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**Early Activation of Th2/Th22 Inflammatory and Pruritogenic Pathways in Acute Canine
Atopic Dermatitis Skin Lesions**

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Abbreviations:

AD: atopic dermatitis

HDM: house dust mites

Th: T-helper

Abstract

Determining inflammation and itch pathway activation in patients with atopic dermatitis (AD) is fraught with the inability of precisely assessing the age of skin lesions, thus affecting the analysis of time-dependent mediators. To characterize inflammatory events occurring during early experimental acute AD lesions, biopsies were collected 6, 24 and 48 h after epicutaneous application of *Dermatophagoides farinae* house dust mites (HDM) to sensitized atopic dogs. The skin transcriptome was assessed using a dog-specific microarray and quantitative PCR. Acute canine AD skin lesions had a significant upregulation of genes encoding T-helper-(Th)2 (e.g. *IL4*, *IL5*, *IL13*, *IL31*, *IL33*), Th9 (*IL9*) and Th22 (*IL22*) cytokines as well as Th2-promoting chemokines such as *CCL5* and *CCL17*. Pro-inflammatory (e.g. *IL6*, *LTB*, *IL18*) cytokines were also upregulated. Other known pruritogenic pathways were also activated: there was significant upregulation of genes encoding proteases cathepsin S (*CTSS*), mast cell chymase (*CMA1*), tryptase (*TPS1*) and mastin, neuromedin-B (*NMB*), nerve growth factor (*NGF*) and leukotriene-synthesis enzymes (*ALOX5*, *ALOX5AP*, *LTA4H*). In conclusion, experimental acute canine HDM-induced AD lesions exhibit an activation of innate and adaptive immune responses and pruritogenic pathways similar to those seen in humans with acute AD, thereby validating this model to test innovative therapeutics modalities for this disease.

INTRODUCTION

Atopic dermatitis (AD) is a common, chronic, recurrent, inflammatory and pruritic allergic skin disease that spontaneously develops with nearly identical clinical phenotypes in humans and dogs (Marsella and Girolomoni 2009). In both of these species, the pathogenesis of AD is

thought to involve a multifaceted—often IgE-mediated— immune dysregulation and skin barrier dysfunction stemming from an increasingly complex interplay of genetic and environmental factors (Mu et al., 2014, Czarnowicki et al., 2014, Malajian and Guttman-Yassky 2015, Bizikova et al., 2015, Pucheu-Haston et al., 2015).

Several studies recently reported microarray-based transcriptome profiles obtained from biopsies of visibly lesional or nonlesional human (for example in (Saaf et al., 2008, Suarez-Farinas et al., 2011, Gittler et al., 2012, Suarez-Farinas et al., 2013a, Gittler et al., 2013, Esaki et al., 2015)) and canine AD skin (Wood et al., 2009, Plager et al., 2012). These studies have provided relevant insight on the inflammatory and structural changes occurring at the time of acute or chronic AD skin lesions, or after immunomodulating treatment with cyclosporine (Rozenblit et al., 2014), narrow-band UVB (Suarez-Farinas et al., 2013b) or the monoclonal antibody dupilumab (Hamilton et al., 2014). Unfortunately, the inability to precisely assess the age of biopsied skin lesions in patients with spontaneous AD potentially limits the validity of characterizing the immunologic cascade that occurs during lesion development, especially in situations when gene transcription is transient or time-dependent.

Alternatively, inducing focal allergic skin reactions via epicutaneous (i.e. patch test) application of offending allergens in hypersensitive permits to study the sequence of immunologic and skin barrier alterations during AD lesion genesis and expansion. To our knowledge, only four studies have reported changes in transcriptome profiles after topical allergen challenges. These were done after patch testing with either *Malassezia sympodialis* (Saaf et al., 2008) or contactants (Correa da Rosa et al., 2015) in humans with AD, or house dust mite (HDM) allergens in

experimentally sensitized dogs (Schamber et al., 2014). While the latter study recently confirmed the expected allergen-induced upregulation of proinflammatory immune pathways and the downregulation of genes involved in epidermal differentiation and barrier function in sensitized dogs, specific immunologic changes were not discussed in details (Schamber et al., 2014).

Our objectives were to study the activation of early immunologic and pruritogenic pathways in an HDM-induced dog acute AD model using mRNA profiling of skin biopsies sequentially obtained following allergen challenge.

RESULTS

Epicutaneous house dust mite challenges in sensitized dogs elicit inflammation characteristic of acute AD

Cutaneous inflammation was not visible in any of the eight dogs before or 6 h after HDM or control patch applications; it consisted of mild erythema and edema 24 and 48 h after HDM challenges (Figure 1a,b). Eosinophilic and mononuclear dermal inflammation with focal epitheliotropism was detected at the HDM—but not control—patches after 6 h in 4/8 dogs (50%); it was strongest in 24 and 48 h HDM-skin biopsies from all dogs (Figure 1c,d). All microscopic inflammation scores were significantly correlated with their respective patch test lesion grades (Spearman's $r = 0.42$ to 0.52 ; $P < 0.05$). Importantly, epidermal hyperplasia was very mild and patchy in 48 h skin biopsies, thereby confirming that these HDM challenges led to acute, but not chronic, atopic skin lesions.

Epicutaneous house dust mite challenges result in time-dependent variability in gene transcription profiles

Skin biopsy mRNA microarray data and expression changes relative to control have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE74136, those determined by quantitative PCR are shown in Table S1.

Microarray and PCR fold changes of mRNA of 23 genes (at the three time points) were significantly correlated (Spearman $r = 0.754$, $P < 0.0001$; Figure S2); such a correlation validates the microarray-determined mRNA expression data.

A principal component analysis was performed on the microarray transcriptome profiles of all biopsies. Outside of a single 24 h-chip that was removed because of aberrant outlying values and failed quality control, transcriptomes from the various time points were generally clustered, which suggested the occurrence of consistent biological processes (Figure S3).

Using a minimum threshold of 1.5 gene expression fold change over values from control biopsies and less than 5% Benjamini-Hochberg false discovery rate (FDR)-corrected P-values, the number of significantly up-/down-regulated genes at 6, 24 and 48 h were 0/90, 104/265 and 710/445, respectively.

The 20 genes most up- and downregulated at each time point based on microarray analysis are listed on Table S4. Only 27 genes were significantly differentially expressed in the HDM-induced skin lesions at all time points compared to the 6 h control samples (Figure S5, Table S6).

Gene ontology categories significantly upregulated at 6 h were those of early signaling events (e.g. membrane receptors, transmembrane transporters, nucleoli), but there was no significant enrichment, as a category, in inflammatory and immune genes at this early time point (Table S7). In contrast, gene expression data from samples collected at 24 and 48 h after HDM challenges showed significant enrichments in immune response genes (Table S7).

Acute canine AD skin lesions are associated with the preferential expression of Type 2 cytokine and Th2-promoting chemokines

At the 6h-time point, the application of HDM to the skin of these sensitized dogs led to the early—but not significant—activation of genes coding for the Th2 cytokines *IL5* and *IL31* and the Th2-promoting chemokine *CCL17* (TARC); the Th22 cytokine *IL22* genes (Figure 2a, Tables S1 and S4) was significantly upregulated. In contrast, and to our surprise, *TSLP* and *IL25* were significantly downregulated.

Twenty-four hour after HDM application, the expression of Th2 and Th22 cytokine genes was markedly upregulated, especially that of *IL5*, *IL13*, *IL31* and *IL22*, while that of *TSLP* and *IL25* was downregulated further. This Th2 bias was corroborated by the strong upregulation of Th2-promoting CC chemokine genes, which were, in decreasing levels of transcription: *CCL17* (TARC), *CCL5* (RANTES), *CCL24* (eotaxin-2), *CCL19* (MIP3-beta), *CCL13* (MCP4), *CCL8* (MCP2), and *CCL27* (CTACK) (Figure 2a, Tables S1 and S4).

By 48 h after starting allergen challenges, there was further amplification of Th2 (*IL4*, *IL5*, *IL13*, *IL31*, *IL33*), Th9 (*IL9*), Th22 (*IL22*) and pro-inflammatory (*LTB*, *IL6*, *IL18*) cytokine gene transcription. The expression of Th2-promoting chemokines was also intensified, with *CCL17* being the most differentially expressed of all genes (Figure 2a, Tables S1 and S4).

In all these samples, the expression of Th1, Th17, regulatory cytokine and CXC chemokine genes did not change markedly (Figure 2a, Tables S1 and S4).

Allergen challenges lead to the upregulation of multiple pruritogenic pathways

In 6 h-HDM patch test skin biopsies, the only three significantly upregulated non-cytokine pruritogens were genes encoding nerve growth factor (*NGF*) and enzymes involved in leukotriene-B4 (*LTB4*) synthesis (5-lipoxygenase [*ALOX5*] and its activating protein FLAP [*ALOX5AP*]) (Figures 2b and 3, Tables S1 and S4).

The Th2 cytokine *IL31* was the most highly transcribed pruritogen 24 h after challenge. At that time point, other significantly upregulated pruritogenic genes encoded the 5-lipoxygenase complex, the proteases cathepsin S (*CTSS*) and C (*CTSC*), chymase (*CMA1*) and the tryptic peptidase mastin, the bombesin neuromedin B (*NMB*) and TrkA (*NTRK1*), the NGF receptor (Figures 2a,b and 3, Tables S1 and S4).

After 48 h, there were few additional changes in pruritogen gene upregulation compared to the 24 h-samples, except for the significant transcription increases in mast cell tryptase *TPS1*, the

pruritogenic cytokines *IL4* and *IL6*, as well as genes encoding two enzymes involved in histamine metabolism: histidine decarboxylase (*HDC*) and histamine N-methyltransferase (*HNMT*) (Figures 2a,b and 3, Tables S1 and S4).

Acute experimental AD skin lesions are associated with few epidermal changes

In this canine acute atopic skin lesion model, the expression of genes encoding epidermal differentiation and stratum corneum barrier genes was generally not significantly changed (Tables S1 and S2). Notable exceptions were the downregulation of claudin-1 (*CLDN1*), ELOVL fatty acid elongase 6 (*ELOVL6*) and sphingosine-1-phosphate lyase 1 (*SGPL1*), and the upregulation of the proliferating cell nuclear antigen (*PCNA*), serpin peptidase inhibitor B4 (*SERPINB4*) and histidine ammonia-lyase (histidase, *HAL*). Interestingly, the calcium-binding proteins *S100A8*, *S100A9* and *S100A12* were all downregulated at the 6 h time point before being increasingly upregulated over time, with the most significant changes occurring 48 h after challenge (Table S1).

Allergen challenge is not associated with very early immune dysregulation

To determine if some of the genes might have been upregulated earlier than what was seen in the 6 h-biopsies, the expression of 14 selected genes perceived to be of importance was quantified by PCR in a second experiment in which biopsies were taken 1 and 3 h after allergen challenge.

Outside of *TNF*, which was significantly upregulated as early as 1 h after HDM challenge, respectively, the expression of the other genes was not significantly changed (Table S8).

DISCUSSION

In this paper, we establish that, in an experimental HDM-sensitized dog model, acute AD skin lesions develop soon after epicutaneous allergen challenge and exhibit the expected Th2 and Th22 cytokine bias. Furthermore, induced lesions are associated with the activation of several itch-promoting pathways, which include known pruritogenic cytokines such as IL31, cathepsin and mast cell proteases, nerve growth factor, neuromedin B and enzymes involved in leukotriene B4 and histamine synthesis.

The predominant increase in the expression of Th2 cytokine genes seen in this model mirrors that found in the transcriptome of acute—but not chronic—human AD skin (Gittler et al., 2012). We did not detect the small upregulation of Th1 and Th17 cytokines seen in chronic human AD lesions, however (Gittler et al., 2012); this difference might be due to the early post-challenge biopsy times in this study, which reflects early acute atopic lesion development but not chronic AD. This existing Th2 cytokine bias of canine AD and experimental HDM-induced skin lesions has been known for over a decade (Olivry et al., 1999, Nuttall et al., 2002, Marsella et al., 2006). Our study confirms the stronger upregulation of *IL13* and *IL5* over that of the classic Th2 cytokine, *IL4*, as previously shown in spontaneous canine AD skin (Schlotter et al., 2011), after HDM patch testing in sensitized dogs (Marsella et al., 2006) or after stimulation of T-lymphocytes collected from canine chronic AD skin (Jassies-van der Lee et al., 2014).

Herein, we document the previously unreported increased transcription of *IL31* after allergen challenge; in fact, *IL31* was the gene most upregulated in 6 and 24 h-samples, and it was second in expression to *CCL17*, 48 h after allergen provocation. This cytokine, a strong pruritogen in dogs as in other species (Gonzales et al., 2013), is also highly expressed in acute and chronic human AD skin (Gittler et al., 2012). While *IL31* had previously been detected in the serum of half of atopic dogs (Gonzales et al., 2013), it was not found to be transcribed in the lesional skin of dogs with AD using PCR (Mizuno et al., 2009). The clear Th2 cytokine bias seen in these acute canine AD lesions was likely enhanced by the preferential—and often strong—upregulation of CC chemokines that recruit cells involved in a type 2 immune response. In fact, *CCL17* was the highest gene expressed in microarray and PCR assays at the 48 h time point. This strong upregulation of *CCL17* has been previously reported in lesional human (Gittler et al., 2012, Choy et al., 2012) and canine AD skin (Plager et al., 2012, Maeda et al., 2002, Maeda et al., 2005), as well as after HDM challenges in sensitized dogs (Marsella et al., 2006).

In this study, a significant expression of *IL22* was found as early as 6 h after allergen challenge; importantly, this was the only cytokine upregulated at that early time point. This cytokine encoding gene has been similarly found to be activated in the skin of humans with acute and chronic AD (Nogales et al., 2009, Gittler et al., 2012), especially that of patients with the intrinsic form of the disease (Martel et al., 2016). Furthermore, skin-homing IL13- and IL22-producing Th2/22 and Tc2/22 were recently found to be elevated in human patients with AD, and their frequency in the peripheral blood correlated with skin lesion scores and IgE levels (Czarnowicki et al., 2015). Such increase in *IL22* has been reported in stimulated T-lymphocytes

collected from both nonlesional and lesional chronic canine AD skin (Jassies-van der Lee et al., 2014). Interleukin-22 might participate in the promotion of AD chronicity, as it induces epidermal hyperplasia, inhibits proteins involved in epidermal differentiation and skin barrier function and upregulates antimicrobial peptides that include S100A8/A9, the latter being also highly expressed in our study (Eyerich et al., 2009, Wang and Xu Landen 2015).

In this paper, we document the significant upregulation of the Th2-promoting cytokine *IL9* and two of its transcription factors *SPI1* and *BATF* after allergen challenge in atopic dogs. This cytokine appears important for the promotion of IL13, IL17 and interferon gamma production by skin-homing T-lymphocytes (Schlapbach et al., 2014). The importance and the specific role of IL9 in the pathogenesis of human AD are currently being investigated (Ciprandi et al., 2013, Ma et al., 2014).

As recently shown in another HDM patch test study in dogs (Schamber et al., 2014), the Th2-promoting cytokine gene *IL33* was also upregulated in our study. This cytokine and its receptor are also overexpressed in human lesional AD skin and after HDM patches in sensitized mice; in the latter, it is activated earlier than in dogs after allergen challenge (Savinko et al., 2012, Savinko et al., 2013). Interleukin-33 might participate in the induction of IL5 and IL13 production by type 2 lymphocytes (Th2) and innate lymphoid cells (ILC2) (Imai et al., 2013, Lott et al., 2015).

In the skin of dogs with AD and after the challenge of canine keratinocytes with HDM allergens, the Th2-promoting cytokine gene *TSLP* was found to be upregulated (Klukowska-Rotzler et al.,

2013). A strong immunostaining for TSLP was also seen focally in spontaneous canine AD lesional skin (our unpublished results). In contrast to these observations, we found the *TSLP* gene to be downregulated as early as 6 h after allergen provocation in mite-sensitized dogs. In human AD skin, there is a similar discrepancy between the lack of significant upregulation of *TSLP* in acute or chronic AD skin (Gittler et al., 2012) and the strong epidermal immunostaining for this protein that was initially reported in human AD lesions (Soumelis et al., 2002). The reasons for this inconsistency are not known, but the hypothesis that an increase in the transcription of *TSLP* could be precocious yet transient was invalidated, as we did not detect such upregulation in biopsies collected 1 and 3 h after HDM application in our sensitized dogs. Alternatively, the observed downregulation of *TSLP* might be that of the constitutively-expressed anti-inflammatory short isoform of TSLP rather than that of its pro-inflammatory long isoform; this has been shown recently in human AD (Fornasa et al., 2015). The carboxy-terminal PCR probe used in our study unfortunately did not allow for the differentiation in the transcription of either *TSLP* isoform.

When looking at the expression of pruritogens in this acute canine AD model, we found the early activation of genes encoding several proteins known to induce itch, such as Th2 cytokines, several proteases, enzymes involved in the synthesis of leukotrienes, the bombesin neuromedin B (NMB) as well as nerve growth factor (NGF).

Th2 cytokines shown to directly induce itch are IL4, -13 and -31 as well as TSLP while a pruritogenic role is only suspected for IL2 and IL6 (reviewed in (Storan et al., 2015)). Recently, IL33 was also found to indirectly induce itch via mast cell degranulation and histamine release in

transgenic mice (Imai et al., 2013). Interestingly, all of these cytokine genes, but *TSLP*, were found to be markedly upregulated in our dog model of HDM-induced acute AD; the early and particularly strong expression of *IL31* was especially remarkable. It is suspected that these Th2 cytokines are involved in the genesis of atopic itch because of the strong antipruritic effect seen with the new JAK inhibitor oclacitinib in atopic dogs (Cosgrove et al., 2013); this drug preferentially blocks JAK1-mediated signal transduction after binding of the pruritogenic cytokines IL2, -4, -6, -13 and -31—and at least theoretically TSLP—to their respective receptors (Gonzales et al., 2014). That IL31 is likely responsible for the generation of clinical signs of canine and human AD is supported by the recent demonstration of substantial relief of pruritus manifestations after injection of therapeutic monoclonal antibodies targeting IL31 in dogs (Michels et al., 2015) or the IL31 receptor A in humans with this disease (Nemoto et al., 2015); in atopic dogs, this monoclonal antibody also reduces the severity of skin lesions, albeit with a smaller magnitude than that of itch (Michels et al., 2015).

In this dog model, HDM induced the upregulation of genes coding for three mast cell proteases that could be involved in itch induction: chymase, tryptase and the tryptase-like mastin; the latter being analogous to mouse mast cell protease-11 (*Prss34*) (note: their human homologue is only a pseudogene). Mastin has been shown previously to be upregulated in acute canine AD skin (Plager et al., 2012), but its itch-inducing effect has not been studied in animal species in which this protein exists. Chymase has been shown to be a pruritogen in a single old publication (Hagermark et al., 1972). While intradermal injections of tryptase induce itch in mice (Ui et al., 2006), they do not appear to do so in dogs (Carr et al., 2009).

Interestingly, we observed in this study the significant upregulation of the pruritogenic cathepsin S, a cysteine protease secreted by keratinocytes after stimulation by proinflammatory cytokines such as tumor necrosis factor-alpha and interferon-gamma (Schonefuss et al., 2010). Even though it was first believed to induce itch via activation of protease-activated receptors (PAR)-2 and -4 (Reddy et al., 2010), it is now known to do so, not by means of PARs, but by the direct activation of MrgprC11 after specific cleavage of its aminoterminal (Reddy et al., 2015). While cathepsin S was not detected in the lesional epidermis of NC/Nga mice with AD (Schonefuss et al., 2010), transgenic mice overexpressing this protein spontaneously develop a chronic AD-like dermatosis (Kim et al., 2012). The expression of CTSS was previously found to be significantly higher in lesional than nonlesional canine AD skin (Plager et al., 2012). Altogether, cathepsin S might be relevant for the induction of atopic skin inflammation and/or its associated itch; the demonstration of its importance awaits trials with interventions specifically targeting this protease.

The bombesin NMB, whose gene was previously shown to be upregulated in lesional acute canine AD skin (Plager et al., 2012), was also overexpressed early after allergen challenge in our study. Neuromedin B induces itch when injected intrathecally in rats (Sukhtankar and Ko 2013). It is possible, at least theoretically, that NMB could be secreted by primary afferent neurons and induce mast cell degranulation and histamine release, as shown for the other mammalian bombesin gastrin releasing peptide (GRP) (Andoh et al., 2011). Whether or not NMB is a skin pruritogen, directly or indirectly via mast cell degranulation, has not yet been shown in any species.

Of interest was the observation that genes encoding LTB₄ synthesis enzymes were among the first to be upregulated after allergen challenge. This leukotriene has long been known to be present in high concentrations in the lesional skin of dogs and humans with AD (Ruzicka et al., 1986, Kietzmann 1990). Intradermal injections of LTB₄ variably induce pruritus in mice (Andoh and Kuraishi 1998), while injections in human skin are mostly not pruritogenic (Camp et al., 1983). This leukotriene appears involved in the pruritus present in NC mice with chronic AD (Andoh et al., 2011), and a positive correlation has been found between LTB₄ concentrations and pruritus in atopic dogs (Kietzmann 1990). Whereas the use of the 5-lipoxygenase inhibitor zileuton appears to have some antipruritic effect in humans with AD (Woodmansee and Simon 1999), the clinical benefit of interventions targeting leukotrienes in atopic dogs appears minimal (Olivry et al., 2010).

In this study, the only known cytokine pruritogen gene significantly upregulated in 6 h biopsies was *NGF*. This neurotrophin has been shown to sensitize nerves to non-histamine-induced itch, especially that induced by cysteine protease-containing cowhage spicules (Rukwied et al., 2013). In humans with AD, the immunoreactivity of NGF is higher in lesional compared to nonlesional AD and healthy skin (Dou et al., 2006); its expression is also high in keratinocytes from atopic patients, and it appears to be directly responsible for neurite outgrowth (Roggenkamp et al., 2012). In contrast to these observations, the dermal concentration of NGF was reported to be lower in microdialysates of nonlesional and lesional AD compared to healthy human skin (Papoiu et al., 2011). A possible explanation for this discrepancy might be that NGF is only transiently expressed during a flare of AD as, in our study, it was upregulated early before being downregulated in samples collected 48 h after allergen challenge.

In summary, this study of the skin transcriptome that occurs early after epicutaneous HDM allergen challenge in sensitized dogs revealed the early expression of Th2/Th22 cytokines and proteases as well as other known non-cytokine pruritogens. As this model recapitulates many of the molecular events seen in acute human AD, it could be used to test new therapeutics developed to treat atopic itch and skin lesions in humans or dogs. These observations should ideally be confirmed with studies of corresponding protein expression and with a higher number of animals (especially for the study of early time points after allergen challenge). Nevertheless, our results suggest that atopic itch is likely due to the combined action of multiple pruritogens. As a result, the successful treatment of existing and active pruritic AD skin lesions is likely to require the use of drugs with broad targets rather than the specific inhibition of single molecules or pathways. In contrast, the prevention of flares might benefit from the identification, and subsequent targeting, of early mediators activating downstream pro-allergic pathways.

MATERIALS AND METHODS

All the experiments involving animals herein had been approved beforehand by the institution's animal care and use committee; animal studies were not terminal.

Animals

Eight Maltese-beagle atopic dogs (five intact males, three intact females; 5.5 to 10.5-year-old), epicutaneously sensitized to *Dermatophagoides farinae* HDM (Olivry et al., 2011), were selected after demonstration of acute AD skin lesions following epicutaneous HDM challenge. This

number of dogs was deemed sufficient for this experiment to have a 100% power to detect a significant two-fold difference in values (mRNA transcription or cell counts) between pre- and post-challenge skin biopsy samples.

House dust mite patch tests

To avoid the skin irritation seen with adhesives surrounding small patches, allergen challenges were done with slight modification of our previously reported method (Olivry et al., 2012).

Herein, allergen challenges consisted of 500 μ g of HDM slurry in 10 μ l of mineral oil covered by 4 x 1.5 cm bands of laboratory film (Parafilm M laboratory film, Pechiney Plastic Packaging, Chicago, IL) then covered with waterproof tape at least 2 cm away from the HDM application sites.

In the first experiment, three HDM and one mineral oil control patches were placed on the lateral thorax of each of the eight dogs. Skin lesions of increasing severity were graded before and then 1 h after removing patches 6 (HDM and control), 24 (HDM) and 48 h (HDM) after application using a 4-point scale, as done previously (Olivry et al., 2006). After heavy sedation and local anesthesia, skin biopsies were collected at each time point. They were bisected, and half was placed in neutral buffered formalin while the other was disposed in RNALater (Ambion, TX) and kept frozen till mRNA extraction.

To detect even earlier mRNA expression changes, HDM patches were later applied onto the thoracic skin of four of the original eight HDM-sensitized dogs (all intact males, 7-years-old).

One and 3 h after HDM application, patches were removed, macroscopic skin lesions were then graded and biopsies collected as described above.

Histopathology

One five-micrometer section of each formalin-fixed paraffin-embedded skin biopsy was stained routinely with hematoxylin and eosin. The entire section was examined, in a manner blinded to the biopsy timing and patch nature; epidermal, dermal, eosinophilic and mononuclear dermal inflammation were graded subjectively as none (0), mild (1), moderate (2) or severe (3). Epidermal and dermal scores were also added to yield a score reflecting total inflammation.

Macro- and microscopic lesions were compared between control and 6, 24 and 48 h HDM patches by repeated-measure ANOVA (Friedman test) and subsequent post-tests (Dunn's multiple comparison tests). Nonparametric correlations were done by calculation of Spearman's coefficients.

Microarray analysis

Skin biopsies disposed in RNA later were minced and placed in a Precellys homogenizer. Total mRNA was extracted using Qiagen RNeasy kit (Qiagen, Valencia, CA)) and quantified by NanoDrop. Samples were normalized to the lowest concentration sample, and cDNA was made using Superscript Vilo cDNA synthesis Master Mix (Invitrogen Life Technologies, Grand Island, NY).

The cDNA samples were then labeled with biotin with the FL-Ovation cDNA Biotin Module V2 and hybridized to a Canine Genome 2.0 Array using a Genechip Hybridization Kit. The microarray chips were washed and stained using a Genechip Hybridization Wash and Stain Kit then scanned using a Genechip Scanner. All reagents and readers were used according to the manufacturer's instruction (Affymetrix, Santa Clara, CA).

The microarray gene expression data were analyzed using ArrayStudio 7.0 (OmicSoft, Cary, NC). Data were normalized using robust multiarray averaging and scaled to a mean target intensity of 150. Differentially expressed transcripts were identified using a Student's t-test to compare each challenged time point to the unchallenged control. Significance thresholds were set at a Benjamini-Hochberg FDR of 5% and at least a 1.5-fold-change difference.

Quantitative PCR analysis

To obtain mRNA expression levels for selected genes of interest that were not assessable with the chosen Affymetrix canine chip, quantitative PCR was performed on an Applied Biosystems Viia7 System (Carlsbad, CA) using Taqman Fast Advanced Master Mix (Invitrogen), the previously prepared cDNA and the primer/probe sets reported in Table S9. The relative expression levels of mRNA were determined using the delta,deltaCT formula; fold changes were calculated as $2^{-\Delta\Delta CT}$.

To verify the validity of the data obtained with the Affymetrix microarray, the expression levels of 23 genes with high and low microarray up- and down-regulation were also tested by

quantitative PCR. The correlation between microarray and PCR fold changes of mRNA of all genes at the three time points was calculated using Spearman rank correlation coefficient.

CONFLICT OF INTEREST

C. Peredo, D. Mayhew, L. Yoon, D. Rajpal, H. Hofland and J. Cote-Sierra are, or were at the time of the study, employees of GlaxoSmithKline.

FIGURE LEGENDS

Figure 1. Macro- and microscopic skin lesions after HDM challenges.

(a) Erythema became visible 24 and 48 h after HDM application. (b) Patch test lesion scores were significantly higher 24 h compared to 6 h control and HDM patches. (c) eosinophilic and lymphocytic dermal inflammation with focal epitheliotropism after HDM challenges. Note that the epidermis only became slightly hyperplastic at the latter time point, hematoxylin-and-eosin bar = 100 μ m. (d) total (epidermal + dermal) inflammation scores were significantly higher 24 and 48 h after HDM application compared to those of 6h biopsies. HDM: house dust mites *: $P < 0.05$; ** $P < 0.01$. Clinical and photomicrograph series are of patches from the same dog.

Figure 2. Expression of selected relevant cytokine, chemokine and pruritogenic genes after HDM challenges.

(a) expression of cytokine and chemokine-encoding genes arranged by dominant function; (b) expression of selected genes encoding known pruritogens, enzymes involved in their metabolism or pruritogen receptors. Genes are arranged by their dominant function or family. In this figure, colors correspond to the level of relative expression compared to 6 h control samples, and bold lettering denotes statistically significant differences at $P < 0.05$. Fold changes are a combination of data from microarray and PCR (starred), which was generally done when the gene was not present in, or to verify its expression using the microarray.

Figure 3. Expression of selected pruritogenic genes after HDM challenges

In this word cloud are only represented genes with a significantly greater than 1.5 fold change in gene expression in HDM compared to control samples. The size is proportional to the level of expression, the color corresponds to that of the pathway involved while an underlined gene name denotes a downregulation of this gene.

FIGURES

Figure 1. Macro- and microscopic skin lesions after HDM challenges.

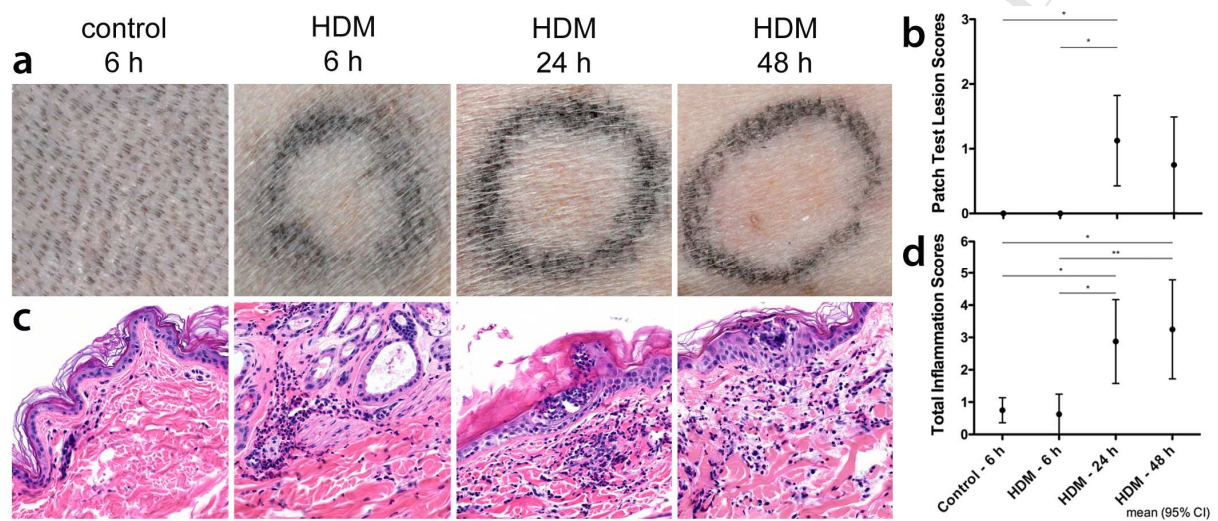


Figure 2. Expression of selected relevant cytokine, chemokine and pruritogenic genes after HDM challenges

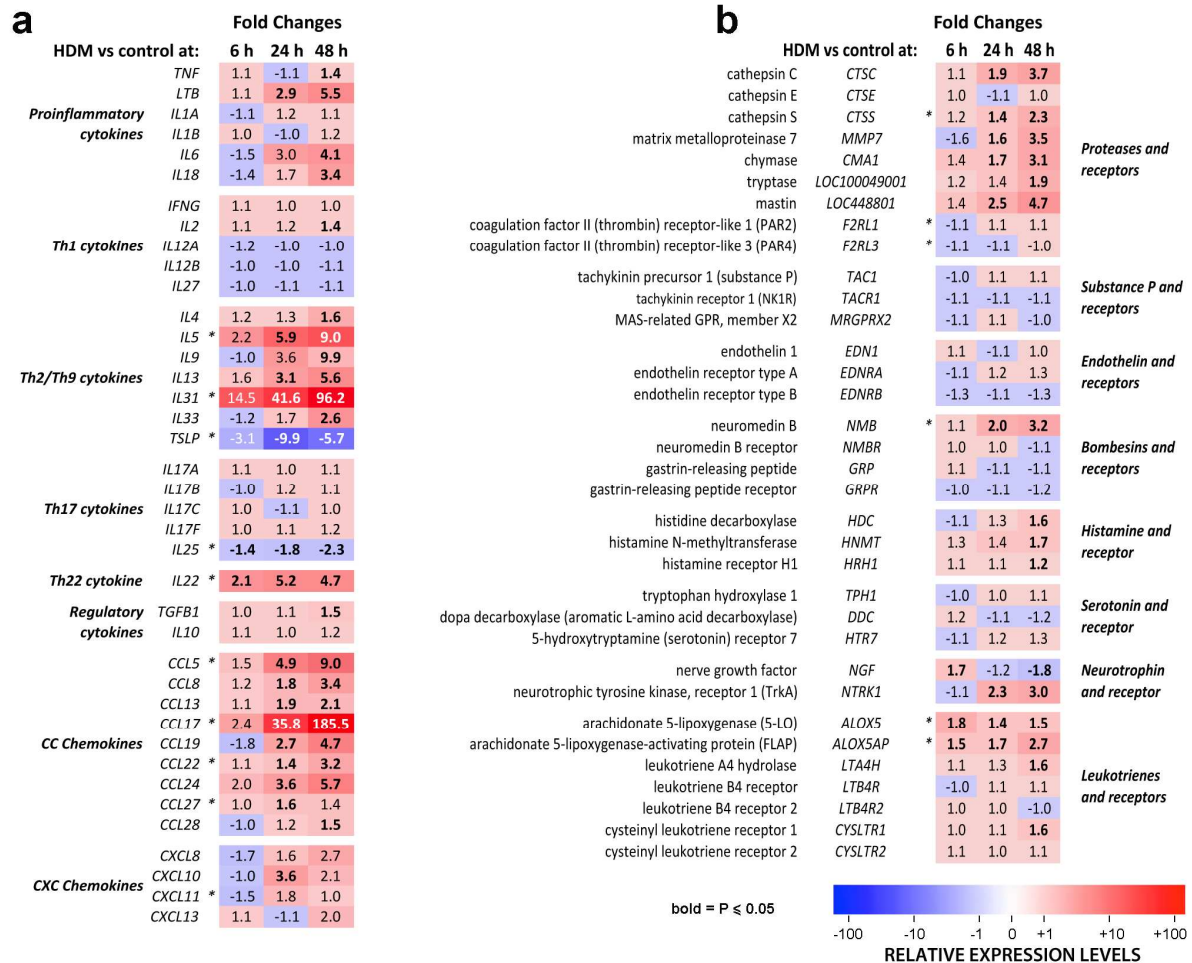
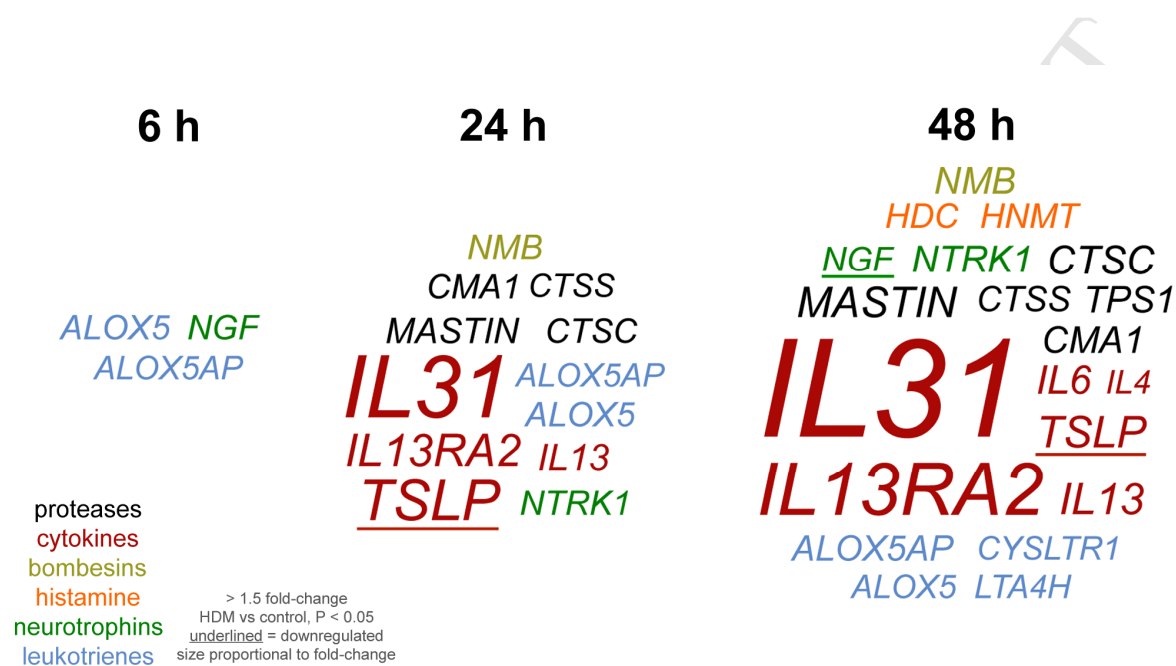


Figure 3. Expression of selected pruritogenic genes after HDM challenges



SUPPLEMENTAL MATERIAL

Table S1. Quantitative PCR mRNA expression levels of selected genes at 6, 24 and 48 h

Figure S2. Correlation between microarray and PCR mRNA expression levels

Figure S3. Principal component analysis (microarray mRNA expression levels)

Principal component analyses confirmed the relative clustering of microarray datasets outside of that of one outlying sample that was removed from further analyses.

Table S4. Most up- and down-regulated genes (microarray mRNA expression levels)

Figure S5. Differentially expressed genes (microarray mRNA expression levels)

Differentially expressed genes between transcriptome profiles from biopsies collected at 6, 24 and 48 h after HDM challenge.

Table S6. Genes commonly differentially expressed at 6, 24 and 48 h (microarray mRNA expression levels)

Table S7. Gene ontology categories (microarray mRNA expression levels)

Table S8. Quantitative PCR mRNA expression levels of selected genes at 1 and 3 h

Table S9. Quantitative PCR probes for selected genes

The assay ID number correspond to Life Science's assay catalog number or new custom-made assay ID numbers (highlighted in yellow).

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