Host and environmental contribution to atopic dermatitis in dogs

The research described in this thesis was performed at the Division of Immunology, Derpartment of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

ISBN: 978-90-889-1893-3

Printed by: Proefschriftmaken.nl || Uitgeverij BOXPress

Cover designed by: Frank Looringh van Beeck

The publication of this thesis was financially supported by Royal Canin.

Host and environmental contribution to atopic dermatitis in dogs

Atopische dermatitis bij de hond:
Onderzoek naar enkele gastheerspecifieke factoren en omgevingscomponenten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 5 juni 2014 des middags te 4.15 uur

door

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geboren op 3 augustus 1978 te Alphen aan den Rijn

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Contents

Chapter 1

General introduction

Introduction

The research presented in this thesis is focussed on atopic dermatitis (AD) in dogs. The International Task Force on Canin Atopic Dermatitis currently defines canine AD as: a genetically predisposed, inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens [1].

Clinical signs and prevalence of canine AD

One of the key symptoms of canine AD is chronic or recurrent pruritus (itch). Usually skin inflammation is observed at typical sites such as ears, face, feet and axillae. AD often develops in young dogs between 1 and 3 years old [2]. It was shown in a recent large scale study that 68% of the dogs develop clinical signs of AD before 3 years of age [3].

Certain dog breeds such as Labrador Retriever, Golden Retriever, German Shepherd Dog and West Highland White Terrier show a higher incidence of AD suggesting that genetic factors play a role [4]. It has been shown that breed predisposition and location of the skin lesions is also influenced by geographical location [5-7].

Several studies have shown that AD is the second most common cause of pruritus in dogs [8, 9]. It has been estimated that 10% of the total dog population is affected with the disease [10]. In the Netherlands there are approximately 2 million dogs indicating that about 200,000 dogs have AD. These dogs will have in most cases a life-long disease with clinical signs varying from relapsing mild pruritus and erythema to severe chronic inflammation of the skin and intense itch.

A short history of canine AD

The first reports describing hypersensitivity reactions in dogs towards food and environmental allergens were published about 80 years ago [11, 12]. In 1967, human IgE antibody was identified as the carrier of the reaginic activity seen in atopic diseases [13]. The antibody was detected in the skin and was linked to allergic diseases such as asthma, rhinitis and atopic dermatitis [14]. AD in humans and dogs was defined as a classical type I hypersensitivity. Allergen-specific IgE antibodies bind to mast cells and initiate mast cell degranulation after cross-linking of two IgE molecules by the allergen. The release of mediators such as histamine exerts pruritus and inflammation of the skin.

The putative role of IgE in AD became less clear when it was found that apparently healthy dogs can also present high levels of allergen-specific IgE and showed a positive response when tested intradermally with allergens [15-17]. Furthermore, in humans it has been found that about 20-30% of the patients have a so-called “intrinsic” form of AD. These patients have identical clinical symptoms but without elevated IgE levels or IgE-mediated skin test reactivity [18]. This form of AD is defined in dogs as canine atopic-like dermatitis [1], but more data is required to determine the prevalence.

Our current knowledge of atopic diseases and atopic dermatitis in particular, has showed that it is a multifactorial disease. Different pathogenic mechanisms may result in the same clinical outcome of AD, and characteristic clinical features associated with AD can also be observed in other skin diseases. The complex

interactions between immune cells like Langerhans cells (LC), dermal dendritic cells (DDC), T- and B lymphocytes, keratinocytes, mast cells and eosinophils and their produced cytokines and chemokines play an important role. Furthermore, environmental exposure, genetic factors and skin barrier defects contribute to the pathogenesis. Each of these components can influence each other (Figure 1) and can be of varying importance in the development of AD between individuals, and are described in more detail in this chapter.

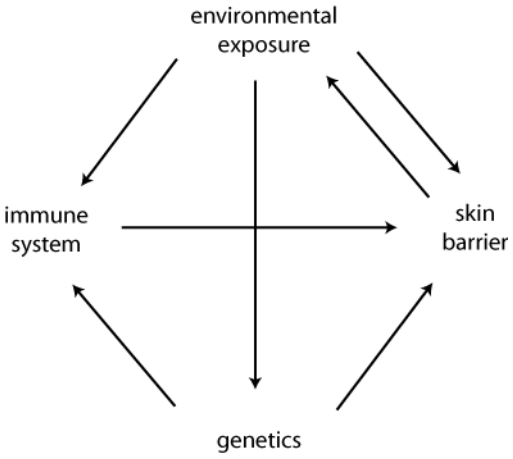

Figure 1. Schematic diagram of the four major components involved in the development of atopic dermatitis. Each of these components can have a direct or indirect effect on the other components and the importance of each component in the development of atopic dermatitis can vary between individuals.

The role of the immune system

There are remarkable similarities in clinical signs and pathogenesis of AD in dogs and humans [19, 20]. Much of our understanding of canine AD has come from human studies. A schematical overview of the immunological pathway including the major cell types considered being important in the development of AD in dogs and human is depicted in figure 2. The left panel shows the penetration of the environmental allergen through the skin and the capture of these allergens by classical epidermal dendritic cells, the Langerhans cells (LC). LC migrate to a draining lymph node and present these cells to naïve T cells, which differentiate to T helper 2 cells. These in turn produce IL-4 and IL-13 leading to isotype switching of B cells, which start to produce IgE antibodies. In the acute phase (middle panel) there is a second exposure of the allergen, which is bound by IgE molecules on mast cells leading to release of mediators such as histamine resulting in itch. The acute inflammation is characterized by influx of Th2 cells, IgE antibodies, dendritic cells and eosinophills. In a chronic state, Th1 cells dominate the T cell population and increased keratinocyte proliferation is observed. Furthermore, the barrier of the chronic inflamed skin is weakened, making it more susceptible for pathogens such as yeasts and staphylococci, which contribute to a perpetuation of the

inflammation. The above described inside-out theory stresses the importance of a disregulated immune system and sees disruption of the skin barrier as an outcome of this [20-22].

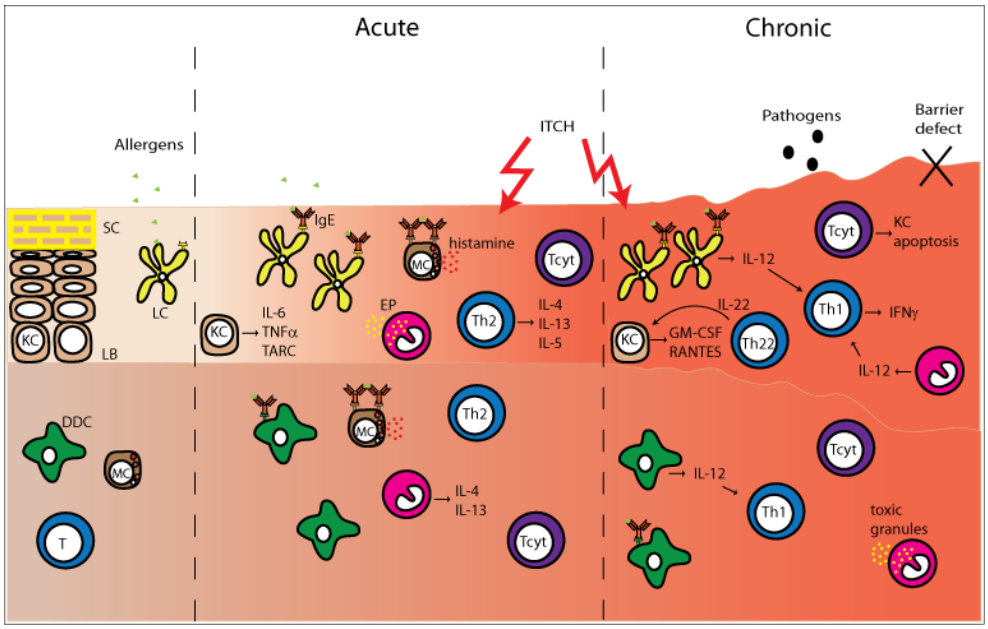

Figure 2. Immunologic pathways in AD. LCs capture allergens which penetrate the epidermis of an atopic individual before the onset of inflammation (left panel). Antigen presentation in a local lymph node by LCs enables the production of allergen specific IgE molecules by B cells. The IgE antibodies are subsequently deposited in the skin via the circulation. A complex of allergen-IgE molecules bind to FcεRI which leads to rapid antigen presentation by LCs and DDCs and activation of MCs which release inflammatory mediators such as histamine. During acute inflammation there is an influx of Th2 cells which produce IL-4 and IL-13 (middle panel). The presence of IL-12 in chronic inflamed skin triggers the switch from Th2 cells to Th1 cells (right panel). Th22 cells produce IL-22 which induces keratinocyte proliferation leading to a thick layered skin. The chronic inflamed skin has a weakened barrier due to inflammation and scratching which leads to skin infections with bacteria and yeast. Abbreviations: SC (stratum corneum); LB (lamina basalis); KC (keratinocyte); LC (Langerhans cell); DDC (dermal dendritic cell); MC (mast cell); IgE (immunoglobulin E); EP (eosinophil); Th2 (T-helper 2 cell); Tcyt (cytotoxic T cell); Th1 (T-helper 1 cell); Th22 (T-helper 22 cell) [22-26].

T lymphocytes

The role of T cells in AD has become increasingly more complex due to the continuous discovery of new T cell subsets. The basic Th1/Th2 balance model comprises of only two antagonising $CD4^+$ T helper subsets. During the acute phase (Figure 2) there is an increase of Th2 cells which release cytokines such IL-4, IL-5 and IL-13 and reduce the activation/proliferation of Th1 cells. Recently it has been shown that Th2 cells also produce IL-31, and that this cytokine may be an important provoker of pruritus seen in AD [27-31]. At the beginning of the chronic

phase IL-12 production by LC, DDC and eosinophils triggers naive T cells to switch to Th1 cells, which maintain the inflammatory environment by secreting IFNγ, and decreasing the activity of Th2 cells [32-35].
The expression profile of Th1 and Th2 cells in canine AD shows similarities with its human counterpart. A clear histological feature of canine atopic inflamed skin is the influx of $CD4^+$ T lymphocytes and higher expression of IL-4 mRNA, whereas in chronic inflamed lesional skin higher levels of INFγ were found [36-38].
More recent studies have shown that regulatory T cells (Treg) can inhibit the development of allergen-specific Th2 and Th1 cell responses [39]. Human Treg were able to suppress B cell IgE production and reduce the effector functions of mast cells and eosinophils [40]. Another study showed that only inducible regulatory IL-10 and TGFβ secreting Treg were found in human atopic skin, whereas natural occurring Treg, expressing CD25 and FoxP3, were not found [41].
The role of Treg or other T cell subsets in canine AD is not well described yet. It was found that canine atopic skin had a significant lower expression of TGFβ which may be caused by a lower Treg population in skin [38]. A recently discovered subset of $CD4^+$ cells, named Th22, produces the cytokine IL-22 which mediates keratinocyte proliferation [42]. In human, the number of Th22 cells is significantly higher in atopic skin compared to healthy skin [25].
In atopic skin there is also an influx of $CD8^+$cytotoxic T cells [36, 37, 43, 44]. T cells infiltrating the skin have also been shown to upregulate the apoptotic Fas receptor on keratinocytes, and induce KC apoptosis by releasing IFNγ and by Fas ligand expression [26], which may contribute to disease pathogenesis in chronic lesional skin.

Mast cells and Eosinophils
Mast cells (MC) are important initiators of the acute inflammatory response and are present in both acute and chronic skin. MC expresses FcεRI which bind IgE antibodies with high affinity. Activation occurs when an allergen binds to IgE on multiple receptors leading to the secretion of pruritic and inflammatory mediators such as histamines, major basic proteins, prostaglandins and leukotrienes [45].
Eosinophils (EP) infiltrate the acute and chronic inflamed skin and contribute to skin inflammation. Activated EP release toxic protein granules which can mediate tissue damage. EP also produce a variety of cytokines including IL-4, IL-13 and IFNγ and can thereby influence the Th1/Th2 balance [46, 47].

Keratinocytes
Keratinocytes were usually regarded as building blocks of the epidermis with no particular immunological function. In the last two decades it has become clear that keratinocytes produce various cytokines and chemokines, are actively involved in the skin immune system, and play an important role in the development of AD.
The keratinocyte-derived thymus and activation-regulated chemokine (TARC) is increased in canine and human lesional atopic skin [48, 49] and may therefore attract Th2 cells expressing the TARC sensitive chemokine receptor CCR4. In cultured canine and human keratinocytes it was shown that stimulation with pro-inflammatory cytokines (TNFα, IL-1β) augmented the mRNA expression of TARC [49, 50].
Keratinocytes from human AD patients synthesize exaggerated amounts of mediators such as GM-CSF and CCL-5 (RANTES), which are important for

enhanced recruitment as well as sustained survival and activation of dendritic cells, T cells and eosinophils [51, 52]. Maeda et al. showed that incubating canine keratinocytes with the Der f1 house dust mite allergen increased pro-inflammatory cytokines such as IL-1, IL-6, TNFα and IL-8, and immunomodulatory cytokines such as IL-12, IL-18, and TGFβ [53]. IL-31-induced keratinocytes produce IL-20 and IL-24 which regulate proliferation and differentiation during inflammation and seem to disregulate the skin barrier [54].
Besides their influence on the adaptive immune response, keratinocytes contribute to innate skin immunity by producing antimicrobial proteins: β-defensins and cathelicidins [55]. These proteins protect the skin against bacteria, fungi and viruses. The role of these proteins in AD is not clear as some studies showed a lower expression of human β-defensins and cathelicidins in atopic skin compared to psoriatic skin [56, 57], whereas in others the opposite was found [55, 58].
The role of antimicrobial peptides in canine atopic dermatitis is also not clear. One study showed an increase in gene expression of canine β-defensin 1 (cBD1) and a decrease of cBD103 expression compared to healthy skin [59]. Others have found that the expression of CBD103 was not decreased in AD and that cBD1,2 and 3 expression increased [60, 61]. The above studies show that the role of antimicrobial peptides in AD need further investigation.

Dendritic cells
Dendritic cells (DC) seem to play an important role in the development of AD in both humans and dogs. There are two to three times more dendritic cells in atopic canine skin compared to normal skin [62]. Epidermal dendritic cells such as Langerhans cells (LC) are able to capture allergens when they pass through the epidermis. After capturing the allergen, LC migrate to local lymph nodes where they present these allergens to T cells. A recent study identified canine epidermal DC as $CD1^{+}CD11c^{+}MHCII^{+}$ cells, whereas dermal DC (DDC) had the same phenotype, but with additional low expression of CD11b [63]. Under inflammatory conditions there was a subset of epidermal and dermal DC positive for IgE. The canine epidermal DC in this study is addressed as LC, however it is currently unclear whether a classical canine LC exist. The tennis-racket shaped Birbeck granule may function as endosomal compartment [64], and is a hallmark feature of LC. It has been suggested that Birbeck granules are not present in canine epidermal DC [65], but more evidence is needed to confirm this. In healthy human skin, LC are $CD1a^{++}Langerin^{+}HLA\text{-}DR^{+}$ cells and DDC are characterized as $CD1a^{+}CD1c^{+}CD11c^{+}$ $HLA\text{-}DR^{+}$ cells [66]. In human AD, an additional subset of DC is found in skin, the inflammatory epidermal DC (IDEC) with a $CD1a^{+}CD1c^{-}CD11c^{+}$ $HLA\text{-}DR^{+}$ phenotype. Whether this population is present in canine atopic skin is unknown.
It was shown that IgE molecules are present on both canine and human epidermal LC in lesional atopic skin and not on LC in healthy non-atopic controls [63, 67]. IgE molecules were bound by the high affinity IgE receptor FcεRI [68] and were able to increase allergen uptake and expression by 100-1000 fold [69]. LC and DDC, when activated via the FcεRI, are able to produce pro-inflammatory cytokines such as IL-1, IL-6, IL-8,TNF-β and IL-12, which may induce T cell differentiation [70].

CD1

In healthy humans, but not mice, one of the hallmarks of skin-resident LC is their very high expression of CD1a. As described in the dendritic cell section above, one of the key features in canine and human atopic skin is the increased number of dendritic cells including LC expressing CD1 [36, 43, 62, 71, 72]. CD1 molecules are lipid antigen presenting molecules which has structural similarities with the peptide presenting MHC class I molecule. Knowledge on canine CD1 molecules is limited, and is based on comparative immunohistochemistry. A substantial part of this thesis comprises the identification and characterization of canine CD1 genes and molecules.

CD1 genes are found in every mammalian species studied so far [73-82], and are also identified in chickens [83]. This indicates that the CD1 system was present in the common ancestor of mammals and birds at least 300 million years ago. The localization of the chicken CD1 genes within the MHC locus suggests that CD1 genes were formed by tandem duplication of MHC class I, followed by translocation of the MHC-paralogous region containing the CD1 locus in early mammals [83-85].

The CD1 molecule has the same structure as MHC class I, an α chain that folds into three domains of which the α3 domain binds non-covalently with β2-microglobulin and is continued into a transmembrane region and a cytoplasmic tail (Figure 3). The groove of CD1 molecules is formed by the α1- and α2 domain and is in contrast to the peptide-presenting MHC class I molecules, deeper, narrower and contains a hydrophobic lining which makes it suitable to harbour lipid acyl chains [86].

The CD1 family contains five isoforms which can be divided based on gene homology and functionality into two groups, group 1 consists of CD1a, -b, -c, and -e, and with CD1d as only representative of group 2. CD1a, -b and -c have been shown to present foreign and self (glyco-)lipid antigens to polyclonal T cells [87-92]. CD1e does not present lipid antigens to T cells. It stays intracellular and facilitates the processing of complex glycolipids which are presented by CD1b, -c and -d [93, 94]. CD1d presents foreign and self (glyco-)lipid antigens to a subset of T cells with a limited T cell repertoire known as invariant natural killer T cells (NKT) [95]. It was shown that NKT cells depend on the expression of CD1d for their development in the thymus [96].

Due to the differences in groove morphology and different intracellular trafficking, CD1 molecules are able to present a wide variety of self and foreign lipid antigens. Intracellular trafficking is mediated by the cytoplasmic tail of CD1 molecules. Human CD1a has no known trafficking motif in its cytoplasmic tail and has been shown to traffic to early endosomes, where it samples lipid antigens and translocates back to the cell surface. Human CD1b, CD1c, and CD1d have a cytoplasmic tail containing a tyrosine based motif and traffic to early or late endosomal compartments or lysosomes, where they encounter other types of lipid antigens [97-99].

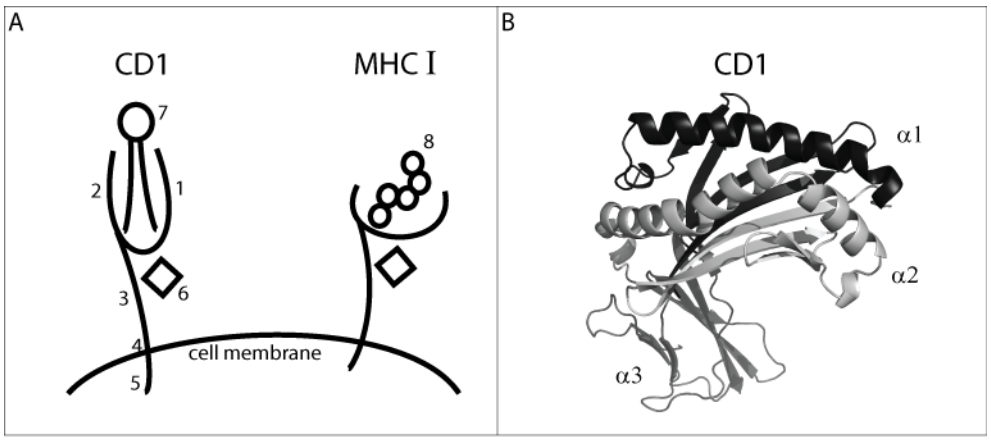

Figure 3. (A) The structure of the CD1 lipid antigen presenting molecule. The groove is formed by the α1 and α2 domain (1,2), which is continued by the α3 domain (3), a transmembrane region (4) and a cytoplasmic tail (5). The CD1 molecule forms a heterodimer with β2-microglobulin (6). The CD1 molecule has a deep and narrow groove with an apolar lining, which enables the binding of lipid antigens (7). In contrast, MHC class I molecules has a broader polar groove suitable for peptide binding (8), (B) A top-side view of the extracellular part of the CD1 molecule (canine CD1a8.2) containing the α1 ,α2 and α3 domain.

Another difference between CD1 isoforms is their differential expression pattern on cells. Group 1 CD1 molecules (CD1a, CD1b, and CD1c) are expressed on professional antigen presenting cells (APC). Human Langerhans cells in the skin are known for their high expression of CD1a, while dermal dendritic cells express CD1b and CD1c [100]. CD1c is also expressed on a subset of B cells [101]. CD1d is expressed on epithelial cells including KCs, gut and liver cells, APCs and B cells [99].
There is a large variety in the number of CD1 isoforms and homologs per isoform between mammalian species. Mice and rats express only CD1d [74, 75] whereas guinea pigs and cows show an extended CD1 family containing multiple homologs per isoform [76, 82]. Mutations in the murine CD1D gene have enabled broader endosomal trafficking and antigen presentation compared to human CD1d, and may compensate for the lack of murine group 1 isoforms [102]. It is unclear which mechanisms are responsible for this diversity in isoforms, however, at least one CD1 isoform is present in each studied species [103] and suggests that CD1 molecules have become a necessary extension of the immune system coping with pathogens by presenting their lipid antigens.

CD1 and atopic dermatitis
Limited information is available regarding the functional role of CD1 molecules in the pathogenesis of atopic dermatitis or other allergic diseases. Several canine and human studies report about a significant increase of $CD1a^+$ DC in atopic skin [36, 43, 62, 71]. In these studies, CD1a molecules were used as markers for

Langerhans cells or DC subsets, and antigen presenting aspects of these molecules were not analyzed.
In contrast to dogs and humans, murine atopic skin is characterized by an influx of dendritic cells which lack the expression of CD1a due to the absence of group 1 CD1 genes in mice. Murine models are therefore not suitable to study the role of CD1a-restricted T cells in the pathogenesis of AD.
The functional aspects of CD1 molecules on the development of allergic diseases was described in a study of Agea et al. it was shown that lipid components from cypress pollens may be recognized as antigens by human T cells through a CD1-dependent pathway [104]. Phospholipids from cypress grains, such as phosphatidyl-choline and phosphatidyl-ethanoloamine, were recognized by mostly $CD4^+$ T cells expressing $\alpha\beta^+$ T cell receptor (TCR), but also by some $CD4^-CD8^-$ $TCR\gamma\delta^+$, and rarely invariant natural killer T cells. These T cells required $CD1a^+$ or $CD1d^+$ antigen presenting cells for stimulation and produced IFNγ and IL-4. Furthermore, it was observed that asthmatic patients in the study had cypress lipid-specific IgE antibodies in contrast to the control group.

CD1a
A recent paper shows that CD1a-autoreactive T cells belong to the normal human T cell repertoire [105]. These polyclonal T helper cells with $\alpha\beta^+$ TCR home to normal healthy skin and produce IL-22 (Th22) in response to CD1a on Langerhans cells. In human AD skin an increase of IL-22 producing T cells and IL-22 expression was found, and may contribute to the pathogenesis by mediating hyperplasia of keratinocytes [25].

CD1d
Human and murine CD1d-restricted invariant NKT cells can rapidly secrete large amounts of Th1 (IFNγ) and/or Th 2 cytokines (IL-4, IL-13) [106-109]. It was shown that IL-4 secreting NKT cells were able to promote the differentiation of naïve $CD4^+$ cells into IL-4 producing Th2 cells, and therefore may be responsible for isotype switching of B cells to produce IgE antibodies [110].
In human lesional atopic skin it was shown that the cellular infiltrate contained NKT cells. About 5% of the $CD3^+$ cells were NKT cells, and these cells expressed IFNγ and IL-4 [111]. Magnan et al. showed that the number of $CD4^+$ NKT cells was significantly higher in peripheral blood of AD patients compared to controls, and was correlated to IL-4 expression [112]. In contrast, another study found no difference for $CD4^+$ NKT cells but a lower count of $CD4^-$ NKT cells was found in the blood of AD patients, which may result in a lower expression of IFNγ and contribute to a Th2 deviation of the Th1/Th2 balance [113].
Another study reported no role for CD1d-restricted NKT cells in the development of AD. In this study, sensitized NKT-deficient CD1d knock-out mice showed similar eosinophil and $CD4^+$ skin infiltrate and increased Th2 cytokine expression level in skin as wild-type mice [114].
In normal human skin, keratinocytes express low levels of CD1d, which is in contrast with the high CD1d-expression of keratinocytes from acute and chronic psoriatic skin [115]. It was shown that IFNγ increased CD1d-expression on keratinocytes and leads to clustering of NKT cells which produced large amounts of IFNγ. The investigators did not look at expression of CD1d in AD skin, but a similar process could be present in chronic lesions which show high expression of IFNγ.

The role of CD1d/NKT system has also been studied in other allergic diseases such as asthma. In CD1d-deficient and NKT receptor-deficient mice it was impossible to develop an allergen-induced airway hyperreactivity (AHR) [116, 117]. The administration of anti-CD1d mAb blocked ability of eosinophil recruitment and production of Th2 cytokines and IgE in allergen-challenged wild type mice. In these mice it was still possible to induce AHR by administration of IL-13, and these mice were able to display a normal Th2 response, indicating that the CD1d/NKT system is necessary for the initiation of allergen-induced AHR.
In mice it was shown that the CD1d/NKT system plays an important role in the induction of contact hypersensitivity (CHS). It was shown, that CD1d-restricted T-cell-null mice and wild type mice treated with a CD1d antagonist, have diminished CHS responses [118]
The above studies showed that different CD1 molecules expressed on APC and keratinocytes are able to activate different T cell subpopulations, which may play a role in the development of atopic dermatitis and other allergic diseases in humans and mice. Activated T cells produce cytokines which regulate the inflammatory response and affect tissue such as skin. The functional aspects of CD1 molecules in canine AD need further investigation.

Barrier defect
Earlier studies claimed that a skin barrier defect seen in AD, was one of the outcomes of a dysregulated inflammatory immune response in the skin, known as the inside-out theory (figure 2) [21]. More recent studies have shown that even in unaffected atopic skin the barrier may be defective, and therefore can be a prerequisite for the development of AD: the outside-in theory [119].
The epidermis is the outer layer of the skin and acts as a barrier preventing the entrance of pathogenic compounds and water loss by evaporation. Keratinocytes are the major building blocks of the epidermis and comprise about 90% of the cells. Keratinocytes proliferate at the stratum basale, are pushed upwards and differentiate into mature enucleated corneocytes which form the stratum corneum (SC), the outermost layer of the epidermis (left panel figure 2). The stratum corneum is organised in a so-called bricks-and-mortar structure with dense fibrous protein packed corneocytes (bricks) and endogenous lipids (mortar), such as ceramides, cholesterol and fatty acids.
One important protein that plays a role in the barrier formation is filaggrin. Filaggrin is short for filament-aggregating protein and is able to bind keratin fibers during the formation of corneocytes. It has been shown that non-lesional and lesional skin from humans with AD have a decreased expression of filaggrin compared to control skin [120]. More recent genetic studies have shown that two independent loss-of-function genetic variants (R510X and 2282del4) in the gene encoding filaggrin are very strong predisposing factors for atopic dermatitis [121, 122]. Others found that the presence of a Th2 cytokine milieu (IL-4 and IL-13) reduced the expression of filaggrin in differentiated keratinocytes [123].
There is little known about the role of filaggrin in canine AD. In a pilot study using an experimental model of AD, it was reported that unaffected skin of atopic beagles had a lower expression of filaggrin than skin of control dogs [124]. Furthermore it was shown with immunohistochemical staining for filaggrin that in atopic skin of dogs expression was lower than in control dogs [19]. It was also found that mRNA expression of filaggrin was lower in non-lesional atopic skin compared to healthy

canine skin [125]. In contrast, in another study increased filaggrin mRNA expression was found in lesional skin compared to control skin [126]. This indicates that the role of filaggrin in canine atopic dermatitis needs further investigation.
Ceramides are produced by keratinocytes and play an important role in the maintenance of the skin barrier as they fill up the intercellular space between the corneocytes and enable adhesion. A marked reduction was found in the amount of ceramides in non-lesional and lesional human atopic skin compared with control skin [127, 128]. Sphingomyelinase is an enzyme which is responsible for hydrolysis of glucosylceramide to ceramide [129]. The activity of this enzyme was reduced in human non-lesional and lesional skin and correlated to the ceramide content in the skin [130]. In dogs similar results were found; it was observed that the amount of ceramides was significantly lower in non-lesional skin of AD dogs compared to controls [131-135]. Transmission electron microscopy of canine atopic skin tissue revealed a decreased lipid deposition in the stratum corneum compared to control dogs, and intracellular lipid lamellae in AD skin were thin and discontinuous, which may reduce the barrier function of the epidermis [136].
One of the methods to investigate barrier function is to measure transepidermal water loss (TEWL) [137]. In human, atopic skin showed reduced water content and an increased TEWL [138]. In house dust mite-sensitized atopic beagle dogs TEWL was increased after allergen challenge, whereas no effect on TEWL was found in control dogs [139]. Improvement of the barrier of atopic skin by treating the skin with ointments and creams reduced the TEWL [140-142]. In *in vitro* cultured keratinocytes from healthy dogs nutritional components, such as pantothenate, choline, nicotinamide, histidine, proline, pyridoxine and inositol, were able to increase epidermal lamellar lipid synthesis [143]. Supplementing the diet with these components lead to a reduced TEWL in dogs within 9 weeks and may be beneficial in preventing AD in predisposed dogs.

Epidemiology and environmental exposure
Atopic dermatitis is a common skin disease in dogs and humans. An interesting aspect is the increased prevalence of human atopic dermatitis and other atopic diseases in developed countries in the last decades [144-146]. Whether this phenomenon also occurred in the canine population is unknown as no epidemiological study on geographical or time variations in the prevalence of atopic diseases has been performed.
The increased prevalence of atopic diseases shows that besides the genetic constitution, which cannot dramatically change in a few decades, other factors like environmental exposure are important for the development of the disease. The “hygiene hypothesis” postulates that the improved sanitary conditions of western people resulted in the lack of exposure towards infectious agents, microbes and parasites, which drives the immune response towards a state which enhance allergic responses [147].
It was shown that exposure to environmental endotoxin, a cell wall component of gram-negative bacteria, was inversely related to the occurrence and development of hay fever, atopic asthma, and atopic sensitization in children [148-151]. Expression of cytokines such as TNFα, IFNγ, IL-10 and IL-12 measured in leukocytes was inversely correlated to endotoxin exposure levels. This may indicate a down-regulation of the immune response in endotoxin exposed children. In contrast, other studies found that higher environmental endotoxin exposure was

associated with symptoms and severity of asthma [152, 153]. It is important to consider the dosage, time of exposure, and type of endotoxin in studying its effect, so it may act preventively, but can also augment allergic symptoms and inflammation when the allergic disease is already present [154]. In earlier studies, the effects of endotoxins were usually addressed in-line with the Th1/Th2 paradigm. It was stated that endotoxins increased the levels of the classical Th1 cytokine IFNγ [155, 156], which could reduce the levels of the Th2 cytokine IL-4 and prevents the development of a Th2-mediated allergic immune response. A murine study showed that daily low-dose intranasal administration of endotoxin (LPS) blocked Th2 cells without increasing Th1 cells, probably by inducing IL-10 producing T regulatory cells (Treg) which reduced the development atopic immune response [157]. It was also shown that high environmental endotoxin exposure in farmers children influenced the innate immune system by increasing the CD14 receptor and Toll-like receptor 2 on PBMC, which may modulate the development of allergic diseases [158]. No studies have been performed that investigated the role of endotoxins on the development of canine AD.

Besides bacterial endotoxins also other components can alter the immune response. The (1→3)-β-D-glucans are cell wall constituents of most fungi and yeasts and have been used as a marker for mould exposure [159]. It was reported that (1→3)-β-D-glucans enhanced the production of pro-inflammatory cytokines such as IL-6, IL-1 and TNFα by macrophages [160]. Results of several studies indicate a protective effect of exposure of mould components to atopic disease [161-164].

The effect of environmental allergen exposure on natural occurring AD in human and dogs is not clear. House dust mite (HDM) species *Dermatophagoides pteronyssinus* and *D. farinae* are a major source of indoor environmental allergens [165-167] and sensitization towards HDM is commonly associated with AD, both in dogs and humans [168, 169]. The relation between HDM exposure level and development of AD remains controversial. Studies report about a positive correlation between the level of HDM exposure and atopic eczema in children [170]. In a study by Ricci et al. (1999), high HDM exposure was only correlated to sensitization of HDM and not with the development of AD [171], whereas other studies even failed to determine an effect of HDM exposure [172, 173]. Besides acting as an allergen during the allergic immune response, HDM allergens can also exert enzymatic activity. The most studied HDM allergens are Der p1 and Der f1, which can be found at high concentrations in mite faecal pellets. It was shown that the proteolytic activity of Der f1 reduced the skin barrier formation and may lead to a higher penetration of allergens and microbes [174].

The role of environmental exposure on the development of AD in dogs is unclear. It will be interesting to address this issue because humans and (pet) dogs share the living environment and show similar pathophysiological mechanisms.

Scope of the thesis

Previous studies show that atopic dermatitis is a multifactorial, heterogeneous disease, in which pathological immune responses, genetic factors, epidermal barrier defects, environmental exposure and likely other as yet unknown factors are important for the development of the disease.

In this thesis we describe several host and environmental aspects which directly or indirectly seem to play a role in canine atopic dermatitis.

Chapter 2 describes the identification and characterization of the canine CD1 genes. This chapter reports about differential expression of three canine CD1A genes in skin, recognition of canine CD1a by antibodies. Based on protein modelling, we predict that both canine CD1a proteins can bind different glycolipids.

In Chapter 3 we provide further insight in canine *CD1D* gene, in which three different tandem repeats were found. We show that these repeats modify the structure of the canine *CD1D* gene, which seems to prevent expression of the canine CD1d protein *in vivo*.

In Chapter 4 we characterize the CD1d/NKT system in mammalian species including dog. The CD1d/NKT system in pig and horse are highly homologous to the human complex in contrast to various ruminant species and dogs.

A pilot study on the expression of filaggrin, pro-inflammatory cytokines and chemokines in *in vitro* cultured keratinocytes from dogs with atopic dermatitis and control dogs, is reported in Chapter 5. Our findings seem to suggest that local skin conditions affect filaggrin expression of keratinocytes. We show increased IL-8 expression in cultured keratinocytes from atopic after stimulation with Th1 and Th2 cytokines, which may contribute to the influx of inflammatory cells in atopic skin during acute and chronic inflammation.

The relation between exposure to environmental factors like house dust mites, bacterial endotoxins and fungal glucans and atopic dermatitis in dogs is described in Chapter 6. We showed that there was an inverse association between endotoxin exposure and atopic dermatitis.

In chapter 7 the main findings are summarized and discussed in the context of the current knowledge on canine atopic dermatitis.

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Chapter 2

Two canine CD1a proteins are differentially expressed in skin

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Immunogenetics 2008; 60(6):315-324

Abstract

Lipid antigens are presented to T cells by the CD1 family of proteins. In this study we characterize the complete dog (*Canis lupus familiaris*) CD1 locus which is located on chromosome 38. The canine locus contains eight CD1A genes (canCD1A), of which five are pseudogenes, one canCD1B, one canCD1C, one canCD1D, and one canCD1E gene. *In vivo* expression of canine CD1 proteins was shown for canCD1a6, canCD1a8, and canCD1b, using a panel of anti-CD1 mAb. CanCD1a6 and canCD1a8 are recognized by two distinct mAb. Furthermore, we show differential transcription of the three canCD1A genes in canine tissues. In canine skin the transcription level of canCD1A8 was higher than of canCD1A6, and no transcription of canCD1A2 was detected. Based on protein modeling and protein sequence alignment, we predict that both canine CD1a proteins can bind different glycolipids in their groove. Besides differences in ectodomain structure, we observed the unique presence of three types of cytoplasmic tails encoded by canCD1A genes. cDNA sequencing and EST sequences confirmed the existence of a short, human CD1a-like cytoplasmic tail of four amino acids, of an intermediate length form of 15 amino acids, and of a long form of 31 amino acids.

Introduction

Antigen presenting molecules play an important role in the adaptive immune system. The well known MHC class I and class II antigen presenting molecules have been found in all jawed vertebrates studied so far. In mammals and birds an additional antigen presenting lineage has been identified: the non-polymorphic CD1 family of glycoproteins consisting of CD1a, CD1b, CD1c, CD1d, and CD1e. The remarkable difference between CD1 and MHC is that CD1 molecules present foreign and self-lipid antigens, rather than peptides to T cells [1-5]. It has been shown that different classes of lipid antigens are presented by different CD1 isoforms. The small binding groove of CD1a allows binding of mycobacterial lipopeptides and sulfatide sphingolipid [6-8], whereas CD1b has the largest binding groove of all CD1 isoforms and is capable to bind lipid antigens with much longer alkyl chains, such as mycolyl lipids [1, 9-11]. Not only the size and shape of the CD1 groove structure but also intracellular trafficking determines which lipid antigens are presented by which CD1 isoform. Human CD1a has been shown to traffic to early endosomes were it samples lipid antigens and translocates back to the cell surface. Human CD1b, CD1c, and CD1d traffic to lysosomes and/or late endosomal compartments within the cell where they encounter other types of lipid antigens [12-17].

Another difference between CD1 isoforms is their expression pattern and the type of T cell they activate. Group 1 CD1 molecules (CD1a, CD1b, and CD1c) are expressed on professional antigen presenting cells. Human Langerhans cells in the skin are known for their high expression of CD1a, while dermal dendritic cells express CD1b and CD1c [18]. It has been shown that group 1 CD1 molecules present mycobacterial antigens to T cells [1, 7, 19, 20] . Group 2 CD1 molecules (CD1d) are expressed on several types of antigen presenting cells but are also present on a wide range of non-hematopoietic cells. CD1d is the restricting element that selects and activates invariant NKT cells [21-24]

The human CD1 locus contains one gene for each of the different CD1 isoforms [25, 26]. However, as a result of CD1 gene duplication and deletion during mammalian evolution, the number of CD1 molecules and their genes is different in other species. For example, the guinea pig CD1 locus consists of four CD1B genes and three CD1C genes. It is thought that the loss of CD1 isoform diversity and their specific function is at least partly recovered by gene duplication and neo-functionalization of CD1 molecules [27].

In this study the complete dog CD1 locus is characterized. We show the differential expression of CD1A genes in canine skin and the unique *in vivo* expression of two distinct CD1a proteins.

Materials and methods

In silico identification of canine CD1 genes
Blast searches in the canine genome (Broad Institute of MIT/Harvard, CanFam 2.0 version 46.2d; coverage 7.5), available at www.ensembl.org/Canis_familiaris, were performed with α1 and α2 domain nucleotide sequences of all human CD1 isoforms. To compare canine CD1A genes with other mammalian CD1A genes we used the known CD1A genes in GenBank of human (NM001763), rabbit (AF276977 and AF276978), pig (AF059492), cattle (DQ192541). To identify cat CD1A genes we performed a blast search with human α1 and α2 domain nucleotide sequence of human CD1A in the feline genome (Agencourt Bioscience, CAT version 46.1b; coverage 2). Blast searches for EST sequences were performed at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Canine tissue collection and cDNA synthesis
Tissue samples were obtained from healthy adult dogs (Beagles). PBMCs were isolated from dog blood by standard Ficoll-Hypaque gradient centrifugation. Skin samples were processed using a Biopulverizer and homogenized using a mini-Turrax (IKA). Intestine tissue (duodenum, jejunum, and colon) was washed and incubated in PBS with 0,75 mg/ml EDTA, 5% FCS, and 50µg/ml gentamicin at 37°C for 1 hour to obtain intestinal cells. Single-cell suspensions were freshly prepared from liver, spleen, lymph node and thymus. Canine tissue was collected according to the regulations of the Animal Ethical Committee of the University of Utrecht, the Netherlands (protocol number 2007.II.06.152). From all collected tissues RNA was isolated using the RNeasy kit (Qiagen) followed by cDNA synthesis with Multiscribe reverse transcriptase (Applied Biosystems).

PCR, cloning, and sequence analysis
PCRs were performed with Pfu Turbo polymerase (Stratagene) according to the protocol of the manufacturer under the following cycling conditions: an initial denaturation of 7 min. at 95°C, followed by 40 cycles of 15 sec. at 95°C, 45 sec. at a primer-specific annealing temperature, 30 sec. at 72°C, followed by a final elongation step of 5 min. at 72°C. PCR for full length CD1 genes was performed using thymus cDNA as template and the following primers: canCD1A6 forward 5'-GCAAGAGAAAGACATCTGCAAACACG and reverse 5'-CCCTGGACTGACCTCAAGGCA at annealing temperature (Ta) of 61°C; canCD1A8 forward 5'-GCAAGAGAAAGACATCTGCAAACACG and reverse 5'-CACRGGGAGTGGCCAGGAC at Ta of 61°C; canCD1B forward 5'-CAGCCCACTGTCCGGGGAG and reverse 5'-AAGAAGAGTTCATGAGATGGCGAGGG at Ta of 61°C; canCD1C forward 5'-GGGAGCGGGGAAGCATCTGC and reverse 5'-GGGAGGCATGGTGACGAAGC at Ta of 59°C. The CD1D gene was not amplified in full length due to unknown sequence upstream from the α2 domain sequence, therefore canCD1D forward was designed in the α2 domain 5'-GATCCTGAGTTTCCAAGGGTCTCAC and reverse 5'-GCTGAGGGTGAAGAAAGGCTGC in the 3'UTR region at Ta of 65°C. To determine the mRNA expression of the different CD1A genes in dog tissue, gene specific forward primers and a general reverse primer were designed: canCD1A2 forward in α2 domain 5'-AGCTGCGCATTGGAGAACCTTTC;

canCD1A6 forward in α1 domain 5'-GGACAGTGACTCTGGCACTTTCTTG; canCD1A8 forward in α1 domain 5'-CATACACCATCCGATCCCCTTCC; canCD1A general reverse primer in α3 domain 5'-AGACTGCTGTGTCTCACACGGC at a Ta of 59°C. Gel electrophoresis (10% agarose gel) was performed for 1 hour at 100V. After PCR, full length CD1 PCR products were isolated from gel. Products were ligated in a pCR4Blunt-TOPO vector (Invitrogen) to transform One Shot TOP10 cells (Invitrogen). Vector DNA of single colonies was sequenced by BaseClear (Leiden, Netherlands). The obtained sequences were compared to the canine CD1 sequences obtained from the ensemble database (www.ensembl.org/Canis_familiaris). The ExPASy Translate Tool was used (www.expasy.ch/tools/dna) for translation into amino acids. All alignments were performed with ClustalW (align.genome.jp).

Monoclonal antibodies and staining procedures

Expression of CD1 by canine thymocytes, 030-D histiocytosis cell line [28], and 293T cells transfected with canCD1 was measured using a FACScalibur flow cytometer (Becton Dickinson). Transfection of 293T was performed with full length CD1 gene sequences in pcDNA3.1+ vector (Invitrogen) using FuGENE6 (Roche) as transfection reagent. Anti-canine CD1 mAbs CA13.9H11 (IgG1), CA9.AG5 (IgG1), anti-feline CD1a Fe1.5F4 (IgG1) and anti-feline CD1c Fe5.5C1 (IgG1) were provided by Dr. P.M. Moore (School of Veterinary Medicine, University of California, Davis CA). Anti-human CD1a (OKT6; IgG1), CD1b (BCD1b3; IgG1), CD1c (F10/21A3; IgG1) were provided by Dr. D.B. Moody (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). Anti-human CD1a (WM-35; IgG2b) was obtained from ImmunoTools. Anti-bovine CD1 CC20 (IgG2a) was provided by Dr. C.J. Howard (Institute for Animal Health, Compton, U.K.); CC14 (IgG1) was provided by Dr. J.C. Hope (Institute for Animal Health Compton, U.K.); 20.27 SBU-T6 (IgG1) was obtained from the European Collection of Cell Cultures. GAM-FITC was used as secondary antibody. Primary antibodies were diluted in PBS with 0.1% sodium-azide and 2% FCS. Canine thymocytes, 030-D cell lines and CD1-transfected 293T cells were incubated with primary antibody for 30 minutes at 4°C, followed by incubation with GAM-FITC (BD Biosciences) for 30 minutes at 4°C.

Protein modeling

Canine CD1a protein models were generated using the Swiss Model Server with human CD1a (PDB code 1ONQ) as a template. The model was visualized using PyMol (pymol.sourceforge.net). The program APBS [29] was used to calculate the electrostatic surface potentials with electronegative in red and electropositive in blue (-30 to +30 kT/e).

Results

Characterization of the canine CD1 locus

The canine CD1 (canCD1) genes are located close to the telomeric end of chromosome 38 between 26.30 Mbp and 26.55 Mbp. The boundaries of the canCD1 locus are formed by the CD1-unrelated Kirrel and olfactory receptor gene group of which OR10T2 was the first gene adjacent to the CD1 locus. The canCD1 locus contains six gaps of 61-578 nucleotides which are located between the CD1 genes and one gap of 2852 nucleotides that lies between bp 26,503,057 and 26,505,908 of which 75% of the nucleotide sequence was present in a draft sequence deposited by the Broad Institute (AC183576). The canCD1 locus consists of the following genes from telomeric end to centromere: one canCD1D gene, eight canCD1A genes, one canCD1C, one canCD1B, and one canCD1E gene (Figure 1). The canine CD1 genes have the same orientation as human CD1 genes except for CD1B which in the human CD1 locus has an opposite orientation compared to the other human CD1 isoforms.

Of the eight CD1A genes, three CD1A genes (canCD1A2, canCD1A6, canCD1A8) revealed a complete sequence without obvious characteristics of pseudogenes like internal stop codons, frameshift mutations, and splice site mutations. Five CD1A genes were considered to be pseudogenes (canCD1A1, canCD1A3, canCD1A4, canCD1A5, canCD1A7). During the *in silico* study we found a fourth full length CD1A gene however, due to its high nucleotide identity with canCD1A8 (93%) and because it was not assigned to chromosome 38 by Ensemble (CanFam 2.0, www.ensembl.org), we assume that this is an allelic variation and named these two CD1A8 genes canCD1A8.1 and canCD1A8.2 accordingly. Furthermore, full length genes for canCD1B, canCD1C and canCD1E were present in the dog CD1 locus. In the draft sequence (AC183576) we found a part of the canine CD1D gene. In this sequence it was not possible to identify the leader fragment, the first intron, and a small part of the α1 domain of canCD1D because nucleotide sequence was unknown, but most of the α1 domain, and the complete 3'part of the canCD1D gene were intact. Because the map of the locus is complete in the sense that the locus between the adjacent non-CD1 Kirrel and olfactory receptor genes did not contain any gaps big enough to contain a CD1 gene, we assume that we identified all canCD1 genes.

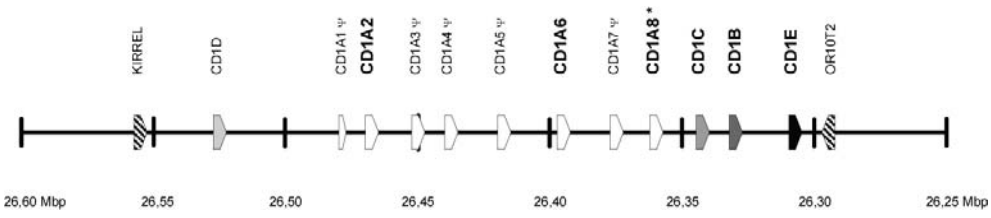

Figure 1. Complete gene map of the canine CD1 locus located on chromosome 38. Full length canine CD1 genes without characteristics of pseudogenes are given in bold. Pseudogenes are marked with Ψ. Exact location of CD1 genes: CD1D: unknown-26502267; CD1A1: 26479022-26477829; CD1A2: 26470445-26467658; CD1A3: 26452485-26449619; CD1A4: 26439630-26436644; CD1A5: 26418868-

26416544; CD1A6: 26398237-26395316; CD1A7: 26378486-26375657; CD1A8: 26363063-26360148; CD1B: 26332684-26329120; CD1C: 26344187-26341100; CD1E: 26310804-26308062. *The location of the allelic variant of CD1A8 is: 81854142-81857071 on unknown chromosome (www.ensembl.org).

Cloning of full length canCD1 cDNAs

Full length cDNAs were cloned using a PCR based strategy. The forward primer was designed to anneal upstream of the start codon, based on the available genomic sequences, and the reverse primer was designed to bind downstream of the predicted stop codon. Full length canCD1A6 (GenBank EU373508 / protein_id ACB12088) and canCD1A8.2 (GenBank EU373509 / protein_id ACB12089) transcripts were cloned. We have not been able to obtain full length CD1A2, which may be due to a low transcription level in thymus cDNA and high homology with canCD1A8 at the sites where the primers were designed to bind. The ectodomain of the canCD1A6 transcript was 99% identical at amino acid level to the predicted canCD1A6 transcript from the *in silico* study. The canCD1A8 transcript showed greater amino acid identity to the predicted canCD1A8.2 transcript than to the predicted canCD1A8.1 transcript (98% and 88% respectively). A single full length canCD1B transcript was cloned (GenBank EU373510 / protein_id ACB12090) and showed 99% amino acid identity with the predicted canCD1B. Two different full length canCD1C transcripts were obtained from thymus-derived cDNA of a single dog (Beagle), which was named dog A. The two different transcripts canCD1C clone 1 and clone 2 (GenBank EU373511 / protein_id ACB12091 and EU373512 / protein_id ACB12092) both showed 98% amino acid identity to the predicted canCD1C transcript. These small nucleotide alterations compared to the predicted sequence might be caused by PCR artefacts and/or allelic variation. A third unique full length canCD1C, clone 3, (GenBank EU373513 / protein_id ACB12093), was obtained from cDNA derived from the 030-D canine histiocytosis cell line, and showed 99% identity with the predicted canCD1C. From thymus-derived cDNA, a partial canCD1D transcript was cloned containing a fragment of the α2 domain, full length α3 domain, a transmembrane region, and a cytoplasmic tail.

Recognition of canCD1 molecules by monoclonal antibodies

To determine which antibodies recognize the canine CD1 isoforms, we tested a panel of mAb specific for CD1 in humans, cattle, sheep, and cat. We also tested the two anti-canine CD1 mAb CA13.9H11 and CA9.AG5 for their specificity for canCD1. Results are summarized in Table 1. Freshly isolated thymocytes of dog A were recognized by the anti-canine mAb CA13.9H11 and CA9.AG5, the anti human mAb BCD1b3, the anti-bovine mAb CC20 and SBUT6, and the anti feline mAb Fe1.5F4 and Fe5.5C1, but not by the anti-human CD1a mAb OKT-6 and anti human CD1c mAb F10/21A3. Using 293T cells transfected with full length canCD1 transcripts, we found that canCD1a6 is recognized by the anti-feline CD1a Fe1.5F4 but not by anti-canine CD1 CA13.9H11. The opposite was true for recognition of canCD1a8.2 (Figure 2). This implies that two different mAb recognize two different *in vivo* expressed canCD1a proteins. The anti-canine CD1 antibody CA9.AG5 did not recognize canCD1a6, canCD1a8.2, and canCD1b protein on transfected 293T cells, however it did recognize an unidentified epitope on canine thymocytes.

293T cells transfected with the full length canCD1C transcript clone 1 and 2, derived from dog A, or canCD1C transcript clone 3, derived from the 030-D cell line, were not recognized by any of the mAbs in this panel. Unfortunately we have not been able to confirm expression of the three full length canCD1c cDNAs by staining with any mAb or by alternative methods. The mAb CA13.9H11 and CA9.AG5 have both been suggested to recognize canCD1 [30]. Here we show that CA13.9H11 recognizes canCD1a8.2. We have not been able to show CD1 recognition by CA9.AG5. The mAb CA9.AG5 recognizes a molecule expressed on thymocytes of approximately 60% of all dogs, suggesting that this mAb is specific for an allotype or allelic variation of an unidentified gene product. In our study, the same animal (dog A) was used for flowcytometric analysis of *ex vivo* thymocytes and for cDNA synthesis from thymocytes. The *ex vivo* thymocytes of this animal were recognized by CA9.AG5, but the two slightly different CD1c cDNAs were not. This makes it unlikely that the lack of recognition by the anti-canine CD1 antibodies CA13.9H11 and CA9.AG5 is caused by allelic differences in canCD1c.

mAb	canine thymocytes	030-D	canCD1a6	canCD1a8.2	canCD1b	Published reactivity	Ref.
CA13.9H11	+	+	-	+	-	canCD1	[30]
CA9.AG5	+	-	-	-	-	canCD1	[30]
WM-35	-	+	-	-	-	huCD1a	
BCD1b3	+	+	-	+	+	huCD1b	
SBUT6	+	+/-	-	+	+	boCD1a/boCD1b3	[31]
CC20	+	+	-	+	-	huCD1b/boCD1b3	[32]
Fe1.5F4	+	-	+	-	-	felCD1a	[33]
Fe5.5C1	+	+	-	-	-	felCD1c	[33]

Table 1. A panel of anti-CD1 mAb was tested for recognition of canCD1 on transfectant 293T cells, canine thymocytes, and 030-D canine histiocytosis cell line.

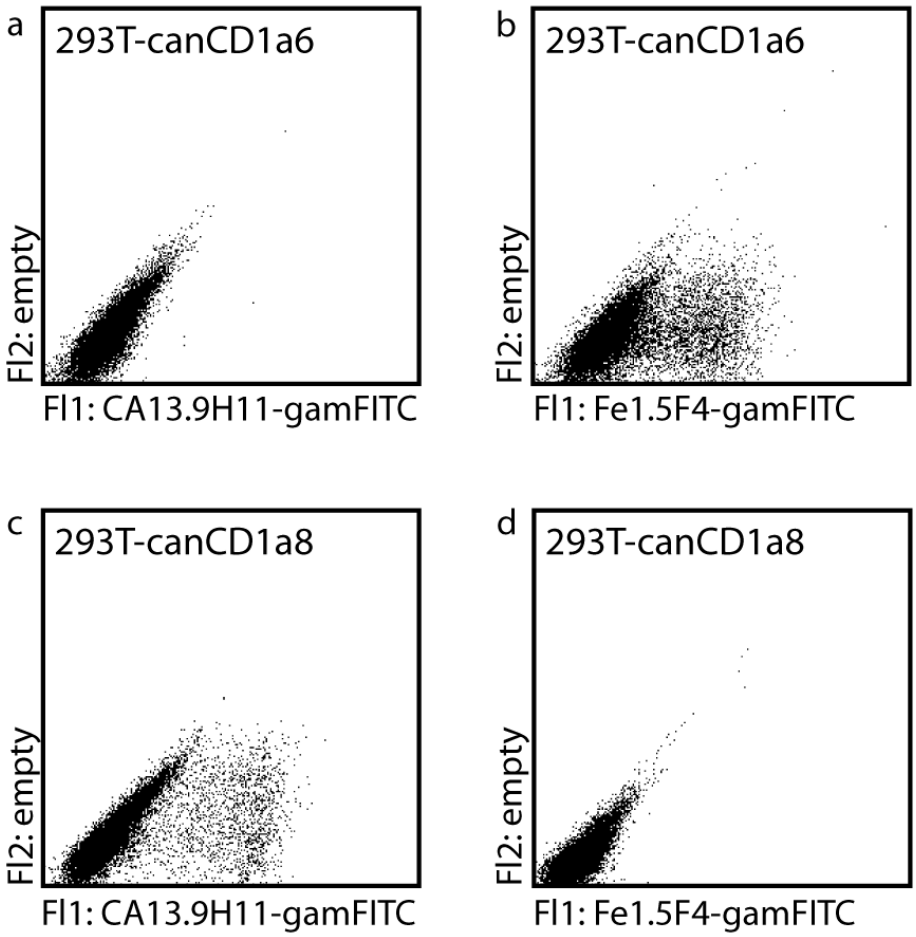

Figure 2. Flow cytometric analysis of expression canCD1 on tranfectant 293T cells. Cells are transfected with canCD1A6 (a-b) or with canCD1A8.2 gene transcripts (c-d). Staining was performed with anti-canine CD1 mAb CA13.9H11, and with anti-feline CD1a mAb, followed by goat-anti-mouse FITC.

Transcription of CD1A genes in different tissues

Transcription levels of the three different CD1A genes were determined in different tissues of a healthy Beagle. Expression of different CD1A genes in canine lymphoid and non lymphoid tissue is shown in Figure 3. Transcription of canCD1A2 was only found in thymus whereas canCD1A6 and canCD1A8 were transcribed in thymus, lymph node, spleen, liver, skin, and PBMC. Interestingly, in skin, canCD1A8 is clearly expressed at a much higher level than the other CD1A genes. Results are representative of three independent experiments.

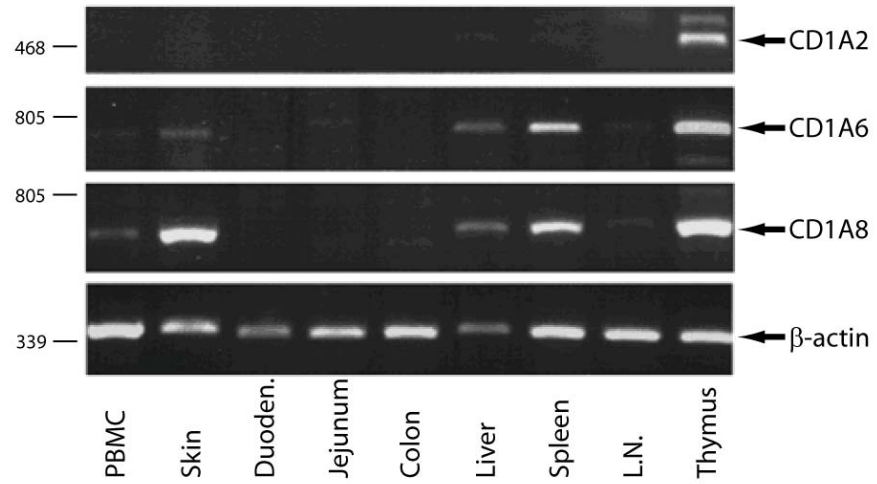

Figure 3. Transcription of three canCD1A genes in different dog tissues. To be able to compare the quantity of input cDNA, β-actin was included.

Comparison of canCD1a6 and canCD1a8 models, and canine CD1 cytoplasmic tails

The protein sequence of the two different canCD1a molecules of which a full length transcript was derived is very similar. However, single amino acid differences at key positions in the binding groove can result in noticeable differences in the structure of the binding groove and, moreover, in the ability to bind and present lipids. To predict whether the overall architecture of the antigen binding grooves of canCD1a is similar to that of human CD1a, we generated models of the two *in vivo* expressed canCD1a molecules using the Swiss Model server [34] and compared these to their human counterpart (Figure 4a). Both the structural models and the sequence comparison (Figure 4b) suggest that both canCD1a6 and canCD1a8, like human CD1a [8], have a hydrophobic binding groove with a buried A' pocket and a more exposed F' pocket. Key structural features such as the A' pole are conserved (Phe70 and Val/Ile12, as well as an A' roof is predicted (residues 66, 69, 161, 165). In addition, the A' pocket of canCD1a8 is predicted to be open-ended (Gly28 *vs.* Val or Ala), similar to mouse CD1d. Therefore, the length of bound lipids in that pocket could be longer than C_{18}, as observed for human CD1a [35]. Clearly the shape of the individual pockets will be slightly different, as not all groove-lining residues are conserved. However, the overall size of the CD1a grooves appears to be similar. Furthermore, the groove opening of canCD1a8 appears slightly larger than the opening of canCD1a6. Also, the residues lining the opening to the CD1a groove (F' portal) are the least conserved between the CD1a molecules. As these residues were shown to bind the glycolipid headgroup, we speculate that the three CD1a proteins have the capacity to bind different glycolipid antigens.

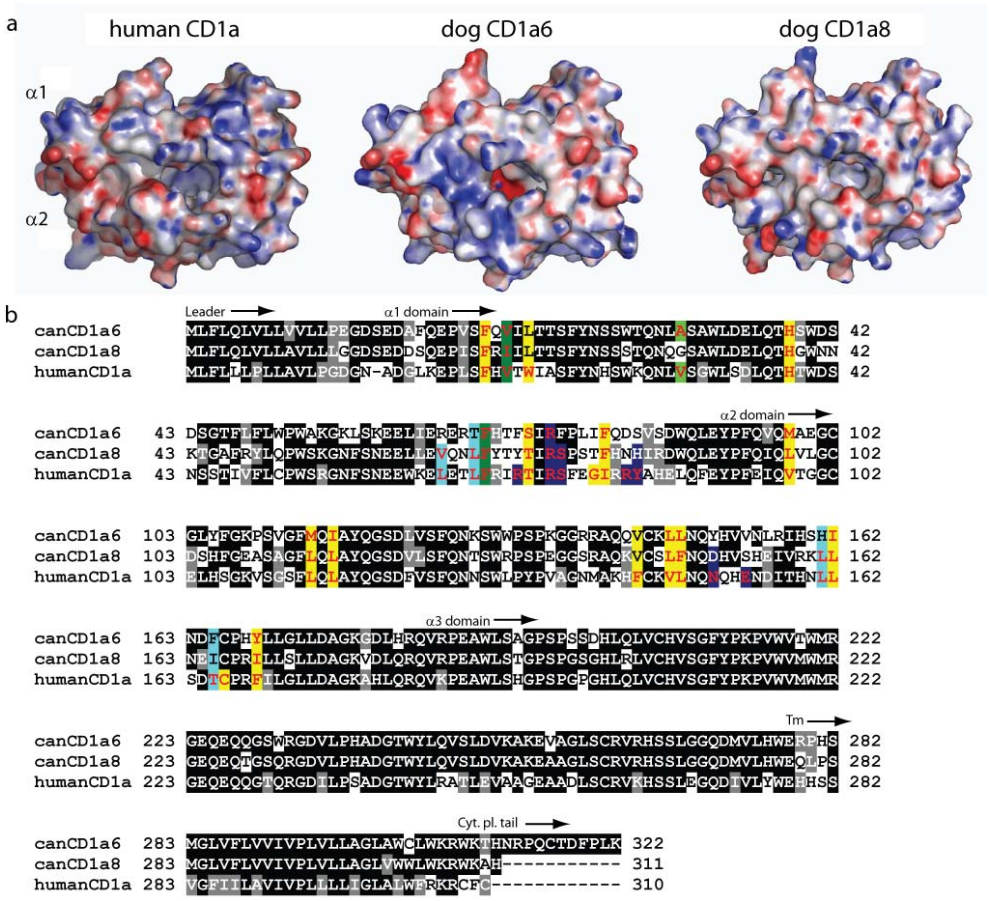

Figure 4. (a) Molecular surface of human CD1a, canine CD1a6, and canine CD1a8. The α1 and α2 domains are depicted and the opening to the binding groove is in the center of each molecule. Note that the bottom of the canCD1a6 binding groove appears electronegative due to the presence of Glu100, however, overall the binding grove is hydrophobic. Also residue 100 is not highlighted in Figure 5B, as it was not a residue contacting the sulfatide ligand in the human CD1a-sulfatide structure (1onq). Electrostatic potentials were calculated using APBS and PyMol, red is electronegative and blue is electropositive (-30 to +30 kT/e). (b) Multiple sequences alignment (CLUSTAL W 1.83) of canine CD1a isoform 6 and 8 and human CD1a, colored by sequence identity (black) and structural features (in color). Residues contributing to the CD1a binding groove of human CD1a are highlighted in yellow. These residues were originally identified base on their interaction with the sulfatide ligand (PDB code 1onq). Darkgreen, A'pole; green, terminal residue restricting the length of the A' pocket; cyan, A' roof; blue, residues at the opening to the CD1a groove (F' portal). Residues colored in red or gray are similar. Residues that are highlighted but not colored in red are predicted not to dramatically alter the structural feature. Canine residues not highlighted according to their human counterparts are predicted to have an altered structural element.

Furthermore, different antigens are presented by CD1 proteins due to sampling in different endocytic compartments. This intracellular trafficking of CD1 is directed by the presence or absence of a sorting motif in the cytoplasmic tail. We aligned cytoplasmic tail sequences of the three canine CD1a proteins with the closely related carnivore species cat and to other, more distantly related mammalian species (Figure 5a). To make this alignment we performed a blast search in the cat genome and found four CD1A genes of which two contained the nucleotide sequence of the cytoplasmic tail and we used known CD1a cytoplasmic tail sequences of human (NM001763), rabbit (AF276977 and AF276978), pig (AF059492), and cattle (DQ192541). Except for human CD1a and canCD1a8.2, all other known mammalian CD1a cytoplasmic tails are long. CanCD1a2 and canCD1a8.1 have an even longer cytoplasmic tail of 31 amino acids, which was confirmed by the identification of transcripts of canCD1A8.1 (DN877612) and canCD1A2 (DN409579) that included these cytoplasmic tails in the EST databases. No known sorting motif was observed in any of the canine CD1a cytoplasmic tail sequences.
The cytoplasmic tails of the other canine CD1 isoforms were aligned with the amino acid sequences of cytoplasmic tails of their human homologs (Figure 5b). Like in their human counterparts, the tyrosine trafficking motif YXXZ was present in all three canine sequences. However, the human CD1c tail contains a dileucine motif which is not present in canCD1c.

a

humanCD1a	CFC
canCD1a8.2	WKAH
canCD1a2	WKAHWRPQCMDFPSEQEPSSPSSSTYLNPAQH
canCD1a8.1	WKAHWRPQCTDFPSEQEPSSPGSSTYLNPAQH
canCD1a6	WKTHNRPQCTDFPLK
felCD1a1 (p)	WKSHETPQCTGLPLE
felCD1a2 (p)	WKSHWRHQCTGLPLE
rabbitCD1a1	WIHHG.PLETLLPLQ
rabbitCD1a2	WSHHGSPN.SLLPLK
pigCD1.1	WK.HCDPSSALHRLE
boCD1a	WTHRESPSS.VLPLE

b

canCD1b	RS**YQSI**S
humanCD1b	RS**YQNI**P
canCD1c	CS**YQDI**P
humanCD1c	CS**YQDI**L
canCD1d	RS**YQDI**L
humanCD1d	TS**YQGV**L

Figure 5. (a) Alignment of the CD1a cytoplasmic tail sequences of different mammalian species. Tail sequences that were not confirmed by cDNA sequencing but only predicted from the genome are marked accordingly (p). (b) Alignment of cytoplasmic tail sequences of canCD1b, canCD1c, and canCD1d with their human orthologs. The tyrosine trafficking motif (YXXZ) is underlined.

Discussion

The canine CD1 locus located on chromosome 38 contained all known CD1 isoforms. We identified a remarkable large number of CD1A homologs, three of which were shown to be full length canCD1A genes and five were considered pseudogenes. Intensive duplication of CD1A genes leading to presence of two or possibly three distinct CD1a proteins is characteristic for the canine CD1 locus. This is the first study showing differential transcription of two CD1A genes for which *in vivo* protein expression is confirmed. These differences in expression might indicate differences in function between the CD1a molecules in canine skin. So far it is unknown whether these different CD1a molecules are present on the same antigen presenting cell in the canine skin or that different antigen presenting cells express different canCD1a molecules. Stationary epidermal Langerhans cells as well as migrating dermal Langerhans cells have a high CD1a expression. Besides Langerhans cells, also a sub-population of dermal DCs has been reported to express CD1a [36]. These professional antigen presenting cells play an important role in the initiation of the immune response and are able to activate T cells in a CD1a-restricted manner [19, 37].

Increased numbers of $CD1c^+$ Langerhans cells have been described in lesional skin of dogs with atopic dermatitis [38-40]. In these two studies the primary monoclonal antibody CA13.9H11 was used to detect canCD1c. However, from our study using 293T cells transfected with the different CD1 isoforms, we know that CA13.9H11 recognizes canCD1a8.2. It is possible that CA13.9H11 recognizes both CD1a8.2 and canCD1c, but we have not been able to demonstrate recognition of canCD1c by this mAb so far. Therefore, it is possible that the reported expression of CD1 on Langerhans cells in lesional canine skin is reflecting expression of canCD1a8 rather than canCD1c. The current assignment of canCD1c being the molecule recognized by anti-canine CA13.9H11 has not been invalidated in this study. However, 293T cells transfected with three different full length canCD1C transcripts were not recognized by CA13.9H11. The mAb CA9.AG5 has been used to determine canCD1a expression on Langerhans cells [38]. In our study, 293T cells transfected with canCD1A6, canCD1A8.2, canCD1B, or the three canCD1C sequences were not recognized by CA9.AG5. It is possible that the mAb CA9.AG5 does not recognize canCD1 but an unknown epitope on canine thymocytes.

The current study shows the expression of two different types of canine CD1a proteins. We found differences in ectodomain between the two molecules and the unique presence of two different cytoplasmic tails which might contribute to a greater scope of glycolipid antigen presentation and improved skin immunity.

Rabbits are the only other mammalian species which are known to have two different CD1a proteins [41]. However, cytoplasmic tails of the two rabbit CD1a proteins show higher sequence identity compared to dogs. Based on the presence of long cytoplasmic tails in all mammalian species other than primates, it is likely that the canCD1A gene encoding short cytoplasmic tail (canCD1A8.2) is derived from a paralog with a long cytoplasmic tail. Single nucleotide point mutations may have introduced a stop codon within the long tail sequence resulting in a shorter tail. Indeed, the 31 amino acid long cytoplasmic tails of canCD1a2 and canCD1a8.1 can be changed into the short, four amino acid long cytoplasmic tail

like the one in CD1a8.2, by a single point mutation. The cytoplasmic tail of the human CD1a ortholog does not contain any known endosomal targeting sequences [16]. However, whether this also holds for CD1a molecules of other mammalian species, and whether they traffic like their human counterpart is unknown. Like their human homologs, a tyrosine motif was present in the cytoplasmic tail of canCD1b and canCD1d. This might indicate that these canine CD1 molecules traffic to similar compartments as human CD1b and human CD1d compartments to sample lipid antigens. Canine CD1c also contained a tyrosine trafficking motif within its cytoplasmic tail, but unlike human CD1c, a dileucine motif was not present.

CD1 gene duplication might be driven by the lack of certain CD1 isoforms. One of the four CD1B genes found in guinea pig seems to replace the loss of a guinea pig CD1A gene [42]. The extensive canine CD1A isoform duplication found in this study seems not to be driven by the limited repertoire of other CD1 isoforms because all isoforms are present within the canine CD1 locus. This suggests that for canCD1A, gene duplication was driven by another reason than replacement of loss of other CD1 isoforms.

The presence of multiple CD1A genes in dogs, their differential transcription in healthy skin and upregulation at least for canCD1a8 during allergic skin diseases [38, 39] in combination with their potential to present different glycolipid antigens suggests different functions in skin immunity.

Acknowledgements

We thank H. van Engelen and A. van der Lee for their technical assistance. The financial support for this study was provided by Royal Canin, France.

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Chapter 3

Tandem repeats modify the structure of the canine *CD1D* gene

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Animal Genetics 2013; 44(3):352-355

Summary

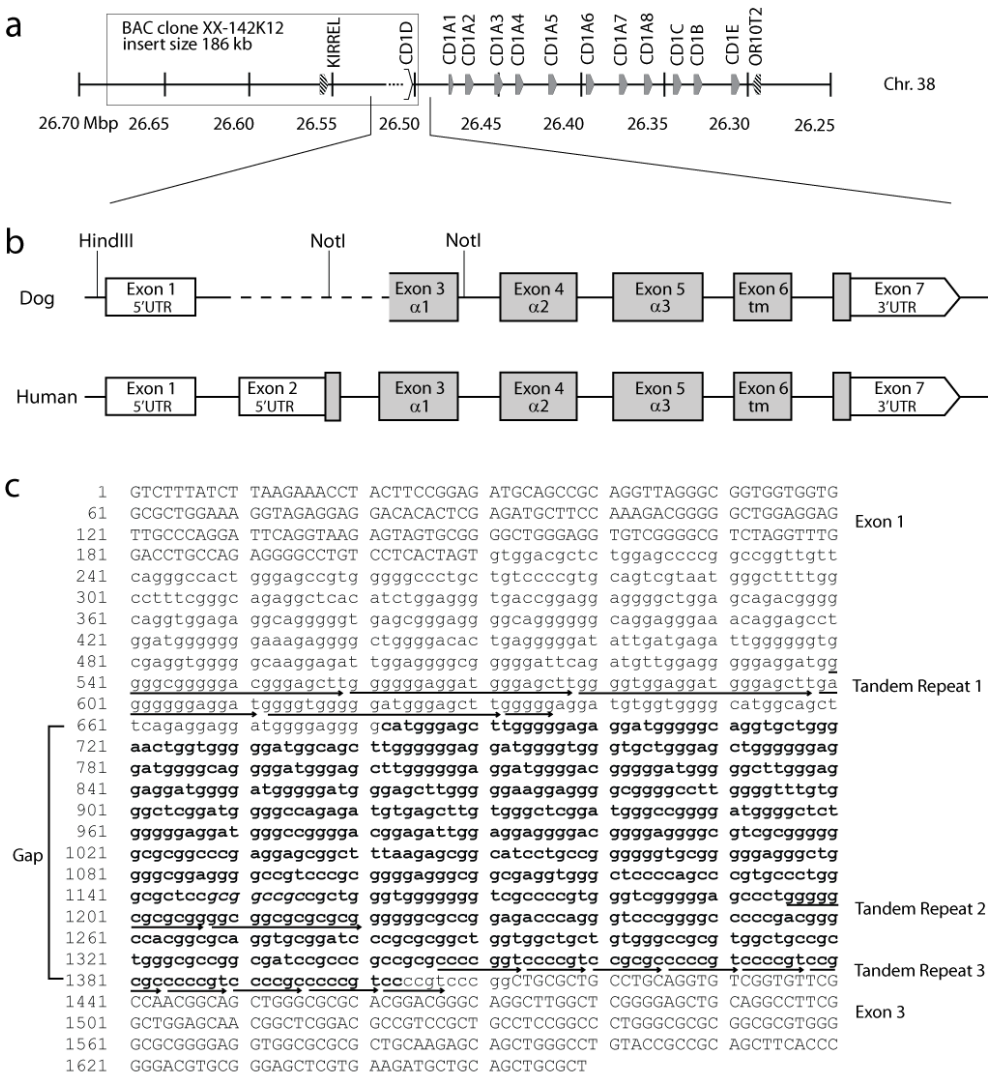
Among the CD1 proteins that present lipid antigens to T cells, CD1d is the only one that stimulates a population of T cells with an invariant T cell receptor known as NKT cells. Sequencing of a 722 nucleotide gap in the dog (*Canis lupus familiaris*) genome revealed that the canine *CD1D* gene lacks a sequence homologous to exon 2 of human *CD1D*, coding for the start codon and signal peptide. Also, the canine *CD1D* gene contains three different short tandem repeats that disrupt the expected gene structure. Because canine *CD1D* cDNA lacks sequences homologous to human exon 2 and 3, the functionality of canine CD1d protein may be affected and this could have consequences for the development and activation of canine NKT cells.

Short communication

The CD1 family is a group of non-polymorphic glycoproteins which present lipid antigens to T cells [1-3]. Like MHC class I proteins, CD1 proteins are heterodimers of β2 microglobulin and a heavy chain consisting of three extracellular α domains, a transmembrane region, and a cytoplasmic tail, each encoded by a separate exon, preceded by an exon that encodes the start codon and signal peptide, and sometimes an additional exon that consists of 5' UTR only.

Large variation exists in the number of *CD1A*, *CD1B*, and *CD1C* genes between mammalian species, while one or two *CD1D* genes are present in all mammalian species studied to date [4-7]. CD1d is crucial for the selection [8] and activation [9] of a subset of T cells known as natural killer T (NKT) cells. The canine CD1 locus is located on chromosome 38, and contains one *CD1D* gene, eight *CD1A* genes, one *CD1C*, one *CD1B* and one *CD1E* gene (Fig. 1a) [10]. In the canine genome (CanFam 3.1) and in the sequence of BAC clone XX-14K12 AC183576.27, the canine *CD1D* gene is incomplete due to an internal gap of unknown length. Upstream from this gap we detected a sequence with 62% nt identity to exon 1 of human *CD1D* and downstream from the gap a sequence with 68% identity to human exon 3 (ENST00000368171; www.ensembl.org) (Fig. 1b). No canine homolog of the human *CD1D* exon 2 was found. Putative full length canine exons were found for *CD1D* exon 4, 5, 6 and 7, with identities of respectively 76%, 84%, 58% and 56% to the corresponding human exons.

To be able to fill the gap in the genomic DNA sequence between exon 1 and exon 3 of canine *CD1D*, we performed a two-step digestion on BAC clone XX-14K12 DNA using restriction endonuclease *Hpa*I followed by *Hind*III and *Not*I (Fig. S1). The two restriction fragments containing the gap were subsequently cloned and sequenced. The complete DNA sequence of the gap was obtained and consists of 722 nt (GenBank GU930707) (Fig. 1c). No canine homolog of the human *CD1D* exon 2 was found in this DNA sequence.

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   1  GTCTTTATCT TAAGAAACCT ACTTCCGGAG ATGCAGCCGC AGGTTAGGGC GGTGGTGGTG
  61  GCGCTGGAAA GGTAGAGGAG GACACACTCG AGATGCTTCC AAAGACGGGG GCTGGAGGAG
 121  TTGCCCAGGA TTCAGGTAAG AGTAGTGCGG GGCTGGGAGG TGTCGGGGCG TCTAGGTTTG
 181  GACCTGCCAG AGGGGCCTGT CCTCACTAGT gtggacgctc tggagccccg gccggttgtt
 241  cagggccact gggagccgtg ggggccctgc tgtccccgtg cagtcgtaat gggcttttgg
 301  cctttcgggc agaggctcac atctggaggg tgaccggagg aggggctgga gcagacgggg
 361  caggtggaga ggcagggggt gagcgggagg ggcaggggag caggagggaa acaggagcct
 421  ggatgggggg gaaagagggg ctgggacac tgaggggat attgatgaga ttgggggtg
 481  cgaggtgggg gcaaggagat tggaggggcg ggggattcag atgttggagg gggaggatgg
 541  gggcgggga cgggagcttg gggggaggat gggagcttgg ggtggaggat gggagcttga
 601  gggggagga tggggtgggg gatgggagct tgggggagga tgtggtgggg catggcagct
 661  tcagaggagg atggggaggg gcatgggagc ttgggggaga ggatgggggc aggtgctggg
 721  aactggtggg ggatggcagc ttgggggggag gatggggtgg gtgctgggag ctgggggggag
 781  gatggggcag gggatgggag cttgggggga ggatggggac gggggatggg ggcttgggag
 841  gaggatgggg atgggggatg ggagcttggg ggaaggaggg gcggggcctt ggggtttgtg
 901  ggctcggatg gggccagaga tgtgagcttg tgggctcgga tgggccgggg atggggctct
 961  gggggaggat gggccgggga cggagattgg aggaggggac ggggaggggc gtcgcggggg
1021  gcgcggcccg aggagcggct ttaagagcgg catcctgccg gggggtgcgg gggagggctg
1081  gggcggaggg gccgtcccgc ggggagggcg gcgaggtggg ctccccagcc cgtgccctgg
1141  gcgctcc*gcg gccgc*cgctg ggtgggggag tcgccccgtg ggtcgggggga gccctgggggg
1201  cgcgcgggggc ggcgcgcgcg gggggcgccg gagacccagg gtcccggggc ccccgacggg
1261  ccacggcgca ggtgcggatc ccgcgcggct ggtggctgct gtgggccgcg tggctgccgc
1321  tgggcgccgg cgatccgccc gccgcgcccc ggtccccgtc cgcggccccg tccccgtccg
1381  cgccccgtc cccgccccg tccccgtccc ggCTGCGCTG CCTGCAGGTG TCGGTGTTCG
1441  CCAACGGCAG CTGGGCGCGC ACGGACGGGC AGGCTTGGCT CGGGGAGCTG CAGGCCTTCG
1501  GCTGGAGCAA CGGCTCGGAC GCCGTCCGCT GCCTCCGGCC CTGGGCGCGC GGCGCGTGGG
1561  GCGCGGGGAG GTGGCGCGCG CTGCAAGAGC AGCTGGGCCT GTACCGCCGC AGCTTCACCC
1621  GGGACGTGCG GGAGCTCGTG AAGATGCTGC AGCTGCGCT
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Figure 1. Canine CD1 locus and *CD1D* gene

a. Map of the canine CD1 locus containing eight *CD1A* genes, one *CD1C* gene, one *CD1B* gene, one *CD1E* gene and one *CD1D* gene as previously published [10]. The gap in the genomic sequence is indicated by a dashed line. BAC clone XX-142K12 that covers the gap and the *CD1D* gene is indicated by a box. **b.** Schematic representation of the alignment of canine and human *CD1D* showing exons (boxes) and introns (line) and the gap (dashed line). Restriction sites for *Hind*III and *Not*I in the canine *CD1D* gene are shown. **c.** Partial genomic sequence of canine *CD1D*, including: gap sequence (bold, lower case), canine homologs of human exon1 and 3 sequence (upper case), three types of tandem repeats (arrows) and the *Not*I-site within the gap (italic).

Using Tandem Repeat Finder, three different types of tandem repeats were identified in the sequence. Tandem repeats occur more frequently in the canine

genome compared to other mammalian genomes as a result of an impaired DNA replication and repair mechanism in dogs [11]. The repeat fragment size of the first tandem repeat (TR1) is 20 nt, and was found five times. The second tandem repeat (TR2) has a fragment size of 12 nt and was repeated twice. The region containing TR1 and TR2 is characterized by a high guanine content (59%), which may explain why this part had not been sequenced before. The motif TR3 consists of a hexanucleotide repeat of the consensus DNA sequence CCCCGT. This tandem repeat was present ten times, and based on alignment with human *CD1D*, is located at the intron-exon boundary of canine *CD1D* homolog of human *CD1D* exon 3. To determine the presence and the copy number of the tandem repeats in dog breeds other than the Boxer from which BAC clone XX-142K12 was derived, PCR was performed on genomic DNA of three Beagles, three Labrador retrievers and a wolf. Using a primer set located outside TR3 (primer set 1, Supplementary Materials and Methods), PCR products were generated and sequenced (Fig. S2). We found a $(CCCCGT)_6$ sequence in wolf genomic DNA (GenBank GU930708). In the PCR products of the three Beagles, we only detected the $(CCCCGT)_8$ sequence (GenBank GU930709). In one Labrador retriever we found both $(CCCCGT)_8$ and $(CCCCGT)_{10}$, whereas in the other two Labrador retrievers only $(CCCCGT)_{10}$ was found (GenBank GU930710). These findings indicate that TR3 is a variable number tandem repeat with at least three different alleles. This suggests that TR3 is still an active site with tandem duplication events before and after the divergence of domestic dogs from the grey wolf between 15,000-100,000 years ago [12].

Using primer set 5 (Supplementary Materials and Methods), we observed differential transcription of *CD1D* in cDNA from various Beagle tissues (Fig. 2a). The highest transcription levels were found in thymus, lymph node, spleen, and PBMC, showing that tissue distribution of transcription of *CD1D* in dogs is comparable to other species, and not hampered by the presence of the tandem repeats. Sequencing of *CD1D* PCR products generated from Beagle thymus-derived cDNA, generated with primer set 6, located outside the full length coding sequence of human CD1d, revealed that canine *CD1D* transcript contained exon 1 and exon 4-7 (Genbank JX139112). Longer PCR products were not obtained using this primer set. Figure 2b shows the structure of the human (NM_001766.3; coding sequence 1008 bp) and canine *CD1D* cDNA sequences (Genbank JX139112; coding sequence 726 bp). Consistent with the absence of an exon 2 homolog in canine genomic DNA, the canine transcript lacks a sequence similar to human exon 2 encoding the signal sequence of CD1d. However, an alternative start codon in the CD1d reading frame was present in exon 1. No signal peptide could be identified. Exon 3, encoding the α1 domain, was also absent in the cDNA. The deletion of nucleotides flanking a repeat is common and it is likely that TR3 is responsible for the deletion of the first part of canine *CD1D* exon 3, and its acceptor splice site, explaining the absence of exon 3 in the cDNA. The introns between exon 4-7 were spliced out as expected and no additional splice variants were found. Because a homolog of exon 3, encoding the α1 domain, was absent, it is not expected that translation of this transcript will lead to a functional CD1d protein.

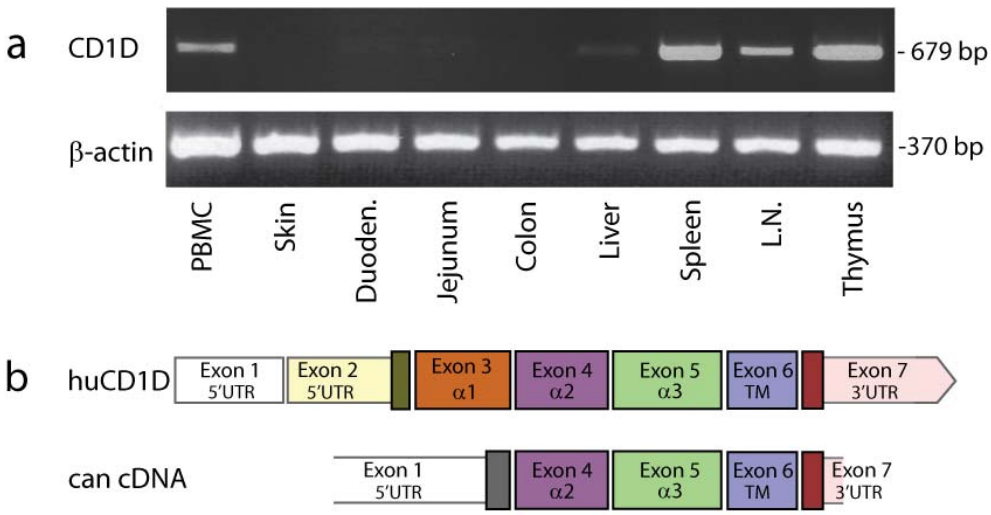


Figure 2. Canine *CD1D* transcription

a. Transcription of canine *CD1D* in different dog tissues. Primer set 5 was used for the detection of *CD1D* transcription in different dog tissues. To compare the quantity of input of cDNA, a PCR for *β*-actin was performed. L.N.: Lymph node. **b.** Structure of human and canine *CD1D* transcripts, with the untranslated parts represented by narrower boxes in lighter shades, and the coding sequence represented by broader boxes in darker shades. The canine transcript was obtained using primer set 6 and lacks a sequence homologous to human *CD1D* exon 2 and 3. TM: Transmembrane domain. Primer sequences are provided in the Supplementary Materials and Methods.

Even though only Beagle CD1d cDNA was analyzed and we can not formally rule out that other breeds have normal CD1d transcripts, the Beagle CD1d cDNA is consistent with the Boxer *CD1D* genomic sequence, and we have confirmed the presence of TR3 in Labrador and wolf. Therefore we expect that other dog breeds show the same aberrant CD1d transcript. Together, our findings suggest that despite gene transcription, expression of the canine CD1d protein is possibly absent or altered *in vivo*. Consistent with this, a panel of anti CD1d antibodies, raised against different species, did not stain canine thymocytes (Fig. S3). Our findings raise the question of how the recently described canine NKT cells [13] can be positively selected and activated in the absence of CD1d protein. One possibility is that one of the many canine *CD1A*, *CD1B*, or *CD1C* genes might encode a restriction element for these cells.

Acknowledgements

IVR was supported by an NWO Meervoud subsidy, Netherlands. FALVB was supported by Royal Canin, France.

Supplementary Materials and methods

In silico sequence analysis
A partial canine CD1D gene was detected by BLAST searches in the canine genome assembly CanFam3.1 (Broad Institute) using Ensembl (www.ensembl.org), trace files on the NCBI site (www.ncbi.nlm.nih.gov), and the CHORI-82 Canine Boxer BAC library (bacpac.chori.org). Alignments were performed using ClustalW (http://www.genome.jp/tools/clustalw/). To determine the presence of tandem repeats we analyzed the full genomic length of the canine CD1D gene with Tandem Repeat Finder (http://tandem.bu.edu/trf/trf.html) [14] using default settings. The presence of splice sites in this sequence was predicted using NetGene2 server (www.cbs.dtu.dk/services/NetGene2), signal peptide analysis was performed with SignalP (www.cbs.dtu.dk/services/SignalP) [15].
Blast searches in the canine genome (Broad Institute of MIT/Harvard, CanFam 2.0 version 46.2d; coverage 7.5), available at www.ensembl.org

BAC Clone DNA sequencing
BAC clone XX-142K12 from the CHORI-82 Canine BAC library was obtained from BACPAC Resources Center (bacpac.chori.org). The CHORI-82 library formed the basis for the canine genome and consists of genomic DNA of a Boxer cloned into the pTARBAC2.1 vector [16]. Rapid alkaline lysis miniprep method (Qiagen) was used followed, by precipitating BAC clone DNA with ice cold isopropanol. BAC DNA was digested using endonuclease *Hpa*I, succeeded by excision and elution of the band containing the gap in the *CD1D* gene with subsequent digestion with *Hind*III and *Not*I (Stratagene) (Fig. S1). The two fragments containing the gap were ligated into pcDNA3.1+ vector (Invitrogen) and sequenced, using a method for GC rich sequences, by BaseClear (Leiden, Netherlands).

Genomic DNA and cDNA sequencing
Blood from three Beagles, three Labrador retrievers and a wolf (*Canis lupus*) was collected according to the regulations of the Animal Ethics Committee of the University of Utrecht, Netherlands (protocol number 2008.II.11.106). Genomic DNA was isolated from EDTA blood from all seven animals, using the salt extraction method [17]. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized Beagle blood by standard Ficoll-Hypaque gradient centrifugation. From one Beagle also skin, intestinal tissue, liver, spleen, lymph node and thymus were collected. RNA was isolated from tissues using the RNeasy kit (Qiagen) followed by cDNA synthesis using the iScript kit (Bio-Rad). All genomic DNA and cDNA was frozen at -20˚C until use. To determine the genomic conservation of the CCCCGT-repeat across dog breeds, we performed a hot-start touch down polymerase chain reaction (PCR) using primer set 1 with genomic DNA as template. PCR was performed with *Pfu* Turbo polymerase (Stratagene) and 4% dimethyl sulfoxide under the following conditions: 7 min at 98˚C, followed by 35 cycles of 15 s at 95˚C, 15 s at annealing temperature, 30 s at 72˚C, followed by a final elongation of 5 min at 72˚C. After each of the first 20 cycles, the annealing temperature was decreased with 0.5˚C, starting at 69˚C. PCR products were ligated in a pCR4Blunt-TOPO vector (Invitrogen) and vector DNA was sequenced, using a method for GC rich sequences, by BaseClear (Leiden, Netherlands). The expression of canine *CD1D* was determined in various tissues using PCR conditions as described above with

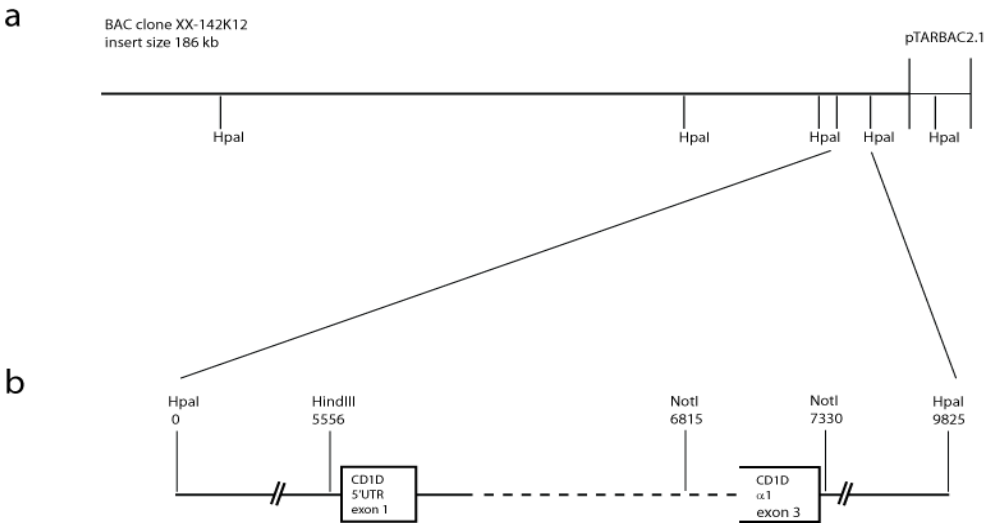
an annealing step at 65˚C for 60 s with primer set 5. To check the quantity of cDNA in each PCR, we performed *β*-actin-specific PCR in parallel.

Staining canine thymocytes with CD1d mAbs

Canine thymocytes were stained with biotinylated anti-rat CD1d (IgG2a) antibodies WTH-1, WTH-2, WTH-3, the latter has also been designated as 58 [18], and anti-human CD1d (IgG1) antibody CD1d42. Raji cells transfected with rat CD1D cDNA and human PBMC were used as positive control, and stainings without primary antibody as negative control. WTH-1 and WTH-2 have been shown to cross-react with murine CD1d, but have never been tested for reactivity with canine CD1. To confirm CD1 expression on canine thymocytes, we used anti-canine CD1a8 (IgG1) antibody CA13.9H11, which was kindly provided by Professor P.F. Moore, University of California, Davis, CA, USA.

The cells were stained with primary antibody for 30 min at 4˚C followed by incubation with streptavidin-R-phycoerythrin or Goat Anti Mouse Ig -R-phycoerythrin (BD Biosciences) for 30 min at 4˚C. Fluorescence was measured using a FACScalibur flow cytometer (Becton Dickinson).

Supporting information



Supplemental Figure 1. Endonuclease restriction map of BAC clone XX-142K12
Map showing HpaI restriction sites of BAC clone XX-142K12 which as an insert size of 186kb in vector pTARBAC2.1 (a). Map showing *HpaI*-fragment of 9825 nt, numbered after retrieving the full length sequence of the gap, containing the gap which was subsequently digested with *HindIII* and *NotI* leading to two fragments which were both cloned into pcDNA3.1+ vector (b).

```
boxer           CGCGGCTGGTGGCTGCTGTGGGCCGCGTGGCTGCCGCTGGGCGCCGGCGATCCGCCCGCC 60
labrador        CGCGGCTGGTGGCTGCTGTGGGCCGCGTGGCTGCCGCTGGGCGCCGGCGATCCGCCCGCC 60
beagle          CGCGGCTGGTGGCTGCTGTGGGCCGCGTGGCTGCCGCTGGGCGCCGGCGATCCGCCCGCC 60
wolf            CGCGGCTGGTGGCTGCTGTGGGCCGCGTGGCTGCCGCTGGGCGCCGGCGATCCGCCCGCC 60
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boxer           GCGCCCCGGTCCCCGTCCGCGCCCCCGTCCCCGTCCGCGCCCCCGTCCCCGCCCCCGTCC 120
labrador        GCGCCCCGGTCCCCGTCCGCGCCCCCGTCCCCGTCCCCGTCCCCGTCCCCGCCCCCGTCC 120
beagle          GCGCCCCGGTCCCCGCCCGCGCCCCCGT------------CCCCGTCCCCGTCCCCGTCC 108
wolf            GCGCCCCGGTCCCCGTCCGCGCCCCCGT------------------------CCCCGTCC 96
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boxer           CCGTCCCGGCTGCGCTGCCTGCAGGTGTCGGTGTTCGCCAACGGCAGCTGGGCGCGCACG 180
labrador        CCGTCCCGGCTGCGCTGCCTGCAGGTGTCGGTGTTCGCCAACGGCAGCTGGGCGCGCACG 180
beagle          CCGTCCCGGCTGCGCTGCCTGCAGGTGTCGGTGTTCGCCAACGGCAGCTGGGCGCGCACG 168
wolf            CCGTCCCGGCTGCGCTGCCTGTAGGTGTCGGTGTTCGCCAACGGCAGCTGGGCGCGCACG 156
                ******************** ************************************

boxer           GACGGGCAG 189
labrador        GACGGGCAG 189
beagle          GACGGGCAG 177
wolf            GACGGGCAG 165
                *********
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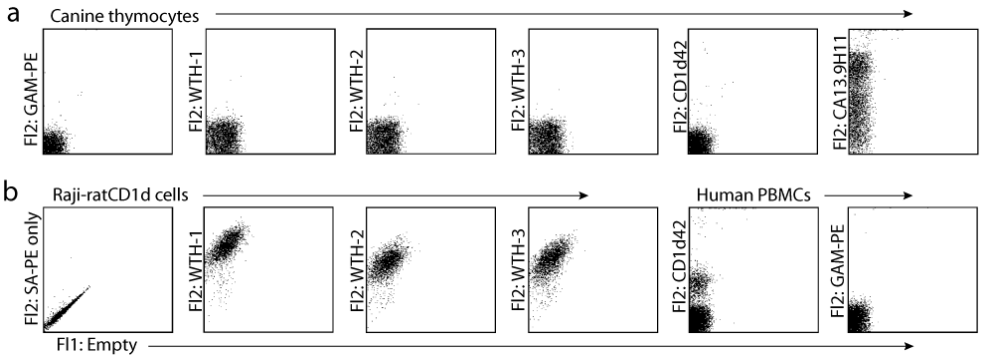
Supplementary Figure 2. Alleles of tandem repeat 3
Alignment of the 3'part of the gap in the genomic sequence of the *CD1D* gene of one Boxer, one Beagle, one Labrador retriever, and one wolf containing the hexanucleotide CCCCGT tandem repeat TR3. The repeat was shown to be a variable number tandem repeat with 6 – 10 repeat units. Arrows indicate the location of the CCCCGT repeat in the canine sequence. Identical nt are indicated by an asterisk.



Supplementary Figure 3. Flow cytometric analysis of expression of CD1d on canine thymocytes
Canine thymocytes were stained with biotinylated anti-rat CD1d antibodies (WTH-1, WTH-2 or WTH-3), anti-human CD1d (CD1d42) or anti-canine CD1a8 (CA13.9H11) as a positive control for CD1 expression by thymocytes, followed by SA-PE or GAM-PE (a). As a negative control we omitted the primary antibody, and incubated cells only with secondary antibody. Staining of ratCD1d-transfected Raji cells and human PBMC was used as a positive control for respectively WTH-1,-2, -3 and CD1d42 (b).

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Chapter 4

Functional CD1d and/or NKT cell invariant chain transcript in horse, pig, African elephant and guinea pig, but not in ruminants

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Molecular Immunology 2009; 46(7):1424-1431

Abstract

CD1d-restricted invariant natural killer T cells (NKT cells) have been well characterized in humans and mice, but it is unknown whether they are present in other species. Here we describe the invariant TCR α chain and the full length CD1d transcript of pig and horse. Molecular modeling predicts that porcine (po) invariant TCR α chain/poCD1d/α-GalCer and equine (eq) invariant TCR α chain/eqCD1d/α-GalCer form complexes that are highly homologous to the human complex. Since a prerequisite for the presence of NKT cells is the expression of CD1d protein, we performed searches for CD1D genes and CD1d transcripts in multiple species. Previously, cattle and guinea pig have been suggested to lack CD1D genes. The CD1D genes of European taurine cattle (*Bos taurus*) are known to be pseudogenes because of disrupting mutations in the start codon and in the donor splice site of the first intron. Here we show that the same mutations are found in six other ruminants: African buffalo, sheep, bushbuck, bongo, N'Dama cattle, and roe deer. In contrast, intact CD1d transcripts were found in guinea pig, African elephant, horse, rabbit, and pig. Despite the discovery of a highly homologous NKT/CD1d system in pig and horse, our data suggest that functional CD1D and CD1d-restricted NKT cells are not universally present in mammals.

Introduction

CD1d proteins are expressed on the surface of a variety of antigen presenting cells and non hematopoietic cells, and present cellular self lipids and exogenous lipids with an α-anomerically linked sugar to T cells with a highly conserved, invariant TCR, NKT cells. CD1d-/- mice have no detectable mature NKT cells [1, 2], showing that functional CD1D genes are a prerequisite for their development. NKT cells have been implicated in oral tolerance, autoimmunity, dendritic cell maturation, tumor surveillance, and anti-microbial immunity. Natural exogenous ligands for NKT cells presented by CD1d have been identified, such as GSL-I from *Sphingomonas* species [3] and BbGL-II from *Borrelia burgdorferi* [4]. The entire population of NKT cells can be activated strongly by the synthetic ligand α-galactosylceramide (α-GalCer) [5], which is considered a universal super agonist for NKT cells. It has been suggested that the CD1d/NKT system evolved to cope with pathogens that produce antigens with α-glycosidic linkages [3], but there is only limited supportive data available.

Sphingomonas species contain antigens that are presented by CD1d to NKT cells. *Sphingomonas*, a genus that does not include highly pathogenic bacteria, belongs to the class of α-proteobacteria. This class of bacteria contains peptidoglycan and LPS-negative bacteria, including pathogenic tick-borne genera: *Rickettsia*, *Anaplasma*, and *Ehrlichia,* all causing morbidity and mortality in livestock. Unfortunately, none of these bacteria has been studied closely enough to determine whether they contain antigens for NKT cells. *Ehrlichia ruminantium* (formerly named *Cowdria ruminantium* [6]), causes heartwater (cowdriosis), *Anaplasma bovis* (formerly named *Ehrlichia bovis*) causes bovine ehrlichiosis, and *A. marginale* and *A. centrale* cause bovine anaplasmosis, and these diseases are major problems in the livestock industry in sub-Saharan Africa. Some indigenous African breeds of cattle are more resistant to heartwater and anaplasmosis than other breeds, but this can be explained by higher resistance to the vector (ticks of the genus *Amblyomma*). All breeds of cattle will develop clinical disease once they get infected. Since the aforementioned bacterial pathogens do not carry the signature danger molecules LPS and peptidoglycan, recognition by the innate immune system other than TLRs, like the CD1d/NKT system, may be of crucial importance in the early defense against these pathogens.

It has been suggested that the group 1 CD1 proteins (CD1a, CD1b, CD1c) are not universally present in all species, whereas group 2 CD1 proteins (CD1d) are. CD1D genes have indeed been found in most mammalian species studied, including primates like humans and chimpanzees (*Pan troglodytes*), African green monkeys (*Chlorocebus sabaceus*) and rhesus macaques (*Macaca mulatta*) [7], mice (*Mus musculus*) [8], rats (*Rattus norvegicus*) [9], cottontail rabbits (*Sylvilagus floridanus*) [10], sheep (*Ovis aries*) [11], and pigs (*Sus scrofa*) [12]. However, not all of these genes have been shown to lead to functional transcripts or proteins yet. CD1 genes have also been discovered in chickens (*Gallus gallus*) [13-15], but chicken CD1 genes could not be classified according to the existing isoforms, and are therefore named CD1.1 and CD1.2. There are two species that have till now been suggested to have no functional CD1D genes. Before the availability of its genome, the guinea pig (*Cavia porcellus*) family of CD1 genes had been well characterized, but a CD1D gene was not identified [16]. In cattle, two CD1D genes

have been identified, named CD1D1 and CD1D2, but these are in fact pseudogenes [17]. The two CD1D pseudogenes that were identified both contain a mutated start codon and an unspliceable intron. In this paper we describe CD1D pseudogenes in N'Dama cattle, and five other ruminants, including sheep, which had previously been assumed to have functional CD1D genes. Functional CD1d transcripts were identified in guinea pig, African elephant, horse, rabbit, and pig.

NKT cells can be distinguished by their highly conserved invariant TCR. The NKT cell population can be visualized by flow cytometric analysis using fluorescently labeled CD1d tetramers loaded with α-GalCer that interact with the NKT cell TCR. Human and murine CD1d tetramers are known to stain human and murine NKT cells, also in a species cross-reactive manner, so it is possible that these tetramers also recognize NKT cells in other species. However, the lack of species cross-reactive staining does not prove absence of NKT cells. Alternatively, evidence for the existence of NKT cells in a species might come from TCR α chain sequences. Recent data on the molecular interactions between α-GalCer-loaded CD1d and the invariant TCR [18-20] have provided clear insights in these interactions and allow detailed predictions on whether CD1d and TCR protein sequence homologs that are found in other species, like dog and horse as described in this paper, are likely to be true functional homologs.

Our data provide supportive evidence that functional CD1d transcripts and/or NKT cells are present in several mammalian species, but not in ruminants. This shows that the CD1d/NKT system is not universally present as previously thought. The CD1d/NKT system may be lacking in ruminants altogether, providing a possible explanation for their high sensitivity to *Rickettsia*, *Anaplasma*, and *Ehrlichia*.

Materials and methods

Animals and nucleic acid samples
Genomic DNA from sheep (*Ovis aries*), African buffalo *(Syncerus caffer)*, bushbuck (*Tragelaphus scriptus)*, and bongo *(Tragelaphus eurycerus)*, was freshly isolated from whole, heparinized blood, using the Wizard Genomic DNA Purification Kit (Promega). Genomic DNA from N'Dama cattle (*Bos taurus*) and roe deer (*Capreolus capreolus*) were isolated at least 10 years ago. DNA from N'Dama cattle was a gift of Dr. Bradley (Trinity College, Dublin, Ireland). First strand cDNA was synthesized with Multiscribe reverse transcriptase (Applied Biosystems) from RNA isolated with the Qiagen RNAEasy kit from freshly isolated PBMC from cattle, sheep, pig *(Sus scrofa)*, horse *(Equus caballus)*, guinea pig *(Cavia porcellus)*, cat *(Felis catus)*, rabbit *(Oryctolagus cuniculus)*, and African elephant *(Loxodonta africana)*. Animal-derived materials were obtained and collected according to the regulations of the Animal Ethical Committee of the University Utrecht, the Netherlands (protocol number 2007.II.06.152/Vervolg1).

Searches in genomes and EST databases
BLAST searches were performed in selected genomes (www.ensembl.org) and EST databases (www.ncbi.nlm.nih.gov/BLAST) with the nucleotide sequence of the α1 and the α2 domains of human CD1D (NM_001766) and with the human TRAV10 segment (also called Vα24) used by NKT cells (AE000659). The results of the CD1D searches were included in a phylogenetic tree together with the α1 and the α2 domains of the known CD1 isoforms to assess with which CD1 isoforms they group. Also, the obtained potential CD1D genes were checked for the presence of a leader peptide, α1, α2, and α3 domain, and a transmembrane region. The nucleotide sequence of the α1 domain of the published sheep CD1d cDNA (AJ006722) was used to perform a BLAST search in the NCBI sheep EST database (www.ncbi.nlm.nih.gov/BLAST). The predicted amino acid sequences of the hits obtained from BLAST searches with the human TRAV10 segment in the genomes of selected species were all aligned and evaluated as described in the results section.

Primer design, PCR and sequencing
Universal ruminant CD1D primers (Invitrogen) were designed based on the sequences of bovine pseudogene CD1D1 (accession number DQ192544). Forward primers "K" (5'-GGCGGAGATTCGAGGTCCG-3') and "S" (5'-CGTTGGGGGAGGACGGAGG-3') ended approximately 20 and 50 base pairs respectively upstream of the start codons. The reverse primers "N" (5'-CTTCTCCAGCTCMGACTTCCC-3') and "M" (5'-CAGGCCCAGGACTGGGGCCACTGG-3') were designed to anneal to the end of the α2 domain and the beginning of the α3 domain respectively. Primers for the full length CD1d transcripts of guinea pig, African elephant, rabbit, and horse were designed based on the predicted sequences in their genomes: CporCD1dFor#1 (5'-TGCTCAGAAGTCGCGGTCCC-3'), CporCD1dRev#2 (5'-AAATCTTAGCTACTCACAGGATATCTTGATAG-3'), LafrCD1dFor#1 (5'-GAGGCCAGGGTAGGACTTCAG-3'), LafrCD1dRev#1 (5'-CTTGGGCACTTGACGTCCTGAG-3'), RabbCD1dFor#2 (5'-

GGGAGTGCAGCGCTAGTTTG-3'), RabbCD1dRev#1 (5'-TCACAAGATGCCTTGGTAGGAGC-3'), EcabCD1dFor#1 (5'-CTCCACACGCAGCGACATGAG-3'), EcabCD1dRev#1 (5'-TTGAGGTCGTGAGTTCCAGACAG-3'). TCR α chains were amplified using a V segment-specific forward primer and a reverse primer in the constant segment: CporTCRaVFor#1 (5'-CTTCTGAATAGGTGTGAATGGCAACC-3'), CporTCRaCRev#1 (5'-CTGACAGATTGACGTTAGAATCAAAATCG-3'), LafrTCRaVFor#1 (5'-CTTTCTGAATAGGGGTGAATGGCAAC-3'), LafrTCRaCRev#1 (5'-GTGACACGTTGGCTTTAGAATCGAAATC-3'), FcatTCRaVFor#1 (5'-GCAAAAACCAAGTGGAACAGCACC-3'), FcatTCRaCRev#1 (5'-CACTGTCAGGTTGTCGAAATTTAGGTTC-3'), EcabTCRaVFor#1 (5'-GAGGGAGAGAACTGCACGTTTC-3'), EcabTCRaCRev#1 (5'-TGCGGAACCCAATCAGTGACA G-3'), SscrTCRaVFor#1 (5'-GGTGAATGGCAAAAACCAAGTGGAAC-3'), SscrTCRaCRev#1 (5'-CT GAC AGG TTT TGG AGG TTG AGG TTC-3'), OariTCRaVregFor#1 (5'-CAATTATACAGTGAGCCCCTTTAACAAC-3'), OariTCRaCRev#1 (5'-GAACACGGTCACTGACAGGTTTTG-3'), BtauTCRaFor (5'-CAAATGGACGGTACACAGCGAC-3'), BtauTCRaCRev "J" (5'-TTTCAAAGCTTTTCTCTACCAGCTTGG-3'). PCR was performed with PFU Turbo polymerase (Stratagene) according to the protocol of the manufacturer using 20 ng genomic DNA or 10 ng cDNA in a reaction volume of 20 µl under the following cycling conditions: an initial denaturation of 7 min. at 95°C, followed by 40 cycles of 30 sec. at 95°C, 45 sec. at different annealing temperatures (60 °C, 57 °C, 54.5 °C), 1 min. at 72°C, followed by a final elongation step of 7 min. at 72°C. PCR products were cut from a 1.5% agarose gel, purified with Gel DNA recovery kit (Zymo), and ligated in a Topo4blunt vector which was used to transform one shot Top10 cells (Invitrogen). DNA inserts of single colonies was sequenced by Baseclear (Leiden, Netherlands).

Sequence analysis and homology modeling

Homology models of pig *(Sus scrofa)* and horse *(Equus caballus)* CD1d, as well their α chain of the invariant NKT cell TCR were modeled using the Swiss Model Server [21], using both human CD1d and Vα24 TCR crystal structures as templates. The obtained CD1d and TCR models were superimposed onto their corresponding human counterparts in the CD1d/α-GalCer/Vα24 TCR crystal structure (PDB code 2PO6) [20]. No reorientation of the TCR was necessary to accommodate the TCR CDR loops, due to their similar orientation in both models. The CD1d surface residues in all three CD1d orthologs are mostly conserved, except for a glycine residue instead of the human tryptophan (W153), which is responsible for tilting the galactose of α-GalCer when bound to human CD1d [22] in comparison to mouse CD1d. Therefore, we manually modeled this galactose in the orientation that it adopts when bound to mouse CD1d, as mCD1d also has this conserved glycine residue [23]. The models were visualized using PyMol (pymol.sourceforge.net). The Translate Nucleic Acid Sequence Tool was used (http://biotools.umassmed.edu) for translation into amino acids. Alignments were performed and trees generated with ClustalW and Phylip. SignalP, available at http://www.cbs.dtu.dk/services/SignalP/ was used to predict leading fragments and cleavage sites.

Results

Invariant α chain analysis

The human TRAV10 V segment (also called Vα24) that is used by the human NKT invariant TCR was used to identify TCR α chain V segments in the genomes of cat, dog, horse, pig, cattle, guinea pig, African elephant, rabbit, and sheep. All resulting V α segment sequences were translated and aligned with the human TRAV10 segment. We considered all V segments with higher sequence homology to TRAV10 than to any other human V segment as candidate V segments for the NKT invariant chain in other species. Because the CDR1 region is encoded by the V segment and known to interact with α-GalCer [18-20], we only included V segments in which at least two residues, including the P that was indicated as crucial in all studies, were identical to the human TRAV10 CDR1 region (VSPFSN). According to these criteria we identified one candidate V segment in cat, dog, horse, pig, guinea pig, African elephant, rabbit, and sheep, and three in cattle (Table Ia).

Using a forward primer before or at the CDR1 region of the candidate V segments and a reverse primer in the constant segment, we amplified partial TCR α chains covering the CDR1, CDR2, CDR3, and part of the constant domain. For this purpose, PBMC-derived cDNA was available from cat, dog, horse, pig, guinea pig, African elephant, rabbit, sheep and cattle. CDR3 sequences that were highly homologous to the human and murine NKT CDR3α were obtained from horse (two out of four sequences) and pig (one out of nine sequences). Six out of nine sequences obtained from cat had a two amino acid deletion in the CDR3 compared to the human and murine sequences. From cattle, one out of 15 sequences showed high homology to the human CDR3, but it had one extra amino acid. None of 15 sheep sequences, eight guinea pig sequences, eight rabbit sequences, and one African elephant seqence showed homology to the human invariant CDR3α (Table Ib). We were not able to derive TCR α chain sequences from dog. To predict whether the obtained CDR1α and CDR3α loops would be able to interact with a CD1d/α-GalCer complex, we generated models using the Swiss Model server [21], and compared these to the available human data [20]. The horse and pig invariant TCR α chain/CD1d/α-GalCer models suggest that these α chain sequences are fully functional invariant NKT cell TCR sequences, capable of binding α-GalCer, when presented by its species-matched CD1d molecule (Figure 1). Even though otherwise highly conserved, the differences in CDR3α length of the obtained bovine and feline sequences make it difficult to predict whether the residues that normally interact with α-GalCer do so in these species, and therefore we can not conclude that these sequences represent the bovine or feline NKT invariant chain.

a V segments homologous to hu Vα24

Species	V segment sequence
Human	GKNCTLQCNYTVSPFSNLRWYKQDTGRGPVSLTIMTFSENTKSNGRYTATLDADTKQSSLHITASQLSDSASYICVV
Mouse	GENCVLQCNYSVTPDNHLRWFKQDTGKGLVSLTVLVDQKDKTSNGRYSATLDKDAKHSTLHITATLLDDTATYICVV
Afr. elephant	GENCTFQCNSSVSPFNNLRWYKEDAGRGPVSLIIMTYSESQKSNGRYTATLDANAKRSFLHLTGSQLSDSASYICAV

Guinea pig	GESCSLQCDYTVSPVNNVRWYRQDPGRGPVLVIIIASTKSKTSDGRYTVTLDVAAKHSSLHITASQPSDSASYICVV
Pig	GENCTIHCNYTVSPFNNLRWYKQDTERGLISLIIMTFSESKKSNGRYTATLDATTMHSTLHITAAWLSVPATYICVV
Sheep	GENCTFQCNYTVSPFNNLRWYTQDTGKGLVSLITMTYSDNKKSNGRYTATMDATAKHSFLHLTAAQLSDLAFYICVV
Dog	GKNYTIQCNYTVNPFGSLRWYKHSTGTGPASLIGMTYSDSKKSYGSYTVTLDANSKQSSLHITAAQLSDSASYICVV
Cat	GENCTFQCNYTVSPFSHLKWYKQGTGRSPAFLIIMTDDDSKNPSGRYTVTLDATSKHSFLHITAAQLSDSAVYICVV
Horse	GENCTFQCNYTVSPFNNLRWYKWDTGRGPVSLIIMSSSERRRSNGRYTATLDTANKGSSLHITAAQLSDAAVYICVV
Rabbit	GGNCTLQCRYTVTPVNNVRWYRQDVGRGPVLLIVMTSFESKKSNGRYTATLESTAGHNSLHIRASQLSDSASYICVV
Cattle	GENCTFQCNFTVSPFNNLRWYTQDTGRGLVSLITMTYSDNKKSNGRYTATVDATAKHSSLHITAAQLSDPAFYICVV
	GENCTFQCNYTVSPF-KLRWYTQDTGRGLVSLITMTYSDNKKSNGQYTATVDATAKHSSLHLTAAQLSDPAFYLCVV
	GENCTFQSNYTVRPFNNLRWYTQDTGRGLVSLITMTYSDNKKSNGRYTATLVVTSKHSSLHLTAAQLSNPAFYICVV

b CDR3α with high homology to human and mouse invariant CDR3α

Species	Clone name	CDR3 sequence	Frequency
Human		CVVSDRGSTLGRLYFG	
Mouse		CVVGDRGSALGRLHFG	
Pig	susscr6	CVVGDRGSRLGRLYFG	1x
Horse	clone1	CVVGDRGSSVGKLYFG	2x
Cat	feliscat2	CVV--RGSTLGKLYFG	6x
Cattle	PRK11	CVVGAGRGSSLGRFYFG	1x

CDR3α with low homology to human and mouse invariant CDR3α

Species	Clone name	CDR3 sequence	Freq	Species	Clone name	CDR3 sequence	Freq.
Pig	susscr1	CVVGRYSGVGSSQLTFG	1x	Cattle	PRK12	CVVGVGSAYGKLMFG	1x
	susscr3	CVVGAPGATTGKLIFG	1x		PRK9	CVVGGDGNTLIFG	1x
	susscr4	CVVGVDSSYQLIWG	1x		PRK25	CVVGPSGNTLIFG	1x
	susscr7	CVVGGPQSANKIVFG	1x		PRK13	CVVGEAGNTLIFG	1x
	susscr10	CVVDYGANRLVFG	1x		PRK7	CVVGALYNQGGKLIFG	1x
	sus2_clone1	CVVGSTSGGNYRPIFG	2x		PRK23	CVVGRYGNTGRLTFG	1x
	sus2_clone3	CVVGRTGANRLVFG	1x		PRK17	CVVANTGSGGKVIFG	1x
	sus2_clone4	CVVGALNTGYQKLVFG	1x		PRK21	CVVANTGSGGKVIFG	1x
	sus2_clone5	CVVGGPGKLIFG	1x		PRK1	CVVDDMNYGGAANQLIFG	1x
Guinea pig	c1	CVVGGYFGSSANKLIFG			PRK22	CRGHNNNNLRFG	1x
	c8	CVVGAWRAAKLVFG			PRK26	CVVKGNNNNNLRFG	1x
	c3	CVVGVSGSGSYRLTFG			PRK4	CVVAEEGQGFSLVFG	1x
	c4	CVVGVSGSGSYRLTFG			PRK19	CVVLDQGGNALVFG	1x
	c11	CVVGYNYGNNRLTFG			PRK6	CVVRSSGNYKYVFG	1x
	c6	CVVGAGSADYKLSFG		Sheep	ovisari3	CVVGSGTGKLTFG	1x
	c9	CVVGARAFGGMLHFG			ovisari4	CVVGRDVGSSQGKLIFG	1x
	c10	CVVFIMLAPAVSFG			ovisari5	CVVEGGNYKYVFG	1x
Cat	feliscat6	CVVSANNNNDLRFG			ovisari6	CVVGDNYKFTFG	1x
	feliscat11	CVVRPISSGSSWQLTFG			ovisari7	CVVGDNTGGAYGKLMFG	1x
Rabbit	PRE11	CVVVHAGAGQGKLTFG	1x		ovisari8	CVVGARDNYGQGLIFG	1x
	rab7	CVVAYYRDTGGRQLVFG			ovisari9	CVVGSYNNDLRFG	1x

	rab9	CVVVDTGGRQLVFG	
	rab11	CVVGYIGNNQKLIWG	
	PRU1	CVVVAVRGVAGNQLTFG	1x
	PRU2	CVVENVGSSNKLTFG	1x
	PRU3	CVVVEDGSSNKLTFG	1x
	PRU7	CVVVQMTSCNTWTFG	1x
Elephant	c18	CAVSPLLFNYQLIWG	2x

	ovisari10	CVVDGNYRLIWG	1x
	ovisari12	CVVDGNYRLIWG	1x
	ovisari13	CVVADSGNYKYVFG	1x
	ovisari15	CVVDGNCQLIWG	1x
	ovisari17	CVVGRDVGSSQGKLIFG	1x
Horse	clone3	CVVGYGGSQAKLIFG	2x

Table 1. a Amino acid sequences of TRAV10-homologous V segments in several species, identified by searching the available genomic data. In green: CDR1. b TCR α chain sequences using the TRAV10 homologs were derived from PBMC from multiple species. The CDR3 of these TCR α chains that are highly homologous to the CDR3 of the human and mouse NKT TCR α chain are aligned (top panel). The human and mouse sequences that are included were derived from literature. CDR3 that were not homologous to the CDR3 of the human and mouse NKT TCR α chain, but were used by TRA10 homologous V segments are shown for comparison (lower panel). Green: CDR1; Grey: the first two amino acids of the FGXG motif, forming the end of the CDR3.

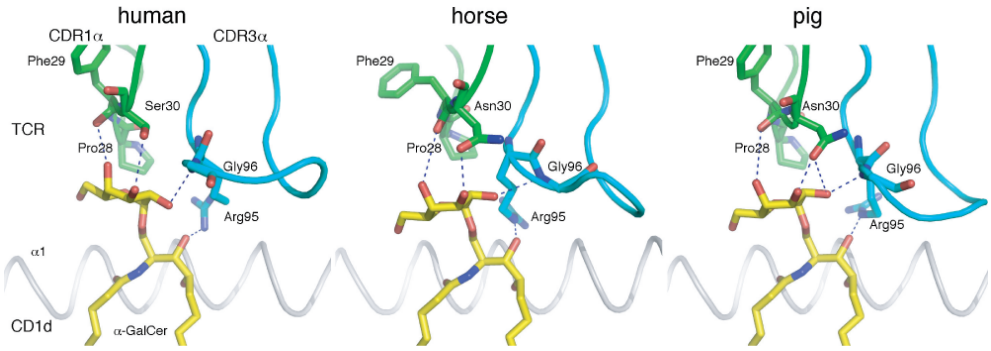

Figure 1. NKT cell receptor α chain binding to CD1d-bound α-GalCer.
Residues of CDR1α (green) and CDR3α (cyan) that directly interact through hydrogen-bonding with α-GalCer, are represented as stick, colored by atoms (oxygen in red, nitrogen in blue). The α-GalCer ligand is shown as yellow sticks, while the CD1d α1-helix is shown in grey. The α2-helix of CD1d was removed for clarity. Hydrogen-bonds are depicted as blue dashed lines. Only one residue in the porcine and equine CDR1α sequence (Asn30) differs from the human counterpart (Ser30) but the model suggests that it can still hydrogen-bond with the α-GalCer ligand. Several other TCR residues that are involved in binding to CD1d residues are also conserved or similar but not shown. See sequence alignment of CD1d (Figure 3) and NKT TCR (Table 1) for detailed sequence conservation.

CD1D pseudogenes in ruminants

PCR products were generated using genomic DNA from N'Dama cattle *(Bos taurus)*, African buffalo *(Syncerus caffer)*, sheep *(Ovis aries)*, roe deer *(Capreolus capreolus)*, bushbuck (*Tragelaphus scriptus)*, and bongo *(Tragelaphus eurycerus)*, using heterologous CD1D primers. Subsequent cloning of PCR products and sequencing of at least four independent bacterial colonies of each species resulted in CD1D sequences available at Genbank with accession numbers EU247610-EU247617 and FJ028651-FJ028652. In case of small differences between sequences derived from one species, the sequence that was closest to the

consensus sequence was submitted to Genbank. Alignment of the newly derived ruminant sequences with previously published CD1D sequences of humans and cattle (Figure 2a) revealed that all newly derived ruminant CD1D sequences have the same disrupting mutations as the bovine CD1D genes. The start codon is mutated and the donor splice site of the first intron (the intron after the leading fragment) is mutated, rendering it an unspliceable intron. Interestingly, the mutated donor splice site of that intron forms ATG in all ruminant CD1D genes, and might function as an alternative start codon. This ATG is in the right reading frame and does in most cases not lead to any premature stop codons. However, the protein that would be synthesized is not predicted to contain a leading fragment by the SignalP program and can thus not be expressed at the cell surface (Signal peptide probability: 0.001, Signal anchor probability: 0.000). In N'Dama cattle and bongo we found one gene homologous to bovine CD1D1 and another gene homologous to bovine CD1D2. The obtained African buffalo and bushbuck sequences are homologous to bovine CD1D1. The roe deer and sheep sequences could not be classified as CD1D1 or CD1D2 (Figure 2b).

a

b

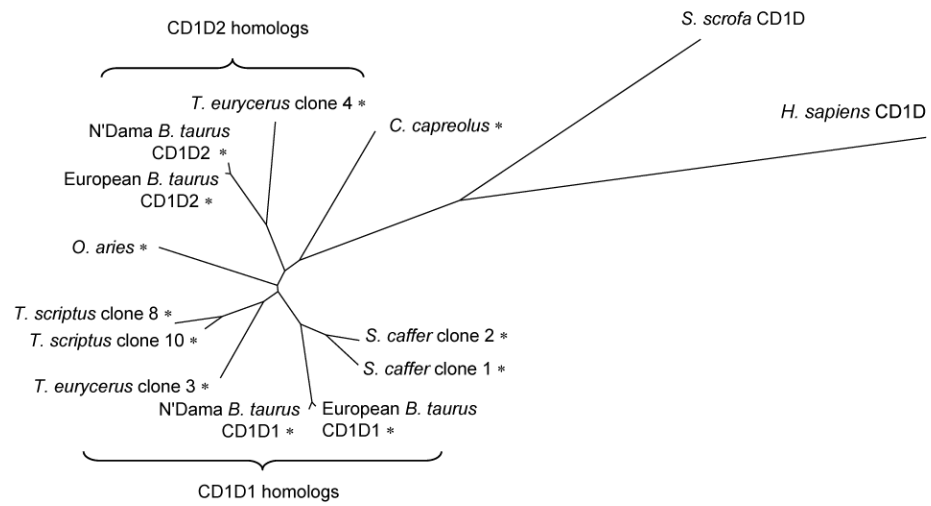

Figure 2. Ruminant CD1D pseudogenes

a Alignment of the 5' end of newly derived ruminant genomic CD1D sequences (full length sequences available under accession numbers EU247610-EU247617 and FJ028651-FJ028652) with the previously published sequences of bovine CD1D1 (DQ192544), bovine CD1D2 [17], porcine CD1D (AB221037), and human CD1D (NM_001766.3). Functional human and porcine start codons: underlined; the mutated ruminant equivalent codons: underlined, bold; possible alternative start codon formed by the mutated donor splice site in ruminants: box; donor and acceptor splice sites in the human and porcine sequence: gray shade. The intron of the human and porcine CD1D sequence is shown in lower case and the translation of the human exons is shown on the lowest line. Btau: *Bos taurus*; Bnda: N'Dama breed of *B. taurus*; Scaf: *Syncerus caffer*; Teur: *Tragelaphus eurycerus*; Tscr: *Tragelaphus scriptus*; Oari: *Ovis aries*; Ccap: *Capreolus capreolus*; Sscr: *Sus scrofa*; Hsap: *Homo sapiens*. b Neighbor joining unrooted tree of porcine CD1D, human CD1D, and the newly derived ruminant genomic CD1D genes. *: pseudogene.

The published sheep CD1d mRNA sequence with accession number AJ006722 [11] does not show disruptive mutations, while the sheep CD1D pseudogene we describe here does. Comparison of exons 1-3 of these two sequences, revealed that they were >98% identical at nucleotide level, suggesting that AJ006722 may be a transcript of the gene we report here. To obtain additional data on the status of the sheep CD1D gene, we investigated CD1D transcripts in the sheep EST database. A BLAST search with the nucleotide sequence of exon 2, encoding the α1 domain of the AJ006722 sequence resulted in five hits that were >98% identical at nucleotide level, suggesting that they were transcripts of the same gene. Three of these hits (EE803429, DY491833, and DY491595) contained a mutated start codon and an unspliceable intron between the leading fragment and the α1 domain. The other two hits did not contain any sequence upstream of the α1 domain. From this we conclude that in the EST database there are no functional CD1D transcripts corresponding to the AJ006722 sequence, but there are transcripts of the pseudogene we describe in this paper. The only sheep CD1 proteins that have been demonstrated at protein level were CD1b and CD1e, isolated by immunoprecipitation with an antibody that recognizes multiple ruminant CD1 molecules [11].

CD1D genes and CD1d transcripts in non ruminant species

CD1D sequences were identified in the genomes of dog, cat, pig, guinea pig, horse, African elephant, rabbit, nine-banded armadillo, small Madagascar hedgehog, European shrew, and northern tree shrew (Table 2). A full length CD1D sequence without any of the characteristics of pseudogenes could be found in pig, horse, and nine-banded armadillo. The CD1D sequences of the other mammals were incomplete because of gaps in the genomic sequences. However, the available parts of the sequences did not show any of the characteristics of pseudogenes.

Species	**NCBI accession or ensemble gene ID**	**Functionality**
Dog (*Canis familiaris*)	AC183576	incomplete[1,4]
Cat (*Felis catus*)	ENSFCAG00000012532	incomplete[1,5]
Pig (*Sus scrofa*)	NM_001102680.1	functional[2,4]
Guinea pig (*Cavia porcellus*)	ENSCPOG00000013837	functional[1, 2,5]
Horse (*Equus caballus*)	NW_001800080.1	functional[2,5]
African elephant (*Loxodonta africana*)	ENSLAFG00000013716	functional[1, 2,5]
Nine-banded armadillo (*Dasypus novemcinctus*)	ENSDNOG00000018694	functional[3,5]
Madagascar hedgehog (*Echinops telfairi*)	ENSETEG00000015412	incomplete[1,5]
European shrew (*Sorex araneus*)	ENSSARG00000000647	incomplete[1,5]
Northern tree shrew (*Tupaia belangeri*)	ENSTBEG00000001753	incomplete[1,5]

Table 2. Searches for CD1D sequences in genome of mammalian species

[1] The genomic sequence contained gaps. The available part does not contain any of the characteristics of a pseudogene.

[2] Full length transcripts of this gene that are predicted to translate into a functional protein have been described in this paper

[3] The gene is complete and did not contain any of the characteristics of a pseudogene, but it is unknown whether the gene is transcribed and translated in vivo.

[4] Location on internet: www.ncbi.nlm.nih.gov

[5] Location on internet: www.ensembl.org

In order to obtain the full length coding sequence of the incomplete genes, and proof that the CD1D genes are transcribed and properly spliced in vivo, we successfully cloned full length CD1d transcripts from guinea pig, rabbit, horse, and African elephant PBMC (accession numbers FJ028653-FJ028656). Alignment of these sequences with the human and murine CD1d sequences (Figure 3) shows that the residues on the surface of CD1d that interact with the NKT TCR are highly conserved. Contrary to all other CD1d sequences the African elephant CD1d sequence has a truncated cytoplasmic tail and lacks a YXXZ motif. The YXXZ motif in the tail sequence of murine and human CD1d is needed for interaction with AP-2 and thus trafficking to the late endosome [24, 25].

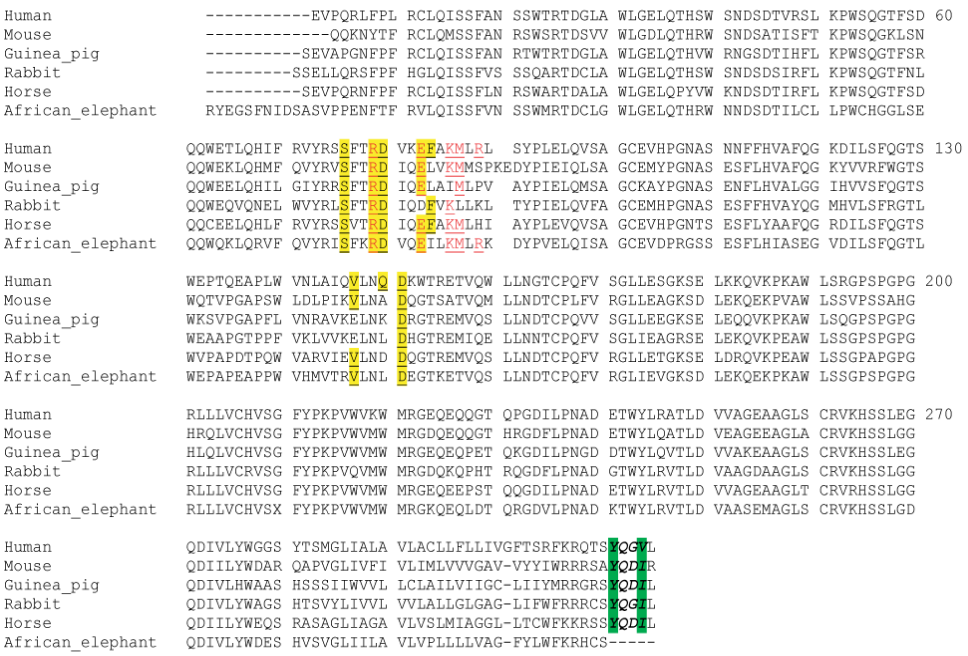

Figure 3. Comparison of CD1d sequences

The human and murine CD1d sequences were aligned with the newly derived guinea pig, rabbit, horse, and African elephant sequences (accession numbers FJ028653-FJ028656). Residues that are in the human CD1d sequence known to interact with the human NKT TCR CDR3α are in yellow/underlined, and with the human CDR2β in red/underlined [20]. The YXXZ motif in the tail sequence is shown in green/bold/italics.

Discussion

In this paper we show that the NKT/CD1d system is present in horse and pig. Equine and porcine NKT invariant α chains and CD1d transcripts are sequenced and their models suggest that they are likely to function as their human and murine counterparts. In addition, we sequenced full length CD1d cDNA of African elephant, guinea pig, and rabbit, and we show that in the genomes of dog, cat, African elephant, nine-banded armadillo, Madagascar hedgehog, European shrew, and Northern tree shrew, (partial) functional CD1D genes are present, suggesting that these species may also have a functional NKT/CD1d system. However, in the six ruminant species we studied here, all CD1D genes we identified were non functional, which strongly suggests that ruminants may not have NKT cells.

Human and murine CD1d-restricted NKT cells can be detected using α-GalCer-loaded CD1d tetramers. Even though human and murine CD1d tetramers cross-react between these two species, lack of detection of NKT cells in ruminants using murine or human CD1d tetramers is not conclusive because these reagents may not cross react with ruminants. Using the same molecular approach that led to the identification of the invariant NKT α chain in pig and horse we were unable to identify invariant NKT α chain homologs in guinea pig, cat, rabbit, African elephant, cattle and sheep. So, even though these species do express V segments homologous to TRAV10 (Vα24), we have not found these V segments in combination with the canonical NKT CDR3α. It is possible that we did not obtain invariant NKT α chain sequences because the TRAV10-homologous V segment is used often by other, non-NKT cells in these species, our sample size is not big enough, and/or the NKT cells are under represented in PBMC. Therefore, based on TCR α chain sequences only, we can not conclude that NKT cells are absent in these species. However, the combination of the fact that ruminants lack functional CD1D genes, and the observed absence of an invariant α chain sequence among 26 different ruminant (bovine and ovine) TRAV10 homolog-containing TCR α chain sequences points strongly to absence of NKT cells in ruminants.

CD1d presents lipids with an α-glycosidic linkage to NKT cells and may therefore be an important molecule to stimulate the immune system in response to α-proteobacteria that contain these compounds. Ruminants are very sensitive to infection with these pathogens. Previously we have shown that European cattle lack functional genes for CD1D. Because we found CD1D pseudogenes and no functional CD1D genes in African N'Dama cattle (*Bos taurus*), two other species of the family *Bovinae* (bongo, bushbuck, and African buffalo), a member of the superfamily *Bovidae* that does not belong to the family of *Bovinae* (sheep), and a ruminant that is a member of the superfamily *Cervidae* (roe deer), we conclude that CD1d proteins are probably absent in all ruminants, though we are aware that we have not formally proven this. In the absence of a fully finished and assembled genome, it is difficult to prove that a functional CD1D gene is absent in a certain species. Southern blotting detects hybridizing sequences, but does not discriminate between functional genes and pseudogenes and between comigrating restriction fragments. Especially the latter poses problems because homology of CD1 genes can be exceptionally high, and these genes will be cut in an identical way by the restriction enzymes used, leading to an underestimation of the real number of CD1 genes. This probably explains that the guinea pig was previously suspected of

having no CD1D gene [16] based on Southern blot data, while we report a guinea pig CD1D gene and transcript here.
Our data on the presence of a CD1D pseudogene in sheep, carrying a mutated start codon and an unspliceable intron, seem to be in contradiction with published data on sheep CD1d. A full length cDNA sequence of sheep CD1d has been published and is predicted to translate into a normal CD1d protein [11]. However, this cDNA sequence has been assembled in silico from partial PCR products. The full length cDNA sequence in which the first intron was properly spliced out has never been obtained (S. Rhind, personal communication). In the sheep EST database we could only find CD1D pseudogene transcripts, and no functional transcript analogous to the published one. Together, this suggests that the published sheep CD1d cDNA may derive from transcripts of a sheep CD1D pseudogene, and are consistent with the possibility that sheep do not have functional CD1d.
The artiodactyl pig (*S. scrofa*) is the closest relative of the ruminants that we studied, and it has a functional gene and no CD1D pseudogene. This dates the loss of a functional CD1D gene by point mutations, and thus the emergence of a CD1D pseudogene approximately 65 million years ago, when the ancestors of *Suidae* and *Ruminantia* diverged [26]. This is consistent with the fact that we have only found CD1D pseudogenes in all ruminants studied and argues against the presence of a functional sheep CD1D gene.
To emphasize the special status of group 2 CD1 molecules as compared to group 1 CD1 molecules, it has often been stated that CD1d molecules and NKT cells are universally present in mammals, while this is not the case for group 1 CD1 molecules. Lack of functional CD1D genes in a considerable group of animals as we show here would suggest that there is no reason for a special status for CD1d proteins based on universal distribution among mammals. In addition to different expression patterns and being slightly separated based on sequence homology, group 2 CD1 molecules (CD1d) are thought to differ fundamentally from group 1 CD1 molecules (CD1a, CD1b, and CD1c) in that they stimulate an invariant T cell population. However, in addition to being able to activate NKT cells with an invariant TCR, it has been shown that CD1d can also stimulate other, non-invariant T cells [27-30]. Whether CD1d is the only member of the CD1 family of proteins that can stimulate an invariant T cell population, remains open: it is possible that in the future invariant group 1 CD1-restricted T cell populations will be discovered, and if so, this would question whether group 1 and group 2 CD1 proteins really perform fundamentally different functions.

Acknowledgements
Ildiko Van Rhijn is supported by the Wellcome trust [GR078283]. Dirk M. Zajonc is a recipient of a CRI investigator award. We would like to thank Jackie Picard and Basil Allsopp (University of Pretoria, Onderstepoort, South Africa) for expert advice, Dan Bradley (Trinity College, Dublin, Ireland), Astrid Rijkenhuizen, and Ton Willemse (University Utrecht, Utrecht, Netherlands) for kindly sharing DNA and/or tissue samples.

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Chapter 5

Expression of filaggrin, pro-inflammatory cytokines and chemokines by keratinocytes cultured from skin of dogs with atopic dermatitis and healthy dogs – preliminary results

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Abstract

The role of keratinocytes in the pathogenesis of atopic dermatitis (AD) in dogs and humans is unclear. Recent studies in humans have shown that mutations in the filaggrin gene lead to impaired filaggrin production by keratinocytes, cause intrinsic skin barrier defects and are associated with AD. Others have shown production of a large variety of pro-inflammatory cytokines and chemokines by keratinocytes, which indicates potential contribution of these cells to the inflammatory environment of atopic skin. In the current study expression of filaggrin, as wells as pro-inflammatory cytokines and chemokines, was investigated in keratinocytes cultured from non-lesional and lesional skin of dogs with AD and from skin of healthy control dogs. Keratinocytes were stimulated with Th1 or Th2 type cytokines (IFNγ, respectively IL-4 and IL-13) or PMA. There was no difference in filaggrin expression in non-lesional or lesional keratinocytes compared to control keratinocytes, but expression was significantly lower in lesional keratinocytes compared to non-lesional keratinocytes. Significant higher expression of IL-8 was found in non-lesional canine keratinocytes compared to control keratinocytes when stimulated with IFNγ or IL-4 and IL-13. IL-8 gene expression was significantly higher in lesional keratinocytes compared to control keratinocytes when stimulated with IFNγ.
In conclusion, this study pilot showed that keratinocytes cultured from lesional sites have a reduced filaggrin production compared to keratinocytes from non-lesional sites, but it is not clear whether this is caused by an intrinsic factor or regulated by cytokines. Our findings seem to suggest that local skin conditions affect filaggrin expression of keratinocytes. In addition, we found that after stimulation with Th1 or Th2 type cytokines, keratinocytes from atopic dogs upregulate IL-8 expression, which may contribute to the influx of inflammatory cells in atopic skin during acute and chronic inflammation.

Introduction

Canine atopic dermatitis is a genetically predisposed, inflammatory and pruritic allergic skin disease with characteristic clinical features associated with IgE antibodies most commonly directed against environmental allergens [1]. It affects about 10 per cent of the dog population in general, and shows remarkable similarities in clinical signs and immunopathogenesis with the disease in humans [2-7]. Both a dysregulated immune response and a defective skin barrier have shown to be important in the development of atopic dermatitis in dogs and humans [2, 8-11].

The epidermal barrier protects the skin to access of environmental pathogens and prevents excessive transepidermal water loss [12-14]. Keratinocytes are the main constituents (95%) of the epidermis which consists of 3-5 cell layers of these cells that proliferate at the *lamina basale* and mature to become terminally differentiated cornified keratinocytes (corneocytes) at the surface. Filament aggregating protein (filaggrin) plays an important role in this cornification process, as it links keratin filaments into a strong dense intracellular matrix [15-17].

Several studies have shown that a loss-of-function mutation in the human filaggrin gene results in a defective skin barrier, which is a significant risk factor for the development of atopic dermatitis [18-23]. Besides an impaired function due to a genetic defect, downregulation of filaggrin expression by Th2 cytokines may play a role [21, 24]. Cultured human keratinocytes show reduced filaggrin mRNA and protein expression when incubated with medium containing both IL-4 and IL-13. It is not clear yet whether lack of filaggrin expression also plays a role in the development of canine AD. One study showed a reduced profilaggrin expression in 15 of 18 dogs with AD [25]. In another study, the skin of Beagles with experimentally-induced atopic dermatitis had lower filaggrin protein expression compared to healthy skin [26].

In a study by Chervet *et al.* [25] the reduced profilaggrin expression in 4 of the 15 dogs seems to be caused by a loss-of-function mutation, as indicated by the absence of staining with a C-terminus specific antibody. A genome study in a group of West Highland White Terriers (WHWT) with atopic dermatitis did however not show penetrance of a filaggrin haplotype [27], suggesting that a canine equivalent of filaggrin null mutations such as R501X and 2282del4, does not play a role in development of AD in WHWT. This is as yet unknown for other breeds.

Keratinocytes do not merely act as skin barrier cells, but also contribute actively to the regulation/dysregulation of the local skin immune response [28, 29]. Keratinocytes may produce pro-inflammatory cytokines, expression of which was shown to be increased in lesional AD skin of both dogs and humans [28, 30-33]. In addition, keratinocytes may also induce a Th2-cell influx, a major histological feature of AD skin during the acute phase, by producing chemokines such as thymus and activation regulated chemokine (TARC) [34, 35], of which high levels were found in canine lesional skin, but not in non-lesional or control skin [33]. The chemokines produced by keratinocytes are IL-8, which attracts neutrophils [30], CCL-5 also known as RANTES to attract T cells, eosinophils and basophils[36], and GM-CSF which recruits and activates dendritic cells [10, 37].

Th2 cytokines are characteristic of the acute phase of inflamed atopic dermatitis skin [38], whereas the chronic phase is dominated by Th1 cytokines [38]. In human cultured keratinocytes it was shown that Th2 cytokines were able to decrease the

expression of filaggrin [21, 24]. It is not known whether Th2 has a similar effect on canine keratinocytes. Therefore, we investigated if Th1- or Th2-type cytokines are able to downregulate filaggrin (FLG) gene expression in canine keratinocytes, and as a consequence contribute to a defective skin barrier. It was shown that a nutrient combination of pantothenate, choline, nicotinamide, histidine and inositol, when fed at supplemented concentrations, was able to upregulate epidermal lipid synthesis and significantly improved skin barrier function in dogs [39]. In this study we investigated the ability of these skin supportive dietary supplements (SSD) to upregulate FLG expression in keratinocytes. Furthermore, in both canine and human lesional skin upregulation of pro-inflammatory cytokines and chemokines has been found [28, 30-33]. To understand if T cells actively contribute to the regulation of pro-inflammatory cytokines and chemokine, we studied whether Th1- or Th2-type cytokines stimulate the production of pro-inflammatory cytokines and chemokine by keratinoctytes.

Materials and methods

Animals

Three client-owned dogs with AD referred to the Utrecht University Faculty of Veterinary Medicine, were included in this study after consent was given by the owner. The diagnosis of AD was based on standard criteria including: pruritus, an early age of onset, the presence of a corticosteroid-responsive dermatitis, involvement of at least the feet, face and pinnae, immediate skin test reactivity to environmental allergens including *Dermatophagoides pteronyssinus* and *D. farinae*, and the presence of allergen-specific IgE in serum [40, 41]. Three randomly selected, healthy adult Beagle dogs owned by the university, served as control animals. The study fulfilled the requirements set by the Utrecht University Animal Ethics Committee.

Keratinocyte culture

Four millimeter punch biopsies were taken under general anaesthesia (medetomidine 20 ug/kg and propofol 1-3 mg/kg IV on effect), and after thorough skin disinfection, from non-lesional and lesional skin of dogs with AD, whereas control biopsies were collected from the flanks of healthy dogs. Directly after collection, biopsies were submerged in cold medium. Biopsies were dissected under a light microscope and the dermis and subcutis were separated from the epidermis using fine tweezers and a scalpel. The remaining epidermal skin was cut in small 1x1 mm explants and stored in cold medium on ice until all epidermis was processed (max. 2 hours).

Explants were allowed to adhere for several minutes to wells of a 24-well culture plate, CnT-09 keratinocyte proliferation medium from CellnTEC Advanced Cell Systems (Bern, Switzerland) was added, and plates were subsequently incubated at 37°C, 5% CO_2. CnT-09 is a proliferation medium specially designed for culturing canine keratinocytes. Keratinocyte outgrowth from the explants was monitored daily. After around 5 days, wells containing proliferating keratinocytes (33-50% of the wells) were washed with PBS and incubated with 0,25% trypsin at 37°C, until all cells detached. Keratinocytes (10^6 cells/10 ml) were further cultured in T75 flasks until 70-80% confluence and passaged to a new flask at 10^6 cells/10 ml up to passage 5. Keratinocytes were suspended in CnT-09 medium (2-10 x 10^6 cells/ml)

containing 20% DMSO/10%FCS and stored at -140°C. For subsequent experiments, lesional, non-lesional and control keratinocyte suspensions were thawed and cultured in CnT-09 in T75 flasks until 70-80% confluence and transferred to 6-well culture plates in CnT-09 medium.
To study fillagrin (FLG) expression, CnT-09 proliferation medium was removed at 70-80% confluency in 6-well culture plates and cells were washed with PBS. Thereafter, the keratinocytes were kept under proliferating or differentiating conditions. To induce differentiation, keratinocytes were cultured for five days in CellnTEC Advanced Cell Systems Calcium-free, serum-free medium CnT-02CF with 1,3 mmol/L $CaCl_2$. To maintain keratinocytes in proliferation phase, the same CnT-02CF medium was used without additional $CaCl_2$ [24] for five days. During the entire five days, keratinocytes were incubated with or without bioactive recombinant canine IFNγ (10ng/ml; R&D Systems), bioactive recombinant canine IL-4 and IL-13 (50ng/ml; R&D Systems), or the skin supportive dietary supplements (SSD) containing choline, histidine, inositol, nicotinamide, pantothenate, proline and pyridoxine, all at 100 µmol/L [39].
To study cytokine and chemokine expression, CnT-09 proliferation medium was removed at 70% confluency in 6-well culture plates and cells were washed with PBS. Thereafter, keratinocytes were cultured in CnT-02CF medium without additional $CaCl_2$ to maintain keratinocytes in proliferation phase, and were incubated with or without IFNγ (10ng/ml; R&D Systems), IL-4 and IL-13 (both at 50ng/ml; R&D Systems), or phorbol 12-myristate 13-acetate (PMA) (10ng/ml) for 6, 24 and 48 hours. PMA has been used in other studies as a potent stimulus for gene expression in cultured keratinocytes [37, 42].

Quantitative real time RT-PCR
From all cultured cells RNA was isolated using the RNeasy kit (Qiagen) followed by cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) both according to manufacturer's instructions. The quantitative real-time RT-PCR was performed with a MyiQ Sing-Color Real-Time PCR Detection System (Bio-Rad) using the high affinity double-stranded DNA-binding dye iQ SYBR Green. Primers (Eurogentec) were designed based on the canine genome sequences in Ensembl (www.ensembl.org/Canis_familiaris) using Oligo Explorer 1.1.0 software. PCR products of FLG and GM-CSF were sequenced by BaseClear (Leiden, The Netherlands). Primers for ribosomal protein RPS19 a canine endogenous reference gene [43] and the target genes IL-6, TNFα, IL-8, TARC and RANTES were obtained from literature [44, 45]. Gene specific primers are listed in Table 1.
Quantitative real-time RT-PCR reactions were performed under the following conditions: an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 20 s at 95°C and 30 s at primer specific annealing temperature (Table 1).

Gene	Primer	Primers sequences	Product length (bp)	Tm (°C)
RPS19	Fwd	CCTTCCTCAAAAA/GTCTGGG	95	61.0
	Rev	GTTCTCATCGTAGGGAGCAAG		
Filaggrin	Fwd	GCCAATCCTGAAGAATCCAG	173	61.0
	Rev	TTTGCTCTGATGCTTGGG		

IL-6	Fwd	GAGCCCACCAGGAACGAAAGAGA	123	65.0
	Rev	CCGGGGTAGGGAAAGCAGTAGC		
TNFα	Fwd	CCCCGGGCTCCAGAAGGTG	84	64.0
	Rev	GCAGCAGGCAGAAGAGTGTGGTG		
IL-8	Fwd	CTTCCAAGCTGGCTGTTGCTC	172	60.0
	Rev	TGGGCCACTGTCAATCACTCTC		
TARC	Fwd	GGAGCCATTCCTATCAGCAG	109	64.5
	Rev	GGTCGGAACAGATGGACTTG		
RANTES	Fwd	ATATGCCTCAGACACCACAC	124	59.0
	Rev	GGTGACAAAGACGACTGC		
GM-CSF	Fwd	TCACTCGGCCCTCTCAG	153	61.0
	Rev	GTAGGCGGGTCTCCAGG		

Table 1. Primer sequences, products lengths and Tm used for mRNA expression analysis.

Statistical analysis

Relative gene expression was calculated using the Pfaffl method [46] and data were analyzed using SPSS 17.0. A linear mixed model was used to analyse FLG expression. The explanatory factors in the model were group (control, non-lesional and lesional) and treatment (IFNγ, IL-4 and IL-13, SSD) in both proliferating and differentiating conditions. The subject variable 'dog' was used as a random factor. The model was designed to study main effects (group and treatment) and interactions between explanatory factors by performing multiple comparisons. For the expression of cytokines and chemokines a repeated measures general linear model was used including four time groups (0, 6, 24, 48 hours). As within subject factors, group (control, non-lesional and lesional) and treatment (IFNγ, IL-4 and IL-13, or PMA) were used as fixed factors. A Bonferroni post-hoc analysis was done to compare between group and treatment to determine if main effects were significant. Expression levels below the detection level (DL) were given an arbitrary value of 2/3 of the lowest measured expression level of the particular gene in order not to exclude these samples from the model. For TNFα 2 of 90 samples were below DL, for GM-CSF 10 of 90 samples were below DL.

Results

The relative filaggrin gene expression

To understand the significance of a lower filaggrin protein expression as observed in atopic canine skin [25] better, we studied the FLG gene expression of proliferating keratinocytes and differentiated keratinocytes cultured form lesional, non-lesional and control biopsies with or without stimulation with the Th1-cytokine IFNγ , the Th2-cytokines IL-4 and IL-13 or skin supportive dietary supplements (SSD).

At the end of the 5-day culture a clear morphological difference was observed between proliferating and differentiating keratinocytes. However, there was no morphological difference between keratinocytes cultured from healthy dogs and dogs with atopic dermatitis (non-lesional and lesional). Under the light microscope, proliferating keratinocytes cells were small, well- defined, cuboidal cells with a nucleus, whereas the differentiated keratinocytes had a variable morphology, no clearly visible nucleus and they showed as stratified islands (Fig. 1).

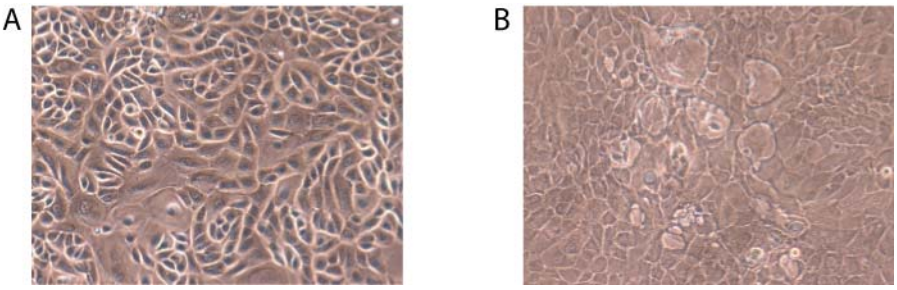

Figure 1. Morphology of keratinocytes after 5 days of culture deprived of $CaCl_2$ to promote proliferating cells (A) and in the presence of 1,3 mmol/L $CaCl_2$ to induce differentiation (B).

It was demonstrated that keratinocytes cultured from lesional skin had a lower expression of FLG compared to keratinocytes from non-lesional skin of atopic dogs ($P < 0.001$). No difference was found in FLG expression in keratinocytes from lesional or non-lesional skin compared to control keratinocytes (Fig. 2A), nor between proliferating and differentiating keratinocytes (Fig. 2B). Stimulation of the control, non-lesional and lesional groups of proliferating or differentiating keratinocytes with the Th1cytokine IFNγ, the Th2 cytokines IL-4 and IL-13, or SSD for five days, did not increase FLG expression (Fig. 2B).

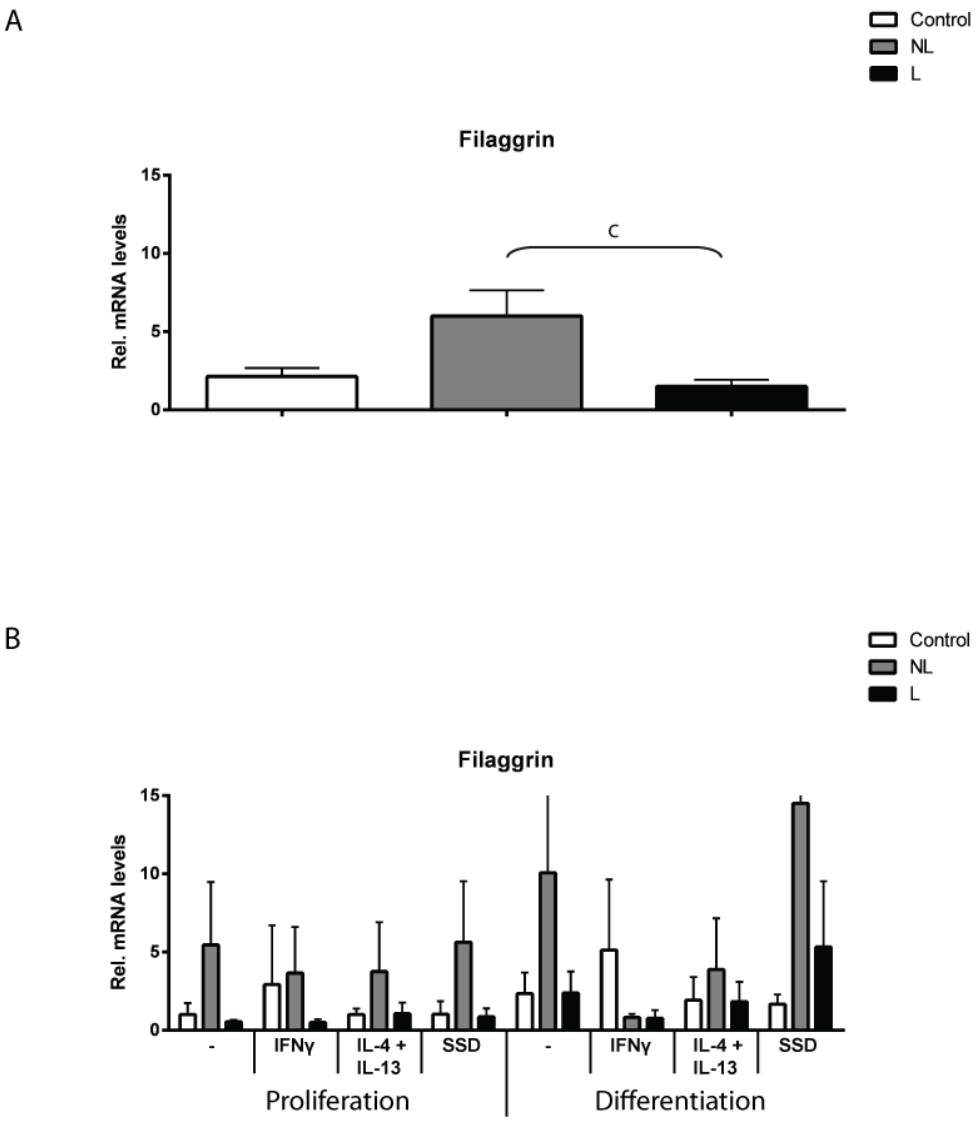

Figure 2. Relative filaggrin gene expression of proliferating and differentiating keratinocytes cultured from control dogs and from non-lesional and lesional biopsies from dogs with atopic dermatitis. Keratinocytes were cultured with or without the stimulation of IFNγ, IL-4 and IL-13, or Skin Supportive Dietary supplements (SSD). In panel A the different stimulation groups are clustered, panel B shows each stimulation group separately. Difference between groups was indicated with: c = $P < 0.001$.

The relative gene expression of pro-inflammatory cytokines

To explore whether canine keratinocytes can contribute to the inflammatory environment seen in atopic skin, we studied gene expression of pro-inflammatory cytokines IL-6 and TNFα by real time RT-PCR in control, non-lesional and lesional proliferating keratinocytes, cultured for 0, 6, 24 or 48 hours in the presence of Th1 (IFNγ), Th2 (IL-4 and IL-13) cytokines, or PMA (Fig. 3).

There were no significant differences in IL-6 mRNA levels between unstimulated control skin derived keratinocytes (t=0) and non-lesional or lesional keratinocytes. Significant differences were neither found between control keratinocytes and non-lesional or lesional keratinocytes, when stimulated with Th1 (IFNγ) or Th2 (IL-4 and IL-13) cytokines for 6, 24 or 48 hours. Stimulating control keratinocytes with PMA for 6 hours showed a significant higher IL-6 mRNA level compared to non-lesional and lesional keratinocytes. This effect was not observed at 24 or 48 hours. Looking at the effect of Th1, Th2, or PMA stimulation within a group (control, non-lesional or lesional), we found a significant increase of IL-6 mRNA levels in control keratinocytes stimulated with PMA for 6 hours compared to unstimulated (t=0) expression in control keratinocytes ($P < 0.05$). No effects were found for stimulation within the non-lesional group or lesional group.
No significant differences were found in TNFα mRNA levels between unstimulated control skin derived keratinocytes (t=0) and non-lesional or lesional keratinocytes.
Stimulating non-lesional keratinocytes with IFNγ for 24 hours showed a significant higher TNFα mRNA level compared to lesional keratinocytes, but not to control keratinocytes ($P < 0.05$). No effects of stimulation with IFNγ were seen at 6 or 48 hours between the groups. Non-lesional keratinocytes showed a significant higher TNFα mRNA level compared lesional keratinocytes when stimulated with IL-4 and IL-13 for 6 hours ($P < 0.05$), but not at 24 or 48 hours. No differences were found with Th2 stimulation between control keratinocytes and non-lesional keratinocytes or between control keratinocytes and lesional keratinocytes. Control keratinocytes stimulated with PMA for 6 and 24 hours showed a significant increase in TNFα mRNA levels compared to lesional keratinocytes (resp. $P < 0.01$; $P < 0.01$). This effect was not observed at 48 hours. No effect was found after PMA stimulation between control keratinocytes and non-lesional keratinocytes or between non-lesional keratinocytes and lesional keratinocytes. Within the control group it was found that stimulation with Th1 or Th2 cytokines had no effect compared to unstimulated control keratinocytes (t=0), but PMA stimulation for 6, 24 and 48 hours significantly increased TNFα mRNA levels compared to unstimulated keratinocytes (resp $P < 0.01$; $P < 0.05$; $P < 0.05$). No effects were found of stimulation with Th1 or Th2 cytokines or PMA within the non-lesional group or lesional group. To summarize, IL-6 expression was only upregulated in keratinocytes from control dogs. Th1 and Th2 cytokines do not markedly increase TNFα gene expression in keratinocytes.

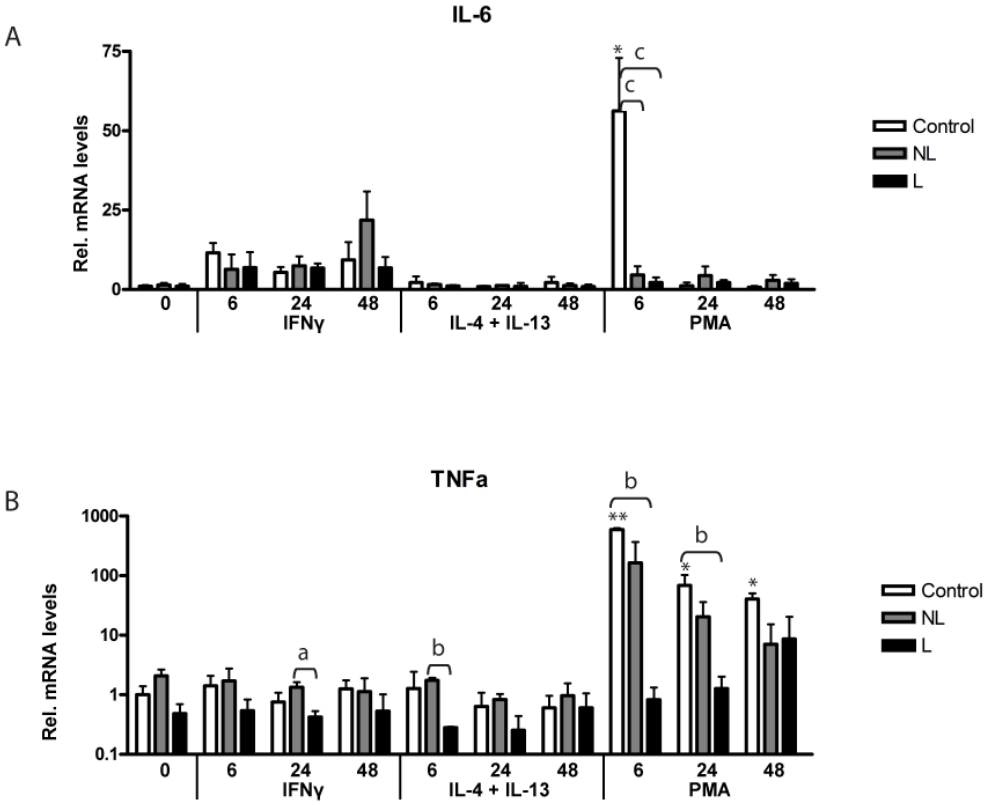

Figure 3. Relative gene expression levels of pro-inflammatory cytokines. Keratinocytes from control and atopic dogs were stimulated with or without IFNy, IL-4 and IL-13, or PMA. Expression levels of IL-6 (A) and TNFα (B) are depicted. Mean expression is shown with standard error of the mean, asterisks indicate level of significance within groups with unstimulated keratinocytes as reference category, * P < 0,05; ** P < 0.01; *** P < 0.001. Difference between groups was indicated with: a = P < 0.05; b = P < 0.01; c = P < 0.001.

The relative gene expression of chemokines and GM-CSF

To determine whether canine keratinocytes can contribute to the cellular influx seen in atopic skin, we studied gene expression of chemokines IL-8, TARC and RANTES, and gene expression of GM-CSF by real time RT-PCR in control, non-lesional and lesional keratinocytes cultured for 0, 6, 24 or 48 hours in the presence of Th1- (IFNγ), Th2- (IL-4 and IL-13) cytokines or PMA (Fig. 4).

There were no significant differences in IL-8 mRNA levels between unstimulated control skin derived keratinocytes (t=0) and non-lesional or lesional keratinocytes. IFNγ stimulation in both non-lesional (24 and 48 hours) and lesional keratinocytes (24 hours) increased expression of IL-8 mRNA compared to control keratinocytes (P < 0.01; P < 0.05; P < 0.01). IL-8 gene expression in non-lesional keratinocytes was higher when stimulated with IL-4 and IL-13 for 24 hours compared to both control keratinocytes (P < 0.01) and lesional keratinocytes (P < 0.05). No significant differences in IL-8 gene expression were found between control, non-lesional and lesional keratinocytes when stimulated with PMA. Looking at the effect

of Th1, Th2, or PMA stimulation within a group (control, non-lesional or lesional), we found a significant increase of IL-8 mRNA levels in control keratinocytes stimulated with PMA for 6 hours compared to unstimulated (t=0) expression in control keratinocytes ($P < .01$). No effects were found for PMA stimulation within the non-lesional group or lesional group.

No significant effects in gene expression of TARC, RANTES and GM-CSF were found between control, non-lesional and lesion keratinocytes when cultured for 0, 6, 24 or 48 hours in the presence of Th1 (IFNγ) or Th2 (IL-4 and IL-13) cytokines, or PMA. RANTES gene expression of control keratinocytes stimulated for 48 hours with PMA showed to be higher compared to that in unstimulated control keratinocytes ($P < 0.05$). This was not observed for stimulated non-lesional or lesional keratinocytes compared to unstimulated non-lesional keratinocytes or lesional keratinocytes. The above results show that Th1 and Th2 cytokines can increase IL-8 expression in keratinocytes, which may result in the influx of inflammatory cells such as neutrophils and eosinophils.

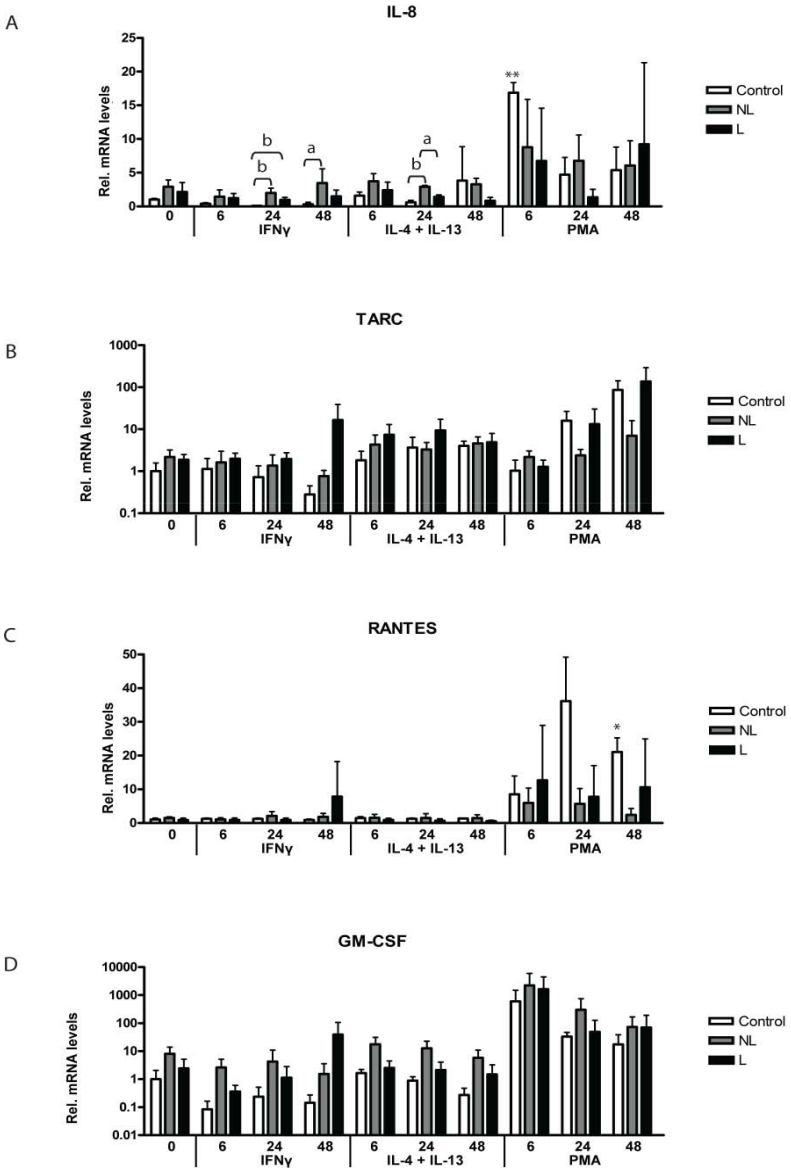

Figure 4. Relative gene expression of chemokines and GM-CSF. Keratinocytes from control and atopic dogs were stimulated with or without IFNγ, IL-4 and IL-13, or PMA. Expression levels for IL-8 (A), TARC (B), RANTES (C) and GM-CSF (D). Mean expression is shown with standard error of the mean, asterisks indicate level of significance within groups with unstimulated keratinocytes as reference category, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Difference between groups was indicated were indicated with: a = $P < 0.05$; b = $P < 0.01$; c = $P < 0.001$.

Discussion

The present study was designed to determine the potential role of keratinocytes in the pathogenesis of AD by investigating differential expression of filaggrin, pro-inflammatory cytokines and chemokines between cultured keratinocytes from dogs with atopic dermatitis and from control dogs.

Filaggrin is important in the formation of the skin barrier. A reduction in filaggrin protein expression due to genetic mutations in the filaggrin gene [18-23] or as a consequence of an inflammatory environment [21, 24, 47] seem to contribute to the development of AD. We did not find a significant lower expression of the filaggrin gene in unstimulated atopic keratinocytes compared to control keratinocytes. Furthermore, we were not able to show a direct effect of a Th1 or Th2 inflammatory cytokine environment on canine filaggrin gene expression. However, we did find that lesional keratinocytes had a lower filaggrin expression than non lesional keratinocytes. This seems to suggest that the inflammatory environment at the lesional site causes a downregulation of filaggrin expression in keratinocytes even after several culture passages. It remains unclear whether Th1 or Th2 inflammatory cytokines are indirectly related to filaggrin expression or not, but there seems no direct role in canine keratinocytes. In this study, we were not able to demonstrate a similar role for the canine filaggrin gene in atopic dermatitis as it was shown for human filaggrin. We measured filaggrin expression in keratinocytes from 3 dogs with AD and 3 control dogs and found large variance in filaggrin gene expression between the samples within a group, which makes it difficult to demonstrate potential differences between AD and control keratinocytes from dogs. In one canine study, filaggrin protein expression in the skin of atopic dogs was lower than in healthy control skin in line with human findings [2], but another study reported higher mRNA levels in atopic skin compared to control skin [48]. These contradicting findings indicate that further studies are needed to elucidate the role of filaggrin in canine atopic dermatitis.

In the current study, expression of pro-inflammatory cytokines such as IL-6 and TNFα was measured to determine the contribution of keratinocytes to inflamed atopic skin. We found lower TNFα expression in lesional keratinocytes after culturing with IL-4 and IL-13 or IFNγ compared to non-lesional keratinocytes. This finding seems to suggest that CD4 T cells downregulate the TNFα production of keratinocytes at the lesional site during the acute and chronic phase of the inflammation. Our *in vitro* results do not correlate with expression profiles measured in lesional skin. In one study, similar TNFα protein expression levels were found in non-lesional and lesional skin biopsies from dogs with atopic dermatitis [49], but no comparison was made with keratinocytes from healthy dogs. Other studies report higher gene expression of TNFα in lesional skin compared to non-lesional and control canine skin [32, 33, 50]. We did find that keratinocytes from dogs with atopic dermatitis and control dogs were able to upregulate TNFα expression after stimulation with PMA. This may indicate, that the higher TNFα gene expression seen in non-lesional and lesional skin biopsies is from keratinocytes, but not induced by stimulation with IL-4 and IL-13 or IFNγ, or that keratinocytes are not a major contributor to TNFα expression in skin. It is possible that other inflammatory cells in lesional skin such as CD4 T cells produce the majority of TNFα and that the Th1 or Th2 cytokines produced by these cells reduce the expression of TNFα by keratinocytes. Alternatively, other factors e.g. pro-

inflammatory stimuli such as bacterial superantigens, may induce TNFα expression by keratinocytes [51].
In this study, we measured the expression levels of chemokines such as IL-8, TARC and RANTES of keratinocytes to determine whether keratinocytes play a role in the influx inflammatory cells observed in atopic skin. There is a higher expression of IL-8 in non-lesional and lesional atopic keratinocytes stimulated with IFNγ compared to control keratinocytes. We also found increased IL-8 gene expression in non-lesional keratinocytes when stimulated with IL-4 and IL-13 compared to control keratinocytes. In humans, cultured keratinocytes from non-lesional skin and control keratinocytes upregulate IL-8 mRNA after exposure to IL-4 or IFNγ [52] . Our findings suggest that during the acute and chronic phase of inflammation, CD4 T cells induce IL-8 secretion by atopic keratinocytes leading to the attraction of neutrophils and eosinophils in skin. In a study with experimentally-induced atopic dogs, it was found that intradermal allergen exposure induced an influx of neutrophils in the dermis during the late phase reaction [53, 54]. Another study reported that increased IL-8 gene expression was found when a canine keratinocyte cell line was cultured with house dust mite allergen Der f1 [45]. Nevertheless, one of the striking findings in human and canine atopic skin is the absence of neutrophils in lesional skin, even when there is a secondary infection [3, 55, 56]. In contrast to neutrophils, eosinophils are found in the epidermis and dermis of lesional skin of atopic dogs [5] and our findings suggest that keratinocytes may play a role in attracting these cells.
Upregulation of TARC and RANTES have been found in both atopic skin of dogs and humans [33, 57-59]. TARC expression by keratinocytes leads to chemotaxis of Th2 cells towards the skin [35], whereas RANTES attracts eosinophils, basophils, monocytes/macrophages and CD4 T cells [60, 61].
In our study we could not confirm that canine keratinocytes upregulate expression of these chemokines suggesting that local factors/cytokines other than Th1 and Th2 cytokines, may be important for the upregulation in keratinocytes. Furthermore, our study did not show a difference in GM-CSF expression between atopic and control keratinocytes. GM-CSF is a cytokine which enables dendritic cells development and seems to play an important role in the high number dendritic cells found in lesional atopic skin [62]. In a canine keratinocyte cell line, upregulation of GM-CSF protein was found when cells were stimulated with Der f1 [63], which is in line with the observation of upregulation of GM-CSF gene and protein in keratinocytes cultured from lesional skin of atopic patients [37].
In summary, this pilot study showed that expression of canine filaggrin in keratinocytes from lesional sites is lower than from non-lesional sites and suggests that local skin conditions affect filaggrin expression of keratinocytes. In addition, we found that keratinocytes from atopic dogs may contribute to the influx of inflammatory cells by secreting IL-8 when exposed to Th1 and Th2 cytokines.

Acknowledgements
This work was financially supported by Royal Canin, France.

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Chapter 6

Inverse association between endotoxin exposure and canine atopic dermatitis

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The Veterinary Journal 2011; 190(2):215-219

Abstract

The development of atopic dermatitis in dogs may be related to exposure to mite allergens, bacterial endotoxin and/or fungal glucans. In this study, indoor exposure levels of house dust mite allergens, endotoxins and fungal glucans were measured to determine their possible association with canine atopic dermatitis. A case-control study including adult Labrador retrievers with (n = 28) and without atopic dermatitis (controls; n = 65) was conducted. Dust samples were collected from the living room floor and the bedding and the coat of the dog and these were analyzed for house dust mite allergens Der p1 and Der f1, endotoxin and (1→3)-β-D-glucan levels. Dog owners were also required to return a questionnaire regarding their home characteristics.

The endotoxin exposure level in the coat of dogs was significantly inversely associated with atopic dermatitis (odds ratio, 0.38; 95% confidence interval, 0.15-0.97; $P < 0.05$). No significant difference was found in exposure levels to house dust mite allergens and fungal glucans. The results indicated that endotoxin exposure is inversely associated with canine atopic dermatitis, suggesting a protective effect of high indoor endotoxin exposure towards the development of the condition.

Introduction

The rise of human atopic diseases in Western countries in the last decades has been described by several large-scale epidemiological studies. There are currently no data available to show whether the phenomenon also occurs in pets exposed to the same indoor living environment. Atopic diseases naturally develop in dogs and cats, and atopic dermatitis in dogs in particular shows remarkable similarity in disease pathogenesis and clinical symptoms to its human counterpart [1-4].

Canine atopic dermatitis (cAD) is a genetically predisposed, inflammatory and pruritic allergic skin disease with characteristic clinical features associated most commonly with IgE antibodies to environmental allergens. As the result of pruritus, dermatitis is commonly noticed at the feet, face, ears, and axillae. Approximately 75% of dogs show the first symptoms before the age of three years. About 10% of the canine population is affected with cAD, and it is more common in certain breeds including Labrador retrievers [5, 6].

Exposure to environmental allergens is crucial for the development of cAD. Most frequently sensitization to house dust mite (HDM) allergens is reported [7, 8], in particular allergens from the species *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) and *Dermatophagoides farinae* (*D. farinae*). Although it is clear that exposure to environmental allergens is a prerequisite for sensitization, the relationship between the level of exposure and sensitization and development of AD remains elusive. It has been shown that there is a positive relationship between the level of HDM exposure and atopic eczema in children [9]. In addition, some found this effect only with sensitization to HDM and without development of AD [10], whereas other studies failed to determine a clear relationship [11, 12]. Several environmental factors other than HDM allergens are also commonly associated with sensitization and clinical atopy in humans or, in contrast, with an inhibitory effect on disease development.

Endotoxin is a strong pro-inflammatory response-inducing agent produced from the cell walls of Gram-negative bacteria. High exposure to endotoxin in early life is associated with low incidence of atopic eczema and other atopic diseases in children with a parental history of asthma or allergies [13-17]. Depending on timing and dose of exposure, mould components such as fungal glucans may have adjuvant or protective effects on atopy [18, 19].

In this case-control study, Labrador retriever owners were asked to collect dust samples from the living room floor and from the bedding and coat of the dog to explore the possible association between cAD and exposure to HDM allergens, bacterial endotoxin and fungal glucans.

Materials and methods

Study population
We analyzed dust samples from households with an adult Labrador retriever with (cases; n = 28) or without (controls, n = 65) cAD. The diagnosis of cAD was based on the standard clinical criteria such as pruritus, early age of onset, presence of a corticosteroid-responsive dermatitis (at least on feet, face, and pinnae), immediate skin test reactivity to environmental allergens including *D. pteronyssinus* or *D. farinae*, and the presence of allergen-specific IgE in serum [20, 21]. Control dogs were healthy and did not have a history or clinical signs compatible with cAD. The adult Labrador retrievers were randomly selected from the cAD-database of the university hospital (cases) or from among members of the Dutch Labrador Retriever Kennel Club (controls).

Dust collection
Dust samples were collected from the owners' living room and from the dogs' bedding and coats. All samples were collected between March and October 2007. Dust samples were collected using nylon sampling socks (Allied Filter Fabrics, Hornsby, Australia) and a vacuum cleaner according to the protocol used in the PARSIFAL study [19]. Briefly, for the collection of dust samples, sampling time and area depended on the type of floor covering of the room: for fully carpeted floors, 1 m^2 was vacuumed for 2 min; for smooth floors with a rug larger than $4m^2$, $1m^2$ of rug was vacuumed for 2 min; for smooth floors with no rug or with a rug smaller than $4m^2$, $4m^2$ of smooth floor was vacuumed for 4 min. The surface of the dogs' bedding was vacuumed for 2 min. For the dust sample from the dogs' coats, the whole dog was evenly vacuumed for 1 min. After collection, dust samples were sealed in plastic bags and stored at -20°C for 0 – 5 months until extraction.

Dust extraction and analysis
Dust was transferred from the nylon filter bag into a pyrogen-free Greiner tube, weighed and then extracted in a volume of 5 - 40 ml of pyrogen-free water with 0.05% Tween20 (<0.5 g in 5 ml, 0.5-1.0 g in 10 ml, 1.0-2.0 g in 20 ml and >2.0 g in 40 ml). Endotoxins, allergens and glucans were extracted sequentially [18]. For endotoxin, suspensions were incubated using an end-over-end roller for 1 h at room temperature (RT), and after centrifugation (15 min at 1000 *g*), dust-free supernatant was removed and stored at -20°C. For allergen extraction, the removed volume supernatant was replaced with exactly the same volume 10x concentrated phosphate-buffered saline (PBS). After re-suspending the dust sediment, the tubes were incubated at RT for 1 h followed by centrifugation (15 min at 3000 *g*). The supernatant was transferred and stored at -20°C. For glucan extraction, the removed supernatant was replaced with the same volume PBS-Tween (0.05%). After re-suspending the settled dust, the tubes were placed on end-over-end roller for 15 min at RT, followed by 1 h heat incubation in an autoclave (1 bar, 120°C). Thereafter, tubes were again placed for 15 min on end-over-end roller at RT and centrifuged (15 min at 1000 *g*). The dust-free supernatant was collected and stored at -20°C.
Concentrations of HDM allergens (Der p1 and Der f1; ng/ml) were measured with a sandwich enzyme immunoassay (EIA) using a dust mite ELISA kit (Indoor Biotechnologies, Warminster, UK) [22]. The quantitative kinetic chromogenic

Limulus amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, USA) was used to determine the amount of endotoxin in the dust samples. Concentrations were expressed as endotoxin units per ml. Levels of (1→3)-β-D-glucans (μg/ml) were measured with an inhibition EIA [23]. Measured levels of house dust mite allergens and microbial agents were converted to concentrations per gram of collected dust and per square meter of surface area.

Questionnaire data

All participants completed a questionnaire concerning household characteristics such as type of housing, type of floor covering, age of carpeting/rug, floor level, ventilation system, isolation of floors and windows, temperature and use of heating, dampness of the house and number and type of indoor pets. The questionnaire also contained questions about the dogs' health (allergic symptoms, allergies diagnosed by veterinarian) and housing conditions.

Statistical analyses

Differences between cases and controls in the general data of the study population and home characteristics were determined using the Mann–Whitney U test and Chi-square test. Dust samples that had undetectable amounts of allergen, endotoxin, or (1→3)-β-D-glucan were given a value of two-thirds of the detection limit. These substituted values were included in the statistical analyses in order to prevent the loss of data and not to overestimate the values above the detection level [24]. The detection limit for HDMs was 10 ng Der p1/g of dust and 22 ng/g for Der f1; endotoxin and (1→3)-β-D-glucan levels were detected above 1159 EU/g and 77 μg/g, respectively. Data were not normally distributed even after ln-transformation, and therefore the Mann–Whitney U test was used to compare the means between cases and controls.

Logistic regression analyses were performed to calculate associations between cAD and exposure to HDM allergens, endotoxin, or (1→3)-β-D-glucan. The odds ratios (OR) with 95% confidence intervals (CI) were calculated with and without adjustment for age and sex. Logistic regression analyses on continuous exposure data did not reveal any interpretable results. Further logistic regression analyses were performed after dichotomizing exposure data at the median or at the limit of detection when more than half the samples yielded no detectable results. Division was made at the detection level to have an as much as possible equal distribution between the cells. Correlation between exposure levels of samples taken from the living room and the dogs' bedding and coat were analyzed using the Spearman rank correlation test. All statistical analyses were performed with SPSS statistical software (version 16.0 for Windows, SPSS Inc.).

Results

The characteristics of the study population are shown in Table 1. Dogs with cAD were older, more often male than control dogs, and were washed more frequently. Home characteristics regarding the number of dogs, the presence of other pets (such as cats, rodents and birds), the age of the house, and the living room conditions (such as type of floor cover, temperature and type of windows) were not significantly different between the two groups. About 50% of the houses were built after 1975, and it was observed that the majority of dogs had lived their entire life in their present homes. The type of home in which the dog lived was usually a single-family home with more cases living in an upper-floor flat.

Characteristics	Cases (n = 28)	Controls (n = 65)
Age in years, median (range)*	7.0 (2.5-13.0)	3.5 (1.0-13.5)
Age at diagnosis, median (range)	3.6 (1.6-8.0)	-
Male (%)**	18 (64)	15 (23)
Wash frequency		
0-3 times/year**	13 (46)	57 (88)
4-52 times/year**	12 (43)	2 (3)
unknown	3 (11)	6 (9)
Season of sampling (%)		
Spring*	0 (0)	14 (21)
Summer*	18 (64)	20 (31)
Autumn	10 (36)	31 (48)
Household		
Number of dogs	1.5	1.9
Other pet present	7 (25)	19 (29)
House built		
Before 1920	3 (11)	7 (11)
1920 - 1975	10 (36)	24 (37)
After 1975	14 (50)	33 (51)
Date moving into present house	1996	1995
Type of home (%)		
Single-family home	23 (82)	56 (86)
Ground-floor flat	0 (0)	2 (3)
Upper-floor flat*	4 (14)	2 (3)
Farmhouse	0 (0)	5 (8)
Type of floor cover in living room		
Smooth floor	16 (57)	38 (58)
With rug >4m^2 or carpet	11 (39)	27 (42)
Age of carpet/rug (years)	9.2	8.1
Double-glazed windows LR (%)	27 (96)	62 (95)
Indoor temperature in winter (°C)	20	20

Table 1. Characteristics of the study population and home characteristics

* P-value < 0.05; ** P-value < 0.01

The number of samples with a detectable amount was higher for Der p1 than Der f1, and samples taken from the coat of the dog were more frequently below the level of detection compared to samples from the living room floor or from dogs' bedding. Almost all dust samples contained detectable amounts of endotoxin and (1→3)-β-D-glucans.

The endotoxin exposure levels in dust from the living room floor, bedding, and coat of the dog are shown in Fig. 1. The median endotoxin exposure was higher for controls than cases with largest difference found in coat samples. However, these differences were not significant. There were no significant differences in exposure levels of Der p1 or Der f1 and (1→3)-β-D-glucans between cases and controls (data not shown) in samples from all three sample locations. The concentration of Der p1 measured in all samples of house dust was significantly higher than Der f1 ($P < 0.01$), with 42 ng/g being the median concentration for Der p1, whereas the median concentration for Der f1 was below the level of detection (data not shown). The same distribution was found when exposure levels in the three different sample locations were analysed per square meter (data not shown). Exposure levels were stratified by type of floor (smooth floor, carpet/rug) and season, and analysis of these stratified data did not show significant differences (data not shown).

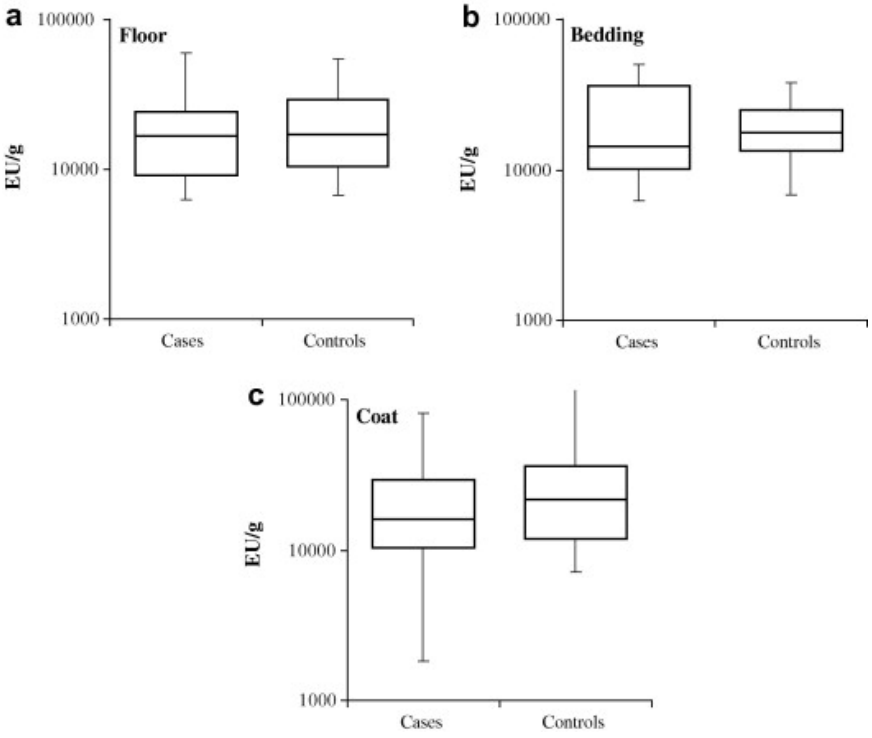

Figure 1. Endotoxin exposure levels

Exposure levels of endotoxin expressed as endotoxin units (EU) per gram of dust from the floor in the living room (a) and from the bedding (b) and coat of the dog (c). Boxplots showing median and interquartile range (box); the whiskers indicate 10th and 90th percentiles.

Owing to the high number of samples below the detection level, house dust mite exposure levels were divided in samples below and above the detection level. No significant relationship between house dust mite exposure in each of the three sample locations and cAD was found (Table 2), all confidence intervals contained 1 for both unadjusted (cOR) models and those adjusted for age and sex (aOR). In contrast to house dust mites, endotoxin exposure measured in the coat (Table 3) was found to be significantly and inversely associated with cAD (cOR, 0.38; 95% CI, 0.15-0.97; P < 0.05) indicating that high (above median) endotoxin levels are less likely to be found in the coats of dogs with cAD. After adjusting for age and sex the association between endotoxin exposure and cAD was not significant (aOR 0.45; 95% CI, 0.15-1.40; P = 0.17. Endotoxin exposure levels from the floor in the living room and bedding were not significantly associated with cAD. No relationship was found for (1→3)-β-D-glucan exposure in all three sample locations and cAD. A post hoc power analysis on the non-significant results showed that this could be due to low power (<50%) instead of a small effect of indoor exposure.

	Cases	Controls	cOR[a] (95% CI)	aOR[a] (95% CI)
Der p1 floor (ng/g)				
< LOD	15	29	0.70	0.57
10-32886	13	36	(0.29-1.70)	(0.19-1.67)
Der p1 bedding (ng/g)				
< LOD	15	30	0.74	0.59
10-25919	13	35	(0.31-1.81)	(0.20-1.76)
Der p1 coat (ng/g)				
< LOD	21	45	0.64	0.23
10-3362	6	20	(0.22-1.83)	(0.05-1.02)
Der f1 floor (ng/g)				
< LOD	20	47	1.04	1.93
22-1767	8	18	(0.39-2.79)	(0.56-6.59)
Der f1 bedding (ng/g)				
< LOD	21	52	1.09	0.44
22-4010	6	13	(0.37-3.24)	(0.10-1.85)
Der f1 coat (ng/g)				
< LOD	21	55	1.51	1.65
22-1301	6	10	(0.51-4.87)	(0.39-6.91)

Table 2. Exposure of Der p1 and Der f1 per gram of dust for cases and controls

[a] Crude OR (cOR) and adjusted OR (aOR) with 95% confidence intervals are shown. ORs are adjusted for age and sex. Exposure levels are dichotomized at the level of detection (LOD) to generate nominal data.

	Cases	Controls	cOR[a] (95% CI)	aOR[a] (95% CI)
Endotoxin floor (EU/g)				

773-17104	14	32	0.90	0.75
17237-625049	13	33	(0.37-2.21)	(0.24-2.28)
Endotoxin bedding (EU/g)				
773-17354	16	30	0.64	0.73
17433-148916	12	35	(0.26-1.57)	(0.25-2.13)
Endotoxin coat (EU/g)				
773-18345	18	28	0.38*	0.45
19205-1616531	9	37	(0.15-0.97)	(0.15-1.41)
Glucan floor (µg/g)				
51-1220	12	34	1.46	1.54
1222-10706	16	31	(0.60-3.57)	(0.53-4.51)
Glucan bedding (µg/g)				
51-1382	16	30	0.64	0.39
1385-5047	12	35	(0.26-1.57)	(0.13-1.19)
Glucan coat (µg/g)				
51-706	14	32	0.90	0.76
766-7960	13	33	(0.37-2.21)	(0.25-2.29)

Table 3. Exposure of endotoxin and (1→3)-β-D-glucan per gram of dust for cases and controls

[a] Crude OR (cOR) and adjusted OR (aOR) with 95% confidence intervals are shown. ORs are adjusted for age and sex. Exposure levels are dichotomized at the median to generate nominal data.

* P-value < 0.05.

Discussion

In this case-control study, we have shown that low endotoxin levels in the coats of dogs were associated with cAD. Our findings indicate that environmental exposure to endotoxins may be protective against the development of atopic dermatitis in dogs. This is the first study to address the association between endotoxin exposure and atopic diseases in dogs. No associations were found between cAD and exposure to house dust mite allergens or fungal glucans.

Our observations are supported by several human studies demonstrating that high exposure to endotoxins may have a protective effect against allergic diseases. Endotoxin levels found in dust from living rooms or mattresses were shown to be inversely related to the occurrence of atopic dermatitis, atopic asthma, or atopic sensitization in children[13, 14, 16-19, 25].

The suggested protective effect of endotoxins may be explained by their immunoregulatory capabilities. Exposure to Gram-negative bacteria, as a source of endotoxins, especially during early childhood will augment the stimulation of helper T cell type 1 (Th1). These Th1 cells will in turn inhibit the generation of helper T cell type 2 (Th2), which play an important role in the development of allergic diseases[26]. Besides the classical Th1/Th2 balance, regulatory T cells are also stimulated by endotoxin exposure and may suppress the allergic inflammatory reaction[27, 28].

Interbreed variations may be an important form of bias especially for determining environmental exposure levels in the coat. For this reason, we only included only Labrador retrievers in this study. Another potential confounding factor could be how often dogs are being washed. We have shown that dogs with AD were washed more frequent than healthy dogs. However, there were no associations between washing frequency and exposure levels of mites, endotoxins and fungal glycans, or with the amount of dust collected in the coat samples (data not shown). Only adult dogs were selected to reduce the proportion of false negative selection in the control group as allergy develops within the first years of a dog's life. One of the potential limitations of the case-control study design has been that only present exposure is determined and not exposure before sensitization. However, the majority of dogs in this study lived their entire life in the same houses, and it has been shown by others that indoor exposure levels are relatively stable[29]. Therefore the measurements in our study may be representative for exposure levels in early life.

We saw no clear correlation between samples taken from the floor of the living room and from the bedding and coats of the dogs (data not shown). Levels of endotoxin were significantly higher in the coat of a dog living in a house with a smooth floor in the living room, whereas no differences in endotoxin levels were found between smooth and carpeted floors. For carpeted floors, we found significantly higher levels of Der p1 and glucans compared to smooth floors, as has also been reported in other studies[30, 31], but no higher levels of these two compounds were found in the coats and bedding of dogs living in carpeted living rooms.

It has been assumed that the major route of allergen is through the epidermis [32], and therefore we would expect that coat samples give a good representation of exposure at the level of the epidermis. The lack of correlation between sample locations indicates that it is advisable for both human and veterinarian

epidemiological studies to obtain dust samples from different sample locations to get an overall picture of actual exposure. It also shows that efforts to reduce indoor mite allergen exposure by removing carpets in the living area do not result in lower allergen exposure levels in the coat of the dog.

Conclusion

Endotoxin exposure in the coats of dogs is inversely associated with cAD. This finding suggests that reduced endotoxin exposure may play a role in pathogenesis of atopic dermatitis in dogs.

Acknowledgements
The authors would like to thank all dog owners for collecting dust samples, including the Dutch Labrador Retriever Kennel Club for giving the opportunity to recruit participants for this study. We thank Marieke Oldenwening, Siegfried de Wind, Mirian Boeve, Gert Buurman and Jack Spithoven for their assistance with the analysis of dust samples. This study was funded by Royal Canin France.

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Chapter 7

General discussion

General discussion

The chapters of this thesis directly or indirectly focus on atopic dermatitis in dogs. A substantial part, chapters 2-4, concerns the canine CD1 family. CD1 molecules are nonpolymorphic glycoproteins which are able to present foreign and self-lipid antigens to T cells. One of the characteristics of canine and human epidermis is the presence of a dendritic network of CD1a-expressing Langerhans cells ready to capture invading pathogens. In skin of dogs with atopic dermatitis the CD1a expression is increased and can contribute to local T cell activation. In chapter 5 we describe the role of Th1 and Th2 cytokine modulation in keratinocytes. In this chapter we assess whether cytokines produced by activated T cells in atopic skin are able to impair barrier functions of the skin by down-regulating keratinocyte filaggrin expression, and if these cytokines influence pro-inflammatory cytokine and chemokine expression of keratinocytes. The skin is an important route of entry for allergens, bacterial and fungal components, especially when the barrier function is reduced. In chapter 6, we analyze the association between environmental exposure, such as house dust mite allergens, endotoxins and fungal components, and atopic dermatitis in dogs.

Canine CD1

In a paper of Olivry et al. 1996 [1] canine CD1-specific antibodies were used as markers of Langerhans cells and dermal dendritic cells. The number of CD1 positive cells in both lesional and non-lesional canine atopic skin, was found to be about twofold higher than in control skin. Their main conclusion was that hyperplasia of Langerhans cells and dermal dendritic cells occur in canine atopic dermatitis, suggesting an active role for these antigen presenting cells in this disease.

It was shown by Beckman et al. 1994 [2], that human CD1 molecules were not just markers on Langerhans cells or other dendritic cells, but in fact were antigen presenting molecules able to present different classes of lipid antigens to T cells.

CD1 genes are found in every mammalian species studied so far [3-10]. However, the number and type of CD1 genes varies considerably among these species due to gene duplication and genomic deletion during mammalian evolution [11]. Our basic aim was to identify CD1 genes present in the canine genome. In chapters 2 and 3 we characterized the canine CD1 genes. We observed that all canine CD1 genes are present on chromosome 38 in one locus marked by specific locus boundary genes. The locus contains eight CD1A genes (canCD1A), of which five are pseudogenes, one canCD1B, one canCD1C, one canCD1D, and one canCD1E gene. Of the three full length CD1A genes (CD1A2, CD1A6 and CD1A8), we found *in vivo* expression for CD1a6 and the two CD1a8 alleles (CD1a8.1 and CD1a8.2). A reason for the conservation of the three canine CD1A genes (CD1A2, CD1A6, CD1A8) may be that they have different functions. Our modeling data predicted that CD1a8 molecule had an open binding pocket and a larger groove opening in contrast to the CD1a6 molecule. This suggests that the different canine CD1a molecules are able to bind different glycolipid antigens, which in turn indicate a broader range of glycolipid antigen presentation in the skin.

The other interesting part about the canine CD1a molecules is their differences in cytoplasmic tail lengths. It will be interesting to find out whether the dissimilar

cytoplasmic tails will result in a various trafficking patterns and sampling of antigens. The CD1a isoform is divergent compared to the other CD1 isoforms, because it has a cytoplasmic tail which lacks a tyrosine-based motif (YXXZ)[12] or other trafficking motif. The human CD1a molecule is mainly present on the cell surface but can traffic to early endosomes [12, 13]. If we compare the cytoplasmic tail of human CD1a (and other primates) to that of other mammals, it is clear that most mammalian species have a long CD1a tail. Based on comparison of the genomic sequence containing the CD1a cytoplasmic tail exon between mammalian species, it is clear that splicing of this exon is different and may be the result of a splice site mutation. It is hard to predict if there are other trafficking motifs present in the non-primate CD1a cytoplasmic tail. Of interest is the finding that a tryptophan residue is conserved at the beginning of each non-primate CD1a cytoplasmic tail found so far. This residue was also found in the cytoplasmic tail of the three canine CD1a molecules. In a study of Jarousse et al. 2003 [14] it was shown that a single tryptophan mutation in the cytoplasmic tail of a Synaptotagmin 1 strongly reduced internalization. Constructing cells expressing the wild-type non-human CD1a cytoplasmic tails and constructs with a truncated tail would give insight if this tryptophan-based motif also plays a role in CD1a trafficking.

High expression of CD1a is a characteristic of human Langerhans cells. In dogs both CD1a8.1 and CD1a8.2 follow a similar expression pattern [1]. Our study showed for the first time a full length CD1a molecule (CD1a6) which is less expressed in skin and seems to suggest that CD1a2 is not expressed in skin. We did find CD1A2 gene expression in thymocytes. Obviously the evidence for *in vivo* expression of CD1a2 on thymocytes is required, but it would be interesting to find out why this is present on developing thymocytes. It has been shown that CD1 presentation on cortical thymocytes is critical for the positive selection of T lymphocytes [15]. Our study showed that to understand more of the immunological role of CD1a molecules it may be important to study this molecule in other mammalian species than humans, and the dog would be a good candidate.

Besides the “expansion” of CD1A genes, another remarkable finding of the canine CD1 locus was the presence of a gap in the canine genome sequence at the 5’ part of the canine CD1D gene. In chapter 3 we unravel the gap sequence and characterize the canine CD1D gene. We found three different short tandem repeats which disrupted the canine CD1D gene structure and seem to be responsible for deleting exon 2 and the 5’ part of exon 3. The finding of a full length canine CD1D transcript containing exon 1, 4, 5, 6 and 7 confirmed this genomic finding. The deletion of homologous exons and the lack of alternative exons seem to suggest that dogs do not express CD1d.

CD1d has shown to be a restricting element for the development and activation of invariant Natural Killer T cells in humans and mice [16, 17]. In a paper of Yasuda et al. 2009 [18] it was shown that the canine genome contained sequences which were homologous to the human invariant Vα and Jα gene and that the recombination of these two genes occurred. However, a full length transcript of the invariant T cell receptor was not obtained. The paper shows very low percentage of cells in PBMC (0,004%), hepatic mononuclear cells (0,045%) and splenic cells (0,028%) which stain positive for murine CD1d-tetramer loaded with αGalCer. The data show that there are cells which have transcription of the invariant T cell receptor. It is unclear whether the tetramer-positive cells are the cells that actually express an invariant T cell receptor. Furthermore, there is no data displayed with

an unloaded CD1d-tetramer and all data shown is generated from 1 dog. Combining the data from our canine CD1d study and the canine iNKT study gives plenty of room for speculation. It could be that there are no iNKT cells due to the lack of a functional canine CD1d molecule. It is also possible that other members of the canine CD1 family replace the loss of canine CD1d functionality.
In every other eutherian mammal studied so far at least one functional CD1D gene is found. In chapter 4 we describe the presence of functional CD1D genes and invariant alpha chain in several mammalian species. We showed that both pig and horse have a highly homologous NKT/CD1d system compared to humans. In this chapter we also suggest that several ruminant species seem to lack a functional CD1D gene due to a point mutation in the start codon, in a similar way as was earlier described in cows [10]. In a more recent study, it was found that a leader segment with an alternative start codon is present in a region which is homologous to human CD1D intron sequence and that bovine CD1d was expressed on the cell surface[19]. Therefore the dog seems to be the only species found so far, which seems to have a non-functional CD1D gene.
Of interest is that in the genome of three other species, which are like dogs members of Carnivora order (Giant panda , cat and ferret; www.ensembl.org) a homologous sequence is found for CD1D exon 1 and exon 3 (unpublished personal observation). In all three genomes there is a gap sequence in the area between these two exons similar as was the case for the canine genome. For both cat and ferret the gap seems to cover the 5' part of exon 3 just as we found in the dog. However, for the Giant panda there seems to be a full sequence of exon 3 without the short tandem repeats as found in dogs. It will be interesting to characterize the CD1 locus in these three species and unravel the gap sequence to determine whether functional CD1d molecules and invariant NKT cells are present in the Carnivora order.

CD1 and atopic dermatitis
Since in lesional atopic skin of both humans and dogs a high expression of CD1 molecules has been observed [20, 21], it is suggested that besides proteins (enzymes) also lipid components of allergens may play a role in the pathogenesis of atopic dermatitis. A recent study in humans showed that lipid components from cypress pollens were able to stimulate T cells [22]. Moreover, allergic patients displayed circulating specific IgE and positive skin test reactivity to pollen-derived phospholipids. Cells expressing CD1 molecules were shown to be required for presentation of pollen lipids to T cells.
Sensitization to allergens derived from house dust mite species *Dermatophagoides pteronyssinus* and *D. farinae* is reported for both humans [23] and dogs with atopic dermatitis [24, 25]. We have tried to determine whether lipid components *of D. pteronyssinus* and *D. farinae* were able to stimulate canine peripheral blood mononuclear cells (PBMC) and provoke immediate skin test reactivity in dogs with atopic dermatitis which were sensitive for *D. pteronyssinus* and/or *D. farinae*. We were able to extract phospholipids and free fatty acids from both house dust mite species, but we failed to show a distinct stimulation of PBMC with both a lipid extraction or a protein extract or skin test reactivity in four dogs (data not presented in this thesis). The ^{3}H thymidine proliferation assays used in our study is not very sensitive, other assays such as an ELISPOT assay may be more sufficient to

determine whether lipid allergens presented by CD1 molecules play a role in canine atopic dermatitis.
The role of CD1 as an antigen presenting molecule for foreign lipid allergens was only studied by Agea et al (2005). To the author's knowledge there are no other papers which describe this. In contrast, recent findings have shown that CD1 may play a role in atopic dermatitis by presenting autoantigens. It was shown that human CD1a-autoreactive polyclonal T cells homed to skin and responded to CD1a-expressing Langerhans cells by producing IL-22 [26]. In this study it was shown that CD1a-autoreactive T cells were abundantly present in the blood and skin of healthy donors and seem to be part of the normal T cell population. IL-22 induces keratinocyte proliferation and reduces genes which are involved in keratinocyte differentiation [27]. It was shown that IL-22 is able to induce hyperplasia in reconstituted human epidermis. In a dose depending manner IL-22 is also able to induce STAT-3 activation and upregulates pro-inflammatory molecules such as S100A7, S100A8, S100A9, and matrix metalloproteinase 3, platelet-derived growth factor A and the CXCL5 chemokine. CD1a-autoreactive T cells have been shown to produce cytokines such as IL-2, IL-13 and IFNγ [26]. Interestingly, it has been found that IL-22-producing CD4+ and CD8+ T-cell populations were significantly increased in AD skin compared to healthy skin [28], and that IL-22+CD8+ T-cell frequency correlated with AD disease severity [29].
The above findings suggest that increased expression of CD1a molecules on dendritic cells in skin and the presence of IL-22-producing CD1a-autoreactive T cells may play an important role in epidermal hyperplasia and skin inflammation in human atopic dermatitis. So far no studies have described the expression of IL-22 in canine atopic skin, and it is also not clear if canine IL-22-producing CD1a-autoreactive T cells exist. The increased expression of CD1a8.1 and CD1a8.2 in lesional skin of dogs with atopic dermatitis and the remarkable similarities in atopic dermatitis pathophysiology between humans and dogs [30], make it worthwhile to study this new T cell lineage in dogs.

Canine keratinocytes and atopic dermatitis
In humans it has been shown that T cells in atopic skin produce cytokines which reduce the barrier function and activate keratinocytes to excrete pro-inflammatory cytokines and chemokines [31-36]. In canine atopic skin increase in the expression of Th1 and Th2 cytokines has been described [37-39], however it is not clear what the effect of these T cell cytokines was on canine keratinocytes.
In chapter 5 we investigated differential expression of pro-inflammatory cytokines, chemokines and filaggrin, between cultured keratinocytes from dogs with atopic dermatitis and healthy control dogs. In order to understand the role of keratinocytes in canine atopic dermatitis we determined the spontaneous expression of filaggrin, pro-inflammatory cytokines and chemokines by keratinocytes and after stimulation with cytokines produced by Th1 or Th2 type cells.
The role of the skin barrier in the development of atopic dermatitis has gained more interest in the last decade. One of the major functions of keratinocytes is to form a functional skin barrier to prevent the entrance of pathogens and avoid loss of body fluids. Several studies have shown that the impaired skin barrier observed in atopic dermatitis seems to be a driver for the development of atopic dermatitis rather than the outcome of the local skin inflammation [40, 41]. One of the proteins which seem

to play a crucial role in skin barrier formation is filament aggregating protein (filaggrin). Several studies have showed that genetic mutations in the filaggrin gene lead to reduction in filaggrin protein expression, and are associated with atopic dermatitis [32, 42-46]. Other studies have found that cytokines produced by Th2 cells were able to down-regulate human filaggrin expression [31, 32, 47] contributing to the development of AD. Our study (chapter 5) did not show lower expression of filaggrin in keratinocytes from atopic dogs nor did stimulation with Th2 cytokines lower the filaggrin expression. In other studies contradicting results were found regarding the expression of filaggrin in skin of dogs with atopic dermatitis. In one canine study, filaggrin protein expression in the skin of atopic dogs was lower than in healthy control skin[30], which is similar to what was found in humans, but other studies reported higher filaggrin expression in atopic skin compared to control skin [48, 49]. The effect of filaggrin on canine skin barrier formation and the relation with atopic dermatitis clearly needs to be studied in more detail before it is possible to determine similarity with human filaggrin function.

Several studies have shown that keratinocytes seem to actively contribute to skin inflammation in atopic dermatitis by producing pro-inflammatory cytokines [50-52]. In addition, keratinocytes may also induce a Th2-cell influx, a major histological feature of AD skin during the acute phase, by producing chemokines such as thymus and activation regulated chemokine (TARC) [35, 36]. In chapter 5 we describe the expression of pro-inflammatory cytokines and chemokines, by canine keratinocytes from dogs with atopic dermatitis or control dogs. We showed that IL-8 expression was higher in non-lesional canine keratinocytes compared to control keratinocytes when stimulated with IFNγ or IL-4 and IL-13. IL-8 gene expression was significantly higher in lesional keratinocytes compared to control keratinocytes when stimulated with IFNγ. This seems to indicate that Th2 cells and Th1 cells in the epidermis are able to induce IL-8 secretion by keratinocytes, which may lead to the attraction of neutrophils and eosinophils in skin [53-56]. This is in line with one study in humans where increased levels of IL-8 are found in lesional atopic skin [57], whereas another study only found a tendency of increase of IL-8 in lesion atopic skin [58]. In addition, the level of IL-8 expression in non-lesional human skin was not increased compared to control skin [58].

Despite of the high levels of IL-8 expression by stimulated keratinocytes in our cultures, neutrophils are usually absent in canine atopic skin as in human atopic skin, even when there is a secondary infection [21, 59, 60]. It is possible that the keratinocytes in culture conditions react differently compared to keratinocytes in atopic skin. However, it is also possible that keratinocytes in atopic skin produce IL-8 but that it does not lead to an influx of neutrophils in the skin due to other neutrophil inhibiting factors. In contrast to neutrophils, eosinophils are found in the epidermis and dermis of lesional skin of atopic dogs [61] and our preliminary findings suggest that canine keratinocytes may play a role in attracting these cells.

In our cultures no difference was found between the spontaneous expression of filaggrin, pro-inflammatory cytokines and chemokines of keratinocytes from dogs with atopic dermatitis and healthy control dogs. It is possible that due to the culturing process the keratinocytes from atopic dogs lose their disease expression profile. However, in keratinocytes cultured from human atopic skin and healthy skin there was a clear difference in expression profile in unstimulated keratinocytes even after several passages of culture [50].

As described above, the expression profiles in our keratinocyte cultures are not always in line with the expression profiles in canine atopic skin biopsies reported in other studies [30-32, 35, 36, 42-47, 50-58]. We selected the cytokines from both Th1 and Th2 cells for stimulation of keratinocytes. The influx of Th2 cells and expression of IL-4 and IL-13 cytokines are characteristic during the acute phase of inflamed atopic dermatitis skin [20], whereas the chronic phase is dominated by Th1 cells producing IFNγ [20]. However, during skin inflammation, also other T cell subsets are present and can alter expression of filaggrin, pro-inflammatory cytokines and chemokines by keratinocytes. At the time of the experiments the human Th22/Tc22 subset was not as well defined as it is now[27, 29, 62-64], and its relation with atopic dermatitis was not clear. It would be interesting to stimulate canine keratinocytes with IL-22 to determine whether expression profiles are more in line with expression found in skin tissue.

The effect of house dust mite allergens, bacterial and fungal components on canine atopic dermatitis

The prevalence of atopic dermatitis in humans and other atopic diseases has increased in the last decades. Several studies have shown that the life-style of individuals and therefore the indoor/ outdoor environmental exposure was associated with atopic dermatitis [65] [66, 67].

Due to the lack of data it is unclear whether the prevalence of atopic dermatitis in dogs has also increased in the last decades, and whether environmental exposure plays a role. Two aspects make it interesting to study this in dogs: canine atopic dermatitis shows remarkable similarities in pathophysiology and dogs share a similar living environment and therefore a comparable environmental exposure. In chapter 6, we describe a case-control study of Labrador retrievers with and without atopic dermatitis. We determined the association between canine atopic dermatitis and the environmental exposure to house dust mite allergens, bacterial endotoxin and fungal glucans and the association of this exposure with atopic dermatitis. We could not detect an association between house dust mite allergen or fungal glucan exposure and atopic dermatitis. However, the endotoxin exposure level in the coat of dogs was significantly inversely associated with atopic dermatitis. This finding suggests a protective effect of high indoor endotoxin exposure towards the development atopic dermatitis in dogs. Our findings are in line with several human studies [68-71].

The mechanism behind such protective effect is unclear. There are several studies which have shown that endotoxin, also known as lipopolysaccharide which is a constituent of the cell wall of Gram-negative bacteria, activates the innate immune system by binding to Toll-like receptor-2 (TLR-2) and TLR-4 on granulocytes, dendritic cells and macrophages. These cells in turn produce pro-inflammatory cytokines such as IL-12 and TNFα leading to a shift towards Th1 cells and a decrease of Th2 cells, which play an important role in the development of allergic disease [72-74]. It was shown that the timing of the exposure is crucial, and that endotoxin exposure during pregnancy and early childhood, when the immune system is developing, let it shift towards a Th1 phenotype [75] [76]. The above findings seems to indicate that breeders of sensitive dog breeds such as Labrador Retriever, Golden Retriever, German Shepherd Dog and West Highland White Terrier may be able to reduce the number of puppies with atopic dermatitis by not

making the kennel too sterile and consequently enhancing endotoxin exposure during pregnancy and early life.

Concluding remarks

The development of atopic dermatitis is a complex mechanism in which four factors play an important role: immune system, skin barrier, environmental exposure and genetics. Each of these four components can be at the start of the development of atopic dermatitis in an individual dog and these components also influence each other (see Figure 1 of chapter 1). We revealed that the immune system of the dog contains three different CD1a molecules. Our work enables further investigation of the potential role of canine CD1a molecules in atopic dermatitis by activating skin resident T cells as seen in human atopic dermatitis.

Our findings also suggest a non-functional canine CD1d, which may have consequences for the development of NKT cells in dogs.

We could not detect an effect of Th1 and Th2 cytokines on filaggrin expression in canine keratinocytes, which implies that our *in vitro* model could not provide evidence for the inside-out theory in which immunologic disturbance initiates skin barrier dysfunction. We showed that IL-8 expression was higher in non-lesional canine keratinocytes compared to control keratinocytes when stimulated with IFNγ or IL-4 and IL-13. This seems to indicate that stimulated canine keratinocytes may contribute to inflammatory conditions in canine atopic skin.

Finally we observed an inverse association between endotoxin exposure and canine atopic dermatitis. This finding can be useful to for breeder management to prevent the development atopic dermatitis in newly born dogs.

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Nederlandse samenvatting

Atopische dermatitis

Atopische dermatitis is een inflammatoire, allergische huidziekte waarbij erfelijke aanleg een belangrijke rol lijkt te spelen. Kenmerkend voor een hond met atopische dermatitis is dat deze last heeft van chronische of terugkerende jeuk en dat de huidontsteking zich manifesteert op karakteristieke plaatsen zoals de snuit, oren, poten, oksels, buik en liezen. Bij veel honden met atopische dermatitis heeft de blootstelling aan omgevingsallergenen, zoals huisstofmijten, pollen van bomen en gras, het immuunsysteem geactiveerd en zijn er IgE-antilichamen aangemaakt tegen deze allergenen.

Atopische dermatitis is een van de meest voorkomende huidziekten bij honden, en geschat wordt dat ongeveer 10% van de hondenpopulatie de ziekte heeft. Opvallend is ook dat atopische dermatitis vaker voorkomt bij bepaalde rassen zoals de Labrador retriever, Golden retriever, Duitse herder en West Highland White Terriër, wat lijkt aan te geven dat ook genetische factoren een rol spelen. De ziekte ontwikkelt zich voornamelijk in jonge honden tussen de 1 en 3 jaar oud en zal in veel gevallen niet volledig te genezen zijn. De huidige therapie richt zich voornamelijk op het terugdringen van de jeuk en het beperken van de huidontsteking.

Er bestaan duidelijke parallellen tussen de pathofysiologie van atopische dermatitis bij de hond en mens. Ons huidige beeld van de ziekte is, dat deze ontstaat door een complex van interacties tussen het immuunsysteem, de huidbarrière, genetische factoren en omgevingsblootstelling aan allergenen en niet-allergische componenten (zie Figuur 1; Hoofdstuk 1). De inside-out theorie beschrijft dat een ontregeld immuunsysteem en huidontsteking de eerste trigger zijn voor het ontstaan van atopische dermatitis en dat de verstoring van de huidbarrière een gevolg is van de ontstekingsreactie, terwijl de outside-in theorie juist beweert dat een verminderde huidbarrière centraal staat bij de ontwikkeling van atopische dermatitis.

In dit proefschrift worden studies gepresenteerd waarin gekeken wordt naar bepaalde facetten van het immuunsysteem, de huidbariere en de omgevingsblootstelling. In hoofdstuk 2 en 3 karakteriseren we de CD1 genen en de lipide-antigeen presenterende CD1 eiwitten in de hond. De aanwezigheid van het CD1d/Natural Killer T cel (NKT) systeem in meerdere zoogdiersoorten waaronder de hond, wordt beschreven in hoofdstuk 4. Het effect van Th1 en Th2 cytokinen op de expressie van filaggrine, pro-inflammatoire cytokinen en chemokinen door gekweekte keratinocyten van honden met atopische dermatitis en controle honden wordt beschreven in hoofdstuk 5. In hoofdstuk 6 presenteren we een patiënt-controle onderzoek waarbij de associatie wordt bepaald tussen atopische dermatitis en de blootstelling in de omgeving aan huisstofmijtallergenen, bacteriële en schimmelcomponenten.

Karakteriseren van CD1 genen en moleculen in de hond

De CD1 familie bestaat uit een groep van vijf non-polymorfe lipide-antigeen presenterende moleculen (CD1a-e), die lichaamseigen en lichaamsvreemde (glyco-)lipide antigenen aan T lymfocyten presenteren.

Onze belangstelling voor CD1 in de hond werd geïnitieerd door een artikel van Olivry et al. 1996, waarin werd aangetoond dat er in de lesionale huid een significante toename was aan CD1 positieve dendritische cellen, zoals Langerhans cellen en dermale dendritische cellen.

Tot nu toe zijn er in het genoom van ieder bestudeerd zoogdiersoort (mens en andere primaten, muis, rat, cavia, varken, schaap en rund) CD1 genen gevonden. Echter, er is een grote variatie in het aantal en type CD1 genen tussen deze soorten waarschijnlijk als het gevolg van gen duplicatie en genoom deletie tijdens de evolutie van zoogdieren. Ons eerste doel was om de CD1 genen te karakteriseren in het genoom van de hond. Alle gevonden hond CD1 genen lagen op chromosoom 38 in een locus, die werd gemarkeerd door bekende CD1 locus specifieke grens genen. Het hond CD1 locus bevat acht CD1A genen, waarvan er vijf pseudogenen zijn, een CD1B gen, een CD1C gen, een CD1D gen en een CD1E gen. We hebben drie volledige CD1A genen gevonden (CD1A2, CD1A6 en CD1A8), waarvan we voor CD1A8 twee allelen vonden (CD1A8.1 en CD1A8.2). *In vivo* expressie is aangetoond voor CD1a6 en CD1a8.1 en CD1a8.2. Een mogelijke reden voor het nog aanwezig zijn van deze drie CD1A genen kan zijn dat er een verschil zit in functionaliteit tussen de moleculen. De voorspelde modelstructuur van CD1a8 liet zien dat van een van de twee allelen de antigeen-bindende pockets een open einde heeft, waardoor er ruimte is voor het binden van lipiden met een langere koolstofketen, terwijl deze pocket gesloten is in het CD1a6 molecuul. Daarnaast was de groeve van het CD1a8 molecuul meer open dan bij CD1a6, en dit kan mogelijk leiden tot een verschil in lipiden presentatie tussen de beide moleculen.
Daarnaast verschillen de cytoplasmatische staarten van de hond CD1a moleculen in aminozuurlengte. Het is opmerkelijk dat de korte cytoplasmatische staart van het humane CD1a zowel in lengte als aminozuursamenstelling afwijkend is ten opzichte van die van in andere zoogdieren. De cytoplasmatische staarten zijn betrokken bij het sturen van de interne trafficking pathway van de CD1 moleculen. Van het humane CD1a is bekend, dat deze zich voornamelijk op het celoppervlak bevindt en dat deze ook naar vroege endosomen kan transporteren waar het antigenen kan opnemen. Het is nog onduidelijk of niet humane CD1a moleculen zich op dezelfde manier gedragen. In alle niet-humane CD1a cytoplasmatische staarten is tryptofaan het eerste aminozuur en dit zou een intern trafficking motief kunnen zijn. Toekomstige studies bij de hond moeten aantonen welk trafficking motief deze CD1a moleculen hebben en of ze mogelijk ook in staat zijn om lipide antigenen te binden uit andere endosomen of lysosomen.
Bij de mens wordt hoge CD1a expressie gevonden in de huid op Langerhans cellen en dermale dendritische cellen; een vergelijkbaar expressiepatroon is gevonden voor CD1a8.1 en CD1a8.2 in hondenhuid. Onze studie toonde aan dat CD1a6 een lagere expressie heeft in de huid en dat CD1a2 niet in de huid tot expressie lijkt te komen, maar alleen op thymocyten. Dit suggereert dat deze moleculen functioneel anders zijn dan CD1a8.1 en CD1a8.2.
De bovenstaande bevindingen geven aan dat om de biologische functie van CD1a moleculen te begrijpen het noodzakelijk is om deze moleculen ook in andere soorten dan de mens te bestuderen; de hond zou een zeer geschikte kandidaat zijn voor dit soort onderzoek.
Naast de expansie van de CD1A genen, hebben we ook een afwijkende structuur van het CD1D gen gevonden. We waren in staat om het onbekende stuk in het CD1D gen (gap sequence) te sequencen. In deze sequentie bevonden zich drie soorten tandem repeats die waarschijnlijk hebben gezorgd voor de verstoring van de oorspronkelijke genstructuur. We konden geen sequentie vinden die homoloog was aan humaan CD1D exon 2 en het 5' gedeelte van exon 3. We waren wel in

staat om een volledig transcript te maken met exon 1, 4, 5, 6 en 7, dat deze genomische bevindingen onderbouwt. Het bovenstaande suggereert dat de hond geen CD1d tot expressie brengt. Het CD1d molecuul is essentieel voor de ontwikkeling en activatie van NKT cellen met een invariante T cel receptor (iNKT). Het ontbreken van het CD1d zou daarom ook consequenties kunnen hebben voor de ontwikkeling van iNKT cellen in de hond.
In hoofdstuk 4 laten we zien dat varken en paard functionele genen hebben voor CD1D en de invariante α-keten. Een model van een α-Galactosyl-ceramide bindend CD1d-iNKT complex van varken en paard voorspelt een grote overeenkomst in de structuur van dit complex bij de mens. Het ontbreken van een functioneel CD1D gen in de hond betekent dat dit mogelijk de enige soort is waarbij het CD1d-iNKT complex ontbreekt. Aangezien iNKT cellen in staat zijn om de immuun response te sturen door het snel produceren van grote hoeveelheden cytokinen, is het interessant om te onderzoeken hoe de hond zich heeft aangepast aan het niet functionele CD1D gen.

CD1 en atopische dermatitis in de hond
Na het in kaart brengen van de CD1 genen in de hond wilden we gaan kijken naar de functionaliteit van deze lipide-presenterende moleculen bij atopische dermatitis in de hond. Zoals eerder aangegeven is er een verhoogde expressie van CD1a8.1 en CD1a8.2 in de lesionale huid van een hond met atopische dermatitis ten opzichte van de huid van een gezonde hond. Atopische dermatitis wordt geassocieerd met allergenen zoals huisstofmijt, dus onze initiële onderzoeksvraag was of het mogelijk was om lipiden uit huisstofmijt te extraheren om vervolgens te kijken of deze lipiden instaat waren om perifere bloed mononucleaire cellen (PBMC) te stimuleren, en of het mogelijk was om een intradermale huidreactie te genereren om IgE antilichamen aan te tonen tegen lipide allergenen van huisstofmijt. De lipiden extracten van beide huisstofmijtsoorten *Dermatophagoides pteronyssinus* en *D. farinae* bevatte fosfolipiden en vrije vetzuren. Echter bij de PBMC stimulatieassay en de intradermale huidtest was er geen significante reactie aantoonbaar. De resultaten van deze pilot studie zijn niet toegevoegd aan het proefschrift.
Recent is bij de mens aangetoond dat de CD1a moleculen een belangrijke rol kunnen spelen bij atopische dermatitis door het stimuleren van specifieke subset van IL-22 producerende T lymfocyten met autoantigenen en dat het aantal IL-22 producerende T lymfocyten significant hoger was in atopische huid ten opzichte van gezonde huid. Het zou interessant zijn om te onderzoeken of een dergelijke parallel ook aanwezig is bij de hond.

Keratinocyten en atopische dermatitis in de hond
Keratinocyten spelen een belangrijke rol bij de formatie van de huidbarrière. Een van de kenmerken van de atopische huid is dat de barrièrefunctie verstoord is, waardoor vocht makkelijk via de huid verdampt en er allergenen en pathogenen de huid kunnen binnendringen. In sommige humane patiënten met atopische dermatitis lijkt het dat een defecte huidbarrière de oorzaak is voor het ontstaan van de ziekte, terwijl in andere patiënten het defect ontstaat als gevolg van een ontspoorde schadelijke immuunreactie in de huid.
In hoofdstuk 5 van dit proefschrift presenteren we een onderzoek met gekweekte keratinocyten van honden waarin we kijken naar het effect van Th1- en Th2

cytokinen (IFNγ en IL-4 /IL-13) op de expressie van filaggrine, een essentieel eiwit voor een normale barrière functie in de huid. Th1 en Th2 cytokinen hadden geen effect op de filaggrine expressie in keratinocyten en er was geen significant lagere filaggrine expressie in gekweekte keratinocyten van atopische honden ten opzichte van die van gezonde honden. Het is goed mogelijk dat de *in vitro* opstelling niet overeenkomt met keratinocyten in de huid. Er is tot op heden nog geen duidelijke bewijs voor de rol van filaggrine bij de ontwikkeling van atopische dermatitis bij honden.
De keratinocyten hebben naast hun barrièrefunctie ook een belangrijke rol bij de immuunreactie in de huid door het produceren van cytokinen en chemokinen. Wij hebben gekeken naar het effect
van Th1- en Th2 cytokinen op de expressie van pro-inflammatoire cytokinen en chemokinen. In de biopten van lesionale hondenhuid is een overexpressie gevonden van pro-inflammatoire cytokinen zoals TNFα. In onze studie vonden we geen toename maar juist een afname in de expressie van TNFα in keratinocyten gekweekt van lesionale huid ten opzichte van normale controle huid. Dit kan betekenen dat de keratinocyten geen bijdrage leveren aan de verhoogde TNFα in de atopische huid. Het is ook mogelijk dat een andere stimulans dan Th1 en Th2 cytokinen zorgen voor een verhoogde expressie van TNFα in keratinocyten.
We toonden wel aan dat de IL-8 expressie hoger was in non-lesionale en lesionale keratinocyten vergeleken met controle keratinocyten, als we deze stimuleerden met Th1 of Th2 cytokinen. Dit lijkt aan te geven dat Th1 en Th2 cellen in de epidermis van een hond met atopische dermatitis IL-8 secretie kunnen induceren door keratinocyten. Het is onbekend of IL-8 ook verhoogd is in de atopische huid van de hond. IL-8 is een chemokine die zorgt voor het aantrekken van neutrofielen en eosinofielen. Echter, een van de kenmerken van atopische huid is juist het ontbreken van neutrofielen, wel worden er eosinofielen gevonden in de huid.
De resultaten die we hebben gevonden in de gekweekte keratinocyten van honden met atopische dermatitis kwamen niet altijd overeen met wat er gevonden is in huidbiopten. Wel hebben we gevonden dat gestimuleerde keratinocyten in staat zijn om pro-inflammatoire cytokinen en chemokinen te induceren bij keratinocyten, waardoor de keratinocyt zeker kan bijdrage aan de immuunreactie in atopische huid. Het is mogelijk dat er andere componenten dan Th1 en Th2 cytokinen nodig zijn om de keratinocyten te activeren.

Het effect van de blootstelling aan huisstofmijt allergenen, bacteriële en schimmel componenten op atopische dermatitis in de hond
In de laatste dertig jaar is er in de Westerse populatie sprake van een toename in atopische ziekten zoals astma, allergische rhinitis (hooikoorts) en atopische dermatitis. Een mogelijke oorzaak zou kunnen liggen in het feit dat onze leefomgeving en manier van leven in de laatste decennia sterk zijn veranderd. Het is aangetoond in enkele epidemiologische studies dat kinderen die opgroeien op het platteland/boerderij een kleinere kans hebben op het ontwikkelen van atopische ziekten. Het is onduidelijk of deze toename in atopische dermatitis ook heeft plaatsgevonden in de hondenpopulatie. In hoofdstuk 6 beschrijven we een patiënt-controle onderzoek waarin we kijken naar de blootstelling van huisstofmijtallergenen, endotoxinen afkomstig van Gram-negatieve bacteriën en bèta-glucanen van schimmels. Wij hebben aan hondenbezitters met een gezonde hond of met een hond met atopische dermatitis gevraagd of ze monsters wilden

nemen van de vloer in de woonkamer, de mand van de hond en van de vacht van de hond. We hebben geen associatie kunnen vinden tussen atopische dermatitis en de blootstelling aan huisstofmijtallergenen of schimmelcomponenten. Echter, een omgekeerde associatie werd gevonden in de blootstelling aan endotoxinen gemeten in de vacht en het voorkomen van atopische dermatitis bij de hond. Dit suggereert dat de blootstelling aan endotoxinen mogelijk de hond beschermt tegen de ontwikkeling van de ziekte. Het is nog onduidelijk hoe de endotoxinen dit kunnen bewerkstelligen. Het is mogelijk dat endotoxinen het aangeboren immuunsysteem (granulocyten, dendritische cellen en macrofagen) activeert, waardoor het adaptieve immuunsysteem in de richting van Th1 cellen wordt gestuurd. Deze shift kan zorgen voor een afname van Th2 lymfocyten, en zo ook mogelijk de ontwikkeling van atopische dermatitis voorkomen. Deze bevindingen kunnen nuttig zijn voor fokkers, vooral van de gevoelige rassen, door de leefomgeving van de zwangere teefjes en de opgroeiende pups niet te steriel te maken en zo de kans op het ontwikkelen van atopische dermatitis bij deze jonge honden mogelijk te verkleinen.

Dankwoord

Allereerst wil ik graag mijn beide promotoren Ton en Victor en co-promotoren Ildiko en Femke bedanken. Ton, je hebt dit project mogelijk gemaakt, ik heb veel aan je te danken. Hartelijk dank voor je supervisie tijdens dit promotieonderzoek en het begeleiden van het schrijven van dit proefschrift. Daarnaast wil ik je bedanken voor de mogelijkheden die je me hebt gegeven tijdens mijn aio-tijd in Utrecht. Ik heb kennisgemaakt met onderwijs, de colleges geven en studenten begeleiden waren erg leerzaam en leuk om te doen. Ik heb tijdens dit onderzoek vaardigheden ontwikkeld die ik in mijn verdere carrière zal toepassen.
Victor, hartelijk dank voor je belangrijke inbreng tijdens mijn promotieonderzoek. Jouw enthousiasme en kennis over immunologie waren erg belangrijk. Ik wil je hartelijk danken voor jouw bijdrage en commentaar bij het schrijven van de hoofdstukken in dit proefschrift
Ildiko, het begon met een klein regeltje in mijn onderzoeksplan, kijken naar CD1 in de hond, het werd uiteindelijk het grootste deel van dit proefschrift. Dit is mede tot stand gekomen door jouw enorme gedrevenheid en wetenschappelijke kennis over CD1 genen en moleculen. Daarnaast heb ik je waardevolle commentaar en snelle response bij het reviewen van de geschreven stukken zeer gewaardeerd. Hartelijk dank daarvoor.
Femke, hartelijk dank voor je bijdrage aan mijn promotieonderzoek. Ik ben uiteindelijk niet de Treg-kant opgegaan met mijn onderzoek maar je hebt toch altijd je interesse getoond voor hetgeen wat ik aan het doen was. Ik wil je bedanken voor je feedback tijdens het schrijven van mijn proefschrift.

I would like to thank Vincent Biourge for making it possible to perform this PhD project. I really enjoyed working with you on the diet-trial. Furthermore, your interest in the rest of my project, the warm welcomes and nice atmosphere at Aimargues and Sommieres have left an indelible impression. I also would like to thank Margriet Bos, William van Stiphout and Adrian Watson for their contribution in the diet trial. I would like to thank the employees at Waltham Center for Pet Nutrition, and in particular Claire Baker and Julie Bailey for their enthusiasm and patience in learning me how to grow canine keratinocytes.

Ik wil iedereen van de afdeling Immunologie hartelijk bedanken, en in het bijzonder Willem van Eden. Wat fijn dat ik mijn onderzoek kon uitvoeren op deze afdeling. Ik denk met veel plezier terug aan de leuke labuitjes, kerstvieringen en andere momenten. Ook de grappen onder de mannen op het blauwe lab met Peter, Aad, Wiebren en anderen waren super, lekker de radio hard en pipeteren. Daarnaast de mede-aio's, post-docs, kamergenoten en studenten voor alle bijdragen en gezelligheid: Lotte, Teun, Sylvia, Eveline, Kim Anh, Annette, Marij, Chantal M. en K., Rachel,Bram Christine, Willemien, Dietmar, Alice, Lonneke, Ger, José, Jeroen S en H., Peter R. en van der H., Mariëlle, Roel, Hilde, Daphne, Ellen, Willem en vele anderen. In het bijzonder mijn PCR-buddy Natascha, wat hebben wij veel gePCRed samen. Vooral het lachen om de vele bandjes of de juist zwarte laantjes heeft erg geholpen, en dan nog maar weer een PCRtje er tegenaan gooien om het af te leren!

Bij de afdeling Gezelschapsdieren wil ik graag Annette, Yvette en Marinus bedanken voor hun bijdrage aan dit proefschrift, het afnemen van de huidbiopten, het uitvoeren van de bloedafnames en de huidtesten.
Ik wil Peter Leegwater bedanken voor zijn enthousiasme en hulp bij het zoeken naar het *CD1D* gen van de hond. Het was leuk om met je te discussiëren over moleculaire technieken die “onze” gap in het hondengenoom mogelijk zou kunnen blootleggen.

Voor het mogelijk maken van de endotoxine studie, wil ik bij het IRAS graag de volgende mensen bedanken: Bert Brunekreef, Marieke Oldenwening, Siegfried de Wind, Mirian Boeve, Gert Buurman and Jack Spithoven, en natuurlijk Hilde Hoekstra voor alle hulp bij het analyseren van de monsters. Ook wil ik de Nederlandse Labrador Vereniging bedanken voor de bijdrage aan deze studie en de hondeneigenaren voor nemen van de stofmonsters.

George Postuma van de afdeling Celbiologie UMC Utrecht, wat was het gaaf om met jou naar huidcellen te kijken door een elektronenmicroscoop. Ik kan me herinneren dat de WK kwartfinale Nederland-Brazilië in 2010 werd gespeeld terwijl we de cellen zaten te bekijken, jouw enthousiasme was aanstekelijk en zorgde er voor dat ik geen moment aan die wedstrijd dacht. Het was mooi geweest als we ook daadwerkelijk een CD1-gelabelde Langerhanscel van de hond hadden kunnen aantonen.

Ik wil collega’s, vrienden en familie bedanken voor jullie interesse in mijn project en jullie steun bij het afronden van mijn proefschrift. In het bijzonder wil ik mijn ouders bedanken. Mijn studententijd zit er dan echt eindelijk op, jullie hebben er voor gezorgd dat ik kon studeren wat ik wilde en daar heb ik zeer dankbaar gebruik van gemaakt.
Fransje, Niek, Koen en Chris, het leven is mooi, dank voor jullie liefde, op naar ons volgende avontuur!

Curriculum Vitae

Frank Looringh van Beeck werd geboren op 3 augustus 1978 te Alphen aan den Rijn. De middelbare school werd doorlopen aan het Christelijk Lyceum in Arnhem en deze werd in 1996 afgesloten met het havo-diploma. In september van dat jaar werd er begonnen met Hogerlaboratoriumonderwijs aan de HAN in Nijmegen. Na het behalen van het propedeuse, werd de studie voortgezet in 1997 met Biomedische Gezondheidswetenschappen aan de Radboud Universiteit van Nijmegen. Tijdens deze studie liep hij stage bij de afdeling Reumatologie van het Universitair Medisch Centrum St. Radboud in Nijmegen. Het onderzoek, onder begeleiding van dr. Peter van Lent, richtte zich op FcγR modulatie op macrofagen door cytokinen en de fysiologische response na stimulatie met immuuncomplexen. Zijn tweede stage werd uitgevoerd op de afdeling Fysiologie van Mens en Dier aan de Wageningen Universiteit en werd begeleid door dr. Katja Teerds. In dit onderzoek werd het effect van luteïniserend hormoon op Fas-geinduceerde apoptose bij ovariumkankercellen bestudeerd. Het masterdiploma werd behaald in 2004. In 2002 werd er een tweede masterstudie gestart, Resource Ecology, aan de Wageningen Universiteit. Onder begeleiding van dr. ir. Ignas Heitkonig, deed hij onderzoek naar het effect van bomen in de savanne op gras- en bodemnutriënten, en de gevolgen voor herbivoren in the Greater Makalali Conservancy in Hoedspruit, Zuid-Afrika. In 2005 werd het masterdiploma behaald. Hij startte in 2006 als assistent in opleiding bij de afdeling Immunologie van de Faculteit Diergeneeskunde aan de Universtiteit van Utrecht. Het promotieonderzoek leidde tot dit proefschrift. Vanaf 2011 is hij werkzaam als Clinical Research Associate bij PPD in Bennekom.

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