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7 **Tumor necrosis factor alpha-mediated inhibition of erythropoiesis involves GATA-**
8 **1/GATA-2 balance impairment and PU.1 over-expression**
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Abstract

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2 Many physiological perturbations can cause anemia. In cancer patients, activation of
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4 the immune system leads to the production of proinflammatory cytokines including tumor
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6 necrosis factor alpha (TNF α), that have been shown to inhibit red-cell production *via* poorly
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8 understood mechanisms. Treatment of anemia by human recombinant erythropoietin (EPO) is
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10 strongly suspected to induce tumor growth.
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14 This study focuses on the mechanisms involved in TNF α -mediated inhibition of
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16 erythropoiesis. CD34⁺ hematopoietic stem/progenitor cells (HSPC) were isolated from human
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18 cord blood. Erythropoiesis was achieved *in vitro* by stimulating cells with EPO. We show that
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20 TNF α clearly affected erythroid development, as assessed by May-Grünwald/Giemsa
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22 staining, flow cytometry analysis and fluorescent microscopy. The amount of hemoglobin-
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24 producing cells as well as the expression of GATA-1 target erythro-specific genes (EPO
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26 receptor, glycoporphin A and globins) was found decreased after TNF α treatment of HSPC. In
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28 correlation, TNF α induced the expression of the transcription factors GATA-2 and PU.1,
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30 described as inhibitors of erythropoiesis. In this regard, TNF α promoted the formation of the
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32 GATA-1/PU.1 complex that has been reported to block the transcriptional activity of GATA-
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34 1. Our results clearly demonstrate that TNF α prevents EPO-mediated erythropoiesis of HSPC
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36 as an early event, by directly affecting erythroid cell development.
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47 **Keywords:** anemia; inflammation; TNF α ; GATA-1; GATA-2; PU.1
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1 Introduction

The majority of tumors are largely infiltrated by inflammatory cells, such as myelomonocytes and macrophages [1]. In response to the inflammatory environment, these cells produce inflammatory mediators including chemokines and cytokines. These mediators are tightly associated with cancer progression in combination with genetic alterations [2]. One of them, the Tumor Necrosis Factor alpha (TNF α), is widely found in the inflammation-associated cancer microenvironment [3-6]. Interestingly, because TNF α is commonly present in cancer and inflammatory diseases, it has been implicated in cancer- and inflammation-related anemia. Indeed, patients suffering from cancer and chronic inflammation are often anemic [7]. Moreover, *in vitro* and *in vivo* studies have led to the conclusion that TNF α inhibits hematopoietic progenitors from undergoing erythroid differentiation [8-15]. Anti-TNF α treatment is effectively used for the treatment of chronic inflammatory diseases, but leads to increased risk of infection [16, 17]. Despite the beneficial effects of anti-TNF α , this treatment may promote different types of cancers [18, 19].

Several events may trigger anemia, including iron deficiency, hemolysis and hemorrhage. In non-hematopoietic cancer, anemia can also result from the activation of the immune system with the release of inflammatory cytokines that affect red-cell production in patients. So far, one well described effect of cytokine release is the reduction of erythropoietin synthesis by kidneys [20]. In this specific case, anemia is usually treated with human recombinant EPO (EPO). Nevertheless, this treatment addresses only the symptoms, not the cause of the anemia. Furthermore, EPO can provoke cardiovascular and thrombo-embolic side effects, as well as possible tumor progression [21-24]. Indeed, cancer cells in many tumor types express the Erythropoietin receptor (EPOR) [25], and the interaction between Erythropoietin and the EPOR can activate the expression of genes implicated in angiogenesis

1 and cell proliferation [26]. Additionally, anemic patients without Erythropoietin deficiency do
2 not respond to EPO treatment [27, 28].
3

4 Erythropoiesis is a tightly regulated process by which red blood cells are generated from
5 hematopoietic stem and progenitor cells (HSPCs). The development and maturation of
6 erythroid cells requires Erythropoietin, which activates several signaling pathways by acting
7 on its specific receptor, EPOR [29, 30]. These pathways are involved in cell proliferation,
8 survival and differentiation [31-34]. Erythropoiesis is regulated by the combined action of
9 transcription factors [35, 36]. Indeed, the expression of erythroid specific genes is regulated
10 by the major erythroid transcription factor, GATA-1 [37], which is involved in the terminal
11 maturation and survival of erythroid cells. As previously shown, erythropoiesis is arrested
12 when GATA-1 is inactivated, which leads to increased susceptibility of mouse embryos to
13 death by anemia [38, 39]. GATA-2, another member of the GATA family of transcription
14 factors, acts as a balance to GATA-1 and is crucial during the earliest stages of erythropoiesis
15 [40]. In addition, GATA-2 controls the self-renewal of erythroid progenitors in bone marrow
16 [40-42]. These two factors act dynamically on hematopoietic progenitors during erythroid
17 commitment [43, 44].
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38 GATA-1 transcriptional activity is also modulated by co-factors [37] and post-
39 translational modifications [42]. One GATA-1 co-factor of interest is PU.1/SP1, from the
40 Ets-family of transcription factors, that plays a critical role in the balance between myelo-
41 monoipoiesis and lymphopoiesis on the one side, and erythropoiesis on the other side [45].
42 Indeed, PU.1 and GATA-1 can physically interact in progenitor cells, leading to inactivation
43 of GATA-1 and therefore the down-regulation of erythroid specific genes [46].
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53 The mechanisms involved in TNF α -mediated inhibition of erythropoiesis are not fully
54 understood. Our previous studies showed that TNF α inhibits erythroid differentiation of
55 human leukemia cell lines in culture. This suggests that the cytokine can act directly on
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1 hematopoietic cells independently from Erythropoietin deficiency. We reported that TNF α
2 prevented the induction of erythroid differentiation in correlation with decreased expression
3 of erythroid-specific genes and perturbations to the balance between GATA-1 and GATA-2
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10 In the present work, we studied the effect of TNF α on the development of CD34⁺
11 HSPCs in the presence of EPO. In this model, different steps of erythropoiesis were
12 successfully reproduced *in vitro*. We show that, in the heterogeneous cell population that
13 constitutes the CD34⁺ HSPC culture, TNF α acts on EPO-treated HSPCs by perturbing
14 erythroid development. This effect was found to correlate with changes in the key
15 transcription factors, GATA-1, GATA-2 and PU.1. Moreover the direct effect of TNF α on
16 erythroid progenitors has been confirmed by analyzing erythrospecific markers in isolated
17 erythroid (GPA⁺) cells.
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2 Materials and methods

2.1 Cell culture and treatment

Human umbilical cord blood was obtained from the Clinique Bohler in Luxembourg. For ethical reasons, cord blood cells were collected with written informed consent for use in research in agreement with the National Committee of Research Ethics in Luxembourg. The blood was collected in heparinized tubes. The mononucleated cell fraction was isolated using Ficoll™ (GE Healthcare, Roosendaal, The Netherlands) density gradient medium. CD34⁺ HSPCs or GPA⁺ cells were selected using magnetic cell sorting. Purification was performed following the manufacturer's instructions (MACS Miltenyi, Utrecht, The Netherlands). The average purity of the cells obtained was around 96% of CD34⁺ cells.

After isolation, the cells were cultured in serum-free culture medium for cell expansion and erythroid differentiation (Stem Cell II, Sigma-Aldrich, Bornem, Belgium), to which a cytokine cocktail containing Interleukin 3 (IL3, Reliatech, Wolfenbüttel, Germany) and stem cell factor (SCF, Reliatech) was added. Interleukin 3 (10 ng/mL) was added together with SCF (10 ng/mL) for 3 days following CD34⁺ cell enrichment. Erythroid differentiation was achieved by adding 2 U/mL human recombinant erythropoietin (EPO) (Eprex, a kind gift of Janssen-Cilag) to the medium. EPO was added on day 0, whereas IL3 was removed on day 4 and SCF was removed on day 6. The medium was also supplemented with L-glutamine and penicillin/streptomycin (Lonza, Vervier, Belgium). The cells were maintained at 2×10^5 cells/mL. TNF α (20 ng/mL; ReliaTech) was added 1 h before EPO (**Figure S1A**).

K562 and TF1 cells were provided by DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen; Braunschweig, Germany). These cells were cultured and treated as previously described [13, 15]. All cells were kept in 5% CO₂ at 37 °C.

2.2 Assays of cell proliferation, viability and morphology and erythroid differentiation

Cell number and viability were assessed using trypan blue dye exclusion. For evaluation of cell morphology, cytospin preparations were stained with May-Grünwald/Giemsa solution (Merck, Leuven, Belgium). Benzidine staining was used to detect the pseudoperoxidase activity of hemoglobin in cells. Images were collected with a Leica DM 2000 microscope.

2.3 Flow cytometry

HSPCs (2×10^5) were washed three times in PBS, before incubation with 5 μ L of antibody for 90 min at 4 °C. FITC-labeled CD34 (clone 8G12) and CD41a (clone HIP8), PE-labeled CD11b (clone D12), CD36 (clone CB38), and CD38 (clone HB7) and PE-CyTM5-labeled GPA (235a; clone HIR2) antibodies were used (BD Biosciences, Erembodegem, Belgium). The corresponding isotype control antibodies (BD Biosciences) were used to set the gating levels. The samples were fixed with 2% formaldehyde after being washed twice with 1X PBS. Analysis was performed on a FACS Calibur flow cytometer (BD Biosciences). The statistical analysis was based on 10,000 events per sample using FlowJo[®] software (version 8.8.7, Tree Star, Ashland Oregon, USA).

2.4 Fluorescence microscopy

HSPCs (5×10^5) were washed three times in PBS. Cell pellets were then resuspended in 100 μ L of 1X PBS containing 3 μ L of GPA antibody (235a; clone HIR2; BD Biosciences) and incubated for 2 h at 4 °C. After washing three times with 1X PBS, cells were fixed with 2% formaldehyde and stained with Hoechst 33342 (1 μ g/mL; Merck, Leuven, Belgium). Five microliters of stained cells were mounted on slides and observed with an Olympus CellM fluorescent microscope (Aartselaar, Belgium).

2.5 Western blotting

Up to 4×10^6 HSPCs were used to prepare nuclear, cytoplasmic and total protein

1 extracts, as previously described [15]. Denatured proteins (10 μ g or 20 μ g) were separated on
2 an SDS-PAGE gel. The proteins were transferred to a PVDF membrane that was then
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4 saturated for 1 h in 5% BSA or non-fat dry milk in PBS-Tween20 (PBS-T). The membranes
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6 were probed with antibodies against GATA-1 (C20X), GATA-2 (H-116), PU.1 (T-21), EPOR
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8 (M20), α -globin, β -globin, γ -globin (Santa Cruz Biotechnology, Tebu Bio, Boechout,
9
10 Belgium) and β actin (AC-15, Sigma-Aldrich). After washing with PBS-T, the membranes
11
12 were incubated with peroxidase-conjugated antibodies (Santa Cruz Biotechnology). The
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14 proteins were then visualized using chemiluminescence detection (ECL, GE Healthcare) and
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16 were revealed by autoradiography. Chemiluminescence was analyzed with the Kodak image
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18 station 440 CF (Kodak, Analis, Suarlée, Belgium) and quantified with the Kodak 1D image
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20 analysis software. The fold change of relative protein expression was normalized to the
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22 internal control, β actin.
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30 **2.6 Immunoprecipitation assays**

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32 Immunoprecipitation assays were performed using MACS protein G microbeads from
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34 MACS Miltenyi. Two hundred micrograms of nuclear and cytoplasmic proteins obtained
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36 from primary hematopoietic cells were incubated with 100 μ L of protein G beads and 2 μ g of
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38 GATA-1 (C20X) antibody (Santa Cruz Biotechnology). This mixture was mixed gently
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40 overnight at 4 °C. The immune complex was immobilized in a microMACS separation
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42 column and isolated according to the manufacturer's instructions. Immunoprecipitated
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44 proteins were loaded in an SDS-PAGE electrophoresis gel followed by western blot with
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46 GATA-1 (N1) and PU.1 (T-21) antibodies (Santa Cruz Biotechnology).
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53 **2.7 Electrophoretic mobility shift assay**

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55 Ten micrograms of nuclear extracts from TF1 or HSPCs was incubated for 30 min on
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57 ice with [³²P]ATP-labeled oligonucleotides in a reaction mixture containing protease
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59 inhibitors, 10 mM Tris-HCl, pH 8.5, 5% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM
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DTT, 1 mM MgCl₂, 2.5 mM poly(dI-dC), 0.2 mg/mL BSA and 4 mg/mL spermidine. For supershift the nuclear extracts and labeled oligonucleotide probes were incubated with the reaction mixture for 30 min on ice prior to 30 min incubation with 2 µg p50 (H119X) and p65 (C20X) antibodies (Santa Cruz Biotechnology). The sequence of the NFκB probe was: sense 5'-AGTTGAGGGACTTCCCAGGC-3'. The corresponding binding site is underlined. Annealing and labeling was performed as described previously [15].

2.8 Real time PCR

RNA was extracted from 3 x 10⁶ EPO-stimulated CD34⁺ cells treated with TNFα or left untreated, using Trizol (Invitrogen, Merelbeke, Belgium) and cleaned using RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). RNA quantity was measured by spectrophotometry (Nanodrop[®] ND-1000).

The RT² PCR Array First Strand Kit (SuperArray, Tebu-Bio, Boechout, Belgium) was used to synthesize cDNA from 1.5 µg of RNA according to the manufacturer's instructions. Real time PCR was performed using a customized RT² Profiler PCR Array System kit and the primers listed in **Table S1** according to the manufacturer's instructions. The samples were analyzed in a 7300 Real Time PCR system (Applied Biosystems, Halle, Belgium). The results were calculated using the ΔCT method and were normalized to the GAPDH housekeeping gene.

2.9 Transient transfection assays and plasmids

TF1 cells (3.75 x 10⁶) were electroporated using the Gene Pulser (BioRad, Nazareth, Belgium). Each pulse was made at 250 V and 950 µF with 5 µg of DNA in a medium added to 0.1% SVF. The reporter plasmids under the control of GATA-1 is pGL3-GATA-Luc [47] whereas the *Renilla*-expressing pHRG-TK plasmid (Promega, Leiden, The Netherlands) was used as an internal control. The pXM-GATA1 expressing vector was used to express

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exogenous GATA-1 in TF1 cells [48]. Twenty-four hours after transfection, the cells were seeded at 1×10^6 cell/mL and treated with 20 ng/mL of TNF α for 2 h. Dual-Glo™ Luciferase Reagent and Dual-Glo™ Stop&Glow Reagent (Promega) were used according to the manufacturer's instructions. Luciferase activities (Firefly and Renilla) were measured with an Orion microplate luminometer (Berthold). Firefly activity was normalized to Renilla and results were expressed as a ratio in arbitrary units.

2.10 Statistics

Data are expressed as mean \pm S.D. and were analyzed by Student's t-test. Results were considered statistically significant when *P < 0.05, **P < 0.01 and ***P < 0.001.

3 Results

3.1 TNF α affects EPO-mediated erythroid development of CD34⁺ cells

CD34⁺ HSPCs were isolated from umbilical cord blood to an average purity of approximately 96% (**Figure 1**). Following CD34⁺ isolation, cell populations were characterized by analyzing specific hematopoietic markers with flow cytometry. As expected, results show a heterogeneous population with erythro-, myelo-, megakaryo- and lymphoblastic features (**Figure 1**). The cell culture procedure used to study the effect of TNF α on EPO-mediated erythropoiesis was designed as described in **Figure S1A**. The culture medium was supplemented with 20 ng/mL TNF α on days 0, 4 and 6, 1 h before EPO stimulation. As shown in the control EMSA experiment, NF- κ Bp50/p65 was activated in TNF α -treated cells, indicating that CD34⁺ cells are sensitive to TNF α (**Figure S1B**). Moreover, the percentage of CD34⁺ cells significantly decreased from day 4 to day 9 of treatment with EPO alone and EPO/TNF α (**Figure S1C**). This suggests that cells underwent differentiation.

As shown by May-Grünwald/Giemsa staining, EPO successfully induced erythropoiesis *in vitro* (**Figure 2A**). The different stages of cell maturation during erythropoiesis were observed, including reticulocyte-like cells and enucleation at days 5 and 8. TNF α treatment prevented EPO-mediated development of erythropoiesis so that the different stages of erythroblast maturation were not observed. Moreover, TNF α treatment triggered a 50% decrease in the percentage of hemoglobin producing cells (benzidine positive cells) after 4 days of treatment (**Figure 2B**). Flow cytometry confirmed the effect of TNF α on the erythroid pathway. The early erythroid marker, CD36, was significantly down-regulated in TNF α -treated cells in comparison to the control EPO-treated cells on day 9. Likewise, the

1 expression of GPA, another erythro-specific marker, was significantly decreased by 2.8-fold
2 on day 9 of TNF α treatment (**Figure 2C**). This down-regulation was also observed by
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4 fluorescent microscopy, as fluorescent intensity was lower in TNF α -treated cells (**Figure S2**).
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6 Similarly, flow cytometry showed a decrease in the percentage of GPA⁺ cells after 4 and 9
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8 days of TNF α treatment (**Figure 2C**). Together, results showed that TNF α reduced both the
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10 percentage of GPA⁺ cells and the expression level of GPA in these cells. Moreover,
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12 megakaryocytic CD41a and myelo-monocytic CD11b, as well as neutrophil CD10, eosinophil
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14 CD15 and macrophage CD16 markers were not affected (data not shown). Furthermore, we
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16 observed that TNF α increased cell proliferation (**Figure 2D**). Altogether, our results provide
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18 evidence that TNF α significantly prevents EPO-mediated erythroid differentiation of HSPCs
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20 in our culture system.
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28 **3.2 TNF α inhibits GATA-1 transactivation in TF1 cells**

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30 GATA-1, a key transcription factor involved in erythroid differentiation, regulates the
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32 expression of erythroid-specific genes. The ability of TNF α to inhibit GATA-1
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34 transactivation was assessed by transient transfection assay in an established cell line to
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36 circumvent the heterogeneity of HSPC culture. The human erythroleukemia TF1 cell line was
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38 co-transfected with the pXM-GATA1 expression vector together with a reporter plasmid
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40 containing GATA sequences within the promoter upstream of the luciferase reporter gene
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42 (pGL3-GATA-Luc). After 24 h of transfection, the cells were treated with TNF α or left
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44 untreated for 2 h in medium containing granulocyte macrophage-colony stimulating factor
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46 (GM-CSF). Luciferase activity was increased 2-fold in cells transfected with pXM-GATA1.
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48 Results show that the exogenous GATA-1-mediated luciferase activation was significantly
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50 reduced in the presence of TNF α compared to basal levels, suggesting a direct effect of the
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52 cytokine on GATA-1 transactivation (**Figure 3A**). This effect may be considered to be
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1 independent from the expression level of endogenous GATA-1 in these culture conditions
2 because GATA-1 had low basal expression in TF1 cells (**Figure 3B**).
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5 **3.3 TNF α affects the expression of GATA-1, GATA-2 and PU.1**

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8 The effect of TNF α on GATA-1 expression, as well as other transcription factors
9 critical for erythroid development, was then studied in EPO-differentiated HSPCs. Despite the
10 inhibiting effect of TNF α on erythropoiesis, real-time PCR revealed a 1.5-fold increase in
11 GATA-1 mRNA expression after 4 days of TNF α treatment. However, a 2.7-fold increase in
12 GATA-2 and 2.3-fold-increase in PU.1 were also observed after TNF α treatment (**Figure**
13 **4A**). GATA-2 and PU.1 over-expression on the one hand and a perturbation of the balance
14 between GATA-1 and GATA-2 on the other hand, are in accordance with inhibition of
15 erythropoiesis. Moreover, GATA-2 over-expression was correlated with increased cell
16 proliferation (**Figure 2D**). Western blot analysis of the transcription factors validated the real-
17 time PCR results as GATA-1 expression was increased (up to 2.9-fold) in TNF α -treated
18 HSPCs on days 2, 3 and 4. Likewise, GATA-2 and PU.1 showed a 2.8- and 2.3-fold maximal
19 increase in expression, respectively (**Figure 4B**). These results suggest that TNF α mediates
20 its inhibiting effect by affecting transcription factors associated with GATA-1, rather than
21 GATA-1's expression level.
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44 **3.4 Effect of TNF α on the interaction between GATA-1 and PU.1**

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46 PU.1 and GATA-1 are described as physically interacting with each other, which results
47 in the inhibition of GATA-1 transactivation and, therefore, the down-regulation of erythroid
48 specific genes [49]. To assess the possible capacity of TNF α to affect GATA-1 activity *via*
49 PU.1 over-expression, the interaction between GATA-1 and PU.1 was studied by
50 immunoprecipitation assay in HSPCs that had undergone EPO-mediated differentiation in the
51 presence or absence of TNF α . The results show that PU.1 co-immunoprecipitated with
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1 GATA-1 in the nuclear protein extracts from TNF α -treated HSPCs after 4 days, whereas it
2 did not in control cells (**Figure 4C**). This result suggests that PU.1 contributes to TNF α -
3 mediated inhibition of erythropoiesis by directly interacting with GATA-1.
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8 **3.5 TNF α down-regulates erythro-specific gene expression**

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10 To correlate the inhibiting effect of TNF α on GATA-1 activity with GATA-1 target
11 gene expression, we analyzed glycoporphin A (GPA), EPOR and globin (α , β and γ) gene
12 expression in EPO- and EPO/TNF α -treated HSPCs at both the mRNA and protein levels. All
13 the genes studied were found to be down-regulated in the presence of TNF α in comparison to
14 EPO-differentiated cells that were not treated with TNF α . Real-time PCR analysis showed a
15 2-fold decrease in GPA, EPOR, α -globin, β -globin and γ -globin mRNA levels after 4 days of
16 TNF α treatment. Conversely, the expression of CD11b, a monocyte/macrophage
17 differentiation-related gene and CD41b, a megakaryocyte differentiation-related gene, were
18 not significantly affected (**Figure 5A**). Western blot analysis showed a 2-fold reduced
19 expression of EPOR protein at days 3 and 4. Globins were not detectable or only slightly
20 expressed after two days of TNF α treatment in comparison to EPO-stimulated cells without
21 TNF α treatment. The expression of globins progressively increased in EPO-treated and
22 EPO/TNF α -treated cells over the 4 days of treatment. However, α - and β -globin expression
23 levels remained lower in the presence of TNF α . The inhibition of γ -globin observed on day 2
24 of TNF α treatment was abolished on days 3 and 4 (**Figure 5B**). These results are in
25 agreement with the decrease in erythroblastic cell population in culture, supporting the
26 inhibitory effect of TNF α on erythroid cell development *via* a down-regulation of GATA-1
27 erythro-specific target genes.
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57 **3.6 TNF α acts directly on erythroid cells**

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To verify the direct effect of TNF α on erythroid progenitors, HSPC were stimulated by EPO with or without TNF α treatment. Cells were treated for 9 days based on the results of flow cytometry. This result showed a more important inhibitory effect of TNF α on GPA expression level at day 9 compared to day 4 (**Figure 2C**, GPA, MFI=f (days of treatment)). GPA⁺ cells were isolated and Western blot analysis showed that GATA-1, GATA-2 and PU.1 expression remained over-expressed in TNF α -treated cells. Moreover, globin expression remained lower in these cells compared to EPO-stimulated cells (**Figure 6**). This is in agreement with a delay of erythroid differentiation and correlates with the low expression of GPA as shown in figure 2C and S2. Results demonstrate that TNF α inhibits erythroid development through a direct effect on early erythroid progenitors.

4 Discussion

We used CD34⁺ HSPCs purified from umbilical cord blood, which are commonly used *in vitro* to mimic erythropoiesis, in order to investigate the effect of TNF α on erythropoiesis at the cellular and molecular levels. Cytometry revealed a heterogeneous CD34⁺ cell population detected in the culture prior to the addition of EPO or EPO/TNF α . In this study, we successfully reproduced the different steps of EPO-mediated erythropoiesis, as assessed by May-Grünwald/Giemsa staining after several days of culture. The addition of TNF α to the medium clearly repressed erythroid development of CD34⁺ cells as confirmed by several methods. In contrast, the myeloid (granulo-monocytic) and megakaryocytic pathways were not affected. This experimental model is a simplification of the *in vivo* situation since it omits other mediators of inflammation that are involved in the development of anemia [50]. Nonetheless, the action of TNF α was observed in a heterogeneous hematopoietic cell population in which anemia was successfully mimicked. Indeed, TNF α specifically inhibited erythropoiesis by acting directly on HSPCs, likely by affecting EPO activity.

At the molecular level, this study indicates the effect of TNF α on the combination of transcription factors that leads to physiological hematopoietic lineage commitment [35-37]. In erythropoiesis, the GATA-1 and GATA-2 transcription factors have essential roles, and a quantitative balance in their protein levels is required to achieve erythroid lineage development [51]. GATA-2 plays a role in early progenitor cell proliferation, and activates GATA-1 gene expression, which, conversely, silences GATA-2 expression in erythroid progenitors [52]. GATA-1 deficiency leads to death due to severe anemia [38], while GATA-2 deficiency leads to embryonic death [41]. Moreover, Ikonomi *et al.* reported that GATA-2 over-expression induced the arrest of erythroid differentiation in K562 cells [53]. However, it has also been reported that over-expression of GATA1 in erythroid cells, both *in vitro*, and *in*

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in vivo, inhibits erythroid differentiation [54, 55]. In our *in vitro* culture system, GATA-1, and GATA-2 were both over-expressed, suggesting a deregulation of the balance between GATA-1 and GATA-2 after TNF α treatment. The increase in GATA-2 expression could explain the over-expression of GATA-1 [43]. However, we previously reported increased expression of GATA-2 in combination with decreased expression of GATA-1 in EPO-induced TF1 cells in the presence of TNF α [13]. Observations were then in favor of a role for GATA-2 over-expression in the effect of TNF α on HSPCs. However, GATA-2 up-regulation by TNF α has also been described in the hepatocarcinoma cell line (HepG2) [56]. This effect was involved in the inhibition of Erythropoietin gene expression at the level of the kidney and liver, which could represent an alternate mechanism by which pro-inflammatory cytokines trigger anemia. Here, we provide evidence that TNF α can also inhibit erythropoiesis by directly affecting EPO-responsive HSPC erythroid differentiation.

To evaluate the effect of TNF α on GATA-1 transcriptional activity, exogenous GATA-1 was transiently transfected together with the pGL3-GATA-Luc reporter plasmid in TF1 cells. The use of this cell line circumvented the heterogeneity of HSPC culture and the activation of the GATA responsive element within the reporter gene promoter by other members of the GATA family of transcription factors, including endogenous GATA-1 itself. Indeed, GATA-1 expression remains at a very low basal level in TF1 cells when cultured in GM-CSF containing medium. In the presence of TNF α , the transcriptional activity of GATA-1 was significantly reduced, such that the inhibiting effect was attributed to a direct alteration of GATA-1 transactivation by TNF α . This effect could involve impairments in the posttranslational regulation of GATA-1, as we previously showed that TNF α reduced GATA-1 acetylation in TF1 cells [13].

Moreover, GATA-1 activity is also dependent on interaction with co-factors. It has been reported that PU.1 inhibits erythroid differentiation by physically interacting with GATA-1

1 and inhibiting its binding to the DNA of erythro-specific genes [46, 49, 57]. We show in this
2 study that TNF α induced increased expression of the myeloid and lymphoid transcription
3 factor, PU.1 [58, 59]. The analysis of GATA-1/PU.1 interaction by immunoprecipitation
4 showed that the TNF α -mediated increase in PU.1 expression was associated with the
5 formation of the GATA-1/PU.1 complex in the nuclear extracts from EPO-induced HSPCs.
6 These results support a critical role for PU.1 in TNF α -mediated inhibition of erythropoiesis,
7 likely by affecting GATA-1 activity in HSPCs. In correlation with this proposal, TNF α -
8 mediated inhibition of erythropoiesis was associated with the down-regulation of only
9 GATA-1 target genes. Furthermore, persistent over-expression of GATA-2 is in accordance
10 with the decreased activity of GATA-1, as GATA-2 has been reported to be up-regulated in
11 the absence of GATA-1 [60]. However, over-expression of GATA-2 might contribute to
12 progenitor renewal.

13 Altogether, our results indicate that TNF α affects distinct regulatory steps that are
14 implicated in erythroid maturation and differentiation. We show that TNF α plays a critical
15 role in the impairment of the balance between GATA-1 and GATA-2. Moreover, we provide,
16 for the first time, the evidence that TNF α induces PU.1 protein over-expression in HSPCs and
17 perturbs the normal pattern of erythroid-implicated factors, thus promoting deregulation of
18 lineage commitment despite the presence of EPO. Furthermore, the analysis of globin and
19 transcription factor expression in GPA⁺ erythroid cells after TNF α treatment of HSPCs
20 supported the hypothesis of a direct effect of TNF α on erythroid progenitors.

21 Therefore, we suggest that cancer-related anemia results in part, from a direct
22 interaction between HSPCs and pro-inflammatory cytokines released in the bone marrow and
23 tumor microenvironment. According to our results this interaction triggers modifications in
24 specific transcription factor networks implicated in the regulation of the different lineages
25 commitment. While EPO is efficient for treating anemia associated with Erythropoietin

1 deficiency, alternative therapies are obviously required in the case of direct TNF α -mediated
2 injury of erythroid development in the presence of functional Erythropoietin.
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Figure Legends

Figure 1: Characterization of the CD34⁺ isolated population. The purity of CD34⁺ cells obtained after isolation was assessed by flow cytometry. The average purity was approximately 96%. In parallel, the presence of hematopoietic markers, such as CD36, GPA, CD41a, CD11b, CD24, and CD38 were evaluated. One representative experiment out of three is shown here.

Figure 2: *In vitro* erythropoiesis and the effect of TNF α on erythroid morphology, development and differentiation. **A.** Giemsa staining at days (D) 2, 3, 5, and 8 of culture in EPO-differentiated cells treated with TNF α (20 ng/mL) or left untreated. Untreated cells from day 0 were used as a control. Red arrows: proerythroblasts; black arrows: polychromatic erythroblasts; blue arrows: orthochromatic erythroblasts; green arrows: enucleating reticulocytes; orange arrows: reticulocyte-like cells. Control, D2 and D3 images were captured using a 40x objective, and D5 and D8 were captured using a 100x objective. **B.** Benzidine staining was performed on EPO-differentiated cells treated with 20 ng/mL of TNF α or left untreated for 3 and 4 days. (*: P<0.05). **C.** Flow cytometry was used to evaluate the evolution of the erythroid specific markers, CD36 and GPA/CD235a, after 4 and 9 days of TNF α treatment. **D.** Cell proliferation of HSPCs in the presence of EPO only or EPO/TNF α .

Figure 3: Effect of TNF α on GATA-1 transcriptional activity **A.** TF1 cells were co-transfected with the GATA-1-expressing vector, pXM-GATA1 and the reporter luciferase plasmids, pGL3-GATA-Luc and pHRG-TK. Transfected cells were treated for 2 h with TNF α or left untreated prior to analysis of luciferase activity. Firefly activity was normalized to Renilla, and the results are expressed as a ratio in arbitrary units. The data are presented as mean \pm S.D. of three independent experiments. (**: P \leq 0.01 and ***: P<0.001). **B.** GATA-1

1 protein expression in TF1 cells cultured in the presence of GM-CSF (GM) or GM-CSF and
2 TNF α (GM/TNF) was assessed by western blot analysis. Nuclear extracts from human
3 erythroleukemia HEL cells were used as a positive control. β actin was used as an internal
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12 **Figure 4: Effect of TNF α on erythroid-specific transcription factors. A.** EPO-
13 differentiated cells were treated with TNF α or left untreated for 4 days. Total RNA was
14 extracted and subjected to reverse transcription and real-time PCR. Results were calculated by
15 Δ CT method and normalized to the housekeeping gene, GAPDH. **B.** EPO-differentiated cells
16 were treated with TNF α or left untreated, and nuclear factors were extracted on days 2, 3 and
17 4. GATA-1, GATA-2 and PU.1 expression were analyzed by western blot. β actin was used
18 as an internal control. One experiment out of three independent experiments is shown. **C.**
19 EPO-stimulated CD34⁺ cells were treated with TNF α or left untreated for 4 days. Nuclear
20 (nucl) and cytoplasmic (cyto) proteins were extracted and GATA-1 was immunoprecipitated
21 using GATA-1 (C20X) antibody. GATA-1 (N1) and PU.1 (T-21) antibodies were then used
22 to reveal GATA-1 and PU.1 by immunoblotting. One out of three independent experiments is
23 shown.
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45 **Figure 5: Effect of TNF α on erythroid-specific gene expression A.** EPO-stimulated CD34⁺
46 cells were treated with TNF α or left untreated for 4 days. Total RNA was extracted and
47 submitted to reverse transcription and real-time PCR using specific primers for GPA
48 (glycophorin A), EPOR (erythropoietin receptor), HB (hemoglobin), CD41b and CD11b
49 genes. Δ CT values were calculated and normalized to the GAPDH housekeeping gene. (*: P
50 values<0.05; **: P values<0.01). **B.** Western blot analysis of α -globin, β -globin, γ -globin and
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1 EPOR in total protein extracts from CD34⁺ cells treated with TNF α or left untreated for 2, 3
2 and 4 days. β actin was used as an internal control. One out of three independent experiments
3 is shown.
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10 **Figure 6: Effect of TNF α on isolated GPA⁺ cells.** HSPC cells were treated with TNF α or
11 left untreated for 9 days. GPA⁺ cells were then isolated and the nuclear and cytoplasmic
12 protein extracts were used to perform western blot analysis of the transcription factors
13 GATA-1, GATA-2 and PU.1 and the α -, β - and γ -globin. β actin was used as an internal
14 control. One out of three independent experiments is shown.
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Figure 1

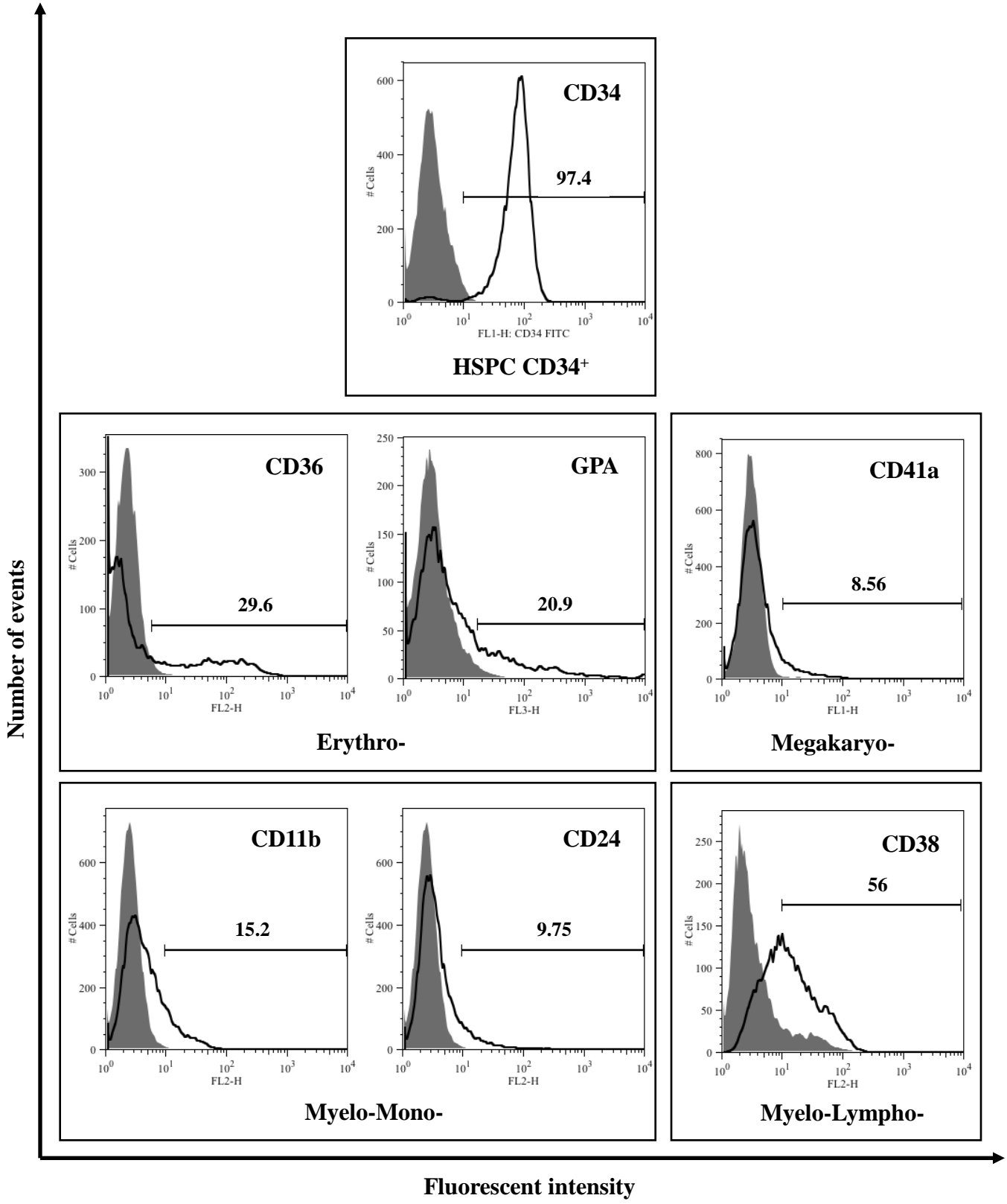


Figure 2

C

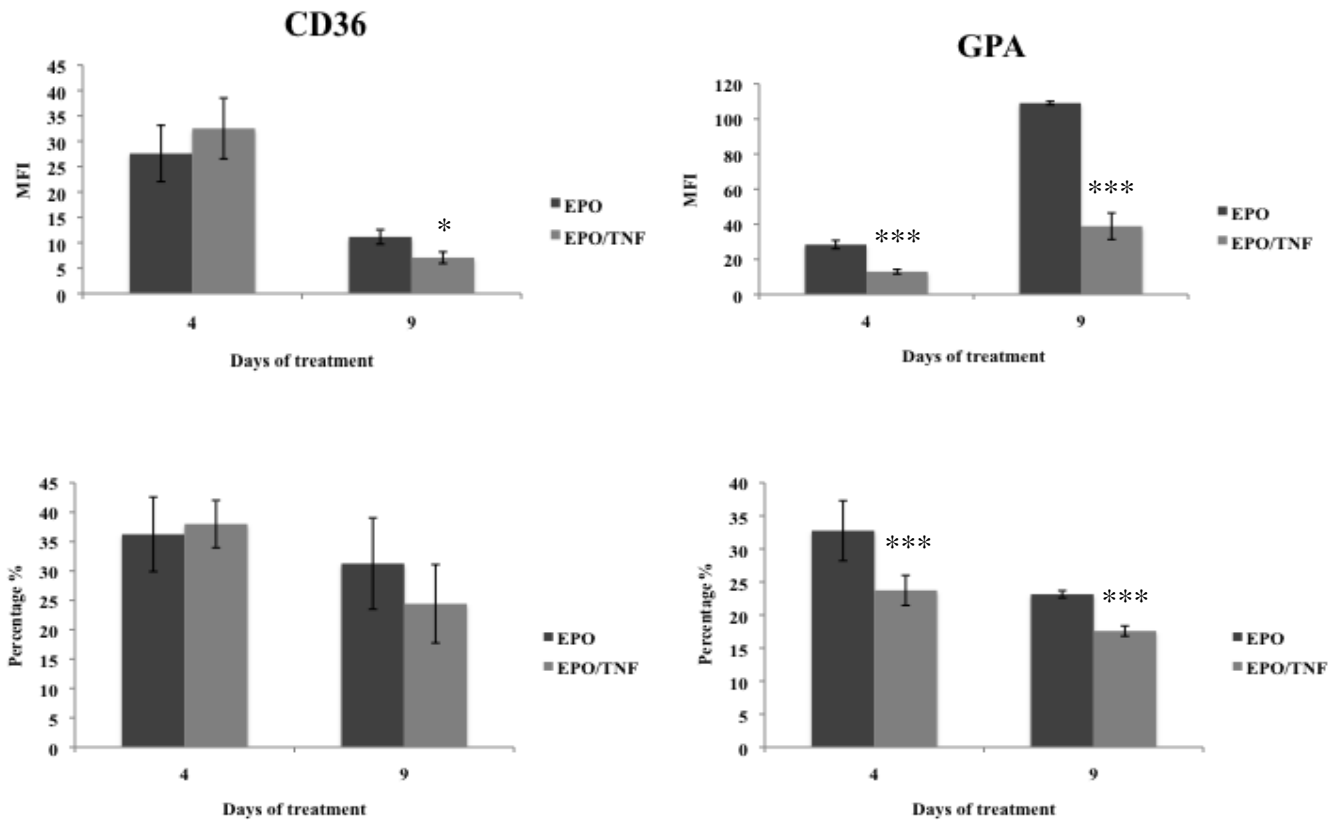
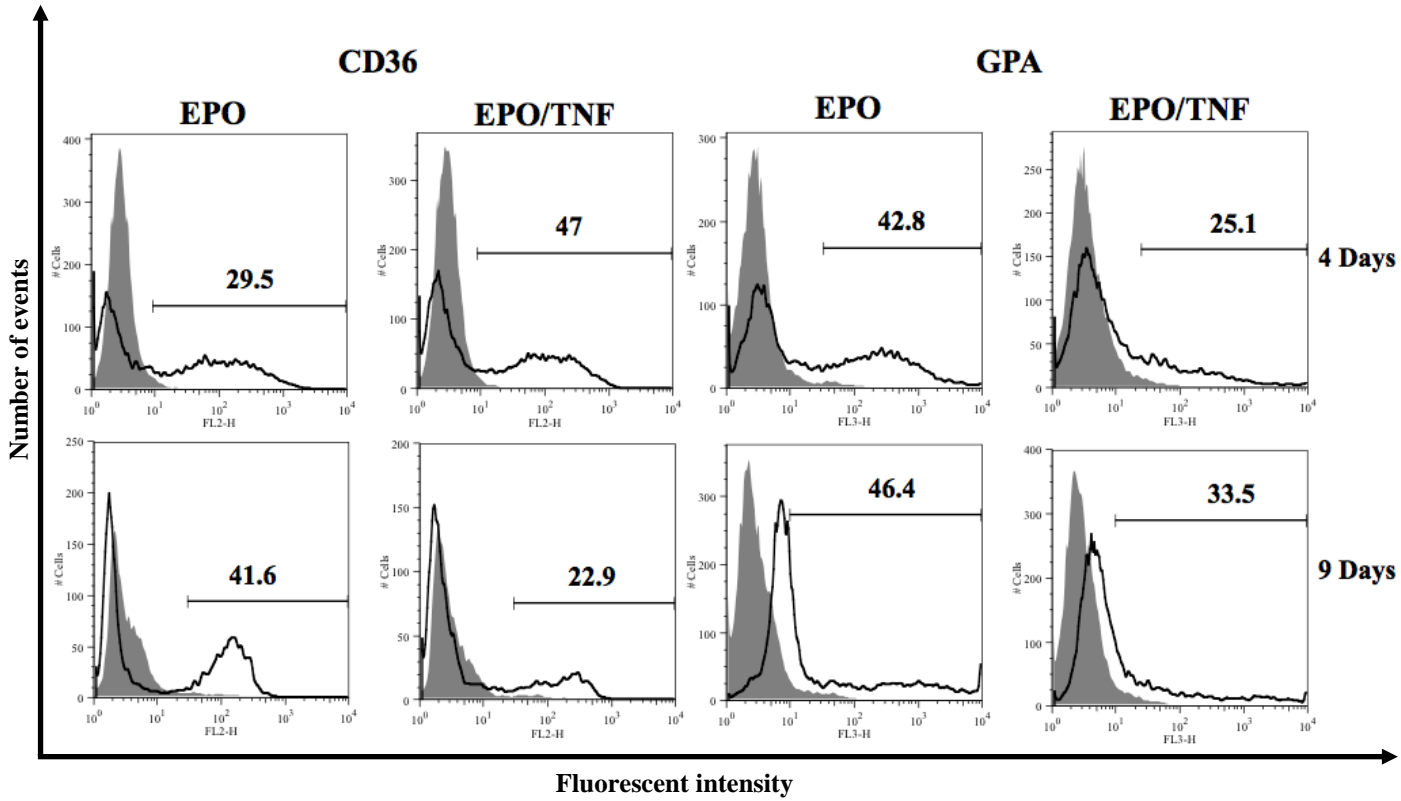
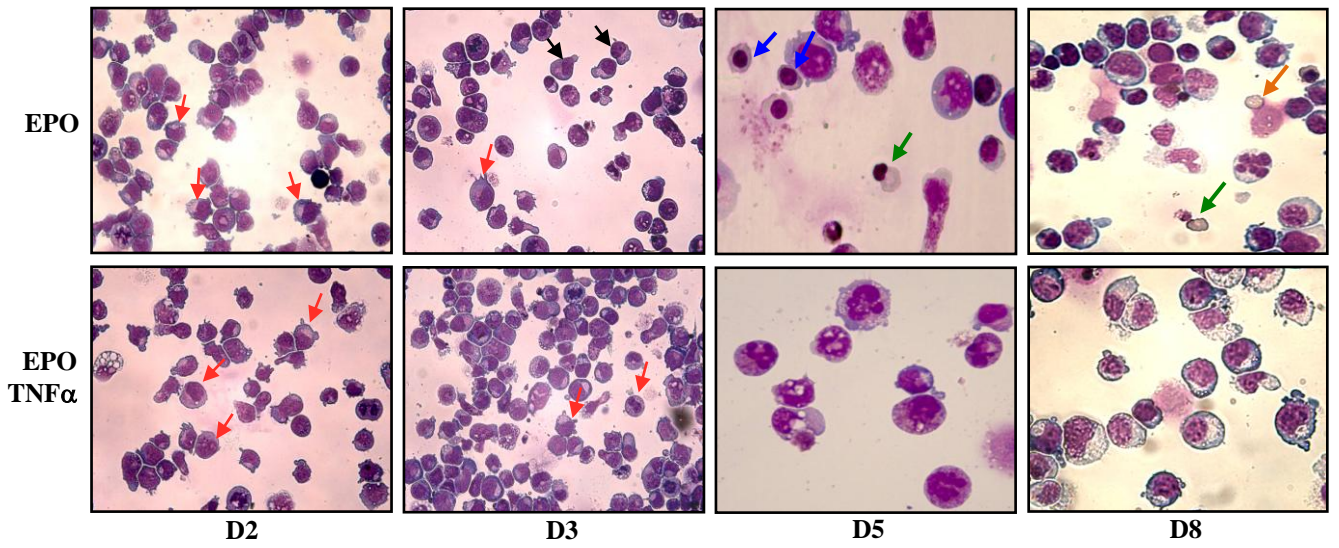
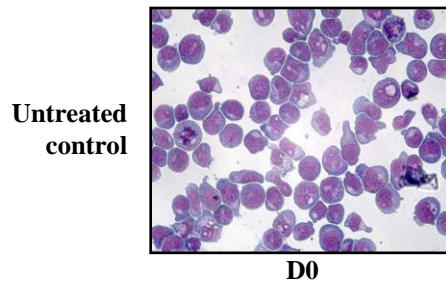


Figure 2

A



B

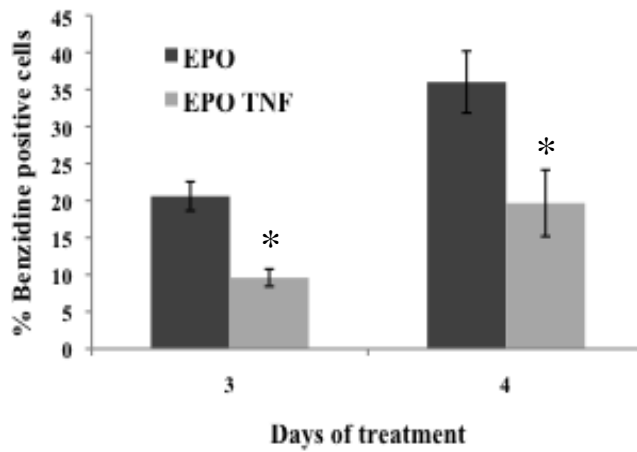


Figure 2

D

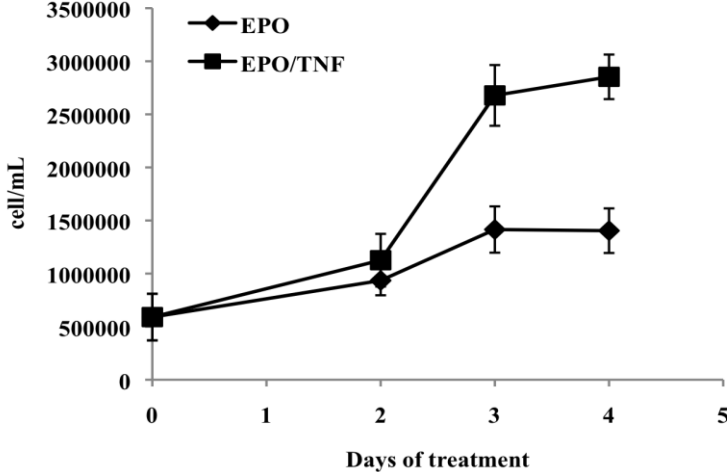
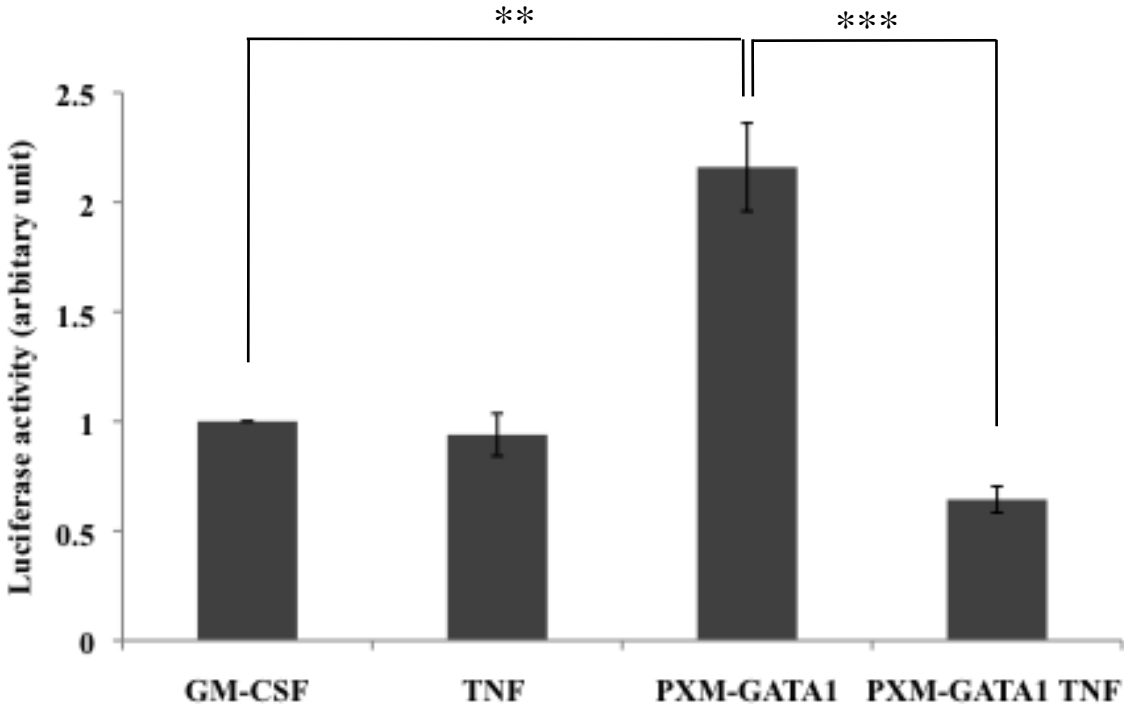


Figure 3

A



B

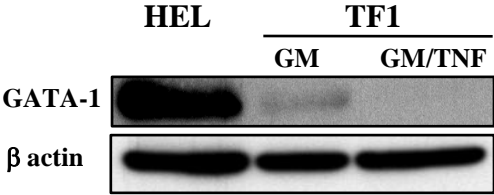
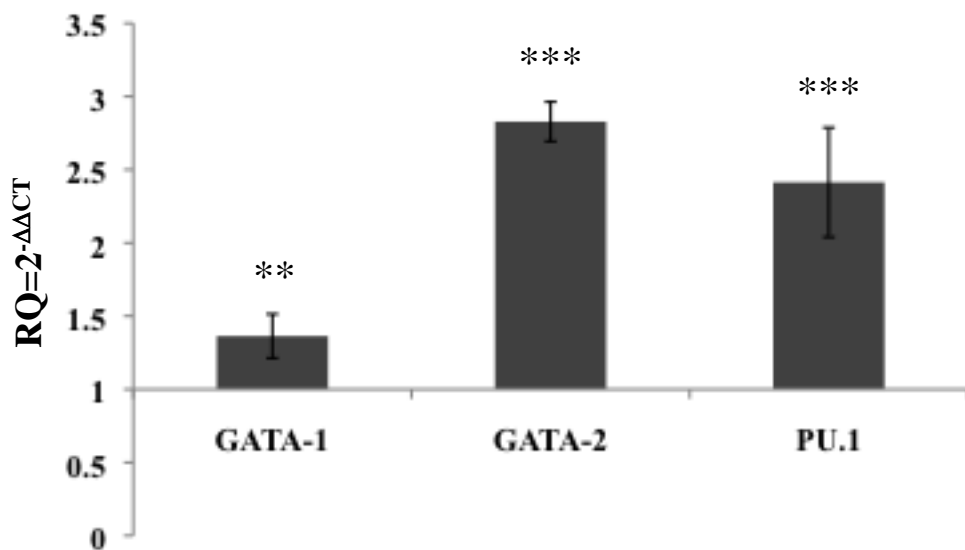
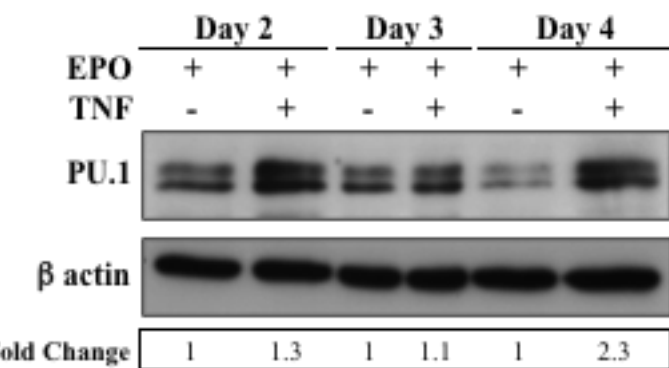
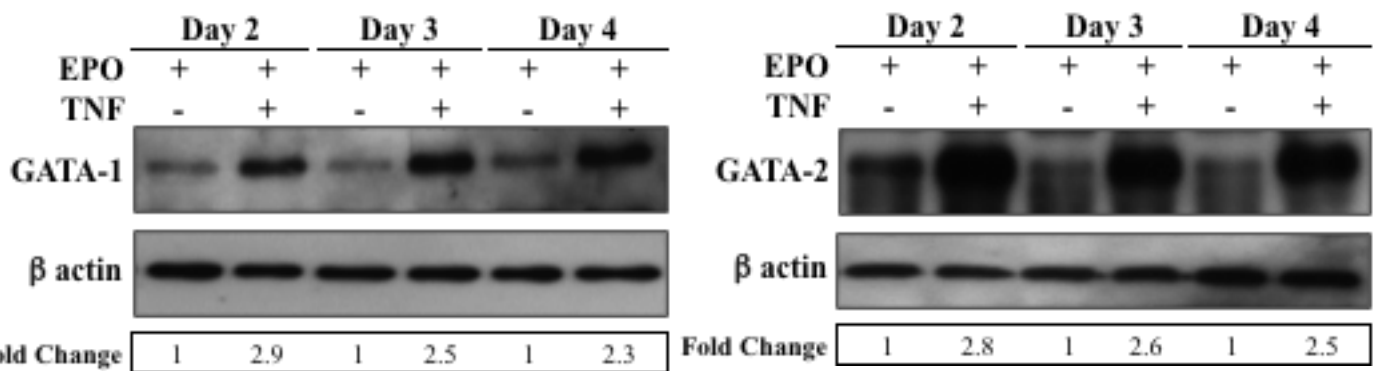


Figure 4

A



B



C

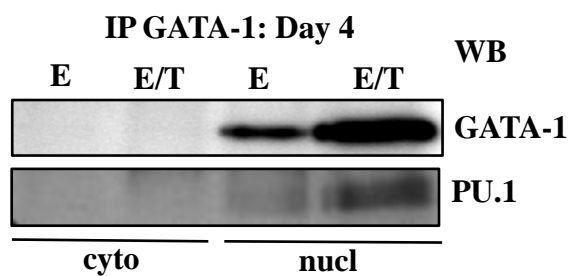
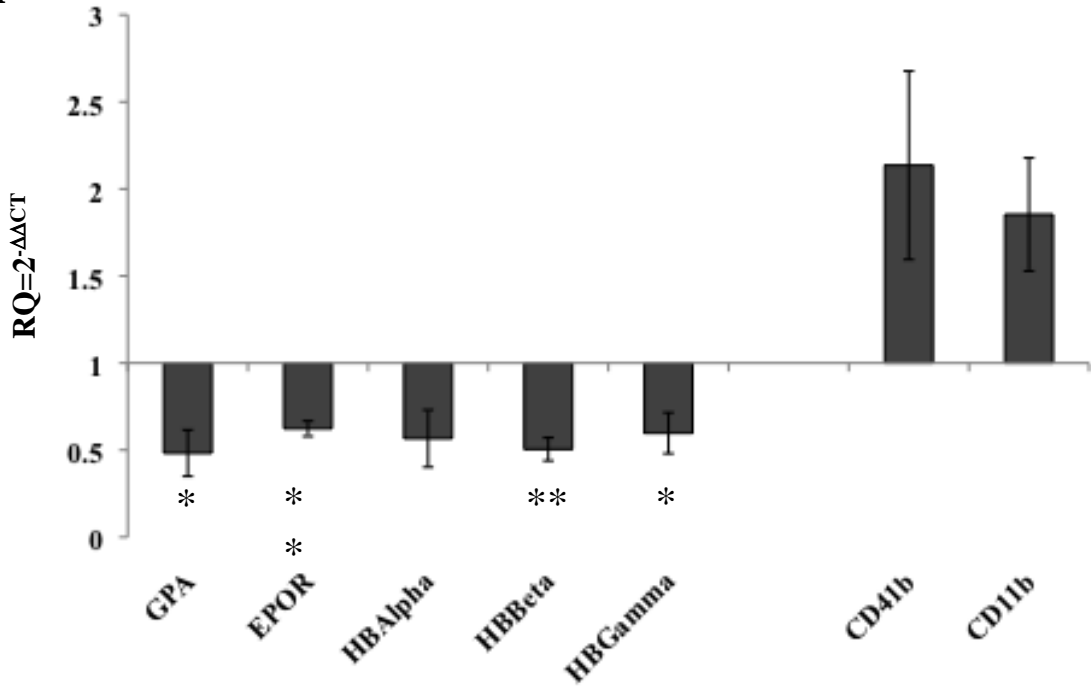


Figure 5

A



B

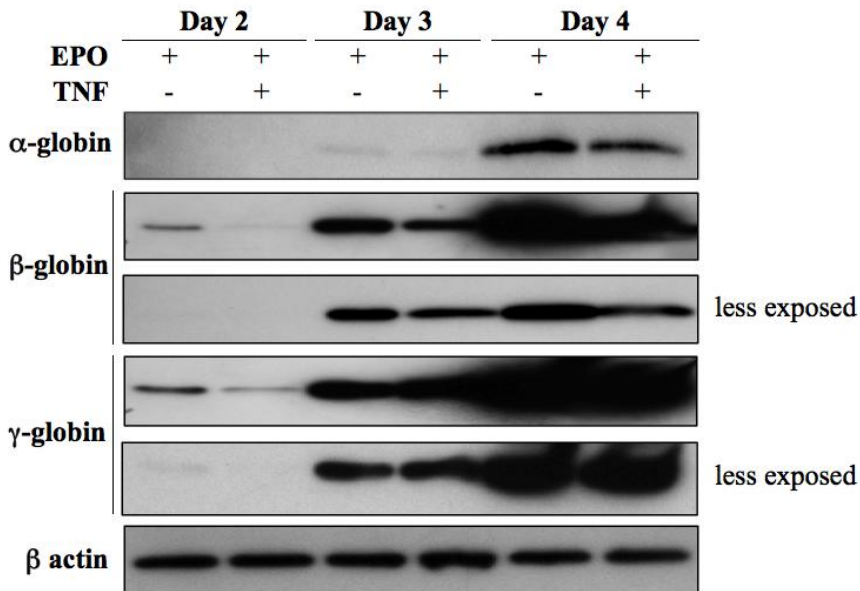
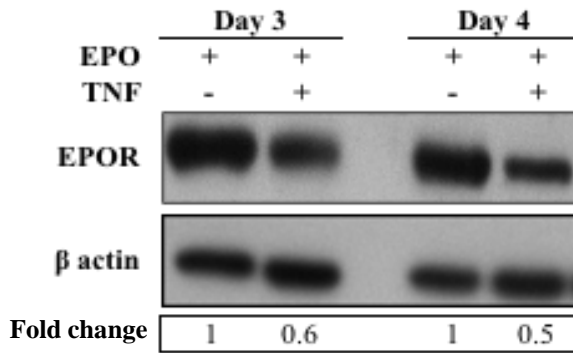


Figure 6

