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

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ABSTRACT

Cultures of human CD34^{pos} cells stimulated with erythroid growth factors plus dexamethasone, a model for stress erythropoiesis, generate numerous erythroid cells plus a few macrophages (approx. 3%; 3:1 positive and negative for CD169). Interactions occurring between erythroblasts and macrophages in these cultures and the biological effects associated with these interactions were documented by live phase-contrast videomicroscopy. Macrophages expressed high motility interacting with hundreds/thousands of erythroblasts per hour. CD169^{pos} macrophages established multiple rapid 'loose' interactions with proerythroblasts leading to formation of transient erythroblastic island-like structures. By contrast, CD169^{neg} macrophages established 'tight' interactions with mature erythroblasts and phagocytosed these cells. 'Loose' interactions of CD169^{pos} macrophages were associated with proerythroblast cytokinesis (the M phase of the cell cycle) suggesting that these interactions may promote proerythroblast duplication. This hypothesis was tested by experiments that showed that as few as 103 macrophages significantly increased levels of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide incorporation frequency in S/G2/M and cytokinesis expressed by proerythroblasts over 24 h of culture. These effects were observed also when macrophages were co-cultured with dexamethasone directly conjugated to a macrophage-specific CD163 antibody. In conclusion, in addition to promoting proerythroblast proliferation directly, dexamethasone stimulates expansion of these cells indirectly by stimulating maturation and cytokinesis supporting activity of macrophages.

Introduction

Clinical observations and loss-of-function studies in mice have established the important role exerted by the glucocorticoid receptor (GR) in eliciting the response to erythroid stress.[1,2] GR activation directly promotes proliferation of erythroid progenitor cells by blocking terminal erythroid maturation.[3-6] This effect is mediated, at least in part, by the ZFP36L2 gene.[6] However, whether GR may also favor the response to stress indirectly by modulating the activity of supporting cells in the marrow microenvironment has not yet been investigated.

Macrophages have been identified as the resident cells of the microenvironment that interact with maturing erythroid cells since 1958, when Marcel Bessis described for the first time the erythroblastic island.[7] Indirect evidence that this structure may regulate erythropoiesis *in vivo* was provided in 1978 by Narla and colleagues[8] who used tridimensional electron microscopy to show that suppression of erythropoiesis by hypertransfusion in rats is associated with big reductions in the numbers of erythroblastic islands in the marrow. These observations were followed by additional studies that identified macrophages as the 'niche' that provides erythropoietin (EPO)[9] and other erythroid stimulating agents[10,11] [stem cell factor (SCF)[12] and the erythroid macrophage protein (Emp)[13]] nurture, physical support and other still poorly defined instructions required for the maturation of erythroid cells.[14,15]

In 1991, Rich and colleagues determined that the marrow of mice recovering from hemolytic anemia contain increased numbers of macrophages and suggested that these cells may play an important role in the activation of erythropoiesis in response to stress.[16] Recently, two papers proved this hypothesis by demonstrating that macrophage depletion in mice, either by clodronate treatment or by genetic ablation of cells expressing CD169, has no effect on steady state erythropoiesis but greatly impairs the response to a variety of erythroid challenges, including EPO stimulation and anemia following treatment with phenyl-hydrazine, 5-fluorouracyl or radiation.[17,18]

Whether the ability of macrophages to stimulate erythropoiesis under stress conditions is an intrinsic property of macrophages or is activated by GR is not known.

Liquid cultures in which human hematopoietic progenitor cells are stimulated with SCF, low levels of interleukin-3 (IL-3) and EPO generate a synchronous wave of unilineage erythroid differentiation which produces orthochromatic erythroblasts (orthoErys) within 14-16 days.[19,20] These cultures are considered a model for steady state erythropoiesis. Addition to these cultures of dexamethasone (Dex), a synthetic glucocorticoid, blocks the ordered progression of erythroid maturation allow-

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ing amplification of great numbers of proerythroblasts (proErys).[21-23] These cultures are defined as human erythroid massive amplification (HEMA) cultures and are considered a model for stress erythropoiesis in humans. Both adult proErys generated in culture with Dex[4] and those generated in mice under stress conditions[3,24] acquire a GR activation-dependent self-renewal state.

In 1991, Allen and Testa[25] for the first time used lapse videomicroscopy to detail the complexity of interactions occurring among murine macrophages and erythroid cells leading to formation of erythroblastic islands in long-term cultures. The accompanying commentary by Dr. Bessis praised the physiological relevance of this information and raised the question as to whether the technology was suitable to clarify the role played by macrophages during the stress response.[25] Here we use time-lapse videomicroscopy to determine the role exerted by Dex on the erythroid promoting activity of macrophages in HEMA culture. The results provide evidence that, in addition to its direct effects, Dex sustains proliferation of human proErys indirectly by promoting maturation of $CD169^{pos}$ macrophages that are then instructed by Dex to facilitate progression of proErys through the cell cycle.

Methods

Human specimens and cell preparation

Buffy-coats from 12 de-identified blood donations were obtained according to guidelines established by institutional ethical committees. Mononuclear cells (MNC) and $CD14^{neg}CD34^{pos}$ cells (>98% $CD34^{pos}$ cells) were isolated as previously described.[4,23]

Amplification of human erythroid cells

$CD34^{pos}$ cells (104 cells/mL) or MNC (10^6 cells/mL) were cultured for 10-14 days with SCF (100 ng/mL, Amgen, Thousand Oaks, CA, USA), IL-3 (1 ng/mL, RD System, Minneapolis, MN, USA) and EPO (5 U/mL, Janssen, Raritan, NJ, USA) either without or with Dex (10^{-6} M, Sigma).[4,20]

Cell numbers, viability and phenotype

Cell numbers and viability were determined by trypan blue staining (Boston Bioproducts, Ashland, MA, USA). Cell maturation was assessed by flow cytometry on the basis of CD235a (glycophorin A) and CD36 (thrombospondin receptor) expression and confirmed by visual examination of cytospins. Macrophages were identified on the basis of CD14 (co-receptor for the bacterial lipopolysaccaride), CD16 (receptor for the Fc portion of IgG), CD163 (scavenger receptor for the hemoglobin/haptoglobin complex) and CD169 (sialoadhesin) expression (BD-Pharmigen, San Diego, CA, USA). Size and cell cycle distributions were determined as previously described.[4,26,27]

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide proliferation assay

Day 10 proErys (105 $CD36^{pos}CD235a^{neg}$ cells/100 μL/well) and macrophages (600-10,000 $CD14^{pos}$ cells/100 μL/well) were isolated by FACS and cultured either alone or in combination in HEMA. After 24 h, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL, Sigma) was added and colorimetric reactions read as optical density (OD) at 570 nm (Victor^{3}TM 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA).

Time-lapse videomicroscopy

Time-lapse videomicroscopy was performed in the 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 seconds (s) with the Hamamatsu digital CCD camera (Hamamatsu Photonics KK, Hamamatsu City, Japan), and processed with Cell_R software (Olympus). Images were validated by cytofluorimetrical and morphological analysis (*Online Supplementary Figures S1 and S2*). Quantitative data were obtained with the open source software ImageJ v.1.46 (*http://imagej.nih.gov/ij*).

Transmission electron microscopy

Ultrathin sections of cells embedded in Spurr resin (Poliscience, Warrington, PA, USA) were mounted on 300 mesh nickel grids, counterstained with lead citrate and uranyl acetate, observed with a 109 transmission electron microscope (Zeiss, Oberkochen, Germany) and photographed with SC200 transmission electron microscopy CCD Orius camera (GATAN, Munchen, Germany).[28]

Reverse-phase protein array and Western blot analyses

Reverse-phase protein array (RPPA) of Erys expanded with and without Dex were constructed and analyzed as previously described.[29,30] For Western blot (WB) analyses, cell extracts (30 μg/lane) were separated on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with GRα (total or phosphorylated) or GAPDH antibodies and then with horseradish peroxidase-coupled secondary antibodies that were detected by enhanced chemiluminescence (Amersham, Buckingamshire, UK).

Statistical analysis

Statistical analysis was performed by Anova (Origin 6.0 for Windows, Microcal Software, Inc., Northampton, MA, USA), Kruskal-Wallis or Wilcoxon-Mann-Whitney test (two-sided, P=0.05) (SAS software v.9.2, SAS Institute, Cary, NC, USA). Unsupervised hierarchical clustering of protein levels and Wilcoxon rank-test for pair comparison were performed with JMP v.6 (SAS Institute, Cary, NC, USA).

Further information about the methods used is available in the *Online Supplementary Appendix.*

Results

The presence of Dex in HEMA culture facilitates generation of highly motile macrophages and promotes establishment of Ery-Ery as well as Ery-macrophage interactions

In order to define the role of macrophages present in HEMA cultures, frequency (by FACS) and behavior (by time-lapse videomicroscopy) of macrophages present in HEMA cultures of adult blood stimulated with and without Dex were first compared (Figure 1).

By day 6, macrophages were no longer detectable in HEMA cultures seeded with either $CD14^{pos}$ cell-depleted $CD34^{pos}$ cells or with MNC from adult blood (*E van den Akker and AR Migliaccio, unpublished results, 2014*). As previously reported,[4,19,20] by day 10-14 of HEMA, no macrophages were observed in the single cell FACS gate. We hypothesized that this failure could be a bias due to the tendency of macrophages to form aggregates. In agreement with this hypothesis, macrophages were readily detected in the aggregate gate, which is excluded in routine FACS analyses (*Online Supplementary Figure S1*). In HEMA stimulated with Dex, clusters containing macrophages, some of which expressed high levels of CD235a, and, therefore,

containing also Erys, represented approximately 1.4% of all the events detected by FACS (Figure 1A and *Online Supplementary Figure S1*). By contrast, parallel cultures established without Dex contained limited numbers of clusters (0.3±0.1%) expressing barely detectable levels of CD235a. Generation of macrophages in cultures of human CD34^{pos} cells stimulated with erythroid-specific growth factors and Dex may be related to the fact that GR activation, by blocking the GATA2/GATA1 switch[31] necessary to retard erythroid maturation,[32] retains cells at a high GATA2 state that may allow some of them to mature along the macrophage lineage[33] at an IL-3 concentration (1 ng/mL) 50 times lower than the minimal concentration required to induce macrophage differentiation in colony assay (50 ng/mL).[34]

Time-lapse observations confirmed that, by day 14, HEMA cultures without Dex contain very few macrophages (Figure 1B and *Online Supplementary Movie 1*). Moreover, both macrophages and Erys generated in culture without Dex had low motility. Erys moved in a co-ordinated fashion but changed direction frequently and established few interactions among themselves. Cell density remained homogeneously low (approx. 600 cells/frame) (Table 1). The rare macrophages moved independently from Erys, establishing few tight contacts with mature Erys lasting 30 min.

By contrast, both macrophages and Erys generated with Dex were extremely motile. Erys moved toward each other establishing areas in which cell density reached approximately 2700 cells/frame (P<0.05 compared to cultures without Dex) (Table 1). This great motility facilitated formation of stable clusters composed by numerous Erys (Figure 1B and *Online Supplementary Movie 2*). Macrophages appeared as 'fat' highly motile cells that established interactions with hundreds of Erys over 1 h.

Addition of the GR inhibitor RU486[35] or the FAK inhibitor PF-562271[36] reduced motility and cell interactions observed in cultures with Dex down to levels observed in those without Dex (Table 1).

Dynamics of cell interactions occurring between macrophages and Erys in HEMA culture

Both in 'snap shot' images of cytospins, and in still images of time lapse recording of cells at day 10 of HEMA, culture macrophages were observed associated with Erys in structures resembling either 'erythroblastic islands' or 'erythroblast laden' macrophages (Figure 2). Time-lapse videomicroscopy was instrumental in elucidating the dynamics of interactions leading to the formation of these structures.

Based on the extent of contact area and duration, interactions among macrophages and Erys were classified into 'loose' and 'tight'.

'Loose' interactions

'Loose' interactions involved limited areas of the cell surface with boundaries between cell types remaining clearly distinguishable (*Online Supplementary Figure S4A* and *Online Supplementary Movie 3*), as confirmed by electron microscopy (*Online Supplementary Figure S5*). 'Loose' interactions lasted from 30 s to 2 min and were reiterated many times with the two cells sequentially touching each other. They were usually established between one macrophage and pre-formed proEry clusters, and led to formation of structures resembling erythroblastic islands. At day 14, no 'loose' interactions were identified in cultures without Dex but represented approximately 33% of those recorded in cultures with Dex (Table 1).

'Tight' interactions

'Tight' interactions involved large areas of cell surface with the membranes of the two cells appearing partially coalescent (*Online Supplementary Figure S4B* and *Online Supplementary Movie 4*). These interactions lasted, on average, 5-30 min and were established through macrophage protrusions that caught mature Erys passing in the proximity. They led to formation of structures resembling macrophages that had phagocyted Erys. The presence in HEMA of macrophages that had phagocyted mature Erys was confirmed by electron microscopy (*Online Supplementary Figure S5*). In addition, time-lapse recording of CD235a labeled cells provided visual evidence of macrophages phagocytosing fluorescent cells (*Online Supplementary Figure S4C* and *Online Supplementary Movie 5*). Quantification of phagocytosis was hampered by the fact that these events were not always unequivocally recognized. By day 10-14, 'tight' interactions represented 100% and 23% of the interactions occurring in HEMA without and with Dex, respectively (Table 1). Addition to cultures with Dex of either RU486 or PF-562271 did not significantly affect the total number of macrophage/Ery interactions but greatly reduced the frequency of 'loose' interactions and increased that of the 'tight' ones (Table 1).

In HEMA cultures with Dex, 'loose' and 'tight' interactions were not mutually exclusive and in 43% of the cases macrophages were observed to be simultaneously engaged in both (Table 1).

Table 1. Quantitative analyses of events identified by time-lapse videomicroscopy at day 14 of HEMA cultures established with and without Dex, or with Dex plus the GR inhibitor RU486 (5 μM) or the FAK inhibitor PF-562271 (2 μM).

Culture conditions	Cell numbers/ frame	N. of cells involved	ϕ/Ery interactions/frame/hour Loose (%)	Mixed (loose/tight) (%)	Tight (%)
–Dex	634±100	10.0±7.0	0	0	100
+Dex	2772±203*	80.7±22.5*	34.3±12.0*	42.4±17.4*	23.2±7.1*
+Dex +RU486	762±94#	52.7±11.0	10.7±9.6#	17.2±4.5#	72.0± 12.5#
+Dex +PF-562271	934±28#	66.0±19.6	2.0±4.0#	14.7±10.6#	83.2±13.6#

All the cultures were initiated with 4x10^5 cells/mL/35 mm^2 dish. Results are presented as mean (±SD) of those observed in time-lapse recording of at least 3 separate experiments (5 frames per experiment). Values statistically different by Wilcoxon-Mann-Whitney test (P<0.05) with respect to those observed without Dex (*) or with Dex (#) are indicated. ΦIndicates macrophage.

'Loose' interactions are associated with cytokinesis of proEry doublets

Time-lapse videomicroscopy allowed unequivocal identification of cytokinesis events, i.e. the microscopic visualization of the M phase, occurring at day 10-14 in HEMA cultures and to assess how these events were related to macrophage interactions.

Single proErys underwent cytokinesis without apparent physical association with macrophages. During this process, a round-shaped proEry developed a progressively tighter restriction of its equatorial zone and divided into two daughter cells that remained in contact for some time through a tiny filament. This process lasted approximately 15 min and occurred at a significantly different frequency in HEMA cultures without and with Dex (0.47 or 3.17 event/frame/h with and without Dex, respectively; $P<0.05$) (Table 2, *Online Supplementary Figure S6A* and *Online Supplementary Movie 6*). Addition of RU486 or PF-562271 significantly reduced the frequency of cytokinesis in cultures with Dex (Table 2).

In cultures with Dex, proErys were often associated in doublets, some of which underwent cytokinesis (*Online Supplementary Figure S6B* and *Online Supplementary Movie 7*). In this case, the two proErys divided in sequence leading first to the formation of a 3-cell cluster and then to 4 cells that remained connected for some time through a tiny filament. Only when the cytokinesis of the first proEry was completed did the second begin to divide. Each division lasted 15 min and the entire process, which was 30 min long, occurred in proximity of one mature Ery and one macrophage. The macrophage was observed to engage in 'loose' interactions with the proEry undergoing cytokinesis through a protrusion that gently touched and 'cuddled' the dividing cell. At the end of the process, the macrophage continued to touch the 4 daughter cells until they finally became separated. Although these events occurred at low frequency (0.16 events/frame/h), the fact that they took place suggested that macrophages may facilitate proEry duplication.

Macrophages generated in HEMA culture synergize with Dex in promoting proliferation of proErys by accelerating their cytokinesis

Preliminary experiments confirmed that Dex directly stimulates proliferation (measured as MTT incorporation) of purified proErys in a concentration-dependent fashion with maximal effects observed at 10^{-6} M (i.e. the concentration used in HEMA) (*Online Supplementary Figure S7A*). Exposure of Erys to both Dex and RU486 prevented GR activation (*Online Supplementary Figure S7B*) and greatly reduced levels of MTT incorporation (*Online Supplementary Figure S7C*). The direct effect exerted by Dex on Ery proliferation was further documented by culturing cells purified on the basis of CD36, CD235a and CD44 (the receptor for hyaluronic acid/osteopontin the expression of which decreases during maturation of murine Erys[37]) expression. When used alone, CD44 expression did not correlate with the maturation state of human Ery (*data not shown*).

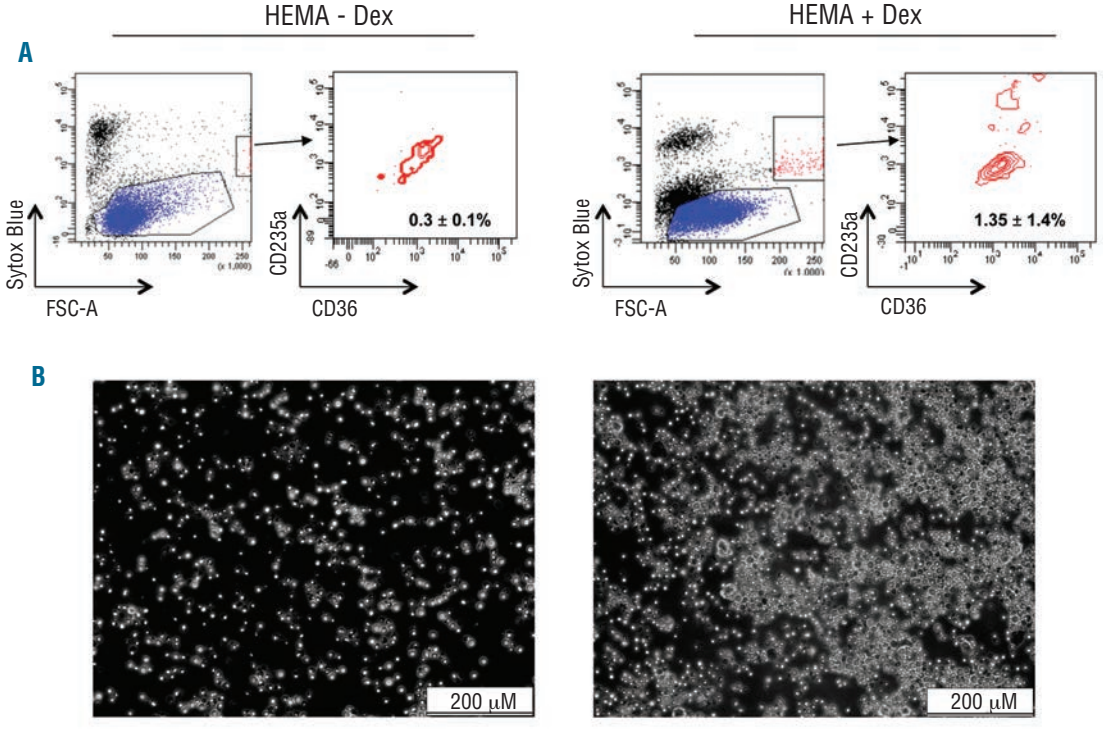


Figure 1. Dex promotes both generation of macrophages and cell-cell interactions in HEMA culture. (A) FACS analyses for CD36/CD235a expression of cells in the aggregate gate of day 14 HEMA cultures with or without Dex, as indicated. Results are representative of those obtained in 5 separate experiments. (B) Phase-contrast observations of cells present at day 14 in parallel HEMA cultures established with (right panel) or without (left panel) Dex, as indicated ($4x10^5$ cells/mL in both cases). The apparently greater cell density observed in the right panel is due to the fact that Erys obtained with Dex moved toward each other establishing large cell aggregates, while those obtained without Dex did not (see Table 1, *Online Supplementary Movies 1 and 2*).

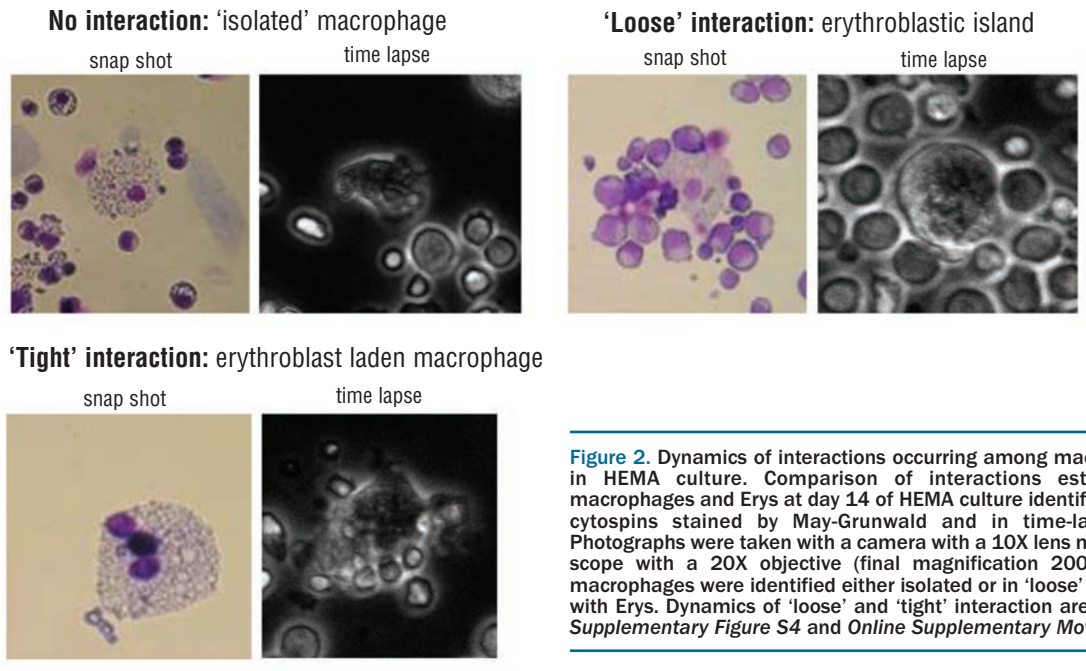


Figure 2. Dynamics of interactions occurring among macrophages and Erys in HEMA culture. Comparison of interactions established between macrophages and Erys at day 14 of HEMA culture identified in snap shots of cytospins stained by May-Grunwald and in time-lapse observations. Photographs were taken with a camera with a 10X lens mounted on a microscope with a 20X objective (final magnification 200X). In both cases, macrophages were identified either isolated or in 'loose' and 'tight' contacts with Erys. Dynamics of 'loose' and 'tight' interaction are depicted in *Online Supplementary Figure S4* and *Online Supplementary Movies 3 and 5*.

However, CD44 staining of $CD36^{pos}$ cells clearly divided these cells into five classes of progressive maturity (*Online Supplementary Figure S7D*). In limiting dilution cultures (2-6x10^4/mL) with Dex, $CD36^{pos}CD235a^{neg}$ Erys expressing CD44 with a mean fluorescent intensity (MFI) of over 2000 proliferated by 2-3 fold within two days, while those expressing CD235a and CD44 with an MFI under 1500 did not proliferate at any concentration (*Online Supplementary Figure S7E*).

To determine whether macrophages may induce proEry proliferation in addition to Dex, proliferation potential of prospectively isolated proErys cultured either alone or with macrophages for 24 h was assessed. Cells purified as described in Figure 3A were co-cultured in ratios equivalent to those present in HEMA culture at day 10-14 (105 proErys/600-10,000 macrophages) (Figure 1). Cultured alone, macrophages did not incorporate MTT with the exception of some incorporation at the highest concentration while proErys cultured alone incorporated robust levels of MTT (Figure 3B). Co-culture with 2000-6000 macrophages significantly increased the levels of MTT incorporated by proErys. Addition of the FAK inhibitor significantly reduced levels of MTT incorporated by purified proErys at a concentration of 200 nM and of pro-Erys co-cultured with 2000 macrophages at a concentration of 2 nM (Figure 3C).

To characterize the mechanism underlying the ability of macrophages to promote proEry proliferation, cell cycle determinations of proErys cultured in HEMA for 24 h either alone or with macrophages were performed (Figure 3D). Cell cycle profiling of proErys present in unfractionated HEMA culture was used as control. The majority of proErys in unfractionated HEMA cultures were either in S (45%) or G2/M (20%). When cultured alone, proErys were mostly in G1 (53%) and when co-cultured with macrophages, acquired a cell cycle profile similar to that of proErys in the unfractionated population with the majority of cells in S (42%) and G2/M (15%). The great numbers of proErys in S/G2/M observed in cultures containing macrophages suggest that these cells may accelerate proEry transition from G1 to S/G2/M, shortening the overall length of the cell cycle. In order to test this hypothesis, time-lapse videomicroscopy of prospectively isolated proErys cultured in HEMA either alone or with macrophages was performed (Figure 4 and *Online Supplementary Movies 8 and 9*). In four separate experiments in which proErys were cultured alone for 7 h, cytokinesis was rarely recorded (0.5

Table 2. Numbers of cytokinesis events observed over time in cultures of day 10 Erys stimulated without Dex, with Dex (10^{-6} M), with Dex plus the GR inhibitor RU486 (5 μM) or with Dex plus the FAK inhibitor PF-562271 (2 nM), as indicated.

	Cytokinesis/frame/hour			
Time (h)	- Dex	+ Dex	+Dex +RU486	+Dex +PF-562271
1	0	0	0	0
2	0	4	0	0
3	0	3	0	0
4	1	1	1	0
5	0	2	0	–
6	1	–	0	–
7	0	–	0	–
8	0	–	0	–
9	0	–	0	–
10	0	–	0	–
Mean (±SD)	0.2±1.4	2.0±1.8	0.1±2.1	0

*Before the analyses, Erys were growth factor deprived for 4 h. All the cultures contained optimal concentrations of SCF, IL-3 and EPO. *Values statistically different by Wilcoxon-Mann-Whitney test (P<0.05). Results are representative of those obtained in 2 different experiments*

events/frame/h). Based on cell density (approx. 340 proErys/frame), numbers of frames analyzed/experiment (five) and numbers of experiments performed (four), it can be calculated that when cultured alone, proErys undergo cytokinesis with a frequency lower than 1/6800 cells per hour. By contrast, approximately 2 cytokinesis/frame/h were detected when proErys were co-cultured with 103 macrophages. These events were often associated with

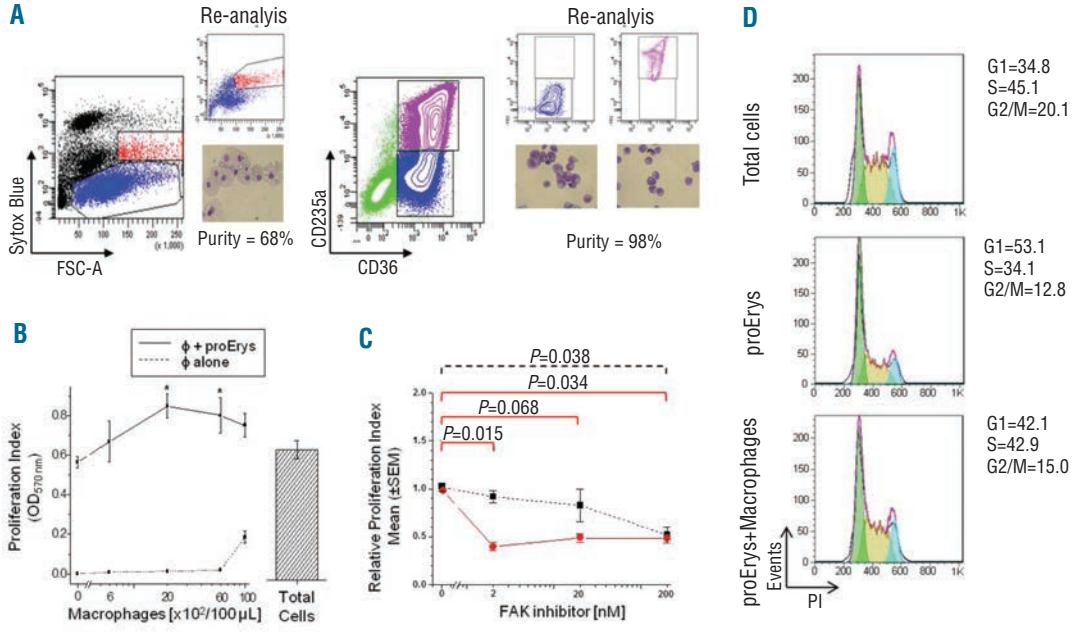
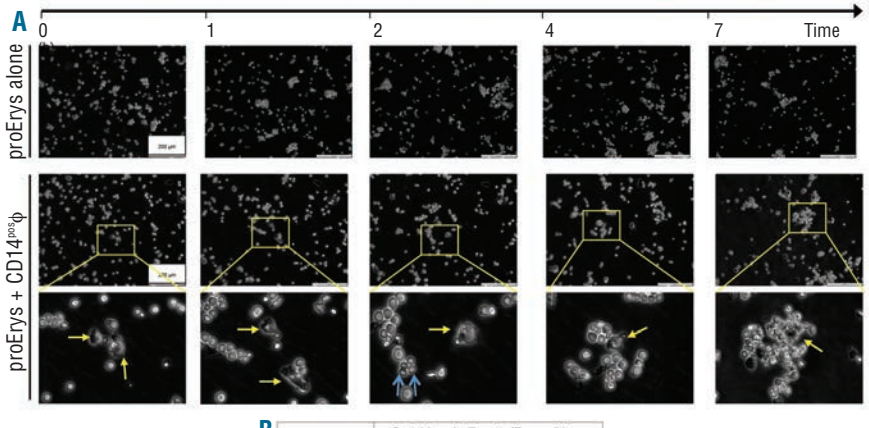


Figure 3. Co-culture with low numbers of macrophages increases proliferation and frequency in G2/S of prospectively isolated proErys. (A) Cell purification strategy. FSC-A/Sytox Blue and CD36^{pos}/CD235a^{neg} gating used to prospectively isolate macrophages (red dots), proErys (blue contour) and mature Erys (purple contour) from cells obtained at day 10-11 of HEMA culture. The purity of sorted cells was assessed by FACS re-analysis and May-Gruwald staining. Prospectively isolated cells were used in co-culture experiments presented in B-D. (B) MTT incorporation by proErys cultured in HEMA for 24 h either alone or with increasing numbers of macrophages (straight line). Levels of MTT incorporated by increasing numbers of macrophages cultured alone (dotted line) and by non-purified cell populations (2x10^5 cells/well) (bar graph) are reported for comparison. Results are presented as mean (±SD) of those obtained in 4 separate experiments performed in duplicate. *P<0.01 with respect to proErys cultured without macrophages. (C) Levels of MTT incorporated by proErys alone (dotted line) or by proErys co-cultured with macrophages (straight line) without or with increasing concentration of the FAK inhibitor PF-562271. Results are presented as mean (±SD) of 3 experiments performed in triplicate. (D) Cell cycle analysis of proErys present in the total population generated at day 10 in HEMA culture (top panel) and of proErys prospectively isolated from these cells cultured either alone (middle panel) or with 10^3 macrophages (bottom panel). All the cells had been cultured for 24 h under standard HEMA conditions. The frequency of cells in G1 (green peak), S (yellow peak) and G2/M (blue peak) was calculated with FlowJo software.



B

	Cytokinesis Events/Frame/Hour	
Time (hrs)	proErys Alone	proErys + CD14^{pos} Φ
1	0	6
2	0	3
3	1	0
4	1	2
5	0	2
6	0	1
7	0	2
8	1	0
9	1	1
10	1	0
Mean (±SD)	0.5 ± 0.5	1.7 ± 1.8

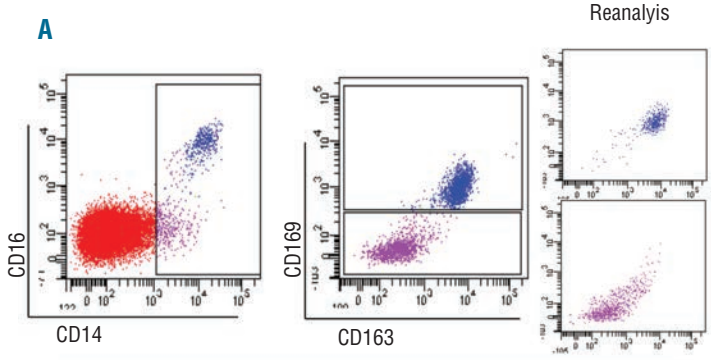
Figure 4. Macrophages promote proEry cytokinesis in HEMA culture. (A) Dynamics of cell interactions and of cytokinetic events occurring in cultures of prospectively isolated proEry (2x10^5/mL) cultured for 7 h in HEMA either alone (top panels) or with prospectively isolated macrophages (2x10^3/well)(bottom panels). Areas indicated by rectangles are presented at greater magnification in the panels below (original magnification 100 x and 150 x, respectively). Frames selected from 7h of image recording. The yellow and blue arrows indicate macrophages and cytokinesis events of proEry doublets, respectively. Similar results were observed in one additional experiment (*see Online Supplementary Movies 8 and 9*). (B) Number of cytokinesis events observed over time in cultures of purified proErys cultured either alone or with 2x10^3 CD14^{pos} macrophages (Φ) purified from the same HEMA culture. All the cultures were stimulated with concentrations of SCF, IL-3, EPO and Dex that stimulate optimal growth of proErys.[38]

'loose' Ery/macrophage interactions, indicating that macrophages favor proEry proliferation by accelerating their transition from G1 to G2/S/M.

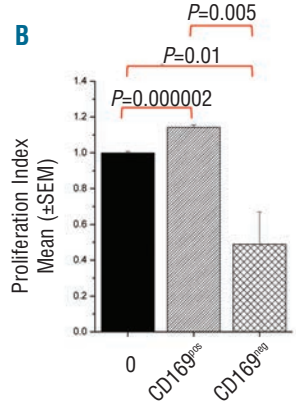
Macrophages that promote proEry duplication express CD169

FACS analyses for CD163/CD169 expression identified that by day 8, HEMA culture contained two types of macrophages: one triple positive for CD16, CD163 and CD169 and one triple negative for these markers (indicated CD169^{pos} and CD169^{neg}) (Figure 5A). CD169^{pos} macrophages were predominant until day 10 and became 20%-30% of CD14^{pos} cells from day 12 to day 17 (*Online Supplementary Figure S8*). By contrast, the rare macrophages detected in HEMA without Dex were all CD169^{neg} (*data not shown*).

To identify the phenotype of the macrophages capable of promoting proEry cytokinesis, MTT incorporation assays and time-lapse videomicroscopy of co-cultures of prospec-



	CD14^{pos}	CD163^{pos} CD169^{pos}	CD163^{neg} CD169^{neg}
AB (n=7)	3.5±1.1	77.7±10.1	22.4±10.2



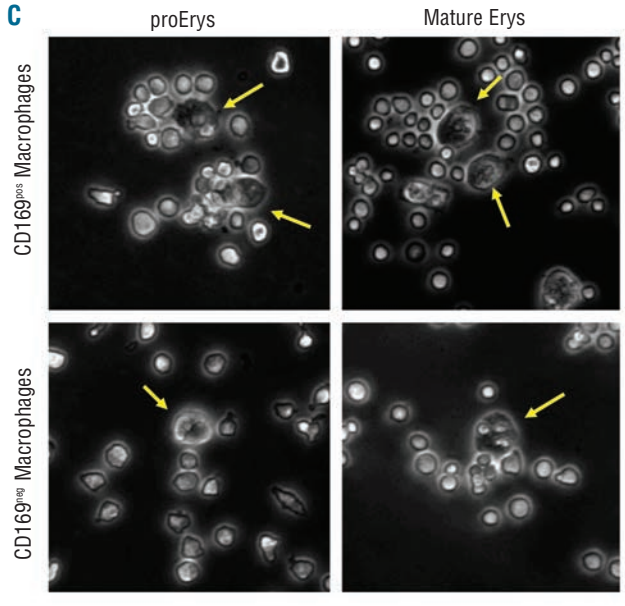


Figure 5. Macrophages that stimulate proEry proliferation express CD169 and CD163. (A) Flow cytometry determinations of the frequency of macrophages at day 10 of HEMA culture. Macrophages were characterized on the basis of CD16, CD14, CD163 and CD169 expression. (B) Levels of MTT incorporated by proErys cultured either alone or in the presence of CD169^{pos} or CD169^{neg} macrophages, as indicated. Results are presented as mean (±SD) of those observed in 2 separate experiments performed in triplicate. (C) Cell interactions occurring when prospectively isolated proErys or mature Erys (2x10^5/mL for both) are co-cultured in HEMA with prospectively isolated CD169^{pos} or CD169^{neg} macrophages (2x10^3/mL for both), as indicated. Yellow arrow: macrophage. (D) Number of cytokinesis events observed over time in cultures of purified proErys cultured either alone or with 2x10^3 CD169^{pos} or CD169^{neg} macrophages (Φ) purified from the same HEMA cultures. *Values statistically different by Wilcoxon-Mann-Whitney test (P<0.05). Cultures with CD169^{neg} macrophages were observed only for 2 h because the macrophages lost motility and appear dead after the first hour of observation. Results are representative of those obtained in 3 different experiments.

D

	Cytokinesis Events/Frame/Hour		
Time (hrs)	proErys Alone	proErys + CD169^{pos} Φ	proErys + CD169^{neg} Φ
1	0	0	0
2	0	8	0
3	1	5	-
4	1	3	-
5	0	4	-
6	0	1	-
7	0	0	-
8	1	2	-
9	1	2	-
10	1	1	-
Mean (±SD)	0.5 ± 0.5	2.6 ± 2.5	0

*

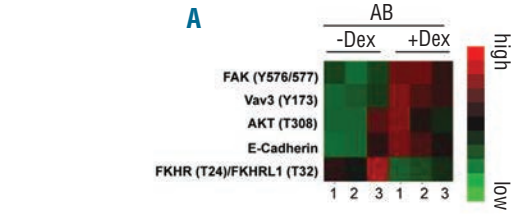
tively isolated proErys (and mature Erys as control) with either CD169^{pos} or CD169^{neg} macrophages were performed. Addition of CD169^{pos}macrophages significantly increased the levels of MTT incorporated by proErys while CD169^{neg} macrophages significantly decreased MTT incorporation (Figure 5B). Time-lapse microscopy identified that CD169^{pos} macrophages promptly established 'loose' interactions with proErys and induced cytokinesis events, often occurring in proximity of macrophages (Figure 5, *Online Supplementary Figure S9* and *Online Supplementary Movie 10*), at a frequency similar to that detected in co-cultures containing the total macrophage population (compare Figures 4 and 5). CD169^{pos} macrophages reacted poorly with mature Erys. By contrast, CD169^{neg} macrophages established 'tight' interactions leading to phagocytosis and died within 2 h when co-cultured with either proErys or mature Ery (Figure 5).

Dex directly stimulates the ability of macrophages to promote proEry proliferation

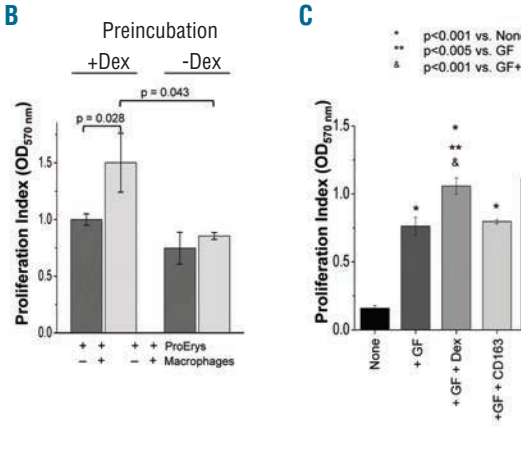
By phosphoproteomic profiling, Erys obtained with and without Dex express significantly different levels of 52 proteins, 31 up-regulated and 21 down-regulated by Dex (*http://capmm.gmu.edu/data*). The array included proteins that favor homologus cell interaction (e-cadherin) and intracellular proteins (FAK and its upstream partners Vav3, AKT and FOXO1/O3) that up-regulate cell surface expression of integrins necessary for heterologous cell interactions.[36,39] Dex significantly increased the levels of expression/activity of FAK, VAV3, AKT and FOXO1/O3 (the T24/T32 modification suppressed by Dex inhibits FOXO1/O3 activity) but had no effect on expression of e-cadherin (Figure 6A). These analyses suggest that Dex primes Erys for interactions with macrophages.

To clarify whether Dex also induces the proliferation-supportive activity of macrophages is also Dex induced, MTT incorporation by proErys cultured either alone or with macrophages that had been pre-incubated for 30 min with and without Dex were compared (Figure 6B). Macrophages incubated for as little as 30 min without Dex became unable to promote MTT incorporation by proErys while those cultured in parallel with Dex proved able to support proEry proliferation.

Additional experiments directly assessed whether GR activation promotes the ability of macrophages to stimulate proEry proliferation by exploiting the observation that the supportive macrophages express CD163 and the availability



Gene ID	Protein	Adult blood	
		FC -Dex/+Dex	Prob > ChiSq
PTK2	FAK (Y576/577)	0.60	0.0495
VAV3	Vav3 (Y173)	0.57	0.0495
AKT1	AKT (T308)	**0.42**	0.2683
CDH1	E-Cadherin	0.40	0.1266
FOXO1/O3	FKHR (T24)/FKHRL1 (T32)	2.87	0.0495



D

Time (hrs)	Cytokinesis events/Frame/Hour			
	- Dex	+ Dex	+ anti-CD163	+ Dex-anti-CD163
1	0	3	1	2
2	0	5	0	5
3	2	1	0	4
4	1	4	1	4
5	0	2	0	2
6	1	-	0	5
7	0	-	0	3
8	0	-	1	6
9	0	-	0	7
10	0	-	-	4
	0.2 ± 0.7	3.0 ± 1.6	0.3 ± 0.5	4.2 ± 1.6

Figure 6. Dex stimulates macrophages to sustain Ery proliferation. (A) Reverse-phase protein array for proteins involved in cell interaction in Erys expanded from 3 different adult blood donors (1, 2 and 3) with or without Dex. The differentially enriched signal intensities were analyzed with Wilcoxon rank test and then expressed as ratio between values observed without Dex and those expressed with Dex (FC: fold change). Signals significantly (P<0.05) up-regulated (FC<1) or down-regulated (FC>1) by Dex are indicated in red and green fonts, respectively. In the case of the inhibitory T24/T32 modification, the FC<1 indicates that Dex activates FOXO1/O3. (B) Levels of MTT incorporated by 10^5 proErys cultured either alone or with $2x10^3$ macrophages. Before being added to the co-culture, macrophages were incubated for 30 min with SCF, IL-3 and EPO supplemented or not with Dex. Results are expressed as relative MTT incorporation using the levels incorporated by proErys preincubated with Dex cultured alone as 1 and are presented as mean (±SD) of 4 independent experiments performed in triplicate. (C) Levels of MTT incorporated by cells harvested from day 10 of HEMA culture, growth factor deprived for 4 h and then cultured for additional 24 h either in media (None) or with SCF, IL-3 and EPO (+GF) or with GF plus Dex (10^{-6} M), CD163 or CD163 directly conjugated with Dex (Dex/CD163, at a concentration equivalent to 10^{-6} M Dex). The amount of natural glucocorticoids present in these experiments was minimized by the use of media containing charcoal-treated FBS. Results are presented as mean (±SD) of 3 independent experiments performed in triplicate and P values calculated with Anova. (D) Numbers of cytokinesis events observed over time when the unfractionated population present at day 10 in HEMA culture is cultured without Dex, with Dex, with free CD163 antibody (anti-CD163) or with a CD163 antibody directly conjugated with four molecules of Dex (Dex-anti-CD163). *Values statistically different by Wilcoxon-Mann-Whitney test (P<0.05). Results are representative of those obtained in 2 separate experiments.

of a compound in which 4 Dex molecules are directly conjugated to the antibody for CD163 (Dex-anti-CD163).[40] Levels of MTT incorporation and number of cytokinesis occurring in 24 h in HEMA cultures with Dex or with Dex-anti-CD163 were then compared. In cultures containing macrophages, Dex-anti-CD163 increased the levels of MTT incorporation at levels similar to those observed with Dex (Figure 6C) while this compound was ineffective in cultures containing proErys alone (*data not shown*). Similar numbers of proEry cytokinesis (>3/h) were detected in HEMA cultures with Dex and with Dex-anti-CD163 already during the first 3 h of time-lapse observation, when macrophages were unlikely to have released in the media significant amounts of Dex cleaved from the CD163 antibody. Cytokinesis was barely detected in cultures without Dex or with anti-CD163 (Figure 6D).

Discussion

The presence of Dex in HEMA culture stimulates adult hematopoietic progenitor cells to generate great numbers of proErys and 1%-3% macrophages, which we demonstrate here play a key role in erythropoiesis *in vitro*. Time-lapse videomicroscopy allowed the recognition of 'loose' and 'tight' interactions between Erys and macrophages with different biological significance.

Cultured macrophages established 'loose' and 'tight' interactions with Erys. The first were established with proEry clusters and allowed formation of structures resembling 'erythroblastic islands'; these were associated with proEry cytokinesis. Co-culture experiments of prospectively isolated cells indicated that these interactions increase proEry proliferation by a novel mechanism involving acceleration of their transition through cytokinesis. Moreover, we showed that macrophages responsible both for 'loose' interactions and for promoting proEry proliferation are CD169^{pos}. Cultured CD169^{pos} macrophages identified in this study may correspond to the resident CD169^{pos} macrophages that specifically elicit the stress response in the spleen of mice.[17,18]

'Tight' interactions involving macrophages and clusters of mature Erys led to the establishment of structures resembling erythroblast-laden macrophages. By contrast with 'loose' interactions, 'tight' interactions were also observed without Dex. Co-culture experiments indicated that macrophages responsible for 'tight' interactions are CD169^{neg}. These CD169^{neg} macrophages died rapidly when co-cultured with proErys releasing factors that may inhibit their growth. Further studies are necessary to clarify whether CD169^{neg} exert a quality control function by phagocytosing Erys undergoing defective maturation and whether this function is regulated by Dex.

Our data have several implications for the role of macrophages in stress erythropoiesis. An electron microscopy study comparing the differentiation state of erythroblasts in islands adjacent or not to sinusoids identified that non-adjacent islands contain mainly proErys while those neighboring sinusoids are enriched for differentiated acidophilic erythroblasts.[41] Although recognizing the bias of physiological considerations based on static morphological evaluations, Narla and Chasis used this information to hypothesize that islands may be motile structures migrating toward sinusoids as their Erys become more differentiated.[14] Our time-lapse observations confirm the dynamic structure of erythroblastic islands and provide new information on the interactions that likely take place within them. Our results suggest that, in cultures containing Dex, Erys migrate in a co-ordinate fashion, establishing areas of 'high' cell density that are permissive for homotypic interactions. Macrophages position themselves at the center of these areas establishing transient erythroblastic islands with a turnover of 30 min. Narla and Chasis also suggested that interaction dynamics and specificity for developmental stages represent the two most important issues in proEry/macrophage relashionships.[14] Our data clearly show that Ery/macrophage interactions, as well as homotypic interactions between Erys, are dynamic processes affected by Dex. In addition, they show that macrophages can establish different interactions according to Ery maturation stage. CD169^{pos} macrophages establish 'loose' interactions with proErys associated with cytokinesis while CD169^{neg} macrophages establish 'tight' interactions with mature Erys leading to phagocytosis. By contrast with results obtained with purified cells, video recording of unfractionated cell populations identified that approximately 40% of macrophages in HEMA cultures with Dex are engaged both in 'loose' and 'tight' interactions. It is possible that these mixed 'loose'/'tight' interactions, which were not identified in cultures of purified cells, represent a novel type of interaction triggered by the presence of Erys at multiple maturation stages, the physiological significance of which has not yet emerged. Additional experiments are necessary to clarify whether Erys may educate macrophages to establish 'niches' which allow completion of functions appropriate to their maturation state.

Synthetic ligands of GR are used as erythroid stimulating agents to treat the EPO-resistant Diamond Blackfan anemia.[42] Data obtained in murine and human experimental systems indicate that GR exerts its erythroid-stimulating activity directly by blocking the maturation of proErys, conferring to these cells a self-renewal state,[3,4,6] and up-regulating the expression of FAK and of its upstream elements that activate expression of integrins required for macrophage interaction (see above). Although previous studies had demonstrated that Dex, in combination with other cytokines, increases the number of macrophages generated by murine hematopoietic progenitors[43] and modulates their immunological properties,[44] it was less clear whether Dex also instructs these cells to promote Ery proliferation. Our data indicate that Dex directly promotes maturation of stress-specific macrophages and instructs them to exert their erythroid niche function, acting, therefore, as a master regulator of stress erythropoiesis.

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