However, Theurl *et al*. showed that resident Kupffer cells in the liver contain a mixture of *de novo* hematopoiesis-derived and embryonic-derived macrophages. They identified an on-demand mechanism to facilitate quick and transient increases in cells that can function as Kupffer cells but originate from classical monocytes.[52] Taken together with our work, we hypothesize a new scenario in which specific macrophages originate from different sources depending on the need of a specific tissue or process. These processes may also occur in other tissues in response to stress, like the BM. The origin and homeostasis of human BM resident macrophages is presently ill-defined, if described at all. Elevated glucocorticoid levels may lead to direct differentiation of monocytes and elevated numbers of nursing central macrophages to facilitate the increased erythroid output in analogy to Kupffer cells. Active research is aimed at unraveling the origin of tissue resident macrophages, which is important in order to understand not only homeostatic but also pathogenic erythropoiesis in which a driving role of macrophages has been implicated, such as polycythemia vera and β-thalassemia. Herein, we provide evidence that monocytes can indeed differentiate *in vitro* to macrophages that support erythropoiesis, providing a model to study such erythroid-macrophage interactions.

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