

**p38 Mitogen-activated Protein Kinase  
and Nuclear Factor- $\kappa$ B Collaborate to Induce  
Interleukin-6 Gene Expression and Release:**

**Evidence for a Cytoprotective Autocrine  
Signaling Pathway in a Cardiac Myocyte Model System**

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**Running Head:** IL-6 induction by p38 and NF- $\kappa$ B

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**Abstract:**

In cardiac myocytes the stimulation of p38 MAP kinase by the MAPKK, MKK6, leads to the activation of the transcription factor, NF- $\kappa$ B, and to protection from apoptosis. In the present study it was found that in primary neonatal rat cardiac myocytes, constitutively active MKK6, MKK6(Glu), bound to I $\kappa$ B kinase(IKK)- and stimulated its ability to phosphorylate I $\kappa$ B. This phosphorylation step is required for translocation of NF- $\kappa$ B to the nucleus, where it can activate NF- $\kappa$ B-sensitive genes. Expression of the interleukin (IL)-6 gene requires NF- $\kappa$ B; IL-6 transcription was induced in cardiac myocytes in response to MKK6(Glu) in a p38-dependent manner. MKK6(Glu) also induced the release of IL-6, a cytokine which was found to protect myocardial cells against apoptosis. TNF- $\alpha$ , which activates both NF- $\kappa$ B and p38, also induced IL-6 expression and release in a manner partially dependent upon p38, and protected myocytes from apoptotic. Activation of the transcription factor, STAT, has been associated with cytokine-mediated cytoprotection. While, TNF- $\alpha$  was relatively ineffective, IL-6 activated myocardial cell STAT3 by about 8-fold, indicating a probable role for this transcription factor in IL-6-mediated protection from apoptosis. The induction of IL-6 by TNF- $\alpha$  was inhibited by a kinase-inactive form of the MAPKKK, TGF- $\beta$  activated protein kinase (Tak1), which is known to activate p38 and NF- $\kappa$ B in other cell types. These results indicate that in cardiac myocytes, Tak1-activating cytokines, like TNF- $\alpha$ , can induce IL-6 expression and release in a manner that involves both p38 and NF- $\kappa$ B. Moreover, the released IL-6 may then function in an autocrine

fashion to provide protection from apoptosis, acting in part, through the STAT3 pathway. Thus, cardiac myocyte-derived cytokines may comprise an intricate autocrine signaling system in the heart that participates in the regulation of myocardial cell growth and survival.

**Introduction:**

Cytokines derived from either infiltrating cells, such as macrophages, or sometimes from cells comprising the tissue itself, play important roles in the wound-healing process (1). Since some injuries, such as myocardial infarction, are frequently life-threatening, a better understanding of the roles of cytokines in tissues such as the heart is critical. Following a myocardial infarction there is an accumulation of tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 at or near the affected region (2-5). Previously, production of these cytokines in the heart was attributed to infiltrating macrophages or leukocytes, and endothelial cells (e.g. 6). However, recent findings have demonstrated the expression of all three of these cytokines in cardiac myocytes following ischemic stress (7-9). Moreover, certain cytokines are thought to promote cardiac tissue recovery after a brief ischemic insult (4; 9-11). Accordingly, there is renewed interest in studying the possible beneficial effects of cytokines in the heart, as well as elucidating the signal transduction pathways and mechanisms responsible for their induction.

The induction of most cytokine genes requires activation of the transcription factor, nuclear factor (NF)- $\kappa$ B (12-15). NF- $\kappa$ B is activated and cytokine expression is increased in rat hearts submitted to ischemic stress; interestingly, these events appear to require the mitogen-activated protein kinase (MAPK), p38 (16). Additionally, in cultured cardiac myocytes, NF- $\kappa$ B-dependent reporter gene expression is activated following the selective stimulation of p38 by its upstream

activator, MKK6 (17). Moreover, this p38-dependent NF- $\kappa$ B activation contributes to protecting cultured myocardial cells from undergoing apoptosis (17). However, while p38 and NF- $\kappa$ B are both important for the maintenance of heart function following stress, neither the mechanism by which p38 activates NF- $\kappa$ B, nor the role of p38-activated NF- $\kappa$ B in myocardial cell survival are well understood. Accordingly, the present study was undertaken to begin addressing these questions.

Many signaling pathways can interact with each other through biochemical cross-talk, however, such interactions between p38 and NF- $\kappa$ B are not well understood. Like the other MAPK family members, p38 is part of a cascade of kinases. One of the best studied activators of p38 is the MAPKK, MKK6, which lies directly upstream of p38; among ERK, JNK and p38, MKK6 activates only p38 (18,20). Although MKK6 itself can be activated by several upstream kinases, a particularly interesting finding is that in some cells, MKK6 can be activated by the MAPKKK, TGF- $\beta$ -activated protein kinase (Tak1) (18,20). Tak1 was originally named for its ability to be activated by TGF- $\beta$ . However, Tak1 can also be activated by cytokines, such as interleukin-1 or TNF- $\alpha$  (20-22) (see Fig. 7 for reference).

In comparison to the p38 pathway, the NF- $\kappa$ B pathway is activated through a series of events which are also mediated by a cascade of kinases, many of which are believed to be unique to that pathway. NF- $\kappa$ B, which is comprised of two subunits

(p65 and p50), is retained in the cytoplasm by virtue of its interaction with the inhibitor of  $\kappa$ B (I $\kappa$ B). I $\kappa$ B is phosphorylated in response to NF- $\kappa$ B-activating signals; this phosphorylation leads to the ubiquitination and subsequent degradation of I $\kappa$ B. This then allows NF- $\kappa$ B to translocate to the nucleus where it binds to critical elements in cytokine genes and increases their transcription (23,24). The kinases responsible for the phosphorylation of I $\kappa$ B belong to the I $\kappa$ B kinase family, or the IKKs (25-29). NF- $\kappa$ B-inducing kinase, or NIK, phosphorylates and activates IKK (26,30,31). Although it preferentially activates IKK, NIK, which bears strong sequence homology to-, and is now considered a member of the MAPKKK family, can also activate the JNK MAPK pathway (32). Interestingly, in addition to being activated by NIK, IKK can also be activated by MEKK1 (27), a MAPKKK that is also known to activate Jnk in some cell types (33) and by Tak1 (refer to Fig. 5). Thus, at least at the MAPKKK level, there exists the potential for cross-talk between the NF- $\kappa$ B- and MAPK pathways.

In the present study we examined the nature of the cross-talk between the NF- $\kappa$ B- and p38 pathways. Because of the clear importance of these pathways in promoting the recovery- or maintenance of heart tissue function following stress or injury, we used cardiac myocytes as the model system. To this end, we explored whether NF- $\kappa$ B and p38 collaboratively activate the IL-6 gene in cardiac myocytes,

and further, whether IL-6 itself has functions that are consistent with functional recovery of the tissue following stress. We have found that

- 1) agonists, such as TNF- $\alpha$ , can activate IL-6 transcription in cardiac myocytes in a manner that requires both NF- $\kappa$ B and p38,
- 2) the coordinate activation of the NF- $\kappa$ B- and p38 pathways by TNF- $\alpha$  takes place largely through the MAPKKK, Tak1,
- 3) the p38 pathway can influence NF- $\kappa$ B activation, at least partly, through the physical association of MKK6 and IKK $\beta$ , and
- 4) IL-6 can protect cardiac myocytes from undergoing apoptosis induced by sphingosine, a signaling lipid known to increase in the heart following ischemic stress.

**Methods:**

**Cell Culture:** Primary ventricular myocytes were prepared from 1 to 4 day old Sprague-Dawley rats as described (34, 35). Briefly, hearts were dissected in DMEM/air; the apical two thirds of the ventricles were dissected away from the atria. After mincing and washing the ventricles twice with air-compatible DMEM, cells were isolated by multiple rounds of 10 minute-long tissue dissociation with 0.001% trypsin. After each incubation with trypsin the supernatant was added to an equal volume of DMEM/F12 (1:1) containing 20% fetal bovine serum, and all the supernatants were combined. Plastic wells were treated for at least 1 hour with 5 ng fibronectin/ml DMEM/F12 (1:1). Myocytes were plated in DMEM/F12 (1:1) containing 10% fetal bovine serum for approximately 16 hours. After washing with DMEM/F12 (1:1) the cultures were incubated in DMEM/F12 (1:1) ± any test agents for the indicated times.

**Transfection by Electroporation:** Immediately following the final dissociation step, myocardial cells were resuspended in serum free DMEM:F12 (1:1). Between 5- and 12 x 10<sup>6</sup> cells were combined with the combinations of plasmids indicated in the Figure Legends in a total volume of 300 µL of DMEM/F12 (1:1). The total amount of plasmids used for each electroporation was equalized using pCMV6. Dose-response experiments were carried out to determine optimal quantities of plasmids for transfection, and to verify that the results obtained were consistent over a range of plasmid levels. Generally, the dose-response experiments led to using the following

quantities of each plasmid type in each electroporation: 1  $\mu\text{g}$  each of pCMV6 (Control) or p38  $\alpha$ ; 15  $\mu\text{g}$  of MKK6(Glu), Tak1 or Tab1; 20  $\mu\text{g}$  each of luciferase- and -galactosidase reporters; 45  $\mu\text{g}$  of IKK  $\beta$ -M, MKK6-M1, MKK6-M2, Tak1-M. Cells were electroporated at 500 volts, 25  $\mu\text{farads}$ , and 100 ohms in a 0.2 cm gap electroporation cuvette (Bio-Rad Laboratories) using a Gene Pulser II (Bio-Rad Laboratories). Under these conditions only cardiac myocytes are transfected (34, 36). For reporter assays,  $1.5 \times 10^6$  cells were plated per 24 mm well, whereas  $4.5 \times 10^6$  myocytes per 35 mm well were plated to perform kinase assays.

***Plasmids:***

***MKK6(Glu), MKK6-M1 and MKK6-M2:*** pcDNA3 Flag-MKK6 (Glu) and pcDNA3 Flag-MKK6(K82A), the latter of which we call MKK6-M1, code for activated- and kinase-inactive human MKK6, respectively (19) and were obtained from R. J. Davis (University of Massachusetts, Worcester MA). In some cases, instead of MKK6-M1 we used MKK6-M2 [Flag-MKK6(K82A; S207A; T211A)] which is a kinase-inactive form of MKK6 that also has the sites normally phosphorylated by an upstream MAPKKK, S207 and T211, mutated to Ala. MKK6-M2 produced similar inhibitory effects on the p38 pathway as MKK6-M1.

***p38:*** Sr 3 HA-p38-2, which codes for wild type human p38-2, was obtained from B. Stein (37) (Signal Pharmaceuticals, Inc., San Diego CA). p38-2 is distinct

from p38 $\alpha$ , p38 $\beta$  and p38 $\gamma$ , however, it is identical to human p38 $\beta$  (38). In the present manuscript we have adopted the p38 $\beta$  nomenclature.

***IKK $\beta$  and IKK $\beta$ -M:*** pRK5 C-Flag-IKK $\beta$  and pRK5 C-Flag-IKK $\beta$  (K44A), which code for wild type human IKK $\beta$ , and kinase-dead human IKK $\beta$ , were obtained from M. Rothe (26) (Tularik, San Francisco CA).

***NF- $\kappa$ B-luc:*** p2X NF- $\kappa$ B, which codes for a luciferase reporter driven by a minimal prolactin promoter with 2 nearby, upstream NF- $\kappa$ B consensus sites, was obtained from M. Karin (39) (University of CA, San Diego CA).

***IL-6-luc, IL-6 (NF- $\kappa$ B mut)-luc and NF- $\kappa$ B/IL-6-luc:*** p1168hu.IL6-luc and pmut1168hu.IL6-luc, which code for 1168 nts of the wild type or NF- $\kappa$ B-mutated human IL-6 promoter driving luciferase in pGL3, respectively (15), were obtained from G. Haegeman (University of Gent, Belgium). NF- $\kappa$ B/IL-6-luc was prepared by ligating three IL-6 NF- $\kappa$ B elements upstream of the non-inducible IL-6 minimal promoter, which is comprised of 50 nts of IL-6 5'-flanking sequence. This construct was also obtained from G. Haegeman.

***Tak1, Tab1 and Tak1-M:*** pFLAG-Tak1 and pFLAG-Tak1-M encode human, full-length wild type Tak1, and human full-length Tak1 K63W, respectively. pHA-Tab1 encodes human, full-length Tab1 (22). All Tak1 and Tab1 constructs used in this study were obtained from T. Sugita (Tanabe Seiyaku Co., Ltd., Osaka, Japan).

***Preparation of Recombinant Adenovirus:***

***AdV-p38 and AdV-MKK6(Glu):*** The AdEasy system was used for preparing recombinant adenoviral strains using previously described methods (40). Briefly, human MKK6(Glu) and human p38  $\alpha$  were PCR-amplified from the parent templates (see above) such as to create restriction sites that would facilitate cloning into pAdTrack-CMV, an adenoviral shuttle vector which harbors CMV-driven GFP, and a CMV-flanked multiple cloning site for the insertion of the gene of interest. PCR-amplified p38  $\alpha$  and MKK6(Glu) were cloned into the EcoRI and NotI sites of pGEX-6P-1 (Pharmacia Biotech.), which served as a shuttle cloning vector. pGEX-6P-1/p38  $\alpha$  and pGEX-6P-1/MKK6(Glu) were then digested with BamHI and NotI and the resulting products of interest were cloned into the BglII and NotI sites of pAdTrack-CMV, to create pAdTrack-CMV-p38  $\alpha$  and pAdTrack-CMV-MKK6(Glu). pAdTrack-CMV-p38  $\alpha$ , or pAdTrack-CMV-MKK6(Glu) were linearized and then co-transformed with the adenoviral vector, pAdEasy-1, into *E. coli* strain BM5183. This strain of *E. coli* allows for homologous recombination of pAdEasy-1 and the pAdTrack-CMV shuttle vector containing the gene of interest. Recombinants were selected on kanamycin and screened by restriction digestion with PacI. Recombinant plasmids were then re-transformed into *E. coli* DH5 for propagation purposes. Recombinant adenoviral plasmids were linearized with Pac1 and then transfected into 293 human embryonic kidney cells, using Lipofectamine® (Gibco BRL). Transfection efficiency was determined by observing GFP fluorescence, as

previously described (40). The recombinant virus were then harvested 7- to 10 days post-infection. Viral titers were determined by observing GFP fluorescence of primary neonatal cardiac myocytes; the minimum quantity of viral stock that afforded 100% transfection efficiency was selected for the experiments in this study.

**Reporter Assays:**

**$\beta$ -galactosidase:** Following the appropriate time in culture, each well of myocytes was washed twice with PBS and then lysed in ice cold 500  $\mu$ l lysis buffer (25 mM gly-gly pH 7.8, 15 mM MgSO<sub>4</sub>, 4 mM EDTA, 0.25% triton-X 100) containing 1 mM dithiothreitol. The cell debris was removed by centrifugation. To measure  $\beta$ -galactosidase activity, 200  $\mu$ l of the cell extract was added to 400  $\mu$ l  $\beta$ -galactosidase buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1mM MgSO<sub>4</sub>) containing 1 mg/ml chlorophenolred- $\beta$ -D-galactopyranoside and 50 mM  $\beta$ -mercaptoethanol. The reaction was incubated for two hours at 37°C. After stopping each reaction by adding 100  $\mu$ L of 1M Na<sub>2</sub>CO<sub>3</sub>, the absorbance was measured at 570 nm.

**Luciferase:** To measure luciferase activity, 100  $\mu$ l of the following buffer (25 mM gly-gly pH 7.8, 15 mM MgSO<sub>4</sub>, 4 mM EGTA, 45 mM KPO<sub>4</sub> pH 7.8, 1 mM DTT, 0.3 mM D-luciferin, 3 mM ATP) were added to 100  $\mu$ l of cell lysate. Light emission of each sample was measured by a BioOrbit 1251 luminometer for 30 seconds. The relative luciferase activities were determined by dividing the relative luciferase activity by the relative  $\beta$ -galactosidase activity.

***Cytokine ELISAs:***

***IL-6:*** To measure IL-6 secretion, IL-6 ELISAs were carried out using a kit according to the manufacturer's protocol (Biosource International). After the myocardial cells were pre-plated, as described (17) to eliminate non-cardiac myocytes, the myocytes were plated at a density of 1,300 cells/mm<sup>2</sup> in 10% FCS for 24 hours. Cells were washed, and cultured in media  $\pm$  rTNF- (Genzyme),  $\pm$  5  $\mu$ M SB203580, or the cells were infected with AdV-MKK6(Glu), AdV-p38<sub>2</sub> or AdV-Control. Following 48 hours incubation, media were collected and assayed using the IL-6 ELISA kit.

***Apoptosis:***

***TUNEL:*** Myocardial cells were cultured  $\pm$  rIL-6 (1 ng/ml) (Biosource International) supplemented DMEM/F12  $\pm$  5  $\mu$ M SB203580 for 48 hours prior to addition of 10  $\mu$ M sphingosine (Calbiochem) for 6 hours. TUNEL analyses of fragmented DNA were performed on cultured cardiac myocytes as previously described (17), and according to the manufacturers protocol (Boehringer Mannheim). Cells were scored for TUNEL-positive nuclei in a researcher-blinded manner.

***DNA Laddering:*** Assessment of apoptosis was performed by DNA laddering essentially as previously described (17). Approximately 10<sup>6</sup> cardiac myocytes were lysed in a digestion buffer containing ProteinaseK, scraped, pipeted several times and incubated at 55°C for 5-14h. Samples were then extracted with phenol/chloroform/isoamyl alcohol, and then DNA was isopropanol-precipitated and

washed several times with ethanol and allowed to air-dry. After dissolving the DNA and incubating with RNase A, DNA fragments were fractionated on an agarose gel.

***Immunocytofluorescence:***

***p65 Immunocytofluorescence:*** To study the cellular localization of the p65 subunit of NF- $\kappa$ B, myocardial cells were co-transfected with test expression constructs [1  $\mu$ g each of pCMV6, p38 $\alpha$  or 15  $\mu$ g of MKK6(Glu)] and 5  $\mu$ g of a plasmid encoding green fluorescent protein (GFP). Following 48 hours culture in minimal media, cells were fixed as described (35) and immunocytofluorescence was carried out using a polyclonal antibody raised against p65/NF- $\kappa$ B (Santa Cruz Biotechnology) followed by Texas-red-conjugated anti-rabbit IgG (Molecular Probes). Transfected cardiac myocytes were identified as GFP-positive cells, and p65 localization was visualized in only the transfected cells.

***Kinase Assays:***

***MAPKAP-K2 Assay:*** Myocardial cells were treated  $\pm$  TNF- $\alpha$  [1 ng/ml] for 10 minutes, then extracted in 400  $\mu$ l of Buffer A (50 mM Tris [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 5 mM Na pyrophosphate, 10 mM Na glycerophosphate, 50 mM NaF, 0.5mM Na o-vanadate, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, 1ug/ml Aprotinin, and 1ug/ml Leupeptin). Following removal of debris by centrifugation, MAPKAP-K2 was immunoprecipitated using 1.5  $\mu$ g of anti-MAPKAP-K2 (Upstate Biotechnology, Lake Placid, NY #06-534) and submitted to a

kinase assay using  $\gamma$ - $^{32}\text{P}$ -ATP and hsp27 as the substrates, as described by the manufacturer's protocol. Labeled hsp27 was then resolved by 12% SDS-PAGE and the gel was submitted to phosphorimage analysis.

**IKK $\beta$  Assay:** IKK kinase activity was assessed in myocardial cells by co-transfecting C-Flag-IKK  $\pm$  test expression constructs  $\pm$  SB203580 (5  $\mu\text{M}$ ). After the appropriate times, cultures were extracted in a buffer containing 20 mM Tris pH 7.6, 20 mM  $\beta$ -glycerolphosphate, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 0.5% NP40, 0.1 mM Na *o*-vanadate, 10  $\mu\text{g}/\text{ml}$  aprotinin, 2 mM DTT, 1 mM PMSF, 1 mM PNPP and 10  $\mu\text{g}/\text{ml}$  of leupeptin. After brief centrifugation, extracts were incubated for 2 h at 4°C with anti-IKK antibody (Santa Cruz Biotechnology), followed by protein G-Sepharose (Pharmacia Biotech) precipitation. Immunocomplex kinase assays were carried out using 1  $\mu\text{g}$  of recombinant I B- (1-317) (Santa Cruz Biotechnology) per sample and 10  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP (5000 Ci/mmol) in a final volume of 30  $\mu\text{L}$  of kinase buffer (30 mM HEPES pH 7.4, 10 mM  $\text{MgCl}_2$ , 1 mM DTT) at 25°C for 15 minutes. The reactions were terminated by the addition of Laemmli sample buffer and the phosphorylation level of I B- was evaluated by SDS-PAGE followed by autoradiography and phosphorimage analyses. The quantity of IKK in each sample was determined by Western analysis; the observed levels were used to normalize relative I B- phosphorylation levels found in phosphorimage analyses.

**Western Analysis:**

**p38, JNK and ERK:** Cultures (approximately  $2 \times 10^6$  myocytes) were lysed in 100  $\mu$ l of supplemented Laemmli Sample Buffer supplemented with 0.1 mM Na o-vanadate, 10  $\mu$ g/ml Aprotinin, 2 mM DTT, 1 mM PMSF, 1 mM PNPP and 10  $\mu$ g/ml leupeptin and boiled for 5 minutes, and then submitted to 10% SDS-PAGE and transferred to a nitrocellulose membrane in methanol transfer buffer at 60V for 5 hours or 30V overnight. Membranes were blocked for 30 minutes in 5% non-fat milk dissolved in TBS-Tween(0.01%) at room temperature. Western analyses were then performed using 1:1000 dilutions of antisera specific for either phospho-p38 (New England BioLabs, Beverly, MA #9211S), phospho-JNK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA #SC6254) or phospho-ERK (New England BioLabs, Beverly, MA #9101S). Blots were subsequently stripped with 6.25mM Tris, 2% SDS, and 100mM 2-mercaptoethanol for 30 minutes at 50°C, washed for 1 hour in TBS-Tween and re-probed with a 1:1000 dilutions of antisera specific for either p38, (Stressgen Biotechnologies Corp., Victoria BC, Canada #KAP-MAOO9E), JNK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA #SC-474), or ERK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA #SC-093) for normalization purposes.

**STAT3 and P-STAT3:** Myocytes were cultured as described above and then treated  $\pm$  various test agents for 15 min. The cells from 3-35 mm culture wells ( $4.5 \times 10^6$  myocytes) were collected by scraping into 450  $\mu$ l of lysis buffer which consisted

of 20 mM Tris-HCl (pH 7.6), 20  $\mu$ M Na-glycerophosphate, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 0.5% NP-40, 0.25 mM PMSF, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 50 mM NaF and 2 mM  $\text{Na}_3\text{VO}_4$ . Extracts were centrifuged for 15 sec at 14,000 rpm in a microfuge, and the supernates were pre-cleared by incubating with protein A sepharose beads for 30-60 min at 4°C. Extracts were then incubated for 12-18h at 4°C with anti-STAT3 antibody (New England Biolabs, Inc. Beverly MA; kit #9130) at 1:100. Immunoprecipitates were collected on protein A sepharose beads, eluted with Laemmli buffer, and analyzed by SDS-PAGE followed by blotting on nitrocellular paper, as described above. Blots were then probed with either anti-STAT3 antibody or with anti-Y-705-STAT3 antibody and washed according to the manufacturer's protocol (New England Biolabs, Inc., Beverly MA). Upon visualizing using chemiluminescence, blots were digitized using a Phosphorimager and the bands representing Y-705-STAT3 and total STAT3 were quantitated using Image Quant software (Molecular Dynamics).

***IKK $\beta$ /MKK6 Association:*** Myocytes were cultured for 72 hours in DMEM/F12 containing 1% fetal bovine serum.  $9 \times 10^6$  myocytes were lysed in 500 mL extraction buffer containing 20 mM Tris pH 7.6, 20 mM  $\beta$ -glycerolphosphate, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 0.5% NP40, 0.1 mM Na o-vanadate, 10  $\mu$ g/ml Aprotinin, 2 mM DTT, 1 mM PMSF, 1 mM PNPP and 10  $\mu$ g/ml Leupeptin. The cell debris was removed by brief centrifugation. Lysates were incubated at 4°C with anti-IKK polyclonal antibody (Santa Cruz) or with anti-MEK-6 polyclonal

antibody (Stressgen) at a dilution of 1:250 for 2 hours. Thirty  $\mu$ L of a protein-G-sepharose slurry were then added to each sample and incubated for 30 minutes at 4°C. Following centrifugation, Lammeli buffer was added to the pellet which was then boiled for 5 minutes. Proteins were resolved by a 12% SDS-PAGE and transferred to a nitrocellulose membrane in methanol transfer buffer at 100V for 2 hours. Blots were blocked for 30 minutes in 5% non-fat milk dissolved in TBS-Tween (0.01%) at room temperature, then probed with anti-FLAG M2 monoclonal antibody (Sigma) at a dilution of 1:300 in 5% non-fat milk for 2 hours at room temperature. After washing for 1 hour in TBS-Tween(0.01%) the membrane was incubated in 1:2000 dilution of goat anti-mouse IgG horseradish peroxidase (Jackson ImmunoResearch Laboratories). Visualization of immune complex was carried out by an enhanced chemiluminescence (ECL) method using ECL western blotting detection reagents (NEN Life Sciences) according to the manufacturers instructions.

**Results:**

***MKK6 and p38 Induce NF- $\kappa$ B Translocation and Transcription:*** Previous studies have demonstrated that constitutively active MKK6 [MKK6(Glu)], activates myocardial cell p38, but does not activate the ERK or JNK MAPK pathways (35, 41). MKK6(Glu) was also shown to protect cardiac myocytes from apoptosis (17). Since NF- $\kappa$ B is known to protect several other cell types from apoptosis (e.g. 12), we explored whether p38 could activate NF- $\kappa$ B in primary cardiac myocytes. Accordingly, myocardial cell cultures were transfected with constructs that code for the expression of wild type p38  $\alpha^1$  and/or MKK6(Glu). NF- $\kappa$ B activation was estimated qualitatively by visualizing the extent of nuclear translocation of one of the NF- $\kappa$ B subunits, p65/NF- $\kappa$ B, by immunocytofluorescence, and quantitatively by examining the activation of NF- $\kappa$ B-dependent transcription of a co-transfected reporter gene. In cells transfected with a control plasmid, p65/NF- $\kappa$ B cross-reactivity was distributed in a punctate pattern mostly in the cytoplasm of cardiac myocytes (**Fig. 1A**). In cells transfected with a plasmid encoding wild type p38  $\alpha^2$  only there was a small increase in cross-reactive p65/NF- $\kappa$ B in the nucleus (**Fig. 1B**), compared to control cells. However, in cells transfected with a plasmid encoding MKK6(Glu), there were clear increases in nuclear p65/NF- $\kappa$ B (**Fig. 1C**), which was even more pronounced upon co-transfection with plasmids encoding p38  $\alpha^2$  and MKK6(Glu) (**Fig. 1D**). Consistent with the nuclear accumulation of p65/NF- $\kappa$ B

were the abilities of overexpressed p38 $\beta$  and/or MKK6(Glu) to increase luciferase expression from an NF- $\kappa$ B-dependent luciferase reporter plasmid (**Fig. 1E**). Overexpression of either p38 $\beta$  or MKK6(Glu) resulted in approximately 2- and 3-fold induction of NF- $\kappa$ B/luciferase, respectively. However, co-expression of p38 $\beta$  and MKK6(Glu) conferred a robust, 16-fold induction of reporter expression. The p38-specific inhibitor<sup>2</sup>, SB203580, diminished this induction by about 70%. Thus, upon overexpression of wild type p38 $\beta$  and/or MKK6(Glu), the extent of NF- $\kappa$ B-dependent luciferase induction correlated well with the nuclear accumulation of p65/NF- $\kappa$ B. These results are consistent with the hypothesis that in cardiac myocytes, activation of the p38 pathway can lead to the nuclear translocation of NF- $\kappa$ B and to the induction of NF- $\kappa$ B-sensitive genes.

***MKK6 and p38 Induce NF- $\kappa$ B-dependent IL-6 Transcription:*** Since p38 is activated during the stress response in many cells types, and since NF- $\kappa$ B is known to facilitate cytokine gene induction during cell stress, we explored the hypothesis that together, p38 and NF- $\kappa$ B could mediate cytokine induction in cardiac myocytes. Accordingly, myocardial cells were transfected with a construct which possesses 1186 nts of the IL-6 gene regulatory region and promoter driving luciferase, IL-6 (wt) (**Fig. 2A**). Overexpressing wild type p38 $\beta$  or MKK6(Glu) resulted in approximately 2- or 3-fold reporter induction, respectively (**Fig. 2B**). However, co-expression of p38 $\beta$  and MKK6(Glu), together, conferred an approximately 7-fold induction of

reporter expression (**Fig. 2B**). These results indicate that the IL-6 promoter is transcriptionally active in cardiac myocytes<sup>3</sup> and that myocardial cell IL-6 transcription can be stimulated by p38  $\alpha$  and MKK6. IL-6 transcriptional induction in response to p38  $\alpha$  and/or MKK6(Glu) was decreased by 70 to 100% upon addition of SB203580 (**Fig. 2B**). Notably, IL-6 transcriptional induction was completely dependent upon NF- $\kappa$ B, since a point mutation which abolishes the only NF- $\kappa$ B binding site in the IL-6 gene (IL-6-M in **Fig. 2A**; ref. 15) resulted in a nearly complete loss of p38  $\alpha$ - and/or MKK6(Glu)-inducible IL-6 reporter activity. Interestingly, the abilities of p38 and/or MKK6(Glu) to induce the IL-6 promoter were also inhibited by overexpression of a kinase-inactive, dominant-interfering form of IKK  $\alpha$ , IKK  $\alpha$ -M (**Fig. 2B**). Taken together, these results indicate that IL-6 transcriptional induction in cardiac myocytes depends on the activation of NF- $\kappa$ B and is increased upon stimulation of the p38 pathway in a manner that is dependent upon IKK  $\alpha$ .

TNF- $\alpha$  stimulates p38 in other cell types (Bayaert et al., 1996; Yamakawa, 1999). Consistent with those results, we found that in cardiac myocytes, among the MAPKs, TNF- $\alpha$  preferentially stimulated p38 by about 6-fold, compared to approximately 2-fold for either JNK or ERK (**Fig. 3A & 3B**). Accordingly, we evaluated whether TNF- $\alpha$  could augment NF- $\kappa$ B-dependent transcription and, if so, whether this enhancement was sensitive to the inhibition of p38. For these

experiments, we employed a reporter gene comprised of the minimal IL-6 promoter flanked only by NF- $\kappa$ B binding sites (NF- $\kappa$ B/IL-6 in Fig. 2A). Indeed, TNF- $\alpha$  conferred a robust, 10-fold induction of NF- $\kappa$ B-dependent IL-6 transcription in cardiac myocytes (Fig. 2C) which was diminished by about 30-40% by SB203580, or by a dominant-negative, kinase-inactive MKK6 (MKK6-M1), indicating a partial requirement for p38. TNF- $\alpha$ -mediated induction of IL-6 transcription was not inhibited by treating cells with 5  $\mu$ M of the MEK-inhibitor, PD098059, or by co-transfecting cells with a dominant-negative form of MEK (not shown), indicating that ERK is probably not involved. Transfection with IKK-M resulted in an approximately 40-50% reduction in TNF- $\alpha$ -mediated induction of IL-6 transcription, and, SB203580 and IKK-M together completely blocked the effects of TNF- $\alpha$ . The effectiveness of SB20380 as an inhibitor of p38 was assessed by assaying MAPKAP-K2, a kinase that lies downstream of p38. When cultures were treated with 5  $\mu$ M, SB203580, there was a complete blockade of the ability of TNF- $\alpha$  to stimulate MAPKAP-K2, supporting the contention that at this concentration, SB20380 is an effective inhibitor of myocardial cell p38 (Fig. 3C). These results are consistent with a signaling process whereby TNF- $\alpha$ -activated p38 and NF- $\kappa$ B constitute the major pathways responsible for TNF- $\alpha$ -mediated NF- $\kappa$ B-dependent IL-6 transcriptional induction.

Recently, it has been shown that in HeLa cells, TNF- $\alpha$  stimulates TGF- $\beta$ -activated protein kinase 1 (Tak1), a newly discovered, multifunctional MAPKKK capable of binding to- and activating IKK $\alpha$  and MKK6 (Fig. 5; ref. 22). To determine whether this MAPKKK can mediate NF- $\kappa$ B- and p38-dependent IL-6 induction in myocardial cells, Tak1, and its required activator protein, Tak1 binding protein-1 (Tab1) (44), were both expressed in cardiac myocytes, along with the NF- $\kappa$ B/IL-6 luciferase reporter. This combination of Tak1 and Tab1 resulted in an approximate 5-fold activation of NF- $\kappa$ B-dependent IL-6 reporter gene expression<sup>5</sup>, which was inhibited by about 50-60% by SB203580, by kinase-inactive MKK6 (MKK6-M2), or by kinase-inactive IKK $\alpha$  (IKK $\alpha$ -M) (Fig. 2D). Together, SB203580 and IKK $\alpha$ -M completely blocked Tak1-stimulated NF- $\kappa$ B-dependent IL-6 reporter gene expression. These results are consistent with the capacity of Tak1 to stimulate both the IKK $\alpha$ /NF- $\kappa$ B- and the MKK6/p38 pathways in cardiac myocytes, the latter of which we have observed<sup>6</sup>. Moreover, in conjunction with results shown in Figure 2C, these findings indicate that the p38 pathway is required for optimal TNF- $\alpha$ - and Tak1-mediated NF- $\kappa$ B activation in cardiac myocytes.

To evaluate whether TNF- $\alpha$ -mediated IL-6 induction involves endogenous myocardial cell Tak1, which is present in neonatal rat heart (45), a kinase-inactive Tak-1 mutant (Tak1-M) was expressed. Tak1-M strongly inhibited TNF- $\alpha$ -induced

IL-6 transcription by about 70% (**Fig. 2E**). As expected from the results in Figure 2C, the p38 inhibitor, either SB203580 or MKK6-M1 blocked TNF- $\alpha$ -inducible IL-6 transcription by about 50%. Further, SB203580 and the Tak1-M together nearly completely inhibited the effects of TNF- $\alpha$  (**Fig. 2E**). These results indicate that in cardiac myocytes, TNF- $\alpha$  signals through Tak1 to activate IL-6 transcription in a partially p38-dependent manner. This is consistent with the ability of Tak1 to activate MKK6 and p38.

**MKK6 and p38 MAP Kinase Activate IKK $\beta$ :** Since MKK6 and p38 can activate NF- $\kappa$ B and, thus, lead to the induction of IL-6, a cytokine with potentially important functions in the heart, we carried out experiments to begin elucidating the mechanism of cross-talk between the p38- and NF- $\kappa$ B pathways. Initially, the effects of p38 $\alpha$  and/or MKK6(Glu) on the activity of IKK $\alpha$  in cultured myocardial cells was assessed. While expression of wild type p38 $\alpha$  alone had little effect on the kinase activity of IKK $\alpha$ , expression of MKK6(Glu) or MKK6(Glu) together with p38 $\alpha$  increased IKK $\alpha$  kinase activity by about 2- and 5-fold, respectively (**Fig. 4A**). The IKK $\alpha$  activation mediated by p38 $\alpha$  and MKK6(Glu) was decreased by about 50% upon addition of SB203580, indicating a requirement for p38.

**MKK6 Associates with IKK $\beta$ :** To pursue possible mechanisms by which MKK6(Glu) and p38 $\alpha$  can influence IKK $\beta$  activity, experiments were carried out to

evaluate whether p38 $\beta$  and/or MKK6 can interact directly with IKK $\beta$ . Myocardial cells were transfected with constructs encoding Flag-IKK $\beta$  and Flag-MKK6(Glu) and/or Flag-p38 and the abilities of these proteins to interact with each other were assessed using immunoprecipitation followed by Western blotting. While transfection of Flag-IKK $\beta$  together with Flag-p38 $\beta$  did not result in any apparent complex formation (not shown), co-expression of Flag-IKK $\beta$  and Flag-MKK6 did result in complex formation. Immunoprecipitating with either IKK $\beta$ - or MKK6-specific antisera resulted in the pull-down of both IKK $\beta$  and MKK6 (Fig. 4B), consistent with the abilities of the two proteins to interact *in vivo*. Taken together, the results of these kinase- and association experiments indicate that MKK6 can directly interact with IKK $\beta$ , and that this interaction may position p38 in the NF- $\kappa$ B pathway, resulting in an enhancement in the activity of IKK $\beta$ .

***MKK6 and p38 MAP Kinase Induce IL-6 Release from Cardiac Myocytes:*** To evaluate whether IL-6 promoter induction correlates with increased expression of IL-6 protein, itself, cardiac myocytes were treated with various doses of TNF- $\alpha$  and the quantity of IL-6 released into the culture medium was assessed by ELISA. TNF- $\alpha$  increased the quantity of IL-6 cross-reactive material in the medium in a dose-dependent manner by up to about 3-fold (Fig. 5A). The quantity of IL-6 in the medium was reduced significantly when SB203580 was added, consistent with a role for p38 in TNF- $\alpha$ -simulated IL-6 release from myocardial cells.

To further determine the role of the p38 pathway in myocardial cell IL-6 induction and release, cardiac myocytes were treated with recombinant adenoviral strains harboring the genes for p38  $\alpha$  or MKK6(Glu). The levels of virus were adjusted so that all of the myocytes expressed the transgenes. Shown in **Figure 5B** is an example using AdV-MKK6(Glu), a strain of adenovirus which possesses sequences encoding both MKK6(Glu) and green fluorescent protein (GFP) under the control of separate CMV promoters. Cultured myocytes were infected with virus, and then photographed under phase- and fluorescence microscopy 48h later (see Methods). Upon inspection of low power micrographs by phase- and fluorescence microscopy, it was apparent that essentially all of the cells visible in the phase field expressed GFP (compare Phase-Low to GFP-Low in **Fig. 5B**). Upon inspection of higher power micrographs it was apparent that AdV-MKK6(Glu)-infected myocytes displayed the visual characteristics typical of hypertrophic cells, such as enlarged cell area and extensive sarcomeric organization (**Fig. 5B: GFP-High and Phalloidin-High**). This is consistent with previous studies of the effects of MKK6(Glu) on cardiac myocyte morphology (35, 41). Cells infected with AdV-MKK6(Glu) and AdV-p38  $\alpha$  display similar morphology (not shown).

Infecting myocytes with AdV-p38  $\alpha$  alone did not significantly increase medium levels of IL-6 compared to control (**Fig. 5C**), consistent with the relatively weak activation of IL-6 transcription afforded by p38 alone (see Fig. 2A). However, infecting myocytes with AdV-MKK6(Glu) alone, or with both AdV-p38  $\alpha$  and AdV-

MKK6(Glu) together, increased the release of IL-6 by about 2- and 8-fold, respectively (Fig. 5C), which are very similar to their abilities to induce IL-6 transcription (see Fig. 2A). Moreover, MKK6(Glu)- and p38/MKK6(Glu)-dependent IL-6 release were both effectively inhibited by SB203580 (Fig. 5C), indicating that the selective activation of the p38 pathway confers significant IL-6 induction and release from cultured cardiac myocytes.

***IL-6 Protects Cardiac Myocytes from Apoptosis:*** To explore whether IL-6 might exert functions consistent with myocardial recovery following stress, the effect of IL-6 on myocardial cell apoptosis was determined. When cardiac myocytes were treated for a limited time with sphingosine, a lipid second messenger known to mediate apoptosis in many cell types (46-48), there was an approximate 3-fold increase in the number of apoptotic cells, as determined using TUNEL analysis (17). Strikingly, IL-6 exerted potent, dose-dependent, anti-apoptotic effects, affording complete protection from sphingosine-induced apoptosis at doses above 0.1 ng/ml (Fig. 6A). TNF- $\alpha$  also conferred protection against sphingosine-induced apoptosis, again, with nearly maximal effects being observed at 0.1 ng/ml of cytokine. These results were confirmed using DNA-laddering, where the fragmentation of DNA to create discrete increments of approximately 180 bp is diagnostic of apoptosis. Treating cells with sphingosine resulted in an approximate 6-fold increase in the intensity of the DNA bands observed, and incubation with either IL-6 or TNF- $\alpha$  at 1 ng/ml resulted in complete protection from apoptosis, as determined by DNA

laddering (Fig. 6B & 6C). The mechanisms by which TNF- $\alpha$  and IL-6 confer protection from apoptosis remain to be determined. However, several recent studies have suggested that certain cytokines that signal through the gp130 receptor can activate the Janus kinase (Jak)/signal transducer and activator of transcription (STAT) and protect against apoptosis in cardiac myocytes (49, 50). Moreover, one recent study has shown that, even though it does not couple through gp130, TNF- $\alpha$  can activate STAT3 in 3T3-L1 adipocytes (51). Accordingly, we tested the abilities of TNF- $\alpha$  and IL-6 to activate myocardial cell STAT3 by evaluating the level of Jak-mediated phosphorylation of Y-705 on immunoprecipitated STAT3. Treatment with TNF- $\alpha$  resulted in a small, 2-fold increase in STAT3 activation, while treatment with IL-6 induced a robust 8.2-fold increase in STAT3 activation (Fig. 6D). Thus, while it is conceivable that TNF- $\alpha$  could induce STAT3-sensitive genes involved in protection from apoptosis, it seems more likely that TNF- $\alpha$  exerts its cardioprotective effects in this cultured cell model via the activation of NF- $\kappa$ B and the subsequent induction of cytokines, such as IL-6, which may signal to cardiac myocytes in an autocrine manner.

**Discussion:**

The way the heart responds to stress is critical for maintaining proper cardiac function. One of the stress responses that has attracted recent attention is the release of cardiac-derived cytokines. Although it is widely believed that these cytokines serve important functions in the heart, the precise nature of those functions remains unclear. Our findings indicate that the myocardial IL-6 system serves an important autocrine signaling role that contributes to myocyte survival following certain cardiac stresses. Moreover, it appears that the optimal induction of IL-6 in cardiac myocytes involves both the NF- $\kappa$ B and p38 pathways. Our results indicate further that a potential point of collaboration between these signaling pathways resides in the ability of MKK6 to augment the activity of IKK in a p38-dependent manner (Fig. 7).

The possibility that p38 plays a role in NF- $\kappa$ B activation has been explored in cardiac myocytes (8,17) and in other cell types (e.g. 42), however, the mechanism of interaction between the two pathways has not been fully resolved and has been addressed in only a few studies. To address whether p38/NF- $\kappa$ B cross-talk could occur at the transcriptional level, Vanden Berghe et al. used a Gal4-based two-hybrid assay and presented data indicating that p38 increased the transcriptional activation potential p65/NF- $\kappa$ B (15). In contrast to that study, in preliminary experiments, we were unable to demonstrate such *trans*-activation of p65/NF- $\kappa$ B in

cardiac myocytes<sup>6</sup>. Moreover, while phosphorylation of p65/NF- $\kappa$ B augments its ability to bind to DNA (52), and is apparently required for its transcriptional activity (53), recent data indicate that p38 itself does not phosphorylate p65/NF- $\kappa$ B (54). To our knowledge, the results in the present study constitute the first demonstration that p38/NF- $\kappa$ B cross-talk may exist at the level of MKK6 and IKK.

Although the mechanism by which MKK6 augments IKK kinase activity is yet to be determined, our results showing that MKK6 and IKK can physically interact provide some clues. For example, it is formally possible that MKK6, which has never been shown to phosphorylate any protein other than p38 (19,38), might phosphorylate IKK and, in so doing, stimulate its kinase activity. However, we were unable to observe any change in the phosphorylation status of IKK upon incubation with MKK6(Glu) *in vitro* (not shown). Alternatively, in addition to activating p38, MKK6 might serve to position p38 near IKK, and other members of the NF- $\kappa$ B signaling pathway. This positioning might then lead to the observed p38-dependent increases in NF- $\kappa$ B activity. Interestingly, we have been unable to observe the binding of p38 itself to IKK, which further supports an anchoring role for MKK6. IKK and MKK6 are both members of the MAPKK family, and IKK is known to associate with other MAPKKs in the NF- $\kappa$ B signaling pathway (28,29). Thus, while it has not previously been shown that MKK6 can bind to other MAPKKs, it seems probable that IKK could form dimers with MKK6 via the same binding

domains involved in the formation of IKK homodimers. Future studies directed toward mapping such association domains in the IKKs, MKK6 and other MAPKKs will be required to address this provocative yet very feasible hypothesis.

To the best of our knowledge, the present study is also the first to demonstrate that a functional consequence of p38-mediated NF- $\kappa$ B activation by MKK6(Glu) is the induction of the IL-6 gene and release of IL-6 from cardiac myocytes. Consistent with roles for both pathways in IL-6 induction were our findings that either TNF- $\alpha$  or Tak1, both of which activate p38 and NF- $\kappa$ B in other cell types (22) and in cardiac myocytes<sup>4</sup>, induced the IL-6 promoter. In agreement with results showing that the activation of p38 and NF- $\kappa$ B by MKK6(Glu) leads to protection from apoptosis in cardiac myocytes (17), was our finding that IL-6 is anti-apoptotic.

Although many studies have been carried out, clear physiological functions for IL-6 and other cytokines in the stressed heart remain elusive. For example, the expression of IL-6 in the heart has been associated with acute ischemia and cardiac failure, findings which have been interpreted as indicative of either a productive or a deleterious role for the cytokine (2-5). Interestingly, in addition to the findings reported in this study, there is considerable evidence, mostly in non-cardiac myocytes, that supports a cytoprotective- and even growth-promoting role for IL-6. For example, IL-6 has been shown to protect hepatocytes and myeloma cells from apoptosis (55, 56). Additionally, ET-1, which is a well known myocardial cell growth

factor (57), is believed to mediate some of its cardiac growth-promoting effects through IL-6 (58). Interestingly, IL-6 was found to improve survival in an animal model of viral myocarditis, in part by reducing myocardial cell death (59). Moreover, the induction of IL-6 upon myocardial ischemia (6,8), and the finding that p38 can activate NF- $\kappa$ B during cardiac ischemia (16), suggest that upon such stresses the heart responds by producing cytokines that may serve important roles as barriers against apoptosis.

Consistent with this hypothesis was our finding in the present study that IL-6 afforded significant protection from apoptosis. The mechanism by which IL-6 might confer such protection remains to be elucidated. However, IL-6 belongs to the superfamily of trophic factors which includes cardiotrophin (CT-1), ciliary neurotrophic factor, leukemia inhibitory factor (LIF) and oncostatin M (60), all of which activate ERK and STAT3 and foster the growth and survival of cardiac myocytes, neurons and other cells types, via a ubiquitously expressed gp130 signal transduction mechanism. Thus, it is reasonable to speculate that it is through a related, gp130 signaling mechanism that IL-6 confers the protection from apoptosis observed in the present study. Supporting this view are recent findings demonstrating defects in cardiac growth and development in gp130 knock-out mice (61). It is conceivable, therefore, that in some circumstances, the induction of IL-6 in the heart following ischemia, or perhaps some other stresses, may not always represent a cytotoxic response, but may confer a cytoprotective response to the injury

(4). Indeed, cardiac myocytes in the region surrounding a myocardial infarct express IL-6 which may have an autocrine cytoprotective effect on the myocytes that survive the initial ischemic insult (9). While the precise mechanism by which cytokines, like IL-6, might confer protection from apoptosis is unknown, it is of interest to note that both TNF- $\alpha$  and IL-1 $\beta$  can induce several cytoprotective proteins in the heart, the free-radical scavenger, Mn superoxide dismutase (62) and several of the heat shock proteins (63).

In summary, the results of the present study provide evidence for a signaling pathway whereby stimulators of p38, such as TNF- $\alpha$ , can augment the activity of the NF- $\kappa$ B pathway via cross talk at the level of IKK (Fig. 7). Much remains to be understood about this interesting convergence of the NF- $\kappa$ B- and p38 MAPK pathways. For example, what is the mechanism by which MKK6 and p38 collaborate to enhance the activity of IKK? Do MKK6 and other known activators of p38, such as MKK3, occupy positions in the rapidly growing list of proteins comprising the NF- $\kappa$ B signalsome? Do multiple isoforms of p38, such as p38 $\alpha$ , p38 $\beta$ , p38 $\delta$ , p38 $\gamma$ , all participate in this form of crosstalk? Perhaps most interesting are recent findings indicating that bone morphogenic proteins (BMPs), which are required for directing the cardiac lineage during embryogenesis, operate through Tak1 to enhance cardiac-specific gene induction and growth (64). Since we found that Tak1 is a potent activator of p38 and NF- $\kappa$ B in cardiac myocytes, which serve to enhance the

transcription and release of IL-6, and probably other cytokines, it is tempting to speculate that in some ways, the myocardial stress response recapitulates the early cardiac development program. For example, perhaps during early cardiac embryogenesis, BMP-mediated activation of p38 and NF- $\kappa$ B augments the expression of genes sensitive to both pathways. Indeed, we and others have found that p38 can enhance the expression of a variety of cardiac genes that are normally upregulated during early development and during myocardial hypertrophy. Thus, it seems possible that in collaboration with p38, NF- $\kappa$ B may play an important, as yet unappreciated role in the developing myocardium, serving to induce NF- $\kappa$ B-sensitive genes, such as IL-6, which could contribute to myocardial cell growth and the associated protection from apoptosis. Addressing these fascinating ideas will undoubtedly reveal more detail about the interesting connection between the developing- and the stressed myocardium.

**Footnotes:**

<sup>1</sup>We found that when co-transfected with MKK6(Glu), p38 induced apoptosis in cultured cardiac myocytes, while p38<sub>2</sub> (same as p38-2) did not. This is consistent with a previous study indicating that p38 is pro-apoptotic in cardiac myocytes (41). Accordingly, all experiments in this study that required the overexpression of p38 employed only constructs encoding p38<sub>2</sub>.

<sup>2</sup>At the levels used in this study (5  $\mu$ M), SB203580 has been shown to be a selective inhibitor of p38. The effectiveness of 5  $\mu$ M SB203580 as a p38 inhibitor is also demonstrated in Figure 3C.

<sup>3</sup>The electroporation parameters used in this study have been shown to allow for the transfection of plasmid DNA into cardiac myocytes only, and not any other cell types that may be present in the cultures (34, 36). Accordingly, any reporter expression observed in culture extracts is from cardiac myocytes, only.

<sup>4</sup>Unpublished observation, Martindale and Glembotski

<sup>5</sup>Experiments using various doses of pFLAG-Tak1 or pHA-Tab1 in various ratios showed qualitatively similar results, with optimal activation of NF- $\kappa$ B/luciferase being observed using 15  $\mu$ g of pFLAG-Tak1 and 15  $\mu$ g of pHA-Tab1 per transfection.

<sup>6</sup>Unpublished observation, Craig and Glembotski

<sup>7</sup>Kim et al., manuscript in preparation.

<sup>8</sup>Dose response experiments were carried out with all plasmids to verify that the effects shown are representative of those obtained using a variety of plasmid levels and to determine optimal plasmid doses.

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**References:**

1. Hawkins, H.K., Entman, M.L., Zhu, J.Y., Youker, K.A., Berens, K., Dore, M., and Smith, C.W. (1996) *Am. J. Pathol.* **148**: 1957-1969.
2. Guillen, I., Blanes, M., Gomez-Lechon, M.J. and Castell, J.V. *Am. J. Pathol.* **269**: R229-R235.
3. Neumann, F.J., Ott, I., Gawaz, M., Tichardt, G., Holzapfel, H., Jochum, M., and Schoemig, A. *Circulation* **92**: 748-755.
4. Mann, D.L. (1996) *Cytokine & Growth Factor Reviews* **7**:341-354.
5. Meldrum, D.R. (1998) *Am. J. Physiol.* **274**: R577-R595.
6. Kukielka, G.L., Smith, W., Manning, A.M., Youker, K.A., Michael, L.H. and Entman, M.L. (1995) *Circ* **92**: 1866-1875.
7. Kapadia, S., Lee, J.R., Torre-Amione, G., Birdsall, H.H., Ma, T.S. and Mann, D.L. (1995) *J. Clin. Invest.* **96**: 1042-1052.
8. Yamauchi-Takahara, K., Ihara, Y., Ogata, A., Yoshizaki, K., Azuma, J. and Kishimoto, T. (1995) *Circ.* **91**: 1520-1524.
9. Gwechenberger, M., Mendoza, L.H., Youker, K.A., Fangogiannis, N.G., Smith, W., Michael, L.H. and Entman, M.L. (1999) *Circulation* **99**: 546-551.
10. Hirota, H., Yoshida, K., Kishimoto, T., and Taga, T. (1995) *Proc. Natl. Acad. Sci USA* **92**: 4862-4866.
11. Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J.H. and Chien KR (1997) *J. Biol. Chem.* **272**: 5783-5791.

12. Beg, A.A. and Baltimore, D. (1996) *Science* **274**(5288): 782-784.
13. Wang, C.Y., Mayo, M.W. and Baldwin, A.S. Jr (1996) *Science* **274**(5288): 784-787.
14. Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R. and Verma, I.M. (1996) *Science* **274**(5288): 787-789.
15. Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M.L., Fiers, W. and Haegeman, G. (1998) *J. Biol. Chem.* **273**: 3285-3290.
16. Maulik, N., Sato, M., Price, B.D. and Das, D.K. (1998) *FEBS Lett.* **429**: 365-369.
17. Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC. (1998) *J. Biol. Chem.* **273**: 8232-8239.
18. Moriguchi, T., Kuroyanagi, N., Yamaguchi, K., Gotoh, Y., Irie, K., Kano, T., Shirakabe, K., Muro, Y., Shibuya, H., Matsumoto, K., Nishida, E. and Hagiwara, M.. (1996) *J. Biol. Chem.* **271**: 13675-13679.
19. Raingeaud, J., Whitmarsh, A.J., Barrett, T., Derijard, B. and Davis R.J. (1996) *Mol Cell Biol* **16**: 1247-1255.
20. Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Ioshi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995) *Science* **270**: 2008-2011.
21. Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) *Nature* **398**: 252-256.
22. Sakurai, H., Miyoshi, H., Toriumi, W. and Sugita, T. (1999) *J. Biol. Chem.* **274**: 10641-10648.
23. Chen, Z.J., Parent, L. and Maniatis, T. (1996) *Cell* **84**: 853-862.

24. DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. and Karin M (1996) *Mol. Cell Biol.* **16**: 1295-1304.
25. Regnier, C.H., Song, H.Y., Gao, X., Goeddel, D.V., Cao, Z. and Rothe, M. (1997) *Cell***90**: 373-383.
26. Woronicz, J.D., Gao, X., Cao, Z., Rothe, M.. and Goeddel DV. (1997) *Science* **278**: 866-869.
27. Mercurio, F., Zhu, H., Murray, B.W., Shevchenko, A., Bennett, B.L., wu Li, J., Young, D.B., Barbosa, M., and Mann, M. (1997) *Science* **278**: 860-866.
28. Zandi, E., Rothwarf, D.M., Delhase, M., Hayakawa, M., and Karin, M. (1997) *Cell***91**: 243-252.
29. Karin M. (1998) *J. Biol. Chem.* **274**: 27339-27342.
30. Malinin, N.L., Boldin, M.P., Kovalenk,o A.V. and Wallach, D. (1997). *Nature* **385**: 540-544.
31. Song, H.Y., Regnier, C.H., Kirschning, C.J., Goeddel, D.V. and Rothe, M. (1997) *Proc. Natl. Acad. Sci. USA* **94**: 9792-9806.
32. Su, Y.C., Han, J., Xu, S., Cobb, M. and Skolnik, E.Y. (1997) *EMBO J.* **16**: 1279-1290.
33. Lu, X., Nemoto, S. and Lin, A. (1997) *J. Biol. Chem.* **272**: 24751-24754.
34. Sprengle, A.B., Murray, S.F. and Glembotski, C.C. (1995) *Circ. Res.* **77**: 1060-1069.
35. Zechner, D., Thuerauf, D.J., Hanford, D.S., McDonough, P.M. and Glembotski, C.C. (1997) *J. Cell Biol.* **139**:115-127.

36. LaPointe, M.C., Wu, G., Garami, M., Yang, X.P. and Gardner, D.G. (1996) *Hypertension* **27**: 715-722.
37. Stein, B., Yang, M.X., Young, D.B., Janknecht, R., Hunter, T., Murray, B.W. and Barbosa, M.S. (1997) *J. Biol. Chem.* **272**: 19509-19517.
38. Enslin, H., Raingeaud, J. and Davis, R.J. (1998) *J. Biol. Chem.* **273**: 1741-1748.
39. DiDonato, J.A., Mercurio, F., Karin and M. (1995) *Mol. Cell Biol.* **15**: 1302-1311.
40. He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W. and Vogelstein, B. (1998) *Proc Natl Acad Sci U S A* **95**: 2509-2514.
41. Wang, Y., Huang, S., Sah, V.P., Ross, J. Jr, Brown, J.H., Han, J. and Chien KR (1998) *J. Biol. Chem.* **273**: 2161-2168.
42. Beyaert, R., Cuenda, A., Vanden Berghe, W., Plaisance, S., Lee, J.C., Haegeman, G., Cohen, P. and Fiers, W. (1996) *EMBO J.* **15**: 1914-1923.
43. Yamakawa, T., Eguchi, S., Matsumoto, T., Yamakawa, Y., Numaguchi, K., Miyata, I., Reynolds, C.M., Motley, E.D. and Inagami, T.. (1999) *Endocrinol.* **140**: 3562-3572.
44. Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, K., Nishida, E. and Matsumoto, K. (1996) *Science* **272**: 1179-1183.
45. Kim, S.O., Irwin, P., Katz, S., and Pelech, S.L. (1998) *J. Cell. Biochem.* **71**: 286-301.
46. Ohata, H., Yatomi, Y., Sweeney, E.A., Hakomori, S. and Igarashi, Y. (1994) *FEBS Lett.* **355**: 267-270.

47. Krown, K.A., Page, M.T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K.L., Glembotski, C.C., Quintana, P.J. and Sabbadini, R.A. (1996) *J. Clin. Invest.* **98**: 2854-2865.
48. Alessenko, A.V. and Khrenov, A.V. (1999) *Lipids* **34**: S75-S76.
49. Pennica, D., King, K.L., Shaw, K.J., Luis, E., Rallamas, J., Luoh, S-M., Darbonne, W.C., Knutzon, D.S., Yen, R., Chien, K.R., Baker, J.B. and Wood, W.I. (1995) *Proc. Natl. Acad. Sci. USA* **92**:1142-1146.
50. Matsui, H., Fujio, Y., Kunisada, K., Hirota, H. and Yamauchi-Takihara, K. (1996) *Commun. Molec. Pathol. Pharmacol.* **93**:149-162.
51. Guo, D., Dunbar, J.D., Yang, C.H., Pfeffer, L.M. and Donner, D.B. (1998) *J. Immunol.* **160**:2742-2750.
52. Naumann, M. and Scheidereit, C. (1994) *EMBO J.* **13**:4597-4607.
53. Schmitz, M.L., dos Santos Silva, M.A. and Baeuerle, P.A. (1995) *J. Biol. Chem.* **270**:15576-15584.
54. Carter, A.B., Knudtson, K.L., Monick, M.M., and Hunninghake, G.W. (1999) *J. Biol. Chem.* **274**:30858-30863.
55. Chen, R.H., Chang, M.C., Su, Y.H., Tsai, Y.T. and Kuo, M.L. (1998) *J. Biol. Chem.* **274**: 2301-2309.
56. Lichtenstein, A., Tu, Y., Fady, C., Vescio, R., and Berenson, J. (1995) *Cell Immunol.* **162**:248-255.

57. Shubeita, H.E., McDonough, P.M., Harris, A.N., Knowlton, K.U., Glembotski, C.C., Brown, J.H. and Chien, K.R. (1990) *J. Biol. Chem.* **265**: 20555-20562.
58. Saito, S., Aikawa, R., Shiojima, I., Nagai, r., Yazaki, Y., and Komuro, I. (1999) *FEBS Lett.* **30**: 103-107.
59. Kanda, T., Sakamoto, H., Mcmanus, B.M., Sakamaki, T., Nagai, R., Suzuki, T., and Kobayashi, I. (1996) *Life Sci.* **58**: 1705-1712.
60. Pennica, D., Wood, W.I. and Chien, K.R. (1996) *Cytokine & Growth Factor Reviews* **7**: 81-91.
61. Hiroto, H., Chen, J., Betz, U.A.K., Rajewsky, K., Gu, Y., Ross, J.R., Jr., Muller, W., and Chien, K.R. (1999) *Cell* **97**:189-198.
62. Nelson, W.K., Wong, G.H.W., and McCord, J.M. (1995) *J. Mol. Cell. Cardiol.* **27**:223-229.
63. Nakano, M., Knowlton, A.A., Yokoyama, T., Lesslauer, W. and Mann, D.L. (1996) *Am. J. Physiol.* **270**:H1231-H1239.
64. Monzen, K., Shiojima, I., Hiroi, Y., Kudoh, S., Oka, T., Takimoto, E., Hayashi, D., Hosoda, T., Habara-Ohkubo, a., Nakaoka, T., Fujita, T., Yazaki, Y. and Komuro, I. (1999) *Mol. Cell. Biol.* **19**: 7096-7105.

**Figure Legends:**

**Figure 1: Effects of p38 and MKK6(Glu) on NF- $\kappa$ B Translocation and NF- $\kappa$ B-mediated Transcription-**

**Panels A-D:** Myocardial cells were transfected with 1  $\mu$ g of plasmids encoding either no protein, pCMV6 (Control; 1  $\mu$ g<sup>8</sup>), p38  $\alpha$  (1  $\mu$ g) and/or MKK6(Glu)[15  $\mu$ g], as shown. Cultures were plated onto glass slides and incubated for 48h in serum-free media, after which they were fixed and stained for p65/NF- $\kappa$ B and visualized using a Texas-red conjugated second antibody. Shown are single cells that are representative of the population of cells observed following the treatments indicated.

**Panel E:** Myocardial cells were co-transfected with p2X NF $\kappa$ B (20  $\mu$ g<sup>8</sup>) and pCH110 (-gal; 20  $\mu$ g)  $\pm$  plasmids encoding p38  $\alpha$  (1  $\mu$ g), and MKK6(Glu)[15  $\mu$ g], and treated  $\pm$  SB203580 (5  $\mu$ M), as indicated. After 48h in serum-free medium, culture extracts were assayed for luciferase and -galactosidase activities. Relative luciferase (Rel Luc = luciferase/ -gal) was normalized to the maximum Rel Luc value in each experiment and then displayed as the % of that value. Each bar represents the mean of the results obtained from 3 identically treated cultures  $\pm$  standard error. Each experiment was replicated at least three times; the results shown are from one representative experiment.

**Figure 2: Effects of p38, MKK6(Glu), IKK $\beta$ M, Tak1, Tak1-M and TNF- $\alpha$  on IL-6 Promoter Activity-**

**Panel A: Reporter Diagram-** The three IL-6 reporter constructs used in this study

are diagrammed. *IL-6 (wt)* comprised of 1168 nts of the native human IL-6 5'-flanking sequence driving luciferase; *IL-6-M* is comprised of the same 1168 nts of the human IL-6 5'-flanking sequence except that the sole promoter-proximal NF- $\kappa$ B binding site was mutated so it no longer binds NF- $\kappa$ B; *NF- $\kappa$ B/IL-6* is comprised of a concatomer of three IL-6 NF- $\kappa$ B sites ligated directly to 50 nts of the human IL-6 5'-flanking sequence, a non-inducible, minimal promoter driving luciferase. Each of these IL-6 promoter/reporter constructs is described in more detail in the Methods (see ref. 15).

**Panels B-E: IL-6 Promoter Induction-** Myocardial cells were co-transfected with 20  $\mu$ g<sup>8</sup> of either IL-6 (wt), IL-6-M [**Panel B**] or with NF- $\kappa$ B/IL-6 [**Panels C-E**] and pCH110 (-gal). Some cultures were also co-transfected with combinations of plasmids which encode the following proteins: p38 $\alpha$  (1  $\mu$ g), MKK6(Glu) [15  $\mu$ g], IKK $\beta$ -M (45  $\mu$ g), MKK6-M (45  $\mu$ g), Tak1 (15  $\mu$ g), Tab1 (15  $\mu$ g), and Tak1-M (45  $\mu$ g), as indicated in the figure (see Methods for plasmid details). Cultures were treated  $\pm$  SB203580 (5  $\mu$ M), as indicated. After 48h, culture extracts were assayed for luciferase and -gal. Relative Luciferase values are expressed as % of maximum, as described in the legend to Figure 1. Each bar represents the mean of 3 cultures  $\pm$  standard error. Each experiment was replicated at least three times; the results shown are from one representative experiment.

**Panel F: Assessment of transgene expression** - Duplicate myocardial cell cultures were transfected as described above with plasmids encoding the proteins

shown. Cultures were then extracted, submitted to immunoprecipitation with either anti-flag (MKK6, IKK- $\alpha$ , IKK- $\beta$ -M, Tak, and Tak-M), or with anti-HA (p38) antisera and then Western blotting to assess the approximate levels of expression of each of the test proteins. As can be seen, each of the transgenes are expressed at approximately the same levels.

**Figure 3: Effects of TNF- $\alpha$  on the MAPKs and MAPKAP-K2-**

**Panels A and B: MAPK Activity-** Triplicate cultures were treated  $\pm$  TNF- $\alpha$  (1ng/ml) for 5 min and then extracted and submitted to Western analyses to determine P-p38, P-JNK, P-ERK, total p38, total JNK or total ERK, as described in the Methods. The phosphorimage from each triplicate culture set is shown in Panel A, and the results of the densitometric analysis of each image, carried out using Molecular Dynamics Image Quant software, are shown in Panel B. Each bar represents the mean of 3 cultures  $\pm$  standard error.

**Panel C: MAPKAP-K2 Activity-** Triplicate cultures were treated  $\pm$  TNF- $\alpha$  (1ng/ml),  $\pm$  SB203580 (5  $\mu$ M) for 10 min and then extracted and the activity of MAPKAP-K2 was determined as described in the Methods. The phosphorimage from each triplicate culture set is shown at the top of Panel C, and the results of the densitometric analysis of each image, carried out using Molecular Dynamics Image Quant software, are shown at the bottom of Panel C. Each bar represents the mean of 3 cultures  $\pm$  standard error.

**Figure 4: Interaction of IKK $\beta$  and MKK6-**

**Panel A: IKK $\beta$  Kinase Activity-** Duplicate myocardial cultures were transfected with IKK $\beta$  and the combinations of plasmids encoding p38 $\alpha$  and/or MKK6(Glu)  $\pm$  SB203580, as shown. Following 72h, extracts were submitted to immunoprecipitation using a IKK $\beta$ -specific antibody. IKK $\beta$  kinase assays were then carried out using I $\kappa$ B $\alpha$  as the substrate (see Methods). The phosphorylation of I $\kappa$ B $\alpha$  was assessed by SDS-PAGE followed by autoradiography. The relative levels of I $\kappa$ B $\alpha$  phosphorylation following each treatment were assessed using Molecular Dynamics Image Quant Software. Shown above each autoradiogram is the fold stimulation normalized to total IKK $\beta$  protein levels as determined by subsequent Western analysis of each kinase assay.

**Panel B: IKK $\beta$  MKK6 Association-** Myocardial cells were transfected with plasmids encoding IKK $\beta$  and/or MKK6(Glu). After 72h, culture extracts were submitted to immunoprecipitation using IKK $\beta$ - or MKK6-specific antibody. Immunoprecipitates were then submitted to SDS-PAGE and electroelution onto a nitrocellulose membrane. Western blotting was then carried out using a FLAG-specific antibody. This experiment was repeated at least three times; the results shown are from one representative experiment.

**Figure 5: Effect of TNF- $\alpha$ , p38 $\beta_2$  and MKK6(Glu) on IL-6 Cytokine Secretion -**

**Panel A: TNF- $\alpha$  Induction of IL-6 Secretion-** Myocardial cells were incubated with various levels of recombinant rat TNF- $\alpha$   $\pm$  SB203580, as shown. After 24h, the levels of IL-6 in media samples were determined by ELISA.

**Panel B: Characterization of Adenoviral-MKK6(Glu)-** Myocardial cells that had been in culture for 24h in serum free medium, were infected with AdV-MKK6(Glu) [see Methods]; this virus strain expresses both GFP and MKK6(Glu) under the control of separate CMV promoters (see Methods and ref. 40). After 48h, cultures were visualized under low magnification (20X) phase contrast [**Phase-Low**], low magnification green fluorescence [**GFP-Low**] (**note:** reference arrow in Phase-Low and GFP-Low panels points to the same cell in each field), high magnification (100X) green fluorescence [**GFP-High**], or high magnification red fluorescence [**Phalloidin-High**].

**Panel C: Effects of Adenoviral-MKK6(Glu) on IL-6 Secretion-** Myocardial cells were infected with AdV-(Con), AdV-p38 $\beta_2$ , and/or AdV-MKK6(Glu) such that 100% of the cells were transfected, and they were treated  $\pm$  SB203580, as shown and as described for Panel B and in the Methods. After 48h, media samples were assayed for the presence of IL-6 by ELISA. Each value represents the mean of 3 identically treated cultures  $\pm$  standard error.

**Figure 6: Effects of TNF- $\alpha$  and IL-6 on Apoptosis and STAT3 Activation-**

**Panel A: TUNEL Analyses-** Myocardial cells were plated onto glass slides and cultured for 48h in serum-free medium  $\pm$  IL-6 (1 ng/ml) or TNF- $\alpha$  (1 ng/ml), as indicated. Cultures were then treated  $\pm$  sphingosine (10  $\mu$ M), as indicated, and after 4h they were fixed for further TUNEL analysis. The number of TUNEL-positive cells observed in each field was determined as described in the Methods (see ref. 17), and then normalized to the total number of cells in each field on each slide. Each value represents the mean of 10 separate fields  $\pm$  standard error.

**Panels B: Ladder Analyses-** Myocardial cells were treated for 24h with media lacking cytokine [Lanes 2-4], with TNF- $\alpha$  (1 ng/ml) [lanes 6 and 7], or with IL-6 (1 ng/ml) [Lanes 8 and 9]. Sphingosine (10  $\mu$ M) was then added to some cultures [lanes 4-9], and after 4h they were assessed for apoptosis by DNA ladder analysis, as described in the Methods. Standard DNA ladders consisting of fragments comprised of 100 bp increments are shown in Lanes 1 and 10. The 400 bp standard is shown.

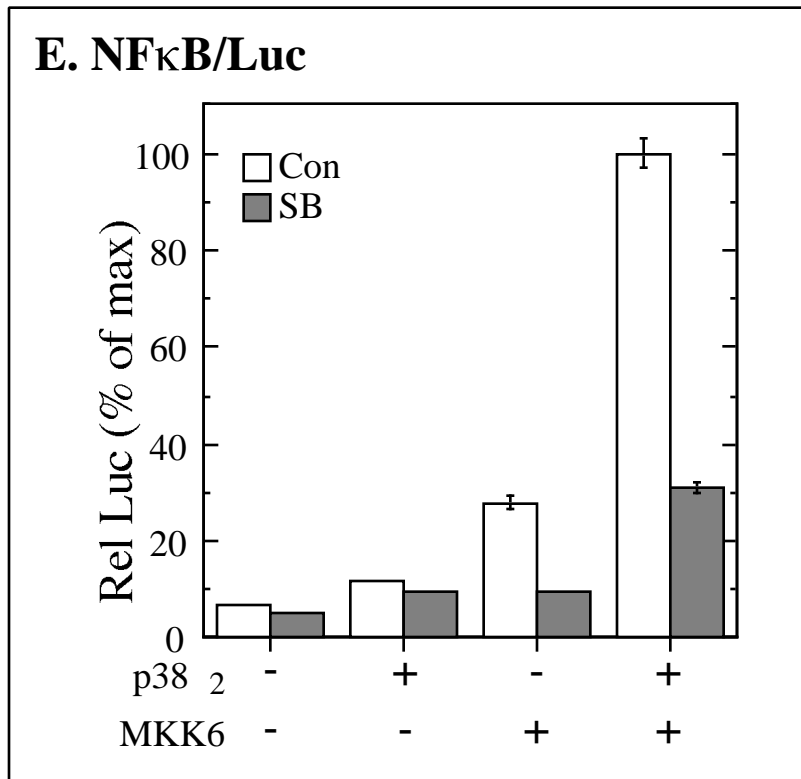
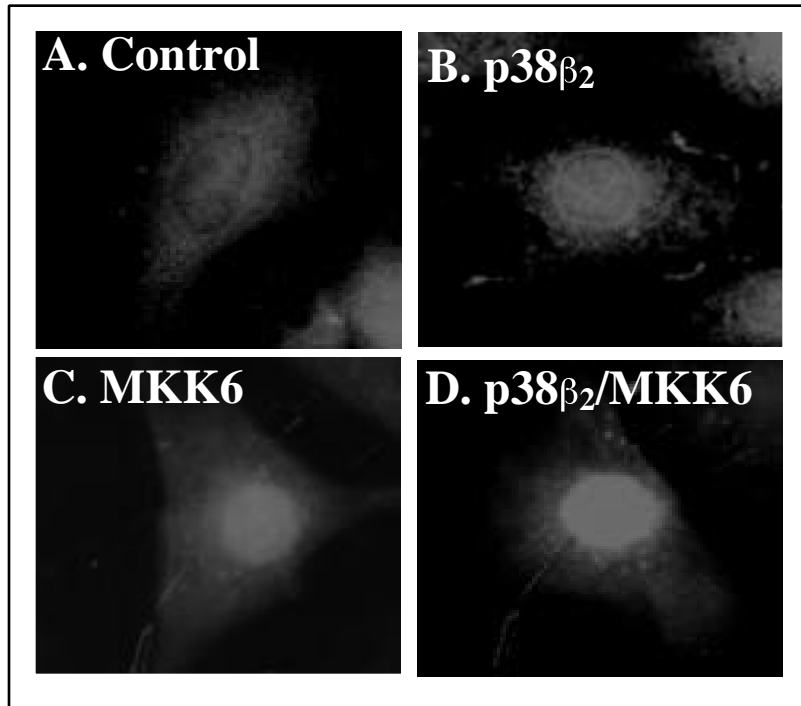
**Panel C: Ladder Quantitation-** Densitometric analysis of the 360 bp bands from Panel E was carried out and the average  $\pm$  SD of the results of each treatment are shown relative to the control (no cytokine, no sphingosine), which was set at 1.0.

**Panel D: STAT3 Phosphorylation-** Duplicate cultures were treated for 15 min  $\pm$  10 ng/ml of each test compound shown, and then extracts were assessed for the

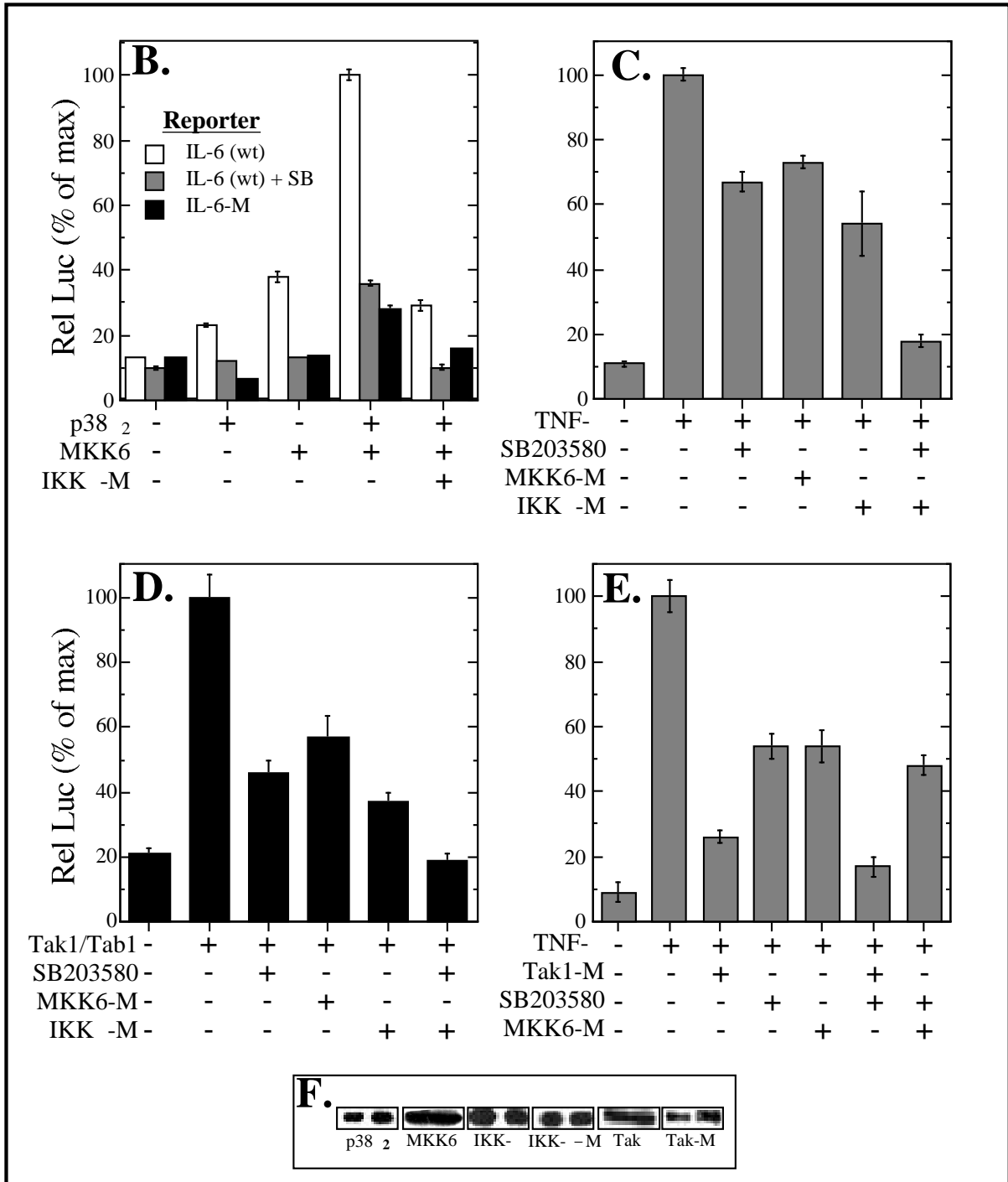
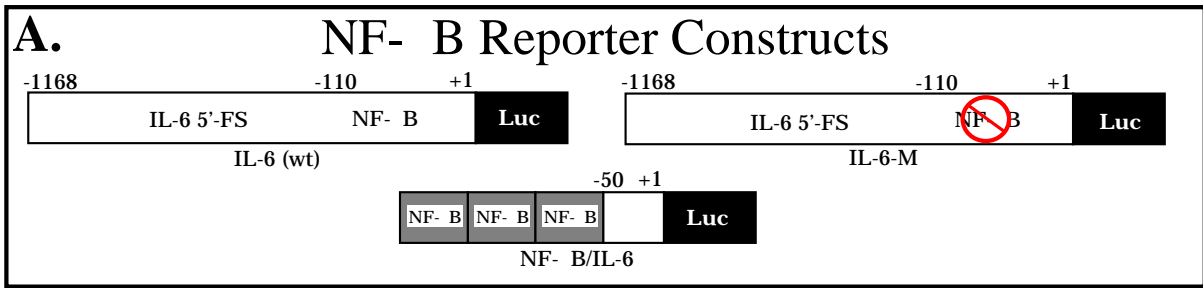
phosphorylation of STAT3 on Y-705 (P-STAT3) or for total STAT3 levels as described in the Methods. The controls are extracts of A431 cells that have been treated with (+) or without (-) EGF, which is known to induce STAT3 phosphorylation on Y-705. The fold increase in STAT3 phosphorylation was determined by densitometry and then normalizing each P-STAT3 value to the total STAT3 in that sample. Values shown are mean  $\pm$  SD; n = 2 cultures/treatment. This blot is representative of 3 similar experiments.

***Figure 7: Diagram of IKK $\beta$ /NF- $\kappa$ B- and the MKK6/p38 Pathways Relevant to this Study-*** Shown is a simplified diagram depicting the signaling pathways under study in the present report. Tak1 is a MAPKKK which requires a co-activator, Tab, for optimal activity. IKK and MKK6 are MAPKK family members that can be activated by Tak1. The shaded arrows indicate possible cross-talk between the p38- and the NF- $\kappa$ B pathways that is supported by the present study.

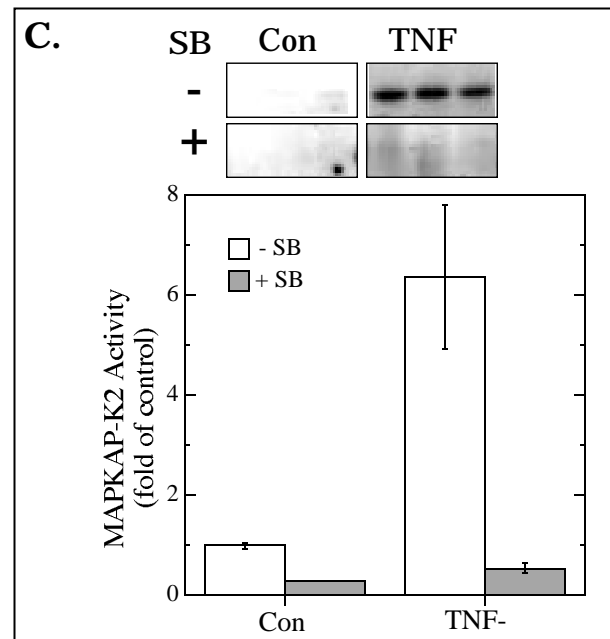
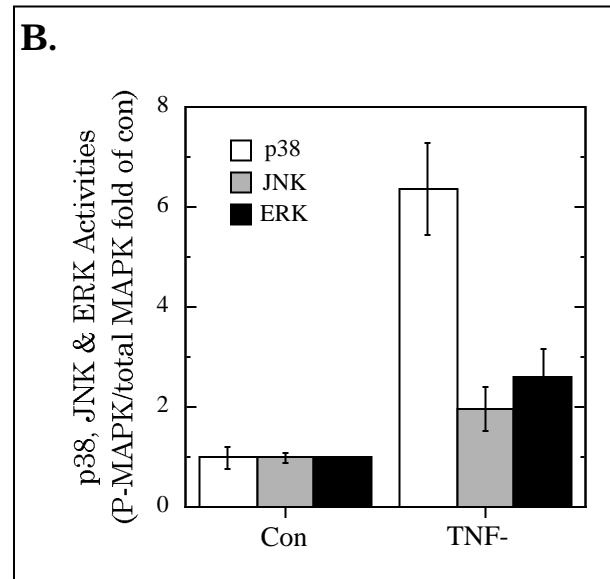
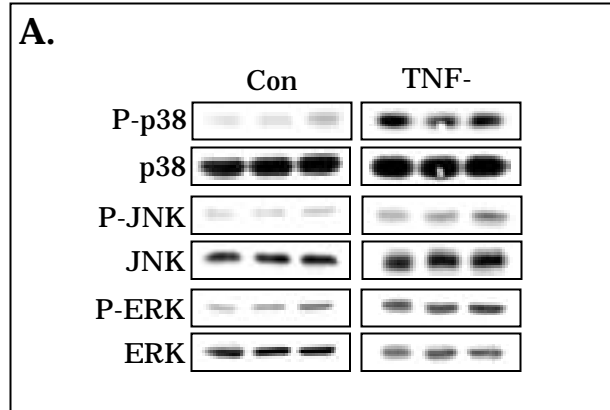
**Figure 1**



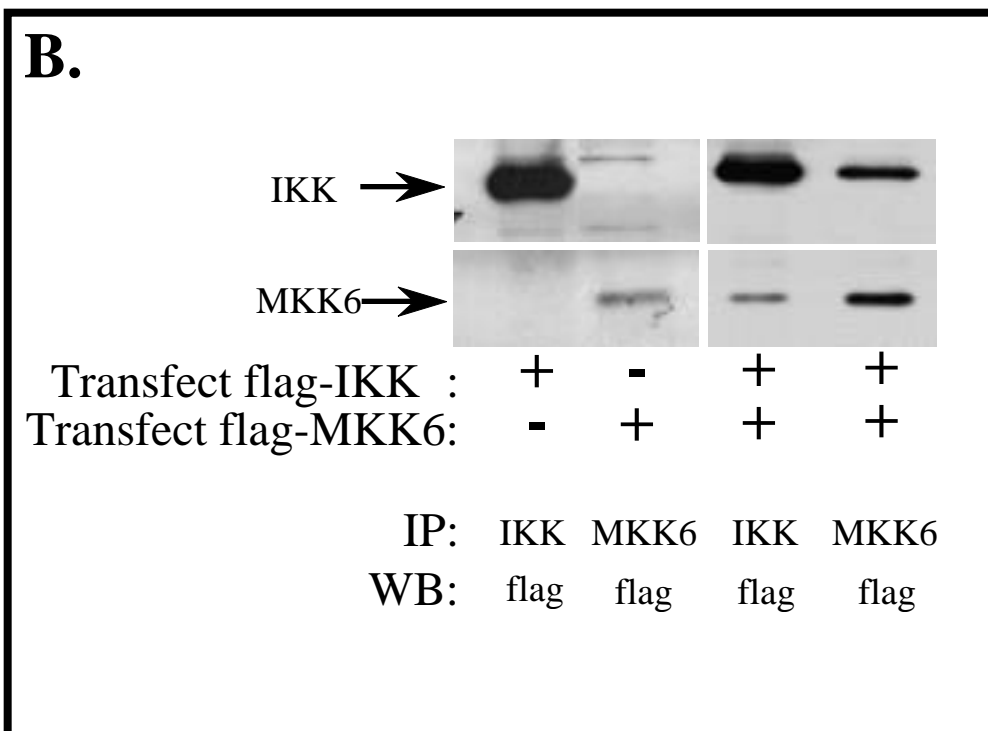
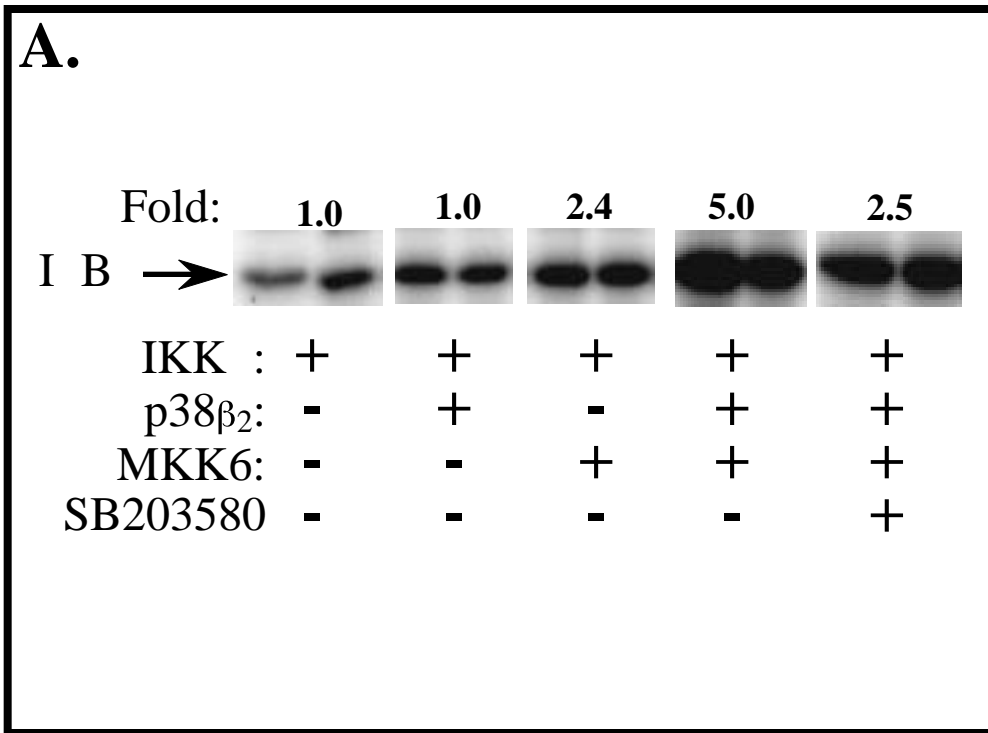
# Figure 2



**Figure 3**

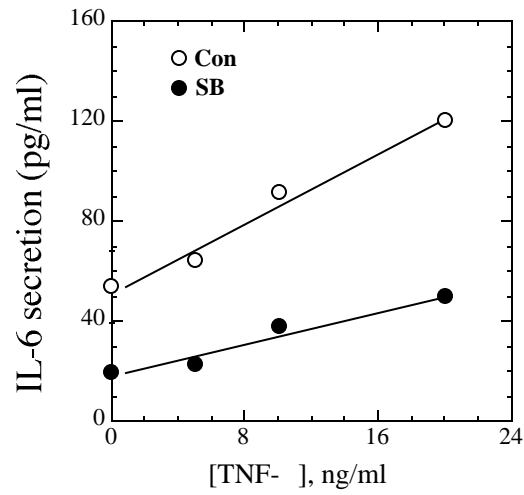


# Figure 4

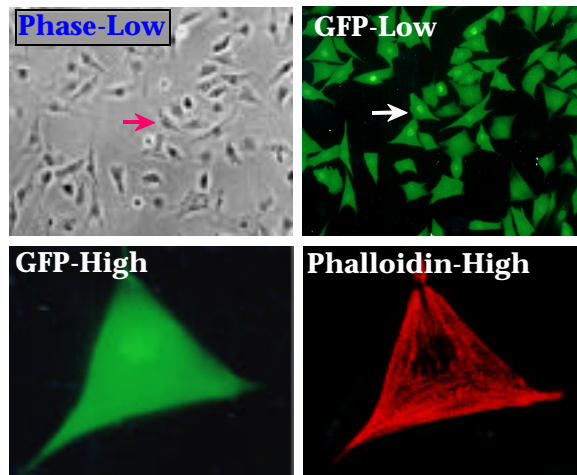


# Figure 5

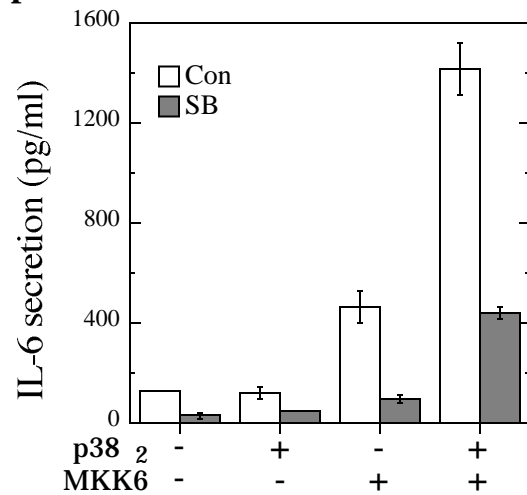
## A. TNF-activated IL-6 Secretion



## B. AdV-MKK6 Transfection

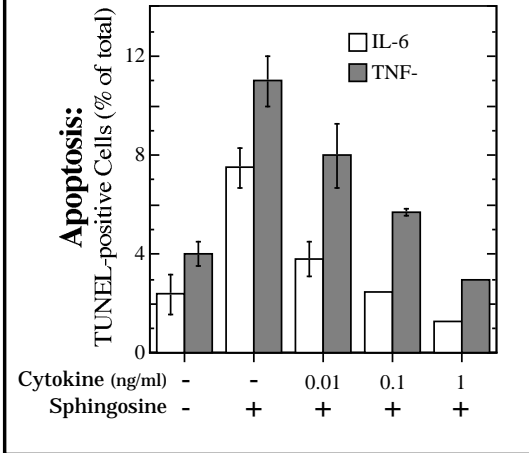


## C. p38/MKK6-activated IL-6 Secretion

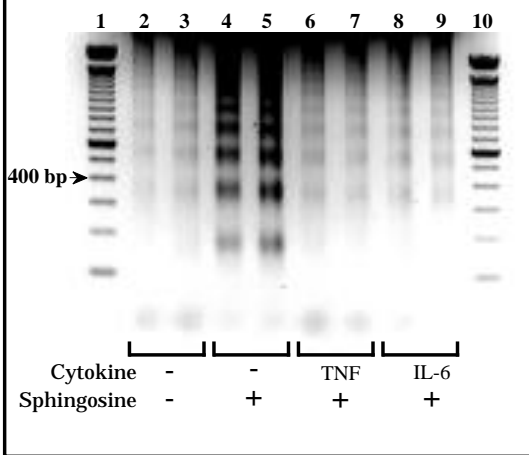


**Figure 6**

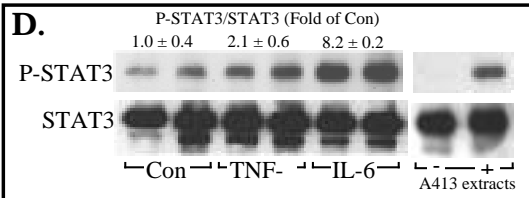
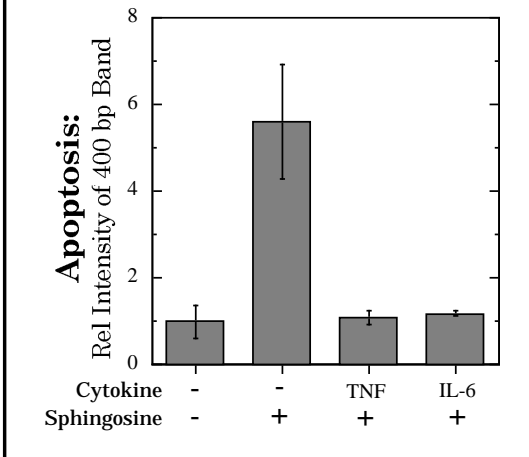
**A. IL-6 and TNF: TUNEL**



**B. IL-6 and TNF: DNA Ladders**



**C. DNA Ladder Quantitation**



**Figure 7**

