TNF1 Promotes Tumor Necrosis Factor-mediated Mouse Colon Epithelial Cell Survival through RAF Activation of NF-κB

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Tumor necrosis factor (TNF) is a therapeutic target in the treatment of inflammatory bowel disease; however, the exact role of TNF signaling in the colon epithelium remains unclear. We demonstrate that TNF activation of TNF receptor (R)1 stimulates both pro- and anti-apoptotic signaling pathways in the colon epithelium; however, TNFR1 protects against colon epithelial cell apoptosis following TNF exposure. To investigate anti-apoptotic signaling pathways downstream of TNFR1, we generated an intestinal epithelial-specific Raf knock-out mouse and identified Raf kinase as a key regulator of colon epithelial cell survival in response to TNF. Surprisingly, Raf promotes NF-κB p65 phosphorylation, independent of MEK signaling, to support cell survival. Taken together, these data demonstrate a novel pathway in which Raf promotes colon epithelial cell survival through NF-κB downstream of TNFR1 activation. Thus, further understanding of colon epithelial cell-specific TNFR signaling may result in the identification of new targets for inflammatory bowel disease treatment and define novel mediators of colitis-associated cancer.

In the gastrointestinal tract, increased levels of tumor necrosis factor (TNF) promote the pathogenesis of several diseases, including inflammatory bowel disease (IBD) (1, 2), celiac disease (3), graft-versus-host disease (4), and nonsteroidal anti-inflammatory drug enteropathy (5). Clinical studies indicate that circulating serum levels of TNF are increased in Crohn disease patients (6), and neutralizing TNF monoclonal antibodies have been shown to reverse disease activity in IBD patients (7, 8), further supporting the pathogenic role of TNF in the colon epithelium. The exact role of TNF signaling in the colon epithelium remains unclear as TNF overexpression causes inflammatory bowel disease in a mouse model (9), whereas mice deficient in TNF production develop worse colitis in response to dextran sulfate sodium than their wild type counterparts (10). Therefore, further study is required to delineate the role of TNF signaling in the colon epithelium.

TNF binds two cell surface receptors as follows: TNF receptor (R)1 (p55/60) or TNFR2 (p75/80) (reviewed in Refs. 11, 12), with a higher ligand affinity for TNFR2 (13, 14). Each receptor promotes different cellular responses in a cell context-dependent manner (15, 16). In the colon epithelium, TNF signals through TNFR2 to promote proliferation (17) and migration (18), whereas sustained activation of mitogen-activated protein kinase (MAPK) signaling through TNFR1 induces growth arrest (19). In contrast, in intestinal myofibroblasts, TNFR2 stimulates proliferation through MAPK activation (20). Although it is known that TNFR1 can promote apoptosis through death domain-dependent signaling (21), several reports indicate that TNF promotes anti-apoptotic signaling pathways in a cell context-dependent manner (22–26). Considering this single ligand induces signaling of both cell survival and cell death, cell-specific signal transduction regulation must occur to determine at which point a cell irreversibly progresses toward apoptosis.

TNF stimulation is insufficient to induce apoptosis in a number of cell types, demonstrating a complex balance between cell survival and apoptotic pathways. For example, in the colon epithelial cells, TNF-induced anti-apoptotic signaling requires the serine/threonine kinase, kinase suppressor of Ras (KSR), which promotes MAPK, Akt, and NF-κB activation. Loss of KSR shifts cells toward an apoptotic program following TNF exposure (22). These findings suggest that during the maintenance of epithelial homeostasis, TNF tightly modulates the apoptotic response by inducing both pro- and anti-apoptotic pathways.

Raf-1, the only well characterized substrate of KSR (22, 27, 28), is a serine/threonine kinase that regulates several cellular functions from proliferation and differentiation to survival (29).
Raf Promotes Cell Survival through TNFR1

Canonical Raf activation through receptor tyrosine kinase signaling is well characterized (30); however, little is known about the mechanism of Raf activation in cytokine signaling. A requirement for Raf in cell survival was demonstrated in Raf knock-out mice, which are embryonic lethal because of Fas-mediated hepatocellular apoptosis and placental defects (31, 32). Conditional tissue-specific Raf deletion shows that Raf is required for both cardiomyocyte, through the regulation of apoptosis signal-regulating kinase 1 (ASK1) (33), and macrophage survival following Salmonella typhimurium infection (34). Furthermore, in inducible intestinal epithelium-specific Raf knock-out mice (Raf \(^{flx/flx}\); villin-Cre ERT2), Raf enhances colon epithelial cell survival during acute colitis through a nuclear factor-κB (NF-κB)-dependent mechanism (35).

TNF is a potent inducer of NF-κB, which regulates cell survival and the production of inflammatory cytokines (36). NF-κB p50/p65 dimers are sequestered in the cytoplasm through binding interactions with inhibitor of κB (IκB). TNF induces phosphorylation of IκB by the IκB kinases (IKK), leading to IκB ubiquitination and proteasomal degradation and subsequent NF-κB nuclear translocation. In the nucleus, NF-κB is phosphorylated and functions as a transcription factor to induce expression of both pro- and anti-apoptotic target genes, including Bcl family members and inhibitor of apoptosis proteins (37).

Although NF-κB promotes both cell survival and cell death in a cell context-dependent manner (36), conditional inactivation of the NF-κB pathway shows a requirement for NF-κB in colon epithelial cell survival following inflammatory stimuli (38, 39).

In this study we show that TNFR1 is required for colon epithelial cell survival and anti-apoptotic signaling following exposure to TNF. Our data indicate that TNF stimulation promotes activation of Raf in colon epithelial cells via a novel Ras-independent mechanism. Through the generation of an intestinal epithelium-specific knock-out mouse (Raf \(^{KO}\)B), we provide the first evidence that Raf expression is required for TNFR-induced cell survival both in vivo and in vitro. Although inhibition of the Raf kinase target, MEK, had no effect on TNF-stimulated cell death, blockade of a novel Raf-1 target, NF-κB activation, resulted in increased apoptosis. Consistent with this observation, constitutive activation of NF-κB rescues mouse colon epithelial cells from TNF-induced apoptosis in the absence of Raf. Interestingly, Raf promoted TNF-induced NF-κB p65 phosphorylation and target gene expression in vivo, suggesting that Raf mediates colon epithelial cell survival through activation of NF-κB in a MEK-independent manner downstream of TNFR1.

Experimental Procedures

Mice and TNF Injections—TNFRI, TNFRII knock-out (KO), and TNFR double knock-out mice were a gift from Jacques Peschon (Amgen). Raf intestinal epithelium-specific knock-out (Raf \(^{KO}\)B) mice were generated by crossing Raf \(^{flx/flx}\) mice (gift from Manuela Baccarini, University of Vienna, Austria) and villin-Cre mice (gift from Sylvie Robine, Curie Institute, Paris, France). Mice were genotyped as described previously (34, 40). All mice were maintained on a C57BL/6 background, and these mice were used as wild type controls for all TNFR KO mouse experiments. At 6 weeks of age, mice were injected intraperito-
times indicated. MEK inhibitors PD98059 (20 ng/ml) or U0126 (10 ng/ml) were obtained from Calbiochem.

**Generation of TNFR1 Addback Colon Epithelial Cells—**TNFR1 addback cells were generated by infection of TNFR1 KO colon epithelial cells with a green fluorescent protein-containing LZR5 retrovirus containing vector alone or an HA-tagged mouse TNFR1 sequence. TNFR1 cDNA was generated by amplifying total RNA isolated from YAMC cells with TRIzol reagent (Invitrogen) with a TaqMan reverse transcription-PCR oligo(dT) kit (Applied Biosystems) using 5'-TGT CAA TTG CTG CCC TGT C-3' and 5'-GGG CAT CTA GCA GAA TGG TC-3' primers. A C-terminal HA epitope tag was added to TNFR1 using 5'-ATG TCA TGG GTC TCC CCA CCG TGC C G-3' and 5'-GTCAGG CAT AGT CTG GGA CGT CAT ATG GAT ATC GTC CCG GGA GGC GGG TCG TG-3' primers. Following ligation into pGEM-T-easy vector (gift from Albert Reynolds, Vanderbilt University, Nashville, TN), digested with EcoRI, and treated with calf intestinal phosphatase (Promega). Empty vector or vector containing the HA-TNFRI construct was transiently transfected using Lipofectamine 2000 (Invitrogen) into Phoenix ecotropic viral packaging cells (43). Virus-containing supernatant was harvested and placed on TNFR1 KO cells for 6 h, after which the media were replaced with regular culture media. The infection protocol was repeated for 3 consecutive days and then sorted based on green fluorescent protein expression to obtain empty vector or HA-TNFRI stably integrated cell populations.

**Cell Lysates, SDS-PAGE, Western Blot Analysis—**Cell lysates were prepared by scraping cells in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% phosphatase and protease mixtures) (Sigma). Epithelial and stromal fractions were isolated from mice by manually shaking 1-cm pieces of colon or small intestine following incubation in Cell Recovery Solution (BD Biosciences) at 4 °C overnight. Lysis buffer was added separately to liberated crypts pelleted by centrifugation and to the remaining stroma. Cell lysates were boiled in Laemmli sample buffer, run on 10 or 15% SDS-PAGE, and transferred onto nitrocellulose membranes. Polyclonal antibodies used for Western blot analysis include phosho-MAPK (ERK1/2) (Promega), phospho-MEK, MEK, phospho-p38, phospho-JNK, IκBα, active caspase-3, active caspase-9, cleaved poly(ADP-ribose) polymerase (Cell Signaling), Raf-1, B-Raf, NF-κB p65 (Santa Cruz Biotechnology), and HA (Invitrogen). Monoclonal antibodies used include actin (Sigma) and Ras (Upstate). Anti-rabbit and mouse horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) were used when appropriate.

**Activation Assays—**YAMC cells were treated with TNF (100 ng/ml) or EGF (10 ng/ml) for the times indicated. Activated Ras was precipitated from whole cell lysates with agarose conjugated to the Ras-binding domain of Raf (Raf-RBD), which binds the active GTP-bound form of Ras with high affinity (Upstate). For Raf kinase assays, endogenous Raf was immunoprecipitated from whole cell lysate and washed once in 1 M NaCl and twice with PBS. Kinase assays were performed with Raf-1 kinase assay cascade kit according to manufacturer's directions (Upstate).

**Statistical Analyses—**For each experiment, the analysis of variance model was applied to test if there were any differences in the primary outcome variable. The overall effect was tested at 5% level to control the overall type I error rate for the experiment. Pairwise comparisons were warranted only when there was an overall effect. The reported p values are unadjusted for multiplicity, and they are all below adjusted type I error rate for each experiment except for Fig. 1D. When the normality assumption did not seem to hold (Fig. 1D), a nonparametric Kruskal-Wallis test was used instead. Each individual data point with the horizontal bar at the group mean are shown in the figures. All hypothesis tests were two-sided, and all data analyses were performed using R2.6.0 (44).

**RESULTS**

**TNFR1 Is Required for Cell Survival in the Presence of TNF—**Previous findings from our laboratory indicate that TNFR2 promotes proliferation and migration responses in vitro, whereas TNFR1 activation inhibits both of these processes (18). To further delineate the role of TNFR1 in the intestinal epithelium, wild type, TNFR1 KO, TNFR2 KO, and TNFR1/2 double knock-out mice were injected intraperitoneally with PBS or TNF (10^4 units). After 24 h, mice were sacrificed, and apoptosis was assessed by ISOL. TNFR1 knock-out mice exhibited a significant increase in apoptosis following TNF treatment compared with wild type (Fig. 1, A and B, supplemental Fig. 1) (p < 0.01). Neither TNFR2 KO nor double knock-out mice showed enhanced TNF-induced apoptosis.

These findings were confirmed in a TNFR1 KO cell culture model. TNFR1 KO colon epithelial cells were either mock-infected or infected with retrovirus expressing HA-tagged TNFR1, and then treated with TNF (100 ng/ml). Following 6 h of TNF exposure, TNFR1 KO cells showed higher levels of apoptosis compared with the untreated control and TNFR1 add-back cells treated with TNF (Fig. 1, C and D). To investigate the signaling pathways underlying this response, TNFR1 KO and addback cells were exposed to TNF for 15 min. Western blot analysis of whole cell lysates demonstrated that in the absence of TNFR1, TNF-induced activation of pro- and anti-apoptotic signaling pathways was blocked; however, re-expression of TNFR1 in these cells restored activation of these signaling pathways (Fig. 1E). Activation of caspase-3 and -9 were detected in TNFR1 KO cells following longer TNF exposure, and caspase activation was absent in cells re-expressing the receptor (Fig. 1F). These data indicate that TNFR1 is required for acute TNF stimulation of both pro- and anti-apoptotic signaling in colon epithelial cells at the times studied, but in the absence of TNFR1 the balance of TNF signaling shifts toward cell death.

**TNFR2 Promotes Both Apoptosis and Proliferation in Response to TNF Treatment in Vivo—**The individual TNFR receptors demonstrate opposing functions in the colon epithelium, and TNFR2 promotes cell proliferation and migration, whereas TNFR1 inhibits both of these processes (18). As shown in Fig. 1, A and B, loss of TNFR1, but not TNFR2, resulted in increased susceptibility to TNF-induced colon epithelial cell apoptosis. To address the requirement for TNFR2 in cell proliferation in vivo, wild type, TNFR1, and TNFR2 KO mice were injected intraperitoneally with PBS or TNF for 24 h, and prolif-
FIGURE 1. TNFR1 mediates colon epithelial cell survival signaling. Wild type (wt), TNFR1, TNFR2, and TNFR double knock-out mice (DKO) were treated with TNF for 24 h. Apoptosis was assessed by ISOL staining (scale bar = 62 μm) (A) and quantified as the number of positive cells per 100 crypts (B). Bars represent the mean value. C, TUNEL staining of TNFR1 KO colon epithelial cells and TNFR1 addback cells treated with TNF for 6 h. (Scale bar = 100 μm). D, graphical representation of the percentage of TUNEL-positive cells. E, Western blot analysis of TNFR1 knock-out cells expressing vector or TNFR1 treated with TNF for 15 min. Whole cell lysates were blotted for phospho-MAPK, IκB, phospho-Akt, phospho-p38, phospho-JNK, and actin. F, Western blot analysis was performed on TNFR1 knock-out cells expressing vector or TNFR1 following 30 min and 6 h of TNF exposure for cleaved poly(ADP)-ribose polymerase (PARP) and active caspase-3 and -9. Total caspase-9 was used as a loading control.
Raf Promotes Cell Survival through TNFR1

To confirm that TNF promotes Raf activity in our system, an in vitro kinase assay was performed with endogenous Raf immunoprecipitated from YAMC cells following TNF or EGF treatment. Raf isolated from TNF-stimulated cells phosphorylated recombinant MEK in vitro, although to a lesser extent than with EGF treatment (Fig. 4). Taken together, these data demonstrate that TNF promotes MAPK activation in a Ras-independent manner.

TNFR-induced Cell Survival Is Raf-dependent—Because Raf knock-out mice are embryonic lethal (31), we generated conditional Raf knock-out mice by crossing mice harboring a Raf floxed allele (34) with those expressing a Cre transgene under control of a villin promoter (35). Western blot analysis was performed on epithelial and stromal fractions isolated from wild type and Raf KOIE mice colon to confirm loss of Raf expression (Fig. 5A). Raf-1 was absent from the colon epithelium but not the underlying stroma, indicating that Raf ablation is specific to the epithelium. Knockdown of Raf-1 had no effect on expression of B-Raf, another Raf isoform expressed in the colon.

To determine whether Raf is required for cell survival in the presence of TNF in vivo, we treated wild type and Raf KOIE mice with PBS or TNF for 24 h and performed ISOL staining to detect apoptotic cells in the colon epithelium. Following 24 h of TNF treatment, Raf KOIE mice exhibited increased apoptosis compared with wild type (Fig. 5, B and C) (p < 0.01), which was comparable with levels of apoptosis detected in TNF-treatedTNFR1KO mice (Fig. 1B). Similar findings were obtained by immunostaining for active caspase-3 (data not shown).

RNA interference was used in vitro to confirm the role of Raf in colon epithelial cell survival in response to TNF. Nontargeting or Raf siRNA duplexes were transfected into YAMC cells, and Western blot analysis indicated that Raf siRNA was specific to Raf-1 and had no effect on B-Raf expression (Fig. 5D). Following 6 h of TNF treatment, siRNA-transfected YAMC cells were incubated with a cell-permeable, fluorescent-tagged activated Ras was determined by Ras-GTP pulldown assay. Western blot analysis from the pulldown indicated that EGF, but not TNF, strongly stimulated Ras activation, suggesting that Ras is not involved in TNF activation of MAPK. To test this, YAMC cells were transfected with a dominant-negative H-Ras construct (S17N), which blocks the access of Ras guanine nucleotide exchange factors (47). Consistent with Ras activation patterns, expression of dominant-negative Ras blocked EGF- but not TNF-stimulated ERK activation (Fig. 3B). These findings demonstrate a novel mechanism by which ERK is activated in a Ras-independent manner.

NF-induced Cell Survival Is Raf-dependent—Because Raf knock-out mice are embryonic lethal (31), we generated conditional Raf knock-out mice by crossing mice harboring a Raf floxed allele (34) with those expressing a Cre transgene under control of a villin promoter (40) to specifically delete Raf from the intestinal epithelium. These mice develop normally and unchallenged mice do not display a detectable intestinal phenotype (35). Western blot analysis was performed on epithelial and stromal fractions isolated from wild type and Raf KOIE mice colon to confirm loss of Raf expression (Fig. 5A). Raf-1 was absent from the colon epithelium but not the underlying stroma, indicating that Raf ablation is specific to the epithelium. Knockdown of Raf-1 had no effect on expression of B-Raf, another Raf isoform expressed in the colon.

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Raf Promotes Cell Survival through TNFR1

Although MEK, the kinase directly upstream of MAPK in the canonical signaling cascade, is the best characterized downstream target of Raf activation, novel substrates for Raf are being investigated (49). To address whether TNF-mediated anti-apoptotic signaling requires MAPK signaling, YAMC cells were pretreated with two MEK inhibitors, PD98059 (20 μM) or U0126 (10 μM), followed by 6 h of TNF treatment. Western blot analysis showed that both inhibitors effectively blocked MAPK phosphorylation after 10 min of TNF stimulation (Fig. 6A). However, IκB degradation was independent of MAPK activation, demonstrating that MAPK activation is not required to promote NF-κB signaling in response to TNF in mouse colon epithelial cells. Consistent with this result, TNF did not stimulate apoptosis as detected by TUNEL in the presence of either MEK inhibitor compared with a phosphoinositide 3-kinase inhibitor, wortmannin (42) (Fig. 6B). Taken together, these data indicate that Raf promotes cell survival in the presence of TNF through a MEK-independent mechanism.

MEK Activation Is Not Required for Cell Survival Downstream of TNFR1—As shown in Fig. 1E, TNF stimulates MAPK activation in mouse colon epithelial cells through TNFR1. (7A). In nontargeting siRNA-transfected cells, TNF promotes nuclear translocation of NF-κB after 30 min; however, knockdown of IκB expression resulted in constitutive NF-κB p65 nuclear translocation as shown by immunocytochemistry (Fig. 7B). siRNA-transfected cells were treated with TNF for 6 h, and apoptosis was detected by multicaspase activation assay (Fig. 7C). TNF stimulated apoptosis in the absence of Raf or NF-κB p65; however, constitutive activation of NF-κB through inhibition of IκB expression protected colon epithelial cells from TNF-induced apoptosis in the absence of Raf expression (p < 0.01).

caspase inhibitor (SR-VAD-fluoromethyl ketone) to detect cells actively undergoing apoptosis. TNF-induced apoptosis was significantly increased in the absence of Raf (Fig. 5, E and F), similar to our findings in the in vivo model (p < 0.01). These data were confirmed by TUNEL assay (data not shown). Thus, Raf protects against TNF-induced apoptosis in colon epithelial cells both in vivo and in vitro.

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FIGURE 5. Raf protects against TNF-stimulated colon epithelial cell apoptosis. A, epithelial and stromal fractions were isolated from colons of wild type (wt) and Raf KOIE mice and subjected to Western blot analysis for Raf, B-Raf, and actin. B, ISOL was performed on colon sections from wild type and Raf KOIE mice treated with PBS or TNF. (Scale bar = 125 μm.) C, graph represents the number of ISOL-positive cells per 100 colonic crypts. Bars represent the mean value. D, Western blot analysis was performed on whole cell lysates of nontargeting and Raf siRNA-transfected YAMC cells following TNF treatment for 6 h. Caspase activation is shown in red and DAPI-stained nuclei in blue. (Scale bar = 20 μm.) F, number of caspase-positive cells is represented.

FIGURE 6. Anti-apoptotic signaling downstream of TNF receptors is MEK-independent. A, YAMC cells were pretreated with DMSO, PD98059, or U0126 for 30 min followed by TNF for 10 min or EGF for 5 min. Western blot analysis was performed to detect phosho-MAPK, IκB, and actin. B, TUNEL assay was performed in YAMC cells pretreated with DMSO, PD98059, U0126, or wortmannin for 30 min and then treated with TNF for 6 h. Percent TUNEL-positive cells are shown. Bars represent the mean value.
Raf Promotes Cell Survival through TNFR1

To determine whether Raf expression is required for TNF-induced NF-κB activation in vivo, wild type, TNFR1 KO, and Raf KOIE mice were injected with PBS or TNF for 1 or 2 h, and immunohistochemistry was performed to detect phosphorylated NF-κB p65 (S276) on colon sections (Fig. 8A). TNF treatment at both time points stimulated increased NF-κB p65 phosphorylation in wild type but not TNFR1 KO or Raf KOIE mice (Fig. 8B) \((p<0.001, p=0.02, p=0.03)\). Furthermore, Raf was required for increased expression of NF-κB-induced increases in cIAP1, IκB, and COX-2 protein expression in the colon epithelium following 24 h of TNF exposure (Fig. 8 and supplemental Fig. 2) \((p=0.03)\). cIAP1 inhibits apoptosis by preventing activation of both pro-form and mature caspasases (21), whereas IκB expression is up-regulated as part of a feedback response that inhibits further NF-κB signaling (50). TNF promotes NF-κB-induced COX-2 expression (51), and COX-2 protects intestinal epithelial cells from apoptosis (52, 53). These data indicate that Raf is required for activation of anti-apoptotic NF-κB target genes to promote cell survival downstream of TNFR1.

DISCUSSION

In this study, we demonstrate an essential role of Raf-1 in cell survival in response to inflammatory cytokines. Furthermore, TNFR1 is required for ERK and NF-κB activation and colon epithelial cell survival in response to TNF stimulation both in vitro and in vivo. We have previously shown that KSR promotes anti-apoptotic signaling, and we now demonstrate that Raf-1, a KSR substrate, is also required for cell survival in response to TNF. Interestingly, inhibition of the Raf substrate MEK did not induce apoptosis in TNF-treated colon epithelial cells; however, loss of NF-κB signaling resulted in increased cell death. Furthermore, the loss of Raf inhibits TNF-induced NF-κB phosphorylation and NF-κB-induced expression of cIAP1, IκB, and COX-2. Taken together, these data demonstrate Raf promotes colon epithelial cell survival through NF-κB downstream of TNFR1 activation.

In contrast to EGF, TNF stimulates Raf activation in a Ras-independent manner (Figs. 3 and 4). Interestingly, previous reports have shown that Ras is not required for Raf-mediated MAPK signaling involved in calcium-induced differentiation pathways in keratinocytes (54). In addition, lipopolysaccharide-stimulated macrophage differentiation occurs through Ras-independent, protein kinase C, or phosphatidylcholine-specific phospholipase C activation (55). KSR directly interacts with Raf at the plasma membrane (22, 56), and thus we propose in the absence of Ras activation, KSR promotes recruitment of Raf to the membrane. Following membrane translocation, TNF-induced KSR kinase activity is required for Raf threonine phosphorylation and subsequent MAPK activation in colon epithelial cells (57).

Although Raf is best characterized with regard to MEK activation, our data clearly indicate a MEK/ERK-independent role for Raf signaling. Although inhibition of KSR kinase activity toward Raf results in decreased MAPK activation (57), we now show that the TNF-mediated cell survival response does not require MEK signaling (Fig. 6). Similar findings were reported for anti-apoptotic signaling in the cardiac-specific Raf knockout mouse, where deletion of Raf resulted in increased ASK1 activity in a MEK-independent manner (33). However, Raf knockdown in YAMC cells by siRNA shows no change in the phosphorylation of ASK1 or other pro-apoptotic kinases such as JNK or p38 (data not shown).

The Raf mutant (T481A) activates an NF-κB reporter plasmid independent of MEK (58), consistent with our findings that MEK inhibition does not block TNF-induced NF-κB activation (Fig. 7). Taken together, these data provide the first evidence that Raf mediates NF-κB activation through a novel MEK-independent mechanism in intact cells.

We have shown that the absence of KSR impairs TNF-stimulated NF-κB nuclear translocation both in vitro and in vivo (22, 57, 59). Surprisingly, NF-κB p65 nuclear translocation following TNF treatment of colon epithelial cells does not require Raf (Fig. 7B); however, Raf expression is necessary for TNF-stimulated NF-κB p65 serine 276 phosphorylation in vivo (Fig. 8B). Thus, KSR promotes NF-κB nuclear translocation...
through canonical pathways upstream of Raf-mediated NF-κB p65 phosphorylation through Raf-independent targets. These data demonstrate independent roles of KSR and Raf in NF-κB nuclear translocation and phosphorylation.

NF-κB p65 subunit phosphorylation on Ser-276 in the Rel homology domain enhances DNA binding as well as the interaction between p65 and CBP/p300 (60, 61). Overexpression of a p65 S276A mutant in p65 KO mouse embryonic fibroblasts shows that TNF-induced interleukin-6 expression and cell survival require phosphorylation at this site (62). Further analysis of the requirement for Ser-276 phosphorylation in NF-κB transcriptional activation indicated that the phosphorylation state of p65 may target NF-κB to distinct cis-acting elements to activate gene expression in a cell context-dependent manner (63).

These studies raise the important question of whether Raf can directly phosphorylate NF-κB p65 at serine 276. Although protein kinase A can phosphorylate this site (60), it has been demonstrated that mitogen and stress-activated kinase-1 (MSK1) phosphorylates Ser-276 in the nucleus following TNF stimulation (64). Vanden Berghe et al. (65) showed that the upstream activators of MSK1, p38 and ERK MAPK, were required for NF-κB activation in TNF-treated fibroblasts; however, our results demonstrate that ERK MAPK is not required for TNF-induced NF-κB activation. Therefore, further investigation is necessary to determine the exact mechanism by which Raf stimulates NF-κB p65 phosphorylation in the colon epithelium following TNF exposure.

Raf promotes NF-κB p65 Ser-276 phosphorylation in dendritic cells following activation of a c-type lectin, DC-SIGN, in a protein kinase A- and MSK1-independent manner (66), indicating that Raf may activate NF-κB in additional cell types. Similar to our findings, Raf promotes Ser-276 phosphorylation in dendritic cells following canonical NF-κB nuclear translocation through TLR4. Our data demonstrate a novel regulatory mechanism through which Raf promotes TNF-stimulated NF-κB p65 Ser-276 phosphorylation independent of MEK activation.

Based on our current results and previous reported findings, we propose a model (Fig. 9) in which binding of TNF to TNFR1 promotes recruitment of FAN (factor-associated with neutral sphingomyelinase (N-SMase) activation) (67) to the N-SMase domain of TNFR1 (68). N-SMase hydrolyzes sphingomyelin in the plasma membrane to increase intracellular ceramide production (67, 69). Ceramide can then bind to and activate KSR, which phosphorylates Raf on threonine residues in response to TNF and EGF treatment (27, 28, 57, 70–72). Through an unknown mechanism, Raf enhances NF-κB Ser-276 phosphorylation in a MEK-independent manner to protect against colon epithelial cell apoptosis.

Our results position Raf and NF-κB as important factors supporting cell survival signaling downstream of TNFR1, consist-
ent with the notion of NF-κB being a key target in response to inflammation. Mice with a deletion of the IKKγ/NEMO subunit in the intestinal epithelium are more susceptible to TNF-induced apoptosis. These findings support our model by demonstrating that the loss of an anti-apoptotic signaling molecule downstream of TNFR1, such as NF-κB, Raf, or KSR, disrupts intestinal homeostasis by down-regulating anti-apoptotic signaling and shifts cells toward an apoptotic program (38). NEMO KO mice develop spontaneous colitis because of decreased barrier integrity, whereas crossing these mice into a TNFR1 KO background rescues the colitis phenotype. Although the NEMO KO/TNFR1 KO mice show no sign of decreased proliferation in response to colitis. Taken together, these findings show that Raf functions as a novel Ras-independent mechanism, likely involving KSR based on our prior results. Furthermore, Raf promotes NF-κB phosphorylation independent of MAPK signaling to support cell survival. In the absence of Raf, TNF stimulation of TNFR1 results in increased apoptosis, which may lead to disruption of epithelial barrier function and mucosal inflammation. These data demonstrate an important role for Raf in the maintenance of intestinal homeostasis. Further understanding the role of Raf in cytokine signaling in the colon epithelium under noninflammatory conditions may lead to the development of novel therapeutics for the treatment of IBD.

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