UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Deletion of Epidermal Caspase-8 as a Model for Atopic Dermatits

A Thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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2009

The thesis of Christopher Kuok Choi Li is approved and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2009

# DEDICATION

I dedicate this thesis to my family, my parents for their teaching, money, and efforts they have spent on me and my siblings for all the insights and experiences that they taught me.

Don’t worry, be happy!

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ABSTRACT OF THE THESIS

**Deletion of Epidermal Caspase-8 as a Model for Atopic Dermatitis**

by

Christopher Kuok Choi Li

Master of Science in Biology

University of California, San Diego, 2009

Professor Colin Jamora, Chair

Atopic dermatitis (AD) is a chronic inflammatory skin disease that features eczematic skin lesions, pruritus (itch), spongiosis (skin edema) and is triggered by a complex genetic and environmental background. The deletion of epidermal caspase-8 in mouse shares similarities with the AD pathology and a rare genetic cause of AD. This caspase-8 KO mouse model display skin barrier dysfunction, recruitment of immune/inflammatory cells, gene expression of genetic signatures related to AD, elevated immunoglobulin levels and trans-epidermal water loss which are all very similar to the pathophysiology of AD. Using this mouse model, we have identified proteases (MMPs – matrix metalloproteases) that may play a role in the skin barrier dysfunction and play a possible mechanism in spongiosis.

# Introduction

One of the interesting questions in biology is how tissue homeostasis is maintained and how they are regenerated and repaired when damaged. One tissue with remarkable regenerative ability is the skin, which is in contact with the outside environment. Accounting for approximately 7% of our body weight, the skin is composed of two distinct compartments, the dermis and the outermost epidermis separated by a basement membrane. The integrity of the skin functions to protect the deeper organs from various extremities such as scrapes, wounds, heat, cold, ultraviolet rays and dehydration. These epidermal cells within the skin have the abilities to confront these issues by continual regeneration, repair and self-renewal to replace damaged and/or old cells.

The dermis is composed of connective tissue and many nerve endings that cushions the body as well as sensing pressure and temperature. The dermis contains important appendages such as hair follicles, sebaceous glands and sweat glands; all these appendages contribute to the normal functions of the skin such as thermoregulation. One important component in the dermis is the blood vessels. The circulation provides nutrients and waste removal from the dermis while providing a way of recruiting important cell types that is required during wound healing.

The epidermis functions as a protective barrier that prevents microbial infection and fluid loss from the body. The epidermis is a stratified epithelium of keratinocytes that is composed of biochemically distinct layers (Figure 1). Attached to the basement membrane is the stratum basale (basal layer). The keratinocytes in the basal layer are mitotically active stem cells capable of undergoing self-renewal to maintain a pool of progenitor cells. Among the biochemical markers denoting the basal layer is keratin 5 (K5). Immediately above the basal layer is the stratum spinosum (spinous layer). The

keratinocytes in this layer are flattened cells that have exited the cell cycle and entered into the terminal differentiation program. In the spinous layer, intermediate filaments polymerization provides structural integrity for the tissue. Among the biochemical markers denoting the spinous layer is keratin 1 (K1). As keratinocytes further progress through the differentiation program, they form the stratum granulosum (granular layer). The granular layer is an important layer for terminal differentiation and cell death. In this layer, late differentiation markers can be observed such as filaggrin, an intermediate filament-associated protein found in keratin fibers and loricrin, an important component of cornified envelopes that are found in the subsequent layer of the epidermis. The outermost layer of the epidermis is the stratum corneum. The stratum corneum is composed of cornified envelopes – enucleated polyhedral cells (corneocytes) that are extensively crosslinked and filled with keratin. This network of crosslinked proteins and lipids provide the barrier function of the skin. The surface corneocytes are shed and thus, the epidermis must be replenished every few weeks. Furthermore, another small but important population of cells within the epidermis are dendritic cells and Langerhans cell, which are located in the basal and spinous layer and are generated in the bone marrow and migrate to the skin. Here, these cells act as surveillance of epidermal integrity by acting as antigen-presenting cells during infections and immune response.

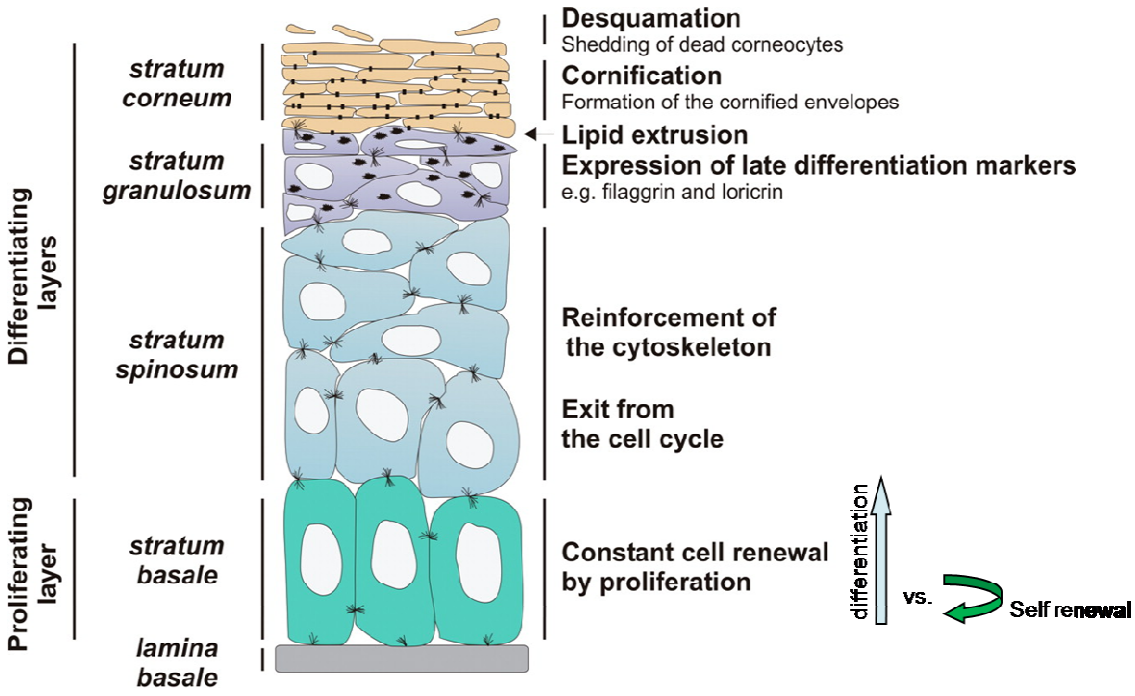



**Figure 1:** Overview of the different layers in the epidermis. Adapted from (Denecker et al., 2008).

Since the epidermis turns over every few weeks, this tissue has two main mechanisms to maintain tissue homeostasis. The first major site of regulation occurs between the basal and spinous layer where the balance between constant self-renewal and differentiation is controlled by multiple signaling pathways (Fuchs and Raghavan, 2002). Towards the surface of the epidermis, there is a balance between cell survival and cell death and numerous factors are known to regulate these processes (Raj et al., 2006). Promoting cell survival is the activation of growth factors such as epidermal growth factor (EGF), Akt/protein kinases B, and NFκB. Stimulating cell death are the activation of death receptors and caspases. Disruption of this equilibrium towards cell survival can lead to various forms of skin cancer, psoriasis, and the thickening of the epidermis. This may occur by the mutations of cell death promoting enzymes. On the

other hand, defects in cell survival contribute to sunburn, toxic epidermal necrolysis, or graft-versus-host disease (Raj et al., 2006).

The formation of dead cells in the stratum corneum resembles the process of apoptosis (Bragulla and Homberger, 2009). Apoptosis, the process of program cell death, is part of normal development in multicellular organisms. In the skin, it has been proposed that the apoptotic balance between cell survival and cell death plays a crucial role in the granular layer and the stratum corneum. One group of genes that play a role in cell death in epidermal homeostasis are the caspases (Raj et al., 2006). Caspases are cysteine-aspartic acid proteases that are synthesized as inactive pro-enzymes and are activated when dimerization/cleavage occurs at the activation site. Caspase-8 functions in the initiation phase of apoptosis upon binding of the death receptor to its ligand. This leads to the activation of caspase-8 which turns on downstream caspases and promotes the execution phase of apoptosis (Kaufmann et al., 2009).

Under normal physiological conditions, caspase-8 is found to be expressed in the granular layer of the epidermis (Lee et al., 2009). This localization suggests that caspase-8 may play a role in the formation of the dead cells in the stratum corneum. However, deletion of epidermal caspase-8 does not affect the formation of the stratum corneum which implies that this protease is dispensable in the terminal differentiation program of the epidermis (Lee et al., 2009).

A possible role of epidermal caspase-8 was reported in a study which demonstrated that elevated levels of caspases led to an impaired wound healing response (Al-Mashat et al., 2006). Moreover, humans with a systemic decrease in caspase-8 exhibited the symptoms of eczema which is reminiscent of a chronic wound healing response (Chun et al., 2002). Thus, these reports suggest that proper regulation of caspase-8 expression plays a role in a proper wound healing response.

In order to determine whether dynamic expression of caspase-8 is a normal feature of a wound healing response we analyzed the caspase-8 RNA levels during the closure of an excisional wound. Caspase-8 expression is downregulated at sites of the wound and is restore upon completion of the wound healing program (Lee et al., 2009).

To understand the skin specific physiological role of the gene, epidermal caspase-8 knockout (KO) mouse model was generated. Gross examination of the caspase-8 KO mice reveals rough, scaly and dehydrated skin consistent with a thickening of the epidermis (Lee et al., 2009) (Figure 2). Epidermal markers reveal that the expansion of the epidermis in the knockout animal was due to the expansion of both the basal and spinous layers. Interestingly, the formation of the stratum corneum was unaffected.

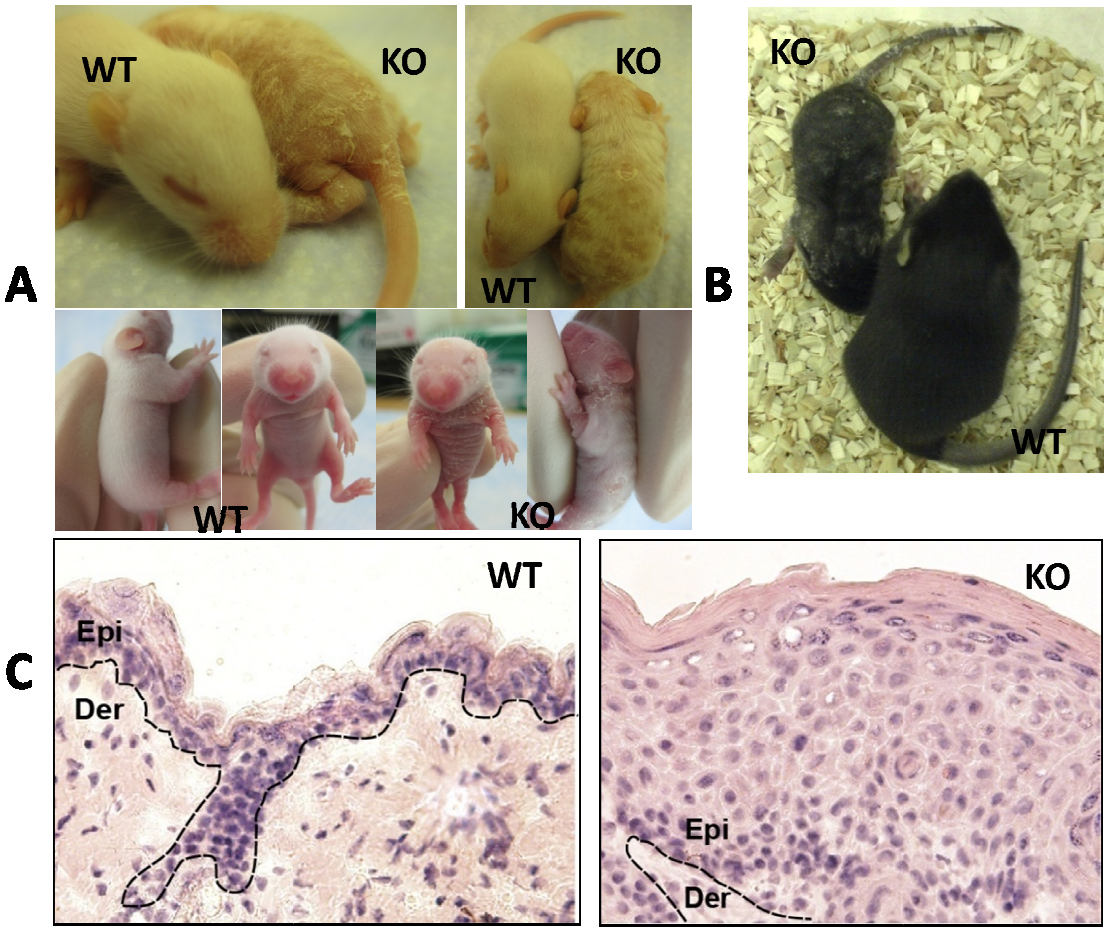



**Figure 2:** Gross and histological phenotype of epidermal specific caspase-8 null (KO) mice and their WT littermate. 10 days old (A) and 21 days old (B). (C) H&E (Hematoxylin and eosin) staining of a 5 days old mouse; epidermis (Epi) and dermis (Der) is separated by the basement membrane (black dotted line).

Upon trauma to the skin, the downregulation of epidermal caspase-8 initiates a complex series of signaling events that mediate a proper wound healing response. The downregulation of caspase-8 causes keratinocytes to release their reservoirs of IL-1α (interleukin-1 alpha) (Lee et al., 2009) (Figure 3). The interleukin-1 proteins are cytokines that have a variety of effects on different cells in the skin. IL-1α can stimulate both neighboring keratinocytes and underlying dermal fibroblast to release of pro-inflammatory cytokines to provide immune defense against infection (Barland et al.,

2004) resulting in an inflammatory response. Additionally, IL-1α can cause dermal fibroblast to synthesize and secrete growth factors that have the ability to signal back to the epidermis to induce proliferation of the keratinocytes in the basal layer. Furthermore, IL-1α also causes the nuclear translocation and activation of the transcriptional factor NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) in keratinocytes of the suprabasal layer (Lee et al., 2009). In epidermal keratinocytes, activation of the NFκB leads to growth arrest and cell survival (Perkins, 2007) (Figure 3).

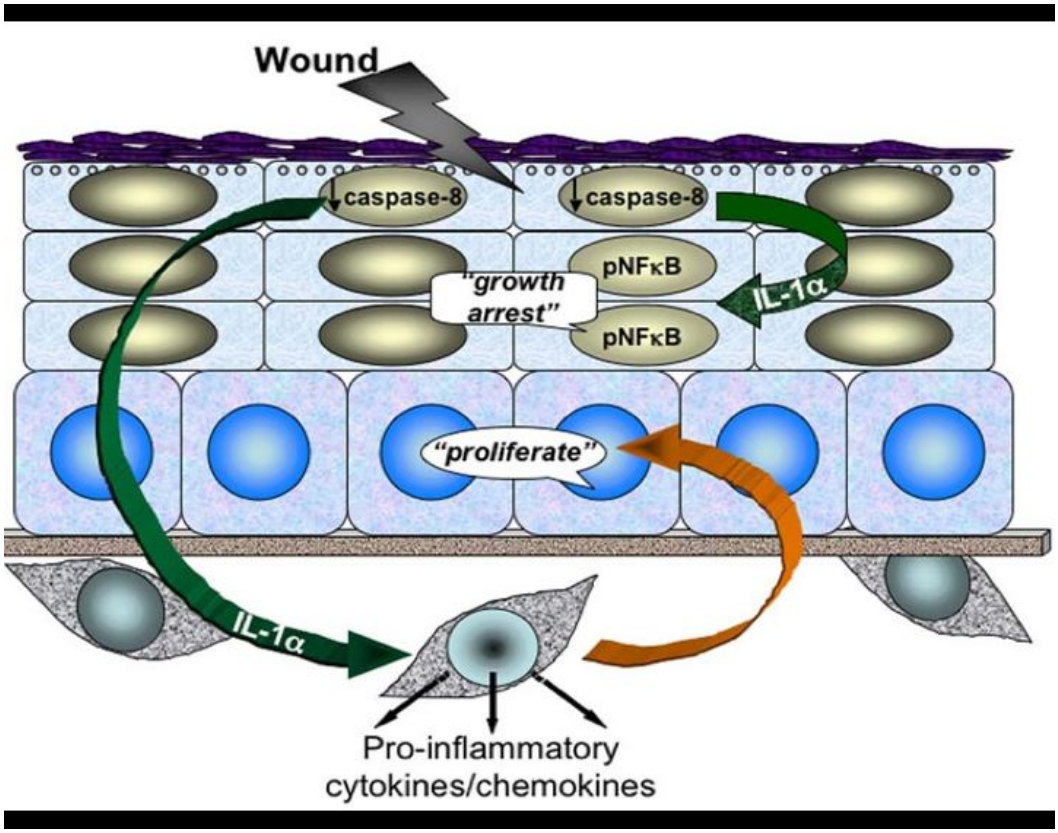



**Figure 3:** Model of IL-1α mediated crosstalk in the epidermis and the underlying dermis during a downregulation of caspase-8. Adapted from Lee et al., 2009.

We then focused on the contribution of the downregulation of caspase-8 to the different phases of a wound healing response. Wound healing response consists of

three overlapping phases: inflammation, proliferation and remodeling phases. The inflammation phase involves the recruitment of immune cells and the clotting cascade in the dermis to prevent excessive blood loss. Immune cells such as macrophages and neutrophils function to fight off infections, prevent further damages at the site of the wound and secret growth factors, cytokines and proteases required for the proliferation phase. In the proliferation phase, closure of the wound and repair of the tissue occurs. During this phase, angiogenesis (the growth of new blood vessels) and reepithelialization (the production of new keratinocytes from cutaneous stem cells and migration of keratinocytes) contribute to wound closure. In the remodeling phase, extracellular matrix and cellular remodeling occurs, and is mediated by various proteases that aid in cleaving and forming new intercellular and cell-substratum adhesions. Despite the fact that these three phases have different functions, they are all interdependent in that signals from one phase affects the activity of the others.

Asides from being an intricate part of self-defense and repair, the wound healing response may be a model for the control of epidermal proliferation in other contexts (Fuchs, 2007). In development, low basal layer cell density signals the stem cells to proliferate. The proliferation of these cells increases cell density as well as creating newly form intercellular cell-cell adhesion complexes known as adherens junctions. These adherens junctions have the ability to inhibit further growth factor signaling pathways such as the Ras/MAPK, which would otherwise promote cell division. In a wound response, injury causes a transient loss of adherens junctions which promotes cell proliferation and the activation of NFκB. This activation of NFκB causes two things, the recruitment of immune cells to fight against infections and further stimulation of cell proliferation and migration via the Ras/MAPK pathway. In cancer and chronic inflammation, a mutation and/or permanent change in adherens junctions causes a

similar wound-like response where the activation of NFκB and Ras/MAPK growth factor signaling occurs. However, constitutively active NFκB causes a chronic immune response which promotes growth factor signaling. This continuous migration and cell proliferation contributes to tumorigenesis. This is a prime example of the conservation of the wound-related inflammatory and proliferative responses in different contexts (Figure 4).

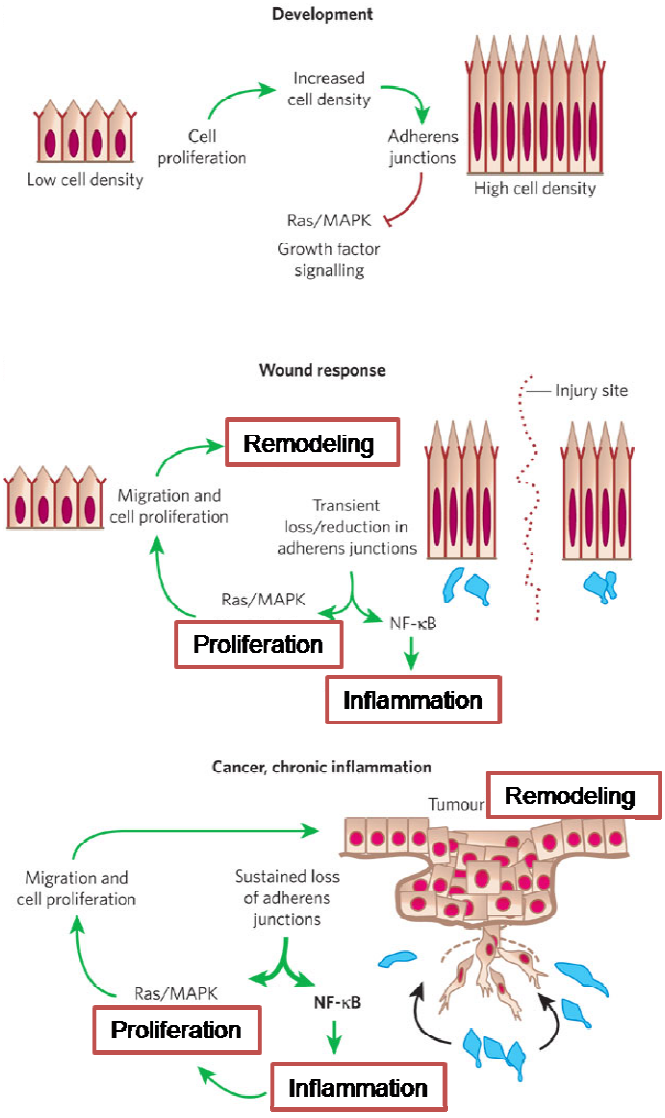



**Figure 4:** Conservation of mechanisms controlling epidermal proliferation. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, MAPK, mitogen-activated protein kinases. Adapted from Fuchs, 2007.

Given the conservation of the immune response in both wound healing and cancer/chronic inflammation, it is interesting to note that humans with a decrease in caspase-8 levels suffer from eczema , a form of dermatitis with a range of clinical symptoms such as asthma and elevated serum immunoglobulin (Chun et al., 2002). Dermatitis is the general term for any inflammation of the skin; typical phenotypes of dermatitis are rashes, skin edema (the abnormal accumulation of fluids under the skin), itchiness, and dehydration/dryness. Among the most common types of dermatitis are psoriasis and eczema. Psoriasis is a non-contagious autoimmune disease that affects the skin and joints of the body. It is a chronic inflammatory skin disease that can affect anyone regardless of age or sex (Bolognia et al., 2008). Clinical features of psoriasis are scaly red patches that appear in the skin induced by an excess of skin formation known as psoriatic plaques. The causes of psoriasis is still unknown, but evidence suggests that it may be part of a genetic disorder in which certain T-cells are chronically active, resulting in the release of various cytokines causing a rapid production of skin cells. Treatments do exist for psoriasis, but effectiveness varies. Typical treatment is topical ointment to reduce the scalps, but since the disease is chronic, it may reappear later and requires different treatments such as laser procedure and drugs/injections.

Eczema has a broad range of chronic skin conditions. Symptoms shared with psoriasis include redness, skin edema, itching, dryness, cracking, blistering, and/or bleeding (Bolognia et al., 2008). The most common type of eczema is atopic eczema, which is also known as atopic dermatitis (AD). This non-contagious pruritic (itchy) skin disease affects about 10-20% of the children/infants in the world, though adults do get AD at a much lower rate. Causes of AD are still unknown, but studies have suggested that because the barrier functions of the skin is compromised leading to increased susceptibility to infection and dehydration. The chronic inflammatory response in AD

patients may compromise the barrier function of the skin and can be initiated by environmental triggers such as allergens, microbes and/or viruses. Another hallmark of AD is a phenotype known as spongiosis. The barrier defect caused by spongiosis results from gaps between the cells of the tissue allowing infiltration of fluids in the epidermis. However, the underlying cause of this gap formation remains unknown. There is currently no known treatment for AD; however, there are drugs that control the symptoms of AD such as topical corticosteroids to reduce inflammation, moisturizers to relieve itchiness and antibiotics to stop bacterial infections.

Given the remarkable similarities between the caspase-8 conditional knockout mouse and the symptoms of AD, my aim was to determine whether an epidermal specific deletion of caspase-8 is sufficient in developing the eczematic phenotype.

**Acknowledgement**

This chapter, in part, is being prepared for publication of the material as it may appear as Li, Christopher K., Jamora, Colin, 2009. The thesis author was the primary investigator and author of this paper.

# Results

Extensive research has been done on the clinical features of atopic dermatitis (AD). The prominent pathological features of AD are shown in Table 1.

**Table 1:** Atopic Dermatitis Pathology

| |
|---|
| Skin hyperreactivity to environmental and immunologic triggers such as food, allergens, microbes and autoantigens |
| Disruption of skin barrier function |
| Acute AD – systemic Th2 response and immunoglobulin productions |
| Chronic AD – Th1 response and skin remodeling |
| Expression of chemokines and cytokines leading to a skin inflammatory response |
| Decrease filaggrin gene expression |
| Cell type involved in AD: T cells, dendritic cells, keratinocytes |

In AD, various environmental and immunologic triggers such as microbes, viruses, scratching and allergens cause the stimulation of an innate and adaptive immune response and the production of cytokines and chemokines from the keratinocytes in the epidermis (Leung et al., 2004). AD is biphasic and exhibits an acute and a chronic phase. In the acute response, mast cells and basophils are recruited to the dermis and aid the production and activation of cytokines such as interleukin-4, interleukin-10 and the epidermal-derived TSLP (thymic stromal lymphopoietin) (Bolognia et al., 2008). Adaptive T-cells recruited from the circulation can be activated by Langerhans cells (a resident skin dendritic cell that acts as antigen presenting cells) to converts themselves to Th2 cells in the dermis. These Th2 cells can produce cytokines

including interleukin-4 (IL-4), which influences IgE production from B-cells and mast cells, IL-5 (for recruitment of eosinophils) and IL-13, which modulate keratinocyte and epithelial cell process in allergic diseases (Schmidt-Weber et al., 2007). During the chronic response, chemokine and cytokine production causes activation of IDEC (inflammatory dendritic epidermal cell) – a specialized dendritic cell that only appears in an immune response in the skin. With the aid of dermal immune and inflammatory cells like eosinophils and macrophages, the primary role of IDEC is the conversion of adaptive T-cells into Th1 cells via the release of IL-12. The activation of Th1 cells then regulates downstream cytokines such as IL-2 and IFN-γ (interferon-gamma) which is known to induce cell death in keratinocytes and cause the spongiform pathology/spongiosis (a dissolution of intercellular adhesions in the epidermis allowing infiltration of fluids in the tissue), and a chronic immune response (Bolognia et al., 2008; Leung et al., 2004; Scharschmidt and Segre, 2008) (Figure 5).

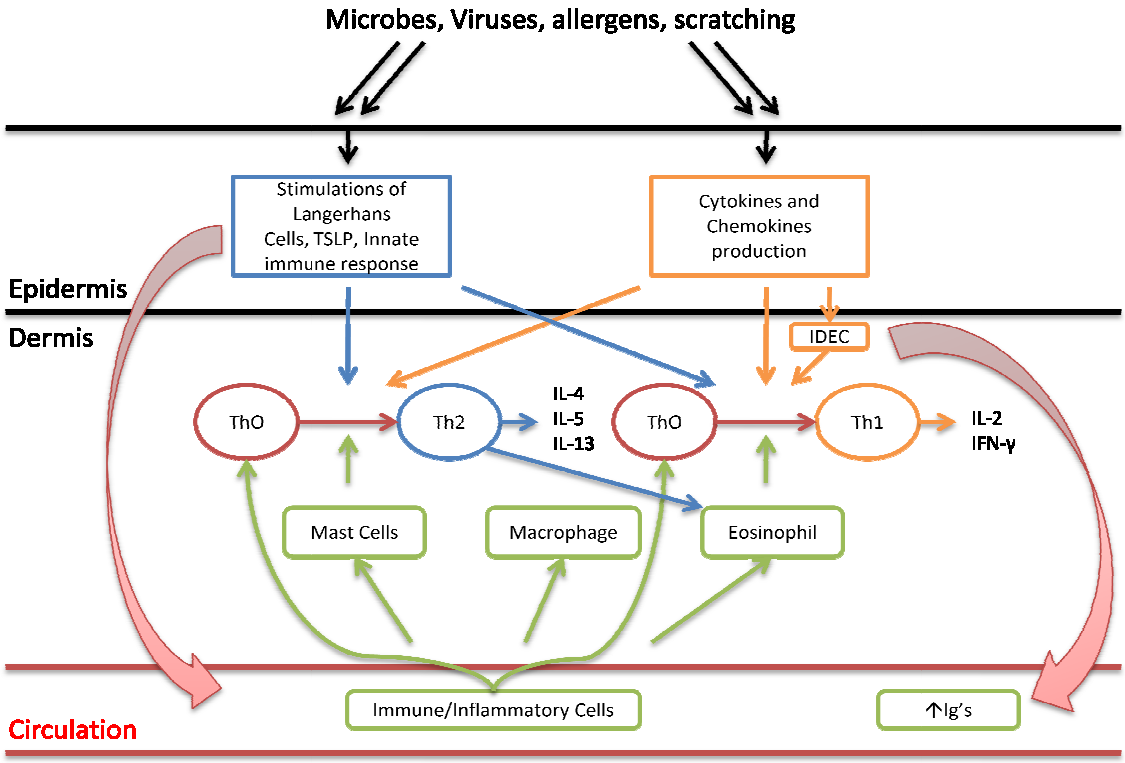



**Figure 5:** Pathology of acute and chronic AD. Induction by microbes, viruses, allergens, and/or scratching leads to the stimulation of Langerhans cells, TSLP, innate immune response (acute) and cytokines/chemokines production (chronic) in the epidermis. Innate immune response in the epidermis causes the recruitment of immune/inflammatory cells to the dermis. As shown, mast cells, macrophages, eosinophils and innate T cells are recruited into the dermis from blood circulation. Th2 cell development is assisted by antigen presenting cells such as Langerhans cells, TSLP, cytokines/chemokines, and mast cells. Mature Th2 cells results in the production of eosinophils and elevated Ig levels, which is promoted by both Langerhans cells and IDEC. Similarly, Th1 development are caused by cytokines/chemokines production in the epidermis, eosinophils, langerhans cells and as well as IDECs. Both mature Th1 and Th2 cells in the dermis recruit further downstream cytokines such as IL-4, 5, 13, 2, and IFN-γ. TSLP (thymic stromal lymphopoietin), IDEC (inflammatory dendritic epidermal cell), IL (interleukin), IFN (interferon), Ig (immunoglobulin)

In addition to the pathology of AD, there are also genetic contributions and predisposing factors for AD. Previous studies have shown that a common loss-of-function modification of the epidermal barrier protein filaggrin is the leading factor for atopic dermatitis (Palmer et al., 2006). Filaggrin, an important polypeptide that aggregates the keratin cytoskeleton, forms a dense layer of cornified cell envelope through the crosslinking of proteins and lipid matrix. Filaggrin null mutation was found to cause ichthyosis vulgaris, a skin disease which is closely associated with atopic dermatitis. In addition, case studies revealed patients with AD have decreased levels of the filaggrin gene expression. 17.5% of all individuals with AD are carriers of the filaggrin null allele. Additionally it was reported that the decrease in filaggrin expression may be a result of interleukins released by Th2 cells (Palmer et al., 2006).

Since a systemic deletion of caspase-8 develops eczema, I set out to test the hypothesis that the epidermal specific deletion of caspase-8 is sufficient to cause AD-like features in the murine skin (Chun et al., 2002). In order to determine the extent to which the caspase-8 conditional knockout (cKO) recapitulated the pathological features of AD, I compared the gross phenotype and histology of the caspase-8 KO mice to phenotypes of atopic dermatitis patients as well as other mouse models for AD. This analysis includes the profiling of cytokines, chemokines and genetic signature of AD on the caspase-8 cKO background. Lastly, I determined possible target genes that play a role in one of the phenotype of the caspase-8 KO – the spongiotic-like epidermal gaps between each cell in the epidermis. My goal in the latter is to understand how the barrier integrity is disrupted in caspase-8 KO and the possible molecular underpinnings behind AD's sensitivity to environmental factors and dehydration in the skin.

Among the most prominent phenotypes of the caspase-8 KO mice was the skin defect that the mutant mice developed. Scaling is observed in the caspase-8 KO skin

(Figure 2). Comparing the wildtype (WT) to the knockout (KO) littermate, skin scaling was first recognized at approximately 5 days after birth and became obvious at postnatal day 10 (Figure 2a). The scaling of the skin occurred in a mosaic pattern throughout the body of the mouse and progressively worsened with age. The total body size of the KO mouse is relatively smaller than its WT counterpart (Figure 2b). The scaling phenotype suggests a thickening of the epidermis which was verified by histological analysis of skin sections (Figure 2c).

Histological analysis also revealed cellular infiltration in the dermis in the knockout mouse which suggested an inflammatory response. Caspase-8 KO revealed an infiltration of both innate and adaptive immune cells in the dermis including macrophages, and T-cells (Lee et al., 2009). Since mast cells are an important mediator of AD, I tested for their presence in the caspase-8 KO skin. Using toludine blue staining, I found that there is a dramatic elevation of mast cells in the dermal layer of the caspase-8 KO when compared to the WT littermate (Figure 6).

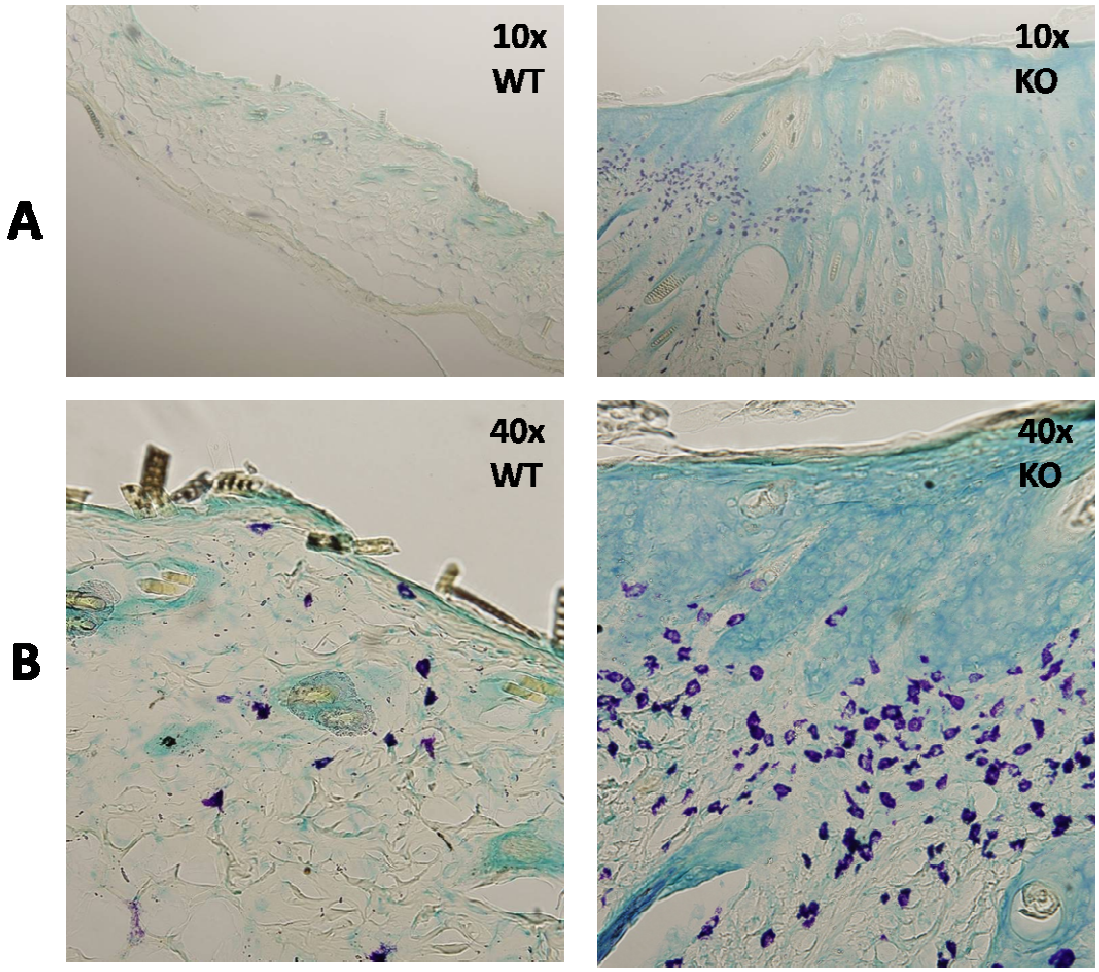



**Figure 6:** Elevated infiltration of mast cells in caspase-8 KO. Toluidine blue staining of caspase-8 WT (left column) and KO (right column) with 10x magnification (A) and 40x magnification (B). Light blue color stains the epidermis and hair follicles, dark purple color stains mast cells.

There are currently various mouse models for AD ranging from transgenic to knockouts and spontaneous mutations to study the molecular mechanisms underlying this disease. In addition, there is also a characteristic pattern of highly expressed genes that form an AD "signature". Interleukin-4 (IL-4) has previously been shown to play an important role in Th2 cell activation and initiates an inflammatory response. A transgenic (Tg) mouse overexpressing IL-4 features chronic lesions that are similar to human AD as

well as elevated IgE and IgG1 levels (Chan et al., 2001). In the caspase-8 KO model, real-time polymerase chain reaction (qPCR) reveals an elevated level of IL-4 expression compared to the WT skin (Figure 4a). TSLP, thymic stromal lymphopoietin, is normally expressed in the epidermis and is highly upregulated in AD phenotypic skin and plays a role in Th2 activation. TSLP transgenic mouse develops AD like phenotypes with increased levels of IgE, IgG1, and decrease IgG2a (Soumelis et al., 2002; Yoo et al., 2005). Like IL-4, TSLP gene expression was also increased in the caspase-8 KO (Figure 4a). In addition, there are other mutant mice that are used to model AD such as caspase-1 Tg and IL-18 Tg. Caspase-1, a proteolytic enzyme which acts as a precursor for other cytokines like IL-1β and IL-18, mimics AD-like phenotype when overexpressed in the skin and features elevated IgE and IgG1 levels in the serum. In addition, caspase-1 Tg was shown to have elevated IL-18 in its serum (Konishi et al., 2002). Interestingly, overexpression of caspase-1 in a STAT6 deficient mice still exhibits chronic dermatitis, but without the elevation of IgE. This suggests that IgE may play a dispensable role in the development and progression of the AD phenotype. IL-18 is known to enhance IL-4, IL-13, and IFN-γ production. Similar to the caspase-1 Tg model, IL-18 transgenic mice features AD-like characteristics as well as elevated IgE and IgG1. On the contrary, overexpression of caspase-1 in the skin of mice lacking IL-18 and STAT6 caused no dermatitis and normal production of IgE in the serum. Furthermore, overexpression of both caspase-1 and IL-18 with the deficient of IL-1 took the skin longer to develop dermatitis than caspase-1 Tg alone. This finding reveals the essential role IL-18 plays in causing skin inflammation with the additional acceleration of IL-1 (Konishi et al., 2002). When caspase-1 and IL-18 was probed for relative gene expression in the caspase-8 KO, there was a consistent increase of expression of both genes compared to wild type skin (Figure 4a).

Recent knockout AD models were generated and studied which includes, but is not limited to, cathepsin E, PPAR-α, and RelB. Cathepsin E, an endolysosomal aspartic proteinase, is predominantly expressed in APC (antigen presenting cell) and plays a role in the activation of APCs during immune responses. Deletion of this gene leads to an elevation of IgE and Th2 cell response (Tsukuba et al., 2003). However, Th1 cell responses such as IFN-γ and IL-2 secretion are unaffected. In addition, these cathepsin KO mice show increased amounts of dermal infiltration with eosinophils, lymphocytes and macrophage. One possibility for the decreased Th1 cell response might be due to the absence of dendritic cell function in these KO mice which are required for Th1 cell activation. In our qPCR analysis of the caspase-8 KO mouse, there was no difference in cathepsin E levels between the KO and the WT (Figure 7b). PPAR-α (peroxisome proliferator-activated receptor-α) belongs to a nuclear receptor superfamily that are ligand-activated transcription factors and these receptors play a role in anti-inflammatory response. This may also result in the ability of PPAR-α to inhibiting proinflammatory NFκB pathway (Staumont-Salle et al., 2008). Furthermore, PPAR-α also plays a role in keratinocytes proliferation/differentiation during wound healing (Dahten et al., 2008). Mice that are deficient in PPAR-α developed AD-like features such as recruitment of inflammatory cells, increased IgE and IgG2a production, and increased Th2 and Th1 cell responses. In addition, PPAR-α expression was decreased in eczematous skin from human AD patients. Deletion of PPAR-α from the epidermis caused the mutant mice to develop AD-like dermatitis in the skin. Furthermore, TSLP is strongly expressed and induced in this knockout model (Staumont-Salle et al., 2008). Interestingly, PPAR-α is decreased (Figure 7b) and TSLP is increased (Figure 7a) in the caspase-8 KO skin relative to the wild type tissue. This finding supports our previous observation that the caspase-8 conditional knockout has significantly more NFkB activity (Lee et al., 2009)

and may be due to the relief of the NFkB inhibition by PPARa. RelB, which is a component of the NF-κB transcription factor complex, plays a key role in immune response during an infection. RelB KO mice develop AD-like thickening of the skin and hair loss 4-10 weeks after birth. Phenotypic skin in the knockout animal displays epidermal hyperplasia, infiltration of T cells, eosinophils, neutrophils, dendritic cells and masts cells into the dermis and an increase of Th2 cytokines expression (Barton et al., 2000). However, when RelB KO mice were crossed with Tg mice that lack peripheral T cells, no dermatitis developed, suggesting this phenotype was T-cell dependent. Consistent with the RelB KO mouse model, the caspase-8 KO skin has lower levels of RelB compared to the wild type tissue (Figure 7b).

Both acute and chronic atopic dermatitis reveal a characteristic profile of changes in cytokine and chemokine expression and activity. In AD, numerous CCL's (chemokines C-C motif ligand), CXCL (chemokines C-X-C motif ligand) and IL's (interleukin) are activated during both acute and chronic phases. Chemokines such as CCL-5, 11, 17 and CXCL-9, 10, 11 are derived from keratinocytes in the epidermis. The release of these ligand aid the activation of Th2 and Th1 cells in the dermis by binding to their respective receptors CCR's and CXCR's. Ligand-receptor interactions results in the activation of cytokines such as IL-4, IL-5, IL-12, IL-13 and IFN-γ to produce an immune response and recruitment of immune/inflammatory cells to the dermis. My analysis revealed an increase of AD-associated cytokines, chemokines, and chemokines receptors listed above in the caspase-8 KO skin relative to wild type tissue (Figure 7c).

Transforming growth factor (TGF) is a well-known cytokine that is capable of inducing keratinocytes proliferation and plays a role in tumor progression. TGF is produced by multiple cell types including macrophages and keratinocytes. Given its role in processes present in AD, I analyzed whether TGF levels are affected by the loss of

caspase-8. I found that TGF RNA levels in the caspase-8 knockout epidermis were increased substantially compared to wild type tissue (Figure 7c).

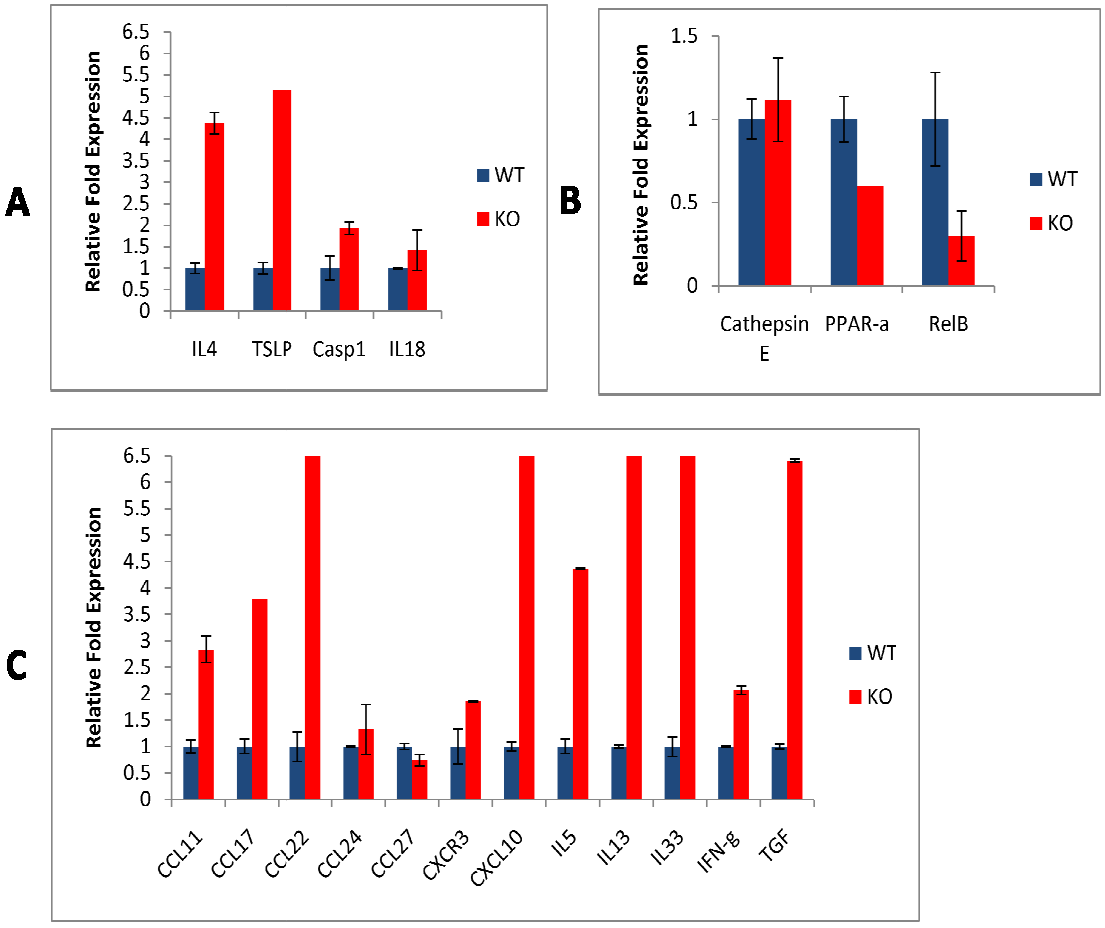



**Figure 7:** Real-time PCR of genes related to current molecular models and genetic phenotype of AD. Caspase-8 WT and KO complementary DNA (cDNA) samples were prepared from RNA extracted from the skin of a 10 day old mouse. Gene expression of transgenic (A), KO's (B) and AD related genes (C) were amplified using qPCR and KO expression was compared to the WT reference (set at 1). β-actin was used as a calibrator reference.

Many of the mutant mouse models displayed elevated levels of IgG1 and IgE in their serum. One of the clinical features of AD in humans is the increased levels of immunoglobulin in circulation. Nearly 80% of human AD patients have increased levels

of IgE and IgG1. To determine if the caspase-8 KO exhibits the same feature, serum was collected from caspase-8 KO and wild type mice and isotyped for immunoglobulin levels. As shown in figure 8, as the mice age, there is trend of increasing IgG1 levels in the caspase-8 KO compared to WT (Figure 8).

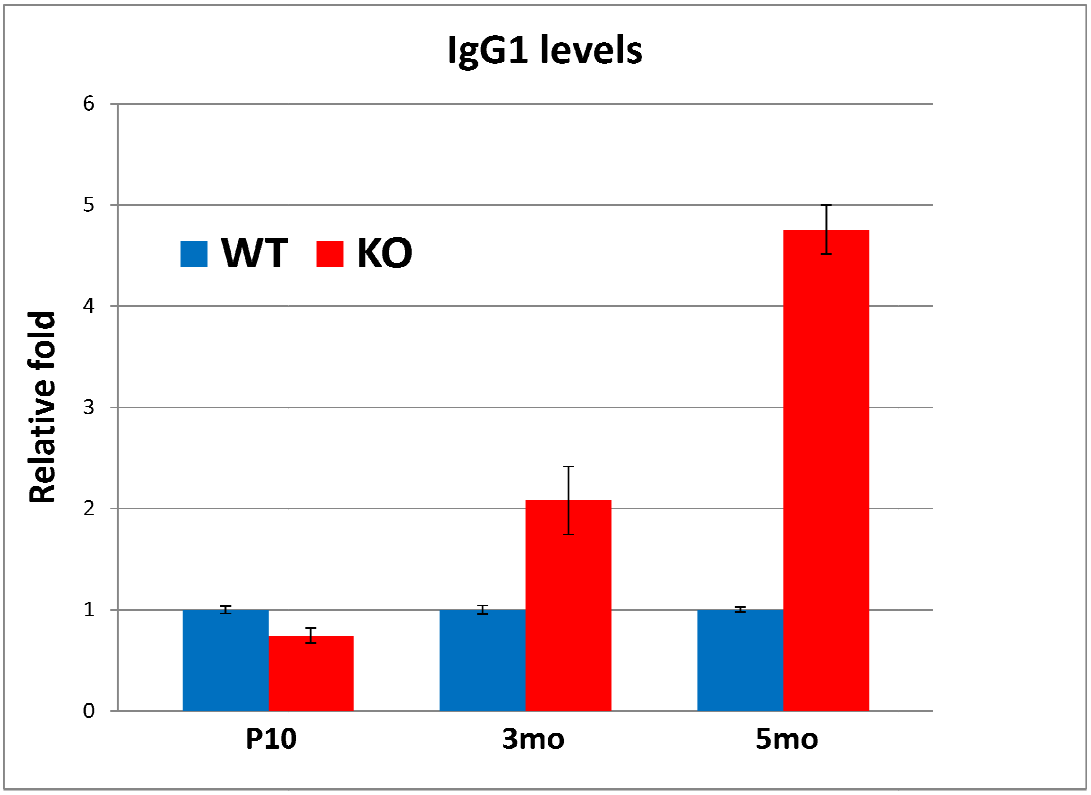



**Figure 8:** Caspase-8 WT and KO immunoglobulin G1 levels. Whole blood was collected from pair of WT and KO littermate mice at age 10 day, 3 month and 5 month old. The whole blood was centrifuged at 14,000 rpm two times for 10 minutes to separate the supernatant serum. Serum was analyzed by ELISA isotyping kit for antibody IgG1. Each KO serum samples was compared to their respective WT reference which is set at 1.

In addition to environmental contributions, there are genetic factors that play a role in AD. One predisposing genetic factor of AD is the decreased level of filaggrin

gene expression (Palmer et al., 2006; Weidinger et al., 2006). Immunofluorescent analysis revealed a decrease in filaggrin expression in regions of the caspase-8 knockout skin with an expanded epidermis (Figure 9a). The change in filaggrin expression level was verified by western blot analysis. In caspase-8 KO, there is a significant decrease of pro-filaggrin compared to the WT (Figure 9b).

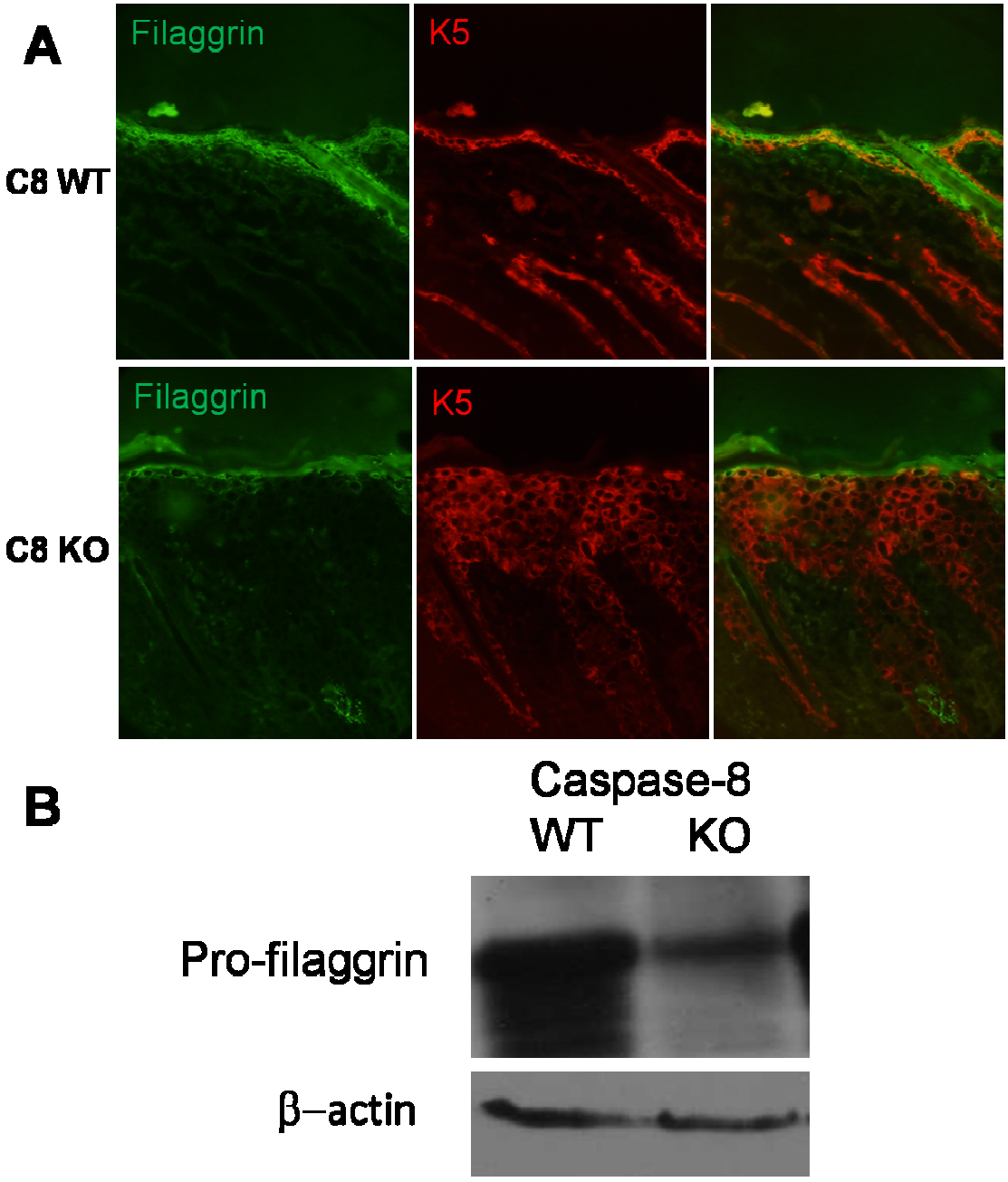



**Figure 9:** Caspase-8 KO lesional skin areas have decreased levels of filaggrin. Immunofluorescent staining (A,B) of caspase-8 WT and KO using the filaggrin marker (green) and basal layer/hair follicle marker (K5 – red). Panel A shows lesional KO and WT skin, panel B shows a non-lesional KO skin. Western blot analysis of caspase-8 WT and KO (C) probed with pro-filaggrin antibody and β-actin as loading control.

Another clinical feature of AD in humans is the development of spongiosis. The spongiotic phenotype is characterized by the detachment of cells within a tissue from one another thus allowing fluid to infiltrate in the intercellular space. Our current data suggests caspase-8 cKO recapitulates many of the AD-associated immune responses and I now focused our investigation on the effect of the loss of caspase-8 on epidermal integrity. Histological analysis of skin sections reveals caspase-8 KO mice exhibiting a spongiotic-like phenotype (Figure 10a). Within the expanded epidermis, gaps were observed between suprabasal keratinocytes of the epidermis, a feature reminiscent of human spongiotic skin (Figure 10b). Maintaining an intact and functional skin barrier is crucial for the prevention of dehydration. A trans-epidermal water lost (TEWL) assay was performed on the WT and KO skin to determine if epidermal barrier integrity of the skin is compromised as a result of the loss of caspase-8 expression. A microsensor housed in a closed measuring chamber is applied to skin where the measurements of water vapors emitted is measured and calculated. Results reveal an increase water loss in the caspase-8 KO compared to the WT, revealing barrier integrity dysfunction in the KO and may be caused by the spongiotic phenotype (Figure 10c).

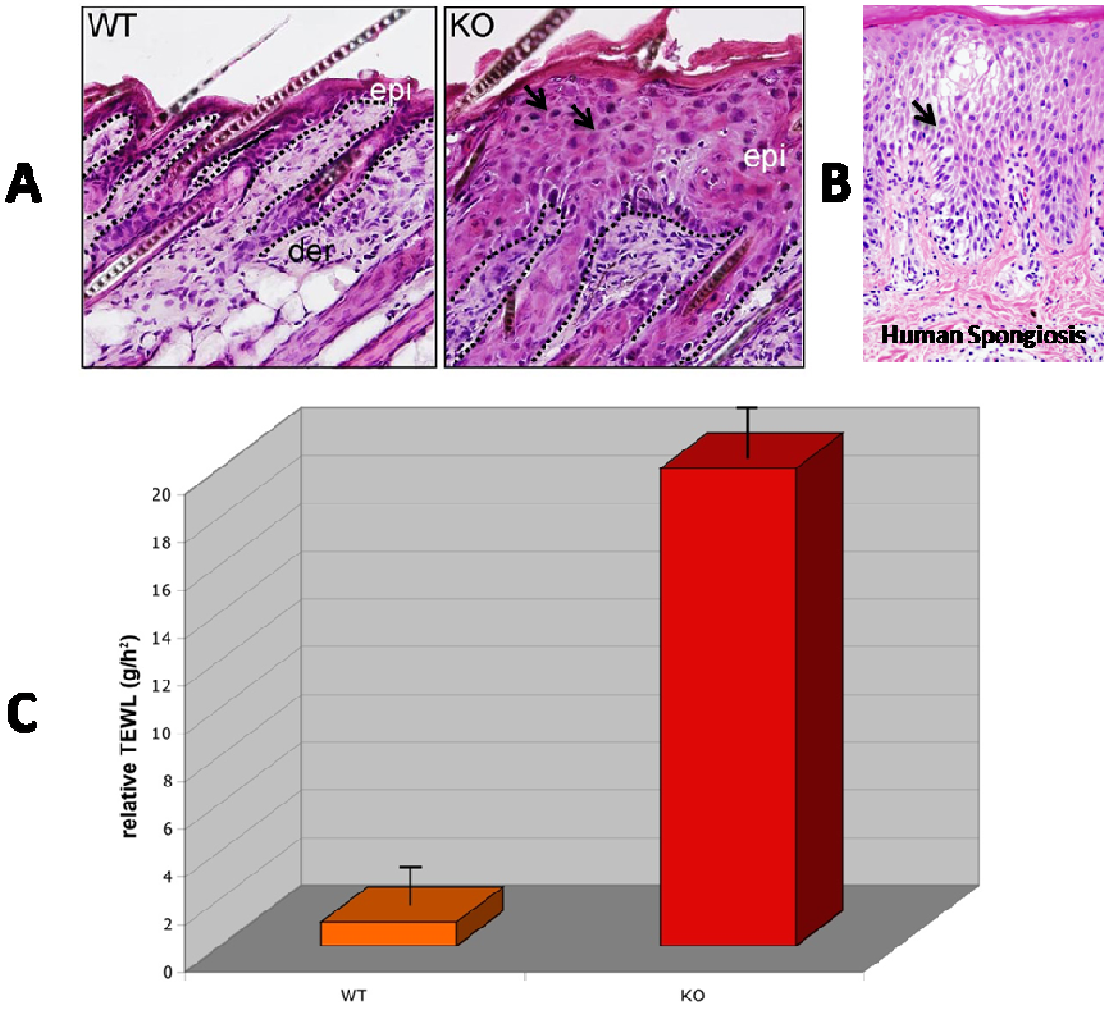



**Figure 10:** Barrier integrity is compromised in the caspase-8 KO. Epidermal gap formation was observed in the caspase-8 KO compared to the WT (A), the similar gap can be observed in human spongiosis skin section (B); black arrows represents examples of epidermal gaps – figures were adapted from Lee et al., 2009. Trans-epidermal water loss (TEWL) assay reveals epidermal water loss in the caspase-8 KO is higher relative to the WT reference set at 1 (A). TEWL values are calculated from the growing rate of the humidity and moisture created from the water vapor emitted from the skin.

To determine the molecular basis of the spongiotic phenotype in the caspase-8 null epidermis, I assessed the integrity of adherens junction apparatus in the skin. Adherens junctions (AJs) are protein complexes that mediate intercellular adhesion and

help seal cells together to form epithelial sheets (Jamora and Fuchs, 2002). The adherens junction in epithelial cells is nucleated by a single pass transmembrane protein called E-cadherin (E-cad). E-cadherin is linked to the actin cytoskeleton via β and α-catenin (Figure 11a). To investigate the status of AJs, I analyzed whether E-cadherin levels were changed in the caspase-8 KO vs. the wild type epidermis. I found that E-cadherin staining along the cell borders on the caspase-8 null epidermis was significantly diminished whereas the wild type epidermis exhibited E-cadherin along the cell-cell contacts between the keratinocytes (Figure 11b). This observation was verified by Western blot of protein extracts from the skin of wild type and knockout animals. I found that the knockout skin had decreased full-length E-cadherin and there was a concomitant increase in the amount of cleaved E-cadherin (detected with an antibody recognizing the C-terminal fragment [CTF]). However, the protein levels of β-catenin, which helps to link E-cadherin to the actin cytoskeleton, remain unchanged in the caspase-8 KO (Figure 11c).

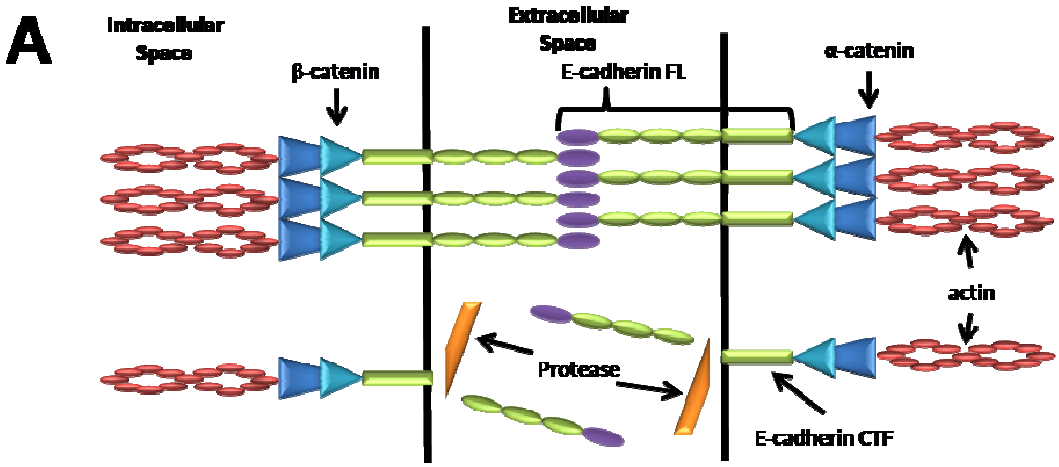



**Figure 11:** Structure of E-cadherin adherens junction. (A) Full length E-cadherin (E-cad FL) is a transmembrane protein linking cells to each other. The extracellular domain undergoes hemophilic interaction with the ectodomain of another E-cadherin protein on the opposing membrane. The cytosolic domain (E-cad CTF) binds to β-catenin and this cadherin/catenin complex is linked to the actin cytoskeleton through α-catenin. Proteases have the ability to cleave E-cadherin in extracellular region to shed the ectodomain while the E-cad CTF remains in the cell . (B) Immunofluorescent staining of caspase-8 WT and KO using an antibody recognizing the extracellular domain of E-cadherin (red). The white dotted line refers to the basement membrane that separates the epidermis (epi) from the dermis (der). (C) Western blot analysis of E-cad FL, E-cad CTF and β-catenin levels in the caspase-8 WT and KO. Protein lysates were generated from the skin of 5 month old caspase-8 WT and KO littermates. β-actin was probed as loading control.

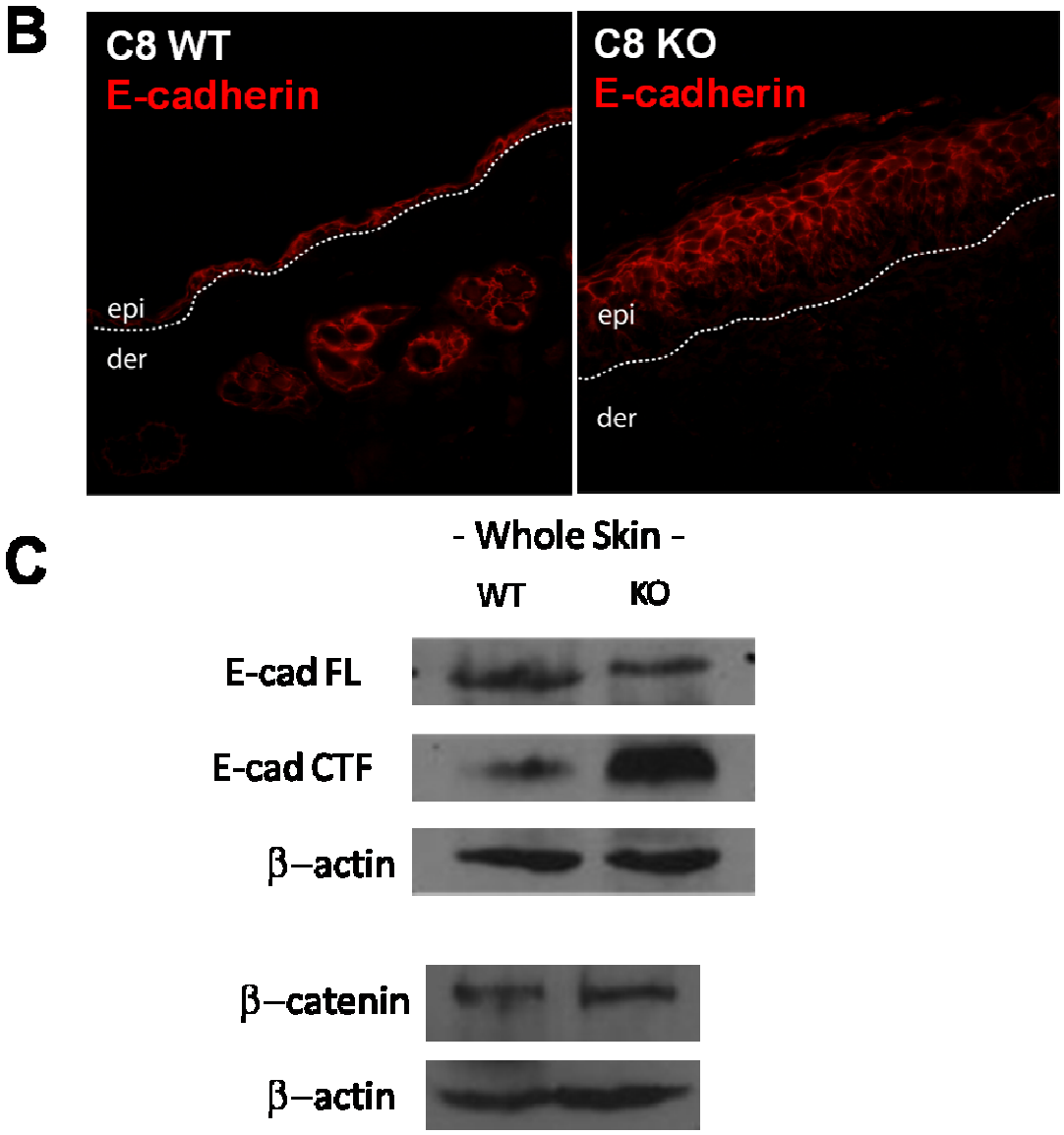
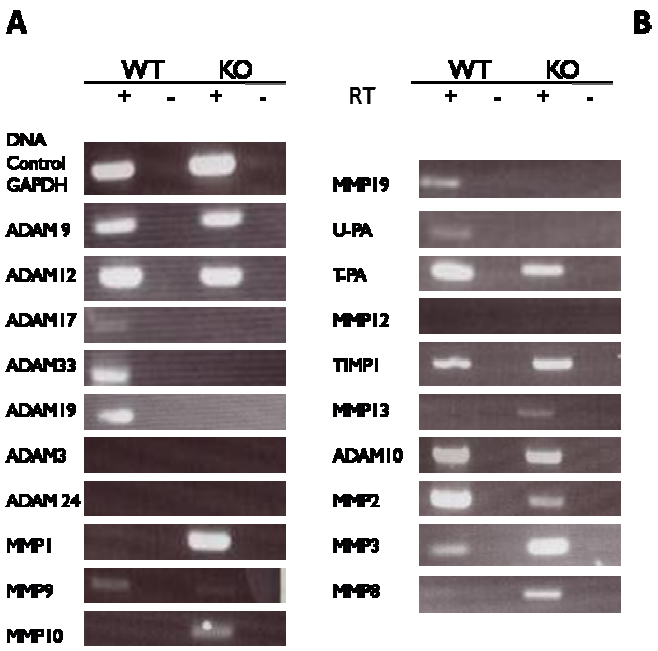



Figure (B) and (C) are continuation of **Figure 11** on previous page.

The cleavage of E-cadherin in the caspase-8 KO suggests the involvement of proteases that may shed the extracellular domain of this protein from the surface of the keratinocytes. Since caspase-8 KO is involved in a wound healing response, I screened a select group of proteases that are differentially expressed during excisional wound healing (Madlener et al., 1998). These proteases may have the ability to cleave E-

cadherin causing cell detachment intercellularly and explains for the gaps formation and spongiotic phenotype in the caspase-8 KO epidermis (Figure 10a, 11a). Among the proteases that are involved in wound healing are ADAMs (a disintegrin of metalloproteases), MMPs (matrix metalloproteases), TIMPs (tissue inhibitor of metalloproteases), and various plasminogens such as tissue (T-PA) and urokinase plasminogen (U-PA) (Madlener et al., 1998). Interestingly, many of these enzymes have been found to shed E-cadherin in different diseases such as tumor metastasis. In order to determine the expression of these proteases, I isolated RNA from postnatal day 10 skin from either wild type or caspase-8 knockout mice. RNA was then subjected to reverse-transcriptase PCR, and I found the following proteases that are present in caspase-8 KO are ADAM9, 10, 12, MMP1, 2, 3, 8, 9, 10, 13, TIMP1, and T-PA (Figure 12).




B

| Proteases | Caspase-8 WT | Caspase-8 KO |
|---|---|---|
| mMMP1 | - | + |
| mMMP2 | + | + |
| mMMP3 | + | + |
| mMMP8 | - | + |
| mMMP9 | + | + |
| mMMP10 | - | + |
| mMMP12 | - | - |
| mMMP13 | - | + |
| mMMP19 | + | - |
| mADAM3 | - | - |
| mADAM9 | + | + |
| mADAM10 | + | + |
| mADAM12 | + | + |
| mADAM17 | + | - |
| mADAM19 | + | - |
| mADAM24 | - | - |
| mADAM33 | + | - |
| mTIMP1 | + | + |
| mT-PA | + | + |
| mU-PA | + | - |

**Figure 12:** Reverse transcriptase PCR (RT-PCR) screen of proteases that are present during wound healing that are also expressed in the Caspase-8 KO. cDNA was generated from RNA extracted from the skin of 10 day old caspase-8 WT and KO. RT- indicates negative control where no transcriptase was added in generating the cDNA and RT+ indicates the presence of reverse transcriptase. GAPDH (glyceraldehydes 3-phosphate dehydrogenase) was used as a DNA loading control.

If the proteases are functional in the caspase-8 KO, the inhibition of these proteases would hypothetically abrogating the formation of gaps between keratinocytes of the epidermis. Caspase-8 KO skin samples were treated with a pan-MMP inhibitor and its vehicle control (DMSO). In the sample with vehicle control, the spongiotic phenotype is unaffected as gaps between epidermal cells are still present. However, treatment of the caspase-8 KO skin with the pan-MMP inhibitor resulted in fewer detectable gaps between cells in the expanded caspase-8 KO epidermis (Figure 13).

The blocking of MMPs using the pan-MMP inhibitor reveals the possibility that MMPs may be the cause of epidermal gaps in the caspase-8 KO spongiotic skin.

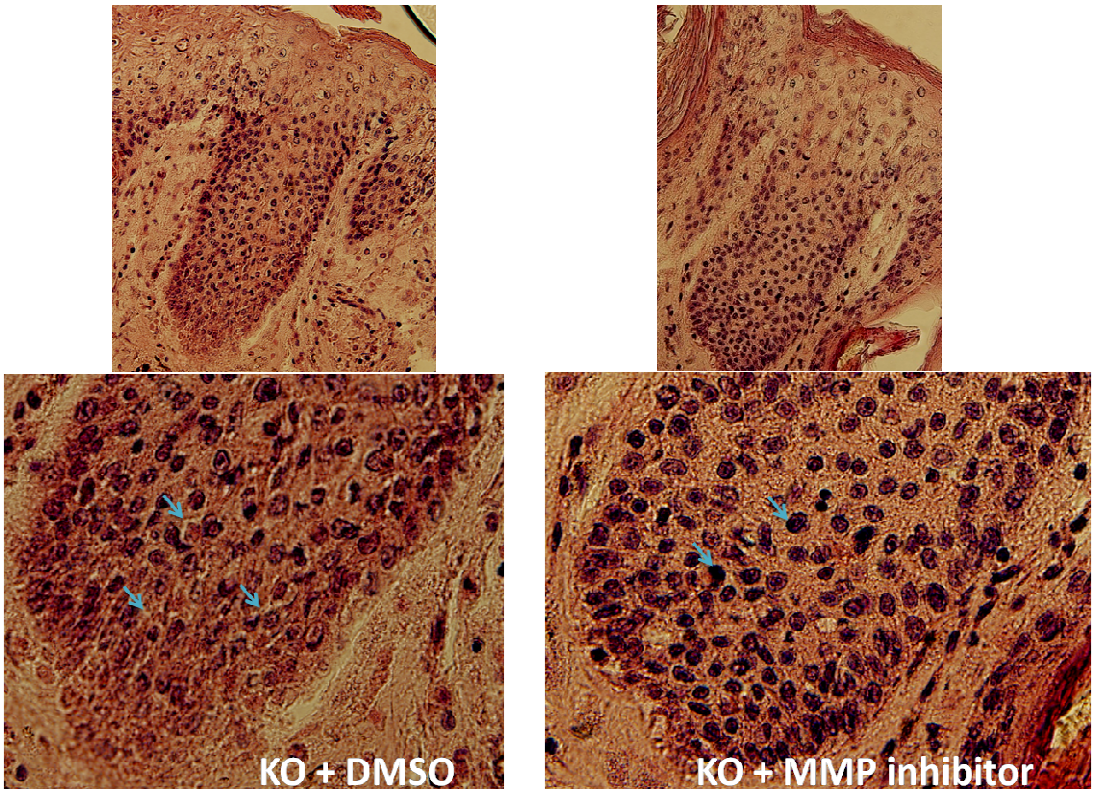



**Figure 13:** Blocking of MMPs on caspase-8 KO skin reduces spongiotic phenotype. Hematoxylin and Eosin staining of caspase-8 knockout skin sections at 40x magnification (top panels) and 100x magnification (bottom panels). Blue arrows indicate epidermal gap comparison. Skin explants were extracted from a 5 month old caspase-8 KO mouse. The extracted skin was place in cell culture dish with DMEM (Dulbecco's modified Eagle's medium) with FBS (Fetal bovine serum). Explants were treated with either vehicle control, DMSO (left panels) or a pan- MMP inhibitor (right panels) for 48 hours.

**Acknowledgement**

This chapter, in part, is being prepared for publication of the material as it may appear as Li, Christopher K., Jamora, Colin, 2009. The thesis author was the primary investigator and author of this paper.

# Discussion

My studies reveal that the epidermal deletion of caspase-8 KO is an attractive model for human atopic dermatitis. Comparison of the pathology (Table 1), gross phenotype (Figure 2) and genetic expression (Figures 7 and 9) of the caspase-8 KO mouse model to the established characteristics of atopic dermatitis, demonstrates a strong correlation between the disease and the mutant mouse phenotype.

Clinical atopic dermatitis patients feature scaling and spongiosis in the skin. Gross examination of our caspase-8 KO mouse model reveals similar scaling defects in the skin starting as early as 5 days after birth (Figure 2). Using histological analysis, we observed that the expanded epidermis in the knockout animal also exhibited a spongiotic phenotype. This spongiotic phenotype was observed as gaps between epidermal cells in our caspase-8 KO mice model, suggesting a disruption of barrier integrity that also occurs in atopic dermatitis (Figure 10a, c, Table 1). Studies have established that an altered skin barrier with increased trans-epidermal water loss in the skin is a common symptom of AD patients (Allam et al., 2005). This skin barrier dysfunction in chronic AD is thought to be the mechanism by which antigens, bacteria and viruses penetrate into the immunocompetent cells in the epidermis and the dermis and exacerbates the severity of the disease (Maintz and Novak, 2007). In the caspase-8 KO model, trans-epidermal water loss was greatly increased when compared to the WT, revealing water loss in the KO skin is a shared characteristic with human AD patients (Figure 10).

In addition to the trans-epidermal water loss observed in AD patients, there has also been a report done that reveals filaggrin gene variants may play an important risk factors in severe AD cases (Weidinger et al., 2006). Filaggrin is crucial for maintaining normal barrier function in the upper layers of the skin (granular layer) by orchestrating the development of the cornified envelope. I therefore tested whether the caspase-8

KO model has any perturbation in filaggrin expression. In the phenotypic epidermis, there is a noticeable decrease of filaggrin expression (Figure 9a) and pro-filaggrin protein levels were decreased in the KO compared to the wild type skin (Figure 9b). This decrease of filaggrin expression in the caspase-8 KO is consistent with previous findings that AD patients have a deficiency of filaggrin peptides (Brown and McLean, 2009; Palmer et al., 2006). The overall decrease of filaggrin protein level in the KO is most likely an underestimate due to the mosaicism of the phenotype in the caspase-8 KO.

Keratinocytes can play an important role in both innate and adaptive immunity. Keratinocytes have the ability to initiate an innate immune response by inducing a host defense program via NFκB (Kaufman and Fuchs, 2000). NFκB can induce the activation and expression of inflammatory cytokines in cells of the epidermis as well as in dermal fibroblasts. In addition, keratinocytes have the ability to induce adaptive immune response by differentiating circulating T-cells into active Th1 and Th2 cells (Figure 5). One such gene that is highly expressed in patients with AD is TSLP (Thymic stromal lymphopoietin), which resides in the keratinocytes of the epidermis and have the ability to activate resident dendritic cells of the skin. This activation of dendritic cells allows the recruitment and release of chemokines and cytokines such as IL-5, IL-13 and CCL17 to the skin. These active chemokines/cytokines bind their respective receptors such as CCR4-Th2 lymphocytes and aids the conversion of innate T-cells to Th2 cells (Figure 5). My findings demonstrate that the loss of epidermal caspase-8 induces a similar immune response - TSLP, IL-5, IL-13 and CCL17 gene expression levels were increased in the KO when compared to the WT, suggesting that the caspase-8 KO models exhibits a similar chemokine/cytokine response as AD patients (Figure 7a, c).

A characteristic infiltration of leukocytes occurs at the site of infection in AD patients. The loss of epidermal caspase-8 results in a similar immune cell infiltration. There is an increased immune cells expression such as macrophages, mast cells and T-cells in the caspase-8 KO model (Figure 6) (Lee et al., 2009). Chemokines that play a role in the recruitment of these immune cells are CCL5/RANTES (regulated upon activation, normally T cell expressed and secreted), CCL11/Eotaxin, CCL17/TARC (Thymus and activation regulated chemokines), and CCL22/MDC (macrophage-derived chemokines), all of which are unregulated in our caspase-8 KO model (Figure 7c) (Lee et al., 2009).

Interestingly, my analysis of the caspase-8 KO model suggests that both acute and chronic responses are present in the skin. mRNA expressions of chemokines and cytokines for both acute/Th2 and chronic/Th1 phases are elevated in the KO sample (Figure 7). While the deletion of epidermal caspase-8 leads to immune cell infiltration in the skin and an acute response, the chronic response might be initiated by the pruritus (itch) from the histamine that the masts cell produces. (Maintz and Novak, 2007; Novak et al., 2004) This scratching behavior of the mice may be one of the environmental triggers that the chronic response of AD (Figure 5) (Maintz and Novak, 2007) (Leung et al., 2004).

Asides from elevated immune cell infiltration and increased expression of cytokines/chemokines, AD also features increased levels of immunoglobulin in blood circulation. AD patients as well as other mouse models commonly feature elevated levels of IgE and IgG1s. The primary cause of immunoglobulin elevation is gene products of chemokines and cytokines such as CCL5, IL-4, IL-13 and IL-18. These gene products have the ability to stimulate Ig synthesis via mast cells and increase modification of B-cell (lymphocytes) to produce antibodies (Maintz and Novak, 2007).

My current findings and analysis of the caspase-8 KO model reveals an overall increase of IgG1 levels in the KO (Figure 8). Despite the mouse having no difference of IgG1 levels at postnatal day 10, there is a remarkable difference as the mice ages to 3 months and 5 months. This phenomenon may be explained by the increasing severity of the phenotype that the mouse attains with age – as more immune cells are recruited and chemokines/cytokines released, more immunoglobulins may be produced.

The current dogma in the field of dermatology is that the spongiotic phenotype is the result of the accumulation of fluids in the skin (edema). Consistent with this, the trans-epidermal water loss assay and spongiotic-like phenotype in the expanded epidermis suggested that barrier integrity is compromised in the caspase-8 KO (Figure 10). Here we analyzed a potential mechanism of epidermal gap formation. Cell-cell adhesion is regulated by intercellular complexes known as adherens junctions. Our findings reveal a disruption of the adherens junction, specifically, E-cadherin was cleaved at the extracellular domain. Loss of the protein mediating intercellular adhesion may explain the increase trans-epidermal water loss and spongiotic phenotype (Figure 10c). I found that the underlying mechanism of E-cadherin shedding was through the cleavage of this protein by members of the MMP family of proteases. The ability to circumvent epidermal gap formation by blocking MMP-mediated shedding of E-cadherin suggests that edema is not the sole factor in the manifestation of the spongiotic phenotype. My current findings revealed a list of possible proteases that may involved in compromising intercellular adhesion and we are attempting to narrow down the list of possible candidate MMP proteins responsible for spongiosis in the caspase-8 KO mouse as well as human AD patients.

Work is still needed to verify the degree to which the caspase-8 KO is a model for atopic dermatitis. Among the outstanding questions that need to be resolved are the

levels of serum IgE in the wild type vs. caspase-8 KO mouse, expression of important cytokines such as IL-2, 12, 16, 31 and chemokines/receptors CCL2, 5, CXCL9, 11, CCR3, 4, 5. The detection of IgE levels is a crucial concept in AD should be addressed as a majority of AD patients (~80%) develop elevated IgE levels in their blood circulation. The elevated levels of IgE may be important to facilitate AD progression due to the presence of high affinity receptors for IgE found on Langerhans cells (LC) and inflammatory dendritic epidermal cells (IDEC) (Allam et al., 2005). My model proposes a similar expression of LC and IDEC in the caspase-8 KO mice and I would expect a similar elevated level of IgE in the blood serum.

Further profiling of the cytokines present in the caspase-8 KO mouse will further establish the validity of the mutant mouse as a model for human AD. Important regulatory cytokines that should be investigated include IL -2, 12, 16, 31, CCL2, CXCL9, 11. These cytokines and chemokines mediate leukocyte activation by increasing the affinity for ligands on leukocyte receptors. This activation ultimately plays a major role in the immune response of AD (Allam et al., 2005). Lastly, filaggrin mutation is not the only predisposing factor for AD, there are also many other gene polymorphism that leads to similar atopic inflammatory diseases. One of such polymorphism is the SPINK5 mutation (Walley et al., 2001). SPINK5 (Serine peptidase inhibitor, Kazal type 5) is a gene that encodes a serine protease inhibitor which plays a role in skin/hair morphogenesis. A mutation in this gene was found to cause Netherton syndrome, a form of ichthyosis diseases which features atopy (hypersensitivity) and dry, scaly skin similar to AD. Understanding the pathology of this closely associated gene could lead to new insights for current and future atopic dermatitis models like epidermal deletion of caspase-8.

**Acknowledgement**

This chapter, in part, is being prepared for publication of the material as it may appear as Li, Christopher K., Jamora, Colin, 2009. The thesis author was the primary investigator and author of this paper.

# Materials and Methods

## Reagents

Primary antibodies used were against: E-cadherin (M. Takeichi, Kyoto University, Japan); β-actin, β-catenin (Sigma, St. Louis, Missouri, United States), and K5 (Jamora Lab). MMP inhibitor used were from Calbiochem GM 6001 (Cat #364205). Dilutions were according to the manufacturer's recommendation.

## Mice

Generation of caspase 8 conditional knockout mice. Epidermal-specific knockouts were obtained by crossing mice carrying the floxed caspase 8 allele (Casp8fl/fl)4 to K14-Cre mice3 (Lee et al., 2009).

## Western blot

Protein extracts from skin tissue: Frozen tissue was pulverized in a liquid nitrogen-cooled Gevebesmascher and the powder scraped into a chilled microcentrifuge tube. RIPA buffer (1% Triton X-100 in PBS with 10 mM EDTA, 150 mN NaCl, 1% sodium deoxycholate, and 0.1% SDS) and protease inhibitors or Laemmli buffer was added. The cell suspension was sonicated three times for 15 s and centrifuged at 14,000 rpm at 4 ℃. The supernatant was separated from the pellet and used in the experiments. Samples were run on SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell Bioscience, Keene, New Hampshire, United States). Western blot signals were developed using the enhanced chemiluminescence solution generated in the lab.

**Immunofluorescence and immunohistochemistry**

Mouse skin isolated from 5days/10 days/5 months old wild-type and knockout animals were either frozen in OCT (Tissue-Tek) or fixed overnight in Bouin's fixative and embedded in paraffin, depending on the application. 5 days old skin paraffin sections were prepared for the staining of hematoxylin and eosin. Epidermal differentiation markers K5, and filaggrin were stained on 10 days old in 8-μm frozen sections after the tissues were fixed for 10 min in 4% paraformaldehyde. Immunofluorescence was detected using rhodamine-X or FITC-conjugated secondary antibodies (Jackson Immunoresearch). Toluidine blue staining were performed on 5 month old skin in 8-μm frozen sections. 1% Toluidine Blue Stock Solution was prepared from Toluidine blue O (Sigma) in 70% ethanol; working solution was a mixture of 5mL Toluidine blue stock solution in 45mL of 1% sodium chloride. OCT tissues were fixed at 10 min in 4% PF, washed in water and stained in toluidine blue working solution for 3 minutes, rinse with 3 changes of water. All sections were coversliped with mounting medium.

**Skin explants**

Whole skin explants were extracted from a 5 month old caspase-8 KO or wild type mice. The extracted skin was cultured in DMEM with 10% FBS (HyClone). Samples were treated with inhibitor for 48 hours at 37$^{o}$C. Skin sections were prepared via Bouin's fixation and embedded in paraffin and prepared for H&E staining as previously described (Lee et al, 2009).

**Reverse Transcriptase PCR (RT-PCR)**

Total RNA was extracted from whole skin of wild-type and knockout mice at P10 using Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNA was

synthesized by reverse transcription using oligo-dT as primers ( Superscript III kit, Invitrogen). The primer pairs used for Reverse transcriptase PCR are listed below.

**Real Time PCR (qPCR)**

Total RNA was extracted from whole skin of wild-type and knockout mice at P10 using Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesized by reverse transcription using oligo-dT as primers ( Superscript III kit, Invitrogen). Real-time PCR analysis was performed with primer pairs listed below using a Stratagene Mx-3000 system. Reactions were performed using the Brilliant SYBR Green QPCR reagent mix (Stratagene) and experiments were carried out in duplicates. Data shown is a representative example. The specificity of reactions was determined by dissociation curve analysis and quantification analysis was performed using the Mx-3000 software.

**Primer Pairs**

**Table 2:** Primer pairs

| | |
|---|---|
| GAPDH(RT-PCR)for | CGTAGACAAAATGGTGAAGGTCGG |
| GAPDH(RT-PCR)rev | AAGCAGTTGGTGGTGCAGGATG |
| mADAM3(RT-PCR)for | TAACAAAGAGAATTCTGAGGATAAAGA |
| mADAM3(RT-PCR)rev | AAAGCCCTCCACAGCTAACGTCTTTGG |
| mMMP9(RT-PCR)for | GGACAGCCAGACACTAAAGGCC |
| mMMP9(RT-PCR)rev | AAACCCCACTTCTTGTCAGT |
| mMMP10(RT-PCR)for | AGGGACCAACTTATTCCTGG |
| mMMP10(RT-PCR)rev | CAG TAT GTG TGT CAC CGT CC |

**Table 2:** Primer pairs - continued

| | |
|---|---|
| mMMP1(RT-PCR)for | TGAACATCCATCCCGTGACC |
| mMMP1(RT-PCR)rev | ATCTCCACCCCACCCCCACC |
| m t-PA(RT-PCR)for | GGGAGGTTCAGAAGAGGAGCCCGG |
| m t-PA(RT-PCR)rev | GCGTTTCCCTACAAATCCATCAGGG |
| m u-PA(RT-PCR)for | TGCCCAAGGAAATTCCAGGG |
| m u-PA(RT-PCR)rev | CAATCTGCACATAGCACCAG |
| mMMP12(RT-PCR)for | ACAGCATCTTAGAGCAGTGC |
| mMMP12(RT-PCR)rev | ATGAGGCAGAAACGTGGACT |
| mMMP19(RT-PCR)for | GCCCATTTCCGGTCAGATG |
| mMMP19(RT-PCR)rev | CAAAGTGTATACTCCCAAGTTC |
| mADAM10(RT-PCR)for | GCCAGCCTATCTGTGGAAACGGG |
| mADAM10(RT-PCR)rev | TTAGCGTCGCATGTGTCCCATTTG |
| mMMP13(RT-PCR)for | CATCCATCCCGTGACCTTAT |
| mMMP13(RT-PCR)rev | GCATGACTCTCACAATGCCA |
| mMMP2(RT-PCR)for | CTCTGCGTCCTGTGCTGCCTGTTG |
| mMMP2(RT-PCR)rev | AAAGTGAGAATCTCCCCCAACACC |
| mTIMP1(RT-PCR)for | GTGGGAAATGCCGCAGATATC |
| mTIMP1(RT-PCR)rev | GACCTGATCCGTCCACAAAC |
| mMMP8(RT-PCR)for | GTTACCCCAAAAGCATACCAAGCA |
| mMMP8(RT-PCR)rev | AAACGGCTTGCTCTCTTTGTGATG |
| mMMP3(RT-PCR)for | CAGACTTGTCCCGTTTCCAT |
| mMMP3(RT-PCR)rev | GGTGCTGACTGCATCAAAGA |
| mADAM33(RT-PCR)for | CCACTACAGGCCAGATGGGCAT |

**Table 2:** Primer pairs - continued

| | |
|---|---|
| mADAM33(RT-PCR)rev | GAGAATCTGGTCCACGCAAT |
| mADAM12(RT-PCR)for | CTTGACTGTAGGAATCCTGG |
| mADAM12(RT-PCR)rev | CTCACCAAGGCACTAGTGAG |
| mADAM9(RT-PCR)for | GTGTGTACTCAGTTATTGGC |
| mADAM9(RT-PCR)rev | TCCCAGAGACCAGTATAATT |
| mADAM17(RT-PCR)for | CACTTTTGGGAAGTTTCTGG |
| mADAM17(RT-PCR)rev | CTCTGTCTCTTTGCTGTCAAC |
| mADAM19(RT-PCR)for | TCGGGAGCAGGTTCGGC |
| mADAM19(RT-PCR)rev | CCCTGGGACTGCACTTCC |
| cathepsinE for(qPCR) | TCGAGTGTCAATGAACCCCTC |
| cathepsinE rev(qPCR) | GGTGCAGTACACAGAAGGGAC |
| CCL11 for(qPCR) | GCT TTC AGG GTG CAT CTG TT |
| CCL11 rev(qPCR) | CAC GGT CAC TTC CTT CAC CT |
| CCL17 for(qPCR) | GCT CGA GCC ACC AAT GTA GG |
| CCL17 rev(qPCR) | ACA CGA TGG CAT CCC TGG A |
| CCL22 for(qPCR) | TGG TGC CAA TGT GGA AGA CA |
| CCL22 rev(qPCR) | GGC AGG ATT TTG AGG TCC AGA |
| CCL24 for(qPCR) | GAC CAT CCC CTC ATC TTG CTG |
| CCL24 rev(qPCR) | GGT GAT GAA GAT GAC CCC TGC |
| CCL27 for(qPCR) | CAT GGA ACT GCA GGA GGC TG |
| CCL27 rev(qPCR) | TTG GCG TTC TAA CCA CCG AG |
| CXCL10 for(qPCR) | ACC CAA GTG CTG CCG TCA T |
| CXCL10 rev(qPCR) | CAT TCT CAC TGG CCC GTC AT |

**Table 2:** Primer pairs - continued

| | |
|---|---|
| CXCR3 for(qPCR) | TGA GCA GCA CGG ACA CCT TC |
| CXCR3 rev(qPCR) | AAC CCA CTG GAC AGC AGC AT |
| IFNgamma for(qPCR) | ACC CTG TCG TAT GCT GGG AA |
| IFNgamma rev(qPCR) | GTT GGT GCA GGA ATC AGT CCA |
| IL13 for(qPCR) | GACCAGACTCCCCTGTGCAA |
| IL13 rev(qPCR) | TGGGTCCTGTAGATGGCATTG |
| IL18 for(qPCR) | ACGTGTTCCAGGACACAACA |
| IL18 rev(qPCR) | ACAAACCCTCCCCACCTAAC |
| IL31for(qPCR) | GAC GAA TCA ATA CAG CTG CCG |
| IL31 rev(qPCR) | GGT TAA TGC TTC CCG GTC CA |
| IL33 for(qPCR) | TCC TTG CTT GGC AGT ATC CA |
| IL33 rev(qPCR) | TGC TCA ATG TGT CAA CAG ACG |
| IL4 for(qPCR) | CGGAGATGGATGTGCCAAAC |
| IL4 rev(qPCR) | GCACCTTGGAAGCCCTACAG |
| IL5 for(qPCR) | TCACCGAGCTCTGTTGACAA |
| IL5 rev(qPCR) | CCACACTTCTCTTTTTGGCG |
| PPARalpha for(qPCR) | ATG CCA GTA CTG CCG TTT TC |
| PPARalpha rev(qPCR) | CCG AAT CTT TCA GGT CGT GT |
| TGF for(qPCR) | TAC TCT GGA GAC GGT TTG CCA |
| TGF rev(qPCR) | CAT GAA GAA AGT CTC GCC CG |
| TSLP for(qPCR) | TAT CCC TGG CTG CCC TTC A |
| TSLP rev(qPCR) | TGT GCC ATT TCC TGA GTA CCG |

**ELISA/Isotyping**

Whole blood was collected from mice at age 10 days old, 3 months old and 5 months old mice via cheek-bleeding method or drawn from heart. Serum was separated from whole blood by centrifuging at max at 14,000 rpm for 10 minutes and taking the supernatant. Serum was analyzed via ELISA Clonotyping kit from SouthernBiotech (Cat #5300-04) using IgG1 purified antibody standards (SouthernBiotech Cat #0102-01). Dilutions of the serum varies depends on mice and protocol was followed as instructed from the Clonotyping kit.

**TEWL (trans-epidermal water loss) assay**

We determined TEWL and stratum corneum conductance by biophysical methods to examine whether our patients exhibited changes in barrier function and stratum corneum hydration(Hashimoto-Kumasaka et al., 1993; Pinnagoda et al., 1990). Skin hydration and TEWL were measured noninvasively with a Tewameter TM210 (Courage & Khazaka, Cologne, Germany) in lesional and healthy mouse skin.

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