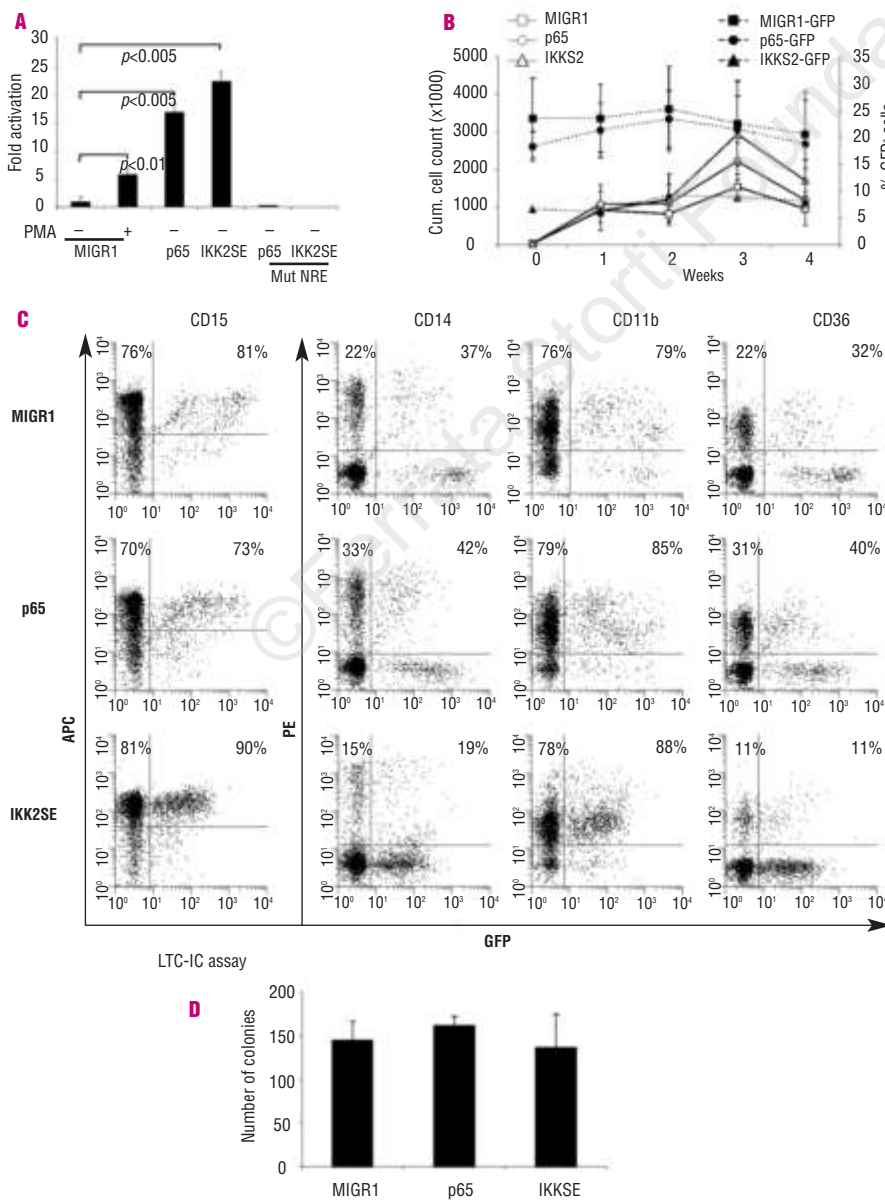


**Constitutive activation of NF-κB is not sufficient to disturb normal steady-state hematopoiesis**

**Since nuclear factor-κB (NF-κB) is frequently activated in acute myeloid leukemia, we questioned whether active NF-κB can affect the cellular properties of cord blood CD34<sup>+</sup> cells. The results demonstrated that NF-κB activation did not influence growth or differentiation properties of these cells. Furthermore, NF-κB activation was not sufficient to induce changes in stem- and progenitor cell numbers.**

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Constitutive activation of nuclear factor-κB (NF-κB) has been observed in a number of patients with acute myeloid leukemia (AML),<sup>1</sup> predominantly in the myelomonocytic and monocytic subtypes,<sup>2</sup> rather than in normal CD34<sup>+</sup> cells.<sup>3</sup> With a multiplicity of signal transduction pathways converging on NF-κB, this protein is therefore suggested to play a relevant role in disturbed hematopoiesis in AML. Various reports have indicated that constitutive activation of NF-κB is sufficient to induce cellular transformation,<sup>4,5</sup> however, limited information is available on the role of NF-κB in regulating normal hematopoiesis.<sup>6,7</sup> Its role in primary hematopoietic cells has been investigated mainly by inactivation studies using chemical inhibitors, gene knock-outs or overexpression of dominant negative constructs.<sup>6,7</sup> Furthermore, these studies largely focused on the anti-apoptotic properties of NF-κB, and its effects on other important characteristics of cell biology such as proliferation, differentiation and self-renewal were rarely considered. We, therefore, established a model for studying the



**Figure 1.** NF-κB activation does not alter growth, differentiation or self-renewal capacity of cord blood (CB) CD34<sup>+</sup> cells on MS-5 long-term co-cultures. **(A)** 293T HEK cells were co-transfected with MIGR1, p65 or IKK2SE mutant constructs together with a luciferase vector containing three NF-κB responsive elements (NRE). PMA-stimulated (50 nM, 16 hrs) cells were used as a positive control and Mut NRE (in which the three NRE are mutated)-luciferase vector transfected cells as a negative control. Experiments were performed in triplicate (n=3). A representative example is shown. Data are presented as averages with standard errors of the mean (SEM). A Student's t-test was performed to calculate significance levels. **(B)** An MS5 co-culture experiment with MIGR1, p65 and IKK2SE transduced cord blood CD34<sup>+</sup> cells. Dotted lines indicate GFP percentages, with error bars representing the SEM, (n=3). Solid lines indicate cumulative cell counts. An average of three independent experiments is shown. **(C)** FACS analysis of a representative week 3 MS5 co-culture experiment with MIGR1, p65 or IKK2SE transduced cord blood CD34<sup>+</sup> cells. The percentages of CD15, CD14, CD11b and CD36 positive cells in either untransduced cells or in transduced GFP<sup>+</sup> cells are shown (n=3). **(D)** LTC-IC assay with MIGR1, p65 or IKK2SE transduced cord blood CD34<sup>+</sup> cells. After 5 weeks of MS5 co-culture, the medium was changed to methylcellulose for another 2 weeks. At week 7 colonies were scored. An average of three independent experiments is shown. Error bars denote the SEM.

influence of constitutive NF- $\kappa$ B activation on hematopoiesis of primary human CD34<sup>+</sup> cells.

Transfection experiments in 293T HEK cells demonstrated that overexpression of wild-type (wt) p65 or a constitutively active IKK-2 (IKK2SE), a kinase upstream of NF- $\kappa$ B,<sup>8</sup> is sufficient to activate an NF- $\kappa$ B responsive luciferase reporter construct (Figure 1A), which is not activated in control-transfected cells (MIGR1). A TF-1 cell-line, stably transfected with p65, demonstrated enhanced DNA-binding of p65 in EMSA as well as enhanced NF- $\kappa$ B luciferase reporter activity (2.6-fold greater DNA binding for p65 compared to the level in mock transfected cells, *data not shown*). In addition, quantitative polymerase chain reaction analysis for the NF- $\kappa$ B target gene *IL8* demonstrated a 12-fold increase in *IL8* mRNA expression in p65-expressing TF-1 cells (*data not shown*). Together these data demonstrated that overexpression of either p65 or IKK2SE results in an activated NF- $\kappa$ B signal transduction pathway.

Long-term co-cultures of transduced cord blood (CB)-derived CD34<sup>+</sup> stem- and progenitor cells on MS-5 stromal cell layers indicated that neither p65 nor IKK2SE-transduced cells had a proliferative advantage, as demonstrated by stable percentages of green fluorescence protein (GFP) (Figure 1B, dotted lines) compared to MIGR1-transfected cells. Cumulative cell counts from these cultures gave comparable results (Figure 1B, solid lines). Parallel flow-cytometric analysis demonstrated no significant changes in the percentage of GFP<sup>+</sup> cells positive for the myeloid differentiation markers, CD11b, CD14, CD15 and CD36, when compared to either GFP<sup>+</sup> cells within the same culture or to MIGR1-transfected cells (N=3, a representative example is shown in Figure 1C). In addition, progenitors were enumerated in limiting dilution CFC assays. IKK2SE transduction produced no significant advantage compared to MIGR1 transduction in progenitor frequencies (14.5% vs. 13.7%; respectively; *data not shown*). In separate CFU-GM and BFU-E assays also no differences were observed (*data not shown*).

In order to investigate whether active NF- $\kappa$ B affected the self-renewal capacity of hematopoietic stem cells, LTC-IC assays were performed with MIGR1-, p65- and IKK2SE-transduced CB CD34<sup>+</sup> cells, by adding methyl-cellulose to a week 5 MS-5 co-culture (as described previously)<sup>9</sup> and determining the number of colonies at week 7. No significant differences in colony formation were detected between p65-, IKK2SE- or MIGR1-transduced cells (Figure 1D). Together these findings indicate that constitutive activation of NF- $\kappa$ B is not sufficient to change the differentiation potential of human CD34<sup>+</sup> cells. Others have shown that a reduction of NF- $\kappa$ B activity in murine fetal liver cells or ES-derived hematopoietic progenitors results in severely disturbed myeloid differentiation.<sup>6,7</sup> Apparently NF- $\kappa$ B activation is required for normal myelopoiesis, but its increased activity is not sufficient to impair differentiation.

In normal and leukemic stem and progenitor cells it has been demonstrated that NF- $\kappa$ B antagonizes (ROS-mediated) apoptosis.<sup>3,7</sup> In contrast, we did not detect lower levels of apoptosis (assessed by annexin V staining, *data not shown*) in p65- or IKK2SE-transfected CB CD34<sup>+</sup> cells compared to control cultures. Additionally, interleukin-3 deprivation of p65-expressing stable TF-1 cell lines did not cause reduced apoptosis (*data not shown*). This is in line with data from Romano *et al.*,<sup>10</sup> demonstrating that constitutive NF- $\kappa$ B activity is not relevant for sustained basal cell survival of

CB CD34<sup>+</sup> and AML cells, but only for cells triggered with a stress response, e.g. exposure to chemotherapy.

Collectively, these data demonstrate that constitutive activation of NF- $\kappa$ B as a single *hit* is not sufficient to induce changes in steady-state hematopoiesis with regard to proliferation, differentiation, self renewal and apoptosis during steady-state hematopoiesis, which could potentially shift cells towards a more leukemogenic phenotype. Whether constitutive NF- $\kappa$ B activation in concert with additional triggers has other effects on hematopoiesis needs to be further defined in order to gain insight into their respective and potentially additive roles in events leading towards AML.

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