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Molecular mechanisms leading to the inhibition of erythroid differentiation by the proinflammatory cytokine tumor necrosis factor alpha

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1 Summary

Erythropoiesis is considered as a multistep and tightly regulated process under the control of a series of cytokines including erythropoietin (Epo). Epo activates specific signaling pathways and key transcription factors such as GATA-1, in order to ensure erythroid differentiation. Dysregulation leads to a decreased number of red blood cells, a hemoglobin deficiency, thus a limited oxygen-carrying capacity in the blood. Anemia represents a frequent complication in various diseases such as cancer or inflammation related disease. Tumor necrosis factor alpha (TNFα) was described to be involved in the pathogenesis of inflammation and cancer related anemia, which reduces both quality of life and prognosis in patients. Blood transfusions and erythroid stimulating agents (ESAs) including human recombinant Epo (rhuEpo) are currently used as efficient treatments. However, the recently described conflicting effects of ESAs in distinct studies require further investigations on the molecular mechanisms involved in TNFα-caused anemia.

The aim of this study was to reveal the molecular mechanisms linked to the inhibition of erythroid differentiation by the proinflammatory cytokine TNFα. In order to achieve this goal, we used three different hematopoietic cell lines (K562, HEL, and TF-1) as well as purified CD34+ hematopoietic progenitor cells from umbilical cord blood. For K562 and HEL cells, distinct chemical compounds such as Aclacynomicin (Acla), Doxorubicin (Dox), or Hemin (He) were used to induce erythroid differentiation, whereas TF-1 and CD34+ cells were treated with Epo. Results showed an inhibitory effect of TNFα on hemoglobin synthesis in the different cellular models, independently of the inducer used. This effect was correlated with a decrease of the major erythroid transcription factor GATA-1 and its coactivator Friend of GATA-1 (FOG-1). We further demonstrated that the reduction of the GATA-1/FOG-1 complex was partly due to a proteasome-dependent degradation of the interaction partners. Moreover, an unsettling of the complementary expression profiles of GATA-1 and GATA-2 in the three cell lines tested was observed, which is in disfavor of final erythroid differentiation. The observed abolishment of the acetylation status of GATA-1 by TNFα in He-induced K562 cells even suggested an impact of the cytokine on GATA-1 transcriptional activity. As assessed by transfection experiments, TNFα had also an inhibitory effect on GATA-1 transactivation activity, independently of the inducer used. Then we analyzed the expression of specific marker genes partly known as GATA-1 target genes. Results revealed a decrease in Epo receptor (EpoR), α- and γ-globin, erythroid-associated factor (ERAF), hydroxymethylbilane synthetase (HMBS), and glycophorin A (GPA) expressions after TNFα treatment. Furthermore, we showed that p38 is involved in the TNFα-mediated inhibition of Epo-triggered erythroid differentiation, as the p38 inhibitor SB203580 reverses the inhibition of hemoglobin production, γ-globin gene and GATA-1 expression.

These data contribute to a better understanding of the molecular mechanisms involved in cytokine-dependent anemia both by revealing modulations of key erythroid transcription factors as well as potential diagnostic markers. Overall this study gives first hints of the key players involved in TNFα-mediated inhibition of erythroid differentiation, which can be seen as foundation for future investigations.

1.1 Zusammenfassung

Die Erythropoese stellt einen mehrstufigen, streng regulierten Prozess dar, der durch Erythropoetin (Epo) und andere Zytokine gesteuert wird. Epo aktiviert bestimmte Signaltransduktionswege und Transkriptionsfaktoren (TF), wie z.B. TF GATA-1, der eine entscheidende Rolle als Regulator der Erythrozytendifferenzierung spielt. Eine Deregulierung dieser Schlüsselfaktoren kann zur Reduzierung der roten Blutkörperchen führen, was mit einer Hämoglobindefizienz und somit einer verminderten Sauerstoff-Transportkapazität im Blut einhergeht. Die Anämie stellt eine häufig auftretende Komplikation bei Krebs- oder Entzündungserkrankungen dar. Der Tumornekrosefaktor alfa (TNFα) ist ein Hauptmediator von Entzündungskrankheiten und wurde in der Pathogenese von diversen entzündungs- sowie bei krebsbedingten Anämien beschrieben, wodurch sowohl die Lebensqualität als auch die Prognose des Krankheitsverlaufes beeinträchtigt wird. Vor der Verwendung von Erythropoese stimulierenden Agenzien (ESAs) waren Bluttransfusionen die einzige verfügbare Therapie. Die kürzlich diskutierten, z.T. widersprüchlichen Therapieergebnisse der ESAs machen die Erforschung der molekularen Mechanismen, die mit der genannten Anämie assoziiert sind, notwendiger denn je.

Ziel dieser Studie ist es, molekulare Mechanismen, zu entschlüsseln, die der Hemmung der Erythropoese durch das proinflammatorische Zytokin TNFα zugrunde liegen. Hierfür wurden verschiedene zelluläre Modelle verwendet. Hämatopoetische Zelllinien (K562, HEL) wurden durch chemische Verbindungen wie Aclacynomicin (Acla), Doxorubicin (Dox) oder Hemin (He) zur Erythropoese angeregt, wohingegen TF-1 Zellen oder aus Nabelschnurblut isolierte, hämatopoetische Vorläuferzellen (CD34+) mit Epo behandelt wurden. Nach Zugabe von TNFα wurde dessen inhibitorischer Effekt auf die Hämoglobinsynthese in allen Modellen sichtbar. Dieser Effekt ging in allen untersuchten Zelllinien mit einer Reduktion der GATA-1- und FOG-1-Proteine einher. Der Rückgang des GATA-1/FOG-1 Interaktionskomplexes nach TNFα-Zugabe ließ sich auf einen Proteasom-abhängigen Abbau der Interaktionspartner zurückführen. Ferner wurde eine Veränderung der komplementären Expressionsprofile von GATA-1 und GATA-2 beobachtet, was dem Differenzierungsprozess entgegensteht. Eine Veränderung des Acetylierungsstatus von GATA-1 in He-behandelten K562 Zellen nach TNFα Zugabe ließ einen Einfluss des Zytokins auf die transkriptionelle Aktivierung von GATA-1 vermuten. Diese Theorie unterstützend zeigte TNFα, unabhängig vom benutzten Induktor, einen inhibitorischen Effekt auf die transkriptionelle Aktivität von GATA-1. Eine erythroidbezogene Markergen-Analyse zeigte nach TNFα-Zugabe eine Expressionsreduzierung folgender Gene: Erythropoetin Rezeptor, α- und γ-Globin, Erythroid-assoziierter Faktor, Hydroxymethylbilan Synthetase und Glykophorin A. Gleichzeitig konnte in TF-1 Zellen mit Hilfe eines p38-Inhibitors die Beteiligung von p38 an der TNFα-getriggerten Hemmung auf verschiedenen Ebenen (Hämoglobinsynthese, GATA-1- und γ-Globin-Proteinexpression) gezeigt werden.

Die Ergebnisse führen zu einem besseren Verständnis der molekularen Mechanismen, die der Zytokin-abhängigen Anämie zugrunde liegen. So wurden Veränderungen im Transkriptionsfaktorprofil, sowie in der Expression verschiedener potentieller diagnostischer Markergene festgestellt. Insgesamt enthüllt diese Studie erste vielversprechende Erkenntnisse über verschiedene Schlüsselelemente, die in der Hemmung der Erythropoese durch TNFα eine entscheidende Rolle spielen.

2 Introduction

2.1 Erythropoiesis

2.1.1 Regulation of erythropoiesis

Erythropoiesis is commonly considered as a multistep event leading from hematopoietic stem cells (HSC) to the formation of erythrocytes. These reside in the bone marrow and have the unique ability to give rise to the different mature hematopoietic cells, which are usually classified into two distinct lineages, the lymphoid and the myeloid.

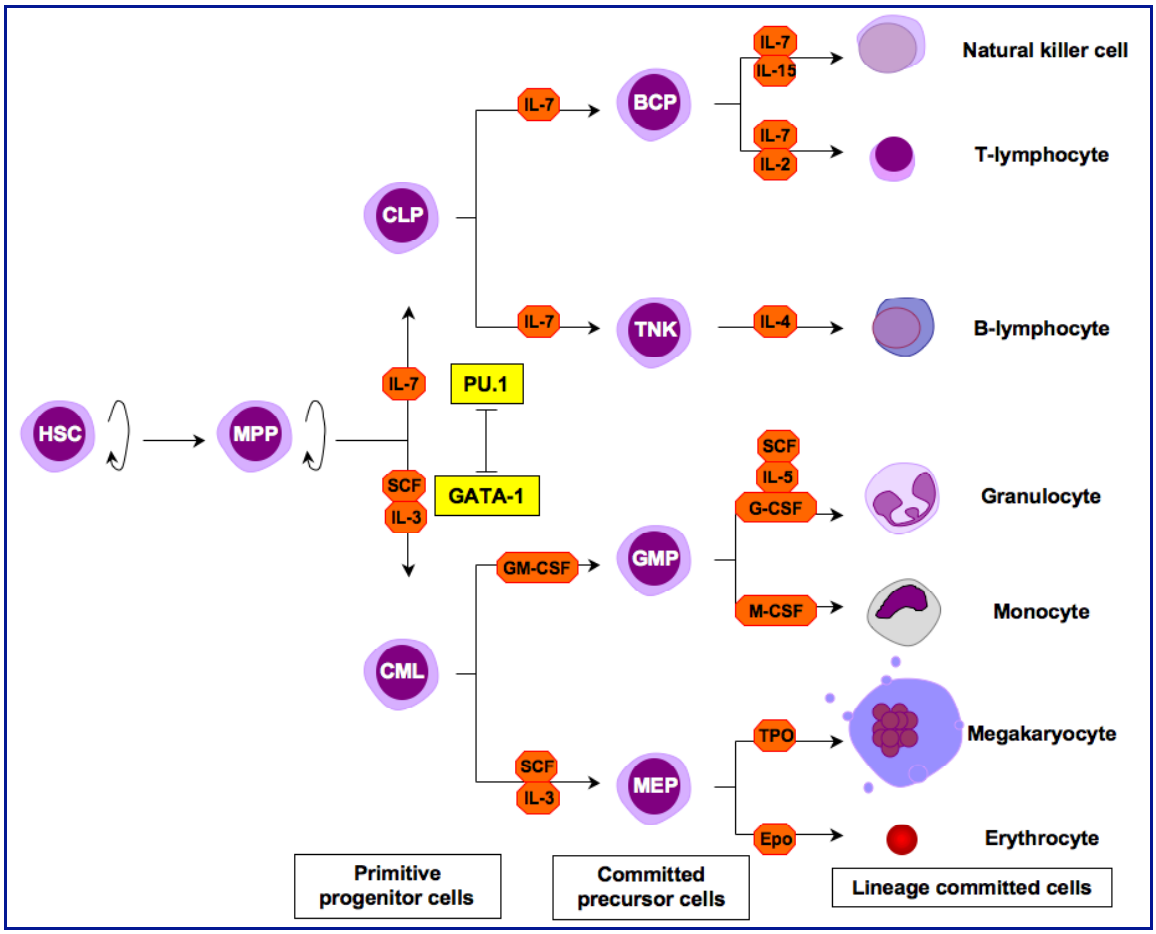


Figure 1: Hematopoiesis and the role of cyokines. *Cytokines act both on multipotential progenitors and their committed offspring. Yellow squares emphasize the cross-antagonistic role of the transcription factors GATA-1 and PU.1 in myeloid versus lymphoid commitment. BCP, B-cell progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HSC, hematopoietic stem cell; GMP, granulocyte–macrophage progenitor; MEP, megakaryocyte erythroid progenitor; MPP, multipotent progenitor; TNK, T-cell natural killer cell progenitor; IL, Interleukin; SCF, stem cell factor; GM-CSF, granulocyte-macrophage colony stimulating factor; G-CSF, granulocyte colony stimulating factor; M-CSF, macrophage colony stimulating factor; TPO, thrombopoietin, Epo, Erythropoietin. (Adapted from Robb et al. and Wickrema et al.* [1,2]*)*

Erythroid differentiation arises from the myeloid root and is phenotypically characterized by the production of hemoglobin synthesis and expression of erythroid markers such as hydroxymethylbilane synthase (HMBS), Erythroid-associated factor (ERAF), transferrin receptor (TFRC), globins and glycophorin A (GPA). Hematopoiesis is regulated by distinct cytokines acting on both multipotential progenitors and their committed offspring [1,2]. Ferrous iron (Fe^{2+}) is also essential for erythropoiesis as a major component of heme in hemoglobin and in the redox system of the respiratory chain. Hepcidin, a 25-amino acid peptide, is the main regulator of iron transport. During differentiation from a multipotent common myeloid progenitor (CMP) to a bipotent megakarocyte erythroid progenitor (MEP), burst-forming units erythroid (BFU-E) and colony forming units erythroid (CFU-E) are the earliest identifiable erythroid progenitors in culture and are characterized by their *in vitro* ability to form colonies [3] (Fig. 1).

Erythropoiesis is a very dynamic and tightly regulated process by which $2*10^{11}$ erythrocytes (lifespan of 100 days) are produced every day [4]. A feedback loop involving the major cytokine for human erythropoiesis, erythropoietin (Epo), is regulating this physiological process. The kidney and the liver are the main sites that produce the glycoprotein hormone Epo in adult humans. The rate of expression of the Epo gene depends on the level of tissue oxygen through the availability of the hypoxia inducible factor (HIF), which acts as a global transcriptional regulator, thus as a sensor of oxygen homeostasis. Indeed, there are essential HIF binding sites in the Epo enhancer, which in hypoxic conditions are bound by the HIF heterodimer, consisting of the oxygen sensitive HIF-1α and the constitutively expressed HIF-1β subunit. Changes in circulating Epo, its function, or its action can lead to major changes in the number of erythrocytes. Oxygen-dependent prolyl hydroxylases control Epo variations in the kidney by regulating the stability of HIF-1α.

Erythroid differentiation can be considered as a finely triggered balance between positive signals constituted by Epo and stem cell factor (SCF) and negatively influenced by death receptor ligands and inhibitory cytokines. Epo acts through its receptor (EpoR) in order to stimulate various underlying cell signaling pathways including the Phosphotidylinositol 3 kinase (PI3K), the Janus kinase/Signal-transducer and activator of transcription (JAK/STAT) and the mitogen-activated protein kinase (MAPK)/ extracellular signal-related kinase (ERK) pathways [5]. Like Epo and EpoR, GATA-1 is

considered as an essential transcription factor for the survival of erythroid precursors and their terminal differentiation into red blood cells. It has been reported that Epo modulates GATA-1 function in erythroid cells [6] (Fig. 1).

Dysregulation of Epo or other key factors of erythroid differentiation can either lead to major changes in red blood cell number and subsequently the oxygen-carrying capacity of the blood. Erythrocytoses are disorders resulting in an excessively high level of erythrocytes, whereas anemia is characterized by a qualitative or quantitative deficiency of hemoglobin and is clinically defined as an hemoglobin (Hb) level <12g/dL.

2.1.2 Transcription factors involved in erythropoiesis

Erythrocytes were described to result from passage through cellular hierarchies dependent on differential gene expression under the control of complex transcription factor networks responsive to changing niches [7]. In this regard, a key regulator of erythroid development that plays a central role in red cell gene expression is the transcription factor GATA-1. GATA-1 null mouse embryos die between E10.5 and E12.5 from severe anemia due to a complete ablation of embryonic erythropoiesis [8], and GATA-1-/- embryonic stem cells cannot contribute to definitive erythropoiesis [9].

GATA-1 is a member of the GATA family, which includes 6 members (GATA-1 to GATA-6). These transcription factors recognize the same DNA consensus sequence (A/T)GATA(A/G) and present two characteristic zinc finger motifs specific to the GATA family [10-13]. The GATA family can be divided in two subfamilies on the basis of the expression of the individual transcription factors. GATA-1, GATA-2, and GATA-3 belong to the hematopoietic, whereas GATA-4, GATA-5, and GATA-6 belong to the nonhematopoietic subfamily [14,15]. GATA-1 was first identified as a protein with binding capacity to the β-globin promoter [16]. It is expressed in various cells such as primitive and definitive erythroid cells [8,17], megacaryocytes [18,19], eosinophils [20], mast cells [18], and Sertoli cells from testis [21]. GATA-1 protein revealed at least three functional domains: the N-terminal activation domain, the N-terminal Zinc finger (N-finger) and the C-terminal Zinc finger (C-finger). The C-finger is essential for binding to the GATA consensus sequence of the DNA [10,22]. The N-finger supplies the stabilization and specificity of DNA binding, and is responsible for the interactions with cofactors such as Friend of GATA-1 (FOG-1) for example [10,13,23-25].

Regulation of erythroid and megakaryocytic gene expression requires a combination and cooperation of specific hematopoietic transcription factors including NF-E2 (Nuclear Factor Erythroid-2), described to act as a major regulator of hemoglobin synthesis during erythropoiesis [26], and EKLF (erythroid Krüppel-like factor), a crucial factor in erythroid and megakaryocytic differentiation and maturation (Fig. 2).

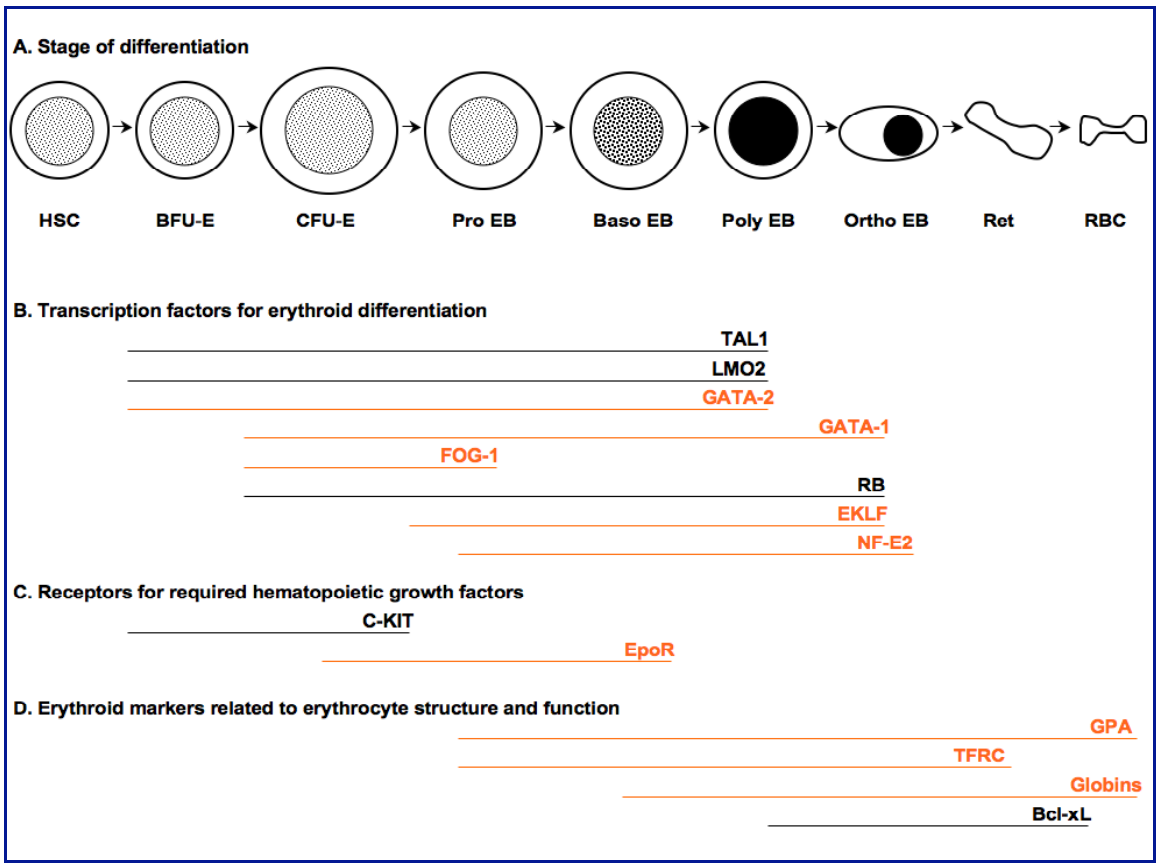


Figure 2: Stages of mammalian hematopoiesis: *(A) The relative sizes and the known or presumed morphologic appearances of erythroid cells at various stages of differentiation: pluripotent hematopoietic stem cell (HSC), burst-forming units-erythroid (BFU-E), colony-forming units-erythroid (CFU-E), proerythroblasts (Pro EB), basophilic erythroblasts (Baso EB), polychromatophilic erythroblasts (Poly EB), orthochromatic erythroblasts (Ortho EB), reticulocytes (RET), and erythrocytes (RBC). (B) Erythroid transcription factors: basic helix-loop-helix factor (TAL1); Lim-domain partner of TAL1 (LMO2); zinc finger factors that bind GATA sequences (GATA-1, GATA-2); GATA-1 cofactor, friend of GATA (FOG-1); retinoblastoma protein (RB); erythroid Kruppel-like factor (EKLF); and nuclear factor erythroid2 (NF-E2). (C) Receptors for hematopoietic growth factors: stem cell factor-receptor/c-KIT, erythropoietin receptor (EpoR). (D) Proteins related to erythrocyte structure and function: periods of expression for erythroid-specific forms of proteins are shown; transferrin receptors (TFRC) are present in all stages, but period of large upregulation is shown. For each transcription factor, growth factor receptor, and erythrocyte-related protein, the degree of expression can vary significantly during the period shown. Expression of factors analyzed in this study are highlighted in orange. (Adapted from Koury et al. [27])*

Positive and negative interactions with transcriptional cofactors as well as autoregulatory mechanisms contribute to GATA-1 activity leading to transcription of hematopoietic lineage-specific genes including globins, Epo receptor (EpoR), porphobilinogene deaminase, GPA and transcription factors such as erythroid Kruppel-like factor (EKLF) and GATA-1 itself (Fig. 2). Thus, FOG-1 [28,29], CBP/p300 [30], Friend leukemia integration [31] are positive cofactors of GATA-1 activity whereas PU.1 (Sp1) and pRB [32], the hairy-enhancer-of-split-related factor [33] and Ski [34] have been described as negative regulators of GATA-1. FOG-1 is expressed simultaneously with GATA-1 in erythroid and megakaryocytic lineages and cooperates with this major erythroid transcription factor during erythroid and megakaryocytic development [28]. FOG-1/GATA-1 has been shown to be essential for GATA-1 function during erythroid differentiation, since GATA-1 mutants were defective in FOG-1 binding [35]. Furthermore FOG-1 was shown to differentially modulate GATA-1 activity depending on the promoter context [28,36,37]. PU.1 is an *Ets* family member of transcription factors responsible for the myeloid lineage commitment. GATA-1 and PU.1 have cross-antagonistic relationships, thus they seem to functionally antagonize each other via direct physical interaction of their DNA-binding domains [38,39] (Fig. 1). GATA-1 inhibits PU.1 by preventing its interaction with c-Jun, while PU.1 impairs GATA-1 by inhibiting its binding to DNA [37,40].

GATA-1 and GATA-2 are both expressed in erythrocyte and megacaryocyte 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 [41]. On the other hand, transcription factors such as nuclear factor, erythroid derived 2 (NF-E2) or EKLF, which act downstream of GATA-1 are required for globin gene expression [7,42,43].

GATA-1 is also regulated by posttranslational mechanisms. GATA-1 has been shown to be acetylated by the ubiquitously expressed acetyltransferases p300 [44] and CREB-binding protein [45]. The function of GATA-1 acetylation remains partially unclear. The interaction between p300/CBP and GATA-1 and the subsequent acetylation of GATA-1 seems to stimulate transcriptional activity [30,44]. GATA-1 can also be phosphorylated [46] and sumoylated [47]. Hernandez-Hernandez et al. suggested that acetylation of GATA-1 positively signals ubiquitination, and that MAPK phosphorylation then assists with acetylation to perform definitive protein degradation [48]. GATA-1 protein degradation,

another possible regulatory mechanism, has also been previously reported *via* caspase cascade or the proteasome [49,50].

2.2 Tumor necrosis factor alpha

Tumor necrosis factor alpha (TNFα), also known as cachectin or differentiation inducing factor, is a proinflammatory multifunctional cytokine, which is mainly produced by macrophages, but also by neutrophiles, fibroblasts, keratinocytes, astrocytes, Kupffer cells, smooth-muscle cells, T and B cells. It was initially described to induce hemorrhagic necrosis in transplanted tumors [51]. Its effects are principally mediated through two distinct receptors TNFα receptor I (TNFRI) (p55) and TNFα receptor II (TNFRII) (p75) before stimulating various underlying cell signaling pathways including nuclear factor kappa B (NF-κB), c-Jun N-terminal kinase (JNK), p38, or caspase activation. Thus TNFα simultaneously activates both apoptotic and anti-apoptotic or cell survival signals depending on the factors present in the receptor complex [52]. TNFα is known as the most powerful activator of NF-κB, which was discovered as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin [53]. The NF-κB family consists of Rel-domain-containing proteins: Rel A (also called p65), Rel B, c-Rel, p50 (also called NF-κB1), and p52 (also called NF-κB2). Inactive NF-κB is kept in the cytoplasm of cells by a family of anchorin-domain containing proteins, inhibitor of NF-κB family (IκB family). When TNFα binds to and trimerizes its receptor, distinct factors are recruited forming a receptor complex, which activates the Inhibitor of κB kinase (IKK) [54]. Activated IKK then phosphorylates IκBα, which is ubiquitinated and degraded, thus releasing p50-p65 heterodimer, which then translocates to the nucleus, binds its specific ten base pair consensus binding site and regulates over 200 immune, growth, and inflammation genes [55].

As TNFα as well as other TNFα superfamily members play a role in hematopoiesis, host defense, immune surveillance, and proliferation, its deregulation leads to numerous diseases, including cancer [52,56,57]. Indeed, TNFα expression has been confirmed in the tumor microenvironment of various malignancies [58]. Indeed, this cytokine is paradoxically able to induce necrosis and to promote tumor development, depending on the levels of TNFα in distinct settings [59]. When TNFα is secreted by tumors and tumor-

associated macrophages at physiological levels, it promotes tumor growth and stimulates angiogenesis, whereas when administered therapeutically at high doses, it induces an increased permeability of tumor vasculature. Thus, recombinant TNFα, as a tumor regressing agent, is approved in Europe to be administered locoregionally at supraphysiological levels as a therapy for soft tissue sarcoma [60-63].

2.2.1 TNFα and inflammation

A TNFα overproduction generates numerous chronic inflammatory diseases, such as rheumatoid arthritis [64-66] chronic hepatitis C [67], or Crohn's disease [68,69]. An increase in the TNFα level was described in diabetic patients to cause retinopathies [70-75], while during pancreatitis, released TNFα leads to inflammation and cellular damage [76].

Currently, three marketed TNFα antagonists [etanercept (Enbrel®), infliximab (Remicade®), and adalimumab (Humira®)] are indicated in a variety of diseases characterized by abnormally elevated TNFα levels such as rheumatoid arthritis (RA), psoriatic arthritis, ankylosing spondylitis, Juvenile rheumatoid arthritis, and Crohn's disease, whereas the effectiveness of the treatments varies with agent and disease [77,78]. TNFα is thus leading to various biological phenomena implying different molecular mechanisms and is involved in various cellular responses. Although TNFα is considered to act as a proinflammatory cytokine, it was described both as a positive and negative regulator of myeloid cell proliferation and differentiation [79-87]. Effects of TNFα can be mediated either directly [83,88,89] or indirectly by inducing other cells to produce cytokines, including hematopoietic growth factors [90-94].

2.2.2 Link between TNFα, cancer and inflammation

Furthermore TNFα plays an interesting as well as complex function in cancer. Considering its role as a proinflammatory cytokine, the investigation of a murine model of inflammation-associated hepatocellular carcinogenesis involved activation of the tumor promoter NF-κB via production of inflammatory TNFα [95]. Moreover several reports associated detection of abnormally high levels of TNFα protein and/or constitutively active NF-κB in cancer patients with a wide range of tumor types [96], including kidney [97], breast [98,99], asbestosis induced lung [100], and prostate cancers [101,102]. Suppression of constitutively active NF-κB resulted in cell proliferation arrest and apoptosis indicating a crucial role for NF-κB in proliferation and survival [55]. Furthermore chronic bioaccessibility of TNFα has been correlated with enhanced

invasive activities as well as survival of neoplastic cells [59]. Within groups of patients with the same tumor type, higher levels of TNFα have been correlated with advanced tumor stage, greater complications, and shorter survival time [103]. Moreover various cytokines, including TNFα, are overexpressed in pancreatic cancer cells, leading to an NF-κB activation and as a consequence, to cell growth by inhibiting apoptosis [104,105]. TNFα also appears as a growth factor regulated by NF-κB in Hodgkin's lymphoma, T cell lymphoma and glioma [106].

As Rudolf Virchow already suspected in 1863, inflammation and cancer cannot be handled separately anymore [107-109]. It was demonstrated that cancer-associated inflammation could even promote tumor growth [95,107,110]. Indeed cancer-associated inflammation includes the expression of cytokines such as TNFα or IL-1 by tumor-associated macrophages, stimulating tumor growth [111]. Indeed, as TNFα was even categorized as a tumor promoter, it is not astonishing that cytokine antagonists as well as NF-κB inhibitors are already used in cancer therapy and prevention [59,112-115].

2.3 Link between erythropoiesis and TNFα

Besides the proinflammatory, proliferative and apoptotic properties, TNFα was also described as an inhibitor of the erythroid differentiation in *vitro* and in *vivo* [80,81,84,89,116-122]. Its expression is associated with several hematologic diseases such as Fanconi anaemia (FA) [123,124], myelodysplastic syndromes [125], aplastic anemia [123,126] and anemia due to chronic diseases [94,127,128]. Indeed, in FA patients, TNFα is significantly overexpressed in stimulated marrow mononuclear cells, which leads to a suppression of erythropoiesis. In bone marrow cultures, the addition of anti-TNFα increases the size and the number of CFU-E and BFU-E grown from FA patients but not from healthy controls. This indicates that FA subjects have a marrow TNFα activity that inhibits erythropoiesis *in vitro*. TNFα plays a relevant role in the pathogenesis of erythroid failure in FA patients [124]. Several *in vitro* studies revealed the inhibitory effects of this cytokine on hematopoietic progenitor cell growth [80,81,84,89,118,124,129]. It was shown that the inhibition of human CFU-E by TNFα requires beta interferon, which is produced by macrophages in response to TNFα [94]. TNFα was also shown to have a direct inhibitory effect on Epo-induced generation of GPA positive cells from CD34+ progenitors, leading to a suppression of erythropoiesis and a reduction of the proliferation capacity of GPA+ cells [129].

Interestingly, TNFα is also believed to play a critical role in many forms of cancer [130,131] and inflammation related anemia [132-135]. Indeed anemia is considered as a common symptom induced by inflammation and cancer pathologies. In patients with B-cell chronic lymphocytic leukemia suffering from anemia, the serum levels of TNFα were significantly higher than in those without anemia [136]. The incidence of anemia was shown to vary with tumor type, stage and patient age. Up to one third of patients are suffering from anemia at diagnosis [137], a number, which increases after chemotherapy [138]. Cancer-associated anemia can be considered as a negative prognostic factor for survival regardless of tumor type [139] (Fig. 3). Moreover, the quality of life is considerably affected and is associated with a range of symptoms including fatigue, depression, and dizziness [140]; thus pro-inflammatory cytokines were recently suggested as the common denominator for cancer related fatigue [141].

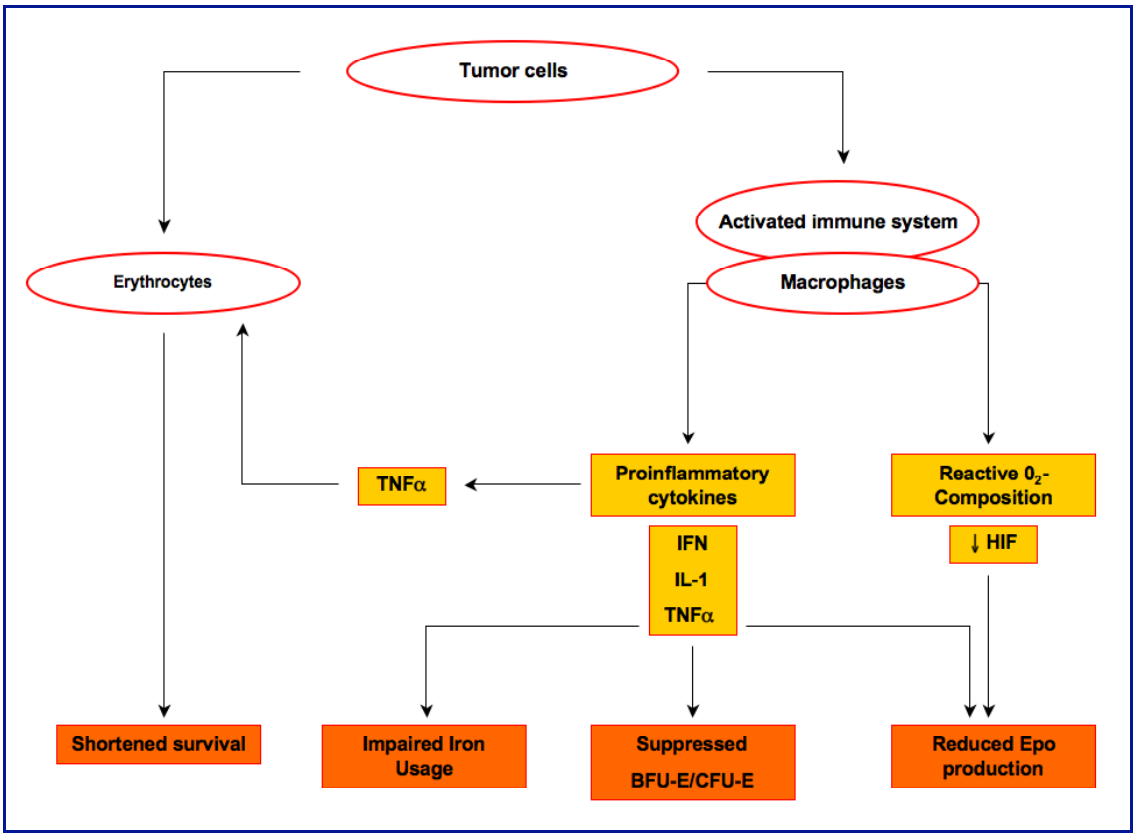


Figure 3: Pathophysiology of anemia. *Tumor cells act on erythrocytes by affecting cell survival and trigger cytokine release through macrophages, which leads to impaired erythropoiesis. TNFα, tumor necrosis factor alpha; IFN, Interferon; IL, Interleukin; HIF, hypoxia inducible factor; BFU-E, burst-forming units-erythroid; CFU-E, colony-forming units-erythroid, Epo, Erythropoietin. (Adapted from Engert et al. [142])*

Regarding anemia due to inflammation, this sign of ineffective erythropoiesis was described as a clinical entity of patients with inflammatory disorders [143]. Prior to the use of erythroid stimulating agents (ESAs), blood transfusions were the only treatment for cancer related anemia. However, the recently described conflicting effects of ESAs in distinct studies [144-146] as well as the tremendous costs related to this treatment, make it necessary to further investigate the molecular mechanisms involved in anemia and to identify new targets for drug development as well as to detect more significant predictors for anemia. In order to improve quality of life, several drugs are under investigation for the treatment of different forms of anemia. In this context Jelkmann reviewed several anti-anemic drugs and techniques based on Epo gene expression [147,148].

3 Aim

The proinflammatory cytokine TNFα was linked to the pathogenesis of inflammation and cancer related anemia, which reduces both quality of life and prognosis of patients.

In order to investigate the mechanisms involved in the inhibition of erythroid differentiation by TNFα, the objectives of the present study were:

- to investigate the effect of TNFα on human leukemia cell lines K562, HEL, TF-1 as well as umbilibal cord blood CD34+ cells stimulated by distinct chemical agents or Epo
- to analyze the effect of NF-κB induction in our cellular models
- to study the expression profiles of major erythroid transcription factors after TNFα addition
- to evaluate the effect of TNFα transactivation activity of the key erythroid transcription factor GATA-1
- to examine the variations of GATA-1 target genes after cytokine treatment
- to reveal possible signaling pathways involved in the TNFα-mediated inhibition of erythroid differentiation

4 Materials and methods

4.1 Materials

Chemicals	
1-bromo-3-chloro-propane	Sigma
2-mercaptoethanol	Merck
Acetic acid	MP Biomedicals
Agarose	MP Biomedicals
Alexa Fluor® 488 donkey anti-goat IgG (H+L)	Invitrogen
Ammonium persulfate	MP Biomedicals
Amphotericin	MP Biomedicals
Ampicillin	Sigma
Antibody anti-acetyl-Lysine (4G12) monoclonal mouse IgG	Upstate
Antibody anti-β-actin mouse monoclonal IgG (A5441)	Sigma
Antibody anti-EpoR rabbit polyclonal IgG M-20 (sc-697)	Santa Cruz Biotechnology
Antibody anti-EpoR rabbit polyclonal IgG C-20 (sc-695)	Santa Cruz Biotechnology
Antibody anti-FOG-1 goat polyclonal IgG M-20 (sc-9361)	Santa Cruz Biotechnology
Antibody anti-γ-globin mouse monoclonal IgG (51-7) (sc-21756)	Santa Cruz Biotechnology
Antibody anti-GATA-1 goat polyclonal IgG C-20 (sc-1233X)	Santa Cruz Biotechnology
Antibody anti-GATA-1 rat monoclonal IgG N-1 (sc-266)	Santa Cruz Biotechnology
Antibody anti-GATA-2 rabbit polyclonal IgG (H-116) (sc-9008)	Santa Cruz Biotechnology
Antibody anti-GPA goat polyclonal IgG (E-18) (sc-19451)	Santa Cruz Biotechnology
Antibody anti-phospho-p38 rabbit polyclonal IgG (9211)	Cell signaling
Antibody anti-p38 rabbit polyclonal IgG (9212)	Cell signaling
Antibody anti-p50 rabbit polyclonal IgG (H-119) (sc-7178X)	Santa Cruz Biotechnology
Antibody anti-p65 rabbit polyclonal IgG (C-20) (sc-372X)	Santa Cruz Biotechnology

Antibody anti-PU.1 rabbit polyclonal IgG (Spi-1, T-21) (sc-352)	Santa Cruz Biotechnology
Aprotinine	Roche
Benzidine	Fluka
Bradford solution	Bio-Rad
Bromophenol blue	LKB
BSA	MP Biomedicals
CD34 Microbead Kit human	Miltenyi Biotec
CD-34 monoclonal mouse anti-human reagent (FITC)	BD Biosciences
CD-45 monoclonal mouse anti-human reagent (PerCP)	BD Biosciences
CO_2	Air liquide
Complete Protease Inhibitor Cocktail Tablets	Roche
Cytofix/Cytoperm kit	BD Biosciences
Donkey anti-goat IgG HRP (sc-2020)	Santa Cruz Biotechnology
Donkey anti-goat Alexafluor 488	Molecular probes
Donkey anti-rabbit IgG HRP (sc-2313)	Santa Cruz Biotechnology
1,4-Dithiothreitol (DTT)	MP Biomedicals
Dual Glo Luciferase Assay System	Promega
ECL + Lumigen PS-3 detection reagent	GE healthcare
EDTA	MP Biomedicals
Ethylene glycol-bis-N,N,N', N'-tetraacetic acid (EGTA)	Sigma
Ethanol	Prolabo
Ether	Merck
ethidium bromide aqueous solution	Sigma
FCS	Cambrex
Ficoll-Paque Premium	GE healthcare
Formaldehyd	MP Biomedicals
Formamid	MP Biomedicals
γ-^{32}P-phosphate	MP Biomedicals
Glycerol (87%)	Merck
Glycerol bidistilled	VWR International
Glycine electrophoresis grade	MP Biomedicals
Goat anti-mouse IgG-HRP (sc-2005)	Santa Cruz Biotechnology
Goat anti-rat IgG-HRP (sc-2006)	Santa Cruz Biotechnology
H_2O_2	Sigma

Heparin	Braun
Hepes free acid	MP Biomedicals
Igepal	MP Biomedicals
Isopropanol	VWR international
KCl	Merck
KH_2PO_4	VWR International
KOH	Merck
LB Agar, Miller	MP Biomedicals
LB Broth, Miller	MP Biomedicals
Leupeptine	Merck
Liquid-gel 29:1	MP Biomedicals
Liquid-gel 37.5:1	MP Biomedicals
Methanol	MP Biomedicals
Milk	group Auchan
Na_2HPO_4	MP Biomedicals
NaCl	MP Biomedicals
NF-κB p50 rabbit polyclonal IgG	Santa Cruz Biotechnology
NF-κB p65 rabbit polyclonal IgG	Santa Cruz Biotechnology
Nucleobond AX	Macherey-Nagel
O-phenylanthroline	Sigma
Orthovanadate	Calbiochem
Penicillin	Sigma
Penicillin-streptomycin-fungizone mixture	Cambrex
Phenylmethylsufonylfluoride (PMSF)	Roche
Poly-dIdC	Roche-Prophac
Protease inhibitor cocktail tablets	Roche
Qiaquick Removal Nucleotide kit	Qiagen
Renilla Luciferase reporter vectors (phRL-SV40)	Promega
RNeasy Mini kit	Qiagen
RPMI medium	Cambrex-Lonza
RT-PCR kit	Invitrogen
RT^2PCR Array First strand kit (C-02)	SuperArray
SDS 20%	MP Biomedicals
Spermidine	MP Biomedicals
Stem line medium II	Sigma
Streptomycin	Sigma
T4 Polynucleotide kinase & kinase buffer	Roche
Tetrametylethyldiamine (Temed)	MP Biomedicals

TransAM kit	Active Motif
Tris	MP Biomedicals
Triton X-100	Merck
TRIzol	Invitrogen
Trypan Blue	Cambrex
Tween 20	MP Biomedicals
Xylene cyanole	MP Biomedicals

Erythroid differentiation inducers and treatment agents	
Aclacynomicin A	Sigma
Aclacynomicin A Hydrochloride	Sigma
Bay11-7082 (inhibitor of nuclear factor κB (NF-κB) activation)	Calbiochem
DMSO	MP Biomedicals
Doxorubicin Hydrochloride	MP Biomedicals
Epo (Eprex)	Jansson-Cilag
Granulocyte/macrophage colony stimulating factor (GM-CSF)	Pepro Technology
Hemin	MP Biomedicals
IL-3	Pepro Technology
JNK inhibitor (SP600125)	Calbiochem
Lactacystin	Sigma
MEK inhibitor (PD98059)	Calbiochem
MEK inhibitor (U0126)	Promega
P38 inhibitor (SB203580)	Promega
PI3K inhibitor (Ly 294002)	Calbiochem
Remicade	Centocor
SCF	Pepro Technology
TNFα	Pepro Technology

DNA markers	
100 base pair ladder	Invitrogen
Kaleidoscope Prestained Standards	Bio-Rad

Instruments	
7300 real-time PCR system	Applied Biosystems
96-well plate	Greiner
96-well plate reader	Packard, Perkin Elmer

Autoradio cassette	GE healthcare
Autoradiography development machine Curix 60	AGFA
Balance	Ohaus
Bioanalyzer	Agilent
Biorad Gene Pulser II	Bio-Rad
Centrifuge 5415	Eppendorf
Centrifuge 5415 D	Eppendorf
Centrifuge 5810	Eppendorf
Centrifuge KR 25	Jouan
CO_2 incubator	Jouan
Electrophoresis apparatus	Biometra
FACS Calibur	BD Biosciences
Freezer -80 °C	Jouan
Gel dryer	Bio-Rad
Generator	Biometra
Heating plate	IKA
Hood	Microflow/FluFrance/ClearAir
Liquid-nitrogen tank	Taylor-Wharton
μMACS Separator Sarting Kit	Miltenyi Biotec
MACS μ Columns	Miltenyi Biotec
MACS pre-Separation Filters	Miltenyi Biotec
MACS protein G Microbeads	Miltenyi Biotec
MACS (MidiMACS) Starting Kit	Miltenyi Biotec
Malassez chamber	Marienfeld
Material for gelshift	Bio-Rad
Material for western blot	Bio-Rad
Microscope	Leica
Microscope	Nikon
Microwave	Severin
MilliQ machine	Millipore
Mini centrifuge	National Labnet Co.
Nanodrop ND-1000	Nanodrop technologies
Nucleofector	Amaxa
Orion microplate luminometer	Berthold
PCR Mastercycler	Eppendorf
Petri dish	Greiner
PH meter	Eutech Instruments
PhosphorImager Cyclone	Packard, Perkin Elmer

Photo film (GS)	Kodak
Photo film (western blot)	GE healthcare
Pipet-aid	Falcon
Pipettes	Gilson
Plexiglas protection shields	Nalgene
Precision balance	Ohaus
PVDF membrane	GE healthcare
Roller Mixer SRT2	Stuart Scientific
Speedvac	Jouan
Spectrophotometer	Pharmacia Biotech
Transfert instrument (western blot)	Bio-Rad
Vortex	Scientific industries
Water bath	GFL

Software	
Elisa reader	Reader
Gel camera	LabWorks Gel-Pro application
Gelshift phosphoimager	Cyclone OptiQuant
Quantification program	Kodak 1D 3.5
Microscope	Leica, Image J

Cell lines and bacteria	
HEL	DSMZ
K562	DSMZ
TF-1	DSMZ
TOP 10F' one shot bacteria	Invitrogen

4.2 Cells

4.2.1 Cell lines

• The human chronic myeloid leukemia (CML) K562 cell line (Deutsche Sammlung von Microorganismen und Zellkulturen, DSMZ). In 1970 this cell line was established from the pleural effusion of a 53-year-old woman with CML [149]. The cells bear the Philadelphia chromosome and show spontaneous and inducible globin gene expression [150].

• The HEL cell line (DSMZ). It was established in 1980 from a 30-year-old man who suffered from erythroleukemia, a form of acute myeloid leukemia (AML), which is classified as acute nonlymphocytic leukemia of the M6 type. These cells were described to differentiate spontaneously towards the erythroid pathway [150].

• The TF-1 cell line (DSMZ). In 1987 this cell line was established from the bone marrow of a 35-year-old man with erythroleukemia (AML M6) [151,152]. The cells are proliferatively responsive to several hematopoietic growth factors including Epo, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFNγ), Interleukin 3 (IL-3), IL-4, IL-5, IL-6, TNF-α, and others.

4.2.1.1 Freezing and thawing of the stocks

Cell stock aliquots are deep-freezed in a RPMI solution containing 20% fetal calf serum (FCS) and 10% dimethyl sulfoxide (DMSO). They are stored in liquid nitrogen. The thawing is done by heating the cells rapidly to 37 °C and putting them in their usual cell culture conditions (see maintenance of the cell culture). The number of cell passages is restricted to 30 before thawing a new cell stock.

4.2.1.2 Maintenance of the culture

K562 and HEL cells are grown in RPMI 1640 Medium supplemented with 10% of FCS and 1% of a mixture of antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL) and an antifungal agent (amphotericin B, 25 µg/mL).

TF-1 cells are cultured in RPMI 1640 Medium supplemented with 20% of FCS and 1% of a mixture of antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL) and an antifungal agent (amphotericin B, 25 µg/mL) as well as 5 ng/mL of GM-CSF. Before

treating the TF-1 cells with Epo, GM-CSF is discarded overnight (O/N). Cells cultured with GM-CSF were used as controls (C).

Cells are routinely cultured in an incubator at 37 °C with 95% humidity and 5% CO_2. Every 3 days, the cells are subcultured and seeded at a concentration of 200.000 cells per mL. Cell counts were performed with a Malassez chamber using Trypan Blue exclusion method.

4.2.2 Magnetic cell sorting of CD34+ cells from umbilical cord blood

Umbilical cord blood samples were collected from healthy donors after informed consent under protocols approved by the Bohler Hospital Ethics committee. The cord blood is collected in 50 mL tubes containing an anticoagulant (20 U/mL Heparin in 5 mL phosphate buffered saline (PBS), and then diluted 1:4 with PBS containing 2 mM ethylene-diamin-tetra-acetat (EDTA) within 4 hours. The diluted cell suspension (35 mL) is then carefully layered of over 15 mL of Ficoll-Paque Premium. The mononuclear cells are isolated by centrifugation (35 min, 400 g, 20 °C) in a swinging-bucket rotor without brake. Interphase cells are carefully collected and washed twice with 50 mL PBS containing 2 mM EDTA. Cells are centrifuged 10 min at 200 g at 20 °C. Cell counts are performed with a Malassez chamber using Trypan Blue exclusion method. The cell pellet is then resuspended in a final volume of 300 µL magnetic cell sorting (MACS) buffer/10^8 cells (MACS buffer: PBS pH7.2, supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA).

MACS of CD34+ cells is performed using the MidiMACS manufacturer's protocol. The purity of the eluted CD34+ cells, resuspended in 60 µL MACS buffer (MACS buffer: PBS pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA) is then analyzed by FACS calibur using 3 µL fluorescein isothiocyanate (FITC) conjugated anti-CD34, as well as 3 µL peridinin chlorophyll A protein (PerCP) conjugated anti-CD45. The suspensions were incubated for 10 min on ice in the dark and then washed by adding 1 mL PBS. The cells were processed in the flow cytometer. Statistics were elaborated in 50000 events/sample by WinMDI software.

4.2.2.1 CD34+ cell culture

The CD34+cells were cultured in serum-free liquid medium, supplemented with 10 U/mL penicillin/streptomycin and a cocktail of cytokines as follows: days 0–3, 10

ng/mL IL-3 and 10 ng/mL SCF; days 3–7, 10 ng/mL IL-3, 10 ng/mL SCF, and 2 U/mL EPO; days 7–9, 10 ng/mL SCF and 2 U/mL EPO; and after day 9, 2 U/mL EPO [153].

4.2.3 Viability assay

Trypan Blue is a vital dye. This chromophore is negatively charged and therefore not able to interact with the cell membrane unless the cell is damaged. Viable cells remain unstained, while non-viable cells are stained blue. The ratio gives the percent of viability for each coverslip.

4.2.4 Exponential growth phase

Before any treatment, the cells are seeded at 200.000 cells/mL. After 24 hours (h), at the beginning of the exponential growth phase, the cells are recounted to have a concentration of 200.000 cells/mL at the moment of treatment.

4.3 Erythroid differentiation inducers, TNFα and inhibitors

4.3.1 Erythroid differentiation inducers

Different pharmacologic agents were used as erythroid differentiation inducers. They are known to induce the erythroid differentiation pathway by different, not fully understood mechanisms.

All solution preparations and treatments were done under sterile conditions. Preparation as well as dilutions steps of Hemin (He), Aclacynomicin A (Acla) and Doxorubicin (Dox) were done in the dark.

4.3.1.1 Aclacynomicin A

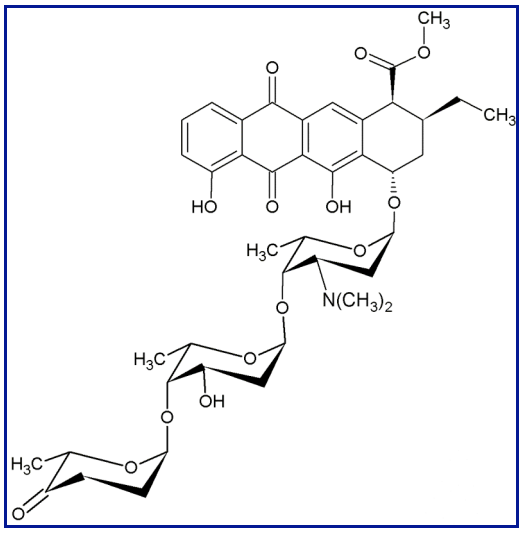


Figure 4: Chemical structure of Aclacynomicin A

Aclacynomicin A (Acla) is a cytotoxic anthracycline used as anticancer agent isolated from *Streptomyces galilaeus* [154]. This cell-permeable agent acts as a catalytic inhibitor of topoisomerase I/II [155] and of the degradation of ubiquitinilated proteins [156]. Acla stock solution (10^{-3} M) is prepared by dissolving 5 mg of Acla (molecular weight (MW): 812 g/mol) powder in 6.2 mL DMSO. Aliquots are stored at -20 °C. The treatment solutions are diluted using RPMI culture medium.

4.3.1.2 Doxorubicin

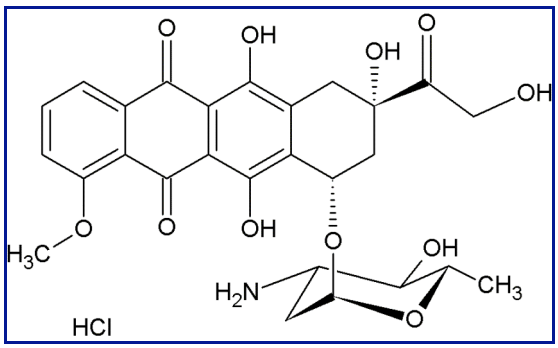


Figure 5: Chemical structure of Doxorubicin Hydrochloride

Doxorubicin (Dox) is a cytotoxic anthracycline antibiotic isolated from *Streptomyces peucetius var. caesius* [157]. It is used alone or in combination with other drugs as treatment against a wide range of cancers including solid tumors, leukemias, and lymphomas [158]. The exact mechanism of this antineoplastic agent remains unknown but

it may involve inhibition of the nuclear enzyme DNA topoisomerase II [159,160] inducing double-strand DNA breakage [161,162], and generation of superoxide, hydrogen peroxide and hydroxyl radicals [163]. The stock solution (10^{-3} M) is prepared by dissolving 5 mg of Dox (MW: 812 g/mol) powder in 8.6 mL sterile water. Aliquots are stored at -20 °C. The treatment solutions are diluted in RPMI culture medium.

4.3.1.3 Hemin

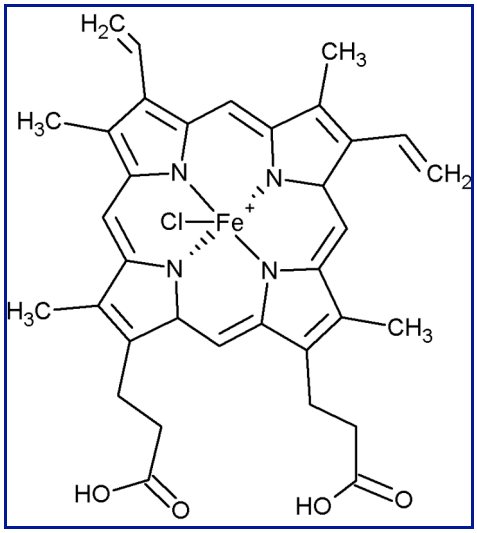


Figure 6: Chemical structure of Hemin

Hemin (He) or Ferriprotoporphyrin IX is a porphyrine. To prepare the He stock solution (10^{-3} M), 65.2 mg (MW: 652 g/mol) are dissolved in 2.5 mL absolute ethanol added to 2.5 mL KOH 0.2 M. After addition of 95 mL PBS 1X [from a solution PBS 10X (pH 7.4, 1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4] dissolved in H_2O), the solution is filtered (0.22 µm) and aliquots are stored at -20 °C. The treatment solutions are diluted in RPMI culture medium.

4.3.1.4 Erythropoietin

Erythropoietin (Epo) is a 30.4 kDa glycoprotein growth factor produced primarily in the adult kidney under the control of an oxygene-senitive mechanism that triggers erythrocyte production [164]. Epo regulates the red blood cell production in the bone marrow to sustain the oxygen – bearing capacity of the peripheral blood under physiologic conditions [165]. The local oxygen tensions are controlled by HIF [166]. HIF cannot be hydroxylated under hypoxia conditions, is therefore stabilized and thus upregulates Epo production [167]. Recombinant Epo has been widely used in the clinic to

prevent or treat malignancy-associated anemia [168]. Epo stock solution (10000U/mL) was diluted to 10 U/mL (TF-1 cells) or 2 U/mL (CD34+ cells) in RPMI culture medium.

4.3.2 TNFα

TNFα stock solution (10 μg/mL) is prepared by dissolving 10 μg of TNFα (T) in 1 mL of PBS 1X (from a PBS 10X solution (pH 7.4, 1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4) dissolved in H_2O) supplemented with 0.5% BSA and stored at 4 °C. The treatment solutions are diluted in RPMI culture medium. 20 ng/mL T were added to cell suspension as a pretreatment for 1 hour prior to the addition of differentiating inducers.

4.3.3 Inhibitors

These inhibitors have to be protected from light and are given to the cells one hour prior to TNFα or any inducer treatment.

4.3.3.1 Remicade

Remicade (R) (generic name: Infliximab) is an IgG_1 monoclonal anti-TNFα antibody composed of human constant and murine variable regions. It is used to eliminate the excess of TNFα from the circulation and from the sites of inflammation in various diseases such as Crohn's disease, rheumatoid arthritis, psoriasis, and ankylosing spondylitis [169-178]. The stock solution, which is also used for patients' treatment, has a concentration of 100 mg/10 mL H_2O. The treatment solutions are diluted to a concentration of 100 μg/mL in RPMI culture medium.

4.3.3.2 Lactacystin

Lactacystin is an organic compound naturally synthesized by bacteria (Streptomyces) that is widely used as a selective inhibitor of the 20 S proteasome [179,180]. For a stock solution of 13.35 mM lactacystin, 2 mg lactacystin were dissolved in 398 μL sterile H_20. The treatment solutions are diluted to a concentration of 1 μM lactacystin in RPMI culture medium.

4.3.3.3 Bay11-7082

Bay11-7082: an inhibitor of NF-κB activation, which prevents a step of the phosphorylation of inhibitory protein IκBα bound to NF-κB. Bay11-7082 stock solution (10 mM) was diluted to 1 μM in RPMI culture medium.

4.3.3.4 U0126

U0126 is a chemically synthesized organic compound, which inhibits both active and inactive MAPK kinase (MEK) thus blocks the ERK pathway. It has a 100-fold higher potency than PD98059. The stock solution (20 mM in DMSO) is diluted to a concentration of 10 μM in RPMI culture medium.

4.3.3.5 PD98059

PD98059 is a potent MAPK kinase inhibitor, which only inhibits activation of inactive MEK. PD98059 does not inhibit MAPK itself. The stock solution (20 mM in DMSO) is diluted to a concentration of 10 μM RPMI culture medium.

4.3.3.6 SB203580

SB203580 is a highly selective inhibitor of p38. It binds within the ATP pocket of the active kinase. SB203580 selectively inhibits the p38α and β isoforms. SB203580 stock solution (10 mM in DMSO) was diluted to 10 μM μM in RPMI culture medium.

4.3.3.7 SP600125

SP600125 is a selective JNK inhibitor. It competitively and reversibly inhibits JNK1, 2 and 3. SP600125 stock solution (10 mM in DMSO) was diluted to 10 μM μM in RPMI culture medium.

4.3.3.8 Ly294002

Ly294002 is a selective phosphatidylinositol 3-kinase (PI3K) inhibitor. Ly294002 blocks the catalytic activity of PI3K. Ly294002 stock solution (10 mM in DMSO) was diluted to 10 μM μM in RPMI culture medium.

4.4 Benzidine staining

The benzidine reagent is used to identify Hemoglobin-containing cells, as described previously [181,182]. It forms a blue precipitate upon oxidation by the heme group of hemoglobin in the presence of hydrogen peroxide (H_2O_2). Therefore it serves as a histochemical stain specific for differentiated red blood cells.

Phosphate Buffered Saline (PBS) 10X (pH 7.4)	1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
PBS 1X	1:10 dilution of PBS 10X in H_2O
NaCl (0.9%)	1.54 mL 1M NaCl, *ad* 10 mL H_2O
benzidine chlorhydrate solution	0.2 g benzidine chlorhydrate, 2.85 mL acetic acid, *ad* 100 mL H_2O (in the dark)
H_2O_2	

- spin $3x10^5$ cells down [5 minutes (min) at room temperature, 600 rcf (relative centrifugal force, g)]
- rinse the cells with 1 mL PBS 1X
- spin cells down (5 min, 600 rcf)
- carefully pour off the supernatant
- mix 1 mL benzidine solution with 20 µL H_2O_2 in a separate tube
- resuspend the cell pellet with 100 µL NaCl (0.9%) and 50 µL benzidine/H_2O_2
- leave 20 minutes in the dark
- add 300 µL NaCl (0.9%)
- observe and count the percentage of differentiated (blue) cells using the microscope

4.5 Total RNA extraction

The extraction is realized from 10^7 cells by using the TRIzol reagent. This is a monophasic solution of phenol and guanidine isothiocyanate for isolating total RNA. In fact this reagent is lysing the cells. Adding chloroform separates the organic and aqueous phases. Phenol removes the proteins from nucleic acid samples during isolation.

TRIzol
1-Bromo-3-Chloro-Propane
Isopropanol
Ethanol (75%)
H_2O DEPC (diethylpyrocarbonate)

- spin 10^7 cells down (5 min, 600 rcf)
- wash the cell pellet with PBS 1X
- spin cells down (5 min, 600 rcf)
- homogenize by adding 1 mL TRIzol, hold 5 min at room temperature
- add 200 µL 1-Bromo-3-Chloro-Propane, vortex for 15 sec until the emulsion turns to pale rose
- incubate for 5 min at room temperature
- centrifuge (15 min, 12000 rcf, 4 °C)
- remove the colorless upper phase containing the RNA in a separate tube
- add 500 µL Isopropanol and incubate for 10 min at -20 °C or O/N at 4 °C
- centrifuge (10 min, 12000 rcf, 4 °C)
- recover the translucent pellet by removing the supernatant
- wash the pellet by adding 1 mL Ethanol 75%
- centrifuge (5 min 30 sec, 11000 rcf, 4 °C)
- recover the translucent pellet by removing the supernatant
- dry the pellet for 5-10 min
- add 50-100 µL H_2O DEPC depending on the size of the pellet
- put the tubes for 5 min in the water bath at 55 °C
- for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, RNA was cleaned using RNeasy Mini Kit from Quiagen, and RNA integrity and purity was controlled using a Bioanalyzer (Agilent).

▪ quantify the RNA by measuring the optical density (OD) 260 nm using the Nanodrop ND1000, considering the dilution and multiplying by 40 (1 OD 260 = unit of single stranded RNA = 40 ug)

4.6 PCR analysis

The polymerase chain reaction (PCR) is a technique that amplifies a DNA template to produce specific DNA fragments *in vitro*. The PCR method was initially developed to amplify segments of a longer DNA molecule [183,184]. The amplification includes the target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension.

4.6.1 Reverse transcription (RT)-PCR

In order to apply PCR to the study of RNA, the RNA sample must first be reverse transcribed to cDNA to provide the necessary DNA template for the thermostable polymerase. This process is called reverse transcription (RT). The RT-PCR is realized with substances from Invitrogen.

10 mM dNTP mix	
Oligo(dT) primers	
10X RT buffer	
25 mM $MgCl_2$	
0.1 M DTT 1 (1,4-Dithiothreitol)	
RNaseOUT	
Superscript II RT	
RNase H	
10X PCR buffer	
50 mM $MgSO_4$	
Platinum Taq polymerase	
H_2O DEPC	
TBE 10X	0.9 M Trishydroxymethylaminomethane (TRIS) base, 0.9 M boric acid, 25 mM EDTA (pH 8.0) *ad* 1l H_2O
loading buffer	Glycerol 33%, TBE 2X, Bromophenol Blue 0.005%, Xylene cyanole 0.005%
Gel 2%	agarose 2%, TBE 1X, 2% ethidium bromide
GATA-1	(s) : 5'-TCAATTCAGCAGCCTATTCC-3'
	(as) :5'-TTCGAGTCTGAATACCATCC-3'

GATA-2	(s) : 5'-TGTTGTGCAAATTGTCAGACG-3'
	(as) : 5'-CATAGGTGCCATGTGTCCAGC-3'
γ-globine	(s) : 5'-GGCAACCTGTCCTCTGCCTC-3'
	(as) : 5'-GCCAGGAAGCCTGCACCTCA-3'
EpoR	(s) : 5'-GGTCCTCCGTGAAGGGGGTGC-3'
	(as) : 5'-AGCCTGTGTCGCTGCTGACGC-3'
S14	(s) : 5'-GGCAGACCGAGATGAATCCTC-3'
	(as) : 5'-CAGGTCCAGGGGTCTTGGTCC-3'

- put RNA samples for 8 min in the water bath at 65 °C
- centrifuge and put on ice
- mix following reagents in an 0.5 mL tube:
  - 5 µg RNA
  - 1 µL 10 mM dNTP mix
  - 1 µL Oligo(dT) (0.5 µg/µL)
  - *ad* 10 µL H_20 DEPC
- incubate for 5 min at 65 °C
- put on ice for at least 1 min
- add 9 µL MIX:
  - 2 µL 10X RT buffer
  - 4 µL 25 mM MgCl2
  - 2 µL 0.1 M DTT
  - 1 µL RNaseOUT
- incubate at 42 °C for 2 min
- add 1 µL Superscript II RT (50 U)
- incubate at 42 °C for 50 min
- incubate at 70 °C for 15 min
- add 1 µL RNase H
- incubate at 37 °C for 20 min
- store at -20 °C

Then the PCR is performed:

- in an 0.2 mL tube mix following reagents:
  - 10X PCR buffer

- 2 - 3 µL 50 mM $MgCl_2$
- 1 µL 10 mM dNTP mix
- 1 µL sense (s) primer
- 1 µL antisense (as) primer
- 0.2 µL Platinum Taq (5 U/µL)
- 2 µL cDNA
- *ad* 50 µL DEPC H_2O

▪ The following program is performed:

- 5 min at 94 °C initial denaturation
- 2 min at 94 °C denaturation
- 1 min at 60 °C primer-hybridization } x cycles depending on the primers used
- 2 min at 68 °C polymerization
- 2 min at 68 °C final extension
- hold at 4 °C

primers	number of cycles (x)
S14	20
GATA-1	22
γ-globin	20
EpoR	26

▪ run a 2% agarose gel

▪ add 6 µL loading buffer to the samples and run the gel

▪ take a photo under UV-light, and quantify the bands using the quantification progam Kodak 1D 3.5 and compare these results with the ones obtained with the reference gene S14 (40S ribosomal protein).

4.6.2 Quantitative real-time PCR (qRT-PCR)

1.5 µg of total RNA were used to perform cDNA synthesis using RT2 PCR Array First Strand Kit (C-02) (SuperArray) according to the manufacturer's instructions. qRT-PCR was performed with SuperArray primers according to manufacturers using a custom RT2 Profiler PCR Array System (table 1) using a 7300 real-time PCR System (Applied Biosystems). Results were evaluated using an Excel-based data analysis template from SuperArray.

Table 1: Genes used for custom RT² Profiler PCR Array System

Symbol	Unigene	Refseq No	Gene name
GATA1	Hs.765	NM_002049	GATA binding protein 1
GATA2	Hs.367725	NM_032638	GATA binding protein 2
NF-E2	Hs.75643	NM_006163	Nuclear factor (erythroid-derived) 2
KLF1	Hs.37860	NM_006563	Kruppel-like factor 1 (erythroid) (EKLF)
MYB	Hs.531941	NM_005375	Myb oncogene-like
TAL1	Hs.73828	NM_003189	T-cell acute lymphocytic leukemia 1
ZFPM1	Hs.507185	NM_153813	Friend of GATA-1 (FOG-1)
SPI1	Hs.502511	NM_003120	SFFV proviral integration 1 (PU.1)
TFRC	Hs.529618	NM_003234	transferrin receptor
HBA1	Hs.449630	NM_000558	Alpha globin (α-globin)
HBG1	Hs.567283	NM_000559	Gamma globin (γ-globin)
EPOR	Hs.127826	NM_000121	Erythropoietin receptor
GYPA	Hs.434973	NM_002099	Glycophorin A (GPA)
HMBS	Hs.82609	NM_000190	Hydroxylmethylbilane synthase
SLC4A1	Hs.443948	NM_000342	solute carrier family 4, anion exchanger, member 1
ERAF	Hs.274309	NM_016633	erythroid associated factor
KIT	Hs.479754	NM_000222	kit oncogene
AKT1	Hs.525622	NM_005163	v-akt murine thymoma viral oncogene homolog 1
ITGAM	Hs.172631	NM_000632	integrin, alpha M
ITGA2B	Hs.411312	NM_000419	integrin, alpha 2b
BCL2L1	Hs.516966	NM_138578	BCL2-like 1
MRPS14	Hs.247324	NM_022100	mitochondrial ribosomal protein S14
GAPDH	Hs.544577	NM_002046	glyceraldehyde-3-phosphate dehydrogenase
GATA6	Hs.514746	NM_005257	GATA binding protein 6

4.7 Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic proteins are extracted from 10^7 cells with or without treatment using the method described by Schreiber *et al.* [185]. During this procedure the cell membranes are lysed in a hypertonic detergent medium containing protease inhibitors. The extraction is realized on ice to avoid denaturation of the proteins.

PBS 10X (pH 7.4)	1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
buffer A	Hepes (pH 7.9) 10 mM, KCl 10 mM, EDTA 0.1 mM, EGTA 0.1 mM, DTT 1 mM, PMSF 0.5 mM, 0-Phenylanthroline 1 mM, Aprotinine 2 µg/mL, Leupeptine 30 µg/mL, Pepstatine 15µg/mL
buffer B	Hepes (pH 7.9) 20 mM, NaCl 0.4 mM, EDTA 1 mM, EGTA 1 mM, DTT 1 mM, PMSF 1 mM, 0-Phenylanthroline 1 mM, Aprotinine 2 µg/mL, Leupeptine 30 µg/mL, Pepstatine 15 µg/mL, Glycerol 20%
Igepal 10%	
BSA	20 µg/mL
Bradford solution	

- spin 10^7 cells down (5 min, 600 rcf)
- wash the cell pellet with PBS 1X
- spin cells down (5 min, 600 rcf)

- resuspended the pellet with 400 µL buffer A, keep on ice for 15 min
- add 25 µL Igepal 10%
- vortex during 10 seconds (sec)
- centrifugate (30 sec, 15700 rcf, 4 °C)
- transfer the supernatant with the cytoplasmic proteins is in a second tube
- resuspend the pellet containing the nuclei in 50 µL buffer B
- vortex the solution horizontally for 15 min at 4 °C
- pool the supernatants and divide them in aliquots of 10 µL (4 °C)
- quantify the protein concentration by establishing a BSA reference curve according to Bradford [186].

4.8 Western Blot

For western blot analysis a sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) is realized to separate the proteins by size, which are transferred from the gel to a PVDF membrane via electro transfer. Finally, this blot is processed for the detection of specific proteins with a primary antibody (ab), detected by a chemifluorescent secondary ab.

denaturation & loading buffer (2X)	Tris-HCl (pH 6.8) 0.125 M, Glycerol 20%, SDS 4%, bromophenol blue 0.005%, 2-Mercaptoethanol 5%
electrophoresis buffer (10X)	Tris 30 g, Glycine 144 g, SDS 10 g → *ad* 1 l H_2O
concentration gel (4%)	liquid gel (37.5:1) 4%, Tris-HCl (pH 6.8) 0.125 M, SDS 0.1%, ammonium persulfade 0.1%, Temed 0.05%
separation gel (10%)	liquid gel (37.5:1) 10%, Tris-HCl (pH 8.8) 0.375 M, SDS 0.1%, ammonium persulfade 0.1%, Temed 0.05%
transfer buffer (1X)	Tris 3 g, Glycine 14.4 g, Methanol 50 mL → *ad* 1 l H_2O
PBS 10X (pH 7.4)	1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
PBS-T	PBS 10X 100 mL, 1 mL Tween 20 → *ad* 1 l H_2O
BSA	
milk	
antibody anti-acetyl-Lysine monoclonal mouse IgG	1:500 in PBS-T-milk 5%

antibody anti-GATA-1 N-1 rat monoclonal IgG (200 µg/0.1 mL)	1:5000 in PBS-T-BSA 5%
antibody anti-GATA-1 C-20 goat polyclonal IgG (200 µg/0.1 mL)	1:5000 in PBS-T-BSA 5%
antibody anti-GATA-2 goat polyclonal IgG (200 µg/mL)	1:8000 in PBS-T-milk 5%
antibody anti-EpoR M-20 rabbit polyclonal IgG (200 µg/mL)	1:1000 in PBS-T-milk 5%
antibody anti-β-actin mouse monoclonal IgG (200 µg/0.1 mL)	1:5000 in PBS-T-milk 5%
antibody anti-FOG-1 goat polyclonal IgG M-20	1:500 in PBS-T-milk 5%
antibody anti-γ-globin mouse monoclonal IgG (51-7)	1:8000 in PBS-T-milk 5%
antibody anti-phospho-p38 rabbit polyclonal IgG	1:1000 in PBS-T-BSA 5%
antibody anti-p38 rabbit polyclonal IgG	1:1000 in PBS-T-BSA 5%
antibody anti-PU.1 rabbit polyclonal IgG (Spi-1, T-21)	1:500 in PBS-T-milk 5%
goat anti-mouse IgG-HRP conjugated (200 µg/0.5 mL)	1:10000 in PBS-T-milk 5%
goat anti-rat IgG-HRP conjugated (200 µg/0.5 mL)	1:5000 in PBS-T-milk 5%
donkey anti-goat IgG-HRP conjugated (200 µg/0.5 mL)	1:3000 in PBS-T-milk 5%
donkey anti-goat IgG-HRP conjugated (200 µg/0.5 mL)	1:4000 in PBS-T-milk 5%
donkey anti-goat IgG-HRP conjugated (200 µg/0.5 mL)	1:5000 in PBS-T-milk 5%
donkey anti-rabbit IgG-HRP conjugated (200 µg/0.5 mL)	1:5000 in PBS-T-milk 5%
Ecl + Lumigen PS-3 detection reagent	

- add the volume of 5-20 µg proteins to an equal volume of denaturation & loading buffer (2X)
- incubate in the water bath for 5 min at 100 °C
- put on ice
- centrifuge (5 sec, 1900 rcf) and put the samples on the SDS-PAGE
- migrate at 10 mA/gel in the concentration gel and at 20 mA/gel in the separation gel using electrophoresis buffer (1X)

▪ after separation, transfer the proteins on a PVDF membrane at 100 mA/gel using transfer buffer 1X

▪ saturate the membrane either in PBS-T-milk 5% during the night at 4 °C or in BSA during 1 hour at room temperature depending on the primary antibody ab used

▪ wash the membrane 2 x 5 min, 1 x 10 min with PBS-T

▪ incubate the membrane with the first ab for 1 h at room temperature or O/N at 4 °C depending on the primary ab used

▪ wash the membrane 2 x 5 min, 1 x 10 min with PBS-T

▪ incubate the membrane with the secondary ab for 1 h at room temperature

▪ wash the membrane 2 x 5 min, 1 x 10 min with PBS-T

▪ visualize the protein using the chemiluminescent Ecl-detection reagent, the photo film and the development solutions and machinery

4.9 Flow cytometry analysis

The expression of GPA of differentiated cells is analyzed by using flow cytometry analysis. Glycophorins are sialoglycoproteins of the human erythrocyte membrane, which bear the antigenic determinants. The genetic array of expressed glycophorin surface antigens on erythrocytes defines the blood group phenotype of the individual.

PBS 10X	1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
Cytofix/Cytoperm	
Perm/Wash buffer	
antibody anti-GPA goat polyclonal IgG E-18	1:50 in Perm/Wash buffer
Alexa Fluor® 488 donkey anti-goat IgG (H+L)	1:200 in Perm/Wash buffer

▪ spin $10*10^6$ cells down (350 rcf, 7 min)

▪ rinse the cells with 1 mL PBS 1X, centrifuge (450 rcf, 7 min)

▪ discard supernatant

▪ add 250 µL Cytofix/Cytoperm

- incubate for 20 min at 4 °C
- rinse the cells with 1 mL Perm/Wash buffer solution 1X, centrifuge (450 rcf, 7 min)
- incubate the cell pellet with 60 µL GPA ab solution for 1 h shaking
- rinse the cells with 1 mL Perm/Wash buffer solution 1X, centrifuge (450 rcf, 7 min)
- incubate the cell pellet with 60 µL secondary ab (488 donkey anti-goat IgG) for 30 min shaking in the dark
- rinse the cells with 1 mL Perm/Wash buffer solution 1X, centrifuge (450 rcf, 7 min)
- add 5 mL PBS
- Cells are then processed in a FACS calibur flow cytometer. Statistics were elaborated in 50000 events/sample by WinMDI software.

4.10 Immunoprecipitation

Protein-protein interactions between GATA-1 and FOG-1, and acetylated GATA-1 were determined by immunoprecipitation (IP) experiments based on the protocol of Ribeil and coworkers [187].

lysis buffer	Igepal 1%, 150 mM NaCl, 5 mM EDTA, 65 mM TrisHCl pH 8, 50 mM Hepes, 3% glycerol, 1 mM orthovanadate, 1 mM PMSF, 1 mM DTT, 40 µg/mL complete
low salt buffer	20 mM Tris HCl pH 7.5
elution buffer	50 mM TrisHCl pH 6.8, 50 mM DTT, 1% SDS, 0.005% bromphenolblue, 10% glycerol
PBS 10X	1.4 M NaCl, 27 mM KCl, 15 mM KH_2PO_4, 65 mM Na_2HPO_4
2 µg anti-GATA-1 ab (sc-1233X)	

- spin $10*10^6$ cells down (350 rcf, 7 min)
- rinse the cells with 10 mL PBS 1X, centrifuge (450 rcf, 7 min)
- discard supernatant
- add 1 mL lysis buffer, mix vigorously for 20 min
- centrifuge (30 min, 15700 rcf, 4 °C)
- preheat heating block (95 °C)

- whole cell lysates (500 µg) were diluted 1:3 in H_2O and incubated on ice for 1.5 hours with 2 µg anti-GATA-1 ab (sc-1233X) in the presence of 100 µL Protein G Microbeads (Miltenyi)
- place µColumn in magnetic field
- rinse column with 200 µL lysis buffer
- place elution buffer (90 µL) in preheated block
- immune complex was immobilized to the µColumn
- rinse 4x with 200 µL lysis buffer
- rinse with 200 µL low salt buffer
- apply 20 µL of preheated (95 °C) elution buffer
- incubate for 5 min
- place a new tube under the column
- apply 50 µL of preheated (95 °C) elution buffer
- perform immunoblotting (see section 3.9) using the GATA-1 (sc-266X), FOG-1 (sc-9361), and acetyl-Lysine (4G12) antibodies.

4.11 Electrophoretic Mobility Shift assay (EMSA)

EMSA is a technique for studying DNA-protein interactions *in vitro*. Double stranded oligonucleotides are used to examine binding affinity of cell extracts for specific DNA elements. Nuclear extracts are incubated with a radioactively labeled probe, which contains the specific recognition sequence for NF-κB or GATA. The binding occurs under precise salt and pH settings in a binding buffer. Poly-dIdC is used to prevent unspecific binding of proteins to the probe and thus reduce background. After binding, the samples are separated on a non-denaturating gel, DNA-protein complexes migrate more slowly than the unbound radioactive probe and consequently they are visualized as distinct bands of radioactivity on an acrylamide gel image detected by autoradiography.

In order to determine which proteins are responsible for the observed bands, a "supershift" can be realized by adding specific antibodies to the binding reactions. The antibodies will bind to the corresponding protein, leading to a protein-probe-ab ternary complex. Consequently the electrophoretic mobility of this complex is lower than that of

the protein-probe complex so that a supershifted band can be observed. Occasionally an ab does not result in a shifted band even if its corresponding subunit is present: instead, by binding to its subunit, the ab prevents the association of the subunit and the labeled probe so that the intensity of the primary shifted signal decreases. In this case, we talk about immunodepletion.

TBE 10X	0.9 M TRIS base, 0.9 M boric acid, 25 mM EDTA (pH 8.0) *ad* 1l H_2O
GATA consensus	(s) :5'-GGCAGTGCCTTATCTCTGCGGCG-3'
	(as) :5'-CGCCGCAGAGATAAGGCACTGCC-3'
NF-κB consensus	(s) : 5'-AGTTGAGGGGACTTTC**C**CAGGC-3'
	(as) : 5'-GCCTGGGAAAGTCCCCTCAACT-3'
binding buffer 5X (hybridization)	Tris (pH 7.5) 335 mM, $MgCl_2$ 65 mM, DTT 33 mM, EDTA 6.5 mM
ethanol (100%)	
T4 Polynucleotide kinase & kinase buffer 10X	
Poly-dIdC	
gamma-^{32}P-phosphate	7000 Ci/mmol
Qiaquick Removal Nucleotide kit	
binding H_2O (+ protease-inhibitors)	DTT 0.5 mM, PMSF 0.5 mM, Aprotinine 1 µg/mL, O-phenanthroline 1 mM, Leupeptine 30 µg/mL
binding buffer 5X (MIX)	Tris-HCl (pH 8.0) 10 mM, NaCl 50 mM, EDTA (pH 8) 1 mM, Glycerol 5%, DTT 1 mM, PMSF 2.5 mM
BSA	2 mg/mL
Spermidine	80 mg/mL
antibody anti-GATA-1 rat monoclonal IgG (200 µg/100 µL)	2 µg
antibody anti-GATA-2 goat polyclonal IgG (200 µg/100 µL)	2 µg
NF-κB p50 rabbit polyclonal IgG (200 µg/100 µL)	2 µg
NF-κB p65 rabbit polyclonal IgG (200 µg/100 µL)	2 µg
loading buffer	Glycerol 33%, TBE 2X, Bromophenol Blue 0.005%, Xylene cyanole 0.005%
polyacrylamide gel (29 :1) 5%	TBE 0.5X, ammonium persulfate 0.2%, Temed 0.1%

4.11.1 Hybridization and labeling of the oligonucleotide probe

In a PCR tube (0.5 mL), add following components:

- 1500 ng oligonucleotide sense

- 1500 ng oligonucleotide antisense
- 12 µL binding buffer 5X
- *ad* 60 µL H_2O MilliQ

The hybridization is realized at the following temperatures:

- 90 °C during 5 min
- 65 °C during 10 min
- 37 °C during 10 min
- 20 °C during 10 min
- 4 °C until the precipitation of the probe

After hybridization, transfer the double-stranded probe in a 1.5 mL tube:

▪ add 3X volume 100% ethanol and 0.1 volume Natrium acetate

▪ incubate for 1 h on ice (4 °C)

▪ centrifuge (5 min, 15700 rcf, 4 °C)

▪ wash the pellet with 200 µL ethanol 75%

▪ centrifuge (5 min, 15700 rcf, 4 °C)

▪ dry the pellet using the Speedvac (5-10 min)

▪ resuspend the pellet with 15 µL H_2O MilliQ

▪ store at – 20 °C

The probe labeling is realized by using the T4 Polynucleotide kinase which catalyzes the transfer of the gamma-^{32}P-phosphate from ATP to the 5'-OH group of DNA and RNA [188-190].

The labeling is realized in the following mixture:

- 2 µL double stranded oligonucleotide
- 2 µL γ-^{32}P-phosphate ATP
- 1 µL T4 Polynucleotide kinase
- 2 µL kinase buffer 10X
- 13 µL H_2O

▪ incubate for 30 min at 37 °C

▪ purify the probe using the kit “Qiaquick Removal Nucleotide”

▪ elute with 35 µL H_2O

▪ store the labeled probes in a lead chest at -80 °C

4.11.2 protocol

- Assemble plates, spacers, clamps
- run the 5% polyacrylamide gel
- incubate 10 µg nuclear extracts with the following MIX:
  - 0.25 µL radioactive probe
  - 7 µL binding H_2O
  - 2.5 µL binding buffer (MIX)
  - 2.5 µL Poly dIdC
  - 2 µL BSA (2 mg/mL)
  - 1 µL Spermidine (80 mg/mL)
- [incubate for 30 min at 4 °C with 2 µg of specific ab (anti-p50, anti-p65 for the NF-κB subunits, anti-GATA-1, anti-GATA-2) before adding the radioactive probe to realize a supershift]
- incubate for 20 (GATA1/-2) and 45 (p50, p65) min on ice (4 °C)
- add 6 µL loading buffer
- put the whole MIX on the gel
- run the gel (3 h, 16 mA/gel)
- dry the gel
- results are analyzed using an autoradiography or the PhosphorImager (Cyclone OptiQuant)

4.12 TransAM

TransAM assays were performed according to the manufacturer's instructions (Active Motif, Belgium). The GATA-1 binding assay consists of a multiwell plate coated with DNA trappers containing the GATA-1 motif. These kits contain a 96-stripwell plate to which the consensus binding site oligonucleotide has been immobilized. Activated nuclear extract is added to each well and the transcription factor of interest binds specifically to the oligonucleotide. A primary ab specific for an epitope of the transcription factor is then added followed by incubation with a secondary ab and Developing Solution to provide a quantified and colorimetric readout at 450 nm with a reference wavelength of 655 nm.

4.13 Plasmids and transient transfection assays

4.13.1 Recombinant vectors used

- expression plasmid: pXM-GATA-1, containing the gene which encodes the protein GATA-1 (gift from L. Collavin).
- reporter plasmid: pGL3-GATA-luc, in this plasmid the luciferase gene is associated to three consensus GATA binding sites under the control of the minimal metallothionein promoter in the basic pGL3-vector [30].

4.13.2 Bacteria transformation and purification of plasmids

The aim of this procedure is to amplify plasmid DNA. This step is necessary to prepare a sufficient quantity of vectors for the transfection experiments. All experiments were realized under sterile conditions and following standard protocols.

LB Broth, Miller	25 g *ad* 1 l H_2O, autoclaved
LB Agar, Miller	32 g *ad* 1 l H_2O, autoclaved
ampicillin	100 µg/mL dissolved in H_2O
TOP 10F' one shot bacteria	

▪ cool down autoclaved LB Agar to 40-50 °C

▪ add 100 µg/mL ampicillin and mix gently

▪ pour LB Agar into petri dishes using sterile technique

▪ allow to solidify

▪ store the petri dishes upside down at 4 °C until use (stable for 4 weeks covered by aluminum foil)

▪ 200 µL competent bacteria (TOP 10F' one shot) are incubated with 5 ng recombinant plasmid during 15 min at 4 °C

▪ apply a heat shock to this mixture (45 sec at 42 °C)

▪ put on ice (4 °C)

▪ sterilely place the transformed cells on the agar surface and spread it out uniformly over the entire surface with a sterile pipette tip

▪ incubate at 37 °C for 10-18 h

- control colony density and size (stable at 4 °C until further notice)
- pick single colony from the agar using a pipette with sterile tip
- put the colony in the preculture (5 mL LB Broth supplemented with 100 µg/mL ampicillin)
- vortex during 10 sec
- put the preculture on the agitator at 37 °C for 10-18 h
- put 250 µL of preculture in main culture (250 mL LB Broth supplemented with 100 µg/mL ampicillin)
- put the main culture on the agitator at 37 °C for 10-18 h
- harvest bacteria from the LB Broth culture by centrifugation (15 min, 6000 rcf, 4 °C)
- plasmid DNA isolation is performed according to the manufacturer's instructions (Nucleobond Ax, Machinery-Nagel)
- quantify plasmid DNA by measuring the optical density (OD) at 260 nm (OD_{260} = 1 corresponds to 50 µg/mL). Purity is evaluated by measuring the ratio OD260/280. Values should lie between 1.8 and 2.1.

4.13.3 Transient transfection assays

The concept of electroporation is based on the relatively weak structure of the phospholipid bilayer's and its ability to reassemble after disintegrating. After breaking areas of the membrane by applying a quick voltage shock, a transient, permeabilized state can be used to charge cells with different molecules, either through simple diffusion in the case of small molecules, or through an electrophoretically driven process allowing passage through the damaged membrane – as is the case for DNA transfer. Transfections of K562 cells are performed by electroporation using a Bio-Rad gene Pulser. TF-1 and HEL cells were transfected using a nucleofector from Amaxa following manufacturer's instructions using KIT V. Cells in the exponential growth phase are resuspended in RPMI 1640 Medium.

For electroporation:

$3.75*10^6$ cells at a concentration of $1.5*10^7$ cells/ mL are electroporated at 250 V and 500 μF.

For nucleofection:

$5*10^6$ cells at a concentration of $5*10^7$ cells/ mL nucleofector solution, are electroporated using program T03 for TF-1 cells and X05 for HEL cells.

- Five micrograms of luciferase reporter gene construct and 5μg phRL-SV40-plasmid expressing Renilla are used for each pulse. For co-transfection assays, 5 μg of expression plasmid are added to this mixture.
- After 24 h, cells are harvested and resuspended in growth medium (RPMI/FCS 10%) at a final concentration of 10^6 cells/mL with or without treatments (48h).
- Dual-GloTM Luciferase Reagent (75μL) and Dual-GloTMStop&GlowReagent (Promega) are added according to the manufacturers recommendations. Firefly and Renilla luciferase activities are measured using a Orion microplate luminometer (Berthold) by integrating light emission for 10 s. Results are expressed as a ratio of arbitrary units of firefly luciferase activities normalized to Renilla luciferase activities.

4.14 Statistics

Data were expressed at the mean +/- standard deviation (SD). Statistical significance was assessed by Students t-test P-values below 0.05 were considered as statistically significant (*$P \leq .05$, **$P \leq .01$, ***$P \leq .001$).

5 Results

In the nineteenth century, TNFα was identified as a negative regulator of erythropoiesis [116,121,122]. In the meantime, a critical role for TNFα has been established in many forms of cancer [130] and inflammation related anemia [134]. In order to elucidate and generalize the inhibiting effect of TNFα on erythroid differentiation, we used the hematopoietic cell lines K562, HEL, and TF-1 as well as purified CD34+ cells as cellular models to mimic anemia in the presence of TNFα. Due to their differentiating potential, erythroleukemia cell lines present a good experimental system to study erythroid differentiation. Various agents can induce K562 and HEL towards the erythroid differentiation pathway [191-194]. In this study we used Acla, Dox, He, and Epo to induce erythroid differentiation. TF-1, a factor dependent, Epo-sensitive erythroleukemia cell line, was shown to produce hemoglobin after Epo treatment [151,152,195]. Moreover, the primary umbilical CD34+ cells are known to differentiate towards the erythroid differentiation pathway using Epo.

The aim of this study is to decode the effect of the proinflammatory cytokine TNFα on the hemoglobinization level, the RNA and protein levels of erythroid specific genes as well as the regulation by the major erythroid transcription factor GATA-1 and involved cell signaling pathways.

5.1 Effect of TNFα on hemoglobin synthesis

5.1.1 Effect of TNFα on hemoglobin synthesis, proliferation and mortality in cell lines

In order to measure the effect of TNFα on induced erythroid differentiation, we investigated the effect of the cytokine on hemoglobin synthesis, cell growth, and cell death. At least 100 cells were counted. The cells were retained as “benzidine positive” (benzidine +), if they were colored in green-blue as shown on figures 7, 8, or 9.

Effect of TNFα in K562 cells

- Hemoglobin synthesis

When TNFα was added to the cell culture medium 1 hour prior to Acla, Dox, or He treatment, a reduction of the number of benzidine + cells was observed, whereas a TNFα or Remicade pretreatment alone, used as a control, did not influence hemoglobin synthesis in K562 cells (Fig. 7A).

The K562 cells turned out to be inducible towards the erythroid pathway with all substances: Acla, Dox and He. Results show a reduction of hemoglobinization whenever cells were treated with TNFα and this independently of the chemical inducer used (Fig. 7B).

Acla (10 nM) induced a hemoglobinization rate of 48%, which was significantly reduced to 23% after addition of TNFα (Fig 7B). Moreover, the TNFα inhibitor Remicade (R) (100 μg/mL) significantly reduced the inhibitory effect of TNFα, resulting in a final hemoglobinization rate of 47% (Figure 7B).

Following addition of 40 nM Dox, 31% of cells were benzidine +, and after addition of TNFα, a significant decrease could be observed. Compared to other chemical inducers, Dox had also an effect on cell size. Furthermore, Remicade abolished the effect of the cytokine whenever added in combination with Dox (25%) (Fig. 7B).

After 3 days of treatment with He (30 μM), 78% of cells were benzidine +, which was significantly decreased to 57% by retreating the cells with 20 ng/mL TNFα. This inhibitory effect of TNFα was reversed by the addition of Remicade to 77% (Fig. 7B).

- cell growth

Treatment of K562 cells with He reduced the cell growth by 23%, and the addition of TNFα alone or in combination with He decreased cell growth of 22% and 31%. Using Acla alone reduces the proliferation by 18%. In combination with TNFα, the proliferation is restricted by 44%. Treating the cells during 3 days with Dox induces a reduction of 52%, taking TNFα as supplement stopped proliferation of 68% (Fig. 7B).

- viability

To analyze the toxicity of the different treatments, the Trypan viability assay was realized. K562 Trypan mean values did not exceed 7% of mortality rate after the different treatments (Fig. 7B).

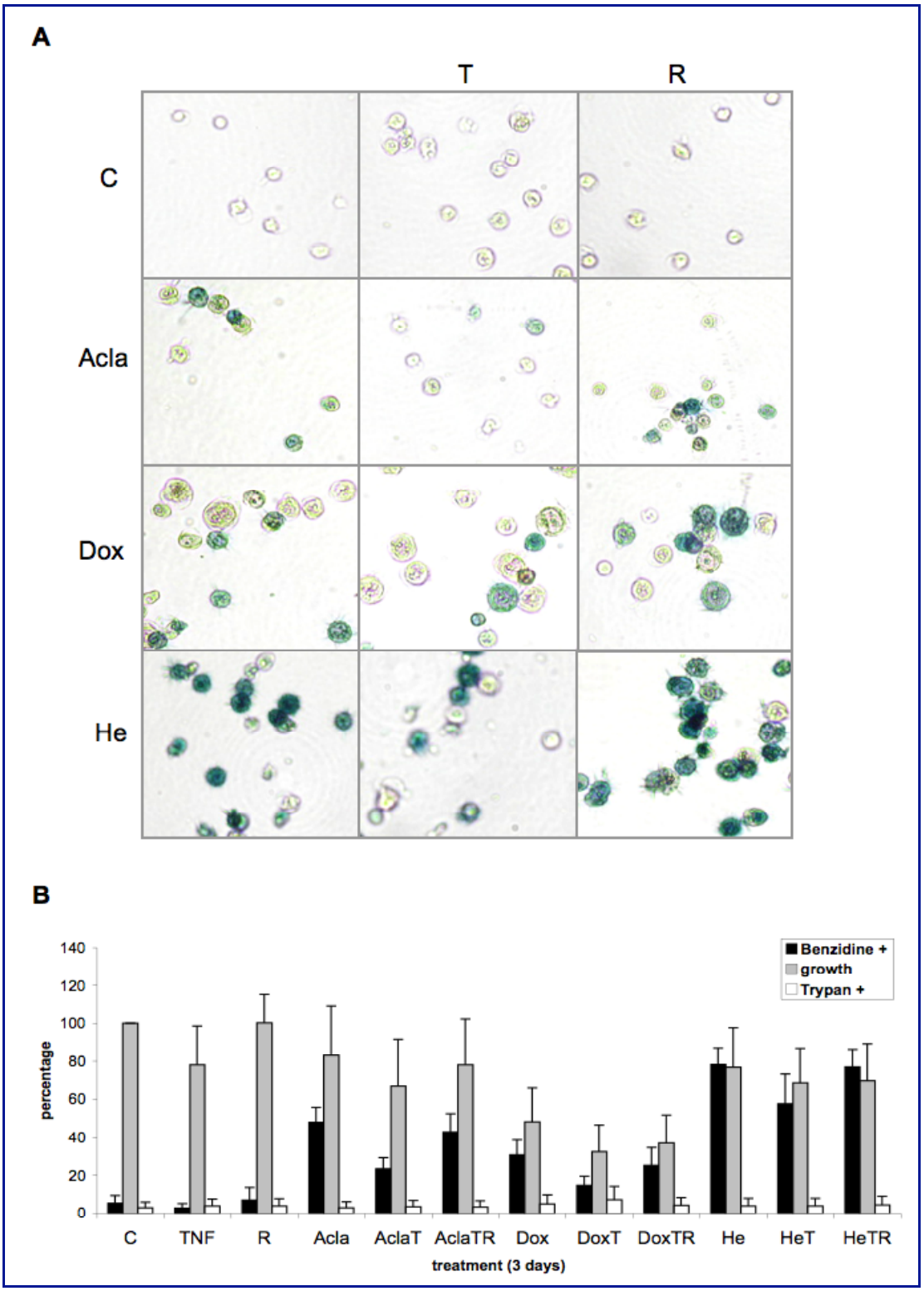


Figure 7: Effect of TNFα on hemoglobin synthesis in K562 cells. *Cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla, 40 nM Dox, or 30 µM He. A) Hemoglobinized cells were stained on day 3 using the benzidine method. B) Percentages of benzidine + cells, cell growth and Trypan + cells. Untreated cells were used as control (c). Values are reported as the means (+/- standard deviation, SD) of 7 independent experiments. All benzidine results were significant. (***P ≤ .05)*

Effect of TNFα in HEL cells

- Hemoglobin synthesis

HEL cells were induced towards the erythroid pathway using He (Fig. 6A). Results showed that 20 ng/mL of TNFα treatment 1 hour prior to 3 days of treatment with 30 μM He reduced the hemoglobinization rate to 39% when compared to He treatment alone (61%) (Fig. 8B). Remicade alone had no influence on hemoglobinization rate, but rescued the inhibitory effect of TNFα when treated in combination with He (60%) (Fig. 8B).

- cell growth

In HEL cells, the different treatments had no significant effect on cell growth (Fig. 8B).

- viability

To analyze the toxicity of the different treatments, the Trypan viability assay was realized. Trypan mean values did not exceed 7% of mortality rate after the different treatments (Fig. 8B).

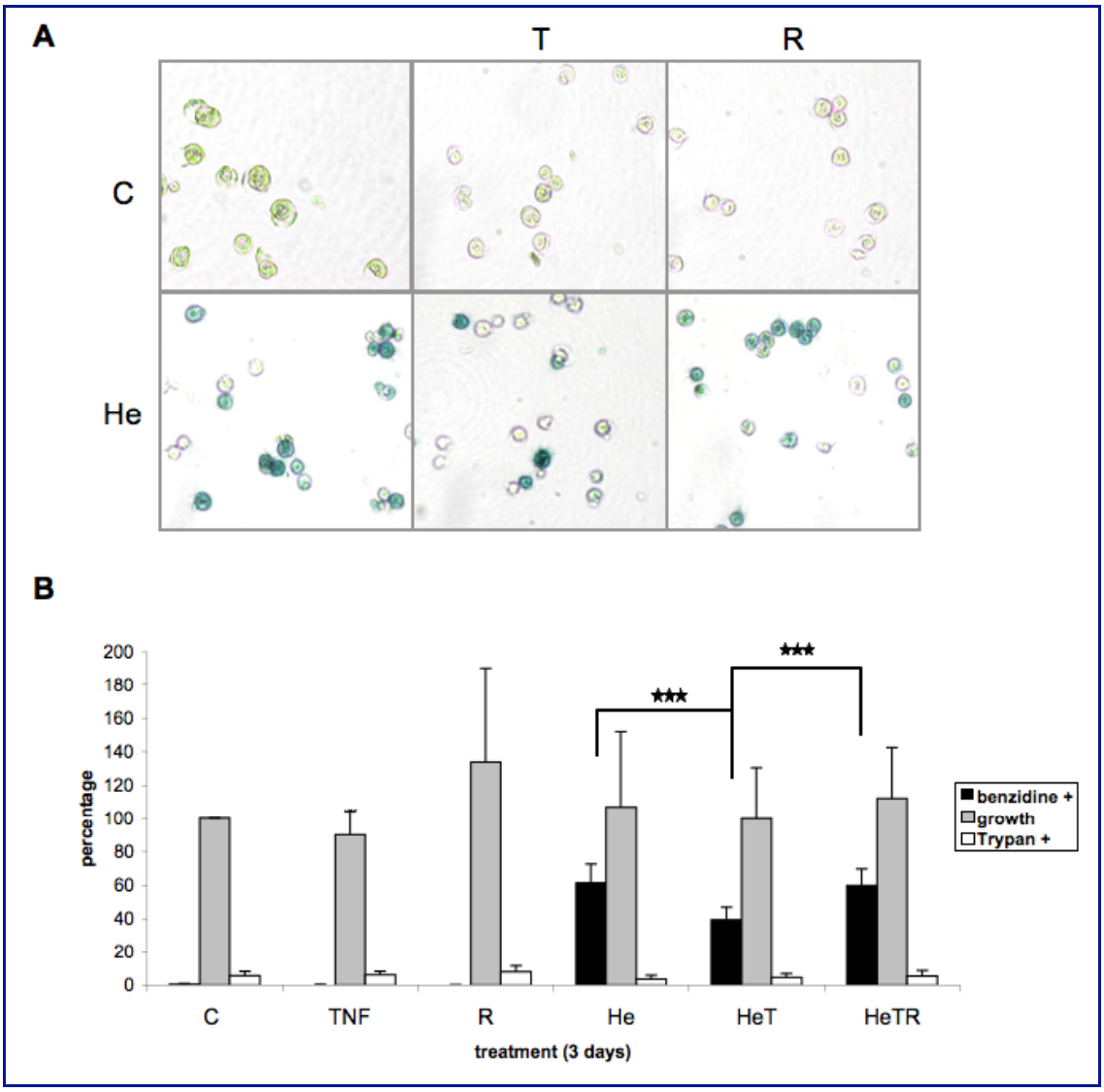


Figure 8: Effect of TNFα on hemoglobin synthesis in HEL cells. *Cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 µM He. A) Hemoglobinized cells were stained on day 3 using the benzidine method. B) Percentages of benzidine + cells, cell growth and Trypan + cells. Untreated cells were used as control (c). Values are reported as the means (+/- standard deviation, SD) of 7 independent experiments. (***P ≤ .001)*

Effect of TNFα in TF-1 cells

- Hemoglobin synthesis

Benzidine staining of control TF-1 cells revealed no benzidine positive cells (Fig. 9). After treating the cells with 10 U/mL Epo, benzidine staining revealed hemoglobinized green-blue colored cells, and TNFα had a visible inhibitory effect on this differentiation. The addition of Remicade in combination with TNFα rescued the inhibitory effect of the cytokine (Fig. 9A).

Quantifications of benzidine positive cells showed up to 44% of hemoglobin producing cells in the presence of Epo alone and only 24% after the pre-treatment with TNFα providing evidence that erythroid differentiation was significantly inhibited during TNFα treatment. Moreover, the TNFα inhibitor Remicade (R) (100 μg/mL) significantly reduced the inhibitory effect of TNFα, resulting in a final hemoglobinization rate of 35% (Figure 9B).

- cell growth

TNFα did not show any effect on the viability of Epo-induced TF-1 cells (Fig. 9B).

- viability

To analyze the toxicity of the different treatments, the Trypan viability assay was realized after each treatment. Trypan mean values did not exceed 3% of mortality rate after the different treatments (Fig. 9B).

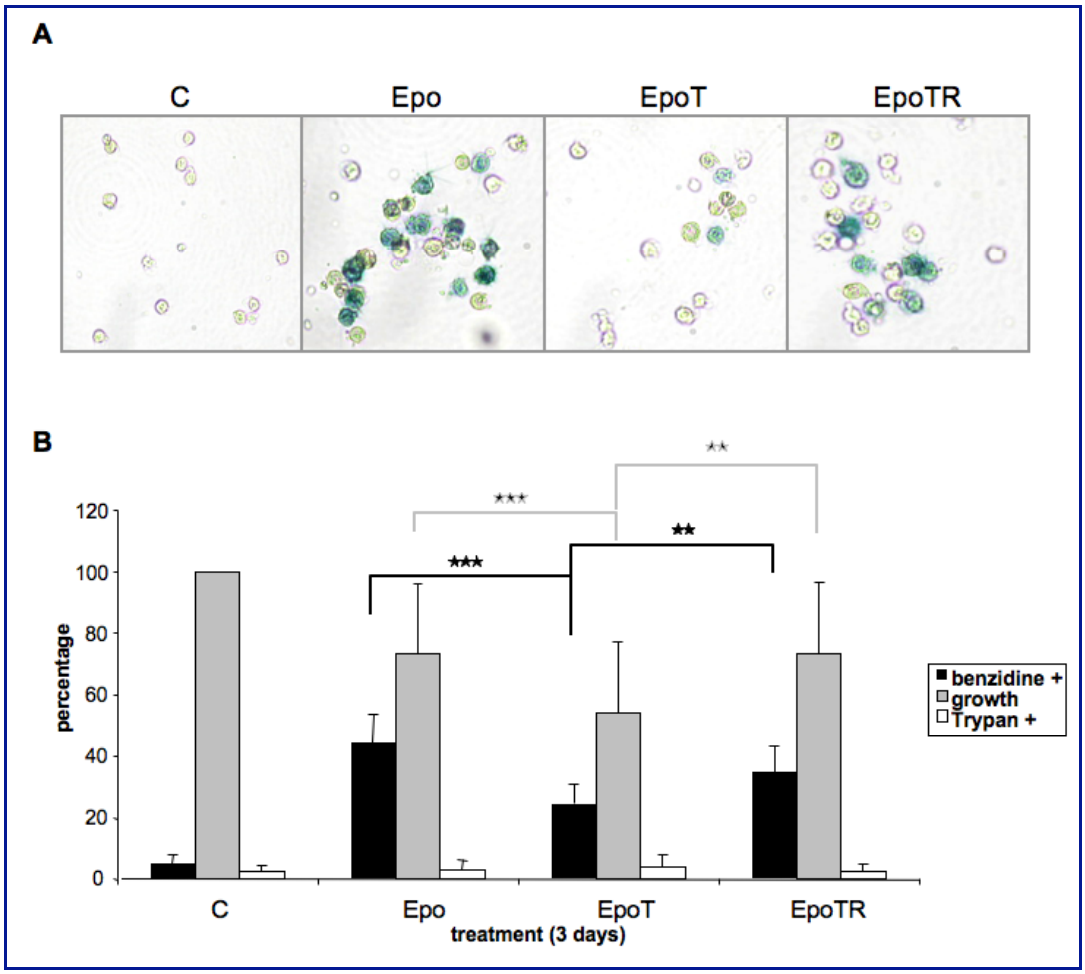


Figure 9: Effect of TNFα on hemoglobin synthesis in TF-1 cells. *Cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. A) Hemoglobinized cells were stained on day 3 using the benzidine method. B) Percentages of benzidine + cells, cell growth and Trypan + cells. Untreated cells were used as control (c). Values are reported as the means (+/- standard deviation, SD) of 5 independent experiments. (**P ≤ .01, ***P ≤ .001)*

K562, HEL, and TF-1 cells were able to differentiate towards the erythroid pathway using He, Acla, Dox, or Epo depending on the cell line used. TNFα decreased the hemoglobinization rate independently of the inducer used. TNFα had an inhibitory effect on both chemical as well as Epo-induced erythroid differentiation. The different erythroid inducers did not affect cell viability and cell growth varied depending on the substances used.

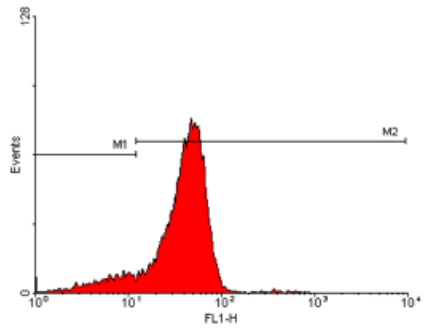
5.1.2 Effect of TNFα on hemoglobin synthesis in CD34+ hematopoietic progenitor cells

5.1.2.1 CD34+ purity

In order to generalize the previous findings of the inhibitory effect of TNFα on the three cell lines, we wanted to consolidate these results by using healthy human early progenitors, which can be differentiated along the erythroid differentiation pathway by Epo.

In order to handle CD34+ cells gained from cord blood, we performed magnetic cell labeling and cell sorting. In order to assess CD34+ purity we performed flow cytometry analysis. The purity of the eluted CD34+ cells showed that an average purity of 92% varying between 89% and 96% (Fig. 10).

A



B

	initial volume (umbilical cord blood)	cell number	CD34+ purity
1	15 ml	425 000	89 %
2	32 ml	304 000	90 %
3	32 ml	360 000	96 %
4	43 ml	306 000	94 %

Figure 10: Purity assessment of CD34+ cell fraction after CD34+ magnetic cell sorting*. A) Histogram of CD34+ cells after magnetic cell sorting by flow cytometry. The CD34+ cell fraction M2 is 89% pure. One representative result of 4 independent experiments. B) Initial volume, final CD34+ cell number and cell purity of four umbilical cord blood samples.*

5.1.2.2 CD34+ differentiation

In order to differentiate CD34+ cells gained from cord blood, we supplemented our cells with a cocktail of cytokines as mentioned in Material and Methods. 12 different cord blood cultures were processed and benzidine staining was realized at different time points (Fig. 11). Quantifications of these benzidine results showed an increase in hemoglobinized cells in a time dependent matter.

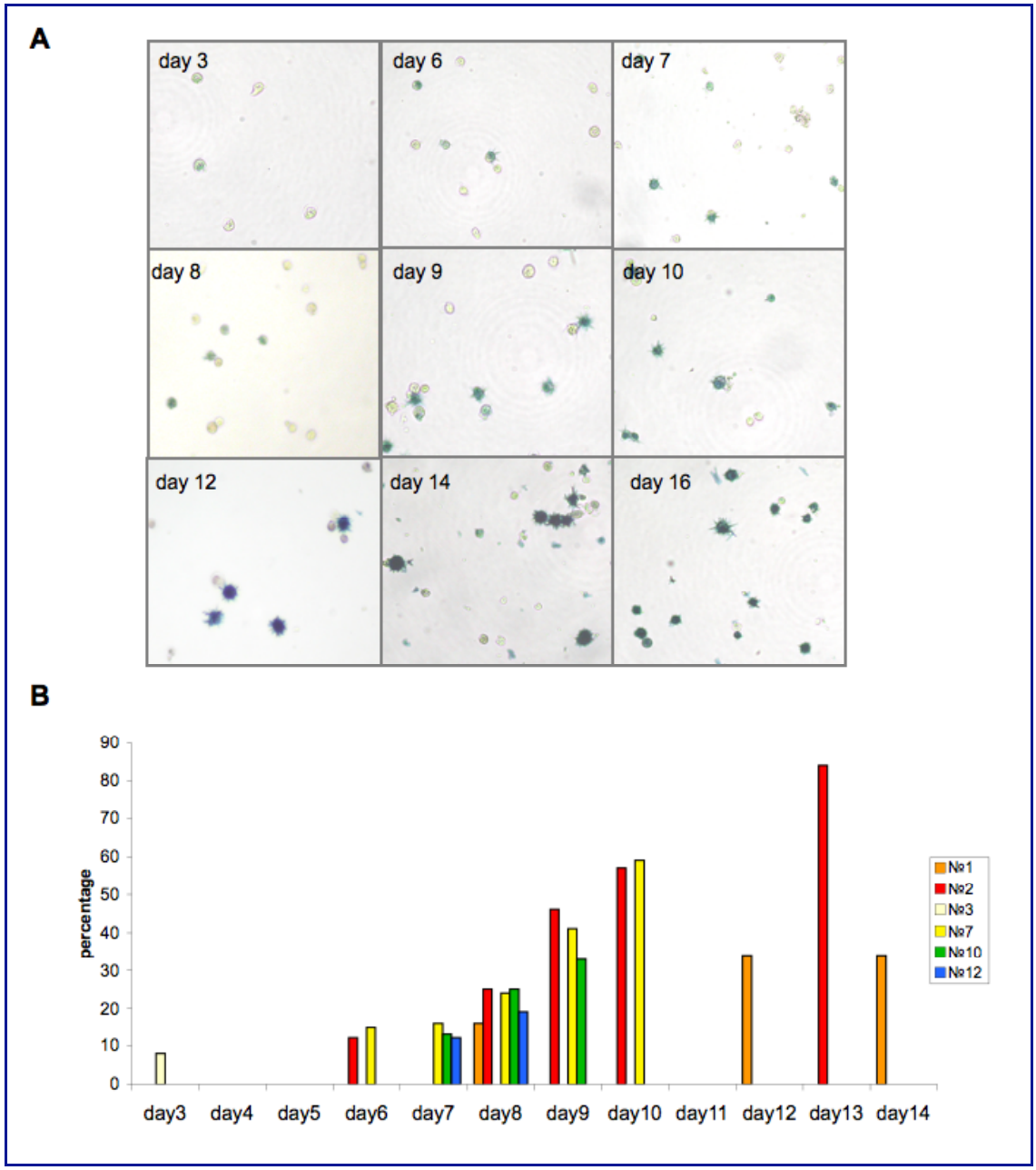


Figure 11: Benzidine staining of CD34+ cells treated with a cocktail of cytokines. *(days 0–3, 10 ng/mL IL-3 and 10 ng/mL SCF; days 3–7, 10 ng/mL IL-3, 10 ng/mL SCF, and 2 U/mL EPO; days 7–9, 10 ng/mL SCF and 2 U/mL EPO; and after day 9, 2 U/mL EPO days 0 to 3, 10 with 2 U/mL Epo). A) Hemoglobinized cells were stained on indicated days using the benzidine method. B) Percentages of benzidine + cells of distinct cord blood cultures on indicated days.*

5.1.2.3 Effect of TNFα on Epo-differentiated CD34+ cells

Treating CD34+ cells for 7 or 10 days with Epo alone or in combination with TNFα showed differences in the number of benzidine + cells over the time (Fig. 12). Thus

TNFα had a similar inhibitory effect on human hematopoietic CD34+ progenitor cells than in the three cell lines. This inhibitory effect was even enhanced over time (Fig. 12).

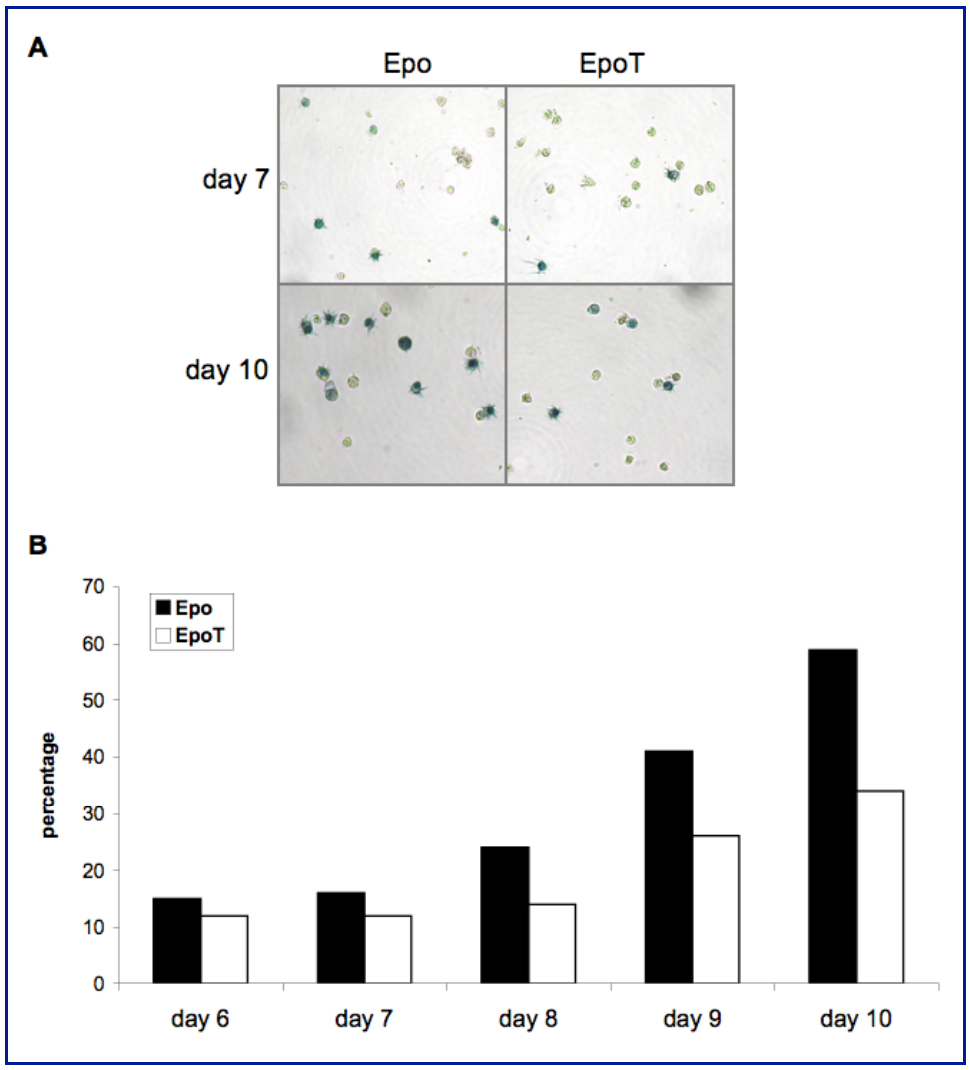


Figure 12: Benzidine staining of CD34+ cells treated or not with 20 ng/mL TNFα and a cocktail of cytokines *(days 0–3, 10 ng/mL IL-3 and 10 ng/mL SCF; days 3–7, 10 ng/mL IL-3, 10 ng/mL SCF, and 2 U/mL EPO; days 7–9, 10 ng/mL SCF and 2 U/mL EPO) A) Hemoglobinized cells were stained on day 7 and day 10 using the benzidine method. B) Percentages of benzidine + cells of distinct cord blood cultures on day 7 and day 10. (One representive result of 3 independent experiments)*

A CD34+ cell purity of 89-96% was gained from umbilical cord blood. The effect of the cytokine TNFα revealed a first insight of the inhibitory effect of this cytokine on these differentiating CD34+ human progenitor cells.

5.2 Effect of TNFα on NF-κB induction

In order to ascertain that TNFα is functional in our experimental conditions, EMSA assays were performed using 10 μg of nuclear extracts and a labeled NF-κB consensus probe. Indeed, TNFα is a well-known inducer of the canonical NF-κB pathway leading to the activation and translocation of NF-κB (p50/p65) dimer into the nucleus. Moreover, induction of NF-κB binding activity has been shown to be involved in the repression of globin gene expression [196].

Effect of TNFα on NF-κB induction in K562 cells

Results show an increased NF-κB (p50/p65) binding activity after treating K562 cells with TNFα. Using the erythroid differentiation inducers alone, no stimulation of the NF-κB pathway was observed (Fig. 13), whereas pretreating the K562 cells with TNFα induces the binding of the p50/p65 dimer as shown by immunodepletion assays. The use of Remicade abrogated the TNFα-induced NF-κB binding activity independently of the inducer used (Fig. 13).

Effect of TNFα on NF-κB induction in HEL cells

Analysis of the NF-κB binding capacity in HEL cells shows an increase of the binding capacity after treating the cells with TNFα alone, or in combination with He, which is decreased after simultaneous addition of Remicade (Fig. 13).

Effect of TNFα on NF-κB induction in TF-1 cells

Analysis of the NF-κB binding capacity in TF-1 cells showed an increase of the binding capacity after treating the cells with TNFα in combination with Epo, which is decreased after simultaneous addition of Remicade (Fig. 13).

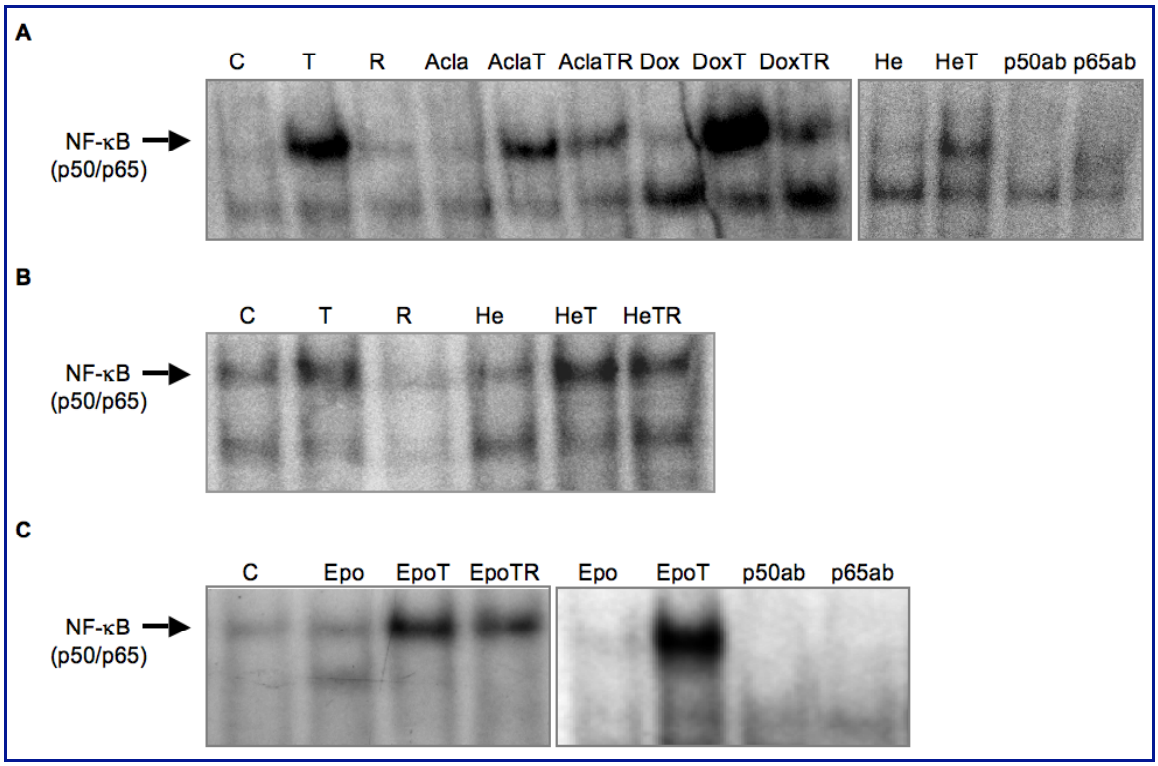


Figure 13: Effect of TNFα and Remicade on NF-κB DNA binding activity analyzed by EMSA. *EMSA assay was performed on 10 µg nuclear extracts using NF-κB consensus (NF-κBc)-^{32}P-labeled probe and the use of p50 and p65 antibodies (ab) revealed a p50/p65-DNA complex after K562 cells treated with TNFα and Hemin. Nuclear extracts were prepared from K562 (A), HEL (B), and TF-1 (C) cells treated with 10 nM Acla, 40 nM Dox, 30 µM He, or 10 U/mL Epo for 3 days in the presence or absence of 20 ng/mL TNFα (T) and/or 100 µg/mL of Remicade (R). Untreated cells were used as controls (c). (One representative result of 3 independent experiments)*

In our experiments, TNFα activation of NF-κB was efficient and concomitant to inhibition of differentiation in the three cell lines, independently of the inducer used. The use of Remicade reversed this effect.

5.3 Effect of TNFα on major erythroid transcription factors

5.3.1 Effect of TNFα on mRNA expression

We then investigated the effect of TNFα on erythroid transcription factors. First we investigated the effect of the cytokine on the major hematopoietic transcription factor GATA-1. Moreover, other important erythroid transcription factors such as NF-E2 or EKLF were also investigated in this study as they were shown to act downstream or together with GATA-1 [7,26,197], and to be also required for specific gene expression such as globin or HMBS [42,43,198-200]. Indeed, GATA-1 is known to be regulated by a number of

coregulators acting as coactivator (FOG-1) or as corepressor (PU.1) [201]. Thus FOG-1 was shown to act as a cofactor of GATA-1 in erythroid differentiation [35], since mice lacking FOG-1 die during mid-embryonic development with severe anemia [202]. Moreover, it is known that GATA-1 and GATA-2 expression are inversely expressed during erythropoiesis. Indeed, for terminal differentiation, GATA-2 has to be suppressed [203].

In order to evaluate whether TNFα affects mRNA level of transcription factors known to play a role in erythropoiesis, we analyzed GATA-1 as well as other key erythroid transcription factor mRNA expression after TNFα treatment. mRNA levels were analyzed by semi-quantitative RT-PCR, or qRT-PCR.

Effect of TNFα on mRNA expression in K562 cells

After quantifications of qRT-PCR analysis of 5 independent experiments, we observed a significant 1.7 fold increase of constitutive GATA-2 mRNA after TNFα treatment and a simultaneous decrease in GATA-1 and EKLF mRNA expression (Fig.14A). Similarly, in Acla-induced K562 cells, a significant increase in GATA-2 and a parallel significant decrease in GATA-1, FOG-1, NF-E2 and EKLF mRNAs' expression was observed after TNFα treatment in Acla-treated cells (Fig. 14B).

Effect of TNFα on mRNA expression in HEL cells

In HEL cells, RT-PCR analysis showed a decrease of the constitutive amount of GATA-1 mRNA in the cells (0.69 fold) after TNFα treatment. GATA-1 mRNA expression was reduced after TNFα addition in He-treated cells (Fig. 14C).

Effect of TNFα on mRNA expression in TF-1 cells

In TF-1 cells, TNFα addition resulted in a decrease of GATA-1 and NF-E2 and a parallel increase in GATA-2 mRNA expression when compared to Epo-treated cells alone (Fig. 14D).

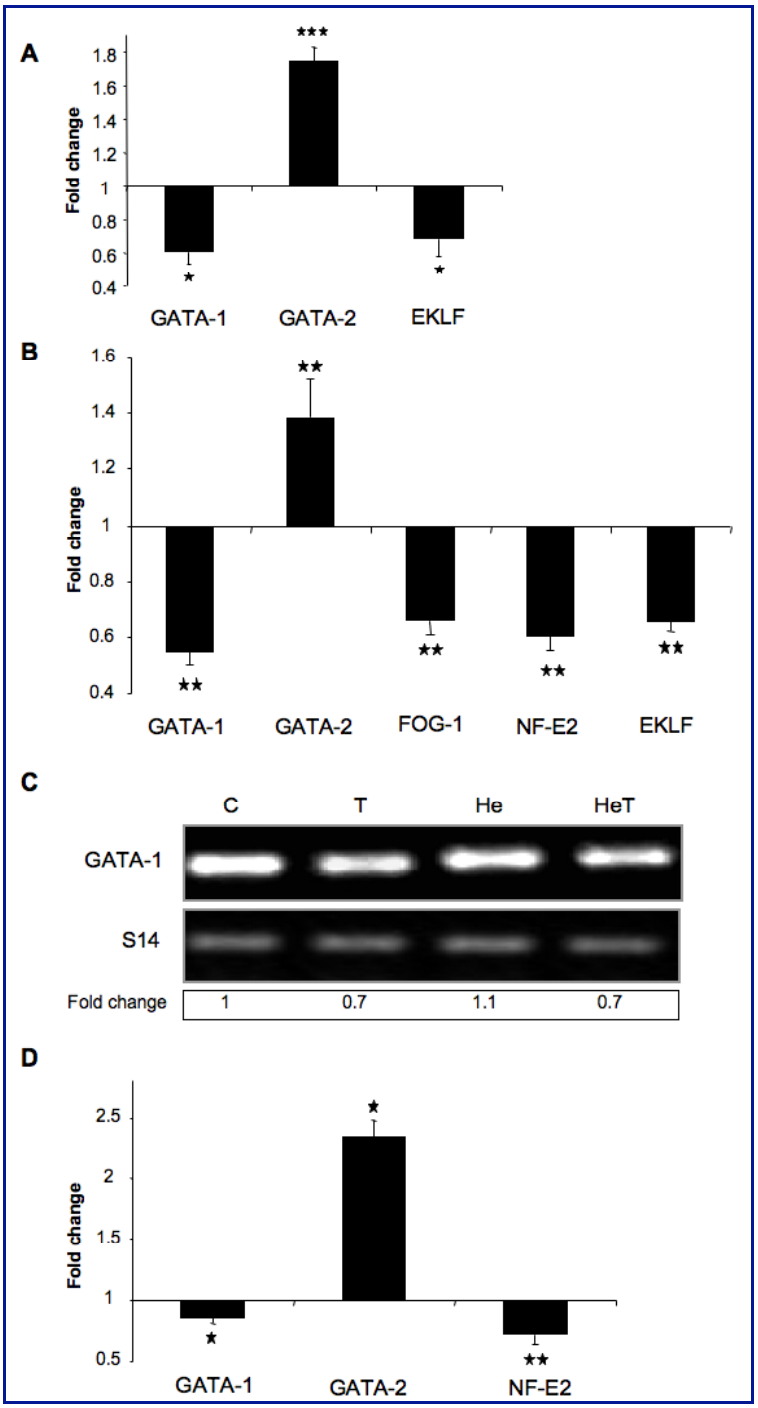


Figure 14: Effect of TNFα on erythroid transcription factor mRNA expression. *A) qRT-PCR analysis expressed in fold change of erythroid transcription factor mRNA expression after TNFα treatment of uninduced K562 cells compared to control cells. B) qRT-PCR analysis expressed in fold change of erythroid transcription factor mRNA expression after TNFα treatment of Acla-induced K562 cells compared to Acla treatment alone. C) RT-PCR of total RNA was prepared from treated (30 µM He) or untreated HEL cells in the presence or absence of 20 ng/mL TNFα (T). (One representative result of 3 independent experiments) D) qRT-PCR analysis expressed in fold change of erythroid transcription factor mRNA expression after TNFα treatment of Epo-induced TF-1 cells compared to Epo treatment alone. The relative amounts of mRNA were normalized to the housekeeping gene MRPS14 and for qRT-PCR results; data are means +/- SD of 5 independent experiments. (*P ≤ .05, **P ≤ .01, ***P ≤ .001)*

5.3.2 Effect of TNFα on protein analysis

5.3.2.1 Effect of TNFα on GATA-1 protein expression

We then performed GATA-1 western blot analysis on nuclear extracts from TNFα-treated constitutive or induced cells.

Effect of TNFα on GATA-1 protein expression in K562 cells

We investigated the effect of TNFα on the constitutive and induced GATA-1 protein expression after 3-day treatment. Compared to control, a 2.4 fold induction was observed after 3 days of Acla treatment, which is in turn decreased after addition of TNFα (1.2 fold induction) (Fig. 15A). Treating K562 cells with Dox and TNFα sowed a reduction when compared to Dox treatment alone. The results of He treatments showed a decrease of GATA-1 protein expression, when treated in combination with TNFα. Cotreatment with Remicade abrogated the reductive effect of TNFα on Acla- or Dox-induced GATA-1 protein expression (Fig. 15A), suggesting that the inhibiting effect of TNFα on hemoglobinization occurs via GATA-1 transcription factor activity. Remicade abrogated the inhibitory effect of TNFα on GATA-1 protein expression.

Effect of TNFα on GATA-1 protein expression in HEL cells

In HEL cells, TNFα treatment resulted in a decrease of GATA-1 protein compared to the control after TNFα treatment. In He-induced cells, we could observe an inhibitory effect of TNFα on GATA-1 protein expression. Remicade abrogated this effect in both non-treated and induced cells (Fig. 15B).

Effect of TNFα on GATA-1 protein expression in TF-1 cells

In TF-1 cells, analysis of GATA-1 protein expression using the C-20 ab revealed an increase of GATA-1 protein expression, when compared to control cells (GM-CSF). TNFα addition resulted in a reduction of the protein level, and the use of Remicade reversed this effect of the cytokine (Fig. 15C).

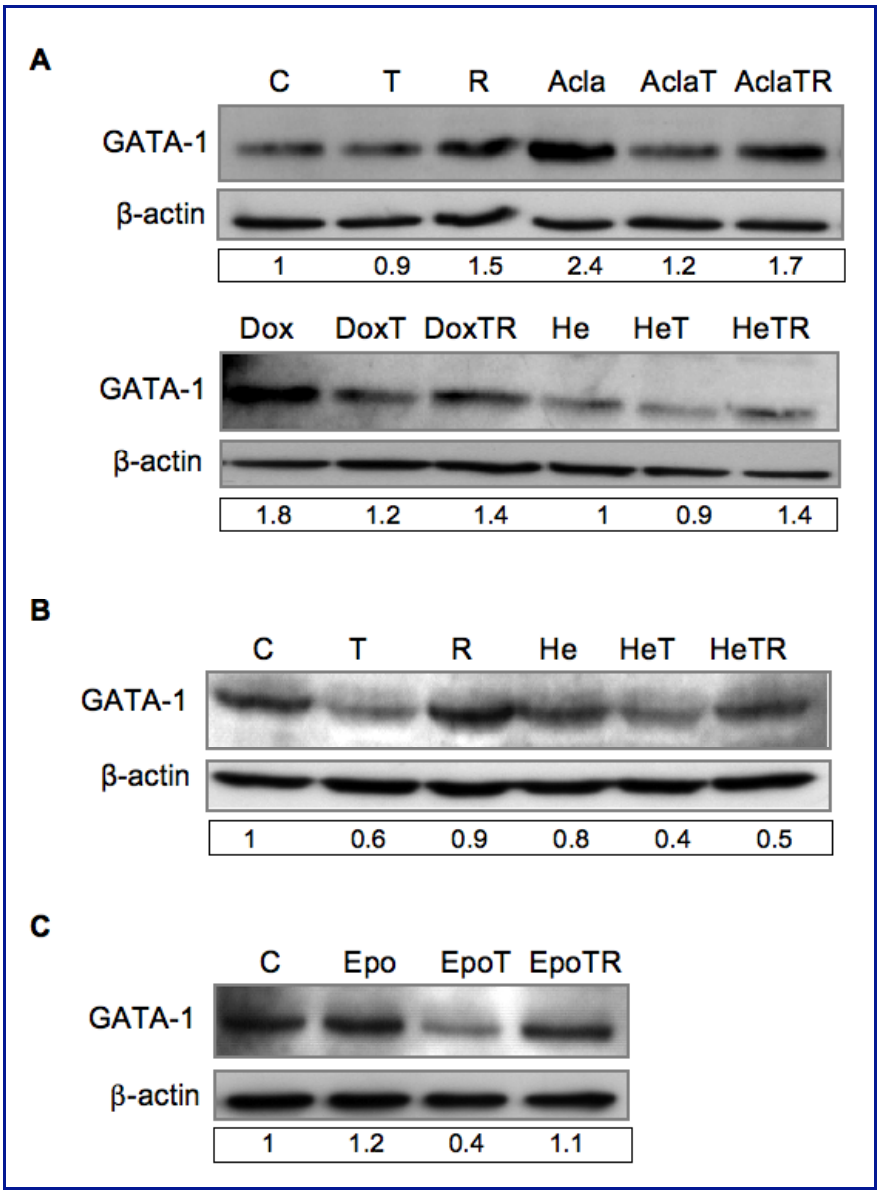


Figure 15: Western blot analysis of GATA-1 protein expression. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla, 40 nM Dox, or 30 µM He. B) HEL cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20ng/mL) prior to three days of differentiation with 30 µM He. C) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

TNFα had an inhibitory effect on GATA-1 mRNA as well as on induced GATA-1 protein expression in all three cell lines, both after chemical induction by Acla, Dox, or He and after Epo-induction. The anti-TNFα ab neutralized the inhibitory effect of TNFα.

5.3.2.2 Effect of TNFα on FOG-1 protein expression

FOG-1 encodes a protein of 995 amino acids containing multiple Zn fingers [28]. FOG-1-/- mice are dying early in embryonic development from severe anemia [202]. These mice show a block in erythroid development at a stage similar to that observed in GATA-1 deficient mice. This fact provides evidence that FOG-1 and GATA-1 function as cooperators in a coordinate way in erythroid differentiation [204]. In order to investigate the role of TNFα on the level of FOG-1 we analyzed this protein expression by western blot.

Effect of TNFα on FOG-1 protein expression in K562 cells

First western blot results showed an inhibitory effect of TNFα on both constitutive as well as induced FOG-1 protein expression. Results showed an increase after treating the cells for 3 days with Acla. This effect is reduced to after TNFα addition (Fig. 16A).

Effect of TNFα on FOG-1 protein expression in HEL cells

Results showed an increase of FOG-1 protein expression in He-induced HEL cells, which was reduced after TNFα addition (Fig. 16B).

Effect of TNFα on FOG-1 protein expression in TF-1 cells

In Epo-induced TF-1 cells we could observe an increase in FOG-1 protein expression compared to control. This induction was reduced after TNFα treatment and the use of Remicade reversed this inhibitory effect (Fig. 16C).

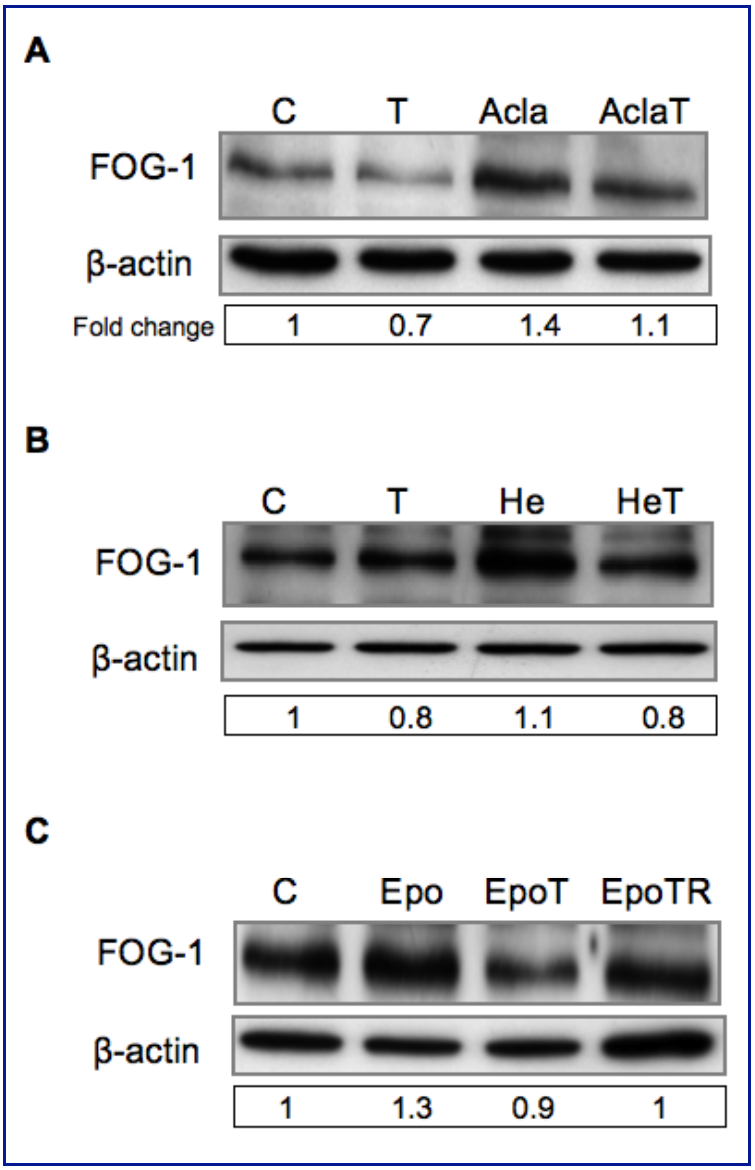


Figure 16: Western blot analysis of FOG-1 protein expression. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with or without Remicade (R) (100µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla. B) HEL cells were pretreated with or without TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 µM He. C) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior 3 days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

TNFα showed an inhibitory effect on FOG-1 protein expression in K562, HEL, and TF-1 cells independently of the inducer used.

5.3.2.3 Effect of TNFα on NF-E2 protein expression

After observing the inhibitory effect of TNFα on NF-E2 mRNA expression, we then analyzed the effect of TNFα on the protein expression of the erythroid transcription factor NF-E2.

Effect of TNFα on NF-E2 protein expression in K562 cells

By investigating the role of TNFα on NF-E2 protein expression, we saw an inhibitory effect of TNFα on constitutive NF-E2 protein expression in K562 cells (Fig. 17A).

Effect of TNFα on NF-E2 protein expression in HEL cells

Treating HEL cells for 3 days with TNFα resulted in a strong downregulation of the protein in untreated and He-induced cells (Fig. 17B).

Effect of TNFα on NF-E2 protein expression in TF-1 cells

In TF-1 cells NF-E2 protein expression analysis showed a downregulation of NF-E2 protein expression after TNFα treatment in Epo-stimulated TF-1 cells. Remicade reversed this inhibitory effect (Fig. 17C).

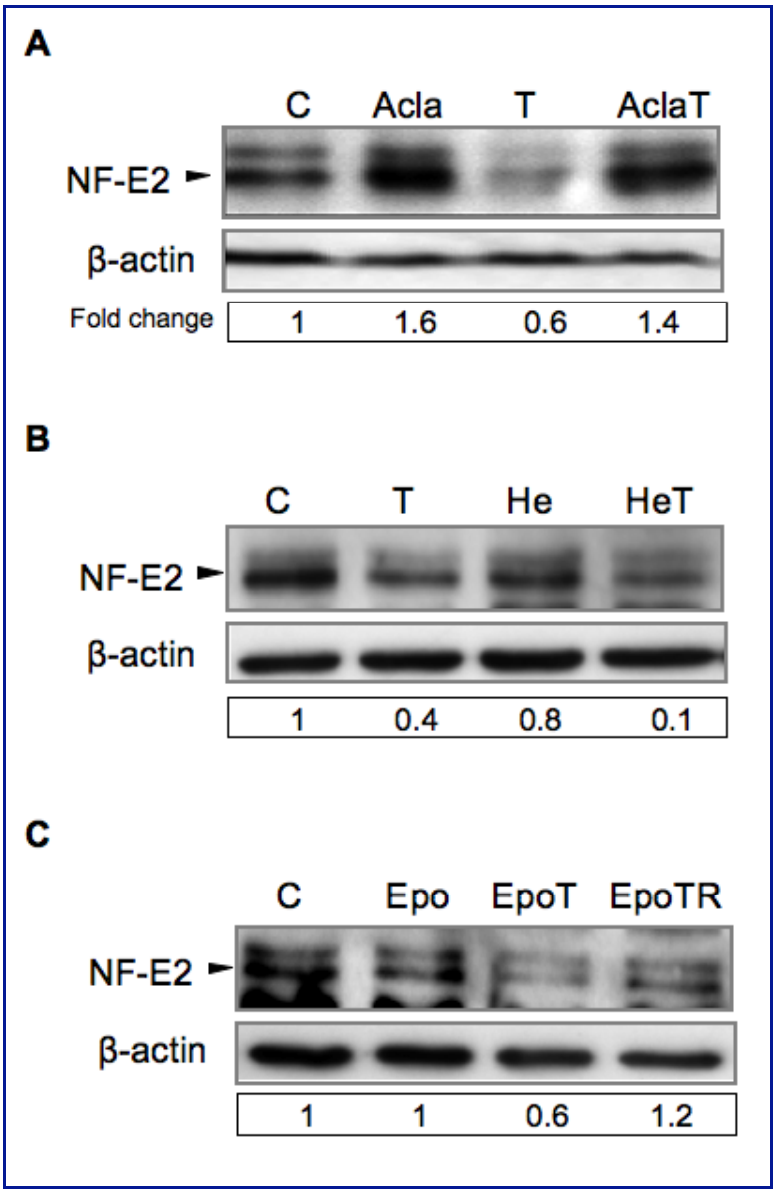


Figure 17: Western blot analysis of NF-E2 protein expression. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with or without 20 ng/mL TNFα (T) prior to three days of differentiation with 10 nM Acla. B) HEL cells were pretreated with or without 20 ng/mL TNFα (T) prior to three days of differentiation with 30 µM He. C) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior 3 days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

In all three cellular models, TNFα had an inhibitory effect on the erythroid transcription factor NF-E2.

5.3.2.4 Effect of TNFα on GATA-2 protein expression

We investigated the effect of TNFα on GATA-2 protein expression. Indeed GATA-2 is mainly expressed during early hematopoiesis, whereas GATA-1 has been shown to be responsible for terminal differentiation [203].

Effect of TNFα on GATA-2 protein expression in K562 cells

In order to investigate the effect of TNFα on GATA-2 protein expression, K562 cells were treated for three days with the different inducers in the presence or absence of TNFα. Results show an upregulation of GATA-2 protein expression after TNFα treatment independent of the inducer used (Fig. 18A). Moreover, TNFα showed an inducing effect on constitutive GATA-2 protein expression

Effect of TNFα on GATA-2 protein expression in HEL cells

In HEL cells were treated for 3 days with He in the presence or absence of TNFα. Results show an increase of GATA-2 protein expression compared to the control (Fig. 18B).

Effect of TNFα on GATA-2 protein expression in TF-1 cells

In TF-1 cells GATA-2 protein expression analysis showed the same expression pattern. A 1.7 fold increase in GATA-2 protein expression was already observed after 8 hours of TNFα treatment and was maintained for up to 72 hours in Epo-treated TF-1 cells. This inductive effect for GATA-2 was partially abrogated by the use of Remicade (R) after 3 days of treatment (Fig. 18C).

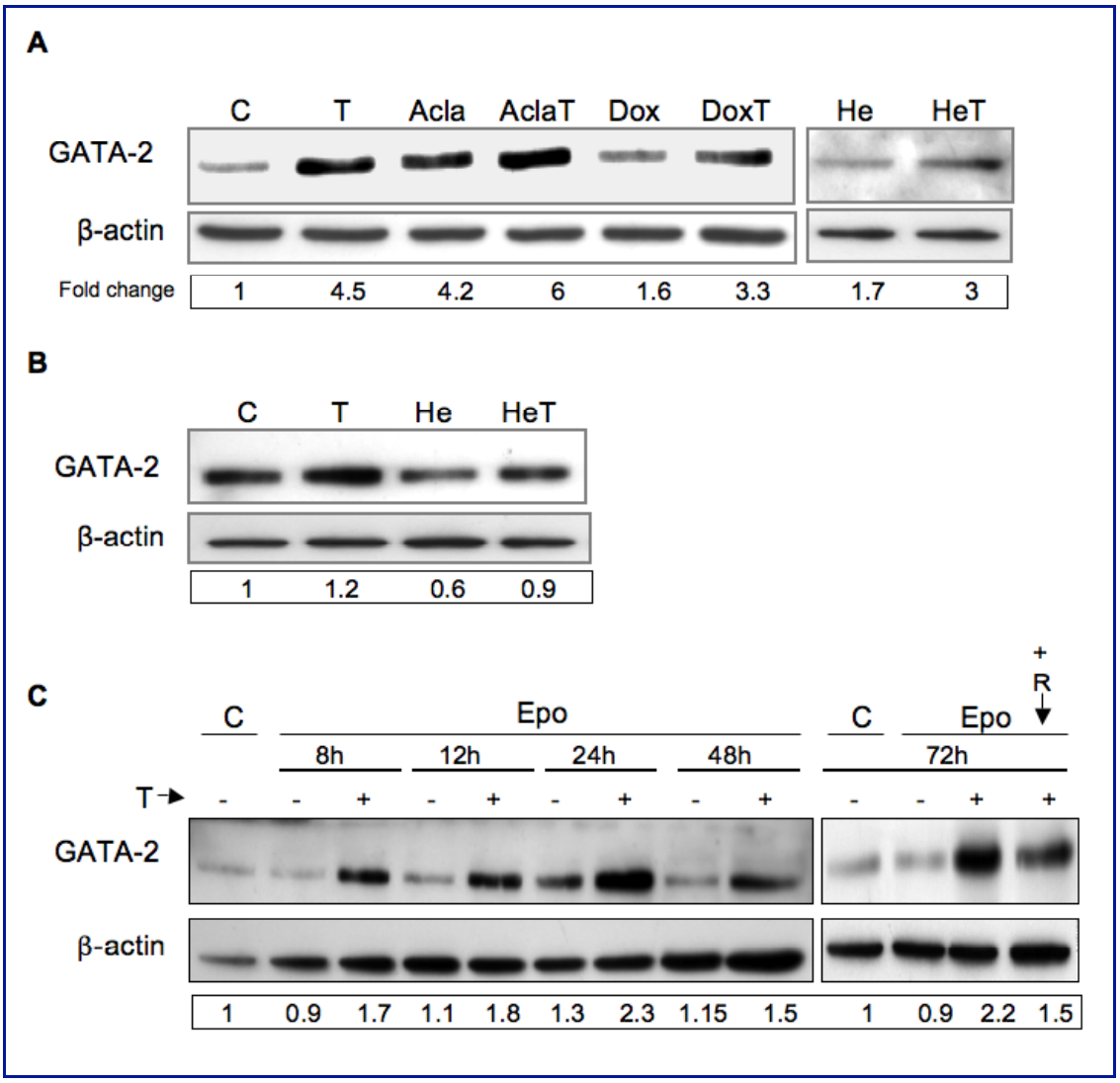


Figure 18: Western blot analysis of GATA-2 protein expression. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with or without 20 ng/mL TNFα (T) prior to three days of differentiation with 10 nM Acla, 40 nM Dox, or 30 µM He. B) HEL cells were pretreated with or without 20 ng/mL TNFα (T) prior to three days of differentiation with 30 µM He. C) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to 8, 12, 24, 48, or 72 hours of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

Independently of the nature of the cell line or the inducer used, TNFα reversed the physiological balance between GATA-1 and GATA-2 normally present during erythropoiesis.

5.3.2.5 Effect of TNFα on PU.1 protein expression

After analyzing the effect of TNFα on the transcription factor GATA-1, we investigated the effect of this cytokine on the transcription factor PU.1, which is described in the literature as a potent inhibitor of GATA-1. An antagonism between GATA-1 and PU.1 has already been described, which is important for determining lineage commitment during hematopoiesis (erythroid *versus* myeloid) [205]. qRT-PCR analysis did not show any significant variations after TNFα treatment. In order to investigate the role of TNFα on the level of PU.1 we analyzed PU.1 protein expression by western blot.

Effect of TNFα on PU.1 protein expression in K562 cells

TNFα as well as Remicade treatment resulted in a reduction of constitutive PU.1 protein expression in K562 cells. Compared to control, a reduction was observed after 3 days of Acla treatment, which is not changed after addition of TNFα (Fig. 16A). Results of He treatments showed a decrease of PU.1 protein expression, whenever treated in combination with TNFα (0.7 fold) or not (0.8 fold) (Fig. 19A).

Effect of TNFα on PU.1 protein expression in HEL cells

HEL cells were treated for 3 days with He in the presence or absence of TNFα and/or Remicade. Results show a similar increase of PU.1 protein expression compared to the control whatever treatment was realized (Fig. 19B).

Effect of TNFα on PU.1 protein expression in TF-1 cells

In TF-1 cells PU.1 protein expression analysis did not reveal any important variations compared to the controls (Fig. 19C).

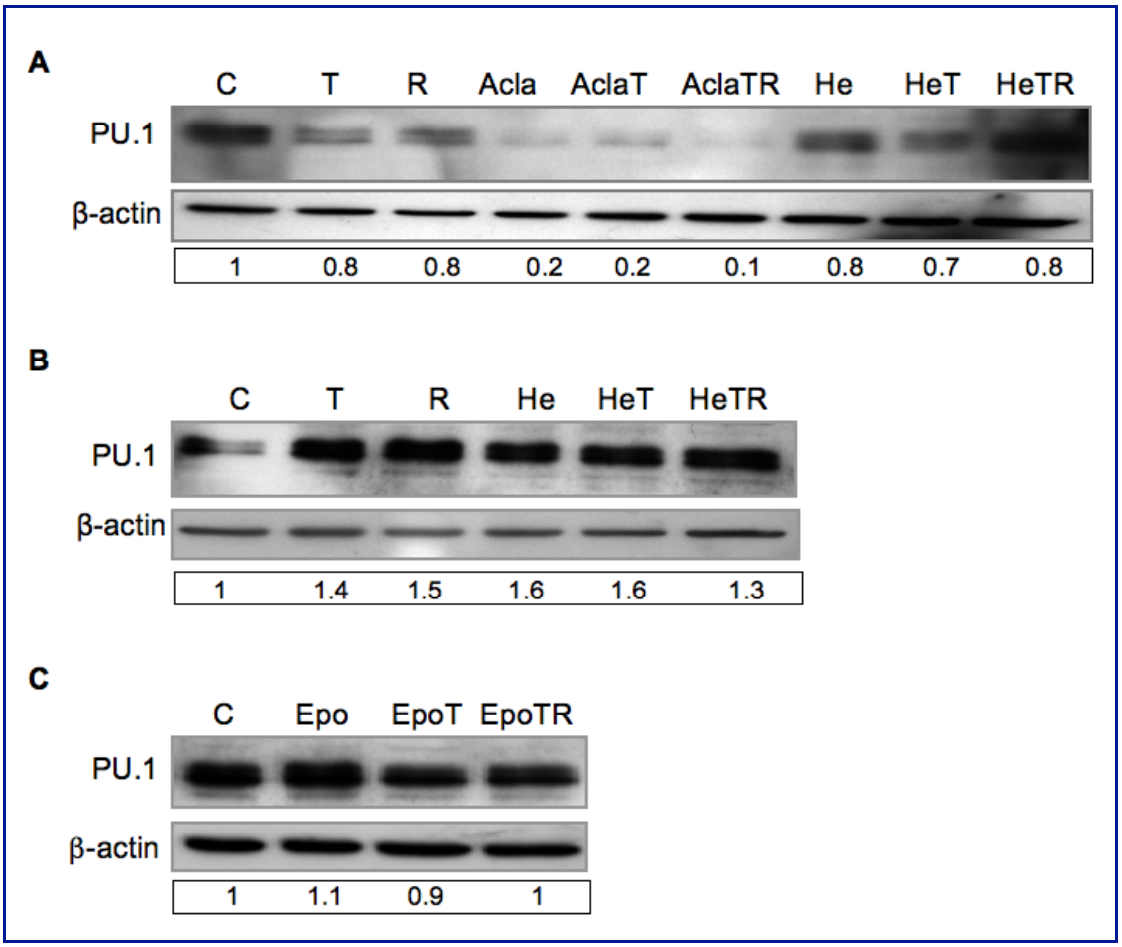


Figure 19: Western blot analysis of PU.1 protein expression. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with or without Remicade (R) (100µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla or 30 µM He. B) HEL cells were pretreated with or without Remicade (R) (100µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 µM He. C) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior 3 days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

No general conclusions can be made for PU.1, since the effect of TNFα on PU.1 protein expression varied with the inducer and the cell line used.

5.3.3 Effect of TNFα on GATA-1/FOG-1 interaction

To assess whether TNFα treatment could further affect the interaction between GATA-1 and FOG-1, besides both proteins downregulation, GATA-1 was immunoprecipitated using the C-20 GATA-1 ab. GATA-1 was revealed by western blot using the N1 GATA-1 ab.

As previously shown a decrease in GATA-1 protein expression was observed in cells treated with TNFα independently of the inducer used. Co-immunoprecipitated FOG-1 was present in induced HEL cells confirming its interaction with GATA-1 during erythroid cell fate decision (Fig. 20A). After TNFα treatment, immunoblots showed a complete abrogation of the constitutive GATA-1/FOG-1 complex in untreated HEL cells (Fig. 20A). Moreover, in He-induced HEL cells, we could observe a slight decrease of FOG-1 and GATA-1 proteins in the complex (Fig. 20A). Similarly TNFα decreased the amount of both interaction partners in Epo-induced TF-1 cells (Fig. 20B). The persistence of FOG-1 in the immunoprecipitation showed that GATA-1/FOG-1 interaction was not abrogated by TNFα in differentiated cells. The reduction of co-immunoprecipitated proteins was then rather due to a decrease in the amount of the GATA-1/FOG-1 complex in correlation with the cofactors downregulation than to a prevented physical interaction. However, this decrease in GATA-1/FOG-1 complex might lead to a less efficient transactivation of erythroid genes (Fig. 20A, B).

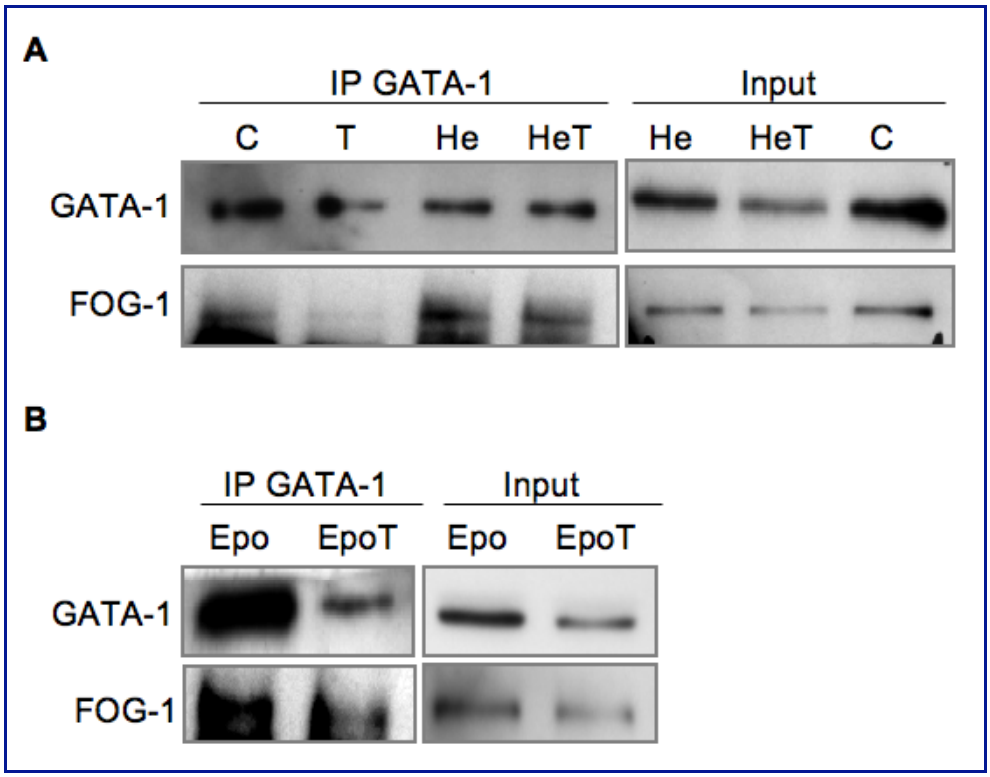


Figure 20: Immunoprecipitation analysis of GATA-1/FOG-1 protein interaction. *500µg total protein extracts were immunoprecipitated using the GATA-1 antibody C-20. 20 µg nuclear protein extracts were used for input. A) HEL cells were pretreated with or without TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 µM He. B) TF-1 cells were pretreated with or without TNFα (T) (20 ng/mL) prior 3 days of differentiation with 10 U/mL Epo.*

TNFα abrogates constitutive GATA-1/FOG-1 complex formation in untreated HEL cells, whereas its inhibiting effect in differentiated cells is correlated with the downregulation of GATA-1 and FOG-1 protein expression in HEL and TF-1 cells.

5.3.4 Effect of TNFα on posttranslational modifications of GATA-1

5.3.4.1 Effect of TNFα on GATA-1 and FOG-1 degradation

As we saw that TNFα had an inhibitory effect on the amount of GATA-1 and FOG-1, we investigated the possibility that the decrease in GATA-1 and FOG-1 expression resulted in protein degradation.

Effect of TNFα on GATA-1 degradation in K562 cells

Results showed that by using lactacystin, a proteasome inhibitor, we were able to abrogate the inhibitory effect of TNFα on both constitutive as well as induced GATA-1 protein expression in K562 and HEL cells (Fig. 21A).

Effect of TNFα on GATA-1 and FOG-1 degradation in HEL cells

Results showed that by using lactacystin, a proteasome inhibitor, we were able to abrogate the inhibitory effect of TNFα on both constitutive as well as induced GATA-1, and induced FOG-1 protein expression in HEL cells (Fig. 21B).

Effect of TNFα on GATA-1 and FOG-1 degradation in TF-1 cells

Results showed that lactacystin prevented the inhibiting effect of TNFα on FOG-1 but not on GATA-1. This allowed us to conclude that the reduction of FOG-1 expression in TF-1 resulted at least in part in its degradation by the proteasome in TNFα treated TF-1 cells (Fig. 21C).

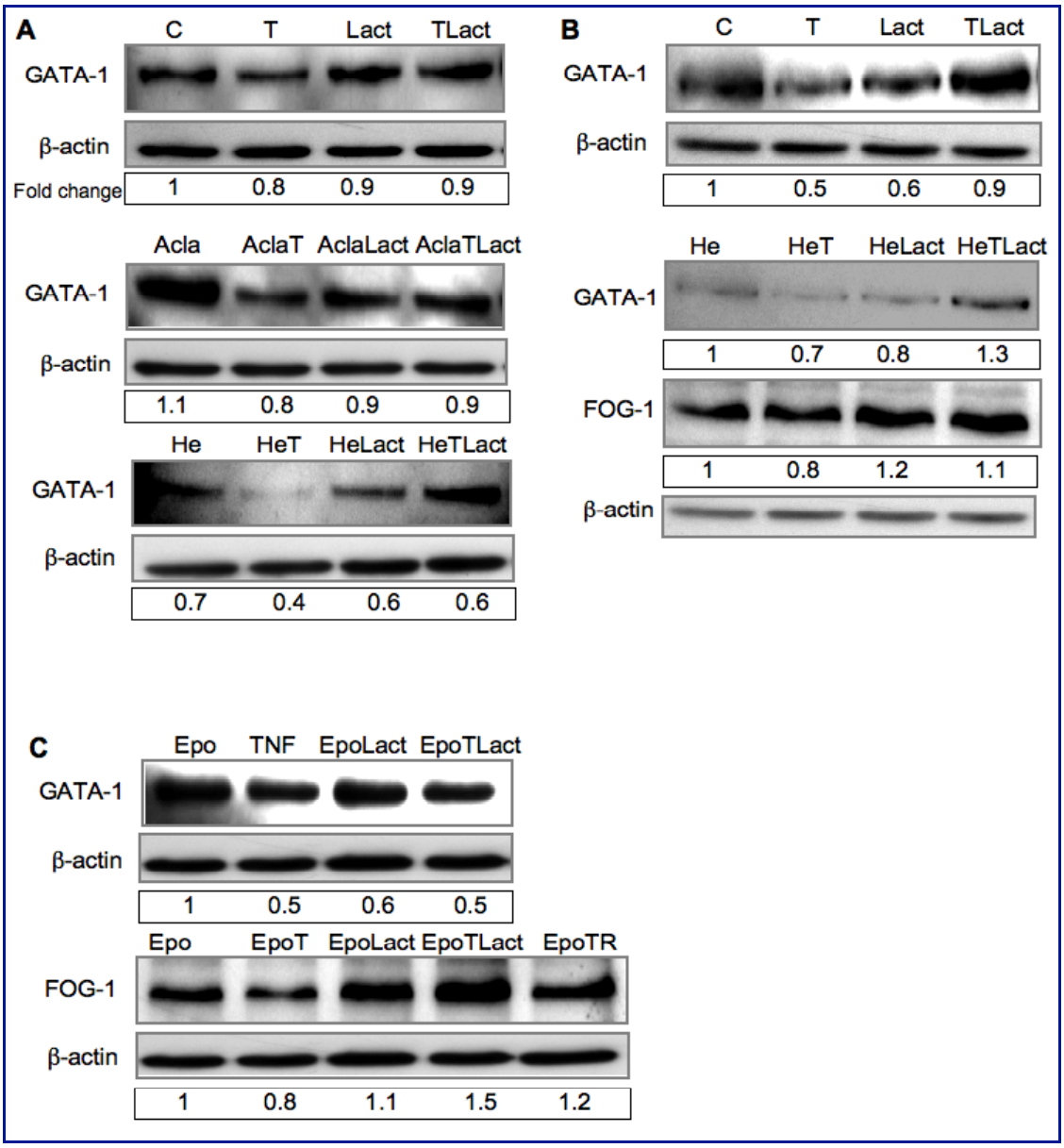


Figure 21: Western blot analysis of GATA-1 and FOG-1 protein degradation. *20 µg nuclear protein extracts were used. β-actin was used as internal control. A) K562 and B) HEL cells were pretreated with or without lactacystin (Lact) (1µM), and/or TNFα (T) (20 ng/mL) prior to one day of incubation with 10nM Acla, or 30µM He. C) TF-1 cells were pretreated with or without 1µM lactacystin (Lact), Remicade (R) (100µg/mL) and/or TNFα (T) (20 ng/mL) prior to one day of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

Analyzing the effect of an inhibitor of proteasomal degradation, lactacystin, on the inhibition of erythroid differentiation by TNFα, pointed to a proteasomal dependent degradation of GATA-1 and/or FOG-1 protein expression in the different cell lines.

5.3.4.2 Effect of TNFα on GATA-1 acetylation

Besides the above-discussed degradation of GATA-1, this transcription factor might underlie other posttranscriptional modifications such as phosphorylation, acetylation, and sumoylation. As acetylation was shown to clearly affect GATA-1 transcriptional activation, we then assessed its role after TNFα treatment. As acetylation of GATA-1 is described to be essential for binding to DNA *in vivo*, and thus activation of target genes, we analyzed the acetylation status of GATA-1 after TNFα treatment. After immunoprecipitating GATA-1, the use of an anti-acetylated antibody revealed a complete absence of acetylation after TNFα treatment in He-induced K562 cells, which would suggest a possible loss of GATA-1's ability to bind DNA after TNFα treatment (Fig. 22).

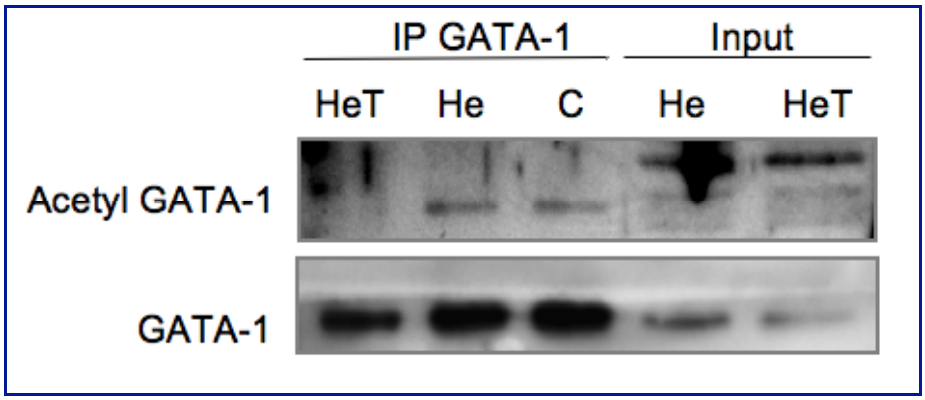


Figure 22: Immunoprecipitation analysis of acetylated GATA-1 protein. *500µg total protein extracts were immunoprecipitated using the GATA-1 antibody C-20. 20 µg nuclear protein extracts were used for input. K562 cells were with or without TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 µM He.*

TNFα showed an inhibitory effect on the acetylation status of GATA-1 protein expression in He-induced K562 cells. This result gives first hints about an effect of TNFα on GATA-1 binding capacity *in vivo*.

5.4 Effect of TNFα on GATA-1 transcriptional regulation mechanisms

5.4.1 Effect of TNFα on GATA-1 binding capacity

5.4.1.1 Effect of TNFα on GATA-1 binding capacity analyzed by EMSA assay

Since TNFα treatment was shown to inhibit erythroid transcription factor expression, we investigated its effect on the hematopoietic transcription factor GATA-1 binding activity. Ten micrograms of nuclear extracts were analyzed by EMSA using a GATA consensus probe as described in Materials and Methods.

Effect of TNFα on GATA-1 binding capacity in K562 cells by EMSA assay

Independent of the inducer of erythroid differentiation, EMSA experiments showed a decrease in GATA-1 binding activity after 3 days of TNFα treatment (Fig. 23A). Moreover, TNFα had an effect on constitutive binding in K562 cells. Acla induced an increase in GATA-1 binding activity, which in turn was diminished by TNFα. The GATA-1 forming complex in EMSA was identified by immunodepletion experiments using a GATA-1 ab. Remicade abrogated the inhibitory effect of the cytokine on induced GATA-1 binding capacity.

Effect of TNFα on GATA-1 binding capacity in HEL cells by EMSA assay

In HEL cells, the constitutive binding capacity of GATA-1 is decreased after TNFα addition. Using He as an erythroid inducer, we saw an increase of GATA-1 binding to its DNA sequence. This effect was slightly reduced after supplementing with TNFα (Fig. 20B). The use of Remicade abrogated the inhibitory effect of TNFα on constitutive GATA-1 binding activity (Fig. 23B).

Effect of TNFα on GATA-1 binding capacity in TF-1 cells by EMSA assay

Results obtained with TF-1 cells showed an important constitutive GATA-1 binding activity in Epo-treated cells. TNFα had an inhibitory effect on Epo-induced GATA-1 binding activity, which is abrogated after Remicade addition (Fig. 23C). The GATA-1 forming complex in EMSA was confirmed by immunodepletion experiments using a GATA-1 ab.

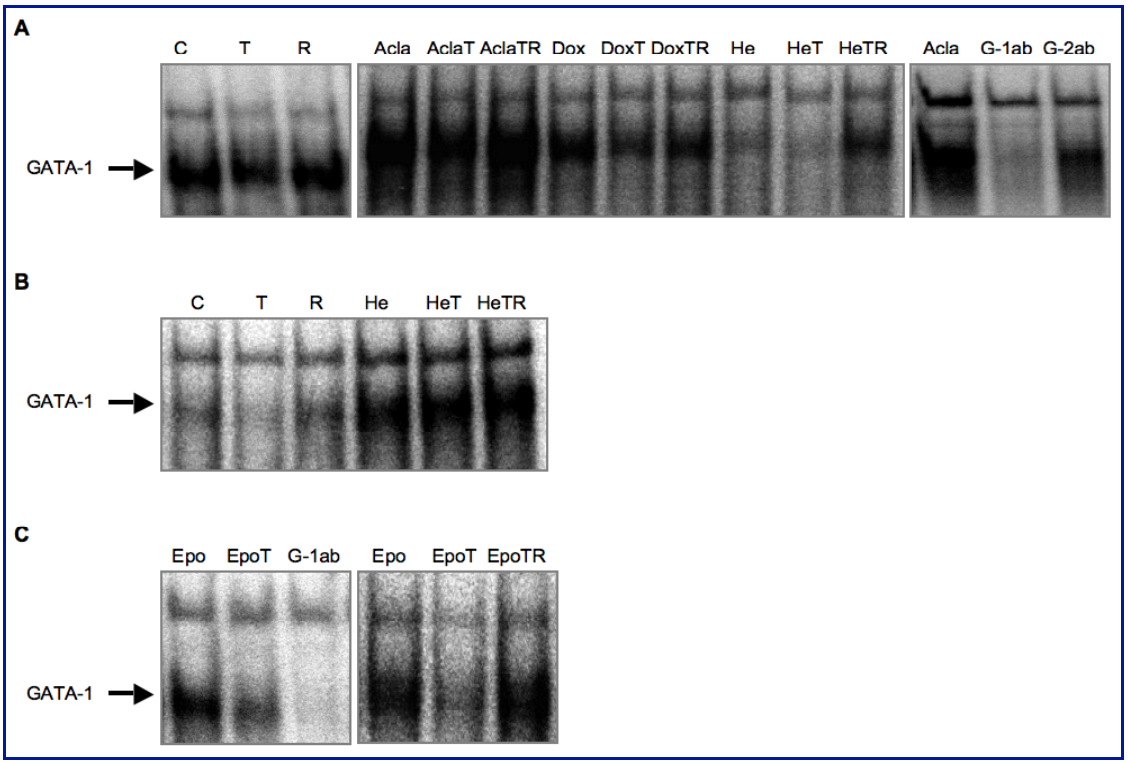


Figure 23: Effect of TNFα and Remicade on GATA-1 DNA binding activity analyzed by EMSA. *EMSA assay was performed on 10 µg nuclear extracts using GATA-1 consensus (GATA-1)-^{32}P-labeled probe and the use of GATA-1 and GATA-2 antibodies (G-1ab, G-2ab) revealed a GATA-1-DNA complex after TNFα treatment. Nuclear extracts were prepared from K562 (A), HEL (B), and TF-1 (C) cells treated with 10 nM Acla, 40 nM Dox, 30 µM He, or 10 U/mL Epo for 3 days in the presence or absence of 20 ng/mL TNFα (T) and/or 100 µg/mL of Remicade (R). (One representative result of 3 independent experiments)*

5.4.1.2 Effect of TNFα on GATA-1 binding capacity analyzed by TransAM assay

In order to confirm and quantify the effect of TNFα on GATA-1 binding activity, we performed the TransAM assay. Nuclear extracts from different cell lines (0.25 µg/µL) were loaded into each well, allowing GATA-1 to bind to its target sequence which has been immobilized in the 96-stripwell plate. The bound fraction was then quantified using a spectrocolorimetric assay (see materials and methods).

Effect of TNFα on GATA-1 binding capacity in K562 cells by TransAM assay

To investigate the effect of TNFα on constitutive GATA-1 binding, we treated K562 cells for three days with increasing concentrations of TNFα (20 ng/mL; 40 ng/mL; 60 ng/mL; 100 ng/mL). Results showed that binding activity decreased in a concentration dependent manner (Fig. 24A).

Effect of TNFα on GATA-1 binding capacity in TF-1 cells by TransAM assay

Then we analyzed the effect of TNFα on GATA-1 binding activity in TF-1 cells. GATA-1 binding activity was significantly downregulated after TNFα treatment in Epo-induced cells. Remicade abrogated the inhibitory effect of the cytokine (Fig. 24B).

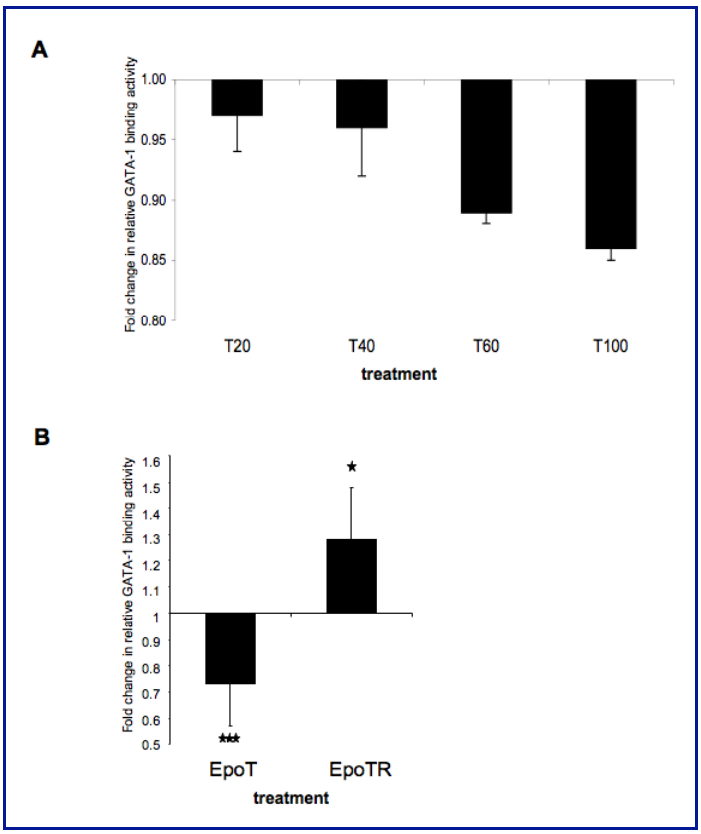


Figure 24: TransAM analysis of constitutive and induced GATA-1 binding to the GATA-1 trapper. *(A) 10 µg nuclear extracts were prepared from K562 treated for three days with 20, 40, 60, or 100 ng/mL TNFα (T). (B) 10 µg nuclear extracts were prepared from TF-1 cells treated for three days with 10 U/mL Epo in the presence or absence of 20 ng/mL TNFα (T) and/or 100 µg/mL Remicade (R). Quantification analyses of the TransAM results are expressed in fold changes of the average of 3-5 independent experiments. (*P ≤ .05, ***P ≤ .001)*

Binding activity of GATA-1 in TNFα-treated cells was reduced as shown by EMSA and TransAM experiments. When compared to western blot results, this result was not astonishing *per se*, suggesting that the decrease in GATA-1 binding activity is a result of its downregulation. Nevertheless the downregulation of GATA-1 affected the level of GATA-1 binding capacity suggesting an effect on transactivation of target genes.

5.4.2 Effect of TNFα on transactivation activity of GATA-1

As erythropoiesis is mainly regulated at a transcriptional level, we then assessed the effect of TNFα on transcriptional regulation. We performed transient transfection assays using a luciferase construct driven by three consensus GATA-1 sites (pGL3-GATA-Luc). Renilla luciferase reporter vector used were used as an internal control reporter.

Effect of TNFα on transactivation activity of GATA-1 in K562 cells

Results showed an inhibitory effect of TNFα alone on luciferase activity ass well as in combination with Acla. The use of Remicade partially abrogated the inhibitory effect of TNFα on transcriptional activation in both untreated and treated cells. Cells, which were co-transfected with a GATA-1 expression vector, showed an increase of GATA-1-driven reporter gene activity. After TNFα addition, this induction was blocked and thus gave hints about a direct effect of TNFα on GATA-1 transactivation (Fig. 25A).

Effect of TNFα on transactivation activity of GATA-1 in HEL cells

Whereas He induced an increase in luciferase expression compared to untreated HEL cells, TNFα pretreatment clearly abolished this induction (Fig. 25B). Moreover, TNFα had a similar effect on transcriptional activity in non-induced cells. Similar results were obtained after transient overexpression of GATA-1 by co-transfection of the pXM-GATA-1 construct (Fig. 25B). TNFα showed an inhibitory effect on GATA-1 transactivation.

Effect of TNFα on transactivation activity of GATA-1 in TF-1 cells

In TF-1 cells, nucleofection was performed. Results showed an inhibitory effect of TNFα on luciferase activity in Epo-treated cells. Cells, co-transfected with a GATA-1 expression vector, revealed an increase GATA-1-driven reporter gene activity. After TNFα addition, this induction is blocked and thus gives us clues about a direct effect of TNFα on GATA-1 transactivation (Fig. 25C).

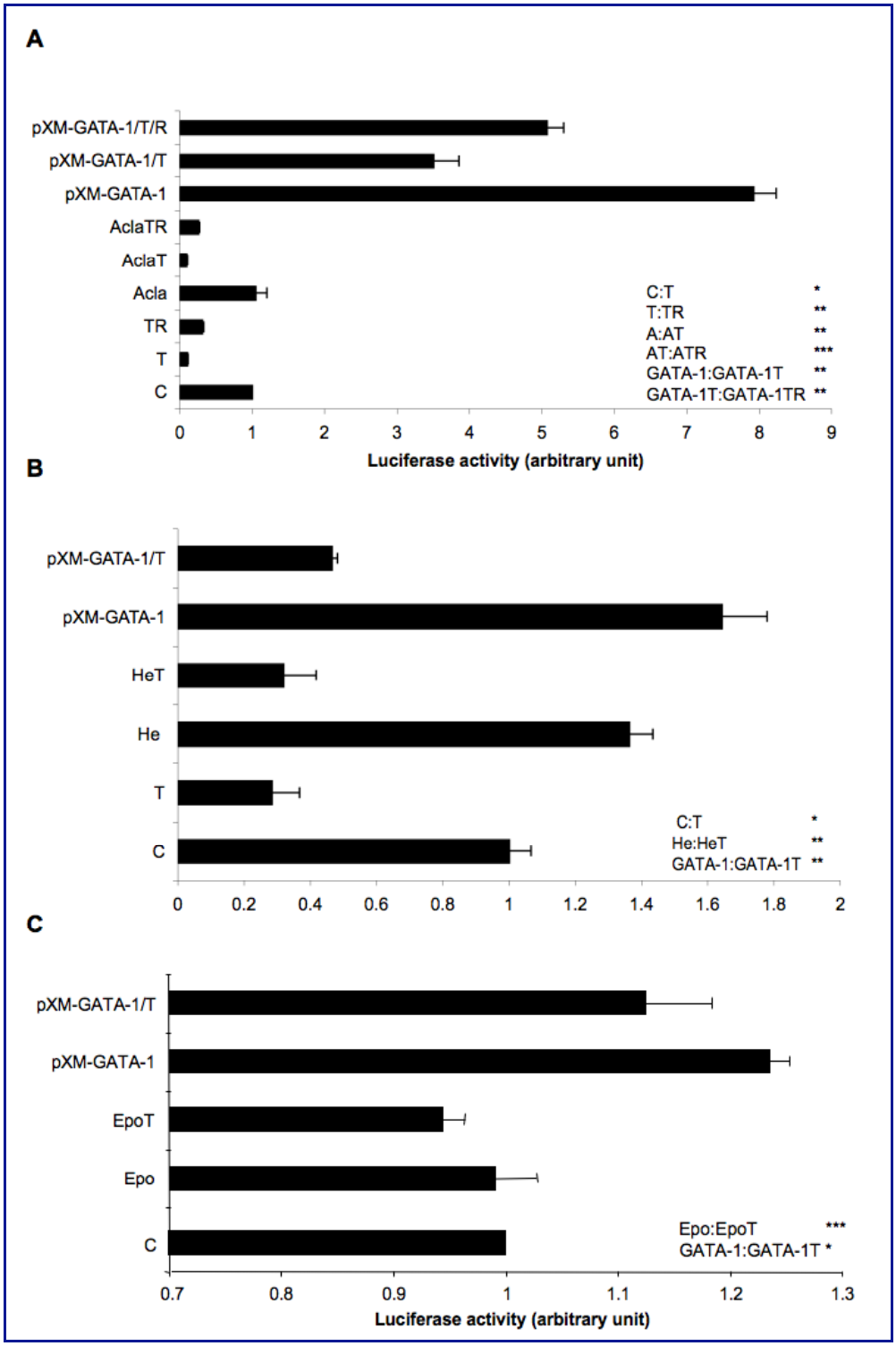


Figure 25: Inhibition of GATA-1 driven promoter activity by TNFα. *Standardized luciferase activity of cells transfected with pGL3-GATA-Luc or in combination with the expression vector pXM-GATA-1 in the presence or absence of 20 ng/mL TNFα (T) and 100 µg/mL Remicade (R) prior to 48 hours of treatment with 10 nM Acla in K562 cells (A), 30µM He in HEL cells (B), or 10 U/mL Epo in TF-1 cells (C). Renilla luciferase reporter vector phRL-SV-40 was used as an internal control reporter. Values are reported as the means of three independent experiments. (*P ≤ .05, **P ≤ .01, ***P ≤ .001)*

In agreement with the downregulation of GATA-1 expression and its decreased DNA binding activity, TNFα had a negative effect on the transactivation of luciferase reporter gene by a GATA transcription factor. The use of GATA-1 expression vector showed that TNFα inhibited the transactivation activity of GATA-1 in the three cell lines independently of the inducer used. This suggested that TNFα could affect target genes.

5.5 Effect of TNFα erythroid-specific marker gene expression

As erythroid specific genes are known target genes of the major erythroid transcription factor GATA-1 [206-209], and as we saw the inhibitory effect of TNFα on the transcriptional regulation of GATA-1, we investigated the effect of the cytokine on some erythroid markers. mRNA and protein expressions were analyzed by RT-PCR or qRT-PCR analysis and western blot.

5.5.1 Effect of TNFα on erythroid marker mRNA expression

Effect of TNFα on erythroid marker mRNA expression in K562 cells

In K562 cells, Acla induced a 4.6 fold increase in EpoR mRNA expression compared to the control value, which was decreased after addition of TNFα (Fig. 26A). The results of γ-globin mRNA analysis showed a 0.7 fold decrease after TNFα treatment when compared to Acla alone.

Effect of TNFα on erythroid marker mRNA expression in HEL cells

In HEL cells, TNFα decreased the constitutive amount of EpoR and γ-globin mRNA in uninduced cells. In He-induced HEL cells, a pretreatment with TNFα resulted in a decrease of EpoR and γ-globin mRNAs (Fig. 26B).

Effect of TNFα on erythroid marker mRNA expression in TF-1 cells

The effect of TNFα on erythroid specific genes expression was analyzed by performing qRT-PCR analysis using primers for distinct erythroid specific markers. Figure 26C shows that EpoR, α-globin, γ-globin, ERAF, HMBS, and GPA were significantly downregulated, while TFRC mRNA was significantly upregulated after cytokine treatment (Fig. 26C).

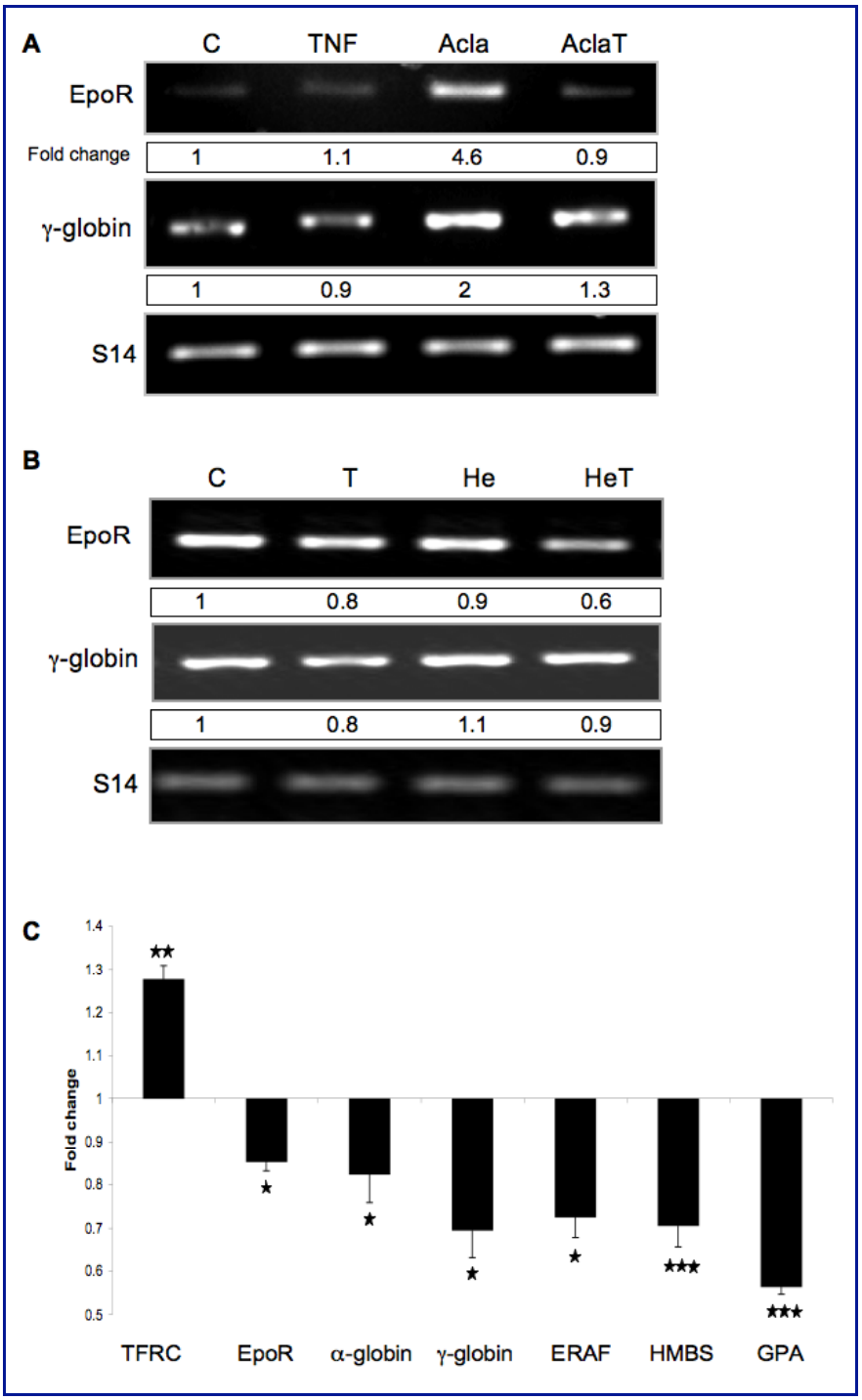


Figure 26: Effect of TNFα on erythroid marker mRNA expression. *A) RT-PCR of EpoR and γ-globin mRNA expression prepared from treated (10 nM Acla) or untreated K562 cells in the presence or absence of 20 ng/mL TNFα (T). (One representative result of 3 independent experiments) B) RT-PCR of EpoR and γ-globin mRNA expression prepared from treated (30 µM He) or untreated HEL cells in the presence or absence of 20 ng/mL TNFα (T). (One representative result of 3 independent experiments) C) qRT-PCR analysis expressed in fold change of erythroid marker mRNA expression after TNFα treatment of Epo-induced TF-1 cells compared to Epo treatment alone. Data are means +/- SD of 5 independent experiments. Significance was evaluated by Student t-test. (The relative amounts of mRNA were normalized to the housekeeping gene MRPS14) (*P ≤ .05, **P ≤ .01, ***P ≤ .001)*

5.5.2 Effect of TNFα on erythroid marker protein expression

In order to confirm mRNA results, we investigated the effect of TNFα on erythroid marker protein expression (Fig. 27-29). We analyzed the effect of TNFα and Remicade on γ-globin, EpoR and GPA protein expressions in different cell lines treated with distinct erythroid inducers.

5.5.2.1 Effect of TNFα on γ-globin protein expression

As it is known that K562 and HEL cells do not express β-globin in K562 and HEL cells, we analyzed γ-globin expression [150,210].

Effect of TNFα on γ-globin protein expression in K562 cells

We first analyzed its effect on constitutive γ-globin protein expression. K562 cells were treated for 2 to 12 hours with 20 ng/mL TNFα. Our findings showed a decrease of constitutive γ-globin protein expression after addition of TNFα in the first 6 hours. This effect is even enhanced after 8, 10, or 12 hours (Fig. 27A). Moreover, Acla caused an important increase in γ-globin protein, whereas after addition of TNFα, γ-globin expression was decreased. Three days of treatment with Dox did not stimulate γ-globin protein expression, whereas a simultaneous TNFα addition diminished the γ-globin level. He treatment resulted in a γ-globin increase, which was downregulated after cytokine addition. In combination with each of the 3 inducers, Remicade reversed the inhibiting effect of TNFα, confirming the observed effect of TNFα (Fig. 27B).

Effect of TNFα on γ-globin protein expression in HEL cells

In HEL cells, TNFα had a reductive effect on γ-globin protein expression in both untreated and He-induced HEL cells (Fig. 27C). He treatment alone showed an important increase (2.9 fold) in γ-globin protein expression compared to control. Remicade reversed the inhibitory effect on both constitutive as well as on induced γ-globin protein expression compared to the TNFα treated cells (Fig. 27C).

Effect of TNFα on γ-globin protein expression in TF-1 cells

Analyzing TF-1 cells for γ-globin variations revealed an induction of γ-globin protein expression after a 3-day treatment with Epo compared to the control. This induction is reduced after pretreatment with TNFα (Fig. 27D). The use of Remicade reversed this inhibitory effect of the cytokine.

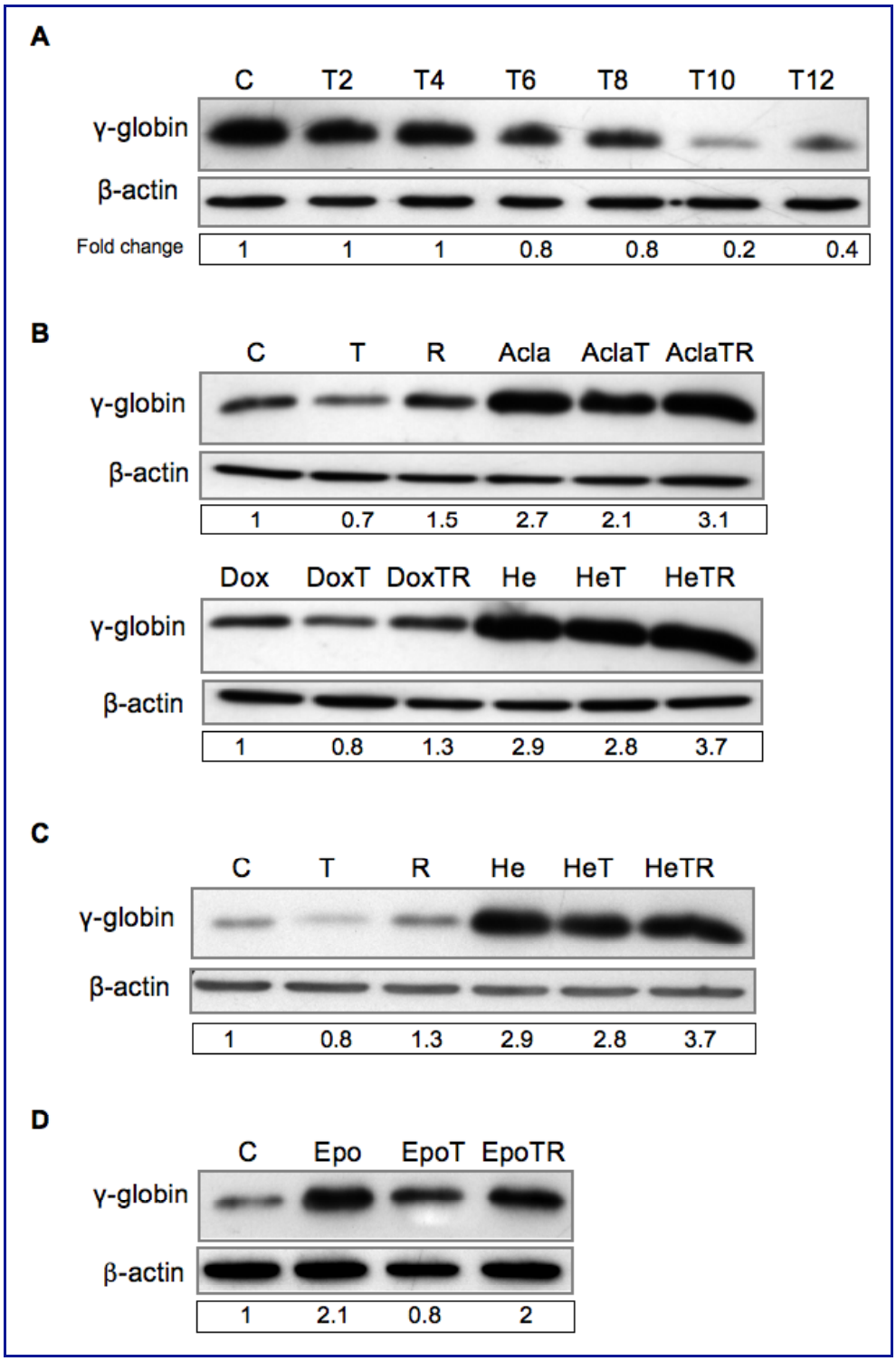


Figure 27: Western blot analysis of γ-globin protein expression. *10 µg cytoplasmic protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with 20 ng/mL TNFα (T) for 2, 4, 6, 8, 10, or 12 hours. B) K562 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla, 40 nM Dox, or 30 µM He. C) HEL cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20ng/mL) prior to three days of differentiation with 30 µM He. D) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

5.5.2.2 Effect of TNFα on EpoR protein expression

Effect of TNFα on EpoR protein expression in K562 cells

In order to investigate the inhibitory role of TNFα on constitutive EpoR protein expression, K562 cells were treated for 2 to 12 hours with 20 ng/mL TNFα. Indeed TNFα had an inhibitory effect on constitutive EpoR protein expression in a time dependent manner (Fig. 28A).

This inhibitory effect of the cytokine persisted for three days as observable for EpoR protein expression. Moreover, no matter which inducer used, western blot analysis showed a reduction of EpoR protein expression in K562 cells (Fig. 28B). Indeed, in Acla treated cells, TNFα revealed a 0.6 fold decrease in EpoR protein (Fig. 28B). Three days treatment with Dox did not stimulate EpoR protein expression, whereas a simultaneous TNFα addition strongly diminished the EpoR protein level. Cytokine treatment resulted also in a decrease of EpoR protein expression in He-treated cells. In combination with each of the 3 inducers, Remicade reversed the inhibiting effect of TNFα (Fig. 28B). Interestingly in some cases the use of Remicade even enhanced the expression when compared to the corresponding treatment.

Effect of TNFα on EpoR protein expression in HEL cells

In HEL cells, TNFα had a reductive effect on EpoR protein expression in both untreated and He-induced HEL cells (Fig. 28C). Remicade rescued the inhibitory effect on both constitutive as well as on induced EpoR protein expression (Fig. 28C).

Effect of TNFα on EpoR protein expression in TF-1 cells

Analyzing Epo-induced TF-1 cells for EpoR protein variations revealed a reduction after pretreatment with TNFα (Fig. 28D). The use of Remicade permitted to reverse this inhibitory effect of TNFα on EpoR protein expression.

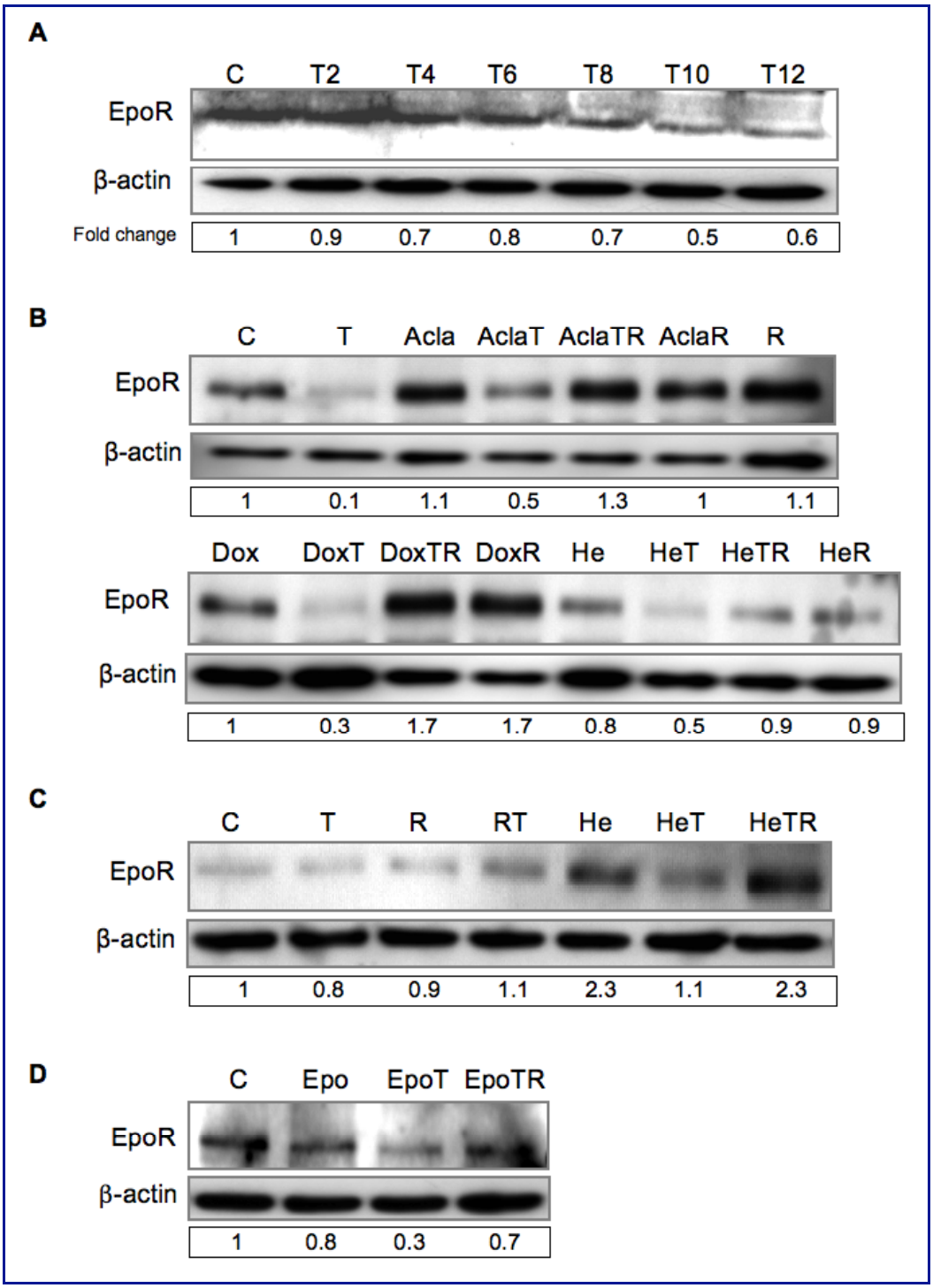


Figure 28: Western blot analysis of EpoR protein expression. *20 µg cytoplasmic protein extracts were used. β-actin was used as internal control. A) K562 cells were pretreated with 20 ng/mL TNFα (T) for 2, 4, 6, 8, 10, or 12 hours. B) K562 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla, 40 nM Dox, or 30 µM He. C) HEL cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20ng/mL) prior to three days of differentiation with 30 µM He. D) TF-1 cells were pretreated with or without Remicade (R) (100 µg/mL) and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

5.5.2.3 Effect of TNFα on GPA protein expression

Glycophorins are sialoglycoproteins of the human erythrocyte membrane, which bear the antigenic determinants. The goal of investigating the expression of GPA is to see whether the TNFα treatment has an effect on the expression of this erythroid specific marker. The expression of GPA on differentiated cells is analyzed by using flow cytometry analysis.

Effect of TNFα on GPA protein expression in K562 cells

FACS results showed that GPA expression in K562 cells did not vary significantly after TNFα treatment in Acla-, or Dox-treated cells, but TNFα significantly decreased GPA protein expression in He-induced K562 cells (Fig. 29A).

Effect of TNFα on GPA protein expression in HEL cells

In HEL cells, TNFα significantly decreased the amount of GPA protein expression after He treatment. The mean of three independent experiments revealed a significant 20% diminution of He-induced EpoR protein expression after TNFα treatment comparing to control cells (Fig. 29B).

Effect of TNFα on GPA protein expression in TF-1 cells

Compared to the control, Epo-induction revealed an increase of 30% of EpoR protein in TF-1 cells, which was significantly reduced after addition of TNFα to 16%. The addition of Remicade rescued this inhibitory effect to 32% (Fig. 29C).

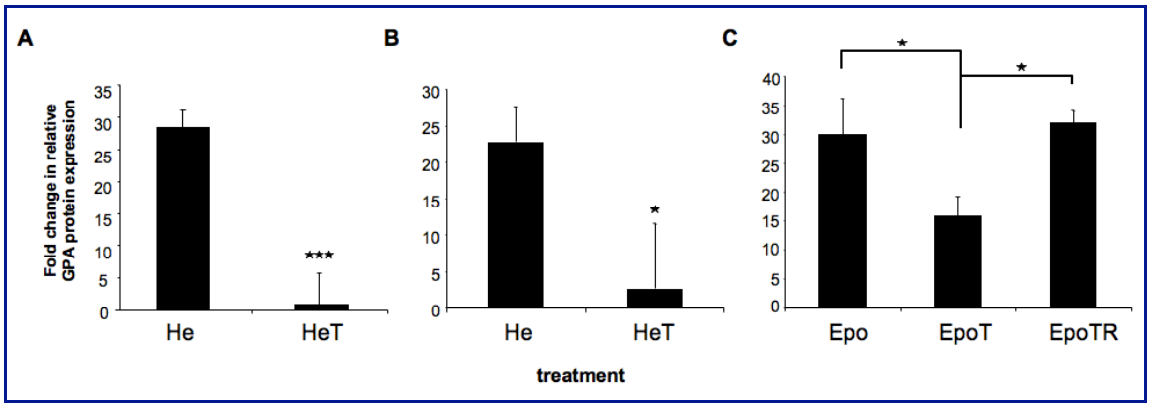


Figure 29: Flow cytometry analysis of GPA protein expression. *Histograms showing the relative percentage of GPA fluorescence compared to untreated controls (c). Pretreatments with 100μM Remicade (R) and/or 20 ng/mL TNFα (T) for 1 hour were followed by three days of differentiation with 30 μM He in K562 (A), and HEL (B) cells, or 10 U/mL Epo in TF-1 cells (C). Values are reported as the means (+/- standard deviation, SD) of three independent experiments. (*P ≤ .05, ***P ≤ .001)*

TNFα had an inhibitory effect on EpoR and γ-globin mRNA as well as on constitutive and induced EpoR, GPA, and γ-globin protein expression in all three cell lines independently of the inducer used. Moreover, TNFα had an effect on other erythroid markers' mRNA such as TRFC, ERAF, and HMBS, as shown by qRT-PCR analysis in TF-1 cells. The anti-TNFα antibody neutralized the inhibitory effect of TNFα. In conclusion, TNFα inhibits erythroid specific gene expression whose regulation is known to involve the major erythroid transcription factor GATA-1.

5.6 Effect of TNFα on signaling pathways involved in erythroid differentiation

In order to investigate the implication of distinct cell signaling pathways in the inhibition of erythroid differentiation by TNFα, we investigated the effect of different inhibitors on TNFα treated cells, which mainly blocks MEK (U0126, PD98059), PI3K (Ly294002), JNK (SP600125), NF-κB (Bay11-7082), and p38 (SB203580) pathways.

Effect of TNFα on signaling pathways in K562 cells

After 3-day treatment with the distinct inhibitors, results showed that the use of PD98059, a MEK inhibitor resulted in an increased rate of benzidine positive cells, independently of the inducer used. Moreover, by using this inhibitor alone or in combination with the inducer, an increased hemoglobinization rate could be observed when compared to control or induced cells (Fig. 30A, B, C).

In Dox-induced TNFα treated cells, the use of another MEK inhibitor, U0126, also increased the percentage of benzidine positive cells when compared to DoxT treated cells (Fig. 30B). Moreover, in He-induced TNFα treated cells, we also observed an induction of hemoglobinized cells after the following inhibitors: U0126 (MEK inhibitor), Ly294002 (PI3K inhibitor), SP600125 (JNK inhibitor), Bay11-7082 (inhibitor of NF-κB activation) (Fig. 30C).

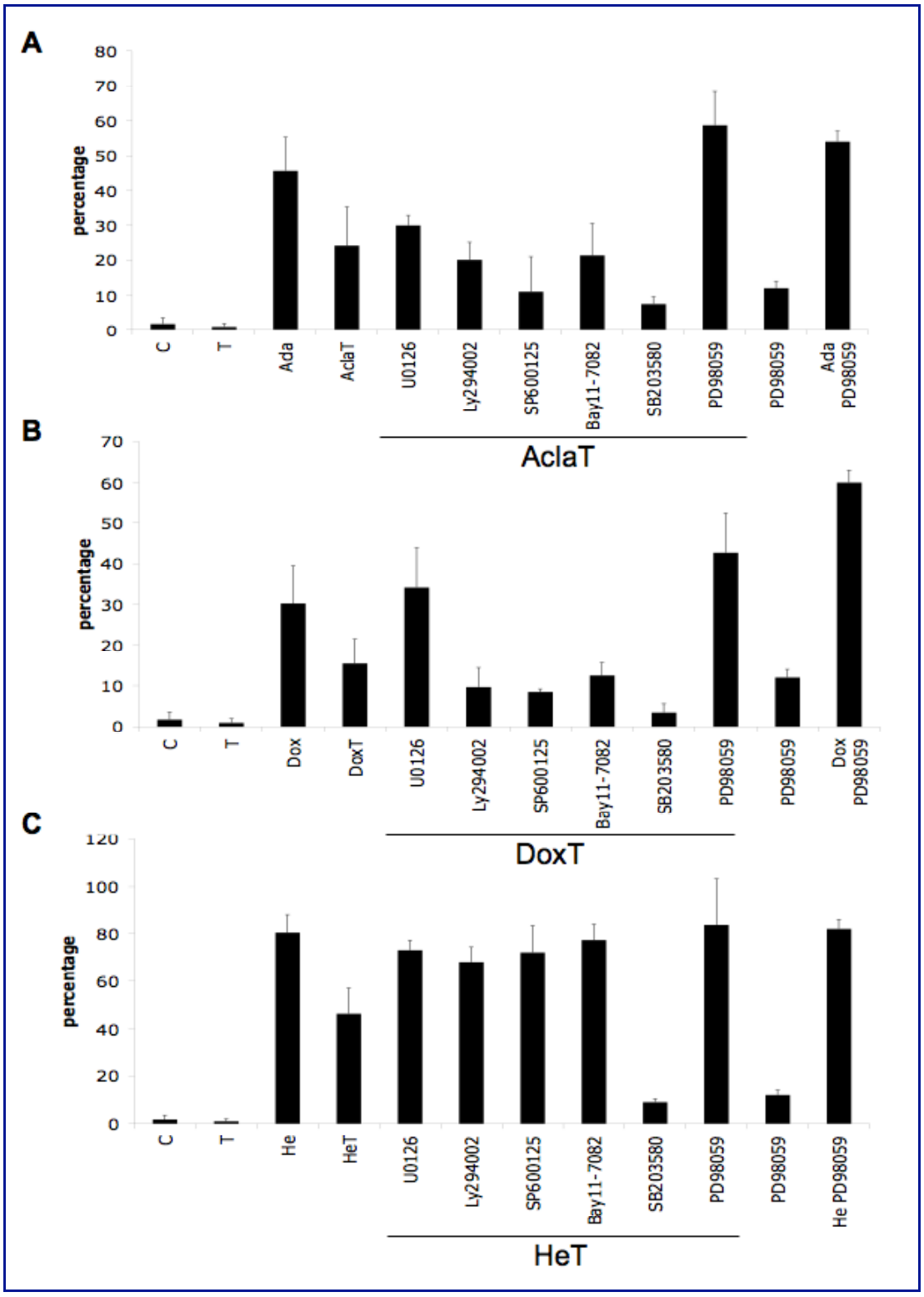


Figure 30: Effect of signaling pathway inhibitors on the inhibitory effect of TNFα on hemoglobin synthesis in K562 cells. *Cells were pretreated with or without U0126 (10µM), Ly294002 (10µM), SP600125 (10µM), Bay11-7082 (µM), SB203580 (10µM), PD98059 (10µM), and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 nM Acla (A), 40 nM Dox (B), or 30 µM He (C). Values are reported as the means (+/- standard deviation, SD) of 3 independent experiments.*

Effect of TNFα on signaling pathways in HEL cells

In HEL cells, the use of distinct cell signaling inhibitors in HeT treated cells, showed an increase of benzidine positive cells when compared to HeT treated cells alone. Thus an increase was observed after the use of the MEK inhibitor (PD98059), or the NF-κB inhibitor (Bay11-7082) and the highest rate of Benzidine positive cells were obtained after the use of the PI3K inhibitor (Ly294002) (Fig. 31).

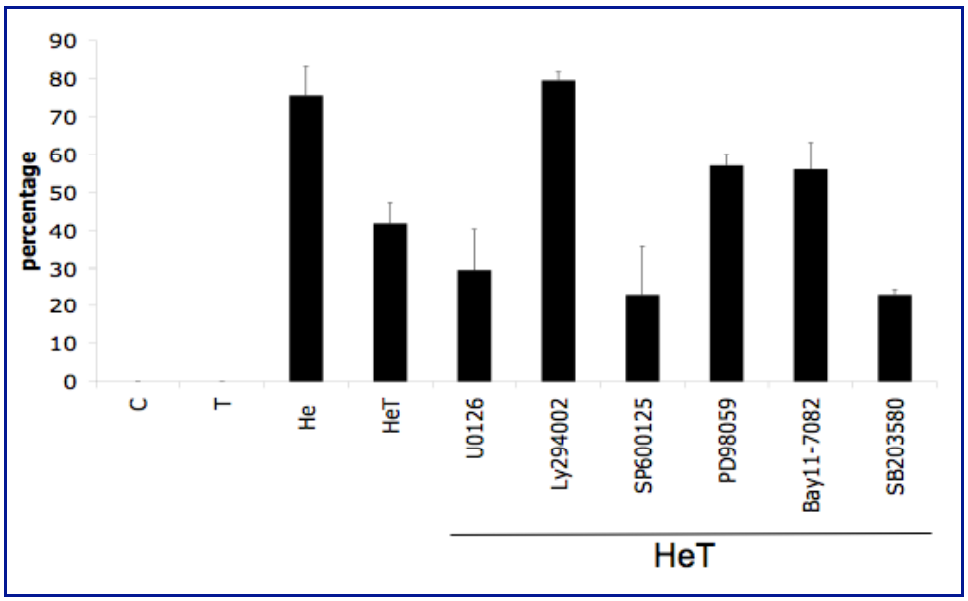


Figure 31: Effect of signaling pathway inhibitors on the inhibitory effect of TNFα on hemoglobin synthesis in HEL cells. *Cells were pretreated with or without U0126 (10μM), Ly294002 (10μM), SP600125 (10μM), PD98059 (10μM), Bay11-7082 (1 μM), SB203580 (10μM), and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 30 μM He. Values are reported as the means (+/- standard deviation, SD) of 3 independent experiments.*

Effect of TNFα on signaling pathways in TF-1 cells

In order to validate the involvement of distinct signaling pathways in the TNFα inhibiting effect of erythroid differentiation in Epo-treated TF-1 cells, we used distinct cell signaling inhibitors in co-treatment with TNFα. In the presence of the p38 inhibitor SB203580, we observed an abrogation of the inhibitory effect of TNFα on hemoglobin production as assessed by benzidine staining (Fig. 32).

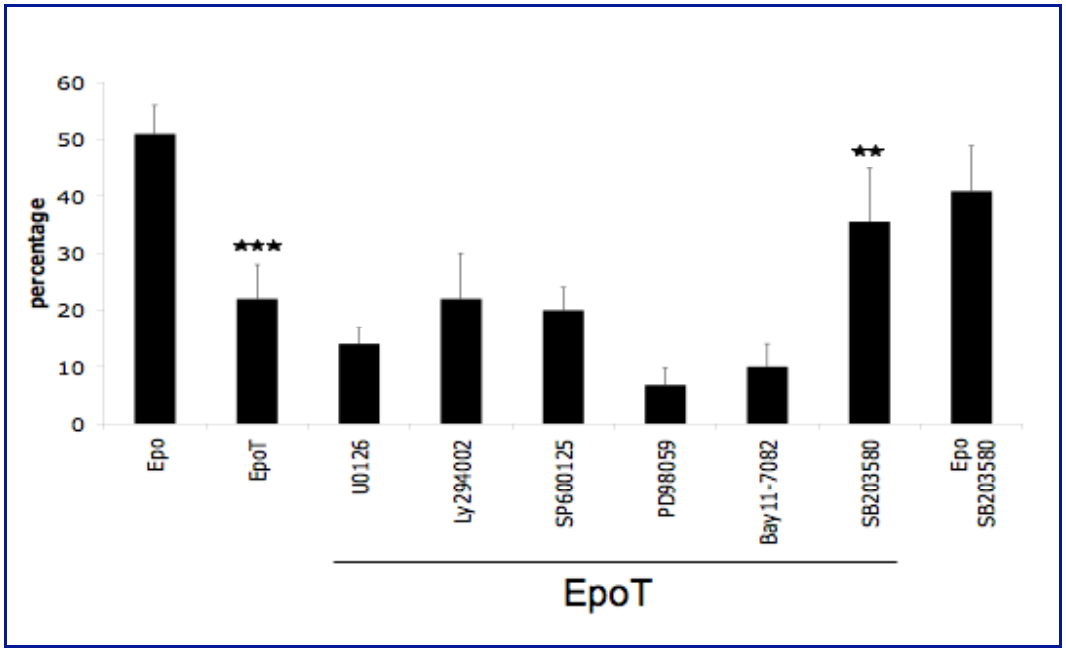


Figure 32: Effect of signaling pathway inhibitors on the inhibitory effect of TNFα on hemoglobin synthesis in TF-1 cells. *Cells were pretreated with or without U0126 (10µM), Ly294002 (10µM), SP600125 (10µM), PD98059 (10µM), Bay11-7082 (1 µM), SB203580 (10µM), and/or TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. Values are reported as the means (+/- standard deviation, SD) of 3 independent experiments. (**P ≤ .01, ***P ≤ .001)*

By using inhibitors of distinct signaling pathways, it seems like there is more than one pathway involved in the inhibition of erythroid differentiation by TNFα depending on the inducer and the cell line used. As our goal is to understand the molecular mechanisms leading to anemia, we will especially focus our attention on the signaling pathway involved in the inhibition of erythroid differentiation by TNFα in Epo-treated cells. Thus, the p38 inhibitor SB203580 partly reversed the inhibitory effect of TNFα on the hemoglobinization rate of Epo-treated TF-1 cells.

5.6.1 Effect of p38 inhibitor on the inhibition of erythroid differentiation by TNFα in TF-1 cells

5.6.1.1 Effect of SB203580 on p38-phosphorylation and total p38 protein in TF-1 cells

We evaluated the role of p38 signaling pathway in TNFα-mediated inhibition of erythroid differentiation in Epo-differentiated TF-1 cells. TNFα treatment alone showed a

1.8 fold activation of p38 phosphorylation compared to control or Epo treated cells after 30 min (Fig. 30, lane 8), confirming its ability to rapidly activate p38 phosphorylation. Conversely, for short times p38 was not phosphorylated in TF-1 cells in the presence of Epo alone. Furthermore, in the presence of Epo, p38 phosphorylation after 10 minutes was due to TNFα treatment. Thus, phosphorylation was persistent up to 48 hours (Fig. 33). Moreover, we noticed a progressive increase in phosphorylated p38 (p38-P) protein expression after 8 hours of TF-1 culture in the presence of Epo, without TNFα. Nevertheless, p38-P expression remained higher in the TNFα-treated cells during the time course experiment.

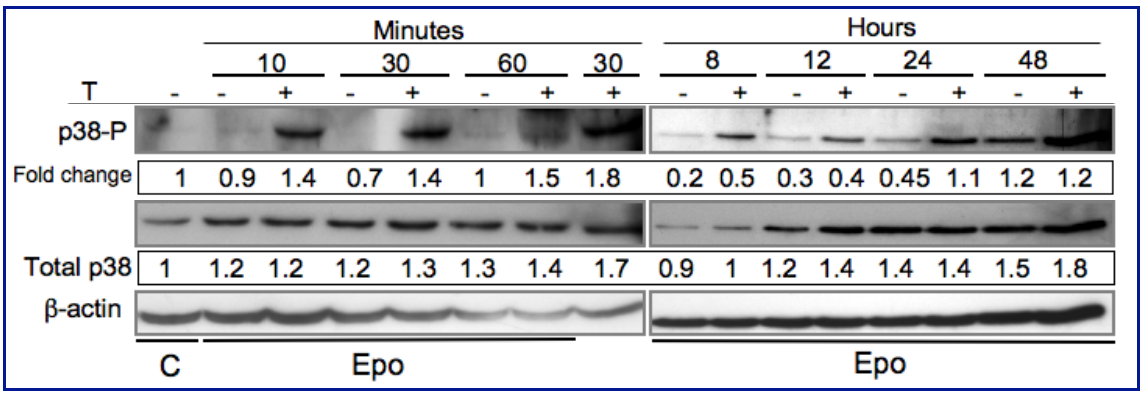


Figure 33: Western blot analysis of phosphorylated p38 (p38-P) and total p38 protein expression in Epo-induced TF-1 cells in the presence or absence of TNFα after indicated treatment time. *10 µg cytoplasmic protein extracts were used. β-actin was used as internal control. As a positive control, cells were treated by TNFα alone for 30 min. (One representative result of 3 independent experiments)*

5.6.1.2 Effect of SB203580 on GATA-1 protein expression

Then we analyzed the effect of the p38 inhibitor on the major erythroid transcription factor GATA-1. Results show a partial abrogation of the inhibitory effect of TNFα on GATA-1 protein expression after the use of the p38 inhibitor. Thus, the use of the p38 inhibitor (SB203580) pointed to an implication of the p38 pathway in the inhibition of erythroid differentiation by TNFα (Fig. 34).

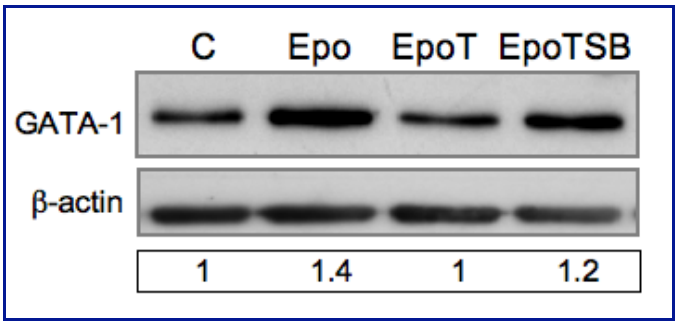


Figure 34: Effect of p38 inhibitor (SB203580) on GATA-1 protein expression in Epo-induced TF-1 cells. *20 µg nuclear protein extracts were used. β-actin was used as internal control. TF-1 cells were pretreated with or without TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

5.6.1.3 Effect of SB203580 on γ-globin protein expression

In order to confirm the results obtained for GATA-1 protein expression, we assessed γ-globin protein expression. Results showed a rescue of the inhibitory effect of TNFα on γ-globin expression after the use of the p38 inhibitor (SB203580) (Fig. 35).

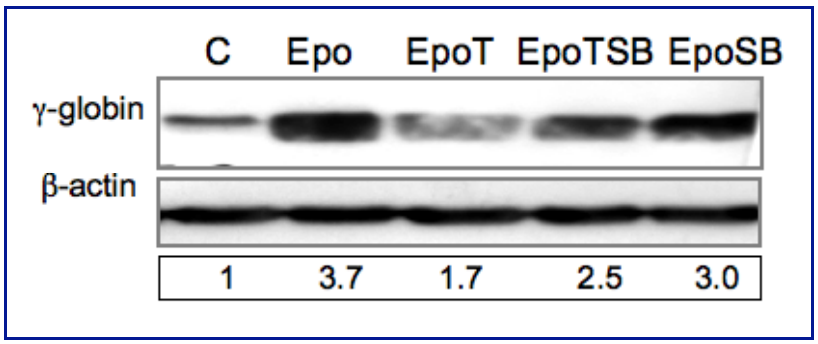


Figure 35: Effect of p38 inhibitor (SB203580) on γ-globin protein expression in Epo-induced TF-1 cells. *10 µg cytoplasmic protein extracts were used. β-actin was used as internal control. TF-1 cells were pretreated with or without TNFα (T) (20 ng/mL) prior to three days of differentiation with 10 U/mL Epo. (One representative result of 3 independent experiments)*

These results showed an activation of p38 by TNFα in our cellular model. Moreover, it gives first hints about an involvement of this MAPK in the inhibitory effect of TNFα on GATA-1 and γ-globin protein expression in Epo-induced TF-1 cells.

6 Discussion

6.1 TNFα reduces hemoglobin synthesis

In order to investigate the molecular mechanisms involved in cytokine-dependent erythroid inhibition, we investigated the effect of TNFα in three different erythroleukemia cell lines, K562, HEL and TF-1. These erythroleukemia cell lines are widely used as cellular models to study erythroid differentiation thanks to their ability to terminally differentiate *in vitro* in response to distinct agents.

K562 and HEL cells were induced to differentiate towards erythroid differentiation using three different cellular inducers, including two anthracyclins (Acla, Dox) as well as a porphyrine (He), all known as inducers of erythroid differentiation [191,194,211]. By benzidine staining, we could notice the highest differentiation level for He, followed by Acla and Dox. In the HEL cell line hemoglobinization was achieved by He. The rate of hemoglobinized K562 and HEL cells induced to differentiate by chemicals was comparable to previous results [192,212]. In order to work with Epo-responsive cells, we also we used the growth factor dependent erythroleukemia cell line TF-1 [151,152]. Indeed, TF-1 represented a good cellular model to study erythroid differentiation pathways affected by TNFα, because these cells differentiate towards erythroid pathway when cultured in the presence of the physiological cytokine Epo [195,213,214]. In our differentiation model, 10 U/ml Epo induced erythroid differentiation in TF-1 cells.

Then we investigated the inhibitory effect of TNFα on erythroid differentiation in these three cell lines after induction with the corresponding agents. Independently of the chemical inducer (Acla, Dox, He) or the cell line used, we could reveal a decrease of the hemoglobin synthesizing cells after TNFα treatment. Moreover, in TF-1 cells, TNFα prevented hemoglobin synthesis induced by Epo signaling pathway. In order to validate and generalize our hypothesis that TNFα has an inhibitory effect on erythroid differentiation, we also started to explore hematopoietic progenitor cells from umbilical cord blood. Using a magnetic cell sorting mechanism, we selected CD34+ cells, which are pluripotent hematopoietic stem cells. These can give rise to both the myeloid and lymphoid lineage. Treating these cells with a distinct cytokine cocktail including SCF, IL-3 and Epo [153], we were able to differentiate them towards the erythroid differentiation pathway as shown by benzidine staining. Carlile and colleagues showed the appearance

of pronormoblasts at day 7 as well as an increasing level of GPA positive cells, which correlated with our results the detection of hemoglobin synthesizing cells after 6-7 days of culture [153]. Moreover, by investigating the effect of TNFα on these hematopoietic progenitor cells, we could see a more and more pronounced inhibition of erythroid differentiation over time. In previous studies, indications were already provided that TNFα had an inhibitory effect on erythropoiesis *in vivo* and *in vitro* [116,121,122], implicating roles for both TNFα receptors [87,89,119,215]. Similarly, it was shown that another proinflammatory cytokine IFN, playing a similar role than TNFα in anemia of chronic disease, inhibited human CFU-E cells [216]. Moreover, Felli and colleagues confirmed this inhibitory effect of IFNγ on CD34+ cells of peripheral blood cells implicating also TNF superfamily members in the IFNγ-mediated inhibition of erythroid differentiation [217]. Interestingly, the transplantation of a Chinese hamster ovary cell line transfected with the TNFα gene, led to anemia in nude mice, with a significant decrease in erythroid progenitors [120].

The use of Remicade, a clinically applied anti-TNFα ab, allowed us to restrain the inhibitory effect of TNFα on erythroid differentiation to TNFα alone. In patients with chronic diseases an inhibition of erythroid precursor cells was reversed by the use of an anti-TNFα ab [132,134,218]. Furthermore, elevated levels of TNFα and IFNγ were detected in the bone marrow of patients with aplastic anemia and the use of an anti-TNF ab resulted in an increase in corresponding erythroid colonies [219]. These results suggested that TNFα inhibited differentiation by affecting key regulators of erythropoiesis, common to the three different cell lines and the hematopoietic progenitors, rather than disturbing specific molecular mechanisms and signaling pathways activated by inducing agents.

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6.2 TNFα induced NF-κB activity

TNFα plays a role in various physiological processes why it is implicated in numerous diseases, including cancer [52,56,57]. The proinflammatory cytokine TNFα is produced by

many cell types, but mainly by macrophages in response to inflammation. Indeed, TNFα, activated by a broad range of stimuli, is considered as one of the major mediators of inflammation by orchestrating inflammatory responses. As already mentioned, its effects are principally mediated through two distinct receptors, before stimulating various underlying cell signaling pathways [52]. As TNFα was shown to be the most powerful activator of NF-κB, which is considered as the link between inflammation and cancer [55,220], we assessed whether TNFα was able to normally activate the NF-κB transcription factor in our cellular model. Results showed that NF-κB binding activity was induced in K562, HEL and TF-1 cells after TNFα treatment, whatever the inducer used, and the use of Remicade partially or completely inhibited this activation. The most frequently described active dimer is p50-p65, which leads to the activation of the so-called canonical pathway, and is mainly involved in primary inflammatory responses in response to inflammatory cytokines [220]. The use of the p50 and p65 antibodies permitted us to identify the heterodimer p50/p65, known to be constitutively activated in many tumors [55].

6.3 TNFα modulates the expression and regulation of major erythroid transcription factors

During the development of erythrocytes from hematopoietic progenitor cells, cells progressively restrict their differentiation potential and establish their lineage-specific gene expression profiles, relying on lineage-specific transcription factors. The transcription factors important for erythroid lineage development include GATA-1, GATA-2, NF-E2, and EKLF. However, Blobel suggested that tissue-specific and developmentally correct expression of a given gene is not achieved by a single transcription factor, rather unique combinations of cell-type specific and widely expressed nuclear factors account for the enormous specificity and diversity in gene expression profiles [221]. So, in order to decode the effects of TNFα on erythroid differentiation, we analyzed the expression patterns of distinct erythroid factor transcription factors as well as cofactors.

The importance of the major erythroid transcription factor GATA-1 is emphasized by the fact that knock out mice for GATA-1 gene and mutations result in a maturational arrest

and apoptosis of erythroid precursors [8,9,222]. Moreover, as GATA-1 was reported to be involved in the regulation of virtually all erythroid genes, since they all present several GATA sequences as specific binding sites for the GATA family of transcription factors in their cis-regulatory regions [223,224], we first investigated the effect of TNFα on this major erythroid transcription factor. Results showed a reductive effect of the cytokine on constitutive and induced GATA-1 mRNA and protein expression in the three cell lines. Indeed, GATA-1 is weakly expressed in quiescent erythroid progenitors, but the expression is upregulated during differentiation towards the erythroid pathway [225,226]. In He-treated K562 or HEL cells, we saw a decrease of GATA-1 expression after TNFα treatment, but no overexpression when compared to untreated cells. In fact, our group previously showed that He-induced GATA-1 expression was associated to lower and transient factor activation [194].

On the other hand, the transcription factor GATA-2 is also involved in the regulation of erythropoiesis. Our study demonstrated a reversal of the erythroid expression pattern of GATA-1 and GATA-2 after TNFα treatment in uninduced and induced 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. GATA-2 regulates the development of hematopoietic precursors [227], whereas GATA-1 is essential for terminal maturation of erythroid cells [9]. Furthermore it was shown that GATA-1-dependent repression of GATA-2 was regulated via disruption of positive autoregulation and chromatin remodeling [228]. Epo gene regulation was shown to be negatively controlled by GATA-2 as well as NF-κB in HepG2 cells in response to hypoxia [229]. Moreover, cytokines were already shown to stimulate GATA-2 expression in primary human hematopoietic progenitor cells [230]. Together with our findings, these data suggest that cytokines could exert their inhibitory effect on erythropoiesis by up-regulating GATA-2 expression. On the other hand, Nakano and colleagues suggested the GATA-2 inhibitor K-11706 as therapeutic purposes against anemia of inflammatory diseases and cancer [128]. 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.

The transcription factor NF-E2 plays a role in hemoglobin synthesis, by controlling genes essential for globin and HMBS [26,42,199]. EKLF is also required for globin gene

expression and was shown to be essential for definitive erythropoiesis since EKLF deficient mice succumb to fatal anemia due to a defect in hemoglobin accumulation [43,231]. Interestingly, our results showed a reductive effect of TNFα on both transcription factor expressions, which was observed in parallel to the GATA-1 protein reduction in all three cell lines. In this context EKLF was shown to be act synergistically with the major erythroid transcription factor GATA-1 in erythroid complexes [232].

We then investigated the effect of TNFα on the corresponding GATA-1 cofactors. In fact, GATA-1 is known to be regulated by a number of coregulators, which can act either as coactivator, such as FOG-1 or as repressor such as PU.1. FOG-1 is essential for normal erythroid development as a cofactor of GATA-1 [9,202]. It was shown that mice lacking FOG-1 die during mid-embryonic development from severe anemia [202]. Interestingly, analysis of FOG-1 expression showed a decrease in FOG-1 protein level after TNFα treatment in differentially induced K562, HEL, and TF-1 cells, which correlated with erythroid differentiation arrest. PU.1, which is described as the antagonistic master regulator of GATA-1, is responsible for the myeloid lineage commitment [18,205,233]. Moreover, chromatin immunoprecipitation assays showed that GATA-1 and PU.1 can colocalize on promoters, and that these transcription factors physically interact by distinct motifs [32,234,235]. In order to investigate whether TNFα had an influence on erythroid/myeloid cell fate decision, we analyzed its effect on PU.1 protein expression, but the results did not allow us to reveal any common expression patterns in the differentially expressed cell lines.

The N-finger of GATA-1 not only supplies the stabilization and specificity of DNA binding [13,24,25], but is also responsible for the interaction with the cofactor FOG-1 [23]. As the analysis of GATA-1 mutants, defective in FOG-1 binding and subsequent identification of compensatory mutations in FOG-1, provided definitive proof that the FOG-1/GATA-1 interaction is essential for GATA-1 function during erythropoiesis [35], we investigated the effect of TNFα on GATA-1/FOG-1 interaction by immunoprecipitation. Results showed that GATA-1 and FOG-1 were linked together in the untreated or He-induced HEL or Epo-treated TF-1 cells since they co-immunoprecipitated. After TNFα addition, the co-immunoprecipitation revealed a decrease in the amount of both interaction partners and a suppression of the GATA-1/FOG-1 complex in uninduced HEL cells after TNFα addition. This suggested that the

interaction between GATA-1 and FOG-1 was not significantly affected in differentiated cells. The decrease of the complex could be due to an inhibition of the expression of the interaction partners, which could involve an inhibitory effect of TNFα on the transcriptional level and/or an increase of protein degradation. The use of the proteasome inhibitor lactacystin confirmed an ubiquitin-proteasome signaling pathway dependent degradation of FOG-1 and/or GATA-1 after TNFα treatment in differentiated cells. To our knowledge, no data were reported concerning FOG-1 degradation by proteasome and this phenomenon should be investigated in the future. On the other hand, it is reasonable to think that the inhibitory effect of TNFα occurs at the transcriptional level of GATA-1 gene expression since GATA-1 mRNA and protein amount is reduced in the presence of TNFα, and that GATA-1 protein decreased even when proteasome was inhibited. Interestingly, Lurie and colleagues recently described that differential GATA-1 and GATA-2 factor stabilities regulated by proteasomal regulation represent an important determinant of chromatin target site occupancy and thereby altering the gene expression profile of erythroid precursors as an essential step in erythropoiesis [50]. Cantor and colleagues recently suggested a cross-antagonistic regulatory loop between GATA-2 and FOG-1, where high GATA-2 levels lead to FOG-1 repression [236], what could be in line 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 [237]. Another interesting study shows that GATA factor exchange reconfigures higher chromatin organization involving chromatin loop changes [238].

Another important posttranslational modification of GATA-1 consists of its acetylation by CREB-binding protein (CBP) [30,45]. Interestingly, previous results showed that acetylation-defective GATA-1 binds DNA normally *in vitro* as shown by EMSA assay but fails to bind physiologic target genes *in vivo* [239]. Moreover, Hernandez-Hernandez and colleagues even proposed a model showing that GATA-1 acetylation is essential for DNA binding and a subsequent GATA-1 phosphorylation marks the protein for subsequent proteasomal degradation [48]. Our immunoprecipitation results showed an inhibition of GATA-1 acetylation by TNFα in He-induced K562 cells, suggesting that TNFα could have an influence on erythroid gene transcription.

Overall, the reduction of the amount of GATA-1/FOG-1 complex as well as the modulation of GATA-1 acetylation should have consequences on erythroid gene

transcription since GATA-1, NF-E2, EKLF, and FOG-1 were downregulated while GATA-2 transcription factor was over-expressed in TNFα treated cells.

6.4 TNFα inhibits GATA-1 transactivation activity

Acla was previously shown to act by activating transcription of erythroid specific genes in association with prolonged increase of GATA-1 DNA binding activities [191,192,211]. In our study GATA-1 binding activity was reduced after TNFα addition independent of the inducer or the cell line used. TransAM confirmed the inhibitory effect of TNFα on erythroid differentiation and showed a concentration dependent reductive effect of TNFα on constitutive GATA-1 binding activity in K562 cells. EMSA and TransAM results indicated that Dox did not induce GATA-1 binding when compared to the control, whereas the binding activity was reduced when treated in combination with TNFα. This observation is in agreement with prior results, where the existence of a Dox-specific differentiating mechanism was proposed, based on posttranscriptional regulation mechanisms of erythroid gene expression [192,211]. Moreover, EMSA results obtained after He treatment were similar to the western blot results, where no increase of GATA-1 binding activity was noted in He-induced K562 cells after three days of treatment suggesting a transient GATA-1 activation [194]. By adding Remicade, this inhibitory effect of TNFα was abrogated. Furthermore, TNFα abolished GATA-1 transcription activity as shown by transfections. The results of this study using three different cell lines could indicate a general trend of TNFα to inhibit erythroid differentiation.

6.5 TNFα has an effect on erythroid markers

Thus, in order to confirm this transcriptional modulation by TNFα on a phenotypic level, we analyzed distinct erythroid markers, known to be regulated by GATA-1, and other erythroid transcription factors such as NF-E2 or EKLF [26,42,199]. In Epo-induced TF-1 cells, analysis of a panel of erythroid genes showed a significant decrease in EpoR, α-globin, γ-globin, ERAF, HMBS and GPA erythroid specific marker mRNAs after TNFα addition. The TFRC gene expression was increased in agreement with its current use as an index of tissue iron deficiency in anemic patients with cancer or inflammation [240]. The inhibitory effect of TNFα on erythroid gene expression was confirmed on Epo receptor

(EpoR), γ-globin and GPA protein levels in all three cell lines independently of the inducer used. After long discussions on the detection of EpoR in distinct studies, the M-20 ab permitted us to observe reproducible decreases in EpoR protein expression after TNFα treatment [241]. Interestingly, after Remicade addition, the analysis of EpoR or γ-globin protein expression showed sometimes an overexpression of the protein, when compared to corresponding treatments without Remicade. This observation could be due to the fact that erythroid cells were shown to express TNFα. Indeed Jacobs-Helber and colleagues already reported that TNFα is constitutively expressed in erythroid cells [242]. Xiao and colleagues showed that GPA positive generation was inhibited in TNFα treated human CD34+ progenitor cells [129]. Overall we can conclude that these three erythroid genes (GPA, globin and EpoR) could represent efficient erythroid markers in the detection of cytokine dependent anemia.

6.6 TNFα involves the p38 pathway in the inhibition of Epo-induced erythroid differentiation

By using inhibitors of distinct signaling pathways, results showed that depending on the inducer and the cell line used, more than one pathway seemed to be involved in the inhibition of erythroid differentiation by TNFα depending on the inducer and the cell line used. The use of the inhibitor of NF-κB, Bay11-7082, did not permit us to draw any general conclusions in the differentially induced cell lines despite of some data suggesting an involvement of the NF-κB signaling pathway during erythroid differentiation [196], as well as during the inhibition of erythroid differentiation [129,229].

As the aim of this study is to understand the molecular mechanisms leading to anemia, we wanted to especially focus our attention on the signaling pathway involved in the inhibition of erythroid differentiation by TNFα in Epo-treated cells. During erythroid differentiation, p38 isoforms p38α and p38γ were already reported to be expressed [243], which is in agreement with our results concerning total p38 expression. Moreover, p38 as well as JNK, are known to be activated by Epo and are required for Epo-induced erythropoiesis [242,244]. Moreover, Tamura and colleagues showed a defect in definitive erythropoiesis in p38-/- mice embryos due to an Epo deficiency [245]. TNFα is known to activate various signaling pathways leading, amongst others, to p38 activation through TNFRI/II, TNFR-associated death domain (TRADD), TNFR-associated factor 2

(TRAF2), receptor-interacting protein (RIP), MAP/ERK kinase kinase 3 (MKK3) [52,246]. Using the p38α isoform inhibitor SB203580, we observed an abrogation of the inhibitory effect of TNFα on erythroid differentiation as shown by hemoglobin, γ-globin and GATA-1 upregulation. Interestingly, the differentiating inhibitor TNFα and the activator Epo are both described being capable to stimulate p38 phosphorylation. Results showed that TNFα treatment combined to Epo induced strong p38 phosphorylation after only 10 min, while in the presence of Epo alone, this phosphorylation in only observed after 8 hours. The effect of Epo was amplified over time, an effect already reported in differentiating Apicidin-induced K562 cells [247]. Moreover, TNFα-induced phosphorylation was also observed after 30 min in the absence of Epo, eliminating the hypothesis that cotreatment would amplify p38 activation. The inhibition of erythroid differentiation by TNFα seems to be dependent on p38 pathway in TF-1 cells and to occurs in correlation with an early activation of p38. Interestingly, inhibition of GATA-1 expression was shown to be dependent on p38 signaling pathway, and probably independent of proteasomal degradation. Stassen and collegues even postulated GATA-1 as a target for p38 mediated phosphorylation in mast cells [248], a posttranslational modification, which is still controversially debated concerning its importance in DNA binding [46,249]. Interestingly, Miwatashi and collegues already used a novel p38 inhibitor, N-[4-[2-Ethyl-4-(3-methylphenyl)-1,3-thiazol-5-yl]-2-pyridyl] benzamide (TAK-715), as an anti-TNFα drug for the treatment of rheumatoid arthritis patients [250], known to be susceptible for anemia [134].

In conclusion, our results provide first evidence that TNFα inhibits erythroid differentiation by downregulating key transcription factors such as GATA-1, NF-E2, and EKLF. Furthermore, we showed that TNFα-mediated inhibition of GATA-1 involved the p38 MAPK pathway. Downregulation of GATA-1 and its cofactor FOG-1 lead to a diminished GATA-1/FOG-1 forming complex. This inhibition correlated with a proteasomal degradation of both interaction partners. Moreover, TNFα abolished the acetylation status of GATA-1, which was correlated to an inhibition of GATA-1 transcriptional activity resulting in a reduction of GATA-1 regulated erythroid genes. In addition we observed a simultaneous upregulation of GATA-2 in accord with a reduced hemoglobin production as well as decreased erythroid marker genes expression in both chemical- and Epo-induced cells. Overall these results give first insights of the major changes involved in the inhibition of erythroid differentiation by a key mediator of inflammation. This might serve as a solid base for future investigations in finding potential therapeutic targets for inflammation and cancer related anemia.

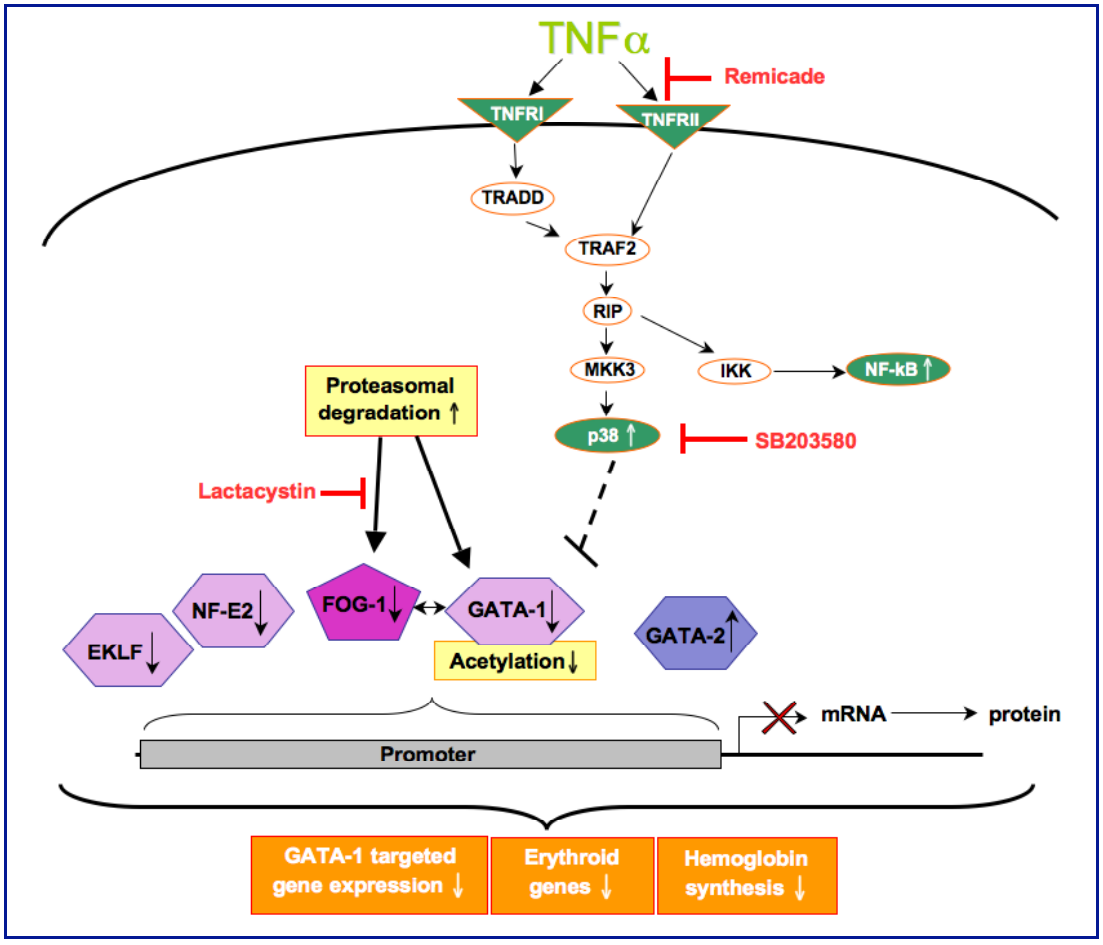

Figure 36: Scheme summarizing the effect of TNFα on erythroid differentiation based on the results obtained for TF-1 cells.

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8 Table of figures

9 Abbreviations

ab	antibody
Acla	Aclacynomicin A
AML	acute myeloid leukemia
Baso EB	basophilic erythroblasts
BCP	B-cell progenitor
BFU-E	erythroid burst-forming units
BSA	bovine serum albumin
C	control
CFU-E	erythroid colony-forming units
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
Dox	Doxorubicin
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	1,4-Dithiothreitol
EDTA	ethylene-diamin-tetra-acetat
EKLF	erythroid Kruppel-like factor
EMSA	electrophoretic mobility shift assay
Epo	erythropoietin
EpoR	erythropoietin receptor
ERK	extracellular signal-related kinase
FA	Fanconi anemia
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FOG-1	Friend of GATA-1
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte–macrophage progenitor
GPA	Glycophorin A
He	Hemin
H_2O_2	hydrogen peroxide
HCl	chlorhydric acid
HEL	human erythroleukemia cell line
HIF	hypoxia-inducible factor
HSC	pluripotent hematopoietic stem cell
IκB	inhibitor of NF-κB
IKK	Inhibitor of kappa B kinase
IFN	Interferon
IL	Interleukin

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony stimulating factor
MEK	mitogen-activated protein kinase MAPK kinase
MEP	megakaryocyte erythroid progenitor
MKK	MAP/ERK kinase kinase
MPP	multipotent progenitor
MW	molecular weight
NF-E2	nuclear factor erythroid-2
NF-κB	nuclear factor kappa B
O/N	overnight
OD	optical density
Ortho EB	orthochromatic erythroblasts
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PerCP	peridinin chlorophyll A protein
PI3K	phosphoinositide-3 kinase
Poly EB	polychromatophilic erythroblasts
Pro EB	proerythroblasts
qRT-PCR	quantitative real-time RT-PCR
R	Remicade
RBC	erythrocytes
RET	reticulocytes
rcf	relative centrifugal force
rhuEpo	Recombinant human erythropoietin
RIP	receptor-interacting protein
RT	reverse transcriptase
SCF	stem cell factor
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide electrophoresis
STAT	signal transducer and activator of transcription
T	Tumor necrosis factor alpha
TFRC	transferrin receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
TNK	T-cell natural killer cell progenitor
TPO	thrombopoietin
TRADD	TNFR-associated death domain
TRAF2	TNFR-associated factor 2
Tris	Trishydroxymethylaminomethane

10 Publications and scientific activities

publications

Tumour necrosis factor alpha inhibits Aclacinomycin A-induced erythroid differentiation of K562 cells via GATA-1.
Morceau F, Schnekenburger M, Blasius R, **Buck I**, Dicato M, Diederich M (Cancer Letters, 2006 Aug 28;240(2):203-12. Epub 2005 Nov 7)

Tumor necrosis factor α inhibits erythroid differentiation in human Erythropoietin-dependent cells involving p38 MAPK pathway, GATA-1, FOG-1 downregulation and GATA-2 upregulation
Buck I, Morceau F, Cristofanon S, Heintz C, Chateauvieux S, Reuter S, Dicato M, Diederich M (Biochem Pharmacol, in press)

The inhibitory effect of the proinflammatory cytokine TNFα on erythroid differentiation involves erythroid transcription factor modulation.
Buck I, Morceau F, Dicato M, Diederich M (Int J Oncol, submitted August 2008)

posters

Télévie conference 2006 (30.11.2005) Liège, Belgium:
Inhibition de la différenciation érythroïde par le TNFα: Rôle de GATA-1
Buck I, Morceau F, Dicato M, Diederich M

Molecular basis for targeted therapy for leukaemia (3-5. 2. 2006) Cascais, Portugal:
Effect of TNFα on chemically-induced erythroid differentiation in three different human leukaemia cell lines
Buck I, Morceau F, Dicato M, Diederich M

ESH - Club du Globule Rouge et du Fer Euroconference
Disorders of iron homeostasis, erythocytes and erythropoiesis (2-4. 11. 2007) Athens, Greece:
Mimicking anaemia: Inflammatory cytokine TNFα inhibits erythroid differentiation in human Epo-dependent TF-1 cells
Buck I, Morceau F, Jauch A, Dicato M, Diederich M
(Award: a European Commission's Marie Curie Actions scholarship)

oral presentations

Congrès annuel et XXXIIème forum des jeunes chercheurs de la SFBBM (24. – 26. 10. 2005) Nantes, (France): Effet du TNFα sur la différenciation érythroïde.
Buck I, Morceau F, Dicato M, Diederich M

Télévie seminar (30.11.2005) Liège, (Belgique): Effet du TNFα sur la différenciation érythroïde.
Buck I, Morceau F, Dicato M, Diederich M

11th International conference on differentiation therapy and innovative therapeutics in Oncology (4-8.11.2006) Versailles (France): Tumor necrosis factor alpha inhibits chemically induced differentiation of human erythroleukemia.
Morceau F, **Buck I**, Schnekenburger M, Dicato M, Diederich M

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on ne voit bien qu'avec le cœur, l'essentiel est invisible pour les yeux

Antoine de Saint Exupéry