The role of macrophages in human erythropoiesis

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

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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.
Introduction

In human bone marrow (BM) and fetal liver (FL), the production of erythrocytes through erythropoiesis occurs on erythroblastic islands. These erythroblastic islands consist of a central macrophage surrounded by erythroblasts at different stages of terminal differentiation and support proliferation, differentiation and phagocytose the extruded nuclei (or pyrenocytes) of erythrocyte cells. 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). Their absence leads to mobilization of HSPC, reduced BM erythropoiesis and the inability to properly respond to anemia. 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. 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. These macrophages display a tissue-resident profile expressing CD14 (lipopolysaccharide [LPS]-receptor), CD16 (FcgRIII), scavenger receptor CD163, CD169, CD206 (mannose receptor), CXCR4 and minimal expression of dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). 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. 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.

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. Chow et al. described human BM macrophages as also express VCAM1. However, Ulyanova et al. has shown that Vcam⁻/⁻ mice do not display an erythroid phenotype during homeostasis or phenylhydrazide-induced stress. 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. 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 human 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 consult, 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 TCC, 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, increased EPO (10U/ml, ProSpec, East Brunswick, NJ, USA) and addition of 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 30 min at 4°C, 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 Supplementary methods).

Mass spectrometry
See Supplementary methods.

ImageStreamX and IncuCyte
GC-macrophages or unstimulated cells were incubated with 100 µg/ml fluorescein isothiocyanate (FITC)-labeled zymosan (S. cerevisiae; MP Biomedicals, Solon, OH, USA) for 40 min at 37°C. Zymosan was removed and cells were fixed in 4% paraformaldehyde (PFA) for 20 min at 4°C. 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 7 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 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 described previously. 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 Supplementary methods).

Results
Glucocorticoid stimulation directs monocyte differentiation to CD16+CD163+CD169+CXCR4+CD206+ macrophages
We have 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. 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). 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 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 (Figure S1B). Monocyte differentiation increases expression of DC-SIGN independent of dexamethasone, albeit to expression levels that are significantly lower compared to dendritic cells (Figure 1E and 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 (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 (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 (Figure S2A,B).

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 3210 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 (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 up- or
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 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; ND: not detected; ns: not significant.
downregulation of functionality-linked protein networks, based on 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 FcgRlla (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 macrophages27, while ABCA1 was also upregulated, which has been highly associated with hemoglobin-associated macrophages28. 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 IFNg-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, CTS1, 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, 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 factor29,30, while CD44 has been expressed on pro-inflammatory tissue macrophages31. 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-αβ116,32, integrin-α5β1-ICAM433,34, erythroblast macrophage protein (EMP)-EMP435, or EphrinB2-EphrinB436. 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 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 exhibit a 10-fold reduction in ITGB1 expression and low expression of ICAM1 and PECAM, whereas VCAM1 was not detected (Figure 3A). When differ
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.
entiating erythroblasts towards reticulocytes (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 in GC-macrophages compared to non-GC macrophages (Figure 3E and Figure S3D). Nonetheless, no difference in interaction duration between erythroblasts and macrophages from both conditions was observed (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.
Modeling human erythroid-macrophage interactions

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 macrophages (-Dex) and 749 (+Dex), **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.

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

As CD169+/CD163+ macrophages promote erythropoiesis8, 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 manner18. 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 (T-cell immunoglobulin and mucin-domain containing-3), STAB (CMRF35-like molecule 8). TIM3 mRNA levels were increased, albeit independently of dexamethasone (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 iden-
tified, 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\textsuperscript{40}, was significantly induced in GC-macrophages compared to unstimulated cells. RT-PCR confirmed increased lactadherin mRNA levels but this was dexamethasone-independent (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\textsuperscript{41}.

Expression of TAM-receptors and other PS-receptors on GC-macrophages may be a pre-requisite to phagocytose particles, cells or pyrenocytes in case of erythropoiesis. Figure 4B showed 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 (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 Figure S4C). Note that all nucleated erythroid cells are specifically aligned with their nucleus towards the macrophage as observed \textit{in vivo} (Figure 4C). Pyrenocytes, however, were almost solely phagocytosed by GC-macrophages (Figure 4F and Figure S4D). Importantly, GC-macrophages and unstimulated cells did not overtly phagocytose nucleated cells or reticulocytes (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\(^\text{+}\) 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\(^\text{+}\)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\(^\text{+}\) FL macrophages compared to BM (3.3\% vs. 0.5\%). Further characterization shows only subtle differences in expression of macrophage markers (Figure 5D and 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
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 macrophages (-Dex) and 530 (+Dex), ****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.
CXCR4, whereas CD163+ FL macrophages have higher expression of CD16. 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 suggests 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 HSPC12. 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\textsuperscript{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\textsuperscript{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\textsuperscript{42}. EPO, SCF and glucocorticoids induce erythroblasts to proliferate whilst inhibiting differentiation\textsuperscript{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 cytopsin 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\textsuperscript{47}. Via live cell imaging we analyzed the interaction between GC-macrophages and erythroblasts, which revealed GC-macrophages are more mobile compared to cells that were cultured in the absence of dexamethasone, and 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\textsuperscript{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\textsuperscript{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 \textit{Vcam1} knockout mice do not display a compromised erythroid stress response in spleen and BM\textsuperscript{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.
Figure 5. CD163+ macrophage populations in human BM and FL. (A) Representative dot plots of erythroid cells characterized by the expression of CD71<sup>hi</sup> 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 cluster formation of erythroid cells (CD71<sup>hi</sup>/CD235a<sup>+</sup>) with CD163<sup>+</sup> 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.
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 monocytes⁵⁰, recent parabiosis and fate-mapping studies showed that most resident macrophages are maintained independently of monocytes⁵¹. 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 monocytes⁵². 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 aimed at unraveling the origin of tissue resident macrophages, which is important 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.

Author contributions
EH performed the experiments. LAH-O and EH performed the IncuCyte experiments and SJC and LAH-O did IncuCyte data analysis. FPJA and MB performed the mass spectrometry data acquisition and analysis. EH and EA designed the experiments and analyzed the data. EH and EA wrote the manuscript. AMT contributed to experiment design, analysis and writing of the manuscript. The manuscript was critically revised by all authors. The authors declare no competing financial interests.

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References

43. 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 Sep;95(9):1594-1598.
Supplementary methods

Flow cytometry
Antibodies used: AbD Serotec (Bio-Rad, Veenendaal, The Netherlands): anti-CD54, ICAM1 (AF647 1:400), anti-CD209, DC-SIGN (PE 1:100); Acris (Herford, Germany): anti-CD235a (FITC 1:450; PE 1:450); BD Biosciences: anti-CD11c, ITGAX (PE 1:10), anti-CD14 (Pacific Blue 1:150), anti-CD18, ITGB2 (FITC 1:20), anti-CD49d, ITGA4 (APC-H7 1:80), anti-CD62L, L-selectin (V450 1:300), anti-CD106, VCAM1 (APC 1:10), anti-CD169 (APC 1:100), anti-CD206 (APC 1:100); Beckman-Coulter (Fullerton, CA): anti-CD29, ITGB1 (FITC 1:70); Bender MedSystems (Vienna, Austria): anti-CD62E, E-selectin (FITC 1:10); Biolegend (ITK Diagnostics, The Netherlands): anti-CD11a, ITGAL (APC 1:50), anti-CD11b, ITGAM (BV421 1:50); eBioscience (Vienna, Austria): anti-CD184, CXCR4 (PE 1:100); Miltenyi Biotec: anti-CD71 (VioBlue 1:200), anti-CD235a (VioBlue 1:200), propidium iodide (PI; 1:100); Pelicluster (Amsterdam, The Netherlands): anti-CD16 (FITC 1:100), anti-CD31, PECAM (FITC 1:10).

Mass spectrometry data acquisition and analysis
Cells were lysed in 100μl 4% SDS, 100mM DTT, 100mM Tris.HCl pH7.5 and processed into tryptic peptides using Filter Aided Sample Preparation1. Peptides were desalted and concentrated using Empore-C18 StageTips2 and eluted with 0.5%(v/v) acetic acid, 80%(v/v) acetonitrile. Sample volume was reduced by SpeedVac and supplemented with 2% acetonitrile, 0.1% TFA to a final volume of 12μl. 3μl was injected for mass spectrometry analysis. Tryptic peptides were separated by nanoscale C18 reverse phase chromatography coupled on line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific) via a nanoelectrospray ion source (Nanospray Flex Ion Source, Thermo Scientific). Peptides were loaded on a 20cm 75–360μm inner-outer diameter fused silica emitter (New Objective) packed in-house with ReproSil-Pur C18-AQ, 1.9μm resin (Dr Maisch GmbH). The column was installed on a Dionex Ultimate3000 RSLC nanoSystem (Thermo Scientific) using a MicroTee union formatted for 360μm outer diameter columns (IDEX) and a liquid junction. The spray voltage was set to 2.15kV. Buffer A was composed of 0.5% acetic acid and buffer B of 0.5% acetic acid, 80% acetonitrile. Peptides were loaded for 17min at 300nl/min at 5% buffer B, equilibrated for 5min at 5% buffer B (17-22min) and eluted by increasing buffer B from 5-15% (22-87min) and 15-38% (87-147min), followed by a 10min wash to 90% and a 5min regeneration to 5%. Survey scans of peptide precursors from 400-1500m/z were performed at 120K resolution (at 200m/z) with a 1.5x105 ion count target. Tandem mass spectrometry was performed by isolation with the quadrupole with isolation window 1.6, HCD fragmentation with normalized collision energy of 30, and rapid scan mass spectrometry analysis in the ion trap. The MS2 ion count target was set to 1.5x104 and the max injection time was 35ms. Only those precursors with charge state 2-7 were sampled for MS2. The dynamic exclusion duration was set to 60s with a 10ppm tolerance around the selected precursor and its isotopes. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 3s cycles. All data were acquired with Xcalibur software. The RAW mass spectrometry files were processed with the MaxQuant3 computational platform, version 1.5.2.8. Proteins and peptides were identified using the Andromeda search engine by querying the human
Uniprot database (release 2015-02, 89796 entries). Standard settings with the additional options match between runs, Label Free Quantification (LFQ), and only unique and razor peptides for quantification were selected. The generated ‘proteingroups.txt’ table was filtered for potential contaminants, reverse hits and ‘only identified by site’ using Perseus, version 1.5.1.6. The LFQ values were transformed in log2 scale and proteins were filtered for four valid values in at least one of the experimental groups. Missing values were imputed by normal distribution (width=0.3, shift=1.8), assuming these proteins were close to the detection limit. Quantitative significance (Principal Component Analysis and Volcano plot using an FDR of 5% and SO of 0.4) was performed by Perseus software. Interaction network analysis of the most differentially expressed proteins was performed using STRING (version 10) using all parameters and Score: 0.400. The identified network was uploaded into Cytoscape, version 3.5.1. Enrichment analysis of the most differentially expressed proteins was performed using the Cytoscape (version 3.5.1) plug-in BiNGO (version 3.0.3) with an FDR threshold of 0.05 and enrichment mapper (version 2.1.0) with a P-value cut-off of 0.005, an FDR Q-value cut-off of 0.005, an overlap coefficient of 0.5 and a combined constant of 0.5.

**IncuCyte data acquisition and analysis**

1.5x10^6 monocytes/well were seeded in a 12-well plate and cultured in the presence or absence of dexamethasone. After three days, cells were gently washed with PBS and incubated for 45min with CellTracker (ThermoFisher Scientific, Waltham, USA) Green CMFDA (5-chloromethylfluorescein diacetate) to monitor cell movement and location. Cells were co-cultured with 3x10^6 erythroblasts for 6hrs. The wells were gently washed to remove excess erythroblasts. For imaging, medium was changed into Iscove’s modified Dulbecco’s medium (IMDM without phenol red) supplemented with erythropoietin (4U/ml), holotransferrin (700µg/ml) and 30% human serum. Plates were mounted on the IncuCyte Zoom (Essen Biosciences) and once per hour real-time images at 25 spots per well were taken for a 68-hour time period. The spatial relationship between erythroblasts and macrophages was characterised using Fiji. Initially, lateral drift in the phase-contrast and fluorescence images over time was corrected using the StackReg plugin. A difference of Gaussian filter (approximating the equivalent Laplacian of Gaussian) was then applied to the phase-contrast channel to enhance features with diameters matching those expected for erythroblasts. Erythroblasts were subsequently identified with the TrackMate plugin using the Laplacian of Gaussian feature detector. Fluorescence channel images were processed with rolling-ball and Gaussian filters to remove inhomogeneity of illumination and high frequency noise, respectively. The images were then thresholded using the Otsu method with a user-defined fixed multiplier offset and passed through the ImageJ particle analyser to identify macrophages. Macrophages were tracked between frames using the Apache HBase (v1.3.1; Apache Software Foundation, https://hbase.apache.org) implementation of the Munkres algorithm with costs assigned based on object centroid separation. Instances where objects in the phase-contrast channel coincided with macrophages identified in the fluorescence channel were removed, as these likely corresponded to accidental detection of macrophages. Finally, spatial relationships between erythroblasts and macrophages were determined based on the maximum separation of object perimeters. Multiple erythroblasts could be assigned to a single macrophage.
RT-PCR analysis

The following primer sets were used: S18 (forward: 5’-GGACAACAAGCTCCGTGAAGA-3’, reverse: 5’-CAGAAGTGACGCAGCCCTCTA-3’), HPRT (forward: 5’-ATGGGAGCCCATCACATTGT-3’, reverse: 5’-ATGTAATCCAGCCCTCTA-3’), MERTK (forward: 5’-ACCTCTGTCGAATCCGCTGAAAG-3’, reverse: 5’-GTACTGTCCTCGGCCAAAG-3’), TYRO3 (forward: 5’-CAGCCGGTGAAAGCTCAACT-3’, reverse: 5’-TGGGCACCTTCTACCGTGAGA-3’), Tim3 (forward: 5’-GACTCTCAGCAGCCTTTCC-3’, reverse: 5’-GATCCCTGCTCCGATGTAGA3’), Lactadherin (forward: 5’-GACAAGCAGGGCAACTTCAAC-3’, reverse: 5’-CAGGATGGGCGTCTCAAACA-A3’), CD16 (forward: 5’-ACAGGTGCCAGACAAACCTC-3’, reverse: 5’-TTCCAGCTGTGACACCTCAG-3’), CD163 (forward: 5’-AATGGAAAAGGAGGCCATTC-3’, reverse: 5’-TGCTCCATTCAATAGTCCAGG-3’), CD169 (forward: 5’-GGGAGTACAAGTGCTCAGCC-3’, reverse: 5’-GCTTCTGCAGCTCAGTGTCA-3’), CXCR4 (forward: 5’-AGCAGGTACGCAAAGTGAC GC3’, reverse: 5’-ATAGTCCCTGAGCCCATTT-3’), and CD206 (forward: 5’-TCCTGGTTTTTGCTCGTGC T-3’, reverse: 5’-GCACTGGGACTCAGCTCAT-3’).

Supplementary references

Supplementary data

Online Supplementary Table S1. List of proteins expressed in GC-macrophages compared to non-glucocorticoid stimulated cells. In blue downregulated proteins, in red upregulated proteins, n=4 (see www.haematologica.org/content/103/3/395)

Online Supplementary Table S2. BiNGO analysis of GC-macrophages combined up and down regulated GOBP GCC GOMF. FDR<0.05, n=4 (see www.haematologica.org/content/103/3/395).

Table S3. Flow cytometry analysis of MFI macrophage marker expression on FL, BM, unstimulated cells (non-GC) and GC-macrophages. Unpaired t-test, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (n=3-7).

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<th>GC vs. non-GC</th>
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Figure S1. Macrophage population characteristics. (A) Representative images of cytospins (in 100x magnification) of CD14+ monocytes directly after isolation, cultured CD14+ cells in the absence (-Dex) or presence (+Dex) of dexamethasone. Note that monocytes are smaller compared to macrophages. (B) Relative mRNA expression of CD16, CD163, CD169, CXCR4, and CD206 on CD14+ cells (D0) cultured for three days (D3) in the presence or absence of dexamethasone (Dex) and/or 20µM mifepristone (Mif) as indicated. Mean ± SEM (ratio paired t-test, *P<0.05, **P<0.01, ***P<0.001). (C) Representative histograms belonging to Figure 1E showing CD16, CD163, CD169, CXCR4, CD206 and DC-SIGN expression on monocytes (n=3) directly after isolation (Day 0) and after culture in the presence or absence of dexamethasone (Dex) and/or mifepristone (Mif1, 1µM; Mif20, 20µM) compared to isotype control. (D) Gating strategy of multi-color flow cytometry experiments showing cells, single cells, and expression of CD16, CD163, CD169, CXCR4 and CD206. (E) CD14+ monocytes were cultured for three days in the presence of dexamethasone and subsequently treated with 1-20µM mifepristone for 4 or 24 hours. Graphs displaying the geometric mean fluorescence intensity (MFI) of CD16, CD163, CD169, CXCR4, CD206 and DC-SIGN (n=2-4). Mean ± SEM (ratio paired t-test, **P<0.01), values normalized to day 0 in Figure 1E.
Figure S2. Long-term macrophage cultures with dexamethasone. (A) Representative histograms of CD16, CD163, CD169, CXCR4, CD206 expression on differentiated CD14+ monocytes at day 3, 7 and 8 of culture in the presence of dexamethasone (n=4). (B) Representative dot plots show that CD14+ monocytes cultured for 17 days in the presence of dexamethasone maintain CD163, CD169, and CD206 expression (n=3).
Figure S3. Integrin expression on GC-macrophages and erythroid differentiation in co-culture with GC-macrophages. (A) Expression of ITGB2, ITGAL, ITGAM, ITGAX, E-selectin and L-selectin on GC-macrophages belonging to Figure 3A-B (n=6). Mean fluorescence intensity (MFI) has been normalized to the isotype control. Mean ± SEM. (B) Representative dot plots of erythroblasts at day 0, 1, and 7 of differentiation (Dif). Upon differentiation CD71 expression is reduced and CD235a expression is increased (left panel) while cells also start to enucleate as DRAQ5 stains DNA (right panel) (n=4). (C) Representative ImageStreamX images of the maturation process of erythroblasts at day 7 of differentiation where the nuclei (red, DRAQ5 staining) is expelled from the erythroid cell (n=3). (D) Co-culture of GC-macrophages (+Dex) or unstimulated cells (-Dex) with erythroblasts for 24 hours (n=4). Bars present the percentage of unbound macrophages or macrophages (Mφ) bound to 1 to 6 erythroblasts. Mean ± SD (paired t-test, *P<0.05, **P<0.01, ***P<0.001). (E) Co-culture of GC-macrophages or unstimulated cells with erythroblasts (unpaired t-test of 1153 macrophages (-Dex) and 749 (+Dex), n=5). Images were taken every hour during 64 hours of analysis. Plot showing the mean duration of contact between macrophages and erythroblasts.
Figure S4. Binding and phagocytosing capacity of GC-macrophages and unstimulated cells in co-culture with differentiated erythroid cells. Relative mRNA expression of TIM3 (A) and lactadherin (B) in cells from Figure 4A (n=4). Mean ± SEM (ratio paired t-test). (C-F) GC-macrophages or unstimulated cells were co-cultured for 24 hours with erythroid cells at day 6 of differentiation. Cytospins were analysed of 370 macrophages (-Dex) and 313 (+Dex) macrophages. (C) Percentage of macrophages (Mφ) that are unbound, or bound to nucleated cells, reticulocytes or pyrenocytes or a combination of erythroid cells. Mean ± SD (unpaired t-test, *P<0.05, n=3). (D) Percentage of macrophages that bind or phagocytose pyrenocytes. Mean ± SD (unpaired t-test, n=3). (E) Percentage of macrophages phagocytosing nucleated cells. Mean ± SD (paired t-test, n=3). (F) Percentage of macrophages phagocytosing reticulocytes. Mean ± SD (paired t-test, n=3).
Figure S5. Characterization of human BM and FL macrophages. Representative histograms belonging to Figure 5D showing the expression of macrophage markers CD14, CD16, CD163, CD169, CXCR4, CD206 and VCAM1 (filled) on human BM (A) and FL (B) compared to isotype control (unfilled).