CHARACTERIZATION OF SKIN TRANSCRIPTOME AND SERUM CYTOKINES/CHEMOKINES IN FELINE ATOPIC SKIN SYNDROME

by

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(Under the Direction of Frane Banovic)

ABSTRACT

Feline atopic skin syndrome (FASS) is a pruritic and inflammatory skin disease commonly encountered in cats. Studies investigating skin lesion pathogenesis in feline atopic disease (FAD) are limited and generally centered on cell-type histopathologic evaluations. Whether FAD and FASS mirror the allergic immune responses seen in the skin and blood of humans and dogs with atopic dermatitis requires further investigation. This study aims to characterize the molecular pathways in the skin and serum of cats diagnosed with FASS using quantitative real-time polymerase chain reaction (qRT-PCR) and multiplex cytokine/chemokine assay. Determining the immunologic pathways in FASS is warranted to further our understanding of the pathogenesis of FASS, which will then allow for the development of more targeted and beneficial therapies for FASS to be achieved.

INDEX WORDS: Feline atopic skin syndrome, Feline atopic syndrome, Feline, Cat, Cytokines, Chemokines, Th1 and Th2

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by

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Thesis Structure

This thesis follows a manuscript style format and includes an introduction and literature review chapter, an objectives chapter, two articles included as chapters, and discussion and concluding chapters. Each chapter has its own references section with discussion and conclusion references combined. Some material within the introductory and concluding chapters will unavoidably be duplicated as the included articles are reprinted here in their original full published proof. The objectives presented are each addressed by the article chapters immediately following them.

Terminology

The nomenclature of feline allergies has been recently updated. In the past, feline hypersensitivity dermatitis (HD) has widely been used for cutaneous allergic reactions due to flare factors of flea, food and environmental allergens. The terminology of feline HD is now replaced with feline atopic syndrome (FAS), which broadly includes feline allergic skin disease, food allergy and/or asthma.[1] Within the new FAS nomenclature, feline atopic skin syndrome (FASS) refers explicitly to allergic skin disease induced by environmental allergens (e.g., dust mites, pollens). In a large multicentre study, FASS represented 20% of the study cats and one of the most common skin diseases in cats.[1-2] Pruritus is a major component of FASS in addition to clinical lesion patterns consisting of miliary dermatitis (MD), self-induced symmetrical alopecia (SIA), facial head neck pruritus with excoriations and erosions (HNP), and eosinophilic

granuloma complex (EGC).[2,3-4] Miliary dermatitis features multiple erythematous papules covered by small crusts typically centered around the neck but can be generalized (Figure 1a).[5] Self-induced symmetrical alopecia results from overgrooming and is commonly seen on the abdomen and flanks. (Figure 1b).[4] Excessive scratching around the head and neck results in the third clinical lesion pattern HNP; alopecia and MD can also be associated with this clinical presentation (Figure 1 c).[4] Eosinophilic granuloma complex consists of three clinical forms: indolent ulcer, eosinophilic plaque and granuloma. An indolent ulcer, also frequently referred to as a rodent ulcer, presents as an ulcerative lesion of the upper lip; the lesion may be unilateral or bilateral and may become quite large with chronicity.[4] An eosinophilic plaque (EP) is an elevated, erythematous, and often eroded or ulcerative lesion most commonly found on the ventral abdomen and medial thighs (Figure 1 (d)).[4] Eosinophilic granuloma (EG) can appear either as a linear, firm lesion, most often on the caudal aspect of the hindlimbs, diffuse chin swelling, and/or a proliferative lesion in the mouth.[4] In most studies, cats diagnosed with FASS were around three years of age and younger, and females appeared to be overrepresented.[4] Seasonality often plays a role in FASS, but many cats develop nonseasonal signs over time.[4]

Diagnostics and Differential Diagnosis

The diagnosis of FASS is based on history, the clinical examination findings, the exclusion of other pruritic dermatoses and fulfillment of published diagnostic criteria for FASS.[3-4] There is no specific finding or test for the diagnosis of FASS. Instead, the diagnostics of FASS involves a systematic approach in excluding other differentials for pruritus along with the four clinical lesion patterns. Ectoparasites such as Notoedres, Demodex (i.e., *D. gatoi*) and Otodectes can result in pruritus; diagnostics such as superficial skin scrapings, hair plucks and examination of otic debris should be performed where appropriate.[4] Flea allergy dermatitis is a crucial

differential for FASS. Effective flea control is essential for affected patients, any in-contact animals and the patient's environment for a minimum of nine weeks.[4] Both bacterial pyoderma and Malassezia overgrowth can be a complicating feature in FASS cats and can contribute to pruritus; skin cytology is simple to perform and identification and treatment of secondary infections aids in the overall management of these patients.[4]

A set of criteria has been established (Table 1) to assist in diagnosing FAS (previously described as non-flea-induced hypersensitivity dermatitis); when six of the 10 criteria are met, the sensitivity and specificity are 90% and 83%, respectively.[3] Although this helps differentiate FASS from other causes of pruritus, there is no current means of discriminating FASS from a food allergy.[3-4] A food allergy can mimic any of the four clinical lesion patterns. A minimum-eight-week elimination diet trial must be performed using either a novel limited ingredient or a hydrolyzed diet.[4,6] Extracutaneous signs involving the gastrointestinal system (vomiting, diarrhea, flatulence) and/or conjunctivitis and/or an older patient that suddenly develops one or more of the clinical lesion patterns may be more suggestive of a food allergy.[4] During the first 5-6 weeks of the elimination diet trial, treatment of any secondary skin/ear infections and the resolution of pruritus is necessary to assess the return of clinical signs when the medication is discontinued and the patient is solely on a diet. If the patient does not relapse clinically within two weeks, a rechallenge with the old diet should be performed. Return of clinical signs within seven days of rechallenge diagnoses 90% of cats with a food allergy; almost all cats will relapse within 14 days.[7] Although FASS and a food allergy can be present; a recent review reported a very low incidence of 2.4% for both conditions in the same patients.[4]

Treatment of FASS

Feline atopic skin syndrome frequently requires medical therapy, which can pose challenges to both treatment and owner compliance. While several treatments are proposed for managing FASS in a recent extensive review,[8] glucocorticoids, cyclosporine, oclacitinib, maropitant and allergen-specific immunotherapy will be reviewed here.

Glucocorticoids

Systemic glucocorticoids are the most commonly used drug in FASS management due to their broad-range anti-inflammatory effects. Systemic glucocorticoids rapidly and effectively ameliorate pruritus and skin lesions in FASS.[8] However, both dosage and formulation of glucocorticoid can impact treatment efficacy.[8-12] Four studies have critically evaluated the efficacy of glucocorticoids in FAS cats. In the most recent study, oral dexamethasone sodium phosphate (DexSP, DexajectSP, Henry Schein, USA; 4 mg/mL) at 0.2 mg/kg for 20-31 days induced a marked reduction in pruritus and lesional skin scores (SCORing Feline Allergic Dermatitis, SCORFAD) of greater than 50% in 12 allergic cats.[9] Four cats had mild side effects, including one cat with increased appetite and sneezing; however, all adverse events were self-limiting and none required veterinary medical intervention.[9] The results of this study were particularly significant since DexSP is a liquid formulation and can be used in patients who don't tolerate tablet-type medications.[9] In a separate study, triamcinolone was approximately seven times more potent than methylprednisolone in treating pruritus in 32 client-owned cats diagnosed with environmental and/or food allergy dermatitis.[10] In this study, once-daily oral mean induction dosages of 0.18 mg/kg of triamcinolone and 1.41 mg/kg of oral methylprednisolone (16 cats in each group) resulted in a 90.6% reduction in pruritus within 14 days and clinical remission in 29 out of 32 cats.[10] Pruritus and lesion scores were assessed using the pruritus visual analog scale

(pVAS)[11-12] and the feline erythema, excoriation and alopecia score (FEEAS), respectively.[10] After tapering both drugs, the mean maintenance dose administered every other day to cats was 0.08 mg/kg for triamcinolone and 0.54 mg/kg for methylprednisolone.[10] Although three cats were withdrawn at the end of the induction phase for laboratory abnormalities, these parameters resolved following the cessation of glucocorticoids.[10] Another study found oral prednisolone at 1 mg/kg/day for 28 days showed lower anti-pruritic efficacy than the previous two studies; only five out of 11 FASS cats had an improved pruritus score and only four cats had a greater than 25% improvement in lesional score.[13] The pruritus and lesion scores assessment differed in this study as a linear analog scale (LAS) from 1 to 5 and the Canine Atopic Dermatitis Extent and Severity Index (CADESI 02) were used, respectively.[13] A fourth study observed a mean improvement of 69% in owner-assessed pVAS and 67% in SCORFAD after 28 days in 20 FASS cats (16 SIA, nine MD, 12 HNP, three EP) with oral methylprednisolone at a mean total daily dose of 0.77 mg/kg.[14] Interestingly, all MD and EP cats attained clinical remission at the end of the study (100% improvement in SCORFAD).[14] Once clinical remission is achieved, the glucocorticoid dosage is slowly tapered to the lowest effective dose, resulting in no new lesion formation and/or increase in pruritus. In all four studies, glucocorticoids were generally well tolerated and monitoring of cats on long-term glucocorticoid therapy with periodic bloodwork (complete blood count, serum biochemistry), urinalysis and monitoring for adverse effects (for example, weight loss, changes in appetite) is recommended.[8]

Two retrospective studies have evaluated the treatment response of subcutaneous injections of methylprednisolone acetate (MA) (Depo-Medrol, Zoetis, NY, USA) in managing FAS cats.[15-16] In the first study, MA at 20 mg/cat administered subcutaneously every two weeks (total of two injections) demonstrated a clinical remission of 100% in seven (of 55) treated cats.[15]

In the second study with 194 FAS cats, MA at 20 mg/cat administered subcutaneously every two weeks (total of two to three injections) achieved clinical remission in 66 treated cats.[16] Injectable MA may appear as an attractive treatment option for feline patients not tolerating oral administration of tablets; however, the lack of prospective controlled studies evaluating the dosages, frequency of administration and efficacy in FASS cats[17] as well as possible side effects, including iatrogenic hyperadrenocorticism[18], suggests this treatment should be utilized carefully.

Ciclosporin

Ciclosporin (Atopica, Elanco, USA) is a calcineurin inhibitor that inhibits T-cell activation[19] and is another drug commonly used to manage FASS. The recommended dosage of 7 mg/kg orally once daily is based on two randomized double-blinded placebo-controlled studies.[20-21] In the first study, 100 client-owned allergic cats were randomized to receive oral ciclosporin (CsA) at 7 mg/kg once daily, 2.5 mg/kg once daily, or a placebo for six weeks.[20] Clinical lesions were scored using a total lesion score (TLS), pruritus was evaluated using an owner pruritus score (OPS) based on VAS along with an investigator pruritus scale (IPS) and a global assessments of improvement (GAI) scale was utilized to evaluate overall clinical status at day 21 and day 42 compared to baseline.[20] The most common clinical presentations in descending order were SIA (85%), HNP (77%), MD (48%) and eosinophilic plaques (46%).[20] Improvements were seen in all evaluated parameters with individual improvements in TLS by >50% in 70% of the 7.0 mg/kg CsA group, compared with 47% in the 2.5 mg/kg group and 23% in the placebo group, improvement in IPS by 54%, 32%, 21% and GAI was "excellent" or "good" in 61%,47% and 23% respectively.[20] The most common side effect seen in all three groups was vomiting and the number of vomiting cats was higher in cats receiving CsA than placebo.[20] Additionally, weight loss was recorded in the first three weeks in all treatment groups;

however, this was reversed in the second three weeks.[20] Despite statistically significant reductions in total white blood cell counts, eosinophils and increases in serum biochemistry values (total bilirubin, glucose, urea) in the 7 mg/kg CsA group as compared to placebo, all reported values were still within the normal reference range.[20] In the second study, 144 FASS cats received 7 mg/kg oral CsA and 73 FASS cats received a placebo for a total of six weeks.[21] As a result of gastrointestinal upset, lack of efficacy, the owner's inability to medicate and lack of owner compliance, 120 CsA and 61 placebo FASS cats completed the study.[21] Outcome measures evaluated were identical to the previous report using TLS, OPS, IPS and GAI.[21] Ciclosporin at 7 mg/kg orally once daily showed a significant reduction in the TLS; 65.1% as compared to 9.2% with placebo.[21] All other variables showed significant improvement, and 78.3% of owners rated their FASS cat's treatment with ciclosporin as a success.[21] Although there were more CsA-treated cats (84) than placebo-treated cats (31) with side effects, the percentage of cats in both groups with gastrointestinal upset (vomiting, diarrhea and hypersalivation), which was the most common side effect, was not significantly different; 40% and 39% respectively.[21] Similar to the preceding study, weight loss was also seen in the CsA-treated group and increases in serum biochemistry values (total bilirubin, glucose, urea and creatinine), although all remained within the normal reference range.[21] Prior studies assessed both the human,[13,19, 22-23] and veterinary formulation[22] of oral ciclosporin for the treatment of feline allergic disease at dosages ranging from 2.1 to 13.3 mg/kg once daily, including a prospective open pilot study,[19] two retrospective studies[22-23] and one randomized, controlled, double-blind study[13] for a total of 61 cats. Clinical lesion scoring systems were recorded utilizing a feline eosinophilic granuloma and eosinophilic plaque extension and severity index (FEGEPESI),[19] a clinical score ranging from 1 to 10[23], CADESI 02[13], and pruritus by means of pVAS[19], a scale consisting of mild,

moderate, severe[22], a score ranging from 1 to 10[23] and LAS[13] respectively. Improvements in clinical lesions varied from 38% of cats showing a greater than 25% improvement (13/18 cats) after four weeks,[13] 50% showing a greater than 50% improvement (5/10 cats) after four weeks[19] to all cats being cured (15/15 cats)[23] after 12 weeks and 10 out of 10 cats having a good clinical response with CsA therapy respectively; the later study did not specify the length of time to clinical remission.[22] Interestingly, pruritus worsened in five out of 18 cats, with no improvement in pruritus in two additional cats administered 5 mg/kg/day of CsA for four weeks.[13] In contrast, a reduction to complete resolution of pruritus was observed in the remaining studies.[19, 22-23] Side effects were considered mild in three studies[13, 19, 23] with gastrointestinal signs (intermittent vomiting, soft feces to diarrhea) being the most commonly reported and serious adverse events were seen in three cats in the fourth study.[22] All cats in the latter study had been administered CsA for a minimum of 12 months; one cat developed localized nasal cryptococcosis (4.1 mg/kg once daily to every second day), one cat developed clinical Toxoplasmosis (doses ranged from 2.1 to 4.2 mg/kg/day) and a single cat died in hospital due to unknown causes (~5 mg/kg/day).[22]

Two studies totaling 222 cats have specifically evaluated clinical efficacy in tapering CsA to every other day (EOD) and twice-weekly (TW) in managing FAS.[24-25] Both investigations had previously established 7 mg/kg of CsA orally once daily as an effective treatment for FAS[24-25] and following four weeks of once-daily CsA administration, the two studies were similarly successful in tapering the drug with up to 70% of cats in one study[24] and greater than 70% in the second study[25] to EOD and 57% and 53% of cats to TW respectively. Gastrointestinal upset was the most common side effect and lessened both with continued use and decreasing frequency.[24-25] In the study where all 15 allergic cats underwent clinical remission with CsA administration, all cats were able to be maintained on alternate-day CsA

therapy; seven of these cats had eosinophilic granuloma, eosinophilic plaque, indolent ulcer and/or linear granuloma and the remaining eight cats had idiopathic pruritus.[23]

Oclacitinib

Oclacitinib (Apoquel, Zoetis, USA), a Janus kinase inhibitor registered for use in dogs with allergic dermatitis[26] has recently garnered a lot of attention in treating FASS. Although this drug is not labeled for use in felines, there are three prospective studies[27-29] and one case report[30] documenting the efficacy of oclacitinib in FAS and FASS cats, along with a safety study[31] and a pharmacokinetic study of oclacitinib in six cats.[32] In the most recent prospective study, oclacitinib prescribed at 1 mg/kg twice daily for two weeks followed by 1 mg/kg once daily for two weeks resulted in improvement in pVAS and SCORFAD by greater than or equal to 50% in 61% (11/18) and 89% (16/18) of 18 client-owned FASS cats respectively.[27] Plasma oclacitinib levels were measured and no correlation was found between drug concentrations and clinical response.[27] At the start of the study, 28 client-owned FASS cats had been enrolled; however, 10 cats dropped out; five for difficulties in administrating the oral tablet, two for vomiting, two for perceived lack of treatment efficacy and one cat for developing anemia that resolved after the drug was stopped.[27] Two of the study cats had mild elevations in alanine aminotransferase (ALT); in both patients, the ALT returned to normal after cessation of the drug.[27] No medical intervention was required for any of the cats. The clinical lesion patterns were not reported in this study. In the second prospective pilot study,[28] four of 12 FASS cats (eight SIA, three HNP, three eosinophilic plaques, one eosinophilic granuloma, two lip ulcerations, one MD and one having diffuse erythema; some cats presented with multiple lesion patterns) showed improvement in both SCORFAD and owner pVAS after being administered 0.47 mg/kg of oclacitinib orally twice daily for two weeks followed by once daily for two weeks. An additional

two cats showed improvement, one each for pruritus and SCORFAD, respectively.[28] Three cats did not complete the study; two showed a poor response and the third cat was lost to follow-up.[28] Importantly, no side effects were documented in any of the cats.[28] In the third study presented as an abstract, a similar dosing schedule was prescribed to 15 cats with head and neck pruritus that had been unresponsive to systemic corticosteroids or ciclosporin; all cats received 2.7 mg (0.5-0.8 mg/kg) of oclacitinib orally twice daily for two weeks and then once daily for two weeks.[29] Owner pVAS and SCORFAD clinically improved by 66.6% (10/15 cats); two cats had a poor response and three cats dropped out due to difficulty in administrating the drug.[29] Reporting of any side effects was lacking. A higher oclacitinib dosing regimen was prescribed in a double-blinded, randomized methylprednisolone-controlled study where 20 FASS cats (20 SIA, eight MD, 14 HNP, four EP) received 0.7-1.2 mg/kg of oclacitinib and 20 FASS cats received 0.5-1 mg/kg methylprednisolone orally twice daily for 28 days.[14] No statistically significant differences in improvements in mean SCORFAD and pVAS were detected between the two groups; for oclacitinib 61% and 54% and methylprednisolone 69% and 67%, respectively.[14] The number of cats that had minimal or no response was, however, higher in the oclacitinib group at five versus three cats in the methylprednisolone group.[14] Furthermore, two of 14 cats had neutropenia, one cat had thrombocytopenia, three cats had increased ALT and four cats had mild elevations in renal values on oclacitinib treatment; as to whether the underlying renal disease was present at study enrollment is unknown as diagnostics such as urinalysis and symmetrical dimethylarginine (SDMA) were not performed.[14] Despite these findings, no cats were reported to require medical intervention. Interestingly, the 10-year-old female feline detailed in the case report had been diagnosed with stage one chronic kidney disease (CKD) at the start of oclacitinib treatment and over 300 days, the CKD remained stable and no side effects were noted during the

duration of treatment.[30] This cat had a 10-month history of pruritic dermatitis and diarrhea and, after being diagnosed with FAS was placed on 1 mg/kg of oral oclacitinib twice daily for 14 days followed by 1 mg/kg once daily as maintenance.[30] Pruritus resolved after 30 days of treatment and then relapsed at 300 days; the dosage of oclacitinib was increased back to 1 mg/kg twice daily, which resulted in remission of clinical signs.[30] In the blinded, randomized, placebo-controlled trial evaluating the safety of oclacitinib in cats,[31] two groups of 10 cats received either 1 mg/kg or 2 mg/kg of oclacitinib orally twice daily for 28 days, with the third group of 10 cats receiving placebo tablets.[31] The drug was well tolerated with no significant laboratory abnormalities aside from a small increase in fructosamine levels, which remained within the normal reference range.[31] At the higher dose of oclacitinib, two cats each were noted to have vomiting and soft stools, respectively, with one cat in the placebo group also having soft stools.[31] The significance of soft stools in the oclacitinib-treated group was questionable as fecal samples from both cats tested positive for *Giardia* sp.[31] Additionally, of the two cats that vomited, one cat only vomited once while the other cat vomited several times throughout the study; however, the vomiting resolved without necessitating medical attention.[31] Prospective studies evaluating the safety profile of oclacitinib beyond 28 days are lacking.

Twice daily administration of oclacitinib will likely be necessary in the cat based on a recent pharmacokinetic study showing the drug's half-life to be 2.3 hours compared to 3.45 hours in the dog.[32] Currently, the recommended oclacitinib dose is 1 mg/kg once to twice daily with informed owner consent[8] and monitoring of blood parameters (hematology, serum biochemistry) is recommended. Long-term prospective studies looking at the efficacy and safety of oclacitinib are desperately needed.

Maropitant

There is a single open-labeled, uncontrolled pilot study evaluating maropitant in treating FASS patients.[33] In this study of 12 FASS cats, 11 cats showed improvement in both pruritus and SCORFAD when treated with maropitant at 2.22 mg/kg/day for four weeks; one cat showed improvement in pruritus only and five cats were on concurrent antipruritic medication.[33] Maropitant is a neurokinin-1 receptor (NK-1R) antagonist and can inhibit the signaling pathway of neurotransmitter substance P, which in humans and dogs has been shown to induce wheals, flare reaction and pruritus at the site of intradermal injections.[33-34] In the author's experience, maropitant alone or in combination with other antipruritic medication has not resulted in improvement of pruritus and/or SCORFAD in FASS cats.

Allergen-Specific Immunotherapy

Allergen-specific immunotherapy (ASIT) is the only treatment for FASS that has the potential to induce tolerance and enable the reduction or complete elimination of other long-term treatments.[35] Mechanisms of tolerance include induction of Treg cells leading to increased levels of IL-10 and transforming growth factor-beta (TGFß), resulting in decreases in Th2 cytokines.[36] Lower levels of Th2 cytokines lead to decreased degranulation and infiltration of mast cells, basophils and eosinophils in tissues.[36] Additionally, ASIT induces the production of IgG_4, which leads to reduced production of allergen-specific IgE.[36] Allergen-specific immunotherapy has traditionally been administered subcutaneously or sublingual (SLIT) in cats and in a recent study that evaluated SLIT in 22 allergic cats for one year, all 16 cats that completed the study showed marked improvement in skin lesions after only three months.[35] Furthermore, three of seven cats that required oral methylprednisolone at the beginning of the study were able to have the

medication discontinued and the remaining four cats had over a 50% reduction in methylprednisolone dosage at the end of the study period.[35]

Other therapies that have been used in the treatment of FASS with variable success include antihistamines, essential fatty acids, and palmitoylethanolamide.[8] A recent study that evaluated repeated intradermal injections of heat-killed actinomycetes as a treatment for feline atopic syndrome reported a successful response to treatment (defined as a percentage reduction of SCORFAD of 50%) in five of six cats administered five injections with *Gordoni bronchialis* over one year.[37] Ultimately, treatment for FASS requires a partnership with the client and treatments tailored to the individual feline.

Pathogenesis of FASS

In contrast to humans and dogs with allergic dermatitis, there remains a lack of information regarding the cytokine and chemokine involvement in the skin and/or blood of cats diagnosed with FASS. Most investigations into the pathogenesis of FAD and FASS have been limited to histopathologic cell-type evaluations of skin lesions.[38-39] In one study that evaluated T cells in the skin of 10 FASS (MD) cats using immunohistochemistry, higher numbers of CD4+ T cells were present in both the epidermis and superficial dermis of lesional and nonlesional skin of FASS cats as compared to five healthy cats.[38] Additionally, an increased CD4+/CD8+ ratio in the lesional skin of FASS cats was seen, whereas no CD8+ T cells were present in any of the skin biopsies of the healthy cats.[38] Similar to the findings of T cells, increased numbers of CD1a+ Langerhans cells were documented in the epidermis and dermis of lesional skin of nine FASS cats compared to nine healthy cats; there were three times as many in the epidermis and twice as many in the dermis respectively.[39] Higher numbers of both T cells and Langerhans cells have been observed in the skin of humans and dogs with atopic dermatitis (AD).[40-44]

Mast cells (MCs) are another cell type that has increased numbers in the lesional skin of FASS cats compared to healthy cats.[45] In a study of skin biopsies from 10 FASS cats (MD) and nine control cats, MCs were found to be significantly increased in the FASS cats; in both groups, MCs were primarily detected in the superficial dermis. [45] Interestingly, there were more coarse-granulated chymase-positive MCs in the lesional skin versus the nonlesional skin of the FASS cats with no differences in tryptase-positive MCs; coarse granules have not been described before in cat skin; thus the significance of this finding is unknown.[45] A separate study that utilized a double-labeling procedure identified the MC populations in the superficial and deep dermis of eight cats with eosinophilic conditions (three EP, two eosinophilic granuloma, three eosinophilic dermatitis) as tryptase-positive/chymase-positive (81.2% superficial, 73.8% deep dermis), which is a subtype that had not been recognized previously.[46] Chymase-positive/tryptase-negative MCs had a higher percentage (12.8%) than tryptase-positive/chymase-negative MCs (5.9%) in the superficial dermi. In contrast, no differences were noted in the deep dermis (12.8% for each subtype) of these cats.[46] Finally, in a large retrospective study that included skin biopsies from 143 allergic cats, 228 nonallergic cats and 31 normal cats, MC numbers were increased in both the allergic and nonallergic cats as compared to the healthy cats with no apparent differences between allergic and nonallergic skin samples.[47] As with the prior study, median MC numbers were increased in the superficial dermis versus the deep dermis.[47]

Pathogenesis of FASS: IgE

The role of allergen-specific IgE in the pathogenesis of FASS remains unclear. In one study that measured serum levels of IgE along with performing a serum allergy panel (49 allergens) in 179 pruritic cats (60 FASS, 15 food allergic, 70 undetermined, 16 flea-bite hypersensitivity, 18 nonallergic pruritic cats, 20 healthy controls), there were no significant

differences between the allergic cats and the healthy control cats.[48] Additionally, half of the FASS cats had a negative serum IgE level.[48] In this same study, increasing age corresponded with increasing serum levels of IgE and the absence of deworming and residing outdoors.[48] The lack of significance of IgE in allergic cats was further demonstrated in a separate study that measured serum IgE levels to house dust mites (HDM; *Dermatophagoides farina*, *Dermatophagoides pteronyssinus*) in 59 allergic cats (22 SIA, seven MD, seven EGC, 16 HNP, seven combination group, 10 of 59 were diagnosed with FASS) and 59 normal cats.[49] In a third study, seven of 15 specific pathogen-free cats had positive IgE levels to pollens and/or HDM despite an HDM-confirmed free environment.[50] Evidence suggestive of IgE involvement include favorable responses to ASIT[22,35] along with similar findings between the lesional skin of FASS cats and atopy patch testing.[51] Whether an intrinsic form (i.e., IgE is not detectable) exists in FASS felines as in humans[52] and canines[53] is unknown; further investigations are needed.

Pathogenesis of FASS: Histopathology

The histopathology of FASS will vary depending upon the biopsied clinical lesion pattern and the skin lesions' chronicity. Of the four clinical lesion patterns in FASS, SIA closely resembles normal skin on histopathological examination; the epidermis may be normal or mildly hyperplastic with a mild superficial perivascular infiltration of mast cells, eosinophils and lymphocytes.[54] In contrast, MD skin lesions reveal variable degrees of epidermal hyperplasia, serocellular crusting, intraepidermal pustules and epidermal spongiosis (including adjacent hair follicles) along with a superficial perivascular infiltrate containing variable numbers of eosinophils, mast cells, neutrophils and lymphocytes.[5,38] Of the EGC, indolent ulcers, depending on the chronicity, can have vacillating numbers of eosinophils, mast cells, macrophages and neutrophils; eosinophils are the predominant cell type and fibrosis be seen in chronic lesions.[55]

Eosinophilic granulomas (EG) may be eroded, ulcerated and/or exudative, and the epidermis is often acanthotic.[55] Flame figures, which consist of collagen fibers coated with degranulated eosinophilic material, are predominant in the dermis and may be surrounded by macrophages and giant cells.[55] Diffuse dermal infiltration of eosinophils, mast cells, macrophages and lymphocytes are present with fewer eosinophils in chronic lesions of EG.[55] Eosinophilic plaques (EP) is characterized by erosions, ulcerations, moderate to severe acanthosis, mucinosis and spongiosis, along with a perivascular to diffuse, moderate to severe infiltration of eosinophils that can extend to the superficial panniculus.[55] Mast cells, lymphocytes, macrophages and neutrophils are also present, typically in smaller numbers.[55] Flame figures, while described in cats with eosinophilic plaques,[56] are not considered distinctive for this clinical lesion subtype.[55] Interestingly, in a study of 16 cats with allergic skin disease[54] (eight with EP, 12 with SIA, one with EG and two with MD; some cats exhibited more than one lesion pattern), specific phenotypes (i.e., MD, EGC, etc.,) could not be determined based on histopathology, rather six histopathological patterns were described suggesting that the pathogenesis of FASS may vary depending upon which clinical lesion pattern is present and/or is predominant.

<u>Pathogenesis of FASS: Cytokines and Chemokines</u>

Only two reports evaluated gene expressions (mRNA) of multiple cytokines in the skin of cats diagnosed with allergic dermatitis.[57-58] In the first study, interleukin (IL)-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p35 and p40), IL-18, tumor necrosis factor (TNF-α), transforming growth factor-beta (TGF-β) and interferon-gamma (IFN-γ) mRNA was measured using quantitative real-time polymerase chain reaction (q RT-PCR) in the lesional and nonlesional skin of 16 allergic cats (eight with EP, 12 with SIA, one with EG and two with MD; some cats exhibited more than one lesion pattern) and the normal skin of 10 healthy control cats; no significant differences in

mRNA expression were found between lesional, nonlesional and normal skin.[57] In the second study, mRNA expression of IL-5, IL-31, and IL-33 from formalin-fixed, paraffin-embedded skin biopsies of cats with and without allergic dermatitis were either low or not detected using qPCR (IL-31) and RNA-scope (IL-5, Il-31, Il-33); the number of skin biopsies examined in each group was not specified.[58] In a third study that evaluated the cytokine expression of interleukin (IL)-4 via immunohistochemical staining in skin biopsies of five allergic cats (MD), CD4+ T cells were determined to be the primary source and the number of these cells was significantly increased, in the lesional and nonlesional skin of the allergic cats as compared to the skin of five healthy cats.[38] Increased CD4+ T cells and IL-4 were also demonstrated via immunohistochemical staining in skin biopsies of allergic cats that underwent patch testing.[51]

There have been only three reports evaluating serum cytokine immune activation in FAD.[58, 59-60] In the earliest study that examined a single serum cytokine, no significant differences in IL-5 serum levels were detected amongst 54 allergic cats as compared to 11 control cats; phenotypes were not described.[59] Additionally, no correlation was found between the levels of IL-5 and the number of eosinophils in the blood.[59] This was an unexpected finding considering that eosinophils play an important role in FAD and IL-5 is an essential cytokine for all stages of eosinophil development (growth, differentiation, maturation), in addition to acting as a chemoattractant for the migration of eosinophils to areas of allergic inflammation.[59]

A second study evaluating IL-31 serum levels in 73 cats with presumed allergic disease found mean serum levels to be higher than that of 17 age-matched controls.[60] The opposite result was found in a separate third study, where no significant differences in serum IL-31 levels were detected between 18 allergic cats, 18 asthmatic cats, and 17 healthy cats.[58] Interleukin-31 has been demonstrated to induce pruritus in both cats[61] and dogs[62,] and serum levels in one canine

study were found to be elevated in just over half of the dogs diagnosed with AD.[62] In addition to assessing serum IL-31 levels, this third feline study evaluated a Luminex panel of 19 cytokines and found no significant upregulations between the allergic cats, asthmatic cats and healthy controls.[58] These results are in contrast to human[63] and canine[64] patients with AD.

Two reports examined two different chemokines in cats with EP. The CC chemokine ligand 5 (CCL5), also known as regulated upon activation, normal T-expressed and secreted (RANTES) is a potent chemoattractant for eosinophils which are the predominant cell type in EP.[55,65] Increased expression of CCL5 mRNA was demonstrated in the lesional skin of seven cats with EP as compared to nonlesional skin using RT-PCR analysis.[65] Similar to CCL5, the CC chemokine ligand 17 (CCL17), also formerly known as thymus and activation-regulated chemokine (TARC), was found to have a higher expression in EP skin of five cats than nonlesional skin.[66] This chemokine is a chemoattractant for T helper 2 (Th2) cells.[66] Results of both of these chemokines are comparable to that seen in the skin of human and canine patients with atopic dermatitis.[67-68]

Study Rational

In summary, there is a need to further characterize the immunologic pathways in FASS to expand our understanding of the pathogenesis of FASS, which can lead to the development of more targeted and beneficial therapies for our FASS patients. Therefore, the objectives of the present studies were to characterize the molecular pathways in the skin and serum of cats diagnosed with FASS using qRT-PCR and multiplex cytokine/chemokine assay.

Table 1.1. Clinical diagnostic criteria for feline atopic syndrome; adopted from Favrot C et al. Establishment of diagnostic criteria for feline nonflea-induced hypersensitivity dermatitis. *Vet Dermatol* 2011;23:45-e11. The terminology of feline nonflea-induced hypersensitivity dermatitis has now been replaced with the feline atopic syndrome (FAS).

Diagnostic criteria for the feline atopic syndrome (FAS; food and/or environmental-induced)
Presence of pruritus at the onset
Presence of at least two of the following classical clinical reaction patterns:
• Symmetrical Alopecia
• Miliary dermatitis
• Eosinophilic dermatitis
• Head and neck erosions/ulcerations
Presence of at least two sites affected
Presence of miliary dermatitis as a dominant pattern
Presence of eosinophilic dermatitis or symmetrical alopecia or erosions/ ulcerations on the head, face, lips, ears or neck
Presence of nonsymmetrical alopecia on the rump, tail or hindlimbs
Presence of symmetrical alopecia on the abdomen
Absence of erosions/ulcerations on the forelimbs
Absence of lesions on the sternum or axilla
Absence of nodules or tumors
Fulfillment of six of these 10 criteria gives a sensitivity of 90% and a specificity of 83% for the diagnosis of FAS

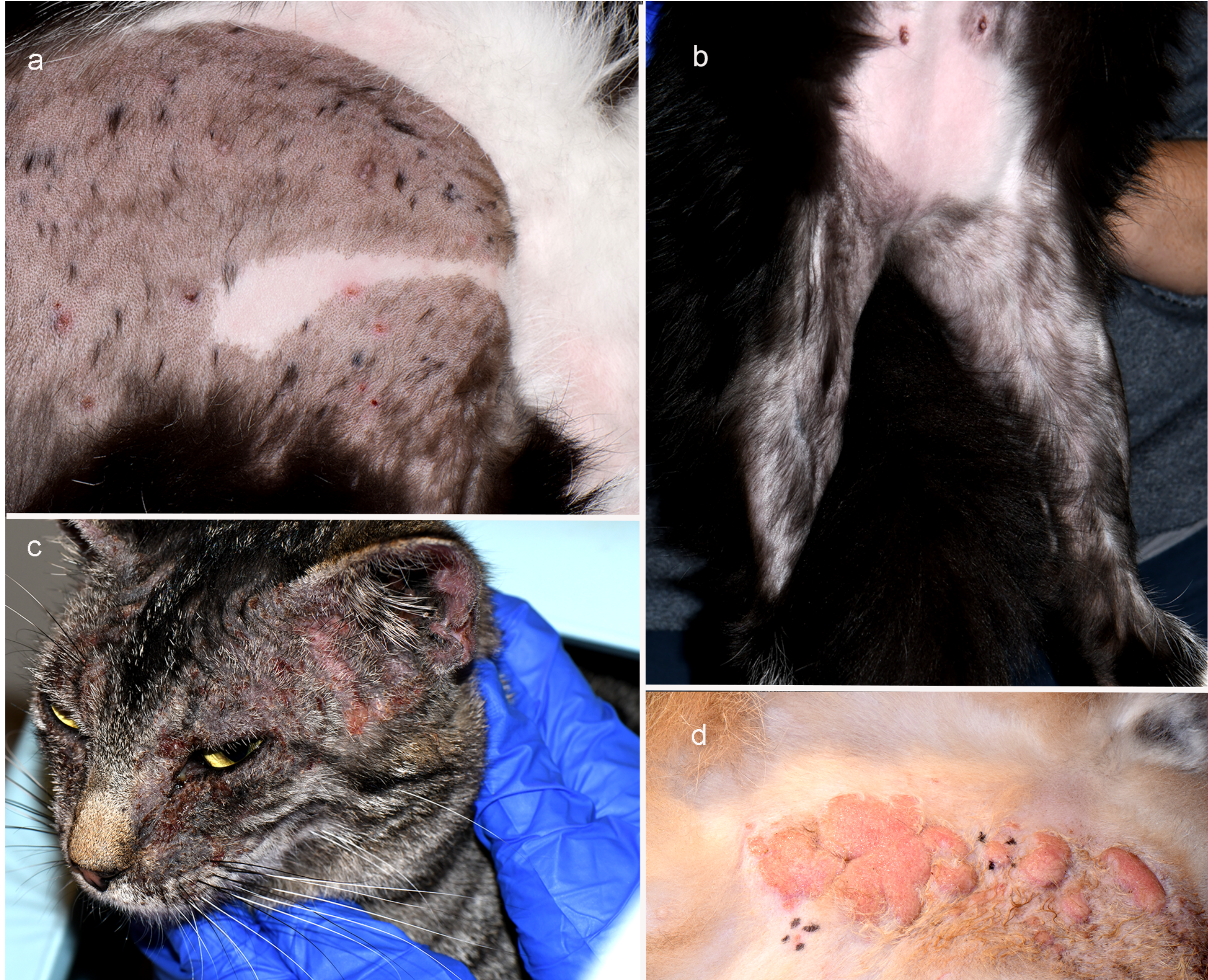

Figure 1.1. Clinical patterns in feline atopic syndrome (FAS) and feline atopic skin syndrome (FASS); miliary dermatitis (a); symmetrical alopecia (b); facial head and neck pruritus with excoriations and erosions (c); and eosinophilic granuloma complex; eosinophilic plaques (d).

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CHAPTER 2

OBJECTIVES

The two sections below, objective 1 and objective 2, correlate with the two included articles, respectively.

Objective 1

Hypothesis: Serum cytokine profile in FASS would demonstrate significant upregulation of the T helper 2 (Th2) inflammatory pathway with an elevation of interleukin (IL)-4 and IL-13 cytokines compared to control cats.

Objective 1: To evaluate differences in the serum cytokine profile of cats diagnosed with FASS compared to healthy cats and correlate serum markers with the extent of FASS skin disease using clinical scoring systems (pVAS and SCORFAD).

Objective 2

Hypothesis: Gene expression (mRNA) in the skin of cats with FASS with eosinophilic plaques would demonstrate upregulation of the Th2 pathway with the elevation of interleukin (IL)-4 and IL-13 compared to control skin samples of healthy cats.

Objective 2: To characterize the molecular signature in the skin of five cats diagnosed with feline atopic skin syndrome (FASS) using quantitative RT-PCR analysis.

CHAPTER 3

CHARACTERISATION OF THE SERUM CYTOKINE PROFILE IN FELINE ATOPIC SKIN SYNDROME [1]

[1] Vargo, C., Gogal, R., Barber, J., Austel M., and Banovic, F. 2021. Veterinary Dermatology. 32:485-491. Reprinted here with permission of the publisher.

Abstract

Background – Feline atopic skin syndrome (FASS) is a pruritic and inflammatory skin disease commonly encountered in cats. Three previous reports evaluated cytokine immune activation in cats diagnosed with feline allergic dermatitis. However, no significant upregulations were observed in allergic cats compared to healthy controls. **Hypothesis/Objective –** To evaluate differences in the serum cytokine profile of cats diagnosed with FASS compared to healthy cats and correlate serum markers with the extent of FASS skin disease using clinical scoring systems. **Animals –** Thirteen client-owned FASS cats and 12 healthy control cats. **Materials and methods –**Thirteen cytokine and chemokines from the serum of FASS cats and healthy controls were analyzed using a commercially available feline-specific multiplex assay. **Results –** Patients with FASS had a significant increase in serum concentrations of interferon-gamma (IFN-γ), interleukin (IL)-2, IL-13 and IL-18. In addition, cytokine/chemokines involved in inflammation and chemotaxis (IL-8, C-C Motif Chemokine Ligand (CCL) 5, CCL2 and CXCL12, as well as growth factors, stem cell factor and Fms-related tyrosine kinase 3 ligand (Flt3L), were also significantly elevated. A significant positive correlation ($r = 0.64$) between the serum levels of Flt3L and Scoring Feline Allergic Dermatitis (SCORFAD) score was observed. **Conclusions –** These results demonstrate the activation of a broad array of immune secretory cytokines in the serum of cats with FASS, which are largely associated with a mixed Th1 and Th2 inflammatory response along with specific growth factors. Further larger-sample studies are needed to assess the modulation of serum biomarkers in FASS by pharmacological/therapeutic interventions.

Introduction

Feline atopic syndrome (FAS) is a broad term that includes feline allergic skin disease, food allergy and/or asthma.[1] Feline atopic skin syndrome (FASS) specifically refers to allergic skin disease incited by environmental allergens, and in one large multicentre study represented 20% of the study cats .[1-2] Pruritus is a major component of FAS (excluding asthma), FASS and flea allergy dermatitis (FAD) in addition to clinical lesion patterns consisting of head neck pruritus with excoriations and erosions, self-induced symmetrical alopecia, miliary dermatitis and eosinophilic granuloma complex.[2,3-4] Feline atopic skin syndrome frequently requires medical therapy, which can pose challenges to both treatment and owner compliance. Glucocorticoids are the most commonly used drug in the management of FASS and provide rapid and effective amelioration of pruritus and skin lesions.[5,6] Other therapies include ciclosporin,[7] antihistamines,[8] oclacitinib,[9] maropitant,[10] palmitoylethanolamide[11], and allergen immunotherapy;[12] all of these therapeutic modalities have the potential for mild to significant side effects.

In contrast to humans and dogs with allergic dermatitis, there remains a paucity of information regarding the cytokine involvement in cats diagnosed with feline allergic dermatitis (FAD). The majority of investigations into the pathogenesis of FAD have been limited to histopathologic cell-type evaluations of skin lesions.[13-17] At present, there have been only three reports evaluating serum cytokine immune activation in FAD. [18-20] However, no significant upregulations were found in allergic cats compared to healthy controls.[18-20] The findings of these three immunological studies in the serum of feline allergic cats are in contrast to those from human[21] and canine[22] patients with atopic dermatitis (AD). Understanding the cytokine involvement in human and canine AD has led to novel therapies, including narrow-targeted

therapeutics such as monoclonal antibodies.[23] Therefore, determining the immunologic pathways in FASS is needed to further our understanding of the pathogenesis of FASS.

We hypothesized that the serum cytokine profile in FASS would demonstrate significant upregulation of T helper 2 (Th2) inflammatory pathway with an elevation of interleukin-4 (IL-4) and IL-13 cytokines compared to control cats. The first objective of this study was to evaluate differences in the serum cytokine profile of cats diagnosed with FASS compared to healthy control cats. The second objective of this study was to correlate significantly changed serum markers with FASS clinical scoring systems, Scoring Feline Allergic Dermatitis (SCORFAD)[24] and the pruritus Visual Analog Scale (pVAS), validated for dogs with descriptors adapted to the cat.[6,25]

Methods and Materials

Animals

We determined that a minimum of 10 cats in each group would be sufficient for a 90% power to detect a significant two-fold difference in values between allergic and healthy cat samples (SD of 30% at $P = 0.05$; 1 sided: healthy < allergic; http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html).

Thirteen cats diagnosed with FASS and 12 healthy cats were prospectively enrolled at University Veterinary Teaching Hospital's Dermatology service between May 2018 and May 2020. Signed informed consent was obtained from all owners. All aspects of the study were approved and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC).

Cats were diagnosed with FASS based on history, the clinical examination findings, exclusion of other pruritic dermatoses and fulfillment of published diagnostic criteria for FASS.[3-4] All allergic cats were deemed systemically healthy except for the clinical signs of FASS. All cats diagnosed with FASS showed no clinical improvement after receiving ectoparasite control for at least 3 months and all cats underwent a minimum 8-week elimination diet trial as previously described.[26,27] The presence of bacterial pyoderma and/or Malassezia overgrowth in contributing to pruritus was ruled out with skin cytology.[3] Clinical lesions and pruritus were assessed in all enrolled cats by Scoring Feline Allergic Dermatitis (SCORFAD)[24] and a 10 cm-long Visual Analog Scale (VAS).[6,25] To be included in the study, cats with FASS had to have a minimum pVAS of 4/10.[6]

Twelve privately-owned cats without any history and evidence of recent or chronic medical conditions served as controls; these cats were classified as healthy based on history, physical examination, complete blood count and serum biochemistry results.

In order to limit the influence of previously administered medications on serum cytokines of enrolled FASS and healthy cats, drug withdrawal times for all cats were two weeks for antihistamines, four weeks for topical (skin and ear), injectable and oral glucocorticoids and four weeks for all other immunosuppressants.[21]

Serum multiplex cytokine assays

Samples were processed and immediately frozen at −80°C until cytokine analyses were performed. Serums were analyzed according to manufacturer's recommendations using a commercial feline-specific multiplex bead-based assay (FCYTMAG-20K-PMX Feline Cytokine/Chemokine Magnetic Bead Panel Premixed 19-Plex; EMD, Millipore, Billerica, MA USA).[28]

The following cytokines of interest were evaluated:

(i) Th1 cytokines; interferon gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and Il-2; and

(ii) Th2 cytokines IL-4 and IL-13; and

(iii) cytokines IL-1β, IL-6, IL-8, IL-12p40, IL-18; and

(iv) chemokines: regulated upon activation, normal T cell expressed and secreted (RANTES; CCL5), monocyte chemoattractant protein-1 (MCP-1; CCL2), stromal cell-derived factor-1 (SDF-1; CXCL12), C-X-C Motif Chemokine Ligand 1 (CXCL1); and

(v) growth factors: stem cell factor (SCF) and fms-related tyrosine kinase 3 ligand (Flt3L).

The observed concentration of each analyte for each sample was calculated using a standard curve generated from the standards and a blank per manufacturer. For each analyte, multiplex data recorded out of range were assigned the respective lowest or highest extrapolated values as previously described[28-30]; the values were reported in pg/mL.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software; La Jolla, CA, USA). Descriptive statistics were calculated and expressed as median (min-max) or mean with standard deviation. Normality of data for groups was determined using D'Agostino-Pearson test. Non-parametric statistics using Mann-Whitney-U (Wilcoxon rank-sum) test were used to compare cytokines between different groups (healthy vs. cats affected by feline atopic skin syndrome). Based on preliminary next generation transcriptome sequencing data which showed significant upregulation of of IL-4 (45-fold) and IL-13 in lesional skin of cats affected by FASS (unpublished data by the authors), all analyses were performed as one-tailed tests, with 5% level of significance ($P < .05$). To assess the influence of FASS disease severity

on the cytokine concentrations, a Spearman correlation test was performed between significantly changed serum markers and clinical score values (pVAS, SCORFAD).

Results

Thirteen client-owned cats diagnosed with FASS were enrolled in this study (Supplementary Table 1). The ages of the cats ranged from 1.8 to 11.5 years (mean 6.7 years). Seven cats were spayed females, one was an intact female and five neutered males. Breeds included twelve domestic short haired and one Siamese cat. The ages of the 12 privately owned healthy control cats ranged from 2.2 to 12.7 years (mean age 5.9 years) (Supplementary Table 1). Six cats were spayed females, one intact female and five neutered males. Breeds included eight domestic short haired, one domestic medium haired and three domestic long haired cats.

Feline atopic skin syndrome was characterized by a significant systemic upregulation of cytokines belonging to Th1 (IFN-γ, $P = 0.030$; IL-2, $P = 0.016$) and Th2 (IL-13, $P = 0.016$) immune response (Figure 1). In addition, the following cytokine and chemokines (IL-8, $P = 0.030$, IL-18, $P = 0.049$; CCL5, $P < 0.001$; CCL2, $P = 0.016$; CXCL12, $P = 0.043$), as well as the growth factors (SCF, $P = 0.010$; Flt3L, $P = 0.001$), were significantly increased (Figure 2). There was no significant changes in the Th2 cytokine IL-4 ($P = 0.130$) and the following 5 analytes (TNF-α, $P = 0.155$; IL-1β $P = 0.500$; IL-6 $P = 0.240$; IL-12p40 $P = 0.100$; CXCL1 $P = 0.310$). Assay internal controls for all analytes were within manufacturer's reference intervals.

Among the significantly upregulated serum markers in FASS, only the growth factor Flt3L ($r = 0.64$; $P = 0.01$) showed a moderate significant positive correlation with SCORFAD, the inflammatory severity score (Figure 3).

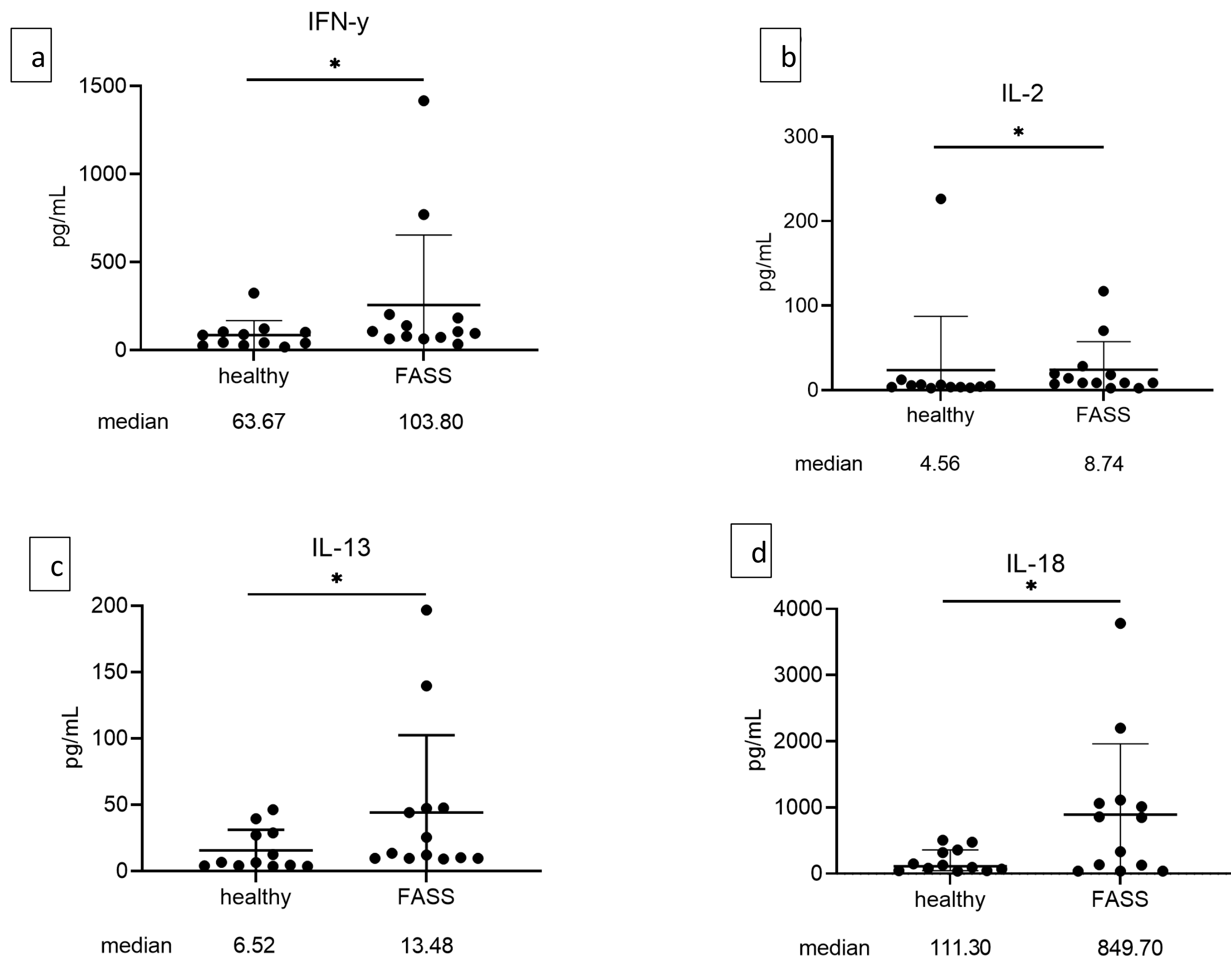

Figure 3.1. Scatter plots showing significant upregulation of cytokines belonging to (a) T helper (Th) 1 [interferon-gamma (IFN-y], (b) interleukin (IL)-2, (c) Th2 (IL-13) and (d) cytokine IL-18 immune responses in 13 cats affected by feline atopic skin syndrome compared to 12 healthy controls using the Mann-Whitney U-test. Data are expressed as mean ± standard deviation (SD). Median values for serum markers are shown below the plots. the *$P < 0.05$.

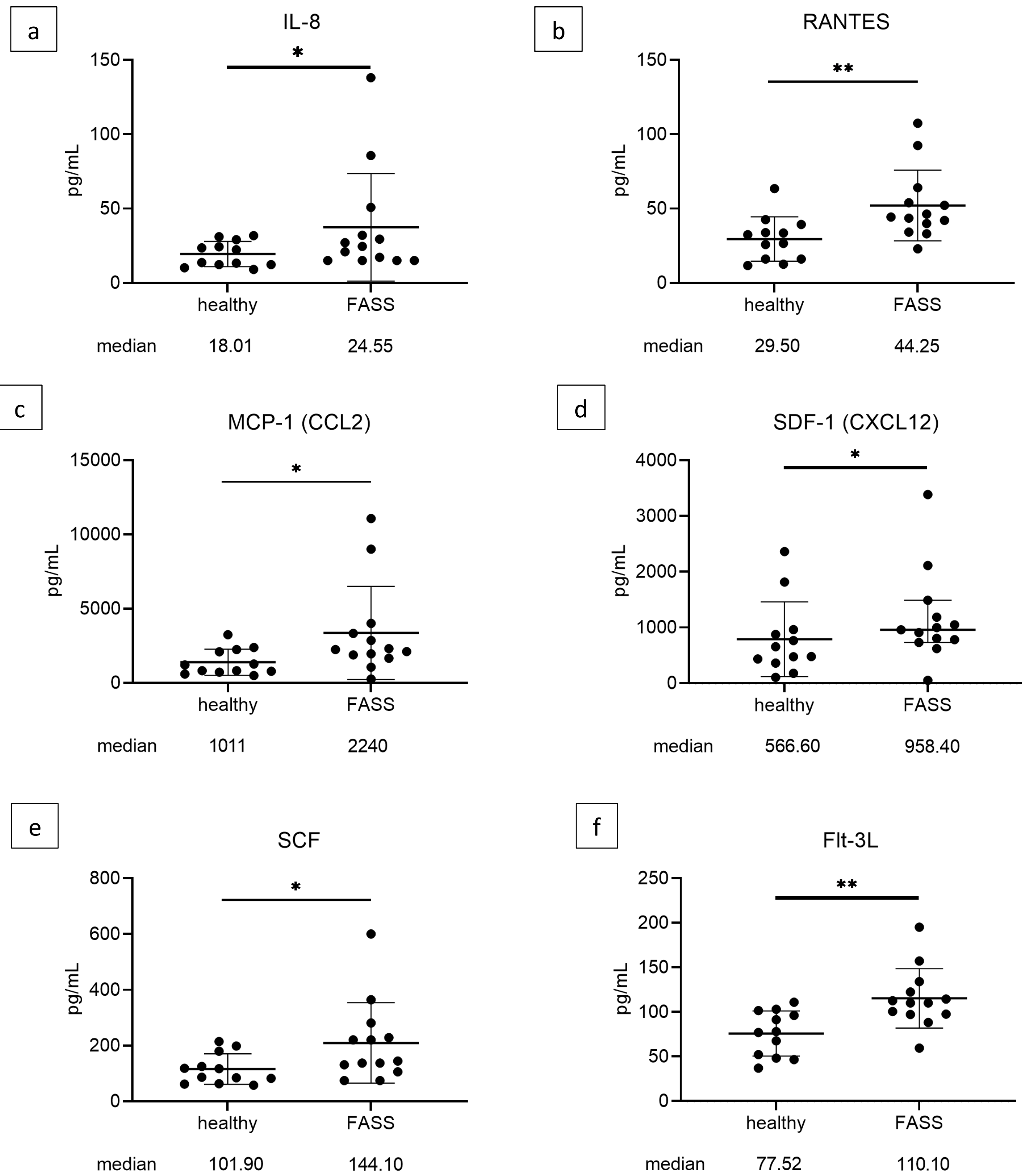

Figure 3.2. B Scatter plots showing significant upregulation of: (a) cytokine [interleukin (IL-8)], chemokines; (b) regulated upon activation, normal T cell expressed and secreted (RANTES; CCL5); (c) monocyte chemoattractant protein-1 (MCP-1; CCL2); (d) stromal cell-derived factor-

1 (SDF-1;CXCL12) and growth factors; (e) stem cell factor (SCF) and (f) Fms-related tyrosine kinase 3 ligand (Flt3L) in 13 cats affected by feline atopic skin syndrome compared to 12 healthy controls using the Mann-Whitney U-test. Data are expressed as mean ± standard deviation (SD). Median values for serum markers are shown below the plots. *$P < 0.05$, **$P < 0.01$.

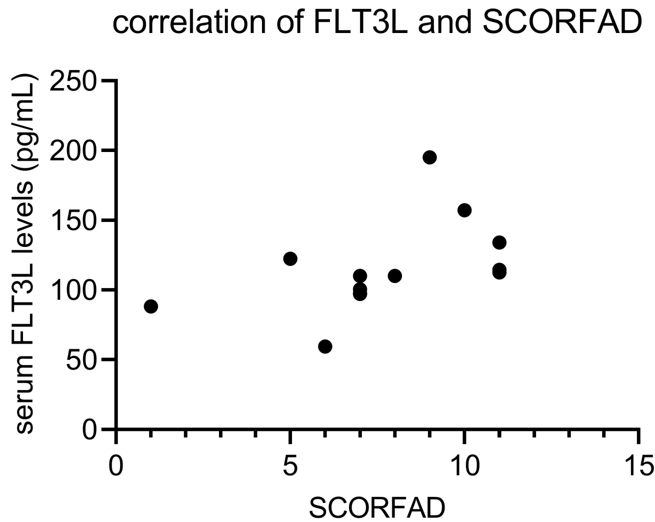

Figure 3.3. The growth factor Fms-related tyrosine kinase 3 ligand (Flt3L) showed a moderate significant positive correlation (r = 0.64; P = 0.01) with Scoring Feline Allergic Dermatitis (SCORFAD) using the Spearman correlation test.

Discussion

The results of our study show that cats affected by FASS experience a predominant upregulation of a broad array of immune secretory cytokines associated with a mixed Th1 and Th2 inflammatory response in addition to specific growth factors. Our findings contrast with data reported in abstract form which showed lack of elevated circulating immune markers in FAS using the identical commercially available multiplex bead-based assay.[20] This result could plausibly reflect the different protocols for and timing of serum collection, isolation and storage,

diagnostic criteria, medication effects, the severity of disease and recruited etiologic clinical phenotypes of FAS and FASS patients in both studies. In the aforementioned abstract,[20] included cats featured allergic dermatitis without specifying the etiologic factor, whereas in our study, only cats affected by FASS were enrolled.

Although a limited number of serum cytokines were evaluated in our study, the results show a systemic mixed Th1 and Th2 inflammatory response in the pathogenesis of FASS, which also has been shown in the serum of human[21] and canine[22] patients affected by spontaneous AD. Similar to our study results, a significant upregulation of proinflammatory and Th1 cytokines IL-8,[31-33] IFN-γ[21] and IL-18[34-35] and chemokines CCL2[36-37] and CCL5[36] has been demonstrated in the serum of human AD patients. These immune markers also are upregulated in eosinophilic plaques (i.e., CCL5)[16] of cats affected by FASS as well as in human and canine AD skin lesions.[38-45] These findings may suggest that skin-focused allergic inflammation can extend into the circulation and lead to systemic inflammation. In addition, we observed elevated levels of a clonal activator cytokine IL-2 in this study; IL-2RB, the high-affinity IL-2 receptor involved in transduction of mitogenic signals from IL-2, is upregulated in the blood of human AD patients.[46-47]

Our data show that circulating IL-13 was a significantly upregulated Th2 cytokine, which is consistent with previous serum studies performed in human and canine AD.[48,21] Interleukin-13 is considered one of the key mediators of multiple proinflammatory and allergic processes in the development of human and canine AD; IL-13 has been demonstrated to impact the epidermal barrier negatively, decrease the production of antimicrobial peptides and contribute to pruritus in human AD patients.[48-51]

Mast cell infiltration in skin lesions of FAS is considered to play an important role in the pathogenesis of the disease.[14,52] Stem cell factor stimulates growth, migration and differentiation of mast cells.[53-54] The increase in serum SCF levels has been observed in human AD patients[53] and higher levels of SCF production were shown in the skin of atopic dogs.[54] In the present study, serum SCF levels were significantly higher in the cats affected by FASS than those in the healthy control. However, additional studies are needed to determine whether the serum SCF elevation is the consequence of enhanced SCF production in the skin lesions of FASS cats.

Serum levels of CXCL12 were observed to be elevated in our cats affected by FASS. Stromal cell-derived factor-1 (SDF-1; CXCL12) recruits monocytes and lymphocytes to areas of inflammation and has been shown to further exacerbate inflammation in a mouse model of allergic airway disease.[55] To the best of the author's knowledge, there have not been any investigations of circulating CXCL12 levels in human and canine AD.

The Fms-related tyrosine kinase 3 (Flt3) receptor tyrosine kinase and its ligand Flt3L, regulate dendritic cell development and activation.[56] Fms-related tyrosine kinase 3 belongs to the class III receptor tyrosine kinase group; this group also contains other cytokine receptors, such as the c-kit, the receptor for SCF. The present study demonstrated a significant increase in serum Flt3L levels in FASS as well as a positive correlation between serum levels of Flt3L and SCORFAD. Systemic Flt3L administration in mice induced a significant increase in mature dermal dendritic cells.[57] Significant hyperplasia of epidermal Langerhans cells and dermal dendritic cells has been shown in the skin of allergic cats compared to healthy feline controls.[17] Taken together, this suggests that elevated serum Flt3L levels in FASS patients may play a role in the pathogenesis of allergic dermatitis lesions and warrants further investigation as a potential diagnostic clue or a therapeutic marker.

The limitations in the present study include the small sample size and the utilization of an established commercial feline-specific multiplex bead-based assay containing a limited number of 19 markers (cytokines, chemokines and growth factors). By contrast, the pathogenesis of circulating markers in human inflammatory skin diseases has been evaluated using the OLINK high-throughput proteomic platform, which assesses a panel of 257 immunological and cardiovascular risk proteins.[21] Another limitation of our study is that we evaluated only markers in the serum. Previous investigations of human inflammatory skin diseases have shown that not all pathogenic markers in lesional skin sites are circulating markers.[58] Interestingly, the two previous studies that evaluated cytokines and chemokines in the skin of allergic cats failed to show any upregulation of markers.[14,20] Further investigations should include a larger number of serum samples and compare the serum markers to the lesional skin markers of cats affected by FASS.

Conclusion

In conclusion, this study evaluated a broad array of serum secretory immune cytokines in cats affected by FASS. These secretory immune markers were of a mixed Th1 and Th2 inflammatory response similar to findings in human and canine patients with AD. Additional studies with larger sample sizes are needed to advance our understanding of the pathogenesis of feline atopic skin syndrome.

<u>Supplemental</u>

Table S3.1. Signalment and clinical scores for all healthy and cats affected with feline atopic skin syndrome (FASS) included in the study. DSH, domestic short haired; DMH, domestic medium haired; FS, female spayed; MN, male neutered; F, intact female; pVAS, pruritus visual analog scale; SCORFAD; scoring feline allergic dermatitis; N/A, not applicable; FASS, feline atopic skin syndrome; SA, self-induced alopecia; EP, eosinophilic plaque; MD, miliary dermatitis; EX, excoriations

Patients	**Breed**	**Sex**	**Age (years)**	**pVas (0/10)**	**SCORFAD (0/16)**	**Skin lesion type**
Healthy						
1	DSH	FS	7.7	0	0	N/A
2	DSH	FS	6	0	0	N/A
3	DSH	MN	10	0	0	N/A
4	DSH	FS	4.3	0	0	N/A
5	DSH	MN	2.8	0	0	N/A
6	DMH	MN	2.2	0	0	N/A
7	DLH	FS	1.8	0	0	N/A
8	DLH	MN	12.7	0	0	N/A
9	DSH	FS	3.2	0	0	N/A
10	DLH	F	6	0	0	N/A
11	DSH	MN	4	0	0	N/A
12	DSH	FS	9.8	0	0	N/A

Patients	**Breed**	**Sex**	**Age (years)**	**pVas (0/10)**	**SCORFAD (0/16)**	**Skin lesion type**
FASS CATS						
1	DSH	MN	10	7.5	6	SA/EP
2	DSH	MN	3.5	7	11	SA/EP
3	DSH	FS	3	10	11	MD/EP
4	DSH	FS	11.5	7	11	SA/EP/MD
5	DSH	FS	2	7.5	7	SA/EX
6	Siamese	FS	10	7.9	10	SA/EP/MD
7	DSH	FS	10.5	7.9	7	SA/EP
8	DSH	FS	3	7.4	7	MD/EP
9	DSH	MN	4	9.8	7	MD/EP
10	DSH	MN	9	8	5	SA/MD
11	DSH	F	9.5	10	8	SA/EX
12	DSH	FS	2.5	6	1	EX
13	DSH	MN	1.5	7	9	SA/EP

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CHAPTER 4

TRANSCRIPTOME ANALYSIS OF SELECTED CYTOKINE AND CHEMOKINES IN THE EOSINOPHILIC PLAQUES OF CATS WITH ATOPIC SKIN SYNDROME [2]

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[2] Vargo, C., Howerth, E.W., and Banovic, F. 2023. Veterinary Dermatology. 34:40-45.

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Abstract

Background - Previous evaluations of cytokine and chemokine gene expressions (mRNA) in the skin of allergic cats were mostly unsuccessful in detecting the T helper 2 (Th2) pathway, which is associated with the major effector cytokines interleukin (IL)-4, IL-5 and IL-13. **Hypothesis/Objective** – To evaluate differences in the mRNA expression in eosinophilic plaques of cats diagnosed with feline atopic skin syndrome (FASS) compared to healthy controls.**Animals** – Four client-owned FASS cats with eosinophilic plaques and 5 healthy control cats. **Methods** – Gene expressions (mRNA) of 14 cytokines and chemokines from eosinophilic plaque skin of FASS cats and site-matched skin samples from healthy controls were analyzed using quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis. **Results** - Eosinophilic plaques were characterized by upregulation of Th2 cytokines IL-4 ($P \leq 0.01$), IL-5 ($P \leq 0.01$) and IL-13 ($P \leq 0.01$) and Th2-attracting chemokine CCL17 ($P \leq 0.05$). Moreover, there was higher expression of S100 calcium-binding protein A 8 ($P \leq 0.01$) as well as C-X-C Motif chemokine ligand 10 (CXCL10; $P \leq 0.01$), IL-10 ($P \leq 0.05$) and the Th17 cytokine IL-17A ($P \leq 0.01$) in lesional skin compared to healthy samples. There was no difference in gene expressions of IL-12A, IL-31, IL-33, thymic stromal lymphopoietin (TSLP), tumor necrosis factor-α (TNF-α) and CCL5. **Conclusions** - Results demonstrate that eosinophilic plaques feature dominant Th2 and IL-17A inflammatory responses in the skin. Further larger-sample transcriptome studies are needed to advance our understanding of the pathogenesis of different skin lesions in FASS.

Introduction

Feline atopic skin syndrome (FASS) is a pruritic and inflammatory allergic skin disease induced by environmental allergens (e.g., dust mites, pollens, molds).[1,2] Cats with FASS typically exhibit one or more of the following clinical lesion patterns: miliary dermatitis (MD), self-induced symmetrical alopecia (SIA), facial head neck pruritus with excoriations and erosions (HNP), and eosinophilic granuloma complex (EGC).[3] Eosinophilic plaques are grouped within the eosinophilic granuloma complex (EGC) and are characterized as an elevated, intensively pruritic, erythematous and often eroded or ulcerative lesion most commonly found on the ventral abdomen and medial thighs.[2,3]

Only a few studies have reported the type of inflammatory mediators in the different skin lesions (e.g., MD, SA, HNP, EGC) of cats with FASS.[4] Two large gene expression studies involving 38 allergic cats showed no apparent difference in several pro-inflammatory and pro-allergic cytokine messenger RNA (mRNA) transcript expressions in lesional skin compared to healthy controls.[5,6] Based on these results,[5,6] the authors proposed that cats with allergic dermatitis lack the common allergy-associated T-helper 2 (Th2) immunopathogenesis observed in other species (e.g., human, canine, equine).[7-9] Further understanding of the pathogenesis and immunologic pathways in skin lesions of FASS cats is warranted to develop mechanism-based therapy for this disease.[4]

In this pilot study, we hypothesized that the gene expression (mRNA) in the skin of FASS cats with eosinophilic plaques would demonstrate upregulation of the Th2 pathway with the elevation of interleukin (IL)-4, IL-5 and IL-13 compared to control skin samples of healthy cats.

Methods and Materials

Animals

All aspects of the study were approved and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC). Signed informed consent was obtained from all owners.

Four cats diagnosed with FASS[3,10] and eosinophilic plaques[3] (e.g., raised pruritic, erythematous, erosive to ulcerated lesions with skin cytology revealing eosinophilic inflammation without bacteria) were prospectively enrolled at the University Veterinary Teaching Hospital between January 2019 and June 2020. Cats were diagnosed with FASS based on history, clinical examination findings, exclusion of other pruritic dermatoses and fulfillment of published diagnostic criteria for FASS.[3,10]

All allergic cats were deemed systemically healthy except for the clinical signs of FASS. All cats diagnosed with FASS showed no clinical improvement after receiving ectoparasite control for at least three months. In addition, all cats underwent a minimum 8-week elimination diet trial, as previously described,[11,12] without clinical improvement. Clinical lesions and pruritus were assessed in all enrolled cats by Scoring Feline Allergic Dermatitis (SCORFAD)[13] and a 10 cm-long Visual Analog Scale (VAS).[14]

Five privately-owned cats without any history and evidence of recent or chronic medical conditions were enrolled as controls; these cats were classified as healthy based on history, physical examination, complete blood count and serum biochemistry results.

This number of cats was deemed sufficient for this experiment to have a 100% power to detect a significant 2-fold difference in values (mRNA transcription) between healthy and FASS skin.

In order to limit the influence of previously administered medications on mRNA expression of cytokines/chemokines in the skin of enrolled FASS and healthy cats, drug withdrawal times for all cats were two weeks for all immunomodulating drugs (e.g., antihistamines, glucocorticoids, ciclosporin).[15]

Sample collections

All cats were sedated at the discretion of the clinician with either dexmedetomidine hydrochloride (5 µg/k; Dexdomitor, Zoetis, Kalamazoo, MI, USA) or a combination of dexmedetomidine hydrochloride (5 µg/kg), ketamine (3 mg/kg; Dechra Veterinary products, Overland Park, KS, USA) and butorphanol (0.3 mg/kg; Torbugesic[T], Zoetis); all drugs were administered by intramuscular injection. An injection of lidocaine hydrochloride 2% (Hospira Inc., Lake Forest, IL, USA) was administered subcutaneously at the biopsy site to provide additional local anesthesia.

One 8 mm skin biopsy sample was taken from lesional skin of eosinophilic plaques and bisected: one half was placed in 10% neutral buffered formalin for paraffin embedding and routine histopathology and the second half was immediately placed in RNALater solution (Ambion, Austin, TX, USA) and stored at –80°C for subsequent transcriptome analysis. Two 6 mm skin biopsy samples from site-matched areas (e.g., abdomen, lateral thorax) were obtained from healthy cats and immediately placed in RNALater solution for subsequent transcriptome analysis.

Histopathological evaluation

Formalin-fixed paraffin-embedded skin biopsy samples were routinely processed and stained with hematoxylin and eosin. Histological preparations were subjectively evaluated by

two of the authors (CV and EH) blinded to the type of gross lesion sampled. The entire skin section was scanned under low magnification and then scored over approximately three 40X magnification fields. Epidermal changes were evaluated for the presence of crusts, hyperplasia, spongiosis, and exocytosis. The dermal infiltrate was subjectively scored as follows: mild = 0-100 cells per field; moderate – 100-200 cells per field; severe = > 200 cells per field. Cell types were listed in the area of highest cellular density and dermal inflammation was noted to be predominantly superficial dermis, deep dermis, or superficial to deep dermis.

RNA extraction and quantitative reverse-transcription (qRT)-PCR analysis

Total mRNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) and RNA quantification was evaluated by spectrophotometry (NanoDrop 2000/2000c, Thermo Fischer Scientific, Wilmington, Delaware, USA). All RNA samples had a 260/280 ratio of ~1.8-2.0 and RNA quality evaluation revealed an average ribosomal integrity number (RIN) for samples of 8.1, indicating high-quality RNA for gene expression data.

The RT-qPCR analysis was performed as previously described with slight modifications.[16] Total RNAs from skin biopsy samples were reverse-transcribed into complementary DNA (cDNA) by using the qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) and the PTC-200 Gradient Thermal Cycler (MJ Research, Watham, MA, USA). The cDNA of selected genes using forward and reverse primers (Integrated DNA Technologies, San Diego, CA, USA) was amplified with PerfaCTa SYBR Green FastMix (Quantabio, Beverly, MA, USA) in accordance with the instructions of the manufacturer. The list of utilized primers is available in Supplementary Table 1.

The selected genes evaluated, IL-4, IL-5, IL-10, IL-12A, IL-13, IL-17A, IL-31, IL-33, S100 calcium-binding protein A8 (S100A8), C-C Motif Chemokine Ligand (CCL) 17, CCL5, tumor necrosis factor-α(TNF-α), C-X-C Motif Chemokine Ligand 10 (CXCL10), and thymic stromal lymphopoietin (TSLP), were chosen based on pathogenesis studies that evaluated mRNA expression of these molecules in the skin of humans and dogs with atopic dermatitis.[7,8]

All the primers were validated and confirmation of the correct size of the PCR product for all primers was performed by agarose gel electrophoresis. For each primer pair, the PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and sequenced. Using the ABI Sequencing analysis software, sequences were aligned, matched and confirmed using the Basic Local Alignment Search Tool (BLAST). The qRT-PCRs were performed in a total volume of 50 μL with 5 μL of cDNA used per reaction; wells with no cDNA served as negative controls.

Cycle threshold (Ct) values were standardized to the normalizing gene ribosomal protein L17 (RPL17), which has been documented to be a stably expressed gene in feline skin[17] and then converted to fold change using the $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software; La Jolla, CA, USA). As the data were not normally distributed, the Mann-Whitney-U (Wilcoxon rank-sum) test was used to compare values from cats affected by eosinophilic plaques from those from healthy control cats. All analyses were performed as two-tailed tests and the level of significance was $P \leq 0.05$. Fold changes are expressed as median values.

Results

Animals

The signalment and clinical scores for cats affected by FASS and healthy cats are provided in Supplementary Table 2. Four client-owned cats diagnosed with FASS and eosinophilic plaques were enrolled in this study (Figure 1a). The ages of the FASS cats ranged from 1.5 to 10 years (mean 5.5 years). Two cats were spayed females and two cats were neutered males. Breeds included three domestic short-haired cats and one Siamese cat. The ages of the five privately owned healthy control cats ranged from 0.8 to 9.8 years (mean 4.5 years). Three cats were neutered males, and two were spayed females; all were domestic short-haired cats.

Histopathological evaluation

The histological scoring and cell types for all samples is available in Supplementary Table 3. All samples had mild orthokeratotic hyperkeratosis with mild to moderate epidermal hyperplasia and spongiosis (Figure 1b). In two of the four FASS cats, the epidermis had extensive areas of ulceration. Dermal inflammation extended into the deep dermis in the three FASS cats. Numbers of cells infiltrating the dermis varied amongst the FASS cats, but was consistently composed of mast cells, eosinophils and lymphocytes. Flame figures were absent in all skin sections examined.

qRT-PCR analysis

Expression of mRNAs for all transcripts were detected in all skin biopsies. Eosinophilic plaques from FASS cats were characterized by upregulation of mRNA expression of cytokines (Figure 2, 3) belonging to Th2 markers IL-4 (125.3-fold, $P \leq 0.01$), IL-5 (57-fold, $P \leq 0.01$), IL-13 (3.7-fold, $P \leq 0.01$) and Th2-attracting chemokine CCL17 (23.5-fold, $P \leq 0.05$). Moreover, there was higher expression of S100 calcium-binding protein A 8 (3977.3-fold; $P \leq 0.01$) as well

as CXCL10 (6.8-fold, $P \leq 0.01$), IL-10 (5.9-fold, $P \leq 0.05$) and the Th17 cytokine IL-17A (116.4-fold, $P \leq 0.01$) in lesional skin compared to healthy samples. There was no significant change in mRNA expression in the following 6 transcripts (IL-12A, $P = 0.28$; IL-31, $P = 0.28$; IL-33, $P = 0.91$; CCL5, $P = 0.92$; TNF-α, $P = 0.92$; TSLP, $P = 0.99$).

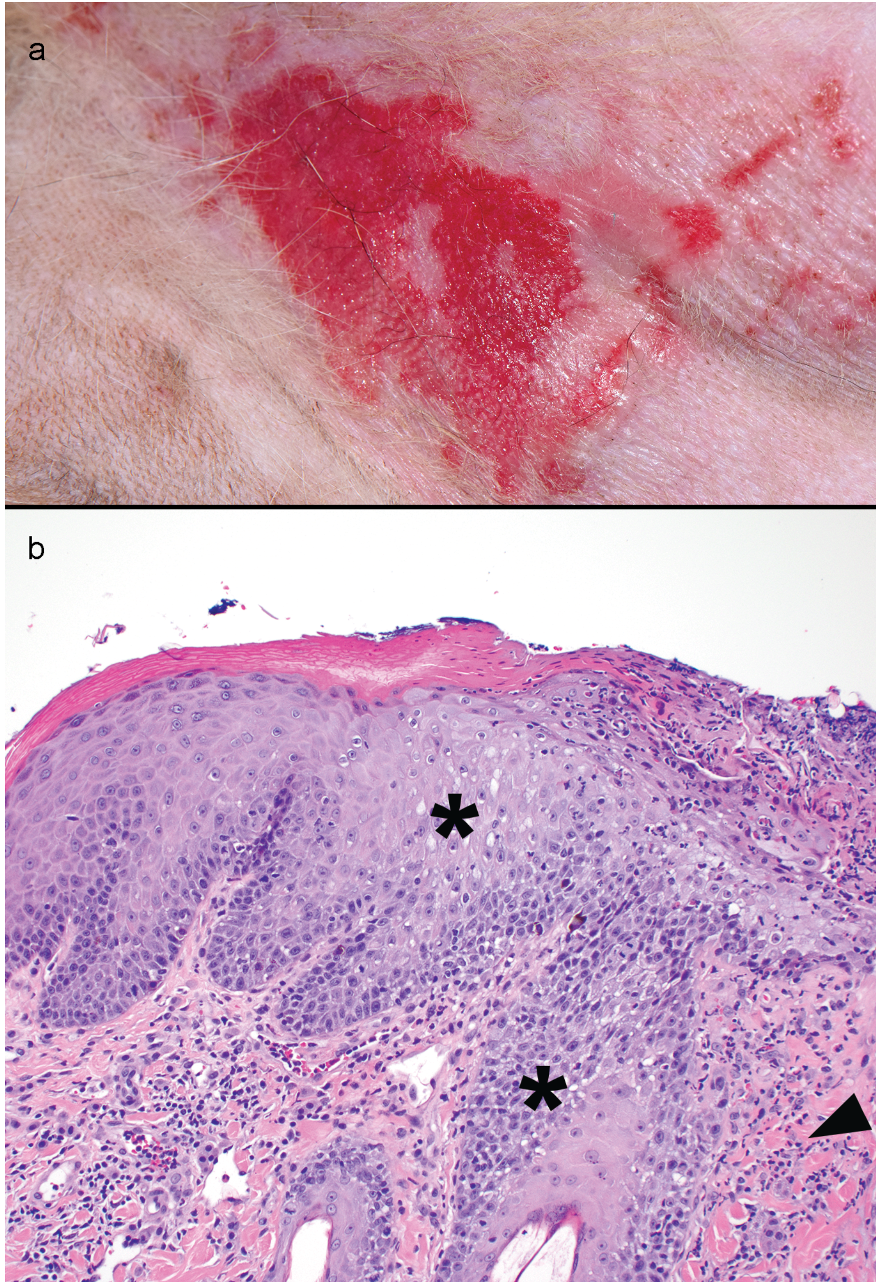
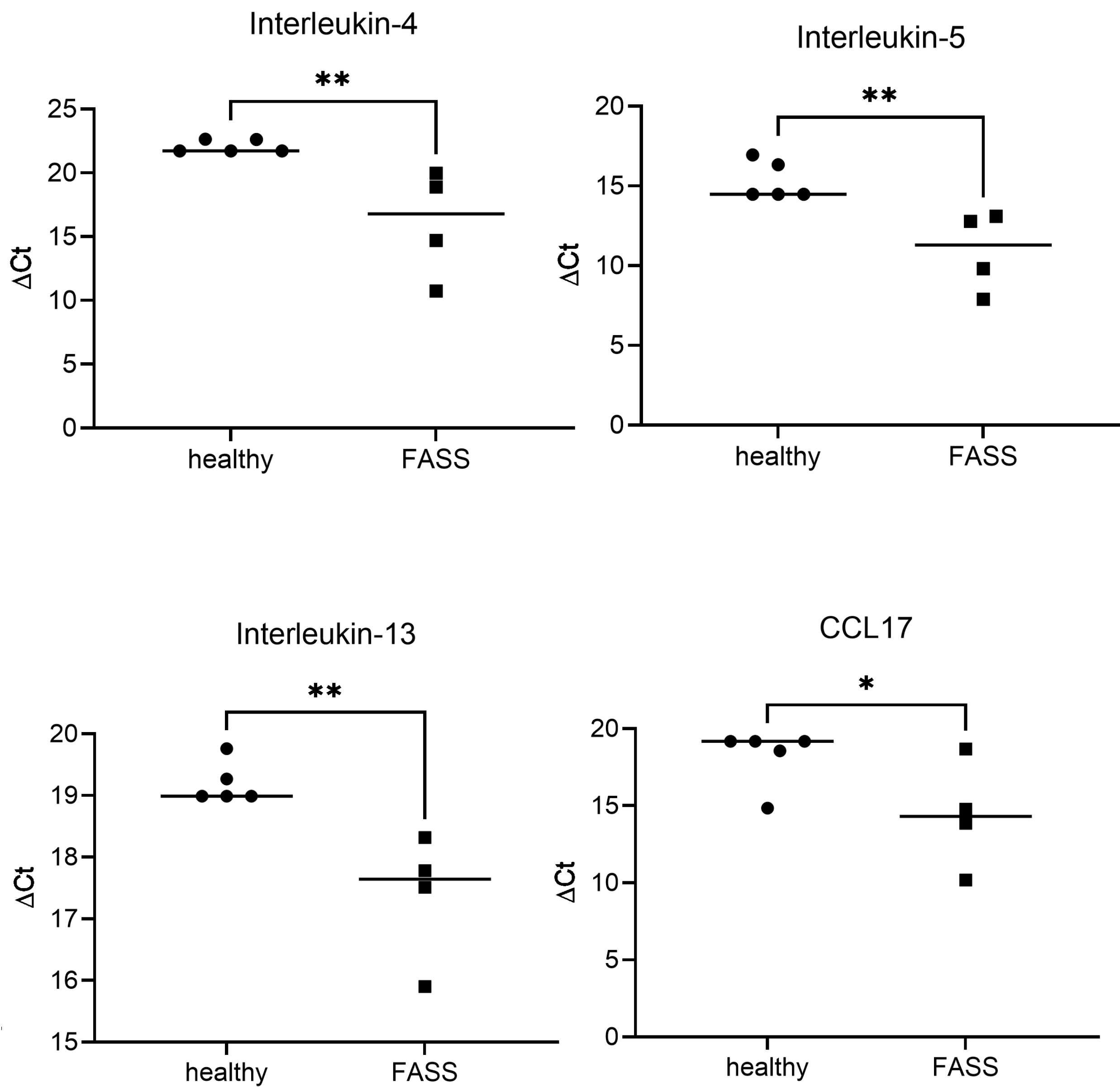


Figure 4.1. Clinical images of well-demarcated, erythematous ulcerative and alopecic eosinophilic plaque in cat 1 (a). (b) Histopathology of an eosinophilic plaque showing erosion, epidermal hyperplasia and spongiosis (asterisk) and dermal infiltration of mast cells, eosinophils and lymphocytes (arrowhead). Haematoxylin and eosin stain; x20 magnification.



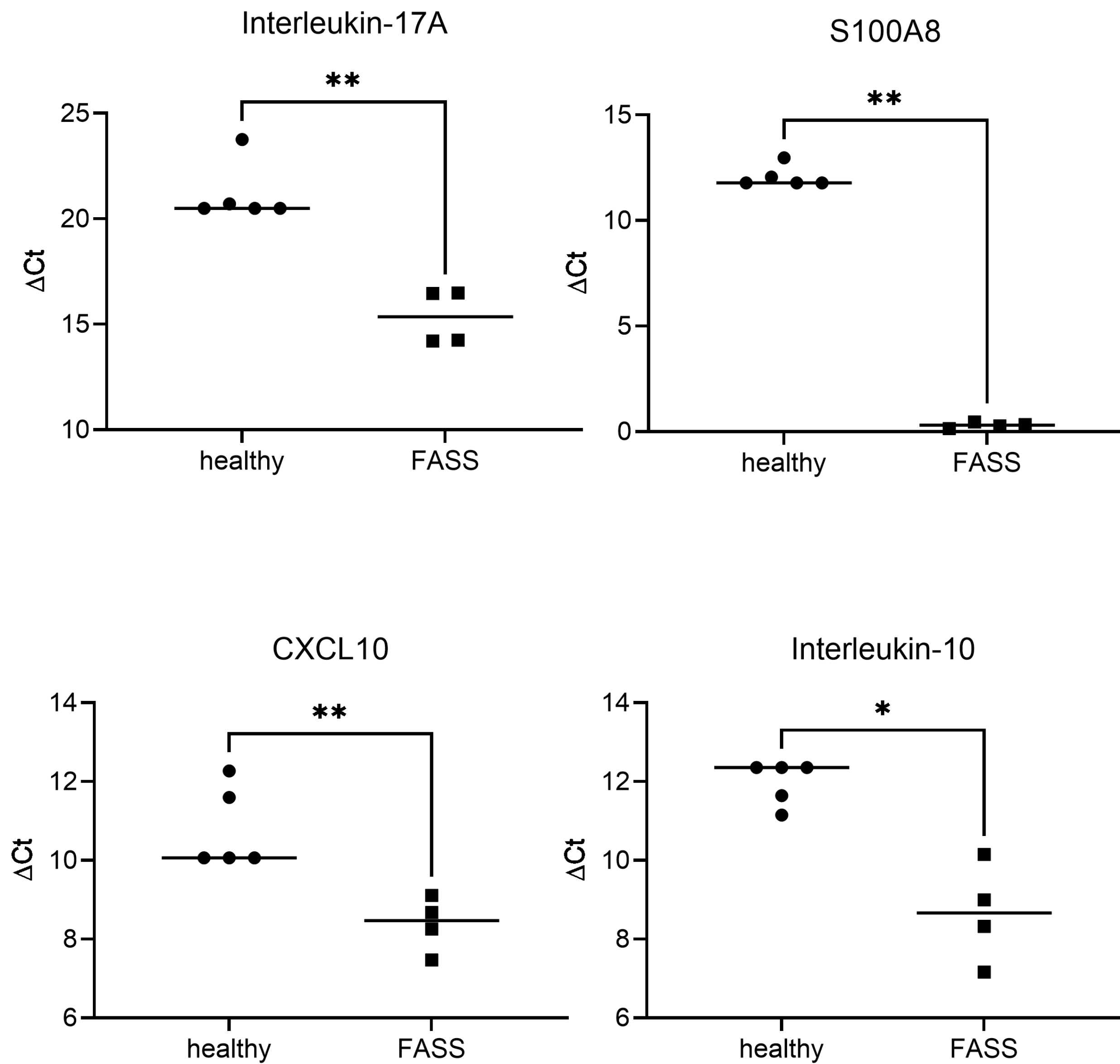


Figure 4.2. Gene expression delta cycle threshold values (ΔCt) of selected markers in lesional eosinophilic plaques of cats with feline atopic skin syndrome (FASS) were assessed by quantitative real-time RT-PCR and normalized to the expression of the RPL17 gene; healthy feline skin served as control. (* $P \leq 0.05$; ** $P \leq 0.01$).

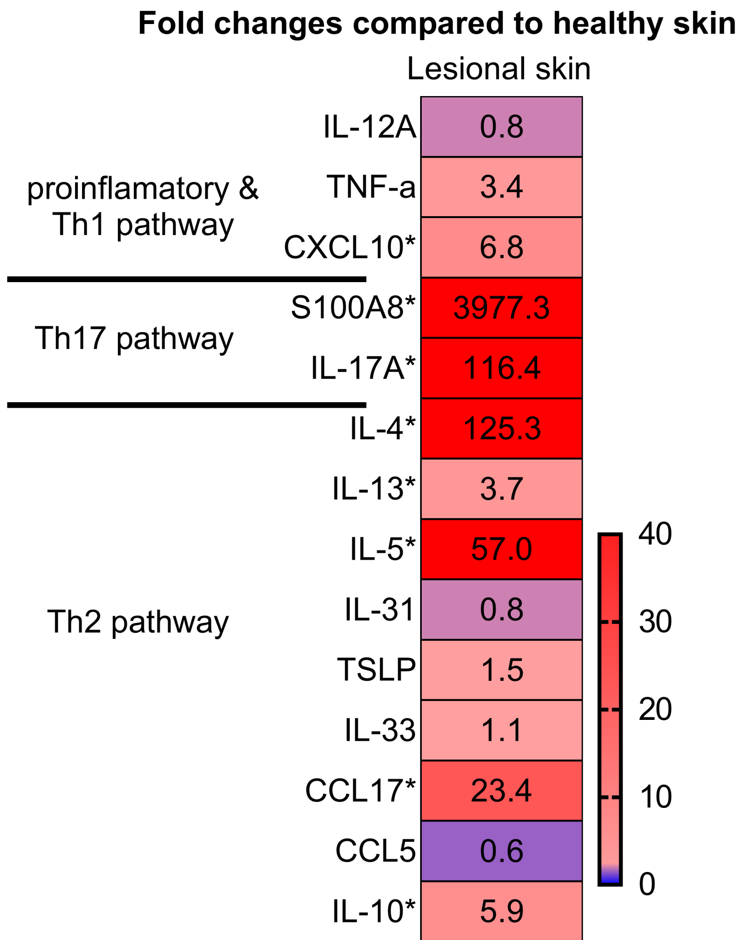


Figure 4.3. Median fold changes of quantitative reverse-transcription polymerase chain reaction (RT-qPCR) analysis for selected markers in eosinophilic plaque skin of cats diagnosed with feline atopic skin syndrome (FASS). IL, interleukin; S100A8, S100 calcium-binding protein; CCL17, C-C chemokine ligand 17; CCL5, C-C chemokine ligand 5; TNF-α, tumor necrosis factor-alpha; CXCL10, C-X-C Motif Chemokine Ligand 10; TSLP, thymic stromal lymphopoietin. *$P \leq 0.05$

Discussion

The results of our study show that eosinophilic plaques in FASS cats exhibit a predominantly Th2 inflammatory response in the skin. Our study findings expand from the conclusions of two previous large transcriptome studies where a Th2 skewing of cytokines in

lesional skin of allergic cats was absent or minimally observed.[5,6] These contrasting results could reflect the different protocols in inclusion criteria (e.g., diagnostic criteria, withdrawal times for medication effects, the severity of disease, including both feline atopic syndrome and FASS cats, FASS clinical phenotypes) and the processing of the skin biopsies for RNA isolation. Both previous transcriptome studies evaluated mRNA expressions without providing any data on the quality of RNA (i.e., RIN),[5,6] with one study[6] utilizing formalin-fixed, paraffin-embedded samples that are susceptible to RNA degradation.[18] In addition, elimination diet trials were not performed in the majority of allergic cats in one study[5] and the other investigation enrolled samples from allergic cats based on histological descriptions without detailed clinical phenotypes.[6] Compared to the two previous studies,[5,6] we evaluated a larger number of cytokines in the lesional skin of FASS cats, the results of which build upon the limited body of knowledge regarding the immunologic pathways in skin lesions of FASS cats. Furthermore, to the best of the authors knowledge, our study provides the most comprehensive assessment of cytokine and chemokine expression of a single clinical phenotype of FASS to date.

Human[19] and canine[8,20] atopic dermatitis (AD) are no longer strictly considered a Th2/Th1 disease. Non-lesional and lesional skin of human and canine AD feature a heterogeneous inflammatory profile with upregulation of multiple T-cell subsets (e.g., Th2/Th22/Th17/Th1) compared to healthy controls.[8,19] In human AD, the major Th1, Th2, and Th17 responses are progressively heightened from non-lesional to acute and then chronic AD skin lesions.[19] Although a limited number of eosinophilic plaque samples and selected immune markers were evaluated in our study, the results of mRNA expression in our study demonstrated Th2 polarization (elevated IL-4, IL-5, IL-13) and Th17 inflammatory responses.

Interleukin-4 and IL-13 represent the two key Th2 cytokines that have been shown to be important in the development of human AD[19,21] via several mechanisms such as the increased expression of chemoattractants for eosinophils, basophils and Th2 cells, inhibition of keratinocyte production of antimicrobial peptides, impairment of skin barrier function, promotion of lipid abnormalities in the epidermis, stimulation of itch-sensory neurons in skin and skin remodeling.[22] Both cytokines, IL4 and IL-13, in conjunction with CCL17, a potent chemoattractant for Th2 cells, were elevated in skin lesions of FASS cats in this report.

Interleukin 17A has multiple physiological functions.[23,24] In epithelium, IL-17A stimulates human keratinocytes to secrete IL-8 and increase the production of antimicrobial peptides and S100 family of proteins, which triggers keratinocyte inflammatory responses and induces migration and activation of neutrophils.[23,24] These IL-17A-driven epithelial responses are part of the innate cutaneous immune responses against pathogens. [23,24] In our study and per inclusion criteria, eosinophilic plaques were ulcerated and the increased IL-17A response could represent activated innate immune responses when the skin barrier is defective. The S100 proteins have the calcium-binding capacity and can induce a variety of inflammatory responses. S100A8, produced by keratinocytes, neutrophils, monocytes and macrophages, possesses both antimicrobial and inflammatory properties.[25] Expression levels of S100A8 were strongly upregulated in the eosinophilic plaques of this study; elevated S100A8 transcripts have been reported in human and canine lesional AD skin.[7,8,19]

Interleukin-5 is recognized as a key mediator for all stages of eosinophil development (growth, differentiation, maturation) and recruitment of eosinophils to areas of allergic inflammation in various organs, where they act as important effector cells.[26] Eosinophils are the predominant cell type in eosinophilic plaques; however, a heterogeneous cell population could

be observed depending on the age of biopsied skin lesions.[27] Interleukin-5 was 57-fold increased in eosinophilic plaques analyzed in this study, corresponding to the increased eosinophilic infiltrate observed on histopathological examination.

Conclusion

In conclusion, this study demonstrates that eosinophilic plaques in FASS cats feature dominant Th2 inflammatory responses in the skin. The main limitations of our pilot study are the small number of samples, the limited number of cytokines and chemokines that were analyzed and the lack of evaluation of protein expressions. Further larger-sample transcriptome and proteomic studies are needed to advance our understanding of the pathogenesis of different clinical phenotypes in FASS.

Supplemental

Table S4.1. List of primers used for quantitative RT-PCR; F, forward; R, reverse; TM, annealing temperature; IL, interleukin, RPL7, ribosomal protein L17, S100A8, S100 calcium binding protein A8; CCL17, C-C chemokine ligand 17; CCL5, C-C chemokine ligand 5; TNF-α, tumour necrosis factor-alpha; CXCL10, C-X-C Motif Chemokine Ligand 10; TSLP, thymic stromal lymphopoietin.

Primers	Accession Number	Sequence	TM (℃)	Product Length
RPL17 F	NM_001128842.1	5'- CTC TGG TCA TTG AGC ACA TCC -3'	59.1	109
RPL17 R		5'- TCA ATG TGG CAG GGA GAG C -3'		
IL-4 F	NM_001043339	5'- CTA TAC ACA TCA CAA CTG -3'	55.8	102
IL-4 R		5'- CAC TTC TTG ACT TCA TTC -3'		
IL-5 F	NM_001009845	5'- AAC CTG ATG ATT CCT ACT C -3'	58.6	90
IL-5 R		5'- AGT GCG ATT CTT TAA TGT G -3'		
S100A8 F	XM_003999782	5'- TTA CCA CAA ATA CTC CTT -3'	53.6	140
S100A8 R		5'- TAT TGA CAT CCA ACT CTT -3'		
IL-10 F	NM_001009209	5'- CTA GGA CAT AAA TTG GAG AT -3'	50	120
IL-10 R		5'- GTT GAG GTA TCA GAA GTA AT -3'		
CCL17 F	NM_001009849	5'- GCC ATA GTG TTT GTA ACT -3'	55.8	85
CCL17 R		5'- GCA AAT ATC TGA CTG TCT -3'		
IL-12A F	NM_001009833	5'- TCT CTC TGA TAA CTA ATG G -3'	53.6	91
IL-12A R		5'- TTC AAG TCC TCA TAG ATA C -3'		
IL-13 F	XM_019831874	5'- AAC AAG GTA ACT AAC AAG -3'	51.8	102
IL-13 R		5'- ATG CTC TAA TAC TAT TAT CG -3'		
CCL5 F	NM_001009827	5'- TTG GAG ATG AAC TAG GAT -3'	50.6	80
CCL5 R		5'- GTT AGG ACA TAA ACA AGA AG -3'		
IL-17A F	XM_006931816	5'- CAC AAT CTC ATC CTT CTC -3'	58.6	106
IL-17A R		5'- TAT AGC CAT CAG ACA GAG -3'		
IL-31 F	XM_011287838	5'- CCA TCC TGC CTT ATT TCA -3'	57.6	179
IL-31 R		5'- CGC TGA GAA CTG TTG TAA -3'		
IL-33 F	XM_006939157	5'- CAC AGG ATT AAC ATC ACA AT -3'	57.6	145
IL-33R		5'- TGG GAA GAA AGT ATT AGA AGA -3'		
CXCL10 F	XM_003985274	5'- ACC TGT ATC AAG ATT AGT G -3'	50.6	151
CXCL10 R		5'- TCT TAG ACT CTG GAT TCA -3'		
TNF F	NM_001009835	5'- CAA CTA ATC AAC CCT CTG -3'	59.1	79
TNF R		5'- CTA CTA CAT GGG CTA CTG -3'		
TSLP F	XM_003981241	5'- GAC CTC ATT ACC TTC TAT -3'	50	77
TSLP R		5'- GTA GCG TTA GTT CTT ATC -3'		

Table S4.2. Signalment and clinical scores for all healthy and cats affected with feline atopic skin syndrome (FASS) included in the study; DSH, domestic short haired; FS, female spayed; MN, male neutered; pVAS, pruritus visual analog scale; SCORFAD; scoring feline allergic dermatitis; N/A, not applicable; FASS, feline atopic skin syndrome

Patients	Breed	Sex	Age (years)	pVAS (0/10)	SCORFAD (0/16)	Skin lesion type	Site of lesion
Healthy							
1	DSH	MN	0.8	0	0	N/A	N/A
2	DSH	FS	3.2	0	0	N/A	N/A
3	DSH	MN	6	0	0	N/A	N/A
4	DSH	MN	2.8	0	0	N/A	N/A
5	DSH	FS	9.8	0	0	N/A	N/A
FASS cats							
1	DSH	MN	3.5	7	11	Eosinophilic plaque	Abdomen
2	Siamese	FS	10	7.9	10	Eosinophilic plaque	Abdomen
3	DSH	FS	3	10	11	Eosinophilic papule; miliary dermatitis	Neck
4	DSH	MN	1.5	7	9	Eosinophilic plaque	Limb
5	DSH	FS	7	7	4	Eosinophilic plaque	Inguinal

Table S3. Histological scoring of hematoxylin and eosin-stained sections of biopsies of eosinophilic plaques from four cats with feline atopic skin syndrome (FASS). Dermal infiltrate was scored as mild = 0-100 cells per field; moderate – 100-200 cells per field; and severe = > 200 cells per field. Cell types are listed in area of highest cellular density.

FASS cats	Epidermal changes					Dermal changes
	Crusts	**Ulcerations/ erosions**	**Hyperplasia**	**Spongiosis**	**Exocytosis**	**Infiltrate and Cells**
1	focal	ulceration 90% of epidermis	moderate; rete pegs	mild	present; mainly neutrophils, intraepidermal pustule	diffusely moderate; mast cells > neutrophils > plasma cells > lymphocytes > eosinophils extends superficial and deep dermis
2	none	ulceration 95% of epidermis	moderate; rete pegs	moderate including hair follicles	present; eosinophils, neutrophils	diffusely severe; eosinophils > mast cells > neutrophils > lymphocytes extends superficial to deep dermis
3	present; bacteria rare	ulceration 40% of epidermis	mild to moderate	moderate	present; mainly neutrophils, intraepidermal pustules	diffusely severe; neutrophils>mast cells > eosinophils > lymphocytes pockets of bacteria noted extends superficial to deep dermis
4	focal	erosions 20% of epidermis	mild	mild	intraepidermal pustule	focally moderate; mast cells > lymphocytes > plasma cells > eosinophils predominantly superficial dermis

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CHAPTER 5

DISCUSSION

The results of our two studies show that cats affected by FASS experience a predominant Th2 and Th17 inflammatory response in the skin and a mixed Th1 and Th2 inflammatory response in addition to specific growth factors in the blood. Our skin findings contrast, however, with the study that evaluated multiple cytokines in the lesional skin of allergic cats and found no significant differences in mRNA expression between lesional, nonlesional and normal skin[1] and a recent study showing a lack of upregulation of selected cytokines in lesional skin of feline allergic patients.[2] These results could plausibly reflect the different protocols in processing of the skin biopsies, RNA isolation, diagnostic criteria, medication effects, the severity of disease and recruited clinical phenotypes of FAS and FASS patients. In the multiple cytokine skin study,[1] total mRNA was extracted without specifying the average RIN for samples and only four of 16 cats completed an elimination diet trial. In contrast, in our study, the average RIN was 8.1, indicating high-quality RNA and only cats affected by FASS were enrolled. In the recent study that evaluated selected cytokines in the lesional skin of feline allergic patients,[2] formalin-fixed, paraffin-embedded skin biopsies were used for qPCR and RNAScope versus in our study, skin biopsies for qRT-PCR were immediately placed in RNALater solution; the former method can potentially be problematic as the fixation process degrades nucleic acids.[3] Our serum cytokine results also diverged with this study;[2] all of the previously mentioned possible explanations for differences in our results for skin are applicable, including the fact that the etiologic factor was

not specified for the allergic cats, whereas only cats affected by FASS were recruited in our study.

Although a limited number of cytokines were evaluated in both of our studies, the results of mRNA expression of cytokines in EP skin demonstrated Th2 polarization and Th17 inflammatory responses in the pathogenesis of FASS, which also has been shown in the lesional skin of humans[4-6] and dogs[7-8] with spontaneous and experimentally induced AD. Similar to our study results, a significant upregulation of IL-4, IL-5, CCL17, S100A8 and IL-17A has been demonstrated in the lesional skin of human AD patients.[4,6] Human AD is no longer strictly considered a Th2/Th1 disease rather multiple T-cell subsets (Th2/Th22/Th17/Th1) are involved and Th2 immune responses intensify with chronicity of skin lesions;[4] these immune pathways may also be analogous in the pathogenesis of FASS. The mRNA expression of IL-4, secreted by Th2 lymphocytes, type 2 innate lymphoid cells (ILC2), mast cells and basophils,[9] was significantly increased in the EP skin of our four FASS cats. Although IL-4 is central to the production of IgE,[10] the role of IgE in FASS is unclear. Interleukin-4 is elevated in the skin of humans[79] and dogs[11] with AD and amplifies the allergic inflammatory response by decreasing skin barrier function, decreasing the production of antimicrobial peptides and playing a pivotal role in chronic pruritus.[4, 12-15] This cytokine, however, was not found to be elevated in the serum of the 13 FASS cats, which is similar to a recent human study that analyzed an extensive array of cytokines and chemokines in patients with moderate to severe AD.[16]

Upregulation of IL-5 mRNA expression was expected as all our FASS cats had EP; however, surprisingly, CCL5 did not reach significance; this may have been due to our smaller sample size. As further evidence for the potential impact sample size may have had on our skin

results, CCL5 was significantly elevated in the serum of our 13 FASS cats; significant upregulations of CCL5 have also been reported in the serum of human AD patients.[17]

The marked elevation in CCL17 mRNA expression in our skin study replicated the findings of an earlier study examining the skin of five cats with EP; etiologies for each of the cats were not described.[18] Produced by epidermal keratinocytes, CCL17 directs the migration of Th2 cells in allergic inflammation and the receptor for this chemokine, CC chemokine receptor 4 (CCR4) is preferentially expressed on Th2 cells.[19] In addition to being upregulated in the skin and blood of human and canine AD patients, CCL17 has also been correlated with AD disease severity.[16,19-21]

The S100 protein, S100A8, which is produced by keratinocytes, neutrophils, monocytes and macrophages, possesses both antimicrobial and inflammatory properties and, similar to our findings, is upregulated in the skin of human AD patients.[4,6,22] Additionally, S100A8 has also been shown to be higher in the serum of canine AD patients compared to healthy controls.[23] Furthermore, IL-17A has been demonstrated to enhance S100A8 transcription in human keratinocytes *in vitro*.[24] Interleukin-17A is produced by Th17 cells, and as with our feline skin study where significant upregulation of this cytokine was demonstrated, IL-17A is increased in human AD skin.[4] It has also been shown that IL-17A -down-regulates the expression of filaggrin and proteins important in keratinocyte adhesion, resulting in skin barrier dysfunction.[25] The role of 100A8 and IL-17A and their effect on the skin barrier in cats in the pathogenesis of FASS requires further examination.

In contrast to our first study, the results of our serum cytokine study in the pathogenesis of FASS showed a systemic mixed Th1 and Th2 inflammatory response, which has also been shown in the serum of human[16] and canine[26] patients affected by spontaneous AD. Analogous to

our serum study results, a significant upregulation of proinflammatory and Th1 cytokines IL-8,[27-29] IFN-γ[16] and IL-18[30-31] and the chemokine CCL2[19,32] has been demonstrated in the serum of human AD patients as well as in human and canine AD skin lesions.[33,11,34-39] These findings suggest that skin-focused allergic inflammation can extend into the circulation and lead to a potential systemic inflammation. Unfortunately, due to limited amounts of cDNA for each of our FASS cats, IL-18 and IFN-γ were not evaluated; validated primers for IL-8 and CCL2 remain incomplete. However, a recent human study supports our theory that the cytokine signature in the blood originates from the skin and not vice versa based on significantly higher expression of cytokines in the skin as compared to blood.[6]

Circulating IL-13 was a significantly upregulated Th2 cytokine in our serum study, which is consistent with previous serum studies performed in human and canine AD.[16,26] Interleukin-13 is considered one of the key mediators of multiple pro-inflammatory and allergic processes in the development of human and canine AD; similar to IL-4, IL-13 has been demonstrated to impact the epidermal barrier negatively, decrease the production of antimicrobial peptides and contribute to pruritus in human AD patients.[9,13-15] Although IL-13 did not reach significance in our skin study, we suspect significance would have been achieved with a larger number of FASS cats.

The main limitations of both of our studies include the small sample size, the limited number of cytokines and chemokines analyzed, and the absolute requirement for high-quality RNA for the skin study. Originally, we had enrolled 5 additional FASS cats with EP; however, the average RIN for these five tissue samples was only 3.2, far below the 8.2 that was utilized for qRT-PCR. Preliminary data from Dr. Banovic's laboratory strongly indicates that RNA isolation from feline skin is very challenging and several methods, including snap frozen in liquid

nitrogen, have been attempted. Another limitation of our skin study is the lack of evaluation of protein expression; upregulation of mRNA may or may not result in a functional protein. A strength of our skin study is that we limited our evaluation of cytokines and chemokines to one phenotype of FASS, unlike the other two studies.[1-2] Further investigations should include a larger number of serum and skin samples and compare the serum markers to the lesional skin markers of cats affected by FASS.

CHAPTER 6

CONCLUSION

In conclusion, these two studies evaluated a broad array of cytokines and chemokines in the skin and serum in cats affected by feline atopic skin syndrome. These immune markers showed a predominant Th2 and Th17 inflammatory response in the skin and a mixed Th1 and Th2 inflammatory response in the serum, which are similar to findings in human and canine patients with atopic dermatitis. Further larger-sample studies are needed to continue advancing our understanding of FASS's pathogenesis so that more targeted and beneficial therapies for FASS can be developed.

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