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Tumor necrosis factor α inhibits erythroid differentiation in human erythropoietin-dependent cells involving p38 MAPK pathway, GATA-1 and FOG-1 downregulation and GATA-2 upregulation

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ABSTRACT

The proinflammatory cytokine tumor necrosis factor α (TNFα) has been linked to inflammation- and cancer-related anemia, which reduces both quality of life and prognosis of patients. The aim of this study was to reveal molecular mechanisms linked to the inhibition of erythroid differentiation by TNFα. In this study, we showed that the inhibition of erythropoietin (Epo)-mediated differentiation by TNFα lead to a downregulation of hemoglobin synthesis and was correlated to a modulation of key erythroid transcription factors. Thus, a reverse of the transcription factor GATA-1/GATA-2 balance normally present during erythropoiesis, as well as a downregulation of the cofactor of GATA-1, friend of GATA-1 (FOG-1), and the coregulating transcription factor nuclear factor erythroid 2 (NF-E2) was observed after TNFα treatment. Moreover, we showed a reduction of GATA-1/FOG-1 interaction due to a reduced transcription of GATA-1 and a proteasome-dependent FOG-1 degradation after TNFα treatment. These changes led to an inhibition of erythroid gene expression including Epo receptor (EpoR), α- and γ-globin, erythroid-associated factor (ERAF), hydroxymethylbilane synthetase (HMBS), and glycophorin A (GPA). An analysis of distinct signaling pathway activations then revealed an activation of p38 by TNF, as well as a corresponding involvement of this mitogen-activated protein kinase (MAPK) in the cytokine-dependent inhibition of erythroid differentiation. Indeed the p38 inhibitor, SB203580, abrogated the inhibitory effect of TNFα on the major erythroid transcription factor GATA-1 as well as erythroid marker expression in Epo-induced TF-1 cells. Overall, these data contribute to a better understanding of cytokine-dependent anemia, by giving first hints about key erythroid transcription factor modulations after TNFα treatment as well as an involvement of p38 in the inhibition of erythroid differentiation.

1. Introduction

Anemia is a widespread complication in diseases linked to inflammation and cancer. The incidence of anemia varies with tumor type, stage and patient age and affects quality of life. Up to one-third of patients are suffering from anemia at diagnosis [1], a number, which is even increased after chemotherapy [2]. Cancer-associated anemia can be considered as a negative

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prognostic factor for survival regardless of tumor type [3]. Regarding anemia due to inflammation, this sign of ineffective erythropoiesis was described as a clinical entity of patients with inflammatory disorders [4]. Prior to the use of erythroid-stimulating factors (ESAs), blood transfusions were the only treatment for cancer-related anemia. However, the recently described conflicting effects of ESAs in distinct studies [5] make it necessary to further investigate the molecular mechanisms involved in anemia. Tumor necrosis factor α (TNFα) is an important factor in many different forms of anemia and fatigue related to cancer [6,7] as well as inflammation [8–10]. This cytokine is mainly produced by macrophages in response to inflammation [11] and its expression has been confirmed in the tumor microenvironment of various malignancies [12]. TNFα was even categorized as a tumor promoter [13] and several *in vitro* studies revealed the inhibitory effects of this cytokine on hematopoietic progenitor cell growth and erythroid markers such as glycophorin A (GPA) [14,15]. TNFα acts through its receptors and activates distinct underlying cell signaling pathways leading to activation of nuclear factor kappa B (NF-κB) or p38 mitogen-activated protein kinase (MAPK) depending on the factors present in the receptor complex [11].

The hormone erythropoietin (Epo) is a key factor for human erythropoiesis regulation and acts through its receptor (EpoR) in order to stimulate underlying cell signaling pathways [16]. Another important regulator of erythroid development is the transcription factor GATA-1. Like Epo and EpoR, GATA-1 is essential for the survival of erythroid precursors and their terminal differentiation into red blood cells. GATA-1 binds to a consensus GATA motif present in the *cis*-regulatory elements of most erythroid genes [17]. GATA-$1^{-/-}$ embryonic stem cells cannot contribute to definitive erythropoiesis [18]. Furthermore, GATA-1 activity is regulated by a large number of cofactors, which can act either as coactivators, namely friend of GATA (FOG-1), CREB-binding protein (p300/CBP) or as repressors such as PU.1 [19]. GATA-1 and GATA-2 are both expressed in erythrocyte and megakaryocyte lineages and present overlapping but distinct expression patterns. During early hematopoiesis, GATA-2 is involved in expanding progenitor cells, while GATA-1 is required for terminal erythroid maturation [18,20,21]. GATA-1 and GATA-2 activities are also modulated by posttranslational modifications [22]. On the other hand, transcription factors such as nuclear factor erythroid-derived 2 (NF-E2) or erythroid Krüppel-like factor 1 (EKLF), which act downstream of GATA-1, are required for globin gene expression [23–25].

In a previous study, we showed that TNFα inhibited aclacinomycin-induced erythroid differentiation of K562 cells in correlation with a decrease in GATA-1 expression [26]. In order to better understand the inhibiting effect of TNFα on erythroid differentiation, we used the erythroleukemia cell line TF-1 as a cellular model. We analyzed the effect of this proinflammatory cytokine on erythroid-specific transcription factors, as well as erythroid gene expression and signaling pathways in Epo-differentiated TF-1 cells. We showed that TNFα induced a reversal of the GATA-1/GATA-2 balance as well as a decrease of the GATA-1/FOG-1 protein complex formation through a reduced transcription of GATA-1 and a proteasomal degradation of FOG-1 leading resulting in a reduction of erythroid marker genes. Moreover, this study gave first hints about a p38 involvement in the inhibition of erythroid differentiation.

2. Materials and methods

2.1. Cell culture and treatments

The human erythroleukemia cell line TF-1 was purchased from DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells were cultured (37 °C, 5% CO_2) in RPMI 1640 (Cambrex, Verviers, Belgium) supplemented with 10% fetal bovine serum (Cambrex), a penicillin–streptomycin mixture (Cambrex), and 5 ng/mL granulocyte–macrophage colony-stimulating factor (GM-CSF) (PeproTech, London, UK). These culturing conditions were used for untreated controls (U). Prior to the induction of differentiation by 10 U/mL Epo (Eprex, donated by Janssen-Cilag, Berchem, Belgium), cells were cultured without GM-CSF overnight. Cell density was maintained at 2×10^5 cells/mL. 100 μg/mL Remicade® (R) (generic name: infliximab; Centocor, Leiden, The Nederlands), 1 μM lactacystin (Lact) (a 26S proteasome-specific inhibitor; Sigma, Bornem, Belgium), 10 μM p38MAPK inhibitor (SB203580, Promega, Leiden, The Nederlands), 1 μM NF-κB inhibitor (Bay 11-7082, Calbiochem, Leuven, Belgium), 10 μM Jun N-terminal kinase (JNK) inhibitor (SP600125, Calbiochem), 10 μM mitogen-activated protein kinase kinase 1 (MEK) inhibitor (U0126, Promega), 10 μM MEK inhibitor (PD098059, Calbiochem), 10 μM phosphoinositide-3-kinase (PI3K) inhibitor (LY 294002, Calbiochem), and/or 20 ng/mL TNFα (PeproTech) were added to cell suspension prior to the addition of Epo. Cell viability was analyzed using Trypan blue. Erythroid differentiation was scored by benzidine staining as described [26].

2.2. Real-time PCR

RNA was extracted from TF-1 cells using Trizol (Invitrogen, Merelbeke, Belgium), and cleaned using RNeasy Mini Kit (Qiagen, Westburg, Venlo, The Netherlands). RNA integrity was controlled using a Bioanalyzer 2100 (Agilent, Belgium). 1.5 μg of total RNA was used to perform cDNA synthesis using RT^2PCR Array First Strand Kit (SuperArray, Tebu-Bio) according to the manufacturer's instructions. Real-time PCR with SuperArray primers for GATA-1, GATA-2, NF-E2, FOG-1, PU.1, EpoR, α- and γ-globin, erythroid-associated factor (ERAF), hydroxymethylbilane synthetase (HMBS), and GPA was performed, according to manufacturers using a custom RT^2Profiler PCR Array System and a 7300 real-time PCR System (Applied Biosystems, Lennik, Belgium). Results were evaluated using an Excel-based data analysis template from SuperArray.

2.3. Western blot

Nuclear and cytoplasmic extracts were prepared from 10^7 TF-1 cells as previously described [26]. 20 μg of denaturated TF-1 nuclear or cytoplasmic proteins was resolved in a SDS-PAGE. Membranes were saturated for 1 h in 5% BSA or milk in a 0.005% Tween 20-PBS (PBS-T) solution, and incubated for 1 h or

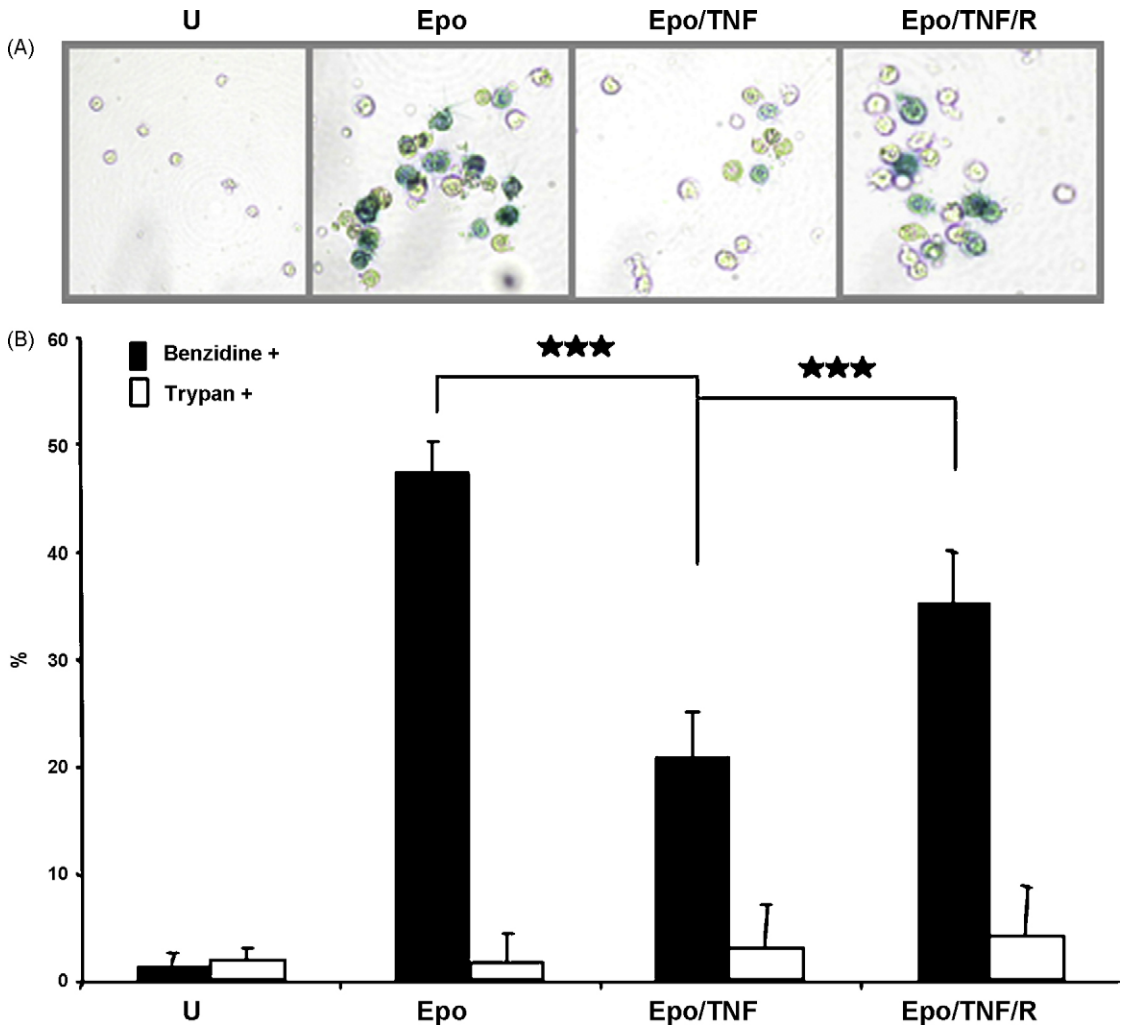

Fig. 1 – Effect of TNFα on hemoglobin synthesis. TF-1 cells were induced to differentiate for 3 days with 10 U/mL Epo in the presence or absence of 100 μg/mL Remicade (R) and/or 20 ng/mL TNFα (TNF) for 1 h prior to 3 days of differentiation. Untreated cells were used as controls (U). (A) Hemoglobinized cells were stained on day 3 using benzidine method. (B) Percentages of benzidine-positive cells and Trypan-positive cells. Data are means ± S.D. of five independent experiments. Asterisks (*) denote statistical significance by Student's *t*-test of $^{***}P \leq .001$.

overnight with the following antibodies: GATA-1 (sc-1233X), Phospho-p38 MAPK (P-p38; 9211), p38 MAPKinase antibody (9212), γ-globin (sc-21756), EpoR (sc-697), GATA-2 (sc-9008), FOG-1 (sc-9361), NF-E2 (sc-291), and β-actin (A5441). After washing with PBS-T, membranes were incubated for 1 h with peroxidase IgG conjugates, the immunoreactive proteins were visualized using enhanced chemiluminescence system by autoradiography (ECL, GE Healthcare, Diegem, Belgium). Antibodies used for western blot analysis were purchased from Santa Cruz except for p38 antibodies (Cell Signaling Technology, Leiden, The Nederlands) and β-actin antibody (Sigma).

2.4. *Electrophoretic mobility shift assay (EMSA)*

Nuclear extracts (10 μg) were prepared from 10^7 TF-1 cells as previously described [26] and incubated for 30 min on ice with [γ-^{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 DTT, 1 mM $MgCl_2$, 2.5 mM poly(dI-dC), 0.2 mg/mL BSA, and 4 mg/mL spermidine. In immunodepletion experiments, nuclear extracts and labeled oligonucleotide probes were incubated in the reaction mixture for 30 min on ice prior to 30 min incubation with 2 μg antibody (GATA-1 (sc-1233X), p50 (sc-7178X), p65 (sc-372X); Santa Cruz, Tebu-Bio, Boechout, Belgium) on ice. Sequences of DNA sense strand oligonucleotides used as probes were as follows: probe 'GATA-consensus' from Tal-1 (T-cell acute lymphocytic leukemia 1) gene promoter (sense: 5′-GGCAGTGCCTTATCTCTG-CGGCG-3′), and probe 'NF-κBc' from the immunoglobulin light kappa chain gene containing consensus NF-κB-binding sequence (sense: 5′-AGTTGAGGGGACTTTCCCAGGC-3′). Annealing and labeling was performed as described [26].

2.5. *TransAM*

GATA-1 activation was assayed using an ELISA-based transactivation TransAM kit following the manufacturer's protocol

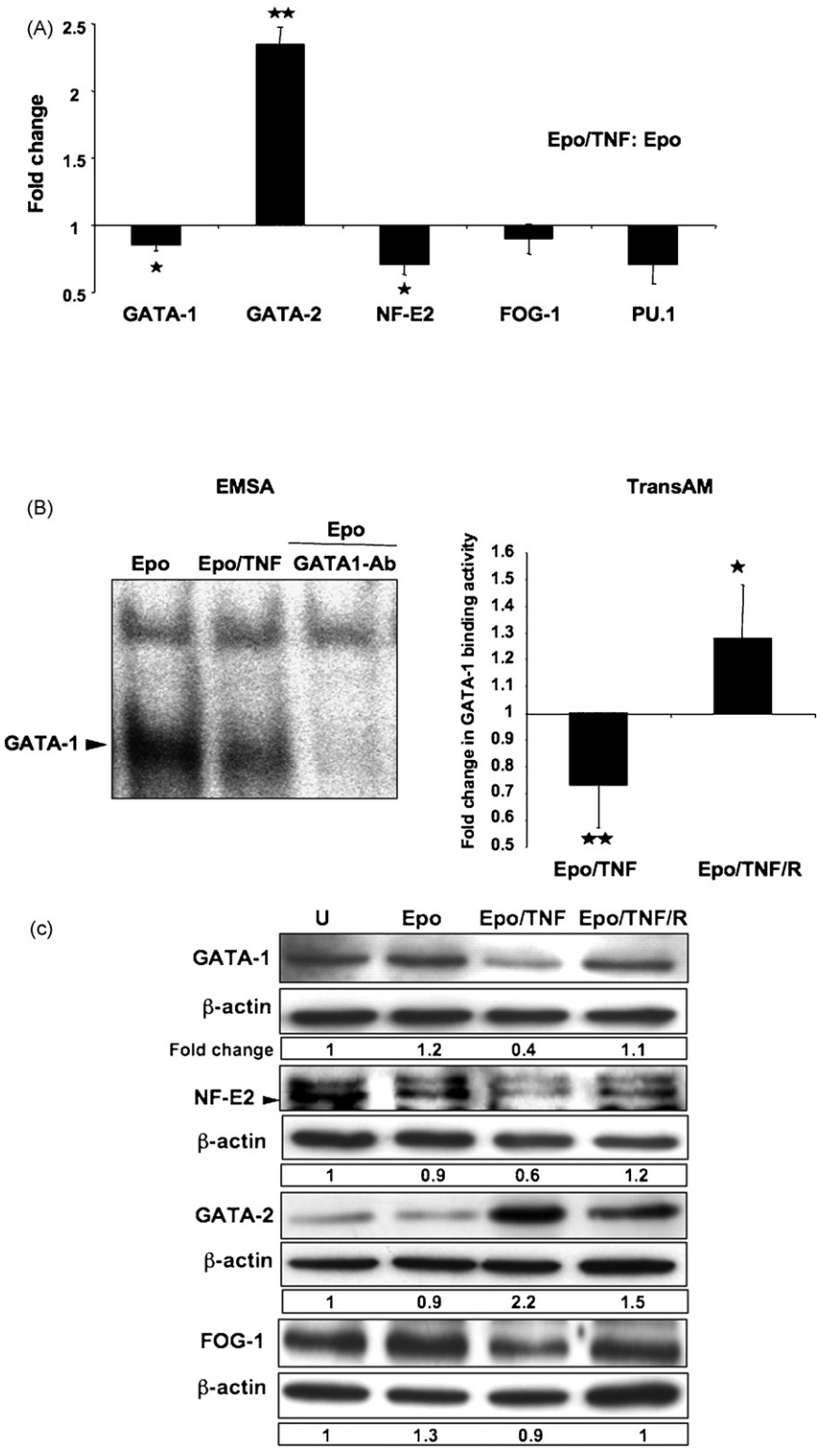

Fig. 2 – Effect of TNFα on erythroid transcription factors. TF-1 cells were induced to differentiate for 3 days with 10 U/mL Epo in the presence or absence of 100 μg/mL Remicade (R) and/or 20 ng/mL TNFα (TNF) for 1 h prior to 3 days of differentiation. (A) Fold change of mRNA expression of genes after TNFα (TNF) treatment compared to Epo induction alone (Epo = 1) using real-time PCR. The relative amounts of mRNA were normalized to the housekeeping gene MRPS14 and data are means ± S.D. of five independent experiments. Asterisks (*) denote statistical significance by Student's t-test of $^{*}P \leq .05$ and $^{**}P \leq .01$. (B) Effect of TNFα on GATA-1 DNA-binding capacity in TF-1 cells. EMSA using the GATA consensus ^{32}P-Labeled oligonucleotide, immunodepletion assay was performed using a GATA-1 antibody (one representative result of three independent experiments). TransAM assay analyzing GATA-1 binding to the GATA-1 trapper in Epo-induced TF-1 cells (=1). Data are means ± S.D. of five independent experiments compared to Epo-treated TF-1 cells. Asterisks (*) denote statistical

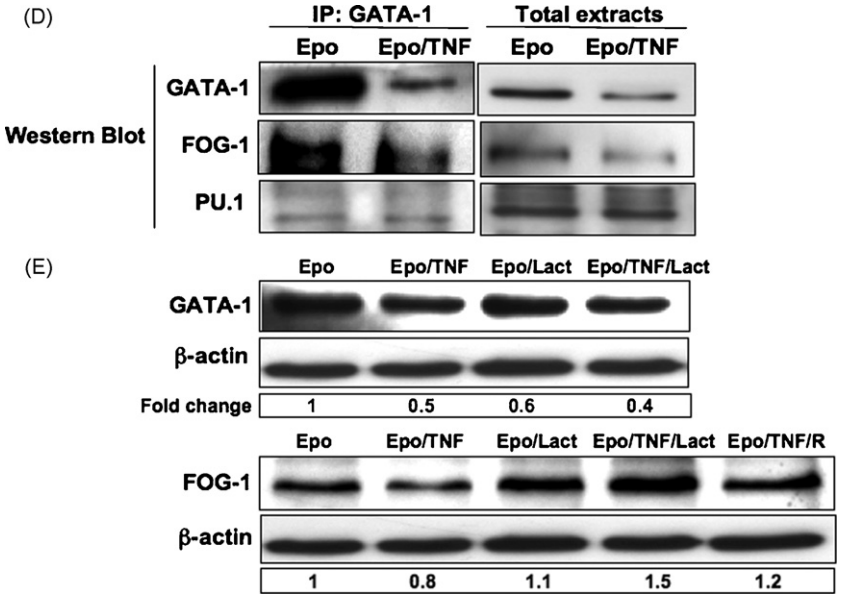

Fig. 2. (Continued).

(Active Motif, Rixensart, Belgium). A horseradish peroxidase (HRP)-conjugated secondary antibody provides a sensitive colorimetric readout that is quantified by a spectrophotometer (Pharmacia Biotech, Freiburg, Gemany) at 450 nm with a reference wavelength of 655 nm.

2.6. *Immunoprecipitation*

Protein–protein interactions between GATA-1 and FOG-1 and ubiquitinated GATA-1 and FOG-1 analysis were determined by immunoprecipitation (IP) experiments based on the protocol of Ribeil et al. [27]. 10^7 cells were lysed on ice in an IP buffer (1% NP40, 150 mM NaCl, 5 mM EDTA, 65 mM Tris–HCl, pH 8, 50 mM Hepes, 3% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM DTT, and 10 μg/mL of aprotinin/leupeptin/pepstatin) for 20 min, spun (15,700 g, 4 °C, for 30 min) and the supernatant was collected. Whole lysates (500 μg) were diluted 1:3 and incubated on ice for 1.5 h with 2 μg anti-GATA-1 antibody (sc-1233X) in the presence of 100 μL ProteinG Microbeads (Miltenyi, Bergisch Gladbach, Germany). The immune complex was immobilized to a μColumn (Miltenyi), isolated according to the manufacturer's instructions and analyzed by immunoblotting using the GATA-1 (sc-266X), FOG-1 (sc-9361), and PU.1 (sc-352) antibodies.

2.7. *Flow cytometry*

After the different treatments, 10^7 cells were washed once with PBS 1×, fixed, and permeabilized with BD Cytofix/Cytoperm Kit according to manufacturer's instructions (BD, Becton Dickinson, San José, CA, USA). Primary antibody incubation was performed in BD Perm/Wash solution (BD) with the anti-GPA (Santa Cruz) antibody. Cells were incubated with Alexafluor488 (Molecular Probes, Eugene, OR). The fluorescence of the cells was measured by a flow cytometer (FacsCalibur; BD), events were recorded statistically and 10,000 cells/sample were gated on an FL1 channel.

2.8. *Statistics*

Data were expressed at the mean ± S.D., and analyzed by the Student's *t*-test. *P*-values below 0.05 were considered as statistically significant ($^*P \leq .05$, $^{**}P \leq .01$, and $^{***}P \leq .001$).

3. Results

3.1. *TNFα inhibits hemoglobin synthesis in Epo-dependent TF-1 cells*

Compared to control cells, Epo-induced hemoglobinization of TF-1, as shown by benzidine staining, whereas TNFα inhibited this differentiation (Fig. 1A). Quantifications of benzidine-positive cells showed up to 47% of hemoglobin producing cells in the presence of Epo alone and only 21% after the pretreatment with TNFα, providing evidence that erythroid differentiation was significantly inhibited during TNFα treatment. Moreover, the TNFα inhibitor Remicade (R)

significance by Student's *t*-test of Epo/TNF: Epo $^{**}P \leq .01$; Epo/TNF/R: Epo/TNF $^*P \leq .05$. (C) Western blot analysis of GATA-1, NF-E2, GATA-2, and FOG-1 protein expression. Untreated cells were used as controls (U). β-Actin was used as internal control (one representative result of three independent experiments). (D) Immunoprecipitation (IP) of GATA-1 in whole cell extracts in Epo-induced TF-1 cells in the presence or absence of TNFα after 3 days of treatment and immunoblotting for indicated proteins. (E) Western blot of the effect of 1 μM lactacystin (Lact) on GATA-1 and FOG-1 protein expression in the presence or absence of 100 μg/mL Remicade (R) and/or 20 ng/mL TNFα (TNF) prior to 1 day of Epo treatment in TF-1 cells. β-Actin was used as internal control (one representative result of three independent experiments).

significantly reversed the inhibitory effect of TNFα, resulting in a final hemoglobinization rate of 35% (Fig. 1A and B). Additionally, TNFα did not show any effect on the viability of Epo-induced TF-1 cells, as observed by Trypan blue staining (Fig. 1B).

3.2. TNFα modulates erythroid transcription factor expression and shifts the physiological GATA-1/GATA-2 balance

Real-time PCR analysis showed that GATA-1 and NF-E2 mRNAs expressions, two erythroid transcription factors playing a role in erythroid differentiation, were significantly decreased in TNFα-treated cells when compared to Epo-treated TF-1 cells (Fig. 2A). EMSA and TransAM experiments showed that GATA-1-binding activity decreased after 3 days of TNFα treatment, suggesting that the decrease in GATA-1-binding activity could be a result of its downregulation (Fig. 2B). Indeed, western blot results demonstrated that TNFα treatment triggered a decrease in GATA-1 and NF-E2 protein expression (Fig. 2C). Remicade abrogated the inhibitory effect of the cytokine on induced GATA-1 and NF-E2 protein expression (Fig. 2B and C).

Moreover, results revealed a significant increase of 2.3-fold in GATA-2 mRNA expression after cytokine treatment (Fig. 2A), which was confirmed by protein analysis (Fig. 2C). These reversal effects on the GATA-1/GATA-2 balance were partially abrogated by the use of Remicade (Fig. 2B and C).

3.3. TNFα decreases GATA-1/FOG-1 interaction

While having no significant effect of TNFα on the mRNA of the GATA-1 cofactor, FOG-1, or the corepressor, PU.1 (Fig. 2A), we then performed western blot analysis showing that a 3-day TNFα treatment of TF-1 cells in Epo containing culture medium decreased FOG-1 protein expression when compared to control (Fig. 2C). Remicade prevented the decrease of FOG-1 (Fig. 2C). To assess whether TNFα treatment could further affect the interaction between GATA-1 and FOG-1, GATA-1 was immunoprecipitated (Fig. 2D). Indeed, FOG-1 co-immunoprecipitated in Epo-treated cells confirming its interaction with GATA-1, but no complete abrogation of this interacting complex was observed after TNFα treatment. Results merely showed a decrease of the GATA-1/FOG-1 complex formation in TNFα-treated cells, whereas PU.1 was not affected by TNFα treatment (Fig. 2D).

3.4. TNFα induces FOG-1 degradation by the ubiquitin–proteasome pathway

After observing a decrease in GATA-1 protein expression, resulting at least partly of a transcriptional downregulation of GATA-1 (Fig. 2A), we then investigated the possibility that the decrease in FOG-1 protein expression could be a result of its degradation. The effect of proteasome inhibition on both proteins was analyzed using lactacystin. Results showed that lactacystin prevented the inhibiting effect of TNFα on FOG-1 but not on GATA-1 protein expression (Fig. 2E); confirming that the reduction of FOG-1 expression resulted in its degradation by the proteasome in TNFα-treated TF-1 cells (Fig. 2E).

3.5. TNFα reduces different erythroid-specific marker expression

After analyzing the effect of TNFα on major erythroid transcription factors, we then analyzed its effect on erythroid-specific gene expression by performing real-time PCR analysis using primers for distinct erythroid-specific markers. Fig. 3A shows that EpoR, α-globin, γ-globin, ERAF, HMBS, and GPA mRNA expressions were significantly downregulated after TNFα treatment (Fig. 3A). Moreover, protein analysis confirmed, compared to Epo-treated cells, a significant reduction of GPA protein by flow cytometry analysis after addition of TNFα (Fig. 3B). In addition, EpoR and γ-globin mRNA results were validated by a decrease in both protein expressions after TNFα addition compared to Epo alone (Fig. 3C). The inhibitory effect of TNFα was completely reversed after Remicade addition (Fig. 3B and C).

3.6. The inhibitory effect of TNFα on erythroid differentiation involves the p38 MAPK pathway

As both the NF-κB and the p38 MAPK pathways can be activated by the proinflammatory cytokine TNFα in proliferating conditions [11], and as we did not see any significant effect of TNFα on cell survival (Fig. 1B), we investigated corresponding activations. TNFα is a well-known inducer of the canonical NF-κB pathway leading to the activation and translocation of the NF-κB p50/p65 dimer to the nucleus. EMSA assays were performed and showed that pretreating TF-1 cells with TNFα, induced the binding of the p50/p65 dimer as confirmed by immunodepletion assay (Fig. 4A). We then evaluated the activation of p38 by TNFα. Western blot results showed a strong phosphorylation of p38 (p38-P) after TNFα treatment in the presence (Fig. 4B, lane 3, 5, 7, 10, 12, 14, and 16) or absence of Epo (Fig. 4B, lane 8). Indeed, in the presence of Epo, p38-P was visible after 10 min of TNFα treatment and was persistent up to 48 h (Fig. 4B, lane 3, 5, 7, 10, 12, 14, and 16). Moreover, we noticed a progressive increase in phosphorylated p38 (p38-P) protein expression after 8 h of TF-1 culture in the presence of Epo alone (Fig. 4B, lane 9, 11, 13, and 15). Nevertheless, p38-P expression remained higher in the TNFα-treated cells throughout the experiment (Fig. 4B). Total p38 protein analysis showed an increment over differentiation time up to 48 h of corresponding treatment (Fig. 4B).

In order to further investigate the involvement of the p38 MAPK pathway as well as other signaling pathways in the TNFα inhibiting effect of erythroid differentiation, we used distinct cell signaling inhibitors in co-treatment with TNFα. Only in the presence of the p38 inhibitor SB203580, we observed a significant abrogation of the inhibitory effect of TNFα on hemoglobin production as assessed by benzidine staining (Fig. 4C). In order to confirm this result, we then assessed GATA-1 and γ-globin protein expressions (Fig. 4D). Results showed a rescue of the inhibitory effect of TNFα, which pointed to an implication of the p38 MAPK pathway in the inhibition of erythroid differentiation by TNFα (Fig. 4D).

4. Discussion

TNFα is a proinflammatory cytokine closely linked to inflammation and cancer. Moreover, this cytokine is believed

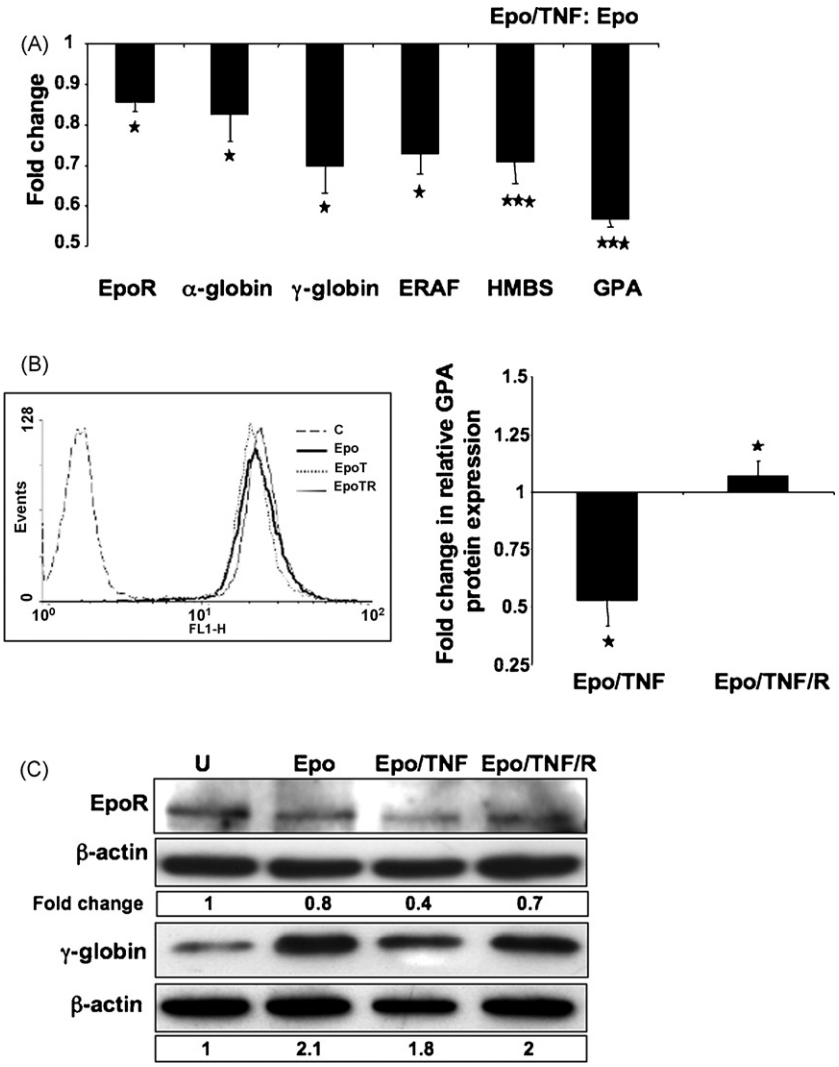

Fig. 3 – Effect of TNFα on erythroid marker gene expression. TF-1 cells were induced to differentiate for 3 days with 10 U/mL Epo in the presence or absence of 100 μg/mL Remicade (R) and/or 20 ng/mL TNFα (TNF) for 1 h prior to 3 days of differentiation. (A) Fold change of mRNA expression of genes after TNFα (TNF) treatment compared to Epo induction alone (=1) using real-time PCR. The relative amounts of mRNA were normalized to the housekeeping gene MRPS14 and data are means ± S.D. of five independent experiments. Asterisks (*) denote statistical significance by Student's t-test of $^{*}P \leq .05$ and $^{***}P \leq .001$. (B) Effect of TNFα on GPA protein expression in TF-1 cells analyzed by flow cytometry. One representative histogram shown, unstained cells were used as control (C). Data are means ± S.D. of four independent experiments compared to Epo-treated TF-1 cells alone (Epo = 1). Asterisks (*) denote statistical significance by Student's t-test of Epo/TNF: Epo $^{*}P \leq .05$; Epo/TNF/R: Epo/TNF $^{*}P \leq .05$. (C) Western blot analysis of EpoR and γ-globin protein expression. Untreated cells were used as controls (U). β-Actin was used as internal control (one representative result of three independent experiments).

to play a critical role in many forms of cancer-related [6] and inflammation-related anemia [10]. However, the molecular basis of the TNFα-mediated inhibition of erythropoiesis remains to be elucidated.

In our differentiation model, Epo-induced differentiation in TF-1 cells, which was significantly downregulated after TNFα treatment demonstrating that TNFα prevented hemoglobin synthesis induced by the Epo signaling pathway in TF-1 cells. The use of Remicade, a clinically applied anti-TNFα antibody, allowed us to restrain the inhibitory effect on erythroid differentiation. Interestingly, in patients with chronic diseases an inhibition of erythroid precursor cells could be reversed by the use of an anti-TNFα antibody [8,10]. Moreover, the transplantation of a Chinese hamster ovary cell line transfected with the TNFα gene, led to anemia in nude mice [28].

We then analyzed the effect of the cytokine on transcription factors specifically involved in the regulation of erythropoiesis. mRNA and protein levels of the major erythroid transcription factor GATA-1 were downregulated by TNFα, and correlated to a reduced synthesis of EpoR and γ-globin. Indeed, GATA-1 was already shown to directly act on the EpoR promoter [29]. The transcription factor NF-E2 is known as a

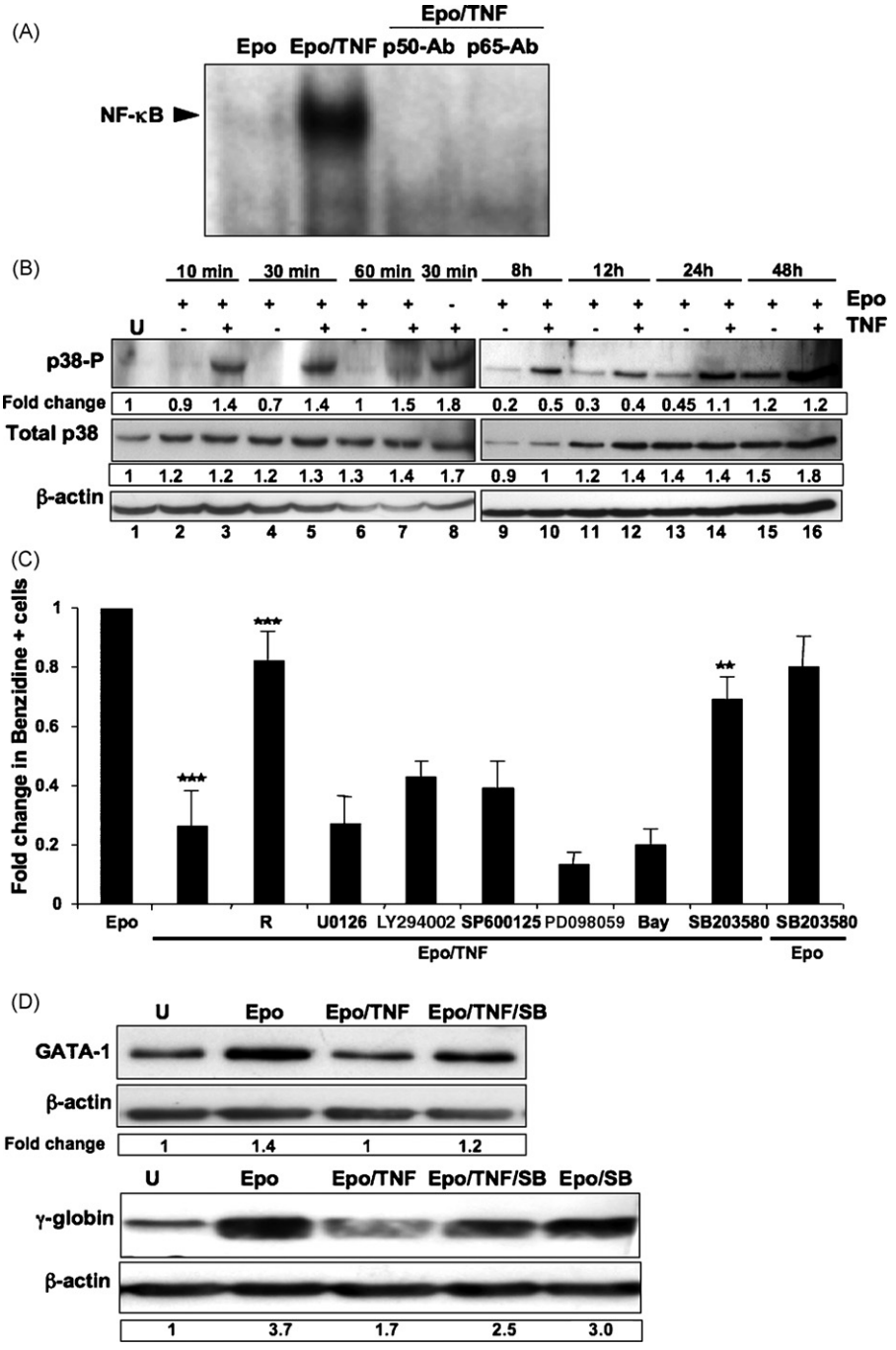

Fig. 4 – Activation and involvement of distinct signaling pathways in the TNFα-mediated inhibition of erythroid differentiation. (A) Effect of TNFα on NF-κB activation in TF-1 cells. Cells were induced to differentiate for 3 days with 10 U/mL Epo in the presence or absence of 20 ng/mL TNFα (TNF) for 1 h prior to 3 days of differentiation. EMSA using the NF-κB consensus ^{32}P-labeled probe, immunodepletion assay was performed using a p50 and a p65 antibody (one representative result of three independent experiments). (B) Effect of TNFα on p38 activation in TF-1 cells. Western blot analysis of phosphorylated p38 (p38-P) and total p38 in Epo-induced TF-1 cells in the presence or absence of 20 ng/mL TNFα (TNF) after indicated treatment times. Untreated cells were used as controls (U). β-Actin was used as internal control (one representative result of three independent experiments). (C) Effect of signaling pathway inhibitors on hemoglobin synthesis in TNFα-treated Epo-induced TF-1 cells. Cells were induced to differentiate for 3 days with 10 U/mL Epo, 100 μg/mL Remicade (R), and/or 20 ng/mL TNFα in the presence or absence of distinct signaling pathway inhibitors (10 μM p38MAPK inhibitor (SB203580), 1 μM NF-κB inhibitor (Bay 11-7082), 10 μM JNK inhibitor (SP600125), 10 μM MEK inhibitor (U0126), 10 μM MEK inhibitor (PD098059), and 10 μM PI3K inhibitor (LY 294002)) for 1 h prior to 3 days of differentiation. Data are means ± S.D. of five independent experiments and are expressed as fold change of benzidine-positive cells compared to Epo-induced TF-1 cells. Asterisks (*) denote statistical significance by Student's *t*-test of Epo/TNF: Epo ***$P \leq .001$; Epo/TNF/R: Epo/TNF ***$P \leq .001$; Epo/TNF/SB203580: Epo/TNF **$P \leq .01$. β-Actin was used as internal control (one

regulator of hemoglobin synthesis, by controlling genes coding for globins and HMBS [23,30]. Indeed, the analysis of NF-E2 revealed a decrease in its mRNA and protein expression after TNFα treatment in correlation with the downregulation of the corresponding marker genes. On the other hand, the transcription factor GATA-2 is also involved in the regulation of erythro/megakaryopoiesis. Our study demonstrated a reversal of the erythroid expression pattern of GATA-1 and GATA-2 after TNFα treatment in Epo-induced TF-1 cells. Indeed, we showed an increase in GATA-2 expression, which parallels a decrease in GATA-1 expression. The balanced expression of GATA-1 and GATA-2 during erythropoiesis is an important step of red blood cell differentiation [18,21]. Furthermore, it was shown that GATA-1-dependent repression of GATA-2 was regulated *via* disruption of positive autoregulation and chromatin remodeling [31]. Epo gene regulation was shown to be negatively controlled by GATA-2 as well as NF-κB in HepG2 cells in response to hypoxia [32]. Together with our findings, these data suggest that cytokines could exert their inhibitory effect on erythropoiesis by upregulating GATA-2 expression and inducing NF-κB. Nakano et al. suggested the GATA-2 inhibitor K-11706 as therapeutic purposes against anemia of inflammatory diseases and cancer [33]. This compound partially reverses the inhibition of the Epo gene expression by inflammatory cytokines. Nevertheless, as this drug is not specific for GATA-2, GATA-1 might also be inhibited and thus final erythroid maturation would not be effective.

Moreover, it was shown that mice lacking FOG-1 died during mid-embryonic development from severe anemia [34]. Analysis of FOG-1 expression showed that TNFα induced a decrease in the protein level of Epo-induced TF-1 cells, which correlated with erythroid differentiation arrest. By analyzing the effect of TNFα on GATA-1/FOG-1 interaction by immunoprecipitation, results showed that GATA-1 and FOG-1 were linked together in Epo-treated TF-1 cells since they co-immunoprecipitated, while a decrease in the amount of both proteins was observed after TNFα addition. This suggested that the interaction between GATA-1 and FOG-1 was not considerably affected. Obviously, the decrease resulted from the inhibition of both proteins. The use of the proteasome inhibitor lactacystin confirmed a proteasome-dependent degradation of FOG-1 after TNFα treatment. On the other hand, results showed that the inhibitory effect of TNFα occurs for GATA-1 at the transcriptional level since GATA-1 mRNA and protein amount is significantly reduced in the presence of TNFα. Nevertheless, the reduction of the amount of GATA-1/FOG-1 complex, as well as the modulation of GATA-2 and NF-E2 had consequences on erythroid marker gene transcription since all erythroid-specific genes were downregulated. Cantor et al. recently suggested a cross-antagonistic regulatory loop between GATA-2 and FOG-1, where high GATA-2 levels lead to FOG-1 repression [35], which is consistent with our results. Furthermore, it was shown that FOG-1 facilitates GATA-1 chromatin occupancy and GATA-2 removal at chromatin sites bound by GATA-2 [36].

During erythroid differentiation, p38α and p38γ were already reported to be expressed [37], which is in agreement with our results concerning total p38 expression. Moreover, p38 is known to be activated by Epo and required for Epo-induced erythropoiesis [38,39]. Tamura et al. even showed a defect in definitive erythropoiesis in p38−/− mice embryos due to an Epo deficiency [40]. TNFα, on the other hand, is also known to activate various signaling pathways leading to, among others, NF-κB and p38 activation [11,41]. Thus, the differentiating inhibitor TNFα and the activator Epo are interestingly both described as being capable to activate p38. In our cellular model, the effect of Epo on p38 was amplified over time, which was already reported in apicidin-induced K562 cells [42]. Moreover, TNFα-induced phosphorylation was also observed after 30 min in the absence of Epo, eliminating the hypothesis that co-treatment would amplify p38 activation. Interestingly, in our cellular model, by using distinct pathway inhibitors, only the use of the p38α isoform inhibitor SB203580 resulted in an abrogation of the inhibitory effect of TNFα on erythroid differentiation as shown by hemoglobin, γ-globin, and GATA-1 upregulation. Thus, the inhibition of GATA-1 expression was shown to be dependent on p38 MAPK signaling pathway, as discussed before independent of proteasomal degradation. GATA-1 was also described as a target for MAPK-mediated phosphorylation [43,44], a posttranslational modification, which is still controversially debated concerning its importance in DNA binding or even serving as degradation signal [45–47]. Moreover, as the decrease of GATA-1 seems to be more prominent at the protein than at the mRNA level, p38 could also play a role in affecting, directly or indirectly, GATA-1 stability through activation of caspases [27,48], or its interaction with the numerous coregulators such as p300/CBP, retinoblastoma (RB), EKLF, Tal-1 [22]. Interestingly, Miwatashi et al. already used a novel p38 MAPK inhibitor, TAK-715, as an anti-TNFα drug for the treatment of rheumatoid arthritis patients [49], known to be susceptible for anemia [10].

As our group already observed a decrease of the major erythroid transcription factor GATA-1 expression after TNFα treatment in chemically induced K562 cells, we further analyzed here the modulations of key erythroid factors around GATA-1 after cytokine addition in an Epo-stimulated cell model. Thus, by mimicking a TNFα-caused anemic condition, we detected inhibited erythroid marker expression in correlation with a reversed pattern of the GATA-1/GATA-2 balance. Moreover, the inhibitory effect on GATA-1 gene transcription as well as the induction of the proteasome-dependent FOG-1 degradation by TNFα contributed to a reduced GATA-1/FOG-1 complex formation. Furthermore, a signaling pathway analysis gave first hints about a p38 MAPK involvement in the TNFα-mediated inhibition of GATA-1. Overall, these data contribute to a better understanding of the molecular mechanisms

representative result of three independent experiments). (D) Western blot of the effect of 10 μM p38MAPK inhibitor (SB203580) on GATA-1 and γ-globin protein expression in the presence or absence of 20 ng/mL TNFα (TNF) prior for 3 days of Epo treatment in TF-1 cells. β-Actin was used as internal control (one representative result of three independent experiments).

underlying the cytokine-dependent inhibition of erythropoiesis.

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