Myosin II Light Chain Phosphorylation Regulates Membrane Localization and Apoptotic Signaling of Tumor Necrosis Factor Receptor-1

Yijun Jin[1], Simon J. Atkinson[2], James A. Marrs[2], and Patricia J. Gallagher[1#]

[1]Department of Physiology
[2]Department of Medicine
Indiana University School of Medicine
Indianapolis, IN 46202

#Address Correspondence to:
Patricia J. Gallagher
Department of Physiology
635 Barnhill Drive
Indianapolis, IN 46202-5120

317.278.2146 PHONE
317.274.3318 FAX
pgallag@iupui.edu

Total number of characters: 43,902

Key Words: tumor necrosis factor receptor, myosin, myosin light chain kinase

Summary

Activation of myosin II by myosin light chain kinase (MLCK) produces force for many cellular processes including muscle contraction, mitosis, migration and other cellular shape changes. Results in this study show that inhibition or potentiation of myosin II activation via over-expression of a dominant negative or wild type MLCK can delay or accelerate TNF-induced apoptotic cell death in cells. Changes in the activation of caspase-8 that parallel changes in RLC phosphorylation levels reveal that myosin II motor activities regulate TNFR-1 signaling at an early step in the TNF death signaling pathway. Treatment of cells with ionomycin or endotoxin (LPS) both lead to activation of myosin II and increased translocation of TNFR-1 to the plasma membrane independent of TNF signaling. The results of these studies establish a new role for myosin II motor activity in regulating TNFR-1 mediated apoptosis through the translocation of TNFR-1 to or within the plasma membrane.

Running Title

Myosin II regulation of TNFR-1 apoptotic signaling

Introduction

The inflammatory cytokine tumor necrosis factor-α(TNF)[1] has an important role in many diverse cellular events, including cell proliferation and apoptosis (1-4). TNF signals through two receptors, TNFR-1 and TNFR-2, which are members of the TNF receptor superfamily. Of the two receptors for TNF, TNFR-1 is principally associated with signaling which results in either apoptosis or activation of the transcription factor NF-kB (5). Binding of TNF to TNFR-1 initiates death signaling by inducing trimerization of TNFR-1, aggregation of the cytoplasmic death domains and recruitment of TNF receptor-associated death domain protein (TRADD) to the cytoplasmic death domain of TNFR-1 (3,6-9). Subsequently, Fas-associated death domain protein (FADD/MORT1) is recruited to form a death-inducing signaling complex (DISC), which initiates apoptosis through recruitment and activation of procaspase-8 (FLICE/MACH/Mch5)(3,10-12). Auto-activation of the initiator caspase, caspase-8, occurs upon oligomerization following its recruitment to FADD (13) and is a key step in the execution of the death receptor pathway for apoptosis. Caspase-8 then activates downstream effector caspases, which cleave structural and regulatory proteins necessary for cell survival (14-16).

While many of the molecules involved in transducing TNF mediated cell death *in vivo* have been identified, there are still aspects of TNF signaling that are not understood, particularly with respect to the cellular mechanisms that regulate trafficking and translocation of TNFR-1 and DISC components to form an active death signaling complex. In unstimulated cells TNFR-1 is primarily localized to the trans-Golgi network (17) and therefore must move to the plasma membrane to be accessible to TNF.

Because Golgi vesicles are known to be associated with actin, myosin IIA, and myosin IIB, it is likely that myosin motor activities are an important regulatory component of this translocation step (18-20). A role for myosin II motor activities in TNF has been suggested in a previous study where an inhibitor of myosin light chain kinase (MLCK), the principal protein kinase responsible for activation of myosin II, delayed TNF induced apoptotic DNA fragmentation (21). In this report we describe studies examining the role of myosin II motor activities in regulating the intracellular trafficking of TNFR-1 and its associated DISC proteins, during TNF-induced apoptosis. The results presented in this study highlight a new role for myosin II motor activities at an early step of apoptotic signaling that regulates translocation of TNFR-1 to or within the plasma membrane.

Materials and Methods

Reagents and Antibodies. In all experiments, murine or human TNF-α (Calbiochem, La Jolla, CA) was used at 10 ng/mL and gave the same results. Ionomycin and lipopolysaccharide (LPS, endotoxin) were from Calbiochem (La Jolla, CA). Polyclonal antibodies to TRADD and poly-ADP ribose polymerase (PARP) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA Cruz). Anti-procaspase-8 and FADD were from Calbiochem (La Jolla, CA). Polyclonal antibody to TNFR-1 was from Stressgen (Victoria, B.C., Canada). A polyclonal antibody to purified myosin II regulatory light chains (RLC), was generated and characterized in this laboratory.

Cell lines and cell culture. MDCK cell lines expressing either wild type (WT) or a kinase dead (KD) MLCK under the control of a tetracycline-repressible transactivator were constructed by co-transfection of pTRE-MLCK plasmids and pTK-Hyg into MDCK cells already expressing Tet-VP16 transactivator (22,23). Stable neomycin / hygromycin resistant cell lines were selected and characterized for tetracycline-regulated expression of MLCK. The exogenously expressed rabbit 150 kDa WT-MLCK and a mutant with an in-frame deletion of Lys 725 have been previously characterized (24,25). Both MLCK cDNAs have a C-terminal “flag” epitope (DYKDDDDK). MDCK cell lines expressing MLCK were routinely maintained in DMEM media supplemented with 10% (vol/vol) fetal calf serum (DMEM/FCS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, containing 2 μg/ml Dox to suppress exogenous MLCK expression. Expression of MLCK in the MDCK cell lines was induced by plating cells at low density in DMEM/FCS media containing either 0 ng/mL Dox (KD-MLCK) or 0.2 ng/mL Dox (WT-

MLCK) for 24 hours. These conditions allow for stable, approximately equal expression levels of the MLCKs. Cells that do not express exogenous MLCK and maximally repressed MDCK cells (2 μg/mL Dox) served as controls for all experiments. Over-expression of either MLCK did not have a deleterious effect on growth, doubling time, or morphology. Western blotting and immunofluorescence were used routinely to monitor the expression of the exogenous WT or KD MLCK to ensure that both cell lines were expressing each MLCK at equal levels. Cells were maintained at sub-confluent levels (approximately 30-50% density) during analysis.

Cell death quantitation. MDCK cells were seeded at 5 X 10^4 cells per well in 6-well tissue culture dishes. Viable, attached cells were identified and counted using trypan blue-exclusion and counted. ML-7 and ML-9 were added to culture media 30 minutes before TNF treatment at 10 μM or 20 μM respectively and were present during the incubation with TNF. An equal amount of vehicle (DMSO, <0.1% final concentration) was added to control cells at the same time. Cell viability is expressed as the % of the surviving TNF treated cells compared to the surviving control cells not treated with TNF.

Western blotting. Cellular proteins were extracted with NP-40 lysis buffer (1% NP-40, 300mM NaCl, 0.5mM EGTA, 50 mM $MgCl_2$, 10% glycerol and 20 mM MOPS, pH 7, plus protease inhibitors) and analyzed by western blotting as described previously (26).

DNA fragmentation. Flow cytometric analysis of cells was performed on a Becton-Dickinson (Mountain View, CA) FACStar plus. Adherent cells were trypsinized, pooled with floating cells, fixed in 5% acetic acid / 95% ethanol at -20°C and stained with 50

μg/ml propidium iodide (Sigma, St. Louis, MO)**(27)**. At least 10,000 cells were counted for each analysis.

Myosin II RLC phosphorylation. Phosphorylation of RLCs in attached and floating cells was determined as described (28). Briefly, the cellular proteins are precipitated with 10% trichloroacetic acid; the pellets are washed with acetone and dissolved in 8M urea, 20mM Tris, 23 mM glycine and 10 mM DTT. Western blotting with an anti-myosin II RLC antibody was used to identify un-phosphorylated, mono-phosphorylated, and di-phosphorylated forms of RLC after fractionation through a 10% glycerol-polyacrylamide gel and transfer to nitrocellulose. The relative abundance of each RLC band was determined by scanning densitometry. The scan data was used to calculate the myosin II RLC phosphorylation index using the formula: mol phosphate / mol RLC = P1 + 2 (P2) / U + P1 + P2, where U=% unphosphorylated RLC, P1 = % monophosphorylated RLC and P2 = % diphosphorylated RLC.

Caspase-8 activity. MDCK cells (1 X 10^6 cells / 100 mm dish) were washed with PBS and lysed with CHAPS lysis buffer (1% CHAPS, 100mM NaCl, 100 μM EDTA, 10mM DTT and 50 mM HEPES, pH7.4). After centrifugation, cell extracts were incubated at 25°C in assay buffer (CHAPS lysis buffer plus 10% glycerol) with 200 μM Ac-IETD-pNA (Calbiochem, La Jolla, CA), a colorimetric caspase-8 specific peptide substrate. The time dependent change in absorbance at 405 nm was monitored by spectrophotometry and converted to caspase-8 activity (pmol/min/mg total protein). Pure p-nitroanilline (pNA, Calbiochem, La Jolla, CA) was used for a standard curve and the caspase-8

specific inhibitor, Ac-IETD-CHO (Calbiochem, La Jolla, CA) was added to each cell lysate as negative control.

Fractionation and biotin labeling of membrane proteins. Cells were washed 4 times with PBS and incubated in 0.5 mg/mL biotin-X-NHS (Calbiochem, La Jolla, CA) in PBS containing 1 mMM MgCl2 and 0.1 μM CaCl2 (PBS/CM) for 30 min at 4 °C. After washing 5 times with PBS C/M, cells were lysed in SDS lysis buffer. (1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH7.4). Soluble proteins were collected after centrifugation at 16,000 x g for 10 min and then incubated for 1 hour at room temperature with 0.2g avidin-agarose beads (Calbiochem, La Jolla, CA). The avidin-agarose beads were pre-treated by incubation with 20 mg/ml BSA in SDS lysis buffer. The beads were washed 5 times in SDS lysis buffer to remove unbound, non-biotinylated proteins and boiled in 2X protein gel sample buffer for 5 min to solubilize biotinylated, bound membrane proteins prior to analysis by SDS-PAGE and western blotting. Conditions for biotin labeling and subsequent fractionation of labeled cell surface proteins by avidin-agarose chromatography were optimized using Western blotting to confirm that cytoplasmic or nuclear proteins were absent in the biotin labeled fraction.

Immunofluorescence labeling and microscopy. Cells were fixed and processed for immunofluorescence in the presence of 3.7% paraformaldehyde and 0.1% Triton X-100. Photomicrographs were adjusted using Photoshop. Each image was treated identically.

Results

Inhibition of myosin II RLC phosphorylation delays the progression of TNF induced apoptosis in MDCK cells. To learn if myosin II motor activities have a role in TNF induced cell death, myosin II RLC phosphorylation and thus myosin II activation was inhibited through the use of two MLCK specific inhibitors, ML-7 (Ki=0.3 μM) or ML-9 (Ki=3.8 μM). The effects of MLCK inhibitors on TNF mediated cell death were examined in MDCK cells, which are sensitive to the apoptotic effects of TNF in the absence of cyclohexamide. Treatment of MDCK cells with ML-7 (Figure 1A) or ML-9 (data not shown) slows the rate of MDCK cell death, and the $t_{1/2}$ for cell death (time at which 50% cell death occurs) is extended from 44 h to 60 h in the presence of ML-7. Paralleling the delay in cell death, were delays in the appearance of fragmented DNA and the 89 kDa poly-ADP ribose polymerase (PARP) cleavage product (not shown). These MLCK inhibitors also result in a decrease of the peak of TNF-induced RLC phosphorylation at 30 min from 0.94 $\pm$ 0.05 mol Pi / mol RLC to 0.51 $\pm$ 0.02 mol Pi / mol RLC suggesting that inhibiting myosin II RLC phosphorylation delays TNF-induced apoptotic cell death (Figure 1B).

To further determine the role of myosin II motor activities in TNF induced cell death, MDCK cell lines over-expressing an inactive, kinase dead (KD) or wild type (WT) Ca^{2+}/calmodulin-dependent MLCK under the control of the tetracycline promoter were generated. Based upon previous studies, KD MLCK was expected to act as a dominant negative to compete with the endogenous MLCK and reduce RLC phosphorylation and myosin II activation (25). Characterization of these cell lines revealed that continuous over-expression of either maximal or moderate levels of these MLCKs does not result in

alterations in survival or growth rates in the absence of TNF. However, in the presence of 10 ng/mL TNF, the temporal progression of cell death for MDCK cells expressing KD MLCK is significantly delayed compared to that of the parental cell line or MDCK cells over-expressing WT MLCK (Figure 2A). The levels of RLC phosphorylation were quantitated following 15 or 30 min of TNF treatment, when RLC phosphorylation is observed to peak in the parental MDCK cells (Figure 1). These results showed that the levels of RLC phosphorylation in MDCK cells expressing KD MLCK are significantly lower at both time points (0.71 $\pm$ 0.06 and 0.80 $\pm$ 0.4 mol Pi / mol RLC, respectively) compared to the parental MDCK cells (0.86 $\pm$ 0.06 and 0.95 $\pm$ 0.03 mol Pi / mol RLC, respectively) (Figure 2B). In contrast, the levels of RLC phosphorylation in the MDCK cells expressing WT MLCK were elevated to 1.4 $\pm$ 0.1 at 15 min or 1.2 $\pm$ 0.1 at 30 min (Figure 2B).

DNA fragmentation, PARP cleavage and the rate of cell death were also compared in parental MDCK cells and MDCK cells over-expressing KD or WT MLCK. Degradation of high molecular weight chromosomal DNA into smaller fragments was examined using flow cytometry (Figure 2C). Comparison of the DNA fragmentation occurring in the parental MDCK cells and MDCK cells expressing KD or WT MLCK showed that following 30 hr of TNF treatment over-expression of the dominant negative KD MLCK decreases TNF-induced DNA fragmentation (<5% of cells). In contrast, greater than 30-40% of the MDCK cells expressing WT MLCK were found in the hypodiploid, sub-G1 peak (Figure 2C) as compared to 15-20% of the parental MDCK cells.

To determine when myosin II motor activity is important in the TNF-induced apoptotic pathway, the activation of distal execution caspases such as caspase-3 were examined by monitoring the appearance of an 89 kDa cleavage fragment of PARP (29,30). The 89 kDa PARP fragment is first detectable by western blotting in the parental MDCK cells after 24 h of TNF treatment (Figure 2D). However, the appearance of the PARP cleavage product is delayed until 48 h in MDCK cells expressing the dominant negative, KD MLCK. In contrast, in MDCK cells expressing WT MLCK, the 89 kDa PARP cleavage fragment is rapidly detectable within 8 hours of TNF treatment. This result demonstrates that activation of receptor distal proteases such as caspase-3 is delayed by decreasing MLCK activity and places the role of Ca^{2+}/calmodulin-dependent MLCK activity and RLC phosphorylation at a point upstream of activation of caspase-3 in the TNF-induced apoptotic signaling cascade.

The rates of intracellular movement of TNFR-1 associated DISC proteins are regulated by changes in myosin II motor activity.

Examination of the intracellular distribution of both FADD and caspase-8 in permeabilized MDCK cells in response to TNF, demonstrated that within 15 min of TNF stimulation, a dramatic alteration in the appearance of death signaling proteins occurred (Figure 3). In unstimulated MDCK cells, FADD had an indistinct cytosolic pattern and reorganized within 15 min to form visible complexes resembling small filamentous perinuclear aggregates in MDCK cells over-expressing WT MLCK. In comparison, in MDCK cells over-expressing KD MDCK, aggregates did not become visible until at least 3 h of TNF treatment. Procaspase-8 was also found to re-organize into visible

aggregates in MDCK cells over-expressing WT MLCK within 15 min of TNF treatment, but is delayed until approximately 3 h in MDCK cells expressing the dominant negative KD MLCK (Figure 3). The decrease in the pro-caspase-8 reactive protein observed at 3 h for MDCK cells over-expressing WT MLCK is consistent with the fact that the antibody used to detect procaspase-8 in fixed cells does not react with the cleaved form of active caspase-8 (31). These results show that within 15 min of TNF treatment, a dramatic change in the intracellular distribution of FADD and pro-caspase-8 occurs and this re-organization is dependent on the relative levels of MLCK activity. Together these results demonstrated that the rate of aggregation and activation of the apical death signaling caspase, procaspase-8 can be modulated by changes in MLCK activity.

Consistent with the proposal that FADD and caspase-8 aggregation is dependent on myosin motor activity, we determined that the percent increase in TNF-induced activation of caspase-8 is significantly abrogated at all time points between 1 and 8 h in MDCK cells expressing the dominant negative KD MLCK. However, by 24 h the increase in caspase-8 activity approaches that of the parental MDCK cells. In contrast, within 8 h of TNF treatment, the caspase-8 activity in WT MDCK cells rapidly increases to surpass the maximal activity level determined for the parental MDCK cells (Figure 4). Following this accelerated peak, the caspase-8 activity levels decline as the population of WT MDCK cells is decimated by apoptosis. For these experiments a colorimetric assay that results from cleavage of a caspase-8 specific substrate, Ac-IETD-pNA was used to directly determine caspase-8 activity in cell extracts. Together, the physical aggregation and changes in activity of capase-8 reflect the relative levels of MLCK activity in these TNF-treated MDCK cells and is consistent with the suggestion that

myosin motor activities may have a role in the intracellular distribution of components of the TNFR-1 signaling cascade. However, these experiments do not address the possibility that the intracellular aggregates are associated with TNFR-1 at the cell surface membrane.

Intracellular trafficking of TNFR-1 is regulated by myosin II motor activity.

To determine if myosin II motor activity has a role in movement and activation of death signaling by TNFR-1, proteins located on the plasma membrane were labeled by treating the intact cell monolayer with biotin-X-NHS. Following biotin labeling at 4^{o}C, the cells were lysed and the biotin labeled proteins were purified using avidin-agarose chromatography as described in the methods. Western blotting of the avidin-agarose fractions using an anti-TNFR-1 antibody revealed that at the zero time point, in the absence of TNF stimulation, only a small fraction of TNFR-1 is on the plasma membrane (Figure 5). In response to TNF, the relative amount of TNFR-1 that becomes biotin-labeled rapidly increased in MDCK cells and MDCK cells over-expressing WT MLCK and is readily detectable within 15 min. In contrast, TNFR-1 is not detected on the plasma membrane of MDCK cells that over-express the dominant negative, KD MDCK until between 3 and 8 hr.

LPS or ionomycin both result in the TNF-independent translocation of TNFR-1 to the plasma membrane.

To see if translocation of TNFR-1 requires TNF or if activation of MLCK and myosin II motor activities alone is sufficient for translocation, MDCK cells were stimulated either

with ionomycin or lipopolysaccharide (LPS, endotoxin) and the levels of RLC phosphorylation and TNFR-1 in the biotin-labeled fraction determined. Ionomycin is expected to elevate intracellular calcium, leading to activation of MLCK and myosin motor activities. In support of a TNF-independent translocation mechanism, the parental MDCK cells and the WT MLCK over-expressing cell line treated for 5 min in the presence of 10 μM ionomycin have significantly increased levels of RLC phosphorylation compared to DMSO treated controls (Figure 6A). In addition, the amount of TNFR-1 in the biotin-labeled fraction of the parental and WT MLCK expressing MDCK cells is also increased (Figure 6B). MDCK cells over-expressing KD MLCK have no significant increase in RLC phosphorylation and little detectable TNFR-1 on the plasma membrane in response to ionomycin. Finally, the MLCK inhibitor, ML-7, abrogates the ionomycin stimulated increase in TNFR-1 on the plasma membrane in the parental and MDCK cells over-expressing WT MLCK to levels that are similar to that found in the unstimulated control cells (Figure 6B).

LPS or endotoxin is a component of the cell walls of gram-negative bacteria that stimulates macrophages and other cells to release pro-inflammatory cytokines including TNF. In addition to stimulating cytokine expression, bacterial sepsis also induces contractile responses and loss of barrier function in cells like endothelial cells through activation of myosin II motor activities (32). This LPS induced activation of myosin motor activities could provide a mechanism for translocation of vesicles containing TNFR-1 to the plasma membrane where TNF binding can initiate signaling. To determine if LPS stimulates TNFR-1 translocation to the plasma membrane, experiments were conducted to evaluate the level of apoptosis in cells that were not pretreated LPS (TNF only) or

were treated simultaneously with TNF and LPS. The results of these experiments show that the presence of LPS does not alter the extent of apoptosis in KD or WT MLCK expressing cells or in the parental MDCK cells. However, pretreatment of MDCK cells for 8 h with LPS (1 μg/mL) enhanced the extent of apoptosis in the MDCK cell lines expressing KD MLCK and the parental MDCK cells from approximately 48% or 62% to 80% and 93% respectively. Similar results were obtained whether or not LPS was maintained in the presence or not of TNF. LPS pretreatment had little or no effect on the extent of apoptosis in MDCK cells expressing WT MLCK most likely because these cells already exhibit maximal levels of apoptosis by 48 h in the absence of LPS. Control experiments utilizing MDCK cell lines treated for 56 h with LPS only showed that LPS does not induce apoptosis by itself (data not shown). To further establish that LPS can stimulate myosin motor activities and subsequent translocation of TNFR-1 to the plasma membrane the levels of RLC phosphorylation and surface TNFR-1 in response to LPS treatment were determined (Figure 7B). RLC phosphorylation in MDCK cells expressing KD MLCK gradually increases from 0.5 $\pm$ 0.05 mol Pi/mol RLC to a peak level of approximately 1.1 $\pm$ 0.17 mol Pi/mol RLC in response to LPS and this level appears to be maintained between 3-8 h. In contrast, RLC phosphorylation in MDCK cells expressing WT MLCK rapidly increases from 0.5 $\pm$ 0.1 to 1.7 $\pm$ 0.1 mol Pi/mol RLC after 15 min of LPS and then slowly declines to approximately 1.15$\pm$0.1 mol PI/mol RLC by 8 h. The surface levels of TNFR-1 also correspond to the changes in RLC phosphorylation and gradually increase in KD MDCK to approximate the surface level of TNFR-1 detected in WT expressing MDCK cells. Together these results suggest that

myosin motor activities are important for translocation of TNFR-1 to the plasma membrane.

Discussion

While a detailed characterization of the signaling pathways emanating from the TNFR-1 has been compiled, little is known about signaling mechanisms that regulate the intracellular or intra-membrane trafficking of this death receptor. The pivotal importance of myosin II motor activities in other contractile processes like mitosis, migration, and cellular shape changes including muscle contraction are well established. In this study we show that myosin II motor activity also has an important role in regulating the apoptotic signaling cascade activated by TNF and is directly involved in translocation of TNFR-1 to a biotin-accessible location on the cell membrane. The experiments in this report utilized both direct and indirect modulation of myosin II motor activities by pharmacological agents or the expression of a dominant negative or WT MLCK, to link changes in RLC phosphorylation to changes in the rate of TNF-induced apoptotic cell death in MDCK cells. These results show that translocation of TNFR-1 to a biotin-accessible membrane location is a myosin II-dependent motor process that can occur in the absence of TNF in response to either increased intracellular calcium or to LPS, a physiological activator of cytokine signaling.

Myosin II motor activities regulate TNF induced apoptosis.

MLCK is the primary regulator of myosin II ATPase activity, and in contrast to most other ser/thr protein kinases, MLCK has a single, well-characterized physiological

substrate, the myosin II RLC. Phosphorylation of myosin RLC by MLCK directly leads to activation of myosin II ATPase and myosin II force production. Based upon this pathway we hypothesized that if myosin II motor activities have a role in regulating TNF signaling, then changes in the total MLCK activity in cells will directly impact the progression of apoptosis. Consistent with this proposal, our results demonstrate that increasing or decreasing the total MLCK activity in cells, either by over-expression of WT MLCK or dominant negative KD MLCK or by the use of a specific inhibitor of MLCK, ML-7, leads to a corresponding change in RLC phosphorylation and either potentiation or abrogation of TNF-induced apoptosis.

By examining the signaling pathway that is initiated by TNF, we show that the downstream activities of the caspases that are activated by TNF signaling respond in parallel to changes in myosin motor activity. In addition, we have noted a rapid re-organization of both FADD and caspase-8 into small aggregates in response to TNF. Although it is unclear whether or not the aggregates are associated with TNFR-1, the data suggest that the formation of FADD and caspase-8 aggregates is linked to myosin II motor activities, as evidenced by the difference in the rates of TNF-induced aggregation observed in MDCK cells expressing WT and KD MLCK. The rapid aggregation and coordinated increase in caspase-8 activity are consistent with the recently proposed model of proximity-induced auto-activation (13,33). However, in contrast to other studies we do not see formation of large cytoplasmic filamentous aggregates, called death effector filaments (DEF) that have been described for FADD and caspase-8 (34-36). This distinction could be due to the fact that we are examining changes in intracellular distribution by endogenous FADD and caspase-8, which may

only form smaller aggregates and suggests that the larger, filamentous DEFs are a consequence of over-expression.

Finally, we also show that the translocation of TNFR-1 from a biotin inaccessible to a biotin-accessible plasma membrane location is linked to myosin motor activities. This observation is consistent with previous studies showing that the bulk of TNFR-1 is localized to the trans-Golgi (17) and suggests that myosin II forces can power the translocation of a Golgi-derived vesicle containing TNFR-1 to the plasma membrane (18,37). Consistent with this suggestion are several recent reports showing that nonmuscle myosin IIA and possibly IIB as well as F-actin are associated with distinct golgi vesicles. The association of acto-myosin filaments with golgi-derived vesicles that contain TNFR-1 could provide the necessary forces for the intracellular trafficking of these vesicles (18,19,37,47,48). Alternatively, or in addition, these results suggest that myosin II motor activities may translocate TNFR-1 within the plasma membrane from a biotin-inaccessible to a biotin-accessible location.

Is TNF required for translocation of TNFR-1 to the plasma membrane?

These results raised the issue as to whether stimulation of cells with TNF is required for receptor translocation or whether acto-myosin motor activities alone can promote translocation of TNFR-1 to the cell membrane. Experiments using either LPS or ionomycin both showed that activation of myosin II motor activities is sufficient to result in translocation of TNFR-1 to the plasma membrane in the absence of TNF. Treatment of MDCK cells with ionomycin, a calcium ionophore, increases RLC phosphorylation and this is paralleled by a rapid increase in the level of biotinylated

TNFR-1 detected on the plasma membrane. Similarly, treatment of MDCK cells with LPS, an inflammatory response mediator, also results in increased RLC phosphorylation and a parallel increase in surface expression of TNFR-1. Rapid and sustained increases in intracellular calcium have been demonstrated in septic shock induced by endotoxin (LPS)(38-41). In addition, LPS-induced septic shock is known to cause a rapid, contractile response in endothelial cells through increased RLC phosphorylation (32). Therefore, it is reasonable to expect that increases in intracellular calcium occur in response to LPS. This would lead to activation of MLCK, to result in increased RLC phosphorylation, stimulation of myosin II motor activity and translocation of TNFR-1 to the plasma membrane where ligand binding would activate TNFR-1 signaling. We also considered the possibility that the response to LPS for these MDCK cells occurs via LPS-induced synthesis of TNF. This does not appear to be a likely possibility as increases in RLC phosphorylation and translocation of TNFR-1 to the plasma membrane are detected within 15 min of LPS addition to the cultures, making it unlikely that de novo expression and autocrine stimulation by TNF is involved in translocation of TNFR-1. Finally, we show that pretreatment of the cells with LPS can increase the extent of apoptosis as well as lead to a sustained accumulation of TNFR-1 on the plasma membrane. Together these findings are consistent with the suggestion that LPS can activate myosin II motor activities to result in the translocation of TNFR-1. In addition, these results suggest that LPS activation of myosin motor activities may serve to prime cells for TNF induced signaling.

Is TNFR-1 translocation dependent on MLCK activity?

Several experiments in this report show that the surface level of TNFR-1 is decreased in TNF-stimulated MDCK cells expressing KD MLCK. In addition, MDCK cells treated with ionomycin in the presence of the MLCK, inhibitor ML-7 also have decreased surface levels of TNFR-1. Together these experiments suggest that the Ca^{2+}/CaM dependent MLCK may directly involved in the activation of myosin II motor activity for TNFR-1 translocation. However, even the over-expression of a dominant negative KD MLCK is insufficient to completely block the apoptotic effects of TNF. One reason for this finding may be that the exogenous expressed 150 kDa KD MLCK is unable to completely compete with the endogenous 220 kDa MLCK expressed in MDCK cells because these two forms of MLCK have distinct intracellular locations (Gallagher, unpublished). Alternatively, other protein kinases known to phosphorylate myosin II RLC, such as rho-dependent kinase (RHOK), p21activated kinase (PAK) or the Ca^{2+}/CaM-dependent death associated protein kinase (DAP kinase) may act either independent of or in addition to MLCK (42-46) to activate myosin II motor activities for translocation of TNFR-1 to the plasma membrane. Additional studies will be required to define the relative contributions of the two MLCK forms as well as other RLC protein kinases to receptor trafficking in cells.

The growing list of myosin II motor activities that includes contractile processes such as migration, cytokinesis and muscle contraction illustrates the pivotal importance of myosin functions. In this report we show that myosin II motor activities are important at an early step in TNF signaling and power the translocation of TNFR-1 to or within the plasma membrane. We also show that activation of myosin II motor activities independent of TNF stimulation is sufficient for translocation of TNFR-1 to a biotin-

accessible membrane location and can occur as a result of increasing intracellular calcium. Together these results suggest a model by which TNF receptor movements may be regulated (Figure 8). In this model the relationship between calcium, MLCK, myosin II activity and the intracellular distribution of TNFR-1 are emphasized. Stimulation of cells with TNF, ionomycin or LPS is postulated to cause an increase in intracellular calcium and result in activation of myosin II motor activity through RLC phosphorylation by the Ca^{2+}/calmodulin dependent MLCK. The activation of acto-myosin motor activities provides the force generating activity to translocate TNFR-1 containing vesicles to the plasma membrane where the vesicles fuse with and deliver the receptor it can bind its ligand, TNF. Alternatively or in addition, myosin II motor activities may drive re-distribution of TNFR-1 within the plasma membrane. Following movement to or within the plasma membrane, the binding of TNF then induces trimerization of TNFR-1, followed by the recruitment of TRADD, FADD, and caspase-8 to result in activation of the cell death pathway. Activation of TNFR-1 may also lead to a sustained increase in intracellular Ca^{2+} to provide additional myosin driven translocation of the receptor. Overall these studies show that myosin II motor activities are important for the translocation and regulation of the surface level of TNFR-1 and ultimately the response of the cell to TNF.

Acknowledgements

The authors wish to thank Bradley A. Poteat for his technical assistance in preparation of the cell lines. NIH HL54118 to PJG and an American Heart Association - Midwest Affiliate Pre-doctoral fellowship to YJ, supported this work.

References

1. Tracey, K. J., and Cerami, A. (1993) *Annu Rev Cell Biol* **9,** 317-43

2. Ashkenazi, A., and Dixit, V. M. (1998) *Science* **281**(5381)**,** 1305-8

3. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) *Cell* **81**(4)**,** 495-504

4. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) *Cell* **84**(2)**,** 299-308

5. Goeddel, D. V. (1999) *Chest* **116**(1 Suppl)**,** 69S-73S

6. Hsu, H., Huang, J., Shu, H. B., Baichwal, V., and Goeddel, D. V. (1996) *Immunity* **4**(4)**,** 387-96

7. Itoh, N., and Nagata, S. (1993) *J Biol Chem* **268**(15)**,** 10932-7

8. Tartaglia, L. A., and Goeddel, D. V. (1992) *J Biol Chem* **267**(7)**,** 4304-7

9. Tartaglia, L. A., Ayres, T. M., Wong, G. H., and Goeddel, D. V. (1993) *Cell* **74**(5)**,** 845-53

10. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) *Cell* **85**(6)**,** 817-27

11. Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995) *J Biol Chem* **270**(14)**,** 7795-8

12. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) *Cell* **81**(4)**,** 505-12

13. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) *J Biol Chem* **273**(5)**,** 2926-30

14. Kumar, S., and Colussi, P. A. (1999) *Trends Biochem Sci* **24**(1)**,** 1-4

15. Nicholson, D. W., and Thornberry, N. A. (1997) *Trends Biochem Sci* **22**(8)**,** 299-306

16. Thornberry, N. A. (1998) *Chem Biol* **5**(5)**,** R97-103

17. Jones, S. J., Ledgerwood, E. C., Prins, J. B., Galbraith, J., Johnson, D. R., Pober, J. S., and Bradley, J. R. (1999) *J Immunol* **162**(2)**,** 1042-8

18. Heimann, K., Percival, J. M., Weinberger, R., Gunning, P., and Stow, J. L. (1999) *J Biol Chem* **274**(16)**,** 10743-50

19. Stow, J. L., and Heimann, K. (1998) *Biochim Biophys Acta* **1404**(1-2)**,** 161-71.

20. Valderrama, F., Luna, A., Babia, T., Martinez-Menarguez, J. A., Ballesta, J., Barth, H., Chaponnier, C., Renau-Piqueras, J., and Egea, G. (2000) *Proc Natl Acad Sci U S A* **97**(4)**,** 1560-5.

21. Wright, S. C., Zheng, H., Zhong, J., Torti, F. M., and Larrick, J. W. (1993) *J Cell Biochem* **53**(3)**,** 222-33

22. Barth, A. I., Pollack, A. L., Altschuler, Y., Mostov, K. E., and Nelson, W. J. (1997) *J Cell Biol* **136**(3)**,** 693-706

23. Gaush, C. R., Hard, W. L., and Smith, T. F. (1966) *Proc Soc Exp Biol Med* **122**(3)**,** 931-5

24. Gallagher, P. J., Herring, B. P., Griffin, S. A., and Stull, J. T. (1991) *J Biol Chem* **266**(35)**,** 23936-44.

25. Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P., and Cheresh, D. A. (1997) *J Cell Biol* **137**(2)**,** 481-92

26. Gallagher, P. J., Garcia, J. G., and Herring, B. P. (1995) *J Biol Chem* **270**(49)**,** 29090-5

27. Esser, C., Gottlinger, C., Kremer, J., Hundeiker, C., and Radbruch, A. (1995) *Cytometry* **21**(4)**,** 382-6

28. Goeckeler, Z. M., and Wysolmerski, R. B. (1995) *J Cell Biol* **130**(3)**,** 613-27

29. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) *Cell* **81**(5)**,** 801-9

30. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995) *Nature* **376**(6535)**,** 37-43

31. Cock, J. G., Tepper, A. D., de Vries, E., van Blitterswijk, W. J., and Borst, J. (1998) *J Biol Chem* **273**(13)**,** 7560-5

32. Essler, M., Staddon, J. M., Weber, P. C., and Aepfelbacher, M. (2000) *J Immunol* **164**(12)**,** 6543-9

33. Martin, D. A., Siegel, R. M., Zheng, L., and Lenardo, M. J. (1998) *J Biol Chem* **273**(8)**,** 4345-9

34. Siegel, R. M., Martin, D. A., Zheng, L., Ng, S. Y., Bertin, J., Cohen, J., and Lenardo, M. J. (1998) *J Cell Biol* **141**(5)**,** 1243-53

35. Perez, D., and White, E. (1998) *J Cell Biol* **141**(5)**,** 1255-66

36. Tang, D., Lahti, J. M., Grenet, J., and Kidd, V. J. (1999) *J Biol Chem* **274**(11)**,** 7245-52

37. Musch, A., Cohen, D., and Rodriguez-Boulan, E. (1997) *J Cell Biol* **138**(2)**,** 291-306

38. Baldwin, T. J., Ward, W., Aitken, A., Knutton, S., and Williams, P. H. (1991) *Infect Immun* **59**(5)**,** 1599-604

39. Song, S. K., Karl, I. E., Ackerman, J. J., and Hotchkiss, R. S. (1993) *Proc Natl Acad Sci U S A* **90**(9)**,** 3933-7

40. Sayeed, M. M., and Maitra, S. R. (1987) *Am J Physiol* **253**(4 Pt 2)**,** R549-54

41. Portoles, M. T., Ainaga, M. J., Municio, A. M., and Pagani, R. (1991) *Biochim Biophys Acta* **1092**(1)**,** 1-6

42. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) *J Biol Chem* **271**(34)**,** 20246-9

43. Goeckeler, Z. M., Masaracchia, R. A., Zeng, Q., Chew, T. L., Gallagher, P., and Wysolmerski, R. B. (2000) *J Biol Chem* **275**(24)**,** 18366-18374

44. Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., and Williams, L. T. (1997) *Proc Natl Acad Sci U S A* **94**(25)**,** 13642-7

45. Van Eyk, J. E., Arrell, D. K., Foster, D. B., Strauss, J. D., Heinonen, T. Y., Furmaniak-Kazmierczak, E., Cote, G. P., and Mak, A. S. (1998) *J Biol Chem* **273**(36)**,** 23433-9

46. Chew, T. L., Masaracchia, R. A., Goeckeler, Z. M., and Wysolmerski, R. B. (1998) *J Muscle Res Cell Motil* **19**(8)**,** 839-54

47. Ikonen, E., de Almeid, J. B., Fath, K. R., Burgess, D. R., Ashman, K., Simons, K., and Stow, J. L. (1997) *J Cell Sci* **110**(Pt 18)**,** 2155-64.

48. Stow, J. L., Fath, K. R., and Burgess, D. R. (1998) *Trends Cell Biol* **8**(4)**,** 138-41.

Footnotes

[1]Abbreviations used are: Myosin light chain kinase, MLCK; tumor necrosis factor - alpha, TNF; tumor necrosis factor receptor-1, TNFR-1; myosin II 20 kDa regulatory light chain, RLC; TNF receptor-associated death domain protein (TRADD); Fas-associated death domain protein, FADD; LPS, lipopolysaccharide (endotoxin).

Figure Legends

Figure 1. An inhibitor of MLCK activity delays TNF induced apoptosis and decreases RLC phosphorylation in MDCK cells. MDCK cells were treated with TNF (10 ng/mL) for indicated times in the presence of ML-7 (10 μM) or vehicle (0.1% DMSO). Apoptosis (A) and RLC phosphorylation (B) were determined at the indicated time points. The percent apoptosis (%) was determined by counting the number of viable attached cells after the indicated times of TNF treatment and comparing them to the number of viable cells in untreated controls. The relative levels of total phosphate incorporation into myosin II RLC were determined following urea-glycerol gel electrophoresis and western blotting using a specific anti-RLC antibody. The extent of myosin II RLC phosphorylation was calculated as moles of phosphate incorporated per mole of RLC (mol Pi/ mol RLC). Results show ML-7 delays TNF induced apoptosis and decreases RLC phosphorylation in MDCK cells. Results represent the mean $\pm$ SEM of at least 6 independent experiments.

Figure 2. A dominant negative, kinase dead MLCK abrogates TNF induced apoptosis in MDCK cells. (A) Time course of TNF induced apoptosis in the parental (MDCK) and MDCK cells expressing KD MLCK (KD) or WT MLCK (WT). Equivalent numbers ($5x10^4$) of cells were seeded and percent apoptosis was determined at the indicated times of treatment with TNF (10ng/ml). (B) Myosin II RLC phosphorylation levels in the parental (MDCK) and in MDCK cell lines expressing inactive, kinase dead (KD), or wild-type MLCK (WT). The levels of total phosphate incorporation into myosin II

RLC were determined at 0, 15 and 30 min of TNF treatment using urea-glycerol gel electrophoresis and western blotting with a specific anti-RLC antibody. The extent of myosin II RLC phosphorylation was calculated as moles of phosphate incorporated per mole of RLC (mol Pi/ mol RLC). Results shown in (A) and (B) represent mean $\pm$ SEM from 6 experiments. (C) Flow cytometric analysis of TNF treated MDCK cells and MDCK cell lines expressing WT or KD MLCK. Following 30 h of TNF treatment cells were dissociated, fixed, and treated with propidium iodide (PI). The DNA content of 10,000 cells was analyzed by flow cytometry as described in methods. Results shown are representative of 3 independent experiments. The approximate locations of the peaks representing diploid DNA content (G1), tetraploid (G2), or hypodiploid (<G1; arrow) are indicated. (D) Parental MDCK or MDCK cells expressing KD MLCK or WT MLCK were treated with TNF (10ng/ml) for indicated times and full-length (116 kDa) and caspase-cleaved (89 kDa) PARP were detected by western blotting. Each lane represents 50 μg total protein.

Figure 3. TNF treatment causes FADD and Caspase-8 to form aggregates. Indirect immunofluorescence with an anti-FADD or anti-pro-caspase-8 antibodies was used to detect formation of FADD or pro-caspase-8 aggregates in MDCK cells in response to TNF treatment (10 ng/mL). The pro-caspase-8 antibody is specific only for the pro-form and does not react with mature caspase-8.

Figure 4. Caspase-8 activity is rapidly activated in MDCK cells expressing WT MLCK. MDCK cells were treated with TNF (10ng/ml) for indicated times and caspase-8 activity was measured as a change in absorbance at 405 nm using a colorimetric caspase-8 substrate, IETD-pNA.

Figure 5. Changes in MLCK activity alter translocation of TNFR-1. TNFR-1 present on the plasma membrane in MDCK cells expressing WT or KD MLCK were labeled at indicated times by treating intact cells with biotin. The biotin labeled proteins were isolated by avidin-agarose fractionation and the TNFR-1 present in the biotin labeled membrane fraction (surface) was detected by western blotting using a TNFR-1 antibody.

Figure 6. Ionomycin stimulates TNFR-1 translocation to the plasma membrane. MDCK cell lines either over-expressing WT or KD MLCK or parental (MDCK) cells were treated for 5 min with ionomycin or with vehicle (DMSO). A, the levels of phosphate incorporation into myosin II RLC (mol Pi/ mol RLC) were determined following urea-glycerol gel electrophoresis and densitometry of western blots using anti-RLC antibody to detect RLC. B, the relative amounts of TNFR-1 in the biotin-labeled fraction were determined by western blotting using anti-TNFR-1 antibody. The amount of TNFR-1 in the biotin labeled fraction was also examined in the presence or absence of the MLCK inhibitor, ML-7. Results shown represent mean $\pm$ SE from 4 experiments.

Figure 7. LPS stimulates TNFR-1 translocation to the plasma membrane.

A, MDCK cell lines either over-expressing WT or KD MLCK or parental (MDCK) cells were treated with TNF(10 µg/mL) in the absence or presence of LPS (1 µg/mL) for the indicated times and the relative amounts of biotin-labeled TNFR-1 were determined as in Figure 6. B, MDCK cell lines were treated with LPS (1 µ/mL) for up to 8 h. At the indicated times, the levels of phosphate incorporation into myosin II RLC (mol Pi/ mol RLC) and surface expression of TNFR-1were determined. Results shown represent mean $\pm$ SE from 4 experiments.

Figure 8. Pathway for myosin II regulation of TNFR-1 translocation to the plasma membrane. Stimulation of cells to increase intracellular Ca^{2+} leads to activation of MLCK and phosphorylation of myosin II RLC. RLC phosphorylation increases actin-associated myosin II ATPase activity and provides the motor activity to translocate a TNFR-1-associated vesicle from the Golgi to the plasma membrane or within the plasma membrane, where TNF binds and receptor trimerization occurs. Activated TNFR-1 recruits TRADD, FADD and pro-caspase-8. Continued TNF stimulation may amplify or prolong increased intracellular Ca^{2+} signaling by activation of membrane calcium channels.

Figure 1

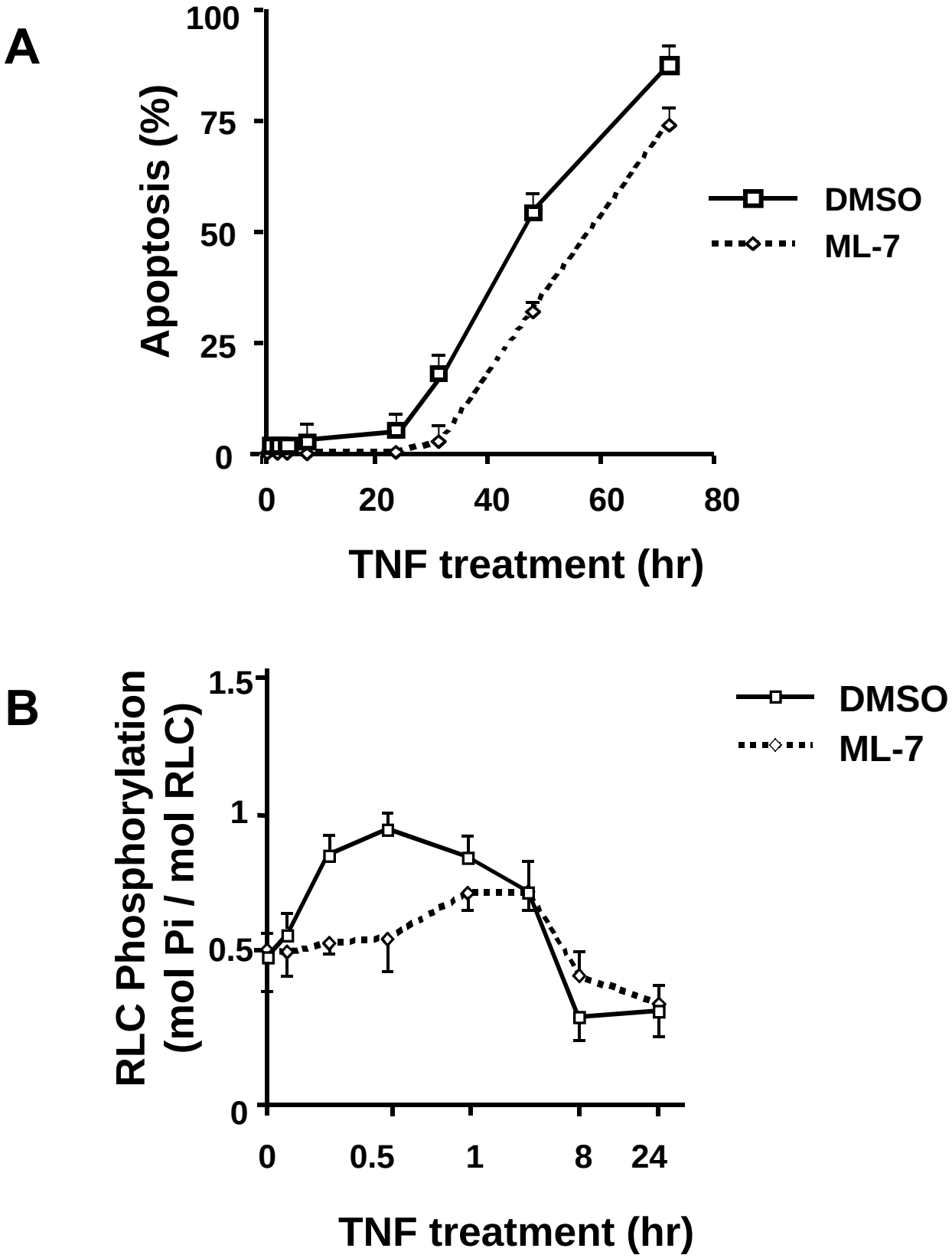
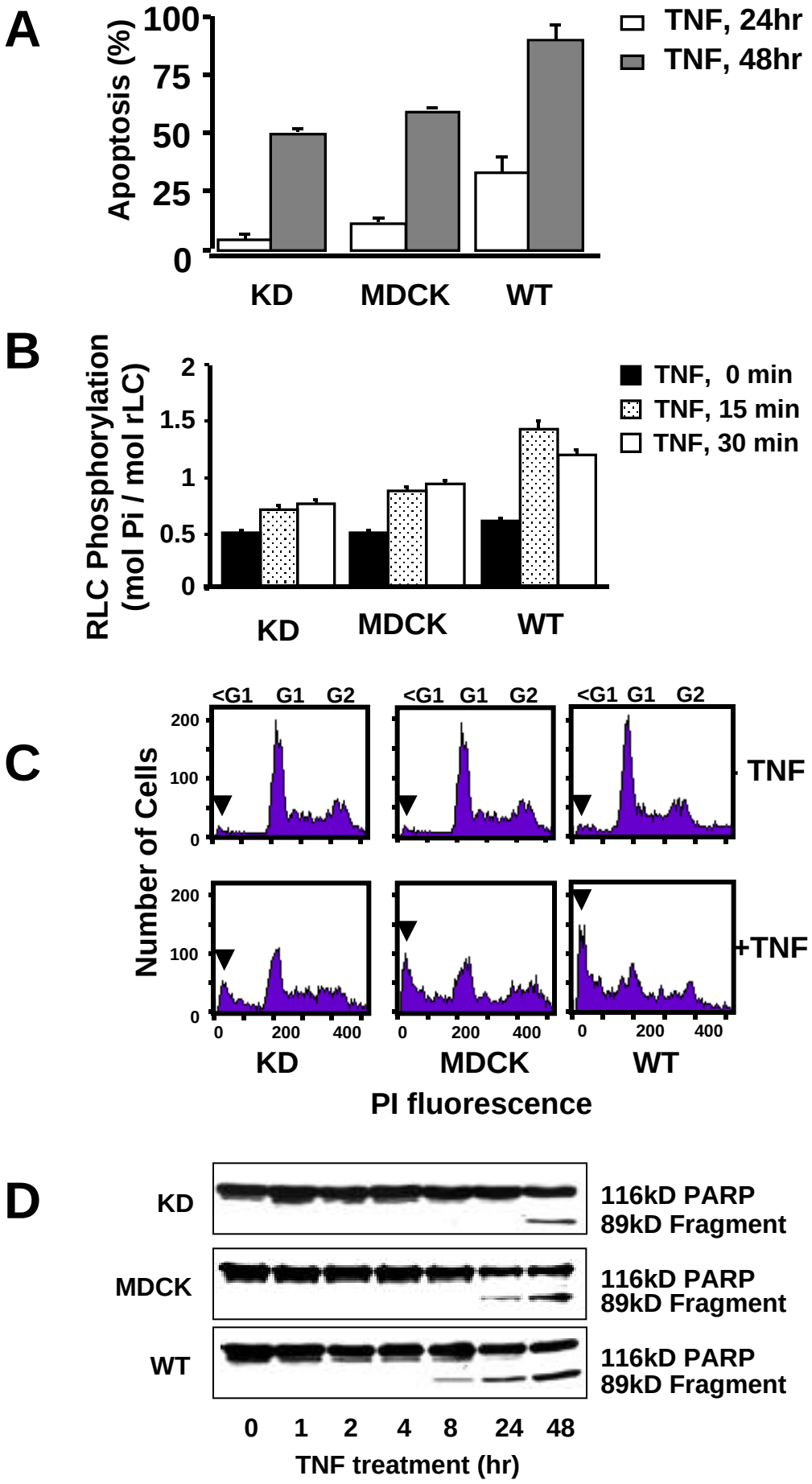

A
100
75
50
25
0
Apoptosis (%)
0
20
40
60
80
TNF treatment (hr)
DMSO
ML-7
B
1.5
1
0.5
0
RLC Phosphorylation
(mol Pi / mol RLC)
0
0.5
1
8
24
TNF treatment (hr)
DMSO
ML-7

Figure 2


A
Apoptosis (%)
100
75
50
25
0
TNF, 24hr
TNF, 48hr
KD
MDCK
WT
B
RLC Phosphorylation
(mol Pi / mol rLC)
2
1.5
1
0.5
0
TNF, 0 min
TNF, 15 min
TNF, 30 min
KD
MDCK
WT
C
<G1 G1 G2
Number of Cells
200
100
0
TNF
+TNF
0 200 400
KD
MDCK
WT
PI fluorescence
D
KD
MDCK
WT
116kD PARP
89kD Fragment
0 1 2 4 8 24 48
TNF treatment (hr)

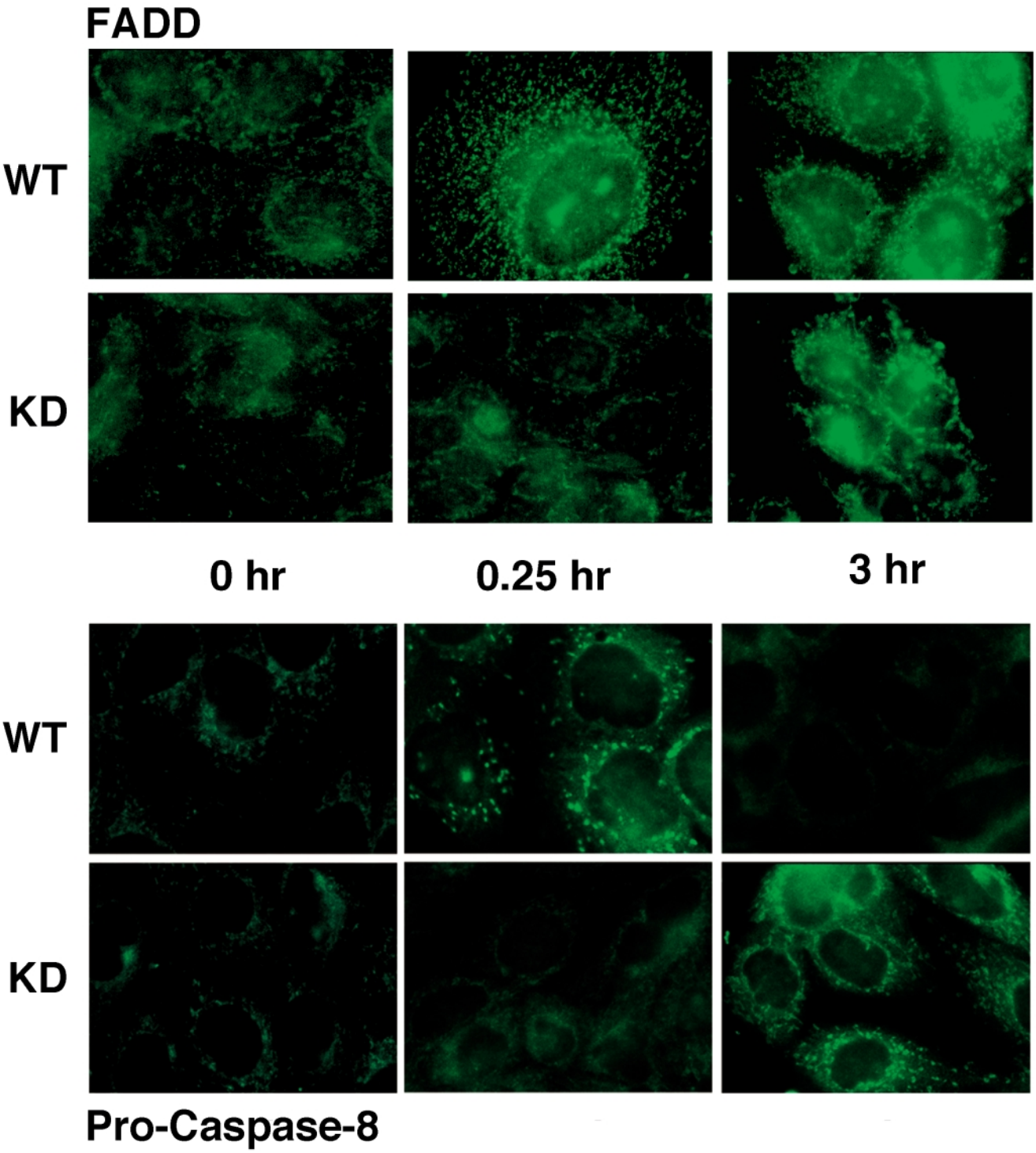

FADD
WT
KD
0 hr
0.25 hr
3 hr
WT
KD
Pro-Caspase-8

Figure 4

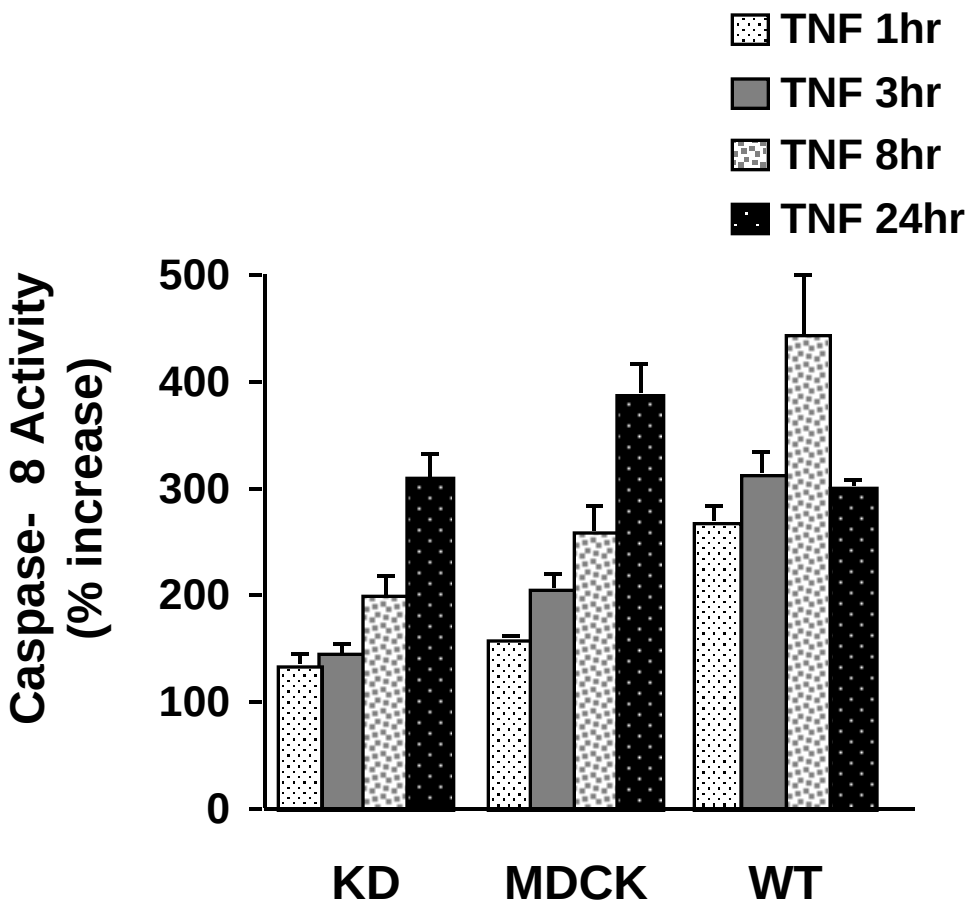
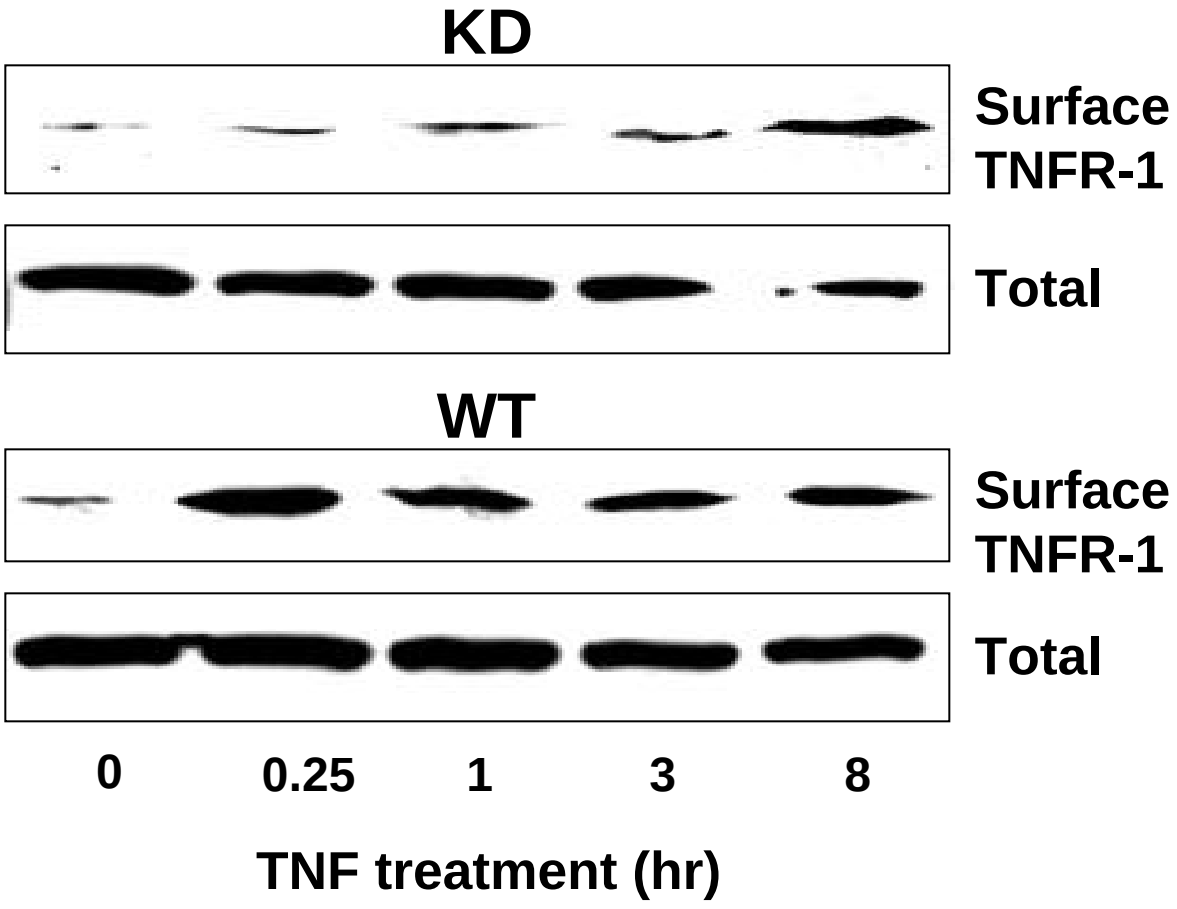

TNF 1hr
TNF 3hr
TNF 8hr
TNF 24hr
Caspase- 8 Activity
(% increase)
500
400
300
200
100
0
KD
MDCK
WT

Figure 5


KD
Surface TNFR-1
Total
WT
Surface TNFR-1
Total
0
0.25
1
3
8
TNF treatment (hr)

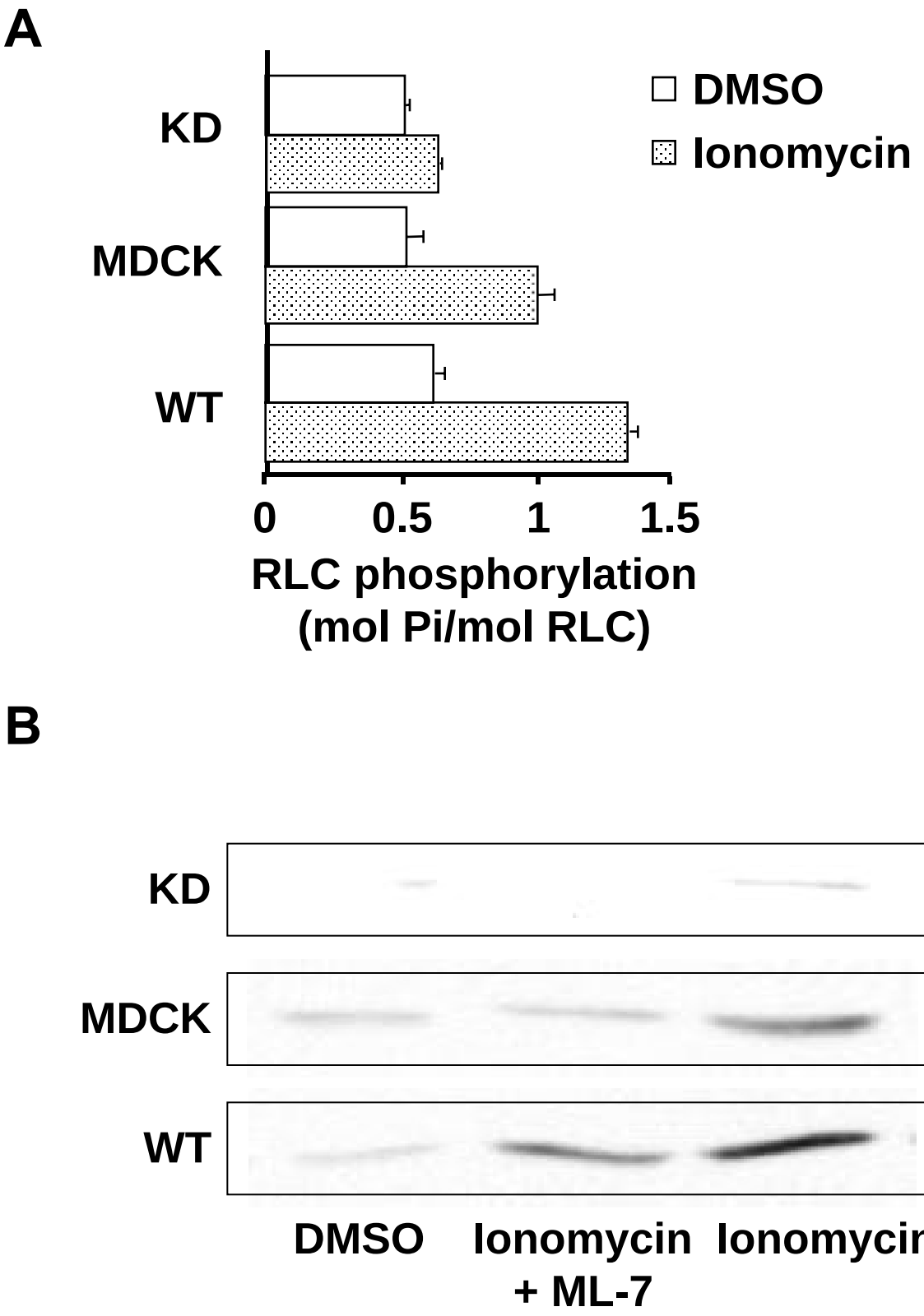

A
KD
MDCK
WT
DMSO
Ionomycin
0
0.5
1
1.5
RLC phosphorylation
(mol Pi/mol RLC)
B
KD
MDCK
WT
DMSO
Ionomycin
Ionomycin
+ ML-7

Figure 7

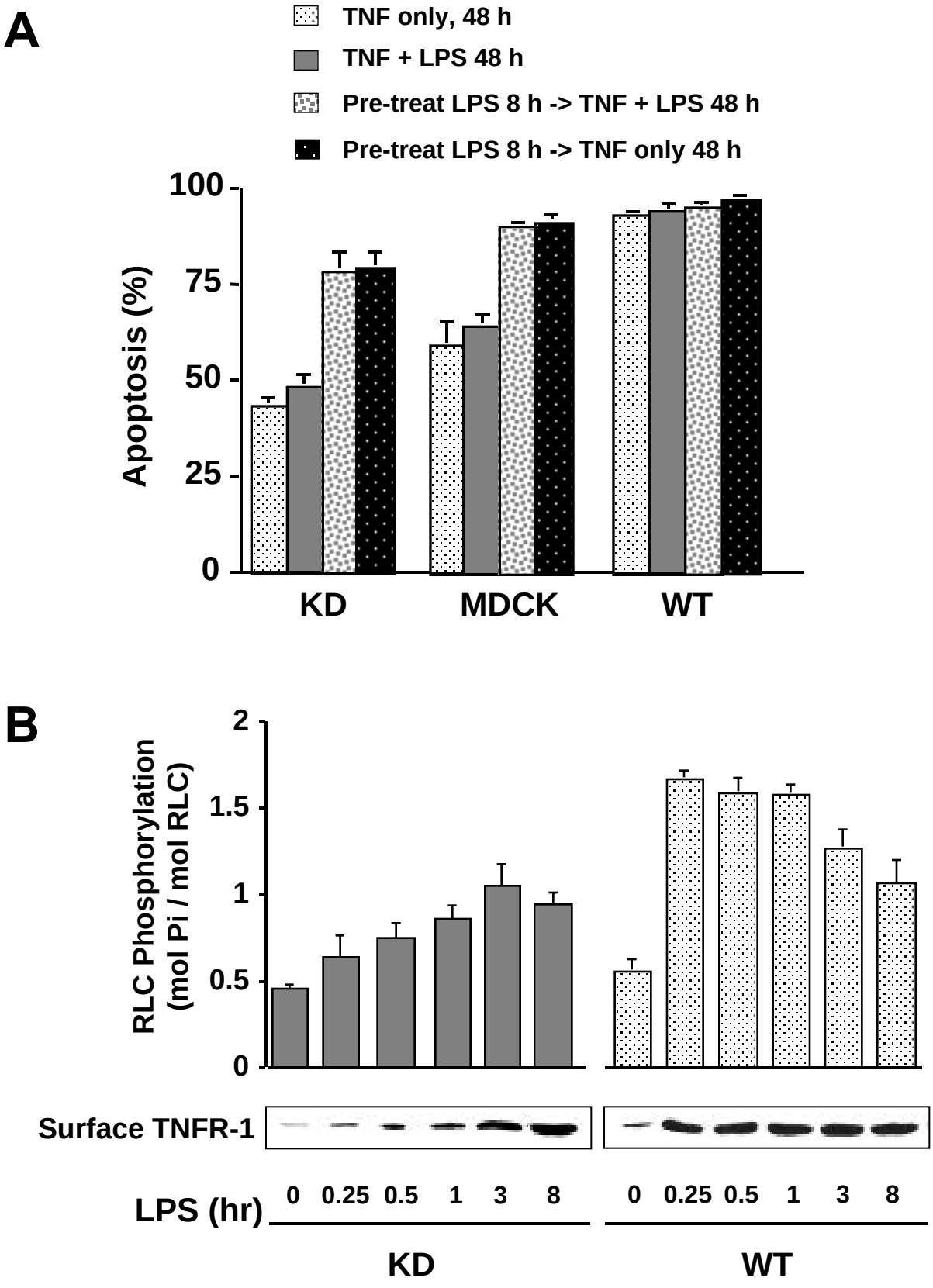
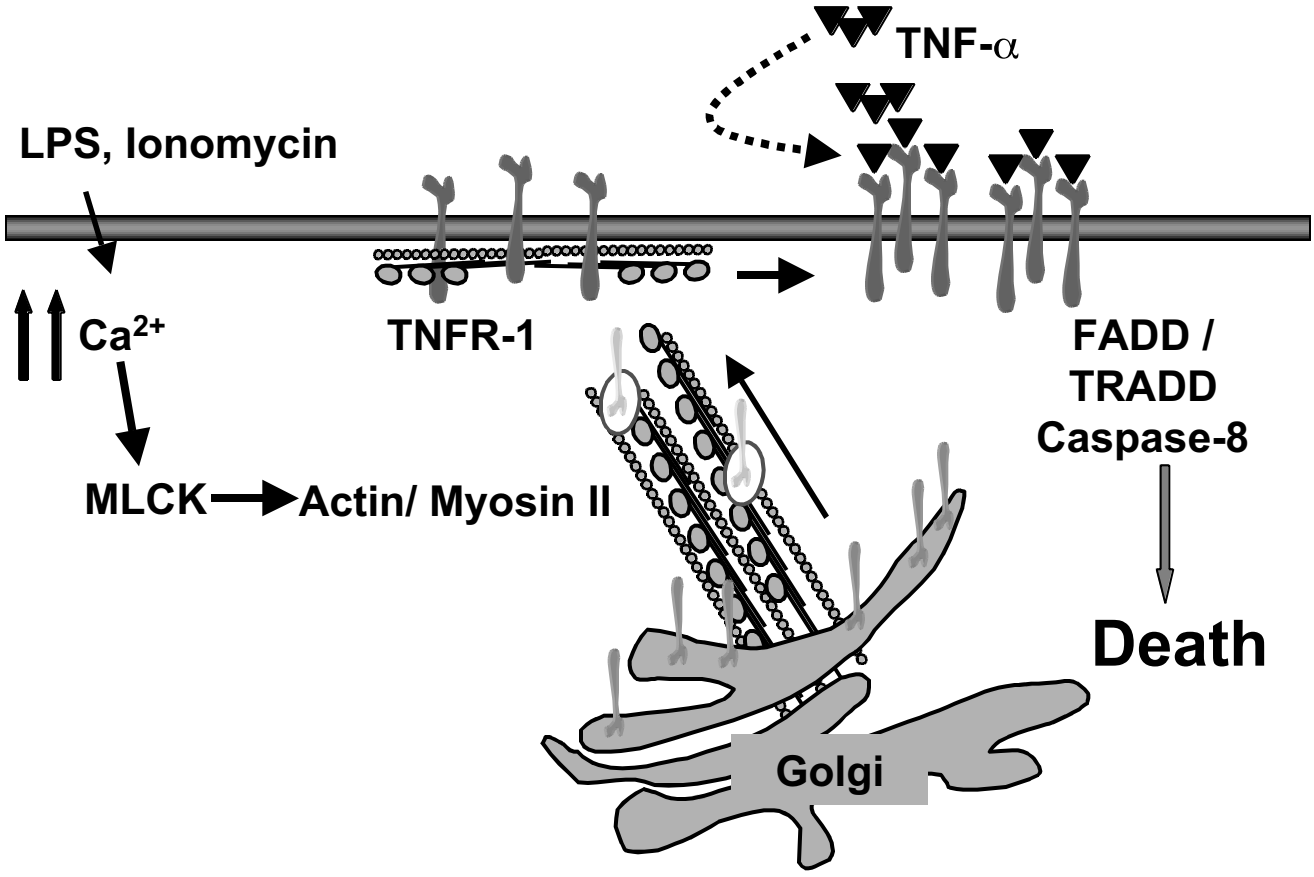

A
TNF only, 48 h
TNF + LPS 48 h
Pre-treat LPS 8 h -> TNF + LPS 48 h
Pre-treat LPS 8 h -> TNF only 48 h
Apoptosis (%)
100
75
50
25
0
KD
MDCK
WT
B
RLC Phosphorylation (mol Pi / mol RLC)
2
1.5
1
0.5
0
Surface TNFR-1
LPS (hr)
0 0.25 0.5 1 3 8
0 0.25 0.5 1 3 8
KD
WT

Figure 8


TNF-α
LPS, Ionomycin
Ca2+
TNFR-1
FADD /
TRADD
Caspase-8
MLCK
Actin/ Myosin II
Death
Golgi