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

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Abstract

Many physiological perturbations can cause anemia. In cancer patients, activation of the immune system leads to the production of proinflammatory cytokines including tumor necrosis factor alpha (TNF α), that have been shown to inhibit red-cell production *via* poorly understood mechanisms. Treatment of anemia by human recombinant erythropoietin (EPO) is strongly suspected to induce tumor growth.

This study focuses on the mechanisms involved in TNF α -mediated inhibition of erythropoiesis. CD34⁺ hematopoietic stem/progenitor cells (HSPC) were isolated from human cord blood. Erythropoiesis was achieved *in vitro* by stimulating cells with EPO. We show that TNF α clearly affected erythroid development, as assessed by May-Grünwald/Giemsa staining, flow cytometry analysis and fluorescent microscopy. The amount of hemoglobin-producing cells as well as the expression of GATA-1 target erythro-specific genes (EPO receptor, glycophorin A and globins) was found decreased after TNF α treatment of HSPC. In correlation, TNF α induced the expression of the transcription factors GATA-2 and PU.1, described as inhibitors of erythropoiesis. In this regard, TNF α promoted the formation of the GATA-1/PU.1 complex that has been reported to block the transcriptional activity of GATA-1. Our results clearly demonstrate that TNF α prevents EPO-mediated erythropoiesis of HSPC as an early event, by directly affecting erythroid cell development.

Keywords: anemia; inflammation; TNF α ; GATA-1; GATA-2; PU.1

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

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

Erythropoiesis is a tightly regulated process by which red blood cells are generated from hematopoietic stem and progenitor cells (HSPCs). The development and maturation of erythroid cells requires Erythropoietin, which activates several signaling pathways by acting on its specific receptor, EPOR [29, 30]. These pathways are involved in cell proliferation, survival and differentiation [31-34]. Erythropoiesis is regulated by the combined action of transcription factors [35, 36]. Indeed, the expression of erythroid specific genes is regulated by the major erythroid transcription factor, GATA-1 [37], which is involved in the terminal maturation and survival of erythroid cells. As previously shown, erythropoiesis is arrested when GATA-1 is inactivated, which leads to increased susceptibility of mouse embryos to death by anemia [38, 39]. GATA-2, another member of the GATA family of transcription factors, acts as a balance to GATA-1 and is crucial during the earliest stages of erythropoiesis [40]. In addition, GATA-2 controls the self-renewal of erythroid progenitors in bone marrow [40-42]. These two factors act dynamically on hematopoietic progenitors during erythroid commitment [43, 44].

GATA-1 transcriptional activity is also modulated by co-factors [37] and post-translational modifications [42]. One GATA-1 co-factor of interest is PU.1/SP1, from the Ets-family of transcription factors, that plays a critical role in the balance between myelopoiesis and lymphopoiesis on the one side, and erythropoiesis on the other side [45]. Indeed, PU.1 and GATA-1 can physically interact in progenitor cells, leading to inactivation of GATA-1 and therefore the down-regulation of erythroid specific genes [46].

The mechanisms involved in TNF α -mediated inhibition of erythropoiesis are not fully understood. Our previous studies showed that TNF α inhibits erythroid differentiation of human leukemia cell lines in culture. This suggests that the cytokine can act directly on

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
4 [13-15].
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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

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

2.6 Immunoprecipitation assays

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

2.7 Electrophoretic mobility shift assay

Ten micrograms of nuclear extracts from TF1 or HSPCs was incubated for 30 min on ice with [32 P]ATP-labeled oligonucleotides in a reaction mixture containing protease inhibitors, 10 mM Tris-HCl, pH 8.5, 5% glycerol, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM

1 DTT, 1 mM MgCl₂, 2.5 mM poly(dI-dC), 0.2 mg/mL BSA and 4 mg/mL spermidine. For
 2 supershift the nuclear extracts and labeled oligonucleotide probes were incubated with the
 3
 4 reaction mixture for 30 min on ice prior to 30 min incubation with 2 µg p50 (H119X) and p65
 5
 6 (C20X) antibodies (Santa Cruz Biotechnology). The sequence of the NFκB probe was: sense
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 8 5'-AGTTGAGGGACTTCCCAGGC-3'. The corresponding binding site is underlined.
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 10 Annealing and labeling was performed as described previously [15].
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15 **2.8 Real time PCR**

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 17 RNA was extracted from 3 x 10⁶ EPO-stimulated CD34⁺ cells treated with TNFα or left
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 19 untreated, using Trizol (Invitrogen, Merelbeke, Belgium) and cleaned using RNeasy Mini Kit
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 21 (Qiagen, Venlo, The Netherlands). RNA quantity was measured by spectrophotometry
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 23 (Nanodrop[®] ND-1000).
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 28 The RT² PCR Array First Strand Kit (SuperArray, Tebu-Bio, Boechout, Belgium) was
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 30 used to synthesize cDNA from 1.5 µg of RNA according to the manufacturer's instructions.
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 32 Real time PCR was performed using a customized RT² Profiler PCR Array System kit and the
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 34 primers listed in **Table S1** according to the manufacturer's instructions. The samples were
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 36 analyzed in a 7300 Real Time PCR system (Applied Biosystems, Halle, Belgium). The results
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 38 were calculated using the ΔCT method and were normalized to the GAPDH housekeeping
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 40 gene.
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45 **2.9 Transient transfection assays and plasmids**

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 47 TF1 cells (3.75 x 10⁶) were electroporated using the Gene Pulser (BioRad, Nazareth,
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 49 Belgium). Each pulse was made at 250 V and 950 µF with 5 µg of DNA in a medium added
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 51 to 0.1% SVF. The reporter plasmids under the control of GATA-1 is pGL3-GATA-Luc [47]
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 53 whereas the *Renilla*-expressing pHRG-TK plasmid (Promega, Leiden, The Netherlands) was
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 55 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 $\text{TNF}\alpha$ 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

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

3.2 TNF α inhibits GATA-1 transactivation in TF1 cells

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

independent from the expression level of endogenous GATA-1 in these culture conditions because GATA-1 had low basal expression in TF1 cells (**Figure 3B**).

3.3 TNF α affects the expression of GATA-1, GATA-2 and PU.1

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

3.4 Effect of TNF α on the interaction between GATA-1 and PU.1

PU.1 and GATA-1 are described as physically interacting with each other, which results in the inhibition of GATA-1 transactivation and, therefore, the down-regulation of erythroid specific genes [49]. To assess the possible capacity of TNF α to affect GATA-1 activity *via* PU.1 over-expression, the interaction between GATA-1 and PU.1 was studied by immunoprecipitation assay in HSPCs that had undergone EPO-mediated differentiation in the presence or absence of TNF α . The results show that PU.1 co-immunoprecipitated with

GATA-1 in the nuclear protein extracts from TNF α -treated HSPCs after 4 days, whereas it did not in control cells (**Figure 4C**). This result suggests that PU.1 contributes to TNF α -mediated inhibition of erythropoiesis by directly interacting with GATA-1.

3.5 TNF α down-regulates erythro-specific gene expression

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

3.6 TNF α acts directly on erythroid cells

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*

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

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

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

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

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

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References

- [1] Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest* 2007;117:1155-66.
- [2] Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature* 2008;454:436-44.
- [3] Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, et al. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004;431:461-6.
- [4] Friess H, Guo XZ, Nan BC, Kleeff O, Buchler MW. Growth factors and cytokines in pancreatic carcinogenesis. *Ann N Y Acad Sci* 1999;880:110-21.
- [5] Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer* 2004;4:540-50.
- [6] Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, et al. Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 1999;5:828-31.
- [7] Knight K, Wade S, Balducci L. Prevalence and outcomes of anemia in cancer: a systematic review of the literature. *Am J Med* 2004;116 Suppl 7A:11S-26S.
- [8] Johnson RA, Waddelow TA, Caro J, Oliff A, Roodman GD. Chronic exposure to tumor necrosis factor in vivo preferentially inhibits erythropoiesis in nude mice. *Blood* 1989;74:130-8.
- [9] Papadaki HA, Kritikos HD, Valatas V, Boumpas DT, Eliopoulos GD. Anemia of chronic disease in rheumatoid arthritis is associated with increased apoptosis of bone marrow erythroid cells: improvement following anti-tumor necrosis factor-alpha antibody therapy. *Blood* 2002;100:474-82.

- [10] Xiao W, Koizumi K, Nishio M, Endo T, Osawa M, Fujimoto K, et al. Tumor necrosis factor-alpha inhibits generation of glycophorin A+ cells by CD34+ cells. *Exp Hematol* 2002;30:1238-47.
- [11] Dufour C, Corcione A, Svahn J, Haupt R, Poggi V, Beka'ssy AN, et al. TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. *Blood* 2003;102:2053-9.
- [12] Dubey S, Shukla P, Nityanand S. Expression of interferon-gamma and tumor necrosis factor-alpha in bone marrow T cells and their levels in bone marrow plasma in patients with aplastic anemia. *Ann Hematol* 2005;84:572-7.
- [13] Buck I, Morceau F, Cristofanon S, Heintz C, Chateauvieux S, Reuter S, et al. Tumor necrosis factor alpha inhibits erythroid differentiation in human erythropoietin-dependent cells involving p38 MAPK pathway, GATA-1 and FOG-1 downregulation and GATA-2 upregulation. *Biochem Pharmacol* 2008;76:1229-39.
- [14] Buck I, Morceau F, Cristofanon S, Reuter S, Dicato M, Diederich M. The inhibitory effect of the proinflammatory cytokine TNFalpha on erythroid differentiation involves erythroid transcription factor modulation. *Int J Oncol* 2009;34:853-60.
- [15] Morceau F, Schnekenburger M, Blasius R, Buck I, Dicato M, Diederich M. Tumor necrosis factor alpha inhibits aclacinomycin A-induced erythroid differentiation of K562 cells via GATA-1. *Cancer Lett* 2006;240:203-12.
- [16] Haraoui B. Differentiating the efficacy of tumor necrosis factor inhibitors. *J Rheumatol Suppl* 2005;74:3-7.
- [17] Vilcek J, Feldmann M. Historical review: Cytokines as therapeutics and targets of therapeutics. *Trends Pharmacol Sci* 2004;25:201-9.
- [18] Yan L, Anderson GM, DeWitte M, Nakada MT. Therapeutic potential of cytokine and chemokine antagonists in cancer therapy. *Eur J Cancer* 2006;42:793-802.

- [19] Scheinfeld N. A comprehensive review and evaluation of the side effects of the tumor necrosis factor alpha blockers etanercept, infliximab and adalimumab. *J Dermatolog Treat* 2004;15:280-94.
- [20] Spivak JL. The anaemia of cancer: death by a thousand cuts. *Nat Rev Cancer* 2005;5:543-55.
- [21] Engert A. Recombinant human erythropoietin in oncology: current status and further developments. *Ann Oncol* 2005;16:1584-95.
- [22] Rizzo JD, Somerfield MR, Hagerty KL, Seidenfeld J, Bohlius J, Bennett CL, et al. Use of epoetin and darbepoetin in patients with cancer: 2007 American Society of Hematology/American Society of Clinical Oncology clinical practice guideline update. *Blood* 2008;111:25-41.
- [23] Bohlius J, Schmidlin K, Brillant C, Schwarzer G, Trelle S, Seidenfeld J, et al. Recombinant human erythropoiesis-stimulating agents and mortality in patients with cancer: a meta-analysis of randomised trials. *Lancet* 2009;373:1532-42.
- [24] Tonelli M, Hemmelgarn B, Reiman T, Manns B, Reaume MN, Lloyd A, et al. Benefits and harms of erythropoiesis-stimulating agents for anemia related to cancer: a meta-analysis. *CMAJ* 2009;180:E62-71.
- [25] Fandrey J. Erythropoietin receptors on tumor cells: what do they mean? *Oncologist* 2008;13 Suppl 3:16-20.
- [26] Yasuda Y, Fujita Y, Matsuo T, Koinuma S, Hara S, Tazaki A, et al. Erythropoietin regulates tumour growth of human malignancies. *Carcinogenesis* 2003;24:1021-9.
- [27] Cooper AC, Mikhail A, Lethbridge MW, Kemeny DM, Macdougall IC. Increased expression of erythropoiesis inhibiting cytokines (IFN-gamma, TNF-alpha, IL-10, and IL-13) by T cells in patients exhibiting a poor response to erythropoietin therapy. *J Am Soc Nephrol* 2003;14:1776-84.

- [28] Macdougall IC, Cooper AC. Hyporesponsiveness to erythropoietic therapy due to chronic inflammation. *Eur J Clin Invest* 2005;35 Suppl 3:32-5.
- [29] Tan CC, Eckardt KU, Firth JD, Ratcliffe PJ. Feedback modulation of renal and hepatic erythropoietin mRNA in response to graded anemia and hypoxia. *Am J Physiol* 1992;263:F474-81.
- [30] Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, et al. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. *Cell* 1993;74:227-36.
- [31] Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. *Blood* 2001;98:3261-73.
- [32] Bouscary D, Pene F, Claessens YE, Muller O, Chretien S, Fontenay-Roupie M, et al. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. *Blood* 2003;101:3436-43.
- [33] Myklebust JH, Blomhoff HK, Rusten LS, Stokke T, Smeland EB. Activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34(+) hematopoietic progenitor cells. *Exp Hematol* 2002;30:990-1000.
- [34] Carroll MP, Spivak JL, McMahon M, Weich N, Rapp UR, May WS. Erythropoietin induces Raf-1 activation and Raf-1 is required for erythropoietin-mediated proliferation. *J Biol Chem* 1991;266:14964-9.
- [35] Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 2008;132:631-44.
- [36] Friedman AD. Transcriptional control of granulocyte and monocyte development. *Oncogene* 2007;26:6816-28.

- [37] Morceau F, Schnekenburger M, Dicato M, Diederich M. GATA-1: friends, brothers, and coworkers. *Ann N Y Acad Sci* 2004;1030:537-54.
- [38] Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, et al. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991;349:257-60.
- [39] Fujiwara Y, Browne CP, Cunniff K, Goff SC, Orkin SH. Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proc Natl Acad Sci U S A* 1996;93:12355-8.
- [40] Tsai FY, Orkin SH. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. *Blood* 1997;89:3636-43.
- [41] Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* 1994;371:221-6.
- [42] Ferreira R, Ohneda K, Yamamoto M, Philipsen S. GATA1 function, a paradigm for transcription factors in hematopoiesis. *Mol Cell Biol* 2005;25:1215-27.
- [43] Ohneda K, Yamamoto M. Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage. *Acta Haematol* 2002;108:237-45.
- [44] Leonard M, Brice M, Engel JD, Papayannopoulou T. Dynamics of GATA transcription factor expression during erythroid differentiation. *Blood* 1993;82:1071-9.
- [45] Tenen DG. Disruption of differentiation in human cancer: AML shows the way. *Nat Rev Cancer* 2003;3:89-101.

- [46] Zhang P, Zhang X, Iwama A, Yu C, Smith KA, Mueller BU, et al. PU.1 inhibits GATA-1 function and erythroid differentiation by blocking GATA-1 DNA binding. *Blood* 2000;96:2641-8.
- [47] Blobel GA, Nakajima T, Eckner R, Montminy M, Orkin SH. CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. *Proc Natl Acad Sci U S A* 1998;95:2061-6.
- [48] Collavin L, Gostissa M, Avolio F, Secco P, Ronchi A, Santoro C, et al. Modification of the erythroid transcription factor GATA-1 by SUMO-1. *Proc Natl Acad Sci U S A* 2004;101:8870-5.
- [49] Liew CK, Simpson RJ, Kwan AH, Crofts LA, Loughlin FE, Matthews JM, et al. Zinc fingers as protein recognition motifs: structural basis for the GATA-1/friend of GATA interaction. *Proc Natl Acad Sci U S A* 2005;102:583-8.
- [50] Buck I, Morceau F, Grigorakaki C, Dicato M, Diederich M. Linking anemia to inflammation and cancer: the crucial role of TNF α . *Biochem Pharmacol* 2009;77:1572-9.
- [51] Ferreira R, Wai A, Shimizu R, Gillemans N, Rottier R, von Lindern M, et al. Dynamic regulation of Gata factor levels is more important than their identity. *Blood* 2007;109:5481-90.
- [52] Grass JA, Boyer ME, Pal S, Wu J, Weiss MJ, Bresnick EH. GATA-1-dependent transcriptional repression of GATA-2 via disruption of positive autoregulation and domain-wide chromatin remodeling. *Proc Natl Acad Sci U S A* 2003;100:8811-6.
- [53] Ikonomi P, Rivera CE, Riordan M, Washington G, Schechter AN, Noguchi CT. Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. *Exp Hematol* 2000;28:1423-31.

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- [54] Whyatt D, Lindeboom F, Karis A, Ferreira R, Milot E, Hendriks R, et al. An intrinsic but cell-nonautonomous defect in GATA-1-overexpressing mouse erythroid cells. *Nature* 2000;406:519-24.
- [55] Whyatt DJ, Karis A, Harkes IC, Verkerk A, Gillemans N, Elefanty AG, et al. The level of the tissue-specific factor GATA-1 affects the cell-cycle machinery. *Genes Funct* 1997;1:11-24.
- [56] La Ferla K, Reimann C, Jelkmann W, Hellwig-Burgel T. Inhibition of erythropoietin gene expression signaling involves the transcription factors GATA-2 and NF-kappaB. *Faseb J* 2002;16:1811-3.
- [57] Stopka T, Amanatullah DF, Papetti M, Skoultschi AI. PU.1 inhibits the erythroid program by binding to GATA-1 on DNA and creating a repressive chromatin structure. *EMBO J* 2005;24:3712-23.
- [58] DeKoter RP, Singh H. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 2000;288:1439-41.
- [59] Ito T, Nishiyama C, Nishiyama M, Matsuda H, Maeda K, Akizawa Y, et al. Mast cells acquire monocyte-specific gene expression and monocyte-like morphology by overproduction of PU.1. *J Immunol* 2005;174:376-83.
- [60] Weiss MJ, Keller G, Orkin SH. Novel insights into erythroid development revealed through in vitro differentiation of GATA-1 embryonic stem cells. *Genes Dev* 1994;8:1184-97.

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

protein expression in TF1 cells cultured in the presence of GM-CSF (GM) or GM-CSF and TNF α (GM/TNF) was assessed by western blot analysis. Nuclear extracts from human erythroleukemia HEL cells were used as a positive control. β actin was used as an internal control.

Figure 4: Effect of TNF α on erythroid-specific transcription factors. **A.** EPO-differentiated cells were treated with TNF α or left untreated for 4 days. Total RNA was extracted and subjected to reverse transcription and real-time PCR. Results were calculated by Δ CT method and normalized to the housekeeping gene, GAPDH. **B.** EPO-differentiated cells were treated with TNF α or left untreated, and nuclear factors were extracted on days 2, 3 and 4. GATA-1, GATA-2 and PU.1 expression were analyzed by western blot. β actin was used as an internal control. One experiment out of three independent experiments is shown. **C.** EPO-stimulated CD34⁺ cells were treated with TNF α or left untreated for 4 days. Nuclear (nucl) and cytoplasmic (cyto) proteins were extracted and GATA-1 was immunoprecipitated using GATA-1 (C20X) antibody. GATA-1 (N1) and PU.1 (T-21) antibodies were then used to reveal GATA-1 and PU.1 by immunoblotting. One out of three independent experiments is shown.

Figure 5: Effect of TNF α on erythroid-specific gene expression **A.** EPO-stimulated CD34⁺ cells were treated with TNF α or left untreated for 4 days. Total RNA was extracted and submitted to reverse transcription and real-time PCR using specific primers for GPA (glycophorin A), EPOR (erythropoietin receptor), HB (hemoglobin), CD41b and CD11b genes. Δ CT values were calculated and normalized to the GAPDH housekeeping gene. (*: P values<0.05; **: P values<0.01). **B.** Western blot analysis of α -globin, β -globin, γ -globin and

EPOR in total protein extracts from CD34⁺ cells treated with TNF α or left untreated for 2, 3 and 4 days. β actin was used as an internal control. One out of three independent experiments is shown.

Figure 6: Effect of TNF α on isolated GPA⁺ cells. HSPC cells were treated with TNF α or left untreated for 9 days. GPA⁺ cells were then isolated and the nuclear and cytoplasmic protein extracts were used to perform western blot analysis of the transcription factors GATA-1, GATA-2 and PU.1 and the α -, β - and γ -globin. β actin was used as an internal control. One out of three independent experiments is shown.

Figure

Figure 1

Figure 2

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Figure 2

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Figure 2

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Figure 3

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Figure 4

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Figure 6