KINASE SUPPRESSOR OF RAS 1 IS A FUNCTIONAL PROTEIN KINASE AND PROTECTS FROM EXPERIMENTAL COLITIS IN MICE BY REGULATING T LYMPHOCYTE IFN-γ PRODUCTION

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"In coming to understand anything we are rejecting the facts as they are for us,

in favour of the facts as they are." – C.S. Lewis

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LIST OF ABBREVIATIONS

AKT ..... v-akt murine thymoma viral oncogene homolog

AOM ..... Azoxymethane

APC .....Antigen presenting cell

ATP ..... Adenosine-5'-triphosphate

BM .....Bone marrow

c-IAP.....cellular inhibitor of apoptosis protein

C-TAK1..... Cdc25C-associated kinase 1

CA .....Conserved area

CD ..... Clusters of differentiation

DD .....Death domain

DNA..... Deoxyribonucleic acid

DSS ..... Dextran sulfate sodium

E. coli..... *Escherichia coli*

ERK ..... Extracellular signal-regulated kinase

FADD.....Factor-associated death domain

FAP .....Familial adenomatous polyposis

GFP .....Green fluorescent protein

GPCR ..... G protein-coupled receptor

GTP .....Guanosine-5'-triphosphate

H&E ..... Hematoxylin and Eosin

HA ..... Hemagglutinin

HEK .....Human embryonic kidney

HIS ..... Hexahistidine

Hsp ..... Heat shock protein

IBD ..... Inflammatory bowel disease

IEL ..... Intraepithelial lymphocytes

IFN-γ ..... Interferon-gamma

IgG ..... immunoglobulin G

IKK ..... IκB kinase

IL ..... Interleukin

JNK ..... c-jun N-terminal kinase

kDa ..... Kilodalton

KSR1 ..... Kinase suppressor of Ras 1

MAPK ..... Mitogen-activated protein kinase

MBP ..... Myelin basic protein

MEK ..... MAPK/ERK kinase

MHC ..... Major histocompatibility compex

Mkp3 ..... MAP kinase phosphatase 3

N ..... Amino

NF-κB ..... Nuclear factor kappaB

NK ..... Natural killer

NOD2 ..... Nucleotide-binding oligomerization domain containing 2

NSAID ..... Non-steroidal anti-inflammatory drugs

PAK ..... p21-activated kinase

PCR ..... Polymerase chain reaction

PKB ..... Protein kinase B

PP2A ..... Protein phosphatase 2A

PP2B ..... Protein phosphatase 2B

Raf ..... v-raf-1 murine leukemia viral oncogene homolog 1

Rag2 ..... Recombination activating gene 2

RIP ..... Receptor-interacting protein

RTK ..... Receptor tyrosine kinase

SH3 ..... Src homology 3

SPF ..... Specific pathogen free

TACE ..... TNF-alpha converting enzyme

Th ..... T helper

TLR ..... Toll-like receptor

TNBS ..... Trinitrobenzene sulfonic acid

TNF ..... Tumor necrosis factor

TNFR1 ..... Tumor necrosis factor receptor 1

TNFR2 ..... Tumor necrosis factor receptor 2

TRADD ..... TNF receptor-associated death domain

TRAF2 ..... TNF-R-associated factor 2

TUNEL ..... Terminal deoxynucleotidyl transferase dUTP nick end labeling

V(D)J ..... Variable, (diversity), joining

VDR ..... Vitamin D receptor

WASP ..... Wiskott-Aldrich Syndrome Protein

WNK1 ..... With no lysine 1

WT ..... Wild-type

YAMC ..... Young adult mouse colon

CHAPTER I

INTRODUCTION

Protein kinase signal transduction

Encoded among an estimated 20,000-25,000 protein-coded genes in humans (Reviewed in (Stein, 2004)) is an important group of proteins that comprise the protein kinase superfamily. Protein kinases catalyze the transfer ATP γ-phosphate onto hydroxyl groups of tyrosine, serine, or threonine amino acid residues of substrate proteins in a process called phosphorylation. While protein phosphorylation and protein kinases were originally described in the mid 1950's by Fischer & Krebs (Fischer, et al., 1955), and Sutherland & Wosilait (Sutherland, et al., 1955), the significance of protein phosphorylation as a regulatory mechanism wasn't realized until 14 years later (Linn, et al., 1969). Currently there are 518 known protein kinases accounting for approximately 2% of genes in humans (Manning, et al., 2002). Based on amino acid alignment, the protein kinase domain contains 11 highly conserved sub-domains consisting of 250-300 amino acids divided into an N-terminal ATP binding lobe and C-terminal peptide binding and phosphotransfer lobe (Figure 1.1) (Hanks, et al., 1988; Hanks, 2003). The conservation of particular residues and motifs across most protein kinases was hypothesized, and later demonstrated, to be critical for enzymatic activity (Hanks, et al., 1988). Specifically, subdomain II contains a lysine that is important for binding and orienting the ATP molecule whereas aspartic acid and asparagine residues in the HR**D**LKxx**N** motif of subdomain VIb, combined with the aspartic acid in the **D**FG motif of subdomain VII, are important for phosphotransfer (Hanks, et al., 1988). Since protein phosphorylation is a reversible post-translational modification, it is a dynamic mechanism that can regulate protein function and gene expression by way of protein-protein interactions, localization, and enzymatic activity. Hence, many cancer therapies

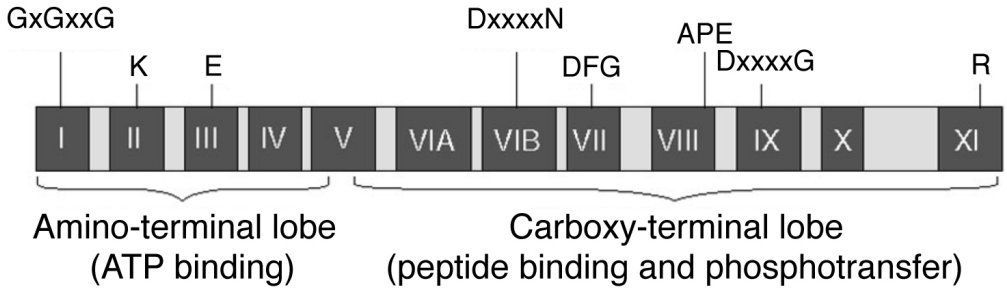

Figure 1.1: Conserved subdomains and key amino acid residues within the protein kinase domain The protein kinase domain consist of 11 subdomains highly conserved in all protein kinases. Within these subdomains lie key amino acid residues, usually invariant, critical for enzymatic function. In particular, the lysine residue in subdomain II is important for ATP binding and the aspartic acid residues in subdomains VIb and VII are involved in phosphotransfer of ATP γ-phosphate. Letters represent amino acids as follows: Glycine (G), Lysine (K), Glutamic acid (E), Aspartic acid (D), Alanine (A), Proline (P), Arginine (R), and any amino acid (x). Figure from (Hanks, 2003).

utilize selective kinase inhibitors to inhibit pathways in which mutations have resulted in aberrant or dysregulated kinase signaling.

TNF signaling

The pro-inflammatory cytokine TNF was originally described in 1975 by Carswell and characterized as a necrosis-inducing agent in mouse and human sarcomas (Carswell, et al., 1975; Haranaka, et al., 1981; Haranaka, et al., 1984). The major source of TNF is produced by macrophage and monocytes in response to infection. TNF transcripts are translated as membrane anchored precursors that are cleaved into soluble peptides by the activity of TNF converting enzyme (TACE) (Black, et al., 1997). While TNF can bind either of two TNF receptors (TNFR1 & TNFR2), much attention has been given to TNFR1 signaling due to its role in cell death pathways via its conserved death domain (DD). Thus, TNFR1 is the prototypic member among several death receptors in the larger TNFR superfamily. Like many cell surface receptors, signaling through TNFR1 is initiated at the cell surface, which activates signaling pathways that control cellular processes including growth arrest, cell survival, and apoptosis (Edelblum, et al., 2008; Kaiser, et al., 1999). Since TNFR1 lacks intrinsic kinase activity, signal transduction is mediated by the recruitment of DD-containing adaptor proteins to the TNFR1 DD (Figure 1.2). Ligation of TNF to TNFR1 recruits the DD-containing adaptor molecule TNF receptor-associated death domain (TRADD). TRADD recruits other adaptor proteins including the protein kinase receptor-interacting protein (RIP), TNF-R-associated factor 2 (TRAF2), and Fas-associated death domain (FADD). While FADD couples to the apoptotic pathway through the recruitment and activation of caspase-8, TRAF2 binds cellular inhibitor of apoptosis protein-1 (cIAP-1), inhibiting caspase activity (Uren, et al., 1996). In addition, TRAF2 and RIP activate IκB kinase (IKK), which phosphorylates IκB promoting its degradation and subsequent release of nuclear factor-κB (NF-κB). Upon NF-κB release and nuclear translocation, cIAP is upregulated, contributing to cell survival responses (Devin, et al., 2000). Furthermore, the cytoplasmic tail of TNFR1 contains a neutral sphingomyelinase (N-SMase) domain that stimulates N-SMase activity, which hydrolyzes

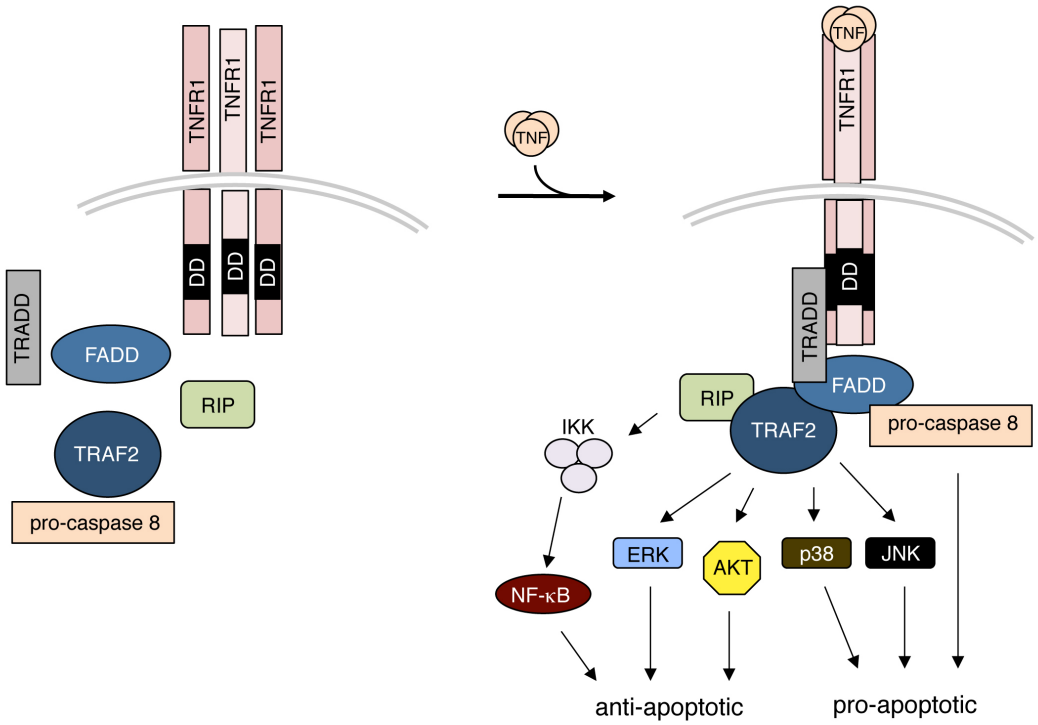

Figure 1.2: Pro- and anti-apoptotic signaling through TNFR1 Binding of TNF to homotrimeric TNFR1 triggers assembly of the death domain containing proteins TRADD and FADD to the TNFR1 death domain. FADD recruits pro-caspase 8 that undergoes auto-cleavage and activates effector caspases promoting apoptosis. TRAF2 also binds FADD and activates pro-apoptotic signaling cascades through p38 and JNK. In addition, TRAF2 promotes activation of anti-apoptotic targets ERK and AKT and RIP-mediated activation of the NF-κB pathway.

the membrane lipid sphingomyelin to generate ceramide. Ceramide promotes activation of kinase suppressor of Ras 1 (KSR1) and stimulates KSR1 kinase activity towards Raf-1 (Zhang, et al., 1997). Activation of Raf-1 leads to extracellular-signal-regulated kinase (ERK) activation, which promotes cell survival by phosphorylating the mitochondrial membrane protein Bcl-2 on Ser87, blocking cytochrome c release (Deng, et al., 2004; Tamura, et al., 2004). Previous data from our lab indicate that the activities of both ERK and NF-κB pathways function synergistically to promote cell survival in colon epithelial cells following TNF treatment (Yan, et al., 2001). Given the pleiotropic nature of TNF in a variety of cell types, understanding the mechanisms that contribute to the biological consequences of TNFR1 activation has implications for inflammatory diseases in which TNF is upregulated.

The ERK pathway

Canonical activation of the ERK/mitogen-activated protein kinase (MAPK) cascade occurs via a three-tiered kinase module that is initiated by many G protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs) (Figure 1.3*A*). Downstream of GPCRs and RTK activation, a membrane-anchored small GTPase known as Ras recruits the MAPK kinase kinase Raf-1 to the plasma membrane. Raf-1 translocation to the plasma membrane occurs following release of 14-3-3 proteins upon dephosphorylation of Ser259 by protein phosphatase 2A (PP2A) (Abraham, et al., 2000). While the complete mechanism of Raf-1 activation is not entirely understood, phosphorylation by KSR1 on Thr269 and phosphorylation of Ser338 by p21-activated protein kinase (Pak) are reported to stimulate Raf-1 kinase activity (King, et al., 1998; Xing, et al., 2001). Activated Raf-1 propagates signal transduction by phosphorylating and activating the dual specificity MAPK/ERK kinase (MEK) by direct phosphorylation of Ser217 and Ser221 in the MEK activation loop (Yan, et al., 1994). Activated MEK phosphorylates ERK1/2 on both threonine and tyrosine residues resulting in ERK activation (reviewed in (Roux, et al., 2004)). Following activation, ERK translocates into the nucleus where it phosphorylates a number of transcription

factors to regulate gene expression. While the ERK pathway has been extensively studied for the past 15 years, new data continues to emerge about the how this pathway is regulated. For instance, in TNFR1 signaling, Raf-1 activation was shown to occur in a Ras-independent manner (Edelblum, et al., 2008). In addition, recent evidence suggests that Raf-1 activation is enhanced via a dimerization-dependent mechanism with KSR1 (Rajakulendran, et al., 2009). These data suggest that alternative mechanisms are involved in pathway activation that is likely cell-type and context-dependent.

Kinase suppressor of Ras 1

Identification

Oncogenic mutations that confer constitutive activity in any of three Ras genes (*H-Ras*, *K-Ras*, or *N-Ras*) are found in many human adenocarcinomas (90% of pancreatic and 50% of colon cancers) (Bos, 1989). Therefore, much effort has been made to identify molecules and understand the biochemical processes that regulate the Ras pathway. To elucidate the mechanism by which the Ras effector protein Raf-1 becomes activated, three independent groups carried out forward genetic screens using chemical mutagenesis in *Drosophila melanogaster* and *Caenorhabditis elegans* to identify modifiers of the Ras pathway. In *Drosophila*, ectopic expression of a $Ras1^{V12}$ constitutively activated Ras allele in the developing eye results in a readily observable rough eye phenotype. Therefore, mutant alleles that disrupt the constitutive $Ras1^{V12}$ signaling would allow for normal eye development. One particular mutant that was able to suppress the activated $Ras1^{V12}$ was given the name kinase suppressor of Ras 1 because of its putative C-terminal kinase domain (Figure 1.3*Bii*) (Kornfeld, et al., 1995; Sundaram, et al., 1995; Therrien, et al., 1995). Likewise, the multi-vulva phenotype induced by the constitutively active *let-60 ras* gene in *C. elegans* was suppressed by mutations in *ksr-1* (Kornfeld, et al., 1995; Sundaram, et al., 1995). To determine where KSR functioned in the Ras pathway, Therrien and colleagues crossed a KSR loss-of-function allele with an ectopically expressed constitutively

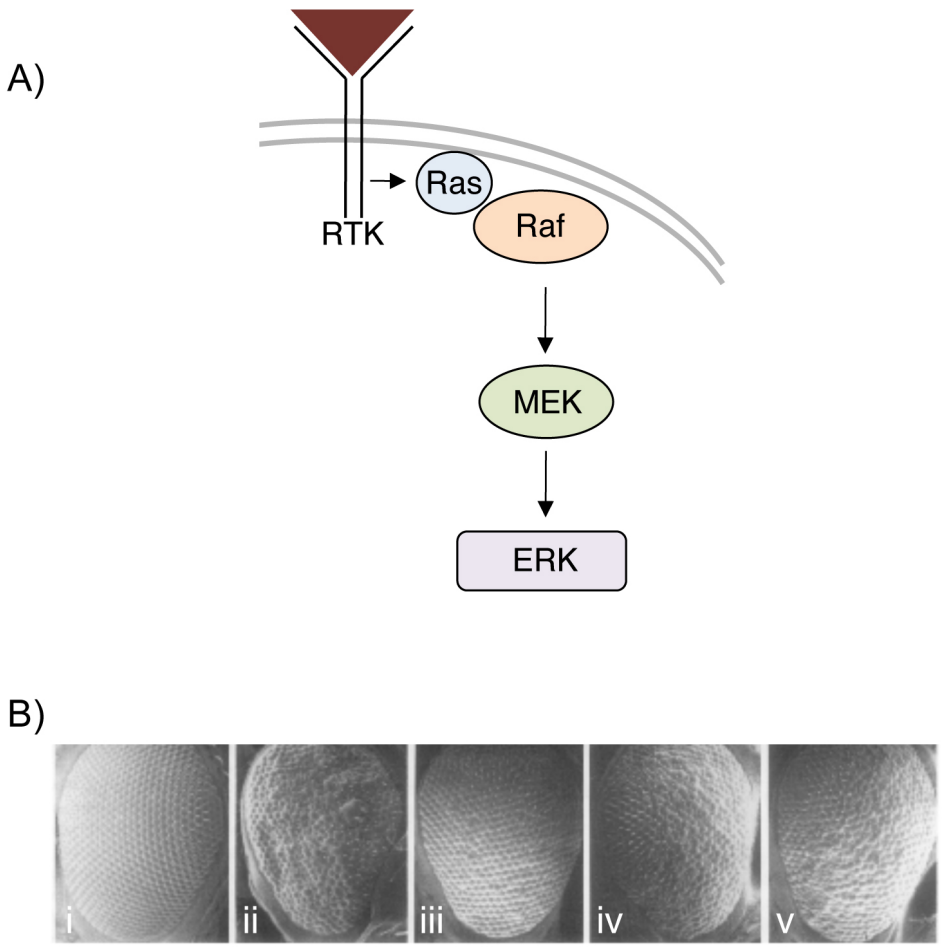

Figure 1.3: Kinase suppressor of Ras 1 (KSR1) identified as a modifier of the Ras signaling pathway A) Canonical activation of the Ras-Raf-MEK-ERK signaling pathway downstream of receptor tyrosine kinases (RTKs). Activation of RTKs promotes activation of Ras, which binds and promotes Raf activation. Activated Raf phosphorylates and activates MEK, which then phosphorylates and activates ERK. Bi) Topography of a wild-type *Drosophila* eye Bii) *Drosophila* rough eye phenotype resulting from ectopic expression of a constitutively active Ras^{V12}. Biii) Suppression of Ras^{V12} activity as a result of a mutant that mapped to the *ksr-1* locus. Biv) Rough eye phenotype resulting from ectopic expression of constitutively active $raf^{tor4021}$. Bv) Mutant *ksr-1* is unable to suppress activated $raf^{tor4021}$ phenotype.

active Raf-1 mutant that also produces a rough eye phenotype in *Drosophila*. This genetic epistasis analysis placed KSR1 directly upstream or parallel to Raf-1 since the KSR1 loss-of-function allele was unable to suppress the active Raf-1 phenotype (Figure 1.3*Bv*) (Therrien, et al., 1995). In the years following the initial discovery of KSR1, a brain-specific isoform (B-KSR1) has also been described (Müller, et al., 2000) as has a second KSR gene, named KSR2, which is found in *C. elegans* and mammals to have both unique and redundant functions with KSR1 (Dougherty, et al., 2009; Ohmachi, et al., 2002).

Structure of KSR1

Amino acid alignments comparing KSR1 across multiple species revealed that KSR1 contained five conserved areas (CA1-CA5) and is related to the Raf family of tyrosine kinase like (TKL) protein kinases (Figure 1.4) (Therrien, et al., 1995). The CA1 domain is unique to KSR1 proteins and in the original screen, a weak loss-of-function allele mapped to CA1, implicating a functional role for this domain in Ras signaling. More recently it was reported that the CA1 domain was involved in binding Raf proteins (McKay, et al., 2009). This interaction with Raf is dependent on the ability of MEK to bind KSR1 since mutation of Cys809 in the KSR1 kinase domain, a position essential for MEK binding, abolished KSR1-Raf dimers (McKay, et al., 2009). This raises the possibility that an intramolecular interaction may exist between KSR1 CA1 and kinase domain and contribute to KSR1 structure and/or function.

The second and third conserved areas consist of a proline-rich (CA2) and cysteine rich (CA3) domains respectively. The proline rich CA2 domain corresponds to a src homology 3 (SH3) recognition site while the cysteine rich CA3 domain is highly similar to both the CR1 domain of Raf (while lacking the Ras binding domain), and the lipid-binding domain of protein kinase C-ζ (PKCζ). In fact, the KSR1 CA3 domain facilitates membrane translocation and is hypothesized to bind the second messenger lipid ceramide, though results are conflicting (Michaud, et al., 1997; van Blitterswijk, 1998; Zhou, et al., 2002).

The serine/threonine rich CA4 domain is similar the CR2 domain in Raf-1, and the CA5 domain contains the eleven subdomains conserved in all protein kinases. The kinase domain of KSR1 has received much attention due to its unique features and conflicting reports surrounding catalytic activity. The first unique features are found in subdomains VIb and VIII that are indicative of serine/threonine (S/T) or tyrosine kinases. Typically S/T kinases contain HRDLKxxN in subdomain VIb while tyrosine kinases contain HRDLR/AxA/RN. For KSR1, the *Drosophila* homolog possesses an arginine in VIb indicative of a tyrosine kinase, while mammalian KSR1 contains a lysine corresponding to a S/T kinase. Another interesting feature for all KSR1 genes is that subdomain VIII contains a WxxY motif instead of T/SxxY/F for S/T kinases or PxxW found in tyrosine kinases. Even still, the most divergent feature occurs in subdomain II of the mammalian KSR1 kinase domain. Typically, an invariant lysine residue in subdomain II is critical for binding and orienting the ATP molecule and necessary for catalytic function (Hanks, et al., 1995). While the *Drosophila* KSR1 homolog has this lysine present, mammalian KSR1 contains an arginine in this position. Most protein kinases are rendered catalytically inactive when this lysine is substituted with arginine or methionine (Cotten, et al., 2003; Ebina, et al., 1987; Gibbs, et al., 1991; Snyder, et al., 1985). However, KSR1 is not the first protein kinase with variations in conserved residues, as the proteins with-no-lysine (WNK) and p53-related protein kinase (PRPK) contain variations in conserved sequences and possess enzymatic activity (Abe, et al., 2001; Xu, et al., 2000).

Function and regulation of KSR1

The contribution of KSR1 in the ERK/MAPK signal transduction pathway has been demonstrated for a variety of cellular functions including *Xenopus* oocyte maturation, monocyte differentiation, NK cell-mediated lysis, and colon epithelial cell survival (Therrien, et al., 1996; Wang, et al., 2004, Giurisato, et al., 2009; Yan, et al., 2004). The majority of studies conclude that the predominant function of KSR1 is to scaffold ERK/MAPK cascade components Raf-1, MEK, and ERK (Denouel-Galy, et al., 1998; Therrien, et al., 1996). As mentioned earlier,

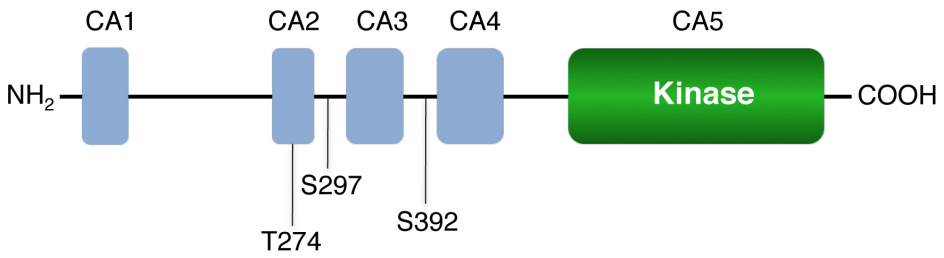

Figure 1.4: Linear representation of KSR1 conserved domains and regulatory phosphorylation sites KSR1 contains five conserved regions termed CA1-CA5. CA1 is a domain unique to KSR1 and is involved in binding Raf proteins. CA2 is a polyproline region corresponding to SH3 domain recognition. CA3 is a cysteine rich motif involved in lipid binding and membrane translocation. CA4 is a serine/threonine rich region and CA5 is a kinase domain. Phosphorylation sites demonstrated to be involved in regulation of KSR1 are depicted as serine (S) or threonine (T) with corresponding amino acid position. Ser297 and Ser392 are C-TAK1 phosphorylation sites that mediate 14-3-3 binding. Phosphorylation of Ser274 and Ser392 regulate the nucleocytoplasmic distribution of KSR1.

transient B-Raf binding occurs in the KSR1 CA1 domain (McKay, et al., 2009), whereas MEK is constitutively bound to KSR1 in the KSR1 kinase domain. KSR1 association with MEK is completely abolished when KSR1 Cys809 is mutated to tyrosine. Since this cysteine residue is conserved even for Raf family members, it likely contributes to the structural integrity of the kinase domain itself. Since substitution of KSR1 Arg589 in the ATP binding cleft of the kinase domain also reduces MEK binding, this position may also be critical for structural integrity (Yu, et al., 1998). While KSR1 contains an FxFP consensus ERK binding site in CA4, the binding of KSR1 to ERK appears to be transient and only induced upon Ras activation (Cacace, et al., 1999; Müller, et al., 2000). Thus, signal transduction through the ERK module is greatly enhanced by the ability of KSR1 to bind each component and bring them together to allow signal propagation from Ras to ERK. Just as KSR1 facilitates ERK pathway activation, it is also involved in attenuating ERK pathway activation. Recently KSR1 was found to possess a DExD caspase cleavage site just upstream of the kinase domain. This allows for rapid termination of survival cues stemming from ERK signaling once an apoptotic program is initiated (McKay, et al., 2007).

While the role of KSR1 association with ERK cascade components has been well studied, other KSR1 interacting proteins including 14-3-3, Cdc25C-associated kinase 1 (C-TAK1), $p50^{cdc37}$, heat shock protein 70 (Hsp70), γ subunit of G proteins, and Hsp90 also regulate KSR1 function (Stewart, et al., 1999). As with many proteins, phosphorylation regulates KSR1 function by modulating dynamics of the ERK signaling cascade. KSR1 is constitutively phosphorylated on 10 sites outside of the kinase domain by a number of protein kinases when overexpressed in HEK 293T cells (Volle, et al., 1999). Of these sites, phosphorylation at Ser297 and Ser392 by C-TAK1 mediates 14-3-3 binding and functions to sequester KSR1 in the cytosol (Müller, et al., 2001). Following stimulation, these sites are dephosphorylated by protein phosphatase 2A (PP2A) allowing for KSR1 membrane translocation and interactions with Raf (Ory, et al., 2003; Therrien, et al., 1996). It is during this stimulus-induced association with Raf that MEK, and subsequently ERK, are activated. Activated ERK phosphorylates KSR1, promoting dissociation

of KSR1 and B-Raf in a negative feedback loop releasing KSR1 from the plasma membrane (McKay, et al., 2009). In addition, phosphorylation of Thr274 and Ser392 on KSR1 regulates nuclear export and possibly regulates the duration of ERK activation in the absence of signaling at the level of Raf-1 (Brennan, et al., 2002).

While the kinase activity of KSR1 has been contentious, work from our lab and others support a catalytic role for KSR1 in the MAPK cascade. Prior to KSR1 being discovered, data from Kolesnick's group show that TNF treatment stimulates a ceramide activated protein kinase (CAPK) to undergo autophosphorylation and direct phosphorylation of Raf-1 (Liu, et al., 1994; Yao, et al., 1995). Following the cloning of KSR1, data from this same group demonstrate that KSR1 recapitulates previous results for CAPK, suggesting that these two proteins were one and the same (Xing, et al., 2000; Zhang, et al., 1997). Though it is still unclear if autophosphorylation is a prerequisite for KSR1 activity towards Raf-1, phosphorylation of Raf-1 on Thr269 by KSR1 increases Raf-1 activity towards MEK (Yan, et al., 2004; Zafrullah, et al., 2009; Zhang, et al., 1997). While our lab has recapitulated some of the findings surrounding the ability of KSR1 to phosphorylate Raf-1 *in vitro* (Yan, et al., 2004), others have suggested that the observed kinase activity is attributed to contaminating protein kinases complexed with KSR1 in these *in vitro* assays (Michaud, et al., 1997; Volle, et al., 1999). Thus, the controversy surrounding KSR1 catalytic activity has not been resolved since evidence for KSR1 enzymatic function has relied upon *in vitro* kinase assays utilizing immunoprecipitated KSR1 from mammalian cells. Therefore, interpretation of previous data remains inconclusive and warrants new approaches to determine if KSR1 functions as a protein kinase.

Role of KSR1 in cell survival

Previous data from our lab show that TNF-induced ERK, AKT, and NF-κB activation is impaired in *KSR1*$^{-/-}$ colon epithelial cells. Since ERK and NF-κB act synergistically to promote cell survival downstream of TNFR1 in young adult mouse colon (YAMC) epithelial cells (Yan, et al., 2001), it is consistent that cultured *KSR1*$^{-/-}$ colon epithelial cells exhibited increased apoptosis in

response to TNF (Yan, et al., 2004). The impairment in signaling is rescued by transient expression of wild-type KSR1 but not a kinase-inactive KSR1 mutant containing double aspartic acid to alanine substitutions in the kinase domain (D683A/D700A) (Yan, et al., 2004). In addition, *KSR1*$^{-/-}$ mice displayed increased epithelial cell apoptosis following intraperitoneal TNF injections (Yan, et al., 2004). In neuronal cells, KSR1 is required to protect against DNA damage-induced apoptosis by promoting ERK activation (Szatmari, et al., 2007). Collectively, these data indicate that KSR1 contributes to activation of signal transduction pathways important for cell survival during both extrinsic and intrinsic apoptotic programs. Since TNF is upregulated in many inflammatory diseases including those of the gastrointestinal tract, studying the role of KSR1 within this context will better elucidate the function of KSR1 in health and disease.

Regulation of gastrointestinal tract homeostasis

The human digestive system is a dynamic and complex multi-organ system. Stretching from mouth to anus, the gastrointestinal tract is a hollow tube where ingested food particles are digested, nutrients are absorbed, and waste is eliminated. Lining the surface of the small and large intestine is a single cell layer of columnar epithelial cells that facilitate nutrient uptake, water absorption, ion transport, and barrier functions. In the small intestine, this epithelial layer is maintained by stem cells that reside near the base of invaginated crypts, that divide and give rise daughter stem cells and proliferating progenitor cells that differentiate into four distinct lineages: enterocytes, entero-endocrine cells, goblet cells, or paneth cells. These cells migrate along the crypt-villus axis where they initiate an apoptotic program upon reaching the top of the villi and are ejected into the lumen (Figure 1.5). A similar process occurs for the colonic epithelium, barring the presence of protruding villi and paneth cells. Since the entire epithelial surface is renewed about every 5 days (Hall, et al., 1994), homeostasis requires tight regulation over proliferation, migration, differentiation, and apoptosis to maintain barrier integrity and protect the host from potentially harmful luminal contents. When epithelial damage or ulceration occurs, the epithelial cells at the wound margin quickly migrate to re-establish the epithelial barrier in a process known

as restitution. Simultaneously, cells of the immune system located in the sub-mucosa survey for and clear antigens that have penetrated the epithelial barrier. Critical to this process are specialized leukocytes called dendritic cells that identify pathogenic entities and present processed antigens to T helper lymphocytes to initiate host defense responses as necessary (As reviewed in (Laffont, et al., 2009; Tezuka, et al.)). The interplay between the epithelium and immune system is critical for ensuring that the host is protected from the outside world, while also directing immunotolerance to beneficial microbes and self-antigens.

Immune function in the gastrointestinal tract

Innate and adaptive immunity

The immune system, comprised of white blood cells called leukocytes, is divided between two branches consisting of innate and adaptive immunity. While functionally distinct, these two systems often cooperate in host defense. Leukocytes differentiate from myeloid or lymphoid precursors in the bone marrow into the various immune cell types. The innate immune system is comprised of neutrophils, macrophage, basophils, eosinophils, dendritic cells, and natural killer cells. Often the first lines of defense, innate immune cells utilize pattern recognition receptors (PPRs) that recognize invariant molecular patterns common to classes of pathogens. The different types of PPRs allow tailored innate responses directed towards the type of extracellular or intracellular pathogen. Some PPRs are able to activate transcriptional programs including NF-kB downstream of cell surface toll-like receptors (TLRs) and are sufficient to invoke adaptive immune T and B lymphocyte responses (Palm, et al., 2009).

A specific type of leukocyte termed lymphocytes function in the adaptive immune response. There are two branches of adaptive immunity comprising a humoral response involving B lymphocyte antibody production, and cell-mediated immunity involving macrophages, $CD8^+$ cytotoxic T lymphocytes, and $CD4^+$ helper T cells. As the name implies, adaptive immunity

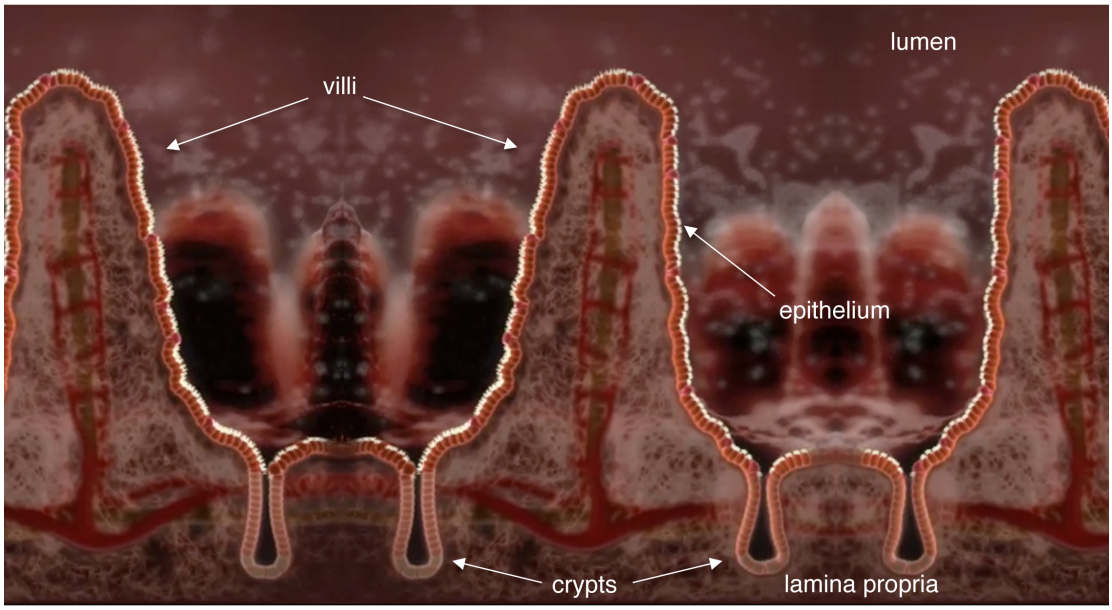

Figure 1.5: Regulation of gastrointestinal tract homeostasis Separating the lumen from the lamina propria is a single layer of columnar epithelial cells. This layer is maintained by stem cells residing near the base of the crypt that divide and give rise to proliferating progenitor cells. These progenitors migrate up the crypt-villus axis where they undergo differentiation into absorbent enterocytes, secretory enteroendocrine, mucin producing goblet cells, or paneth cells (small or intestine only). Once these cells reach the villus tip, they are ejected into the lumen and undergo apoptosis. Image adapted from (Keller, 2007).

is not restricted to a defined set of genes but rather utilizes recombination of both T cell receptor (TCR) and immunoglobulin (Ig) to acquire antigen-specific responses. Both TCR beta chain and Ig genetic loci contain multiple variable, diversity, and joining (V(D)J) gene segments that are rearranged through recombination, allowing for a vast repertoire of combinations (Roth, et al., 1992). This efficient mechanism eliminates the need to encode every possible combination within the genome. Development of T cells begins in the thymus where positive and negative selection ensures T cells do not mount immunological responses to self-antigens, while being competent to recognize major histocompatibility complex (MHC) molecules during antigen presentation. Following this process, T cells will become activated when TCRs on naïve T cells recognize specific peptides presented by antigen presenting cells (APCs). Consequently this triggers rapid T cell proliferation and, within days of an infection, the adaptive system mounts an effective defense against a specific pathogen. Since the ERK pathway promotes T cell proliferation and differentiation, understanding the contribution of MAPK cascade components in these processes will be important to develop therapies to control aberrant T cell cytokine production associated with chronic inflammatory diseases (Chang, et al., 2008).

Th1-Th2-Th17

Within the $CD4^+$ helper T cell populations there are functionally distinct subtypes, three of which are Th1, Th2, and Th17. The development of naïve Th0 cell precursors into one of these three specific lineages depends on the type of pathogenic infection, which drives the production and release of small proteins called cytokines from other immune cells (Figure 1.6). For instance, for Th0 cells to become polarized into Th1 cells requires the cytokine interleukin-12 (IL-12). Th1 cells then produce pro-inflammatory cytokines interferon-γ (IFN-γ), lymphotoxin-α (LT–α), and interleukin-2 (IL-2) that often cooperate with macrophage-produced tumor necrosis factor (TNF) to clear intracellular pathogens. Conversely, the cytokine intertleukin-4 (IL-4) directs naïve T cells towards the Th2 subtype. Th2 cells produce IL-4, interleukin-5 (IL-5), interleukin-6 (IL-6),

interleukin-10 (IL-10), and interleukin-13 (IL-13) to direct antibody production and combat parasitic infections. In addition, the Th2 cytokine IL-10 counteracts the pro-inflammatory effects of Th1 cytokines and restricts Th1 development. In recent years a new class of helper T cells was discovered in which TGFβ and IL-6 drive T cell differentiation into an interleukin-17 (IL-17) producing subtype termed Th17. While the role of IL-17 in IBD is less clear, it is commonly associated with autoimmune diseases because of its pro-inflammatory function. Since elevated cytokine production is a hallmark of inflammatory bowel disease (IBD), the balance of Th1/Th2/Th17 development is critical for maintaining immune homeostasis and an ideal target for immune modulating therapies.

Inflammatory bowel disease

The delicate balance between the appropriate immunological responses that protect the host, and the detrimental effects of chronic inflammation, highlight the necessity for understanding immune regulation. Transient activation and expansion of leukocytes is critical for clearance of intracellular or extracellular pathogens through innate and/or adaptive immune responses. However, sustained activation results in chronic inflammation often concurrent with elevated levels of cytokines and chemokines. Diseases including rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease (IBD) are characterized by increased cytokines and dysregulated immune responses (Kalyan, et al., 2009; Pletneva, et al., 2009; Stockinger, et al., 2007). While the etiology of IBD is not fully understood, both genetic and environmental elements have been attributed to disease pathogenesis (Hampe, et al., 2001; Hampe, et al., 2007; Hugot, et al., 2001; Ogura, et al., 2001). The two predominant forms of IBD affecting 1.4 million Americans are Crohn's disease and ulcerative colitis. These two diseases are largely thought to result from dysregulated immunological responses to enteric antigens following epithelial ulceration or invading pathogens (Figure 1.7). While both forms of IBD can present with similar symptoms, each disease has unique characteristics. Originally described in 1932, Crohn's disease can affect any part of the gastrointestinal tract and often is discontinuous in nature

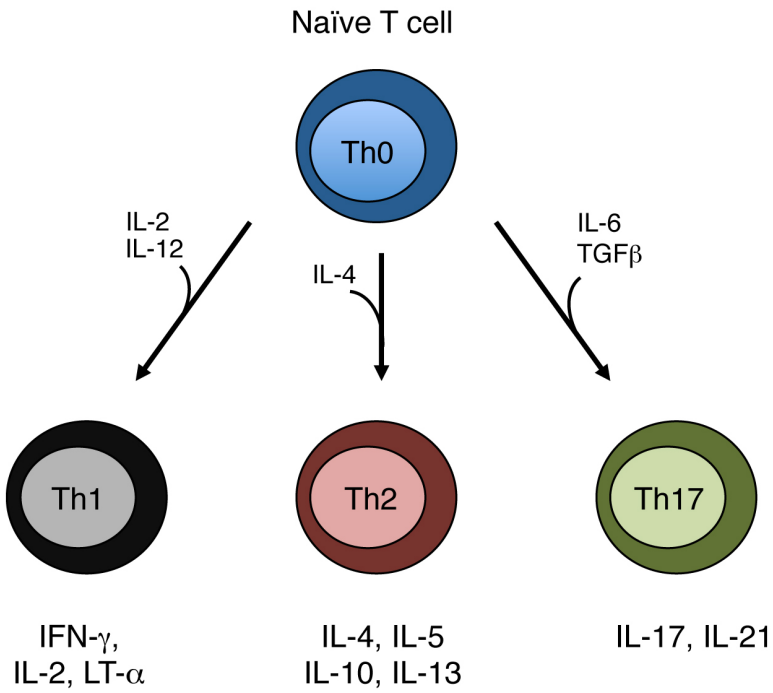

Figure 1.6: Differentiation of Naïve CD4$^+$ T cells Naïve T cells receiving a mitogenic stimulus differentiate along different developmental paths based on the cytokine milieu surrounding them. Naïve Th0 cells develop into a Th1 subtype in the presence of IL-12 and IL-2. Th2 development is dependent on the presence of IL-4, whereas IL-6 and TGFβ drive Th17 differentiation.

(Crohn, et al., 1932). While symptoms of ulcerative colitis have been noted for over millennia, Wilks and Moxon formally described the disease characteristics in 1875 following post-mortem examination of patients who succumbed to dysentery. Unlike Crohn's disease, ulcerative colitis usually begins at the rectum and progresses proximally in the large intestine. Both diseases involve immunological responses that largely define the condition, albeit with distinct features and likely varying causes. To date there is no cure for either disease and alleviating symptoms with current therapeutic strategies risk long-term complication. Furthermore, for patients suffering from IBD, inflammation-associated carcinogenesis is increased over the course of their lifetime (Itzkowitz, et al., 2004). Thus, the development of new chemical and biologic strategies that modulate inflammatory responses holds promise for treating patients with IBD and reducing the associated cancer risk.

Role of cytokines in the pathogenesis of IBD

Patients with IBD exhibit elevated levels of cytokines that are associated with disease pathogenesis. Crohn's disease is traditionally classified as a Th1-mediated disease with increased TNF, IFN-γ and IL-2 (Mullin, et al., 1992; Niessner, et al., 1995), which induce tissue damage and epithelial apoptosis (Figure 1.7) (Francoeur, et al., 2004). Ulcerative colitis is characterized as a Th2-mediated disease displaying elevated IL-6 and IL-13 that can increase epithelial barrier permeability and apoptosis (Funakoshi, et al., 1995; Heller, et al., 2005). While IL-17 is primarily associated with autoimmune diseases including multiple sclerosis and rheumatoid arthritis (Kalyan, et al., 2009; Pletneva, et al., 2009; Stockinger, et al., 2007), recent evidence on the pathogenic effects of Th17 cells in Crohn's disease and ulcerative colitis has been described (Liu, et al., 2009). While there is currently no cure for IBD, inhibition of TNF has proved to be an effective therapeutic strategy that induces remission in patients with Crohn's disease (Plevy, et al., 1997). However, the long-term consequences of immunosuppressive therapies are not without complications and side effects. Therefore, identifying the molecules

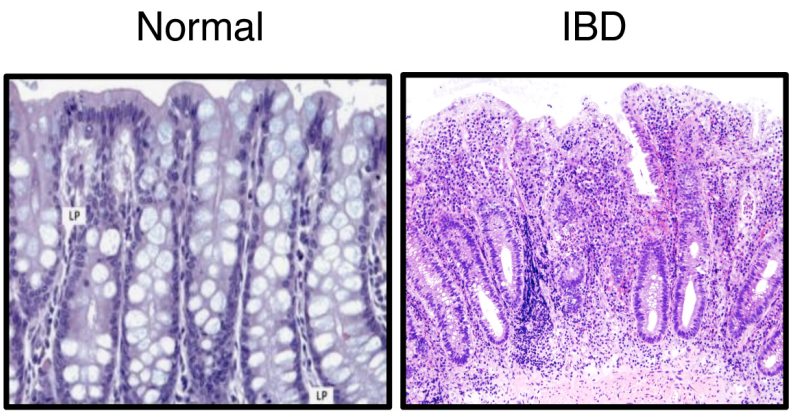

Figure 1.7: Colon section histopathology from normal and IBD tissue Normal architecture of the colonic crypts resemble that of “test tubes in a rack”. The base of the glands contacts the lamina propria and the top of the crypt faces the lumen. In IBD, infiltrating granulocytes, macrophage, and mononuclear cells disrupt the tight arrangement of colonic glands leading to epithelial barrier dysfunction, translocation of luminal antigens, and infiltrating immune cells.

and the signaling pathways involved in regulating immunological responses are key for developing new therapeutic approaches for treating patients with IBD.

Mouse models of IBD

Many human diseases such as cystic fibrosis, sickle cell anemia, Tay-Sachs disease, and hemophilia arise from inheritable mutations that result in disease (Ashley-Koch, et al., 2000; Bell, et al., 1995; Kerem, et al., 1989; Mahuran, et al., 1990). Although no single gene has been found to be causative for IBD, mutations in *NOD2* and *CD39* elevate risk suggesting that multiple genes may be involved in disease pathogenesis (Friedman, et al., 2009; Hampe, et al., 2002). Technological advances in manipulation of the mouse genome have allowed for reverse genetic studies of protein function and have revolutionized the study of several human diseases. In fact, the 2007 the Nobel Prize in Physiology or Medicine was awarded to Mario R. Capecchi, Martin J. Evans, and Oliver Smithies for their discoveries on genetic manipulation of mice. In the past 20 years, utilization of genetic deficiency mouse models including Wiskott-Aldrich Syndrome protein *(WASP)*$^{-/-}$, interleukin-10 (*Il10)*$^{-/-}$, and T cell receptor (*TCR)*$^{-/-}$, or chemically-induced models such as dextran sulfate sodium (DSS) or trinitrobenzene sulfonic acid (TNBS) have been used to study IBD since these mouse models develop characteristics similar to human IBD. While no one model fully recapitulates human IBD, insight into the molecules involved in inflammation, tissue damage, and epithelial restitution has been learned (As reviewed in (Hibi, et al., 2002)). Identifying the mechanisms that drive disease in animal models may yield new therapeutic targets and strategies to treat patients with IBD and is worthy of further investigation.

Interleukin-10 deficiency model of colitis

One cytokine involved in immune homeostasis and suppresses the activity of Th1 responses is IL-10 (Fiorentino, et al., 1989; Maynard, et al., 2007). It is known that decreased IL-10 levels are associated with IBD and recently polymorphisms were found in the IL-10 receptor locus of IBD patients (Glocker, et al., 2009; Ishizuka, et al., 2001). Consistent

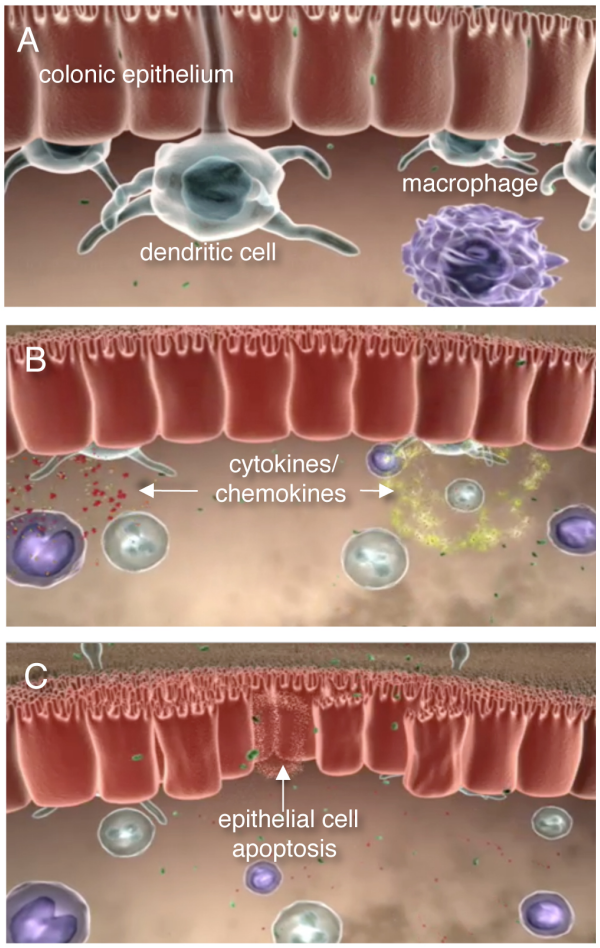

Figure 1.8: Effects of cytokines in IBD pathogenesis For patients with IBD, dysregulated innate and adaptive immune responses to commensal luminal flora lead to epithelial barrier breakdown. A) Bacterial translocation resulting from increased barrier permeability in Crohn's disease. B) Hyperactive immune responses trigger cytokine and chemokine release that then recruit and activate additional immune cells. C) Persistent elevation of cytokine levels, including TNF and IFN-γ, result in additional epithelial cell apoptosis and increased barrier permeability. Image adapted from (Gil, 2008).

with an immunosuppressive role for IL-10, *Il10*$^{-/-}$ mice develop spontaneous enterocolitis as a result of dysregulated immune responses to enteric microbial antigens (Kühn, et al., 1993). Disease progression and penetrance in *Il10*$^{-/-}$ mice is highly dependent on mouse strain and vivarium conditions. Since IL-10 deficiency-induced colitis is a Th1-mediated disease responsive to anti-TNF therapy, it is considered a valid model for studying human Crohn's disease (Rennick, et al., 1995; Scheinin, et al., 2003). Unfortunately, administration of recombinant IL-10 to IBD patients has not proven to be a successful therapeutic strategy. Thus, the need for identifying other immunomodulatory molecules that can influence Th1-mediated diseases including Crohn's disease is necessary (Li, et al., 2004).

Dextran sulfate sodium (DSS) model of colitis

Another mouse model developed for studying IBD is the chemically induced DSS model of colitis (Okayasu, et al., 1990). DSS is routinely administered via the drinking water at a concentration ranging from 2.5-5% (v/v) over a period of several days. While the efficacy for inducing colitis with DSS is highly strain dependent as well (Mahler, et al., 1998), the resulting ulcerations are characterized by epithelial cell loss and increases in barrier permeability (Cario, 2005). This disruption in epithelial barrier results in mucosal immune activation and the recruitment of neutrophils, macrophage, and lymphocytes, which promotes the release of pro-inflammatory cytokines including TNF (Dieleman, et al., 1998). Unlike other models of IBD, DSS-induced colitis exhibits both Th1 and Th2 cytokine profiles depending on the stage of disease (Dieleman, et al., 1998). While DSS is able to induce colitis in *Rag2*$^{-/-}$ mice, abrogating a requirement for the adaptive arm, Th1 cytokines exacerbate disease pathogenesis in the DSS model (Dieleman, et al., 1994; Kim, et al., 2006). The DSS model is a powerful tool to investigate the acute and chronic phases of disease as well as epithelial restitution due to its ulcerating effects.

Objectives

Previous work from our lab and others demonstrate that immunoprecipitated KSR1 phosphorylates and activates Raf *in vitro* (Yan, et al., 2004; Zafrullah, et al., 2009; Zhang, et al., 1997). In addition, expression of kinase-inactive KSR1 in YAMC cells sensitizes cells to TNF-induced apoptosis *in vitro* (Yan, et al., 2001). However, interpreting data from *in vitro* kinase assays using immunoprecipitated KSR1 proteins is inconclusive since KSR1 associates with several known protein kinases. To date there have been no published data demonstrating that bacterially expressed recombinant KSR1 possesses kinase activity. Furthermore, since *KSR1*$^{-/-}$ mice have increased apoptosis in colon epithelial cells following TNF treatment (Yan, et al., 2004), and TNF levels are increased in patients with IBD, the role of KSR1 in inflammatory disease warrants further investigation. The first objectives of this study were to determine if KSR1 possesses catalytic activity by expressing and recovering KSR1 from *E. coli*, a system devoid of serine/threonine protein kinases. Secondly, we investigated the role of KSR1 during chronic inflammation using the *Il10*$^{-/-}$ mouse model of spontaneous enterocolitis.

To test the hypothesis that KSR1 is a functional protein kinase (Chapter III), we established a bacterial expression system in *E. coli* and recovered recombinant KSR1 (rKSR1). *In vitro* kinase assays utilizing [γ-^{32}P]ATP were performed on rKSR1 to screen for intrinsic kinase activity. Kinase assays were also performed assessing the ability of mammalian KSR1 and recombinant KSR1 to phosphorylate bacterially expressed recombinant MEK. We also examined if KSR1 kinase activity towards MEK promoted epithelial cell survival in response to TNF.

The second objective of this study was to investigate the role of KSR1 during chronic inflammation. We tested the hypothesis that KSR1 is protective against a mouse model of colitis by promoting epithelial cell survival (Chapter IV). To do this we crossed a *KSR1*$^{-/-}$ mouse to the *Il10*$^{-/-}$ mouse model of spontaneous colitis. Mice were examined for external and histological signs of colitis. Changes in epithelial cell turnover were determined by immunohistochemical staining for apoptotic and proliferative markers. Bone marrow transplants were performed to determine if KSR1 functioned in the immune system to suppress colitis. Primary lymphocytes

were isolated and assayed for Th1/Th17 polarization defects *in vitro*. Neutralizing antibodies were administered intraperitoneally to investigate the involvement IFN-γ and IL-17A in *KSR1*$^{-/-}$*Il10*$^{-/-}$ disease pathogenesis. We also examined the role of KSR1 in a second model of Th1-mediated colitis using DSS.

CHAPTER II

MATERIALS AND METHODS

Cell culture and generation of cell lines

Culture conditions and procedures

The conditionally immortalized *KSR1*$^{-/-}$ colon epithelial cell line was generated by crossing a *KSR1*$^{-/-}$ mouse with the H-2K^{b}–tsA58 ImmortoMouse (Charles River Laboratories International Inc., Wilmington, MA), as previously described (Corredor, et al., 2003; Whitehead, et al., 1993; Yan, et al., 2004). Conditionally-immortalized colon epithelial cell lines were maintained under permissive conditions consisting of 5 units/ml of murine interferon-γ (BD Bioscience, San Jose, California) at a temperature of 33º C 5% CO_2 in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 5% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 units/mL penicillin and streptomycin (Invitrogen, Carlsbad, California), 5 mg/mL insulin, 5 mg/mL transferrin, 5 mg/mL selenous acid (ITS) (BD Bioscience). Prior to each experiment, cells were placed overnight at non-permissive conditions at 37^{o} C 5% CO_2 in RPMI 1640 containing 0.5% FBS, 100 units/mL penicillin and streptomycin, and without interferon-γ.

Retroviral infections

Murine wild-type KSR1 or murine KSR1$^{D683A/D700A}$, both FLAG-tagged at the N-terminus, were a generous gift from Richard Kolesnick (Memorial Sloan-Kettering Cancer Center, New York, NY). These constructs were subcloned into the bicistronic pLZRS-IRES-GFP retroviral vector and transfected into Phoenix 293 ecotropic viral packaging cells. Viral supernatants were collected and *KSR1*$^{-/-}$ colon epithelial cells were infected with virus containing empty vector (+vector), FLAG-tagged wild-type KSR1 (+KSR1), or FLAG-tagged KSR1$^{D683A/D700A}$ (+D683A/D700A). Infected cells were then sorted based on GFP expression by fluorescence-

activated cell sorting (FACS). Sorted cell lines were screened for KSR1 protein expression and those expressing near endogenous levels of KSR1, when compared to young adult mouse colon (YAMC) epithelial cells, were used for subsequent cell culture experiments.

Cell lysate preparation

Cell lysis and immunoprecipitation

Cell lysates were prepared by washing adherent cells in ice cold PBS followed by scraping cell into cell lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% protease and phosphatase I and phosphatase II inhibitor cocktails (Sigma) on ice. Total cell lysates were analyzed for protein concentration, boiled in Laemmli sample buffer at 95º C for 5 minutes, separated by SDS-PAGE, and analyzed by Western blot analysis. For immunoprecipitations, 30 μl of agarose-linked anti-FLAG M2 antibody (Sigma) were washed 3X in TBS (50 mM Tris pH 7.4, 150 mM NaCl) followed by a final wash in cell lysis buffer. Unless specifically stated, immunoprecipitations were performed on whole cell lysate from 1 mg of total protein and rocked overnight at 4º C. Immunoprecipitates were washed 3X in lysis buffer, boiled in 30 μl Laemmli sample buffer at 95º C for 5 minutes, and analyzed by Western blot analysis.

SDS-PAGE and Western blot analysis

Whole cell lysates or immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Membranes were blocked in 5% non-fat dry milk in TBS-Tween (0.05%) (TBST) at room temperature for 1 hour, washed in TBST, and incubated in primary antibody at room temperature for 1 hour. Membranes were then washed for 5 minutes 3X in TBST, incubated with the appropriate HRP-conjugated secondary antibody for 1 hour at room temperature, and washed for 5 minutes 3X in TBST. Total and phospho-proteins were detected by chemiluminescence using a luminol HRP substrate. Antibodies used in these studies include: KSR1 (BD Bioscience, #611577), KSR1 C-terminus

(Santa Cruz Biotechnology, #sc-1837, Santa Cruz, CA) Raf-1 (Santa Cruz Biotechnology, #sc-133), B-Raf (Santa Cruz, #sc-166), MEK1/2 (Cell Signaling, #9122, Danvers, MA), phospho-MEK1/2 (Cell Signaling, #9121), ERK1/2 (Cell Signaling, #4695), phospho-ERK1/2 (Promega, #V803A, Madison, Wisconsin), phospho-p38 (Cell Signaling, #9211), p38 (Cell Signaling, #9212), actin (Sigma).

Recombinant protein expression

FLAG-tagged wild-type KSR1 (rKSR1), FLAG-tagged kinase-inactive KSR1 (rD683A/D700A), FLAG-tagged rKSR1DCA1, HA-tagged wild-type KSR1 kinase domain (rKSR1ΔN521), FLAG-tagged wild-type Raf-1 (rRaf-1), FLAG-tagged kinase-inactive Raf-1 (rRaf-1 K375M), and His-tagged kinase-inactive (rMEK K97M) constructs were cloned into the pET 30 bacterial expression vector and were transformed into *E. coli* BL21(DE3) (Novagen). Protein expression was induced upon the addition of 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 25º C. Bacteria were lysed using CelLytic B Plus Kit (Sigma, St Louis, MO) following the manufacturer's protocol. Recombinant KSR1 and Raf-1 proteins were recovered from insoluble inclusion bodies under denaturing conditions using 8 M urea and dialyzed into buffer (50 mM Tris pH 7.4, 100 mM NaCl). His-MEK K97M protein expression was induced as before and bacteria supernatant containing recombinant proteins were purified using Ni-MAC column according to the manufactures protocol (Novagen).

***In vitro* kinase assays**

MEK phosphorylation assay

FLAG immunoprecipitates from KSR1 expressing cells lines were washed 3X in lysis buffer containing 1 M NaCl, equilibrated in kinase assay buffer (20 mM MOPS pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA) (Millipore) for 20 minutes, then incubation with rMEK K97M in the presence Mg^{2+}/ATP (Millipore) at 30º C for 30 minutes. The kinase reaction was stopped

upon addition of Laemmli sample buffer and heated to 95º C for 5 minutes. Western blot analysis was performed on separated samples to determine MEK phosphorylation. For *in vitro* kinase assays using recombinant proteins, rMEK K97M was incubated with rKSR1, rD683A/D700A, or rRaf-1 proteins as before at 30º C for 30 minutes. The kinase reaction was stopped upon addition of Laemmli sample buffer and heated to 95º C for 5 minutes. Samples were then analyzed by Western blot analysis.

KSR1 autophosphorylation assay

In vitro kinase assays were performed on rKSR1 and rD683A/D700A in the presence of 10 μCi [γ-^{32}P]ATP (Perkin-Elmer, Waltham, Massachusetts) at 30º C for 30 minutes. The kinase reaction was stopped upon addition of Laemmli sample buffer and heated to 95º C for 5 minutes. Samples were separated using SDS-PAGE, transferred to PVDF membrane, and incorporation of ^{32}P was assayed by autoradiography. Total KSR1 protein was then determined by Western blot analysis.

KSR1 Phosphoamino acid analysis

Following autoradiography, PVDF bound ^{32}P-KSR1 was excised and amino acids were hydrolyzed using 6 M HCl at 110º C for 1 hour. Amino acids were then lyophilized and rehydrated with pH 1.9 buffer containing PAA standards. Amino acids were spotted at a single origin on a TLC plate and were run in the first dimension at 1500 V for 25 minutes. The TLC plate was then rotated 90º and run in the second dimension in pH 3.9 buffer at 1300 V for 20 minutes. The TLC plate was dried and then sprayed with 0.25% Ninhydrin solution and incubated at 100º C until PAA standards were detectable by development of Ruhemann's purple (Duclos, et al., 1991). rKSR1 ^{32}P-phosphoaminio acids were determined via autoradiography and aligning the exposed Kodak BioMax film against the Ruhemann's purple phosphoamino acid standards visible on the TLC plate.

KSR1 2D-phosphopeptide mapping

Briefly, following an *in vitro* kinase assay using [γ-^{32}P]ATP and exposure to film, the ^{32}P-rKSR1 was excised from the PVDF membrane and MeOH was added to wet to the membrane. The PVDF membrane was then incubated at 37° C for 30 minutes in 50 mM NH_4HCO_3 containing 0.1% Tween-20. Following incubation, membrane was rinsed 5X with H_2O, 2X with 50 mM NH_4HCO_3 buffer, then resuspended in 200 μl NH_4HCO_3 buffer containing 10 μg trypsin and digested for 3 hours at 37° C. Following digestion, 400 μl water was added, vortexed, and spun 5' at 13k RPM. Supernatant was removed and lyophilized in speed-vac. Lyophilized sample was resuspended in 400 μl of pH 1.9 buffer and lyophilized again. Lyophilized sample was resuspended in 6 μl of pH 1.9 buffer and spotted on the origin of a pre-marked TLC plate and subjected to electrophoresis at 1000 V for 30 minutes. TLC plate was dried and placed in a chromatography buffer (75 vols. n-Butanol, 50 vols. pyridine, 15 vols. acetic acid, and 60 vols. water) chamber overnight oriented such that migration in the second dimension is towards the top of the glass. TLC plate was dried and phosphopeptides determined by autoradiography.

MBP phosphorylation assay

MBP phosphorylation assays were performed using the MBP Kinase Flex Assay Kit (Millipore) with rKSR1, rD683/D700A, rKSR1ΔN521, rRaf-1, or rRaf-1 K375M proteins following manufactures protocol. Whole cell lysates from EGF treated YAMC cells were used as a positive control for MBP phosphorylation. Relative MBP phosphorylation was calculated by comparing recombinant proteins incubated with MBP to MBP phosphorylation when no additional recombinant proteins were present (MBP only).

MEK activation assay

In vitro MEK activation assay with recombinant KSR1 proteins were conducted using unactive recombinant wild-type MEK1 (rMEK) (Millipore), and recombinant kinase-dead ERK2 (rERK) (Millipore) incubated for 30 minutes at 30° C. Kinase reaction was stopped by the addition

of Laemmli sample buffer and boiled for 5 minutes at 95º C. Total and phospho-proteins were determined by Western blot analysis. For *in vitro* MEK activation assays utilizing immunoprecipitated FLAG-KSR1, FLAG-D683A/D700A, and FLAG-Raf-1 proteins, KSR1 and Raf-1 immunoprecipitated protein concentrations were determined using linear regression based on Western blot analysis of a standard curve with FLAG-BAP (Sigma), for KSR1 determination, or recombinant Raf-1 (Millipore), for Raf-1 determination. FLAG-KSR1 and FLAG-D683A/D700A were immunoprecipitated from 1 mg of whole cell lysate to obtain 1 pmol KSR1 protein. FLAG-Raf-1 was immunoprecipitated form 0.6 mg of whole cell lysate to obtain 1 pmol Raf-1 protein. Immunoprecipitated proteins were washed 3X in cell lysis buffer containing 1M NaCl. Immunoprecipitates were then washed 1X with lysis buffer and equilibrated in kinase assay buffer for 20 minutes at 4º C. Samples were incubated with 0.5 pmol rMEK1 and 1 pmol rERK in the presence of ATP/Mg^{2+} in assay dilution buffer (ADB) for 30 minutes at 30º C. MEK activity was determined by Western blot analysis using an anti-phospho-ERK antibody (Promega).

Cell loss assays

TUNEL assay

Cell lines expressing +vector, +KSR1, and +D683A/D700A were seeded in 4-well chamber slides at $5x10^4$ cells (4-well chamberslide, Lab-Tek), and placed under non-permissive conditions overnight prior to experiments. Cells were then treated with 100 ng/ml murine TNF (Peprotech, Rocky Hill, NJ) for 8 hours. Apoptotic cells were labeled using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Millipore, Billerica, MA) following the manufacturer's protocol. The percentage of TUNEL positive cells was determined for a minimum of 500 cells counted using differential interference contrast microscopy at 20X magnification.

Nucleocount assay

6-well dishes were seeded at $4x10^5$ cells/well and maintained under permissive conditions for 24 hours. Following 16 hours under non-permissive conditions, cells were then pre-treated with vehicle (DMSO) or 10 μM of U0126 (MEK kinase inhibitor) (Calbiochem, Darmstadt, Germany) for 45 minutes followed by 100 ng/ml TNF for 24 hours. Cells were then washed 3X in PBS, incubated in cell lysis buffer, and the total number of adherent cells was determined by counting propidium iodide stained nuclei using a NucleoCounter (New Brunswick Scientific, Edison, New Jersey). Cell loss was calculated by comparing 24-hour treated cell numbers to 24-hour untreated control.

Animals

Generation of mice

KSR1$^{-/-}$ mice were crossed with *Il10*$^{-/-}$ mice to generate *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Since *KSR1*$^{-/-}$ *Il10*$^{-/-}$ female mice did not produce litters, maintenance of the colony required a breeding strategy using *KSR1*$^{-/-}$*Il10*$^{-/-}$ male mice mated with *KSR1*$^{-/-}$*Il10*$^{+/-}$ female mice. BALB/c (WT), *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were bred and maintained on a BALB/c background, conventionally housed under SPF conditions, and fed a standard rodent chow diet. *Rag2*$^{-/-}$ mice (BALB/c) were purchased from Taconic and housed in autoclaved cages and fed autoclaved rodent chow (Albany, NY).

Conformation of genotype

Alleles corresponding to targeted disruption in the *Il10* or *KSR1* loci were confirmed by PCR using isolated genomic DNA from tail clippings obtained by digesting mouse tails in DirectPCR (Tail) buffer (Viagen Biotech, Inc., Los Angeles, CA) containing 250 μg/ml proteinase K (MACHEREY-NAGEL, Germany) overnight at 55º C followed by a 1-hour incubation at 85º C.

PCR was performed on genomic DNA to confirm the presence of wild-type or knock-out alleles for *Il10* and/or *KSR1* using specific primers and corresponding PCR program outlined in Table 1.

Table 1: Genotyping primers and PCR programs

Primer	Sequence	PCR Program
KSR1 WT Fwd	5'-TATCTCCATCGGCAGTCT	Step 1: 94ºC -3m Step 2: 94ºC-30s, 56ºC-45s, 72ºC-30s (Repeat 32X) Step 3: 72ºC-5m Step 4: 4ºC-hold
KSR1 WT Rev	5'-TCGACGCTCACACTTCAA	
KSR1 KO Fwd	5'-CTGACCGCTTCCTCGTG	Step 1: 94ºC -3m Step 2: 94ºC-30s, 56ºC-45s, 72ºC-30s (Repeat 32X) Step 3: 72ºC-5m Step 4: 4ºC-hold
KSR1 KO Rev	5'-ATAGAGCCCACCGCATCC	
IL10 WT Fwd	5'-GTGGGTGCAGTTATTGTCTTCCCG	Step 1: 94ºC -3m Step 2: 94ºC-30s, 56ºC-45s, 72ºC-45s (Repeat 35X) Step 3: 72ºC-2m Step 4: 4ºC-hold
IL10 KO Fwd	5'-GCCTTCAGTATAAAAGGGGGACC	
IL10 WT/KO Rev	5'-CCTGCGTGCAATCCATCTTG	

Histological assessment

Mice were sacrificed by administration of CO_2 followed by cervical dislocation. Mouse colon and small intestine were harvested and "swiss-rolled", fixed in 10% formalin, and embedded in paraffin blocks. 5 μm sections were mounted and stained with Hematoxylin and Eosin (H&E). Histology was scored for inflammation and injury by M.K.W. who was blinded to the genotypes and/or treatment conditions based on a scoring method previously described (Kennedy, et al., 2000).

Administration of treatment

Azoxymethane (AOM)

6-week old mice were administered a single intraperitoneal injection of 12.5mg/kg AOM.

Cytokine neutralization

3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were given intraperitoneal injections of 100 μg/mouse anti-IgG (eBioscience, cat#16-4301, San Diego, CA), anti-IFN-γ (eBioscience, clone XMG 1.2), or anti-IL-17A (eBioscience, clone: eBioTC11-18H10.1) neutralizing antibodies twice per week for 3 weeks. Mouse colons were harvested and scored for inflammation and injury by Vanderbilt pathologist M.K.W.

Dextran sulfate sodium (DSS)

Mice were placed in cages where regular drinking water was replaced with water containing 3% (w/v) DSS (36–50 kilodaltons; MP Biomedicals, Solon, OH) for 7 days. Subsets of mice were given regular drinking water for a period of 4 days following DSS treatment to determine the ability to recover from injury. Mice were monitored daily for changes in weight as well as tested for fecal occult blood (Hemoccult; Beck- man Coulter, Fullerton, CA). Following DSS or the recovery period, mice were sacrificed, colons removed, fixed in 10% formalin, and

embedded in paraffin blocks. 5 μm sections were cut and stained with H&E and colon histology was scored by pathologist M.K.W. based on a method for DSS-induced injury previously described (Dieleman, et al., 1998).

Immunohistochemistry

Mouse colon sections were de-paraffinized, rehydrated, and antigen unmasked by boiling in a citrate-containing buffer (Vector Laboratories, Burlingame, CA). Slides were blocked with 10% goat serum (Zymed Laboratories Inc., Carlsbad, CA) for 30 min and proliferation was assayed by staining with Ki67 (Lab Vision, Fremont, CA) or phospho-histone-H3 (Cell Signaling, Boston, MA) antibody at 4° C overnight. Slides were washed and incubated with anti-rabbit horseradish peroxidase (Dako, Denmark) for 30 minutes. HRP-conjugated secondary were developed using a DAB substrate kit (Vector Laboratories), counterstained with Hematoxylin and viewed using a Nikon Eclipse E800 microscope. Apoptotic cells were detected by *in situ* oligonucleotide ligation (ISOL) (Chemicon, Billerica, MA) or terminal deoxynucleotidyltransferase nick-end labeling (TUNEL) peroxidase staining (Chemicon) following the manufacture's protocol and viewed using a Nikon Eclipse E800 microscope.

Barrier permeability

Mice were sedated for 30 minutes using a single intraperitoneal injection of ketamine (5 mg/ml)/xylazine (0.5 mg/ml) solution (100 μl/10g body weight). Fluorescein-dextran (Sigma cat# FD4, St. Louis, MO) (0.6 mg/gram mouse weight) was administered per rectum using a 3.5 Fr catheter. Peripheral blood was collected 30 minutes later and serum fluorescence was measured using a fluorescent plate reader and compared to preinjection serum fluorescence concentration using linear regression analysis using a FITC-dextran concentration standards.

Bone marrow transplantation

3-week old WT or *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipient mice were placed on water containing neosporin G.U. irrigant (Monarch Pharmaceuticals, Bristol, TN) 1 week prior to irradiation. Recipient mice were given a single total body lethal dose of 9 Gy from a ^{137}Cs γ-radiation source 5 hours prior to transplant. Bone marrow was isolated from femurs of WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, or *KSR1*$^{-/-}$*Il10*$^{-/-}$ donor mice and 5 x10^{6} bone marrow cells/mouse were injected via the retro-orbital venus plexus of irradiated recipients. For transplantation of *KSR1*$^{-/-}$*Il10*$^{-/-}$ bone marrow into *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipients, sex mismatched male donor cells were injected into female recipients and engraftment was screened by PCR to detect the male *SRY* gene locus. Mice were sacrificed 6 weeks post-transplant and colitis assessed by scoring colon inflammation and injury as before.

Leukocyte differentiation

Mouse peripheral blood was drawn from the saphenous vein and collected in heparin coated Microvette tubes (Sarstedt, Germany). Blood was analyzed using a HemaVet 950FS (Drew Scientific Inc., DallasTX). Leukocyte populations were recorded as percentage of total leukocytes.

T cell proliferation

Primary T cells were isolated from the spleens of mice. Red blood cells were lysed and 2 x 10^{5} cells in RPMI 1640 media containing 10 mM HEPES (Sigma, St Louis, MO), 10 μg/mL gentamycin (GIBCO, Carlsbad, CA), 100 U/mL penicillin/100 μg/mL streptomycin (GIBCO) and 10% heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA) were seeded in round bottom 96-well plates pre-coated with 5 μg anti-CD3ε (BD Bioscience cat # 557306, San Jose, CA) and 5 μg anti-CD28 (BD Bioscience cat # 557393). Cells were grown for 72 hours at 37° C then pulsed for 18 hours with 1 μCi/well [^{3}H]thymidine (ICN, Solon, OH). Incorporated [^{3}H]thymidine was detected using a TopCount plate reader (Perkin Elmer, Waltham, MA).

Colonic cytokine profiling

RNA isolation and quantitative real-time PCR analysis

Total RNA was collected from whole mouse colons that were flushed of contents with ice-cold PBS, homogenized and lysed on ice, and purified using Qiagen RNA isolation kit and treated with RNase-free DNase as directed (Qiagen, Valencia, CA). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). For real-time RT-PCR, we used the relative gene expression method (Giulietti, et al., 2001). GAPDH served as the normalizer. All cDNA samples were analyzed in triplicate, along with "no reverse transcriptase" controls, using Applied Biosystems 7000 or Step One Plus real-time PCR instruments. Levels of cytokine expression are indicated as relative units, based on comparison of RNA pooled from 6 WT mouse colons (calibrator tissue) (Giulietti, et al., 2001). Primer and probe sets were purchased as Taqman Gene Expression Assays (Applied Biosystems).

Intracellular cytokine staining

Flow cytometry reagents

Fluorochrome coupled anti-CD4, -CD45RB, -TCRb, -IFN-γ, and anti-IL-17 as well as unconjugated anti-CD3, -CD28, -IFN-γ, -IL-4 and rmIL-6 were all purchased from BD Pharmingen (San Jose, CA). rhTGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Generation of Th17 and Th1 cells in vitro

Th17 cells were generated as described (Lee, et al., 2009) with some modifications. Briefly, total splenocytes were lysed of red blood cells and plated at a density of $2.5x10^6$ cells per well (12-well plate) in RPMI containing 10% FCS in the presence of plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (2.5 μg/ml), anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml), rhTGF-β1 (5

ng/ml), rmIL-6 (20 ng/ml), and supplemented with sodium pyruvate and non-essential amino acids. Two days after culture, cells received 1 ml of fresh medium containing anti-IFN-γ, anti-IL-4, rhTGF-β1, rmIL-6, sodium pyruvate, and non-essential amino acids. At day 6, cells were analyzed for cytokine production. Th1 cells were generated by using same isolation as above, cultured in RPMI containing 10% FCS in the presence of plate-bound anti-CD3 (5 μg/ml), soluble anti-CD28 (2.5 μg/ml), anti-IL-4 (10 μg/ml), IL-2 (20 units/ml), IL-12 (5 ng/ml), supplemented with sodium pyruvate and non-essential amino acids, and analyzed as before on day 6.

Naïve T cell activation

Primary splenocytes were isolated and single cell suspensions were incubated with PMA/Ionomycin (20 ng/ml and 1 μM respectively) and GolgiPlug (BD Biosciences) for 5 hours. Cells were then washed and stained with the appropriate surface markers. After fixing, cells were washed with Perm/Wash solution (BD Biosciences) followed by intracellular staining with anti-cytokine antibodies. Cells were acquired in a FACSCalibur instrument and data analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

Adoptive transfer of colitogenic T cells

Total splenocytes from donor mice were cleared of red blood cells by lysis in sterile water briefly then brought up in RPMI 1640 media and depleted of B cells using magnetic beads and columns as described by the manufacturer (Miltenyi, Germany). Cells were stained with anti-CD4 APC and anti-CD45RB FITC and sorted to obtain a fraction (~99% purity) of $CD4^{+}CD45RB^{high}$ cells as previously described (Powrie, et al., 1994). Sorted cells were thoroughly washed with sterile PBS and 0.75×10^{6} cells were adoptively transferred, i.v. per recipient *Rag2*$^{-/-}$ mice. Hosts were followed twice a week for weight change and signs of colitis, such as diarrhea, rectal bleeding, and scruffiness.

Lymphocyte isolation

Splenocytes and cells from mesenteric lymph nodes were isolated using conventional procedures. Colon IEL and lamina propria lymphocytes were obtained as previously described (Olivares-Villagómez, et al., 2008). Briefly, colons were dissected and the intestinal content flushed out with cold Hanks' balanced salt solution (HBSS). Colons were cut into 0.5 cm pieces and incubated with medium force shaking (~200 rpm) for 30 minutes in HBBS/5% FCS at 37° C. Supernatant was recovered, filtered through a glass wool column and after washing, IEL were separated in a 70% - 40% Percoll discontinuous gradient. The remaining tissue was incubated again for 30 minutes as before in HBSS/5% FCS/2mM EDTA. Supernatant was discarded and the tissue was cut into smaller pieces and digested for 20 minutes at 37° C in 5ml of HBSS/5% FCS containing 1.5 mg/ml collagenase VIII (Sigma) and 100 units of bovine pancreatic DNase I (Sigma). The slurry was filtered and collected, and the tissue was again digested in fresh medium for an additional 20 minutes. Slurries were pooled, washed and lamina propria cells were resuspended in fresh medium.

Statistical analysis

For the experiments involving KSR1-MEK binding and cell loss assays, a one-way analysis of variance (ANOVA) model was used to determine significance. The *P* value was adjusted for multiple comparisons with the Dunnett's post-test using a 95% confidence interval for three independent experiments. Histopathology, immunohistochemistry, and qRT-PCR statistical analysis were compared using one-way ANOVA followed by Dunnett's post-test. Bone marrow transplant, Th1/Th17 polarization, intracellular cytokine staining and cytokine neutralization statistical analysis were performed using an unpaired Student's t test. *P*-values of ≤ 0.05 were considered significant. All data was analyzed using Prism 5 (GraphPad Software, Inc.).

CHAPTER III

KSR1 IS A FUNCTIONAL PROTEIN KINASE CAPABLE OF SERINE AUTOPHOSPHORYLATION AND DIRECT PHOSPHORYLATION OF MEK1

Introduction

Many cellular responses to external stimuli utilize MAPK pathways to carry out a diverse range of biological processes. Activation of these pathways, which are conserved in all eukaryotes, is often initiated by GTPases downstream of cell surface receptors, followed by sequential signal transduction through a three-component kinase system. One particular MAPK module consists of the protein kinases Raf, MEK, and ERK. Canonical activation of the Raf/MEK/ERK cascade occurs downstream of the small GTPase Ras to elicit a variety of cellular responses including proliferation, differentiation, and cell survival (Cheng, et al., 2008; Kaiser, et al., 1999; Kanakura, et al., 1991; Qui, et al., 1992). Since the ERK pathway is integral for many cellular events, and constitutive pathway activation is frequently concurrent with many cancers, understanding the precise mechanisms that contribute to pathway activation are essential for developing therapeutic targets that modulate this pathway (Andreyev, et al., 2001). KSR1, first identified through genetic screens in *D. melanogaster* and *C. elegans,* is an evolutionarily conserved protein that positively regulates the Raf/MEK/ERK cascade by functioning either upstream or in parallel with Raf-1 (Kornfeld, et al., 1995; Sundaram, et al., 1995; Therrien, et al., 1995). KSR1 functions as a molecular scaffold by binding several signaling components of the ERK cascade; and thus can enhance MAPK activation by regulating the efficiency of these interactions (Denouel-Galy, et al., 1998; Stewart, et al., 1999; Yu, et al., 1998). In addition to its scaffolding role, there is evidence that KSR1 functions as a protein kinase. The KSR1 C-terminus contains the twelve subdomains that are conserved in all protein kinases including the conserved aspartic acid and asparagine residues within subdomain VIb (HRDLKxxN motif) and the aspartic acid in subdomain VII (DFG motif) (Hanks, et al., 1995; Hanks, et al., 1988). However, the

catalytic function of KSR1 remains controversial since mammalian KSR1 contains an arginine in place of the invariant lysine residue in subdomain II. This lysine positioned in subdomain II is involved in binding and orienting the ATP molecule to facilitate phosphotransfer of ATP γ-phosphate (Kamps, et al., 1986). While lysine to arginine mutations in this position disrupt ATP binding and render many protein kinases inactive (Cotten, et al., 2003; Ebina, et al., 1987; Gibbs, et al., 1991; Snyder, et al., 1985), a KSR1 splice variant is able to bind ATP when the arginine was substituted with lysine or methionine (Müller, et al., 2000). This suggests that KSR1 might utilize a different lysine, as seen with the protein kinase with no lysine-1 (WNK1) (Xu, et al., 2000), or may have a structurally unique ATP-binding cleft compared to other protein kinase domains. Therefore, further investigation into KSR1 catalytic function is warranted.

Initial reports of KSR1 protein kinase activity suggest that immunoprecipitated KSR1 autophosphorylates, as well as phosphorylate and activate Raf-1, *in vitro* (Xing, et al., 2000; Yan, et al., 2004; Zhang, et al., 1997). However, immunoprecipitated KSR1 contains additional co-precipitating protein kinases making it difficult to delineate KSR1 protein kinase activity from that of other contaminating kinases in the assay (Michaud, et al., 1997; Volle, et al., 1999). Therefore, to resolve KSR1 kinase activity from other protein kinases *in vitro* requires isolating recombinant proteins expressed in a system with no known serine/threonine protein kinases, such as *E. coli* (Rahmsdorf, et al., 1974).

Here we report that bacterially-derived KSR1 underwent serine autophosphorylation, phosphorylated myelin basic protein (MBP) as a generic substrate, and phosphorylated recombinant kinase-inactive MEK1 (rMEK K97M). We also demonstrate that both a functional KSR1 kinase domain and MEK protein kinase activity are required for resistance to TNF-induced cell death in colon epithelial cells. Taken together, these data indicate that in addition to a scaffold, KSR1 is indeed a functional protein kinase in the ERK pathway downstream of TNF signaling.

Results

KSR1 protection from TNF-induced cell death requires a functional KSR1 kinase domain and MEK kinase activity.

The pleiotropic pro-inflammatory cytokine, tumor necrosis factor, (TNF) elicits various cellular responses in a variety of cell types *in vitro* and *in vivo* and plays a pivotal role in chronic gastrointestinal disorders including celiac and inflammatory bowel diseases (Breese, et al., 1994; Kontakou, et al., 1994; Zhou, et al., 2008). Previous studies from our lab show that the colon epithelium of *KSR1*$^{-/-}$ mice is sensitive to TNF-induced apoptosis compared to wild-type mice (Yan, et al., 2004). Therefore, to determine if KSR1 kinase activity is required to prevent TNF-induced cell death, we produced stable *KSR1*$^{-/-}$ colon epithelial cell lines expressing either empty vector (+vector), FLAG-tagged wild-type KSR1 (+KSR1), or FLAG-tagged kinase-inactive KSR1 containing alanine substitutions at aspartic acid residues in the HRDLKxxN and DFG motifs involved in phosphotransfer (+D683A/D700A) via retroviral infection with bicistronic GFP expression constructs. These cells were then sorted based on GFP expression to obtain lines expressing KSR1 at near endogenous levels compared to YAMC epithelial cells (Fig. 3.1*A*). Since a previous report indicates that a kinase-inactivating mutation in the KSR1 kinase domain reduces KSR1 association with MEK (Yu, et al., 1998), we performed FLAG immunoprecipitation on +vector, +KSR1, and +D683A/D700A cell lines and compared MEK binding. Both KSR1 proteins were equally competent to co-precipitate endogenous MEK (Fig. 3.1*B*). This suggests that the D683A/D700A kinase-inactivating mutation in KSR1 had no deleterious effects on MEK binding. To determine if KSR1 was required for TNF-induced cell survival in cultured colon epithelial cells, apoptosis was assessed in TNF-treated +vector, +KSR1, and +D683A/D700A cell lines by TUNEL assay. Unlike cells expressing KSR1, those expressing either +vector or +D683A/D700A were sensitive to TNF-induced apoptosis (Figs. 3.2*A*-*B*). Previous data from our lab indicate that TNF-mediated ERK activation was impaired in cells lacking KSR1 or expressing

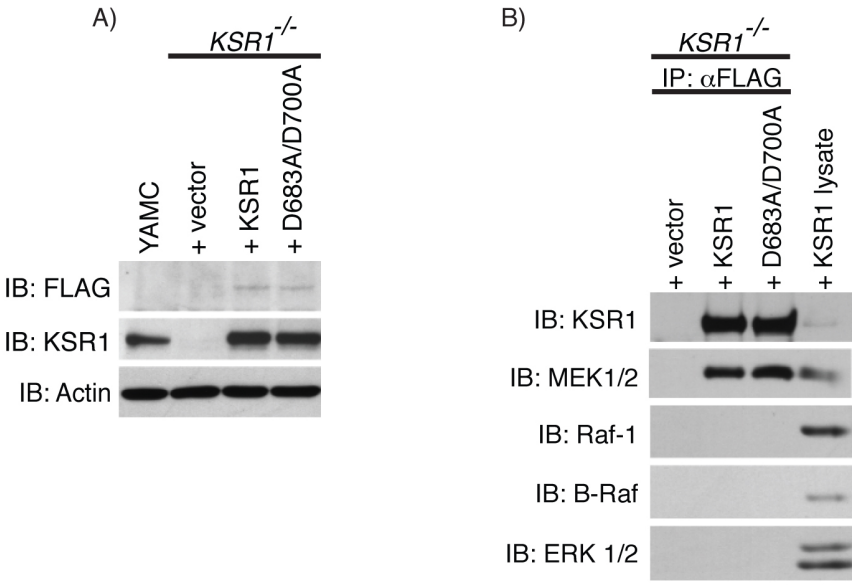

Figure 3.1: Wild-type KSR1 and kinase-inactive KSR1 associate with MEK. A) KSR1 protein expression was determined by Western blot analysis on whole cell lysates from *KSR1*$^{-/-}$ mouse colon epithelial cells expressing +vector, +KSR1, or +D683A/D700A, or from YAMC cells using the indicated antibodies. B) Co-immunoprecipitation of MEK was determined by Western blot analysis on FLAG-immunoprecipitates from +vec, +KSR1, and +D683A/D700A expressing cells. Whole cell lysate from +KSR1 cells was used as a control for detecting each protein probed. Immunoblots are representatives of at least 3 independent experiments. *IP, immunoprecipitation; IB, immunoblot.*

kinase-inactive KSR1 (Yan, et al., 2004). Therefore, we investigated whether MEK kinase activity was required for protection against TNF-induced cell death. An equal number of cells were pre-treated with vehicle or the MEK inhibitor U0126 and then treated with TNF. Cells lacking KSR1 or expressing the D683A/D700A mutant KSR1 were sensitive to TNF-induced cell loss, whereas +KSR1 cells were not (Fig. 3.2*C*). However, the +KSR1 cells were sensitized to TNF-induced cell loss following MEK inhibition, whereas U0126 did not potentiate cell loss in cells expressing +vector or +D683A/D700A. Interestingly, treatment of +D683A/D700A cells with U0126 caused increased cell loss in the absence of TNF. These data suggest that the protective effect of wild-type KSR1 is mediated through MEK activation. In a parallel experiment, U0126 effectively blocked TNF-induced ERK phosphorylation, but not p38 MAPK phosphorylation, in +KSR1 cells, confirming its specificity for MEK (Fig. 3.2*D*). Taken together, these observations demonstrate that TNF-induced colon epithelial cell survival requires a functional KSR1 kinase domain, which in turn relies on MEK activity.

KSR1 phosphorylates recombinant kinase-inactive MEK1

The observation that inhibiting MEK activation sensitized +KSR1 cells, but did not potentiate cell loss in +D683A/D700A or +vector cells, combined with the prior observation that kinase-inactive KSR1 expression attenuates TNF-induced ERK activation (Yan, et al., 2001), suggests that KSR1 protein kinase activity may effect MEK activation. Since MEK activation requires phosphorylation on two serine residues in the MEK activation loop (Alessi, et al., 1994; Zheng, et al., 1994), we used a phospho-specific antibody to these MEK serine residues (S218/S222) to determine the phosphorylation state of KSR1-associated MEK. FLAG immunoprecipitations were performed on serum-starved +vector, +KSR1, and +D683/D700A cells. In cells expressing +KSR1, KSR1-associated MEK was phosphorylated 5-fold over +D683A/D700A-associated MEK (Fig. 3.3*A*), demonstrating that MEK interaction with wild-type KSR1 enhances MEK phosphorylation.

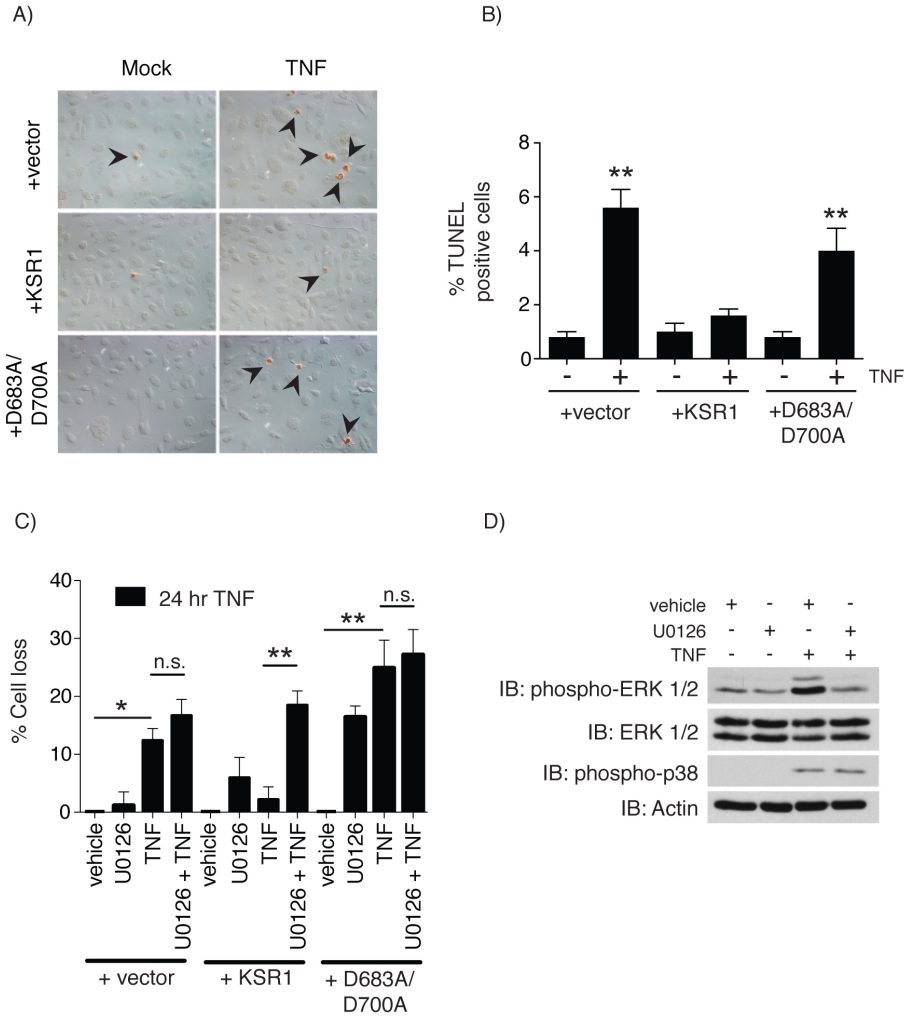

Figure 3.2: KSR1-mediated protection against TNF-induced apoptosis requires both a functional KSR1 kinase domain and MEK kinase activity. A) TNF-induced apoptosis was determined by treating serum-starved +vector, +KSR1, and +D683A/D700A cells with TNF (100 ng/ml) for 8 hours. Apoptotic nuclei were labeled using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). B) Apoptotic cells from panel A were quantified and reported as the percentage of TUNEL positive cells out of 500 cells. Solid bars represent the mean for each condition and the error bars the SEM. C) TNF-induced cell loss was determined for cells that were pre-treated with vehicle or the MEK inhibitor U0126 (10 μM) for 45 minutes followed by TNF (100 ng/ml) for 24 hours. The total number of adherent cells was quantified and cell loss calculated as the percent of the vehicle treated control for each cell line. Assays were performed a minimum of 3 times. D) The specificity of U0126 was confirmed in +KSR1 cells pretreated with vehicle or U0126 (10 μM) for 45 minutes followed by TNF for 15 minutes and assayed for phosphorylated ERK. Protein phosphorylation and total protein were determined by Western blot analysis using the indicated antibodies. Immunoblots are representatives of at least 3 independent experiments. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$

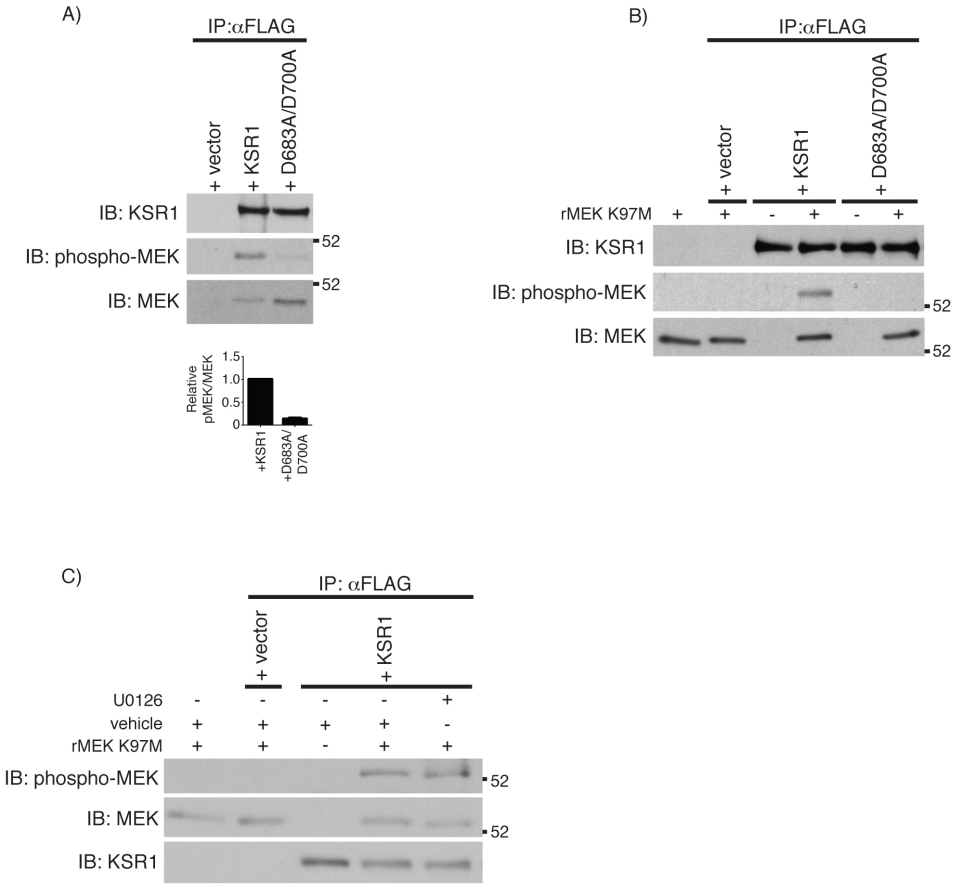

Figure 3.3: KSR1 phosphorylates MEK1 A) FLAG immunoprecipitation was performed using whole cell lysates from *KSR1*$^{-/-}$ cells expressing +vector, +KSR1, or +D683A/D700A. MEK phosphorylation was determined by Western blot analysis using the indicated antibodies. Densitometric analysis was performed on total and phosphorylated co-precipitated MEK protein and represented as the ratio of phosphorylated MEK/total MEK. Solid bars represent the mean ratio from 3 independent experiments and error bars represent the SEM. B) *In vitro* kinase assay using immunoprecipitated FLAG-tagged KSR1 proteins incubated with kinase-inactive rMEK K97M. Total protein and phosphorylated rMEK K97M were determined by Western blot analysis. C) *In vitro* kinase assay performed as before following a 30 minutes pre-incubation with vehicle or U0126 (10 mM). D) Efficacy of inhibiting an active Raf/MEK/ERK cascade *in vitro* was assayed using recombinant constitutively active Raf-1 (rCA-Raf-1), recombinant MEK1 (rMEK) and recombinant ERK (rERK) in the presence of vehicle or U0126 (10 μM). Total and phosphorylated proteins were determined by Western blot analysis. *IP, immunoprecipitation; IB, immunoblot.*

Based on our observation that MEK phosphorylation was increased when complexed with wild-type KSR1, we investigated whether MEK was a KSR1 substrate in an *in vitro* kinase assay. For these assays we utilized recombinant kinase-inactive MEK1 harboring an amino acid substitution at the conserved lysine residue (rMEK K97M) in the MEK kinase subdomain II. FLAG immunoprecipitates from each cell line were incubated with rMEK K97M in the presence of ATP. Immunoprecipitates from cells expressing +KSR1, but not +D683A/D700A or +vector were able to phosphorylate rMEK K97M, as determined by Western blot analysis (Fig. 3.3*B*). Since endogenous MEK co-precipitates with KSR1 under non-stringent conditions, we determined if endogenous MEK activity was responsible for phosphorylating rMEK K97M. FLAG immunoprecipitates from +KSR1 cells were incubated with either vehicle or U0126 then subjected to an *in vitro* kinase assay using rMEK K97M. U0126 was unable to block rMEK K97M phosphorylation by +KSR1 immunoprecipitates *in vitro* (Fig. 3.3*C*). The efficacy of U0126 in these experiments was confirmed by the ability of U0126 to block recombinant wild-type MEK (rMEK) phosphorylation of recombinant ERK (rERK) when stimulated by a constitutively active recombinant Raf-1 (rCA-Raf-1) (Fig. 3.3*D*). These data further indicate that MEK is a substrate for KSR1 protein kinase activity.

Recombinant wild-type KSR1 is a functional protein kinase capable of serine autophosphorylation.

Our data utilizing FLAG immunoprecipitation of KSR1 expressed in cell culture for *in vitro* kinase assays suggest that MEK is a substrate of KSR1. However, these data cannot exclude the possibility that KSR1 co-precipitates additional protein kinases. Ther46efore, to directly assay KSR1 kinase activity, we produced recombinant wild-type KSR1 (rKSR1) or recombinant kinase-inactive KSR1 (rD683A/D700A) in *E. coli*. We first examined the ability of rKSR1 proteins to co-precipitate with rMEK K97M. Consistent with our cell culture data, both rKSR1 and rD683A/D700A were equally competent in MEK binding (Fig. 3.4*A*). To determine if rKSR1 is capable of autophosphorylation, we performed *in vitro* kinase assays using [γ-^{32}P]ATP with

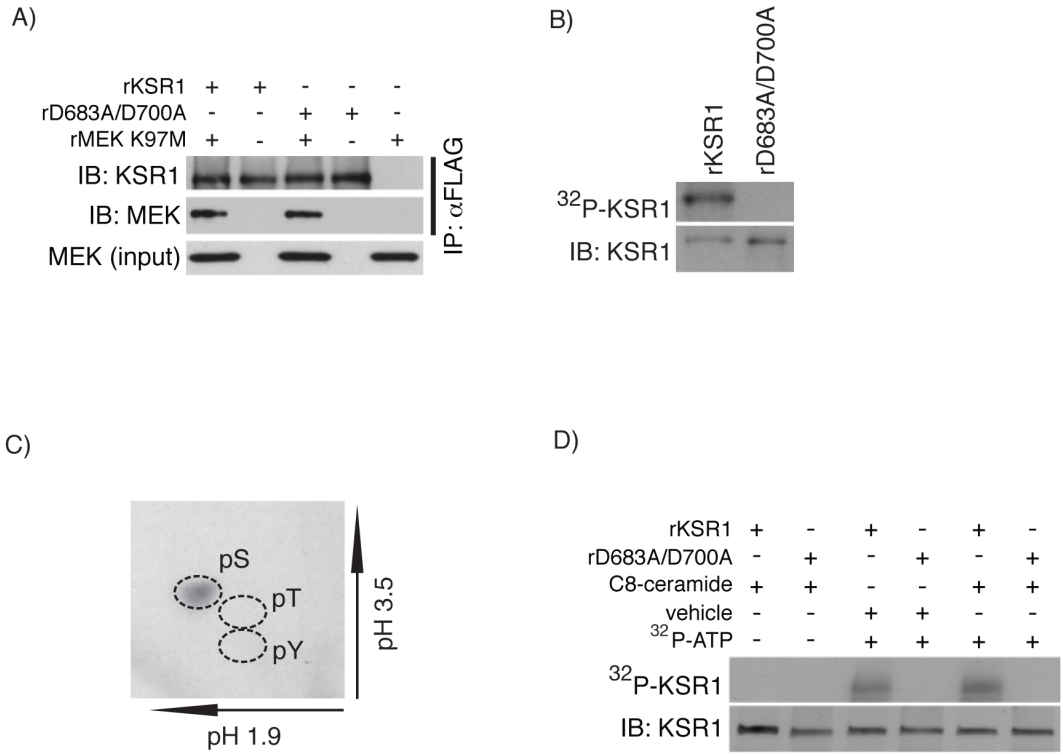

Figure 3.4: Recombinant KSR1 undergoes serine autophosphorylation. A) rKSR1 and rD683A/D700A proteins were incubated with rMEK K97M for 2 hours an then immunoprecipitated with aFLAG antibody. MEK co-precipitation was determined by Western blot analysis using the indicated antibodies. B) KSR1 autophosphorylation was assayed using *in vitro* kinase assays in which rKSR1 or rD683A/D700A were incubated in the presence of [γ-^{32}P]ATP at 30^{o} C for 30 minutes. rKSR1 and rD683A/D700A samples were then separated by SDS-PAGE and ^{32}P incorporation was determined by autoradiography and total KSR1 protein detected by Western blot analysis. C) Phosphoamino acid analysis was conducted on ^{32}P-rKSR1 following an *in vitro* kinase assay. Phosphorylated amino acids were determined by autoradiography. Dashed lines indicate the migration of phosphoamino acid standards. D) *In vitro* kinase assay with rKSR1 and rD683A/D700A in the presence of [γ-^{32}P]ATP with vehicle or C8-ceramide (3 μM). KSR1 autophosphorylation was determined as before. Total KSR1 protein was detected by Western blot analysis. Autoradiography and immunoblots are representatives of 3 independent experiments. *IP, immunoprecipitation; IB, immunoblot.*

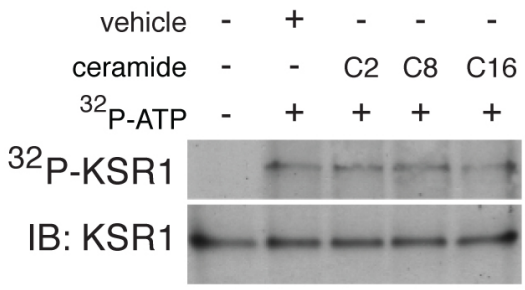

Figure 3.5: rKSR1 autophosphorylation is not enhanced by ceramide *in vitro*. *In vitro* kinase assay using recombinant KSR1 (rKSR1) in the presence of vehicle or 3mM C2-, C8-, or C16-ceramide and [g-^{32}P]ATP. Phosphorylated KSR1 was detected by autoradiography and total KSR1 protein was determined by Western blot analysis. Immunoblot and autoradiography is a representative of 3 independent experiments. *IB, immunoblot.*

bacterially derived KSR1 proteins. We found that rKSR1 but not rD683A/D700A incorporated ^{32}P, indicating that rKSR1 undergoes autophosphorylation (Fig. 3.4*B*). To determine which amino acid residue(s) were autophosphorylated we performed phosphoamino acid analysis on rKSR1 following the [γ-^{32}P]ATP *in vitro* kinase assay. We found that rKSR1 autophosphorylation occurred exclusively on serine residue(s) (Fig. 3.4*C*). Further analysis using 2D tryptic phosphopeptide mapping indicated that KSR1 autophosphorylation likely occurs on a single or limited number of serine residues (data not shown).

Since previous studies from our lab and others describe KSR1 as a ceramide-activated protein kinase, we determined if rKSR1 autophosphorylation was modulated by ceramide (Yan, et al., 2001; Zhang, et al., 1997). *In vitro* kinase assays were performed using rKSR1 or rD683A/D700A in the presence or absence of ceramide. The addition of C8-ceramide did not enhance rKSR1 autophosphorylation in this assay (Fig. 3.4*D*). Since ceramide bioactivity varies depending on the structure and length of the hydrocarbon chain (Siskind, et al., 2000), we tested the effect of various ceramide analogs in augmenting rKSR1 kinase activity. The addition of C2-, C8-, or C16-ceramide to the kinase reaction had no effect on rKSR1 autophosphorylation (Fig. 3.5). Collectively, these data demonstrate that rKSR1 is a functional protein kinase that undergoes serine autophosphorylation likely on a limited number of serine(s) in a ceramide-independent manner.

KSR1 phosphorylates Myelin basic protein (MBP) in vitro

MBP is commonly used as a generic substrate to assay for protein kinase activity *in vitro* (Erickson, et al., 1990; Kishimoto, et al., 1985; Shoji, et al., 1987). To determine if rKSR1 is able to phosphorylate MBP, we performed *in vitro* kinase assays with rKSR1 and rD683A/D700A. Similar to recombinant Raf-1 (rRaf-1) rKSR1, but not rD683A/D700A, was able to phosphorylate MBP (Fig. 3.6*A*). We also tested if the KSR1 kinase domain (rKSR1ΔN521) was sufficient to phosphorylate MBP. Consistent with previous reports, rKSR1 kinase activity requires full length KSR1 (Fig. 3.6*A*) (Xing, et al., 2000). As a positive control for MBP phosphorylation, we

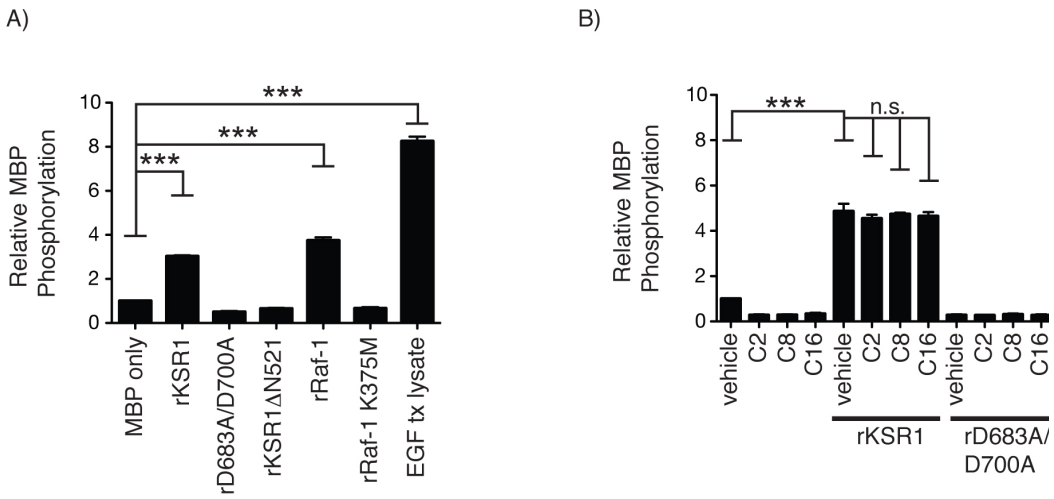

Figure 3.6: Recombinant KSR1 phosphorylates Myelin basic protein (MBP). A) Recombinant rKSR1, rD683A/D700A, rKSR1DN521, rRaf-1, or rRaf-1 K375M proteins, or lysate from EGF-treated cells (EGF tx lysate) were incubated with MBP in an *in vitro* kinase assay. Solid bars are the average relative MBP phosphorylation compared to MBP alone and error bars represent the SEM from 3 independent experiments. B) *In vitro* MBP kinase assay performed as before with rKSR1 or rD683A/D700A incubated with vehicle, C2-, C8-, or C16-ceramide (3 μM). MBP phosphorylation was determined as before. Solid bars are the average relative MBP phosphorylation compared to MBP alone and error bars represent the SEM from 3 independent experiments. Statistical analysis was performed using a one-way ANOVA with Dunnett's post-test. *** = $P < 0.001$

incubated MBP with whole cell lysate from EGF treated YAMC cells (EGF tx) (Fig. 3.6*A*). These findings indicate that rKSR1 can phosphorylate MBP as a generic substrate.

We next tested if rKSR1 phosphorylation of MBP was augmented by ceramide. rKSR1 and rD683A/D700A were incubated with MBP in the presence of C2, C8, or C16-ceramide. Phosphorylation of MBP by rKSR1 was not potentiated by any of the ceramide analogs suggesting that ceramide does not regulate rKSR1 kinase activity *in vitro* (Fig. 3.6*B*). The activity of ceramide was confirmed by treating YAMC cells with each ceramide analog and ERK activation was detected by Western blot analysis (data not shown). Together, these observations indicate that only full-length wild-type rKSR1 can directly phosphorylate MBP as a generic substrate and that ceramide does not appear to regulate rKSR1 kinase activity.

Recombinant KSR1 phosphorylates recombinant kinase-inactive MEK1.

Since +KSR1 cells showed enhanced endogenous MEK phosphorylation and FLAG immunoprecipitation from these cells phosphorylated rMEK K97M (Figs. 3.3*A*-*B*), we assessed whether MEK was a substrate for recombinant KSR1. *In vitro* kinase assays were performed incubating rMEK K97M with rKSR1, rD683A/D700A, or rRaf-1 as a positive control. Similar to rRaf-1, rKSR1 phosphorylated rMEK K97M (Fig. 3.7*A*). Furthermore, no kinase activity was detected in a second kinase-inactive KSR1 mutant where the putative ATP-binding arginine was substituted with methionine (rR589M) (Fig. 3.7*A*). Since previous data indicate that mutations in the *D. melanogaster* KSR1 CA1 domain reduced KSR1 function (Therrien, et al., 1995), we generated a recombinant amino-terminal deletion of KSR1 (rKSR1ΔCA1). rKSR1ΔCA1 was unable to phosphorylate rMEK K97M, suggesting that the CA1 domain may be required structurally or facilitates KSR1 enzymatic activity through an unknown mechanism (Fig. 3.7*A*). Taken together, these data demonstrate that rKSR1 can phosphorylate rMEK K97M as a substrate consistent with the observations for KSR1 immunoprecipitated from colon epithelial cells.

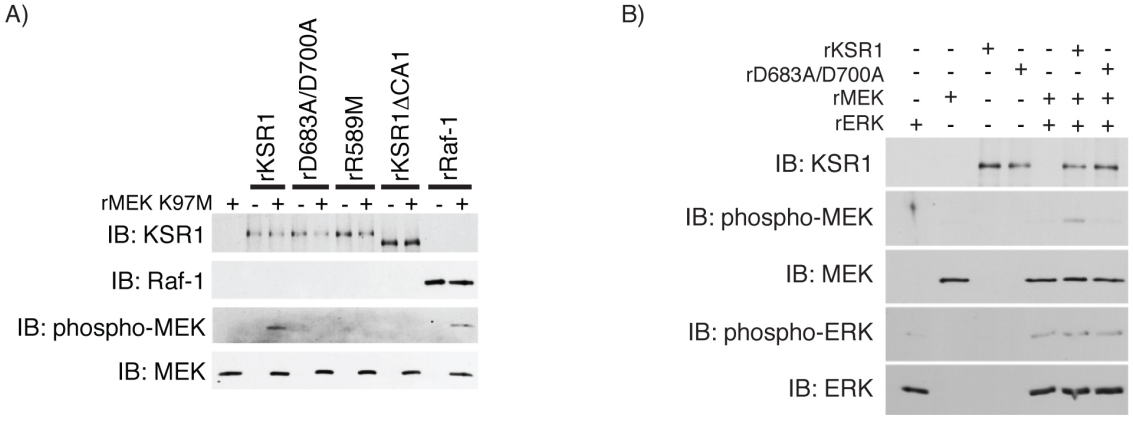

Figure 3.7: Recombinant KSR1 phosphorylates rMEK K97M. A) Phosphorylation of rMEK K97M was assayed using *in vitro* kinase assays by incubating rMEK K97M with rKSR1, rD683A/D700A, rR589M, rKSR1DCA1, or rRaf-1. Phosphorylated rMEK K97M was assayed by Western blot analysis using the indicated antibodies. B) rKSR1-stimulated recombinant ERK (rERK) phosphorylation was determined using *in vitro* kinase assays in which rKSR1 or rD683A/D700A was incubated with rMEK in the presence rERK. Protein phosphorylation and total protein were determined by Western blot analysis using indicated antibodies. Immunoblots are representative of 3 independent experiments.

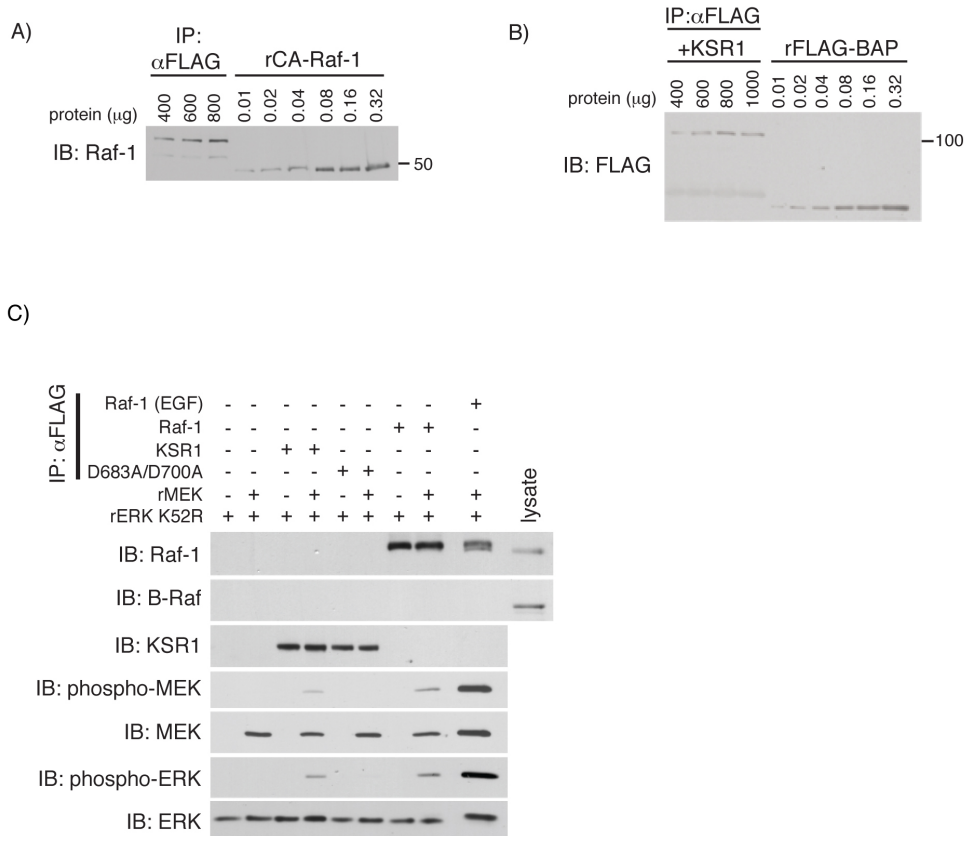

Figure 3.8: Immunoprecipitated KSR1 promotes rMEK activation A) FLAG-tagged Raf-1 was immunoprecipitated from increasing quantities of total protein (400-800 μg) from whole cell lysate, gel-loaded along with increasing amount of purified recombinant CA-Raf-1 (0.01-0.32 μg), and separated by SDS-PAGE. Densitometric measurements were obtained from Western blot analysis using an aRaf-1 antibody. Linear regression using rCA-Raf-1 as the protein standard determined the amount of immunoprecipitated FLAG-tagged Raf-1. Immunoblot is a representative of 3 different experiments. B) FLAG-tagged KSR1 was immunoprecipitated from increasing quantities of total protein (400-1000 μg) from whole cell lysate, gel-loaded along with increasing amount of purified recombinant FLAG-tagged bacterial alkaline phosphatase (BAP) (0.01-0.32 μg), and separated by SDS-PAGE. Densitometric measurements were obtained from Western blot analysis using an αFLAG antibody. Linear regression using rFLAG-BAP as the protein standard determined the amount of immunoprecipitated FLAG-tagged KSR1 protein. C) The ability of mammalian FLAG-KSR1, FLAG-D683A/D700A, or FLAG-Raf-1 to promote rMEK kinase activity towards ERK was determined by immunoprecipitating 1 pmol of each FLAG-tagged protein and performing an *in vitro* kinase assay with recombinant kinase-dead ERK (rERK K52R) in the presence or absence of rMEK. Total and phospho-proteins were determined by Western blot analysis as indicated. Immunoblots are representatives of 3 independent experiments. *IP, immunoprecipitation; IB, immunoblot.*

While rKSR can phosphorylate rMEK K97M, utilizing kinase-inactive MEK does not allow for assaying rKSR-mediated MEK activation. Therefore, we determined if rKSR1 is able to phosphorylate unactive recombinant wild-type MEK (rMEK). *In vitro* kinase assays were performed incubating unactive rMEK with rKSR1, rD683A/D700A, or rRaf-1. To determine if rKSR1 phosphorylation of rMEK is sufficient to activate rMEK, we performed an *in vitro* kinase assay using unactive recombinant wild-type ERK (rERK) as the rMEK substrate. While rMEK phosphorylation by rKSR1 was readily detected, it was insufficient to induce rERK phosphorylation (Fig. 3.7*B*). We then investigated whether KSR1, D683A/D700A, or Raf-1 proteins immunoprecipitated from mammalian cells promoted rMEK kinase activity towards recombinant kinase-dead ERK (rERK K52R). We first determined the quantity of immunoprecipitated proteins to ensure equimolar amounts of KSR1 and Raf-1 proteins to be used in the kinase assay (Fig. 3.8*A*-*B*). We then performed *in vitro* kinase assays incubating rERK K52R with immunoprecipitated FLAG-KSR1 (1 pmol), FLAG-D683A/D700A (1 pmol), FLAG-Raf-1 (1 pmol), or FLAG-Raf-1 (1pmol) from EGF treated cells as a positive control in the presence or absence of unactive rMEK. We found that FLAG-KSR1 and FLAG-Raf-1, but not FLAG-D683A/D700A, immunoprecipitated from mammalian cells promoted rERK K52R phosphorylation by rMEK (Fig. 3.8*C*). As a positive control for MEK activation, we performed the same kinase assay using rMEK and ERK K52R incubated with FLAG-Raf-1 immunoprecipitated from EGF treated cells. Collectively, these data suggest that while MEK is a KSR1 substrate *in vitro*, activation of rMEK requires KSR1 isolated from intact mammalian cells.

Discussion

Our findings indicate that KSR1 is a functional protein kinase that, along with MEK1 protein kinase activity, is required for protection against TNF-induced cell death. The observation that MEK associated with wild-type KSR1 has increased phosphorylation, together with our *in vitro* kinase assays using recombinant proteins, suggests that KSR1 is capable of direct

phosphorylation of MEK. With the prevailing controversy surrounding KSR1 as a functional protein kinase, the results presented here showing that recombinant KSR1 is serine autophosphorylated and is capable of phosphorylating MBP, solidifies KSR1 as a functional protein kinase. Furthermore, we have found that both recombinant KSR1 and KSR1 immunoprecipitated from colon epithelial cells are able to phosphorylate recombinant kinase-inactive MEK1. Interestingly, while phosphorylation of rMEK by rKSR1 occurs within the MEK activation loop, only mammalian KSR1 was able to promote rMEK activation. Collectively, the data presented here support a role for KSR1 as a catalytically active protein kinase that functions to promote cell survival in the presence of TNF. This finding has implications for chronic inflammatory diseases, such as inflammatory bowel disease (IBD), in which the cytotoxic effect of TNF contributes to epithelial cell loss and epithelial barrier dysfunction.

Activation of the ERK cascade is a key component for cell survival in a number of cell types (Erhardt, et al., 1999; Le Gall, et al., 2000; McKay, et al., 2007; Ripple, et al., 2005; Shimamura, et al., 2000). While the ability of KSR1 to scaffold and sensitize the ERK pathway is well-established (Fusello, et al., 2006; Lin, et al., 2009; Xing, et al., 2000), the catalytic contribution of KSR1 has remained inconclusive. We found that colon epithelial cells lacking KSR1, or stably expressing a kinase-inactive KSR1, are sensitive to TNF-induced apoptosis (Fig. 3.2*A*). Interestingly, inhibition of MEK protein kinase activity sensitized +KSR1 cells to TNF-induced cell loss whereas +vector and +D683A/D700A cells had no potentiation in cell loss (Fig. 3.2*C*). This suggests that the protective role of KSR1 kinase activity requires MEK kinase activity. The importance of MEK1 is established from early development, as *MEK1*$^{-/-}$ mice are embryonic lethal (Giroux, et al., 1999). In fact, a study utilizing an inducible tissue-specific deletion of MEK1 in the epidermis causes keratinocyte apoptosis (Scholl, et al., 2007). It is conceivable that wild-type KSR1 augments MEK kinase activity through protein-protein interactions independent of catalytic function in a manner similar to Raf-1 (Michaud, et al., 1997; Rajakulendran, et al., 2009). This seems unlikely given that +D683A/D700A-binding affinity for MEK is equivalent to wild-type KSR1, yet +D683A/D700A cells are just as sensitive to TNF-

induced apoptosis as cells lacking KSR1 (Fig. 3.2*C*). Alternatively, expression of wild-type KSR1 may induce a different protein expression profile compared to the protein profiles of +vector and +D683A/D700A cells such that MEK activity is required.

Previous studies using immunoprecipitated KSR1 from cultured cells reveal that KSR1 autophosphorylates and phosphorylates Raf-1 in response to ceramide (Yan, et al., 2004; Zhang, et al., 1997). While our results indicate that recombinant KSR1 autophosphorylates exclusively on serine, consistent with previous results (Liu, et al., 1994), ceramide was not required for recombinant KSR1 protein kinase activity *in vitro* (Figs. 3.4*D* & 3.5). We cannot rule out a role for ceramide in activating endogenous KSR1. The requirement for ceramide in KSR1 activation observed by others may provide insight into KSR1 regulation. For instance, ceramide could function to relieve a negative regulatory mechanism mediated by other associated proteins such as the 14-3-3 family, similar to Raf-1, through activation of PP2A (Cacace, et al., 1999; Dobrowsky, et al., 1992; Hartsough, et al., 2002; McPherson, et al., 1999; Müller, et al., 2001; Volle, et al., 1999; Xing, et al., 1997). It is attractive to speculate that since recombinant KSR1 does not require activation by ceramide, KSR1 must adopt an active conformation or be allosterically modulated through an intramolecular interaction. Regulation of endogenous KSR1 might occur by sequestering these interacting regions within the molecule. In fact, the original mutagenesis screen in *D. melanogaster* detailed a weak loss of function allele that mapped to a double amino acid substitution within the KSR1 CA1 domain. This domain is a highly conserved domain unique to the KSR1 family and is important for interactions with B-Raf (McKay, et al., 2009). Interestingly, recombinant KSR1 with an N-terminal truncation of this domain (rKSR1ΔCA1) did not function as an active kinase (Fig. 3.7*A*). Additionally, a construct containing only the KSR1 kinase domain (rKSR1ΔN521) was unable to phosphorylate MBP (Fig. 3.6*A*). This finding is consistent with a report that the N-terminus is required for KSR1 protein kinase activity (Xing, et al., 2000). This is unlike Raf1 where expression of the kinase domain alone results in a constitutively active enzyme (Boerth, et al., 1994; Hecht, et al., 1996).

The finding that MEK1 is a KSR1 substrate raises new questions about the potential mechanisms that modulate the ERK pathway. The data presented here show that rKSR1 can phosphorylate rMEK1 on at least one serine residue within the MEK1 activation loop (Figs. 3.3*B* & 3.7*A*). These serine residues in the MEK activation loop are reported to be both necessary and sufficient for phosphorylation of ERK (Alessi, et al., 1994; Zheng, et al., 1994). Interestingly, while rKSR1 phosphorylation of rMEK was insufficient to stimulate phosphorylation of rERK2 (Fig. 3.7*B*), KSR1 immunoprecipitated from mammalian cells did promote rMEK activation (Fig. 3.8*C*). It is conceivable that KSR1 expressed in mammalian cells may possess post-translational modifications that may promote more efficient activation of rMEK. We cannot rule out the possibility that immunoprecipitated wild-type KSR1 promotes rMEK activation through an allosteric mechanism in a manner similar to KSR1-stimulated Raf-1 activation (Rajakulendran, et al., 2009). In addition, previous observations with MEK kinase-1 (MEKK1) also indicate a level of complexity in MEK activation. While MEKK1 transfected into 293 cells readily phosphorylated MEK1 and MEK2 at these serine residues, it did not result in phosphorylation of ERK2 (Xu, et al., 1995). Our data presented here, combined with previous data for MEKK1, suggests that MEK activation is regulated, at least in part, by additional factors that likely vary with the type of stimulus or expression system. Nonetheless, the *in vivo* contribution of KSR1 kinase activity towards MEK may function to sensitize MEK activation by Raf-1, or regulate the spatiotemporal phosphorylation state of MEK. In fact, the spatiotemporal control of ERK activation is able to alter the physiological responses downstream of both TNF receptor-1 and staphylococcal enterotoxin E-mediated T cell activation (Kaiser, et al., 1999; Lin, et al., 2009). Thus, KSR1 kinase activity towards MEK may function to make MEK activation by Raf or other MEK kinases more efficient and/or lower the threshold for activation of the ERK pathway.

For over a decade, KSR1 has been appreciated as a scaffold of the ERK pathway that coordinates pathway activation, yet the enzymatic contributions of KSR1 have remained controversial (Denouel-Galy, et al., 1998; Michaud, et al., 1997; Sugimoto, et al., 1998; Therrien, et al., 1996; Yu, et al., 1998). The data presented here demonstrate that KSR1 contains intrinsic

protein kinase activity *in vitro* and suggests a mechanism by which KSR1 can sensitize the ERK signaling cassette through direct phosphorylation of MEK1. KSR1 and other protein kinases have been classified as pseudokinases based on variation in conserved amino acid residue(s) within the kinase domain (reviewed in (Boudeau, et al., 2006)). Even so, the maintenance of a highly conserved protein kinase domain throughout evolution, and the demonstration of protein kinase activity from other unique protein kinases (Abe, et al., 2001; Xu, et al., 2000), imparts significance to these proteins and their potential enzymatic function. Our observation that the catalytic activity of KSR1 protects colonic epithelial cells from TNF-induced apoptosis provides a potential mechanism by which the intestinal epithelial barrier can be maintained in the presence of elevated levels of TNF. For patients suffering from IBD, anti-TNF therapy is beneficial and able to induce remission. Yet, the long-term side effects, and concomitant immunosuppressive therapy, highlight the need for novel strategies that reduce inflammation by promoting epithelial cell survival within the inflammatory microenvironment (reviewed in (Rutgeerts, et al., 2006)). Therefore, understanding the precise mechanisms that modulate the ERK pathway downstream of TNF could lead to new therapeutic targets for patients afflicted with chronic inflammatory diseases.

CHAPTER IV

KSR1 PROTECTS AGAINST INTERLEUKIN-10 DEFICIENCY-INDUCED COLITIS IN MICE BY SUPPRESSING T LYMPHOCYTE INTERFERON-γ PRODUCTION

Introduction

Pathogen defense mechanisms, such as innate and adaptive immunological responses, have greatly contributed to the success of vertebrate evolution. While acute inflammatory responses are beneficial to the host, chronic inflammation can be deleterious. Thus, identification of the molecules involved in regulating these responses is critical for understanding the mechanisms that can contribute to immunological disorders. For patients suffering from IBD, dysregulated immune responses result in sustained immune cell activation and elevated cytokine production, which can lead to an increased risk for developing colon cancer over their lifetime (Balkwill, et al., 2001; Itzkowitz, et al., 2004). Therefore, it is imperative to develop strategies and identify novel targets that modulate immune responses to complement current therapeutic options for IBD patients.

Much of our current understanding of the molecular mechanisms involved in IBD has come from knockout, transgenic, and chemically-induced mouse models. Although mouse models of IBD do not fully recapitulate the human disease, interleukin-10 deficient (*Il10*$^{-/-}$) mice display similar characteristics to that of human Crohn's disease (Rennick, et al., 1995; Scheinin, et al., 2003). Since the anti-inflammatory effects of IL-10 are required to regulate Th1 cytokine production and promote immune homeostasis (Fiorentino, et al., 1989; Maynard, et al., 2007), loss of IL-10 in mice results in spontaneous enterocolitis driven by an aberrant immunological response to enteric antigens (Kühn, et al., 1993; Sellon, et al., 1998).

KSR1 functions as a kinase or molecular scaffold of the Raf/MEK/ERK signaling module to regulate proliferation, apoptosis or function in a cell/tissue context-dependent manor (Michaud, et al., 1997; Zafrullah, et al., 2009; Zhang, et al., 1997). For example, KSR1 is required for colon

epithelial cell survival downstream of TNF signaling both *in vitro* and *in vivo* (Yan, et al., 2001; Yan, et al., 2004). Given that pro-inflammatory cytokines, including TNF, are elevated in *Il10*$^{-/-}$ mice, we hypothesized that loss of KSR1 in *Il10*$^{-/-}$ mice would exacerbate colitis. Therefore, we crossed *KSR1*$^{-/-}$ mice with *Il10*$^{-/-}$ mice to generate *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice and examined the role of KSR1 as a protective mediator during chronic inflammation.

In the present study, we found that *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice developed an early onset form of severe colitis resulting from loss of KSR1 expression in hematopoietic lineages. Specifically, KSR1 deficient T cells produced more IFN-γ and had a greater propensity to polarize along the Th1 axis *in vitro*. The data presented here, along with recent reports, reveal an emerging role for KSR1 in immune function.

Results

KSR1$^{-/-}$*Il10*$^{-/-}$ *mice develop accelerated spontaneous colitis*

Although KSR1 inhibits epithelial cell apoptosis downstream of TNF signaling (Yan, et al., 2004), the role of KSR1 during chronic inflammation has not been examined. Since *Il10*$^{-/-}$ mice on the BALB/c (WT) strain under SPF conditions develop spontaneous colitis between 12-16 weeks of age, we examined the effect of KSR1 deficiency on colitis in *Il10*$^{-/-}$ mice. The clinical onset of disease was assessed for each group of mice by examination of fecal occult blood and monitoring whole body weight from 3 to 10 weeks. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice frequently suffered from chronic diarrhea and failed to thrive beginning at 5 weeks of age (Fig. 4.1*A*). By colonoscopic examination, the mucosa of 10-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice appeared thickened with loss of normal vascular pattern compared to the colonic mucosa of WT, *KSR1*$^{-/-}$, and *Il10*$^{-/-}$ mice (Fig. 4.1*B*). Since colon weight per unit length is often indicative of inflammation, colon length-to-weight (l/w) ratio was quantified for each group. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had an average l/w ratio of 15.03 ± 1.5 whereas WT, *KSR1*$^{-/-}$, and *Il10*$^{-/-}$ mice had ratios of 44.36 ± 2.96, 41.64 ± 3.24, and

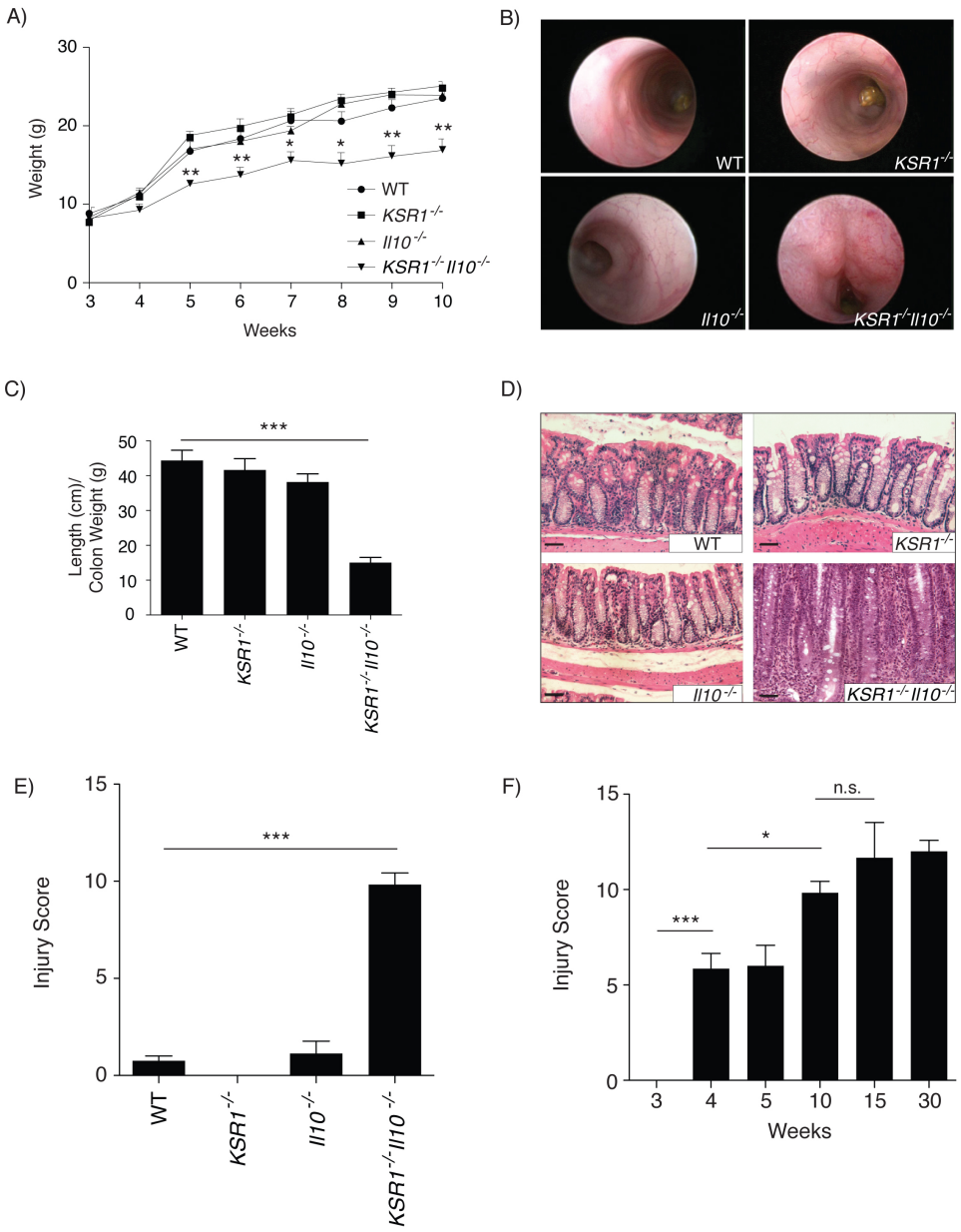

Figure 4.1: *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice develop accelerated spontaneous colitis. A) WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were weighed weekly from 3 weeks of age to 10 weeks. Plotted data are the mean weight for each group (n ≥ 5). Error bars represent the SEM. B) Mouse colonoscopic images taken on 10-week old mice. C) Colons were removed from 10-week old mice flushed, weighed, and measured from the cecum to anus. Solid bars represent the mean (n ≥ 3) of the length/weight ratios. Error bars represent the SEM. D) Paraffin embedded colon sections from 10-week old mice stained with Hematoxylin and Eosin (H&E). Images were taken at 20X magnification (*scale bars, 50 μm*). E) H&E stained 10-week old mouse colon sections were scored by a pathologist blinded to the genotype. Solid bars represent the mean injury and inflammation score for each group (n ≥ 6) and error bars are the SEM. F) *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were sacrificed at each time point indicated and scored for inflammation and injury as before. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

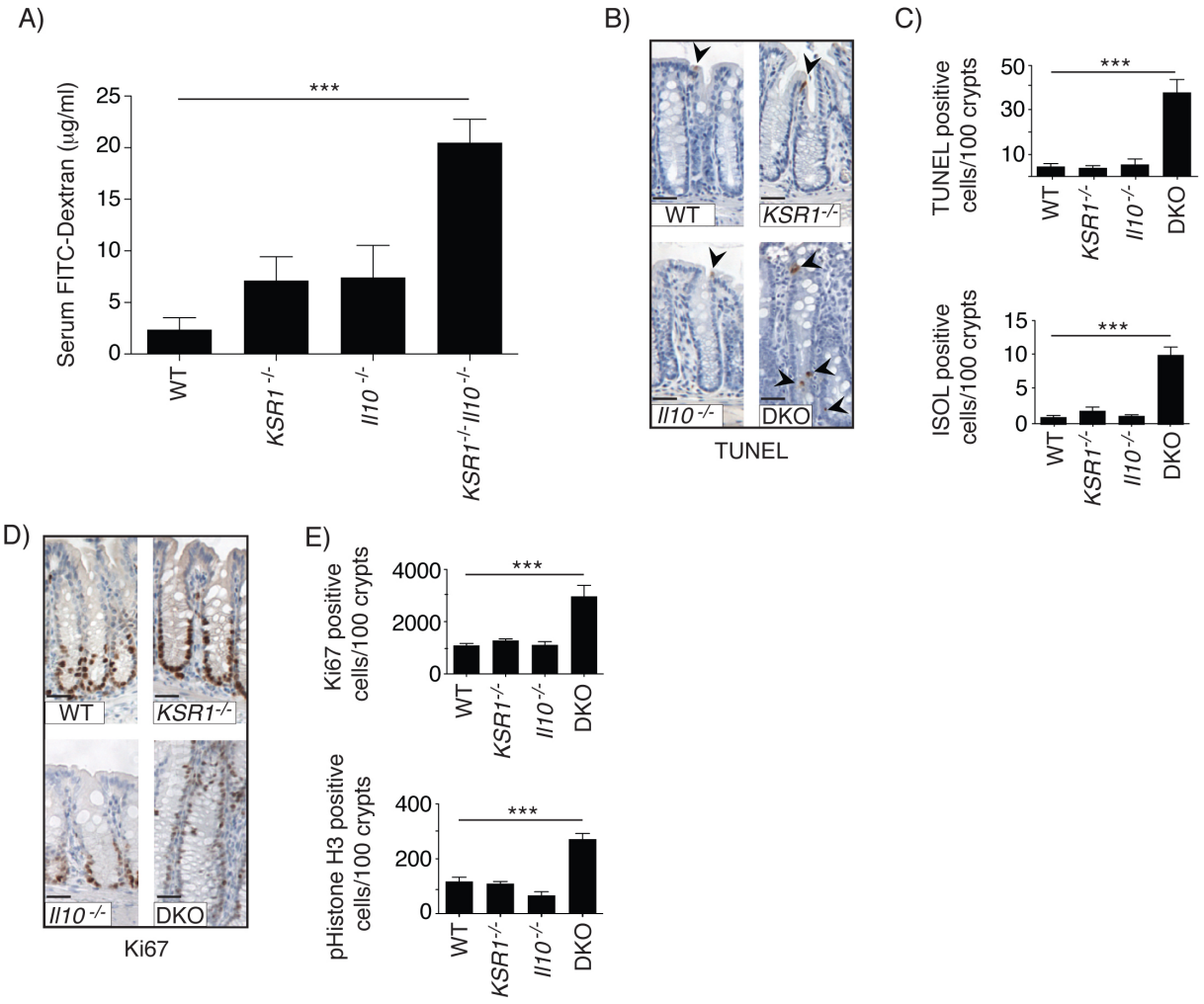

Figure 4.2: *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice have increased colon epithelial barrier permeability and increased epithelial cell turnover. A) Colonic barrier permeability was assayed by the administration of fluorescein isothiocyanate (FITC)-dextran enemas in 10-week old mice. Mouse serum was collected after 1 hour and FITC in peripheral blood serum was determined using a fluorescence microplate reader. Solid bars represent the mean and error bars represent the SEM from three independent experiments. B) Paraffin embedded colon sections from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (DKO) mice were labeled using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Black arrows indicate TUNEL positive cells. All images were taken at 20X magnification (*scale bars, 50 μm*). C) Colon epithelial cell apoptosis was quantified by counting the number of TUNEL and *in situ* oligo ligation (ISOL) labeled epithelial cells per 100 crypts. Bars represent the mean and error bars the SEM ($n \geq 3$) from three independent experiments. D) Immunohistochemical staining performed on paraffin embedded colon sections from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and DKO mice using an anti-Ki67 antibody. Images were photographed at 20X magnification (*scale bars, 50 μm*). E) Epithelial cell proliferation was quantified by counting the number of Ki67 or phospho-histone H3 positive cells per 100 crypts, as indicated. Solid bars represent the mean and error bars represent the SEM ($n \geq 3$) from three independent experiments. *** $P < 0.001$

38.19 ± 2.34 respectively (Fig. 4.1*C*). Paraffin-embedded colon sections from 10-week old mice were stained with Hematoxylin and Eosin (H&E) and a blinded pathologist scored each section for inflammation and injury. 10-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice suffer from severe colitis (score of 9.83 ± 0.6) (Figs. 4.1*D-E*). Barrier permeability was assed by administering FITC-dextran enemas to 10-week old mice, peripheral blood serum collected one hour later, and quantified for the presence of FITC. *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had increased barrier permeability, possibly due to the increased epithelial cell turnover as determined by apoptotic and proliferative markers (Fig. 4.2). We then evaluated the developmental time course of colitis and found at 4 weeks of age, *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice already have histological signs of disease (Fig. 4.1*F*). These data indicate that loss of KSR1 in *Il10*$^{-/-}$ mice results in accelerated and severe spontaneous colitis with retarded growth similar to findings in children with Crohn's disease (Heuschkel, et al., 2008).

Expression of KSR1 in hematopoietic lineages is protective against colitis

The *KSR1*$^{-/-}$ and *Il10*$^{-/-}$ knockout mice in this study were engineered as global deletions. Since the disease that develops in *Il10*$^{-/-}$ mice is attributed to immune hypersensitivity to enteric microflora (Kühn, et al., 1993; Sellon, et al., 1998), we investigated whether KSR1 expression in hematopoietic lineages mediated protection from disease in *Il10*$^{-/-}$ mice. To test this, we performed bone marrow transplantation on irradiated 4-week old WT or *KSR1*$^{-/-}$*Il10*$^{-/-}$ recipient mice. Following transplant, mice were sacrificed at 10 weeks of age and the inflammation and injury in the colon was scored as before. Irradiated *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice reconstituted with WT bone marrow (BM) developed mild colitis scores (2.8 ± 1.12) while reconstitution using *KSR1*$^{-/-}$*Il10*$^{-/-}$ bone marrow resulted in severe colitis as expected (11 ± 1) (Figs. 4.3*A* and 4.3*B* panels 1 & 4). Interestingly, restoring IL-10 to hematopoietic lineages in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice did not ameliorate colitis (8.1 ± 1.72) (Figs. 4.3*A* and 4.3*B* panel 3). However, restoring KSR1 to hematopoietic cells in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (5.5 ± 1.25) attenuated the disease (Figs. 4.3*A* and 4.3*B* panel 2). Interestingly, reconstitution of WT mice with *Il10*$^{-/-}$ BM was insufficient to cause disease, while

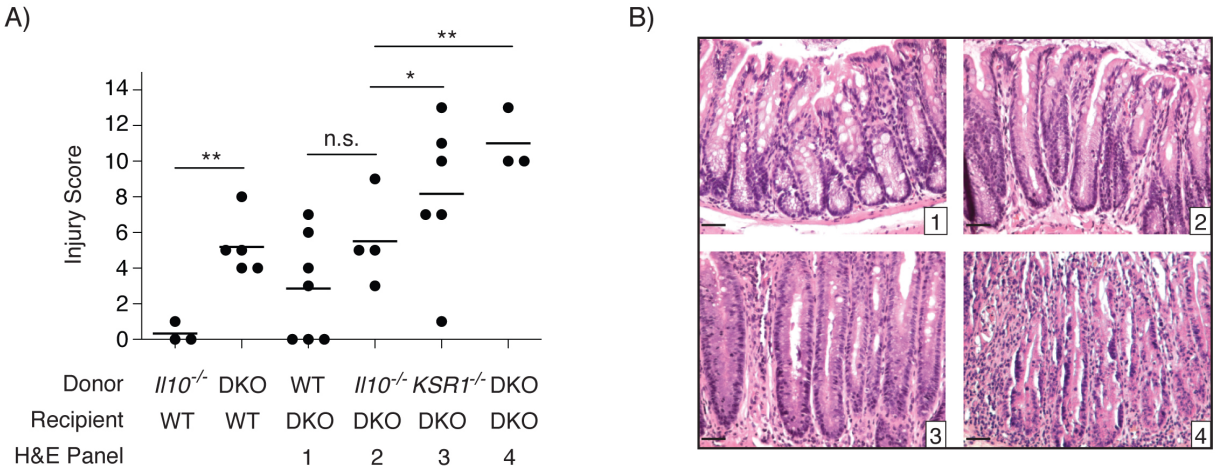

Figure 4.3: KSR1 in hematopoietic lineages suppresses colitis in *Il10*$^{-/-}$ mice. A) 4-week old WT and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (DKO) recipient mice were irradiated with 9 Gy ^{137}Cesium. Bone marrow transplants using the indicated donor mice were performed and mice were sacrificed at 10-weeks of age. Each individual colonic injury and inflammation score is plotted with a solid line indicating the mean score for each group (n ≥ 3) pooled from three independent experiments and error bars are the SEM. B) Representative H&E stained paraffin embedded colon sections from recipient DKO mice transplanted with WT (1), *Il10*$^{-/-}$ (2), *KSR*$^{-/-}$ (3), or *KSR1*$^{-/-}$*Il10*$^{-/-}$ (4) bone marrow as indicated. * $P < 0.05$, ** $P < 0.01$

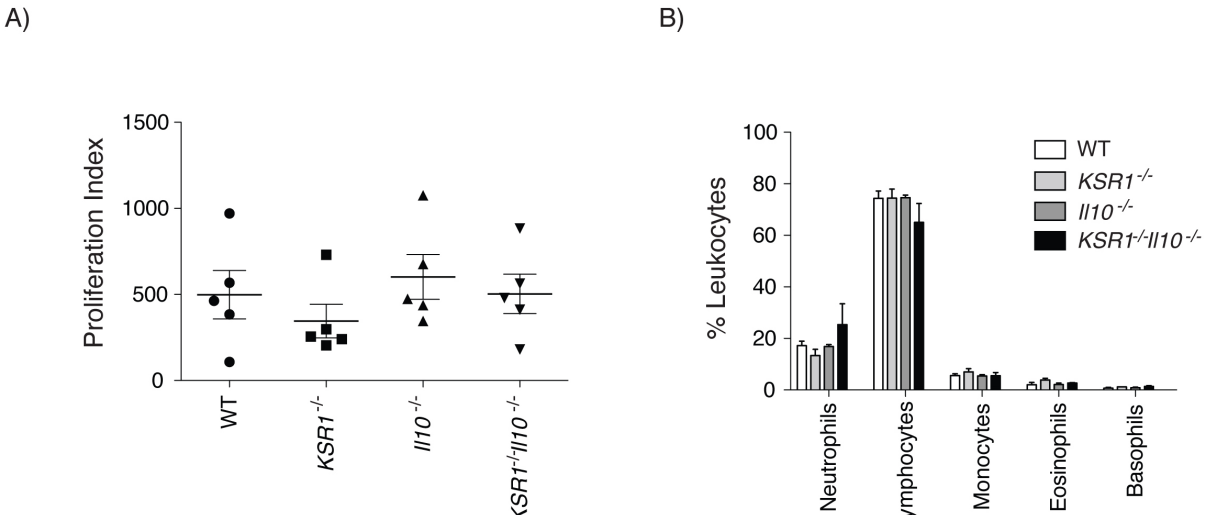

Figure 4.4: KSR1 is not required for stimulated T cell proliferation or leukocyte differentiation. A) Splenocytes isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were plated in 96-well plates containing plate-bound anti-CD3/anti-CD28 for 72 hours at 37° C then pulsed for 18 hours with 1 μCi/well [^{3}H]thymidine. Proliferation was recorded as [^{3}H]thymidine incorporation of stimulated wells relative to unstimulated. Solid line is the mean (n = 5) for each group and error bars the SEM from three independent experiments. B) Peripheral blood leukocyte populations were analyzed using an automated HemaVet counter and recorded as percent leukocytes. Solid bars represent the mean and error bars represent the SEM from 3 independent experiments (n ≥ 3).

KSR1$^{-/-}$*Il10*$^{-/-}$ BM injected into irradiated WT mice was sufficient to drive colitis (5.25 ± 0.84) (Fig. 4.3*A*). While hematopoietic cells require KSR1 to suppress colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice, KSR1 was not required for leukocyte differentiation or stimulated T cell proliferation *in vitro* (Figs. 4.4*A-B*). Taken together, our data suggest that KSR1 expression in hematopoietic lineages plays a significant role in suppressing colitis in *Il10*$^{-/-}$ mice.

Stimulated CD4^{+}*TCRβ*$^{+}$ *splenocytes from KSR1*$^{-/-}$ *and KSR1*$^{-/-}$*Il10*$^{-/-}$ *mice have increased IFN-γ production*

Pro-inflammatory cytokines including TNF, IFN-γ, and IL-17 are increased in the intestinal mucosa of Crohn's disease patients and in mouse models of IBD (Breese, et al., 1993; Cua, et al., 2003; Langrish, et al., 2005; MacDonald, et al., 1990; Murphy, et al., 2003). Examination of Th1 and Th17 gene expression in the colonic mucosa of WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice revealed that IFN-γ and IL-17A transcripts were increased KSR1 deficient mice (Fig. 4.5). Splenocytes were isolated and stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin and intracellular IFN-γ and IL-17A production examined by flow cytometry. CD4^{+}TCRβ^{+} lymphocytes isolated from *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had increased intracellular IFN-γ (12.80% ± 1.16% and 13.5% ± 2.87% respectively) compared *Il10*$^{-/-}$ mice (6.83% ± 2.03%) and WT mice (7.02% ± 1.58%) (Fig. 4.6). Interestingly, only splenocytes isolated from *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had elevated IL-17A compared to WT mice (1.82% ± 0.4% vs. 0.32% ± 0.03%) (Fig. 4.6). Thus, KSR1 expression modulates IFN-γ production in CD4^{+}TCRβ^{+} T lymphocytes.

Th1 polarization is enhanced while Th17 polarization is impaired in KSR1 deficient CD4^{+}*TCRβ*$^{+}$ *T cells in vitro*

IL-17A production by Th17 effector cells has been implicated in the pathogenesis of diseases including rheumatoid arthritis, multiple sclerosis, and Crohn's disease (Kebir, et al., 2007; Koenders, et al., 2006; Yagi, et al., 2007). Our previous observations that IFN-γ and IL-17A transcripts were increased in mice lacking KSR1 raised the possibility that KSR1 plays a role in

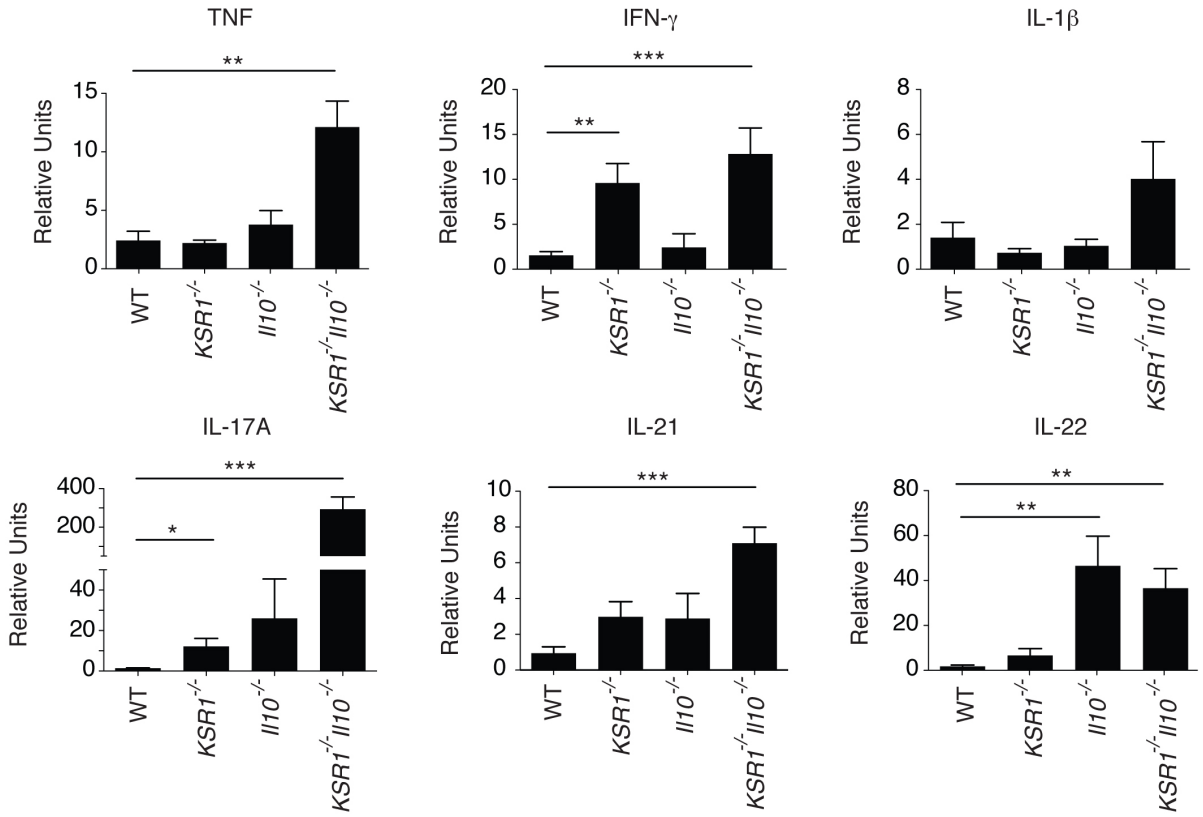

Figure 4.5: IFN-γ and IL-17A gene expression is increased in the colon of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Total RNA was isolated from homogenized whole colon tissue from 10 week-old WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Th1 and Th17 cytokine transcript levels were analyzed by quantitative real-time PCR for TNF, IFN-γ, IL-1β, IL-17A, IL-21, and IL-22. Solid bars represent the mean (n ≥ 6) for each genotype and error bars represent the SEM pooled from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

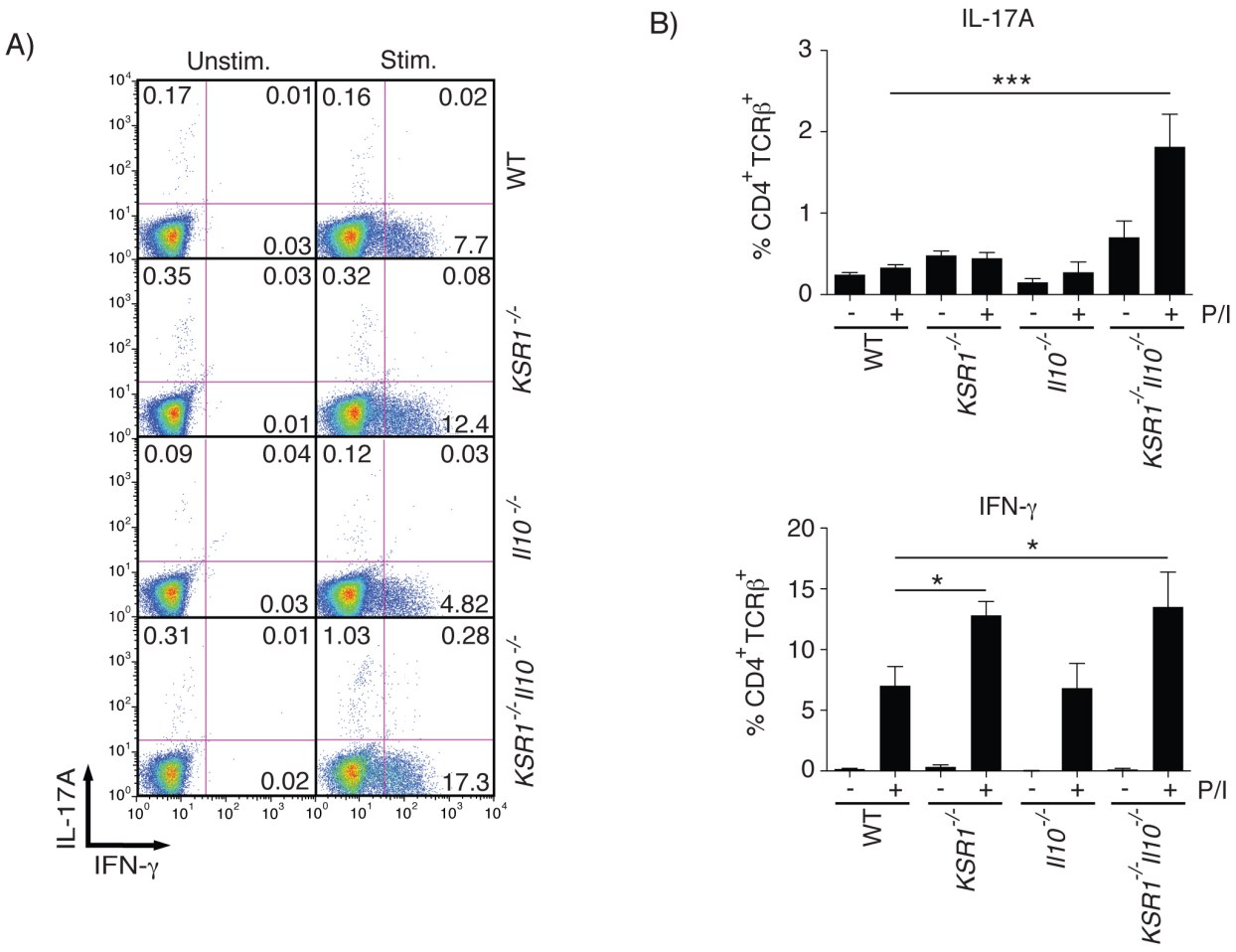

Figure 4.6: Stimulated splenocytes from *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice have increased IFN-γ production. Splenocytes isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were cultured for 5 hours in the presence of the protein transport inhibitor GolgiPlug and treated with or without PMA/Ionomycin. Lymphocytes were stained for CD4 and TCRb cell surface markers followed by intracellular IFN-γ and IL-17A. Samples were analyzed by flow cytometry gated on lymphocyte geometry and CD4^{+}TCRb^{+} staining. A) Flow cytometry dot plots of unstimulated and stimulated WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ T cells stained for intracellular IFN-γ and IL-17A. B) Intracellular cytokine staining was quantified and solid bars represent the mean (n ≥ 4) for each cultured T cell population pooled from three independent experiments and error bars are the SEM. ** $P < 0.01$

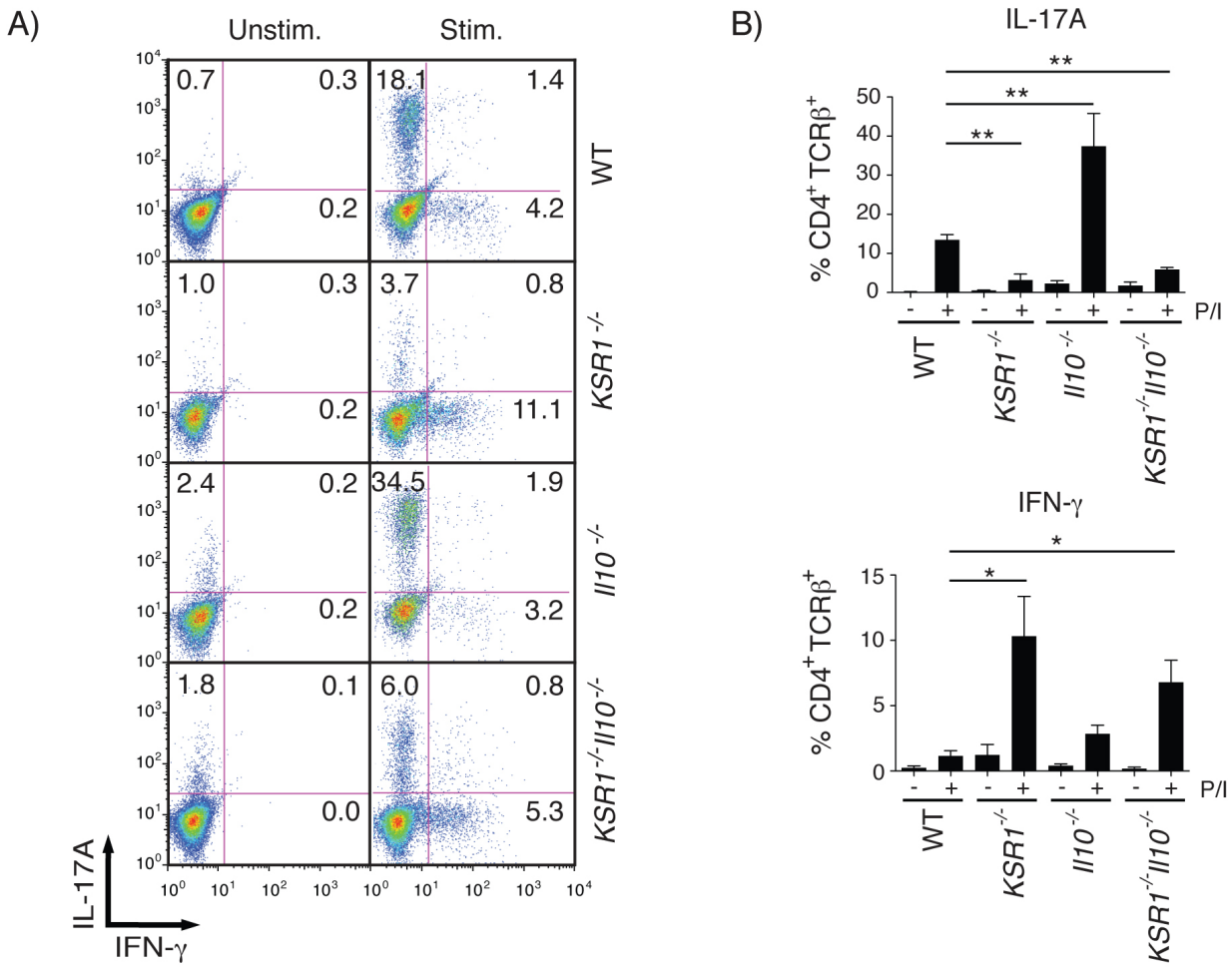

Figure 4.7: *In vitro* Th17 polarization is impaired in *KSR1*$^{-/-}$ T cells. Splenocytes isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were cultured under Th17 polarizing conditions as described. Lymphocytes were stained for cell surface CD4 and TCRβ and intracellular IFN-γ and IL-17A. Samples were analyzed by flow cytometry gated on lymphocyte geometry and CD4^{+}TCRβ^{+} cell surface staining. A) Flow cytometry dot plots of unstimulated and stimulated T cells cultured under Th17 polarizing conditions. B) Intracellular cytokine staining on Th17 polarized cells was quantified and reported as the percent CD4^{+}TCRβ^{+} cells staining positive for IL-17A or IFN-γ. Bar graphs represent the mean (n ≥ 4) for each cultured T cell population pooled from two independent experiments. Error bars are the SEM.

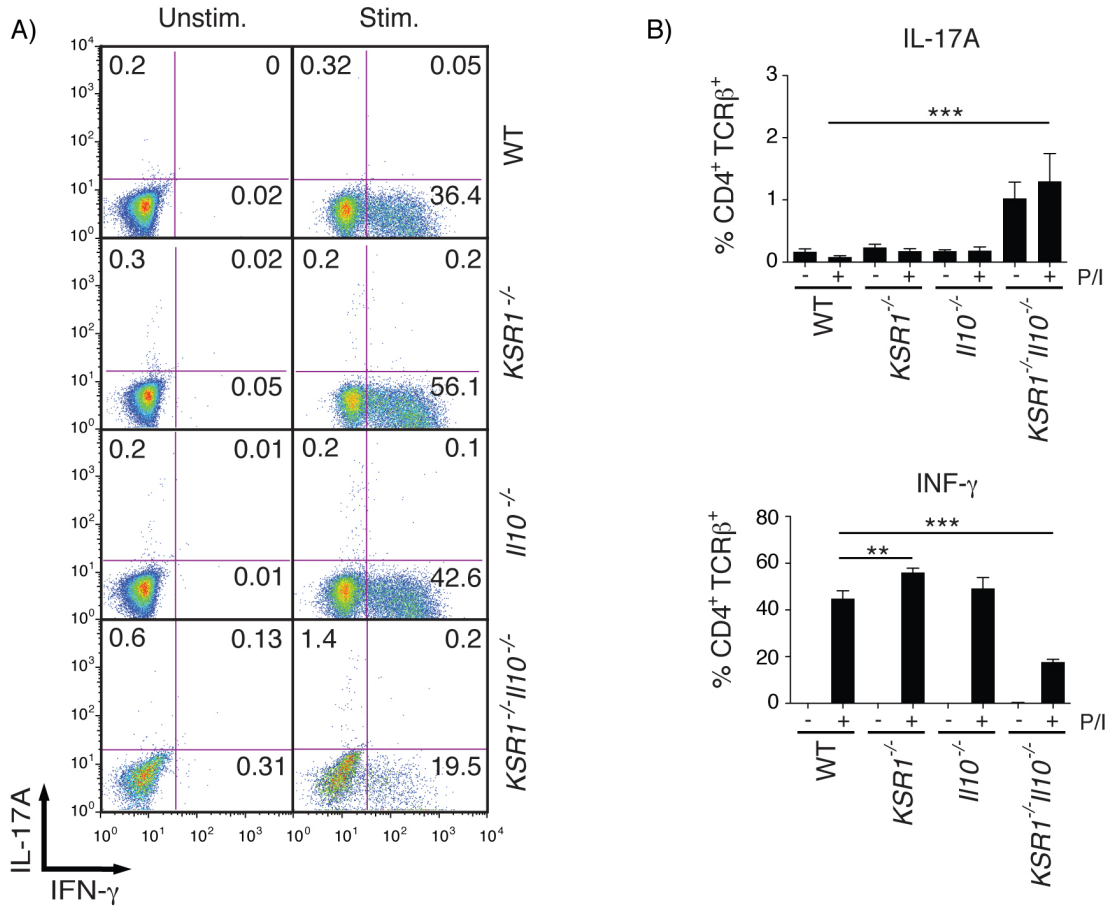

Figure 4.8: *In vitro* Th1 polarization is increased in *KSR1*$^{-/-}$ T cells. Splenocytes isolated from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice were cultured under Th1 polarizing conditions as described. Lymphocytes were stained for cell surface CD4 and TCRβ and intracellular IFN-γ and IL-17A. Samples were analyzed by flow cytometry gated on lymphocyte geometry and CD4^{+}TCRβ^{+} cell surface staining. A) Flow cytometry dot plots of unstimulated and stimulated T cells cultured under Th1 polarizing conditions. B) Intracellular cytokine staining on Th1 polarized cells was quantified and reported as the percent CD4^{+}TCRβ^{+} cells staining positive for IL-17A or IFN-γ. Bar graphs represent the mean (n ≥ 4) for each cultured T cell population pooled from two independent experiments. Error bars are the SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

T cell development along the Th1/Th17 axis. To test this, we isolated splenocytes from WT, *KSR1*$^{-/-}$, *Il10*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice and cultured them under Th1 or Th17 polarizing conditions. We then stimulated with PMA/ionomycin for 5 hours in the presence of GolgiPlug and measured intracellular IL-17A and IFN-γ production by flow cytometry. We found that under Th17 polarizing conditions, IL-17A expressing cells were reduced for *KSR1*$^{-/-}$ (3.20% ± 1.5%) and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (5.9% ± 0.48%) compared to WT (13.48% ± 1.34%) and *Il10*$^{-/-}$ (37.43% ± 8.3%) CD4^{+}TCRβ^{+} T cells (Figure 5*A-B*). In fact, the number of CD4^{+}TCRβ^{+} T cells producing IFN-γ was increased in *KSR1*$^{-/-}$ (10.32% ± 3.0%) and *KSR1*$^{-/-}$*Il10*$^{-/-}$ (6.8% ± 1.7%) when compared to WT (0.89% ± 0.4%) and *Il10*$^{-/-}$ (2.84% ± 0.7%) even under Th17 polarizing conditions (Fig. 4.7*A-B*). We then determined if loss of KSR1 promoted T cell development along the Th1 axis *in vitro*. Splenocytes were isolated as and cultured under Th1 polarizing conditions and analyzed as before. We found that cell expressing IFN-γ under Th1 polarization was increased in *KSR1*$^{-/-}$ CD4^{+}TCRβ^{+} T cells (56.1% ± 1.9%) compared to WT (44.8% ± 3.4%) (Fig. 4.8*A-B*). Interestingly, culturing splenocytes from *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice under Th1 polarizing conditions resulted in reduced T cell viability. These findings suggest that *KSR1*$^{-/-}$ T cells have a greater propensity to develop along the Th1 axis *in vitro*.

IFN-γ production is increased in lymphocytes isolated from Rag2$^{-/-}$ *mice previously transferred with KSR1*$^{-/-}$ *or KSR1*$^{-/-}$*Il10*$^{-/-}$ *naïve T cells*

Adoptive transfer of CD4^{+}CD45RB^{high} naïve T cells into immunodeficient recombination activating gene-2 (*Rag2*$^{-/-}$) mice causes colitis (Powrie, et al., 1993; Shinkai, et al., 1992). Since KSR1 deficient T cells had increased IFN-γ production, we hypothesized that adoptive transfer of KSR1 deficient CD4^{+}CD45RB^{high} naïve T cells into *Rag2*$^{-/-}$ mice would accelerate colitis. To test this, we injected WT, *KSR1*$^{-/-}$, or *KSR1*$^{-/-}$*Il10*$^{-/-}$ CD4^{+}CD45RB^{high} naïve T cells into 8-week old *Rag2*$^{-/-}$ mice. After 4 weeks, proximal and distal colon histological sections were scored for colitis. Interestingly, the colitis that developed in *Rag2*$^{-/-}$ mice transferred with CD4^{+}CD45RB^{high} T cells was similar regardless of donor genotypes (Fig. 4.9). Concurrently we isolated spleen,

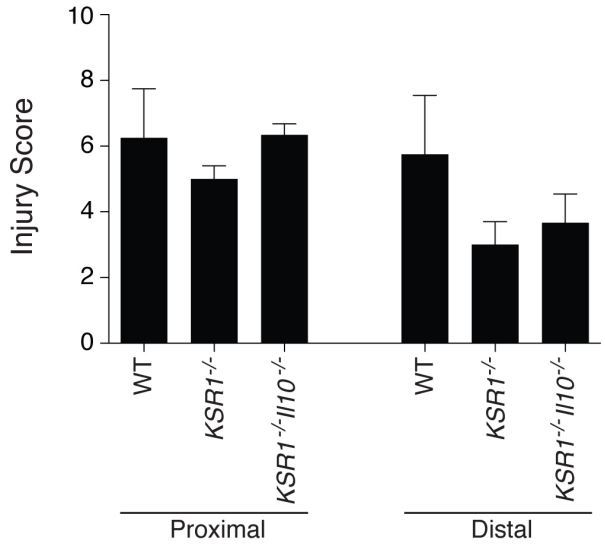

Figure 4.9: KSR1 deficiency does not exacerbate colitis following adoptive transfer of $CD4^{+}CD45RB^{high}$ T cells into *Rag2*$^{-/-}$ mice. Mouse colons were removed and a 2 mm section cut from proximal and distal colon was fixed and paraffin embedded. H&E stained sections were scored by a pathologist blinded to the groups as previously described (Corazza, et al., 1999). Solid bars represent the mean colitis score for each group (n ≥ 6) and error bars are the SEM from two independent experiments.

mesenteric lymph node (MLN), and intraepithelial lymphocyte (IEL) donor T cells, stimulated with PMA/Ionomycin, and stained for intracellular IFN-γ and IL-17A and analyzed by flow cytometry. The percentage of IFN-γ producing $CD4^+TCR\beta^+$ T cells isolated from spleens of *Rag2*$^{-/-}$ mice transferred with *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ naïve T cells was increased (39.4% ± 5.3% and 41.2% ± 1.5% respectively) compared to transferred WT naïve cells (24. 6% ± 1.6%) (Figs. 4.10*A, 4.10C*). Additionally, donor *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ $CD4^+$ T cells isolated from the spleen had fewer T cells expressing IL-17A (1.0% ± 0.1% and 0.6% ± 0.4%) compared to WT (2.5% ± 0.4%) (Figs. 4.10*B-C*). Similar results were obtained for *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ T cells isolated from the MLN of *Rag2*$^{-/-}$ mice. Interestingly, IFN-γ in T cells isolated from IEL fractions was not increased and only *KSR1*$^{-/-}$*Il10*$^{-/-}$ IEL exhibited a decrease in IL-17A production (Figs. 4.10*B-C*). These data suggest that the effect of KSR1 deficiency in colitis may require loss of KSR1 in innate immune cells to exacerbate disease, or that the increased IFN-γ production by $CD4^+TCR\beta^+$ T cells is insufficient to increase colitis severity in the *Rag2*$^{-/-}$ transfer model.

Neutralization of IFN-γ attenuates severity of disease in KSR1$^{-/-}$*Il10*$^{-/-}$ *mice*

Our data showing that colons of *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice had increased in IFN-γ mRNA levels, together with the data indicating *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ $CD4^+$ T cells have enhanced IFN-γ production, suggest a potential mechanism for accelerated disease in *KSR1*$^{-/-}$ *Il10*$^{-/-}$ mice. Therefore, we determined if IFN-γ expression was involved in disease pathogenesis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. To test this, we administered intraperitoneal injections of α-IgG, α-IFN-γ, or α-IL-17A neutralizing antibodies in 3-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice twice per week for 3 weeks. Three days following the last injection, mice were sacrificed and H&E stained colon sections were scored for inflammation and injury as before. While neutralization of IL-17A caused no discernable decrease in colitis severity over isotype control (8.75 ± 1.5 vs. 9.75 ± 1.0), treatment with anti-IFN-γ significantly reduced the severity of colitis (6.3 ± 1.1) in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Figs. 4.11*A-B*). As expected, α-IL-17A reduced KC expression indicating IL-17A inhibition (Fig. 4.11*C*).

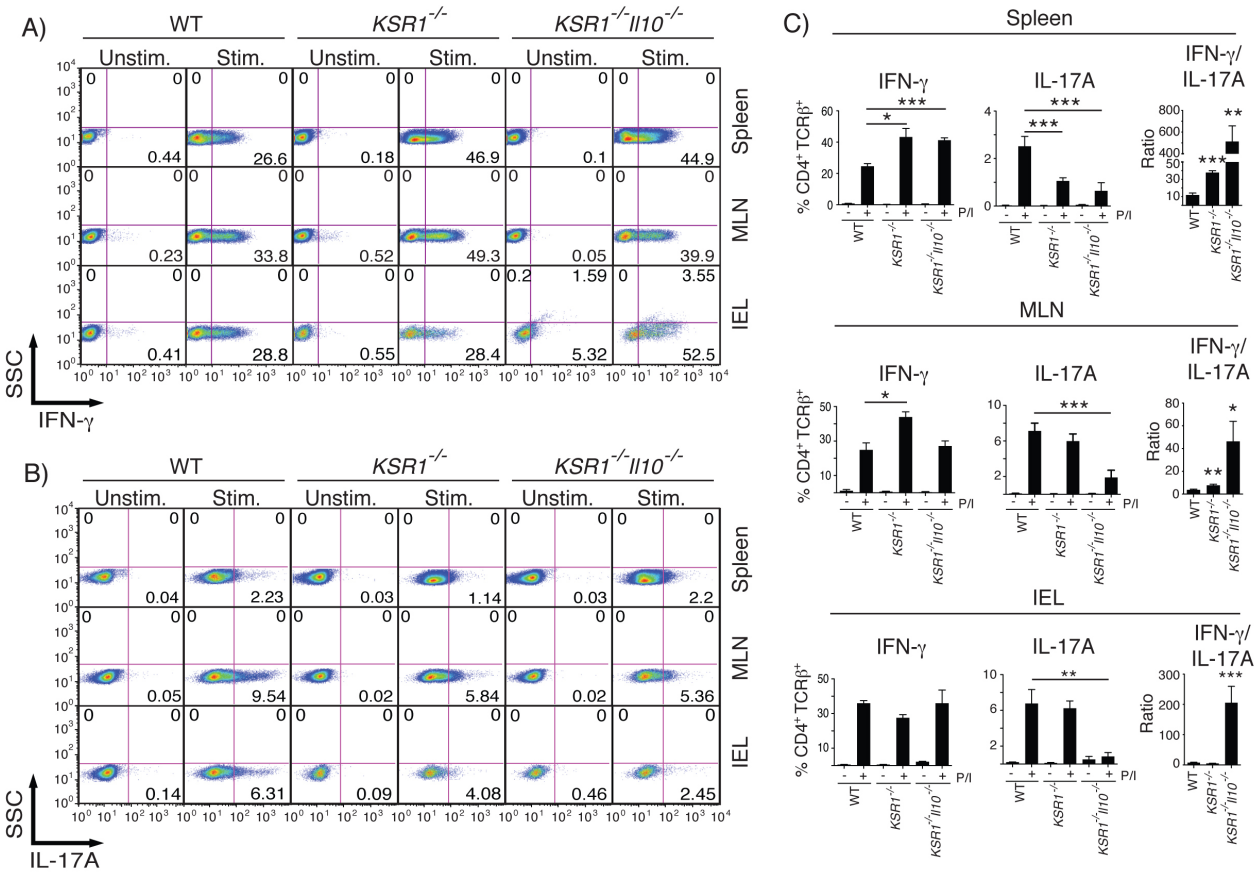

Figure 4.10: *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ T lymphocytes re-isolated from *Rag2*$^{-/-}$ mice have increased IFN-γ production and reduced IL-17A production. Naïve CD4^{+}CD45Rβ^{high} T lymphocytes sorted from WT, *KSR1*$^{-/-}$, and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mouse spleens were injected into 8-week old *Rag2*$^{-/-}$ mice and followed for 4 weeks post-injection. T cells isolated from the spleen, MLN, or IEL populations were cultured for 5 hours in the presence of the protein transport inhibitor GolgiPlug and treated with or without PMA/Ionomycin. Intracellular A) IFN-γ and B) IL-17A staining of CD4^{+}TCRβ^{+} T cells were analyzed by flow cytometry. C) Intracellular cytokine staining for IFN-γ and IL-17A was quantified for each isolated T cell population and the mean is represented by solid bars for each indicated group as well as the mean ratio of T cells positive for intracellular IFN-γ compared to T cells positive for intracellular IL-17A. Error bars represent the SEM pooled from two independent experiments (n ≥ 4). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

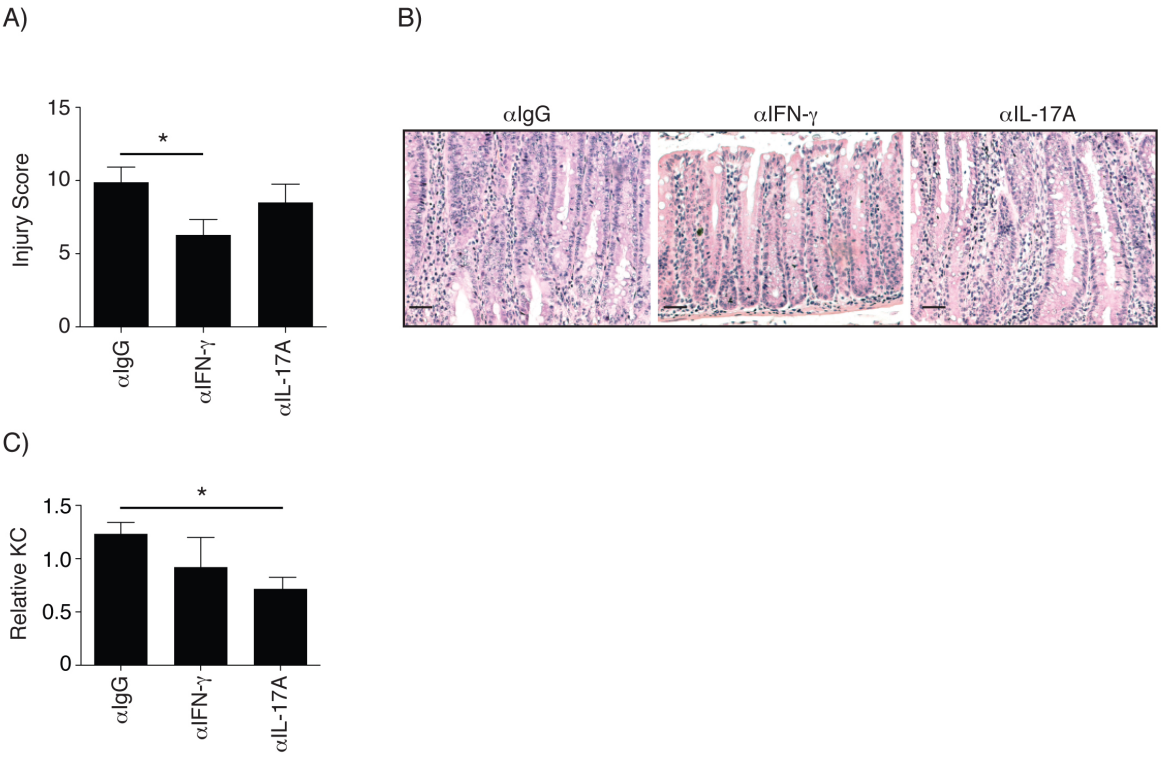

Figure 4.11: Neutralizing IFN-γ attenuates colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. 3-week old *KSR1*$^{-/-}$ *Il10*$^{-/-}$ mice were administered intraperitoneal injections of 100 μg/mouse neutralizing antibodies against IgG, IFN-γ, or IL-17A twice per week for a period of 3 weeks and assessed for colitis. A) Colon sections were scored as before for inflammation and injury. Solid bars represent the mean (n = 5) pooled from three independent experiments with the error bars representing the SEM. B) Representative H&E stained colon sections from *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice administered IgG, IFN-γ, or IL-17A neutralizing antibody. C) Efficacy of IL-17A neutralizing antibody was examined by determining KC transcript levels using quantitative real-time PCR analysis on RNA isolated from colons of *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice administered IgG or IL-17A neutralizing antibody. Solid bars are the mean (n=5) and error bars represent the SEM from three independent experiments. * $P < 0.05$

These data indicate that the severity of colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice is mediated, at least in part, by the increase of IFN-γ associated with loss of KSR1.

KSR1$^{-/-}$ *mice have increased susceptibility to DSS-induced experimental colitis*

Oral administration of DSS results in colitis characterized by Th1 cytokines during the acute phase of disease (Dieleman, et al., 1998). While lymphocytes are not required for DSS-induced injury (Dieleman, et al., 1994), Th1 lymphocytes play detrimental roles in DSS-induced colitis (Kim, et al., 2006). Since colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice is, in part, mediated by IFN-γ, combined with the observation that *KSR1*$^{-/-}$ $CD4^+TCR\beta^+$ T cells have enhanced IFN-γ production, we hypothesized that KSR1 is protective against DSS-induced experimental colitis. To assess the role of KSR1 in DSS-induced colitis, 8-week old WT and *KSR1*$^{-/-}$ mice were administered 3% DSS in the drinking water for a period of 7 days. Some mice were allowed to recover following the DSS-induced injury by administering regular drinking water for 3 days following DSS treatment. While the percent initial body weight was relatively unchanged in WT and *KSR1*$^{-/-}$ given water or DSS, *KSR1*$^{-/-}$ mice continued weight loss during the recovery period (88.8% ± 1.6%) compared to WT mice (96.9% ± 1.2%) (Fig. 4.12*A*). We then examined for histological changes using H&E staining paraffin embedded mouse colon sections (Fig. 4.12*B*) which were scored by a pathologist blinded to the genotype and treatment group. While the injury scores for *KSR1*$^{-/-}$ mice following the 7-day DSS treatment were statistically indistinguishable from WT (7.4 ± 1.1 vs. 9.1 ± 1.0), *KSR1*$^{-/-}$ mice had increased injury scores following the 3-day recovery period (13.3 ± 0.68) compared to WT (8.9 ± 0.71) (Fig. 4.12*C*). Interestingly, while injury scores in *KSR1*$^{-/-}$ mice increased following the recovery phase compared to the 7-day injury, injury in WT mice was similar for both 7-day injury and 3-day recovery (Fig 4.12*C*). Thus, KSR1 appears to be an important molecule that protects against Th1 experimental models of colitis.

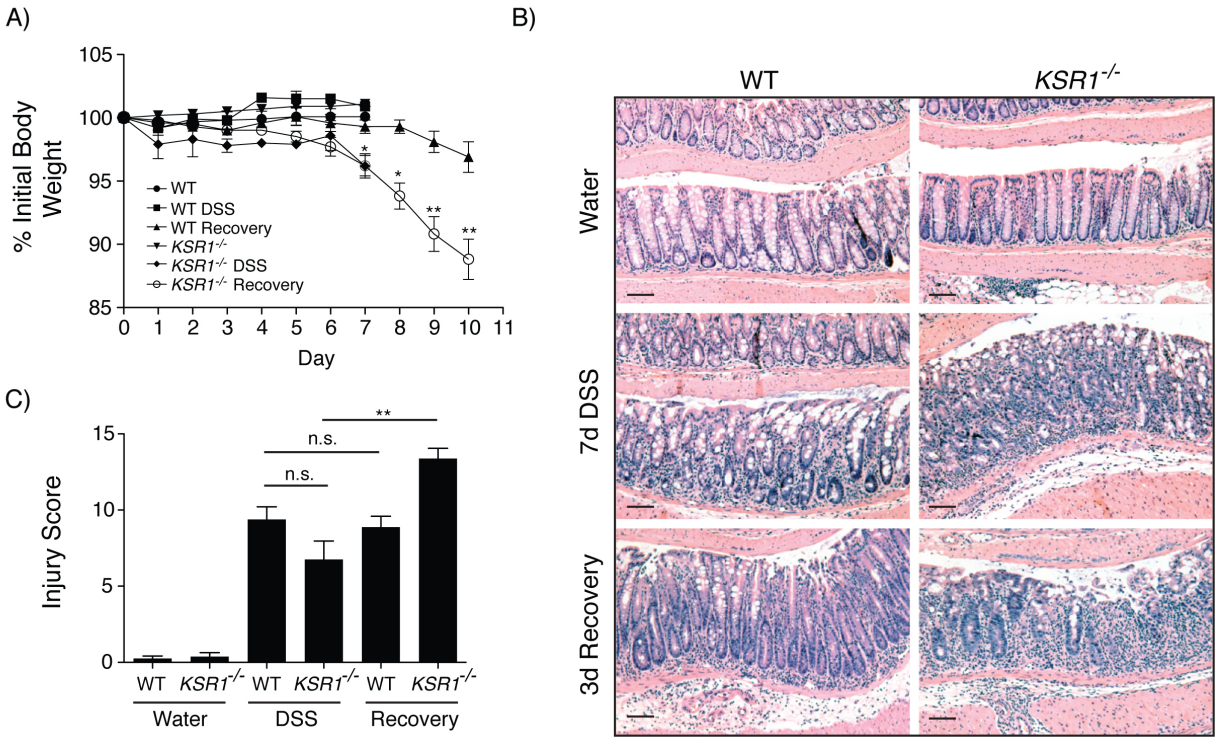

Figure 4.12: *KSR1*$^{-/-}$ mice have increased sensitivity to DSS-induced colitis. 8-week old WT and *KSR1*$^{-/-}$ mice were administered 3% DSS (w/v) in the drinking water for a period of 7 days. Subsets of mice were provided regular drinking water for 3 days following DSS treatment. A) Mice were weighted daily and recorded as a percent of initial body weight. Data are plotted as the mean percentage with error bars representing the SEM from two independent experiments (n = 8). * $P < 0.05$, ** $P < 0.01$ B) Paraffin embedded mouse colon sections were H&E stained. Images were taken at 10X magnification (*scale bars, 20 μm*). C) H&E stained colon sections were scored for inflammation and injury by a pathologist blinded to the genotype and treatment group. Solid bars represent the mean injury and inflammation score for each group (n = 8) and error bars are the SEM from two independent experiments. ** $P < 0.01$

Discussion

While distinct roles for KSR1 have been reported for intestinal epithelial cells (Yan, et al., 2001; Yan, et al., 2004), T cell proliferation (Nguyen, et al., 2002), T cell differentiation,(Wang, et al., 2004; Wang, et al., 2006) and recently NK cell-mediated cytolysis (Giurisato, et al., 2009), the role of KSR1 in inflammatory diseases has not been defined. In this study, we utilized the *Il10*$^{-/-}$ mouse model of spontaneous experimental colitis to investigate the role of KSR1 during chronic inflammation. We found that *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice developed accelerated severe colitis with 100% penetrance by 4 weeks of age. The pathogenesis of the disease was predominantly attributed to loss of KSR1 in hematopoietic lineages. We found that splenocytes isolated from *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice exhibited increased IFN-γ production. In fact, KSR1 deficient splenocytes had a greater propensity to develop along the Th1 axis while Th17 development was impaired *in vitro*. Finally, administration of α-IFN-γ neutralizing antibody attenuated colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Collectively, the data presented here implicate KSR1 as a regulatory molecule that functions to suppress IFN-γ production in T cells and promotes Th1/Th17 developmental homeostasis.

Spontaneous colitis in *Il10*$^{-/-}$ mice requires interaction with luminal bacteria (Kühn, et al., 1993; Sellon, et al., 1998). Since *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice develop colitis by 4 weeks of age, and that enteric microbial populations are established just post-weaning (Savage, et al., 1968), we initially suspected that KSR1 was involved in epithelial barrier integrity. Though epithelial barrier permeability was detected in 10-week old *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice, *KSR1*$^{-/-}$ and *Il10*$^{-/-}$ mice were similar to that of WT mice at 10 weeks of age (Fig. 4.2*A*). Although KSR1 protects from TNF-induced apoptosis in colon epithelial cells (Yan, et al., 2001; Yan, et al., 2004), it does not appear to be required for barrier function in the absence of challenging conditions.

We were surprised to find that the protective role of KSR1 was mostly attributed to cells of the immune system (Fig. 4.3). Previous data indicate that *Il10*$^{-/-}$ BM is insufficient to cause disease in WT mice, consistent with our findings (Bamba, et al., 2006). The fact that *KSR1*$^{-/-}$*Il10*$^{-/-}$ BM was sufficient to cause disease in WT recipients established a clear role for KSR1 in immune cell function. Since IFN-γ and IL-17A transcripts were elevated in the colon of healthy *KSR1*$^{-/-}$

mice and diseased *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Fig. 4.5), it seemed plausible that KSR1 was involved in T cell development or in Th1/Th17 responses. Though current data on the pathogenesis of IL-17 in inflammatory diseases remains unclear (Fujino, et al., 2003; Haak, et al., 2009; Komiyama, et al., 2006; Nakae, et al., 2003), our data revealed that colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice was attenuated by inhibiting IFN-γ and not IL-17A (Fig. 4.11). In a second colitis model involving Th1-mediated pathogenesis (Kim, et al., 2006), *KSR1*$^{-/-}$ mice also exhibited greater sensitivity to DSS-induced injury compared to WT mice (Fig. 4.12), further implicating KSR1 in pathogenic Th1 responses.

The observation that *in vitro* Th1 polarization was enhanced in *KSR1*$^{-/-}$ CD4^{+}TCRβ^{+} cells (Fig. 4.8) while Th17 polarization was impaired (Fig. 4.7), suggest KSR1 is involved in Th1 development and suppresses IFN-γ production. While the exact mechanism by which KSR1 suppresses T cell IFN-γ expression is presently not yet understood, scaffolding proteins including KSR1 can modulate signaling pathways by localization of protein phosphatases. KSR1 interacts with protein phosphatase 2B (PP2B) to uncouple ERK activation from the transcriptional activity of Elk-1 (Sugimoto, et al., 1998) as well as associates with PP2A (Ory, et al., 2003). It is attractive to speculate that an antigenic stimulus in the absence of KSR1 perturbs signaling pathways normally regulated by phosphatases. For instance, suppression of PP2A activity increases IFN-γ production in NK cells in the innate immune system (Trotta, et al., 2007). Future analysis on KSR1-interacting proteins in T lymphocytes will be important to identify genes involved in this response.

The crosstalk between innate immune defenses and acquired immunity is actively being investigated for autoimmune diseases and IBD (Kalyan, et al., 2009; Pletneva, et al., 2009; Stockinger, et al., 2007). While our data support a role for KSR1 in lymphocyte function, we cannot rule out a role for KSR1 in innate immunity. The observation that *Rag2*$^{-/-}$ mice transferred with CD4^{+}CD45RB^{high} naïve T cells from WT, *KSR1*$^{-/-}$, or *KSR1*$^{-/-}$*Il10*$^{-/-}$ developed comparable disease with similar histological colitis scores (Fig. 4.9) was surprising given that isolated donor *KSR1*$^{-/-}$ and *KSR1*$^{-/-}$*Il10*$^{-/-}$ naïve T cells produced more IFN-γ (Fig. 4.10*A*). One possibility is that WT colitogenic T cells express appreciable levels of IFN-γ and further increases due to KSR1

deficiency may be insufficient to exacerbate the disease. Alternatively, KSR1 deficiency in the innate immune system may be an important component for increasing susceptibility to colitis mice. Since *Rag2*$^{-/-}$ mice possess KSR1 in cells of the innate immune system independent of transferred T cell genotype, colitis would develop independent of the genotype of transferred $CD4^{+}CD45RB^{high}$ naïve T cells. Consistent with a functional role for KSR1 in innate immune system, we observed decreased nitric oxide production from stimulated bone marrow derived *KSR1*$^{-/-}$ macrophages (unpublished observations). While emerging data implicate KSR1 in innate immune cell function (Giurisato, et al., 2009), further investigations are necessary to elucidate what role, if any, KSR1 plays in innate immune responses and the susceptibility to colitis.

Finally, there may be a direct connection between vitamin D and KSR1 in immunity. Vitamin D receptor (VDR) signaling regulates T cell development and helps maintain immunological tolerance with implications for multiple sclerosis, type-1 diabetes mellitus, and IBD (Cantorna, 2006; Ginanjar, et al., 2007). Recently, polymorphisms at the *VDR* locus were linked to IBD and other autoimmune disorders (Naderi, et al., 2008). Vitamin D supplementation holds promise as a therapeutic agent in the treatment of Crohn's disease by increasing NOD2 expression, which then couples to the expression the antimicrobial peptide defensin β2 (Wang, et al., 2010). Interestingly, the *KSR1* promoter contains a vitamin D responsive element and moreover, KSR1 protein is upregulated by 1, α25-dihydroxyvitamin D_3 (Wang, et al., 2006). In fact, *VDR*$^{-/-}$*Il10*$^{-/-}$ mice develop severe accelerated spontaneous colitis harboring many phenotypic similarities to those observed in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice including increased levels of IFN-γ (Froicu, et al., 2003; Froicu, et al., 2006). It is attractive to speculate that vitamin D-mediated suppression of pathogenic immune responses is, in part, regulated by KSR1 expression and suppression of IFN-γ production in Th1 effector cells.

We conclude that KSR1 expression suppresses IFN-γ production in T lymphocytes by promoting T cell homeostasis along the Th1/Th17 axis. Therefore, induction of KSR1 expression may be an ideal strategy for modulating IFN-γ in Th1-mediated diseases.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary of findings

The results of the work comprised in this dissertation contribute to our understanding of KSR1 as a functional protein kinase in the ERK pathway and describe a new role for KSR1 in immune regulation *in vivo*. While the kinase activity of KSR1 has remained contentious over the past 15 years, the data in Chapter III demonstrate that the kinase activity of KSR1, together with MEK kinase activity, promote colon epithelial cell survival in response to TNF. We show that recombinant KSR1 expressed and recovered from bacteria possesses intrinsic kinase activity and autophosphorylates on serine independent of ceramide. In addition, recombinant KSR1 phosphorylates MBP as a generic substrate and directly phosphorylates recombinant MEK. The kinase activity of KSR1 required full length KSR1 protein since an N-terminal deletion (KSR1ΔCA1) and expression of the kinase domain alone (KSR1ΔN521) had no detectable catalytic activity. Interestingly, while recombinant KSR1 is insufficient to promote MEK activation, phosphorylation of recombinant MEK by immunoprecipitated KSR1 is able to promote MEK activity towards recombinant ERK.

In Chapter IV we described a new role for KSR1 in protection from a Th1-mediated mouse model of colitis. Though *KSR1*$^{-/-}$ mice were phenotypically normal, loss of KSR1 in the *Il10*$^{-/-}$ mouse model of spontaneous colitis resulted in accelerated severe colitis that was characterized by increased epithelial cell turnover and elevated mucosal IFN-γ and IL-17A. We show that KSR1 deficiency in CD4^{+} T lymphocytes resulted in enhanced IFN-γ production. This is attributed to a greater propensity of *KSR1*$^{-/-}$ naïve T cells to develop along the Th1 lineage *in vitro.* Not only was Th1 polarization enhanced in KSR1 deficient T cells; *in vitro* Th17 polarization was impaired. Furthermore, neutralization of IFN-γ attenuated colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Consistent with a role in protecting against Th1-mediated colitis, *KSR1*$^{-/-}$ mice exhibited greater

sensitivity to the DSS-induced injury model of colitis. While elevated IFN-γ increased severity of colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice, IFN-γ expressing T cells did not cause increased colitis in an adoptive transfer model using lymphocyte deficient *Rag2*$^{-/-}$ mice. This suggests that, in addition to a role in the adaptive immune system, KSR1 may also function in the innate immune system to suppress colitis. We conclude that KSR1 expression protects from Th1-mediated colitis by suppressing IFN-γ production and promotes T cell developmental homeostasis along the Th1/Th17 axis.

Future directions

KSR1 as a functional protein kinase

Our data detailed in Chapter III indicate that KSR1 is a functional protein kinase involved in the colon epithelial cell survival response downstream of TNF signaling. While we demonstrated that recombinant KSR1 autophosphorylates on serine, we have yet to identify the specific autophosphorylation site. Using 2D tryptic phosphopeptide mapping we found that KSR1 autophosphorylation occurs on a single tryptic peptide suggesting a single or limited number of autophosphorylation sites. Since many protein kinases are activated by phosphorylation within or near the kinase domain, it will be important to identify this site and determine if KSR1 autophosphorylation is a prerequisite for KSR1 kinase activity towards MEK and for TNF-mediated cell survival. KSR1 contain two serines in the kinase domain activation loop, Ser707 and Ser722, which are good candidates for future analysis. KSR1 also contains a serine bracketed by an F-S-L-F motif C-terminal to the kinase domain. This is highly similar to the C-terminal hydrophobic F-x-x-F/Y-S/T-Y/F motif found in many AGC protein kinases such as protein kinase B (PKB). For PKB, protein stability, kinase activity, and PDK1 binding in the kinase domain are increased when the serine following the FxxF motif is phosphorylated (Romano, et al., 2009). It will be interesting to see if KSR1 is autophosphorylated at this serine residue and what effect a phosphomimetic mutant would have on KSR1 stability, MEK association, and protein kinase activity. In addition, MEK activation requires phosphorylation at two serines in the MEK

activation loop. We observed MEK activation *in vitro* using KSR1 immunoprecipitated from mammalian cells but not recombinant KSR1. This could be expected if KSR1 in mammalian cells has higher catalytic activity due to post-translational modifications. KSR1 is phosphorylated at a number of sites outside the kinase domain by other protein kinases (Volle, et al., 1999). Therefore, site-directed mutagenesis at known KSR1 phosphorylation sites would determine if any modulate KSR1 kinase activity. Alternatively, if KSR1 preferentially phosphorylates one MEK serine residue over the other, it would suggest a mechanism by which KSR1 could sensitize the pathway by reducing the threshold for Raf-1-medaited ERK activation. However, if KSR1 phosphorylates both sites equivalently, KSR1 might sustain MEK activation following Raf-1 dissociation (Figure 5.1*A*-*B*). Lastly, the observation that KSR1 kinase activity required full-length KSR1 protein may offer clues into the tertiary structure of KSR1. For many kinases, expression of the kinase domain alone results in a constitutively active enzyme. Since the KSR1 kinase domain alone or a deletion of the CA1 domain resulted in a non-functional kinase, studies should be conducted to determine if the CA1 and kinase domain of KSR1 interact and what impact this interaction has on catalytic activity.

Role of KSR1 in colitis

In Chapter IV we describe the physiological characteristics that exacerbate colitis in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Specifically, increased IFN-γ in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice resulted in a severe early onset colitis that could be attenuated by blocking IFN-γ *in vivo*. In mouse models of autoimmune disorders such as Rheumatoid arthritis and multiple sclerosis, blocking IFN-γ is an effective therapeutic strategy (Skurkovich, et al., 2007). We demonstrated that CD4^{+} T cells lacking KSR1 had a greater propensity to develop along the Th1 axis and produced more IFN-γ upon stimulation compared to WT CD4^{+} T cells. Yet, the mechanism by which KSR1 suppresses IFN-γ production remains unknown. Since IFN-γ functions in a positive feedback loop, KSR1 could regulate induction of IFN-γ by IL-12 (Figure 5.2). Work from others offer insight into how KSR1 might play a role in this process as vitamin D suppresses IFN-γ synthesis (Cippitelli, et al., 1998).

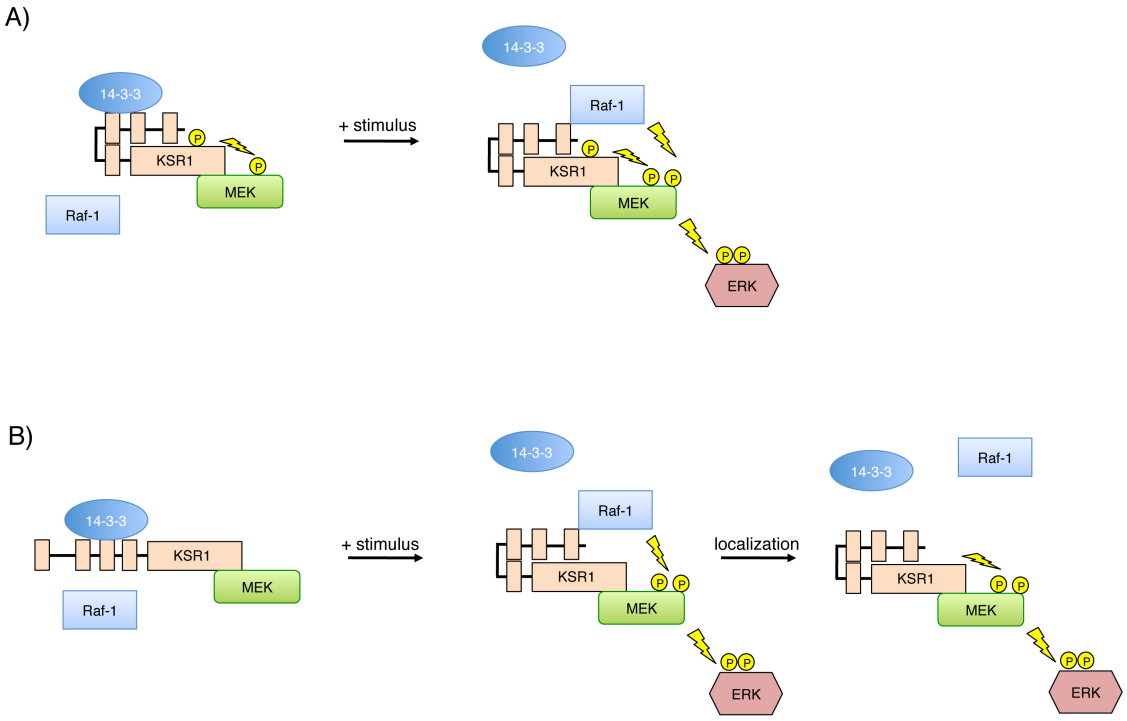

Figure 5.1: Models of KSR1 kinase activity in MEK activation A) KSR1 N- and C-terminus interact to stabilize an active state of the KSR1 kinase domain. KSR1-assocaited MEK is phosphorylated by KSR1 constitutively to lower the threshold of Raf-1-mediated activation of MEK in response to stimulus. B) KSR1 kinase activity is inhibited by sequestration of the N- and C-terminus by an inhibitory protein such as 14-3-3. Upon stimulation, inhibition by 14-3-3 is relieved allowing for the intramolecular interaction of the KSR1 CA1 and kinase domains. This interaction promotes KSR1 kinase activity that function to maintain MEK activation following the dissociation of Raf-1.

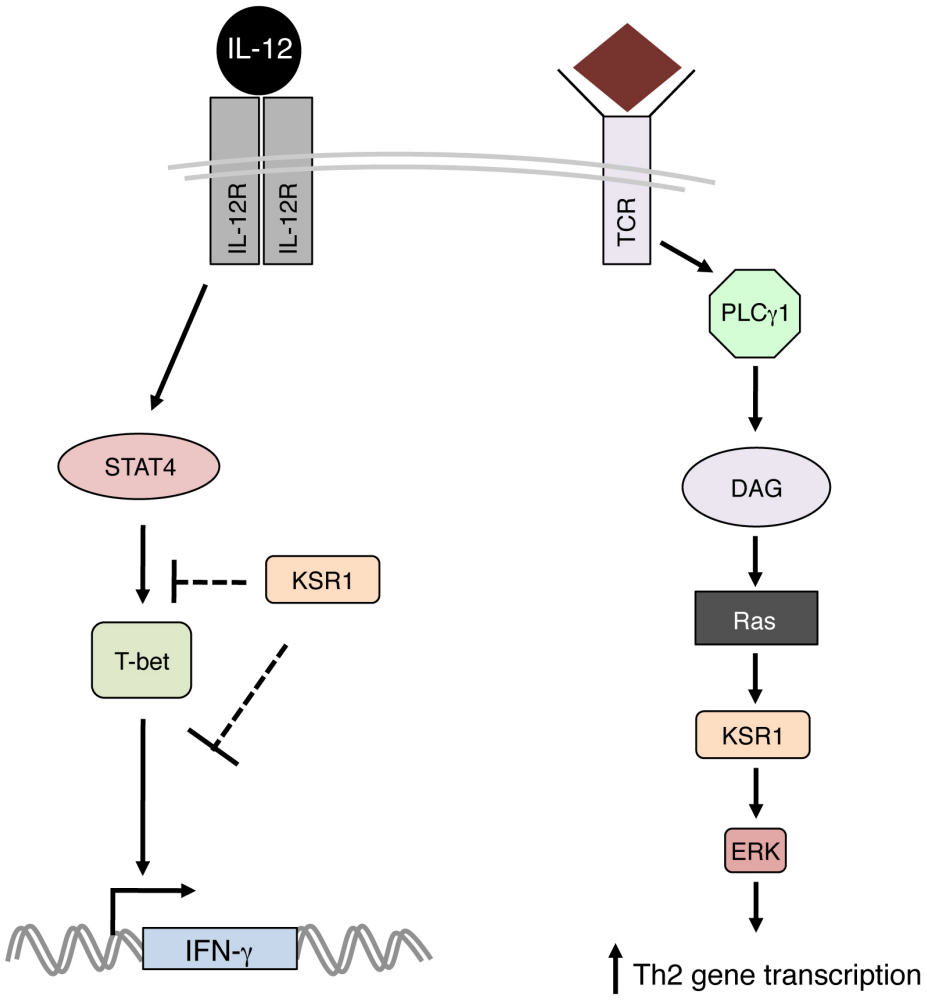

Figure 5.2: Model of KSR1 regulation of T cell IFN-γ production and Th1 development Differentiation of naïve $CD4^+$ T lymphocytes into Th1 subtypes requires ligation of TCR in the presence of IL-12. IL-12 signals through IL-12R to drive IFN-γ production and positively regulates Th1 development. TCR signals through PLCγ1 to activate the Ras-MAPK cascade and promote transcription of Th2 related genes. Adapted from {Liew, 2002 #422} and {Yamashita, 2005 #423}

Work from Studzinski's group demonstrated that KSR1 expression is under the control of a vitamin D responsive element (Wang, et al., 2006). They also found that KSR1 expression modulates monocyte differentiation induced by vitamin D (Wang, et al., 2004). In fact, vitamin D receptor (VDR) knockout mice crossed with *Il10*$^{-/-}$ mice develop similar disease characteristics as *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice (Froicu, et al., 2006). Perhaps *VDR*$^{-/-}$*Il10*$^{-/-}$ mice develop disease due to reduced KSR1 protein levels. While it appears these two components intersect in IL-10 deficiency-induced colitis, further work is needed to determine how KSR1 functions in suppression of IFN-γ production in T cells. Nevertheless, data from our adaptive transfer experiments into *Rag2*$^{-/-}$ mice indicate that, in addition to the adaptive immune response, KSR1 may also function in innate immunity to suppress disease. Recent data demonstrate that KSR1 is required for proper NK cell-mediated lysis (Giurisato, et al., 2009), and preliminary data from our lab found that bone marrow-derived macrophage from *KSR1*$^{-/-}$ mice are impaired in *H. pylori*-stimulated nitric oxide production. Performing the adoptive transfer experiments on *KSR1*$^{-/-}$*Rag2*$^{-/-}$ mice would determine the requirement for KSR1 in the innate immune system to protect against colitis. In addition, characterization of KSR1 function in macrophage is needed to assess KSR1 involvement in macrophage activation by other stimulus such as LPS. Defect in macrophage ability to clear invading pathogens may also contribute to the exacerbated disease in *KSR1*$^{-/-}$*Il10*$^{-/-}$ mice. Nevertheless, our data in Chapter III indicates that KSR1 promotes epithelial cell survival in colon epithelial cells. Since our *in vivo* inflammation studies utilized a KSR1 deficient mouse that was generated as a global deletion, generation of a mouse containing floxed alleles at the KSR1 locus will need to be crossed with the transgenic mouse expressing Cre recombinase under the control of an epithelial-specific villin promoter to investigate tissue-specific contributions of KSR1 in colitis.

Ever since Rudolf Virchow made a correlation between inflammation and tumorigenesis nearly 150 years ago, the consequences emanating from chronic inflammation have been appreciated. The effects of chronic inflammation on colonic tumor progression are mediated in part through epithelial cell survival (Greten, et al., 2004). Since our data show KSR1 to be

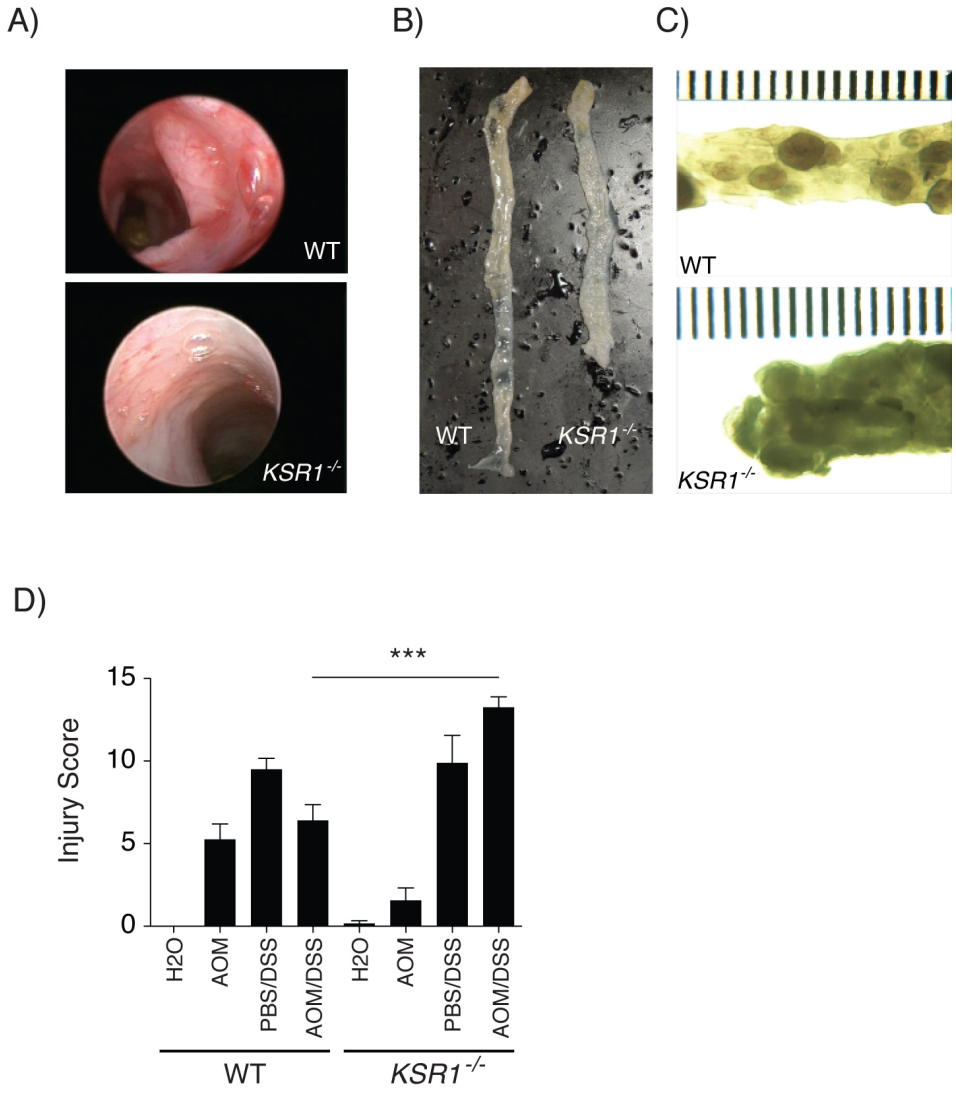

Figure 5.3: AOM-DSS treatment promotes adenomas in WT mice and flat adenomas with regenerative alterations in *KSR1*$^{-/-}$ mice WT and *KSR1*$^{-/-}$ mice were given a single injection of PBS or AOM. 7 days later, a subset of these mice were administered DSS in the drinking water for 5 days followed by a 16-day recovery period on normal drinking water. The cycle was repeated 2 more times and mice were sacrificed at the end of the last recovery period. A) Colonoscopy images taken from AOM-DSS treated WT mice (top) and *KSR1*$^{-/-}$ mice (bottom) following the final cycle. B) Image of whole colons isolated from WT mice (left) or *KSR1*$^{-/-}$ mice (right) treated with AOM-DSS. C) Macroscopic images of the distal colon architecture from AOM-DSS treated WT mice (top) and *KSR1*$^{-/-}$ mice (bottom). D) Total injury scores for WT mice and *KSR1*$^{-/-}$ mice treated with water, AOM, PBS-DSS, or AOM-DSS. Solid bars are the mean and the error bars are the SEM from two independent experiments ($n \geq 8$). *** $P < 0.001$

involved in TNF-mediated epithelial cell survival and pro-inflammatory Th1 responses, we investigated the role of KSR1 in colitis-associated cancer using the mutagen azoxymethane (AOM) followed by three rounds of DSS treatment. Our preliminary data show that WT mice administered AOM-DSS develop classic adenoma polyps whereas *KSR1*$^{-/-}$ mice had no distinguishable polyps, but instead displayed large ulcerations with regenerative crypt changes (Figure 5.3). These characteristics are similar to those seen for mice expressing oncogenic K-Ras in the colonic epithelium in combination with a second tumor-promoting mutation in APC (Haigis, et al., 2008). These mutations contribute to the grade and metastatic potential of tumor progression that is partially attributed to a reduction in ERK activation (Haigis, et al., 2008). Further analysis comparing the mutations arising in AOM-DSS treated WT mice vs. AOM-DSS treated *KSR1*$^{-/-}$ mice by Laser capture microdissection will determine if there is a disparity in the occurrence of particular oncogenic mutations between the two distinct types of tumor growth. In addition, for the oncogenic K-Ras mouse model, the MAP kinase phosphatase 3 (Mkp3) was upregulated and functioned to reduce ERK signaling. Mkp3 expression in AOM-DSS treated *KSR1*$^{-/-}$ mice should be examined to determine if loss of KSR1 contributes to regenerative changes due to Mpk3 gene expression. Alternatively, enhanced inflammatory responses and slow restitution in response to DSS-induced injury might increase the instance of inflammation-associated mutagenesis.

Concluding remarks

Initial observations nearly 30 years ago began to unravel a basic sequential signal transduction mechanism utilized by all eukaryotes known as the MAPK cascade (Cobb, et al., 1983). The ERK/MAPK pathway is one of several MAPK signaling modules that utilizes a tiered signaling cascade to instruct a diverse range of cellular functions from yeast to mammals. Since this pathway is activated downstream from a variety of extracellular signals, specific responses are often determined by regulation over signal duration and/or activation threshold. Scaffolding proteins such as Ste5 in yeast, or KSR1 and MEKK1 in mammals, facilitate activation of the

MAPK cascade by association with each MAPK cascade component. However, recent evidence points to more complex mechanisms by which scaffolding proteins fine-tune pathway activation to coordinate diverse signaling output. An elegant set of experiments demonstrated that dimerization of Raf and KSR1 enhances Raf activation through an allosteric mechanism (Rajakulendran, et al., 2009). A similar mechanism was described for Fus3 activation by the yeast scaffolding protein Ste5 (Bhattacharyya, et al., 2006). Furthermore, the diverse system output in response to ERK activation suggests that the pathway itself is highly plastic. This can partly be attributed to the fact that activation of the ERK pathway can occur as a graded or digital response based on the type of stimulus. The response to stimulus can also be changed by the sensitivity to a given ligand-induced signal. In many ways KSR1 functions like a variable rheostat that enhances the sensitivity to a particular stimulus by altering the ERK activation threshold (Lin, et al., 2009). However, the exact mechanism by which KSR1 functions to achieve this sensitization is not entirely clear. Based on our *in vitro* data using recombinant KSR1 protein, we propose two models in which KSR1 kinase activity contributes to MEK activation. In the first model the KSR1 N-terminus directly interacts with the C-terminal kinase domain to allosterically modulate KSR1 kinase activity towards MEK and lower the threshold for MEK activation by Raf-1 (Figure 5.1*A*). Alternatively, inhibitory proteins such as 14-3-3 proteins may sequester the N- and C-terminus of KSR1 to suppress KSR1 activity. Ligation of TNF promotes dissociation of 14-3-3 proteins, allowing an intramolecular interaction to occur between the KSR1 CA1 domain and the KSR1 kinase domain. Dissociation of 14-3-3 from KSR1 also allows for KSR1 association with Raf-1, which leads to Raf-1 activation of MEK. Following Raf-1 dissociation from KSR1, KSR1 could function to sustain MEK activation in the absence of Raf-1 spatially within the cell to maintain ERK activation (Figure 5.1*B*).

In many human cancers, activation of the ERK pathway due to constitutive signaling from oncogenic mutations in RTK and Ras are common occurrences (Kranenburg, 2005; Meshinchi, et al., 2003). While these cell-autonomous mutations are necessary for tumorigenesis, often times they are not sufficient. One key factor that elevates risk for carcinogenesis is inflammation.

Under normal function the immunosurveillance can recognize and remove transformed cells (Smyth, et al., 2006). However, free radicals generated by immune cells can result in oxidative damage to genomic DNA, promoting tumorigenesis. Evidence for the tumor-promoting consequence of inflammation comes from patients with familial adenomatous polyposis (FAP). These individuals develop colonic tumors due to inherited mutations in the adenomatous polyposis coli (APC) gene. Fewer polyps are observed when these patients are treated with non-steroidal anti-inflammatory drugs (NSAIDs) (Ulrich, et al., 2006). In a similar manner, patients with IBD have a greater risk for developing colon cancer with higher mortality rates than that for sporadic colon cancer due to repeated bouts of inflammation (Ekbom, et al., 1990; Richards, et al., 1989). Animal models have provided clues into the potential mechanisms driving inflammation-associated cancer. One key factor found to mediate the tumor promoting effects of inflammation in the colon is the anti-apoptotic transcription actor NF-κB expressed in colon epithelial cells (Greten, et al., 2004). TNF, predominantly produced by monocytes, activates NF-κB downstream of TNFR1 (Edelblum, et al., 2008). By promoting colon epithelial cell survival in the presence of DNA damaging byproducts from immune cells, epithelial cells acquire mutations that circumvent an apoptotic program and instead, pass along oncogenic mutations to progenitor cells. Since KSR1 promotes TNF-mediated colon epithelial cell survival, as detailed in Chapter III, KSR1 expression may actually be tumor promoting in the context of inflammation. Targeting KSR1 expression in early stages of tumor development may reduce the overall tumor burden and progression towards metastasis. In fact, loss of KSR1 expression disrupts oncogenic Ras signaling and prevents pancreatic carcinoma xenografts in nude mice (Xing, et al., 2003).

For patients with chronic inflammation, dysregulated immune responses not only increase discomfort and impact quality of life; the risk of developing inflammation-associated cancer is increased. With the data presented in Chapter IV, promoting KSR1 expression in the immune system may be a viable strategy to reduce IFN-γ gene transcription and promote T cell developmental homeostasis and decrease inflammation-associated cancer risk. These data position KSR1 an attractive molecule to suppress IFN-γ production in Th1 lymphocytes, as well as

to disrupt cell survival cues in Ras-dependent tumors. Thus, modulating KSR1 protein expression in a tissue specific manner may be a viable strategy to attenuate Th1 inflammatory responses and decrease cancer risk associated with chronic inflammation.

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