Ectopic Expression of Protein Tyrosine Kinase Bcr-Abl Suppresses TNF-Induced NF-κB Activation and IκBα Phosphorylation: Relationship With Downregulation of TNF Receptors

Asok Mukhopadhyay, Shishir Shishodia, Jill Suttles¹, Katherine Brittingham¹, Betty Lamothe, Ramdevi Nimmanapalli², Kapil N. Bhalla² and Bharat B. Aggarwal†

From the Cytokine Research Laboratory, Department of Bioimmunotherapy, Box 143, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030; ¹Department of Microbiology and Immunology, University of Louisville, Louisville KY 40292
²Lee Moffitt Cancer Center and Research Institute, Tampa, FL

Running title: Bcr-Abl protein downregulates TNF signaling

† To whom correspondence should be addressed:
Phone: 713-792-3503 / 6459 Fax: 713-794-1613
Email: aggarwal@utmdacc.mda.uth.tmc.edu
Abbreviations used: Abl, Abelson tyrosine kinase; ALL, acute lymphocytic leukemia; ALLN, N-acetylleucylleucylnorlucinal; Bcr, breakpoint cluster region gene; CML, chronic myelogenous leukemia; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage colony-stimulating factor; IκB, inhibitory subunit of NF-kB; IL-6Rα, interleukin-6 receptor α; NF-κB, nuclear transcription factor-κB; PARP, poly (ADP) ribose polymerase; RPA, RNase protection assay; TNF, tumor necrosis factor; TGFβR II, transforming growth factor b receptorII, TRAF2, TNF receptor associated factor 2
Summary

Bcr-Abl, the product of the protooncogene bcr-abl, is a constitutively active protein tyrosine kinase that is highly expressed in chronic myelogenous leukemia and in acute myeloid leukemia cells. Because Bcr-Abl is known to provide mitogenic signals through suppression of apoptosis, we investigated the effect of this oncogene product on signaling by TNF, a proapoptotic cytokine. We used a bcr-abl-deficient human megakaryocytic leukemia cell line MO7E and an isogeneic MBA cell line stably transfected with bcr-abl. Electrophoretic mobility shift assay revealed that TNF activated the nuclear transcription factor NF-κB in MO7E cells but not in MBA cells. The impaired NF-κB activation in Bcr-Abl-expressing cells was not due to absence of the NF-κB proteins p65, p50, or p100 or of IκBα or IκBβ. Okadaic acid-induced NF-κB activation was unaffected by Bcr-Abl expression. TNF induced IκBα phosphorylation and degradation in MO7E cells but not in MBA cells. The suppression of TNF-induced NF-κB activation by Bcr-Abl was not restricted to MBA cells, because ectopic expression of Bcr-Abl in human acute myeloid leukemia HL-60 cells also blocked TNF-induced NF-κB activation. When examined for the TNF receptors by the radio-receptor assay, flow cytometry, or by western blot analysis, we found that Bcr-Abl expression downregulated the expression of the TNF receptors. The RNase protection assay and northern blot analysis revealed the transcriptional downregulation of the TNF receptor by Bcr-Abl protein. Overall, these results indicate that ectopic expression of Bcr-Abl interferes with the TNF signaling pathway through the downregulation of TNF receptors.
Introduction

The chimeric oncogene \textit{bcr-abl} is formed by the reciprocal translocation (Philadelphia translocation) that fuses part of the breakpoint cluster gene (\textit{bcr}) on chromosome 22 upstream of the Abelson tyrosine kinase (\textit{abl}) gene on chromosome 9 (1). Depending on the chromosomal fusion point, Bcr-Abl proteins are expressed in three different molecular sizes, 185, 210, and 230 kDa, and are believed to be responsible for acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), and chronic neutrophilic leukemia (CNL), respectively (2-4).

Bcr-Abl is a deregulated tyrosine kinase that transforms fibroblasts and immature hematopoietic cells \textit{in vitro}, and the transformed cells are tumorigenic (5-7). The introduction of a retrovirus vector expressing p210$^{\text{Bcr-Abl}}$ and p185$^{\text{Bcr-Abl}}$ into growth-factor (GM-CSF/IL-3)-dependent human (MO7E) and mouse (32D) cell lines respectively converted them rapidly to growth factor-independent cell lines (8, 9). Bcr-Abl expression has been implicated in the induction of resistance of CML to apoptosis induced by antileukemic drugs (10, 11). This oncogene has been shown to block apoptosis induced by various stimuli through suppression of mitochondrial release of cytochrome $c$ and by blocking the cytosolic pathway that leads to activation of caspase-3 (12-14). Additionally Bcr-Abl has been shown to regulate c-jun gene expression, activation of c-jun N-terminal kinase, and the ras pathway, which may also contribute to suppression of apoptosis, transformation and tumorigenesis (15-18). It is thus apparent that t(9,22) Philadelphia translocation modulates cellular signaling. In mammalian cells various signal transduction pathways leading to survival or death are activated depending upon extracellular stimuli.

How Bcr-Abl expression affects signaling to cytokines that either stimulate or inhibit cell growth is poorly understood. It has been shown that Bcr-Abl affects cell
growth via autocrine production and action of IL-3 and granulocyte colony-stimulating factor in chronic myeloid leukemia (19). Furthermore it was recently shown that p210Bcr-Abl interacts with the IL-3 receptor beta(c) subunit and constitutively induces its tyrosine phosphorylation (20). Here, we investigated the effect of ectopic expression of Bcr-Abl on TNF signaling using the human megakaryocytic leukemic cell line the MO7E, which lacks Bcr-Abl, and isogeneic MBA, which expresses Bcr-Abl ectopically. Our results indicate that the expression of Bcr-Abl downregulates TNF-induced NF-κB activation and IκBα phosphorylation through the downregulation of TNF receptors. We also demonstrate that this effect is not unique to megakaryocytic leukemic cells but also occurs in human T cells and in acute myelogenous leukemia cells. The downregulation of the TNF receptor by Bcr-Abl occurred at the transcription level.
Experimental Procedures

Cell lines and culture: U-937 cell line was procured from American Type Culture Collection (Rockville, MD). The human megablastic leukemic cell line MO7E, a growth factor-dependent cell line, was obtained from the Genetics Institute, Boston, MA. We used isogeneic MO7E cell line transformed with retrovirus vector containing the chimeric bcr-abl gene (MBA cell) which is growth factor independent (8). The stable transfection of human promyelomonocytic HL-60 with neo or with bcr-abl plasmids has been previously described from our laboratory (14). We also transfected Jurkat cells with doxycycline-inducible Bcr-Abl plasmid. For this, Bcr-Abl p210 cDNA, was cloned into BamH1 site of the plasmid vector pSTAR (21). The resulting plasmid pSTAR Bcr/Abl was stably transfected into Jurkat cells using lipofectamine (Gibco BRL, Grand Island, NY). Stable clones were screened using 500 ug/ml of G 418 sulfate. After selecting the single clone by limiting-dilution method, Bcr-Abl gene was induced using 5 ug/ml doxycycline. All these cells were regularly grown in RPMI-1640 containing 10% FBS and antibiotics-antimycotics, except the medium for MO7E was supplemented with 200 U/ml human granulocyte-macrophage colony stimulating factor (GM-CSF).

Materials: Polyclonal antibodies against IκBα, IκBβ, p50, p52, p65, and PARP raised in rabbits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against the phospho-IκBα (Ser-32) and phospho-p42/44 MAPK were obtained from New England BioLabs, Inc. (Beverly, MA). Biotinylated anti-phosphotyrosine monoclonal antibody, anti-biotin IgG-HRP, and β-actin antibody were procured from Sigma Chemicals (St. Louis, MO). Monoclonal Bcr antibody was obtained from Oncogene Research Products.
RiboQuant multi-probe RNase protection assay kit was purchased from Pharmingen (San Diego, CA). Anti-p60 and anti-p80 polyclonal antibodies were raised in rabbits, and purified by ligand-affinity column chromatography. Bacterium-derived recombinant human TNF and GM-CSF purified to homogeneity with a specific activity of about $5 \times 10^7$ units/mg, were kindly provided by Genentech Inc. (South San Francisco, CA). RPMI-1640, fetal bovine serum (FBS), and antibiotics-antimycotics were obtained from Life Technologies Inc. (Grand Island, NY).

**Identification of Bcr-Abl protein, and its phosphorylated form:** Fifty micrograms of whole-cell lysates were resolved on 6 % SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to nitrocellulose membrane, blocked with 2% BSA, and probed with anti-Bcr antibody (1:1000) for 1 h. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent. To detect the phosphorylated form of Bcr-Abl, 15 µg of lysate proteins were resolved on 6 % SDS-PAGE gel. The proteins were electrotransferred to nitrocellulose membrane, blocked with 2% BSA, and probed with anti-phosphotyrosine biotin monoclonal antibody (1:2000). The blot was then treated with anti-biotin-HRP conjugate and detected by ECL reagent.

**Electrophoretic mobility shift assay (EMSA):** NF-κB activation was analyzed by EMSA as described previously (22). In brief, 8-µg nuclear extracts prepared from TNF- treated or untreated cells were incubated with $^{32}$P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5’-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGG- 3’; underlined are NF-kB binding sites) for 15 min at 37 °C, and
the DNA-protein complex resolved in a 6.6% native polyacrylamide gel. The specificity of binding was examined by competition with unlabeled 100-fold excess oligonucleotide and with mutant oligonucleotide. The composition and specificity of binding were also determined by supershift of the DNA-protein complex using specific and irrelevant antibodies. The antibody-treated samples of NF-κB were resolved on a 5.5% native gel. The radioactive bands from the dried gels were visualized and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

**Western blot analysis of NF-κB proteins**: Thirty to fifty micrograms of cytoplasmic protein extracts, prepared as described (23), were resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to nitrocellulose membrane, blocked with 5% nonfat milk, and probed with IκBα, IκBβ, p50, p52, p65, and ser-32-phosphorylated IκBα polyclonal antibodies (1:3000) for 1 h. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by chemiluminescence (ECL, Amersham Pharmacia Biotech. Arlington Heights, IL).

**c-Jun NH2-terminal kinase assay**: The c-Jun kinase assay was performed by a modified method as described earlier (24). Briefly, whole-cell extracts were prepared from TNF- treated cells, and 100 µg cytoplasmic extracts were treated with anti-JNK1 antibodies. The immune complexes were precipitated with protein A/G-Sepharose beads (Pierce, USA). The kinase assay was performed using washed beads as source of enzyme and glutathione S-transferase-Jun (1-79) as substrate (2 µg/sample) in the presence of 10 µCi [32P]ATP per sample. The kinase reaction was carried out by incubating the mixture at 30 °C, in kinase assay buffer for 15 min. The reaction was stopped by boiling beads in SDS.
sample buffer. Finally, protein was resolved on 10% SDS-PAGE gel. The radioactive bands of the dried gel were visualized and quantitated by phosphorImager as mentioned earlier.

**Receptor-binding assay:** Human recombinant TNF was labeled with Na$^{125}$I using the IODOGEN procedure as described (25). The specific activity of the labeled TNF was 38 µCi/µg. The binding assays were performed by using the 96-well method as previously described (26). Briefly, cells (0.5 x 10$^6$ /ml) were incubated in a binding buffer (RPMI-1640 containing 10% FBS) in a flexible 96-well plate (Falcon 3911) in the presence of 2 µg/ml anti-p60 or anti-p80 antibodies for 1 h at 4 ºC. Cells were then exposed to 50 nM unlabeled TNF in the presence of $^{125}$I-labeled TNF (0.2 x 10$^6$ cpm/sample) in a total volume of 0.1 ml. Thereafter, cells were washed three times with 200 µl of ice-cold medium. Cell-bound radioactivity was then measured by a gamma counter (Packard Instruments Co., Downers Grove, IL). The binding of TNF to the p60 or p80 receptor was calculated by subtracting TNF-specific binding in the absence of antibody from that in the presence of either anti-p80 or anti-p60 receptor antibodies, respectively. All results were determined in triplicate and expressed as the mean ± S.E.

**Flow cytometric analysis of TNFR expression:** For analysis of TNFR expression, MO7E and MBA cell lines were harvested, centrifuged, and resuspended in Dulbecco’s phosphate buffered saline (DPBS) containing 10% FBS and 0.1% sodium azide. The cells were incubated with polyclonal, affinity purified rabbit anti-p60 and p80 antibodies (27). Following 1 h incubation at 4ºC, the cells were washed and incubated for an additional 1 h with biotin conjugated anti-rabbit Ig monoclonal antibody (Jackson ImmunoResearch, West Grove, PA).
The cells were washed and incubated for 1 h at 4°C with phycoerythrin conjugated streptavidin (Molecular Probes, Eugene, OR). Thereafter the cells were analyzed using a FACS Vantage flow cytometer and CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA).

**TNF receptor western blot analysis:** To prepare the cell extracts, MBA, MO7E, U937 and KBM-5 cells (2x10^6) were incubated for 30 min on ice in 100 µl of lysis buffer (20 mM HEPES; pH 7.4, 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1mM PMSF, 0.5 µg/ml benzamidine, and 1mM DTT). The lysates were then centrifuged for 4 min at 14000 rpm at 4°C, the supernatants collected and measured the protein by the Bradford method. The cell extract (100 µg) was resolved on a 10% SDS-polyacrylamide gel, electrotransferred onto nitrocellulose membrane and blocked with blocking solution containing 5% non-fat milk in phosphate buffered saline (PBS) containing 0.5 % tween-20. The membrane was blotted first with anti-p80 TNF receptor antibody (1: 1000 dilution in blocking solution) for 1 h, washed 3x (10 minutes each) with PBS-tween and then blotted with horseradish peroxidase-conjugated IgG as the secondary antibody for 1 h, washed 3x (10 minutes each) and then detected by chemiluminescence (ECL, Amersham).

**RNase protection assay:** The expression of p60 and p80TNF receptor mRNA was determined by RNase protection assay using human cytokine receptor multi-probe template sets (hCR-4) as per manufacturer’s instruction. Briefly, each cell type (5 x 10^6) was washed 2 times with PBS to remove medium protein. Total RNA was isolated using TRizol Reagent (Life Technologies, Rockville, MD). Ten-microgram RNA samples were hybridized with ^32^P-labeled antisense mRNA probes against IL-1RI, IL-1RII, p60TNFR, p80TNFR, IL-6Rα, gp130, TGFβRI,
TGFβRII, L32, and GAPDH, and digested with RNase and T1 nuclease. The protected hybridized probe fragments were resolved on 5% TBE urea polyacrylamide gel (BioRad, Richmond, CA. The radioactive bands were visualized using a BioRad Personal Molecular Imager Fx and the associated Quantity One software. Band density was quantitated using the UN-SCAN-IT gel automated digitizing system (Silk Scientific Corp., Orem, UT). The relative mRNA levels were determined by normalizing band intensities of p60 and p80TNFR with that of L32 probe.

**Northern blot Analysis:** Cell cultures seeded at 1 x 10^6 cells/ml were incubated in 75-cm² flasks. Total RNA was extracted from cells using TRI-zol Reagent. For electrophoresis, 30µg of RNA was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde at 50-100 V for approximately 3 h. Thereafter, the gels were rinsed with DEPC and the RNA was transferred to Hybond nylon membranes (Amersham Corp. Arlington Heights, IL). After alkaline transfer (overnight), the filter was stained with methylene blue to visualize 28s RNA. Prehybridization was carried out at 65°C for 1 h in a buffer containing 7% SDS, 50 mM sodium phosphate, 1mM EDTA, pH 7.2 (Church buffer or hybridization buffer). Filters were then hybridized for 16-20 h with p60 or p80 cDNA probes (approximate specific activity 2 x 10^8 cpm/µg DNA) in a hybridization buffer containing denatured salmon sperm DNA (200 µg/ml). After hybridization, membranes were washed several times at 65°C with 40mM sodium phosphate containing 1% SDS. The filters were exposed to Kodak xAR-5 film with intensifying screens at −70°C for 1-3 days. Equal loading of lanes was demonstrated by examination of gels after methylene blue staining of the 28s rRNA.
Results

In this report we investigated the effect of ectopic expression of Bcr-Abl protein on TNF-mediated cellular responses in the human megakaryoblastic cell line MO7E, which was originally derived from an acute megablastic leukemia patient. To confirm that the effects of Bcr-Abl on these cellular responses are not unique to one cell line, we also used the human acute myelogenous leukemia HL-60 cell line.

Differential expression of $p210^{Bcr-Abl}$ protein and kinase activity in MO7E and MBA cells: We first examined the expression of Bcr-Abl protein by western blot using Bcr-specific antibodies. As shown in Fig. 1A, MO7E cells did not express Bcr-Abl protein, whereas MBA cells expressed a large amount of $p210^{Bcr-Abl}$ protein. We next examined whether this protein exhibited protein tyrosine kinase activity. The chimeric oncogene product was tyrosine phosphorylated as shown by phosphotyrosine western blot analysis (Fig. 1A, lower panel).

Bcr-Abl downregulates TNF-induced NF-$\kappa$B activation: The activation of NF-$\kappa$B is one of the earliest cellular responses to TNF in most cells (29). Whether expression of Bcr-Abl modulates TNF-mediated NF-$\kappa$B activation was investigated. MO7E and MBA cells were treated with 0.1 and 1 nM TNF for 30 min, and nuclear extracts prepared and analyzed by DNA-binding assay using EMSA. The results in Fig. 1B show that TNF activated NF-$\kappa$B in MO7E cells almost to the maximum with 0.1 nM but in MBA cells not even at 1 nM TNF had any effect. These results indicate that Bcr-Abl downregulates TNF-induced NF-$\kappa$B activation (Fig. 1B).
To ensure that the activated NF-κB in MO7E cells was composed of transcriptionally active heterodimers of p50 and p65 subunits, the TNF-treated nuclear extracts were incubated with anti-p65 or anti-p50 antibodies before EMSA. EMSA result showed that the NF-κB/DNA complex was either abrogated or supershifted when nuclear extract was treated with p50/p65 antibodies (Fig. 1C). The DNA binding was not prevented by treatment of nuclear extracts with irrelevant cyclin D1 antibodies or pre-immune sera, indicating specificity of the heterodimer. The specificity of the TNF-induced NF-κB/DNA complex was further confirmed by demonstrating that the binding was disrupted in the presence of a 100-fold excess of unlabeled κB-oligonucleotide, but not by mutant oligonucleotide (Fig. 1C).

**Bcr-Abl does not affect the expression of various NF-κB proteins:** It is possible that Bcr-Abl downregulated the expression of NF-κB proteins, making MBA cells unable to respond to TNF-induced NF-κB activation. To determine this, we prepared cytoplasmic extracts from both cell types and examined the expression of p65 (c-rel), p50, p100, IκBα, and IκBβ by western blot analysis using specific antibodies. Fig. 2A shows that all the NF-κB proteins are expressed to a similar level in both cell types, thus suggesting that Bcr-Abl had no effect on the expression of various NF-κB proteins.

**Bcr-Abl does not affect the NF-κB activation induced by okadaic acid:** Since Bcr-Abl did not affect the expression of various NF-κB proteins, we examined if it affected the NF-kB activation induced by other agents. For this cells were treated with 0.5 μM okadaic acid for 4 h, prepared the nuclear extracts and examined the NF-κB activation by EMSA. Activation by TNF was used as a
control. As shown in Fig. 2B, okadaic acid activated NF-κB in both MO7E and MBA cell lines, indicating that ectopic expression of Bcr-Abl has no effect on NF-κB activation by other agents.

**Bcr-Abl suppresses TNF-induced IκBα phosphorylation:** TNF-induced NF-κB activation requires phosphorylation of IκBα at serine residue 32 and 36 (30). Whether Bcr-Abl suppresses TNF-induced NF-κB activation through suppression of IκBα phosphorylation was investigated. Cells were treated with TNF in the presence of ALLN (a proteasome inhibitor), which prevents the degradation of the phosphorylated form of IκBα, and then examined for non-phosphorylated and phosphorylated forms of IκBα using specific antibodies. Fig. 2C shows that the phosphorylated form of IκBα appeared in MO7E cells but not in MBA cells, thus suggesting that Bcr-Abl prevents the phosphorylation of IκBα.

**Bcr-Abl activates JNK and p44/p42MAPK activation:** TNF is also a potent activator of JNK and MAPKK (29). Whether Bcr-Abl also suppresses the TNF-induced activation of JNK and MAPKK was examined. Cells were treated with 0.1 and 1 nM TNF, and whole-cell extracts prepared and analyzed for JNK by the immunocomplex kinase assay and for p44/p42MAPK by western blot analysis using specific antibodies. As Fig. 2D shows JNK was constitutively active in MBA cells and not in MO7E cells, suggesting that Bcr-Abl expression leads to JNK activation. Interestingly, TNF failed to activate JNK in MO7E cells, whereas in MBA cells no further enhancement was found.

The results in Fig. 2E show that MAPKK, which is an upstream kinase to MAPK, is also constitutively active in MBA cells but not in MO7E cells, suggesting that Bcr-Abl expression leads to MAPKK activation. Similarly, TNF was unable
to activate MAPKK in MO7E cells, whereas in MBA cells the constitutive expression of MAPK was not further increased by the ligand.

**Downregulation of TNF-induced cellular responses by Bcr-Abl is not specific to MO7E:** It is possible that the effect of Bcr-Abl on TNF-mediated cellular responses is unique to megakaryoblastic cells. To determine whether Bcr-Abl downregulates TNF responses in other cell types, we employed human acute myelogenous leukemia HL-60 cells. These cells were transfected with *bcr-abl* plasmid and then examined for TNF-mediated cellular responses. As shown in Fig. 3A, normal HL-60 cells did not express Bcr-Abl but transfected cells did. As was the case for MBA cells, expression of Bcr-Abl in HL-60 cells downregulated TNF-induced NF-κB activation (Fig. 3B) without any significant loss of NF-κB proteins (Fig. 3C). Thus the effects of Bcr-Abl on TNF signaling were not cell type specific.

TNF-induced cytotoxicity is known to require activation of caspases which cleave various cellular substrates including PARP (31). To determine whether Bcr-Abl affects TNF-induced PARP cleavage, we treated cells with TNF in the presence and absence of cycloheximide (which suppresses the synthesis of antiapoptotic proteins), prepared cell extracts, and analyzed them by western blot using anti-PARP antibodies. The results showed that TNF induced PARP cleavage in HL-60 cells but not in HL-60 cells transfected with Bcr-Abl, thus suggesting that Bcr-Abl also suppresses TNF-induced activation of caspases (Fig. 3D).

We also examined the effect of doxycyline-inducible Bcr-Abl in Jurkat cells on TNF-induced NF-κB activation. The results showed that TNF activated NF-κB in control cells but not in Bcr-Abl expressing cells (data not shown).
**Bcr-Abl downregulates TNF receptors:** Our results so far indicated that most of the TNF-induced cellular responses were downregulated by Bcr-Abl irrespective of cell type. It is possible that Bcr-Abl may have suppressed TNF-induced cellular responses through downregulation of TNF receptors. Most leukemic cells express two types of TNF receptor; viz, p60 and p80 (32). It is known that most of the TNF signals are mediated through the p60 receptor. We examined the effect of Bcr-Abl expression on the cell surface expression of these two receptors using radioreceptor assays and receptor-specific antibodies. Because both types of TNF receptor are well characterized on U-937 cells, we used these cells as a control. As shown in Fig. 4A, U-937 cells expressed almost 65% p80 and 35% p60 TNF receptors. Similarly, most of the ligand binding in MO7E cells was due to p60 receptor; very little p80 receptor was found. Amazingly, MBA cells were found to lack any specific TNF binding. Thus these results indicate that Bcr-Abl downregulates TNF receptors in megakaryoblastic cells. The cell surface of TNF receptors was also examined by flow cytometry. These results also showed that MO7E cells express both p60 and p80 form of the TNF receptors, but MBA cells expressed neither of the receptors (Fig. 4B). Whether Bcr-Abl expression downregulates the TNF receptor protein, was examined by western blot analysis. As shown in Fig. 4C MO7E cells expressed significant levels of TNF p80 receptor protein and these levels were comparable with other myeloid cell lines such as U-937 and KBM-5 cells. Two different bands observed suggest a breakdown of the p80 receptor. In contrast, MBA cells did not express TNF p80 receptor protein, suggesting that Bcr-Abl expression downregulates the TNF receptor protein. No antibody was found sensitive enough to detect the p60 receptor by the western blot analysis.
**Bcr-Abl downregulates the mRNA for TNF-receptor:** Whether Bcr-Abl downregulates the expression of TNF-receptors at the transcriptional level was determined by isolating the mRNA from different cell types and performing the RNase protection assay using specific probe kits. Fig. 5 A and 5B show that MO7E cells expressed the mRNA for p60 and p80 receptor and the expression for p60 was higher than p80. The expression of Bcr-Abl in MO7E eliminated the expression of mRNA for both p60 and p80 receptors. The effects of Bcr-Abl were not unique to TNF receptors, in as much as the mRNA for IL-6Rα and TGFβIIR were also completely downregulated in Bcr-Abl expressing MBA cells. These results indicate that Bcr-Abl can downregulate the mRNA for various cytokine receptors.

The downregulation of the mRNA by Bcr-Abl expression was further confirmed by northern blot analysis. The results in Figure 5C indicate that MO7E expressed the mRNA for both p60 and p80 form of the TNF receptors whereas MBA cells did not express either of the receptor mRNA.
Discussion

In this report, we investigated the effect of Bcr-Abl on TNF-mediated NF-κB activation. Our results show that Bcr-Abl suppresses TNF-induced NF-κB activation, IκBα phosphorylation, and caspase-mediated PARP cleavage, and this suppression correlates with downregulation of TNF receptor expression both at the mRNA and protein levels. Our results also indicate that these effects are not cell type specific, as Bcr-Abl was effective both in megakaryoblastic, in acute myelogenous leukemia cells and in Jurkat T cells.

Our results indicate that Bcr-Abl by itself had no effect on constitutive NF-κB activation in either of the human leukemic cell lines. These results, however, differ from two earlier reports of Reuther et al (33) and Hamdane et al (34) which showed that Bcr-Abl causes constitutive NF-κB activation. Both of these investigators used murine myeloid 32D and DA1 cell lines. Whether the difference in our results from those previously reported is due to the cell line is not clear. We used three different human cell lines and found similar results. Rather than stimulating NF-κB by itself, Bcr-Abl suppressed TNF-induced NF-κB activation in our study. This suppression occurred through the inhibition of IκBα phosphorylation needed for NF-κB activation. This is the first report to our knowledge to indicate that Bcr-Abl can modulate the signaling of any cytokine, other than IL-3. IL-3 is known to be produced by Bcr-Abl-expressing leukemic cells and acts as an autocrine growth factor (19). Furthermore, Bcr-Abl can interact with the IL-3 receptor beta chain and induce constitutive tyrosine phosphorylation (20).
We found that ectopic expression of Bcr-Abl by itself activated JNK. This result is in agreement with previous reports, one by Raitano et al (16) on human embryonic kidney cells, and one by Burgess et al (15) on MO7E cells. While Bcr-Abl expression leads to constitutive activation of JNK in MO7E cells, TNF did not. That these cells are insensitive to TNF-induced JNK activation is not due to lack of TNF receptors. Furthermore, these receptors are functional, as they activated NF-κB in MO7E cells. It is possible that TRAF2, which is needed for JNK activation but not for NF-κB activation is either not expressed, or not functional due to the expression of TRAF2 inhibitors in parental MO7E cells.

Our results also indicate that Bcr-Abl suppressed TNF-induced cytotoxicity. TNF was not highly cytotoxic to MO7E cells. The suppression of TNF-induced cytotoxicity in our studies was consistent with downregulation of TNF-activated caspase activation. Several reports indicate that Bcr-Abl provides a growth advantage to the cells by blocking apoptosis (35-38), thus promoting transformation and tumorigenesis. However, there is very little known about how Bcr-Abl modulates cytokine signaling. IL-3 is known to be produced by Bcr-Abl-expressing leukemic cells and acts as an autocrine growth factor (19). Additionally, Bcr-Abl may also provide growth advantage to the leukemic cells through suppression of cytokine-mediated apoptosis. Alternatively, it is possible that Bcr-Abl may induce antiapoptotic proteins, such as Bcl-2, as reported previously (39) which could mediate the suppression of TNF-induced apoptosis. Indeed, we did find that Bcr-Abl-expressing MBA cells co-express Bcl-xL, whereas MO7E did not (unpublished data).

Our results indicate that most of the effects of Bcr-Abl on TNF signaling can be explained through the downregulation of TNF receptors.
downregulation of death receptors is a novel mechanism through which Bcr-Abl could provide a proliferative advantage to the leukemic cells. Our preliminary studies indicate that it is not the TNF receptor alone, but the mRNA for TGF-βRII and IL-6 receptor, that are also downregulated by Bcr-Abl. IL-6 has inhibitory effects on human and murine leukemic cell lines in vitro (40). By suppressing transcription of IL-6Rα, Bcr-Abl-expressing cells escape the growth inhibitory effects of IL-6. The role of TGFβ as a negative autocrine growth factor for tumorigenesis has been reported (41). Most normal cells are growth-inhibited by TGFβ. However, tumor cells lose their responsiveness to TGFβ in several ways (42). One of the possible ways by which tumor cells protect themselves from inhibitory effects of TGFβ is by losing TGFβIRs (43, 44). We believe that Bcr-Abl-expressing leukemic cells could escape suppression of growth also by TGFβ by down-modulation of TGFβ receptors.

Previously, Bcr-Abl had been shown to interact with the IL-3 receptor β-chain and induce constitutive tyrosine phosphorylation (20). Our studies suggest that downregulation of receptors involved in antiproliferative effects may be another mechanism through which Bcr-Abl provides a growth advantage. Overall, the studies described here provide a novel mechanism through which Bcr-Abl may interfere with cytokine signaling, especially those involved in suppression of cell growth. Whether TNF receptors and TNF signaling are downregulated in samples from CML or AML patients should be investigated in the future.

**Acknowledgment:** This research was conducted with support from The Clayton Foundation for Research. We would like to thank Dr. Jian Ni, and Bharati Matta
for assistance with Northern and RPA analysis.
References

17. Shi C-S, Tuscano JM, Witte ON, Kehrl JH. Blood 93:1338, 1999


44. Arteaga CL, Tandon AK, von Hoff DD, Osborne CK. *Cancer Res* 48:3898, 1988
Figure Legends

Fig. 1. A. Western blot analysis of Bcr-Abl protein. Whole-cell extracts of MO7E and MBA (60 µg) were resolved on 6% SDS-PAGE gel and probed with anti-Bcr antibody (upper panel). Same extracts (15 µg each) were resolved on 6% SDS-PAGE gel and probed with anti-phosphotyrosine biotin antibodies (lower panel). As a loading control, stripped nitrocellulose membrane was probed with β-actin antibodies. B. Dose response effect of NF-κB activation in MO7E and MBA cells by TNF. Two millions cells per milliliter were treated with 0.1 and 1 nM TNF for 30 min, and nuclear extracts were prepared and assayed for NF-κB. C, Composition of NF-κB induced by TNF. Nuclear extracts prepared by treating MO7E cell with 0.1 nM TNF was incubated at 37°C for 15 min with either none, or anti-p50 antibodies, or anti-p65 antibodies, or mixture of anti-p50 and anti-p65 antibodies, or pre-immune sera, or anti-cyclin D1, or unlabeled oligo, or mutant oligo, and then assayed for NF-κB as described in the Experimental Procedures. NSB denotes nonspecific binding.

Fig. 2. A, Western blot analysis of various NF-κB proteins in MO7E and MBA cells. Cytoplasmic proteins (30-50 µg) of MO7E and MBA cells were resolved on SDS-PAGE gel and probed with p65, p50, p100, IκBα, and IκBβ. As a loading control, one of the blots was stripped and probed with β-actin antibodies. B, Okadaic acid activates NF-κB activation in MO7E and MBA cells. Two millions cells per milliliter were treated with either 0.1 nM TNF for 30 min, or with 0.5 uM okadaic acid for 4 h, and nuclear extracts were prepared and assayed for NF-κB. C, Western blot analysis of Ser32 phosphorylated IκBα. Two million MO7E and
MBA cells per ml were pretreated with none, 100 µg/ml ALLN for 1 h. The cells were treated with 0.1 nM TNF for 15 min. Forty microgram cytoplasmic extracts were resolved on 10% SDS-PAGE gel, electrotransferred on a nitrocellulose membrane and first probed with Ser32 phosphospecific IκBα antibodies, and then with IκBα antibodies. **D, Activation of JNK by TNF in MO7E and MBA cells.** Two million cells per milliliter were treated with 0.1 and 1 nM TNF for 15 min, and whole-cell extracts were analyzed for kinase assay as mentioned in the experimental procedures.  **E, Activation of MAPK kinase by TNF in MO7E and MBA cells.** Two millions cells per milliliter were treated with 0.1 and 1 nM TNF for 15 min, and whole-cell extracts were analyzed by western blot using p42/44 MAPK specific antibodies.

**Fig. 3. Suppression of TNF signaling by Bcr-Abl in HL-60 cells. A, western blot analysis of Bcr-Abl protein.** Whole-cell extracts of HL-60 (Neo) and HL-60 (Bcr-Abl) (60 µg) were resolved on 6% SDS-PAGE gel and probed with anti-Bcr antibody (top panel). Same extracts (15 µg each) were resolved on 6% SDS-PAGE gel and probed with anti-phosphotyrosine biotin antibodies (middle panel). As a loading control, stripped nitrocellulose membrane was probed with β-actin antibodies (lower panel). **B, TNF-induced NF-κB activation.** Two millions HL-60 (Neo) and HL-60 (Bcr-Abl) cells per milliliter were treated with 0.1 and 1 nM TNF for 30 min, and nuclear extracts were prepared and assayed for NF-κB. **C, western blot analysis of various NF-κB proteins.** Cytoplasmic proteins (30-50 µg) of HL-60 (Neo) and HL-60 (Bcr-Abl) cells were resolved on SDS-PAGE gel and probed with p65, p50, p100, IκBα, IκBβ, and β-actin antibodies. **D, TNF-induced apoptosis.** One million cells per milliliter were pretreated with 5 µg/ml cycloheximide for 1 h (as indicated in the figure),
followed by the treatment with 10 nM TNF for 2 h. After treatment, whole cell extracts were prepared, 40 µg protein was resolved on SDS-PAGE gel and probed with PARP antibodies, as described in the Experimental Procedures.

Fig. 4. A, Effect of Bcr-Abl protein on the expression of TNF-receptors. Five hundred thousand cells (U937, MO7E and MBA) per 0.1 milliliter were pre-incubated with none, anti-p60 or anti-p80 antibodies for 1h at 4 °C. Following incubation, cells were treated with 50 nM TNF and 125I-TNF (2 x 10^5 cpm/sample) for another 1h. After washing, the bound TNF was counted in a gamma counter. B. Flow cytometric analysis of TNFR expression. One million MO7E and MBA cells were harvested and assayed for expression of p60 (dotted lines) and p80 (solid lines). Labeling was performed via a three-step stain consisting of sequential incubations with rabbit anti-TNFR antibodies, followed by biotin-conjugated anti-rabbit Ig, followed by phycoerythrin-conjugated streptavidin. Negative controls, shown as shaded histograms, consisted of cells labeled with second step antibodies, alone. The histograms shown depict analysis of 10,000 cells. C, Analysis of TNF receptor protein western blot. Cell lysates were prepared from two million MBA, MO7E, U937 and KBM-5 cells. 100 µg protein was resolved by 10% SDS PAGE, transferred to nitrocellulose membrane and analyzed for TNF receptors by western blot using p80TNFR-specific antibodies as described in Experimental Procedures.

Fig. 5. A. Evaluation of mRNA expression of TNF receptors by RNase protection assay. Ten microgram RNA samples were hybridized with 32P-labeled antisense mRNA probes, and digested with RNase and T1 nuclease. The protected hybridized probe fragments were resolved on 5% polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by
PhosphorImager. The relative mRNA levels were determined by normalizing band intensities of p60 and p80TNFR with that of L32 probe. The quantitation of the mRNA data normalized with the intensity of band for L32 mRNA is shown in panel B. C, Northern blot analysis of the TNF receptor in MO7E and MBA cells. The total RNA was isolated, resolved on the gels, electrotransferred on to the membrane and probed with cDNA for p60TNFR and p80TNF receptor. 28s rRNA was used as a loading control.
A

![Bar chart showing TNF receptors (Specific ^125I-TNF Binding, CPM) for different cell types.](chart)

B

![Flow cytometry blot for MO7E and MBA cells.](flow_cytometry)

C

![Western blot analysis for MBA, MO7E, U937, and KBM-5 cells.](western_blot)
Ectopic expression of protein tyrosine kinase Bcr-Abl suppresses TNF-induced NF-κB activation and IκBα phosphorylation: Relationship with downregulation of TNF receptors

Asok Mukhopadhyay, Shishir Shishodia, Jill Suttles, Katherine Brittingham, Betty Lamothe, Ramdevi Nimmanapalli, Kapil N. Bhalla and Bharat B. Aggarwal

J. Biol. Chem. published online June 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204748200

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts