Nucleoside Diphosphate Kinase-3 (NME3) Enhances TLR5-Induced NFκB Activation

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Abstract

Bacterial flagellin is a potent activator of NFκB signaling, inflammation, and host innate immunity, and recent data indicate that flagellin represents a novel antitumor ligand acting through toll-like receptor 5 (TLR5) and the NFκB pathway to induce host immunity and aid in the clearance of tumor xenografts. To identify innate signaling components of TLR5 responsible for these antitumor effects, a loss-of-function high-throughput screen was employed utilizing carcinoma cells expressing a dynamic NFκB bioluminescent reporter stimulated by Salmonella typhimurium expressing flagellin. A live cell screen of a siRNA library targeting 691 known and predicted human kinases to identify novel tumor cell modulators of TLR5-induced NFκB activation uncovered several interesting positive and negative candidate regulators not previously recognized, including nucleoside diphosphate kinase 3 (NME3), characterized as an enhancer of signaling responses to flagellin. Targeted knockdown and overexpression assays confirmed the regulatory contribution of NME3 to TLR5-mediated NFκB signaling, mechanistically downstream of MyD88. Furthermore, Kaplan–Meier survival analysis showed that NME3 expression correlated highly with TLR5 expression in breast, lung, ovarian, and gastric cancers, and furthermore, high-level expression of NME3 increased overall survival for patients with breast, lung, and ovarian cancer, but the opposite in gastric cancer. Together, these data identify a previously unrecognized proinflammatory role for NME3 in signaling downstream of TLR5 that may potentiate cancer immunotherapies.

Implications: Proinflammatory signaling mediated by innate immunity engagement of flagellin-activated TLR5 in tumor cells results in antitumor effects through NME3 kinase, a positive downstream regulator of flagellin-mediated NFκB signaling, enhancing survival for several human cancers.

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Introduction

Harnessing host immunity constitutes a promising cancer therapeutic strategy (1–3), and agonists of Toll-like receptors (TLR) have been actively pursued for their antitumor potential, either as monotherapy or as adjuvants to vaccination or other therapeutic modalities (4–6). In this context, Salmonella typhimurium, a common flagellated facultative intracellular bacterial pathogen, has been identified as a potential tumor therapeutic, capable of inducing tumor regression (7–13). While the exact mechanisms behind Salmonella therapeutic efficacy remain unclear, the effects are thought to be mediated by bacterial antigenicity and activation of host innate immune responses mediated by the TLR family of pattern recognition receptors (14, 15). Several groups have shown that mouse tumor xenografts are uniquely sensitive to treatment with bacterial flagellin, resulting in tumor regression (16–19). Likely mediated by TLR5, which specifically binds flagellin (20), the induced proinflammatory signals appear to reside within the cancer cells per se as cancer cells lacking TLR5 fail to respond to treatment with flagellin in vivo (16).

TLR5 is an important conventional component of innate immunity and, interestingly, also may be involved in maintaining genomic stability through its integration into the DNA damage p53 regulatory network (21). The enhancement of TLR5-mediated recruitment of the innate immune response by p53 points toward an important crossroad between cancer progression and treatment (22, 23). TLR5 acts by recognizing flagellated bacteria, such as Salmonella, alerting the host of their presence (24, 25). Once bound by activating ligand, TLR5 initiates a signaling cascade that ultimately impacts transcription in host cells resulting in activation of proinflammatory pathways, which include NFκB (16, 24). To initiate NFκB signaling, activated TLR5 induces recruitment of the cytosolic adapter, MyD88, which interacts directly with the Toll-interleukin-1 receptor (TIR) domain (24). MyD88 then recruits the serine/threonine kinase IRAK (I1 receptor-associated kinase), and via phosphorylation, IRAK activates TNF receptor–associated factor (TRAF6), which in turn activates TRAF-associated kinase (TAK; ref. 26). TAK links TRAF6 to the IKK complex, which phosphorylates IκBα, the negative regulator of NFκB (24). Canonical ubiquitination and proteasome-mediated degradation of IκBα frees NFκB to translocate into the nucleus and activate downstream transcriptional programs that promote inflammation and immune responses (24). This robust activation
of host innate immunity signaling has been postulated to mediate chemokine secretion and tumor regression (16).

In NFκB signaling, various induction signals, inclusive of, but not limited to, pathogen-associated ligands, reactive oxygen species, cytokines and DNA damage, lead to activation of transcriptional programming specifically designed to shape responses to each cellular stress (27). As multiple different triggers are capable of inducing NFκB, multiple cellular outcomes exist in response to each insult (27, 28). To further complicate the scenario, the resulting transcriptional profile and downstream effects of NFκB signaling may differ based on specific cell contexts (29). For example, a beneficial proinflammatory signal in an acute exposure may transition to a tumorigenic signal in a chronic context. Indeed, acute activation of NFκB in an individual tumor cell may activate host defenses to isolate transformed cells, but in T cells, flagellin has been shown to upregulate PD-1 expression, an immune checkpoint receptor, which may enable tumor cells to escape immune surveillance (30). Interestingly, interactions between commensal microbiota in the gut and TLR5 have been recently reported to modulate systemic inflammatory responses that may suppress or accelerate distal cancers depending on context (31).

Using a bioluminescence reporter, we herein confirmed a robust activation of proinflammatory TLR5-mediated NFκB signaling in HCT116 human colon carcinoma cells by bacterial flagellin. Then, to identify novel regulatory components of innate immunity and TLR5 signaling that may contribute to the proinflammatory signals mediating the antitumor effects of flagellin, we employed a high-throughput siRNA screen. After assessing the contribution of 691 human kinases, we identified a nucleoside diphosphate kinase, NME3, as a positive regulator of NFκB signaling in response to flagellin. Expression of this kinase, which we demonstrate to be required for maximal TLR5 signaling by bacterial flagellin, is also correlated with enhanced survival rates in lung and breast cancer patients. In addition to NME3, this investigation also identified multiple candidates that may serve as adjuvant targets to support the antitumor properties of flagellin.

Materials and Methods

Cell lines and culture conditions

HCT116 human colon carcinoma cells (TP53 WT) were a gift of Bert Vogelstein (2009) and cultured according to ATCC instructions (11). Mycoplasma testing was performed routinely. Further cell line authentication was not performed. All stably transfected HCT116 cells were cultured in 0.5 μg/mL puromycin.

Salmonella strains

Salmonella typhimurium strain SL1344 was used for all experiments, except where noted. All mutants (flIC, flIB, and flIC/flIB) were constructed using a lambda red recombinase strategy (32). First, primers were designed to amplify the kana-mycin- or chloramphenicol-resistance cassette in pKD4 or pKD3 with tails flanking the targeted locus of the Salmonella genome to be deleted. PCR fragments were then electroporated into SL1344 bacteria expressing plasmid-encoded red recombinase. Following electroporation, growth on kanamycin or chloramphenicol plates at 37°C selected for strains that had lost the temperature-sensitive recombinase plasmid and inserted the chloramphenicol-resistant cassette into the targeted genomic locus. The double mutant strain was created in a step-wise manner, by individually deleting each gene. Deletion of target genes was confirmed by PCR.

Creation of a ρB5:IkBe-FLuc-expressing HCT116-stable cell line

HCT116 cells at 95% confluency were cotransfected with 10 μg of ρB5:IkBe-FLuc and 3 μg of pIRES-puro plasmid DNA using FuGene 6 (Roche) in 10-cm dishes (33). After 24 hours, the media was replaced with fresh cell media. Twenty-four hours later, the cells were split at multiple dilutions into media containing 0.5 μg/mL puromycin to select for stable transformants. After two weeks, isolated cell colonies were imaged to confirm reporter gene expression and bioluminescent colonies were harvested and expanded. Reporter cells were continuously cultured in the presence of 0.5 μg/mL puromycin to maintain expression of the reporter plasmid.

Transient transfections of HCT116 cells

HCT116 cells were transiently transfected where noted. Cells were plated in 24-well (50,000–60,000 cells/well) or 96-well (10,000 cells/well) plates and transfected with FuGene 6 and 200 ng of plasmid DNA (24-well) or 50 ng of plasmid DNA (96-well) per well. In the case of NME3 overexpression experiments, reporter cells were transfected with FuGene 6 and 100 ng of target (pCMV6:NME3) or vector control (pCMV6) plasmid in each well of a 96-well plate. Alternatively, 100 ng of reporter plasmid and 200 ng of target (pCMV6:NME3) or vector control (pCMV6) plasmid were used in each well of a 24-well plate. Cells were allowed to recover for 48 hours prior to imaging.

Dynamic imaging of NFκB signaling

Thirty minutes prior to imaging, cell media were aspirated and replaced with colorless DMEM or McCoy 5A supplemented with 10% heat-inactivated FBS and 150 μg/mL β-luciferin. Cells were imaged at baseline and after stimulation as indicated in an IVIS 100 or IVIS 50 imaging system, with images being acquired every 5 minutes for 6 hours, unless otherwise indicated. Cells were maintained in the imaging chamber by a heated stage (37°C) and 5% CO2 air flow. Acquisition parameters were: acquisition time, 60 seconds; binning, 4–8; filter, open; f stop, 1; FOV, 12–23 cm. Stimuli included: SL1344 Salmonella typhimurium, or indicated mutants, processed from confluent culture (final dilution 1:100 in well) or SL1344 matched for OD600, heat-killed by boiling 10 minutes and diluted 1:10, 1:100, or 1:1,000 into each well (where noted); lipopolysaccharide (1 μg/mL Sigma); peptidoglycan (Sigma); iEDAP (10 μg/mL InvivoGen); MDP (10 μg/mL InvivoGen); Tnf (20 ng/mL; R&D systems); flagellin (InvivoGen); anti-hTLR5-IgA (10 μg/mL InvivoGen); or shTLR5-FC (5 μg/mL) InvivoGen). Bioluminescence photon flux (photons/sec) data represent the mean of triplicate wells for the indicated number of independent experiments, and were analyzed by region of interest (ROI) measurements with Living Image 3.2 (Caliper Life Sciences). Data were imported into Excel (Microsoft Corp.), averaged, and normalized to both initial (t=0) values (fold-initial) and vehicle-treated controls (fold-vehicle) for presentation in dynamic plots (34).

High-throughput screen

siRNA screening in triplicate was performed in white, clear-bottomed, 96-well culture plates using a Beckman-Coulter Core Robotics system, including an FX liquid handler, controlled by the Sagian graphical method development tool (SAM1 scheduling software). HCT116 cells stably expressing ρB5:IkBe-FLuc were seeded at 15,000 cells per well in a 96-well plate and cells

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were allowed to attach for 24 hours. Forward transfection was performed with a 96 multichannel head on the FX liquid handler, adding 0.5 µL/well of media-complexed R1 Transpass (NEB) to the aliquoted siRNA library (Kinase siRNA set v2; Qiagen Inc.) in a 96-well reaction plate and allowed to incubate for 15 minutes. After incubation of siRNA complexes, 100 µL was added to each well of a plate with cells (×3 plates) using the FX liquid handler, yielding a final concentration of approximately 50 nmol/L siRNA/well. Experimental siRNA oligos were arrayed in columns 2–11 of each plate and individual controls comprising vehicle-treated wells, a nontargeting control sequence (Qiagen Allstar Negative control), TLR5-targeting siRNA sequences (IDT), and a firefly luciferase-targeting PGL3 siRNA (Dharmacon Research Inc.) were placed manually in columns 1 and 12. Plates were maintained at 37°C and 5% CO2 for 48 hours. At this time, media were aspirated and replaced with 180 µL imaging media (colorless DMEM supplemented with 10% heat-inactivated FBS and 150 µg/mL t-luciferin) and the cells were allowed to equilibrate for 45 minutes. After equilibrating, 20 µL of stimulus (1:100 dilutions of heat-killed Salmonella cultures) or control (LB broth) were added to each well. Bioluminescent readings were obtained on an EnVision plate reader (PerkinElmer) immediately following the stimulus, at 45 minutes poststimulation and at 245 minutes post-stimulation. After the final luminescent reading, 20 µL of reazurin dye was added to all wells, allowed to incubate for 2 hours at 37°C and monitored on a FLUOstar OPTIMA fluorescence reader for cell viability (BMG Labtech; excitation, 544 nm; emission, 590 nm).

Statistical analysis
Initially, signal in each well was normalized to a plate-matched control well containing a nontargeting siRNA sequence at each time point to facilitate experiment-wide analysis. Then, the differences in the log2 values of the normalized data between 0 minutes and 45 or 245 minutes were averaged across triplicate siRNA experimental replicates. Then, screening hits were selected by quartile analysis of the normalized kinase library data. To perform the quartile analysis, median (Q2), and lower boundaries for hit selection were calculated as Q3 – Q2 and Q1 – 2c(Q3 – Q2) and Q1 – 2c(Q2–Q1), respectively, for c = 1.2245 corresponding to a high-stringency targeted error rate (α = 0.02) and for c = 0.7193 corresponding to a low-stringency targeted error rate (α = 0.1; ref. 35).

siRNA knockdown validation
Knockdown of NME3 or MyD88 was performed utilizing heterogeneous mixtures of siRNAs targeting NME3 or MyD88 mRNA sequence, respectively. Reporter HCT116 cells were plated in 96-well plates at 15,000 cells/well and allowed to incubate overnight. Twenty-four hours later, cells were transfected with X-tremeGene siRNA transfection reagent (Sigma) and 25 nmol/L esiRNA (Sigma). Cells were incubated in media for 72 hours prior to imaging. Additional siRNA knockdown of NME3 was performed utilizing 4 separate targeting sequences. Reporter HCT116 cells were treated as before with the exception that cells were transfected with R1 Transpass (NEB) and 25 nmol/L siRNA (Qiagen) as per R1 Transpass instructions. Cells were incubated in media for 72 hours prior to imaging.

shRNA lentiviral knockdown cell line construction
Lentivirus-expressing constructs (pLKO.1 puro) were obtained presynthesized from the Genome Institute at Washington University. The targeting sequences for the 3 shNME3 constructs are as follows:

#7 - 5’-GAGGTTGCAAGAACCCTGATT
#8 - 5’-GGCTTGGCAAGATATGCGGCT
#9 - 5’-CGAAGGAATGGTGGGCCT

Additionally, a scrambled shRNA construct was utilized as a negative control. To generate lentivirus containing hairpins, 293T cells (5 × 10⁴) were preplated in 60-mm dishes and cotransfected the following day with 1 µg of hairpin construct, 900 ng packaging plasmid pCMV:ΔR8.2, and 100 ng of envelope plasmid pVSVG using Fugene 6. Two days after transfection, virus-containing supernatant was collected from 293T cells and filtered through a 0.45-µm filter, mixed with 5 µg/mL protamine sulfate, and added to HepG2 cells at 50% confluency in a 10-cm² dish. Media were replenished 12 hours posttransduction, and cells were subsequently maintained in media supplemented with 500 ng/mL puromycin hydrochloride to retain expression of the hairpins. Following transduction, shNME3 or shSCRAMBLED cells were plated in parallel for mRNA knockdown confirmation and transient transfection and subsequent imaging measurements with the bB2:JeBa-Fluc reporter or the bB2:Fluc reporter as described previously.

Semi-quantitative RT-PCR
HCT116 cells transfected with siRNA targeting either NME3 or MyD88 were lysed and total RNA was purified using the Qiagen RNeasy kit (Qiagen). cDNA synthesis was performed using Script Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad) using 800 ng of total RNA per reaction. To perform quantitative PCR, 100 ng of cDNA per sample were amplified with primers specific for either human NME3 or human MyD88 (PrimePCR SYBR Green, Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). PCR cycling conditions were: 95°C for 30 seconds for polymerase activation and DNA denaturation, followed by 42 cycles of denaturation (95°C for 10 seconds), and annealing/extension/plate read (60°C for 30 seconds).

HCT116 cells transfected with shNME3 or shSCRAMBLED hairpins were lysed and total RNA was purified using the Qiagen RNeasy kit (Qiagen). Samples were then treated with DNase I at room temperature for 15 minutes, after which EDTA was added and samples were incubated for 10 minutes at 65°C to inactivate the DNase. Samples were then ethanol precipitated and resuspended in water. Reverse transcription was performed using the PrimeScript II Reverse Transcriptase and 300 ng random primers as per the manufacturer’s instructions (Invitrogen). To perform semi-quantitative PCR, samples were amplified using 2 µL of RT reaction and primers specific to NME3 or GAPDH. PCR cycling conditions were: 95°C for 5 minutes, 35 cycles (or 25 cycles for GAPDH reactions) of denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute. PCR products were fractionated on 1% agarose gel.

Survival analysis
The univariate Cox proportional hazard model was used to assess the correlation of gene expression with patient overall survival, and the likelihood ratio test P values were reported.
Datasets from TCGA, GEO, and caBIG (36–38) were used for determining overall survival of patients with breast cancer, lung cancer, ovarian cancer, and gastric cancer. Gehan–Breslow–Wilcoxon and Log-rank tests were used to estimate P values between high and low expression groups (using top and bottom 25% as the cutoff for grouping). χ² tests were used to test the association between two gene expression profiles with high (>75%), median (<75%–>25%) and low (<25%) expression levels for the entire cohort of each cancer type (39). A two-tailed P < .05 was considered significant. Analyses were performed using R 3.2.3.

Results
To assess TLR5-induced activation of NFκB by Salmonella in real time in living cells, we utilized a bioluminescent transcriptionally coupled κB-κBα-FLuc fusion reporter, consisting of the negative regulator of NFκB, κBα, directly fused to firefly luciferase. In this case, baseline activity of the fusion reporter protein serves as a direct readout of basal IKKβ kinase activity in the host cell (33). As activated IKKβ phosphorylates endogenous IkBα, the reporter fusion protein is similarly phosphorylated, ubiquitinated, and targeted for degradation, resulting in a reduction in bioluminescence reporter activity, which can be monitored in real time (33). Liberation of endogenous NFκB from its inhibitor allows translocation to the nucleus and transcriptional activation of NFκB response elements. Because the reporter construct is linked on its 5’ end to five of these response elements in tandem, NFκB nuclear transactivation drives transcription/translation of new fusion proteins resulting in an increase in bioluminescent signal (33). Thus, stimulation of HCT116 cells with heat-killed Salmonella robustly initiated degradation and the subsequent resynthesis of the IkBα-FLuc reporter fusion, which could be imaged periodically to visualize changes in reporter photon output (Fig. 1A) and

Figure 1.
Real-time activation of NFκB signaling by TLR5 using heat-killed Salmonella typhimurium. HCT116 cells in 96-well format transiently transfected with pκBα-κBα-FLuc were stimulated with heat-killed Salmonella at t = 0 and bioluminescence activity imaged every 5 minutes for 6 hours. A, Representative images show pseudocolor-coded radiance maps superimposed on black-and-white photographs of the assay plate every 20 minutes. B, Graphical representation of the changes in photon flux as a function of time after vehicle, TNFα, or Salmonella addition. Data are plotted as fold-initial. Bioluminescence photon flux (photons/sec) data represent the mean of triplicate wells. C–E, HCT116 cells stably expressing pκBα-κBα-FLuc were stimulated with the indicated ligand at t = 0 and bioluminescence activity imaged every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). F, HCT116 cells stimulated with heat-killed Salmonella wild-type, flIC-, flJB-, or flIC~/flJB~ double mutant strains at t = 0 and bioluminescence activity imaged every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle).
represented graphically (Fig. 1B). Heat-killed bacteria were preferred as a stimulus over live bacteria. When performing the assay with live Salmonella, replication and overgrowth of the bacteria quickly changed media conditions, ultimately leading to complete attenuation of the luciferase signal. After stimulation with heat-killed Salmonella, the reporter signal initially decreased to 60% of its steady-state level prior to activation. Following this decrease, which corresponded to IkBα degradation, Salmonella-induced reporter activity rebounded to >3× the original bioluminescence level, corresponding to transcriptional activation and translation of the IkBα-FLuc fusion protein.

**Reporter responsiveness to flagellin-mediated activation of TLR5**

To isolate the specific moiety of the bacteria that activated NFκB signaling in HCT116 carcinoma cells, we tested individual activities of known immunostimulatory components of bacteria. In HCT116 cells stably expressing the kB5:IκBα-FLuc reporter, bacterial LPS, purified peptidoglycan (PG), and NOD ligands (MDP and iEDAP) were all incapable of inducing significant bioluminescent reporter activity (Fig. 1C–E). To determine the contribution of the two Salmonella flagellin proteins to NFκB pathway activation in HCT116 cells, we constructed Salmonella strains mutated singly or in both flagellin genes flIC and flIB. Both single mutants still activated NFκB signaling, albeit to a lesser extent than that of wild-type bacteria, but the Salmonella flIC/ flIB double mutant was incapable of activating NFκB signaling (Fig. 1F), indicating that flagellin was the predominant ligand inducing NFκB signaling in HCT116 cells.

Flagellin is the principal component of bacterial flagella, a major virulence factor recognized by mammalian TLR5 receptors (20), which, upon binding activates NFκB in a MyD88-dependent manner (40). To further define flagellin-mediated activation of the TLR5 pathway in HCT116 cells, we utilized two different bioluminescent reporters to visualize signaling in real time. First, cells containing the transcriptionally coupled kB5:IkBα-FLuc fusion reporter were treated with increasing concentrations of Salmonella typhimurium-derived flagellin (10, 20, 50, 100, 200, and 1,000 ng/mL), which induced progressive increases in reporter degradation kinetics as well as transcriptional responses (Fig. 2A). Both maximal reporter degradation and resynthesis showed different kinetics compared to stimulation with TNFα, differing infers in the signal transduction mechanism induced by the different stimuli. Similarly, concentration-dependent responses were observed when cells were treated with heat-killed Salmonella (Fig. 2B). To support these findings, a standard transcriptionally activated kB5:FLuc reporter was utilized as our second bioluminescent reporter in HCT116 cells. A concentration-dependent activation of the kB5:FLuc reporter was confirmed when cells were treated with increasing concentrations of flagellin (5, 10, 20, 50, 100, 200 ng/mL; Supplementary Fig. S1A and S1B), and by heat-killed Salmonella (Supplementary Fig. S1C and S1D).

Next, we confirmed that activation of the kB5:IkBα-FLuc fusion reporter in HCT116 cells was mediated by flagellin binding to TLR5 receptors. First, we preincubated flagellin (at two concentrations: 100 ng/mL and 1 μg/mL) with 5 μg/mL of soluble human TLR5 (shTLR5) (molar ratios 1:27 and 1:3) for 30 minutes prior to addition of the activation mixture to cells. Preincubation of flagellin with shTLR5 abrogated flagellin-induced activation of the kB5:IkBα-FLuc fusion reporter at both concentrations (Fig. 2C). Reporter activation could be competitively rescued by preincubation with a 10× increase in flagellin concentration (10 μg/mL) in the presence of the same concentration of shTLR5 (Supplementary Fig. S2). As expected, preincubation of shTLR5 with TNFα had no significant effect on TNFα-induced activation of the kB5:IkBα-FLuc fusion reporter (Fig. 2C). These data confirmed the specificity of the flagellin-mediated activation of the kB5:IkBα-FLuc fusion reporter through TLR5 receptor binding. Second, we tested whether an antibody directed against the extracellular domain of TLR5 (hTLR5 Ab) also inhibited flagellin-mediated activation of the kB5:IkBα-FLuc fusion reporter. Cells were preincubated for 1 hour with hTLR5 Ab (10 μg/mL) followed by treatment with flagellin at two concentrations that readily activated the reporter under standard conditions (100 ng/mL or 10 μg/mL). At the low flagellin concentration (100 ng/mL), hTLR5 Ab preincubation completely blocked degradation or activation of the reporter, while at the high flagellin concentration (10 μg/mL), the overall blockade was less, but the activation phase was still reduced by 2.5-fold at 6 hours (Fig. 2D). Conversely, while cells preincubated with hTLR5 Ab and then activated with TNFα (20 ng/mL) showed a shallower and extended degradation phase of IkBα compared with cells treated only with TNFα (20 ng/mL), consistent with cross-talk between the two pathways converging on IkBα, the transcriptional activation response was unaffected at 6 hours (Fig. 2D).

**Live-cell siRNA library screen**

With the knowledge that HCT116 colon carcinoma cells were robustly activating proinflammatory signaling responses to Salmonella flagellin, we set out to identify novel cancer cell kinases regulating TLR5-mediated innate immune responses. We utilized a siRNA library to knockdown all known and predicted human kinases and screen for involvement of each in Salmonella-induced activation of the TLR5 pathway. The screen utilized HCT116 cells colon carcinoma cells stably expressing the kB5:IkBα-FLuc construct. Cells were plated and transfected with siRNA targeting 691 host kinases arrayed in the 10 center columns of 96-well plates with each well containing two sequences targeting a single host kinase. Forty-eight hours following siRNA transfection, cells were stimulated with heat-killed Salmonella and the reporter signal was measured immediately (baseline), at 45 minutes and at 245 minutes following exposure to Salmonella (Fig. 3A and B). Normalized bioluminescence data at 45 and 245 minutes were plotted individually (Fig. 3C and D; Supplementary Fig. S3A and S3B), or on x-y coordinates representing the normalized signals at 45 and 245 minutes, respectively (Fig. 4).

High-throughput screening hits were determined using a quartile-based analysis. Figures 3 and 4 display the quartile-identified values for low- and high-stringency hit selection. siRNA-induced loss-of-function of cellular kinases that positively affect TLR5 signaling will demonstrate reduced responsiveness to Salmonella. In contrast, wells with siRNA targeting a negative regulator of TLR5-induced NFκB signaling will show enhanced reporter response. Figure 4 demonstrates the four possible effects kinases in the screen may have had on reporter activity (insert). Kinase knockdowns further enhancing the loss of photon flux signal at 45 minutes, that is, increasing IkBα degradation, indicated that a negative regulator of early TLR5-induced NFκB signaling had been targeted. Meanwhile, kinase knockdowns attenuating the early degradation phase of signal at 45 minutes indicated that siRNA targeted a positive regulator of early TLR5-induced NFκB...
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Figure 2.
Dose-dependent activation of the transcriptionally coupled αB2:κBα-Fluc fusion reporter by flagellin and heat-killed Salmonella in HCT116 colon carcinoma cells and TLR5 receptor antagonists inhibit flagellin-mediated activation of the reporter. (A and B) HCT116 cells containing the αB2:κBα-Fluc fusion reporter were stimulated with the indicated ligand at time 0 and bioluminescence activity measured every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). A, HCT116 cells containing αB2:κBα-Fluc were treated with increasing flagellin concentrations: 10 ng/mL (n = 4), 20 ng/mL (n = 4), 50 ng/mL (n = 4), 100 ng/mL (n = 5), 200 ng/mL (n = 5), 10 μg/mL (n = 3); TNFα 20 ng/mL (n = 5). B, HCT116 cells expressing αB2:κBα-Fluc were treated with different dilutions of heat-killed Salmonella typhimurium from confluent culture grown at 37°C. 1,000-fold dilution (error bars represent range of n = 2); 10-fold dilution (error bars represent range of n = 3); TNFα 20 ng/mL (n = 5). C and D, HCT116 cells containing the αB2:κBα-Fluc fusion reporter were treated with two different hTLR5 antagonists and stimulated with the indicated ligand at time 0. Bioluminescence activity was measured every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). C, Prior to cell stimulation with flagellin or TNFα, flagellin or TNFα were preincubated with or without soluble hTLR5 receptor (shTLR5, 5 μg/mL), then added to cells: TNFα 20 ng/mL (n = 5); TNFα 20 ng/mL with shTLR5 5 μg/mL (n = 4); shTLR5 5 μg/mL alone (n = 5). D, Prior to stimulation with flagellin or TNFα, cells were preincubated with antibody against hTLR5 (hTLR5 Ab) for an hour: TNFα 20 ng/mL (n = 5); TNFα 20 ng/mL with hTLR5 Ab 10 μg/mL (n = 5); flagellin 100 ng/mL (n = 5); flagellin 1 μg/mL with shTLR5 5 μg/mL (n = 4); shTLR5 5 μg/mL alone (n = 5). Error bars represent SEM for the indicated number of independent experiments.

signaling. Conversely, at the 245-minute time point, decreased values represented a low signal during the resynthesis phase, which indicated a lack of full NFκB transactivation and therefore siRNA knockdown of a positive regulator of NFκB-induced transcription. Greater photon flux values corresponded to overactivation of NFκB transcriptional activity, and therefore siRNA treatment targeted a negative transcriptional regulator. Therefore, in Fig. 4, a siRNA that targets a positive regulator of both the degradation and resynthesis phases will fall in the lower right quadrant, while a negative regulator of both phases will be found in the top left portion of the scatter plot. The positive control well containing siRNA targeting TLR5, for example, should prevent IκBα degradation at 45 minutes, which in turn, will inhibit reporter transcriptional activation at 245 minutes. This is shown by the blue triangle corresponding to TLR5 siRNA-treated wells, which falls in the lower right quadrant of the scatter plot. Table 1 lists the top ten positive and negative regulators from each phase of the screen. In addition, Supplementary Table S1 details all of the low- and high-stringency hits from the screen. Of note, IRAK1 and AKT1, both known activators of NFκB signaling, were identified as hits in the screen, further verifying the validity of the strategy. The screen also identified MAP2K2, MAP2K3, and PIK3CG, PIK3CD, among others, as kinases involved in positively regulating flagellin-induced NFκB signaling. However, targeting known downstream MAP kinases with chemical inhibitors (U0126, SB203580) showed no consistent effect in this system.
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Figure 3.
A schematic of the high-throughput screening technique and high-throughput screening data. A, HCT116 cells stably expressing βB2;IkBαFLuc were plated into 96-well plates. After a 24-hour incubation, cells were transfected with siRNA from the library and incubated for 48 hours more. To image, cells were transferred into α-luciferin-containing media, allowed to equilibrate for 45 minutes, stimulated with heat-killed Salmonella and imaged for reporter activity at 0, 45, and 245 minutes. B, Each siRNA library plate contained targeting siRNA in columns 2–11 and control siRNA constructs in columns 1 and 12, as indicated. Control wells included: mock-transfected cells (blue, A1), vehicle-treated wells (yellow; E1, F1, G1, H1), three nontargeting control sequences (turquoise; Qiagen scrambled, A12, B12), and a firefly luciferase-targeting PGL3 siRNA (purple; Dharmacon Research Inc, A12, B12). C and D, Normalized photon flux data for 691 targeted kinases are shown at 45 minutes (C) and 245 minutes (D) after Salmonella stimulation. Data are the average of three replicates. Dashed red and dotted blue lines show significance cutoffs for low (α = 0.1) and high (α = 0.02) stringency targeted error rates, respectively.

and given this complexity, MAP kinase pathways were not further pursued. Notably, CDK6, a known anticancer drug target (41), CDK5R2, and TYRO3 were affirmed as negative regulators of TLR5-induced NFκB signaling.

Interestingly, one of the novel hits identified in the screen was NME3, a nucleoside diphosphate kinase that catalyzes the formation of other nucleoside triphosphates from ATP. NME3 was originally named on the basis of the discovery of the gene by preferential expression in non-metastatic (nme) cells in a cancer model and has subsequently been demonstrated to possess anticancer properties in multiple model systems upon overexpression (42–45). In the screen, knockdown of NME3 attenuated reporter signal at 45 minutes and caused reduced TLR5-induced transcriptional activation at 245 minutes, indicating that NME3 was behaving as both a positive regulator of degradation and transcription of NFκB signaling (Fig. 4).

Given that MyD88 is an essential signal adaptor of TLR5-mediated activation of NFκB signaling (46), we first confirmed the signature of MyD88 siRNA knockdown on Salmonella-stimulated activation of the xB2;IkBα-FLuc fusion reporter in this system. Figure 5A shows that knockdown of MyD88 by siRNA markedly reduced both the degradation and resynthesis phases of NFκB signaling compared with scrambled control. The signal showed virtual, but not complete abrogation, likely due to incomplete knockdown of MyD88. Quantitative PCR confirmed that NME3 mRNA levels were indeed reduced in cells treated with pooled NME3 siRNA sequences compared with cells treated with scramble siRNA sequence (Fig. 5A, top insert). Semiqualitative analysis of cell viability showed no significant effect on cell growth/survival in NME3 knockdown compared with scrambled siRNA controls.

To further determine the contribution of NME3 to TLR5-induced activation of NFκB, we produced stable knockdown of NME3 in HCT116 cells using lentiviral shRNA constructs.
Table 1. siRNA knockdown screen of flagellin/TLR5-induced NF-κB signaling. Top positive and negative regulatory kinases identified in the degradation and resynthesis phases of the reporter screen.

<table>
<thead>
<tr>
<th>Negative regulators of degradation</th>
<th>Positive regulators of degradation</th>
<th>Negative regulators of resynthesis</th>
<th>Positive regulators of resynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rank</td>
<td>Gene target</td>
<td>Rank</td>
<td>Gene target</td>
</tr>
<tr>
<td>1</td>
<td>TYRO3</td>
<td>1</td>
<td>MAP2K3</td>
</tr>
<tr>
<td>2</td>
<td>TGFBR2</td>
<td>2</td>
<td>MAP2K2</td>
</tr>
<tr>
<td>3</td>
<td>PRKAR2A</td>
<td>3</td>
<td>ITPKA</td>
</tr>
<tr>
<td>4</td>
<td>CDK5R2</td>
<td>4</td>
<td>TESK1</td>
</tr>
<tr>
<td>5</td>
<td>RPS6KA5</td>
<td>5</td>
<td>PIK3CD</td>
</tr>
<tr>
<td>6</td>
<td>PIM1</td>
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<td>PKM2</td>
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<tr>
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<td>8</td>
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<tr>
<td>9</td>
<td>TNK2</td>
<td>9</td>
<td>EPHA3</td>
</tr>
<tr>
<td>10</td>
<td>RAF1</td>
<td>10</td>
<td>KDR</td>
</tr>
</tbody>
</table>

Survival analysis

NF-κB signaling is well known to have both tumor-promoting and tumor-suppressive roles through cell autonomous, innate proinflammatory signaling (47–49). Having identified NME3, which has previously been shown to have tumor suppressor-like functions (43, 45, 50), as a novel enhancer of TLR5-induced NF-κB activation, we hypothesized that NME3 may participate in tumor suppression through positive activation of this proinflammatory signaling pathway. To begin to explore the clinical relevance of this proposed mechanism, we utilized cancer patient clinical expression profiles and mutational analysis tools. Kaplan–Meier survival analysis for breast ($n = 3,951$; relapse-free survival; ref. 36) and lung ($n = 1,926$; overall survival; ref. 37) cancer patients showed that patients with high level expression of NME3 correlated with increased survival compared with patients with low levels of NME3 ($P < 0.0001$; Fig. 6A and B). For ovarian cancer, median survival increased with higher expression of NME3, but long-term survival did not; overall survival differences were not significant ($n = 1,435$; progression-free survival; $P < 0.22$; ref. 38; Fig. 6C). In contrast, gastric cancer patients ($n = 876$)
with higher NME3 expression showed decreased overall survival \( (P < 0.002; \text{Fig. 6D}) \).

Consistent with our hypothesis, TLR5 clinical survival analysis correlated with NME3 survival analysis. Breast \( (n = 3,951); \) relapse-free survival; ref. 36), lung \( (n = 1,926); \) overall survival; ref. 37), and ovarian \( (n = 1,435); \) progression-free survival; ref. 38) cancer patients with higher TLR5 expression levels showed enhanced survival \( (P < 0.0001; \) ref. 0.0001, and \( P < 0.01, \) respectively; Supplementary Figs. S5A–S5C), whereas gastric cancer patients \( (n = 876); \) overall survival with higher TLR5 expression levels showed decreased survival \( (P < 0.02; \) Supplementary Fig. S5D).

Indeed, overall TLR5 expression profiles significantly associated with NME3 expression profiles for breast \( (n = 3,951); \) lung \( (n = 1,926); \) ovarian \( (n = 1,435); \) and gastric \( (n = 876); \) cancer patients (Pearson residual; Fig. 7A–D). Of interest, higher expression of \( NF-kB1 \) \( (P < 0.0001); \) correlated with better survival for breast cancer \( (n = 3,951); \) relapse-free survival; \( P < 0.0001; \) Supplementary Fig. S6A), but not for lung \( (n = 1,926); \) overall survival; ref. 37), ovarian \( (n = 1,435); \) progression-free survival) or gastric cancer \( (n = 876); \) overall survival; ref. 0.11, and \( P < 0.27, \) respectively; Supplementary Fig. S6B–S6D). However, low, median, and high \( NF-kB1 \) expression was significantly associated with NME3 and TLR5 expression profiles of breast \( (n = 3,951); \) lung \( P < 0.0001; \) and \( n = 3,951, \) \( P < 0.0001); respectively; lung

Figure 5.

NME3 regulates TLR5-induced NFκB signaling. A, NME3 and MyD88 knockdown inhibits NFκB signaling. HCT116 cells stably expressing \( \gamma B_{A,B} F_{L u c} \) and transfected with the indicated heterogeneous shRNA sequences were stimulated with heat-killed Salmonella and imaged for reporter activity every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). Inserts, quantitative PCR verifies knockdown of NME3 (top) or MyD88 (bottom) mRNA in corresponding shRNA-treated HCT116 cells. B–D, Targeting NME3 by shRNA reduces NFκB responsiveness. B, HCT116 cells were subjected to lentiviral knockdown with the indicated shRNA constructs and transfected with the \( \gamma B_{A,B} F_{L u c} \) plasmid. Cells were then stimulated with heat-killed Salmonella and imaged for reporter activity every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). C, HCT116 cells were subjected to lentiviral knockdown with the indicated shRNA constructs and transfected with the \( \gamma B_{A,B} F_{L u c} \) plasmid. Imaging was performed at 0, 2, 4, 6, and 7 hours following stimulation with Salmonella. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle). Error bars represent SD with propagated error. D, Semi-quantitative PCR verifies knockdown of NME3 mRNA in shRNA-expressing HCT116 cells. GAPDH mRNA levels are shown as a control. Primer dimers are observably cut at the bottom of the gel (right). E, NME3 over-expression (insert) enhances NFκB transcriptional activation. HCT116 cells stably expressing \( \gamma B_{A,B} F_{L u c} \) were transfected with the indicated plasmid constructs, stimulated with heat-killed Salmonella and imaged for reporter activity every 5 minutes for 6 hours. Data are displayed as normalized photon flux values (fold-initial, fold-vehicle).
Salmonella effects (7, 8, 10). Furthermore, recent studies have shown that high and low expression groups (using top and bottom 25% as the cutoff for grouping). NME3 mRNA levels correlate with overall patient survival in various cancers. Gehan–Breslow–Wilcoxon test were used to estimate the P values between high and low expression groups (using top and bottom 25% as the cutoff for grouping). A, Kaplan–Meier survival analysis in breast cancer patients (n = 3,951) indicates that high NME3 expression correlates with longer patient survival (P < 0.0001). B, Kaplan–Meier survival analysis of lung cancer patients (n = 1,926) indicates that high NME3 expression correlates with longer patient survival (P < 0.0001). C, Kaplan–Meier survival analysis of ovarian cancer patients (n = 1,435) shows overall no significant difference (P < 0.22) in survival between patients with higher or lower NME3 expression levels. D, Higher NME3 expression in gastric cancer patients (n = 876) significantly correlates (P < 0.002) with poorer survival.

Discussion

Salmonella engagement of TLR5 and activation of NFkB serves primarily to alert the host of invading pathogens, which is accomplished by activating specific signaling pathways, release of cytokines and recruitment of host immune cells to the local microenvironment. Agonists of TLRs have been actively pursued for their antitumor potential, and in this context, coopting evolutionarily conserved pathways for tumor colonization by Salmonella has been explored due to the capacity of the bacterium to specifically localize to tumors in vivo with minimal host side-effects (7, 8, 10). Furthermore, recent studies have shown that Salmonella flagellin provides potent antitumor effects capable of suppressing tumor growth (7–13). Taken together, these data provide a basis for development of a Salmonella-based treatment strategy taking advantage of TLR5-mediated activation of NFkB following tumor-specific flagellin delivery.

Herein, using HCT116 colon carcinoma cells, we demonstrated that stimulation of TLR5 with flagellated Salmonella robustly activated proinflammatory NFkB signaling in a tumor cell-autonomous manner, and that the bacterial flagellin, and not other known immunostimulatory moieties, were necessary for this proinflammatory signaling event. Note that our results stand in contrast with another study (15) that reported that stimulation in vitro with bacterial flagellin (FlaB) failed to elicit a NFkB response in HCT116 cells stably expressing firefly luciferase. At least two possible differences may account for this discrepancy. First, HCT116 cell lines are not all isogenic. However, in our experiments, we utilized two different HCT116 cell lines stably carrying different NFkB reporter constructs and both responded concordantly. Also, the TLR5 stimulus was different. We used either heat-killed Salmonella or flagellin derived from Salmonella as a TLR5 stimulus, whereas the other study (15) used flagellin derived from Vibrio vulnificus.
HCT116 cells have previously been shown to express TLR5, but not TLR2 and TLR4 (51), suggesting an important distinguishing attribute of this system. TLR2 and TLR4 have been shown to be predominantly protumorigenic compared with the noted antitumorigenic properties of the flagellin–TLR5 pathway (52–55). Furthermore, TLR5 is the only known TLR family member to bind flagellin. Therefore, with HCT116 cells, we were able to utilize a siRNA high-throughput screen to isolate the contribution of individual host kinases to the regulation of TLR5-mediated NFκB signaling specifically in response to bacterial flagellin.

Multiple kinases linked to the NFκB signaling pathway were identified as modulators of TLR5-induced reporter activity in the screen. Notably, IRAK1, a kinase central to TLR signal transduction, was revealed in the screen as a positive regulator at 45 minutes and this correlated with its known role in IKK activation (56). At 245 minutes, AKT was identified by the screen as a positive regulator of NFκB, and indeed, AKT has been shown to contribute to full NFκB activation and to promote nuclear NFκB transactivation (57, 58). Although siRNA-mediated knockdown of both MAP2K2 and MAP2K3 gave reproducible modulation of NFκB in our screen, targeting known downstream MAP kinases with chemical inhibitors showed no consistent effect in this system. This could be explained by a model wherein the identified kinases act on other downstream proteins, as opposed to their typical MAP kinase targets, in the context of flagellin-induced signaling. Recent work identified MAP kinases as important modulators of NFκB-induced cytokine production in intestinal epithelial cells with constitutively active NFκB, further supporting our observation (59). Because intestinal cancers often display high levels of active NFκB, MAPK activation in these cells may be
required for full inflammatory-mediated NFκB transcriptional activation (60). Perhaps this effect is the underlying reason for the seemingly important contribution of MAP2K2 and MAP2K3 to TLR5-induced signaling in HCT116 colon carcinoma cells seen herein. In this regard, it has been recently reported that TLRs represent upstream effectors of a nucleotide receptor signaling network, specifically, one lying upstream of MAP kinase Erk1/2 activation (26).

Despite the above confirmation hits, numerous other kinases with expected functions in the NFκB pathway did not appear as hits in our high-throughput screen. In these instances, such as IKKβ, there are several possible reasons for lack of detection. First and foremost, such kinases may be more specific to other modes of NFκB signaling not relevant to Salmonella-induced signaling. Second, as has been established with published screens (e.g., ref. 61), not all physiologically important kinases necessarily appear as high-stringency hits, revealing the complexity of systems and their regulation. Third, the specific kinase may be expressed at high levels or possess such a long half-life that siRNA knockdown was inadequate to reduce protein levels sufficiently to affect signaling. And alternatively, for parallel pathways, host cells may have compensating collateral mechanisms to cope with loss of one kinase, thus preventing any phenotypic change.

Herein, targeted siRNA sequences against the nucleotide diphosphate kinase NME3 had a dramatic effect on downregulating TLR5-induced NFκB reporter activity. NME3 is one of eight human nucleotide diphosphate kinase genes (44). These genes are capable of utilizing ATP to form non-ATP NTPs through their catalytic kinase domain, but also have been attributed with a catalytic kinase, thus preventing any phenotypic change. However, the knockdown of these genes was inadequate to reduce protein levels sufficiently to affect signaling. And alternatively, for parallel pathways, host cells may have compensating collateral mechanisms to cope with loss of one kinase, thus preventing any phenotypic change.

In this context, these data further support the significance of NME3 as a negative regulator of NFκB signaling. Indeed, a CDK4/6 inhibitor has been recently approved by the FDA for treatment of breast cancer (41). Given that NFκB is a downstream effector of TLR5 signaling, it is not surprising that NFXB1 expression was significantly associated with NME3 and TLR5 expression profiles in breast, lung, ovarian, and gastric cancer patients. However, the finding that high NFXB1 expression correlated with enhanced survival for breast cancer patients may reflect the context-dependent differences in NFXB1 signaling inputs between these cancers. Alternatively, but not mutually exclusively, differences in mutational landscape among these cancers may contribute to these differences. Furthermore, six NME3 nonsense mutations were identified in various cancer types predicted to have a high impact on protein function (68, 69). Five of these mutations reside within or adjacent to the predicted active site of NME3 based on conserved NME1 and NME2 active site domains (Supplementary Fig. S8A and S8B; ref. 70). Collectively, our data and past studies indicate that NME3 is a putative tumor suppressor and may evoke these functions via enhancement of NFκB signaling downstream of TLR5 activation.

In summary, these results shed light on the downstream signaling mechanisms that govern the recently characterized antitumor effects of flagellin-mediated TLR5 signaling. For this work, we utilized HCT116, a cell line whose NFκB pathway specifically responds to flagellin and not other bacterial components, such as LPS, peptidoglycan, and NOD ligands, which negates the contribution of other TLR signaling components to our system. A live cell high-throughput screen to identify individual candidate kinases mediating the tumor cell-autonomous antitumor effects of flagellin identified several kinases not previously recognized, in particular, NME3. Future studies will further determine the mechanisms of how NME3 reinforces the antitumor activity of the TLR5-induced NFκB pathway in tumor cells and provide insight to optimize cancer immunotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K. Flentie, C. Gonzalez, B.A. Kocher, D. Prewicza-Worms
Development of methodology: K. Flentie, C. Gonzalez, B.A. Kocher, D. Prewicza-Worms
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Flentie, C. Gonzalez, B.A. Kocher, J. Marasa
References
Nucleoside Diphosphate Kinase-3 (NME3) Enhances TLR5-Induced NF κB Activation

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