Molecular Pharmacology Fast Forward. Published on February 5, 2008 as doi:10.1124/mol.107.042176

MOL # 042176

TNF-α REGULATES INFLAMMATORY AND MESENCHYMAL RESPONSES VIA MEK, p38, NF-κB IN HUMAN ENDOMETRIOTIC EPITHELIAL CELLS

Eric M. Grund, David Kagan, Cam Anh Tran, Andreas Zeitvogel, Anna Starzinski-Powitz, Selvaraj Nataraja, and Stephen S. Palmer

EMD-Serono Research Institute, Rockland, Massachusetts 02370, USA (EMG, DK, CAT, SN, SSP); and Humangenetik fuer Biologen, Johann Wolfgang Goethe-Universitat, D-60054 Frankfurt, Germany (AZ, AS)

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: TNF-α regulates inflammatory response in endometriotic cells

Corresponding Author:

Stephen S. Palmer

EMD-Serono Research Institute

One Technology Place

Rockland, Massachusetts 02190

Tel. 781-681-2795

Fax 781.681.2910

E-mail: Stephen.Palmer@EMDSerono.com

Number of text pages: 34

Number of tables: 0

Number of figures: 9

Number of references: 40

Number of words in the Abstract: 247

Number of words in the Introduction: 736

Number of words in the Discussion: 1410

Nonstandard Abbreviations: TBP, TNF binding protein; Erk, Extracellular signal-regulated kinase; PI3K, Phosphatidylinositol 3-kinase; GM-CSF, granulocyte monocyte colony stimulating factor; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; HPRT, hypoxanthine phosphoribosyltransferase; ICAM-1, intracellular adhesion molecule-1.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Tumor Necrosis Factor-alpha (TNF-α) is central to the endometriotic disease process. TNF-α receptor signaling regulates epithelial cell secretion of inflammation and invasion mediators. Since epithelial cells are a disease-inducing component of the endometriotic lesion, we explored the response of 12Z immortalized human epithelial endometriotic cells to TNF-α. This report reveals the impact of disruption of established TNF-α induced signaling cascades on the expression of biomarkers of inflammation and EMT (Epithelial-Mesenchymal Transition) from endometriotic epithelial cells. Importantly, we show the molecular potential of sTNF-R1 (TBP) and a panel of small molecule kinase inhibitors to block endometriotic gene expression, directly. TNF-α receptor is demonstrated to signal through IKK2>IκB>NFκB, Erk>MEK, p38, and PI3K>Akt1/2. TNF-α induces the expression of transcripts for inflammatory mediators IL-6, IL-8, RANTES, TNF-α, GMCSF, MCP-1 and also invasion mediators MMP-7, MMP-9, and ICAM-1. Indeed, TBP inhibits the TNF-α induced expression of all the above endometriotic genes in 12Z endometriotic epithelial cells. The secretion of IL-6, IL-8, GMCSF, and MCP-1 by TNF-α is blocked by TBP. Interestingly, MEK, p38, and IKK inhibitors block TNF-α induced IL-8, IL-6 and GM-CSF secretion and 12z invasion while the PI3K inhibitors does not. The only inhibitor to block MCP-1 expression is the p38 inhibitor. Lastly, TBP, MEK inhibitor, or p38 inhibitor also block cell surface expression of N-cadherin, a marker of mesenchymal cells. Taken together these results demonstrate, that interruption of TNF-α induced signaling pathways in human endometriotic epithelial cells results in decreased expression and secretion of biomarkers for inflammation, EMT, and disease progression.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Endometriosis is a female disease presenting with painful and persistent lesions within the peritoneum. Retrograde menstruation of the endometrial cells into the peritoneal cavity cause endometriosis in ten percent of all women (Sampson, 1927). For the majority of women the immune response in the peritoneum that follows is sufficient to suppress attachment and transformation of refluxed tissue. In those women that develop the disease, endometrial cells become attached to the mesothelial cell layer lining the peritoneal cavity, and initiate a cascade of events that includes localized invasion and transformation of cells within the lesion (Sampson, 1927).

In endometriosis, activated peritoneal leukocytes responding to ectopic menstrual effluent secrete TNF-α, which elicits inflammatory and necrotic gene expression from epithelial cells (Szyllo *et al.*, 2003;Braun *et al.*, 2002). TNF-α mRNA is upregulated in both the endometrium and peritoneum of women with endometriosis when compared to normal women (Kyama *et al.*, 2006). Importantly, TNF-α receptors are expressed by endometrial cells in women with endometriosis (Kharfi *et al.*, 2003). Therefore both TNF-α and its receptor are expressed in the epithelial endometriotic cells.

Experimental evidence supporting a critical regulatory role of inflammation, and particularly TNF-α, in endometriosis is strong (Berkkanoglu and Arici, 2003). A targeted approach to controlling TNF-α driven inflammation in endometriosis was achieved in the rat model with administration of TNF-α soluble receptor (D'Antonio *et al.*, 2000). Two independent results of use of soluble TNF-α receptor in baboon models of endometriosis indicate that therapies targeting the TNF-α mediated inflammatory cascade have potential to treat endometriosis (D'Hooghe *et al.*, 2006;Barrier *et al.*, 2004). These in-vivo efficacy studies in animal models

of endometriosis further validate the importance of inhibiting TNF-α receptor signaling in treatment of endometriosis.

TNF-α mediates inflammation in the surrounding tissue, in part, by inducing the expression of IL-6, IL-8, GM-CSF, and MCP-1. Epithelial cells are a major source of these cytokines in the human endometrium (Giacomini *et al.*, 1995;Meter *et al.*, 2005;Fahey *et al.*, 2005). IL-6 is a chemoattractant for monocytes while IL-8 activates angiogenisis and neutrophil migration and differentiation. GM-CSF stimulates granulocyte and monocyte differentiation from haematopoietic stem cells (Hamilton and Anderson, 2004). MCP-1 mediates both acute and chronic inflammation through recruitment of mast cells, eosinophils, and macrophages to the site of inflammation (Conti and DiGioacchino, 2001). These inflammatory innate immune cells induce an inflammatory and EMT responses in surrounding epithelial cells.

Endometriotic epithelial cells have also been shown to express mesenchymal markers (N-cadherin) amidst diminishing levels of epithelial markers (E-cadherin); further supporting that a population of endometriotic cells undergo the dedifferentiation process of EMT (epithelial-mesenchymal transition) (Gaetje *et al.*, 1997). Endometriotic lesions express aberrant or elevated levels of matrix metalloproteinases (MMPs) (Osteen *et al.*, 2003). TNF-α induces the expression of MMP-9 from endometrial cells (Curry, Jr. and Osteen, 2003). Interestingly, active MMP-9 is increased in the eutopic and ectopic endometrium of women with endometriosis when compared with normal endometrium (Liu *et al.*, 2002).

Absence of good cell model for endometriosis has hindered development of therapies that target lesions preferentially over eutopic endometrium. Recently, a set of immortalized endometriotic epithelial cells and stromal cells have been developed and shown to exhibit many characteristics of primary epithelial and stromal endometriotic cells (Zeitvogel *et al.*, 2001). The availability of these cells has enabled investigation of direct pharmacologic

responses of isolated endometriotic epithelial and stromal cells. The remarkable change that occurs in epithelial endometriotic cells compared to stromal endometriotic cells suggests that a major contribution to the disease occurs within epithelial cells (Banu *et al.*, 2007b).

We report here the ability of TNF-α binding protein (TBP; TNF-R1) and kinase inhibitors to reverse TNF-α induced and/or TNF-α independent effects on 12Z endometriotic epithelial cells. TBP competes with TNF-α receptor for TNF-α binding to the receptor, and so is used as a positive control to attribute the direct inhibitory effects on specific signaling pathways within endometriotic epithelial cells. TNF-α is shown to stimulate production of endometriotic biomarkers from 12Z cells similar to those within the peritoneal cavity of women with Endometriosis. TBP and novel kinase inhibitors reverse the effects of TNF-α induced expression of cellular adhesion markers and MMPs that define mesenchymal endometriotic cells. Lastly, kinase inhibitors also cause reversion of the invasive phenotype. These studies clearly link TNF-α with maintenance of the inflammatory and mesenchymal endometriotic properties. These results could lead to development of drugs for endometriosis that target signaling pathways uniquely modified in ectopic lesions over eutopic endometrium.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Cell Culture, Cytokines, and Inhibitors

The SV40 T-antigen-transformed human ectopic endometrial epithelial cell line 12Z was maintained in DMEM/F12 medium supplemented with 10% fetal calf serum, and penicillin-streptomycin at 37°C and 5% CO_2 (Zeitvogel *et al.*, 2001). Cells were passaged at 75% confluence in T-150 culture flasks to a 1:50 dilution. For stimulation experiments, cells (20,000/well) were seeded in 96-well plates; the following day cells were washed once with PBS and serum free media added. Stimulation with various factors was carried out in serum free media for 24hr. Culture supernatant was stored at –80°C until used for determination of cytokine levels. For blocking signaling pathways, cells were incubated with inhibitors for 30 minutes prior to the addition of TNF-α. Culture media, antibiotics and serum were obtained from Invitrogen (Carlsbad, CA). TNF-α was purchased from R&D Systems. The MEK inhibitor PD98059, the p38 inhibitor SB203580, and the PI3K inhibitor Wortmannin were purchased from BIOMOL Research Labs, Inc. The IKK2 inhibitor (AS602868) was synthesized at Serono (Frelin *et al.,* 2003). TNF-Binding Protein (TBP/Onercept) was produced at Serono and was used at 100 μg/ml (McKenna *et al.,* 2007;D'Antonio *et al.,* 2000).

Antibodies

Phospho- Akt, Erk, IκB, NFκB, p38 and unphosphorylated Erk and NFκB were purchased from Cell Signaling. Akt1/2, IκB-a, p38 and immunoflorescence antibodies for E-cadherin and N-cadherin were purchased from Santa Cruz Biotechnology. Goat Anti-Human IgG (gamma)-HRP Conjugate used as secondary antibody was obtained from Bio-Rad Laboratories.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Western Blot

For production of cell lysate one million treated cells were lysed in 1 ml RIPA buffer with 250nM NaOVan with 1/10 of a tablet of Protease Inhibitors(Complete Mini, Roche). Protein concentration was determined by BCA Assay according to the manufacturers protocol (Biorad). 30µg of protein was loaded per well of a 10% Tris Bis Gel in a Xcell Sure Lock System (Invitrogen). Gels were run at 120V until the dye front was at the bottom. Proteins were tranfered from gels to Immobilon Membrane (Millipore) at 25 volts for 1.5 hours. The membranes were then incubated overnight at 4°C with primary antibody in 5% Milk/TBST/0.02% NaAz at the manufacturor recommended dilution while rocking in a square petri dish. Membranes were washed in TBST three times for five minutes and then incubated while rocking slowly at room temperature for 1 hour in a 1:5000 dilution of HRP conjugated secondary antibody in 10ml of TBST. The membranes were washed, as above, and were placed on glass and semidried by gentle blotting with a Kimwipe. 1ml ECL/Luminol (Santa Cruz) was added to the membrane, left for 1 minute and then removed. Membranes were semidried and covered with plastic wrap to prevent complete drying. The plastic covered semidry membranes were exposed to film in the darkroom for 10 seconds to 1 minute and developed. The resultant film was scanned for images.

Quantification of phosphoproteins and secreted cytokines by Electrochemiluminescence

Electrochemiluminescence assays were performed on biological triplicate samples using capture antibody precoated 96-well Multispot™ plates from Meso Scale Discovery (MSD). 25µl of supernatant or calibrator was added to each well and incubated with shaking for one hour at room temperature. Specific protein levels were quantitated by adding 25µl of 1µg/ml of specific detection antibody labeled with MSD SULFO-TAG™ reagent to each well and incubated with shaking for one hour at room temperature. The plate was then washed 3 times with PBS/0.05% Tween-20 and 150µl of 2X read buffer was added to each well. Plates were

immediately read using the SECTOR Imager 6000 and data quantitated using Discovery Workbench and SOFTmax PRO 4.0 software.

Quantitative Real Time-PCR

RNA was isolated from 12Z cells on a 60mm cell culture dish using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase I (Qiagen) and purity of RNA increased using an RNeasy kit (Qiagen) before cDNA synthesis. One microgram of total RNA was reverse transcribed using Oligo DT priming and the Superscript™ III First-strand cDNA synthesis kit (Invitrogen). RT-minus samples served as a control to exclude the possibility that the amplified product was derived from contaminating undigested genomic DNA. CDNA corresponding to 100 ng of input RNA was amplified in duplicate with the TaqMan Universal PCR Master Mix on a custom Taqman Low Density Array (Applied Biosystems, Foster City CA). Real-time PCR was conducted with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Differential target gene expression was calculated according to the $2^{-\Delta\Delta C_T}$ method using HPRT as an endogenous control (Fleige *et al.*, 2006). Interarray reproducibility was proven by repeated measurements of control cDNA samples on three different arrays. C_T values were approximately normally distributed. Target gene CT values were normalized to the mean of HPRT values. P-values were computed by nonparametric one-way ANOVA with a 95% confidence interval and Tukey post-test using Sigmastat software.

Immunofluorescence

Following treatment, monolayers of 12Z cells in 96-well plates were washed with PBS, 30µl of 10% Formalin/PBS was added, and incubated for one hour at room temperature. After incubation the cells were washed three times in PBS. To permeabilize and block, the cells were treated overnight at 4°C in 0.2% Tween 20 / 0.1% BSA / PBS. Permeabilization and blocking solution was aspirated, replaced with a 1:100 dilution of primary antibody in 0.2% Tween 20 / 0.1% BSA / PBS, and incubated overnight at 4°C. Cells were then washed 5

times in PBS. A 1:1000 dilution of secondary antibody in 0.2% Tween 20 / 0.1% BSA / PBS was added and incubated for one hour at room temperature. After incubation cells were washed and flourescence visualized on a Nikon Eclipse TE 2000-S.

Invasion Assay

Cells were cultured at not greater than 70% confluence. Cells were serum starved in 0% serum media for 5 hours. At 2.5hrs of serum starvation the 24-well invasion plate (Becton Dickinson, 08-774-122) was removed from -20°C storage and allowed to come to room temperature for 30 minutes. Once the plate was warmed, 500 µL of 37°C DMEM was added to each apical chamber. The plate was allowed to rehydrate for 2 hours at 37°C in a 5% CO_2 environment. After 5 hours of serum starvation, cells were trypsinized and the cell concentration adjusted to $4e^4$/500ul in serum free media. The DMEM medium was carefully removed from apical chambers of rehydrated plates without disturbing the layer of BD Matrigel™ Matrix on the membrane. Media containing chemoattractant (10% serum) or no chemoattractant (no serum) was added to each basal chamber. Membranes were inserted into wells making sure no air bubbles were trapped under the membrane. 500 µL of cell suspension ($4e^4$ cells) was added to the apical chambers. The BD BioCoat™ Tumor Invasion System was then incubated for 24 hours at 37°C, 5% CO_2. Following incubation, medium was carefully removed from the apical and basal chambers by inverting the plates and gently dabbing plates on absorbent paper. All non-invading cells were removed from the Matrigel membrane using a cotton swab. The invasive cells on the lower surface were stained using a Diff-Quick kit (Dade Behring, Newark De). 500 µL of each of the three Diff-Quick solutions was added in succession to each well, membranes were incubated for 2 minutes with each solution, aspirating between each solution. The membranes were then washed two times with water. The number of invasive cells (purple nuclei, pink cytoplasm) in each membrane was counted with a disecting microscope.

Statistics

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Data were analyzed by ANOVA followed by Tukey tests (Sigmastat, VA). Differences between groups were considered significant when $P < 0.05$.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

TNF-α signals through the, IκB/NFκB, MEK/Erk, p38, and PI3K/Akt kinase cascade in Epithelial Endometriotic cells

In this first set of experiments 12Z cells were validated as a model system to study TNF-α receptor regulation of kinase signaling in endometriotic cells in vitro. The 12Z cell line is immortalized, inherently endometriotic, and invasive (Zeitvogel *et al.*, 2001). To measure the ability of TNF-α to signal through various kinase cascades recombinant TNF-α receptor (TBP) was used to block the binding of endogenous or exogenous TNF-α to the TNF-α receptor. Specific intracellular kinase signaling pathways downstream of the receptor were blocked by the use of small molecule inhibitors. The Western blot in figure 1a shows that TNF-α receptor phosphorylates(ser32/36) and degrades the IκB (NFκB inhibitor), and subsequently phosphoryles NFκB (ser536) whereas TBP and an IKK2 inhibitor block this change. TNF-α also induces kinase signaling through MEK to Erk (Thr202/Tyr204) and through p38 (Thr180/Thyr 182). Indeed, a MEK inhibitor blocks the TNF-α induced phosphorylation of MEKs target Erk and not p38 phosphorylation (Figure 1b).

Multiplexed electrochemoluminescent assays were used to further confirm and expand on the TNF-α induced kinase signaling pathways in 12Z epithelial endometriotic cells seen by Western Blot. TBP specifically blocks TNF-α induced phosphorylation of p-38 (Thr421/Tyr182), Akt (Ser473), NFκB (Ser468), and Erk (Thr202/Tyr204 and Tyr185/Thr187) (Figure 2a-d). TBP completely inhibits the phosphorylation of NFκB by the peak of activity at 5 minutes (Figure 2c). In comparison to TBP, , the PI3K inhibitor Wortmannin completely inhibits the TNF-α induced phosphorylation of Akt while TBP reduces the phosphorylation after the initial burst in P-Akt (Figure 2b). The MEK inhibitor PD98059 and TBP significantly reduce the phosphorylation of Erk at 15 minutes post

treatment (Figure 2d). These data demonstrate a direct effect of TNF-α on 12Z endometriotic cells and antagonism of TNF-α receptor signaling in epithelial endometriotic cells by TNF-α-Binding Protein. Small molecule kinase inhibitors specifically block their enzymatic targets in 12Z cells. Taken together these data show that the TNF-α receptor signals through a known array of kinase pathways.

TBP and Kinase Inhibitors block transcription of inflammatory cytokines involved in Endometriosis.

The next aim was to determine if 12Z cells respond to TNF-α in a manner consistent with its proposed pro-inflammatory role. In this set of experiments the effects of TNF-α on inflammatory cytokine mRNA expression was evaluated by qPCR with and without kinase inhibitors or TBP. As shown in figure 3a-f; IL-8, IL-6, MCP-1, GM-CSF, RANTES, and TNF-α mRNA expression was increased 40-900 fold upon addition of TNF-α. TBP blocked the induction of all 6 cytokines while PI3K inhibitor did not. Expression of IL-6, MCP-1, and GM-CSF mRNA were reduced to basal levels by MEK, p38, and IKK2 inhibitors (Figure 3 B and D). Both RANTES and TNF-α mRNA were reduced by p38 and IKK2, but not by the MEK inhibitor (Figure 3 e & F), while IL-8 mRNA was reduced by MEK and IKK2 inhibitor but not by p38 inhibitor (Figure 3a). Expression of mRNA for ICAM-1 by endometriotic (12Z) was increased by TNF-α (Fig 3c), consistent with previous observations in primary endometriotic tissues (Gonzalez-Ramos *et al.*, 2007). Although TBP inhibited expression of TNF-stimulated ICAM-1 mRNA to untreated levels, the IKK2 inhibitor alone was capable of reducing ICAM-1 mRNA. This is consistent with previous studies that demonstrate TNF-α signaling through IKK2 and NFκB regulates ICAM-1 expression and monocyte adhesion (Chen *et al.*, 2001).

TBP inhibits TNF-α induced IL-8, IL-6, GM-CSF, and MCP-1 Secretion.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

To determine if the increases in mRNA levels of IL-8, IL-6, GMCSF, and MCP-1 also result in increased protein secretion, 12Z cells were treated with different doses of TNF-α (0.01 to 100 ng/ml) for 24 hours. Cytokine secretion in the culture supernatant was evaluated by multiplex electrochemiluminescence assays. As shown in figure 4a-d; TNF-α stimulated dose-dependent secretion of IL-6, IL-8, GM-CSF, and MCP-1 in 12Z culture supernatant. To evaluate the ability of TBP to reverse the effects of TNF-α on cytokine production, increasing concentrations of TBP were added to cultures containing 15 ng/ml TNF-α. Following 24 hours incubation in the presence of 15 ng/ml TNF-α;GMCSF, IL-6, MCP-1, and IL-8 secreted by 12Z cells were elevated by 200, 1000, 900, and 32-fold over basal respectively (Fig. 4). Addition of TBP to cell cultures in the absence of added TNF-α did not cause a significant change on cytokine levels. As expected, TBP dose-dependently reduced TNFα-mediated increase in cytokines when added to TNF-α treated 12Z cultures. At a concentration of 10ug/ml, TBP reduced the secretion of IL-8, IL-6 and GMCSF to the baseline, while to inhibit MCP-1 production a 10-fold higher concentration of TBP was required (Fig. 4c). These results confirm that 12Z cells produce very little, if any TNF-α in culture, and that TBP effectively abolishes the direct effect of exogenous TNF-α on endometriotic cells.

Kinase Inhibitors differentially effect the secretion of IL-8, IL-6, GMCSF and MCP-1.

In an effort to develop orally bioavailable therapies for endometriosis we have evaluated the effects of known and novel inhibitors of intracellular signaling pathways reported to be involved in TNF-α receptor function. In the experiments reported here we present the effects of kinase inhibitors on cytokine protein expression. As shown in Figure 5 A-D, TNF-α stimulation increased IL-6, IL-8, GM-CSF, and MCP-1 secretion, and kinase inhibitors tested had specific effects on each of the cytokines. PI3K inhibitor did not block secretion of any of the cytokines. MEK, p38, and IKK2 inhibitors blocked GM-CSF and IL-6 secretion from endometriotic epithelial cells (Figure 5a & b), and only MEK and p38 inhibitors blocked IL-8 and MCP-1 ,respectively. These results suggest that the IKK, MEK, and p38

pathways and not the PI3K pathway regulate TNF-α stimulated GM-CSF, IL-6, IL-8, and MCP-1 secretion in endometriotic epithelial cells. Interestingly, the TNF-α induced levels of IL-8 or MCP-1 protein were not affected by p38 and IKK2 inhibitor or by MEK and IKK2 inhibitor respectively, although the RNA levels byof these cytokines were reduced by these inhibitors. The RNA levels were not induced in the presence of inhibitor and TNF-α, but the protein levels were. It is possible that protein degradation or translation rates are not blocked but the RNA degradation or transcription rates are affected.

TBP and Kinase Inhibitors block transcription of extracellular matrix remodeling mediators involved in endometriosis.

To validate the direct effects of TNF-α and inhibitors on expression of genes involved in EMT of endometriotic cells, 12Z cells were exposed to TNF-α in the presence or absence of TBP. TNF-α caused a dramtic increase in expression of MMP-7 and MMP-9 mRNA. Levels of MMP-7 and MMP-9 mRNA were reduced 6 and 300 fold respectively in the presence of TBP as measured by qPCR (Fig. 6a). MMP-7 mRNA levels were not significantly effected by the panel of kinase inhibitors while MMP-9 mRNA levels were reduced by MEK, P38, and IKK2 but not PI3K inhibitor. Indeed the expression of MMPs is indicative of the EMT process. These results validate that 12Z cells serve as an appropriate in vitro model to evaluate effects of inflammatory cytokines and their inhibitors on invasive and inflammatory gene expression in endometriotic cells.

Inhibition of MEK, p38, or IKK reduces invasion of human endometriotic epithelial cells (12Z)

12Z cells are inherently invasive as endometriotic epithelial cells (Zeitvogel *et al.*, 2001). Addition of TNF-α alone (serum-free conditions) was unable to increase the inherent invasive activity of 12Z cells. Addition of TNF-α moderately increased serum-induced invasion, although neither the effect of TNF-α nor the addition of TBP significantly affected 12Z

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

invasion. However, TNF-α and serum-induced invasion was significantly reduced by inhibiting MEK, p38, and IKK signaling, but not by inhibiting PI3K signaling (Figure 7). PI3K is involved in TNF-α induced cell survival while the IKK>IκB>NFκB, p38, and ERK>MEK pathways induce invasiveness of cells. While TNF- α didn't increase the number of invasive epithelial cells it was considered that the invasive potential of 12Z cells that express elevated levels of MMP-2 and MMP-9 could be dependent on changes in cell-cell adhesion that are not observed in invasion assays. It was considered that TNF-α might be responsible for reducing cell-cell adhesion of 12Z cells and endometriotic cells by modifying N-cadherin expression .

TBP, MEK inhibitor, and p38 inhibitor block TNF-α induced N-cadherin expression

A shift from expression of E-cadherin to N-cadherin is a proven marker for the cellular shift from epithelial to mesenchymal phenotype. Previously 12Z cells were characterized to express N-cadherin over E-cadherin . To investigate whether TNF-α receptor signaling altered their cellular phenotype, 12Z cells were immunostained for N-cadherin, E-cadherin and cytokeratin after treatment with TNF-α with and without TBP or kinase inhibitors. Nuclear stainingwith Hoechst was used to monitor changes in cell numbers. E-cadherin was not detected in 12Z culture, but was detected in cell line controls (data not shown). As demonstrated earlier, 12Z cells normally express low levels of N-cadherin at cellular junctions and in the cytoplasm (Fig 8A). 12Z cells express significantly higher levels of N-cadherin at cellular junctions and in the cytoplasm after 24-hours of treatment with TNF-α (Fig 8B). Addition of 100 μg/ml TBP with TNF-α (15 ng/ml) significantly reduced N-cadherin expression (Fig. 8C). Interestingly, exogenous MEK or p38 inhibitor significantly blocked TNF-α induced N-cadherin expression (Fig. 8D & E) while PI3K and IKK2 inhibitors did not reduce N-cadherin staining (Data not shown). E-cadherin expression was still undetectable with any treatment (data not shown). Taken together these data indicate that TNF-α promotes invasive potential of endometriotic cells while treatment of endometriotic

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

epithelial cells with TBP, MEK inhibitor, or p38 inhibitor lowers the expression of mesenchymal markers of the endometriotic phenotype and reverts the cells to an expected phenotype for normal endometrial epithelial cells.

DISCUSSION

The findings presented here indicate that MEK>Erk, p38, and IKK2>IκB>NFκB phosphorylation of downstream targets of TNF-α receptor results in the regulation of inflammation and invasion mediators independent of PI3K>AKT. Indeed microarray analysis of eutopic endometrium identified up-regulation of several genes in two important signaling pathways: RAS/RAF/MAPK and PI3K in patients with endometriosis vs. controls (Matsuzaki *et al.*, 2005). Our data shows that interruption on PI3K signaling does not significantly affect the presented biomarkers of the epithelial endometriotic phenotype. While the design of these studies was limited to the use of kinase inhibitors which restricts the breadth of interpretations of these results, the concentration of these inhibitors used was selected to minimize the effect of the compound on off-target pathways. Nevertheless, the clinical benefit of TBP, etanercept, and infliximab on experimental endometriosis in baboons confirms the relevance of interrupting TNF-α signaling as a means of disease treatment. The model in figure 9 depicts a simplified diagram of TNF-α signaling through kinase pathways and inducing the expression of proteins involved in inflammation and invasion in endometriosis.

Evaluation of novel therapies for treatment of endometriosis has been difficult because of the difficulty of the cellular and animal models for this disease. Following their initial description several years ago, 12Z cells have been increasingly used by investigators to model cellular pathophysiology of endometriosis because in many ways they recapitulate the physiology of endometriotic epithelial cells. 12Z cells demonstrate some similar attributes as primary endometriotic cells (Zeitvogel *et al.*, 2001) and also properties similar to fibroblast-derived endometrial stromal cells (Banu *et al.*, 2007b). 12Z cells and endometriotic cells express high levels of COX-2 and PGE2 that are associated with the pain of endometriosis (Banu *et al.*, 2007a). Moreover, TNF-α caused methylation of the progesterone receptor

promoter in 12Z cells in a manner that resembles primary endometriotic cells (Wu *et al.*, 2006;Wu *et al.*, 2007a). 12z cells were prepared from red lesions of AFS stage I-II endometriosis. Early stage I and II endometriotic lesions have also been described as the more invasive cell type compared to cells from later stage III – IV endometriotic lesions. Inhibiting TNF signaling at stage I or II has been proposed to be potentially more effective strategy than at later stages of disease (AFS stage III – IV). Previous studies demonstrated that 12Z cells possessed similar invasive properties as primary endometriotic cells, and that this invasiveness correlated with expression of N-cadherin in greater amounts than E-cadherin (Zeitvogel *et al.*, 2001). These previous studies suggested that 12Z cells offered a unique opportunity to investigate processes of endometriosis disease progression with a specific focus on endometriotic epithelial cells and how the molecular endometriotic disease phenotype can be pharmacologically treated.

The therapeutic potential of rhTNFR (TBP-1, Onercept; Enbrel; and infliximab [c5N]) has previously been demonstrated in primate models of endometriosis (D'Hooghe *et al.*, 2006;Barrier *et al.*, 2004;Falconer *et al.*, 2006). The therapeutic activity of these molecules in vivo comprises responses of the immune system as well as responses specific for endometrial cells. Detailed analysis of the effects of pro-inflammatory cytokines on endometrial components of endometriosis lesions has been hampered in the past by the availability to lesions, and the ability to separate cellular fractions of lesions. This is the first study to investigate cellular mechanisms of TNF-α neutralization or inhibition specific for endometriotic epithelial cells using a previously characterized endometriotic epithelial cell line (12Z cells).

Peritoneal fluid from women with endometriosis has been found to contain elevated levels of IL-6, IL-8, and TNF-α. Primary cultures of endometriotic epithelial cells have been shown to

produce IL-6, IL-8, and TNF-α (Luk *et al.*, 2005;Bergqvist *et al.*, 2000). MCP-1 and RANTES have also been found elevated in peritoneal fluid of women with endometriosis, and previously their production has been reported to originate from stromal cells (Bersinger *et al.*, 2006;Kalu *et al.*, 2007). Results from our studies clearly demonstrate an effect of TNF-α, a primary effector of inflammatory responses, to increase production of IL-6, IL-8, MCP-1, and GM-CSF from 12Z epithelial endometriotic cells. Furthermore, our results confirm that neutralization of the effect of TNF-α with TBP suppresses production of these cytokines. Interestingly various kinase inhibitors effect various biomarkers of endometriosis. TBP followed by MEKi and then p38i has the most significant normalization to a non-inflammatory and non-mesenchymal molecular and cellular phenotype.

The studies presented here demonstrate that cytokines commonly found in the peritoneal cavity of endometriotic patients (IL-6, IL-8, GM-CSF, and MCP-1) were also secreted by 12Z cells stimulated with TNF-α. Indeed, TBP completely reversed the effects of TNF-α on IL-6, IL-8 and GM-CSF production from 12Z cells, while inhibition of the TNF-α response by compounds that block downstream kinase signaling from TNF-α receptor demonstrated pathway specific patterns of inhibition. Expression of mRNAs for cytokines and cellular adhesion markers that are indicative of an inflammatory and mesenchymal phenotype were increased by the presence of TNF-α and differentially decreased in the presence of inhibitors. MEKi blocks IL-6, IL-8, and MCP-1 while p38i blocks IL-6, GM-CSF, and MCP-1. Inhibition of IKK2 inhibited all four cytokines placing IKK2>IκB>NFκB signaling as a key pathway of TNF-α signaling to inflammatory cytokine secretion from endometriotic epithelial cells.

Endometrial epithelial cells normally secrete MMPs during normal endometrial breakdown during menstruation. The pathogenesis of endometriosis is partially due to secretion of ectopic endometrial cells into the peritoneum. It has been well established in cellular and animal models that MMPs are expressed at higher levels in cycle-matched samples of ectopic

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

endometrium than in eutopic endometrium (Zhou and Nothnick, 2005). TNF-α has been shown to induce epithelial-mesenchymal transformation of several cell types, and long-term exposure to TNF-α leads to unrecoverable transformation (Chaudhuri *et al.*, 2007). In the present study, it is presumed that 12Z cells have already undergone some aspects of epithelial-mesenchymal transformation, and others have been induced experimentally by short-term exposure to TNF-α. TBP and inhibitors of TNF-α signaling caused reversion of the mesenchymal endometriotic phenotype of the cells as measured by changes in MMP and N-cadherin expression. In the case of MMP expression, MMP-7 and MMP-9 expression were induced by TNF-α. MMP-7 expression was reduced by TBP and marginally affected by p38i, while MMP-9 was also dramatically blocked by TBP, p38-, IKK2- inhibitors and to a lesser extent by MEK inhibitor. The redundancy of the TNF-α induced kinase signaling to MMP-9 indicates MMP-9 could be important for the pathogenesis of epithelial endometriotic cells.

Expression of cellular adhesion molecules cytokeratin, E-cadherin and N-cadherin has previously been used to distinguish endometriotic epithelial cells from normal endometrial cells, and as well 12Z endometriotic cells from eutopic endometrium (Gaetje *et al.*, 1997). In the present experiments we measured the ability of TNF-α to exacerbate the endometriotic phenotype of these cells. Addition of TNF-α caused elevation of N-cadherin. Neutralization of the TNF-α effect with TBP reversed the change in cadherin expression to a less mesenchymal phenotype (lower expression of N-cadherin) previously observed in endometrial cells rather than endometriotic cells. These results suggest there may be a direct beneficial effect of TBP or specific kinase inhibitors, during treatment for endometriosis on reversion of an endometriotic phenotype.

The lack of E-cadherin and increased expression of N-cadherin marks an epithelial-mesenchymal transition with loss of adherins junctions. Decreased expression of E-cadherin

in epithelial cells was found in peritoneal endometriosis as compared with normal endometrium (Poncelet *et al.*, 2002). It has been proposed that E-cadherin negative invasive endometriotic cells represent the cell population that causes endometriosis in-vivo (Gaetje *et al.*, 1997). These previous studies examing cadherin expression in eutopic and ectopic tissues support our findings with TNF-α signaling and N-cadherin expression. Consistent with our findings, additional invasion promoting factors shrew-1 and CD147 are found elevated in endometriotic cells and in 12Z cells relative to eutopic endometrium (Schreiner *et al.*, 2007;Bharti *et al.*, 2004). Treatment of the immortalized endometriotic cells with trichstatin A, a histone deactelase inhibitor reduces the invasivenss ans reactivate E-cadherin expression in these cells (Wu *et al.*, 2007b).

The present study highlights various signaling pathways modulating TNF-α-mediated effects in human endometriotic cell line. These results, though obtained from immortalized endometriotic cells but not primary cells, provide us evidence to explore the use of specific kinase inhibitors to treat endometriosis. Clinically, a preferred therapeutic would balance the pharmacologic consequences of broad TNF-α antagonism by neutralizing agents, with selective agents that target specifically sites of inflammation in endometriotic lesions. Future studies will focus on inhibition of preferred kinase targets in animal models of endometriosis to further validate these pathways for developing novel therapeutics in human.

REFERENCES

Banu SK, Lee J, Speights V O, Jr., Starzinski-Powitz A and Arosh J A (2007a) Cyclooxygenase-2 regulates survival, migration and invasion of human endometriotic cells through multiple mechanisms. *Endocrinology*. (In press)

Banu SK, Lee J, Starzinski-Powitz A and Arosh J A (2007b) Gene Expression Profiles and Functional Characterization of Human Immortalized Endometriotic Epithelial and Stromal Cells. *Fertil Steril*. (In press)

Barrier BF, Bates G W, Leland M M, Leach D A, Robinson R D and Propst A M (2004) Efficacy of Anti-Tumor Necrosis Factor Therapy in the Treatment of Spontaneous Endometriosis in Baboons. *Fertil Steril* **81 Suppl 1**: 775-779.

Bergqvist A, Nejaty H, Froysa B, Bruse C, Carlberg M, Sjoblom P and Soder O (2000) Production of Interleukins 1beta, 6 and 8 and Tumor Necrosis Factor Alpha in Separated and Cultured Endometrial and Endometriotic Stromal and Epithelial Cells. *Gynecol Obstet Invest* **50**: 1-6.

Berkkanoglu M and Arici A (2003) Immunology and Endometriosis. *Am J Reprod Immunol* **50**: 48-59.

Bersinger NA, von Roten S, Wunder D M, Raio L, Dreher E and Mueller M D (2006) PAPP-A and Osteoprotegerin, Together With Interleukin-8 and RANTES, Are Elevated in the Peritoneal Fluid of Women With Endometriosis. *Am J Obstet Gynecol* **195**:103-108.

Bharti S, Handrow-Metzmacher H, Zickenheiner S, Zeitvogel A, Baumann R and Starzinski-Powitz A (2004) Novel Membrane Protein Shrew-1 Targets to Cadherin-Mediated Junctions in Polarized Epithelial Cells. *Mol Biol Cell* **15**:397-406.

Braun DP, Ding J and Dmowski W P (2002) Peritoneal Fluid-Mediated Enhancement of Eutopic and Ectopic Endometrial Cell Proliferation Is Dependent on Tumor Necrosis Factor-Alpha in Women With Endometriosis. *Fertil Steril* **78**:727-732.

Chaudhuri V, Zhou L and Karasek M (2007) Inflammatory Cytokines Induce the Transformation of Human Dermal Microvascular Endothelial Cells into Myofibroblasts: a Potential Role in Skin Fibrogenesis. *J Cutan Pathol* **34**:146-153.

Chen C, Chou C, Sun Y and Huang W (2001) Tumor Necrosis Factor Alpha-Induced Activation of Downstream NF-KappaB Site of the Promoter Mediates Epithelial ICAM-1 Expression and Monocyte Adhesion. Involvement of PKCalpha, Tyrosine Kinase, and IKK2, but Not MAPKs, Pathway. *Cell Signal* **13**:543-553.

Conti P and DiGioacchino M (2001) MCP-1 and RANTES Are Mediators of Acute and Chronic Inflammation. *Allergy Asthma Proc* **22**:133-137.

Curry TE, Jr. and Osteen K G (2003) The Matrix Metalloproteinase System: Changes, Regulation, and Impact Throughout the Ovarian and Uterine Reproductive Cycle. *Endocr Rev* **24**:428-465.

D'Antonio M, Martelli F, Peano S, Papoian R and Borrelli F (2000) Ability of Recombinant Human TNF Binding Protein-1 (r-HTBP-1) to Inhibit the Development of Experimentally-Induced Endometriosis in Rats. *J Reprod Immunol* **48**:81-98.

D'Hooghe TM, Nugent N P, Cuneo S, Chai D C, Deer F, Debrock S, Kyama C M, Mihalyi A and Mwenda J M (2006) Recombinant Human TNFRSF1A (r-HTBP1) Inhibits the Development of Endometriosis in Baboons: a Prospective, Randomized, Placebo- and Drug-Controlled Study. *Biol Reprod* **74**:131-136.

Fahey JV, Schaefer T M, Channon J Y and Wira C R (2005) Secretion of Cytokines and Chemokines by Polarized Human Epithelial Cells From the Female Reproductive Tract. *Hum Reprod* **20**:1439-1446.

Falconer H, Mwenda J M, Chai D C, Wagner C, Song X Y, Mihalyi A, Simsa P, Kyama C, Cornillie F J, Bergqvist A, Fried G and D'Hooghe T M (2006) Treatment With Anti-TNF Monoclonal Antibody (C5N) Reduces the Extent of Induced Endometriosis in the Baboon. *Hum Reprod* **21**:1856-1862.

Fleige S, Walf V, Huch S, Prgomet C, Sehm J and Pfaffl M W (2006) Comparison of Relative MRNA Quantification Models and the Impact of RNA Integrity in Quantitative Real-Time RT-PCR. *Biotechnol Lett* **28**:1601-1613.

Frelin C, Imbert V, Griessinger E, Loubat A, Dreano M and Peyron J F (2003) AS602868, a Pharmacological Inhibitor of IKK2, Reveals the Apoptotic Potential of TNF-Alpha in Jurkat Leukemic Cells. *Oncogene* **22**: 8187-8194.

Gaetje R, Kotzian S, Herrmann G, Baumann R and Starzinski-Powitz A (1997) Nonmalignant Epithelial Cells, Potentially Invasive in Human Endometriosis, Lack the Tumor Suppressor Molecule E-Cadherin. *Am J Pathol* **150**: 461-467.

Giacomini G, Tabibzadeh S S, Satyaswaroop P G, Bonsi L, Vitale L, Bagnara G P, Strippoli P and Jasonni V M (1995) Epithelial Cells Are the Major Source of Biologically Active Granulocyte Macrophage Colony-Stimulating Factor in Human Endometrium. *Hum Reprod* **10**:3259-3263.

Gonzalez-Ramos R, Donnez J, Defrere S, Leclercq I, Squifflet J, Lousse J C and Van Langendonckt A (2007) Nuclear Factor-Kappa B Is Constitutively Activated in Peritoneal Endometriosis. *Mol Hum Reprod* **13**:503-509.

Hamilton JA and Anderson G P (2004) GM-CSF Biology. *Growth Factors* **22**:225-231.

Kalu E, Sumar N, Giannopoulos T, Patel P, Croucher C, Sherriff E and Bansal A (2007) Cytokine Profiles in Serum and Peritoneal Fluid From Infertile Women With and Without Endometriosis. *J Obstet Gynaecol Res* **33**:490-495.

Kharfi A, Labelle Y, Mailloux J and Akoum A (2003) Deficient Expression of Tumor Necrosis Factor Receptor Type 2 in the Endometrium of Women With Endometriosis. *Am J Reprod Immunol* **50**:33-40.

Kyama CM, Overbergh L, Debrock S, Valckx D, Vander P S, Meuleman C, Mihalyi A, Mwenda J M, Mathieu C and D'Hooghe T M (2006) Increased Peritoneal and Endometrial Gene Expression of Biologically Relevant Cytokines and Growth Factors During the Menstrual Phase in Women With Endometriosis. *Fertil Steril* **85**:1667-1675.

Liu XJ, He Y L and Peng D X (2002) Expression of Metalloproteinase-9 in Ectopic Endometrium in Women With Endometriosis. *Di Yi Jun Yi Da Xue Xue Bao* **22**:467-469.

Luk J, Seval Y, Kayisli U A, Ulukus M, Ulukus C E and Arici A (2005) Regulation of Interleukin-8 Expression in Human Endometrial Endothelial Cells: a Potential Mechanism for the Pathogenesis of Endometriosis. *J Clin Endocrinol Metab* **90**:1805-1811.

Matsuzaki S, Canis M, Vaurs-Barriere C, Boespflug-Tanguy O, Dastugue B and Mage G (2005) DNA Microarray Analysis of Gene Expression in Eutopic Endometrium From Patients With Deep Endometriosis Using Laser Capture Microdissection. *Fertil Steril* **84 Suppl 2**:1180-1190.

McKenna SD, Feger G, Kelton C, Yang M, Ardissone V, Cirillo R, Vitte P A, Jiang X and Campbell R K (2007) Tumor Necrosis Factor (TNF)-Soluble High-Affinity Receptor Complex As a TNF Antagonist. *J Pharmacol Exp Ther* **322**:822-828.

Meter RA, Wira C R and Fahey J V (2005) Secretion of Monocyte Chemotactic Protein-1 by Human Uterine Epithelium Directs Monocyte Migration in Culture. *Fertil Steril* **84**:191-201.

Osteen KG, Yeaman G R and Bruner-Tran K L (2003) Matrix Metalloproteinases and Endometriosis. *Semin Reprod Med* **21**:155-164.

Poncelet C, Leblanc M, Walker-Combrouze F, Soriano D, Feldmann G, Madelenat P, Scoazec J Y and Darai E (2002) Expression of Cadherins and CD44 Isoforms in Human Endometrium and Peritoneal Endometriosis. *Acta Obstet Gynecol Scand* **81**:195-203.

Sampson, JA (1927) . Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obst Gynecol **14**:442-469.

Schreiner A, Ruonala M, Jakob V, Suthaus J, Boles E, Wouters F and Starzinski-Powitz A (2007) Junction Protein Shrew-1 Influences Cell Invasion and Interacts With Invasion-Promoting Protein CD147. *Mol Biol Cell* **18**:1272-1281.

Szyllo K, Tchorzewski H, Banasik M, Glowacka E, Lewkowicz P and Kamer-Bartosinska A (2003) The Involvement of T Lymphocytes in the Pathogenesis of Endometriotic Tissues Overgrowth in Women With Endometriosis. *Mediators Inflamm* **12**:131-138.

Wu Y, Starzinski-Powitz A and Guo S W (2007a) Prolonged Stimulation With Tumor Necrosis Factor-Alpha Induced Partial Methylation at PR-B Promoter in Immortalized Epithelial-Like Endometriotic Cells. *Fertil Steril*. (In Press)

Wu Y, Starzinski-Powitz A and Guo S W (2007b) Trichostatin A, a Histone Deacetylase Inhibitor, Attenuates Invasiveness and Reactivates E-Cadherin Expression in Immortalized Endometriotic Cells. *Reprod Sci* **14**:374-382.

Wu Y, Strawn E, Basir Z, Halverson G and Guo S W (2006) Promoter Hypermethylation of Progesterone Receptor Isoform B (PR-B) in Endometriosis. *Epigenetics* **1**:106-111.

Zeitvogel A, Baumann R and Starzinski-Powitz A (2001) Identification of an Invasive, N-Cadherin-Expressing Epithelial Cell Type in Endometriosis Using a New Cell Culture Model. *Am J Pathol* **159**:1839-1852.

Zhou HE and Nothnick W B (2005) The Relevancy of the Matrix Metalloproteinase System to the Pathophysiology of Endometriosis. *Front Biosci* **10** :569-575.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Fig. 1. **Pharmacological Inhibition of TNF-α mediated kinase signaling in Endometriotic Epithelial cells.** A, 12Z cells were treated with 15ng/ml TNF-α for 0, 15, or 30 minutes with or without 100μg/ml TBP, 1μM, 10μM IKK2 inhibitor, or treated with carrier control for a 30 minute pretreatment. P-IκB (Ser 32/36) and P-NFκB (Ser 536) levels were determined by Western blot analysis, and unphosphorylated forms were used as a loading control. B, 12Z cells were treated with 15ng/ml TNF-α for 15 minutes with or without 100μg/ml TBP, 10μM PD98059, μM SB203580 or treated with carrier control for a 30 minute pretreatment. P-Erk (Thr202/Tyr204) and P-p38 (Thr180/Tyr182) levels were determined by Western blot analysis, and unphosphorylated forms were used as a loading control. Representative results of at least three experiments are shown.

Fig. 2. **TBP and kinase inhibitors block TNF-α signaling through multiple kinase pathways.** A, 12Z cells were treated with 15ng/ml TNF-α for 0, 5, 15, or 30 minutes with or without 100μg/ml TBP or treated with carrier control for a 30 minute pretreatment. P-p38 (Thr180/Tyr182) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. B, 12Z cells were treated with 15ng/ml TNF-αfor 0, 5, 15, or 30 minutes with or without 100μg/ml TBP, 1μM Wortmannin, or treated with carrier control for a 30 minute pretreatment. P-Akt (Ser473) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. C, 12Z cells were treated with 15ng/ml TNF-α for 0,

5, 15, or 30 minutes with or without 100μg/ml TBP or treated with carrier control for a 30 minute pretreatment. P-NFκB (Ser468) mean signal levels were determined by MSD analysis. D, 12Z cells were treated with 15ng/ml TNF-α for 0, 5, 15, or 30 minutes with or without 100μg/ml TBP, 10μM PD98059, or treated with carrier control for a 30 minute pretreatment. P-Erk (Thr202/Tyr204) levels were determined by MSD analysis, and unphosphorylated forms were used as a control for percent phosphorylated determination. Representative results of at least three experiments are shown.

Fig. 3. **TBP blocks expression of TNF-α induced transcripts involved in inflammation and invasion.** Total RNA was isolated from serum starved 12Z cells or from cells after the addition of 100 μg/ml TBP, 1μM Wortmannin, 10μM PD98059, 50μM SB203580, 1μM IKK2 inhibitor, and 15 ng/ml TNFα or vehicle for 24 hours. Inflammation or invasion associated transcript levels were determined by LDA (Low density Array) qPCR using HPRT as the endogenous control/reference. All treated groups were normalized to the untreated 12Z/calibrator. Data is the mean of three replicate. Standard deviation is shown. A, Relative quantity of IL-8 transcript is shown for all groups. B, Relative quantity of IL-6 transcript is shown for all groups. C, Relative quantity of MCP-1 transcript is shown for all groups. D, Relative quantity of GM-CSF transcript is shown for all groups. E, Relative quantity of RANTES transcript is shown for all groups. F, Relative quantity of TNF-α transcript is shown for all groups. G, Relative quantity of ICAM-1 transcript is shown for all groups. Representative results of at least three experiments are shown. . a, $P < 0.05$; b, $P < 0.01$; compared to TNF-α treated cells, ANOVA followed by Tukey test.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4. **TBP blocks TNF-α induced inflammatory cyto/chemokine secretion.** 12Z cells were treated with various concentrations 100 μg/ml TBP or TNF-α, or TBP in the presence of 15 ng/ml of TNF-α for 24 hours. Cyto/chemokines were measured in the supernatant using the MSD multiplex assay as described in the methods. A, TNF-α dose response induced GM-CSF kinetics graph and TBP blocking TNF-α dose response induced GM-CSF kinetics graph are shown, B, TNF-α dose response induced IL-6 kinetics and TBP blocking TNF-α dose response induced IL-6 kinetics graph are shown, C, TNF-α dose response induced MCP-1 kinetics and TBP blocking TNF-α dose response inducedMCP-1 kinetics graph are shown, and D, TNF-α dose response induced TNF-α dose response induced IL-8 kinetics and TBP blocking TNF-α dose response induced IL-8 kinetics graph are shown kinetics are shown. Data is the mean of three replicates. Standard deviation is shown.

Fig. 5 **Effect of Inhibitors on TNF-α induced cytokine secretion.** 12Z cells were treated with 1μM Wortmannin, 10μM PD98059, 50μM SB203580, 1μM AS602868 (IKK2 inhibitor), or vehicle; with or without 15 ng/ml TNF-α for 24 hours in the absence of serum. Concentrations of secreted proteins were measured by MSD. A, Inhibitors blocking TNF-α induced GM-CSF kinetics graph is shown, B, Inhibitors blocking TNF-α induced IL-6 kinetics graph is shown, C, Inhibitors blocking TNF-α induced IL-8 kinetics graph is shown, and D, Inhibitors blocking TNF-α induced MCP-1 kinetics graph is shown. Data is the mean of three replicate. Standard deviation is shown. a, $P < 0.05$; b, $P < 0.01$; compared to TNF-α treated cells, ANOVA followed by Tukey test.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6 **TBP and kinase inhibitors block expression of TNF-α induced Matrix Metalloproteinases involved in Epithelial Mesenchymal Transition.** Total RNA was isolated from serum starved 12Z cells or from cells after the addition of 100 μg/ml TBP, 1μM Wortmannin, 10μM PD98059, 50μM SB203580, 1μM IKK2 inhibitor, and 15 ng/ml TNF-α or vehicle for 24 hours. Inflammation or invasion associated transcript levels were determined by LDA (Low density Array) qPCR using HPRT as the endogenous control/reference. All treated groups were normalized to the untreated 12Z/calibrator. Data is the mean of three replicate. Standard deviation is shown. A, Relative quantity of MMP-7 transcript is shown for all groups. B, Relative quantity of MMP-9 transcript is shown for all groups. Representative results of at least three experiments are shown. . a, $P < 0.05$; b, $P < 0.01$; compared to TNF-α treated cells, ANOVA followed by Tukey test.

Fig. 7 **MEKi, IKKi, and p38i inhibit 12Z invasion into Matrigel.** 12Z cells were treated with vehicle, 100 μg/ml TBP, 1μM Wortmannin, 10μM PD98059, 50μM SB203580, or 1μM AS602868 (IKK2 inhibitor); with or without 15 ng/ml TNF-α for 30 hours in the absence of serum. Treated 12Z were allowed to invade Matrigel for 24 hours as described in the methods. Invasive 12Z cells were stained and counted at 24hrs post treatment. a, $P < 0.05$; compared to TNF-α treated cells, ANOVA followed by Tukey test.

Molecular Pharmacology Fast Forward. Published on February 5, 2008 as DOI: 10.1124/mol.107.042176
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8 **TBP, MEK Inhibitor, and p38 inhibitor reduce the expression of N-Cadherin induced by TNF-α in 12Z cells.**

Serum starved 12Z cells were stimulated with various agents for 24 hr and fixed with paraformaldehyde. Fixed cells were stained with primary antibody against N-cadherin and Hoechst as described in the methods. Fluorescence images were captured at a specific wavelength for each dye. Images were merged to obtain cell surface expression of N-cadherin. A, Vehicle treated 12Z cells show basal levels of N-Cadherin expression. B, TNF-α15 ng/ml) treated 12Z cells shows elevated levels of N-Cadherin expression. C, TNF-α + TBP (100 μg/ml) shows normalized levels of N-Cadherin expression. D, TNF-α + PD98059 reduces levels of N-Cadherin expression below steady state levels. E, TNF-α + SB203580 also reduced levels of N-Cadherin expression below steady state levels. Representative results of at least three experiments are shown.

Fig. 9 **Schematic representation of TNF-α induced signaling in epithelial endometriotic cells.** TNF-α signals through the TNF-α Receptor. TNF-α activates pathways involved in regulating expression of inflammation and invasion mediators, including the PI3K, MEK, JNK, p38, and IKK pathways. The findings presented here indicate that TNF-α regulates components of inflammation and invasion through diverse signaling cascades in epithelial endometriotic cells.

Fig. 1

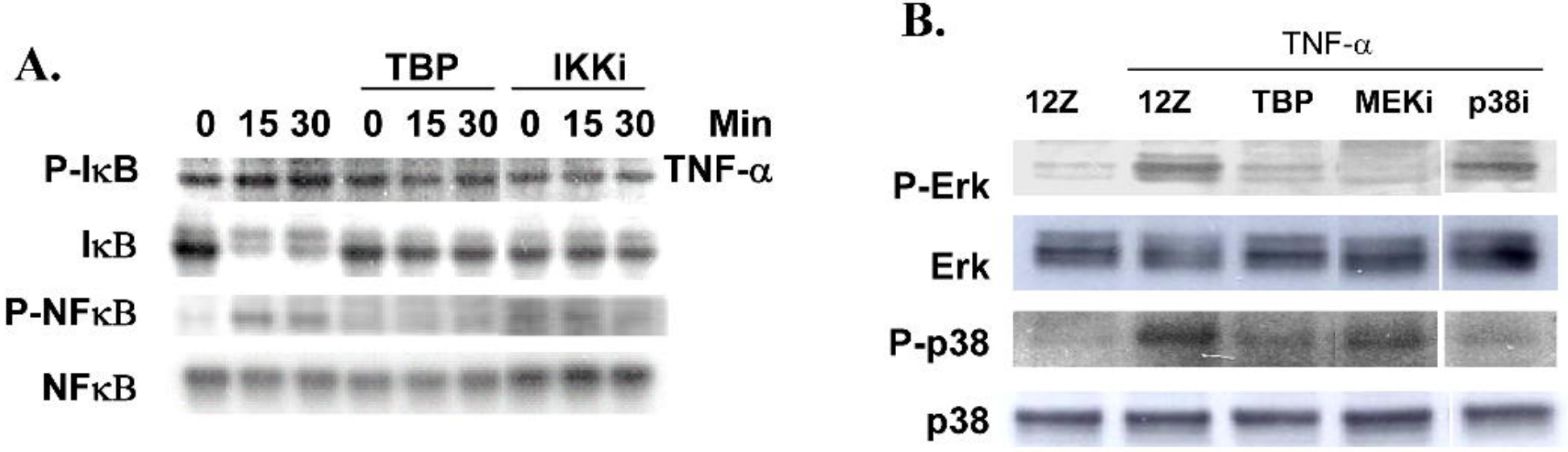

A.
TBP
IKKi
0 15 30 0 15 30 0 15 30 Min
P-IκB
TNF-α
IκB
P-NFκB
NFκB
B.
TNF-α
12Z
12Z
TBP
MEKi
p38i
P-Erk
Erk
P-p38
p38

Fig. 2

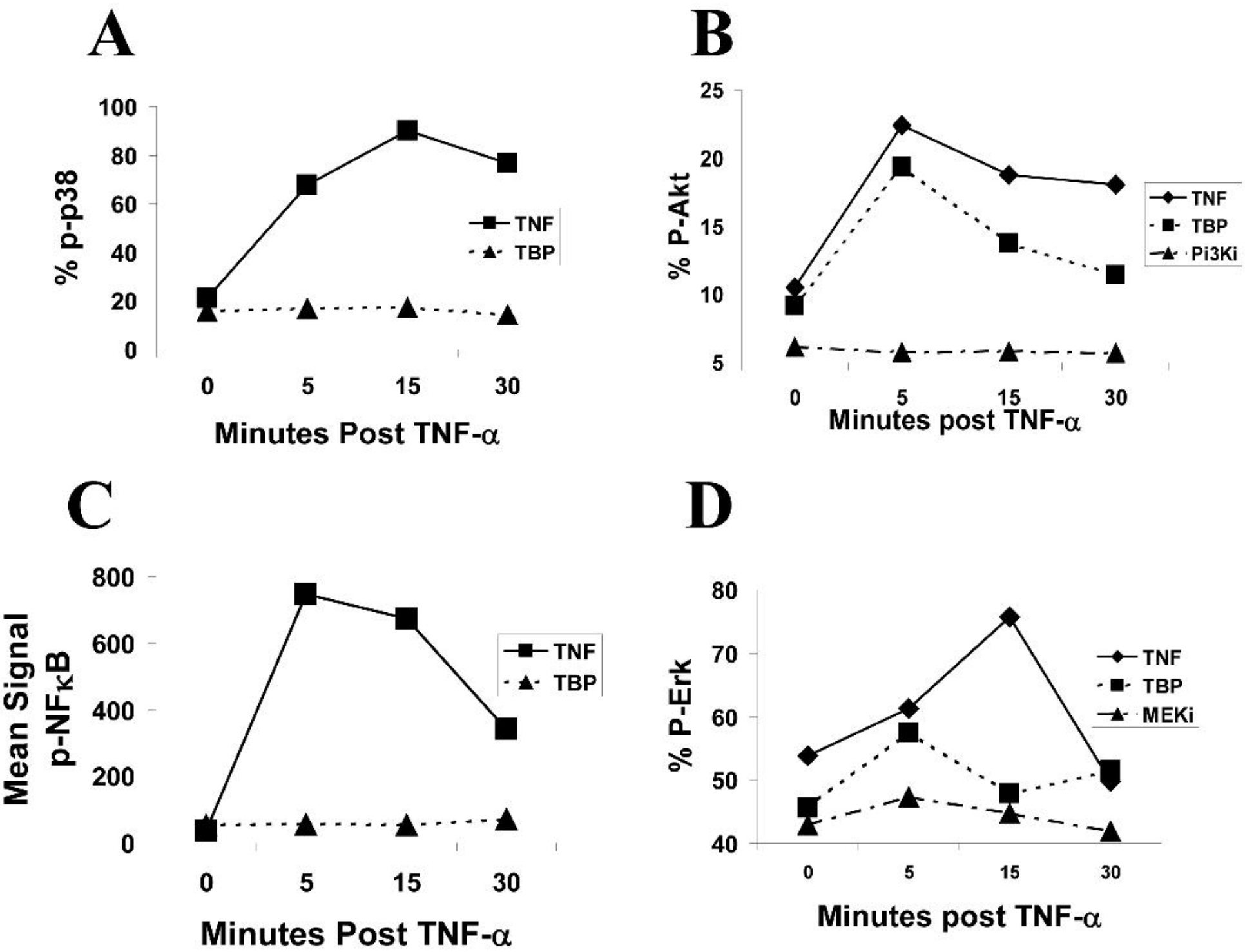

A
% p-p38
100
80
60
40
20
0
TNF
TBP
0
5
15
30
Minutes Post TNF-α
B
% P-Akt
25
20
15
10
5
TNF
TBP
Pi3Ki
0
5
15
30
Minutes post TNF-α
C
Mean Signal
p-NFκB
800
600
400
200
0
TNF
TBP
0
5
15
30
Minutes Post TNF-α
D
% P-Erk
80
70
60
50
40
TNF
TBP
MEKi
0
5
15
30
Minutes post TNF-α

Fig. 3

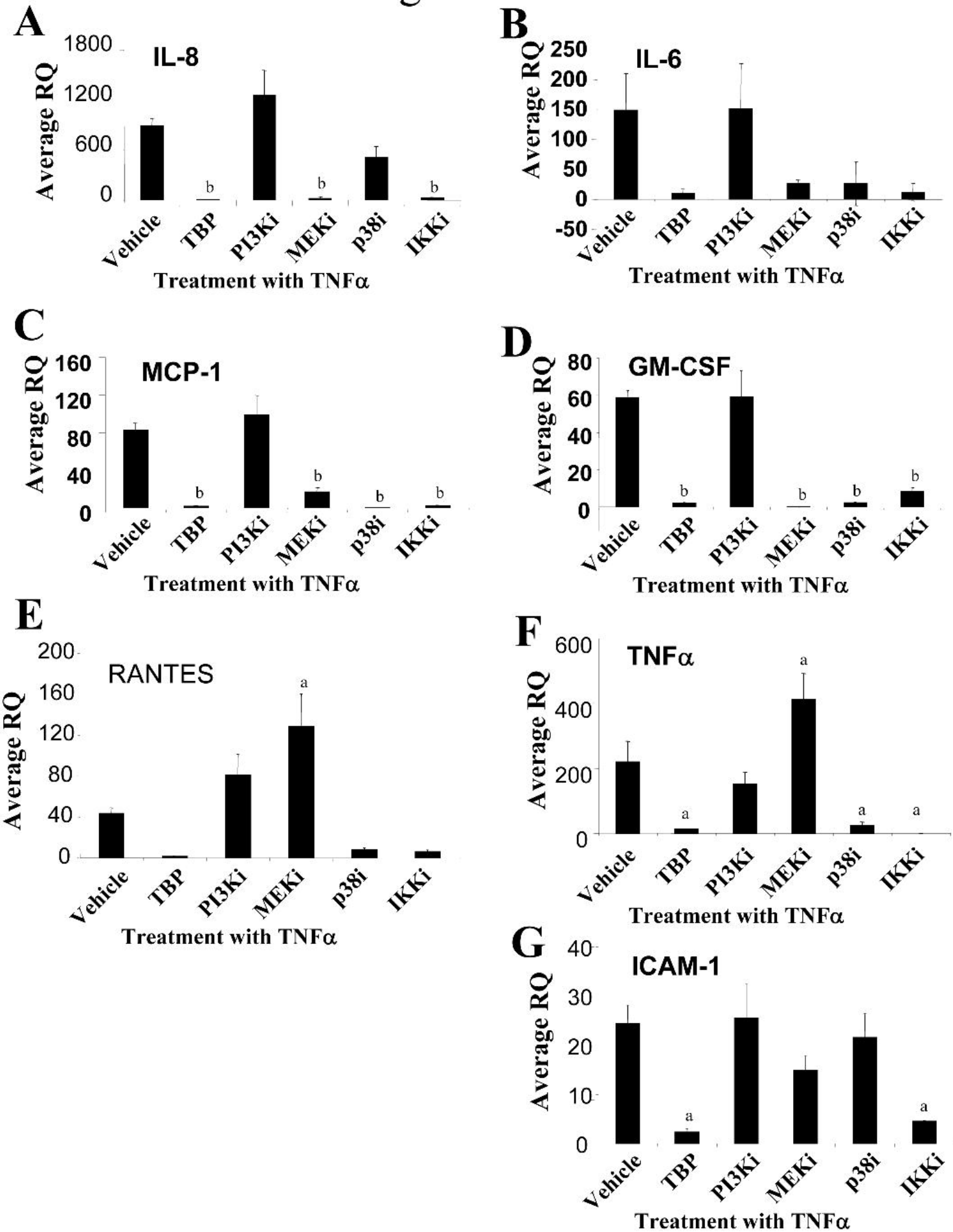

A
IL-8
Average RQ
1800
1200
600
0
b
Vehicle
TBP
PI3Ki
MEKi
p38i
IKKi
Treatment with TNFα
B
IL-6
Average RQ
250
200
150
100
50
0
-50
Treatment with TNFα
C
MCP-1
Average RQ
160
120
80
40
0
Treatment with TNFα
D
GM-CSF
Average RQ
80
60
40
20
0
Treatment with TNFα
E
RANTES
Average RQ
200
160
120
80
40
0
a
Treatment with TNFα
F
TNFα
Average RQ
600
400
200
0
a
Treatment with TNFα
G
ICAM-1
Average RQ
40
30
20
10
0
a
Treatment with TNFα

Fig. 4

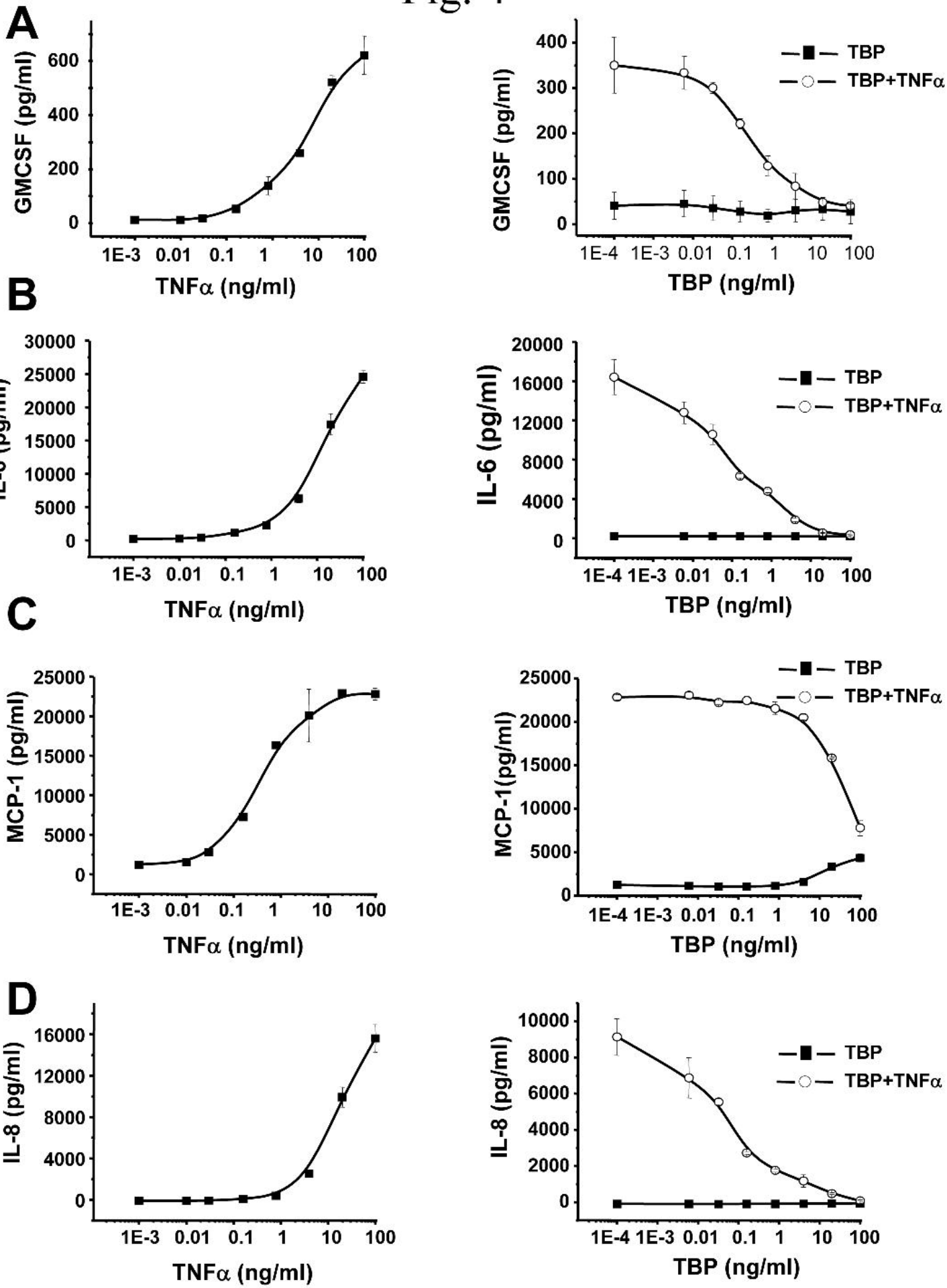

A
GMCSF (pg/ml)
600
400
200
0
1E-3 0.01 0.1 1 10 100
TNFα (ng/ml)
GMCSF (pg/ml)
400
300
200
100
0
TBP
TBP+TNFα
1E-4 1E-3 0.01 0.1 1 10 100
TBP (ng/ml)
B
IL-6 (pg/ml)
30000
25000
20000
15000
10000
5000
0
1E-3 0.01 0.1 1 10 100
TNFα (ng/ml)
IL-6 (pg/ml)
20000
16000
12000
8000
4000
0
TBP
TBP+TNFα
1E-4 1E-3 0.01 0.1 1 10 100
TBP (ng/ml)
C
MCP-1 (pg/ml)
25000
20000
15000
10000
5000
0
1E-3 0.01 0.1 1 10 100
TNFα (ng/ml)
MCP-1(pg/ml)
25000
20000
15000
10000
5000
0
TBP
TBP+TNFα
1E-4 1E-3 0.01 0.1 1 10 100
TBP (ng/ml)
D
IL-8 (pg/ml)
16000
12000
8000
4000
0
1E-3 0.01 0.1 1 10 100
TNFα (ng/ml)
IL-8 (pg/ml)
10000
8000
6000
4000
2000
0
TBP
TBP+TNFα
1E-4 1E-3 0.01 0.1 1 10 100
TBP (ng/ml)

Fig. 5

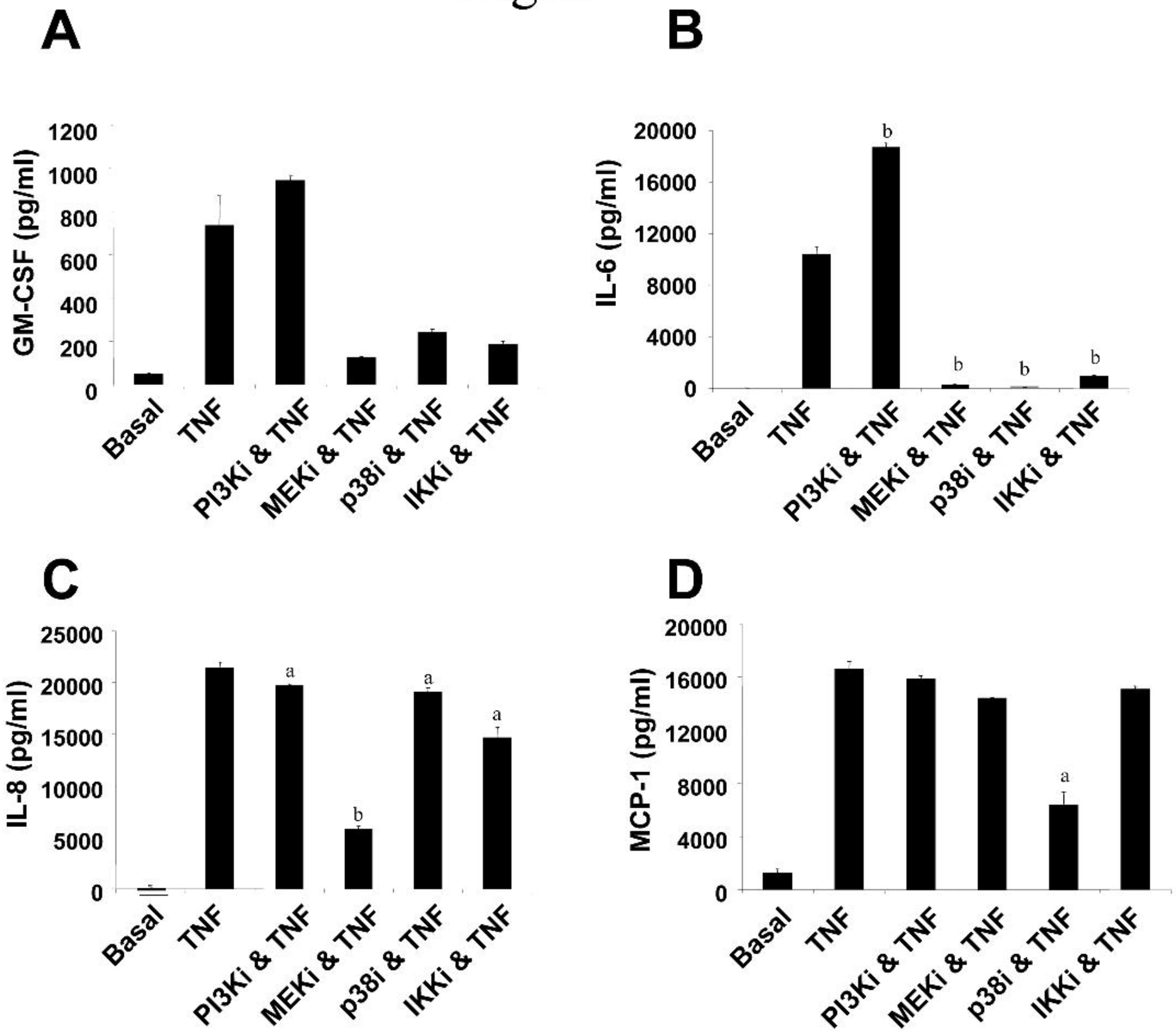
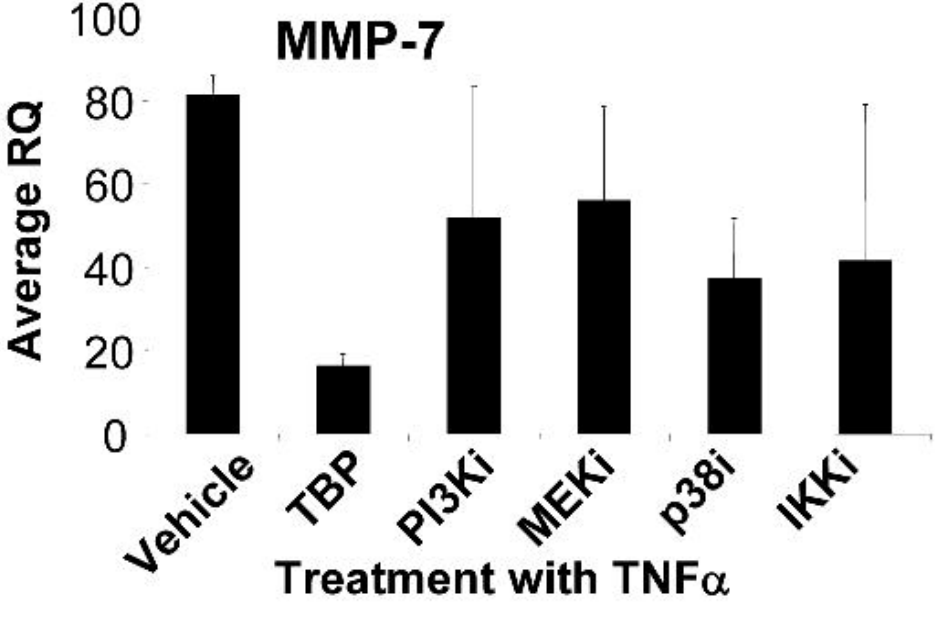

A
GM-CSF (pg/ml)
1200
1000
800
600
400
200
0
Basal
TNF
PI3Ki & TNF
MEKi & TNF
p38i & TNF
IKKi & TNF
B
IL-6 (pg/ml)
20000
16000
12000
8000
4000
0
b
C
IL-8 (pg/ml)
25000
20000
15000
10000
5000
0
a
D
MCP-1 (pg/ml)
20000
16000
12000
8000
4000
0
a

Fig. 6


MMP-7
Average RQ
100
80
60
40
20
0
Vehicle
TBP
PI3Ki
MEKi
p38i
IKKi
Treatment with TNFα

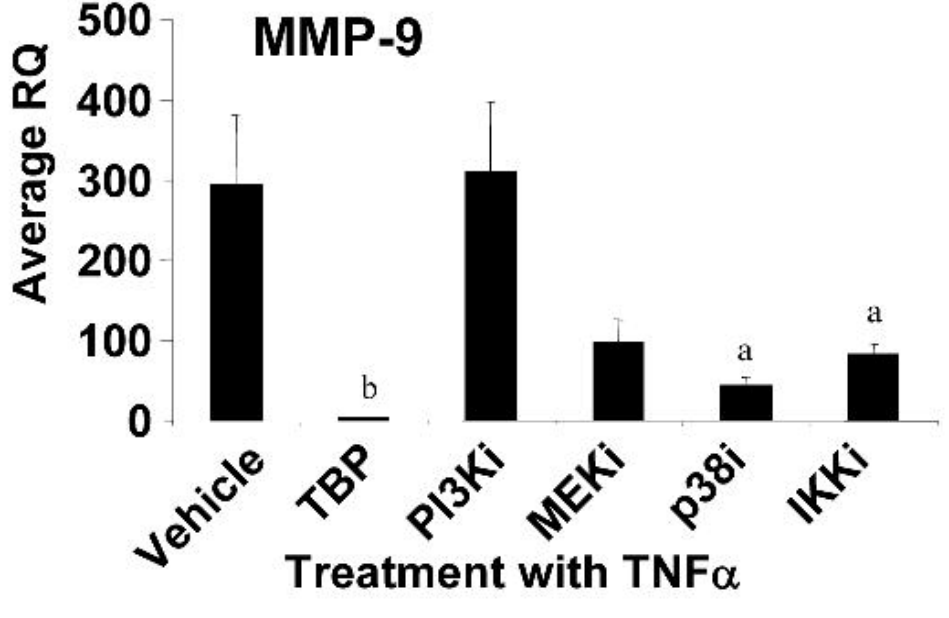

MMP-9
Average RQ
500
400
300
200
100
0
b
a
a
Vehicle
TBP
PI3Ki
MEKi
p38i
IKKi
Treatment with TNFα

Fig. 7

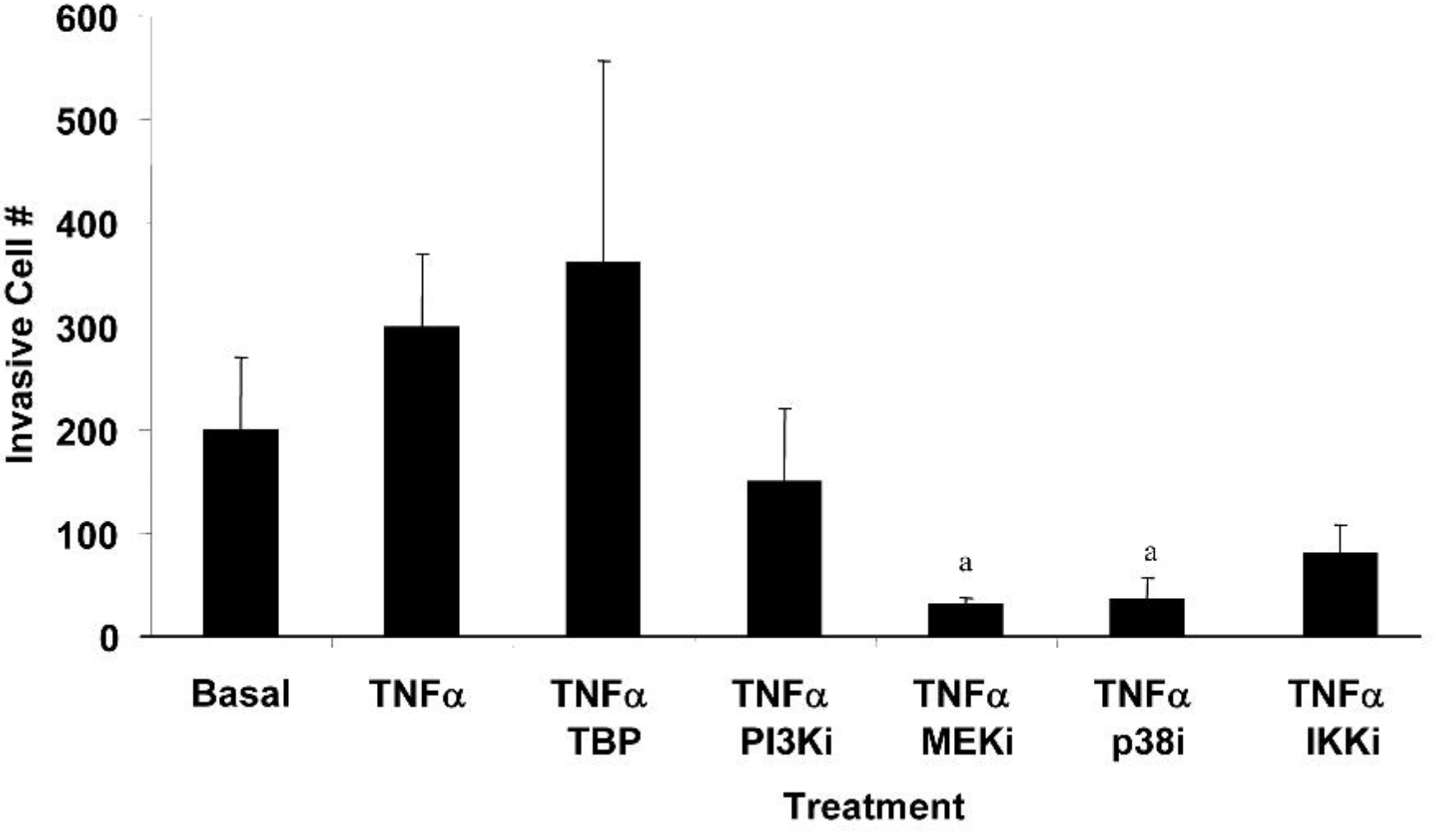

600
500
400
300
200
100
0
Invasive Cell #
a
a
Basal
TNFα
TNFα TBP
TNFα PI3Ki
TNFα MEKi
TNFα p38i
TNFα IKKi
Treatment

Fig. 8

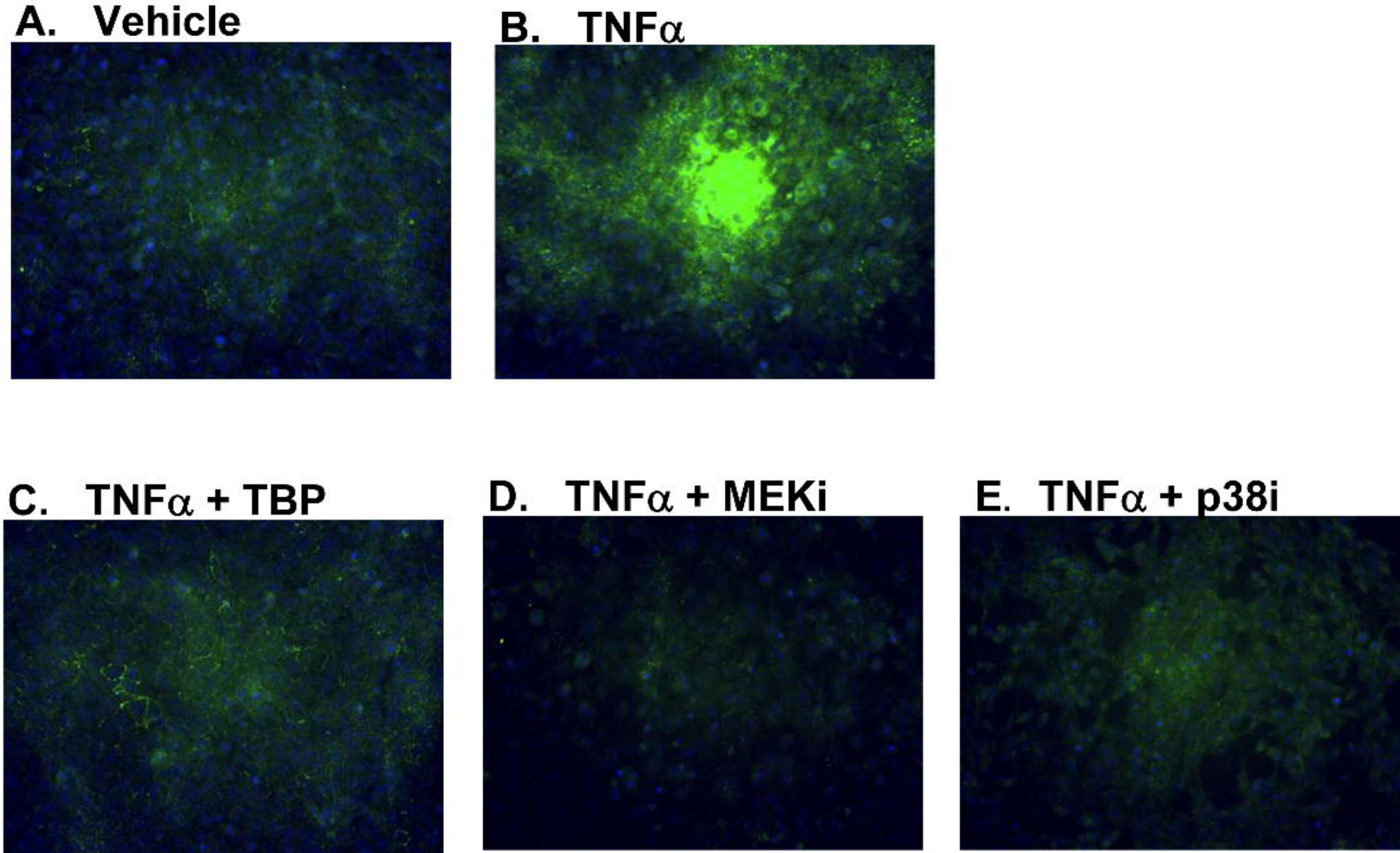
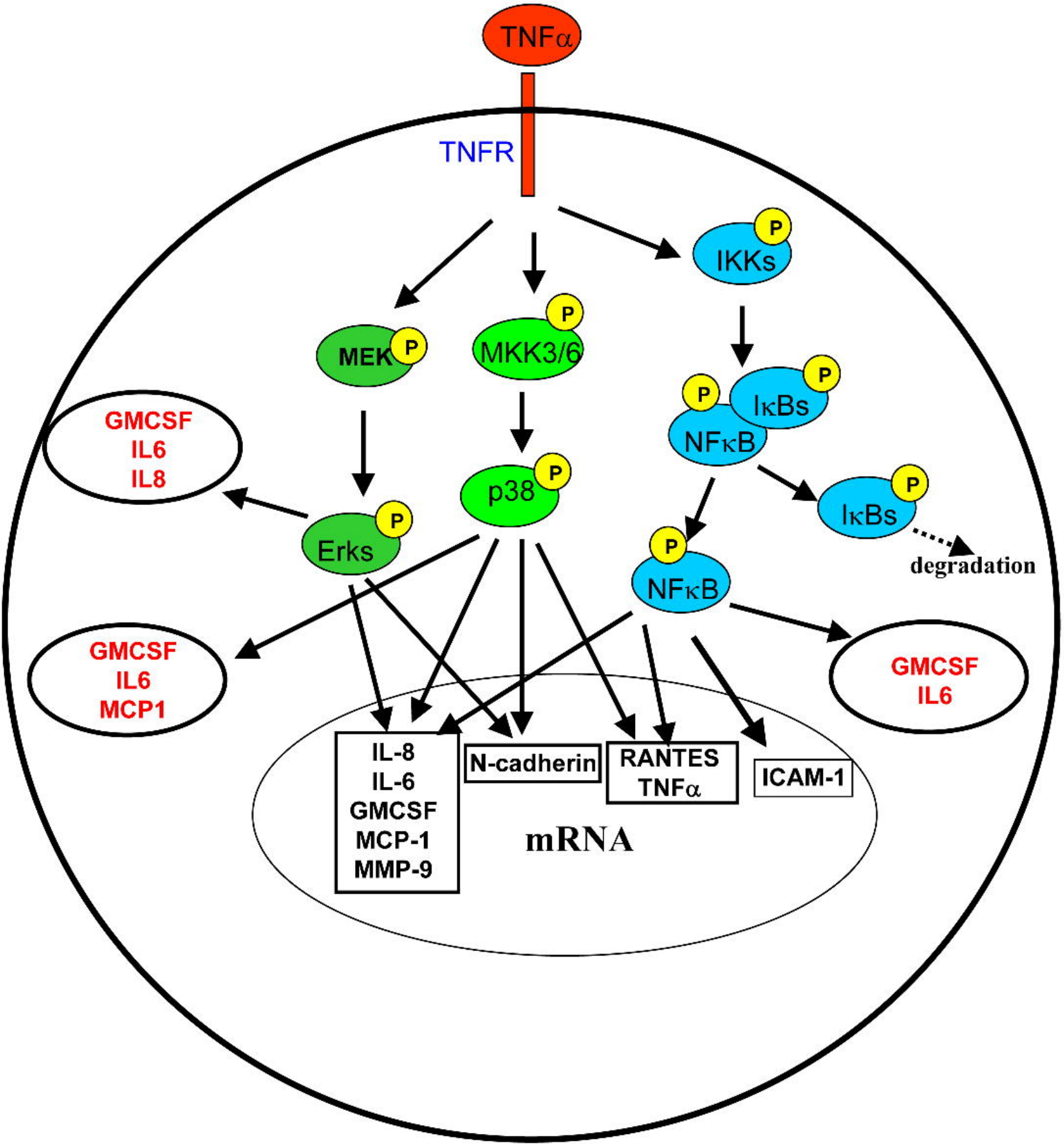


Fig. 9


TNFα
TNFR
IKKs
P
MEK
MKK3/6
IκBs
NFκB
GMCSF
IL6
IL8
p38
Erks
degradation
GMCSF
IL6
MCP1
GMCSF
IL6
IL-8
IL-6
GMCSF
MCP-1
MMP-9
N-cadherin
RANTES
TNFα
ICAM-1
mRNA