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Role of NFKB2 on the early myeloid differentiation of CD34+ hematopoietic stem/progenitor cells ☆

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ABSTRACT

To better understand the early events regulating lineage-specific hematopoietic differentiation, we analyzed the transcriptional profiles of CD34+ human hematopoietic stem and progenitor cells (HSPCs) subjected to differentiation stimulus. CD34+ cells were cultured for 12 and 40 h in liquid cultures with supplemented media favoring myeloid or erythroid commitment. Serial analysis of gene expression (SAGE) was employed to generate four independent libraries. By analyzing the differentially expressed regulated transcripts between the un-stimulated and the stimulated CD34+ cells, we observed a set of genes that was initially up-regulated at 12 h but were then down-regulated at 40 h, exclusively after myeloid stimulus. Among those we found transcripts for NFKB2, RELB, IL1B, LTB, LTBR, TNFRSF4, TGFB1, and IKBKA. Also, the inhibitor NFKBIA (IKBA) was more expressed at 12 h. All those transcripts code for signaling proteins of the nuclear factor kappa B pathway. NFKB2 is a subunit of the NF-κB transcription factor that with RELB mediates the non-canonical NF-κB pathway. Interference RNA (RNAi) against NFKB1, NFKB2 and control RNAi were transfected into bone marrow CD34+HSPC. The percentage and the size of the myeloid colonies derived from the CD34+ cells decreased after inhibition of NFKB2. Altogether, our results indicate that NFKB2 gene has a role in the early commitment of CD34+HSPC towards the myeloid lineage.

1. Introduction

Hematopoiesis is a highly regulated process resulting in the formation of all blood lineages from the multipotential hematopoietic stem/progenitor cells (HSPCs) which possess the potentials of self-renewal, proliferation, and differentiation towards different lineages of blood cells(Mao et al., 1998).

The CD34 antigen is a surface glycophosphoprotein expressed on developmentally early lymphohematopoietic stem and progenitor cells, endothelial cells and embryonic fibroblasts and it is a hallmark of HSPC (Mayani and Lansdorp 1998; Krause et al., 1996). The earliest multilineage stem cell is CD34+, MDR-1+, c-kit+, and CD45RO+, but negative for CD38 and HLA-DR (Burt, 1999). CD34 antigen is found on progenitor cells that are already committed towards lineage specificity; within this CD34+ population resides a subset of multipotent stem cells capable of myeloid, lymphoid, erythroid, or megakaryocyte commitment (Sutherland et al., 1993). Progenitor cells expressing the surface antigen CD34 are also capable of long-term B lymphopoiesis and myelopoiesis *in vitro* and mediate T, B, and myeloid repopulation of human tissues implanted into SCID mice (DiGiusto et al., 1994; Gao et al., 2001).

Although much knowledge about hematopoietic stem cells has been gained, we still do not know much about the genetic mechanisms determining their development. One of the major questions in stem cell biology remains that the identification of the mechanisms by which a multipotent cell selects a particular differentiation pathway.

Several studies have been carried out to address the role of hematopoietic cytokines in this process (Phillips et al., 2000). However, it has been shown that the whole process of hematopoietic differentiation is orchestrated at the molecular level by a complex network of transcription factors that act by regulating the expression of a large set of target genes (Testa, 2004). As the phenotype of any given cell is ultimately the

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product of the genes, which is or has been expressed during the course of its development, describing the complete gene expression programs of self-renewing and differentiating cells is an approach that addresses how self-renew and differentiation are regulated (Liu et al., 2007).

Efforts to understand the molecular mechanisms underlying the differentiation of hematopoietic stem/progenitor cells into mature blood cells have focused so far on late events that largely reflect the differentiated state of the cell (Ferrari et al., 2007; Komor et al., 2005). In order to evaluate early changes of the gene expression profile of HSPC subjected to differentiation stimuli, we used serial analysis of gene expression (SAGE) to generate transcriptional profiles of immuno-magnetically sorted bone marrow CD34+ HSPC, before and after 12 and 40 h of culture in conditions favoring either myeloid or erythroid commitment. We observed a set of genes that was initially up-regulated at 12 h but then it was down-regulated at 40 h, exclusively after myeloid stimulus. Functional studies confirmed the early involvement of the NF-κB pathway in the commitment of CD34+ cells towards the myeloid differentiation.

2. Materials and methods

2.1. CD34+ hematopoietic and progenitor cells

Bone marrow iliac crest aspirates from healthy adult donors were collected after an informed consent was obtained, approved by the local Institutional Review Board. CD34+ cells were isolated to $>95\%$ purity by MACS Indirect CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec), following the manufacturer's instructions. Cell purity was determined by flow cytometry using anti-CD34-PE and anti-CD45-PerCP (BD Pharmingen) and data were acquired on a FACScan system (Becton Dickinson).

2.2. Differentiation assay

We used two different culture conditions to induce the differentiation process. For myeloid differentiation we used StemSpan (SteamCell Technologies, No. 09600) supplemented with 20% FBS, SCF (100 ng/ml), Flt-3lig (50 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml) and GM-CSF (20 ng/ml), whereas for erythroid differentiation we used StemSpan supplemented with 20% FBS, IL-3 (0.1 ng/ml), GM-CSF (1 ng/ml) and erythropoietin (3 U/ml). After 12 and 40 h of liquid culture, cells were collected and an aliquot of each sample was cultivated for 14 days in MethoCult-GF H4434 (StemCell Technologies, No. 04444) in order to examine the proportion of each type of colonies formed: BFU-E (burst forming unit-erythroid), CFU-GM (colony forming unit-granulocytic/monocytic) and CFU-Mix (mixed colony). All colonies containing more than 30 cells were scored as a colony-forming unit.

2.3. RNA extraction

The remaining cells, collected at 12 or 40 h of differentiation culture assays, were submitted to RNA extraction to construct the SAGE libraries. Four distinct libraries were constructed: myeloid for 12 h (M12) and 40 h (M40), erythroid for 12 h (E12) and 40 h (E40). Total RNA was prepared using TRizol® LS Reagent (Invitrogen Corporation), following manufacturer's instructions; RNA was obtained from a total of 8×10^6 polled cells for each library. Additionally, RNA obtained from CD34+ cells without any differentiation stimulus was used as a control (Panepucci et al., 2007).

2.4. SAGE transcriptomes

Twenty-eight micrograms of total RNA was used for each SAGE library, carried out by the I-SAGE™ Kit (Invitrogen Corporation, Cat no. T5001-01), following the manufacturer's instructions. Tag frequency tables were obtained from sequences by the SAGE™ analysis software with minimum tag count set to 1 and maximum ditag length set to 28 bp; the other parameters were set as default. The annotation was based on CGAP SAGE Genie (http://cgap.nci.nih.gov/SAGE). A SAGE library of un-stimulated CD34 BM cells, previously constructed in our laboratory (Panepucci et al., 2007), was used as a reference to compare the stimulated CD34 samples. For the analyses, the number of tags in each library were normalized to a total count of 300,000 tags. Statistical analyses were carried using the software SAGEstat (Ruijter et al., 2002) and a maximum p-value of 0.01 was used to consider a gene as differentially expressed. The SAGE data were further analyzed, through the use of PathwayStudio 4.0 (Ariadne Genomics Inc.) to search for meaningful biological interactions.

2.5. RNA-interference (RNAi) assay

siRNA mediating inhibition of gene expression was carried out using Stealth RNAi duplex oligoribonucleotides for NFKB1 and NFKB2 genes (Invitrogen corporation, Cat. no. 1299003—set of three oligos for each gene) and Stealth RNAi Negative Control (Invitrogen corporation, Cat. no. 12935-200); transfection was performed using Lipofectamine 2000 (Invitrogen Corporation, Cat. no. 11668-019). All the procedures were carried out following the manufacturer's instructions.

siNFKB1, siNFKB2 and negative control siRNA were transfected into CD34+ HSPC. RNAi assay was carried out in duplicate. Cells submitted to transfection with siRNAs and intact cells were cultured in the same condition that stimulates the myeloid differentiation process; after 72 h of culture they were subjected to evaluation by real-time PCR and an aliquot of the cells were cultured on methylcellulose.

2.6. Quantitative polymerase chain reaction

To confirm SAGE data and to measure gene expression of the siRNA experiments, real-time polymerase chain reaction was performed. Total RNA (0.5 μg) was processed directly to cDNA by reverse transcription using High Capacity cDNA Archive Kit (Applied Biosystems) following manufacturer's instructions.

Real-Time PCR to validate the SAGE libraries was performed in duplicates for 13 genes and, were carried out with TaqMan probes and Master Mix (Applied Biosystems). To normalize sample loading, the differences of threshold cycles (ΔCt) were derived by subtracting the Ct value for the internal reference (GAPDH), from the Ct values of the evaluated genes. The relative fold value was obtained by the formula $2^{-\Delta\Delta Ct}$ using the ΔCt value of un-stimulated CD34+ sample as a reference, ΔΔCt was calculated by subtracting the reference ΔCt from the ΔCt values of the samples.

Real-time PCR to confirm the knock-down efficiency was performed for NFKB1 and NFKB2 genes. TaqMan probes and Master Mix (Applied Biosystems) were also employed as well as the $2^{-\Delta\Delta Ct}$ method and GAPDH as internal reference. The ΔCt value of CD34+ without siRNA transfection was used as a reference, ΔΔCt was calculated by subtracting the reference ΔCt from the ΔCt values of the siRNA transfected samples. Statistical significance of difference between the siRNA samples and the control siRNA was determined by unpaired Student's t-test.

2.7. Western blotting

For Western blotting analysis, proteins were extracted from TRizol® LS Reagent (Invitrogen Corporation), following manufacturer's instructions. Protein concentration was determined using the BCA Protein Assay Kit (Pierce). Equal amounts (50 μg) of total cellular protein were separated into 5–20% gradient SDS-PAGE and electrophophoretic transferred to PVDF membrane (Amersham Biosciences). The membrane was blocked with 5% nonfat dry milk in TBST for 1 h at room temperature, incubated overnight at 4 °C with primary antibodies anti-NFKB1, anti-NFKB2 (1:1000, Santa Cruz Biotechnology), and anti-γ-tubulin (1:2000, Sigma) then, incubated for 1 h at room temperature with the peroxidase-conjugated secondary antibody. The protein band was revealed by chemiluminescence (Western Lightning™, Perkin-Elmer) and signals were visualized using radiographic films. Western blotting experiments were done in duplicate.

3. Results

3.1. Colony-forming assay

CD34+ HSPC cells generate about equal percentages of erythroid (BFU-E) and myeloid (CFU-GM) colonies (Van Epps et al., 1994), as observed here when we cultured un-stimulated CD34+ cells in semi-solid cultures, and observed 52.4% of BFU-E and 47.6% of CFU-GM colonies. Table 1 shows the percentages obtained in the colony-forming assay. The results indicate that after 12 and 40 h of treatment, the genetic program of those cells were shifted towards the expected differentiation pathway. Additionally, after stimulation not only the number of colonies increased, but we observed colonies with more cells as well. Fig. 1 shows the morphological aspect observed in the colony-forming assay.

3.2. SAGE analysis

A total of four SAGE libraries were analyzed. More than 60,000 tags were sequenced for each library, representing more than 19,000 unique tags that could be mapped to more than 12,000 distinct UniGenes (full data available at the Gene Expression Omnibus site). Gene expression profiles of the cells submitted to 40 h of induction were compared to those obtained with 12 h of induction and to un-stimulated CD34 BM cells (Panepucci et al., 2007). A total of 12 sets of transcripts (SAGE tags) were selected based on their behavior at different treatment conditions or different time points (Fig. 2 and supplemental file 1). In this report we focused on the set of genes that were initially up-regulated (12 h) followed by down-regulation (40 h) upon myeloid stimulation. We focused on this set of early modulated genes in order to identify primary signaling mechanisms involved with the development and commitment, instead of genes involved with specialized functions of the cells from the myeloid lineage.

3.2.1. Transcripts exclusively modulated upon myeloid differentiation

Upon myeloid differentiation, transcripts of many inter-related proteins are initially up-regulated followed by a latter down-regulation. The majority of these transcripts are involved in the NF-κB signaling pathway. Table 2 shows the expression values obtained by the SAGE technique for the transcripts of the NF-κB signaling pathway; it is worthy to notice the up-regulation at 12 h for the majority of the NF-κB pathway genes and for some of its related genes. These results suggest an involvement of this pathway with the early commitment of BM HSPC to the myeloid differentiation process. NF-κB complexes are composed of regulatory subunits, NFKB1 and NFKB2, and transcriptionally active subunits RELA and RELB (Beinke and Ley, 2004). NFKB1 and NFKB2 were found more expressed at 12 h followed by a down-regulation at 40 h. Furthermore, NFKB2 showed a more significant differential expression between these two culture times. When analyzing the other subunits, RELB was the only expressed active subunit found in our myeloid libraries. The set of differentially expressed genes in myeloid libraries included both subunits of the non-canonical NF-κB pathway, NFKB2 and RELB, responsible for the sustained activation of the NF-κB signaling (Beinke and Ley, 2004). Factors known to be involved with the non-canonical NF-κB signaling pathway were found following the same pattern of being up-regulated at 12 h and down-regulated at 40 h in the myeloid libraries. Among these transcripts we found IL1B, TNFRSF1B, TNFRSF4 and TNFSF13B. Agonist factors like TGFB1, LTB, FGF2 also presented the same up-regulation at 12 h as well as the receptor for LTB, LTBR. NFKBIA and NFKBIB, inhibitors of NF-κB signaling pathway, were, respectively, down-regulated at both time points and not expressed; in spite of NFKBIA being down-regulated, this gene showed important expression at 12 h.

Table 1
Percentages of erythroid and myeloid colonies obtained in colony-forming assays following different treatments on the CD34+ cells and siRNA experiments.

Differentiation treatment (duration)	Colonies at day 14, differentiation assay			
	Total number of colonies	BFU-E % (std. dev.)	CFU-GM % (std. dev.)	CFU-Mix % (Std Dev)
Purified CD34+ cells[a]				
Un-stimulated CD34+	600	52.4 (1.9)	47.6 (1.9)	0 (0)
Myeloid (12 h)	1190	40.9 (7.2)	58.0 (7.3)	1.1 (1.4)
Myeloid (40 h)	2506	23.4 (3.6)	62.3 (2.9)	14.3 (1.2)
Erythroid (12 h)	1128	61.8 (4.1)	37.9 (4.5)	0.3 (0.6)
Erythroid (40 h)	3046	86.3 (4.8)	13.7 (4.8)	0 (0)
siRNA transfected CD34 cells—experiment 1[b]				
Control siRNA	871	417 (47.9)	454 (52.1)	0
siNFKB2	665	447 (67.2)	218 (32.7)	0
siNFKB1	1115	540 (48.4)	575 (51.5)	0
siRNA transfected CD34 cells—experiment 2[b]				
Control siRNA	539	256 (47.4)	283 (52.5)	0
siNFKB2	476	296 (62.1)	180 (37.8)	0
siNFKB1	462	203 (43.9)	259 (56.0)	0

[a] The percentage values for purified CD34+ samples represent the average of four independent experiments and standard deviation between parentheses.
[b] Total colony counts for both siRNA experiments are shown and percentages between parentheses.

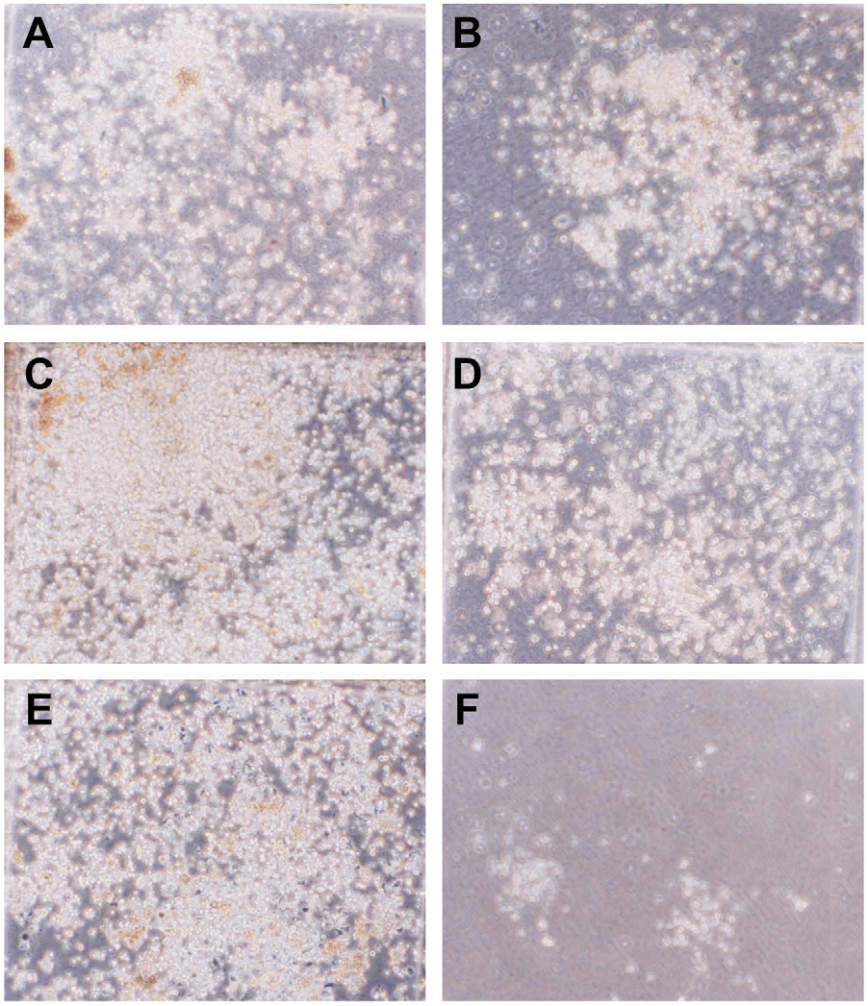

Fig. 1. Bone marrow CD34+ HSPC colony forming assay. Aspects of the colonies formed after 14 days of culture in methylcellulose: (A) CD34+ cells cultured without any previous differentiation treatment, (B) CD34+ cells cultured for 12 h inducing myeloid differentiation, (C) CD34+ cells cultured for 40 h inducing myeloid differentiation, (D) CD34+ cells transfected with negative control siRNA were cultured in medium allowing myeloid differentiation for the RNAi experiments; (E) CD34+ cells transfected with siNFKB1 were cultured in medium allowing myeloid differentiation for the RNAi experiments and (F). CD34+ cells transfected with siNFKB2 were cultured in medium allowing myeloid differentiation for the RNAi experiments. All pictures are shown in 40 × magnification.

The differential expression observed between the 12 and 40 h culture times was validated by real-time PCR on additional BM CD34+ samples obtained separately that have received the same culture treatments as the samples used for the SAGE technique. Both for myeloid and erythroid samples, all the evaluated genes followed the same expression pattern showed by the SAGE technique; we observed the same 12 h up-regulation followed by the 40 h down-regulation for the myeloid samples (Fig. 3) and, a smaller expression variation when analyzing erythroid samples (data not shown).

3.2.2. *Other genes, functional categories and signaling pathways of transcripts modulated upon myeloid differentiation*

Among the transcripts initially up-regulated at 12 h and down-regulated at 40 h under myeloid differentiation, we find many coding for ribosomal proteins. In addition, TPT1 (TCTP/p23) and EEF1B2 transcripts, which are involved in the process of translation elongation (Cans et al., 2003), also followed this pattern, indicating an up-regulation of the translation machinery, preceding differentiation. A similar expression pattern was also saw for Nucleophosmin (NPM or Nuclear Phosphoprotein B23), a protein initially characterized as a nucleolar protein functioning in the processing and transport of ribosomal RNA (Borer et al., 1989). NPM is found to be more abundant in growing cells and is down-regulated in cells undergoing differentiation or apoptosis and was recently found to antagonize stress-induced apoptosis by negatively modulating p53 protein in hematopoietic precursors through a direct interaction (Li et al., 2005). Interestingly, the activation of p53 leads to a strong down-regulation of TPT1 (Cans et al., 2003). Both, the activation of p53 or the targeted down-expression of TPT1 (by interfering RNA), lead to apoptosis (Cans et al., 2003). Thus, our data is in agreement with a common response to differentiation stimuli involving an initial proliferative phase associated with protective anti-apoptotic machinery. In addition, CCL2 (MCP-1), a NF-kB transcription target (Martin et al., 1997) that is up-regulated and depends on the interaction with IL1B (Gavrilin et al., 2000), is also initially up-regulated after granulocytic differentiation stimuli. Furthermore, the receptor for CCL2, CCR2, is also co-regulated at these time points. CCL2 is known to exert an inhibitory effect on the proliferation of myeloid progenitors, but also enhances their survival (Reid et al., 1999).

Using ProbFAST (Silva et al., 2010) we performed the functional classification for the genes that were initially up-regulated followed by down-regulation upon myeloid stimulation. Under the Cellular Component term, the genes most commonly expressed were involved in nucleus (546 genes), cytoplasm (516 genes), membrane (308 genes), integral to

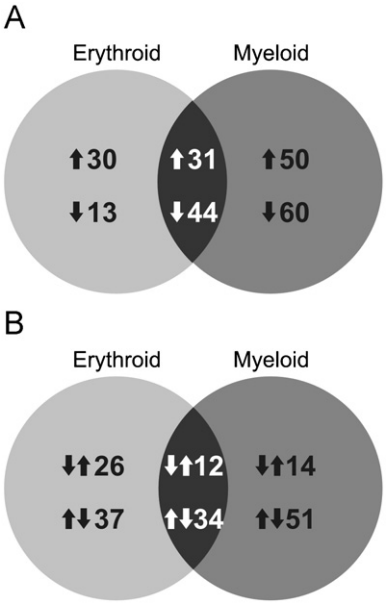

Fig. 2. Twelve gene sets were selected based on the SAGE data. The gene sets of transcripts (SAGE tags) were selected based on their behavior at different treatment conditions (myeloid or erythroid) or different time points (12 or 40 h): (A) up-regulated and down-regulated at both time points and (B) alternated expression between 12 and 40 h. The number of tags for each gene set is shown between parentheses. ↑: up-regulated; ↓: down-regulated.

membrane (252 genes) and intracellular (183 genes); under the Molecular Function term, the genes most commonly expressed were involved in protein binding (592 genes), metal ion binding (264 genes), zinc ion binding (233 genes), nucleotide binding (220 genes) and ATP binding (154 genes); under the Biological Process term, the genes most commonly expressed were involved in regulation of cellular transcription (107 genes), signal transduction (98 genes), regulation of cellular transcription, DNA dependent (67 genes), cell cycle (54 genes) and oxidation reduction (53 genes). Kim et al. (2009) carried out a similar Gene Ontology (The Gene Ontology Consortium, 2000) analysis as they performed a global functional classification for the genes expressed in $CD34^+$ cells. They reported that the broad functional categories found in CD34+ cells is due to the fact that these cells execute many basic biological activities. On the other hand, the Gene Ontology profile found at our differentiated $CD34^+$ cells indicated more specialized functions/processes which is expected for the stimulated CD34+ cells reflecting the commitment of these cells to the myeloid differentiation process.

We also identified other signal transduction pathways involved in the myeloid differentiation. The signaling pathways involving more expressed genes were: the MAPKinase, the EGF, the IGF-1, the IL-4, the FGF, the Sonic Hedgehog and the Sterol Biosynthesis signaling pathways. Kim et al. (2009) searched for genes involved in signal transduction pathways in $CD34^+$ cells. We have found in common the MAPKinase and the Sonic Hedgehog signaling pathways. Again, the difference observed in

Table 2
Gene expression values obtained by SAGE technique for the NF-κB signaling pathway components (gene list were obtained from BioCarta Pathways at CGAP).

Symbol	Unigene (Hs)	Best tag	Number of SAGE tags (normalized to 300,000)				
			CD34+	Myeloid 12	Myeloid 40	Erythroid 12	Erythroid 40
NFKB1	654,408	GTTACAATCA	9.79	28.98	0	4.89	10.24
NFKB2	73,090	GGAAGGGGAG	39.15	173.88	14.27	53.8	56.32
RELA	502,875	GCTGAAGGAA	4.89	0	0	0	10.24
RELB	654,402	TGGGGGCACC	14.49	19.58	4.76	4.89	25.6
TNFRSF1B	256,278	ATGGAGCGCA	0	4.83	0	0	5.12
TNFRSF4	129,780	CATACCTCCT	0	53.13	0	4.89	0
IL1A	1722	GTGGTAGTAG	0	0	4.76	0	0
IL1B	126,256	CAATTTGTGT	9.79	86.94	0	14.67	15.36
TLR4	174,312	GTGCACTAGA	4.89	4.83	0	0	0
LTB	376,208	GCAGTGGGAA	112.56	241.5	23.79	122.28	112.63
LTBR	1116	CAAATAAAAA	0	33.81	19.03	53.8	30.72
TNF	241,570	TAGCCCCCTG	14.68	0	0	4.89	0
TGFB1	645,227	GGGGCTGTAT	39.15	83.72	63.43	39.12	71.67
TNFAIP3	591,338	AGCTTGTGTA	0	4.83	0	0	0
TNFRSF1A	279,594	TTACACTAAT	14.68	4.83	14.27	0	0
TNFSF13B	525,157	TCTGTAGCTA	0	3.22	0	0	0
IL8	624	TGGAAGCACT	44.04	53.13	4.76	14.67	10.24
CHUK	198,998	AAACCATTCT	0	9.66	0	0	0
FADD	86,131	GCTGGGGTGG	4.89	0	0	0	15.36
NFKBIA	81,328	TAACAGCCAG	205.54	82.11	9.52	44.02	25.6
NFKBIB	9731	GGTGGTGGGC	4.89	0	0	0	0
IKBKB	656,458	GGGCTGCCTG	0	4.83	0	0	0
IKBKG	43,505	CTATTCCATT	0	9.66	0	9.78	5.12
IL1R1	701,982	CATCACGGAT	0	9.66	0	0	15.36
IRAK1	522,819	CCCCCGTGAA	53.83	72.45	71.37	58.69	76.8
MAP3K1	657,756	CCCACCACAT	0	0	0	0	5.12
MAP3K14	404,183	GCTCCGGAGG	4.89	0	0	4.89	0
MAP3K7	702,174	CTCATTGTGT	0	0	0	0	5.12
MAP3K7IP1	507,681	GGCCTCTTCT	0	0	0	4.89	5.12
MYD88	82,116	TTTTGTACGC	0	4.83	0	0	5.12
RPAIN	462,086	AGCTGTCTGG	9.79	0	0	0	5.12
TRADD	460,996	AAGCACCTTG	0	4.83	0	4.89	5.12
TRAF6	591,983	TTGCAGCAGC	0	0	4.76	0	5.12
ICAM1	700,573	GTTCACTGCA	9.79	14.49	0	4.89	0

the gene expression of differentiated CD34$^+$ cells compared to their data may be associated to the early events of our *in vitro* myelopoiesis.

3.3. NFKB1 and NFKB2 knock-down

3.3.1. NFKB1 gene

RNA-interference was employed to confirm whether NFKB1 gene may be or not related to the decrease in the emergence of the myeloid colonies. When comparing the siNFKB1 transfected cells to the negative control siRNA transfected cells, the qPCR fold relative reduction for NFKB1 was four and three in experiments 1 and 2, respectively (Fig. 4). Western blotting data corroborated the NFKB1 silencing as we noticed a reduction in the protein band when comparing the optical densities from the siNFKB1, control siRNA and CD34+ samples upon normalization against the endogenous protein (Fig. 5). We observed that the inhibition of this gene neither reduced the percentage of the myeloid colonies (Table 1) nor caused smaller colonies formation (Fig. 1E); the average percentage of CFU-GM and BFU-E colonies of the two independent experiments shifted from 52.3% and 47.2% on the

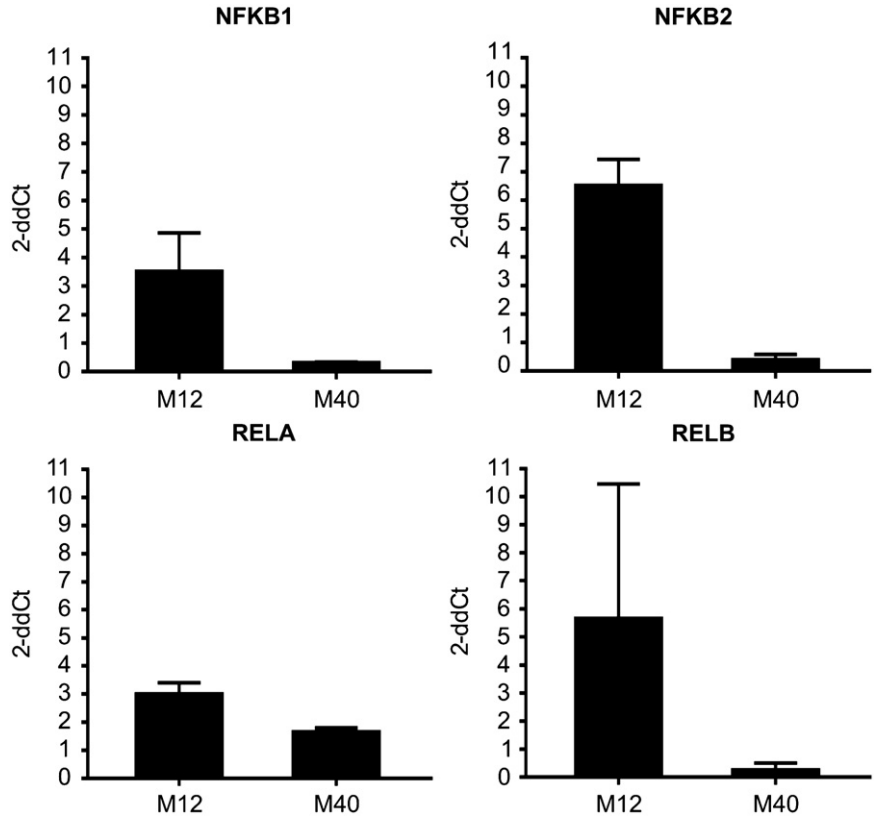

Fig. 3. Validation of the NF-κB pathway genes by Real-Time PCR. Quantitative PCR was carried out to validate SAGE data on samples cultured for 12 h (M12) and 40 h (M40). qPCR was performed in the same samples used for SAGE technique and repeated on new BM CD34+ samples that have received the same culture treatment as the samples used by the SAGE technique (called N samples in the figure). ΔΔCt was calculated by subtracting the un-stimulated CD34+ ΔCt from the ΔCt values of the stimulated samples.

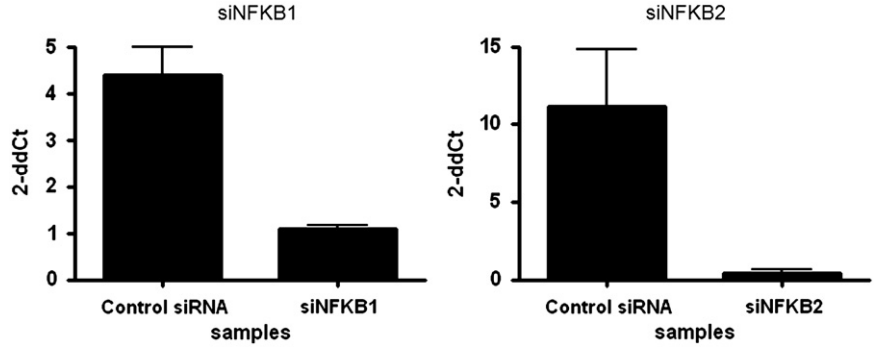

Fig. 4. Real-time PCR results for the siRNA assay. siNFKB1, siNFKB2 and negative control siRNA were transfected into CD34+ HSPC. RNAi assay was carried out in duplicate (experiments 1 and 2). Cells submitted to transfection with siRNAs and intact cells were cultured in the same condition that stimulates the myeloid differentiation process; after 72 h they were subjected to evaluation by real-time PCR. Experiments 1 and 2 revealed for siNFKB1 an average fold relative reduction of 3.5 which is statistically significant ($P < 0.05$). Experiments 1 and 2 revealed for siNFKB2 an average fold relative reduction of 10.0 which is also statistically significant ($P < 0.05$).

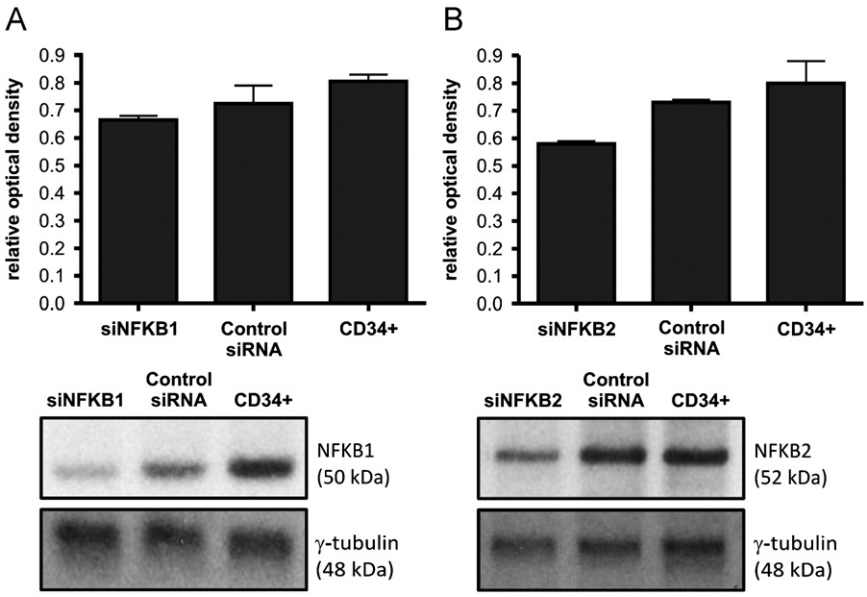

Fig. 5. Western blotting data for the siRNA assay. Western blotting assay confirmed the reduction in the siRNA protein band when comparing the densitometric values of the siNFKB1, siNFKB2, control siRNA and CD34+ samples upon normalization against the γ-tubulin protein: (A) relative protein expression of siNFKB1/γ-tubulin and (B) relative protein expression of siNFKB2/γ-tubulin.

control siRNA cells, respectively, to 53.75% and 46.15% on the siNFKB1 cells (Table 1).

3.3.2. *NFKB2 gene*

siNFKB2 was employed to confirm the role of the NFKB2 gene on the myeloid differentiation process. Real-time PCR on cells submitted to the RNAi assays confirmed, in two independent experiments, the reduction in NFKB2 transcript levels when comparing the siNFKB2 transfected cells to the negative control siRNA transfected cells. The fold relative reduction for experiments 1 and 2 was fourteen and seven, respectively (Fig. 4). As previously reported, Western blotting data also corroborated the NFKB2 silencing as we verified a similar reduction in the protein band when comparing the optical densities from the siNFKB2, control siRNA and CD34+ samples upon normalization against the endogenous protein (Fig. 5). Additionally, we observed that after the inhibition of the NFKB2 gene expression, the average percentage of CFU-GM and BFU-E colonies of the two independent experiments shifted from 52.3% and 47.2% on the control siRNA cells, respectively, to 35.25% and 64.65% on the siNFKB2 cells (Table 1). We also observed that the majority of the CFU-GM colonies after the gene inhibition were smaller with less than 30–50 cells (Fig. 1F).

4. Discussion

In this report, we suggest a novel role for NF-κB pathway in the differentiation of the primitive hematopoietic progenitors towards the myeloid lineage. We observed an early up-regulation of the central components of the alternative or non-canonical NF-κB pathway as a major characteristic of the transcripts of CD34 cells stimulated to differentiate along the myeloid pathway. Moreover, the initial up-regulation of these transcripts at 12 h was followed by an impressive down-regulation at 40 h. The inhibition of NFKB2 by siRNA decreased considerably the percentage and the size of the myeloid colonies derived from the CD34+ cells while inhibition of NFKB1 showed no effect on the emergence of the myeloid colonies.

The Rel/NF-κB family consists of five members, NFKB1, NFKB2, RelA, RelB, and c-Rel, which are maintained in the cytoplasm as dimers composed of one regulatory subunit (NFKB1 or NFKB2) and one active subunit (RelA or RelB). Dimers bind to one of the several inhibitors proteins of the IκB family. Phosphorylation of the NF-κB inhibitor protein targets it for degradation, thus enabling NF-κB dimers to translocate to the nucleus and activate gene transcription (Beinke and Ley, 2004). NF-κB signaling comprises two pathways: the classical or canonical, mediated by NFKB1 and RelA, and alternative or non-canonical, mediated by NFKB2 and RelB. NF-κB is an inducible transcription factor complex which regulates the expression of a variety of genes which are involved in the immune, inflammatory and acute-phase responses (Liptay et al., 1994). Gene-inactivation studies suggest that the genetic program influenced by the canonical NF-kB pathway codes for inflammation and protection from cell death, whereas the non-canonical NF-kB pathway seems to control genes that are essential for the correct and coordinated development of lymphatic tissues (Bonizzi and Karim, 2004).

The NF-κB transcription factors are also major regulators of programmed cell death whether via apoptosis or necrosis. NF-kB generally protects cells by inducing the expression of genes encoding anti-apoptotic and antioxidizing proteins (Dutta et al., 2006). NF-kB pathway has effects on preventing apoptosis, either by reducing the cell oxidative stress or by activating the expression of anti-apoptotic genes (Nakata et al., 2004; Grumont et al., 1998; Pyatt et al., 1999; Kucharczak et al., 2003). Also, NF-kB has already been reported as having a role in cell proliferation (Karin et al., 2002).

The colony forming assay data summarized at Table 1 showed that whereas the treatment with the siNFKB2 had little effect on the total number of BFU-E, a significant reduction of the total number of CFU-GM colonies was noticed. Thus, the reduction observed in the percentage of CFU-GM colonies reflected, in fact, a positive effect of the NFKB2 gene on myeloid differentiation, rather than a negative effect on erythroid differentiation. It remains uncovered whether the regulation of the early stage of myeloid commitment and differentiation goes through directly by the induction of myeloid progenitors or, by the protection of early myeloid progenitors from apoptosis.

This may be the first report correlating NFKB2/RelB with the early commitment of the myeloid differentiation. RelB was already reported as essential for the dendritic cell maturation (Zanetti et al., 2003) as well as a biological requirement for NFKB2 in the negative regulation of RelB-induced dendritic cell maturation was also demonstrated (Speirs et al., 2003). Furthermore, RelB/NF-κB proteins play important roles in various biologic phenomena such as immune responses, stress responses, and inflammation (Dutta et al., 2006); these proteins also control cell growth and survival of B- and T-lymphocytes (Nakata et al., 2004). Few reports describe so far the relationship between NF-κB components and differentiation of hematopoietic stem/progenitor cells. Zhang et al. (1998) suggested the possibility of Rel/NF-κB playing a role in terminal erythropoiesis, and Pyatt et al. (1999) described a role for NF-κB in maintaining survival and function in HSPCs. Nakata et al. (2004) found that Rel/NF-κB activity was required for terminal differentiation of erythrocytes, granulocytes, and megakaryocytes, and Panepucci et al. (2007) described a role for the NF-κB components on the molecular and functional differences between BM and UCB HSPCs.

Our data revealed the coordinated expression of many NF-kB pathway components, indicating an early role of this pathway in the myeloid commitment and differentiation from CD34+ HSPC. In fact, components of the NF-κB pathway, such as, TNFRSF4, IL1B, LTB, LTBR, IKBKA (CHUK), and TNFSF13B (BAFF) followed the same temporal up and down regulation pattern suggesting that all these genes may act in a coordinated way. Furthermore, recent experimental data described the role of these genes in the development of the lymphoid tissue and the differentiation of T, B and NK-cells, as well as their interactions with the non-canonical NF-κB pathway (Lian and Kumar, 2002; Gommerman and Browing, 2003; Song et al., 2004; Hauer et al., 2005; Huddelston et al., 2006; Hacker and Karin, 2006; So and Croft, 2007; Wu and Chakravarti, 2007; Giuliani et al., 2008).

The activation of the non-canonical NF-κB pathway is solely regulated by the control of the NFKB2 processing to active p52 isoform (Dejardim, 2006). The control of this process is dependent upon the presence of NF-κB inducing kinase (NIK) or the presence of a subset of receptors such as TNF-family, LTBR, CD40 (Keats et al., 2007). Looking for these receptors expression among our SAGE libraries we found out that some of the TNF-family members and LTB-R showed the up and down myeloid expression. In addition, studies using IKBKB and IKBKG (NEMO) deficient mice have demonstrated that the non-canonical NF-κB pathway functions in the absence of these two complex subunits (Solt et al., 2007). On the other hand, intact IKBKA (CHUK) is absolutely critical for activation of the non-canonical pathway in response to ligands such as BAFF, CD40, LTB (Hacker and Karin, 2006). Our data show that IKBKA and all these ligands are up-regulated at myeloid 12 h and BAFF (TNFSF13B) showed exclusively myeloid 12 h expression. Thus, the coordinated expression of these NF-kB pathway components strongly indicates a role of the NF-kB pathway in myeloid commitment and differentiation.

Based on our SAGE data we have hypothesized a model where two levels drive the early myeloid differentiation directly inducing the differentiation program or, alternatively, protecting early myeloid progenitors from apoptosis. One level would be through the canonical pathway being controlled by IKB proteins via NFKBIA binding to NFKB1-RELA complex and keeping it latent in the cytoplasm. The other level is the activation of the non-canonical pathway, which would occur in response to ligation of LTB-LTBR or TNF-member, and IKK action via IKBKA protein.

In summary, the early commitment of the myeloid differentiation process may result from an activation of the non-canonical NF-kB pathway in response to differentiation stimulus. We showed here the role of the gene NFKB2 on the myeloid differentiation process; however, further studies are required to elucidate precisely how the non-canonical NF-kB pathway really acts in order to drive the differentiation process towards the myeloid commitment and differentiation.

Author disclosure statement

No competing financial interests exist.

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Appendix A. Supplemental material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.diff.2010.07.004.

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