High Throughput Short Interfering RNA (siRNA) Screening of the Human Kinome Identifies Novel Kinases Controlling the Canonical Nuclear Factor-κB (NF-κB) Activation Pathway

Sanjeev Choudhary, Kevin P. Rosenblatt, Ling Fang, Bing Tian, Zhao-Hui Wu, and Allan R. Brasier

From the °Sealy Center for Molecular Medicine, †Department of Internal Medicine, ‡Department of Biochemistry and Molecular Biology, and §Institute for Translational Sciences, University of Texas Medical Branch, Galveston, Texas 77555, the †Department of Pathology, University of Tennessee Health Science Center, Memphis, Tennessee, and the §Center for Clinical and Translational Sciences, Brown Foundation Institute of Molecular Medicine, and **Department of Pathology and Laboratory Medicine, University of Texas Health Science Center, Houston, Texas 77030

Nuclear factor-κB (NF-κB) is an inducible cytoplasmic transcription factor that plays a role as a master regulator of airway mucosal inflammation. The prototypical (‘canonical’) NF-κB pathway controls cytoplasmic to nuclear translocation in response to stimulation by the mononuclear cytokine, TNF. Despite intensive investigation, the spectrum of kinases involved in the canonical NF-κB pathway has not yet been systematically determined. Here we have applied a high throughput siRNA-mediated loss-of-function screening assay to identify novel kinases important in TNF-induced NF-κB signaling. Type II A549 epithelial cells stably expressing an IL-8/luciferase reporter gene optimized for high throughput siRNA format (Z’ score of 0.65) and siRNAs for 636 human kinases were reverse transfected and screened in the assay. 36 candidate genes were identified that inhibited TNF signaling with a Z-score deviation of < −1.3 in replicate plates. From this group, 11 kinases were selected for independent validation, of which eight were successfully silenced. Six kinases were validated, including ATM, CDK2, -5, and -7, CALM3, MAPAKP5, and MAP3K/MEKK3. The surprising functionality of ATM in TNF signaling was confirmed where reduced NF-κB/RelA translocation and Ser-276 phosphorylation were seen in ATM−/− mouse embryo fibroblasts. These data indicate that ATM is a key regulatory kinase that may control global NF-κB activation in the TNF-induced canonical pathway.

Eukaryotic tissues respond to signals in their extracellular environment through the induction of long term phenotypic plasticity. An important mechanism for this plasticity is through the activation of cell surface receptors coupled to intracellular signal transduction networks, the activation of which, in turn, induces a reactive gene expression. One specific example occurs in airway mucosal surfaces that normally promote gas exchange, facilitate particulate matter clearance through the mucus-ciliary escalator, and protect against oxidative damage (1). Within this surface, sentinel epithelial cells are responsible for activating the innate inflammatory response upon stimulation with macrophage-derived cytokines via the plasma membrane-anchored TNF/death receptor (TNFR)2 superfamily (2, 3). Liganded TNFRs signal intracellularly through nuclear factor-κB (NF-κB), an inducible cytoplasmic transcription factor that temporally coordinates expression of gene networks, including interferon, cytokine, and anti-apoptotic genes (4–6). As a result, the mucosal surface assumes a pathogen-resistant and proinflammatory phenotype. Because of its central role in airway inflammatory disease, the NF-κB signaling pathway has been extensively investigated.

Under resting conditions, NF-κB is inhibited in the cytoplasm by binding to members of the 1κB family of inhibitory proteins, resulting in cytoplasmic retention and inactivation of its latent DNA binding activity. TNFR-ligand binding results in membraneous recruitment of death domains containing adapter molecules, including the TNF receptor-associated factors, and the receptor-interacting kinase recognized by Lys-63-linked polyubiquitylation (7). Lys-63-linked TNF receptor-associated factor and receptor-interacting kinase are recognized by the ubiquitin binding domain of the 1κB kinase γ (IKK-γ) adapter subunit (8–11), triggering recruitment of the associated IKK complex and the subsequent Ser-170/172 phosphorylation of the catalytic IKKα/β subunits in their activation loops (12). Ser-phosphorylated IKK dissociates from the activated receptor and enters the cytoplasm to phosphorylate 1κBα in its NH2-terminal regulatory domain, converting it to a binding substrate for the β-transducin repeat-containing (βTRCP) SKP1-cullin-F-box ligase complex, resulting in Lys-48-linked ubiquitylation and subsequent proteolysis (13).

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1 To whom correspondence should be addressed: MRB 8.138, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1060. Fax: 409-772-8709; E-mail: sachoudh@utmb.edu.

2 The abbreviations used are: TNFR, TNF/death receptor; 1κB, κB inhibitor; Q-RT-PCR, Quantitative real-time PCR; ATM, ataxia telangiectasia mutated; MEF, mouse embryo fibroblast; HT-siRNA, high throughput siRNA; CDK, cyclin-dependent kinase; ROS, reactive oxygen species.

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Liberated from its IκBα inhibitor, the major transcriptional NF-κB subunit (RelA) rapidly enters the nucleus. Recently, it has been demonstrated that a parallel activation pathway is required for NF-κB transactivation, initiated by RelA phosphorylation at Ser-536 or -276 (14, 15). RelA phosphorylation is required for binding the p300/CBP coactivator, stimulating Lys-310 acetylation (16), a post-translational switch that enhances formation of an active enhanceosome (15) that modifies histones of target gene promoters and induces transcriptional elongation (17–19). As a result, temporally dependent waves of gene expression are induced (5) that are subsequently terminated by proteolytic clearance of promoter-bound RelA through the ubiquitin proteosome pathway (20). In this way, subcellular localization, transcriptional activity, and protein turnover of NF-κB subunits are processes controlled by coupled inducible phosphorylation that affect protein-protein interactions.

Despite the intensive investigation of the canonical NF-κB pathway mediated by the TNFR network, the kinases controlling this pathway are still largely unknown. High throughput, tandem affinity purification studies have elucidated novel protein interactions in the NF-κB network and demonstrated novel roles of TNF receptor-associated factor isoforms (1). However, few regulatory kinases were discovered, perhaps because kinase-substrate interactions are transient, and low affinity binding interactions may not have been captured. The recent development of high throughput siRNA screening assays offers an opportunity for new insights into this and other signaling pathways. Here, we apply a high throughput siRNA-mediated screen targeting 636 human kinases to identify novel kinases controlling canonical NF-κB pathway activation.

EXPERIMENTAL PROCEDURES

Materials—The Stealth RNAi human kinase library, in a 96-well microtiter plate format (eight master plates), which targets 636 human kinase genes, was purchased from Invitrogen. Ku-55933 and wortmannin were purchased from Calbiochem.

Cell Culture—Human A549 pulmonary epithelial cells stably transfected with IL-8 luciferase reporter gene were commercially obtained (A549-Luc, Panomics) and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO2 incubator. Hygromycin (100 μg/ml; Invitrogen) was added to the culture medium to maintain stable cellular expression of NF-κB luc. Serum starvation involved replacing growth medium with DMEM containing 0.5% bovine serum albumin (Sigma). For the ataxia telangiectasia mutated (ATM) inhibitor experiments, wortmannin (30 μM) or Ku 55933 was added at 40 μM for 1 h prior to TNF stimulation.

High Throughput siRNA Screening—Prior to the high throughput screen, RNAi and transfection reagent concentrations and transfection efficiency in A549 cells were optimized. The stealth RNAi master plate collection consisted of three non-overlapping RNAi duplex siRNAs to each target; aliquots from the three sets were pooled into one master plate and diluted to 250 nM individual RNAi in deep well 96-well plates (Axygen, Union City, CA). Each plate consisted of three negative-universal siRNA controls (Invitrogen) containing low, medium, and high GC content. NF-κB-inducing kinase and IKKγ siRNAs were included as positive controls. For siRNA screening, 25 μl of combined, diluted RNAis were robotically (Biomek FXp, Beckman, Brea, CA) aliquoted into a 96-well plate (Nunc, Rochester, NY), mixed with 25 μl of diluted transfection reagent (0.3 μl/well of Siquiet reagent, Mirus, Madison, WI), and reverse transfected by dispensing A549-Luc stable cells (10,000 cells in 100 μl) into each well using the Titerpak, multidoor 384 cell dispenser (effective concentration of 40 nM for each RNAi from the three duplexes for each target). Each plate was transfected in duplicate to deduce reproducible inhibition. Forty-eight h later, cells were incubated in DMEM/BSA and serum-starved overnight, followed by TNF treatment (20 ng/ml) for 6 h. Cells were washed twice with 1× PBS using the Bio-Plus PI wash station (Bio-Rad) before adding 50 μl of luciferase lysis buffer and freezing the lysates at −80 °C.

Luciferase Assay—Twenty μl of cell lysate from each well in the transfection plates was transferred and mixed with 80 μl of luciferin reagent for measurement of relative luminescence (96-well plate; Nunc). Luciferase activity was measured by a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA).

Protein Assay—Five μl of lysate was transferred, and protein concentration was measured using CBB binding relative to BSA standards. Liquid handling was performed by the Biomek FX Robotic system (Beckman Coulter, Brea, CA).

Nuclear and Cytoplasmic Fractionation—Cells were harvested in phosphate-buffered saline and centrifuged, and the pellets were resuspended sequentially in low salt, sucrose, and high salt solutions to obtain cytosolic and highly purified nuclear extracts as described previously (21, 22). Protein concentrations were measured by Coomassie dye binding (protein reagent; Bio-Rad).

Statistical Analysis—Z’ score calculations were performed in 96-well plates as described (23). The absorbance of Coomassie Brilliant Blue binding was fitted to a regression curve, and exact protein concentrations were determined using least squares regression analysis (Sigma Plot, version 11, Sys). The luciferase activity RLU was normalized to absolute the protein concentration for each well where indicated. Normalized luciferase activity was further normalized using the standard Z score method relative to each plate’s mean value (24). The Z score is calculated by subtracting each luciferase value from the plate sample mean and dividing by the plate S.D. value. The criteria for identification of potential hits used a Z score cut-off of less than −1.65, which corresponded to a p value of 0.05, for both replications.

Quantitative real-time PCR (Q-RT-PCR)—Total RNA was extracted using acid guanidium phenol extraction (Tri Reagent; Sigma-Aldrich). One μg of RNA was reverse transcribed using SuperScript III (Invitrogen) in a 20-μl reaction mixture. One μl of cDNA product was amplified in a 20-μl reaction mixture containing 12.5 μl of SYBR Green Supermix (Bio-Rad) and 0.4 m each of forward and reverse gene-specific primers; aliquoted into 96-well, 0.2-mm thin wall PCR plates; and covered with optical quality sealing tape. The plates were denatured for 90 s
NF-κB pathway, we performed loss-of-function analyses using HT-siRNA screening for 636 human kinases. In preliminary assay development, we optimized culture conditions, cell number/well, siRNA and stimulation doses, and timing of TNF exposure; the Z′ factor served as a measure of the effect size relative to the assay variation. A Z′ score of 0.65 was achieved, indicating that the assay was sufficiently robust for HT-siRNA screening (23) (supplemental Fig. S1). The strategy for HT-siRNA screening is shown in Fig. 1. 10,000 A549 NF-κB/Luc stable cells were plated into the transfection reagent/siRNA mixture, containing the combination of the three separate siRNA duplexes to each kinase, in each well. Cells were stimulated with TNF, and luciferase activity was subsequently measured. The data were analyzed for relative differences in TNF-inducible luciferase activity. In initial pairwise plots to survey the plate-to-plate reproducibility, we found that there was significant signal variation (Fig. 2, top). This variation was probably the result of differences in cell number in each well as a result of plate manipulations (plating, washing, and serum starvation); subsequent normalization of the luciferase signals to protein signals in each well significantly reduced the plate-to-plate variability, with Pearson correlation coefficients exceeding 0.9 (Fig. 2, bottom).

To identify kinases involved in the TNF-activated canonical NF-κB pathway, wells corresponding to positions where the normalized luciferase activity was significantly different using the standard Z score method relative to each plate’s median value were chosen (24). Briefly, this method assumes that the majority of kinases do not have an effect on the signaling pathway and that the plate serves as its own control. This approach reduces edge effects produced as a result of controls on the outside wells (26). Plate signals are rescaled relative to within-plate variation by subtracting the average of the plate values and dividing the difference by the S.D. value estimated from all measurements of the plate (24).

Analysis of the Positive Controls—The luciferase activity for siRNA targeting IKKα demonstrated a Z score deviation of −1.3 and −0.75 in separate replicates (data not shown). The data were rank order-filtered by the mean Z score, and candidates were identified as those with Z score deviations of <−1.3, a stringent cut-off that we intentionally selected to reduce the number of false positives (Fig. 3). Using this cut-off, we identified those hits corresponding to siRNAs that produced a mean Z score inhibition of >−1.3. IKKα, another well established kinase involved in TNF-induced canonical NF-κB activation pathway, had an inhibition score similar to that of IKKγ; however, it also missed the stringent filter cut-off. We then identified 36 candidate genes, which produced a greater inhibition than that produced by the positive control IKKγ siRNA (Table 1). In addition, a number of siRNAs produced enhanced reporter activity (Fig. 3); the analysis and validation of this group of kinases is beyond the scope of the present study. Focusing on the kinases required for TNF-inducible reporter activity, we observed an enrichment of cyclin-dependent...
kinases 2, 5, 7, and 11B (CDK2, -5, -7, and -11B, respectively), CDC2-like kinases, and mitogen-activated protein kinases (MAPKs). Although some of these kinases have already been shown to be involved in canonical NF-kB signaling, including protein kinase Cζ (PKCζ) (27), MAP3K3 (28), and MAPK-activated protein kinase 5 (MAPKAPK5) (29, 30), the remainder of the kinases in Table 1 have not been previously implicated in canonical NF-kB pathway signaling.

To validate this candidate set, 11 kinases were randomly selected for independent validation by knockdown and examination of the effect on the NF-kB canonical pathway. These kinases included ATM, CDK isozymes 2, 5, and 7, eukaryotic elongation factor-2 (EEF2), MAPKAPK5, MAP3K12, bone morphogenetic protein R 1A (BMPR1A), calmodulin kinase-3 (CALM3), phosphorylase kinase γ2 (PHKG2), and PKCζ. A set of duplex siRNAs (separate synthesis of the same siRNA sequences used in the HTP screen) for each gene were reverse transfected into wild-type A549 cells (Table 2). We found that greater than 50% inhibition was observed for ATM, MAPKAPK5, CALM-3, and CDK7, -5, and -7 (Fig. 4A). Efficiency of siRNA knockdown of the selected kinases at the protein level was quantified by Western blot analysis. Here the abundance of CDK2, CDK5, and ATM proteins were almost completely abolished by siRNA. CDK7 was reduced by ~50% (Fig. 4B).

The effect of successfully down-regulated kinases was assessed on endogenous NF-kB-induced gene expression. A549 cells were transfected with specific siRNAs to ATM, EEF2, MAPKAPK5, MAP3K12, CALM-3, and CDK isoforms 2, 5, and 7. 48 h later, cells were stimulated in the absence or presence of TNF, and the expression of TNFAIP3/A20, a highly inducible NF-kB-dependent gene (5), was measured via Q-RT-PCR. Relative to scrambled controls, 25% inhibition of inducible TNFAIP3/A20 was observed after ATM knockdown; 50% inhibition was produced by MAP3K12, PKCζ, and IKKγ; and a >75% inhibition was observed for CDK5, CDK7, and CALM3, respectively (Fig. 5A). No significant inhibition of inducible TNFAIP3/A20 expression was observed for MAPKAPK5 or EEF2, and an apparent induction was observed for CDK2, indicating that these latter kinases may have been false positives generated from the screening assay.

Similar qualitative effects on TNF-induced TNFAIP3/A20 expression was observed in HEK293 cells, with the exception of CDK2 and MAP3K12, which did not affect TNFAIP3/A20 expression in HEK293 cells (supplemental Fig. S2). These results suggest that the role of ATM, MAP3K12, PKCζ, CALM-3, and CDK7, -5, and -2 in TNF signaling is not specific to A549 cells.

To determine whether these kinases were important in inducible transcription of any stimulus type, we examined the effect of siRNA knockdown on IL-6-Jak-STAT3 signaling. Silencing ATM, MAP3K12, PKCζ, CALM-3, and CDK7, -5, and -2 did not affect IL-6-induced SOCS3 expression (supplemental Fig. S3).

We next sought to initially identify the regulatory step that each kinase had in NF-kB canonical activation. For this purpose, the magnitude of RelA translocation was assayed after siRNA-mediated silencing of CDK2, -5, and -7, CALM3, PKCζ, and ATM. TNF-inducible RelA translocation was significantly inhibited for the CDK2, -5, and -7 isoforms, with a smaller effect seen for CALM3, PKCζ, and ATM (supplemental Fig. S4).

The finding that ATM had a weak inhibitory activity on TNFAIP3/A20 expression was difficult to interpret, but this may have occurred because the level of ATM knockdown produced by the siRNA transfection was only ~50% (see Fig. 4A). ATM is a member of the PI3K family that has been implicated in double-stranded break-induced NF-kB activation in response to genotoxic stress; this mechanism involves its nuclear activation (phosphorylation at serine 1981), association with IKKγ, and consequent export into the cytoplasm (31).

Because the role of ATM in extracellular TNF signaling is unknown, we sought to further explore its role in the canonical NF-kB signaling pathway. We first sought to ask whether TNF activates ATM by measuring formation of phospho-ATM in a time course of TNF stimulation. Fig. 6A shows that TNF (30 ng/ml) treatment increases ATM phosphorylation starting from 15 min in A549 cells. To understand which regulatory step ATM affects in the canonical NF-kB activation pathway, we asked if ATM is required for IκBα degradation and/or RelA nuclear translocation, the two key steps in the NF-kB activation pathway. Although TNF stimulation resulted in an increased IκBα degradation and RelA nuclear translocation in ATM wild-type mouse embryonic fibroblasts (MEFs), both of these processes were significantly reduced in ATM−/− MEFs, especially at 15 min of TNF treatment (Fig. 6B and C). Additionally, ATM deficiency leads to decreased RelA phosphorylation. Here, TNF-induced RelA Ser-536 phosphorylation at 15 min in both ATM+/+ and ATM−/− MEFs, but RelA Ser-276 phosphorylation was only detected in ATM−/+ cells (Fig. 6D). Taken together, these results clearly suggest that ATM is involved in a regulatory step controlling the initial phase of IκBα degradation, RelA release, and Ser-276 phosphorylation in the TNF-induced NF-kB activation pathway.
To separately confirm the effect of ATM siRNA, A549-Luc cells were pretreated with the PI3K inhibitor wortmannin or Ku-55933, a specific ATM inhibitor (32). We observed that a TNF-inducible NF-κB reporter activity (750-fold) was now almost completely inhibited by either Ku-55933 or wortmannin (Fig. 7).

To quantify the transcriptional effect of ATM on the canonical pathway, ATM$^{+/+}$ and ATM$^{-/-}$ MEFs were stimulated with TNF for 0–1 h prior to the analysis of NF-κB-dependent gene expression by Q-RT-PCR. We observed that 1 h of TNF treatment induced a 16-fold induction of TNFAIP3/A20 mRNA expression in ATM$^{+/+}$ MEFs but was induced less than 4-fold in ATM$^{-/-}$ MEFs (Fig. 8A). TNF stimulation induced a 5-fold increased expression of IκBα, a level that was not significantly different between the ATM$^{+/+}$ and ATM$^{-/-}$ cells (Fig. 8B). Similar to the findings for TNFAIP3/A20, Groβ and KC gene expression was increased, in a TNF-dependent fashion to 28- and 9-fold, respectively, but the expression was significantly reduced in the TNF-stimulated ATM$^{-/-}$ MEFs (Fig. 8, C and D). Previous work from our group has shown that the NF-κB-dependent genes are inducible by several distinct mechanisms, with a subgroup of early response genes requiring activating phosphorylation of NF-κB/RelA at Ser-276, a site required for activation of a subgroup of inflammatory genes (29). Together, these data indicate that ATM is a regulator of NF-κB, mediating the canonical NF-κB activation pathway that activates a subset of dependent genes.
FIGURE 4. A, siRNA knockdown of validation set. A549 cells were transfected with indicated siRNAs (EEF2, BMPR1A, CALM3, CDK7, ATM, CDK2, CDK5, PKCγ, PKH2, MAP3K12, and MAP3K5). 72 h later, total RNA was extracted, and the level of each target was measured by Q-RT-PCR. Shown is the mRNA expression for each gene target as a percentage of untransfected control (mean ± S.D. (error bars) of triplicate plates). Data were analyzed by Student’s t test. *, p < 0.05 significantly different from scrambled siRNA-treated samples. B, Western blot to validate protein knockdown by their respective siRNA after 72 h of transfection. β-Actin was probed as a protein loading control; lamin B was protein loading control for ATM due to its large size. Scr, scrambled siRNA.

FIGURE 5. siRNA knockdown on TNF-inducible endogenous NF-κB-dependent gene expression. A549 cells were reverse transfected with siRNAs to the indicated kinases for 72 h prior to TNF stimulation and RNA extraction. The x axis shows -fold change of TNF-stimulated relative to unstimulated value (mean ± S.D. (error bars) of triplicate plates), and the y axis shows the target gene (TNFAIP3/A20 mRNA) mRNA. Data were analyzed by Student’s t test. *, p < 0.05, significantly different from scrambled siRNA-treated samples. Scr, scrambled siRNA.

DISCUSSION

NF-κB is a major regulatory arm of the innate immune response in airway epithelial cells that mediates temporally coordinated expression of gene networks by a mechanism involving its nuclear translocation and activating phosphorylation on the Ser-276 residue (5, 33). Despite intensive study of this pathway, the complete spectrum of kinases involved in its regulation is not fully understood. In this study, we standardized and applied an HT-siRNA screen to identify new kinase candidates that control the canonical NF-κB pathway; we further sought to validate a subset of the identified kinases. Thirty-six serine-threonine kinases were identified within our screen that had effects significantly greater than that produced by siRNA-mediated knockdown of IKKγ/NEMO, which served as a positive control within our screen. Of the 11 kinases selected for validation, we were able to achieve sufficient knockdown of eight. From these eight, the knockdown of six kinases demonstrated significant effects on TNF-inducible and NF-κB-dependent gene expression, indicating that our duplicate screening strategy has uncovered several genuine candidates involved in the canonical NF-κB pathway.

Of the kinases identified in our screen, a few have been implicated in NF-κB signaling in prior studies. For example, PKCγ, an atypical PKC isoform, has been reported as a regulator of NF-κB transcriptional activity in certain cell types (34). Consequently, PKCγ deficiency results in reduced cell proliferation and TNF-inducible NF-κB-dependent gene expression (34). Furthermore, lack of PKCδ in B cells and embryonic fibroblasts does not affect the ability of TNF or IgM to activate the IKK complex or the DNA-binding activity of NF-κB, respectively. However, PKCζ mediates Ser-311 phosphorylation of RelA and thus regulates diacylglycerol kinase-induced TNF-dependent NF-κB activation (35). Thus, PKCζ can directly associate with and efficiently phosphorylate RelA, and, more importantly, TNF-α-induced phosphorylation of RelA in vivo was shown to be dramatically inhibited in pkcζ−/− embryonic fibroblasts (34). Moreover, PKCζ has also been implicated in LPS-induced NF-κB activation within human peripheral blood monocytes and macrophages through its association with both RhoA and activating transforming growth factor β-activated kinase-1 (TAK1) (36). Our findings also implicate a role for PKCζ in TNF signaling in airway epithelial cells.

Several CDKs were also identified in our screen. The CDKs are a family of related serine-threonine kinases best known, perhaps, to be responsible for the orderly progression of cells through various phases of the cell cycle; nevertheless, they also play an important role in transcription and mRNA processing (37–39). The enzymatic activity of CDKs is regulated by interactions with their cyclin protein partners to form an active heterodimer complex. CDK2 and cyclin E have been shown to interact with RelA via p300/CPB and, thus, negatively regulate phorbol 12-myristate 13-acetate-induced and NF-κB-dependent gene expression (40). On the other hand, we and others have shown that recruitment of transcriptional elongation factor b (P-TEFb), a heterodimer of CDK9 and cyclin T1, is essential for the expression of selected subgroups of NF-κB-dependent genes (33). Activated NF-κB associates and recruits P-TEFb to target genes, and CDK9 mediates increased phosphorylation of negative elongation factors and phosphorylation of Ser residue 2 of the heptad repeat in the RNA polymerase II COOH-terminal domain. In turn, there is an initiation of a transcriptional elongation phase in NF-κB-dependent gene expression (41). Consistent with these findings, we have reported that flavopiridol, a pan-selective CDK inhibitor, potently inhibits NF-κB-dependent gene expression (33).
Additionally, murine 264.7 macrophages treated with olomoucine and roscovitine, selective inhibitors of CDK1, -2, -5, and -7, exhibit a reduction in lipopolysaccharide-induced inflammatory responses by down-regulating NF-κB-dependent gene expression (42). Taken together, these studies suggest that CDKs regulate NF-κB-dependent gene expression by various mechanisms and at multiple steps in the transcriptional activation process. However, with the exception of CDK2 and -9, the roles of the other CDKs uncovered here have not been clearly implicated in TNF-induced NF-κB activation. Therefore, our finding that siRNA-mediated knockdown of CDK5 and -7 significantly inhibited TNF-induced NF-κB promoter activity suggests that additional CDKs may also have additional roles as transcriptional regulators of TNF-induced NF-κB signaling.

The more profound effect of CDK7 knockdown on TNF-dependent transcription may also be explained by its additional role in general transcription as a component of universal transcription factor TFIIH (43).

FIGURE 6. TNF-induced ATM activation and role in NF-κB signaling. A, A549 cells treated with TNF (30 ng/ml) for various time intervals and immunoblotted for phospho-ATM (Ser1981). Western blot analysis shows increased ATM phosphorylation after TNF treatment. The bottom panel shows quantification of Western blots from three independent experiments. B, wild type (ATM+/+) and ATM-deficient (ATM−/−) MEFs were stimulated in the absence or presence of TNF (30 ng/ml) for the indicated times. Shown is normalized signal of the specific band to β-actin loading control. The top panel shows a representative blot, whereas the bottom panel shows quantification of Western blots from three independent experiments. C, a representative Western blot showing TNF-induced nuclear translocation of RelA in ATM+/+ and ATM−/− cells (top). Data represent percentage fold change as compared with untreated cells after normalizing to total RelA. Shown is the mean ± S.D. (error bars) of three independent experiments. Overall significance was determined using two-way analysis of variance. *, p < 0.05, significantly different from untreated (0 h) samples; †, p < 0.01, significantly different from ATM+/+ using a post hoc t test. Dark bars, ATM+/+; light bars, ATM−/− cells. ns, nonspecific. D, a representative Western blot showing the effect of ATM knock-out on RelA phosphorylation. ATM+/+ and ATM−/− cells were treated with TNF (30 ng/ml) for the specified time intervals, lysed in radioimmune precipitation assay buffer, and immunoblotted for Ser-phosphorylated RelA (Ser-536 (top) or Ser-276 (bottom)). Total RelA was quantified as a loading control.

FIGURE 7. ATM kinase inhibition on TNF-inducible reporter activity. NF-κB/Luc cells were pretreated in the absence or presence of Ku55933 (40 μM) or wortmannin (30 μM). Cells were then stimulated in the absence or presence of TNF (30 ng/ml, 6 h) and lysed, and normalized luciferase reporter activity (to protein) was measured. Shown is the mean ± S.D. (error bars) of replicate plates. Overall significance was determined using one-way analysis of variance. *, p < 0.01, significantly different from control samples; †, p < 0.01, significantly different from TNF-treated samples.

Additionally, murine 264.7 macrophages treated with olomoucine and roscovitine, selective inhibitors of CDK1, -2, -5, and -7, exhibit a reduction in lipopolysaccharide-induced inflammatory responses by down-regulating NF-κB-dependent gene expression (42). Taken together, these studies suggest that CDKs regulate NF-κB-dependent gene expression by various mechanisms and at multiple steps in the transcriptional activation process. However, with the exception of CDK2 and -9, the roles of the other CDKs uncovered here have not been clearly implicated in TNF-induced NF-κB activation. Therefore, our finding that siRNA-mediated knockdown of CDK5 and -7 significantly inhibited TNF-induced NF-κB promoter activity suggests that additional CDKs may also have additional roles as transcriptional regulators of TNF-induced NF-κB signaling. The more profound effect of CDK7 knockdown on TNF-dependent transcription may also be explained by its additional role in general transcription as a component of universal transcription factor TFIIH (43).

Similarly, MAP3K3/MEKK3 and MAPKAPK5/p38-regulated and -activated kinase have been reported earlier to regulate NF-κB activity (28–30). Of these, MAP3K3/MEKK3 is required for lysophosphatidic acid signaling to the IκB kinase complex through the BCL10-, MALT lymphoma translocation.
gene 1-, and CARD and MAGUK domain-containing protein complex. Interestingly, this signaling pathway is independent of TAK1, the IKK effector kinase that mediates PKC-α/H9256-induced NF-κB signaling.

ATM is a serine-threonine kinase of the PI3K family involved in cell cycle regulation in response to DNA damage. ATM has been reported to activate NF-κB in response to DNA damage (31); identifying ATM as a requirement for NF-κB signaling within our screen was a surprising finding. We therefore decided to explicitly validate this result. Previous work has shown that ATM mediates a nuclear stress response signaling pathway whereby activated ATM forms a complex with nuclear IKKγ, inducing its SUMOylation and promoting nuclear export (31). In the cytoplasm, the ATM-IKKγ complex associates with the cytoskeletal ELKS-IKKα/β complex, resulting in IκBα phosphorylation and release of NF-κB/RelA sequestered in the cytoplasm (44).

Our comparison of the kinetics of IκBα proteolysis and NF-κB translocation indicates that ATM participates in the initial steps of NF-κB release. Several reports previously suggested that ATM may not play a role in TNF-mediated NF-κB activation; these conclusions have been made after measuring the effect of ATM inhibition on its DNA binding ability using electrophoretic mobility shift assays (EMSAs) (45). Because EMSAs do not measure the activated (Ser-276-phosphorylated) form of NF-κB, an effect on the separate transactivation pathway may have been missed. More detailed experiments are required to ascertain the precise role of ATM in the NF-κB transactivation pathway.

Although the predominant mechanism by which ATM is activated occurs in response to double-stranded DNA breaks in the genome, it is also known that ATM is activated by an ancillary pathway in response to reactive oxygen species (ROS)-induced stress, which is double strand break-independent (46). In this regard, we note that TNF stimulation induces ROS stress and oxidative DNA damage measured by the inducible formation of 8-oxoguanine adducts in stimulated cells (15). The kinetics of ROS formation in epithelial cells, peaking 15 min after TNF stimulation, are consistent with the activation profile of ATM that we have observed in this study. More detailed work will be required to determine whether TNF signaling induces a nuclear signal or whether ATM is somehow involved in cytoplasmic IKK activation in response to extracellular TNF receptor signaling.

Another interesting finding from our study is that ATM affects only a subset of NF-κB-dependent genes. Earlier we have observed that the subset of NF-κB-dependent genes, including Groβ, TNFAIP3/A20, and IL-8, are downstream from an ROS-PKAc-CDK9 pathway that controls phosphorylation of the CCOH-terminal domain of RNA polymerase II (5, 6). Here, Ser-276 RelA phosphorylation is a key activating step, enabling RelA to form a stable complex with CDK9, recruiting it to target chromatin. The finding that RelA Ser-276 phosphorylation is impaired in ATM−/− MEFs suggests that ATM participates in

FIGURE 8. Effect of TNF stimulation in ATM−/− MEFs. Wild type (ATM+/+) and ATM-deficient (ATM−/−) MEFs were stimulated in the absence or presence of TNF (30 ng/ml) for the indicated times or with VP16. Total RNA was extracted, and the expression of TNFAIP3/A20 (A), IκBα (B), Groβ (C), and KC (D) was determined by Q-RT-PCR. Shown is fold change mRNA expression relative to unstimulated ATM+/+ cells as calibrator. Data were analyzed by two-way analysis of variance with multiple comparison (time and treatment) and Tukey’s post hoc test for significance between time intervals and the treatment groups. *, p < 0.01, significantly different from control samples; †, p < 0.05, significantly different from TNFα-treated samples. Error bars, S.D.
controlling transcriptional elongation of the Groβ, TNFAIP3/A20, and IL-8 genes. By contrast, IkBa is an NF-κB-dependent gene that does not require RelA Ser-276 phosphorylation of CDK9 for its inducible expression (5, 6). Here we note that IkBa is also insensitive to ATM deficiency (Fig. 7). Together, these findings suggest that ATM may participate in the ROS-PKAc-CDK9 pathway important in the activation of rapidly responding inflammatory genes.

In summary, by using an HT-siRNA screening approach, we identified and validated six kinases in TNF-induced NF-κB activation, including a novel role for CDK5 and -7 and ATM. Our functional data complement the protein interaction map previously determined by tandem affinity purification proteomics for a more complete understanding of the regulatory kinases controlling this complex signaling network. We further selected and validated the role of ATM in TNF signaling using selective inhibitors and ATM−/− MEFs. Although ATM is an established mediator of double-stranded DNA break-induced signaling and NF-κB release from cytoplasmic sequestration, our findings in TNF signaling suggest that the role of ATM extends to other stimulus types and affects the transactivation step of NF-κB during pathway activation. These exciting findings link the nuclear cell stress-responsive ATM kinase to the canonical NF-κB pathway classically mediated by cytokine signaling through the TNF receptors.

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