Impact of C-rel inhibition of cord blood-derived B-, T-, and NK cells

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\textbf{ABSTRACT}

The c-Rel transcription factor is a unique member of the nuclear factor (NF)-\textit{kB} family that has a role in curtailing the proliferation, differentiation, cytokine production, and overall activity of B- and T-cells. In addition, c-Rel is a key regulator of apoptosis in that it influences the expression of anti-apoptotic genes such as \textit{Bcl-2} and \textit{Bcl-XL}; conversely, inhibition of c-Rel increases cell apoptosis. To better understand the relationship between c-Rel expression and effects on B- and T-cell expansion, the current study evaluated c-Rel expression in cord blood mononuclear cells. This particular source was selected as cord blood is an important source of cells used for transplantation and immunotherapy, primarily in treating leukemias. As stem cell factor (SCF) and FLT3 are important agents for hematopoietic stem cell expansion, and cytokines like interleukin (IL)-2, -7, and -15 are essential for T- and B- (and also NK) cell development and proliferation, the current study evaluated c-Rel expression in cord blood mononuclear cells and CD34\textsuperscript{+} cells, as well as effects on B-, T-, and NK cells associated with alterations in c-Rel expression, using flow cytometry and PCR. The results showed c-Rel expression increased among cells cultured in the presence of SCF and FLT3 but was reduced when IL-2, IL-7, and IL-15 were used all together. Further, inhibition of c-Rel expression by siRNA reduced cord blood-derived B-, T-, and NK cell differentiation and expansion. These results indicated that with cells isolated from cord blood, c-Rel has an important role in B-, T-, and NK cell differentiation and further, that agents (select cytokines/growth factors) that could impact on its expression might not only affect immune cell profiles in a host but could potentially also limit apoptotic activities in (non-)immune cells in that host. In the context of cancer (immune)therapy, in particular, when cord blood is used an important source in stem cell transplantation in leukemia patients, such down-regulating changes in c-Rel levels could be counter-productive.

\textbf{Introduction}

C-Rel as a member of the NF-\textit{kB} transcription factor family has a limited role in the proliferation, differentiation, cytokine production, and activity of B- and T-cells (Hilliard et al. 2002; Gerondakis et al. 2006; Pasparakis 2009; Gilmore & Gerondakis 2011; Visekruna et al. 2012). In mature B-cells, c-Rel regulates cell division and survival through promotion from G1 to S phase during the cell-cycle progression. In addition, c-Rel is a key regulator of apoptosis by influencing anti-apoptotic genes such as \textit{Bcl-2} and \textit{Bcl-XL} (Kucharczak et al. 2000; Liou & Hsia 2003). In addition, a growing number of reports now indicate that c-Rel has important functions in many tumors, although studies in mice suggest that it may not always function as an oncogene. Moreover, c-Rel is a critical regulator of fibrosis and inflammation that could provide an environment for tumor development in many settings (Hunter et al. 2016).

Among the many cytokines in the body, interleukin (IL)-2, -7, and -15 are essential for hematopoiesis. Specifically, IL-2 is important for the development and proliferation of T-cells (Nelson 2004). IL-7 is essential for the development of human B cells; inhibition of IL-7 completely blocks B-cell progenitors in the pro-B stage (Grabstein et al. 1993; Parrish et al. 2009). IL-15 plays a predominant role in the survival, expansion, and proliferation of natural killer (NK) cells and also acts as an anti-apoptotic factor (with conservation by \textit{Bcl-2} and activation of nuclear factor (NF)-\textit{kB}) (Mrzek et al. 1996; McDonald et al. 1998; Giron-Michel et al. 2003; Ranson et al. 2003). Similarly, stem cell factor (SCF) and \textit{fms}-Related Tyrosine Kinase 3 (FLT3/Flik2/Stk-2; which encodes a Class III receptor tyrosine kinase) are important in hematopoietic stem cell expansion, survival, and differentiation (Hassan & Zander 1996; Ueda et al. 2000; Hofmeister et al. 2007). Several studies have shown that over-expression of FLT3 caused up-regulation of NF-\textit{kB} in acute myeloid leukemia (AML) and FLT3 inhibition induces cell apoptosis through the NF\textit{kB} pathway in AML patients (Griessinger et al. 2008, Grosjean-Raillard et al. 2008).

In previous studies, \textit{c-Rel} gene inhibition was considered a potential therapeutic approach in treating cancers (Turco et al. 2003; Pepper et al. 2009; Tian & Liou 2009). Along those lines, \textit{c-Rel} small interfering RNA (siRNA) has been shown in \textit{in vitro} studies to have a potential to be an effective tool in treating inflammation and autoimmune diseases (Pizzi et al. 2005; Sandy et al. 2005; Aigner 2006; Lorenz et al. 2014).
While it remains uncertain if c-Rel inhibition might also be useful in treating leukemias or other types of cancers, a few studies have been undertaken. For example, Shono et al. (2016) showed that a bioactive naphthalene-thiobarbiturate derivative that inhibited c-Rel (IT-901) suppressed graft-versus-host (GVH) reactions while preserving graft-versus-lymphoma effects in allogeneic transplants and during preclinical analyses for the treatment of human B-cell lymphoma. Such anti-tumor properties in vitro and in vivo were attributed to modulated redox homeostasis in lymphoma cells [but not in normal leukocytes] – resulting in oxidative stress. Those particular studies built upon earlier ones that assessed effects of another small-molecule c-Rel inhibitor (IT-603) to reduce allo-activation of T-cells without compromising their utility in subsequent anti-tumor activities (Shono et al. 2014).

Allogeneic hematopoietic stem cell transplantation (allo-HSCT), originally developed to treat patients with chemo-/radio-therapy-related bone marrow failure, represents a potent therapy for malignant/non-malignant diseases (Appelbaum 2003; Locatelli & Pagliara 2012; Holtick et al. 2014). Over the years, the concept of allo-HSCT has shifted toward a strategy of graft-versus-tumor (GVT) activity (Jenq & van den Brink 2010; Ghosh et al. 2013, 2014). Since GVH outcomes are often a complication of allo-HSCT, resulting in significant morbidity and mortality, and strategies to suppress GVH often result in broad immunosuppression (leading to immune deficiency/compromised anti-tumor surveillance), it has been important to explore targeting of molecular pathways that could accomplish separation of GVHD from GVT activity.

Cord blood is an important source in stem cell transplantation for leukemia patients (Gluckman et al. 1997; Smith & Wagner 2009). However, to date, it remains unclear whether cord blood transplantation in combination with c-Rel inhibition could potentially be applicable (or beneficial) for treatment of leukemias. Previous findings with different inhibitors of NF-kB/c-Rel activity – such as nuclear localization signal-competing peptides, decoy oligonucleotides (decoy ON), or proteasome inhibitors were conflicting (Pyatt et al. 1999; Romano et al. 1999) as the effects of some of these inhibitors often impacted on other systems apart from those related to NF-kB/c-Rel (Adams 2002). Some studies showed that phosphorothioate decoy oligodeoxynucleotides (ODN; at high doses) were able to reduce NF-kB/c-Rel nuclear levels and so enhance spontaneous/inducible apoptosis of cord blood CD34+ progenitors or acute myeloid leukemia cells (Romano et al. 2003). Unfortunately, enhanced CD34+ cell apoptosis impacted on hematopoietic cell survival and bone marrow recovery post-chemotherapeutic treatments (Romano et al. 2003), even though it was subsequently shown the NF-kB inhibition did not affect normal CD34+ hematopoietic precursors (Frelin et al. 2005).

Based on the above, it remains unclear what types of agents could successfully be used to modulate c-Rel levels in transplantable cells and, further, if such changes might yield unintentional untoward outcomes. Accordingly, unlike many of the above-cited studies utilizing natural products, decoy ON, ODN, etc., this study evaluated c-Rel expression in cord blood mononuclear cells cultured in the presence of various cytokines/growth factors known to impact on sub-populations that evolve from this source itself. The in vitro study here also investigated some of the consequences of how any induced changes in c-Rel expression could impact on apoptosis, proliferation, and expansion among cord blood-derived B-, T-, and NK cells. It was hoped such studies might help to guide future studies that could be done to amplify use of allo-HSCT and concurrently increase the potential success for GVT over any GVH arising from the transferred cells.

Materials and methods

Cell isolation

Cord blood samples were collected from a total of 22 full-term normal deliveries at the Al Zahra Hospital (Tabriz). Each sample had its volume measured and then the materials were diluted in 1:2 with phosphate-buffered saline (PBS, pH 7.4). Subsequently, mononuclear cells (MNC) were isolated by centrifugation over Ficoll (ρ = 1.077 g/ml; Sigma, St. Louis, MO). The MNC were collected, washed twice with PBS (supplemented with 10% fetal bovine serum [FBS, Gibco, Grand Island, NY]), and then re-suspended in RPMI 1640 (Gibco) supplemented with 10% FBS either for use in cultures or for freezing at −80°C. Isolates were counted and checked for viability in a hemocytometer using trypan blue staining. Cells were not pooled.

To obtain enriched CD34+ cells for use in some assays, aliquots of isolated MNC were co-incubated with 100 μl of anti-CD34-coated microbeads (Miltenyi Biotec, Berlin, Germany) for 30 min at room temperature and then passed through an LS MACS column (Miltenyi Biotec). Enriched CD34+ populations were then flushed from the column with kit-provided buffer. Purity of the CD34+ cells was evaluated by flow cytometry. Data routinely indicated that enrichment led to yields of ≈95% CD34+ cells. Again, cells were not pooled.

Monoclonal antibodies and flow cytometry

Monoclonal antibodies used in these studies were anti-CD3 (UCHT1; R&D, Minneapolis, MN) for T-cells, anti-CD20 (clone 2H7; BD Biosciences, San Jose, CA) for B-cells, anti-NKP46 (clone 9E2BD, eBioscience, San Diego, CA) for NK cells, anti-Ki67 (clone: 20Raj1, eBioscience) for proliferation, and anti-caspase-3 (BD Biosciences, San Diego, CA) for apoptosis. When analyses required flow cytometry, a FACSCalibur system (BD Biosciences, San Diego, CA) was employed and all data were analyzed using FlowJo software (Ashland, OR). In many cases, propidium iodide (1.0 mg PI/ml; Invitrogen, Carlsbad, CA) was also added to the aliquots of cells to help eliminate dead cells from assignment/counting during data analysis. All resulting FACS plots were generated based on 10 000–30 000 events/sample.

Cell cultures

The MNC from each collected sample were seeded into 96-well culture plates (at 5 × 10^5 cells/well) in 250 μl complete RPMI 1640 (RPMI containing 10% FBS, and 1% penicillin-streptomycin [Gibco, Grand Island, NY]), supplemented with SCF, FLT3 ligand (FL), IL-2, IL-7, and IL-15 (all PeproTech, Rocky Hill, NJ) – each at 50 ng/ml final concentration in well. The cells were then cultured at 37°C for 21 d; half the culture medium/well was replaced weekly. At indicated days (i.e. 7, 14, or 21), cells were harvested and RNA was isolated for use in real-time PCR analysis. In parallel, sets of MNC-bearing wells only received SCF and FLT3L.

C-rel inhibition

Inhibition of c-Rel expression in cells was obtained using siRNA (Santa Cruz Biochemi-cals, Santa Cruz, CA). In brief, MNC
and enriched CD34+ cells were cultured (in the same manner as noted above) for 14 d in the presence of SCF and FLT3L. For the inhibition of c-Rel, C-Rel siRNA was then delivered into the cells by electroporation using either of two doses (100 nM or 600 nM) of the siRNA. Standard electroporation was performed using an Amaxa Cell Line Nucleofector Kit L (Lonza, Basel, Switzerland) according to the protocols of the manufacturer. Here, 1.5 x 10⁵ cells/sample were suspended in 100 μl Nucleofector solution, siRNA was added to the suspension, and electroporation then done at 140 V (250 ms/pulse). Thereafter, the cells were harvested cells were evaluated using qPCR and flow cytometry (see below).

RNA and DNA extraction
Total RNA was extracted from harvested MNC and CD34+ cells at each timepoint using RNX-Plus Solution (RN7713C, Sinacolon, Karaj, Iran) according to the instructions of the manufacturer. In brief, 1 ml ice-cold RNX-PLUS solution was added to a tube containing 5 x 10⁵ cells and the suspension was then vortexed and incubated at room temperature for 5 min. Consequently, 200 μl chloroform was added, the sample was mixed, and the suspension incubated for 5 min on ice before undergoing centrifugation [12,000 rpm, 4°C, 15 min]. The aqueous phase was transferred to new RNase-free tube and an equal volume of isopropanol added. After mixing and incubation on ice for 15 min, the sample was centrifuged at 12,000 rpm [4°C, 15 min]. The resulting supernatant was discarded and 1 ml 75% ethanol was added to dislodge the pellet. After another centrifugation (4°C, 8 min, 7500 rpm), the supernatant was discarded and the pellet then air-dried at room temperature before being re-dissolved in 20 μl DEPC-treated water. Spectro-photometry (260 and 280 nm) was used to assess purity of the isolated RNA samples.

cDNA synthesis and real-time PCR
Reverse transcription was carried out using a Thermo Scientific RevertAid™ First Strand cDNA Synthesis Kit (K1622; Fermentas, Waltham, MA). For each reaction, 1 μg RNA was used for the first-strand cDNA synthesis (in a total volume of 20 μl) according to manufacturer guidelines. In brief, each reaction tubes was incubated at 42°C for 60 min, then at 70°C for 5 min, and then at 4°C for 5 min. For every reaction set, one RNA sample was prepared without RevertAidMMuLV reverse transcriptase (RT reaction) to provide a negative control in the subsequent PCR. All PCR were performed using a RotorGene™ 6000 HRM system (Corbett Research, Brisbane, Australia) in a total volume of 20 μl containing Power SYBR Green master mix (2X) (Takara, Tokyo, Japan), primer (0.4 μM), cDNA (20 ng/μl), plus nuclease-free water. The mRNA-specific primers were designed using Oligo 7 software (v. 7.52, Molecular Biology Insights, Cascade, CO). Sequences of the primers used are listed in Table 1. Both GAPDH (endogenous housekeeping gene) and c-REL amplification were done in triplicate for each sample.

| Number          | Gene   | Primer pair sequence (5'3')   | Product length (bp) |
|-----------------|--------|-------------------------------|--------------------|
| XM_011533010.1  | c-REL  | GAATCAATCCATCTAATGGTCCC       | 125                |
| NM_001289746.1  | GAPDH  | CAGATGATCAAAGCCCGTCC          | 166                |

Forty thermal cycles were performed in the following order: 10 min at 95°C (holding temperature), 40 cycles, 95°C for 15 s, 59°C for 35 s, and 72°C for 20 s. PCR data were analyzed using Rotor-Gene 6000 Software (version: 1.7) to determine CT values. Delta CT (ΔCT) values were calculated in relation to GAPDH CT values by the 2−ΔΔCT method, in which ΔCT represents the difference between the CT value of c-REL genes and the CT value of GAPDH.

Statistical analysis
All data are presented as means ± SD. Data were analyzed using a by one-way analysis of variance (ANOVA) and a Tukey test. All analyses were performed using by Prism software (v.6.0, GraphPad Software, San Diego, CA). Significance was assigned at a p value <0.05.

Figure 1. c-Rel expression, qPCR was used to evaluate c-rel expression at different timepoints. (A) Cells from individual donors were cultured in the presence of SCF + FLT3 + IL-2 + IL-7 + IL-15. (B) Cells were also cultured in presence of SCF + FLT3 only. Values shown are means ± SD from four independent experiments; each experiment was performed (in triplicate) using cells from 22 separate individual samples isolated from cord blood (a total of 66 wells analyzed for [A] and 66 wells analyzed for [B]). *p < 0.05, **p < 0.01, or ***p < 0.001.

Figure 2. Inhibition of c-rel by c-Rel siRNA. Cord blood MNC were cultured for 14 days in the presence of SCF + FLT3 only before (A) 100 or (B) 600 nM c-Rel siRNA was transferred into the cells by electroporation. After a 24-h incubation, c-Rel mRNA expression was evaluated by qPCR. Values shown are means ± SD from independent siRNA treatments of separate sets of MNC isolated from five different/randomly selected cord blood samples.
Results

Effects of select cytokines/factors on c-Rel expression

With blood mononuclear cells, when all the tested cytokines/factors (i.e. SCF, FLT3, IL-2, -7, and -15) were used in culture at the same time, a decrease in c-Rel gene expression was observed over the period from Day 7 to Day 21 (Figure 1(A)). c-Rel expression was reduced from 2.48 on Day 7 to 0.50 on Day 14 to 0.05 on Day 21. However, expression among cells that had received SCF and FLT3 only was 0 on Day 7, increased to 6.7 on Day 14, but then declined to 2.4 on Day 21 (Figure 1(B)). In both exposure scenarios, these values all significantly differed from one another (at a minimum of \( p < 0.05 \)).

c-Rel siRNA impact on c-Rel expression in cord blood parent MNC

As was shown in Figure 1(B), the expression of c-Rel in the isolated MNC (when only receiving SCF and FLT3) was highest on day 14. When small interfering RNA (siRNA) was used to inhibit c-Rel expression in these cells at that day of culture, i.e. by adding 100 nM c-Rel siRNA to cultures for 24 hr, relative expression of the c-Rel gene was reduced by \( \approx 70\% \) (Figure 2(A)). In comparison, c-Rel expression was even more repressed in the cells due to 24 h treatment with 600 nM c-Rel siRNA (reduction from control was now \( \approx 93\% \); Figure 2(B)).

c-Rel siRNA impact on cord blood B-, T-, and NK cell development

To clarify the effects of c-Rel siRNA on B-, T-, and NK cell development, the levels of each cell type in cultures were evaluated using CD20, CD3, and NKp46 antibodies, respectively. As can be seen in Figure 3, blocking of c-Rel expression led to decreases in relative levels of B-cells among all the cells from 12.2 \( \pm 0.9\% \) in cultures without siRNA to 2.9 \( \pm 1.6\% \) in cultures with siRNA treatment. For the T-cells, the reduction was from 33.7 \( \pm 1.0\% \) in cultures without siRNA to 6.4 \( \pm 1.2\% \) in cultures with siRNA. For NK cells, the reduction was dramatic, from 11.9 \( \pm 0.9\% \) in cultures without siRNA to 0.6 \( \pm 0.2\% \) in cultures with siRNA. Each change was significant (\( p < 0.05 \)).
Effects of c-Rel status on B-, T-, and NK cell differentiation among cord blood CD34<sup>+</sup> cells

To determine the effect of c-Rel silencing on cord blood stem cells, isolated/enriched CD34<sup>+</sup> cells were cultured for 14 d with SCF and FLT3L. As above, the cells then were or were not treated with c-Rel siRNA for 24 h. As can be seen in Figure 4, blocking of c-rel expression led to significant decreases in relative levels of B-cells among all the cells, from 12.6 ± 0.7% in cultures without siRNA treatment to 5.6 ± 0.7% in cultures with siRNA treatment. For T-cells, the level of reduction was significant, from 30.8 ± 1.2% in cultures without siRNA to 10.2 ± 0.4% in cultures treated with siRNA. For NK cells, the reduction was not significant, i.e. from 13.0 ± 0.1% in cultures without siRNA to 10.4 ± 0.6% in cultures treated with siRNA.

Effect of c-rel siRNA on proliferations and apoptosis

To identify the impact of c-Rel inhibition on proliferation and/or apoptosis of cord blood mononuclear and CD34<sup>+</sup> cells, each cell type was cultured as above with SCF and FLT3L for 14 d and then treated with 100 nM or 600 nM c-Rel siRNA. After 24 h, anti-Caspase-3 and anti-Ki67 antibodies were used to assess levels of apoptosis and proliferation, respectively, among the treated cells (Figure 5(A)).

The data also showed that when 100 nm siRNA was used to treat the starting MNC, Ki67 expression was unchanged (consistently values of 6–8%) (Figure 5(B)), indicating no effect on proliferation of the cells. In contrast, caspase 3 expression (indicator of apoptosis) decreased from 13.7 ± 1.0% to 7.7 ± 1.0% among the MNC (Figure 5(C)). On one hand, results were almost identical with the enriched CD34<sup>+</sup> cells (data not shown). When 600 nM siRNA was used, Ki67 expression was reduced to <4% among the starting MNC (Figure 5(C)). On the other hand, and unlike what was seen at the 100 nM dose, caspase 3 levels did not change significantly from control values (Figure 5(C)).

Discussion

The c-Rel transcription factor is a unique member of the NF-κB family that has important roles in many aspects of lymphoid cell
function. This study showed an increase in the expression of c-Rel in the presence of SCF (stem cell factor) and Flt3, but a reduction if a certain combination of cytokines (i.e. IL-2, -7, and -15) was present. Inhibition of c-Rel expression reduced cord blood-derived B-, T-, and NK cell levels among the isolated MNC populations; however, the inhibition of c-rel seemed to be related to whether the changes were either due to impacts on proliferation versus any due to apoptosis. It has been shown that Flt3 can lead to activation of NF-κB transcription factors (via IKK (IkB kinase) phosphorylation) via the canonical NF-κB activation pathway (Takahashi et al. 2005). Studies have also shown that FLT3 and SCF are important in myeloid/lymphoid progenitor cell development and promote their proliferation, differentiation, and survival (Keller et al. 1995; Griessinger et al. 2007). According to one study, activation of SCF leads to activation of NF-κB via MAP kinase pathways (da Silva et al. 2003).

Previous studies have shown the role of c-Rel in B- and T-cell development as well as in growth and cell survival (Gilmore et al. 2004). It was also shown that c-Rel plays an important role in the induction of cytokines, including the production of IL-2 from T-cells (Kontgen et al. 1995). IL-2 and IL-15 cytokines stimulate the proliferation of B- and T-cells and are involved in the development of NK cells (Waldmann et al. 2001). Previous studies showed that IL-7 is an essential cytokine for T- and B-cell development; a lack of IL-7 leads to a severe reduction in the numbers of T- and B-cells in situ (Kikuchi et al. 2008).

Localized c-Rel in the cytoplasm stays active by binding IκB; however, activation of IkB kinase through pro-inflammatory cytokines stimulates c-Rel activity. This activation of c-rel transcription factors could be an important reason underlying the abilities of the c-Rel to impact on proliferation and apoptosis – most likely via induction of MnSOD, Bcl2, TRAF1, TRAF2, and Bcl-x proteins whose genes have been previously identified as Rel targets (Bernard et al. 2001, 2002).

The present results showed that due to the role of c-Rel in cell cycle and cell division following stimulation of B-cell receptors (BCR) and T-cell receptors (TCR) (Gilmore & Gerondakis 2011), an inhibition of c-Rel expression (here by silencing of its gene with siRNA) led to reductions in levels (percentages) of B- and T-cells among cultured cord blood MNC. Somewhat similar findings were also noted among enriched isolated cord blood CD34+ cells. As these are the first studies of this kind, at this time we have no literature against which to compare our outcomes.

Figure 5. Effects of c-Rel inhibition on proliferation and apoptosis among MNC. Cells were cultured with SCF + FLT3 + IL2 + IL7 + IL15 and treated on day 14 with 100 or 600 nM siRNA (or not), and then evaluated 24 h later. (A) Representative FACS profile; (B) Percentage of proliferating cells (based on Ki67 expression). (C) Percentage of apoptosis/likely apoptotic cells (caspase3). Values shown are mean ± SD from experiments using separate sets of cells isolated from 12 different randomly selected cord blood samples. *p < 0.05.
Genomic and cytogenetic studies showed there was an overexpression of c-rel in different types of lymphoma and some leukemia (Hunter et al. 2016). It is thus possible that inhibition of c-Rel could play an important role in cancer therapy. As cord blood transplantation is often used for treatment of differing types of leukemia, it is also interesting to wonder what an inhibition of c-Rel among cord blood-derived cells might have in such treatments. Certainly, based on the results here, further studies are well deserved with regard to moving this potential approach forward.

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Disclosure statement

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References

Adams J. 2002. Proteasome inhibition: A novel approach to cancer therapy. Trends Mol Med. 8:549–554.

Aigner A. 2006. Delivery systems for the direct application of siRNAs to induce RNA interference (RNAi) in vivo. J Biomed Biotechnol. 10:1110–1115.

Appelbaum F. 2003. The current status of hematopoietic cell transplantation. Annu Rev Med. 54:491–512.

Bernard D, Monte D, Vandenbunder B, Abbodie C. 2002. The c-Rel transcription factor can both induce and inhibit apoptosis in the same cells via the upregulation of MnSOD. Oncogene. 21:4392–4402.

Bernard D, Quatannens B, Begue A, Vandebunder B, Abbodie C. 2001. Anti-proliferative and anti-apoptotic effects of c-Rel may occur within same cells via up-regulation of manganese superoxide dismutase. Cancer Res. 61:2656–2664.

da Silva C, Heilbock C, Kassel O, Frossard N. 2003. Transcription of stem cell factor (SCF) is potentiated by glucocorticoids and interleukin-1β through concerted regulation of a GRE-like and an NF-κB response element. FASEB J. 17:2334–2336.

Frelin C, Imbert V, Greggisser E, Peyron AC, Rochet N, Philip P, Dageville C, Sirvent A, Hummelsberger M, Bédard E, et al. 2005. Targeting NF-κB activation via pharmacologic inhibition of IKK2-induced apoptosis of human acute myeloid leukemia cells. Blood. 105:804–811.

Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura J, Ho W, Banerjee A. 2006. Unravelling the complexities of the NF-κB signalling pathway using mouse knockout and transgenic models. Oncogene. 25:6781–6799.

Ghosh A, Dogan Y, Moroz M, Holland A, Yim N, Rao U, Young L, Tannenbaum D, Masih D, Velardi E, et al. 2013. Adoptively transferred TRAIL− T-cells suppress GVHD and augment antitumor activity. J Clin Invest. 123:2654–2662.

Ghosh A, Holland A, van den Brink M. 2014. Genetically engineered donor T-cells to optimize graft-versus-tumor effects across MHC barriers. Immune Rev. 257:226–236.

Gilmore T, Gerondakis S. 2011. The c-Rel transcription factor in development and disease. Genes Cancer. 2:695–711.

Gorez T, Kalaitzidou D, Liang M, Starczynowski D. 2004. The c-Rel transcription factor and B-cell proliferation: A deal with the devil. Oncogene. 23:227–228.

Giron-Michel J, Caingnard A, Fogli M, Brouty-Boye D, Briard D, van Dijk M, Meazza R, Ferrini S, Lebourne-Kerdiles C, Clay D, et al. 2003. Differential STAT3, STAT5, and NF-κB activation in human hematopoietic progenitors by endogenous IL-15: Implications in the expression of functional molecules. Blood. 102:109–117.

Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquinli R, Ortega J, Souillet G, Ferreira E, Laporte J, et al. 1997. Outcome of cord-blood transplantation from related and unrelated donors. Eurocord Transplant Group and the European and Marrow Transplantation Group. New Engl J Med. 337:373–381.

Grabstein K, Waldschmidt T, Finkelman F, Hess B, Alpert A, Boiani N, Namen A, Morrissey PJ. 1993. Inhibition of murine B- and T-lymphopoeisis in vivo by an IL-7 monoclonal antibody. J Exp Med. 178:257–264.

Griessinger E, Frelin C, Cuburu N, Imbert V, Dageville C, Hummelsberger M, Sirvent N, Dreano M, Peyron J. 2008. Preclinical targeting of NF-κB and FLT3 pathways in AML cells. Leukemia. 22:1466–1469.

Griessinger E, Imbert V, Lagadec P, Genthier N, Dubreuil P, Romanelli A, Dreano M, Peyron J. 2007. AS602868, a dual inhibitor of IKK2 and FLT3 to target AML cells. Leukemia. 21:877–885.

Grojescou-Revjard J, Ades L, Boehrer S, Tailler M, Fabre C, Braun T, de Botton S, Israel A, Fenaux P, Kroemer G. 2008. Fh3 receptor inhibition reduces constitutive NF-κB activation in high-risk myelodysplastic syndrome and acute myeloid leukemia. Apoptosis. 13:1148–1161.

Hassan H, Zander A. 1996. Stem cell factor as a survival and growth factor in human normal and malignant hematopoiesis. Acta Haematol. 95:257–262.

Hilliard B, Mason N, Xu L, Sun J, Lamhamedi-Cherradi S, Liou H, Hunter C, Chen Y. 2002. Critical roles of c-Rel in autoimmune inflammation and helper T-cell differentiation. J Clin Invest. 110:843–850.

Hofmeister C, Zhang J, Knight K, Le P, Stiff P. 2007. Ex vivo expansion of umbilical cord blood stem cells for transplantation: Growing knowledge from the hematopoietic niche. Bone Marrow Transplant. 39:11–23.

Holstic U, Albrecht M, Chemnitz J, Theurich S, Skoetz N, Scheid C, van Bergwelt-Baildon M. 2014. Cochrane Database Syst Rev. 4:CD010189.

Hunter J, Leslie J, Perkins N. 2016. c-Rel and its many roles in cancer: An old story with new twists. Br J Cancer. 114:1–6.

Jenq R, van den Brink M. 2010. Allogeneic haematopoietic stem cell transplantation: Individualized stem cell and immune therapy of cancer. Nat Rev Cancer. 10:213–221.

Keller J, Ortiz M, Ruscetti F. 1995. Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. Blood. 86:1757–1764.

Kikuchi K, Kasai H, Watanabe A, Lai A, Kondo M. 2008. IL-7 specifies B-cell fate at the common lymphoid progenitor to pre-proB stage by maintaining early B-cell factor expression. J Immunol. 181:383–392.

Kontgen F, Grumont R, Strasser A, Metcalf D, Li R, Tarlinton D, Gerondakis S. 1995. Mice lacking the c-rel proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and IL-2 expression. Genes Dev. 9:1965–1977.

Kucharcz J, Simmons M, Fan Y, Gelinas C. 2000. To be, or not to be: NF-κB activation and IL-8 production in human neutrophils. Blood. 95:2841–2848.

Liou H, Hsia C. 2003. Distinctions between c-Rel and other NF-κB proteins in immunity and disease. BioEssays. 25:767–780.

Locatelli F, Pagliara D. 2012. Allogeneic hematopoietic stem cell transplantation in children with sickle cell disease. Pediatr Blood Cancer. 59:372–376.

Lorenzo V, Schon M, Seitz C. 2014. c-Rel down-regulation affects cell cycle progression of human keratinocytes. J Invest Dermatol. 134:415–422.

McDonald P, Russo M, Ferrini S, Cassatella M. 1998. IL-15 induces NF-κB activation and IL-8 production in human neutrophils. Blood. 92:4828–4835.

Mrozek E, Anderson P, Caligiuri M. 1996. Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells. Blood. 87:2633–2640.

Nelson B. 2004. IL-2, regulatory T-cells, and tolerance. J Immunol. 172:3983–3988.

Parrish Y, Baez I, Milford T, Benitez A, Galloway N, Rogerio J, Sahakian E, Kagoda M, Huang G, Hao Q, et al. 2009. IL-7 dependence in human B-lymphopoiesis increases during progression of ontogeny from cord blood to bone marrow. J Immunol. 182:4252–4266.

Paspakis M. 2009. Regulation of tissue homeostasis by NF-κB signalling: implications for inflammatory diseases. Nat Rev Immunol. 9:778–788.
Pepper C, Hewamana S, Brennan P, Fegan C. 2009. NF-κB as a prognostic marker and therapeutic target in chronic lymphocytic leukemia. Future Oncol. 5:1027–1037.
Pizzi M, Sarnico I, Boroni F, Benarese M, Steinberg N, Mazzoleni G, Dietz G, Bahr M, Liou HC, Spano P. 2005. NF-κB factor c-Rel mediates neuroprotection elicited by mGlu5 receptor agonists against amyloid b-peptide toxicity. Cell Death Differ. 12:761–772.
Pyatt D, Stillman W, Yang Y, Gross S, Zheng J, Irons R. 1999. An essential role for NF-κB in human CD34 progenitor cell survival. Blood. 93:3302–3308.
Ranson T, Vosshenrich C, Corcuff E, Richard O, Muller W, Di Santo J. 2003. IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood. 101:4887–4893.
Romano M, Lamberti A, Bisogni R, Garbi C, Pagnano A, Auletta P, Tassone P, Turco M, Venuta S. 1999. Amifostine inhibits hematopoietic progenitor cell apoptosis by activating NF-κB/Rel transcription factors. Blood. 94:4060–4066.
Romano M, Petrella A, Bisogni R, Turco M, Venuta S. 2003. Effect of NF-κB/Rel inhibition on spontaneous vs chemotherapy-induced apoptosis in AML and normal cord blood CD34 progenitor cells. Leukemia. 17:1190–1192.
Sandy P, Ventura A, Jacks T. 2005. Mammalian RNAi: A practical guide. BioTechniques. 39:215–224.
Shono Y, Tuckett A, Liou H, Doubrovina E, Derenzini E, Ouk S, Tsai J, Smith O, Levy E, Kreines F, et al. 2016. Characterization of a c-Rel inhibitor that mediates anti-cancer properties in hematologic malignancies by blocking NF-κB-controlled oxidative stress responses. Cancer Res. 76:377–389.
Shono Y, Tuckett A, Ouk S, Liou H, Altan-Bonnet G, Tsai J, Oyler J, Smith O, West M, Singer N, et al. 2014. A small-molecule c-Rel inhibitor reduces allo-activation of T-cells without compromising anti-tumor activity. Cancer Discov. 4:578–591.
Smith A, Wagner J. 2009. Alternative hematopoietic stem cell sources for transplantation: Place of umbilical cord blood. Br J Hematol. 14:261–267.
Takahashi S, Harigae H, Ishii K, Inomata M, Fujiwara T, Yokoyama H, Ishizawa K, Kameoka J, Licht J, Sasaki T, et al. 2005. Over-expression of FLT3 induces NF-κB pathway and increases the expression of IL-6. Leuk Res. 29:893–899.
Tian W, Liou H. 2009. RNAi-mediated c-Rel silencing leads to apoptosis of B-cell tumor cells and suppresses antigenic immune response in vivo. PLoS One. 4:e1932.
Turco M, Romano M, Petrella A, Bisogni R, Tassone P, Venuta S. 2003. NF-κB/Rel-mediated regulation of apoptosis in hematologic malignancies and normal hematopoietic progenitors. Leukemia. 18:11–17.
Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, Mitsui T, Manabe A, Tanaka R, Kobayashi K, et al. 2000. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. J Clin Invest. 105:1013–1021.
Visekrana A, Volkov A, Steinhoff U. 2012. A key role for NF-κB transcription factor c-Rel in T-lymphocyte differentiation and effector functions. Clin Dev Immunol. 2012:239368.
Waldmann T, Dubois S, Tagaya Y. 2001. Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy. Immunity. 14:105–110.