Over-expression of MAP kinase phosphatase-2 enhances adhesion molecule expression and protects against apoptosis in human endothelial cells.

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Running Title: MKP-2 over-expression in human endothelial cells.

Summary

Background and Purpose: In this study we used adenovirus infection to over-express the dual specific phosphatase, MAP kinase phosphatase-2 (MKP-2), in human umbilical vein endothelial cells and examined inflammatory protein expression and apoptosis, two key features of endothelial dysfunction in disease.

Experimental approaches: We generated an adenoviral version of MKP-2 (Adv.MKP-2) and infected HUVECs for 40 h. TNFα stimulated MAP kinase phosphorylation and protein expression was measured by Western blotting. Cellular apoptosis was assayed by FACS.

Key results: Infection with Adv.MKP-2 selectively abolished TNFα-mediated JNK activation and had little effect upon ERK or p38 MAP kinase. Adv.MKP-2 abrogated COX-2 expression whilst induction of the endothelial cell adhesion molecules ICAM and VCAM, two NFκB-dependent proteins, were not affected. However, when ICAM and VCAM expression was partly reduced by blockage of the NFκB pathway Adv.MKP-2 was able to reverse this inhibition. This correlated with enhanced TNFα-induced IκBα loss, a marker of NFκB activation. TNFα in combination with NFκB blockade also increased HUVEC apoptosis; this was significantly reversed by Adv.MKP-2. Protein markers of cellular damage and apoptosis, H2AX phosphorylation and caspase-3 cleavage, were also reversed by MKP-2 over-expression.

Conclusions: These results show that over-expression of MKP-2 has differential effects upon the expression of inflammatory proteins due to a reciprocal effect upon JNK and NFκB signalling and is also able to prevent TNFα mediated endothelial cell death. These properties may make Adv.MKP-2 a potentially useful future therapy in cardiovascular diseases were endothelial dysfunction is a feature.

Key words

MAP kinase phosphatase-2, endothelial cell dysfunction, caspase, JNK, apoptosis

Abbreviations:

HUVECs, human umbilical vein endothelial cells; MKP-2, MAP kinase phosphatase-2, JNK; c-Jun-N-terminal kinase, IKK, inhibitory kappa B kinase; Adv., adenovirus, ICAM, inter-cellular adhesion molecule; VCAM, vascular cell adhesion molecule; TNFα, Tumour necrosis factor alpha.

Introduction

It is now accepted that perturbation in normal endothelial cell function is a key initiator of cardiovascular disease including atherosclerosis, renal failure and diabetes (Cines *et al.*, 1998; Esper *et al.*, 2006). Endothelial cells activated by factors such as cytokines, free radicals and hypoxia increase the expression of a number of pro-inflammatory proteins such as IL-6 and MCP-1, COX-2 which produces prostaglandins, and adhesion molecules such as E-selectin, ICAM and VCAM. This results in a functional shift towards reduced vasodilatation and a pro-inflammatory state (Esper *et al.*, 2006). In addition, endothelial cell apoptosis is also implicated in a number of cardiovascular conditions. This includes atherosclerosis (Rossig *et al.*, 2001; Tricot *et al.*, 2000), myocardial ischaemia (Scarabelli *et al.*, 2001; Scarabelli *et al.*, 2002; Zhao *et al.*, 2009), heart failure (Rossig *et al.*, 2000) and lower limb ischaemia (Xie *et al.*, 2006). Pharmacological approaches to prevent either of these events may therefore be of benefit in certain disease conditions.

The mitogen-activated protein (MAP) kinases play key roles in the regulation of endothelial cell function through phosphorylation of a number of cytosol and nuclear targets (Yu *et al.*, 2007). There are three main families of MAP kinases; the extracellular regulated kinases (ERKs), the p38 MAP kinases and the c-Jun N-terminal kinases (JNKs). Whilst ERK is associated with endothelial cell survival (Mavria *et al.*, 2006; Pintus *et al.*, 2003; Yu *et al.*, 1999), both p38 MAP kinase and JNK are strongly associated with inflammation and endothelial cell apoptosis (Nakagami *et al.*, 2001; Wadgaonkar *et al.*, 2004). Whilst JNK is linked to expression of COX-2, and E-selectin (Min *et al.*, 1997; Naderer *et al.*, 2008; Nakagami *et al.*, 2001), a larger body of evidence implicates JNK in the regulation of cell death (Sabapathy *et al.*, 1999; Tournier *et al.*, 2000). In endothelial cells, JNK is associated with apoptosis mediated by agents such as cytokines, hydrogen peroxide (Wang *et al.*, 1999) and high glucose concentration (Ho *et al.*, 2000). Recently the JNK inhibitor SP600125 (Bennett *et al.*, 2001) have been shown to reverse apoptosis

in endothelial cells (Fu *et al.*, 2006; Karahashi *et al.*, 2009), however, SP600125 may not be as selective as first indicated (Cameron *et al.*, 2003). Thus, novel approaches to selectively inhibit JNK activation in endothelial cells could be useful.

Thus, in an attempt to negatively regulate JNK activity and prevent endothelial cell inflammation and death, we developed an adenoviral version of mitogen-activated protein kinase phosphatase-2 (MKP-2), (Misra-Press *et al.*, 1995). MKP-2 is a member of the dual specific phosphatase family, (Keyse, 2008) which functions to dephosphorylate the mitogen-activated protein (MAP) kinases. It is nuclear targeted due to the presence of two nuclear location sequences (Sloss *et al.*, 2005) and although specific for ERK and JNK *in vitro* (Chu *et al.*, 1996), we have demonstrated selectivity for JNK in a number of cell systems (Cadalbert *et al.*, 2005; Robinson *et al.*, 2001). We used this adenovirus to over-express MKP-2 in endothelial cells and determine the profile of kinase inhibition and the effect upon a number of end points associated with endothelial cell activation. In this study we show that over-expression of MKP-2 abolished JNK activity in HUVECs and abrogated COX-2 expression in response to TNFα. In contrast, ICAM and VCAM expression were not affected by Adv.MKP-2. However, when ICAM and VCAM expression was reduced by blocking the NFκB pathway, MKP-2 partly reversed inhibition of ICAM and VCAM expression. This effect correlated with enhanced IκBα loss, indicative of cross talk regulation of the NFκB pathway by MKP-2. Apoptosis induced by TNFα in combination with NFκB blockage, was also inhibited following Adv.MKP-2 expression. This effect correlated with a reduction in the phosphorylation of the nuclear protein H2AX and cleavage of caspase-3, a cytosolic effector caspase of the apoptosis pathway. These data suggests that MKP-2 may be a useful mode of therapy in conditions were endothelial cell apoptosis is a feature, not only by inhibiting JNK signalling, but by enhancing NFκB activation. We also show for the first time that MKP-2 can regulate cytosolic initiated apoptotic signalling events despite being a nuclear enzyme.

Methodology

Reagents

All reagents were from Sigma (Poole UK), unless otherwise stated. Tumour necrosis factor-α (TNF-α) was obtained from Santa Cruz Biotechnology (CA, USA). Antibodies were purchased as follows: p65 NFκB, IκBα, phospho and total ERK, MKP-2, and IKKβ from Santa Cruz Biotechnology (CA, USA) ; phospho-p38 and -JNK from Invitrogen, Paisley UK; COX-2 from Cayman Chemicals (Michigan, USA); anti-human VCAM-1 and ICAM-1 from R & D systems (Abingdon, UK); H2AX (Ser-139) from Upstate Biotechnology Inc, (Lake Placid, NY,USA); Cleaved Caspase-3 from Cell Signalling Biotechnology Inc, (Beverly, MA); FITC anti-Rabbit and HRP-conjugated secondary antibody from Jackson Immuno Research laboratories Inc. (West Grove, PA, USA).

Cell culture

Cryopreserved primary HUVECs were purchased from Lonza (Slough UK), and were grown in endothelial basal media (EBM-2), supplemented with endothelial growth media (EGM-2 Lonza, Slough, UK) containing single aliquots of defined supplements (2% foetal bovine serum, 0.2 ml hydrocortisone, 2 ml rhFGF-B, 0.5 ml VEGF, 0.5 ml R^3-insulin like growth factor-1, 0.5 ml ascorbic acid, 0.5 ml rhEGF, 0.5 ml GA-1000 and 0.5 ml heparin (concentrations not disclosed by the company). Cells were incubated at 37˚C in humidified air with 5% CO_2. All experiments were performed between passages 2 and 5.

Adenoviral infections

An adenoviral vector encoding dominant negative IKKβ (Adv.DN-IKKβ) and MKP-2 (Adv. MKP-2) were created in-house using the Adeno-X virus purification kit from Clontech laboratories, Inc (CA, USA). The DN-IKKβ plasmid was originally a gift from Dr D. Goeddel (Tularik Inc., CA, USA). Large-scale production of high titre

recombinant adenovirus was performed by routine methods (Nicklin *et al.*, 2004) HUVECs, when approximately 50-60% confluent, were incubated with adenovirus up to 300p.f.ucell^{-1} for 40 h in endothelial growth media. Cells were stimulated with agonist for the indicated times in complete medium.

Western blotting

Proteins (15μg) were separated by either 8.5% or 10% SDS–PAGE and transferred onto nitrocellulose. The membranes were blocked for non-specific binding for 2 h in 2% BSA (w:v) diluted in NATT buffer (20 mM Tris, 150 mM NaCl, 0.2% (v:v) Tween-20, pH 7.4). Membranes were then incubated overnight with primary antibody diluted in 0.2% BSA (w:v) in NATT buffer at room temperature. The membranes were then washed with NATT buffer for 90 min and incubated with HRP-conjugated secondary antibody for 2 h. After a further 90 min wash, the membranes were subjected to ECL reagent and exposed to Kodak X-ray film for appropriate time.

JNK activity

Cells were stimulated as appropriate and the reaction terminated by rapid aspiration and the addition of ice-cold PBS. The cells were solubilised in 20 mM HEPES buffer, pH 7.7, containing 50 mM NaCl, 0.1 mM EDTA, 0.1mM Na_3VO_4, 0.1 mM PMSF, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, and 1% (w/v) Triton X-100. Lysates were clarified by centrifugation for 5 min at 13,000 rpm and equal amounts of protein were incubated with 20 μg of GST-c-Jun-(5–89) immobilized on glutathione-Sepharose at 4°C for 3 h. Beads were then washed three times in solubilisation buffer and twice in 25 mM HEPES buffer, pH 7.6, containing 20 mM β-glycerophosphate, 0.1 mM NaV_3O_4 and 2mM dithiothreitol. Precipitates were then incubated with the same buffer containing 25 μM/0.5 μCi of ATP/ [γ-^{32}P] ATP in a final volume of 30 μl at 30 °C for 30 min. The reactions were terminated by the addition of 4 x SDS-sample buffer and aliquots of each sample subjected to electrophoresis on 11% SDS-polyacrylamide gel electrophoresis. Phosphorylation of GST-c-Jun was then determined by autoradiography.

Immunofluorescence

HUVECs were grown to 50-60% confluency on No.0 glass cover slips (Merck Biosciences, Nottingham, England). After adenovirus infection (40 h) cells were washed 3x with ice-cold phosphate-buffered saline (PBS) prior to fixation with 1 ml of 3% paraformaldehyde solution. Cover slips were then incubated with 0.1% triton X-100 in PBS for 5 min at room temperature. Cover slips were washed 3 times in PBS and 3 times with 1% BSA diluted in PBS (w:v) for 5 min followed by incubation with primary monoclonal MKP-2 antibody (1:200 in 1% BSA) for 1 h. Again cover slips were washed with ice cold PBS and incubated with secondary monoclonal antibody (1:400 in 1% BSA) mixed with 100 $ngml^{-1}$ of DAPI for 45 min. Following three washes with ice cold PBS, Mowiol was used to mount cover slips onto slides for visualization by EP-1 fluorescence light microscopy.

Flow cytometry assay of apoptosis

Cells were infected for 40 h then stimulated for a further 24 h prior to analysis. Cells were trypsinised and then pelleted at 1000 rpm for 2 min. The pellet was then resuspended in 500µl of 1x annexin binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Phycoerthyrin-Annexin V and 7-AAD were added to the cells according to the manufacturer's instructions and the samples were read in the FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data was analysed using FACS Diva (Becton Dickinson, Oxford, UK) and RCS Express (De Novo Software, Canada) software. A total of 10,000 events were measured per sample. Gating was determined using PE-Annexin V FL-2 and 7–AAD FL-3 standards attached to beads (Becton Dickinson, Oxford, UK) and preliminary experiments conducted using paraformaldehyde and serum deprivation to define apoptotic and necrotic populations as outlined by the manufacturer's instructions.

Data analysis

Each figure represents one of at least four separate experiments. Western blots were scanned on a Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software. For gels, densitometry measurement was performed using the Scion Image program. Data were expressed as mean ± s.e.m. Statistical analysis was performed by One-way ANOVA with Dunnett's Post test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

The effect of adenoviral infection with MKP-2 (Adv.MKP-2) on tumour necrosis factor alpha (TNFα)-stimulated JNK activation was assessed in HUVECs (Figure 1). Imunofluorescent staining (Panel A) showed no endogenous MKP-2 expression in LacZ-infected control cells, however following Adv.MKP-2 infection at a maximum concentration of 300 m.o.i., greater than 95% of the cells stained for nuclear MKP-2. Under these conditions, Adv.MKP-2 caused a significant inhibition of JNK phosphorylation, stimulated by TNFα (20 $ngml^{-1}$), the response being virtually abolished at 200-300 $p.f.u.cell^{-1}$ (Panel B, fold stimulation: TNFα = 24.4 ± 4.5, TNFα + Adv.MKP-2 (300 $p.f.u.cell^{-1}$) = 1.6 ± 0.3, $P<0.01$). Both p46 and p54 isoforms were similarly affected. Adv.MKP-2 also reduced TNFα -stimulated JNK *in vitro* kinase activity (Panel C, $P<0.01$ compared to TNFα stimulation) and phosphorylation of c-Jun, an immediate downstream target of JNK (Panel D, $P<0.01$).

We also examined ERK and p38 MAP kinase phosphorylation to confirm that the effect of MKP-2 was specific to JNK signalling (Figure 2). TNFα stimulated ERK phosphorylation was maximal at 15- 30 min, 2-3 fold of basal values. Neither this response, or basal levels, were affected by MKP-2 infection up to 300 $p.f.u.cell^{-1}$ (panel A). TNFα stimulated a substantial increase in p38 MAP kinase phosphorylation however, this response was not significantly affected by Adv.MKP-2 infection (Panel B).

We next sought to determine if by a selective effect upon JNK, MKP-2 was able to regulate the expression of ICAM, VCAM and COX-2 (Figure 3). For both ICAM and VCAM, infection with 300 $p.f.u.cell^{-1}$ of Adv.MKP-2 had no inhibitory effect upon TNFα stimulated induction and in fact, a minor non-significant enhancement of both ICAM and VCAM expression could be observed (Panels A & B). In contrast, TNFα-induced COX-2 expression was significantly reduced following infection with Adv.MKP-2 (Panel C, % inhibition = 99.66 ± 0.2, $P<0.001$). A similar

finding was also observed for HUVECs stimulated with LPS (not shown) suggesting that the effect of MKP-2 was not agonist-dependent.

Studies suggest that in HUVECs, COX-2, ICAM and VCAM are regulated by NFκB signalling, so we sought to assess possible synergy effects of MKP-2 over-expression and NFκB inhibition (Figures 4-6). Initially, we utilised a adenoviral dominant negative form of inhibitory kappa B kinase β (DN-IKKβ) a kinase which plays a key role in regulating NFκB (Gomez *et al.*, 2005; MacKenzie *et al.*, 2007). We demonstrated that infection of HUVECs with 300 p.f.u.cell^{-1} of DN-IKKβ resulted in a substantial, almost complete inhibition of the expression of all three proteins induced in response to TNFα (Figure 4). Conditions were then established to allow simultaneous infection with both viruses and we then assessed the effects of the two interventions in combination (Figures 5 & 6). We found that for COX-2 (Figure 5), Adv.DN-IKKβ and Adv.MKP-2 infection alone substantially reduced protein expression (% inhibition; Adv.MKP-2 = 88.3 ± 0.11, Adv.DN-IKKβ = 64.52 ± 8.94). In combination the inhibition was not significantly greater (% inhibition 98.0 ± 3.2%) than for either agent alone. For ICAM and VCAM however, the situation was markedly different (Figure 6). Whilst DN-IKKβ substantially reduced ICAM and VCAM, co-expression of MKP-2 was able to almost completely reverse this inhibition, expression of both proteins where similar to LacZ controls. As shown previously, MKP-2 alone did not significantly affect ICAM or VCAM expression. Control Western blotting also indicated that the reversal was not due to an inhibitory effect of MKP-2 upon DN-IKKβ expression nor vice versa, neither protein was affected by infection with the other adenovirus.

The potential for MKP-2 to influence NFκB-dependent gene expression was further examined at the level of NFκB signalling (Figure 7). Pre-incubation with Adv. MKP-2 had a minor effect upon basal IκBα levels in HUVECs, however infection with Adv.MKP-2 was able to markedly potentiate submaximal TNFα-induced IκBα loss (Panel A). As expected pre-incubation with DN-IKKβ partially reversed the agonist-stimulated IκBα loss, however, further infection with Adv.MKP-2 restored the loss. Individual expression of MKP-2 and DN-IKKβ was not influenced by co-infection and LacZ at higher concentrations (600 p.f.u.cell^{-1}) did not mimic the effects of the combination, suggesting a bona fide effect of Adv. MKP-2.

We also examined the potential for MKP-2 to reverse endothelial cell apoptosis (Figure 8). We adopted the strategy to use TNFα in combination with DNIKKβ not only to mirror conditions for ICAM and VCAM expression but because inhibition of NFκB signalling is a requisite in most cell types, including endothelial cells, for TNFα induced apoptosis. Cells stimulated alone with TNFα alone showed a minor, insignificant increase in apoptosis (% apoptosis; control = 1.73 ± 1.23, TNFα = 5.23 ± 1.04, ns). However, in the presence of DN-IKKβ apoptosis was marked, rising to 28.23 ± 7.15 %. (Panels A & B). Co-expression of MKP-2 significantly reversed cell death by approximately 50% (% apoptosis +Adv.MKP-2 = 14.26 ± 4.86, $p<0.05$). Again control Western blotting demonstrated no difference in relative expression of either DN-IKKβ or MKP-2 alone or in combination at this longer time point (Panel C).

Finally we sought to confirm that MKP-2 reversal may have an effect upon JNK regulated nuclear proteins involved in regulating apoptosis and nuclear damage (Figure 9). TNFα alone gave a minor increase in the phosphorylation of H2AX a nuclear histone 2A family protein, whilst in combination DN-IKKβ phosphorylation was markedly increased (Panel A, fold simulation; TNFα/ DN-IKKβ = 12.33±2.36). We found that Adv.MKP-2 was able to reverse TNFα/DN-IKKβ-mediated phosphorylation of H2AX (Fold stimulation; TNFα/DN-IKKβ + Adv.MKP-2 = 3.64 ± 0.47, $P<0.05$). As an additional negative control we also assessed the formation of cleaved caspase-3 products p19 and p17, pro-apoptotic proteins generated in the cytosol (Panel B). To our surprise however, we found that MKP-2 over-expression resulted in a complete reversal of caspase-3 cleavage stimulated again by TNFα in combination with DN-IKKβ.

Discussion

In this study we utilised a dual specific nuclear phosphatase, MKP-2, as an experimental pharmacological tool to reverse JNK mediated responses in HUVECs and thus determine its potential use in clinical conditions involving endothelial cell dysfunction. At the cellular level our studies reveal a novel point of cross talk between MKP-2 and the NFκB signalling pathway. We also found that MKP-2 over-expression can inhibit apoptosis and that this effect correlated not only with the dephosphorylation of the pro-apoptotic histone protein H2AX, but with caspase-3 degradation, a cytosolically mediated event, despite MKP-2 being a nuclear phosphatase. This unique combination of effects suggests that Adv.MKP-2 could represent a novel experimental approach for future treatment of cardiovascular disease in which endothelial cell apoptosis is a feature.

Initially, we demonstrated that HUVECs infected with Adv MKP-2 resulted in the expression of a 42 KDa protein and as assessed by immunofluorescence, a protein strictly targeted to the nucleus (Sloss *et al.*, 2005). Previous studies have shown that HUVECS express MKP-2 protein endogenously (Wadgaonkar *et al.*, 2004) but no function was assigned for MKP-2 in endothelium. Under our conditions of culture we found no basal nor agonist induced expression of MKP-2. The lack of MKP-2 expression, in contrast to rodent systems, is a common feature of human cellular studies and it is at present unclear as to what manipulations are required to promote protein expression. Nevertheless, it allowed the use of Adv.MKP-2 without the potential of contaminating endogenous protein. Our results clearly demonstrated a selective effect of MKP-2 over-expression, the abrogation of JNK phosphorylation (Figure 1). This finding is consistent with previous studies in our laboratory examining TNFα stimulation (Robinson *et al.*, 2001) and genotoxic stress (Cadalbert

et al., 2005). Studies *in vitro* show MKP-2 to be selective for JNK and ERK (Chu *et al.*, 1996), lack of effectiveness against ERK following cellular stimulation may reflect the poor ERK stimulation in response to TNFα.

In our hands we found Adv.MKP-2 to be effective against the expression of COX-2, consistent with previous studies showing expression of this protein to be regulated by the JNK pathway (Nieminen *et al.*, 2006; Nieminen *et al.*, 2005; Wu *et al.*, 2006). By contrast, we found no inhibition of ICAM and VCAM a finding consistent with some but not all studies (De Cesaris *et al.*, 1999; Kobuchi *et al.*, 1999) suggesting cell type specific differences in the role of JNK in the regulation of adhesion molecule expression. The predominant pathways regulating ICAM and VCAM in most cell types are p38 MAP kinase, which is not a substrate for MKP-2 (Rahman *et al.*, 2004; Yan *et al.*, 2002), and also NFκB (Rahman *et al.*, 1999). However, using MKP-2 we have identified, for the first time, the potential of JNK inhibition to enhance the expression of ICAM and VCAM, revealed only in conditions of partial NFκB inhibition. This effect was specific for ICAM and VCAM, COX-2 expression, despite also being NFκB-dependent, was also strongly reduced by Adv.MKP-2 alone and in combination with DN-IKKβ did not show any reversal of inhibition. This suggests the potential for JNK to play a negative role in the expression of ICAM and VCAM. This phenomenon was implied from a single study by (Hosokawa *et al.*, 2006) whereby pre-treatment with the JNK inhibitor SP600125 resulted in slightly enhanced VCAM expression. Indeed, preliminary results in our laboratory showed a similar phenomenon using SP600125 (not shown), indicating a JNK mediated effect. However, in this study we extend the work and show this phenomenon to be correlated with an effect upon NFκB activation, MKP-2 was able to enhance TNFα induced IκBα loss or reverse the inhibitory effect of DN-IKKβ. This present study is one of the few demonstrating that long term JNK down-regulation can enhance NFκB activation (Sanchez-Perez *et al.*, 2002). Overwhelmingly, studies indicate the reverse phenomenon, NFκB limits the kinetics of JNK signalling (Papa *et al.*, 2006).

We then determined if MKP-2 had an effect upon HUVEC apoptosis, a process also influenced by the interplay between NFκB and JNK signalling. TNFα has been shown to be mediated apoptosis in HUVECs but only in certain conditions such as following cycloheximide pre-treatment or NFκB inhibition (Wadgaonkar *et*

al., 2004). A number of studies have shown that in endothelial and other cell types NFκB is able to mediate cellular protection through the enhanced expression of a number of protective proteins including Gadd45b (De Smaele *et al.*, 2001) and XIAP (Tang *et al.*, 2001) which function to suppress JNK activation. Recent studies have also shown that several antioxidant enzymes are under NFκB regulation and may also contribute to limiting JNK activity (Papa *et al.*, 2006) by inhibiting ROS generation. Under conditions of NFκB inhibition, TNFα was indeed found to sustain JNK activation in HUVECs (not shown) and cause a significant increase in apoptosis, results consistent with previous findings in endothelial cells (Stehlik *et al.*, 1998). MKP-2 significantly reversed apoptosis however the reversal was not total, suggesting the potential of both JNK -dependent and -independent pathways controlling apoptosis. A role for JNK in endothelial cell apoptosis has been previously established using pharmacological inhibitors (Fu, Yin et al. 2006; Karahashi, Michelsen et al. 2009) however, only one previous study has demonstrated that MKP-2 can mediate the same inhibition of JNK and thus regulate cell death (Cadalbert *et al.*, 2005).

In investigating the correlation between reversal of cell death and effects upon pro-apoptotic proteins we made a series of novel observations. Initially we found that TNFα in combination with DN-IKKβ results in increased phosphorylation of H2AX, a histone protein involved in regulating cellular responses to DNA damage (Kinner *et al.*, 2008). Initial studies indicated that this protein, was regulated by PIKKs. (Fernandez-Capetillo *et al.*, 2004), however our work suggests phosphorylation by JNK in agreement with a recent study in HeLa cells (Lu *et al.*, 2006). Interestingly, we also found that MKP-2 over-expression was also able to abolish the formation of cleaved caspase-3 proteins p19 and p17. Caspases are implicated in endothelial cell apoptosis mediated in response to TNFα (Daniel *et al.*, 2004) and a number of stressful agents such as high glucose and hydrogen peroxide (Ho *et al.*, 2000; Ramachandran *et al.*, 2002). In some of these models caspase-3 cleavage is regulated by JNK (Ho *et al.*, 2000; Ramachandran *et al.*, 2002). Translocation of caspase fragments is dependent upon prior cleavage of procaspase 3 in the cytosol and association with recognised substrates (Kamada *et al.*, 2005). Thus, as MKP-2 is strictly nuclear located, even at high viral concentrations, this suggests that to regulate caspase-3 cleavage in endothelial cells, JNK must be firstly phosphorylated within the

nucleus prior to translocation to the cytosol. Current dogma suggests roles for JNK both within the nucleus and mitochondria (Dhanasekaran *et al.*, 2008) however, a functional link between each compartment has not been established. Alternatively, it may be that a pool of MKP-2 is located in the mitochondria, as is the case with MKP-1 (Rosini *et al.*, 2004), and functions to directly regulate JNK activity within this compartment. In addition is the possibility that long term down regulation of JNK has a rebound effect upon the expression of anti-apoptotic proteins such as cFLIP, (Albrecht *et al.*, 2009; Suzuki *et al.*, 2003) XIAP, (Tang *et al.*, 2001) or A20 (Daniel *et al.*, 2004).

Overall, our studies have identified the potential for over-expression of MKP-2 to down regulate JNK-dependent COX-2 expression and up regulate NFκB dependent expression of ICAM and VCAM. We also show that MKP-2 is able to negate TNFα stimulated apoptosis by regulating not only nuclear events such as phosphorylation of H2AX but also cytosolic events such as caspase 3 cleavage. Thus, Adv.MKP-2 has a potential therapeutic use in clinical conditions were JNK mediated effects upon endothelial apoptosis is a feature. A number of previous studies have already utilised adenovirus infection to express proteins such TIMP-3 and p53 to inhibit vascular remodelling *in vitro* (George *et al.*, 2001; George *et al.*, 1998a; George *et al.*, 1998b) and *in vivo* (George *et al.*, 2000). Whilst these approaches are associated primarily with inhibiting smooth muscle cell migration and mediating apoptosis, other studies have delivered adenoviral eNOS to enhance re-endothelialisation and inhibit restenosis (Sharif *et al.*, 2008; Tanner *et al.*, 2004). Thus, the anti-apoptotic effects of Adv.MKP-2 over-expression in endothelial cells may be a useful outcome which can be exploited *in vivo*. It will be complicated however, by effects upon adhesion molecule expression which may enhance migration of macrophages to the sub-endothelial space. This may limit its clinical use.

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Figures

Figure 1. Adv.MKP-2 expression inhibits TNFα-stimulated JNK phosphorylation and activation in HUVECs.

HUVECs were infected with 300 p.f.u.cell^{-1} of Adv.MKP-2 or Lac Z (Panels A, C and D) or increasing concentrations as indicated (Panel B) for 40 h prior to stimulation with TNFα for a further 30 min (Panels B-D). In panel A cells were stained for MKP-2 expression. Samples were also assessed for phospho-JNK (Panel B) JNK activity (panel C) and phospho-c-Jun content (Panel D) as outlined in the Methods section. Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least 4 experiments. ***P<0.001 compared to TNFα stimulation alone.

Figure 2. Effect of Adv.MKP-2 expression on TNFα-stimulated ERK or p38 MAP kinase phosphorylation

HUVECs were infected with 300 p.f.u.cell^{-1} of Adv.MKP-2 or LacZ control for 40 h prior to stimulation with TNFα for a further 30 min. Samples were assessed for phospho-ERK (Panel A) and phospho-p38 MAP kinase (Panel B) as outlined in the Methods section. Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least 4 experiments. Ns –not significant compared to TNFα stimulation alone.

Figure 3. Differential effect of Adv.MKP-2 on TNFα-stimulated ICAM and VCAM and COX-2 expression in HUVECs

HUVECs were infected with 300 p.f.u.cell^{-1} of Adv.MKP-2 or LacZ control for 40 h prior to stimulation with TNFα for a further 24 h. Samples were assessed for MKP-2 levels, ICAM (Panel A), VCAM (Panel B) or COX-2 (Panel C) by Western blotting.

Results from gels were quantified using scanning densitometry. Each value represents the mean ± SEM of at least 4 experiments. ***P<0.001 compared to agonist stimulation alone.

Figure 4. Inhibition of NFκB signalling inhibits TNFα-stimulated ICAM, VCAM and COX-2 expression in HUVECs.

HUVECs were infected with 300 p.f.u.cell^{-1} Adv.DN-IKKβ or lacZ control for 40 h prior to stimulation with TNFα for a further 24 h. Samples were assessed for ICAM (Panel A), VCAM (Panel B) and COX-2 (Panel C). Results from gels quantified using scanning densitometry. Each value represents the mean ± SEM of at least 4 experiments. **P<0.01 and ***P<0.001 compared to agonist stimulation alone.

Figure 5. Combined effect of DN-IKKβ and MKP-2 co-expression on TNFα-stimulated COX-2 induction in HUVECs.

HUVECs were infected with 300 p.f.u.cell^{-1} Adv.DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation with TNFα for a further 24 h. Samples were assessed for COX-2 expression (Panel A) as outlined in the Methods section. Gels were quantified by densitometry (Panel B) and each value represents the mean ± S.E.M. from four experiments. *P<0.05 and **P<0.01 compared to agonist stimulation alone, ns-not significant compared to Adv.MKP-2 inhibition.

Figure 6. MKP-2 co-expression reverses DN-IKKβ inhibition of ICAM and VCAM expression in TNFα-stimulated HUVECS.

HUVECs were infected with 600 p.f.u.cell^{-1} Lac Z, 300 p.f.u.cell^{-1} Adv.DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation with TNFα for a further 24 h. Samples were assessed for ICAM or VCAM expression (Panel A) as outlined in the Methods section. Data from blots where quantified by densitometry (Panel B) and each value represents the mean ± S.E.M. of at least four experiments. *P<0.05 compared to agonist stimulation alone, ns-not significant compared to either control or Adv.MKP-2 inhibition.

Figure 7. MKP-2 expression reverses DN-IKKβ inhibition of TNFα - stimulated IκBα loss in HUVECs.

HUVECs were infected with 600 p.f.u.cell^{-1} Lac Z, or 300 p.f.u.cell^{-1} Adv.DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation with TNFα (1ngml^{-1}) for a further 30 min. Samples were assessed for IκBα or total p65 NFκB expression as outlined in the Methods section. Each blot is representative of three others. Data from blots where quantified by densitometry (Panel B) and each value represents the mean ± S.E.M. of four experiments. *P<0.01 compared to TNFα/DN-IKKβ alone. +P<0.05 compared to MKP−2 inhibition.

Figure 8. Adv.MKP-2 infection reverses TNFα-mediated apoptosis in HUVECS.

HUVECs were infected with 600 p.f.u.cell^{-1} Lac Z, or 300 p.f.u.cell^{-1} Adv. DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation with TNFα for a further 24 h. Samples where assessed for Annexin V and 7-AAD staining as outlined in the Methods section (Panels A & B) whilst in parallel control experiments cell samples were Western blotted for IKKβ and MKP-2 expression (Panel C). In Panel B each value represents the mean ± S.E.M. of at least 4 experiments. *P<0.05 compared to TNFα/DN-IKKβ stimulation alone.

Figure 9. MKP-2 over-expression reverses TNFα-induced H2AX phosphorylation and caspase-3 cleavage in HUVECs.

HUVECs were infected with either 600 p.f.u.cell^{-1} Lac Z, or 300 p.f.u.cell^{-1} Adv.DN-IKKβ or Adv.MKP-2 alone or in combination for 40 h prior to stimulation for a further 24 h. Samples were assessed for phospho-H2AX (Panel A), caspase 3 (Panel B), IKKβ, or MKP-2 expression as outlined in the Methods section. Data from blots where quantified by densitometry. Each value represents the mean ± S.E.M. of at least 4 experiments. **P<0.01 compared to TNFα/DN-IKKβ alone. T-p38 represents a loading control.

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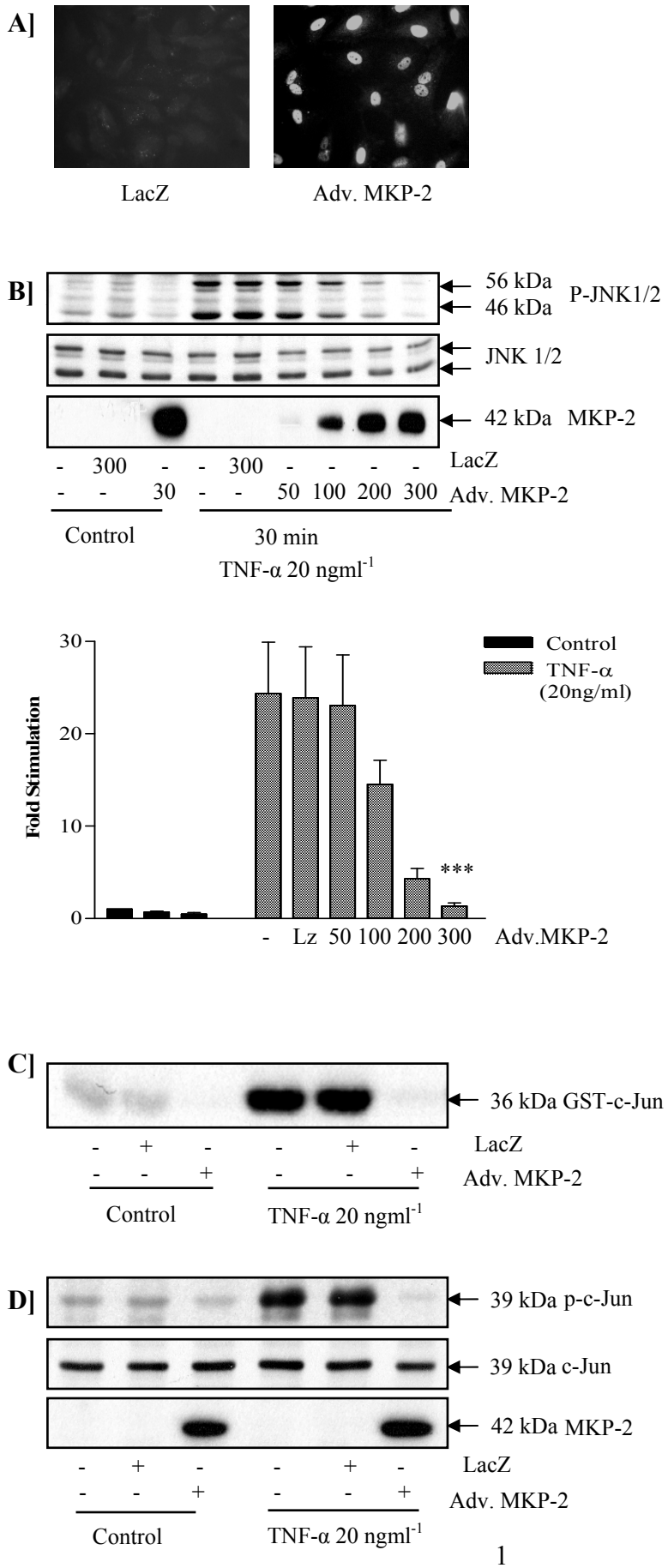
Figure 1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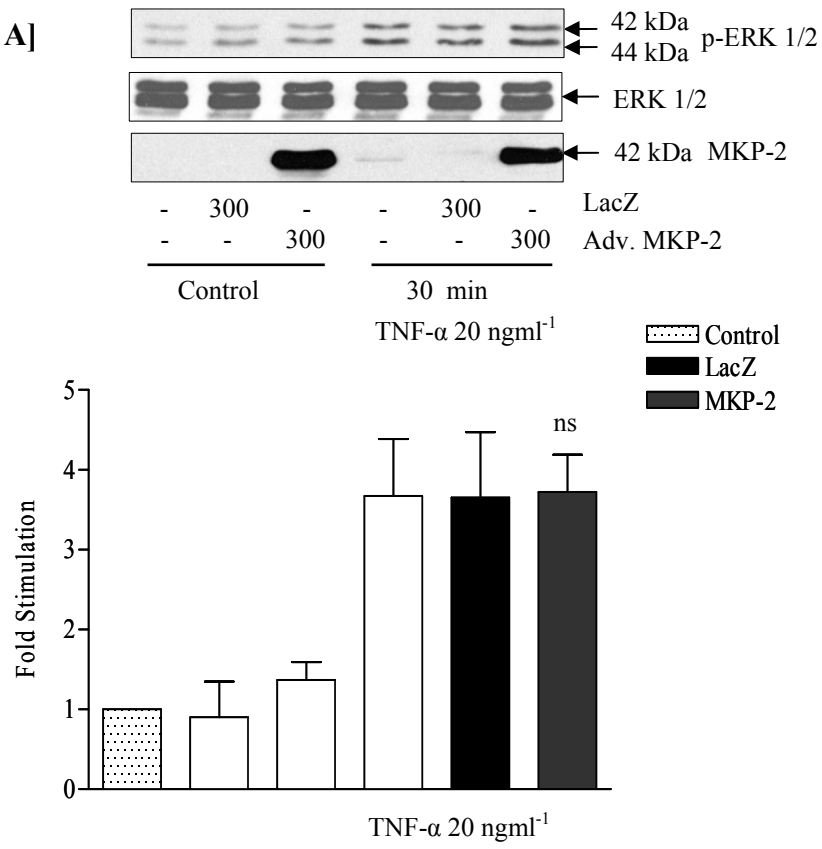
LacZ
Adv. MKP-2
B]
56 kDa
46 kDa
P-JNK1/2
JNK 1/2
42 kDa MKP-2
- 300 - - 300 - - - - LacZ
- - 30 - - 50 100 200 300 Adv. MKP-2
Control
30 min
TNF-α 20 ngml-1
Fold Stimulation
30
20
10
0
Control
TNF-α (20ng/ml)
***
- Lz 50 100 200 300 Adv.MKP-2
C]
36 kDa GST-c-Jun
- + - - + - LacZ
- - + - - + Adv. MKP-2
Control
TNF-α 20 ngml-1
D]
39 kDa p-c-Jun
39 kDa c-Jun
42 kDa MKP-2
- + - - + - LacZ
- - + - - + Adv. MKP-2
Control
TNF-α 20 ngml-1

Figure 2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42 kDa
44 kDa
p-ERK 1/2
ERK 1/2
42 kDa MKP-2
- 300 - - 300 - LacZ
- - 300 - - 300 Adv. MKP-2
Control
30 min
TNF-α 20 ngml-1
Control
LacZ
MKP-2
ns
Fold Stimulation
0
1
2
3
4
5
TNF-α 20 ngml-1

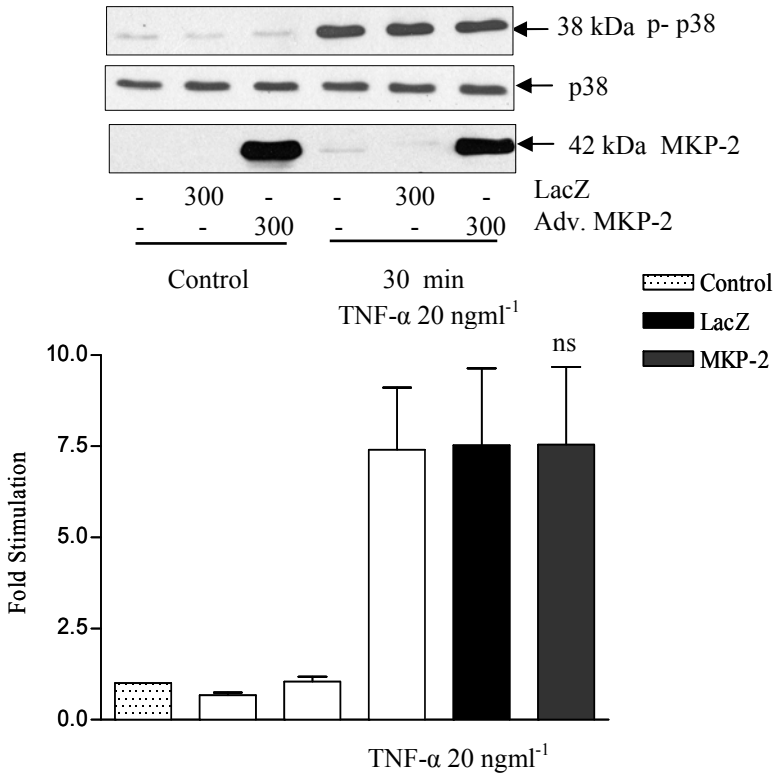
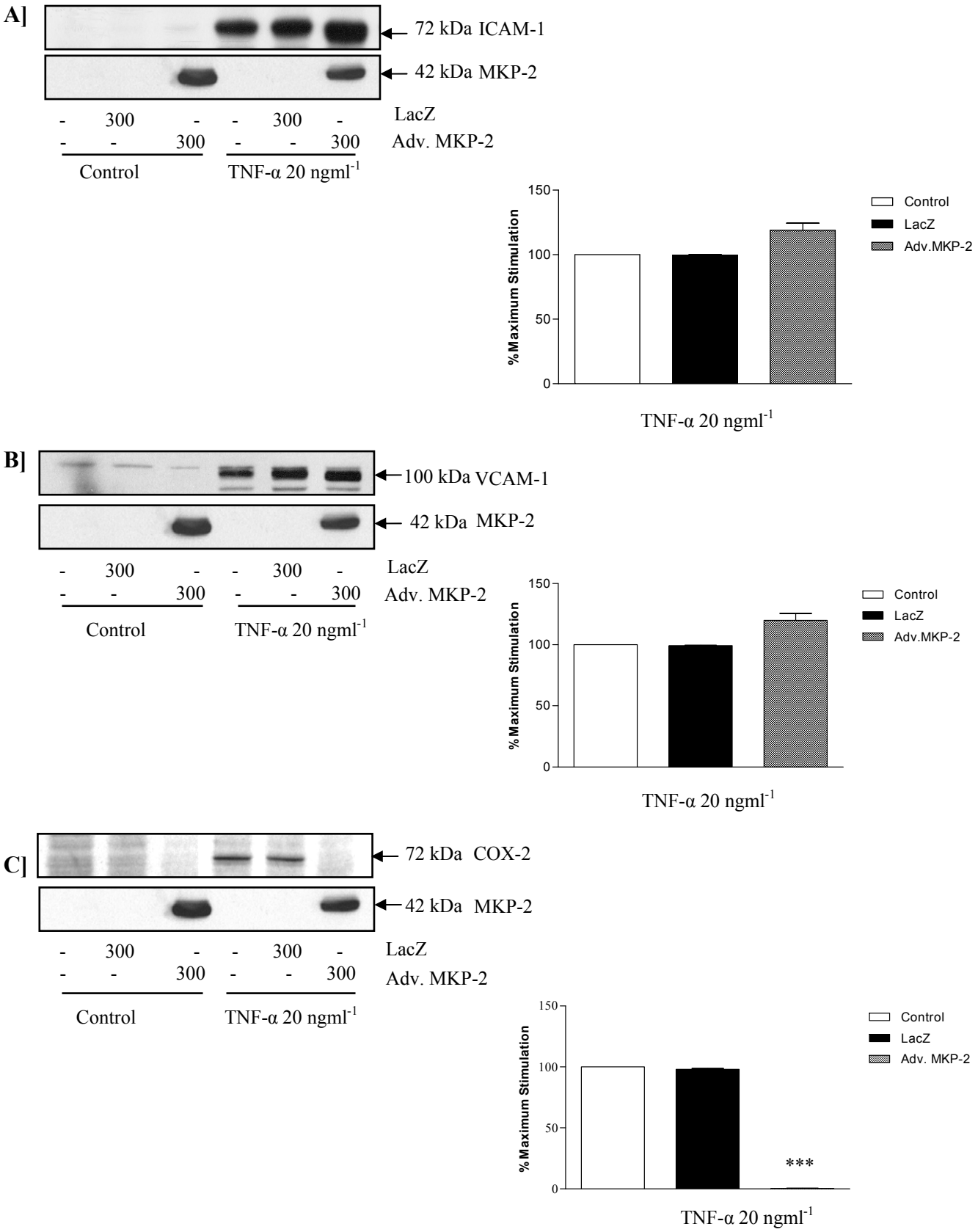

B]
38 kDa p- p38
p38
42 kDa MKP-2
- 300 - - 300 - LacZ
- - 300 - - 300 Adv. MKP-2
Control
30 min
TNF-α 20 ngml-1
Control
LacZ
MKP-2
ns
Fold Stimulation
0.0
2.5
5.0
7.5
10.0
TNF-α 20 ngml-1

Figure 3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72 kDa ICAM-1
42 kDa MKP-2
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv. MKP-2
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
Control
LacZ
Adv.MKP-2
TNF-α 20 ngml-1
B]
100 kDa VCAM-1
42 kDa MKP-2
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv. MKP-2
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
Control
LacZ
Adv.MKP-2
TNF-α 20 ngml-1
C]
72 kDa COX-2
42 kDa MKP-2
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv. MKP-2
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
***
Control
LacZ
Adv. MKP-2
TNF-α 20 ngml-1

Figure 4

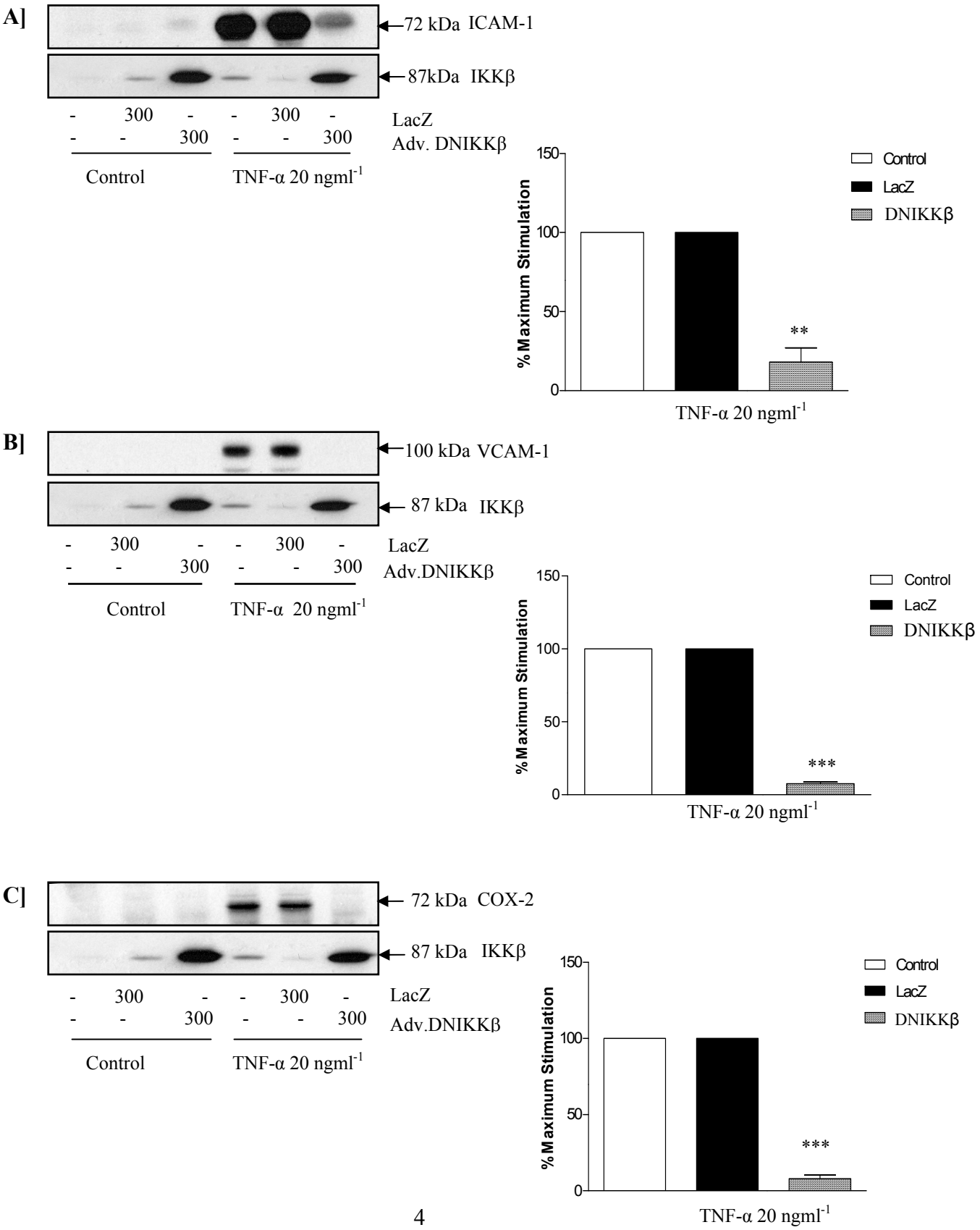
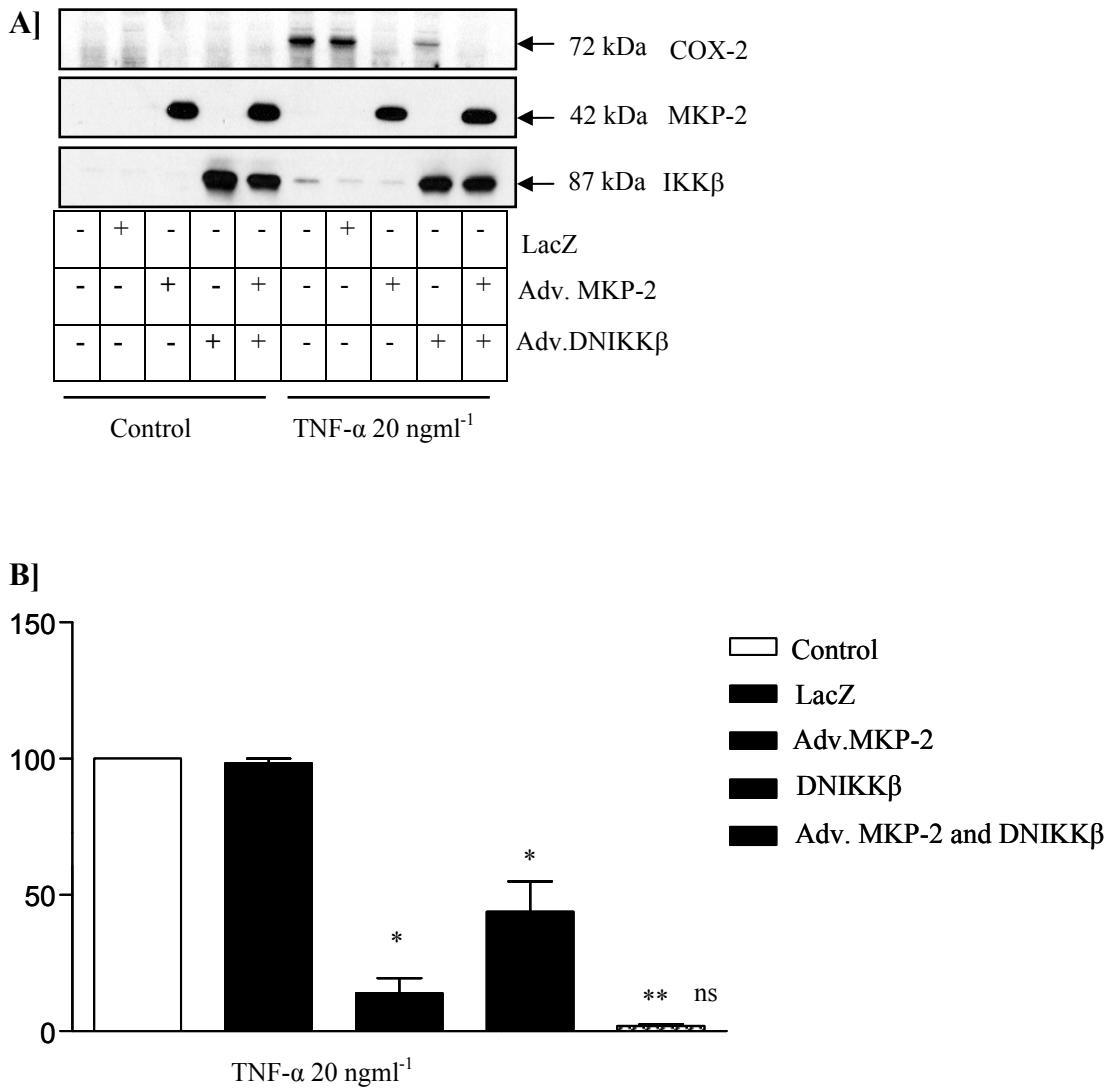

A]
72 kDa ICAM-1
87kDa IKKβ
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv. DNIKKβ
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
**
Control
LacZ
DNIKKβ
TNF-α 20 ngml-1
B]
100 kDa VCAM-1
87 kDa IKKβ
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv.DNIKKβ
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
***
Control
LacZ
DNIKKβ
TNF-α 20 ngml-1
C]
72 kDa COX-2
87 kDa IKKβ
- 300 - - 300 -
- - 300 - - 300
LacZ
Adv.DNIKKβ
Control
TNF-α 20 ngml-1
% Maximum Stimulation
150
100
50
0
***
Control
LacZ
DNIKKβ
TNF-α 20 ngml-1

Figure 5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72 kDa COX-2
42 kDa MKP-2
87 kDa IKKβ
LacZ
Adv. MKP-2
Adv.DNIKKβ
Control
TNF-α 20 ngml-1
B]
% Maximum Stimulation
150
100
50
0
Control
LacZ
Adv.MKP-2
DNIKKβ
Adv. MKP-2 and DNIKKβ
*
*
**
ns
TNF-α 20 ngml-1

Figure 6

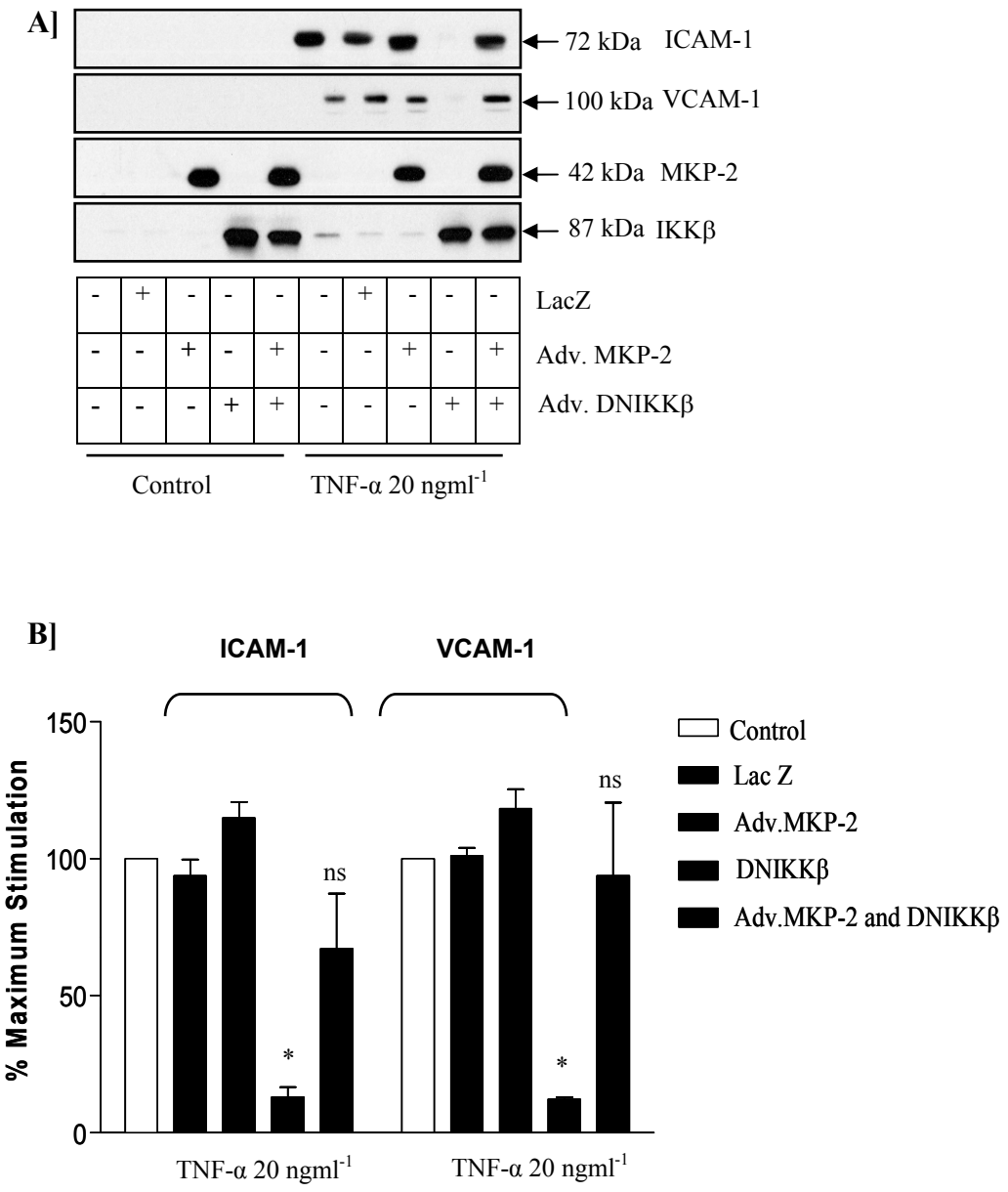
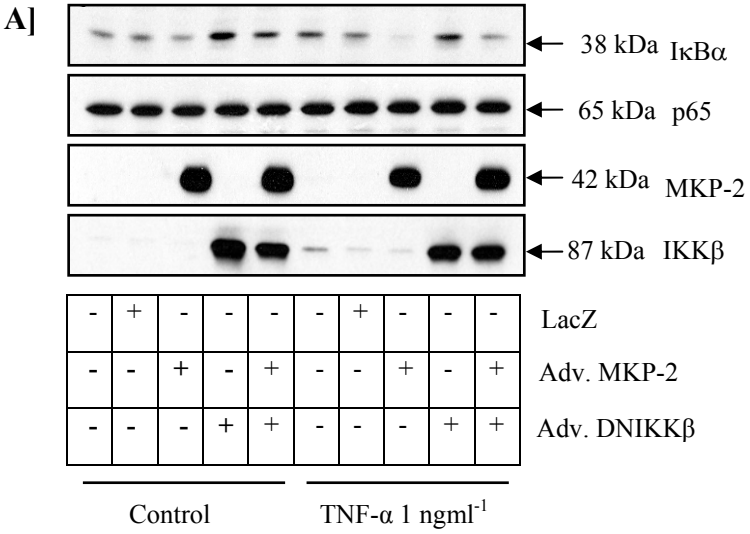

A]
72 kDa ICAM-1
100 kDa VCAM-1
42 kDa MKP-2
87 kDa IKKβ
LacZ
Adv. MKP-2
Adv. DNIKKβ
Control
TNF-α 20 ngml-1
B]
ICAM-1
VCAM-1
% Maximum Stimulation
150
100
50
0
ns
*
Control
Lac Z
Adv.MKP-2
DNIKKβ
Adv.MKP-2 and DNIKKβ
TNF-α 20 ngml-1
TNF-α 20 ngml-1

Figure 7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38 kDa IκBα
65 kDa p65
42 kDa MKP-2
87 kDa IKKβ
LacZ
Adv. MKP-2
Adv. DNIKKβ
Control
TNF-α 1 ngml-1

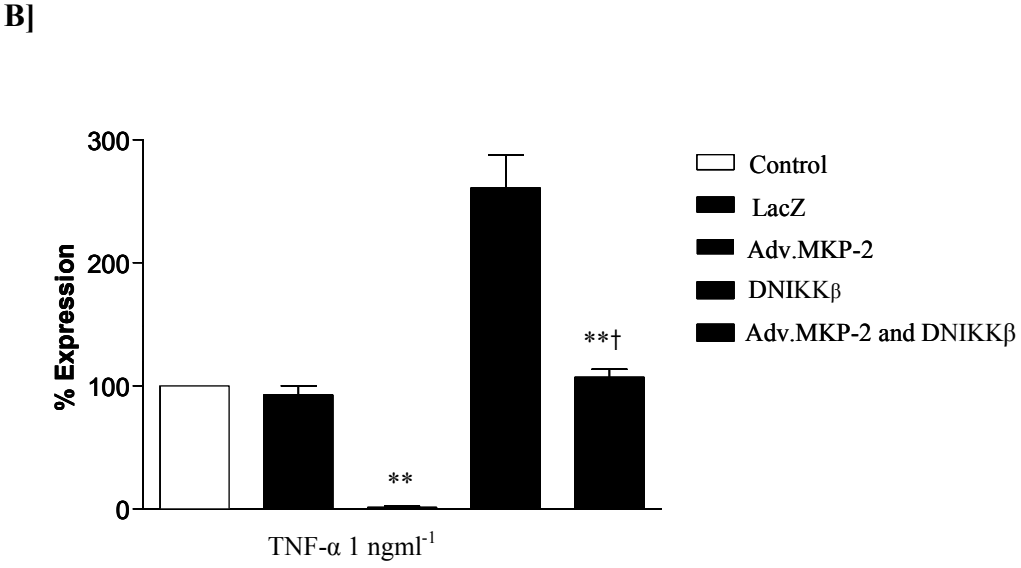
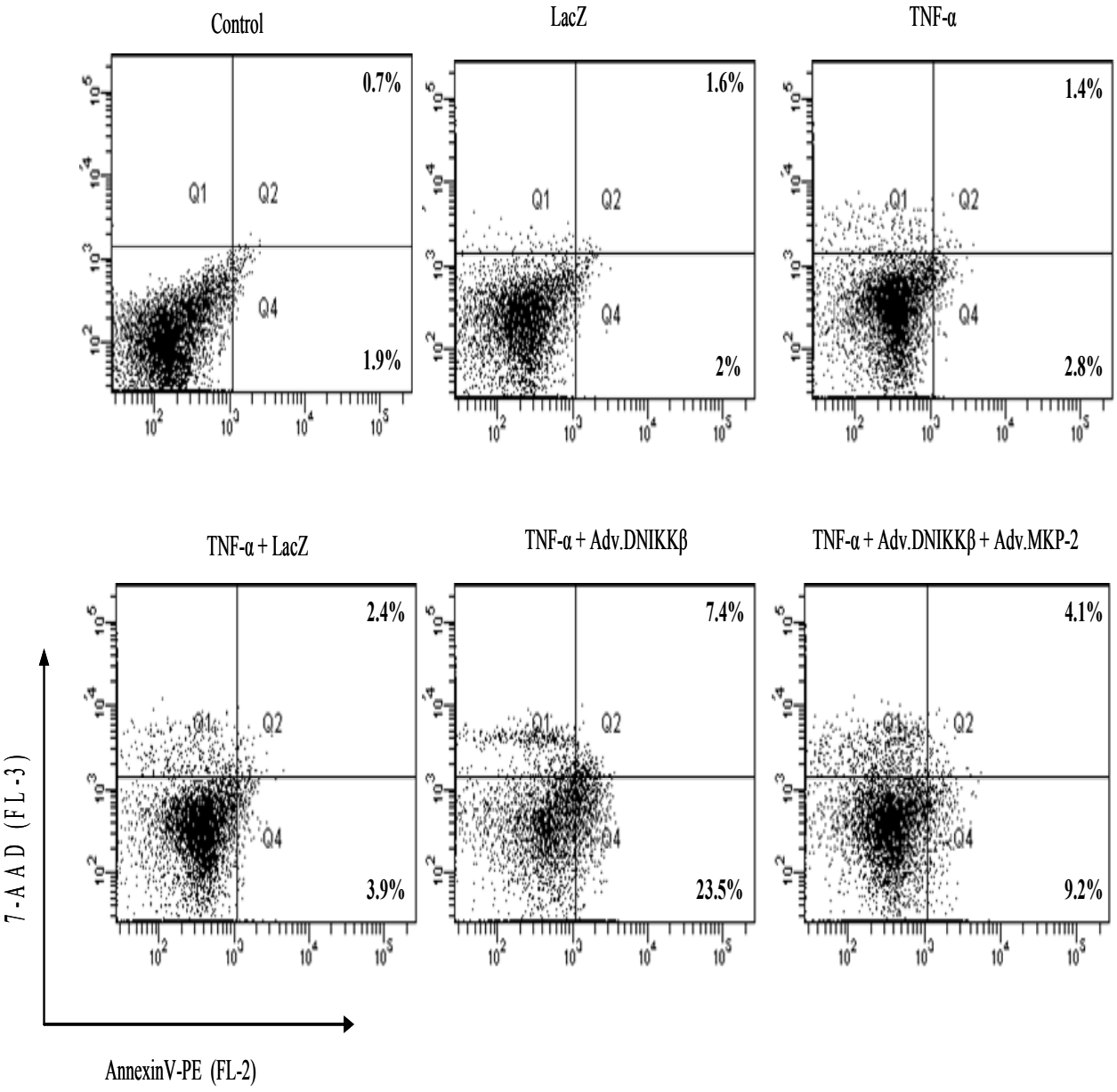
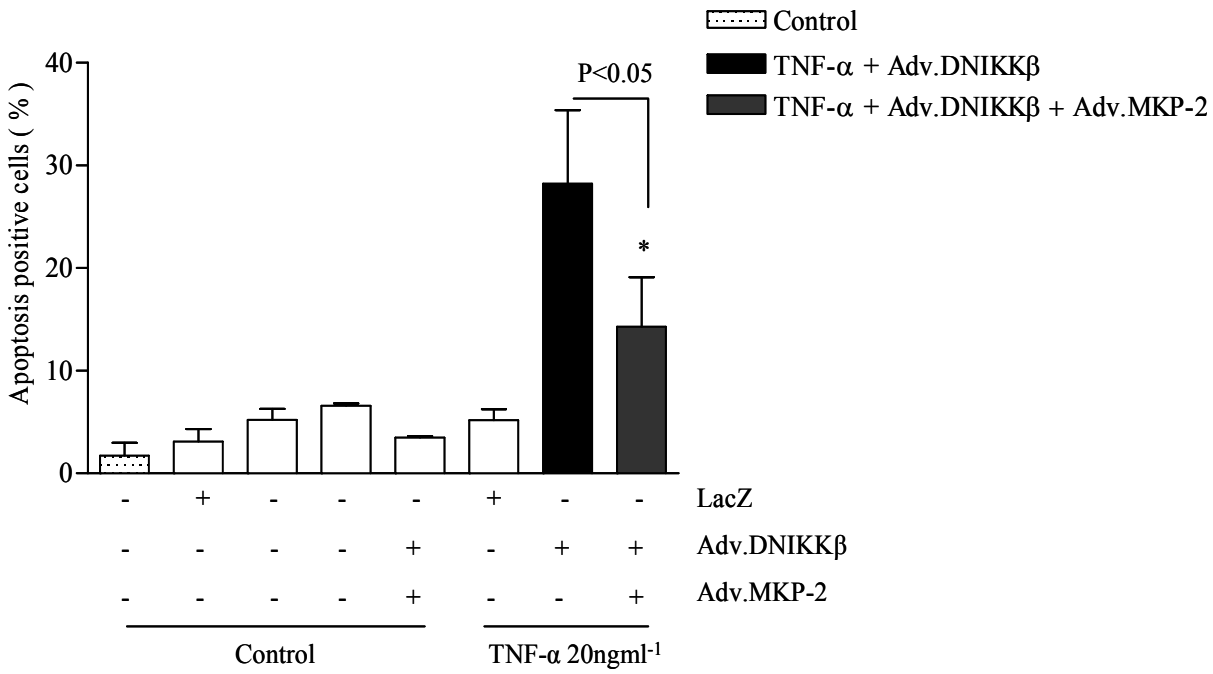

B]
% Expression
300
200
100
0
**
**†
Control
LacZ
Adv.MKP-2
DNIKKβ
Adv.MKP-2 and DNIKKβ
TNF-α 1 ngml-1

Figure 8

A]


Control
LacZ
TNF-α
0.7%
1.9%
1.6%
2%
1.4%
2.8%
TNF-α + LacZ
TNF-α + Adv.DNIKKβ
TNF-α + Adv.DNIKKβ + Adv.MKP-2
2.4%
3.9%
7.4%
23.5%
4.1%
9.2%
Q1
Q2
Q4
7-AAD (FL-3)
AnnexinV-PE (FL-2)

B]


Control
TNF-α + Adv.DNIKKβ
TNF-α + Adv.DNIKKβ + Adv.MKP-2
Apoptosis positive cells (%)
40
30
20
10
0
P<0.05
*
- + - - - + - - LacZ
- - - - + - + + Adv.DNIKKβ
- - - - + - - + Adv.MKP-2
Control
TNF-α 20ngml-1

C]

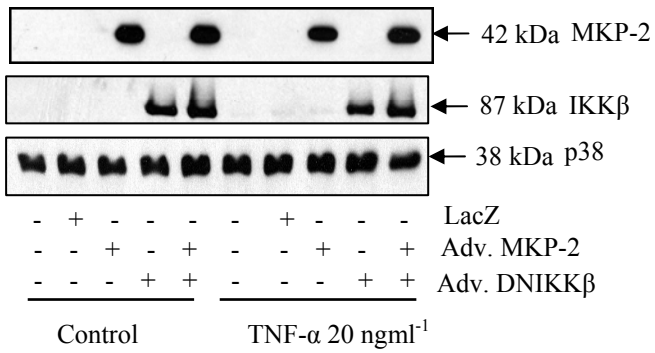
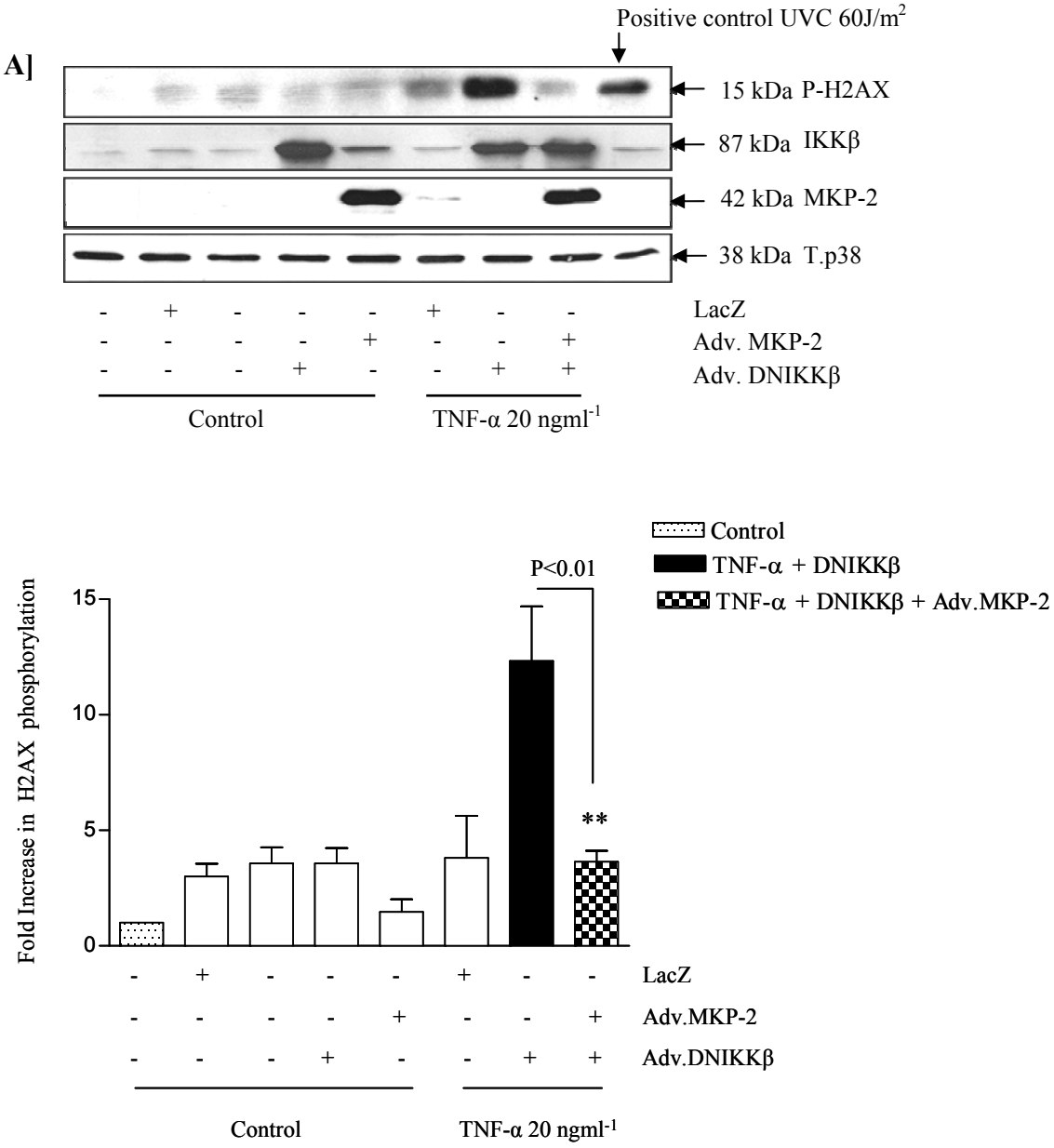
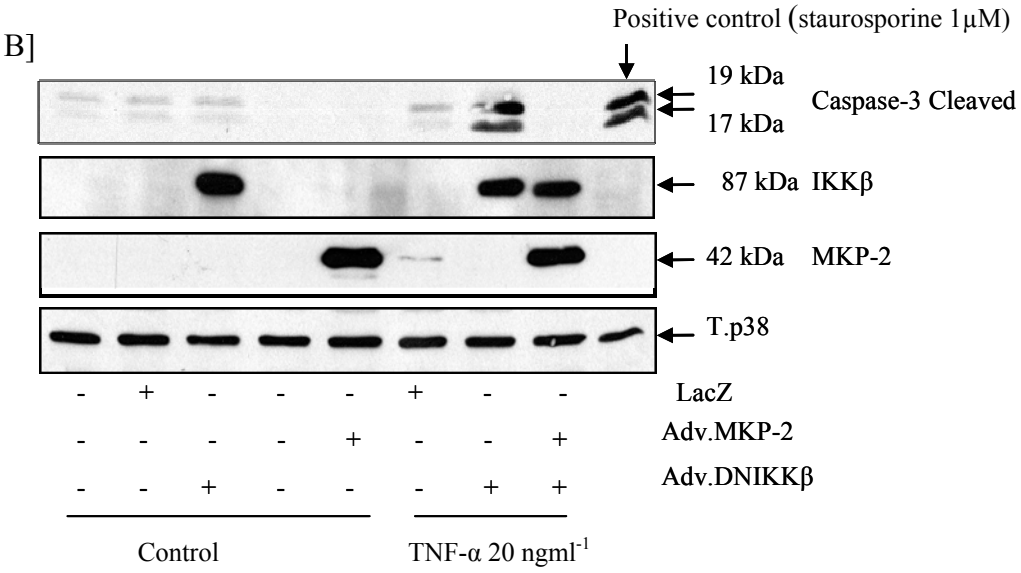
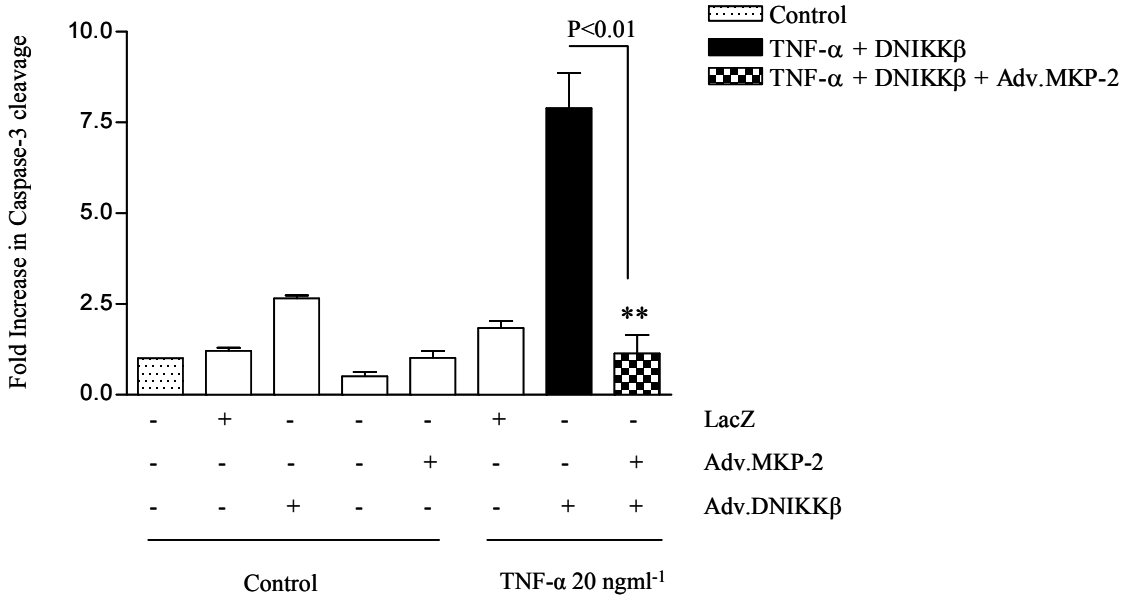

42 kDa MKP-2
87 kDa IKKβ
38 kDa p38
- + - - - - + - - - LacZ
- - + - + - - + - + Adv. MKP-2
- - - + + - - - + + Adv. DNIKKβ
Control
TNF-α 20 ngml-1

Figure 9


A]
Positive control UVC 60J/m2
15 kDa P-H2AX
87 kDa IKKβ
42 kDa MKP-2
38 kDa T.p38
LacZ
Adv. MKP-2
Adv. DNIKKβ
Control
TNF-α 20 ngml-1
Control
TNF-α + DNIKKβ
TNF-α + DNIKKβ + Adv.MKP-2
P<0.01
**
Fold Increase in H2AX phosphorylation
15
10
5
0
LacZ
Adv.MKP-2
Adv.DNIKKβ
Control
TNF-α 20 ngml-1


B]
Positive control (staurosporine 1μM)
19 kDa
17 kDa
Caspase-3 Cleaved
87 kDa IKKβ
42 kDa MKP-2
T.p38
LacZ
Adv.MKP-2
Adv.DNIKKβ
Control
TNF-α 20 ngml-1


Control
TNF-α + DNIKKβ
TNF-α + DNIKKβ + Adv.MKP-2
Fold Increase in Caspase-3 cleavage
10.0
7.5
5.0
2.5
0.0
P<0.01
**
LacZ
Adv.MKP-2
Adv.DNIKKβ
Control
TNF-α 20 ngml-1