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

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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-CD163 (PE 1:100), 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 Preparation[1]. Peptides were desalted and concentrated using Empore-C18 StageTips[2] 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 400-1500*m*/*z* were performed at 120K resolution (at 200*m*/*z*) with a 1.5×10^5 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 MS^2 ion count target was set to 1.5×10^4 and the max injection time was 35ms. Only those precursors with charge state 2-7 were sampled for MS^2. 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 MaxQuant[3] 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[4], 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 S0 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[5]. The identified network was uploaded into Cytoscape[6], version 3.5.1. Enrichment analysis of the most differentially expressed proteins was performed using the Cytoscape (version 3.5.1) plug-in BiNGO[7] (version 3.0.3) with an FDR threshold of 0.05 and enrichment mapper[8] (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[9,10]. Initially, lateral drift in the phase-contrast and fluorescence images over time was corrected using the StackReg plugin[11]. A difference of Gaussian filter (approximating the equivalent Laplacian of Gaussian12) 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[13]. 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[14] 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[15]. 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'-ATGGGAGGCCATCACATTGT-3', reverse: 5'-ATGTAATCCAGCCAGGTCAGCAA-3'), MERTK (forward: 5'-ACCTCTGTCGAATCAAAGCCC-3', reverse: 5'-GCACACTGGTTATGCTGA-AGGA-3'), AXL (forward: 5'-GTGGGCAACCCAGGGAATATC-3', reverse: 5'-GTACTGTCCCGTGTCGGAAAG-3'), TYRO3 (forward: 5'-CAGCCGGTGAAGCTCAACT-3', reverse: 5'-TGGCACACCTTCTACCGTGA-3'), TIM3 (forward: 5'-GACTTCACTGCAGCCTTTCC-3', reverse: 5'-GATCCCTGCTCCGATGTAGA3'), Lactadherin (forward: 5'-GACAAGCAGGGCAACTTCAAC-3', reverse: 5'-

CAGGATGGGCGTCTCAAACAA3'), 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'-AGCAGGTAGCAAAGTGACGC-3', reverse: 5'-ATAGTCCCCTGAGCCCATTT-3'), and CD206 (forward: 5'-TCCTGGTTTTTGCCTCTGTC-3', reverse: 5'-GCACTGGGACTCACTGCAT-3').

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Online Supplementary Table Legends

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).

Online Supplementary Table S2. BiNGO analysis of GC-macrophages combined up and down regulated GOBP GOCC GOMF. FDR<0.05 (n=4).

Online Supplementary 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).

Table S3				
	MFI mean±SEM	**Experiments**	**P-value**	**Significance**
CD14				
BM vs FL	5178±907.7 / 6642±2900	N=5 / N=4	0.6127	NS
GC vs non-GC	25545±1825 / 21606±4664	N=4 / N=4	0.4616	NS
GC vs BM	25545±1825 / 5178±907.7	N=4 / N=5	<0.0001	****
GC vs FL	25545±1825 / 6642 ± 2900	N=4 / N=4	0.0015	**
Non-GC vs BM	21606±4664 / 5178±907.7	N=4 / N=5	0.0060	**
Non-GC vs FL	21606±4664 / 6642±2900	N=4 / N=4	0.0344	*
CD16				
BM vs FL	1803±342.4 / 8748±1695	N=4 / N=4	0.007	**
GC vs non-GC	3380±658 / 206.4±70.64	N=6 / N=5	0.0019	**
GC vs BM	3380±658 / 1803±342.4	N=6 / N=4	0.1062	NS
GC vs FL	3380±658 / 8748±1695	N=6 / N=4	0.0092	**
Non-GC vs BM	206.4±70.64 / 1803±342.4	N=5 / N=4	0.0014	**
Non-GC vs FL	206.4±70.64 / 8748±1695	N=5 / N=4	0.0007	***
CD163				
BM vs FL	49689±7048 / 38955±6209	N=7 / N=5	0.3036	NS
GC vs non-GC	947777±24328 / 3334±1151	N=7 / N=5	0.0107	*
GC vs BM	947777±24328 / 49689±7048	N=7 / N=7	0.1004	NS
GC vs FL	947777±24328 / 38955±6209	N=7 / N=5	0.0891	NS
Non-GC vs BM	3334±1151 / 49689±7048	N=5 / N=7	0.0003	***
Non-GC vs FL	3334±1151 / 38955±6209	N=5 / N=5	0.0005	***
CD169				
BM vs FL	2053±972 / 739.6±270.1	N=7 / N=5	0.2948	NS
GC vs non-GC	3705±1272 / 186.7±107.4	N=6 / N=5	0.0341	*
GC vs BM	3705±1272 / 2053±972	N=6 / N=7	0.3168	NS
GC vs FL	3705±1272 / 739.6±270.1	N=6 / N=5	0.0675	NS
Non-GC vs BM	186.7±107.4 / 2053±972	N=5 / N=7	0.1418	NS
Non-GC vs FL	186.7±107.4 / 739.6±270.1	N=5 / N=5	0.0937	NS
CXCR4				
BM vs FL	11415±3563 / 2155±1368	N=3 / N=3	0.0723	NS
GC vs non-GC	19596±4359 / 9769±4426	N=7 / N=4	0.1774	NS
GC vs BM	19596±4359 / 11415±3563	N=7 / N=3	0.2896	NS
GC vs FL	19596±4359 / 2155±1368	N=7 / N=3	0.0362	*
Non-GC vs BM	9769±4426 / 11415±3563	N=4 / N=3	0.7956	NS
Non-GC vs FL	9769±4426 / 2155±1368	N=4 / N=3	0.2147	NS
CD206				
BM vs FL	2618±1672 / 7569±3026	N=4 / N=5	0.2263	NS
GC vs non-GC	15144±1103 / 55±30.74	N=4 / N=4	<0.0001	****
GC vs BM	15144±1103 / 2618±1672	N=4 / N=4	0.0008	***
GC vs FL	15144±1103 / 7569±3026	N=4 / N=5	0.0713	NS
Non-GC vs BM	55±30.74 / 2618±1672	N=4 / N=4	0.1764	NS
Non-GC vs FL	55±30.74 / 7569±3026	N=4 / N=5	0.0647	NS
VCAM1				
BM vs FL	4524±2338 / 898±439.1	N=4 / N=4	0.1783	NS
GC vs non-GC	30.33±11.22 / 0±0	N=3 / N=3	0.0538	NS
GC vs BM	30.33±11.22 / 4524±2338	N=3 / N=4	0.1652	NS
GC vs FL	30.33±11.22 / 898±439.1	N=3 / N=4	0.1558	NS
Non-GC vs BM	0±0 / 4524±2338	N=3 / N=4	0.1629	NS
Non-GC vs FL	0±0 / 898±439.1	N=3 / N=4	0.1445	NS

Online Supplementary Figure Legends

Online Supplementary 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.

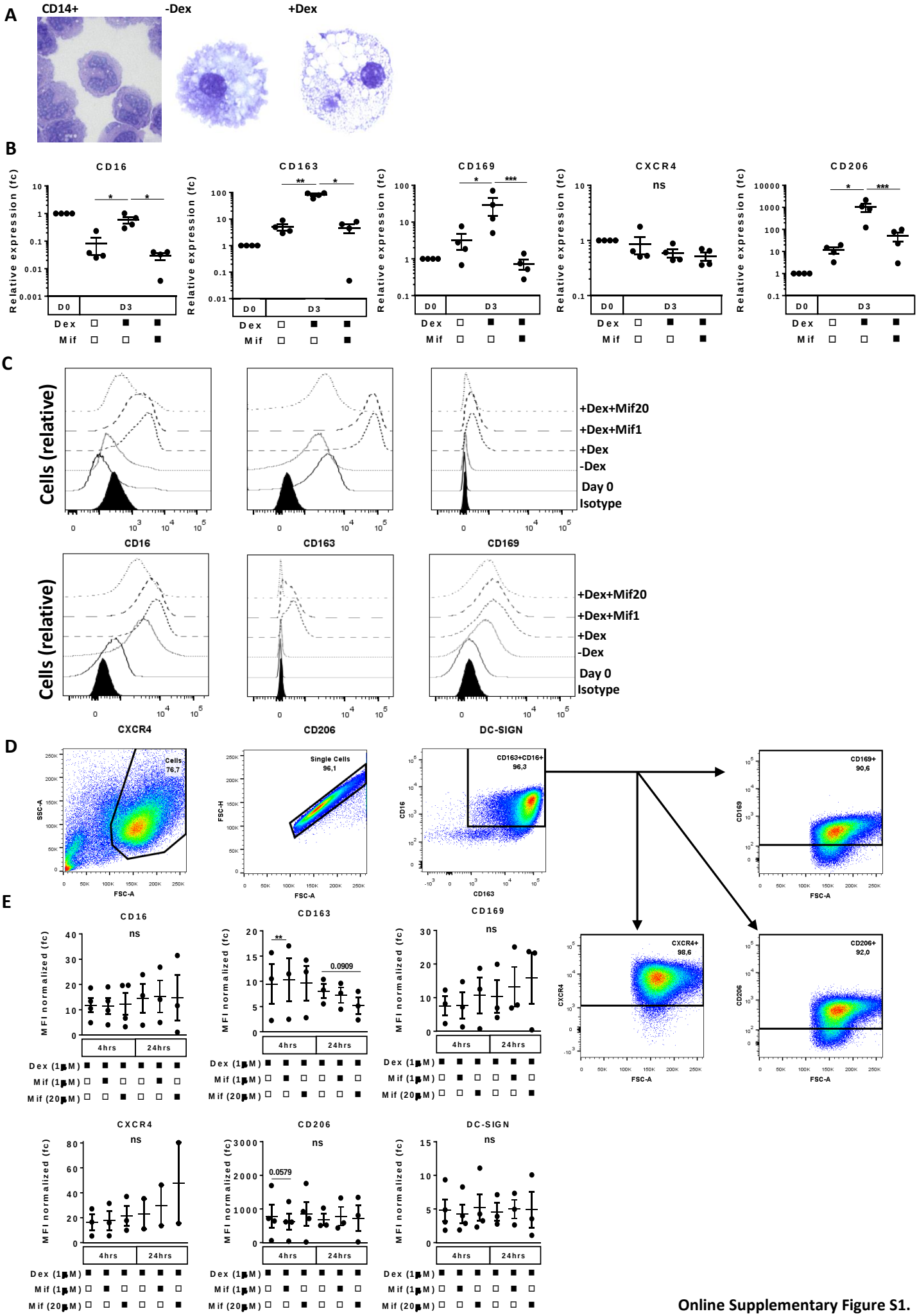
Online Supplementary Figure S2. Long-term macrophage cultures with dexamethasone. (A) Representative histograms of CD16, CD163, CD169, CXCR4, CD206 expression on differentiated $CD14^+$ monocytes at day 3, 7 and 8 of culture in the presence of dexamethasone (n=4). **(B)** Representative dot plots show that $CD14^+$ monocytes cultured for 17 days in the presence of dexamethasone maintain CD163, CD169, and CD206 expression (n=3).

Online Supplementary 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.

Online Supplementary 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

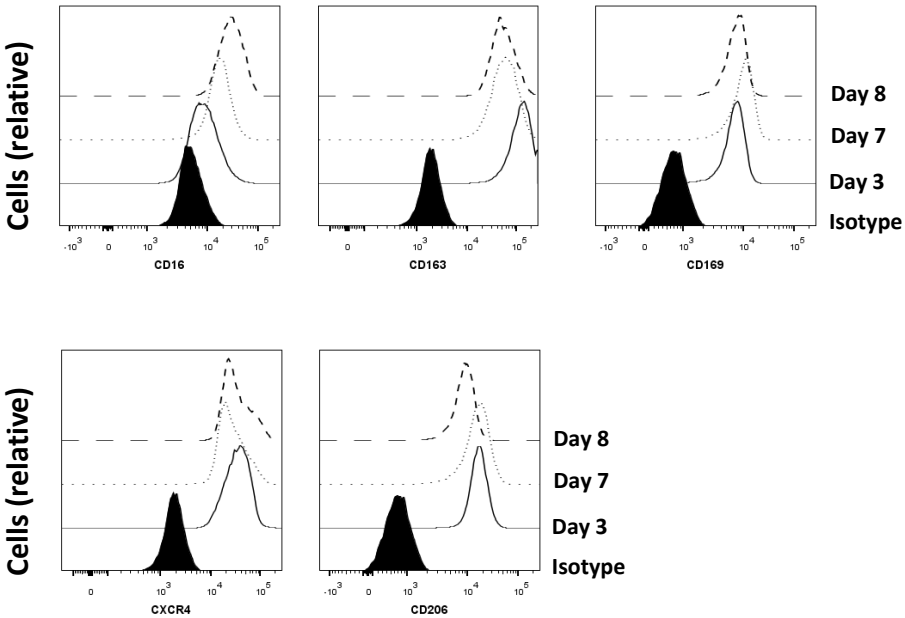
macrophages (Mφ) that are unbound, or bound to nucleated cells, reticulocytes or pyrenocytes or a combination of erythroid cells. Mean ± SD (Unpaired T-test, *p<0.05, n=3). **(D)** Percentage of macrophages that bind or phagocytose pyrenocytes. Mean ± SD (Unpaired T-test, n=3). **(E)** Percentage of macrophages phagocytosing nucleated cells. Mean ± SD (Paired T-test, n=3). **(F)** Percentage of macrophages phagocytosing reticulocytes. Mean ± SD (Paired T-test, n=3).

Online Supplementary 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).



Online Supplementary Figure S1.

A



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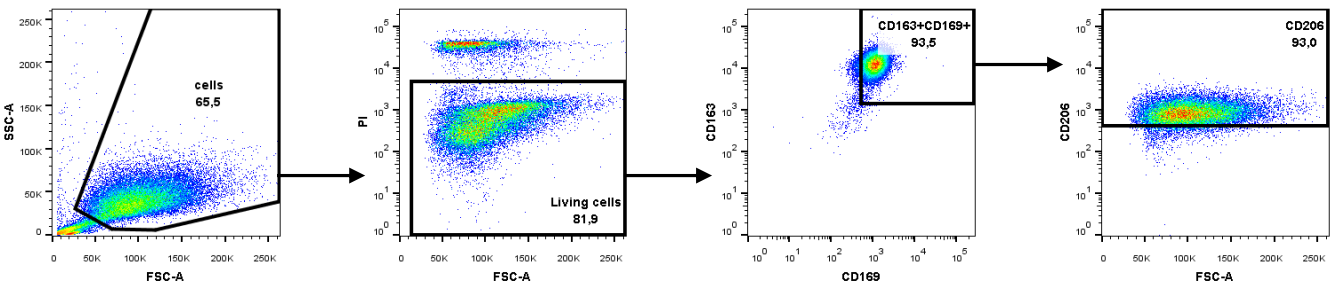

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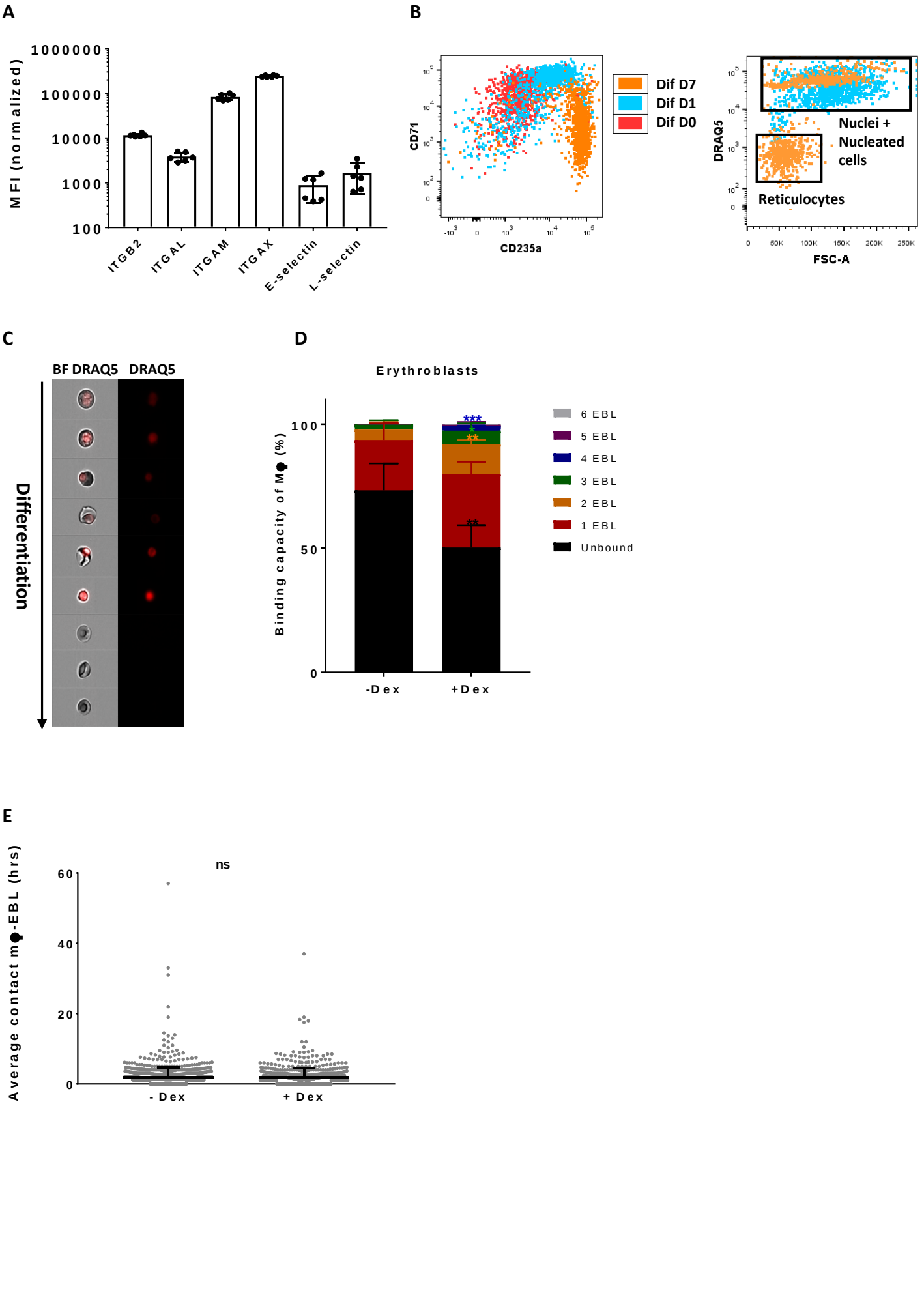

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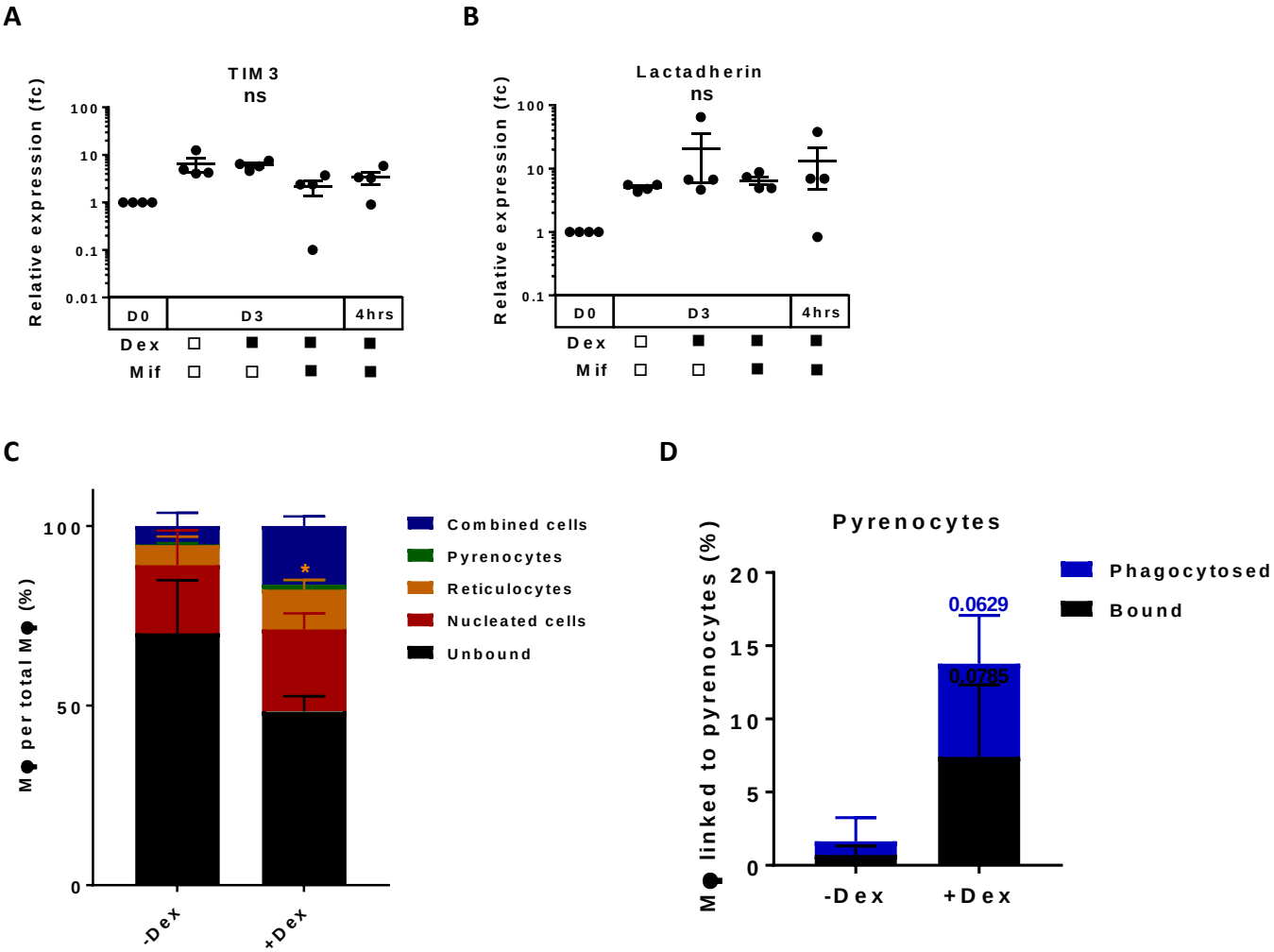

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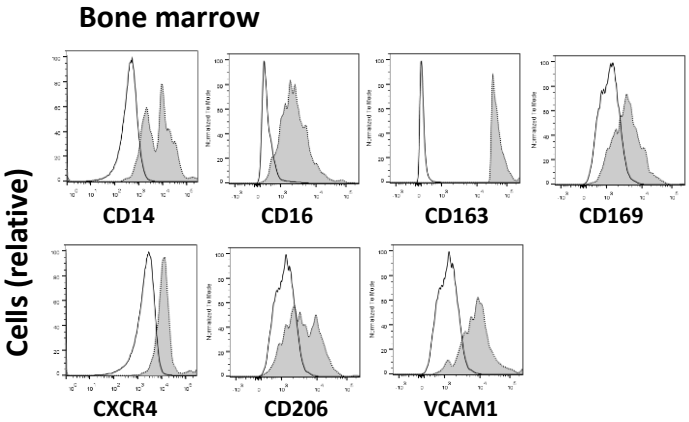
A



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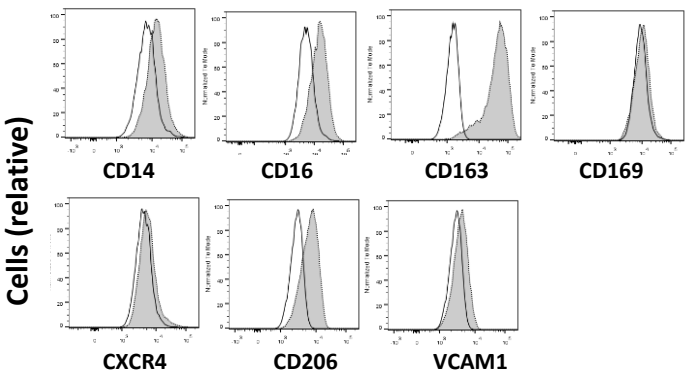

Online Supplementary Figure S5.