RAF-1 KINASE REGULATES INTESTINAL EPITHELIAL CELL SURVIVAL IN RESPONSE TO PRO-INFLAMMATORY STIMULI

By

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To those that possess courage and strength in the face of great odds,

and succeed because of those challenges

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APC – antigen presenting cell

ASK1 – apoptosis signal-regulating kinase

CR – conserved region

CRD – cysteine-rich domain

DAPI – 4',6-diamidino-2-phenylindole

DD – death domain

DSS – dextran sulfate sodium

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ERK – extracellular signal-regulated kinase

FADD – Fas-associated death domain

FBS – fetal bovine serum

H&E – hematoxylin and eosin

IAP – inhibitor of apoptosis protein

IBD – inflammatory bowel disease

IFN – interferon

I□B – inhibitor of □B

IKK – I□B kinase

IL – interleukin

i.p. – intraperiteoneal

ISOL – *in situ* oligo ligation

JNK – c-Jun N-terminal kinase

KO – knockout

KO^{IE} – intestinal epithelium-specific knockout

KSR – kinase Suppressor of Ras

MAPK – mitogen-activated protein kinase

NF-□B – nuclear factor □B

NSD – neutral sphingomyelinase domain

PBS – phosphate buffered saline

RBD – Ras binding domain

RHD – Rel homology domain

RTK – receptor tyrosine kinase

S – serine

siRNA – small interfering RNA

T – threonine

TNF – tumor necrosis factor

TNFR – tumor necrosis factor receptor

TRADD – TNFR-associated death domain

TRAF – TNFR-associated factor

TUNEL – terminal UTP-nucleotide end labeling

Y – tyrosine

YAMC – young adult mouse colon

CHAPTER I

INTRODUCTION

A single layer of epithelial cells serves as the sole interface between the organism and the contents of the gastrointestinal tract, underlining the importance of regulating cellular viability in a luminal environment full of pathogens, toxins and cytokines. A balance between cellular proliferation and apoptosis is necessary for maintenance of this critical barrier. The molecular mechanisms by which intestinal epithelial cells are able to survive in this environment and how loss of normal regulatory processes may lead to inflammatory bowel disease (IBD) are just beginning to be understood. The experiments described in this thesis will examine the molecular mechanism by which Raf kinase mediates colon epithelial cell survival in response to injury and inflammatory stimuli, including dextran sulfate sodium (DSS) colitis and tumor necrosis factor (TNF), respectively. First, the structure and function of Raf in cell signaling pathways will be presented followed by a brief summary of cell signaling involved in apoptosis downstream of TNF activation. Lastly, the contribution of these cell survival signaling pathways and colon epithelial apoptosis to the pathogenesis of IBD will be reviewed.

Raf family of kinases

In 1983, Ulf Rapp first identified oncogenic *v-Raf* (named for *ra*pidly growing *f*ibrosarcoma), the gene responsible for murine sarcoma virus 3611-mediated transformation (1). The same year, another group identified *v-mil*, a similar gene sequence capable of inducing transformation in the naturally occurring avian retrovirus MH2 (2). Soon afterwards the cellular homolog, c-Raf or Raf-1, was mapped to mouse chromosome 6 (3), and human chromosomes 3 and 4 (4). Together, these groups identified *v-raf* as the first oncoprotein with serine/threonine kinase activity (5).

Raf isoforms and knockout mice

Identification of the cellular counterparts to *v-raf* and *v-mil* resulted in the discovery of the Raf family of kinases, consisting of three mammalian isoforms, A-Raf, B-Raf, and Raf-1 (6). Raf is a serine/threonine protein kinase that serves as a key regulator of cell proliferation, differentiation, and survival. Whereas Raf-1 is ubiquitously expressed, A-Raf is most highly expressed in muscle and urogenital tissues, and B-Raf is primarily located in the brain and nervous system (7). Although the structure and function of the Raf kinases are highly conserved, studies using knockout mice for each Raf isoform have demonstrated that Raf family members are not functionally redundant in adult mice (8-11). A-Raf knockout (KO) mice are viable; however, at days 7-21 post partum these mice display various neurological and enteric nervous system defects depending on the genetic background (8). B-Raf KO mice die at embryonic day 10.5-12.5 due

to vascular hemorrhage induced by endothelial apoptosis (9). Further studies demonstrated that loss of B-Raf inhibits VEGF production resulting in decreased angiogenesis in the placenta (12). Raf-1 KO mice are also embryonic lethal due to placental defects and increased hepatocellular apoptosis (13). The generation of B-Raf and Raf-1 double KO mice demonstrated that Raf expression is required for embryonic development past the two-cell stage and that both isoforms are functionally redundant for germ layer specification during the first half of embryonic development (14). Of the three isoforms, Raf-1 has been the most extensively studied and characterized both in structure and in function.

Structure of Raf-1 kinase

Although the precise sequence of events governing Raf activation remains unclear, protein binding and phosphorylation of multiple residues including S259, S338, Y340/341, and S621 are required for Raf kinase activity. The importance of this complex regulation of Raf is demonstrated in oncogenic Raf variants, which are constitutively active due to the lack of appropriate regulatory mechanisms.

All Raf isoforms contain three conserved structural and functional domains. The amino terminal domains consist of conserved regions (CR) 1 and 2, which through intramolecular interactions inhibit the activity of the carboxy-terminal kinase domain (CR3) (Figure 1.1)(6). The inhibition of the

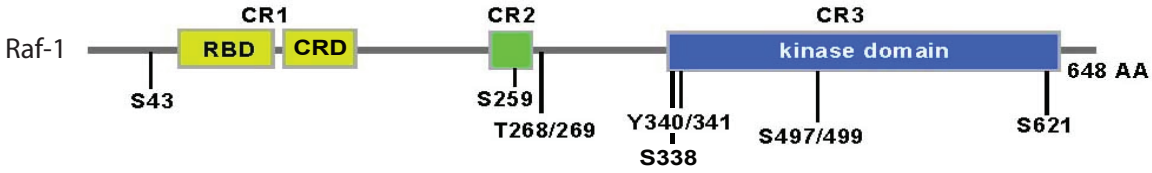

Figure 1.1: Schematic representation of Raf-1 functional domains and phosphorylation sites. Raf is a serine/threonine kinase containing three conserved regions. CR1 contains a Ras-binding domain and a cysteine-rich domain, CR2 is a serine/threonine-rich region, and CR3 contains the kinase domain. Serine (S), threonine (T), and tyrosine (Y) phospho-sites are indicated.

Raf kinase domain by the N-terminal region prevents the phosphorylation of Raf substrates in the absence of appropriate stimuli. Oncogenic variants of Raf contain deletions of the N-terminal domain, resulting in constitutive activation of the kinase domain and increased transforming capacity (15,16).

Interaction with the small GTPase Ras and changes in Raf phosphorylation state relieve the conformational inhibition of the kinase domain. The CR1 domain contains two distinct regions, a Ras binding domain (RBD) and a cysteine-rich domain (CRD) (17,18). The effector domain Ras possesses high affinity for the RBD (amino acids 51-131), and deletion of this region abolishes Ras/Raf interaction (19-23). A second Ras binding domain on Raf, located within the CRD domain of CR1 (amino acids 139-184), becomes unmasked once Ras binds to the RBD. This shift relieves the autoinhibition of the C-terminal domain allowing activating kinases to access and phosphorylate multiple residues necessary for kinase activation (24).

In addition to serving as a secondary Ras binding site, the Raf CRD contains a zinc finger domain, which possesses significant sequence homology to the C1 domains of protein kinase C (PKC) and kinase suppressor of Ras (KSR) (25,26). This domain in Raf, KSR, and PKC has been implicated in binding ceramide and other membrane lipids including phosphatidylserine (27,28), which suggests that this protein-lipid interaction may be required for kinase activation.

Another key regulatory domain is CR2, a serine-threonine rich domain containing one of two serine residues recognized by the 14-3-3 family of proteins.

Although Raf is constitutively associated with 14-3-3, phosphorylation of S259 induces a conformational change in the Raf/14-3-3 interaction, effectively sequestering Raf in an inactive conformation in the cytoplasm (29). Dephosphorylation of this serine enables kinase activation by relaxing the autoinhibitory interaction between Raf N- and C-terminal domains. Although the mechanism by which S259 is dephosphorylated remains unknown, subunits of protein phosphatase 2A (PP2A) have been identified in a complex with Raf (30,31).

Several kinases phosphorylate Raf to promote kinase activation. Yao et al. (1995) reported that Raf-1 is a substrate of KSR through the use of an in gel kinase assay in which activated KSR from ceramide- or sphingomyelinase-treated HL-60 cells increased Raf activity toward its substrate MEK. Tryptic digestion and phosphopeptide analysis of KSR-stimulated Raf indicated that KSR phosphorylates Raf on threonines 268 and 269. Although KSR was unable to activate a Raf T268/269V mutant *in vitro*, mutation of these sites to alanine showed that T269 was the primary site of KSR phosphorylation (32), demonstrating that T269 was a significant target residue for activation by KSR.

In addition to T269, several of the molecule's activating phosphorylation sites are located in the catalytic domain, CR3. Phosphorylation of Y340/341 by Src-family kinases is required for kinase activation, and mutation of these residues to aspartate results in constitutive activation and increased transforming capacity (33). Phosphorylation of S338 by p21-activated kinase (PAK) kinases (34) cooperates with Src phosphorylation of Y340/341 (33,35) to promote optimal

activation of Raf kinase (36). As a second 14-3-3 recognition site, phosphorylation of S621 may prevent Raf dephosphorylation by protein phosphatases (37). S621 may be an essential autophosphorylation site since it is phosphorylated in both resting and active states (29,38). Mutation of this residue to alanine completely abolishes Raf kinase activity (29).

In summary, activation of Raf kinase is dependent on multiple regulatory events to ensure appropriate activation of downstream signaling pathways. However, it is important to consider that the majority of the observations concerning requirements for Raf activation were made in *in vitro* model systems using overexpression of recombinant proteins. Future studies analyzing endogenous Raf kinase activation will provide further insight into the mechanism by which Raf is activated in response to a broader variety of stimuli in specific cell types.

Raf-mediated signaling pathways

Canonical Raf activation has been primarily characterized through receptor tyrosine kinase activation of mitogen-activated protein kinase (MAPK). However, recent literature indicates that Raf may play an important role in cell survival signaling downstream of tumor necrosis factor (TNF) receptor (R) family members, including Fas and TNFR1. The requirement for Raf in cell signaling and cellular functions will be discussed further in this section.

Activation of MAPK

As an upstream component of the MAPK cascade (Figure 1.2), Raf functions as a MAP kinase kinase kinase, which phosphorylates and activates a dual-specificity MAP kinase kinase, MEK (MKK1). MEK phosphorylates ERK MAPK, which can then translocate to the nucleus to promote transcriptional activation of several genes such as activator protein-1 (AP-1) and ELK-1 (39-42). ERK also regulates cytoplasmic targets involved in cell migration including paxillin (43) and components of microtubule assembly (44).

ERK activation plays an important role in cell proliferation through the induction of nucleotide synthesis, chromatin phosphorylation, activation of transcription factors, and promotion of cyclin D/CDK4 complex formation (45-47). The diverse functions of MAPK signaling were first described in PC12 neuroendocrine cells [reviewed in (48)], in which nerve growth factor (NGF) promotes sustained ERK activation resulting in differentiation, whereas transient ERK stimulation by epidermal growth factor (EGF) induces cell proliferation (49).

In addition to being an integral component of the MAPK cascade, Raf has been shown to function outside of the Ras/Raf/MAPK pathway. Studies using a Raf construct that is unable to bind to MEK (T481A) demonstrated that only a subset of cellular functions, including cell growth and transformation, are impaired when Raf is uncoupled from MEK activation; however, Raf-mediated nuclear factor (NF)-□B reporter activation and neurite differentiation are unaffected (50).

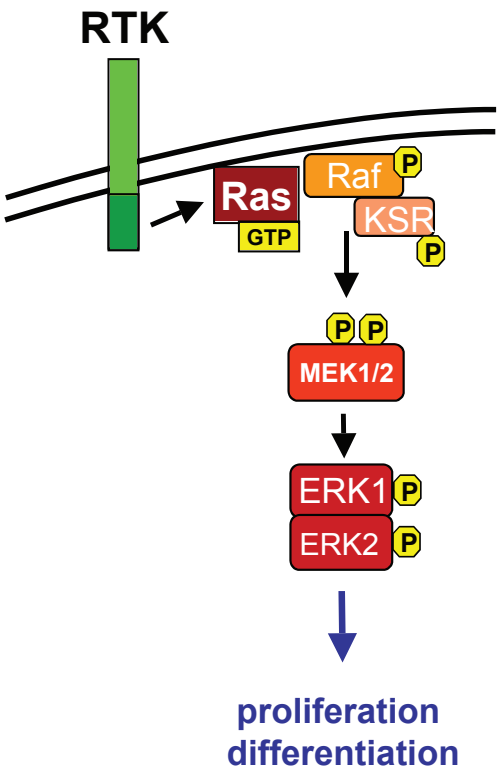

Figure 1.2: Model of canonical MAPK signaling.
Receptor tyrosine kinase (RTK) signaling promotes activation of Ras-GTPase, which recruits Raf to the plasma membrane through a high affinity binding interaction in the N-terminus of Raf. Subsequent phosphorylation of Raf by kinase suppressor of Ras (KSR) and other kinases, promotes its kinase activity toward MEK, and MEK then phosphorylates ERK MAPK, which can activate cytoplasmic targets or translocate to the nucleus to promote transcription of AP-1 or ELK. In this model, KSR functions as both a kinase to phosphorylate Raf and a scaffold to bring Raf, MEK and ERK in close proximity to one another.

Raf in cell survival

Following the initial identification of *v-raf*, the discovery that the Raf oncogene could cooperate with Myc to promote tumorigenesis was one of the first indications of Raf involvement in cell survival (51). Whereas oncogenic Myc promotes cell death, expression of an activated Raf (gag-*v*-Raf) postpones cell death following growth factor removal in an interleukin (IL)-3-dependent promyeloid 32D cell line (52).

Similar observations were made in Raf-1 knockout mice which are embryonic lethal due to increased hepatocellular apoptosis (11). Further studies investigating the mechanism by which Raf regulates cell survival demonstrated that Raf plays an essential role in inhibiting Fas-induced apoptosis by regulating Fas expression and internalization (53,54). In fibroblasts, Raf antagonizes activation of the pro-apoptotic kinase, MST2, (55) indicating that the mechanism by which Raf blocks Fas-induced apoptosis varies with cell type.

Due to the embryonic lethality of Raf KO mice, conditional knockout mice have been generated to better address the role of Raf in specific cell types. Using Cre/loxP technology, Raf has been shown to be anti-apoptotic in cardiomyocytes through the regulation of apoptosis signal-regulating kinase 1 (ASK1), an upstream activator of c-Jun N-terminal kinase (JNK) and p38 MAPK (56). In macrophage-specific Raf knockout mice, Raf was shown to protect macrophages from Salmonella-induced apoptosis through inhibition of caspase-1 (57). *In vitro* studies have demonstrated that Raf-1 blocks intrinsic apoptotic pathways through binding Bcl-2 at the mitochondrial membrane and directly

phosphorylating Bad to release Bcl-2 from inhibition (58-60). Interestingly, these studies demonstrated a novel mechanism through which Raf promotes cell survival independent of MEK/ERK activation.

In regard to the colon epithelium, KSR, an upstream activator of Raf, is a key regulator of cell survival in response to TNF both *in vivo* and *in vitro*. KSR knockout mice and colon epithelial cells expressing a dominant-negative kinase-inactive KSR or a KSR antisense construct show increased sensitivity to TNF-induced apoptosis. Furthermore, disruption of KSR kinase activity inhibits TNFR activation of ERK MAPK and NF-□B, while pro-apoptotic signaling through p38 and JNK pathways is not impaired. Since Raf has been reported to be a downstream effector of KSR kinase activity, these findings suggest that KSR activation of Raf may promote anti-apoptotic signaling in response to TNF stimulation (61,62).

Taken together, these studies illustrate the cell type-dependent manner in which Raf promotes cell survival in response to various stimuli. Both cell culture models and tissue-specific Raf knockout mice have shown that Raf can inhibit apoptosis in several tissue types. In addition, Raf activation of different cell survival signaling pathways is independent of MEK activation.

Cell signaling involved in apoptosis

Cell death occurs through several processes including: necrosis, apoptosis, anoikis and autophagy. During necrosis, the cytoplasmic and nuclear content of cells is released into the intercellular space resulting in inflammation

[reviewed in (63)]. However, in 1972, Kerr et al. demonstrated that cells undergo an evolutionarily conserved, programmed cell death. This genetically controlled form of cell death was termed apoptosis, based on the Greek word for “a falling off” (64). In multicellular organisms, apoptosis functions to remove senescent, damaged or redundant cells by breaking down cell components into apoptotic bodies, which are phagocytosed by macrophages (65). Apoptosis occurring after loss of anchorage to the extracellular matrix is termed anoikis (66). Autophagy, a process by which cells sequester organelles and cytoplasmic contents for delivery to lysosomes, can either promote or protect from cell death depending on the stimulus (67). Further investigation is required to determine under what conditions extensive autophagy results in cell killing, or whether autophagy contributes to known cell death processes such as apoptosis and necrosis. This section will focus on the initiation of apoptosis in response to a variety of cellular cues, through the activation of intrinsic pathways from intracellular signals or by an extrinsic pathway responsive to extracellular events.

Intrinsic apoptosis

Intrinsic apoptosis occurs following DNA damage or toxic stress, in which mitochondria release cytochrome c from their intramembrane space (Figure 1.3). Mitochondrial membrane permeability is controlled by both pro- and anti-apoptotic members of the Bcl-2 family of proteins, all of which contain at least one conserved Bcl-2 homology domain (BH) [reviewed in (68)]. Bax and Bad

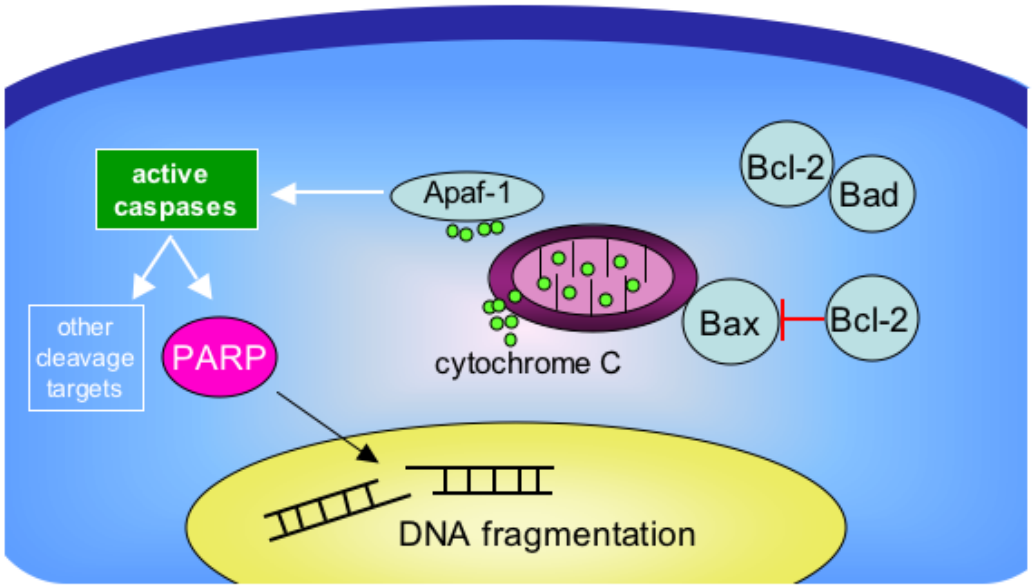

Figure 1.3: Intrinsic apoptotic pathways
Intracellular signals, such as DNA damage or genotoxic stress, induce apoptosis through activation of pro-apoptotic Bcl-2 family members which promote cytochrome c release from the mitochondria. Cytochrome c binds to and activates Apaf-1, which stimulates caspase activation. Caspases cleave various cellular substrates including endonucleases, such as poly ADP-ribose polymerase (PARP), leading to DNA fragmentation.

oligomerize with additional pro-apoptotic BH-3 motif-containing proteins to promote cytochrome c release. Located at the outer mitochondrial membrane, Bcl-2 and Bcl-Xl can bind to these BH-3 containing proteins to inhibit pore formation and cytochrome c release, thereby inhibiting apoptosis. Once cytochrome c is released, it binds to apoptotic protease-activating factor (Apaf-1) promoting activation of cysteine proteases (caspases), which target several proteins essential for cellular function (69,70). Inhibitors of apoptosis proteins (IAPs), can prevent apoptosis via selective inhibition of specific caspases, allowing these proteins to block both intrinsic and extrinsic apoptotic pathways (71).

Extrinsic apoptosis – TNF receptor family

Extrinsic apoptotic signaling occurs following activation of death receptors belonging to the tumor necrosis factor receptor family. TNF was first described in 1975 as a potent, pro-inflammatory cytokine expressed by several cell types, such as macrophages, T-cells, B-cells, and natural killer T-cells (NKT) (72). The TNF family of ligands include lymphotoxin-□ (LT-□), Fas ligand, receptor activator of nuclear factor kappa B ligand (RANKL), CD40L and TNF-related apoptosis-inducing ligand (TRAIL), all of which are homotrimeric and share some sequence homology primarily within residues responsible for trimerization (73). TNFR1, TNFR2 and Fas are among several receptors for TNF family ligands (74). Both TNFR1 and Fas contain death domains within the cytoplasmic portion of the receptor, whereas TNFR2 does not (75). Recruitment of several adaptor

proteins, including TNF receptor-associated death domain (TRADD), Fas-associated death domain (FADD), TNFR associated factor-2 (TRAF2) and receptor interactive protein (RIP) (76-79) to the death domain of TNFR1 activates pro-apoptotic signaling pathways (Figure 1.4). Each adaptor protein promotes apoptosis through different pathways: RIP induces NF-□B activation, FADD stimulates caspase-8 and 3 activation (80), and TRAF2 activates several MAPK pathways (81).

In addition to inducing apoptotic signaling, TNFR1 can also promote cell survival pathways. While TRAF2 stimulates p38 and SAPK/JNK to promote apoptosis, TRAF2-mediated ERK activation is anti-apoptotic in response to TNF in several cell types (81). Similarly, RIP-induced NF-□B activation stimulates cell survival or apoptosis in a cell-context dependent manner (82). The role of NF-□B in cell survival will be further addressed in the next section. In summary, TNFR1 can stimulate both pro- and anti-apoptotic signaling pathways dependent upon the cell type.

While the role of TNFR1 and its associated adaptor proteins in apoptotic signaling is well-established, the role of TNFR2 in cell death remains unclear. Although there is no death domain in TNFR2, the cytoplasmic tail of the receptor contains a TRAF-interacting domain, which recruits TRAF2 and cIAP to the receptor resulting in NF-□B activation (83). However, overexpression of TRIP (TRAF-interacting protein) prevents TRAF2-mediated NF-□B activation leading to apoptosis (84). A recent report suggests that TNFR2 may induce apoptosis in

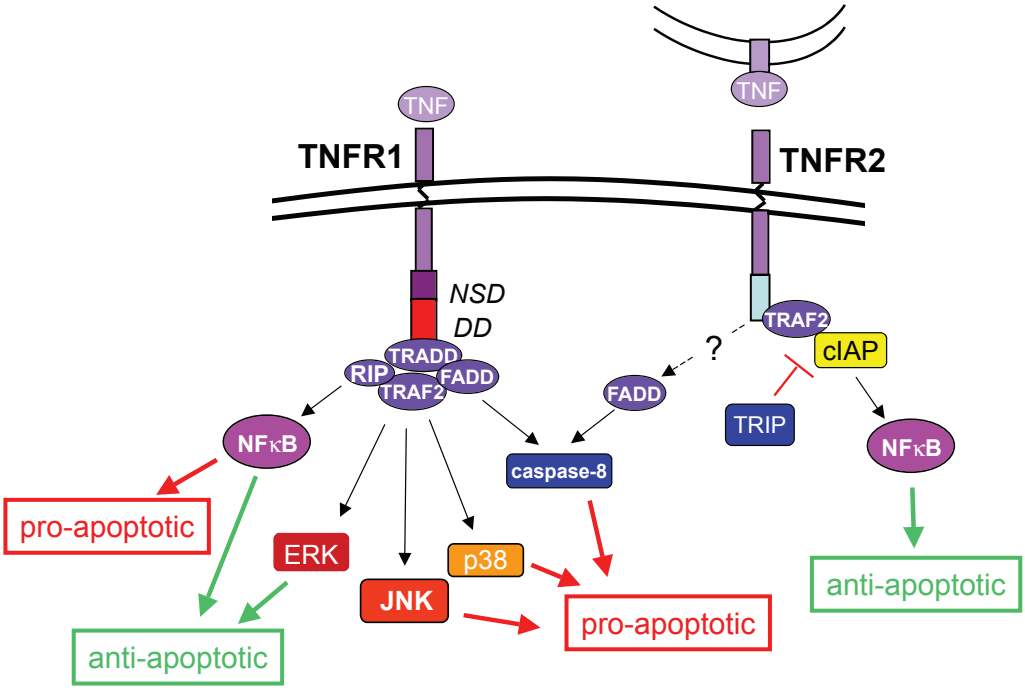

Figure 1.4: TNF receptors stimulate both pro- and anti-apoptotic signaling pathways. TNFR1 promotes apoptotic signaling through recruitment of adaptor proteins to the death domain (DD). RIP, TRAF2, and FADD stimulate downstream signaling pathways to induce cell death. However, activation of ERK and NF-κB is anti-apoptotic in some cell types. Since TNFR2 lacks a death domain, the role of TNFR2 in apoptosis remains unclear. TNFR2 is thought to induce cell death through binding of membrane-bound TNF or through a TRAF2-interacting domain in the cytoplasmic tail. TRAF-interacting protein (TRIP) has been shown to promote apoptosis by inhibiting TRAF2/cIAP interactions in response to TNFR2 stimulation.

antigen-presenting cells after binding to membrane-bound TNF (85). Other groups have demonstrated a requirement for TNFR2 in T-cell apoptosis through activation of FADD and downregulation of TRAF2 (86,87). Therefore, these data indicate that each TNF receptor can independently induce pro-apoptotic signaling.

NF-κB signaling

NF-κB is a significant mediator of inflammatory responses and a regulator of target genes involved in cell proliferation, apoptosis, and oncogenesis. The five NF-κB family members, p65/RelA, RelB, NF-κB1 (p50/p105), NF-κB2 (p52/p100), and c-Rel share a Rel homology domain (RHD) involved in dimerization, DNA-binding, and association with IκB inhibitory proteins (88). Of these family members, the NF-κB p65/p50 heterodimer is the most prevalent. In resting cells, binding to IκB in the cytoplasm masks the nuclear localization signal of NF-κB dimers. TNFR signaling promotes activation of two MAP kinase kinase kinases, NF-κB-inducing kinase (NIK) and MEKK1, which in turn activate the IκB kinases (IKKs) (Figure 1.5). The IKK complex contains two catalytic IKKα/β subunits and a regulatory subunit, IKKγ (NEMO) (89-91). IKK phosphorylates IκB on serines 32 and 36 leading to IκB poly-ubiquitination by the E3 ubiquitin ligase complex (92-94). Degradation of IκB by the 26S proteasome releases NF-κB for translocation to the nucleus (95).

NF-κB regulates gene activation by binding to the promoter region of genes encoding several cytokines, chemokines, adhesion molecules, and other

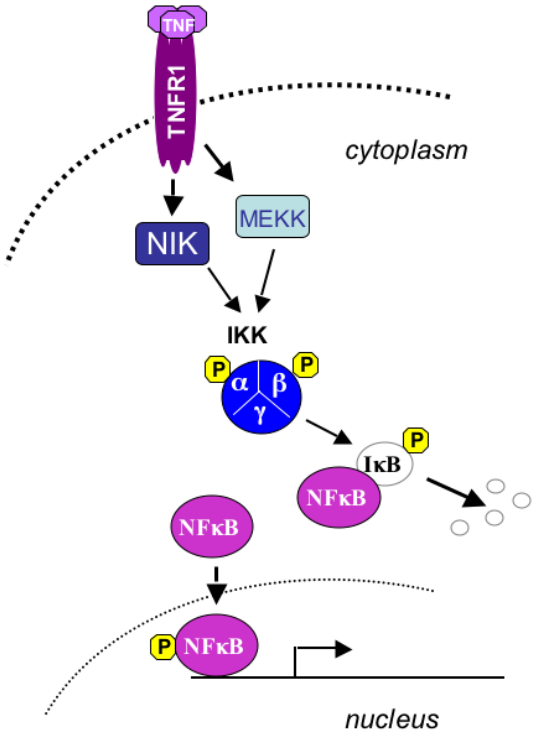

Figure 1.5: NF-κB signaling pathway
TNFR1 activates MAPKKKs, NF-κB-inducing kinase (NIK) and MEKK, to phosphorylate IκB kinases (IKK). The catalytic IKKα and β subunits phosphorylate IκB promoting its ubiquitination and proteasomal degradation. Degradation of IκB allows NF-κB to translocate to the nucleus, where it is subsequently phosphorylated and promotes transcription of genes involved in pro- and anti-apoptotic signaling.

pro-survival proteins including IAPs. The effect of NF-κB activation on apoptosis is largely dependent on cell type. In lymphocytes, NF-κB promotes cell death by working in conjunction with AP-1 to induce FasL expression (96), while in the intestinal epithelium NF-κB potently induces inflammatory responses and inhibits cell death (97,98).

The importance of NF-κB activation in preventing apoptosis was demonstrated in p65 KO mice (99). Similar to the phenotype seen in Raf-1 KO mice, p65 KO animals show increased apoptosis in the liver and were highly sensitive to pro-inflammatory stimuli. In most cells, TNF stimulation alone is unable to induce cell death; however, TNF treatment is sufficient to induce apoptosis in p65 KO fibroblasts (100).

In summary, the biological outcome of signaling through TNFR is highly dependent upon cell type and context. While TNFR signaling can promote apoptosis in certain cell types, it remains unclear as to why some cell types are more susceptible to TNF-induced apoptosis compared to others. Furthermore, little is understood as to how or why TNFR1 activates both pro- and anti-apoptotic signaling in a single cell. Similar questions arise in regard to NF-κB activation, which promotes transcription of genes involved in both cell survival and cell death. These questions are important because both TNF and NF-κB are critical mediators of intestinal epithelial cell survival and apoptosis, both in the context of maintaining intestinal homeostasis and in response to inflammation.

The intestinal epithelium

The gastrointestinal epithelium is a dynamic tissue characterized by a high cellular turnover rate leading to renewal of the entire intestinal epithelium every 3 to 5 days (101). This unique system is maintained by a balance of intestinal epithelial proliferation, differentiation and apoptosis. This interplay is already established early in vertebrate development as the endoderm is converted from a stratified cell layer into a differentiated intestinal epithelial monolayer.

Intestinal architecture

The mature intestinal epithelium consists of rapidly proliferative, columnar epithelial cells located in crypts, from which cells differentiate and move up the crypt-villus axis. To maintain a constant number of cells and a consistent epithelial architecture, apoptosis serves as a mechanism to counterbalance rapid proliferation (Figure 1.6)(101). Under normal conditions, colon epithelial cell apoptosis occurs at the surface epithelium and is associated with cell shedding into the intestinal lumen (102). Beneath the epithelium is the lamina propria, a layer of loose connective tissue containing blood vessels, fibroblasts, and lymphoid tissues. Immune cells within the lamina propria provide immune surveillance and enable host defense mechanisms in response to foreign antigens. Together, the epithelium and the lamina propria serve as a barrier and coordinated defense against bacteria and other toxins present in the intestinal

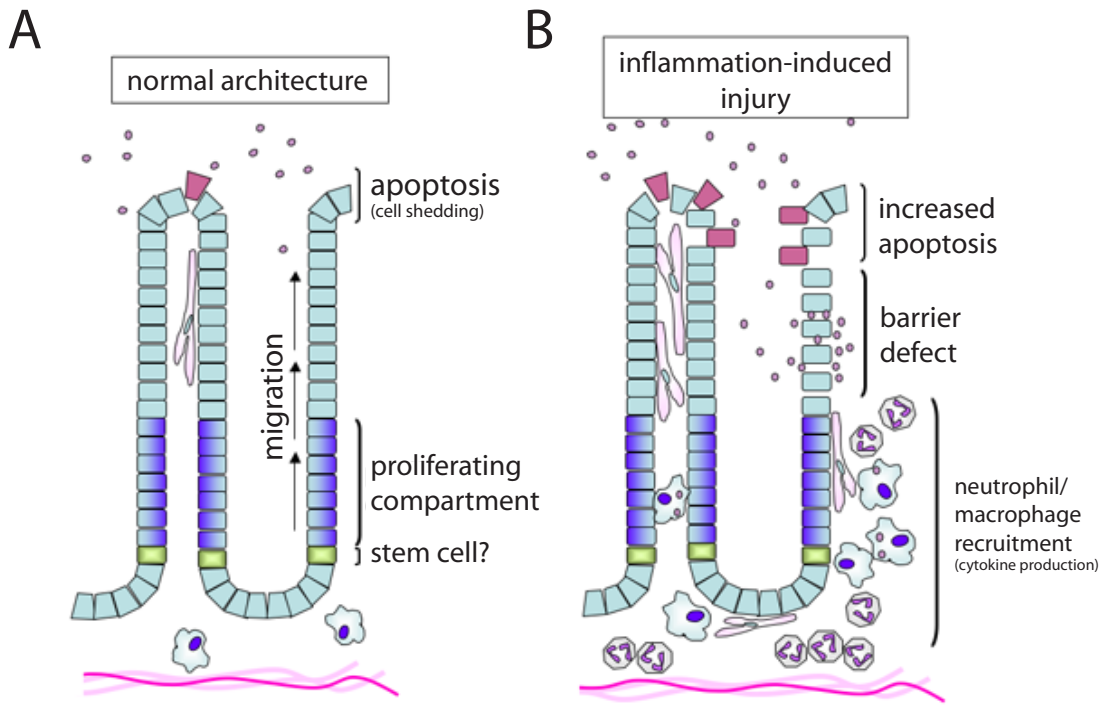

Figure 1.6: Colon epithelial crypt architecture under normal and inflamed conditions. (A) Cells migrate from the base of the crypt toward the surface epithelium where cells undergo apoptosis and are shed into the lumen. (B) During inflammation-induced injury, epithelial apoptosis is increased, resulting in decreased barrier integrity. This barrier defect allows migration of pathogens from the lumen into the lamina propria leading to recruitment of neutrophils and macrophages. Pro-inflammatory cytokines produced from immune cells can further inhibit barrier function and induce cell death, leading to cyclical inflammation and epithelial damage.

lumen. Defects in epithelial integrity and dysregulation of host immune responses lead to the development of gastrointestinal diseases.

Inflammatory bowel disease

The major forms of inflammatory bowel disease (IBD) are Crohn's disease and ulcerative colitis. Ulcerative colitis is characterized by extensive, superficial mucosal ulcerations extending proximally from the rectum. Increased numbers of neutrophils are present in the lamina propria and crypts. In contrast, Crohn's disease primarily affects the ileum, although it may occur anywhere in the gastrointestinal tract, and is characterized by an accumulation of macrophages often resulting in transmural inflammation.

Although the cause of IBD remains unknown, three factors are known to contribute to the pathogenesis of these chronic inflammatory diseases, including defects in barrier function, dysregulation of adaptive and innate immunity, and impaired microbe recognition (Figure 1.6).

When the epithelial barrier is compromised, the immune system promotes inflammatory cytokine production and upregulation of host responses to the enteric microbiota (103). The current understanding of function and contribution of each immune cell type in the pathogenesis of IBD is described in Table 1.1. Innate immune cells such as neutrophils and macrophages depend on cytokines, chemokines, and adhesion molecules for recruitment to the site of injury to amplify the local immune response (104). This increased production of cytokines and chemokines can result in loss of tight junction stability, leading to a further

Table 1.1: Immune cell function in IBD

Type of immune cell		Function	Involvement in IBD	ref
Neutrophils		Phagocytose bacteria and produce cytokines	Exhibit an extended lifespan due to defective apoptosis; cause tissue damage through release of non-specific inflammatory mediators	(105)
Macrophages		1. Regulate inflammatory responses to bacteria and antigens 2. Scavenge dead cells and foreign debris	Enhance abnormal inflammatory response to luminal antigens	(106)
Dendritic cells		Primary APC to prime naïve T-cells	1. Prime abnormal T-cell responses to flora 2. Maintain T-cell inflammatory responses by remaining in contact with T-cells in inflamed regions resulting in increased cytokine production	(107)
T-cells	CD4+	Regulate suppression of immune responses during homeostasis and upregulate inflammatory response	Produce sustained levels of pro-inflammatory cytokines	(105)
	Regulatory T-cells (Tregs)	1. Maintain immune homeostasis in gut and self-tolerance 2. Suppress T-cell proliferation	Decreased levels in IBD; IL-10 and TGF-□ suppress pro-inflammatory cytokine production	(108)
	Natural Killer T-cells (NKT)	1. Cytolytic activity through secretion of perforin and granzyme 2. Produce Th2 cytokines directly or through regulation of conventional T-cells	1. Cytolytic activity directed toward intestinal epithelial cells resulting in injury 2. Cytolytic activity toward dendritic cells to decrease immune responses 3. Increase Th2 cytokine production	(109)
B-cells		Produce antibodies to bacterial and non-bacterial antigens	Loss of tolerance to commensal bacteria	(105)

disruption of epithelial integrity. While the innate immune system promotes a rapid response to bacterial and luminal antigens, adaptive immunity is necessary to establish a sustained inflammatory response to following continuous epithelial damage.

Components of the adaptive immune system also play an important role in chronic inflammation. Dendritic cells are involved in both innate and adaptive immunity, and are recognized as the dominant antigen-presenting cell in the lamina propria. Live imaging studies show that dendritic cells form networks under the epithelium and extend long processes between crypts to sample the intestinal flora (110). In the absence of inflammation, dendritic cells constitutively express IL-10, which suppresses TNF production (111). Several mouse models of colitis demonstrate a requirement for B-cell and regulatory T-cell function in response to chronic inflammation [reviewed in (103)]. Therefore, cells involved in innate and adaptive immunity are necessary to maintain intestinal homeostasis and protect from bacterial infection when the epithelial barrier is compromised.

TNF and NF-□B signaling in IBD

TNF is a primary mediator of chronic inflammation in IBD. Increased levels of TNF are recovered from serum and stool of patients (112,113), and in situ hybridization in colon sections showed increased TNF mRNA expression in macrophages (114). Importantly, neutralizing monoclonal antibodies against TNF reverse disease activity and induce remission in some IBD patients (115,116).

Both innate and adaptive immune cells produce increased levels of pro-inflammatory cytokines including TNF and interferon-γ, which can promote epithelial apoptosis (117). Increased apoptosis is seen throughout the length of the colonic crypt in IBD patients (118,119). In a study by Marini et al., anti-TNF antibody treatment reduces epithelial cell apoptosis and Fas/CD95 expression in the intestinal epithelium of Samp1/Yit mice, which develop spontaneous ileitis (120). Furthermore, lamina propria mononuclear cell apoptosis is increased resulting in decreased chronic inflammation in these mice. These findings demonstrate an important role for TNF signaling in the pathogenesis of IBD, by regulating host immune responses, barrier function, and intestinal epithelial cell survival.

Among known intracellular signaling pathways downstream of TNF, IKK inhibition has been suggested as a therapeutic target for IBD (121). Elevated NF-κB activation is commonly seen in inflamed colon mucosa of IBD patients (122), prompting investigations into the role of NF-κB activation during intestinal epithelial damage. In fact, 5-aminosalylic acid (5-ASA), an effective therapy for ulcerative colitis, inhibits IKK activity in intestinal epithelial cells (123,124). Furthermore, use of an IKK inhibitor peptide, which blocks association of IKKα and with IKKγ/NEMO, reduced the severity of colitis and production of pro-inflammatory cytokines (125). However, complete deletion of IKKα in the intestinal epithelium does not protect against colitis (126), indicating that NF-κB could be a therapeutic target in one cell type but not in another, complicating the utility of generalized NF-κB targeting in IBD. However, the continued study of

NF-□B is still a key to understand the mechanism and identify potential targets for IBD treatment.

DSS colitis model

Several mouse models of colitis have been developed to better understand the contribution of various signaling pathways in the pathogenesis of IBD. In one well-characterized model, dextran sulfate sodium (DSS) is administered via drinking water to induce acute colitis (127). Although the exact mechanism by which DSS induces colitis remains unknown, studies show that it directly induces crypt injury (128,129), pro-inflammatory cytokine production (111,130), and decreases barrier function (131). Secretion of both TNF and IL-6 are enhanced in response to DSS-induced injury, and are thought to be the primary mediators of tissue damage in this model [reviewed in (132)].

During recovery from acute DSS colitis, epithelial hyperproliferation occurs, presumably to restore barrier integrity. Several studies have begun to investigate the requirement for crosstalk between the epithelium and immune cells in the regulation of these repair processes during recovery. This model is a useful tool to address how cell signaling pathways modulate acute inflammation and crypt injury, as well as the role of signaling molecules in re-epithelialization of colon following epithelial damage.

YAMC cells

Most immortalized cell culture models of the colon epithelium are transformed cell lines originating from colon adenocarcinomas. To better understand the role of signaling pathways in the normal colon epithelium, the conditionally-immortalized young adult mouse colon (YAMC) cell line was used in this study. YAMC cells were isolated from a transgenic mouse expressing a simian virus 40 (SV40) large T-antigen with a temperature-sensitive mutation (tsA58) driven by a major histocompatibility complex (MHC) H-2kb class I promoter (133,134). Interferons increase SV40 T-antigen expression, therefore the promoter was engineered with an interferon-inducible element (134). These cells are immortalized at 33ºC in the presence of interferon-□; when the cells are shifted to 37ºC in the absence of interferon, the SV40 T-antigen is inactivated. Under these non-permissive conditions, YAMC cells behave like primary cells, including onset of senescence and cell death after several passages. Previous results from our laboratory have characterized TNF and MAPK signaling in YAMC cells and have demonstrated that this cell model reproduces proliferative and apoptotic responses similar to conditions observed *in vivo*, making YAMC cells an ideal model for these studies (61,135).

Objectives

Previous work in this laboratory has demonstrated that TNF promotes both pro- and anti-apoptotic signaling pathways in colon epithelial cells. In the absence of KSR kinase activity, cell survival signaling downstream of TNF is

inhibited resulting in TNF-induced apoptosis (136). Furthermore, KSR activates Raf both *in vitro* and *in vivo* following TNF stimulation. While studies in other cell types indicate that Raf is an important mediator of cell survival, little is known about the role of Raf downstream of cytokine stimulation in the colon epithelium. The objectives of this study were to investigate the role of Raf in colon epithelial cell survival in response to acute colitis and in TNFR signaling using intestinal epithelium-specific Raf knockout mice (Raf KO^{IE}).

For studies describing the role of Raf in inflammation and epithelial damage (Chapter III), wildtype and Raf KO^{IE} mice were subjected to DSS colitis. Apoptosis and cell proliferation were measured in these mice following DSS-induced injury and recovery. We established an *in vitro* DSS model to determine whether Raf expression altered cell survival or activation of anti-apoptotic signaling pathways in the colon epithelium in response to DSS. Genetic and pharmacological inhibitors were used to identify Raf downstream signaling pathways involved in the observed responses, which were then confirmed *in vivo*.

Results from this study demonstrate that Raf protects against epithelial injury and inflammation and promotes recovery from acute DSS-induced colitis by both MEK-dependent and –independent pathways. While we show that Raf may mediate MEK-dependent ERK activation to promote rapid proliferation of the colon epithelium, Raf promotes anti-apoptotic signaling in response to injury through mediating NF-□B nuclear translocation in a MEK-independent manner.

Since TNF is a primary mediator of DSS-induced epithelial injury (129), the second objective of this study addressed the requirement for Raf in colon epithelial cell survival in response to TNF (Chapter IV). Raf KO^{IE} mice were utilized to assess colon epithelial cell apoptosis in response to TNF exposure. Pharmacological inhibitors and RNA interference strategies were used to investigate the requirement for Raf downstream signaling pathways involved in cell survival *in vitro*. Once these targets were identified, short-term TNF treatment of Raf KO^{IE} mice confirmed downregulation of the observed pathways *in vivo*. Further examination demonstrated receptor specificity for TNF-induced cell survival signaling through Raf.

Our results indicate that activation of TNFR1 stimulates both pro- and anti-apoptotic signaling pathways in the colon epithelium; however, TNFR1 is required for cell survival following TNF exposure. Additional studies with Raf KO^{IE} mice identified Raf kinase as a key regulator of colon epithelial cell survival in response to TNF. Interestingly, Raf promoted NF-□B p65 phosphorylation and subsequent expression of an NF-□B anti-apoptotic target gene, cIAP1, independent of MEK signaling to support cell survival. Taken together, these results identify a novel pathway in which Raf promotes colon epithelial cell survival through NF-□B downstream of TNFR1 activation.

Overall, the findings of this study indicate that Raf protects against acute colitis by promoting colon epithelial cell survival in response to TNF and other pro-inflammatory stimuli. In both studies, we reveal a novel anti-apoptotic signaling pathway in which Raf promotes cell survival through NF-□B,

independent of MEK activation. Furthermore, we show that Raf activation downstream of TNFR1 is required for colon epithelial cell survival in response to TNF treatment. In the absence of Raf activation, cells shift toward an apoptotic program, revealing a mechanism that counterbalances TNF-mediated pro-apoptotic signaling.

CHAPTER II

MATERIALS AND METHODS

Mice and genotype confirmation

Transgenic mice and genotyping

Mice harboring a loxP-flanked Raf exon 3 (Raf $^{flx/flx}$) (gift from Manuela Baccarini, Vienna Biocenter, Austria) were crossed with mice expressing Cre recombinase driven by a villin promoter [vilCre (constitutive) or vilCre ERT2 (inducible) mice (gift from Sylvie Robine, Curie Institute, Paris, France)] to generate Raf intestinal epithelium-specific knockout (Raf KO^{IE}) mice. All mice were maintained on a C57BL/6 background. Polymerase chain reaction was performed to confirm the genotype of homozygous $Raf^{flx/flx}$; vilCre ERT2 mice. PCR primers and programs for genotyping are listed in Table 2.1. TNFR1 and TNFR2 KO mice (gift from Jacques Peschon, Amgen, Seattle, WA) were mated to generate TNFR double knockout mice. C57BL/6 mice were used as wildtype controls for all TNFR KO mouse experiments. Immortomice (H-$2K^b$-tsA58) were a gift from Robert Whitehead (Vanderbilt University, Nashville, TN).

Tamoxifen injections

Cre recombination in Raf $^{flx/flx}$; vilCre ERT2 mice was induced at 6 weeks of age by intraperitoneal injection of 1 mg tamoxifen (Sigma) daily for 5 days. Sunflower oil (Sigma) vehicle was used as the negative control. Tamoxifen was

Table 2.1: Genotyping primers and PCR programs

primer		sequence	ref	PCR program
Raf DS-lox		AACATGAAGTGGTGTTCTCCGGGCGC	(57)	95°C - 5m, 95°C - 30s, 55°C – 30s, 72°C – 1m (step 2, 35 times), 72°C – 7m, 4°C - hold
Raf US-lox		TGGCTGTGCCCTTGGAACCTCAGCAC		
Louvard vilCre F		CGCGAACATCTTCAGGTTCT	(137)	
Louvard vilCre R		CAAGCCTGGCTCGACGGCC		
Immorto F		CAATGCCTGTTTCATGCC	(134)	94°C – 5m, 94°C – 1m, 54°C – 2m, 72°C – 3m (step 2, 30 times), 72°C – 7m, 4°C - hold
Immorto R		CCTGGAATAGTCACCATG		
R1KO	p60B	GGATTGTCACGGTGCCGTTGAAG	(138)	94°C –3m, 94°C – 20s, 64°C – 30s (-0.5°C per cycle), 72°C – 35s (step 2, 12 times), 94°C – 20s, 58°C – 30s, 72°C – 2m (step 5, 25 times), 4°C hold
	p60E	TGACAAGGACACGGTGTGTGGC		
	p60spe	TGCTGATGGGGATACATCCATC		
Use for R1 & R2	pgk5'-66	CCGGTGGATGTGGAATGTGTG	(138)	
R2KO	p80Kas F	AGAGCTCCAGGCACAAGGGC		95°C – 4m, 95°C – 1m, 65°C – 1m, 72°C – 30s (step 2, 34 times), 72°C – 7m, 4°C - hold
	p80Kas R	AACGGGCCAGACCTCGGGT		

solubilized in sunflower oil by sonication at room temperature for 10 min. Experiments began ten days after the last tamoxifen injection to allow effective Cre induction and recombination of the floxed allele.

Mucosal isolation

Mucosal scrapings were prepared by scraping the colon or small intestine with a glass slide as previously described (139). Protein lysates were extracted in 10 volumes of RIPA buffer (1% triton X-100; 1mM EDTA, 1mM EGTA, 10mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 0.2% sodium deoxycholate) with protease and phosphatase inhibitor cocktails (Sigma) on ice for 10 min. Epithelial and stromal fractions were isolated by incubating several 1 cm pieces of opened colon or small intestine in Cell Recovery Solution (BD Biosciences) for 16 h at 4°C followed by manual shaking. The liberated crypts and remaining stroma were centrifuged separately and lysed with RIPA buffer. RIPA lysates were cleared by centrifugation, assayed for protein concentration with DC Protein Assay (Biorad), and boiled in Laemmli sample buffer (140).

Mouse treatment administration

DSS treatment

A 3% aqueous solution of dextran sulfate sodium (DSS) (36-50 kD, MP Biomedicals, Solon, OH) was filtered and administered *ad libitum* to mice for four days. A subset of these mice was sacrificed at four days and others were allowed to recover on regular drinking water for three days. As a control, some

mice were given regular drinking water throughout the treatment period. Mice were weighed and stool samples tested with guaiac (Hemoccult, Beckman Coulter) for fecal blood daily. Colon length was measured at the time of sacrifice. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

TNF injections

Mice were injected intraperitoneally (i.p.) with PBS or murine TNF (10^4 U) (Peprotech, Rocky Hill, NJ) for 1, 2, or 24 h. TNF was diluted in 200 ml PBS with 2% fetal bovine serum (FBS) as a carrier protein. PBS control injections also contained 2% FBS.

Histological assessment

Mice were euthanized by cervical dislocation under anesthesia. Swiss-rolled colon and small intestine were fixed in 10% formalin, paraffin-embedded, sectioned at 4-6mm, and hematoxylin and eosin (H&E) stained as previously described (136). Histology was scored blinded to genotype and condition based on a scale described by Dieleman et al. (129), which categorizes the severity and extent of colonic crypt damage and inflammation. The sections were graded separately as to the amount and depth of inflammation with a range 0 to 3. The amount of crypt damage or regeneration and the percentage involvement in disease process were graded independently with a range 0 to 4.

Organ culture

Colons were flushed with PBS and placed in a 10cm dish with DMEM containing 0.5% FBS and 1% penicillin/streptomycin. Small rings of colon were cut from the distal end, opened longitudinally, and cut into three segments. Using a small, flat spatula, each segment was placed flat with the lumen facing up in the bottom of a 500□m transwell insert in a 6-well plate (Corning) containing 1.8 ml of media. 2-4 explants were plated per transwell for each condition. Explants were placed at 37°C for 30 min to equilibrate and treated with TNF (50 ng/ml) for 6 h. Following treatment, explants were placed in an Eppendorf tube containing 4% paraformaldehyde in PBS overnight at 4°C.

Cell culture procedures and generation of cell lines

Cell culture procedures

Conditionally-immortalized young adult mouse colon cells (YAMC) (133) and TNFR1 KO cells (141) were cultured on rat tail collagen (BD Biosciences) coated plates and maintained in RPMI 1640 (5% FBS, 100 U/ml penicillin/streptomycin, 5 □g/ml insulin, 5 ug/ml transferrin, 5 ng/ml selenous acid (BD Biosciences), and 5 U/ml mouse IFN-□(Intergen) at 33°C (permissive conditions) in a 5% CO_2 humidified incubator. Prior to all assays, cells were serum-starved (RPMI 1640, 0.5% FBS, 1% penicillin/streptomycin) and shifted to 37°C (non-permissive conditions) for 16 h. Cells were treated with murine TNF 100 ng/ml (Peprotech), murine EGF (10 ng/ml) (R&D Systems), or 5% DSS for

the times indicated. MEK inhibitors, PD98059 (used at 20□M) and U0126 (used at 10□M), Raf kinase inhibitor (GW5074, 5-20□m), and a PI3-kinase inhibitor, wortmannin (100nM) were obtained from Calbiochem (Gibbstown, NJ).

Transient transfections

For transient transfections, cells were plated at a density of $2x10^5$ (6-well plate) or $5x10^4$ (4-well chamber slide, Lab-Tek) and transfected with 50 nmol NF-□B p65, Raf-1 and/or I□B□ siRNA SMARTpool or non-targeting siRNA duplexes (Dharmacon) with Lipofectamine RNAi/ MAX (Invitrogen) according to manufacturer's directions. The siRNA transfection reagent was incubated with the cells overnight.

The cDNA clone for dominant-negative Ras (S17N) was obtained from the Missouri S&T cDNA Resource Center (www.cdna.org) transfected using Lipofectamine 2000 (Invitrogen) for 6 h, at which point media was replaced with RPMI growth media.

Generation of TNFR1 addback colon epithelial cells

TNFR1 addback cells were generated by infection of TNFR1 KO colon epithelial cells (141) with a GFP-containing LZRS retrovirus containing vector alone or a HA-tagged mouse TNFR1 sequence. Total RNA was isolated from YAMC cells with Trizol reagent (Invitrogen) and TNFR1 cDNA amplified with a TaqMan RT-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 AAT TCA TGG GTC TCC CCA CCG TGC C G-3' and 5'-GTCAGG CAT AGT CTG GGA CGT CAT ATG GAT ATC GCG GGA GGC GGG TCG TG-3' primers. Following ligation into pGEM-T-easy vector (Promega) and digestion with EcoR1 (New England Biolabs), HA-TNFR1 DNA was ligated into the bi-cistronic LZRS-GFP retroviral vector (gift from Albert Reynolds, Vanderbilt University, Nashville, TN) digested with EcoR1 and treated with calf intestinal phosphatase (Promega). Empty vector or vector containing the HA-TNFR1 construct was transiently transfected using Lipofectamine 2000 (Invitrogen) into Phoenix ecotropic viral packaging cells (142). Virus containing supernatant was harvested and placed on TNFR1 KO cells for 6 hours, after which media was replaced with regular culture media. The infection protocol was repeated for three consecutive days and then sorted based on GFP expression to obtain empty vector or HA-TNFR1 stably-integrated cell populations.

Generation of conditionally-immortalized Raf ^{flx} cell line

Raf ^{flx} mice were crossed with an Immortomouse to generate Raf $^{flx/flx}$;Immorto mice. Colon and stomach were isolated from these mice, washed in PBS and incubated separately in 0.04% sodium hypochlorite in PBS for 15 min. Following a rinse with 0.5mM dithiothreitol (DTT)/3mM EDTA in PBS, tissue was incubated in 25 ml DTT/EDTA solution for 90 min at room temperature. Organs were then vigorously shaken in 15 ml PBS to liberate crypts, which were then transferred to a 15 ml tube and centrifuged at 400 rpm for 5 min to pellet crypts. Crypts were

resuspended in LHC-9 (Invitrogen) with 5 U/ml IFN-□(7.5 ml/stomach, 30 ml/colon) and plated in minimal volume in Cellcoat 24-well plates (Greiner Bio-one). After 24 h, 1 ml of media was added to the wells. When the cells attached, LHC-9 was replaced with RPMI containing 5% FBS, 1% pen/strep, and interferon-□and media was changed once a week. Cells were allowed to become completely confluent before passage into a larger collagen-coated plate.

Cell lysate preparations

Cell lysates, SDS-PAGE, Western blot analysis

Cell lysates were prepared by scraping in a buffer containing (20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, and 0.1% phosphatase and protease cocktails (Sigma)). 0.1% sodium dodecyl sulfate (SDS) and 0.2% sodium deoxycholate was added to lysates from cells used in DSS experiments, which were then passed through a 27g needle to shear DNA. Cell lysates were boiled in Laemmli sample buffer, run on 10 or 15% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS-Tween (0.05%) and incubated in primary antibody overnight at 4°C. Polyclonal antibodies used for Western blot analysis include: anti-Raf-1, B-Raf, E-cadherin, NF-□B p65 (Santa Cruz, Santa Cruz, CA), phospho-ERK (Promega), I□B□, phospho-p38, phospho-JNK, phospho-Akt (Cell Signaling, Boston, MA) and HA (Invitrogen). Monoclonal antibodies used include: Ras (Upstate) and actin (Sigma). Following 3 washes

with TBS-Tween (0.05%) x 5 min, membranes were incubated with secondary antibodies, anti-rabbit- or anti-mouse-HRP (Cell Signaling). Membranes were washed again (3 x 5 min) with TBS-Tween and developed with Western Lightning (PerkinElmer).

Subcellular fractionation

TNFR1 KO cells containing vector alone or HA-TNFR1 were scraped with lysis buffer (20mM Tris pH 7.4, 2mM EDTA, 2mM EGTA, 200mM NaCl, and 0.5 mg/ml digitonin including protease and phosphatase inhibitor cocktails) and incubated on ice for 5 min. Cytoplasmic extracts were isolated by ultracentrifugation using a Beckman ultracentrifuge at 55,000 rpm for 40 min at 4°C. The cytoplasmic fraction was removed, boiled in Laemmli buffer, and the membrane fraction was incubated with lysis buffer containing 1% Triton X-100 on ice for 30 min. Following brief centrifugation at 14,000 rpm for 5 min, the triton-soluble membrane fraction was removed and boiled in Laemmli buffer.

Activation assays

Ras activation assay

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, Billerica, MA).

Agarose beads were washed twice with PBS and boiled in Laemmli sample buffer. Western blot analysis was performed to detect Ras bound to the Raf-RBD beads.

Raf kinase assay

To immunoprecipitate endogenous Raf, 200□g whole cell lysate was pre-cleared with protein A/G beads (Santa Cruz), incubated with 600ng anti-Raf-1 antibody for 2 h, followed by 35□l protein A/G beads for 1 h. Immunocomplexes were washed once in 1M NaCl, twice with PBS, and once in assay dilution buffer. Kinase assays were performed with Raf-1 kinase assay cascade kit according to manufacturer's directions (Upstate). Briefly, immunoprecipitated Raf was incubated with 75mM magnesium chloride, 500□M ATP and 0.4 □g inactive recombinant MEK1 for 30 min at 30°C. Supernatants were removed and boiled in Laemmli sample buffer. Beads were washed once with PBS and boiled in sample buffer.

Immunocytochemistry

Apoptosis assays

Apoptosis in colon sections was determined by Apoptag *in situ* oligo ligation (ISOL) (Chemicon) or immunostaining for active cleaved caspase-3 (Cell Signaling) (136). The number of positive-staining cells was determined by counting the number of positive cells per 100 colonic crypts. Apoptosis in cell

culture was assessed by terminal deoxynucleotidyl transferase nick end labeling (TUNEL) peroxidase staining (Chemicon) or by multi-caspase activation assay (Biomol) using a cell permeable, sulforhodamine-labeled caspase inhibitor (SR-VAD-FMK). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI)(Vector Labs) and percent active caspase- or TUNEL-positive cells were determined. Caspase assays were visualized by immunofluorescence on an Axiovert 200 microscope with Apotome (Zeiss) and TUNEL assays were visualized using differential-interference contrast (DIC) microscopy on a Leica DM-IRB microscope.

NF-κB staining

Cells plated in 4-well chamberslides (Lab-Tek, Nunc) were fixed with 1% freshly de-polymerized paraformaldehyde. Following post-fixation in ice-cold methanol, cells were quenched with 3% hydrogen peroxide and solublized with PBS with 0.2% Triton X-100. Slides were blocked with 10% goat serum for 20 min, incubated with NF-κB p65 antibody (sc-372, Santa Cruz) for 1 h at room temperature followed by Cy3-labeled goat anti-rabbit or 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-conjugated donkey anti-rabbit secondary antibodies (Jackson Immunoresearch) for 30 min. Slides were mounted using Vectashield with (Cy3) or without (AMCA) DAPI (Vector Labs) and visualized on a Leica DM-IRB microscope.

Immunohistochemistry

Antibodies and staining protocol

Sections were de-paraffinized, rehydrated, and antigen unmasked by boiling in a citrate-containing buffer (Vector Labs). Slides were blocked with 10% goat serum (Zymed) for 30 min and incubated with primary antibody overnight at 4°C. Antibodies used for immunohistochemistry include: rabbit anti-human Ki-67 (Vector Labs), rabbit monoclonal active caspase-3, phospho-specific antibodies against ERK, NF-□B p65 phospho-S276 and Ser10 Histone H3 (Cell Signaling). Slides were incubated with goat anti-rabbit-HRP (Dako) and developed using DAB substrate kit (Vector Labs). Slides were counterstained with methyl green or Mayer's hematoxylin and viewed on a Nikon Eclipse E800 microscope. Phospho-ERK staining was quantified by determining the average number of positive cells per crypt in 50 midcolonic crypts. The number of NF-□B phospho-p65-positive cells was determined by counting the number of stained cells per 100 colonic crypts. The percentage of Ki-67 positive cells was determined by analysis with the Ariol SL-50 automated image analysis system (Applied Imaging, San Jose, Calif). Nuclear Ki-67 staining was processed with the NuclearHighRes script and cells were classified as positive or negative based on pre-determined thresholds that evaluate color and intensity of staining, as well as cell size, axis length, roundness, and compactness.

X-gal staining

Opened colon or small intestine was fixed in 2% paraformaldehyde for 30 min and washed 3 times in PBS. Tissue was incubated in a PBS-buffered solution containing 100mM ferrocyanide, 100mM ferricyanide, 1M $MgCl_2$, 10% Nonidet P (NP)-40, and 20mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) overnight at 37°C. Following X-gal staining, tissue was washed twice in PBS and Swiss-rolled (136) for sectioning.

***In situ* hybridization**

In situ hybridization followed the protocol as previously described (143). Antisense ^{35}S-labeled cRNA clones for Lgr5 was generated using T7 RNA polymerases. Frozen sections (12□m) were prehybridized and hybridized at 45°C for 4 hours in 50% formamide hybridization buffer containing ^{35}S-labeled antisense cRNA probe (470bp coding sequence + 300bp 3'UTR) (gift from Hans Clevers, University of Utrecht, Netherlands). RNase A–resistant hybrids were detected by autoradiography. Sections were post-stained with H&E.

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. Pair-wise 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 (Figure 4.2). The comparisons of interest and their expected direction of the effect were pre-specified in some cases; otherwise a two-sided test was conducted. When the normality assumption did not seem to hold (Figures 3.5 & 4.2), a nonparametric Kruskal-Wallis test was used instead. When replicates were available (Figures 3.3 & 3.7), linear mixed-effect models were used to incorporate the additional information. The comparisons between the genotypes within a treatment group were carried out by specifying appropriate contrasts. All data analyses were performed using R 2.6.0 (144).

CHAPTER III

RAF PROTECTS AGAINST COLITIS BY PROMOTING MOUSE COLON EPITHELIAL CELL SURVIVAL THROUGH NF-κB

Introduction

The intestinal epithelium is a single cell layer that forms the only barrier between the body and the luminal gastrointestinal contents. Maintenance of this cell layer, which turns over every 3-5 days, is dependent upon tight regulation of proliferation and apoptosis (101,145). Coordinated activation of signaling molecules involved in the regulation of these processes is essential to maintain intestinal homeostasis.

In the current study, we test the hypothesis that Raf-1 protects the colon epithelium during injury and inflammation through activation of an NF-κB-dependent survival mechanism. The most widely studied family member of the Raf family of serine/threonine kinases, Raf-1, is involved in key cellular processes including proliferation, differentiation, and survival. Its function is best understood in the context of the highly conserved mitogen-activated protein kinase (MAPK) cascade, in which Raf phosphorylates its substrate MEK, which in turn phosphorylates and activates ERK to promote proliferation and differentiation. In addition, Raf regulates MAPK-independent responses to promote cell survival through various cell context-dependent mechanisms. Raf appears essential for mammalian development as Raf-1 knockout mice die between days E9.5-10.5 due to placental defects and Fas-mediated apoptosis in

the fetal liver (13,54). In contrast to the Fas-dependent mechanism shown in the liver, Raf-null macrophages are more sensitive to *S. typhimurium*-induced apoptosis through caspase-1 activation (57). In cardiac-specific Raf knockout mice, Raf functions as an anti-apoptotic regulator through inhibition of the pro-apoptotic kinase, ASK-1 (56).

Using the IL-$10^{-/-}$ mouse model, which develops spontaneous colitis associated with unchecked TNF production (145), we have shown that Raf threonine phosphorylation is increased during active disease (136). In the current study, we utilize a well-characterized model of chemically induced colitis (127,128,146) to better understand the role of Raf in regulating response to epithelial injury and mucosal inflammation. Short-term administration of dextran sulfate sodium (DSS) induces acute colitis characterized by epithelial damage, disruption of crypt architecture, and severe infiltration of inflammatory cells (128,129). While the exact mechanisms by which DSS induces colitis are not well characterized, the physiological response involves increased local production of cytokines (129,130) resulting from increased apoptosis due to loss of barrier function(147) or failure to recognize bacterial-derived signals(148,149). Recovery from the acute colitis involves a process during which the damaged epithelium undergoes hyperproliferation to re-establish the epithelial barrier (149).

Our data show that intestinal epithelium-specific Raf knockout (Raf KO^{IE}) mice develop worse colitis than their wildtype counterparts. Raf KO^{IE} mice exhibit significantly higher levels of colon epithelial apoptosis and decreased

enterocyte proliferation following DSS treatment compared to wildtype. Furthermore, Raf expression is required for ERK and NF-κB activation in DSS-treated mouse colon epithelial cells. While MEK activation is insufficient to promote colon epithelial cell survival, constitutive NF-κB activation rescues DSS-induced apoptosis in the absence of Raf expression. NF-κB activation is attenuated in colon epithelial cells following DSS-induced injury in Raf KO^{IE} mice compared to wildtype. These results demonstrate that Raf regulates NF-κB activation and cell survival in a MEK-independent manner to protect against intestinal epithelial injury and inflammation.

Results

Raf protects against DSS-induced crypt injury and inflammation.

Raf is a key regulator of cell proliferation and differentiation, and appears to be an important mediator of cell survival (150). As Raf-1 deletion is embryonic lethal (13), we tested the role of Raf in colitis using intestinal epithelium-specific Raf knockout (Raf KO^{IE}) mice generated by crossing $Raf^{flx/flx}$ mice (wildtype)(gift from Manuela Baccarini)(57) with mice expressing a tamoxifen-inducible Cre recombinase under the control of a villin promoter (gift from Sylvie Robine)(151). To test the efficiency of Cre recombination in the inducible Cre model, vilCre ERT2 mice were crossed with Rosa26 reporter mice, which express a beta-galactosidase (LacZ) gene interrupted by a loxP-flanked stop codon. During active recombination of the allele, the stop codon is excised permitting LacZ

expression. X-gal staining was used to detect LacZ expression in tamoxifen-treated Rosa26R; vilCre ERT2 mice (Figure 3.1A). A majority of crypts in both the colon and small intestine stained LacZ-positive (Figure 3.1B), indicating successful recombination of the floxed allele in the intestinal epithelium.

Recombination of the Raf allele in the inducible villin-Cre ERT2 system was detected by Western blot analysis of isolated epithelial and stromal fractions. Raf expression was effectively knocked out in the intestinal epithelium, but not the stroma, of both the colon and small intestine in Raf KO^{IE} mice (Figure 3.2). In contrast, B-Raf expression in the intestinal epithelium was not affected.

While other conditional Raf knockout mice have striking phenotypic changes (56), Raf KO^{IE} mice show normal architecture of the intestinal and colonic epithelium (Figure 3.3A). Unchallenged Raf KO^{IE} mice also display no basal defects in cell proliferation or migration up the crypt-villus axis (data not shown). B-Raf expression is detectable in the colon epithelium, both in cultured cells and in vivo, indicating that regulation of Raf signaling may be functionally redundant in the context of cell maintenance.

Injury models have been used to delineate the requirement for specific gene products and/or signaling pathways in response to tissue damage and disease. To assess the role of Raf in intestinal injury and inflammation, 3% DSS was administered via drinking water to 8-week-old wildtype and Raf KO^{IE} mice. Mice received either water alone (control) or 3% DSS for four days to induce colitis. Following four days of acute injury, some DSS-treated mice were

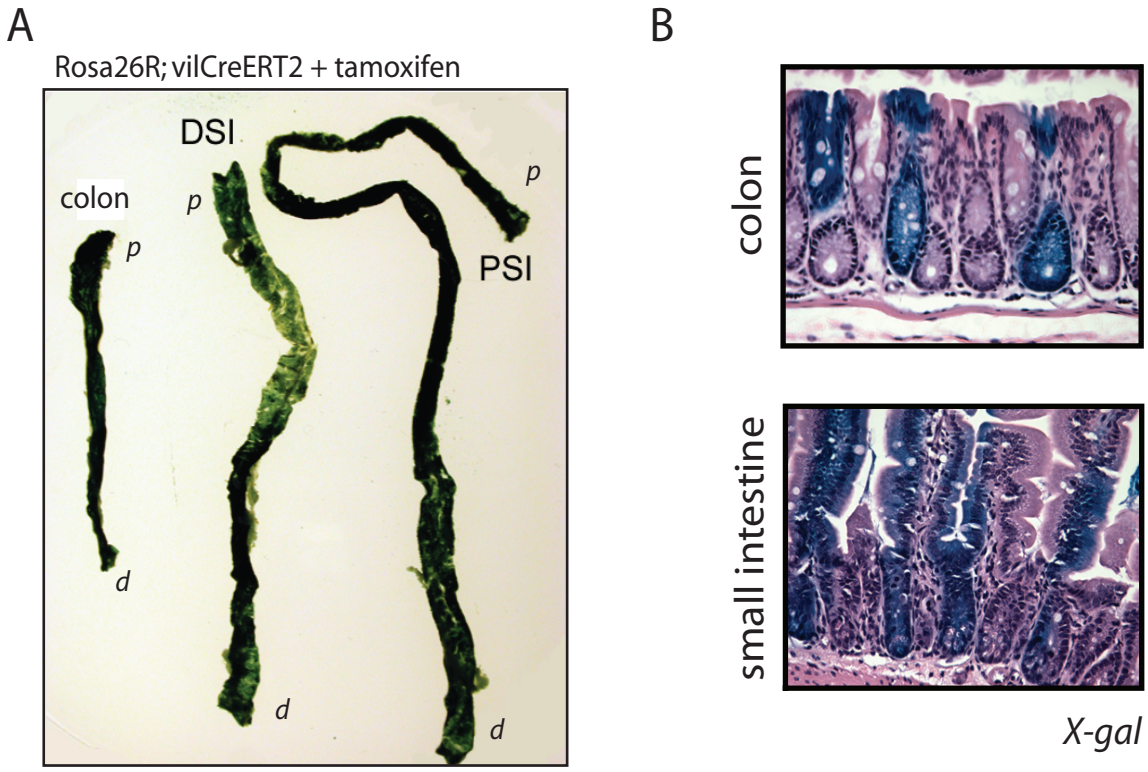

Figure 3.1: Inducible villin-Cre ERT2 expression induces LacZ recombination. (A) Whole mount images of colon, distal small intestine (DSI), proximal small intestine (PSI) from Rosa26R; vilCreERT2 mice 2 weeks after injection with oil or tamoxifen stained with X-gal. (*d*, distal; *p*, proximal) (B) Representative images of X-gal-stained colon and small intestine from tamoxifen-injected mice.

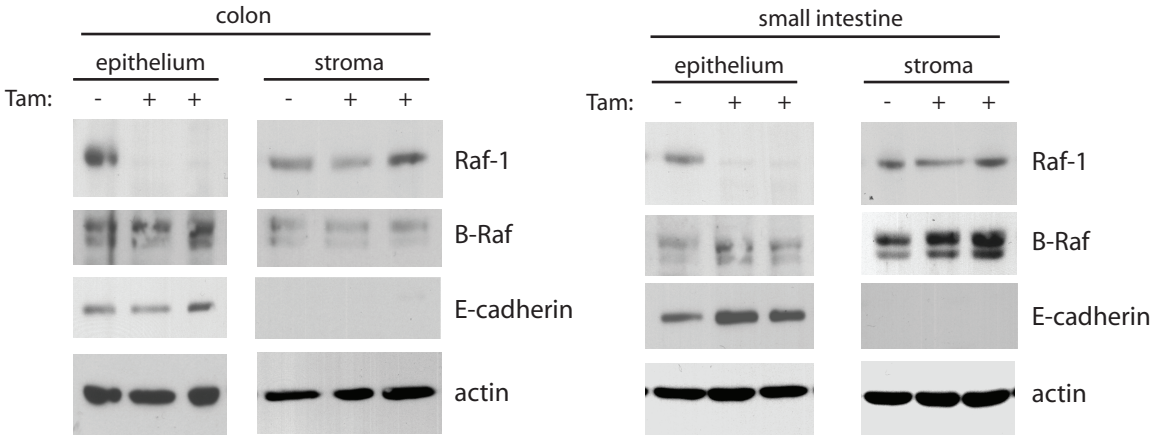

Figure 3.2: Raf expression is absent in inducible Raf intestinal epithelium-specific knockout mice. Epithelial and stromal fractions were isolated from the colon and small intestine of oil- or tamoxifen-injected Raf $^{flx/flx}$; villin-Cre mice. Western blot analysis was performed with antibodies against Raf-1, B-Raf, E-cadherin, and actin. Raf $^{flx/flx}$ mice were a gift from Manuela Baccarini (Vienna Biocenter, Austria) and villin-Cre mice were a gift from Sylvie Robine (Curie Institute, Paris, France).

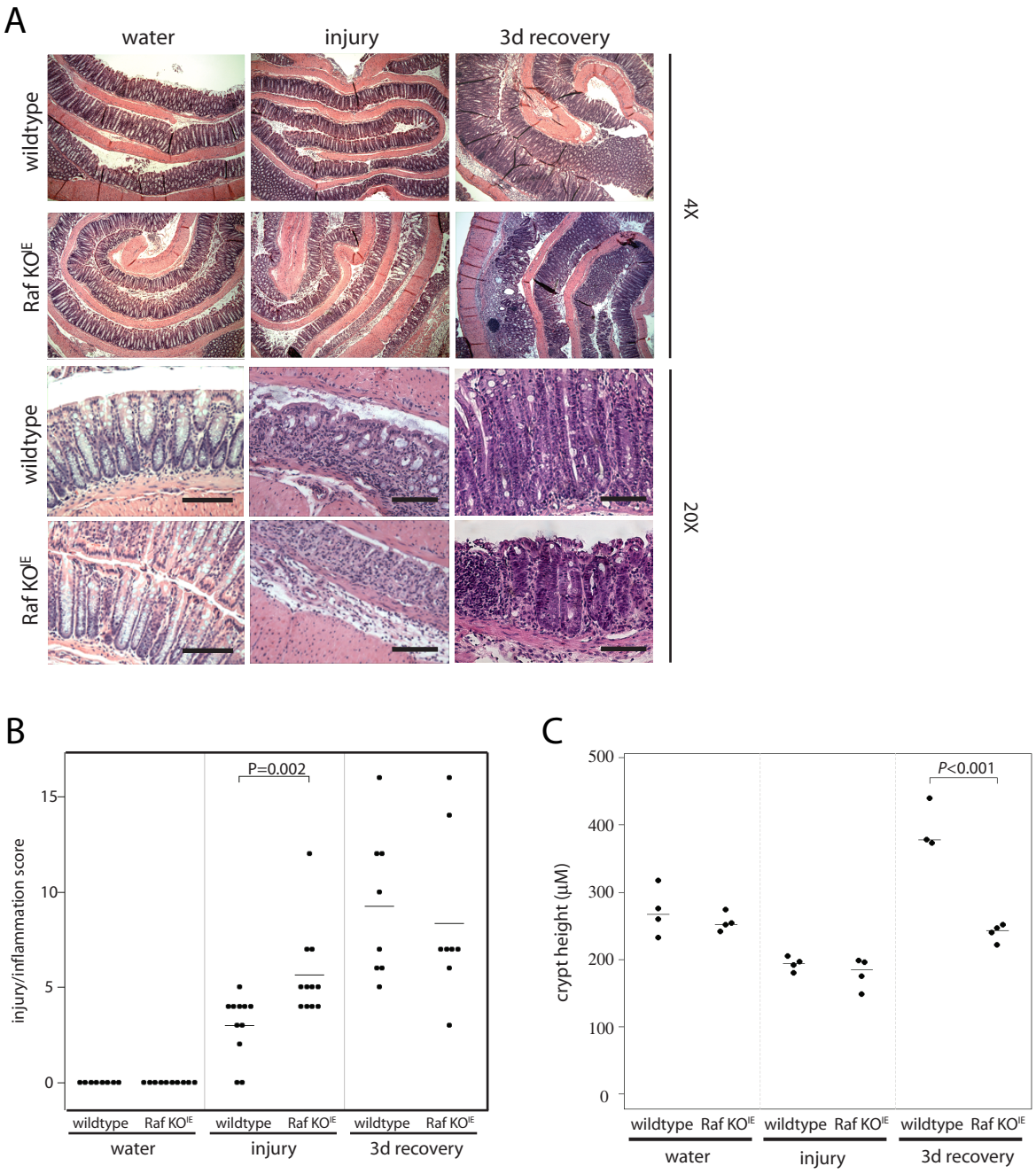

Figure 3.3: Raf KO^{IE} mice exhibit increased crypt injury and inflammation resulting from colitis. (A) H&E staining of colon sections from wildtype and Raf KO^{IE} mice treated with water, 3% DSS for 4 days, or 4 days plus a 3 day recovery period. Scale bar = 500μm or 125μm (B) Combined scores grading severity of inflammation and extent of crypt damage in wildtype or Raf KO^{IE} mice following DSS treatment. Bars represent the mean value. (C) Crypt height in wildtype and Raf KO^{IE} mice following DSS treatment. Bars represent the median.

maintained on water alone for a three-day recovery period. In response to DSS, both wildtype and Raf KO^{IE} colonic epithelium suffered loss of crypt architecture and mucosal inflammation primarily in the midcolon (Figure 3.3A). The proximal and terminal colon showed little crypt damage. However, injury and inflammation scores were significantly higher in Raf KO^{IE} mice (P=0.002) (Figure 3.3B). Raf expression contributed to attenuation of DSS-induced inflammatory response as Raf KO^{IE} mice had increased inflammatory infiltrate, greater depth of inflammation, and higher percent colon involved in inflammation following acute injury. We observed that Raf KO^{IE} mice were 2.5 (95% CI (1.2,5.4)) times more likely to have blood present in the stool compared to wildtype during days 2-4 of DSS treatment (data not shown). Wildtype mice showed increased crypt height compared to Raf KO^{IE} during recovery (P<0.001), whereas both genotypes had shortened crypts following injury (Figure 3.3C).

No clinical signs of colitis were apparent in wildtype or Raf KO^{IE} mice after 4 days of DSS treatment. To assess if loss of Raf resulted in clinical differences in response to a more severe injury, the length of DSS adminstration was extended to 8 days. In response to prolonged DSS exposure, both wildtype and Raf KO^{IE} mice demonstrated extensive crypt injury and inflammation (Figure 3.4A). Interestingly, mice began losing weight at treatment day 6, and by day 8, Raf KO^{IE} mice had lost significantly more weight than their wildtype counterparts (Figure 3.4B)(P=0.023). Furthermore, Raf KO^{IE} mice had decreased colon length compared to wildtype mice, another indication of colonic inflammation (Figure3.4C)(P<0.01). These data indicate that Raf expression in the intestinal

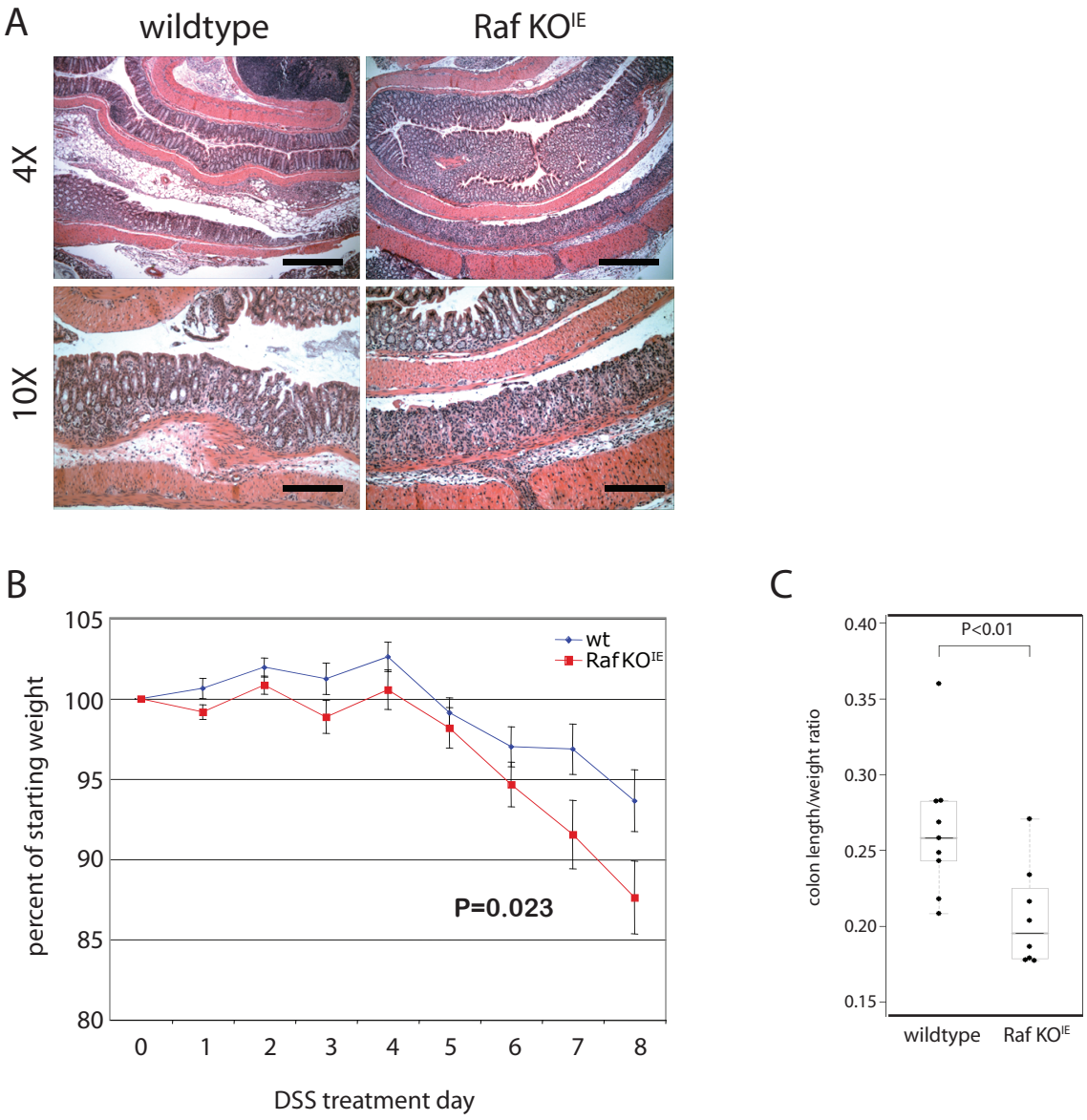

Figure 3.4: Loss of Raf expression results in worsened clinical signs of colitis. (A) H&E staining of wildtype and Raf KO^{IE} mice following 8 day DSS treatment. Scale bars = 500µm and 250µm (B) Graphical representation of the average percent of weight loss for wildtype and Raf KO^{IE} mice compared to the weight at the start of DSS treatment. (C) Colon length is represented in a ratio compared to the starting weight of mice prior to DSS administration. Horizontal bars represent the mean value.

epithelium protects against epithelial damage and the associated inflammatory response after acute DSS treatment.

Raf expression confers intestinal epithelial cell survival during DSS colitis.

Maintenance of the intestinal epithelial barrier is dependent upon the tight regulation of proliferation and apoptosis. To assess whether the increased severity of crypt damage in Raf KO^{IE} mice was due to decreased intestinal epithelial survival, we measured DSS-induced apoptosis in intact crypts surrounding regions of ulceration in wildtype and Raf KO^{IE} mice by ISOL (Figure 3.5). Acute DSS exposure induced colon epithelial cell death in both wildtype and Raf KO^{IE} mice; however, apoptosis was significantly higher in the absence of Raf expression (P=0.046). These data were confirmed by active-caspase-3 staining (P=0.008, data not shown). Taken together, the results indicate that Raf plays an anti-apoptotic role during DSS-induced injury and inflammation.

Raf promotes hyperproliferation during epithelial regeneration.

Previous studies have demonstrated that hyperproliferation of enterocytes occurs to regenerate damaged epithelium during recovery from acute colitis (149). To determine the role of Raf in this phenomenon, we examined colonic epithelial cell proliferation during the recovery phase of DSS colitis in wildtype and Raf KO^{IE} mice by immunohistochemistry for Ki-67 and phospho-histone H3. During recovery, wildtype mice have long, highly proliferative colonic crypts compared to Raf KO^{IE}, which display fewer proliferative cells in shorter, poorly

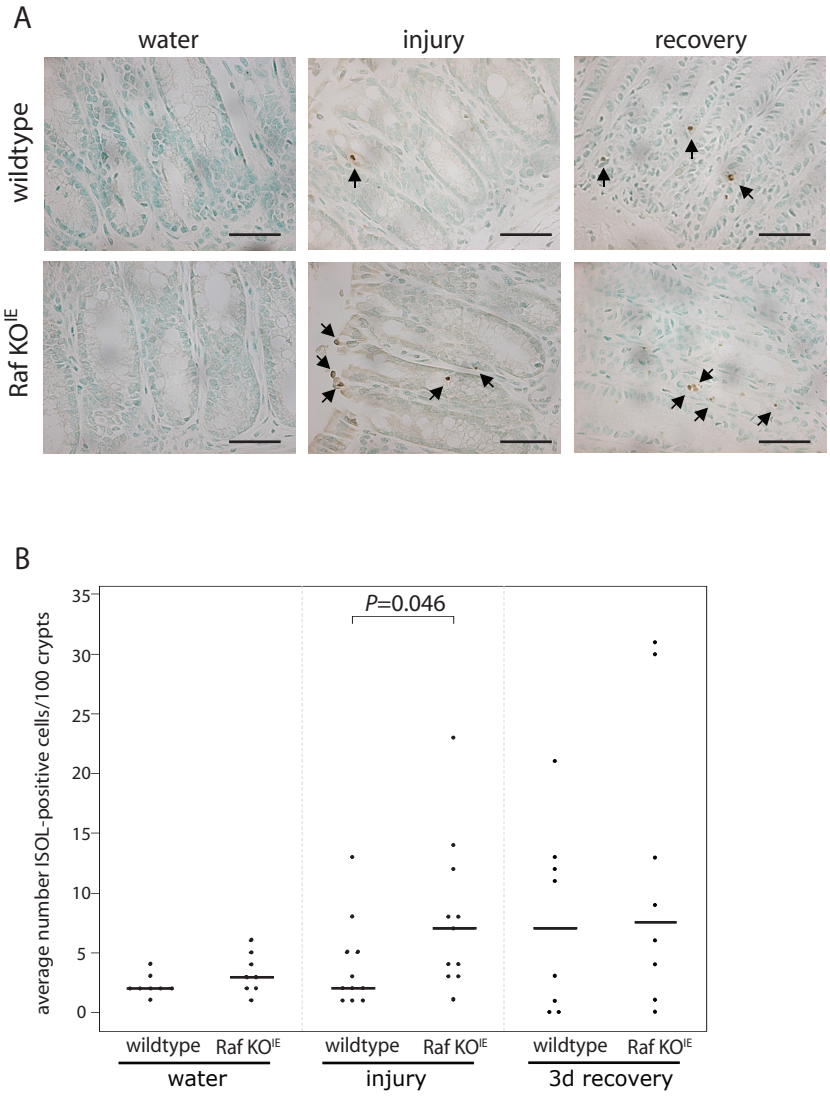

Figure 3.5: Raf protects from apoptosis in response to damage in the intestinal epithelium. (A) Apoptotic cells were detected by *in situ* oligo ligation (ISOL) in colon sections of DSS-treated wildtype and Raf KO^{IE} mice. Scale bar = 50μm (B) The average number of ISOL-positive cells were represented graphically. Horizontal lines represent the median for each data set.

organized crypts (Figure 3.6A). Quantification of Ki-67 positive cells demonstrated hyperproliferation in the midcolon of wildtype mice in response to initial injury (P=0.036, P=0.003); in contrast, the number of proliferating cells in Raf KO^{IE} mice during recovery is similar to that of mice receiving water alone (Figure 3.6B). The number of cells per hemicrypt was also increased in wildtype mice compared to Raf KO^{IE} mice (P<0.001) (Figure 3.6C). *In situ* hybridization for Lgr5 demonstrated that Raf expression does not drastically effect the stem cell population in unchallenged mice or during epithelial regeneration (Figure 3.7).

To determine the role of canonical Raf signaling in cell proliferation, we assessed the localization of active ERK in wildtype and Raf KO^{IE} mice during recovery. In both wildtype and Raf KO^{IE} untreated mice, phosphorylated ERK is localized to the nucleus of differentiated cells lining the surface epithelium (Figure 3.8A). In the hyperproliferating crypts of recovering wildtype, but not Raf KO^{IE}, mice active ERK is visible as nuclear and diffuse cytoplasmic staining throughout the crypt axis (P=0.001) (Figure 3.8B). Western blot analysis confirmed the absence of Raf expression in the epithelium following injury, eliminating the possibility that Raf signaling is restored during recovery (data not shown). These data indicate that in addition to playing a protective anti-apoptotic role, Raf is required for rapid epithelial regeneration following DSS colitis.

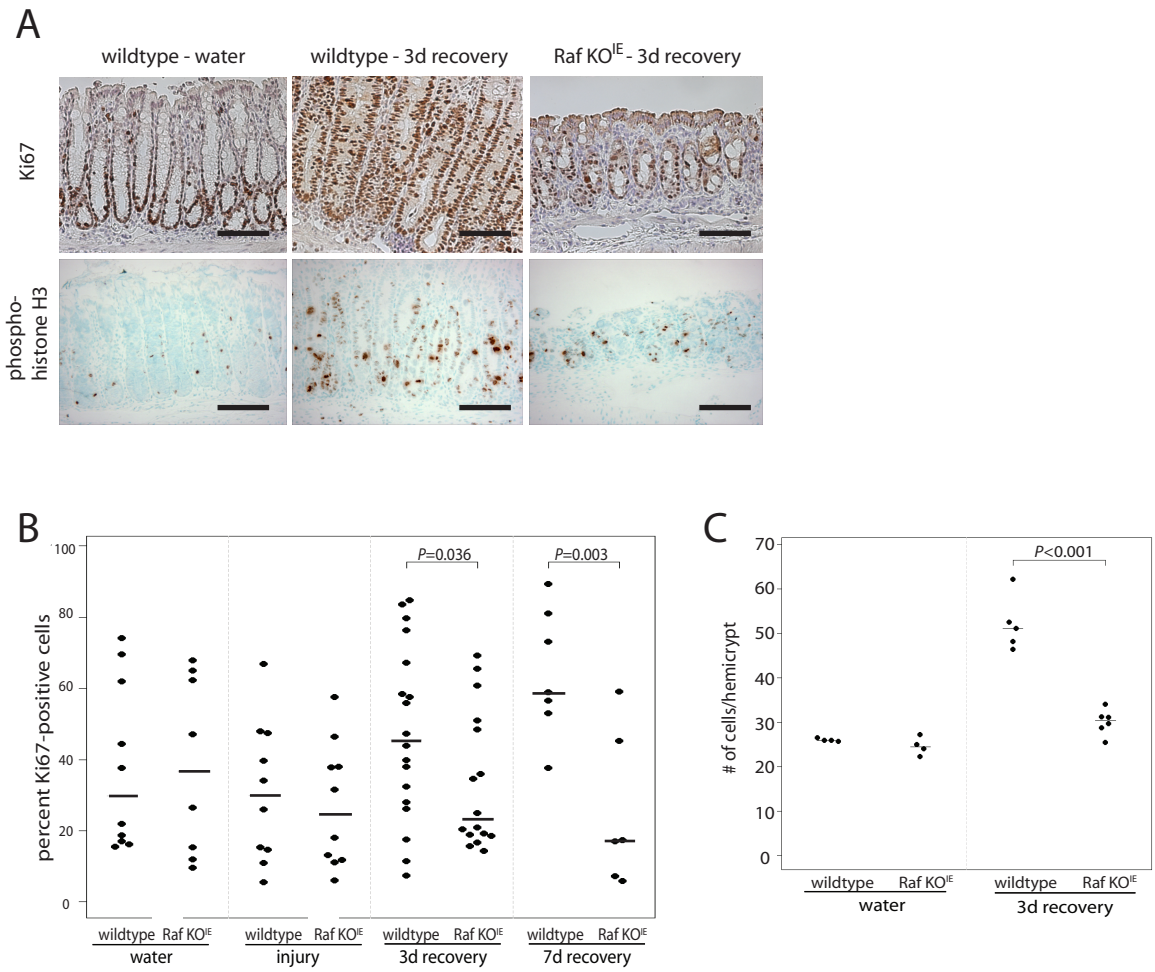

Figure 3.6: Raf is required for the epithelial hyperproliferative response following DSS-induced injury. (A) Proliferating cells were detected by Ki67 and phospho-histone H3 staining in colon sections of wildtype and Raf KO^{IE} mice during recovery from DSS-induced colitis. (B) The percentage of Ki67-positive cells in the midcolon are represented graphically. (C) The number of cells per hemicrypt are represented graphically.

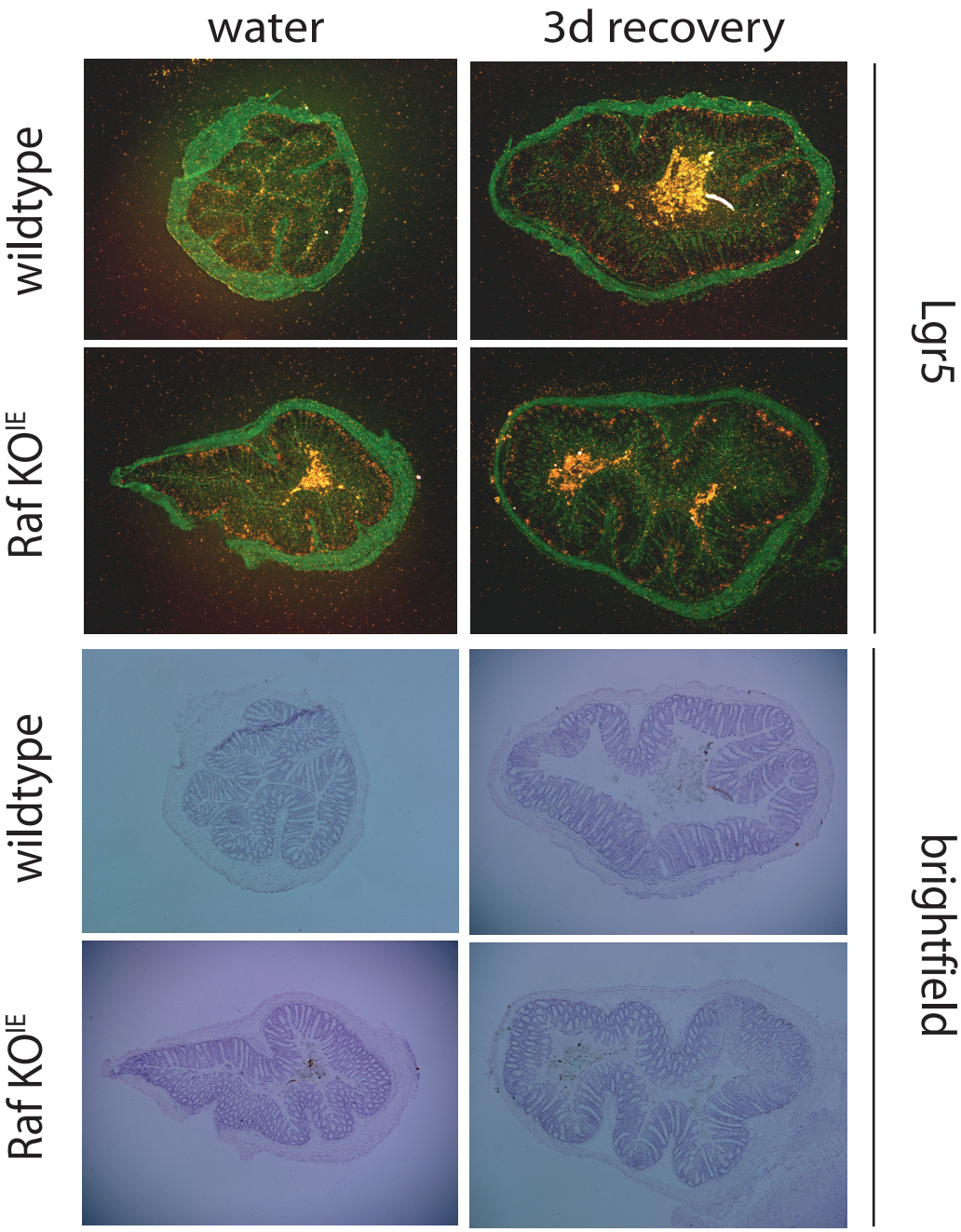

Figure 3.7: Raf expression does not affect the number of Lgr5-positive cells in the colon epithelium. *In situ* hybridization was performed for Lgr5 in colon sections of wildtype and Raf KO^{IE} mice following water treatment and 3 day recovery following DSS-induced injury. Darkfield images show Lgr5 (orange) and eosin (green).

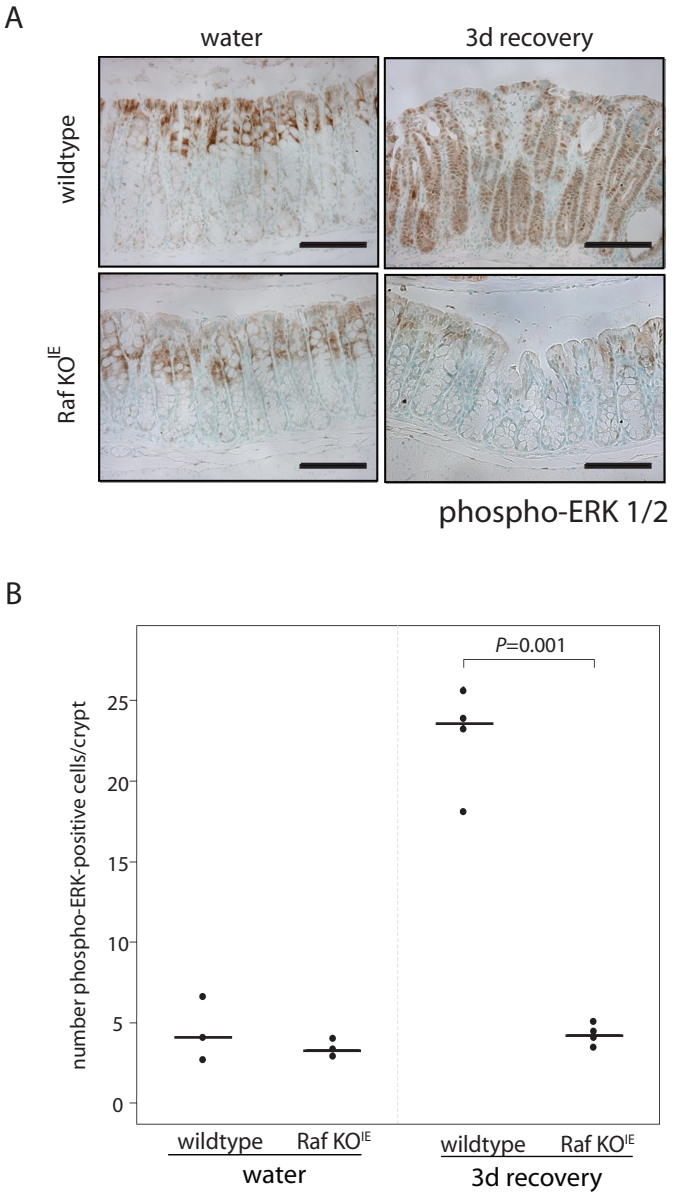

Figure 3.8: Loss of Raf results in decreased ERK activation in the colon epithelium during recovery. (A) Phospho-ERK staining of colon sections from control and Raf KO^{IE} mice during recovery. Scale bars = 125μm. (B) The average number of phospho-ERK-positive cells per crypt are represented graphically. Horizontal lines represent the median of each data set.

Raf protects from DSS-induced apoptosis through NF-κB activation.

To explore the underlying mechanism of Raf promoting colon epithelial cell survival during injury and inflammation, we utilized RNA interference in cell culture to assess the role of Raf on the activation of pro- and anti-apoptotic signaling pathways. YAMC cells were transfected with non-targeting or Raf-1 siRNA and then treated with 3 or 5% DSS for 7 h. A time course was performed to determine at which time point, DSS induced cell loss (data not shown). TUNEL staining showed that apoptosis was significantly increased in response to 5% DSS in YAMC cells transfected with Raf siRNA compared to non-targeting siRNA (P=0.03) (Figure 3.9A&B). To confirm that these apoptotic effects were specific to our DSS treatment, siRNA-transfected YAMC cells were treated with 5% high molecular weight DSS (500kD), which did not induce colitis or intestinal epithelial apoptosis in wildtype or Raf KO^{IE} mice (data not shown). Similar to our in vivo findings, high molecular weight DSS treatment did not induce significant apoptosis in cell culture compared to treatment with 5% low molecular weight DSS (P=0.01)(Figure 3.10). IL-13 was used as a negative control to demonstrate Raf-independent apoptosis (152).

By five hours, a majority of DSS-treated cells underwent apoptosis and detached from the plate. To address the mechanisms involved in this effect, lysates from cells treated for time points leading up to five hours were subjected to Western blot analysis for pro- and anti-apoptotic signaling pathways. TNF, which promotes both cell survival and death in these cells (135), served as a positive control. Raf siRNA is specific to Raf-1 and does not block expression of

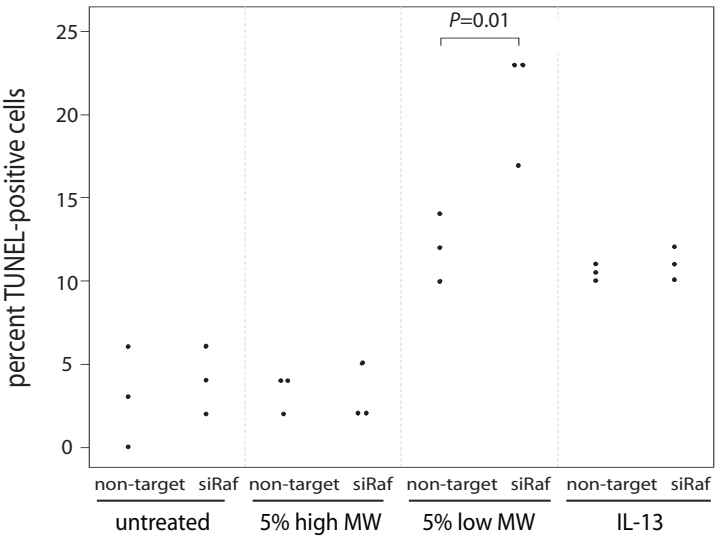

Figure 3.9: 36-50 kD DSS induces apoptosis in vitro. Transfected cells were treated with IL-13 (10 ng/ml), 5% high (500kD) or low (36-50kD) molecular weight DSS for 7 h and the number of apoptotic cells were quantified by TUNEL assay.

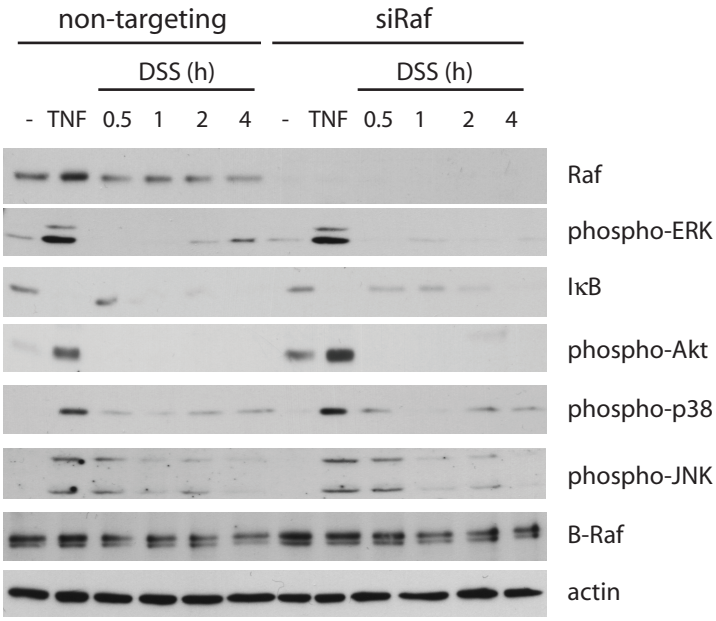

Figure 3.10: Raf is required for DSS-induced ERK and NF-κB activation. Non-targeting or Raf siRNA-transfected YAMC cells were treated with 5% DSS for the times indicated. Western blots with whole cell lysates from DSS- or TNF- (100 ng/ml, 15 min) treated siRNA-transfected cells were probed for Raf, B-Raf, phospho-ERK, IκB, phospho-Akt, phospho-p38, phospho-JNK, and actin antibodies.

B-Raf isoform (Figure 3.11). 5% DSS stimulated the pro-apoptotic pathways p38 and JNK regardless of Raf expression. In contrast, DSS-induced ERK phosphorylation and I□B degradation required Raf (Figure 3.11). Interestingly, DSS did not induce Akt phosphorylation, suggesting that in this model Raf is only required for ERK and NF-□B activation.

To determine whether Raf activation of MEK/ERK signaling or NF-□B mediates cell survival in response to DSS, TUNEL was performed on YAMC cells either co-treated with a MEK inhibitor, PD98059 (20□M) or U0126 (10□M), and 5% DSS for 7 h or transfected with siRNA directed against I□B□ to induce activation of NF-□B. While both MEK inhibitors blocked ERK activation in response to DSS (Figure 3.12B), neither increased susceptibility to DSS-induced apoptosis (Figure 3.12A), indicating that MEK activation is not required for cell survival after DSS exposure. Inhibition of MEK using U0126 did not block DSS-induced apoptosis in the absence of Raf expression (Figure 3.13) further confirming that Raf-mediated cell survival occurs independent of MEK activation.

Using siRNA to knock down expression of NF-□B p65, Raf and/or I□B□, we showed that NF-□B signaling is involved in Raf-induced cell survival (Figure 3.14&15). Deletion of I□B□ promoted constitutive translocation of NF-□B p65 subunit to the nucleus (Figure 3.14). Furthermore, DSS-induced NF-□B p65 translocation was partially inhibited in the absence of Raf. Following treatment with 5% DSS for 7 h, TUNEL staining shows that either absence of NF-□B p65 or Raf absence promotes DSS-induced apoptosis to a similar extent (Figure 3.15)(P=0.005, P=0.002). Furthermore, constitutive activation of NF-□B with

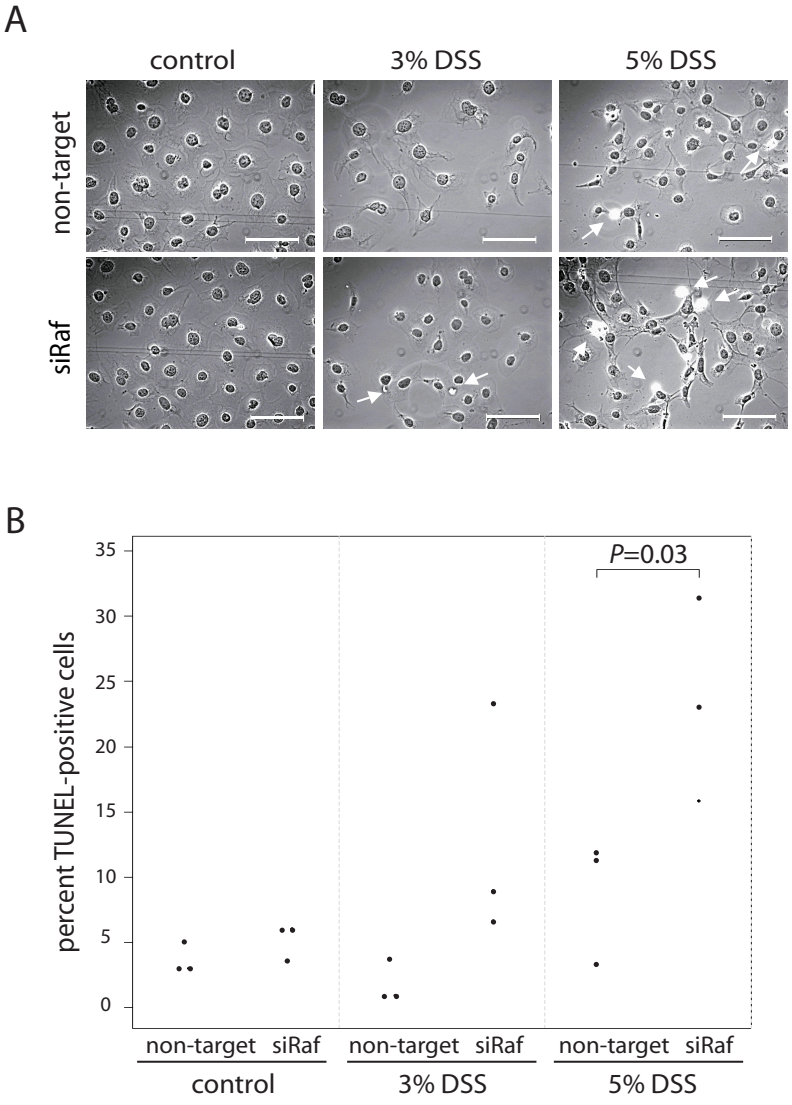

Figure 3.11: Raf is required for intestinal epithelial cell survival in response to DSS. YAMC cells were transfected with non-targeting or Raf siRNA and treated with 3 or 5% DSS for 7 h. (A) Apoptosis was determined by TUNEL assay (Scale = 100μm) and (B) the percent of TUNEL-positive cells was assessed.

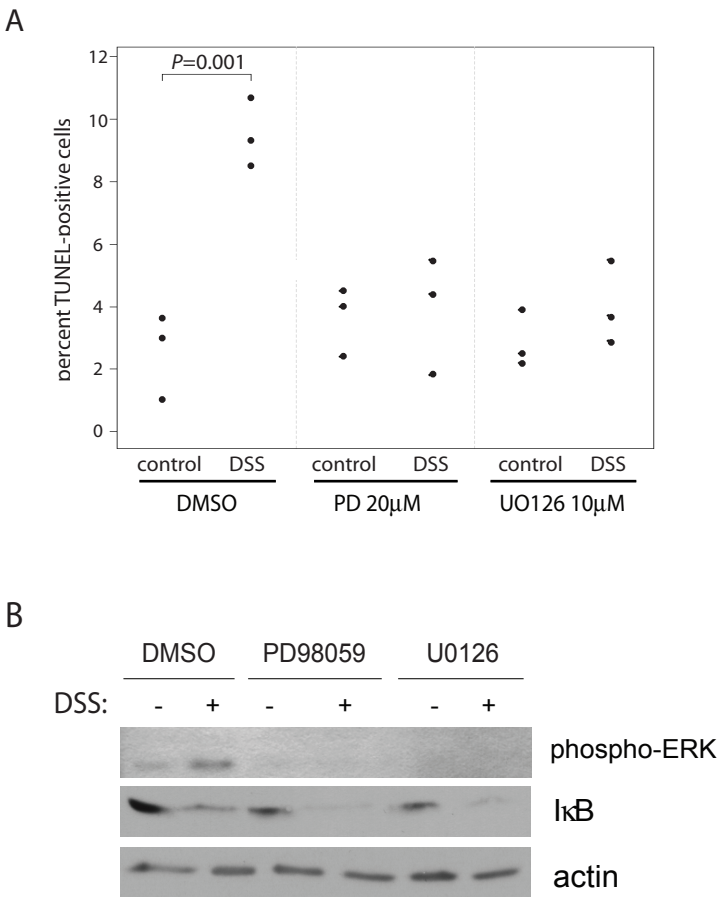

Figure 3.12: MEK activation promotes DSS-induced apoptosis. YAMC cells were treated with MEK inhibitors, PD98059 (20 μM) or U0126 (10 μM), and 5% DSS for 7 h. (A) Apoptosis was detected by TUNEL and (B) whole cell lysates were blotted with anti-phospho-ERK, IκB, and actin.

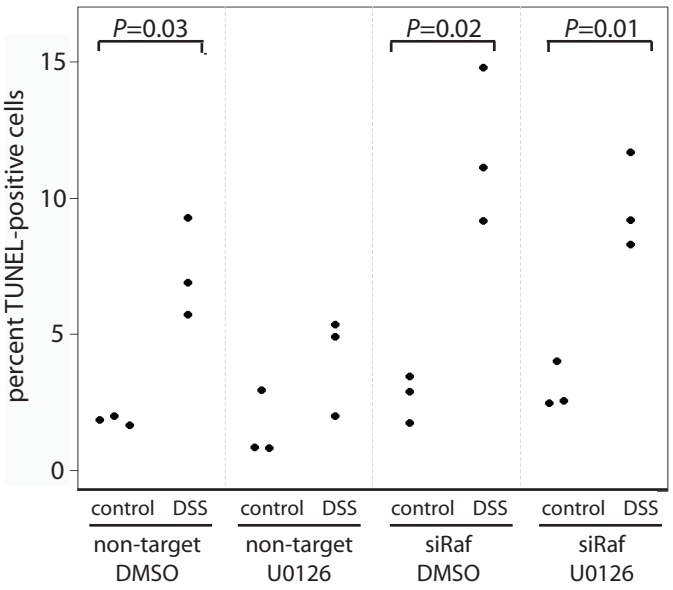

Figure 3.13: MEK inhibition does not block DSS-induced apoptosis in the absence of Raf. YAMC cells were transfected with non-targeting or Raf siRNA and treated with U0126 (10μM) and 5% DSS for 7h.

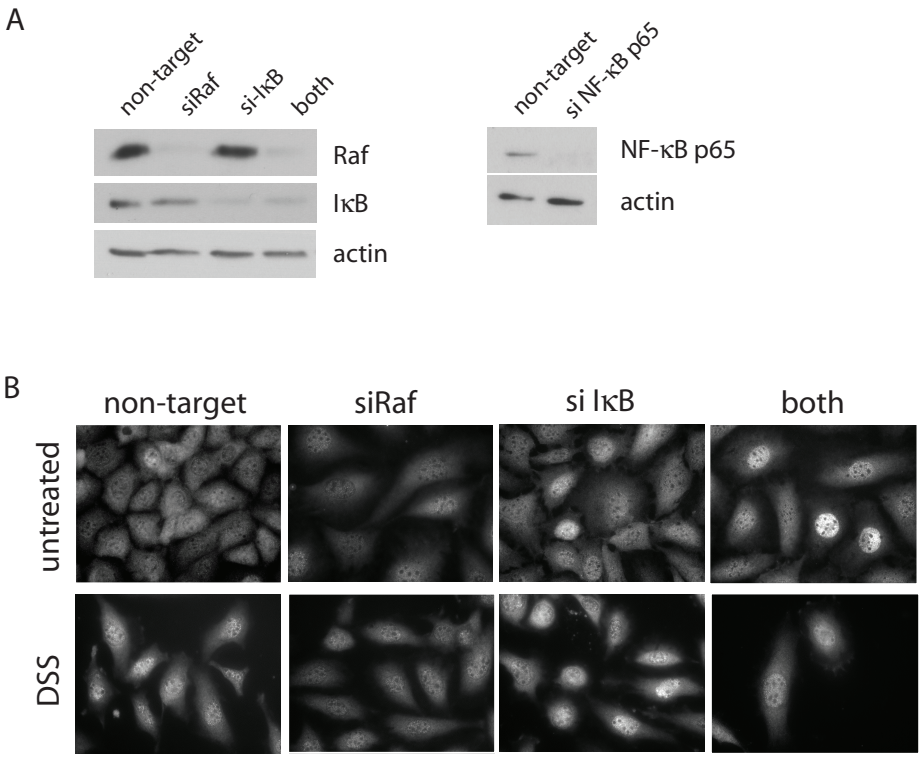

Figure 3.14: Raf is required for NF-κB nuclear translocation in response to DSS treatment. (A) Western blot analysis was performed on NF-κB p65, Raf or IκBα siRNA-transfected YAMC cells to detect Raf, IκB, NF-κB p65 and actin expression. (B) NF-κB p65 nuclear translocation was detected in untreated or 5% DSS-treated (2 h) non-targeting, IκBα, or Raf siRNA-transfected cells by immunocytochemistry. Scale bar = 20μm

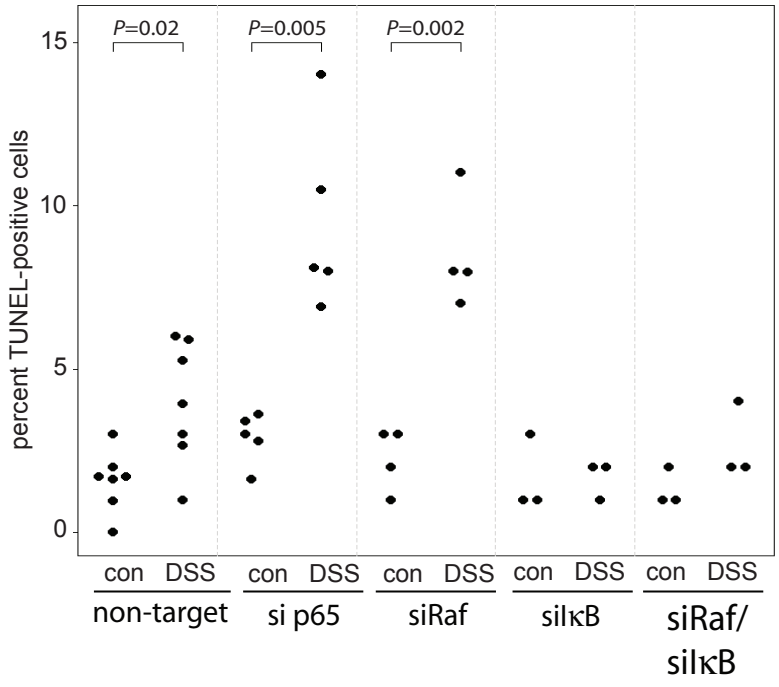

Figure 3.15: NF-κB activation rescues DSS-induced apoptosis in the absence of Raf expression. Apoptosis in 5% DSS-treated NF-κB p65, Raf and/or IκBα siRNA-transfected YAMC cells was assessed by TUNEL assay.

I□B□ siRNA rescues Raf siRNA-transfected cells from DSS-induced apoptosis. This requirement for Raf in NF-□B activation was confirmed in vivo, as nuclear NF-□B p65 was increased in the colon epithelium following DSS injury in wildtype, but not Raf KO^{IE} mice (Figure 3.16). Taken together, these data demonstrate that Raf regulates NF-□B activation in a MEK-independent manner to promote colon epithelial cell survival in response to injury.

Discussion

In this study, we demonstrate a MEK-independent requirement for Raf in anti-apoptotic signaling in response to colonic epithelial injury. DSS-induced cell damage promotes Raf-dependent activation of both MEK/ERK/MAPK and NF-□B signaling in mouse colon epithelial cells both *in vivo* and *in vitro.* In the absence of Raf expression, DSS-induced apoptosis is increased in cell culture and in intact colon epithelium. Interestingly, ERK activation promotes apoptosis in response to DSS-induced injury, suggesting that this pathway is not involved in Raf-stimulated cell survival. However, Raf may directly or indirectly promote ERK activation to enhance epithelial cell proliferation during recovery from colitis. In contrast, loss of NF-□B p65 expression induced apoptosis following DSS exposure, and constitutive activation of NF-□B signaling rescued Raf-null colon epithelial cells from apoptosis. *In vivo* studies indicate that NF-□B nuclear translocation is decreased in Raf KO^{IE} mice following DSS-induced injury

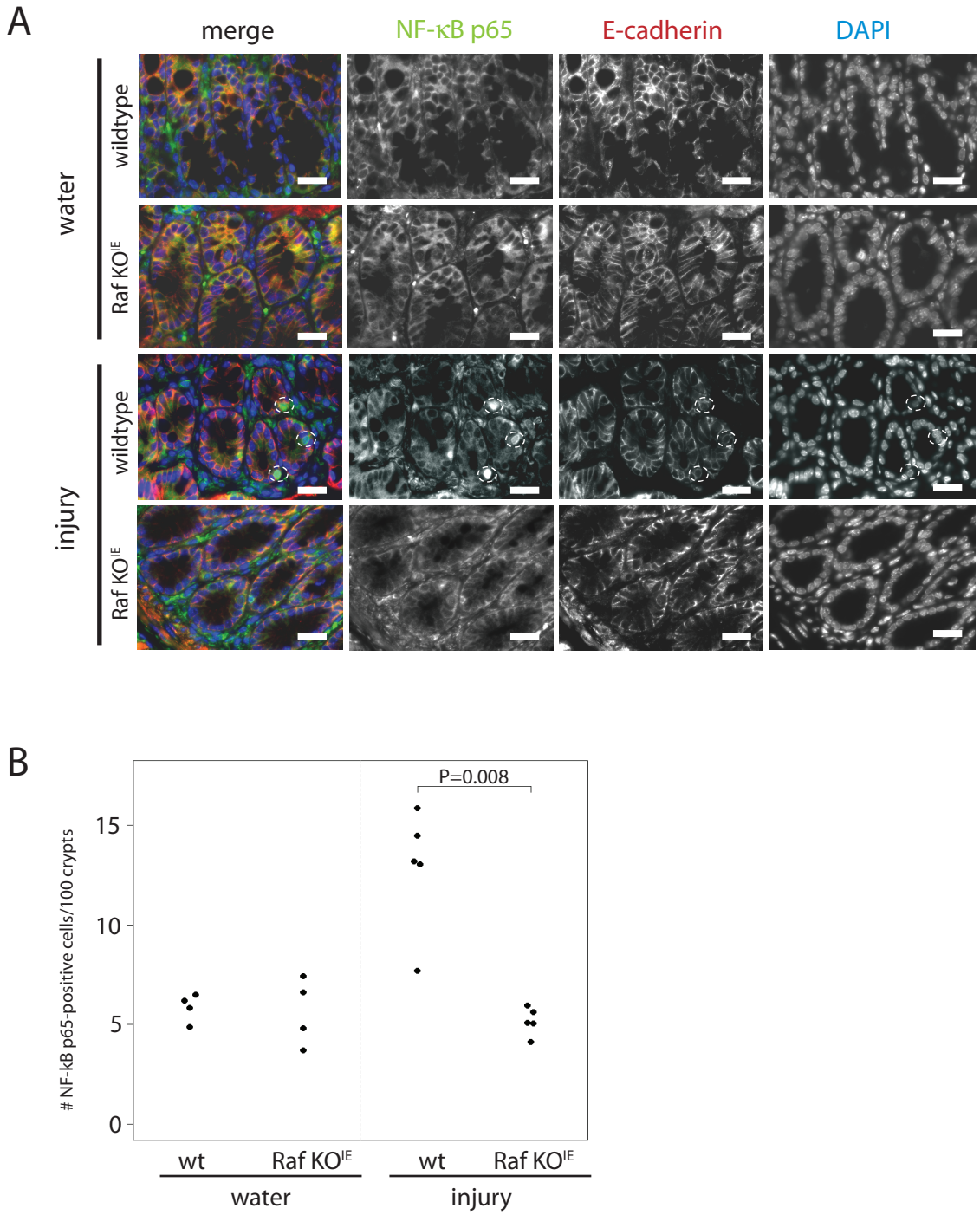

Figure 3.16: Raf promotes NF-κB activation in the colon epithelium in response to DSS-induced injury. Representative images are shown of immunofluorescence for NF-κB p65 (green), E-cadherin (red) and DAPI-stained nuclei (blue) in colon sections of wildtype and Raf KO^{IE} mice following water treatment or DSS-induced injury. Scale bars = 20μM

compared to wildtype. Thus, MEK-independent Raf anti-apoptotic signaling is mediated through NF-κB to protect against acute colitis.

Raf KO^{IE} mice develop more severe colitis, with more extensive crypt damage and inflammation during acute injury, than wildtype mice. Furthermore, Raf prevents colon epithelial cell apoptosis during DSS colitis. To address the molecular mechanism by which Raf promotes cell survival, we used a cell culture model of DSS treatment previously used to characterize Caco-2/bbe-mediated barrier function (153). Knockdown of Raf expression decreased both ERK and NF-κB activation in response to DSS (Figure 3.8). Given previous reports of MEK/ERK anti-apoptotic function (136,154), we hypothesized that Raf activation of this pathway was responsible for promoting cell survival following DSS treatment. However, our findings show that MEK activity is required for DSS-induced apoptosis (Figure 3.12). These data are consistent with previous studies showing ERK promotes hydrogen peroxide-induced cell death by activating caspase-3 in renal epithelial cells (155,156). In the same cell type, *E. coli* toxin promoted ERK-induced apoptosis in a caspase-independent manner (157). These results are consistent with *in vivo* experiments using the MEK inhibitor, U0126, showing decreased inflammation and epithelial apoptosis during cisplatin-induced acute renal failure (158). Our findings expand the known functions of MAPK signaling in the colon epithelium by demonstrating that Raf stimulates colon epithelial cell proliferation in response to injury, and that ERK signaling triggers a pro-apoptotic response to DSS. Thus, Raf promotes colon epithelial cell survival in a MEK-independent manner.

In addition to directly or indirectly activating MEK/ERK signaling, Raf expression is required for I□B degradation and subsequent NF-□B activation following DSS exposure (Figures 3.11, 3.14 and 3.16). Several studies have targeted NF-□B as a potential therapeutic target for IBD; however, NF-□B promotes both apoptosis and cell survival in a cell context-dependent manner. NF-□B p65 antisense oligonucleotides decreased the severity of colitis in trinitrobenzene sulfonic acid and IL-10$^{-/-}$ colitis models (159). Mice deficient in A20, a cytoplasmic ring finger protein that inhibits NF-□B activity, develop spontaneous colitis at 4 weeks of age (160). In contrast, disruption of NF-□B activation specifically in the intestinal epithelium demonstrates that NF-□B has a protective effect against apoptosis and colitis. Intestinal deletion of I□B kinase □ (IKK□), one of three IKK isoforms involved in NF-□B activation, increases susceptibility to DSS colitis and apoptosis, yet targeted deletion of IKK□ in myeloid cells did not affect cell survival (126). Mice deficient for intestinal epithelial cell NEMO (IKK□) develop spontaneous colitis and NEMO-deficient intestinal epithelial cells are highly susceptible to TNF-induced apoptosis (161). RelA-intestinal epithelial-deficient mice are more sensitive to DSS colitis and with increased epithelial apoptosis in response to injury (162), further supporting a role for NF-□B in cell survival in response to injury and inflammation.

In these studies we show that Raf regulates NF-□B activation in a MEK-independent manner in intestinal epithelial cells (Figures 3.12 & 3.15). Our findings are supported by previous reports that a Raf mutant (Raf-BXB T481A)

which cannot bind MEK, and thus blocks ERK activation, still activates a NF-κB reporter construct in NIH 3T3 cells (50).

Baumann et al. demonstrated that NF-κB is required for Raf-mediated oncogenic transformation of fibroblasts. In T-cells, Raf promoted activation of a NF-κB reporter gene independent of MEK/ERK activation, but dependent upon Raf kinase activity (163). In our study, IκB degradation, NF-κB p65 nuclear translocation, and colon epithelial cell survival in the presence of DSS were Raf-dependent. We hypothesize that Raf may function in conjunction with other known upstream regulators of NF-κB signaling such as NIK or IKK, since knockdown of Raf expression did not completely block NF-κB p65 nuclear translocation (Figure 3.14). Thus, these data provide the first evidence that Raf mediates NF-κB activation using a disease model and *in vitro*, using non-transformed cells.

During recovery from initial injury, we found that wildtype and Raf KO^{IE} mice injury and inflammation scores were similar. This may be attributed to the significant increase in apoptosis seen in both wildtype and Raf KO^{IE} mice during remodeling of the injured colon epithelium (Figure 3.5). In fact, increased infiltrating inflammatory cells appear necessary for epithelial regeneration in the DSS model. Pull et al. showed that activated macrophages are required to promote regeneration of the damaged epithelium by eliciting colon epithelial progenitor cell responses (164). No apparent difference in the number of macrophages (anti-F4/80) or neutrophils were detected by immunohistological staining between wildtype and Raf KO^{IE} mice following injury or recovery (data

not shown). Although similar defects in injury repair have been reported in the DSS colitis model for TLR4 and Cox-2 knockout mice (148,149), we found no change in expression of either protein in the colon in the absence of Raf or in response to DSS (data not shown).

The proliferation defect during recovery in Raf KO^{IE} mice is likely due to impaired MEK-dependent pathways in the colonic crypt. Increased levels of phosphorylated ERK are present in the cytoplasm of wildtype mouse cells throughout the crypt axis during recovery, whereas activated ERK is restricted to the surface epithelium in Raf KO^{IE} mice similar to untreated mice (Figure 3.8). These data are consistent with reports in various cell types in which transient ERK activation in the cytoplasm promotes proliferation, while sustained ERK activation in the nucleus induces growth arrest and differentiation (135,165,166). Thus, Raf-1 specific activation of MEK may be required for maximal proliferation following DSS-induced injury. Supporting this conclusion, a recent report by Scholl et al. shows that even in the presence of activated Raf, loss of MEK1/2 expression inhibits hyperproliferation in the mouse epidermis (167). Taken together, these findings suggest that Raf directly or indirectly regulates MEK/ERK-mediated colon epithelial proliferation in response to injury.

Overall, these findings establish a novel role for Raf kinase in both the anti-apoptotic and hyperproliferative epithelial responses to injury in the colon. Our model suggests Raf initiates MEK-independent NF-□B activation to reduce the apoptotic response to injury, while potentially increasing MEK-dependent ERK activation to enhance proliferation and rapidly restore the colon epithelium

during acute colitis. Given the link between these responses and colitis-associated carcinogenesis, our findings suggest future studies are needed to better define the role of Raf-1 in response to chronic injury. In this regard, pharmacological inhibition of Raf signaling is a current target of cancer therapeutic drug development (168). These findings have important implications for human disease, as patients with inflammatory bowel disease are at an increased risk to develop colon cancer with greatest risk to those with repeated cycles of inflammation (169). A key challenge is to understand how Raf activates NF-□B-dependent pathways in intestinal epithelial cells following injury and to determine if there is a pathogenic role in colitis-carcinogenesis transition.

Acknowledgements

Raf $^{flx/flx}$ mice were provided by Manuela Baccarini (Vienna Biocenter, Austria) and villin-Cre mice were provided by Sylvie Robine (Curie Institute, Paris, France). M. Kay Washington assessed and scored the histology following colitis treatment. ARIOL image analysis was performed assistance with from Frank Revetta and the Vanderbilt Ingram Cancer Center. Tatsuki Koyama provided assistance with the statistical analysis of the data.

CHAPTER IV

TNFR1 PROMOTES TNF-MEDIATED MOUSE COLON EPITHELIAL CELL SURVIVAL THROUGH RAF ACTIVATION OF NF-□B

Introduction

In the gastrointestinal tract, increased levels of tumor necrosis factor (TNF) promote the pathogenesis of several diseases including, inflammatory bowel disease (IBD)(170,171), celiac disease (172), graft-versus-host disease (173), and non-steroidal anti-inflammatory drug (NSAID) enteropathy (174). Clinical studies indicate that circulating serum levels of TNF are increased in Crohn's disease patients (175), and neutralizing TNF monoclonal antibodies have been shown to reverse disease activity in IBD patients (115,116), 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 (176), while mice deficient in TNF production develop worse colitis in response to dextran sulfate sodium (DSS) than their wildtype counterparts (177). Therefore, further study is required to delineate the role of TNF signaling in the colon epithelium.

TNF binds two cell surface receptors: TNF receptor (R)1 (p55/60) or TNFR2 (p75/80) (reviewed in (178,179)), with a higher ligand affinity for TNFR2 (180,181). Each receptor promotes different cellular responses in a cell context-dependent manner (182,183). In the colon epithelium, TNF signals through TNFR2 to promote proliferation (184) and migration (185), whereas sustained

activation of mitogen-activated protein kinase (MAPK) signaling through TNFR1 induces growth arrest (135). In contrast, in intestinal myofibroblasts, TNFR2 stimulates proliferation through MAPK activation (186). Although it is known that TNFR1 can promote apoptosis through death domain-dependent signaling (81), several reports indicate that TNF promotes anti-apoptotic signaling pathways in a cell context-dependent manner (86,136,187-189). Considering this single ligand induces signaling 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-□B activation. Loss of KSR shifts cells toward an apoptotic program following TNF exposure (136). 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 (32,136,190), is a serine/threonine kinase which regulates several cellular functions from proliferation and differentiation to survival (150). Canonical Raf activation through receptor tyrosine kinase signaling is well-characterized (191); however, little is known about the mechanism of Raf activation in cytokine signaling. A

requirement for Raf in cell survival was demonstrated in Raf knockout mice, which are embryonic lethal due to Fas-mediated hepatocellular apoptosis and placental defects (11,53). Conditional tissue-specific Raf deletion shows that Raf is required for both cardiomyocyte, through the regulation of apoptosis signal-regulating kinase 1 (ASK1) (56), and macrophage survival following *S. typhimurium* infection (57). Furthermore, in inducible intestinal epithelium-specific Raf knockout mice ($Raf^{flx/flx}$; villin-Cre ERT2), Raf enhances colon epithelial cell survival during acute colitis through an nuclear factor □-B (NF-□B)-dependent mechanism (Edelblum and Polk, unpublished observations).

TNF is a potent inducer of NF-□B, which regulates cell survival and the production of inflammatory cytokines (192). NF-□B p50/p65 dimers are sequestered in the cytoplasm through binding interactions with inhibitor of □B (I□B). TNF induces phosphorylation of I□B by the I□B kinases (IKK), leading to I□B ubiquitination and proteasomal degradation, and subsequent NF-□B nuclear translocation. In the nucleus, NF-□B 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 (IAPs)(82). Although NF-□B promotes both cell survival and cell death in a cell context-dependent manner (192), conditional inactivation of the NF-□B pathway shows a requirement for NF-□B in colon epithelial cell survival following inflammatory stimuli (126,161).

In this report 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 knockout mouse (Raf KO^{IE}), we demonstrate that Raf expression is required for TNFR-induced cell survival both *in vivo* and *in vitro*. While 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. Raf was required for TNF-induced NF-κB p65 phosphorylation and cIAP1 expression *in vivo*, suggesting that Raf mediates colon epithelial cell survival through activation of NF-κB in a MEK-independent manner downstream of TNFR1.

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 (185). To further delineate the role of TNFR1 in the intestinal epithelium, wildtype, TNFR1 KO, TNFR2 KO, and TNFR 1/2 double knockout (DKO) mice were injected i.p. with PBS or TNF (10^4 units). After 24 h, mice were sacrificed and apoptosis was assessed by *in situ* oligo ligation (ISOL). TNFR1 knockout mice exhibited a significant increase in apoptosis following TNF

treatment compared to wildtype (Figure 4.1) (P<0.01). Neither TNFR2 KO nor DKO mice showed enhanced to 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 TNF exposure, TNFR1 KO cells showed higher levels of apoptosis compared to the untreated control and TNFR1 addback cells treated with TNF (Figure 4.2). 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 not blocked; however, re-expression of TNFR1 in these cells restored activation of these signaling pathways (Figure 4.3A). Activation of caspase-3 and 9 were detected in TNFR1 KO cells following longer TNF exposure, caspase activation was absent in cells re-expressing the receptor (Figure 4.3B). Subcellular fractionation was used to confirm expression of HA-tagged TNFR1 at the plasma membrane of the TNFR1 addback cells (Figure 4.3C). 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 towards cell death.

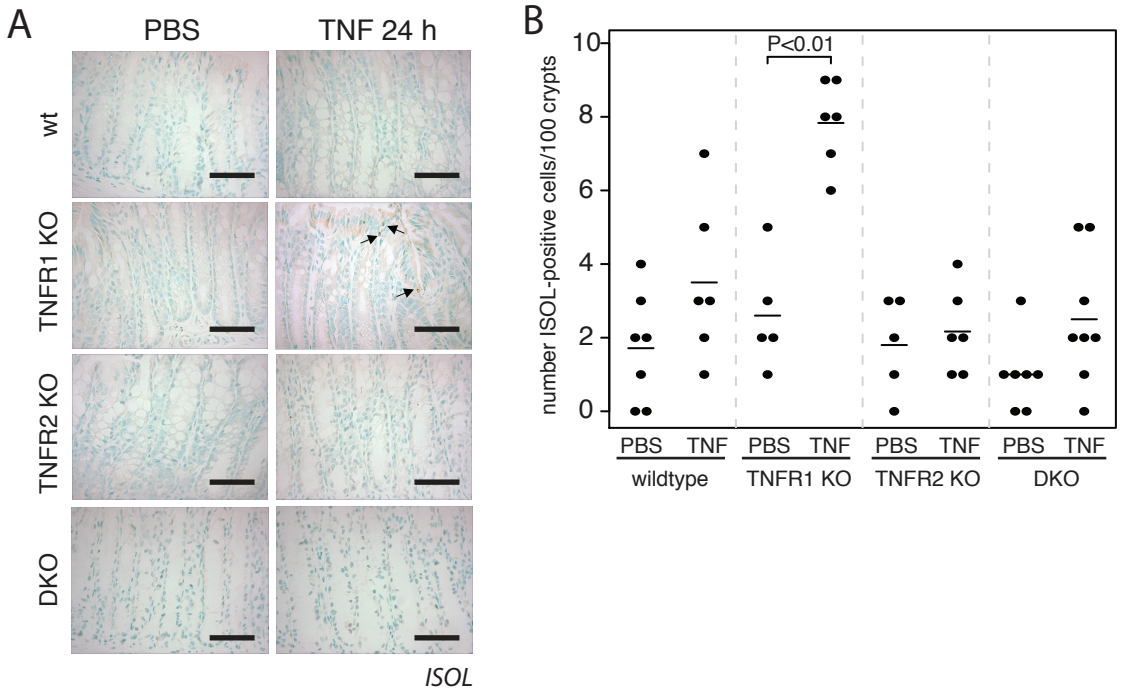

Figure 4.1: TNFR1 mediates colon epithelial cell survival signaling. TNFR1, TNFR2, and TNFR double knockout (DKO) mice were treated with TNF for 24 h. (A) Apoptosis was assessed by ISOL staining (Scale bar = 62μM) and (B) quanitified as the number of positive cells per 100 crypts. Bars represent the mean.

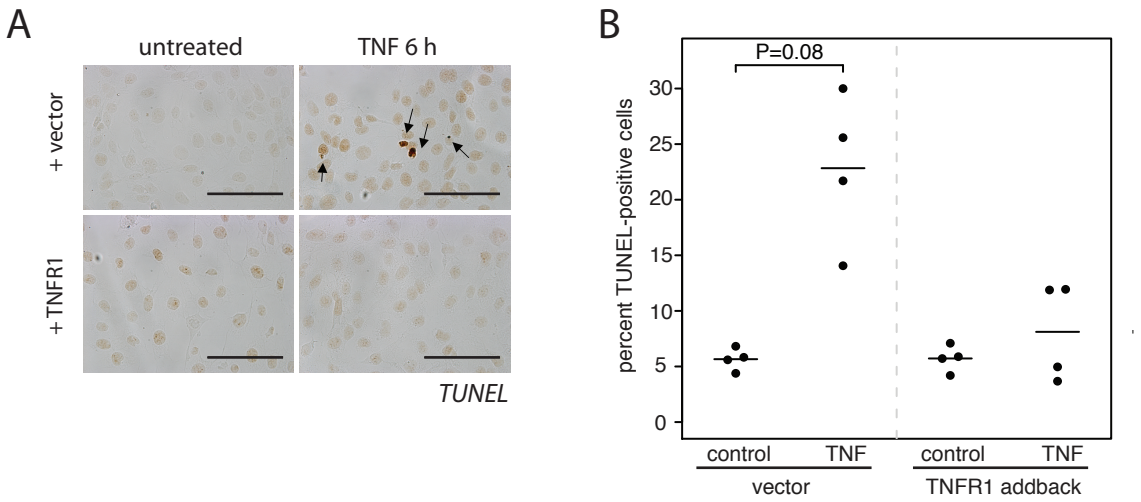

Figure 4.2: TNFR1 is required for TNF-mediated cell survival in vitro.
(A) TUNEL staining of TNFR1 KO colon epithelial cells and TNFR1 addback cells treated with TNF (100 ng/ml) for 6 h. Scale bar = 100μM (B) Graphical representation of the percentage of TUNEL-positive cells.

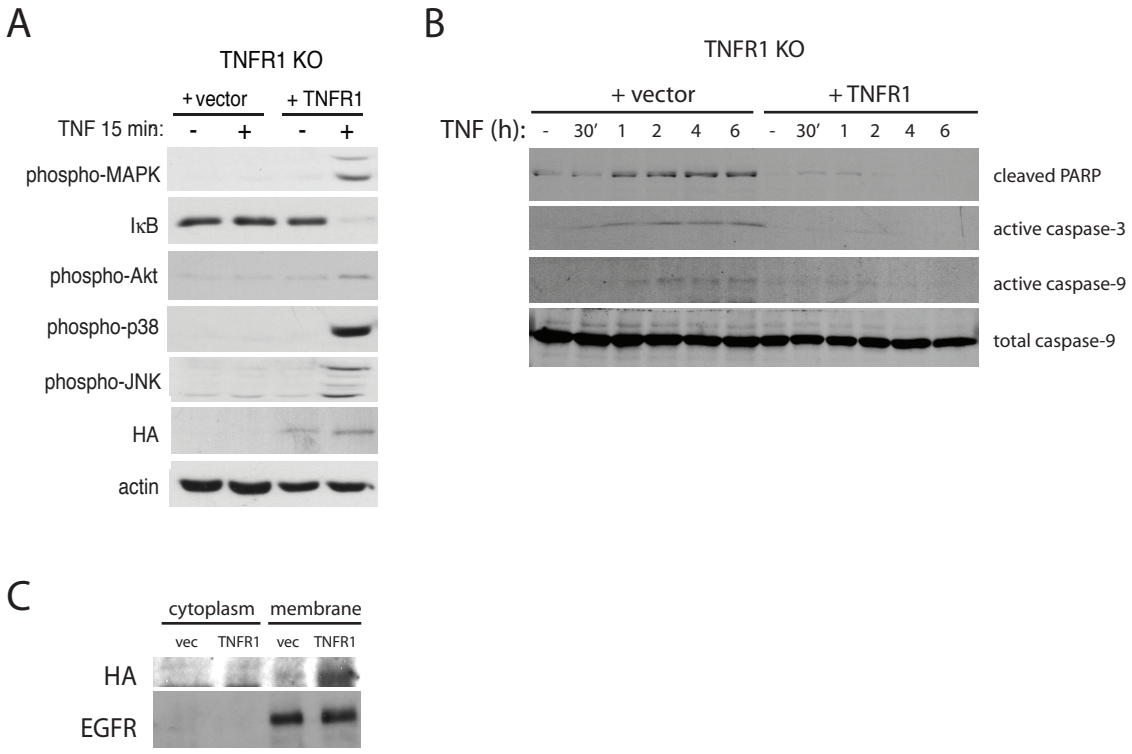

Figure 4.3: TNF-induced activation of TNFR1 promotes both pro- and anti-apoptotic signaling pathways. (A) Western blot analysis of TNFR1 knockout cells expressing vector or TNFR1 treated with TNF (100 ng/ml) for 15 min. Whole cell lysates were blotted for phospho-MAPK, IκB, phospho-Akt, phospho-p38, phospho-JNK, and actin. (B) Western blot analysis was performed on TNFR1 knockout cells expressing vector or TNFR1 following 30 min-6 h TNF exposure for cleaved poly-ADP ribose polymerase (PARP), active caspase-3 and -9. Total caspase-9 was used as a loading control. (C) Western blot analysis for HA was performed on cytoplasmic and membrane fractions from TNFR1 knockout cells expressing vector or TNFR1. EGFR was used as a positive control for membrane proteins.

TNF promotes MAPK activation in a Ras-independent manner.

Raf kinase activation requires membrane translocation and activation by phosphorylation at several sites (29). The canonical mechanism by which Raf is recruited to the membrane involves Ras GTPase (193). To assess whether TNF stimulation promotes Ras activation in colon epithelial cells, YAMC cells were treated with TNF or EGF (Figure 4.4A) and the level of activated Ras determined by Ras-GTP pulldown assay. Western blot analysis from the pulldown assay 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 (194). Consistent with Ras activation patterns, expression of dominant-negative Ras blocked EGF-, but not TNF-stimulated ERK activation (Figure 4.4B). These findings demonstrate a novel mechanism by which ERK is activated in a Ras-independent manner.

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 (Figure 4.5). Taken together, these data demonstrate that TNF promotes MAPK activation in a Ras-independent manner.

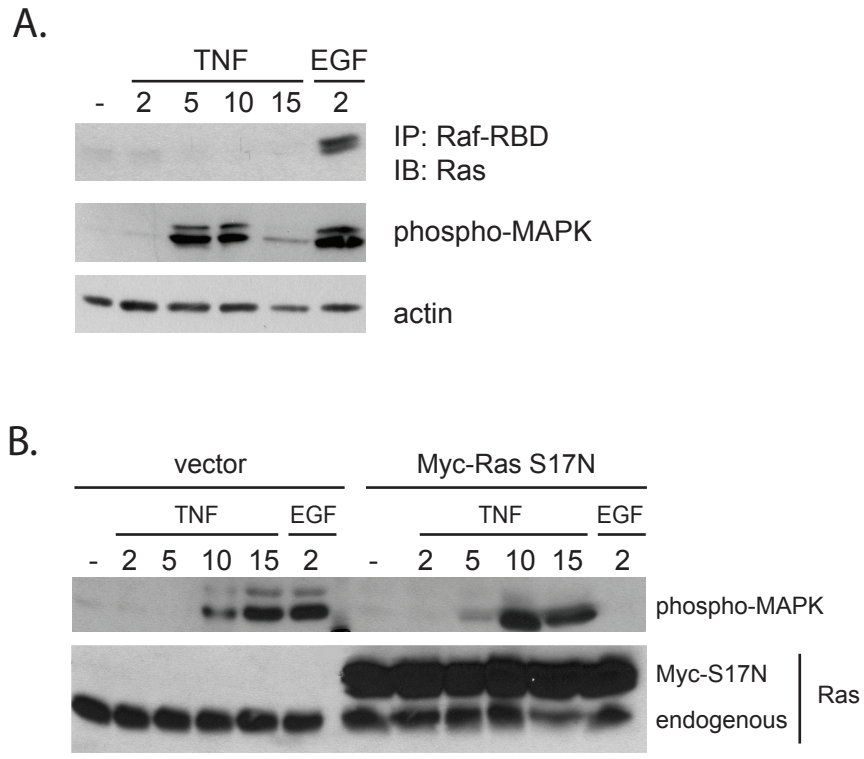

Figure 4.4: TNF promotes MAPK activation in a Ras-independent manner. (A) YAMC cells were treated with TNF or EGF and activated Ras was pulled down using Raf-RBD-conjugated agarose beads. Western blot analysis was used to detect GTP-bound Ras or phospho-MAPK and actin in whole cell lysates. (B) YAMC cells were transfected with vector or Myc-tagged dominant-negative Ras (S17N) and treated with TNF or EGF. Whole cell lysates were subjected to Western blot analysis for phospho-MAPK and Ras.

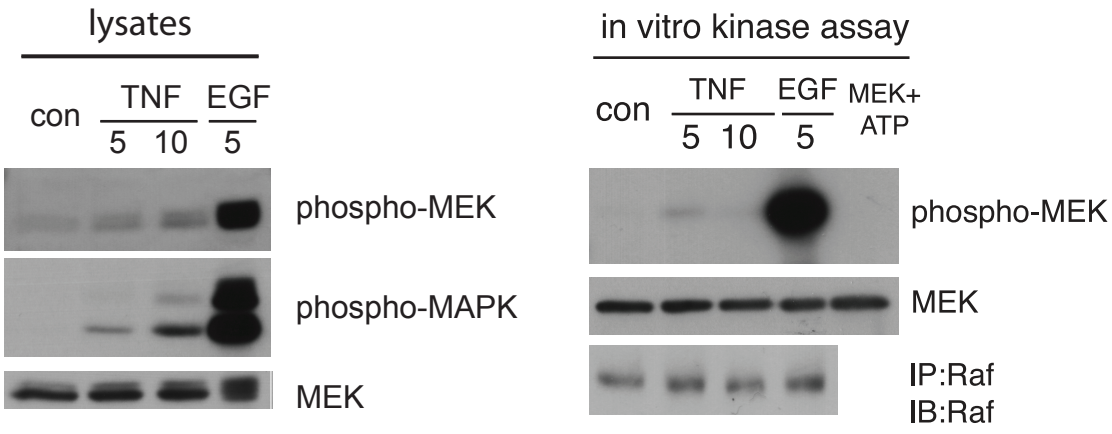

Figure 4.5: TNF stimulates Raf kinase activity.
Endogenous Raf was immunoprecipitated from YAMC cells treated with TNF (100 ng/ml) or EGF (10 ng/ml) and incubated with recombinant MEK and ATP *in vitro*. Western blot analysis was performed for Raf, phospho-MEK and phospho-MAPK. MEK was used as a loading control.

TNFR-induced cell survival is Raf-dependent.

Since Raf knockout mice are embryonic lethal (13), we generated conditional Raf knockout mice by crossing mice harboring a Raf floxed allele (57) with those constitutively expressing Cre recombinase under control of a villin promoter (137) to specifically delete Raf from the intestinal epithelium. Cre expression in the intestinal epithelium of constitutive villin-Cre mice was confirmed by immunohistochemistry (Figure 4.6). To characterize the extent of Cre recombination, constitutive villin-Cre mice were crossed with Rosa26 reporter mice. As previously described in Chapter III, X-gal staining was performed to assess LacZ expression in the colon and small intestinal epithelium. Every crypt was LacZ-positive demonstrating successful Cre recombination in the colon and small intestine (Figure 4.7). $Raf^{flx/flx}$;vilCre (Raf KO^{IE}) mice develop normally and unchallenged mice do not display a detectable intestinal phenotype. Western blot analysis was performed on epithelial and stromal fractions isolated from wildtype and Raf KO^{IE} mice colon to confirm loss of Raf expression (Figure 4.8). 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 if Raf is required for cell survival in the presence of TNF *in vivo*, we treated wildtype and Raf KO^{IE} mice with PBS or TNF for 24 h and performed ISOL staining to detect apoptotic cells in the colon epithelium. Following 24 h TNF treatment, Raf KO^{IE} mice exhibited increased apoptosis compared to wildtype (Figure 4.9)($P<0.01$), which was comparable to levels of apoptosis

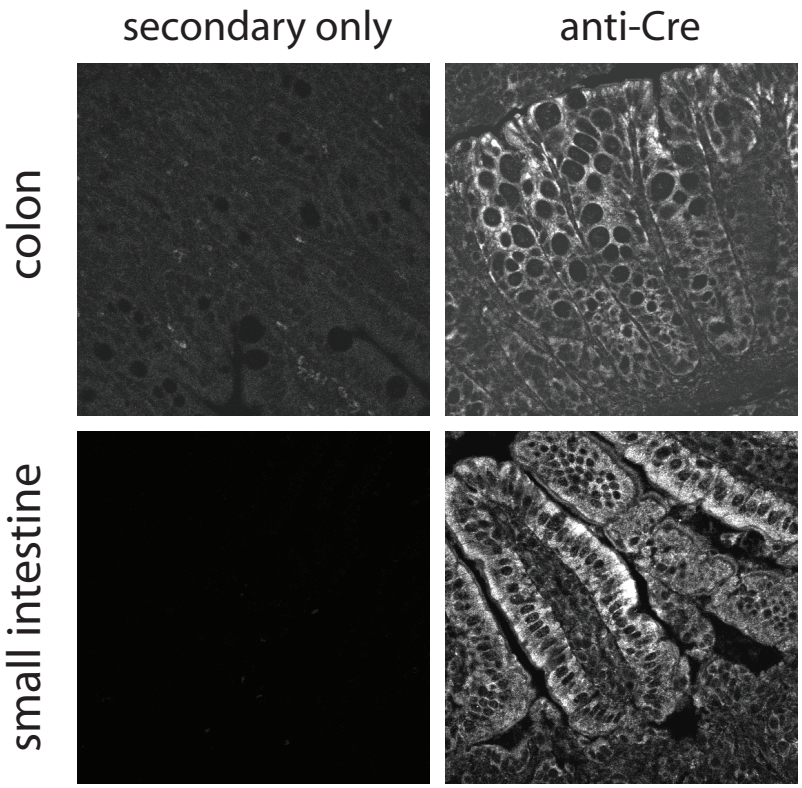

Figure 4.6: Cre expression is restricted to the intestinal epithelium. Immunohistochemistry for Cre was performed on colon sections from constitutive villin-Cre expressing mice. Sections were incubated without primary antibody as a negative control.

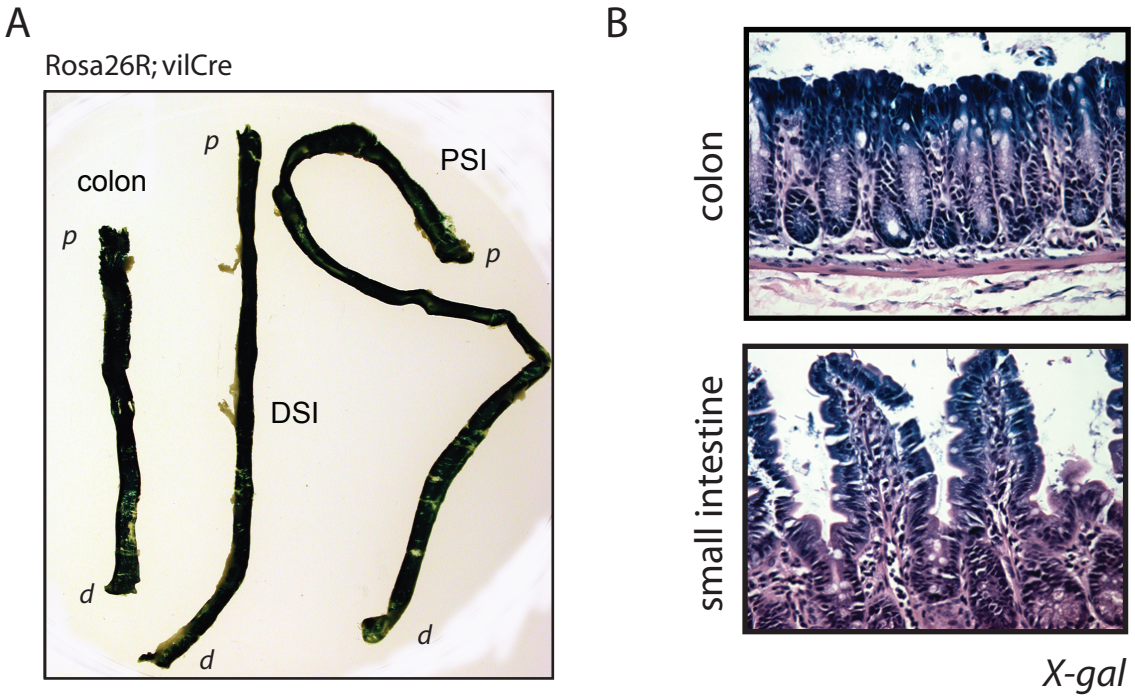

Figure 4.7: Constitutive villin-Cre expression induces LacZ recombination. (A) Whole mount images of colon, distal small intestine (DSI), proximal small intestine (PSI) from Rosa26R; vilCre mouse stained with X-gal. (*d*, distal; *p*, proximal) (B) Representative images of X-gal-stained colon and small intestine.

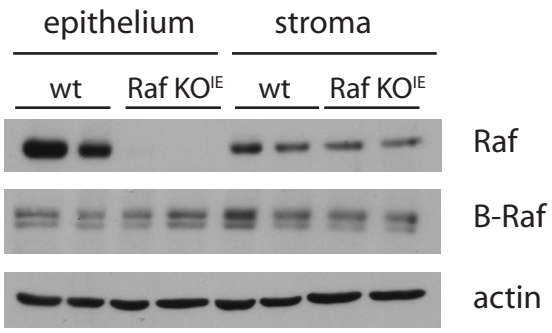

Figure 4.8: Raf expression is absent in constitutive conditional Raf knockout mice. Epithelial and stromal fractions were isolated from colons of wildtype and Raf KO^{IE} mice and subjected to Western blot analysis for Raf, B-Raf and actin.

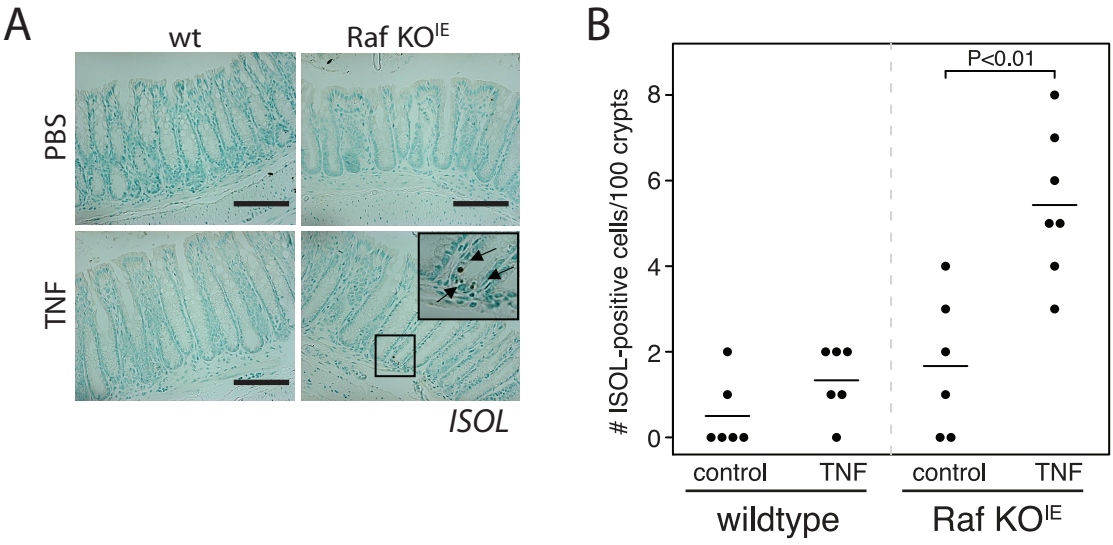

Figure 4.9: Raf protects against TNF-stimulated colon epithelial cell apoptosis. (A) ISOL was performed on colon sections from wildtype and Raf KO^{IE} mice treated with PBS or TNF. Scale bar = 125μM (B) The graph represents the number of ISOL-positive cells per 100 colonic crypts. Bars represent the mean value.

detected in TNF-treated TNFR1 KO mice (Figure 4.1). Similar findings were obtained by immunostaining for active caspase-3 (data not shown).

In addition to *in vivo* studies, an organ culture model was used to assess the role of Raf in TNF-induced cell survival. Colon explants from wildtype and Raf KO^{IE} mice were treated with TNF (50 ng/ml) for 24 h and apoptosis was detected by ISOL staining. Cell death increased over four-fold in Raf KO^{IE} explants compared to explants from wildtype mice (Figure 4.10), further demonstrating a requirement for Raf in cell survival following TNF exposure.

While Raf protects colon epithelial cells from TNF-induced apoptosis *in vivo*, we wanted to develop a Raf-null epithelial cell line to characterize downstream signaling events which are altered in the absence of Raf expression. To this end, colon and gastric epithelial cells were isolated from a Raf^{flx}; Immortomouse (Figure 4.11A). A Raf^{flx} cell line was established in culture and immunostaining was performed for E-cadherin, cytokeratin, and smooth muscle actin. While Raf^{flx} cells stained positive for E-cadherin and cytokeratin, these cells did not express the fibroblast marker, smooth muscle actin, demonstrating that the isolated cells were derived from the epithelium (Figure 4.11B). Unfortunately, expression of lentiviral and retroviral Cre in these cells failed to induce recombination of the Raf^{flx} allele.

As an alternative approach to knock down Raf expression, RNA interference was used *in vitro* to confirm the role of Raf in colon epithelial cell survival in response to TNF. Non-targeting or Raf siRNA duplexes were transfected into YAMC cells, and Western blot analysis indicated that Raf siRNA was specific to

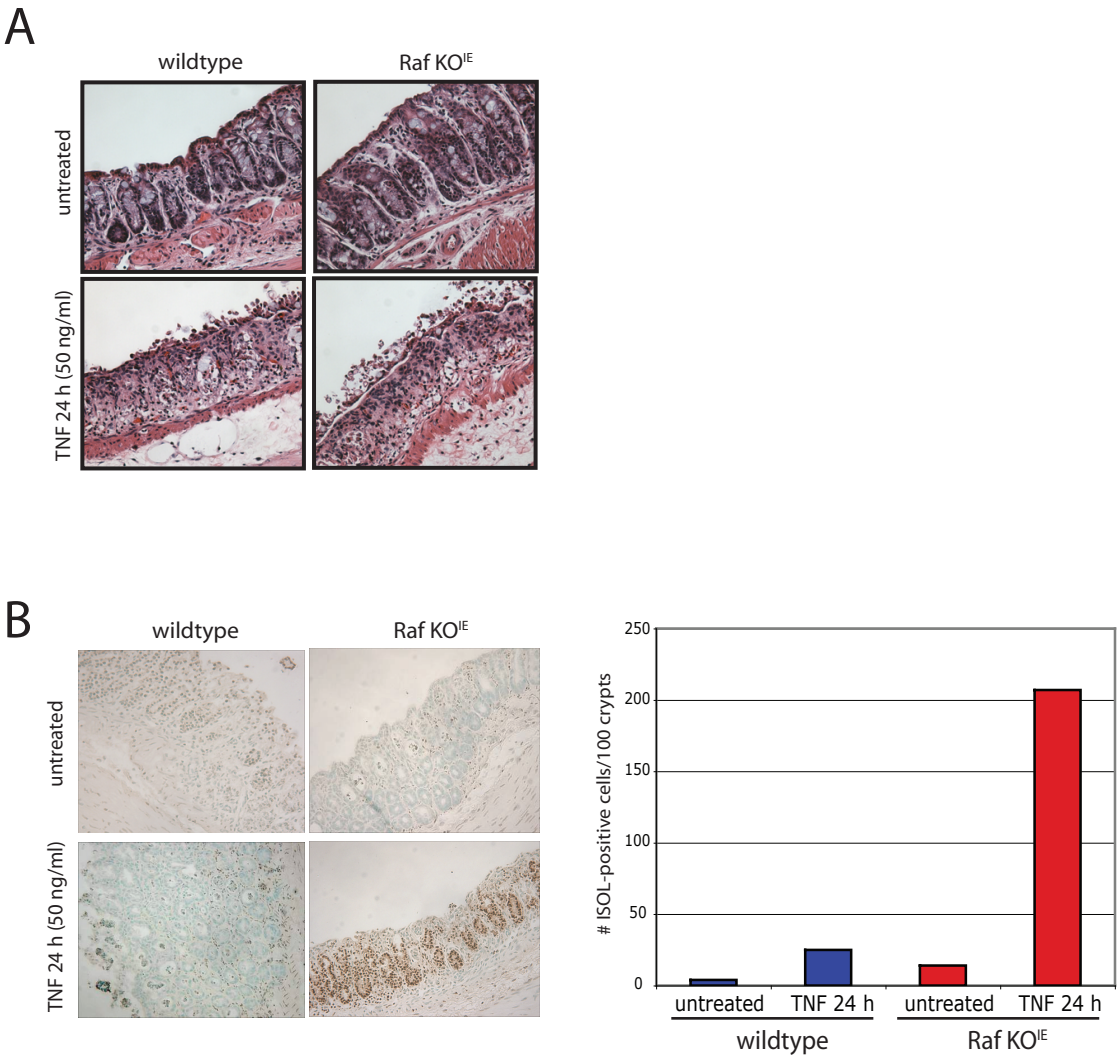

Figure 4.10: Raf promotes cell survival in response to TNF treatment in an organ culture model. Colon explants from wildtype and Raf KO^{IE} mice were treated with TNF (50 ng/ml) for 24 h. (A) H&E staining and (B) ISOL staining of colon explants. The number of ISOL-positive cells/100 crypts is represented graphically.

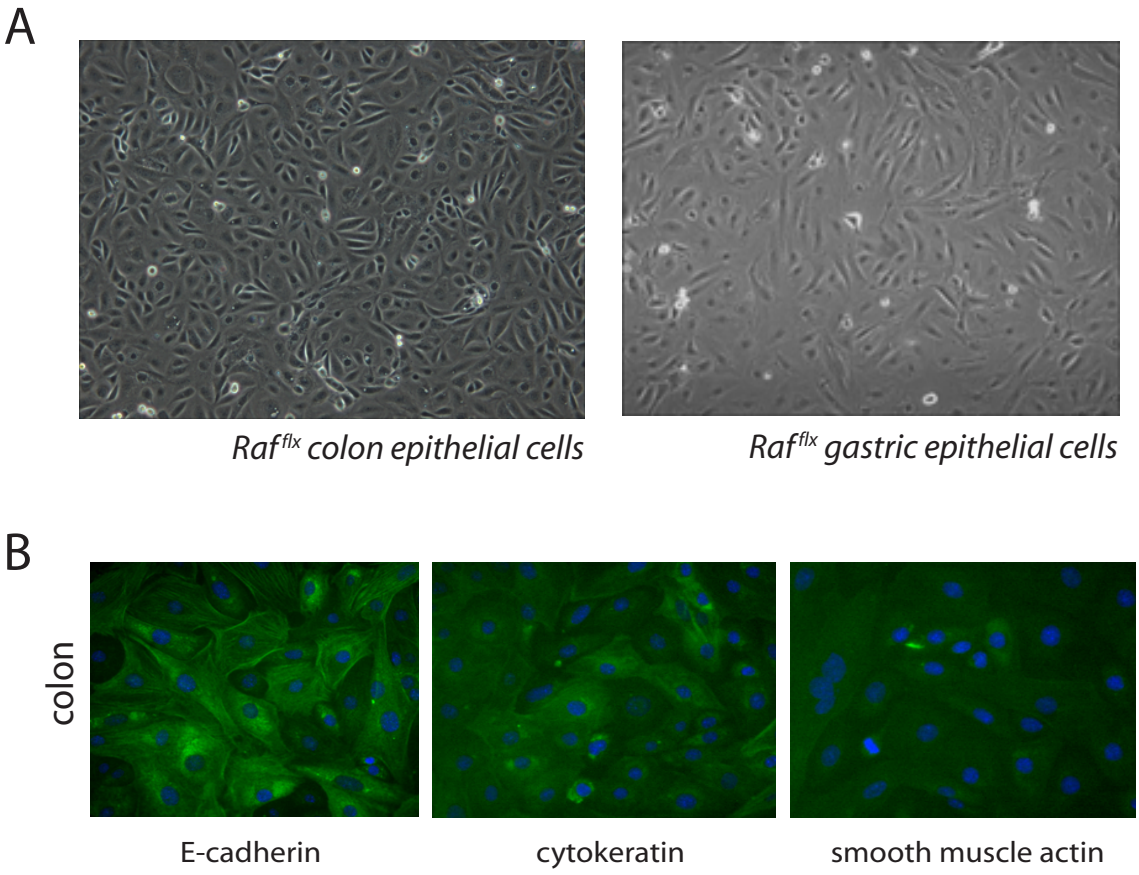

Figure 4.11: Isolation and characterization of Raf ^{flx} colon and gastric epithelial cells. (A) Phase contrast image of Raf ^{flx} colon and gastric epithelial cells isolated from Raf ^{flx};Immortomice. (B) Epithelial cell lineage of Raf ^{flx} colon cells was confirmed by immunostaining for E-cadherin, cytokeratin, and smooth muscle actin.

Raf-1 and had no effect on B-Raf expression (Figure 4.12A). Following 6 h TNF treatment, siRNA-transfected YAMC cells were incubated with a cell-permeable, fluorescent-tagged caspase inhibitor (SR-VAD-FMK) to detect cells actively undergoing apoptosis (Figure 4.12B&C). TNF-induced apoptosis was significantly increased in the absence of Raf, 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*.

To determine if Raf kinase activity is required for cell survival in response to TNF, TUNEL assays were performed on YAMC cells pretreated with DMSO or a Raf-1 kinase inhibitor (GW5074) for 30 min, followed by TNF for 6 h. While TNF or the Raf kinase inhibitor alone did not have an effect on colon epithelial cell survival, inhibition of Raf kinase activity promoted TNF-induced apoptosis in a dose-dependent manner (Figure 4.13). Treatment with TNF and wortmannin, a PI-3 kinase inhibitor, was used as a positive control for the assay. These data indicate that Raf kinase activity is required for cell survival in response to TNF *in vitro*.

MEK activation is not required for cell survival downstream of TNFR1.

As shown in Figure 4.3, TNF stimulates MAPK activation in mouse colon epithelial cells through TNFR1. While 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 (60). To

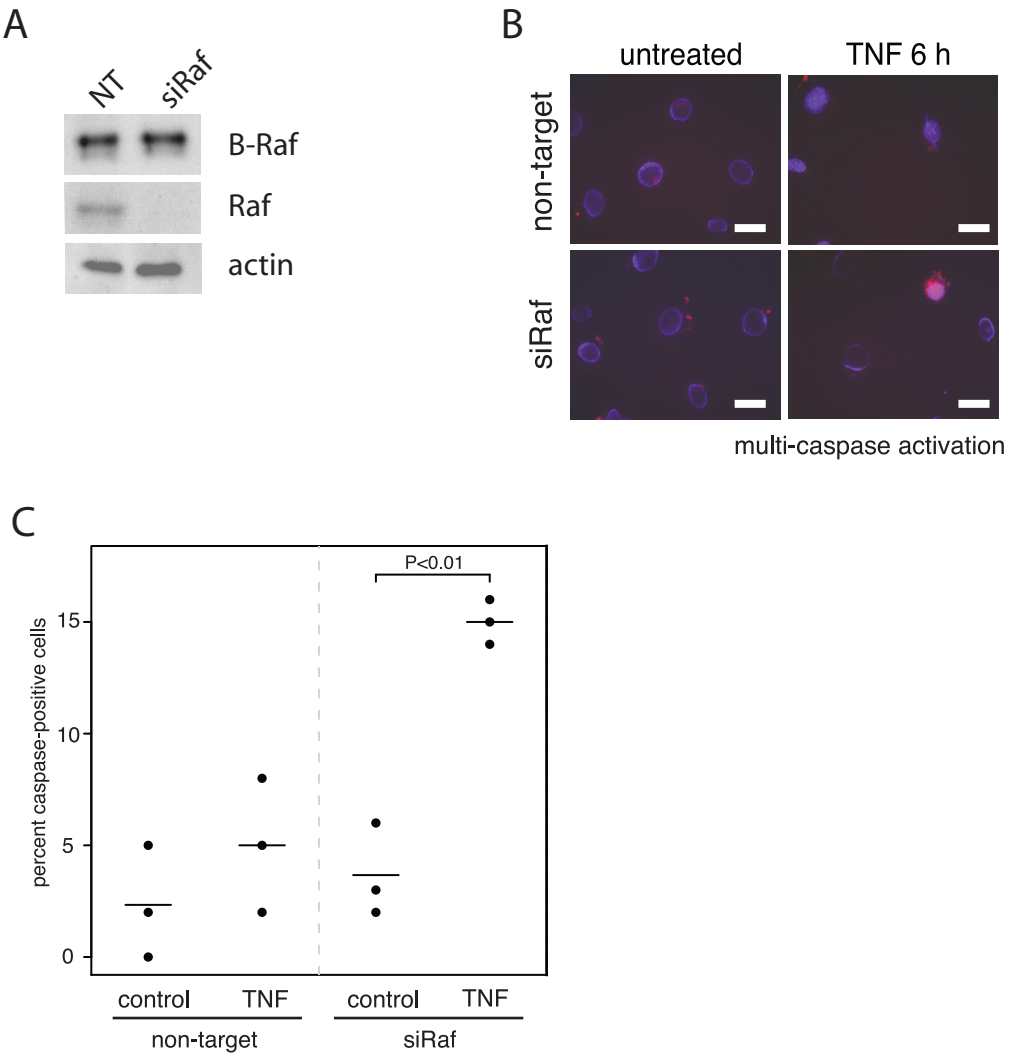

Figure 4.12: Raf is required for colon epithelial cell survival in response to TNF. (A) Western blot analysis was performed on whole cell lysates of non-targeting and Raf siRNA-transfected cells for Raf, B-Raf, and actin. (B) Multi-caspase activation assay was performed on non-targeting and Raf siRNA-transfected YAMC cells following TNF treatment for 6 h. Caspase activation is shown in red, DAPI-stained nuclei in blue. Scale bar = 20μM (C) The number of caspase-positive cells is represented. Bars represent the mean value.

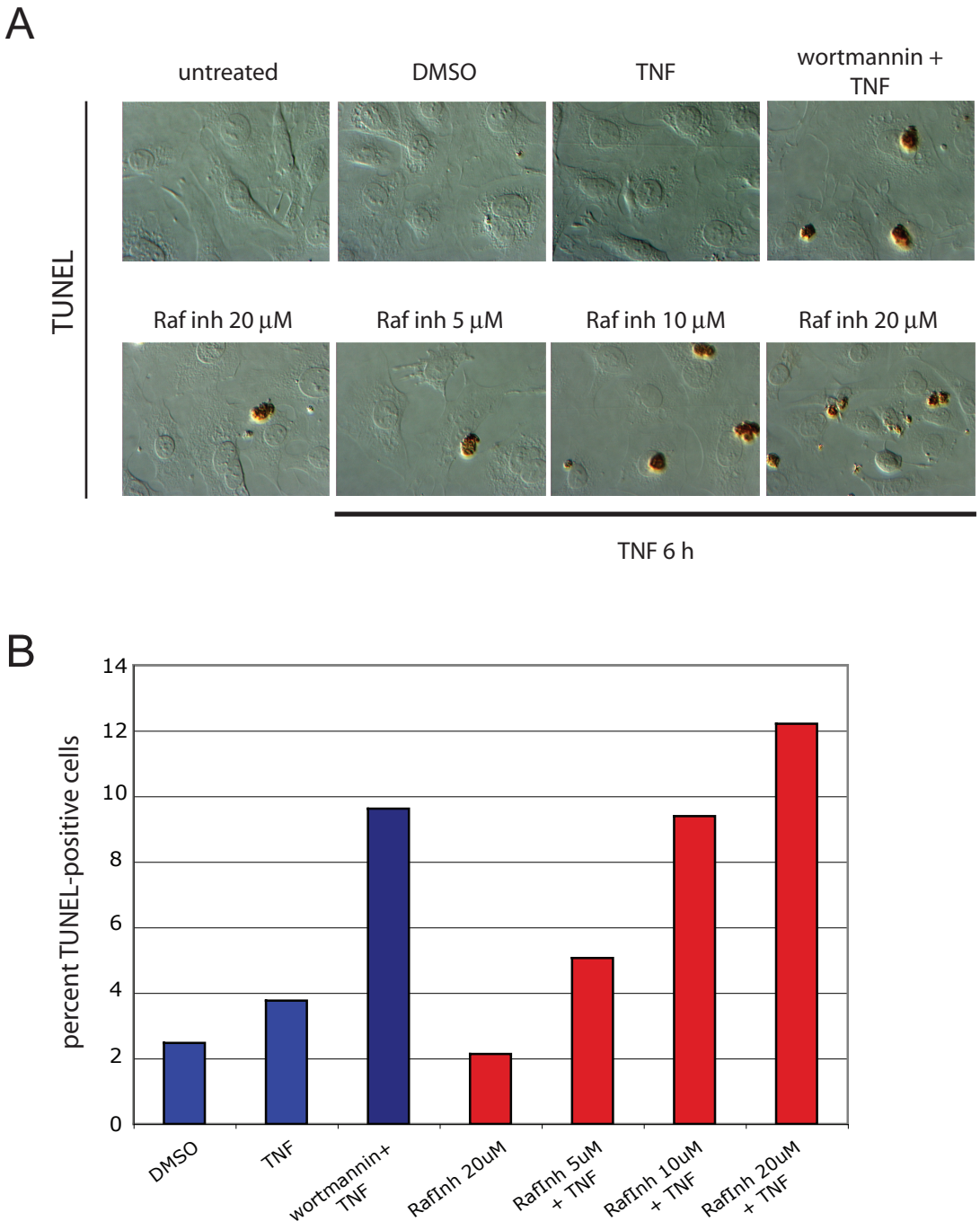

Figure 4.13: Raf kinase inhibitor induces apoptosis in colon epithelial cells following TNF exposure. YAMC cells were pre-treated with Raf kinase inhibitor (5-20 μM) or wortmannin (100 nM) followed by 6 h TNF treatment (100 ng/ml). (A) Apoptosis was detected by TUNEL assay and (B) percent TUNEL-positive cells were represented graphically.

address whether TNF-mediated anti-apoptotic signaling requires MAPK signaling, YAMC cells were pre-treated with two MEK inhibitors, PD98059 (20□M) or U0126 (10□M), followed by 6 h TNF treatment. Western blot analysis showed that both inhibitors effectively blocked MAPK phosphorylation after 10 min TNF stimulation (Figure 4.14A). However, I□B degradation was independent of MAPK activation, demonstrating that MAPK activation is not required to promote NF-□B 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 to a phosphoinositide 3 (PI3)-kinase inhibitor, wortmannin (Figure 4.14B)(141). Taken together, these data indicate that Raf promotes cell survival in the presence of TNF through a MEK-independent mechanism.

Raf mediates cell survival through activation of NF-□B.

Since inhibition of MEK kinase activity had no effect on TNF-mediated cell survival or TNF-induced I□B degradation, we hypothesized that Raf might promote cell survival through activation of NF-□B. To address this hypothesis, YAMC cells were transfected with siRNA against Raf, NF-□B p65, I□B, or both Raf and I□B. Western blot analysis for Raf, I□B, NF-□B p65, or actin was performed on whole cell lysates to confirm the respective inhibition of protein expression by siRNA (Figure 4.15A). In non-targeting siRNA-transfected cells, TNF promotes nuclear translocation of NF-□B after 30 min; however, knockdown of I□B expression resulted in constitutive NF-□B p65 nuclear translocation as

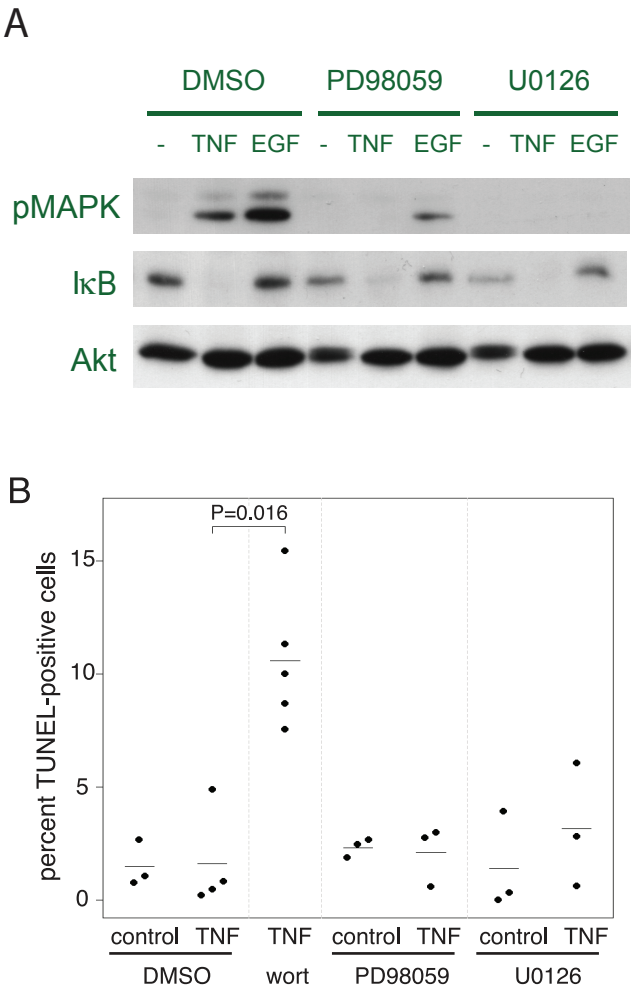

Figure 4.14: Anti-apoptotic signaling downstream of TNF receptors is MEK-independent. (A) YAMC cells were pre-treated with DMSO, PD98059, or U0126 for 30 min followed by TNF for 10 min or EGF for 5 min. Western blot analysis was performed to detect phospho-MAPK, IκB, and actin. (B) TUNEL assay was performed in YAMC cells pre-treated with DMSO, PD98059, U0126, or wortmannin for 30 min and then treated with TNF for 6 h. Percent TUNEL-positive cells shown. Bars represent the mean value.

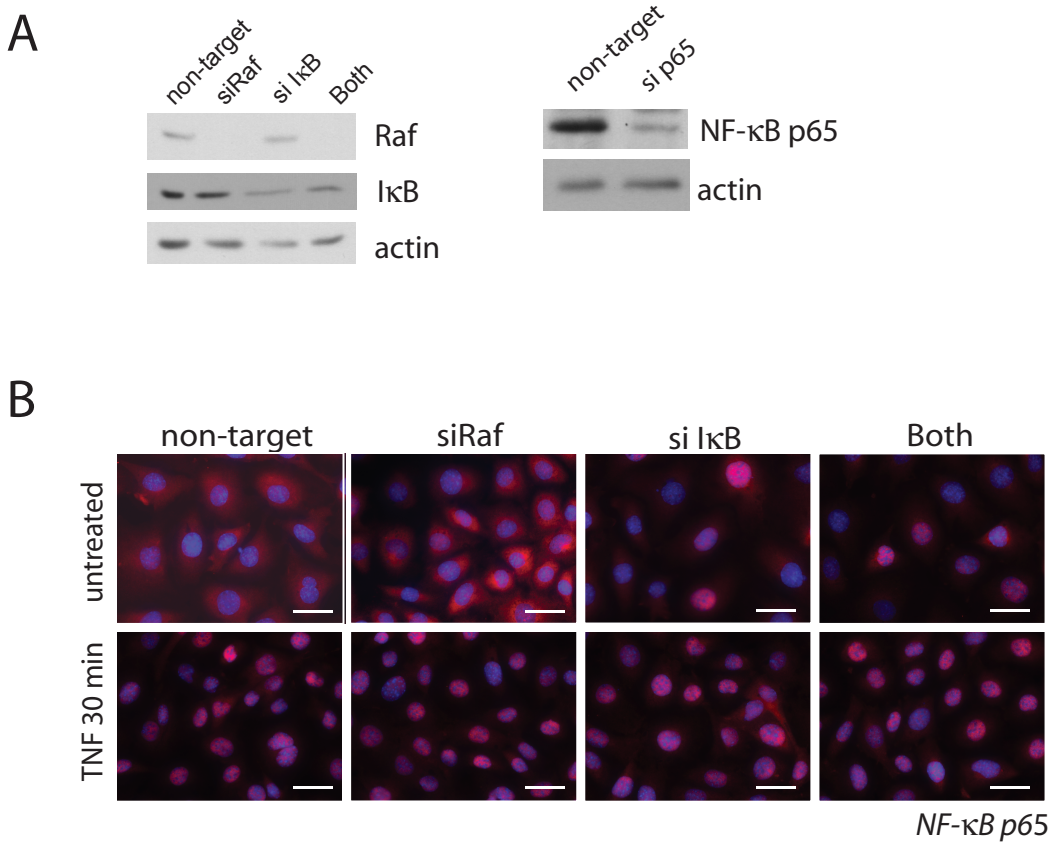

Figure 4.15: Loss of IκB expression results in constitutive NF-κB nuclear translocation. YAMC cells were transfected with non-targeting, NF-κB p65, Raf and/or IκBα siRNA. (A) Western blot analysis was performed for NF-κB p65, Raf, IκB, and actin. (B) Immunofluorescence for NF-κB p65 subunit (red) was performed on siRNA-transfected cells treated with TNF for 30 min. Nuclei were stained with DAPI (blue). Scale bars = 20μm

shown by immunocytochemistry (Figure 4.15B). siRNA-transfected cells were treated with TNF for 6 h and apoptosis detected by multi-caspase activation assay (Figure 4.16). 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).

To determine whether Raf expression is required for TNF-induced NF-κB activation *in vivo*, wildtype, TNFR1 KO, and Raf KO^{IE} mice were injected with PBS or TNF for 1 or 2 h, and immunohistochemistry performed to detect phosphorylated NF-κB p65 (S276) on colon sections (Figure 4.17). TNF treatment at both time points stimulated increased NF-κB p65 phosphorylation in wildtype, but not TNFR1 KO or Raf KO^{IE} mice (P<0.001, P=0.02, P=0.03). Furthermore, Raf was required for increased expression of an anti-apoptotic NF-κB target gene, cIAP1, in the colon epithelium following 24 h TNF exposure (Figure 4.18) (P=0.03). cIAP1 inhibits apoptosis by preventing activation of both pro-form and mature caspases (81). 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 that TNFR1 is required for ERK and NF-κB activation and colon epithelial cell survival in response to TNF stimulation both *in*

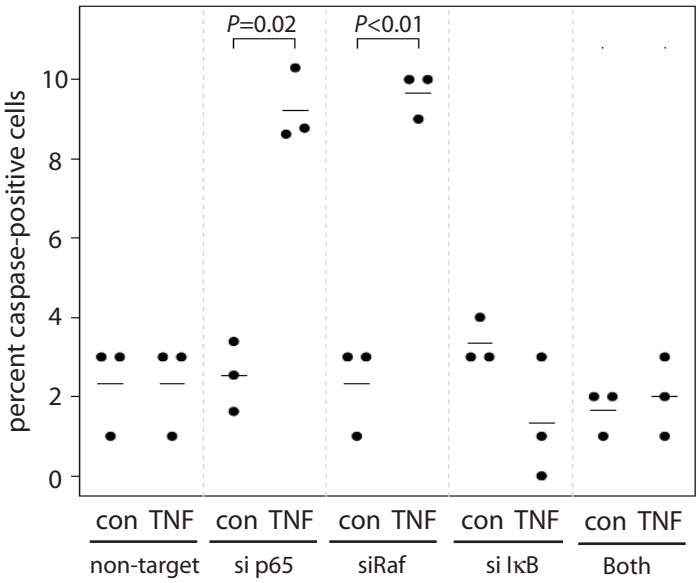

Figure 4.16: NF-κB activation rescues TNF-induced apoptosis in the absence of Raf. YAMC cells were transfected with non-targeting, NF-κB p65, Raf and/or IκBα siRNA. Percent of caspase-positive cells following treatment with TNF for 6 h. Bars represent the mean value.

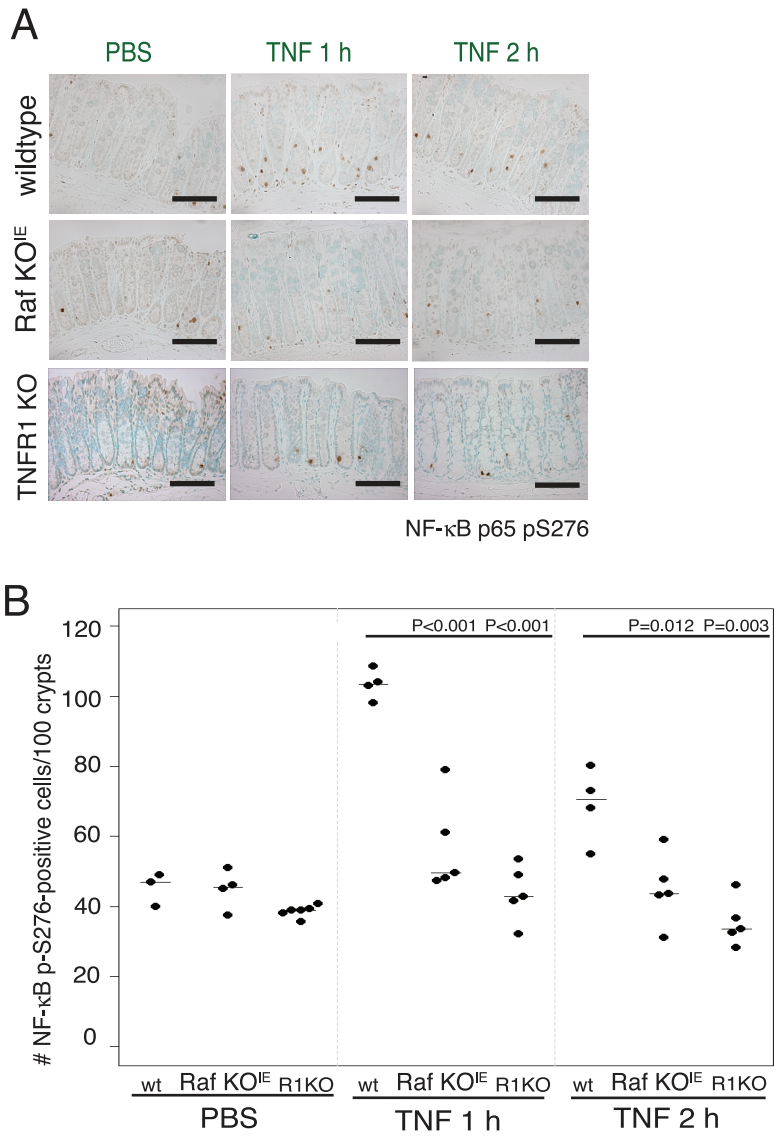

Figure 4.17: Raf is required for TNF-stimulated NF-κB activation *in vivo*. (A) Immunostaining for NF-κB p65 phospho-Ser276 was performed on colon sections of wildtype, Raf KO^{IE}, and TNFR1 KO (R1KO) mice injected with TNF for 1 or 2 h. Scale bar = 125μM (B) Number of NF-κB p65 phospho-Ser276-positive cells per 100 crypts. Bars represent the mean value.

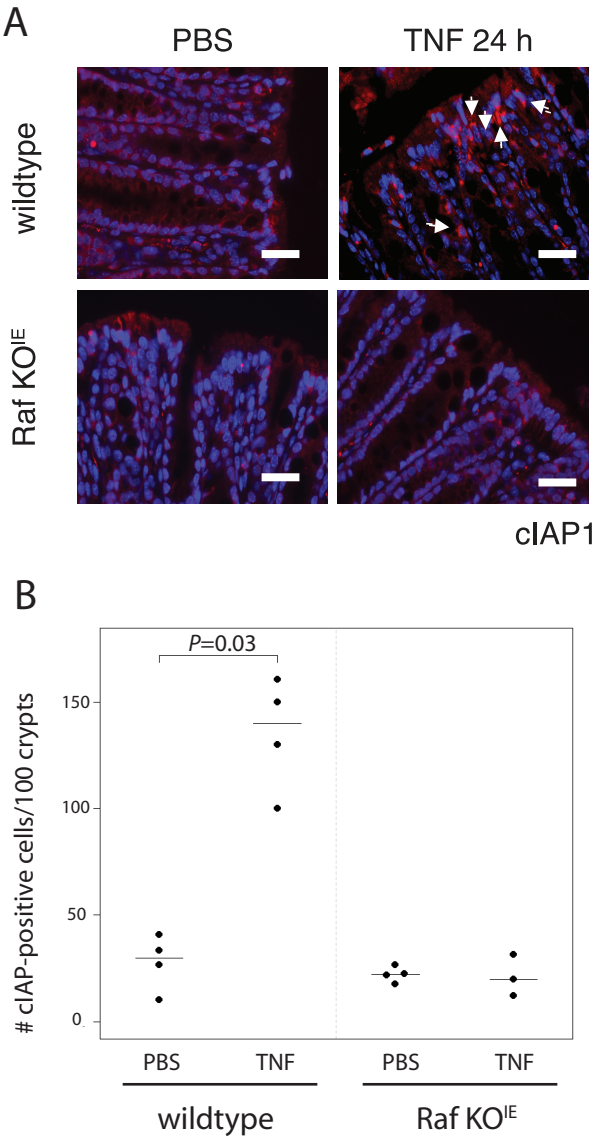

Figure 4.18: Raf is required for increased expression of cIAP1 in response to TNF stimulation. (A) Immunostaining for cIAP1 (red) was performed on colon sections of wildtype and Raf KO^{IE} mice injected with TNF for 24 h. DAPI-stained nuclei are blue. Scale bar = 20μM (B) Number of cIAP1-positive cells per 100 crypts are represented graphically. Bars represent the mean value.

vitro and *in vivo*. We have previously shown that KSR promotes anti-apoptotic signaling, and now demonstrate that Raf-1, a KSR substrate, is also required for cell survival in response to TNF. 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 absence of Raf expression inhibits TNF-induced NF-κB p65 phosphorylation and expression of an NF-κB anti-apoptotic target gene, cIAP1. 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 (Figure 4.4). Interestingly, previous reports have shown that Ras is not required for Raf-mediated MAPK signaling involved in calcium-induced differentiation pathways in keratinocytes (195). In addition, lipopolysaccharide (LPS)-stimulated macrophage differentiation occurs through Ras-independent, protein kinase C (PKC) or phosphatidylcholine-specific phospholipase C activation (196). KSR directly interacts with Raf at the plasma membrane (136,197), 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 (61).

Although inhibition of KSR kinase activity toward Raf results in decreased MAPK activation (61), we now show that the TNF-mediated cell survival response does not require MEK signaling (Figure 4.14). 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 (56). 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 a NF-κB reporter plasmid independent of MEK (50), consistent with our findings that MEK inhibition does not block TNF-induced NF-κB activation (Figure 4.14). Taken together, these data suggest that Raf mediates NF-κB activation through a novel MEK-independent mechanism.

We have shown that the absence of KSR impairs TNF-stimulated NF-κB nuclear translocation both *in vitro* and *in vivo* (61,62,136). Surprisingly, NF-κB p65 nuclear translocation following TNF treatment of colon epithelial cells does not require Raf (Figure 4.15); however, Raf expression is necessary for TNF-stimulated NF-κB p65 serine 276 phosphorylation *in vivo* (Figure 4.17). Thus, KSR activates NF-κB upstream of Raf-mediated NF-κB p65 phosphorylation, demonstrating that both KSR and Raf are necessary for TNF-induced NF-κB signaling.

NF-κB p65 subunit phosphorylation on S276 in the Rel homology domain (RHD) enhances DNA binding as well as the interaction between p65 and CBP/p300 (198,199). Overexpression of a p65 S276A mutant in p65 KO mouse embryonic fibroblasts (MEFs) shows that TNF-induced IL-6 expression and cell survival require phosphorylation at this site (200). Further analysis of the requirement for S276 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 (201). Raf promotes NF-κB p65 S276 phosphorylation in dendritic cells following activation of a c-type lectin, DC-SIGN (202), indicating that Raf may activate NF-κB in additional cell types. Our data demonstrate a novel regulatory mechanism through which Raf promotes TNF-stimulated NF-κB p65 S276 phosphorylation independent of MEK activation.

Based on our current results and previous reported findings, we propose a model (Figure 4.19) in which binding of TNF to TNFR1 promotes recruitment of FAN [factor-associated with neutral sphingomyelinase (N-SMase) activation] (203) to the N-SMase domain (NSD) of TNFR1 (204). N-SMase hydrolyzes sphingomyelin in the plasma membrane to increase intracellular ceramide production (203,205). Ceramide can then bind to and activate KSR, which phosphorylates Raf on threonine residues in response to TNF and EGF treatment (32,61,190,206-208). Through an unknown mechanism, Raf enhances NF-κB S276 phosphorylation in a MEK-independent manner to protect against colon epithelial cell apoptosis.

In regard to the colon epithelium, TNF has been implicated in promoting cell survival through NF-κB in response to inflammation *in vivo*. 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 downregulating anti-

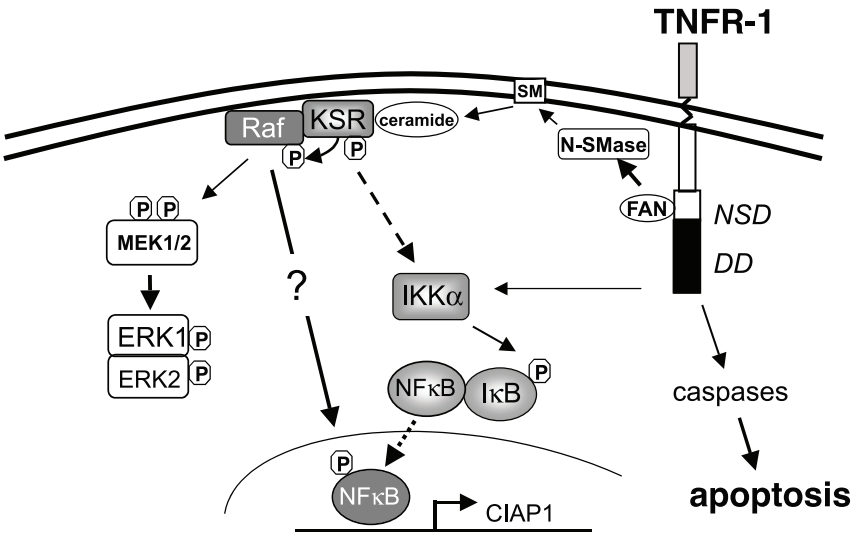

Figure 4.19: TNFR1 regulation of both pro- and anti-apoptotic signaling in colon epithelial cells. Binding of TNF to TNFR1 induces production of neutral sphingomyelinase (N-SMase) to generate intracellular ceramide, which can bind to and activate KSR. Raf, a KSR substrate,promotes NF-κB phosphorylation through a MEK-independent mechanism, whereas KSR stimulates NF-κB through IKKα activation. Both Raf and KSR are required to induce anti-apoptotic signaling to balance pro-apoptotic pathways activated downstream of the TNFR1 death domain.

apoptotic signaling and shifts cells toward an apoptotic program (161). NEMO KO^{IE} mice develop spontaneous colitis due to decreased barrier integrity, whereas crossing these mice into a TNFR1 KO background rescues the colitis phenotype. Although the NEMO KO^{IE}/TNFR1 KO mice show no sign of inflammation, it is unclear whether TNFR1 signaling in the colon epithelium or immune cells contributes to the development of colitis.

A recent study showed that blocking TNF reduced initiation and progression of inflammation-associated tumorigenesis (209), which is consistent with data demonstrating that IKKβ inhibition results in decreased tumor multiplicity following azoxymethane (AOM)/DSS exposure (126). Raf kinase inhibitors are currently used in the treatment of hepatocellular and renal cell carcinoma (168), suggesting that further investigation into the role of TNF activation of Raf signaling may lead to the identification of novel mediators of colitis-associated cancer.

In summary, these findings show that Raf functions as a molecular cell survival switch to enhance anti-apoptotic signaling in response to TNF activation of TNFR1 by promoting NF-κB. Our model suggests that TNF stimulates Raf activity through 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 non-inflammatory conditions may lead to the development of novel therapeutics for the treatment of IBD.

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CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary of findings

Previous studies in our lab demonstrated that an upstream activator of Raf kinase, KSR, is required for cell survival in response to TNF stimulation (136). Additionally, KSR-mediated Raf threonine phosphorylation is increased during active colitis in the IL-10 KO mouse model, suggesting that Raf activation may regulate cellular responses to inflammation. The studies presented here begin to define the mechanism by which Raf regulates colon epithelial cell survival. In response to epithelial injury and pro-inflammatory cytokine production, Raf activates NF-κB through a novel MEK-independent mechanism.

The data demonstrate that Raf protects against inflammation and crypt injury during acute colitis. Loss of Raf promotes colon epithelial cell apoptosis following injury, and inhibits the proliferative response necessary to regenerate the epithelial barrier during recovery. Furthermore, Raf enhances epithelial proliferation through activation of MAPK, whereas Raf promotes cell survival through a MEK-independent mechanism. Activation of Raf or NF-κB is required to protect against DSS-induced apoptosis in YAMC cells. However, constitutive NF-κB activation rescued cells from DSS-induced cell death *in vitro* in the absence of Raf, positioning NF-κB downstream of Raf activation. Furthermore, Raf expression was required for NF-κB p65 nuclear translocation in the colon

epithelium during DSS-induced injury *in vivo*. Taken together, these data demonstrate that Raf stimulates NF-κB to protect against colon epithelial cell apoptosis in DSS colitis (Figure 5.1).

Previous reports characterizing cytokine expression during DSS colitis showed that increased TNF and IL-6 production contribute to epithelial injury (129). Experiments conducted in the present study to address the role of Raf in TNFR signaling indicate that Raf is a key regulator of cell survival both *in vitro* and *in vivo*. Although Raf is not required for TNF-stimulated NF-κB nuclear translocation, short term TNF treatment of Raf KO^{IE} mice demonstrated that Raf promotes NF-κB p65 phosphorylation (Figure 4.17). In addition, TNF-induced expression of an NF-κB anti-apoptotic target gene, cIAP1, was blocked in the absence of Raf. Further analysis showed that Raf promotes NF-κB phosphorylation downstream of TNFR1 activation (Figure 5.1). Interestingly, pharmacological inhibition of MEK kinase activity did not induce apoptosis in YAMC cells following exposure to TNF. These data indicate that Raf protects against TNF-induced apoptosis through NF-κB activation in a MEK-independent manner.

This is the first report characterizing the role of Raf in the colon epithelium. Although Raf is not required for maintenance of the intestinal epithelium in unchallenged mice, these findings demonstrate that it is essential for promoting colon epithelial cell survival during inflammation. Furthermore, the identification

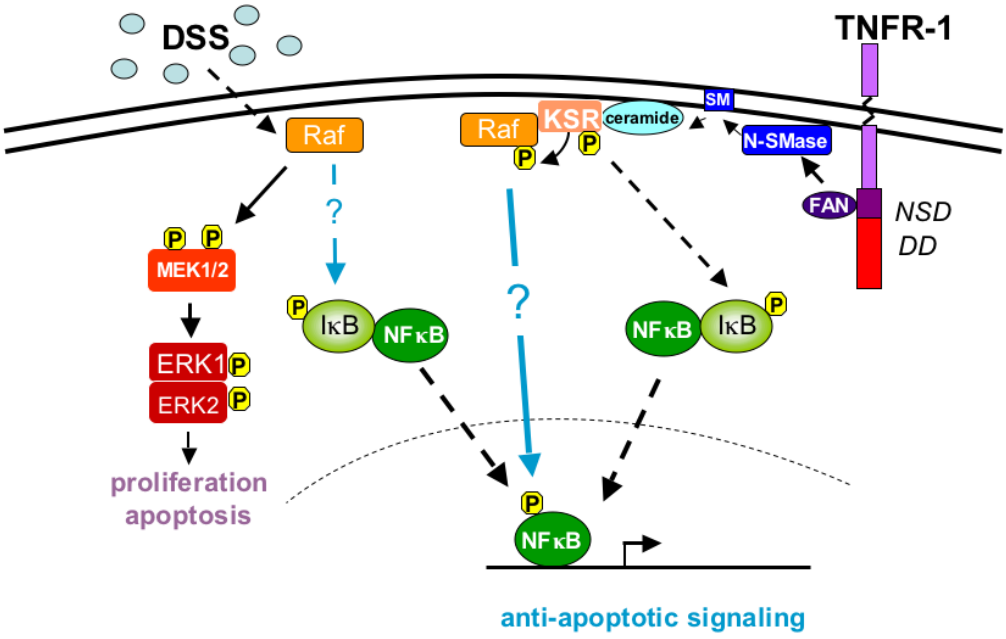

Figure 5.1: Raf promotes colon epithelial cell survival through NF-κB in a MEK-independent manner. Raf stimulates IκB degradation resulting in NF-κB nuclear translocation following DSS exposure. While Raf activates ERK to promote epithelial hyperproliferation during recovery from DSS colitis, MEK activation is also pro-apoptotic in response to DSS exposure. TNF stimulation of TNFR1 results in increased intracellular ceramide generation which activates kinase suppressor of Ras (KSR), and its substrate, Raf. Following TNF exposure, KSR induces NF-κB nuclear translocation, whereas Raf promotes NF-κB p65 phosphorylation. Raf activation of NF-κB occurs through a novel MEK-independent mechanism.

of additional MEK-independent Raf signaling pathways involved in cell survival complements and expands the known functions of Raf in the literature.

Raf in DSS-induced cytokine signaling

Our data indicate that Raf is an essential regulator of colon epithelial cell survival in response to pro-inflammatory stimuli. While several studies have highlighted the importance of TNF production in the pathogenesis of colitis, it remains unclear as to which cytokines promote Raf activation in response to DSS colitis. Elevated TNF levels play an important role in DSS-induced epithelial injury (129,130); however, TNF KO mice develop more severe colitis in response to DSS compared to wildtype controls (177). These findings emphasize the importance of cytokines other than TNF in promoting inflammation and crypt damage associated with DSS colitis.

One example of another cytokine that may promote Raf activation is IL-1. IL-1 is elevated in lamina propria macrophages following DSS treatment (210) and in inflamed mucosa of IBD patients (211). Similar to our proposed model of TNFR-induced Raf activation, Huwiler et al. demonstrated that IL-1 stimulates Raf through generation of endogenous ceramide in fibroblasts (28), indicating IL-1 may promote KSR activation of Raf through increased ceramide production. IL-1 is also a potent activator of NF-□B signaling; therefore, investigating the role of Raf in IL-1-induced NF-□B activation may extend our current findings to additional cytokine signaling pathways.

TNF and TNFR signaling in the colon epithelium

TNF activation of TNFR can promote cell survival or apoptosis in a cell context-dependent manner; however, the role of TNFR in colitis is not clearly defined. Studies performed to evaluate the role of TNFR1 in acute colitis showed that immune progenitor reconstitution by transplanting wildtype bone marrow in TNFR1 KO mice results in protection against severe DSS colitis (212). While these data demonstrate that TNFR1 expression in bone marrow-derived cells contributes to maintenance of intestinal homeostasis, it is important to consider that these experiments were performed in the absence of adaptive immunity. Whereas TNFR1 promotes cell survival in the colon epithelium, loss of TNFR1 inhibited macrophage apoptosis indicating that TNF signaling through TNFR1 is pro-apoptotic in this cell type. Unfortunately, it is difficult to determine the exact contribution of TNF signaling in inflammation because of paracrine signaling between myeloid and epithelial cells. The generation of conditional TNFR KO mice is necessary to definitively address the cell-specific role of TNFRs in inflammation-induced injury. DSS treatment of conditional intestinal epithelium-specific TNFR KO mice would allow us to examine the role of each TNFR in colon epithelium in response to injury and inflammation. In addition, generation of these mice would enable further investigation of the requirement for TNFR1 in colon epithelial cell survival.

As discussed in Chapter IV, TNF binding to TNFR1 promotes both pro- and anti-apoptotic signaling pathways in the colon epithelium. We hypothesize that activation of TNFR1 signaling may occur in a dose-dependent manner

following TNF stimulation. Therefore, the amount of soluble TNF available in the system may alter cellular responses downstream of TNFR1. Previous studies demonstrated that TNF-induced signaling pathways through TNFR1 can be coordinated in a temporal manner (213). In B-cells, TNF stimulates N-SMase activation through TNFR1 NSD prior to the generation of acid SMase (A-SMase) through activation of the TNFR1 death domain (213). Induction of N-SMase may contribute to cell survival (214-216), while generation of A-SMase results in apoptosis (214,217,218). Thus, we hypothesize that activation of neutral SMase to promote KSR- and Raf-mediated cell survival signaling may counterbalance acid SMase-induced apoptotic signaling in the colon epithelium; however, the role of ceramide in Raf activation has not yet been addressed. The period between activation of neutral and acid SMases may allow the cell to activate a negative-feedback loop to inhibit further TNFR signaling. One example of feedback inhibition is the proteolytic cleavage of TNFR at the plasma membrane by metalloproteinases, which results in the shedding of soluble TNFR to prevent further activation of downstream signaling pathways (219). Therefore, TNF-induced anti-apoptotic signaling through NF-□B may function as a first response to permit epithelial cells (220) or innate immune cells (221) to shed decoy receptors as a negative feedback mechanism to inhibit TNF-induced colon epithelial cell apoptosis.

Raf involvement in NF-κB signaling

As demonstrated in Chapters III and IV, Raf can promote activation of NF-κB at multiple steps of the NF-κB signaling pathway. In response to DSS, Raf is required for NF-κB nuclear translocation, whereas TNF stimulates Raf-dependent NF-κB phosphorylation, but NF-κB nuclear translocation is Raf-independent. One possible explanation for this difference may be that DSS stimulates Raf through cytokine receptors other than TNFR to promote Raf-dependent NF-κB activation, as discussed earlier in this section. Regardless of the stimulus, the mechanism by which Raf induces NF-κB nuclear translocation or phosphorylation remains unknown. While overexpression studies using constitutively activated Raf showed increased NF-κB activation in a transformed cell line (222), this is the first demonstration of endogenous Raf mediating NF-κB activation in cell culture and *in vivo*. Although Raf is a MAPKKK, similar to MEKK or NIK, there is no evidence that Raf directly phosphorylates the IKKs (163). One report shows that Raf and IκBα may physically interact in a yeast two-hybrid screen (222); however, Raf expression was not required for IκB phosphorylation in our studies. Rather, Raf may indirectly promote NF-κB translocation to the nucleus through the activation of NF-κB binding partners or other kinases involved in NF-κB signaling.

Similarly, it is unknown whether Raf can directly phosphorylate NF-κB p65 at serine 276. Although protein kinase A (PKA) can phosphorylate this site (198), it has been demonstrated that mitogen and stress-activated kinase-1 (MSK1) phosphorylates S276 in the nucleus following TNF stimulation (223). Vanden

Berghe et al. showed that the upstream activators of MSK1, p38 and ERK MAPK, were required for NF-κB activation in TNF-treated fibroblasts (224); 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.

Recently, Raf was shown to promote NF-κB p65 S276 phosphorylation and subsequent acetylation through an unknown mechanism in dendritic cells following activation of a c-type lectin, DC-SIGN, which functions to mediate dendritic cell survival (202). Interestingly, lipopolysaccharide (LPS)-induced stimulation of Toll-like receptor (TLR)-4 was required for NF-κB nuclear translocation following which Raf-dependent NF-κB p65 phosphorylation induced transcription of NF-κB target genes. These data suggest multiple signals from cell surface receptors cooperate to stimulate NF-κB activation. A similar series of receptor-driven events may be required for NF-κB activation in the colon epithelium in response to DSS treatment, in contrast, TNFR-initiated signaling appears to be sufficient to promote both TNF-induced NF-κB nuclear translocation and subsequent Raf-dependent p65 phosphorylation,.

Although NF-κB nuclear translocation and phosphorylation are critical for transcriptional activation of NF-κB target genes, recent studies have revealed

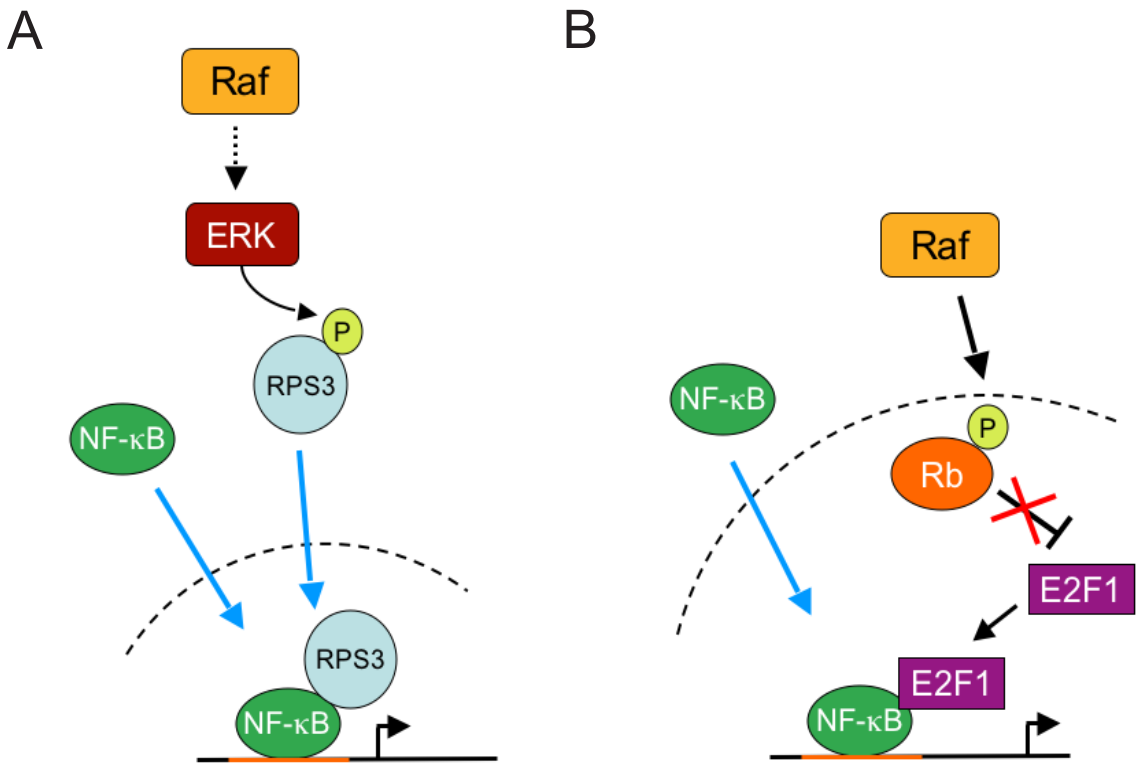

Figure 5.2: Potential involvement of Raf in regulating NF-κB transcriptional co-activators. (A) Raf may mediate ERK phosphorylation of ribosomal protein subunit 3 (RPS3) resulting in RPS3 nuclear translocation where it associates with NF-κB/DNA complexes to enhance transcription of NF-κB target genes. (B) During cell cycle progression, Raf-induced Rb phosphorylation relieves repression of E2F1. Raf may also regulate E2F1 availability and subsequent association with NF-κB at DNA promoter regions through a similar mechanism.

additional components of the NF-κB/DNA complex (Figure 5.2)(225). Among these findings is a report showing that ribosomal protein S3 (RPS3) binds to the NF-κB p65 Rel homology domain (RHD) and enhances the binding association between p65 and DNA resulting in expression of NF-κB-dependent target genes. In this model, ERK phosphorylation of RPS3 in the cytoplasm results in RPS3 nuclear translocation (226,227) where it can then associate with the NF-κB/DNA complex. While initial studies show that TNF stimulates RPS3 translocation to the nucleus in T-cells (225), it would be of interest to test the requirement for Raf in RPS3 nuclear translocation in the colon epithelium following various stimuli.

Following nuclear translocation, NF-κB also recruits E2F1 to promote transcriptional activation of target genes (228). Not only are NF-κB and E2F1 required for expression of several LPS-induced target genes, but both proteins can occupy the same DNA promoter regions. E2F1 is sequestered through binding interactions with retinoblastoma (Rb) protein to inhibit activation of transcription. During regulation of cell cycle progression, Raf can directly phosphorylate and inactivate Rb, reversing Rb-mediated repression of E2F1 (229), suggesting that Raf may regulate E2F1 availability to promote NF-κB transcriptional activation.

Raf in colon cancer development

These studies indicate an essential role for Raf in maintaining colon epithelial integrity through regulation of both cell survival and proliferation in response to acute colitis. These biological processes also play a significant role

in tumor development. Tumorigenesis occurs following the unrestricted proliferation of a cell with genetic mutations (230,231). Defects in the initiation of apoptotic signaling and progression past cell cycle checkpoints allow the mutated cell to escape normal regulatory processes resulting in tumor formation.

IBD patients are at a higher risk to develop colon cancer due to repeated cycles of inflammation and wound repair (169). This link between chronic inflammation and cancer in the colon was confirmed experimentally through the development of an inflammation-associated cancer model in mice (126). In this model, mice receive a single dose of carcinogen (azoxymethane, AOM) followed by multiple rounds of DSS administration to induce chronic inflammation. This protocol induces adenomas in treated mice with high penetrance and frequency. Greten et al. showed that inhibition of NF-κB signaling by IKKβ deletion in the colon epithelium resulted in increased tumor formation following AOM/DSS treatment (126). Since loss of Raf expression in the colon epithelium results in increased inflammation and impaired NF-κB signaling, increased tumorigenesis in IKKβ KO^{IE} mice suggests that Raf may function as a tumor suppressor. In contrast, tumorigenesis may be inhibited due to increased cell death and decreased proliferation in Raf KO^{IE} mice following inflammation-induced injury. Regardless of whether Raf promotes or suppresses tumorigenesis, elucidating the role of Raf in inflammation-associated carcinogenesis would provide valuable insight in determining the course of cancer therapy. Sorafenib, a multikinase inhibitor that also inhibits Raf kinase, is currently used in the treatment of renal cell and hepatocellular carcinoma (232), with clinical trials in process for other

types of cancer (233). Thus, it would be beneficial to determine whether Raf inhibition promotes tumor formation in inflammation-associated colon cancer.

Concluding remarks

Raf kinase and its role in MAPK activation have been extensively studied for the last twenty years; however, much about Raf function remains unknown. Only recently have we begun to characterize and identify the role of Raf in specific cell types and the diversity of cellular stimuli that leads to Raf activation outside of receptor tyrosine kinase signaling. The involvement of Raf in several disease states, including cancer, illustrates the need for further investigation into the mechanism by which Raf regulates cellular processes, including cell survival. We demonstrate that Raf promotes anti-apoptotic signaling through a MEK-independent mechanism, indicating a role for novel Raf substrates in colon epithelial cell survival. The studies described here begin to demonstrate the importance of Raf signaling in maintenance of the colon epithelium and link Raf to a known mediator of inflammatory signaling, NF-κB.

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