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TRADD, TRAF2, RIP1 and TAK1 are required for TNF-α-induced pro-labour mediators in human primary myometrial cells

Running Title: TNF-α signalling in human myometrium

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ABSTRACT

Problem: TNF-α plays a central role in the processes of human labour and delivery. This study sought to determine the role of the adaptor proteins TNFR1-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), receptor interacting protein 1 (RIP1) and transforming growth factor beta-activated kinase 1 (TAK1) in TNF-α-induced formation of pro-labour mediators.

Method of Study: Human primary myometrial cells were transfected with siRNA against TRADD (siTRADD), TRAF2 (siTRAF2), RIP1 (siRIP1) or TAK1 (siTAK1), treated with TNF-α, and assayed for pro-inflammatory mediators expression.

Results: siTRADD, siTRAF2, siRIP1 and siTAK1 significantly decreased TNF-α-induced IL-1α, IL-1β, IL-6, IL-8, MCP-1 mRNA expression and release of IL-6, IL-8 and MCP-1; and cyclooxygenase (COX)-2 expression and release of prostaglandin $PGF_{2\alpha}$. There was a significant attenuation of TNF-α-induced expression of adhesion molecules ICAM-1 and VCAM-1 mRNA with siTRADD, siTRAF2 or siRIP1. siTRADD and siRIP1 significantly attenuated TNF-α-induced MMP-9 mRNA expression and release and nuclear factor κB (NF-κB) transcriptional activity. There was a significant increase in TNF-α-induced sVCAM-1 release, MMP-9 mRNA expression and NF-κB activity with siTAK1.

Conclusions: TRADD, TRAF2, RIP1 and TAK1 are involved in TNF-α signalling in human myometrium. Further studies are required to determine if inhibition of these proteins can prevent preterm birth.

INTRODUCTION

Preterm birth occurs in approximately 10% of all births worldwide [1]. The ensuing prematurity of the newborns is the biggest contributing factor to perinatal morbidity and mortality [2]. Many children born preterm will suffer life-long neurological and developmental problems. In addition to emotional stress on families faced with preterm birth and having a newborn in intensive care, the financial costs for individuals and health care systems are enormous. There are no therapeutics that can reduce the high incidence of preterm birth and prevent its adverse outcomes. Thus, a greater understanding of the mechanisms regulating term and preterm parturition are required.

TNF-α, released from leukocytes invading the intrauterine cavity during parturition [3], plays an important role in the processes of human labour and delivery. Studies in humans demonstrated higher amniotic fluid concentrations of TNF-α in patients who subsequently delivered preterm than

in patients who delivered at term [4]. Likewise, high concentrations of TNF-α in the vaginal or cervical secretions in women with symptoms of preterm labour are associated with early preterm delivery [5]. Elevated TNF-α levels are also detected in amniotic fluid during pregnancies complicated by infection and preterm delivery in humans [6]. In non-human primates, injection of the amniotic cavity with TNF-α induces preterm labour [7]. *In vivo*, intraamniotic infusion of TNF-α is associated with significant elevations in pro-inflammatory cytokines, chemokines, prostaglandins, MMP-9 and leukocytes in amniotic fluid [7]. Likewise, *in vitro*, TNF-α can amplify or initiate the process of parturition by further increasing cytokine production [8]; promoting the synthesis of cyclooxygenase (COX)-2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and the $PGF_{2\alpha}$ receptor (FP) [9-11] which are important regulators of myometrial contractility; activating the extracellular matrix remodelling enzyme matrix metalloproteinase (MMP)-9 [12]; and induce the expression of adhesion molecules [11] that can further recruit leukocytes into the uterus. Therefore, understanding the intermediates involved in the TNF-α signalling pathway is critical for the development of therapeutics to manage or prevent preterm birth.

The canonical (or classical) NF-κB pathway has a major role in the control of innate immunity and inflammation [13]. The NF-κB pathway also plays a central role in the terminal processes of human labour and delivery [14]. In human gestational tissue, NF-κB is activated by a broad range of stimuli including TNF-α. Following stimulation, IκB-α, which typically holds the NF-κB RelA–p50 heterodimer in the cytoplasm, is phosphorylated by IκB kinase (IKK), leading to its rapid degradation. This allows free NF-κB to translocate to the nucleus where it binds to κB sites, leading to gene transcription. In human myometrium, NF-κB has been shown to control the transcription of a number of pro-labour genes including IL-6, IL-8, COX-2 and MMP-9.

The adaptor proteins TNFR1-associated death domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1 and cIAP2), receptor interacting protein 1 (RIP1) and transforming growth factor beta-activated kinase 1 (TAK1) have emerged as critical signalling intermediates of TNF-α-induced inflammation [15-18]. TNF-α binds to its cell surface receptor TNFR1 which leads to the recruitment of the intracellular death domain (DD)-containing adaptor TRADD. Recruitment of TRADD can i) promote the association of the TNFR1 complex with Fas-associated DD (FADD) which induces caspase activation and cell death; or ii) recruit TRAF2, RIP1, cIAPs and TAK1, which trigger NF-κB activation and resultant pro-inflammatory responses [19].

We have previously shown cIAP1 and cIAP2 are required for TNF-α-induced expression of pro-labour mediators in human myometrium [9]. The role, however, of TRADD, TRAF2, RIP1 and TAK1 in the genesis of pro-inflammatory and pro-labour mediators induced by TNF-α in myometrium is not known. Thus, the aim of this study was to use siRNA to determine if TRADD, TRAF2, RIP1 and TAK1 are involved in TNF-α-induced expression and secretion of pro-inflammatory cytokines; COX-2 expression and subsequent prostaglandin release; expression and secretion of adhesion molecules ICAM-1 and VCAM-1; and expression of the ECM remodelling enzyme MMP-9 in primary human myometrial cells.

MATERIALS AND METHODS

Tissue collection

The Research Ethics Committee of Mercy Hospital for Women approved this study. Written, informed consent was obtained from all participating women. All myometrial samples were obtained from women who delivered healthy, singleton infants at term (37-41 weeks gestation) undergoing elective Caesarean section in the absence of labour. All tissues were brought to the research laboratory and processed within 15 mins of the Caesarean delivery. Women with any underlying medical conditions such as diabetes, asthma, polycystic ovary syndrome, preeclampsia and macrovascular complications were excluded. Additionally, women with multiple pregnancies, obese women, and fetuses with chromosomal abnormalities were excluded.

Primary myometrial cell culture

Cells were isolated and cultured as previously described [11]. Briefly, myometrium was minced and digested for 1 h in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) with 3 mg/ml type 1 collagenase (Worthington Biochemical, Freehold, USA) and 80 μg/ml DNase 1 (Roche Diagnostics, Castle Hill, Australia) at 37°C. Cells were centrifuged at 400 × g for 10 min and grown in DMEM/F-12 enriched with 10% heat-inactivated FCS (containing 100 U/ml penicillin G and 100 mg/ml streptomycin).

TRADD, TRAF2, RIP1 and TAK1 siRNA transfection

Transfection of primary myometrial cells was performed as we have previously described [11]. Briefly, cells at approximately 50% confluence were transfected using Lipofectamine 3000 according to manufacturer's guidelines (Life Technologies; Mulgrave, Victoria, Australia). TRADD siRNA (siTRADD), TRAF2 siRNA (siTRAF2), TAK1 siRNA (siTAK1) and negative control siRNA (siCONT) were obtained from Ambion (Thermo Fisher Scientific; Scoresby, Vic, Australia).

RIP1 siRNA (siRIP1) was obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were transfected with 50 nM siRNA (TRADD, TRAF2 or TAK1) or 100 nM siRNA (RIP1) in DMEM/F-12 for 48 h followed by treatment with or without 10 ng/ml TNF-α (PeproTech; Rocky Hill, NJ, USA) for 24 h. Cells were collected and stored at −80°C until assayed for mRNA expression by qRT-PCR as detailed below. Media was collected and stored at −80°C until assayed for cytokine and prostaglandin release as detailed below. Cell viability was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) proliferation assay as we have previously described [11]. The response to TNF-α between patients varied greatly, as we have previously reported [11]. Thus, data is presented as fold change in expression relative to the expression level in TNF-α-stimulated siCONT transfected cells, which was set at 1. Experiments were performed from myometrium obtained from five patients.

NF-κB luciferase assay

A luciferase assay was also used to determine the possible interactions between TRADD, TRAF2, RIP1 and NF-κB as previously described [11]. Primary myometrial cells were transfected with 300 ng/ml NF-κB reporter construct (Qiagen; Chadstone Centre, Vic, Australia) using FuGENE HD transfection reagent (Promega; Alexandria NSW, Australia). After 6 h, cells were transfected with 50 nM siRNA (TRADD, TRAF2 or TAK1) or 100 nM of siRIP1 or siCONT (as detailed above) for 48 h. The medium was then replaced with DMEM/F-12 (containing 0.5% BSA), with or without 10 ng/ml TNF-α and the cells incubated at 37°C for an additional 20 h. After final incubation, cells were harvested in lysis buffer, and luminescence activity was measured using a Luciferase Reporter Assay Kit (Life Research; Scoresby, Vic, Australia) and Renilla Luciferase Flash Assay kit (Thermo Fisher Scientific; Scoresby, Vic, Australia) as instructed. The ratio of the firefly luciferase level to the Renilla luciferase level was determined and the results are expressed as a ratio of normalised luciferase activity. The experiments were performed from myometrium obtained from five patients.

RNA extraction and qRT-PCR

RNA extractions and qRT-PCR was performed as previously described [11]. RNA quality and integrity were measured using a NanoDrop ND1000 and determined via the A_{260}/A_{280} ratio. RNA (0.2 μg) was converted to cDNA using the high-capacity cDNA reverse transcription kit according to the manufacturer's instructions (Applied Biosystems; Waltham, MA, USA). RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia) using 100 nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen; Chadstone Centre, Vic, Australia). Average gene Ct values were

normalised against two housekeeping genes (β2-Microglobulin (B2M) and succinate dehydrogenase complex subunit A (SDHA)). Fold differences were determined using the comparative Ct method.

Enzyme immunoassays

Assessment of cytokine and chemokine release of IL-6 and IL-8 was performed using the CytoSet™ sandwich ELISA according to the manufacturer's instructions (Life Technologies; Mulgrave, Vic, Australia). The release of MCP-1, sICAM-1 and sVCAM-1 was performed by sandwich ELISA from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. The release of $PGF_{2\alpha}$ into the incubation medium was assayed using a commercially available competitive enzyme immunoassay kit according to the manufacturer's specifications (Kookaburra Kits from Sapphire Bioscience, NSW, Australia). The interassay and intraassay coefficients of variation for all assays were less than 10%.

Gelatin zymography

Incubation media was also collected and assessment of MMP-9 was performed by gelatin zymography as previously described [20]. Proteolytic activity was visualized as clear zones of lysis on a blue background of undigested gelatin. Gels were scanned using a ChemiDoc XRS system (Bio-Rad Laboratories; Gladesville, NSW, Australia), inverted, and densitometry performed using ~~using~~ Quantity One image analysis software (Bio-Rad Laboratories; Gladesville, NSW, Australia). Fold change was calculated relative to TNF-α, which was set at 1.

Statistical analysis

All statistical analyses were undertaken using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The homogeneity of data was assessed by the Bartlett's test, and when significant, the data were logarithmically transformed before further analysis using a one-way ANOVA (with LSD post-hoc testing to discriminate among the means). Statistical significance was ascribed to a P value <0.05. Data were expressed as mean ± SEM.

RESULTS

Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on pro-inflammatory cytokines and chemokines

The efficacy of siRNA transfection is demonstrated in Supplementary Figure 1. When compared to siCONT transfected cells, there was ~85% decrease in TRADD mRNA with siTRADD; 60% decrease in TRAF2 mRNA expression with siTRAF2; 70% decrease in RIP1 mRNA expression

with siRIP1; and 90% decrease in TAK1 mRNA expression with siTAK1. A MTT cell viability assay showed no difference in absorbance between cells transfected with siCONT or siTRADD, siTRAF2, siRIP1 or siTAK1 (Supplementary Figure 1).

For subsequent experiments, after siRNA transfection, cells were treated with TNF-α. As expected, in siCONT transfected cells, TNF-α significantly increased IL-6, IL-8 and MCP-1 mRNA expression and secretion (Figure 1). This increase was significantly decreased in siTRADD (Figures 1A-F), siTRAF2 (Figures 1A-F), siRIP1 (Figures 1G-L) or siTAK1 (Figures 1M-R) transfected cells. In addition, IL-1α and IL-1β mRNA expression was also increased by TNF-α treatment in siCONT transfected cells. The effect of siTRADD, siTRAF2, siRIP1 or siTAK1 was a significant decrease in IL-1α and IL-1β mRNA expression (Table I). As we have previously reported, IL-1α and IL-1β levels are not detectable in the incubation media from human primary myometrial cells [10] and thus not assessed.

Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on COX-2-prostaglandin pathway

Treatment of primary myometrial cells with TNF-α significantly increased COX-2 (Figures 2A,D,G) and FP (Figures 2B,E,H) mRNA expression, and subsequent $PGF_{2\alpha}$ release (Figures 2C,F,I). The effect of siTRADD (Figures 2A-C), siTRAF2 (Figures 2A-C), siRIP (Figures 2D-F) or siTAK1 (Figures 2G-I) was a significantly attenuation of TNF-α-induced COX-2 and FP mRNA expression and $PGF_{2\alpha}$ release.

Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on adhesion molecules

As expected, ICAM-1 and VCAM-1 mRNA expression, and sICAM-1 and sVCAM-1 were significantly increased in siCONT cells incubated with TNF-α (Figure 3). TNF-α-induced ICAM-1 mRNA expression and sICAM-1 release was significantly attenuated in cells transfected with siTRADD (Figures 3A,B), siTRAF2 (Figures 3A,B), siRIP1 (Figures 3E,F), or siTAK1 (Figures 3I,J). TNF-α-induced VCAM-1 mRNA expression was significantly suppressed by siTRADD (Figures 3C), siTRAF2 (Figures 3C), siRIP1 (Figures 3G), or siTAK1 (Figures 3K). Further, TNF-α-induced sVCAM-1 release was also significantly decreased by siTRADD (Figure 3D) or siRIP1 (Figure 3H). There was no effect of siTRAF2 on TNF-α-induced sVCAM-1 release (Figure 3D), while siTAK1 significantly augmented TNF-α-induced sVCAM-1 release (Figure 3L).

Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on MMP-9

Figure 4 demonstrates the effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression of the ECM degrading and remodelling enzyme MMP-9. In siCONT transfected cells, TNF-α

significantly increased MMP-9 mRNA expression (Figures 4A,C,E) and pro MMP-9 secretion (Figures 4B,D,F). The effect of siTRADD (Figure 4A), siTRAF2 (Figure 4A) or siRIP1 (Figure 4C) was a significant suppression of TNF-α-induced MMP-9 mRNA. In addition, siTRADD (Figure 4B and siRIP1 (Figure 4D) also significantly reduced TNF-α-induced pro MMP-9 secretion. There was, however, no effect of siTRAF2 on pro MMP-9 secretion (Figure 4B). Unexpectedly, siTAK1 significantly augmented MMP-9 mRNA expression (Figure 4E) and pro MMP-9 secretion (Figure 4F).

Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on NF-κB activation

The final aim of this study was to investigate if TRADD, TRAF2, RIP1 and TAK1 elicits their effects via interfering with NF-κB activation. As expected, TNF-α significantly increased NF-κB transcriptional activity (Figure 5). The result of siTRADD (Figure 5A) and siRIP1 (Figure 5C) was a significant suppression of TNF-α induced NF-κB activation. On the other hand, there was no effect of siTRAF2 on TNF-α induced NF-κB activation (Figure 5B) while siTAK1 further augmented TNF-α induced NF-κB activation (Figure 5D).

DISCUSSION

The novel findings of this study are that in human myometrium, TRADD, TRAF2, RIP1 and TAK1 are required for TNF-α-induced expression of pro-labour mediators. Specifically, loss-of-function studies demonstrated that in the absence of TRADD, TRAF2, RIP1 and TAK1, the expression and or secretion of pro-inflammatory cytokines, chemokines, prostanoids and cell adhesion molecules to TNF-α was, for the most part, significantly blunted. These findings are in concordance with studies in non-gestational tissues [15-18].

TNF-α exists in two biologically active forms, a membrane-bound form (mTNF-α) and a soluble form (sTNF-α), which bind to their cognate receptors TNFR1 and TNFR2. Engagement of TNFR1 rapidly leads to the assembly of a membrane associated protein complex (termed complex I) consisting of TRADD, TRAF2 and RIP1. Activation of TNFR1 recruits the adapter protein TRADD and the serine–threonine kinase RIP1. TRADD can further recruit the adapter protein TRAF2 via its N-terminal TRAF-binding domain. Complex 1 can activate numerous signalling pathways to induce inflammation, activate both pro-apoptotic and anti-apoptotic pathways or promote cell growth [21]. Pro-inflammatory signalling, however, is the most favoured pathway through the recruitment of TAK1 and the IKK complex resulting in NF-κB activation. Complex 1

also activates activator protein (AP)-1 and mitogen-activated protein kinase (MAPK) signalling by recruitment of TAK1.

In this study, knockdown of TRADD, TRAF2, RIP1 or TAK1 in primary myometrial cells significantly suppressed TNF-α-induced expression and secretion of pro-inflammatory cytokines and chemokines. These findings are of significance given the central role of pro-inflammatory cytokines and chemokines in the processes of labour and delivery~~, both at term and preterm~~ [8]. Concomitant with increased macrophage and neutrophil infiltrate [3], myometrium of healthy labouring women is associated with an upregulation of mRNA expression of numerous pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) and chemokines (e.g. IL-8, MCP-1) [22]. Pro-inflammatory cytokines can then further induce: (i) cytokine release through positive feed forward mechanism; (ii) the expression of adhesion molecules; (iii) COX-2 expression and subsequent prostaglandin production, and (iv) ECM matrix remodelling enzymes such as MMP-9. Collectively, these events facilitate human labour and delivery.

Prostaglandins play an important role in the regulation of parturition. COX-2 is the rate limiting enzyme involved in the conversion of arachidonic acid into prostaglandins. Increased expression of COX-2 observed in myometrium before labour onset [23] is responsible for the increased synthesis of prostaglandins observed during labour. $PGF_{2\alpha}$ is an important mediator of uterine contractility [24], exerting its actions via its receptor, FP [25] whose expression is also increased at term labour in myometrium [26]. TNF-α has been shown to induce the expression of COX-2 and FP [9-11] and stimulate the synthesis of prostanoids [9, 10] in human myometrial cells. In this study, we show that TRADD, TRAF2, RIP1 or TAK1 are required for TNF-α-induced COX-2 and FP mRNA expression and resultant prostaglandin secretion.

The expression of the cell adhesion molecules such as ICAM-1 and VCAM-1 are increased in human cervix and myometrium during pregnancy and parturition [27, 28]; in part due to the expression of infiltrating leukocytes in the myometrium, cervix and fetal membranes at parturition [3, 29]. These adhesion molecules can then attract neutrophils, macrophages and T cells to invade these tissues. Cytokines, induced by stretch, play an essential role in leukocyte migration [30]. We have previously shown that TNF-α can induce ICAM-1, VCAM-1 and MMP-9 mRNA expression and secretion from human myometrial cells [11]. TNF-α can also induce the expression of MMP-9 [11, 31] which is involved in tissue remodelling of human myometrium during labour. In this study, we demonstrated a requirement for TRADD, TRAF2 and RIP1 in TNF-α-induced ICAM-1, VCAM-1 and MMP-9 expression and/or secretion in primary myometrial cells. Of note, while VCAM-1 and MMP-9

mRNA expression were decreased by siTRAF2, the secretion of sVCAM-1 and pro MMP-9 were unchanged; possibly due to post-translation modifications. Furthermore, and in contrast to siTRADD, siTRAF2 or siRIP1, siTAK1 significantly augmented MMP-9 mRNA expression and pro MMP-9 secretion. The reason for the increase in MMP-9 is not known; but ~~may~~ it may be due to activation of pathways downstream of TAK1 (discussed below).

Mice with genetic knockout of TRADD, TRAF2, RIP1 or TAK1 have clearly demonstrated their roles in TNF-α-induced NF-κB signalling [17, 32-34]. *In vitro* studies, using siRNA, have confirmed the role of these proteins in TNF-α signalling in a numbers of different cells. For example, TRADD, TRAF2 or TAK1 are required for TNF-α-induced NF-κB activation in mouse embryonic fibroblasts (MEFs) and RIP1-deficient Jurkat T cells are specifically defective in the activation of NF-κB in response to TNF-α stimulation [15-18]. In support, this study reports that TRADD and RIP1 are required for TNF-α-induced NF-κB signalling in human myometrial cells. On the other hand, there was no effect of siTRAF2 on NF-κB transcriptional activity while NF-κB activity was increased by siTAK1. TRAF2 [35] and TAK1 [36], in addition to NF-κB, can also transduce signals to c-Jun N-terminal kinase (JNK) and or p38 mitogen-activated protein kinase (p38 MAPK) of the mitogen-activated protein kinase (MAPK) pathway. Ultimately, other transcription factors downstream of p38 MAPK and JNK are activated including c-Jun and ATF2, resulting in the transcription of genes important for inflammatory and immune responses. p38 MAPK is involved in the regulation of pro-inflammatory and pro-labour mediators in fetal membranes [37] while specific inhibitors of JNK delays preterm labour and decreases inflammation in a mouse model of preterm birth [38]. It is thus possible that TRAF2 and TAK1 regulate pro-inflammatory and pro-labour mediators in human myometrial cells via the p38 MAPK and/or JNK signalling pathways. Alternatively, only one time point was analysed in this study; it is possible that longer or shorter incubations are required with TNF-α to see an effect on NF-κB transcriptional activity.

An avenue of further research, is the effect of siTRADD, siTRAF2, siRIP1 or siTAK1 on TNF-α-induced uterine contractions. The myometrium is maintained in a relatively quiescent state during pregnancy but is transformed into a contractile state at term labour. TNF-α has been shown to enhance contractions in human primary uterine smooth muscle cells [39]. A number of proteins, termed contractions associated proteins (CAPs), have been shown to be important in this process, including FP. Given that we showed that TRADD, TRAF2, RIP1 or TAK1 are required for TNF-α-induced FP mRNA expression, it would also be of interest to determine if they play a role in regulating myometrial contraptions in response to TNF-α treatment.

In conclusion, for the first time, we report that TRADD, TRAF2, RIP1 and TAK1 play a role in the regulating TNF-α signalling in human myometrium. These findings are of significance given the central role of TNF-α in the processes of human labour and delivery. Further studies are required to elucidate the significance of TRADD, TRAF2, RIP1 and TAK1 in human labour and delivery, and whether inhibition of these proteins may be able to prevent preterm birth. Notably, inhibition of TAK1 has been reported to block inflammation in sheep. Specifically, intra-amniotic treatment with the TAK1 inhibitor 5z-7-oxozeaeno reduced amniotic fluid levels of PGE_2 and fetal membrane leukocyte infiltration induced by the bacterial product LPS [40].

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DISCLOSURE SUMMARY

The authors have nothing to declare.

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Table I. Effect of siTRADD, siTRAF2, siRIP and siTAK1 on IL-1α and IL-β mRNA expression

	IL-1α mRNA expression	IL-1β mRNA expression
siCONT	0.04±0.02*	0.00±0.00*
siCONT + TNF-α	1.00±0.00	1.00±0.00
siTRADD + TNF-α	0.50±0.16*	0.12±0.03*
siTRAF2 + TNF-α	0.50±0.10*	0.03±0.02*
siCONT	0.02±0.00*	0.00±0.00*

siCONT + TNF-α	1.00±0.00	1.00±0.00
siRIP1 + TNF-α	0.41±0.07*	0.28±0.12*
siCONT	0.05±0.03*	0.03±0.01*
siCONT + TNF-α	1.00±0.00	1.00±0.00
siTAK1 + TNF-α	0.23±0.09*	0.09±0.03*

For all data, the fold change was calculated relative to TNF-α-stimulated siCONT transfected cells, and displayed as mean ± SEM.

*P<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

FIGURE LEGENDS

Figure 1. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on pro-inflammatory cytokines and chemokines.

Human primary myometrial cells were transfected with **(A-F)** 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, **(G-L)** 100 nM siRIP1 or 100 nM siCONT, or **(M-R)** 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF-α for an additional 24 h (n=5 patients). **(A,C,E,G,I,K,M,O,Q)** IL-6, IL-8 and MCP-1 mRNA expression was analysed by qRT-PCR. **(B,D,F,H,J,L,N,P,R)** IL-6, IL-8 and MCP-1 concentration in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to TNF-α-stimulated siCONT transfected cells, and displayed as mean ± SEM. *P<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

Figure 2. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the COX-2-prostaglandin pathway.

Human primary myometrial cells were transfected with **(A-C)** 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, **(D-F)** 100 nM siRIP1 or 100 nM siCONT, or **(G-I)** 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF-α for an additional 24 h (n=5 patients). **(A,B,D,E,G,H)** COX-2 and FP mRNA expression was analysed by qRT-PCR. **(C,F,I)** $PGF_{2\alpha}$ concentration in the incubation medium was assayed by ELISA. For all data, the fold change was calculated relative to TNF-α-stimulated siCONT transfected cells, and displayed as mean ± SEM. *P<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

Figure 3. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression and secretion of adhesion molecules.

Human primary myometrial cells were transfected with **(A-D)** 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, **(E-H)** 100 nM siRIP1 or 100 nM siCONT, or **(I-L)** 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF-α for an additional 24 h (n=5 patients). **(A,C,E,G,I,K)** ICAM-1 and VCAM-1 mRNA expression was analysed by qRT-PCR. **(B,D,F,H,J,L)** sICAM-1 and sVCAM-1 concentrations in the incubation media was assayed by ELISA. For all data, the fold change was calculated relative to TNF-α-stimulated siCONT transfected cells and data displayed as mean ± SEM. **P*<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

Figure 4. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on the expression and secretion of MMP-9 expression.

Human primary myometrial cells were transfected with **(A,B)** 50 nM siTRADD, 50 nM siTRAF2 or 50 nM siCONT, **(C,D)** 100 nM siRIP1 or 100 nM siCONT, or **(E,F)** 50 nM siTAK1 or 50 nM siCONT for 48 h and then treated with 10 ng/ml TNF-α for an additional 24 h (n=5 patients). **(A,C,E)** MMP-9 mRNA expression was analysed by qRT-PCR. **(B,D,F)** The incubation medium was assayed for pro MMP-9 expression by gelatin zymography. Representative zymography image from 1 patient is shown. For all data, the fold change was calculated relative to TNF-α-stimulated siCONT transfected cells and data displayed as mean ± SEM. **P*<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

Figure 5. Effect of siTRADD, siTRAF2, siRIP1 and siTAK1 on NF-κB activation.

Human myometrial cells were transfected with 300 ng/ml NF-κB reporter construct. After 6 h, cells were transfected with **(A)** 50 nM siTRADD or 50 nM siCONT, **(B)** 50 nM siTRAF2 or 50 nM siCONT, **(C)** 100 nM siRIP1 or 100 nM siCONT, or **(D)** 50 nM siTAK1 or 50 nM siCONT for 48 h, then treated with 10 ng/ml TNF-α for an additional 20 h (n=5 patients). Promoter activity is expressed as a ratio of luciferase activity of TNF-α stimulated siCONT transfected cells. All data displayed as mean ± SEM. **P*<0.05 vs. TNF-α-stimulated siCONT transfected cells (one-way ANOVA).

Figure 1

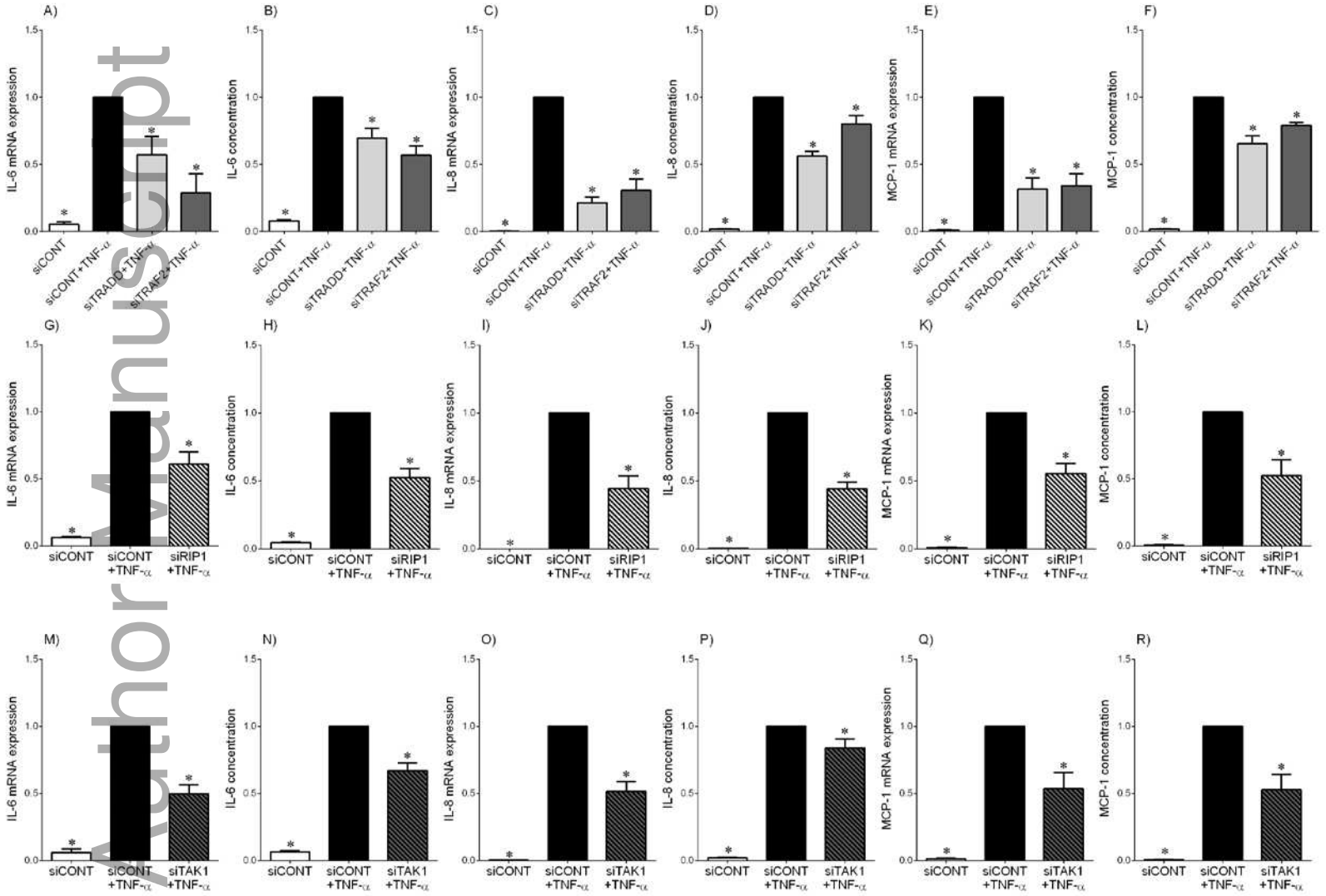

Figure 2

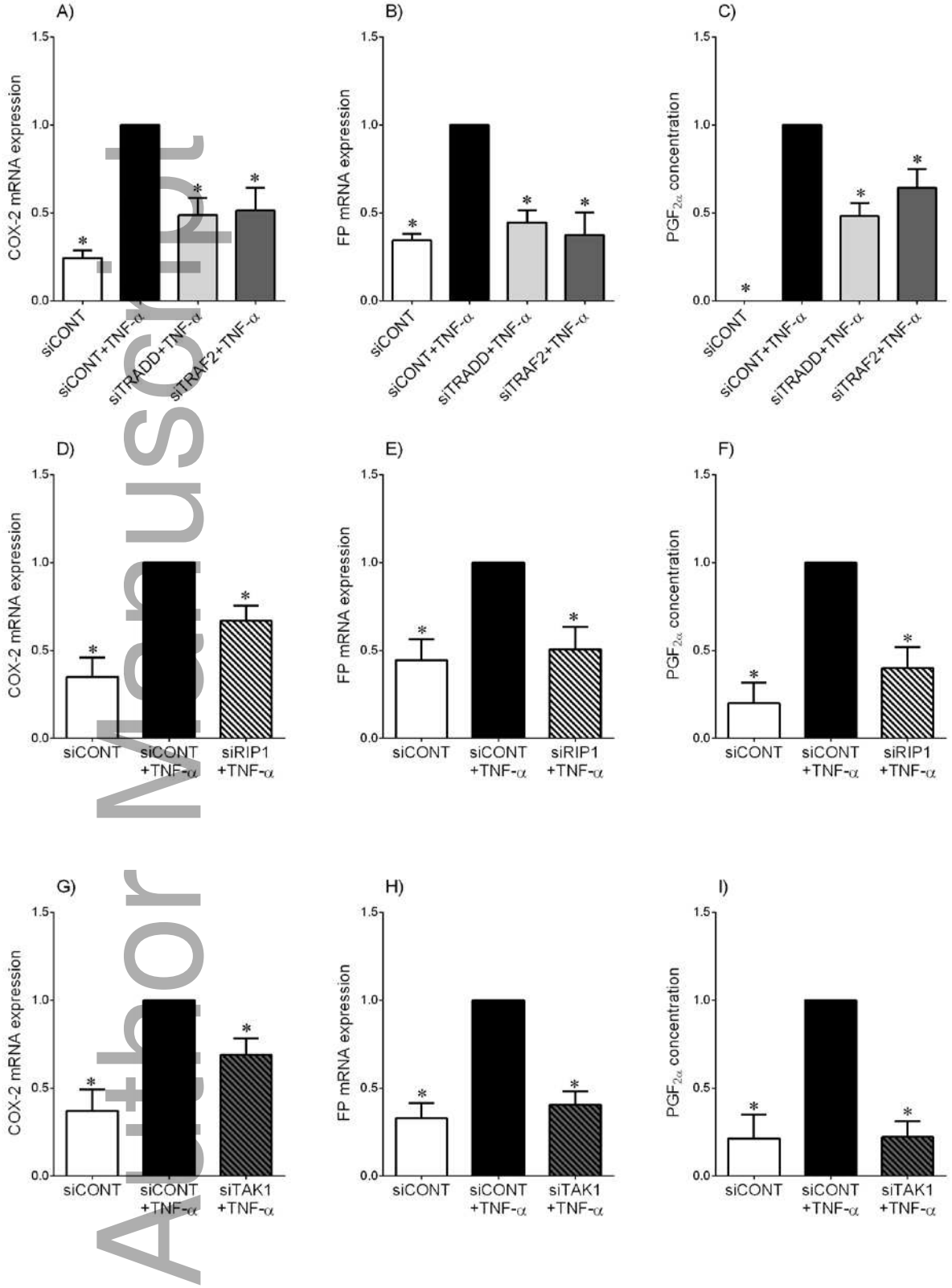

Figure 3

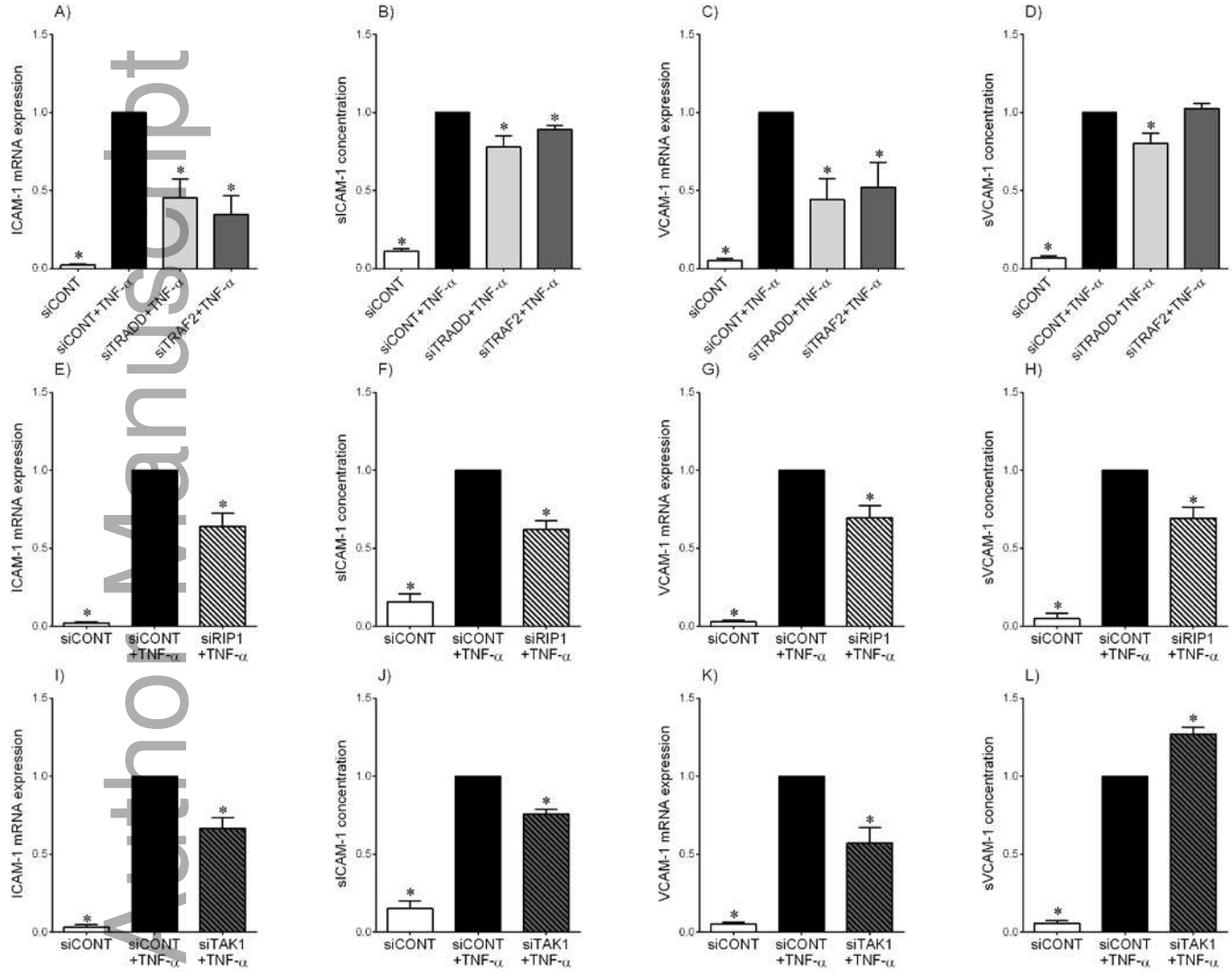

Figure 4

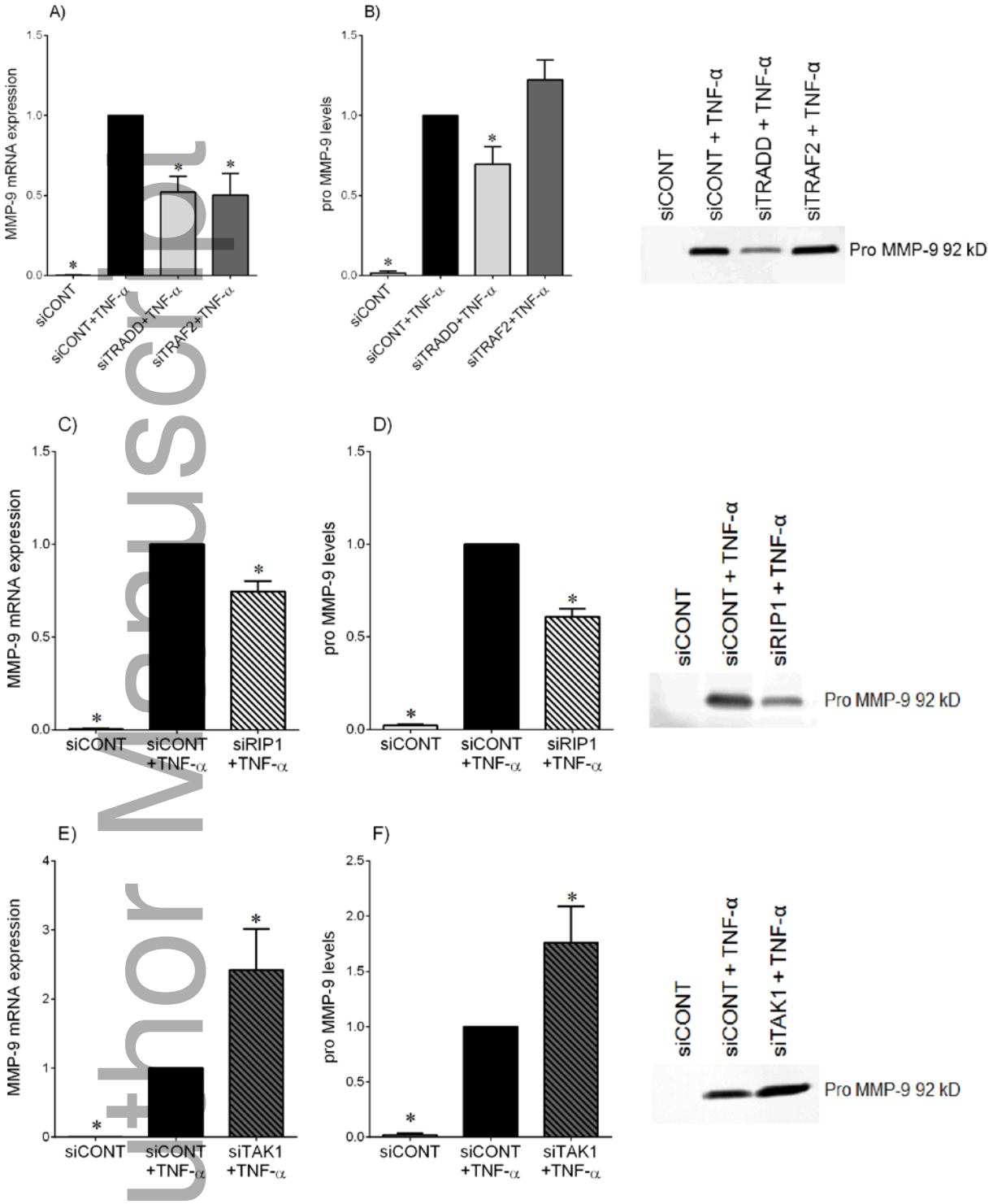

Figure 5