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Non-Muscle Myosin IIB Helps Mediate TNF Cell Death Signaling Independent of Actomyosin Contractility (AMC)

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ABSTRACT

Non-muscle myosin II (NM II) helps mediate survival and apoptosis in response to TNF-alpha (TNF), however, NM II's mechanism of action in these processes is not fully understood. NM II isoforms are involved in a variety of cellular processes and differences in their enzyme kinetics, localization, and activation allow NM II isoforms to have distinct functions within the same cell. The present study focused on isoform specific functions of NM IIA and IIB in mediating TNF induced apoptosis. Results show that siRNA knockdown of NM IIB, but not NM IIA, impaired caspase cleavage and nuclear condensation in response to TNF. NM II's function in promoting cell death signaling appears to be independent of actomyosin contractility (AMC) since treatment of cells with blebbistatin or cytochalasin D failed to inhibit TNF induced caspase cleavage. Immunoprecipitation studies revealed associations of NM IIB with clathrin, FADD, and caspase 8 in response to TNF suggesting a role for NM IIB in TNFR1 endocytosis and the formation of the death inducing signaling complex (DISC). These findings suggest that NM IIB promotes TNF cell death signaling in a manner independent of its force generating property. J. Cell. Biochem. 110: 1365–1375, 2010.

KEY WORDS: NON-MUSCLE MYOSIN II; TUMOR NECROSIS FACTOR; ACTOMYOSIN CONTRACTILITY; REGULATORY MYOSIN LIGHT CHAIN; APOPTOSIS; ACTIN CYTOSKELETON

TNF-alpha (from here on referred to as TNF) is a cytokine that can activate a variety of cellular processes including the ability to promote survival or initiate apoptosis depending on the environmental context and molecular state of the cell [Tracey et al., 2008]. The signaling initiated by TNF is complex and the cellular factors that mediate its diverse biological effects remain to be fully elucidated. Tumor necrosis factor receptor1 (TNFR1) and TNFR2 are cell surface receptors that play central roles in initiating TNF signaling. TNFR2 is expressed mostly by cells of the immune system while TNFR1 is expressed ubiquitously on nearly all cell types and has the ability to initiate both survival and apoptotic signals due to the presence of a death domain (DD) on its cytoplasmic tail [Tartaglia et al., 1993; Legler et al., 2003]. Recruitment of adaptor proteins such as TNFR associated death domain (TRADD), receptor interacting protein 1 (RIP1), and others to the activated TNFR1 forms a prosurvival signaling complex at the plasma membrane that initiates NFkappaB signaling [Micheau and Tschopp, 2003]. Shortly thereafter TNFR1 is internalized and the prosurvival signaling complex is modified to allow recruitment of Fas-associated death domain (FADD) and caspase 8 to form the death inducing signaling complex (DISC). Activation of caspase 8 at the DISC initiates the execution of apoptosis, however, NFkappaB can induce the expression of antiapoptotic proteins such as FLIP to inhibit the cleavage of caspase 8 [Bai et al., 2004]. Therefore, the potential of the cell to promote survival and prevent apoptosis in response to TNF is mainly determined by its ability to activate NFkappaB signaling.

The actin cytoskeleton and non-muscle myosin II (NM II) have been shown to play an important role in mediating TNF signaling [Jin et al., 2001; Petrache et al., 2003b]. NM II is a hexamer made up of two heavy chains, two essential light chains, and two regulatory light chains (MLC) [Vicente-Manzanares et al., 2009]. Phosphorylation of NM II's MLC converts NM II into an active state allowing it to bind actin filaments and generate a force known as actomyosin contractility (AMC) [Tyska and Warshaw, 2002; Sellers and Knight, 2007]. Myosin light chain kinase (MLCK) and Rho kinase are

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activated in response to TNF and help regulate actin cytoskeletal dynamics by phosphorylating the MLC resulting in the generation of force [Kim et al., 2002; McKenzie and Ridley, 2007]. This generation of force by NM II has been suggested to play an important role in signaling pathways mediating survival and apoptosis in response to TNF, but how it does so is unclear [Petrache et al., 2001; Wadgaonkar et al., 2005].

The human genes *MYH9*, *MYH10*, and *MYH14* encode for the NM II isoforms; NM IIA, NM IIB, and NM IIC respectively. Differences in their localization and activation are associated with isoform-specific roles of NM II [Conti and Adelstein, 2008; Vicente-Manzanares et al., 2008, 2009]. There is also evidence that isoform specific functions of NM II may correlate with activation by specific kinases which is seen when Rho kinase activates NM IIA to facilitate thrombin-induced cell rounding of MDA-MB-231 cells [Sandquist et al., 2006]. Activation of NM II isoforms by different upstream kinases may be an important mechanism of how the cell utilizes the NM II isoforms for distinct functions in response to different signals from the extracellular environment. In addition to activating kinases the enzymatic properties of the NM II isoforms themselves may determine their biological function. Unique enzymatic properties of NM IIB include a high duty ratio making it more suited to have structural roles within the cell as compared to NM IIA, since NM IIB can exert tension on actin filaments for a longer period of time without processing as much ATP. Indeed, NM IIB has been shown to play an important structural role in forming an apical meshwork in neuroepithelial cells that is crucial in preventing hydrocephalus [Ma et al., 2007]. Although isoform specific roles for NM II have been reported, little work has been focused on distinct roles during cell death. Therefore, to gain a better understanding of how NM II and AMC may be involved in cell death signaling events in response to TNF we focused our studies on the NM IIA and NM IIB isoforms.

The MLC is an important substrate for a number of the kinases activated by TNF such as death associated protein kinase (DAPK), Rho kinase, and MLCK [Cohen et al., 1999; Kim et al., 2002; Kuo et al., 2003; McKenzie and Ridley, 2007]. Interestingly, signal transduction pathways leading to NM II activation have been shown to be involved in both apoptotic and survival signaling in response to TNF. Inhibiting NM II activation by impairing MLCK activity through pharmacological and genetic means has been shown to impair both NFkappaB signaling and caspase activation [Petrache et al., 2001; Wadgaonkar et al., 2005]. Therefore, we have hypothesized that the NM II isoforms are being utilized in separate and distinct manners to mediate the life and death response to TNF. This paper focuses on the involvement of the NM II isoforms in TNF induced cell death. We present evidence that NM IIB has an isoform specific role in mediating TNF induced cell death signaling and that its function is independent of force generation.

MATERIALS AND METHODS

CELL CULTURE

Hela cells were cultured in Dulbeco's modification of Eagles medium Cat #10-017-CV (Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% of penicillin/streptomycin and grown in 5% CO_2 at 37°C.

ANTIBODIES AND REAGENTS

Rabbit anti-cleaved caspase 3 (Asp175)(5A1), anti-phospho-MLC (ser18/thr19), anti-TNFR1 (C25C1), anti-phospho-I-kappa-B-alpha (Ser32), anti-I-kappa-B-alpha, anti-clathrin heavy chain, and mouse anti-cleaved caspase 8 (1C12) antibodies were all purchased from Cell Signaling (Danvers, MA). Rabbit anti-MLC2, anti-TNFR1 (H-271), anti-Clathrin HC (H-300), anti-FADD (H-181), and mouse anti-caspase 8 p20 (D-8) antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-nonmuscle myosin IIA heavy chain and anti-nonmuscle myosin IIB heavy chain antibodies were purchased from Covance (Emeryville, CA). Recombinant human TNF-alpha was purchased from R&D Systems. ML-7 and Blebbistatin were purchased from BioMol International, LP. Cycloheximide was purchased from Sigma. Cytochalasin D was purchased from Calbiochem. 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen.

siRNA

The siRNA oligonucleotides used to knockdown myosin IIA and IIB expression were purchased from Dharmacon; the antisense sequence for Myosin IIA (MYH9) is 5′-AAAUCGGUCACAUUGAUA-CUU-3′. The antisense sequence for Myosin IIB (MYH10) is 5′-UAUUCUCAGAGUAAAUUGGUU-3′. The protocol recommended by the supplier Dharmacon was followed. Briefly, cells were seeded overnight so that they were approximately 50% confluent at the time of siRNA transfection. siRNA was suspended in the supplied RNase free buffer at a concentration of 20 μM then incubated with the dharmafect transfection reagent (dharmafect 1) at a 11:1 ratio of siRNA to transfection reagent in a total of 400 μl of serum free medium (SFM) for 20 min. This mixture was then added to 1.6 ml of complete medium that did not contain any antibiotics. This mixture totaling 2 ml was then added to one well of a six-well plate in a drop wise manner. The cells were incubated with the medium containing the siRNA transfection complex for 24 h. After this time the siRNA containing medium was replaced with new fresh complete medium not containing any antibiotics. Seventy-two hours post-transfection was the optimal time for myosin IIA and IIB knockdown as determined through Western blot analysis. At this time TNF-alpha and cycloheximide (CHX) was added to the conditioned medium for approximately 2–3 h to analyze the effects knockdown of IIA and IIB had on TNF-alpha + CHX induced apoptosis.

IMMUNOFLUORESCENT STAINING

Cells were plated on glass coverslips for 24 h. After treatments the cells were fixed in 3% paraformaldehyde (PFA) for 15 min, washed 3× in PBS, then permeabilized with 0.1% Triton X-100 in PBS for 15 min, washed 3× with PBS, and blocked with 5% BSA at room temperature for 30 min. The cells were then incubated with myosin IIB Ab from Covance at a 1:500 dilution in 5% BSA for 30 min. Cells were then washed three times with PBS and then incubated with Alexa Fluor 594 goat anti-rabbit IgG secondary (Invitrogen, Molecular Probes, Eugene, OR) at a 1:500 dilution in 5% BSA for 30 min. The 5% BSA also included Oregon green 488 Phalloidin (Molecular Probes). Cells were then washed three times with PBS and incubated in a solution containing DAPI nuclear staining for 5 min then washed two more times. The coverslips containing the cells

were placed on glass slides containing ProLong Gold anti-fade reagent (Invitrogen, Molecular Probes) overnight at room temperature. Cells were viewed and pictures were taken on a Zeiss Axiovert 200M microscope.

APOPTOSIS ASSAY

Apoptosis was assessed by viewing DAPI stained nuclei through an immunofluorescent scope for the presence of nuclear condensation. The percentage of apoptotic cells present was determined by performing a blind count in which the number of cells with nuclear condensation was divided by the total number of cells counted. Each treatment was done in triplicate and a minimum of 200 cells were counted for each treatment.

ISOLATION OF CELL SURFACE TNFR1

Isolation of cell surface TNFR1 was accomplished by using the cell surface labeling accessory pack from Pierce (Rockford, IL) and following the manufacture's protocol. Briefly, after TNF-alpha treatment (5 ng/ml) cells were washed with cold PBS and incubated in a biotin/PBS solution for 30 min after which time a quenching reagent was added to the dish and cells were harvested by gentle scraping. Cell pellets were spun down and washed once in TBS and then lysed for 30 min on ice with vortexing every 5 min along with pippeting of samples through a $25\frac{1}{8}$ needle. The samples were spun down at 10,000 × g and then the supernatant was added to a column containing streptavidin beads that were previously incubated in 5% BSA for 30 min to help reduce non-specific binding. The supernatant containing the biotinylated protein and the streptavidin beads were rotated in a column for 2 h at 4°C. After the incubation the supernatant was spun through the column's filter and the beads/streptavidin/protein complex was washed three times. Laemlli sample buffer + DTT was then added in order to allow proteins to be spun through the column's filtered bottom and gathered in an eppendorf tube. Samples were then ran on SDS-PAGE gel to analyze the presence of TNFR1.

CO-IMMUNOPRECIPITATION

Cells were seeded to obtain 90% confluency at the time of treatment. The dosage and time of treatments with TNF-alpha, CHX, and drugs are indicated in the figure legends. After treatment cells were kept on ice and washed with ice cold PBS two times then immunoprecipitation (IP) lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, and 0.5% SodiumDeoxycholate was added to cells at a 1.2× concentration. The cells were rocked back and forth for 15 min at 4°C and were then gathered into eppendorf tubes through gentle scraping. The cell lysis was then inverted several times and then gently pippetted through a $25\frac{1}{8}$ needle three times. Next, the lysis sample was spun down at an RCF of 16,000*g* for 12 min to form a solid pellet. The supernatant was carefully separated from the pellet and placed in an eppendorf tube containing washed protein A/G agarose beads and rotated for 1 h at 4°C in order to pre-clear the supernatant. The pre-cleared supernatant was then removed from the beads and placed in a new eppendorf tube. Then, either myosin IIB from Covance, TNFR1(H-271) from Santa Cruz Biotechnology, FADD (H-181) from Santa Cruz Biotechnology, or caspase 8 p20 (D-8) from Santa Cruz Biotechnology was added to the supernatant and allowed to rotate overnight. The next day TruBlot anti-Rabbit or anti-Mouse IgG IP beads were added to the supernatant and the Beads/Ab/Supernatant mixture were rotated for 2–3 h at 4°C. After 2–3 h the mixture was spun down and the supernatant was removed so that the beads could be washed three times with IP lysis buffer. After the washing SDS-Lamelli sample buffer was added and the samples were vortexed, spun down, and then boiled for 10 min.

WESTERN BLOT ANALYSIS

Cells were harvested in 1× SDS Laemmli sample buffer from Biorad, Hercules, CA (cat#161-0737) containing beta-mercaptoethanol and Halt phosphatase (Thermo Scientific) and protease inhibitor cocktail (Sigma). Samples were placed in boiling water for 5 min and loaded into the appropriate percentage of SDS-PAGE gel. After running the lysate samples for approximately 2 h to get adequate separation of proteins, the samples were transferred to nitrocellulose membrane at 40 V for 90 min. The nitrocellulose membrane (Hybred-ECL 0.2 μM, Amersham Bioscience) was incubated in Ponceau (Sigma) stain for 3 min in order to view the levels of protein present on the membrane, this helps determine if even loading has been achieved. The membrane was then blocked in 5% milk for 1 h. Membranes were then washed and incubated with appropriate primary antibody at a 1:1,000 ratio in 5% BSA (Calbiochem) overnight. Membranes were then washed three times for 5 min in TTBS at which time the appropriate HRP-conjugated secondary antibody (Cell Signaling) was added to the membrane at a 1:1,000 ratio in 2.5% milk for 1 h. The membrane was then washed three times for 5 min then exposed to Super signal west pico chemilumenescent reagent (Pierce). The membrane was then exposed to Blue lite autorad film (ISC Bioexpress). Densitometry analysis of Western blots was performed using Image J which was downloaded for free from the National Institute of Health (NIH) website.

RESULTS

NM IIB HELPS MEDIATE TNF CELL DEATH SIGNALING

Previous works demonstrate that NM II activation is involved in mediating both survival and apoptosis in response to TNF, however, the mechanism in which NM II is involved in these signaling pathways is not yet known. Western blot analysis determined there was no detectable NM IIC expression in our Hela cells (data not shown), therefore, we wanted to determine if NM IIA or NM IIB were being utilized in an isoform specific manner to mediate TNF induced cell death signaling. In order to address this question the use of siRNA against NM IIA (siIIA), NM IIB (siIIB), and a scrambled siRNA control (siCon) was employed. Although NM II isoforms share a great deal of amino acid identity [Bresnick, 1999] the siRNA knockdown of NM IIA and IIB is isoform specific (Fig. 1A). Once the specificity of the isoform knockdown was verified Hela cells were treated with siCon, siIIA, or siIIB and then exposed to TNF + CHX to determine the ability of these cells to carry out TNF induced cell death signaling. The Western blot in Figure 1B illustrates that cells treated with siIIB had a significant decrease in the amount of caspase 3 cleavage in response to TNF as compared to the siCon and siIIA treated cells. The graph in Figure 1B illustrates that cells treated with siIIB had a 38% decrease in the amount of caspase 3 cleavage in

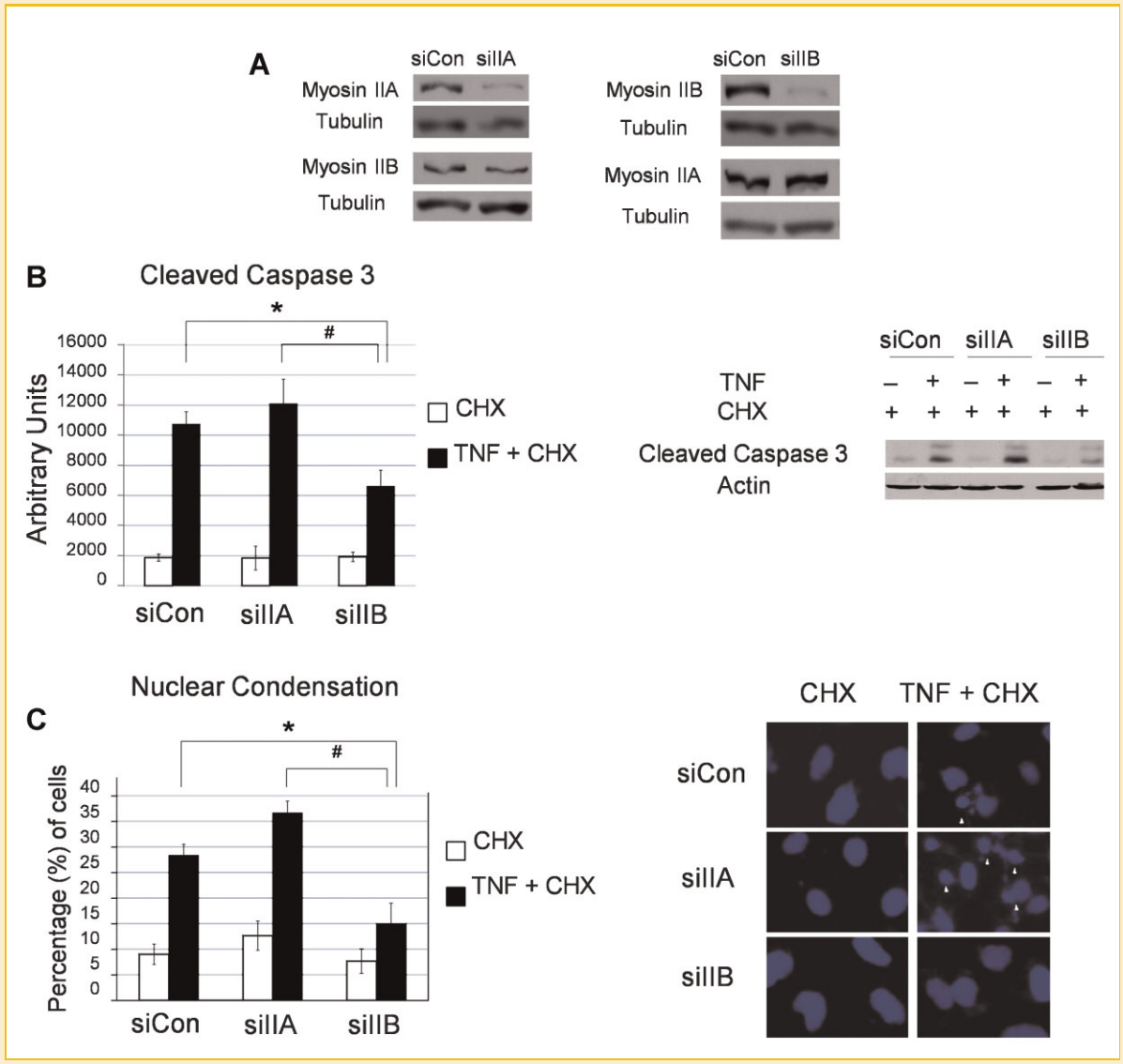

Fig. 1. Knockdown of NM IIB impairs cell death signaling in response to TNF. A: Hela cells were treated with siRNA against NM IIA (siIIA), NM IIB (siIIB), and siRNA control sequence (siCon) as described in experimental procedures. Isoform specific knockdown was assessed by Western blotting for the expression of the NM IIA and NM IIB protein. B: Graph represents cells treated with siIIB having significantly less cleavage of caspase 3 as compared to the siCon (*) and siIIA (#) treated cells in response to 10 ng/ml TNF + 10 μg/ml cycloheximide (CHX) for 3 h. Two-tailed student's *t*-test determined values were significant with *P* values <0.05 ($n = 3$). A representative Western blot corresponding to the graph is displayed on the right. C: Graph represents cells treated with siIIB having significantly less nuclear condensation as compared to the siCon (*) and siIIA (#) treated cells in response to 10 ng/ml TNF + 10 μg/ml CHX for 3 h. Two-tailed student's *t*-test determined values were significant with *P* values <0.05 ($n = 3$). Immunoflourescent (IF) images representing nuclear condensation that was seen are displayed on the right (white arrowheads).

response to TNF as compared to the siCon treated cells and a 45% decrease as compared to the siIIA treated cells.

Caspase 3 plays an important role in nuclear condensation during the execution phase of apoptosis by breaking down components of the nuclear matrix [Kivinen et al., 2005]. Since knockdown of NM IIB impairs caspase 3 cleavage we wanted to determine if siIIB treatment was also able to impair nuclear condensation. Immunofluorescence images in Figure 1C illustrate that cells treated with siIIB had much less nuclear condensation as compared to the siCon and siIIA treated cells in response to TNF (white arrowheads). The graph in Figure 1C illustrates that the siCon and siIIA treated cells had a 19% and 24% increase in nuclear condensation in response to TNF as compared to their respective CHX controls whereas siIIB treated cells only had a 7.4% increase as compared to its CHX control. Taken together these data suggest an important role for NM IIB, but not IIA, in mediating TNF induced cell death.

TNF CELL DEATH SIGNALING DOES NOT REQUIRE AMC

Previous works implicate a role for AMC in mediating TNF signaling based on increases seen in MLC phosphorylation. However, MLC phosphorylation resulting in activation of NM II has been shown to effect cell signaling events independent of AMC [Zang and Spudich, 1998; Arora et al., 2008]. Therefore, in order to gain a better understanding of the role NM II plays in TNF induced apoptosis we

wanted to determine if AMC was required for TNF cell death signaling. In order to address this question we treated Hela cells with the pharmacological agent blebbistatin which specifically inhibits NM II motor activity without impairing MLC phosphorylation [Kovacs et al., 2004; Limouze et al., 2004; Allingham et al., 2005; Ponsaerts et al., 2008]. Blebbistatin impairs the ability of NM II to process ATP by preventing the release of inorganic phosphate from ADP [Kovacs et al., 2004]. This stabilizes NMII in a transition state that disrupts its association with actin filaments leading to actin cytoskeletal destabilization (Fig. 2A). Treatments with blebbistatin from 5 to 100 μM were unable to prevent caspase 3 and 8 cleavage in response to TNF (Fig. 2B). Although blebbistatin treatment is established as an effective method to impair AMC, an alternative means of impairing AMC that does not directly target the NM II protein is through treatment with cytochalasin D. This pharmacological agent disrupts the formation of actin filaments (Fig. 2A) providing another approach in which to examine the involvement of AMC and actin filament re-organization in TNF cell death signaling. Although cytochalasin D disrupts actin filaments thus impairing force generation by NM II, it was unable to inhibit the cleavage of caspases 3 and 8 in response to TNF. It is important to note that MLC phosphorylation in response to TNF was not inhibited by blebbistatin or cytochalasin D treatments (Fig. 2D). However, treatment with 10 μM of the MLCK inhibitor ML-7 impaired MLC phosphorylation by 16% with a concomitant decrease in the cleavage of caspase 3 and 8 by 15% and 19% respectively and these inhibitions increased with the use of 20 μM ML-7 (Fig. 2E,F). Therefore, these data suggest a possible role for activated NM II in TNF cell death signaling independent of AMC.

KNOCKDOWN OF NM IIB EXPRESSION IMPAIRS TNFR1 INTERNALIZATION

One crucial upstream event that is required for TNF induced apoptosis is the internalization of the TNFR1 receptor which has been shown to occur within minutes of TNF binding [Schutze et al., 1999; Schneider-Brachert et al., 2004, 2006]. Therefore, we wanted to determine if NM II was helping to mediate the TNF cell death response through an involvement in TNFR1 internalization. Figure 3A shows that treatment with TNF causes a significant decrease in the amount of cell surface TNFR1 by the 30 min time point. The presence of TNFR1 in whole cell lysate samples indicates that the decrease in cell surface TNFR1 in response to TNF was due to internalization and not to release of the receptor from the cell membrane. To determine if NM IIA and IIB have isoform specific roles in mediating TNFR1 internalization we silenced their expression through siRNA treatment before addition of TNF to the medium. The Western blot in Figure 3B is representative of three independent experiments showing that knockdown of NM IIB impairs TNFR1 internalization as compared to the internalization seen for siIIA and siCon treated cells. In addition, the quantified results demonstrate a twofold increase in the amount of cell surface TNFR1 seen in cells treated with siIIB as compared to the siIIA or siCon treatments. These results suggest a role for NM IIB, but not IIA, in promoting the internalization of the TNFR1 receptor.

The contractile forces generated by NM II as well as myosin VI and myosin 1E have been suggested to play varying roles to support receptor endocytosis [Samaniego et al., 2007; Ungewickell and Hinrichsen, 2007]. In order to determine if AMC produced by NM II was involved in TNFR1 internalization we treated cells with blebbistatin to specifically inhibit the ATPase function of NM II [Kovacs et al., 2004; Limouze et al., 2004; Allingham et al., 2005]. Figure 3C illustrates that blebbistatin treatment was unable to prevent the decrease seen in cell surface TNFR1 in response to TNF. This result indicates that AMC produced by NM II is not required for TNFR1 internalization and is consistent with blebbistatin treatment being unable to prevent caspase cleavage in response to TNF.

NM IIB ASSOCIATES WITH PROTEINS INVOLVED IN TNF SIGNALING

Since internalization of TNFR1 has been shown to be clathrin mediated [Mosselmans et al., 1988] we wanted to determine if there was an association between clathrin and TNFR1 in Hela cells treated with TNF. Internalization of TNFR1 occurs as early as 7.5 min with a concomitant increase in NM II activation (Fig. 4A). Therefore, we performed an immunoprecipitation (IP) against TNFR1 at 15 min and Western blotted for its interaction with clathrin. The representative blot in Figure 4B illustrates an increased association between TNFR1 and clathrin at the 15 min time point. Based on our data NM IIB was the isoform implicated in TNFR1 internalization, therefore, we wanted to determine if there was any interaction between NM IIB with clathrin or TNFR1. We performed an IP against NM IIB and found that there was an increased association with clathrin in response to TNF (Fig. 4C). Surprisingly, no actin was found in the IP samples indicating that clathrin was not indirectly associating with NM IIB through binding to actin filaments. Western blot analysis of NM IIB IP samples also revealed that there was no detectable association between NM IIB and TNFR1 (data not shown).

In addition to TNFR1 internalization the formation of the DISC complex is another event that is crucial for initiating TNF induced apoptosis. For this reason we wanted to examine the possibility of NM IIB interacting with key members of the DISC complex such as FADD and caspase 8. Figure 4D illustrates that at the 3.5 h time point in which cleavage of caspases 3 and 8 are detectable there is also an increased association of FADD and caspase 8 with NM IIB. Increased associations of FADD with NM IIA were inconsistent and when interactions were detected between FADD and IIA they were significantly less then what was seen with NM IIB (data not shown). It is worth noting that attempts to co-IP FADD and caspase 8 in their associated state to detect the formation of the DISC complex were unsuccessful. Therefore, it is still unclear whether or not NM IIB is an actual member of the DISC complex, however, these observations do suggest a possible role for NM IIB in DISC formation.

DISCUSSION

The ability of NM II to generate force on actin filaments has been shown to play a role in various processes during the execution phase of apoptosis including membrane blebbing, nuclear disintegration, and cellular contraction [Cotter et al., 1992; Coleman et al., 2001; Croft et al., 2005]. Although the role of NM II in the execution phase of apoptosis has been established its involvement in apoptotic

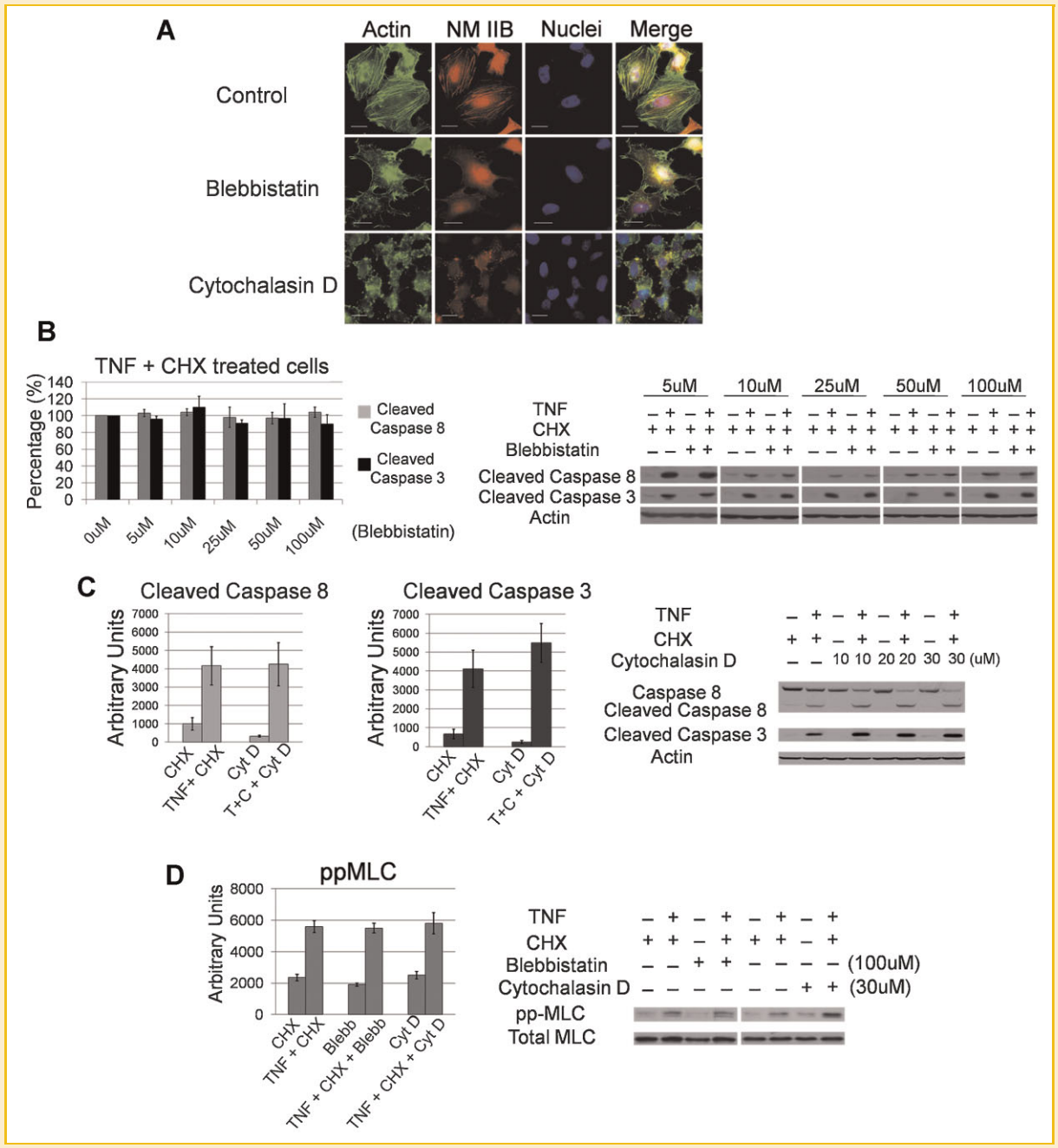

Fig. 2. Inhibitors of AMC do not prevent cell death signaling in response to TNF. A: Immunoflourescent images of the effects that a 30 min treatment with 100 μM blebbistatin and 10 μM cytochalasin D have on the structure of actin filaments (green) and distribution of NM IIB (red) in Hela cells (Scale bar 20 μM). B: Hela cells were pretreated with increasing amounts of blebbistatin for 30 min then 10 ng/ml of TNF + 10 μg/ml of cycloheximide (CHX) was added to the medium for 3.5 h. The graph represents the percentage of caspase cleavage that was determined by dividing the values obtained for TNF-alpha + CHX cells that were pretreated with blebbistatin by the values obtained for TNF-alpha + CHX control cells. These values were arbitrary units obtained through densometric analysis of Western blots (n = 3). C: Hela cells were pretreated with increasing amounts of cytochalasin D (Cyt D) from 10 to 30 μM for 30 min then given the same TNF treatment as above. The graph represents arbitrary units of caspase cleavage from three experiments using 10 μM Cyt D (similar results were found for 20 and 30 μM Cyt D). A representative Western blot illustrates that increasing amounts of Cyt D from 10 to 30 μM was unable to inhibit the cleavage of caspases 3 and 8. D: Hela cells were pretreated with 100 μM blebbistatin and 30 μM Cyt D for 30 min then MLC di-phosphorylation in response to TNF + CHX was measured at the 15 min time point (graph; n = 3). A representative Western blot illustrates that 100 μM blebbistatin and 30 μM Cyt D was unable to inhibit pp-MLC in response to TNF (similar results were found for lower concentrations of blebbistatin and Cyt D; data not shown). E: Hela cells were pretreated with ML-7 for 30 min then 10 ng/ml of TNF + 10 μg/ml of cycloheximide (CHX) was added to the medium for 3.5 h. NM II activation was assessed by Western blotting for di-phosphorylation of the MLC (Thr18/Ser19). Graph represents percentage of pp-MLC and two-tailed student's *t*-test determined that the decrease seen in pp-MLC (*) is statistically significant with *P* values <0.05 (n = 3). Representative Western blots for 10 and 20 μM illustrate the decrease in pp-MLC as the concentration of ML-7 increases. F: Hela cells received the same TNF + CHX and ML-7 treatment as above and the graph represents percentage of caspase cleavage. Two-tailed student's *t*-test determined that the decrease seen in caspase cleavage (*) is statistically significant with *P* values <0.05 (n = 3). Representative Western blots illustrate the decrease seen in caspase cleavage as ML-7 concentration increases.

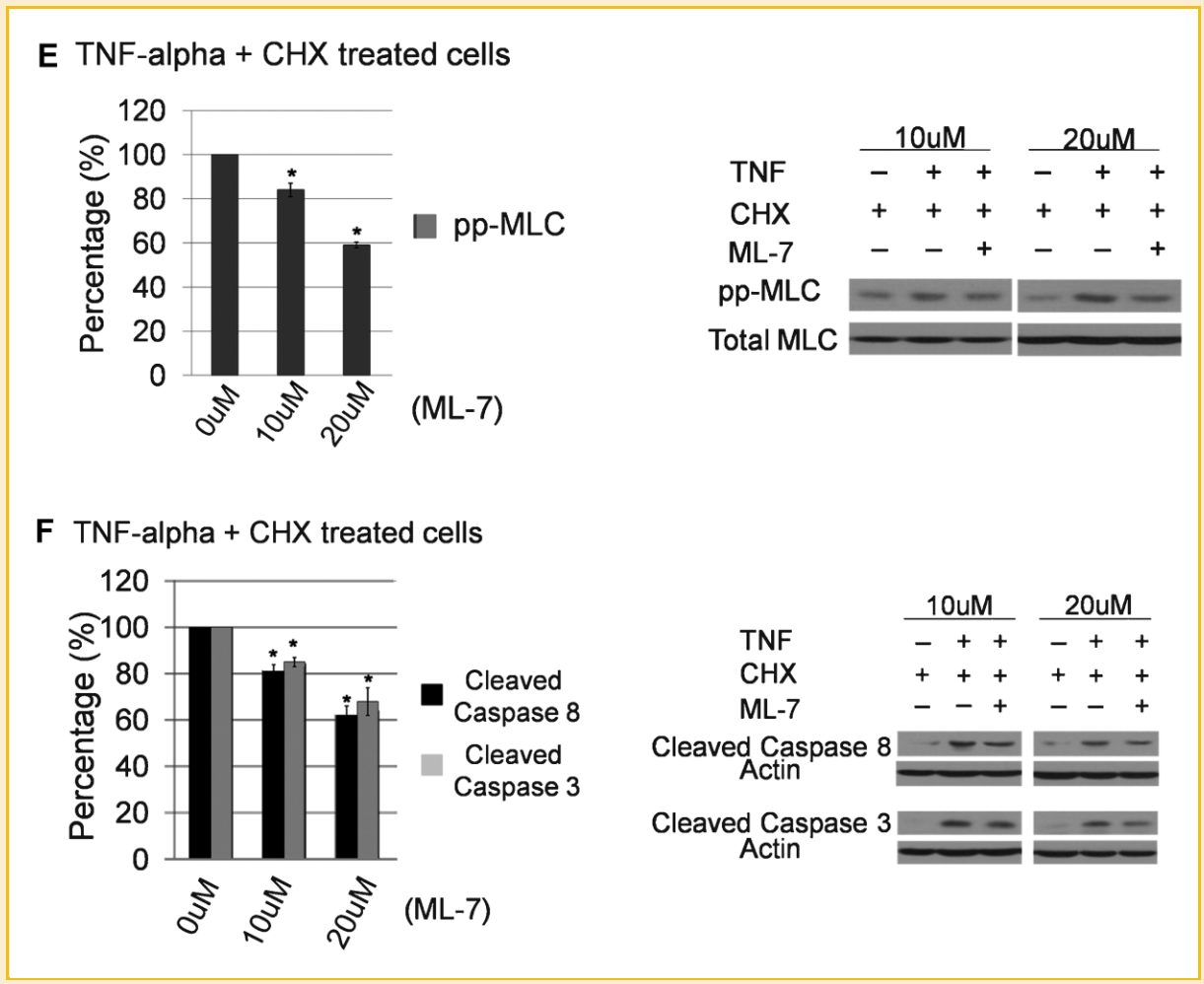

Fig. 2. (*Continued*)

signaling prior to the execution phase has not been as thoroughly studied. Previous works suggest a role for NM II involvement in cell death signaling prior to the execution phase by showing that inhibition of MLCK or Rho kinase can impair apoptosis in response to TNF [Jin et al., 2001; Petrache et al., 2001, 2003a]. Our investigation examined the role of specific NM II isoforms in mediating the initial TNF cell death response by impairing the expression of NM IIA and IIB through siRNA treatment. The results show knockdown of NM IIB expression, but not NM IIA, impairs caspase 3 cleavage in response to TNF. These data suggest an isoform specific function for NM IIB in mediating TNF cell death signaling prior to the execution phase. Preventing early cell death signaling events can impair the ability of the cell to carry out the execution phase of apoptosis. For example, interfering with the initial activation of caspase 3 impairs a cell's ability to breakdown components of the nuclear matrix to allow AMC forces to promote nuclear condensation [Croft et al., 2005; Kivinen et al., 2005]. Our results show that knockdown of NM IIB impairs caspase 3 cleavage which is consistent with the inability of these same cells to undergo nuclear condensation providing further evidence of a central role for NM IIB in TNF cell death. We also observed that knockdown of NM IIA or NM IIB does not appear to effect NFkappaB signaling as measured by IkBalpha phosphorylation (data not shown). It is important to note that there was no NM IIC detected in our Hela cells thereby eliminating the possibility it is playing a role in TNF induced survival or apoptotic signaling. Whether NM IIA and IIB have redundant functions in NFkappaB signaling remains to be investigated and is beyond the scope of the present study. Although the roles for NM IIA and IIB in TNF survival signaling are yet to be determined, to our knowledge these data provide the first report of a NM IIB isoform specific function in mediating cell death signaling prior to the execution phase.

Previous studies have proposed AMC produced by NM II facilitates TNF cell death signaling prior to the execution phase. Published works have suggested an involvement for AMC in TNFR1 translocation to the plasma membrane as well as a possible role in DISC formation [Jin et al., 2001; Petrache et al., 2003b]. The involvement of AMC in these events is based on increases seen in MLC phosphorylation in response to TNF, which indeed can lead to increases in AMC. Although MLC phosphorylation is required to initiate AMC it is not sufficient to do so on its own. Activation of the NM II motor domain through ATP hydrolysis and binding to actin

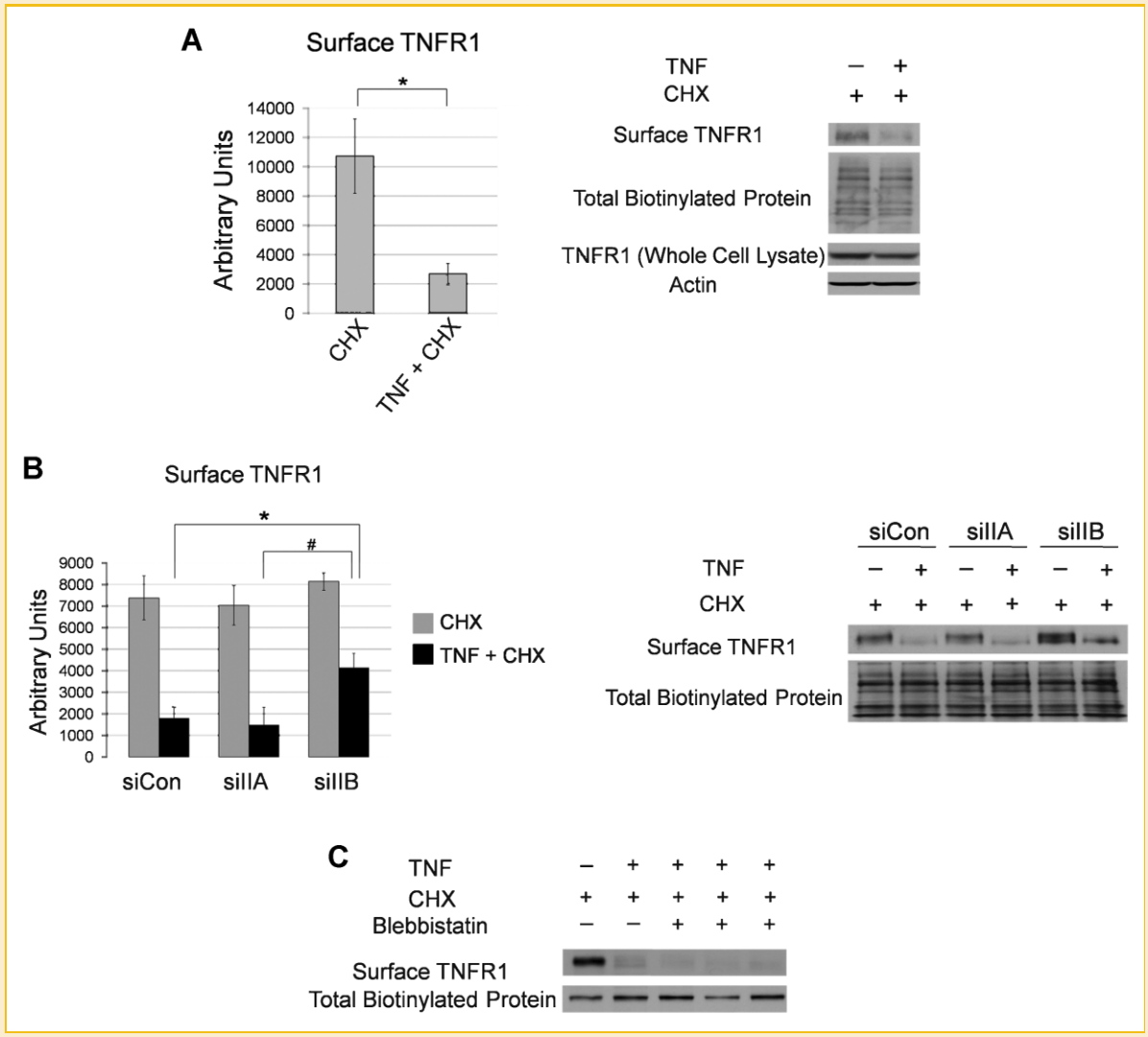

Fig. 3. Knockdown of NM IIB impairs TNFR1 internalization while treatment with blebbistatin does not. A: Graph represents the decrease seen in cell surface TNFR1 after treatment with 10 μg/ml of TNF + 10 μg/ml cycloheximide (CHX) for 30 min; (*) *P*-value <0.05 with $n = 3$. Western blot illustrates decrease seen in cell surface TNFR1. Whole cell lysates were ran to ensure that the decrease in cell surface TNFR1 was due to internalization and not the cleavage and release of the receptor from the plasma membrane. B: Graph represents cells treated with siIIB having significantly less TNFR1 left on the cell surface after treatment with the same TNF + CHX concentrations for 30 min as compared to the siCon (*) and siIIA (#) treated cells. Two-tailed student's *t*-test determined values were significant with *P* values <0.05 ($n = 3$). A representative Western blot corresponding to the graph is displayed on the right. C: Cells were pretreated with 100 μM of blebbistatin for 30 min then the above concentrations of TNF + CHX was added to the medium for an additional 30 min at which time cell surface TNFR1 was isolated and ran out on Western blot to measure the amount of TNFR1 internalization.

filaments must also be achieved to initiate AMC [Tyska and Warshaw, 2002; Sellers and Knight, 2007]. Therefore, MLC phosphorylation, hydrolysis of ATP, and binding to actin filaments can be considered three distinct events all leading to the production of AMC. Further evidence that MLC phosphorylation can be considered a distinct event from force generation come from works showing NM II functions that are activated by MLC phosphorylation and are independent of AMC [Ben-Ya'acov and Ravid, 2003; Arora et al., 2008; Vicente-Manzanares et al., 2008]. For example, NM IIA filaments have been shown to form docking sites for the signaling protein RAP1 which promotes integrin activation. Impairing NM II activation through treatment of cells with ML-7 prevented NM IIA association with RAP1 thereby disrupting RAP1's localization and signaling at the plasma membrane. However, inhibition of NM II motor activity through treatment of cells with blebbistatin did not affect RAP1localization or signaling [Arora et al., 2008]. This work demonstrates how activated NM II can effect a cellular process independent of AMC. Therefore, we wanted to determine if the function of NM II in TNF signaling involved AMC. In the present study we show that inhibition of AMC with blebbistatin or cytochalasin D did not prevent the cleavage of caspases in response to TNF. However, data using 10 μM and 20 μM of ML-7 showed that decreases in MLC phosphorylation correlated with impaired caspase cleavage indicating a role for activated NM II in mediating TNF cell death signaling. Therefore, given that a decrease in MLC phosphorylation impairs caspase cleavage while inhibition of AMC

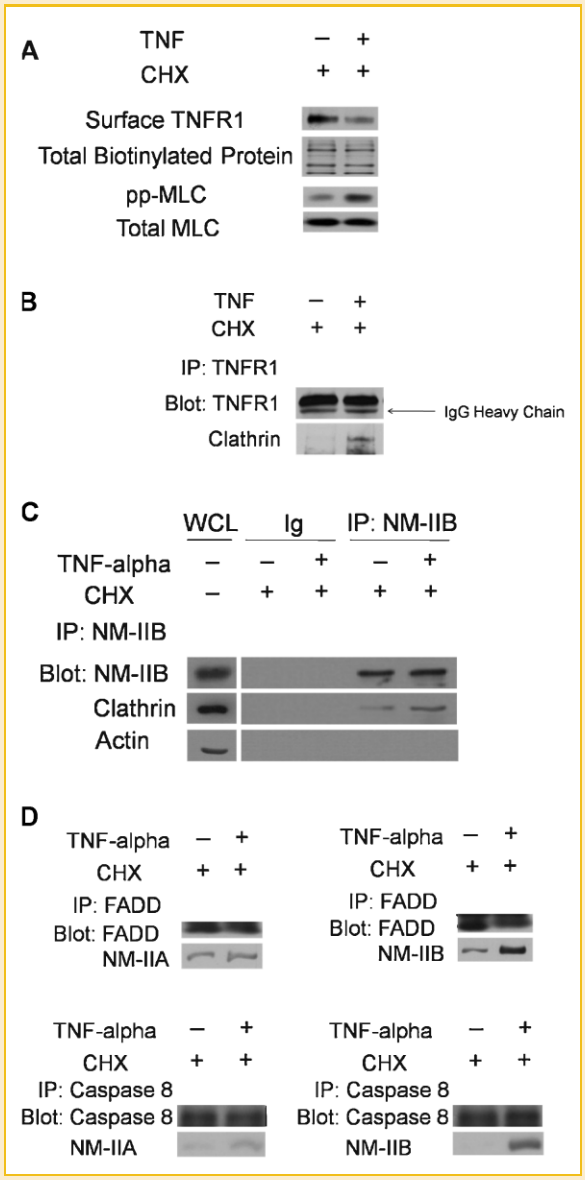

Fig. 4. NM IIB associates with endocytic and pro-apoptotic proteins in response to TNF. A: Hela cells were treated with 10 ng/ml TNF + 10 μg/ml cycloheximide (CHX) for 7.5 min at which time cell surface TNFR1 was isolated and the amount present was analyzed through Western blot. A Western blot illustrating a concomitant increase in di-phosphorylated MLC (pp-MLC) is also shown for the 7.5 min time period. B: Hela cells were treated with above-mentioned concentrations of TNF + CHX for 15 min. Cells were harvested for immunoprecipitation (IP) of TNFR1 and its association with clathrin in response to TNF was analyzed by Western blot (n = 3). C: The above procedure was repeated for IP of NM IIB to examine its association with clathrin in response to TNF + CHX at the 15 min time point (n = 3). D: Immunoprecipitations for FADD and caspase 8 were performed at 3.5 h after treatment with the above-mentioned concentrations of TNF + CHX. Association of FADD and caspase 8 with NM IIA and IIB was analyzed through Western blot (n = 3).

does not suggests there is a role for activated NM II in promoting cell death, independent of AMC.

Activated NM II can participate in the formation and stabilization of actin microfilament bundles. This property of NM II has been suggested to play an important role in TNF induced apoptosis since pharmacologic and genetic inhibition of MLCK and Rho kinase impairs actin filament formation while preventing cell death. In our investigation we disrupted actin filament dynamics by directly targeting the actin cytoskeleton through treatment with cytochalasin D. Our data along with work done in NIH3T3 cells [Croft et al., 2005] suggests that increases in actin microfilament formation and rearrangement are not required for cell death since treatment with cytochalasin D does not inhibit caspase cleavage in response to TNF. It should be noted that although cytochalasin D treatment does destabilize the proper formation and rearrangement of actin microfilament bundles in response to TNF the presence of filamentous aggregates or actin foci have been reported [Schliwa, 1982] and could be playing a role in apoptosis. Nevertheless, cytochalasin D disrupts actin dynamics, but not MLC phosphorylation, in response to TNF suggesting that there may be a function for activated NM II during cell death that is independent of actin filament formation and AMC.

One crucial upstream event of TNF induced apoptosis is the internalization of the TNFR1 receptor which has been shown to occur within minutes of TNF binding. This internalization process is necessary for the formation of the DISC complex and the activation of caspase 8 [Schneider-Brachert et al., 2004]. Published work has demonstrated that preventing endocytosis of the activated TNFR1 receptor through treatment with monodansylcadeverine (MDC) inhibits the induction of apoptosis. MDC is an inihibitor of transglutaminase which is a membrane bound enzyme that is involved in the internalization of a number of receptor systems such as clathrin mediated endocytosis (CME) [Schutze et al., 1999]. CME is involved in the internalization of a variety of ligand-receptor complexes such as the CXCR4 receptor in response to SDF-1alpha. This internalization process has been suggested to rely upon NM IIA to couple the activated receptor to vital endocytic proteins such as B-arrestin [Rey et al., 2007]. Also, NM II has also been shown to be important for the positioning of clathrin coated endocytic structures to the uropod of moving T-lymphocytes for the uptake of transferrin suggesting a role for NM II in supporting CME [Samaniego et al., 2007].

Endocytosis of TNFR1 has been shown to be clathrin mediated and its internalization is required for TNF induced apoptosis [Schneider-Brachert et al., 2004]. Indeed, we found that TNFR1 and clathrin association increased in response to TNF indicating a role for CME. NM II has been implicated in CME and our results show that knockdown of NM IIB, but not IIA, impairs internalization of TNFR1. The exact mechanism in which NM IIB helps promote TNFR1 internalization is not known, however, IP data indicate that it may involve the increased association of NM IIB with clathrin in response to TNF. Interestingly, inhibition of AMC was unable to impair caspase cleavage or TNFR1 internalization suggesting that AMC is not required for the initiation of TNF induced apoptosis. Taken together, these data implicate an AMC independent role for NM IIB in mediating TNFR1 internalization that may involve its association with clathrin.

Although our data suggests that NM IIB plays a role in TNFR1 endocytosis, we wanted to determine if NM IIB had additional functions downstream of TNFR1 internalization. Formation of the

DISC complex follows TNFR1 endocytosis and involves the association of FADD with caspase 8 to initiate TNF induced apoptosis. Our data illustrates that internalization of TNFR1 occurs as early as 7.5 min but caspase cleavage is not detected until the 3 h time period. Therefore, we wanted to examine the protein interactions of NM IIB that were occurring after a time in which cell death signaling was detectable. IPs of the pro-apoptotic proteins FADD and caspase 8 demonstrated an isoform specific association with NM IIB at a time in which caspase cleavage was occurring. Although there were times in which NM IIA association was detected with FADD its association was significantly less then that of NM IIB. Attempts to pulldown TRADD, FADD, and caspase 8 in their associated state to determine the involvement of NM IIB in the formation of the DISC complex was unsuccessful. Since Hela cells have been shown to express viral proteins of the human papillomavirus (HPV) it is possible that these viral proteins interfere with IP of the DISC complex [Rodier et al., 2000]. Although NM IIB associates with FADD and caspase 8 in response to TNF further work will be needed to determine the significance of these interactions.

In conclusion, our studies provide evidence that TNF promotes cell death by utilizing NM IIB in an isoform specific manner. NM II is ubiquitously expressed and its force generating property is crucial to many cellular processes, however, this investigation presents a possible role for NM IIB in early cell death signaling events independent of AMC. Furthermore, our study demonstrates that NM IIB may be involved in multiple facets of TNF cell death signaling from receptor internalization to cleavage of caspases through its associations with clathrin, FADD, and caspase 8. Further work will be required to establish the mechanism by which NM IIB participates in apoptotic signaling.

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