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Novel role of caspase-8 in the maintenance of epidermal regeneration and repair

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Novel Role of Caspase-8 in the Maintenance of Epidermal Regeneration and Repair

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Biology

by

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Professor Wendy Havran
Professor Stephen Hedrick
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2011
The Dissertation of Pedro Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011
DEDICATION

To my dear mother:

In life, you instilled in me the confidence to carry on even in the most difficult times. Your presence in my life was the force that guided me on the right path even when I went astray. In death, I know that you are by my side always and keeping watch over me. I love you mother.
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The text in Chapter II is a modified version of a manuscript published in the journal Nature in 2009. Pedro Lee, Dai-Jen Lee, Carol Chan, Shih-Wei Chen, Iren Ch’en and Colin Jamora. Dynamic expression of epidermal caspase 8 simulates a wound healing response. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Novel Role of Caspase-8 in the Maintenance of Epidermal Regeneration and Repair

by

Pedro Lee

Doctor of Philosophy in Biology

University of California, San Diego, 2011

Professor Colin Jamora, Chair

Mammalian skin plays an essential role in protecting the body from environmental insults and as a result exhibits remarkable healing capabilities. Skin repair is characterized by the expression of three distinct but overlapping phases: inflammation, proliferation and remodeling. Successful restoration of tissue function after damage depends on the interactions among these phases. Despite the wealth of information on the wound healing process, little is known about the molecular mechanisms that are responsible for the crosstalks between the different phases. I present data demonstrating that the loss of epidermal caspase-8, an important mediator
of apoptosis, recapitulates multiple features of a wound healing response and acts as the switch that turns on the repair process. The epidermal hyperplasia in the caspase-8 null skin is the cumulative result of signals exchanged between epithelial and mesenchymal cells and leukocytes.

I show in chapter II that the reciprocal interaction between epidermal and mesenchymal cells is initiated by the paracrine activity of interleukin-1α (IL-1α) which stimulates both skin stem cell proliferation and cutaneous inflammation. Secretion of IL-1α is a consequence of the assembly of a functional NALP3 inflammasome mediated by p38 MAPK activation. Interestingly, this increased proliferation is counterbalanced by IL1α-dependent NFκB signaling which causes keratinocyte growth arrest and cell survival. In chapter III, I present data on the differential proliferation of the skin stem cells pools. I show that IL-1α and IL-7 have synergistic effects in expanding the number of activated γδ T-cells. These epidermal resident immune cells then preferentially promote the proliferation of hair follicle stem cells.
Chapter I:

General Introduction
**Introduction to the Mammalian Skin**

The mammalian skin undergoes continuous self-renewal and differentiation during the lifetime of the animal and is a great model system to study the mechanisms that govern stem cell behavior. The skin is comprised of two major layers, the epidermis and dermis (Fig. 1), which are separated by an extracellular matrix referred to as the basement membrane. During mouse development, the embryonic ectoderm starts as a single sheet of epithelial cells that starts to stratify at embryonic age 13.5 (E13.5) to make the epidermis and in the process forms biochemically distinct layers [1]. Mitotically active skin stem cells (keratinocytes) reside in the basal layer of the epidermis and have the ability to replenish the pool of progenitor cells. These basal layer cells express the biochemical marker keratin-5 (K5) and once they leave the basal compartment, they are committed to a terminal differentiation program. Keratinocytes that exit the basal compartment form the spinous layer, which is characterized by a flattened morphology and exit from the cell cycle. Spinous layer cells display increased structural integrity provided in part by the polymerization of the intermediate filaments keratin-1 (K1) and keratin-10 (K10). Further along the differentiation program are the granular layer cells expressing the late differentiation markers filaggrin and loricrin, which have important roles in providing added structural integrity to the skin. The terminal differentiation program culminates with the formation of a network of highly crosslinked, dead and enucleated cells (corneocytes) that form the squamous layer. The resulting crosslinking of proteins and lipids in this layer is responsible for providing the barrier function of the skin.

Owing to its protective function, the epidermis turns over every few weeks and this tissue depends on two modes of regulation to maintain homeostasis. The first mode of regulation is observed in the basal layer, where the stem cells must decide whether to
differentiate in order to maintain the integrity of the tissue or self-renew to replenish the spent pool of stem cells [2]. The second mode of regulation is observed in the granular layer, where these cells must decide whether to survive or undergo death. The equilibrium of keratinocyte proliferation and cell death is critical for the proper maintenance of epidermal homeostasis [3]. Disruption of this balance to favor proliferation has been observed in pathological conditions such as hyperkeratosis, psoriasis and skin cancer. On the other hand, excessive cell death is characteristic of conditions such as sunburn, toxic epidermal necrolysis and graft-versus-host disease [4].

Overview of the Wound Healing Response

In multicellular organisms, the ability to repair damaged tissue is a fundamental property that ensures the survival of the individual. Wound healing is one of the most complex processes in nature and despite the great advances in the field of skin biology in recent years the molecular details remain poorly understood [5]. Upon physical disruption of its barrier function, the skin initiates a repair or wound-healing program that is typically characterized by the distinct yet overlapping phases of inflammation, proliferation and remodeling. Since the skin is a complex tissue, a full-thickness wound damages many different cells types and structures. The resulting responses from the various cell types in the skin guide the repair program through the different phases of the wound healing process [6].

A. Inflammation

One of the immediate effects of wounding is the rupturing of local blood vessels, which causes platelet activation and aggregation. Activated platelets are then
responsible for the formation of a network of fibrin fibers known as a fibrin clot that is important for stopping hemorrhage. In addition, this fibrin clot serves as a surface on which keratinocytes and other cells migrate to reseal the wound. Through the release of growth factors and chemokines, activated platelets also promote various aspects of the repair process, which include inflammation, angiogenesis, keratinocyte and fibroblast migration [7].

During the inflammatory phase of the wound healing response immune cell infiltration from the peripheral blood, particularly neutrophil migration, is observed within minutes after trauma [8]. These cells play important roles in the killing of invading microorganisms as well as influencing many features of the repair process. Shortly thereafter, monocyte infiltration and differentiation into macrophages is observed where they clear up cellular debris through their classical phagocytic activity [5]. Macrophages are a potent source of inflammatory cytokines that influence the cellular behavior of other cells near the wound. For example, macrophages promote angiogenesis and enhance keratinocyte proliferation [9]. Despite these crucial roles, deficiency of either neutrophils or macrophages can be overcome by a redundancy in the inflammatory response, which has brought into question the necessity of these cells during wound healing [6]. In addition to neutrophils and macrophages, skin resident immune cells such as γδ T cells and Langerhans cells are also rapidly activated upon wounding and in turn release cytokines, chemokines and growth factors. Of particular importance are γδ T cells, as wound healing experiments with mice deficient of these cells show impaired wound healing ability, which may be due in part to the fact that γδ T cells are important sources of factors that stimulate keratinocyte proliferation and differentiation such as
fibroblast growth factor-7 and -10 (FGF-7 and FGF-10) and insulin growth factor-1 (IGF-1) [10, 11].

From the molecular perspective, the rupturing of cells at the edge of the wound release endogenous signals that activate “stress” signaling pathways in neighboring cells. For example, keratinocytes are a rich source of the proinflammatory cytokine interleukin-1α (IL-1α), which induces local inflammation and stimulates the release of keratinocyte growth factors from dermal fibroblasts [12, 13]. Similarly, the roles of the Jun amino terminal kinase (JNK) and p38 MAPK are well established as mediators of the stress signals, where they serve to amplify and/or propagate damage signals [9, 14]. In addition, release of damaged-associated molecular patterns (DAMPs) molecules serve to activate other signaling pathways and also act as chemotactic signals for immune cell recruitment [15]. The signaling cascades initiated by the stress kinases, proinflammatory cytokines and DAMPs lead to physiological changes and alterations in gene expression to ensure cell survival, recruitment of other cell types and cell migration, which are important for proper wound closure.

B. Proliferation and re-epithelialization

Some of the features of the repair process, such as re-epithelialization, have striking similarities to morphological events observed during development such as dorsal closure in Drosophila melanogaster [16]. At the molecular level, some of the same components used during development are also found in epithelial wound closure [17]. The use of dorsal closure in D. melanogaster embryos and ventral closure in Caenorhabditis elegans to model tissue movement, a process analogous to re-epithelialization, resulted in the identification of the JUN amino terminal kinase (JNK) as a pivotal signaling pathway that is also recapitulated during repair [18].
In mammalian skin following mechanical trauma, the process of re-epithelialization is characterized as a combination of cell migration and proliferation. Cell migration at the leading edge of the wounded epidermis occurs as collective cell movements following cell-cell and cell-matrix adhesion changes [19]. Migrating keratinocytes change their preference from laminin V to collagen IV based adhesion and upregulate the expression of matrix metalloproteinases to navigate through the clot and seal the wound [20, 21]. The sources of cells needed for re-epithelialization come from the proliferation of epidermal resident and hair follicle stem cells [22]. The latter population resides in an anatomical region of the hair follicle known as the bulge. Bulge stem cells have multipotent capacity that enables them to give rise to hair follicle, sweat and sebaceous glands [23]. During homeostasis, hair follicle stem cells participate in the formation of hair follicles and its associated appendages, however, in wound healing conditions, hair follicle stem cells can contribute to the formation of epidermis [22].

C. Remodeling

The final phase of the wound healing response entails the ECM/cellular remodeling and it can last from months to years. Increased proliferation of dermal fibroblasts populates the damaged tissue and deposit large amounts of extracellular matrix (ECM). Several days post-wounding, some of the fibroblasts in the wounded area differentiate into myofibroblasts marked by the expression of α-smooth muscle actin (αSMA). The main role of these myofibroblasts is to contract the wound and secrete additional matrix proteins [24]. In addition, bone marrow derived cells differentiate into collage producing fibroblasts in the wounded area and together with dermal fibroblasts degrade the initial fibrin clot by secreting proteinases [25]. The fibrin network is eventually replaced with granulation tissue, which is an ECM composed of fibronectin,
collagen I and III, proteoglycans and glycosaminoglycans [24]. Upon completion of the wound repair, myofibroblasts, macrophages and most of the endothelial cells undergo apoptosis or exit the wound [26].

**Roles of Caspases**

The end stages of terminal differentiation in skin are reminiscent of apoptosis in that the cells become enucleated and die [27]. Apoptosis or programmed cell death is a key modulator of morphogenesis that is essential in sculpting organs during development, turnover of tissues and elimination of damaged cells. Thus, balancing the rate of keratinocyte proliferation and apoptosis is critical in the maintenance of epidermal tissue. The main players involved in this process of cellular death are the family of cysteine dependent aspartate specific proteases called caspases. Apoptosis can occur in two ways, through extrinsic or intrinsic pathways. The focus of my dissertation was on the role of epidermal caspase-8, a key regulator of apoptosis in the extrinsic pathway. Upon activation of death receptors by extracellular signals such as TNFα, caspase-8 initiates a cytosolic cascade of proteolytic activation of the death machinery. However, conditional knockout studies have suggested that this protein may have non-apoptotic functions as well. Some caspases have been found to mediate pro-inflammatory responses, but their mechanisms of action remain largely undefined [28]. Caspase-8 and other members of the caspase family have been reported to participate in the regulation of cytoskeletal dynamics [29, 30]. Recently, caspase-11 has been reported to modulate actin dynamics through its interaction with coflin [31]. Caspase-14 was shown to be required for the formation of the differentiated layers of the epidermis and the barrier function of the skin [32].
Figure 1. Anatomical organization of the mammalian skin. Biochemical markers represented are: Keratin-5 (K5), Keratin-14 (K14), Keratin-1 (K1), Keratin-10 (K10).
Chapter II:

Epidermal Caspase-8 Knockout as a Model of Wound Healing
INTRODUCTION

As one of the few organs that undergo continual renewal, the mammalian skin is a powerful tool to study the regulation of organ regeneration. Because of this life-long renewal process, stem cells in the skin must constantly balance the need to replenish itself and maintain a pool of progenitor cells with the requirement to differentiate to reconstruct a tissue that turns over every few weeks. During periods of epidermal repair, such as wound healing, this equilibrium shifts dramatically toward proliferation to increase the pool of epidermal cells necessary to restore the damaged section of the skin. The two basic components of the skin are an epithelial compartment and an underlying mesenchymal compartment separated by a basement membrane (Figure 2A). The epithelial compartment is derived from a layer of multipotent progenitor cells which can give rise to the epidermis, hair follicle and sebaceous gland [1]. The epidermis is a stratified structure comprised of a basal layer of proliferative stem cells (keratinocytes) that are the building blocks of this tissue. Select keratinocytes cease proliferating and enter into a terminal differentiation program to form the distinct layers of the epidermis which are responsible for protecting the body against external milieu. The completion of the terminal differentiation program culminates in the death of keratinocytes that are sloughed off from the surface of the skin which requires that the epidermis be constantly renewed. Thus the balance between keratinocyte survival and death is another important factor regulating epidermal homeostasis [4]. Dysregulation of this equilibrium in either direction can lead to various diseases and a molecular understanding of this balance will provide important insights on how to contend with them.

The turnover of keratinocytes at the skin surface shares features of apoptosis which plays a critical role in sculpting organs by helping to determine tissue size and
shape during development and repair [33]. A signaling cascade mediated by a family of cysteine proteases called caspases controls this process of cell death. Exciting new roles are being uncovered for different caspase family members which expands their functional repertoire and their roles in multiple cellular behaviors [34] [35] [36] [32].

RESULTS

Epidermal caspase-8 is downregulated during wound healing

In the course of characterizing proteins that potentially affect epidermal homeostasis I discovered an unexpected expression profile for epidermal caspase-8. Caspase-8 RNA is initially detected at embryonic day 18.5 (E18.5) by RT-PCR (data not shown) and via in situ hybridization is localized near the surface of the epidermis where it persists throughout the life of the animal (Figure 2B). I exploited the unique biochemical markers of each layer of the epidermis to pinpoint its exact location within this tissue. Contrary to the notion of its ubiquitous expression, caspase-8 protein is exclusively expressed in cells of the granular layer of the epidermis where it colocalizes with loricrin (Figure 2C). The observation that the full complement of epidermal layers is present prior to the onset of caspase-8 expression implies that it is not involved in epidermal morphogenesis. Instead its perinatal expression suggests that it functions as a sentinel protein by which epidermal homeostasis is monitored when the skin is exposed to the environment.

Consistent with this hypothesis, microarray profiling of injured diabetic mice correlated impaired wound healing with elevated levels of proapoptotic genes including caspase-8 [37]. Moreover, humans deficient in caspase-8 have clinical symptoms
including eczema, which has features of a chronic wound response [38] [39]. On this basis I sought to determine whether epidermal caspase-8 expression normally fluctuates during the course of a wound healing response. Adult mice were subjected to an excisional wound 2mm in diameter and skin samples were taken immediately after wounding and for the next 14 days at which point the wound was completely healed. In situ hybridization revealed that sites proximal (<1mm) to the wound displayed a thickened epidermis coincident with a downregulation of caspase-8 RNA (Figure 2D). However at sites distal (~5mm) to the wound in which epidermal thickness appeared normal, caspase-8 RNA was expressed in the granular layer. By day 14, when the wound is fully closed and the hyperplasia subsides, caspase-8 RNA expression is restored at the region proximal to the wound site. Consistent with its canonical role in apoptosis, the loss of caspase-8 wound be predicted to suspend the penultimate steps of the terminal differentiation program in order to promote the re-epithelialization of a wound.

**Deletion of epidermal caspase-8 induces keratinocyte hyperproliferation**

In order to probe the contribution of reduced caspase-8 to a wound healing response, I generated an epidermal specific knockout of this gene by mating the mouse carrying the floxed caspase-8 alleles [40] with the K14-Cre transgenic mouse [41]. Visual alterations in the conditional knockout animal first become apparent at postnatal day 5 (P5) and the mice become noticeably lethargic at P15 and are sacrificed soon thereafter. As shown in Figure 3A, the P10 knockout mouse has flaky skin throughout its body and is slightly runted relative to its wild type littermate. Histological analysis revealed a dramatic increase in the thickness of the epidermis of the knockout animal
(Figure 3B). This hyperplasia is consonant with the prediction that loss of caspase-8 in the granular layer would abrogate the natural turnover of the epidermal keratinocytes. However, investigation of the terminal differentiation program revealed an unexpected expansion of the keratin 5 (K5) expressing cells of the epidermis (Figure 3C). In wild type epidermis K5 demarcates the basal layer, which is the residence of epidermal stem cells and transit amplifying cells. In the knockout epidermis, the K5 expressing domains extends 6-7 layers from the basement membrane. Though the onset of differentiation is delayed as noted by the induction of keratin 1 (K1), there was also a concomitant increase in the number of suprabasal layers that express K1 in the knockout animal (Figure 3C). Surprisingly, the granular layer (in which caspase-8 normally resides) was only mildly affected in the absence of this protein.

The thickened epidermis in the knockout animal may be the result of increased keratinocyte proliferation and/or an obstruction of their natural death at the conclusion of the terminal differentiation process. Immunohistochemistry with cyclin D1 revealed that cells of the basal layer in the knockout epidermis have a higher proliferation index relative to the wild type epidermis (Figure 3D). Expression of the proliferation antigen Ki67 not only confirmed this basal cell proliferation but also revealed that cells near the bulge region and infundibulum of the hair follicle were proliferating (Figure 3E). This is particularly intriguing, as it has recently been shown that during a wound healing response follicular stem cells residing in the bulge are mobilized to help reseal the epidermis [42] [22, 43-45]. In light of this observation I assessed whether other markers of epidermal hyperproliferation that occur during wound healing are expressed in the caspase-8 knockout tissue [46, 47]. In wild type skin, keratin 6 (K6) is restricted to the hair follicle, but is robustly expressed in the caspase-8 null epidermis (Figure 3F).
During wound closure β4 integrin (which is usually confined to the basal layer) is likewise expressed suprabasally. Consistent with a role in wound healing, the caspase-8 null epidermis exhibits suprabasal expression of β4 integrin (Figure 3G).

Recently, another related cysteine protease in the granular layer, caspase-14, was found to play a critical role in the formation of the subsequent squamous layer [32]. Invoking an analogous role for caspase-8, the thickening of the knockout epidermis may be due to a block in the formation of the cornified envelope. I tested this possibility by isolating the stratum corneum which is comprised of a sheet of dead, enucleated cells (corneocytes) enveloped within detergent insoluble lipids. The corneocytes extracted from the knockout were indistinguishable from those isolated in the wild type epidermis (Figure 3H). Moreover, parakeratosis was not readily apparent in the knockout epidermis (Figure 3B), which would be expected if caspase-8 were playing its classical apoptotic role in the granular layer. As the primary interface between the body and one’s environment, the skin must repel a variety of invasions. Both wild type and knockout skin exhibited a protective barrier (data not shown), which is functional indication that the anatomy of the skin is intact. Thus, even though the knockout epidermis is hyperproliferative, the terminal differentiation is nevertheless carried out to completion. Altogether these observations suggest that the loss of caspase-8 in the granular layer of the epidermis elicits a paracrine signal(s) to stimulate epidermal and follicular stem cells to proliferate to fuel wound closure.

Loss of epidermal caspase-8 induces an inflammatory response

The increased dermal cellularity present in the knockout skin (Figure 3B) is indicative of an immune cell infiltration. Analysis with different leukocyte markers
demonstrates that the caspase-8 knockout skin shows a dramatic increase in the population of granulocytes and macrophages (Figure 4A). I also assayed for the presence of T-cells using the pan T-cell marker, CD3 and not only found a robust increase in the number of T-cells in the dermis but also elevated numbers in the epidermis (Figure 4B). Both CD4 helper T-cells and CD8 cytotoxic T-cells were elevated in both the epidermal and dermal compartments of the knockout skin relative to its wild type counterpart. I also probed for the status of γδ T-cells owing to the pivotal role these cells have been found to play in the wound healing process by secreting factors that regulate keratinocyte proliferation [48]. In wild type skin, these cells are restricted to the basal layer and hair follicle (Figure 4B). In the caspase-8 knockout skin, however, the number of cutaneous γδ T-cells is increased in the suprabasal layers and dermis. Interestingly, the normal dendritic shape of these cells found in the wild type skin is altered towards a spherical morphology in the knockout tissue which is indicative of γδ T-cell activation [10]. There are two sources of γδ T-cell that could contribute to their increased presence in the knockout skin – the epidermis or peripheral vasculature. In order to determine the derivation of the γδ T-cells found in the dermis, I stained for the expression of Vγ3 which is exclusively expressed on epidermal γδ T-cells (Figure 4B). I found that KO dermis had a high level of Vγ3 expressing γδ T-cells demonstrating that not only can the loss of caspase-8 induce their expression but also stimulate their migration from one tissue compartment to another. Interestingly, all of these immune cells which have infiltrated the caspase-8 knockout skin have also been documented to be present and activated in wounded skin. [6] [48]

The recruitment of these immune cells into the knockout skin implies the occurrence of a cutaneous inflammation reaction. To probe this further I analyzed the
expression levels of a select panel of NFκB target genes that are known to stimulate an inflammatory response using quantitative real-time PCR (Figure 4C). Among the genes I analyzed, the majority was increased between 5-10 fold higher in the knockout skin relative to those found in its wild type counterpart and closely mimicked the expression profile of wounded skin. Interestingly, the most highly expressed genes, S100A8 and S100A9, are secreted from both keratinocytes and macrophages and is associated with both wound healing, psoriasis (which manifests as a chronic wound response), and arthritis[49] [50]. Unlike other mutant mice with hyperplasia and inflammation phenotypes[51] [52], suppression of the immune cell infiltration did not completely abrogate cell proliferation (Figure 5). The diminished hyperplasia in the presence of immunosuppressants implies that epidermal and immune cell signaling cooperate to maximally stimulate keratinocyte proliferation.

**Loss of caspase-8 stimulates epithelial-mesenchymal interactions through IL-1α**

The question then arises as to how the loss of caspase-8 near the epidermal surface stimulates cell proliferation only in the basal layer and hair follicle. To unravel this mechanism we analyzed the expression of active MAPK (pMAPK) whose nuclear localization is commonly used as a read-out of a signaling pathway controlling proliferation. The knockout epidermis exhibited higher pMAPK expression than the wild type epidermis (Figure 4A) and, in addition to its nuclear localization, the pMAPK staining was found in the cytoplasm of cells of the granular layer (Figure 6B). This staining pattern is noteworthy as a transgenic mouse designed to express active MEK1 (the upstream activator of MAPK) phenocopies the caspase-8 knockout mouse [53]. Additionally, targeted suprabasal expression of integrins, which we document in the
knockout mouse (Figure 3G), has also been shown to mediate epidermal hyperplasia and cutaneous inflammation in the transgenic mouse [54]. A conserved theme in these mouse models is an increased level of active interleukin-1 (IL-1). IL-1 is an attractive candidate to mediate the phenotype of the caspase-8 KO mouse owing to its well-established role in regulating keratinocytes proliferation and triggering cutaneous inflammation [55, 56]. Both IL-1α and the nearly identical IL-1β are upregulated in the caspase-8 knockout and wounded skin (Figure 4C). Since IL-1β is primarily secreted from leukocytes I focused my investigation on the activity of IL-1α which is highly expressed in keratinocytes [57]. As shown in Figure 4C, the knockout epidermis expresses higher levels of secreted IL-1α compared to wild type epidermis. I was able to distinguish the secreted cytokine from the large pool of intracellular pro-IL-1α by removing the detergent from the immunofluorescence protocol.

It has been proposed that epidermally derived IL-1α mediates keratinocyte proliferation by first stimulating fibroblasts of the underlying dermis to secrete cytokines which then incite keratinocyte reproduction [56]. To test whether this epithelial-mesenchymal interaction was functional in the caspase-8 knockout skin, I tested whether conditioned media from epidermal and dermal explants generated from both wild type and knockout animals had an effect on the proliferation rate of cultured primary keratinocytes. I employed explants from newborn mice which had no detectable increase in immune cell infiltration between wild type and knockout animals (data not shown). In agreement with this scenario, I found that conditioned media from the dermis of knockout animals stimulated keratinocytes to proliferate at a faster rate than those exposed to wild type conditioned media or normal culture media (Figure 6D). Moreover,
this enhanced proliferation over control cells did not require serum starvation of the keratinocytes.

Since the dermal explants are a heterogeneous population of cells we investigated whether dermal fibroblasts are singularly competent to stimulate keratinocyte proliferation. Given my hypothesis that IL-1α is provided by the epidermis we first primed the dermal fibroblasts with media exposed to epidermal explants and then treated keratinocytes with this conditioned media. I found that priming the dermal fibroblast with KO epidermal media ultimately caused a 40% increase in keratinocyte growth compared to wild type epidermal primed dermal fibroblast or regular keratinocyte culture media (Figure 6E). Adding recombinant IL-1α directly to the dermal fibroblast was sufficient to induce them to secrete proliferation-stimulating cytokines. Furthermore, inhibition of IL-1α activity with a neutralizing antibody impaired the ability of the knockout epidermis to prime the dermal fibroblasts (Figure 6F). This data suggests that IL-1α is a required component secreted by the epidermal explant to render the dermal fibroblast competent to stimulate keratinocyte proliferation.

**Epidermal IL-1α induces NFκB signaling and keratinocyte growth arrest**

If fibroblast derived cytokines are stimulating keratinocytes to proliferate, why is this effect limited to cells along the basement membrane? Interestingly, I found that either epidermal conditioned media alone or recombinant IL-1α can directly inhibit keratinocyte growth (Figure 7A). I investigated the possibility that IL-1α may induce NFκB signaling which promotes growth inhibition and cell survival in keratinocytes [57, 58]. In the caspase-8 null epidermis, there is a gradient pNFκB expression (Figure 7B).
It is strongly expressed in the nucleus of granular layer cells and decreases in cells closer to the basement membrane (Figure 7C). This suprabasal expression of pNFκB is likewise present in an excisional wound though the gradient is not as steep. Concomitant with pNFκB expression is the induction of Bcl-XL, a known target gene of NFκB (Figure 7C) as well as other transcriptional targets (Figure 8). To determine whether this profile was due to IL-1α signaling, I stained for nuclear translocation of NFκB in response to cells treated with conditioned media from wild type and knockout epidermal explants (Figure 7D). Keratinocytes treated with wild type conditioned media had NFκB predominantly localized in the cytoplasm whereas cells treated with knockout media showed nuclear localization. The nuclear translocation could be inhibited when knockout conditioned media was added together with an IL-1α neutralizing antibody. A control antibody did not prevent nuclear translocation while the recombinant human IL-1α, which is not recognized by the inhibitory antibody, could rescue the effect. Moreover, addition of knockout dermis conditioned media can overcome the effects of the knockout epidermal conditioned media and block the nuclear translocation of NFκB, which is consistent with its ability to stimulate keratinocyte proliferation. Although IL-1β RNA is elevated in wounded skin (Figure 4C), inhibition of this cytokine does not block NFκB translocation, which suggests that leukocytes (and not keratinocytes) are the source of this cytokine. Thus IL-1α has cell specific effects to mediate the apparently contradictory processes of proliferation and growth arrest/cell survival possibly to promote re-epithelialization while maintaining the pool of surviving cells in the presence of the trauma.
Stimulation of IL-1 secretion by p38 MAPK-dependent caspase-1 activity

Given the widespread and complex effects of extracellular IL-1\(\alpha\), how does the loss of caspase-8 regulate the secretion (and therefore the activity) of this cytokine which lacks a signal peptide? Two proteases have been implicated in the conversion of the interleukin-1 precursor into its biologically active form – caspase-1 and calpain [57]. Interestingly, in both caspase-8 null epidermis and wounded skin there is an upregulation of caspase-1 RNA (Figure 9A). The protein in both of these contexts is localized primarily to the suprabasal layers, while caspase-1 levels in the wild type epidermis is quite low (Figure 9B). In order to determine whether the increase in caspase-1 expression in the null mouse is relevant to IL-1\(\alpha\) secretion, I tested whether pharmacological inhibition of its catalytic activity would alter the amount of cytokine released from epidermal explants. As shown in Figure 9C, the amount of IL-1\(\alpha\) secreted from epidermal explants is significantly higher in the knockout mouse than its wild type littermate. This increase is lost when the explants are treated with a caspase-1 inhibitor. Interestingly, even though calpain is considered the protease responsible for interleukin processing in keratinocytes, blocking the activity of this enzyme had no effect on the elevated secretion of IL-1\(\alpha\) from caspase-8 knockout epidermis.

Caspase-1 is itself synthesized as a proenzyme and must also be processed into its mature/active form. Among the candidate regulators of caspase-1 activation which were screened, I found that phosphorylated p38 MAPK (phospho-p38 MAPK) is dramatically upregulated in both the caspase-8 knockout and wounded skin (Figure 9D). In these situations, phospho-p38 MAPK is nuclear in the basal and spinous layers of the epidermis and is cytoplasmic in the granular layer. This induction of phospho-p38 MAPK converges upon the intracellular signaling pathway as inhibition of its activity abolishes
the secretion of IL-1α from the knockout epidermis (Figure 9C). The p38 MAPK inhibitor
did not alter caspase-1 protein expression (Figure 9E), but did efficiently restrain
caspase-1 catalytic activity (Figure 9F). One effect of blocking p38 MAPK-mediated
activation of caspase-1 is the reduction in the paracrine signaling mediated by
epidermally derived IL-1α (Figure 9G). Therefore, compromising the p38 MAPK-
caspase-1 – IL-1α signaling cascade anywhere along this axis hinders the ability of this
network to stimulate the activation of NFκB in keratinocytes.

In order to probe the mechanistic link between p38 MAPK and caspase-1, we
analyzed the status of the NALP3 inflammasome, a multiprotein complex which is
required for activation of inflammatory caspases such as caspase-1 [59]. This complex
is composed of the adaptor protein Asc and pro-caspase-1, both of which have a basal
expression level in keratinocytes, and NALP3 whose expression must be induced [59].
Interestingly, we found that NALP3 RNA is markedly upregulated in the suprabasal
layers of both the knockout and wounded epidermis (Figure 10A). This expression
pattern coincides with active p38 MAPK expression in the knockout epidermis and we
found that NALP3 induction is dependent upon the activity of this kinase (Figure 10B).
Furthermore, treatment of epidermal sheets with the p38MAPK chemical activator
anisomycin is sufficient to induce NALP3 expression in wild type epidermal sheets
(Figure 10C). The human NALP1 protein can also form an inflammasome complex with
caspase-1 to mediate its proteolytic activation, but its closest murine relative, NALP1b, is
unaffected in the caspase-8 knockout epidermis and is unaltered in response to
modulating p38 MAPK activity (Figure 10A-C). Consistent with its role in stimulating
inflammasome-dependent activation of caspase-1, the p38 MAPK activator anisomycin
can increase the amount of IL-1α secreted from epidermal sheets (Figure 10D). The
ability of both UV-irradiated [60] and anisomycin treated [61] keratinocytes to expel their inflammasome components prompted us to determine whether p38 MAPK can also impact the secretion of caspase-1. Conditioned media from caspase-8 null epidermal sheets contained elevated levels of released pro-caspase-1 which was reduced to wild type levels when p38 MAPK activity was compromised (Figure 10E). The p20 activated form of caspase-1 was also higher in the extracellular media from the knockout epidermis and both p38 MAPK and caspase-1 inhibition abolished the release of this protein from the cell.

DISCUSSION

This data suggests a model in which wound healing downregulates caspase-8 expression in the granular layer of the epidermis, initiating a cascade of paracrine signaling (Figure 11). The loss of caspase-8 leads to the secretion of active IL-1α from a large reservoir of proIL-1α in keratinocytes. This IL-1α binds to its receptor on epidermal keratinocytes to stimulate NFκB translocation and transcriptional activation of NFκB responsive genes. In keratinocytes, NFκB activity leads to growth arrest (Figure 7A) and promotes survival [58]. This cytokine also crosses the basement membrane [62] to stimulate dermal fibroblasts to secrete secondary factors such as KGF and GM-CSF which signal back to the epidermis to induce keratinocytes proliferation [56]. Moreover, IL-1α stimulates NFκB expression in the dermal fibroblast and keratinocytes which has the effect of activating pro-inflammatory genes [55] (Figure 4C). In addition to mediating an inflammatory response these genes can also promote the proliferation of keratinocytes to potentiate the epidermal hyperplasia phenotype.
The caspase-8 conditional knockout mouse highlights several possible cell biological functions for this protein. My data implies that caspase-8 regulates secretion of cytokines and may be analogous to the recently identified role of caspase-1 in mediating unconventional secretion of pro-inflammatory proteins [63]. Moreover, the ability of Drosophila caspase to inhibit migration [36] suggests that the decreased expression of caspase-8 may be important in stimulating cell motility and invasion. This is an important function in the context of wound healing where rapidly closing the gap in the tissue is a top priority. The caspase-8 conditional knockout model also sheds light on the mechanisms regulating epidermal homeostasis. The completion of the terminal differentiation program in the absence of caspase-8 lends support to the notion that the formation of dead keratinocytes at the skin surface (cornification) is not a canonical apoptotic process [64]. This alternate form of cell death may explain the difficulty in detecting markers of apoptosis in normal skin [65] [66].

The distinct pattern of proliferative vs. growth arrested epidermal cells established by IL-1α in the knockout epidermis may be the result of cytokine gradients. Primary keratinocytes extracted from the basal layer are competent to respond to IL-1α as evidenced by the translocation of NFκB (Figure 7D) and the growth arrest (Figure 7A) in response to this cytokine. In vivo, however, these same basal keratinocytes are positive for proliferation markers (Figures 3D and E) despite being exposed to IL-1α (Figure 6C). It is possible that the concentration of cytokines emanating from the dermis such as GM-CSF and KGF are higher in the basal layer than suprabasal layers. In this scenario, the dermal factors are able to better compete with the opposing growth arrest effect of IL-1α in keratinocytes. Consistent with this, I found that the presence of IL-1α in the dermal conditioned media promoted proliferation (Figure 6E) suggesting that...
the proliferative signals derived from the fibroblasts can dominate over the effects of keratinocyte produced IL-1α.
Figure 2. Caspase-8 expression during skin development and wound healing.

A. Anatomy of the skin. Histological staining of newborn wild-type mouse skin with hematoxylin and eosin. Dotted line denotes the basement membrane that separates the epidermis (epi) from the underlying dermis (der). The table shows the contents and biochemical markers of different layers of the epidermis.

B. Expression pattern of caspase-8 in skin. In situ hybridization with antisense cRNA probe reveals the localization of caspase-8 restricted to the epidermis at embryonic day 18.5 (E18.5) but not at E16.5. Specificity of the probe is shown in sense control on E18.5 skin.

C. Epidermal localization of caspase-8 protein. Caspase-8 is detected in the granular layer via colocalization with the granular layer marker loricrin (lor), but is absent in the basal and spinous layers.

D. Expression dynamics of caspase-8 RNA during wound closure. Caspase-8 is downregulated at the excisional wound site and re-expressed in epidermis after the wound is completely closed after 14 days. The expression of caspase-8 proximal and distal to wounded area was examined by in situ hybridization on skin sections from different days after excision. Arrow denotes location of wound. Distal (>5mm) and proximal (<1mm) refer to the distance from the wound site.
Figure 3. Cutaneous phenotype of caspase-8 conditional knockout mouse.

A. Gross phenotype of wild type (WT) vs. caspase-8 knockout (KO) mouse. Caspase-8 KO mice show flaky skin and are smaller than WT littermate at postnatal day 10 (P10).

B. Hematoxylin and eosin staining of P10 WT and KO skin sections. The KO skin shows hyperthickened epidermis and increased dermal cellularity.

C. Immunofluorescence of differentiation markers in KO skin. The altered staining in the KO skin shows a notable expansion of K1 and K5 expressing layers.

D & E. Detection of proliferating cells. Hyperproliferation of the epidermis in caspase-8 KO skin is revealed by increased expression of cell cycle-regulating protein, cyclin D1, and proliferation marker Ki67 in the basal layer and areas around the hair follicle (hf). Inset shows magnified view of boxed area.

F & G. Markers of a wound healing response. Signs of wound healing are shown by epidermal induction of K6 and abnormal β4 integrin expression in the suprabasal layers of the KO epidermis. The basement membrane is marked by laminin (lam) in red.

H. Terminal differentiation is not compromised as shown by normal cornified envelope formation in WT and KO skin.
Figure 4. Characterization of the inflammatory response in the KO skin.

A. Increased number of granulocytes and macrophages are detected in KO skin by immunofluorescent staining for Gr-1 and MAC-1 respectively. Tissue is counterstained with keratin 5 (K5) to denote basal layer and hair follicle and boundary between epidermis (epi) and dermis (der).

B. Different subpopulations of T-cells are increased in the KO skin and show infiltration (arrowhead) into the epidermis as seen by staining with the pan T-cell marker (CD3), helper T-cell marker (CD4), cytotoxic T-cell marker (CD8), and γδT-cell receptor marker (γδTCR). γδT cells are activated in the KO skin as noted by a switch from a dendritic (in WT epidermis) to a spherical morphology. Insets are magnified views of γδT-cell morphology. Vγ3 exclusively marks the epidermal pool of γδT cells, and its numbers are increased in the KO tissue.

C. Quantification by real-time PCR of pro-inflammatory NFκB target genes in WT and KO skin of 10 day old mice and wounded skin samples three days after wounding. The data depicted in the histograms are from 3 independent sets of experiments done in triplicate and the error bars denote the standard error of the mean. RNA from WT skin is in orange, KO is in blue, and wounded skin is in purple.
Figure 5. Blocking immune cell activity does not abolish keratinocyte proliferation.

A. The effect of dexamethasone/indomethacin (DI) on the WT and KO skins. The newborn mice were treated with daily injections of these immunosuppressant over a five-day period. The skin of treated mice demonstrated that immune cell infiltration was abolished with DI treatment to levels that resemble wild type littermates injected with vehicle control.

B. T-cell population in the treated WT and KO skins. The increased number of CD3 expressing cells is the KO skin is suppressed by the injection of DI but not in the PBS treated control skin.

C. Expression of cytokines and chemokines. The highly upregulated cytokines and chemokines in the knockout mouse were reduced to the wild type levels by the effect of DI injection. The data depicted in the histograms are from 3 independent sets of experiments done in triplicate and the error bars denote the standard error of the mean. RNA from WT skin treated with PBS is in yellow, WT skin treated with DI is in blue, and KO skin treated with PBS is in orange, and KO skin treated with DI is in dark blue.

D. Proliferation pattern in the skins. DI injection did not inhibit the higher number of Ki67 expressing proliferating cells in the KO skin but hyperkeratosis is diminished.
Figure 6. Control of hyperproliferation through epithelial-mesenchymal interactions.

A. Elevated pMAPK levels in the epidermis KO skin. Protein levels of total MAPK and active MAPK (pMAPK) are detected by Western blot of lysates from isolated epidermis from WT and KO skin. The ratio of pMAPK to total MAPK for each sample is listed below the panel.

B. Suprabasal expression of pMAPK in the epidermis of caspase-8 null skin. Localization of pMAPK was determined with immunohistological staining of WT and KO skin from P10 mice and skin three days post wounding. Serial sections were stained with loricrin (lor) and keratin 5 (K5) to reveal that cytoplasmic pMAPK is predominantly in the granular layer. In all panels the basement membrane is denoted by a dotted line separating the epidermis (epi) and hair follicle (hf) from the dermis (der).

C. Increased secretion of IL-1α in the KO skin. Extracellular IL-1α is detected by staining in the absence of detergent (denoted as unpermeabilized [unperm.] samples). Detergent was incorporated in the staining protocol to visualize total IL-1α levels in the skin (denoted as permeabilized [perm.] samples). The basement membrane is denoted by the white dotted line and IL-1α is stained in green and nuclei in blue with DAPI.

D. Dermal cells from caspase-8 KO skin stimulates mouse keratinocyte proliferation. The conditioned media from dermal explants of knockout mice (KO der CM; red) was added to primary keratinocytes and growth rate was measured over four days in comparison to WT dermal conditioned media (WT der CM; blue). The baseline keratinocytes growth rate was determined by culturing cells in normal growth media (media; green). The data is representative of three independent experiments done in triplicate.

E. Media incubated with knockout epidermis can prime wild type dermal fibroblasts to stimulate keratinocytes proliferation. Conditioned media was collected from dermal fibroblasts (df) that were primed with either wild type epidermis exposed media (WT epi + df CM; light blue) or media incubated with caspase-8 null epidermis (KO epi + df CM; red [***]). The normal keratinocyte growth rate was determined with cells incubated with normal media (media; green). Normal media supplemented with recombinant human IL-1α (media + rhIL-1α; orange) showed maximum growth rate. The wild type epidermal media can mimic the effect of KO epidermis and dermal fibroblast by addition of rhIL-1α (WT epi + rhIL-1α + df CM; dark blue [*]). The data is representative of three independent experiments done in triplicate. (*p<0.05; ***p<0.001 compared to the media which was used as a control)

F. IL-1α is an active component secreted by KO epidermis to stimulate proliferation. Keratinocytes cultured in normal media (media; green) was used as a baseline for keratinocytes growth rate. Conditioned media from dermal fibroblasts primed with knockout epidermis and either control antibody (KO epi + df + con Ab; red) or IL-1α inhibitory antibody (KO epi + df + inh. Ab; purple) was added to primary keratinocytes and growth rate was determined. Blocking IL-1α activity decreased proliferation rate but was restored when recombinant human IL-1α (which is not inhibited by the antibody) was added (KO epi + df + inh. Ab + rhIL-1α; pink).
Figure 7. IL-1α induces keratinocyte growth arrest via NFκB.

A. IL-1α secreted from the knockout epidermis inhibits keratinocytes growth. Conditioned media from wild type epidermal explants (WT epi CM; light blue) or KO epidermal explants (KO epi CM; dark blue) was added to primary keratinocytes. The reference growth rate was determined by keratinocytes cultured in normal media (media; green). Addition of recombinant human IL-1α to normal media (media + rhIL-1α; red) is sufficient to inhibit growth.

B. Expression of pNFκB is elevated in the knockout epidermis. Western blot of epidermal extracts demonstrate higher expression of pNFκB in the lysates from KO vs. WT epidermis. The ratio of pNFκB to total NFκB is noted.

C. Suprabasal expression of pNFκB and Bcl-XL in the KO and wounded epidermis. Skin sections from 10 day old caspase-8 wild type (WT) and knockout (KO) mice and adult mouse skin three days after excisional wounding were stained for pNFκB, its target gene Bcl-XL (green) and keratin 5 (K5, red). The black dotted line denotes the basement membrane separating the epidermis (epi) from the underlying dermis (der). The hair follicle is denoted by hf and the site of the wound is marked by the arrowhead.

D. NFκB nuclear translocation is induced by IL-1α secretion from caspase-8 null epidermis. Conditioned media from wild type (WT epi CM) and knockout (KO epi CM) epidermal explants were added to primary keratinocytes and stained for NFκB. Inhibitory IL-1α antibody (Inh. Ab) which specifically neutralizes the mouse cytokine and/or recombinant human IL-1α not neutralized by the antibody (rhIL-1α) was added to the KO epi CM. Primary keratinocytes were also treated with knockout epidermis conditioned media (KO epi CM) and wild type dermis conditioned media (WT der CM), knockout dermis conditioned media (KO der CM) or a neutralizing antibody specific for mouse IL-1β.
A graph showing fold increase over time for different conditions.

B: Western blots showing pNFκB and NFκB levels in WT and KO samples.

C: Images of C8 WT, C8 KO, and d3 wound samples with annotations indicating "der," "epi," and "Bcl-XL." K5 is also highlighted.

D: Immunofluorescence images showing NFκB levels in various conditions, such as WT epi CM, KO epi CM, WT epi CM + con. Ab, and KO epi CM + IL-1α inh. Ab.
Figure 8. Induction of NFκB target genes in the caspase-8 null epidermis.
Epidermal keratinocytes were isolated from 3 day old wild type (WT) and knockout (KO) mice. This time point was chosen as it precedes the recruitment of immune cells into the skin as well as the activation of resident dendritic cells found in the knockout animal, both of which is first detected at postnatal day 5. RNA was extracted and real-time PCR was performed using primers for known NFκB target genes. Results are representative of three independent experiments done in triplicate.
Figure 9. IL-1α secretion is mediated by p38 MAPK activation of caspase-1.

A. Real time PCR reveals that caspase-1 RNA levels are increased in the 10 day old caspase-8 knockout (KO) and wounded skin (wound) three days after excision relative to the wild type control (WT). Data is representative of three independent experiments done in triplicate.

B. Expression of caspase-1 in skin. Immunofluorescence with an antibody recognizing the precursor and the active p20 form of caspase-1 demonstrates that it is expressed in the suprabasal layers of the caspase-8 knockout skin (KO) of a 10 day old mouse and in the wounded skin (wound) three days after excision, whereas the 10 day old wild type skin (WT) has low expression. The white dotted line denotes the basement membrane separating the epidermis (epi) and hair follicle (hf) from the dermis (der).

C. Secretion of IL-1α is dependent on caspase-1 activity. 1cm² epidermal explants from three day old wild type (WT) and caspase-8 knockout (KO) mice were used to condition media for 24 hours. Explants were treated with either DMSO, or inhibitors for caspase-1, p38MAPK, or calpain during conditioning. The amount of IL-1α secreted into the media was determined by ELISA. Data is representative of three independent experiments done in triplicate. Note: the efficacy of the calpain inhibitor was verified by its ability to inhibit processing of filaggrin in differentiating keratinocytes (data not shown).

D. Expression of phospho-p38 MAPK in the skin. Immunohistochemistry with an antibody recognizing the phosphorylated form of p38 [(P)-p38] demonstrates that phospho-p38 MAPK is expressed in the epidermis of a 10 day old caspase-8 knockout (KO) mouse and in the wounded epidermis (wound) three days after excision whereas the 10 day old wild type skin (WT) has low expression. The black dotted line denotes the basement membrane separating the epidermis (epi) and hair follicle (hf) from the dermis (der).

E. Caspase-1 protein levels are unaffected by the pharmacological inhibitors. Protein lysates were generated from the epidermal explants in (C), run on SDS-PAGE and probed with antibodies for caspase-1 and actin.

F. Caspase-1 activity is dependent upon p38 MAPK. Epidermal lysates generated as in (C) were incubated with a caspase-1 specific peptide substrate which emits light upon cleavage by caspase-1. The amount of cleavage was measured every 30 minutes over a two hour incubation period. The standard deviation for each data point is <5%. The graph is a representative of three independent experiments done in triplicate.

G. Nuclear translocation of NFκB requires caspase-1 activity. Primary mouse keratinocytes were treated with conditioned media from (C) and stained for NFκB.
Figure 10. Inflammasome assembly in the caspase-8 KO epidermis.

A. In situ hybridization of NALP3 RNA on P10 wild type, knockout, and 3 day post-wounded skin sections.
B. Quantitative RT-PCR of inflammasome components using RNA isolated from epidermal sheets of wild type and knockout skin treated with DMSO vehicle control or p38 MAPK inhibitor (p38 inh) for 16 hours.
C. Quantitative RT-PCR on RNA from wild type epidermal sheets treated with DMSO vehicle control, anisomycin (aniso), or anisomycin + p38 MAPK inhibitor (aniso + p38 inh) for two hours.
D. Measurement of IL-1α secretion into conditioned media from wild type epidermal sheets treated with DMSO, anisomycin (aniso), p38 inhibitor (p38 inh), and/or caspase-1 inhibitor (casp1 inh). Knockout epidermal sheets treated with DMSO (KO DMSO) is used as comparison.
E. Western blot of caspase-1 secreted into the extracellular medium from epidermal sheets.
Figure 11. Model of IL-1α dependent epithelial-mesenchymal crosstalk regulating the caspase-8 knockout phenotype. IL-1α is secreted from epidermal cells and has cell-type specific effects in the skin. In keratinocytes it stimulates NFκB activation which leads to growth arrest and cell survival. Dermal fibroblasts stimulated with IL-1α secrete growth factors which promote keratinocyte proliferation. These opposing effects restrict proliferation along the basal layer of the epidermis and contiguous structures. The secretion of IL-1α is mediated in the caspase-8 null keratinocytes through the upregulation of inactive caspase-1 expression. The activation of the caspase-1 by elevated p38 MAPK leads to the proteolytic cleavage of pro-IL-1α and secretion of the signaling competent form of the cytokine.
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Chapter III:

Regulation Of Stem Cell Proliferation In The Caspase-8 Conditional Knockout
INTRODUCTION

One of the main functions of the skin is to provide the body with a barrier from external assaults while preventing excessive loss of moisture. As a result, the skin is constantly regenerating itself but once this barrier is breached through injury, a wound healing response is rapidly mobilized to restore this barrier. The wound healing response is a complex process that relies on the careful orchestration of signals coming from various cell types. Following injury, three sequential but overlapping phases are initiated commencing with inflammation, followed by proliferation of stem cells and concluding with tissue remodeling. Despite their temporal differences there is significant interdependence among these phases that enable the restoration of tissue function [26]. One such interdependency is the interaction between the inflammation-proliferation phases that can be mediated by interleukin-1 (IL-1), which can induce local inflammation [13].

Epidermal keratinocytes are a rich source of IL-1α, which is active in its pro-form and released upon tissue damage [67]. Once released, it plays key roles in the inflammatory phase of the wound healing response [15]. Additionally IL-1α plays an important role in mediating the reciprocal crosstalk between cells within the epidermis and dermis to stimulate the secretion of stem cell proliferation factors [12, 68]. The proliferative phase of the healing program rests on the ability of various epithelial stem cell pools such as interfollicular epidermal stem cells (IFESC) and hair follicle stem cells (HfSC) to contribute progenitor cells to seal the breached epidermis [69, 70]. Using the epidermal caspase-8 knockout as a model, which has a uniform wound response throughout the skin, I sought to investigate the mechanism by which IL-1 signaling contributes to the proliferative phase of the wound-healing program [68].
RESULTS

Impact of IL-1 signaling on the proliferative phase of wound healing

In order to determine the functional relevance of IL-1 signaling to the proliferative phase of wound healing, wound closure rates were determined from excisional wounds on the back skins of interleukin-1 receptor type I (IL1R) -/- and wild type (WT) littermates [71]. IL-1 deficient mice displayed impaired wound healing as measured by a delay in wound closure of approximately 2 days (Figure 12A). To confirm a previous study showing that IL-1 deficient mice had decreased epidermal stem cell proliferation following injury, I quantified the number of proliferating basal layer cells stained with the proliferation maker Ki67 and found a significant decrease in proliferation in IL-1 deficient skin, most noticeably at days 5 and 7 post-wounding (Figure 12A, B) [72]. A major obstacle in dissecting the signaling pathways regulating the wound response is the limited amount of cells that respond to the trauma. I addressed this constraint by employing a genetic wound-healing model based on the epidermal ablation of caspase-8 (C8cKO) that converts the entire skin to a wound-like state [68, 73]. The epidermal caspase-8 conditional knockout (C8cKO) displayed a thicker epidermis compared to WT skin and skin lacking both caspase-8 and IL-1 receptor (Figure 13A). These results confirm my previous finding that one mechanism by which epidermal stem cell proliferation is achieved is through IL-1α induction of keratinocyte proliferation factors from dermal fibroblasts [68]. The thickened epidermis phenotype observed in the C8cKO mice was due to enhanced proliferation of IFESC stem cells and in the absence of IL-1 signaling this proliferation was reduced (Figure 13B).

In order to determine if HfSC were also playing in a role in epidermal thickening in the C8cKO, the proliferation of these cells was measured by 5'-Bromo-2'-deoxyuridine (BrDU) incorporation assays [23]. After dissociation of the skin into single
cell suspensions, the samples were analyzed by fluorescence activated cell sorting (FACS) for the number of BrDU positive cells. The HfSC were then gated using the α6-integrin and CD34 antibodies [23]. Based on this analysis, the C8cKO skin had more than a 2-fold increase in proliferating HFSC compared to WT skin (Figure 13C). In the absence of IL-1 signaling, the number of proliferating HfSC in the C8cKO skin decreased by ~30% (Figure 13C, Figure 11C). Despite the significant contributions of IL-1 signaling to the development of the epidermal thickening in the C8cKO, removing IL-1 signaling was not sufficient to reduce the epidermal thickness to WT levels. This is likely due to the contribution of other factors in a wound microenvironment such as leukocyte-derived signals that are capable of promoting stem cell proliferation [5].

**Loss of γδ T-cells phenocopies the loss of IL-1 signaling in the epithelial compartment of the wound-like skin**

Since IL-1 signaling is classically associated with the establishment of localized inflammation, I focused my attention on how IL-1 signaling may be mediating the cross-talk between the inflammatory and proliferative phases of the wound healing response. Among the earliest inflammatory cells that respond to injury are macrophages and granulocytes, which are known to enhance the proliferation of epidermal stem cells [74, 75]. In the C8cKO skin, the number of macrophages and granulocytes were increased and a marked reduction of these cells was observed in the absence of IL-1 signaling (Figure 14A). Similarly, another well-established contributor to the wound healing program are epidermal resident γδ T-cells, also known as dendritic epidermal T-cells (DETC). Following a stress signal such as a wound, γδ T-cells become activated and promote stem cell proliferation by secreting keratinocyte growth factor (KGF, also known as FGF7) [48]. In γδ T-cell deficient mice wound healing is impaired, which underscores
the important role of these immune cells in the repair process [10]. Furthermore, γδ T-cells play an important role in the recruitment of other immune cells such as macrophages during wound healing [74]. In the caspase-8/γδ T-cell double knockout (dKO) mouse, the number of granulocytes and macrophages were decreased similar to the caspase-8/IL1R dKO (Figure 14A).

Based on their shared effect on immune cell recruitment, I investigated whether there is an epistatic relationship between IL-1 signaling and γδ T-cell activity. Interestingly, γδ T-cell numbers are increased in the C8cKO mice [68], but in the absence of IL-1 signaling, there is a dramatic reduction of these cells in the skin (Figure 14B). Given the ability of γδ T-cells to stimulate stem cell proliferation, I hypothesized that removal of these cells would reduce the epidermal hyperplasia in the C8cKO mouse in a similar manner as the loss of IL-1 signaling. By measuring the epidermal thickness in the caspase-8/γδ T-cell dKO mouse I found that it was significantly reduced in comparison to the C8cKO mouse (Figure 14C, Figure 15A). Similar to the caspase-8/IL1R dKO, this reduction in epidermal thickness was accompanied by a significant decrease in IFESC and HiSC proliferation (Figure 14C). These observations suggest that IL-1 signaling converges upon γδ T-cells to mediate epithelial stem cell proliferation in the skin.

**Activation of γδ T-cells by IL-1**

I then focused on the nature of the relationship between IL-1 signaling and γδ T-cell function. The remarkable ability of γδ T-cells to perform a variety of effector functions depends on their ability to recognize stress signals coming from keratinocytes [48]. Unlike αβ T-cells, which recognize protein fragments processed by antigen
presenting cells, the effector function of γδ T-cells depend on a combination of a yet unknown antigen recognized by the γδ TCR and a variety of co-stimulatory molecules [76]. In particular, stressed keratinocytes during wound healing increase the expression of the coxsakie and adenovirus receptor (CAR) that acts as a co-stimulatory ligand that is recognized by the junctional adhesion molecule-like protein (JAML) on the surface of γδ T-cells [77]. Upon activation by a wound, γδ T-cells undergo a variety of changes that include the loss of dendritic extensions, increased proliferation and secretion of cytokines and growth factors essential for wound healing [48]. Among the functional consequences of γδ T-cell activation is enhanced proliferation of stem cells [10]. Since epithelial stem cell proliferation was decreased in the caspase-8/IL1R dKO, I hypothesized that IL-1 signaling was necessary for optimal γδ T-cell activation.

Using the C8cKO and caspase-8/IL1R dKO mice, I investigated whether a parameter of γδ T-cell activation, namely proliferation, is dependent upon IL-1 signaling. I assayed γδ T-cell proliferation by BrDU and FACS analysis and found that the number of proliferating γδ T-cells was increased 3-fold in the C8cKO skin compared to WT controls, and a significant decrease was observed in the absence of IL-1 signaling (Figure 16A and Figure 15B). It is noteworthy, however, that γδ T-cells in the caspase-8/IL1R dKO still displayed increased proliferation compared to the WT. Furthermore, γδ T-cell activation was intact in the caspase-8/IL1R dKO mice as observed by TNFα expression suggesting that IL-1 signaling was mainly participating in enhancing γδ T-cell proliferation and not directly involved in their initial activation (Figure 16B). In the newborn thymus, IL-1 signaling has been shown to have a synergistic effect with IL-7 to enhance the proliferation of γδ T-cells [78]. Epidermal keratinocytes constitutively express IL-7 and its expression is required for γδ T-cell development [48]. In the C8cKO, IL-7 expression is
upregulated and this is not affected by the absence of IL-1 signaling (Figure 15C). Based on this observation, I hypothesized that IL-1α released from the keratinocytes in the C8cKO mouse can act synergistically with IL-7 to enhance γδ T-cell proliferation.

In order to validate the contributions of IL-1 and IL-7 signaling to γδ T-cell proliferation, I established short-term γδ T-cell in vitro cultures from WT mice [77]. Using this in vitro approach allowed us to reconstitute the different components required to stimulate γδ T-cell proliferation. I collected conditioned media (CM) from WT and C8cKO keratinocytes [68] and applied them to WT or IL1R -/- γδ T-cells and monitored their growth rates. γδ T-cells were cultured in activating conditions using anti-CD3 and anti-JAML as previously described [77] and then incubated for 2 days in the presence of CM, recombinant human IL-1α (rhIL-1α), recombinant human IL-7 (rhIL-7), or IL-7 inhibitory antibody. The number of γδ T-cells increased 2.5-fold when treated with C8cKO CM versus WT CM (Figure 16C). Conversely, removing IL-1 signaling by treating IL1R -/- γδ T-cells with C8cKO CM resulted in significantly reduced proliferation. Similarly, blocking IL-7 signaling in the C8cKO CM with an anti-IL-7 inhibitory antibody blocked γδ T-cell proliferation. This effect was further substantiated when both IL-1 and IL-7 signaling was perturbed. Since epidermal keratinocytes constitutively secrete IL-7, adding recombinant human IL-1α (rhIL1α) to WT CM was sufficient to cause an increase in γδ T-cell proliferation. Culture media supplemented with rhIL-1α and rhIL-7 was also sufficient to reconstitute the γδ T-cell proliferation observed in the C8cKO CM (Figure 16C). Altogether these results support the model that in conjunction with γδ T-cell activation, IL-1 and IL-7 signaling amplifies the proliferation of these cells in response to stresses that alter epidermal homeostasis.
Contributions of keratinocyte-fibroblast and keratinocyte-γδ T-cell interactions to stem cell proliferation

Since IL-1 signaling can stimulate different pools of epithelial stem cells in the skin, I sought to interrogate whether activated fibroblasts and γδ T-cells can induce proliferation within distinct epithelial stem cell niches. Due to a lack of definitive markers to distinguish IFESC from HfSC, assaying proliferation of these cells within the skin is a challenge. On the other hand, in vitro cultures of IFESC or HfSC are well established and grafting experiments have shown that these cells maintain their progenitor properties even after several passages in vitro [23, 79]. In order to delineate the contributions of the keratinocytes-fibroblast interactions to epithelial stem cell proliferation, I activated dermal fibroblasts (df) with C8cKO CM or rhIL-1α as evidenced by their expression of FGF7 and GM-CSF (Figure 17A). CM was then collected from these activated df and applied to IFESC or HfSC to test their effect on the proliferation of these two different stem cells. Treatment of with CM from activated df caused a dramatic increase in proliferation of IFESC and a modest increase in HfSC proliferation (Figure 17B). However, in the absence of IL-1 signaling, the proliferation of both IFESC and HfSC was reduced to that of control levels (Figure 17B, Figure 18A). These observations suggest that IFESC stem cells preferentially respond to signals from mesenchymal cells.

Treatment of primary γδ T-cell cultures with C8cKO CM resulted in their activation as demonstrated by their increased expression of FGF7 and TNFα (Figure 17C). Moreover, abrogating IL-1 or IL-7 signaling was able to reduce FGF7 and TNFα expression, while rhIL-1α and rhIL-7 was sufficient to induce maximal FGF7 expression. Exposure of HfSC to CM from activated γδ T-cells caused a significant increase in the proliferation of the HfSC (Figure 17D). Though activated γδ T-cells can also stimulate
IFESC proliferation, these were 40% lower than the effect on HfSC. Similarly, CM from rhIL-1α and rhIL-7 treated γδ T-cells was sufficient to recapitulate the increased proliferation of the HfSC (Figure 17D).

Since the CM from df and γδ T-cells can enhance the proliferation of the different stem cell pools, I sought to investigate whether the effects on epithelial stem cell proliferation would be additive. Indeed, treatment of both IFESC and HfSC with a combination of CM from activated df and γδ T-cells substantially increased their proliferation rates (Figure 17E). One likely reason for the differential proliferation of the IFESC and HfSC is the different components present in the cytokine cocktail secreted from either df or γδ T-cells. For instance, even though both activated df and γδ T-cells can secrete the mitogenic factor FGF7 only γδ T-cells secrete IL1F8, which was recently shown to cause proliferation of stem cells [80]. IL1F8 does not signal through the same IL-1 receptor and it is possible that HfSC are more susceptible to the mitogenic effects of this cytokine (Figure 18B and C). Based on these observations, I conclude that enhanced activation of γδ T-cells preferentially regulates Hf stem cell proliferation.

DISCUSSION

One of the goals of regenerative medicine is to restore the functional state of the tissue following trauma and/or disease. A major obstacle to manipulating this process in mammalian tissues is the shear number of cells that participate in the regenerative/repair process. Therefore, understanding the complexities of how multiple cell types must interact with each other in order to orchestrate a successful repair program is of utmost importance. The successful repair of damaged tissue depends on the interactions between the inflammatory, proliferative and remodeling phases of the wound healing response [26]. The focus of this study was to understand how one of this
signaling nodes, IL-1 signaling, could affect a specialized subset of immune cells to enhance the proliferation of the different stem cell pools in the skin. IL-1α release is an early response to an epidermal wound which causes the downregulation of caspase-8. IL-1 mediated activation of dermal fibroblasts preferentially stimulates epidermal stem cells. Additionally, IL-1 signaling can partner with IL-7 that is constitutively secreted from epidermal keratinocytes to expand the population of active γδ T-cells. These activated γδ T-cells can stimulate proliferation of stem cells within the bulge of the hair follicle and mobilize them for epidermal wound repair. As the wound-healing response progresses, other infiltrating cells can contribute (or take over the duties) of stimulating epithelial stem cell proliferation.

IL-1 signaling has been studied at length and found to play critical roles in a variety of cellular processes from mediating immune responses to proliferation of epidermal stem cells [13]. Despite its pleiotropic effects in various cell types and important function during wound healing, blocking IL-1 signaling does not completely impair the repair process. This can be attributed in part to compensatory mechanisms involving other IL-1 family members. IL-1 family members such as IL-1F5, IL-1F6 and IL-1F9 are largely found in epithelial tissues and transgenic mice overexpressing IL-1F6 in the basal layer of the epidermis develop severe cutaneous inflammation, hyperkeratosis and acanthosis [81]. Recently, work from Yang et al. showed that activated γδ T-cells released IL-1F8, which had the ability to promote the proliferation of epidermal stem cells [80, 81]. Since IL-1F6 and IL-1F8 signal through the same receptor and are able to induce the production of mitogenic factors, it is likely that abrogating IL-1 signaling may not be sufficient to block the epidermal hyperproliferation observed in the C8cKO skin [82]. Moreover, in a system as complex as the wound-healing program, removal of an
individual component such as IL-1 signaling will likely impede the kinetics of the process rather than completely inhibit it.

In addition, IL-1 and IL-7 synergy to increase \( \gamma\delta \) T-cell proliferation is observed in newborn thymus where these cells compete with \( \alpha\beta \) T-cells for survival [78]. In the case of injury, this synergistic interaction is most likely recapitulated as a response to trauma to quickly seal the wound. My findings suggest that in the presence of a wound-like state, potentiating \( \gamma\delta \) T-cells proliferation may be a way to increase the pools of cutaneous epithelial stem cells. Given the inherent differences between IFESC and HfSC, it is not surprising to see that they respond differently to signals from activated dermal fibroblasts and \( \gamma\delta \) T-cells. This observation is supported by the fact that in the backskin of WT mice, \( \gamma\delta \) T-cells are found in close association with the HfSC niche [68].

Once the integrity of the skin is compromised and IL-1\( \alpha \) is released, one could predict that the \( \gamma\delta \) T-cells in close association with the HfSC could locally cause proliferation of this stem cell population to contribute to wound repair. However, it remains to be seen whether the main reason for this proliferation difference is due to HfSC being able to respond to IL-1F8 preferentially over IFESC.

The results of these findings shed new insight into the complex nature of the repair process, which is a recurring theme in some pathological conditions such as cancer. I showed that the increased proliferation observed in the C8cKO can be attained through reciprocal keratinocyte-fibroblast or keratinocyte-\( \gamma\delta \) T-cell interactions. The keratinocyte-fibroblast mediated proliferation is akin to the interaction between the tumor cell and stromal cells [83]. With regards to the keratinocytes-\( \gamma\delta \) T-cell interaction it has recently been shown that activation of \( \gamma\delta \) T-cells promotes tumorigenesis [84]. Thus an understanding of the molecular mechanisms underlying the proliferation of stem cells in
a wound microenvironment holds the promise of shedding light on the complex signaling pathways that mediate tumor initiation.
Figure 12. Effect of IL-1 signaling in wound-healing and keratinocyte proliferation.

A. Wound closure rate in WT and IL1R -/- skin. 5 mm excisional wounds were performed on 8 weeks old IL1R -/- and WT control males. Wound closure was determined as the percentage size reduction compared to day 0.

B. Quantification of proliferating epidermal cells at the wound site was determined by Ki67 analysis. Results are the average of 6 wounds per genotype.

C. BrDU positive cells were analyzed by FACS for the expression of proliferating bulge stem cells isolated with CD34 and α6 integrin antibodies.
Figure 13. IL-1 signaling affects the proliferation of the caspase 8 null epidermis.

A. H&E staining of postnatal day 4 (P4) skin showing epidermal thickness differences (denoted by white line) between the various genotypes, WT= wild type, C8 KO = caspase cKO and C8/IL1R= caspase 8/IL1R dKO. The dotted line represents the basement membrane separating epidermis (epi) and dermis (der), hf denotes hair follicle. Scale bar = 50 µm. Epidermal thickness quantification is represented on the histogram as the average +/- SEM.

B. Hyperproliferation of P4 epidermis in the KO skin is revealed by increased expression of Ki67 and it is reduced in the caspase 8/IL1R dKO. Quantitation of proliferating basal layer cells is shown on the right panel as the average +/- SEM.

C. Hair in the bulge is quantified by counting the number of BrDU positive cells and shown as the fold difference in the different genotypes. Data shown in A-C are from 6 different mice per genotype. ** p < 0.001, *** p<0.0001
Figure 14. Inflammatory cell infiltration is reduced in the absence of IL-1 signaling or γδ T cells.

A. Staining of keratin 5 (K5, red) and macrophages (green, Mac1) or granulocytes (green, Gr-1) in the different genotypes shows a similar reduction in the absence of IL-1 signaling or γδ T cells. Bar = 50 µm, white dotted line denotes basement membrane.

B. Presence γδ T cells (green) and keratin 5 (K5, red).

C. Quantification of epidermal thickness, proliferating basal layer cells or hair follicle stem cells (WT= blue, C8 KO= red, C8/γδTCR dKO= orange). Data is the average +/- SEM from 4 different mice per genotype. ** p<0.001, *** p<0.0001.
Figure 15. Effect of γδ T-cell in epidermal proliferation

A. H&E staining of P4 skin showing epidermal thickness differences denoted by the white line. The dotted line denotes the basement membrane separating epidermis (epi) and dermis (der), hf denotes hair follicle. Bar = 50 µm.

B. Measurement of proliferation of γδ T cells by FACS. Mice from each genotype were injected with BrDU and γδ T cells were isolated. Representative plot of experiments from 5 mice per genotype.

C. qPCR measurements of levels of IL-7 expression by keratinocytes. Data is the fold difference +/- SEM of 3 samples per genotype done in triplicate. The p-values between C8 KO and C8/IL1R dKO are not statistically significant (N.S.).
Figure 16. IL-1 signaling contributes to γδ T cell proliferation in the caspase 8 cKO skin.

A. γδ T cell proliferation quantified from the number of BrDU positive cells in the skin are reported as the fold difference from WT. ** p<0.001.

B. Measurement of γδ T cell (green) activation by expression of TNFα (red). Nuclei (blue) are stained with DAPI. Arrowheads mark the activated γδ T cells. The histogram shows the quantification of activated γδ T cells per squared millimeter. Bar = 50 µm, *** p<0.0001.

C. Measurement of WT or IL1R-/ (where indicated) γδ T cell proliferation from primary short-term cultures were primed with anti-CD3 and anti-JAML and treated with WT or C8 KO conditioned media (CM). Inhibitory antibody against IL-7 or recombinant human IL-1α or IL-7 were added as indicated. Histogram is shown as the fold difference from WT CM treated γδ T cells. Data is representative of at least three experiments done in triplicate. ** p <0.001, *** p<0.0001.
Figure 17. Conditioned media from dermal fibroblasts and activated $\gamma\delta$ T cells differentially enhance epithelial stem cell proliferation.

A. Activation of dermal fibroblasts (df). df were treated with CM and expression of FGF7 and GM-CSF were assessed by qPCR.

B. Effect of activated df on stem cell proliferation. Proliferation rates of interfollicular epidermal (IFE) and hair follicle stem cells (Hf SC) were determined by cell counting with trypan blue exclusion. IFE and Hf SC were treated with CM from df treated as described in the figure.

C. Potentiation of $\gamma\delta$ T cell activation by CM. WT or IL1R-/- $\gamma\delta$ T cells were treated with CM as described in the figure and FGF7 and TNF$\alpha$ were assessed by qPCR.

D. Effect of activated $\gamma\delta$ T cells on stem cell proliferation. Proliferation rates of IFE and Hf SC were determined by trypan blue exclusion cell counting. IFE and Hf SC were treated with CM from $\gamma\delta$ T cells treated as described in the figure.

E. Effects of activated df and $\gamma\delta$ T cells on stem cell proliferation. Proliferation rates of IFE and Hf SC were determined by trypan blue exclusion cell counting. IFE and Hf SC were treated with CM from df and $\gamma\delta$ T cells treated as described in the figure. Data A and C was obtained from triplicates of at least three experiments and represented as the fold difference +/- SEM. Experiments in B, D and E are the average +/- SEM three independent experiments done in triplicate. ** p<0.001, *** p<0.0001.
Figure 18. Epithelial stem cell proliferation by activated dermal fibroblast and γδ T-cell CM.

A. Proliferation rates of interfollicular epidermal and Hf stem cells determined by trypan blue exclusion cell counting. IFE and Hf SC were treated with CM from γδ T cells treated as described.

B. Expression of other IL-1 family members in the dermis. qPCR of P4 dermis from the different genotypes are shown as the fold difference +/- SEM. Data was collected from 5 mice per genotype.

C. Expression of IL-1 family members by activated γδ T cells. qPCR of short-term γδ T cell cultures. Data shown were done in triplicate from 3 independent experiments.
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The text in this chapter is a modified version of a manuscript to be submitted in 2011.

Pedro Lee, Eve Kandyba, Krzysztof Kobiela and Colin Jamora. Unraveling the impact of IL-1 signaling on stem cell activation in a wound healing model. The dissertation author was the primary investigator and author of this paper.
Chapter IV:

General Discussion
The results obtained during the course of my doctoral dissertation have established a new paradigm in the field of skin biology. Tissue homeostasis and regeneration are regulated by the intricate balance of seemingly competing processes – proliferation vs. differentiation and cell death vs. survival. Prior to my work, the role of caspase 8 was thought to be solely in initiating the signaling cascade during apoptosis, here I demonstrate that the loss of epidermal caspase-8 recapitulates multiple features of a wound healing response. In Chapter II, I show that the epidermal hyperplasia in the caspase-8 null skin is the cumulative result of signals exchanged between epithelial and mesenchymal cells and leukocytes. This reciprocal interaction is initiated by the paracrine activity of IL-1α which stimulates both skin stem cell proliferation and cutaneous inflammation. I demonstrated that in the absence of injury, IL-1α secretion is mediated by the NALP3 inflammasome, which is dependent on the activation of the p38 MAPK pathway. Interestingly, this increased proliferation is counterbalanced by IL1α-dependent NFκB signaling which causes keratinocyte growth arrest and cell survival.

In Chapter III, I show that caspase 8 downregulation in the skin initiates an important crosstalk between keratinocytes and γδ T-cells that influence hair follicle stem cell proliferation. Since the cutaneous wound-healing program is a product of a complex interplay among diverse cell types within the skin, I investigated how intercellular crosstalks affect epidermal repair. One fundamental process mediated by these reciprocal interactions is the mobilization of local stem cells pools to promote tissue regeneration and repair. Using the conditional ablation of epidermal caspase-8 as a genetic model of wound healing, I analyzed the signaling components responsible for epithelial stem cell proliferation. I found that IL-1α and IL-7 secreted from keratinocytes work in tandem to expand the activated population of resident epidermal γδ T-cells. A
downstream effect of this \( \gamma \delta \text{T-cell} \) activation is the preferential proliferation of hair follicle stem cells. On the other hand, IL-1\( \alpha \) dependent stimulation of dermal fibroblasts optimally stimulates epidermal stem cell proliferation. Altogether these data provide important insights into how the loss of caspase-8 can have an impact beyond programmed cell death to affect the local microenvironment and elicit processes common to many neoplastic skin disorders. These findings provide new mechanistic insights into the regulation and function of epidermal-immune cell interactions and how components classically associated with inflammation can differentially influence distinct stem cell niches within a tissue (Figure 18).

In light of the proliferation, inflammation, and cell survival induced by the loss of caspase-8, it is interesting that various tumors have been documented to reduce their caspase-8 expression[85] [86] [87] [88] [89] [90]. Not only does this confer drug resistance against chemotherapeutics targeting the apoptotic pathway, but downregulation of caspase-8 may contribute to tumor growth by creating a microenvironment conducive to cancer progression [91]. Consequently, the epithelial-mesenchymal-leukocyte interaction stimulated by the loss of epidermal caspase-8 may be a paradigm for tumor-stroma interactions and other hyperplastic skin diseases such as eczema and psoriasis.
Figure 19. Model of the mechanisms that stimulate epithelial proliferation after caspase-8 downregulation.
Materials And Methods
### Mice Breeding

Epidermis specific knockouts were obtained by crossing mice carrying the floxed caspase-8 allele (C8 fl/fl) (kind gift from Dr. Stephen Hedrick) [40] to K14-Cre [41] mice. IL1R -/- mice were purchased from Jackson labs and backcrossed to C57Bl/6 mice for at least 8 generations. γδ TCR -/- mice were a kind gift from Dr. Wendy Havran. All animal work was approved and adhered to the guideless of IACUC.

### Histology, In Situ Hybridization and Immunohistochemistry.

Mouse skin isolated from KO and WT animals at different developmental stages were either fresh frozen in OCT (Tissue-Tek) or fixed overnight in Bouin’s fixative depending on the application. Paraffin sections were prepared for in situ hybridization and histology, and counterstained with hematoxylin and eosin (H&E). In situ hybridization was performed using a digoxigenin-UTP (Roche) labeled probe against the 3'-UTR of caspase-8. Staining for anti Cyclin D1 (Zymed), phospho-MAPK (Cell Signaling), phospho-NFκB (Ser276, Cell Signaling), phospho-AKT (Cell Signaling) and Ki67 (Vector Labs) was performed on 8 μm paraffin sections after antigen-retrieval in 10 mM sodium citrate buffer pH 6.0 for 30 sec using a microwave oven [ref]. Epidermal differentiation markers K5, K1, Loricrin, Filaggrin and the wound healing marker K6 (Covance) were stained in 8 μm frozen sections after the tissues were fixed for 10 min in 4% paraformaldehyde (PFA). Immune cell infiltrates were stained using FITC-conjugated anti MAC-1, Gr-1, CD3, CD4, CD8, γδTCR and Vγ3 (BD Biosciences). For nuclear staining H33342 (Calbiochem) was added in a final concentration of 1 mg ml⁻¹ to the secondary antibody dilution. Immunofluorescence expression was detected using
rhodamine-X or FITC conjugated secondary antibodies (Jackson Immunoresearch) or expression was developed using the Vectastain ABC kit (Vector Labs).

**Keratinocyte Proliferation Assay**

Epidermis of newborn caspase-8 WT and KO mice were separated in dispase overnight at 4°C. Isolated epidermis or dermis was incubated in 3 ml of keratinocyte media [92] for three days. For figures 4D and 5A these conditioned media were diluted 1:5 with keratinocytes media and applied to a well containing 10,000 keratinocytes. Cells were collected by trypsinization every 24 hours for four days and counted on a hemocytometer. To prime dermal fibroblasts, 400 ml of epidermal-conditioned media was diluted in 1.6mL keratinocytes media and added to a confluent 6 cm dish of fibroblasts and incubated for three hours at 37°C. This epidermal primed, dermal fibroblast conditioned media was then diluted 1:2 in keratinocytes media and added to primary keratinocytes with 2 ng/mL IL-1α neutralizing antibody/control antibody, or recombinant human IL-1α.

**NFκB Translocation Assay.**

Diluted (1:5) wild type and knockout epidermal and dermal conditioned media described above was applied to coverslips seeded with 30,000 primary keratinocytes 16 hours earlier. Cells were incubated with this conditioned media and control goat antibody, 2 ng/mL IL-1α neutralizing antibody, or 10 pg/mL recombinant IL-1a for 3 hours at 37°C. Cells were stained with an antibody recognizing NFκB (Santa Cruz).
IL-1α ELISA.

Isolated epidermis from three day old (P3) caspase-8 WT and KO mice were incubated for 24 hours in 2 mL of keratinocyte media containing DMSO or inhibitors of AKT (20 mM), p38 MAPK (SB 203580) (2.4 mM), caspase-1 (80 mM) or calpain (200 mM) (all from Calbiochem). Secreted mouse IL-1α levels were detected using the Quantikine kit from Stratagene according to manufacturer’s instructions.

Caspase-1 Activity Assay.

Explants were treated as described above and following 24 hours of incubation with vehicle control (DMSO) or inhibitors they were trypsinized for 20 minutes at room temperature. Protein lysates were extracted from isolated epidermal cells and 100 mg of protein was analyzed for caspase-1 activity as previously described [29].

Cornified Envelope Assay.

New born mice skins from caspase 8 cKO and WT littermates were removed and incubated in dispase at 4°C overnight. Epidermis was separated and placed in a 1.7 ml microcentrifuge tube containing 1 ml of extraction buffer I (EBI, 0.1 M Tris pH 8.5, 2% SDS, 5 mM EDTA, 20 mM DTT). The samples were then boiled at 95°C for 10 min. Tubes were centrifuged at 5,000 x g for 15 minutes at room temperature. Supernatant was removed and pellet was resuspended with 1 ml of fresh EBI. The samples were centrifuged at 5,000 x g for 15 minutes. Supernatant was removed again and the pellet was resuspended with 1 ml of EBII (EBI, 0.1 M Tris pH 8.5, 2% SDS, 5 mM EDTA, 20 mM DTT, 2% Ficoll). The samples were centrifuged again at 5,000 x g for 15 minutes. The purified cornified envelope floated to the top of the tube and a drop of this fraction
was placed on a slide and mounted. The status of the corneocytes was then imagined by light microscopy.

**RNA Extraction, cDNA synthesis and Quantitative Real Time–PCR.**

Epidermis and dermis from WT (n=6), caspase 8 cKO (n=9), caspase 8/IL1R dKO (n=7) and caspase 8/γδ T dKO (n=5) P4 mice were separated with dispase treatment for 1 hr at 37°C and RNA isolated using Trizol reagent (Invitrogen) according to manufacturer’s instructions. cDNA was synthesized by reverse transcription using the iScript kit (Biorad) and real-time quantitative PCR analysis was performed using the Ssofast EvaGreen mix in a Biorad CFX96 system with primers listed in the supplemental section. Experiments were carried out in triplicate with cDNA isolated from 5 different animals. Data is presented as the fold difference +/- SEM.

**Conditioned media, IL-1α inhibition and IL-1α rescue.**

Epidermis of new born caspase-8 WT and KO mice were separated in dispase overnight at 4°C. Epidermis was incubated in 3 ml of keratinocyte media for three days, conditioned media was collected and applied to primary mouse keratinocytes seeded at 30,000 cells per well in round glass coverslips at a 1:5 dilution for 3 hours and stained for nuclear translocation of NFκB (Santa Cruz). Secreted IL-1α in conditioned media was blocked using a neutralizing goat antibody against IL-1α and human IL-1α (R&D Systems) was used to recover the activity. Goat IgG was used a control.
Wound closure kinetics

8 week old male IL1R-/ and WT control (C57Bl/6) mice were anesthetized by intraperitoneal injections with pentobarbital at 50mg/kg. 5 mm punch biopsies were used to make full-thickness excisional wounds. At each day, images of the wounds were taken and analyzed using ImageJ software to measure wound area closures.

In vivo labeling and FACS analysis

P5 mice were injected intraperitoneally with 1-2 mg of 5'-Bromo'-2'-deoxyuridine (BrDU, BD Pharmingen) dissolved in sterile PBS and skins were collected 4 hrs after. Epidermis and dermis were separated after dispase treatment for 1 hr at 37°C. Dermal portions were incubated for 30 min with constant shaking at 37°C in collagenase IV (Invitrogen) and neutralized collagenase by adding 2 mM EDTA. Epidermal portions were gently incubated in trypsin for 20 min at 37°C and neutralized trypsin with keratinocyte media [93]. Cell were filtered through a 70 µm cells trainer and collected by centrifugation fro 10 min at 4°C. Hair follicle stem cells were detected as described [94], γδ T cells were detected with anti-γδTCR (GL3, BD Biosciences) and BrDU-APC flow kit was used to assess proliferation (BD Pharmingen). All FACS analyses were performed using a FACS Aria flow cytometer (BD) and data was analyzed using FlowJo software (TreeStar).

γδ T cell proliferation assays

Short-term cultures of WT and IL1R-/ γδ T cells were established and 30,000 cells were plated in 96-well plates coated with 0.1 µg/ml anti-CD3 and 10 µg/ml anti-JAML (eBioscience, clone eBio4E10) as described [77]. Cells were treated with conditioned media from WT and caspase 8 cKO keratinocytes collected in RPMI. γδ T
cells were treated with KO CM or KO CM with 4 µg/ml anti-IL-7 inhibitory antibody (R&D Systems) or IL1R-/- γδ T cells were treated with KO CM. γδ T cells were incubated with WT CM, WT CM treated with 300 pg/ml rhIL-1α or media supplemented with rhIL-1α and 2 ng/ml rhIL-7. Cell numbers were assessed by cell counting using trypan blue exclusion.

**Epidermal stem cell proliferation assays**

Unipotent epidermal (IFE) stem cells were isolated as described [93]. Hair follicle stem cells were a kind gift of Drs. Eve Kandyba and Krzysztof Kobielak at USC and prepared as described [23]. Dermal fibroblasts were isolated from 6 week old C57bl/6 WT and IL1R-/- mice. The hair follicles were removed by shaving and treatment with a hair removal agent (Nair®, Church & Dwight Co., Princeton, NJ). Epidermis and dermis were separated after overnight incubation with trypsin solution at 4°C. Dermal portions were then incubated in collagenase IV for 1 hr at 37°C with shaking and collagenase was neutralized with 2mM EDTA. Cell suspensions were filtered through a 70 µm cell strainer and pelleted by centrifugation at 400 x g for 10 min at 25°C. The cells were then plated in DMEM with 10% FBS, 1% penicillin/streptomycin and 2 mM L-Glutamine. WT and IL1R-/- dermal fibroblasts were treated with WT and caspase 8 KO CM in the presence of rhIL-7, rhIL-1α or anti-IL-7 inhibitory antibody for 24 hrs. 5,000 IFE and Hf stem cells were plated in 48-well plates and incubated with CM from treated dermal fibroblasts for 4 days. Cell counts were assessed by trypan blue exclusion.

**Activated γδ T cell assay in vivo**

P4 WT, caspase 8 cKO and caspase 8/IL1R dKO mice were injected with 0.2 mg of Brefeldin A (BFA, Sigma-Aldrich, St. Louis, Mo) subcutaneously and samples
collected after 5 hours. Skins were frozen with OCT compound (Tissue-Tek) and 10 µm sections were made. Tissues were fixed for 10 min in 4% PFA, blocked (2.5% goat serum, 2.5% donkey serum, 1% BSA, 0.3% triton-X in PBS) and incubated for 1 hr at 25°C with antibodies against γδ TCR-FITC (GL3, BD Biosciences) and TNFα-PE (eBiosciences, San Diego, CA). Images were obtained as described [95].

Oligos used

β-actin-F: GGGCTATGCTCTCCCTCAC
β-actin-R: GATGTCACGCACGATTCCC
TCRVγ3-F: GCAGCTGGAGCAAACTGAAT
TCRVγ3-R: GTTTTTGGCCGTTACCAATGT
FGF7-F: GTGAGAAGACTTTCTGTCGC
FGF7-R: CCACGGTCTCTGATTTCCATGA
FGF10-F: GTGTCTGGAGATAACATCAGTG
FGF10-R: AGCCATAGAGTTTCCCCCTTCTT
TNFα-F: CTGTGAAGGGAATGGGTGTT
TNFα-R: GGTCACTGTCCCAGCATTT
IL1F6-F: CACGTACATGGGAGTGCAA
IL1F6-R: GCAGCTCCCTTTAGACGAG
IL1F8-F: GGTATGGGTGCTGACTGGA
IL1F8-R: CCTCCATCTCAACACAGCAG
IL7-F: TGGAATTCCTCCACTGAG
IL7-R: TGGCTCATTCAGGGCAAT
mNALP3F-Q: CGAGACCTCTGGGAAAAAGCT
mNALP3R-Q: GCATACCATAGGAATGTGATGACA
mNALP1bF-Q: CAACAAGACTTGAACACAAACGAG
mNALP1bR-Q: CTCTCAATGACTGTGCTGGGTA
mNALP1cF-Q: GACAAAGGCGAGTCAGAATTGAGATT
mNALP1cR-Q: GTAAAGGTGAAAGGTGAATCATCTCG
mASCF-Q: ATGGGGGCGGGCAGCAGATG
mASCR-Q: GCTCTGCTCCAGGTCATCAC
IL1α-F: CTCTAGAGCACCATGCTACAGAC
IL1α-R: TGGAAATCCAGGGAAACACTG
RANTES-F: CCTCACCATCATCCTCACTGCA
RANTES-R: TCTTCTCTGGGGTGGGACACAC
IFN-γ-F: CTTCTCATGGCTGGTTT
IFN-γ-R: CCAGTTCCTCCAGATATC
IL4-F: CGAGGTCAAGGAGAGGGA
IL4-R: AAGCCCTACAGACGAGCTCCT
IL12-F: AGTTTGCCAGGGTCATTCC
IL12-R: TCTCTGCGGTCTTCACCAT
COX2-F: GGTGAGAGGTGTATCCC
COX2-R: ACTTCCTGCCCCACACAGCA
ICAM1-F: CACCCCAAGGACCCCAAGGAT
ICAM1-R: CGACGCGGCTCAGAAGAACA
MCP1-F: ACTGAAGCCAGCTCTCTCTCCTC
MCP1-R: TTCCCTCTTGGGGTCAGCAGAC
IL-22-F: TCCGAGGAGTCAGTGCTAAA
IL-22-R: AGAACGTCTTCCAGGGTGGA
Ptx3-F: CCTGAGGCGAGTGCGAGCT
Pttx3-R: ACAGAGTGACTTTTGCCATCTC
Cxcl5-F: AGCTCGCCATTCAATGCGGAT
Cxcl5-R: CACTGCAGTGATTCATCGGCT
Tgm2-F: TGATGACCGGGAGGAGCATCA
Tgm2-R: TGTTCCTCAGAGTAGGATCC
Ptgs2-F: GACAGTCCACCTACTTAGAT
Ptgs2-R: GTCGACACTCTGTGTTGCT
IKBα-F: GGAGCGCTTGGTGGACGATC
IKBα-R: GCCCTGCTCACAGGCAAGAT
Tnfaip3-F: ACCGATAACACGCTGGAGATG
Tnfaip3-R: AGTCCTGTTTCCACAATTC
Tnfaip6-F: AGTGACGCGGTGGGAAGCCTA
Tnfaip6-R: GTCATGACATTTCCTGTGCT
S100a8-F: TGAACGTGGAAGGCTTGTA
S100a8-R: ATTCTGTAGACATATCCAGGG
S100a9-F: ACAAAGCACCTTCTCAGATG
S100a9-R: TTTGCCATGACTGTGGCCAT
Igf1-F: ACCTCTTCTACCTGGCGCTC
Igf1-R: TTCTGAGTCTTGGGCATGTC
Casp8-geno-F: ATATTTCCCCAAATCCTCGCATC
Casp8-geno-R: GGCTCACTCCCAGGGCTTCC
Cre-F: TGCTGTTTCACTGTTATGGG
Cre-R: TTGCCCCCTGTTTCACTATCCAG
TCRδ1-geno-F: CTTGGGTTGGAGAAGGCTATTC
TCRδ1-geno-R: AGGTGAGATGACAGGAGATC
TCRd2-geno-F: CAAATGTTGCTTGCTGTTG
TCRd2-geno-R: GTCAGTCGAGTGCAATGTTGCTTGTCTGTTG

IL1RM-geno-F: CTGAATGAACGTGAGGACGA
IL1RM-geno-R: ATACTTTCTCGGCAGGAGCA

IL1RW-geno-F: CCACATATTCTCCATCATCTCTGCTGGTA
IL1RW-geno-R: TTTCAATCTCAGTTGCAAGTGTGTGCCC
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