SUPPLEMENTARY APPENDIX

Dexamethasone targeted directly to macrophages induces macrophage niches that promote erythroid expansion

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SUPPLEMENTAL INFORMATION

MATERIALS AND METHODS.

Human specimens and cell preparation. Buffy-coats from 12 whole blood donations were obtained as by-products of platelet production and provided as de-identified material according to guidelines established by the institutional ethical committees. Mononuclear cells (MNC) were isolated by Ficoll-Paque (Sigma, St. Louis, MO) centrifugation, cryopreserved in Iscove's modified Dulbecco's medium (IMDM, 40% vol/vol, Gibco-Invitrogen, Carlsbad, CA), fetal bovine serum (FBS, 50% vol/vol, Sigma) and dimethylsulphoxide (10% vol/vol, Sigma) and stored in liquid nitrogen. Thawed MNC were cultured either as such or after CD14 depletion and CD34 enrichment with the FACS Aria (Becton Dickinson, Franklin Lakes, NJ) (>98% $CD34^{pos}$ cells by re-analyses), as described[17,18].

Human Erythroid Massive Amplification (HEMA) culture. $CD34^{pos}$ cells (10^4 cells/mL) or MNC (10^6 cells/mL) were cultured in IMDM plus FBS and stimulated with SCF (100 ng/mL, Amgen, Thousand Oaks, CA), IL-3 (1 ng/mL, RD System, Minneapolis, MN), EPO (5 U/mL, Janssen, Raritan, NJ), dexamethasone and estradiol (both 10^{-6} M, Sigma) for 10-14 days at 37 °C and 5% pCO_2 in a fully humidified incubator, as described[19]. In selected experiments, Erys were cultured in HEMA supplemented with either the GR inhibitor RU486 (5-50 μM)[35], or the FAK inhibitor PF-562671 (2, 20 or 200 μM)[36], or to CD163 or to Dex directly conjugated to CD163 (at a concentration equivalent to 10^{-6} M of free Dex)[38].

Determinations of cell numbers, viability and phenotype. Cell numbers and viability were determined by microscopic evaluation of cells stained with trypan blue (Boston Bioproducts, Ashland, MA) in a Burker chamber. Maturation of erythroid cells was assessed by flow cytometry on the basis of CD235a (glycophorin A) and CD36 (the thrombospondin receptor) expression and confirmed by visual examination of cytospins (Cytospin 3, Shandon, Astmoor, England). Macrophages were characterized on the basis of CD14/CD16/CD163/CD169 expression. FACS criteria used to positively identify macrophages in the aggregate gate of HEMA cultures are described in the legend to **Figure S1**. All antibodies were from BD-Pharmigen (San Diego, CA). Size determinations were performed by comparing the mean forward scatter amplitude (FSC-A) of the cells with that of calibration beads (Flow Cytometry Size Calibration Kit, Invitrogen Molecular Probes, Eugene, OR)[4,27]. Dead cells were excluded by Sytox Blue staining (0.002 mM, Molecular Probes). Cell cycle distribution was evaluated on the basis of propidium iodide (PI, Sigma) incorporation by cells fixed overnight with 70% ice-cold ethanol. Fluorescence was measured with a FACS Aria and data analyzed with the FlowJo software (Tree Star, Inc., Ashland, OR).

(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. ProErys (10^5 $CD36^{pos}CD235a^{neg}$ cells/100 μL/well) and macrophages (600-10,000 $CD14^{pos}$ cells/100 μL/well) were isolated by FACS from day 10 HEMA culture and cultured either alone or in combination for additional 24h in HEMA. After 24h, MTT (5 mg/mL, Sigma) was added to each well and the colorimetric reaction read as optical density (OD) at 570 nm with Victor (Victor^{3}TM, 1420 Multilabel Counter, Perkin Elmer, Waltham, MA).

Time lapse video microscopy. Cells were placed in 32 mm dishes and cultured for 3-24h in an Okolab incubator chamber (Okolab, Naples, Italy) equipped with an Olympus IX 81 fully motorized inverted microscope (Olympus, Tokyo, Japan). Phase-contrast observations were performed with 10x or 20x objectives, recorded every 30'', unless specified otherwise, with the Hamamatsu digital CCD camera (Hamamatsu Photonics KK, Hamamatsu City, Japan) and processed with the Cell_R software (Olympus). Criteria used for cell identification by phase-contrast microscopy were validated by the excellent correlation existing between size, morphology and frequency of cells identified by phase-contrast microscopy and by FACS **(Figures S1 and S2).** Quantitative data were obtained with the open source software ImageJ (http://imagej.nih.gov/ij vers. 1.46).

Trasmission Electron Microscopy (TEM). Cells were fixed and embedded in Spurr resin (Poliscience, Warrington, PA) according to standard procedures[28]. Ultrathin sections were mounted

on 300 mesh nickel grids, counterstained with lead citrate and uranyl acetate, observed with a 109 TE microscope (Zeiss, Oberkochen, Germany) and photographed with SC200 TEM CCD Orius camera (GATAN, Munchen, Germany) .

Reverse-Phase Protein Array (RPPA) and Western Blot (WB) Analysis. RPPA were constructed and analyzed as previously described[29,30]. Briefly, all samples were printed in triplicate spots on nitrocellulose-coated glass slides (GRACE Bio-Labs, Bend, OR) using an Aushon 2470 equipped with 185-µm pins (Aushon Biosystems, Billerica, MA), according to the manufacturer's instructions. Reference standard lysates, comprised of HeLa + Pervanadate (BD, Franklin Lakes, NJ), Jurkat + Etoposide (Cell Signaling, Danvers, MA) and Jurkat + Calyculin A (Cell Signaling) were printed in 14-point or 10-point dilution curves as procedural controls and as positive controls for antibody staining. Each reference standard curve was printed in triplicate at concentrations of 0.5 µg/µL and 0.125 µg/µL. A selected subset of the printed array slides were stained with Sypro Ruby Protein Blot Stain (Invitrogen, Carlsbad, CA) to estimate sample total protein concentration, and the remaining slides were stored under desiccated conditions at −20°C. Immediately prior to antibody staining, printed slides were treated with 1x RE-Blot Mild Solution (Chemicon, Temecula, CA) for 15', washed 2 x 5' with PBS (Invitrogen, Carlsbad, CA) and incubated for 1h in blocking solution (2% I-Block, (Applied Biosystems, Foster City, CA), 0.1% Tween-20 in PBS). Immunostaining was carried out using a signal amplification kit (DAKO, Carpinteria, CA). Arrays were probed with a library of almost 180 antibodies against antigens to total, cleaved and phosphorylated protein targets. Primary antibody binding was detected using a biotinylated goat anti-rabbit IgG H+L (1:7500) (Vector Laboratories, Burlingame, CA) or rabbit anti-mouse IgG (1:10) (DAKO) followed by streptavidin-conjugated IRDye680 fluorophore (LI-COR Biosciences, Lincoln, NE). Primary antibodies were extensively validated for single band specificity by western blot using complex cellular lysates. Negative control slides were incubated with secondary antibody only. All Sypro and immunostained slides were scanned using a Tecan power scanner™ (Tecan Group Ltd, Switzerland). Acquired images were analyzed with MicroVigene v5.0. (VigeneTech, Carlisle, MA) for spot detection, local background subtraction, negative control subtraction, replicate averaging and total protein normalization. The software package JMP v6 (SAS Institute, Cary, NC) was used to carry out internal standardization, two-way hierarchical clustering using Wards method, and two-groups Wilcoxon Rank-Test (significance cut off p-val<=0.05) . For WB analyses, whole cell protein extracts (30 µg/lane) were separated on SDS-PAGE and transferred to nitrocellulose membranes which were incubated with antibodies for either total (#sc-8992, Santa Cruz) or phosphorylated form (#4161, Cell Signaling) of GRα or GAPDH (#CB1001, Calbiochem, San Diego, CA) as loading control. Membranes were then incubated with appropriate horseradish peroxidase-coupled secondary antibodies (Calbiochem) and immune complexes detected with an enhanced chemiluminescence kit (Amersham, Buckingamshire, UK).

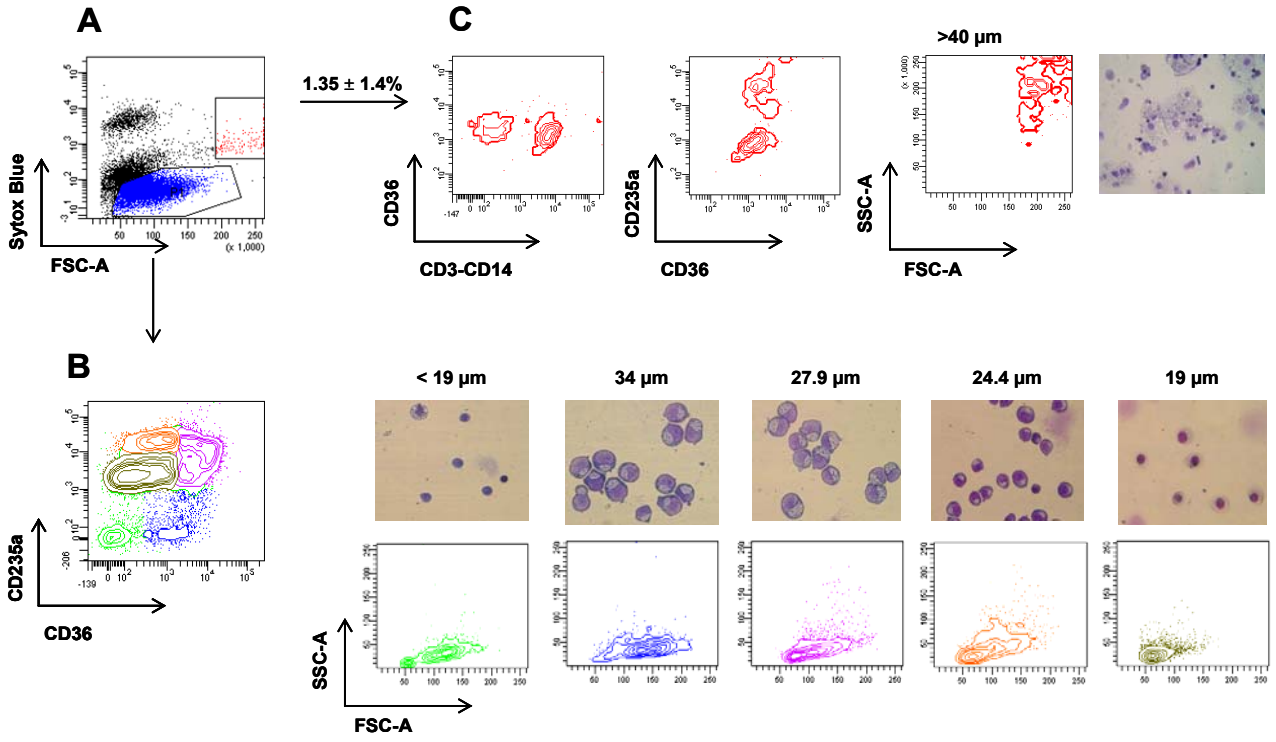
SUPPLEMENTARY FIGURES

Figure S1: Validation of FACS criteria for positive identification of macrophages in HEMA culture.

A) Forward scatter (FSC-A) and Sytox Blue staining of cells present at day 14 in HEMA culture of adult blood. The analysis identifies three populations: Sytox Blue positive cells (in black), corresponding to dead cells; Sytox Blue negative/high FSC-A cells, corresponding to cell aggregates (in red) and Sytox Blue negative/low-medium FSC-A cells (in blue), corresponding to single cells.

B) FACS analyses for CD36/CD235a expression of Sytox Blue negative/low-medium FSC-A cells. Cells were prospectively isolated on the basis of levels of CD36/CD235a expression and their identity confirmed by morphological analyses (after May-Grunwald staining). Their size was also determined (FSC-A/SSC-A analyses against beads of expected size). Events in the single cell gate include five populations: $CD36^{neg}CD235a^{neg}$ cells, lymphocytes (bright green); $CD36^{pos}CD235a^{neg}$ cells, proErys (blue); $CD36^{pos}CD235a^{high}$ cells, basoErys (purple); $CD36^{neg}CD235a^{high}$ cells, polyErys (orange) and $CD36^{neg}CD235a^{low}$ cells, orthoErys (green) but not macrophages. The size of the different cell populations is indicated above the quadrants.

C) FACS analyses for CD3-CD14/CD36/CD235a expression of events in the aggregate gate (Sytox Blue negative/high FSC-A cells). Events were re-analyzed for FSC-A/SSC-A for size determination and prospectively isolated for morphological identification by May-Grunwald staining. Events in this gate express CD14, CD36 and CD235a, are >40 µM in size and have the morphology of macrophages some of which surrounded by Erys. Results are representative of those obtained in at least 4 separate cultures two of which seeded with CD14-depleted $CD34^{pos}$ cells purified from adult blood.



Previous studies have failed to detect the presence of macrophages by day 10-14 of HEMA culture[4,19,20]. By contrast, we regularly observed few macrophages often clustered with Erys, on cytospin preparations of these cultures. We hypothesized that failure to detect macrophages by FACS could be a bias introduced by routine analyses of events included only in single cell gates. This hypothesis was tested by the FACS analysis of events present both in the single cell gate and in the aggregate gate presented in this figure. As previously reported[4,19,20], the majority of cells in the single cell gate expressed the erythroid markers CD36 and CD235a and was composed by Erys

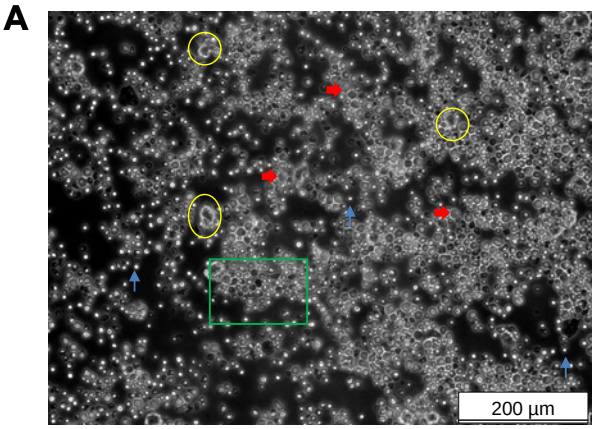
at all stages of maturation ranging in diameter from 34 to 19 μm (**Figure S1B**). The non erythroid ($CD36^{neg}CD235a^{neg}$) cells included in this gate were lymphocytes. Few (1.3±1.4%), events were consistently detected in the aggregate gate which is routinely excluded from FACS analyses. These events included clusters expressing both macrophagic (CD14) and erythroid (CD235a) markers and had an average diameter >40 μm (**Figure S1C**). These clusters expressed CD235a at levels greater than those expressed by single erythroid cells (compare CD235a expression in **Figure S1B** and **1C**), suggesting that they may include more than one Ery each. Morphological analyses of prospectively isolated clusters confirmed that they are formed by macrophages surrounded by several Erys at all stages of maturation. These static determinations do not allow assessment of whether these clusters existed already in culture or were formed ex-novo during the cell preparation for FACS analyses. This point is clarified by data presented in **Figure 1** and **Movies 1** and **2**

Figure S2. Validation on the basis of size, morphology and frequency of the identity of cells observed by phase-contrast microscopy in HEMA culture.

A) Photograph of a representative phase-contrast microscopic recording of cells at day 14 of HEMA culture. Ovals, rectangle, red and blue arrows indicate fields shown at greater magnifications in B.

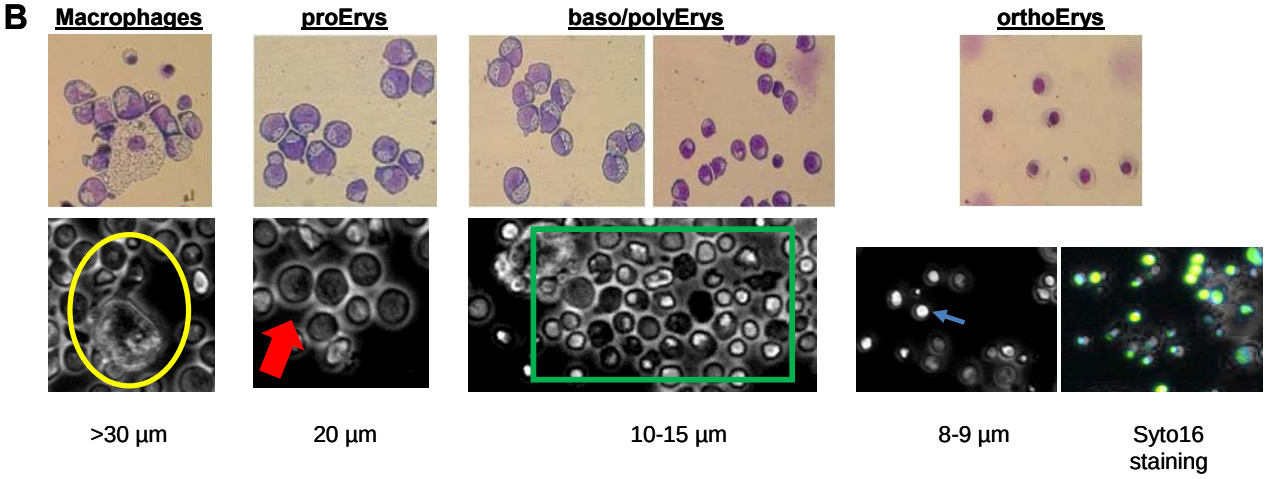
B) Representative May-Grunwald (top panels) and phase-contrast fields (bottom panels) shown at the same magnification (final magnification 200x). The numbers below the panels indicate the average cell diameter calculated on the basis of phase-contrast determinations. Cells 8-9 µm in diameter were confirmed to be orthoErys by Syto-16 staining.

C) Comparison of the frequencies of macrophages, proErys, baso-polyErys and orthoErys as determined by FACS and phase-contrast microscopy. Results are presented as mean (±SD) of three-five separate experiments.



C

	FACS %	Microscopy %
Macrophages	1.3±1.4	2.3±1.1
proErys	12.0±4.1	18.5±5.1
baso/polyErys	49.4±2.5	48.1±8.9
orthoErys	42.6±3.5	31.0±12.2



The extensive characterization of size, morphology and frequency of the cells present in HEMA culture over the years[4,26] was instrumental to validate the identity of cells identified by phase-contrast microscopy (**Figure S2A and B**). Four populations were positively recognized on time-lapse recording of day 10-14 of HEMA culture:

- Macrophages: cells larger than Erys (>30 µm) representing ≅ 3% of the total population. Additional morphological features of these cells were high motility and ability to continuously change shape.
- ProErys: cells approximately 20 µm in diameter representing 12-18% of the total population. These cells had a round shape and lacked a distinctive nuclear area.
- Basophylic/polychromatophylic (baso/poly) Erys: cells 10-13 µm in diameter representing 48-49% of the total population. These cells had various shapes and contained a clearly recognizable brilliant nuclear area, an indication of partial chromatin condensation, localized at one pole.

- Orthochromatic (Ortho) Erys: cells 8-9 μm in diameter representing ≅ 30-40% of the total population. These cells were round with a very brilliant nuclear area that reacted with Syto-16, a property reflecting highly condensed chromatin state.

Good correlations existed among morphology and frequency determined by FACS and phase-contrast microscopy (**Figure S2C**). Cell size determinations obtained by phase-contrast microscopy were instead systematically lower than those obtained by FACS (**Figure S1B** and S**2B**). This apparent discrepancy may reflect the lower resolution of cell boundaries by visual compared to laser determinations.

Figure S3. CD14 depleted CD34^{pos} cells generate consistent numbers of macrophages under erythroid-restricted culture conditions supplemented with Dex. May-Grunwald staining and FACS analyses of the cells present at day 17 in HEMA cultures seeded with CD14^{neg}CD34^{pos} cells purified from adult blood.

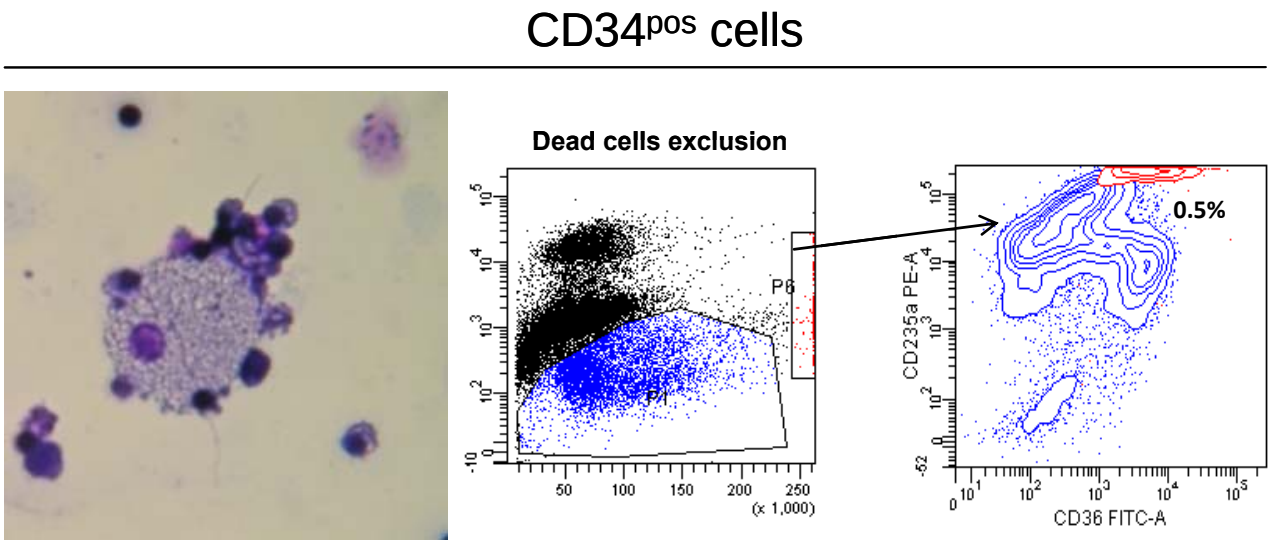
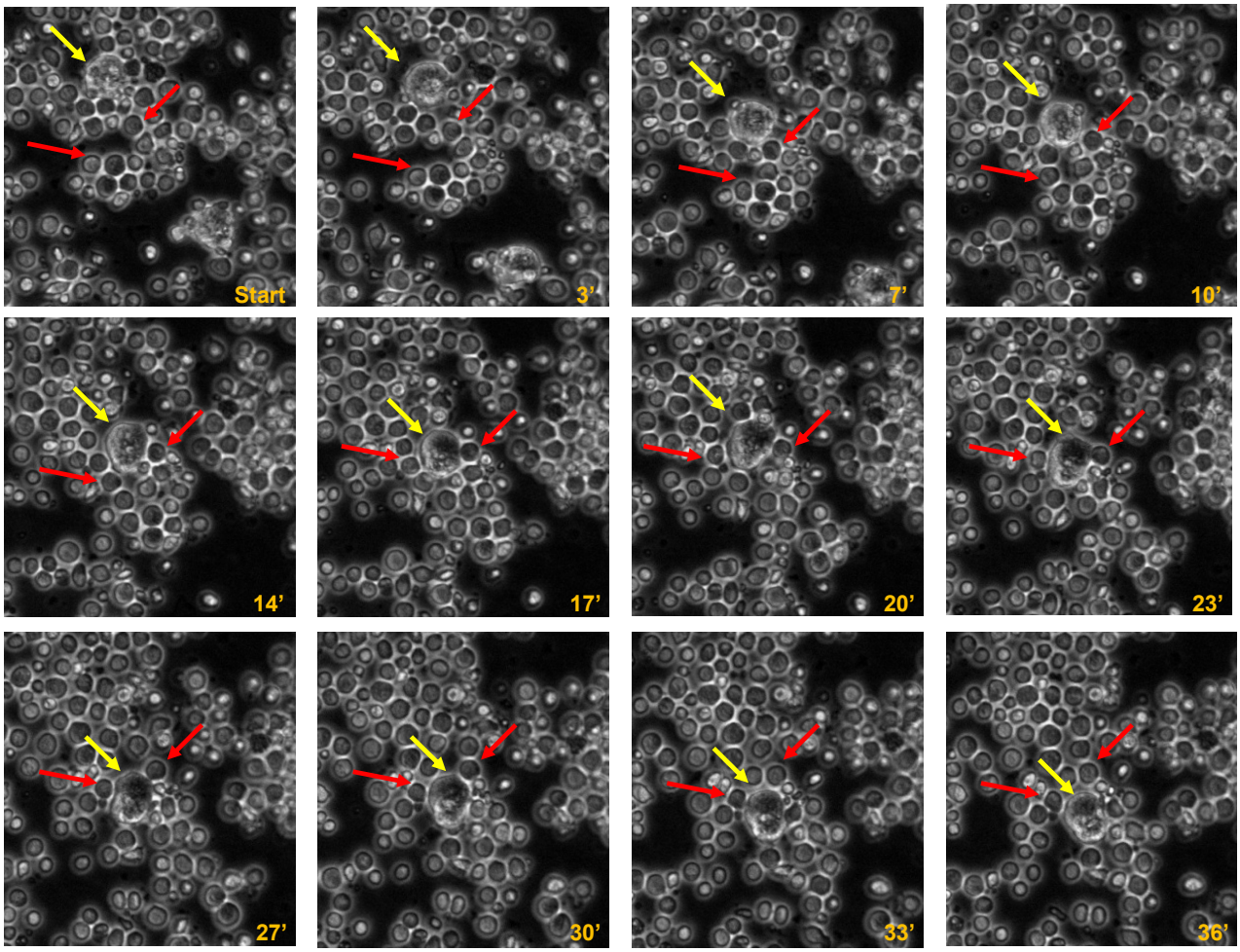
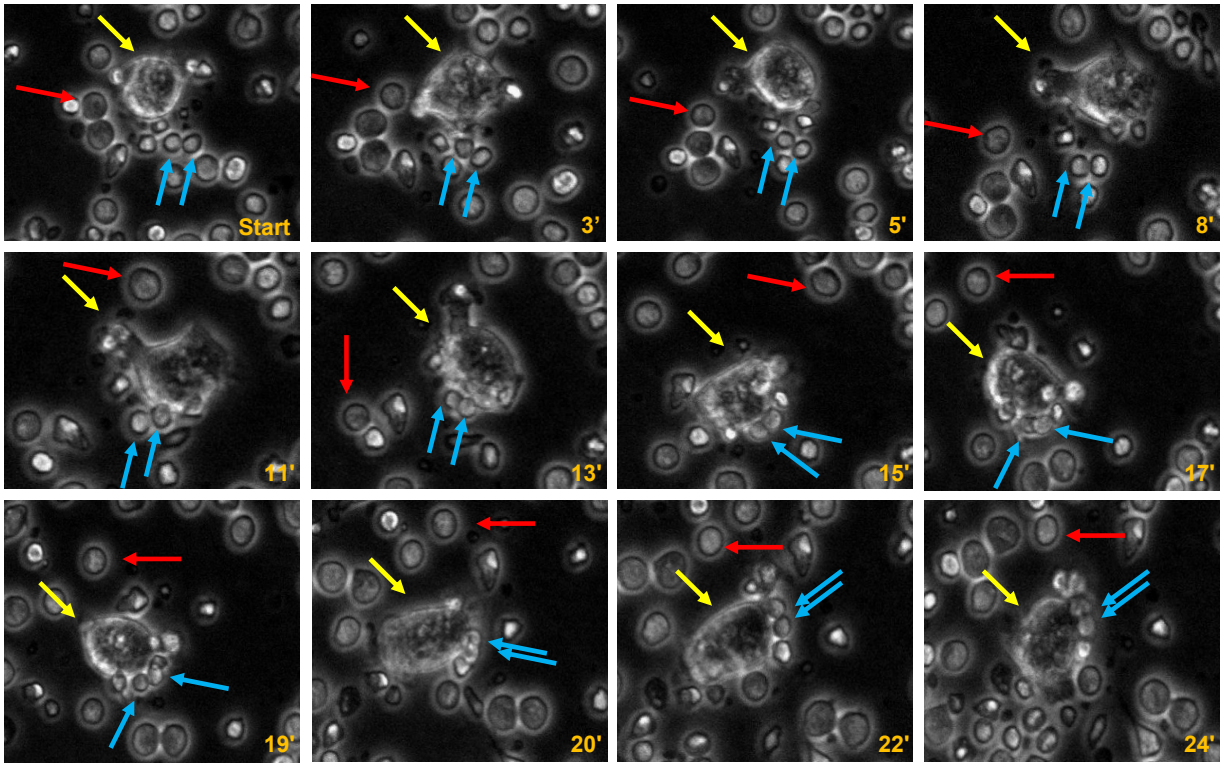


Figure S4. Dynamics of interactions occurring among macrophages and Erys in HEMA colture. A) Dynamics of "loose" interactions between one macrophage (yellow arrow) and preformed proEry clusters (red arrows). Frames selected from 1h of image recording (see **Movie 3)**. B) Dynamics of "tight" interactions occurring between one macrophage (yellow arrow) and mature Erys (blue arrows). Red arrows indicate by-standing proErys which are not participating in the process. Frames selected from 2h of image recording (see **Movie 4**). Timing is reported at the bottom of each frame. C) Dynamics of "tight" interactions occurring between one macrophages (yellow arrow) and mature Erys labeled with FITC-CD235a leading to Ery phagocytosis. Red arrows indicate by-standing proErys undergoing cytokinesis. Frames selected from 2h of image recording (see **Movie 5**). Timing is reported at the bottom of each frame.

S4A



S4B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17'
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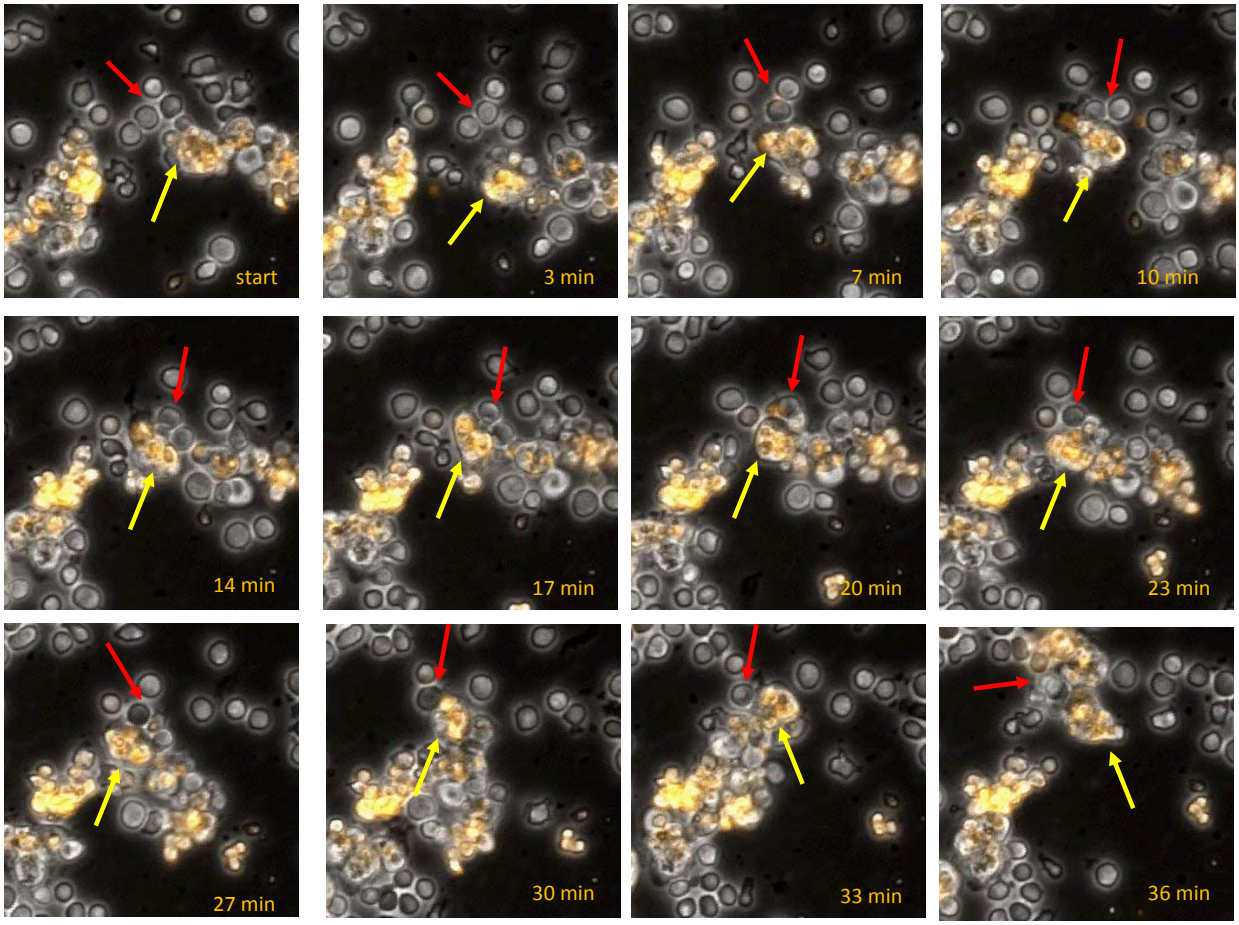
S4C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14 min
17 min
20 min
23 min
27 min
30 min
33 min
36 min

Figure S5. During "loose" interactions, macrophages and proErys maintain distinctive cell membrane boundaries with a clearly, although tight, recognizable intercellular space while "tight" interactions led to phagocytosis of mature Erys by macrophages. Representative TEM photographs depicting "loose" (on the right) and "tight" (on the left) interactions occurring among cells at day 14 of HEMA culture. The area with the rectangle depicting the cell contact in a loose interaction is shown at greater magnification to highlight the membrane boundaries between the two cells. M = macrophage; yellow arrow = proEry; red arrows = Erys embedded within the macrophage cytoplasm.

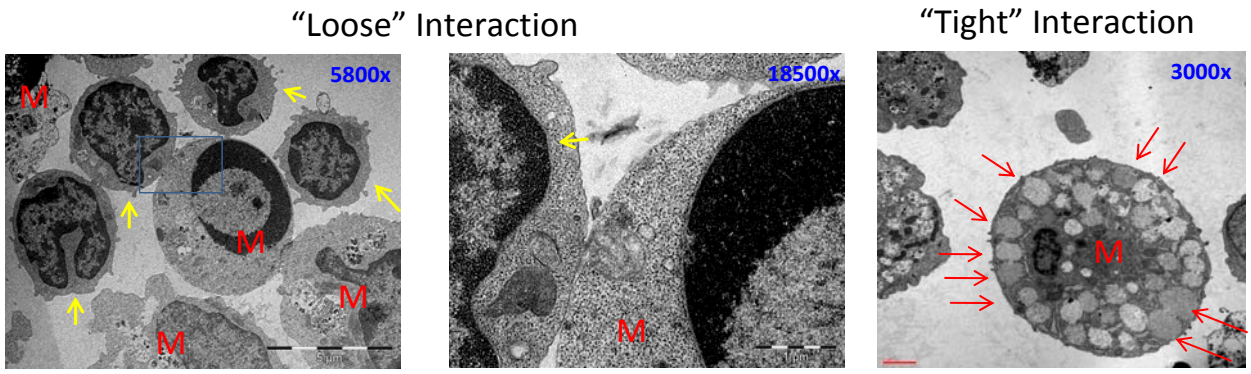
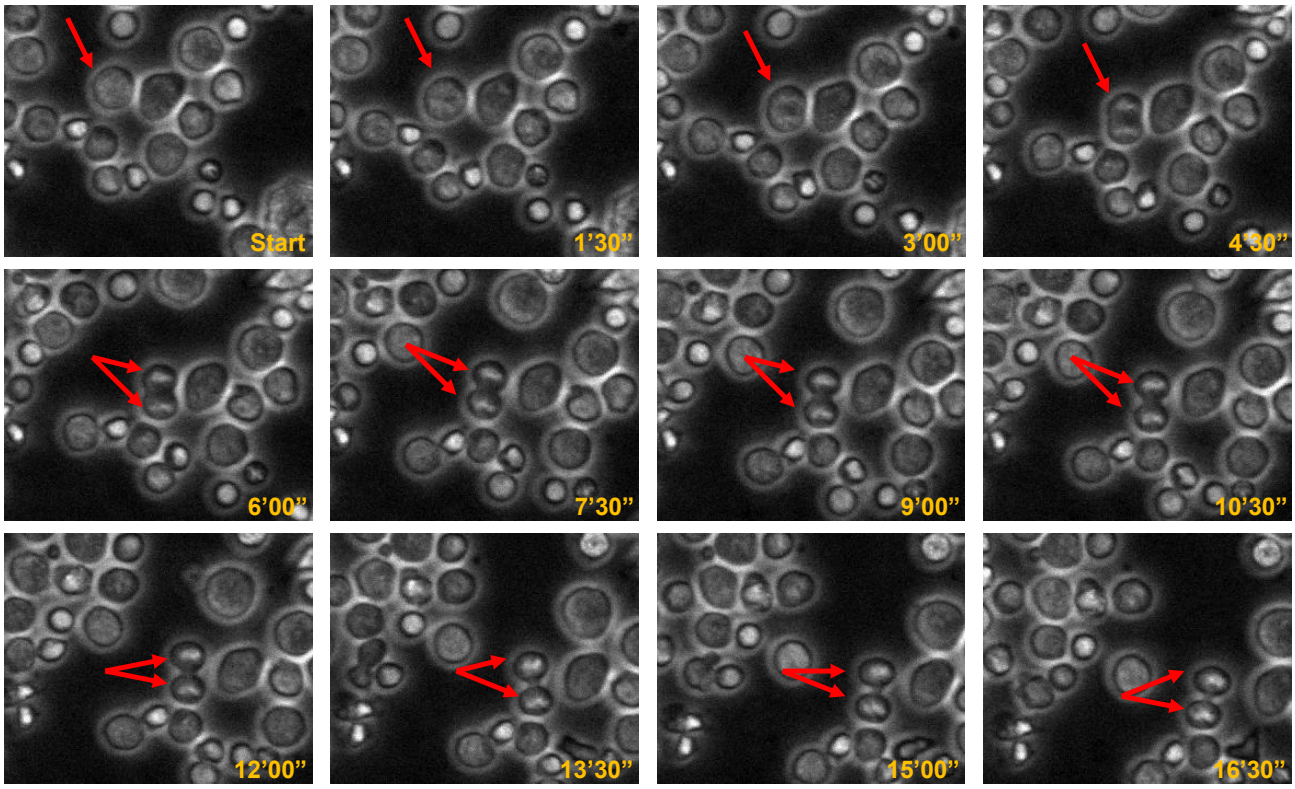


Figure S6. Dynamics of events leading to cytokinesis identified by time lapse videomicroscopy in HEMA culture. A) Cytokinesis of an isolated proEry. Frames are selected from 15' of image recording (see **Movie 6).** B) Cytokinesis of one proEry doublet. Frames selected from 30' of image recording (see **Movie 7).** Timing is reported at the bottom of each frame. Legend: yellow arrow: macrophage; red arrow: proEry; blue arrow: orthoEry; green and white arrowhead: first and second cytokinesis of the proEry doublet.

A



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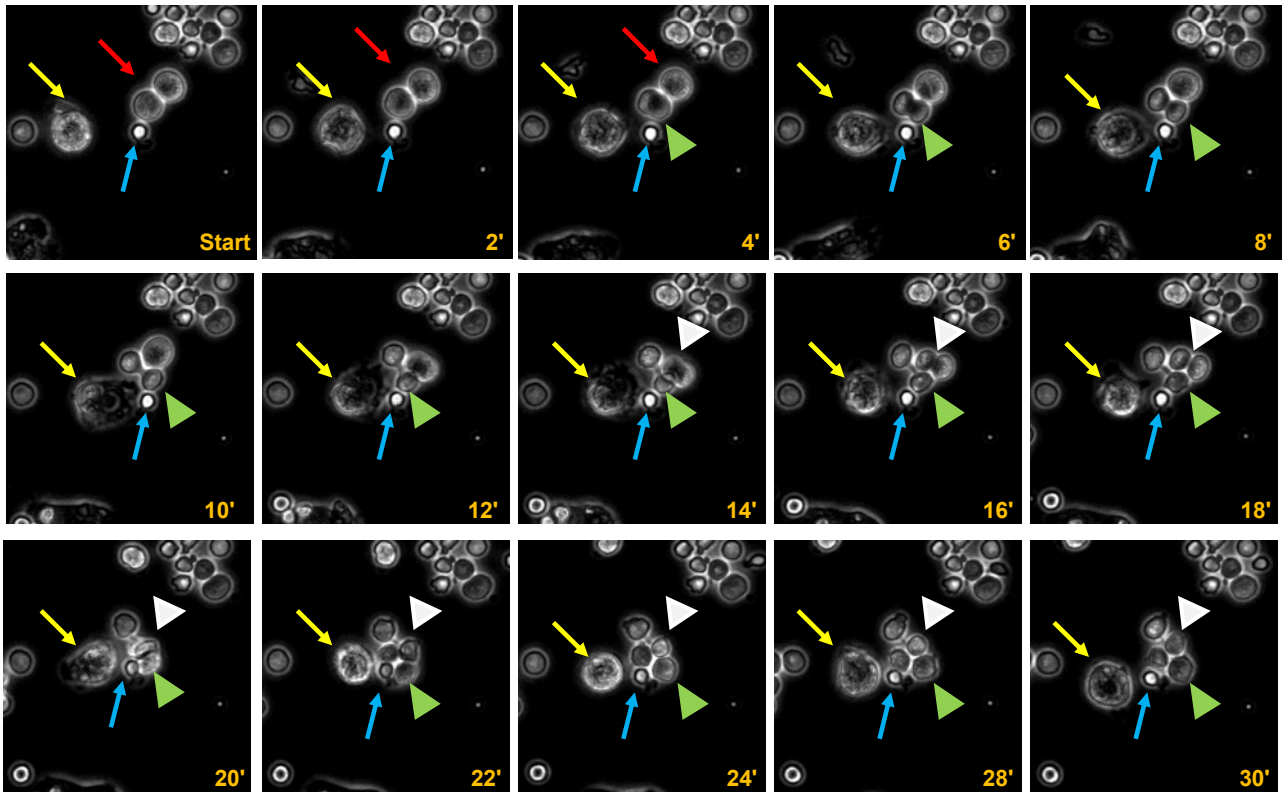
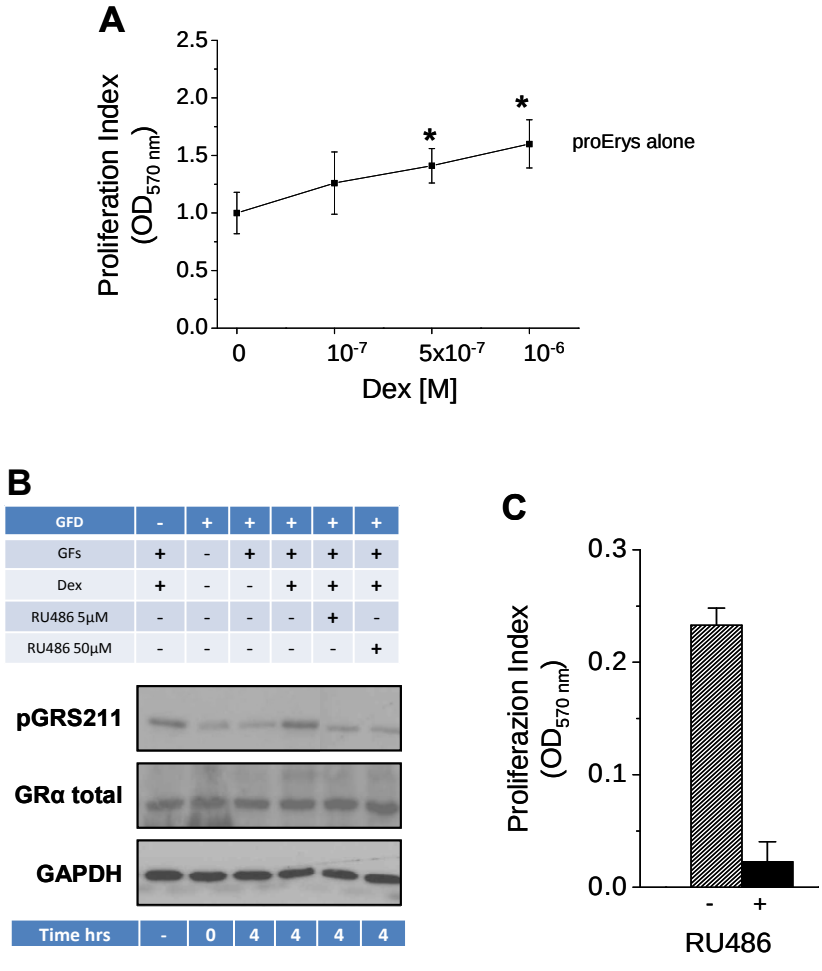
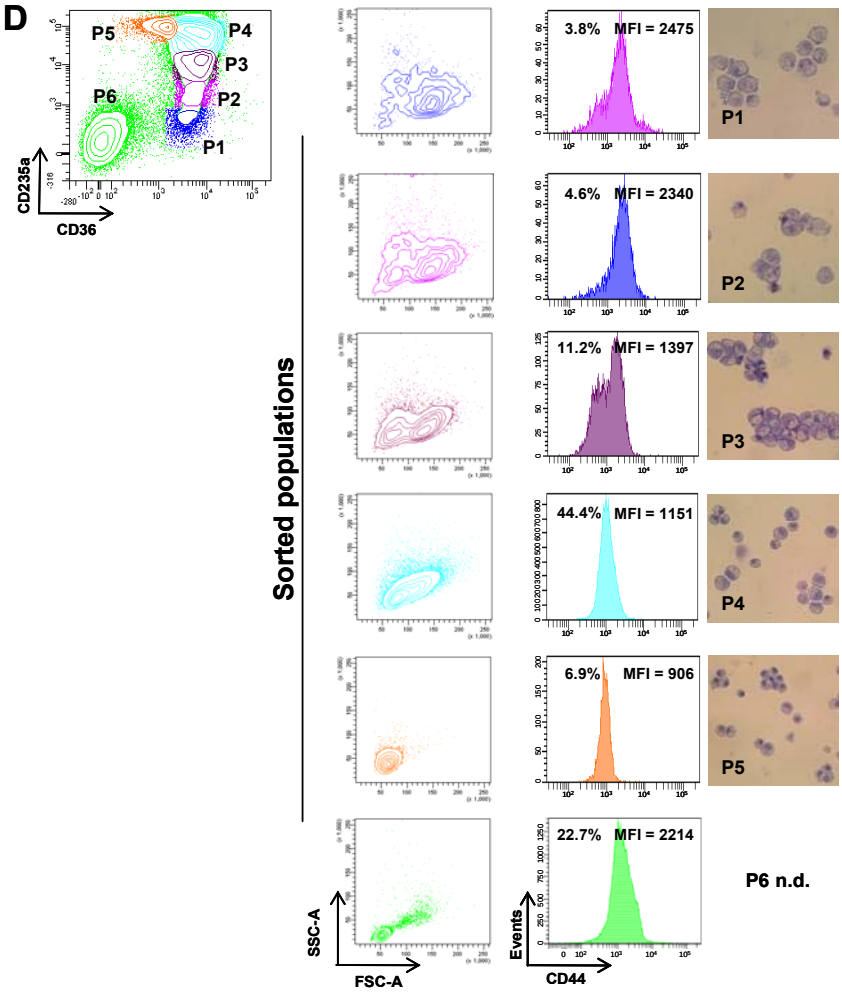


Figure S7. Dex directly promotes proliferation of proErys in HEMA culture. A) Dex concentration/MTT incorporation response curve by prospectively isolated proErys (10^5 cells/well) cultured in HEMA for 24h. Results are expressed as mean (±SD) of those obtained in three separate experiments performed in duplicate. Values statistically different ($p<0.05$) from those obtained in the absence of Dex are indicated with *. B) WB analyses for the activated form of GR phosphorylated at Serine 211, GR total and GAPDH of Erys obtained in HEMA culture and then growth factor deprived for 4 h (GFD) and exposed for 4 additional h to either SCF, IL-3 and EPO (growth factors, GFs) alone or plus Dex, or Dex plus RU486 at 5 or 50 µM concentration. Manipulations and duration of treatments are indicated on the top and bottom of the panels, respectively. C) Levels of MTT incorporated by Erys exposed for 24 h to GFs and Dex in the presence (+) or not (-) of RU486 [5 µM]. Results are presented as Mean (± SD) of those obtained in three experiments performed in triplicate. Values observed with and without RU486 are statistically different ($p<0.001$). D) Gates used to purify Erys obtained from adult blood at day 12 of HEMA culture into 5 classes (P1-P6) on the basis of the levels of CD36, CD235a and CD44 expression. P6 does not contain erythroid cells. Reanalysis for size (FSC-A/SSC-A), levels of CD44 expression (as mean fluorescent intensity, MFI) and morphology of the sorted populations are also shown. Morphology is not available for P6. D) Proliferation under conditions of limiting dilution (2, 4 and $6x10^6$ cells/mL) of cells in the P1-P6 gate. Proliferation was determined by measuring the cell content on day 2, 4 and 7 of culture and expressed as fold increase (FI). Cultures were stimulated with SCF, IL-3, EPO and Dex. The starting population (TOT, grey bars), cultured for comparison, proliferated only at the highest cell density. By contrast, during the first 48 h of culture, cells in the P1 and P2 gate proErys increased in numbers with similar FI at all the concentration tested while cells in the P3, P4, and P5 gate did not increase in numbers and died by day 7. Similar results were obtained in two additional experiments.




D
P5
P4
P3
P6
P2
P1
CD235a
CD36
Sorted populations
3.8% MFI = 2475
4.6% MFI = 2340
11.2% MFI = 1397
44.4% MFI = 1151
6.9% MFI = 906
22.7% MFI = 2214
P1
P2
P3
P4
P5
P6 n.d.
SSC-A
FSC-A
Events
CD44

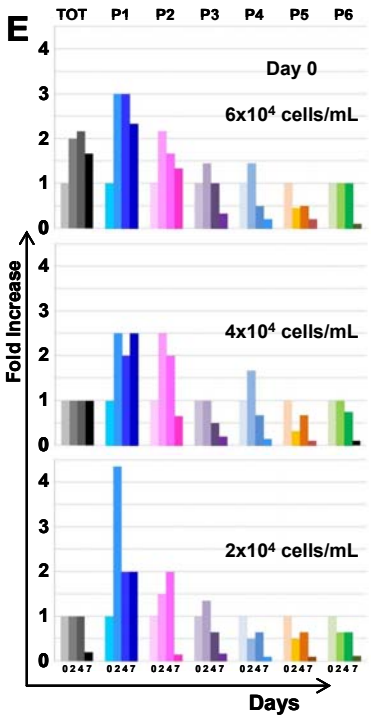

E
TOT
P1
P2
P3
P4
P5
P6
Day 0
6x10⁴ cells/mL
4x10⁴ cells/mL
2x10⁴ cells/mL
Fold Increase
0 2 4 7
Days

Figure S8. Frequency of total ($CD14^{pos}$) and CD163/CD169 double positive and double negative macrophages observed over time in replicate HEMA cultures. Results are presented as mean (±SD) of frequencies observed in separate experiments, indicated by n.

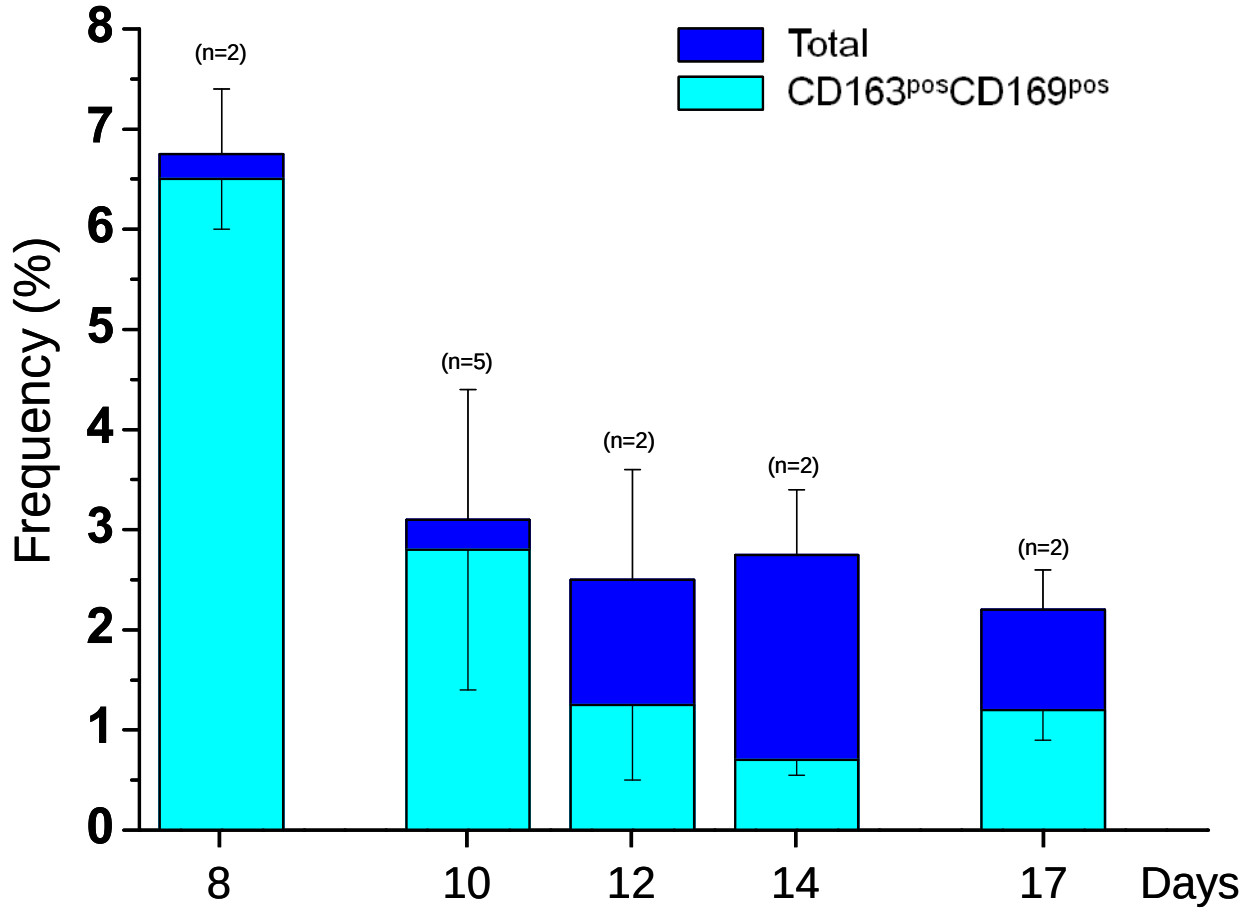
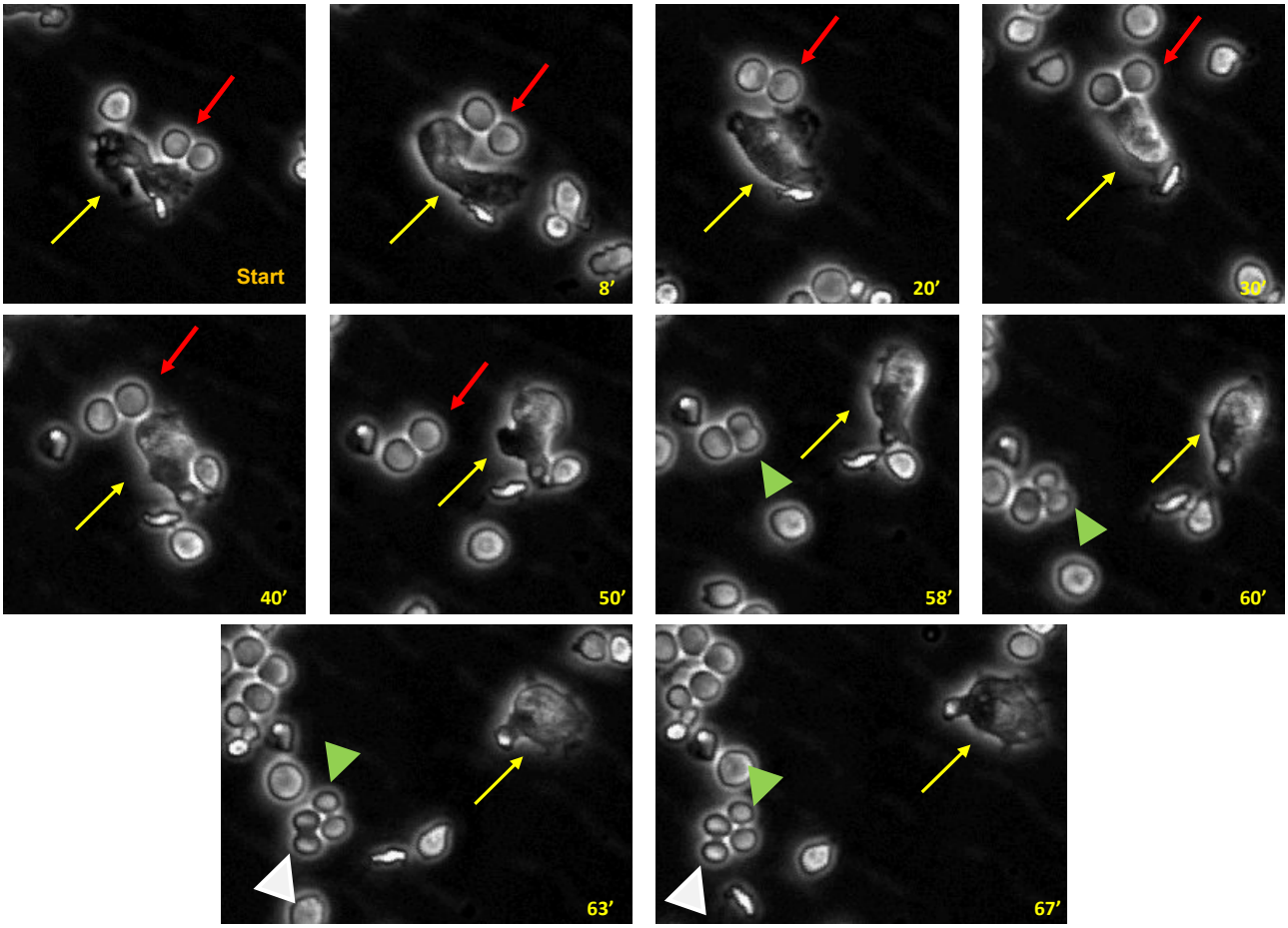


Figure S9. Macrophages interacting with proErys undergoing cytokinesis are CD169^{pos}. Dynamics of cell interactions and cytokinetic events occurring when prospectively isolated proErys ($2x10^5$/mL) were co-cultured in HEMA with CD169^{pos} macrophages ($2x10^3$/mL). Frames selected from 70' of image recording. Yellow arrow = macrophage; red arrow = proEry doublet; green and white arrowheads = first and second cytokinetic event of the proEry doublet (see **Movie 10**).



List of movies included as Supplemental Material

Movie 1. Time lapse observations of cells obtained at day 14 in HEMA culture without Dex. Images captured with 10X objective each 30" for about 2 hours; reproduction speed at 10 frames per second.

Movie 2. Time lapse observations of cells obtained at day 14 in HEMA culture with Dex. Images captured with 10X objective each 30" for about 4 hours; reproduction speed at 10 frames per second.

Movie 3. Dynamics of "loose" interactions occurring among macrophages and proErys in HEMA culture with Dex leading to the formation of erythroblastic islands. Images captured with 10X objective each 30" for about 15'; digitally zoomed x2; reproduction speed at 3 frames per second.

Movie 4. Dynamics of "tight" interactions occurring among macrophages and mature Erys leading to the formation of "glutted" macrophages. Images captured with 10X objective each 30" for about 2 hours; digitally zoomed x2; reproduction speed at 10 frames per second.

Movie 5. Dynamics of "tight" interactions occurring among macrophages and mature x Erys in cells labeled with FITC-CD235a showing phagocytosis of fluorescent cells. Images captured with 10X objective each 30" for about 2 hours; digitally zoomed x2; reproduction speed at 10 frames per second.

Movie 6. Cytokinesis leading to duplication of an isolated proEry. Images captured with 10X objective each 30" for about 20'; digitally zoomed x2; reproduction speed at 10 frames per second. The rectangle labels the area where the cytokinesis of two single proErys is occurring to highlight the spatial distance between these cells and two macrophages present in the same frame.

Movie 7. Cytokinesis leading to duplication of one proEry doublet associated with macrophage "cuddling". Images captured with 20X objective each 30" for about 15'; reproduction speed at 10 frames per second.

Movie 8. Time lapse observations of prospectively isolated proErys cultured in HEMA alone. Images captured with 10X objective each 30" for about 4 hours; reproduction speed at 10 frames per second.

Movie 9. Time lapse observations of prospectively isolated proErys cultured in HEMA with macrophages**.** Images captured with 10X objective each 30" for about 4 hours; reproduction speed at 10 frames per second.

Movie 10. Time lapse observations of prospectively isolated proErys cultured in HEMA with $CD169^{pos}$ macrophages. Images captured with 10X objective each 30" for about 4 hours; reproduction speed at 10 frames per second.