

Cellular and cytokine kinetics after epicutaneous allergen challenge (atopy patch testing) with house dust mites in high-IgE beagles

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Abstract

The cellular and cytokine dynamics of reactions triggered by atopy patch testing with house dust mites were studied in six high-IgE beagles. Sites were scored and biopsied at 6, 24, 48, and 96 h, and samples were processed for histopathology, immunohistochemistry, and polymerase chain reaction (PCR).

All dogs developed positive reactions at some point in time. Mean clinical scores were significantly higher than baseline at 24, 48, and 96 h. Clinically, one of six dogs had a positive reaction at 6 h; two of six reacted at 24 and 48 h, and five of six at 96 h.

Histologically, superficial perivascular mononuclear and granulocytic dermatitis developed (5/6) after 6 h, and progressed in severity at 24 h (6/6). Additionally, at 48 h epidermal spongiosis, hyperplasia and pustules developed (5/6), and were marked at 96 h (6/6). At and beyond 6 h, progressive CD1c-positive epidermal Langerhans cell hyperplasia with cluster formation and dermal dendritic cell infiltration was noted. Cutaneous infiltration of CD3-positive T lymphocytes with epidermal clusters developed over time.

mRNA expression for the cytokines gamma-interferon (γ -IFN), interleukin-6 (IL-6), IL-12p35, IL-13, IL-18, and thymus and activation regulated chemokine (TARC) exhibited significant increases during the challenge compared to baseline, but there was no appreciable alteration in expression for tumour necrosis factor-alpha (TNF- α), IL-12p40, IL-10, regulated on activation normal T-cell expressed and secreted (RANTES), IL-5, IL-2, IL-4, and IL-8. No correlation was detected between clinical scores and cytokines. It is concluded that IL-6 plays a role in early reactions followed by an increase of TARC and IL-13, while IL-18 progressively increases in later reactions.

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Introduction

Canine atopic dermatitis (cAD) is the second most common allergic skin disease of dogs, affecting 10% of the canine population.^{1,2} Traditionally, cAD has been defined as a type I hypersensitivity and is currently described as a 'genetically predisposed inflammatory and pruritic allergic skin disease with characteristic clinical features, associated most commonly with IgE antibodies to environmental allergens'.³ The specific role of IgE, however, is not clear. Disease expression and allergen-specific IgE do not always correlate, thus, additional factors besides IgE production and type I hypersensitivity are involved in the pathogenesis.

T-lymphocyte function abnormalities are demonstrable in both atopic dogs⁴ and people.⁵ In human AD, it is currently accepted that imbalances in lymphocyte populations and cytokine production play an important role in the pathogenesis of the disease.^{5,6} Atopic lesions are dynamic, and a biphasic pattern of cytokine expression has been demonstrated after epicutaneous allergen challenge or atopy patch testing (APT).^{7,8} Acute skin lesions are characterized by CD4⁺ Th2 lymphocytes and eosinophils and the release of Th2-type cytokines (e.g. interleukin-4 [IL-4], IL-5, and IL-13).^{7,8} Chronic lesions show a predominance of Th1 lymphocytes, macrophages, and Th1-type cytokines (e.g. IL-2, IL-12, gamma-interferon [γ -IFN], and IL-18). Proinflammatory cytokines (e.g. IL-6 and tumour necrosis factor-alpha [TNF- α]) are also increased in patients with AD, although the specificity of their role has not been completely elucidated.^{9,10} Other cytokines (e.g. IL-10) have 'regulatory' or inhibitory functions. While IL-10 was traditionally considered a strong Th2 cytokine, it does not have the effector functions of the other cytokines in this group. Recent investigations have highlighted its 'regulatory' properties such as the suppression of cytokine production by macrophages and the inhibition of the accessory function of macrophages in T-cell activation. Reports in the literature regarding the role of IL-10 in AD are conflicting. While IL-10 was found to be elevated in some AD models and antibodies against it have demonstrated clinical benefit,^{11,12} its production has also been reported to be impaired in children with AD.¹³ Importantly, inducible populations of allergen-specific IL-10-secreting T regulatory cells appear to be crucial for successful allergen-specific immunotherapy.¹⁴

A particular group of cytokines, called chemokines due to their strong chemotactic properties (contraction of chemotactic cytokines), has been intensively investigated

for its role in the selective migration of leucocytes in lesional atopic skin. In particular, a chemokine called CCL17 or TARC (thymus and activation regulated chemokine), selectively recruits Th2 lymphocytes expressing its receptor, CCR4 (chemokine receptor 4). TARC is overexpressed in patients with AD and is currently considered the most reliable marker of AD, as its levels closely correlate with disease severity.¹⁵ Another chemokine, called CCL5 or RANTES (regulated on activation normal T-cell expressed and secreted), attracts CD4+ lymphocytes and eosinophils and, similarly, is overexpressed in patients with AD.¹⁶ Also, CXCL8 or IL-8, a chemokine that attracts neutrophils, is increased in atopic individuals.¹⁷

Canine AD shares many clinical and immunological similarities with its human counterpart.^{18,19} There are few studies evaluating cytokine expression in the skin of dogs with AD. Due to the limited availability of antibodies suitable for immunohistochemical staining of cytokines, all studies have used polymerase chain reaction (PCR) techniques. The results are varied but have mostly shown findings similar to the human disease. IL-4 mRNA was overexpressed in atopic dogs when compared to controls,^{20,21} as well as γ -IFN, TNF- α , and IL-2 mRNA.²¹ Additionally, TARC²² and CCR4²³ were found to be selectively expressed in lesional skin of atopic dogs, suggesting a specific role of TARC in the pathogenesis of AD lesions.

It is important to note, however, that all these studies have evaluated dogs with naturally occurring disease. Most had chronic AD lesions, thus, evaluation of the early phases of AD was not possible. Additionally, as the age of the individual lesions biopsied was unknown, a kinetic study of developing lesions was not feasible. As atopic lesion development is a dynamic process, evaluation of lesions of unknown age at one point in time provides limited information.

A reliable model for cAD is critical for evaluation of the pathogenetic mechanisms and identification of targeted treatments. Numerous, unsuccessful attempts to establish such a model have been made in the past.^{24,25} A suitable one has been recently identified in a group of high-IgE beagles,²⁶ which develop a dermatitis after environmental allergen challenge with house dust mites (HDM). Cutaneous lesions are clinically, histologically, and immunohistochemically similar to the natural-occurring cAD and can be elicited after either epicutaneous allergen challenge or APT.^{27–29}

The epicutaneous route of allergen exposure is currently considered to play a crucial role in cAD. This is based on the clinical distribution of the lesions (e.g. contact areas) and the presence of IgE+ Langerhans cells in lesional atopic skin.³⁰ Atopy patch testing offers the great advantage of allowing sequential evaluation of the lesions, as the exact time of initial allergen exposure is known. In humans, APT reactions actually resemble natural-occurring AD lesions more than the late phase reactions triggered by intradermal injection of the allergen.³¹ As APT shows a high specificity for AD, it provides a good model for studying the onset of the allergic response to allergens in the skin.

The purpose of this study was therefore to evaluate the cellular and cytokine dynamics of APT reactions in a model of cAD. Reactions were evaluated clinically, by histopathology, and by immunohistochemistry (IHC) for the assessment of cell types, and by PCR for cytokine expression. The general hypothesis tested was that, similar to human AD,

there would be varying skin expression of cytokine genes depending on lesion age. More specifically, it was hypothesized that the cytokine profile would switch from a general proinflammatory and Th2-dominated environment of acute lesions to a Th1-driven milieu of chronic lesions. Furthermore, it was anticipated that early lesions (6–24 h after allergen challenge) would be associated with high transcription and translation of proinflammatory cytokines (IL-6, TNF- α), chemokines (TARC, RANTES, and IL-8), and Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) to facilitate the recruitment of lymphocytes and eosinophils in the area of allergen challenge. Late lesions (48–96 h after allergen challenge) were predicted to have high expression of Th1 cytokines (IL-2, IL-12, γ -IFN, and IL-18) to promote the switch to the chronic phase of the disease, characterized by accumulation of lymphocytes and macrophages. The identification of key cytokines and chemokines of these reactions is of crucial importance for the design of future, targeted treatments.

Materials and methods

Animals

Six high-IgE-producing beagles previously sensitized to HDM epicutaneously were used. The colony was composed of five intact females and one intact male aged 5 years. Dogs were housed in a research facility where no exposure to HDM was allowed other than during experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida.

APT procedure

An area of 10 × 15 cm (lateral thorax) was shaved 48 h beforehand to minimize irritant reactions. Approximately 20 μ L of saline and HDM (milled, natural *Dermatophagoides farinae*, 100 mg mL⁻¹, Greer Laboratories Inc, Lenoir, NC, US) were applied to adhesive bandages (Band-Aid Clear Spots, Johnson & Johnson, Skillman, NJ, USA). The concentration and formulation of HDM were based on previous experiments.²⁹ Once APT patches were applied, the area was wrapped with bandaging tape (VetRap, 3M Animal Products, St. Paul, MN, USA) and elastic tape (Elastikon, Johnson & Johnson Medical, division of Ethicon, Arlington, TX, USA). Each dog received four applications of saline and eight applications of HDM. Dogs wore a dog-specific T-shirt at all times to protect the area and prevent trauma.

Clinical evaluation of APT sites

APT sites were evaluated at 6, 24, 48, and 96 h after initial application (counted as time 0 h). Sites were assessed for the presence of erythema, papules, macules, and pustules. Each sign was scored from 0 to 3 (0 = absent, 1 = mild, 2 = moderate, and 3 = severe). Total score at each site was calculated and the mean was used for statistical analysis. Two HDM sites (2 × 2 cm/each) were scored at each evaluation time, starting with the most caudal sites on the dog. These sites were the most easily accessible for lifting the T-shirt and bandage, and minimizing movement of other patches, which stayed in place until the 48-h evaluation. Once evaluated, sites were sampled by biopsy. All remaining patches were removed after 48 h but the T-shirt was kept on to prevent trauma.

Skin sampling

Prior to APT application, samples of normal skin were collected by biopsy and used as baseline samples (0 h). After the 6, 24, 48, and 96 h clinical evaluations, samples of the two HDM/APT sites were taken. Saline areas were used as negative controls for clinical evaluations but were not biopsied at evaluation times to minimize the number of samples required. This limitation was imposed by the Institutional Animal Care and Use Committee. Biopsy is considered to be a surgical procedure, therefore a maximal number is allowed in the lifetime of each research dog.

Prior to skin sampling the area was anaesthetized using lidocaine (2% lidocaine HCl, Phoenix Pharmaceuticals, Inc., Belmont, CA, USA), and the samples were processed for histopathology, IHC, and PCR analysis. Due to the different requirements, one sample was taken using a sterile, disposable 6-mm biopsy punch (Miltex Instrument Company, Bethpage, NY, USA) and the second using an 8-mm biopsy punch.

Immediately after collection, the 6-mm sample was placed in a sterile microcentrifuge tube (Fisherbrand, Fisher Scientific, Pittsburgh, PA, USA) and snap frozen in liquid nitrogen. All 6-mm samples were stored at -80°C until shipped to the University of Tokyo, Japan, on dry ice for PCR analysis (CCR4 and IL-18).

The 8-mm biopsy was cut in half immediately after collection. One-half was embedded in OCT and frozen in isopentane cooled to freezing in liquid nitrogen. These samples were shipped to North Carolina State University for histology and IHC. The second half was placed in a sterile microcentrifuge tube and snap-frozen in liquid nitrogen. Samples were stored at -80°C until shipped to the University of California, Davis on dry ice for PCR analysis.

Assessment of cytokine mRNA expression in skin samples

Two-step real-time quantitative TaqMan PCR at UC Davis (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p35, IL-12p40, IL-13, TNF- α , g-IFN, TARC, and RANTES)

Total RNA was extracted from the skin samples by an automated workstation (ABI PRISM 6700 Automated Nucleic Acid Workstation, Applied Biosystems, Foster City, CA, USA). To synthesize cDNA from the isolated total RNA, reverse transcription (RT) was carried out using a commercially available kit (SuperScript III First-Strand System SuperMix, Invitrogen, Carlsbad, CA, USA). Transcription of cytokines was quantified by two-step real-time PCR (TaqMan Universal PCR Master Mix, Applied Biosystems) at the UC Davis Lucy Whittier Molecular Core Facility, using methodologies previously validated.³² Expression of hypoxanthine phosphoribosyltransferase 1 (HPRT1) mRNA was used as internal control. Sequences of the primer and probe pairs used are shown in Table 1.

Quantification of mRNA expression was undertaken using the comparative C_T method (C_T = threshold cycle). With this method, the relative transcription of the target gene (cytokines) is reported as the n -fold difference relative to that of the calibrator gene (HPRT1). For this purpose, C_T values of the calibrator were subtracted from the C_T values of the target cytokine (ΔC_T). All samples were examined in duplicate and the mean value of ΔC_T was calculated. The amount of mRNA was calculated by $2^{-\Delta C_T}$ resulting in the evaluation of the samples as an n -fold difference relative to the calibrator.

One-step real-time quantitative TaqMan PCR at University of Tokyo (IL-18)

Total RNA was extracted from the skin samples by a commercially available kit (SV Total RNA Isolation System, Promega, Madison, WI, USA). Transcription of IL-18 was quantified by one-step real-time PCR (TaqMan OneStep RT-PCR Master Mix Reagents Kit, Applied Biosystems) at the University of Tokyo, using methodologies previously validated.³³ Expression of β -actin mRNA was used as internal control. Sequences of the primer and probe pairs used are shown in Table 1. A comparative C_T method was used for quantification of IL-18 mRNA expression. For each sample, the C_T values for the target amplicon (IL-18) and the calibrator (β -actin) were determined to report the relative transcription of the amplicon cDNA against calibrator cDNA, respectively. The C_T value of the calibrator was subtracted from the C_T value of the target cytokine (ΔC_T) to normalize for differences in the amount of total nucleic acid added to each reaction and the efficiency of the RT step. All samples were examined in duplicate and the mean value of ΔC_T was calculated. The amount of IL-18 mRNA was calculated by $2^{-\Delta C_T}$, resulting in the evaluation of the samples as an n -fold difference relative to that of β -actin mRNA.

Reverse-transcription PCR at University of Tokyo (CCR4)

Transcription of CCR4 was qualitatively evaluated by RT-PCR using a commercially available kit (RNA PCR Kit, Applied Biosystems) as

previously described.³⁴ Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a house-keeping gene. Sequences of primers for CCR4 and GAPDH are shown in Table 1. PCR products were analysed by electrophoresis through a 3% agarose gel and stained with ethidium bromide for visualization. With this methodology no quantification was performed.

Histopathology (NCSU)

Five-micrometre frozen sections were stained using haematoxylin-eosin for visualization of cutaneous inflammation. The severity of dermal cell inflammation was graded using a 10-point visual analogue scale (VAS), from 'none - (0)' to 'very high - (10)'. Immunohistochemical staining for CD1c, CD3, CD4, and CD8alpha was used to determine the phenotype of infiltrating mononuclear cells.^{35,36} The intensity of dermal infiltration with cells labelled by each monoclonal antibody was estimated on the VAS, as described above.

Statistics

Clinical scores were analysed using least squares analysis of variance (LSANOVA) with all main effects and interactions included in the model. Differences among doses were analysed using orthogonal contrast analysis. Because clinical scores are not normally distributed, they were rank transformed prior to analysis. All P -values are representative of the rank-transformed data. In addition, tests for heterogeneity of regression were conducted to evaluate time-trends between treatments. The analysis was performed at the highest significant order of regression.

Correlation between individual cytokines and clinical scores was evaluated using Spearman rank-order correlation. As clinical scores were nonparametric, they were rank transformed, and the Pearson's correlation coefficient was calculated on the ranked data. All analyses were performed using the statistical software package SAS System for Windows version 8.2 (SAS Institute, Cary, NC, USA). A $P < 0.05$ was considered significant.

Results

Clinical scores of APT on saline vs. HDM sites (Fig. 1)

There were no differences in the clinical scores of the saline APT site over time, whereas the HDM site clinical scores were significantly higher than baseline at 24, 48, and 96 h ($P = 0.0019$, 0.0002 , and 0.0001 , respectively). HDM scores were significantly higher than saline at 24, 48, and 96 h ($P = 0.0019$, 0.0002 , and 0.0016 , respectively). One dog (1/6, 16%) had a positive reaction (> 1 clinical score) at 6 h, two dogs (2/6, 33%) at 24 and 48 h, and five (5/6, 83%) at 96 h. No significant correlation was found between clinical scores and any of the cytokines (data not shown).

Cytokines analysis: mRNA expression

Results are presented as linear values of gene expression relative to the internal controls. Values of $2^{-\Delta C_T}$ were calculated at each time point and divided by the values at 0 h, resulting in an n -fold difference relative to preprovocation. Mean and standard error of the mean (SEM) are illustrated in the figures.

Proinflammatory cytokines and chemokines mRNA expression (Fig. 2)

Expression of IL-6 mRNA peaked at 24 h when it was significantly higher than baseline ($P = 0.007$). No significant

Table 1. Primers, probes, and PCR methodology used for each cytokine mRNA analysis

Primer set		Primer sequence	Probe sequence	PCR method	5' Fluorophore	3' Quencher	Institution
HPRT1	Forward	GAGATGACCTCTCAACTTTAACTGAAAA	CTTGATTGTTGAAGATCTCATTGACACAGGCA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAAGGGAAGCAAGGTTTGCA					
Beta-actin	Forward	TGACCCTGAAGTACCCCATTTG	ATCGTCACCAACTGGGACGACATGG	One-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	University of Tokyo
	Reverse	GTTGTAGAAGGTGTGGTGCCAG					
GAPDH	Forward	CTCATGACCACAGTCCATGC		RT-PCR			University of Tokyo
	Reverse	TGAGCTTGACAAAGTGGTCA					
IL-2	Forward	CAACTCTCCAGGATGCTCACATT	TTACACGCCCAAGAAGGCCACAGAA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	TTCTGCTAGACATTGAAGGTGTGTAA					
IL-4	Forward	CTCACCAGCACCTTTGTCCA	CCATGCACGAGTCGTTTCTCGCTGT	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	GCAGTGAAGACGTCCTTGACAGT					
IL-5	Forward	TGCTCTCCACTCATCGAACTTG	CTGATAGCGCATGGGAACCTGATG	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAGTTGGTGATTTTATTTTCAGGAGTA					
IL-6	Forward	TTAAGTACATCCTCGGCAAAATCTC	CTTGTTAAACTTGTCACACATCTCCTTCTCAGTGC	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAGTGCCCTCTTGCTGTCTTCA					
IL-8/CXCL-8	Forward	GTGAAAACTCAGAAATCATTTGTAAGCT	TCCAGGCACACCTCATTTCCATTGAA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAACCTTTTGTAACCATTTTTCCT					
IL-10	Forward	TGAAGGACAAGCTGGACAACAT	CAACCCAGGTAACCTCTTAAAGTCTCCAGCA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	GGGCATCACCTCCTCCAAGTA					
IL-12 p35	Forward	GTGCCTCAACCACTCCCAA	CAACACGCTTCAGAAGGCCAGACAAAC	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAATCTCTTCGGAAGTGCAGG					
IL-12 p40	Forward	AAAACCTACACCAGCAGCTTCTTCA	AGAGACATCATCAAACAGACCCACCCA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	GAGAATTTTCAATGGCTTCAGC					
IL-13	Forward	ATCACCAGCAATCAGGCATCC	AGCATGGTGTGGAGCGTCAACCTGA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CGGAGACATTGATCAGAGATTCTAGA					
IL-18	Forward	CTCTCCTGTAGAACAACAACTATTTCTT	CAGAAAATGAGTCCTCCGGATAGTATCAATGATG	One-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	University of Tokyo
	Reverse	GAACACTTCTCTGAAAGAATATGATGTCA					
TNF-alpha	Forward	TCTCGAACCCCAAGTGACAAG	AGTAGCTCATGTTGTAGCAAACCCGAAGCT	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAACCCATCTGACGGCACTA					
IFN-gamma	Forward	AGTAATCCAGATGTATCGGACGGT	TCACTCTCCTCTCTCCATTTCTTCAAAATATCTACGAA	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	CAATTTGGCTCTGAATGATTGTTTT					
TARC/CCL-17	Forward	CATTCTATCAGCAGGCTGACAA	TGTCCTCAAGGATGCCATCGTGTG	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	GATGGACTTGCCTTGACAGTT					
RANTES/CCL-5	Forward	GCCAGCAGTCGTCTTTGTCA	ACCGCCAGGTGTGTGCCAAC	Two-step real-time PCR	6-carboxyfluorescein	6-carboxytetramethylrhodamin	UC-Davis
	Reverse	TCCCGCACCCATTTCTTCT					
CCR4	Forward	TACCTGGTGGGCTTTTACAGTGCATCTTC		RT-PCR			University of Tokyo
	Reverse	GCCAGGGTCTCTGTGGCCTGAATAGCGTAG					

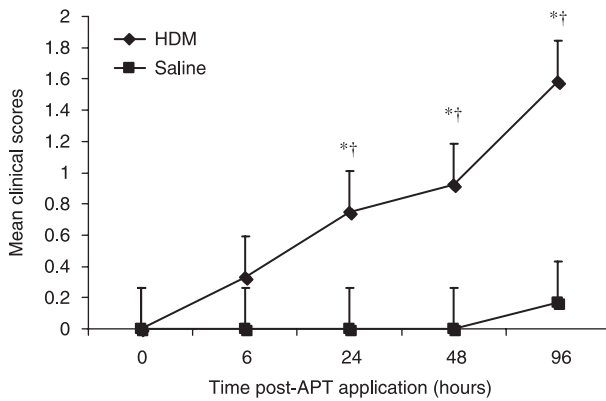


Figure 1. Clinical scores after saline and HDM application (100 mg mL^{-1}). Scores are means $\pm$ standard error of the mean (SEM). The symbol * indicates significant difference from baseline (0 h), whereas + indicates HDM scores significantly different from saline ones. Clinical scores of HDM patches increased progressively over this time course.

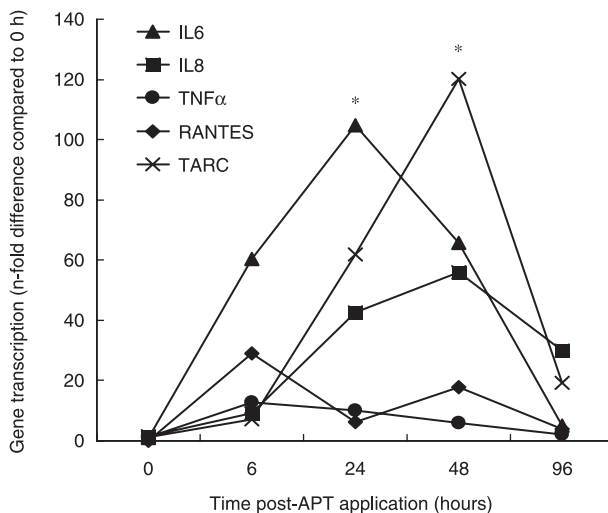


Figure 2. Proinflammatory cytokines and chemokines. Values of IL-6, IL-8, TNF- α , TARC, and RANTES mRNA after APT with HDM. The symbol * indicates values that are significantly different from baseline (0 h). IL-6 mRNA was significantly increased at 24 h and TARC at 48 h.

changes were detected for the expression of TNF- α mRNA. Transcription of mRNA encoding for TARC peaked at 48 h when it was significantly higher than baseline at 48 h ($P = 0.009$). No significant changes were detected over time for the expression of RANTES and IL-8 mRNA.

Th2 cytokines mRNA expression (Fig. 3)

Expression of IL-13 mRNA was significantly higher than baseline at 24 and 48 h ($P = 0.04$). No significant differences were detected for mRNA expression of IL-5 and IL-10. There was not enough information to statistically compare the linear values of IL-4 mRNA to baseline.

Th1 cytokines mRNA expression (Fig. 4)

Transcription of mRNA encoding for γ -IFN was significantly higher than baseline at 6 h ($P = 0.04$) and that for IL-12p35 mRNA expression at 24 h ($P = 0.04$). IL-18 mRNA expression was significantly higher than baseline

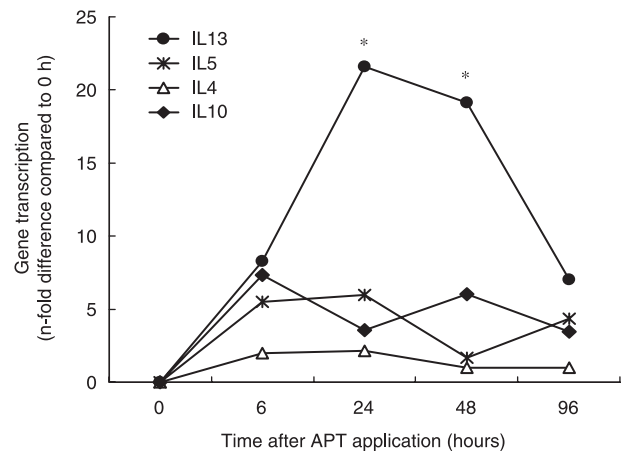


Figure 3. Th2 cytokines. Values of IL-4, IL-5, IL-10 and IL-13 mRNA after APT with HDM. The symbol * indicates values that are significantly different from baseline (0 h). IL-13 mRNA expression had a significant and sustained increase, being significantly different from baseline at both 24 and 48 h.

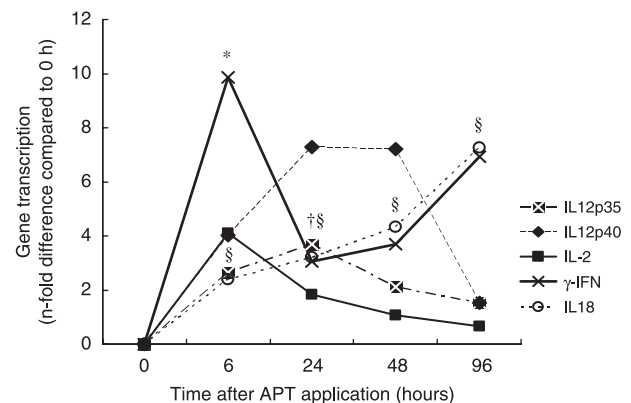


Figure 4. Th1 cytokines. Values of IL-2, γ -IFN, IL12p35, IL12p40, and IL-18 mRNA after APT with HDM. γ -IFN mRNA expression was significantly higher than baseline at 6 h (*), whereas the mRNA expression of the p35 subunit of IL-12 was significantly higher than baseline at 24 h (+). IL-18 mRNA expression increased throughout the experiment being statistically different from baseline starting at 6 h (§).

starting at 6 h and progressively increased throughout the challenge. No statistically significant differences were detected for IL-12p40 and IL-2 mRNA expression.

CCR4 mRNA expression (Fig. 5)

CCR4 mRNA was strongly expressed at 48 or 96 h after the provocation in three of six dogs, whereas the band intensity was relatively weak in two of six dogs when compared with that of GAPDH. Furthermore, it was found that CCR4 mRNA was weakly expressed at 0 and 24 h after the provocation in one dog.

Histopathology and immunohistochemical staining

Prior to the challenge, skin sections from four of six dogs exhibited a superficial mononuclear dermatitis (mean VAS: 1.2), while those from two dogs had no inflammation. At 6 h, skin sections from five dogs had a superficial perivascular mononuclear and granulocytic dermatitis (average VAS: 2.3). At 24 h, sections of all dogs exhibited a superficial perivascular mononuclear and granulocytic dermatitis, and

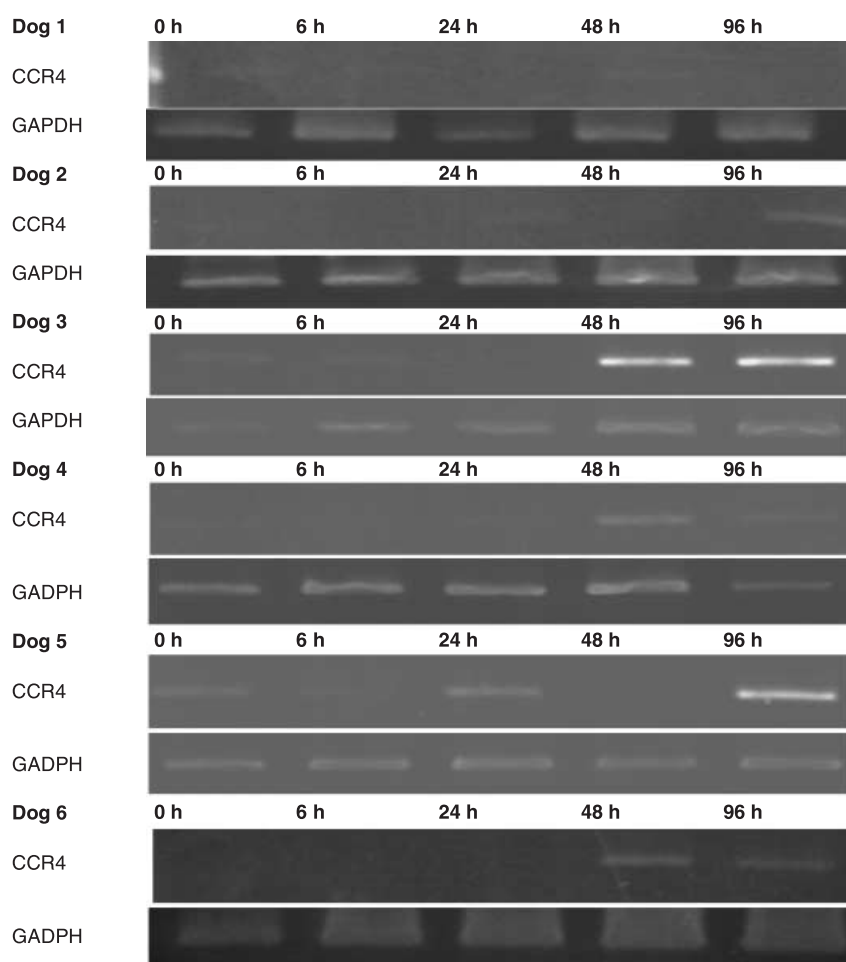


Figure 5. Qualitative representation of CCR4 mRNA expression from cutaneous samples of APT reactions. CCR4 mRNA was strongly expressed in dogs 3, 4, 5, and 6 at 48 or 96 h after the provocation, whereas band intensity was relatively weak in dogs 1 and 2 when compared with that of the internal control (GAPDH).

in one dog, intraepidermal pustules also were seen (mean VAS: 4.6). At 48 h, the examination of sections revealed, in addition to the superficial perivascular dermatitis seen previously, epidermal spongiosis, hyperplasia, and pustule formation in five dogs (mean VAS: 6.8). Finally, at 96 h, a superficial perivascular to diffuse mononuclear dermatitis with epidermal spongiosis and pustule formation was seen in all subjects (mean VAS: 7.8).

At and beyond 6 h, there was increasing CD1c-positive epidermal Langerhans cell hyperplasia with cluster formation and coexisting dermal dendritic cell infiltration and aggregation. Over time, there was a progressive increase in the cutaneous infiltration with CD3-positive T lymphocytes. Epidermal T-lymphocyte clusters became evident in sections collected at and beyond 24 h. Over the course of the study, there was also a progressive increase in the skin infiltration with CD4 and CD8-alpha-positive cells. As both activated dermal dendritic cells and neutrophils,³⁶ besides lymphocytes, can express CD4 and double stains were not undertaken, CD4:CD8 cellular ratio was not calculated.

Discussion

In this study, APT reactions in HDM-sensitive high-IgE beagles were characterized by a progressive hyperplasia of Langerhans cells and superficial perivascular to diffuse granulocytic and mononuclear dermatitis. Significant increases of IL-6, IL-13, IL-12p35, IL-18, and TARC mRNA expression were also detected. While other studies have

evaluated the cell types of APT reactions in dogs,^{27,28} this appears to be the first study investigating the kinetics of cytokine expression in APT reactions in dogs.

The cellular dynamics observed confirm an immunological response in which allergens appear to be epicutaneously captured by epidermal Langerhans cells triggering a progressive infiltration of granulocytes and lymphocytes. These results are similar to reports in both the veterinary and the human literature, in which eosinophilic microabscesses and lymphocyte infiltration of the epidermis are demonstrated after allergen challenge.^{27,30,35,37,38} It is interesting to note that, although only one macroscopic reaction was detected at 6 h, all sites were characterized by immunological reactions, as demonstrated by histopathology and IHC. These findings are similar to previous reports in human literature, in which negative patch test sites contained evidence of mononuclear cell infiltration.³⁹

All dogs developed a positive APT reaction at some point in time. This result is different from previous studies, in which a lower rate of positive APTs was found in dogs with AD.^{40,41} It is important, however, to note that the dogs used in this study were specifically selected for their ability to develop a positive APT and that these results do not suggest an irritant reaction. In irritant reactions, neutrophils dominate; while in allergic reactions, eosinophils are more prevalent.⁴¹ Although this study was unable to discriminate between eosinophils and neutrophils due to the use of frozen sections, cutaneous reactions on allergen challenge had been previously biopsied in these dogs and

found to have epidermal eosinophilic infiltrations.²⁶ Interestingly, in humans, irritant reactions are characterized by high mRNA expression of IL-8,⁴² cytokine whose expression did not significantly change in the course of this study.

Double stains for IHC or *in situ* hybridization were not undertaken; therefore, the source of transcription remains speculative. By combining the findings, the authors hypothesize that, similar to what is reported in human AD, the initial inflammatory response may be promoted by keratinocytes and Langerhans cells through the release of cytokines and chemokines, which in turn lead to the recruitment of granulocytes and lymphocytes in the area of allergen challenge.

Keratinocytes, as well as Langerhans cells, have been demonstrated to release IL-6 in response to allergen challenge.^{43–45} As IL-6 is an important co-stimulator of T-lymphocyte activation, these cells may play an important role in aiding the initial T-cell activation. Of all the proinflammatory cytokines investigated, IL-6 was indeed the only one that had a significant increase compared to baseline. These findings are similar to patch testing with HDM in humans.⁴³

Keratinocytes, as well as dendritic cells, are also the most important source of TARC in atopic people and dogs.^{46,47} As expected, TARC mRNA was extensively expressed after allergen challenge. Its increase was the largest of all the chemokines and cytokines investigated, reaching a peak at 48 h. This finding is in line with previous reports in dogs and humans with AD.^{15,22,47–49} Interestingly, in humans, TARC is not produced by normal keratinocytes.⁵⁰ It is only produced by 'atopic' keratinocytes, confirming a specific role in AD.⁴⁶ With its ability to recruit Th2 CCR4+ lymphocytes, TARC plays a crucial role in allergic reactions.⁵¹ This seemed to be confirmed by the strong detection of TARC receptor, CCR4, at 48 and 96 h in 50% of the dogs.

Langerhans cells appear to be heavily involved in APT reactions, as indicated by the progressive hyperplasia. Their function does not appear to be limited to the initial, allergen capture.^{30,35} Langerhans cells have been demonstrated to release IL-13 and could therefore help promote the initial Th2 response.⁵² As they can also produce IL-12,^{53–55} it is conceivable that they could orchestrate, later on, the switch to the Th1, mononuclear, chronic phase of AD. IL-13mRNA expression was significantly elevated at 24 and 48 h indicating an early, yet sustained role. Our findings are in line with previous reports in patients with AD.^{56,57} Similarly, other investigators have also found that, after HDM challenge, IL-13 is produced earlier and for a much longer period of time than IL-4.^{58–60} It could be hypothesized that the transcription of IL-13 might have been initiated by Langerhans cells⁵² and later sustained by infiltrating lymphocytes,^{56,57} attracted in the skin by the release of TARC. Although most of IL-13 biological activities overlap with those of IL-4, recent *in vitro* investigations have shown that IL-13 is the dominant IgE-inducing cytokine in situations where IL-4 is either absent or present at a low level.^{61,62} This situation could have applied to the present colony. The lack of increase of IL-4 differs from Nuttall's report of overexpression of IL-4 mRNA in canine atopic skin,²¹ but is similar to reports in human literature, which demonstrate that IL-13, and not IL-4 mRNA, is released by peripheral T cells of patients with AD⁶³ and in lesional skin.⁶⁴

In the human literature it is currently hypothesized that the switch of the lymphocytic response from the acute phase to the chronic, monocytic phase is largely mediated by IL-12.^{8,43,54} IL-12 is a heterodimer consisting of two polypeptide chains, and both chains are required for biological activity. Both Langerhans cells and eosinophils have been demonstrated to produce biologically active IL-12.^{53–55,65–67} Thus, both cell types may play a key role in the progression of the allergic reaction. In our study, a statistically significant increase was only detected for the mRNA expression of the p35 subunit of IL-12 while no statistically significant changes were detected for the p40 subunit. These findings are similar to previous reports.^{8,43}

As lymphocytes progressively accumulate in the skin, it can be hypothesized that the Th1 response could be further promoted by IL-18 release (a strong γ -IFN-inducing factor). In this study, IL-18 mRNA expression progressively increased over time. To the best of authors' knowledge, this is the first report of cutaneous IL-18 mRNA expression in the dog. Interleukin-18 is a proinflammatory cytokine that has recently attracted great attention for its potential role in AD as its levels seem to correlate directly with the severity of the clinical disease.^{68,69} IL-18 is stored as a biologically inactive precursor form in various cell types, including macrophages and keratinocytes. It is therefore reasonable to hypothesize that keratinocytes might be responsible for the initial release of IL-18, and that the levels may be later increased and sustained by the progressive accumulation of lymphocytes.

Some results in this study are unexpected. The lack of significant increase of TNF- α mRNA expression is in contrast to the results of Nuttall *et al.*,²¹ who reported higher levels of TNF- α mRNA in lesional atopic skin compared with nonlesional and healthy skin. This difference may be due to lesion age or a combination of variability and a limited number of dogs. In Nuttall's study lesion, age was unknown and, therefore, it is possible that more chronic lesions, possibly with a secondary bacterial component, might have contained higher levels of TNF- α mRNA. The other interesting finding was that γ -IFN mRNA showed a biphasic response with an early peak at 6 h. The early rise of γ -IFN could be potentially explained by the presence of some T-cell infiltration even prior to any allergen challenge, as indicated by the presence of a mononuclear infiltrate in both baseline and 6 h biopsies. After the initial peak, γ -IFN showed another increase in later reactions, possibly linked to the increase of IL-18, although these changes were not statistically significant. Another cytokine that is intriguing and that was not reported to significantly change was IL-10. These results are in line with previous reports in veterinary literature.²¹ Further investigation on the role of IL-10 is needed.

In summary, APT reactions after epicutaneous application of HDM in high-IgE beagles were characterized by a superficial perivascular mononuclear and granulocytic dermatitis with progressive hyperplasia of CD1c-positive epidermal Langerhans and cutaneous infiltration of CD3-positive T-lymphocytes. As hypothesized, varying skin expression of cytokine genes depending on lesion age was found. Early lesions were overall associated with high transcription and translation of proinflammatory cytokines (e.g. IL-6) and Th2 cytokines (e.g. IL13), while later lesions were accompanied by progressive increase of Th1 cytokines

(e.g. IL-18). mRNA expression of the chemokine TARC was also significantly increased, confirming its important role in the recruitment of lymphocytes in the area of allergen challenge. It is therefore proposed that this model mimics both human and canine AD. Future studies using specific cytokine-blocking agents are needed to evaluate the pathogenetic role of each of these cytokines and allow identification of suitable targets for therapy.

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Résumé La dynamique des réactions cellulaires et cytokiniques secondaires à un test épicutané avec les acariens des poussières de maison a été étudiée chez six Beagles haut répondeurs en IgE. Les sites ont été scorés et biopsiés à 6, 24, 48, et 96 h et les échantillons ont été techniqués pour examen histopathologique, immunohistochimique et PCR.

Tous les chiens ont montré des réactions positives à un moment. Les scores cliniques moyens étaient significativement plus élevés qu'au départ à 24, 48, et 96 h. Cliniquement, 1 des 6 chiens avait une forte réaction positive à 6 hr; 2/6 ont réagi à 24 et 48 h, et 5/6 à 96 h.

Histologiquement, une dermatite périvasculaire superficielle mononucléée et granulocytaire s'est développée (5/6) après 6 h, et a augmenté en sévérité à 24 h (6/6). En outre, à 48 h une spongiose épidermique, une hyperplasie et des pustules sont apparues (5/6), et étaient marquées à 96 h (6/6). A et après 6 h, une hyperplasie des cellules de Langerhans épidermiques CD1c-positives avec regroupements a été notée, ainsi qu'une infiltration par des cellules dermiques dendritiques. Une infiltration cutanée par des lymphocytes CD3-positifs avec regroupements s'est également développée avec le temps.

L'étude de l'expression de mRNA pour les cytokines suivantes: gamma Interferon (γ -IFN), Interleukin-6 (IL-6), IL-12p35, IL-13, IL-18 et Thymus and Activation Regulated Chemokine (TARC) a montré des augmentations significatives pendant la provocation par rapport aux données de base, mais il n'y avait pas d'altération pour le Tumor Necrosis Factor- α (TNF- α), IL-12p40, IL-10, Regulated on Activation Normal T-cell-Expressed and Secreted (RANTES), IL-5, IL-2, IL-4 et IL-8. Aucune corrélation n'a été observée entre les scores cliniques et les dosages de cytokines. Nous concluons que l'IL-6 joue un rôle dans les réactions précoces suivie, par une augmentation du TARC et d'IL-13, alors que l'IL-18 augmente progressivement dans les réactions retardées.

Resumen Se estudio la dinámica celular y de citoquinas de las reacciones iniciadas durante la prueba de atopia en parche frente a ácaros del polvo casero, en seis perros Beagle que presentaban altos niveles de inmunoglobulina E (IgE). A las zonas testadas se les dio un valor clínico y se tomaron biopsias a las 6, 24, 48 y 96 horas. Las muestras se procesaron para histopatología, inmunohistoquímica y reacción de polimerasa en cadena (PCR).

Todos los perros desarrollaron reacciones positivas en algún momento del estudio clínico. Los valores clínicos medios fueron significativamente más elevados que el valor basal a las 24, 48 y 96 horas. Clínicamente, uno de 6 perros tuvo una reacción positiva a las 6 horas; 2/6 a las 24 y 48 horas y 5/6 a las 96 horas.

En el examen histológico, 5/6 animales desarrollaron dermatitis superficial perivascular con células mononucleares y granulocitos a las 6 horas, y progresaron en intensidad a las 24 horas (6/6). Además, a las 48 horas se observó espongiosis de la epidermis, hiperplasia y pústulas (5/6), que fueron de marcada intensidad a las 96 horas (6/6). A partir de las 6 horas en adelante se observó hiperplasia progresiva de células de Langerhans positivas para CD1c con formación de agregados, así como infiltración de células dendríticas en la dermis. Asimismo a lo largo del tiempo se desarrollaron agrupamientos epidermales de linfocitos T positivos para CD3.

La expresión de RNA mensajero de las citoquinas interferon γ (γ -IFN), interleuquina 6 (IL-6), proteína 35 de la interleuquina 12 (IL-12p35), interleuquina 13 (IL-13), interleuquina 18 (IL-18) y la quemoquina regulada en el timo y por activación (TARC), demostraron un aumento significativo durante la reexposición al antígeno en comparación con el nivel basal, pero no se observó ninguna alteración en el nivel del Factor de Necrosis Tumoral- α (TNF- α), proteína 40 de la interleuquina 12 (IL-12p40), interleuquina 10 (IL-10), quemoquina regulada en activación con expresión y secreción normal en linfocitos T (RANTES), interleuquina 5 (IL-5), interleuquina 2 (IL-2), interleuquina 4 (IL-4) ni interleuquina 8 (IL-8). No se observó correlación entre el nivel de citoquinas y los valores clínicos. Así pues concluimos que la IL-6 juega un papel importante en las reacciones tempranas, seguido por un incremento en TARC e IL-13, mientras que la IL-18 aumenta progresivamente en reacciones más tardías.

Zusammenfassung Die dynamischen Reaktionen, die durch den Atopie Patch Test mit Hausstaubmilben ausgelöst worden waren, wurden auf der zellulären sowie auf der Zytokinebene an sechs Beagles mit hohem IgE Gehalt untersucht. Die Hautstellen wurden nach 6, 24, 48 und 96 Stunden beurteilt und biopsiert und die Proben mittels Histopathologie, Immunhistochemie und Polymerase Chain Reaction (PCR) analysiert. Alle Hunde zeigten zu irgendeinem Zeitpunkt positive Reaktionen. Die durchschnittlichen klinischen Bewertungen waren nach 24, 48 und 96 Stunden signifikant höher als zu Beginn. Klinisch zeigte 1 von 6 Hunden nach 6 h eine positive Reaktion; 2/6 reagierten nach 24 und 48 h, und 5/6 nach 96 h. Histologisch entstand nach 6 h eine oberflächliche perivaskuläre, mononukleäre und granulozytäre Dermatitis (5/6), deren Schweregrad nach 24 h (6/6) zunahm. Zusätzlich entstanden nach 48 h epidermale Spongiose, Hyperplasie und Pusteln (5/6), die nach 96 h (6/6) sehr ausgeprägt waren. Zum Zeitpunkt von 6 h und danach wurde eine ausgeprägte CD1c-positive epidermale Langerhanszellhyperplasie mit Anhäufung in Gruppen und dermalen Dendritenzellinfiltration festgestellt. Mit der Zeit entstand eine kutane Infiltration von CD3-positiven T-Lymphozyten mit epidermaler Anhäufung. Die mRNA Expression für die Zytokine Gamma Interferon (γ -IFN), Interleukin-6 (IL-6), IL-12p35, IL-13, IL-18 und Thymus and activation-regulated chemokine (TARC) zeigte signifikante Zunahmen während der Provokation im Vergleich zu den Ausgangswerten. Andererseits bestand keine bemerkenswerte Veränderung in der Expression von mRNA für Tumor Nekrose Faktor-alpha (TNF- α), IL-12p40, IL-10, Regulated on activation normal T cell-expressed and secreted (RANTES), IL-5, IL-2, IL-4 und IL-8. Es wurde keine Korrelation zwischen den klinischen Bewertungen und den Zytokinwerten festgestellt. Es wird daraus geschlossen, dass IL-6 bei den frühen Reaktionen eine Rolle spielt, gefolgt von einer Zunahme von TARC und IL-13, während IL-18 in späteren Reaktionen zunehmend erhöht ist.