INTRINSIC ALTERATIONS OF THE SKIN BARRIER FUNCTION PROMOTE THE PATHOGENESIS OF
ATOPIC DERMATITIS IN DOGS

BY

DOMENICO SANTORO

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in VMS - Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Professor Karen L. Campbell, Chair
Professor Mariangela Segre, Director of Research
Professor David Bunick
Professor Thomas K. Graves
Professor Rosanna Marsella
Associate Professor Richard I. Tapping
Professor Federico Zuckermann
ABSTRACT

Canine atopic dermatitis (cAD) is a chronic inflammatory skin disease affecting up to 10% of the canine population, it is the second most common allergic disease in dogs. Canine AD has many similarities to human AD and for this reason the dog has been suggested to be a good animal model to study AD in people. Like people, dogs with AD have a high susceptibility to cutaneous secondary infections. In the past decades, it has been suggested that the reason for the high incidence of cutaneous infection in atopic people is due to defects in the skin barrier function such as deficiency of keratinocyte-secreted natural antimicrobial peptides (AMPs) and filaggrin.

Antimicrobial peptides are small proteins secreted by many mammalian epithelial cells. Their main functions include: defense against external pathogens, orchestration of the innate and adaptive immunity, and regulation of the normal local homeostasis.

Filaggrin is a structural protein present in keratinized epithelia. Its main functions are flattening of keratinocytes, forming a strong barrier against external agents, and producing natural moisturizing factors that lower cutaneous pH and increase skin hydration.

Our studies have been focusing on the alterations of AMPs and filaggrin in the skin of atopic dogs to better understand the function that such proteins play in the pathogenesis of cAD. Our studies have shown that both AMPs and filaggrin mRNA expressions are increased in canine atopic skin when compared with healthy controls. However, this increase in mRNA transcription is not followed by an increase in protein expression suggesting that translational defects occur in atopic dogs. This alteration in expression could be due to intrinsic or extrinsic
alterations that may lead an abnormal immunological response to common environmental stimuli such as house dust mites.

Based on such hypotheses we isolated canine keratinocytes from healthy and atopic dogs and exposed them to several immunological stimuli (cytokines, bacterial antigens, house dust mite extract, and vitamin D₃) to mimic the milieu present in inflamed skin. We were able to show that keratinocytes harvested from atopic dogs behave differently from keratinocytes harvested from healthy dogs. In particular, the former express a higher production of some AMPs (cBD3-like) at baseline, but they are not able to increase such production following stimulation with several immunostimulators. These results suggest that the lack of positive stimulation of some AMPs could have a role in the high incidence of recurrent cutaneous infection in atopic dogs.

Our studies also demonstrated that canine AMPs are able to effectively and rapidly kill the most common cutaneous canine pathogens at variable concentrations. The data showed that different AMPs have different affinity against microorganisms. Canine AMPs were more effective in killing bacteria more commonly involved in canine skin infections (Staphylococcus pseudintermedius and Malassezia pachydermatis) rather than pathogens involved in human skin diseases (Staphylococcus aureus and Candida albicans).

In conclusion, we demonstrated that an altered expression of two markers of the skin barrier function analyzed, AMPs and filaggrin, is present in canine atopic skin. Our results suggest that such alterations may be the results of intrinsic abnormal cellular mechanisms that do not allow the “atopic” keratinocyte to respond promptly to external stimuli. This intrinsic defect of the skin barrier function may play a major role in the increased incidence of
cutaneous infections present in atopic patients and contribute to the severity of allergic disease.
To my family for all their support and love

To my parents for all their love, teachings, and motivation

To Cindy for her love, support, and for teaching me to never give up

To my friends for their support, and for being there when I needed them
ACKNOWLEDGMENTS

I would like to thank my family, my friends, and my colleagues who stimulated and supported me over the past several years of my career. Without them this series of projects would not have been possible. Their involvement made me a better scientist, a better veterinarian, and most importantly a better human being. I would like to thank my research advisor, Mariangela Segre, for welcoming me in her laboratory and for all the time and help that she provided me with over the past three years. I would also like to thank my committee chair, Karen Campbell, for the time and effort she provided to help me succeed in completing my graduate program. In addition, I would especially like to thank Rosanna Marsella and Thierry Olivry for their friendship, moral support, scientific insights and suggestions regarding interpretation of data and experimental troubleshooting. I would also like to thank David Bunick, Carol Maddox, and Tom Graves for allowing me the opportunity to work in their laboratories, for their continuous moral support, and scientific suggestions for experimental troubleshooting. I would also like to thank the other two members of my committee, Richard Tapping and Federeico Zuckermann, for their continuous support and suggestions on how to improve my studies.

I am also very grateful for the Brasley fellowship which allowed my participation in and completion of the graduate program here at the University of Illinois.

Finally, I would like to thank the many clinicians, faculty, technicians, and veterinary students who have made my experience at the University of Illinois – College of Veterinary Medicine a wonderful and rewarding experience.
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CHAPTER 1

INTRODUCTION

Over the past decades, the recognition of the skin as an active component of the immune system has triggered the remarkable interaction between two branches of the veterinary medicine: dermatology and immunology. Such interaction has brought about an outstanding advancement of knowledge on the etiopathogenetic mechanisms of several immune-mediated disorders in animals. Of those, probably the best examples are allergic diseases, like atopic dermatitis (AD), and autoimmune diseases, like pemphigus foliaceus.

The skin is the first physical, chemical, and immunological barrier between the milieu interieur and the external environment. Furthermore, the skin acts as a biological laboratory where the biochemical and immunological interactions necessary to guarantee internal homeostasis occur. The physical protection provided to the body by the skin, is primarily a function of the epithelial cells (keratinocytes) held together by desmosomes and tight junctions. The latter act as mechanical seals against penetration by elements from the external environment such as pathogens and allergens. When this barrier is breached, infections and hyper-stimulation of the immune-system may occur. The chemical barrier is formed by the secretion of chemical substances which act as natural antimicrobials. Such molecules, covering and/or actively secreted by keratinocytes, include enzymes such as lysozyme and phospholipase A present in the saliva, fatty acids, cholesterol, and ceramides secreted in the sebum or by keratinocytes, and antimicrobial peptides like β-defensins (BDs), cathelicidin (Cath), dermcidin and S100 proteins secreted by keratinocytes. Along with these molecules,
other small peptides have been recognized as having indirect antimicrobial action. An example of the latter are peptides derived from the degradation of the structural protein filaggrin. The degradation products of filaggrin (trans-urocanic and pyrrolidone carboxylic acid) lower the cutaneous pH and represent the major portion of natural moisturizing factors produced within the stratum corneum (Rawlings AV, et al. 2004). Finally, the immunological component of the skin is composed of actively secreted peptides such as BDs and Cath which have chemotactic and immunostimulatory effects, a myriad of cytokines and chemokines, and also by the presence of immune cells such as Langerhans’ and dendritic cells, lymphocytes, and keratinocytes.

This triade of defense mechanisms does not always work properly. In allergic patients, innocuous substances penetrate the physical barrier and stimulate an excessive immune-reaction triggering the consequent secretion of small proteins (alarmins) and cytokines which stimulate and exacerbate an inflammatory reaction (Reedy LM, et al. 1997; Scott DW, et al. 2001). The incidence of allergic conditions has been greatly increasing over the past decades making AD the most common allergic disorder in people and the second most common allergy in dogs living in temperate regions, second only to flea bite hypersensitivity (Chalmers SA, et al. 1994; Scott DW, et al. 2001).

Atopic dermatitis is a severe, chronic disease with the potential to significantly decrease the quality of life of people and animals due to constant itchiness and recurrent skin infections. As in people, allergic canine patients are also at an increased risk for skin infections, which represent one of the most important trigger factors for pruritus in AD. Furthermore, the high prevalence of bacterial infections in atopic patients has been leading physicians and
veterinarians to an increased and more prolonged use of antimicrobials. It is possible that this elevated use of antimicrobials has contributed to the recently observed increased incidence of drug resistant bacterial strains worldwide.

Recently abnormal levels of naturally occurring antimicrobial peptides (AMPs), for example BD and Cath, have been suspected to be a root cause of the high incidence of bacterial infection in atopic people. When atopic patients were compared to patients affected by psoriasis a significant reduction in AMP production was measured. Psoriasis is an immune-mediated disorder characterized by a T helper cell (Th) type 1 disorder. This is different from AD which is a classic Th2–characterized disorder in people. These observations motivated many researchers to study the role of AMPs in AD, producing controversial results. The data indicated that some AMPs have a lower expression in the skin of atopic people when compared to healthy controls. However, other AMPs have an increased or an equally expressed level of expression. Based on those studies, the most supported hypothesis was that a particular immunological local environment, Th2 predominant (IL-4 and IL-13), inhibits the production of AMPs as opposed to a more Th1 predominant (TNF-α, IL-1, and IL-12) environment that stimulates them (Howell MD, et al. 2006; Kanda N & Watanabe S 2008).

In dogs, the study of AMPs is only beginning. There is only one study which investigated the possible relationship between some BDs and atopic dogs (Van Damme CM, et al. 2009). The first canine BDs (cBDs) were identified in the canine genome and synthesized for the first time in canine tissue extracts in 2005 (Sang Y, et al. 2005). In 2007 the first and only Cath was identified and synthesized in canine tissue extracts (cCath) (Sang Y, et al. 2007). Only more recently have several cBDs and cCath been confirmed and identified in canine skin (Van Damme
CM, et al. 2009; Wingate KV, et al. 2009; Santoro D, et al. 2011). However, no studies have analyzed the relationship between AMPs and the immunological milieu in dogs (Th1 vs. Th2).

The work described in this dissertation addresses several questions regarding the relationship between defects in the skin barrier and the exacerbation of AD in dogs as well as the microbiological activity of AMPs. We designed a series of experiments to evaluate two important markers of the skin barrier in dogs, AMPs (cBDs and cCath) and filaggrin. The goals of these studies was threefold: 1) Analyze the expression of cAMPs and filaggrin in vivo and ex vivo using molecular biology techniques (PCR) and/or protein evaluation (ELISA). 2) Evaluate the antimicrobial properties of cAMPs. 3) Characterize any differences between keratinocytes harvested from healthy beagles and atopic beagles. After we demonstrated the presence of AMPs and filaggrin in the skin of healthy dogs, we used a colony of experimentally-induced atopic beagles to study the possible difference in expression and distribution of these markers in cAD. The colony of atopic beagles, used for these studies, has been previously observed and documented to develop clinically, histologically, and immunohistochemically lesions similar to naturally occurring canine AD after environmental challenge with house dust mite (HDM) (Marsella R, et al. 2006; Marsella R & Girolomoni G 2009). We also analyzed the expression of AMPs in naturally-affected atopic dogs to confirm the data obtained by using the canine model. We also isolated keratinocytes from healthy and atopic beagles and tested the production of AMPs in primary keratinocytes cell cultures. This last experiment was performed to evaluate if keratinocytes isolated from atopic beagles, and expanded in culture, will exhibit differences in AMP production. Additionally, in order to demonstrate that keratinocytes isolated from atopic dogs do behave differently from keratinocytes isolated from healthy dogs, we tested each cell
culture for several markers including, but not limited to, cytokine production pre and post pro-inflammatory stimulation.
2.1 Atopic dermatitis

Canine atopic dermatitis (cAD) is defined as “a genetically-predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features, most commonly associated with Immunoglobulin (Ig)E antibodies to environmental allergens” (Olivry T, et al. 2001; Halliwell REW. 2006). cAD is one of the most common allergies in dogs; it affects up to 10% of the canine population (Bevier DE. 1990; Reedy LM, et al. 1997; Scott DW, et al. 2001; Hillier A, et al. 2001). Several risk factors have been associated with cAD. These include breed, age, season of the year when born, geographic location, presence of endoparasites, viral and bacterial infections, and type of vaccination administered. (Van Stee EW. 1983; Frick OL, et al. 1983; Halliwell REW, 1990; Hill PB & DeBoer DJ. 2001; Halliwell RE & DeBoer DJ. 2001; Sousa CA & Marsella R. 2001; Hillier A & Griffin CE. 2001; Scott DW, et al. 2001). Age of onset varies between four months and seven years. However, more than 70% of the atopic canine population develops AD between one and three years of age. There are few exceptions which have been reported with Akita, Chow chow, Golden Retriever, Shar pei and West Highland white terrier in which cAD can develop as early as two to four months of age (Scott DW, et al. 1995; White SD & Bourdeau P. 1995; Scott DW, et al. 2001).

It has been proposed, in both people and dogs that both an “extrinsic form” of AD and an “intrinsic form” of AD (or atopic-like cAD) exist (Halliwell R. 2006). It is impossible to differentiate between the two forms based solely on clinical signs (Halliwell R. 2006). However,
clinical signs associated with the “extrinsic form” of AD are associated with detectable circulating specific IgE as well as positive intradermal testing (Halliwell R. 2006). With the “intrinsic form” of AD, no specific IgE or intradermal test reactions are detectable (Halliwell R. 2006). Recently it has been reported that the “intrinsic form” of AD (atopic-like AD) is present in 25.6% of the atopic canine population, with a higher incidence in specific breeds (French Bulldog) and less responsiveness to immunomodulating drugs commonly used in the management of cAD (cyclosporine) (Prelaud P & Cochet-Faivre N. 2007).

Clinically cAD is characterized by chronic, generalized pruritus and papules associated with secondary skin lesions including excoriations, erythema and scaling. Bilateral, chronic otitis externa is present in 86% of dogs with AD, while up to 60% of atopic dogs can be affected by associated ocular disease (conjunctival hyperemia, ocular pruritus, chemosis, ocular discharge, epiphora, corneal involvement) (Scott DW, et al. 2001; Lourenco-Martins AM, et al. 2011). The majority of atopic dogs also develop superficial pyoderma and/or Malassezia dermatitis characterized by pustules, crusts, seborrhea, hyperpigmentation, and lichenification. The body areas mainly affected include muzzle, interdigital spaces (front paws more affected than the rear paws), ears, axillae, inguinal area, antibrachial flexor areas, as well as flexor areas of both carpi and tarsi. The clinical signs are most commonly non seasonal in appearance, with only 20-30% of dogs showing a seasonal onset of the disease. In addition, contrary to people, cAD tends to get worse with age and if characterized by a seasonal onset, to become non-seasonal due to the sensitization to multiple other allergens (Scott DW. 1981; Scott DW, et al. 1995; Reedy LM, et al. 1997; Chandoga P, et al. 1998; Scott DW, et al. 2001).
cAD is very commonly associated with other allergies such as food allergy (FA) and/or flea allergy dermatitis (FAD). Although cAD and FAD are ease to differentiate, cAD and FA present the same clinical signs with only few slight differences (Favrot C, et al. 2010; Bruet V, et al. 2012). For this reason, and because no accurate allergy tests are available to diagnose AD, it is fundamental to rule out all other pruritic diseases in order to confirm AD in canine patients.

Today there are many therapeutic options are available to treat AD in dogs. Therapeutic options include immunomodulating drugs, antimicrobial treatments, and the use of topical moisturizers. The most common immunomodulating drugs used include low potency glucocorticoids (e.g. prednisone, prednisolone and methylprednisolone), calcineurin inhibitors (e.g. cyclosporine and tacrolimus), as well as allergen specific immunotherapy. The latter is generally based on intradermal testing and/or allergen-specific serum IgE for the identification of allergens to include in the vaccine. Antimicrobial treatments are critical to reduce the presence of microorganisms (e.g. *Staphylococcus pseudintermedius* and *Malassezia pachydermatis*). These microbes chronically stimulate the immune system leading to a worsening of the allergic disease. Finally, the use of moisturizers is beneficial to repair the skin barrier and improve protection against the penetration of the epidermis by environmental allergens (see below “Pathogenesis of canine AD” for details).
2.1.1 Etiology

Clinical signs of cAD are due to an excessive immune response to normally innocuous allergens such as house dust mites (HDM), storage mites, pollens and molds (Hill PB & DeBoer DJ. 2001; Griffin CE & DeBoer DJ. 2001). Contrary to people in which allergens penetrate through the respiratory system (inhalant allergy), in dogs the most common site of entry is the skin (percutaneous penetration) (Mueller RS, et al. 2000). This explains the amelioration of the clinical symptoms in dogs that receive frequent baths. Other common “allergens” are the commensal cutaneous microflora as demonstrated by the detection of specific IgE against *Staphylococcus* spp. and *Malassezia* spp. Furthermore, it has been strongly suggested that there is an interaction between FA and AD in human and canine patients. Highly allergenic foods can exacerbate the clinical signs in atopic patients.

Many of the immunological and structural abnormalities associated with human AD have been postulated and/or observed to be present in dogs. Such abnormalities include an imbalance between the Th lymphocyte populations, an excessive cellular immune response, an increased cutaneous reactivity to histamine and substance P injection, increased histamine release, and a decreased response to β-adrenergic stimuli (Turner CR, et al. 1989; Nimmo Wilkie JS, et al. 1991; Emala CW, et al. 1996; Marsella R, et al. 2001). Additionally, there is an alteration of the skin barrier primarily observed and demonstrated in people with AD, but also strongly suggested in dogs with AD.
2.1.2 Pathogenesis

Research from the past decades shows that AD is a disease characterized by a defect in the skin barrier which leads to dry skin as well as IgE mediated sensitization to environmental allergens and food (Rahman S, et al. 2011). In people, AD is very common in children and less so in adults. Up to 85% of AD in children develops before the age of five years (Rahman S, et al. 2011). In children, early-onset AD (<5 years of age) is characterized by three phases: 1) “Non-AD phase” in which there is an absence of detectable IgE (lack of IgE sensitization). 2) Genetic predisposition to develop IgE against food and/or environmental allergens, present in two-thirds of the patients. 3) Skin barrier damage, exacerbated by scratching, that facilitate the release of autoantigens and “danger signals” (e.g. interleukins [ILs], chemokines, AMPs, etc.) perpetuating the allergic reaction (Rahman, S et al. 2011).

This chapter will summarize the most current hypotheses regarding the pathogenesis of AD in dogs and compare it with the information available regarding the human counterpart. The chapter is divided into three subchapters in which genetic, immunological, and skin barrier defects will be discussed.
2.1.2.1 Genetic alterations

People

Human AD is an inheritable disease in which the phenotype-specific gene alterations are associated with “generic” atopic genes (Larsen FS, et al. 1986; Boguniewicz M & Leung DYM. 2011). In families where one or both parents had AD, the risk for a biological child to develop AD was 3.4 times greater than for a child whose parents did not have a history of AD (Dold S, et al. 1992). The hypothesis of the presence of genetic alterations in AD has been reinforced by many studies which have analyzed the genetic background in AD patients. These studies have demonstrated several genetic alterations of AD patients. Genetic linkage to AD was found on chromosomes 1, 3, 4, 5, 11, 13, 15, 17, 18, 19, and 20; however only the locus 3p24 was found repeatedly altered (Boguniewicz M & Leung DYM. 2011). Similarly, using the genome-wide linkage technology, more than 81 genes were examined and only 46 had at least one positive association with AD, of these 46, only 23 (FLG, IL4, IL4RA, SPINK5, CMA1, IL13, IL5, IL12B, IL12RB1, SOCS3, RANTES, CD14, GATA3, DEFB1, GSTP1, IL18, NOD1, NOD2, TIM1, TLR2, BCL2A1, BDNF, and CSF2) were repeatedly associated with AD (Barnes KC. 2010). Of those the most important and strongly associated with AD is the gene encoding for filaggrin and the SPINK5 gene polymorphisms encoding for the lympho-epithelial kazal-type related inhibitor (LEKTI) (Walley AJ, et al. 2001). Filaggrin and LEKTI are proteins coded by the chromosome 1q21, also called epidermal differentiation complex (EDC) (Rahman S, et al. 2011). This complex also codes for the majority of proteins involved in the formation of the skin barrier and the cornified envelope (see below).
Alterations of filaggrin represent the strongest and most convincing genetic linkage between skin barrier dysfunction and AD. One of the most predominant predisposing factors for the disruption of the skin barrier in atopic patients is an alteration of the transcription/translation of the filament-aggregating protein, filaggrin (Wolf R & Wolf D. 2012; Kubo A, et al. 2012). Filaggrin is one of the most significant proteins involved in the development of the cornified envelope and, after its degradation, is involved in the formation of natural moisturizing factors (Nemes Z & Steinert PM. 1999). Many genetic studies analyzing different human populations (e.g. European, Chinese, Afro-American) have revealed up to 40 different semi-dominant, homozygous or compound heterozygous mutations present in patients affected by AD (Chen H, et al. 2011; Kawasaki H, et al. 2011). In particular, two null-loss-of-function mutations (R501X and 2282del4) have been identified, with 2282del4 being the most common (Lesiak A, et al. 2011). R501X and 2282del4 mutations are more common in patients affected by the extrinsic subtype of AD, in which allergen specific IgE levels are increased. This supports the hypothesis that a barrier defect leads to increased allergen penetration and ultimately more severe allergic sensitization (Weidinger S, et al. 2007; Tokura Y. 2010).

Filaggrin is not the only predisposing factor in AD development. Mutations on the filaggrin gene are present in only 15% of human AD patients, and are associated with the most severe form of AD (Henderson J, et al. 2008; Bieber T & Novak N. 2009). The different immunological environments present in extrinsic vs. intrinsic AD may play a role in the association between severity of AD and filaggrin mutations (Tokura Y. 2010). Intrinsic AD is not characterized by a Th2 predominant environment like extrinsic AD is (Tokura Y. 2010). This
hypothesis has been supported by a study in which normal keratinocyte cell cultures were exposed to Th2 cytokines (IL-4 and IL-13) and their effects on filaggrin production observed. This study found that filaggrin production was inhibited in normal keratinocyte cells after exposure to Th2 cytokines. This indicated that a local Th2 environment has the potential to decrease the expression of filaggrin leading to a disruption of the skin barrier, even in patients without filaggrin gene mutations (Howell MD, et al. 2007). In conclusion, genetic and immunological studies in people have suggested that immunologic and skin barrier-related genetic alterations in barrier function work synergistically in increasing the susceptibility to AD.

**Dogs**

cAD is very similar to its human counterpart. As previously mentioned, both diseases (cAD and human AD) are very complex and very common in the canine and human populations, respectively. A genetic component is present in both species. Similarly to people, atopic dogs also have a “family history” of AD which is considered a risk factor. In addition, there is a strong breed predisposition reported in dogs. There are some breeds in which up to 25% of dogs are affected by AD (e.g. West Highland white terrier) (DeBoer DJ & Hill PB. 2000; Picco F, et al. 2008; Wilhem S, et al. 2011). Furthermore, genetic studies performed in the United Kingdom showed that in Labrador and Golden Retriever the mean heritability is 0.47. This indicates that roughly half of risk of developing AD is due to a genetic background in these breeds (Shaw SC, et al. 2004). Even though there is evidence supporting a genetic component, the severity of the disease and its response to treatment is highly variable. This variability suggests that the cAD
phenotype is based on a complex genotype influenced by environmental conditions (Barnes KC. 2010).

Many studies have been performed to identify which genes may be responsible for AD. Many techniques have been used including genome linkage (Genome-wide linkage studies) and gene association studies (Genome-wide association studies, candidate gene association studies, quantitative reverse transcriptase PCR, and microarray studies). Of those techniques, the microarray studies have shown dysregulation of many genes involved in the inflammation, transport, and transcription pathways. The most consistently dysregulated is the gene encoding for the S100 calcium binding protein A8 (S100A8), a pro-inflammatory molecule located in the EDC (Nuttall T. 2013). The expression of S100A8 along with the cytokine tumor necrosis factor (TNF)-α have been correlated with the severity of AD (Nuttall TJ, et al. 2004; Chung T-H, et al. 2010).

S100A8 is coded by genes located in the EDC with the barrier function gene encoding for filaggrin, loricrin, and involucrin. However, the genes encoding such proteins were not available on microchips in the earlier genomic studies (Nuttall T. 2013). More recently such genes have been included in microarrays and the alteration of filaggrin has been associated with specific breeds in specific locations (Labrador retriever in the UK and small breed dogs [toy poodles, Shih Tzu and pugs] in Thailand) (Wood SH, et al. 2010; Suriyapholl G, et al. 2011), but not in West Highland white terrier (Barros Roque J, et al. 2009; Salzmann CA, et al. 2011). This suggests that the environment is influential on the canine genotype. As in people, also in dogs the inflammatory gene coding for the thymic stromal lymphopoietin (TSLP)-receptor has been associated with AD phenotype in different breeds (Liu YJ. 2006).
2.1.2.2 Immunological alterations

People

In past decades, the main hypotheses regarding the development of AD were based on the presence of several immunological abnormalities observed in patients with AD. Atopic skin is characterized by: increased number of eosinophils, increased activity of Langerhans’ and dendritic cells, infiltration of Th2 lymphocytes and mast cells (Bieber T. 2010; Gittler JK, et al. 2013), increased allergen-specific serum IgE, increased in Th2 cytokine production, increased number of T cells expressing the cutaneous lymphocyte-associated antigen (CLA) which lead T cells to invade the skin, increased expression of the high affinity receptor for IgE (FcεRI) on both Langerhans’ cells and inflammatory dendritic epidermal cells (IDECs) (Boguniewicz M & Leung DYM. 2011).

Cytokine alterations have been frequently demonstrated in patients with AD (Thepen T, et al. 1996). There is a clear biphasic response, characterized by an initial Th2 response in acute AD, followed by a shift to a more predominant Th1 response in chronic cases of AD. Th2 cytokines, such as IL-4 and IL-5, are fundamental to the recruitment of eosinophils and the stimulation of the production of IgE (Akdis M, et al. 2001). Different from Th2 cytokines, the Th1 cytokines (IL-2, IL-12, and Interferon [IFN]-γ) are fundamental for the maturation and activation of T cells as well as antigen presentation by antigen presenting cells (APC). This shift from a Th2 to a Th1 predominant phase is thought to be related to the presence of IDECs responsible for the production of cutaneous IL-12.

Along with the classic Th2 cytokines, such as IL-4 and IL-13, other interleukins have received more attention by researchers. In particular IL-17, along with TSLP and IL-22, has been
frequently associated with acute lesions in atopic patients. IL-17 is produced by Th17 and natural killer cells (NK-22 and Lymphoid tissue inducer [LTi] subtypes). The concentration of IL-17 present in the blood is highly expressed in patients with acute AD compared with chronic AD. Additionally, the presence of this interleukin is induced in the skin of both people and mice after allergen challenge or after exposure to staphylococcal enterotoxin B (Toda M, et al. 2003; He R, et al. 2007; Eyerich K, et al. 2009). In keratinocytes, IL-17 also stimulates the production of human β-defensins (hBDs), hBD2 in particular. The stimulation of hBD2 is counterbalanced by the presence of Th2 cytokines, in particular IL-4 and IL-13 (Boguniewicz M & Leung DYM. 2011).

The association between pruritus and skin levels of IL-31 has recently stimulated further interest in this cytokine. IL-31 is increased in the skin of atopic patients and is associated with the development of chronic AD (Dillon SR, et al. 2004; Sonkoly E, et al. 2006; Sun YG, et al. 2009). There is a correlation between IL-31 blood levels and the severity of AD. IL-31 is stimulated by Staphylococcal superantigens suggesting that bacterial skin colonization may contribute to the pruritus and inflammatory changes associated with AD (Sonkoly E, et al. 2006; Raap U, et al. 2008).

Pro-inflammatory cytokines (IL-1, TNF-α, and granulocyte-macrophage colony stimulating factor [GM-CSF]), and chemokines (CCL1, CCL2/MCP1, CCL3, CCL4, CCL5/RANTES, CCL11/Eotaxin, CCL13, CCL17/TARC, CCL18, CCL20/MIP-3α, CCL22/MDC and CCL27) have been linked to the development of AD (Rahman S, et al. 2011). CCL11/Eotaxin, CCL17/TARC, CCL22/MDC, CCL26, CCL27 and CXC3L1 have been found to also correlate with the severity of AD (Rahman S, et al. 2011).
Involved in the pathogenesis of AD are several cellular components of the innate and adaptive immune system. These components consist of keratinocytes, dendritic cells, lymphocytes (T and B), natural killer cells, and mast cells. Keratinocytes produce several chemokines that recruit effector and memory T cells. Keratinocytes have an increased expression of the human leukocyte antigen (HLA) and intercellular adhesion molecule 1 (ICAM-1). This facilitates a more efficient antigen presentation to T cells (Singer KH, et al. 1989).

Langerhans’ cells are involved in the pathogenesis of AD. Langerhans’ cells’ FcεRI as well as the TSLP-receptor, both of which are known to play a predominant role in the initiation of AD (Rahman S, et al. 2011). These receptors, when stimulated, promote the production of pro-inflammatory cytokines such as IL-8, CCL2, and IL-16. The activation of FcεRI on dendritic cells stimulates T cells to produce Th2 cytokines including IL-4, IL-5, IL-13, and IL-31 (Novak N, et al. 2004). Inflammatory cytokine production is also augmented by the stimulation of IDECs. Stimulated IDECs produce IL-1, IL-16, macrophage inhibitory protein 1α (MIP-1α), IFN-γ, IL-12p70, and IL-18 (Novak N, et al. 2004).

Lymphocytes present in the skin are considered a marker for AD. This is due to the fundamental role of lymphocytes in the production of cytokines which determine the local milieu. In atopic patients, lymphocytes not only produce ILs but also induce apoptosis in keratinocytes. The resulting loss of keratinocytes causes the skin barrier to become compromised. Damage to the skin barrier increases the likelihood of infections and leads to the exacerbation of the atopic disease. Skin barrier dysfunction has been strongly connected with the presence of Th2 cytokines. IL-4 and IL-13 inhibit the production of terminal differentiation proteins such as filaggrin, loricrin, and involucrin, in addition to AMPs (Rahman S, et al. 2011).
Mast cells play a major role in allergic diseases, including AD. After FcεRI stimulation, mast cells produce prostaglandin D2. This induces the upregulation of the chemoattractant receptor-homologous molecule on Th2 cells (CRTH2), a mast cells’ receptor (Boehme SA, et al. 2009). Mast cells also produce histamine, one of the major pruritogenic mediators involved in allergic conditions.

Dogs

Very little is known about the immunology of AD in dogs compared to human AD. While not as extensive as human AD, immunological abnormalities have been described in cAD. These abnormalities involve both innate and adaptive immunity in both cellular and humoral components.

Many studies have analyzed the Th polarization in peripheral blood mononuclear cells (PBMC), but very few analyzed this polarization in the skin of atopic dogs. A decrease in T regulatory (Treg) cytokines and an increase in Th2 cytokines have been demonstrated in both lesional and nonlesional skin, as well as in PBMC collected from atopic dogs (Olivry T, et al. 1999; Nuttall TJ, et al. 2002; Marsella R, et al. 2006; Maeda S, et al. 2007).

In atopic skin, Th2 cytokines (IL-4, IL-5 and IL-13) are mainly present in acute lesional skin, whereas the chronic phase (96 hours post-stimulation) is characterized by a more Th1 pattern with the increase of IL-2 (Olivry T, et al. 1999). In addition, Nuttall et al. recently showed that dogs with AD have increased levels of IL-4 and decreased levels of transforming growth factor beta (TGF-β), suggesting that atopic dogs are not able to regulate the inflammatory reaction triggered by the initial stimulus (Nuttall TJ, et al. 2002). TNF-α has also
been widely studied in cAD (Gosset P, et al. 1992). TNF-α is a pro-inflammatory cytokine with potent chemotactic properties. In addition to other cells, TNF-α is released by keratinocytes and macrophages.

A cutaneous increase in mRNA expression of IL-4, TNF-α, thymus and activation-regulated cytokine (TARC/CCL17), CC chemokine receptor 4 (CCR4), and IFN-γ has been reported in cAD (Maeda S, et al. 2002; Maeda S, et al. 2002a; Maeda S, et al. 2005). Similarly, an increased mRNA expression of IL-4 and CCR4, and a decreased expression of IL-10 have been detected in PBMC collected from atopic dogs (Maeda S, et al. 2004; Shida M, et al. 2004; Keppel KE, et al. 2008).

The role of IL-31 has very recently been investigated. Earlier studies did not detect IL-31 mRNA levels in the skin of atopic dogs (Mizuno T, et al. 2009). However, more recent studies clearly demonstrated IL-31 to have a role in the pathogenesis of pruritus in atopic dogs (Gonzales AJ, et al. 2013). The role of TSLP has been investigated in cAD with results similar to what was observed in humans (Klukowska-Rötzler J, et al. 2013). Klukowska-Rötzler J, et al. (2013) report that TSLP was expressed in canine skin, and that atopic skin (lesional or non-lesional) displayed higher mRNA expression levels of TSLP than healthy skin. The authors tested the expression of TSLP in primary keratinocytes harvested from healthy dogs, showing an increase in TSLP mRNA expression in cells after a four hour stimulation with phorbol 12-myristate 13-acetate (PMA), and an 18 hour stimulation with Toll-like receptor (TLR)-4 ligand lipopolysaccharide (LPS), TLR-3 ligand polyinosinic:polycytidylic acid, and HDM extracts suggesting a similar role for TSLP in cAD as in human AD (Klukowska-Rötzler J, et al. 2013).
An increase in the ICAM1, expressed on endothelial cells, was found to be associated with an increase of T lymphocytes in lesional AD skin, as well as an increase CD4/CD8 ratio and an increase of both αβ and γδ T cells (Olivry T, et al. 1997; Sinke JD, et al. 1997; Prélaud P. 1998).

The important function of IgE in cAD is based on its role in the degranulation of mast cells triggering the release of inflammatory mediators (e.g. histamine, chemokines, cytokines, and leukotrienes). Since IgE has been considered a hallmark of cAD, studies on the use of antihistamines have been numerous. However, the role of IgE is still not completely elucidated in dogs, blood levels of IgE do not correlate with the phenotypic disease and high levels of IgE in the serum are not always associated with AD (healthy dogs may have high allergen-specific IgE and AD dogs may have low allergen-specific IgE levels) (Esch RE, et al. 1997; Mueller RS, et al. 1999). Recently, several studies have suggested the possibility of a high heterogeneity in IgE (IgE⁺ and IgE⁻) with only some serotypes (IgE⁺) involved in AD (Bos JD. 1989; Bruijnzeel-Koumen CAFM. 1989; Halliwell REW. 1990; Middleton E, et al. 1998; Halliwell REW, et al. 1998; Lyon TM & Halliwell REW. 1998; Scott DW, et al. 2001). More recent research has suggested that although IgE plays an important role in AD, it cannot explain all aspects of this disease. cAD can be present in patients without detectable allergen-specific IgE (so called “atopic-like disease”) (Olivry T, et al. 1996; Olivry T, et al. 2001; Marsella R, et al. 2012).

As the role of IgE has become less pivotal in the current models of the development of AD, the role of T lymphocytes has gained increasing interest. This is more evident in human than in canine studies. This is probably because in human AD the differential involvement of Th2 and Th1 in different stages of the disease (acute vs. chronic lesions) has been more clearly
established than in dog studies. In dogs this dichotomization is less evident. Atopic skin is characterized by an increased presence of IL-4 secreting Th2 lymphocytes. It is also characterized by a decrease in TGF-β in nonlesional atopic skin suggesting a possible lack of tolerance (Olivry T, et al. 1999; Nuttall TJ, et al. 2002). In chronic lesional AD skin a more mixed population of T cells is present with an increase of IL-2, INF-γ and TNF-α secreting Th1 lymphocytes, but also IL-4 secreting Th2 cells (Nuttall TJ, et al. 2002).

Finally, similar to people, dogs with AD have an increase of eosinophils and Langerhans’ cells in both lesional and non-lesional skin (Day MJ. 1996; Olivry T, et al. 1996; Olivry T, et al. 1997; Prélaud P & Olivry T. 1998).
2.1.2.3 Skin barrier dysfunction

Numerous, well-characterized systemic and cutaneous immune abnormalities have been reported in AD. These include an increase of serum allergen-specific IgE, elevated Th2 cytokines in acute lesions switching to a Th1 cytokine pattern in chronic cases of AD, increased cutaneous lymphocyte-associated antigen (CLA) T cells, and an increased expression of FcεRI receptors on Langerhans’ cells, dendritic cells, and IDECs. These alterations are not enough to explain the complete pathogenesis of AD in both humans and dogs. More recent studies have hypothesized that a disruption of the skin barrier is a fundamental step in triggering the disease. Currently it is not clear whether skin barrier abnormalities precede the immune dysregulation (“outside-inside” hypothesis) or immune dysregulation precedes barrier dysfunction (“inside-outside” hypothesis) (Elias PM, et al. 2008; Wolf R & Wolf D. 2012).

In 1989 Strachan proposed the so-called “hygiene hypothesis”. This hypothesis tried to explain the reason for the increased presence of allergies in more developed countries. According to the hygiene hypothesis, exposure to high levels of viruses and endotoxin-producing bacteria in young individuals may have a protective effect against the development of allergies. On a cellular level, microorganisms lead to the stimulation of TLRs on the surface of APCs such as Langerhans’ cells, dendritic cells, and keratinocytes. This stimulation triggers the production of Th1 cytokines that antagonize the production of Th2 cytokines, reducing the severity or the development of allergies (Okada H, et al. 2010). The hygiene theory did not comprehensively explain all the alterations observed in atopic patients. To “complete” the hygiene theory, recently researchers have been examining the possibility that alterations of the
skin barrier contribute to the development and/or severity of AD. This has been labeled as the “outside-inside-outside” theory (Elias PM, et al. 2008).

**People**

Primary alterations of the skin barrier due to mutation in genes (EDC) regulating the translation of structural proteins (e.g. filaggrin, loricrin, involucrin, claudin-1, and occludin) are present in more severe cases of AD (Cork MJ, et al. 2009; De Benedetto A, et al. 2011; Elias PM & Schmuth M. 2009; De Benedetto A, et al. 2012). Alterations of structural proteins result in disorganization in the stratum corneum manifested as “holes” in the outermost layer of the skin. This barrier disruption results in the penetration of allergens and bacteria to the lower layers of the epidermis (Cork MJ, et al. 2009; De Benedetto A, et al. 2012). Alteration of filaggrin also leads to a reduced production of urocanic acid with an increase in pH in the stratum corneum. This more basic environment results in an increased activity of exogenous and endogenous peptidases which exacerbate the skin barrier alterations (Cork MJ, et al. 2009; Takai T & Ikeda S. 2011).

Consequences to alteration of the skin barrier include, but are not limited to, a greater penetration of allergens and toxins to the lower levels of the epidermis activating TLRs located on basal cells (Cork MJ, et al. 2009; De Benedetto A, et al. 2012). The impaired skin barrier leads to a change in morphology and maturation of Langerhans’ cells and to a greater proliferation and activation of T cells (Katoh N, et al. 1997; Banchereau J & Steinman RM. 1998; Strid J, et al. 2004). In atopic patients with a disruption of the stratum corneum, Langerhans’ cells mature

Dogs

As with people, it has been proposed that disruption of the skin barrier contributes to the development of cAD. Currently, only few studies have addressed this hypothesis. These studies primarily examined trans-epidermal water loss as an indicator of skin barrier integrity and measured the alteration of lipids present in the stratum corneum. It is still controversial whether the skin barrier is a primary (“outside-inside” theory) or a secondary (“inside-outside” theory) cause of cAD (Marsella R, et al. 2011; Olivry T. 2011).

A decrease in the levels of ceramides 1 and 9 and an increase in epidermal cholesterol have been reported in atopic dogs (Reiter LV, et al. 2009). Decreased expression of the stratum corneum (SC) total free ceramides and the subclasses CER[EOS], CER[NS]/[NDS] (ceramide-2/10), CER[NP] (ceramide-3), CER[AS]/[NH] (ceramides 5/8) and CER[EOP] has been reported in atopic skin (Yoon JS, et al. 2011). These results were confirmed by another study in which an experimental dog model of acute AD was exposed to HDM (Stahl J, et al. 2012). A significant decrease in the amount of ceramides (AH, AP, AS, NP, EOP, NS, and EOS) was observed in lesional stratum corneum compared with samples collected before the HDM challenge. The same study found no significant effect on the amount of epidermal cholesterol and free fatty acids. The ceramide amounts returned to normal within two months after lesion remission (Stahl J, et al. 2012). Increased degradation of ceramides due to an increased enzymatic activity

Single nucleotide polymorphism mutations (SNP) in the filaggrin gene and the association with cAD is controversial. Two studies (Wood SH, et al. 2010; Suriyapholl G, et al. 2011) have identified SNPs, but the findings of those studies were not confirmed by others (Barros Roque J, et al. 2009; Roque JB, et al. 2011; Salzmann CA, et al. 2011). In the former (Wood SH, et al. 2010; Suriyapholl G, et al. 2011) an association between SNPs, breed, and geographic location was suggested. Three SNPs associated with cAD were identified in Labrador retrievers in the UK and in small breed dogs (toy poodles, Shih Tzu and pugs) in Thailand (Wood SH, et al. 2010; Suriyapholl G, et al. 2011). A decreased signal intensity of filaggrin has been reported in atopic humans (O'Regan GM, et al. 2009), whereas different patterns of distribution have been reported in canine skin. Chervet et al. (2010) analyzed the immunofluorescence signal in the skin of 18 naturally-affected atopic dogs (various breeds) and 16 healthy dogs (various breeds), using an anti-canine filaggrin polyclonal antibody against the C-terminus. The results showed four different patterns of distribution: in three out of 18 subjects the filaggrin signal was similar to healthy controls, in seven out of 18 dogs it was visibly decreased, in four out of 18 dogs it was visibly decreased with a more granular and discontinuous distribution and large aberrant cytoplasmic vesicles, and in four out of 18 subjects there was absolutely no immunofluorescence signal.

Similarly to people, a recent study reported a significant difference between atopic and healthy dogs in mRNA expression levels of SPINK5, mast cell protease I, dipeptidyl- peptidase-4, phosphatidylinositol-3,4,5-trisphosphate- 5-phosphatase-2 and sphingosine-1-phosphate lyase-
1 (Wood SH, et al. 2009). Another study reported the unlikelihood that kallikrein 7 (KLK7) is involved in the pathogenesis of AD in boxer and West Highland white terriers (Stenshamn K, et al. 2006).
2.2 Antimicrobial peptides

Antimicrobial peptides are an extremely diverse group of small proteins characterized by a relatively short amino acid sequence (between 12 and 100 amino acids), positive charge (net charge between +2 to +11), and amphiphilic properties (Jenssen H, et al. 2006; Kraus D & Peschel A. 2008). AMPs have been isolated from single-celled microorganisms, invertebrates, plants, amphibians, birds, fish, and mammals, including human beings. Such peptides are systemically expressed in unicellular organisms and some multicellular organisms such as in insects’ hemolymph or vertebrate immune cells. Fish, amphibians, birds, and mammals locally express AMPs mainly in cells or tissues most susceptible to infections (e.g. skin and mucosae) (Zanetti M, et al. 1995; Jenssen H, et al. 2006).

To date over 1,000 AMPs have been identified in diverse species and subdivided into categories, mostly based on their molecular structure (Zanetti M, et al. 1995). In bacteria they are subdivided in lanthionine- (lantibiotics) and non-lanthionine-containing AMPs. Bacterial AMPs’ main function is to kill other bacteria that may compete for the same nutrients in a shared environment (Jenseen H, et al. 2006). In plants the main function is to defend the organism against bacteria and fungi and they are localized in leaves, flowers, seeds, and tubers (Jenseen H, et al. 2006). In vertebrates, AMPs achieve the most complex functionality. They not only protect the vertebrate against microorganisms (viruses, bacteria, fungi and parasites), but also are able to actively interact with the adaptive immune system (Zanetti M, et al. 1995; Oppenheimer JJ, et al. 2003; Metz-Boutigue MH, et al. 2010). Antimicrobial peptides are fundamental components of the innate local immunity as well as important components of the
2.2.1 Antimicrobial activity

The mechanism of killing by AMPs is not completely understood. However, many theories have been hypothesized for viruses, bacteria, fungi and parasites, as described in more detail below.

2.2.1.1 Antiviral activity

The spectra of viruses affected by AMPs include enveloped RNA and DNA viruses, non-enveloped viruses such as adenovirus (Bastian et al. 2001; Horne et al. 2005), feline calicivirus (McCann KB, et al. 2003), and echovirus (Pietrantoni A, et al. 2006). It has been hypothesized that the antiviral effect is a result of the AMPs’ interference with the viral adsorption and entry process (Belaid A, et al. 2002), or through a direct effect on the viral envelope (Aboudy Y, et al. 1994; Robinson WE Jr, et al. 1998). Many researchers looked in detail at the possible effects of the structure and charge of AMPs on antiviral activity. These studies concluded that although the presence of hydrophobic and positively charged residues is necessary, alone these are insufficient for the antiviral activity (Giansanti F, et al. 2005).

Several mechanisms of action have been hypothesized for the antiviral effects of AMPs. The most probable involves the interaction of AMPs with the viral modality of entry. Cationic AMPs are able to interact with heparin sulfate and chondroitin sulfate present on mammalian cell surfaces. Those sulfates are extremely anionic, attracting proteins, enzymes, ILs, and growth factors, and also pathogenic agents, such as viruses (Jenssen H, et al. 2006). Glycosaminoglycan is the most important molecule with respect to viral attachment (Spillmann
D. 2001; Mettenleiter TC. 2002). The interaction between this molecule and cationic AMPs will result in the inhibition of viral attachment. AMPs also function to inhibit the spreading of the virus from one cell to another through the inhibition of tight junction (cell-to-cell spread) or the formation of giant cells (syncytium) similar to alpha and gamma interferons’ mechanism of action (Jenssen H, et al. 2006). Another component of AMPs’ mechanism of action involves the interaction with specific viral receptors present on the host cells. This interaction prevents viral attachment, entry, or intracellular shuttling (Tamamura H, et al. 1996; Cole AM, et al. 2001). AMPs block viral penetration through interaction with viral glycoproteins present on the viral envelope (Yasin B, et al. 2004).

AMPs directly interact either with the membrane or viral envelope or with the host cell membranes. It is established that AMPs interact with lipid membranes (e.g. envelope) leading to destabilization, translocation, pore formation, and lysis (Dathe M & Wieprecht T. 1999; Sitaram N &Nagaraj R. 1999). AMPs also interact with the host cell membranes altering the entry process for viral elements (Lau YE, et al. 2005). AMPs interact with viral replication by targeting intracellular structures. Some AMPs, such as LL-37, penetrate into mammalian cells and nuclei with the potential to stimulate genes or the synthesis of proteins that play a role in the host cell’s antiviral mechanisms or to block the viral gene or protein expression (Wachinger M, et al. 1998).
2.2.1.2 Antibacterial activity

Antibacterial activity is the most prominent antimicrobial function of AMPs. The complete mechanism of action is unclear. It is hypothesized that AMPs interact with the bacterial membrane and disrupt it by creating pores in the membrane. There appears to be a virtual lack of bacterial resistance to AMPs. This lack of bacterial resistance may be linked to the fact that AMPs interact with the basic structural molecules present in the bacterial cell wall/membrane and changes in such structures found to be evolutionarily unfavorable.

Several antibacterial mechanisms have been hypothesized and, although different, there is a commonality with the initial step of interaction between AMPs and bacteria. Cationic peptides have a strong affinity for bacterial membranes and walls. AMPs interact with bacteria via electrostatic forces between the AMPs and the anionic phosphate groups of lipopolysaccharide (LPS) and (Lipo) teichoic acid ([L]TA) present on the outer bacterial envelope of Gram-negative and Gram-positive bacteria, respectively (Fedtke I, et al. 2004). What occurs post-attachment of AMPs to bacteria is still controversial. Five models have been hypothesized: barrel-stave, carpet, detergent, toroidal pore, and aggregate models (Jenssen H, et al. 2006). There are two commonalities among all the models. Some AMPs kill bacteria by inducing membrane lysis through the formation of transient channels, micellarization, dissolution, or translocation. Other AMPs kill bacteria by penetration without major membrane disruption (Jenseen H, et al. 2006).

Mechanisms proposed for AMPs that kill by permealizing the bacterial membrane include four of the model previously mentioned: the aggregate model, the toroidal pore model, the barrel-stave model, and the carpet model. The aggregate model hypothesis states that
AMPs organize themselves to span the membrane as an aggregate with micelle-like complexes of peptides and lipids, but without adopting any particular orientation. This model is characterized by a lack of “formal channel structures” (Wu M, et al. 1999). The toroidal pore model proposes that AMPs insert perpendicular to the plane of the bilayer. The hydrophilic regions of the peptides associate with the phospholipid head groups, and the hydrophobic regions associate with the lipid core. This model predicts an inward curvature of the membrane. However, the formation of a formal channel has not been demonstrated (Matsuzaki K, et al. 1996; Yang L, et al. 2001; Hallock KJ, et al. 2003; Henzler Wildman KA, et al. 2003). According to the barrel-stave model the AMPs insert perpendicular to the plane of the bilayer. This leads to formation of the “staves” in a “barrel”-shaped cluster. The hydrophilic regions of the peptides face the lumen of the pore and the hydrophobic regions interact with the lipid bilayer (Ehrenstein G, et al. 1977; Yang L, et al. 2001). The carpet model postulates that the peptides aggregate parallel to the lipid bilayer, coating local areas in a “carpet”-like fashion. The result of the “carpet coating” is the formation of micelles and membrane pores (Pouny Y, et al. 1992).

Mechanisms proposed for AMPs that do not act through permeating the bacterial membrane involve translocation across the membrane. The peptides accumulate intracellularly and disrupt cardinal cellular processes, and this mediates cell death. These AMPs inhibit nucleic acid synthesis, protein synthesis, enzymatic activity, and cell wall synthesis. (Brogden KA. 2005).

The exception to the above mentioned AMPs is dermcidin and Glu-Asp-rich peptides. Dermcidin and Glu-Asp-rich peptides, are the only anionic AMPs. They carry a negative net charge (Metz-Boutigue MH, et al. 2010; Wiesner J & Vilcinskas A. 2010). The mechanism of
action for those anionic AMPs does not involve permealization of the target cell membrane (Steffen H, et al. 2006). Instead these peptides are thought to bind to defined and not yet identified components of the bacterial cell membrane/wall, resulting in decreased RNA and protein synthesis (Senyurek I, et al. 2009).

2.2.1.2.1 Antibacterial resistance

Bacteria develop resistance to antibacterial agents through evolutionary changes that disrupt the antibacterial mechanism of action. So far AMPs have demonstrated more resistant antibacterial mechanisms of action compared to more traditional antibacterial agents. This is because the antibacterial mechanisms of action of AMPs involve basic, critical structures of the microorganisms. This makes evolution-driven resistance for AMPs less likely as any change to the structure components that interact with AMPs would be determinant to the bacterial survival. However, possible mechanisms for resistance to AMPs have been hypothesized (Peschel A. 2002; Fedtke I, et al. 2004; Peters BM, et al. 2010). Microorganisms are able to modify outer cellular surfaces to reduce negative charges and as a result decrease the affinity for cationic peptides (Peschel A. 2002). The negative charges on the bacterial surface are due to the large number of phosphate groups that confers LTA, TA polyanionic properties. Incorporation of D-alanine allows the free exposure of its positively charged group. This decreases the negative charge of these polymers (Peschel A, et al. 1999). As D-alanine is connected to alditol residues via an ester bond, the positively charged amino group remains free. D-alanine modification of (L)TA is through activation of the dlt operon that encodes for
four proteins (DltA, -B, -C, and -D) (Peschel A, et al. 1999). Another structural modification to decrease the anionic surface charge is the introduction of L-lysine into the structure of phosphatidylglycerol, a phospholipid abundantly present in the membrane of Gram-positive bacteria (Nahaie MR, et al. 1984). In this case the L-lysine modification is triggered by the activation of the MprF gene (Peschel A. 2002). Similarly, in Gram-negative bacteria, the negative charge is due to the presence of lipid A forming the LPS. Lipid A is composed of a glucosamine dimer flanked by phosphate groups and linked to five or more fatty acid chains. An increased concentration of aminoarabinose, a positively charged molecule in the structure of lipid A, decreases the negative charge of the bacterial surface reducing the attachment of AMPs. Similarly to D-alanine and L-lysine introduction, aminoarabinose synthesis is initiated by the activation of pmrE and pmrHFIJKL genes (Gunn JS, et a.l 1998).

Other mechanisms of resistance have been identified in Gram-positive bacteria. Those include increased membrane fluidity (Bayer AS, et al. 2000) as well as an increased presence of QacA efflux pumps, a major facilitator superfamily member. The mechanism of resistance associated with QacA is not yet elucidated. Its function as an efflux pump does not appear to be involved in resistance (Kupferwasser LI, et al. 1999). Bacteria such as S. aureus have the potential to increase the production of staphylokinases, metalloprotease aureolysin and the serine-proteases V8 and SepA. Staphylokinases bind to AMPs and inactivate them (Jin T, et al. 2004). Aureolysin, V8 and SepA cleave AMPs, which results in the inactivation of the peptides (Sieprawska-Lupa M, et al. 2004; Lai Y, et al. 2007). Another mechanism of resistance is evident in biofilms. The production of the cationic polysaccharide intercellular and capsular adhesion molecules prevents the attachment of AMPs to bacterial surfaces (Otto M. 2006).
2.2.1.3 Antifungal activity

The antifungal activity of certain AMPs has been demonstrated in both plants and vertebrates. Although the mechanism of action is not completely understood, it has been proposed that AMPs increase the permeability of the fungal cell membranes. It has been observed that many AMPs disrupt the structures of the fungal cell membranes of *Candida spp.* yeasts in a salt-dependent and energy-independent fashion, suggesting a direct interaction with the lipid bilayer (Lee DG, et al. 2003).

2.2.1.4 Antiparasitic activity

The antiparasitic activity of AMPs was first discovered by Zasloff in the late 1980s using magainin 2 against *Paramecium caudatum* (Zasloff M. 1987). As a result, synthetic and natural AMPs have been tested for antiprotozoal activity. An acylated synthetic AMP, Oct-CA(1-7)-M(2-9), has demonstrated to be both safe and effective against *Leishmania spp.* in dogs (Alberola J, et al. 2004). An analogous of mussel defensins was able to kill *Leishmania major* and *Trypanosoma brucei* in a temperature-, time- and dose-dependent manner through interaction with external epithelium (Roch P, et al. 2004).
2.2.2 Immunological effects

In higher evolved vertebrates, AMPs are among one of the most important mechanisms of defense. AMPs represent an essential component of the innate immunity as AMPs recognize and attack invading microorganisms in non-specific fashions (see previously for mechanisms of action). Additionally, AMPs represent a significant link between innate and adaptive immunity. AMPs enhance phagocytosis, stimulate prostaglandin release, neutralize the specific effects of LPS, promote recruitment and the accumulation of various immune cells at inflammatory sites (e.g. monocytes and T cells), promote angiogenesis, induce wound repair, chemo-attract and activate granulocytes, monocyte/macrophages, mast cells, antigen-presenting dendritic cells, and/or lymphocytes, and influence dendritic cell development with adjuvant and polarizing effects (Yang D, et al. 2004; Jenssen H, et al. 2006). AMPs have been labeled as “endogenous alarmins” due to their ability to chemo-attract immune cells and to respond to danger signals by activating the immune system (Yang D, et al. 2009). The immunological effects of AMPs are the subjects of intense research in both human and canine inflammatory and autoimmune diseases such as AD and psoriasis. In people, an important relationship has been shown to exist between AMPs and cytokines of both Th and T regulatory (Treg) cells in inflammatory skin diseases (Niyonsaba F, et al. 2007).

A strong inverse relationship has been observed between defensins and cathelicidins, and anti-inflammatory Th2 phenotype molecules (IL-4 and IL-13) (Howell MD, et al. 2005; Howell MD, et al. 2006; Howell MD. 2007). Pro-inflammatory cytokines such as IL-1, IL-17, IL-22 and TNF-α, direct bacterial contact, vitamin D, and wound healing have been observed to promote the production of defensins and cathelicidin (Schittek B. 2011). Defensins and
cathelicidin are the two most studied AMPs in the past decades (Zanetti M, et al. 1995; Jenssen H, et al. 2006; Lai Y & Gallo RL. 2009). Together cathelicidins and defensins behave synergistically (Nagaoka I, et al. 2000). This implies a combined role in the orchestration of the host’s innate defense system.

### 2.2.3 Defensins

Defensins are cationic, microbicidal, lacking in glycosyl- or acyl- side chain modifications, amphipathic, and variably arginine-rich peptides. They contain a minimum of six highly conserved cysteine residues. These residues form three or four pairs of intramolecular disulfide bonds (Linde A, et al. 2008; Lai Y & Gallo RL. 2009). Determined by the alignment of disulfide bridges and molecular structure, defensins are characterized into three subgroups (α, β, and θ). α-defensins are characterized by cysteine residues linked in a 1-6, 2-4, 3-5 pattern. β-defensins are distinguished by disulfide bonds that link cysteine residues 1-5, 2-4 and 3-6 (Lai et al. 2009). θ-defensins consist of a circular structure formed by two hemi-α-defensins, each of which contributes three cysteines. This latter defensin form has so far only been identified in the neutrophils and monocytes of rhesus macaque, Old World monkeys, orangutans, and baboons (Tang YQ, et al. 1999; Selsted ME. 2005; Stegemann C, et al. 2010).

Currently, six α-defensins (HDs) have been identified in humans. Four (HD1-HD4) appear to be localized in the azurophilic granules in neutrophils (Ganz T, et al. 1985; Selsted ME, et al. 1985; Lynn DJ, et al. 2007). HDs are abundant in neutrophils, macrophages and Paneth cells. The two cryptdins (HD5 and HD6) are primarily localized in the Paneth cells of the small
intestine (Jones DE & Bevins CL. 1992; Jones DE & Bevins CL. 1993). α-defensins are sensitive to the physiological environment, and their local expression is highly increased under neutrophilic, inflammatory conditions (Yang D, et al. 2009). HDs have both antimicrobial and immunostimulatory properties. HDs act as alarmins through induction of the chemotaxis of naïve T cells and immature dendritic cells. This activity does not involve mature dendritic cells, monocytes, or mast cells (Territo MC, et al. 1989; Yang D, et al. 2000; Grigat J, et al. 2007). An additional function of HDs is to induce the expression of pro-inflammatory cytokines and chemokines such as IL-1, IL-8, TNF-α, and monocyte chemoattractant protein (MCP)-1 (Yang D, et al. 2004).

Human β-defensins (hBDs) are primarily generated in epithelial tissue such as the intestinal mucosa, respiratory tract, and skin (Niyonsaba et al. 2007). Four hBD (1-4) have been identified in human skin, located inside the lamellar bodies containing lipid-rich secretory granules (Oren A, et al. 2003). hBD1 is constitutively expressed in terminally differentiated keratinocytes (Ali RS, et al. 2001). It is not influenced by pro-inflammatory cytokines, but the production of hBD1 is stimulated by peptidoglycans, LPS, and calcium (Harder J, et al. 2004; Sorensen OE, et al. 2005).

hBD2 was first identified in psoriatic scales of differentiated keratinocytes. This hBD is present in low quantities under standard physiological conditions. The levels of hBD2 dramatically increase with infections (especially *Pseudomonas aeruginosa*), wounds, and after incubation with particular cytokines (mainly IL-1) (Butmarc J, et al. 2004; Radek K, et al. 2007; Kanda N & Watanabe S. 2008). It is particularly effective against Gram-negative bacteria (Harder J, et al. 1997). hBD2 stimulates the production of prostaglandins and the release of
histamine from mast cells (Befus AD, et al. 1999). It also has chemotactic effects on immature dendritic cells and memory T cells (Metz-Boutigue MH, et al. 2010).

Similar to hBD2, hBD3 is minimally present in the skin under physiological conditions. Its expression is increased by infections and pro-inflammatory cytokines (Harder J, et al. 2004; Metz-Boutigue MH, et al. 2010). hBD3 has exhibited a broad spectrum of antimicrobial activity against Gram-positive, Gram-negative bacteria, and against some fungi (Harder J, et al. 2001). Also, like hBD2, hBD3 stimulates the expression of pro-inflammatory cytokines IP-10, IL-10, and MCP-1 (Niyonsaba F, et al. 2007).

### 2.2.4 Cathelicidins

Cathelicidins (Cath) are a group of cationic AMPs characterized by the highly conserved N-terminal region known as the “cathelin domain”. The domain was named such because it is able to inhibit the protease cathepsin-L. Similar to defensins, cathelicidins also contain a variable number of cysteine residues linked by disulfide bonds (Lai Y & Gallo RL. 2009). Cath have been identified in many species, there is one genetic commonality conserved among them. All Cath genes are characterized by the presence of a large cathelin domain in the precursor protein (Lai Y & Gallo RL. 2009). In humans, there is a single Cath precursor, designated hCAP18. hCAP18 is processed and then released from the carboxyl terminus as an AMP consisting of 37 amino acids beginning with two leucines, designated LL37 (Zanetti M, et al. 1995). hCAP18 is stored in neutrophil granules, NK-cells, epithelial cells and mast cells (Zanetti M, et al. 1995; Lai Y & Gallo RL. 2009). Prior to secretion, hCAP18 is activated by serine
proteases (e.g. kallikreins in the skin) and neutrophil proteases (e.g. proteinase 3) to form the mature peptide, LL37 (Cole AM, et al. 2001; Yamasaki K, et al. 2006). The latter is rich in lysine and arginine and adopts an α-helical structure in the plasma, interstitial fluid, and intracellular fluid (Zanetti M, et al. 1995; Zanetti M. 2004). Secretion of the AMP is stimulated by pro-inflammatory cytokines, bacteria, wounds, and vitamin D (Zanetti M. 2004; Kamen DL & Tangpricha V. 2010). It has rapid, potent, and broad-spectrum antimicrobial properties (Zanetti M. 2004), and it can be further cleaved to form different derivatives with various functions (e.g. RK-31, KS-30 and K20) (Murakami M, et al. 2004). In addition to antimicrobial activity, LL37 acts as a potent alarmin. It is a chemoattractant for neutrophils, lymphocytes, monocytes, mast cells, and dendritic cells (Yang D, et al. 2009). LL37 induces the activation of dendritic cells, monocytes, macrophages, keratinocytes, and endothelial cells (Yang D, et al. 2009).
2.3 Antimicrobial peptides, dogs and atopic dermatitis

Little information is available for AMPs in regards to veterinary medicine. AMPs have been identified in many domesticated species including: horses, cattle, sheep, goats, pigs, poultry, and cats (Linde A, et al. 2008; Leonard BC, et al. 2012). The majority of the veterinary publications on AMPs reference the canine species, as the dog has been more commonly used as an animal model for human diseases.

In dogs, the most studied AMPs are also defensins and cathelicidins. Seven BDs have been identified in canine skin: cBD1, cBD1-like, cBD2-like/122, cBD3-like, cBD102, cBD103, and cBD127 (Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011). cBD1, cBD2, cBD3, and cBD103 have also been identified in the testes (Sang Y, et al. 2005; Leonard BC, et al. 2012). These studies confirmed that the cBDs have a genetic homology that varies from 51% to 80% when compared to the hBDs, with cBD103 and hBD3/103A being the most similar (Sang Y, et al. 2005; Wingate KV, et al. 2009; Van Damme CM, et al. 2009). As in humans, only one Cath has been identified in dogs (cCath) (Linde A, et al. 2008). The cCath sequence was compared to hCAP18 sequence, and a 68% mRNA sequence similarity and 57% protein sequence similarity were identified (Santoro D, et al. 2011b).

Similar to the testing done for human AMPs, the in vitro antimicrobial activity of four cBDs (common sequence for cBD1, cBD2-like/122, and cBD3-like; cBD103) and cCath has been tested (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K, et al. 2010; Leonard BC, et al. 2012). These studies confirm potent antibacterial activity against numerous pathogens associated with the skin and respiratory tract. Very recently the antibacterial properties of cBD103 have been tested against clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) and
methicillin resistant *Staphylococcus pseudintermedius* (MRSP). cBD103 demonstrated antibacterial activity against these pathogens (Leonard BC, et al. 2012).

Current research regarding the role of AMPs has expanded from antimicrobial activity to also include immune-modulating functions and possible involvement in the pathogenesis of AD in both humans and dogs. Over the past decade, interest in skin barrier defects related to inflammatory skin diseases, such as AD, has notably increased in both human and veterinary medicine.

As components of the skin barrier, AMPs have been widely studied. In people, it has been observed that there is a decreased expression of AMPs in patients with AD compared to patients affected by psoriasis. It also been observed that atopic patients are more susceptible to skin infections (*S. aureus*) compared to healthy subjects or patients affected by psoriasis. It has been suggested that there is a link between the two observations; that a decreased expression of AMPs in atopic patients causes an increased susceptibility to skin infections (Zanetti M, et al. 1995; Fulton C, et al. 1997; Gallo RL, et al. 2002; Ong PY, et al. 2002; Howell MD, et al. 2005; Harder J & Schroder JM. 2005; de Jongh GJ, et al. 2005; Howell MD, et al. 2006; Howell MD. 2007; Stryjewski ME, et al. 2007; Guttman-Yassky E, et al. 2008; Pálffy R, et al. 2009; Wollenberg A, et al. 2010). This hypothesis has been challenged by multiple studies which have demonstrated that at both molecular and protein levels, there is an increased expression of AMPs such as BDs, Cath, psoriasin and ribonuclease7 (RNAase7) (Bellardini N, et al. 2009; Glaser R, et al. 2009; Harder J, et al. 2010). Similar to human AMPs, animal AMPs have been localized in tissues that most frequently come in contact with external agents. Examples of such tissues include testicular and mucosal epithelia, skin and blood (Sang Y, et al. 2005; Jenssen H,

In canine, little information regarding the relationship between AMPs and cAD has been reported. One group of investigators compared the expression levels of cBD103 in dogs with chronic AD and in healthy skin. This study reported a decreased expression of cBD103 in lesional skin compared with non-lesional and healthy skin (Van Damme CM, et al. 2009). It is difficult to investigate the involvement of AMPs in the pathogenesis of AD in dogs with naturally occurring disease due to the presence of multiple confounding factors (e.g. different breeds, living conditions and diet). A model for cAD has been identified in a colony of high IgE atopic beagles which develop clinical, histological, and immunohistochemical lesions similar to naturally occurring cAD, after environmental challenge with HDM allergen (Marsella R, et al. 2006; Marsella R & Girolomoni G. 2009).
Table 1. Risk factors for canine atopic dermatitis

<table>
<thead>
<tr>
<th>Risk of developing atopic dermatitis</th>
<th>Environmental factor</th>
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<tr>
<td>Increased</td>
<td>Urban life</td>
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<td>High human population density</td>
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<td>Increased average annual rainfall</td>
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<td>Living in southern Sweden</td>
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<td>Adoption at the age of 8-12 weeks</td>
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<td>Regular bathing</td>
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<td>Reduced</td>
<td>Rural life</td>
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<td>Living with other animals</td>
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<td>Walking in forests</td>
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<td>Feeding noncommercial foods to lactating bitches</td>
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<td>No effect</td>
<td>Sex</td>
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<td>Vaccination</td>
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Nuttall T. The genomic revolution: will canine atopic dermatitis be predictable and preventable? *Veterinary Dermatology* 2013; 24: 10-e4
Chapter 3

EXPRESSION AND DISTRIBUTION OF CANINE ANTIMICROBIAL PEPTIDES IN THE SKIN OF HEALTHY AND ATOPIC BEAGLES

3.1 Abstract

Antimicrobial peptides (AMPs) are small immuno-modulatory proteins important in defense against pathogenic organisms. Defensins and cathelicidin are the most frequently studied human AMPs. An increase in AMPs in atopic humans has been reported recently. Our goals were to determine the distribution of AMPs and evaluate their mRNA and protein expression in non-lesional (Day 0), acute lesional skin (Day 3) and post-challenged skin after resolution of skin lesions (Day 10) using a canine model of atopic dermatitis (AD). All dogs were environmentally challenged for three consecutive days with house dust mite. Clinical evaluation of atopic beagles was performed using a CADESI score at each time point before and after environmental challenge. Skin biopsies were taken from six healthy and seven atopic beagles before and after allergen challenge (Day 0, Day 3 and Day 10). The transcription of canine cathelicidin (cCath) and beta-defensins (cBD)-1, -2 and -3 mRNA was quantified using quantitative-RT-PCR while the protein distribution of cBD2, cBD3 and cCath was detected by indirect immunofluorescence. A significant effect, over-time, was seen in CADESI score in AD beagles with an increase score after challenge (Day 3). Quantitative analysis showed a significant difference in mRNA transcript levels between groups (with atopic dogs having more than controls) for all AMPs but cBD2. No effect over time was evident for either group. No significant differences were seen for the AMP protein patterns of distribution (homogenous distribution). Although, these results showed no
differences in AMP’s localization after allergen exposure in each group; atopic dogs had a higher mRNA expression of AMPs when compared with healthy dogs, a similar finding to humans.

3.2 Introduction

In the past decade the interest in skin barrier defects in inflammatory skin diseases, like atopic dermatitis (AD), has increased notably in both human and veterinary medicine. As part of the skin barrier, antimicrobial peptides (AMPs), a diverse group of small, mainly cationic endogenous proteins have been largely studied. To date over 1,000 AMPs, sub-classified based on their molecular structure, have been identified in diverse species (Marshall SH, et al. 2003; Jenssen H, et al. 2006). Of such peptides, β-defensins (BD) and cathelicidins (Cath) have been the most studied. They are primarily secreted by keratinocytes, sebocytes, sweat glands, mast cells, neutrophils and natural killer cells (Agerberth B, et al. 2000; Murakami M, et al. 2002; Di Nardo A, et al. 2003; Braff MH, et al. 2005; Lee DY, et al. 2008; Linde A, et al. 2008). Such peptides have two main functions; they have antimicrobial activity against bacteria, fungi and viruses and they also modulate the innate and adaptive immune responses in higher organisms (Gallo RL, et al. 2002; Jenssen H, et al. 2006; Schauber J & Gallio RL. 2008). Due to their immune-modulating activity, they have gained increased interest in inflammatory skin conditions, like AD and psoriasis. In fact, it has been suggested that a decreased expression of AMPs in patients affected by atopic eczema, when compared with humans affected by psoriasis, could be part of the reason why atopic patients are more susceptible to skin infection compared with healthy humans or patients affected by psoriasis (Zanetti M, et al. 1995; Fulton

To date, four BDs (hBD1-4) have been identified in human skin (Schauber J & Gallo RL. 2008), whereas eight BDs have been identified in canine skin (cBD1, cBD1-like, cBD2, cBD3, cBD102, cBD103, cBD122 and cBD127) (Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011). Of these, three (cBD1, 2 and 3) have been also identified in the testis (Sang Y, et al. 2005). From the above mentioned studies it has been assessed that the canine BDs have a homology that varies from 51% to 80% compared with the human peptides, with cBD103 and hBD3/103A being the most similar (Sang Y, et al. 2005; Wingate KV, et al. 2009; Van Damme CM, et al. 2009). Currently, only one Cath has been identified in both humans (LL-37) and dogs (cCath) (Jenssen H, et al. 2006; Linde A, et al. 2008). Comparing the canine
sequence with the human cathelicidin precursor (CAMP18), a 68% mRNA sequence similarity and a 57% protein sequence similarity have been identified by the authors.

Very little information on the relationship between AMPs and AD has been reported in dogs. One group of investigators analyzed the expression of cBD103 in dogs with chronic AD showing a decreased expression of cBD103 in lesional skin compared with non-lesional and healthy skin (Van Damme CM, et al. 2009). Unfortunately, the investigation of such AMPs and its involvement in the pathogenesis of AD in dogs with naturally occurring disease is difficult to assess due to the presence of multiple confounding factors (e.g. different breeds, living conditions and diet). Recently, a model for canine AD has been identified in a colony of high IgE atopic beagles which develop clinically, histologically, and immunohistochemically, lesions similar to naturally occurring canine AD, after environmental challenge with house dust mite allergen (HDM) (Marsella R, et al. 2006; Marsella R & Girolomoni G. 2009).

Thus to gain more insight into the role of AMPs in cAD, we analyzed the mRNA expression of three cBDs (cBD1, cBD2, and cBD3) and cCath in healthy and atopic dogs (lesional and non-lesional skin). The cBDs and cCath used were selected based on previously demonstrated antimicrobial properties. In addition, we evaluated their distribution in the epidermis using Indirect Immunofluorescence (IF).

3.3 Materials and Methods

Animals

Seven experimentally sensitized atopic beagle dogs (3 male, 4 female; age: 4 years) were studied. They are members of a colony that has been validated as a model for canine AD
(Marsella R, et al. 2006; Marsella R, et al. 2006; Marsella R & Girolomoni G. 2009). These dogs are sensitized to the HDM but have minimal or no signs of atopic disease when not under antigenic challenge. Six clinically healthy, female beagle dogs (age range: 4 to 6 years) with no history of skin diseases, skin allergies or pruritus were used as controls. The study protocol was approved by the Institutional Animal Care and Use Committees of the University of Illinois and University of Florida.

**Atopic dogs**

All dogs were housed in a research facility at the University of Florida, housed indoors in temperature- and humidity-controlled cement runs (72–75°F; relative humidity of 68–72%) that are washed daily using high temperature and pressure wash (water and bleach). Filters are changed routinely and stuffed toys, carpets, soft bedding or anything that could trap dust are not allowed in the runs. Walls of the runs and filters are checked monthly to ensure the absence of HDM in the environment in between challenges [results are below detection level of test used (MITE-T-Fast™ Allergen Detection System, Aveho Biosciences]. The dogs were fed maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).

**Clinically healthy dogs**

The six dogs belonging to a research colony at the University of Illinois are housed indoors in a temperature- and humidity-controlled facility (72–75°F; relative humidity of 68–72%). The dogs were housed in individual cement runs cleaned daily using high-temperature and high-pressure
wash. The dogs were fed maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).

**Environmental challenge with HDM in sensitized dogs**

All dogs (AD and control) were challenged for HDM as previously described (Marsella R, et al. 2006). Briefly, the animals were exposed to 50mg of *D. farinae* (Greer®, North Carolina, USA) placed on the bottom of their kennels. All dogs were allowed to stay in the kennels for 3 hours/day, 3 days in a row, as previously described (Marsella R, et al. 2006).

**Clinical evaluation**

Severity of clinical signs was scored using the canine atopic dermatitis extent and severity index scoring system (CADESI) (Marsella R, et al. 2006). Dogs were scored at baseline (before allergen exposure), three days after the beginning of the challenge (Day 3 – acute lesional skin) and seven days after discontinuation of allergen exposure (Day 10 – post-challenged skin with resolution of the skin lesions). In the CADESI scoring system, the dog’s body is divided into small areas and each of them receives a score for erythema, erythematous macules, papules, excoriations, and alopecia. Individual score ranges from 0 (absent) to 3 (severe) for each site and each symptom and the total scores are calculated by adding the scores of all body sites. Total CADESI scores were used in the statistical analysis.
Skin sample collection

One 8-mm biopsy punch from either the inguinal or the ventral thorax area from lesional and not lesional areas was collected following subcutaneous injection of 1mL of lidocaine (Lidocaine HCl 2%, Hospira Inc., Lake Forest, USA). The skin biopsy was immediately divided in two halves, of which one half was fixed in 10% buffered formalin for IIF evaluation, while the other half was further divided in two quarters, placed in 1.5 ml microfuge tubes, and immediately flash frozen in Liquid Nitrogen. These latter samples were stored at –80 °C until processed for molecular biology evaluation (QRT-PCR). The inguinal or ventral thorax was chosen for the skin biopsy as it is an easily accessible area with low level of hair density and since it is the most common area involved in AD.

Quantitative RT-PCR

One quarter of the 8mm biopsy punch was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA) and then processed into RNA using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA) reagents and the according to manufacturer’s protocol as previously described (Santoro D, et al. 2011) Briefly, the total RNA concentrations were determined at 260 nm using UV spectrophotometry then the integrity and quality of the RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc. Santa Clara, CA, USA). After DNAse treatment using a Turbo DNA-free™ kit (Invitrogen, Carlsbad, CA), total RNA (0.5 μg) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Sense and antisense primers for each AMP (Table 1) were generated using Primer Designer software (Scientific and Educational
Software, Inc.). Each primer was designed to cross an exon–exon boundary, maximizing the amplification of the mRNA transcript target and minimizing amplification of any residual contaminating genomic DNA. The primers were generated from previously published Gene Bank (http://www.ncbi.nlm.nih.gov/) AMP sequences (Sang et al, 2005; Sang et al, 2007) All primer sequences were subjected to BLAST analysis for unintended homologies (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The relative mRNA expression levels were quantified using a SYBR® Green assay (Qiagen, Valencia, CA) and ABI (Applied Biosystems Inc, Foster City, CA) quantitative-RT-PCR methodology (User bulletin applied biosystems 75000/75000 fast real-time PCR systems. Available at: www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_050637.pdf. Accessed June 13, 2009). All samples were performed in triplicate 50 μl RT reactions in an ABI 7500 Real Time PCR System (Applied Biosystems Inc, Foster City, CA). PCR amplifications were carried out as followed: 50ºC for 2 minutes; 95ºC for 10 minutes; 40 cycles of 95ºC for 15 seconds, 60ºC for 60 seconds. Amplifications were followed by dissociation (melting) curves to ensure specificity of the primers. In initial tests, dilution curves were generated for each gene primer set to determine their amplification efficiencies (Table 1) (Santoro D, et al. 2011). The results were analyzed using the comparative CT (cycle threshold) method and the relative mRNA expression of each AMP was compared using the ΔΔCT method to compare the defensins and cathelicidin. This method is effective when the amplification efficiencies of the housekeeping gene and target gene are similar. When this criteria is fulfilled the formula is 2\(^{-\Delta\Delta CT}\) where \(\Delta C_T\) is the difference between the target gene and the normalizer gene expression (Pfaffl MW. 2001). All samples were
normalized against the canine ribosomal protein L15 (RPLO). This gene was chosen due to the highly stable and consistent expression, previously shown for ribosomal genes in canine skin (Wood SH, et al. 2008).

**Antibody preparation**

Polyclonal anti-canine-AMP antibodies were generated as previously described (Santoro D, et al. 2011). Briefly, the canine anti-AMP antibodies were produced based on the genetic sequences previously identified (Sang Y, et al. 2005; Sang Y, et al. 2007) and published in GenBank (http://www.ncbi.nlm.nih.gov/). Based on these genetic and amino acid sequences the most immunogenic epitopes were chosen for each protein and synthetic peptides were created. The amino acid sequences were then analyzed against the BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparative evaluation. Since the amino acid sequence of cBD1 overlaps with part of both cBD2 and cBD3 sequences, it was not possible to generate an appropriate polyclonal antibody against it. The specificity of the anti-canine-AMP antibodies was tested by immunoabsorption on tissue sections and by ELISA showing no cross reaction. All animal care and work was done in compliance with federal regulations and Institutional Animal Care and Use Committee of the University of Illinois guidelines.

**Indirect Immunofluorescence**

One-half of the 8-mm skin biopsy was fixed in 10% buffered formalin solution for no more than 48 hours and then placed in phosphate buffer solution until processed for IF (Santoro D, et al. 2011). Briefly, 3-µm skin-tissue sections were processed using the immunohistochemical
polymer procedure. The sections were blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA) followed by an extra wash using normal goat serum (BioGenex, San Ramon, CA) as a blocking solution. Epitope retrieval was not necessary for cBD2 and cBD3, whereas it was required for cCath. In the latter case, the method consisted of using boiled sections, performed at 125-130°C under 17-23 lbs pressure for 30 seconds followed by a 10 seconds treatment at 90°C. The sections were then stained for one hour at room temperature using primary polyclonal rabbit antibodies specific for cBD2, cBD3 and cCath. The primary antibodies were used at 1:200 dilution. Negative controls were established using the pre-immune serum at 1:200 dilution. The sections were washed with a blocking solution (Power Block®; BioGenex, San Ramon, CA) and then incubated for 30 minutes at room temperature with a polyclonal goat anti-rabbit antibody bound with a green fluorochrome (Alexa Fluor® 488, Invitrogen, USA) at 1:1,000 dilution, according to manufacturer’s recommendations. Finally, DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA) was used as a counterstain for nuclear detection. Specimens were mounted on glass slides using Vectashield® Mounting Medium (Vector laboratories, USA). The skin sections were examined using an inverted fluorescence microscope (Nikon Eclipse TE 2000-S®). The images were analyzed using MetaMorph® software (Version 63r1; Molecular Devices, Sunnyvale, CA). Five representative fields at 40x magnification were examined for each section and pictures recorded.

**Statistical analysis**

Mean values and 95% confidence intervals were calculated for all results. Differences between $\Delta C_T [C_T \text{AMP} - C_T \text{RPLO}]$ of each AMP were compared using one-way ANOVA. The Kolmogorov-
Smirnov test of normality was used (alpha = 0.05), and Tukey’s Multiple Comparison Test was used for post-hoc testing. The clinical score obtained over-time in the atopic group was also analysed using one-way ANOVA. The Kolmogorov-Smirnov test of normality was used (alpha = 0.05), and Tukey’s Multiple Comparison Test was used for post-hoc testing. P values of ≤ 0.05 were considered significant. Statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3.4 Results

Clinical scores

For CADESI, the Analysis of Variance showed significant effects of time (p= 0.0118). In particular, the Tukey’s Multiple Comparison Test detected a difference between Day 0 and Day 3 (P<0.05) and between Day 3 and Day 10 (P<0.05); however no difference was detected between Day 0 and Day 10 (P>0.05) (Figure 1).

mRNA expression

QRT-PCR was used to compare AMP expression in healthy versus non-lesional AD skin (day 0), acute lesional AD skin (day 3) and post-challenged AD skin after resolution of the skin lesions (day 10). After statistical analysis, a significant effect over-time was not present for any AMPs in any group (P > 0.05). When we compared mRNA expression of each AMP at each time point, between the two groups, a statistically significant higher expression of cBD1, cBD3 and cCath, but not cBD2 was shown in atopic dogs. At day 0 cBD1, cBD3 and cCath mRNA expressions were 10.0-, 6.0- and 5.0-fold more expressed in AD beagles than in healthy dogs, respectively. At day
3 the differences were of 9.5-, 8.8-, and 6.5-fold, respectively. At day 10 cBD1, cBD3 and cCath were 9.0-, 5.0- and 8.0-fold more expressed, respectively (Figure 2).

**Indirect Immunofluorescence**

We used IF to analyze the distribution of AMPs in the skin of healthy and AD dogs. In both groups the proteins were homogeneously distributed with cBD2 and cBD3 detectable in all layers of the epidermis, whereas cCath was detected predominantly in the stratum granulosum and stratum corneum, as previously reported in healthy dogs (Santoro D, et al. 2011). When the pattern of distribution of each AMP in healthy beagles was compared with the pattern of distribution in AD dogs, there were no differences detected.

**3.5 Discussion**

Low expression of AMPs has been proposed as an explanation for recurrent skin infections in atopic human patients (Zanetti M, et al. 1995; Fulton C, et al. 1997; Gallo RL, et al. 2002; Ong PY, et al. 2002; Harder J & Schroder JM. 2005; Howell MD, et al. 2005; Howell MD, et al. 2006; Howell MD. 2007; Stryjewski ME, et al. 2007; Pálffy R, et al. 2009). In fact, it is well known that atopic patients have an increased risk of staphylococcal pyoderma, Malassezia dermatitis and eczema herpeticum (Wollenberg A, et al. 2010). Investigators have reported altered expression of AMPs in atopic eczema showing a significant reduction of BD and Cath expression compared with patients with psoriasis (Ong PY, et al. 2002; de Jongh GJ, et al. 2005; Guttman-Yassky E, et al. 2008). These results suggested that AMPs were involved in the local immunodeficiency affecting atopic patients. However, more recently, a few papers have

Atopic dogs, like atopic humans, are more susceptible to bacterial and Malassezia skin infection, but only recently has there been an increased interest in the role of AMPs as possible cause of such predisposition (Sang Y, et al. 2005; Sang Y, et al. 2007; Schauber J & Gallo RL. 2008; Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011). Of those, only Van Damme et al. (2009) have analyzed the expression of cBD103 in atopic dogs showing a decrease protein expression of this peptide in AD dogs.

Our results show that some canine β-defensins (cBD1, cBD2 and cBD3) and canine cathelicidin are increased in lesional, non-lesional and post-challenged skin after resolution of the skin lesions in atopic beagles when compared with healthy controls. These results are in accordance with Bellardini et al. (2009) and Harder et al. (2010) in demonstrating a clear increase of LL-37, and BDs (hBD2 and hBD3) in atopic eczema when compared with healthy individuals. In addition, our study showed that there is no difference in epidermal distribution between lesional, non-lesional and post-challenged AD and healthy skin.

According with the most recent studies, the higher expression of AMPs in lesional and non-lesional AD skin in both humans and dogs may contradict with the hypothesis of their decrease as major cause of recurrent skin infection in AD patients. These results also seem to contrast previous works which seemed to clearly show an inverse correlation between AMPs and T-helper 2 cytokines expression (Fulton C, et al. 1997; Nomura I, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006). In fact, AMPs seem to be lower in presence of Interleukin (IL)-
4, IL-10 and IL-13 whereas IL-1, IL-17 and IL-22 seem to stimulate their production (Nomura I, et al. 2003; Liu L, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006). However, Harder et al. (2010) have shown a higher expression of hBD2 and hBD3 in acute AD compared with chronic disease suggesting that maybe a T-helper 2 environment is not enough to inhibit AMPs production. For veterinary medicine, to the authors’ best knowledge, no studies have been published analyzing the AMPs expression in different T-helper milieus, but it is reasonable to think that a similar explanation would be applicable in canine patients as well.

A higher mRNA expression of AMP could be explained by an alteration of the skin barrier occurring after allergen exposure. In fact, recently, Ahrens et al. (2011) have shown an increase of AMPs production after strip test in mice. In addition, an alternative possible explanation for higher expression of AMPs in AD dogs may be the presence of high secretion of non-functional peptides in atopic patients compared with healthy controls or a lack of increased secretion in AD patients as suggested for hBD3 (Harder J, et al. 2010). Another possible scenario could be an increased degradation of AMPs in atopic disease compared with healthy subjects. This latter hypothesis could be due hyper-activation of several endogenous as well as exogenous proteases (EliaPM. 2005; Takai T & Ikeda S. 2010). Finally, another explanation of an inverse correlation between AMPs expression and skin infection may be the lack of sufficient stimulation in AD patients to reach AMPs concentrations above the minimum inhibition concentration (MIC) able to kill pathogenic microorganisms.

In conclusion, we have demonstrated that, similar to humans, a higher mRNA expression of cBDs and cCath is present in atopic beagles when compared with healthy controls. We also demonstrated that no difference appears to exist in the distributional
expression pattern of AMPs between atopic and healthy dogs. Consequently, further studies are needed to examine the functional role of AMPs in inflammatory and/or infectious skin diseases in dogs.
3.6 TABLES AND FIGURES

Figure 1. Average of CADESI-03 score before, during and after environmental challenge. (*: P≤0.05; bars: standard errors of the mean).
Figure 2. mRNA expression for each antimicrobial peptide (canine β-defensin [cBD]1, cBD2, cBD3 and canine cathelicidin [cCath]) in atopic beagles compared with normal age and breed-matched control dogs. Groups were compared using the Tukey’s Multiple Comparison Test (*: P≤0.05; bars: standard error of the mean). (Day 0: non-lesional skin; Day 3: acute lesional skin; Day 10: post-challenged skin).
3.7 REFERENCES


CHAPTER 4

ALTERED mRNA AND PROTEIN EXPRESSION OF FILAGGRIN IN THE SKIN OF A CANINE ANIMAL MODEL FOR ATOPIC DERMATITIS

4.1 Abstract

Filaggrin is a structural protein that has attracted increasing interest over the past decade for its role in the pathogenesis of human atopic dermatitis (AD). Null mutations in its sequence are considered risk factors in the development of AD.

To investigate canine filaggrin mRNA and protein expression in the skin of atopic beagles with experimentally-induced AD compared with breed-matched healthy controls.

All dogs were environmentally challenged for three consecutive days with allergens to which the atopic dogs had been sensitized. Skin biopsy specimens were taken from six healthy and seven atopic beagles before and after allergen challenge. Canine filaggrin mRNA was measured using quantitative-real-time PCR. Indirect immunofluorescence was used to localize the filaggrin protein in canine skin. Analysis of variance (ANOVA) with Tukey’s multiple comparison test (over-time effect) and t-test (treatment effect) were used. P values ≤0.05 were considered significant.

ANOVA showed a significant higher expression of filaggrin mRNA in atopic dogs compared with healthy controls (p = 0.004 on day 3 and 0.01 on day 10) and a decreased mRNA expression on day 3 in healthy controls (effect of time: p = 0.006). On blinded evaluation, filaggrin immunofluorescence was distributed homogenously in the stratum granulosum and the
stratum corneum in healthy dogs. Atopic dogs showed a patchy immunofluorescence pattern which was exacerbated after environmental challenge.

Altered epidermal filaggrin mRNA expression and protein distribution was detected in this experimental model.

4.2 Introduction

Atopic dermatitis (AD) is one of the most common allergic skin diseases in both humans and dogs (Hillier A & Griffin CE. 2001; Bieber T. 2010). The pathogenesis of human AD is very complex and not completely elucidated. The most widely accepted theory (Outside-Inside-Outside) hypothesizes a primary skin defect as a major predisposing factor in the development of AD along with an aberrant immunologic response to common allergens (Elias PM & Steinhoff M. 2008). This theory is supported by multiple studies demonstrating an impairment of the skin barrier in atopic human patients compared with healthy individuals and increased risk for sensitization when skin barrier is defective (Pilgram GS, et al. 2001; Proksch E, et al. 2006).

In people, one of the most important predisposing factors for the disruption of the skin barrier of atopic patients includes an alteration of the transcription/translation of the filament-aggregating protein, filaggrin (Wolf R & Wolf D. 2012; Kubo A, et al. 2012). Filaggrin is one of the most significant proteins involved in the development of the cornified envelope and, after its degradation, participates in the formation of natural moisturizing factors (Nemes Z & Steinert PM. 1999). Many genetic studies, analysing different human populations (e.g. European, Chinese, Afro-American) have revealed up to 40 different semi-dominant, homozygous or compound heterozygous, mutations present in patients affected by AD (Chen H,
et al. 2011; Kawasaki H, et al. 2011). In particular, two null-loss-of-function mutations (R501X and 2282del4) have been identified with 2282del4 being the most common (Lesiak A, et al. 2011). R501X and 2282del4 mutations are more common in patients affected by the extrinsic subtype of AD in which allergen specific IgE levels are increased, confirming that a barrier defect may lead to higher allergen penetration, causing a more severe allergic sensitization (Weidinger S, et al. 2007; Tokura Y. 2010). However, filaggrin is not the only predisposing factor in AD development; mutations of the filaggrin gene are present in only 15% of human AD patients and mainly associated with the most severe form of AD (Henderson J, et al. 2008; Bieber T & Novak N. 2009). One explanation for these differences may be the different immunological environment present in extrinsic vs. intrinsic AD (Tokura Y. 2010); intrinsic AD is not characterized by a T helper (Th)2 predominant environment like extrinsic AD (Tokura Y. 2010). This hypothesis has been supported by a study in which inhibition of filaggrin production has been observed in normal keratinocyte cell culture after exposure to Th2 indicating that a local Th2 environment is able to decrease the expression of filaggrin leading to a disruption of the skin barrier even in patients without filaggrin gene mutations (Howell MD, et al. 2007).

Animal models represent a valuable tool to help increase the knowledge of AD (Marsella R & Olivry T. 2003). Experimentally-induced murine models of AD have been used to investigate immunological and structural alterations in atopic skin. However, the murine model has several limitations, including clinical differences and the fact that mice and human beings do not share the same environment leading to different external stimuli. These differences between the two species lead to the necessity for animal models similar to human atopic disease. Recently, the dog has been shown to be a better model for human AD because of the development of clinical
and immunological alterations more similar to the human disease (Helton Rhodes K, et al. 1987; Willemse T. 1988). In addition, dogs are philogenetically closer to humans than mice and share the same environment and nutrition (Helton Rhodes K, et al. 1987; Willemse T. 1988). A model for canine AD has been identified in a colony of high IgE, experimentally-sensitized atopic beagles able to develop clinically, histologically and immunohistochemically, lesions similar to naturally occurring canine AD after environmental challenge with house dust mite (HDM) (Marsella R, et al. 2006; Marsella R & Girolomoni G. 2009).

In dogs, the disruption of the skin barrier, as a predisposing factor of AD, has only been recently hypothesized. Three studies showed a decreased immunohistochemical staining (Marsella R, et al. 2009; Chervet L, et al. 2010) and mRNA expression (Roque JB, et al. 2011) along with a non-homogeneous distribution of filaggrin protein in canine skin, suggesting a potential involvement of filaggrin in the pathogenesis of canine AD. Chervet et al. (2010) specifically demonstrated that approximately 20% of dogs with naturally occurring AD showed a decreased expression of filaggrin when an antibody directed against the C-terminal portion of the molecule was used supporting the usefulness of dogs as a model for the human disease. Recently, filaggrin single nucleotide polymorphisms (SNPs) in specific canine breeds in both the UK and Thailand have also been demonstrated (Wood SH, et al. 2010; Suriyanphol G, et al. 2011).

Canine models able to mimic the clinical, immunological and structural alteration of the skin barrier in human AD may provide a useful tool to further investigate the pathomechanisms of AD in people (Marsella R & Girolomoni G. 2009; Marsella R, et al. 2011; Olivry T. 2012). Thus, we performed the present study, using a canine model, to gain more insight into the role of
filaggrin in canine AD. To do so, we analyzed the mRNA expression of canine filaggrin in healthy and atopic beagles (lesional and non-lesional skin) using a quantitative reverse transcriptase PCR (qRT-PCR). In addition, we evaluated its protein distribution in the epidermis using Indirect Immunofluorescence (IIF). The use of such a model allowed us to show increased mRNA expression of filaggrin in lesional and nonlesional atopic skin compared with healthy controls. In addition, a non-homogeneous distribution of filaggrin protein was seen in atopic skin compared with healthy control skin. These results confirmed that as in human AD, this colony of atopic beagles have an impairment of the skin barrier associated with alterations in filaggrin production and distribution.

4.3 Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of both University of Florida and University of Illinois.

Animals

Atopic dogs

Experimentally-sensitized atopic beagle dogs belonging to a colony that has been validated as a model for canine AD were used (Marsella R & Olivry T. 2003; Marsella R, et al. 2006; Marsella R & Girolomoni G. 2009). These dogs were sensitized to HDM and show low grade AD when not exposed to HDM, but strongly react when under allergenic challenge. These dogs were also been used as control population in previously published studies on filaggrin expression and AD (Marsella R, et al. 2013; Marsella R. 2013).
Normal dogs

Healthy unrelated beagle dogs with no history of skin disease and pruritus were included in this study as breed-matched controls.

Housing conditions

The atopic dogs were housed indoors in individual temperature- and humidity-controlled cement runs (22–24°C; relative humidity of 68–72%). The runs were washed daily using a high-temperature and high-pressure wash (water and bleach). Air filters were changed routinely and stuffed toys, carpets, soft bedding or anything that could trap dust was not allowed in the runs. MITE-T-Fast™ Allergen Detection System (Aveho Biosciences, Tullahoma, TN, USA) was used monthly, and between challenges, on walls and filters to ensure the absence of HDM in the environment. The dogs were fed a commercial maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).

Similarly, the healthy controls were housed indoors, in individual cement runs, in a temperature- and humidity-controlled facility (22–24°C; relative humidity of 68–72%). The runs were cleaned daily using a high-temperature and high-pressure wash (water and bleach). The dogs were fed a commercial maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).

Acute environmental challenge with HDM

All dogs (AD and control) were challenged for HDM as previously described (Marsella R & Olivry
T. 2003). Briefly, the animals’ intact skin surface was exposed to 50mg of *Dermatophagoides farinae* (Greer®, Lenoir, NC, USA) placed on the bottom of their kennels. All dogs were allowed to stay in the kennels for three hours/day, three days in a row.

**Clinical evaluation**

Severity of clinical signs was scored using the canine AD extent and severity index scoring system (CADESI) (Marsella R, et al. 2010). The CADESI used in the present study is a modified version of the validated CADESI -03 (Olivry T, et al. 2007). The sites evaluated are the same as the CADESI-03 but papules are added as a clinical sign in this modified version. The other difference between the scoring system used in this study and the CADESI-03 is that the scores for each sign range from 0 to 3 rather than from 0 to 5, as done in the CADESI-03. Briefly, the dog’s body was divided into small sections, each of which received a score based on the clinical signs evaluated. The total score was calculated by adding the scores of all clinical signs and body sites. Clinical signs evaluated included diffuse erythema, erythematous macules, papules, excoriations and alopecia. The scoring system ranged from 0 (absent) to 3 (severe) for each site and each sign. Total score was used in the statistical analysis. Dogs were scored at baseline (Day 0 - before allergen exposure [nonlesional skin]), three days after the beginning of the challenge (Day 3 – acute lesional skin [only atopic dogs]) and seven days after discontinuation of allergen exposure (Day 10 – post-challenged skin with resolution of the cutaneous lesions [only atopic dogs]).
**Skin sample collection**

One 8-mm skin biopsy punch from either the inguinal or the ventral thorax area from non-lesional (Day 0) and lesional (Day 3) (erythematous maculo-papular dermatitis) areas was collected following subcutaneous injection of 1 mL of lidocaine (Lidocaine HCl 2%, Hospira Inc., Lake Forest, IL, USA). The skin biopsy specimen was immediately divided in two halves. One half was fixed in 10% neutral buffered formalin for IIF evaluation, while the other half was divided in two quarters, placed in 1.5 ml microfuge tubes and quickly frozen in liquid nitrogen. These latter samples were stored at – 80 °C until processed for qRT-PCR.

**Quantitative Real-Time PCR (qRT-PCR)**

One quarter of the 8mm skin biopsy specimen was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA, USA) and then processed into RNA using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA, USA) reagents, according to manufacturer's protocol. A 260 nm using UV spectrophotometry and an Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc. Santa Clara, CA, USA) were used to determine the total RNA concentrations and its integrity and quality. After DNAse treatment using a Turbo DNA-free™ kit (Invitrogen, Carlsbad, CA, USA), total RNA (0.5 μg) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Forward and reverse primers for filaggrin (Table 2) were generated using Primer Designer software (Scientific and Educational Software Inc., Palo Alto, CA, USA). Each primer was designed to cross an exon–exon boundary, maximizing the amplification of the mRNA transcript target and minimizing amplification of any residual contaminating genomic DNA. The primers were generated from
previously published genomic sequence (ENSCAFT00000023034 [now renamed FLG2 with code ENSCAFT00000049887]). All primer sequences were subjected to Basic Local Alignment Search Tool (BLAST) comparison to the canine genome build 2.0 for unintended homologies. The relative mRNA expression levels were quantified using a SYBR® Green assay (Qiagen, Valencia, CA, USA) and ABI (Applied Biosystems Inc, Foster City, CA, USA) Real-Time quantitative PCR methodology. All samples were performed in triplicate 50 μl RT reactions in an ABI 7500 Real Time PCR System (Applied Biosystems Inc, Foster City, CA). PCR amplifications were carried out as follows: 50ºC for 2 min; 95ºC for 10 min; 40 cycles of; 95ºC for 15 s, 60ºC for 60 s. Amplifications were followed by dissociation (melting) curves to ensure specificity of the primers. In initial tests, dilution curves were generated for each gene primer set to determine their amplification efficiencies (Table 2). The results were analysed using the comparative CT (cycle threshold) method and the relative mRNA expression of filaggrin was calculated using the ΔΔCT method. When the efficiency of both the housekeeping gene and the target gene are similar and close to 100%, the formula to use is $2^{[\Delta CT \text{ experimental sample} - \Delta CT \text{ control sample}]}$ where ΔCT is the difference between the target gene C_T and normalizer gene C_T (Pfaffl MW. 2001). All samples were normalized against the canine ribosomal protein L15 (RPLO). This gene was chosen as member of the ribosomal proteins that showed highly stability and consistent expression in canine skin (Santoro D, et al. 2011).

**Antibody preparation**

Polyclonal anti-canine-filaggrin antibody was synthesized as previously described (Marsella R, et al. 2013). Briefly, the canine anti-filaggrin antibody was produced based on the genetic
sequences previously identified (ENSCAFF0000023034 [now renamed FLG2 with code ENSCAFF0000049887]). The amino acid sequence was subject to BLAST comparison to the canine genome build 2.0 for unintended homologies. Based on the aforementioned genetic and amino acid sequences, the most immunogenic epitope (GRRESSVTESSDTEND) on the C-terminus was chosen and a synthetic peptide was created. Antibody production was carried out using one healthy, 3 months old, female New Zealand rabbit, 3.4 kg in weight, purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA).

The peptide conjugates were mixed with an adjuvant and subcutaneously injected into the rabbit. Titermax (Sigma, St. Louis, MO, USA) was used for the primary immunization and incomplete Freund’s adjuvant for all subsequent immunizations. The rabbit was immunized four times and its blood was tested for an immune response using an ELISA technique. The pre-immune serum was used as negative control. When a satisfactory immune responses (positive up to 1: 51,200 dilution) was achieved, the rabbit was exsanguinated and the crude serum was used. The specificity of the anti-canine-filaggrin antibody was tested by immune-absorption on tissue sections incubating the antibody with a high concentration (20 µg/µl) of the synthetic peptide overnight at 4ºC before being applied to the tissue sections. In addition, a standard western blotting was also performed to verify the antibody specificity.

**Western blotting**

One quarter of the 8 mm skin biopsy specimen was weighed and the protein was extracted using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA, USA) according to manufacturer’s protocol. The protein concentration was determined at 280 nm using UV NanoDrop1000®
spectrophotometer (Thermo Scientific, Wilmington, DE, USA). 50 μg and 80 μg of total skin proteins were loaded in the well number 1 and 2 for gel electrophoresis; 80 μg of total skin proteins were loaded in the well number 3 as a negative control, and then a standard western blot was performed. Briefly, 20 μl of total protein extract was loaded in a 10-well 10% Ready Gel Tris-glycine resolving gel (BioRad, Hercules, CA, USA) and run for 1 h at 100 mAm voltage. A polypeptide standard (Precision Plus Protein™ Dual Color Standards, BioRad, Hercules, CA, USA) was used as marker. After electrophoresis, the gel was blotted to a 0.2μm nitrocellulose transfer membrane (BioRad, Hercules, CA, USA) using an electrophoretic transfer cell apparatus (Mini Trans-Blot®; BioRad, Hercules, CA) for 1 h at 100 mAm voltage. The membranes were blocked with a 3% Dry Milk in tris buffer solution (TBS) for 1 h, then incubated for an additional 60 min with a 1:1,000 dilution of anti-canine filaggrin followed by multiple washes with 0.05% Tween/TBS solution (BioRad, Hercules, CA, USA). Then, the membranes were incubated for 1 h with a goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad, Hercules, CA, USA) as a secondary antibody at 1:3,000 dilution as per the manufacturer’s recommendation, followed by multiple washes with 0.05% Tween/TBS solution (BioRad, Hercules, CA, USA). A final wash was performed with TBS solution to remove the excessive secondary antibody not specifically bound to the membrane. The immunofluorescence signal was graded using a modified corrected total well fluorescence (CTWF) index previously described (Burgess A, et al. 2010). Briefly, CTWF was determined as follows: CTWF = integrated density – (area of selected well x mean fluorescence of background readings). The relative fluorescence was expressed in arbitrary units (a.u.).
**Indirect Immunofluorescence (IIF)**

One-half of the 8 mm skin biopsy specimen was fixed in 10% neutral buffered formalin solution for no more than 48 hs and then placed in phosphate buffer solution (PBS) until processed for IIF. Briefly, 3-µm skin-tissue sections were processed using the immunohistochemical polymer procedure. The sections were blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA, USA). A second blocking step was added using normal goat serum (BioGenex, San Ramon, CA, USA). The sections were then stained for 1 h at room temperature using primary polyclonal rabbit antibodies specific for canine filaggrin. The primary antibodies were used at a 1:200 dilution. The pre-immune serum at a 1:200 dilution was used as negative control. The sections were washed with a blocking solution (Power Block®; BioGenex, San Ramon, CA, USA) and then incubated for 30 minutes at room temperature with a polyclonal goat anti-rabbit antibody bound with a green fluorochrome (Alexa Fluor® 488, Invitrogen, Carlsbad, CA, USA) at a 1:1,000 dilution, according to manufacturer’s recommendations. Finally, the sections were counterstained using DAPI (4’,6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA) for nuclear detection. Specimens were mounted on glass slides using Vectashield® Mounting Medium (Vector laboratories, Burlingame, CA, USA).

The skin sections were examined using an inverted fluorescent microscope (Nikon Eclipse TE 2000-S®, Nikon Inc., Shelton, CT, USA). The images were analyzed using MetaMorph® software (Version 63r1; Molecular Devices, Sunnyvale, CA, USA). Five representative fields from each tissue section were examined at 400x magnification and digital images collected. The intensity of the immunofluorescence signal was graded using a computer imaging program (Image J® 1.41). The stratum granulosum and stratum corneum were traced and a modified
corrected total cell fluorescence (CTCF) index was determined as previously described (Burgess A, et al. 2010). Corrected total cell fluorescence was determined as follows: CTCF = integrated density – (area of selected cells x mean fluorescence of background readings). The relative fluorescence was expressed in arbitrary units (a.u.).

**Statistical analysis**

The Kolmogorov-Smirnov test of normality was used (alpha = 0.05). Mean values and 95% confidence intervals were calculated for all results. Differences between \( \Delta C_T \) \([C_T\text{ filagrin} - C_T \text{RPLO}]\) at each time point (between groups) were compared using unpaired t-test. Differences between \( \Delta C_T \) over time (within group) were compared using repeated measurement ANOVA. Tukey’s Multiple Comparison Test was used for post-hoc testing. P values of ≤0.05 were considered significant. Statistical analysis was performed using SAS® statistical software version 9.2 (SAS® Institute Inc., Cary, NC, USA).

**4.4 Results**

**Animals**

Seven 4-year old atopic beagle dogs (3 males, 4 females) and six 4- to 6-year old clinically healthy female beagle dogs were used.

**Clinical scores**

For the clinical score, the ANOVA showed a significant effect between the time points \((p=0.0118)\). In particular, the Tukey’s Multiple Comparison Test detected a difference between
Day 0 and Day 3 (p=0.03) and between Day 3 and Day 10 (p=0.007); however no difference was detected between Day 0 and Day 10 (p=0.7) (Figure 3).

**mRNA expression**

QRT-PCR was used to compare filaggrin expression between healthy and AD dogs. When the FLG mRNA expression measured within each group at various time points (Day 0 vs. Day 3, Day 0 vs. Day 10, and Day 3 vs. Day 10) was evaluated, ANOVA showed a significant effect of time only in the control group with a decreased mRNA expression on Day 3 (p=0.006) and on Day 10 (p=0.02) compared with Day 0 (Figure 4b). When mRNA expression of filaggrin was compared at each time point, between the two groups (healthy vs. AD), a statistically significant higher expression of filaggrin was found in atopic dogs at Day 3 and Day 10 (p=0.004 and p=0.01, respectively), but not on Day 0 (p=0.2). In particular, filaggrin mRNA expression was 1.5-, 5.7- and 3.9-fold higher in AD beagles than in healthy dogs at Days 0, 3 and 10, respectively (Figure 4a).

**Indirect Immunofluorescence**

IIF was used to analyze the distribution and semi-quantitatively assess the amount of filaggrin in the skin of healthy and AD beagles. In the subjective blinded assessment of the distribution pattern, as expected, filaggrin immunofluorescence was detectable only in the stratum granulosum and deeper stratum corneum in all dogs. When the distribution of filaggrin in healthy beagles was compared with the distribution in AD beagles, a different pattern of distribution was detected (Figure 5). More specifically, in healthy controls, a more continuous
and laminar distribution of the fluorescence was detected at each time point, whereas in AD dogs a more patchy distribution of filaggrin immunofluorescence was seen with complete lack of the immunofluorescence in some areas. In acute lesional skin (Day 3) of AD dogs, the filaggrin immunofluorescence had the highest degree of discontinuity compared with nonlesional skin (Day 0) or atopic skin after resolution of the skin lesions (Day 10) (Figure 5). When the fluorescence intensity was analysed objectively using Image J® 1.41, no significant difference in signal intensity was seen in AD dogs compared with healthy control (data not shown).

**Western Blotting**

The Western blotting revealed a single band with a size of ~54 kDa showing a high specificity of the anti-canine filaggrin antibodies. Relative differences in filaggrin intensity were present between the well number 1 (10 a.u.) and 2 (17 a.u.), as expected (Figure 6).

**4.5 Discussion**

In this study an increased mRNA expression of canine filaggrin in the skin of AD beagle dogs when compared with healthy control dogs was found on Day 3 and Day 10 but not on Day 0 (Figure 4a). The results of our study are different from what is reported in humans, where a decreased mRNA and protein expression of filaggrin has been seen in AD patients (Chen H, et al. 2011; Kawasaki H, et al. 2011; Osawa R, et al. 2011). On the other hand, the results of this study are in agreement with other authors that evaluating mRNA expression in dogs have reported an increased mRNA expression of filaggrin and some keratins (keratin-1, -2e, -5, -14...

In addition, when we compared the filaggrin mRNA and protein expression, we noted that the significant increase in filaggrin mRNA level in atopic dogs is not accompanied by increase in filaggrin protein expression suggesting an increase in protein degradation or a post-transcriptional alteration. Decreased (Marsella R, et al. 2009; Chervet L, et al. 2010) or invariant protein expression of filaggrin in atopic dogs may be due to an increased degradation rather than a decreased synthesis. It has been reported that canine atopic skin has increased degradation of ceramides (Shimada K, et al. 2009; Reiter LV, et al. 2009; Yoon JS, et al. 2011) due to increased enzymatic activity. The same may be true for increased degradation of proteins, such as filaggrin, explaining why most studies in atopic dogs have failed to identify a genetic mutation in the filaggrin gene (Wood SHJ, et al. 2010; Roque JB, et al. 2011). It is also known that an increased cutaneous pH in atopic skin induces an increase in proteases activity (Kubo A, et al. 2012), which may results in excessive degradation of proteins including filaggrin.

On the other hand, another explanation could be the increased activity of antiproteases that does not allow the degradation of profilaggrin into filaggrin monomers, as recently reported in people with AD. Unfortunately, the authors did not report the mRNA expression levels to confirm an increase in filaggrin mRNA expression as well (Tan SP, et al. 2012). A third possible explanation for an increased transcription of filaggrin without an increase in protein translation in the affected tissue could indicate a defect in the translation machinery (e.g. translational efficiency and/or a post-translational dysregulation) leading to increased protein instability followed by increased protein degradation. In this latter case, the increased mRNA expression
could be due to a “feedback mechanism” in which the keratinocyte would increase the transcription signal trying to compensate for the lack of protein expression. Genetic mutations (SNPs) in the filaggrin gene and their association with canine AD are still controversial. Two studies (Wood SH, et al. 2010; Suriyaphol G, et al. 2011) have identified SNPs not confirmed by others (Barros RoqueJ, et al. 2009; Roque JB, et al. 2011; Salzmann CA, et al. 2011). In the former studies (Wood SH, et al. 2010; Suriyaphol G, et al. 2011) an association between SNPs, breed and geographic location has been suggested; three SNPs associated with canine AD have been identified in Labrador retrievers in the UK and small breed dogs (toy poodles, Shih tzu and pugs) in Thailand (Wood SH, et al. 2010; Suriyaphol G, et al. 2011).

The experimental model for canine AD has many advantages. One of these is the potential to monitor filaggrin mRNA expression in lesional and non-lesional skin over time in AD. The present study showed a decrease in filaggrin mRNA expression in healthy and atopic beagles after exposure to HDM (Day 3) followed by a slow trend towards a normalization of the filaggrin mRNA expression after resolution of cutaneous lesions (Day 10); however, the decreased expression was significant only in control beagles. Healthy beagles were similarly exposed to HDM to reduce the variability due to the mite treatment; however a greater decrease in filaggrin mRNA expression in healthy beagles, after exposure to HDM, could lead to the question if the increased expression in atopic skin is real or due to the relative decrease in filaggrin mRNA expression in healthy skin. Unfortunately, due to the nature of the technique used in the present study (relative-quantitative PCR) we cannot answer this question and more studies, using an absolute quantitative technique, are needed.
In terms of the protein expression, using IIF, a patchier distribution of the signal was detected in the lesional skin (Day 3) of AD dogs when compared with healthy controls after HDM exposure (Day 3). In addition, a patchier distribution of filaggrin expression was also seen in the atopic dogs when Day 3 was compared to Day 0 and 10. These results may suggest that the allergen exposure can alter filaggrin protein expression in both healthy and atopic beagles, as suggested in human patients (Howell MD, et al. 2007; McPherson T, et al. 2010; Gutowska-Owsiak D, et al. 2011). In particular, when the skin of healthy or atopic patients is disrupted, experimentally or naturally, an alteration of filaggrin mRNA and protein, as well as other proteins occurs (Howell MD, et al. 2007; McPherson T, et al. 2010; Gutowska-Owsiak D, et al. 2011). This phenomenon is not only due to the mechanical disruption of the skin barrier due to increased activity of exogenous and endogenous proteases, including caspase 14, but also for the interaction between proteases and Th2 cytokines (Howell MD, et al. 2007; McPherson T, et al. 2010; Gutowska-Owsiak D, et al. 2011).

Furthermore, a decrease in filaggrin mRNA levels over-time seen in healthy dogs is most likely due to a skin barrier disruption induced by exposure to HDM proteases. It has been reported recently that a marked decrease in filaggrin gene expression is present in healthy human skin after artificial barrier disruption using sodium lauryl sulphate, followed by an increase in mRNA levels four and seven days post-exposure (Nuttall TJ, et al. 2002). However, once the stimulus was eliminated and resolution of clinical lesions was achieved, a partial recovery of both filaggrin mRNA and protein expression was found. Filaggrin expression is affected by cytokines as demonstrated by in vitro studies using normal human keratinocyte cell cultures and showing decreased filaggrin expression after exposure to Th2 cytokines such as IL-

Examination of filaggrin protein distribution shows some distinct differences between humans and dogs. Whereas only decreased signal intensity has been reported in atopic humans (O’Regan GM, et al. 2009), different patterns of distribution have been reported in canine skin. Chervet et al. (2010) analysed the immunofluorescence signal in the skin of 18 naturally-affected atopic dogs (various breeds) and 16 healthy dogs (various breeds), using an anti-canine filaggrin polyclonal antibody against the C-terminus and showed four different patterns of distribution. The filaggrin signal was similar to healthy controls (3 of 18), or visibly decreased (7 of 18), or visibly decreased with a more granular and discontinuous distribution and large aberrant cytoplasmic vesicles (4 of 18), or with a complete lack of immunofluorescence signal (4 of 18) (Chervet L, et al. 2010). Compared with this study, our results showed a similarity with the third pattern; a discontinuous distribution of filaggrin staining. In addition, in our study a visible alteration of the filaggrin distribution associated with areas of complete loss of the immunofluorescence was mainly visible after allergen exposure (Day 3). The altered immunofluorescence signal was followed by an amelioration of the distribution after clinical resolution of the cutaneous lesions (Day 10), suggesting an increase in protein stability (Hvid M, et al. 2011).

Very recently, a new gene belonging to the filaggrin family, filaggrin-2, has been identified in human and murine skin (Wu Z, et al. 2009; Hansmann B, et al. 2012). This gene shares many similarities in the genomic sequence as well as protein distribution with filaggrin,
and its involvement in skin barrier alterations has been hypothesized (Hansmann B, et al. 2012). In dogs, an updated version of the canine database and its comparison with the human database suggested that the previously identified canine filaggrin gene, reported in this and in previous studies (Barros Roque J, et al. 2009; Chervet L, et al. 2010; Wood SH, et al., 2010; Roque JB, et al. 2011; Suriyaphol G, et al. 2011; Salzmann CA, et al. 2011; Theerawatanasirikul S, et al. 2012; Kanda S, et al. 2013), is more similar to human filaggrin 2 than human filaggrin gene. However, since little information on the human and murine filaggrin-2 gene have been published, and since all the veterinary literature on filaggrin has been based on the same gene, we preferred to use the “filaggrin” rather than filaggrin 2 name for the gene/protein studied here.

In summary, this study demonstrated that, as it is in people, filaggrin expression is altered in the skin of atopic dogs. Based on our results the action of HDM may play a fundamental role in skin barrier defect in canine AD. In fact, increased protein degradation may lead to an increase in filaggrin gene expression due to a feedback mechanism”. More studies are necessary to better understand the possible transcriptional/translational alterations present in atopic dogs and to investigate the presence of germline filaggrin mutations and their relation with canine AD. Finally, we confirmed that canine filaggrin is characterized by a ~54 kD protein identified by western blotting, confirming that canine filaggrin repeats are larger than its human counterpart (Kanda S, et al. 2013).
4.6 TABLES AND FIGURES

Table 2. Primers used

<table>
<thead>
<tr>
<th>Canine gene</th>
<th>Primer sequences</th>
<th>Efficiency (%)</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLG Forward: CTCAGCACAAGGAAGACAGG</td>
<td>110</td>
<td>105</td>
<td></td>
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<tr>
<td>FLG Reverse: TTGTGTTCTGGTGCTGCTGCTGTC</td>
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<tr>
<td>RPLO Forward: TTGTGGCTGCTGCTCCTGTG</td>
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</tr>
<tr>
<td>RPLO Reverse: ATCCTCGTCCGATTCCCTC</td>
<td></td>
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Figure 3. Average of the modified CADESI-03 (mCADESI-03) score before, during and after environmental challenge. (**: P≤0.05; bars: standard errors of the mean). Day 0 (before allergen exposure); Day 3 (acute lesional skin); Day 10 (post-challenged skin with resolution of the cutaneous lesions).
Figure 4. Relative mRNA expression between normal age and breed-matched healthy and atopic beagles for FLG; at each time point (2a) and over-time within each group (2b). (*: P≤0.05; **: P≤0.01; bars: standard errors of the mean). Day 0 (before allergen exposure); Day 3 (acute lesional skin); Day 10 (post-challenged skin with resolution of the cutaneous lesions).
Figure 5. Photomicrograph of canine skin showing indirect immunofluorescence of canine filaggrin. The immunofluorescence signal is detectable only in the stratum granulosum and lower stratum corneum. A more continuous, laminar and homogeneous distribution of the fluorescence is seen in healthy dogs, compared with a patchy and discontinuous pattern seen in AD dogs at each time point. The pictures are representative of all samples. Day 0 (before allergen exposure); Day 3 (acute lesional skin); Day 10 (post-challenged skin with resolution of the cutaneous lesions). Magnification 400x.

Figure 6. Western blotting analysis of the polyclonal anti-canine filaggrin antibody tested on nonatopic canine skin extract. Lane 1 contains 50μg of protein extract; Lane 2 contains 80μg of protein extract; Lane 3 contains 80μg of protein extract and it was used as negative control (no primary antibody).
4.7 REFERENCES


Wood SH, Ollier WE, Nuttall T, et al. Despite identifying some shared gene associations with human atopic dermatitis the use of multiple dog breeds from various locations limits detection


CHAPTER 5

EVALUATION OF CANINE ANTIMICROBIAL PEPTIDES IN INFECTED AND NONINFECTED CHRONIC ATOPIC SKIN

5.1 Abstract

Antimicrobial peptides (AMP) are small immune-modulatory peptides produced by epithelial and immune cells. Beta defensins (BDs) and cathelicidin (Cath) are the most studied AMPs. Recently increased cutaneous AMP expression was reported in atopic humans and in experimentally-induced atopic beagles.

Our goal was to analyse mRNA expression and protein levels of canine (c)BD1-like, cBD2-like/122, cBD3-like, cBD103, and cCath in healthy and naturally-affected atopic dogs with and without active skin infection along with their distribution in the epidermis using indirect immunofluorescence (IIF).

Skin biopsies were taken from 14 healthy and 11 atopic privately-owned dogs.

The mRNA levels of cBD1-like, cBD2-like/122, cBD3-like, cBD103, and cCath were quantified using quantitative real-time PCR. The protein levels of cBD3-like and cCath were analyzed by relative competitive inhibition ELISA, while the distribution of cBD2-like/122, cBD3-like, and cCath was detected by IIF.

Dogs with atopic dermatitis had significantly greater mRNA expression for cBD103 (p = 0.04) than control dogs. Furthermore, atopic skin with active infection had higher cBD103 mRNA expression (p = 0.01) and a lower cBD1-like mRNA expression (p=0.04) than atopic skin without infection. No significant differences in AMP protein level (cBD3-like and cCath) or epidermal distribution (cBD2-like/122, cBD3-like, and cCath) were seen between healthy and atopic dogs.
cBD103 mRNA expression was greater, while cBD1-like mRNA was lower in AD dogs with active infections. Work is needed to clarify the biological mechanisms and possible therapeutic options to maintain a healthy canine skin.

5.2 Introduction

Atopic dermatitis (AD) has been recently redefined by the International Task Force on Canine Atopic Dermatitis as ‘genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens’ (Halliwell R. 2006). In both humans and dogs, AD is extremely common, affecting up to 30% and 10% of the respective populations (Hilier A, et al. 2001; Bieber T. 2010). Recently, in both human and veterinary medicine, researchers have shown altered skin barrier integrity as the main factor involved in the pathogenesis and predisposition to AD (Inman AO, et al. 2001; Proksh E, et al. 2008; Shimada K, et al. 2009; Reiter LV, et al. 2009; Marsella R, et al. 2010; Santoro D, et al. 2010; De Benedetto A, et al. 2011; Irvine AD, et al. 2011; Olivry T, et al. 2011; Marsella R, et al. 2011; Yoon JS, et al. 2011; Marsella R, et al. 2011; Olivry T. 2011; De Benedetto A, et al. 2012; Cornegliani L, et al. 2012). As part of the skin barrier, antimicrobial peptides (AMPs) have been studied in different species including human (Marshall SH, et al. 2003; Jenssen H, et al. 2006; Linde A, et al. 2008; Leonard BC, et al. 2012). To date over 1000 AMPs, sub-classified based on their molecular structure, have been identified in diverse species (e.g. peptides, lipids, histones, etc.) (Marshall SH, et al. 2003; Jenssen H, et al. 2006). Of these peptides, β-defensins (BD) and cathelicidins (Cath) have received the most research attention. Such peptides have many functions; they have
antimicrobial activity against a variety of microorganisms, are potent angioactive and
chemotactic molecules, are involved in wound healing, act as potent “host defense peptides”
able to chemo-attract immune cells and respond to danger signals by alerting the adaptive
immune system, and they also modulate the innate and adaptive immune responses in higher
organisms (Jenssen H, et al. 2006; Shauber J & Gallo RL. 2008; Lai Y & Gallo RL. 2009; Nakatsuji
T & Gallo RL. 2012). An increased expression of AMPs (e.g. BDs, LL-37, psoriasin, and
ribonuclease 7) in some human inflammatory conditions, like AD and psoriasis, has
demonstrated an involvement of such AMPs in inflammatory skin conditions (Asano S, et al.
Schitterk B. 2011).

Few studies are published on the possible involvement of AMPs in the pathogenesis of
AD in dogs. In fact, only two studies have investigated the association of canine AMPs with
canine AD. The first using chronically naturally-affected atopic dogs and the second using
Results were similar to human studies showing increased mRNA expression of some canine
peptides (cBD1, cBD1-like, cBD3-like, and cCath) in lesional and nonlesional skin of AD dogs
when compared to healthy control dogs (Van Damme CM, et al. 2009; Santoro D, et al. 2011b);
however, decreased mRNA expression of cBD103 was only detected in naturally-affected atopic

In the above mentioned studies particular attention was made to avoid cutaneous
infections in order to reduce confounding factors. Although AMPs are known for their anti-
microbial effects, their behavior in actively infected atopic skin has not been reported.
However, the presence of cutaneous infections (bacteria and yeast) is extremely common in AD compared with other skin diseases (DeBoer D & Marsella R. 2001; Howell MD. 2007; Niyonsaba F, et al. 2007; Wollenberg A, et al. 2011), and a decreased production or production of nonfunctional of AMPs has been hypothesized as a possible cause of the higher susceptibility to skin infection in AD (Harder J, et al. 2010).

To gain more insight into the role of AMPs in canine AD, we analysed the mRNA expression of four cBDs (cBD1-like, cBD2-like/122, cBD3-like, and cBD103) and cCath in healthy and atopic dogs with and without active skin infection using a quantitative reverse transcriptase PCR (QRT-PCR). The cBDs and cCath used were selected based on previously demonstrated antimicrobial properties and presence in canine skin (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K & Brownlie J. 2010; Santoro D, et al. 2011a). In addition, we evaluated their distribution in the epidermis using indirect immunofluorescence (IIF) and their skin protein levels using competitive inhibition enzyme-linked immunosorbent assay (ciELISA).

5.3 Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee. All dogs entered the study with the owners’ written informed consent.

Inclusion criteria

Atopic dogs

The diagnosis of canine AD was based on compatible history, clinical findings and the exclusion of possible differential diagnoses for pruritus (e.g. scabies, demodicosis, food allergy, flea...
allergy) as previously reported (DeBoer D & Hillier A. 2001; Favrot C, et al. 2010). In particular, for dogs with nonseasonal pruritus a strict food trial with a novel protein source for at least ten weeks was done. When required, multiple skin scrapings were performed to rule out Demodex spp. and a miticidal drug trial was performed to rule out scabies. Dogs included in this group were divided in two subgroups based on the evidence of active skin infection (bacterial, Malassezia spp., or both). The diagnosis of infections by bacteria or yeast was based on clinical signs, history, and skin cytology with evidence of inflammatory cells, bacteria, yeasts, or both. All the dogs were on commercially available diets at the moment of the enrolment in this study.

**Healthy dogs**

Healthy control dogs were recruited from dogs presented to the Veterinary Teaching Hospital for annual vaccination or were dogs that belonged to the hospital staff. To be included in the study, the dogs had to have no history or presence of any cutaneous or systemic disease.

**Exclusion criteria**

**All dogs**

Dogs were excluded if topical, systemic and deposit glucocorticoids had been used within at least two, four, eight weeks, respectively, or if systemic or topical calcineurin inhibitors had been used within at least four weeks. Dogs were also excluded from the study if systemic or topical antibiotic or antifungal medications had been used within at least two weeks.

**Atopic dogs**
Dogs with AD were excluded from the study if there was a history of administration of allergen specific immunotherapy, or if there were other allergic conditions (e.g. food and flea allergy) or any other skin (e.g. endocrinopathies, neoplasia) or other systemic disease (e.g. parasitic, metabolic, neoplastic disease).

**Healthy dogs**

*Control dogs were excluded from the study if there was evidence of superficial or deep bacterial or Malassezia spp. infection.*

**Skin sample collection**

Two 8 mm skin biopsy samples were obtained from abdominal skin using local anesthesia [subcutaneous injection of 1mL of lidocaine HCl 2% (Hospira Inc., Lake Forest, IL, USA).] It was not necessary to sedate dogs for this procedure. One skin biopsy sample was immediately divided in quarters and placed in 1.5 ml microfuge tubes, and quickly flash frozen in liquid nitrogen, and then stored at –80 °C until processed for molecular evaluation (QRT-PCR). The other skin biopsy sample was immediately fixed in 10% neutral buffered formalin for IIF evaluation. The abdominal region was chosen for the skin biopsy site because it is an easily accessible area with low hair density and a common area involved in canine AD.

**Quantitative RT-PCR**

One quarter of the 8 mm skin biopsy sample was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA, USA) using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA, USA) reagents and then processed into RNA according to manufacturer’s protocol as described
Total RNA concentrations were determined at 260 nm using UV NanoDrop® spectrophotometry (Thermo Scientific, Wilmington, DE, USA), and integrity and quality of the RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc. Santa Clara, CA, USA). After DNase treatment using a Turbo DNA-free™ kit (Invitrogen, Carlsbad, CA, USA), total RNA (0.5 μg) was converted to complementary DNA (cDNA) by reverse transcription of mRNA using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Sense and antisense primers for each AMP (Table 3) were generated using Primer Designer software (Scientific and Educational Software, Inc.) as reported. Each primer was designed to cross an exon–exon boundary, maximizing the amplification specificity of the mRNA transcript target and minimizing amplification of any residual contaminating genomic DNA. The primers were generated from previously published Gene Bank AMP sequences (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K & Brownlie J. 2010). All primer sequences were subjected to Basic Local Alignment Search Tool (BLAST) comparison to the canine genome build 2.0 for unintended homologies. The relative mRNA expression levels were quantified using SYBR® Green (Qiagen, Valencia, CA, USA) and ABI (Applied Biosystems Inc., Foster City, CA, USA) quantitative RT-PCR methodology. All samples were tested in triplicate 25 μl reactions in an ABI 7500 Real Time PCR System (Applied Biosystems Inc, Foster City, CA, USA). PCR amplifications were carried out as followed: 50°C for two minutes; 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Amplifications were followed by dissociation (melting) curves to ensure specificity of the primers. The results were analysed using the comparative CT (cycle threshold) method, and the relative mRNA expression of each AMP was compared using the ΔΔCT method. This method is effective when the amplification efficiencies of the housekeeping
gene and target gene are close to 100%. When this criterion is fulfilled the formula is \(2^{-\Delta CT_{\text{experimental sample} - \Delta CT_{\text{control sample}}}}\) where \(\Delta Ct\) is the difference between the target gene and the normalizer gene expression (Pfaffl MW. 2001). All samples were normalized against the canine ribosomal protein L15 (RPLO). This gene was chosen due to the highly stable and consistent expression, previously shown for ribosomal genes in canine skin (Wood SH, et al. 2008).

**Antibody preparation**

Polyclonal anti-canine-AMPs were synthesized by the Immunological Resource Center at the authors’ Institution (Santoro D, et al. 2011a). Briefly, the canine anti-AMP antibodies were generated from previously published Gene Bank AMP sequences (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K & Brownlie L. 2010). All amino acid sequences were subjected to BLAST comparison to the canine genome build 2.0 for unintended homologies. Based on the aforementioned genetic and amino acid sequences the most immunogenic epitopes were chosen for each protein and synthetic peptides were created. Since the amino acid sequence of cBD1-like overlaps with part of both cBD2-like/122 and cBD3-like sequences, it was not possible to generate an appropriate polyclonal antibody against it. Antibody production was carried out using three female New Zealand white rabbits, 3.2-3.6 kg in weight (about 2-3 months old), purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA). The peptide conjugates were mixed with an adjuvant and subcutaneously injected into three rabbits. Titermax (Sigma, St. Louis, MO, USA) was used for the primary immunization and incomplete Freund’s adjuvant for all subsequent immunizations. Rabbits were immunized four times and their blood was tested for an immune response using an ELISA technique with pre-immune serum as the control.
When a satisfactory immune responses (positive up to 1:51,200 dilution) had been achieved, animals were exsanguinated and the crude sera were used. The antibodies were tested by Immunodot blotting using the synthetic peptide as antigen, resulting in a positive signal in up to a 1:10,000 dilution. In addition, to verify the specificity of the primary antibodies, the anti-cBD2-like/122, anti-cBD3-like and anti-cCath were tested by immune-absorption, incubating each antibody with a high concentration (20 µg/µl) of the respective and other synthetic peptides overnight at 4ºC before being applied to the tissue sections. An ELISA technique was also used to test possible cross-reactions with each of the peptides synthetized. Multiple attempts to generate anti-canine-cBD103 resulted in unsuitable antibodies for ELISA or IIF technique because part of the amino acidic sequence is only predicted.

**Indirect Immunofluorescence**

One half of the 8 mm skin biopsy sample was fixed in 10% neutral buffered formalin solution for no more than 48 h and then placed in phosphate buffer solution (PBS) until processed for IIF (Santoro D, et al. 2011a). Briefly, 3 µm sections were processed using the immunohistochemical polymer procedure. The sections were blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA, USA) followed by an extra wash using normal goat serum (BioGenex, San Ramon, CA, USA) as a blocking solution. Epitope retrieval was not necessary for cBD2-like/122 and cBD3-like, whereas it was required for cCath. In the latter case, the method consisted of using boiled sections, performed at 125-130ºC under 7.7-10.4 kg pressure for 30 s followed by a 10 second treatment at 90ºC. The sections were then stained for 1 h at room temperature using primary polyclonal rabbit antibodies specific for cBD2-like/122, cBD3-like, and cCath. The
primary antibodies were used at 1:200 dilution. Negative controls were established using the pre-immune serum at 1:200 dilution. The sections were washed with a blocking solution (Power Block®; BioGenex, San Ramon, CA, USA) and then incubated for 30 min at room temperature with a polyclonal goat anti-rabbit antibody bound with a green fluorochrome (Alexa Fluor® 488, Invitrogen, Carlsbad, CA, USA) at 1:1,000 dilution, according to manufacturer’s recommendations. Finally, DAPI (4',6-diamidino-2-phenylindole) (Invitrogen, Carlsbad, CA, USA) was used as a counterstain for nuclear detection. Specimens were mounted on glass slides using Vectashield® Mounting Medium (Vector laboratories, Burlingame, CA, USA). The skin sections were examined using an inverted fluorescence microscope (Nikon Eclipse TE 2000-S®, Nikon Inc., Shelton, CT, USA). The images were analyzed using MetaMorph® software (Version 63r1; Molecular Devices, Sunnyvale, CA, USA). Five representative fields at 400x magnification were examined for each section and digital images were recorded.

**Relative competitive inhibition enzyme-linked immunosorbent assay (ciELISA)**

One quarter of the 8 mm skin biopsy specimen was homogenized using a PowerGen 125 (Fisher Scientific, Pittsburgh, PA, USA) and then the proteins were extracted using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA, USA) reagents, according to the manufacturer’s protocol. The protein concentration was determined at 280nm using UV NanoDrop1000® spectrophotometry (Thermo Scientific, Wilmington, DE, USA). The ciELISA was then performed as previously described with some modifications (Muhumuza L, et al. 1998). The 96-well flat-bottom microtiter plates (Immunlon II® HB, Fischer, Pittsburgh, PA, USA) were coated with 50 µl/well of 50ng/ml synthetic cBD3-like or 10ng/ml synthetic cCath in coating PBS (BioRad,
Hercules, CA, USA) (pH 7.4) and left overnight at 4°C. After discarding the coating buffer, the plates were blocked with 100 µl/well of 10% fetal bovine serum (FBS) (Midwest Scientific, St. Louis, MO, USA) in PBS for 2 h at room temperature. The plates were then washed three times using a blocking solution containing 10% FBS and 0.05% Tween-20 (PBS-T) (BioRad, Hercules, CA, USA). A typical assay consisted of three sets of triplicate wells: 1) a triplicate wells (positive controls) receiving 50µl of specific anti-canine AMP peptide polyclonal serum (1:8,000 dilution for anti cBD3-like and 1:16,000 dilution for anti cCath) that would give the maximum optical density (OD). 2) Three duplicate wells (negative controls) receiving 50µl of PBS-T only or 50µl of secondary antibody only or 50µl of each specific pre-immune serum that would give the minimum OD. 3) A triplicate wells receiving 50µl/well of mixtures containing 25µl (50µg) of total protein extract (sample) and 25µl of serum dilution (final protein dilution of 2µg/µl). 4) A series of triplicate wells receiving a mixture of the serum dilution and ten-fold serial dilutions (from 1,000ng/mL to 0.1ng/mL) of the appropriate synthetic peptide, used to generate the inhibition standard curves.

The sera dilutions were chosen among those that gave OD values on the linear portion of the curve obtained from a two-fold serial titration of each serum on plates coated with the appropriate peptide. The protein extract served as a competitive inhibitor for the binding of the antibodies to the synthetic peptides coating the plates. The plates were incubated for 90 min at room temperature, washed five times using the blocking solution and 50µl of a secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG at 1:4,000 dilution) (BioRad, Hercules, CA, USA) was added and incubated in the dark for 1 h at room temperature. The plates were then washed seven times with blocking solution and 100µl/well of ABTS
(Kirkegaard and Perry Laboratories, Fischer, Pittsburgh, PA, USA), a colorimetric substrate, was added. The developed plates were read with an automated MR 500 ELISA reader (Dynatech, Chantilly, VA, USA). The percentage of inhibition obtained from each concentration of peptide was calculated using the average of the absorbance values of each triplicate wells and the average of the absorbance values of the positive control wells. To calculate the relative amount of peptide in the ‘unknown’ wells, the increasing percentage inhibition values were plotted versus the corresponding log concentration of the synthetic peptide used to generate the standard curves. Complete inhibition was obtained using 1,000ng/ml of each synthetic peptide. The cBD2-like/122 and cBD103 protein levels were not quantified due to a lack of antibodies suitable for ELISA-based quantitation. The relative amount of AMPs in the tissue extracts was expressed as the ng/ml of its synthetic peptide giving the same percentage of inhibition. This was then transformed in ng/mm² dividing the ng/ml by the surface area of the skin used for the extraction, one quarter of the 8 mm biopsy sample, as follows: ng/ml/(3.14[r²]).

**Statistical analysis**

Mean values and 95% confidence intervals were calculated for all results. The Kolmogorov-Smirnov test of normality was used (alpha = 0.05). Differences between ΔC_T [C_T AMP - C_T RPLO] of each AMP were compared using two-sample independent t-test. Differences between ΔC_T [C_T AMP - C_T RPLO] of cCath were compared using Mann-Whitney U test since they did not follow a normal distribution. Differences between ODs of each AMP were compared using two-sample independent t-test. Expecting a higher AMP levels for atopic dogs and atopic dogs with active skin infections, we used one-tail test and P values of ≤ 0.05 were considered significant.
Statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

5.4 Results

Dogs

A total of 25 dogs were enrolled in this study. Skin biopsy samples were taken from 14 healthy (nine males [three intact] and five spayed females) with a mean age of 4 ± 2.9 years (range: 1-10 years; median: three years); and 11 atopic dogs (four males [one intact] and six females [two intact]) with an average age of 5.6 ± 4.1 years (range: 1-15 years; median: 5 years). Of the atopic dogs, four of eleven had active skin infection (three of four bacteria and one of four mixed bacteria and yeast). The majority of dogs were mixed breed dogs (n=9). Five were Labrador retriever dogs. One each of the following dog breeds was included: pug, wirehaired dachshund, Australian shepherd dog, Siberian husky, Samoyed, bull terrier, Jack Russell terrier, miniature pincher, mastiff, beagle, and English setter (Table 4). No significant age or sex differences were seen between groups.

mRNA expression

When we compared mRNA expression of each AMP between the AD and healthy controls, a statistically significant higher expression (1.9 times) of cBD103 (P=0.04), but not any other AMP was shown in atopic dogs (Figure 7a). Similarly, cBD103 mRNA expression was significantly higher (3.8 times) in AD dogs with active skin infection when compared with atopic dogs without skin infection (P=0.01) (Figure 7b). This difference was 4.4 times higher when
compared to AD dogs with active skin infection and healthy controls \( (P=0.001) \) (Figure 7c). In contrast, cBD1-like mRNA expression level was significantly lower (0.48 times) in AD dogs with active skin infection compared with AD dogs without skin infection \( (P=0.04) \). (Figure 7b).

**Protein expression**

**Indirect Immunofluorescence**

No differences were noted in the distribution of AMPs in the skin of healthy and AD dogs demonstrated by IIF. The proteins were homogeneously distributed in skin samples from both groups of dogs. The cBD2-like/122 and cBD3-like were detectable in all layers of the epidermis, whereas cCath was detected predominantly in the stratum granulosum and stratum corneum, as previously reported (Figure 8) (Santoro D, et al. 2011a; Santoro D, et al. 2011b).

**ciELISA**

Using the ciELISA, we were able to calculate the relative amount of cBD3-like and cCath per mm\(^2\) of skin. The abdominal skin of healthy dogs had an amount of BD3-like corresponding to 1.1±0.95 ng/mm\(^2\) of synthetic cBD3-like and 1.4±0.99 ng/mm\(^2\) of synthetic cCath (Figure 9a). Abdominal skin of atopic dogs had an amount corresponding to 1.8±1.8 ng/mm\(^2\) and 4.7±8.6 ng/mm\(^2\) of synthetic cBD3-like and synthetic cCath, respectively (Figure 9a). The amounts of corresponding cBD3-like in atopic skin with and without active skin infection were 2.5±1.5 ng/mm\(^2\) and 1.4±1.9 ng/mm\(^2\), respectively (Figure 9b). The amounts of corresponding cCath were 5.15±10.54 ng/mm\(^2\) in dogs without infection, and 4±4.9 ng/mm\(^2\) in dogs with active infection, respectively (Figure 9b). When we compared the amount of cBD3-like and cCath
between the AD and healthy control groups, no statistically significant differences were seen (Figure 9a and 9b). However, a significant increase in cBD3-like and cCath was present when we compared AD dogs with active skin infection and healthy control (p=0.04 and p=0.05, respectively) (Figure 9c). A trend toward a significant increase of AD without skin infection vs. healthy control was also seen (p=0.08) (Figure 9c).

5.5 Discussion

Our data showed a significant increase of *cBD103* mRNA expression levels in skin of naturally-affected AD dogs when compared with healthy controls. These results are in accordance with recent studies by Bellardini *et al.* (2009) and Harder *et al.* (2010) in which a clear increase in mRNA expression and protein level of some AMPs, namely *LL-37, hBD2* and *hBD3/103A* (orthologue of *cBD103*), were demonstrated in the skin of atopic human patients when compared with healthy individuals. The results of the present study are also in accordance with results of a previous study in which we demonstrated increased mRNA expression levels of some AMPs in the skin of experimentally-induced AD beagles (Santoro D, et al. 2011b).

The reason for higher expression and production of AMPs in atopic skin compared with healthy controls is not completely understood. Many hypotheses have been proposed, including the effects of the local immunological milieu, the disruption of the cutaneous barrier, differing rates of protein degradation, and the presence of inflammatory cells (Nomura I, et al. 2003; Liu L, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006; Lai Y & Gallo RL. 2009; Schitteck B. 2011; Ahrens K, et al. 2011; Nakatsuji T & Gallo RL. 2012). The involvement of cytokines in the regulation of local AMPs is a subject of controversy. Although a strong inverse
correlation between human AMPs and T helper 2 cytokines expression, like interleukin (IL)-4, IL-10 and IL-13 has been shown in vitro (Nomura I, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006; Ahrens K, et al. 2011), an in vivo model has demonstrated higher expression of some AMPs in acute Th2-dominant AD lesions (Harder J, et al. 2010). In addition, a strong correlation between alterations of the skin barrier, naturally present in AD skin, and production of AMPs has been largely demonstrated in both mouse and human models (Fulton C, et al. 1997; De Benedetto A, et al. 2009; Ahrens K, et al. 2011). Increased degradation of active AMPs in atopic disease due to hyper-activation of several endogenous as well as exogenous proteases (Elias PM. 2005; Takai T & Ikeda S. 2011) has also been hypothesized in AD skin. In this latter case, the increased mRNA expression could be due to a ‘feedback mechanism’ in which the keratinocyte increases the genetic signal in an effort to repair the lack of protein expression.

The increase in cBD103 mRNA expression showed in this study is in disagreement with VanDamme et al. (2009) who reported a 2-fold decrease in mRNA expression of cBD103 in the skin of naturally-affected AD dogs. Possible explanations for this discrepancy could be differences in skin biopsy location, the different ages of the dogs enrolled in the two studies (older in our study), the treatment with antimicrobials prior to enrolment in the study (avoided in our study), and the potential use of different shampoos and topicals (antimicrobial versus moisturizing products) used before enrolment in the two studies. Different AMP expression has been demonstrated in different body sites in people, with areas more subject to friction and environmental challenge having a higher AMP expression (Ali RS, et al. 2001). Increased AMP expression has also been reported in older patients versus younger human patients (Matsuzaka K, et al. 2006).
We were not able to detect any significant differences in mRNA expression levels of the other AMPs (cBD1-like, cBD2-like/122, cBD3-like and cCath) between the two groups. These results are in contrast with a previous study showing an increase of such AMPs in the skin of experimentally-induced AD beagles (Santoro D, et al. 2011b). One explanation for this discord could be the higher variability of mRNA expression present in this study compared with the previous one. In fact, using a canine animal model allowed to control more efficiently for environmental and intrinsic variables, like shared environment (e.g. indoors versus outdoors), other pets in the household, age and breed (Wingate KV, et al. 2009).

Atopic dogs, like atopic humans, are more susceptible to bacterial and *Malassezia* skin infection, but only recently has there been an increased interest in the role of AMPs in this predisposition (Sang Y, et al. 2005; Sang Y, et al. 2007; Schauber J & Gallo RL. 2008; Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011b). However, no studies have reported expression patterns of AMPs in the skin of atopic dogs with or without active skin infection. The present study is the first to demonstrate higher mRNA expression for cBD103 and lower expression of cBD1-like, but not the other cBDs and cCath, in the skin of naturally-affected atopic dogs with active infections. We showed that mRNA expression of cBD103 was 3.8 times higher in infected skin when compared with noninfected skin. This difference increased to 4.4 times when comparing infected AD skin with the skin of healthy controls. This increased expression could be the direct effect of the bacterial stimulation as demonstrated *in vitro* (Sorensen OE, et al. 2005; Schauber J, et al. 2006). The significant decrease in cBD1-like mRNA expression level in AD dogs with active skin infection when compared with AD dogs
without skin infection could be due to less important antimicrobial effects of cBD1-like compared with other defensins, and for this reason not actively involved in skin infections.

We designed a semi-quantitative ciELISA for cBD3-like and cCath to detect the relative amounts of AMPs in canine abdominal skin. Although no significant differences were found between AD (total or without skin infection) and healthy skin, or between AD skin with and without skin infection, a significantly increased amount of cBD3-like and cCath was seen when infected AD skin was compared to healthy controls. These results may suggest an active stimulation of some AMPs by bacteria and/or yeasts in AD skin as previously demonstrated (Sorensen OE, et al. 2005; Schauber J, et al. 2006; Asano S, et al. 2008; Harder J, et al. 2010). Unfortunately, at this point in time antibodies to detect cBD103 protein expression are not available. It would have been interesting to have a comparison between mRNA expression and protein production to confirm the possible major involvement of this AMP in the skin of AD dogs. Finally, our study showed that there was no difference in cutaneous distribution of the tested AMPs between AD skin, with or without active infection, and healthy skin.

In conclusion, we have demonstrated that, as in people, a higher mRNA expression of cBD103 (orthologue of hBD3/103A) is present in atopic dogs than in healthy controls. This difference was more striking when comparing the skin of normal dogs to AD skin with active infection, showing a potentially active role of cBD103 in cutaneous infection in atopic dogs. In contrast, we showed a lower mRNA expression of cBD1-like in AD skin affected by active skin infection when compared with non-actively infected AD skin. These data may indicate that many AMPs may be involved in the cutaneous innate immunity as defense against external micro-organisms. In addition, it is possible that an alteration of the ratio between AMPs is the
cause of increased infections in atopic patients. This and previous studies have yielded mixed results, raising questions of the regulation and the production of AMPs in cutaneous infections, and which AMP ratio is more important in controlling bacterial and yeast infections. Which AMPs are sufficient to control skin infection in healthy and atopic dogs, at what concentrations and what is the action of topicals (e.g. shampoos, foams, sprays, etc.) on cutaneous AMP production. More work is needed to clarify the biological mechanisms and the possible therapeutic treatments to maintain a healthy canine skin.
### 5.6 TABLES AND FIGURES

**Table 3.** Primer used

<table>
<thead>
<tr>
<th>Canine gene</th>
<th>Primer sequences</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cBD1-like</em></td>
<td>Forward: CGAGTGGAAACTATGCTGT</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGAATCTGCTGAGATCAGAC</td>
<td></td>
</tr>
<tr>
<td><em>cBD2-like/122</em></td>
<td>Forward: AGTGGGAAACTATGCTGTCT</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGCTAAGTGTCAGAATTGC</td>
<td></td>
</tr>
<tr>
<td><em>cBD3-like</em></td>
<td>Forward: CAGACATAAAAACAGACACA</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGTTGACCATAAGGTGTAG</td>
<td></td>
</tr>
<tr>
<td><em>cCath</em></td>
<td>Forward: CACTGTTGCTACTGCTGTCTG</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTTGAAGCCATTACACAGCAC</td>
<td></td>
</tr>
<tr>
<td><em>cBD103</em></td>
<td>Forward: ACCTTGCCATCCAGTCTCAG</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGAACAGGCTCAAGAACAG</td>
<td></td>
</tr>
<tr>
<td><em>RPLO</em></td>
<td>Forward: TTGTGGCTGCTGCTCCTGTG</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Reverse: ATCCTCGTCCGATTCCCTCG</td>
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</tbody>
</table>
Table 4. Details of the 25 dogs enrolled in the study and the presence of active skin infection (M: Male intact; F: Female intact; Mc: Male castrated; Fs: Female spayed; AD: atopic dermatitis).

<table>
<thead>
<tr>
<th>N</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Breed</th>
<th>AD</th>
<th>Infection</th>
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<tr>
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<td>Mc</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>2</td>
<td>Mc</td>
<td>5</td>
<td>Labrador</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Mc</td>
<td>10</td>
<td>Mixed breed</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mc</td>
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<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>Mc</td>
<td>2</td>
<td>Pug</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>1</td>
<td>Labrador</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>7</td>
<td>Wirehaired dachshund</td>
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<td></td>
</tr>
<tr>
<td>8</td>
<td>Fs</td>
<td>4</td>
<td>Mixed breed</td>
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</tr>
<tr>
<td>9</td>
<td>Fs</td>
<td>4</td>
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<td>NO</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Fs</td>
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<td>Yes</td>
</tr>
<tr>
<td>11</td>
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<td>2</td>
<td>Australian shepherd</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>2</td>
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<td>No</td>
</tr>
<tr>
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<td>F</td>
<td>8</td>
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<td>Yes</td>
</tr>
<tr>
<td>14</td>
<td>Fs</td>
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<td>Yes</td>
</tr>
<tr>
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<td>M</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>8</td>
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<tr>
<td>17</td>
<td>Mc</td>
<td>4</td>
<td>Mastiff</td>
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<td>No</td>
</tr>
<tr>
<td>18</td>
<td>Fs</td>
<td>3</td>
<td>Siberian husky</td>
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<td></td>
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<tr>
<td>19</td>
<td>Fs</td>
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<td></td>
</tr>
<tr>
<td>20</td>
<td>Mc</td>
<td>6</td>
<td>Samoyed</td>
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</tr>
<tr>
<td>21</td>
<td>Fs</td>
<td>15</td>
<td>Beagle</td>
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<td>No</td>
</tr>
<tr>
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<td>Mc</td>
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<td>1</td>
<td>Bull terrier</td>
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<tr>
<td>25</td>
<td>Fs</td>
<td>3</td>
<td>Jack Russell terrier</td>
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<td></td>
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</table>
**Figure 7.** Relative mRNA expression for each antimicrobial peptide in atopic dogs with and without skin infection compared with normal control dogs. a) comparison between healthy and AD dogs; b) comparison between AD dogs with and without active skin infection; c) comparison of healthy dogs and AD dogs with and without active skin infection. AD: atopic dermatitis; AD w/o: atopic dermatitis without skin infection; AD with: atopic dermatitis with active skin infection. Groups were compared using the Tukey’s Multiple Comparison Test (* $P \leq 0.05$; **: $P \leq 0.01$; ***$P \leq 0.001$; Bars: standard error of the mean).
**Figure 8.** IIF of cBD2-like/122, cBD3-like, cCath: Photomicrograph of canine skin. cBD2-like/122 and cBD3-like fluorescence is diffusely distributed throughout the epidermis and scattered positive cells are also present in the superficial dermis; cCath is mainly detectable in the stratum granulosum and corneum along with few positive cells in the superficial dermis. The pictures are representative of all samples. AD: atopic dermatitis; AD w/o: atopic dermatitis without skin infection; AD with: atopic dermatitis with active skin infection.
Figure 9. Protein levels for each antimicrobial peptide in atopic dogs with and without skin infection compared with normal control dogs. a) comparison between healthy and AD dogs; b) comparison between AD dogs with and without active skin infection; c) comparison of healthy dogs and AD dogs with and without active skin infection. AD: atopic dermatitis; AD w/o: atopic dermatitis without skin infection; AD with: atopic dermatitis with active skin infection. Groups were compared using the Tukey’s Multiple Comparison Test (*P≤0.05; Bars: standard error of the mean).


CHAPTER 6
EVALUATION OF ANTIMICROBIAL PEPTIDES AND CYTOKINE PRODUCTION IN PRIMARY KERATINOCYTE CELL CULTURES FROM HEALTHY AND ATOPIC BEAGLES

6.1 Abstract
Antimicrobial peptides (AMPs) are a broad group of small proteins present in epithelial tissues. The most studied AMPs are β-defensins (BDs) and cathelicidins (Cath). These are involved in the defense against pathogenic organisms and the orchestration of innate and adaptive immunity through interaction with several cytokines.

The purpose of this study was to evaluate/compare cBD3-like, cCath, and cytokine production in cultured keratinocytes harvested from healthy and atopic beagles.

Canine keratinocytes were collected from seven atopic and five healthy age-matched beagles. Second passage keratinocytes were used. The keratinocytes were stimulated with several immune-stimulants for 24 hours. Supernatant and cell extracts were collected and the presence of AMPs and cytokines measured. P values ≤0.05 were considered significant.

A significantly higher production of cBD3-like was present at baseline in the culture supernatant of keratinocytes obtained from atopic dogs compared with the supernatants of keratinocytes from healthy dogs (p=0.05). After stimulation with IL-17 and lipopolysaccharide, keratinocytes from healthy dogs produced more cBD3-like than those from atopic dogs (p=0.0035 and p=0.035, respectively). Cultures from both healthy and atopic dogs when stimulated with several immunogens, showed a significant increase in production of cCath compared to respective baselines. Cultured keratinocytes from atopic dogs produced higher
amount of IL-8 and keratinocyte-derived chemokine-like than those from healthy dogs. The amount of IL-8 further increased after stimulation with house dust mite extract (HDM), but INF-γ production decreased after stimulation with HDM.

The baseline production of cBD3-like by cultured keratinocytes from atopic dogs was greater than production from healthy dogs (p=0.05). The production did not further increase after stimulation with the immunogens tested. These results may suggest a state of over-stimulation of keratinocytes in atopic subjects that may lead to a failure to respond to immunological stimuli with increased production of AMPs. More studies are needed to better understand if defects in the AMPs’ pathway are present in canine keratinocytes. Healthy and atopic keratinocytes respond differently to immunological stimuli producing different levels of pro-inflammatory cytokines.

6.2 Introduction

Antimicrobial peptides (AMPs) are an extremely diverse group of small proteins that are present in all forms of life, from bacteria to mammals. To date over 1,500 AMPs have been identified in diverse species and subdivided into categories, primarily based on molecular structure (Zanetti M, et al. 1995; Marshall SH & Arena G. 2003; Jenssen H, et al. 2006). AMPs have multiple functions, the most important of which includes defense against external pathogens and the orchestration of the innate and adaptive immune response in higher organisms (Zanetti M, et al. 1995; Gallo RL, et al. 2002; Oppenheim JJ et al. 2003; Jenssen H, et al. 2006; Achauber J & Gallo RL. 2008). AMPs can be expressed systemically in the hemolymph of insects or be localized in the tissues most susceptible to contact with external agents in
amphibians, fish, birds, and mammals (e.g. mucous epithelia and keratinocytes) (Zanetti M, et al. 1995).

In mammals the two most studied families of AMPs include defensins (α and β) and cathelicidins (Oppenheim JJ, et al. 2003). Defensins are characterized by six cysteine residues and are divided into three subgroups based on the alignment of disulfide bridges and their molecular structure. To date, four β-defensins (hBDs) have been identified in human skin (Oppenheim JJ, et al. 2003), whereas seven β-defensins (cBDs) have been identified in canine testes and/or skin (Sang Y, et al. 2005; Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011a; Santoro D, et al. 2011b). Cathelicidins (Cath) contain a similar and evolutionarily conserved “cathelin precursor” domain (Zanetti M, et al. 1995). Cath is characterized by an N-terminal signal peptide, a highly conserved pro-sequence, a structurally variable cationic peptide at the C-terminus (Tang YQ, et al. 1999), and occur in many different species including canine (Oppenheim JJ, et al. 2003; Santoro D, et al. 2011a; Sang Y, et al. 2007; Linde A, et al. 2008).

In people, a significant relationship has been shown between AMPs and cytokines of both T helper (Th) and T regulatory (Treg) cells in inflammatory skin diseases such as atopic dermatitis (AD) and psoriasis (Niyonsaba F, et al. 2007). hBD 1-4 and Cath are increased in psoriasis and in chronic AD, but not in acute AD (Nomura I, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006; Bellardini N, et al. 2009). This latter pattern might be due to an increased expression of Th2 cytokines in the acute AD that may inhibit the production of such AMPs in early stages (Howell MD, et al. 2005; Howell MD, et al. 2006; Howell MD. 2007), followed by a more specific Th1 environment in chronic AD which can induce secretion of...
AMPs. An increased production of AMPs has been observed in keratinocytes exposed to several immunogens including those characteristic of Th1 (e.g. Interferon \([\text{INF}-\gamma]\), pro-inflammatory (e.g. IL-1, IL-17, IL-22) (Schauber J, et al. 2006; Nogales KE, et al. 2008), and Treg cytokines (e.g. IL-10) (Howell MD, et al. 2005), bacterial extracts (e.g. lipopolysaccharide [LPS] and lipotheoic acid [LTA]), and vitamin D3 (Schauber J, et al. 2006). These studies suggest that the secretion of AMPs may be influencial by the cutaneous immunological milieu.

In veterinary medicine there are only few studies published that investigated the expression of AMPs in canine skin (Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011a; Santoro D, et al. 2011b; Leonard BC, et al. 2012). Of those, two have been done using healthy dogs (Wingate KV, et al. 2009; Santoro D, et al. 2011a), while the others have investigated the expression of AMPs in atopic dogs (Van Damme CM, et al. 2009; Santoro D, et al. 2011b; Leonard BC, et al. 2012). The latter studies analyzed the expression of different AMPs in canine AD. One of those studies (Santoro D, et al. 2011b) analyzed the expression of cBD1-like, cBD2-like/122, cBD3-like, and cCath in acute cAD. The results claimed an increased mRNA expression of cBD1-like, cBD3-like, and cCath, but not cBD2-like, in the lesional and nonlesional skin of atopic beagles compared with healthy age- and breed-matched dogs (Santoro D, et al. 2011b). These data were later confirmed by another study in which an increased mRNA expression of cBD103 was observed in naturally affected atopic dogs, with and without an active skin infection, compared with healthy controls (Santoro D, et al. 2013). This latter study is contradictory to two other studies in which researchers reported a decreased mRNA expression of cBD103 in the skin of naturally affected atopic dogs (Van Damme CM, et al. 2009; Leonard BC, et al. 2012). To date, no studies have been published regarding the
production of AMPs and cytokines by canine keratinocytes when exposed to different immunological stimulants. The immunological mechanisms involved in the interactions between cAMPs, cytokine production, and the extracellular environment, still need to be investigated.

Thus, the goal of this study was to quantify the production of cBD3-like and cCath by keratinocytes before and after exposure to different immune-stimulants (cytokines characteristic of Th1 vs. Th2 responses, bacterial molecules, vitamin D₃, and house dust mite extract [HDM]), and also the production of several cytokines (Granulocyte-Monocyte-Colony stimulating factor [GM-CSF], IFN-γ, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, Interferon gamma-induced protein 10 [IP-10/CXCL10], keratinocyte-derived chemokine-like [KC-like/CXCL1], monocyte chemotactic protein-1 [MCP-1/CCL2], and Tumor necrosis factor [TNF]-α) by keratinocytes before and after exposure to the antigen HDM. This study utilized keratinocytes harvested from both healthy and atopic beagles.

6.3 Materials and Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Florida.

Animals

Atopic dogs

Seven experimentally-sensitized atopic beagle dogs (3 male, 4 female; age: 7 years) belonging to a colony that has been validated as a model for canine AD were used (Marsella R & Olivry T.
These dogs were sensitized to HDM and show low grade AD when not exposed to HDM, but strongly react when under allergenic challenge.

**Normal dogs**

Five healthy unrelated beagle dogs (2 male, 3 female; age: 7 years) with no history of skin disease or pruritus were included in this study as age- and breed-matched controls.

**Housing conditions**

The atopic dogs were housed indoors in individual temperature- and humidity-controlled cement runs (22–24°C; relative humidity of 68–72%). The runs were washed daily using a high-temperature and high-pressure wash (water and bleach). Air filters were changed routinely and stuffed toys, carpets, soft bedding or anything that could trap dust were not allowed in the runs. MITE-T-Fast™ Allergen Detection System (Aveho Biosciences, Tullahoma, TN, USA) was used monthly, and between challenges, on walls and filters to ensure the absence of HDM in the environment. The dogs were fed a commercial maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).

Similarly, the healthy controls were housed indoors, in individual cement runs, in a temperature- and humidity-controlled facility (22–24°C; relative humidity of 68–72%). The runs were cleaned daily using a high-temperature and high-pressure wash (water and bleach). The dogs were fed a commercial maintenance diet (Hill’s Science Diet Adult Maintenance kibble formulation; Hill’s Pet Nutrition Inc., Topeka, KS, USA).
Skin sample collection

Three 8-mm skin biopsy punches from either the inguinal or the ventral thorax area from nonlesional areas were collected following subcutaneous injection of 1 mL of lidocaine (Lidocaine HCl 2%, Hospira Inc., Lake Forest, IL, USA). The skin samples were collected using a sterile technique and placed in cold Dulbecco’s phosphate buffered saline (PBS) and transported on ice in the laboratory. The skin samples were then placed in betadine solution for 15 minutes and then washed multiple times in PBS.

Cell culture

After washed in PBS the skin samples were immediately dissected into 0.5–1 mm² pieces while submerged in serum-free epidermal keratinocyte growth medium (CnT-09®, CellnTec, Bern, Switzerland). Then the skin was placed in 1.25 U/ml of dispase (Dispase I, Sigma-Aldrich, St. Louis, MO, USA) solution at 4°C overnight in sterile tubes. The next day the skin was placed in a sterile Petri dish with CnT-09® medium and the epidermis detached from the dermis. The epidermal tissue was then transferred into another Petri dish and submerged in TrypLE™ Select (Invitrogen, Carlsbad, CA, USA) for 30 minutes at 37°C and gently agitated to dissociate basal keratinocytes. Then the cell suspension was collected and centrifuged at 1,100 rpm for 10 minutes at 25°C. The cell pellet was then resuspended in a 1 ml of CnT-09® medium and the cells counted. 1x 10⁵ cells were collected and immediately flash frozen for later use. 1x 10⁴ cells were collected and used for morphological and cytoskeletal evaluation.
The morphological and cytoskeletal characteristics of the keratinocytes were verified using an immunofluorescence technique with a monoclonal antbroad spectrum keratin and a polyclonal antivimentin antibody (BioGenex San Ramon, CA, USA). Briefly, primary keratinocytes were fixed with a 10% buffered formalin solution in PBS for 10 minutes. After washing with PBS three times, cells were blocked using a casein solution (Power Block®; BioGenex, San Ramon, CA, USA). A second blocking step was added using normal goat serum (BioGenex, San Ramon, CA, USA). The slides were then stained for 1 hour at room temperature using primary rabbit polyclonal anticytokeratins antibody (Dako, Carpinteria, CA, USA) at a 1:500 dilution. The slides were then washed with a blocking solution (Power Block®) and incubated for 30 min at room temperature with a polyclonal goat anti-rabbit antibody bound with a red fluorochrome (Alexa Fluor 594; Invitrogen, NY, USA) at a 1:1000 dilution, according to the manufacturer’s recommendations. The cells were then blocked with Power Block® and goat serum again and incubated for 1 hour at room temperature with a prediluted primary mouse monoclonal anti-vimentin antibody (BioGenex, San Ramon, CA, USA). The slides were washed with Power Block® and incubated for 30 min at room temperature with a polyclonal goat anti-mouse antibody bound with a green fluorochrome (Alexa Fluor 488; Invitrogen, NY, USA) at a 1:1000 dilution, according to the manufacturer’s recommendations. Finally, the sections were counterstained using 4’,6-diamidino-2-phenylindole (DAPI; Invitrogen) for nuclear detection. Specimens were mounted on glass slides using Vectashield® Mounting Medium (Vector Laboratories, Burlingame, CA, USA).
Figure 10 shows the keratinocytes stained with antikeratin and antivimentin antibodies. Keratinocytes reacted to antikeratin antibody and exhibited red fluorescence staining, whereas no antivimentin fluorescence staining is visible (Figure 10).

**Subculture of keratinocytes**

$1 \times 10^5$ cells were slowly thawed and placed in T25 flasks (TPP, St. Louis, MO, USA) containing CnT-09® enriched with fetal bovine serum and 8ng/ml of cholera toxin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C, with 5% CO$_2$. Cell outgrowth was monitored daily and the medium changed every third day. Once keratinocyte cultures reached 80% confluence, they were split and placed into 48-well plates (Nalge-Nunc, Pittsburgh, PA, USA) at the density of $2 \times 10^6$ cells/cm$^2$.

Pleated keratinocyte cultures were monitored until there was 60-70% confluence and then the keratinocytes were starved for 24 hours using “basic” CnT-09® without supplements or cholera toxin. Next the supernatant was collected and discarded and fresh, “basic” medium added to each well. Different immune-stimulants were added to the keratinocytes. Each immune-stimulant was assayed in duplicate. The keratinocytes were stimulated for 24 hours with the appropriate concentrations of ultra-pure *E. coli*-derived lipopolysaccharide (1µg/ml) (Klukowska-Rotzler J, et al. 2013), Pam3Cys-Ser-(Lys)4 (10µg/ml) (Klukowska-Rotzler J, et al. 2013), canine recombinant IL-1 (10ng/ml), canine recombinant IL-17 (0.5ng/ml), canine recombinant INF-γ (30ng/ml), 1,25(OH)$_2$VD$_3$ (25µM/ml), canine recombinant IL-4 (50 ng/ml), canine recombinant IL-10 (5ng/ml), HDM crude extract (240ng/ml), and phorbol 12-myristate.
13-acetate (PMA) (50ng/ml) (Klukowska-Rotzler J, et al. 2013), individually. Two wells used as baseline controls which contained only cells and medium.

Similarly, for the detection of cytokine production, a 48-well plate was used. After a 24 hour-starvation period, 60-70% confluent keratinocytes were stimulated with HDM (240ng/ml) for 24 hours and the supernatant collected. Each assay was performed in triplicate. Three wells were used as baseline controls which contained only cells and medium.

**Antibody preparation**

Polyclonal anti-canine-cBD3-like and cCath antibodies were synthesized as previously described by Santoro D, et al. (2011a). Briefly, the canine anti-AMP antibodies were produced based on genetic sequences previously identified. The amino acid sequence was subject to BLAST comparison to the canine genome build 2.0 for unintended homologies. Based on the aforementioned genetic and amino acid sequences, the most immunogenic epitopes were chosen and synthetic peptides created. Antibody production was carried out using two healthy, 3 month old, female New Zealand rabbits, 3.4 and 3.6 kg in weight, purchased from Harlan Laboratories, Inc. (Indianapolis, IN, USA). The peptide conjugates were mixed with an adjuvant and subcutaneously injected into the rabbits. Titermax (Sigma, St. Louis, MO, USA) was used for the primary immunization and incomplete Freund’s adjuvant for all subsequent immunizations. The rabbits were immunized four times and blood samples were tested for an immune response using an ELISA technique. The pre-immune serum was used as a negative control. When a satisfactory immune response was achieved (positive up to 1: 51,200 dilution), the rabbits were exsanguinated and the crude sera were used. The specificity of the anti-canine-
cBD3-like and cCath antibodies was tested by immune-absorption on tissue sections. The antibody was incubated with a high concentration (20 µg/µl) of the synthetic peptide overnight at 4°C before being applied to the tissue sections (Santoro D, et al. 2011a).

Relative competitive inhibition enzyme-linked immunosorbent assay (ciELISA)

After stimulation, the supernatant was collected and immediately frozen in -80°C until assayed. Each well of the 48-well plates was filled with 0.5ml of cell lysing buffer (RLT, Qiagen, Valencia, CA, USA) and the proteins extracted using the AllPrep® RNA/protein kit (Qiagen, Valencia, CA, USA) reagents, according to the manufacturer’s protocol. The protein concentration was determined at 280nm using UV NanoDrop1000® spectrophotometry (Thermo Scientific, Wilmington, DE, USA). The ciELISA was then performed as previously described by Santoro D, et al. (2013). Briefly, 96-well flat-bottom microtiter plates (Immunlon II® HB, Fischer, Pittsburgh, PA, USA) were coated with 50 µl/well of 50ng/ml synthetic cBD3-like or 10ng/ml synthetic cCath in coating PBS (BioRad, Hercules, CA, USA) (pH 7.4) and left overnight at 4°C. After discarding the coating buffer, the plates were blocked with 100 µl/well of 10% fetal bovine serum (FBS) (Midwest Scientific, St. Louis, MO, USA) in PBS for 2 hours at room temperature. The plates were then washed three times using a blocking solution containing 10% FBS and 0.05% Tween-20 (PBS-T) (BioRad, Hercules, CA, USA). A typical assay consisted of three sets of triplicate wells: 1) a triplicate wells (positive controls) receiving 50µl of specific anti-canine AMP peptide polyclonal serum (1:8,000 dilution for anti cBD3-like and 1:16,000 dilution for anti cCath) that would give the maximum optical density (OD). 2) Three duplicate wells (negative controls) receiving 50µl of PBS-T only or 50µl of secondary antibody only or 50µl of each
specific pre-immune serum that would give the minimum OD. 3) one triplicate well receiving 50μl/well of mixtures containing 25μl of supernatant or containing 30μg of total protein extract (sample) and 25μl of specific anti-canine AMP peptide polyclonal serum dilution. 4) A series of triplicate wells receiving a mixture of specific anti-canine AMP peptide polyclonal serum dilution and ten-fold serial dilutions (from 100ng/mL to 0.01ng/mL) of the appropriate synthetic peptide, used to generate the inhibition standard curves.

The sera dilutions were chosen among those that gave OD values on the linear portion of the curve obtained from a two-fold serial titration of each serum on plates coated with the appropriate peptide. The supernatant or the protein extract served as a competitive inhibitor for the binding of the antibodies to the synthetic peptides coating the plates. The plates were incubated for 90 minutes at room temperature, washed five times using the blocking solution and 50μl of a secondary antibody (horseradish peroxidase conjugated goat anti-rabbit IgG at 1:4,000 dilution) (BioRad, Hercules, CA, USA) was added and incubated in the dark for 1 hour at room temperature. The plates were then washed seven times with blocking solution and 100μl/well of ABTS (Kirkegaard and Perry Laboratories, Fischer, Pittsburgh, PA, USA), a colorimetric substrate, was added. The developed plates were read with an automated MR 500 ELISA reader (Dynatech, Chantilly, VA, USA). The percentage of inhibition obtained from each concentration of peptide was calculated using the average of the absorbance values of each triplicate wells and the average of the absorbance values of the positive control wells. To calculate the relative amount of peptide in the ‘unknown’ wells, the increasing percentage inhibition values were plotted versus the corresponding log concentration of the synthetic peptide used to generate the standard curves. The relative amount of AMPs in the cell extracts
and in the supernatant was expressed as the ng/ml of its synthetic peptide giving the same percentage of inhibition.

**Cytokine assays: Milliplex MAP canine Multiplex® assay**

Multiple cytokine analysis was done using the Milliplex MAP canine Multiplex® assay (Millipore, Billerica, MA, USA), according to manufacturer’s protocol. Thirteen canine cytokines (GM-CSF, IFN-γ, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10/CXCL10, KC-like/CXCL1, MCP-1/CCL2, and TNF-α) were tested simultaneously using the Luminex-100 system Version 1.7 (Luminex, Austin, TX). Data analysis was performed using the MasterPlex QT 1.0 system (MiraiBio, Alameda, CA). A five-parameter regression formula was used to calculate the sample concentrations from the standard curves.

**Statistical analysis**

Mean values and 95% confidence intervals were calculated for all results. The Kolmogorov-Smirnov test of normality was used (alpha = 0.05). Differences between ODs of each AMP in each group were compared using two-sample independent t-test (between-group effect). Repeated measurement ANOVA was used to compare the expression of each AMP after exposure to the different immune-stimulators (within-group effect). Paired t-test or Mann-Whitney test were used to compare each cytokine in the two groups (between-group effect) or before and after exposure to HDM (within-group effect). Expecting a higher AMP and cytokine levels for atopic dogs we used one-tail test and P values of ≤ 0.05 were considered significant.
Statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

6.4 Results
cBD3-like and cCath: ciELISA supernatant

Using the ciELISA, we were able to calculate the relative amount of cBD3-like and cCath per ml of supernatant. The concentration of cBD3-like peptide present in the supernatant collected from keratinocytes harvested from healthy beagles at baseline was equivalent to 17.9 ± 4.5 ng/ml of synthetic cBD3-like (Figure 11). The concentration of cCath peptide present in the supernatant collected from keratinocytes harvested from healthy beagles at baseline was equivalent to 1.4 ± 0.33 ng/ml of synthetic cCath (Figure 12). The concentration of cBD3-like peptide present in the supernatant collected from keratinocytes harvested from atopic beagles at baseline was equivalent to 30.5 ± 18.2 ng/ml and 1.7 ± 0.5 ng/ml of synthetic cBD3-like and synthetic cCath, respectively. Statistical analysis of these data indicates that cultured keratinocytes from atopic dogs produced a statistically greater amount of cBD3-like compared with cultured keratinocytes from healthy dogs (p=0.05) (Figure 11). After stimulation with LPS and IL-17 “healthy” keratinocytes produced more cBD3-like than keratinocytes from atopic dogs (p=0.035 and p=0.0035, respectively) (Figure 11). A significant increase of cBD3-like was also observed in the cultures of “healthy” keratinocytes after stimulation with LPS (p<0.01), IL-17 (p<0.001), IL-4 (p<0.05), IL-10 (p<0.01), and PMA (p<0.05) when compared to baseline production (Figure 11). These differences were not statistically significant in the supernatant collected from “atopic” keratinocytes.
There was no statistically significant difference in cCath production between “healthy” and “atopic” keratinocyte cultures (Figure 12). Keratinocytes cultured from healthy dogs did show a statistically significant increase in cCath production compared to baseline production after stimulation with LPS (p<0.01), IL-17 (p<0.001), INF-γ (p<0.001), Vitamin D (p<0.05), IL-4 (p<0.001), IL-10 (p<0.05), HDM (p<0.01), and PMA (p<0.01) (Figure 12). Similarly, an increased secretion of cCath was observed in “atopic” keratinocytes after stimulation with LPS (p<0.001), Pam3 (p<0.001), IL-17 (p<0.001), INF-γ (p<0.001), Vitamin D (p<0.01), IL-4 (p<0.001), IL-10 (p<0.05), HDM (p<0.001), and PMA (p<0.001) (Figure 12).

cBD3-like: ciELISA cell extract

Due to the low amount of protein extract obtained, we had to limit the assay to one AMP. cBD3-like was selected based on the results obtained by the analysis of the supernatant. We compared the amount of protein secreted by the cultures and the proteins retained in the cells to see if differences were present among groups.

Therefore, ciELISA was performed on the cell extract of the control, LPS, IL-17, and HDM cultures of keratinocytes from both healthy and atopic dogs. No statistically significant differences were found and no significant comparison could be made with the data obtained from the supernatants. In all extracts from the stimulated cells, the amount of cBD3-like was higher but not significantly when compared to the extracts of the same cells not stimulated.

The ciELISA showed a significant increase in the percentage of inhibition of cBD3-like in the extracts obtained from cells before and after stimulation with each stimulant. When these results were compared with the supernatants data, we did not find any statistically significant
differences in cBD3-like protein expression before or after stimulation in either “healthy” or “atopic” keratinocytes (within-group effect) or between the two groups (between-group effect) (Figure 13).

**Cytokine expression: Luminex®**

Using Luminex® technology we were able to evaluate 13 cytokines simultaneously. Of those 13 cytokines only seven (IL-7, IL-8, IL-10, IL15, INF-γ, KC-like, and MCP-1) were detected in the collected supernates. When we compared the cytokine levels produced by “healthy” and “atopic” keratinocytes, we were not able to show any significant difference between the two groups at baseline (Figure 14a). However, an increased production of KC-like (p=0.05) and IL-8 (p=0.04) was present in “atopic” keratinocytes after exposure to HDM when compared with “healthy” keratinocytes (Figure 14b).

When we compared the production of cytokines before and after exposure to HDM in the same group, we were able to show a significant increase in production of IL-8 (p=0.01) and MCP-1 (p=0.05) in “atopic” keratinocytes (Figure 15b). A trend (p=0.09) toward an increased expression of IL-8 was present in “healthy” keratinocytes. INF-γ production was significantly decreased (p=0.04) in “healthy” keratinocytes after exposure to HDM, while a change in INF-γ production was not evident in “atopic” keratinocytes (Figure 15a).

**6.5 Discussion**

The results of this study demonstrated that cBD3-like and cCath were produced by second passage primary keratinocytes harvested from both healthy and atopic beagles.
Although a significantly higher production of cBD3-like was measured in “atopic” keratinocytes at baseline when compared with “healthy” keratinocytes, this difference was not observed after stimulation with any of the immunogens tested. On the contrary, after 24 hours of stimulation with LPS and IL-17, the “healthy” keratinocytes significantly increased production of cBD3-like when compared with “atopic” keratinocytes. When we compared the production of cBD3-like before and after stimulation, a significant increase was only seen in “healthy” keratinocytes. These results may indirectly confirm a “hyperactivated” cellular state due to “hyperactivation” of nuclear transcription factors, such as NFκB or AP-1, present in cultured “atopic” keratinocytes at baseline, as previously suggested in dogs by Chervet L, et al. (2010). This may explain the higher production of cBD3-like. However, contrary to “healthy” canine keratinocytes, the “atopic” cells did not react with an increased production of AMPs to any immunological stimuli added to the culture media. This suggests an intrinsic alteration of the epithelial cells in atopic dogs. There are a few reported human studies (Schauber J, et al. 2006; Nogrles KE, et al. 2008), that reported an increased expression of AMPs after stimulation with several immunogens (e.g. Th1, Treg, pro-inflammatory cytokines, and bacterial extracts). Such increases were not observed in this study; however, our results correlate with previous canine studies in which a higher expression of AMPs was seen in the nonlesional skin of experimentally-induced atopic beagles (Santoro D, et al. 2011b; Santoro D, et al. 2013).

We also detected cCath in the supernatant of both “healthy” and “atopic” keratinocytes. However, no significant differences were seen when the two populations were compared either at baseline or after stimulation. When the production of cCath after stimulation was compared with the baseline expression, a significant increase was observed in both groups exposed to
several immunogens. Converse to the cBD3-like results, the cCath results are in agreement with the human literature in which an increase of LL-37, the cCath homologue, was seen after stimulation with immunogens (Schauber J, et al. 2006; Nograles KE, et al. 2008). It was also demonstrated that as LL-37 is stimulated in humans, cCath is stimulated by exposure to Vitamin D₃. This suggests the presence of vitamin D response elements (VDRE) in the canine gene encoding Cath.

When the concentrations (ng/ml) of cBD3-like and cCath present in the supernatants at both baseline and after stimulation were compared, a higher concentration of the former was seen. This can be explained by the fact that cCath is primarily produced in the higher differentiated layers of the epidermis (stratum granulosum and corneum) (Santoro D, et al. 2011a; Santoro D, et al. 2011b; Santoro D, et al. 2013) which are not present in the culture system used in this study. Although in “atopic” keratinocytes, cBD3-like did not increase after stimulation with any of the immunogens tested, cCath did. These data may suggest that there are different activation pathways for cBD3-like and cCath, or that a more specific response for cBD3-like is present in canine keratinocytes.

Contrary to human AMPs, no inhibition of cBD3-like production was seen after stimulation with Th2 or Treg cytokines (Nomura I, et al. 2003; Howell MD, et al. 2005; Howell MD, et al. 2006). A possible explanation for this difference in results is the use of different stimulation procedures. Howell et al. (2006) stimulated the human keratinocytes with the supernatant collected from stimulated peripheral blood mononuclear cells (PBMC) after treatment with anti-CD3 antibodies. Whereas we used pure cytokines, so no competition between Th1 and Th2 was present in the keratinocyte supernatant.
In this study we also analyzed the production of several cytokines before and after exposure to HDM. HDM was chosen because this immunogen represents the primary allergen in both human and canine AD. To have a better and more complete picture of the immunological milieu in atopic skin after stimulation with HDM, we used the Luminex® technology which allows the detection of several cytokines simultaneously using the same sample. Using this technique, we were able to detect only seven out of 13 (IL-7, IL-8, IL-10, IL15, INF-γ, KC-like, and MCP-1) cytokines tested. When we analyzed the different expression of cytokines in “healthy” and “atopic” keratinocytes, we were not able to detect any difference at baseline; however, an increased pro-inflammatory response (increased production of IL-8 and KC-like) was measured in “atopic” keratinocytes after stimulation with HDM. These data, confirm that “atopic” keratinocytes respond excessively to environmental stimuli and that this excessive inflammatory response is not the result of an abnormal cutaneous immunological milieu, but due to intrinsic alterations of the epidermal cells.

In conclusion, the results of this study demonstrated that keratinocytes harvested from atopic beagles behave differently from keratinocytes harvested from healthy beagles. The results further suggest that a state of cellular hyperactivation, demonstrated by an increased production of cBD3-like and pro-inflammatory cytokines, is present in “atopic” keratinocytes. Our study also suggests that intrinsic alterations of keratinocytes may be present in atopic dogs and those alterations may represent a fundamental step in the pathomechanism of canine AD. Finally, we also demonstrated that the secretion of certain AMPs does not increase in “atopic” keratinocytes. This suggests that this may be a mechanism involved in the high frequency of cutaneous infections seen in atopic dogs. Further studies are needed to confirm that a
hyperactivation state is present in “atopic” keratinocytes, to elucidate the major pathways used by the different canine AMPs, and to confirm the intrinsic alterations using more markers of barrier function.
6.6 FIGURES AND TABLES

Figure 10. Cytoskeleton staining in canine keratinocytes (×400). Anti-keratin antibody stained keratinocytes showing red fluorescence staining. Keratinocytes appear unstained with antivimentin antibody (green or yellow).
Figure 11. Protein concentrations for canine β-defensin [cBD]3-like in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hours stimulation using several immunostimulants. LPS: ultra-pure *E. coli*-derived lipopolysaccharide; Pam3: Pam3Cys-Ser-(Lys)4; IL: Interleukin; INF-γ: Interferon-γ; Vit D: Vitamin D₃; HDM: House dust mite; PMA: phorbol 12-myristate 13-acetate. Groups were compared using unpaired t-test and repeated measurements ANOVA with Tukey’s Multiple Comparison Test (*P≤0.05; **P≤0.01; ***P≤0.001); Bars: standard error of the mean.
Figure 12. Protein concentrations for canine cathelicidin (cCath) in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hours stimulation using several immunostimulants. LPS: ultra-pure *E. coli*-derived lipopolysaccharide; Pam3: Pam3Cys-Ser-(Lys)4; IL: Interleukin; INF-γ: Interferon-γ; Vit D: Vitamin D3; HDM: House dust mite; PMA: phorbol 12-myristate 13-acetate. Groups were compared using unpaired t-test and repeated measurements ANOVA with Tukey’s Multiple Comparison Test (*P≤0.05; **P≤0.01; ***P≤0.001); Bars: standard error of the mean.
Figure 13. Protein levels for canine β-defensin [cBD]3-like in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hours stimulation using several immunostimulants. LPS: ultra-pure *E. coli*-derived lipopolysaccharide; IL: Interleukin; HDM: House dust mite. Groups were compared using unpaired t-test and repeated measurements ANOVA with Tukey’s Multiple Comparison Test; Bars: standard error of the mean.
Figure 14. Protein levels for canine cytokines in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hours stimulation using house dust mite. HDM: After exposure to house dust mite; IL: Interleukin; INF-γ: Interferon-γ; KC-like: Keratinocyte-derived chemokine-like; MCP1: monocyte chemotactic protein-1. Groups were compared using unpaired t-test (*P≤0.05); Bars: standard error of the mean.
Figure 15. Protein levels for canine cytokines in the supernatant from keratinocytes harvested from healthy and atopic beagles collected before (control) and after 24 hours stimulation using house dust mite. HDM: House dust mite; IL: Interleukin; INF-γ: Interferon-γ; KC-like: Keratinocyte-derived chemokine-like; MCP1: monocyte chemotactic protein-1. Groups were compared using unpaired t-test (*$P \leq 0.05$; **$P \leq 0.01$); Bars: standard error of the mean.
6.7 REFERENCES


CHAPTER 7

CANINE ANTIMICROBIAL PEPTIDES ARE EFFECTIVE AGAINST RESISTANT BACTERIA AND YEASTS

7.1 Abstract

Antimicrobial peptides (AMPs) are small proteins, present in most forms of life, involved in the defense against pathogenic organisms. β-defensins (BDs) and cathelicidin (Cath) are the most frequently studied AMPs in both people and dogs. The goal of this study was to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of two canine BDs and a Cath against drug-sensitive and drug-resistant bacteria as well as yeasts using a broth microdilution method. The bacteria tested were methicillin-sensitive and resistant *Staphylococcus aureus* (MSSA and MRSA), methicillin-sensitive and resistant *Staphylococcus pseudintermedius* (MSSP and MRSP), *Escherichia coli* and *Pseudomonas aeruginosa*. The yeasts tested were *Candida albicans* and *Malassezia pachydermatis*. Reference microorganisms (ATCC) and field strains were tested. All canine AMPs tested were most effective against the canine specific microorganisms. MSSP and MRSP were more susceptible to canine AMPs than human *Staphylococci* (MSSA and MRSA) and *M. pachydermatis* was more sensitive than *C. albicans*. cBD103 was most effective against *Staphylococci* and *P. aeruginosa* while cCath was the most effective AMP against *E. coli*. Additionally, cBD103 was the most effective AMP for both yeasts studied, with *M. pachydermatis* more susceptible than *C. albicans*. All AMPs tested exhibited killing within two hours of exposure. In conclusion, we demonstrated that natural canine AMPs are more effective against canine-specific pathogens, are equally effective against methicillin-
resistant or susceptible strains, and are more effective against _Malassezia_ spp. than _Candida_ spp. organisms.

### 7.2 Introduction

Over the past decade an increase in antibacterial resistance has been reported, among human pathogens as well as those of dogs. This is very relevant to patients suffering from atopic dermatitis (AD) (DeBoer DJ & Marsella R. 2001; Kedzierska A, et al. 2008; Hill SE, et al. 2011; Tang CS, et al. 2011). Patients with AD have an increased susceptibility to skin infections that often require multiple treatments with systemic and topical antibiotics. This frequent use of antibiotics contributes to an increase in bacterial resistance worldwide (DeBoer DJ & Marsella R. 2001; Kedzierska A, et al. 2008; Hill SE, et al. 2011; Tang CS, et al. 2011).

Antimicrobial peptides (AMPs) are small, predominantly cationic, proteins. There are few exceptions that are anionic. AMPs play a fundamental role in the defense against microorganisms and function as immunoregulators (Jenssen et al., 2006). They are primarily produced by epithelial cells, specifically keratinocytes, but monocytes, dendritic cells, and mast cells are also able to produce AMPs (Braff MH, et al. 2005; Braff MH & Gallo RL. 2006). Positively charged AMPs, such as β-defensins (BDs) and cathelicidin (Cath), have been widely studied in people and dogs. Human BDs and Cath attach to microorganisms based upon a high affinity for anionic molecules on the microbes' surface. Hydrophobic regions enable AMPs to be integrated into the microorganisms’ cellular membrane resulting in membrane damage, disruption of the ion gradient, and subsequent death of the microorganism (Howell MD. 2007). Due to the nature of their mechanisms of action, interaction with the microorganisms’ basic
structures (e.g. bacterial membrane), AMPs have shown very low bacterial, fungal and viral resistance (Jenssen H, et al. 2006).

Four BDs and one Cath have been isolated in human skin, and have demonstrated immunological and antimicrobial properties which extend to bacteria including methicillin resistant *Staphylococcus aureus MRSA* (Reynolds NL, et al. 2010), yeasts, and viruses. Six BDs (cBD1-like, cBD2-like/122, cBD3-like, cBD102, cBD103, and cBD127) and one cathelicidin (cCath) have been identified in canine skin (Wingate KV, et al. 2009; Van Damme CM, et al. 2009; Santoro D, et al. 2011) and cBD103 has been found in the canine respiratory tract (Erles K & Brownlie J. 2010). Only the conserved sequence of four cBDs (cBD1-like, cBD2-like/122, cBD3-like, and cBD102) (Sang Y, et al. 2005), cBD103 (Erles K & Brownlie J. 2010; Leonard BC, et al. 2012) and cCath (Sang Y, et al. 2007) have been shown to demonstrate antimicrobial activity against bacteria such as methicillin-sensitive *Staphylococcus aureus (MSSA)*, methicillin-sensitive and resistant *S. pseudintermedius (MSSP and MRSP), E. coli, Proteus mirabilis*, and *Pseudomonas aeruginosa*, as well as against the yeast *Candida albicans*. From those studies it is clear that cBDs are more effective against yeasts than bacteria, whereas the opposite is true for cCath.

To date there have been no reports of the antimicrobial effects (minimum inhibitory concentration [MIC] and the minimum bactericidal concentration [MBC]) of cAMPs against drug-sensitive or resistant bacteria (including MRSA and MRSP) or against *Malassezia pachydermatis*. Thus the objective of this study was to determine the MIC and MBC of cAMPs for common pathogenic microorganisms (MSSA, MRSA, MSSP, MRSP, *E. coli, P. aeruginosa, C. albicans*, and *M. pachydermatis* [Table 5]) using the broth microdilution method.
7.3 Materials and Methods

**Peptide preparation**

Based on previous studies (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K & Brownlie J. 2010), three peptides were synthesized (Peptide Protein Research Ltd., Fareham, UK) and tested: a 34-amino-acid peptide synthesized from the shared mature peptide regions of cBD1-like, cBD2-like/122, cBD3-like, and cBD102 (cBD), a 45-amino-acid peptide synthesized from the C-terminus of cBD103, and a 38-amino-acid peptide synthesized from the C-terminus of cCath. One milligram (mg) of each lyophilized peptide was diluted in 10 mM 0.01% acetic acid to generate a stock concentration of 4 mg/mL. The 0.01% acetic acid solution was added to activate the bonds between the cysteine residues in the peptides. It has been established that this concentration of acetic acid does not have any adverse effect on fungi or other bacteria (Sang Y, et al. 2005; Sang Y, et al. 2007; Fazakerley J, et al. 2010; Fritz P, et al. 2012). Before use, each peptide was further diluted (1:10) with 10 mM sodium phosphate buffer at pH 7.4 (SPB) as previously described (Fazakerley J, et al. 2010).

**Minimal inhibitory concentration assays (bacteria)**

All assays were carried out in duplicate wells in sterile 96-well polystyrene round-bottomed plates (Costar®, Corning Inc., Corning, NY, USA) using a broth microdilution method adapted from the Clinical and Laboratory Standards Institute (CLSI; previously the National Committee on Clinical Laboratory Standards [NCCLS]) methods (Watts JL, et al. 2008; Rex JH, et al. 2008).
All assays were independently repeated three times, and the MIC was calculated as the median of the triplicates. The MIC was defined as the lowest antimicrobial concentration inhibiting growth of the organism in the well as detected by the unaided eye. The MBC was determined by plating the content of wells with dilutions equal to and below the MIC on Columbia Blood Agar (CBA) (Remel, Lenexa, KS). The experimental error was accepted if falling within the doubling dilution.

The bacteria, maintained at -80°C in 25% glycerol, and 75% tryptic soy broth, were grown overnight on CBA at 37°C and 5% CO₂. Yeasts were grown for 48 hours on Sabouraud Dextrose Agar plates (Remel, Lenexa, KS) at 35°C in an air forced incubator (Biolog Inc., Hayward, CA). Isolated colonies were suspended in sterile double distilled water to achieve an optical density equal to a McFarland 0.5 standard (Sensititre Inc. Westlake, OH).

The bacterial suspension was then diluted 1:500 to obtain a final concentration of 1x10⁶ CFU/mL. Twofold serial dilutions of test peptide in SPB (final concentration from 200 µg/mL to 3.125 µg/mL) were added (100 µL) to each well containing the same amount of inoculum. Negative control wells containing 100 µL of SPB in lieu of the bacteria, and positive control wells containing 50 µL of SPB instead of the test peptides were included. Initial inoculum density was confirmed by plate count and the number of bacteria in the SPB suspension after a two hour incubation at 35°C to assess any adverse effects of the buffer. Each well containing cAMPs was similarly plated on CBA to determine the viability after the two hours exposure. Finally, 100 µL of Muller Hinton Broth with TES (MHB, Sensititre, Westlake, OH) was added to each well and the plates were incubated at 35°C for another 18-20 hours. 96 well plates were examined visually for growth at the various concentrations of cAMPs and the lowest
concentration without visible growth was reported as the MIC. The content of the wells were then spiral plated to determine the MBC.

The same method was used to determine the MIC and MBC for C. albicans and M. pachydermatis with the exception of the medium used, Yeast-One (Trek Diagnostic Systems, Cleveland, OH, USA) instead of MHB. In addition, since M. pachydermatis growth was difficult to detect in the well following overnight incubation, the inoculum/peptide and the control wells were also plated on CBA at 35°C for 24-48 hours to facilitate determination of the MIC/MBC.

Initial bacterial and yeast counts are listed in Table 6.

**Minimal bactericidal concentration assays**

All assays (bacterial and yeast) were carried out in duplicate wells. After 18-20 hours of incubation, 100 µL of inoculum/peptide solution at both the MIC and the concentration above the MIC were spirally plated on CBA and were incubated at 35°C for 24 hours. MBC was defined as the lowest dilution at which microorganism were no longer viable on subculture.

**7.4 Results**

The MIC and MBC of each peptide are summarized in Table 7. Representative bacterial count dilutions are summarized in Table 6. cBD103 was overall the most effective cAMP tested at all concentrations for all the microorganisms except E. coli (Table 7). cCath was the most effective cAMP against E. coli. Most of the MBCs were within the doubling dilution compared to the MICs of each cAMP. At the MIC values no growth was observed after two hours of pre-incubation, suggesting that cAMPs are effective within two hours of contact time. However, even at the
lowest peptide concentration (3.125 µg/ml), bacterial growth was dramatically inhibited after two hours (range: 99.4% to 100%), but growth was visible after MHB was added for 18-20 hours (Table 6). Finally, no difference in bacterial or fungal growth was evident between the positive control in SPB after the pre-incubation and the same microorganism dilution before the pre-incubation, showing no effect of the SPB on the bacteria growth (Table 6).

Eleven field isolates of MSSP were screened for susceptibility to the three cAMPs. The MIC90 for cBD was ≥ 200 µg/ml while the MIC90 for both cBD103 and cCath was 50 µg/ml (Table 8).

7.5 Discussion

In this study we have demonstrated that cBD, cBD103, and cCath (peptide 3) are highly effective against both Gram-positive and Gram-negative bacteria as well as yeasts. In addition, different peptides seem to have a different spectrum of activity with cBD103 being overall the most effective against the microorganisms tested.

Few studies have been published on the antimicrobial effects of cAMPs (Sang Y, et al. 2005; Sang Y, et al. 2007; Erles K & Brownlie J. 2010; Leonard BC, et al. 2012). Due to differences in methods and/or in peptides used, a comparison between them and the present study is impossible. When we tried to compare our results with the above mentioned studies, cCath had similar antibacterial activity to that previously reported (Sang Y, et al. 2007); this was not the case for cBD and cBD103.

In agreement with previously reported results (Sang Y, et al. 2007), cCath was more effective against Gram-negative than Gram-positive bacteria (MSSP the most sensitive). A
different susceptibility was observed for the two yeasts tested. *M. pachydermatis* was highly sensitive to cCath, whereas *C. albicans* was resistant at the highest concentration tested (200 µg/ml).

Results for cBD and cBD103 showed a different spectrum of activity when compared to that reported in previous studies (Sang Y, et al. 2005; Leonard BC, et al. 2012). Previously, cBD was reported to be highly effective against Gram-negative bacteria (*E. coli*: 20 µg/ml), but less effective against *C. albicans* (50 µg/ml), and even less effective against *MSSA* (100 µg/ml) (Sang Y, et al. 2005). However, in our study, cBD was highly effective against both yeasts tested (*M. pachydermatis*: 25 µg/ml; *C. albicans*: 50 µg/ml), *P. aeruginosa* (50 µg/ml), and *S. pseudintermedius* (MSSP>MRS), but it was not effective against *E. coli* or *S. aureus*.

Similar discordant results exist between this and a previous study for cBD103 (Leonard BC, et al. 2012). Leonard et al. (2012) reported that cBD103 was most effective against *E. coli*, followed by *MRSP*, and least effective against *MSSP*. In the present study, cBD103 was the most effective cAMP tested, for all the microorganisms tested, except *E. coli*. Specifically, cBD103 was most effective against *P. aeruginosa* and *M. pachydermatis* (both at 12.5 µg/ml), followed by *MSSP* and *C. albicans* (both at 25 µg/ml), then *MRSP* and *E. coli* (50 µg/ml), and finally both strains of *S. aureus* (both at 100 µg/ml).

Differences between our results and the previously published studies on cBD (Sang Y, et al. 2005) and cBD103 (Leonard BC, et al. 2012) may be attributed to either different ATCC strains and/or to the different methodologies used. Of the ATCC strains used in this study and those by Sang Y, et al. (2005), only the *E. coli* strain (ATCC 25922) was used in both studies. As far as cBD103, Leonard BC, et al. (2012) used field strains only, which may have a different
pattern of resistance to cAMPs than the ATCC strains. It is probable that field strains have been more in contact with AMPs than ATCC strains. This increased exposure to AMPs could change their sensitivity to them. Differences in methodology need to be considered as well. Leonard BC, et al. (2012) used a radial diffusion antimicrobial assay, whereas a broth microdilution assay was used in this study, in accordance with the CLSI guidelines.

In conclusion, in this study we demonstrated that cAMPs effectively kill Gram positive and Gram negative bacteria, including MRSA and MRSP, and yeasts tested within two hours of incubation. The authors recognize that the antimicrobial peptide concentrations tested exceed those reasonable for systemic therapy. However, these cAMP concentrations still have potential for topical applications. Further investigations to establish the MIC90s for bacteria and yeasts commonly involved in dermatitis are necessary and warranted.
### Table 5. Bacteria and yeasts tested for susceptibility to canine antimicrobial peptides

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>ATCC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSSA</td>
<td>29213 + 1 UIUC-VDL clinical isolates</td>
</tr>
<tr>
<td>MRSA</td>
<td>43300</td>
</tr>
<tr>
<td>MSSP</td>
<td>4944 + 11 UIUC-VDL clinical isolates</td>
</tr>
<tr>
<td>MRSP</td>
<td>UIUC-VDL Clinical isolate</td>
</tr>
<tr>
<td>E. coli</td>
<td>25922</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>27853</td>
</tr>
<tr>
<td>C. albicans</td>
<td>16231</td>
</tr>
<tr>
<td>M. pachydermatis</td>
<td>14522</td>
</tr>
</tbody>
</table>

### Table 6. Number of bacteria/yeasts recovered from MIC panels in CFU/µl Row A = starting inoculum; row B = counts after 2 hours in SPB; row C1 – counts at 2 hours in MIC for cBD; C2 = counts at 2 hours in MIC for cBD103; C-3 = counts at 2 hours in MIC for cCath; D1 = counts at 2 hours with 3.125 µg/ml cBD; D2 = counts at 2 hours with 3.125 µg/ml CB103; D3 = counts at 2 hours with 3.125 µg/ml cCath.

<table>
<thead>
<tr>
<th>Solution</th>
<th>MSSA</th>
<th>MRSA</th>
<th>MSSP</th>
<th>MRSP</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>C. albicans</th>
<th>M. pachydermatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6.6X10^9</td>
<td>7.4X10^9</td>
<td>1.55 x 10^9</td>
<td>8.6X10^9</td>
<td>4.9X10^9</td>
<td>1.37 x 10^9</td>
<td>1.5x10^7</td>
<td>5.7x10^10</td>
</tr>
<tr>
<td>B</td>
<td>5.0X10^9</td>
<td>7.2X10^9</td>
<td>5.82 x 10^9</td>
<td>5.6X10^9</td>
<td>3.6X10^9</td>
<td>3.36 x 10^9</td>
<td>5.2x10^7</td>
<td>3.4x10^10</td>
</tr>
<tr>
<td>C1</td>
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<td>3.8x10^5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>C2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>197</td>
</tr>
<tr>
<td>C3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>197</td>
</tr>
<tr>
<td>D1</td>
<td>8.6X10^6</td>
<td>7.9X10^6</td>
<td>5.0 x 10^6</td>
<td>2.9x10^6</td>
<td>7.6X10^6</td>
<td>8.3 x 10^6</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td>D2</td>
<td>1.8X10^6</td>
<td>1.4x10^6</td>
<td>0</td>
<td>0</td>
<td>1.8x10^5</td>
<td>6.0 x 10^4</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
<tr>
<td>D3</td>
<td>6.0X10^6</td>
<td>5.0x10^6</td>
<td>9.0 x 10^4</td>
<td>7.4x10^4</td>
<td>3.8X10^6</td>
<td>3.4 x 10^5</td>
<td>TNTC</td>
<td>TNTC</td>
</tr>
</tbody>
</table>
**Table 7.** Average MIC and MBC for three canine antimicrobial peptides

*Average of three repeats of duplicate wells observed visually and reported as no growth

** Average of three repeats of duplicate wells plated to detect viable growth

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; cBDs: Conserved sequence of three canine β-defensins; cBD103: Canine β-defensin 103; cCath: Canine cathelicidin.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC* (µg/ml)</th>
<th>MBC** (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cBDs</td>
<td>cBD103</td>
</tr>
<tr>
<td>MSSA</td>
<td>&gt;200</td>
<td>100</td>
</tr>
<tr>
<td>MRSA</td>
<td>&gt;200</td>
<td>100</td>
</tr>
<tr>
<td>MSSP</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>MRSP</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>E. coli</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>50</td>
<td>12.5</td>
</tr>
<tr>
<td>C. albicans</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>M. pachydermatis</td>
<td>25</td>
<td>12.5</td>
</tr>
</tbody>
</table>
Table 8. Summary of the field strains of *Staphylococcus* spp. tested and susceptibility to canine antimicrobial peptides

<table>
<thead>
<tr>
<th><em>Staphylococcus</em> spp. strains</th>
<th>UIUC - VDL accession number</th>
<th>MIC (µg/ml)</th>
<th>MBC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cBDs</td>
<td>cBD103</td>
<td>cCath</td>
</tr>
<tr>
<td>#1 - MSSP</td>
<td>12-46786</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>#2 - MSSP</td>
<td>12-46873</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#3 - MSSP</td>
<td>13-30073</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#4 - MSSP</td>
<td>11-43740</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#5 - MRSP</td>
<td>12-49755</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#6 - MSSP</td>
<td>12-41388</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#7 - MSSP</td>
<td>12-37915</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#8 - MSSP</td>
<td>12-37957</td>
<td>&gt;200</td>
<td>50</td>
</tr>
<tr>
<td>#9 - MSSP</td>
<td>12-32217</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>#10 - MSSP</td>
<td>12-3125-</td>
<td>200</td>
<td>50</td>
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<tr>
<td>#11 - MSSP</td>
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<tr>
<td>#12 - MSSP</td>
<td>11-43451</td>
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</table>
REFERENCES


CHAPTER 8
CONCLUSIONS

Atopic dermatitis is an extremely common disease affecting the quality of life of up to 10% of the human and canine populations. One of the most common complications of AD is the high incidence of cutaneous infections secondary to disturbances of the immune-system and the skin barrier function in atopic patients. With a better understanding of why the natural defenses in atopic patients lose their effectiveness, new treatment options to enhance the host’s defense against external pathogens may emerge.

In people, an association with AD and primary and secondary alterations of the skin barrier has been established (Elias PM, et al. 2008; Cork MJ, et al. 2009; Elias PM & Schmuth M. 2009; De Benedetto A, et al. 2011; Takai T & Ikeda S. 2011; Wolf R & Wolf D. 2012; De Benedetto A, et al. 2012). It has been demonstrated that defects in the keratinocytes precipitate abnormalities in the skin barrier, and aberrant activation of the local immune system results in the inhibition of cutaneous immune defenses such as AMPs (Lai Y & Gallo RL. 2009). In this series of experiments, we demonstrated that alterations to the skin barrier, measured by the expression and production of cAMPs and filaggrin, are similarly present in atopic dogs.

We first analyzed the expression of cAMPs and filaggrin in skin samples from a colony of atopic beagles and demonstrated that the expression of these proteins was altered. Specifically, atopic beagles display an increase in the gene expression (mRNA) of cBD1-like, cBD3-like, cCath, and filaggrin when compared with healthy breed- and age-matched dogs. These increased
levels of gene expression were even more evident in lesional atopic skin when compared with healthy controls. However, using indirect immunofluorescence, such increase in gene expression was not associated with an increase of protein expression. These results suggest the possibility of intrinsic alterations in atopic keratinocytes. When we analyzed the distribution of cAMPs and filaggrin in atopic skin, we were unable to evidentiate a difference in the distribution pattern for cAMPs, but did evidentiate a distinct alteration of filaggrin distribution in atopic skin. This latter altered distribution pattern was characterized by dyshomogeneity in the immunofluorescence signal. This signal was completely absent in lesional atopic skin suggesting that the inflammation observed in lesional skin inhibits the production of filaggrin thus exacerbating alterations of the skin barrier.

Based on the data collected from the canine animal model, we then decided to evaluate the same markers of the skin barrier in naturally-affected atopic dogs with and without active skin infection. The goal of this study was not only to confirm our initial data, but to determine which cAMP is the most expressed during bacterial and/or yeast infections. We initially compared the cAMPs previously tested in the canine model (cBD1-like, cBD2-like, cBD3-like, and cCath). Although increased gene expression (mRNA) of such cAMPs was present, this was not significant when compared with gene expression in healthy skin. However, a significant decrease of cBD1-like mRNA was observed in infected atopic skin when compared with non-infected atopic skin. This suggests the possibility that cBD1-like plays a minor role in active skin infections.

When we looked into the gene expression (mRNA) of an additional cAMP, cBD103, we demonstrated not only an increased expression in atopic skin (with skin infection > without skin
infection), but also when compared with healthy controls. To a much greater degree than cBD1-like, these latter data suggest that cBD103 has an active role in cutaneous skin infection in atopic dogs. Unfortunately, such alterations of cAMPs mRNA expression, decrease in cBD1-like and increase in cBD103, could not be compared with protein expression since no antibodies were available for those two cAMPs. In this same experiment, we were able to validate a competitive inhibition ELISA (ciELISA) method for evaluation of the protein expression of selected cAMPs (cBD3-like and cCath) in canine skin. Using the ciELISA, we measured a significant increase in cBD3-like and cCath in the skin of atopic dogs with active skin infection when compared with healthy controls. These latter data suggest that active skin infections do stimulate the production of cAMPs. We were unable to evaluate the gene expression of filaggrin at that time. Therefore, no other data for this skin barrier marker are currently available. This leaves an opportunity for further study in the future.

Due to the higher gene expression (mRNA) of cBD103 in the skin of atopic dogs with and without skin infections, we then investigated its efficacy against common skin pathogens and compared its activity with cCath and other cBDs. We then performed an in vitro study in which we tested the antimicrobial activity of cBD103, cCath, and the common peptide sequence for cBD1-like, cBD2-like, and cBD3-like (cBDs) against relevant cutaneous bacteria and yeasts. We demonstrated that after only two hours of incubation, all peptides tested inhibited some of the microorganisms tested. cBD103 was overall the most effective against all the microorganisms tested followed by cCath. These results support the ex vivo data and suggest a major role of cBD103 against active cutaneous infections in dogs.
Finally, based on the above studies, we hypothesized that an intrinsic alteration of the canine keratinocytes is present in atopic dogs. To further explore this hypothesis, we reverted to the canine model of AD and we harvested and isolated keratinocytes from skin biopsies obtained from atopic and healthy, age- and breed-matching, beagles and cultured them; cultured keratinocytes were used for the study since keratinocytes are the primary cells involved in the skin barrier. The keratinocytes were used to evaluate alterations of cAMP and cytokine productions before and after stimulation with several immunogens. When we analyzed the supernatant collected from non-stimulated canine keratinocytes, we were able to show a significant increase of cBD3-like in “atopic” keratinocytes compared with “healthy” keratinocytes. The secretion of cCath and several cytokines (IL-8, Keratinocyte-derived Chemokine-like, and Monocyte Chemotactic Protein-1) was also increased in atopic keratinocytes, but not enough to reach statistical significance; it is possible that the small sample size influenced this. We compared the amount of cBD3-like secreted by atopic keratinocytes and healthy keratinocytes, and found a higher amount was only seen in healthy keratinocytes after stimulation with LPS or IL-17. In addition, a significant increase in cBD3-like protein secretion, when compared with baseline, was only observed in healthy keratinocytes after stimulation with LPS, IL-17, IL-4, and IL-10.

When we looked at the post-stimulation expression of cCath in keratinocytes, we noticed a significant increase of cCath in both healthy and atopic keratinocytes when compared with baselines. However, no difference was observed between the two types of keratinocyte (healthy and atopic). Finally, we focused on the expression of canine cytokines in the supernatant of atopic and healthy keratinocytes after stimulation with HDM. We demonstrated
a significantly increased production of IL-8 and KC-like in atopic keratinocytes compared with healthy keratinocytes.

Altogether, the data presented in this dissertation suggest: 1) Alterations of skin barrier markers, cAMPs and filaggrin, are more prevalent in the skin of atopic dogs compared to healthy dogs. 2) Such alterations are exacerbated by active skin infections. 3) Dysregulation is present in atopic keratinocytes (e.g. a significant increased production of cBD3-like at baseline and a higher production of pro-inflammatory cytokines and cCath after immunological stimulation). 4) An excessive inflammatory response and the lack of increase of certain cAMPs (e.g. cBD3-like) in atopic keratinocytes may represent fundamental steps in the pathogenesis and exacerbation of cAD. 5) cBD103 is the most effective of the studied cAMPs against cutaneous microorganisms and probably one of the most relevant in course of cutaneous skin infections in atopic dogs.

Our studies suggest that although the surrounding environment may exacerbate the clinical signs and immunological alterations associated with cAD, intrinsic factors may be equally important in the instauration of the atopic condition. Furthermore, these studies suggest that alterations of keratinocytes may lead to altered transcription of both cAMPs and filaggrin. This altered transcription may cause consequent abnormality of the skin barrier and impact the severity of the atopic disease.

This series of experiments presents new questions as to why such basic defenses seem to be defective in atopic patients. One of the most relevant of which is to determine if a hyperactivated cellular state, resulting from the hyperphosphorylation of transcription factors
(e.g. NF-κB, NFAT, and AP1), or an excessive presence of toll like receptors are the underlying reasons why “atopic” keratinocyte are hypersensitive to external stimuli.


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