TNF- α inhibits asbestos-induced cytotoxicity via a NF- κ B-dependent pathway, a possible mechanism for asbestos-induced oncogenesis

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Asbestos is the main cause of human malignant mesothelioma (MM). *In vivo*, macrophages phagocytize asbestos and, in response, release TNF- α and other cytokines that contribute to carcinogenesis through unknown mechanisms. *In vitro*, asbestos does not induce transformation of primary human mesothelial cells (HM); instead, asbestos is very cytotoxic to HM, causing extensive cell death. This finding raised an apparent paradox: How can asbestos cause MM if HM exposed to asbestos die? We found that asbestos induced the secretion of TNF- α and the expression of TNF- α receptor I in HM. Treatment of HM with TNF- α significantly reduced asbestos cytotoxicity. Through numerous technical approaches, including chemical inhibitors and small interfering RNA strategies, we demonstrate that, in HM, TNF- α activates NF- κ B and that NF- κ B activation leads to HM survival and resistance to the cytotoxic effects of asbestos. Our data show a critical role for TNF- α and NF- κ B signaling in mediating HM responses to asbestos. TNF- α signaling through NF- κ B-dependent mechanisms increases the percent of HM that survives asbestos exposure, thus increasing the pool of asbestos-damaged HM that are susceptible to malignant transformation. Cytogenetics supported this hypothesis, showing only rare, aberrant metaphases in HM exposed to asbestos and an increased mitotic rate with fewer irregular metaphases in HM exposed to both TNF- α and asbestos. Our findings provide a mechanistic rationale for the paradoxical inability of asbestos to transform HM *in vitro*, elucidate and underscore the role of TNF- α in asbestos pathogenesis in humans, and identify potential molecular targets for anti-MM prevention and therapy.

cancer | inflammation | mesothelioma

Asbestos refers to a group of naturally occurring hydrated mineral silicate fibers that are causally related to the development of pulmonary and pleural diseases. The various types of asbestos are divided into two major groups: serpentine, represented by chrysotile; and amphibole, which includes crocidolite, amosite, anthophyllite, actinolite, and tremolite (1–3). Crocidolite is often considered the most oncogenic type of asbestos in the causation of malignant mesothelioma (MM), a tumor of the serosal lining of the pleural, pericardial, and peritoneal cavities that causes $\approx 2,500$ deaths per year in the U.S. (3, 4).

The mechanisms by which asbestos induces malignancy are not clear (3, 4). It was found that asbestos can cause malignant transformation of Syrian hamster embryo cells by altering chromosomal morphology and ploidy through mechanically interfering with mitotic segregation (5). However, asbestos does not induce transformation of primary human mesothelial cells (HM) in tissue culture (6, 7). Rather, asbestos is very cytotoxic to HM grown *in vitro* (3, 4).

Because asbestos does not directly induce malignant transformation of HM, indirect mechanisms of carcinogenesis have been studied. It was found that, after asbestos exposure, there is an

inflammatory reaction with a large component of mononuclear phagocytes (reviewed in refs. 3 and 4). Upon differentiation into macrophages, these cells phagocytize asbestos and, in response, release numerous cytokines and reactive oxygen species that are mutagenic (8–15). Among these cytokines, TNF- α has been linked to asbestos pathogenesis, although the mechanisms underlying this effect are unclear (15–18). Long fibers, which generally are considered more carcinogenic and fibrogenic than short fibers, stimulate a greater release of TNF- α than short fibers (19). In animal models of asbestosis, excess TNF- α has been shown to correlate with the development of fibrosis (20), and TNF- α receptor knockout mice do not develop fibroproliferative lesions after asbestos exposure (21). Moreover, there is convincing evidence in different experimental models that TNF- α is a critical mediator of tumor promotion and cell transformation (22–25). Indeed, the existence of a link between chronic inflammation and certain types of cancer is now well established (26), and TNF- α has recently been found to be a crucial effector of this link in various experimental models (27–30). Together, this information raised the possibility that TNF- α might be linked to asbestos carcinogenesis. Here we investigated this hypothesis and revealed a possible mechanism of HM carcinogenesis by asbestos.

Results

Asbestos Is Cytotoxic to Human Mesothelial Cells in Culture. Light microscopy observation of HM in tissue culture, suggested that asbestos was cytotoxic (data not shown). This observation was verified by using both the lactate dehydrogenase (LDH) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. These assays indicated that crocidolite was cytotoxic to HM in a dose-dependent manner (Fig. 1*A* and *B*). This interpretation was further verified by flow cytometry (Fig. 1*C*). For example, $\approx 66\%$ and 40% of the HM were viable after exposure to crocidolite at 0.5 or 5.0 $\mu\text{g}/\text{cm}^2$, respectively, for 24 h, and the percentage of viable HM was ≈ 40 – 50% and 0 – 1% , respectively, 1 week after treatment (data not shown). This finding raised the question of how asbestos could cause malignant transformation of HM when, in tissue culture, asbestos killed HM in a dose-dependent manner.

Asbestos Induces Expression of TNF- α and of the TNF- α Receptor TNF-R1 in HM. In experiments performed during RNA microarray analyses to determine gene expression profiles in MM, the most

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Abbreviations: HM, primary human mesothelial cells; MM, malignant mesothelioma; RT, room temperature; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; shRNA, short hairpin RNA.

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Fig. 1. Asbestos is cytotoxic to HM in tissue culture. HM were exposed to different doses of asbestos (0.2–5.0 $\mu\text{g}/\text{cm}^2$) for 24 h. (A) LDH assay. Cytotoxicity was calculated by measuring the amount of LDH released from the cytosol of asbestos-damaged cells into the supernatant. The results show that the higher the amount of asbestos in tissue culture, the higher the cytotoxicity. (B) MTT assay. The OD value of 595 nm, which reflects metabolically active cells, decreased proportionally when cells were exposed to increasing amounts of asbestos. (C) Flow cytometry assay. Cells that were negative for both annexin V and propidium iodide were considered viable. Increasing amounts of asbestos correlated with a higher percent of cell death. Columns represent the means of three separate experiments; error bars indicate SD. *, Significantly different compared with cells not exposed to asbestos ($P < 0.05$).

notable effect was that HM exposed to crocidolite (5 $\mu\text{g}/\text{cm}^2$ for 2 h) induced TNF- α and TNF- α receptor mRNA (4.07- and 3.5-fold induction, respectively, over untreated controls; data not shown). We verified the induction of TNF- α mRNA in HM exposed to crocidolite by using quantitative real-time PCR (Fig. 2A). Moreover, we found that HM exposed to crocidolite released TNF- α in the tissue culture medium (Fig. 2B). Immunohistochemistry showed that, after crocidolite exposure, most HM in each of three separate primary cultures expressed TNF-R1 (data not shown). Western blot experiments confirmed that TNF-R1 was induced in HM after asbestos exposure in a dose-dependent manner (Fig. 2C).

TNF- α Significantly Reduced Asbestos Cytotoxicity. We tested the hypothesis that TNF- α modulated asbestos-induced cytotoxicity. We found that when HM were pretreated with TNF- α , asbestos cytotoxicity was significantly reduced (Fig. 3A). Addition of 0.5 $\mu\text{g}/\text{ml}$ anti-human TNF- α antiserum to the tissue culture medium of HM 1 h before TNF- α treatment abolished the anticytotoxicity effect caused by TNF- α . A matched IgG control had no protective effects confirming specificity (Fig. 3A). These results were verified by flow cytometry assays, measuring HM viability by annexin V and propidium iodide staining. The percentage of double-negative-staining viable cells was significantly higher in HM exposed to both TNF- α and asbestos ($P < 0.05$; data not shown). MTT assays confirmed that TNF- α protected HM from crocidolite cytotoxicity (Fig. 3B).

Fig. 2. TNF- α and TNF- α receptor TNF-R1 are induced in HM following asbestos exposure. (A) Quantitative real-time PCR. HM were exposed to asbestos at 5.0 $\mu\text{g}/\text{cm}^2$ for 6, 12, or 24 h. The levels of TNF- α mRNA expression in HM was induced ≈ 3.5 , 4.8, or 7.2-fold, respectively, after asbestos exposure. (B) Western blot analysis. Cell culture mediums of HM were collected and concentrated by ultrafiltration. The amount of TNF- α in the medium increased significantly 48 h after asbestos exposure. (C) Western blot analysis. HM were exposed to increasing amounts of crocidolite asbestos (0.2–10.0 $\mu\text{g}/\text{cm}^2$) for 24 h. Total cell extracts (40 μg) were analyzed using an anti-TNF-R1 monoclonal antibody. GAPDH (glyceraldehydes-3-phosphate dehydrogenase) was used as a loading control.

TNF- α Induces Activation of NF- κB in HM. Although TNF- α has the potential of inducing programmed cell death in certain cell types (31), usually cells survive exposure to TNF- α due to potent activation of the NF- κB pathway. The protective effect observed in Fig. 3 suggested that TNF- α could have induced NF- κB in HM; therefore, we tested whether TNF- α induced the translocation of the NF- κB subunit p65 (RelA) from the cytoplasm into

Fig. 3. TNF- α significantly reduces asbestos cytotoxicity. HM were incubated with or without 0.5 $\mu\text{g}/\text{ml}$ anti-TNF- α antiserum or normal rabbit IgG isotype control for 1 h followed by addition of TNF- α (10 ng/ml) for 24 h. Crocidolite at 5.0 $\mu\text{g}/\text{cm}^2$ was added at this time point, and cell cytotoxicity was measured 24 h later. (A) LDH cytotoxicity assay. Pretreatment with TNF- α significantly reduced asbestos cytotoxicity. Addition of anti-TNF- α antiserum abolished the anticytotoxicity effect caused by TNF- α . (B) MTT assay. An OD value of 595 nm, which reflects metabolically active cells, significantly increased when HM were pretreated with TNF- α compared with HM exposed to asbestos only. TNF, TNF- α ; Asb, asbestos; Ab, anti-human TNF- α antiserum; Iso, rabbit purified IgG isotype control. Columns represent the means of three separate experiments, and bars indicate SD. *, Significantly different compared with HM not exposed to asbestos ($P < 0.05$); **, significantly different compared with HM exposed to asbestos without TNF- α pretreatment ($P < 0.05$); #, significantly different compared with HM exposed to TNF- α and asbestos without addition of the anti-TNF- α antiserum ($P < 0.05$).

Fig. 6. RNA interference assays confirm that TNF- α inhibits asbestos-induced cytotoxicity by means of a NF- κ B dependent mechanism. (A) Representative Western blot. The expression of NF- κ B p65 in shRNA-RelA-transfected cells is markedly reduced compared with controls. Lanes: 1, uninfected HM; 2, uninfected HM treated with TNF- α ; 3, HM infected with shRNA nonspecific virus and treated with TNF- α ; 4, HM infected with shRNA-RelA and treated with TNF- α . Histone H1 (nuclear extract) and GAPDH (cytoplasmic extract) were used as loading controls. (B) shRNA-RelA-transfected cells and control cells were treated with TNF- α (10 ng/ml) for 24 h before exposure to asbestos (5.0 μ g/cm²) for 24 h. Cell cytotoxicity was evaluated by LDH assay. The anticytotoxicity effect of TNF- α is abrogated in the shRNA-RelA-transfected cells.

investigators for years (3, 4): How could crocidolite asbestos cause MM if HM encountering crocidolite fibers die?

Our results provide a possible mechanistic rationale for crocidolite-mediated transformation: We found that crocidolite induces HM to release TNF- α and to express the TNF- α receptor. Exposure of HM to TNF- α significantly reduced crocidolite cytotoxicity. This protective effect was caused by

Fig. 7. Metaphases from asbestos-treated HM cell cultures. (A) Representative metaphase from an asbestos-treated HM culture. Some chromosomes have prematurely separated chromatids, and two such chromosomes are indicated by arrows. The arrowhead points to an asbestos fiber. Asbestos fibers were found in most metaphases from both HM plus asbestos cultures and in HM plus asbestos plus TNF- α cultures. (B) Tetraploid metaphase without premature separation of chromatids from asbestos-treated HM culture pretreated with TNF- α .

TNF- α -mediated NF- κ B activation, which in turn inhibited crocidolite-induced cytotoxicity and increased the numbers of HM that survived crocidolite exposure and thus the percentage of cells that could become transformed. In fact, inhibition of NF- κ B with Bay11-7082, a chemical compound that blocks the phosphorylation of I κ B, suppressed the protective effect of TNF- α on crocidolite cytotoxicity. We verified these results with RNA interference assays using shRNA-RelA constructed with lentivirus to knock down NF- κ B-RelA expression. The results confirmed that NF- κ B is required for TNF- α -mediated cytoprotection. Therefore, in the presence of TNF- α , HM that have accumulated genetic damage because of asbestos exposure may be more prone to divide rather than die, and this may lead to MM. Cytogenetic studies supported this interpretation, showing that TNF- α protected HM from asbestos-induced genetic damage.

Milligan *et al.* (36) have observed that NF- κ B was activated by TNF- α in rat mesothelial cells, and, in inhalation studies in rats, crocidolite induced NF- κ B p65 nuclear translocation in mesothelial and lung epithelial cells (37). These findings appear to indirectly support our results.

In vivo, multiple cell types contribute to the extracellular levels of TNF- α , and macrophages are considered the main producers. In animal models, asbestos induces the expression of monocyte chemoattractant protein-1 by mesothelial cells. Monocyte chemoattractant protein-1 expression favors the macrophage inflammatory response within the pleural space that follows asbestos exposure (12, 15). Macrophages phagocytize asbestos but are unable to “digest” these fibers. Possibly because they are damaged by asbestos, these macrophages release TNF- α (prevalently) and other cytokines (8, 12). TNF- α can also be released by HM, for example, when these cells are stimulated with erythromycin (38) or by crocidolite as shown here. Although TNF- α was suspected to be a major player in MM pathogenesis (3, 4, 8, 12) because it has been linked to carcinogenesis in other tumor models (22–25) and to asbestos-induced fibrosis in animals (21), the possible mechanism by which TNF- α may contribute to MM had remained elusive.

TNF- α is a proinflammatory cytokine and a major inducer of NF- κ B, a key regulator of oncogenesis (26–30). In different cell types, TNF- α can induce either cell death or, more frequently, increase cell survival via NF- κ B activation (39, 40). Activation of NF- κ B promotes cellular proliferation and inhibits apoptosis, favoring cancer development (26–30). Numerous studies have shown the protective effects of nonsteroidal antiinflammatory drugs in colon and other types of cancers in humans and in animals (41, 42).

The current hypothesis is that chronic inflammation leads to activation of NF- κ B, which in turn leads to the activation of prosurvival genes and prevention of apoptosis of damaged cells (26, 39). Our results indicate that there may be a rationale to test for possible beneficial preventive effects of nonsteroidal antiinflammatory drugs in cohorts at high risk for MM, such as asbestos workers and genetically predisposed families (43). In addition, blocking TNF- α may lead to novel preventive strategies for MM. Examples might include blocking TNF- α with etanercept (Enbrel), a recombinant decoy receptor for TNF- α that is under investigation in breast cancer patients (44) or disruption of the NF- κ B pathway, for example with Onconase (Ranpirinase), a drug that inhibits the NF- κ B pathway (45) and that has already shown favorable effects in some MM patients (46).

In summary, we propose the following pathogenic model. Crocidolite causes accumulation of macrophages in the pleura and lung. When these macrophages encounter asbestos, they release TNF- α . At the same time, asbestos induces HM to secrete TNF- α (both paracrine and autocrine effects). TNF- α activates NF- κ B that increases HM survival. This allows HM with asbestos-induced DNA damage to divide rather than die

and, if sufficient genetic damage accumulates, to eventually develop into a MM.

Materials and Methods

Cell Cultures. HM were from patients who accumulated pleural fluid because of nonmalignant diseases. HM were identified morphologically and characterized by immunostaining for calretinin and pancytokeratin as described (7). HM were cultured in DMEM with 20% FBS and were used at passages 4–6. HM from three different donors were used in all experiments, and each experiment was done in triplicate, except for cytogenetic analyses in which primary HM from two donors were used.

Materials. Human recombinant TNF- α , anti-human TNF- α antiserum (IgG fraction of antiserum developed in rabbit), normal rabbit purified Ig control, Bay11-7082, and (*E*)-3-(4-methylphenylsulfonyl)-2-propenenitrile were from Sigma.

Asbestos Preparation. Crocidolite asbestos fibers were obtained from the Union Internationale Contre le Cancer (47) and processed as described in ref. 9 (average length, $3.2 \pm 1.0 \mu\text{m}$; average diameter, $0.22 \pm 0.01 \mu\text{m}$). Fibers were baked at 150°C for 18 h, suspended in Hanks' balanced salt solution at 2 mg/ml, triturated 10 times through a 22-gauge needle, and autoclaved. Crocidolite fibers from Union Internationale Contre le Cancer also were characterized previously and shown to be carcinogenic, to cause the release of reactive oxygen species, and to cause DNA damage (9).

LDH Cytotoxicity Assays. Cytotoxicity was assessed with an LDH-cytotoxicity detection kit (Roche Diagnostics), which measures LDH activity released from the cytosol of damaged cells. HM were seeded in a 96-well tissue culture plate at a density of 1×10^4 cells per well and incubated in DMEM with 20% FBS. The next day, we changed the medium to 1% FBS and added 100 μl of crocidolite dilutions at different concentrations per well. After 24 h, 100 μl of supernatant per well was harvested and transferred into a new 96-well, flat-bottom plate. LDH substrate (100 μl) was added to each well and incubated for 30 min at room temperature (RT) protected from light. The absorbance of the samples was measured at 490 nm with an ELISA reader. Cytotoxicity was calculated with the formula: % cytotoxicity = (experimental value – low control) $\times$ 100/(high control – low control), where low control is assay medium plus cells and high control is assay medium (plus 2% Triton X-100) plus cells.

MTT Assays. The cell proliferation kit I (MTT; Roche Diagnostics) was used to check viability. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells.

Flow Cytometry Assays. After exposure to different amounts of crocidolite, HM were harvested, washed twice with cold PBS, and suspended in binding buffer (10 mM Hepes, pH 7.4/140 mM NaCl/2.5 mM CaCl_2) at a concentration of 1×10^6 cells per ml. The binding buffer [100 μl (1×10^5 cells)] was transferred to a new tube, and 5 μl of annexin V-Biotin (BD Biosciences) was added for 15 min at RT in the dark. HM were washed, suspended in 100 μl of binding buffer with 5 $\mu\text{g}/\text{ml}$ streptavidin-FITC (BD Biosciences), and stained with propidium iodide (Sigma) at a final concentration of 5 $\mu\text{g}/\text{ml}$ for 15 min at RT. Binding buffer (400 μl) was added to the HM, and the cells were analyzed by flow cytometry.

Quantitative Real-Time PCR. HM were exposed to asbestos (5 $\mu\text{g}/\text{cm}^2$) for 6, 12, and 24 h. Total cellular RNA of each sample was isolated with the RNeasy kit (Qiagen, Valencia, CA) and treated with RNase-free DNase. For each sample, 3 μg of total RNA was reverse transcribed by using the First-Strand cDNA

synthesis kit (Fermentas, Burlington, ON, Canada). 18S rRNA was used as the internal control. PCRs for TNF- α and 18S rRNA were performed in triplicate in 25- μl total reaction volumes using the SYBR green master PCR mix (Applied Biosystems). The GeneAmp 5700 sequence detection system (PerkinElmer–Applied Biosystems) was used by following standard procedures. The following primers were used: TNF- α , 5'-GCTTGTTCCT-CAGCCTCTTCTC-3' and 5'-CTCAGCTTGAGGGTTTGC-TACA-3', and 18S, 5'-TGATTAAGTCCCTGCCCTTTGT-3' and 5'-TCAAGTTCGACCGTCTTCTCAG-3'. No RT-PCR control was run alongside each sample. Relative TNF- α mRNA expression levels for each sample were normalized to the internal control 18S rRNA.

Nuclear Extract Preparation and EMSA. Cell pellets were resuspended in buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.5 mM PMSF) and placed on ice for 15 min, and 25 μl of 10% Nonidet P-40 was added to each tube followed by vortexing for 10 s. Separation of nuclei was assessed by using a phase microscope. The samples were centrifuged for 5 min at $9,000 \times g$ at 4°C . The nuclear pellet was lysed with 30 μl of lysis buffer (Sigma), and the protein concentration was determined with the Bradford assay. Five micrograms of the nuclear extracts was used for EMSA. The gel shift assay was as described in ref. 48. NF- κB consensus oligonucleotides were from Santa Cruz Biotechnology and labeled with [γ - ^{32}P]ATP (Amersham Pharmacia) using T4 polynucleotide kinase. Unincorporated nucleotides were removed by Chroma Spin column (BD Biosciences Clontech). The ^{32}P -labeled NF- κB probe was incubated with 5 μg of nuclear extract in gel shift binding buffer (Promega). Unlabeled oligonucleotide was incubated with nuclear extract for 10 min before the addition of the probe as a specific competitor. The DNA–protein complex was analyzed in 5% nondenaturing polyacrylamide gel. Lanes with samples were devoid of loading dyes, which can interfere with the binding. Dried gels were exposed to x-ray film.

Western Blots. Total cell protein extraction was prepared by using lysis buffer (Sigma). For each sample, 40 μg of protein was used. The proteins were separated on a 10% polyacrylamide gel and transferred to poly(vinylidene difluoride) membranes. The membranes were blocked in Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk at 4°C overnight. The membranes were then probed with the first antibody at RT for 2 h. Anti-NF- κB p65, anti-TNF- α , anti-TNF-R1, and anti-Histone H1 antibodies were from Santa Cruz Biotechnology; anti-GAPDH was obtained from Chemicon International (Temecula, CA). The membrane was washed four times with Tris-buffered saline containing 0.05% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody (Pierce), and the signal was detected by chemiluminescence (Pierce). To check for the secretion of TNF- α by HM, the cell culture medium was concentrated with an Amicon centrifugal filter (Millipore). A rabbit anti-human TNF- α polyclonal IgG (Santa Cruz Biotechnology) was used as the first antibody, and biotinylated anti-rabbit IgG (Vector Laboratories) was used as the secondary antibody. The membrane was then incubated with the horseradish peroxidase-conjugated streptavidin. Bands were visualized by enhanced chemiluminescence (Pierce).

RNA Interference Assay. The lentiviral vector, pLentiLox37 (pLL), expressing eGFP is described in ref. 49. pLL-*shRelA* and pLL-*shNS*, expressing *RelA*-specific and nonspecific shRNA oligonucleotides, were constructed by ligating to the HpaI and XhoI restriction sites of pLL the following DNA oligonucleotides (sense): *shRelA*, tGGATTGAGGAGAAACGTAAttcaagagaTTACGTTTCTCCTCAATCCtttttttc; *shNS*, tCAGTCG-CGTTTTCGACTGGttcaagagaCCAGTCGCAAACGCGAC-

TGttttttt (the 19-nt target sequences are in uppercase and inserts were verified by sequencing). High-titer lentiviral preparations were produced by calcium phosphate transfection of pLL vectors (10 μ g) and the pMDL (6.5 μ g), pRev (2.5 μ g), and pENV (VSVG) (3.6 μ g) plasmids, encoding packaging proteins, into 293T cells (49). Supernatants were harvested at 36–60 h, virions were concentrated by ultracentrifugation at 23,000 rpm for 1.5 h with a Beckman SW28 rotor and stored at -80°C . HM were seeded in six-well plate at $\approx 70\%$ confluency. The next day, the cells were infected with 10 μ l of shRNA–RelA lentivirus or control virus shRNA-nonspecific. The virus was added directly into the medium of the cell culture. The plate was spun 1 h at $1,300 \times g$, and the cells were incubated at 37°C for 48 h. The expression of the reporter gene GFP was verified by fluorescence microscopy. Then the cells were treated with 10 ng/ml TNF- α for 30 min. HM were harvested, and cytoplasmic and nuclear proteins were extracted. NF- κ B was assessed by Western blot with an antibody (Santa Cruz Biotechnology) specific for the p65 (RelA) NF- κ B subunit.

Cytogenetic Analysis. Chromosome analyses were conducted on two primary HM. Metaphases were arrested by exposing cultured cells overnight to colcemid (0.01 μ g/ml). HM were detached with trypsin and treated with 0.075 M KCl hypotonic solution for 20 min, followed by fixation in a 3:1 mixture of methanol/acetic acid. Metaphase slide preparations were made by a steam-drying technique. Chromosomes were G-banded and analyzed.

Statistical Analysis. Multiple group comparisons were performed with ANOVA followed by Fisher's least significant difference testing. Statistical significance between two groups of interest was evaluated by unpaired Student's *t* test. Differences were considered significant at $P < 0.05$.

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