ABSTRACT

KIM, JAE-YOUNG. TAK1 is a Central Mediator of NOD2 Signaling and is Essential for Intestinal Epithelial Cell Protection against Chemical-induced Colitis. (Under the direction of Jun Ninomiya-Tsuji.)

Innate immunity is the first line of defense against invasive microbial pathogens. It is triggered by several families of pattern-recognition receptors (PRRs), which activate cellular responses including activation of NF-κB and mitogen-activated protein kinases (MAPKs) signaling, and subsequently the induction of proinflammatory genes. There are two groups of PRRs, toll-like receptors (TLRs) and nod-like receptors (NLRs). In contrast to TLRs that recognize microbes via plasma membrane or endosomal receptors, NLRs induce innate immune response by detecting bacterial components released into the cytosol. Nucleotide oligomerization domain 2 (NOD2) is the best characterized member of the NLR family. Upon NOD2 stimulation by muramyl dipeptide (MDP), a NOD2-specific ligand originating from the bacterial cell wall, it associates with an adaptor molecule, RIP-like interacting CLARP kinase (RICK, also called Rip2) and activates both NF-κB and MAPKs signaling pathways, which lead to inflammatory gene expression. However, the molecular mechanism by which NOD2-RICK complex activates its downstream signals remains elusive. Here, we report that TGF β-activated kinase 1 (TAK1) is a central mediator of NOD2 signaling in epidermal cells. TAK1 belongs to MAPKKK family and plays an essential role in activating NF-κB and MAPKs signaling in tumor necrosis factor (TNF), interleukin-1 (IL-1), and TLR signaling pathways. In this study, we found that MDP-induced proinflammatory gene expression as well as the activation of NOD2 downstream signals including NF-κB, c-Jun N-terminal kinase (JNK) and p38 were completely abolished in TAK1-deficient keratinocytes, indicating that TAK1 is essential for eliciting NOD2-induced innate immune responses.

NOD2 and its downstream adaptor, RICK, associated with and activated TAK1. Endogenous TAK1-RICK interaction was enhanced by MDP, suggesting that NOD2-RICK-TAK1 complex is induced in an MDP-dependent manner.

We have previously reported that intestinal epithelium-specific TAK1 deletion mice showed severe inflammation and mortality at postnatal day 1 due to TNF-induced epithelial cell death. In this study, we investigated TNF-independent role of TAK1 by utilizing mice harboring double deletion of TNF receptor 1 (TNFR1) and intestinal epithelium-specific deletion of TAK1 (TNFR1KO/TAK1^{IE}KO). To study the role of TAK1 in the intestinal epithelial barrier, the mice were subjected to acute colitis by administration of dextran sulfate sodium (DSS). We found that loss of TAK1 significantly augments DSS-induced experimental colitis. DSS-induced weight loss, intestinal damage and inflammatory markers were significantly increased in TNFR1KO/TAK1^{IE}KO mice compared to the TNFR1KO control mice. Following DSS exposure, apoptosis was strongly induced and epithelial cell proliferation was decreased in the TAK1-deficient intestinal epithelium. These results suggest that epithelial-derived TAK1 signaling is important for cytoprotection and tissue repair after injury. Finally, we showed that TAK1 is essential for IL-1- and bacterial components-induced expression of cytoprotective factors including IL-6 and cycloxygenase 2 (COX2). Collectively, we propose that homeostatic cytokines and microbes-mediated intestinal epithelial TAK1 signaling is pivotal for protecting the intestinal epithelium against injury by promoting cell survival and maintaining proliferation.

Taken together, this study demonstrates that TAK1 is an essential mediator of NOD2

signaling in epidermal cells, and also suggests that bacterial components- and cytokine-induced TAK1 signaling both play a pivotal role in preventing intestinal inflammation by maintaining the integrity of the intestinal epithelium.

TAK1 is a Central Mediator of NOD2 Signaling and is Essential for Intestinal Epithelial Cell Protection against Chemical-induced Colitis.

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy

Toxicology

Raleigh, North Carolina

2009

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DEDICATION

I dedicate this dissertation to my parents, parents in law and my wife Eunjung for their support and love.

BIOGRAPHY

Jae-Young Kim was born on December 10, 1975 in Seoul, the Republic of Korea. He is an older son of Jin-Sook Choi and Jeong-Gil Kim. He grew up in Seoul and enrolled in Seoul National University in 1994 majoring in Animal Science. He served for Korean Army from 1996 to 1998 during his undergraduate study. That period matured him not only physically but also mentally. After finishing military service, Jae-Young resumed his study and received a Bachelor of Science degree in Animal Science in 2001. Subsequently he started his first step as a scientist by beginning a master's degree course under the direction of Dr. Kwon-Haeng Lee in the Department of Pharmacology, Catholic University in Seoul, Korea. After earning his master's degree in 2003, Jae-Young came to the United States in August, 2004 to pursue his Ph.D. degree in the Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC. In January of 2005, he began to work with Dr. Jun Ninomiya-Tsuji and started his project to characterize the function of TAK1 MAPKKK. During his Ph.D. training, he met his soul mate, Eunjung Kim, who is a graduate student in the Department of Mathematics, North Carolina State University, and married her on the day of Christmas Eve, 2005. He expects to finish all work for his Ph.D. by December, 2008 and will continue his research as a postdoctoral fellow under the direction of Dr. Ze`ev Ronai in Burnham Institute for Medical Research, La Jolla, CA.

ACKNOWLEDGMENTS

This dissertation would not have been possible without support from many people, especially my advisor, Dr. Jun Ninomiya-Tsuji. I greatly thank her for invaluable support and a training experience. She always has been a model of good scientist for me. I also would like to thank Dr. Smart, Dr. Tsuji and Dr. Jobin, who were willing to serve as my advisory committee member and provided me invaluable guidance throughout my Ph.D. training period. I would like to thank Dr. Núñez at University of Michigan for his support to my first project on NOD2. I also extend my gratuity to all toxicology faculty and administrative staff.

I thank all of my lab mates, Maiko, Emily, Rie, Pete and Sho for their help and suggestions for my research. I also thank ex-lab mate, Wei-Chun, now at University of Pennsylvania, who ungrudgingly helped me as a senior toxicology student. I am also greatly thankful to my classmates, especially Richard and Rakesh, for their help and encouragement during demanding course works and preliminary exam periods. I sincerely wish them the best of luck in all of their future endeavors.

I express my sincere thank to my parents, parents in law, brother and brother in law in Korea, who gave me endless support, encouragement, faith and love for me. Finally, my utmost thanks to my wife, Eunjung, who always has been and always will be with me.

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GENERAL INTRODUCTION

1. Host immune response – innate immunity and adaptive immunity

All multicellular organisms are constantly exposed to invasive microbes including fungi, bacteria and virus. Therefore, a proper host defense response to eliminate the pathogenic invaders is essential for survival. The host defense mechanism against invasive microbial pathogens is elicited by two components of the immune system, innate immunity and adaptive immunity. These two components of host immune system cooperate to eliminate infectious agents and play a critical role in the survival of the host.

Innate immunity is the first line of defense against invasive microbial pathogens. Innate immune system exists in all multicellular organisms, whereas adaptive immunity exists only in vertebrates. The first step of the innate immune response is to recognize pathogen-associated molecular patterns (PAMPs), which are highly conserved structures expressed in the microbes. The recognition is mediated by specific receptors expressed in epithelial surfaces including the skin and intestinal epithelium, as well as phagocytic cells including marcrophages and neutrophils. After recognition of PAMPs, the innate immune system activates intracellular signaling pathways to transcribe genes required for activating host defense mechanisms. The molecular mechanisms by which the innate immune system recognizes PAMPs and signaling pathways connecting PAMPs recognition to gene transcription have been long standing interests in the immunology field. It has been established that the recognition of PAMPs is mediated by two groups of receptors – toll-like receptors (TLRs) and nucleotide oligomerization domain (Nod)-like receptors (NLRs). The

characteristics of TLRs and NLRs and the signaling pathways mediated by these two groups of receptors will be discussed in the following sessions.

Adaptive immunity, acquired immunity in other words, is a highly evolved defense mechanism compared to innate immunity. Adaptive immunity is elicited by B cell and T cell lymphocytes which utilize antigen receptors such as immunoglobulins and T cell receptors, respectively, to recognize and eliminate infectious microbes. The characteristics of adaptive immunity are high specificity for distinct molecules and their distinct memory, which allows more efficient response against repeated exposure to the same microbe. Innate and adaptive immunity do not function separately but cooperatively. For example, innate immunity activates adaptive immune system by secreting proinflammatory cytokines which promote lymphocytes activation. Thus, both innate and adaptive immunity function cooperatively to make up an integrated host immune system.

2. Toll-like receptors (TLRs)

TLR was firstly identified in the fruit fly, which has only the innate immune system. A Drosophila protein, Toll, was identified as an essential receptor for host defense against fungal infection (Lemaitre et al., 1996). A year later, a mammalian homolog of the Toll receptor (Now called TLR4) was identified as an activator of a transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a major transcriptional regulator of genes encoding inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Medzhitov et al., 1997). A later study

characterized the function of TLR4 that recongnizes the microbial component, lipopolysaccharide (LPS), using mice haboring mutation in *Tlr4* gene (Poltorak et al., 1998). Now, more than 13 mammlian TLRs have been identified. TLR2 is known to recognize microbial lipopeptides in cooperation with TLR1 (for recognition of triacyl lipopeptides) and TLR6 (for recognition of diacyl lipopeptides). TLR2 also recognizes peptidoglycan, lipoteichoic acid from Gram-positive bacteria (Takeuchi et al., 2001; Takeuchi et al., 2002). TLR4 is a well know receptor for LPS as described above and TLR5 detects flagellin, a component of bacterial flagella (Hayashi et al., 2001). TLR3, 7 and 9 are localized in the endosome and recognize viral dsRNA, ssRNA and CpG DNA respectively (Alexopoulou et al., 2001; Hemmi et al., 2002; Hemmi et al., 2000).

TLRs are transmembrane receptors that contain extracellular leucine-rich repeats (LRR) domain which is responsible for PAMPs recognition and intracelluar Toll/Interleukin-1 receptor (TIR) domain which mediates interaction with downstream signaling molecules. After recognition of PAMPs, TLRs activate signaling pathways by recruiting a set of adaptor proteins including myeloid differentiation primary response gene (MyD88), TIR-containing adaptor protein/MyD88-adaptor like (TIRAP/MAL), TIR-containing adaptor inducing interferon-β/TIR-domain-containing adaptor molecule 1 (TRIF/TICAM1) and TIR-domain-containing adaptor molecule/TRIF-related adaptor molecule 2 (TRAM/TICAM2) (Kawai and Akira, 2007; West et al., 2006). The assembly of these molecules induces the activation of transcription factors, NF-κB and Activator Protein-1 (AP-1) signaling pathways, both of which play an important role in transcriptional regulation of inflammatory genes.

The NF-κB family of transcription factors are composed of dimers that contain Rel-homology domains (RHD), which bind to their specific DNA sequence called κB site (5'-GGGRNNYYCC-3'; R, purine; Y, pyrimidine; N, any nucleotides). NF-κB activates the transcription of genes involved in inflammatory responses as well as cell survival. Before stimulation, NF-κB is associated with its inhibitor protein, called inhibitor of κB (IκB), which sequesters NF-κB in the cytosol by masking the nuclear localization signal of NF-κB. Upon TLR stimulation, TIR domain-containing signaling complex activates kinase cascades, which induce phosphorylation of specific serine residues in IκB (Ser-32 and 36). Phosphorylated IκB is targeted for proteosomal degradation, which allows nuclear translocation and transactivation of NF-κB (Kawai and Akira, 2007). The AP-1 transcription factor is mainly composed of Jun, Fos and ATF protein dimers, which bind to the palindromic AP-1 site (5'-TGAGTCA-3'), also known as TPA-responsive element (TRE). All AP-1 proteins contain a leucine zipper domain which is responsible for dimerization and a basic DNA binding domain. AP-1 activity is upregulated by diverse physiological and pathological stimuli, including cytokines, growth factors, stress signals and bacterial or viral infections. Upon AP-1 activating stimuli, mitogen-activated protein kinase (MAPK) cascades phosphorylate and activate Jun N-terminal kinase (JNK). JNK subsequently phosphorylates Jun, within its N-terminal transactivation domain (Ser63 and Ser73) and thereby enhances its transcriptional activity. JNK also phosphorylates and activates ATF-2. Other MAPKs, ERK and p38, are also involved in AP-1 activation. The transactivation of AP-1 leads to transcriptional upregulation of a subset of genes involved in various cellular responses including inflammation (Hess et al., 2004).

3. Nod-like receptors (NLRs)

Mammalian host cells have evolved microbial recognition system both at the cell surface and in the cytosol. The recognition at the cell surface is mediated by the TLR system as described above and intracellular recognition of microbe is mediated by NLR protein family. The NLR proteins were identified by genomic database search for proteins with homology to the apoptosis regulator, Apaf-1 and its nematode homolog, CED-4. The genomic search revealed two related proteins, NOD1 (CARD4) and NOD2 (CARD15) (Inohara et al., 1999; Ogura et al., 2001b). NOD1 and NOD2 contain N-terminal CARD domain as Apaf-1 does. But, unlike Apaf-1, NOD1 and NOD2 possess a leucine-rich repeats (LRR) domain, which also exists in TLRs, in their C-termini, indicating that they function as a bacterial sensor. Subsequent studies revealed more than 20 members of NLRs with similar molecular structure to NOD1 and NOD2. These proteins consist of central nucleotide oligomerization domain (NOD) which is required for oligomerization, and C-terminal LRR domain for microbial recognition. The amino-terminal region of NLRs mediates interaction with downstream signaling molecules (Inohara and Nunez, 2003).

4. Nucleotide oligomerization domain 2 (NOD2)

NOD2 belongs to a family of NLR proteins. It contains an N-terminal CARD domain, central nucleotide oligomerization domain (NOD) and C-terminal LRR domain. NOD2 senses muramyl dipeptide (MDP), which is a minimal peptidoglycan motif common to both Gram-positive and Gram-negative bacteria, via its C-terminal LRR domain (Girardin et al., 2003). Upon MDP binding to NOD2, NOD2 is oligomerized via central NOD domain. The

oligomerization allows NOD2 to interact with its downstream effecter, RIP-like interacting CLARP kinase (RICK, also called Rip2), by CARD-CARD interaction, and activate NF-κB, a key transcription factor for pro-inflammatory responses. It is known that NOD2 also activates MAPK pathways that cooperate with NF-κB pathway in innate immune signals.

RICK is a serine/threonine kinase which carries CARD domain at its C-terminus (Inohara et al., 1998; McCarthy et al., 1998; Medzhitov and Janeway, 2000; Thome et al., 1998). RICK can associate with various CARD domain-containing proteins via CARD-CARD interaction. In vitro studies have shown that overexpression of RICK activates NF-κB and JNK pathways which play an important role in regulating immune responses (Medzhitov and Janeway, 2000). According to RICK knockout (KO) mice study, RICK is essential for eliciting innate immunity in response to the activation of various TLRs (Kobayashi et al., 2002). RICK is also necessary for NF-κB activation in response to NOD2 activation (Kobayashi et al., 2002). A previous study has shown that RICK has a scaffolding role, bringing NOD2 and IκB kinase (IKK), which is a key signaling molecule for NF-κB activation, into close proximity (Inohara et al., 2000). Besides its scaffolding role, RICK is reported to play an essential role in NOD2-mediated ubiquitination of NEMO/IKKγ, a key component of IκB kinase (IKK) complex (Abbott et al., 2004). MDP-induced ubiquitination of NEMO does not require RICK's kinase activity, indicating that RICK activates NOD signaling independent of its kinase activity. However the exact molecular mechanism by which NOD2-RICK signaling activates IKK-NF-κB pathway remains unanswered.

5. NOD2 mutations and Crohn's disease

Crohn's disease is a common chronic inflammatory disease affecting both the small and large intestine. The symptoms of Crohn's disease can vary among individuals. The most common symptoms are abdominal pain, diarrhea and weight loss. Currently, the etiology of Crohn's disease is poorly understood. It is believed that both environmental and genetic factors are associated with the pathogenesis of the disease. It has been reported that smoking increases this risk (Cosnes, 2004) as well as diets high in sweet, fat or refined foods. Genetic approaches were performed to identify susceptibility genes for Crohn's disease and have revealed that the mutations of human *NOD2* gene are strongly associated with this disease (Hugot et al., 2001; Ogura et al., 2001a). There are three major mutations (Arg702Trp, Gly908Arg, Leu1007fs) found in Crohn's disease patients (van Heel et al., 2005). All these mutations exist in LRR domain of NOD2, which is responsible for detecting MDP, a NOD2 ligand. These mutations greatly decrease NOD2's ability to detect MDP and to activate proinflammatory signaling cascade (van Heel et al., 2005). These studies have proposed a hypothesis that failure of innate immune response to bacterial components (MDP) is associated with the pathogenesis of Crohn's disease. To reveal the in vivo role of NOD2 and its implication in the regulation of intestinal inflammation, NOD2 KO mice have been generated and analyzed. Kobayashi et al. reported that NOD2 KO mice do not develop not only spontaneous but also chemical-induced colitis. However, these mice are highly susceptible to bacterial infection via oral route, probably due to defective expression of a subgroup of cryptdins, defensin-related cryptdin 4 (Defcr4) and Defcr-related sequence 10 (Defcr-rs10), which have antimicrobial activity against pathogenic bacteria implicated in

Crohn's disease (Kobayashi et al., 2005). This study suggested a link between loss of NOD2 function and increased susceptibility to Crohn's disease. According to their model, in the healthy intestine, pathogenic microbe can not invade intestinal epithelium due to effective antimicrobial barrier mediated by NOD2-induced defensin expression. However, in Crohn's disease, loss of NOD2 function disrupts the antimicrobial barrier and allows bacterial invasion through the intestinal barrier. This results in a secondary inflammation mediated by lamina propria immune cells (Wehkamp et al., 2005).

The theory that defective innate immune responses caused by NOD2 mutations are responsible for increased Crohn's disease risk has been challenged by studies that indicate NOD2's role as a negative regulator of TLR signaling. Watanabe et al. found that NOD2 deficiency or Crohn's disease-associated NOD2 mutations increased Toll-like receptor 2 (TLR2)-mediated NF-κB activation and Th1 responses, suggesting that NOD2 is a negative regulator of TLR2 response (Watanabe et al., 2004). They extended this theory to in vivo and found that administration of MDP protected mice from experimental colitis by negatively regulating not only TLR2 but also multiple TLR responses (Watanabe et al., 2008). These studies suggest that loss of negative regulation of TLR signaling caused by NOD2 mutations is one of the causes of dysregulated inflammation in Crohn's disease intestine.

Collectively, it is so far believed that failure of NOD2 to mediate MDP-induced innate responses as well as absence of NOD2-mediated downregulation of multiple TLR responses increase the risk of Crohn's disease. It is likely that epithelial NOD2 signaling is required for

intact barrier function of intestinal epithelium by eliciting antimicrobial activity against pathogenic bacteria as well as stimulating cell survival signaling cascades against epithelium disrupting stresses. In contrast, NOD2 is likely to be responsible for preventing overstimulation of intestinal immune cells by negatively regulating TLR responses in lamina propria immune cells. However, it has not been clearly explained why NOD2 plays two opposite roles for maintaining intestinal homeostasis. Therefore, it is important to identify cell type specific role of NOD2 in the intestinal homeostasis using conditional knockout mice models for better understanding physiological role of NOD2 and the etiology of Crohn's disease.

6. TGF-β-activated kinase 1 (TAK1)

TAK1 belongs to mitogen-activated protein kinase kinase kinase (MAPKKK) family. TAK1 was originally identified as a key mediator of TGF-β-induced MAPK activation (Yamaguchi et al., 1995). However, subsequent studies revealed that TAK1 critically participates in cytokine signaling (e.g. IL-1, TNF, RANKL) as well as TLR signaling (Mizukami et al., 2002; Ninomiya-Tsuji et al., 1999; Sato et al., 2005; Shim et al., 2005). Among different TAK1 stimuli, IL-1β and tumor necrosis factor α (TNFα) signaling pathways have been extensively studied. In IL-1β pathway, stimulation of IL-1 receptor leads to recruitment of downstream signaling molecules including MyD88, interleukin-1 receptor associated kinase (IRAK) and TNF receptor associated factor 6 (TRAF6). The TRAF6 protein contains a Really Interesting New Gene (RING) finger domain E3 ubiquitin ligase, which is involved in protein ubiquitination. Upon IL-1 stimulation, TRAF6 induces autoubiquitination linked

through Lys-63 polyubiquitin chain (Deng et al., 2000). The Lys-63 polyubiquitin chain recruits TAK1 to TRAF6 complex and activates TAK1. Active TAK1 subsequently phosphorylates IKKβ, resulting in NF-κB activation. TAK1 also phosphorylates a members of mitogen-activated protein kinase kinase (MAPKK) family, which in turn phosphorylate and activate JNK and p38 (Wang et al., 2001). In TNFα pathway, activation of TNF receptor recruits TNFR-associated death domain protein (TRADD), TRAF2, TRAF5 and receptor interacting protein (RIP). TRAF2 and TRAF5 also contain ubiquitin ligase activity, which induces Lys-63 polyubiquitination of RIP. The polyubiquitinated RIP recruits TAK1 to the TNFR signaling complex and activates TAK1, leading to NF-κB and MAPK activation (Adhikari et al., 2007). TAK1 is also reported to be an important mediator of a subset of stress signaling pathways. It has been reported that osmotic stress strongly induces TAK1 activity (Huangfu et al., 2006). This study also revealed a novel TAK1 regulator, thousand-and-one amino acid kinase 2 (TAO2), which allows TAK1 to specifically activate JNK, but not IKK-NF-κB pathway. Collectively, TAK1 is a central mediator of immune signaling including TLR and cytokine signaling as well as stress-mediated signal transduction.

TAK1 deficiency results in embryonic lethality, thus several approaches using conditional knockout mice have been made to reveal TAK1's function in vivo. It has been reported that TAK1 is required for proper T cell and B cell development and their activation using T cell and B cell-specific KO mice (Liu et al., 2006; Sato et al., 2005; Sato et al., 2006; Wan et al., 2006). TAK1 is known to be important for maintaining epithelial barrier in the skin and intestine. Loss of TAK1 in the epidermal tissue results in a severe inflammatory skin

condition by postnatal day 6-8. It is due to massive keratinocyte apoptosis by TNF in TAK1 deficient epidermis, suggesting that TAK1 prevents TNF-induced cell death in vivo and ablation TAK1 activity leads to loss of skin barrier and dysregulated inflammation (Omori et al., 2006). A further study demonstrated that reactive oxygen species (ROS) are main mediators of TNF-induced killing in epidermal cells and TAK1 plays a pivotal role in reducing ROS to protect epidermal tissue from TNF (Omori et al., 2008). Intestinal-epithelium specific TAK1 deficient mice show similar but a more severe phenotype than epidermal specific TAK1 KO mice. Enterocyte-specific TAK1-deleted mice spontaneously develop intestinal inflammation and die by postnatal day 1. Similar to epidermal-specific KO mice, the ablation of TNF signaling strongly attenuates intestinal inflammation, indicating that TNF-induced disruption of intestinal epithelial barrier is the main cause of dysregulated intestinal inflammation in intestinal epithelium-specific TAK1 KO mice (Kajino-Sakamoto et al., 2008). Collectively, TAK1 plays a central role in maintaining skin and intestinal barrier function, thereby preventing inflammation. Intact barrier function is important for protecting host from bacterial invasion and the ablation the barrier function is highly associated with chronic inflammatory conditions in the skin and intestine especially. Given that TAK1 maintains barrier function in the mouse model, it is likely that loss of TAK1 activity in epithelial tissues is associated with chronic inflammatory diseases such as psoriasis and Crohn's disease.

HYPOTHESES AND SPEFIC AIMS

Manuscript I : TAK1 is a central mediator of NOD2 signaling in epidermal cells.

NOD2 is a critical mediator of MDP-induced signaling pathways leading to NF-κB and MAPK activation. For activation of both NF-κB and MAPK, their upstream kinases including IKK and MKK must be phosphorylated and activated. It has been reported that a Ser/Thr kinase RICK is an essential downstream mediator of NOD2 signaling (Kobayashi et al., 2002), however its kinase activity is known to be dispensable for activating both NF-κB and MAPK (Lu et al., 2005). This suggests that RICK functions as an adaptor molecule, and that there should be another kinase which phosphorylates IKK and MKK to activate NF-κB and MAPK pathways in NOD2 signaling. TAK1 MAPKKK has been reported to be a binding partner of NOD2 (Chen et al., 2004). Chen et al. showed that overexpression of dominant negative TAK1 abolished NF-κB activation induced by NOD2 overexpression (Chen et al., 2004). However, no study elucidating the role of TAK1 in NOD2 signaling in a physiological setting has been conducted. In this study, we hypothesize that TAK1 is a central mediator of NOD2 signaling. We found that keratinocytes are highly stimulated by MDP, thereby TAK1 WT and KO keratinocytes were utilized as model systems. The experiments were designed to demonstrate that

1. TAK1 is required for MDP-induced innate immune response.
2. TAK1 is required for MDP-induced NF-κB and MAPK activation.
3. TAK1 associates NOD2 and RICK in vivo.
4. The stimulation of NOD2 induces TAK1 kinase activity.

Manuscript II : Intestinal epithelial-derived TAK1 signaling is essential for cytoprotection against chemical-induced colitis.

The integrity of intestinal barrier depends on intact single cell layer of intestinal epithelium. Disruption of intestinal barrier triggers abnormal lamina propria immune cell activation, and thereby it is believed to be highly associated with chronic inflammatory disorders such as Crohn's disease. The molecular or physiological mechanisms regulating the barrier function of intestinal epithelium have been actively investigated. It is evident that commensal microbes critically participate in barrier functions through TLR signaling pathways. In previous study, we found that loss of TAK1 in the intestinal epithelium leads to massive apoptosis of epithelial cells, subsequent intestinal inflammation and mortality by postnatal day 1. The ablation of TNFR1 largely rescued this phenotype, indicating that TAK1 is required for neutralizing cytotoxic effect of TNF to intestinal epithelium. However we found that mice harboring double deletion of TNFR1 and intestinal epithelium-specific deletion of TAK1 still occasionally show increased inflammation. In the intestinal epithelium, it is reasonable to anticipate that commensal bacteria and homeostatic cytokines activate TAK1. Therefore, we hypothesize that TAK1 plays an important role in maintaining intestinal barrier integrity other than preventing TNF-induced apoptosis of intestinal epithelium. We utilized intestinal epithelium specific TAK1 KO mice and control mice under TNFR1 KO background and examined their intestinal barrier function by testing sensitivity to dextran sodium sulfate (DSS)-induced colitis. The experiments were aimed to demonstrate that,

1. TAK1 attenuates clinical and histological signs of DSS-induced colitis.
2. TAK1 protects intestinal epithelium from injury-mediated apoptosis.

3. TAK1 promotes reparative proliferation of intestinal epithelium
4. TAK1 is required for producing reparative factors for intestinal epithelium.

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Manuscript I

TAK1 is a central mediator of NOD2 signaling in epidermal cells

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* This manuscript has been published in *the Journal of Biological Chemistry* (2008).

ABSTRACT

Muramyl dipeptide (MDP) is a peptidoglycan moiety derived from commensal and pathogenic bacteria, and a ligand of its intracellular sensor NOD2. Mutations in NOD2 are highly associated with Crohn disease, which is characterized by dysregulated inflammation in the intestine. However, the mechanism linking abnormality of NOD2 signaling and inflammation has yet to be elucidated. Here we show that transforming growth factor β-activated kinase 1 (TAK1) is an essential intermediate of NOD2 signaling. We found that TAK1 deletion completely abolished MDP-NOD2 signaling, activation of NF-κB and MAPKs, and subsequent induction of cytokines/chemokines in keratinocytes. NOD2 and its downstream effector RICK associated with and activated TAK1. TAK1 deficiency also abolished MDP-induced NOD2 expression. Because mice with epidermis-specific deletion of TAK1 develop severe inflammatory conditions, we propose that TAK1 and NOD2 signaling are important for maintaining normal homeostasis of the skin, and its ablation may impair the skin barrier function leading to inflammation.

INTRODUCTION

Animals are constantly exposed to microorganisms present on the skin and the gastrointestinal tract. Detecting microorganisms and activating host immune systems to prevent their invasion are crucial for animal survival. The innate immune system, the first line of defense against invading microbial pathogens, uses pattern-recognition receptors, such as Toll-like receptors (TLRs) to recognize microorganisms or their products on the cell membranes (Omori et al., 2006; Takeda and Akira, 2005). In addition to TLRs, there is increasing evidence that intracellular recognition of bacteria is equally important in innate immune responses (Inohara et al., 2005; Kobayashi et al., 2003). NOD-like receptors (NLRs) are a family of cytosolic proteins that are involved in the recognition of intracellular bacteria (Inohara et al., 2005; Kobayashi et al., 2003). NOD2 is a member of the NLR protein family that contains a caspase recruitment domain (CARD) in the N-terminal region, a nucleotide-binding and oligomerization domain (NOD) in the central region, and leucine-rich repeats (LRRs) in its C-terminus (Inohara et al., 2005; Kobayashi et al., 2003; Ogura et al., 2001). NOD2 senses muramyl dipeptide (MDP), the minimal peptidoglycan (PGN) motif common to both gram-positive and negative bacteria, via the LRR domain (Girardin et al., 2003; Inohara et al., 2003). Upon MDP stimulation, NOD2 is oligomerized via the central NOD domain and recruits RICK, a serine/threonine kinase carrying a CARD domain at its C-terminus, through CARD-CARD interactions (Inohara et al., 1998; McCarthy et al., 1998; Medzhitov and Janeway, 2000; Thome et al., 1998). The induction of NOD2/RICK signaling leads to activation of pro-inflammatory transcription factors such as NF-κB and AP-1 (Inohara et al., 1998; Medzhitov and Janeway, 2000; Ogura et al., 2001). Studies using mice

deficient in RICK have revealed that this kinase is essential for eliciting innate immunity in response to MDP (Park et al., 2007). RICK has been also reported to function as a scaffold protein bringing NOD2 and IKK into close proximity (Inohara et al., 2000) and to mediate ubiquitination of NEMO/IKKγ, a key component of NF-κB signaling complex (Abbott et al., 2004). However, the exact molecular mechanism by which NOD2-RICK activates IKK-NF-κB and MAPK pathways remains undefined.

Initial studies revealed that NOD2 expression was restricted to monocytes/macrophages (Ogura et al., 2001). However, additional studies showed that NOD2 is also expressed in several epithelial cells including enterocytes (Gutierrez et al., 2002) and keratinocytes (Voss et al., 2006). Both enterocytes and keratinocytes are normally exposed to commensal bacteria and they are activated by bacterial components including MDP (Kobayashi et al., 2005; Voss et al., 2006). Upon stimulation, enterocytes and keratinocytes produce anti-bacterial peptides as well as many cytokines/chemokines to recruit and activate immune cells in the intestine and skin, thereby preventing bacterial invasion and proliferation (Kobayashi et al., 2005; Wehkamp et al., 2004). Loss-of-function mutations of NOD2 are highly correlated with susceptibility of Crohn's disease (CD), a subtype of inflammatory bowel disease, which is characterized by chronic inflammation in the intestine (Bouma and Strober, 2003; Kobayashi et al., 2002). The mechanism by which ablation of MDP-NOD2 signaling can enhance inflammation in vivo has been a subject of much debate (Eckmann and Karin, 2005; Hugot, 2006; Kelsall, 2005). One plausible mechanism is that failure of upregulation of anti-microbial peptides and/or cytokines/chemokines via MDP derived from commensal bacteria

increases susceptibility to bacterial invasion, which may impair the epithelial barrier function and ultimately induce chronic inflammation. However, it has not been established whether loss of NOD2 signaling is causally involved in loss of epithelial barrier function in vivo.

Transforming growth factor-β activated kinase 1 (TAK1) is a member of the MAPKKK family and plays an essential role in tumor necrosis factor (TNF), interleukin 1 (IL-1), and Toll-like receptor (TLR) signaling pathways (Omori et al., 2006; Shim et al., 2005; Takaesu et al., 2003). In response to proinflammatory cytokines or TLR ligands, TAK1 is recruited to TNF receptor-associated factors (TRAFs) and TAK1-binding proteins (TABs), which serve as adaptor proteins, and TAK1 in turn phosphorylates and activates IκB kinases (IKKs) as well as MAPKKs, which subsequently activate MAPKs JNK and p38. These pathways ultimately activate transcription factors NF-κB and AP-1. Besides its well-established role in proinflammatory cytokines and TLR signaling, TAK1 is also reported to be involved in the NOD2 signaling (Chen et al., 2004). TAK1 interacts with NOD2 and overexpression of dominant negative TAK1 inhibits NOD2-induced NF-κB activation. Recently, Windheim et al has reported that TAK1 is important for MDP signaling by using a selective inhibitor of TAK1 as well as in a model system using TAK1 knockout embryonic fibroblasts (Windheim et al., 2007). However, the physiological roles of TAK1 in NOD2 signaling remain to be elucidated.

In this study, we determine the role of TAK1 in NOD2 signaling by utilizing TAK1 FL/FL (floxed) and Δ/Δ (knockout) keratinocytes generated by the Cre-LoxP system (Omori et al.,

2006). We found that NOD2-induced innate immune responses are completely abolished in TAK1 Δ/Δ cells, and that TAK1 was activated upon stimulation of the MDP-NOD2-RICK pathway. In addition, we found that ablation of TAK1 blocked MDP-induced upregulation of NOD2. Our results indicate that TAK1 is not only an essential downstream molecule of NOD2-RICK signaling but also is involved in the regulation of NOD2 expression.

EXPERIMENTAL PROCEDURES

Cells- TAK1 FL/FL and Δ/Δ keratinocytes were isolated from TAK1 FL/FL and epidermal specific TAK1 deletion mice described in our previous publication (Omori et al., 2006). Spontaneously immortalized keratinocytes derived from the skin of postnatal day 0-2 mice were cultured in Ca^{2+}-free minimal essential medium (Sigma) supplemented with 4% Chelex-treated bovine growth serum, 10 ng/ml of human epidermal growth factor (Invitrogen), 0.05 mM calcium chloride, and 1% penicillin-streptomycin at 33 °C in 8% CO_2. Human embryonic kidney 293 and human colorectal adenocarcinoma HT-29 cells were cultured in Dulbeccos's modified Eagle's medium containing 10% bovine growth serum (HyClone). 293 cells were transfected by the standard calcium phosphate precipitation method.

Antibodies, plasmids and reagents- Anti-NF-κB p65 (F-6), anti-NF-κB p50 (H-119), anti-NF-κB p52 (K-27), anti-IKKα (H-744), anti-IKKα/β (H-470), JNK (FL), anti-p38 (N-20) and anti-RICK (H-300) antibodies were purchased from Santa Cruz Biotechnology. Antibodies to phosphorylated JNK, phosphorylated p38 and phosphorylated TAK1 (Thr-187) were purchased from Cell Signaling Technology. Anti-Flag (Sigma) and Anti-HA (Covance) were used. Anti-TAK1 was described previously (Omori et al., 2006). Anti-human NOD2 affinity purified rabbit polyclonal antibody was produced by immunizing a peptide DEEERASVLLGHSPGE (aa 11-26 of human NOD2). Human NOD2 cDNAs were subcloned to pCMV-HA vector. Flag-tagged RICK plasmids were described previously (Kobayashi et al., 2002). pMX-puro-TAK1, -NOD2 and –RICK were generated by inserting

the cDNAs into the pMX-puro vectors (Kitamura, 1998). MDP-LD, MDP-LL and LPS were purchased from Sigma.

Real-time PCR Analysis- Total RNA was prepared from cultured keratinocytes using the RNeasy protect mini-kit (Qiagen). In order to obtain cDNA, 200 ng of each RNA samples were reverstranscribed using TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR analysis was performed using the ABI PRISM 7000 sequence detection system. An Assays-on-Demand gene expression kit (Applied Biosystems) was used for detecting the expression of MIP2, TNF and NOD2. All samples were normalized to the signal generated from glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunoprecipitation and Immunoblotting-Cells were washed once with ice-cold phosphate-buffered saline and whole cell extracts were prepared using lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 20 μM aprotinin, 0.5% Triton X-100). For co-precipitation assay, cell lysates were immunoprecipitated with 1 μg of various antibodies and 15 μl of protein G-Sepharose (GE Healthcare). The immunoprecipitates were washed three times with washing buffer (20 mM HEPES, 10 mM $MgCl_2$, 500 mM NaCl) and resuspended 2X SDS sample buffer and boiled. For detecting endogenous interaction between TAK1 and RICK, HT-29 cells were resuspended in hypotonic buffer (20 mM HEPES-KOH, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (pH 7.5) supplemented with protease inhibitors (10 mM NaF, 1 mM

phenylmethylsulfonyl fluoride, and 20 μM aprotinin). Resuspended cells were lysed by passing through a 22-gauge needle 10 times and adding an equivalent volume of hypotonic buffer containing 0.1% Nonidet P-40 and 300 mM NaCl. Cell lysates were immunoprecipitated with 1.5 μg of control IgG or anti-RICK antibody. For immunoblotting, the immunoprecipitates or whole cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Electrophoretic mobility shift assay (EMSA)-Whole cell extracts were prepared from keratinocytes stimulated with MDP for indicated time points. ^{32}P-Labeled NF-κB oligonucleotides (Promega) were used for generating radiolabeled probe. 30 μg of WCEs were incubated with radiolabeled probe, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 500 ng of poly(dI-dC) (GE Healthcare), and 10 μg of bovine serum albumin in a final volume of 20 μl for 20 min and subjected to electrophoresis on a 4% (w/v) polyacrylamide gel. For supershift assay, the whole cell extracts were incubated with 2 μg of NF-κB antibodies or control IgGs for 15 min prior to the addition of the labeled probe.

In vitro kinase assay- IKK complex was immunoprecipitated with anti-IKKα and the immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]-ATP (3,000 Ci/mmol) and 1 μg of

bacterially expressed GST-IκB in 10 μl of kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$ at 30°C for 30 min. Samples were then separated by 10% SDS-PAGE and visualized by autoradiography.

Retroviral infection- In order to obtain retrovirus carrying TAK1, NOD2 and RICK, EP2-293 cells (BD Biosciences) were transiently transfected with retroviral vectors, pMX-puro-TAK1, -NOD2 and -RICK. After 48 h culture, growth medium containing retrovirus was collected and centrifuged at 1000 rpm for 10 min to remove packaging cells. Keratinocytes were incubated with the collected virus-containing EP2-293 medium with 8 μg/ml polybrene for 24 h. Uninfected cells were removed by puromycin selection.

RESULTS

MDP-mediated innate immune response is impaired in TAK1 Δ/Δ keratinocytes

We have previously generated TAK1 Δ/Δ keratinocytes in which 37 amino acids including the ATP binding region of TAK1 are deleted by the Cre-loxP system resulting in the expression of kinase-dead TAK1 (TAK1 Δ) (Omori et al., 2006). In order to investigate the role of TAK1 in NOD2-mediated immune responses in epithelial cells, we used these TAK1 Δ/Δ and control TAK1 FL/FL keratinocytes as a model system. Keratinocytes were stimulated with MDP (MDP-LD, an active isomer) and the expression of cytokine TNF and chemokine MIP2 (IL-8 in human) were measured using quantitative real-time PCR. We found that MDP was a potent inducer of cytokines/chemokines in keratinocytes (Fig. 1A). MDP-induced TNF and MIP2 expression was impaired in TAK1 Δ/Δ keratinocytes when compared with that observed in FL/FL keratinocytes (Fig. 1A). To confirm whether this impairment is caused by TAK1 deletion, we reintroduced wild-type TAK1 in TAK1 Δ/Δ cells by infection of retrovirus expressing wild type TAK1. TAK1 Δ/Δ keratinocytes expressed the kinase-dead TAK1 at low levels presumably due to the unstable nature of mutant TAK1. The ectopically introduced TAK1 was expressed at levels similar to those found in TAK1 FL/FL keratinocytes (Fig. 2A). Notably, the reintroduction of TAK1 into TAK1 Δ/Δ keratinocytes restored MDP-induced proinflammatory cytokine expressions (Fig. 1A), which demonstrates that TAK1 is essential for MDP-mediated innate immune responses in keratinocytes. To confirm that MDP, but not contaminated bacterial component(s) such as LPS, mediated these responses, we examined the effect of a biological inactive isomer of

MDP, MDP-LL and LPS on keratinocytes. Even at very high concentration, either MDP-LL or LPS did not induce expression of TNF or MIP2 in control TAK1 FL/FL keratinocytes (Fig. 1B). These results indicate that TAK1 is an essential intermediate for MDP signaling in keratinocytes leading to innate immune responses.

MDP-induced activation of NF-κB, JNK, and p38 is impaired in TAK1 Δ/Δ keratinocytes

To investigate the role of TAK1 in MDP-mediated intracellular signaling pathways, we examined the activation of NF-κB and MAPKs in TAK1 FL/FL and Δ/Δ keratinocytes. In TAK1 FL/FL keratinocytes, MDP markedly activated NF-κB DNA binding after 2-6 h incubation (Fig. 2A) and this was associated with translocation of the NF-κB subunit p65 into the nucleus (supplementary Fig. S1). In contrast, MDP-induced NF-κB activation was not observed in TAK1 Δ/Δ keratinocytes (Fig. 2A and supplementary Fig. S1). Notably, the reintroduction of TAK1 restored the activation of NF-κB in TAK1 Δ/Δ keratinocytes (Fig. 2A). Gel supershift assay revealed that p65 homodimer is a major NF-κB complex in MDP-stimulated keratinocytes (Fig. 2B). To further confirm if MDP-induced NF-κB pathway is TAK1 dependent, we examined activation of IKK by in vitro kinase assay (Fig. 2C). IKK was activated at 2-6 h after MDP stimulation in TAK1 FL/FL keratinocytes, whereas no activation was detected in TAK1 Δ/Δ keratinocytes. Activation of JNK and p38 MAPKs was determined by detecting the activated forms of JNK and p38 using phospho-specific antibodies. MDP activated JNK and p38 with a time course similar to that of NF-κB activation in control TAK1 FL/FL keratinocytes, but the activation was completely abolished

in TAK1 Δ/Δ keratinocytes (Fig. 2D and E). The reintroduction of TAK1 restored the activation of JNK and p38 (Fig. 2D and E). These results demonstrate that TAK1 is essential for the activation of both NF-κB and JNK/p38 following MDP stimulation in keratinocytes.

The time course of activation of NF-κB, JNK and p38 in MDP-stimulated keratinocytes was slow compared to that observed in TNF- or IL-1-stimulated cells. They are normally activated within 10-30 min after TNF or IL-1 treatment and downregulated afterwards (Omori et al., 2006; Takaesu et al., 2003). Because MDP induces strongly TNF (Fig. 1) but not IL-1 (data not shown), one possibility is that MDP-induced TNF may be responsible for this delayed activation. However, we found that MDP could activate NF-κB, JNK and p38 even in TNF receptor knockout keratinocytes with a time course similar to that observed in wild type keratinocytes (supplementary Fig. S2). Therefore, it is likely that MDP induces TAK1 activation and subsequent downstream events slower than expected.

NOD2 and RICK interact with TAK1

Our results indicate that TAK1 is a critical downstream target molecule of the MDP-NOD2-RICK signaling pathway. We examined next whether TAK1 can physically interact with NOD2 and RICK. Earlier studies reported that TAK1 interacts with NOD2 (Chen et al., 2004). We confirmed that TAK1 could be co-precipitated with NOD2 when ectopically expressed in 293 cells (Fig. 3A, left panels). The reciprocal precipitation assay verified the TAK1-NOD2 interaction (Fig. 3A, right panels). TAK1 and RICK were also ectopically expressed in 293 cells and co-precipitation assay revealed that RICK associated with TAK1

(Fig. 3B). To further verify this interaction under physiological conditions, we examined the association of endogenous TAK1 with RICK in epithelial cells. For this purpose, we used the human colorectal adenocarcinoma cell line HT-29, because we found that HT-29 cells expressed RICK at higher levels than in keratinocytes (data not shown). Endogenous TAK1 was weakly co-precipitated with RICK in HT-29 cells and the interaction was enhanced by MDP treatment (Fig. 3C). Thus, TAK1 associates with RICK in epithelial cells, and MDP may enhance the interaction.

NOD2 and RICK activate TAK1

Although RICK is a kinase, it has been shown that its kinase activity is dispensable for activation of downstream events (Lu et al., 2005). This suggests that an unidentified kinase is responsible for phosphorylation of IKKs and MAPKKs leading to activation of NF-κB and MAPKs in NOD2-RICK pathway. TAK1 is a kinase that activates both IKK and MAPKKs in IL-1, TNF and TLR signaling pathways (Omori et al., 2006; Shim et al., 2005). Taken together with the results shown above, it is likely that TAK1 is activated by NOD2-RICK, and the TAK1 activation mediates both NF-κB and MAPKs pathways. TAK1 is activated by its autophosphorylation of amino acid residues within its activation loop including Thr-187 (Kajino et al., 2006; Kishimoto et al., 2000). We overexpressed NOD2 or RICK in 293 cells and detected the activated form of TAK1 using phospho-Thr-187 TAK1 antibody. NOD2 is normally activated by MDP-induced oligomerization, which can be mimicked by overexpression of NOD2 or RICK (Inohara et al., 1998; McCarthy et al., 1998; Ogura et al.,

2001). We found that overexpression of both RICK wild type and kinase-dead mutant effectively activated TAK1 (Fig. 4A, lanes 1-3). NOD2 overexpression also induced TAK1 activation although at weaker levels than RICK (Fig. 4A, lane 4). To verify whether NOD2 signaling activates TAK1 under physiological conditions, we stimulated TAK1 FL/FL keratinocytes with MDP and tested whether MDP could activate TAK1. We found that TAK1 was activated in keratinocytes after 2-6 h of MDP stimulation (Fig. 4B). These results indicate that MDP-NOD2-RICK activates TAK1, which leads to subsequent activation of NF-κB and MAPK pathways.

NOD2 and RICK induce innate immune responses in a TAK1 dependent manner

To further verify that TAK1 is an essential downstream effector of NOD2-RICK signaling, we examined the effect of NOD2 or RICK overexpression on cytokine/chemokine expression in TAK1 FL/FL and Δ/Δ keratinocytes. To overexpress NOD2 and RICK in keratinocytes, we generated retroviruses expressing the puromycin resistant gene together with NOD2 or RICK gene. TAK1 FL/FL and Δ/Δ keratinocytes were infected with the retroviruses and pools of keratinocytes expressing NOD2 or RICK were selected by puromycin. Overexpression of either NOD2 or RICK upregulated expression of MIP2 and TNF in TAK1 FL/FL keratinocytes. In contrast, no increase of MIP2 and TNF was detected in TAK1 Δ/Δ keratinocytes (Fig. 5A), even though the levels of NOD2 or RICK expression were similar in TAK1 FL/FL and Δ/Δ keratinocytes (Fig. 5B). Collectively, these results confirm that TAK1

is an essential downstream effector of NOD2-RICK signaling to induce innate immune responses.

TAK1 regulates NOD2 expression

It is known that the amount of NOD2 is upregulated by various stimuli including bacterial components in epithelial cells. Such upregulation of NOD2 results in amplification of the NOD2 signaling, which is probably important for effective responses against bacterial invasion (Gutierrez et al., 2002; Rosenstiel et al., 2003). Because TAK1 plays important roles in MDP signaling, we hypothesized that TAK1 could be responsible for induction of NOD2. The levels of NOD2 mRNA in TAK1 FL/FL and Δ/Δ keratinocytes in response to MDP were examined (Fig. 6). We found that MDP could upregulate NOD2 in TAK1 FL/FL but not in TAK1 Δ/Δ keratinocytes. Thus, TAK1 is involved not only in NOD2-induced expression of cytokines/chemokines but also in the induction of NOD2 upon MDP stimulation.

DISCUSSION

In this study, we determine the role of TAK1 in NOD2 signaling using TAK1 wild type and mutant keratinocytes as model cells. Based on our results, we propose a model of NOD2 signaling and regulation (Fig. 7). Upon MDP stimulation, NOD2 oligomerization induces assembly of a signaling complex including RICK and TAK1, which subsequently facilitates TAK1 autophosphorylation and its activation. TAK1 in turn activates both NF-κB and MAPK pathways leading to induction of innate immune responses. In addition, the TAK1 pathway up-regulates the level of NOD2, which further amplifies NOD2 signaling. Thus, TAK1 is a master regulator of NOD2 signaling in epidermal cells.

Recent studies have shown that K63-linked polyubiquitination of signaling molecules is involved in TAK1 activation in IL-1, TNF and TLR signaling (Chen et al., 2006). Stimulation of IL-1 receptor or Toll-like receptors leads to the activation of TNF receptor associated factor 6 (TRAF6) E3 ubiquitin ligase, which induces K63-linked auto-polyubiquitination of TRAF6. The ubiquitinated TRAF6 is recruited to TAK1 complex containing a TAK1 binding protein, TAB2 or TAB3, through the interaction of the polyubiquitin chain with TAB2/3 (Kanayama et al., 2004; Kishida et al., 2005). This interaction is believed to induce TAK1 autophosphorylation at Thr-187 and its kinase activation in IL-1 and TLR signaling. Therefore, it is likely that TRAF-mediated K63-linked polyubiquitination and TAB2/3 participate in TAK1 activation in NOD2 signaling. During review of this manuscript, Abbott et al reported that NOD2 signaling induces polyubiquitination of TRAF6 (Abbott et al., 2007). However, knockdown of TRAF6 did not

impair NOD2-mediated NF-κB activation (Abbott et al., 2007). We found that MDP could upregulate MIP2 and TNF in TAB2-deficient keratinocytes in a manner similar to that observed in wild type keratinocytes (data not shown). Collectively, the results suggest that multiple TRAF family proteins as well as TAB2 and TAB3 function to activate TAK1 in NOD2 signaling. Further studies are needed to define the molecular mechanism by which TAK1 is activated in NOD2 signaling.

The response of keratinocytes to MDP was somewhat unexpected in that epithelial cells in general including keratinocytes and enterocytes do not respond effectively to bacterial components. In our hands, keratinocytes did not respond to LPS at all. Because epithelial cells are constantly exposed to commensal bacteria, TLR signaling would be always activated if they could respond to LPS and other bacterial components. Therefore, this extremely low sensitivity of epithelial cells to TLR ligands is thought to be important for preventing dysregulated inflammation by commensal bacteria (Lotz et al., 2006). MDP, a moiety of peptidoglycan (PGN), is a common component of Gram-positive and –negative bacteria including commensal bacteria located in the skin and the intestine. However, unlike TLR ligands, PGN needs to be incorporated into the cells and digested into MDP in order to be recognized by NOD2. These processes may reduce bacterium-induced innate immune responses in epithelial cells, but they may be sufficient and important to induce basal levels of cytokines/chemokines to maintain epithelial homeostasis.

MDP is a less potent inducer of cytokines/chemokines than TLR ligands including LPS in monocytes/macrophages (Watanabe et al., 2004). In contrast, we found that MDP is the most potent inducer of cytokines/chemokines in keratinocytes among the tested stimuli including LPS, TNF, IL-6, and IL-1β (data not shown). IL-1β and TNF can induce activation of NF-κB and JNK in keratinocytes in a transient manner peaking the response 10-30 min post-stimulation, whereas MDP activates the same TAK1-dependent pathways in a prolonged manner lasting at least 6 h after stimulation, which is associated with induction of TNF and MIP2 at high levels (Fig. 2 and unpublished data E.O. and J. N-T.). Although NOD2 is expressed in both monocytes/macrophages and epithelial cells, it is thought that, because the levels of NOD2 are generally low in epithelial cells, NOD2 may not play a major role in epithelial cells (Strober et al., 2006). However, our results raise the possibility that MDP-NOD2 signaling represents a major pathway of innate immune responses in keratinocytes.

We have previously shown that TAK1 deletion in keratinocytes results ablation of cytokine/chemokine expression in response to inflammatory stimuli such as IL-1β and TNF (Omori et al., 2006). In this study, we show that MDP-induced cytokines/chemokines are also abolished in TAK1 deficient keratinocytes. According to these results, we speculate that ablation of TAK1 would have a negative effect on inflammation in epithelial tissues. However, to our surprise, mice with epidermal specific deletion of TAK1 develop severe inflammatory conditions in skin at early neonatal stages (Omori et al., 2006). Thus, lack of TAK1 signaling that mediates NF-κB and MAPK signaling is associated with increased inflammation which is similar to that observed in the loss-of-function mutants of NOD2 that

are associated with Crohn's disease. A number of studies using culture cells in vitro have demonstrated that NOD2 mediates pro-inflammatory signaling, however, the loss-of-function mutations of NOD2 are associated with inflammatory diseases in vivo in humans (Eckmann and Karin, 2005; Hugot, 2006; Kelsall, 2005). These suggest that the NOD2-TAK1 signaling is important for the cell autonomous inflammatory signaling in epithelial cells, which appears critical for preventing dysregulated inflammation under the in vivo environment. Why and how cell autonomous inflammatory signaling in epithelial cells prevents overall inflammation? Medzhitov and colleagues have demonstrated that depletion of commensal bacteria causes hyper-susceptibility to epithelial injury (Rakoff-Nahoum et al., 2004). When the commensal bacteria are depleted or not recognized, mice develop severe inflammatory conditions upon injury. They have concluded that basal levels of cytokines/chemokines and possibly antibacterial peptides, which are induced by commensal bacteria under normal steady-state conditions, are essential for preventing dysregulated inflammation. The NOD2-TAK1 pathway may be involved in this steady-state epithelial homeostasis. Indeed, susceptibility to bacterial invasion is significantly increased in NOD2 -/- mice and production of antibacterial peptides in the intestine is reduced by NOD2 deletion (Kobayashi et al., 2005). In conclusion, we propose that TAK1-mediated innate immune pathways including NOD2 pathway recognize commensal bacteria and function to maintain the basal levels of cytokines/chemokines under normal conditions, which is essential for epithelial homeostasis and for preventing bacterial invasion and subsequent dysregulated inflammation.

Similar to the TAK1 mutant skin, epithelium specific ablation of NF-κB pathway causes severe inflammation in the skin as well as in the intestine (Nenci et al., 2007; Nenci et al., 2006). Therefore, NOD2-TAK1-NF-κB may be a major pathway to prevent dysregulated inflammation in the skin and possibly in the intestine. However, while ablation of TAK1 or NF-κB alone causes severe inflammatory conditions (Nenci et al., 2007; Nenci et al., 2006; Omori et al., 2006), NOD2 knockout alone does not cause inflammation under normal conditions in mice (Kobayashi et al., 2005; Pauleau and Murray, 2003). We assume that this difference is because TAK1-NF-κB participates in multiple signaling pathways activated by TLR ligands and proinflammatory cytokines, TNF and IL-1, whereas NOD2 is involved only in the MDP pathway. Interestingly, deletion of TNF receptor I can rescue the inflammatory phenotypes in the all NF-κB mutants and TAK1 mutant mice (Nenci et al., 2007; Nenci et al., 2006; Omori et al., 2006), indicating that TNF is the major mediator of inflammation caused by dysfunction of TAK1-NF-κB signaling. In humans, inhibition of TNF is one of the most effective treatments of CD. These lead us to further speculate that the pathology of CD may be associated with dysregulation of TAK1-NF-κB pathway activated by several stimuli including NOD2 in epithelial cells. Our results warrant further study to determine the relationship between TAK1 pathway and the pathogenesis of CD.

ACKNOWLEDGMENT

We thank Drs. C. Jobin and K. Linder for discussion, and T. Kitamura for providing vectors. This work was supported by Crohn's & Colitis Foundation of America and National Institutes of Health Grant GM068812 (to J. N-T).

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FIGURE LEGENDS

Fig. 1. MDP-induced expression of cytokine/chemokine in keratinocytes

A. TAK1 FL/FL, Δ/Δ keratinocytes and TAK1 Δ/Δ keratinocytes whose TAK1 expression was restored by retroviral infection (TAK1 Δ/Δ+TAK1) were stimulated with increasing concentrations of MDP for 6 h, and then MIP2 and TNF expression was examined by quantitative real-time PCR. mRNA levels were normalized with the levels of GAPDH. Relative mRNA levels were calculated using those of untreated TAK1 FL/FL or TAK1 Δ/Δ as base lines. Data are means ±s.d. of three independent samples and representative from three independent experiments with similar results.

B. TAK1 FL/FL keratinocytes were stimulated with 10 μg/ml of MDP (MDP-LD, active MDP isomer), MDP-LL (inactive MDP isomer) or LPS (100 μg/ml) for 6 h, and the expression of MIP2 and TNF was examined as described above.

Fig. 2. MDP-induced activation of NF-κB and MAPKs in keratinocytes

A. TAK1 FL/FL, TAK1 Δ/Δ and TAK1 Δ/Δ+TAK1 keratinocytes were stimulated with MDP (20 μg/ml) for indicated times. Whole cell extracts were harvested from treated cells and the NF-κB-DNA binding activity was examined by EMSA. p65 immunoblotting was used for loading control for EMSA assay. Asterisk indicates a non-specific band.

B. Whole cell extracts from TAK1 FL/FL cells 6 h after MDP (10 μg/ml) stimulation were incubated with antibodies against NF-κB family proteins as well as control mouse or rabbit IgGs (mIgG, rIgG), and subjected to EMSA.

C. Whole cell extracts were immunoprecipitated with anti-IKKα and the IKK complex was subjected to an in vitro kinase assay using GST-IκB as an exogenous substrate. The amount of IKKα was analyzed by immunoblotting.

D,E. The whole cell extracts used for EMSA were subjected to immunoblotting using phospho-JNK, phospho-p38, JNK, and p38 antibodies. All results are representative of three independent experiments.

Fig. 3. NOD2 and RICK interact with TAK1

A. 293 cells were transiently transfected with FLAG-tagged TAK1 along with HA-tagged NOD2 or equal amount of empty vector. HA-NOD2 was immunoprecipitated, and immunoprecipitates and whole cell extracts (WCE) were analyzed by immunoblotting (left panels). The interaction was confirmed by reciprocal co-immunoprecipitation assay (right panels).

B. 293 cells were transiently transfected with Flag-tagged RICK along with HA-tagged TAK1 or equal amount of empty vector. TAK1-RICK interaction was tested as described above.

C. HT-29 cells were treated with MDP (10 μg/ml) for 6 h or left untreated, and whole cell extracts (WCE) was immunoprecipitated with anti-RICK antibody or same amount of control IgG. The immunoprecipitates were analyzed by immunoblotting.

Fig. 4. NOD2 and RICK activate TAK1

A. 293 cells were transiently transfected with Flag-tagged RICK or NOD2. 48 h after transfection, whole cell extracts were prepared and subjected to immunoblotting using phospho-TAK1 (Thr-187), TAK1 and Flag antibody. Asterisk indicates degraded forms of Flag-NOD2. Wild type RICK migrated as a smear due to its autophosphorylation.

B. TAK1 FL/FL keratinocytes were stimulated with MDP (10 μg/ml) for the indicated times and whole cell extracts were prepared and subjected to immunoblotting using phospho-TAK1 (Thr-187) or TAK1 antibody. All results are representative of three independent experiments. Asterisk indicates a non-specific band.

Fig. 5. TAK1 is essential for the NOD2-RICK pathway in keratinocytes

A. TAK1 FL/FL or Δ/Δ keratinocytes were infected with retrovirus carrying NOD2 or RICK. After puromycin selection, RNAs were isolated and used for quantitative real-time PCR. mRNA levels were normalized with the levels of GAPDH. Relative mRNA levels were calculated using those of the vector virus infected TAK1 FL/FL or TAK1 Δ/Δ as base lines. Data are means of duplicate samples and are representative of three independent experiments with similar results.

B. The levels of overexpressed NOD2 or RICK in TAK1 FL/FL and Δ/Δ keratinocytes were examined by immunoblotting.

Fig. 6. TAK1 regulates NOD2 expression

TAK1 FL/FL, TAK1 Δ/Δ and TAK1 Δ/Δ+TAK1 keratinocytes were treated with increasing concentrations of MDP for 6 h, and RNAs were isolated and used for quantitative real-time PCR. mRNA levels were normalized with the levels of GAPDH. Relative mRNA levels were calculated using those of untreated TAK1 FL/FL or TAK1 Δ/Δ as base lines. Data are means ±s.d. of three independent samples and representative from two or three independent experiments with similar results.

Fig. 7. Model of NOD2 signaling pathway

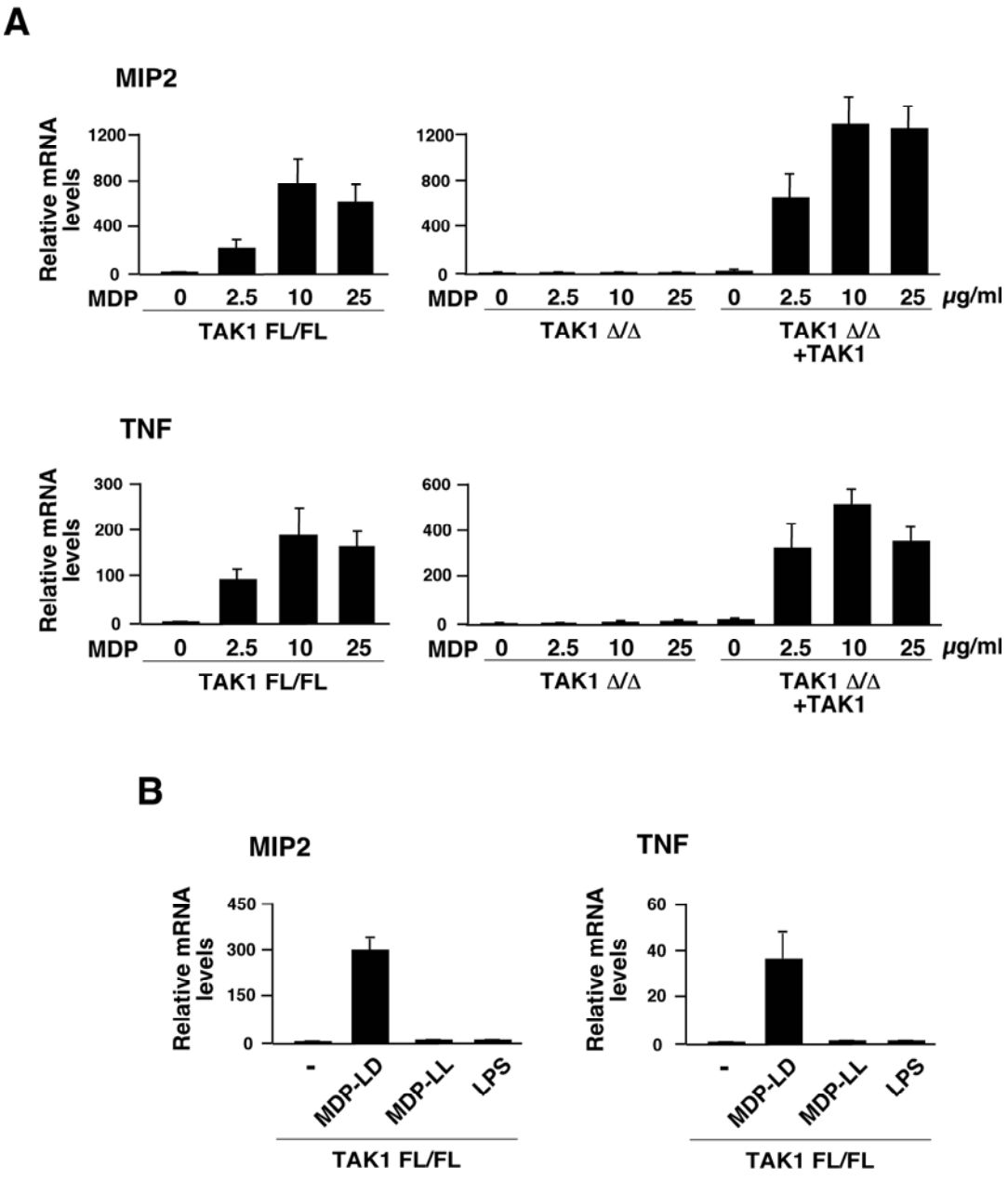


Fig. 1. MDP-mediated innate immune response is impaired in TAK1 Δ/Δ keratinocytes.

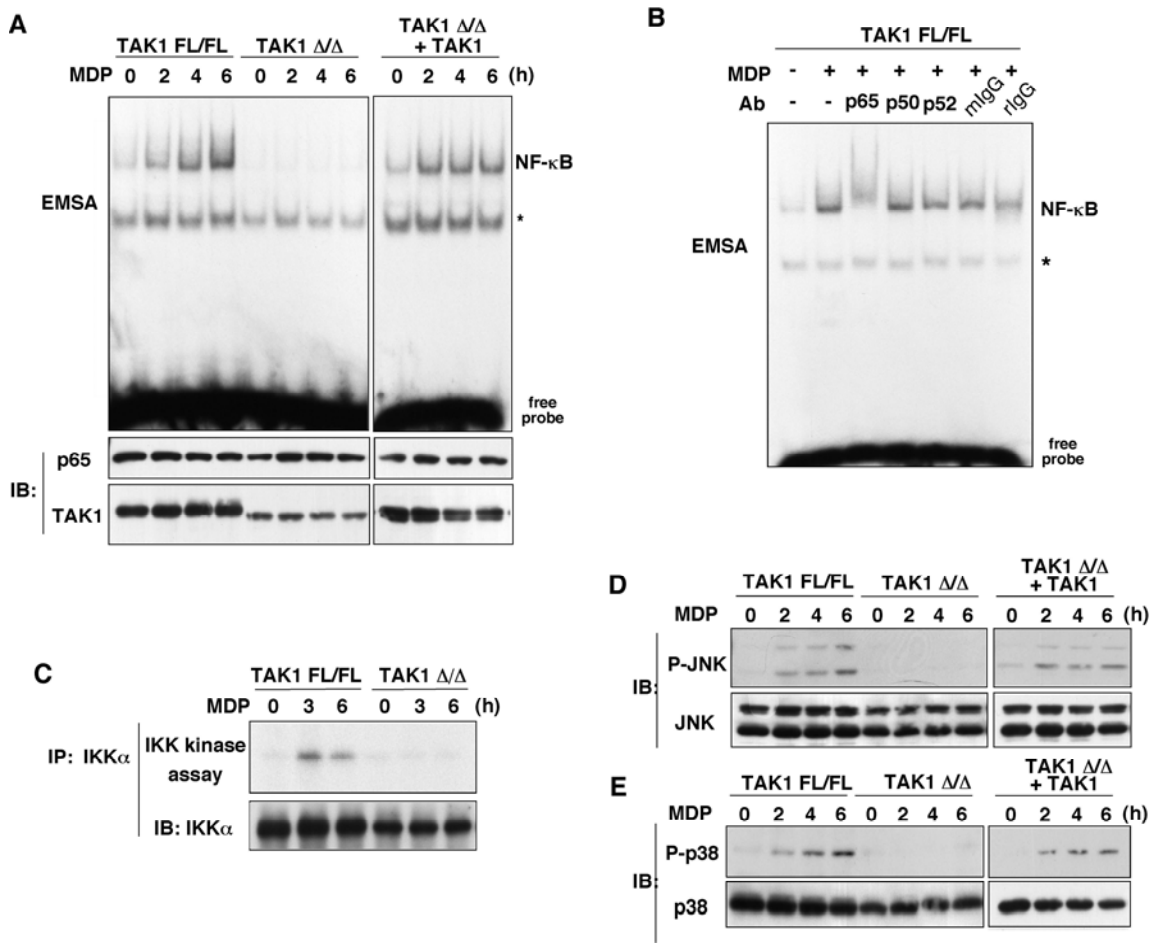


Fig. 2. MDP-induced activation of NF-κB, JNK and p38 is impaired in TAK1 Δ/Δ keratinocytes.

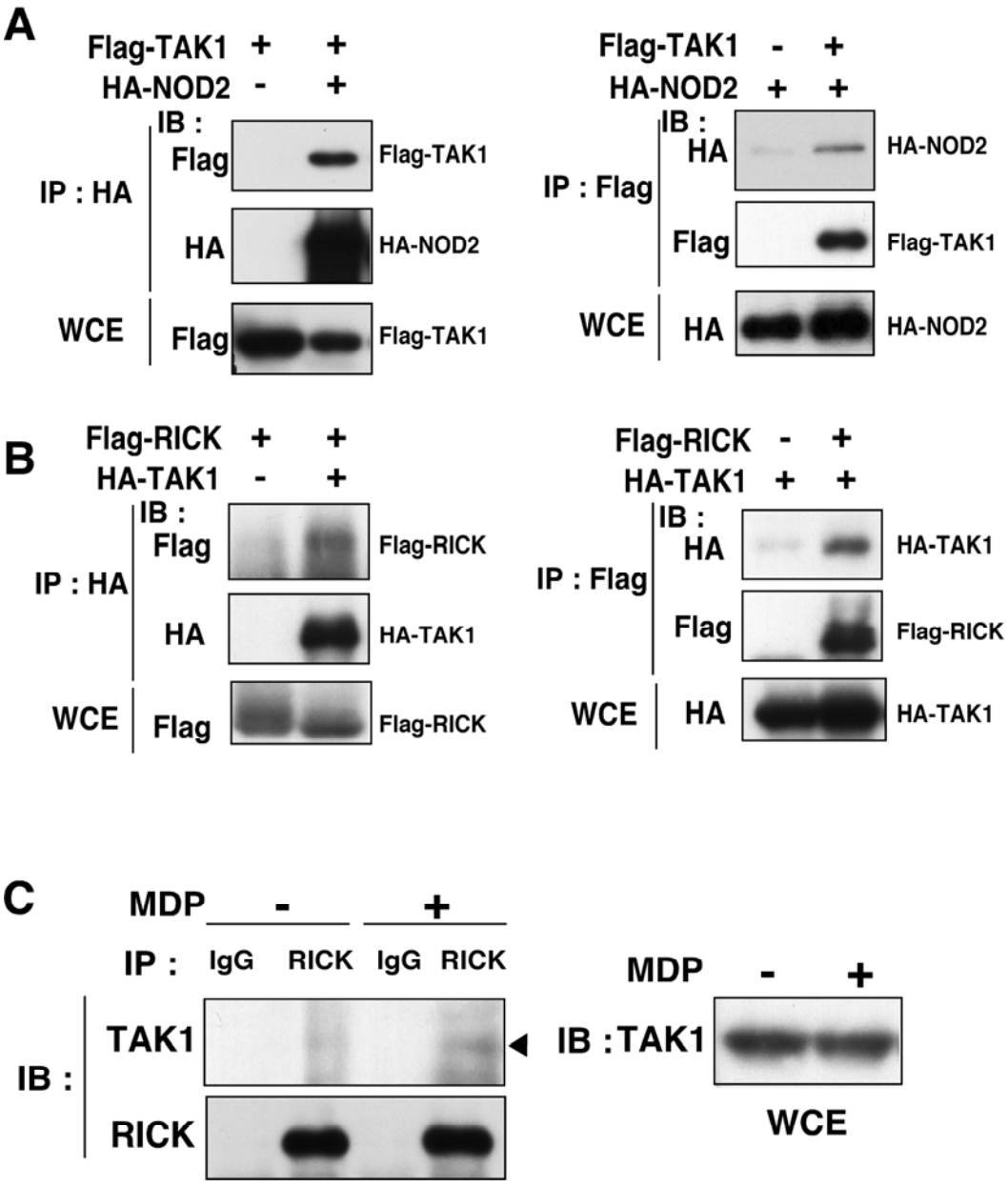


Fig. 3. NOD2 and RICK interact with TAK1.

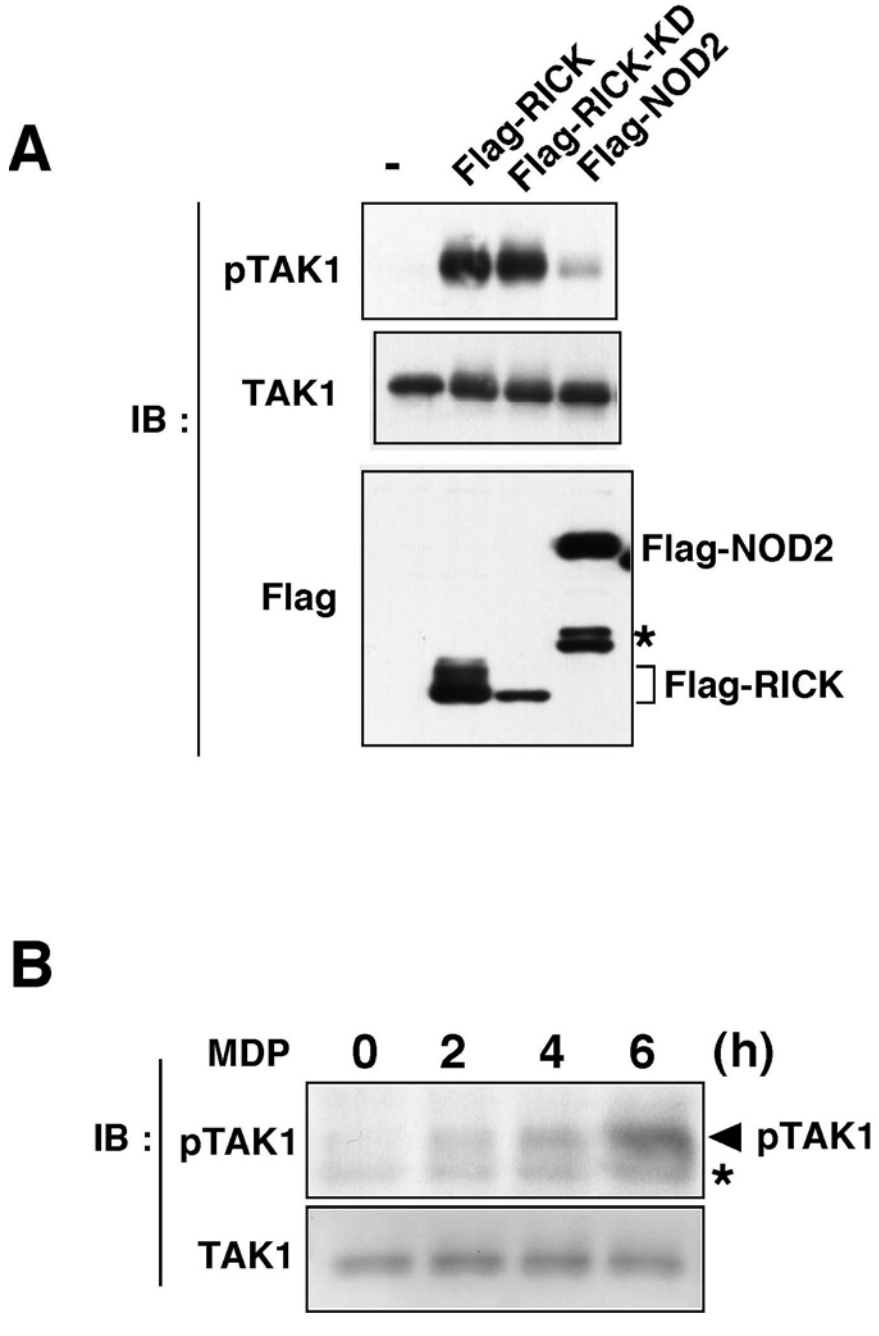


Fig. 4. NOD2 and RICK activate TAK1.

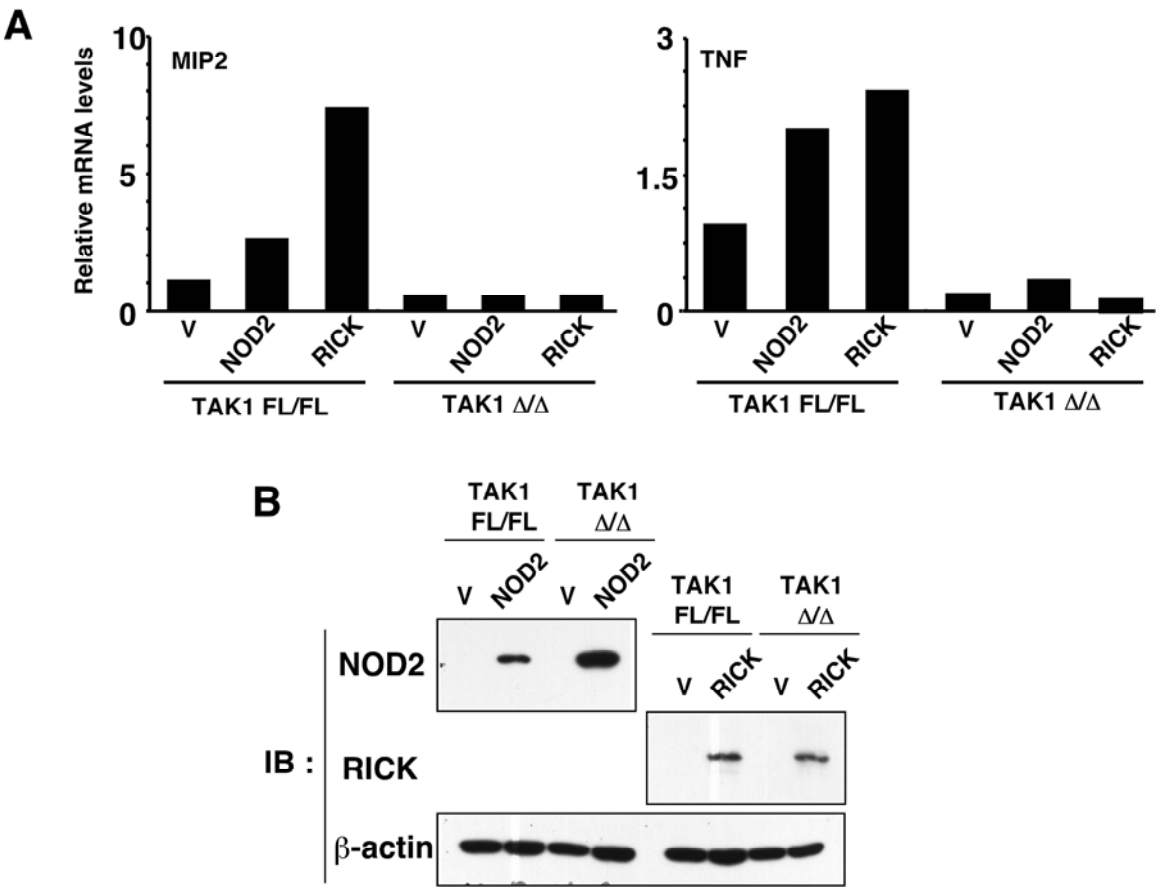


Fig. 5. NOD2 and RICK induce innate immune response in a TAK1 dependent manner.

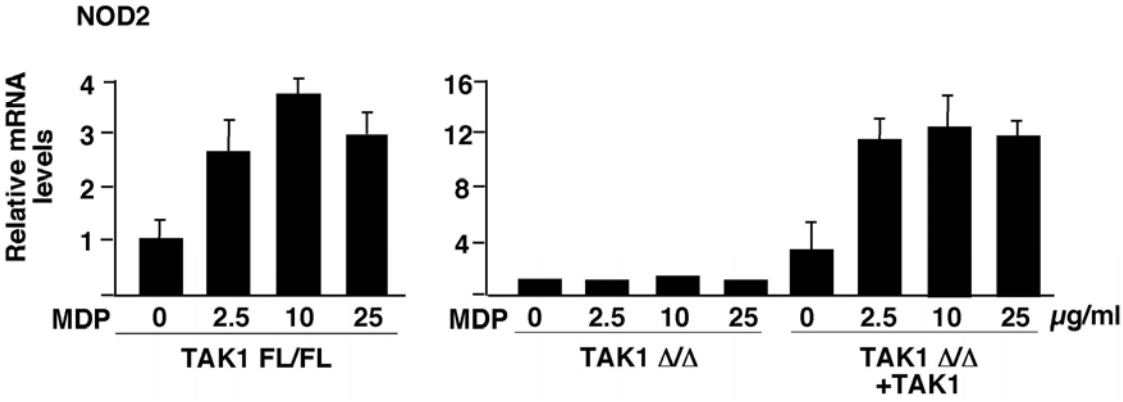


Fig. 6. TAK1 regulates NOD2 expression.

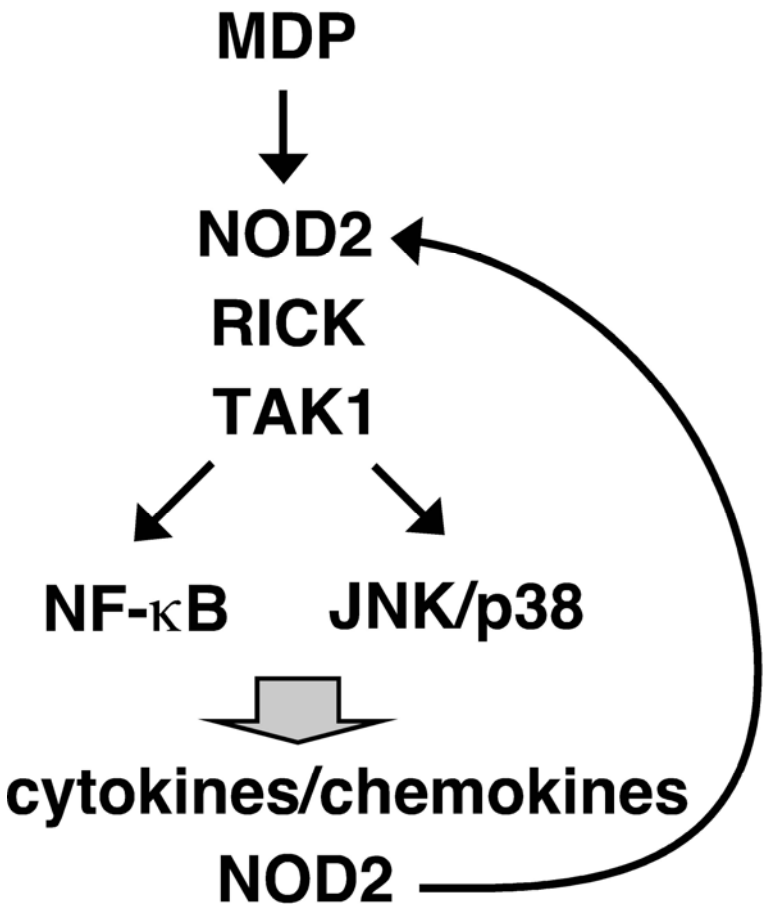


Fig. 7. Model of NOD2 signaling pathway.

SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

Immunocytochemistry- Keratinocytes were plated on Lab-Tek II Chamber Slide (Nalge Nunc International) and treated with MDP or TNF. Cells were fixed with ice-cold 10% formalin and dehydrated by 50% Methaon/50% Acetone. After blocking with 10% bovine serum for 30 min, subcellular localization of p65 was examined by anti-p65 antibody, which was visualized by Cy3-labeled anti-mouse IgG (GE Healthcare).

Cells- TNF receptor I knockout (TNFRI -/-) keratinocytes were isolated from TAK1 FL/FL TNFRI -/- mice(Omori et al., 2006). TNFR-/- mice were from Jackson Lab(Pfeffer et al., 1993). TNFRI -/- keratinocytes were spontaneously immortalized.

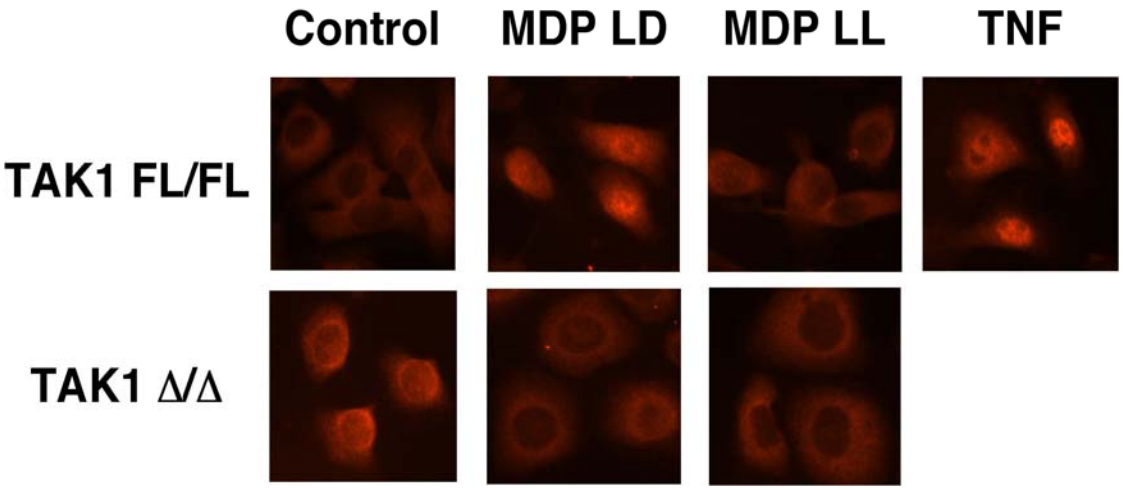
FIGURE LEGENDS

Supplementary Figure 1. Nuclear localization of NF-κB

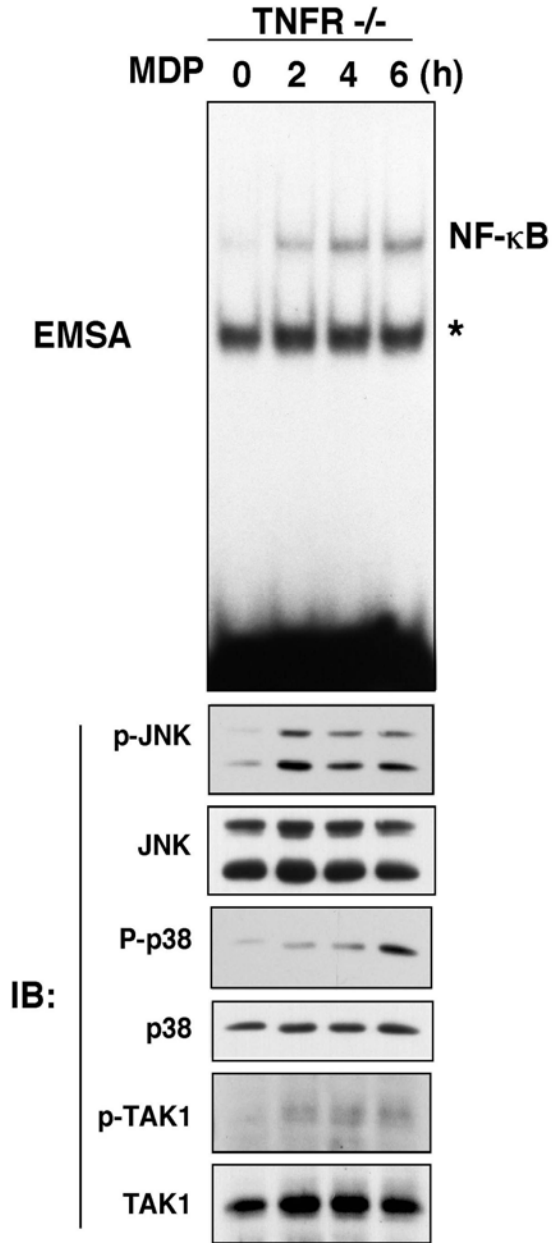
TAK1 FL/FL and TAK1 Δ/Δ keratinocytes were treated with MDP (10 μg/ml) for 6 h, TNF (20 ng/ml) for 15 min or left untreated. NF-κB localization was visualized by the immunofluoresence staining with anti-p65.

Supplementary Figure 2. MDP-induced activation of NF-κB, JNK, p38 and TAK1 in TNFR -/- keratinocytes

TNFR -/- keratinocytes were stimulated with MDP (10 μg/ml) for indicated times. Whole cell extracts were harvested from treated cells and the NF-κB-DNA binding activity was examined by EMSA. The whole cell extracts were also subjected to immunoblotting using phospho-JNK, JNK, phospho-p38, p38, phospho-TAK1 (Thr-187), and TAK1 antibodies. Asterisk indicates a non-specific band.



Supplementary Figure 1. MDP-induced nuclear translocation of p65 is impaired in TAK1Δ/Δ keratinocytes.



Supplementary Figure 2. MDP-induced activation of NF-κB, JNK, p38 and TAK1 in TNFR Δ/Δ keratinocytes

Manuscript II

Intestinal epithelial-derived TAK1 signaling is essential for cytoprotection against chemical-induced colitis

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Running title: TAK1 role in the intestine

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* This manuscript has been submitted to *PLoS ONE* and is in the format for that journal.

ABSTRACT

Background

We have previously reported that intestinal epithelium-specific TAK1 deleted mice exhibit severe inflammation and mortality at postnatal day 1 due to TNF-induced epithelial cell death. Although deletion of TNF receptor 1 (TNFR1) can largely rescue those neonatal phenotypes, mice harboring double deletion of TNF receptor 1 (TNFR1) and intestinal epithelium-specific deletion of TAK1 (TNFR1KO/TAK1^{IE}KO) still occasionally show increased inflammation. This indicates that TAK1 is important for TNF-independent regulation of intestinal integrity.

Methodology/Principal Findings

In this study, we investigated the TNF-independent role of TAK1 in the intestinal epithelium. Because the inflammatory conditions were sporadically developed in the double mutant TNFR1KO/TAK1^{IE}KO mice, we hypothesize that epithelial TAK1 signaling is important for preventing stress-induced barrier dysfunction. To test this hypothesis, the TNFR1KO/TAK1^{IE}KO mice were subjected to acute colitis by administration of dextran sulfate sodium (DSS). We found that loss of TAK1 significantly augments DSS-induced experimental colitis. DSS-induced weight loss, intestinal damage and inflammatory markers were significantly increased in TNFR1KO/TAK1^{IE}KO mice compared to the TNFR1KO control mice. Apoptosis was strongly induced and epithelial cell proliferation was decreased in the TAK1-deficient intestinal epithelium upon DSS exposure. These suggest that epithelial-derived TAK1 signaling is important for cytoprotection and repair against injury.

Finally, we showed that TAK1 is essential for interleukin 1- and bacterial components-induced expression of cytoprotective factors such as interleukin 6 and cycloxygenase 2.

Conclusions

Homeostatic cytokines and microbes-induced intestinal epithelial TAK1 signaling regulates cytoprotective factors and cell proliferation, which is pivotal for protecting the intestinal epithelium against injury.

INTRODUCTION

The intestinal epithelium is a single cell layer that separates lamina propria immune cells from luminal components including food antigens and commensal or pathogenic bacteria [1,2]. The integrity of the intestinal epithelium is essential for the maintenance of intestinal homeostasis. The disruption of intestinal barrier results in hyper-activation of mucosal immune cells, which eventually could lead to the development of chronic inflammatory diseases such as inflammatory bowel disease (IBD) in a susceptible host. Although the etiology of IBD has not been fully elucidated, it is well known that IBD is closely associated with an abnormal response to the normal gut flora caused by the loss of intestinal barrier [3,4]. Maintenance of this barrier depends on tight regulation of epithelial cell proliferation and apoptosis [5]. It has been demonstrated that commensal bacteria-derived signaling is important for intestinal barrier maintenance following chemical-induced injury to intestinal epithelium [6]. Ablation of MyD88, a common signaling intermediate of bacteria-derived signaling pathways, causes dysregulated barrier function [6]. Commensal bacterial components can initiate MyD88 signaling in epithelial cells as well as immune cells in the intestine, both of which can modulate epithelial cell proliferation and survival. It has been well studied that homeostatic activation of immune cells is important for production of cytokines and growth factors, which critically regulate epithelial cell survival and proliferation [7]. However, because epithelial cells are constantly exposed to bacteria and several Toll-like receptors are downregulated [8], it is still unclear whether bacteria-induced signaling in epithelial cells is also important for barrier maintenance in the intestine.

Transforming growth factor-β activated kinase 1 (TAK1) is a member of the MAPKKK family and plays an essential role in tumor necrosis factor (TNF), interleukin 1 (IL-1), and Toll-like receptor (TLR) signaling pathways [9-11]. Furthermore, we and others have recently identified that TAK1 is a central mediator of NOD-like receptor (NLR) signaling [12-14]. In response to proinflammatory cytokines or TLR/NLR ligands, TAK1 activates both NF-κB and AP-1 pathways, which lead to activation of innate immune response. TAK1 deficiency results in embryonic lethality, therefore, conditional knockout systems have been used to reveal the in vivo roles of TAK1. TAK1 is required for T and B cell differentiation and for their activation [10,15-17]. Thus, TAK1 is essential for proinflammatory signaling in vivo as well as in cultured cells.

In addition to this proinflammatory function in immune cells, we have recently demonstrated that TAK1 has a completely opposite role in the skin and intestinal epithelium. TAK1 is important for preventing inflammation in the skin and intestinal epithelium [18,19]. We reported that intestinal epithelium-specific TAK1 deleted mice ($TAK1^{IE}KO$) spontaneously developed intestinal inflammation [18]. The TAK1-deficient intestinal epithelium underwent significant apoptosis that is induced by endogenously expressed TNF in the intestine. The phenotype was strongly attenuated by crossing TNF receptor 1 knockout (TNFR1-/-) mice, indicating that TNF is the major cause of intestinal epithelial cell apoptosis and inflammation. However, a fraction (40-50%) of the double knockout mice (TNFR1KO/ $TAK1^{IE}KO$) developed ileitis and colitis around the age of 14-17-days-old [18], suggesting that TAK1 is also important for preventing TNF-independent inflammation. Because TAK1

is deleted only in epithelial cells in TNFR1KO/ TAK1^{IE}KO mice, this protective signal is derived from epithelial cells. As described above, TAK1 is activated by cytokines and TLR/NLR ligands. Taken together, we hypothesize that intestinal epithelial TAK1 is activated by cytokines and TLR/NLR ligands and maintains the epithelial barrier. In this study, we tested this hypothesis by analyzing TNFR1KO/TAK1^{IE}KO mice. The inflammatory conditions in TNFR1KO/ TAK1^{IE}KO mice at the late neonatal stage were transient and widely varied in each mouse [18]. Although some TNFR1KO/ TAK1^{IE}KO mice lost weight and developed ileitis and colitis at 14-17 days as reported previously [18], more than half of TNFR1KO/ TAK1^{IE}KO mice grew normally and were indistinguishable from TNFR1KO control mice after the age of 6-weeks-old. We speculate that the inflammatory conditions at 14-17 days is associated with stress caused by increased bacterial microflora in the intestine when the mice start having solid food [20]. Thus, we postulate that TAK1 signaling is important for maintenance of intestinal barrier under stress conditions. In order to study the TNF-independent role of TAK1 in the intestinal barrier, we utilized adult TNFR1KO/ TAK1^{IE}KO mice and examined sensitivity to chemical-induced acute colitis. We found that TAK1 deficiency caused hypersensitivity to chemical-induced colitis involving increased apoptosis and dysregulated cell proliferation, suggesting that epithelial TAK1 signaling is cytoprotective.

RESULTS

Intestinal epithelium-specific deletion of TAK1 enhances DSS-induced intestinal damage

In order to investigate the hypothesis that epithelial-derived TAK1 signaling is important for maintenance of intestinal barrier under stress conditions, we exposed adult TNFR1KO/ TAK1^{IE}KO mice to dextran sulfate sodium (DSS), a chemical disruptor of the intestinal epithelium. TNFR1KO/TAK1^{IE}KO and littermate control TNFR1KO mice were fed with 2.5% of DSS and sacrificed at Day 5. The severity of DSS-induced colitis was monitored by daily body weight and observation of clinical signs of acute inflammation such as rectal bleeding. DSS treatment caused little weight loss or no apparent injury phenotype in the control TNFR1KO mice. In contrast, the double mutant TNFR1KO/TAK1^{IE}KO mice showed significant weight loss and rectal bleeding starting from Day 4 (Fig. 1A and data not shown). DSS-induced colon shortening, a marker for intestinal damage, was also observed in TNFR1KO/TAK1^{IE}KO mice (Fig. 1B and C). To further evaluate the role of TAK1 in DSS-induced intestinal injury and inflammation, histological and biochemical analyses were performed. Without DSS treatment, the colon from TNFR1KO/TAK1^{IE}KO showed only slight signs of inflammatory conditions and was almost indistinguishable from TNFR1KO control mice (Fig. 2A and B). After 5 days of DSS treatment, TNFR1KO/TAK1^{IE}KO colon underwent complete loss of crypt architecture and severe ulceration, whereas control mice showed only mild damage characterized by epithelial hyperplasia and immune cell infiltration (Fig. 2A and B). The structural damages in TAK1-deficient colon might be associated with severe inflammation. To verify this possibility, the expression levels of inflammatory genes were measured. After 5 days of DSS treatment, the distal colons were

isolated and the mRNA levels of chemokine MIP2 (IL-8 in human) and chemotactic factor S100A9 were determined by the real-time PCR. Both MIP2 and S100A9 expression were greatly upregulated in TAK1-deficient colon, presumably due to an increased activity of infiltrated immune cell (Fig. 2C). These data indicate that TAK1 signaling in the intestinal epithelium protects against DSS-induced intestinal injury and acute colitis.

TAK1 is required for intestinal epithelial cell survival following DSS exposure

In order to protect the intestinal epithelium from DSS-induced injury, following biological processes are essential; one is suppression of apoptosis; and another is cell proliferation to repair DSS-induced lesions in the intestinal epithelium. We first examined apoptosis in TNFR1KO/TAK1^{IE}KO and control TNFR1KO mice. In the DSS colitis model, those biological events precede clinical signs such as weight loss or rectal bleeding. Therefore, we examined apoptosis in colonic epithelial cells in mice exposed to DSS for 3 days, a time point preceding clinical signs of colitis (Day 4) in the double mutant mice (Fig. 1A). TUNEL staining was performed to assess apoptosis, and the results revealed that DSS exposure induced more pronounced apoptosis in the TAK1-deficient intestinal epithelium compared to the control mice (Fig. 3A and B). In order to confirm this data, the distal colon was isolated from DSS treated mice and the proteins lysates were analyzed for caspase-3 processing using Western blot. Consistent with the TUNEL result, the amount of cleaved caspase-3 was greatly increased by the administration of DSS in the TAK1-deficient colon, but not in control colon (Fig. 3C). These data indicate that TAK1 is required for suppression of apoptosis against DSS-induced intestinal damage.

Loss of TAK1 dysregulates proliferation of the intestinal epithelium

We next examined epithelial cell proliferation. Intestinal homeostasis is maintained by proliferation and differentiation of the intestinal epithelial cells along the crypt axis. It has been reported that commensal bacteria-mediated TLR signaling is important for homeostatic level of proliferation of epithelial cells. Depletion of commensal bacteria or loss of TLR signaling abnormally upregulate the steady-state level of epithelium proliferation and lead to increased sensitivity to chemical and radiation-induced intestinal injury [6]. TAK1 is a key mediator of bacteria-induced signaling pathways, TLR and NLR signalings. Thus, we hypothesize that TAK1-deficient intestinal epithelial cells do not respond to commensal bacteria, which may dysregulate cell proliferation along the crypt axis. This may be associated with increased sensitivity to DSS-induced injury. To address this possibility, we examined the base line proliferating status in the intestinal epithelium of control TNFR1KO and TNFR1KO/TAK1^{IE}KO mice. Proliferating cells were labeled by 5-bromo-2'-deoxyuridine (BrdU) that was injected 2 h prior to harvesting distal colon. Immunohistochemical analysis was performed to detect BrdU labeled cells. Interestingly, we found significantly increased proliferating cells in TAK1 deficient intestinal epithelium (Fig. 4A and B). Of note, whereas proliferating cells were restricted in the bottom regions of the crypts (stem cell area) in control TNFR1KO mice, a large fraction of proliferating cells was found in the middle or upper region of the crypts in the TNFR1KO/TAK1^{IE}KO mice It is known that DSS-induced injury induces compensatory cell proliferation responses in mice [6]. Mice were fed with 2.5% DSS for 3 days and proliferating cells were examined by BrdU.

Cell proliferation was not altered by DSS treatment in control TNFR1KO mice (Fig. 4A and B), which is consistent with the results showing no severe injury in the control mice under 2.5% DSS treatment (Fig. 1). In contrast, we found a dramatic reduction of cell proliferation in TAK1-deficient intestinal epithelium after DSS exposure (Fig. 4A and B). This indicates that reparative cell proliferation is impaired in TAK1-deficient epithelial cells. Taken together, intestinal epithelium-specific TAK1 deficiency results in the disruption of homeostatic epithelial cell proliferation and impairs compensatory epithelial cell proliferation after DSS-induced injury, which might be responsible for greater sensitivity to the injury-mediated inflammation.

TAK1 is required for proinflammatory cytokine- and bacterial components-induced cytoprotective gene expression

We next investigated the mechanism by which epithelial TAK1 signaling protects against DSS-induced injury. We examined cell proliferation and cell survival in cultured wild type and TAK1-deficient cells. Because primary culture of intestinal epithelial cells is technically difficult due to detachment-induced apoptosis (anoikis) during the isolation procedure [21], we utilized skin keratinocytes and dermal fibroblasts as model systems. We found that TAK1-deficient keratinocytes and fibroblasts did not show impaired cell proliferation or increased cell death under unstimulated conditions or stress conditions such as DSS treatment (data not shown). Therefore, TAK1 signaling does not cell-autonomously regulate cell death or proliferation in response to stress conditions including DSS exposure. We postulate that epithelial TAK1 signaling might induce production of cytoprotective factors that modulate

other cell types and eventually facilitate epithelial cell survival and proliferation. To address this hypothesis, we tested whether TAK1 is required for expression of cytoprotective factors, interleukin 6 (IL-6) and cycloxygenase 2 (COX2), following exposure of activators of TAK1 signaling, IL-1, LPS and the NOD2 ligand, muramyl dipeptide (MDP). We found that TAK1 is required for IL-1-induced IL-6 and COX2 expression in fibroblasts (Fig. 5A). LPS also induced IL-6 expression in a TAK1 dependent manner in fibroblasts (Fig. 5A). In wild type keratinocytes, both IL-1 and LPS failed to induce IL-6 and COX2 expression (data not shown), because keratinocytes do not strongly respond to LPS and IL-1 stimulation. However, MDP strongly upregulated the levels of IL-6 and COX2 in wild type keratinocytes, a process completely abolished by the loss of TAK1 (Fig. 5B). These data suggest that epithelial TAK1 signaling is essential for production of cytoprotective factors.

DISCUSSION

In this study, we demonstrated that intestinal epithelium-derived TAK1 signaling plays a pivotal role in preventing injury-induced intestinal inflammation. In the absence of TAK1-derived signaling, the intestinal epithelium is exquisitely sensitive to DSS-induced injury, a process involving increased epithelial cell apoptosis and reduced regenerative proliferative responses. This pathologic response further induces damage-associated inflammation. In addition, ablation of TAK1 leads to an increased expansion of the epithelial cell proliferative zone under steady state conditions, and causes impaired reparative proliferation after injury. Finally, we showed that loss of TAK1 abolishes production of cytoprotective factors in response to proinflammatory cytokines and bacterial components. Taken together, we propose that TAK1 is essential for preventing injury-associated intestinal inflammation by the following two mechanisms. One is that TAK1 is responsible for maintenance of homeostatic proliferation of the intestinal epithelium, and that dysregulated cell proliferation causes hypersensitivity to cytotoxic insults. Intestinal epithelial cells are originated from stem cells located in the bottom of crypts [2]. The intestinal stem cell-derived epithelial cells are proliferating until terminally differentiated. The population of those proliferating cells is tightly regulated. We found that the steady-state level of proliferation is abnormally upregulated in the TAK1-deficient intestinal epithelium. Although increased proliferating cells may be beneficial for tissue repair, proliferating cells are usually hypersensitive to cytotoxic insults. It is well exemplified in the case of cancer therapy. The gastrointestinal tract, oral mucosa and hematopoietic cells, which are all highly proliferative, are the most sensitive to chemotherapy. Another mechanism is that epithelial TAK1 signaling induces

cytoprotective factors, and that ablation of this pathway causes increased apoptosis and degeneration of the intestinal barrier. We found that TAK1 is essential for IL-1, LPS and MDP-induced expression of IL-6 and COX2, both of which are known to play an important role in protecting the intestinal epithelium against DSS-induced injury [22-25]. Commensal bacteria and homeostatic cytokines activate TAK1 in the intestinal epithelium, and the activated TAK1 induces cytoprotective factors including IL-6 and COX2. We propose that this pathway is essential for the maintenance of intestinal barrier integrity.

TAK1 can activate two major transcription factors, NF-κB and AP-1. NF-κB is a key transcription factor implicated in the regulation of both IL-6 and COX2 gene expression [26]. Pharmacological blockage of NF-κB inhibits production of COX2 in intestinal epithelial cells and worsens DSS-induced colitis [23]. Therefore, TAK1-NF-κB signaling pathway in intestinal epithelial cells may be the major pathway for maintenance of the intestinal barrier. However, intestinal epithelial-specific NEMO (IKKγ) deletion mice, which is deficient in NF-κB activation, develop TNF-dependent but not TNF-independent intestinal inflammation [27]. Therefore, whereas TAK1-NF-κB pathway participates in prevention of TNF-induced cell death and inflammation, TAK1-dependent but NF-κB-independent pathways are also important for the intestinal barrier function.

How does TAK1 deletion cause increased cell proliferation in the intestinal epithelium? It has been established that proliferation of the intestinal epithelium depends on the concerted

action of several factors including Wnt (positive regulator of cell cycle) and TGF-β signaling (negative regulator of cell cycle) [2,28-30]. TAK1 is involved in both Wnt (negatively) and TGF-β (positively) signaling pathways [31-34]. Therefore, TAK1 deletion may affect epithelial cell proliferation through dysregulating Wnt or TGF-β signaling. However, we note that intestinal epithelial-specific deletion of TAK1 does not alter epithelial cell proliferation and differentiation at least during embryogenesis [18]. Therefore, TAK1 is not a major signaling intermediate of Wnt and TGF-β signaling in the intestinal epithelium but may somewhat modulate Wnt- and TGF-β-dependent balance of epithelial cell proliferation.

It is noteworthy that TLR- or MyD88-deficient mice and commensal bacteria-depleted mice showed very similar phenotypes observed in our TNFR1KO/TAK1^{IE}KO mice that are characterized by increased susceptibility to DSS-induced injury, dysregulated steady-state levels of cell proliferation and impaired production of cytoprotective factors [6]. Rakoff-Nahoum et al. proposed that commensal bacteria-induced signaling is important for maintenance of the intestinal barrier. Failure to initiate activation of these signaling pathways increases susceptibility of the intestinal epithelium to chemical or radiation-induced injury. However, because these studies have used germline knockout of TLR4, TLR2 and MyD88, it is not clear whether epithelial cell- or immune cell-derived signaling is responsible for maintaining barrier integrity. Our results demonstrated that epithelial-derived TAK signaling is important for the maintenance of intestinal homeostasis. It should also be of interest to determine the contributions of immune cell-derived TAK1 signaling to the barrier maintenance.

Loss of intestinal epithelial barrier is associated with chronic inflammatory diseases such as inflammatory bowel diseases (IBD). Mutations in NOD2 gene are highly associated with IBD susceptibility [3]. Our results indicate that TAK1 signaling is essential for epithelial barrier maintenance. TAK1 is a key mediator of NOD2 signaling [14]. Taken together, loss of NOD2-TAK1 signaling in the intestinal epithelium may be one of the causes to increase susceptibility of IBD. However, further investigation will be needed to clarify the relationship between TAK1 and the pathogenesis of IBD.

METERIALS AND METHODS

Mice and Induction of DSS Induced-Colitis

TAK1^{IE}KO/TNFR1$^{-/-}$ mice and control TAK1$^{FL/FL}$/TNFR1$^{-/-}$ mice in C57BL6 background were generated as described in our previous study [18]. All genotypes were confirmed by genotyping genomic DNA. For inducing acute colitis, 6 to 8 weeks-old TNFR1KO/TAK1^{IE}KO mice and littermate control TNFR1KO (TAK1$^{FL/FL}$ TNFR1$^{-/-}$) were fed with 2.5 % of DSS. Mice were weighed daily and sacrificed at time points indicated. Mice were bred and maintained under specific pathogen-free conditions. All animal experiments were done with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Cells

Dermal fibroblasts were isolated from TAK1$^{FL/FL}$ mice. The cells were spontaneously immortalized and infected with pMX-puro-CRE retroviral vector to delete TAK1. Uninfected cells were removed by puromycin selection and the deletion of TAK1 was confirmed by immunoblotting. The cells were maintained in DMEM containing 10% bovine growth serum (HyClone) and 1% penicillin/streptomycin. TAK1 WT and KO keratinocytes were isolated from epidermis-specific TAK1 deletion mice and maintained in keratinocyte medium as described in our previous publication [19].

Histology and Immunohistochemical analysis

After 5 days of DSS treatment, distal colons were isolated and fixed in 10% formalin.

Paraffin-embedded sections were stained with H&E for histological analysis. Sections were scored in a blinded fashion on a scale from 0 to 4, based on the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural distortion, as previously described [35]. Apoptotic cells were detected after 3 days of DSS treatment using the DeadEnd colorimetric TUNEL system (Promega) according to the manufacturer's instructions. To detect proliferating cells, mice were injected with 0.1 mg/g of BrdU 2 h prior to sacrifice. Immunohistochemical analysis was performed using anti-BrdU antibody (Becton Dickinson).

Real-time PCR analysis

Total RNA was prepared from distal colons or cells using RNeasy minikit (Qiagen). To obtain cDNA, 200 ng of each RNA samples were reverse-transcribed using TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR analysis was performed using the ABI PRISM 7300 sequence detection system (Applied Biosystems). All samples were normalized by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Immunoblotting

After DSS exposure, distal colons were harvested and homogenized in an extraction buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 100 U/ml aprotinin, 0.5% Triton X-100). Proteins from cell lysates were electrophoresed by SDS-PAGE and

transferred to Hybond-P (GE Healthcare). The membranes were immunoblotted with a polyclonal antibody against caspase-3 (Cell signaling) and a mouse antibody against β-actin (Sigma-Aldrich). Bound antibodies were visualized with HRP-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Statistical analysis

Statistical comparisons were made using paired or independent two-tailed Student's t tests assuming equal variance and two-tailed Welch's t tests assuming unequal variance.

ACKNOWLEDGMENT

We thank B.J. Welker and M. Mattmuler for support.

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FIGURE LEGENDS

Fig. 1. Loss of TAK1 in the intestinal epithelium results in worsened clinical signs of DSS-induced colitis.

A Intestinal epithelium-specific TAK1/TNFR1 double knockout mice (TNFR1KO/TAK1^{IE}KO) and control TNFR1 single knockout mice (TNFR1 KO) were fed with 2.5% DSS. Mice were weighed daily to access weight loss. Each point represents a mean value ± SE. (N=8 per group, * = $p<0.05$, ** = $p<0.01$).

B Colons of TNFR1KO/TAK1^{IE}KO and TNFR1 KO were removed at days 0 and 5 of 2.5% DSS administration and the colon length was measured. Each bar represents a mean value ± SE (N=6 for water control; N=9 for DSS, ** = $p<0.01$, NS=not significant).

C Photograph of representative colon at day 5 of DSS administration.

Fig. 2. Loss of TAK1 in the intestinal epithelium leads to severe epithelial damage and upregulates inflammatory gene expression in response to DSS.

A Histologic damage was scored from H&E staining sections of distal colon from TNFR1KO and TNFR1KO/TAK1^{IE}KO mice treated with water or 2.5% DSS for 5 days. Each bar represents a mean value ± SE (N=6 for water control; N=9 for DSS, ** = $p<0.01$, NS=not significant).

B Representative H&E staining. Scale bars, 100 μm.

C Total RNA was isolated from distal colon of TNFR1KO and TNFR1KO/TAK1^{IE}KO mice at day 5 of DSS administration and reverse transcribed. The expression of inflammatory genes was measured using quantitative real-time PCR. Each bar represents

a mean value ± SE (N=5 per group, ** = $p<0.01$).

Fig. 3. TAK1 protects the intestinal epithelium from apoptosis in response to DSS-induced damage.

A Distal colon was isolated from TNFR1KO and TNFR1KO/TAK1^{IE}KO mice treated with water or 2.5% DSS for 3 days. To detect apoptotic cells, TUNEL staining was performed and TUNEL positive cells were counted. Each bar represents a mean value ± SE (N=4 for water control; N=5 for DSS, ** = $p<0.01$, NS=not significant).

B Representative TUNEL staining.

C Distal colon was isolated from TNFR1KO and TNFR1KO/TAK1^{IE}KO mice treated with 2.5% DSS for 3 days. Cell extract was isolated and subjected to immunoblotting using caspase-3 antibody. β-actin was used for loading control.

Fig. 4. Loss of TAK1 disrupts homeostatic proliferation of the intestinal epithelium.

A Distal colon was isolated from TNFR1KO and TNFR1KO/TAK1^{IE}KO mice treated with water or 2.5% DSS for 3 days. Mice were injected with 0.1 mg/g of 5-bromo-2'-deoxyuridine (BrdU) 2 h prior to sacrifice. Immunostaining for BrdU was performed and BrdU-positive cells were counted. Each bar represents a mean value ± SE (N=5 per group, ** = $p<0.01$).

B Representative BrdU staining.

Fig. 5. TAK1 is required for the expression of protective factors in response to

proinflammatory cytokine or bacterial components.

A TAK1 WT and KO dermis fibroblasts (DF) were stimulated with 5 ng/ml of IL-1β or 2 μg/ml of LPS for 6 h. The expression of IL-6 and COX2 was examined by quantitative real-time PCR. mRNA levels were normalized with the levels of GAPDH. Relative mRNA levels were calculated using those of untreated TAK1 WT dermis fibroblasts. Data are mean ±S.E. of three independent samples and representative of three independent experiments with similar results.

B TAK1 WT and KO keratinocytes (KC) were stimulated with 10 μg/ml of MDP for 6 h. The expression of IL-6 and COX2 was examined as described above.

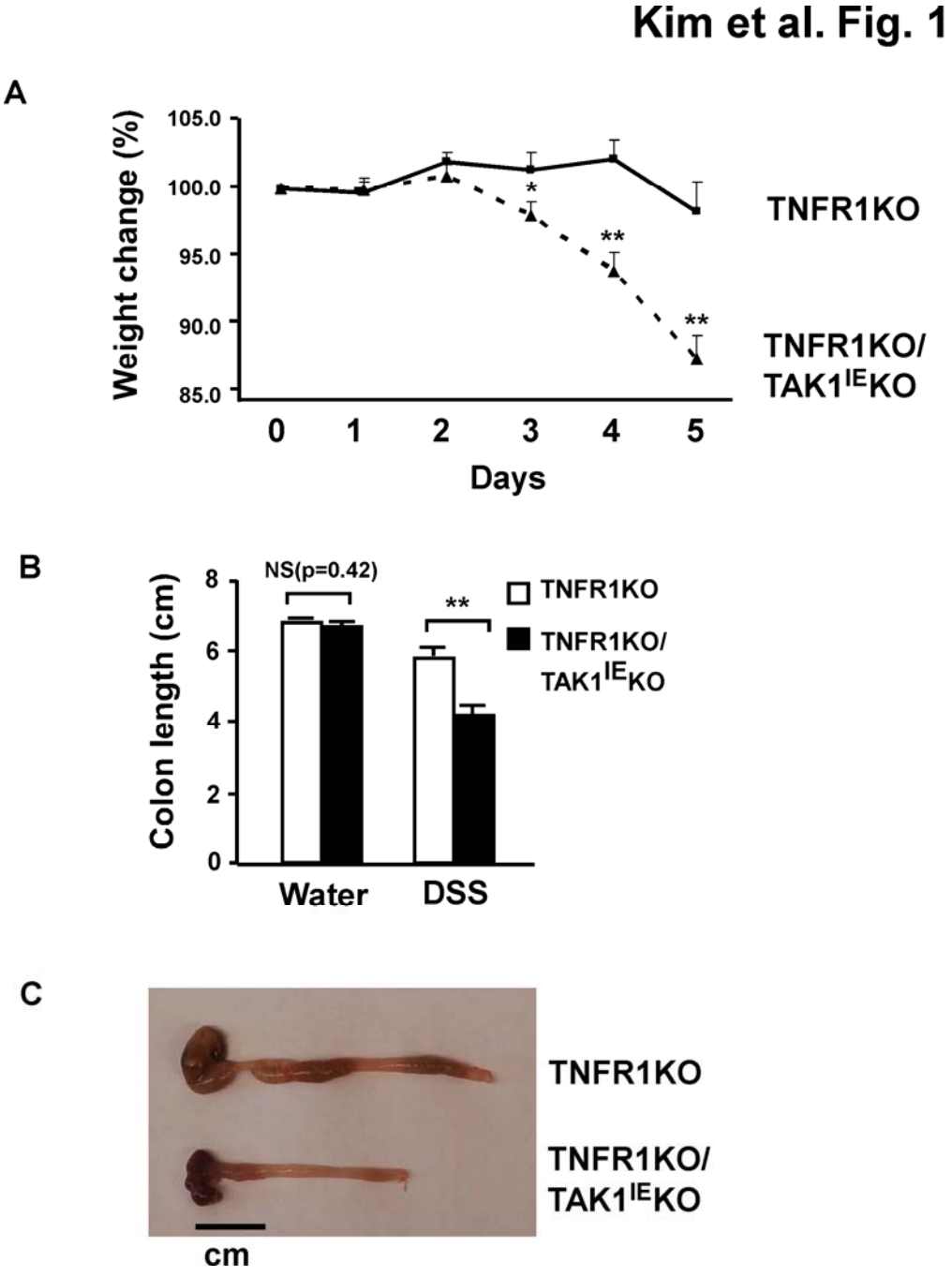

Fig. 1. Loss of TAK1 in the intestinal epithelium results in worsened clinical signs of DSS-induced colitis.

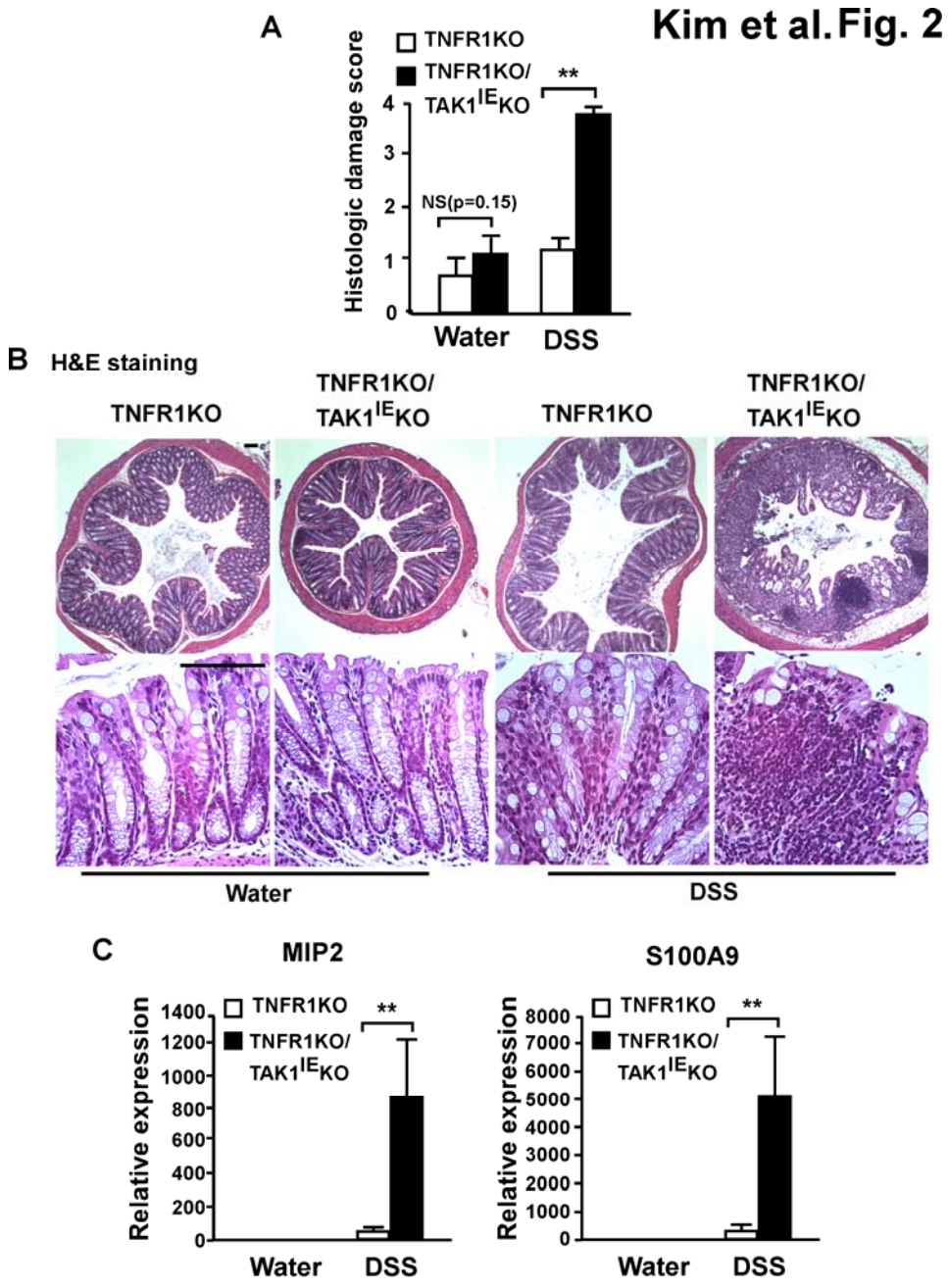

Fig. 2. Loss of TAK1 in the intestinal epithelium leads to severe epithelial damage and upregulates inflammatory gene expression in response to DSS.

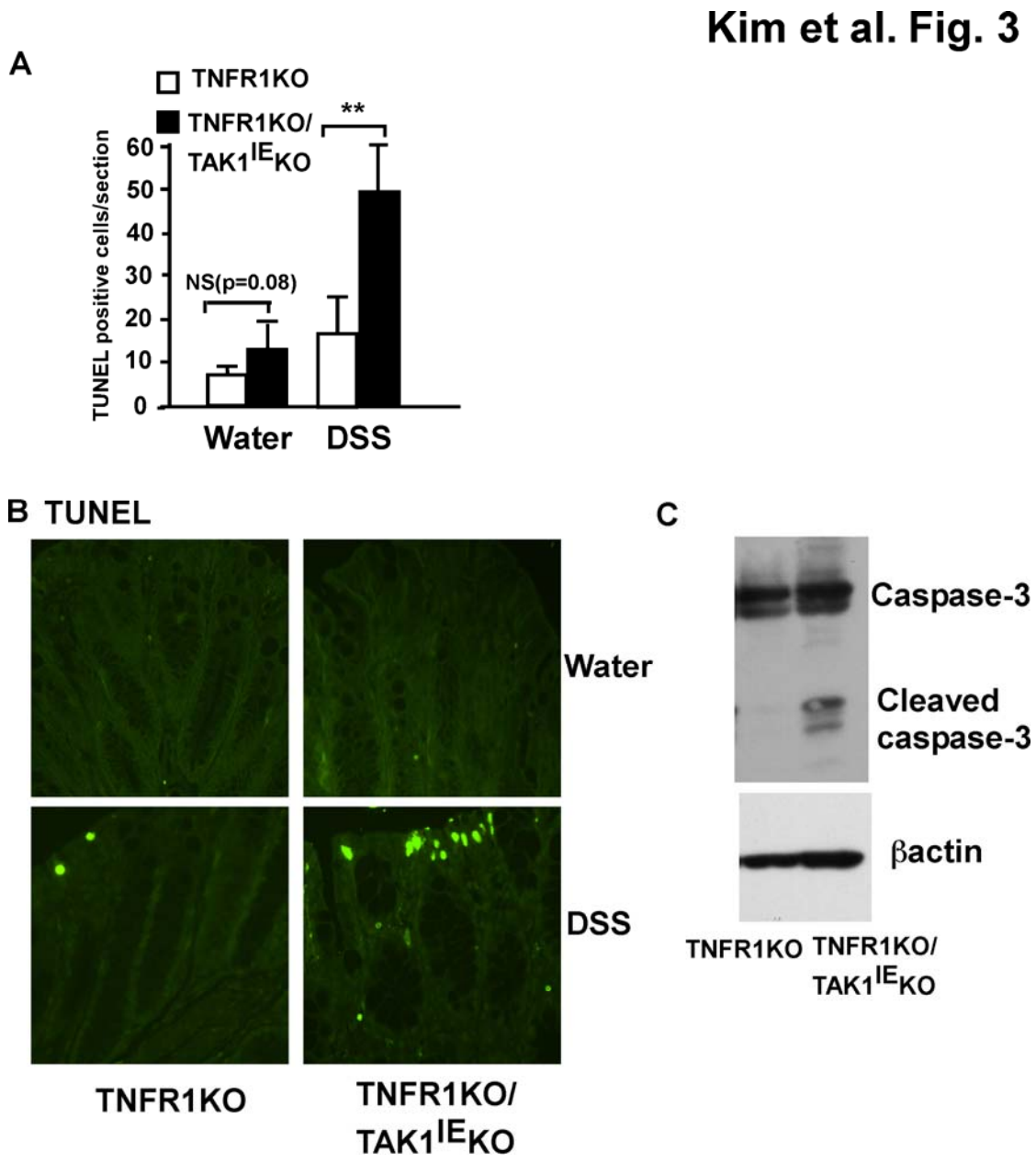

Fig. 3. TAK1 protects the intestinal epithelium from apoptosis in response to DSS-induced damage.

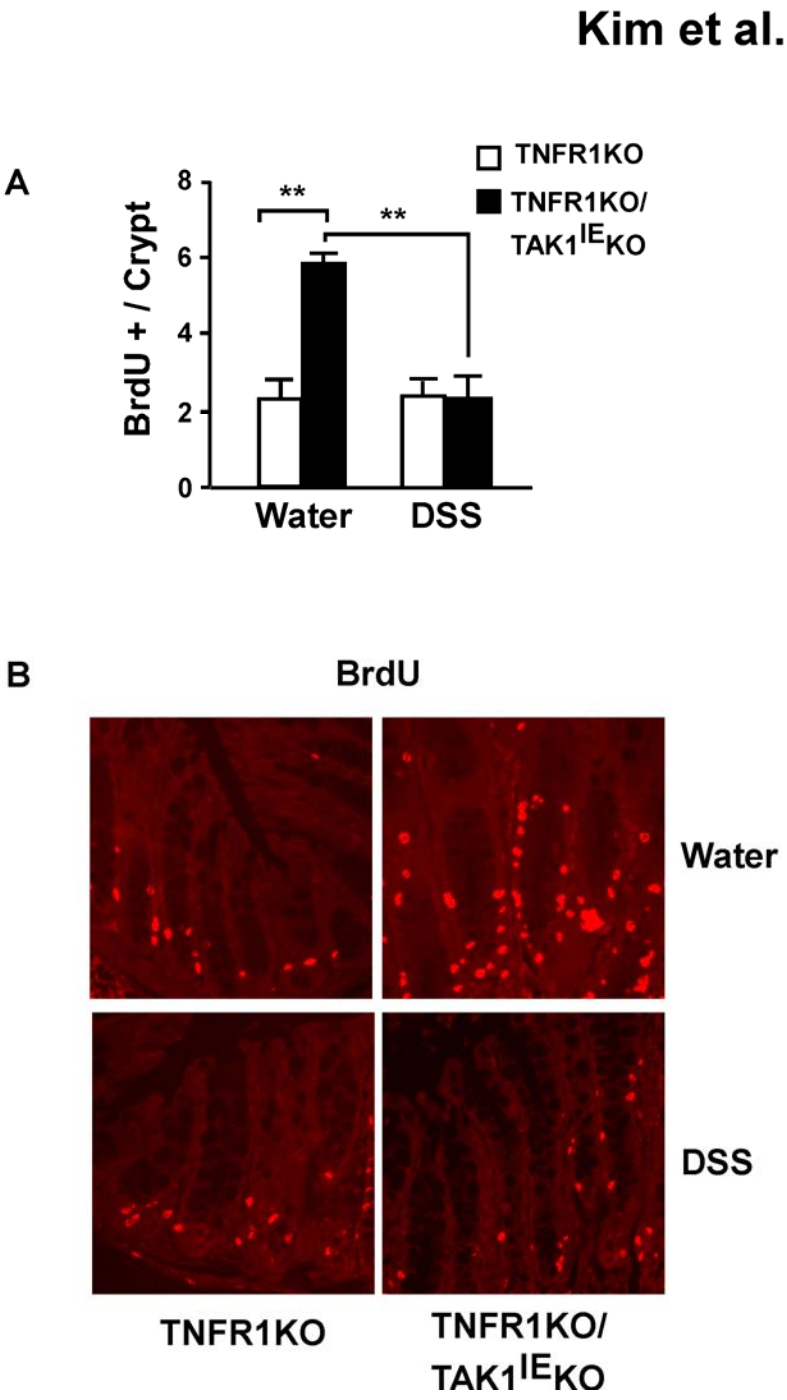

Fig. 4. Loss of TAK1 disrupts homeostatic proliferation of the intestinal epithelium.

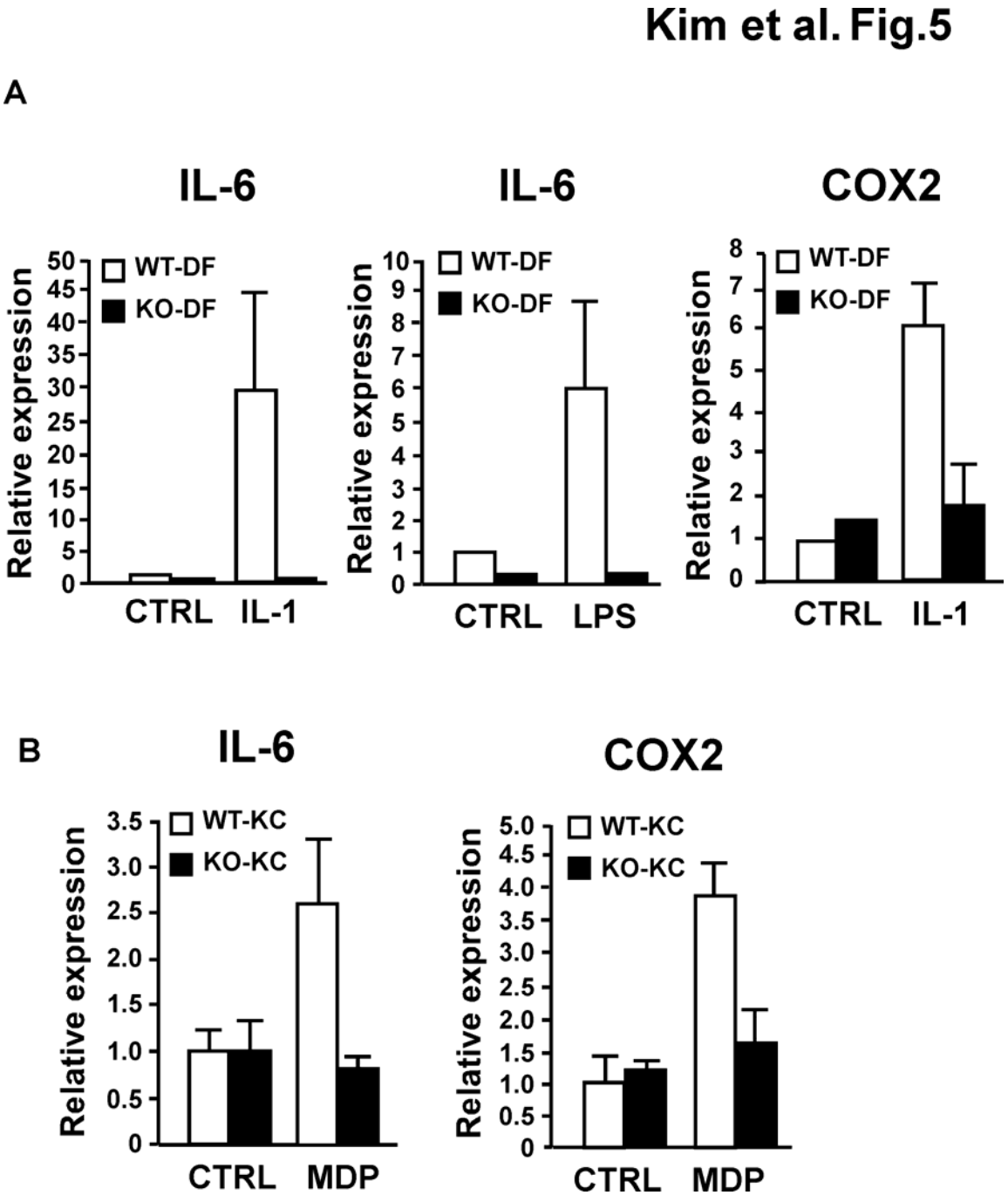

Fig. 5. TAK1 is required for the expression of protective factors in response to proinflammatory cytokine or bacterial components.

GENERAL DISCUSSION

(Some paragraphs are adapted from the manuscript I and II and modified)

1. The role of TAK1 in NOD2 signaling in epidermal cells

The innate immune system detects microbes by extracellular or endosomal Toll-like receptors (TLRs) and intracellular NOD-like receptors (NLRs). Upon detection of bacteria, those receptors activate NF-κB and MAPK pathways to elicit inflammatory responses. The molecular mechanisms by which TLRs activate those signaling cascades have been extensively studied, however the mechanisms of NLR-mediated innate immune signaling have been poorly understood. It was reported that Ser/Thr kinase, RICK, is involved in both NOD1 and NOD2-mediated signaling pathways (Abbott et al., 2004; Kobayashi et al., 2002; Park et al., 2007). However detailed molecular mechanism by which RICK activates NF-κB and MAPK pathways remains to be elucidated.

In the first manuscript of this dissertation, utilizing TAK1-deficient mouse keratinocytes we provided conclusive evidence that TAK1 is an essential mediator of NOD2-mediated innate immune response. We found that TAK1 is essential for activation of NOD2-mediated signaling cascades including both MAPKs (JNK, p38) and NF-κB as well as inflammatory cytokine production (Fig. 1 and 2 in the manuscript #1). The molecular mechanism by which TAK1 activates MAPK and NF-κB pathways is well established. TAK1 is an upstream kinase of MKK4,7 and 3,6, which are upstream MAPKKs for JNK and p38 activation respectively. TAK1 activation is also linked to the activation of IKK complex, which is a key signaling molecule in NF-κB pathway. Here, we showed that overexpression of both NOD2

and RICK induces TAK1 activation. MDP stimulation also activates TAK1 (Fig. 4 in the manuscript #1). RICK is a Ser/Thr kinase, however it is unlikely that RICK directly phosphorylates and activates TAK1 because RICK has been known to function in TLR signaling independent of its kinase activity based on kinase-dead knock-in mice study (Lu et al., 2005). Thus, we propose that upon MDP stimulation, oligomerized NOD2 binds to RICK via CARD-CARD interaction and induces conformational change of RICK, which subsequently facilitates TAK1 autophosphorylation and its activation.

In addition, we also found that MDP upregulates the level of NOD2 possibly through transcriptional activation and it is completely abolished by loss of TAK1 (Fig. 6 in the manuscript #1). It was reported that TNF or LPS stimulation induces NOD2 expression in myelomonocytic cells (Gutierrez et al., 2002). We attempted to examine whether TAK1 is required for TNF- or LPS-induced NOD2 induction in keratinocytes, however we could not compare the effect of TNF because TAK1 deficient keratinocytes were very sensitive to TNF-induced cell death (Omori et al., 2006). LPS failed to induce NOD2 mRNA transcription in mouse keratinocytes (data not shown). LPS did not induce cytokine expression in mouse keratinocytes either (data not shown), while MDP greatly upregulated proinflammatory cytokines (Fig. 1 in the manuscript #1). We think that these observations may be due to defective expression of TLR4 in keratinocytes (Kollisch et al., 2005).

Collectively, our results indicate that NOD2 plays a central role in detecting bacteria in the skin by recognizing a bacterial cell wall component, MDP. MDP binding to NOD2 elicits

NOD2-mediated innate immune responses by activating TAK1-MAPKs and TAK1-NF-κB pathway. In addition, NOD2 signaling pathway activates positive feedback loop to produce more NOD2 for efficient recognition of bacteria via TAK1-dependent pathway.

Keratinocytes are the main constituent of the epidermis. They function not only as a physical barrier between external environment and internal organs, but also produce proinflammatory cytokines and chemokines in response to external stimuli including microbial infections, UV and chemical exposure in order to maintain skin homeostasis (Grone, 2002; Uchi et al., 2000). The role of NOD2 in keratinocytes is not well established. One study showed that NOD2 mediates β-defensin-2 expression in primary keratinocytes (Voss et al., 2006), suggesting that epidermal NOD2 may be associated with bacterial clearance in the skin. Given that TAK1 is a central mediator of NOD2 signaling, we speculate that NOD2-TAK1 pathway is likely to be a major mediator of chemical defense system against microbes for maintaining skin homeostasis. Further analyses using NOD2 deficient mice should enhance our understanding on the signaling pathways maintaining skin homeostasis as well as etiology of chronic inflammatory disorders in the skin.

2. The role of TAK1 in maintaining the integrity of intestinal epithelium

It is widely accepted that TAK1 is a central mediator of cytokine (TNF, IL-1), TLR and NLR signaling pathways. Currently, TAK1 researches focus on elucidating the in vivo role of TAK1 using tissue specific KO mice. Our group has been studying the in vivo role of TAK1 in epithelial barrier function using epidermis- and intestinal epithelium-specific TAK1 KO

mice. In previous publications, we showed that TAK1 plays a pivotal role in maintaining barrier function of the skin and intestine (Kajino-Sakamoto et al., 2008; Omori et al., 2006). In both tissues, TAK1 deletion causes TNF-induced epithelium damage, which leads to lethal inflammation.

In the second manuscript of this dissertation, we attempted to investigate TNF-independent role of TAK1 in the intestinal epithelium. In this study, we demonstrated that intestinal epithelium-derived TAK1 signaling plays a pivotal role in preventing intestinal inflammation against epithelial injury. In the absence of TAK1, DSS significantly induces injury-associated inflammation and epithelial cell apoptosis (Fig. 1, 2 and 3 in the manuscript #2). In addition, we found that ablation of TAK1 leads to abnormally upregulated and mis-localized proliferative epithelial cell before injury and defective reparative proliferation after injury (Fig. 4 in the manuscript #2). Finally, we showed that TAK1 is required for the production of cytoprotective factors in response to proinflammatory cytokine (IL-1) and bacterial components (LPS, MDP) using TAK1 deficient dermis fibroblasts and keratinocytes as model systems (Fig. 5 in the manuscript #2). Taken together, we propose that TAK1 is essential for preventing injury-associated intestinal inflammation possibly by two following mechanisms which are not mutually exclusive.

One mechanism is that TAK1 is responsible for maintenance of normal homeostatic proliferation in the intestinal epithelium. The loss of TAK1 causes dysregulated cell proliferation, which leads to hypersensitivity to cytotoxic insults. Intestinal epithelial cells

originate from stem cells located in the bottom of crypts (Radtke and Clevers, 2005). Intestinal stem cells are actively proliferating until terminally differentiated. The population of the stem cells is tightly regulated. We found that steady-state level of proliferation is abnormally upregulated in TAK1 deficient intestinal epithelium. One can expect that active proliferation can reduce DSS-induced injury because it can help compensatory proliferation. However, TAK1 deficient double mutant mice (TNFR1/TAK1^{IE}KO) showed more severe injury-associated intestinal inflammation. Why are highly proliferating TAK1 deficient cells more sensitive to DSS-induced injury? Although active proliferation is beneficial for tissue repair, it is also accepted that highly proliferating cells are much more sensitive to cytotoxic insults including ionizing radiation (IR). It is well exemplified in the case of cancer therapy that the gastrointestinal tract, oral mucosa and hematopoietic systems, which are all highly proliferating, are most sensitive organs to chemotherapy (Booth and Potten, 2001). It also has been reported that negative regulator of cell cycle can protect intestine. Withers and Elkind reported that administration of TGF-β3, which is an inhibitor of epithelial cell proliferation, increased the survival of crypts after radiation-induced injury (Withers and Elkind, 1970). The loss of TGF-β is known to accelerate tissue damage after DSS administration (Hahm et al., 2001; Sakuraba et al., 2007). The next question is how TAK1 negatively regulates steady-state level of proliferation? It has been established that proliferation of intestinal epithelial cells depends on concerted action of several signaling mechanisms including Wnt (positive regulator of cell cycle) and TGF-β signaling (negative regulator of cell cycle) (Giles et al., 2003; Korinek et al., 1998; Munoz et al., 2006; Radtke and Clevers, 2005; Siegel and Massague, 2003). TAK1 was reported to regulate both of Wnt (negatively) and TGF-β

(positively) signaling (Ishitani et al., 1999; Kajino et al., 2007; Kanei-Ishii et al., 2004). Therefore we speculate that TAK1 regulates the population of self-renewing stem cells by orchestrating these two signals. In the absence of TAK1, the majority of crypts are occupied by actively proliferating cells (progenitors), which are highly sensitive to cytotoxic insults, eventually leading to increased susceptibility to intestinal injury.

Another mechanism is that TAK1 plays an important role in producing cytoprotective factors, which are essential for protecting intestinal epithelium from apoptosis or promoting regeneration of damaged epithelium after injury. In this study, we found that TAK1 is essential for inducing IL-1-, LPS- and MDP-mediated expression of IL-6 and COX2, both of which are known to play an important role in protecting intestinal epithelium against injury. It was reported that IL-6 is important for intestinal wound healing after DSS exposure and loss of IL-6 increases susceptibility to DSS-induced inflammation (Tebbutt et al., 2002). It was also reported that COX2 expression is induced after DSS exposure and its activity is known to be involved in protection of intestinal epithelium against injury-mediated inflammation (Fukata et al., 2006; Karrasch et al., 2007; Morteau et al., 2000). Taken together, we propose that commensal bacteria and homeostatic level of cytokines activate epithelial TAK1, which is required for producing cytoprotective factors including IL-6 and COX2. This pathway is essential for the intestinal barrier function.

TAK1 is a well known upstream activator of NF-κB, which is involved in the transcription of a subset of genes involved in suppressing apoptosis and promoting proliferation. The

phenotypes of intestinal epithelium-specific TAK1 KO mice shown in this study are much similar to that of intestinal epithelium-specific IKK-β KO mice, which showed increased the susceptibility to radiation- and ischemia-reperfusion-induced intestinal injury (Chen et al., 2003; Egan et al., 2004). It was reported that pharmacological blockage of NF-κB inhibits the production of COX2 in the intestinal epithelial cell line and worsens DSS-induced colitis (Karrasch et al., 2007). These results suggest that TAK1-NF-κB signaling pathway in enterocytes is important for protecting intestinal epithelium. It would be of interest to elucidate in vivo link between TAK1 and NF-κB in the intestinal epithelium for future study.

3. TAK1, NOD2 and Crohn's disease

NOD2 mutations are frequently observed in Crohn's disease. Crohn's disease-associated NOD2 mutants are defective in sensing MDP. However, it has long been debated why the loss of NOD2 function results in increased inflammation. One theory is that the lack of NOD2 signaling in the intestinal epithelium is associated with loss of intestinal barrier function and subsequent bacteria invasion, which triggers abnormal inflammatory responses (Wehkamp et al., 2005). NOD2 KO mice study revealed that the ablation of NOD2 leads to impaired expression of a subgroup of cryptdins in the crypts (Kobayashi et al., 2005), suggesting that NOD2 may be required for bacterial clearance, which is essential for preventing overgrowth of pathogenic bacteria in the intestine.

In this dissertation research, we showed that TAK1 plays an essential role in NOD2 signaling in keratinocytes (first manuscript) and is important for maintaining the integrity of intestinal

barrier utilizing intestinal epithelium-specific TAK1 deficient mice (second manuscript). Keratinocytes are similar to enterocytes in that they serve as a barrier to external environment, therefore it is highly possible that TAK1 is an essential mediator of NOD2 signaling in the intestinal epithelium as well. Taken together, we propose that TAK1 is a key mediator of NOD2 signaling and maintains proper expression of cytoprotective factors in the intestinal epithelium, thereby maintains intestinal barrier integrity. In the case of Crohn's disease, defective epithelial TAK1 activity caused by NOD2 mutations is likely to lead to loss of intestinal barrier function and dysregulated intestinal inflammation. There are increasing evidences indicating that crosstalk between intestinal epithelial cells and lamina propria immune cells is closely associated with the maintenance of intestinal epithelial barrier. Thus, we think that it would be important to further investigate the role of NOD2-TAK1 signaling in the intestinal immune cells using tissue specific KO mice. Those approaches should not only enhance our understanding on the etiology of Crohn's disease but also be helpful to identify additional therapeutic targets.

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APPENDIX A

TAK1 is a negative regulator of autophagy and sensitizes cells to starvation-induced apoptosis

-A novel role for TAK1 in cellular aging

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1. This study was supported by student fellowship grant awarded from American Foundation for Aging Research (AFAR).

2. This manuscript was adapted from J-Y Kim's grant proposal for AFAR student fellowship grant application and the research summary submitted for 2008 NC GlaxoSmithKline Foundation and AFAR symposium.

3. This manuscript has not been published in elsewhere.

1. Calorie restriction is associated with prevention of aging

Calorie restriction (CR), defined as a reduction in energy intake, has been shown to enhance longevity of organisms ranging from yeast to mammals (Hursting et al., 2003; Ingram et al., 2004; Lin et al., 2000). Currently, the molecular mechanism underlying the beneficial role of CR in increasing life span is one of the hot areas of aging study. Under CR condition, cells lack energy due to defective ATP synthesis. There are adaptive mechanisms to protect cells from energy depletion caused by various insults such as glucose depletion, hypoxia and oxidative stress. The adaptive mechanisms allow cells to reduce their energy consumption or provide additional energy source until these insults are removed. If the insults overwhelm cells' adaptive ability, they undergo apoptosis. The common view on the relevance of apoptosis to aging has been that apoptosis has primarily a negative impact on aging by destroying essential irreplaceable cells such as neurons and cardiac myocytes. However, it is currently believed that there are two general ways in which apoptosis can play a role in aging ; 1) apoptosis eliminates damaged and dysfunctional cells which can be replaced by cell proliferation (e.g., fibroblasts, hepatocytes), thereby keeps the tissue young and functional; 2) apoptosis is associated with pathogenesis of aging-related diseases such as neurodegeration by destroying cells which can not be replaced (e.g., neurons) (Warner, 1997). Thus, it is possible that CR-induced homeostatic level of apoptosis in non-postmitotic cells is linked to the beneficial effect of CR on aging.

2. Autophagy and aging-related diseases

The word 'autophagy' is derived from the Greek that means to eat (phagy) oneself (auto).

Autophagy is an evolutionally conserved intracellular degradative mechanism and is characterized by the formation of unique double membrane vesicle, called autophagosome, which sequesters long-lived cellular organelles or misfolded proteins. Subsequently the autophagosome is fused with lysosome, in which its contents are degraded and recycled for generation of marcromolecules (Levine and Klionsky, 2004). Autophagy is induced in response to both extracelluar stress conditions (e.g., energy starvation, hypoxia) and intracellular stress conditions (e.g., accumulation of old and damaged cellular organelles or proteins). During starvation, autophagy allows cells to survive by recycling old organelles and proteins. In response to intracellular stress conditions, autophagy sequesters and eliminates the accumulated cellular wastes by lysosomal degradation as described above. What would happen if the autophagy-mediated clearance mechanism is not working properly? It has been reported that the loss of *Atg7*, which is an essential gene for autophagy, in the central nervous system causes neurodegeneration in mice due to abnormal accumulation of ubiquitin-positive inclusion bodies (Komatsu et al., 2006). The ablation of *Atg7* in the liver also causes the accumulation of protein aggregates, hepatic cell swelling and hepatomegaly (Komatsu et al., 2005). These studies suggest that autophagy is a key clearance mechanism for toxic aggregates and is essential for maintaining the homeostasis of post-mitotic cells such as neurons. Defective autophagy is associated with aging-related diseases such as neurodegeneration.

The molecular mechanism of autophagy regulation is actively under investigation. It has been well documented that under nutrient stress condition, AMPK, which is activated by the

increased level of AMP, induces autophagy by inhibiting mTOR, a potent inhibitor of autophagy (Meijer and Codogno, 2006). Several studies reported that MAPK signaling is also involved in autophagy regulation. JNK was reported to be a positive regulator of autophagy by antagonizing Bcl-2 which inhibits an essential autophagic protein Beclin1 under nutrient stress condition (Wei et al., 2008). It was reported that p38 MAPK also positively regulates autophagy by inhibiting mTOR activity (Tang et al., 2008). However, SB202190, which is a pharmacological inhibitor of p38, was reported to induce autophagy in colorectal cancer cells, suggesting that p38 is a negative regulator of autophagy (Comes et al., 2007). Based on these studies, it is plausible that molecular mechanism of autophagy regulation is cell-type and stress-type specific, however more investigation is necessary for better understanding of autophagy regulation.

3. TAK1 MAPKKK is a negative regulator of autophagy and sensitizes cells to nutrient stress-induced apopotosis

Previous studies suggesting the involvement of MAPK pathways in autophagy regulation led us to hypothesize that TAK1 MAPKKK may be involved in autophagy. Firstly, we attempted to compare the autophagy activity of TAK1 WT and KO keratinocytes using the GFP-LC3 assay. LC3 is a mammalian homologue of yeast Atg8, which is an essential protein for autophagy and localizes autophagosome membrane (Kabeya et al., 2000). Autophagy inactive cells show diffuse GFP-LC3 staining, whereas autophagy active cells show punctate GFP-LC3 staining. In this analysis, we found that pronounced GFP-LC3 punctation in TAK1 deficient keratinocytes compared to TAK1 WT cells. Autophagy was also much more

actively induced after starvation in the KO cells (Fig. 1A and B). The data was confirmed by LC3 immunoblotting, which is also widely used to monitor autophagy. LC3 exists in two forms, LC3-I (16 kDa) and LC3-II (14 KDa) and the amount of LC3-II is correlated with the number of autophagosome (Mizushima and Yoshimori, 2007). The immunoblotting results revealed that basal LC3-II level was significantly up-regulated and starvation induced more LC3-II accumulation in TAK1 deficient keratinocytes (Fig. 1C and D). To confirm these findings, similar analyses were performed using TAK1 WT and KO mouse embryonic fibroblast (MEF), and we obtained comparable results (Supplementary Fig. 1). Taken together, we propose that TAK1 is a negative regulator of autophagy.

Autophagy is generally believed to protect cells from energy starvation-induced cell death. Given that TAK1 is a negative regulator of autophagy, TAK1-deficient keratinocytes are likely to be more resistant to nutritional stress-induced apoptosis. To test this, we observed apoptosis in TAK1 WT and KO keratinocytes after starvation. We found that TAK1 deficiency prevented starvation-induced caspase-3 cleavage (Fig. 2A,B and C). Consistent with this data, Annexin V staining, which specifically detects phosphatidylserine on the apoptotic cell membrane, was more pronounced in TAK1 WT keratinocytes after starvation (Fig. 2D), indicating that TAK1 deficiency protects cells from energy starvation-induced cell death presumably due to high activity of autophagy.

A number of studies reported that TAK1 is required for AMPK phosphorylation and its activation (Momcilovic et al., 2006; Xie et al., 2006). AMPK-mTOR pathway is closely

associated with autophagy, thus we next tested whether TAK1 is involved in AMPK activation in the starved condition. However, we found that AMPK activity was not affected by TAK1 (Fig. 3). These data suggest that TAK1 controls energy depletion-induced cell death pathway probably by negatively regulating autophagy in an AMPK-independent manner.

4. Conclusion and significance

Collectively, our results indicate that TAK1 is a negative regulator of autophagy and determines entrance of autophagy or apoptosis under energy depleted conditions. We are currently investigating the molecular mechanisms by which TAK1 regulates autophagy. Increasing studies have suggested that autophagy is closely associated with the pathogenesis of aging-related diseases such as neurodegeneration and cancer, which are worldwide health concern. However, cellular signaling pathways regulating autophagy have been poorly understood. We expect that this study will enhance our understanding on a link among CR, autophagy and longevity, and will contribute to general understanding on molecular biology of aging. We also anticipate that this study will provide molecular basis for therapeutic targets to aging-related diseases.

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FIGURE LEGENDS

Fig. 1. TAK1 is a negative regulator of autophagy

A and B. TAK1 WT and KO keratinocytes stably expressing GFP-LC3 were generated. Cell were left untreated (CT) or starved by glucose and serum free medium (starvation) for 3 h. (A) Representative GFP-LC3 images from 4 independent experiments. (B) Cells with GFP-LC3 puncta dots were quantified. Cells containing more than 20 dots were counted as positive cells and a minimum of 110 cells per sample were counted. Data show means ±SD of 4 independent experiments.

C and D. TAK1 WT and KO keratinocytes were treated with glucose and serum free DMEM (C) or glucose free DMEM supplemented with 10% dialyzed serum (D) for indicated times. The expression of LC3-II was detected by immunoblotting and β-actin (C) and p38 (D) level were used for loading control.

Fig. 2. Loss of TAK1 protects cells from energy starvation-induced apoptosis

A and B. TAK1 WT and KO keratinocytes were treated with glucose and serum free DMEM (A) or Hank's balanced salt solution (B) for indicated times. The expression of Caspase-3 was detected by immunoblotting and β-actin level was used for loading control.

C. TAK1 WT and KO keratinocytes were treated with glucose free DMEM supplemented with 10% dialyzed serum (No Glucose) or Serum free DMEM (No Serum) for 24 hrs. Caspase-3 was detected by immunoblotting and β-actin level was used for loading control.

D. TAK1 WT and KO keratinocytes were treated with glucose and serum free DMEM for 36 hrs and apoptotic cells were detected with Annexin V staining kit (Invitrogen).

Fig. 3. TAK1 is dispensable for energy starvation-induced AMPK activation

A and B. TAK1 WT and KO keratinocytes were treated with glucose and serum free DMEM (A) or glucose free DMEM supplemented with 10% dialyzed serum (B) for indicated times. The expression of phosphor-AMPK was detected by immunoblotting and AMPK level was used for loading control.

Fig. 1 A and B

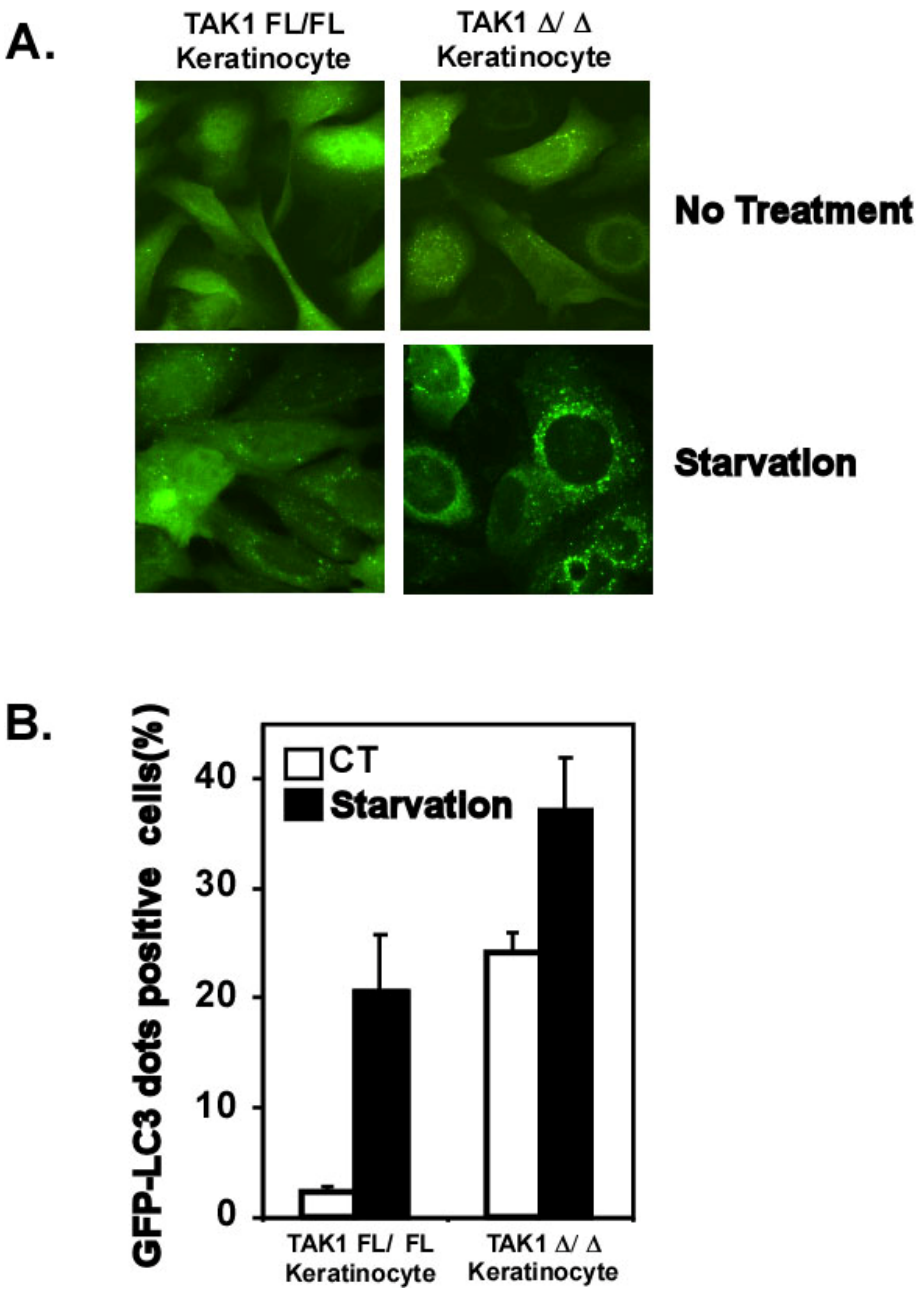


Fig. 1. TAK1 is a negative regulator of autophagy

Fig. 1 C and D

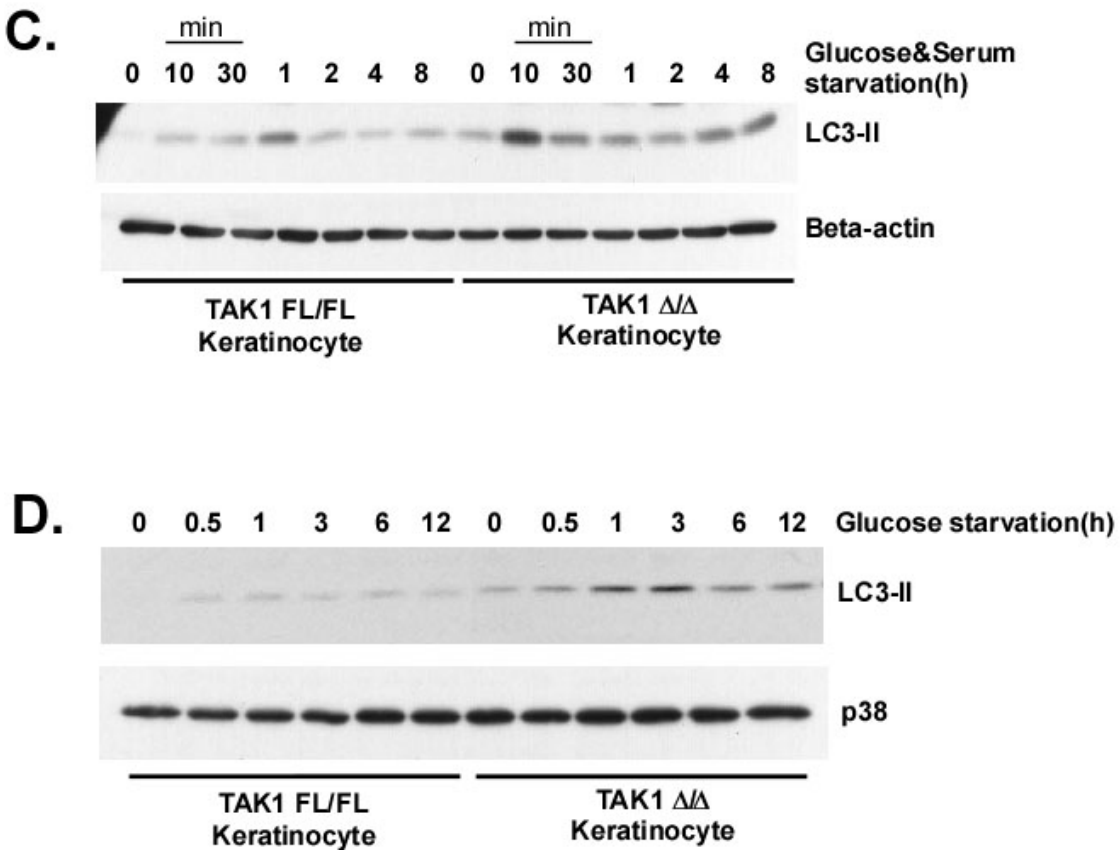


Fig. 1. TAK1 is a negative regulator of autophagy

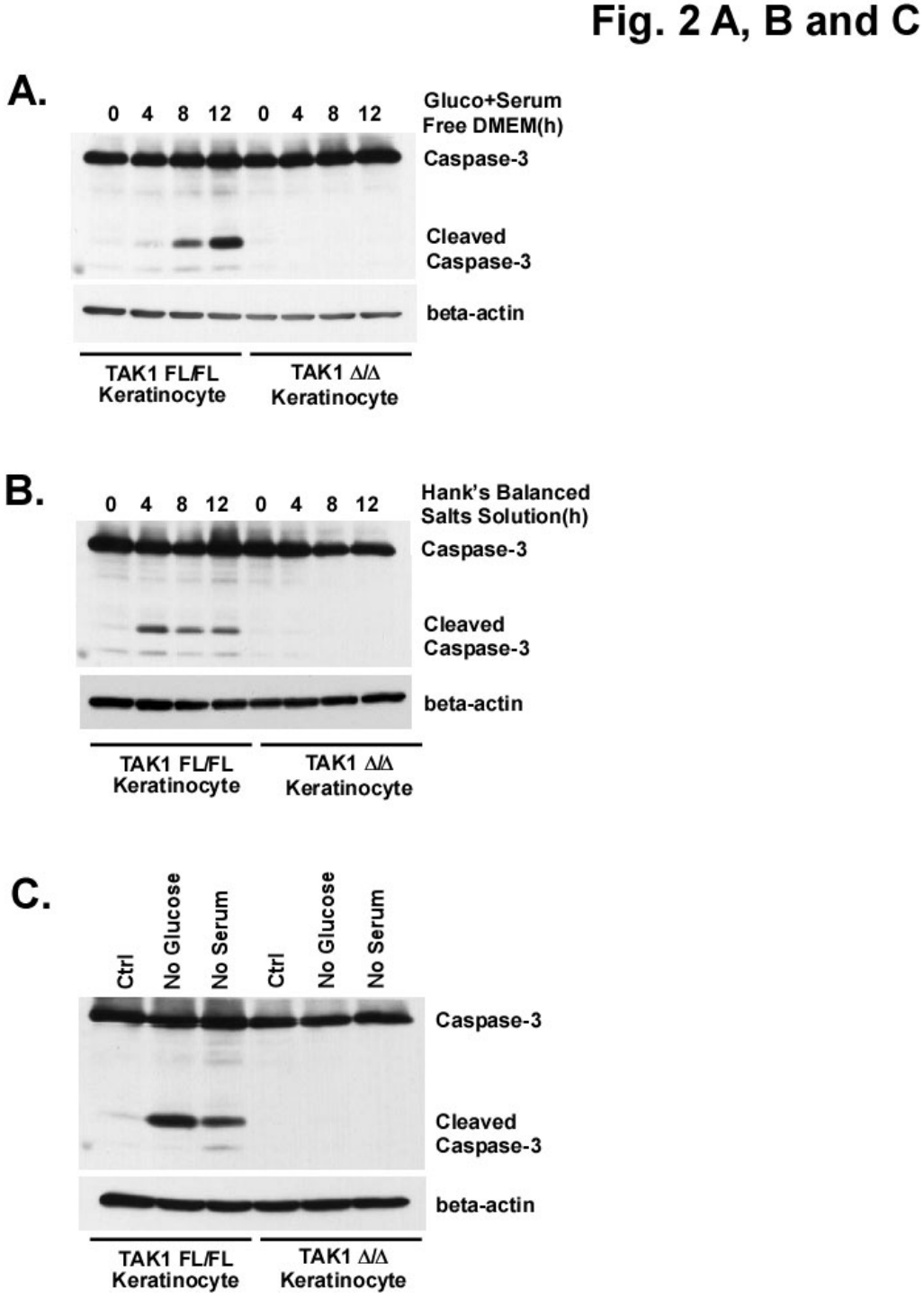


Fig. 2. Loss of TAK1 protects cells from energy starvation-induced apoptosis

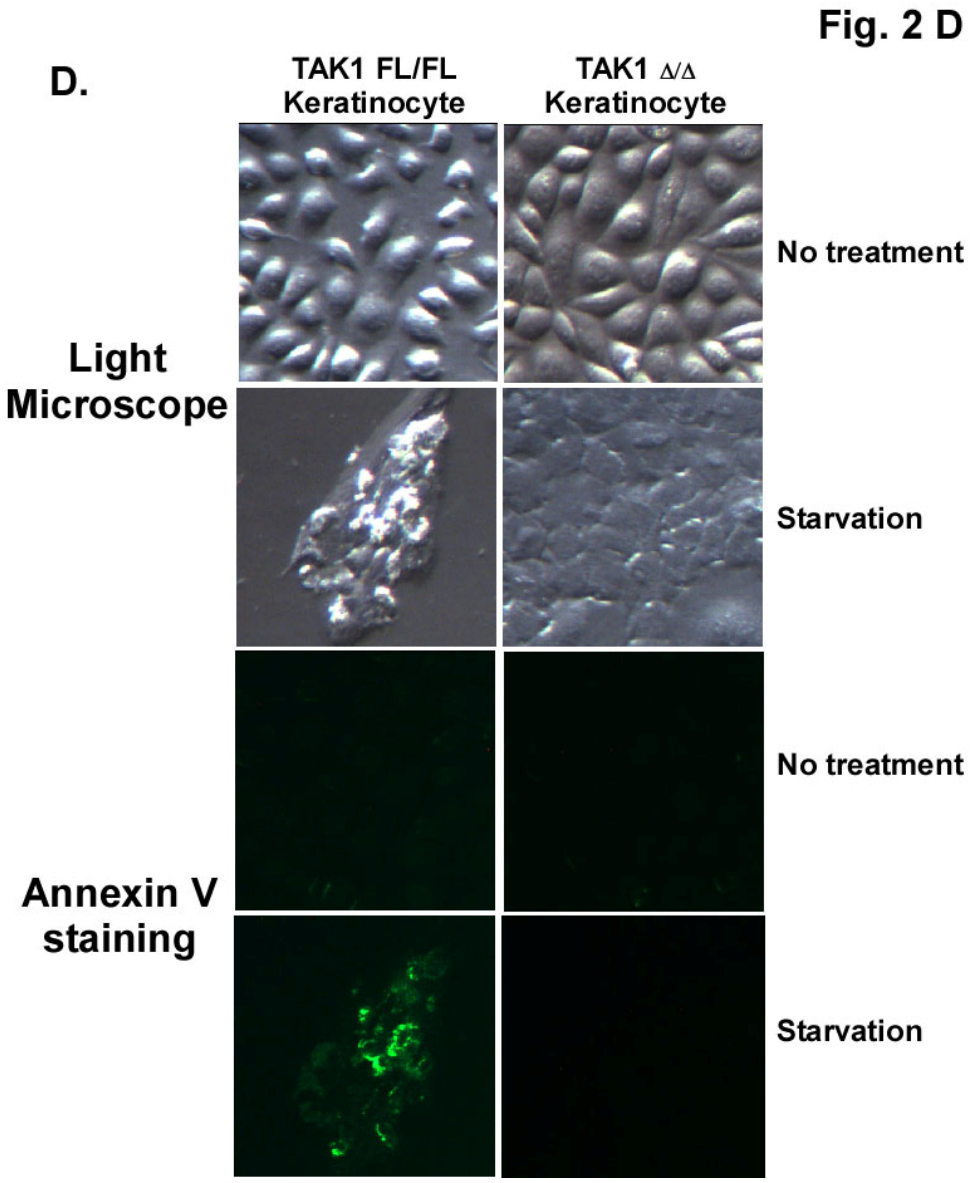


Fig. 2. Loss of TAK1 protects cells from energy starvation-induced apoptosis

Fig. 3 A and B

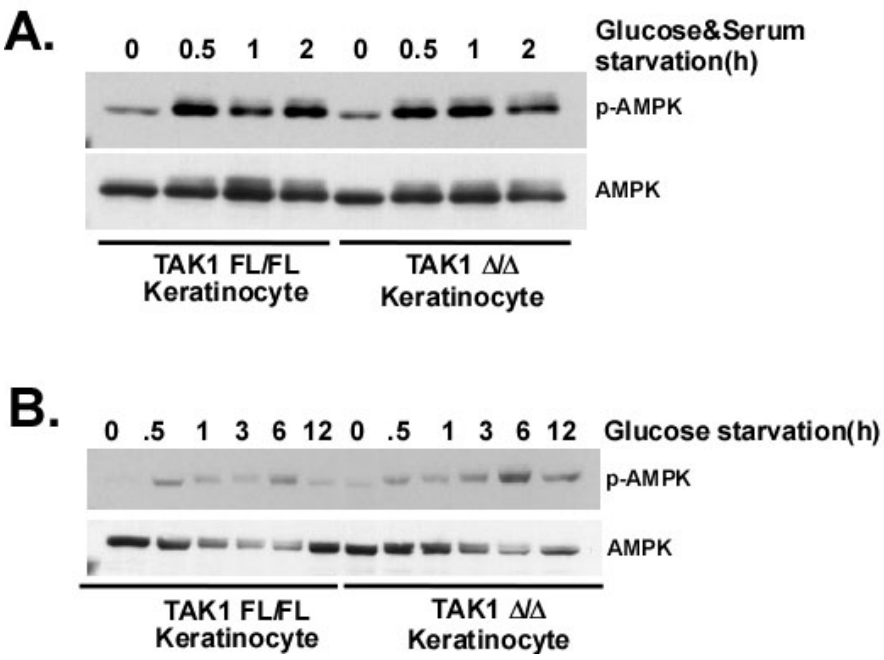


Fig. 3. TAK1 is dispensable for energy starvation-induced AMPK activation

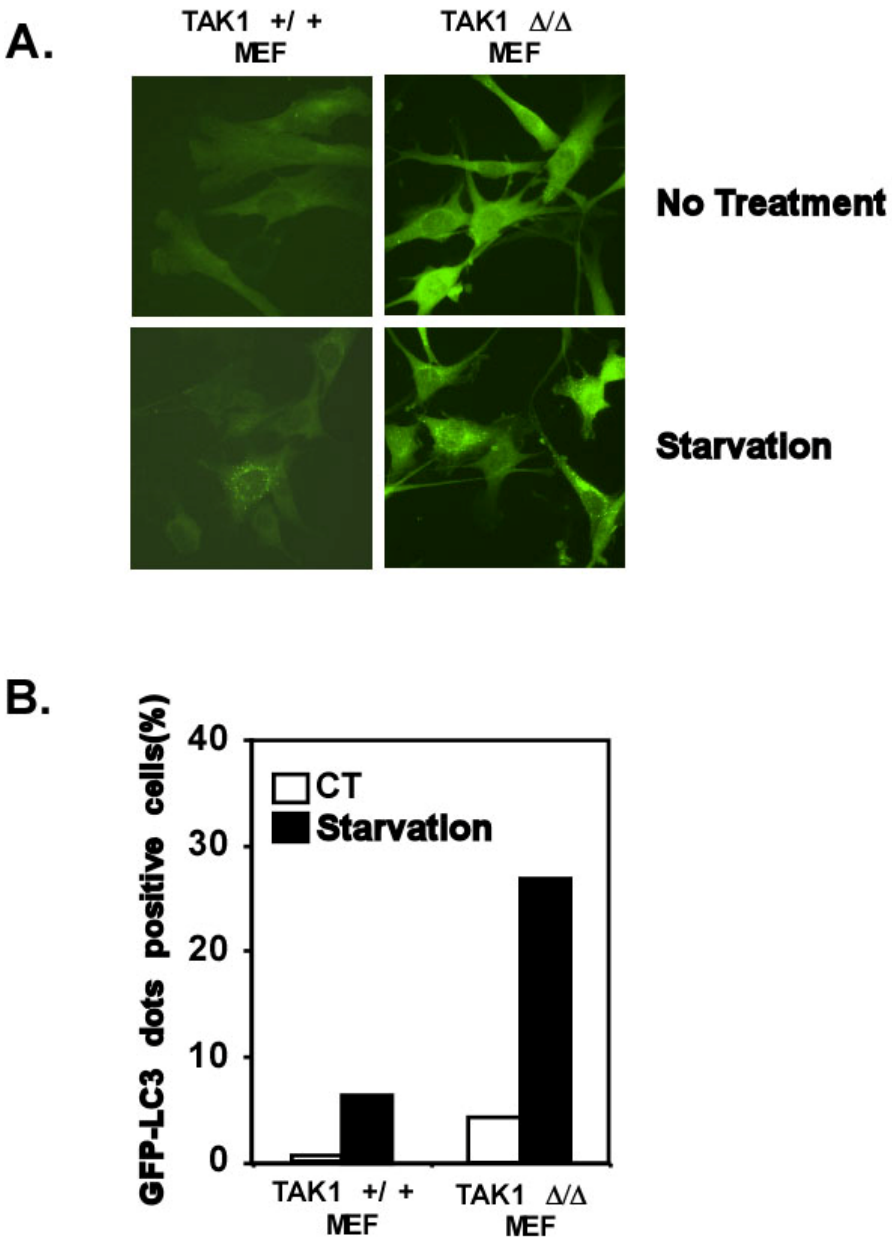
SUPPLEMENTARY DATA

Supplementary Fig. 1. Autophagy in TAK1 WT and KO mouse embryonic fibroblasts (MEF).

A and B. TAK1 WT and KO MEF stably expressing GFP-LC3 were generated. Cell were left untreated (CT) or starved by glucose and serum free medium (starvation) for 1 h. (A) Representative GFP-LC3 images from 2 independent experiments. (B) Cells with GFP-LC3 puncta dots were quantified. Cells containing more than 20 dots were counted as positive cells and a minimum of 110 cells per sample were counted. Data show means of 2 independent experiments.

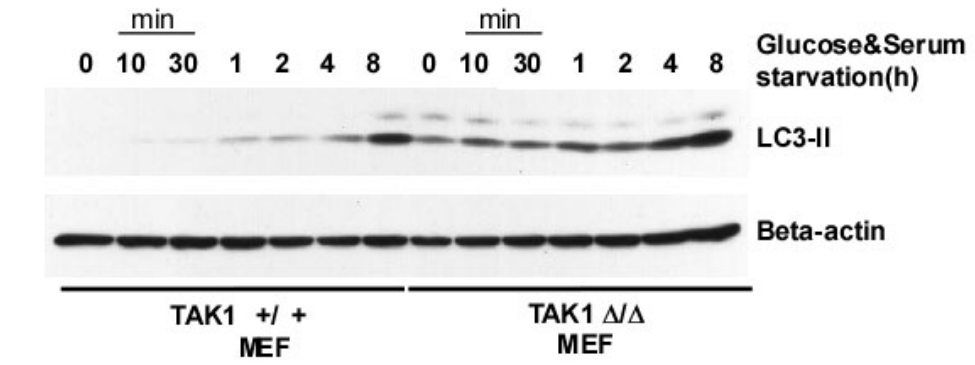
C. TAK1 WT and KO MEF were treated with glucose and serum free DMEM for indicated times. The expression of LC3-II was detected by immunoblotting and β-actin level was used for loading control.

Supplementary Fig. 1 A and B



Supplementary Fig. 1. Autophagy in TAK1 WT and KO mouse embryonic fibroblasts (MEF).

Supplementary Fig. 1 C



Supplementary Fig. 1. Autophagy in TAK1 WT and KO mouse embryonic fibroblasts (MEF).

APPENDIX B

TAK1 binding protein 1, TAB1, mediates osmotic stress-induced TAK1 activation but is dispensable for TAK1-mediated cytokine signaling

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Running title: TAB1 role in TAK1 signaling

1. This manuscript has been published in *the Journal of Biological Chemistry* (2008).
2. Jae-Young Kim has contributed to this study as a coauthor.

JBC Papers in Press. Published on October 1, 2008 as Manuscript M807574200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M807574200

TAK1 binding protein 1, TAB1, mediates osmotic stress-induced TAK1 activation but is dispensable for TAK1-mediated cytokine signaling

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Running title: TAB1 role in TAK1 signaling

TAK1 kinase is an indispensable intermediate in several cytokine signaling pathways including TNF, IL-1 and TGF-β signaling pathways. TAK1 also participates in stress-activated intracellular signaling pathways such as osmotic stress signaling pathway. TAK1 binding protein1 (TAB1) is constitutively associated with TAK1 through its C-terminal region. Although TAB1 is known to augment TAK1 catalytic activity when it is overexpressed, the role of TAB1 under physiological conditions has not yet been identified. In this study, we determined the role of TAB1 in TAK1 signaling by analyzing TAB1 deficient mouse embryonic fibroblasts (MEFs). TNF- and IL-1-induced activation of TAK1 was entirely normal in *Tab1*-deficient MEFs, and which could activate both mitogen activated protein kinases and NF-κB. In contrast, we found that osmotic stress-induced activation of TAK1 was largely impaired in *Tab1*-deficient MEFs. Furthermore, we showed that the C-terminal 68 amino acids of TAB1 were sufficient to mediate osmotic stress-induced TAK1 activation. Finally, we attempted to determine the mechanism by which TAB1 activates TAK1. We found that TAK1 is spontaneously activated when the concentration is increased, and that is totally dependent on TAB1. Cell shrinkage under the osmotic stress condition increases the concentration of TAB1-TAK1, and may oligomerize and activates TAK1 in a TAB1 dependent manner. These results demonstrate that TAB1 mediates TAK1 activation only in a subset of TAK1 pathways, which are mediated through spontaneous oligomerization of TAB1-TAK1.

INTRODUCTION

TAK1 kinase is an indispensable intermediate of several innate immune signaling pathways including cytokines TNF and IL-1 as well as Toll-like receptors and intracellular bacterial sensor NOD-like receptor NOD1/2 pathways (1-5). In those pathways, TAK1 is recruited into the IKK complex through a polyubiquitin chain and activates transcription factor NF-κB (6). The innate stimuli-activated TAK1 also induces activation of transcription factor AP-1 through mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinase (JNK) and p38. NF-κB and AP-1 cooperatively modulate gene expression to induce inflammation and cell survival (7,8). TAK1 is involved in several other signaling pathways, for example, in TGF-β signaling pathways, TAK1 participates in the non-Smad pathway by activating p38 and SnoN degradation (9,10). TAK1 is critically involved in stress-activated cell signaling (11-13). Among the stress conditions, we found that osmotic stress-induced JNK activation requires TAK1 (12).

We have identified several TAK1 binding proteins including TAK1 binding protein 1 (TAB1) (14) and TAK1 binding protein 2/3 (TAB2/3) (15,16). TAB1 and TAB2 are isolated

by the yeast two-hybrid screening using TAK1 protein as a bait, and both endogenous TAB1 and TAB2 are coprecipitated with endogenous TAK1 in many types of cells. TAB2 and its homolog TAB3 are found to bind to ubiquitin and function as an adaptor tethering TAK1 to the IKK complex (17,18). In contrast, the role of TAB1 in TAK1 signaling under the physiological setting has not yet been explored. In culture cells, TAB1 is found to be constitutively associated with TAK1 (19). Ectopic expression of TAB1 together with TAK1 induces TAK1 autophosphorylation and thereby activates TAK1 kinase in vitro (19). Only 68 amino acid residues of C-terminal TAB1 are essential and sufficient for binding to TAK1 and induction of autophosphorylation/activation of TAK1 (20).

Disruption of *Tab1* causes embryonic lethality with several developmental dysregulations including failure of cardiovascular morphogenesis (21). Disruption of *Tak1* also causes early embryonic lethality presumably due to its importance for regulating multiple cytokine signaling pathways (4,22,23). These facts raise the possibility that TAB1 may be involved in TAK1-mediated signaling pathways during development. However, *Tab1*-deficient embryos are grossly normal by embryonic day 14.5 (E14.5) (21), which is different from *Tak1*-deficient embryos that are lethal by E9.5 (4,22,23). This reveals that TAB1 is not essential for all of the TAK1-mediated signaling pathways, or functions of TAB1 can be compensated by other gene products. Earlier study using *Tab1*-deficient mouse embryonic fibroblasts (MEFs) has reported that TNF-, and IL-1-induced activation of NF-κB, JNK and p38 is not affected by *Tab1* deletion (4). However, Mendoza et al. has recently reported that IL-1-induced TAK1 activation may be reduced in *Tab1*-deficient MEFs (24). Our goal is to define the essential roles of TAB1 under physiological conditions. We have recently generated a *Tab1*-floxed mouse line that allows us to investigate the role of TAB1 in several different tissues (25). In the current study, we started determining the TAB1-mediated signaling and investigated which types of TAK1 pathways require TAB1 by using TAB1 deficient MEFs prepared from our newly generated *Tab1*-floxed mice.

EXPERIMENTAL PROCEDURES

Cell culture and transfection- Heterozygous mice for the Cre-recombined allele of *Tab1* were generated by crossing *Sox2-Cre* mice and *Tab1*-floxed (*Tab1*$^{flox/flox}$) mice (25). The heterozygous mice were intercrossed and *Tab1*-control and *Tab1*-deficient MEFs were isolated from wild type and homozygous mutant embryos at embryonic day 14.5, and spontaneously immortalized by the standard method. Preparation of TAK1 +/+ and TAK1 Δ/Δ MEFs were described previously (23). MEFs and 293 cells were cultured in DMEM with 10% bovine growth serum (Hyclone), and penicillin-streptomycin at 37°C in 5% CO2. 293 cells were transfected with expression vectors for hemagglutinin (HA)-tagged TAK1 (pCMV-HA-TAK1) and TAB1 (pCMV-TAB1) as described previously (26).

Reagents- Reagents used were IL-1β (mouse recombinant, Roche), TNFα, TGF-β (human recombinant, Roche), calyculin A and okadaic acid (Calbiochem). The following polyclonal antibodies were used: TAK1 and TAB1 described previously (3), phospho-Thr187 TAK1 (27) (Cell Signaling), JNK1 (FL), p38 (N-20), IκBα, p65 (Santa Cruz), phospho-IκB, phospho-p38 (Thr-180/Tyr182), and AMPK (Cell Signaling). Rabbit monoclonal antibody phospho-AMPK (Cell Signaling) and mouse monoclonal antibodies phospho-JNK (Thr-183/Tyr-185) (Cell Signaling) and Flag-M2 (Sigma) were also used.

Electrophoretic mobility shift assay (EMSA)- The binding reactions contained radiolabeled ^{32}P-NF-κB oligonucleotide probe (Promega), cell extracts, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 500 ng of poly(dI-dC) (GE Healthcare), and 10 μg of bovine serum albumin to a final volume of 10 μl. The reaction mixtures were incubated at 25°C for 15 min, separated by 5% (w/v) polyacrylamide gel, and visualized by autoradiography.

Immunoblotting- Cells were washed once with ice-cold phosphate-buffered saline and whole cell extracts were prepared using lysis buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM

phenylmethylsulfonyl fluoride, 20 μM aprotinin, 0.5% Triton X-100). Cell extracts were resolved on SDS-PAGE and transferred to Hybond-P membranes (GE Healthcare). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using the ECL Western blotting system (GE Healthcare).

Retroviral infection- Retroviral vectors for *Tab1* (pMXs-neo-*Tab1*) was generated by inserting human TAB1 cDNAs into the retroviral vector pMXs-neo (28). To generate retroviral vectors for Flag-TAB1N (pMXs-puro-flag-TAB1N), ClaI-HincII fragment of pCMV-Flag-TAB1 (encodes Flag-tagged TAB1 N-terminus 1-418 a.a.) (29) was inserted into pMXs-puro. To generate retroviral vectors for Flag-TAB1C (pMXs-puro-flag-TAB1C), Flag-tag was fused to C-terminus of TAB1 (437-504 a.a.) and inserted into pMXs-puro. EcoPack293 cells (BD Biosciences) were transiently transfected with pMX-neo-TAB1, pMX-puro-Flag-TAB1N or pMX-puro-Flag-TAB1C. After 48 h culture, growth medium containing retrovirus was collected and filtered with 0.45 μm cellulose acetate membrane to remove packaging cells. MEFs were incubated with the collected virus-containing medium with 8 μg/ml polybrene for 24 h. Uninfected cells were removed by G418 or puromycin selection.

Concentration dependent activation of TAK1– Cell lysates from *Tab1*+/+ and -/- MEFs were incubated and immunoprecipitated with anti-TAK1 at a high protein concentration (10 mg proteins/ml) or at a low protein concentration (2 mg protein/ml) at room temperature for 3 h. Immunoprecipitates were incubated in a kinase buffer (20 mM HEPES (pH 7.4), 1 mM DTT, 10 mM MgCl, 1 mM ATP) at 37 ºC for 20 min, and subjected to an immunoblot analysis and kinase assay described previously (12). Loading amounts of immunoprecipitaes were adjusted to yield the same loading of TAK1.

RESULTS AND DISCUSSION

Conventional *Tab1* knockout mice have demonstrated that TAB1 is essential for proper embryogenesis (21). However, the roles of TAB1 at the molecular levels in cell signaling and morphogenesis are still elusive. To define the in vivo role of TAB1 in several different cell types and tissues, we have recently generated the floxed *Tab1* mouse (25). In this mouse with floxed *Tab1*, Cre-dependent recombination results in deletion of C-terminus amino acid residues 308-504 of TAB1 protein. Because TAB1 C-terminus contains the TAK1 binding domain (20), the C-terminal truncated TAB1, if produced from the Cre recombined allele, should be functionally inactive in TAK1-mediated signaling pathways. To begin characterizing the role of TAB1, Cre-dependent DNA recombination was introduced to generate heterozygous mice for Cre-recombined mice for *Tab1*. Intercross of the resulted heterozygous mice for *Tab1* was set up and subsequently *Tab1*-deficient mouse embryonic fibroblasts (MEFs) were isolated from the homozygous embryos for Cre-recombined *Tab1* allele at E14.5. These cells along with MEFs from control littermates were utilized to determine the essential role of TAB1 in TAK1 signaling pathways.

TAK1 is activated by IL-1 and TNF, and plays a central role in inflammatory responses by activating JNK, p38 and NF-κB (30). We first examined whether TAB1 participates in IL-1- and TNF-induced TAK1 signaling pathway. Because it is a formal possibility that immortalization process may genetically and epigenetically alter cells, we generated *Tab1* restored *Tab1*-deficient MEFs (*Tab1*-restored) to determine if the alteration found is truly TAB1-dependent. To generate *Tab1*-restored cells, we infected *Tab1*-deficient MEFs with retrovirus expressing *Tab*1 and a pool of *Tab*1 expressing cells was used for the following experiments. We treated *Tab1*-control, -deficient and -restored MEFs with IL-1 or TNF and determined activation of JNK, p38 and NF-κB (Fig. 1). In consistent with the earlier study (4), we found that IL-1- and TNF-induced activation of JNK, p38 and NF-κB was not altered by TAB1 deficiency. We asked whether IL-1 and TNF could activate TAK1 in our *Tab1*-deficient MEFs. We detected an active form of TAK1 by using the phospho-Thr187 specific TAK1 antibody (27). The levels of TAK1 activation in response to IL-1 or TNF were not significantly reduced by TAB1 deficiency (Fig. 2A). IL-1 and TNF activate TAK1 in MEFs not as strongly as in other types of cells such as 293 that we have previously shown (27,31). Therefore, to further

confirm the activation of TAK1, we pretreated MEFs with type 2A protein phosphatases inhibitor calyculin A, which inhibits downregulation of TAK1 (27), and examined activation of TAK1 in response to IL-1. The activation of TAK1 in the presence of calyculin A was also not reduced by *Tab1* deletion (Fig. 2B). Thus, TAB1 is dispensable for TNF- and IL-1-induced activation of TAK1.

TAK1 is also activated by stress conditions such as osmotic stress (12) and arsenic treatment (our unpublished results). Among the stress conditions, we have previously found that stringent osmotic stress using 0.5-0.7 M NaCl strongly activates TAK1, and that TAK1 is essential for NaCl-induced JNK activation (12). We examined whether osmotic stress-induced signaling events are altered by *Tab1* deletion, and measured the osmotic stress-induced activation of TAK1 (Fig. 3A). TAK1 was activated at 5-15 min following 0.5 M NaCl treatment in *Tab1*-control MEFs, while osmotic stress-induced activation of TAK1 was greatly impaired in *Tab1*-deficient MEFs (Fig. 3A). Expression of exogenous TAB1 in the *Tab1*-deficient MEFs was able to restore the activation of TAK1. These results demonstrate that TAB1 is essential for osmotic stress-induced TAK1 activation. TAB1 consists of 504 amino acid residues in humans. TAB1 binds to TAK1 through its C-terminal amino acid residues 480-495 (20) (Fig. 3B). TAB1 also binds to and activates p38α through the amino acid residues 373-418 (32). Therefore, besides direct interaction between TAB1 and TAK1, TAB1 might indirectly mediate TAK1 activation through p38α. We next asked which possibility is more likely for activation of TAK1 in osmotic stress signaling. We infected *Tab1*-deficient MEFs with retrovirus expressing the N-terminal amino acid residues 1-418 of *Tab1* (*Tab1*N), which include p38α but not TAK1 binding region, or the C-terminal amino acid residues 437-504 of TAB1 (TAB1C), which only binds to TAK1. Subsequently, pools of the MEFs expressing *Tab1*N or *Tab1*C were treated with 0.5 M NaCl (Fig. 3C). We found that TAB1C but not TAB1N was able to restore the activation of TAK1 in response to the osmotic stress. TAB1C is only 68 amino acids and we could not detect the TAB1C protein by immunoblotting. To confirm whether TAB1C mediates TAK1 activation, we utilized other *Tab1*-deficient MEFs isolated from conventional *Tab1* knockout embryos (21), which are completely different source from the MEFs used in this study, and generated *Tab1*C expressing *Tab1*-deficient. Those *Tab1*C expressing MEFs but not the *Tab1*-deficient MEFs activate TAK1 in response to the osmotic stress (data not shown). These results suggest that TAB1 association with TAK1 is important for osmotic stress-induced activation of TAK1. Moreover, this demonstrates that the C-terminal 68 amino acid residues of TAB1 are sufficient to mediate osmotic stress-induced TAK1 activation.

We next investigated the cellular responses involving osmotic stress induction that are mediated by the TAB1-TAK1 pathway. We examined activation of JNK and p38 in *Tab1*-control and -deficient MEFs (Fig. 4A). Activation of JNK but not of p38 was impaired in *Tab1*-deficient MEFs. We confirmed that TAB1C but not TAB1N was able to restore osmotic stress-induced JNK activation (Fig. 4B). We have previously reported that osmotic stress-induced activation of JNK but not the p38 is impaired in TAK1 deficient cells (12). Therefore, TAB1-TAK1 is predominantly function upstream of JNK but not of p38 in osmotic stress signaling pathway. It has been reported that another MAPKKK, MEKK3, is important for osmotic stress-induced activation of p38 (33). Thus, it is likely that TAB1-TAK1 is the major mediator of JNK activation, while MEKK3 is the major mediator of p38 activation.

We also examined activation of NF-κB (Fig. 4C). Although TAK1 is capable of activating NF-κB and is highly activated under osmotic stress conditions, NF-κB pathway was not activated at all even in wild type MEFs, which is consistent with our previous observation (12). We think that TAK1 is directed to the JNK pathway in response to osmotic stress by binding to TAO2 kinase as described in our previous report (12).

In addition to JNK and p38, many stress conditions activate AMP-activated protein kinase (AMPK) pathway that regulates energy metabolism (34). TAK1 is previously implicated in activation of AMPK (35). We would like to note that AMPK was activated upon 0.5 M NaCl treatment, and the level of activation was not

markedly reduced either by *Tab1* deletion or by *Tak1* deletion (Supplementary Fig. S1). These results demonstrate that osmotic stress activates TAK1 in a TAB1 dependent manner, which is essential for activation of JNK but not of p38 or AMPK.

Our results demonstrated that among the TAK1 stimuli, only osmotic stress signaling utilizes TAB1 to activate TAK1. We then attempted to determine the mechanism by which TAB1 activates TAK1 in response to osmotic stress. In IL-1 and TNF pathways, it has been demonstrated that TAK1 is associated with a large signaling complex consisting of a number of proteins including TAB2/3 and TRAFs (36). TAK1 oligomerization in the signaling complexes is essential for activation of TAK1. In contrast to cytokine signaling, formation of such signaling complexes has not been identified in the osmotic stress pathway. Because oligomerization is one of common mechanisms of enzymatic activation of kinases, we speculated that TAK1 might be oligomerised and activated under an osmotic stress condition in a TAB1 dependent manner. How is TAK1 oligomerized by osmotic stress? There is no specific sensor molecule for osmotic stress in mammalian cells. It has been well known that osmotic stress shrinks cells, and that the reduced cell volume is the trigger of cell signaling (37,38). Thus, we speculated that TAB1-TAK1 complex might be spontaneously oligomerized and activated when the concentration of TAB1-TAK1 is increased. To test this possibility, we examined whether TAB1-TAK1 can be spontaneously activated when protein concentration is increased. We incubated cell lysates from *Tab1*-control or -deficient MEFs at a high (10 mg protein/ml) or low (2 mg protein /ml) concentration, and measured TAK1 activity (Fig. 5A and B). A slowly migrating TAK1 on SDS-PAGE, which was phosphorylated, was detected when incubated at the high concentration in the presence of TAB1. In contrast, TAK1 was not phosphorylated at any concentrations in the lysate from *Tab1*-deficient MEFs. We confirmed that catalytic activity of TAK1 was greatly increased after incubation at the high concentration in a TAB1 dependent manner (Fig. 5B). These suggest that when TAB1-TAK1 concentration is increased, TAK1 is oligomerized and thereby being activated. To further test this idea, we utilized 293 cells to overexpress HA-tagged TAK1 with TAB1 to increase the concentration of TAB1-TAK1. If increase of TAB1-TAK1 concentration causes oligomerization and activation of TAK1, exogenously overexpressed HA-TAK1-TAB1 could activate not only HA-TAK1 but also endogenous TAK1. Because HA-tagged TAK1 is significantly bigger than endogenous TAK1, we were able to detect a slower migrating HA-TAK1 band on SDS-PAGE when HA-TAK1 alone was overexpressed (Fig. 5C, top panel, lane 1). We found that, when cells were transfected with HA-TAK1 and TAB1, both HA-TAK1 and endogenous TAK1 migrated as slower smear bands on SDS-PAGE (Fig. 5C, top panel, lane 2). The slowly migrated HA-TAK1 and endogenous TAK1 were confirmed as phosphorylated forms (Fig. 5C, middle panel). These suggest that exogenously overexpressed TAB1-TAK1 can interact with and induced activation of endogenous TAK1. These results indicate that TAB1 is essential for concentration-dependent spontaneous activation of TAK1, which may be mediated by TAB1-TAK1 oligomerization. We next attempted to determine whether TAB1-TAK1 is oligomerized under osmotic stress conditions. However, the interaction of TAK1 with TAB1 was not altered by osmotic stress (data not shown), and TAB1 was always co-precipitated with TAK1 regardless of treatment of stimuli including osmotic stress and IL-1, which is consistent with our previous observation (19). We assume that TAB1-TAK1 complexes are preformed, and that some of the complexes may be oligomerized upon stimuli challenges. Necessity of overexpression of both TAK1 and TAB1 for activation of TAK1 (Fig. 5C) supports this idea. We speculate that cell shrinkage under osmotic stress conditions increases the TAB1-TAK1 concentration, which may be sufficient to induce oligomerization of some TAB1-TAK1 in the cells.

In summary, TAB1 is essential for osmotic stress-induced activation of TAK1, which may be mediated by concentration-dependent oligomerization of TAK1. In contrast, TAK1 is activated in a TAB1-independent manner in response to IL-1 and TNF, which is presumably because TAK1 is oligomerized through other adaptor molecules including TAB2.

In addition to these pathways, TAB1 is known to be critically involved in TGF-β family signaling pathways. TAB1 is associated with XIAP and thereby recruiting TAK1 to the TGF-β receptor complex (29,39). We examined TGF-β-induced activation of plasminogen activator inhibitor 1 (PAI-1), which is previously identified as one of the TAK1-dependent events in TGF-β signaling pathways (10). We found that the induction of PAI-I mRNA was not impaired in *Tab1*-deficient MEFs (Supplementary Fig. S2). This suggests that TAB1 is dispensable at least for TGF-β-induced PAI-1 induction in MEFs. Because TGF-β family ligand-induced cellular responses are not effectively detected in MEFs, we have not yet determined whether other TGF-β signaling pathways are mediated by TAB1.

Mendoza et al. has recently reported that IL-1-induced TAK1 activation is impaired in *Tab1*-deficient MEFs (24). In contrast, our results demonstrated that TAB1 is dispensable for IL-1-induced TAK1 activation. Mendoza et al. used MEFs from the conventional *Tab1* knockout embryos (21). This *Tab1* knockout embryo lacks exons 10 and 11 of *Tab1*, which presumably express a C-terminal truncated form of TAB1 that is almost identical to the truncated TAB1 generated in our Cre-mediated *Tab1* deletion system. We would like to note that we conducted most of experiments in this study not only using our *Tab1*-deficient MEFs but also MEFs from the conventional *Tab1* knockout embryos, and that all results were the same in both *Tab1*-deficient MEFs. Therefore, we do not know the reason for the discrepancy between their and our results. It might be possible that MEFs respond differently to IL-1 due to genetic or epigenetic alterations generated during immortalization but not due to TAB1 deficiency. In our study, we utilized *Tab1*-restored MEFs to confirm the TAB1-dependent cellular responses and found that *Tab1* deletion does not significantly affect TNF and IL-1 signaling pathways. It has been well documented that TAK1 deletion almost completely abolishes TNF- and IL-1-induced JNK, p38 and NF-κB activation in several types of cells (4,5,23,40). This suggests that there is no compensatory mechanism that can activate JNK, p38 and NF-κB in the absence of TAK1 in IL-1 and TNF signaling pathways, which is consistent with our results that activation of TAK1, NF-κB, JNK, and p38 was all intact in *Tab1*-deficient MEFs. Thus, we conclude that TAB1 is dispensable for TAK1 activation at least in TNF and IL-1 signaling pathways.

In this study, we identified that the C-terminal region of TAB1 is essential and sufficient for osmotic stress-induced activation of TAK1. The C-terminal region of TAB1 binding to TAK1 may be important for oligomerization of TAK1, which in turn activates TAK1. Further studies to define the molecular mechanism by which such small region of TAB1 mediates TAK1 activation in response to osmotic stress will be important. We show here that TAB1 is totally dispensable for IL-1 and TNF signaling pathways but essential for osmotic stress-induced JNK activation. Based on these findings, we assume that TAB1-TAK1 signaling is involved in a subset of stress responses but not in IL-1 or TNF signaling in vivo. Utilizing our *Tab1*-floxed mice, we anticipate that we can explore the role of TAB1-TAK1 signaling in an in vivo setting.

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FOOTNOTE

*We thank S. Akira for materials. This work was supported by the Intramural Research Program of the NIEHS, NIH (ES071003-10) to Y. M. and by a grant (GM068812) from NIH to J. N-T.

The abbreviations used are; AMPK, AMP-activated protein kinase; HA, hemagglutinin; MEF, mouse embryonic fibroblasts; TAB, TAK1 binding protein.

FIGURE LEGENDS

Fig. 1.

TAB1 is dispensable for TNF and IL-1 signaling pathways.

Tab1-control (+/+), *Tab1*-deficient (-/-) and *Tab1*-restored (-/- *Tab1*) MEFs were stimulated with 20 ng/ml TNF or 5 ng/ml IL-1. Activation of JNK and p38 was detected with anti-phospho-JNK and anti-phospho-p38, and the total amounts of JNK and p38 were detected with anti-JNK and anti-p38. Activation of NF-κB was monitored by phosphorylation of IκB with anti-phospho-IκB, degradation of IκB with anti-IκB and EMSA. TAK1 and TAB1 were detected with anti-TAK1 and anti-TAB1. Asterisks indicate non-specific bands.

Fig. 2.

TAB1 is dispensable for TNF- and IL-1- induced activation of TAK1.

(A) MEFs were stimulated with 5 ng/ml IL-1 (left panels) or 20 ng/ml TNF (right panels). Activation of TAK1 was monitored by immunoblotting with anti-phospho-TAK1. TAK1 and TAB1 were detected with anti-TAK1 and anti-TAB1. Asterisks indicate non-specific bands.

(B) MEFs were pretreated with 10 nM calyculin A for 1 h and stimulated with 5 ng/ml IL-1 for 10 min. Activation of TAK1 was analyzed by immunoblots.

Fig. 3.

TAB1 mediates osmotic stress-induced activation of TAK1 through its C-terminus.

(A) MEFs were stimulated with 0.5 M NaCl. Activation of TAK1 was analyzed by immunoblots.

(B) Schematic diagram of TAB1 and truncated mutants of TAB1. p38α and TAK1 binding sites are shown.

(C) *Tab1*-control (+/+), *Tab1*-deficient (-/-) and *Tab1*-deficient but restored by expressing Flag-tagged *Tab1*N (-/- Flag-*Tab1*N) or Flag-tagged *Tab1*C (-/- Flag-*Tab1*C) MEFs were stimulated with 0.5 M NaCl. Activation of TAK1 was analyzed by immunoblots. Expression of Flag-TAB1N was detected by the immunoblot with anti-Flag. Asterisk indicates non-specific bands. Flag-TAB1C was too small to detect by immunoblot analysis.

Fig. 4.

TAB1 is essential for osmotic stress-induced activation of JNK.

(A and B) MEFs were stimulated with 0.5 M NaCl. Activation of JNK and p38 was analyzed by immunoblots.

(C) MEFs were stimulated with 0.5 M NaCl or 5 ng/ml IL-1. Activation of NF-κB was monitored by phosphorylation of IκB with anti-phospho-IκB, degradation of IκB with anti-IκB and EMSA. The amount of p65 is shown as a loading control.

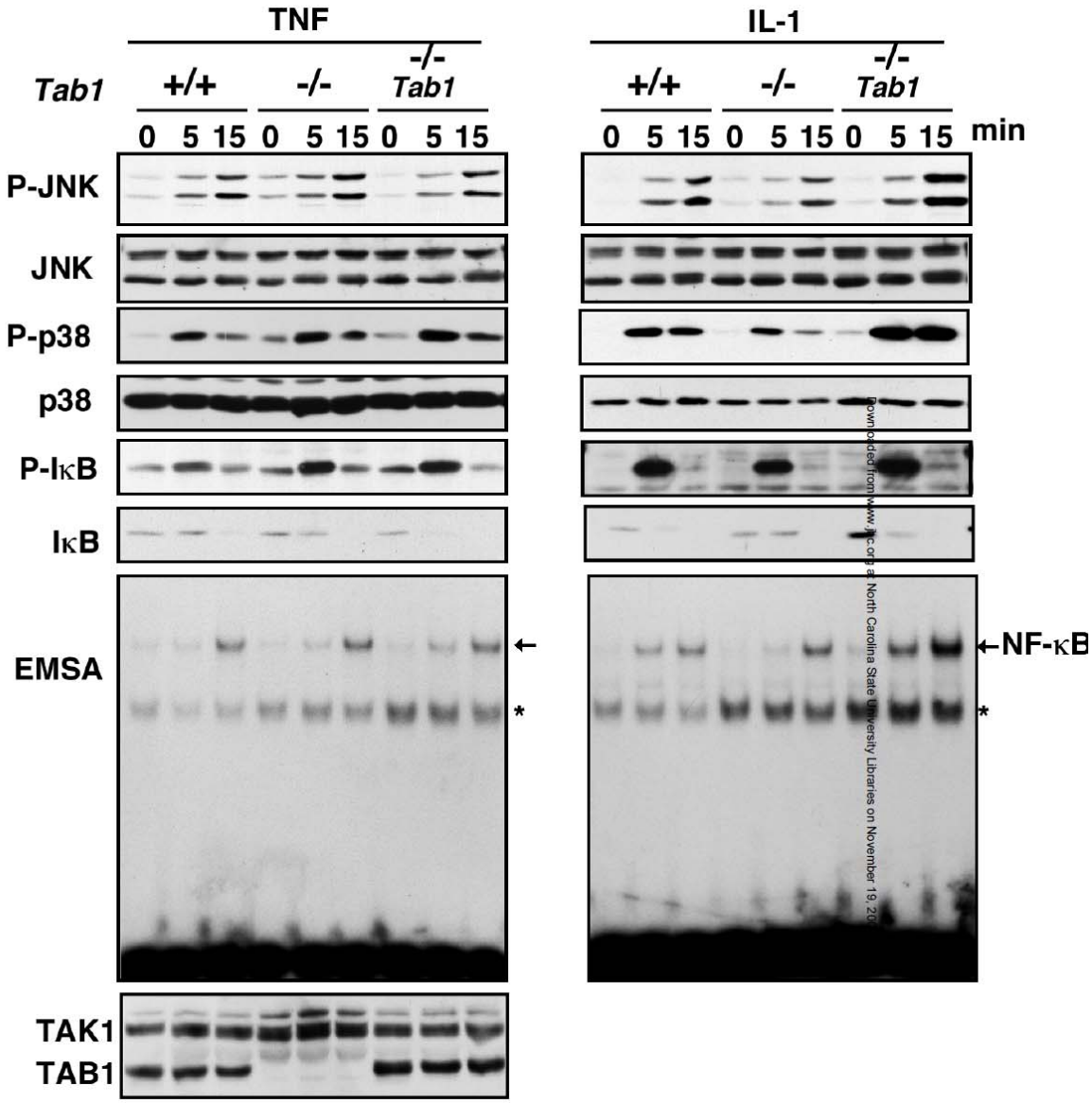
Fig. 5.

TAB1 is essential for concentration-dependent activation of TAK1

(A and B) Cell lysates from *Tab1*-control (+/+) and *Tab1*-deficient (-/-) MEFs were incubated and immunoprecipitated at a high or low protein concentration as described in Experimental Procedures. Immunoprecipitates were incubated in a kinase buffer and analyzed by immunoblots (IB) or by in vitro kinase assay using bacterially expressed MKK6 as an exogenous substrate.

(C) 293 cells were transfected with expression vectors for HA-TAK1 and TAB1. At 48 h post-transfection, approximately 3 μg cell lysates form HA-TAK1 alone (lane 1) or HA-TAK1 + TAB1 (lane 2) transfected cells were loaded onto SDS-PAGE and analyzed by immunoblots. Sizes of upper bands of exogenous and endogenous TAK1 shown in the top panel correspond to the sizes of phosphorylated forms shown in the middle panel.

Inagaki et al. Figure 1



Inagaki et al. Figure 2