UNIVERSITY OF CALIFORNIA, SAN DIEGO

A signaling pathway mediating wound healing responses

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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The Dissertation of Dai-Jen Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my wonderful family.

My parents Sen-Tien Lee and Mei-Liang Lee-Sui,

my brother Chao-Hua Lee,

my sister Ming-Sui Lee,

and my grandmother Tsui-E Lee-Yan.

Without their love and support,

none of this work would have ever been possible.

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LIST OF ABBREVIATIONS

ASC	Apoptosis-Associated Speck-Like Protein
CARD	Caspase Activation and Recruitment Domains
der	Dermis
epi	Epidermis
hf	Hair follicles
Glybu	Glyburide
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
KGF	Keratin Growth Factor
FGF	Fibroblast Growth Factor
IL-1α	Interleukin-1 Alpha
IL-1α inhi Ab	IL-1α Inhibitory Antibody
IKK2-CA	Constitutively Active Mutant of IKK2
TAX	Taxol
NOC	Nocodazole
NFκB	Nuclear Factor κB
p38 MAPK	p38 Mitogen Activated Protein Kinase

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research, and the author of this dissertation was the primary investigator and author of this paper.

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Jeon TJ, **Lee DJ**, Merlot S, Weeks G, Firtel RA. Rap1 controls cell adhesion and cell motility through the regulation of myosin II. J Cell Biol. 2007 Mar 26;176(7):1021-33.

Jeon TJ, **Lee DJ**, Lee S, Weeks G, Firtel RA. Regulation of Rap1 activity by RapGAP1 controls cell adhesion at the front of chemotaxing cells. J Cell Biol. 2007 Dec 3;179(5):833-43.

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ABSTRACT OF THE DISSERTATION

A signaling pathway mediating wound healing responses

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Richard A. Firtel, Co-chair

The goal of tissue repair is to restore the protective barrier function of the skin upon injuries or microorganism infection, and the process relies on a tightly orchestrated system of signaling pathways. The expression of caspase-8, an essential factor in apoptotic signaling cascade, has been reported to play a crucial role mediating wound healing responses. The downregulation of caspase-8 in epidermis recapitulates inflammation and proliferation caused by wounding. Immune cell recruitment and epidermal hyperproliferation cumulatively instigate the expansion of caspase-8 null epidermis. This phenomenon is triggered by the paracrine signaling of interleukin-1α. The secretion of IL-1α is induced by NLRP3

inflammasome, which is a multiprotein complex coordinating the innate immune system. The assembly of NLRP3 inflammasome is the key step for immediate response of inflammation, but the mechanism mediating caspase-1, which is the critical component of NLRP3 inflammasome, is still a mystery. Here we demonstrate that the increased expression of pro-caspase-1 in wound repair process is the limiting factor to coordinate cutaneous inflammation and skin stem cell proliferation regulated by IL-1α, and pro-caspase-1 is transcriptionally regulated by nuclear factor κB (NFκB). Inhibition of NFκB significantly reduces the increased pro-caspase-1 and also prevents the release of IL-1α in caspase-8 knockout and wounded skin. Next we investigated what the primary clue is to initiate the NFκB-caspase-1 pathway. Interestingly, we unveiled that the organization of microtubule is involved in the activation of NFκB, and caspase-8 colocalizes with microtubule filaments in primary keratinocytes. Furthermore, the disorganized microtubule cooresponding to the downregulation of caspase-8 induces the elevation of pro-caspase-1, and stabilization of microtubule in caspase-8 null epidermis inhibits the secretion of IL-1α. Our findings reveal the intricate mechanism regulating inflammation and proliferation by caspase-8 during tissue regeneration, and it provides insights into the cause and the molecular therapy of many inflammatory disorders and cancer.

Chapter I

Introduction

Mammalian skin is the first protective barrier to not only defend outside insults, such as pathogen infection and mechanical damages, but also prevent internal fluid loss and maintain a stable thermal system. As one of the largest organs, skin preserves the ability to proliferate throughout life to maintain the pool of progenitor cells and the capacity to differentiate to different layers for retaining the complex and functional skin structure. Due to its unique capability, skin provides a good environment to study homeostasis and tissue regeneration. There are two main compartments in skin; epidermis and the underlying dermis, which are separated by basement membrane. Basal epidermal cells express intermediate filaments composed of keratins 5 and 14 and fasten through plakin proteins to hemidesmosomes that anchor the cells to basement membrane. Furthermore, keratin filaments also bind to desmoplakin and desmosomal cadherins form cell-cell adhesion structure, desmosomes. Analogously, focal adhesion is also built up between basal cells and basement membrane to regulate cell attachment and motility and it is mainly constructed by actin filaments and integrins. Flanked by adjacent cells, the actin cytoskeleton is linked through adherens junctions that associate through interactions between E-cadherin molecules (Fig 1). Epidermis is composed of proliferating basal layer and three differentiated layers: suprabasal layer, granular layer, and stratum corneum. Skin stem cells, keratinocytes, reside in the basal layer and are responsible for proliferation and differentiation to maintain the capacity of self-renewal and reconstruction followed by turnover and injury. It has been proposed that basal keratinocytes can perform symmetric cell division to produce more

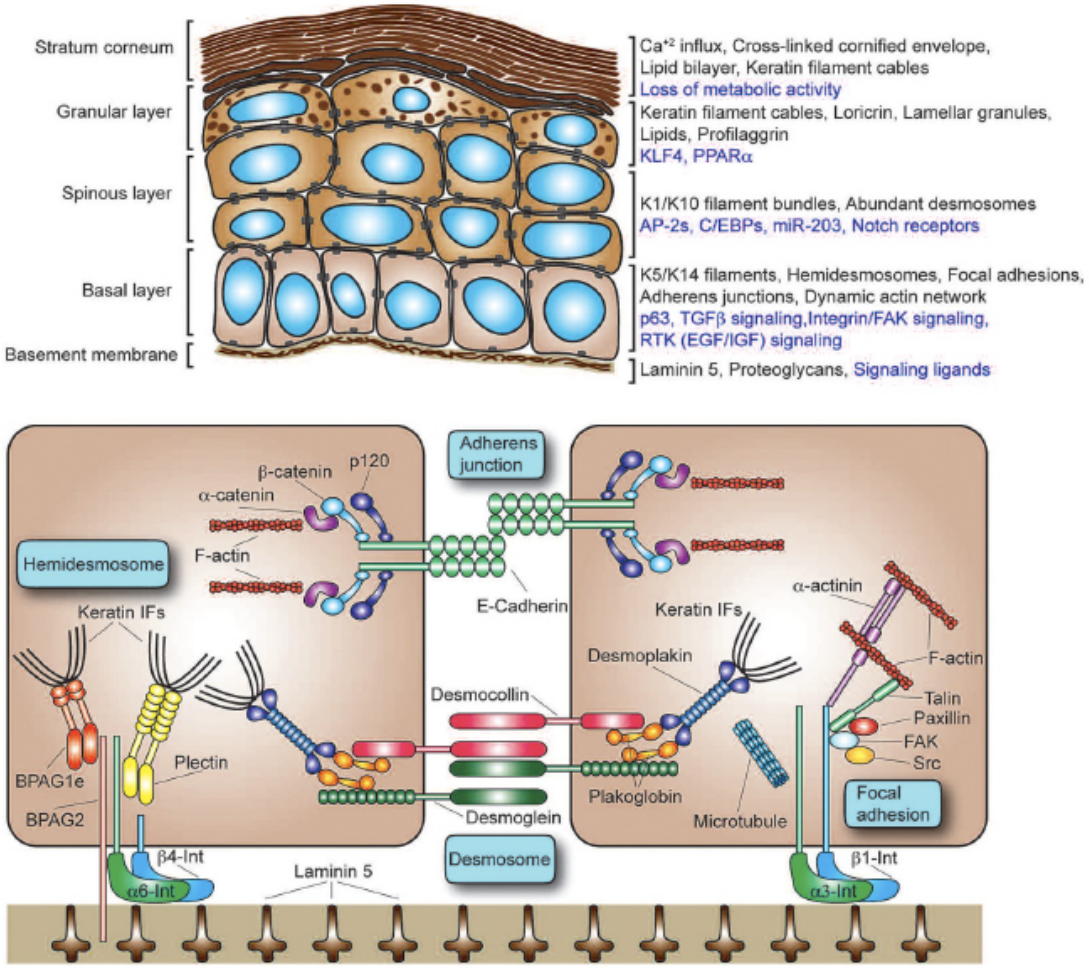

Figure 1. The cellular architecture of the epidermis and major components of the epidermal cytoskeleton. The top panel demonstrates different layers in epidermis and the biochemical markers specifically for each layer. The bottom panel illustrates major elements composed of different adhesion structures in basal keratinocytes.

stem cells for preserving the pool of progenitor cells and asymmetric division to develop stratified layers. Cells that leave the basal layer move first to the spinous layers, where they devote most their time to synthesizing the differentiation-specific keratin filaments, keratin 1 and 10. As cells enter the granular layer, they cease the expression of keratins and instead initiate the expression of loricrin,

which bundles keratin filaments into macrofibrils. Finally cells exit from granular layer and enter terminal differentiation to form stratum corneum, which are merely dead sacs of keratin bundles. Thousands of dead cells slough off from stratum corneum and they are continuously replaced by new cells from granular layers. Dermis is composed of collagens, elastic fibers, and extrafibrillar matrix to form connective tissue and cushions of stress and strain. Hair follicles, sweat glands, sebaceous glands, lymphatic vessels and blood vessels all reside in dermis, and it is also the place where immune cells are recruited upon inflammation to secret growth factors and inflammatory cytokines.

It seems ironic that normal life dependents on ordinary death; skin needs to balance between cellular proliferation and programmed cell death- apoptosis to equilibrate homeostasis. There are several stimuli to initiate apoptosis and it contributes to the development of skin structure, the removal of injured cells, and the elimination of pre-malignant cells. On the other hand, proliferation replaces the loss of skin integrity with new cells and restores the function of protection. The intricate balance relies on the coordination of a large number of different signaling pathways, and once the whole system is off-balanced it causes several skin disorders. It results in skin cancer or psoriasis when cells extend the life cycle instead of entering death termination, or toxic epidermal necrolysis (TEN) and graft-versus-host disease (GVHD) arises if cells are trapped in the decease cycle (REF). Cysteine-aspartic protease (caspase) is one of the critical players to orchestrate this elaborate balance of regeneration and death.

Caspases are well known for their functions on regulating apoptosis, and there two types of caspases: the initiators (caspase-2, caspase-8, caspase-9, and caspase-10) and the effectors (caspase-3, caspase-6, and caspase-7). Caspases are initially synthesized as inactive pro-caspases, including a pro-domain and a catalytic domain, and have post-translational regulation of their functions for rapid responses to stimuli. The regulatory pro-domain of initiator caspases enables them to interact with other proteins leading to the automatic activation and the passage of signals to effector caspases. Additionally, there is another family of caspases, which are involved in several autoimmune diseases, such as caspase-1, caspase-4, and caspase-5. The initiation of apoptosis is classified into 2 different signaling, intrinsic and extrinsic. Fas ligand, tumor necrosis factor (TFN), and related cytokines bind to the extracellular death receptors, which recruit adaptor molecules to activate caspase-8. Consequently caspase-3 and -7 are stimulated to initiate the process of cell death. Alternatively, cytotoxic drug and DNA damage cause mitochondria to release cytochrome-c and promote the intrinsic apoptosis. The potential link of the extrinsic and intrinsic apoptotic pathway is Bid, which is cleaved by caspase-8. Therefore, caspase-8 has a decisive role in mediating apoptosis. Other than the apoptotic function, caspase-8 are also documented to have non-apoptotic functions regulating embryo development, monocyte differentiation, cell motility, and T and B cell proliferation.

The balance of self-renewal and differentiation is well maintained for skin homeostasis, but the equilibrium of skin homeostasis is dramatically shifted to

proliferation upon wounding. Epidermal keratinocytes dedicate to produce new cells and reestablish skin architecture to close the wound. The process of wound repair mimics the microenvironment of skin development and provides a good model to study the mechanism underneath. Wound healing process has been classified into three distinct but overlapping phases: inflammation, proliferation and tissue reconstruction. Although different signaling pathways are responsible for each stage, they are tightly inter-connected. During the inflammatory phase, immune cells are recruited to the wound site for removing pathogens and debris and also secreting inflammatory cytokines and growth factors to promote cell migration and proliferation. Angiogenesis, epithelialization, and wound reconstruction occurr in the proliferative phase. Collagen initiates the remodeling and realignment process in the tissue reconstruction phase to rebuild the intact skin structure. It has been stated that epithelial-mesechymal interactions are critical for pre- and postnatal skin development. These interactions control the formation of limbs, skin architecture, and appendages and they persist in adulthood to regulate skin homeostasis, part of the wound healing responses, or become essentially involved in tumor cell invasion and tumor progression. Furthermore, epidermal proliferation has been proposed to be mediated by a double paracrine signaling: keratinocytes instruct fibroblasts with interleukin-1 (IL-1) to synthesize and secret growth factors and cytokines, such as keratinocyte growth factor (KGF)/fibroblasts growth factor-7 (FGF-7), IL-6, and GM-CSF.

As one of the first growth factors involved in mesenchymal-epithelial interactions, KGF/FGF7 is rapidly induced in fibroblasts after wounding and exerts its effects through binding to its receptor FGFR2IIIb on keratinocytes to initiate proliferation. IL-1, tumor necrosis factor-α (TNF-α), Platelet-derived growth factor (PDGF), and serum were shown to stimulate KGF expression and all these inducers are present at the early stages of wound healing. Moreover, IL-6 deficient mice showed impaired wound repair responses, which suggests that IL-6 can induce epidermal cell proliferation and its expression is strongly upregulated for wounding. Both KGF/FGF7 and IL-6 are primarily produced by mesenchymal cells and act on keratinoctes following the same paracrine pattern. There are also examples where keratinocytes are the primary source of growth factors serving in autocrine and paracrine manners. Oppositely, TGF-α is predominantly expressed in keratinocytes and has profound autocrine effects on keratinocytes. PDGF is another keratinocyte-derived growth factor acting in a paracrine manner to active KGF expression. In brief, these are part of the elements that coordinate epithelial-mesenchymal interactions in wound healing.

There is evidence suggesting that caspase-8 is not only involved in apoptosis, it is also important to wound repair. The expression of caspase-8 is elevated in diabetic mice who have impaired wound healing responses (Al-Mashat et al., 2006), and patients with psoriasis, a form of chronic wound repair, have decreased level of caspase-8 (Chun et al., 2002). We previously discovered that caspase-8 is regularly expressed in the granular layer of murine skin and the level of the protein is downregulated in the wound healing process

(Fig 2). These indicate that the dynamic expression of caspase-8 is important for successful wound healing.

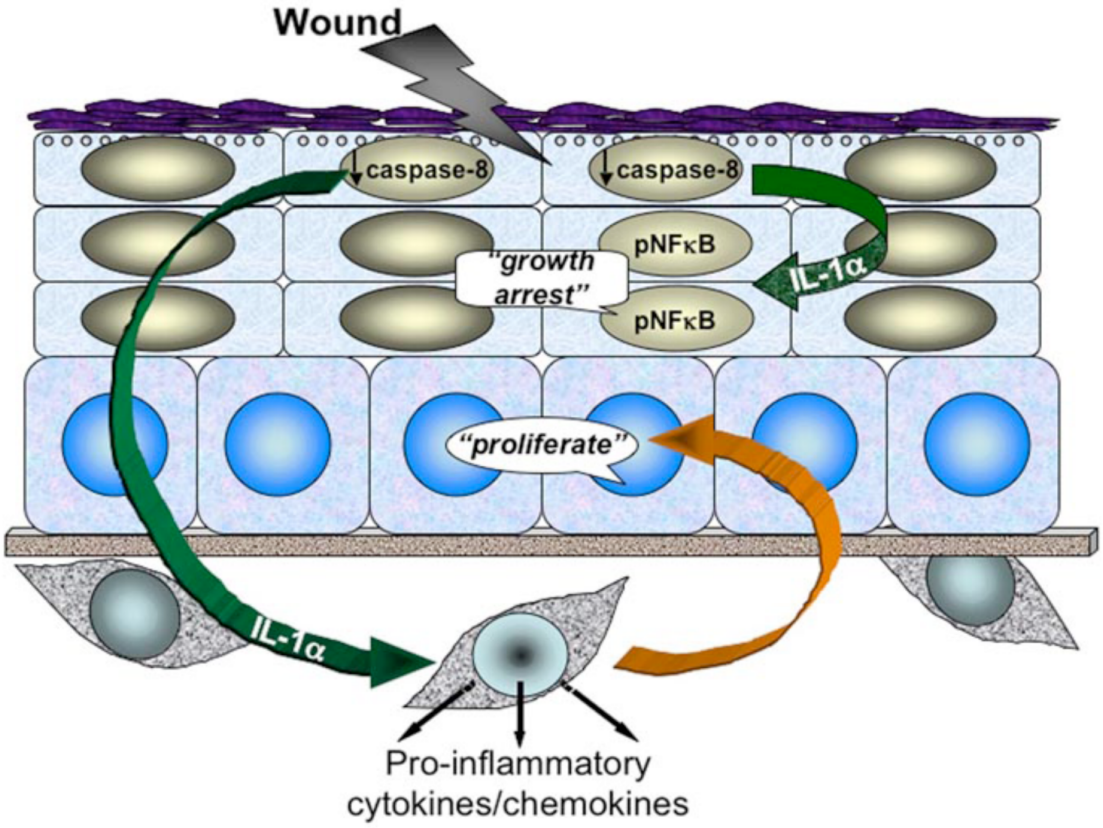

Figure 2. Model of IL-1α dependent epithelial-mesenchymal crosstalk regulating wound healing responses. Downregulation of caspase 8 in the granular layer by wounding initiates the secretion of IL-1α, which stimulates NFκB signaling pathway and the production of pro-inflammatory cytokines and chemokines in dermis. Consequently the proliferation of basal keratinocytes is induced.

To understand how caspase-8 plays its role in responses upon injury, we generated conditional caspase-8 knockout mice and they demonstrate that the loss of caspase-8 recapitulates several phases of wound healing. Immune cells were highly recruited to the dermis and epidermis in caspase-8 null skin compared to wild-type, and NF-κB mediated proinflammatory cytokines were

strongly activated by the loss of caspase-8. In addition, the proliferating basal layer and the suprabasal layer were greatly expanded, and proliferating marker Ki67 and Bcl-x were induced in caspase-8 deficient skin. These observations strongly suggest that the downregulation of caspase-8 mimics inflammatory and proliferative stages of wound healing. Furthermore, we also uncovered that the key factor to synchronize the responses caused by the reduction of caspase-8 was IL-1α. The decrease of caspase-8 in granular layer promoted the secretion of IL-1α, and it sequentially activated the NFκB signaling for cell survival gradually from the granular layer to the basal layer. On the other hand, IL-1α also passed the basement membrane to trigger the expression of growth factors from fibroblasts to epidermal keratinocytes, such as KGF and GM-CSF (Fig 2). Consecutively, keratinocytes begin proliferation after receiving paracrine signals from the mesenchyme. Then we were curious about the connection between downregulated caspase-8 and the release of IL-1α. Interestingly, we found that NLRP3 inflammasomes participate in the regulation of IL-1α secretion, and the expression of NLRP3 is controlled by p38 MAPK (Fig 3). However, there are missing factor explaining the cause of the assembly of NLRP3 inflammasomes and it needs further investigation.

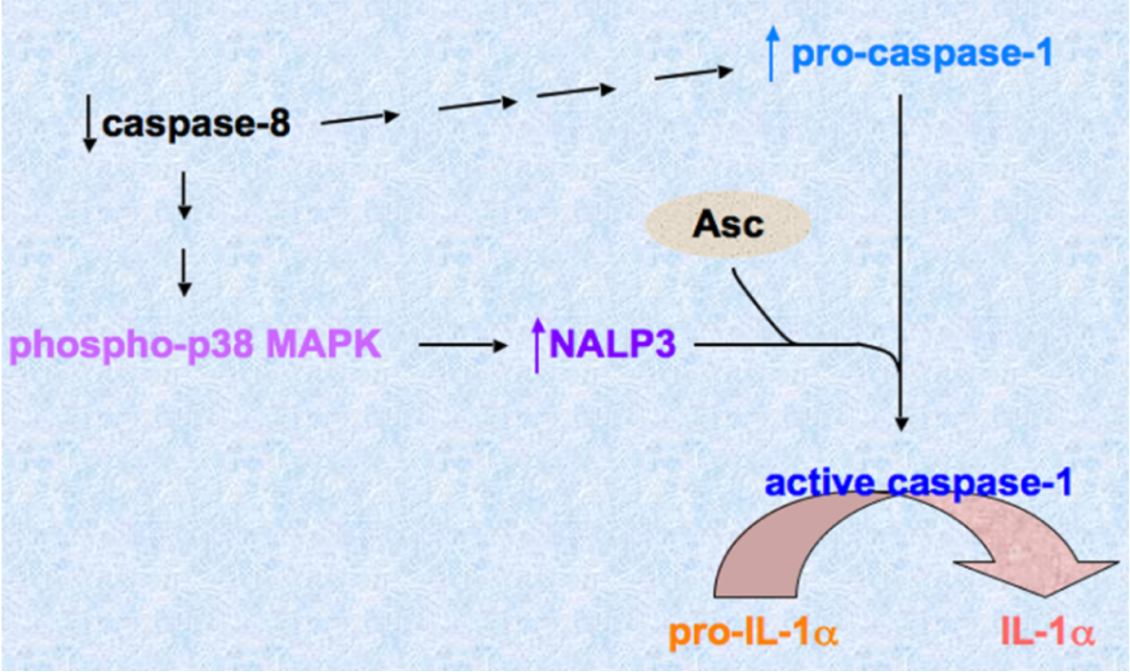

Figure 3. Caspase-8-dependent signaling pathway regulating the secretion of IL-1α. The loss of caspase-8 induces the expression of pro-caspase-1 and NLRP3 through p38 MAPK. NLRP3, ASC, and pro-caspase-1 assemble to NLRP3 inflammasomes, which activate caspase-1 and in turn mediates the secretion of IL-1α.

IL-1α belongs to the IL-1 family, which forms an important part of inflammatory responses against infection. IL-1 family, including IL-1α, IL-1β, IL-18 and IL-33, lacks a signal peptide and they are released by an unconventional, endoplasmic reticulum (ER)/Golgi-independent pathway. IL-1β, IL-18, and IL-33, are expressed as precursor forms and requires to be proteolyticly cleaved to mature forms and then are released out of cells. On the other hand, IL-1α, the functional analog of IL-1β, has recently been shown to bind to the active form of caspase-1 directly, and the activity of caspase-1 is required for IL-1α secretion (Keller et al. 2008). Caspase-1 is expressed as a catalytically inactive zymogen and generally undergoes proteolytic processing to 10kDa (p10) and 20kDa (p20)

subunits upon activation. The activation of Caspase-1 is regulated by several inflammasomes, including, IPAF, NLRP1, and NLRP3. Inflammasomes, which are cytoplasmic multiprotein complexs involved in the activation of inflammatory processes, are composed of the NOD-like receptors (NLRs), the adaptor protein ASC, and inflammatory caspases. There are a number of intracellular receptors coordinating the immune responses upon different stimuli, consisting of Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) (Petrilli, Dostert et al. 2007). The NLR proteins are a family of intracellular sensors not only detecting the presence of pathogens also recognizing endogenous danger or stress signals (Petrilli, Dostert et al. 2007). The NLR family members are characterized by two features, leucine-rich repeats (LRRs) for its autoregulation, pathogen recognition, and protein-protein interaction, and a NACHT nucleotide-binding domain (NBD) for self-oligomerization. Once NLR receptors identify certain antigens, they begin the recruitment of other proteins for further inflammatory signaling pathways, such as the assembly of inflammasomes. NLRP3 is one of the most studied NLRs and forms NLRP3 inflammasome with ASC and pro-caspase-1. ASC binds to NLRP3 through PYD domain and also recruits pro-caspase-1 by its CARD domain. There are increasing evidences demonstrating that NLRP3 inflammasomes respond to a huge number of stimuli. Not only pathogens including fungi, bacteria, viruses and microbial components activate NLRP3 inflammasomes, but also host-derived molecules of injured cells, metabolic stress, sunburn, and environmental irritants induce the assembly of NLRP3 inflammasomes (Faustin

and Reed, 2008; Lamkanfi and Dixit, 2009; Schroder and Tschopp; Schroder et al.; Stutz et al., 2009). Most studies focus on the activation of NLRP3 inflammasomes, but very little is known about the transcriptional regulation of their components. Intriguingly, we previously unveiled that NLRP3 inflammasomes are activated in the absence of caspase 8 and wounding. In addition, p38 MAPK is a specific activator of NLRP3 expression and the level of ACS remains unchanged. As one of the components of NLRP3 inflammasomes, the level of pro-caspase-1 protein becomes a critical factor to mediate the assembly. Although the level of pro-caspase-1 is higher in the absence of caspase-8, there is still a basal level of the protein detected in the wild-type skin. Nevertheless, the regulation of pro-caspase-1 is far from understood and it becomes a challenging question to study.

As discussed above, caspases require the cleavage of their pro-domain to become biologicaly active, and it has been reported that elevated expression of caspase-8 can induce the self-cleaving and auto-activation. Interestingly, although it is well known that the activation of caspase-1 is regulated by inflammasomes, it has also been documented that caspase-1 has spontaneous activation in full-length caspase-1 transgenic mice (Yamanaka et. al). Without any induction of NLR receptors, caspase-1 shows higher protein level of active forms and increased activity of processing IL-18 in caspase-1 transgenic mice. This observation provides a fascinating idea that the expression of caspase-1 may regulate the auto-activation process. However, our current knowledge only

defines a small portion of the mechanism controlling the expression of pro-caspase-1.

Additionally, we previously found that NFκB is induced in the caspase-8 knockout and wounded skin, and has a similar expression pattern as active caspase-1. Members of the NFκB family of transcription factors are critical regulators in the development and maintenance of the immune system and in the coordinated response to infection. For example, the proinflammatory cytokines TNFα and IL-1 signal are secreted to activate NFκB signaling pathway upon stimulation, and NFκB is also involved in many other cellular aspects of differentiation and growth arrest. NFκB is composed of homo- and heterodimers of five members of the Rel family including NFκB1 (p50), NFκB2 (p52), RelA (p65), RelB, and c-Rel. Each Rel protein contains a conserved N-terminal region, called the Rel Homology Domain (RHD), and the RHD contains the DNA-binding and dimerization domains and the nuclear localization signal of the Rel proteins. The C-termial of RHD of RelA (p65), RelB and c-Rel have potent transactivation domains consisting of serine, acidic and hydrophobic amino acids which are essential for transactivation activity. However, p50 and p52 do not possess transactivation domains and therefore cannot act as transcriptional activators by themselves. Nevertheless, the p50 and p52 NFκB members play critical roles in modulating the specificity of NFκB function. Despite that homodimers of p50 and p52 are, in general, repressors of κB site transcription, both p50 and p52 participate in target gene transactivation by forming heterodimers with RelA, RelB, or c-Rel. The dimerization of NFκB subunits is required for the binding of

NFκB to DNA of target genes. The NFκB dimerization domain is located in the C-terminal region of the RHD, whereas the N-terminal part of the RHD contains the DNA-binding domain. Furthermore, Nuclear Localization Signal (NLS), which is essential for the transport of NFκB into the nucleus, is localized at the C-terminal end of the RHD. In unstimulated cells, NFκB dimers are sequestered in the cytosol via non-covalent interactions with a class of inhibitor proteins, called IκBs. There are several members of IκB and they all contain ankyrin repeats mediating the association between IκB and NFκB dimers. The NLS of NFκB is masked by the connection between IκB and NFκB, so NFκB is trapped in the cytosol. Signals that induce IκB kinase (IKK) cause the phosphorylation of IkBs, and subsequently result in ubiquitination and degradation by the 26S proteasome complex. The IKK complex contains two kinase subunits, IKKα and IKKβ, and IKKγ/NEMO, a noncatalytic subunit. Deletion of the inducible subunits has shown that IKKβ and IKKγ/NEMO are required for TNFα- or IL-1-induced phosphorylation of IκB, while IKKα is dispensable (Hu et al.). The dissociation of IκBs from NFκB releases the active form of NFκB which in turn translocates to the nucleus and binds to the κB site of target genes. Once bound to a κB motif, NFκB also interacts with DNA-associated proteins as well as the general transcriptional factors.

Interestingly, NFκB has been reported to transcriptionally regulate the expression of caspase-11, which is also involved in the activation of caspase-1 (Schauvliege et al., 2002) in response to LPS and IFN-γ induction. Moreover, both the level of phospho-IκBα and the expression of caspase-1 are

concomitantly induced in photoreceptor cells after light exposure, which indicates that caspase-1 may be regulated by NFκB. And NFκB and caspase-1 are important for the harmonized responses of inflammation. Increasing evidence has pointed to a potential role for NFκB in the mediation of pro-caspase-1 expression.

NFκB is essential for inflammatory responses due to its capacity of rapid-acting. NFκB is present in cells in an inactive state and does not require new protein synthesis to be activated. This allows NFκB to be the first responder to harmful cellular stimuli. Therefore, NFκB responds to a wide variety of cell-surface receptors, such as RANK, TNFR, and initiates gene expression in a very short period of time. Many bacterial products can activate NFκB through the identification of Toll-like receptors (TLRs) leading to the activation of NFκB. Also, UV radiation and stress are shown to induce the activation of NFκB signaling. Furthermore, microtubule organization is known to be involved in TNF-induced NFκB activation, and the microtubule stabilizing drug, taxol, affects the activity of NFκB. In addition, the inhibitor IκB interacts with a microtubule associated protein, dynein, and influence NFκB activity. On the other hand, Michael Karin and his colleagues demonstrated that depolymerization of microtubule activates NFκB. These provide an intriguing link between cytoskeleton dynamics and gene regulation. There are many possible pathways to induce NFκB upon different stimuli, but the real activator of NFκB in wound healing remains unclear.

In brief, wounding results in the loss of epidermal caspse-8, and both the activity of NFκB and the expression of pro-caspase-1 are induced in the process

of wound repair. In this dissertation, I demonstrate that the downregulation of caspase-8 initiates the activation of NFκB, and active NFκB in turn induces the synthesis of pro-caspase-1. Pro-caspase-1, ASC, and p38 MAPK-mediated NLRP3 assembles to form NLRP3 inflammasomes regulating the secretion of IL-1α. The elevated release of IL-1α stimulates inflammatory and proliferative phases of wound healing, and inhibition of NFκB or caspase-1 abolishes the secretion and effects of IL-1α, which suggests that both NFκB and caspase-1 activities are required for IL-1α secretion. Taken together, we propose a novel model illustrating the regulation and function of the caspase-1 inflammasome in the context of wound healing.

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The text of Chapter I, in part, is the material submitted for publication in 2010, by Dai-Jen Lee, Shih-Wei Chen, Vincent F.-S. Shih, Alexander Hoffmann, and Colin Jamora. Dr. Colin Jamora guided and supervised the writing of the introductory material.

Chapter II

Regulation and Function of Caspase-1 Inflammasomes in Wound Healing

EXPERIMENTAL PROCEDURES

Reagents.

Primary antibodies used were against: NFκB p65 (Santa Cruz, SC-472), caspase 8(Alexis Biochemicals, 1G12), caspase 1(caspase-1 p10, SC-514), phospho-NFκB Ser 276 (Cell Signaling) and HA tag (). FITC-Rhodamine Red-X-, and HRP-conjugated, biotinylated secondary antibodies were from Jackson ImmunoResearch. Dilutions were followed by the instruction of manufacturer's recommandation. DAPI is also applied on cells for nucleus staining. IL-1α inhibitory antibody is from R&D Systems and the usage is calculated by C50. Chemical inhibitors specific for blocking IκB kinase (IKK-I, 80μM), p38 MAPK (p38-I, 2.4 μM), caspase 1 (casp1-I, 40 μM) are all from Calbiochem. Glyburide (200μM) was employed on epidermis for 1 hour at 37°C. Recombinant mouse IL-1α (R&D Systems, 2ng/mL) was added to the media and incubated with wild-type and ikbα knockout epidermis at 37°C for 1 hour, and with primary keratinocytes for 4 hours for chromatin precipitation.

Mice and cell culture.

Generation of caspase-8 conditional knockout mice. Epidermis specific knockouts were obtained by crossing mice carrying the floxed caspase-8 allele (Casp8 fl/fl) to K14-Cre mice. Ikbα knockout mice are the gift from Alexander Hoffmann. Wild-type primary mouse keratinocytes are obtained from newborn pups and cultured in low low-calcium mouse keratinocyte media as described previously (REF).

Tissue preparation and conditioned media collection.

Conditioned media and epidermis was incubated with p38-I and IKK-I overnight and subjected to IL-1α ELISA and RNA preparation. The wash-out of IKK-I treated epidermis was done by washing with PBS once and transferred to fresh mouse keratinocyte media and incubated for another 6 hours. Glyburide was incubated with epidermis for 1 hour, and transferred to fresh media with glyburide for another hour and collected the conditioned media at this step for IL-1α ELISA. IL-1α (2 ng/ul) was added to WT and ikbα KO epidermis for 2 hours, and replaced with fresh media and incubated for another 6 hours for conditioned media collection.

In vivo wounding and injection of mice.

DMSO, IKK-I, and C1-I were injected to the back skin of WT and caspase 8 KO daily for 4 days starting at P0, and back skin were harvested at the 5th day for RNA extraction and OCT embedding. *In vivo* wounding was performed on the back skin of adult CD-1 WT mice with 5mm-punch biopsy, and DMSO and inhibitors were applied to the wound site daily for 3 days. The concentrations of inhibitors are described above. Wounded skin was collected for RNA extraction and OCT embedding.

In vivo wound healing assay.

Excision wound healing experiments were performed as previously described with some modifications (Crowe et al., 2000). Briefly, 2-month-old CD-1 wild-type were anesthesized and two full-thickness skin punch biopsies of 5mm were created using a disposable dermal biopsy punch. Skin wounds were

documented everyday after wounding using a digital camera and the wound area was measured using Image J software (NIH).

Constructs.

Constitutively active form of IKK2 (IKK2-CA) is the gift from Alexander Hoffman, and the pRL-TK containing Renilla luciferase vector is from Promega. DNA within 650 bp of the transcription initiation site of caspase 1 was cloned into pGL3 luciferase reporter vector for luciferase assay, and the mutation on the NFκB binding site is applied to examine the specificity. The primers used are: WT 5'GTTTTTGGTACCCAAGGGCATCCCCTGCCAGG3', 5'GTTTTTCTCGAGCGTTTCTTTTCTACACCGCAG3', and mutant: 5'GTTTTTGGTACCCAAGTTTTTCCCCTGCCAGG, 5'GTTTTTCTCGAGCGTTTCTTTTCTACACCGCAG3'.

Transfection.

TransIT (Mirus) was used to transfect primary mouse keratinocytes with expression vectors. For luciferase reporters, WT-mC1 and mutant-mC1 were cotransfected with pRL-TK and IKK2-CA construct into primary keratinocytes respectively. K14-MCS vector is substituted for mC1 luciferase reporter as the control. For qPCR experiment, IKK2CA is transfected as described above to mouse keratinocytes and after 24 hours, cells were collected in Trizol reagent (Invitrogen) for RNA extraction.

***In vitro* scratch wounding assay.**

Primary mouse keratinocytes were cultured on cover slips in a 24-well Petri dish to confluent and treated with 1.2mM $CaCl_2$ overnight to differentiate

mouse keratinocytes to epithelial sheet. The scratch wound was performed on the cell monolayer with a p200 pipet tip. Remove the debris and smooth the edge of the scratch by washing the cells once with PBS and then replacing with the complete mouse keratinocyte media with higher calcium concentration. Place the dish in a tissue culture incubator at 37°C for 8~16 hours and then fix cells with 4% formaldehyde at RT for immunofluorescence staining or harvest cells for RNA extraction.

Quantitative Real Time–PCR.

Epidermis from wild-type (P5, *n* = 3) and caspase 8 knockout (P5, *n* = 3) mice, iκBα knockout (P0, n=3) was separated with dispase treatment for 1 hour at 37 °C and total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesized by reverse transcription using iScript cDNA Synthesis kit (Bio-Rad). Quantitative real-time PCR analysis was performed by a Bio-Rad CFX96 Real-Time system and reactions were completed using the Sso Fast Green QPCR reagent mix (Bio-Rad). Experiments were done in triplicate from cDNA isolated from three different animals. Melt curve and data quantification were analyzed by Bio-Rad CFX manager software. Primers are described previously (Aravalli et al., 2005; Kobielak and Fuchs, 2006).

Luciferase Reporter Assays.

The pRL-TK vector containing DNA encoding Renilla luciferase was used for the internal control of transfection efficiency. After 24 hour of transfection, cells were subjected to the Dual-Luciferase Kit (Promega) for measuring

luciferase activity. Experiments are performed in triplicate for 3 independent times.

NFκB translocation assay.

For scratch assay, differentiated primary keratinocytes were prepared by calsium switch overnight as described above. Nuclear translocation of NFkB in primary keratinocytes was monitored by an antibody recognizing NFκB p65 from Santa Cruz.

IL-1α ELISA.

Epidermis from postnatal day 3 (P3) caspase-8 WT and KO was incubated with mouse keratinocyte media containing DMSO or the IKK inhibitor (200 μM) from Calbiochem overnight and then collected conditioned media. Secreted mouse IL-1a levels were detected using the Quantikine kit from R&D systems according to manufacturer's instructions.

Chromatin immunoprecipitation and PCR

Wild-type mouse keratinocytes were treated with PBS and recombinant mouse IL-1α (2ng/ml) for 6 hours and cells are harvested for ChIP. Protein-chromatin complexes were crosslinked in keratinocytes with 1% formaldehyde, followed by sonication to fragment genomic DNA to a mid-range of 600 bp (protocols from H. Singh and Cheng CS). DNA from anti-p65 NFκB immunoprecipitation was subjected to PCR using Taq-polymerase (Promega) and primers specific for a 300-bp sequence encompassing the NFκB binding site (NFκB site;
5′AGAGCTATGGGTGTTTGTTGTTTGT3′,5′AAAAAGCATATGAGGCAGGGCAG

TA3′)and a site ~1kb upstream (5′ site;
5′GTAACACCTTGTGGTCATAAATTAA3′,
5′TCCAAGATCCAACTTGAAAAACTTT′). PCR was performed with 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, 72 °C for 30 s. PCR products were separated on 1% agarose gels and quantified by qPCR.

Immunofluorescence and immunohistochemistry staining.

Tissue samples for immunofluorescence were frozen in OCT and sectioned 8 μm thick on a cryostat. Sections were fixed in 4% paraformaldehyde for 10 min at room temperature, blocked, and stained with antibodies. Samples stained with phospho-NFκB and Ki67 were antigen unmasked with 10 mM sodium citrate (pH 6). The DAB substrate kit (Vector Labs) was used according to manufacturer's instructions to develop the signal.

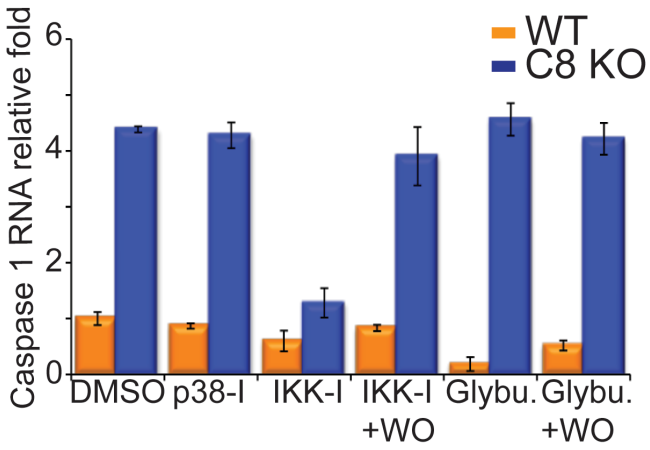
RESULTS

The expression of caspase-1 is induced by the activity of NFκB

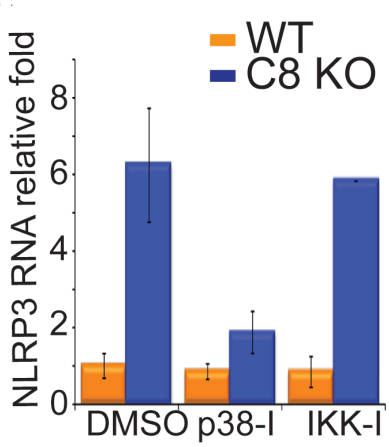
Given its critical role in mediating various features of inflammation and the increased activity during wound healing, we tested the hypothesis that NFκB may regulate the elevated expression of pro-caspase-1. First of all, we investigated the association between NFκB activity and pro-caspase-1 expression in the wounding microenvironment. We took the wild-type and caspase-8 conditional knockout littermates at postnatal day 5 (P5) and blocked the activity of NFκB by the inhibitor of IKK (IKK-I) to examine the mRNA level of pro-caspase-1. Inhibition of IKK hinders the degradation of IκB and consequently inhibits the activation of NFκB. Consistent with the previous observation, the RNA level of pro-caspase-1 from the knockout epidermis is 4-fold higher than the wild-type sample treated with vehicle control ((Lee et al., 2009), Fig. 4A). Interestingly, the increased pro-caspase-1 by the deletion of caspase-8 is diminished by the treatment of IKK-I (Fig. 4A). And the removal of IKK inhibitor from the knockout explants restores the higher expression of pro-caspase-1, which rule out the possibility of off-target effects. Additionally, phosphorylated p38 MAPK is reported to be active and mediates NLRP3 in both the caspase-8 knockout and wounded skin ((Lee et al., 2009) and Fig. 4B), and we checked whether it is also involved in the regulation of pro-caspase-1. In contrast, the p38 MAPK inhibitor affects neither the expression of pro-caspase-1 nor ASC (Fig 4B and C). Furthermore, glyburide has been documented to inhibit the NLRP3

Figure 4. The regulation of pro-caspase-1 expression is NFκB dependent.
A. P5 of WT and caspase-8 KO epidermis were treated with DMSO, inhibitors for p38 MAPK (p38-I) and IKK (IKK-I) overnight and glyburide treatment is performed for 1 hours. Washout (Crepieux et al.) procedure is described in Experimental Procedures. Epidermal samples were then subjected to RNA extraction for quantitative RT-PCR analysis of pro-caspase 1 RNA.
B. Quantitative RT-PCR is probed for the RNA expression of NLRP3 in DMSO, p38-I, and IKK-I treated WT and KO epidermis.
C. Quantitative RT-PCR is probed for the RNA expression of ASC in DMSO, p38-I, and IKK-I treated WT and KO epidermis.

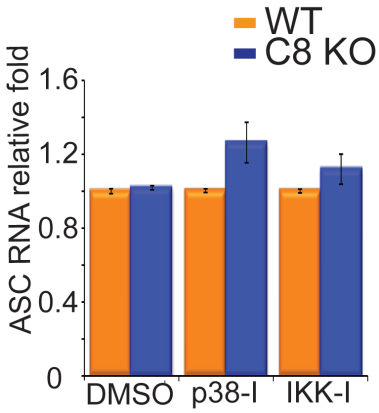
A


WT
C8 KO
Caspase 1 RNA relative fold
0
2
4
6
DMSO
p38-I
IKK-I
IKK-I +WO
Glybu.
Glybu. +WO

B


WT
C8 KO
NLRP3 RNA relative fold
0
2
4
6
8
DMSO
p38-I
IKK-I

C

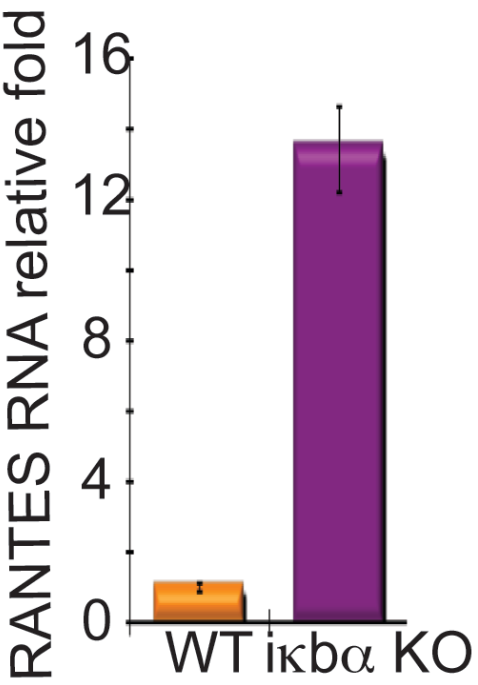

WT
C8 KO
ASC RNA relative fold
0
0.4
0.8
1.2
1.6
DMSO
p38-I
IKK-I

inflammasomes and the release of IL-18 (Lamkanfi et al., 2009). But similar to the effect of p38 MAPK inhibitor, the treatment of glyburide does not affect the expression of pro-caspase-1 instead that the level of pro-caspase-1 maintains at the same level before and after the wash-out (Fig 4A). On the other hand, the activation of NFκB has also been shown to mediate NLRP3 expression responding to LPS stimulation (Bauernfeind et al., 2009). To examine this possibility in wound healing, we probed for NLRP3 in IKK-I treated WT and caspase-8 knockout epidermis. The result in Fig 4B revealed that elevated NLRP3 is not affected by IKK-I and excluded the link between NFκB and NLRP3. The other component of NLRP3 inflammasomes, ASC, has the same expression level in either wild-type and caspase 8 null epidermis upon the application of IKK inhibitor and p38 MAPK inhibitor (Fig 4C). These results suggest a functional role for NFκB in the induction of pro-caspase-1 expression caused by caspase-8 downregulation.

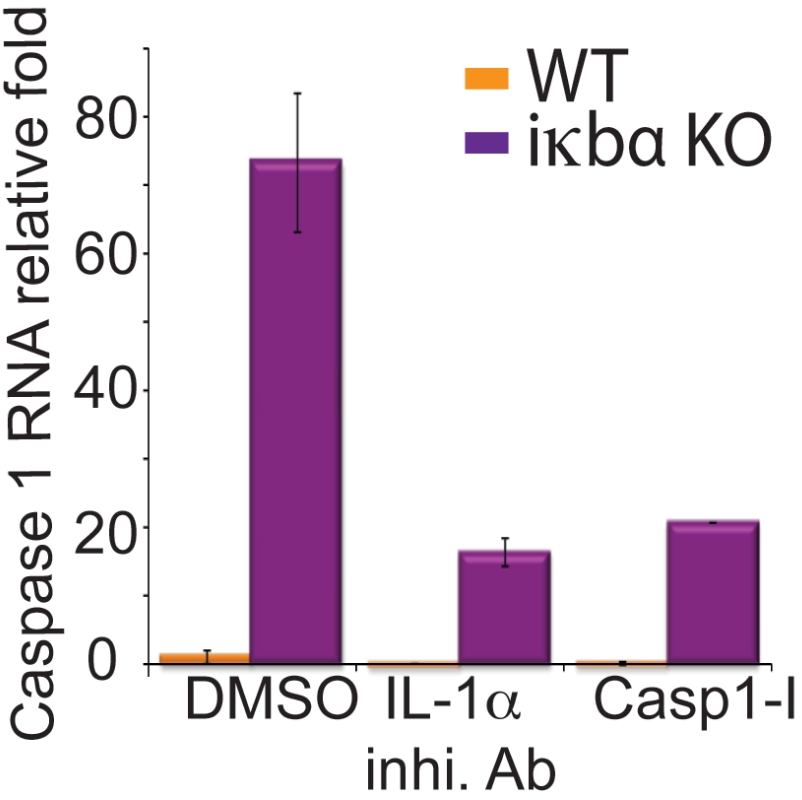
To further study the connection between NFκB and pro-caspase-1, we examined the RNA level of pro-caspase-1 in ikbα deficient mice. IκBα, one of the IκB family members, is the most studied IκB protein, and knocking out IκBα will release active form of NFκB. However, the basal level of NFκB activity in wild-type and ikbα knockout mice are the same (Data not shown). Instead, NFκB has prolonged activation time upon the stimulus of IL-1 or TNF (Klement et al., 1996). Furthermore, we also confirmed that a bona fide NFκB target gene, RANTES, has ~13 fold increased expression in IL-1α induced ikbα deficient epidermis (Fig 5A). Employing the benefit of extended NFκB activation in the loss of IκBα, we

Figure 5. RANTES and pro-caspase-1 are upregulated in ikbα deficient mice.
A. RNA was extracted from P0 of WT and ikbα KO epidermis and examined for the expression of NFκB target gene, RANTES, by quantitative RT-PCR.
B. RNA samples from DMSO, IL-1α inhibitory antibody (IL-1α inhi. Ab), and caspase 1 inhibitor (casp1-I) treated WT and ikbα KO epidermis were probed for the expression of pro-caspase-1.

A


RANTES RNA relative fold
16
12
8
4
0
WT
iκbα KO

B

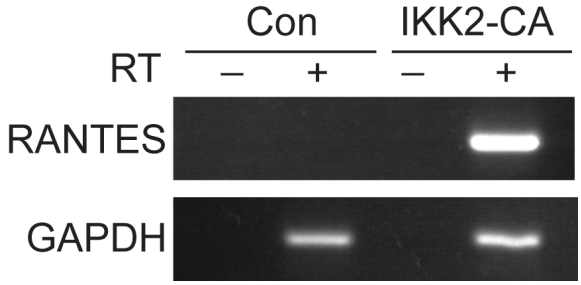

Caspase 1 RNA relative fold
80
60
40
20
0
WT
iκbα KO
DMSO
IL-1α
Casp1-I
inhi. Ab

assayed the expression of pro-caspase-1 in the lack of ikbα. As predicted, pro-caspase-1 is highly upregulated correspondingly with the increased activity of NFκB in ikbα knockout epidermis (Fig 5B). Moreover, secreted IL-1α is not only the product of active caspase-1 but is also an activator of NFκB (Vallabhapurapu and Karin, 2009). We analyzed the possibility of IL-1α working in a positive feedback loop regulating the activation of NFκB by probing pro-caspase-1 expression with IL-1α neutralizing antibody in ikbα deficient mice. The data in Fig 5B indicated that blocking the release of IL-1α significantly reduces the increase of pro-caspase-1 suggesting that IL-1α is released in the loss of ikbα and also stimulates NFκB activation and pro-caspase-1 expression consecutively. In spite of this, application of the IL-1α neutralizing antibody does not completely abolish the increased pro-caspase-1 expression to basal level, which suggests that there should be some initial signals involved in the regulation of pro-caspase-1, excluding IL-1α. The activity of caspase-1 is essential to the secretion of IL-1α, and catalytically inhibiting caspase-1 will reduce the release of IL-1α. Consistent with the hypothetic positive feedback of NFκB activation by IL-1α, the expression of pro-caspase-1 is notably lower in caspase-1 inhibitor treated ikbα knockout epidermis (Fig 5B). The *in vivo* study strongly imply the relationship of NFκB activation and pro-caspase-1 expression, we switched to the *in vitro* system for further research on signaling pathway.

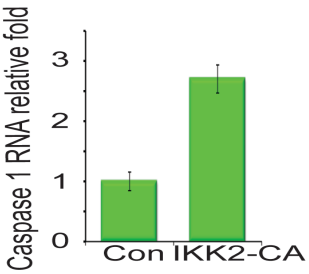
The overexpression of constitutively active IKK2 mutant in primary keratinocytes not only increases the expression of RANTES (Fig 6A), but also translocates NFκB into nucleus (Fig 6C). Additionally, the RNA level of pro-

Figure 6. Constitutively active IKK2 mutant activates NFκB and induces pro-caspase-1 expression.
A. RT-PCR is performed in the control (Con) with empty vector transfection and constitutively active IKK2 (IKK2-CA) transfected mouse keratinocytes to probe for the expression of NFκB target gene, RANTES. GAPDH is for the loading control.
B. The RNA level of pro-caspase-1 is examined in the control and IKK2-CA transfected cells by quantitative RT-PCR.
C. The localization of NFκB and the protein expression of caspase-1 were examined by immunofluorescence staining in IKK2-CA overexpressed primary mouse keratinocytes. Anti-NFκB antibody specifically recognized p65 subunit of NFκB, anti-HA tag antibody recognized transfected HA-IKK2-CA, and DAPI stained for nuclei.
D. The localization of NFκB is visualized by immunofluorescence staining in HA-IKK2-CA transfected cells treated with DMSO, caspase-1 inhibitor (C1-I), and IL-1α inhibitory antibody (IL-1α inhi. Ab). HA antibody localizes the transfected cells.

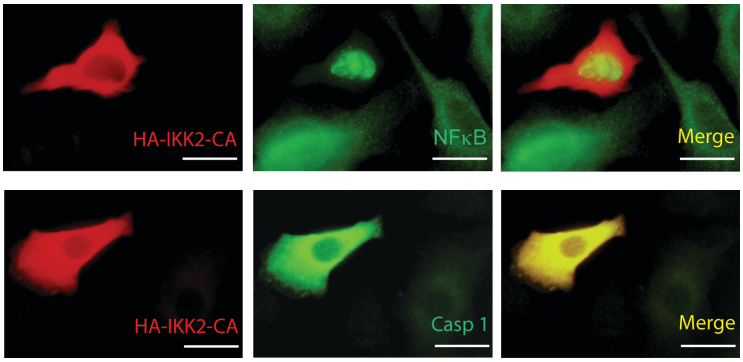
A


Con
IKK2-CA
RT
–
+
–
+
RANTES
GAPDH

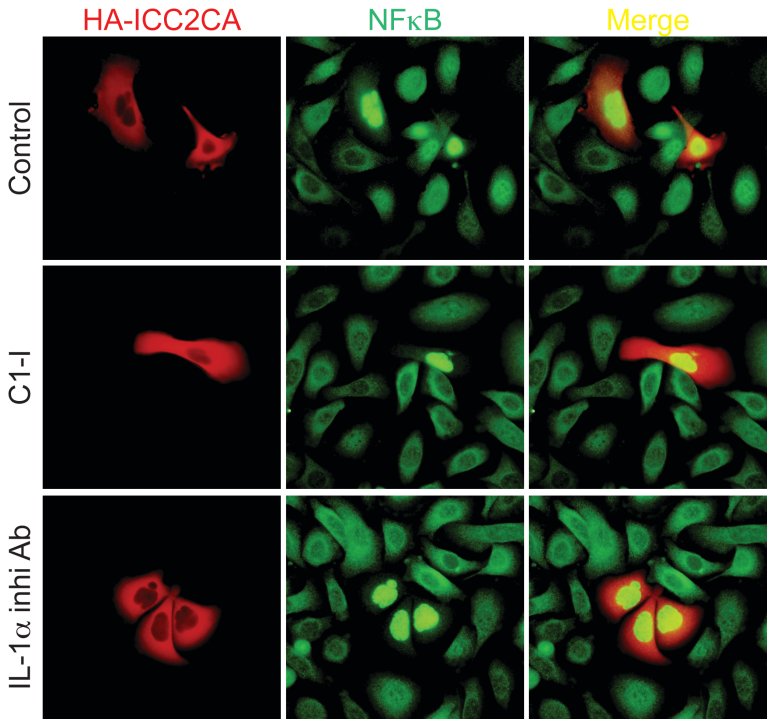
B


Caspase 1 RNA relative fold
3
2
1
0
Con
IKK2-CA

C


HA-IKK2-CA
NFκB
Merge
HA-IKK2-CA
Casp 1
Merge

D


HA-ICC2CA
NFκB
Merge
Control
C1-I
IL-1α inhi Ab

caspase-1 is induced to ~2.5-fold in transfected cells compared to the control with empty vector transfection (Fig 6B). Also, IKK2-CA expressing keratinocytes showed higher protein expression of pro-caspase-1 in comparison with neighboring non-transfected cells (Fig 6C). Due to the potential role of IL-1α to activate NFκB, we examined the localization of nuclear NFκB in IKK2-CA transfected cells in the absence and presence of caspase-1 inhibitor and IL-1α inhibitory antibody to block the secretion of IL-1α. Keratinocytes were transfected with the mutant construct for 2 days to let cells have enough time to release IL-1α, and the first panel of Fig 6D showed that not only transfected cell but also surrounding cells have nuclear NFκB staining, which indicates that the elevated NFκB activity by IKK2-CA discharge some signals to trigger NFκB translocation in neighboring cells. And blocking the secretion of IL-1α either by the inhibitor of caspase-1 or IL-1α inhibitory antibody successfully abolishes the cell autonomous effects on NFκB activation (Fig 6D). Evidence supports the function of IL-1α on activation of the NFκB-caspase-1 pathway, we confirmed the increase of pro-caspase-1 protein in IL-1α stimulated mouse keratinocytes by western blotting (Fig 7A). The result illustrated that the protein level of pro-caspase-1 is higher in response to IL-1α stimulation, and the increase is inhibited by IKK inhibitor. Consistent with our hypothesis, IL-1α induces NFκB activation and consequently promotes the expression of pro-caspase-1. But the protein is not affected by glyburide, which only affects the function of NLRP3 inflammasomes. Altogether, these observations support that NFκB plays an

important role in the regulation of pro-caspase-1 expression in response to wound healing.

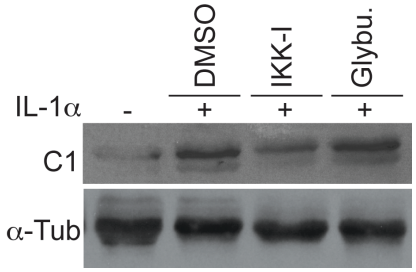
NFκB binds to the promoter of caspase-1 and regulates its expression

The previous results suggest that NFκB is involved in the regulation of caspase-1 expression, but it is not clear whether NFκB is the direct upstream transcription factor of caspase-1 or there are some other intermediate proteins. To address this question, we first did the sequence analysis of putative transcription factors in mouse caspase 1 gene (Genomatix, data not shown). Interestingly, we found a conserved NFκB binding motif resides within 1 kb of the transcription initiation site of caspase-1 and the sequence is shown in Fig 7B, and we also performed a mutation on the consensus site to abolish the binding capacity. Thus, we investigated the promoter activity of the NFκB binding site by the luciferase reporter assay. The influence of NFκB on the promoter activity of caspase-1 was examined by co-transfecting IKK2CA construct with wild-type (WT-mC1) and mutant (mut-mC1) caspase 1 luciferase reporter construct respectively. The activityof wild-type caspase-1 promoter was stimulated by the transfection of IKK2-CA, and mutation of the binding site significantly diminished the response of the mutant promoter (Fig 7C). These numbers support the idea that the putative NFκB binding site on the promoter region of caspase-1 is essential for the transcriptional regulation of its expression.

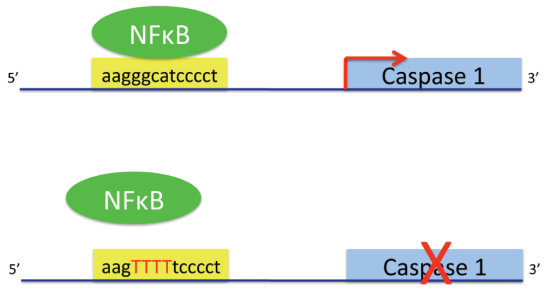
Additionally, the binding of NFκB to the conserved motif is judged by chromatin immunoprecipitation (ChIP) assays performed on mouse keratinocytes

Figure 7. NFκB transcriptionally regulates pro-caspase-1 in response to IL-1α stimulation.
A. Protein level of pro-caspase-1 was examined in IL-1α stimulated mouse keratinocytes, and effects of IKK-I and glyburide on pro-caspase-1 expression was also inspected in stimulated cells. α-Tubulin (α-Tub) was employed as the loading control.
B. The diagram of the conserved NFκB binding site in the 5' region of caspase-1 sequence. Mutation on the binding site eliminates the binding capacity to NFκB.
C. The promoter activity of WT and mutant mouse caspase-1 was analyzed by the luciferase assay, and the activity of NFκB is induced by the transfection of IKK2-CA.
D. The temporal profile of caspase-1 expression in response to IL-1α was examined at different timepoints of IL-1α treatment in primary keratinocytes.
E. ChIP is performed with the NFκB p65 antibody and precipitated DNA is judged by PCR for the sequence encompassing the NFκB binding site (NFκB site) and the sequence around 1 kb upstream of the binding motif as a negative control (upstream). And the loading control is done by the Western blotting probing for immunoprecipitated NFκB (IP NFκB).

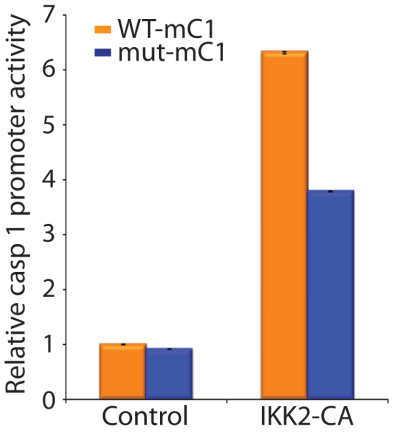
A


DMSO
IKK-I
Glybu.
IL-1α - + + +
C1
α-Tub

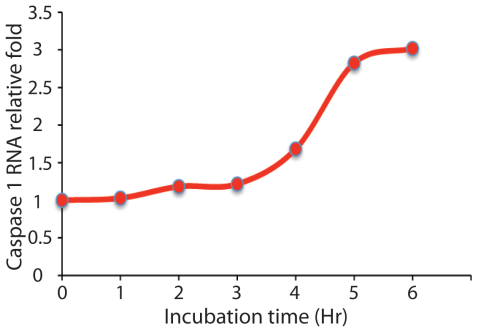
B


NFκB
aagggcatcccct
Caspase 1
5′
3′
NFκB
aagTTTTtcccct
Caspase 1
5′
3′

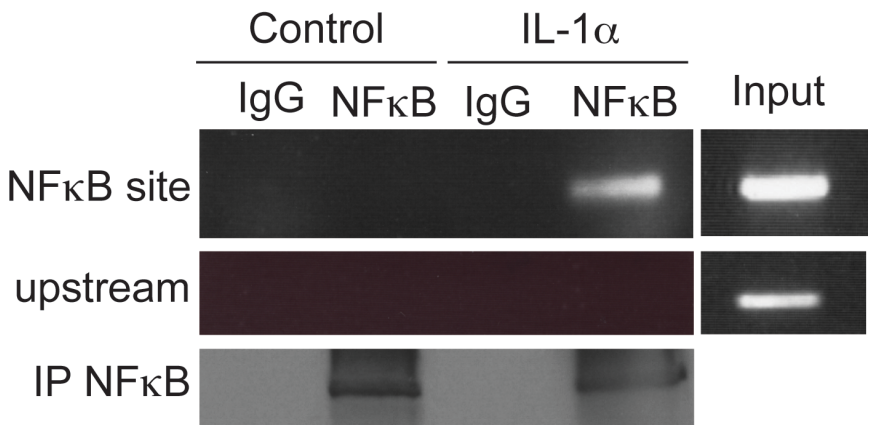
C


Relative casp 1 promoter activity
WT-mC1
mut-mC1
0
1
2
3
4
5
6
7
Control
IKK2-CA

D


Caspase 1 RNA relative fold
0
0.5
1
1.5
2
2.5
3
3.5
0
1
2
3
4
5
6
Incubation time (Hr)

E


Control
IL-1α
IgG
NFκB
IgG
NFκB
Input
NFκB site
upstream
IP NFκB

treated with IL-1α for 4 hours. The expression of pro-caspase-1 starts to climb up at 4 hours after IL-1α induction (Fig 7D), which indicates that the promoter activity of caspase-1 is initiated at that time-point. Only after IL-1α induction were ChIP complexes detected that contained NFκB and the sequences that encompassed the most highly conserved putative NFκB binding site in the caspase-1 promoter (Fig 7E). These complexes were obtained with antibodies against the p65 subunit of NFκB. Conversely, this antibody did not immunoprecipitate DNA from promoter regions that lacked putative NFκB motif. The loading control of ChIP was normalized with the protein level of precipitated NFκB by the western blotting. These results verify that NFκB is a direct activator of caspase-1 expression.

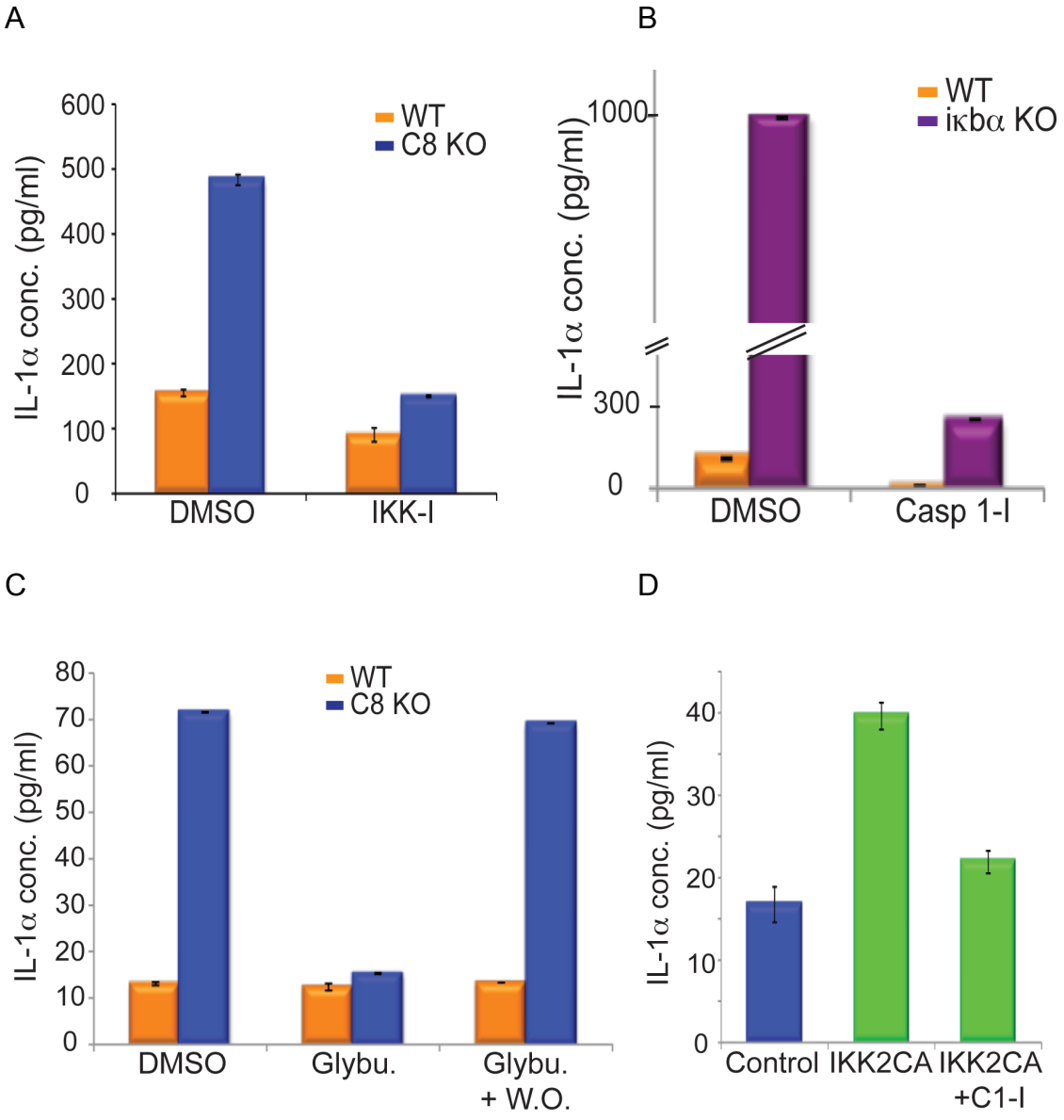
IL-1α secretion depends on the activity of caspase-1.

The expression of pro-caspase-1 is upregulated in the wounding microenvironment, and next I studied the relationship between higher level of protein expression and its activity. To examine the activity of caspase-1, the secretion of IL-1 is routinely used as a read-out for this purpose. IL-1 family is synthesized as a precursor form and caspase-1 is critical for its maturation and secretion (Keller et al., 2008). IL-1α, which belongs to IL-1 family, has a pre-existing pool of its pro-forms in mouse keratinocytes and they are biologically active (Keller et al., 2008). However, the secretion of pro-IL-1α is still in a caspase-1 dependent pathway in the process of wound healing (Lee et al., 2009). To investigate the activity of caspase-1 in response to wounding, I extracted the wild-type and caspase 8 knockout epidermis and incubated in

mouse keratinocyte media with vehicle control and the IKK inhibitor overnight to collect the conditioned media and assayed the amount of secreted IL-1α. In Fig 8A, I detected ~4-fold increase of IL-1α secretion in the knockout conditioned media, which indicates that the increased caspase-1 activity is consistent to the elevated expression of pro-caspase-1. Intriguingly, the inhibition of NFκB by IKK-I not only decreased the expression of pro-caspase-1, but also reduced the secretion of IL-1α back to the wild-type level (Fig 8A). This data suggests that the expression of pro-caspase-1 is a critical factor for its activity. Moreover, the higher activity of NFκB in iκBα deficient mice increased the expression of pro-caspase-1 (Fig 5B), and I next planned to examine whether the release of IL-1α is promoted. For collecting the conditioned media from IL-1α stimulated wild-type and iκbα knockout epidermis, I first induced the epidermis with IL-1α for two hours and transferred it to fresh media and incubated for another 6 hours. In consistent, the quantity of secreted IL-1α was also elevated in the lack of iκbα (Fig 8B). Furthermore, blocking the activity of caspase-1 in iκbα knockout epidermis significantly lowered the amount of secreted IL-1α. However, the inhibition of caspase-1 activity did not fully block the increase of IL-1α secretion, and this may due to the activation of other upstream signaling pathways by the induction of IL-1α.

Glyburide has been reported to effectively prevent the activation of NLRP3 inflammasomes, the processing of pro-caspase-1, and the secretion of IL-18 (Lamkanfi et al., 2009), and our previous data showed that NLRP3 inflammasomes were involved in the release of IL-1α in wounded and caspase 8

Figure 8. The expression of pro-caspase-1 is essential for its activation.
A. Conditioned media harvested from DMSO and IKK-I treated WT and caspase 8 KO epidermis was subjected to ELISA for IL-1α secretion.
B. The conditioned media incubated with WT and ικbα KO epidermis were assayed for IL-1α secretion by ELISA. The effect of caspase-1 inhibitor (Casp 1-I) on IL-1α release is also evaluated.
C. Glyburide treatment is followed by the procedure stated in Experimental Procedures, and conditioned media collected from the treatment is probed for the quantity of IL-1α.
D. The transfection of IKK2-CA induced the activation of caspase-1, and inhibition of caspase-1 activity diminished the elevated release of IL-1α.


A
IL-1α conc. (pg/ml)
600
500
400
300
200
100
0
WT
C8 KO
DMSO
IKK-I
B
IL-1α conc. (pg/ml)
1000
300
0
WT
iκbα KO
DMSO
Casp 1-I
C
IL-1α conc. (pg/ml)
80
70
60
50
40
30
20
10
0
WT
C8 KO
DMSO
Glybu.
Glybu.
+ W.O.
D
IL-1α conc. (pg/ml)
40
30
20
10
0
Control
IKK2CA
IKK2CA
+C1-I

knockout skin (Lee et al., 2009). Subsequently, I examined whether the activation of NLRP3 inflammasomes are required for in the secretion of IL-1α caused by lacking of caspase-8. I also harvested the conditioned media from wild-type and caspase-8 knockout epidermis treated with glyburide and subjected to IL-1α ELISA assay. The results showed that the glyburide treatment abrogated the rise of IL-1α secretion, and the wash-out after the treatment substantially restored the increase of secreted IL-1α, which rules out the off-target effects of glyburide. Even in the *in vitro* system to increase the expression of pro-caspase-1 by NFκB activation, IL-1α was promoted to be secreted more. The reduction of detected IL-1α by caspase-1 inhibitor restated the importance of caspase-1 activity to IL-1α release. Briefly, these data reveals that the expansion of pro-caspase-1 expression induces the activation of caspase-1, and the activation of NLRP3 inflammasomes is required for caspase-1 activity.

The *in vitro* scratch-wounding assay recapitulates the *in vivo* wounding responses

Due to the heterozygous population of cell types in skin, it becomes more difficult to investigate the signaling pathway underneath in the *in vivo* environment. To solve this problem, I isolated primary epidermal keratinocytes from wild-type newborn mice and used for *in vitro* experiments. First, I tested whether *in vitro* wounding recapitulates the downregulation of caspase-8 observed *in vivo*. Differentiated keratinocytes were cultured on glass cover-slips to imitate the epidermal sheet and then the scratch was applied on top to create a wound site, and at different time points, keratinocytes were either fixed for

Figure 9. The *in vitro* scratch wounding recapitulates the *in vivo* wound healing

A. Differentiated keratinocytes were cultured on glass cover slips and a scratch was performed on the cell sheet. After 8 and 16 hours of scratching, the protein expression of caspase 8 is examined by immunofluorescence staining with an antibody specifically recognizing the catalytic of caspase 8. The up-right corner of the right panel is the scratch site.

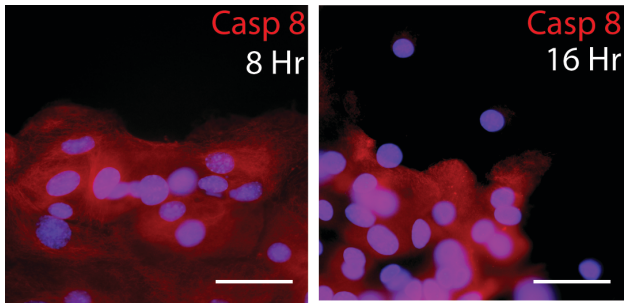
B. The nuclear localization of NF κ B was stained with the NF κ B p65 antibody in the in vitro scratch assay at 8 and 16 hour time points.

C. Scratched cells were harvested for RNA extraction and probed for RANTES expression by quantitative RT-PCR.

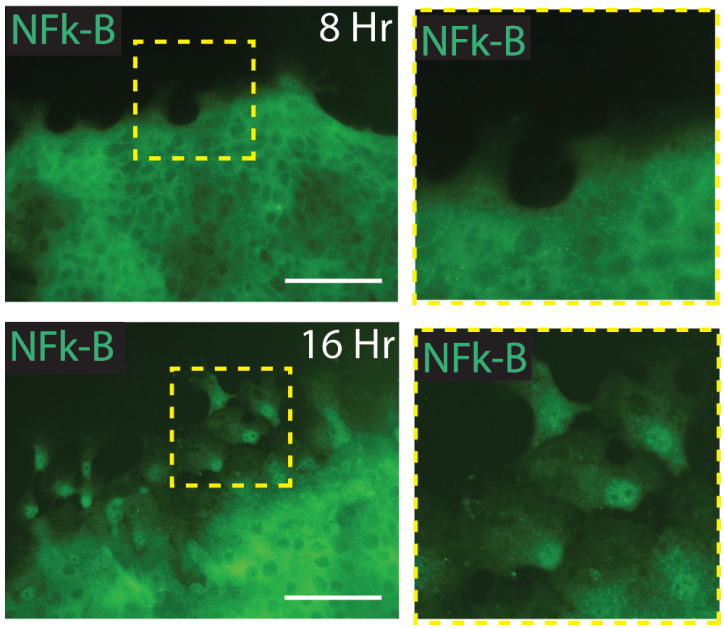
D. RNA samples from C were also probed for the expression of pro-caspase 1.

Scale bar: 20 μ m (A) and 50 μ m (B)

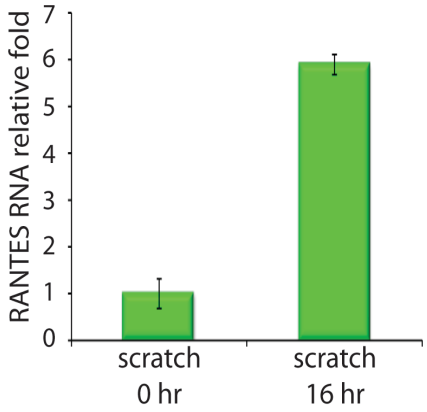
A


Casp 8
8 Hr
Casp 8
16 Hr

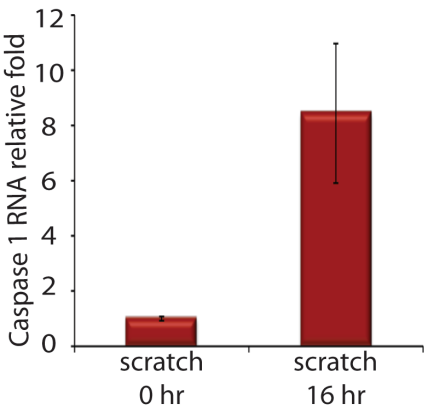
B


NFk-B
8 Hr
NFk-B
NFk-B
16 Hr
NFk-B

C


RANTES RNA relative fold
0
1
2
3
4
5
6
7
scratch
0 hr
scratch
16 hr

D


Caspase 1 RNA relative fold
0
2
4
6
8
10
12
scratch
0 hr
scratch
16 hr

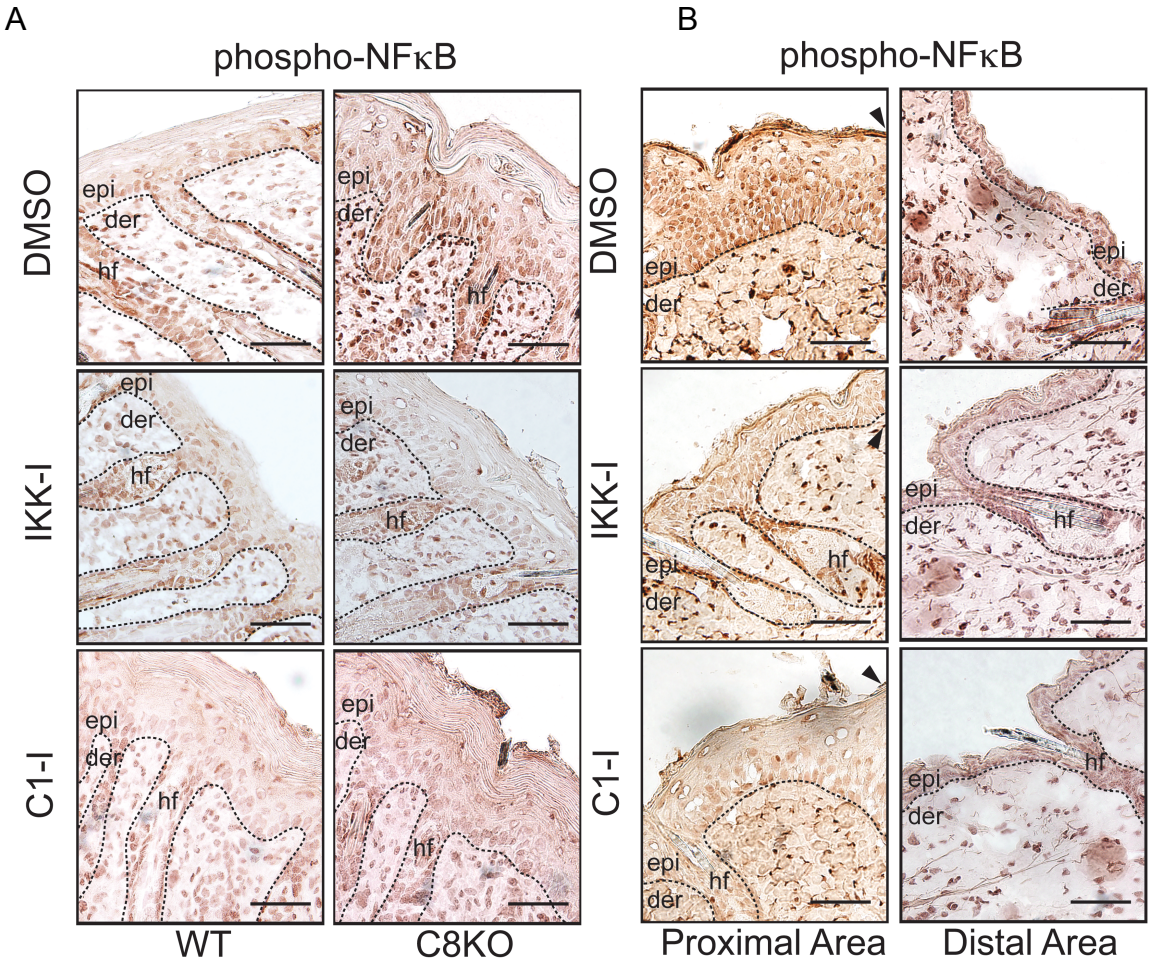
staining or harvested for RNA extraction. Before scratching, caspase-8 was expressed evenly in keratinocytes, and 16 hours after wounding, cells started to migrate out of the cell sheet (Fig 9A). In those moving keratinocytes, caspase-8 protein expression was visibly decreased compared to non-motile cells (Fig 9A). This result mimics the observation of caspase-8 downregulation in the wounded epidermis (Lee et al., 2009). Besides, I also detected nuclear NFκB staining in migrating keratinocytes (Fig 9B), and NFκB stayed in the cytoplasm of non-moving cells and distal cells from the wound site. The activity of NFκB responding to scratching was assessed by probing the expression of RANTES, and it was stimulated in the same kinetics as NFκB translocation (Fig 9C). Furthermore, the RNA expression of pro-caspase-1 was also increased more than 8-fold increase following scratching (Fig 9D). In summary, the *in vitro* scratch-wounding assay on mouse keratinocytes recapitulates several phenotypes of wounding on mammalian skin, and offers a simplified environment to explore the signaling mechanism orchestrating the complicated wound healing processes.

Positive feedback mediation of NFκB activation.

The previous results illustrate that NFκB is activated in the process of wound healing, and this activation results in the increased expression and activity of caspase-1. Therefore, the reservoir of IL-1α is released to synchronize different signaling cascades mediating inflammatory and proliferative responses for wound repair. Furthermore, free IL-1α also works as a positive feedback activator to amply the signals and to relay the information to the whole wounded

system. For further study of the physiological function of NFκB-caspase-1 pathway, I investigated the responses caused by the inhibition of NFκB and caspase-1 during wound healing on animals. First of all, we took the capacity of caspase-8 knockout animals recapitulating wound healing to explore this inquiry. The inhibitor of IKK and caspase-1 were injected to the back skin of wild-type and knockout pups respectively starting at postnatal day 0 (P0) and continued the injection daily for 4 days. Same as the published data, I detected higher level of nuclear phospho-NFκB in caspase-8 knockout epidermis, and a more intriguing observation is that the inhibition of caspase-1 significantly suppressed active NFκB expression but in a less level compared to what IKK-I inhibits (Fig 10A). Blocking the activity of caspase-1 impaired the release of IL-1α, and also restrained the positive feedback effect on NFκB activation. But as previous data suggests the possibility of undefined primary signals to initiate NFκB-caspase-1 pathway, there was still phospho-NFκB detected in C1-I inhibited caspase-8 deficient epidermis. To verify the phenotypes of IKK and caspase-1 inhibition in caspase-8 knockout epidermis, I also applied IKK-I and C1-I to the wounded area of wild-type adult mice, and comparable effects of the inhibitors were observed at proximal sites (<1mm) to wound (Fig 10B). However, at areas distal (~5mm) to the wound, the expression of phospho-NFκB remains unaffected (Fig 10B). Surprisingly, the hyperplasia of epidermis caused by the loss of caspase-8 and wounding was significantly rescued by the application of IKK-I. Although the C1-I didn’t affect the expansion of epidermis as dramatically as IKK-I, the inhibition of

Figure 10. NFκB activation in wound healing is partially mediated by casapse-1.
A. DMSO, IKK-I, and C1-I injected WT and casapase 8-KO back skin were subjected to immunohistochemistry staining for phospho-NFκB. Dotted line marks the basement membrane that separates the epidermis (epi.) from the underlying dermis (der).
B. DMSO, IKK-I and C1-I were applied to the wound site of WT mice, and skin samples proximal and distal to the wound were stained for phospho-NFκB. Scale bar: 20 μ m.


A
phospho-NFκB
DMSO
IKK-I
C1-I
epi
der
hf
WT
C8KO
B
phospho-NFκB
DMSO
IKK-I
C1-I
epi
der
hf
Proximal Area
Distal Area

caspase-1 certainly decreased the phenomenon of epidermal hyperproliferation. These data strengthens the hypothesis of feedback regulation of NFκB by IL-1α.

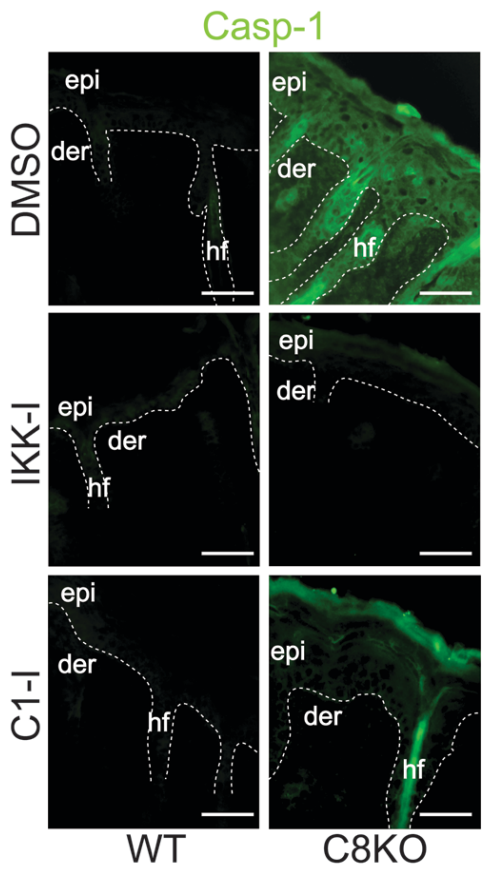
Since the inhibition of IKK and caspase-1 both caused a loss of NFκB activation in the epidermis of caspase-8 deficient and wounded skin, I next examined the influence of the inhibitors on the expression of caspase-1. Cytoplasmic caspase-1 was expressed at a low basal level in wild type epidermis and is significantly upregulated in the caspase-8 null tissue (Fig 11A). This induction of caspase-1 expression is lost when the knockout mice were injected with the inhibitors to IKK or caspase-1. Therefore, abrogation of NFκB activation inhibits the upregulation of caspase-1 expression and inhibition of the latter's proteolytic activity can effectively block the positive feedback loop of NFκB activation mediated by IL-1α and the paracrine signaling of NFκB and caspase-1 expression throughout the epidermis. Consistent data was also observed in wounded skin applied with the inhibitors of IKK and caspase-1 (Fig 11B).

Inhibition of NFκB and caspase-1 delay wound closure rates.

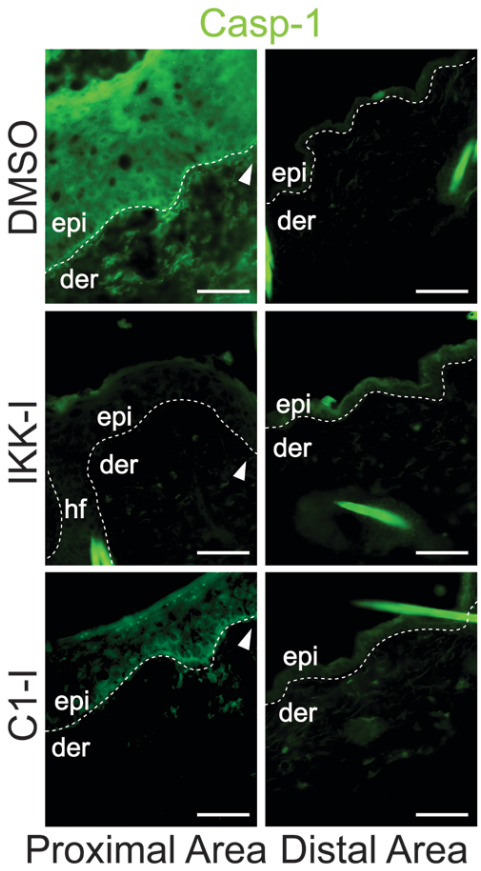
The results of in vivo study on the inhibition of IKK and caspase-1 suggested that the NFκB-caspase-1 pathway is required for the upregulation of pro-caspase-1. To further study on the physiological role of NF-κB-caspase-1 in wound healing, I performed the wound closure assay on DMSO and the inhibitors treated wounded wild-type mice. In Fig. 12A, it showed that there was no much difference between each treatment during the first 3 days, which is usually known as the inflammatory phase. However, the wounds inhibited with IKKI- and C1-I displayed slower wound closuring in the later stages of tissue repair.

Figure 11. NFκB activation is required for the upregulation of caspase-1 in wounding.
A. Expression of caspase-1 is visualized by immunofluorescence staining in WT and caspase-8 null skin injected with DMSO, IKK-I, and C1-I.
B. DMSO, IKK-I, and C1-I treated wounded skin was subjected to immunofluorescence staining of caspase-1. Distal area to the wound is >5mm away form the wound site. Scale bar: 20 μm**.**

A


Casp-1
DMSO
IKK-I
C1-I
epi
der
hf
WT
C8KO

B


Casp-1
DMSO
IKK-I
C1-I
epi
der
hf
Proximal Area
Distal Area

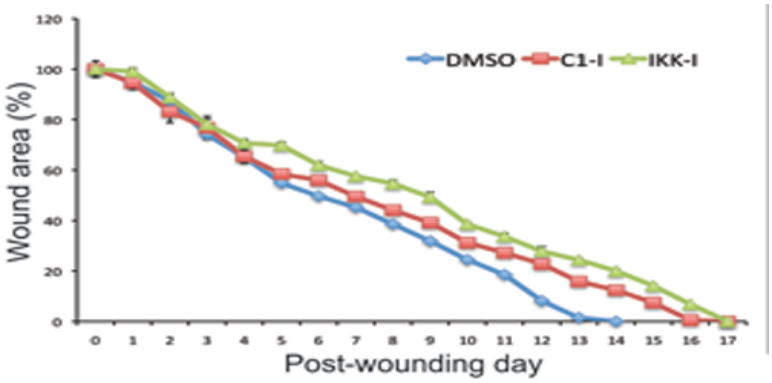
Furthermore, the wounds with the inhibitors were completely healed 2~3 days later than the control. This data suggests that the proliferative phase is impaired by the inhibition of NFκB and caspase-1, which is shown to reduce the activity of caspase-1 inflammasomes (Fig. 8) Combined with the previous results, it exhibits that restraining the release of IL-1α by blocking the inflammasomes significantly suspend the normal responses during wound healing.

NFκB-caspase-1 pathway plays an important role on inflammation in the context of wound healing

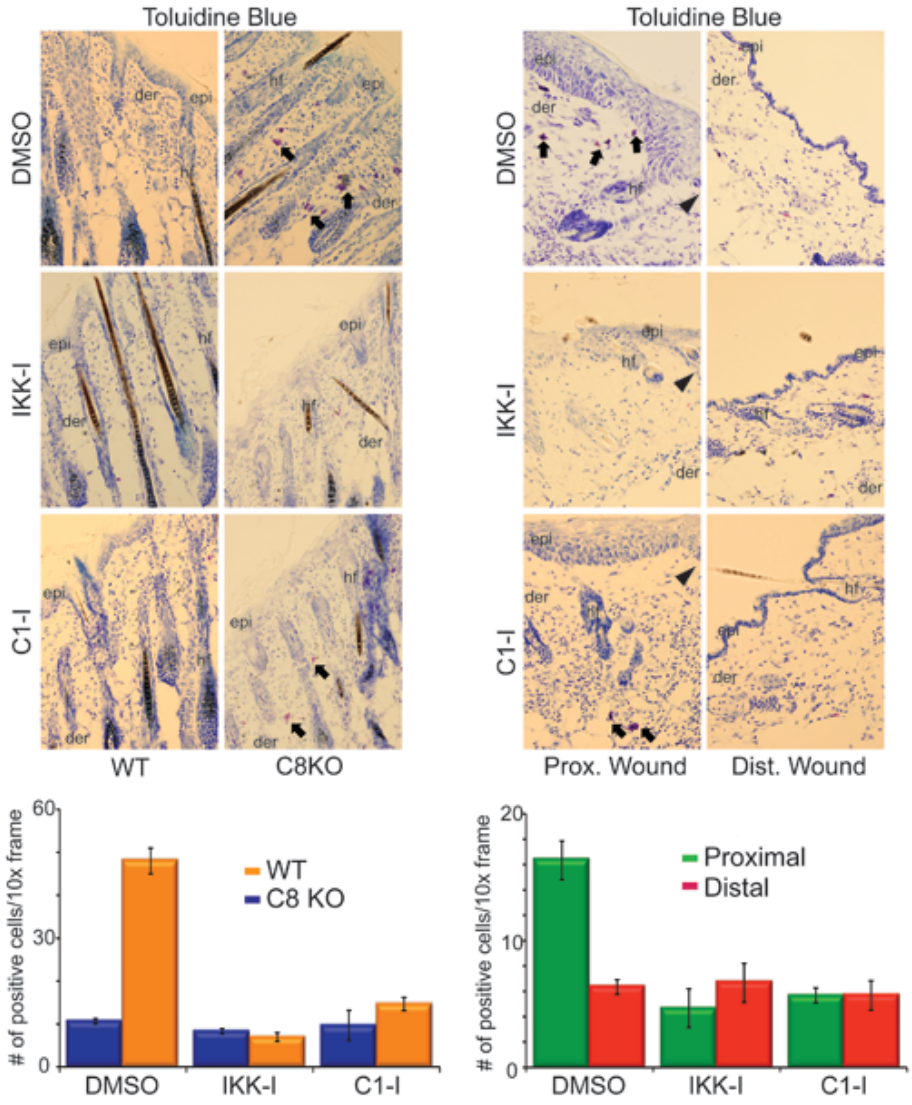
To investigate how inhibition of NFκB and caspase-1 delayed the process of tissue repair, I examined the responses of different phases in wound healing. For inflammation, the recruitment of immune cells to the wound site is critical to activate the production of inflammatory cytokines and chemokines, and proliferative growth factors. To examine the distribution of immune cells in the wounding environment, I used different markers to distinguish each one. First of all, mast cells were visualized by Toluidine blue staining (Fig. 12B) and the number of recruited cells was quantified in the lower panel. According to the quantification of mast cells detected under 10X magnification, the number was 5 times higher in the knockout skin (Fig.12B). Furthermore, as predicted, the elevated population of mast cells caused by the lack of caspase-8 was decreased by the inhibition of NFκB to the basal level. Despite the decrease of Toluidine blue positive cells by caspase-1 inhibitor is significant, there were still some mast cells observed in the dermis of C1-I injected knockout skin. Consistent results were also observed in wounded samples treated with IKK-I

Figure 12. Effects of NFκB and caspase-1 on wound closure.
A. Rates of wound closure in mice treated with DMSO, IKK inhibitor, or caspase-1 inhibitor.
B. Recruitment of mast cells in wild type, caspase-8 knockout, and wounded skin treated with DMSO, IKK inhibitor, and caspase-1 inhibitor. Number of recruited mast cells were quantified and normalized with wild type to 1.
C. Recruitment of T-cells (green) monitored using the pan T-cell marker CD3.
D. Recruitment of macrophages (green) in the wild type, caspase-8 null, and wounded skin treated as in B.

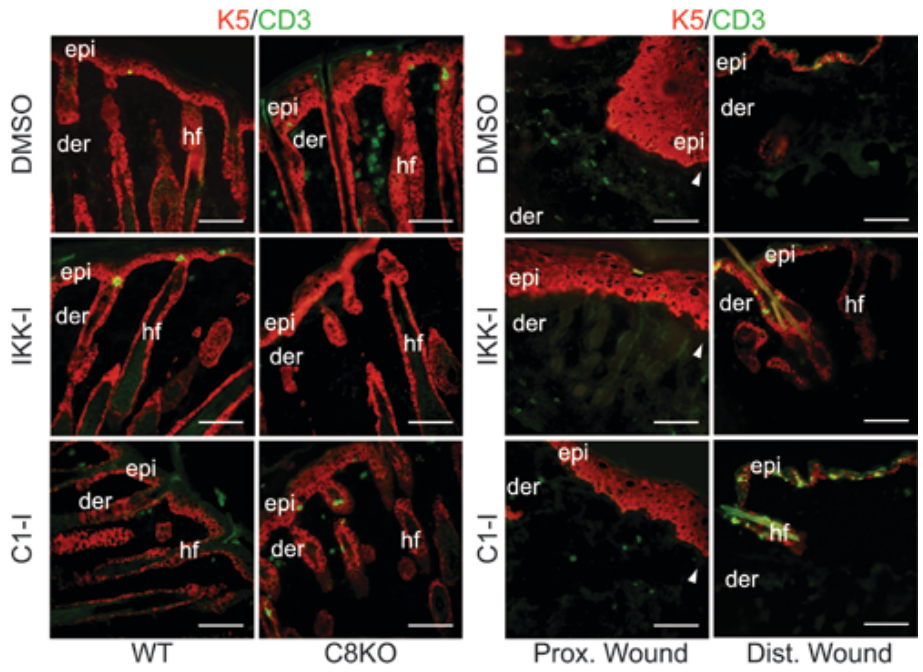
A


DMSO
C1-I
IKK-I
Wound area (%)
Post-wounding day

B


Toluidine Blue
Toluidine Blue
DMSO
IKK-I
C1-I
der
epi
hf
WT
C8KO
Prox. Wound
Dist. Wound
# of positive cells/10x frame
WT
C8 KO
Proximal
Distal
DMSO
IKK-I
C1-I

C



D

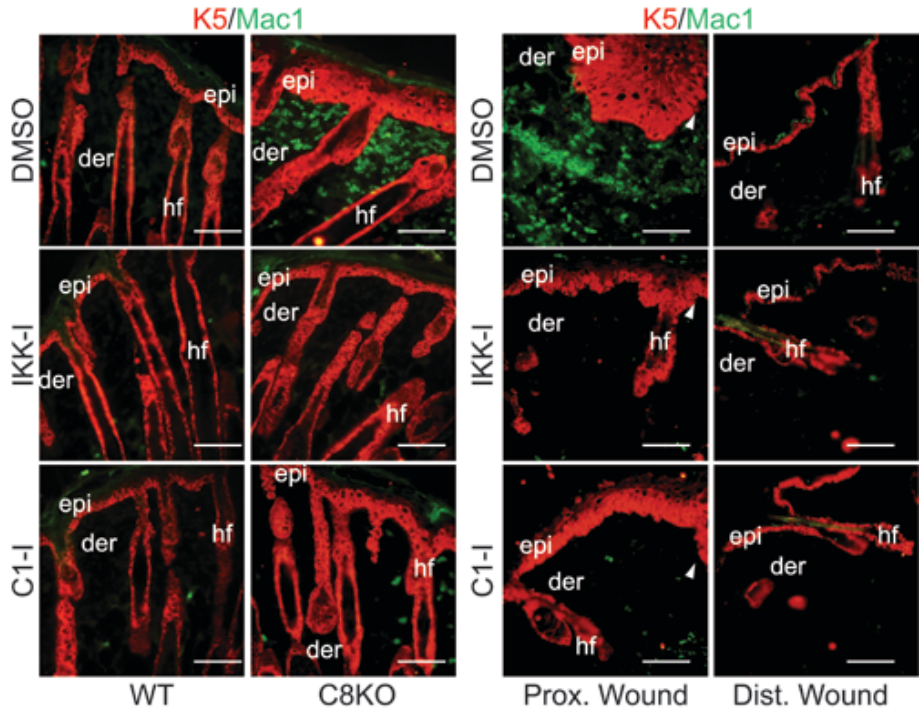


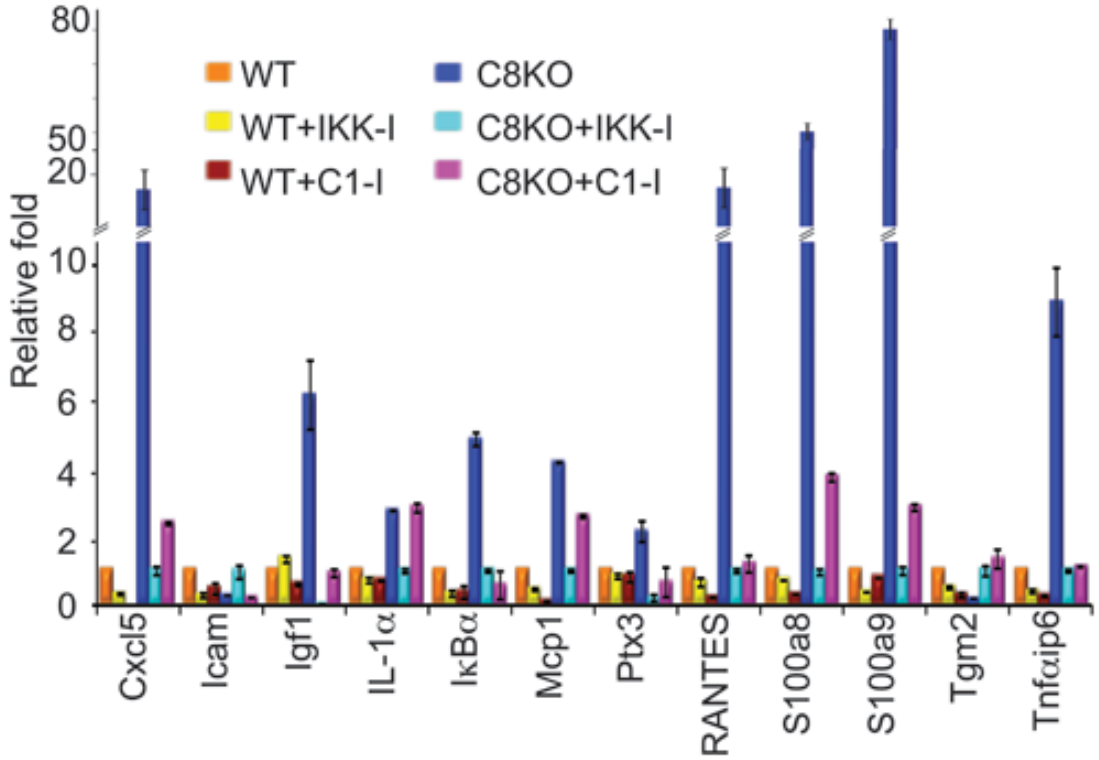
Figure 12 Continued.

and caspase-1 (Fig.12B). As for the distal area to wound sites, there was a low basal level of mast cells but it is not affected by the application of DMSO and the inhibitors. Comparing the effects of IKK-I and C1-I on the recruitment of mast cells, it supports the hypothesis of positive feedback loop of NFκB activation to amplify the signaling pathway of wounding. Furthermore, we previously discovered that T-cells recruitment is activated by the loss of caspase-8 (Lee et al., 2009). Comparably, distinguishable increase of T-cell numbers were seen in the caspase-8 deficient dermis, and even some of them were infiltrated to the basal layer of epidermis and the outer root sheet of hair follicles (Fig. 12C). The inhibition of NFκB prevented the recruitment of T-cells resulted from the loss of caspase-8. As for the influence of C1-I on the distribution of T-cells, it visibly lowered down the detected level of CD-3 positive cells in the knockout dermis but not completely blocked them (Fig. 12C). In consistency, the recruitment of T-cells caused by wounding was also abrogated by IKK-I and C1-I. Lastly, I also examined the number of recruited macrophages by the lack of caspase-8 and wounding. Chemically blocking NFκB and caspase-1 functions prohibited the increased population of macrophages in caspase-8 knockout skin, and the similar phenomenon was detected in the inhibitors treated wounded skin (Fig. 12D). Altogether, the roles of NFκB and caspase-1 are essential to the proper responses of immune cell recruitment in wound healing.

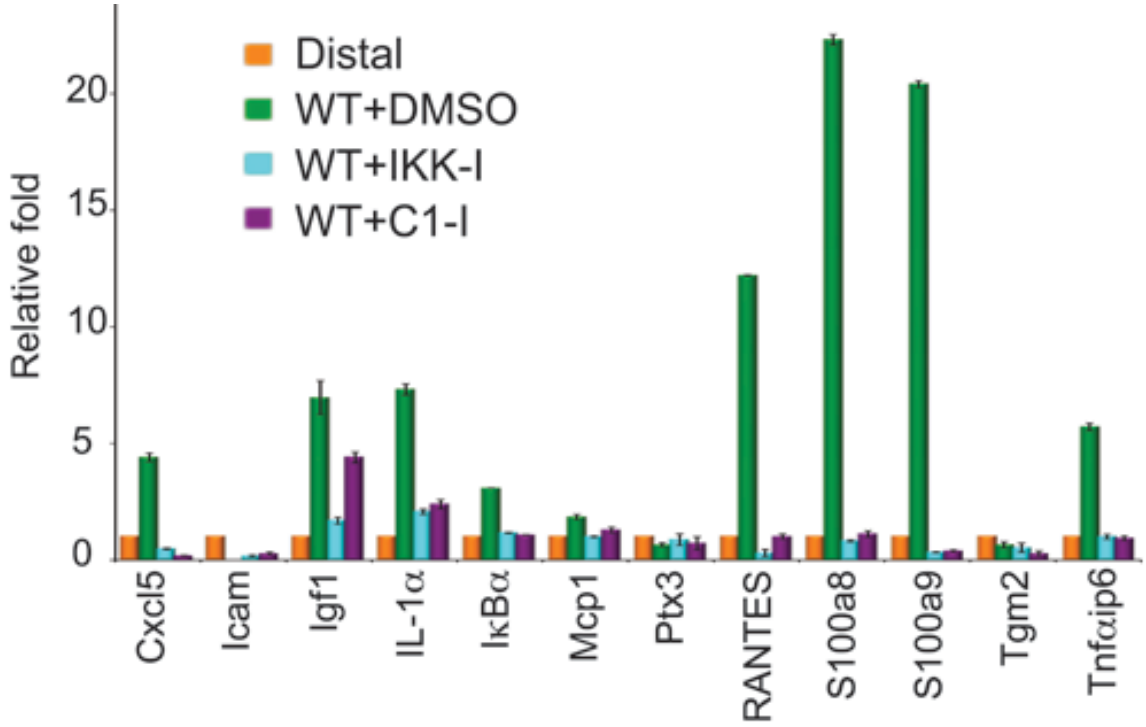
The expression of inflammatory genes is tightly connected to the immune cell recruitment, and the staining result of immune cells strongly suggests that IKK-I and C1-I may also have effects on the production of inflammatory

Figure 13. Profile of inflammatory gene expression activated in wound healing.
A. Expression profile of NFκB dependent genes in wild type and caspase-8 knockout epidermis treated with DMSO, IKK inhibitor, and caspase-1 inhibitor.
B. Wounded skin treated with DMSO, IKK inhibitor, and caspase-1 inhibitor were analyzed for the same expression profile of A.

A


80
50
20
10
8
6
4
2
0
Relative fold
WT
WT+IKK-I
WT+C1-I
C8KO
C8KO+IKK-I
C8KO+C1-I
Cxcl5
Icam
Igf1
IL-1α
IκBα
Mcp1
Ptx3
RANTES
S100a8
S100a9
Tgm2
Tnfαip6

B


20
15
10
5
0
Relative fold
Distal
WT+DMSO
WT+IKK-I
WT+C1-I
Cxcl5
Icam
Igf1
IL-1α
IκBα
Mcp1
Ptx3
RANTES
S100a8
S100a9
Tgm2
Tnfαip6

chemokines and growth factors. To address this possibility, I performed qPCR to screen the expression profile of caspase-8 dependent pro-inflammatory genes which are also NFκB related targets and the result showed in Fig. 13A. Paralleling to the previous reported data, the level of most pro-inflammatory proteins were induced in the wounding microenvironment, such as IL-1α, RANTES, and S100a8 and 9, indicating that inflammation was stimulated by the loss of caspase-8. Intriguingly, the failure to recruit immune cells to the skin by inhibiting NFκB and caspase-1 is also reflected in the diminished expression of inflammatory genes when injected in the caspase-8 null skin, which phenotype is reproducible in wounded animals (Fig. 13B). Not only the production of chemokines and growth factors but also the expression of adhesion molecules are influenced by IKK-I and C1-I. In summary, the data described above suggests that NFκB and caspase-1 pathway is critical to inflammatory responses in several features during wound healing.

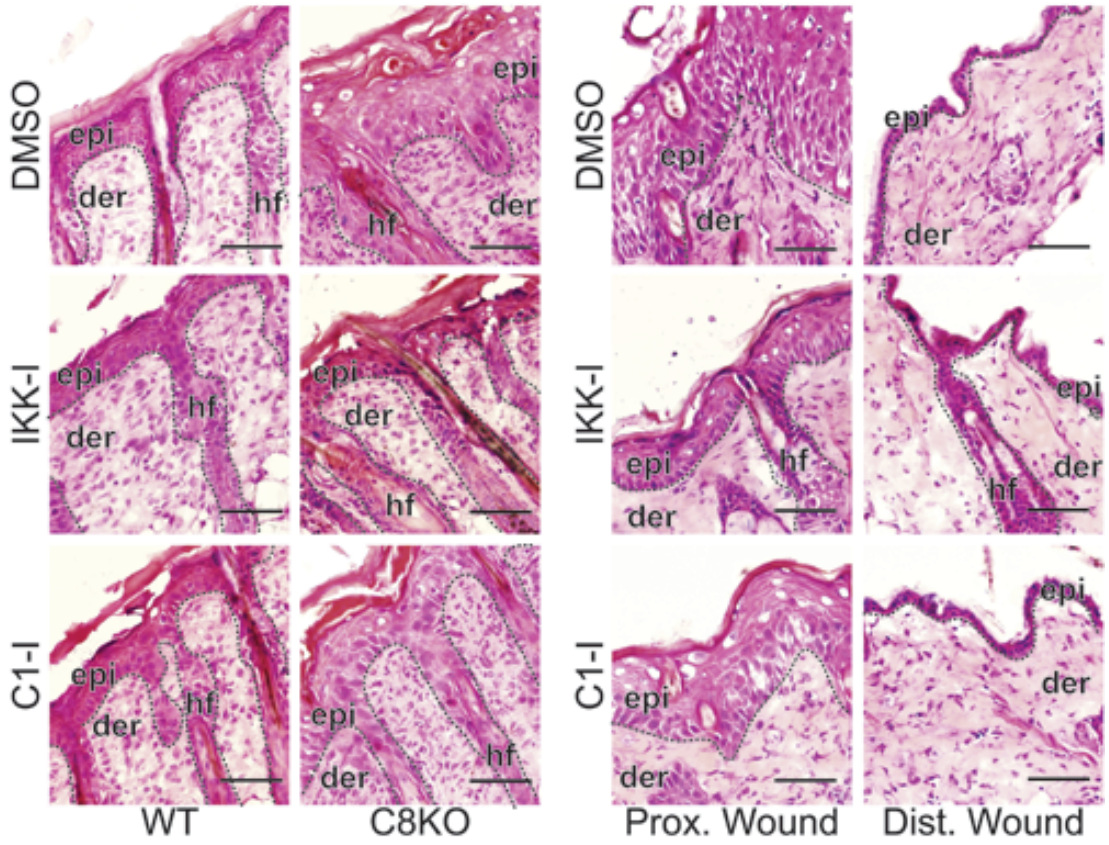
Inhibition of NFκB and caspase-1 affects stem cell proliferation in tissue repair

Epidermal stem cells responded to the stimulation of growth factors will initiate proliferation and re-epithilialization to restore the skin structure after wounding, and also angiogenesis will occur to promote new blood vessel formation. Previously I discussed the effects caused by inhibiting NFκB and caspase-1 in the inflammatory stage of wound healing, and next I planned to inspect the role of NFκB-caspase-1 signaling in proliferation. To address this inquiry, inhibitors injected skin was checked for epidermal expansion by

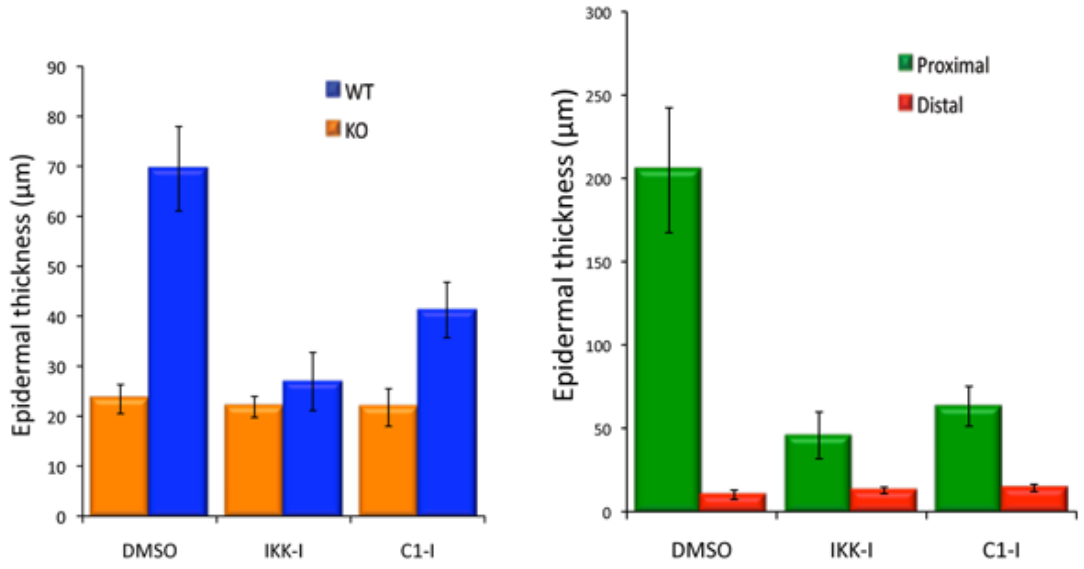
histological staining. Hematoxylin and eosin staining results in Fig. 14A displayed the thickening of epidermis in caspase-8 null skin compared to wild-type, and the expansion is dramatically reduced by the inhibition of IKK and caspase-1. Measurement of the epidermal thickness is accomplished by ImageJ software and the representative data is showed in Fig. 14B. The loss of caspase-8 resulted in 3-fold increase of epidermal thickness and the increase is significantly reduced by inhibiting NFκB and caspase-1. Furthermore, we proved that hyperproliferation is the critical factor to cause the thickening by wounding (Lee, 2009), and an interesting question arises from that phenomenon is whether NFκB-caspase-1 pathway is involved in proliferation. In addition, the differences of would closure rate between DMSO, IKK-I, and C1-I manifested during the proliferative phase of the wound response which commences around day 4 post wounding (Fig. 12A). Then I examined the proliferative responses in the inhibitors applied skin samples, and found that the thickening of the epidermis in the knockout mouse is fueled by an increase in the number of stem/ progenitor cells proliferating in the basal layer of the epidermis as denoted by Ki67 staining (Fig. 14C). Surprisingly, the presence of the IKK and caspase-1 inhibitos in the knockout skin substantially reduces the number of proliferating cells found in the epidermis to near wild type levels (Fig. 14C). Application of the inhibitors on wounded wild-type skin also showed that increased Ki67 expression in the area proximal to the wound site is suppressed, but in the distal areas in which epidermal thickness appeared normal (Fig. 14C). However, the effects of the inhibitors are not applicable to all the phenomena associated with the

Figure 14. Hyperproliferation of the epidermis in caspase-8 KO and wounded skin.
A. Hematoxylin and eosin staining of wild type or caspase-8 knockout skin treated with DMSO or the IKK or caspase-1 inhibitors.
B. Quantification of epidermal thickness in skin sections from A.
C. Proliferating antigen Ki67 (green) and keratin 5 (red) staining in mice treated as in A.
D. Quantification of cell proliferation in skin sections from C.
E. Angiogenesis monitored in wild type or knockout mice treated with DMSO, IKK inhibitor, or caspase-1 inhibitor via staining with CD31 (green).

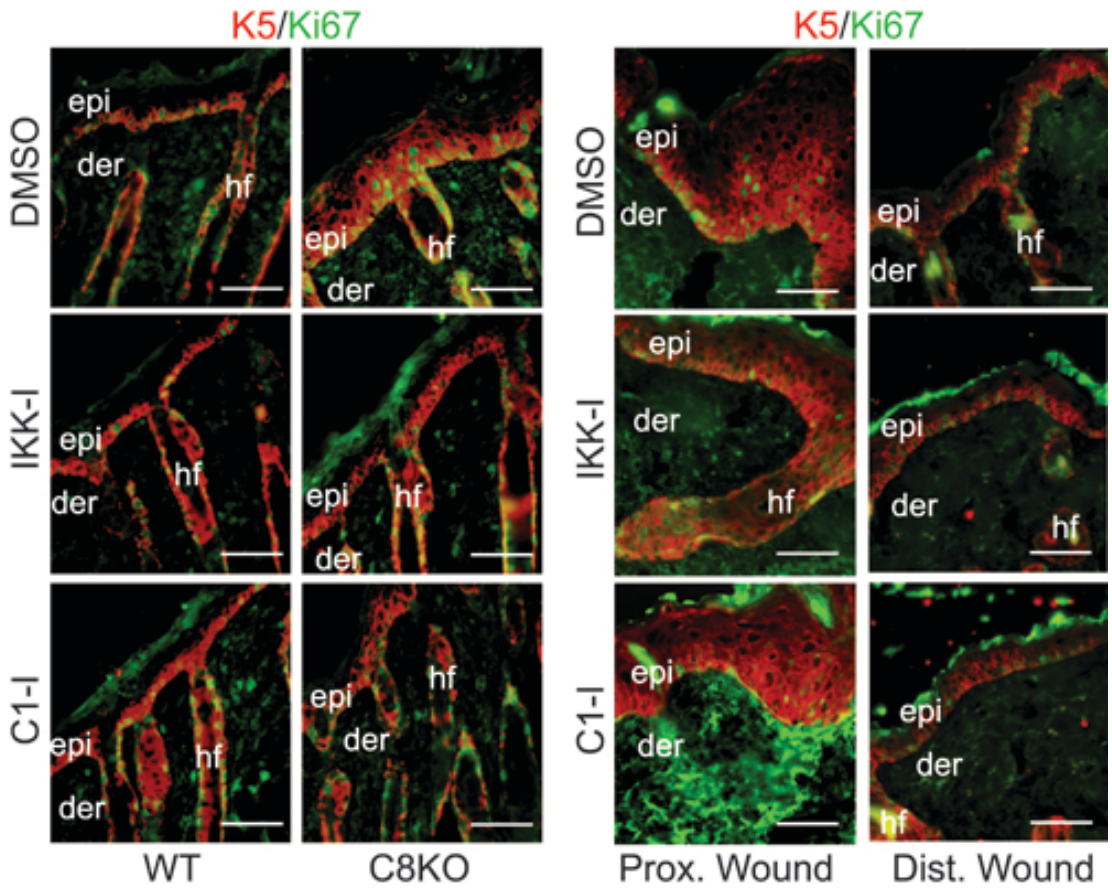
A


DMSO
IKK-I
C1-I
epi
der
hf
WT
C8KO
Prox. Wound
Dist. Wound

B


Epidermal thickness (µm)
WT
KO
DMSO
IKK-I
C1-I
Proximal
Distal

C



D

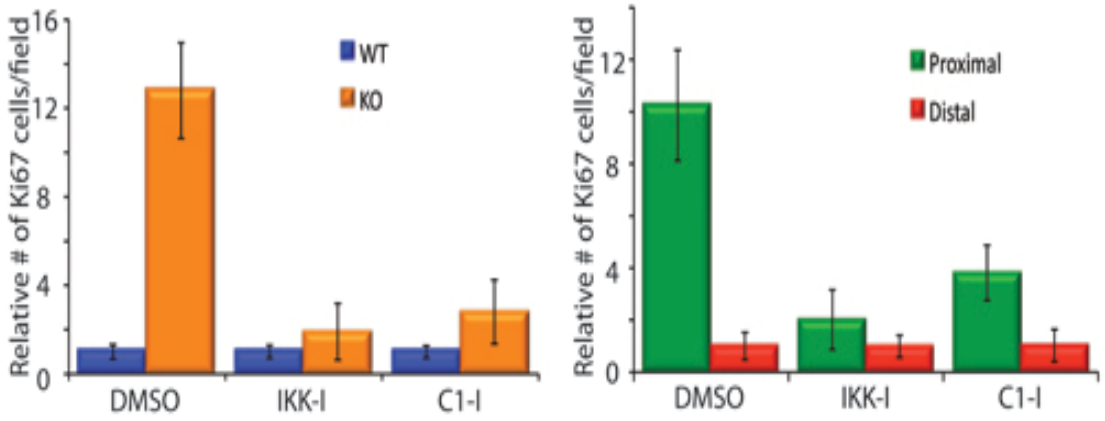


Figure 14 Continued

E

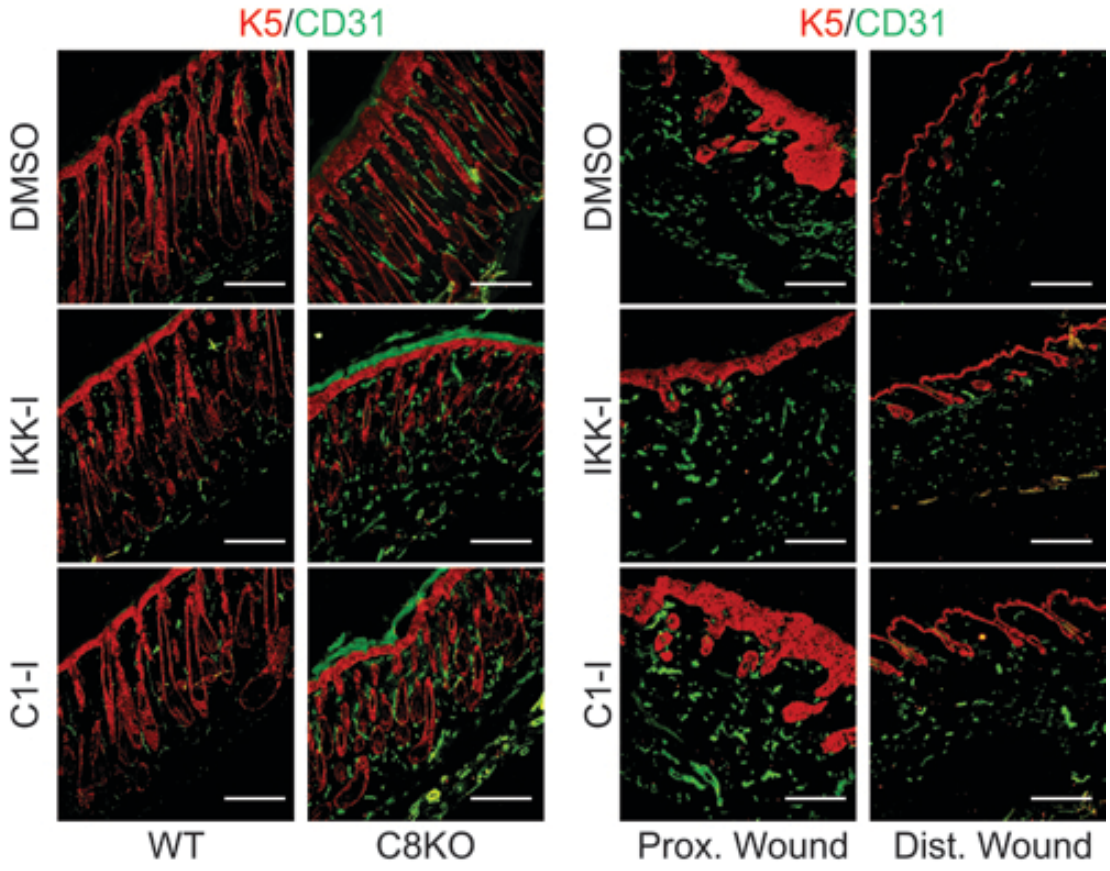


Figure 14 Continued

proliferative phase in wound healing. For instance, new blood vessel formation is a feature of this phase which is not related to the activity of NFκB and caspase-1. The downregulation of caspase-8 and wounding promoted angiogenesis response but it is in a NFκB and caspase-1 dependent manna (Fig. 14D). Inhibition of NFκB and caspase-1 cause similar consequence in caspase 8 knockout and wounded skin, which reinforces that caspase 8 deficient mice are a good model to study wound healing.

Taken together, I proposed a hypothetic model illustrating the signaling mechanism regulating inflammation and proliferation in the wound healing process (Fig. 15). Wounding in mammalian skin causes the downregulation of caspase-8 in the granular layer, and the loss of the protein activates the translocation of NFκB to the nucleus and in turn binds to the promoter region of caspase-1 to initiate transcription. On the other hand, the decrease of caspase-8 also promotes the expression of NLRP3 through the activation of p38 MAPK. With increased NLRP3 and pro-caspase-1, constantly expressed ASC assemble to form NLRP3 inflammasomes which activate caspase-1 and consequently mediate the secretion of IL-1α. The secretion of IL-1α starts in the granular layer, where caspase-8 is usually expressed. However, IL-1α not only have the cell autonomous effect to amplify the wounding signal, it also can relay the message in the paracrine signaling method to neighboring cells and results in a gradient starting from epidermis to dermis. My results support that IL-1α has multi-functions on the regulation of wound healing, including growth arrest, immune

cell recruitment, and epithelial cell proliferation, and the activity of NFκB and caspase-1 is required for the IL-1α dependent regulation.

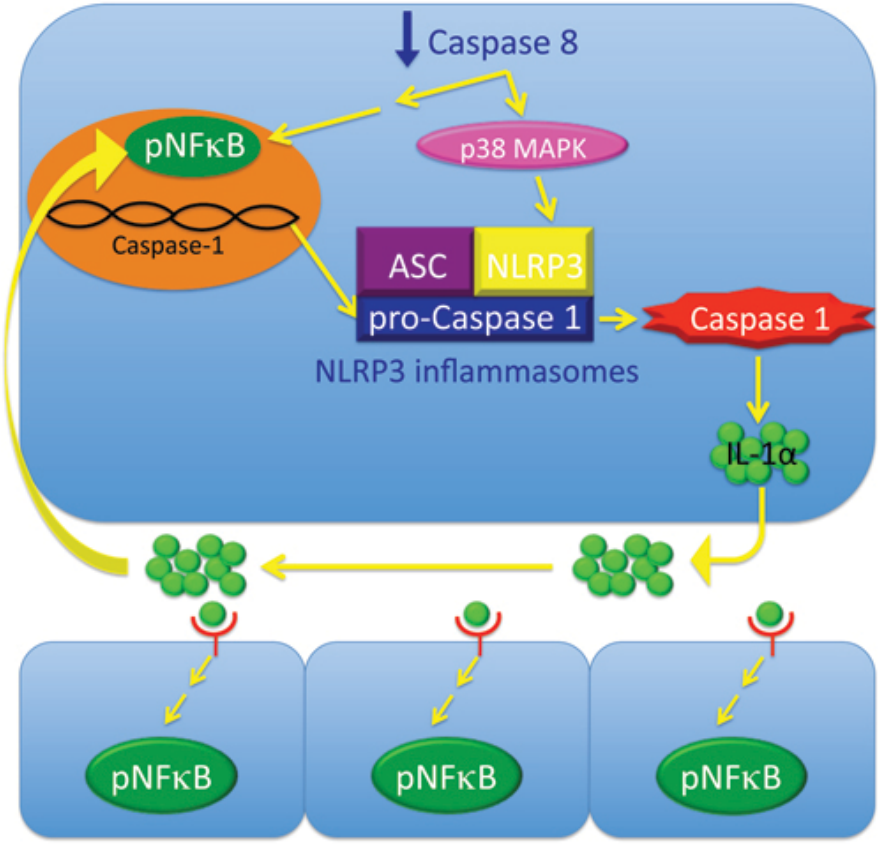


Figure 15. Model of caspase-8 dependent NFκB-caspase-1 pathway mediating IL-1α secretion. Our data suggests a model in which the downregulation of caspase 8 activates NFκB to initiate the transcription of pro-caspase 1. Increased pro-caspase 1 assembles with ASC and NLRP3 to form NLRP3 inflammasomes and in turn facilitates the maturation of caspase 1. Active caspase 1 releases the reservoir of IL-1α and then IL-1α serves as a positive amplifier to the activation of NFκB in a feedback manner.

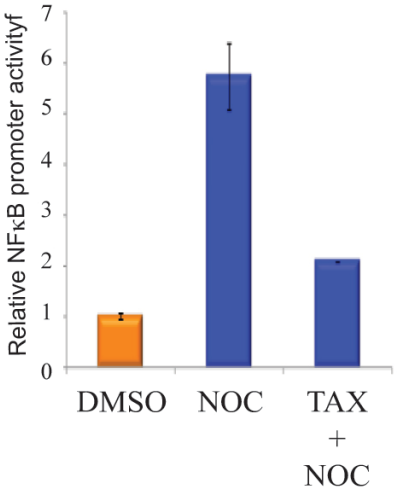
The upstream activator of NFκB

The results documented previously in this dissertation strongly demonstrate that the activity of NFκB is required for wound healing responses, but the mechanism regulating NFκB activation remains uncertain. And the

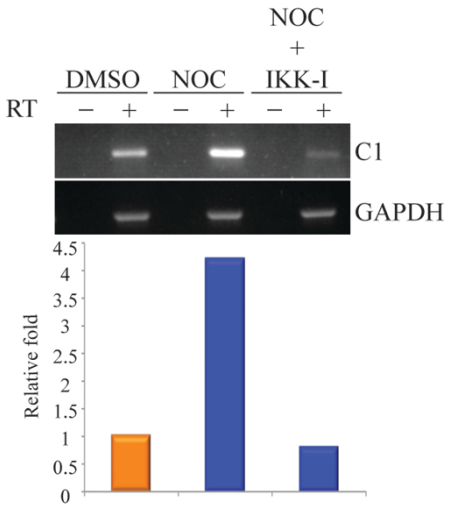
connection of the downregulation of caspase-8 to the activation of NFκB is still undiscovered. To further study how caspase-8 mediates the process of wound repair, I continued the study on the discovery of caspase-8-dependent NFκB activator. Due to the multi-functions of NFκB in several cellular responses, the mechanism regulating its activation is extremely complex. One of the candidates is related to the organization of cytoskeleton which is shown by Michael Karin's lab. Disruption of microtubule networks surprisingly promotes the degradation of IκB and the activation of NFκB signaling, and the activation can be blocked by the microtubule stabilizing drug, Taxol. To investigate the role of microtubule organization on NFκB activation in primary keratinocytes, I treated cells with microtubule depolymerizing reagent, nocodazole, and examine for the promoter activity and localization of NFκB. The luciferase system was applied to test NFκB transcriptional activity, and the result in Fig. 16A showed that nocodazole treatment activates NFκB and the increased activity was significantly reduced by taxol. Furthermore, nuclear NFκB staining was detected in keratinocytes treated with nocodazole, and consistently, taxol blocked the translocation of NFκB to the nucleus. The effects of nocodazole and taxol on microtubule filaments were confirmed by immunofluorescence staining of α-tubulin (Fig. 16B). These data indicates that microtubule organization is critical to the activity of NFκB. The next interesting question arises from this observation is whether disruption of microtubule filaments has influence on the expression of pro-caspase-1. For answering this inquiry, I probed the protein level of pro-caspase-1 in the treatment of nocodazole. Surprisingly, nocodazole not only activated NFκB but

Figure 16. The organization of microtubule is critical to NFκB activation.
A. The activity of NFκB-luciferase reporter was examined in primary keratinocytes treated with DMSO, nocodazole, and taxol+nocodazole.
B. Immunofluorescence staining of NFκB (green) and α-tubulin (red)in primary keratinocytes treated as in A.
C. Caspase-1 RNA level is examined by RT-PCR in keratinocytes treated with nodocazole and the combination of nocodazole and IKK inhibitor. Quantification of gel intensity is assayed by Image J software and GAPDH is probed as a loading control.
D. Caspase-1 RNA level is examined by the same method described in C in several treatments listed in the figure. InhiAb stands for IL-1α inhibitory antibody.

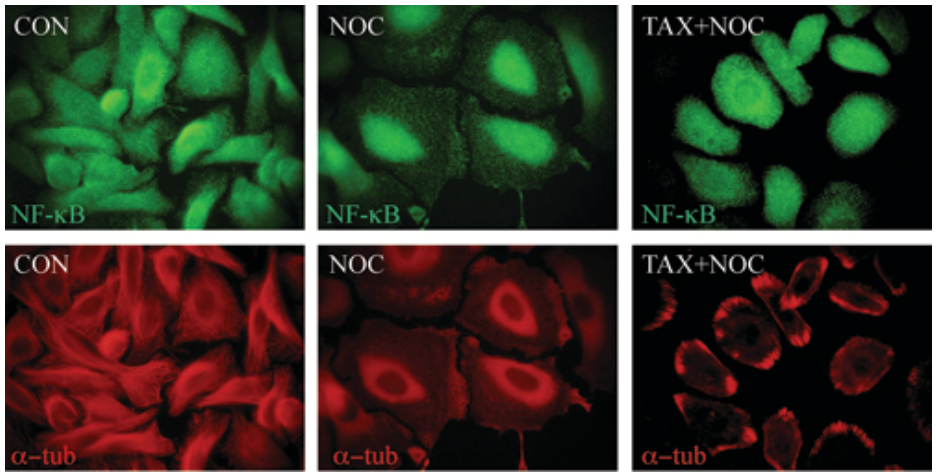
A


Relative NFκB promoter activityf
7
6
5
4
3
2
1
0
DMSO
NOC
TAX
+
NOC

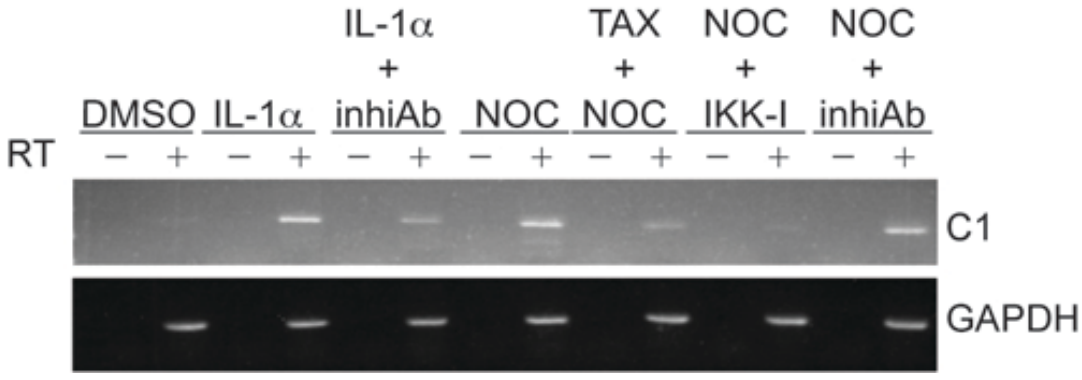
C


NOC
+
DMSO
NOC
IKK-I
RT
– + – + – +
C1
GAPDH
Relative fold
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

B


CON
NOC
TAX+NOC
NF-κB
NF-κB
NF-κB
CON
NOC
TAX+NOC
α–tub
α–tub
α–tub

D


IL-1α
+
TAX
+
NOC
+
NOC
+
DMSO
IL-1α
inhiAb
NOC
NOC
IKK-I
inhiAb
RT
– + – + – + – + – + – + – +
C1
GAPDH

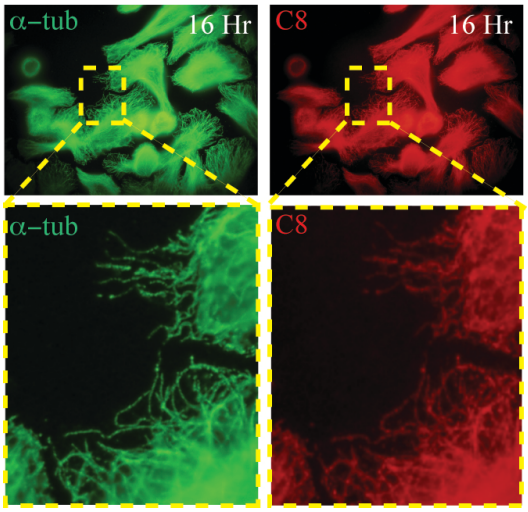
also induced the expression of pro-caspase-1 (Fig. 16C). Additionally, inhibition of NFκB by IKK inhibitor dismissed the induction of pro-caspase-1 expression, which strongly suggests that the dynamic of microtubule filaments plays an essential role of the activation of NFκB-caspase-1 pathway. Moreover, previously I demonstrated that IL-1α works in the positive feedback loop to promotes the activation of NFκB–caspase-1 pathway, and data in Fig. 16C propose another candidate to activate NFκB. To study whether depolymerization of microtubule may be the initial signal to start this signaling, I treated cells with nocodazole and the neutralizing antibody of IL-1α simultaneously. Consistent to our hypothesis, blocking the feedback loop of IL-1α does not completely hinder the increase of pro-caspase-1 caused by nocodazole (Fig. 16D).

Previous data demonstrates that in vitro scratch assay recapitulates several features of in vivo wounding responses, and it is shocking to discover that the organization of microtubule is abnormal in migrating cells (Fig. 17A). Microtubule filaments are fragmented and curved at the leading edge, and, furthermore, the staining of caspase-8 showed similar pattern as microtubule filaments. The correlation of caspase-8 and α-tubulin staining in scratch assay implies the possibility of connection between caspase-8 and microtubule. However, the localization of caspase-8 in resting keratinocytes was mainly detected in the perinuclear area which is totally different from microtubule filaments. But an intriguing phenomenon was observed when the cytosolic pool of cells was washed out. Caspase-8 displayed filamentous structure and was colocalized with α-tubulin. In addition, caspase-8 filaments are susceptible to

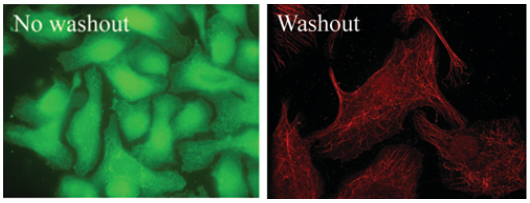
microtubule affecting chemicals, taxol and nocodazole (Fig. 17C). Taxol stabilized caspase-8 structure and formed shorter and thicker filaments in the periphery of keratinocytes, and on the other hand, nocodazole disturbed both caspase-8 and microtubule organization. To verify the effects of caspase-8 on microtubule organization, I extracted and cultured primary keratinocytes from caspase-8 knockout epidermis, and subjected to immunofluorescence staining. As displayed in Fig. 17D, caspase-8 structure disappeared and microtubule network had severe defects, which showed fragmented and short filaments. There are several known microtubule binding proteins, such as katanin and spastin, and they are essential to the dynamic of microtubule. Overexpression of katanin in keratinocytes initiates the severing process of microtubule network and resulted in shorter filaments (Roll-Mecak et al., 2010, Quarmby et al., 2000, and Fig. 17E), which is similar to the phenotype I observed in caspase-8 null keratinocytes. In addition, katanin also disintegrated caspase-8 structure (Fig. 17E), and this suggests that caspase-8 may have an interaction with microtubule filaments. Taken together, the results strongly support the potential role of caspase-8 on the regulation of microtubule organization, and the downregulation of caspase-8 in wound healing can initiate NFκB-caspase-1 signaling cascade.

Figure 17. Caspase-8 has filamentous structure similar to microtubule network.
A. α-tubulin and caspase-8 are visualized by immunofluorescence staining in scratch assay.
B. Staining of caspase-8 in intact and cytosolic washed-out keratinocytes.
C. Staining of α-tubulin and caspase-8 in keratinocytes treated with DMSO, taxol, and nocodazole.
D. Staining of α-tubulin and caspase-8 in caspase-8 knockout primary keratinocytes.
E. Transfection of katanin in keratinocytes disrupts the structure of microtubule and caspase-8.

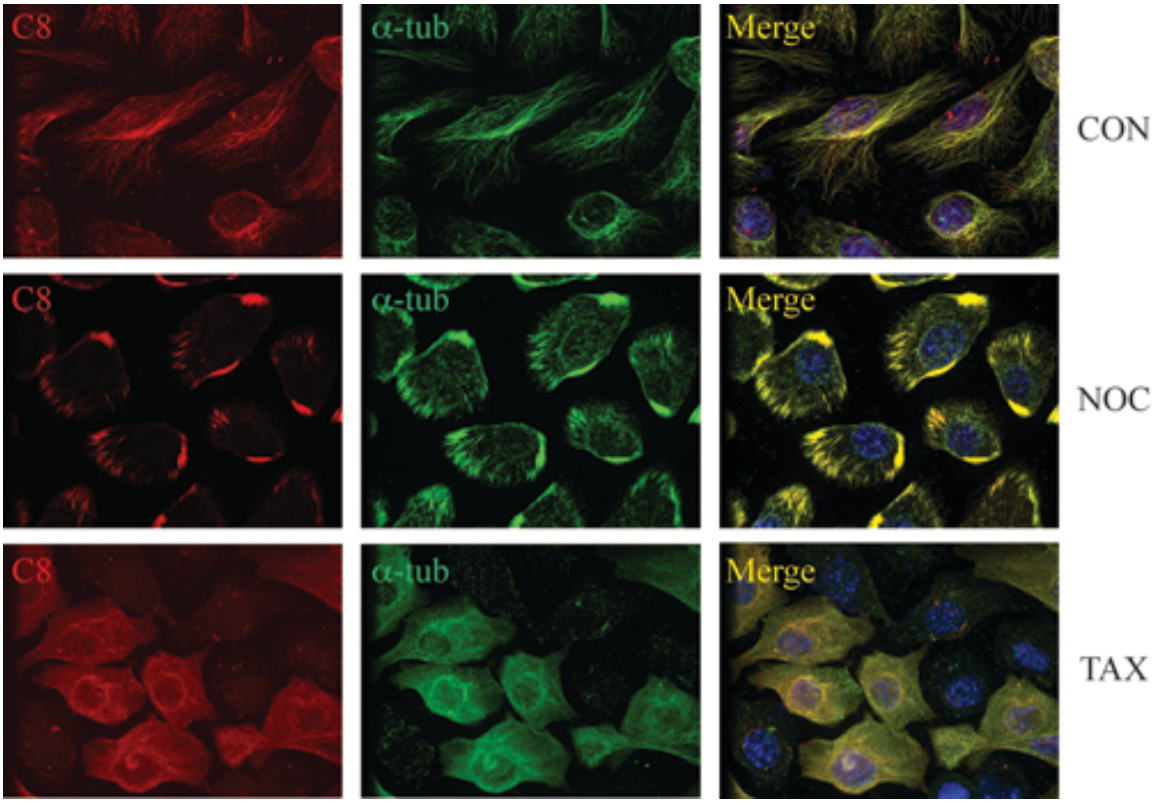
A


α–tub
16 Hr
C8
16 Hr
α–tub
C8

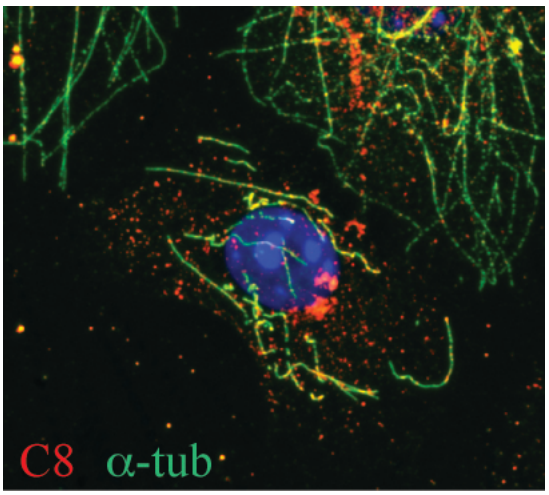
B


No washout
Washout

C


C8
α-tub
Merge
CON
C8
α-tub
Merge
NOC
C8
α-tub
Merge
TAX

D



E

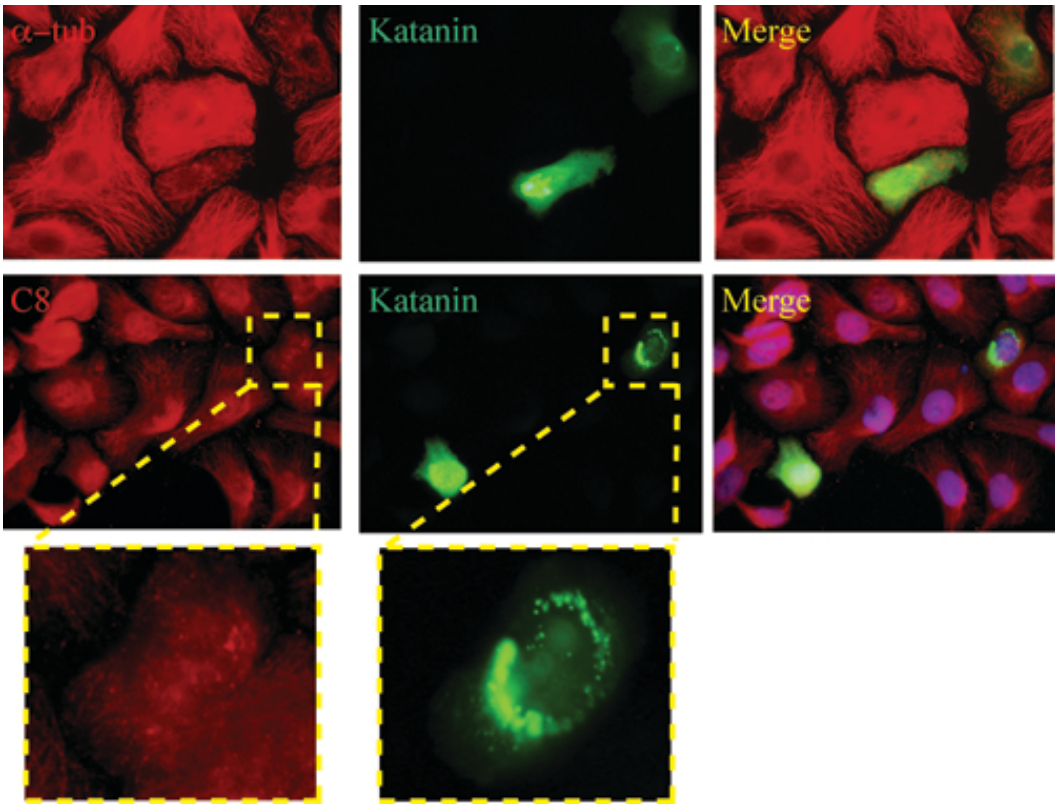


Figure 17 Continued

DISCUSSION AND ANALYSIS

The novel role of caspase-8 in coordinating responses of proliferation and inflammation during wound healing processes has been documented; here we have uncovered the molecular mechanism synchronizing these complex reactions upon the ablation of caspase-8. Caspse-8 is commonly expressed in the granular layer of skin and is downregulated in the process of wound repair (Lee et al., 2009). The loss of caspase-8 in granular layer initiates the release of the reservoir of IL-1α, and establishes a gradient of IL-1α from higher in the granular layer to lower in the basal layer and some of them pass the basement membrane to dermis to induce the production of pro-inflammatory cytokines and growth factors (Lee et al., 2009). But how the deficiency of caspase-8 triggers the secretion of IL-1α becomes the crucial and fundamental question to study. The data described in this paper invokes a new pathway to illustrate the connection of wounding and the release of cytokines. NLRP3 inflammasomes are required for the maturation and secretion of IL-1 family and play an essential role of the activation of caspase-1 in a caspase-8 dependent fashion. Caspase-1 is expressed as a zymogen and its precursor form is one of the components comprised of NLRP3 inflammasomes. Furthermore, the expression of pro-caspase-1 precursor is a critical factor to affect the activation of NLRP3 inflammasomes. We showed that active NFκB initiates the transcription of caspase-1 and in turn influences the formation of NLRP3 inflammasomes. The effects of NFκB in inflammation have been intensely investigated, and it

regulates the expression of many inflammatory genes (Mikenberg et al., 2007). But this is the first time that we discover the link between the transcription factor NFκB and inflammasome assembly.

Inhibition of NFκB activation not only abolishes the increased pro-caspase-1 expression but also affects the release of IL-1α. Although the wild-type skin should have very low basal level of NFκB activity, the application of IKK-I in wild-type epidermis still decreases the RNA level of pro-caspase-1 mediated by NFκB. A reasonable explanation is how we extracted the epidermal sample from animals may initiate wounding responses. And this phenomenon supports that the reduction of pro-caspase-1 expression by IKK-I is wounding specific. Similar impacts of IKK-I on the IL-1α ELISA results are also occurred. Furthermore, glyburide has been proven to impair the activation of NLRP3 inflammasomes, and out data also provide evidence showing it only affects the caspase-1 activity-dependent release of IL-1α but not the protein level of pro-caspase-1.

Caspase family members are first synthesized as inactive pro-caspases, which contain prodomains and the catalytic domains. They are post-translationally regulated to maintain the capacity of rapid responses. Most caspases have the capacity for auto-activation by proteolytic dissociation of the prodomain (Earnshaw et al., 1999; Martin et al., 1998). Caspase-6 has a small prodomain and is capable of self-processing and activation in the hippocampus and cortex in Alzheimer disease (Klaiman et al., 2009), as are caspase-3 and -7. However, the activation of caspase-1 is typically regulated by inflammasomes.

The recruitment of different inflammasome elements facilitates autocatalytic activation of caspase-1 by mediating the apposition of two or more caspase-1 monomers, resulting in cleavage of the pro-enzyme into active subunits (Stutz et al., 2009). Interestingly, Yamanaka and his colleges published a paper stating that full-length caspase-1 transgenic mice have increased caspase-1 activity by inducing the secretion of IL-18. This strongly suggests that the excessive expression of pro-caspase-1 may play a role in activating itself. It is likely that elevated pro-caspase-1 levels causes a denser distribution that augments the chance of oligomerization and self-activation. Despite evidence supporting the contribution of NLRP3 inflammasomes in the activation of caspase-1, we still cannot exclude the possibility of the self-cleavage of caspase-2 through means other than NLRP3 inflammasomes.

The next question that should be focused on is what is responsible for the activation of NFκB. NFκB is present in many different cell types and is found to mediate many different compartments of the immune system during the differentiation of immune cells, development of lymphoid organs, and immune activation (Bauernfeind et al., 2009; Vallabhapurapu and Karin, 2009). NFκB is the central transcription factor of many different receptor-signaling pathways, including tumor necrosis factor, Toll-like receptor superfamilies and IL-1 receptor. The NFκB signaling systems consists of two protein families: the activator, NFκB, and the inhibitor, IκB. NFκB transcription factors are not single proteins but are the results of combinatorial multimerization of five monomers (O'Dea and Hoffmann). In addition, the degradation of IκB is dependent on the activity of IκB

kinase. This elaborate and complex signal network is orchestrated by a cooperation between a large number of proteins. To unwind the puzzle and discover the solution of what activates NFκB in the stimulus of caspase-8 downregulation, further research is required. Although IL-1α is the product of NFκB-caspase-1 pathway, it has also been noted to activate NFκB in a positive feedback loop to amplify the signals of NFκB activation. Restraining the release of IL-1α does not completely block the increase of pro-caspase-1 in iκBα null epidermis, which indicates there are other initial signals to initiate the NFκB-caspase-1 pathway. However, the initial cue to turn on the NFκB cascade remains as a mystery and is the key to explicate the mechanism of inflammasome association and inflammation.

The mechanism regulating pro-caspase-1 expression and the assembly of NLRP3 inflammasomes provides new medical applications to several skin disorders. Psoriasis is a chronic and autoimmune disease that appears on the skin, and the activity of capsase-1 is increased in lesional epidermis of psoriatic patients (Johansen et al., 2007). The increased expression of caspase 8 has been attributed to the abnormal responses of wound healing in diabetic patients (Al-Mashat et al., 2006). However, the reduction of caspase 8 induces the phenotypes of eczema (Chun et al., 2002). Consequently, the molecular regulation of caspase-1 by the abrogation of caspase-8 may furnish knowledge and new insights into therapies for these diseases.

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