NEOPLASIA
www.neoplasia.com

Volume 12 Number 10 October 2010 pp. 766–777 766

Characterization and Mechanistic Studies of a Novel Melanoma-Targeting Construct Containing IκBα for Specific Inhibition of Nuclear Factor-κB Activity[1,2]

Hong Zhou*, Yuying Liu*, Lawrence H. Cheung*, Sehoon Kim*, Weihe Zhang*, Khalid A. Mohamedali*, Preetha Anand†, Walter N. Hittelman‡, Bharat B. Aggarwal† and Michael G. Rosenblum*

*Immunopharmacology & Targeted Therapy Laboratory, Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, USA; †Cytokine Research Laboratory, Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, USA; ‡Laboratory of Cellular Oncology, Department of Experimental Therapeutics, MD Anderson Cancer Center, Houston, TX, USA

Abstract

The transcription factor nuclear factor-κB (NF-κB) is a central mediator of growth and homeostasis for both normal and neoplastic cells. IκBα is the natural intracellular inhibitor of NF-κB and can effectively complex with and thereby inhibit the biologic activity and translocation of NF-κB to the nucleus. We designed a fusion protein designated IκBα/scFvMEL composing of human IκBα and the single-chain antibody scFvMEL, targets melanoma gp240 antigen. Cells treated with IκBα/scFvMEL before irradiation showed specifically inhibition of both constitutive and radiation-induced NF-κB activity on gp240 antigen–positive A375M cells. Pretreatment of A375M cells with IκBα/scFvMEL significantly sensitized melanoma cells to ionizing radiation assessed using a clonogenic survival assay. Mechanistic studies showed that IκBα/scFvMEL, when exogenously added to A375M cells, could be coimmunoprecipitated with the p65 subunit of NF-κB. IκBα/scFvMEL inhibited in a time and/or dose-dependent manner of tumor necrosis factor α– or radiation-induced NF-κB activity *in vitro*. IκBα/scFvMEL was also shown to specifically inhibit the translocation of the NF-κB p65 subunit to the cell nucleus and NF-κB–mediated gene transcription. Further, initial studies showed that mice bearing well-established A375M xenografts were treated (intravenously) with IκBα/scFvMEL and showed a significant suppression of tumor growth. We also observed a decrease in levels of Bcl-2 and Bcl-XL signaling events downstream of NF-κB in the tumor model. These studies demonstrate for the first time that tumor cell–targeted delivery of IκBα may be beneficial for the treatment of melanoma when combined with standard anticancer therapies such as radiation.

Neoplasia (2010) 12, 766–777

Introduction

Recent advances in the understanding of the underlying biology in the progression of melanoma have identified key signaling pathways that are important in promoting melanoma tumorigenesis, thus providing dynamic targets for therapy. One such important target identified in melanoma tumor progression is the nuclear factor-kappa B (NF-κB) pathway [1,2]. The nuclear transcription factor NF-κB is an inducible dimer whose biologic activity is tightly controlled by numerous cellular signal transduction cascades [3,4]. This is a central control pathway responsible for normal and neoplastic cell growth and is involved in various pathways such as cytokine synthesis and release, intracellular

Abbreviations: NF-κB, nuclear factor-kappa B; scFv, recombinant single-chain Fv antibody

Address all correspondence to: Michael G. Rosenblum, PhD, Department of Experimental Therapeutics, University of Texas MD Anderson Cancer Center, Unit 44, 1515 Holcombe Blvd, Houston, TX 77030. E-mail: mrosenbl@mdanderson.org

[1]This research was supported, in part, by the Clayton Foundation for Research.
[2]This article refers to supplementary materials, which are designated by Figures W1 to W5 and are available online at www.neoplasia.com.
Received 25 January 2010; Revised 17 June 2010; Accepted 22 June 2010

DOI 10.1593/neo.10214

signaling, and the inflammatory response in both normal and pathologic conditions. NF-κB complexes are typically retained in the cytoplasm by binding inhibitory IκB proteins (including IκBα) into a ternary, inactive complex. On stimulation, IκBα is rapidly phosphorylated and degraded through the ubiquitin-proteasome pathway and the released dimeric p65/p50 complex becomes active and free NF-κB translocates to and accumulates within the nucleus where it is free to associate with cognate κB elements in target gene promoters.

Many human solid tumor cell lines display increased endogenous nuclear NF-κB levels and/or increased NF-κB transcriptional activity. NF-κB is frequently found to be constitutively activated in Hodgkin lymphoma, head and neck squamous cell carcinoma, non–small cell lung cancer, colorectal cancer, thyroid cancer, pancreatic carcinoma, leukemia, multiple myeloma, prostrate cancer, and breast cancer [5–7]. Several laboratories have also previously shown constitutive NF-κB expression in melanoma cells [8]. In most nontransformed cell types, NF-κB complexes (a heterotrimer composed of p50 and RelA/p65 subunits bound to an inhibitor, IκB) are largely cytoplasmic. Not surprisingly, tumor progression and metastatic spread have been linked to up-regulation of NF-κB activity. Because the NF-κB pathway seems to be a central mediator for growth regulation, cytokine production, and apoptosis modulation. Several studies have suggested that this may represent an excellent potential target for therapeutic intervention [9–13]. Regulation of NF-κB is important for the physiology of inflammation and immune activation, and misregulation of NF-κB activity has been identified as a major culprit of chronic inflammatory diseases and cancer.

The gp240 antigen (also known as the high–molecular weight melanoma-associated antigen) is well expressed on a high percentage of melanoma cell lines and on melanoma tumor biopsy specimens [5–7]. Several monoclonal antibodies (mAbs) that target this antigen have been developed [8,14–16], and a variety of recombinant fusion constructs have been generated from the murine Ab designated ZME-018, which targets this protein [17,18]. A recombinant, single-chain construct designated scFvMEL has been successfully used by our laboratory to deliver a number of therapeutic agents to the cytoplasm of melanoma cells. Because activated NF-κB is a critical mediator of cell growth in melanoma, we proposed that regulation of this pathway in melanoma may be of particular importance. We therefore designed a fusion construct composed of the scFvMEL Ab fused to the natural cellular inhibitor of NF-κB designated IκBα. This article reports the first studies of this novel fusion construct designed to regulate NF-κB activity in tumor-targeted cells, and this represents a potentially new class of targeted therapeutic agents.

In this report, we describe the characterization and mechanism of IκBα/scFvMEL to inhibit NF-κB intracellular activity *in vitro* and *in vivo*. We demonstrated that IκBα/scFvMEL can be specifically internalized into gp240-positive melanoma cells, associate with NF-κB complexes, inhibit NF-κB binding activity and p65 translocation, and inhibit NF-κB–mediated transcription. We demonstrated that targeting treatment with IκBα/scFvMEL inhibited both constitutive and radiation-induced NF-KB activity in melanoma. Clonogenic cell survival assays showed that pretreatment with IκBα/scFvMEL enhanced tumor cell radiosensitivity. Further, *in vivo* studies showed that mice bearing well-established A375M xenografts treated with IκBα/scFvMEL significantly suppressed tumor growth. These studies support the concept of using IκBα as a novel payload in targeted therapeutic constructs for the selective and specific inhibition of NF-κB activity in target cells.

Materials and Methods

Materials

Bacterial strains, pET-32a bacterial expression plasmid, and thrombin were obtained from Novagen (Madison, WI). Hi-Trap chelating HP resin and other chromatography resins were purchased from Amersham Biosciences (Uppsala, Sweden). Abs to anti-IκBα, anti-Bcl2, anti–Bcl-xL, anti-Bax, anti–NF-κB p65, anti–NF-κB p50, anti-PARP, anti–β-actin, and protein G PLUS-agarose were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). FuGENE 6 transfection reagent was obtained from Life Technologies, Inc (Rockville, MD). Recombinant human tumor necrosis factor α (TNFα) were purchased from R&D Systems (Minneapolis, MN). Alexa Fluor 488 goat antirabbit immunoglobulin G (IgG) was purchased from Invitrogen (Grand Island, NY). Fluorescein isothiocyanate (FITC)–coupled antirabbit IgG were purchased from Sigma (St Louis, MO). Alexa Fluor 594 goat antirabbit IgG was purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)–conjugated goat antirabbit IgG was purchased from Bio-Rad (Hercules, CA).

Cell Lines and Cell Culture

The human promyelocytic cell line HL-60 obtained from ATCC were routinely maintained in Iscove's modified Dulbecco's medium with 4 mM L-glutamine and 20% fetal bovine serum. Different human melanoma cell lines A375M, A375SM, AAB527, and TXM-1 were obtained from Dr I. J. Fidler (MD Anderson Cancer Center, Houston, TX). A375M, A375SM, and AAB-527 cells were cultured in Dulbecco's modified Eagle medium, TXM-1 were cultured in minimum essential medium (MEM), and the bladder cell line T-24 was obtained from American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A medium containing 10% fetal bovine serum, with added sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), L-glutamine (2 mM), and MEM vitamins.

Construction of IκBα/scFvMEL Fusion Gene

The human *IκBα* gene was cloned from HL-60 RNA by reverse transcription–polymerase chain reaction (RT-PCR), and DNA was sequenced. The fusion construct was designed in the IκBα-G_4S linker-scFvMEL format. The construction was based on an overlapping PCR method. The *IκBα* gene was amplified by PCR using the primers NTXIÊB (5′-3′) CTGGTGCCACGCGGTTCTTTCCAGGCGGCCGAGCGC and CG4SIÊB (5′-3′) GGAGCCACCGCCACCTAACGTCAGACGCTG. These were designed to insert a thrombin cleavage site at the NH_2 terminus. The *scFvMEL* gene was amplified by PCR from plasmid pET32-scFvMEL/TNF as previously described [17]. The fused *IκBα/scFvMEL* genes were linked together by using primers NTXIÊB and CH3MEL. To clone the fused genes into pET-32a (+) vector with thrombin cleavage site at the NH_2 terminus of *IκBα*, the fragment from pET-32a (+) was amplified by using the primers T_7 promoter (5′-3′) TAATACGACTCACTATAG and CPETTX (5′-3′) AGAACCGCGTGGCACCAGACCAGAAGAATG. The *IκBα/scFvMEL* fusion gene was then cloned into the pET-32a (+) vector at *Xba*I and *Hind*III, designated pET-32 IκBα/scFvMEL.

Expression and Purification of the IκBα/scFvMEL Construct

Escherichia coli Origami DE3 cells (Novagen, Inc) was used to express protein from pET-32 IκBα/scFvMEL. A 5-ml aliquot of a stock culture grown overnight was used to inoculate flasks containing 500 ml

of Luria-Bertani containing 400 μg/ml carbenicillin, 15 μg/ml kanamycin, and 15 μg/ml tetracycline. Cultures were agitated at 240 rpm until $A_{600\ nm}$ = 1.0 at 37°C and then diluted 1:1 with fresh medium plus antibiotics. Expression was induced by addition of isopropyl β-D-thiogalacto-pyranoside to a 100-μM concentration at 18°C overnight. Cells were harvested by centrifuging at 5000 rpm for 20 minutes and were stored frozen.

Cell pellets containing His-tagged IκBα/scFvMEL were resuspended in 20 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Cells were disrupted with Microfluidizer (Newton, MA), and lysates were centrifuged at 40,000 rpm at 4°C for 30 minutes. The supernatant containing only soluble protein loaded onto a cobalt-charged metal-affinity column. The column was washed with 50 column volumes of 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 20 mM imidazole containing 0.1% of Triton X-114 (Sigma-Aldrich, Deisenhofen, Germany) and then washed with 20 column volumes of the same buffer without detergent at 4°C [19]. Elution of the target protein was achieved with 250 mM imidazole. The fusion protein was cut by addition of thrombin (1 U of thrombin cleaves 5 mg of protein when incubated at room temperature for 16 hours) to remove the His-tag. The mixture was further purified using cobalt-charged chelating sepharose resin to remove incompletely digested material and the free His-tag. The final protein product was dialyzed in PBS and stored at 4°C.

Flow Cytometry Analysis

For the analysis of gp240 expression, flow cytometry analysis of cells stained with Abs was performed as described [20]. Briefly, 5×10^5 cells were incubated for 1 hour on ice with anti-gp240 specific ZME-018 IgG2a (2 μg/100 μl in 1% BSA in PBS). Cells were then washed twice with 0.5% BSA in PBS and incubated for an additional 30 minutes on ice with an FITC-conjugated goat antimouse IgG mAb. After two washes, cells were fixed in 3.7% paraformaldehyde and analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA). Use mouse IgG2a as an isotype control.

Enzyme-Linked Immunosorbent Assay (ELISA) and Cytotoxicity Assay of IκBα/scFvMEL to gp240 Positive Melanoma Cells

The 96-well enzyme-linked immunosorbent assay (ELISA) plates containing adherent melanoma and control cells (5×10^4 cells) were used as described previously [21]. To detect the binding activity of IκBα/scFvMEL, cells were incubated with purified IκBα/scFvMEL at various concentrations for 1 hour at room temperature. After they were washed, the cells were incubated with rabbit anti-scFvMEL Ab, followed by the addition of goat antirabbit/HRP conjugate Ab. Finally, the substrate (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid) solution containing 1 μl/ml 30% H_2O_2 was added to the wells. Absorbance at 405 nm was measured with ELISA reader (Bio-Tek Instruments, Inc, Winooski, VT). Cytotoxicity of IκBα/scFvMEL on cells were performed as previously described [21].

Internalization of IκBα/scFvMEL into Cells Expressing gp240 Antigen

The A375M, SK-MEL-5, and T-24 cell lines were added to polylysine-coated 16-well chamber slides (Nunc, Rochester, NY) at 1×10^4 per well and treated with different doses of IκBα/scFvMEL for 4 hours. Or cells were plated at 1×10^6 per well on six-well plates and then treated with IκBα/scFvMEL. Proteins bound to the cell surface were removed by incubating with glycine buffer (500 mM NaCl and 0.1 M glycine, pH 2.5) and neutralizing for 5 minutes with 0.5 M Tris (pH 7.4) followed by washing with PBS. Immunoblot analysis and immunofluorescence laser scanning microscopy were done as previously described [22].

Immunoprecipitation and Western Blot Analysis

For immunoprecipitations and Western blot analysis, cells were lysed in 50 mM Tris (pH 8), 150 mM NaCl, 1% Nonidet P-40 buffer containing protease inhibitors. Aliquots of cell lysates were either resolved in SDS-PAGE or were used for immunoprecipitations with the indicated Abs or with protein G PLUS-agarose (Santa Cruz Biotechnology, Inc). Proteins were transferred onto polyvinylidene difluoride membranes and immunodetected with the indicated Abs. HRP-conjugated secondary Abs and Enhanced Chemiluminescence Detection reagent (Amersham Pharmacia Biotech, Inc, Piscataway, NJ) were used to develop the Western blots as previously described [18,23].

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) were performed by incubating 15 μg of nuclear extracts for 30 minutes at room temperature with α-^{32}P-labeled NF-κB consensus oligonucleotides based on conditions defined previously [24].

Immunocytochemistry for NF-κB p65 Localization

The effect of IκBα/scFvMEL on TNFα-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2; Nikon Corp, Melville, NY) as described previously [25].

NF-κB–Dependent Reporter Gene Expression Assay

The effect of IκBα/scFvMEL on NF-κB–dependent reporter gene transcription induced by TNFα was analyzed by secretory alkaline phosphatase (SEAP) assay as previously described [26]. Briefly, A375M, SK-MEL-5, and T-24 semiconfluent cells (3×10^5 cells per well) were transiently cotransfected with pNF-κB-SEAP (0.5 μg) and the control plasmid pCMV-FLAG1 (0.5 μg) for 24 hours using FuGENE 6 (Life Technologies, Inc). We then treated the cells for 4 hours with IκBα/scFvMEL and/or curcumin and then stimulated them with 1 nM TNFα. The cell culture medium was harvested after 24 hours of TNFα treatment. The culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA) using a Victor 3 microplate reader (Perkin-Elmer, Norwalk, CT).

Clonogenic Survival Assay

The effectiveness of the combination of IκBα/scFvMEL and ionizing radiation was assessed by clonogenic assay. Melanoma cells were pretreated with either PBS or IκBα/scFvMEL (0.3 μM) for 2 hours. Then, cells were irradiated with various doses of ionizing radiation and were then processed for clonogenic cell survival assay [27]. After treatment, cells were trypsinized and counted. Known cell numbers were then replated in triplicate and returned to the incubator to allow macroscopic colony development. Colonies were stained with crystal violet solution and counted after ~14 days. The percentage of plating efficiency and the fraction surviving a given treatment were

calculated based on the survival of nonirradiated cells treated with the agent in question.

Animal Model Studies

A375M xenograft model. Athymic (*nu/nu*) mice, 4 to 6 weeks old, were obtained from Harlan Sprague Dawley (Indianapolis, IN). The animals were maintained under specific pathogen-free conditions and were used at 6 to 8 weeks. Animals were injected subcutaneously (right flank) with 3×10^6 log-phase A375M melanoma cells, and tumors were allowed to establish. Once tumors had reached a measurable size (~30-50 mm^3), animals were treated (intravenously [i.v.], tail vein) with either saline (control) or IκBα/scFvMEL fusion construct daily for 10 days with a total dose of 100 mg/kg.

Localization of IκBα/scFvMEL after systemic administration. Mice bearing A375M xenograft tumors (subcutaneously, flank) were administered IκBα/scFvMEL (100 mg/kg) as an i.v. injection (tail vein). Twenty-four hours after the last administration, animals were killed; tumor tissues were removed, fixed by formalin, and stained by hematoxylin and eosin; and immunohistochemical staining for IκBα/scFvMEL was detected by rabbit anti-scFvMEL Ab from our core laboratory.

Regulation of antiapoptotic proteins. Mice bearing A375M xenograft tumors were administered IκBα/scFvMEL (100 mg/kg). Twenty-four hours after the last administration, animals were killed and tumor tissues were removed, rinsed by ice-cold PBS, and homogenized in ice-cold lysis buffer containing protease inhibitors followed by centrifuge at 14,000 rpm for 10 minutes at 4°C to remove the tissue debris. Protein concentrations of the supernatants were determined, equal amounts of protein were separated by SDS-PAGE, and standard Western blot analysis were performed detected by Bcl-2 and Bcl-xL Abs.

Results

Isolation and Purification of the IκBα/scFvMEL

The IκBα/scFvMEL fusion construct was designed with a short, flexible linker (G_4S) bridging the IκBα and scFvMEL protein (Figure W1*A*). The thioredoxin tag containing the 6-His domain was engineered to contain a thrombin recognition domain for facile, enzymatic removal of the tag, leaving only two amino acid residues (glycine-serine) at the N-terminus of the molecule. Two-phase extraction using a nonionic detergent, such as Triton X-114, is known to be a very effective method for removing endotoxins from purified proteins [28]. We combined affinity chromatography with a nonionic detergent washing step to remove most of the endotoxin contaminants from the end product [19]. An endotoxin reduction of less than 9 to 0.9 EU/mg was achieved with virtually complete protein recovery. The Coomassie-stained SDS-PAGE (Figure W1*B*) demonstrates that one-step binding and elution from immobilized metal affinity chromatography (IMAC) resin resulted in a relatively high level of purity from the bacterial lysate. We were able to generate virtually complete cleavage of the thioredoxin tag from the fusion protein. Re-exposure of the cleavage mixture to fresh IMAC resin allowed the cleaved purification tag to bind, whereas the released IκBα/scFvMEL fusion protein flowed through the column. The final material migrated on SDS-PAGE with a molecular weight of 55 kDa and was essentially free of contaminating proteins.

Analysis of gp240 Antigen (High–Molecular Weight Melanoma-Associated Antigen) Expression in Various Human Melanoma Cell Lines

To examine the expression of the gp240 antigen on melanoma cell lines, melanoma cells were stained with parental mAb ZME-018 IgG2a that specifically binds to the gp240 antigen. As shown in (Figure W2, *A* and *B*), using the anti-gp240 Ab ZME-018, A375M cells show strongly positive (98.9%) and SK-MEL-5 cells show less positive (46.4%) by flow cytometry compared with the isotype-matched control (IgG 2a) murine Ab. In contrast, the TXM-1 cells demonstrated weak binding (4.6%) and the bladder cell line T-24 showed almost no binding to the Ab by flow. Immunohistochemistry analysis confirmed strong, uniform staining on the A375M cells, whereas staining on the TXM-1 cells showed no difference compared with the isotype control (Figure W2*B*). ELISA result also showed the same pattern (Figure W2*C*). These data demonstrated that the gp240 antigen was more highly expressed on A375M, and there was a very low level of expression of the gp240 antigen on TXM-1 and no expression on T-24.

Assessment of Cell-Binding Activity of the scFvMEL Component of IκBα/scFvMEL

Because the gp240 antigen highly presents in melanoma A375M but not in T-24, we further examined whether the IκBα/scFvMEL could bind to antigen-positive cells. ELISA was performed to determine the binding activity of the IκBα/scFvMEL to melanoma cells. The results demonstrate that IκBα/scFvMEL bound to high-level gp240 antigen–expressing melanoma A375M (Figure 1, *A*, *C*, and *D*) cell lines. However, the protein showed very less or did not bind to T-24 (Figure 1, *B* and *D*), in which the gp240 antigen was not detected by ZME-018 mAb (Figure W2, *A* and *C*). To determine whether IκBα/scFvMEL recognize the same antigen of A375M to its parental mAb ZME-018, competitive ELISA was performed by preincubating with a 10-fold higher concentration of ZME-018 before IκBα/scFvMEL was added to the cell surface. Figure 1*C* showed that ZME-018 did compete with IκBα/scFvMEL to bind gp240 antigen on A375M cells.

IκBα/scFvMEL Specifically Is Internalized into gp240 Antigen–Positive Melanoma Cells

We next examined by Western blot and immunostaining whether IκB/scFvMEL was specifically delivered into the cytoplasm of the gp240 antigen–positive melanoma cells. Human melanoma cells expressing high levels of gp240 (A375M), lower levels (AAB527), and antigen-negative cells (TXM-1) were treated with various doses of IκB/scFvMEL for 2 hours. Cell surfaces were washed and stripped by addition of glycine buffer (500 mM NaCl, 0.1 M glycine, pH 2.5) for 5 minutes to remove excess fusion protein. Whole-cell lysates were detected by Western blot using a rabbit anti-IκBα Ab. The migration of authentic IκBα and IκBα/scFvMEL are indicated on Figure 2, *A*, *B*, and *C*, by the *arrows*. There was no internalization of IκBα/scFvMEL observed in the TXM-1 and T-24 cells. In contrast, there were significant intracellular concentrations of IκBα/scFvMEL in the A375M cells within 2 hours of exposure to doses of 50 to 200 nM. Internalization of IκBα/scFvMEL by AAB527 cells showed a higher dose dependence over the range tested. There did not seem to be a breakdown of endogenous IκBα within the 2-hour time course because the basal levels of IκBα did not seem to be affected by the treatment with IκBα/scFvMEL. Figure 2*B* showed that 100 nM IκBα/scFvMEL for approximately 1.5 hours of treatment can mediate the internalization into A375M cells. For the receptor-negative T-24 cells, only a weak band was detected

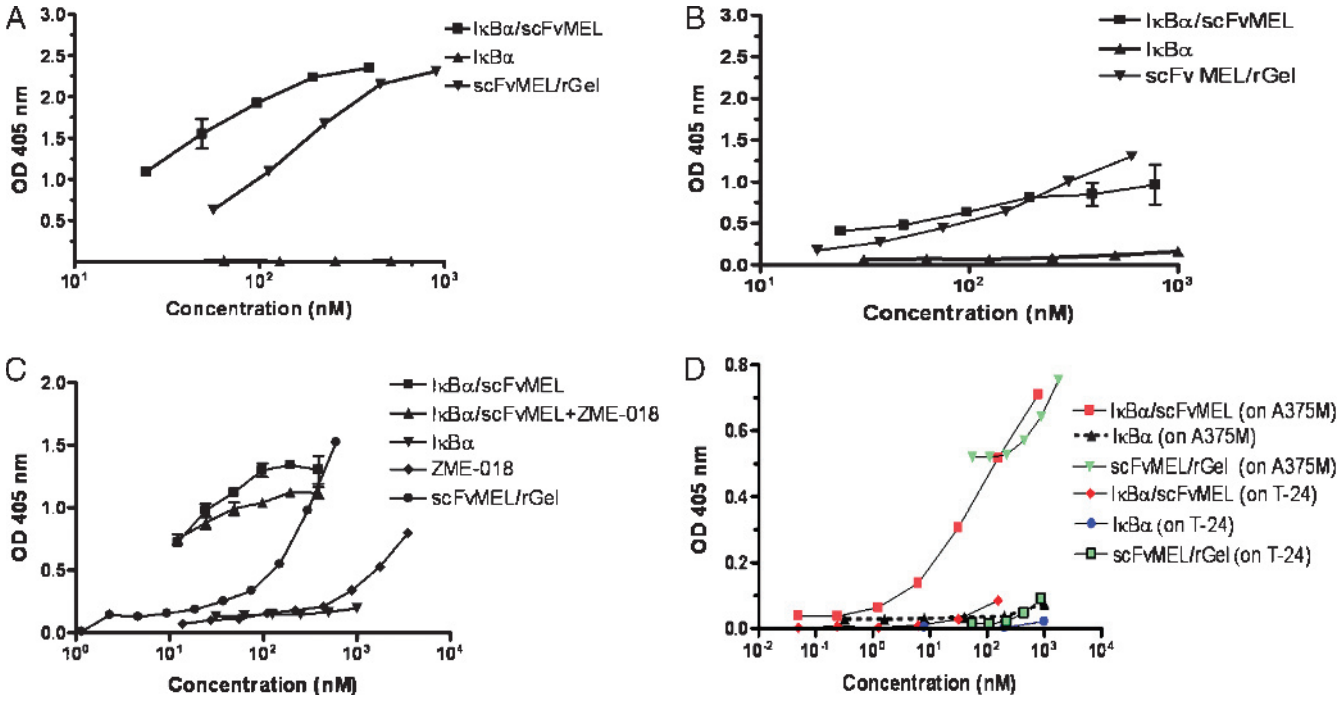

Figure 1. IκBα/scFvMEL specifically binds to gp240 antigen–positive melanoma cells, as assessed by ELISA. IκBα/scFvMEL binds to the (A) gp240 antigen–positive cell line A375M, (B) gp240-negative cell line T-24, (C) A375M but was competed by preadded ZME-018 to the cells, and (D) gp240-positive and –negative cell lines. scFvMEL/rGel as a positive control protein.

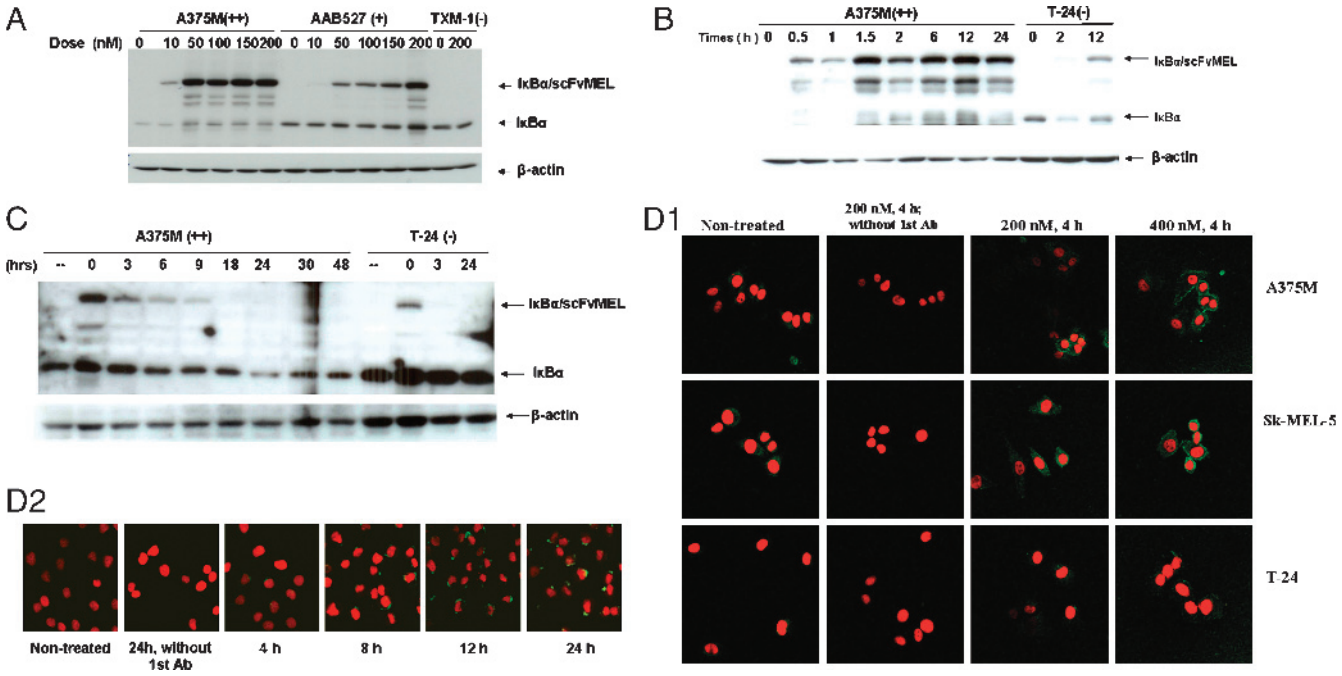

Figure 2. Internalization of IκBα/scFvMEL into antigen-positive and -negative human melanoma cells. (A) IκBα/scFvMEL specifically internalized into gp240-positive cells. Cells were treated with different concentrations of IκBα/scFvMEL for 2 hours. Cell surfaces were washed and stripped to remove excess fusion protein. Whole-cell lysates were analyzed by Western blot using anti-IκBα Ab. (B) Time course of IκBα/scFvMEL internalized into gp240-positive cells. A375M and T-24 cells were treated with 100 nM of IκBα/scFvMEL for the indicated times. Cell surfaces were washed and stripped to remove excess fusion protein. Whole-cell lysates were analyzed by Western blot using anti-IκBα Ab. The arrows demonstrate the migration positions of IκBα/scFvMEL and native IκBα, respectively. (C) Internalization of IκBα/scFvMEL into A375M cells analyzed by pulse chase analysis. A375M and T-24 cells were pretreated with 100 nM of IκBα/scFvMEL for 2.5 hours, then washed with PBS twice, and were added with a fresh medium. After culturing for different times, cells were lysed, and proteins were analyzed by Western blot using anti-IκBα Ab. (D) 1, Internalization of IκBα/scFvMEL into A375M, SK-MEL-5, and T-24 cells was visualized using confocal microscopy (original magnification, ×200). 2, Time-dependent internalization of IκBα/scFvMEL into A375M cells was visualized using confocal microscopy (original magnification, ×200).

up to 12 hours of treatment. Pulse chase analysis showed that after 2.5 hours of 100 nM IκBα/scFvMEL treatment, the fusion construct could be maintained in the cytoplasm up to 9 hours on A375M; however, for T-24, we could only detect the weak signal after immediately removing the protein solution (Figure 2*C*). The IκBα moiety of the fusion construct was efficiently delivered into the cytosol of gp240 antigen–positive A375M, but less was delivered into SK-MEL-5 (because of less gp240 expression level; Figure W2, *A* and *C*) and was not delivered into gp240 antigen–negative T-24 cells showed dose dependent, as visualized by confocal microscope detected using anti-scFvMEL Ab (Figure 2*D1*). Moreover, the internalization effect of IκBα/scFvMEL into A375M was time-dependent as confirmed by confocal microscopy (Figure 2*D2*). These strongly demonstrated that the gp240 expression on the tumor cell surface and the binding of IκBα/scFvMEL to the gp240 antigen are responsible for the internalization of the IκBα/scFvMEL.

IκBα/scFvMEL Associates with the p65 Subunit of NF-κB and Inhibits NF-κB Activation Induced by TNFα

It is known that the inhibitor proteins IκBα tightly regulate the transcriptional activity of p65– and c-Rel–containing NF-κB dimers. In resting cells, IκBα binds extremely tightly to NF-κB, preventing its nuclear accumulation and association with DNA [29]. To assess whether exogenously entered IκBα/scFvMEL can interact with cellular NF-κB, A375M cells were treated with 100 nM IκBα/scFvMEL overnight, and the p65 subunit of NF-κB was immunoprecipitated. IκBα/scFvMEL coimmunoprecipitated with p65, suggesting its association

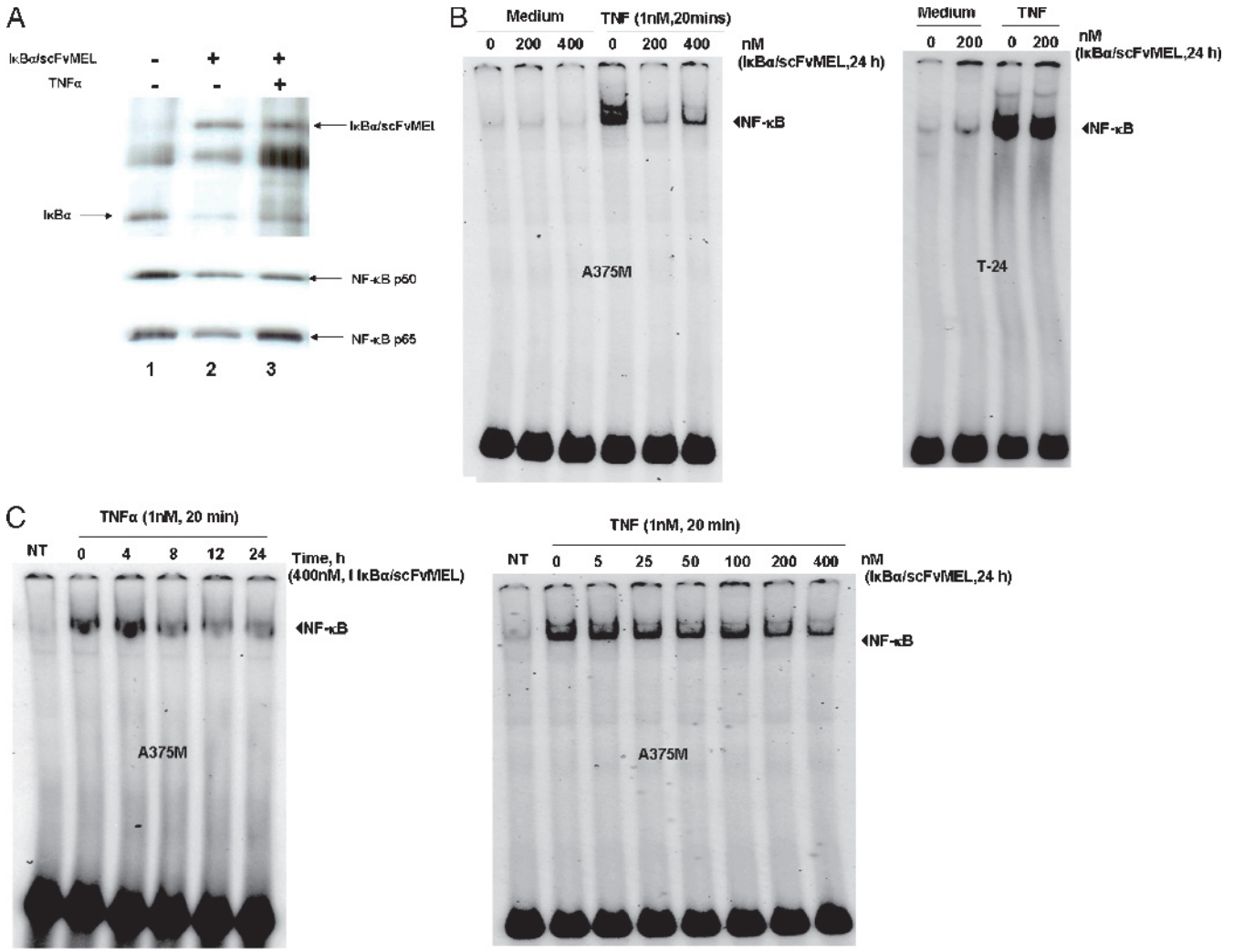

Figure 3. IκBα/scFvMEL associates with NF-κB and inhibits TNFα-induced NF-κB activation. (A) IκBα/scFvMEL associates with NF-κB. A375M cells were left untreated or were incubated with 100 nM IκBα/scFvMEL overnight and then stimulated with or without 0.5 nM TNFα for 30 minutes. Cells were washed and lysed, and the NF-κB p65 was immunoprecipitated. Immune complexes were resolved in SDS-PAGE, transferred onto a membrane, and sequentially probed with anti-IκBα, anti-p50, and anti-p65 Abs. (B) IκBα/scFvMEL specifically inhibits TNFα-induced NF-κB activation on A375M but not T-24 cells. A375M and T-24 cells were pretreated with 200 and/or 400 nM IκBα/scFvMEL for 24 hours at 37°C and then stimulated with 1 nM TNFα for 20 minutes. Nucleus extracts were then prepared and assayed for NF-κB by EMSA. (C) Time- and dose-dependent inhibition of TNFα-induced NF-κB activation by IκBα/scFvMEL. A375M cells were preincubated with 400 nM IκBα/scFvMEL for the indicated times, or A375M cells were treated with different doses (5, 25, 50, 100, 200, or 400 nM) of IκBα/scFvMEL for 24 hours and then induced by 1 nM TNFα for 20 minutes, and nucleus extracts were examined for NF-κB activation by EMSA. NT indicates not treated.

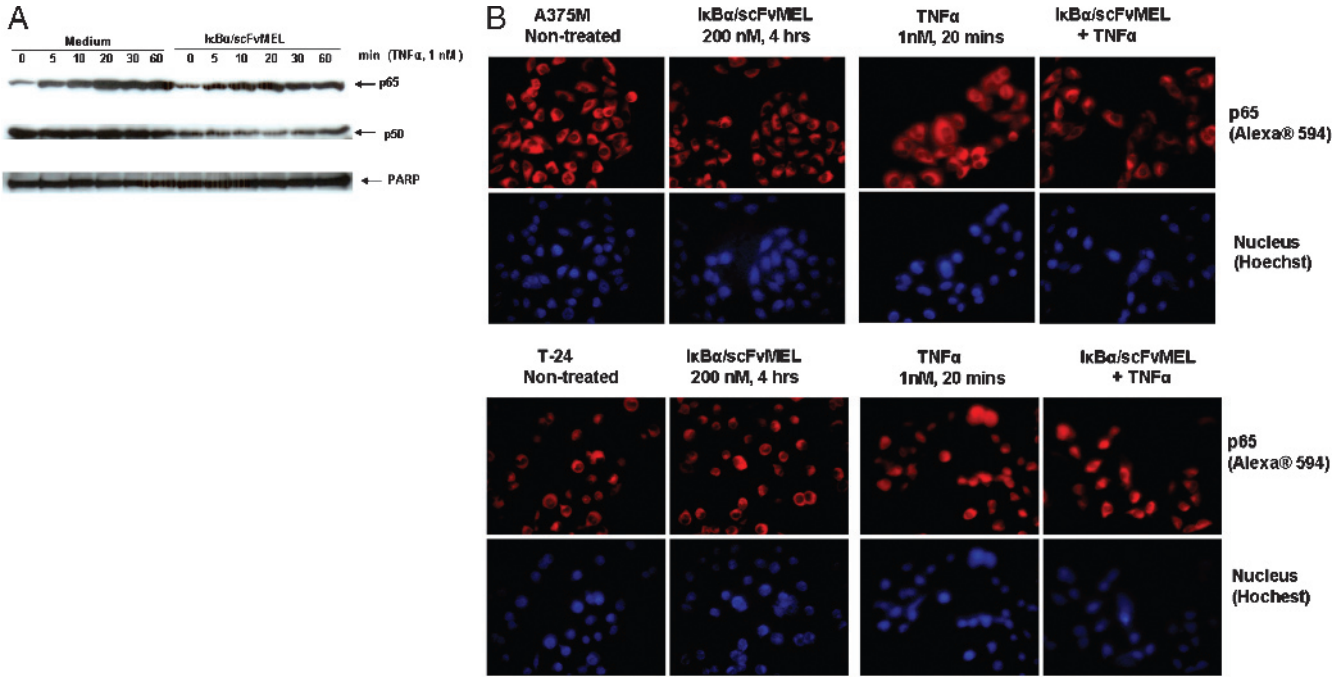

Figure 4. IκBα/scFvMEL inhibits TNFα-induced nuclear translocation of p65. (A) A375M cells were either untreated or pretreated with 200 nM IκBα/scFvMEL for 24 hours and then treated with 1 nM TNFα for the indicated times. Nucleus extracts were prepared and analyzed by Western blot using Abs against p65 and p50. As a nuclear protein loading control, the membrane was blotted with anti-poly (ADP-ribose) polymerase (PARP) Ab. (B) Immunocytochemical analysis of NF-κB p65 localization. A375M and T-24 cells were not treated or pretreated with 200 nM IκBα/scFvMEL for 4 hours, then stimulated with 1 nM TNFα, and subjected to immunocytochemistry analysis. NF-κB p65 were stained red and counterstained for nuclei with Hoechst 33342 (50 ng/ml) for 5 minutes.

with endogenous NF-κB (Figure 3*A*). Because TNFα is one of the most potent activators of NF-κB and the mechanism of activation is relatively well established, we examined the effect of IκBα/scFvMEL on TNFα-induced NF-κB activation in A375M and T-24 cells. A375M stimulated with 1 nM TNFα for 20 minutes can make maximum induction of NF-κB activity (Figure W3). A375M and T-24 cells were pretreated with 200 and/or 400 nM IκBα/scFvMEL for 24 hours and then stimulated with TNFα. As indicated by EMSA, IκBα/scFvMEL can almost specifically fully inhibit NF-κB activity in A375M but not T-24 cells (Figure 3*B*). Incubating with different doses and times of IκBα/scFvMEL on A375M, IκBα/scFvMEL suppressed TNFα-induced NF-κB activation in a dose- and time-dependent manner (Figure 3*C*).

IκBα/scFvMEL Specifically Inhibits TNFα-Induced Nuclear Translocation of p65

In the next step of the NF-κB activation cascade, TNFα induces the phosphorylation of p65, which is required for its transcriptional activity. After phosphorylation, the p65 subunit is translocated to the nucleus. To assess whether IκBα/scFvMEL can inhibit the subunit of NF-κB translocation, A375M cells were either untreated or pretreated with 200 nM IκBα/scFvMEL for 24 hours and were then stimulated with 1 nM TNFα for different times. Nucleus extracts were prepared and analyzed by Western blot using Abs against p65 and p50. Western blot analysis showed that IκBα/scFvMEL partially blocked the TNFα-induced nuclear translocation of p65 in a time-dependent manner in A375M cells (Figure 4*A*). Immunocytochemistry also confirmed this finding (Figure 4*B*), that is, IκBα/scFvMEL alone would not induce the p65 translocation in both cell types. However, IκBα/scFvMEL could specifically inhibit TNFα-stimulated p65 subunit translocation into the nucleus in A375M but not in T-24 cells.

IκBα/scFvMEL Specifically Inhibits NF-κB–Driven Transcription

On stimulation, NF-κB–bound IκBα is specifically phosphorylated (by the IκB kinase), ubiquitinated, and degraded by the proteasome. NF-κB then enters the nucleus, binds DNA, and regulates transcription of its numerous target genes [29]. To determine whether IκBα/scFvMEL could inhibit the transcriptional activity of NF-κB, TNFα-induced NF-κB–dependent reporter gene expression assay was used. We transiently transfected the cells with the NF-κB–regulated SEAP reporter construct, incubated them with IκBα/scFvMEL, and then stimulated with 1 nM TNFα. An almost 12-fold increase in SEAP activity over the cells' control in A375M cells was observed on stimulation with 1 nM TNFα, and IκBα/scFvMEL exogenously added inhibited TNFα-induced SEAP production in a dose-dependent manner on A375M (Figure 5*A*) and SK-MEL-5 (Figure 5*B*) cells. The presence of 10 μM curcumin in the culture medium resulted in only approximately 30% inhibition, but curcumin did a better job on T-24 cells (Figure 5, *A* and *C*). Addition of up to 400 nM IκBα/scFvMEL to the culture medium had less effect on TNFα-induced SEAP production on T-24 cells (Figure 4*C*). These results show that IκBα/scFvMEL specifically represses NF-κB–dependent reporter gene expression induced by TNFα directly related to the gp240 receptor status on the cells. Besides the reporter gene, we also checked the expression of the two important apoptosis-related genes downstream of the NF-κB cascade. Cells were pretreated with IκBα/scFvMEL for 24 hours and then treated with TNFα for different times. Whole-cell extracts were prepared

and analyzed by Western blot analysis for Bcl-2 and Bax expression (Figure 5*D*). IκBα/scFvMEL inhibited TNFα-induced antiapoptotic protein Bcl_2 expression and upregulated proapoptotic protein Bax expression separately. The results further strengthen the validation of our postulate that IκBα/scFvMEL has a role in blocking TNFα-induced NF-κB transcription.

Cytotoxic Effects of IκBα/scFvMEL on Log-Phase Cells

We assessed the effect of IκBα/scFvMEL antigen-positive (A375M, AAB527, and A375SM) and antigen-negative melanoma cells (TXM-1) in log-phase culture for 72 hours of incubation. As shown in Figure W4, there was little toxicity of either IκBα or the IκBα/scFvMEL construct at doses up to 100 nM.

Selective Effect of IκBα/scFvMEL on Sensitivity to Ionizing Radiation

We next treated A375M and TXM-1 cells with IκBα/scFvMEL alone, with radiation exposure (4 Gy) alone, or a combination of the two. As Figure 6*A* shows, radiation exposure induced NF-κB activity above basal levels, and treatment with IκBα/scFvMEL was able to completely abrogate the basal activity and the radiation-induced component. We added a second line (A375SM) that is gp240-positive, a more metastatic variant, and showed a higher induction of NF-κB activity with radiation exposure. Treatment with IκBα/scFvMEL was also able to suppress both basal and radiation-induced activity. TXM-1 cells were found to express low basal levels of NF-κB, and these levels were unchanged with exposure to radiation, to IκBα/scFvMEL, or to a combination of both. In addition, we examined the activity of Bcl-2 and Bcl-xL proteins, which are downstream of NF-κB. As shown in Figure 6*B*, treatment with 200 nM of the IκBα/scFvMEL reduced the activity of both Bcl-2 and Bcl-xL proteins in A375M cells. As expected, there was no effect of treatment on TXM-1 cells.

We next examined the ability of the IκB/scFvMEL to sensitize melanoma cells to ionizing radiation in a clonogenic survival assay. Cells were treated with 300 nM IκBα/scFvMEL and were subjected to ionizing radiation either at a 2- or 4-Gy dose. As shown in Figure 6*C*, there was no effect of IκBα/scFvMEL on the clonogenic potential of antigen-negative (TXM-1) melanoma cells at either 2 or 4 Gy, although the radiation itself was able to reduce survival by almost 90%. Treatment of A375M cells with IκBα/scFvMEL was shown to result in a statistically significant decrease in clonogenic survival at both the 2- and 4-Gy dose levels. The survival factor was reduced from 50.2% ± 1.06% in vehicle-treated A375M cells to 35.4% ± 2.75% in IκBα/scFvMEL-treated cells at 2 Gy ($P < .05$) and from 19.8% ± 2.50% in vehicle–treated cells to 7.4% ± 0.74% in IκBα/scFvMEL–treated cells at 4 Gy ($P < .05$).

In Vivo Antitumor Effects of IκB/scFvMEL on Nude Mice Bearing A375M Xenografts

Mice bearing well-established A375M xenografts were treated (i.v.) with saline or IκB/scFvMEL construct (100 mg/kg total dose) divided

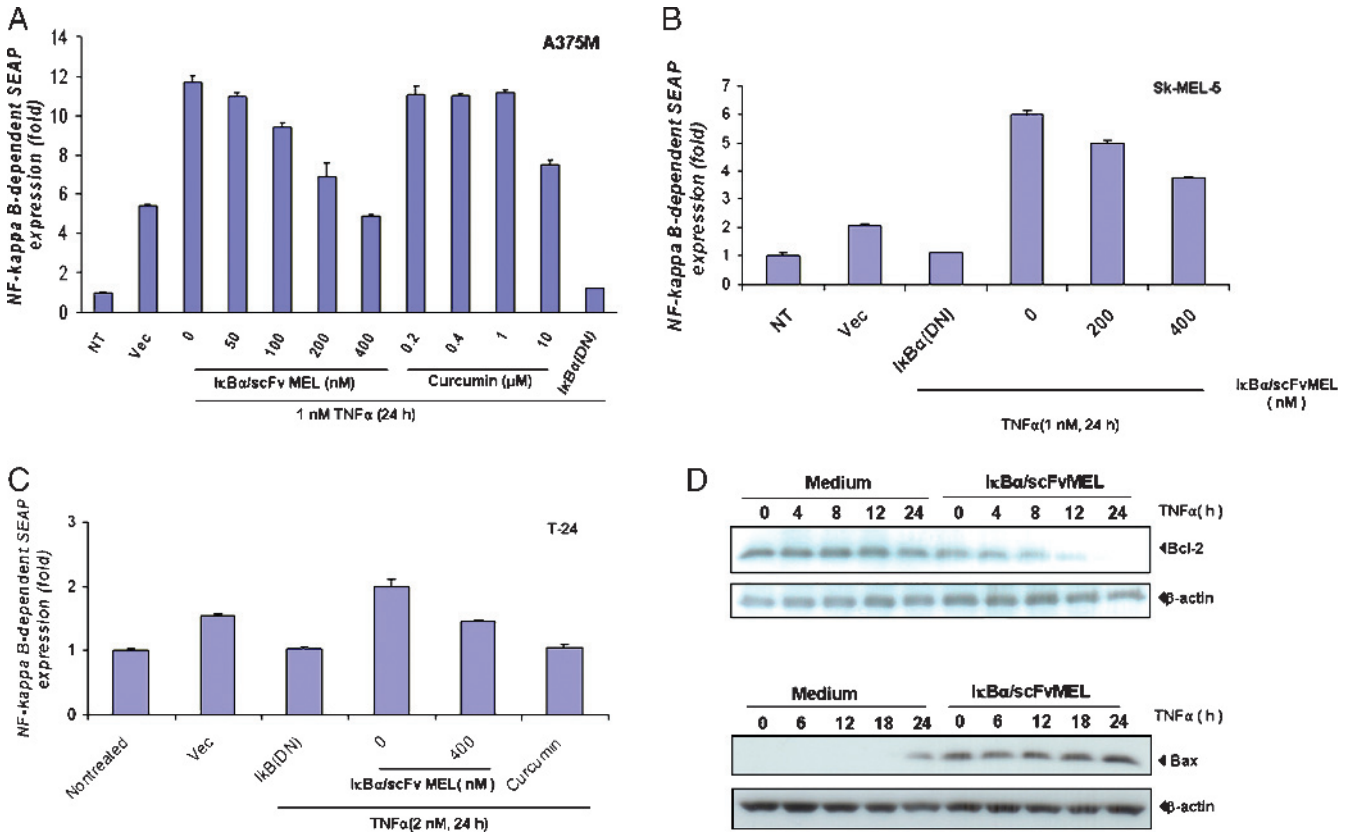

Figure 5. IκBα/scFvMEL inhibits TNF-induced expression of NF-κB–dependent gene. Cells were transiently transfected with a NF-κB–SEAP plasmid and then pretreated with the indicated concentration of IκBα/scFvMEL and/or curcumin for 4 hours and then coincubated with 1 nM TNF for 24 hours. Cell supernatants were collected and assayed for SEAP activity: (A) A375M, (B) SK-MEL-5, and (C) T-24. (D) IκBα/scFvMEL inhibits TNFα-induced expression of NF-κB–dependent antiapoptotic protein Bcl-2 and upregulates the expression of proapoptotic protein Bax. A375M cells were incubated with 400 nM IκBα/scFvMEL for 24 hours and then treated with 1 nM TNFα for the indicated times. Whole-cell lysates were prepared and analyzed by Western blot analysis using the indicated Abs. NT indicates not treated.

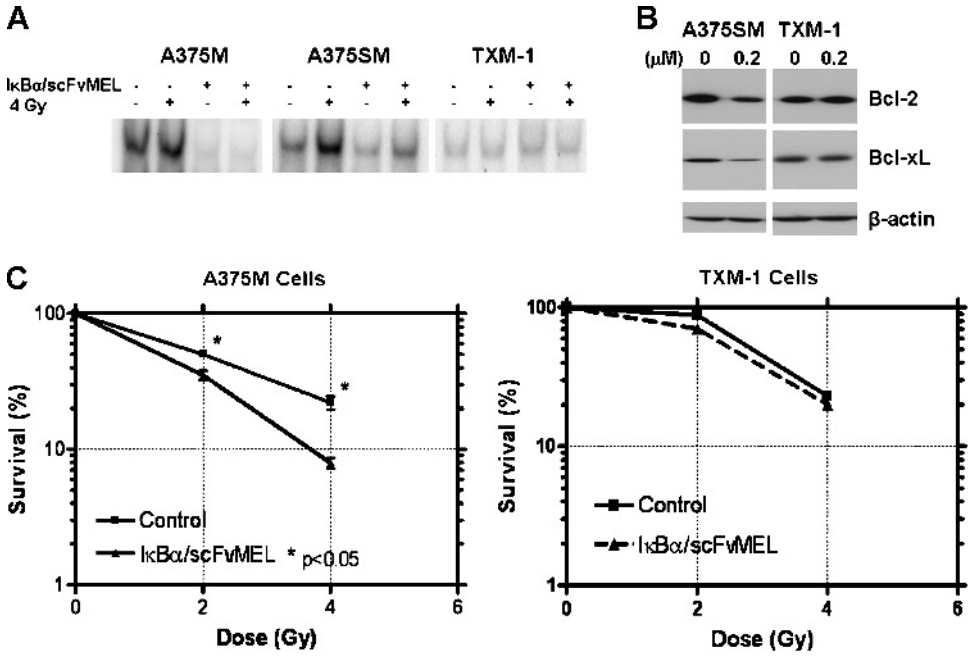

Figure 6. Treatment with IκBα/scFvMEL sensitizes gp240 antigen–positive melanoma cells to ionizing radiation. (A) Treatment with IκBα/scFvMEL specifically blocks constitutive and radiation-induced NF-κB activity in gp240 antigen–positive A375M cells but not on gp240 antigen–negative TXM-1 melanoma cells. A375M, A375SM, and TXM-1 cells were left untreated, exposed to 4 Gy, and pretreated with 300 nM IκBα/scFvMEL for 2.5 hours or a combination of IκBα/scFvMEL and radiation. Cells were harvested 2 hours after irradiation for analysis by EMSA. (B) Decrease in levels of Bcl-2 and Bcl-xL in A375M but not TXM-1 cells after treatment with 200 nM IκBα/scFvMEL. Western blot analysis for Bcl-2 and Bcl-XL in A375M and TXM-1 cells treated with 200 nM IκBα/scFvMEL for 4 hours. (C) Radiosensitization by IκBα/scFvMEL was based on clonogenic cell survival assays. A375M and TXM-1 cells were pretreated with IκBα/scFvMEL (300 nM for 2 hours), the drug was washed off, and cells were irradiated at various doses and plated for clonogenic cell survival assay. Observed sensitizations were statistically significant at 2-, 4-, and 6-Gy dosage groups on A375M cells ($P < .05$; the results were analyzed from three experiments). No statistically significant sensitization was observed in gp240 antigen–negative TXM-1 cells ($P > .05$). Treatment of cells with the construct alone had no effect on survival (data not shown).

into 10 daily doses. As shown in Figure 7*A*, there was a significant suppression of tumor growth in the treated group, which persisted long after the end of i.v. administration. As shown in Figure W5, at the end of the 10-day treatment period, tumors from the control mice were almost four-fold larger than tumors from mice treated with the fusion construct. Thereafter, the saline-treated group continued rapid growth, reaching approximately 2300 mm^3 (mean) compared with the treatment group (130 mm^3) at day 50. The mice were then killed, tumor specimens were assessed by IHC, and the intratumor activities of several signaling events downstream of NF-κB including Bcl-2 and Bcl-xL were assessed by Western blot (Figure 7*B*).

Analysis of tumor xenografts at day 50 (>30 days after the last injection) by immunohistochemistry (Figure 7*C*) showed that the scFvMEL Ab could be easily detected tumor xenografts demonstrating tumor localization of the IκB/scFvMEL construct. There was no detectable change observed in the hematoxylin and eosin stain performed on the same samples. Analysis of various signaling proteins in tumors treated with saline or the IκB/scFvMEL construct (Figure 7*B*) showed no significant change in p53, GADD45, cyclin D1, or BAX levels. We did observe a decrease in levels of Bcl2 and Bcl-xL, consistent with the observations in tissue-cultured cells. In addition, we also found a slight decrease in levels of MDM-2.

Discussion

With the slow progress in finding an effective treatment of melanoma in recent years, the expectation is that novel treatment agents that target signaling pathways essential for melanoma growth and invasion may offer hope for an otherwise dismal disease. It is now clear that melanoma exhibits constitutive activation of NF-κB. This activation of NF-κB leads to endogenous expression of a number of factors associated with escape from apoptosis, tumorigenesis, and metastasis. It is well understood that NF-κB plays a role in tumor growth, metastatic spread, apoptosis, chemoresistance, and radioresistance. Given its dominance in the regulation of a number of growth signals and in the immune and inflammatory response as well as in the growth of neoplastic cells, NF-κB has been the subject of numerous strategies to develop regulators of its activity. Yan Liu et al. [30] have described a small peptide motif capable of inhibiting nuclear transport of NF-κB and, when introduced into mice, was shown to suppress the production of the proinflammatory cytokines TNFα and interferon-γ. Other approaches to regulating NF-κB activity involve regulating IκB phosphorylation [9] and inhibiting the p50-mediated binding to DNA complex [31,32]. Small molecule inhibitors of NF-κB activity include DHMEQ [33], 15-deoxy-$\Delta_{12,14}$-prostaglandin J(2) [34], CP-1158 [35], linoleic acid derivatives [36], triterpenoid derivatives [37], CSA [38], curcumin [39,40], and its derivatives [41]. Reagents like sulindac or other nonsteroidal anti-inflammatory drugs offer potential therapeutic reagents for human tumors [42,43]. Recent progress in developing specific inhibitors of NF-κB has resulted in designing inhibitors of IκB kinases. Another class of NF-κB pathway inhibitors identified is the proteasome inhibitors. Although studies describe a decrease in transcription factor activity, most of these agents target other key regulatory molecules

along with NF-κB, rendering it difficult to discern the specific role of NF-κB in the progression of the disease and its attribute as a therapeutic target. A major concern about using the previously mentioned inhibitors of NF-κB is the lack of specificity or selectivity. The current study differs from previously described molecules in that the effects described are clearly dependent on the cellular targeting and internalization of the fusion construct. These inhibitory effects on NF-κB can clearly be targeted *in vitro* to selected sites using an appropriate construct.

As far as we are aware, there have been no studies that have demonstrated cell-specific delivery of agents capable of inhibiting the NF-κB transcription factor. Tumor-targeting studies have demonstrated proof of concept in this particular fusion construct. The high–molecular weight melanoma-associated glycoprotein gp240 has previously been demonstrated in a majority (80%) of melanoma cell lines and fresh tumor samples [44]. More importantly, the gp240 antigen is either not expressed or expressed in low level in normal cells [45,46], thus making this an interesting target for therapeutic intervention. mAbs targeting the gp240 antigen, such as ZME-018 and 9.2.27, have been extensively studied in melanoma patients and have demonstrated in numerous clinical trials an impressive ability to localize in metastatic tumors after systemic administration [45,46]. In addition, a variety of recombinant fusion constructs have been generated from the murine Ab designated ZME-018, which targets this protein.

The current study describes a fusion construct composed of the natural inhibitor of intracellular NF-κB activity (IκBα) and an anti-melanoma single-chain Ab scFvMEL, IκBα/scFvMEL. We clearly demonstrate that intracellular delivery of the IκBα/scFvMEL molecule can block TNFα-mediated NF-κB activation. And also IκBα/scFvMEL can specifically inhibit both endogenous and radiation-induced up-regulation of NF-κB activity. The inhibitory effect of IκBα/scFvMEL directly correlated with physical association with the subunit of NF-κB complex (Figure 3*A*), and this association could not be disrupted by TNFα stimulation (Figure 3*A*, *lane 3*). As a natural inhibitor of NF-κB, when NF-κB is activated with activating signals, IκBα phosphorylates, ubiquitinates, and degrades IκB by the 26S proteasome. Stimulated cells with TNFα caused the degradation of endogenous IκBα but not IκBα/scFvMEL (data not shown), which indicated that IκBα/scFvMEL most likely exerts its inhibitory function, by associating nonreversibly with the NF-κB components inside the cells. As a super suppressor of NF-κB, we found that IκBα/scFvMEL inhibits TNFα-stimulated NF-κB p65 translocation into the nucleus and also NF-κB–dependent reporter gene expression. And also, we have shown that IκBα/scFvMEL

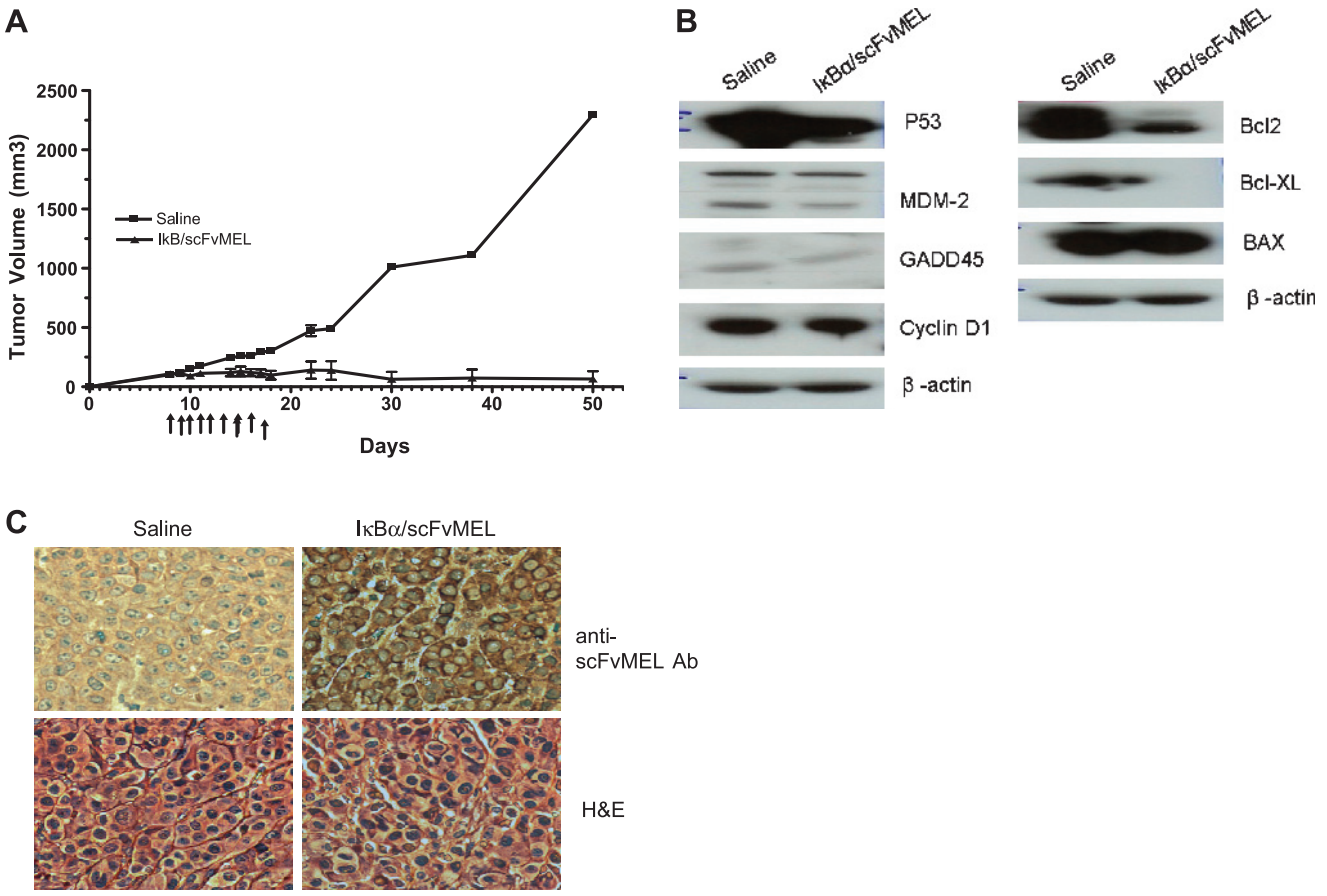

Figure 7. IκBα/scFvMEL demonstrates antitumor activity in a xenograft melanoma model. (A) Nude mice bearing human melanoma (A375M) tumors were treated i.v. with either saline (control) or IκBα/scFvMEL (100 mg/kg; total dose) for 10 consecutive days (arrows). The treatment of mice bearing established (30-50 mm^3) tumors with IκBα/scFvMEL at a total dose of 100 mg/kg resulted in potent tumor suppression and complete tumor regression of all lesions. In contrast, all mice treated with either saline showed rapid tumor growth. (B) Intratumor activities of several signaling events downstream of NF-κB including Bcl-2 and Bcl-xL were assessed by Western blot. (C) IκBα/scFvMEL is localized or internalized in tumor tissues. Twenty-four hours after the last dose, animals were killed, and tumor tissues were removed, fixed, and stained by immunohistochemical staining for IκBα/scFvMEL detected by either anti-scFvMEL Ab. Localization and internalization of IκBα/scFvMEL were observed in tumor tissues in the treatment group but not in the control group.

inhibited tumor growth in the A375M xenograft model (Figure 7*A*). Because NF-κB activation has an antiapoptotic role in tumor cells, it is possible that inhibition of NF-κB activation would regulate the apoptosis-relating gene expression. We demonstrated that IκBα/scFvMEL can downregulate the downstream signals of Bcl-2 and Bcl-xL (Figure 6*B*) and upregulate Bax (Figure 5*D*). We also observed a decrease in levels of Bcl-2 and Bcl-XL signaling events downstream of NF-κB in the tumor model (Figure7*B*). Suppression of NF-κB has been implicated in chemoprevention; studies with the IκBα/scFvMEL demonstrate that the agent can augment the radioresponse of antigen-positive melanoma cells to ionizing radiation.

The relationship of melanoma metastasis and inflammation has been well understood [47]. The pivotal role of NF-κB in inflammation is well established, and considerable efforts have been made to fully understand its regulation and to develop agents that specifically inhibit its action. IκBα, in its wild type and mutated forms, represents a specific inhibitor and has been used in experimental system to inhibit NF-κB activity. In most of the investigations, IκBα was expressed using an adenoviral gene delivery system. However, today, delivery of genes *in vivo* with viral vectors with the aim to regulate signaling pathways has the following disadvantages: inadequate control over expression levels, irreversibility, and introduction into cells irrelevant and in many cases unwanted genetic material. Moreover, the use of this technology for the treatment of inflammatory conditions possesses the additional disadvantage of inflammation and/or immune response elicited by the viral vector, especially in the case of adenoviruses, which may limit their use in the clinic. Here, we describe a novel way to introduce the super-repressor IκBα inhibitor into melanoma cells to inhibit aberrant NF-κB.

Currently, there are several approved treatments for metastatic melanoma, including chemotherapy and biologic therapy as both single treatments and in combination, but none is associated with a significant increase in survival. One of the main problems with melanoma treatment is chemotherapeutic resistance. Melanoma tumors and cultured cell lines are relatively resistant to the cytotoxic effects of ionizing radiation, thereby limiting the use of radiotherapy for the clinical treatment of melanoma. Munshi et al. [48] showed that inhibition of constitutively activated nuclear factor-κB radiosensitizes human melanoma cells. Controlling the transcript factor NF-κB [49] and tumor immunotherapy in melanoma [50] is a promising way of prevention. IκBα/scFvMEL presents a promising targeting molecule to manipulate NF-κB activity in rational combinations with ionizing radiation, and it may improve the efficacy of radiotherapy for melanoma.

References

[1] Ivanov VN, Bhoumik A, and Ronai Z (2003). Death receptors and melanoma resistance to apoptosis. *Oncogene* **22**, 3152–3161.

[2] Nyormoi O and Bar-Eli M (2003). Transcriptional regulation of metastasis-related genes in human melanoma. *Clin Exp Metastasis* **20**, 251–263.

[3] Escarcega RO, Fuentes-Alexandro S, Garcia-Carrasco M, Gatica A, and Zamora A (2007). The transcription factor nuclear factor-κB and cancer. *Clin Oncol (R Coll Radiol)* **19**, 154–161.

[4] Okamoto T, Sanda T, and Asamitsu K (2007). NF-κB signaling and carcinogenesis. *Curr Pharm Des* **13**, 447–462.

[5] Giacomini P, Ng AK, Kantor RR, Natali PG, and Ferrone S (1983). Double determinant immunoassay to measure a human high–molecular-weight melanoma-associated antigen. *Cancer Res* **43**, 3586–3590.

[6] Rentsch M, Cerny T, Geiger L, Gerber H, Brunner KW, Rosler H, and Nachbur B (1989). Radioimmunoscintigraphy with a ^{99m}Tc-labeled F(ab′)2 fragment of a monoclonal antibody (HMW-MAA 225.28S) in 71 patients with malignant melanoma. *Schweiz Med Wochenschr* **119**, 1382–1385.

[7] Wilson BS, Ruberto G, and Ferrone S (1983). Immunochemical characterization of a human high molecular weight–melanoma associated antigen identified with monoclonal antibodies. *Cancer Immunol Immunother* **14**, 196–201.

[8] Kirkwood JM, Neumann RD, Zoghbi SS, Ernstoff MS, Cornelius EA, Shaw C, Ziyadeh T, Fine JA, and Unger MW (1987). Scintigraphic detection of metastatic melanoma using indium 111/DTPA conjugated anti-gp240 antibody (ZME-018). *J Clin Oncol* **5**, 1247–1255.

[9] Briassouli P, Chan F, Savage K, Reis-Filho JS, and Linardopoulos S (2007). Aurora-A regulation of nuclear factor-κB signaling by phosphorylation of IκBα. *Cancer Res* **67**, 1689–1695.

[10] Calzado MA, Bacher S, and Schmitz ML (2007). NF-κB inhibitors for the treatment of inflammatory diseases and cancer. *Curr Med Chem* **14**, 367–376.

[11] Melisi D and Chiao PJ (2007). NF-κB as a target for cancer therapy. *Expert Opin Ther Targets* **11**, 133–144.

[12] Morotti A, Cilloni D, Pautasso M, Messa F, Arruga F, Defilippi I, Carturan S, Catalano R, Rosso V, Chiarenza A, et al. (2006). NF-κB inhibition as a strategy to enhance etoposide-induced apoptosis in K562 cell line. *Am J Hematol* **81**, 938–945.

[13] Pacifico F and Leonardi A (2006). NF-κB in solid tumors. *Biochem Pharmacol* **72**, 1142–1152.

[14] Morgan AC Jr, Galloway DR, and Reisfeld RA (1981). Production and characterization of monoclonal antibody to a melanoma specific glycoprotein. *Hybridoma* **1**, 27–36.

[15] Schulz G, Bumol TF, and Reisfeld RA (1983). Monoclonal antibody–directed effector cells selectively lyse human melanoma cells *in vitro* and *in vivo*. *Proc Natl Acad Sci USA* **80**, 5407–5411.

[16] Schulz G, Staffileno LK, Reisfeld RA, and Dennert G (1985). Eradication of established human melanoma tumors in nude mice by antibody-directed effector cells. *J Exp Med* **161**, 1315–1325.

[17] Liu Y, Zhang W, Cheung LH, Niu T, Wu Q, Li C, Van Pelt CS, and Rosenblum MG (2006). The antimelanoma immunocytokine scFvMEL/TNF shows reduced toxicity and potent antitumor activity against human tumor xenografts. *Neoplasia* **8**, 384–393.

[18] Liu Y, Zhang W, Niu T, Cheung LH, Munshi A, Meyn RE Jr, and Rosenblum MG (2006). Targeted apoptosis activation with GrB/scFvMEL modulates melanoma growth, metastatic spread, chemosensitivity, and radiosensitivity. *Neoplasia* **8**, 125–135.

[19] Reichelt P, Schwarz C, and Donzeau M (2006). Single step protocol to purify recombinant proteins with low endotoxin contents. *Protein Expr Purif* **46**, 483–488.

[20] Holmes K, Lantz LM, Fowlkes BJ, Schmid I, and Giorgi JV (2001). Preparation of cells and reagents for flow cytometry. *Curr Protoc Immunol* **Chapter 5**, Unit 5.3.

[21] Rosenblum MG, Cheung L, Murray JL, and Bartholomew R (1991). Antibody-mediated delivery of tumor necrosis factor (TNF-α): improvement of cytotoxicity and reduction of cellular resistance. *Cancer Commun* **3**, 21–27.

[22] Lyu MA, Cheung LH, Hittelman WN, Marks JW, Aguiar RC, and Rosenblum MG (2007). The rGel/BLyS fusion toxin specifically targets malignant B cells expressing the BLyS receptors BAFF-R, TACI, and BCMA. *Mol Cancer Ther* **6**, 460–470.

[23] Liu Y, Cheung LH, Hittelman WN, and Rosenblum MG (2003). Targeted delivery of human pro-apoptotic enzymes to tumor cells: *in vitro* studies describing a novel class of recombinant highly cytotoxic agents. *Mol Cancer Ther* **2**, 1341–1350.

[24] Suh J, Payvandi F, Edelstein LC, Amenta PS, Zong WX, Gelinas C, and Rabson AB (2002). Mechanisms of constitutive NF-κB activation in human prostate cancer cells. *Prostate* **52**, 183–200.

[25] Aggarwal S, Takada Y, Mhashilkar AM, Sieger K, Chada S, and Aggarwal BB (2004). Melanoma differentiation–associated gene-7/IL-24 gene enhances NF-κB activation and suppresses apoptosis induced by TNF. *J Immunol* **173**, 4368–4376.

[26] Darnay BG, Ni J, Moore PA, and Aggarwal BB (1999). Activation of NF-κB by RANK requires tumor necrosis factor receptor–associated factor (TRAF) 6 and NF-κB–inducing kinase. Identification of a novel TRAF6 interaction motif. *J Biol Chem* **274**, 7724–7731.

[27] Munshi A, Hobbs M, and Meyn RE (2005). Clonogenic cell survival assay. *Methods Mol Med* **110**, 21–28.

[28] Aida Y and Pabst MJ (1990). Removal of endotoxin from protein solutions by phase separation using Triton X-114. *J Immunol Methods* **132**, 191–195.

[29] Kumar A, Takada Y, Boriek AM, and Aggarwal BB (2004). Nuclear factor-κB: its role in health and disease. *J Mol Med* **82**, 434–448.

[30] Yan Liu X, Robinson D, Veach RA, Liu D, Timmons S, Collins RD, and Hawiger J (2000). Peptide-directed suppression of a pro-inflammatory cytokine response. *J Biol Chem* **275**, 16774–16778.

[31] Lee JH, Koo TH, Hwang BY, and Lee JJ (2002). Kaurane diterpene, kamebakaurin, inhibits NF-κB by directly targeting the DNA-binding activity of p50 and blocks the expression of antiapoptotic NF-κB target genes. *J Biol Chem* **277**, 18411–18420.

[32] Pande V, Sharma RK, Inoue J, Otsuka M, and Ramos MJ (2003). A molecular modeling study of inhibitors of nuclear factor κ-B (p50)-DNA binding. *J Comput Aided Mol Des* **17**, 825–836.

[33] Watanabe M, Ohsugi T, Shoda M, Ishida T, Aizawa S, Maruyama-Nagai M, Utsunomiya A, Koga S, Yamada Y, Kamihira S, et al. (2005). Dual targeting of transformed and untransformed HTLV-1–infected T cells by DHMEQ, a potent and selective inhibitor of NF-κB, as a strategy for chemoprevention and therapy of adult T-cell leukemia. *Blood* **106**, 2462–2471.

[34] Thomas RK, Sos ML, Zander T, Mani O, Popov A, Berenbrinker D, Smola-Hess S, Schultze JL, and Wolf J (2005). Inhibition of nuclear translocation of nuclear factor-κB despite lack of functional IκBα protein overcomes multiple defects in apoptosis signaling in human B-cell malignancies. *Clin Cancer Res* **11**, 8186–8194.

[35] Kim BH, Lee KH, Chung EY, Chang YS, Lee H, Lee CK, Min KR, and Kim Y (2006). Inhibitory effect of chroman carboxamide on interleukin-6 expression in response to lipopolysaccharide by preventing nuclear factor-κB activation in macrophages. *Eur J Pharmacol* **543**, 158–165.

[36] Hwang DM, Kundu JK, Shin JW, Lee JC, Lee HJ, and Surh YJ (2007). *cis*-9, *trans*-11–Conjugated linoleic acid down-regulates phorbol ester–induced NF-κB activation and subsequent COX-2 expression in hairless mouse skin by targeting IκB kinase and PI3K-Akt. *Carcinogenesis* **28**, 363–371.

[37] Lee JH, Koo TH, Yoon H, Jung HS, Jin HZ, Lee K, Hong YS, and Lee JJ (2006). Inhibition of NF-κB activation through targeting IκB kinase by celastrol, a quinone methide triterpenoid. *Biochem Pharmacol* **72**, 1311–1321.

[38] Cho ML, Ju JH, Kim KW, Moon YM, Lee SY, Min SY, Cho YG, Kim HS, Park KS, Yoon CH, et al. (2007). Cyclosporine A inhibits IL-15–induced IL-17 production in $CD4^+$ T cells via down-regulation of PI3K/Akt and NF-κB. *Immunol Lett* **108**, 88–96.

[39] Lin YG, Kunnumakkara AB, Nair A, Merritt WM, Han LY, Rmaiz-Pena GN, Kamat AA, Spannuth WA, Gershenson DM, Lutgendorf SK, et al. (2007). Curcumin inhibits tumor growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-κB pathway. *Clin Cancer Res* **13**, 3423–3430.

[40] Zambre AP, Kulkarni VM, Padhye S, Sandur SK, and Aggarwal BB (2006). Novel curcumin analogs targeting TNF-induced NF-κB activation and proliferation in human leukemic KBM-5 cells. *Bioorg Med Chem* **14**, 7196–7204.

[41] Kumar AP, Garcia GE, Ghosh R, Rajnarayanan RV, Alworth WL, and Slaga TJ (2003). 4-Hydroxy-3-methoxybenzoic acid methyl ester: a curcumin derivative targets Akt/NFκB cell survival signaling pathway: potential for prostate cancer management. *Neoplasia* **5**, 255–266.

[42] Berman KS, Verma UN, Harburg G, Minna JD, Cobb MH, and Gaynor RB (2002). Sulindac enhances tumor necrosis factor-α–mediated apoptosis of lung cancer cell lines by inhibition of nuclear factor-κB. *Clin Cancer Res* **8**, 354–360.

[43] May MJ, D'Acquisto F, Madge LA, Glockner J, Pober JS, and Ghosh S (2000). Selective inhibition of NF-κB activation by a peptide that blocks the interaction of NEMO with the IκB kinase complex. *Science* **289**, 1550–1554.

[44] Kantor RR, Ng AK, Giacomini P, and Ferrone S (1982). Analysis of the NIH workshop monoclonal antibodies to human melanoma antigens. *Hybridoma* **1**, 473–482.

[45] Macey DJ, DeNardo SJ, DeNardo GL, Goodnight JK, and Unger MW (1988). Uptake of indium-111–labeled monoclonal antibody ZME-018 as a function of tumor size in a patient with melanoma. *Am J Physiol Imaging* **3**, 1–6.

[46] Rosenblum MG, Levin B, Roh M, Hohn D, McCabe R, Thompson L, Cheung L, and Murray JL (1994). Clinical pharmacology and tissue disposition studies of ^{131}I-labeled anticolorectal carcinoma human monoclonal antibody LiCO 16.88. *Cancer Immunol Immunother* **39**, 397–400.

[47] Melnikova VO and Bar-Eli M (2009). Inflammation and melanoma metastasis. *Pigment Cell Melanoma Res* **22**, 257–267.

[48] Munshi A, Kurland JF, Nishikawa T, Chiao PJ, Andreeff M, and Meyn RE (2004). Inhibition of constitutively activated nuclear factor-κB radiosensitizes human melanoma cells. *Mol Cancer Ther* **3**, 985–992.

[49] Melnikova VO and Bar-Eli M (2008). Transcriptional control of the melanoma malignant phenotype. *Cancer Biol Ther* **7**, 997–1003.

[50] Zigler M, Villares GJ, Lev DC, Melnikova VO, and Bar-Eli M (2008). Tumor immunotherapy in melanoma: strategies for overcoming mechanisms of resistance and escape. *Am J Clin Dermatol* **9**, 307–311.

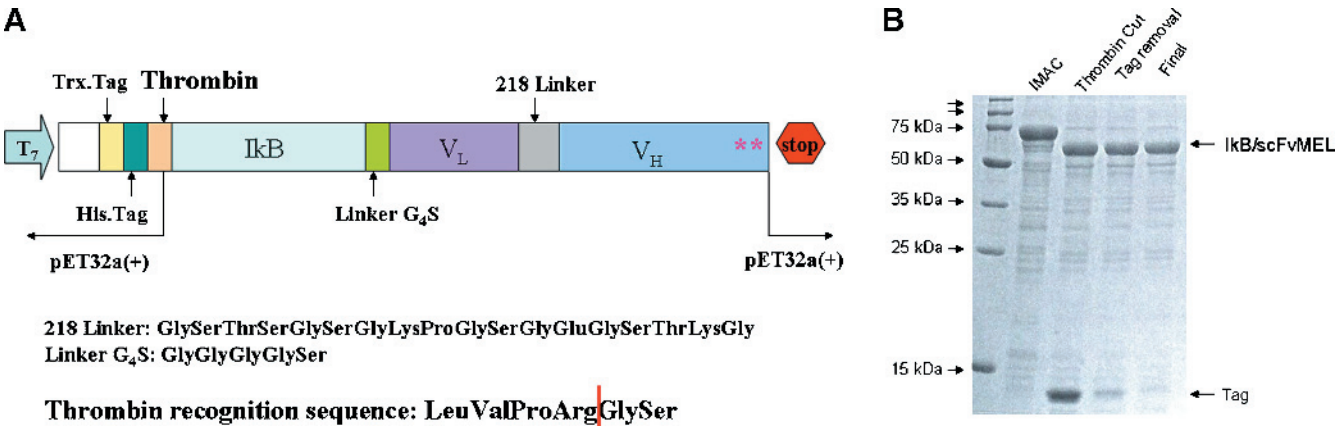

Figure W1. Construction schematic and SDS-PAGE of IκBα/scFvMEL. (A) Construction schematic for the IκBα/scFvMEL fusion protein. A 20-kDa thioredoxin (Trx) containing a His-6 tag was cloned upstream of the target fusion protein. This tag was used for IMAC. The tag also contained a thrombin recognition sequence for enzymatic release of the target protein. The position of the enzymatic cleavage in this sequence is shown by the red line. (B) SDS-PAGE of purified IκBα/scFvMEL. As described in the Materials and Methods, bacterial paste was lysed to release soluble protein and chromatographed on IMAC. The resulting material was then digested by exposing to thrombin, and the resulting fusion protein was purified as described. The final product demonstrated a molecular weight of 55 kDa and was essentially free of the released tag and contaminating proteins.

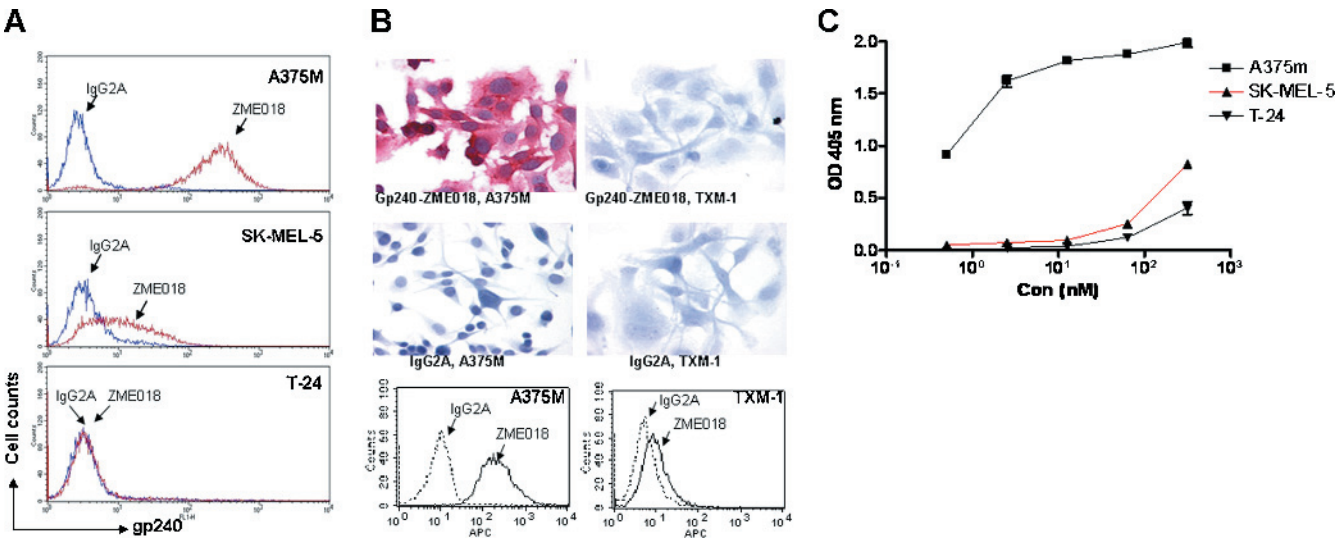

Figure W2. Expression of gp240 antigen on human melanoma cell lines. (A) gp240 expression analysis by flow cytometry. A375M, SK-MEL-5, and T-24 cells were incubated with anti-gp240–specific ZME-018 IgG2a followed by FITC-conjugated goat antimouse IgG mAb and were analyzed with a FACSCalibur flow cytometer. Use mouse IgG2a as an isotype control. (B) Immunohistochemical staining of gp240 on the surface of melanoma cells. A375M and TXM-1 cells were first incubated with ZME-018 and then incubated with an FITC-conjugated goat antimouse IgG mAb. Cells were fixed in 3.7% formaldehyde and permeabilized in 0.2% Triton X-100. The slides were mounted with 1,4-diazobicyclo-{2,2,2}-octane containing 1 μg/ml propidium iodide and analyzed under a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Kingston-upon-Thames, UK). The same samples were analyzed by FACSCalibur flow cytometer. (C) ELISA binding assay of gp240. The 96-well ELISA plates containing adherent A375M, SK-MEL-5 melanoma, and T-24 bladder cells (5×10^4 cells/well) were blocked by the addition of a solution containing 5% BSA for 1 hour. Cells were incubated with ZME-018 at various concentrations for 1 hour at room temperature. After washing, the cells were incubated with an HRP-conjugated goat antimouse IgG mAb. Finally, the substrate solution 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid containing 1 μl/ml 30% H_2O_2 was added to the wells. Absorbance at 405 nm was measured after 10 minutes.

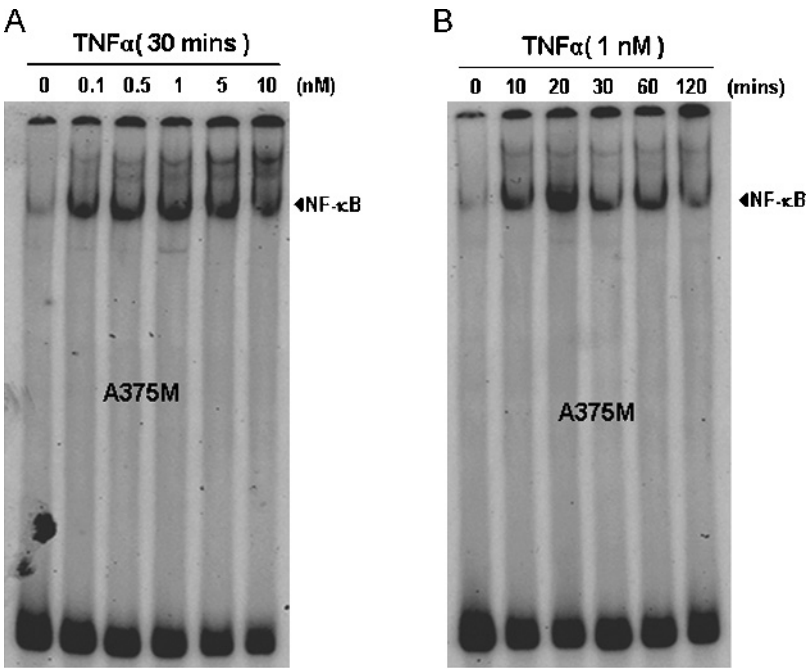

Figure W3. Dose- and time-dependent TNFα-stimulated NF-κB activation on A375M cells. (A) A375M cells were treated with different doses of TNFα for 30 minutes. (B) A375M cells were treated with 1 nM TNFα for the indicated times. Nucleus extracts were then prepared and assayed for NF-κB by EMSA.

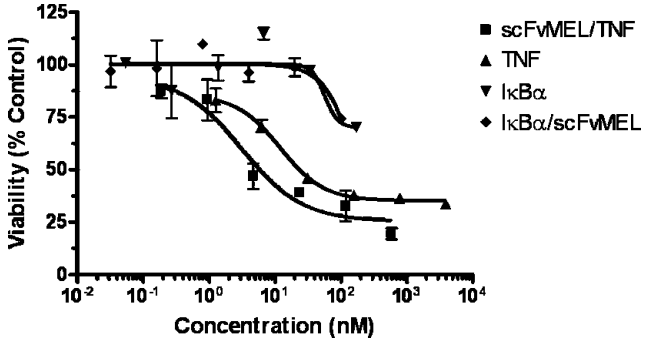

Figure W4. Comparative cytotoxic effects of scFvMEL/TNF, TNF, IκBα, and IκBα/scFvMEL against A375M cells.

Figure W5. At the end of the 10-day treatment period, tumors from control mice were almost four-fold larger than tumors from mice treated with IκBα/scFvMEL construct. Thereafter, the saline-treated group continued rapid growth, reaching approximately 2300 mm^3 (mean) compared with the treatment group (130 mm^3) at day 50.