

Regulation and Function of the Caspase-1 in an Inflammatory Microenvironment

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The inflammasome is a complex of proteins that has a critical role in mounting an inflammatory response in reply to a harmful stimulus that compromises the homeostatic state of the tissue. The NLRP3 inflammasome, which is found in a wound-like environment, is comprised of three components: the NLRP3, the adaptor protein ASC and caspase-1. Interestingly, although ASC levels do not fluctuate, caspase-1 levels are elevated in both physiological and pathological conditions. Despite the observation that merely raising caspase-1 levels is sufficient to induce inflammation, the crucial question regarding the mechanism governing its expression is unexplored. We found that, in an inflammatory microenvironment, caspase-1 is regulated by NF- κ B. Consistent with this association, the inhibition of caspase-1 activity parallels the effects on wound healing caused by the abrogation of NF- κ B activation. Surprisingly, not only does inhibition of the NF- κ B/caspase-1 axis disrupt the inflammatory phase of the wound-healing program, but it also impairs the stimulation of cutaneous epithelial stem cells of the proliferative phase. These data provide a mechanistic basis for the complex interplay between different phases of the wound-healing response in which the downstream signaling activity of immune cells can kindle the amplification of local stem cells to advance tissue repair.

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INTRODUCTION

The major function of the skin is to provide a physical barrier between the body and the external environment. Consequently, it is frequently damaged and mounts a complex wound-healing program to restore tissue homeostasis. This wound-healing response is organized into three interdependent phases: an inflammatory phase leading to the recruitment and activation of immune cells; a proliferative phase comprising the expansion of the progenitor cell pool, angiogenesis, and re-epithelialization (migration of keratinocytes to close the wound); and the remodeling phase wherein alterations in tissue architecture and the extracellular matrix are carried out (Gurtner *et al.*, 2008). Hindering the mech-

anistic understanding of this process is the fact that signaling by cells in one phase of the wound-healing program impacts the behavior of cells associated with another phase.

In response to many different external stimuli, inflammation can be mediated by a multiprotein complex known as an inflammasome, which promotes the maturation and secretion of proinflammatory cytokines. The inflammasome can be comprised of an NLR (nucleotide-binding domain and leucine-rich repeat containing) protein, an adaptor protein ASC (apoptosis-associated speck-like protein), and procaspase-1. The NLR proteins are a family of intracellular sensors that can detect the presence of pathogens and also recognize danger or stress signals (Schroder and Tschopp, 2010). Whereas the levels of the adaptor protein ASC remains unchanged, we found that the stress kinase p38 mitogen-activated protein kinase (MAPK) is responsible for the upregulation of NLRP3 in a wound-like environment (Lee *et al.*, 2009), but the regulation of caspase-1 expression remains poorly understood.

Caspase-1 is a cysteine protease that is synthesized as a zymogen. The assembly of NLRP3 inflammasome induces the cleavage and activation of procaspase-1, which can then process IL-1 family members such as IL-1 α (Keller *et al.*, 2008), IL-1 β , and IL-18 into their mature forms (Yu and Finlay, 2008). In the context of wounding (Lee *et al.*, 2009), as well as diseases with chronic inflammation such as psoriasis (Johansen *et al.*, 2007), heart disease (Merkle *et al.*, 2007), gout, and arthritis (Church *et al.*, 2008), there is an upregulation of procaspase-1 expression. Despite these observations, both the mechanism and the functional relevance of

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Abbreviations: ASC, apoptosis-associated speck-like protein; C8KO, caspase-8 conditional knockout; IKK, I κ B kinase; IKK-I, IKK inhibitor; IKK2-CA, constitutively active mutant of IKK2; MAPK, mitogen-activated protein kinase; NLR, nucleotide-binding domain and leucine-rich repeat containing

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increasing the expression of procaspase-1 have failed to garner much attention. A clue to the importance of the upregulation of procaspase-1 was the development of cutaneous inflammation simply by overexpressing procaspase-1 in epidermal keratinocytes (Yamanaka *et al.*, 2000).

A prime candidate for the transcriptional regulator of caspase-1 is the transcription factor NF- κ B, a well-known mediator of the inflammatory response (Karin, 2009). This dimeric complex is sequestered in the cytosol via its association with I κ B. Activation of NF- κ B is accomplished through the I κ B kinase (IKK), which can phosphorylate I κ B and lead to its dissociation from NF- κ B. Upon activation, NF- κ B dimers are translocated into the nucleus where they modulate the expression of numerous genes involved in the immune response. To investigate the potential link between NF- κ B and caspase-1 in restoring tissue homeostasis, we exploited the conditional deletion of epidermal caspase-8 (C8KO) as a model of wound healing and in which caspase-1 levels are elevated throughout the epidermis (Lee *et al.*, 2009).

RESULTS

The expression of caspase-1 is regulated by NF- κ B

To test the hypothesis that NF- κ B regulates procaspase-1 expression, epidermal explants from wild-type and caspase-8 conditional knockout (C8KO) mice were treated with DMSO (vehicle control) or a pharmacological inhibitor of NF- κ B activation. Inhibition of NF- κ B activation was accomplished with a chemical inhibitor of the upstream activator of NF- κ B (IKK inhibitor (IKK-I)). In the C8KO epidermis, the increased procaspase-1 is diminished to near wild-type levels by the treatment with IKK-I at both the RNA (Figure 1a) and the protein level. This effect is both dose dependent (Supplementary Figure 1 online) and reversible (Figure 1a). Interestingly, IKK-I did not affect the expression of NLRP3 despite suggestions to the contrary (Bauernfeind *et al.*, 2009; Figure 1b). On the other hand, p38 MAPK regulates NLRP3 in the wound-like epidermis (Figure 1b) but did not affect the increase in procaspase-1 (Figure 1a). The third component of many NLRP3-containing inflammasomes is ASC, whose levels were constant in wild-type and C8KO epidermis (Figure 1c). Altogether, these inhibitor studies suggest that expression of the components of the NLRP3 inflammasome is under the regulation of at least two convergent signaling pathways—p38 MAPK regulates NLRP3 expression, whereas NF- κ B controls the expression of procaspase-1.

In order to examine whether NF- κ B activity is sufficient to induce procaspase-1, we utilized newborn epidermal explants lacking I κ b α , a negative regulator of NF- κ B. The basal level of NF- κ B activity in wild-type and I κ b α knockout mice is the same, but there is a prolonged activation of NF- κ B signaling with a pulse of a stimulus such as IL-1 or tumor necrosis factor in I κ b α -null cells (Klement *et al.*, 1996). Approximately 13-fold increase in the level of a *bona fide* NF- κ B target gene, RANTES, verified that the loss of I κ b α induces an extended activation of NF- κ B signaling in epidermal explants (Supplementary Figure 2 online).

Under these conditions, procaspase-1 expression remains ~70-fold higher in the I κ b α knockout epidermis relative to the

wild-type control (Figure 1d), but there is no appreciable effect on NLRP3 or ASC (data not shown). Interestingly, despite the ~70-fold increase in caspase-1 RNA levels in the I κ b α KO relative to the wild-type control, the increase in caspase-8 protein level, although significant, was only ~4-fold. Secreted IL-1 α is not only the product of caspase-1 activity but is also involved in a positive feedback loop to activate NF- κ B in an autocrine or a paracrine manner (Vallabhapurapu and Karin, 2009). In order to determine how much of the increase in caspase-1 expression is due to this positive feedback loop, we tested the effect of blocking the signaling of IL-1 α with a neutralizing antibody.

Neutralizing extracellular IL-1 α markedly reduces the induction of procaspase-1 expression in the I κ b α null background (Figure 1d). The remaining ~15-fold increase in caspase-1 levels in the I κ b α knockout epidermis may reflect the direct intracellular signaling events linking NF- κ B to caspase-1. This theory was substantiated by the effect of the caspase-1 inhibitor, which would effectively short circuit the positive feedback loop resulting from the caspase-1-dependent secretion of IL-1 α . The treatment of I κ b α -null epidermis with the caspase-1 inhibitor likewise results in a ~15-fold difference in caspase-1 RNA levels compared with the wild-type explants (Figure 1d). The decrease in caspase-1 expression by the IL-1 α inhibitor antibody or the caspase-1 inhibitor in the I κ b α KO skin is also reflected at the protein level (Figure 1d).

Reconstitution of caspase-1 regulatory control *in vitro*

In contrast to the heterogeneous population of cells that comprise the epidermis used above, we investigated whether the NF- κ B–caspase-1 relationship is valid within wounded epidermal keratinocytes *in vitro*. Sixteen hours after applying a scratch wound on a confluent sheet of keratinocytes, a decrease in caspase-8 protein levels was only observed in cells migrating from the wound edge (Figure 2a). This occurred in tandem with the activation of NF- κ B (Figure 2b and Supplementary Figure 3A online). Consistent with our proposed pathway, the decrease in caspase-8 in epidermal keratinocytes leading to the activation of NF- κ B resulted in an increase in caspase-1 expression (Figure 2c and Supplementary Figure 3B online). Interestingly, the increase in caspase-1 protein levels was observed in both the cytosol and the nucleus of the migrating cells (Supplementary Figure 3C and D online). This nuclear translocation of caspase-1 has previously been described upon exposure of cells to tumor necrosis factor (Mao *et al.*, 1998), although the function remains unknown. We then examined whether activation of NF- κ B is sufficient to induce caspase-1 expression *in vitro* by transfecting primary keratinocytes with a constitutively active mutant of IKK2 (IKK2-CA). Twelve hours after transfection, about 10% of the cultured cells expressed the IKK2-CA construct leading to the activation of NF- κ B (Figure 2d and Supplementary Figure 3E online) and ~2.5-fold increase in caspase-1 RNA (Figure 2e) and protein (Supplementary Figure 3F online) expression. Moreover, activation of NF- κ B with IL-1 α can elevate the amount of procaspase-1 protein, and this effect can be blocked by inhibiting NF- κ B activation with

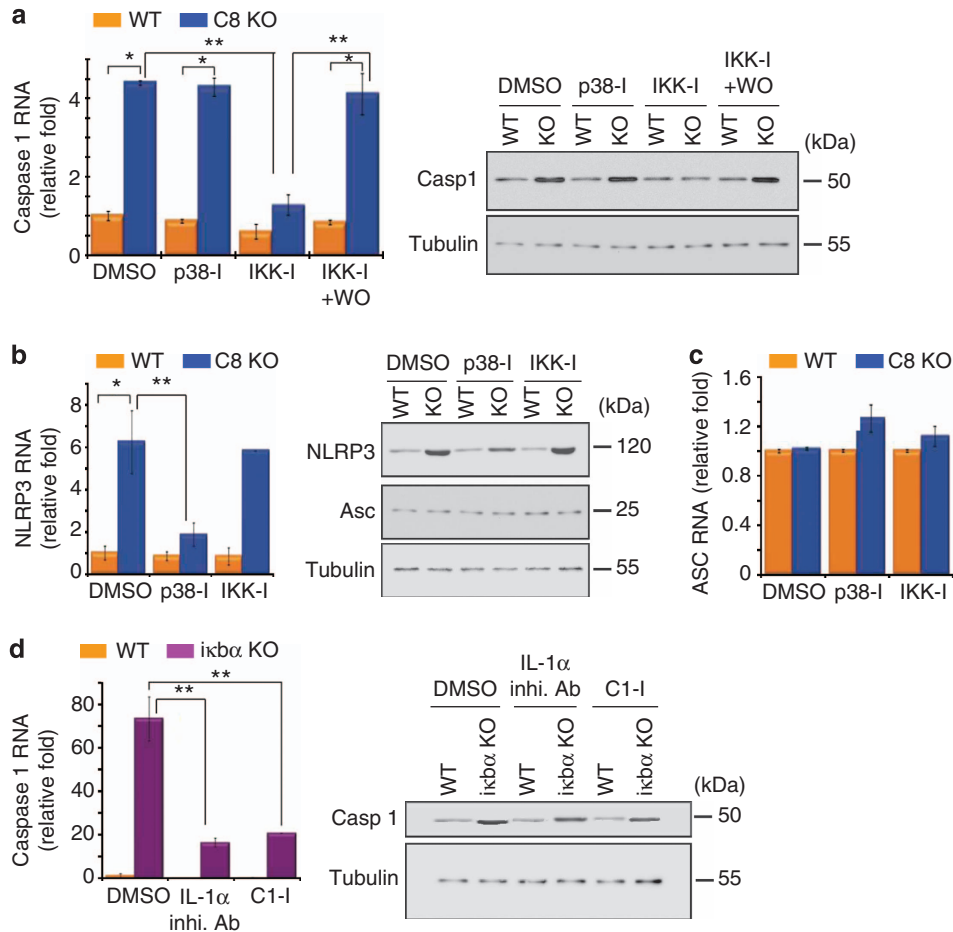


Figure 1. NF-κB regulates caspase-1 expression. Epidermal explants from wild-type (WT) and caspase-8 knockout (C8KO) mice were treated with DMSO, or inhibitors for p38 MAPK (p38-I) or IKK (IKK-I) and qPCR and western blots were performed for (a) caspase-1, (b) NLRP3, and (c) ASC expression. (d) qPCR of caspase-1 expression in the epidermis of newborn wild-type or iκbα knockout mice treated with DMSO, IL-1α inhibitory antibody (IL-1α inhi. Ab), or caspase-1 inhibitor (C1-I). For all experiments $n = 5$ biological replicates, and each sample was run in triplicate for qPCR; error bars are SEM, *denotes $P < 0.001$, and **denotes $P < 0.00001$. β-tubulin level was used as a loading control for western blots. WO, inhibitor washed-out.

the IKK-I or with glyburide, which blocks the inflammasome function (Figure 2f and Supplementary Figure 3G online).

These results provide evidence that NF-κB is involved in the regulation of caspase-1 expression, but it is unclear whether NF-κB is the direct upstream transcriptional activator. To address this question, we cloned a 1 kb fragment of the proximal promoter of caspase-1, which contains a conserved NF-κB-binding motif (Supplementary Figure 4A online), and found that mutating this consensus sequence significantly reduced the ability of active NF-κB to induce caspase-1 transcription (Supplementary Figure 4B online). Moreover, we tested whether this likewise occurs on the conserved motif on the endogenous caspase-1 promoter in primary keratinocytes via chromatin immunoprecipitation. After a 4-hour stimulation with IL-1α (Supplementary Figure 4C online), we were able to co-precipitate the NF-κB-binding motif of the caspase-1 promoter with the p65 subunit of the NF-κB complex (Figure 2g), suggesting that this transcription factor is a direct activator of caspase-1 expression.

Caspase-1 amplifies NF-κB signaling in the epidermis

Upon establishing the NF-κB/caspase-1 regulatory relationship *in vitro*, we then focused our attention on its regulation and function within a tissue. We incubated wild-type or C8KO epidermal explants in media with vehicle control (DMSO) or IKK-I and assayed for the amount of secreted IL-1, which is a readout for activated caspase-1. We previously found that the levels of IL-1α in the C8KO skin is higher than wild type, but its secretion was still dependent on caspase-1 activity (Lee *et al.*, 2009). Reducing caspase-1 expression in the C8KO epidermis through the inhibition of NF-κB also reduces the secretion of IL-1α to wild-type levels (Figure 3a). This occurs concomitant with a blockage of proteolytic cleavage of caspase-1 into its active p20 fragment (Supplementary Figure 5A online). Furthermore, we observed that the prolonged activity of NF-κB in the iκbα-deficient epidermis results in the higher expression of procaspase-1 (Figure 1d), prompting us to examine whether this likewise translates into an increase in IL-1α secretion. The prolonged

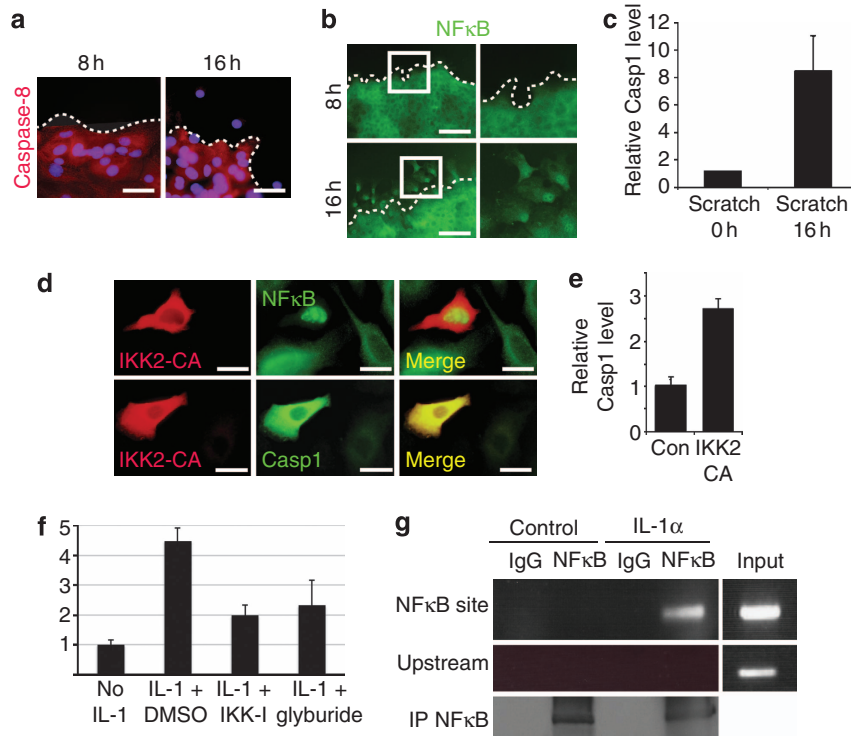


Figure 2. Regulation of caspase-1 by NF- κ B in wounded cells. (a) Immunofluorescence of (a) caspase-8 (red), DAPI (blue), and (b) NF- κ B (green) in primary keratinocytes after 8 or 16 hours of applying a scratch wound (dotted line denotes wound margin). Right panels in **b** are the magnified views of the boxed areas in the left panels. (c) qPCR of caspase-1 RNA levels at 0 and 16 hours after applying scratch wound. (d) Primary keratinocytes were transfected with constitutively active HA-tagged IKK2 (HA-IKK2-CA) and stained for active IKK2 (red) and NF- κ B (green) or caspase-1 (green). Cells expressing IKK2 have nuclear NF- κ B and higher levels of caspase-1. (e) qPCR of relative levels of caspase-1 RNA in keratinocytes transfected with empty vector (Con; normalized to 1) or IKK2-CA. (f) Relative caspase-1 protein levels in keratinocytes exposed to IL-1 and DMSO, IKK-I, or glyburide. Caspase-1 protein levels in cells without IL-1 α stimulation is normalized to 1, and data are presented as fold difference. Data are the mean of three biological replicates, and error bars are SEM. (g) ChIP of NF- κ B upon stimulation with IL-1 α and immunoprecipitation with IgG control antibody or an antibody recognizing the p65 subunit of NF- κ B. *indicates $P < 0.001$. For qPCR experiments (c and e), results are from three biological replicates with each PCR reaction performed in triplicate (error bars are SEM).

NF- κ B signaling in the $\text{I}\kappa\text{B}\alpha$ KO resulted in a substantial increase in activated caspase-1 (Supplementary Figure 5B online) and in IL-1 α secretion (Figure 3b) compared with the wild-type epidermis. Reducing the activity of caspase-1 in the $\text{I}\kappa\text{B}\alpha$ -knockout epidermis (Supplementary Figure 5B online) markedly reduced the amount of secreted IL-1 α (Figure 3b). This effect of activated NF- κ B on raising IL-1 α secretion can be reconstituted *in vitro* by transfecting keratinocytes with the IKK2-CA construct (Figure 3c). Moreover, pharmacological inhibition of caspase-1 activity can also abrogate the stimulation of IL-1 α secretion. The secretion of IL-1 α has been shown to be dependent upon caspase-1 activity (Keller *et al.*, 2008), and the NF- κ B–caspase-1 signaling pathway also yields similar results when tested on the intracellular levels of IL-1 α (Supplementary Figure 5C–F online), as well as the secretion of IL-1 β , a classical readout of caspase-1 function (Supplementary Figure 5G–I online).

The ability of IL-1 α ligand to activate NF- κ B suggests that the release of this cytokine can propagate the NF- κ B–caspase-1–IL-1 α module throughout the epidermis via a paracrine effect. We found that removal of the IL-1 receptor in the wound model diminished the level of secreted IL-1 α compared with the C8cKO epidermis (Figure 3d). We further

tested this paracrine model by transfecting primary keratinocytes with the IKK2-CA. As we have shown, keratinocytes expressing IKK2-CA displayed nuclear NF- κ B 12 hours after transfection (Figure 2d). However, when we allowed the cells to incubate longer (~24 hours), cells adjacent to the transfected cells were induced to activate NF- κ B (Figure 3e). Use of the IL-1 α -neutralizing antibody or the caspase-1 inhibitor restricted NF- κ B activation solely to cells transfected with IKK2-CA.

To test whether this is also valid in wound-like conditions *in vivo*, we subcutaneously injected vehicle control (DMSO) or IKK-I daily in the back skin of newborn wild-type or C8cKO mice for 4 days. Inhibition of NF- κ B activation, which blocks IL-1 α release (Figure 3a), suppresses the expression of active NF- κ B throughout the epidermis, although the dermal activation of NF- κ B is largely unaffected (Figure 4a). This block in epidermal activation of NF- κ B was also present when excisional wounds were treated with IKK-I (Supplementary Figure 6 online). Moreover, genetic removal of caspase-1 expression from the C8cKO epidermis (Figure 4b and c) likewise reduces the expression of active NF- κ B throughout the epidermis.

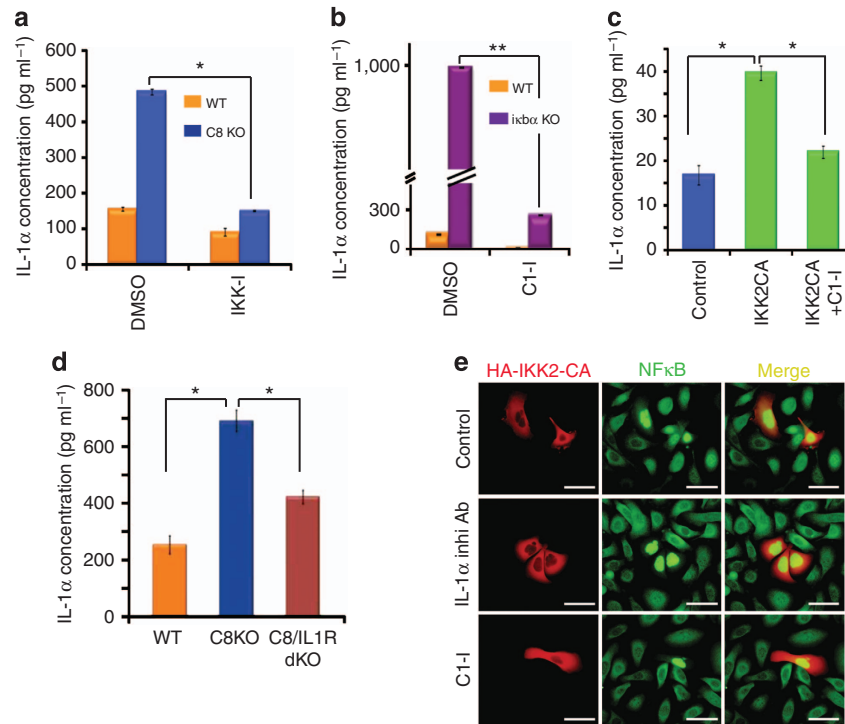


Figure 3. Caspase-1 propagates NF- κ B signaling in epidermal keratinocytes. Secretion of IL-1 α was measured by ELISA from different samples: (a) Wild-type (orange bars) or caspase-8-null (C8KO; blue bars) epidermis treated with DMSO or IKK-I; (b) wild-type or ikb α -null (purple bars) epidermis treated with DMSO or C1-I; (c) primary keratinocytes transfected with empty vector (control), IKK2-CA, or IKK2-CA and treated with C1-I; (d) the epidermis from wild-type, caspase-8-null, and caspase-8/IL-1R double knockout mice. ELISA results are the mean of three biological replicates, and error bars are SEM. *denotes $P < 0.001$; **denotes $P < 0.00001$. (e) Primary keratinocytes were transfected with constitutively active IKK2 (HA-IKK2-CA) and treated with DMSO (control), IL-1 α inhibitory antibody, or caspase-1 inhibitor (C1-I) and stained for IKK2-CA (red) and NF- κ B (green). Data are representative of three independent experiments.

As the inhibition of NF- κ B activity and caspase-1 expression both prevented the spreading of active NF- κ B in the C8cKO epidermis, we predicted that the expression of caspase-1 would likewise be adversely affected. Caspase-1 is expressed at a low basal level in wild-type epidermis, is significantly upregulated in the C8cKO tissue, and this induction is lost in the presence of IKK-I (Figure 4d). Interestingly, application of a pharmacological inhibitor of caspase-1 activity results in a marked reduction in the expression of caspase-1 throughout the tissue, and the remaining protein is restricted to the upper layers of the epidermis (Figure 4d). Moreover, in the absence of IL-1 signaling, caspase-1 expression is not highly induced in the C8cKO epidermis (Figure 4e). Therefore, abrogation of NF- κ B activation inhibits the upregulation of caspase-1 expression and inhibition of the latter's proteolytic activity can effectively block the spreading of NF- κ B and caspase-1 expression from the surface of the epidermis to the lower layers of this tissue, which occurs through IL-1 paracrine signaling.

Inhibition of NF- κ B and caspase-1 delays wound closure rates

We then focused our attention on the functional contribution of these proteins to the wound-healing response. Excisional wounds were made on the back of wild-type mice treated with DMSO or IKK-I, caspase-1 knockout mice, and IL-1R KO mice (Figure 5). Interestingly, inhibition of NF- κ B activation or

caspase-1 expression significantly delayed the closure rate of an excisional wound compared with control samples (Figure 5a). This delay in wound closure by 2 or more days parallels the effects seen when other important factors for wound healing are deleted (Devalaraja *et al.*, 2000; Echtermeyer *et al.*, 2001; Jameson *et al.*, 2002). We verified that both the inhibition of NF- κ B activation and caspase-1 expression indeed impacted inflammation as they were able to decrease the recruitment of immune cells to the skin. For instance, the number of macrophages and T cells recruited to the skin in the C8cKO (Figure 5b and c) or to wounded skin (Supplementary Figure 7A and B online) is severely reduced when NF- κ B activity or caspase-1 expression is suppressed. The failure to recruit immune cells to the skin under these conditions is also reflected in the diminished expression of inflammatory genes (Figure 5d). Together, these data highlight the critical role for the NF- κ B/caspase-1/IL-1 axis in the establishment of the inflammatory phase of the cutaneous wound-healing response.

NF- κ B/caspase-1 signaling affects proliferation during the wound-healing response

Closer examination of the effects of the NF- κ B and caspase-1 inhibition on the rate of wound closure reveals an affect during the proliferative phase of the wound response, which commences around day 3 post wounding. This effect may be

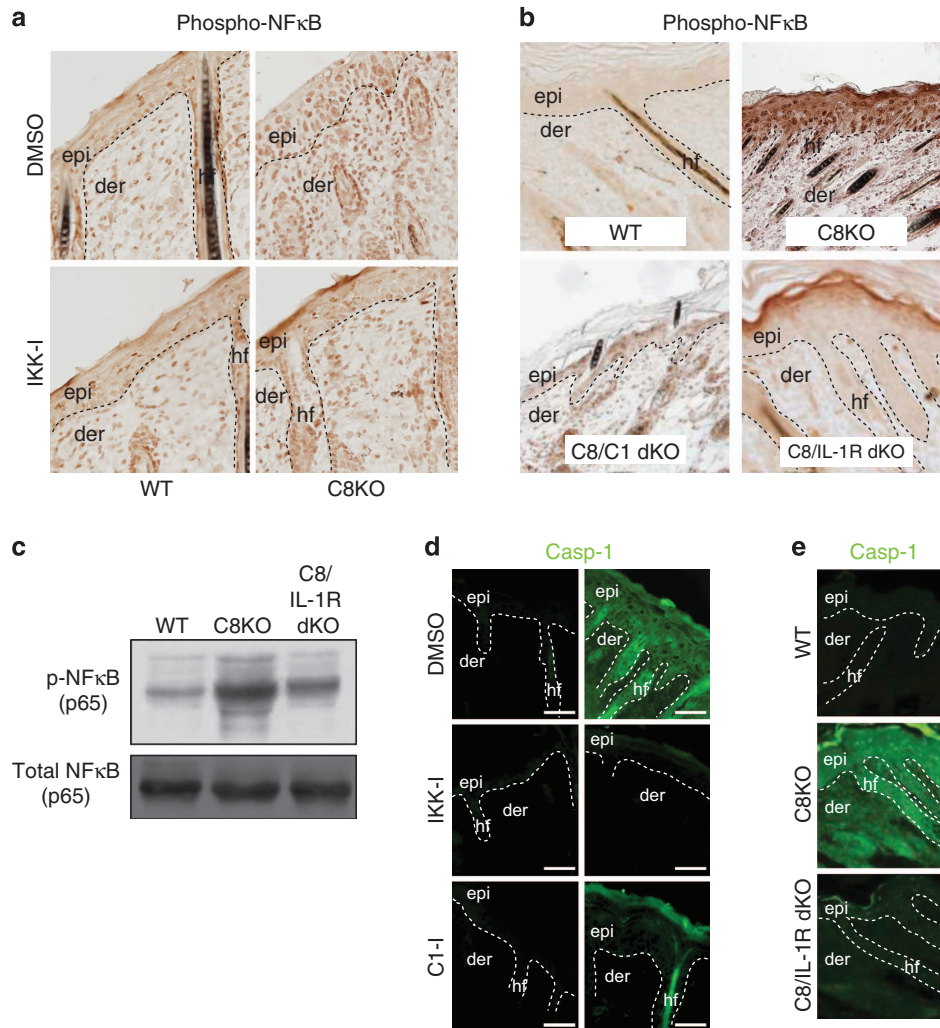


Figure 4. Caspase-1 expands NF-κB activity throughout the epidermis. Immunohistochemistry of phosphorylated NF-κB in (a) wild-type and caspase-8 knockout skin injected with DMSO or IKK-I and (b) in the skin lacking both caspase-8/caspase-1 (C8/C1 dKO) or the skin that is null for caspase-8 and IL-1 receptor (C8/IL-1R dKO). (c) Level of phosphorylated NF-κB, with total NF-κB as loading control. (d) Caspase-1 expression in wild-type or C8KO epidermis treated with vehicle control or chemical inhibitors. (e) Caspase-1 expression in wild-type, caspase-8-null, and caspase-8/IL-1R double knockout skin samples.

attributable to the role that NF-κB and caspase-1 have in the secretion of IL-1. We previously demonstrated that the IL-1-mediated epithelial-mesenchymal signaling loop that stimulates keratinocytes proliferation in a wound is recapitulated in the C8cKO model (Lee *et al.*, 2009). One outcome of this pro-proliferation signaling network is the thickening of the epidermis (epidermal hyperplasia).

Consistent with this model, inhibition of NF-κB activation or loss of caspase-1 expression in the C8cKO skin significantly reduced epidermal hyperplasia (Figure 6a and Supplementary Figure 8A online). This phenomenon was also seen in response to excisional wounds (Supplementary Figure 8B online). The thickening of the epidermis in the C8cKO mouse is fueled by an increase in the number of proliferating progenitor cells found in the basal layer of the epidermis and the hair follicle (Figure 6b). Both are reduced in the presence

of the inhibitor of NF-κB activation or loss of caspase-1 expression (Figure 6b-d). However, these effects are not applicable to all the phenomena associated with the proliferative phase of the wound-healing response. For instance, new blood vessel formation is a feature of this phase of the wound-healing program, but the injection of IKK-I or the loss of caspase-1 expression do not qualitatively decrease angiogenesis in the skin (Figure 6e). Altogether, these observations link a functional inflammatory phase of the wound-healing response with the optimal stimulation of cell proliferation that occurs during a wound-healing response.

DISCUSSION

A major obstacle to understanding wound healing is the untangling of the extensive interdependence of the three classical phases of this process. Using the ablation of

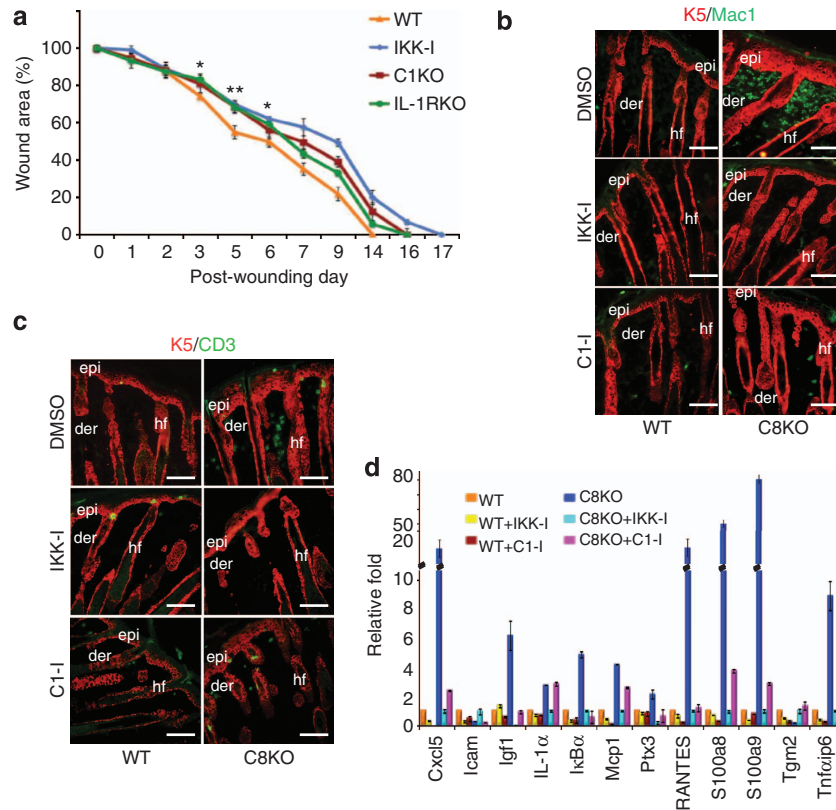


Figure 5. Effect of NF- κ B and caspase-1 on wound closure. (a) Rates of wound closure were measured in wild-type mice treated daily with DMSO or IKK-I or in mutant mice lacking either caspase-1 (C1KO) or IL-1 receptor (IL-1RKO). Data points are averages of six mice with two wounds per mouse (*denotes $P < 0.001$; **denotes $P < 0.00001$ when comparing DMSO versus IKK-I kinetics in wild-type mice). (b) Recruitment of macrophages (green) in the wild-type and caspase-8-null skin treated with DMSO (control) or IKK-I or in the skin lacking caspase-1 and -8. (c) Recruitment of T cells (green) monitored using the pan T-cell marker CD3. The epidermis (epi) and hair follicles (hf) in **b** and **c** are noted in red with an antibody detecting keratin-5. Bar = 50 μ m; der, dermis; (d) Inflammatory gene expression in the wounded/wound-like skin. RNA was extracted from the wounded skin of wild-type mice (treated with DMSO (orange bars) or IKK-I (yellow bars)) or caspase-1 KO mice (C1KO; red bars) 3 days post-excisional wounding. RNA was also extracted from the skin of P5 caspase-8 knockout (C8KO) mice (treated with DMSO (blue) or IKK-I (light blue)) or the caspase-8/caspase-1 (C1KO) double knockout mouse (magenta). Results are from three biological replicates for each mouse genotype/treatment, and each sample was repeated in triplicate. Error bars are SEM. der, dermis.

epidermal caspase-8 as a model of wound healing, we have uncovered a molecular mechanism synchronizing these complex reactions. Upon wounding, caspase-8, which normally resides in the granular layer near the surface of the epidermis, is downregulated and initiates a biochemical program to promote tissue repair (Lee *et al.*, 2009). One effect of this downregulation is the increase in IL-1 α RNA expression, as well as the release of the preexisting reservoir of this cytokine from epidermal keratinocytes (Lee *et al.*, 2009). IL-1 α has a plethora of effects ranging from the promotion of an inflammatory microenvironment to promoting a growth arrest and survival model in suprabasal keratinocytes to the stimulation of stem cell proliferation. Given the profound impact of IL-1 α signaling in the framework of wound healing, the mechanism governing its release is an important issue to resolve. We found that at least two different signaling pathways impact the components of the NLRP3 inflammasome—p38 MAPK regulates NLRP3 expression (Lee *et al.*, 2009) and NF- κ B influences caspase-1 expression levels (Supplementary Figure 9 online).

Together, these pathways lead to IL-1 α secretion that can operate in an autocrine manner to potentiate the NF- κ B signaling cascade or function in a paracrine manner to propagate the NF- κ B signaling to different layers of keratinocytes in the epidermis. This exemplifies the central and complex role that IL-1 α and NF- κ B have in regulating epithelial homeostasis (Swamy *et al.*, 2010).

Recently, however, work from David Wallach's group has raised doubts about the role of IL-1 α in mediating inflammation in the epidermal C8cKO mouse (Kovalenko *et al.*, 2009). This is based on their finding that genetic ablation of both IL-1 α and IL-1 β did not abrogate the cutaneous inflammation stimulated by the loss of epidermal caspase-8. However, as we observed, removal of one signaling pathway such as IL-1 can delay the kinetics of wound closure but not completely abrogate it. The ≥ 2 -day delay in wound healing by blocking components of the NF- κ B/caspase-1/IL-1 axis is consistent with the delay in wound healing found when other important components are deleted such as $\gamma\delta$ T cells (Jameson *et al.*, 2002), syndecan-4 (Echtermeyer *et al.*, 2001), and CXCR2

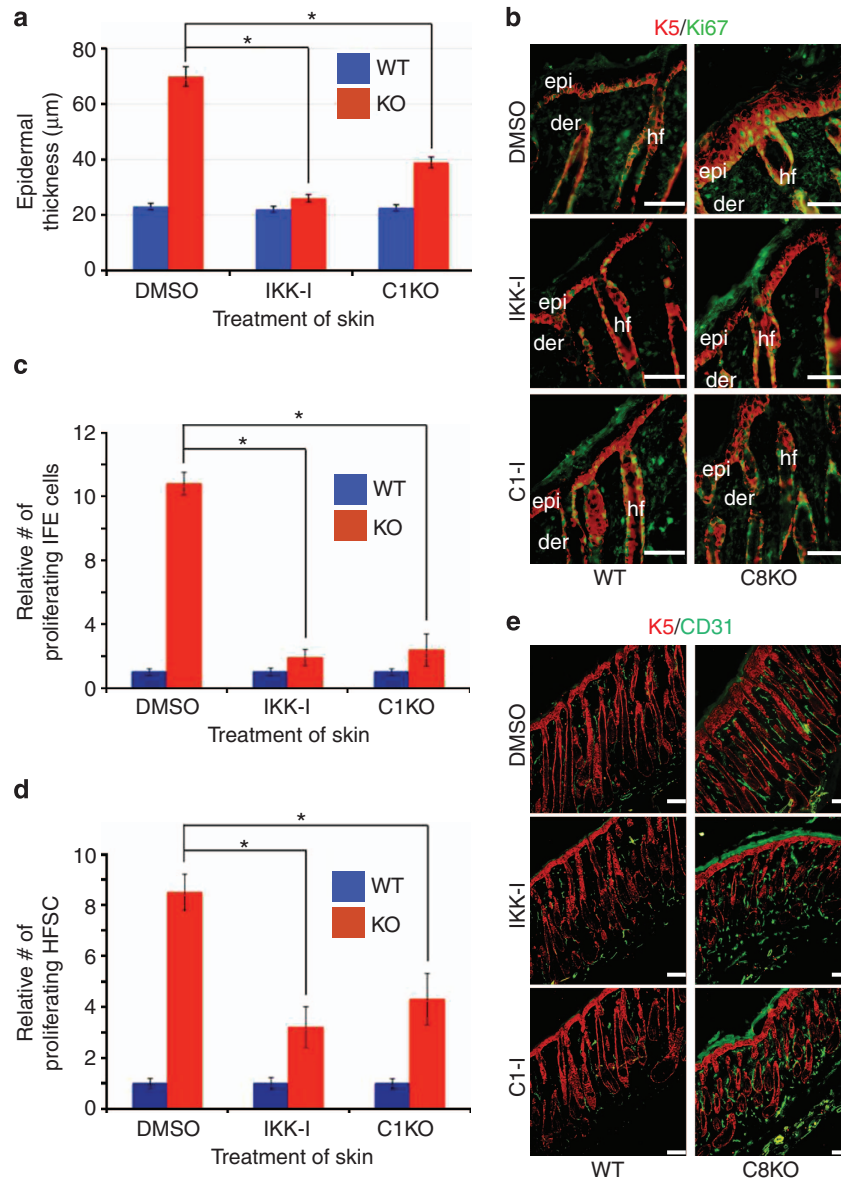


Figure 6. Hyperproliferation of the epidermis in caspase-8 KO and wounded skin. (a) Measurement of epidermal thickness of wild-type (WT) or caspase-8 knockout (C8KO) skin treated with DMSO, or IKK-I, or the skin also lacking caspase-1. (b) Proliferating cells were marked by Ki67 (green) and the epidermis and hair follicle are marked by keratin-5 (red) in mice treated as in a. Bar = 50 μm. Quantification of proliferating (c) interfollicular epidermal (IFE) stem cells and (d) hair follicle stem cell (HFSC) in skin sections from b. Results are the average of six skins (five sections per skin) for each treatment, and error bars are SEM, (*denotes $P < 0.001$). (e) Angiogenesis monitored in wild-type or knockout mice treated with DMSO, or IKK-I, or the skin also lacking caspase-1 via staining with CD31 (green).

(Devalaraja *et al.*, 2000). We also observed that the C8cKO/IL-1 receptor double knockout transiently dampens the phenotype of the C8cKO mouse, which is likely owing to the fact that other components in the inflammatory phase can compensate for the loss of one constituent. Further support for IL-1 signaling in a wound-like environment is provided by the finding of increased nuclear NF-κB when the IL-1 receptor antagonist is missing (Ishida *et al.*, 2006). Thus, we propose that the large reservoir of IL-1α released from damaged keratinocytes is among the earliest responders to epidermal trauma to stimulate the wound-healing response. In particular, IL-1α can induce the inflammatory phase and thereby promote the proliferative phase, and this role is assumed by

infiltrating immune cells, which secretes the more potent IL-1β.

An important question that arises from our model is the link between the downregulation of caspase-8 and the activation of NF-κB prior to the positive feedback loop mediated by IL-1α secretion. Of particular relevance are reports that deletion of RIP3 rescues the caspase-8 knockout phenotype, including cutaneous inflammation (Gunther *et al.*, 2011; Kaiser *et al.*, 2011; Welz *et al.*, 2011). Interestingly, more recent studies have demonstrated that RIP3 can also activate NLRP3 and caspase-1 (Vince *et al.*, 2012; Kang *et al.*, 2013). Thus, the loss of caspase-8 may liberate RIP3 to interact with different partners to stimulate inflammasome activation.

Further delineating the details of inflammasome activation will contribute important new insights on how to combat inflammatory diseases when these signaling pathways are perturbed.

MATERIALS AND METHODS

Reagents

The list of antibodies, inhibitors, and primers and their usage are detailed in the Supplementary Information Section online.

Mice and cell/tissue culture

Generation of C8KO (Lee *et al.*, 2009) and *ikb α* KO (Klement *et al.*, 1996) mice has previously been reported. Wild-type primary mouse keratinocytes are obtained from the epidermis of newborn pups and cultured in low-calcium mouse keratinocyte media as described previously (Lee *et al.*, 2009). Epidermal explants were taken from P0-P3 skin by chemically dissociating it from the dermis with dispase for 1 hour at 37 °C. All animal work was carried out in accordance with the policies and approved procedures of the Institutional Animal Care and Use Committee of University of California San Diego.

Tissue preparation

Epidermal explants were incubated with p38-I and IKK-I overnight and collected in Trizol (Invitrogen) for RNA extraction or in sample buffer for protein extraction. The wash-out of IKK-I-treated epidermis was carried out by washing with phosphate-buffered saline once and transferred to fresh mouse keratinocyte media and incubated for another 6 hours. Glyburide was incubated with the epidermis for 1 hour and transferred to fresh media with glyburide for another hour. IL-1 α (2 ng μ l⁻¹) was added to WT and *ikb α* KO epidermis for 2 hours and replaced with fresh media and incubated for another 6 hours prior to sample collection.

Wounding and injection of mice

DMSO, IKK-I, and caspase-1 inhibitor were injected into the back skin of WT and C8KO mice daily for 4 days starting at P0 prior to the onset of the phenotype, and the back skin was collected on the 5th day for RNA extraction and OCT embedding. *In vivo* wounding was performed on the back skin of adult wild-type mice with 5 mm-punch biopsy, and DMSO and inhibitors were applied to the wound site daily for 3 days. Wounded skin was collected for RNA extraction and OCT embedding. Data presented are averages of two wounds per mouse ($n=6$ mice \pm SEM) for each treatment. For *in vitro* scratch wounds, primary mouse keratinocytes were cultured on coverslips to confluency. The scratch wound was performed on the cell monolayer with a p200 pipet tip. Cells were incubated at 37 °C for up to 16 hours and then fixed for immunofluorescence or collected for RNA extraction.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

REFERENCES

- Bauernfeind FG, Horvath G, Stutz A *et al.* (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183: 787–91
- Church LD, Cook GP, McDermott MF (2008) Primer: inflammasomes and interleukin 1beta in inflammatory disorders. *Nat Clin Pract Rheumatol* 4: 34–42
- Devalaraja RM, Nanney LB, Du J *et al.* (2000) Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol Symp Proc* 115:234–44
- Echtermeyer F, Streit M, Wilcox-Adelman S *et al.* (2001) Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J Clin Invest* 107: R9–14
- Gunther C, Martini E, Wittkopf N *et al.* (2011) Caspase-8 regulates TNF-alpha-induced epithelial necroptosis and terminal ileitis. *Nature* 477:335–9
- Gurtner GC, Werner S, Barrandon Y *et al.* (2008) Wound repair and regeneration. *Nature* 453:314–21
- Ishida Y, Kondo T, Kimura A *et al.* (2006) Absence of IL-1 receptor antagonist impaired wound healing along with aberrant NF-kappaB activation and a reciprocal suppression of TGF-beta signal pathway. *J Immunol* 176: 5598–606
- Jameson J, Ugarte K, Chen N *et al.* (2002) A role for skin gammadelta T cells in wound repair. *Science* 296:747–9
- Kaiser WJ, Upton JW, Long AB *et al.* (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature* 471:368–72
- Kang TB, Yang SH, Toth B *et al.* (2013) Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity* 38:27–40
- Karin M (2009) NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol* 1:a000141
- Keller M, Ruegg A, Werner S *et al.* (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132:818–31
- Klement JF, Rice NR, Car BD *et al.* (1996) IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. *Mol Cell Biol* 16:2341–9
- Kovalenko A, Kim JC, Kang TB *et al.* (2009) Caspase-8 deficiency in epidermal keratinocytes triggers an inflammatory skin disease. *J Exp Med* 206: 2161–77
- Lee P, Lee DJ, Chan C *et al.* (2009) Dynamic expression of epidermal caspase 8 simulates a wound healing response. *Nature* 458:519–23
- Mao PL, Jiang Y, Wee BY *et al.* (1998) Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. *J Biol Chem* 273:23621–4
- Merkle S, Frantz S, Schon MP *et al.* (2007) A role for caspase-1 in heart failure. *Circ Res* 100:645–53
- Schroder K, Tschopp J (2010) The inflammasomes. *Cell* 140:821–32
- Swamy M, Jamora C, Havran W *et al.* (2010) Epithelial decision makers: in search of the 'epimmunome'. *Nat Immunol* 11:656–65
- Vallabhapurapu S, Karin M (2009) Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* 27: 693–733
- Vince JE, Wong WW, Gentile I *et al.* (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. *Immunity* 36:215–27
- Welz PS, Wullaert A, Vlantis K *et al.* (2011) FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature* 477: 330–4
- Yamanaka K, Tanaka M, Tsutsui H *et al.* (2000) Skin-specific caspase-1-transgenic mice show cutaneous apoptosis and pre-endotoxin shock condition with a high serum level of IL-18. *J Immunol* 165:997–1003
- Yu HB, Finlay BB (2008) The caspase-1 inflammasome: a pilot of innate immune responses. *Cell Host Microbe* 4:198–208