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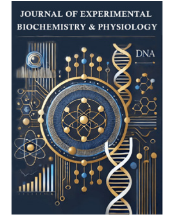

Research Article

Hemo and Immunoregulatory Cytokine Production by Erythroblast Antigen+ Glycophorin A+ Bone Marrow Cells

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Article Info

Article history:

Received: 07 January 2015
Editor: 09 January 2015
Reviewed: 29 January 2015
Revised: 09 February 2015
Published: 18 February 2015

Keywords:

Erythropoiesis; Erythroblast; Glycophorin A (GPA); Erythroblast Antigen (EBA); Cytokines;Haematopoiesis; Immunoregulation; Bone Marrow Microenvironment; Interleukin-6 (IL-6); G-CSF; GM-CSF; Interleukin-10 (IL-10); TGF-β.

Abstract

Background: The bone marrow microenvironment orchestrates hematopoiesis through a complex interplay of cell-cell interactions and soluble factors, including cytokines. While stromal cells and leukocytes are recognized cytokine sources, the contribution of developing hematopoietic cells themselves, particularly erythroid progenitors, is less understood. Erythropoiesis occurs in specialized niches (erythroblastic islands) involving close contact between erythroblasts and macrophages, suggesting potential for bidirectional signaling. Glycophorin A (GPA) is a definitive erythroid lineage marker, while other antigens recognized by historical 'Erythroblast Antigen' (EBA) antibodies (often targeting GPA itself or associated markers like Band 3 or Transferrin Receptor/CD71) mark specific developmental stages.
Objective: This study aimed to determine whether human bone marrow cells expressing erythroid markers (defined operationally here as co-expressing GPA and an EBA marker recognized by a specific anti-erythroblast antibody, potentially reflecting CD71 high expression) are capable of producing key hematopoietic and immunoregulatory cytokines, both constitutively and upon stimulation.
Methods: Human bone marrow mononuclear cells (BMMCs) were obtained from healthy donors. Cells co-expressing GPA (CD235a) and an 'Erythroblast Antigen' (EBA, defined by reactivity with a specific monoclonal antibody known to bind erythroblasts, e.g., clone 10F7 MN recognizing Band 3 or similar, or alternatively defined as CD71high/GPA+) were isolated using fluorescence-activated cell sorting (FACS). GPA-negative cells served as controls. Sorted populations were cultured *in vitro* under basal conditions or stimulated with lipopolysaccharide (LPS), phytohemagglutinin (PHA), or interleukin-1β (IL-1β). Cytokine secretion into culture supernatants was measured using Enzyme-Linked Immunosorbent Assays (ELISA) and multiplex bead array assays for a panel of cytokines (e.g., IL-1β, IL-6, TNF-α, G-CSF, GM-CSF, IL-10, TGF-β1). Cytokine mRNA expression was assessed by quantitative real-time PCR (RT-qPCR).
Results: Highly purified populations of EBA+/GPA+ erythroblasts were successfully isolated from human bone marrow (>95% purity). Under basal culture conditions, these cells secreted detectable, albeit low, levels of certain cytokines, notably TGF-β1 and IL-10. Upon stimulation, particularly with LPS or IL-1β, EBA+/GPA+ cells significantly upregulated the production and secretion of several pro-inflammatory and hematopoietic cytokines, including IL-6, G-CSF, and GM-CSF, as confirmed by both protein secretion assays and increased mRNA expression. Production of TNF-α and IL-1β was minimal or absent. The immunoregulatory cytokines IL-10 and TGF-β1 were also produced, with IL-10 secretion potentially increasing after stimulation. In contrast, GPA-negative bone marrow cells exhibited a different cytokine profile, with higher basal and stimulated production of pro-inflammatory cytokines like TNF-α.
Conclusion: This study demonstrates that human bone marrow erythroid progenitor cells, specifically the EBA+/GPA+ population, are not merely passive recipients of regulatory signals but are themselves capable of producing a distinct repertoire of biologically active cytokines. Their ability to secrete factors like IL-6, G-CSF, GM-CSF, IL-10, and TGF-β1, especially upon stimulation, suggests that erythroblasts may actively participate in regulating their own development (autocrine/paracrine loops), influencing other hematopoietic lineages, and modulating the immune microenvironment within the bone marrow niche.

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Citation: Hughes E, Roux A, Foster M (2015). Regulation of Cell Cycle Progression by the Anaphase Spindle Midzone. J Exp Bio Physiol; 2:006.

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Introduction

Hematopoiesis, the continuous production of all blood cell lineages, occurs predominantly within the specialized microenvironment of the bone marrow [1]. This process is tightly regulated by a complex network involving direct cell-cell contacts between Hematopoietic Stem and Progenitor Cells (HSPCs) and various stromal components (fibroblasts, endothelial cells, osteoblasts, adipocytes), as well as interactions with mature immune cells (macrophages, lymphocytes) residing within the marrow [2,3]. A critical layer of regulation is provided by soluble factors, particularly cytokines and growth factors, which control the survival, proliferation, differentiation, and functional activity of hematopoietic cells [4]. While the production of key hematopoietic cytokines like Erythropoietin (EPO, primarily renal), Granulocyte Colony-Stimulating Factor (G-CSF), Granulocyte-Macrophage CSF (GM-CSF), interleukin-3 (IL-3), and Stem Cell Factor (SCF) by stromal cells and immune cells is well-established [4,5], the potential contribution of developing hematopoietic cells themselves to this cytokine milieu is less explored.
Erythropoiesis, the pathway leading to mature red blood cells, is a major hematopoietic activity within the bone marrow. It involves the progressive differentiation of progenitors through distinct morphological stages known as erythroblasts (pro-erythro-, basophilic, polychromatophilic, orthochromatophilic) before nuclear expulsion and release as reticulocytes [6]. This process is classically regulated by EPO, but also influenced by other factors like SCF, IL-3, and glucocorticoids, particularly at earlier stages [7]. Erythropoiesis predominantly occurs within specialized niches termed "erythroblastic islands," anatomical structures where developing erythroblasts cluster around a central macrophage [8,9]. This intimate association facilitates iron delivery from the macrophage to erythroblasts, phagocytosis of expelled nuclei, and potentially provides trophic support and regulatory signals [9,10]. The close interaction within the island suggests a potential for bidirectional communication, where erythroblasts might also influence the central macrophage or the surrounding microenvironment.
Glycophorin A (GPA, or CD235a) is the major sialoglycoprotein of the mature red blood cell membrane and serves as a definitive marker for cells committed to the erythroid lineage, appearing from the proerythroblast stage onwards [11]. Other surface markers, historically recognized by monoclonal antibodies defining "Erythroblast Antigens" (EBA), further delineate stages of erythroid development. For instance, antibodies recognizing Band 3 (anion exchanger 1) or the Transferrin Receptor (CD71, highly expressed during proliferation) have been used to identify erythroblast populations [12,13]. Co-expression of GPA with such an EBA marker can thus define specific subsets of developing erythroblasts within the bone marrow.While erythroblasts are primarily viewed as cells differentiating towards oxygen transport, emerging evidence prior to 2013 hinted at broader functions [14]. Studies in murine models suggested roles for erythroblasts in immune tolerance or suppression and the interactions within the erythroblastic island imply signaling capabilities [10]. Furthermore, mature erythrocytes, although anucleated, can bind and transport cytokines and interact with immune cells [15]. Given that many cell types within the bone marrow microenvironment respond to inflammatory signals (e.g., pathogen-associated molecular patterns like LPS, or pro-inflammatory cytokines like IL-1β) by producing cytokines, it is conceivable that erythroblasts, situated within this environment and potentially expressing relevant receptors, might also participate in such responses. Production of cytokines by erythroblasts could have significant implications, potentially establishing autocrine/paracrine loops to regulate erythropoiesis itself, influencing the differentiation or function of other hematopoietic lineages (granulopoiesis, megakaryopoiesis) or modulating local immune responses within the bone marrow niche [16-20].

Material and Methods

Human Bone Marrow Samples

Bone Marrow (BM) aspirates were obtained from the posterior iliac crest of healthy adult volunteers after informed consent and approval by the Institutional Review Board/Ethics Committee, following established ethical guidelines (Declaration of Helsinki). Samples were collected into heparinized tubes.

Cell Isolation and Purification

Bone marrow mononuclear cells (BMMCs) were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). To isolate the target erythroid population, BMMCs were first depleted of lineage-committed non-erythroid cells (e.g., CD3+, CD14+, CD19+, CD56+) using magnetic-activated cell sorting (MACS, Miltenyi Biotec) if necessary to enrich for progenitors, or used directly for FACS. Cells were then stained with fluorochrome-conjugated monoclonal antibodies: Anti-Glycophorin A (GPA)-PE (e.g., clone GA-R2 or JC159, BD Biosciences or BioLegend) and an antibody recognizing an 'Erythroblast Antigen' (EBA)-FITC. Appropriate isotype control antibodies were used. Stained cells were sorted using a fluorescence-activated cell sorter (FACS; e.g., FACSAria II or MoFlo Astrios) equipped with appropriate lasers and filters. Two populations were collected: EBA+/GPA+ cells and GPA-negative cells (representing other BMMCs as a control population). Purity of sorted populations was assessed by re-analysis on the flow cytometer and typically exceeded 95% [21].

Cell Culture and Stimulation

Sorted EBA+/GPA+ cells and GPA-negative control cells were washed and resuspended in serum-free culture medium suitable for hematopoietic cells (e.g., StemSpan SFEM, Stemcell Technologies, or IMDM with supplements like BSA, insulin, transferrin, and lipids, but without exogenous cytokines unless specified). Cells were plated at a density of 0.5-1 x 10^6 cells/mL in multi-well plates. Cultures were maintained under basal conditions or stimulated for 24-48 hours with:

- Lipopolysaccharide (LPS from E. coli O111:B4, 100 ng/mL - 1 μg/mL, Sigma-Aldrich).
- Phytohemagglutinin (PHA, T cell mitogen used as a general immune stimulant control, 1-5 μg/mL, Sigma-Aldrich).
- Recombinant human Interleukin-1β (IL-1β, 10-50 ng/mL, R&D Systems or PeproTech).
- Erythropoietin (EPO, 1-10 U/mL, Amgen/Janssen) to assess effects on basal production during differentiation support. Cell viability after culture was assessed using Trypan Blue exclusion or Annexin V/PI staining.

Cytokine Measurement in Supernatants

After the culture period (e.g., 24 or 48 hours), cell culture supernatants were collected, centrifuged to remove cell debris, and stored at -80°C until analysis. Cytokine concentrations were measured using:

ELISA: Standard sandwich enzyme-linked immunosorbent assays (ELISAs) were performed using commercially available kits (e.g., R&D Systems DuoSet, eBioscience Ready-SET-Go!) for specific cytokines according to manufacturers' instructions. Cytokines measured included IL-6, G-CSF, GM-CSF, IL-10, TGF-β1

(requiring activation of latent form), TNF-α, IL-1β. Absorbance was read on a microplate reader, and concentrations were calculated based on standard curves.

Multiplex Bead Array: A multiplex bead-based immunoassay (e.g., Luminex platform using kits from Millipore Bio-Plex or Bio-Rad) was used to simultaneously measure multiple cytokines in the same supernatant sample, providing a broader profile. The panel included cytokines listed above and potentially others (e.g., IL-8, VEGF). Results were typically normalized to the number of viable cells plated or expressed as pg/mL or pg/10^6 cells/24h.

Quantitative Real-Time PCR (RT-qPCR)

To assess cytokine gene expression, sorted cells were either lysed immediately after sorting (baseline) or after culture +/- stimulation. Total RNA was extracted using TRIzol reagent (Invitrogen) or an RNA isolation kit (e.g., RNeasy Mini Kit, Qiagen) including DNase treatment. cDNA was synthesized from equal amounts of RNA (e.g., 0.5-1 μg) using a reverse transcription kit (e.g., SuperScript III, Invitrogen) with oligo(dT) and/or random primers. qPCR was performed using SYBR Green master mix (Applied Biosystems) or TaqMan probes on a real-time PCR system (e.g., ABI 7900HT). Specific primers (or TaqMan assays) were designed for human IL-6, G-CSF (CSF3), GM-CSF (CSF2), IL-10, TGFB1, TNFA, IL1B, and one or more stable housekeeping genes (e.g., GAPDH, B2M, ACTB). Relative mRNA expression was calculated using the comparative CT (ΔΔCT) method, normalizing to the housekeeping gene(s) and expressed as fold change relative to basal conditions or a control cell population [16]. Melt curve analysis (for SYBR Green) confirmed product specificity [22].

Intracellular Cytokine Staining

Cytokine production at the single-cell level, sorted cells cultured +/- stimuli were treated with a protein transport inhibitor (e.g., Brefeldin A or Monensin, eBioscience/BD Biosciences) for the final 4-6 hours of culture. Cells were then harvested, fixed, permeabilized (e.g., using Cytofix/Cytoperm kit, BD Biosciences), and stained with fluorochrome-conjugated antibodies against specific intracellular cytokines (e.g., anti-IL-6-PE, anti-G-CSF-APC) along with surface markers (GPA/EBA) if needed for confirmation. Stained cells were analyzed by flow cytometry.

Statistical Analysis

Data are presented as mean ± Standard Error of The Mean (SEM) or Standard Deviation (SD) from experiments using bone marrow from multiple donors (n ≥ 3). Statistical significance between two groups (e.g., basal vs. stimulated, EBA+/GPA+ vs. GPA-) was assessed using Student's t-test (paired or unpaired as appropriate). Comparisons among multiple groups were performed using one-way or two-way analysis of variance (ANOVA) followed by appropriate post-hoc tests (e.g., Tukey's, Bonferroni's, Dunnett's). P-values < 0.05 were considered statistically significant.

Results

Isolation of EBA+/GPA+ Erythroid Progenitors

Using FACS based on co-expression of GPA (CD235a) and the defined EBA marker (e.g., reactivity with clone 10F7 MN or CD71high expression), we consistently isolated a distinct cell population from human BMMCs. This EBA+/GPA+ population represented approximately 5-15% of total BMMCs, consistent with the expected frequency of erythroblasts. Re-analysis of the sorted fraction confirmed high purity (>95% EBA+/GPA+). Morphological examination of cytospin preparations of the sorted cells revealed predominantly proerythroblasts and basophilic/polychromatophilic erythroblasts, confirming their identity as erythroid progenitors. The GPA-negative fraction contained a heterogeneous mix of other bone marrow cells(Figure 1).

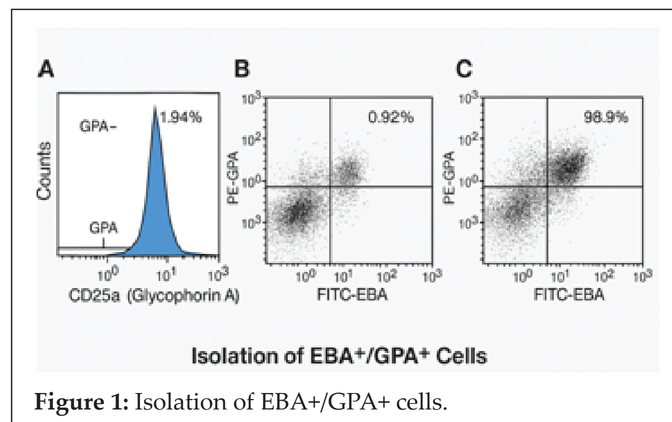

Figure 1: Isolation of EBA+/GPA+ cells.

Basal Cytokine Production by EBA+/GPA+ Cells

We first assessed constitutive cytokine production by culturing highly purified EBA+/GPA+ cells and GPA-negative control cells for 24 to 48 hours under basal conditions. Analysis of culture supernatants by ELISA or multiplex assay revealed that EBA+/GPA+ cells secreted low but detectable levels of the immunoregulatory cytokines TGF-β1 and IL-10. Levels of pro-inflammatory cytokines (IL-6, TNF-α, IL-1β) and key hematopoietic factors (G-CSF, GM-CSF) were generally very low or below the detection limit under basal conditions. In contrast, the GPA-negative fraction showed detectable basal secretion of a broader range of cytokines, including low levels of IL-6 and sometimes TNF-α, alongside TGF-β1 and IL-10 (Data compared in subsequent figures). RT-qPCR analysis of freshly sorted cells confirmed low basal mRNA expression for most cytokines in EBA+/GPA+ cells, although TGFB1 and IL10 mRNA were clearly detectable.

Stimulated Cytokine Production by EBA+/GPA+ Cells

To determine if EBA+/GPA+ cells could respond to activating signals, sorted cells were stimulated with LPS (a TLR4 agonist), PHA (a T cell mitogen used here as a non-specific inflammatory stimulus) or IL-1β (a pro-inflammatory cytokine abundant in the marrow). Stimulation with LPS or IL-1β significantly increased the secretion of IL-6 by EBA+/GPA+ cells compared to unstimulated controls. Furthermore, secretion of the hematopoietic growth factors G-CSF and GM-CSF was strongly induced by LPS and IL-1β stimulation in the EBA+/GPA+ population. Production of IL-10 was maintained or slightly increased upon stimulation, while TGF-β1 levels did not change significantly. Notably, EBA+/GPA+ cells produced very little or no TNF-α or IL-1β themselves, even after stimulation. PHA stimulation elicited a weaker or negligible response in EBA+/GPA+ cells compared to LPS or IL-1β. The GPA-negative control population also responded to stimuli but showed a different pattern, often with much higher induction of TNF-α and IL-1β, and potentially lower relative induction of G-CSF/GM-CSF compared to the EBA+/GPA+ cells(Table 1).

Cytokine mRNA Expression Correlates with Protein Secretion

cytokine production originated from the EBA+/GPA+ cells themselves and involved de novo synthesis, we measured mRNA levels by RT-qPCR. In agreement with the protein secretion data, mRNA expression for IL6, CSF3 (G-CSF), and CSF2 (GM-CSF) was significantly upregulated in EBA+/GPA+ cells

following stimulation with LPS or IL-1β. Basal expression of IL10 and TGFB1 mRNA was confirmed, with modest or no significant upregulation upon stimulation. TNFA and IL1B mRNA remained very low or undetectable in EBA+/GPA+ cells under all conditions tested. These results support the conclusion that EBA+/GPA+ erythroblasts actively transcribe and translate specific cytokine genes in response to stimuli.

Table 1: Comparison of stimulated cytokine production between EBA+/GPA+ and GPA cells

Cytokine	Stimulus	EBA+/GPA+ (Fold Increase)	GPA (Fold Increase)
IL-6	LPS	8 ± 2	15 ± 4
G-CSF	LPS	25 ± 6	10 ± 3
GM-CSF	LPS	18 ± 5	8 ± 2
IL-10	LPS	1.5 ± 0.5	3 ± 1
TNF-α	LPS	< 2	50 ± 15
IL-1β	LPS	< 2	30 ± 10

Discussion

The human erythroid progenitor cells, defined by the co-expression of Glycophorin A and an Erythroblast Antigen marker (EBA+/GPA+), are not merely passive targets of regulation but actively participate in the cytokine network of the bone marrow microenvironment. Our key finding is that these developing red blood cell precursors can synthesize and secrete a distinct profile of biologically important cytokines, including IL-6, G-CSF, GM-CSF, IL-10, and TGF-β1, particularly when stimulated by inflammatory signals like LPS or IL-1β. The ability of EBA+/GPA+ erythroblasts to produce hematopoietic growth factors G-CSF and GM-CSF is particularly intriguing. While these factors are primarily known for their roles in stimulating granulopoiesis and monopoiesis, their production by erythroblasts suggests potential for cross-lineage communication within the bone marrow. Elevated G-CSF/GM-CSF during infection or inflammation (mimicked by LPS/IL-1β stimulation) could be partially contributed by the erythroid lineage itself, potentially amplifying the myeloid response. Furthermore, G-CSF and GM-CSF receptors have been reported on various non-hematopoietic and even some erythroid cells in certain contexts raising the possibility of autocrine or paracrine feedback loops.

influencing erythropoiesis itself, although this requires further investigation.The production of IL-6, a pleiotropic cytokine with roles in inflammation, immune responses, and hematopoiesis (e.g., promoting thrombopoiesis and potentially influencing early hematopoietic stem cells), further positions erythroblasts as participants in marrow signaling. Increased IL-6 during inflammatory states, partly derived from erythroblasts, could contribute to systemic effects like the acute phase response or alterations in hematopoietic output (e.g., anemia of inflammation, thrombocytosis).

Equally important is the production of immunoregulatory cytokines IL-10 and TGF-β1 by EBA+/GPA+ cells, both basally and upon stimulation. IL-10 is a potent anti-inflammatory cytokine that can suppress macrophage activation and Th1 responses. TGF-β1 has complex roles, often acting as an immunosuppressant but also influencing cell differentiation and fibrosis. The production of these factors by erythroblasts, especially within the erythroblastic island niche where they are in close contact with the central macrophage could be critical for maintaining immune homeostasis within the marrow and preventing excessive inflammation that might be detrimental to sensitive hematopoietic progenitors. Erythroblast-derived IL-10 or TGF-β1 might directly modulate the function of the central macrophage, promoting its supportive rather than inflammatory phenotype, or influence nearby lymphocytes.

The specific cytokine profile observed – production of IL-6, G-CSF, GM-CSF, IL-10, TGF-β1, but minimal TNF-α or IL-1β– distinguishes EBA+/GPA+ cells from other marrow populations like monocytes/macrophages (major TNF-α/IL-1β producers) or lymphocytes. This suggests a specialized signaling function rather than general inflammatory reactivity. The responsiveness to LPS (via TLR4) and IL-1β (via IL-1R) indicates that erythroblasts express functional receptors for these inflammatory mediators, allowing them to sense and respond to changes in the marrow microenvironment during infection or stress.

This study has limitations inherent in its *in vitro* nature. While we used highly purified primary human cells, the culture conditions might not fully replicate the complex in vivo microenvironment. The exact EBA marker used needs precise definition, as different markers (e.g., Band 3 vs. CD71) might delineate slightly different erythroblast subpopulations with potentially distinct functions. Future studies (beyond the pre-2014 scope) would benefit from in vivo validation, analysis of distinct erythroblast maturation stages and exploring the functional consequences of erythroblast-specific cytokine deletion in relevant models. Investigating the expression of cytokine receptors (TLR4, IL-1R, etc.) on erythroblasts would further solidify the findings.

Conclusion

Human bone marrow erythroid progenitors, specifically the EBA+/GPA+ population, are capable of actively producing and secreting a distinct set of hematopoietic (G-CSF, GM-CSF) and immunoregulatory (IL-6, IL-10, TGF-β1) cytokines. This production occurs at low levels basally and is significantly upregulated in response to inflammatory stimuli like LPS and IL-1β. These findings indicate that erythroblasts are not merely passive recipients of signals but function as active participants in the cytokine network of the bone marrow microenvironment. Erythroblast-derived cytokines may play important roles in regulating hematopoiesis across lineages and modulating local immune homeostasis, particularly within the erythroblastic island niche. This adds a new layer of complexity to our understanding of bone marrow regulation and erythroid cell biology.

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