

Immune responses in dogs with cutaneous adverse food reactions

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Immune responses in dogs with cutaneous adverse food reactions

Immuunresponsen bij honden met ongewenste cutane reacties tegen voedsel

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Introduction

Adverse food reactions in dogs

Allergy is considered an inappropriate immune response to harmless environmental substances, named allergens. The term allergy is mainly used to describe type I hypersensitivity reactions that are due to activation of mast cells via crosslinking of membrane-bound allergen-specific immunoglobulin E (IgE) by allergens. It is unclear whether the adverse reactions to food antigens in dogs are the result of undesired immune responsiveness, such as type I hypersensitivity, or intolerance to food. Therefore, the generic term adverse food reactions (AFR) is used, rather than food allergy. Although the clinical signs of canine AFR may occur at several locations including the gastrointestinal tract and the respiratory system, they are usually restricted to the skin, hence referred to as cutaneous adverse food reactions (CAFR). The distribution and clinical manifestations are often indistinguishable from those in atopic dermatitis (AD). The prevalence of AFR in the dog population is up to 8%^{1,2} and approximately 7-25 % of all allergic skin disorders are CAFR^{2,3}. Only 10-15 percent of dogs with skin symptoms show concurrent intestinal symptoms, such as vomiting and diarrhea⁴. There is no breed or sex predisposition for CAFR. In 33-48% of cases the age of onset of CAFR is below 1 year, in 51-85% 1-3 years and in 16% 4-11 years of age^{3,5}.

Food consists of proteins, carbohydrates, lipids and water-soluble glycoproteins of which the latter are the most important allergens. Most allergens are resistant to heat, acid and proteases and some even become allergenic after cooking or digestion⁶. Constituents that are not resistant to digestion may, when eaten in large quantities, act as allergens after intact passage through the stomach⁶. Beef, lamb, chicken, wheat, soybean, milk, eggs and corn have been described as causative for AFR^{4,7-9}, however the exact allergenic molecules of these foods, except for cow's milk and beef, have not been identified to date. Martin et al (2004) have shown that in dogs with cow's milk and beef-related AFR, bovine IgG and in beef also phosphoglucomutase are major allergens¹⁰. However, this analysis was based on allergen-specific IgE levels, of which the role in (C)AFR is not yet proven. Currently, CAFR is diagnosed by dietary elimination and provocation tests, using the complete original food for provocation after initial feeding of an elimination diet.

T cell introduction

Food is digested in the gastrointestinal tract and degraded to oligopeptides, which are predominantly adsorbed via M cells in the small intestine and thereafter

internalized by antigen presenting cells (APC). The APC, such as dendritic cells (DC), macrophages and B cells, present antigen via major histocompatibility complex class II (MHC-II) molecules on their surfaces, in conjunction with B7.1 or B7.2 as costimulatory molecules¹¹. The T cell receptor (TCR) is responsible for the specific recognition of the antigen-MHC-complex and CD28 molecules expressed on the T cell bind to the B7 molecule. Interactions between TCR, MHC-antigen complex, CD4 or CD8 and that between B7 and CD28, result in activation of T cells and differentiation to effector T cells¹¹. These effector T cells circulate throughout the body and receptors on their surface enable them to migrate to specific locations directed by chemokines and integrines. CCL17 (also named Thymus and Activation-Regulated Chemokine: TARC) is a chemokine expressed in the skin by dermal cells, and its expression is increased in human AD¹². T cells expressing the CCL17 receptor CCR4 (chemokine [C-C motif] receptor 4) home to the skin^{12,13}. Both CCR4 and CCL17 are also increased in dogs with AD^{14,15}. In addition, the intestinal-derived dendritic cells and mesenteric lymph node stromal cells can induce $\alpha 4\beta 7$ integrin expression on T cells. This integrin binds to MAdCAM-1, an adhesion molecule expressed specifically on intestinal endothelial cells, causing migration of activated T cells into intestinal tissue^{16,17}. In this way the intestine-derived dendritic cells which internalized the antigen, are responsible for return of the antigen-specific T cells to the intestine.

Classically, there are 2 main types of T lymphocytes: CD8⁺ T cells (cytotoxic T cells) and CD4⁺ T cells (T helper cells). CD8⁺ T cells are mainly involved in defense against intracellular pathogens, such as viruses and function by killing the infected cell. The CD4⁺ T cell population, existing of several subtypes, serves to help other immune cells, hence they are called T helper (Th) cells. Th2 as well as Th1 cells are involved in the activation of B-cells and Th1 cells may additionally activate phagocytes to eradicate intracellular pathogens, such as bacteria and fungi. Regulatory T cells (a CD4 subtype named Treg) have an immunomodulatory or suppressive function, whereas Th17 cells are involved in the elimination of extracellular and fungal infections and exert (pro-)inflammatory functions^{18,19}. Important effector molecules of T cells are cytokines, small soluble proteins that influence the functional properties of many cells, including T cells. Typical Th1 cytokines are IFN- γ , IL-18 and TNF- α , Th2 cytokines are IL-4, IL-5 and IL-13, Th17 cytokines are IL-17A, IL-17F and IL-22, and Treg cytokines are TGF- β and IL-10^{20,21}. Regulatory T cells can be further categorized into three types: CD4⁺CD25^{high} Tregs (appearing to suppress T cells in a cytokine-independent manner by cell-cell contact), Th3 cells (immunomodulate and suppress

through TGF- β) and Tr1 cells (immunomodulate and suppress through IL-10)²¹. The selection of the immunoglobulin (Ig) (sub)isotype produced by B cells depends on the T helper subtype and the cytokines they produce. Thus IL-4 stimulates the production of IgG1, IgG3, IgG4 and IgE²²⁻²⁵ and IL-13 stimulates the production of IgG4 and IgE^{24,26,27}. IL-6 stimulates the production of all IgG types²⁸, whereas IL-10 stimulates the production of IgG1, IgG2, IgG3, IgG4 and IgA and inhibits the production of IgE²⁹⁻³². In addition, TGF- β induces IgA³³ production and inhibits that of IgG4 and IgE³⁴, IL-21 stimulates the production of IgG1 and IgG3 in human B cells^{25,35} and IFN- γ can inhibit the overall production of immunoglobulins³⁶⁻³⁸.

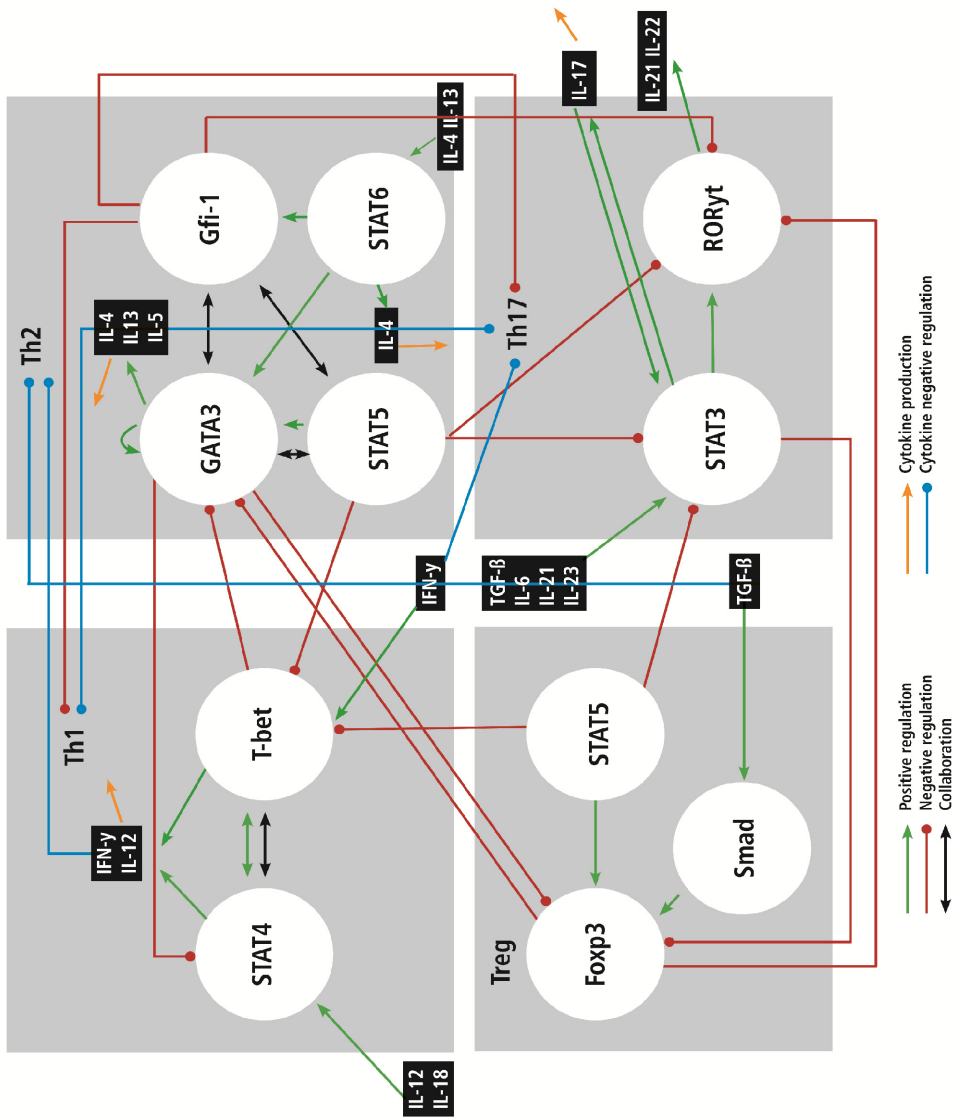
Involvement of various types of CD4⁺ T cells

The differentiation of a naive CD4⁺ T cell to either the Th1, Th2, Th17, or Treg subtype is not fully understood. It is believed to depend on the cytokines present in the environment of the cell during activation, the co-stimulator molecules used to drive the response, antigen dose³⁹, type and differentiation status of APC⁴⁰, and the nature of the antigen-MHC-II molecule and binding strength to TCR^{41,42}. Interactions with T cells lead to the activation of transcription factors within the cell that will bind to promotor sites for cytokine genes specific for a Th subtype. Figure 1 is a simplified schematic overview of the key transcription factors involved in CD4⁺ T cell differentiation.

T helper 1 cell

T helper 1 differentiation can be induced by the cytokines IFN- γ , IL-12, IL-18, IL-23 and IL-27. IL-12, especially in combination with IL-18, acts on T cells to trigger IFN- γ expression. It activates the transcription factor STAT4 which induces and stimulates the IFN- γ promotor⁴³. Next, STAT4 and IFN- γ can regulate the expression of T-bet in T cells, which regulates the IFN- γ and IL-12R β 2-chain gene expression (Figure1). The exact pathway of IFN- γ regulation is however not yet known^{21,44-48}. The T cell differentiation into Th2 cells is inhibited by the Th1-type cytokine expression (IFN- γ and IL-12) and T-bet inhibits the Th2 promoting GATA3 expression^{21,49,50}. After differentiation into Th1 the cell starts to produce IFN- γ and IL-12 (Figure 1).

Figure 1: Simplified schematic overview of key transcription factors and their influences on T helper cell differentiation and function.



T helper 2 cell

T helper 2 cell differentiation can be induced by the Th2 specific cytokines IL-4 and IL-13 (Figure 1). These cytokines trigger the phosphorylation of the STAT6 transcription factor, which triggers the expression of IL-4 and GATA3 genes^{21,51,52}. GATA3 activates the IL-4 promotor, regulates IL-13 and IL-5 expression and can autoregulate its own expression (independent of STAT6)^{21,5,53-55}. Moreover, the T cell differentiation into Th1 cells is inhibited by the Th2-type cytokine expression and GATA3 inhibits the expression of STAT4, a transcription factor involved in Th1 cell differentiation^{49,56}. STAT5 is a transcription factor involved in differentiation into all T helper cell types. Its enhanced expression is needed for differentiation into Th2 or Treg cells, in contrast this enhanced expression inhibits the differentiation into Th1 or Th17 cells²¹. Gfi-1 is a transcription factor involved in T cell development of most CD4⁺ T cell subtypes. It stimulates GATA3^{hi} cells to cell growth and differentiation, suggesting it is selective for Th2 cell differentiation. Moreover it seems to suppress the Th17 and Th1 development²¹. As a consequence of GATA3 activation the cell develops into a Th2 cell (Figure 1).

T helper 17 cell

The exact activation pathway of the recently described Th17 cells is not yet known. In mice, TGF- β , IL-6, IL-21 and IL-23 can induce naive T cells to become Th17 via activation of the STAT3 transcription factor, which triggers ROR γ t^{20,21,57-59}, a transcription factor in mice of which the function is not completely known. In addition, the activation of STAT3 via IL-6 suppresses Foxp3 expression, suggesting that IL-6 may inhibit differentiation into regulatory T cells and stimulates the differentiation into a Th17 cell (Figure 1)^{21,59}. Moreover, both IL-4 and IFN- γ can inhibit the activation of Th17 cells, suggesting that both Th1 and Th2 cells can inhibit Th17 differentiation²¹. In humans, Th17 memory cells have mainly been investigated. The cytokines IL-1 β , IL-23 and IL-6 promote production of IL-17. In contrast to mice, in humans TGF- β inhibited IL-17 when added alone or in combination with IL-1 β , IL-23 or IL-6 (not shown in Figure 1)⁶⁰. Upon differentiation Th17 cells produce IL-17, IL-21 and IL-22 (Figure 1).

Regulatory T cell

The CD4⁺CD25^{high} Treg cells are a natural occurring population which expresses the specific transcription factor Foxp3, the key transcription factor for these cells. It was

long believed that Treg cells could not be induced, however in mice it was shown that induction did occur by TGF- β through activation of Foxp3 via Smad⁶¹. STAT5 activation is also critical for Treg development (Figure 1). Although not proven, it is suggested that STAT5 binds the Foxp3 promoter, thereby influencing Treg cell development²¹. Moreover, Foxp3 can interact with ROR γ t (transcription factor involved in Th17 cells) and herewith suppresses the differentiation into Th17 cells (Figure 1). TGF- β both induces Th17 and Treg in mice, but Treg needs high concentrations of TGF- β to be induced²¹. Suppression of T cells by Tregs is cell-cell contact dependent, but the exact pathway is not known. It possibly depends on IL-2 inhibition, expression of the inhibitory co-stimulatory molecule CTLA-4 or suppression via membrane-bound TGF- β ^{62,63}.

Tr1 and Th3 cells have also been described, however it is unknown whether these cells are specific CD4 subtypes lineage or belong to other Th cell subsets (Th1, Th2, Th17, Treg). Tr1 cells are characterized by secretion of high levels of IL-10, but IL-10 can also be produced by other T cells such as Th1, Th2 and Th17 cells⁶⁴. In addition, oral tolerance induces TGF- β -producing cells, which have been characterized as Th3 cells⁶⁵. However recently, it was observed that approximately 40% of these cells also express Foxp3, thus possibly part of the so-named Th3 cells may be induced Tregs^{66,67}.

Plasticity of T cell differentiation

For years it was believed that the differentiation of the CD4⁺ T cell into a Th1, Th2, Th17 or Treg cell was an irreversible step. Recently, however, it has been shown that differentiated cells can change into other types of T helper cells depending on the cytokine micro-environment²¹. For example, Tregs can produce IL-17 when they are exposed to IL-6, and show an upregulation of ROR γ t^{68,69}. Another example is that Th2 cells can be induced by IL-12 to produce IFN- γ ²¹. This indicates that differentiated CD4⁺ T cells can still be converted into other T helper types through the influence of its micro-environment.

Allergic immune reactions

CD4⁺ T cells and their cytokines play an important role in initiation of allergic reactions. A prominent characteristic of allergy is the production of allergen-specific IgE. IL-4 or IL-13 are produced by Th2 cells and induce class switching in B cells, giving rise to IgE production. The secreted IgE binds to specialized high affinity receptors (Fc ϵ RI) on mast cells, basophils, Langerhans cells and dendritic cells from which it

dissociates only very slowly⁷⁰. When an allergen crosslinks the IgE on the mast cells and basophils, these cells respond by an immediate release of their prestored mediators, which are responsible for the allergic symptoms, that occur rapidly. This process is named Type I hypersensitivity. The substances released from mast cells ensure that inflammatory cells including activated T cells, in course of time enter the tissue and respond to the allergen or the pro-inflammatory environment, hence so-called early and late-phase reactions may be observed.

In humans, food allergy has been associated with Th2 responses, possibly caused by a Th2-like micro-environment resulting in an impaired Th1 response and/or impaired Treg response after allergen challenge. Stimulation of PBMC with cow's milk results in a Tr1 or Th1 response in healthy and non-cow's milk allergic persons and a Th2 response in cow's milk allergic persons⁷¹⁻⁷⁴. Moreover, when PBMC of peanut allergic children are compared with those of children who became tolerant to peanut over time, the tolerant children show a Th1 profile after allergen stimulation rather than a Th2 reaction⁷⁵. In addition, cow's milk allergic children who became tolerant, showed a high level of IL-10⁷⁶, which may have been produced by Tr1 cells or macrophages. In humans, a diagnosis of atopy is supported by the presence of increased levels of total IgE and allergen-specific IgE in blood. In human food allergy, IgE serology needs to be combined with food challenges to confirm allergy and to find the causative allergen, as allergen-specific IgE levels alone are poor predictors for clinical reactions. Atopic dermatitis in dogs is commonly associated with allergen-specific IgE. In contrast, in canine CAFR there is no evidence that it is associated with allergen-specific IgE levels, thus canine CAFR is diagnosed via dietary tests only⁷⁷⁻⁷⁸. Although in some experimental canine models of orally-induced gastrointestinal hypersensitivity high serum concentrations of allergen-specific IgE were found⁷⁹, there is currently no evidence that spontaneous CAFR is caused by type 1 hypersensitivity reactions. Local and systemic immune responsiveness in CAFR are largely unexplored, hence its causative mechanisms remain to be elucidated.

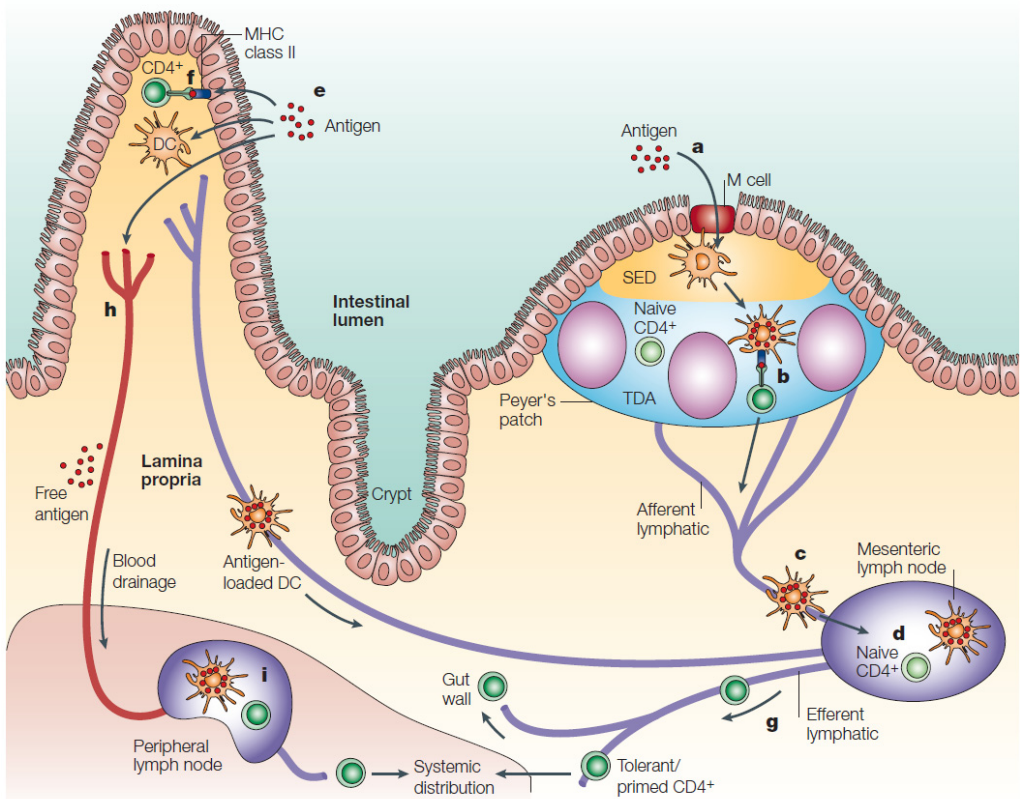
Intolerance reactions

Food allergy is described as an immunological reaction to food proteins and food intolerance is described as an adverse reaction to the chemicals in foods, resulting in e.g. gastrointestinal and skin symptoms. In children food intolerance reactions are more common than true allergic reactions and the signs and symptoms are similar to allergic reactions⁸⁰. One of the differences is that for food allergy often small

amounts of allergen are needed to trigger a fast allergic reaction (within hours), while intolerance reactions are dose-related and cause a slower reaction (within 48 hours). However, there are also exceptions and allergic reactions can occur slower and intolerance reactions faster than 48 hours. Food intolerance reactions may be caused by the absence of specific enzymes needed to digest a food substance, or an abnormality in the absorption of specific nutrients, or by naturally occurring chemicals in food⁸¹. Food intolerance is diagnosed in general by excluding immunological reactions to the food and performing elimination diets and dietary challenges⁸². The presence of immunological reactions to food proteins is not yet proven in dogs with CAFR, thus part of these dogs may actually suffer from intolerance reactions instead of allergic reactions.

Intestine

The mucosal immune system encounters large quantities of antigens daily and generally suppresses immune reactivity to harmless foreign antigens, such as food proteins and commensal bacteria. On the other hand, it may adequately react to pathogens. The induced non-reactivity of immune cells to foreign antigens is called oral tolerance. Antigens may pass the mucosal barrier in four different ways (Figure 2). First, mainly particulate and some soluble antigens are actively transported by microfold cells (M-cells) into the dome region of Peyer's patches. This region contains DC that internalize, process and present the antigen to lymphocytes in the underlying lymphoid structure that contains B cell follicles surrounded by T cells. Second, DC in the lamina propria may sample antigens from the gut lumen by dendrites extruding between the epithelial cells⁸³. Third, epithelial cells may internalize soluble antigens by fluid-phased endocytosis, antigens are transported in small vesicles and are digested when they combine with lysosomes⁸⁴. Fourth, CD23 (low affinity IgE receptor) is expressed on small intestinal epithelial cells in normal and food allergic humans. CD23 can transport allergen into the intestinal tissue with the help of IgE and CD23/IgE-transported allergens seem to be protected from lysosomal degradation. Thus allergens keep their original allergenic properties⁸⁵⁻⁸⁷. Recently, also the high affinity IgE receptor FcεRI has been found on human intestinal cells, however the clinical implication of this finding needs to be investigated⁸⁸. The cellular constitution of the healthy canine intestine is similar to that of human⁸⁹⁻⁹⁴. The epithelium predominantly contains CD8⁺ T cells and the intraepithelial lymphocytes mainly express the αβ T-cell receptors (TCR) and for one-third γδ TCR.



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Figure 2: Overview of the intestinal villus and Peyer's patch and possible antigen pathways. Antigen may enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (a), and after transfer to local dendritic cells (DC), may then be presented directly to T cells in the Peyer's patch (b). Alternatively, antigen or antigen-loaded DC from the Peyer's patch may gain access to draining lymph (c), with subsequent T-cell recognition in the mesenteric lymph nodes (MLN) (d). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLN may occur if antigen enters through the epithelium covering the villus lamina propria (e), but in this case, there is the further possibility that MHC class II⁺ enterocytes may act as local APC (f). In all cases, the antigen-responsive CD4⁺ T cells acquire expression of the $\alpha_4\beta_7$ integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (g) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen may also gain direct access to the bloodstream from the gut (h) and interact with T cells in peripheral lymphoid tissues (i). Reproduced with permission from Nature Publishing Group⁹⁵.

The lamina propria contains more CD4⁺ T cells than CD8⁺ T cells and expresses mostly $\alpha\beta$ TCR^{89-92,94}. The cytokine environment of human healthy intestine differs with the canine intestine. The human healthy intestine has an immunosuppressive profile with a predominance of TGF- β , IL-10 and IL-4^{96,97}, in which IL-4 seems to play an immunosuppressive role and not a Th2-inducer role⁹⁸. The intestinal cytokine profile of healthy dogs has a mixed Th1 (IFN- γ , IL-18) and tolerant profile (TGF- β , IL-10)⁹⁹.

The intestinal homeostasis is largely maintained by the APC and epithelial cells, although the underlying mechanisms are not yet elucidated. Dendritic cells in the lamina propria of the intestine may secrete IL-10 and IL-4, thus contribute to suppression^{84,100}. Moreover, intestinal epithelial cells may present luminal antigens in the context of MHC-II molecules on their surface, but they lack the costimulatory molecules B7.1 and B7.2 rendering them tolerogenic rather than activating^{101,102}. Food allergy can result from a breach in oral tolerance and by exposure of food allergens via other tissues and organs. This has been shown for the respiratory system in humans^{103,104} and for the skin in murine models¹⁰⁵. Moreover, in atopic dermatitis it has been shown that allergens may pass the skin due to skin barrier dysfunction^{78,106}, a possibility that may occur in AFR as well. If so, this may indicate that the intestine may not be the initial exposure site for food allergens. The intestine of CAFR dogs has not been investigated yet, whereas the human food allergic intestine is only sparsely examined. In cow's milk allergic humans with intestinal signs, the intestinal lymphocytes showed a Th2 cytokine profile¹⁰⁷. In addition, decreased TGF- β 1 expression was seen in duodenal epithelial and lamina propria lymphocytes in children with multiple food allergies¹⁰⁸, suggesting a switch from a Th1 to a Th2 environment and/or a failure in oral tolerance with allergy as the outcome.

Skin

Currently, it is unknown how exposure to food can lead to adverse reactions in the skin. There are three hypothetical pathways, one is that free allergen is transported from the intestine into skin or peripheral lymph nodes. The second hypothetical pathway is that allergens within APC or exosomes are transported from the intestine to other tissues^{109,110}. The third option is allergen exposure via the skin^{111,112}. The skin (Figure 3), consisting of epidermis, dermis and subcutis, protects against pathogens, toxin penetration and other damage. Although the skin is protective against pathogen penetration and is water-resistant, the epidermal

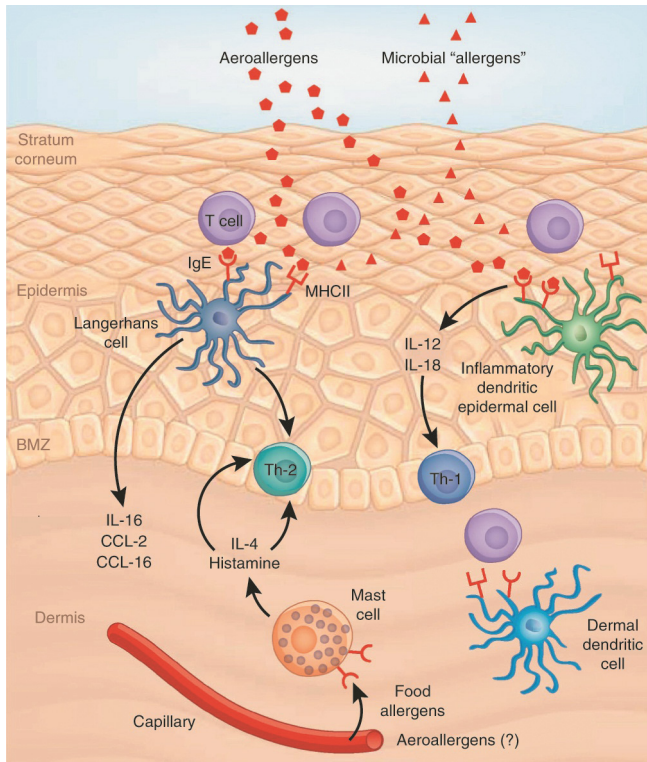


Figure 3: Schematic figure showing the effects of allergens on the cutaneous inflammation in atopic dermatitis. This schematic figure contains more T cells and Langerhans Cells than normally are present in skin for explanatory reasons. Allergens can enter the skin both through the bloodstream (for example, food allergens) or through the stratum corneum (for example, environmental allergens). In the skin, IgE bound on Fc receptors on cutaneous mast cells and on dendritic cells contributes, together with specific T cells, to the inflammatory response. Reproduced by permission from Macmillan Publishers Ltd¹¹³.

permeability in human and canine AD is increased^{78,106}, enabling allergens to enter the skin. This increased permeability process is due to skin barrier dysfunction. Filaggrins are part of the keratin cytoskeleton and together with intracellular lipids and epidermal proteases, influence the skin barrier. Mutations in genes encoding filaggrin have been found in a part of the atopic dermatitis patients, potentially leading to the increased permeability of the skin¹¹⁴⁻¹¹⁶. In addition, the cytokines IL-4 and IL-13 are able to inhibit filaggrin expression¹¹⁷, thus Th2 responses in the skin may also alter the filaggrin-mediated skin barrier and further increase exposure to allergens. Abnormal filaggrin protein expression and decreased ceramide levels are also observed in canine atopic dermatitis and loss-of-function mutations in filaggrin are suggested¹¹⁸. CAFR dogs show cutaneous signs in appearance and distribution often similar as

atopic dermatitis. If food allergens can penetrate the skin, as a result of a decreased skin barrier function, this could explain the presence of cutaneous signs and the lack of intestinal symptoms in the majority of dogs with adverse food reactions.

When allergens penetrate the skin, they can be internalized by APC present in the skin. Langerhans cells (LC) and inflammatory dendritic epidermal cells are two distinct APC in the skin present in lesional and in a lesser extent non-lesional skin. In allergic humans and dogs, both types of APC have the high affinity IgE receptor (FcεRI) on their surface. Allergens penetrating the skin can be efficiently taken up by the FcεRI-bound IgE molecules on the LC^{119,120}, facilitating allergen processing in the skin. APC containing allergen from the skin or intestine can travel via blood and lymph to peripheral lymph nodes. In the lymph nodes naive T and B cells can recognize the allergen and upon recognition interact with the APC; subsequently the B and T cells become activated. It is not completely elucidated yet how T cells are triggered to express specific tissue homing receptors, but the origin of the APC or the location of lymph nodes may play a role¹²¹.

CCR4 and CCR10 are chemokine receptors expressed on the human activated skin homing T cells¹²². Their ligands CCL17 (TARC) and CCL21 (MDC) are chemokines expressed by cutaneous dendritic cells and bind to CCR4¹²³, while CCL27 (CTACK) and CCL28 are expressed by keratinocytes and bind CCR10^{124,125}. With the help of CCR4 T cells can enter the skin at the site of the microvasculature of the dermis and migrate further in the epidermis with the support of CCR10¹²²⁻¹²⁶. Memory T cells may express a skin-specific homing molecule (Cutaneous Lymphocyte Antigen: CLA), leading the cells to the skin^{124,126}. In canine skin CCL28 and CCL17 (TARC) are expressed, and CCR4 is found on canine Th2 cells. The expression of these chemokines and ligands are increased in lesional skin of canine atopic dermatitis^{14,15,127,128}. The presence and role of CCL17, CCL28 and CCR4 in canine CAFR is not investigated yet. The acute lesional skin in AD in humans shows initially a more Th2-skewed reactivity and the skin of chronic AD patients shows a more Th1-skewed reactivity¹²⁹⁻¹³¹. In addition, human lesional skin contains more eosinophils and macrophages than non-lesional skin and their products stimulate or prolong the inflammatory reaction¹³². The inflammatory cell profile in skin of canine AD is similar to that of human AD. Skin inflammation is characterized by an influx of CD4⁺ and CD8⁺ T cells in the lesional skin^{133,134} and a mixed cytokine profile with predominant expression of IL-6, IL-4 and IL-13 in the early stage followed by IFN-γ, IL-12 and IL-18 later on¹³⁵⁻¹³⁷. The T cell subsets and cytokine profiles in the skin of canine CAFR have not been investigated. However, the clinical

symptoms and predilection sites are in general similar to those of AD, suggesting that the T cell profile in CAFR may also be comparable to that in AD.

Aims and Outlines of this thesis

Adverse food reactions (AFR) in dogs are reactions to apparently harmless food antigens, with an unknown etiology i.e. immunopathogenesis. In the majority of dogs with AFR, the signs are only associated with the skin (CAFR). Despite the entry of food allergens via the intestinal tract, they do not generate clinical symptoms at that location. Therefore we investigated the immune responses in the duodenum, the skin as well as PBMC of CAFR dogs with and without clinical symptoms to determine the profile and immunoreactivity in these three tissue types separately and in conjunction.

The genes that can be used as reference genes, showing stable expression in skin tissue for real-time quantitative PCR were investigated in chapter 2. The Th1, Th2 and Treg cell responses characterized by cytokine and transcription factor gene expression in the duodenum after a dietary challenge (provocation and elimination tests) are described in chapter 3. The Th1, Th2 and Treg cell responses as assessed by cytokine and transcription factor expression patterns, in lesional and non-lesional skin after provocation and elimination diets, are described in chapter 4.

The gene expression in PBMC after a provocation dietary test and the allergen-specific proliferation responses of PBMC are investigated in chapter 5.

Finally, the studies described in this thesis are summarized and discussed in the context of our current understanding of T cell responses in allergy (chapter 6).

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2

A GeNorm algorithm-based selection of reference genes for quantitative real-time PCR in skin biopsies of healthy dogs and dogs with atopic dermatitis

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Abstract

Quantitative real time PCR (qPCR) is the method of choice to study mRNA expression levels. Since qPCR is very sensitive, normalisation of the data with stably expressed reference genes is of utmost importance. The stability of reference genes depends on the tissue and the species of interest. Therefore, evaluation of the stability of reference genes must be performed for each new tissue and species under study. The stability of B2M, GAPDH, HPRT, SRPR, hnRNPH, GUSB, RPL8, RPS5, and RPS19 was analyzed with the GeNorm software in snap frozen canine skin biopsies. Healthy dogs (n=7) and dogs with confirmed atopic dermatitis (n=28) were included. Lesional and non-lesional skin was analyzed. The study indicated that the most appropriate reference genes in canine skin are the ribosomal gene products RPL8, RPS5 and RPS19 besides GUSB and HPRT. As little as three reference genes will reveal highly reliable qPCR calculations.

Introduction

Molecular genetic tools are more and more advanced and their applications in the veterinary field are rapidly increasing. This has, amongst others, been greatly improved by the in-depth sequencing of the complete dog genome^{1,2} and a single-nucleotide polymorphism (SNP) data base, which contains 2.5 million SNPs³. Even commercial, dog-specific micro-arrays are available to perform functional genomic studies in order to dissect affected signalling pathways in diseases or to predict the clinical outcomes of a therapy. This kind of high-throughput gene expression profiling requires the use of high quality mRNA and high quality internal controls. Quantitative real time PCR (qPCR) is the method to verify independently the differential expressions as measured with micro-array studies. Moreover, qPCR has been used in most of the mRNA expression studies done in veterinary research the last five to ten years. Since qPCR is an extremely delicate and sensitive technique, numerous variables (e.g. RNA integrity, enzymatic efficiency) need to be controlled in such a gene expression analysis. Evaluation of internal controls that take into account all the variability's, has been limited to internal organs mainly⁴⁻⁶. However, no validation of reference genes in canine skin is available. Therefore, we evaluated nine well-known reference genes in healthy skin, non-lesional and lesional skin of dogs suffering from atopic dermatitis (AD). The stability of B2M, GAPDH, HPRT, SRPR, hnRNPH, GUSB, RPL8, RPS5, and RPS19 was analyzed with the GeNorm software as done previously for companion animal reference genes⁴⁻⁶. This selection was based on reference genes already described and evaluated in companion animals such as dogs and cats^{5,7}, horse skin⁸, dolphin skin⁹ and human skin samples¹⁰. This low number of papers on this specific subject further shows that the evaluation of reference genes for skin specimen is at its infancy.

Materials and methods

Dogs

All privately-owned atopic dogs (n=28) presented to the Department of Clinical Sciences of Companion Animals, Utrecht University, fulfilled the diagnostic criteria for atopic dermatitis^{11,12}. This group included 19 Labradors Retrievers and one of each of the following breeds: Flatcoated Retriever, Gordon Setter, Boxer, Vizsla, French Bulldog, Jack Russell Terrier, Podenco Canario, Dachshund and German Shepherd. Female and male dogs were equally represented. Five healthy

male Beagle dogs and two healthy female mongrel dogs were included as control animals. Punch biopsies (6 mm) were obtained under general anesthesia (medetomidine: 20 ug/kg body weight and propofol: 1-2 mg/kg body weight). Healthy control and non-lesional skin specimens were all taken from the lateral thorax, whereas the lesional biopsies were obtained from affected predilection sites. After collection, the skin biopsies were immediately snap-frozen in liquid nitrogen and stored at -70 °C until used for RNA isolation. All samples were obtained after written consent of the dog owner. The procedures were approved by the Utrecht University Animal Experiments Committee as required under Dutch legislation.

RNA isolation, cDNA synthesis and qPCR.

Total RNA was isolated using a combination of the TRIzol reagent (Invitrogen, Breda, the Netherlands) and the RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. In short, the skin tissue was disrupted and homogenized in TRIzol reagent using a Biopulverizer (Biospec #59013, Biospec Inc., Bartlesville, OK) and Ultra-turrax (T8, IKA® Labortechnik GmbH, Staufen, Germany). The TRIzol manufacturer's instructions were followed until the water-phase was obtained after the chloroform step. Subsequently, the procedure continued with RNeasy columns for clean-up of the RNA including the optional on-column DNase digestion (Qiagen Rnase-free DNase kit). RNA was dissolved in 30 µl of RNase free water and was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, IJsselstein, the Netherlands). cDNA synthesis and qPCR conditions were as described previously⁵. Information about the primers used is depicted in Table 1. To reduce chances to amplify traces of genomic DNA, the primers were positioned in different exons. Calculations to estimate the expression stability and the pair wise variation were performed with the freely available GeNorm program (<http://medgen.ugent.be/~jvdesomp/genorm/>)¹³.

Table 1: Details of primers and reaction conditions used.

Gene	Accessio n number	Forward primer 5' → 3'	Reverse primer 5' → 3'	Product length (bp)	Ta (C°)
β-Glucuronidase (BGLR)	NM_001 003191	AGACGCTTCCAA/GTACCCC	AGGTGTGGTGTAGAGGAGCAC	103	62.0
Ribosomal protein S5 (RPS5)	XM_5335 68	TCCTGGTGAG/AACCCCT	CCTGATTCACACGGCGTAG	141	62.5
Ribosomal protein S19 (RPS19)	XM_5336 57	CCTTCTCAAAAA/GTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	61.0
Hypoxanthine phosphoribosyltransfer ase (HPRT)	AY_2833 72	AG/CTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	114	56.0
Heterogeneous nuclear ribonucleoprotein H (hnRNP H)	XM_5385 76	CTCACTATGATCCACCAG	TAGCCTCCATAAC/CTCCAC	151	61.2
Ribosomal protein L8 (RPL8)	XM_5323 60	CCATGAAT/CCTGTGGAGC	GTAGAGGGTTTGCCGATG	64	55.0
Glyceraldehyde-3- phosphate dehydrogenase (GAPDH)	NM_001 003142	TGTCCTCACCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58.0
b-2-Microglobulin (B2M)	XM_5354 58	TCCTCATCCTCCTCGCT	TTCTCTGCTGGGTGTCG	85	61.2
SRPR	X_03184	GCTTCAGGATCTGGACTGC	GTTCCCTTGGTAGCACTGG	81	61.2

Results and discussion

Samples were screened for contamination with genomic DNA by qPCR of non-reverse-transcribed RNA templates. No-template controls were included to test for other contaminations. All controls were negative. GeNorm-based evaluation of canine reference genes revealed a stable expression of ribosomal gene products (RPS5, RPS19 and RPL8) and HPRT and GUSB as most stably expressed non-ribosomal gene products. B2M and GAPDH turned out to be rather unstably expressed reference genes (Figure 1).

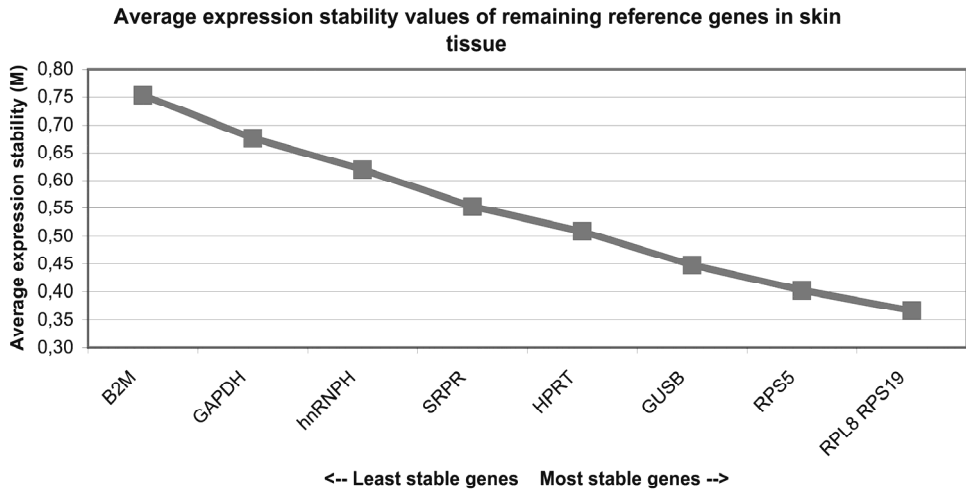


Figure 1: Average expression stability values of remaining reference genes. The geNorm program (<http://medgen.ugent.be/~jvdesomp/genorm>) calculates the gene expression stability (M) of one gene based on the average pair wise variation between all studied reference genes. The highest M values characterize genes with the least stable expression, indicative for a less optimal reference gene. Step-by-step elimination of the least stable gene generates a ranking of reference genes according to their M values and finally results in the identification of the two most stable genes¹³.

Consequently, conclusions in scientific papers based on only one reference gene, especially if it concerns GAPDH, must be read with caution. In contrast, a combination of one or two ribosomal with one or two non-ribosomal gene products will result in highly accurate normalisations. Moreover, determination of the lowest number of reference genes needed for reliable data indicated that little improvement will be obtained with more than four independent reference genes (Figure 2). Furthermore comparing dolphin, horse and human studies on skin reference genes showed conflicting data about the stability of B2M (good in horses, poor in dolphins) and HPRT (poor in dolphins and horses, the best in people). This clearly consolidates our opinion that the use of one single reference gene without prior evaluation of its stability for the tissue/species of interest, can result in misinterpretation of the data.

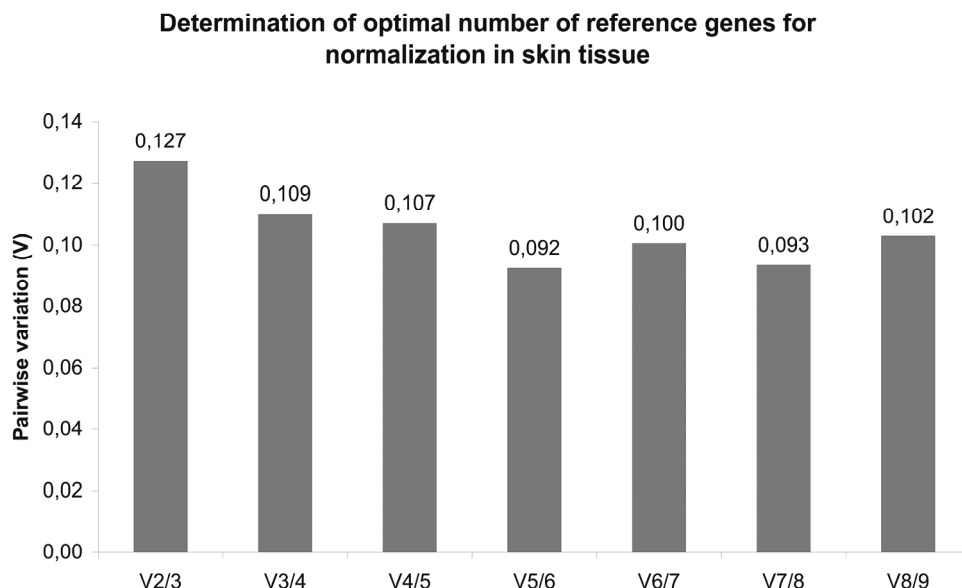


Figure 2: Determination of the optimal number of reference genes for normalization. The geNorm program (<http://medgen.ugent.be/~jvdesomp/genorm>) calculates the normalization factor assessing the optimal number of reference genes for generating the M factor by calculating the pair wise variation V . The pair wise variation between these genes defines the variable V^{13} . The lower the variable V is, the less variation. $V3/4$ indicates the variation in normalization factor with 3 vs. 4 reference genes.

This manuscript is the first, to our knowledge, that describes the evaluation of a large number of well-known canine reference genes in skin tissues. The most appropriate reference genes in canine skin are ribosomal gene products and GUSB and HPRT. As little as three reference genes will reveal highly reliable qPCR calculations. In this respect canine skin tissue is comparable to other canine tissues studied in reference gene evaluations.

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Evaluation of T cell activation in the duodenum of dogs with cutaneous adverse food reactions

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Abstract

Objective: To determine whether skin-related clinical signs in cutaneous adverse food reactions (CAFR) coincide with immune reactivity in the intestine in dogs.

Animals: 11 dogs with CAFR without intestinal clinical signs and 8 healthy control dogs.

Procedures: After a provocation and elimination diet, the duodenal gene expression levels of Th1-, Th2- and Treg-related cytokines and transcription factors were investigated by means of quantitative PCR assay. The presence of CD3⁺, CD8⁺, CD4⁺, CD1c⁺, $\gamma\delta$ T-cell receptor⁺ and major histocompatibility complex II⁺ cells in duodenal epithelium and lamina propria were determined.

Results: The expression of Th1-, Th2-, and Treg-related genes in dogs with CAFR and healthy control dogs was similar. Although clinical signs disappeared, there was no effect of the elimination diet on cytokines, transcription factors, or cellular phenotypes.

Conclusions and Clinical Relevance: No change in T cell phenotypes or a distinct Th1, Th2 or Treg profile was detected in the duodenum of dogs with only cutaneous clinical signs of adverse food reactions. This suggested that the intestinal mucosa is not the primary site of T-cell activation that eventually leads to cutaneous adverse food reactions.

Introduction

Food allergy mostly affecting the gastrointestinal tract and the skin is a serious problem in modern society and in various species. In children suspected to have atopic dermatitis, it is estimated that the prevalence of eczema in combination with intestinal manifestations is 35-40%¹⁻³. The prevalence of food allergy resulting in cutaneous symptoms without intestinal manifestations is unknown in humans, whereas 70-80% of dogs with food allergy have only skin problems.⁴ Because the pathogenesis of adverse reactions to food in dogs has not yet been fully elucidated, we prefer to use the more general term, adverse food reactions. In dogs and humans, adverse food reactions commonly have a juvenile onset. From 33-52% of dogs with adverse food reactions have clinical signs while < 1 year of age^{5,6}, and likewise, most cases of food allergy in humans occur in infants or children⁷. Because the distribution of clinical signs is also comparable⁵ and dogs and humans share the same environmental conditions, dogs with spontaneous adverse food reactions may be a good model to investigate the pathogenesis of this disease.

In humans, the healthy intestine is associated with a Th2 or tolerant environment⁸⁻¹⁰; however, the intestinal immune environment in food-allergic individuals has only been investigated sparsely. Recently, it was found that children with multiple food allergies (immediate and delayed type) had decreased TGF- β 1 expression in duodenal lymphocytes in the epithelial and lamina propria compartments,¹¹ suggesting a failure in oral tolerance with allergy as the outcome.

The cellular constitution of the healthy canine intestine is similar to that of humans¹²⁻¹⁷. In addition, it has been found that the cytokine environment in the intestine of healthy dogs reflects a Th1 (cytokines IFN- γ and IL-18) or tolerant profile (cytokines TGF- β and IL-10)¹⁸. Cytokines have an important regulatory function in immune responsiveness, both in polarization of T cells towards Th1 or Th2 cells and in maintenance of tolerance (Treg). Differential expression of transcription factors that regulate cytokine gene-expression reflects the type of immune response induced: Th1 (IFN- γ , IL-18, T-bet and STAT-4), Th2 (IL-4, IL-13, GATA3 and STAT-6) or Treg (TGF- β , IL-10 and Foxp3). As an example, IL-4 activates STAT-6 and GATA-3 through binding to its receptor on Th2 cells, leading to transcription of additional IL-4 and other Th2 cytokines, and at the same time production of IFN- γ is inhibited¹⁹⁻²². Although the role of transcription factors has not yet been elucidated in food allergy, in individuals with atopic asthma, STAT-6 (a Th2-related transcription factor) is overexpressed, GATA-3 (Th2-related) mRNA expression is increased, and T-bet (Th1-

related) is reduced in lung tissue²³⁻²⁶. The influence and role of transcription factors in T cell responses have been reviewed by Wahl et al²⁷.

Whereas the intestine is the first site of contact with food allergens, we hypothesized that the intestinal immune reactivity and accompanying transcription factor expression of dogs with cutaneous adverse food reactions (CAFR) may be associated with a Th2 profile or a diminished expression of genes involved in oral tolerance. The purpose of the study reported here was to determine whether skin manifestations in CAFR coincide with immune reactivity in the intestine in dogs by evaluation of T-cell phenotypes and cytokine and transcription factor expression in duodenal biopsy specimens of dogs with CAFR after administration of a provocation diet and an elimination diet.

Materials and Methods

Animals and experimental design

Eleven adult client-owned dogs with CAFR, thus without intestinal clinical signs (5 females, 6 males; age, 10 months-8 years [median, 4 years]) referred to the Utrecht University Faculty of Veterinary Medicine and 8 adult healthy control dogs (3 females, 5 males; age, 3-10 years [median, 8 years]) were included in this study. None of the client-owned or control dogs had feces of abnormal consistency or other gastrointestinal clinical signs for at least 6 months before entering the study. The CAFR group consisted of 4 Labrador Retrievers and 1 dog of each of the following breeds: Shorthaired Dachshund, Beagle, mixed-breed, English Bulldog, English Cocker Spaniel, West Highland White Terrier, and German Shepherd Dog. The healthy control dogs were 4 Beagles and 4 mixed-breed dogs, owned by the University and housed in open kennels. Their regular food consisted of commercially available diets similar to those fed to pet dogs. The study was performed according to the guidelines of the Utrecht University Animal Experiments Committee. A diagnosis of CAFR was confirmed by means of dietary testing: first, a minimum of 80% reduction in pruritus and associated clinical signs had to be achieved after a food trial that used a novel protein home-cooked diet of at least 8 weeks' duration; second, a provocative test with the original food had to result in reappearance of the clinical signs; and third, after a second elimination diet the clinical signs had to resolve for a second time. The control dogs underwent a similar dietary regimen for 8, 2 and 2 weeks, which were the mean periods required for elimination, provocation, and second elimination test results, respectively. Prior to this dietary testing, the presence of intestinal parasites

was evaluated by means of a fecal flotation test and microscopy. Moreover, all dogs were treated with paracitidals before inclusion in the study.

Duodenal biopsy specimens were collected within 10 days after the onset of clinical signs caused by the dietary provocation (time 0) and after the disappearance of clinical signs as a result of the second elimination diet (time 1). Food was withheld for 14-18 hours before gastroduodenoscopy. Several duodenal biopsy specimens (2.3-4 mm diameter) were taken endoscopically by use of general anesthesia. Biopsy specimens were snap frozen in liquid nitrogen and stored at -70 °C until processing. Additional biopsy specimens were fixed in neutral buffered 4% formaldehyde (Klinipath b.v., Duiven, the Netherlands) for histopathological evaluation (H&E staining).

RNA isolation

Frozen duodenal biopsy specimens were immersed in a phenol-guanidine isothiocyanate solution (TRIzol, Invitrogen Corp, Carlsbad, California, USA) at 4 °C and homogenized by use of a disperser (T8 Ultra-turrax, IKA Labortechnik GmbH, Staufen, Germany). Total RNA was isolated according to the manufacturer's instructions with the modification that an additional phenol-chloroform-isoamylalcohol (125:24:1; pH, 4.0) purification step was performed after the phenol and chloroform separation in the original protocol. The RNA was dissolved in 30 µL of RNase free water, and the RNA concentration was determined via spectrophotometry (ND-1000, NanoDrop, Wilmington, Delaware, USA). The presence of genomic DNA was evaluated by use of quantitative PCR assay on the isolated RNA samples directly. Subsequently, 3 µg of RNA was transcribed into cDNA by use of an I-script cDNA synthesis kit (BioRad, Veenendaal, the Netherlands) according to the manufacturer's instructions. The cDNA was diluted 1:2 in RNase free water, aliquoted, and stored at -20 °C until use in a quantitative PCR assay.

Primer design and quantitative PCR assay

Oligonucleotide primers (Eurogentec, Maastricht, the Netherlands) were designed for Th1-related genes (IL-12p40, IL-12p35, IL-18, IFN- γ , T-bet, STAT-4 and SOCS-5), Th2-related genes (IL-4, GATA-3, STAT-6, SOCS-3 and IL-13), tolerance-related genes (IL-10, TGF- β and Foxp3) and for IL-2, TNF- α , B7.1, and B7.2 on the basis of the sequences as described in the ensemble project (<http://www.ensembl.org>, version v.32) and sequence analysis (Primer-3 software) software. The primer pairs were

designed in such a way that they spanned an intron as an extra precaution to prevent the transcription of residual genomic DNA in the sample, except those for IFN- γ and SOCS-5. Each primer pair was tested for the optimum annealing temperature and PCR reaction efficiency. Primer sequences, intron overlap, and optimum annealing temperatures were tabulated (Appendix). All PCR products had sizes from 100-150 bp and sequences were verified (ABI PRISM 3100 Genetic Analyser, Applied Biosystems, Foster City, California, USA) before quantitative PCR analyses were started. The genes HPRT, RPS19, and RPS 5 were used as reference genes²⁸.

For each quantitative PCR reaction, 1.5 μ L of cDNA was used in a reaction volume of 25 μ L containing 12.5 μ L of commercial PCR mix containing cyanine dye (iQ SYBR Green Supermix, BioRad, Veenendaal, the Netherlands) and 20 pmol of forward and reverse primers. Each PCR test was performed in triplicate in 96-well quantitative PCR plates (BioRad, Veenendaal, the Netherlands). Each plate contained an internal standard, which was generated by use of 4-fold dilutions of pooled cDNA from a mixture of canine concanavalin A-stimulated and unstimulated peripheral blood mononuclear cells, duodenal biopsy specimens, and lesional skin biopsy specimens. The internal standard was used to check the PCR reaction efficacy and intertest differences. All quantitative PCR assays were performed in a spectrofluorometric thermal cycler (BioRad, Veenendaal, the Netherlands) with a 5-minute polymerase activation step and continued with 40 cycles containing a denaturing step 95 °C for 30 seconds, an annealing step for 30 s, and an elongation step at 72 °C for 30 s with a final extension for 2 min at 72 °C, followed by a meltcurve procedure. All PCR reaction efficiencies were from 95-105%, product melting curves revealed 1 product, and negative controls yielded negative results.

Immunohistochemical staining

Biopsy specimens were mounted, (Tissue-Tek O.C.T. Compound, Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) and 6- μ m cryostat sections were placed on positively charged slides (Superfrost Plus, Menzel-Glaser, Braunschweig, Germany), dried and stored at -70 °C until use. After thawing, the tissue sections were fixed in cold 100% acetone for 10 min at 21 °C. Endogenous peroxidase was eliminated by 20 minutes' incubation in Tris-buffered saline solution (0.05M Tris-HCl, 0.15M NaCl, pH 7.5), supplemented with 0.3% hydrogen peroxidase, followed by washing in Tris-buffered saline solution. Sections were incubated for 25 minutes in blocking reagent (Tris-buffered saline solution with 10% inactivated normal horse

serum) and subsequently incubated for 1 hour at 21 °C with primary canine-specific antibodies diluted in 1% blocking reagent (CD3 [CA17.2A12 (Serotec Ltd, Oxford, England), 1:100], CD4 [CA13.1E4 (Serotec Ltd, Oxford, England), 1:20], CD8 [CA9.JD3 (Serotec Ltd, Oxford, England), 1:10], MHC-II [CA2.1C12 (Serotec Ltd, Oxford, England), 1:60], $\gamma\delta$ TCR [Kindly provided by Peter F. Moore, University of California, Davis, California, USA) 1:50] and CD1c [Kindly provided by Peter F. Moore, University of California, Davis, California, USA, 1:20]). After washing, sections were incubated for 45 min with horse anti-mouse biotin-labelled antibody, washed, and incubated for 45 min with peroxidase-labeled avidin-biotin complex (Vectastain ABC systems, Vector Laboratories, Burlingame, California, USA). Staining was developed with 3-amino-9-ethyl-carbazole, resulting in a red-brownish color and sections were counterstained with hematoxylin. Stained cells in the epithelium and in the lamina propria of 3 randomly chosen villi in each tissue section were counted via light microscopy. Means of the number of stained cells (per square millimeter) were used for statistical analysis.

Statistical analysis

Because the cDNA input may vary among samples, results of quantitative PCR analysis of each sample were normalized to the mean quantities of gene expression measured via PCR assay for the endogenous reference genes (HPRT, RPS19 and RPS5)²⁹ and expressed as relative gene expression, which was used in the statistical tests. The data were normally distributed (Kolmogorov-Smirnov test), as indicated by the combination of residues of the 1-way ANOVA test of the 2 independent groups of dogs (ie, healthy dogs and dogs with CAFR). All variances were homogeneous as indicated by results of the Levene test. A repeated measurements ANOVA test was used because there were 2 independent groups of dogs and 2 dependent groups of data (T0 after food challenge and T1 after the second elimination diet). Multiple comparisons were evaluated by use of the Bonferroni correction, which resulted in $P \leq 0.001$ being considered significant.

The original immunohistochemical results were recorded as discontinuous values, so non-parametric statistical tests were used. For comparison between dogs with CAFR and healthy dogs at time 0, the Mann-Whitney test was used; for comparison between time 0 and time 1, the Wilcoxon signed ranks test was used for both the absolute cell numbers and their ratios. Statistical software (SPSS software, Benelux,

Gorinchem, the Netherlands) was used for all analyses. A value of $P \leq 0.05$ was considered significant.

Results

Analysis of the mRNA expression of grouped Th1-related genes (IL-18, SOCS-5, IL-12, STAT-4, T-bet, TNF- α , and IFN- γ), Th2-related genes (IL-4, IL-13, GATA-3, STAT-6, and SOCS-3), and tolerance-related genes (Foxp3, TGF- β , and IL-10) did not reveal differences in expression between dogs with CAFR and healthy dogs. Expression was not affected by the elimination diet (Figure 1).

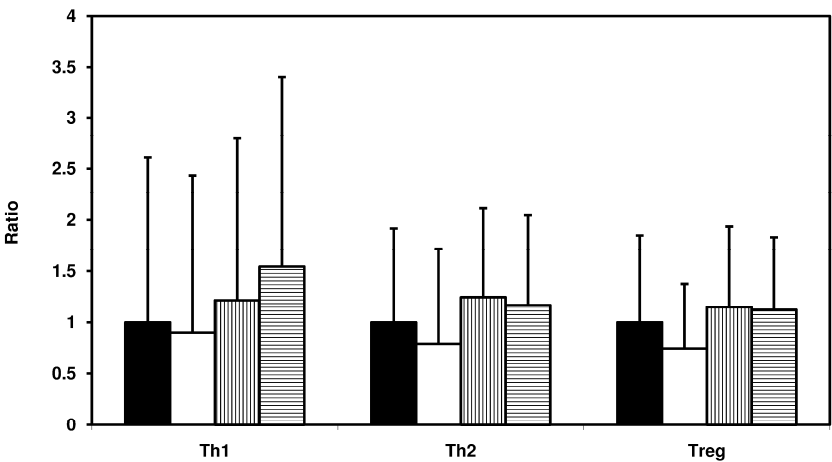


Figure 1—Mean \pm SD expression of grouped Th1-, Th2-, and Treg-related mRNA in duodenal biopsy specimens of dogs after use of a provocation diet (T0) and an elimination diet (T1). Values represent ratios of mRNA expression in each group, compared with control dogs at T0. Black bars = Control T0 group. White bars = Control T1 group. Vertically striped bars = CAFR T0 group. Horizontally striped bars = CAFR T1 group.

Analysis of mRNA expression of the individual Th1-, Th2-, tolerance-related and IL-2, B7.1 and B7.2 genes in duodenal tissue revealed that the expression was not altered in the CAFR group after the elimination diet, and differences were not seen in gene expression between dogs with CAFR and control dogs after provocation (time 0).

As revealed via immunohistochemical staining, the numbers of CD3⁺, CD8⁺, CD4⁺, CD1c⁺, $\gamma\delta$ T cell receptor⁺ and major histocompatibility complex II⁺ cells in duodenal epithelium and lamina propria were similar in dogs with CAFR and healthy dogs (Figures 2 and 3). The dietary changes had no influence on these absolute cell numbers or their ratios. Histologic examination (H&E staining) revealed normal epithelium on the villi and in the crypts with a low number of intraepithelial lymphocytes. The lamina propria had a typical amount of plasma cells and lymphocytes with occasionally one or a few eosinophilic granulocytes in the control dogs and the dogs with only cutaneous manifestations of adverse reactions. The results were similar at both time points.

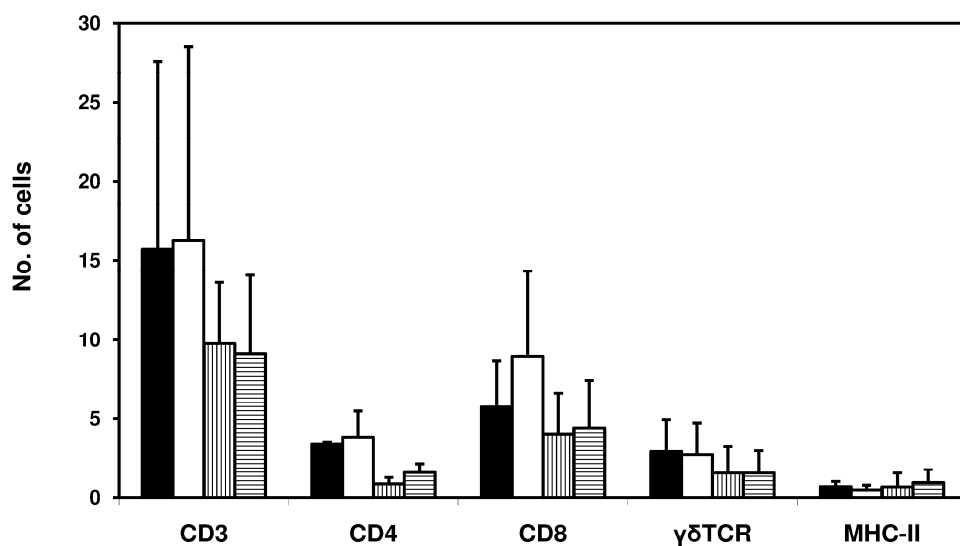


Figure 2—Mean \pm SD cell counts in a 0.1 mm² area of duodenal epithelium of control dogs and dogs with CAFR determined after use of a provocation diet (T0) and an elimination diet (T1). See Figure 1 for key.

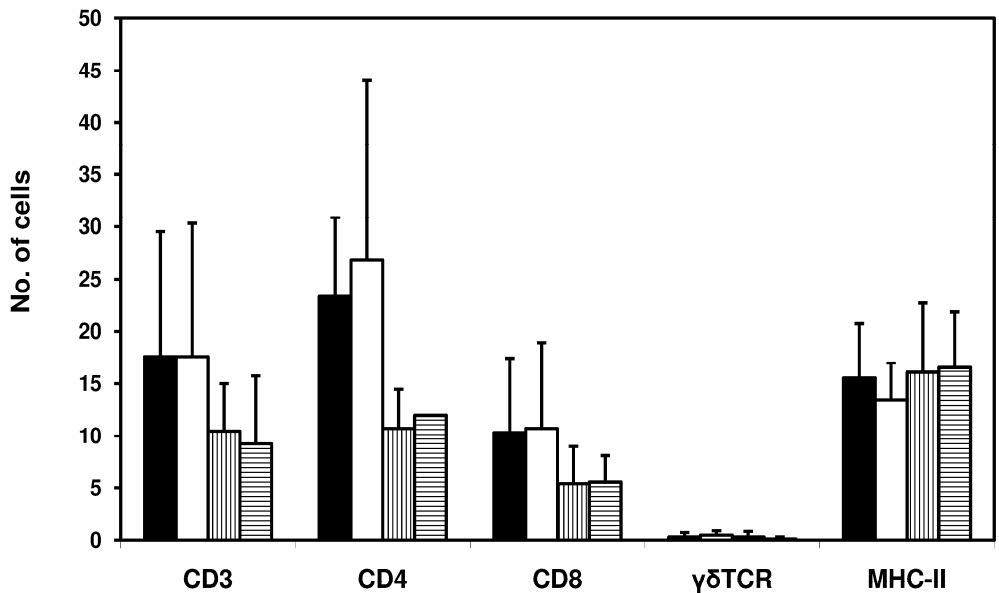


Figure 3— Mean \pm SD cell counts in a 0.1 mm² area of duodenal lamina propria of control dogs and dogs with CAFR determined after use of a provocation diet (T0) and an elimination diet (T1). See Figure 1 for key.

Discussion

To the authors’ knowledge, the present study was the first to investigate the cellular infiltrates and mRNA expression of Th1-, Th2- and Treg-related genes in duodenal biopsy specimens of dogs with adverse food reactions with cutaneous clinical signs only. Although the dogs with CAFR were selected on the basis of absence of clinical intestinal signs, an immune response was still expected to have occurred in the intestine where the first contact with the allergens takes place. The duodenum, rather than ileum or jejunum, was chosen as sampling site because it is the initial place of digestion and absorption of food constituents including allergens. In addition, from a practical point of view, the duodenum may be readily approached via gastroduodenoscopy. The duodenal biopsy specimens were taken after the cutaneous clinical signs were observed and again after the clinical signs disappeared because of the dietary changes. Consequently, in dogs with CAFR, we expected a

higher expression of the Th2-associated genes responsible for the presence of clinical signs of adverse food reactions and a decreased Th2 mRNA expression or increased immune-tolerance-related gene expression when the clinical signs disappeared. In contrast to these expectations, expression of the Th1 and Th2 genes did not differ between dogs with CAFR and healthy dogs at the time the clinical signs were observed and the elimination diet did not influence the duodenal expression of these genes in the dogs with CAFR.

Because dogs with CAFR are characterized by cutaneous clinical signs only, the lack of intestinal signs may be explained by a more tolerant intestinal environment, compared with the healthy dogs. This hypothesis was not supported by our findings, because the CAFR group did not have greater expression of tolerance-related genes (Foxp3, TGF- β and IL-10), indicating that, from the perspective of T cell regulation, dogs with CAFR did not differ from healthy dogs.

No distinct cellular infiltrate or Th1-, Th2- or Treg-related gene expression patterns or an influence by the elimination diet were observed in dogs with CAFR, compared with the healthy dogs, despite cutaneous clinical signs of CAFR. Recently, it was determined in corn-allergic dogs with cutaneous clinical signs only, that despite relapse or disappearance of cutaneous clinical signs, no changes in blood CD4⁺CCR4⁺, corn-activated CD4⁺ cells, or IgE occurred³⁰, supporting the idea that the immunologic changes are focused in the skin. One reason for that finding may be the time at which the duodenal biopsy specimens were taken. For practical reasons, endoscopy was done from 4 to 10 days after pruritis reappeared (as determined via a provocation test) and after it was reduced for > 80% (via an elimination diet) according to the dog owners' information. Although substantial changes may have occurred between relapse or disappearance of clinical signs and endoscopy, we consider this scenario as highly unlikely. In contrast, we expect these changes to persist in this chronic exposure setup, rather than fluctuate or disappear, as long as food exposure is unchanged during such a period.

The change in cutaneous clinical signs without a measurable mucosal immune response suggests that an intrinsic reaction occurs independently of the mucosal allergen exposure or that exposure occurs at a location other than the place where the mucosal biopsy specimens were taken. In the present study, duodenal biopsy specimens were taken, carefully circumventing the Peyer's patches, because we were mainly interested in the effector T cells residing in the mucosa and their reactivity at the end of the elimination diet. The possible pathways of antigen entry into the

intestine and antigen presentation to naive T cells or systemic distribution are not fully elucidated³¹. However, there is increasing evidence in mice that antigen is taken up by the enterocytes and dendritic cells in the mucosa³²⁻³⁴ and lamina propria dendritic cells migrate to the mesenteric lymph nodes^{35,36}. Worbs³⁷ suggest that oral tolerance is regulated solely in the mesenteric lymph nodes and that induction of oral tolerance relies on the lymph that is drained from the intestine, which likely contains antigen transported via lamina propria dendritic cells. Their suggestion implies that allergen recognition occurs in the mesenteric lymph nodes and not in the mucosa, which supports absence of distinct Th1, Th2, or Treg changes in intestinal tissue observed in our study. The allergens or T cells may be distributed throughout the body from the mesenteric lymph nodes via blood or lymph and cause the cutaneous clinical signs.

Results of the present study, which evaluated factors before and after administration of the elimination diet, did not indicate a role for Th subsets in the duodenal tissue in immune regulation or dysregulation in dogs with CAFR with only cutaneous signs. We hypothesize that the immune mechanism in cutaneous food allergy at the mucosal level is different from the patterns that are seen in human intestinal food allergy^{11,38-43}. It has been determined that circulating cells in animals with atopic dermatitis and allergic asthma do not express typical Th1 or Th2 genes despite being primed to home to the skin and express genes involved in activation or proliferation. Upon culture of these cells in the presence of allergen, the cells produced Th2- or Th1-related mRNA⁴⁴⁻⁴⁶. When immune cells are only primed, but do not develop to Th1, Th2 or Treg/Th3 *in vivo* as suggested for peripheral blood mononuclear cells in atopic dermatitis⁴⁵, this may explain the lack of a reaction by intestinal T cells, which normally are memory T cells⁴⁷ in a Th1-like and immunosuppressive environment. Additionally, neither significant differences in the expression of Treg-related genes nor differences in cellular infiltrate were found in the present study, supporting the idea that the primed intestinal cells might not develop into Th1, Th2, or Treg cells in the mucosa upon allergen stimulation. Another possible explanation of the lack of changes at the level of T cells might be that differences in the humoral immune system in dogs with CAFR are involved in this clinical phenotype. Further research on the role of the humoral immune system in dogs with CAFR is therefore justified.

The present study revealed no change in T cell presence or a clear Th1, Th2, or Treg profile at the duodenal mucosal level in dogs with CAFR after dietary

provocation, and this profile did not change after administration of the elimination diet, despite the disappearance of clinical skin signs. Results suggested that the intestinal mucosa is not the primary site of T cell activation that eventually leads to cutaneous adverse food reactions.

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Appendix—Quantitative PCR primer sequences and annealing temperatures used in a study of dogs with CAFR.

Gene	Primer sequence (5'- 3')	Annealing temperature (°C)
IL-4	F: ccaaaagaacacaagcgataaggaa R: gtttgccatgctgctgaggtt	61
IL-10	F: cccgggctgagaaccacgac R: aaatgcgctcttcacctgctccac	63
IL-12p40	F: ggacgtttcacatgctggt R: ccactctgaccctctctgct	59
IL-12p35	F: taatggatcccaagaggcag R: tcaaggaggagatttctgtgg	62.5
IL-2	F: atgagaaccccaactctcc R: gcacttctccaggtttttg	57
IL-18	F: gaggatatgccgattctga R: tccggaggactcatttctg	56
IFN- γ	F: agcgcaaggcgataaatg R: gcggcctcgaacagatt	55.8
TGF- β	F: caaggatctgggctggaagtgga R: ccaggacctgtgtactgcgtgt	65
GATA-3	F: tacgtccccgaatacagctc R: actccctgccttctgtgct	64
T-bet	F: aatcagcaccagacggagat R: gtccacgaacatccggtaat	61.2
STAT-4	F: actggaagaggcgacaacag R: gccttctgagttggaacagg	59
STAT-6	F: aactgcagcggctctatgtc R: catgttgacagagaagggtg	64
SOCS-5	F: tctgccgtgcagtaatctgt R: gccttgactggttctctgttc	61
TNF- α	F: cccgggctccagaagggtg R: gcagcaggcagaagagtgtggtg	64
SOCS-3	F: acaccagcctgcgcctcaagacct R: cgctcgcgcccgctca	63
IL-13	F: gaggagctggtcaacatca R: tgcagtccggagacattga	59
B7.1	F: acagcgaagtggagaacacc R: ctggatgatgcctgaacaga	61
B7.2	F: ctgaagcaagcaatgtgagc R: atcaggggtgggtttctgtat	59
HPRT	F: agcttgctggtgaaaaggac R: ttatagtcaagggcataatcc	56
RPS19	F: ccttctctcaaaaagtctggg R: gttctcatcgtagggagcaag	61

RPS5	F: tcactggtgagaacccct R: cctgattcacacggcgtag	62.5
Foxp3	F: caaatggtgtctgcaagtgg R: gtgctctgcccttctcatct	59

F = Forward. R = Reverse.



Characterization of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions.

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Abstract

The immunopathogenesis of cutaneous adverse food reactions (CAFR) in dogs is unknown. Since the clinical manifestations in the skin are like those found in canine atopic dermatitis (AD), this study investigated the similarity in T cell phenotypes and gene expression of cytokines and transcription factors in CAFR. In addition, the influence of an elimination diet on these parameters was tested. In the skin of canine CAFR, a predominant presence of CD8⁺ T cells and increased expression of the IL-4, IL-13, Foxp3 and SOCS3 genes were observed. IFN- γ gene expression was increased in lesional skin compared to non-lesional skin. The predominance of CD8⁺ T cells indicates that the immunopathogenesis of CAFR is different from that of canine AD. The elimination diet relieved clinical signs, but did not influence T cell phenotypes or expression of the cytokine and transcription factor genes in the skin of dogs with CAFR, indicating a continuously pre-activated immune status in dogs sensitised to food constituents.

Introduction

Skin responses to dietary components in dogs are generally referred to as cutaneous adverse food reactions (CAFR), because the underlying immunological response patterns are unknown¹. Although food allergen exposure occurs first in the digestive tract, only 20-30% of CAFR dogs have gastrointestinal signs¹, whereas the majority of the dogs show cutaneous symptoms only. Signs of CAFR can occur at the same predilection sites and may be clinically indistinguishable from atopic dermatitis (AD). In the most detailed retrospective study, 63 % of dogs diagnosed with food hypersensitivities based on dietary elimination and provocation tests exhibited cutaneous lesions and pruritus suggestive of AD². However, CAFR may also manifest as papular abdominal rash, otitis, seborrhoea or recurrent superficial pyoderma³⁻⁷.

Currently, it is unknown how exposure to food leads to adverse reactions in the skin. In contrast, cell subsets and cytokine production in the skin of dogs with AD have been investigated extensively. In AD, inflammation is characterized by an influx of CD4⁺ and CD8⁺ T cells in the lesional skin^{8,9} and a mixed cytokine profile with predominant expression of IL-6, TARC, IL-4 and IL-13 in the early stage followed by IFN- γ , IL-12 and IL-18 later on¹⁰⁻¹². Whereas most dogs with AD have circulating allergen-specific IgE¹³, there is controversial evidence for a similar reaction phenomenon in dogs with spontaneous manifestations of CAFR¹⁴⁻¹⁶. Increasing our insight in the immunopathogenesis of CAFR, may enable the development of novel modalities to treat them.

Since the clinical manifestations of CAFR are comparable to those of AD, we hypothesized that the cutaneous reaction pattern of dogs with CAFR is comparable to that of dogs with AD. To test this hypothesis, the inflammatory response (T cells and expression of cytokines and transcription factors representative for Th1, Th2 and regulatory T cells; reviewed by Wilson¹⁷; Ozdemir¹⁸) in the skin of dogs with CAFR was investigated after a provocation diet with the animals' original food and a subsequent elimination diet.

Materials and Methods

Animals and experimental design

Ten client-owned CAFR dogs without intestinal symptoms (five females, five males; age 10 months-8 years, median 3.5 years) were referred to the Utrecht University Faculty of Veterinary Medicine. The group consisted of four Labrador retrievers and one of each of the following breeds: shorthaired Dachshund, a German Shepherd, English Bulldog, English Cocker Spaniel, West Highland White terrier, and a mixed-bred dog. There is no known breed predisposition for CAFR. The seven healthy control dogs (three females, four males; age 4-10 years, median 8 years) included in this study were three Beagles and four mixed-bred dogs. The study fulfilled the requirements set by the Utrecht University Animal Experiments Committee.

Prior to dietary testing, flea bite hypersensitivity was ruled out by skin testing, IgE serology and flea control measures with adulticides and insect growth inhibitors at 1-month intervals. Other parasitic disorders were excluded by routine diagnostic methods. Secondary infections (staphylococcal pyoderma, *Malassezia* spp. dermatitis) were treated, if applicable, before and throughout the trial. The CAFR dogs were included based on the following dietary testing procedure. First, a full reduction in pruritus and associated symptoms had to be achieved after an elimination diet using a novel protein (ostrich, turkey, horse or goat meat) home-cooked diet for at least 8 weeks. Second, a provocative test with the original food had to result in reappearance of the pruritus (T0). Third, after a second elimination diet the pruritus had to disappear for a second time (T1). The healthy control dogs underwent a similar dietary regimen for 8, 2 and 2 weeks, being the average periods necessary for dietary responses in CAFR dogs.

Skin samples

Skin biopsies from both lesional (LS) and non-lesional skin (NLS) were taken using a 6 mm disposable punch (Kai Industries) under general anaesthesia (medetomidine 20 µg/kg and propofol 1-3 mg/kg IV on effect) within 10 days following the onset of symptoms due to the dietary provocation (T0) and after the disappearance of symptoms resulting from the second elimination diet (T1). Since clinical signs vanished after the elimination diet (T1) and LS was no longer visible, biopsies were taken at the same locations as at T0. Skin biopsies of the healthy control dogs were collected from the front legs and the thorax. Skin samples were snap frozen in liquid nitrogen for immunohistochemistry and RNA isolation, or fixed in 4% neutral

buffered formaldehyde for routine haematoxylin and eosin (HE) staining.

RNA isolation

Frozen LS and NLS biopsies were immersed in TRIzol at 4 °C (Invitrogen), cut in smaller pieces and homogenized using an Ultra-Turrax dispenser (T8, IKA Labortechnik GmbH). Isolation of total RNA and removal of genomic DNA was performed as previously described¹⁹ and consisted of a combination of TRIzol and RNeasy mini kit (Qiagen) procedures.

Primer design and Quantitative Polymerase Chain Reaction (Q-PCR)

Oligonucleotide primers (Eurogentec) were designed for Th1-related genes (IL-12p35, IL-18, IFN- γ , STAT-4, SOCS-5, TNF- α), Th2-related genes (IL-4, GATA-3, STAT-6, SOCS-3, IL-13, TARC) and tolerance-related genes (IL-10, TGF- β , Foxp3) based on the sequences described in the ensemble project (<http://www.ensembl.org/index.html>, version v.32) using the Primer-3 software. Primer sequences and optimum annealing temperatures are shown in Table 1. cDNA synthesis and Q-PCR conditions were as described previously²⁰ with the following modifications. The Q-PCR program included a 5 min polymerase activation step and continued with 40 cycles consisting of a denaturing step at 95 °C for 30 s, an annealing step for 30 s and an elongation step at 72 °C for 30 s with a final extension for 2 min at 72 °C. All PCR reaction efficiencies were between 95 and 105% and product melting curves showed single products and absence of a product in the negative controls (data not shown). If the expression of a gene was below the detection limit, the lowest detectable value was used for statistical analysis. The results of each sample were normalized to the average amounts of the endogenous reference genes (HPRT and RPS19) of the same sample^{19,21}; the relative gene expression. RPS5 was used as control gene for statistical analysis.

Table 1: quantitative PCR primer sequences and annealing temperature.

Gene	Primer Sequences 5'-3' Forward (F) and Reverse (R)	Annealing temperature
IL-4	F: ccaaagaacacaagcgataaggaa R: gtttgccatgctgctgaggtt	61 °C
IL-10	F: cccgggctgagaaccacgac R: aaatgcgtcttcacgtgctccac	63 °C
TARC	F: ggagccattcctatcagcag R: ggtcggaaacagatggacttg	64.5 °C
IL-12p35	F: taatggatccaagaggcag R: tcaaggaggagatttctgtgg	62.5 °C
IL-18	F: gaggatagccccgattctga R: tccggaggactcatttctg	56 °C
IFN- γ	F: agcgcgaaggcgataaatg R: gcggcctcgaacagatt	55.8 °C
TGF- β	F: caaggatctgggctggaagtgga R: ccaggaccttgctgtactgcgtgt	65 °C
GATA-3	F: tacgtccccgaatacacgctc R: actccctgccttctgtgct	64 °C
STAT-4	F: actggaagaggcgacaacag R: gccttctgagttggaacagg	59 °C
STAT-6	F: aactgcagcggctctatgtc R: catgttgacagcagaaggtgt	64 °C
SOCS-5	F: tctgccgtgcagtaatctgt R: gccttgactggttctcgttc	61 °C
TNF- α	F: ccccgggctccagaaggtg R: gcagcaggcagaagagtgtggtg	64 °C
SOCS-3	F: acaccagcctgcgcctcaagacct R: cgctcgccgcccgtca	63 °C
IL-13	F: gaggagctggtaacatca R: tgcagtcggagacattga	59 °C
Foxp3	F: caaatggtgtctgcaagtgg R: gtgctctgccccttctcatct	59 °C
HPRT	F: agcttgctggtgaaaaggac R: ttatagtaaggcatatcc	56 °C
RPS19	F: ccttctcaaaaagtctggg R: gttctcatcgtagggagcaag	61 °C
RPS5	F: tctactggtgagaacccct R: cctgattcacacggcgtag	62.5 °C

Immunohistochemistry

Biopsies were mounted in Tissue-Tek (Sakura Finetek Europe) and 6 μ m cryostat sections were placed on Superfrost Plus slides (Menzel-Glaser), dried and stored at -70 °C until use. After thawing, the tissue sections were fixed in 100% acetone for 10 min at room temperature (RT). Endogenous peroxidase was eliminated by 20 min incubation in Tris-buffered saline (TBS; 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) supplemented with 0.3% hydrogen peroxide, followed by washing in TBS. Sections were incubated for 25 min in blocking reagent (TBS with 10% inactivated normal dog and normal goat serum) and subsequently for 1 h at RT with primary unlabelled antibodies diluted in 1% blocking reagent (Table 2). After washing, sections were incubated for 30 min with horseradish peroxidase (HRP)-labelled antibodies (Table 2), washed and blocked for 30 min with unlabelled rabbit anti-mouse antibodies (Table 2) to cover any unbound primary antibodies. After washing,

sections were incubated for 45 min with the second primary unlabelled antibodies, washed and incubated for 30 min with alkaline phosphatase (AP)-labelled antibodies (Table 2). Staining was developed with 3-amino-9-ethyl-carbazole (AEC, Sigma-Aldrich) resulting in a red-brownish colour (HRP) and thereafter with Fast Blue (AP), resulting in a blue colour. Double stained cells appeared purple. Cells in the epidermis and dermis from two biopsies were counted using light microscopy. Average numbers of positive cells/mm² were used for statistical analysis.

Table 2: Antibodies and concentration used for immunohistochemistry.

Antibody	Origin	Concentration
CD3 (CA17.2A12) primary antibody	Serotec Ltd.	1:200
CD4 (CA13.1E4) primary antibody	Serotec Ltd.	1:20
CD8 (CA9.JD3) primary antibody	Serotec Ltd.	1:20
γδTCR primary antibody	Monoclonal antibody , P.F. Moore, University of California.	1:50
αβTCR primary antibody	Monoclonal antibody , P.F. Moore, University of California.	1:100
MHC-II (CA2.1C12) primary antibody	Serotec Ltd.	1:60
CD1c primary antibody	Monoclonal antibody, P.F. Moore, University of California.	1:20
Rabbit anti mouse blocking antibody	Polyclonal antibody, Dako	1:50
Goat anti mouse-HRP secondary antibody	Polymer antibody, Envision ⁺ system, Dako	undiluted
Goat anti mouse-AP secondary antibody	Polyclonal antibody, Dako	1:100

Statistical analysis

The relative gene expression and immunohistochemistry results were analysed with the SPSS version 15 software. A linear mixed model²² was done to analyze the relative gene expression as outcome variable. The relative expression of all genes together was analyzed in one model. The explanatory factors in the model are gene, LS/NLS/Control group, T0/T1 group and 3 two-way interactions between the 3

factors. The subject variable dog was used as the random factor to account for the repeated measurements within the dogs and was assumed to have a normal distribution. RPS5 was used as the control gene. Residuals were studied to check the validity of the model. Multiple comparisons were corrected with the False Discovery Rate implying that $P \leq 0.05$ was considered statistically significant.

Since the results for immunohistochemistry were discontinuous values, non-parametric statistical tests were used. For the comparison between skin sections of CAFR and control dogs, the Mann-Whitney U test (non-parametric analog of independent t-test) was used and for the comparison of skin sections between T0 and T1 and between LS and NLS skin the Wilcoxon Signed Ranks test (non-parametric analog of the paired t-test) was used.

Results

Cell phenotypes

Analysis of the T-cell phenotypes and cell numbers in the epidermis of LS showed significantly increased numbers of $CD8^+ \gamma \delta TCR^+$ T cells ($P = 0.036$) compared with control skin during the provocation diet (Figure 1). Moreover, the dermis contained more $CD3^+ CD8^+$ ($P = 0.029$) and $CD8^+ \alpha \beta TCR^+$ ($P = 0.023$) T cells in LS than in the skin of control dogs (Figure 2). The elimination diet did not affect the T-cell phenotypes or cell numbers.

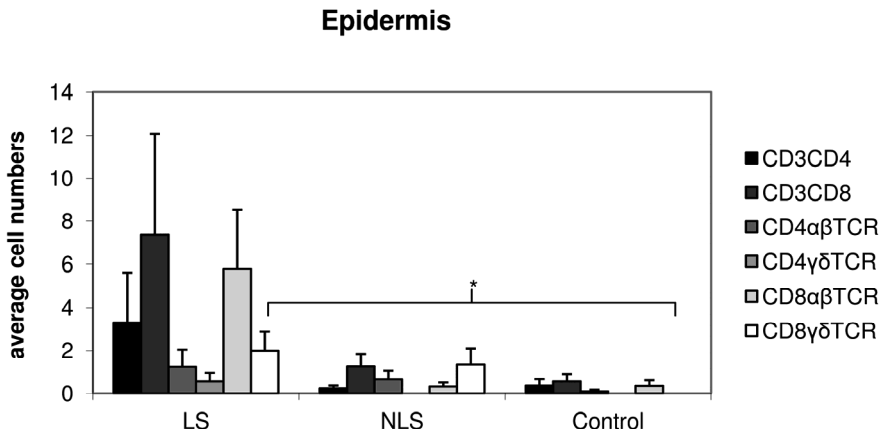


Figure 1: Cell numbers in the epidermis of CAFR and control dogs. Each bar represents the average and SEM of the number of cells/mm length of the epidermis. * $P \leq 0.05$.

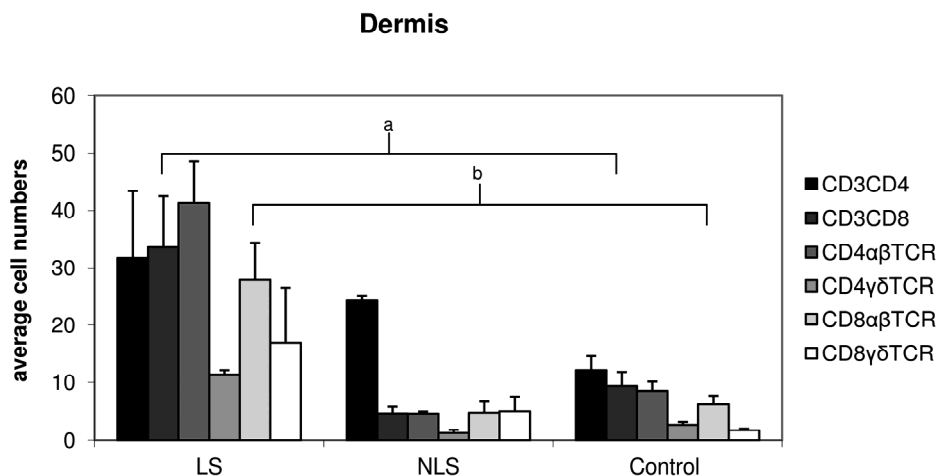


Figure 2: Cell numbers in the dermis of CAFR and control dogs. Each bar represents the average and SEM of the number of cells/mm². a: CD3⁺CD8⁺ LS compared to control skin ($P = 0.028$). b: CD8⁺αβTCR⁺ LS compared to control skin ($P = 0.023$).

The T cell numbers in LS and NLS skin were highly variable in the CAFR dogs (Figures 1 and 2), but no effect of the elimination diet was found. In biopsies of LS skin, the epidermis showed occasional acanthosis and patchy spongiosis. Dermal lesions included a mild to moderate, superficial perivascular inflammation, predominantly characterized by a mixed infiltrate with lymphocytes and histiocytes, a variable amount of mast cells and plasma cells, and few neutrophils and eosinophils.

NLS skin of CAFR dogs had a normal epidermis and a very mild, superficial, mononuclear perivascular infiltrate in the dermis. Eosinophils and mast cells were rare.

Gene expression

Foxp3 and IL-4 were increased in LS ($P = 0.001$; $P = 0.008$) and NLS ($P = 0.002$; $P = 0.001$) and in addition SOCS3 ($P = 0.004$) and IL-13 ($P = 0.002$) showed higher expression in LS compared to control skin during provocation (Figure 3). Finally, IFN-γ was significantly more expressed in LS than in NLS ($P = 0.007$) during provocation. The expression of the remaining genes investigated did not differ. Expression of none of the genes was affected by the elimination diet.

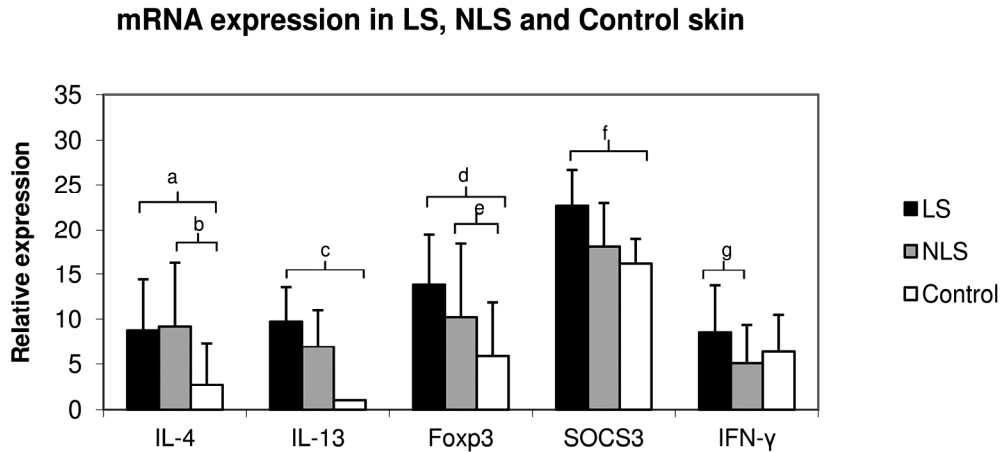


Figure 3: mRNA expression in LS, NLS skin of CAFR dogs and canine control skin. The gene expression level is depicted as the ratio of genes expressed compared to expression of IL-13 in control dogs, which is set to 1. Each bar represents the average and standard deviation. a: IL-4 LS compared to control skin ($P = 0.008$). b: IL-4 NLS compared to control skin ($P = 0.001$). c: IL-13 LS compared to control skin ($P = 0.002$). d: Foxp3 LS compared to control skin ($P = 0.001$). e: Foxp3 NLS compared to control skin ($P = 0.002$). f: SOCS3 LS compared to control skin ($P = 0.004$). g: IFN- γ LS compared to NLS skin ($P = 0.007$).

Discussion

The present study is the first to investigate gene expression and cell phenotypes in the skin of CAFR dogs. We found strong indications for a CD8-dependent inflammatory response in LS skin and observed an intertwined Th1, Th2 and Treg cytokine reaction pattern in the skin. Surprisingly, none of the dogs had a significant alteration in cytokine or transcription factor gene expression or cell phenotype in the skin after the elimination diet, despite full eradication of clinical manifestations.

Cell numbers and phenotypes and gene expression levels observed in the skin did not parallel the clinical alterations resulting from dietary elimination and provocation. The absence of dietary influence may indicate that once an animal is sensitised to develop CAFR, the skin remains in a primed immune status even after dietary elimination. This observation is supported by Olivry²³, who found in experimentally corn-hypersensitized dogs that the corn-diet did not influence

CD4⁺CCR4⁺ cell numbers in blood, despite visible clinical changes. Furthermore, gene expression does not necessarily reflect biological activity of related proteins; this may also explain why mRNA expression of cytokines and transcription factors did not parallel the clinical response to the elimination diet.

T cell numbers were highly variable in LS skin and stable in control skin, indicating that such variability is specific for canine CAFR, especially since the CAFR dogs had the same clinical starting condition and showed similar clinical responses during the dietary tests. Interestingly, the immune cell population in canine CAFR was dominated by increased numbers of CD3⁺CD8⁺, CD8αβ⁺TCR⁺ cells in LS dermis and by increased CD8γδ⁺TCR⁺ cells in LS epidermis. In a mouse model of topical allergen-induced AD, data indicated that allergen-primed CD8⁺ T cells are required for the development of AD-like lesions in mice²⁴. In addition, under different circumstances CD8⁺ T cells are able to express IL-10, TGF-β, IL-4, IL-13, Foxp3, and IFN-γ²⁵⁻²⁸. In canine AD an increased presence of CD4⁺ and CD8⁺ T cells in LS skin is reported, but the ratio of CD4⁺/CD8⁺ T cells measured in the epidermis is debated. Sinke⁹ observed an increased ratio of CD4⁺/CD8⁺ T cells, whereas Olivry⁸ found a preferential presence of CD8⁺ T cells. In addition, we observed that the IFN-γ expression in LS skin was increased in comparison to NLS skin.

We observed an increased expression of IL-4, IL-13, SOCS3 and Foxp3 genes in LS skin compared to control dogs, indicating a more Th2-skewed environment. NLS skin had increased expression of IL-4 and Foxp3, which may indicate that NLS skin is primed to become LS skin. In comparison, the cutaneous inflammation in dogs with spontaneous AD is characterized by expression of IL-6, TARC, IL-4 and IL-13 mRNA in the early stages, and IFN-γ, IL-12 and IL-18 in the chronic stages and no data are available on Foxp3 expression levels¹⁰⁻¹². Regulatory CD4⁺CD25⁺T cells are known to transcribe Foxp3 and function by inhibiting the response of T cells, resulting in a tolerant environment. We suggest that despite the increased expression of Foxp3, they are not sufficiently effective in the LS skin, because the other cytokines and transcription factors assessed did not seem to be affected. The CD4⁺CD25⁺ Foxp3 transcribing cell population has been investigated most extensively, however, a population of CD8⁺ T regulatory cells also exist. CD8⁺ T regulatory cells can produce IL-10, TGF-β, CTLA4 and CCL4 and can express Foxp3 as well^{25,29-33}. It would be interesting to investigate whether the CD8⁺ T cells are responsible for Foxp3 expression in CAFR skin.

Conclusions

In dogs with CAFR, CD8⁺ T cells are important in the initiation and maintenance of skin lesions, which is different from observations in canine AD. In addition, dietary elimination did not influence T cell profiles in the skin of dogs with CAFR, indicating a sustained pre-activated immune status once dogs have been sensitised to food.

Conflict of interest statement

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Peripheral blood mononuclear cells of dogs with cutaneous adverse food reactions show a diminished Th1 response after a provocative diet.

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Abstract

Background: The majority of dogs with adverse food reactions have only cutaneous signs (cutaneous adverse food reactions; CAFR), despite the fact that the contact with food allergens mainly occurs in the gastrointestinal tract. The blood circulation may serve as a means of transport of allergens, antigen presenting cells (APC) or activated lymphocytes to the skin. Therefore, it is assumed that cytokine profiles in peripheral blood mononuclear cells (PBMC) may reflect immune reactivity relevant in immunopathogenesis in CAFR dogs.

Materials and Methods: This study investigates the cytokine profile in PBMC after dietary provocation in CAFR and healthy dogs and the allergen-specific proliferative responses of PBMC from CAFR and healthy dogs.

Results: PBMC of CAFR dogs with clinical symptoms after a provocative dietary test showed a lower expression of Th1 genes, particularly IFN- γ , compared to that of healthy dogs. The allergen-specific proliferative responses were very low and did not differ between CAFR and healthy dogs. One case showed proliferative responses after wheat, cow's milk proteins and soy based dry milk formula stimulation of PBMC. For this CAFR case the immune responsiveness was analysed in more detail and demonstrated variations in IL-10 expression with an increase after in vitro cow's milk and soy-based dry milk stimulation and a decrease after wheat stimulation.

Conclusion: Allergen-specific proliferative responses of PBMC showed in one CAFR case variations in IL-10 reactions, suggesting that in this case IL-10 expression may be the crucial regulating cytokine. However, in general Th1 gene responses are lower in PBMC of CAFR dogs after feeding the causative diet, suggesting a reduced Th1 response is underlying the allergic response.

Introduction

Cutaneous adverse food reactions (CAFR) are considered to be the cause of up to 23% of nonseasonal allergic dermatitis in dogs¹. Among the reported dietary components leading to adverse food reactions, as determined with provocation trials, are beef, cow's milk, lamb, soy, wheat, chicken, chicken eggs and corn^{2,3}. The majority of dogs with CAFR has mainly cutaneous signs, despite the fact that the first contact with food allergen occurs in the alimentary tract. Recently, we showed in CAFR dogs that duodenal T cell phenotypes and cytokine gene expression were comparable to that in healthy dogs⁴, suggesting that the duodenal tissue is not the site where the first allergic immune reactions occur. Currently, the cause of dietary allergen-specific lymphocyte reactivity in the skin is not known. Four hypothetical options exist. One is the transport of free allergens via blood, second the transport of allergens via antigen presenting cells (APC) or exosomes^{5,6}, third allergen exposure through the skin and fourth the influx of activated lymphocytes from the intestinal lymph nodes into the skin.

Human T cells, derived from food allergic individuals, allergen-specific T cell lines and clones, show a Th2 type response after stimulation with the causative allergen, while healthy persons show a regulatory T cell or Th1 type of response after stimulation⁷. The predominance of a Th2 type reaction may be due to decreased Th1 responsiveness or reduced function of regulatory T cells⁷⁻¹¹. In spontaneously occurring CAFR in dogs the response of PBMC has not been investigated yet. In an experimentally-corn allergen-induced dog model the percentages of activated CD4⁺ T cells and CD4⁺CCR4⁺ (skin homing) T cells in corn-allergen stimulated PBMC did not differ after feeding a provocative corn diet or a reduced allergenicity diet¹². In spontaneous as well as experimentally-induced CAFR the cytokine profiles of immunocompetent cells have not been investigated so far.

In the present study we investigated allergen-specific proliferative responses of PBMC from CAFR dogs and their cytokine and transcription factor gene expression, aiming at elucidating the roles of Th2, Th1 or regulatory T cells in this disease.

Materials and Methods

Animals

CAFR in client-owned dogs was diagnosed based on the following dietary testing procedure. First, a home-cooked elimination diet existing of a novel protein (ostrich, turkey, horse or goat meat) fed for at least 8 weeks should result in at least 80%

reduction in pruritus and associated signs. Second, a provocative test with the original food had to result in reappearance of the pruritus within 2 weeks. Third, after a second elimination diet the pruritus and associated signs had to disappear for a second time within 2 weeks. None of the client-owned or control dogs had feces of abnormal consistency or other gastrointestinal clinical signs for at least 6 months before inclusion. Peripheral blood was collected from client-owned CAFR dogs and healthy control dogs in heparinized tubes by venapuncture. Two groups of dogs were used for the present studies. The first group contained 6 CAFR dogs with clinical symptoms after a provocative diet and 6 healthy control dogs. The CAFR-blood was collected after the appearance of clinical signs and was used for analysis of gene expression in PBMC *ex vivo*. The second group consisted of 4 CAFR dogs with clinical symptoms at the time of blood collection, 3 CAFR dogs without clinical symptoms and 6 healthy control dogs. Their PBMC were used for lymphocyte stimulation tests. Based on the results in this study, one case (Labrador Retriever, age 21 months, male) without clinical symptoms at the time of blood sampling was further investigated on the expressed genes and provocation tests were repeated to confirm the observed results in the lymphocyte stimulation test. The healthy control dogs were housed in a laboratory animal research facility and had no previous history of skin or intestinal diseases. The study was approved by the Utrecht University Animal Ethics Committee.

Isolation of peripheral blood mononuclear cells (PBMC)

Heparinized peripheral blood was diluted 1:1 with calcium and magnesium free Hanks-buffered saline solution (CMF-HBSS; Gibco, Invitrogen Life Technologies, Carlsbad, CA), layered on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged for 30 min at 400 g at 18 °C. Cells from the interphase were collected, washed with CMF-HBSS and finally used in a Lymphocyte Stimulation Test (LST) and for RNA isolation. For LST the cells were immediately processed by reconstitution in RPMI-1640 supplemented with 25 mM Hepes and L-Glutamine (Gibco, Invitrogen Life Technologies, Carlsbad, CA), 10% fetal calf serum (FCS; PAA Laboratories GmbH, Pasching, Austria) and 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco, Invitrogen Life Technologies, Carlsbad, CA). For RNA isolation the cells were reconstituted in 1 ml Trizol (Invitrogen Corporation, Carlsbad, CA) and stored at -70 °C until processing.

Isolation of antigens from food ingredients

Wheat gluten, wheat bran and egg powder (gift from Royal Canin, Airmarques, France), cow's milk proteins α -lactalbumin, β -lactoglobulin and casein (Sigma-Aldrich Corporation, Zwijndrecht, The Netherlands), storage mites *Lepidoglyphus destructor*, *Acarus siro*, *Tyrophagus putrescentiae* (ALK-Artu Biologicals, Lelystad, The Netherlands), soybean protein (Surpo500E, the Solae Company, Geneva, Switzerland), and soy-based dry milk formula (Nutrilon soy; Nutricia, Zoetermeer, The Netherlands) were used as antigens. Wheat gluten, egg powder, soy-based dry milk formula (30 mg each), wheat bran, and casein (50 mg each) were stirred in 10 ml cold Tris-HCl buffer of various pH, for 4 hours at 4 °C. The samples were centrifuged for 20 min at 5000 g at 4 °C. The supernatants were filter-sterilized by a 0,22 μ m filter. The other antigens were obtained lyophilised and were dissolved in sterile CMF-HBSS. The protein concentrations were measured using a Lowry-based assay (DC Protein Assay; BioRad, Veenendaal, The Netherlands). The optimum pH for isolation of proteins per ingredient was determined by comparing the isolated protein to the crude extract via SDS-PAGE according to the method of Laemmli¹³. The protein isolates were diluted to 0,25 μ g/ μ l in Laemmli-buffer supplemented with dithiothreitol (Sigma-Aldrich Corporation, Zwijndrecht, The Netherlands), whereas the crude ingredients (wheat gluten, wheat bran, egg powder, soy-based dry milk formula and casein) were directly dissolved at 0,25 μ g/ μ l in Laemmli-buffer supplemented with dithiothreitol, denatured for 3 min at 95 °C and electrophoresed on precast 15% Tris-HCl SDS PAGE polyacrylamide gels (BioRad, Veenendaal, The Netherlands). After electrophoresis the proteins were stained with Coomassie Blue R-250.

Lymphocyte Stimulation Test

PBMC were cultured in triplicate in flat-bottom 96-wells plates at a final concentration of 150.000 cells in 200 μ l RPMI-1640 supplemented with 25 mM Hepes and L-Glutamine (Gibco, Invitrogen Life Technologies, Carlsbad, CA), 10% FCS (PAA Laboratories GmbH, Pasching, Austria), 1:20.000 β -mercaptoethanol, 50 units/ml penicillin and 50 μ g/ml streptomycin (Gibco, Invitrogen Life Technologies, Carlsbad, CA) in the presence or absence of antigen in a concentration of 25 μ g/ml. As a positive control Concavalin A was used at a concentration of 2,5 μ g/ml. After 4 days of culture (37 °C, 5% CO₂ atmosphere) 0.4 μ Ci tritiated thymidine (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) was added per well. After

18 hours of incubation, cells were harvested and the incorporation of tritiated thymidine in DNA was determined with a scintillation counter. Stimulation indices (SI) were calculated as the ratio of counts per minute in antigen-stimulated PBMC and counts per minute in unstimulated PBMC.

Simultaneously, PBMC were cultured in flat-bottom 24-wells plates at a concentration of 500.000 cells at similar conditions as described before. After 2 days of culture, cells were collected, centrifuged and resuspended in 1 ml Trizol (Invitrogen Corporation) for RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated from dogs in group 1 and the individual evaluated case according to the method described by Veenhof¹⁴. Briefly, chloroform was added to the resuspended cells and centrifuged for 15 min at 12000 g at 4 °C, the aqueous phase containing RNA was collected and loaded on a RNeasy mini kit column (Qiagen Benelux BV, Venlo, The Netherlands) and RNA was isolated according to the manufacturers' protocol, including the DNase treatment (Qiagen Benelux BV, Venlo, The Netherlands). RNA was eluted in 30 µl RNase-free water and the concentration was calculated by spectrophotometry (ND-1000, NanoDrop®, Delaware). Three µg RNA was transcribed into cDNA by the I-script cDNA synthesis kit (BioRad, Veenendaal, The Netherlands) according to the manufacturers' instructions, and cDNA was aliquoted and stored at -70 °C until use for quantitative PCR.

Quantitative PCR

Oligonucleotide primers (Eurogentec, Maastricht, The Netherlands) were designed as described previously¹⁴ for IL-4, IL-13, STAT6, STAT4, IL-12p35, IL-10, IL-18, IFN-γ, SOCS5, TNF-α, Foxp3, TGF-β, GATA3, SOCS3, TARC, HPRT, RPS5 and RPS19 genes, and were designed for T-bet (F: aatcagcaccagacggagat, R: gtccacgaacatccggaat, Tm 61.2 °C) and IL-2 (F:atgagaaccccaactctcc, R: gcacttctccagggttttg, Tm 57 °C) genes. Quantitative PCR was done as described previously¹⁴ including an internal standard, which was generated by use of 4-fold dilutions of pooled cDNA from a mixture of canine concavalin A-stimulated and unstimulated PBMC, homogenates of duodenal biopsies and lesional skin biopsies. The results of each sample were normalized to the average amounts of the endogenous reference genes (HPRT, RPS5 and RPS19) of the same sample: the relative gene expression.

Statistical analysis

Expressed genes were analysed both individually and clustered according to T cell type (Th1: SOCS5, STAT4, T-bet, IFN- γ ; Th2: IL-4, IL-13, STAT6, SOCS3, GATA3; Treg: TGF- β , IL-10), with the Mann-Whitney test. All statistical analyses were done with SPSS software (Benelux, Gorinchem, The Netherlands).

Results

PBMC gene expression ex vivo

Analysis of mRNA expression in PBMC ex vivo of CAFR dogs in group 1 with clinical symptoms after a provocative diet (n=6) revealed a significant lower expression of IFN- γ ($P=0.037$) compared to control dogs (n=6). The overall Th1 expression is also significantly lower ($P=0.027$) in these CAFR dogs (Figure 1). Expression of Th2 and regulatory T cell-related genes either single or clustered, did not differ between CAFR and control dogs (data not shown).

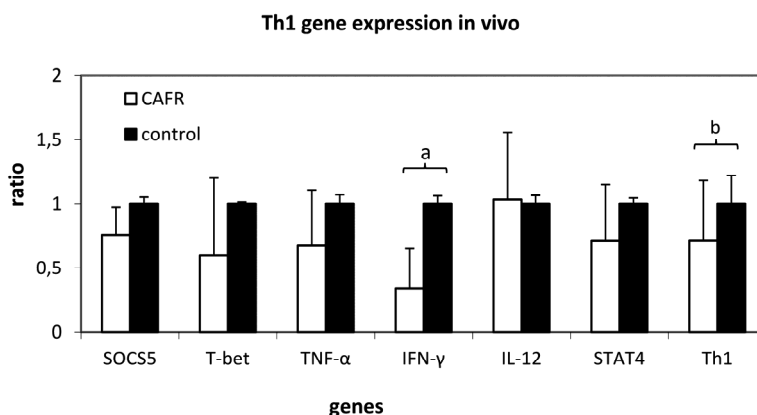


Figure 1: mRNA expression in resting PBMC ex vivo in CAFR and healthy control dogs. The gene expression level in CAFR is depicted relative to the expression in control dogs, which is set to 1. Bars represent averages and standard deviations. a: $P:0.037$; b: $P:0.027$.

Allergen-specific PBMC responses in vitro

Though with considerable variation, PBMC clearly responded to Concavalin A (median stimulation index (SI): 109, range 22-532), as a positive control stimulant. The stimulation indices of allergen-specific (median SI: 1.30, range 0.61-5.64) proliferative assays were low in the CAFR dogs (with (n=4) and without (n=3) clinical symptoms at the time of sampling) and healthy dogs (n=6), and did not differ between both groups (data not shown).

Gene expression in PBMC after in vitro allergen exposure in a CAFR case

PBMC of one dog affected with CAFR without clinical symptoms at the time of blood sampling showed increased lymphocyte responsiveness as compared to the average SI of the control dogs after stimulation with wheat (gluten and bran), cow's milk proteins and soy based dry milk formula (Figure 2).

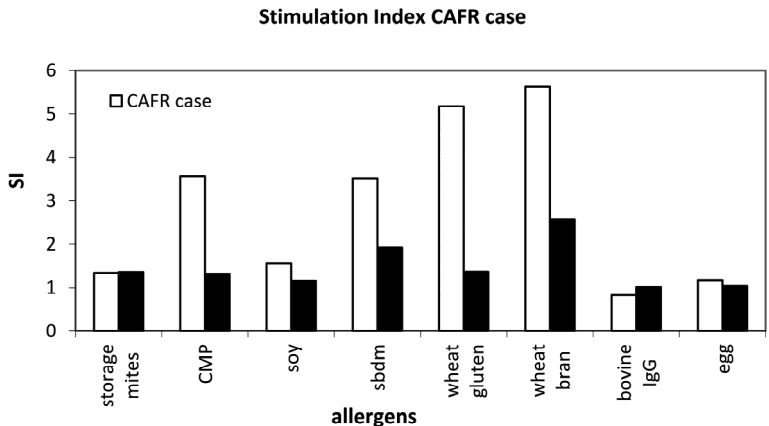


Figure 2: Stimulation Indices of lymphocytes of a CAFR case (white bars) and average of healthy control dogs (black bars) after in vitro stimulation. CMP: cow's milk proteins, sbdm: soy based dry milk.

To investigate whether the dog developed clinical symptoms after exposure to these allergens, the animal was challenged and the response reconfirmed its allergic status. The gene expression of PBMC after stimulation with cow's milk, wheat and soy-based dry milk formula were analysed in this CAFR dog (Figure 3a). Cow's milk protein stimulated PBMC showed increased SOCS3, STAT6 and IL-10 expression compared to the negative control (unstimulated PBMC). The expression of other Th2 and regulatory T cell related genes and all Th1 related gene expression after cow's milk stimulation was similar to that in the unstimulated PBMC (Figure 3a). Stimulation with soy-based dry milk formula resulted in an increased expression of SOCS3 and IL-10; other Th2 and T regulatory cell related genes as well as the Th1 genes did not differ. After wheat stimulation a decreased IL-10 expression compared to the negative control was found. The other genes did not differ. The expression of clustered Th1, Th2 and Treg related genes, revealed that the regulatory related genes were increased after cow's milk protein and soy-based dry milk formula stimulation, but were decreased after wheat bran stimulation (Figure 3b).

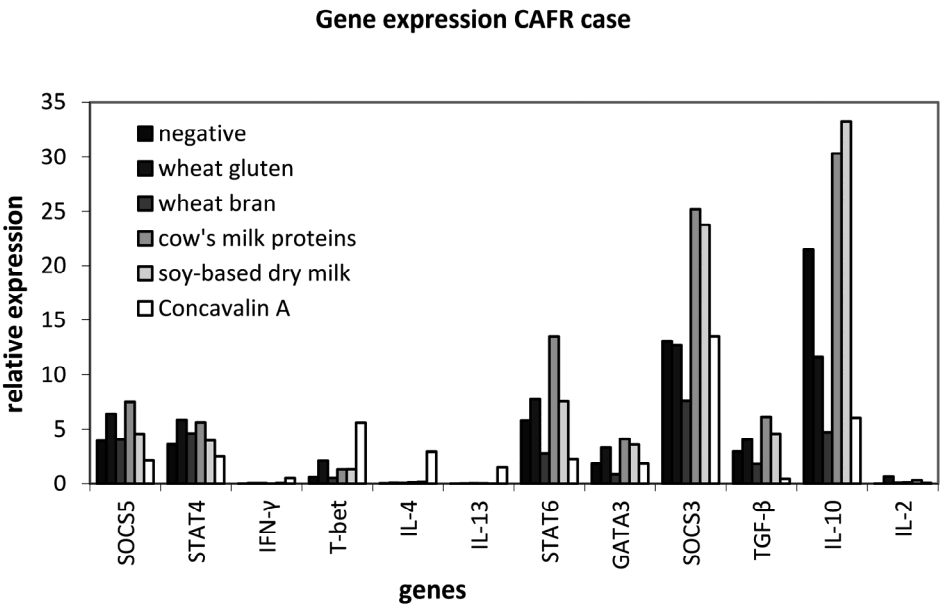


Figure 3a: Relative gene expression of the allergen-stimulated PBMC in a CAFR case.

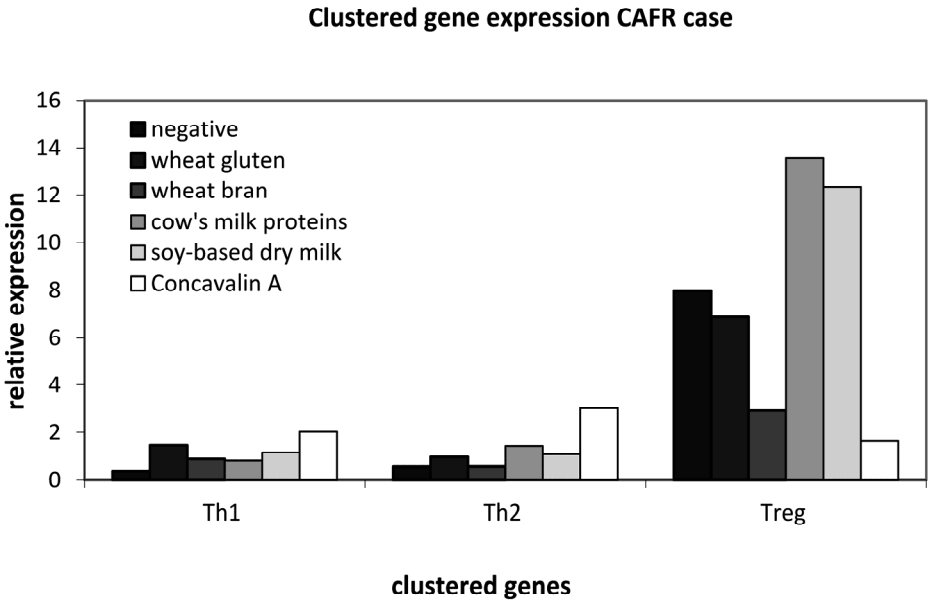


Figure 3b: Relative gene expression of the clustered Th1, Th2 and Treg genes in allergen-stimulated PBMC in a CAFR case.

Discussion

Food hypersensitivity is generally believed to be caused by a diminished Th1 response leading to Th2 reactions, or by a decreased response of regulatory T cells to food ingredients. Analysis of the differential gene expressions in PBMC in CAFR dogs seems to confirm this idea. It did not indicate increased Th2 or decreased T regulatory cell responses to food allergens, but rather decreased expression of IFN- γ , suggesting a defective Th1 response.

In humans, a low or diminished IFN- γ expression in allergy has been observed in allergic patients¹⁵⁻¹⁸. It was observed that children that became tolerant to their allergens and non-allergic children showed increased Th1 responses after stimulation with allergens, while allergic children show Th2 responses^{8,10,11}. Recently a role for a defective regulatory response instead of solely a diminished Th1 response has been shown^{9,19,20}, indicating that the role of T cells in allergy is not straightforward and possibly includes more than one type of deranged T cell reaction (e.g. Th1, Treg). In our study a diminished Th1 response was observed in CAFR dogs and no differences were observed in Th2 or T regulatory gene expression between CAFR and healthy dogs, suggesting that the low Th1 response in this study possibly altered the reactivity of T cells and may have resulted in allergy.

The current diagnostic procedure to identify food hypersensitivity in dogs is limited to dietary elimination and provocation tests, in which the causative allergen is not specified. Previously, Ishida²¹ suggested that Lymphocyte Stimulation Tests (LST) with individual food ingredients in dogs after provocation can be used to help identifying the allergens responsible for the clinical symptoms. To investigate the allergen-specific responses we performed LST on PBMC of CAFR dogs with a range of ingredients known to be involved in CAFR dogs^{2,3}. Our results show that the proliferative response was very limited and responses did not significantly differ from those in control dogs. One of the reasons might be that the control dogs responded to the allergens, a phenomenon also known in human allergy²². One possibility for the poor proliferation can be that the frequency of allergen-specific T cells in blood is very low⁷. Another possibility could be the clinical status of the CAFR dogs, because not all dogs showed clinical symptoms at the time blood was collected. However, another study showed that there were no significant differences in CD4 T cell stimulation indexes between dogs exposed to a corn containing diet and to a hypoallergenic diet after *in vitro* stimulation of PBMC from experimentally induced corn-allergic food allergy in dogs despite the presence or absence of clinical

symptoms¹². Interestingly, one case of the investigated CAFR dogs in this study did show more profound proliferation and this case had no clinical symptoms at the time of PBMC collection. The reason that this dog showed proliferation in contrast to the other CAFR dogs cannot be explained since the dog did not differ in breed, age, predilection sites or clinical diagnosis from the other tested CAFR dogs. The gene expression of the CAFR case, which had no clinical symptoms at the time of testing, but responded to allergen-specific stimulation, was further investigated. In particular, the IL-10 expression was increased after cow's milk protein and soy-based dry milk stimulation and decreased after wheat stimulation. In cow's milk allergic children that have overcome their allergy increased IL-10 has been observed as well⁹. IL-10 is regarded as one of the immunosuppressive cytokines, but the other oral tolerance related genes were not increased. Moreover, the IL-10 expression was decreased after wheat stimulation, indicating that immune responses may be specific for the type of allergen exposure. Some Th2-type transcription factors were increased after cow's milk protein stimulation compared to the unstimulated PBMC of this CAFR case, however the Th2-cytokine expression levels did not change, a discrepancy we can not explain. The decreased Th1 gene expression observed in unstimulated PBMC ex vivo, was not seen in this specific case.

In conclusion, allergen stimulation of PBMC did not show differences in responses between CAFR and control dogs, with the exception of one dog with a higher response to cow's milk proteins, soy-based dry milk and wheat. This dog did not show clinical symptoms at the time of sampling. Its variable IL-10 expression upon allergen-specific PBMC proliferation, suggests a role for this immunomodulatory cytokine. PBMC of CAFR dogs collected after a provocation diet showed decreased expression of Th1-related genes, IFN- γ in particular, indicating a diminished Th1 cell response in CAFR.

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6

Summarizing discussion

Adverse food reactions are undesired reactions in the body to specific food constituents. Common types of adverse food reactions are food allergy and food intolerance. Food allergy is caused by immune reactions to food antigens (also called allergens), food intolerance is caused by pharmacological reactions to food as well as its impaired enzymatic digestion¹. Since the clinical symptoms of food allergy and food intolerance can be quite similar, it is not always possible to differentiate between both conditions. The majority of dogs with adverse food reactions shows cutaneous signs, the cutaneous adverse food reactions (CAFR). In only 10-15 percent of dogs gastrointestinal symptoms are observed as well^{2,3}. Since the first confrontation between food allergens and the local immune system most likely occurs in the intestine, the low incidence of gastrointestinal symptoms in CAFR is remarkable. In contrast, the high incidence of cutaneous signs suggests that the antigens, antigen presenting cells (APC) and/or activated T cells are able to travel to the skin. However, the mechanism behind this trafficking of cells and proteins to skin and other organs remains to be determined.

CAFR in dogs is similar to atopic dermatitis (AD) in its distribution and appearance of clinical symptoms in 65% of the patients⁴. The immunopathogenesis of AD has been investigated more extensively than that of CAFR. AD is associated with increased levels of serum allergen-specific IgE, whereas in CAFR dogs, there is only limited evidence for a role of IgE in the pathogenesis^{2, 5-7}. This suggests that other immune mechanisms are involved in CAFR compared to AD. Canine CAFR is currently diagnosed by dietary elimination and provocation tests. The clinical signs should disappear after feeding an elimination diet for 8-10 weeks. Subsequently, skin signs (mainly pruritus) should re-appear after feeding the original food (provocation) and should disappear again when feeding the elimination diet for the second time. In human juveniles, the immature status of the immune system combined with the 'leaky' immature gut is considered a risk factor to develop intestinal immune reactivity to food antigens when introduced early in life⁸. In these individuals intestinal food allergy is associated with an active Th2 reaction. In T cell cultures and in intestinal lymphocyte populations from allergic persons impaired Th1 or Treg responses have been observed after allergen challenge⁹⁻¹³. This suggests that intestinal food allergy is caused by a loss of tolerance, a failure to induce tolerance or a decreased Th1 responsiveness, giving allergen specific Th2 cells the opportunity to develop. However, the decreased Th1 or impaired Treg responses may also be the outcome rather than the initial cause of development of allergy.

Tissue homeostasis (skin, intestine) is well maintained, involving many cells, cytokines and homing molecules. The immunopathogenesis of CAFR in the intestine and/or the skin is unknown. The aim of the research presented in this thesis was to understand the pathogenesis of CAFR in dogs with and without clinical symptoms by investigating the T cell activity profile in the intestine, skin and blood.

The profile of intestinal T cell populations in CAFR

Food allergy with intestinal symptoms, such as diarrhea, has been investigated in several species. The intestinal lymphocytes of food allergic children with cow's milk allergy show a Th2 cytokine profile¹⁴. In addition, decreased TGF- β -expression was observed in duodenal epithelial and lamina propria lymphocytes and epithelial cells from children with multiple types of food allergies as compared to healthy children¹². In general it is believed that a disruption of the normal intestinal suppressive immune environment may lead to intestinal food hypersensitivity⁹⁻¹³. Remarkably, the intestine of food allergic patients without intestinal signs, has not been investigated yet. In chapter 3 we studied the duodenal T cell responses in CAFR dogs. It was found that both the duodenal epithelium and the lamina propria did not differ in terms of T cell type, and cytokine and transcription factor expression between CAFR and control dogs. Surprisingly, exposure to and withdrawal of the causative diet and the concurrent changes in clinical cutaneous signs (mainly pruritus), did not affect these T cell profiles during the observation periods in this study. Consequently, the Th2 profile and decreased TGF- β -expression indicating a loss of oral tolerance as demonstrated in human intestinal food allergy¹², was not observed in canine CAFR. An explanation is that in food allergic human children intestinal signs are evident, suggesting that changes in immune responses are expected to be found in the duodenum when compared to healthy children, whereas in dogs CAFR is characterized by skin symptoms only. The healthy intestine is characterized as an immunosuppressive environment that generates tolerance to several food constituents and bacterial substances. Thousands of foreign proteins pass the intestine yearly in huge quantities¹⁵, and the intestinal immune environment enables the proteins to pass through without causing adverse reactions. Moreover, billions of bacteria lining the intestinal lumen in healthy individuals and their produced toxins, do not induce immune responsiveness¹⁵. This immunosuppressive environment is largely maintained by dendritic cells (DC) and the intestinal epithelium¹⁶⁻²¹. Moreover, DC in the mesenteric lymph nodes (MLN), which may have migrated from

the lamina propria, can stimulate differentiation of naive T cells into Treg^{18,22,23}. In spite of such an immunosuppressive environment, in CAFR dogs a food allergen is potentially able to induce a Th2 reaction. However, based on our non-conclusive findings we suggest that the local intestinal response is strictly controlled in the intestine. This is not surprising since we do not observe clinical intestinal signs in CAFR dogs. The reason why food allergic humans have clinical intestinal signs is not clear.

The profile of cutaneous T cell populations in CAFR

Since the intestine does not seem to be affected both at the clinical and at the cellular level, we focused on the affected skin tissue, to analyze local immune responsiveness. The skin of CAFR dogs has not been investigated previously, but the clinical signs and predilection sites of CAFR are frequently similar to those of canine atopic dermatitis (AD). Hence, it was hypothesized that the cutaneous immune response in dogs with CAFR might be similar to AD. Canine AD is characterized by an influx of both CD4⁺ and CD8⁺ T cells in the dermis of lesional and non-lesional skin with a predominance of CD4⁺ T cells in the lesional dermis^{24,25}. However, as described in chapter 4 we observed a predominant CD8⁺ T cell influx in the lesional skin of CAFR instead of a CD4⁺ and CD8⁺ T cell influx. Although we found increased CD8⁺ T cell numbers, this was not accompanied by an apparent increase in the cytokine IFN- γ . More precisely, analysis of the lesional skin of CAFR dogs showed an increased expression of IL-4, IL-13, SOCS3 and Foxp3 genes compared to control dogs. In addition, the expression of Foxp3 and IL-4 is already increased in non-lesional skin, which does not contain the CD8⁺ T cell influx like in lesional skin. The only difference in gene expression between these two tissue types is the IL-13 and SOCS3 gene expression. IL-4, IL-13 and SOCS3 are genes mainly expressed by Th2 cells, and since we did not observe an increase in CD4⁺ T cell numbers we suggest that the cytokines are expressed by mast cells, or by T cells, neutrophils and basophils already present in the skin²⁶⁻²⁸. In addition, the epidermal keratinocytes in AD produce TSLP, chemokines and pro-inflammatory cytokines following exposure to environmental factors and the production of these cytokines and chemokines will lead to a predominant Th2-type inflammatory response²⁹. The reduced expression of the suppressive cytokine TGF- β described in AD compared with healthy skin³⁰, was not observed in CAFR. Moreover, the increased expression of the Treg transcription factor Foxp3 in lesional and nonlesional CAFR skin is not seen in AD³¹. Despite the

increase in this immunosuppressive transcription factor, the allergic reaction still develops. Thus we suggest that the regulatory T cells are not sufficiently effective in the CAFR lesional skin to suppress the immune activities of the other cells. An insufficient Treg response has also been observed in autoimmune diseases where the infection continues while Treg numbers are increased³². Beside CD4⁺ regulatory T cells, Foxp3 expressing regulatory CD8⁺ T cells also exist^{33,34}. Since CD8⁺ T cell numbers were raised in CAFR skin, the increase in Foxp3 expression in lesional and non-lesional skin may be due to influx of a CD8⁺ regulatory T cell population in CAFR skin.

In canine atopic dermatitis a predominant Th2 environment is found in the early stages of the disease, as indicated by the expression of TARC, IL-4 and IL-13. In contrast, in the chronic AD stage the skin reaction is dominated by the expression of IFN- γ , IL-12 and IL-18, indicating a switch from a Th2 dominated to predominant Th1 reactivity over time^{30,35,36}. The lesional biopsies of our studies should be regarded as acute, since they were collected within 10 days after onset of pruritus following the dietary provocation. Despite the induction of pruritus, we did not observe the expected concurrent changes in T cell profile in the skin. Although differences in cell numbers and cytokine expression were found between lesional and non-lesional skin, in both skin types a Th2-like gene expression with increased Foxp3 was observed. These results suggest that a switch from a Th2-like acute to a Th1-like chronic reaction may not occur in CAFR. In contrast to our hypothesis that the immunopathogenesis of CAFR and AD is similar, our findings show a significant difference regarding the T cells involved and their cytokines.

In AD it is generally believed that in a large part of the patient population an epidermal barrier defect results, in combination with environmental factors, in an increased exposure to foreign antigens through the skin^{37,38}. In addition, it is possible to induce food allergy in mice by epicutaneous allergen exposure³⁹. It should be realized that in dogs as opposed to humans a direct contact between food components and the skin (muzzle and lips) and the oral mucosa is clear. Consequently, food allergens will be present in saliva⁴⁰ which following licking are distributed over the body surface. Anticipating that a skin barrier defect would also be present in CAFR, this could not only explain an immune response restricted to the skin, but also the clinical distribution pattern. Moreover, the lack of intestinal signs in CAFR can be explained if the exposure to allergen occurs via the skin. Hence, it would be interesting to investigate if allergen uptake via the skin occurs and whether an

epithelial barrier defect is indeed also present in CAFR. In addition, the activation state of T cells and other cell types and the actual cytokine expression rather than gene expression levels, need to be investigated to get a better insight in the immune environment and responses in the skin.

Besides the intestinal and cutaneous route of entry, in humans it has been shown that allergens may enter via other tissues as well. For instance, atopic dermatitis may be induced via inhalation of the relevant allergen⁴¹ and respiratory allergy to lipid transfer protein may induce food allergy to fruit⁴². However, this pathway is unlikely in dogs as respiratory allergies are very uncommon and the so-called ‘atopic march’ is not present in this species.

The link between intestine and skin.

It is highly likely that the majority of food allergens is absorbed through the intestine. Despite this, the most important clinical symptoms of canine CAFR are observed in the skin and the connection between these sites needs elucidation. Most antigens are actively transported via the M cells into the dome region of the Peyer’s patches and become internalized in DC. The DC may present the antigen to T and B cells in the Peyer’s patch⁴³ or migrate to the MLN and present the antigen to T and B cells at that site^{44,45}. In addition to active transportation by M cells, antigens can pass from the lumen into intestinal tissue through the lamina propria via dendritic cells that sample antigens in the intestinal lumen with their dendrites^{46,47}, by direct interaction with epithelial cells⁴⁸ or through internalization of epithelium-derived exosomes^{49,50}. DC containing allergens may be transported to peripheral lymph nodes via blood and lymph⁵¹, but most will migrate to the nearest lymph nodes, the intestinal MLN, first. The majority of the MLN-derived activated T cells are imprinted via the DC to home to the lamina propria, thus will remain in the intestine and not migrate to other sites in the body⁵². This suggests that allergen specific T cells mainly remain in the intestine instead of being transported to other tissues. However, it is believed that activated T cells may migrate to other tissues than the intestine as well, although this pathway is not proven yet. In mice, it was shown that CD8⁺ T-cell activation in the presence of antigen-pulsed DC from non-intestinal tissues leads to expression of skin homing selectin ligands, suggesting that in mice the CD8⁺ T cells activated by DC will by default express skin homing ligands⁵³. Although farfetched, if this is similar in dogs, then the CD8⁺ T cell influx in lesional skin could be explained by skin-homing CD8⁺ T cells that were activated by DC in non-intestinal tissues. An

affected intestinal barrier has been observed e.g. in inflammatory bowel disease⁵⁴, increasing the possibility of foreign allergens to enter the intestinal tissue. However, we did not observe intestinal infection or inflammation in CAFR, thus a role of a leaky intestinal barrier seems unlikely. In addition, we observed that the cutaneous clinical symptoms that were induced by dietary provocation did not result in differences in T cell profile and numbers in the skin and intestine compared to the phase with absent clinical symptoms after following an elimination diet. This suggests that immune cells other than T cells may play a role in flaring up of the disease. However, IgE-mediated mast cell reactions (including late-IgE reactions) are less likely as recurrence of pruritus after provocation was not observed within hours. In such case, a release of mast cells substances after crosslinking the allergen (if free allergen is transported to the skin or enters skin after direct contact of skin with the food) would result in a rapid cutaneous reaction. Even delayed-type reactions, which take more than 24 hours to induce clinical symptoms, are not likely in CAFR. Such reactions would result in changes in T cell profiles after an elimination and provocation diet, but were not observed in our study. Mast cells can also degranulate following exposure to other molecules than IgE, such as anaphylatoxins, bacterial and viral antigens, cytokines, neuropeptides and immunoglobulin-free light chains^{55,56}. Since pruritus is most likely caused by a release of the mast cell substances, but the reaction in CAFR is slow, possibly the above mentioned other molecules can cause a delayed mast cell degranulation in CAFR.

Since free allergen, DC containing allergen or T cells, may migrate to the skin via the blood circulation, we analyzed in Chapter 5 the gene expression of PBMC in CAFR dogs after re-occurrence of clinical signs following a provocation diet. The Th1 related genes, especially IFN- γ were decreased after re-occurrence of the clinical signs, compared to the expression found in the healthy dogs. This apparent decrease in Th1 cytokines may have led to increased skewing to Th2 responsiveness and re-occurrence of the clinical signs. Allergen-specific proliferative responses in PBMC of CAFR dogs were also investigated in chapter 5. The proliferative response was generally very low. In addition, there was no difference between CAFR and healthy dogs, which may be explained by a very low frequency of allergen-specific T cells in blood, as demonstrated before¹⁰.

Conclusions

Cutaneous adverse food reactions (CAFR) in dogs	<ul style="list-style-type: none">- are not the result of intestinal T cell dysfunction.- are characterized by a mixed Th2 and Treg cutaneous gene expression profile.- differ from atopic dermatitis as the cutaneous lesions are dominated by CD8⁺ T cell infiltration.- are paralleled by a decreased Th1 response in peripheral blood.
In addition,	<ul style="list-style-type: none">- clinical signs are not paralleled by changes in intestinal, blood and cutaneous T cell and cytokine profiles.- no immunological relationship between intestine, blood and skin in dogs with CAFR was found.
Future research is needed,	<ul style="list-style-type: none">- to show the potential regulatory function of cutaneous CD8⁺ T cells.- to investigate if an impaired skin barrier is at the basis of CAFR pathogenesis.

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Nederlandse samenvatting

Ongewenste reacties van het lichaam tegen voedselbestanddelen komen bij ongeveer 8% van de honden voor^{1,2}. In de volksmond wordt deze reactie een voedselallergie genoemd. Het is echter vaak onduidelijk of het om een echte allergie of een voedselintolerantie gaat. Voedselallergie is een ongewenste reactie van het afweersysteem tegen voedingsbestanddelen (allergenen), terwijl bij een voedselintolerantie het afweersysteem niet direct betrokken is. Een voorbeeld van voedselintolerantie is een enzymstoornis (zoals lactosedeficiëntie) die de normale afbraak van voedselbestanddelen beïnvloedt en leidt tot klinische verschijnselen³. In honden kunnen de symptomen van voedselallergie en voedselintolerantie dezelfde zijn, dus het is moeilijk om deze twee aandoeningen op grond hiervan te onderscheiden. Vandaar dat in dit proefschrift de algemene term ‘adverse food reactions’ (AFR: ongewenste reacties tegen voedsel) gebruikt wordt. De meeste honden met dergelijke reacties hebben alleen jeuk en huidsymptomen. Slechts bij 10-15% van deze honden komen ook darmproblemen voor^{4,5}. Dit is opvallend, aangezien het grootste gedeelte van het voedsel via de darm wordt opgenomen, zou men juist daar de meeste reacties verwachten. De honden die enkel huidsymptomen en geen darmsymptomen hebben, worden in dit proefschrift aangeduid als CAFR honden (CAFR: cutaneous adverse food reactions; ongewenste huidreacties tegen voedsel). De diagnose CAFR wordt bij honden gesteld door middel van eliminatie- en provocatie dieet testen. Allereerst wordt een eliminatiedieet gegeven gedurende 8-10 weken waarbij de symptomen verdwijnen. Daarna wordt het originele voer gegeven als provocatie, de symptomen keren terug. Vervolgens wordt het eliminatiedieet opnieuw gegeven zodat de symptomen nogmaals verdwijnen. Op deze manier wordt aangetoond dat in het originele voer een component aanwezig is die aanleiding is tot de jeuk en de huidverschijnselen.

De huidsymptomen van honden met CAFR lijken qua uiterlijk en voorkeursplaatsen in 65% van de gevallen sterk op die bij constitutioneel eczeem (atopische dermatitis; AD)². De achtergrond en onderliggende afweerreactie bij AD is veel uitgebreider onderzocht dan die bij CAFR. AD wordt geassocieerd met een verhoogd gehalte aan specifieke antistoffen (immunoglobuline E; IgE) in het bloed, terwijl er bij CAFR slechts beperkte aanwijzingen zijn dat IgE een rol speelt in de ontwikkeling van de allergische reactie⁵⁻⁸. In ieder geval kan door het meten van het allergeen-specifiek IgE in bloed van CAFR honden niet voorspeld worden waarvoor de hond allergisch is. Dit suggereert dat ondanks de overeenkomst in symptomen er

toch verschillen zijn in het ontstaan en de ontwikkeling van de afweerreacties bij CAFR en AD in honden.

Een sub populatie van de witte bloedcellen, de T lymfocyten, speelt een belangrijke rol in het onderhouden van het evenwicht binnen het afweersysteem en bij reacties tegen ziekteverwekkers, allergenen en lichaamseigen moleculen. Dit doen zij door directe interactie met andere cellen en/of door de productie van eiwitten (cytokines of interleukines), als boodschapper moleculen voor andere cellen betrokken bij de afweer. De T cellen kunnen in de volgende hoofdtypen onderverdeeld worden: CD8⁺ T cellen, betrokken bij de afweer tegen bijvoorbeeld virussen, die met name functioneren door de geïnfecteerde cel te doden, en CD4⁺ T cellen (T helper cellen) die betrokken zijn bij het aansturen van andere afweercellen en een rol spelen bij allergische reacties. Er zijn verschillende subtypen CD4⁺ T cellen die elk een speciale functie hebben en specifieke cytokines produceren. Zo produceren T helper type 1 cellen (Th1) IFN- γ , IL-18 en TNF- α ; Th2 maken IL-4, IL-5 en IL-13; Th17 cellen IL-17A, IL-17F en IL-22 en T regulatoire cellen (Treg) TGF- β en IL-10⁹⁻¹¹. Onder normale omstandigheden houden deze subtypen elkaar in evenwicht, maar bij een allergische reactie is dit verstoord. Met name Th2 cellen en hun cytokines zijn betrokken bij allergische reacties. De rol van de T cellen en de ontwikkeling van de afweerreacties bij CAFR in de darm, bloed en/of de huid is onbekend. Het doel van het onderzoek gepresenteerd in dit proefschrift was om inzicht te krijgen in de wijze van ontstaan en ontwikkeling van de afweerreacties bij CAFR en de rol van T cellen daarin in darm, huid en bloed bij honden, in aan- of afwezigheid van symptomen.

Het T cel profiel in de dunne darm bij honden met CAFR

De darm komt dagelijks in aanraking met grote hoeveelheden lichaamsvreemde stoffen, bacteriën en hun toxines¹². Het afweersysteem in de darm onderdrukt normaliter reacties tegen deze stoffen en kiemen; dit wordt orale tolerantie genoemd. In tegenstelling tot de situatie bij honden, wordt bij mensen vaak een reactie tegen voedselbestanddelen gezien in de darmen en is er sprake van een echte (intestinale) allergie. Naar deze specifieke vorm van allergie is veel onderzoek verricht¹³⁻¹⁷. Dit is echter niet het geval voor die vormen van voedselallergie waarbij de darmen niet aangedaan zijn, zoals bij voedsel-gerelateerd eczeem. Algemeen wordt aangenomen dat een verstoring in de normaal tolerante darm kan leiden tot een intestinale voedselallergie of tot andere typen darmontsteking. Bij kinderen met

koemelkallergie wordt een Th2 cytokine profiel gezien¹⁸. Daarnaast is ook een verminderde TGF- β (door Treg geproduceerd) expressie gevonden bij darmlymfocyten afkomstig van kinderen met verschillende soorten voedselallergie in vergelijking met gezonde kinderen¹⁹. Dit suggereert dat intestinale voedselallergie veroorzaakt kan worden door een verminderde Treg activiteit. In hoofdstuk 3 werd onderzocht of er een verschil in de betrokkenheid van T cel subtypen kon worden vastgesteld in de darm van honden met CAFR. Er werd vastgesteld dat T cel subtypen, cytokines en transcriptiefactoren (betrokken bij de sturing van T cel ontwikkeling en cytokine productie) in de twaalfvingerige darm niet verschilden tussen CAFR en controle honden. Bovendien bleek, dat de T cel profielen in de darm van CAFR honden ook niet beïnvloed werden door het provocatie- en eliminatie dieet, ondanks het respectievelijke verschijnen en verdwijnen van de huidsymptomen.

Een verminderde TGF- β of verhoogde Th2 expressie, zoals aangetoond in intestinale voedselallergie bij de mens^{18,19}, is niet waargenomen op darmniveau bij honden met CAFR. Bij de mens zijn darmsymptomen aanwezig bij intestinale voedselallergie, waardoor veranderingen in het intestinale afweersysteem te verwachten zijn, terwijl honden met CAFR alleen huidsymptomen hebben. Een verklaring daarvoor zou kunnen zijn dat bij de hond, door een krachtig gereguleerde afweer in de darmen, de symptomen in de darm worden gecontroleerd of voorkomen.

Het T cel profiel in de huid bij honden met CAFR

Het ontstekingsmechanisme in de huid van CAFR honden is nog niet eerder onderzocht, maar omdat de symptomen in 65% van de gevallen vergelijkbaar zijn met die van AD², kunnen de afweerreacties van beide aandoeningen mogelijk ook overeenkomen. Bij AD wordt de huidreactie gekarakteriseerd door een instroom van zowel CD4⁺ als CD8⁺ T cellen in lesionale (waar eczeem zichtbaar is) en niet-lesionale huid (geen eczeem), waarbij de CD4⁺ T cellen in lesionale huid overheersen^{20,21}. Daarnaast zien we in de lesionale huid in de vroege fase met name Th2-gerelateerde cytokine expressie en in de chronische fase Th1-gerelateerde²²⁻²⁴. Dit wijst op een overgang van een in eerste instantie door Th2 cellen gedomineerde reactie naar een door Th1 cellen gedomineerde reactie in de chronische fase. Verder is de TGF- β expressie verlaagd wat suggereert dat er minder onderdrukking is door Treg activiteit²³. In hoofdstuk 2 is onderzocht welke genen het beste als referentiegenen

gebruikt kunnen worden bij het meten van de genexpressie in de huid. In hoofdstuk 4 zijn T cellen van lesionale en niet-lesionale huid van CAFR honden onderzocht, gedurende perioden van aan- en afwezigheid van de symptomen (met name jeuk). In tegenstelling tot bij AD, werd in CAFR vooral een influx van CD8⁺ T cellen gezien in lesionale huid en overheersten de CD4⁺ T cellen niet. Naast karakterisatie van de cellen werd ook de cytokine expressie onderzocht. De lesionale huid vertoonde een verhoogde expressie van meerdere Th2 type genen (IL-4, IL-13 en SOCS3) en een Treg gen (Foxp3) in vergelijking met huid van controle honden, terwijl de niet-lesionale huid een verhoogde Treg (Foxp3) en één Th2 (IL-4) gen expressie vertoonde in vergelijking met controle huid. Ondanks de aanwezigheid en vervolgens juist afwezigheid van symptomen tijdens de provocatie-eliminatie procedure, zijn er geen veranderingen waargenomen in de onderzochte afweer parameters in lesionale en niet-lesionale huid.

Een cytokine dat onder andere door CD8⁺ T cellen wordt geproduceerd is IFN- γ ²⁵. Ondanks de verhoogde aantallen CD8⁺ T cellen in lesionale huid werd er geen toename van IFN- γ expressie gezien. Daarentegen zijn juist de Th2 en Treg-gerelateerde genexpressie verhoogd, dit terwijl de CD4⁺ T cel populatie niet is toegenomen. Deze waarneming zou verklaard kunnen worden doordat sommige van de in verhoogde mate geproduceerde cytokines ook door andere afweercellen, dan T cellen, geproduceerd kunnen worden²⁶⁻²⁸. De toegenomen Foxp3 expressie wijst op een toename in Treg, aangezien deze transcriptie factor niet door andere afweercellen geproduceerd wordt. Echter Treg-specifieke cytokine genexpressie is niet verhoogd. Bovendien blijft de hond symptomen ontwikkelen. Mogelijk zijn de Treg cellen in lesionale huid van CAFR niet voldoende werkzaam om het afweersysteem te onderdrukken, zoals wel wordt beschreven bij auto-immuun ziekten²⁹. Naast CD4⁺ regulatoire T cellen zouden CD8⁺ Treg ook betrokken kunnen zijn^{30,31}, mogelijk zijn de verhoogde aantallen CD8⁺ T cellen in de huid wel CD8⁺ Treg cellen die niet voldoende werkzaam zijn. Een andere mogelijkheid is dat Foxp3 door CD4⁺ T cellen, zodra ze geactiveerd worden, korte tijd geproduceerd wordt, dus de recent geactiveerde T cellen in de huid markeert³².

Bij AD is in de afgelopen jaren aangetoond dat een groot deel van de patiëntenpopulatie een epidermale barrière defect heeft, gekarakteriseerd door een verhoogde doorlaatbaarheid van de buitenste laag van de huid (epidermis)^{33,34}. Dit defect in combinatie met de invloed van omgevingsfactoren (zoals aanwezigheid van bacteriën en gisten), kan ertoe leiden dat allergenen gemakkelijk via de huid

binnendringen en zo een allergische reactie aanzwengelen. Ook blijkt het mogelijk om voedselallergie bij muizen te induceren door de huid rechtstreeks in contact te brengen met voedsel eiwitten³⁵. De vraag is of een defecte barrière functie van de huid ook een rol zou kunnen spelen bij honden met CAFR. In dat licht bezien is er bij honden immers een direct contact tussen voedselbestanddelen en de huid (snuit en lippen) en het mondslijmvlies, waarbij voedselallergenen bovendien via speeksel door middel van likken over het lichaam verspreid kunnen worden. Indien er bij CAFR (net als bij AD) ook een defecte barrière functie van de huid is, dan zou deze niet alleen verklaren waarom er met name huidsymptomen bij deze groep voorkomen, maar ook de plaatsen waar de huidsymptomen optreden.

De link tussen darm en huid.

Het is zeer waarschijnlijk dat de meerderheid van voedselallergenen door de darm wordt opgenomen en aangezien de symptomen in huid voorkomen, verdient de verbinding tussen darm en huid, naast de eerder genoemde defecte huid barrière, opheldering. De meeste voedselbestanddelen worden na opname in de darm door speciale afweercellen (genaamd antigeen presenterende cellen) opgenomen en aan T cellen gepresenteerd in lymfeknopen in de darm (mesenteriale lymfeknopen)^{36,37}. De meerderheid van deze T cellen zal terugkeren naar de darm en zal niet naar andere lichaamsdelen migreren³⁸, dus de kans dat een allergeen specifieke in de darm geactiveerde T cel naar de huid migreert is klein.

Naast de presentatie van allergeen door antigeen presenterende cellen aan T cellen in de mesenteriale lymfeknopen in de darm, zijn er nog een aantal andere manieren waarop voedselbestanddelen door de darm worden opgenomen, waarna ze via de bloedsomloop naar de huid kunnen migreren³⁹⁻⁴³. In hoofdstuk 5 is de genexpressie beschreven van witte bloedcellen in de bloedcirculatie van CAFR honden op het moment dat ze symptomen vertoonden. Voorlopige resultaten laten zien dat de Th1 gerelateerde cytokine expressie was afgenomen na het optreden van de symptomen in vergelijking met de expressie in de gezonde honden. Deze afname van Th1 cytokine expressie kan aanleiding zijn geweest voor het ontstaan van de klinische symptomen, ondanks het feit dat er geen verhoogde Th2 cytokine expressie in bloed is gevonden. Vervolgens hebben we in hoofdstuk 5 ook bestudeerd of de T cellen uit bloed van CAFR honden zich gingen vermenigvuldigen (celdeling) nadat ze aan allergenen werden blootgesteld. Over het algemeen was de celdeling erg laag en was er geen verschil tussen de CAFR en gezonde honden. Dit kan mogelijk worden

verklaard doordat in het bloed allergeen-specifieke T cellen sporadisch voorkomen⁴⁴. In dit onderzoek kon de bloedsomloop niet nadrukkelijk worden geïdentificeerd als intermediair tussen darm en huid.

Conclusies van het gepresenteerde onderzoek

Ongewenste huidreacties tegen voedsel (CAFR) bij honden	<ul style="list-style-type: none"> - zijn niet het resultaat van een afwijkende T cel functie in de dunne darm. - worden gekarakteriseerd door een gemengd Th2- en Treg cytokine profile in de huid. - verschillen van atopische dermatitis, omdat de lesionale huid gedomineerd wordt door CD8⁺ T cel-infiltratie. - gaan gepaard met een verminderde Th1 respons in bloed.
Daarnaast,	<ul style="list-style-type: none"> - worden veranderingen in symptomen ten gevolge van eliminatie en provocatie dieet niet gereflecteerd in darm, bloed, en huid T cel karakteristieken. - Zijn er geen relaties in afweerrespons tussen darm, bloed en huid bij honden met CAFR gevonden.
Toekomstig onderzoek is nodig,	<ul style="list-style-type: none"> - om de potentieel regulatoire functie van CD8⁺ T-cellen in de huid te laten zien. - om de hypothese te testen dat bij honden met CAFR een verminderde barrièrefunctie van de huid de basis is voor de pathogenese.

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**Dankwoord
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Abbreviations**

Dankwoord

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Curriculum Vitae

Eveline Zwaantje Veenhof was born on 29 May 1978 in Vleuten-De Meern, the Netherlands. She attended secondary school at the St. Gregorius College in Utrecht (1990-1996). In 1996 she started the study Medical Biology at the Free University in Amsterdam, which she finalized in 2001 with a Master of Science degree. During this study two scientific researches were done. The first was focused on the identification of *Helicobacter pylori* genes that were responsible for the biosynthesis of the phase-variable type I epitopes. During the second research the longitudinal analysis of the CD8⁺ T cell response during the evolution of epitope mutations in HIV infected patients was investigated. Furthermore, a literature study was done during this study investigating the effect of infections on the occurrence of allergy, which led to her interest in allergic diseases. In 2002 she started with the present research that resulted in this thesis. In addition, Eveline started working as Clinical Research Associate in 2006 and current holds the position of senior Clinical Research Associate and Local Submission Responsible at a pharmaceutical company.

Abbreviations

AD	Atopic dermatitis/atopische dermatitis
AFR	Adverse food reactions/ongewenste reacties tegen voedsel
APC	Antigen presenting cells/antigeen presenterende cellen
B2M	b-2-microglobulin
bp	basepairs
CAFR	Cutaneous adverse food reactions/ongewenste huidreacties tegen voedsel
CCR4	chemokine [C-C motif] receptor 4
CD23	low affinity IgE receptor
cDNA	complementary deoxyribonucleic acid
CLA	Cutaneous Lymphocyte Antigen
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cells
FAE	follicle-associated epithelium
FcεRI	high affinity IgE receptor
Foxp3	transcription factor; Forkhead box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA3	transcription factor; GATA binding protein 3
Gfi-1	transcription factor involved in T cell development
GUSB	β-Glucuronidase
hnRNPH	heterogeneous nuclear Ribonucleoprotein H
HPRT	Hypoxanthine Phosphorybosyltransferase
IFN	Interferon
Ig	Immunoglobulin/ immunoglobuline
IL	Interleukin/interleukines
LC	Langerhans cells
LS	Lesional skin/lesionale huid
LST	Lymphocyte Stimulation Test
M-cells	microfold cells
MDC	CCL21
MHC-II	Major histocompatibility complex II
Min	minutes
MLN	mesenteric lymph nodes/mesenteriale lymfeknopen
mRNA	messenger Ribonucleic acid

NLS	Non-lesional skin/niet-lesionale huid
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
qPCR	Quantitative real time PCR
RNA	Ribonucleic acid
ROR γ t	RAR-related orphan receptor gamma
RPL8	Ribosomal protein L8
RPS5	Ribosomal protein S5
RPS19	Ribosomal protein S19
RT	Room temperature
S	seconds
SI	Stimulation Index
Smad	Transcription factor; protein homologs of both the drosophila protein, mothers against decapentaplegic (MAD) and the <i>Caenorhabditis elegans</i> protein SMA. The name is a combination of the two.
SNP	single-nucleotide polymorphism
SOCS	Suppressor of cytokine signaling
SRPR	Signal recognition particle receptor
STAT	Signal transducer and activator of transcription
TARC	Thymus and Activation-Regulated Chemokine, CCL17
T-bet	T-box transcription factor 21, T-box expressed in T cells
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TNF	Tumor necrosis factor
Tr1	one of the suppressor T cell population
Treg	Regulatory T cells/T regulatoire cellen



